Functional profiling of the endometrium transcriptome during preimplantation development in Finnsheep, Texel and their F1 crosses

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Carefully coordinated interaction between the endometrium and embryo is critical for the establishment and maintenance of pregnancy in mammals. By exploring the gene expression dynamics of this tissue during preimplantation development, we may be able to get insight into the genetic mechanisms of reproduction during early pregnancy. Here, we have performed comparative transcriptome profiling of the endometrium in response to spherical (Day 7 to Day 12) and elongated (Day 13 to Day 17) embryos in Finnsheep, Texel and their F1 crosses using RNA sequencing (RNA-seq) approach. A total of 21125 genes were expressed in our dataset of which 554 were significantly (absolute log2 fold change > 2.5; adjusted *p*-value < 0.01) upregulated in the endometrium with elongated embryos. Highly abundant autosomal genes in the endometrium were associated with biological processes such as facilitation of maternal recognition of pregnancy, trophoblast elongation and implantation (*LGALS15*, *CST3*, *CST6*, and *EEF1A1*). Several endogenous retroviruses (ERVs) including a novel ERV gene located in a reduced FecL locus potentially associated with sheep prolificacy were expressed in our dataset. Comparative transcriptome profiling of the endometrium having spherical and elongated embryos revealed distinct gene expression patterns. Genes that were upregulated in response to elongated embryos indicated the importance of immune system at the maternal-embryo interface prior to implantation.

Key words: endogenous retrovirus, IFNT, embryo, prostaglandins, immune processes

Introduction

Prolific breeds of sheep such as native Finnsheep are of high economic importance for global sheep industry. Prolificacy is a complex trait mainly defined by ovulation rate and litter size and it is measured by the number of live births (litters). In sheep, the first two to three weeks of pregnancy is the most critical period in determining the litter size because of high embryo mortality during that period (Quinlivan et al. 1966, Bolet 1986, Rickard et al. 2017). Therefore, the endometrium plays equally important role for the high prolificacy in sheep as the ovulation rate.

Establishment of pregnancy in sheep and other domestic ruminants requires continuous reciprocal interaction between the uterus and the conceptus (Geisert et al. 1992, Spencer et al. 2004, Spencer et al. 2007). The endometrium, the lining of the uterus, is the site of implantation. In addition, the outer lining of the endometrium secretes histotroph, a complex mixture of enzymes, growth factors, hormones, transport proteins and other substances that are key to conceptus survival and implantation, pregnancy recognition and placentation (Spencer and Bazer 2004, Forde et al. 2013). Spencer et al. (2004) describe the preimplantation development of sheep as follows: embryos enter uterus around Day 4 after insemination, hatch from zonae pellucidae between Day 8 and Day 9 and form tubular conceptus by Day 11 followed by elongation. The mononuclear cells from the trophectoderm of elongating conceptus synthesize and secrete interferon tau (*IFNT*) and prostaglandins which are essential for coordinating endometrial functions during early pregnancy (Bazer et al. 2009, Dorniak et al. 2012). In the endometrium *IFNT* acts in a paracrine manner to inhibit the expression of oxytocin receptor (*OTR*) thereby preventing luteolysis (Spencer et al. 1995, Spencer and Bazer 2002). In addition, progesterone from the ovarian *corpus luteum* (CL) also regulates the function of the endometrium and plays key role in mediating the actions of *IFNT* to signal pregnancy recognition (Ott et al. 1992, Spencer et al. 2004).

Due to the biological complexity of the underlying physiological processes and to technical difficulties in conducting experiments, embryo implantation is still not well understood (Dorniak et al. 2012, Spencer et al. 2013). Despite a large number of pregnancy-related studies in sheep, (Spencer et al. 2004, Spencer et al. 2007, Mamo et al. 2012, Bazer 2013, Raheem 2017), only a handful of experiments have applied whole transcriptome based RNA sequencing (RNA-seq) approaches to the endometrium. Recently, we compared endometrial gene expression differences between Finnsheep and European mouflon, and identified several genes associated with

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reproductive processes (Yang et al. 2019). Transcriptome analyses of pretransfer embryos and the endometrium identified several genes with potential indicator to pregnancy success in cattle (Salilew-Wondim et al. 2010). Another study reported over expression of several immune-related genes including cytokines in the bovine endometrium as a result of lipopolysaccharide (LPS) treatment (Guo et al. 2019). Brooks et al. (2016) evaluated gene expression profiles of the endometrium during early pregnancy in sheep. Here, we have used RNA sequencing (RNA-seq) approach to compile a list of genes expressed in the endometrium and compared the expression profiles of the endometrium having spherical and elongated forms of embryos during preimplantation development in sheep. We have included native Finnsheep, Texel and their F1 crosses in our study and due to inadequate biological replicates, we were unable to perform breed-wise differential expression analysis. The dataset presented in this paper is part of a larger project which aimed to understand the genetic basis of prolificacy in sheep by conducting experiments on two different time points during establishment of pregnancy: follicular growth phase and early pregnancy prior to implantation.

Materials and methods

Experimental design

Procedures for animal handling and sample collection were approved by the Southern Finland Animal Experiment Committee (approval no. ESAVI/5027/04.10.03/2012). Endometrial samples were collected from the uterine horns with a cytobrush, which was rinsed in a tube containing RNAprotect Cell Reagent (Qiagen, Valencia, CA, USA). The experimental procedures have been described in more detail in an earlier study (Pokharel et al. 2018). RNA-seq libraries of 18 ewes representing Finnsheep (n = 6), Texel (n = 6) and F1-crosses of purebreds (n = 6) were sequenced in the Illumina HiSeq 2000 system following paired-end (2 x 100 bp) strategy. Library preparation and sequencing was done in Finnish Microarray and Sequencing Center, Turku, Finland and have been described previously (Pokharel et al. 2020).

Bioinformatic analyses

The raw sequence reads were assessed for errors and the presence of adapters using FastQC v0.11.6 (https://github.com/s-andrews/FastQC). As we noticed the presence of adapters, Trim Galore v0.5.0 (https://github.com/FelixKrueger/TrimGalore) was used to remove the adapters and low-quality reads and bases. The transcripts were quantified under the quasi-mapping-based mode in Salmon v0.11.2 (Patro et al. 2017). We extracted the FASTA sequences (oar31_87.fa) of the sheep transcriptome (oar31_87.gtf) using the gffread utility (Trapnell et al. 2010) and built the transcriptome index. The resulting index was used for transcript quantification (also known as pseudo alignment) of the RNA-seq reads.

