Cervical immune activation during the luteal phase may compromise subsequent trans-cervical ram sperm transport†

Laura Abril-Parreño¹, Anette Kristine Krogenæs², Xavier Druart³, Paul Cormican⁴, Sean Fair¹,* and Kieran G. Meade⁵

¹Laboratory of Animal Reproduction, Department of Biological Sciences, School of Natural Sciences, Biomaterials Research Cluster, Bernal Institute, Faculty of Science and Engineering, University of Limerick, Limerick, Ireland
²Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway
³UMR-PRC, INRA-85, Université de Tours, IFCE, Physiologie de la Reproduction et des Comportements, Institut National de la Recherche Agronomique, Nouzilly, France
⁴Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Grange, Ireland
⁵School of Agriculture and Food Science, University College Dublin, Dublin 4, Ireland

*Correspondence: Laboratory of Animal Reproduction, School of Natural Sciences, Biomaterials Research Cluster, Bernal Institute, Faculty of Science and Engineering, University of Limerick, Limerick V94 T9PX, Ireland. Tel: +35 361202548; E-mail: sean.fair@ul.ie

†Grant Support: The project was funded through the European Research Area Network, on Sustainable Animal Production (SusAN; Grant no. 16/RD/SusAn/ERA-NET). National funding was provided in Ireland by the Department of Agriculture, Food and the Marine as well as Teagasc (RMIS 0299) and in Norway by The Research Council of Norway (NFR 272338 / E50).

Abstract
Worldwide, cervical artificial insemination using frozen–thawed semen yields low pregnancy rates. The only exception to this is in Norway, where vaginal insemination with frozen–thawed semen yields pregnancy rates in excess of 60% and which has been attributed to the specific ewe breed used. Our previous work demonstrated differences in cervical gene expression at the follicular phase of the estrous cycle in ewe breeds with known differences in pregnancy rates. In this study, we characterized the cervical transcriptome of the same ewe breeds [Suffolk, Belclare, Fur, and Norwegian White Sheep (NWS)] during the luteal phase, as an optimal environment at the luteal phase could better prepare the cervix for sperm migration through the cervix at the subsequent follicular phase. High-quality RNA extracted from postmortem cervical tissue was analyzed by RNA sequencing. After stringent filtering, 1051, 1924, and 611 differentially expressed genes (DEGs) were detected in the low-fertility Suffolk breed compared with Belclare, Fur, and NWS, respectively. Gene ontology analysis identified increased humoral adaptive immune response pathways in Suffolk. Increased expression of multiple immune genes supports the presence of an active immune response in the cervix of Suffolk ewes, which differentiates them significantly from the other three ewe breeds. Inflammatory pathways were upregulated in the Suffolk, resulting in higher expression of the potent pro-inflammatory cytokines. Therefore, higher levels of pro-inflammatory cytokines indicate unresolved inflammation in the cervix of the low-fertility Suffolk breed that could contribute to reduced cervical sperm transport in the next follicular phase.

Summary Sentence
Increased humoral adaptive immune response in the low-fertility Suffolk breed at the luteal phase indicates unresolved inflammation in the cervix of Suffolk that may contribute to reduced cervical sperm transport in the subsequent follicular phase.
Introduction

Cervical artificial insemination (AI) with frozen–thawed semen is not routinely performed in the sheep industry since it usually yields pregnancy rates of less than 30% [1, 2]. The only exception to this is in Norway, where farmers themselves vaginally inseminate their ewes (shot-in-the-dark) using frozen–thawed semen to a natural estrous and routinely achieve pregnancy rates in excess of 60% [3, 4]. Data from our group have demonstrated that these differences in pregnancy rates following cervical AI using frozen–thawed semen are due to the breed of the ewe used in Norway and specifically differences in cervical sperm transport between ewe breeds [5]. A more recent study by our group of six European ewe breeds has shown no relationship between gross cervical anatomy, mucus properties and previously reported pregnancy rates following cervical AI with frozen–thawed semen are due to the breed of the ewe used in Norway and specifically differences in cervical sperm transport between ewe breeds [6]. However, we have identified extensive differences in the cervical gene expression between high- and low-fertility ewe breeds at the follicular phase of both a natural [7] and a synchronized [8] estrous cycle. It has been reported that cervical gene expression fluctuates between the phases of the estrous cycle [9, 10]. Therefore, it is likely that an appropriate environment at the luteal phase could potentially support an optimal and receptive cervix for sperm in the next follicular phase.

The cervix plays a dynamic role in fertility, which is regulated by endogenous hormones (mainly estrogen and progesterone) across the follicular and luteal phases of the estrous cycle. At the follicular phase, there is an increase in mucus production [11, 12] to allow sperm with normal morphology and motility to traverse the cervix [13]. At the luteal phase, mucus production and hydration decrease [14] resulting in more viscous and cloudier mucus than that at the follicular phase [6], thereby providing an effective barrier against pathogens [11, 15]. These cyclic changes in mucus properties are also accompanied by changes in cervical gene expression. At the luteal phase, there is a reduction in the expression of genes involved in mucin synthesis in bovine [16] and human [17] cervical tissue. A recent study by Mukhopadhyay et al. [18] revealed an enrichment of genes in the immune response pathway during the secretory phase of the menstrual cycle.

