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Research article

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Impact of negative energy balance on transcriptomic profiles of three endometrial cell types isolated by laser capture microdissection in postpartum dairy cows

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Abstract

Background: In postpartum dairy cows, the energy needs to satisfy high milk production induces a more or less pronounced Negative Energy Balance (NEB) status. NEB associated with fat mobilization impairs reproductive function. This study investigated the specific impact of NEB on gene expression in the three main types of endometrial cells at time planned for insemination and implantation. Endometrial cell types (stromal, glandular and luminal epithelial cells) were isolated by laser micro-dissection allowing the study of constitutive gene expression and their specific response to NEB.

Methods: Nine Swedish Red cows receiving a control diet or a mild restricted diet to induce differences of energy balance were categorized into mild (MNEB, n = 5) and severe negative energy balance (SNEB, n = 4). The three endometrial cell types: luminal (LE), glandular (GE) epithelium and stroma (ST) were collected by laser microdissection from endometrial biopsies performed at 80 days postpartum.

Results: Transcriptome profiles obtained by RNA sequencing revealed differences in constitutive gene expression between the three cells types and also differences in specific responses related to the severity of NEB. Number of differentially expressed genes between SNEB and MNEB cows was higher in ST than in LE and GE, respectively. SNEB was associated with differential expression of genes related to metabolic processes and embryo-maternal interactions in ST. Under-expression of genes related to cell structure was found in GE whereas genes related to pro-inflammatory pathways were over-expressed. Genes associated to adaptive immunity were under-expressed in LE.

Conclusion: The three different main cells types of the endometrium, have very different patterns of gene expression. The severity of NEB after calving is associated with changes in gene expression at time of breeding. Specific alterations in GEs are associated with activation of pro-inflammatory mechanisms. Concomitantly, changes in the expression of genes related to cell to cell interactions and maternal recognition of pregnancy takes place in ST. The combination of these effects possibly

altering the uterine environment and embryo maternal interactions may negatively influence the establishment of pregnancy.

Keywords: negative energy balance, endometrial cells, transcriptome, laser microdissection, inflammation

Background

The existence of common genetic and epigenetic factors that influence metabolic imbalance, milk production and reproductive performance have been raised for long [1] and are still an important topic in dairy cow industry [2]. A significant decrease in fertility due to genetic improvement for increasing milk production has been reported for decades in dairy cows [3, 4]. Despite a more balanced selection is applied nowadays [5], high milk-yield cows still meet strong negative energy balance (NEB) during the early postpartum period due to the high nutrient and energy demand for body metabolism, milk production, and body weight maintenance [6]. Energy deficiency and excessive lipid mobilization during the postpartum period have been reported to be the cause of unfavorable reproductive performances such as delayed ovarian activity [7], prolonged uterine involution period [8], retained placenta [9], endometritis [10], increased early embryonic losses and decreased conception rates [11]. Previous studies also showed the impacts of metabolic imbalance on gene expression in the endometrium during the early postpartum period [12, 13]. However, these studies were based on RNA prepared from biopsies taken from endometrial tissue sections without discriminating between different cell types. To our knowledge, constitutive gene expression and possible effects of metabolic imbalance on the response of specific endometrial cell types at time of conception remains to be deciphered.

The uterus is the site of intensive tissue remodeling during the estrous cycle, at time of implantation and placental development in response to the developing embryo [14]. Reciprocally, the control of the endometrium on embryo development steps has been recently documented in mice [15]. In the cow, histology of the endometrium shows a complex association of heterogeneous structures mainly consisting of luminal epithelial cells (LE), glandular epithelial cells (GE) as well as fibroblast-like stromal cells (ST) found in different proportions in caruncular and intercaruncular tissues [16]. These three cell types are functionally responsible for the embryo implantation process under the control of steroid hormones and act in different ways [17]. For instance, bovine uterine stromal cells synthesize and release prostaglandin E-2 (PGE-2), involved in maternal recognition of pregnancy, whereas epithelial cells contribute less to such changes in prostaglandin levels [18]. Uterine epithelial cells play key roles for the establishment and maintenance of pregnancy through activation of the innate immune system and secretion of chemokines [19] that support the recruitment and activation of immune cells directed against pathogens. Moreover, LE and GE exhibit unique molecular signatures having cooperative roles at time of establishment of pregnancy [16, 20, 21]. Their morphology [22] and biochemical activity [23] differs at time of implantation. RNA-sequencing of the complete transcriptome for the three cell types has been described for equine cells [24]. Laser capture microdissection (LCM) has also been successfully used to retrieve two different uterine epithelial cell types to define the transcriptome and proteomic analysis of the ovine and porcine endometrium, respectively [25, 26]. However, to our knowledge the transcriptomic profile of bovine endometrial cells has not yet been documented. Previously published research, regarding the impact of NEB on uterine function and endometrial transcriptome, suggests that NEB associated with elevated non-esterified fatty acids (NEFAs) concentrations induces infertility in postpartum cows through dysregulation of immune pathways [12]. However, the understanding of molecular changes induced by NEB from entire endometrial tissues is still unclear and difficult to interpret functionally as responses may be affected by other cell types such as endothelial cells, smooth muscle cells and leukocytes [27]. *In vitro* studies have clearly shown that NEFAs stimulate pro-inflammatory cytokine production and lipid accumulation of endometrial cells [28] and oviductal epithelial cells [29] but the results from these *in vitro* models need to be confirmed *in vivo*.

We hypothesized here that NEB may differentially influence the physiology of three endometrial cell types. The objectives of the present study were *i*) to investigate transcriptomic profiles of luminal epithelial cells, glandular epithelial cells and stromal cells which were harvested by LCM, and *ii*) identify possible differences in the profiles between cows diagnosed with either mild or severe NEB during the postpartum period. The collection of endometrial biopsies was performed at time of planned AI and the observed changes in gene expression suggest the existence of long-term impacts of NEB that are cell type-specific.

Results

Body condition score (BCS) and plasma NEFA concentrations.

The evolution of residual feed intake with post-partum time in the two groups of cows is presented in (Figure 1A). Throughout the full experimental period, the BCS of SRB cows in both NEB groups tended to decrease ($p = 0.08$). Mean BCS was 3.65 ± 0.25 at start of the experiment and 3.05 ± 0.22 at 120 days postpartum. However, NEB did not have a significant effect on BCS (data not shown). Plasma NEFA concentrations did not differ between NEB groups over the full experimental period. However, SNEB cows presented higher NEFA plasma concentrations compared to MNEB cows at Day 14 pre-partum and Day 14 post-partum ($p < 0.05$) (Figure.1B). BCS loss from 30 days pre-calving and 60 days post-calving was associated with the energy balance nadir (r = -0.68, $p < 0.05$). NEFA concentrations tended to be significantly associated with the residual feed intake values (r = -0.28, $p = 0.06$).

RNA-Sequencing of cell type-specific samples collected by LCM.

The sequencing depth of RNA-seq libraries was in the range of 60 to 100 million reads per sample for each endometrial cell type. A total of 22915 transcripts with a unique Identifier were found. Salmon's method provides both read counts and TPM (transcripts per million), and the latter expression is more appropriate when comparing relative abundance between different cell types or tissues [30]. Before comparing the differences in gene expression between the endometrial cell types, transcripts whose average value computed from biological replicates were less than 10 TPM were regarded as biological background noise, partly independent of transcription regulation and discarded. The number of expressed genes detected (higher than 10 TPM) was 6622, 7814 and 8242 for luminal epithelial cells (LE), glandular epithelium (GE) and stromal cells (ST), respectively (Figure 2A). In the RNA-Seq analysis, the highest number of detectable expressed genes (8242) in the LCM datasets was obtained for ST and the lowest number of detectable genes (6622) was observed for LE. As displayed on the Venn diagram (Figure 2A), 5672 genes were expressed by all the three cell types. A total of 1236 genes were expressed exclusively by ST cells, which represents 15% of all genes expressed by this type of cell, while only 551 (7% of all genes expressed) transcripts were specific to the GE cells and 330 (5%) transcripts specific to LE cell. The lists of genes specifically expressed by each cell type are provided in additional file (TableS1_TS1-LE_TS2-GE_TS3-ST.xlsx). An overview of the GO terms associated to genes specifically expressed by each cellular type is visualized in Figure.3. The list of 5672 genes expressed in common between the three cell types was used as a reference list for PANTHER overrepresentation tests. Over and under-represented GO terms for biological process were visualized using REVIGO algorithm to reduce term redundancy (corresponding tables of GO terms are provided in additional file (TableS2_GO-REVIGO05_TS1-TS2-TS3.xlsx). Respectively 97, 14 and 13 clusters of GO terms were over-represented in ST, GE and LE cells whereas 45, 11 and 8 were under-represented. Numerous metabolic processes were under-represented in the three lists of genes specifically expressed by each cell type which means that the genes involved in metabolism are shared genes. For ST, over-represented biological processes included many regulation processes and response to stimulus, cell communication and cell adhesion, extracellular matrix organization as well as developmental process and wound healing. For GE, cilium organization, cilium movement, protein localization to cilium and microtubule-based process were only the four main biological processes enriched. For LE, over-represented biological processes were enzyme linked receptor protein signalling pathway, cell-substrate adhesion, circulatory system process and activation of adenylate cyclase activity.

Heatmap (Figure 2B) illustrates hierarchical clustering obtained with samples and genes. The clustering unambiguously joins samples of each cell-type. The most expressed genes for each cell type are highlighted and framed by boxes (Figure 2B). The corresponding statistical analyses revealed that 8360 genes were differentially expressed (adjusted p value < 0.05) between GE and LE cells (2666 genes greater expressed in GE vs. 5694 in LE). The expression of 10761 genes differs between ST and LE (4298 genes more expressed in ST vs. 6463 more expressed in LE). The level of expression of 10003 genes differs between GE and ST (2900 genes more expressed in GE vs. 7103 in ST).

The principal component analysis also reveals a clear separation of the samples from the three cell types (Figure 2C). The first two dimensions explain 80% of the variability. The first dimension distinguishes epithelial cells from ST whereas the variation associated to the second dimension relates to differences of expression between GE and LE. Supplementary tables (Table_S3_PCA_tables.xlsx; sheets TS4 and TS5 for the first dimension, sheets TS6 and TS7 for the second dimension) show the most characteristic genes according to each dimension (correlation coefficient >|0.9|at $p<0.01$ for dimension 1 and >|0.8| at $p<0.01$ for dimension-2).

