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Research Article

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

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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.

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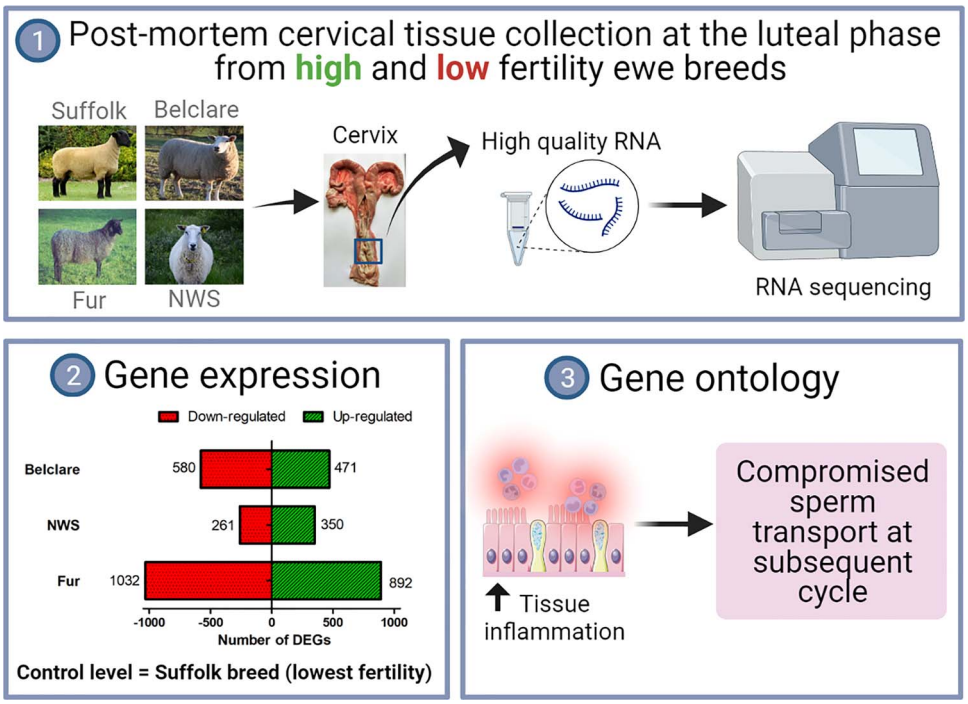
Graphical Abstract



Keywords: sheep, semen, estrous, immunity, sustainability

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 [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, Boxmeer, 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). Postmortem cervical tissue samples were collected from the four ewe breeds at the luteal phase (Day 10) of a 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 Cemitools (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, Täby, 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 *TGBFR3* gene expression. TGFBR3 was chosen because it is a widely expressed membrane-anchored proteoglycan and it plays a central role in the TGFB 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 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 breeds

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 Beclare, 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), *BPIFB1* (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 I), *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 (*FOXC1*), 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