Proteomic analyses of ovine cervical mucus further revealed differences in proteomic composition and concentration across the phases of the estrous cycle, identifying higher levels of protein in luteal mucus samples [19]. Proteins associated with the immune system were also increased at the luteal phase [20, 21] including ceruloplasmin (an acute phase reactant), lactoferrin (bacteriostatic effect), and CD9 antigen (tetraspanin), which emphasize the immunological role of the cervical mucus preventing ascending pathogens during the luteal phase. Interestingly, Maddison et al. [22] showed an absence of the neuraminidase protein (NEU1) during the luteal but not in follicular phase mucus samples. This enzyme cleaves the sialic acid terminals from glycans and thus modifies the distension and flexibility of the mucin protein and decreasing mucus viscosity [23]. Therefore, the lack of this enzyme supports increased mucus viscosity as an effective barrier against pathogens at the luteal phase of the estrous cycle. Relevantly, we have previously reported higher levels of sialic acid in the cervical epithelium [24] and sialylated glycans in the cervical mucus [25] of the low-fertility Suffolk breed.

Despite these limited observations, it is plausible that differences in gene expression during the luteal phase could change the protection toward pathogenic species contributing to altered cervical health in the subsequent follicular phase. Altered cervical gene expression at the luteal phase can also have an impact on the reestablishment of ovarian homeostasis after a recent inflammatory event (ovulation) and on the
establishment of pregnancy. Given the importance of the luteal phase, we hypothesized that there is an intricate interplay between the cervical gene expression patterns at the luteal phase and sperm transport during the next follicular phase may contribute to differences in pregnancy rates. Therefore, the objective of the this study was to characterize for the first time the differential gene expression profiles in cervical tissue collected postmortem at the luteal phase from two Norwegian and two Irish ewe breeds with known differences in pregnancy rates following cervical AI using frozen–thawed semen.

Material and methods

Ethical approval

Protocols were developed in accordance with the Cruelty to Animals Act (Ireland 1876, as amended by European Communities regulations 2002 and 2005) and the European Community Directive 86/609/EC. In Norway, the study was approved by Norwegian Food Safety Authority (FOTS ID 13168). In Ireland, all animal procedures were conducted under experimental license from the Health Products Regulatory Authority (AE19132/P065) and the study was approved by the Teagasc Animal Ethics Committee (TAEC145/2017).

Experimental design and tissue collection

The animal model used in this study has previously been described by Abril-Parreño et al. [6]. In this study, we interrogated the gene expression of the sheep cervix of four ewe breeds across two countries: Ireland (Suffolk and Belclare; low and medium fertility, respectively) and Norway (NWS and Fur; both with high fertility compared with the Irish ewe breeds) at the luteal phase. We used these ewe breeds due to their known different pregnancy rates following cervical/vaginal AI with frozen–thawed semen. Suffolk ewes were the reference level in this analysis since they have the lowest pregnancy rates. Ewes were synchronized using intravaginal progestogen vaginal sponges (20 mg Flugestone Acetate; Chronogest® vaginal sponges, Intervet, The Netherlands) inserted on a random day of the cycle. After 14 days, the sponges were removed and ewes were treated with equine chorionic gonadotropin (400 IU; Intervet, Boxmeer, The Netherlands) inserted on day 10 of the synchronized cycle (n = 8–11 ewes per breed). Following euthanasia, the ovaries were assessed for the presence of an active corpus luteum (luteal phase). The reproductive tracts were then longitudinally opened and two sections were taken from the mid region of the cervix while avoiding folds. All samples were snap-frozen in liquid nitrogen, and subsequently stored at −80°C until RNA isolation. Another cervical tissue biopsy was taken and immersed in formalin to perform immunohistochemical staining.

Tissue processing and RNA extraction

As previously described [7], frozen cervical tissue immersed in TRIzol reagent was homogenized using the homogenizer (Biogen Pro200 Homogenizer, Pro Scientific) in order to lyse the tissue. The RNA extraction was completed using the RNeasy Kit (Qiagen Ltd., Crawley, West Sussex, UK) according to the manufacturer’s instructions. Total RNA concentration was quantified using the Nanodrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Quality of RNA was ascertained with the use of 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA integrity number was greater than 7 in all samples and RNA aliquots were frozen after extraction.

Library preparation and RNA sequencing

RNA libraries were prepared with Illumina® TruSeq® Stranded mRNA library preparation kit to convert mRNA into cDNA libraries for DNA sequencing. Indexes were allocated to specific samples prior to library construction so that each sample within a pool had a unique bar code. Following adapter ligation, DNA fragments were selectively enriched by performing PCR. Quality control checks were performed to assess the quality and quantity of the cDNA libraries. The Agilent 2100 Bioanalyzer (Agilent Technologies) was used to assess purity of the samples, using the Agilent DNA 1000 kit. Library quantity was measured using the Qubit fluorometer. These steps were previously reported by Brewer et al. [26]. All libraries were sequenced using an Illumina NovaSeq sequencer by Macrogen, Inc. (Seoul, Republic of Korea). Sequencing was performed for each sample at 2 × 150 bp paired end reads (50 M reads) as previously described by Abril-Parreño et al. [7].