Dimension-1 corresponds to a significant over-representation in ST of genes involved in extracellular matrix organization (GO: 0043062) and in integrin signalling pathway (P00034). These genes encode proteins that are compounds of the extracellular region (GO: 0005576) and are represented by an important group of collagen coding genes (*COL1A2, COL1A1, COL16A1, COL5A2, COL3A1*) and by *SULF1* and *ECM2*. Genes encoding proteins involved in protein binding, *CDH11, ADAMTS1, FAP, SERPING1* and *SFRP1*, are also associated to ST. Finally, a set of metallopeptidases and other proteases coding genes (such as *ARHGAP10, MMP9, MMP19, C1R* and *C1S*) that are complementary to the previous ones for hydrolase activity (GO: 0016787) and tissue remodeling are also more expressed in ST. Both GE and LE are characterized by an over-representation for a first group of genes involved in cell junction (GO: 0030054) including *EPCAM* (epithelial adhesion molecule), *CDH1* (cadherin-1), *ITGB6* (integrin beta 6), *DSP* (desmoplakin) and *MYO5B* (myosin-VB). Other genes encoding proteins involved in binding are associated with the epithelial type (*RHPN2*, rhophilin-2, *DYNCI1*). Numerous genes over-expressed in epithelial cells are also closely associated to cellular response to stimulus (GO: 0051715) and signal transduction (GO: 0007165; *RAB25*, *F2RL1, ITGB6, LPAR3, KSR2 and ERBB3*). In addition, a large number of genes are involved in catalytic activity (GO: 0003824) such as enzymes of metabolism *GPT2, PLA2G4A, AKR1B* and *IDH1*. Others genes are associated to EGF signalling pathway (P00018), cell proliferation (*MAPK13*, *PEBP4*, *ERBB3*, *CCNA1* and *RAB25*) and transcription regulator activity (GO: 0140110; *DLX5*, *IRF6*, *KLF5, OCLN, HNF1B* and *EHF*).

When analyzing differences in expression between types of cells related to the second dimension, a set of 69 genes is over-represented in GE vs LE. An important part of these genes associates to structural cell organization. This includes genes such as actin-binding *VIL1* (villin-1) and numerous other encoding proteins involved in microtubule organization (GO: 0007017) including members of the dynein complex *DNAH5* (dynein heavy chain 5), *WDR63* (wd repeat containing protein 63), *CCDC65* (dynein regulatory complex subunit), *DRC1* (dynein regulatory complex protein 1) and *RSPH4A* (radial spoke head protein 4). In this category, one gap junction (*GJB5*) and 2 tight junctions (*CLDN10* and *CLDN8*) are specifically over-expressed in glandular epithelial cells. A complementary set of genes over-represented in GE relates also to binding (GO: 0005488) including protein binding (GO: 0005515), signalling receptor binding (GO: 00051102) and calcium binding (*IHH*), *WIF1* and *S100B*. Relatively few genes were more expressed in LE, the majority of them coding for proteins with catalytic activity (GO: 0003824) including hydrolase (*BACE2, RCAN1, TINAGL* and *LCAT*) and transferase (*GPCRC5A* and *LCAT*) activities. LE are also enriched in specific receptor related G-protein such as *HCRTR1* and *GPRC5A* (G-protein coupled receptors for orexin and retinoic acid) and receptor *SFRP4* which modulates Wnt signalling.

Differential gene expression between the three endometrial cell types in NEB cows

The principal component analysis reveals differences in gene expression patterns in MNEB and SNEB cows for the three cell types (Figure 4A). A clear separation between samples issued from the two groups of cows is observed in ST, whereas overlapping gene expression patterns appears in GE and LE. The numbers of differentially expressed genes between MNEB and SNEB cows for each endometrial cell type are given in Table 1 and in the Venn diagram (Figure 4B). The total number of DEGs in ST, GE and LE when comparing SNEB cows to MNEB cows were 1049, 24 and 52.

Expression	Cell types		
	ST	**GE**	**LE**
Over	751	15	1
Under	298	9	51
Total	1049	24	52

Table 1: Number of DEGs, which were identified as being over- or under-expressed, presented in specific endometrial cell types (ST, GE and LE) of SNEB cows when compared to MNEB cows

Seven DEGs are found as common in ST and GE: BTG Anti-Proliferation Factor 2 (*BTG*2), Lymphocyte Antigen 6 Family Member G6C (LY6G6C), C-C Motif Chemokine Ligand 4 (*CCL*4) and JunB Proto-Oncogene, AP-1 Transcription Factor Subunit (*JUNB*), chemokine (C-C motif) ligand 3 (*CCL3*), chromobox protein homolog 1 and one pseudogene (ENSBTAG00000047824). Three DEGs are common between ST and LE: CRK Proto-Oncogene, Adaptor Protein (*CRK*), Plexin Domain Containing 1 (PLXDC) and Myotubularin related protein 10 (*MTMR10*). None of the genes are common to all three cell types. The list of over- and under-expressed mRNAs in ST, GE and LE are given in sheets S8, S9 and S10 respectively of the additional file (TableS4_TS8_TS9_TS10_DEG-SNEBvsMNEB.xlsx). In SNEB animals, a large proportion of DEGs were identified as over-expressed in ST (72%) and GE (63%) whereas almost all DEGs were under-expressed in LE (98%) (Table 1). An overview of the differential patterns of gene expression in ST, GE, and LE obtained by LCM between SNEB and MNEB cows are illustrated in volcano plots (Figure 5A to 5C).

Under-expressed genes in ST (Table 2 and supplemental TableS5_david_ST-underexpressed.pdf)

Either by using the statistical over-representation test from PANTHER with reactome pathways annotation or by browsing pathways ontology classification, the analysis detected four main significant pathways from the 298 under-expressed genes. A first group of genes encode proteins that are involved in the regulation of interferon signalling as well as in inflammation mediated by chemokine and cytokine (P00031) (*RAPGEF1, MX1, EIF2AK2, UBA7, ISG15, PTPN2, MX2,*

DDX58, IL1RAP, IL16, CRK, IFIT1, STAT1, IFNGR2, JAK1, STX3, NFATC1 and *ALOX12*). A second important group of under-expressed genes code for proteins with functions associated with the extracellular matrix and its degradation *(KLK1, TPSB1, COL4A4, COL2A1, MMP19, NID1, COL6A6, COL4A3* and *COL26A1*). A third group of genes code for proteins related to Wnt signalling pathway (P00057) (*CDH11, TLE4, LEF1, NFATC1, PRKCH, SMARCD2* and *FBXW7*). In addition, genes of integrin signalling pathway (P00034) are over-represented including *ITGA5, ITGA10, RAPGEF1, MAP3K5* and *CRK*. Around 10% of under-expressed genes in ST from SNEB animals are genes involved in signal transduction (GO: 0007165) and cellular response to stimulus (GO: 0051716).

	annotation terms		genes (number)
PANTHER pathways	Regulation of IFNG signaling (R-BTA-877312) Cytokine Signaling in Immune system (R-BTA-1280215) Antiviral mechanism by IFN-stimulated genes (R-BTA-1169410)		16*
	Extracellular matrix organisation (R-BTA-1474244) Collagen chain trimerization (R-BTA-8948216)		9*
	Wnt signaling pathway (P00057)		8
	Integrin signaling pathway (P00034)		9
	Cadherin signaling pathway (P00012)		6
	Apoptosis signaling pathway (P00006)		4
PANTHER molecular function	Binding (GO:0005488)		80
		> protein binding (GO: 0005515)	53
		>cytoskeletal protein binding (GO: 0008092)	10
		>signaling receptor binding (GO: 0005102)	9
		>enzyme binding (GO: 0019899)	7
		>cell adhesion molecule binding (GO: 0050839)	5
	Catalytic activity (GO:0003824)		83
		>transferase activity (GO: 0016740)	36
		>hydrolase activity (GO: 0016787)	35
DAVID (6.8) clusters	cluster 1	GO:0005887, integral component of plasma membrane G-protein coupled receptor	9
	cluster 2	Immunoglobulin-like domain	6
	cluster 3	GO: 0016021 integral component of membrane	27

Table 2: Gene Functional Classification Result (PANTHER 14.1 and DAVID 6.8) of under-expressed genes in ST cells from SNEB animals. Main pathways and ontology annotation groups are shown. Asterix * indicates the significant (FDR P<0.05) over-representation statistical test.

Functional classification using DAVID identifies also a first cluster of nine genes encoding proteins including mainly G-protein coupled receptors (GO: 0005887; integral component of plasma membrane), which were under-expressed in ST from SNEB. Six genes encoding membrane proteins with immunoglobulin-like domains and related to cytokine are part of a second cluster and a last group includes 28 genes coding for component of membrane.

Over-expressed genes in ST (Table 3 and additional TableS6_david_ST-overexpressed.pdf)

The analysis from the GO molecular function annotation of PANTHER database indicates that 50% of the over-expressed genes from SNEB ST samples are distributed in three main categories: binding (GO:0005488) (n=186), catalytic activity (GO: 0003824) (n=130) and transporter activity (GO:0005215) (n=52). Binding categories includes cytoskeletal protein binding (GO: 0008092) (n=17), enzyme binding (GO: 0019899) (n=24) and signalling receptor binding (GO: 0005102)(n=21). Catalytic activity class includes genes involved in hydrolase activity (GO: 0016787) (n=57) and transferase activity (GO: 0016740) (n=47). In the transporter activity category 92% of genes are related to transmembrane transporter activity (GO: 0022857) and 8% to lipid transporter activity (GO: 0005319). Considering the PANTHER classification based on biological process annotation, the most frequently reported GO terms are cellular process (GO: 0009987; n=230), cell proliferation (GO: 0008283; n=105), metabolic process (GO: 0008152; n=101) and localization (GO: 0051179; n=72).

The analysis from PANTHER pathways revealed that genes from three significant pathways are over-represented in ST from SNEB vs MNEB cows including: (i) genes related to inflammation mediated by chemokine and cytokine signalling pathway (P00031; *CAMK2B, PLCB4, PRKCZ, PAK4, MYH14, JUNB, ACTA1, MYH11, CCL4, CCL3, ITPR2, PLCH1* and *CCL11*), (ii) genes involved in Wnt signalling pathway (P00057; *FZD5, PLCB4, CDH3, PRKCZ, CDH1, ACTA1, CTBP2, ITPR2, FRZB,* and *ANKRD6*) and (iii) genes associated to integrin signalling pathway (P00034; *ITGB4, FRK, RAP2A, ITGB6, FLNA, COL4A6, ACTA1, FLNB* and *COL4A5*). In addition, a positive enrichment was detected for genes related to the sequestration of calcium ion (GO: 0015278) and for genes related to cytoskeleton, dynein complex and axoneme.