The Salmon-based transcript counts were summarized to gene level using tximport v1.12.3 (Soneson et al. 2016). DESeq2 v1.24.0 (Love et al. 2014) was used for gene expression analyses. We compared gene expression profiles of the endometrium from ewes before (Day 7 to Day 12) and after (Day 13 to Day 17) embryonic elongation phase (see also Fig. 2). All replicates were collapsed before running DESeq. Differentially expressed genes with absolute log2 fold change (abs(log2FoldChange)) > 2.5 and adjusted *p*-value (padj) < 0.01 were considered significant.

All the expressed genes were annotated with Bioconductor biomaRt v2.40.5 (Durinck et al. 2005) to retrieve additional information (gene name, gene description, and chromosome number). Gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with significantly up- and down-regulated genes were derived using Cluego v2.5.5 (Bindea et al. 2009) application in Cytoscape v3.7.2 (Shannon et al. 2003). Similar terms and pathways were grouped together using kappa score and only the terms and pathways that include at least 6 genes and 6% of genes from given GO term or KEGG pathway were taken. The enrichment analysis was performed using right-sided hypergeometric test with Bonferroni stepdown correction method. All the genes expressed in our dataset was used as a reference set for the gene enrichment analysis.

When we noticed that many genes lacked gene annotations, we manually searched for additional information of unannotated genes from the subset of top abundant (n = 25) and significantly differentially expressed (n = 40) genes. First, we extracted the coding sequence (CDS) of each novel gene using Ensembl BioMart. All genes that had CDS were BLASTed against the nonredundant (NR) nucleotide database. For the BLAST-based annotation, we chose the hit with the highest coverage and the highest percentage of sequence identity to the query sequence. Gene IDs that lacked CDS were queried back to the Ensembl database to retrieve existing information. Throughout the paper, the genes that were annotated based on the BLAST results have been marked with an asterisk (*), while those that were annotated based on information available in Ensembl are marked with a hash/pound (#).

Results and discussion

Phenotype observations

Several tissue biopsies were collected at the slaughterhouse of which ovarian CL (Pokharel et al. 2020) and the endometrium were employed for RNA-seq. Based on the visual inspection, we observed hatching on Day 7. Similarly, the shape of the embryos was either spherical (Day 7 to Day 12) or elongated (Day 13 to Day 17). Hereinafter, based on the anatomy of embryos, the two group of the endometrium samples are referred to as spherical (S) and elongated (E). Both of our observations are in disagreement with previous report (Spencer et al. 2004) and need further validation. One possibility for the difference could be due to inaccurate estimation of embryonic age in our samples. Nevertheless, the transition time from spherical to elongated embryo appeared to be very short to classify them into intermediary (tubular) shape which have also been explained by the gene expression results (see also Fig. 2).

RNA-seq data

From the 21 RNA-seq libraries 1065.3 million (M) of paired-end reads were sequenced, and after trimming and quality filtering, approximately 3% of the reads were discarded. On average, 63% of the clean reads were mapped to the ovine reference transcriptome (Ensembl release 92). The raw Fastq data were deposited to European Nucleotide Archive (ENA) and are publicly available under project PRJEB32852 (Table 1).

Table 1. Sample summary

Sample name	Breed	Age of embryo	Shape of embryo	ENA Accession	# of paired end-reads (M)	Mapped reads (M)
1033	TX	7	S	ERR3349024	58.6	37
107A	TX	10	S	ERR3349026	53.1	33
107B	TX	10	S	ERR3349028	44.3	27.5
251	TX	9	S	ERR3349030	46.9	28.1
302	TX	17	E	ERR3349032	53.7	33
312A	FS	11	S	ERR3349034	46.6	28.3
312B	FS	11	S	ERR3349036	37.1	22.6
3609	FS	7	S	ERR3349038	50.8	31.5
379	TX	15	E	ERR3349040	54.2	34.5
4208	F1	9	S	ERR3349042	52.1	32.3
4271A	F1	16	E	ERR3349044	45.6	30.1
4271B	F1	16	E	ERR3349046	44.3	29.3
4519	F1	13	E	ERR3349048	54	35
4563	F1	9	S	ERR3349050	56.3	34.9
4590	F1	11	S	ERR3349052	41.9	25.3
4823	F1	15	E	ERR3349054	54.3	35.5
48	FS	12	S	ERR3349056	53.1	33.4
554	FS	15	E	ERR3349058	56.1	36.6
73	TX	10	S	ERR3349060	52.6	32.5
897	FS	15	E	ERR3349062	51.6	33.2
974	FS	15	E	ERR3349064	58.1	37.6

Sample Name = unique name for each sample including technical replicates (represented by A and B e.g. 107A and 107B); Breed = name of the breeds, Finnsheep (FS), Texel (TX) and F1 (F1 crosses of Finnsheep and Texel); age of embryo represents the number of pregnancy days when slaughtered; shape of embryo was visually inspected and were categorized as spherical (S) and elongated (E). ENA Accession = individual accession codes for all samples; # of paired-end reads = number of paired-end (2 x 100 bp) reads in millions; and Mapped reads (M) = number (in millions) of clean reads aligned to ovine reference transcriptome.

Gene expression in the endometrium

A total of 21125 genes were expressed in the whole data set which represent ~72.5% of known (n = 29118) sheep genes available in Ensembl. The complete list of genes with TPM (transcript count per million of reads) greater than 0.1 is available as a supplementary table (Table S1). The total number of genes expressed in the endometrium was comparatively higher than that in ovaries (Pokharel et al. 2018).

Several immunoglobulins (Igs) were expressed in the endometrium samples. Igs are heterodimeric proteins that belong to the Ig superfamily (IgSF) and are composed of two heavy and two light chains, of which the light chain may further consist of a κ or λ chain (Williams and Barclay 1988). Interestingly, the structure and organization of the genes enable Igs to be receptive to a virtually unlimited array of antigens rather than being limited to a fixed set of ligands (Honjo 1983). This feature is particularly important for adaptation to changing environments and may have contributed to enabling Finnsheep, for example, to survive in the harsh Finnish climate. Studies on human have shown that Igs, in general, improve pregnancy success (De Placido et al. 1994, Coulam and Goodman 2000). In addition to 11 Ig genes representing both the light and heavy chains, the joining chain of multimeric IgA and IgM (*JCHAIN*) was also expressed. *JCHAIN* is a small polypeptide containing eight cysteine residues that makes disulfide (C-C) bonds with IgA and IgM to form multimers. Two of the eight cysteines are linked with cysteines available on the heavy chain of IgA or IgM to result in dimer or pentamer forms, respectively (Bastian et al. 1995).