Differential expression analysis

Quality assessment of the raw sequence data was carried out using the software FastQC (v 0.11.8; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Data were quality and adapter trimmed using the BBduk java package to trim Illumina adapter sequences and any low-quality bases (Phred score < 20) from the 3′ end of sequence read pairs. Reads were aligned to the ovine genome Oar_v3.1 using the Spliced Transcripts Alignment to a Reference (STAR) aligner. A maximum of two mismatches with the reference genome were allowed and only uniquely mapped read pairs were retained for downstream analysis. Read counts overlapping all protein coding genes in the Oar_v3.1 Ensembl (v.95) annotation were estimated using featureCounts. To filter out lowly expressed genes, genes with less than one count per million in at least 10 samples were discarded from the analysis. Remaining gene counts were normalized using the median of ratios method as implemented in DESeq2 (version 1.130.0) [27] to account for varying sequencing depth between samples. Transcript counts were modeled by fitting the data to a negative binomial distribution using genewise dispersion estimates and differentially expressed genes (DEGs) were identified with a generalized linear model likelihood ratio test. Statistical tests were corrected for multiple testing using the Benjamini–Hochberg method. DEGs with an adjusted P < 0.05 and a log2 FC threshold of 1.5 were used for further DEG data exploration and pathway analysis.

Functional and pathway enrichment analysis

We used the gProfiler2 (v.0.2.0) package to identify aggregated functional profiles of genes and gene clusters in the DEG lists. In this analysis, GO terms and Reactome pathways were analyzed with an enrichment threshold cut-off of adjusted P < 0.05. We also used the R package rrvgo (v.1.1.4) to reduce the redundancy of significantly enriched GO terms by grouping similar terms based on their similarity within the GO hierarchy. Gene co-expression network analyses were carried out using the R package Cemttools (v1.14.0). For any modules identified, a gene set enrichment analysis was carried...
out to indicate if each module was induced or repressed in the different ewe breeds. Finally, an over representation analysis was implemented to identify enriched biological functions in each module.

Immunohistochemistry preparation

Immunohistochemistry staining for TGFBR3 was performed using HRP One-Step Polymer anti-Mouse/Rabbit/Rat from Nordic BioSite, Taby, Sweden (cat. no. KDB-SS0BD3-6) on cervical tissue from a subset of ewes from the NWS, Fur, and Suffolk breeds only as these had the most divergent TGFBR3 gene expression. TGFBR3 was chosen because it is a widely expressed membrane-anchored proteoglycan and it plays a central role in the TGFβ signaling pathway, which is implicated in regulating the female immune response. Formalin-fixed cervical tissues were paraffin-embedded, cut into 4 μm sections, rehydrated in graded ethanol, and demasked in a microwave oven 15 min 121°C in 0.01 M citrate buffer (pH 6.0). Nonspecific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 10 min. Blocking was done by incubating the sections with 2% normal goat serum for 20 min at room temperature, before adding 1:400 dilution of TGFBR3 antibody (cat. no. OASG07150, Aviva Systems Biology, San Diego, CA, USA) and incubated 45 min at room temperature. After washing, one drop of HRP One-Step Polymer solution was added to each slide and incubated for 30 min. The immunoreaction was visualized using the chromagen 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, MO, USA) and incubated for 30 min. The immunoreaction was visualized using the chromagen 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, MO, USA) and contrast staining with Mayer's hematoxylin. Staining in the absence of the primary antibody was used as a negative control.

Results

Principal component plot analysis showed the highest separation between the high-fertility Fur and the low-fertility Suffolk

Individual animals of all four ewe breeds were clustered using principal component analysis (PCA; Supplemental Figure S1), which showed the distribution of samples within ewe breed and between ewe breeds (Figure 1). Results showed clear segregation between the two Norwegian ewe breeds compared with the low-fertility Suffolk breed, with Fur being the most divergent.

Differential gene expression analysis identified increased expression of immune genes involved in immune system in the low-fertility Suffolk breed

Using stringent statistical filtering criteria (adjusted $P < 0.05$ and FC > 1.5), RNA sequencing detected 1051 DEGs (580 downregulated and 471 upregulated genes), 1924 (1032 downregulated and 892 upregulated), and 611 (261 downregulated and 350 upregulated) in Belclare, Fur, and NWS, respectively, compared with the Suffolk breed (Figure 2). Fur had the highest difference in terms of number of DEGs compared with Suffolk, which is the reference level due to the lowest reported pregnancy rates for this breed, following cervical AI using frozen–thawed semen. Volcano plots show the log2 fold-change of the top 20 DEGs at the luteal phase in Belclare, Fur, and NWS compared with Suffolk ewes (Figure 3).

The top 5 downregulated DEGs in Belclare compared with Suffolk are shown in Supplemental Table S1. These include TAC3 (Tachykinin Precursor 3), COL9A2 (Collagen Type IX Alpha 2 Chain), GFRA2 (GDNF Family Receptor Alpha 2), and PKIA (CAMP-Dependent Protein Kinase Inhibitor Alpha). The top 5 upregulated DEGs in Belclare compared with Suffolk included MUC3A (Mucin 3A), BPIF1B (BPI Fold Containing Family B Member 1), and SLC27A2 (Solute Carrier Family 27 Member 2). A total of 1924 DEGs were detected in Fur compared with Suffolk, from which the top 5 downregulated DEGs included COX1 (Mitochondrially Encoded Cytochrome C Oxidase 1), OXTR (Oxytocin Receptor), TAC3 (Tachykinin Precursor 3), and SLC6A14 (Solute Carrier Family 6 Member 14 gene). The top 5 upregulated DEGs included R-Spondin 3 gene (RSPO3), Purkinje Cell Protein 4 (PCP4), MAM Domain Containing 2 (MAMDC2), Alpha-2-Macroglobulin (A2M), and Thrombospondin 4 (THBS4; Supplemental Table S2).