Using medium stringency for functional classification of genes, DAVID further identified 15 clusters. According to ranking from enrichment score the top 11 main clusters group include (i) five genes involved in microtubule and axoneme assembly (GO: 0005874, microtubule; cilium; axoneme), (ii) 15 genes related to homeodomain (GO: 0043565), (iii) four genes of myosin complex (GO:0016459), (iv) 6 genes for calcium ion binding, (v) nine genes related to ankirin repeat, (vi) five genes for regulation of Rho protein signal transduction (GO: 0005089), (vii) 13 genes related to extracellular region of the cell, (viii) seven genes for nucleotide and mRNA binding (GO: 0000166), (ix) four genes for protein kinase activity (GO: 0004672), (x) 11 genes related to products being integral components of plasma membrane (GO: 0005887) and (xi) 109 genes coding for membrane associated proteins (GO: 0016021).

	annotation terms	genes (number)
PANTHER pathways	Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	15
	Wnt signaling pathway (P00057)	12
	Integrin signalling pathway (P00034)	11
PANTHER molecular function	protein binding	121
	>cytoskeletal protein binding (GO: 0008092)	18
	>enzyme binding (GO: 0019899)	25
	>signaling receptor binding (GO: 0005102)	22
	catalytic activity	130
	>hydrolase activity (GO: 0016787)	57
	>transferase activity (GO: 0016740)	47
	>oxidoreductase activity (GO: 0016491)	17
	transporter activity (GO: 0005215)	52
	>transmembrane transporter activity (GO: 0022857)	48
	calcium-release channel activity (GO: 0015278)	5 *
	sequestering of calcium ion (GO: 0051208)	8 *
	plasma membrane bounded cell projection cytoplasm (GO: 0032838)	10 *
PANTHER cellular component	cytoskeleton (GO: 0005856)	45 *
DAVID (6.8)	cluster 1 microtubule ; Cilium ; axoneme (GO: 0005874)	5

clusters	cluster 2	sequence-specific DNA binding ; Homeodomain (GO: 0043565)	15
	cluster 3	myosin complex (GO: 0016459)	4
	cluster 4	calcium ion binding (GO: 0005509)	6
	cluster 5	Ankyrin repeat	9
	cluster 6	Rho guanyl-nucleotide exchange factor activity (GO: 0005089)	5
	cluster 7	extracellular region (GO: 0005576)	13
	cluster 8	nucleotide binding ; RNA recognition motif domain (GO: 0000166)	7
	cluster 9	protein kinase activity (GO: 0004672)	4
	cluster 10	integral component of membrane (GO: 0016021)	130
	cluster 11	GTP binding (GO: 000552)	4
	cluster 12	Immunoglobulin-like domain	7

Table 3: Gene Functional Classification Result (PANTHER 14.1 and DAVID 6.8) of over-expressed genes in ST cells from SNEB animals. Main pathways and ontology annotation groups are shown. Asterix * indicates the significant (FDR P<0.05) over-representation statistical test (only positive enrichment is shown)

Differential expression in GE (Table 4, Table 5 and additional TableS7_david_GE-overexpressed.pdf)

Only seven known genes are under-expressed in GE cells from SNEB cows when compared to MNEB ones (*CDH18, PPP1R1C, LY6G6C, MT1E, ASB16, PROM2* and *SESN2*). Four are related to binding functions (*CDH18, PROM2, SESN* and *MT1E*) and/or to cell surface component (*CDH18, PROM2* and *LY6G6C)*. Due to the very small number of under-expressed genes, no functional cluster is identified from DAVID. Among the 15 over-expressed genes, two pathways are over-represented. These genes are equivalently present in two of the three clusters defined by DAVID. Four genes (*JUNB, CCL2, CCL4* and *CCL3*) relates to inflammation mediated by chemokine and cytokine signalling pathway (P00031). Three genes encoding immediate-early transcription factors (*FOS, JUNB* and *ATF3)* are over-expressed and associated with two annotation terms: RNA polymerase II proximal promoter sequence-specific DNA binding (GO: 0000978) and Gonadotropin-releasing hormone receptor pathway (P06664).

	annotation terms	genes (number)
PANTHER molecular function	binding (GO: 0005488)	4
	catalytic activity (GO: 0003824)	1
PANTHER Cellular component	cell surface (GO: 0009986)	3*
DAVID (6.8)	no cluster	

Table 4: Gene Functional Classification Result (PANTHER 14.1 and DAVID 6.8) of under-expressed genes in GE cells from SNEB animals. Main pathways and ontology annotation groups are shown. Asterix * indicates the significant (FDR P<0.05) over-representation statistical test.

		annotation terms	genes (number)
PANTHER Molecular function and pathway		RNA polymerase II proximal promoter sequence-specific DNA binding (GO:0000978)	3*
		Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	4*
		cytokine activity (GO: 0005125); cytokine receptor binding (GO: 0005126)	
		Gonadotropin-releasing hormone receptor pathway (P06664)	3*
		protein binding (GO: 0005515)	7
		heterocyclic compound binding (GO: 1901363)	4
		catalytic activity (GO: 0003824)	5
DAVID (6.8)	cluster 1	positive regulation of inflammatory response (GO: 0050729) ; chemokine-mediated signaling pathway (GO: 0070098)	3
	cluster 2	RNA polymerase II core promoter proximal region sequence-specific DNA binding (GO: 0000978)	4
	cluster 3	integral component of membrane (GO: 0016021)	3

Table 5: Gene Functional Classification Result (PANTHER 14.1 and DAVID 6.8) of over-expressed genes in GE cells from SNEB animals. Main pathways and ontology annotation groups are shown. Asterix * indicates the significant (FDR P<0.05) over-representation statistical test (only positive enrichment is shown)

Differential expression in LE (Table 6 and additional TableS8_david_LE-underexpressed.pdf)

In LE samples, only *B4GALT5* is over-expressed in SNEB. No significant enriched GO terms is related to the 55 under-expressed DEGs at FDR p value <0.05. By raising the FDR p value at 0.25, over-represented DEGs corresponds to biological processes associated with complement activation, B cell mediated immunity, defense response to bacterium, cell differentiation and cellular component link with plasma membrane and organelle.

		annotation terms	genes (number)
PANTHER Molecular Function		binding (GO:0005488)	13
		catalytic activity (GO:0003824)	11
		molecular transducer activity (GO:0060089)	5
		molecular function regulator (GO:0098772)	4
DAVID (6.8)	cluster 1	integral component of membrane (GO:0016021)	11

Table 6: Gene Functional Classification Result (PANTHER 14.1 and DAVID 6.8) of under-expressed genes in LE cells from SNEB animals. Main pathways and ontology annotation groups are shown. Asterix * indicates the significant (FDR P<0.05) over-representation statistical test (only positive enrichment is shown)

cell type	under/over	KEGG Pathway Id	pathway name	genes
Stromal cells	over-expressed	map04020	calcium signaling	P2RX3, ITPKA, ITPR2, CHRM3, ERBB3, CAMK2B, PLN, PLCB4, SLC25A4, HTR2A, MYLK, ADCY8, TACR1, PTGFR, PTGER3, RYR3, GRIN1
		map04530	tight junction	OCLN, IGSF5, MYH14, CLDN23, PRKCZ, MYH11, CGN, TJP3, LLGL2, CLDN8, CLDN3
	under-expressed	map05162 map05164	measles and influenza A	JAK1, DDX58, ADAR, STAT1, IFIH1, EIF2AK2, IFNGR2, MX1, OAS1Z, OAS1Y, IRF7
Glandular cells	over-expressed	map04010	TNF signaling	FOS, SOCS3, JUNB, CCL2

Table 7: The significant KEGG pathways with over- or under-expressed DEGs for three endometrial cell types (ST, GE and LE) were identified using DAVID database (adjusted p-value < 0.05).

***KEGG pathway analysis of the DEGs*.**

Significantly enriched KEGG pathways from DAVID database were found in GE and ST, whereas no significant KEGG pathway was detected in LE. In ST cells, DEGs between SNEB and MNEB cows were significantly enriched in four different KEGG pathways. 25 KEGG pathways were recognized by David with the overexpressed genes. Two were found significantly enriched. They are related to calcium signalling pathway (KEGG map04020, fold enrichment = 3.4; 17 DEGs) and tight junctions (KEGG map04530; fold enrichment = 4.8; 11DEGs. With under-expressed DEGs, two KEGG pathways associated with viral infectious diseases (KEGG “measles” map05162 and KEGG “Influenza A” map05164; fold enrichment respectively = 5.0 and 4.1; 11 DEGs) are overrepresented (Table 7). The names of these two KEGG pathways do not make sense with endometrial physiology. The genes of these pathways are known to be important partners of interferon signalling that is a critical mechanism for establishment of pregnancy (reactome pathways: BTA-913531, BTA-877312). For glandular epithelium, over-expressed DEGs matched to 10 overrepresented KEGG pathways. The KEGG TNF signalling pathway (KEGG map04010) was the only one found to be significantly enriched (Fold enrichment = 21.5). In contrast, no enriched KEGG pathways were found from the set of under-expressed DEGs.

The corresponding STRING-generated interaction network obtained from DEGs belonging to the 5 KEGG pathways associated to ST and GE cells revealed strong interactions (PPI enrichment value < 1.0E-16) between these sets of DEGs that are related to the JAK/STAT signalling (Figure 6).

DISCUSSION

During negative energy balance (NEB), lipolysis in adipose tissue is increased resulting in decreased BCS and increased NEFAs in blood [31]. Changes in BCS and NEFA concentrations were correlated with NEB nadir and plasma NEFA concentrations in SNEB cows were greater than in MNEB cows in the prepartum and early post-partum. Both observations are consistent with earlier findings [32] and shows that the two groups were in a different metabolic status before and during the two first weeks post-partum. The impacts of NEB on bovine reproductive performances are well documented [33]. A wealth of information illustrates the negative effects of NEB and NEFA on ovarian cells [34], embryos [35] and oviduct [36]. On the contrary, relatively few publications have reported effects of NEB on the endometrial tissue or cells. *In vivo* studies showed that NEB had negative impacts on endometrial function through the alteration of immune response and activation of pro-inflammatory and IGF-insulin signalling pathways [37, 38]. However, in those studies information was obtained from full tissue and to our knowledge, the present study is the first time that the specific effects of NEB on the three main cell types of the endometrium are reported.

Transcriptome of the three endometrial cell types

Our results fully confirm that stromal cells, glandular and luminal epithelial cells reveal specific molecular signatures as documented before in studies using LCM in human [39], sheep [26] and horse [40]. Our results based on biopsies collected in the luteal phase, have shown that a higher number of genes with a strong constitutive expression in stromal cells compared with epithelial cells (either glandular or luminal) are different from the expression pattern observed at the beginning of pregnancy [40]. This may result from differences between species but could also reveal the changes induced by the conceptus on the endometrial transcriptome previously reported from full tissue [37, 41] and epithelial cells [26].