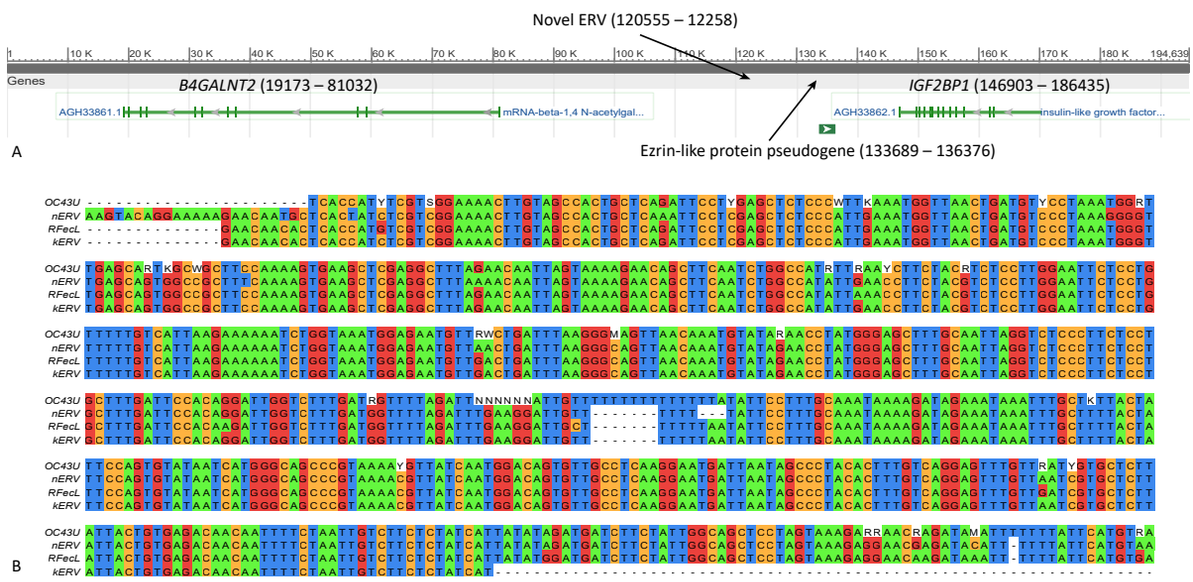

Fig. 1. Multiple sequence alignment (partial) of the novel endogenous retrovirus gene. (A) The novel ERV identified in this study belongs to FecL locus and is located between *B4GALNT2* and ezrin-like protein pseudogene. (B) The multiple sequence alignment was prepared using Clustal Omega (Madeira et al. 2019) based on the novel ERV (nERV, ENSOARG00000009959), ovine endogenous-virus beta-2 pro/pol region, partial sequence (kERV, AY193894.1), *Ovis canadensis canadensis* isolate 43U chromosome 17 sequence (OC43U, CP011902.1) and the reverse complement of reduced FecL locus (RFecL, KC352617). The bases are colored based on the nucleotide coloring scheme in Jalview (Waterhouse et al. 2009).

We also identified several genes associated with endogenous retroviruses (ERVs) in the endometrium samples. ERVs are copies of retroviral genomes that have been integrated into the host genome during evolution. Sheep ERVs share sequence similarity with exogenous and pathogenic Jaagsiekte sheep retrovirus (JSRV) (DeMartini et al. 2003). The genome of sheep contains at least 32 ERVs related to JSRV (Sistiaga-Poveda and Jugo 2014), and these ERVs are essential during pregnancy, including during placental morphogenesis and conceptus elongation (Palmarini et al. 2001, Dunlap et al. 2006a, Spencer and Palmarini 2012). A number of earlier studies have suggested critical roles of ERVs in uterine protection from viral infection, preimplantation conceptus development and placental morphogenesis (Dunlap et al. 2005, Dunlap et al. 2006a, Dunlap et al. 2006b, Denner 2016). Interestingly, one of the novel genes (ENSOARG00000009959) predicted to be an ERV was part of reduced FecL locus which is linked to prolificacy in French Lacaune breed (Drouilhet et al. 2013). Located on the reverse strand of

chromosome X, this gene has 24 paralogs and is absent in 162 (out of 184) species available in the Ensembl database. Although Ensembl lists 71 orthologs of this gene, none of them have even 50% sequence homology. A BLAST search against the NR database showed that 97% of the bases matched to the region of the reduced FecL locus (GenBank ID KC352617.1), which was recently characterized (Drouilhet et al. 2013). So far, only two genes, beta-1,4 N-acetylgalactosaminyltransferase 2 (*B4GALNT2*) and insulin-like growth factor 2 mRNA-binding protein 1 (*IGF2BP1*), and a pseudogene, ezrin-like protein, are known to exist in that region; our results have added one additional gene. In addition to the finding that the best hit was related to the FecL locus, the gene appeared to be an ERV, as we noticed that the query gene had 98% sequence identity with a partial sequence of the endogenous-virus beta-2 pro/pol region (see also Fig. 1). Finally, several lincRNAs were also expressed in the dataset. LincRNAs are long ncRNAs (lncRNAs) that originate from intergenic regions and do not overlap a protein-coding transcript. LincRNAs have a wide array of functions, including transcriptional regulation, biogenesis, epigenetic regulation, tissue specificity and developmental patterning (Pauli et al. 2011, Ulitsky and Bartel 2013, Deniz and Erman 2017, Ransohoff et al. 2018).

Most highly expressed genes

To obtain an overview of the most abundant genes in the endometrium, we selected the top 25 genes (Table 2). We noticed that nine out of the top 25 genes were mitochondrial genes. Mitochondrial genes play prominent roles during reproduction. We also observed high levels of expression of mitochondrial genes in the ovaries during the follicular growth phase (Pokharel et al. 2018). Top expressed autosomal genes appeared to have substantial roles during the preimplantation stage. Translationally controlled tumor protein (*TCTP*) is a highly conserved, multifunctional protein that plays essential roles in development and other biological processes in different species (Tuynder et al. 2002, Chen et al. 2007, Brioudes et al. 2010, Li et al. 2011, Branco and Masle 2019). With a maximum level of expression on Day 5 of pregnancy, this protein has been shown to play a significant role in embryo implantation in mice (Li et al. 2011). Consistent with these earlier studies, *TCTP* appeared to have the highest level of expression during the embryo implantation period. Matrix Gla protein (*MGP*) is a vitamin K-dependent extracellular matrix protein whose expression is known to be correlated with development and maturation processes (Zhao and Nishimoto 1996, Zhao and Warburton 1997) and receptor-mediated adhesion to the extracellular matrix (Loeser and Wallin 1992). Several studies have reported that *MGP* is highly expressed in the bovine endometrium (Spencer et al. 1999, Mamo et al. 2012, Forde et al. 2013). The high level of *MGP* expression in our current and previous (Pokharel et al. 2020) studies is consistent with earlier reports where this gene was found to be elevated during the preimplantation stage in sheep (Spencer et al. 1999, Gray et al. 2006) and cattle (Mamo et al. 2012). Similarly, Casey et al. (2005) reported that *MGP* was significantly upregulated in nonregressed compared to regressed bovine CLs. Our data and supporting results from earlier studies on cattle show that *MGP* is highly expressed in both tissues during the preimplantation stage and plays important roles in superficial implantation and placentation in sheep.