The top 5 DEGs downregulated in NWS compared with Suffolk included the Solute Carrier Family 6 Member 14 gene (SLC6A14), the Forkhead Box C1 (FOXCl), orthologue of the HLA class II histocompatibility antigen, DQ alpha (DQA), and the Oxytocin Receptor (OXTR; Supplemental Table S3). The top 5 upregulated DEGs included R-Spondin 3 gene (RSPO3), Nuclear Receptor Subfamily 4 Group A Member 1 (NR4A1), GTP Binding Protein Overexpressed in Skeletal Muscle (GEM), Alpha-2-Macroglobulin (A2M), and Potassium Voltage-Gated Channel Modifier Subfamily G Member 1 (KCNG1). The mapping information and the full lists of the DEGs for all the three comparisons can be found in Supplemental Tables S4–S7.

Gene ontology analysis identified increased humoral adaptive immune response pathways in the low-fertility Suffolk compared with both Norwegian ewe breeds

Comparing Belclare to Suffolk, only the circulatory system biological process was downregulated (Table 1). For DEGs that had differential expression between Fur and Suffolk ewes, adaptive immune response and cell activation were significantly enriched (Table 1), while there was an upregulation of the muscle contraction and structure development pathways in Fur compared with Suffolk. All the enriched pathways involved in biological processes are listed in Supplemental Tables S8 and S9.

Pathway analysis revealed enriched pathways in NWS compared with Suffolk, the majority of these were biological processes, such as humoral adaptive immune response that were downregulated, while muscle contraction was upregulated in NWS compared with Suffolk (Table 1). All the enriched pathways involved in biological processes are listed in Supplemental Tables S10 and S11.

A more active immune response in the cervix of the low-fertility Suffolk breed

Enhanced expression of genes in multiple functional classes were apparent in the Suffolk relative to the Belclare including cell-surface receptors (including the gene encoding NOD1), cytokines, chemokines, acute phase proteins and genes involved in the antimicrobial response. Genes encoding CD markers such as CD200 (Cluster of Differentiation 200), a
transmembrane glycoprotein, CD244—a signaling Lymphocyte Activation Molecule (SLAM) family immunoregulatory receptor, CD72—a transmembrane protein of the C-type lectin family, CD93—a C-type lectin transmembrane receptor, and CD96—a member of the Ig family were all increased in expression in the cervix of the Suffolk compared with the Belclare. In addition, two chains of the CD8 T-cell receptor (CD8A and CD8B) were also significantly differentially expressed. Inflammatory cytokines including IL1A, IL33, and IL34 as well as the IL1R2 and members of the NFKB (Nuclear factor κB) family were increased in Suffolk. The acute phase protein gene APOE, IGF1, and genes of the nitric oxide synthase family (NOS2 and NOS3), which encode the expression of inflammatory free radicals, were also upregulated in Suffolk.

The inflammatory signal was further apparent when comparing the lists of differentially expressed immune genes in Suffolk relative to Fur ewes. For example, multiple pathogen recognition receptor genes were higher expressed in Suffolk including TLR7, NOD1, CARD11 (Caspase Recruitment Domain Family Member 11), a total of 24 genes encoding different CD receptors including CD14 receptor known to serve as a co-receptor for several TLRs on innate cells as well as CD79A and CD79B, receptors expressed primarily on B cells. Multiple complement protein-encoding genes (C3, C4, C5, and C9) and chemokines (CCL5, CCL20, CCL28, and CXCL16), and chemokine receptors were increased in Suffolk. Expression of IL1A, MYD88, IGF2, TGFB1, Major Histocompatibility Complex–associated genes, and multiple cytokine receptors were also upregulated in Suffolk. However, transforming growth factor beta receptor type 3 (TGFBR3) was downregulated in Suffolk compared with NWS and Fur, and this trend was also clear when assessed using immunohistochemical TGFBR3 staining (Figure 4). Acute phase proteins APOE, SAA1, and LPO as well as antimicrobial protein-encoding genes S100A8, S100A9, and S100A12 were also increased relative to Fur. Very few immune genes were decreased in expression in the Suffolk relative to other breeds, apart from CD55, IL17B, and IL17RB.

**Gene co-expression analysis across all four ewe breeds**

Co-expression analysis identified five modules, from which module 4 revealed different co-expression patterns between Irish (Suffolk and Belclare) and Norwegian ewe breeds (Fur and NWS; Figure 5). Module 4 contained enriched pathways related to the immune system such as humoral immune response, cell surface receptors, activation of complement pathway, adaptive immune response, and phagocytosis ($P < 0.05$; Figure 5). This module 4 had lower expression in Fur and NWS and higher expression in both Irish breeds ($P < 0.05$). A total of 66 genes were in this
module, from which the top 5 of main regulators genes were AL590764.2 (FNDC3A) encoding a Fibronectin Type-III Domain-Containing Protein, EVI2B (Ectropic Viral Integration Site 2B), CD53 (a member of tetraspanin family), FERMT3 (Fermitin Family Member 3), and ARHGAP45 (Rho GTPase Activating Protein 45).