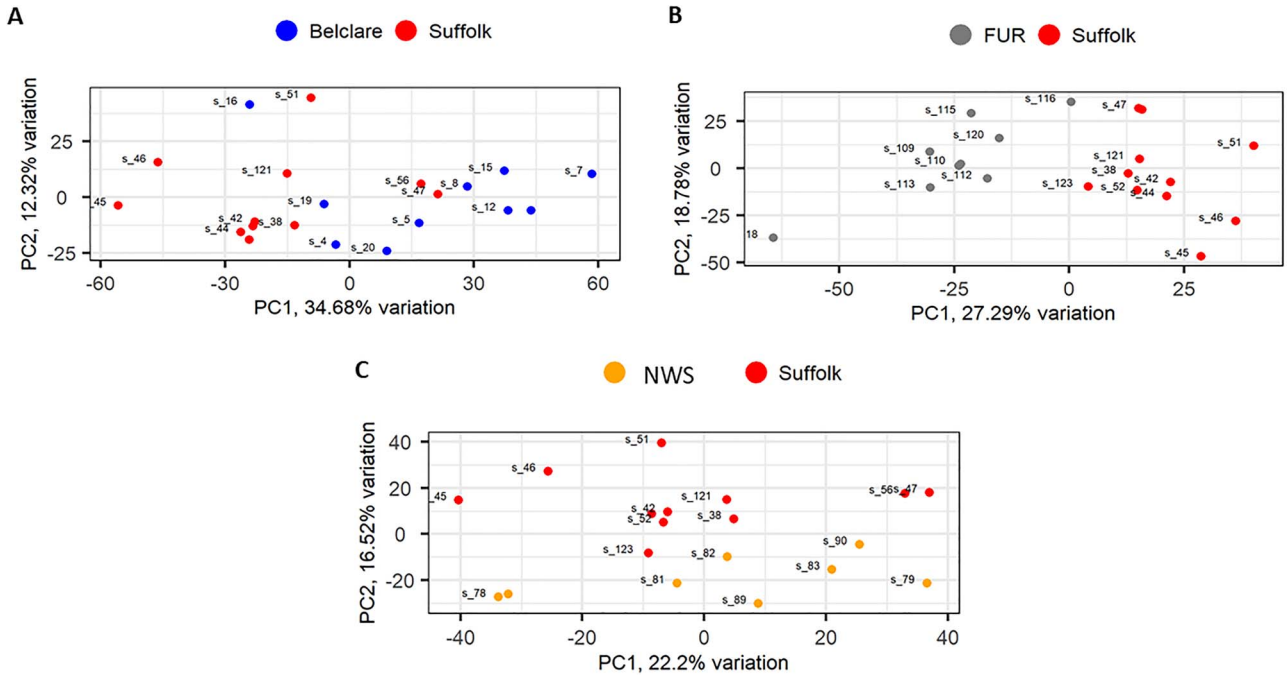


Figure 1. PCA plots show distribution of the transcriptomic profiles resulting from RNA sequencing, where colors indicate the two ewe breeds in each comparison: Belclare (A), Fur (B), and NWS (C) compared with Suffolk at the luteal phase of the estrous cycle. Adjusted $P < 0.05$ and FC > 1.5.

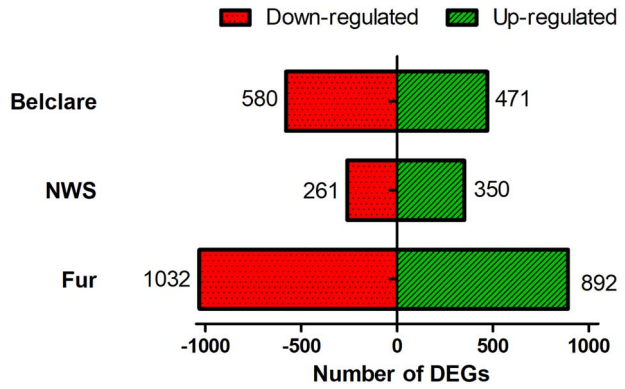


Figure 2. Histogram showing the number of DEGs (including downregulated and upregulated genes) in Belclare, NWS, and Fur compared with the low-fertility Suffolk at the luteal phase of the estrous cycle. Adjusted $P < 0.05$ and FC > 1.5.

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 *NFKβ (Nuclear factor κβ)* 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