Six of the top 25 genes (*NUPR1*, *BCL2L15*, *CST3*, *CST6*, *S100G*, and *OST4*; see Table 2 for descriptions) expressed in the endometrium were also found to be highly abundant in a recent study that compared gene expression changes in the luteal and glandular epithelium during the peri-implantation stage in sheep (Brooks et al. 2016). Galectin 15 (*LGALS15*) is induced by *IFNT* and is involved in conceptus development and implantation (Kim et al. 2003, Gray et al. 2004, Lewis et al. 2007). Furthermore, *LGALS15* facilitates adhesion of the trophectoderm to the endometrial luminal epithelium (Lewis et al. 2007, Spencer et al. 2007). Two cystatin (CST) family members, namely, cystatin C (*CST3*) and cystatin E/M (*CST6*), were highly expressed in the endometrium. Known for their importance during the elongation and implantation of the conceptus, CSTs are protease inhibitors that are initiated by progesterone, and their high expression levels are attributable to stimulation by *IFNT* (Spencer et al. 2008, Spencer et al. 2015). Elongation factor 1-alpha (*EEF1A1*) is an important component of the protein synthesis machinery because it transports aminoacyl tRNA to the A sites of ribosomes in a GTP-dependent manner (Tatsuka et al. 1992, Mateyak and Kinzy 2010). The high level of *EEF1A1* in the endometrium most likely correspond to the production and transport of progesterone and other molecules that are essential during the implantation stage. The exact function of BCL2-like 15 (*BCL2L15*) in the sheep endometrium is not known, nor has it been reported in the endometria of other species, but its high expression has been reported previously (Koch et al. 2010, Brooks et al. 2016, Romero et al. 2017).

Table 2. List of the 25 most abundant genes in the endometrium. The table includes the Ensembl gene ID (GeneID), abundance measured by number of transcripts per million reads (TPM), chromosome number (Chr; MT refers to mitochondrial genome and AMGL01125506.1 is an unplaced scaffold), and gene description. Gene IDs and annotations that were not available in BioMart were retrieved based on a homology search using the nucleotide BLAST (marked with an asterisk, "*") or on information available in Ensembl (marked with a hash, "#").

Gene ID	Abundance (TPM)	Gene name	Chr	Gene description
ENSOARG00000007815	34771.26	*LOC105580399**	6	*Cercocebus atys* 60S ribosomal protein L41-like
ENSOARG00000019088	33284.80	*LGALS15*	AMGL01125506.1	galectin 15
ENSOARG00000000037	25447.87	*Mt tRNA#*	MT	mitochondrial tRNA
ENSOARG00000020724	21124.83	*MGP*	3	matrix Gla protein
ENSOARG00000003793	15373.87	*TSMB4X*	23	thymosin beta-4
ENSOARG00000000016	14147.12	*COX1*	MT	Cytochrome c oxidase subunit 1
ENSOARG00000000023	10341.99	*COIII*	MT	Cytochrome c oxidase subunit 3
ENSOARG00000000022	9327.50	*ATP6*	MT	ATP synthase subunit a
ENSOARG00000007617	9130.00	*TCTP*	10	tumor protein, translationally controlled, 1
ENSOARG00000000021	8535.39	*ATP8*	MT	ATP synthase protein 8
ENSOARG00000003184	8523.87	*NUPR1*	24	nuclear protein 1, transcriptional regulator
ENSOARG00000019924	8089.16	*BCL2L15*	1	BCL2 like 15
ENSOARG00000006202	6230.27	*CST3*	13	cystatin C
ENSOARG00000000035	5973.32	*CYTB*	MT	cytochrome b (mitochondrion)
ENSOARG00000001346	5551.71	*CST6*	21	cystatin E/M
ENSOARG00000016080	5472.76	*ATP5F1E*	13	ATP synthase F1 subunit epsilon
ENSOARG00000021079	5469.74	*S100A11*	1	S100 calcium binding protein A11
ENSOARG00000003341	5184.15	*IFI6*	2	interferon alpha inducible protein 6
ENSOARG00000000006	5179.55	*ND1*	MT	NADH-ubiquinone oxidoreductase chain 1
ENSOARG00000018666	5119.04	*RPLP1*	7	ribosomal protein lateral stalk subunit P1
ENSOARG00000000028	5104.93	*ND4*	MT	NADH-ubiquinone oxidoreductase chain 4
ENSOARG00000019491	4871.67	*OST4*	3	oligosaccharyltransferase complex subunit 4, non-catalytic
ENSOARG00000003782	4816.16	*B2M*	7	Beta-2-microglobulin
ENSOARG00000013018	4768.67	*S100G*	X	S100 calcium binding protein G
ENSOARG00000000019	4710.35	*COX2*	MT	Cytochrome c oxidase subunit 2

Differentially expressed genes in the endometrium having spherical and elongated embryos

Principal component analysis (PCA) based on variance stabilized transformation (VST) of expressed genes clearly indicated two distinct groups (Fig. 2A). Similarly, a heatmap plot of sample distances also based on VST showed a similar pattern (Fig. 2B). The two main clusters are primarily reflecting gene expression dynamics of the endometrium before (Day 7 to Day 12) and after (Day 13 to Day 17) embryonic elongation phase (see also Table 1). The anatomical difference in two groups was verified by manual inspection of the embryos whereby all embryos collected from Day 7 to Day 12 were spherical (S) and the rest were elongated (E).