Discussion

At the luteal phase of the estrous cycle, the main function of the cervix is to protect the upper female reproductive tract against ascending pathogens from the vagina. Cyclic changes in hormonal profiles, mainly estrogen and progesterone, modulate the defense response of the cervix at each phase of the estrous cycle [28]. Here, we assessed for the first time the luteal phase cervical transcriptome of four ewe breeds with divergent cervical frozen–thawed sperm transport to elucidate the optimal environment at the luteal phase that prepares the cervix for sperm around the time of ovulation. The main finding of this study was the presence of an unresolved inflammation in the cervix of Suffolk ewes, which could contribute to reduced cervical sperm transport in the next follicular phase.

In contrast to our earlier findings where only minor differences in gene expression were detected between the Irish ewe breeds (Suffolk and Belclare) compared with both Norwegian ewe breeds during the follicular phase of a natural [7] and synchronized estrous cycle [8], larger numbers of DEGs were expressed at the luteal phase. Although the FC in gene expression was not of a high magnitude for the majority of these genes, there was remarkable consistency in terms of the functional classes and genes differentially expressed, thereby adding important biological insights into mechanisms that may inhibit frozen–thawed sperm transport in the cervix of the Suffolk at the follicular phase.

The biological pathway for muscle contraction was upregulated in Fur and NWS compared with Suffolk. The role of the contractions of the cervix in fertility is still not fully understood; however, it has been reported that the presence of Chlamydia reduced smooth muscle contractions in the cervix and uterus [29]. This could be indicative of a suboptimal microbial environment in the cervix of the low-fertility Suffolk breed. Levels of COX1 were also higher in Suffolk than in Fur (but not in the other ewe breeds) as we reported previously at the follicular phase of a natural [7] and a synchronized [8] cycle. Although its role in the cervix is unknown, high levels of COX1 contribute to gastric mucosal defense [30]. This supports the active immune response showed in the cervix of the Suffolk irrespective of the phase or type of estrous cycle.

The active and increased expression of multiple genes encoding transmembrane receptors suggests differential immune responses to either pathogen-associated molecular patterns or perhaps damage-associated molecular patterns in...
Table 1. The top 5 biological processes enriched pathways down- and upregulated in Belclare (A), Fur (B), and NWS (C) compared with the low-fertility Suffolk at the luteal phase of their estrous cycle

| Term name | Term ID | P-value |
|-----------|---------|---------|
| **A** Term name | Term ID | P-value |
| Multicellular organismal process | GO:0032501 | <0.001 |
| Circulatory system development | GO:0072359 | <0.001 |
| Blood vessel development | GO:0001568 | <0.001 |
| System development | GO:0006936 | <0.001 |
| Regulation of multicellular organismal process | GO:0051239 | <0.001 |
| **B** Term name | Term ID | P-value |
| Immune response | GO:0006955 | <0.001 |
| Immune system process | GO:0002376 | <0.001 |
| Cell activation | GO:0001775 | <0.001 |
| Regulation of immune system process | GO:0002682 | <0.001 |
| Adaptive immune response | GO:0002250 | <0.001 |
| **C** Term name | Term ID | P-value |
| Adaptive immune response | GO:0002250 | <0.001 |
| Immune system process | GO:0002376 | <0.001 |
| Production of molecular mediator of immune response | GO:0002440 | <0.001 |
| Immune response | GO:0006955 | <0.001 |
| Humoral immune response | GO:0006959 | <0.001 |
| Upregulated in NWS compared with Suffolk | GO:0006936 | <0.001 |
| **Figure 4.** Representative images of TGBFR3 expression (stained brown) in cervical tissue from NWS, Fur, and Suffolk at the luteal phase of their cycle using immunohistochemical TGBFR3 staining (magnification: 20×). A representative image of the negative control (without adding TGBFR3 antibody) is also shown. Suffolk had a noticeable lighter TGBFR3 staining of the cervical epithelium than Fur and NWS, in line with the gene expression data.**

TGFBR3 protein was localized in the epithelial cells of the cervical crypts using immunohistochemical TGBFR3 staining. The expression of the TGFBR3 gene was lower in Suffolk compared with NWS and Fur ewes and this was backed up by immunohistochemical staining. TGFBR3, also known as betaglycan, is a co-receptor for the canonical TGFβ signaling...
pathway [37]. Sharkey et al. [38] demonstrated that human ectocervical cells downregulate expression of all three TGFβ receptors, TGFβR1, TGFβR2, and TGFβR3, after exposure to seminal plasma, and at least for TGFβR3, TGFβ contributes to this response. TGFβ is a key signaling agent involved in the induction of cytokines that control cervical tissue immune responses to seminal fluid antigens and influence the quality of the response to skew the balance toward a tolerogenic response. Therefore, lower expression of TGFβR3 in the low-fertility Suffolk breed and thus less binding with its ligands could result in higher female immune response against sperm.