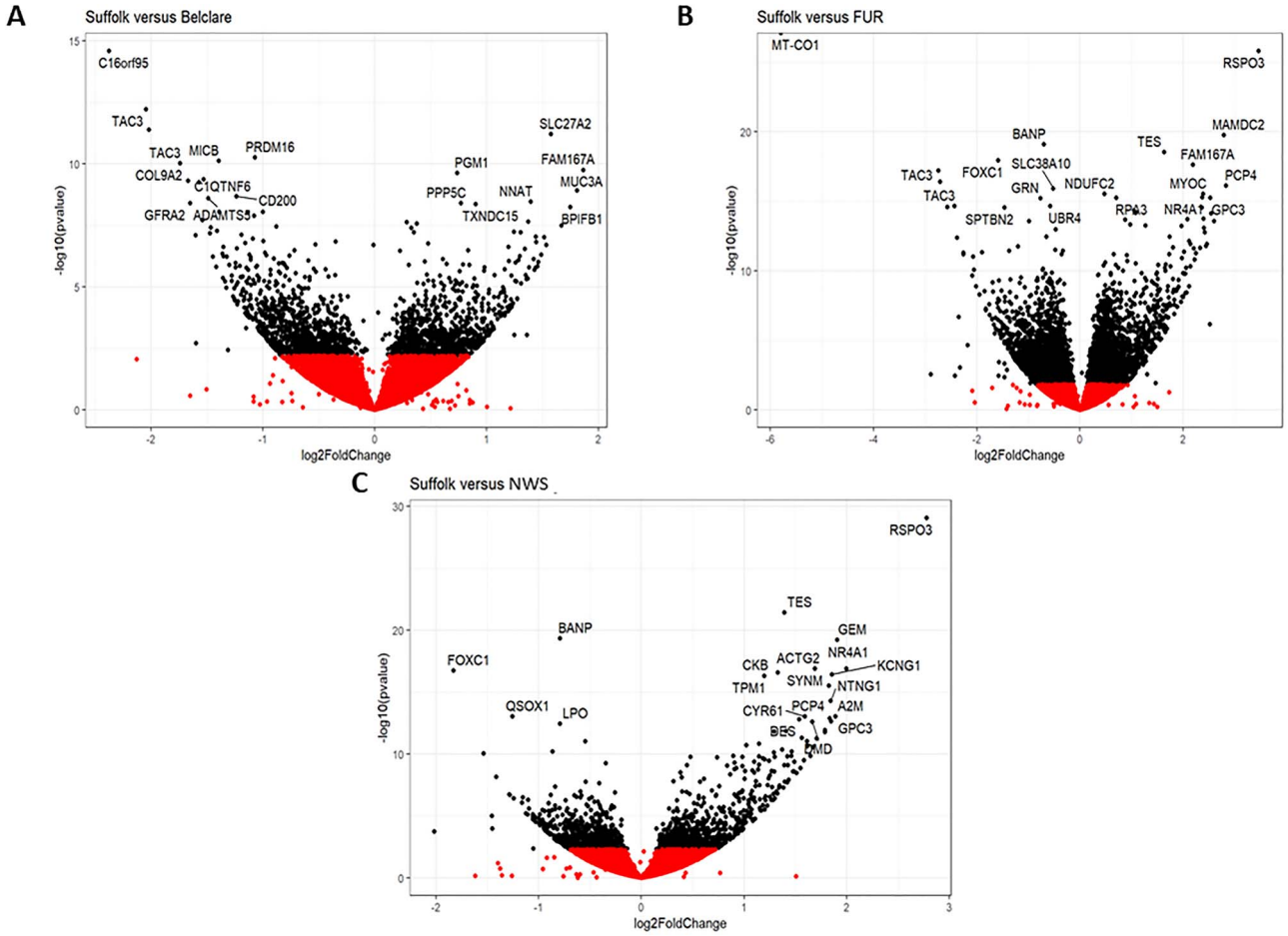


Figure 3. Gene expression data presented as volcano plots at the luteal phase of their cycle for Belclare (A), Fur (B), and NWS (C) compared with the low-fertility Suffolk using log values of the fold change and *P*-value. Each point represents a single gene, with those in black representing genes that survived the cut-off thresholds of adjusted $P < 0.05$ and FC > 1.5 and red points represent genes with a $P > 0.05$.

module, from which the top 5 of main regulators genes were *AL590764.2* (*FNDC3A*) encoding a Fibronectin Type-III Domain-Containing Protein, *EVI2B* (Ecotropic 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

A **Term name** **Down-regulated in Belclare compared with Suffolk**	**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** **Downregulated in Fur compared with Suffolk**	**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
Upregulated in Fur compared with Suffolk		
Muscle contraction	GO:0006936	<0.001
Muscle system process	GO:0003012	<0.001
Muscle structure development	GO:0061061	<0.001
Muscle organ development	GO:0007517	<0.001
System process	GO:0003008	<0.001
C **Term name** **Downregulated in NWS compared with Suffolk**	**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		
Muscle contraction	GO:0006936	<0.001
Muscle system process	GO:0003012	<0.001
System process	GO:0003008	<0.001
Muscle structure development	GO:0061061	<0.001
Muscle organ development	GO:0007517	<0.001

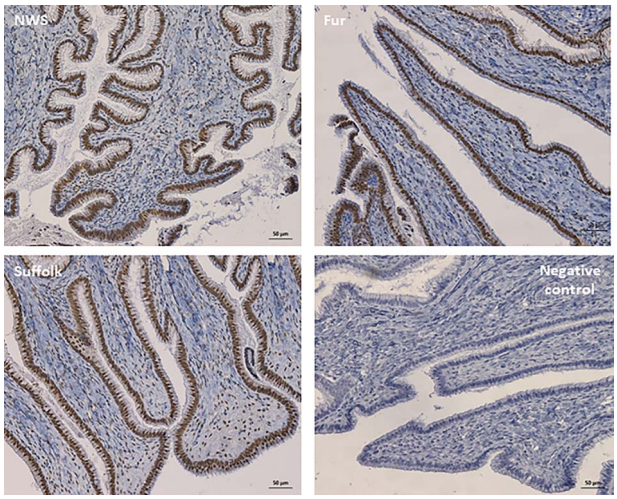


Figure 4. Representative images of TGBFR3 expression (stained brown) in cervical tissue from NWS, Fur, and Suffolk at the luteal phase of their cycle using immunohistochemical TGBFR3 staining (magnification: 20×). A representative image of the negative control (without adding TGBFR3 antibody) is also shown. Suffolk had a noticeable lighter TGBFR3 staining of the cervical epithelium than Fur and NWS, in line with the gene expression data.

the cervix of the Suffolk breed. We identified higher expression in Suffolk ewes compared with Fur of members of two key families of pattern recognition receptors, namely the *TLR7* and the *NOD1*. *TLR7* is expressed throughout the female reproductive tract [31] and recognizes viral RNA [32], including human immunodeficiency virus [33]. In addition to the *TLR7* expression, NOD1 recognizes a component of the cell membrane of Gram-negative bacteria [34]. This is supported by the activation of multiple genes encoding members of these inflammatory pathways, specifically *MYD88* and *NFKB* resulting in pro-inflammatory cytokine expression including *IL1A*. A previous study has identified that the expression of *NOD1* activates NFKB during chlamydial infections [35]. We identified higher expression of *NOD1* and *NFKB* in the low-fertility Suffolk breed, which could indicate the presence of a divergent cervical microbiome, which could contribute to subfertility as it has been reported in women [36].