Using a cut-off of 10 TPM, different numbers of genes were expressed in the three endometrial cell types. ST expressed 5% and 25% more genes than GE and LE, respectively. However, as reported before from a large variety of tissues [42], and the three laser-dissected cell types of porcine endometrium [43], our results confirm that a high number of genes are expressed in common in different endometrial cell types. In the present study, 70 to 85% of genes were expressed in all cells suggesting either "house-keeping" functions or genes encoding proteins with functions common to the endometrium while lower proportions (5%, 7% and 15% for LE, GE and ST, respectively) were restricted to each cell type indicating that they code for proteins supporting the functional specialized signature of each cell type. When compared to porcine endometrium [43], the number of genes showing cell-specific expression is in the same order of magnitude for GE and LE, but appears different for ST cells where this number is ten times higher. These differences in specific expression between cell types, especially the large number of functions enriched in ST are well reflected by the REVIGO analysis (Figure 4).

Regardless of the cut off chosen and related limitations, these studies illustrate huge differences in the gene expression patterns between cell types corresponding to specialized functions. This confirms that separating cell types is more appropriate and possibly less biased to decipher the impacts of any factor on a given tissue than former approaches based on full tissue. The clear clustering obtained when analysing the full transcriptome, indicates that luminal and glandular epithelial cells are closely related. These similarities may reflect common functional properties and/or may be related to the common epithelial nature of these cells. The genes associated with GE and LE, which distinguish these two epithelial cells from stromal cells, are all related to GO terms typical of epithelia (GO: 0030855, epithelial cell differentiation; GO: 0060429, epithelium development; GO: 0045216, cell-cell junction organization). Examples are given below for critical genes previously cited as key regulators of endometrial epithelial cells. *CDH1* is involved in organization of epithelium in mouse and its ablation causes the absence of endometrial glands. Occludin is an important protein for tight junction assembly which preserves the epithelial barrier function. The REVIGO analysis showed that in both epithelial cell types, genes encoding proteins related to metabolism were under-represented.

On the contrary, genes related to cilium function are enriched in GE, whereas those involved in binding/ receptor function and adhesion are over-represented in LE. In addition, LE cells differentiate from GE by the expression of genes like *CYP26A1*, that encodes a key enzyme of trans retinoic acid inactivation, already shown as strongly expressed in luminal epithelial cells of rat endometrium and playing a role in embryo implantation [44]. Endometrial expression of *HCTR1* has been reported to be, with its main ligand orexin-A, an important local regulator of endometrial functions in porcine uterus [45, 46].

As mentioned above for GE and LE, the REVIGO analysis showed that genes involved in metabolism were also under-represented in ST. In contrast, a very large number of functions including but not limited to, cell structure, angiogenesis, extra cellular matrix and immunity are enriched in ST whereas a lack of strong expression of these genes is observed in GE and LE. As awaited, among the genes most discriminating stromal cells, those involved in the production of extracellular matrix and collagen are highly represented in ST. *COL1A2, COL3A1, COL7A1* and *COL3A3* encode proteins that are involved in dynamic remodeling of endometrial extracellular matrix in cattle and regulate embryo receptivity [47]. Our data identified genes associated with extracellular matrix organization that had not been previously described in bovine endometrium including *LOXL2*, responsible for the cross-linking of collagen and elastin [48], *ECM2* involved in the regulation of cell proliferation and differentiation [49] and *CRISPDL* Lknown to regulate extracellular matrix and branching morphogenesis [50]. These genes encode proteins that may have an important role in the formation of glands and vasculature in bovine endometrium as well as *WT1,* already known to be preferentially expressed in stromal endometrial cells [51, 52].

We identify here also original genes related to stromal cell differentiation and cell migration such as *CDH11, PRELP, THY1* (the latter encoding a stem cell marker) [53], *GJA1* [54], *OSR2* [55], *P4HA3. PRLEP* gene expression has been reported to be regulated by the embryo in the bovine oviduct [56]. Contrary to the porcine endometrium where its expression was located in epithelial cells, *NTRK2* was mainly expressed here in stromal cells [57]. The expression of the NTRK2 gene, which encodes the receptor of brain derived neurotrophic factor, is conserved in mammalian uterus but its signalling function is not yet understood in the female reproductive system [58]. Genes known to be key regulators of uterine receptivity in different species such as, *HOXA10* and *HOXA11* belong also to the top list of 50 genes which characterize ST (human [59], mice [60] and goat [61]). This list includes *CALPAIN7* [62] and *SNAI2* [63] which are involved in embryo attachment and implantation and the disintegrins and metalloproteases *ADAMTS1* and *ADAM23* which are genes encoding key molecules for bovine endometrial remodelling [64]. In addition, a group of stromal genes including *SERPING1* [65], *C1R*, *C1S* [66], *SFRP1* and *IGF1* are involved in embryo maternal immune modulation and IFN response.

Finally, among these first 50 genes that best separate ST from epithelial cells, numerous ones have not been described so far in the mammalian endometrium. For instance, we could not find any information on the expression and function in the endometrium of the following genes and their encoded proteins: *MUSTN1, OSR2, TGM2, PCDH9, PGM5, MXRA5, MAMDC2, MRGPRF*, RASD2, *SULF1, RASL11A, ECM2, OLFML3* and *P4HA3*. These results may help to formulate new hypotheses for exploring new biological roles for stromal genes.

Impact of NEB on the three endometrial cell types

Overall, our results show that NEB impacts mainly ST whereas GE and LE cells are less affected. More than 10% (13%) of the total number of genes expressed in ST were impaired by NEB status while less than 1% were affected in GE and LE (0.3% and 0.7% respectively). When considering the sub groups of genes showing a specific expression related to cell type, NEB did not affect any of those in GE and modified only the expression of *TCN1* and *B4GALT5* in LE cells. This number is probably under-estimated in LE due to the comparison restricted to a single sample in the SNEB group. By contrast, a relatively high number of genes (about 8%; n=91) specifically expressed by ST are affected by NEB.

Impact of NEB on genes related to cytoskeleton and cell adhesion. Genes encoding tropomyosins (*TPM1, TPM2*) and myosins (*MYO5C, MYO5B*) proteins which are structural constituents of cytoskeleton (GO: 0016459) were over-expressed in ST of SNEB cows. Similar over-expression of tropomyosins and myosins has been reported in the endometrium of fertile cows [67]. The increased expression of myosins was associated to over-expression of genes of the dynein family (*DNAH5, DNAH7, DNAH11, DYNC1I1* and *DYNLRB2*) which encode proteins that are involved in cell mobility (GO: 0005874). The signification of these changes in the context of fertility deserves further investigations. In contrast, a large set of genes related to cell adhesion and cell-cell and cell-extracellular matrix adhesion [68], such as integrins (*ITGA5*, *ITGA10*), cadherins (*CDH2, CDH11, CDH12*), *AGRN*, *EGFLAM, TGFBI*, type IV collagen (*COL4A4*), type VIII collagen (*COL8A1*), *ODZ3, SCARB2* and *WISP3* were under-expressed in ST of SNEB cows. The lower expression of integrins could be seen as unfavourable to establishment of pregnancy. In humans, *ITGB3* mRNA has been cited as a positive marker associated with pregnancy [69, 70]. In sheep, elevated expression of *ITGAV*, *ITGA4*, and *ITGA5* in GE have been found during pregnancy [71]. E-cadherin (*CDH1*) has been documented as a critical gene for embryo implantation as its under-expression in epithelial cells allows endometrial cells dissociation following blastocyst invasion [72]. Moreover, an increased expression of type IV collagens has been identified in endometrium of low fertility heifers [73], however, the opposite trend was found here in SNEB cows. In ST from the SNEB group, genes belonging to the Wnt pathway (P00057) were either over expressed (*ACTG2, FZD5, PLCB4, CDH3, PRKCZ, CDH1, ACTA1, CTBP2, ITPR2, FRZB, ANKRD6* and *ACTA2)* or under expressed (*CDH11, TLE4, LEF1, NFATC1, PRKCH, SMARCD2* and *FBXW7).* These genes encode proteins that are associated with GO: 0001763 (morphogenesis of a branching structure) GO: 0001944 (vasculature development) including involvement in the morphogenesis and function of the endometrial glands [74, 75] as well as in the development of uterine vasculature [76]. The altered expression of these genes by the NEB can have a critical role in the regeneration of the endometrium during the postpartum period.

Impact of NEB on genes related to energy metabolism. In SNEB cows, among the 700 genes that are over-expressed in ST, a large proportion were genes classified to encode proteins related to metabolic process (GO: 0008152), macromolecule metabolic process (GO: 0043170) and organic substance metabolic process (GO: 0071704). DEGs were most particularly related to catalytic activity (GO:

0003824) revealing the breakdown of nutrient molecules to supply energy to cells. This suggest that SNEB cows still presented an energy deficit in endometrial cells at time planned for breeding, despite that energy balance is progressively restored. SNEB cows presented also an increased expression of many genes encoding proteins with functions related to lipid metabolism (fatty acids, triglyceride and cholesterol metabolic processes) such as *ACSM3, CPT1B, LPL, PPARGC1A, PRKAA2, GGT1, PLA2G10, CYP2B6, CYP2C18, HACD1, SLC27A6* and *PLIN4* in ST. Four of them *CYP2B6, CYP2C18, PLA2G10,* and *GGT1* are involved in arachidonic acid (AA) metabolism. While the release of arachidonic acid following phospholipase activation is usually engaged in the production of endometrial prostaglandins via cyclooxygenases enzymes, the conversion of AA by CYP enzymes contribute to oxidative stress and inflammation and may not be favourable to endometrial function [77]. The receptivity of fibroblasts to prostaglandins could also be modified through their receptors with the observed extreme over-expression of *PTGFR* mRNA (the second top of over-expressed DEGs in ST) and *PTGER3*. The over-expression of *SLC27A6,* a fatty acid binding protein (FABP) [78] and *PLIN4,* which controls intra-cellular lipid droplet-associated proteins, are consistent with earlier findings in obese mice and human [79, 80]. Our data showing associations between over-expression of these genes with increased plasma NEFA concentrations are consistent with the over-expression of genes of the PLIN family found in the endometrium of low fertility heifers [73]. Taken together, this information suggests that up-regulation of genes involved in lipid uptake in ST of SNEB cows, associated with elevated NEFA concentration during the peri-parturient period may not be favourable to fertility in postpartum cows. Increased gene expression from the solute carrier family in ST from SNEB cows (such as *SLC2A12, SLC45A2* and *SLC35A3*), which encode proteins involved in carbohydrate transportation, could be seen as a compensatory mechanism as the under-expression of the glucose transporter (*SLC2A1*) mRNA was detected in endometrial tissue of subfertile dairy cows [81].