Chemokines secreted by the cervical tissue were differentially expressed in Suffolk compared with Fur ewes. CC-chemokine ligand 28 (CCL28) attracts neutrophils and monocytes to the sites where the immune response needs to be strengthened [39]. However, an excessive response can also initiate the formation of neutrophil extracellular traps [40], and thus reducing the number of sperm to get across the cervix at the next follicular phase in the low-fertility Suffolk breed. In addition, we identified higher levels of IL1A in Suffolk compared with Belclare ewes. It has been shown that IL-1 is a potent pro-inflammatory cytokines, previously associated with unresolved inflammation and tissue damage [41]. IL1 was also found in cervicovaginal mucus from cows, showing persistent high levels of IL1 in cows with clinical endometritis [42]. IL33 is a member of the IL1 family of cytokines, which increases the production of other pro-inflammatory cytokines such as IL5, IL13, and the Granulocyte-macrophage colony-stimulating factor [43]. In this study, we identified higher levels of IL33 in Suffolk compared with Belclare ewes that could support higher levels of inflammation in the cervix of the low-fertility Suffolk breed. According to this, high levels of IL33 were found in uterine endometrial tissue from low-fertility heifers thus linking inflammation induced by the effect of pro-inflammatory cytokines to low pregnancy outcomes [44]. Chronic inflammatory conditions of the endometrium such as endometriosis have also been related with high levels of IL33 in women [45]. Therefore, high levels of the IL1 family members in the Suffolk could indicate unresolved inflammation, which could damage sperm. In agreement with this, it has been demonstrated that altered levels of IL1 [46] and other pro-inflammatory cytokines [47] affect sperm membrane lipid per-oxidation and increase reactive oxygen species production, thereby, causing increased level of sperm DNA damage as well as decreased sperm motility [48], which could impede sperm transport across the cervix.

The activation of complement genes and antimicrobial peptides all point toward an environment, which could be potentially hostile to sperm transit. Interestingly, despite the weight of evidence supporting the presence of an inflammatory environment in the cervix, IL17B was consistently significantly increased in expression in the NWS (highest fertility). Although our understanding of the role of this cytokine is limited [49], there are some suggestions that this family member has a regulatory role in inflammation [50]. Elevated IL17 in the vaginal mucosa has been proposed to increase immune protection against infections [51], and...
therefore may indicate a conserved mechanism for improved bacterial clearance in sheep.

In conclusion, this is the first study that provides data on the luteal transcriptome of the cervix from high- and low-fertility ewe breeds. Our results revealed the presence of an active immune response in the cervix of the low-fertility Suffolk breed, which differentiates them significantly from the other three ewe breeds with higher fertility. This led us to conclude that the cervix of the Suffolk may be less responsive to the immune suppressive effect of progesterone. Higher expression of pro-inflammatory cytokines in the cervix of the Suffolk suggests that the presence of an inflammatory environment may explain, in part, the low pregnancy outcomes in this breed. However, more studies are needed to elucidate the optimal environment at the luteal phase that prepares the cervix for sperm transport around the time of ovulation.

**Authors’ contributions**

S.F., A.K.K., and X.D. conceived and designed the experiments, secured funding, and oversaw the work. L.A.-P. and A.K.K. collected the postmortem samples from the ewes. L.A.P. and K.G.M. interpreted the data and contributed to manuscript preparation. L.A.P. performed the RNA extraction and drafted the manuscript. P.C. performed the statistical and bioinformatics analysis. All authors proofread the final manuscript.

**Acknowledgments**

The authors would like to acknowledge the help with the sampling of all the technical staff at Teagasc Research Centre, Athenry, Ireland. In Norway, the personnel working at the Section for Small ruminant research and herd health, NMBU—Faculty of Veterinary Medicine, Sandnes, Norway. The authors would also thank Gunn Charlotte Østby at the NMBU for her work on the immunohistochemistry preparation.

**Conflict of interest**

The authors declare that they have no competing interests.

**Data availability**

The datasets generated and/or analyzed during the current study are available in the NCBI Gene Expression Omnibus https://www.ncbi.nlm.nih.gov/geo/ under accession number GSE179486.

**References**

1. Donovan A, Hanrahan JP, Kummen E, Duffy P, Boland MP. Fertility in the ewe following cervical insemination with fresh or frozen-thawed semen at a natural or synchronised oestrus. *Anim Reprod Sci* 2004; 84:359–368.

2. Maxwell WMC. Artificial insemination of ewes with frozen-thawed semen at a synchronised oestrus. 1. Effect of time of onset of oestrus, ovulation and insemination on fertility. *Anim Reprod Sci* 1986; 10:301–308.

3. Paulenz H, Adnøy T, Soderquist L. Comparison of fertility results after vaginal insemination using different thawing procedures and packages for frozen ram semen. *Acta Vet Scand* 2007; 49:26–26.

4. Paulenz H, Soderquist L, Adnøy T, Nordtoga AB, Andersen BK. Effect of vaginal and cervical deposition of semen on the fertility of sheep inseminated with frozen-thawed semen. *Veterinary Record* 2005; 156:372–375.

5. Fair S, Hanrahan JP, O’Meara CM, Duffy P, Rizos D, Wade M, Donovan A, Boland MP, Lonergan P, Evans AC. Differences between Belclare and Suffolk ewes in fertilization rate, embryo quality and accessory sperm number after cervical or laparoscopic artificial insemination. *Theriogenology* 2003; 63:1993–2005.

6. Abril-Parreño L, Krogenæs AK, Byrne CJ, Donovan A, Stuen S, Caldas E, Diskin M, Drau®t X, Fair S. Ewe breed differences in cervical anatomy and cervicovaginal mucus properties: an international study. *Theriogenology* 2021; 160:18–25.

7. Abril-Parreño L, Meade KG, Krogenæs AK, Drau®t X, Fair S, Cormican P. Conserved and breed-specific differences in the cervical transcriptome of sheep with divergent fertility at the follicular phase of a natural oestrus cycle. *BMC Genomics* 2021; 22:752.