Altogether 1410 genes were significantly (padj < 0.01 and abs(log2FoldChange > 2.5)) differentially expressed between S and E of which 554 were upregulated in E and the rest (n = 856) were upregulated in S. Several of the non-classical *IFNT*-stimulated genes (ISGs) including *LGALS15*, *HSD11B1*, *SLC7A2*, *ISG20*, *CLEC4F*, *CCL8*, *CXCL11*, *CXCL10*, and *CST6* were upregulated in E (Spencer et al. 2008, Brooks et al. 2014). All these ISGs are known to

be induced by the progesterone and are critical for conceptus elongation (Brooks et al. 2014). We observed high abundance of key genes involved in progesterone production in the CL of the studied animals (Pokharel et al. 2020) which complements findings from this data. This is consistent with maximal production of *IFNT* which causes massive changes in the endometrial gene expression starting Day 13 of pregnancy (Gray et al. 2006, Spencer et al. 2007, Forde and Lonergan 2017). Nine chemokines (*CCL2, CCL3, CCL4, CCL8, CCRL2, CXCL9, CXCL10, CXCL11, CXCR1*) were all upregulated in E. Chemokines serve a variety of functions including migration of immune cells to the site of inflammation, chemotaxis, angiogenesis, cell differentiation, and signal transduction (Luther and Cyster 2001, Thelen 2001, Fernandez and Lolis 2002, Griffith et al. 2014). Numerous reports have suggested the role of chemokines and their receptors in establishing proper environment for implantation by facilitating orientation and adhesion of conceptus to the uterine wall (van Mourik et al. 2009, Sakumoto et al. 2017, Imakawa et al. 2017, Złotkowska and Andronowska 2019). In our another study, chemokines (including *CXCL9*) were also expressed in the CL and were upregulated in prolific Finnsheep compared to Texel (Pokharel et al. 2020). Upregulated expression of several chemokines and their receptors indicated their prominent role during early pregnancy. Similarly, several members from the complement system including 10 complements, three galectins (*LGALS3, LGALS7, LGALS15*), two selectins (*SELE, SELP*), mannan binding lectin serine peptidase 1 (*MASP1*), and C-type lectin domain family 4 member F (*CLEC4F*) were upregulated in E. Half of the complement genes (n = 5) significantly upregulated in E are in the unplaced scaffold of the sheep reference genome (Oarv3.1) and *C1QL2* was the tenth most upregulated (LFC = 7.4, Padj = 3.28E-27) gene in E. It has been well established that complement system has important role in successful establishment of pregnancy. *C1Q* in particular is known to have significant role at the implantation site whereby it promotes trophoblast invasion in maternal decidua (Singh et al. 2011, Bulla et al. 2012). While some of the genes (e.g. *LGALS15*, *CLEC4F*) involved in the complement system have been extensively studied, more research will be needed to elucidate the role of complement genes during the implantation window of pregnancy.

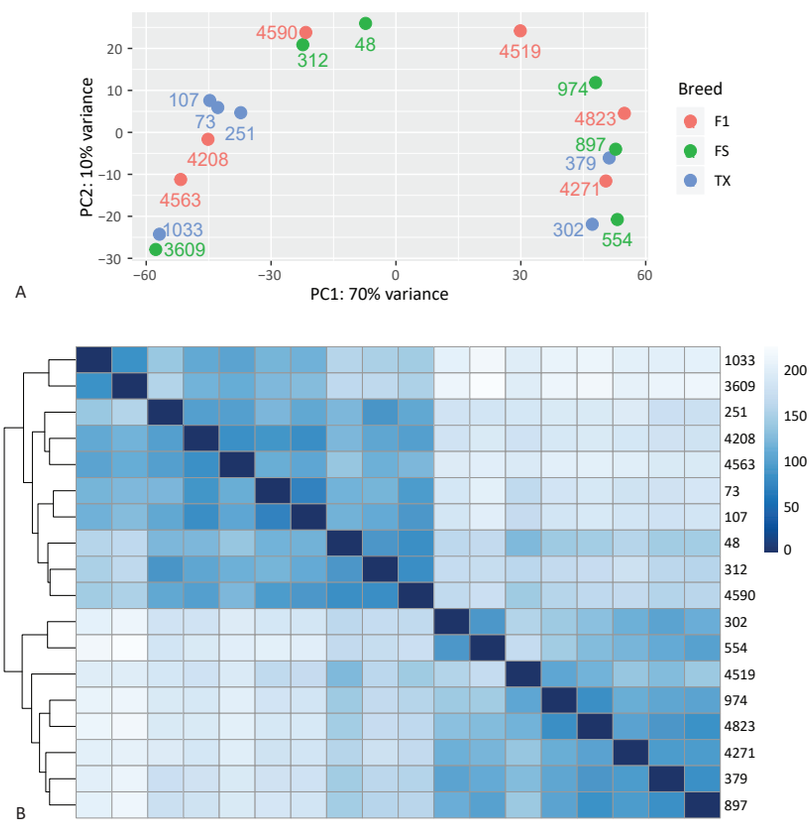

Fig. 2. Sample relatedness. (A) PCA plot of samples representing Finnsheep (FS), Texel (TX) and F1 crosses (F1) based on normalized counts. (B) Heatmap plot of sample distance matrix

Prostaglandin F synthase 1-like (*PGFS1*) was the most significantly upregulated (LFC = 11.4, Padj = 4.51E-77) gene in E group (Table 3). Prostaglandins, mainly secreted by the endometrium are another important regulators of embryo development (Dorniak et al. 2011, Spencer et al. 2013). Two dehydrogenase enzymes (*DHDH* and *HSD3B1*) were among the top 20 upregulated genes in E. Two additional dehydrogenases (*HSD11B1* and *HSD11B2*) were also differentially expressed in our data of which *HSD11B1* was upregulated and *HSD11B2* was downregulated in E.

Table 3. Top 40 significantly differentially expressed genes between the endometrium having spherical (S; Day 7 to Day 12) and elongated (E; Day 13 to Day 16) embryos in sheep. The genes were ranked based on the log2 fold change (LFC) values. First 20 genes (with positive LFC values) were the top upregulated genes in E and the rest (bottom 20 with negative LFC) were upregulated in S. Few genes lacking annotations were derived from homology based BLAST search against the non-redundant (NR) database (marked with *) or from the Ensembl (marked with #). For the complete list of significantly differentially expressed genes, see Table S2.