Impact of NEB on genes related to growth factors. Interestingly, expression of genes associated with IGF-insulin signalling, such as *IGF1R* and *IGF2BP2*, was higher in SNEB cows. On the contrary, *IGFBP2, GDF6, EDIL3* and *TGFBI* were under-expressed in ST of SNEB cows. The expression of

IGFs were detected in the uterine stroma especially the caruncular areas of cyclic cows [82]. As suggested in the above-referred study and by others [38], the dysregulation of genes related to insulin-like growth factors function may delay tissue remodelling during the postpartum period. In our study, the importance of those changes on matrix metalloproteinase (MMP) appeared limited as only one gene of the MMPs family (*MMP19*) was under-expressed in ST of SNEB cows. However, 9 closely related genes involved in the degradation of the cellular matrix and tissue remodelling were also under-expressed in the SNEB cows. On the contrary, growth factor receptors such as *GRB7, GRB14* and *FGFR2*, which are known as stromal-derived paracrine stimulators of epithelial proliferation, were over-expressed in ST of SNEB. This increase may be a mechanism for compensating endometrial epithelial defects in order to achieve uterine receptivity [83]. In bovine species, gene expression of FGFs and their receptors is upregulated during pregnancy and these factors stimulate interferon-tau (*IFN-T*) production during the pre-attachment phase of conceptus development [84]. The increase of transcripts encoding proteins of the cyclin family (*CCND3* and *CCNB1*) in ST of SNEB cows may also be associated with the modifications of proliferative properties and tissue differentiation in the endometrium for preparing embryo implantation [85]. Our results show that NEB status influences both the over-expression and under-expression of different and numerous growth factors. However, further studies are needed to decipher the consequences of these changes and how they may affect fertility.

Impact of NEB on genes related to inflammatory responses. Nearly 20 genes belonging to two pathways [cytokine signalling in immune system pathway (R-BTA-1280215) and inflammation mediated by chemokine and cytokine signalling pathway (P00031)] displayed reduced transcripts in ST of SNEB. Among these genes *JAK1* and *STAT1* have been associated with both IFN-γ and IFNα/β endometrial receptors [86]. It may be hypothesized that the reduced-expression of *JAK1* and *STAT1* may alter JAK/STAT signalling and immune response in stromal cells. Indeed, a large number of IFN-inducible genes (R-BTA-877312), such as *MX1, MX2, IFI44, IFI6, IFIH1, IFIT1, IFITM2* and *IFNGR2* were under- expressed in ST of SNEB cows. These findings are different from previous observations showing over-expression of *MX1* and *MX2* genes in the full endometrium of SNEB cows during early postpartum [37]. The specificity of stromal cell response to SNEB, may explain differences between studies, however due to the lack of effect on GE, these discrepancies may result also from differences in time postpartum and severity of NEB. The glandular epithelium plays a major role in the activation of the innate immune system as reviewed by [87]. In our study, most of the DEGs in GE related to chemokines, immune response processes, TLRs and TNF signalling pathways, such as *CCL2, CCL3, CCL4, CCL11, FOS, JUNB,* and *SOCS3* were strongly over-expressed in SNEB cows. Some of those genes belonged to the C-C motif chemokine ligands (CCLs) family and play an important role in monocyte recruitment in the endometrium [88]. Increased expression of *CCL2* mRNA was found associated with lipid accumulation induced uterine inflammation in obese rats [89]. The present results are similar with previous studies performed with full endometrial tissue, showing the up-regulation of inflammatory response genes in SNEB cows [38]. This is also consistent with several studies in mammals showing that metabolic imbalance, increased lipolysis and most particularly NEFAs, play essential functions in the activation of TNF and TLRs signalling to promote the release of pro-inflammatory molecules [90, 91]. Taken together, these studies and our present findings suggest that SNEB and NEFAs activate pro-inflammatory pathways in the glandular epithelium and stromal cells. On the contrary, in luminal epithelium, the adaptive immune response (B cell-mediated immunity) and innate immunity, was represented by under-expressed genes such as tracheal antimicrobial peptide (*TAP*), a beta-defensin gene, which was associated to the NF-κB pathway [92], and by genes coding for immunoglobulin heavy variable chains that participates in the antigen recognition. These observations need further confirmation. Our results indicate that SNEB induces changes in immune responses, which are different in the three endometrial cell types. They show also that these changes are still present, long after NEB has disappeared suggesting long term effects of metabolic imbalance and NEFAs on the pro-inflammatory status of the glandular epithelium and the stroma.

Effect of NEB on genes related to maternal-conceptus recognition. A large set of IFN-inducible genes such as *MX1, MX2, STAT1, JAK1, IFIH1, IFNGR2, ISG15, LY6G6C, OAS1Y, OAS1Z* and *IRF7* were under-expressed in ST of SNEB cows. A weaker expression of those genes that encode proteins involved in IFN-T signalling could account for the decreased endometrium-related fertility in SNEB cows. In pregnant ruminants, IFN-T is the main pregnancy recognition signal [93], that allows the persistence of the corpus luteum and maintaining elevated progesterone concentrations by blocking oxytocin signalling and PGF2α secretion [94]. Oxytocin signalling has been associated with the maintenance of gap-junctions in luteal tissue [95] and intracellular calcium release in endometrial cells [96]. Differentially expressed genes and our STRING protein-protein network revealed in ST of SNEB cows showed an increase in expression of six genes encoding proteins belonging to the oxytocin signalling pathway namely *PLCB4, ADCY8, CAMK2B, ITPR2,* and *MYLK* (Figure 6). These changes are consistent with the over-expression of 10 genes related to tight junction such as *MYH14, MYH11, PRKCZ, OCLN, CGN, IGSF5, TJP3, CLDN3, CLDN8* and *CLDN23*. Our data suggest that in ST of SNEB cows, the over- representation of oxytocin signalling and tight junction pathways results from the decreased expression of IFN-T inducible genes. The changes in ST are consistent with downstream changes related to PGF2α produced by both endometrial epithelial and stromal cells [97]. Furthermore the deregulation of this signalling pathway in SNEB cows is supported by changes in PTGFR which was over-expressed in ST but under-expressed in GE. In addition, other important genes encoding proteins with established functions critical for implantation such as *IL1RAP, SOSC3* and *AREG* were found differentially expressed in SNEB cows. We observed a lower expression of the *IL1RAP* gene in ST of SNEB cows. The IL1RAP protein is a necessary part of the interleukin 1 receptor complex and is regulated by interleukin 1 beta (IL-1β). The over- expression of *IL1R* and *IL1RAP* under IL-1β regulation has been reported in the pig endometrium at day 12 of pregnancy to stimulate the expression of *PTGS1* and *PTGS2* genes which encode key enzymes for PGE2 and PGF2α synthesis [98]. Blocking IL1R signalling with an IL-1 receptor antagonist led to implantation failure in mice [99]. The reduced expression of *IL1RAP* in ST of SNEB cows may compromise the establishment of pregnancy, but this deserves further investigation in the cow. SOCS family genes (*SOCS1-7*) inhibit cytokine signalling through the JAK–STAT pathway and regulate IFNs, growth factors and hormones which are critical for implantation [100]). *SOCS1-3* mRNAs are over-expressed at time of implantation in the endometrium of pregnant cows and their expression was induced by IFN-tau in endometrial cells in vitro [101]. The over-expression of *SOCS3* mRNA in GE may contribute to down regulate the JAK/STAT pathway in the neighbouring ST cells, as reported above. *AREG* was over-expressed in GE of SNEB cows. *AREG* gene is known as an epidermal growth factor receptor and is involved in cell growth, proliferation, differentiation and migration. It is highly expressed in luminal and glandular epithelium during the secretory phase of menstrual cycle and early pregnancy in human and primate [102]. As for *SOCS3,* it could be speculated that the over-expression of *AREG* mRNAs in GE may be part of a compensatory mechanisms in response to the increased expression of cytokines in these cells. It would be interesting to compare the amplitude of over-expression of *SOCS3* and *AREG* in the present situation (luteal phase under cyclic conditions) and in pregnancy to evaluate possible impacts of NEB on implantation.

Conclusion

The present study provides novel and specific information about gene expression in three endometrial cell types from postpartum dairy cows and illustrates specific signatures in ST, LE and GE cells. We also show that the impacts of negative energy balance on the gene expression of endometrial cells are cell type specific. Major and specific changes in gene expression were observed in stromal cells illustrating dysregulation of metabolic processes especially lipid and carbohydrate metabolism, cytoskeleton and cell adhesion properties. Altered gene expression of endometrial epithelial cells under SNEB condition was related to activation of pro-inflammatory responses via chemokine pathway in GE, whereas down-regulation on adaptive immunity and defence mechanism were found in LE. Strong changes in the expression of genes involved in prostaglandin production and maternal-conceptus recognition was found in ST and in GE. Considering the above and the crucial role of IFN-tau for embryo implantation and maintenance pregnancy, our hypothesis is that the under-expression of IFN-tau responsive genes associated with the increased expression to oxytocin and PGF2α related genes may be detrimental for the establishment of pregnancy in SNEB cows. The changes in gene expression induced by NEB in LE should be considered as preliminary and needs further confirmation whereas the specific response of ST and GE to NEB paves the way for functional studies relating the importance of these changes for the establishment of pregnancy

Abbreviations

BCS:	Body condition score
CIDR:	Controlled Internal Drug Release)
DAVID:	Database for annotation, visualization and integrated discovery
DEG:	Differentially expressed gene
EB:	Energy balance
ECM:	Energy-corrected milk
Elisa:	enzyme-linked immunosorbent assay
FDR:	False discovery rate
GE:	Glandular epithelial cell
GO:	Gene ontology
KEGG:	Kyoto encyclopedia of genes and genomes
LCM:	Laser capture microdissection
LE:	Luminal epithelial cell
MNEB:	Mild energy balance
NEB:	Negative energy balance
NEFAs:	Non-esterified fatty acids
NorFor:	Nordic Feed Evaluation System
OCT:	Optimal cutting temperature coumpound
PANTHER:	Protein analysis through evolutionary relationships
PCA:	Principal Component Analysis
PGE2:	Prostaglandin-E2
PGF2α:	Prostaglandin-F2α
REVIGO:	Reduce, visualize gene ontology

RFI: Residual feed intake

RNA: Ribonucleic acid

RNA-Seq: RNA sequencing

SRB: Swedish Red breed

ST: Stromal cell

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

TPM: Transcripts per million

Declarations

Ethics approval and consent to participate

All experimental protocols were approved by the Uppsala Animal Experiment Ethics Board (application C329/12, PROLIFIC)(Uppsala University, Sweden) and were carried out in accordance with the terms of the Swedish Animal Welfare Act. After the study was completed, all cows were kept alive under normal husbandry conditions.