8. Abril-Parreño L, Meade KG, Krogenæs AK, Drau®t X, Cormican P, Fair S. Ewe breed differences in the cervical transcriptome at the follicular phase of a synchronised oestrus cycle. *BMC Genomics* 2022; 23:363.

9. Thurman AR, Chandra N, Yousefieh N, Zalenskaya I, Kimble T, Asin S, Rollenhagen C, Anderson SM, Herold B, Mesquita PM, Richardson-Harman N, Cunningham T et al. Comparison of follicular and luteal phase mucosal markers of HIV susceptibility in healthy women. *AIDS Res Hum Retroviruses* 2016; 32:547–560.

10. Yildiz-Arslan S, Coon JS, Hope TJ, Kim J. Transcriptional profiling of human endocervical tissues reveals distinct gene expression in the follicular and luteal phases of the menstrual Cycle. *Biol Reprod* 2016; 94:1–13.

11. Bigelow JL, Dunson DB, Stanford JB, Ecochard R, Gnoth C, Colombo B. Mucus observations in the fertile window: a better predictor of conception than timing of intercourse. *Hum Reprod* 2004; 19:889–892.

12. Gipson IK. Mucins of the human endocervix. *Front Biosci* 2001; 6:D1245–D1255.

13. Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. *Hum Reprod Update* 2006; 12:23–37.

14. Katz DF, Slade DA, Nakajima ST. Analysis of pre-ovulatory changes in cervical mucus hydration and sperm penetrability. *Adv Contracept* 1997; 13:143–151.

15. Evans G, WMC M, Salamon S. Salamon’s Artificial Insemination of Sheep and Goats / Gareth Evans. Sydney: Butterworths; 1987.

16. Pluta K, McGgettigan PA, Reid CJ, Browne JA, Irwin JA, Tharmalingam T, Corfield A, Baird A, Loftus BJ, Evans AC, Carrington SD. Molecular aspects of mucin biosynthesis and mucus formation in the bovine cervix during the periestrous period. *Physiol Genomics* 2012; 44:1165–1178.

17. Gipson IK, Moccia R, Sparr-Michaud S, Argueso P, Gargiulo AR, Hill JA 3rd, Offner GD, Keutmann HT. The amount of MUC5B mucin in cervical mucus peaks at midcycle. *J Clin Endocrinol Metab* 2001; 86:594–600.

18. Mukhopadhyay S, Liang Y, Hur H, Villegas G, Calenda G, Reis A, Millen L, Barnable P, Mankima L, Kumar N, Kalir T, Spoerl R et al. Comparative transcriptome analysis of the human endocervix and ectocervix during the proliferative and secretory phases of the menstrual cycle. *Sci Rep* 2019; 9:13494.

19. Maddison JW, Rickard JP, Mooney E, Bernevic NC, Soleihavoup C, Tsikis G, Drau®t X, Leahy T, de Graaf SP, Gerard N, Druart X. Proteomic characterization of the qualitative and quantitative changes in cervical mucus composition during the menstrual cycle. *Anim Reprod Sci* 2016; 172:114–122.

20. Grande G, Milardi D, Vincenzi F, Pompa G, Biscione A, Astorri AL, Fruscella E, De Luca A, Messana I, Castagnola M, Marana R. Proteomic characterization of the qualitative and quantitative differences in cervical mucus composition during the menstrual cycle. *Mol Biosyst* 2015; 11:1717–1725.

21. Soleihavoup C, Rieu G, Tsikis G, Labas V, Harichaux G, Kohneke P, Reynaud K, de Graaf SP, Gerard N, Drau®t X. Proteomes of the female genital tract during the oestrous cycle. *Mol Cell Proteomics* 2016; 15:93–108.

22. Maddison JW, Rickard JP, Bernevic NC, Tsikis G, Soleihavoup C, Labas V, Combes-Soia L, Harichaux G, Drau®t X, Leahy T, de
Immune response gene expression in the cervix, 2022, Vol. 102, No. 4

Graaf SP. Oestrus synchronisation and superovulation alter the cervicovaginal mucus proteome of the ewe. J Proteomics 2017; 155:1–10.

23. Varki A, Schauer R. Sialic acids. In: Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME (eds.), Essentials of Glycobiology, 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009. Chapter 14.

24. Richardson L, Hanrahan JP, Tharmalingam T, Carrington SD,Lonergan P, Evans ACO, Fair S. Cervical mucus sialic acid content determines the ability of frozen-thawed ram sperm to migrate through the cervix. Reproduction 2019; 157:259–271.

25. Abril-Parreño L, Wilkinson H, Krogenæs A, Morgan J, Gallagher ME, Reid C, Drautx X, Fair S, Saldova R. Identification and characterisation of O-linked glycans in cervical mucus as biomarkers of sperm transport: a novel sheep model. Glycobiology 2022; 32: 23–35.

26. Brewer A, Cormican P, Lim JJ, Chapwanya A, O’Farrelly C,Meade KG. Qualitative and quantitative differences in endometrial inflammatory gene expression precede the development of bovine uterine disease. Sci Rep 2020; 10:18275–18275.

27. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010; 26:139–140.