Gene ID	Mean count	LFC	padj	Gene name	Gene description
ENSOARG00000012196	1117.02	11.46	9.12E-77	*PGFS1*	prostaglandin F synthase 1-like
ENSOARG00000011663	271.36	10.53	9.55E-48	*DHDH*	dihydrodiol dehydrogenase 3-like
ENSOARG00000007954	791.69	8.61	2.84E-44	*FGFBP1*	fibroblast growth factor-binding protein 1
ENSOARG00000015726	405.88	8.30	2.26E-39	*DCSTAMP*	dendrocyte expressed seven transmembrane protein
ENSOARG00000016674	1927.54	8.26	2.89E-17	*PLET1*	placenta-expressed transcript 1 protein
ENSOARG00000020537	9334.75	8.22	2.38E-31	*CRYGS*	crystallin gamma S
ENSOARG00000016590	58.03	7.74	8.39E-21	*TRIM29*	tripartite motif containing 29
ENSOARG00000020896	48.99	7.51	3.30E-15	*MUC17**	mucin 17-like
ENSOARG00000008599	6530.38	7.51	6.47E-22	*SLC36A2*	solute carrier family 36 member 2
ENSOARG00000012340	216.26	7.39	3.28E-25	*C1QL2*	complement C1q like 2
ENSOARG00000020029	182.35	7.22	3.19E-47	*FBXO40*	F-box protein 40
ENSOARG00000004524	36.23	7.21	2.17E-14	*SPINK9**	serine peptidase inhibitor, kazal type 9
ENSOARG00000020026	16.37	7.00	2.43E-22	*ARGFX*	arginine-fifty homeobox
ENSOARG00000025059	44.93	6.92	2.17E-24	*SCARNA6**	small Cajal body-specific RNA 6
ENSOARG00000011075	59.03	6.80	1.72E-17	*SELE*	selectin E
ENSOARG00000020398	17.03	6.76	3.63E-16	*HAO2*	hydroxyacid oxidase 2
ENSOARG00000006417	17.41	6.73	1.81E-15	*TTBK1*	tau tubulin kinase 1
ENSOARG00000004790	328.60	6.72	6.40E-10	*MMP13*	matrix metallopeptidase 13
ENSOARG00000020402	13084.60	6.69	6.35E-29	*HSD3B1*	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
ENSOARG00000005877	57.14	6.62	5.44E-13	*TKDP1**	Trophoblast Kunitz domain protein 1
ENSOARG00000018477	72.96	-6.63	1.68E-16	*PRSS16*	serine protease 16
ENSOARG00000016704	159.31	-6.66	1.23E-16	*CNGA1*	cyclic nucleotide gated channel alpha 1
ENSOARG00000009436	52.81	-6.86	2.74E-15	*SLITRK4*	SLIT and NTRK like family member 4
ENSOARG00000001686	38.40	-6.91	4.41E-15	*HAVCR1**	hepatitis A virus cellular receptor 1
ENSOARG00000010567	69.04	-7.05	1.18E-19	*STATH**	statherin
ENSOARG00000003097	433.14	-7.10	6.36E-40	*C8orf34*	chromosome 9 open reading frame, human C8orf34
ENSOARG00000004187	657.19	-7.15	9.22E-13	*SMIM18*	small integral membrane protein 18
ENSOARG00000002992	1741.32	-7.26	3.14E-28	*HAVCR1**	hepatitis A virus cellular receptor 1
ENSOARG00000004092	71.45	-7.30	4.96E-18	*CLDN22**	claudin 22
ENSOARG00000026762	808.19	-7.38	1.21E-30	LincRNA#	
ENSOARG00000006412	126.01	-7.53	1.53E-15	*MGAT5B*	alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase B
ENSOARG00000025314	67.94	-7.54	1.80E-17	LincRNA#	
ENSOARG00000013676	1091.06	-7.54	1.51E-19	*SUSD2**	sushi domain containin 2
ENSOARG00000018755	6302.26	-7.73	1.49E-29	*TNNI1*	troponin I1, slow skeletal type
ENSOARG00000019210	311.45	-7.91	2.80E-19	*CPS1*	carbamoyl-phosphate synthase 1
ENSOARG00000011999	236.10	-8.01	8.12E-31	*JPH3*	junctophilin 3
ENSOARG00000010828	19130.13	-8.03	5.25E-41	*HAVCR1**	hepatitis A virus cellular receptor 1
ENSOARG00000010754	4415.85	-8.08	1.34E-43	*HAVCR1**	hepatitis A virus cellular receptor 1
ENSOARG00000000132	1328.32	-8.09	1.90E-24	*SUSD2**	sushi domain containing 2
ENSOARG00000009890	258.25	-8.13	1.28E-29	*RTL4**	retrotransposon Gag-like protein 4

This finding was in part consistent with earlier study that reported the abundance of *HSD11B1* in the endometrium between Days 12 and 16 while *HSD11B2* was abundant in the conceptus (Simmons et al. 2010). *FGFBP1*, a secreted protein functions as a chaperone of FGFs to their receptors (Tassi et al. 2001, Tassi et al. 2011). *FGF14*, *FGF11*, *FGF10*, *FGF13*, *FGF9* and *FGF21* were significantly differentially expressed between E and S of which *FGF14* and *FGF11* were upregulated in S and the rest were upregulated in E. *DCSTAMP* is a recently characterized protein known to regulate osteoclastogenesis by mediating cell-cell fusion in osteoclasts (Hartgers et al. 2000, Yagi et al. 2005, Chiu and Ritchlin 2016). Although the function of this gene in the endometrium remains to be elucidated, we speculate that it has important role in attachment of conceptus to the uterine wall. *TKDP4*, belongs to a member of rapidly evolving secreted proteins predominantly expressed in the trophoblast of placenta during adhesion of conceptus to the endometrium (Chakrabarty et al. 2006, Clemente et al. 2011).

More than 50% of the top 20 upregulated genes in S lacked gene descriptions indicating that the existing knowledge on endometrial expressed genes with respect to spherical embryos is rather limited. Some of the highly upregulated genes in S such as *CPS1* (Gu et al. 2014), *TNNI1* (Rehman et al. 2003), *PRSS16* (Forde et al. 2013) were expressed in the endometrium of pregnant animals but their precise function was not reported. Notably, four (ENSOARG00000010754, ENSOARG00000010828, ENSOARG00000002992 and ENSOARG00000001686) of the top 20 genes upregulated in S had high sequence similarity to hepatitis A virus cellular receptor 1 (*HAVCR1**). Moreover, two lincRNAs (ENSOARG00000025314, ENSOARG00000026762) were also highly significantly upregulated in S. A recent study on the placental transcriptome of mare reported the expression of *SPINK9* (Loux et al. 2019). Further research and improved genome annotation will be required to get a better picture of the gene expression changes in the endometrium in response to non-elongated embryos.

Biological processes and pathways of differentially expressed genes

Altogether 52 GO terms related to the biological processes category were associated with the genes upregulated in E group (Table S3). Further merging of the terms using kappa score (see methods section) led to 14 groups. “Receptor regulator activity (GO:0030505)” was one of the most significantly enriched GO terms associated with upregulated genes in E. Majority of the 52 significant GO terms associated with upregulated genes in E were related to immune processes, followed by transport related processes (Table 4, Table S3). Other categories include “embryonic placenta development”, “perception of sound” and “monocarboxylic acid metabolic process”.