Consent for publication

« Not applicable »

Availability of data and materials

The data will be deposited pending acceptance of publication. The datasets generated and/or analyzed during the current study will be available in the [NCBI/Gene expression omnibus)] repository, [https://www.ncbi.nlm.nih.gov/geo/info/seq.html]

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

W.C., P.H. and G.C. contributed to the conception and design of the study. S.L., M.R., C.R., C.B. and T.N. contributed to sample collection and preparation. G.C., D.M., Y.G., W.C. and P.H. performed bioinformatics analysis and integration of data. W.C. performed the experiment, sample collection and preparation, data analyses and W.C., G.C., and P.H. drafted the manuscript. All authors provided critical feedback and helped shape research, analyses and manuscript. GC and PH are both senior co-authorship.

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Authors' information (optional)

Methods

Animals and experimental design. This study was approved by the Uppsala Animal Experiment Ethics Board (application C329/12, PROLIFIC). After the study was conducted all cows have been kept in usual farm living conditions. The animals used in this study were second lactation cows of the Swedish Red breed (SRB; n = 12) fed two different diets *i.e.* i) high-energy diet (control, n=6) targeting 35 kg energy-corrected milk (ECM) and ii) low-energy diet targeting (n=6) 25 kg energy-corrected milk (ECM) which was achieved by giving to these cows 50% concentrate. All cows were conducted at the Swedish Livestock Research Centre in Lövsta, Uppsala, Sweden. For each cow, the differential diets were given between 30 days prepartum and 120 days postpartum. The animals were kept in a loose housing barn with a voluntary milking system (VMS, DeLaval, Tumba, Sweden), and had free access to drinking water. The dietary details and management conditions were previously described [32]. During the experiment, consumption of concentrate was individually adjusted with an automatic feeding machine while forage was fed *ad libitum*. At day 60 after calving, estrous was synchronized using an intra-vaginal progesterone device (CIDR, Zoetis, Parsippany, NJ, USA) for a week followed by i.m. injection of 500 µg of prostaglandin analog (Estrumate®, MSD animal health, Madison, NJ, USA) intramuscular as described [103]. Fifteen days after visual oestrus detection, endometrial tissue biopsies were collected under epidural anesthesia with 0.5 mg/kg of 1% lidocaine hydrochloride (1% Xylocaine®, Astra Zeneca, Cambridge, UK). Timeline for samplings and analysis of phenotypic responses are presented in supplemental Figure.S1.

Energy balance (EB) calculation and classification. The energy balance (EB) (residual feed intake (RFI) expressed in MJ/day) was calculated as the difference between energy consumed and energy used for milk production, body maintenance, growth and pregnancy for each individual cow. Calculations were performed once per week from first week after calving to day 120 as described in [104]. All data used were routinely recorded in the university herd and energy balance calculation was performed with NorFor used as the reference system in the Nordic countries. Based on most differentiated EB profiles, nine out of twelve cows were classified into two NEB groups with either a mild negative energy balance (MNEB) group (n = 5) or a severe negative energy balance (SNEB) group (n = 4). Residual feed intake values in the first week postpartum of these nine cows ranged from -52.77 to 21.26 MJ/day and means (± s.e.m.) of 1.30 ± 6.35 and -29.48 ± 7.10 MJ/day were observed in the MNEB and SNEB groups, respectively.

Body condition score (BCS) and plasma NEFA measurements. Body condition score (BCS) was evaluated and recorded by the same person every two weeks, from 30 days prepartum until 120 days postpartum. BCS was used on a 5 point scale with 0.5 point increments, 1 = very lean to 5 = fat [105]. Blood samples were taken every two weeks from the coccygeal vein in EDTA containing tubes (BD Vacutainer, Kremsmünster, Austria) from 30 days prepartum to 56 days postpartum and then centrifuged at 4000 g for 10 min at 4°C. Following centrifugation, plasma samples were distributed into 0.5 mL aliquots and stored in -20° C until NEFA analyses were performed. NEFA concentrations were measured in duplicate by using a non-esterified fatty assay kit (Bio Scientific Corporation, Austin, TX, USA) with detection range 0 – 4 mM. The intra- and inter-assay variability was 4.19 ± 3.99% and 2.63 ± 1.08%, respectively.

Milk progesterone measurements and estrous cycle stage at time of biopsies. Whole milk samples were collected by the automatic milking machine, VMS (DeLaval) three times per week from Day 7 to Day 120 after calving. Milk progesterone concentrations were measured with a commercial enzyme-linked immunosorbent assay (ELISA) (Ridge way 'M' kit, Ridgeway Science, Gloucester, UK) as previously published [32]. The progesterone concentration profile was used to determine the estrous cycle stage at the time of biopsy sampling. All cows selected were in the luteal phase at time of endometrial biopsy as shown by their mean (± s.e.m.) progesterone concentration (8.78 ± 2.12 ng/mL; range from 6.66 to 10.90 ng/ml).

Collection of endometrial biopsies. Endometrial biopsies were collected from the uterine horn ipsilateral to the corpus luteum by using Kevorkian-Younge uterine biopsy forceps (Alcyon, Paris, France). Biopsies were cut into 3 pieces (sizes ≈ 4×4 mm^2). One of them was snap frozen in cold isopentane (2-Methylbutane, Sigma Aldrich, Saint Louis, MO, USA) previously placed in liquid nitrogen for 5 min, and immediately embedded in ≈1 cm^3 optimal cutting temperature (OCT) compound (VWR, Radnor, PA, USA). OCT conditioned biopsies were then put into dry ice and kept at -80°C until sectioning. Tissue blocks were 8 µm sectioned with a cryostat (Leica CM1860 Cryostat, Wetzlar, Germany) at -20°C under RNA-free conditions. Tissue section slices were mounted on Super Frost slides RNA-free which were chilled on ice, following immersion in ice-cold 75% RNA-free ethanol and stored at -80°C until staining [106].

Laser capture microdissection (LCM) and RNA isolation. All procedures used were those previously published [106]. Tissue sections were mounted on RNAse-free glass slides which were chilled on ice, following immersion in cold 75% RNA-free ethanol at -20°C in the cryostat and then transferred into 75% ethanol at RT (30 sec), stained with 1% cresyl violet in ethanol (15 sec), rinsed successively with 75% ethanol (30 sec), 95% ethanol (2 x 1 min), and 100% ethanol (2 x 1 min) (anhydrous Ethanol absolute). Finally, the slides were completely dehydrated by immersion in pure xylene (M-xylene, Sigma-Aldrich, Saint-Quentin-Fallavier, France) for 2 × 5 min. Stained tissue sections were then immediately air dried. The LCM process was performed by using an ArcturusXT™ Laser Capture Microdissection System and software (Applied Biosystems®, Arcturus, ThermoFisher Scietific, Waltham, MA, USA), within 1 h to avoid RNA degradation. Luminal epithelial cells (LE), glandular epithelial cells (GE) and stromal cells (ST), were harvested in sufficient numbers to obtain at least 5 ng of total RNA for each endometrial cell type. Briefly, cells were captured from the slide onto LCM plastic caps (CapSure®Macro LCM Caps, Arcturus) by using infrared laser with the following settings: power range 75 to 90mW, time 1300 to 3500µsec and 200mV intensity. Collected cells were then placed in a RNAse-free 0.5 mL microcentrifuge tube with 25 µL extraction buffer (provided together with the PicoPure™RNA isolation kit; KIT0202, Arcturus) and incubated for 30 min at 42°C. The histology of each endometrial cell type before and after capture with LCM is presented in Figure 7. Captured cells in PicoPure extraction buffer were frozen at -80°C before processing samples for RNA isolation. Total RNA from LCM samples was isolated and mRNA purified using the PicoPure™RNA isolation kit (KIT0202, Arcturus) following the manufacturer's protocol. RNA integrity value (RIN values) and quantity were evaluated using the Pico RNA chip on the Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA, USA). Mean RNA integrity (RIN) values obtained from LCM samples and from the full tissue samples issued from the same biopsy were similar (paired T-test; Table S9).

RNA sequencing and data analysis. RNA sequencing libraries prepared from 24 samples (number of samples in each NEB group and endometrial cell types presented in Table S9) were prepared and sequenced on GenomEast Platform (IGBMC, Cedex, France; http://genomeast.igbmc.fr/). Libraries were built using the Clontech SMART-Seq v4 Ultra Low Input RNA kit for Sequencing. Full length cDNA were generated from 4 ng of total RNA using Clontech SMART-Seq v4 Ultra Low Input RNA kit for Sequencing (Takara Bio Europe, Ozyme, Montigny-Le-Bretonneux, France) according to manufacturer's instructions, with 10 cycles of PCR for cDNA amplification by Seq-Amp polymerase. Then, 600 pg of pre-amplified cDNA were then used as input for Tn5 transposon tagmentation using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA) followed by 12 cycles of library amplification. Following purification with Agencourt AMPure XP beads (Beckman-Coulter, Roissy, France), the size and concentration of libraries were assessed by capillary electrophoresis. Sequencing was performed on an Illumina HiSeq 4000 with 50 bp paired-end reads. Image analysis and base calling were performed using RTA 2.7.3 and bcl2fastq 2.17.1.14. Gene level exploratory analysis and differential expression were performed using the RNAseq workflow described by [107] and the update version https://bioconductor.org/help/course-materials/2017/CSAMA/labs/2-tuesday/lab-03-rnaseq/rnaseqGene_CSAMA2017. html). The Salmon method [108] was used to quantify transcript abundance. The cDNA sequence database for *Bos taurus* was obtained from Ensembl (release-98; Bos_taurus.ARS-UCD1.2.cdna.all.fa) and was used to build a reference index for the bovine transcriptome (see details in [108]. After quantifying RNA-seq data, tximport method [109] (R package version 1.8.0) was used to import Salmon's transcript-level quantifications to the downstream DESeq2 package (R package, version 1.20.0) for analysis of differential expressed genes (DEGs) with the statistical method proposed [110]. Principal component analysis was performed with DESeq2 and with FactoMineR (R package, version 1.4.1) using the variance stabilizing transformation output files from DESeq2. Heatmap was generated in R software using the pheatmap package (version 1.0.12) and Venn diagrams were plotted with VennDiagram package (1.6.20). DEGs of specific-endometrial cell samples were identified in comparison between SNEB and MNEB group with an adjusted *p*-value of 0.05. Volcano plot was applied to gene lists of each endometrial cell type considering the log2 fold change between SNEB and MNEB on the *x* axis and the negative log10 of the adjusted *p*-value on the *y* axis.