28. Lee S, Kim J, Jang B, Hur S, Jung U, Kil K, Na B, Lee M, Choi Y, Jung J, Park S, Lee S, Jang B, Yi H. PBAE: a bioinformatics method for analysis of transcriptome data. Bioinformatics 2019; 35:1457–1465.

29. Lee JM, Mayall JR, Chevalier A, McCarthy H, Van Helden D, Hansbro PM, Horvat JC, Jobling P. Chlamydia muridarum infection differentially alters smooth muscle function in mouse uterine horn and cervix. Am J Physiol Endocrinol Metab 2020; 318: E981–E994.

30. Wallace JL, Devchand PR. Emerging roles for cyclooxygenase-2 in gastrointestinal mucosal defense. Br J Pharmacol 2005; 145: 275–282.

31. Hart KM, Murphy AJ, Barrett KT, Wira CR, Guyre PM, Piotl PA. Functional expression of pattern recognition receptors in tissues of the human female reproductive tract. J Reprod Immunol 2009; 80: 33–40.

32. Barton GM. Viral recognition by Toll-like receptors. Semin Immunol 2007; 19:33–40.

33. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004; 303:1526–1529.

34. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, Tedin K, Tata MK, Labigne A, Zahringer U, Coyle AJ, DiStefano PS et al. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 2003; 300: 1584–1587.

35. Welter-Stahl L, Ojcius DM, Viala J, Girardin S, Liu W, Delarbre C, Philpott D, Kelly KA, Darville T. Stimulation of the cytosolic receptor for peptidoglycan, Nod1, by infection with Chlamydia trachomatis or Chlamydia muridarum. Cell Microbiol 2006; 8: 1047–1057.

36. den Hartog JE, Morré SA, Land JA. Chlamydia trachomatis-associated tubal factor subfertility: immunogenetic aspects and serological screening. Hum Reprod Update 2006; 12:719–730.

37. Blobe GC, Schiemann WP, Pepin MC, Beauchemin M, Moustakas A, Lodish HF, O’Connor-McCourt MD. Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. J Biol Chem 2001; 276:24627–24637.

38. Sharkey DJ, Macpherson AM, Tremellen KP, Motterness DG, Gilchrist RB, Robertson SA. TGF-β mediates proinflammatory seminal fluid signaling in human cervical epithelial cells. J Immunol 2012; 189:1024–1035.

39. Bischof P, Cohen M. Endometrial factors and blastocyst implantation. Indian J Physiol Pharmacol 2010; 54:7–16.

40. Alghamdi AS, Lovassa BJ, Bird SL, Lamb GC, Rendahl AK, Taube PC, Foster DN. Species-specific interaction of seminal plasma on sperm-neutrophil binding. Anim Reprod Sci 2009; 114:331–344.

41. Islam R, Kumar H, Nandi S, Mehrotra S. Circulatory level of interleukin-1 in periparturient cows with or without postpartum reproductive diseases. Asian Pac J Reprod 2013; 2:316–320.

42. Kim H, Kang HG, Jeong JK, Hur TY, Jung YH. Inflammatory cytokine concentrations in uterine flush and serum samples from dairy cows with clinical or subclinical endometritis. Theriogenology 2014; 82:427–432.

43. Barksby HE, Lea SR, Preshaw PM, Taylor JJ. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin Exp Immunol 2007; 149:217–225.

44. Killeen AP, Morris DG, Kenny DA, Mullen MP, Diskin MG, Waters SM. Global gene expression in endometrium of high and low fertility heifers during the mid-luteal phase of the estrous cycle. BMC Genomics 2014; 15:234–234.

45. Miller JE, Monsanto SP, Ahn SH, Khalaj K, Fazleabas AT, Young SL, Lessey BA, Koti M, Tayade C. Interleukin-33 modulates inflammation in endometriosis. Sci Rep 2017; 7:17903.

46. Martínez P, Proverbio F, Camejo MI. Sperm lipid peroxidation and pro-inflammatory cytokines. Asian J Androl 2007; 9:102–107.

47. Chyra-Jach D, Kaletka Z, Dobrakowski M, Machoń-Grecka A, Kasperczyk S, Birkner E, Kasperczyk A. The associations between infertility and antioxidants, proinflammatory cytokines, and chemokines. Oxid Med Cell Longev 2018; 2018:8354747.

48. Dada R, Mahfouz RZ, Kumar R, Venkatesh S, Shamsi MB, Agarwal A, Talwar P, Sharma RK. A comprehensive work up for an asthenozoospermic man with repeated intracytoplasmic sperm injection (ICSI) failure. Andrologia 2011; 43:368–372.

49. Bie Q, Jin C, Zhang B, Dong H. IL-17B: a new area of study in the IL-17 family. Mol Immunol 2017; 90:50–56.

50. Reynolds JM, Lee YH, Shi Y, Wang X, Angkasekwinai P, Nallaparaju KC, Flaherty S, Chang SH, Watarai H, Dong C. Interleukin-17B antagonizes Interleukin-25-mediated mucosal inflammation. Immunity 2015; 42:692–703.

51. Logerot S, Figueiredo-Morgado S, Charmeteau-de-Muylder B, Sandou D, Drillet-Dangeard A-S, Bomsel M, Bourgault-Villada I, Couedel-Courteille A, Cheynier R, Rancez M. IL-7-adjuvanted vaginal vaccine elicits strong mucosal immune responses in non-human primates. Front Immunol 2021; 12:614115.