Table 4. Selected GO terms associated with genes that were upregulated in endometrium having elongated (E) embryos (for more details and complete list, see Table S5)

GO ID	GO Term	Associated genes
GO:0001892	embryonic placenta development	*EPAS1, GCM1, IL10, LEF1, LIF, SOCS3*
GO:0002684	positive regulation of immune system process	*ACOD1, C5, CARD11, CDKN1A, CXCL10, DCSTAMP, DPP4, FCN1, FGF10, FGR, GATA1, GPLD1, IL6, ITGB3, LEF1, LGALS3, LGMN, LIF, LOC443475, LYN, MASP1, PGC, PGLYRP4, S100A14, SELP, SEMA7A, SERPING1, TLR2, TLR5, TNFSF18, TNIP3, TTBK1*
GO:0052548	regulation of endopeptidase activity	*AHSG, C5, CAST, COL28A1, CRYAB, CST3, CST6, CSTB, FABP1, LEF1, LGMN, LOC101123265, LOC101123672, LXN, LYN, SERPINB5, SERPING1, TNFSF10, TTBK1*
GO:0032787	monocarboxylic acid metabolic process	*ACOT7, ACOX2, ACSM1, ACSM3, ADH1C, ADIPOQ, ALDH8A1, ANGPTL3, CYP1A1, FABP3, GATM, HAL, HAO2, PCK1, PLA2G1B, SDS, SLC27A2*
GO:0046651	lymphocyte proliferation	*CARD11, CDKN1A, CTPS1, FGF10, GPNMB, IL10, IL6, LEF1, LGALS3, LYN, TNFSF18*
GO:0009617	response to bacterium	*ACOD1, ADIPOQ, CUBN, CXCL10, CXCL9, FAM20A, FGR, IL10, IL6, LOC443322, LY6G6C, LYN, NUGGC, PCK1, PGC, PGLYRP4, PLA2G1B, S100A14, SLC10A2, TLR2, TLR5, TNFSF4, TNIP3*
GO:0022804	active transmembrane transporter activity	*ATP12A, ATP2B2, ATP2B3, LOC101106534, LOC101109370, LOC101113819, SLC10A2, SLC13A1, SLC15A1, SLC22A4, SLC23A1, SLC28A2, SLC36A2, SLC4A5, SLC6A13, SLCO1C1*
GO:0006935	chemotaxis	*C5, CCRL2, CXCL10, CXCL11, CXCL9, CXCR1, DOCK4, FGF10, GBX2, ISL2, LEF1, LGALS3, LGMN, LOC101114285, LOC101114535, LOC101119572, LOC101119832, LYN, MATN2, S100A14, SEMA6A*
GO:0030545	receptor regulator activity	*ADIPOQ, ANGPTL3, CXCL10, CXCL11, CXCL9, FGF10, FGF13, FGF21, FGF9, FST, GPNMB, IL10, IL6, LGALS3, LIF, LOC101110855, LOC101114285, LOC101114535, LOC101119572, LOC101119832, LY6G6D, RETN, TNFSF10, TNFSF4*

GO terms associated with transporter activities and metabolic processes were also enriched in E. Majority (n = 6) of the 19 KEGG pathways associated with the genes upregulated in E were signaling pathways such as "chemokine signaling pathway", "JAK-STAT signaling pathway", "PPAR signaling pathway". In addition pathways related to diseases ("malaria", "pertussis", "*Staphylococcus aureus* infection", "rheumatoid arthritis"), secretions ("bile-secretion", "salivary secretion", "pancreatic secretion") and metabolism ("pyrimidine metabolism", "retinol metabolism") were also in the list (Table S4). The importance of robust, adaptive and responsive immune system during early pregnancy has been well documented. However, several of the GO terms and KEGG pathways associated with early pregnancy should be redefined. In particular, categories related with diseases and bacterial infections may not be relevant in the context of implantation and placentation as all animals included in the study were healthy. In recently published report on human placenta, authors did not find any evidence of bacterial infection (de Goffau et al. 2019). Moreover, it should also be noted that many of the immune related genes have characteristics that are well suited for the processes leading to implantation such as apposition, adhesion and interaction between the conceptus and the endometrium.

Biological processes and pathways enriched by upregulated genes in S group were substantially different than those from upregulated genes in E. Functional analysis of 856 genes upregulated in S revealed 143 GO terms that were further categorized into 25 groups (Table S5). Transport related GO terms were the most common and included ion (e.g. potassium, sodium, chloride, organic, and inorganic) transports, amino acid transport, carboxylic acid transport, channel activities and transmembrane transporter activities (Table 5, Table S5). Several GO terms associated with development (limb, connective tissue, neural crest cell, mesenchyme, ear, eye, sensory system, heart, neural tube, muscle tissue, etc.) and morphogenesis (cardiac chamber, sensory organ, epithelial tube, embryonic limb, tissue, etc.) were associated with the upregulated genes in S. These tissue and organ development related GO terms are not relevant and may rather point to general development related processes in the endometrium. For example, as shown in Table S5, many of the genes present in ear or eye development categories are also present in tissue morphogenesis. In the context of this study, tissue morphogenesis makes better sense than organ development. Similarly, several signaling pathways, and those related to "cholesterol metabolism", "ECM-receptor interaction", "axon guidance", "GABAergic synapse", "cell adhesion molecules (CAMS)" etc. were enriched by genes upregulated in S (Table S6). Wnt signaling pathway is known to have important role in conceptus elongation (Mohamed et al. 2005).

Table 5. Selected GO terms associated with the genes that were upregulated in the endometrium having spherical (S) conceptus (for more details and complete list, see Table S5)