Gene ontology and KEGG Pathway Analysis. Lists of genes expressed by the three types of endometrial cells as well as sets of over- or under-expressed DEGs between SNEB and MNEB were annotated into three categories of Gene Ontology (GO) pathways such as biological process (BP), cellular component (CC) and molecular function (MP) using PANTHER classification system (Protein Analysis THrough Evolutionary Relationships version 14.0, http://pantherdb.org). PANTHER overrepresentation tests were performed using all genes from the whole Bos taurus genome or from specified list. Lists of GO terms were summarized and visualized in semantic space by REVIGO (http://revigo.irb.hr/) [111]. The SimRel semantic similarity score was used and the threshold was set at 0.15. Moreover, the analysis of enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed using Database for Annotation, Visualization and Integrated Discovery software (DAVID version 6.8, https://david.ncifcrf.gov/summary.jsp). If a KEGG pathway was determined to be significantly enriched (Benjamini- adjusted *p*-value < 0.05), this significant process/pathway was reported. By using DEGs which are involved in significant KEGG pathways, a molecular interaction network analysis was generated by using STRING database (STRING version 10.5, http://string-db.org/) (Szklarczyk et al. Nucleic Acids Res. 2015 43(Database issue):D447-52) at medium confidence level (0.4) for giving an overview of the genes networks and their interactions.

Statistical analysis

The statistical analyzes for phenotype parameters (BCS, NEFA concentrations) were performed using the Statistical Analysis System Software (SAS® version 9.4, SAS Institute Inc., Cary, NC, USA) and analyzed by mixed models with repeated measurement (Proc MIXED). All variables were checked for normality and data were log10-transformed if needed. The effect of the cow was considered as random when running the models. The model included NEB group, diet group and time of sampling defined as fixed effects and their second order interactions. Non-significant effects were progressively removed from models. Scheffe's post hoc test was used for multiple comparisons and also the "estimate" and "contrast" statements under Proc MIXED were used for pairwise comparisons. Individual BCS loss from start of experiment until a nadir (the lowest postpartum value) and a nadir of feed residual intake (RFI) value after calving were recorded. Pearson correlation coefficients between the different variables were calculated using the Proc CORR function. The results of BCS, NEFA's concentration, and milk progesterone concentration are presented as LSmeans ± S.E.M. Differences with associated *p*-value < 0.05 were considered to be significant. In the statistical analysis of transcriptome profiles, generalized linear model was fitted and Wald test were performed to determine which of the observed fold changes were significantly different between severe and mild negative energy balance groups. *p*-values < 0.05 were considered to identify DEGs according to procedures described by [107].

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Legends of figures

Figure 1:

Residual feed intake (A) and plasma NEFA concentrations (µmol/l; LSmeans ± s.e.m.) (B) of LCM-selected SRB cows between observed start of the experiment and 56 days after calving in MNEB (■ solid line; n = 5) and SNEB (○ dashed line; n = 4) group. Significant differences were observed at 14 days before (a vs b; $p < 0.05$), and 14 days after calving (c vs d ; $p < 0.05$).

Figure 2: Transcriptomic analysis of endometrial cell types

(A) Venn diagram from genes expressed more than 10 TPM in specific endometrial cells (LE, luminal epithelial cells; GE, glandular epithelial cells; ST, stromal cells) (numbers of identified genes are indicated).

(B) Heat map of genes expressed by ST, GE and LE cells and clustering of the three cellular types (the colors show the relative level of expression. Boxes highlight the more expressed genes for each cell type [(a): stromal cells; (d): luminal epithelial cell type; (c): glandular epithelial cells: (d): epithelial cell type].

(C) Principal component analysis for clustering expressed genes of the three endometrial cell types. Confidence ellipses around the barycenter of each cell type are shown.

Figure 3: Scatterplot representation of biological process GO terms in semantic space using REVIGO. GO terms overrepresented in the list of genes specific to the three different cell-types of bovine endometrium (ST: stromal cells; GE: glandular epithelial cells; LE: luminal epithelial cells). Each circle corresponds to log 10 p-values according to the color scale shown at the bottom left of each figure. The size of each circle is proportional to the size of GO terms.

Figure 4: Effect of energy balance on transcriptome of endometrial cell types

(A) Principal component analysis of all three cell types: stromal cells (ST), glandular epithelium (GE), and luminal epithelium (LE) among two groups of cow (severe negative energy balance; SNEB and moderate negative energy balance; MNEB).

(B) Venn diagrams from differentially expressed genes differentially expressed (DEGs) between SNEB and MNEB in each endometrial cell types (ST, GE and LE).

Figure 5: Volcano plots of distribution of differentially expressed genes between SNEB and MNEB for the three endometrial cell types ST (A), GE (B) and LE (C). The dotted lines in green and blue represent the cut-off, respectively for the statistical significance [−Log10 (P-value), y-axis] and for +/- 2 log2fold change of gene expression [x-axis]. Differentially expressed genes are shown in red dots.

Figure 6: STRING-generated protein-protein network at medium confidence level (0.4) from DEGs of ST and GE endometrial cell types selected from significant KEGG pathways (Table 8) in comparison between SNEB and MNEB cows.

Figure 7: Isolation of the three bovine endometrial cell types by LCM: stromal cells (ST), glandular epithelial cells (GE) and luminal epithelial cells (LE), before [(1): left column and arrows)] and after [(2): right column] capture by LCM. (400x magnification)

Additional Files

Additional file 1 (TableS1_TS1-LE_TS2-GE_TS3-ST.xlsx):

Title of data: List of genes specifically expressed by the three endometrial cell types (excel file):

Sheet 1: list of genes specifically expressed by luminal cells

Sheet 2: list of genes specifically expressed by glandular cells

Sheet 3: list of genes specifically expressed by stromal cells

Additional file 2 (TableS2_GO-REVIGO05_TS1-TS2-TS3.xlsx):

Title of data: List of GO term for under and over expressed genes three endometrial cell types (excel file):

Sheet 1: over-represented GO terms for ST

Sheet 2: under-represented GO terms for ST

Sheet 3: over-represented GO terms for GE

Sheet 4: under-represented GO terms for GE

Sheet 5: over-represented GO terms for LE

Sheet 6: under-represented GO terms for LE

Additional file 3 (TableS3_PCA_tables.xlsx):

Title of data: List of genes expressed by endometrial cells according to the first two dimensions of the Principal Component Analysis (excel file):

Sheet 1: TS4_prolificPCAdim1_r+0.9_p0.01; genes positively correlated to first dimension

Sheet 2: TS5_prolificPCAdim1_r-0.9_p0.01; genes negatively correlated to first dimension

Sheet 3: TS6_prolificPCAdim2_r+0.8_p0.01; genes positively correlated to second dimension

Sheet 4: TS7_prolificPCAdim2_r-0.8_p0.01; genes negatively correlated to second dimension

Additional file 4 (TableS4_TS8_TS9_TS10_DEG-SNEBvsMNEB.xlsx):

Title of data: List of differentially expressed genes between SNEB and MNEB (excel file):

Sheet 1: list of DEG for stromal cells between SNEB vs MNEB

Sheet 2: list of DEG for glandular cells between SNEB vs MNEB

Sheet 3: list of DEG for luminal cells between SNEB vs MNEB

Additional file 5 (TableS5_david_ST-underexpressed.pdf):

Title of data: Gene Functional Classification Result (DAVID 6.8) of under-expressed genes in ST cells from SNEB animals

Additional file 6 (TableS6_david_ST-overexpressed.pdf):

Title of data: Gene Functional Classification Result (DAVID 6.8) of over-expressed genes in ST cells from SNEB animals

Additional file 7 (TableS7_david_GE-overexpressed.pdf):

Title of data: Gene Functional Classification Result (DAVID 6.8) of over-expressed genes in GE cells from SNEB animals

Additional file 8 (TableS8_david_LE-underexpressed.pdf):

Gene Functional Classification Result (DAVID 6.8) of under-expressed genes in LE cells from SNEB animals

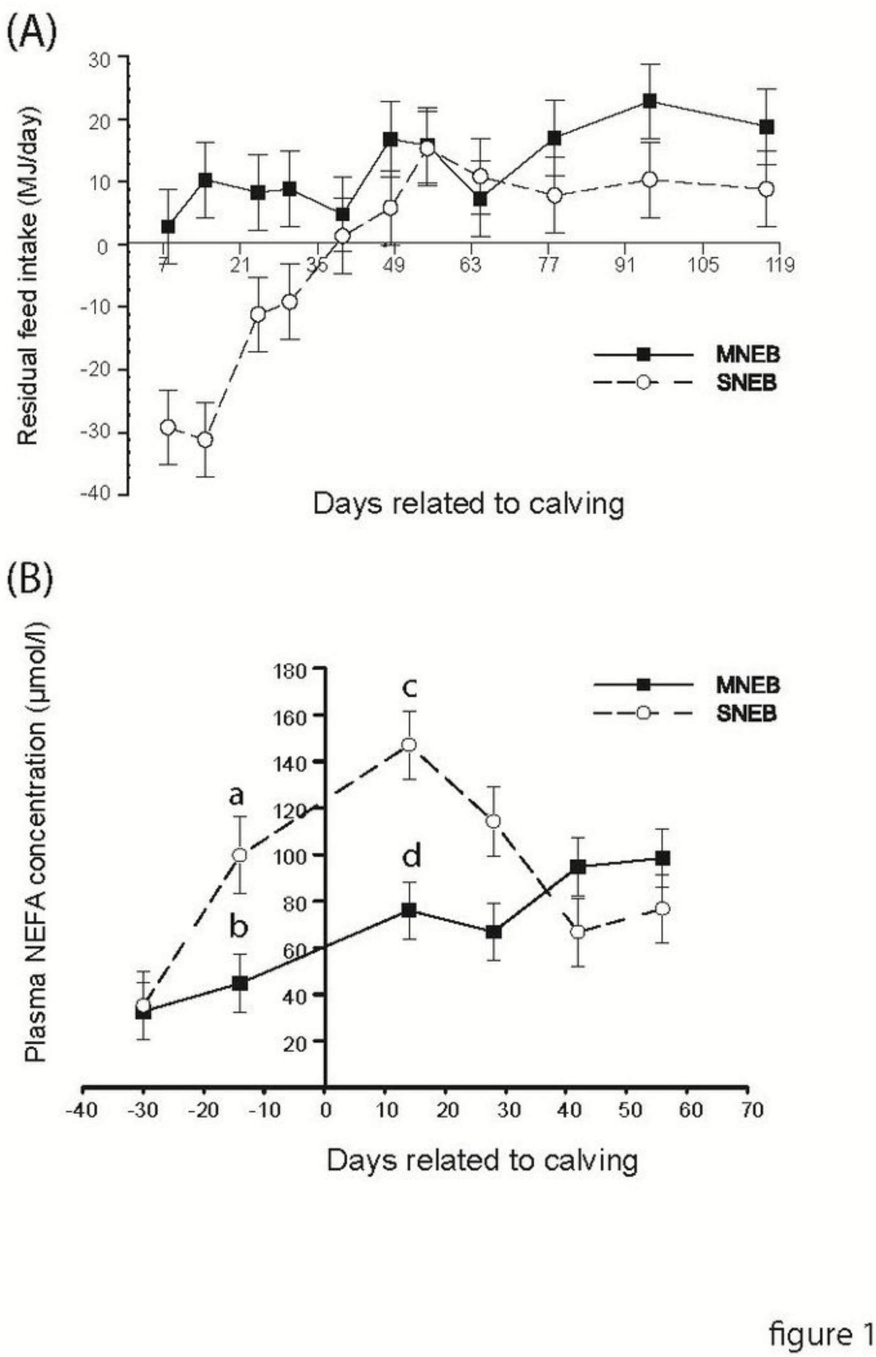
Additional file 9 (TableS9.pdf):

Title of data: Number of samples of each cell type from MNEB and SNEB group. RNA Integrity Number (RIN)] [mean value (± s.e.m)] and average number of tissue sections required to obtain at least 10 ng of total RNA in each endometrial cell type.