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 TGFB signaling

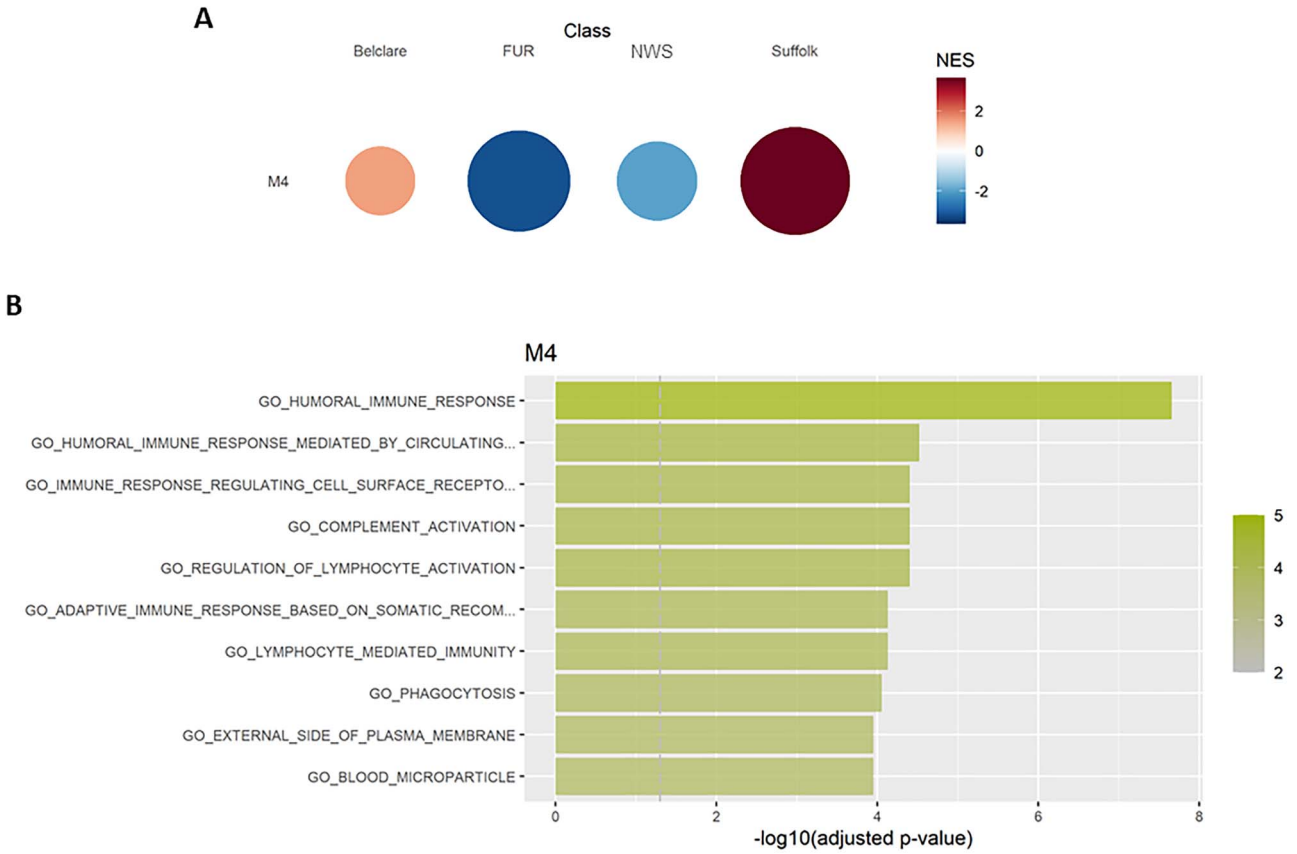


Figure 5. Gene co-expression analysis across the four ewe breeds (Suffolk, Belclare, Fur, and NWS) at the luteal phase of their cycle. (A) Gene set enrichment analysis identified module 4 to differ between Irish and Norwegian ewe breeds. (B) Over-representation analysis of genes showed the gene ontology terms in module 4. The size of the circle is proportional to its normalized enriched score (NES) value.

pathway [37]. Sharkey et al. [38] demonstrated that human ectocervical cells downregulate expression of all three TGFB receptors, TGFBR1, TGFBR2, and TGFBR3, after exposure to seminal plasma, and at least for TGFBR3, TGFB contributes to this response. TGFB 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 TGFBR3 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 and Fur 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.

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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.

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