GOID	GO Term	Associated genes
GO:0030509	BMP signaling pathway	*CHRDL1, LRP2, SFRP2, SMPD3, SOSTDC1, TMPRSS6*
GO:0050727	regulation of inflammatory response	*ADORA1, ENPP3, IL1RL2, ISL1, LPL, METRNL, PTGIS, SMPDL3B, SPHK1, TMEM173, TREM2*
GO:0018108	peptidyl-tyrosine phosphorylation	*AFAP1L2, BANK1, CASS4, EFEMP1, EPHB1, EPHB6, FLT4, ISL1, PKDCC, RET, ROS1, SFRP2, SRCIN1, TDGF1, TREM2*
GO:1905475	regulation of protein localization to membrane	*ACSL3, CDK5R1, KCNB1, PKDCC, PLS1, SLC7A11, TREM2, VIL1*
GO:0032872	regulation of stress-activated MAPK cascade	*CD40LG, DACT1, EDN1, EPHB1, FLT4, GADD45G, PLCB1, SFRP2, SH3RF2, SPHK1*
GO:0016042	lipid catabolic process	*ABCD2, ACACB, AIG1, CIDEA, CPS1, HSD17B11, LPL, PLCB1, PNPLA1, SMPD3, SMPDL3B, SPP1*
GO:0061448	connective tissue development	*COL11A1, DGAT2, EDN1, EFEMP1, OSR1, PKDCC, SFRP2, SLC39A13, SMPD3, SOX5*
GO:0010876	lipid localization	*ABCA2, ACACB, ACSL3, ANO6, BDKRB2, CIDEA, DGAT2, LIPG, LOC101121414, LOC101122517, LPL, PLTP, PTCH1, SPP1, VSTM2A*
GO:0010975	regulation of neuron projection development	*BRSK1, CAMK1D, CDK5R1, KEL, NEFL, NRCAM, PLXNB3, RAP1GAP2, RASAL1, RET, SEMA3F, SFRP2, SRCIN1, ZNF804A*
GO:0048638	regulation of developmental growth	*ACACB, CDK5R1, EDN1, GLI1, PLCB1, PLS1, PTCH1, RASAL1, SEMA3F, SFRP2, VIL1*
GO:0060173	limb development	*FREM2, MKS1, OSR1, PKDCC, PTCH1, RDH10, SEMA3C, SFRP2, SLC7A11, SMOC1*
GO:0042391	regulation of membrane potential	*ABCB5, ADORA1, AKAP6, ANK2, BOK, CACNB4, DGKI, GABBR1, GPD1L, GRIN3A, KCNB1, NRCAM, RNF122, SCN1A, SLC26A3, STX1A, STX1B*
GO:0022804	active transmembrane transporter activity	*ABCA2, ABCB5, ABCD2, ATP2A3, ATP6V1G2, LOC101119236, LOC101122517, SLC1A1, SLC26A3, SLC47A2, SLC4A11, SLC6A12, SLC6A20, SLC9C1*

GO:0050808	synapse organization	*CACNB4, CDK5R1, CDKL5, COL4A5, EPHB1, INA, L1CAM, NEFL, NRCAM, SEMA3F, SLC7A11, SLITRK4, SRCIN1, SYNDIG1, WASF1*
GO:0044057	regulation of system process	*ADORA1, ANK2, EDN1, GPD1L, HOMER3, LOC101120030, NMU, NOS1, SLC1A1, SPHK1, SPX, STX1A, STX1B, THRB, TMEM98, TNNC1, TNNT1, TPM1*
GO:0007389	pattern specification process	*CDON, DNAH5, EDN1, GLI1, GRHL3, HOXB1, ISL1, LRP2, MKS1, OSR1, PTCH1, SEMA3C, SEMA3F, SFRP2, SOSTDC1, SUFU*
GO:0007267	cell-cell signaling	*ADORA1, ANK2, ANXA9, BRSK1, CACNB4, DACT1, DGKI, EDN1, EPHB1, GABBR1, GLI1, GRHL3, GRID2IP, GRIN3A, HMGN3, HOMER3, ILDR1, ISL1, JADE1, JPH3, JPH4, KCNB1, LGR5, LOC101119591, LOC443240, MC4R, MKS1, MME, NOS1, PLAT, PLCB1, PLEKHG5, PLK2, PYGO1, RAB26, RNF43, SFRP2, SHC3, SLC7A11, SMPD3, SNCAIP, SOSTDC1, SPP1, STX1A, STX1B, SV2C, SYN2*
GO:0014070	response to organic cyclic compound	*ABAT, ABCA2, AKAP6, BGLAP, CIDEA, ESR2, KCNJ8, LOC101122517, LY6D, NR0B1, NR2E3, NR2F1, PADI2, PPP1R1B, PTCH1, RORB, RORC, RXRG, SLC26A3, SLC7A11, SPP1, SRARP, THRB, TMEM173, VDR*
GO:0048729	tissue morphogenesis	*BRSK1, COL11A1, EDN1, FAT4, FREM2, GRHL3, ISL1, KRT27, LGR5, LRP2, MKS1, MTHFD1L, OSR1, PTCH1, RDH10, RET, SEMA3C, SFRP2, SOSTDC1, SUFU, SYNE4, TNNC1, TPM1, VDR*
GO:0099537	trans-synaptic signaling	*ADORA1, ANXA9, BRSK1, CACNB4, DGKI, EDN1, EPHB1, GABBR1, GRID2IP, GRIN3A, HOMER3, JPH3, JPH4, KCNB1, LOC101119591, MME, NOS1, PLAT, PLCB1, PLEKHG5, PLK2, RAB26, SHC3, SLC7A11, SNCAIP, STX1A, STX1B, SV2C, SYN2*
GO:0007507	heart development	*ACACB, ADAMTS6, ANK2, COL11A1, DNAH5, EDN1, FAT4, FREM2, GATA4, GLI1, ISL1, KCNJ8, LRP2, MKS1, MYO18B, OSR1, PDLIM3, PTCH1, SEMA3C, SFRP2, SUFU, TDGF1, TNNC1, TPM1*
GO:0006811	ion transport	*ACSL3, AKAP6, ANK2, ANO6, ATP2A3, ATP6V1G2, BDKRB2, CACNB4, CDH23, CLDN10, CLIC5, CNGA1, CP, GABRR2, GPD1L, GRIN3A, JPH3, JPH4, KCNB1, KCNG4, KCNJ8, KCNK12, KCNK2, KCNN1, KEL, LOC101108019, LOC101111082, LOC101116002, LOC101116975, LOC101119236, LOC101122517, LOC101122774, LRP2, MCOLN2, MCOLN3, NOS1, OSR1, PLTP, RHCG, ROS1, RYR3, SCN1A, SCN2A, SCN3A, SCNN1A, SLC1A1, SLC25A42, SLC26A3, SLC38A3, SLC39A13, SLC4A11, SLC6A12, SLC6A20, SLC7A11, SLC7A7, SLC9C1, SLCO5A1, STX1A, TRPV6, VDR*

Conclusion

Collectively, the data uncovered the pattern of endometrial gene expression in relation to spherical and elongated embryos during preimplantation window of early pregnancy. To that end, the endometrium having spherical embryos displayed a distinct transcriptome profile compared to endometrium compared to those with elongated embryos. The roles of retroviruses during early pregnancy were indicated in our data. A novel gene sharing similarity with an ERV was identified in the FecL locus. The importance of adaptive immune system at the embryo-maternal interface was clearly indicated in our data. Due to lack of enough replicates, we were unable to perform breed-wise gene expression comparison. Moreover, it would be very interesting to see how gene expression during pregnancy development changes with respect to the number of viable embryos (Finnsheep have higher number of embryos than Texel).

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