Additional file 10 (FigS1.pdf):

Title of data: Experimental design including 12 cows. From energy balance profiles 9 cows were selected for LCM of endometrial tissue biopsies (5 mild NEB and 4 severe NEB cows). An arrow with dash line indicate a timing for BCS measurement and blood sampling for NEFA measurement

Figures



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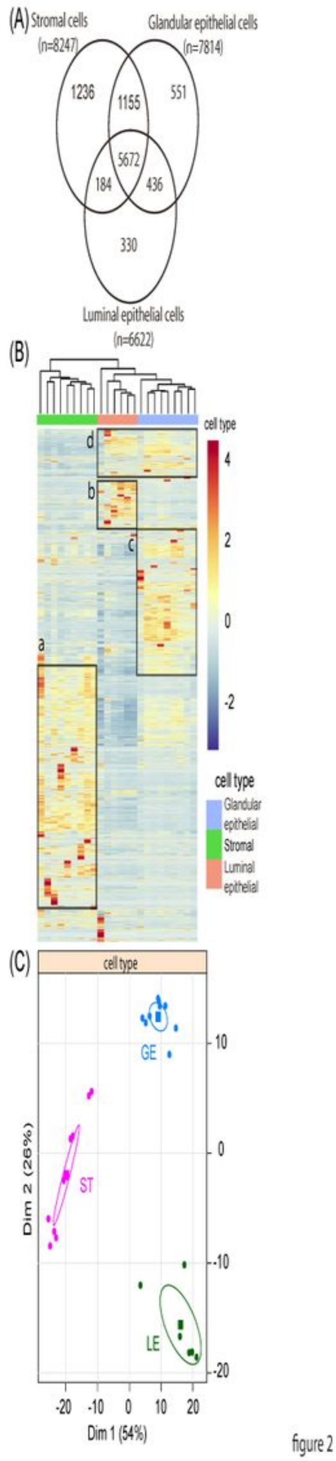

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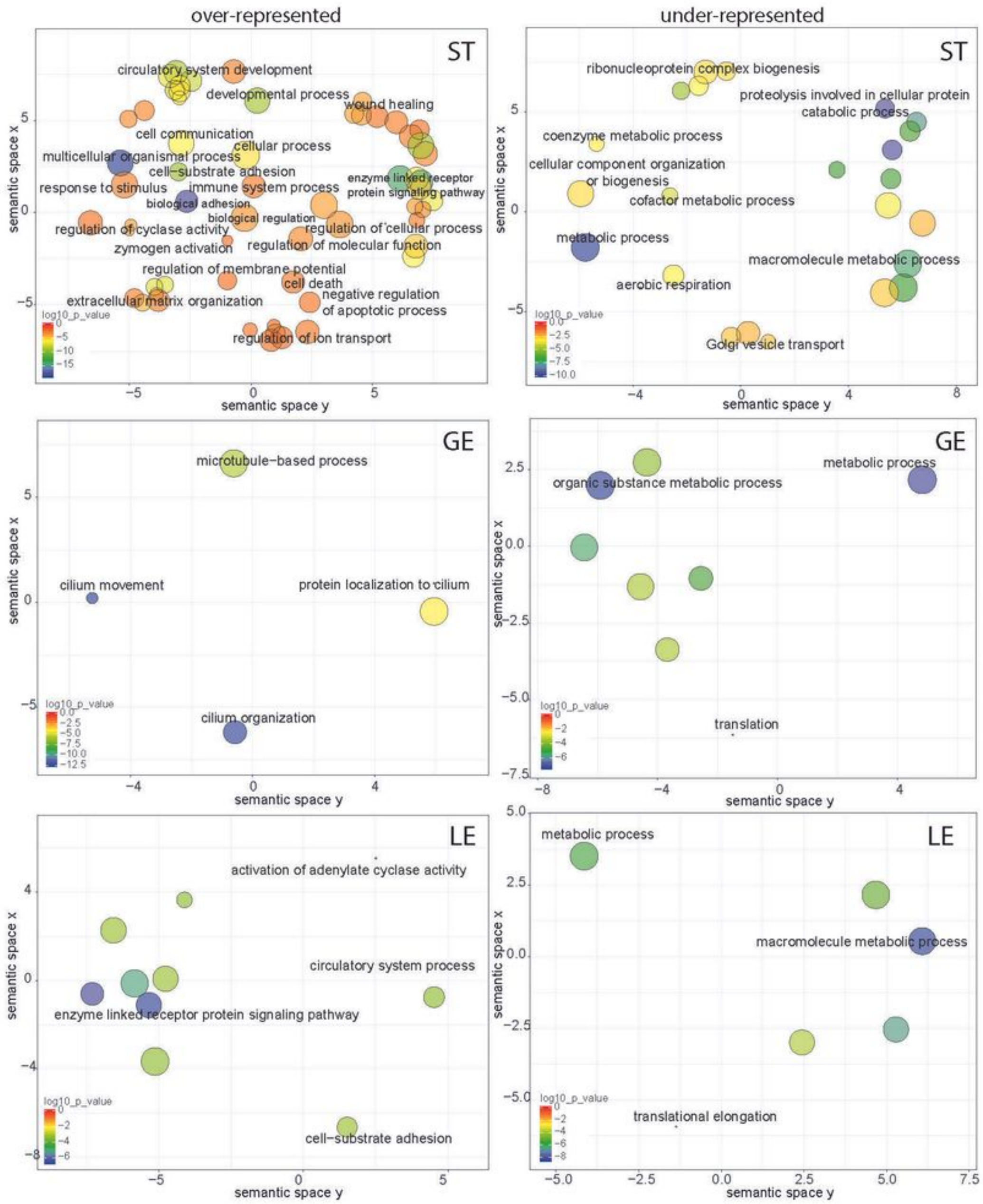

figure 3

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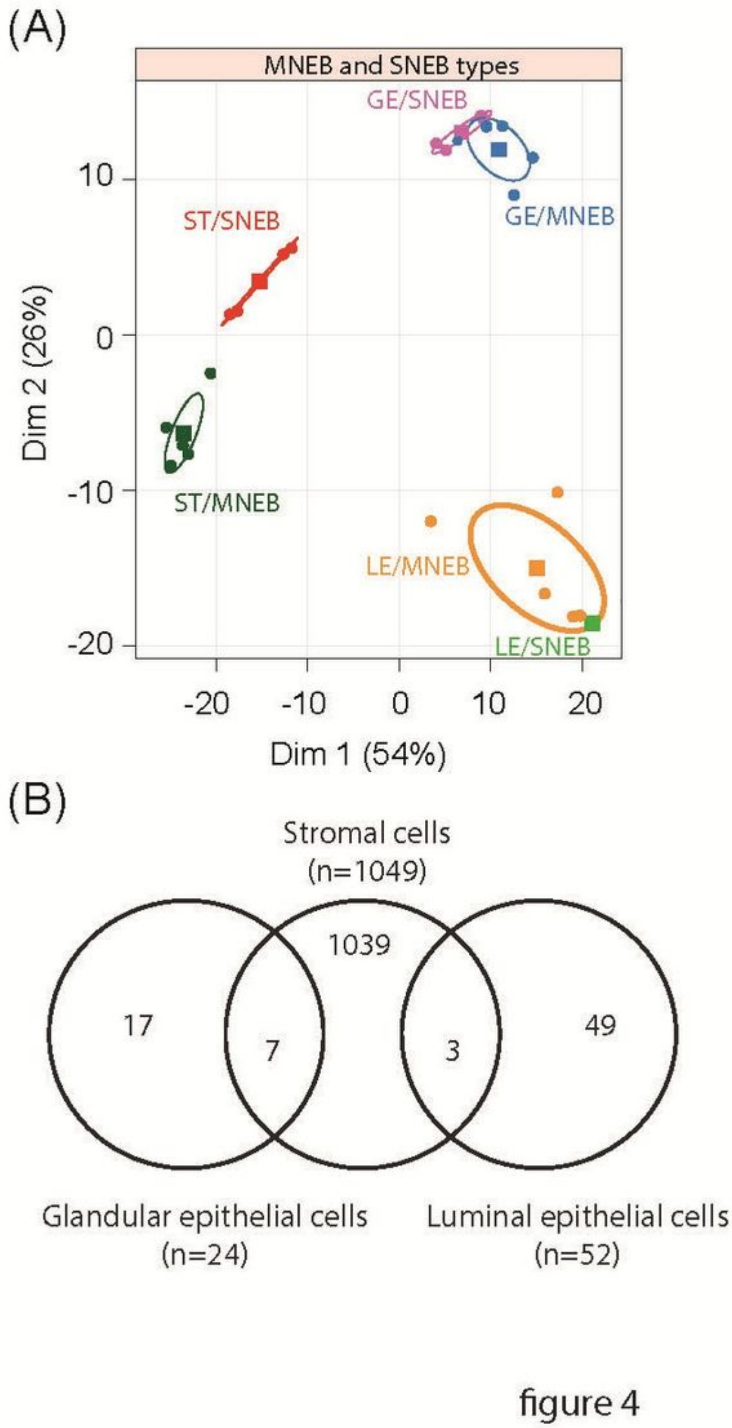

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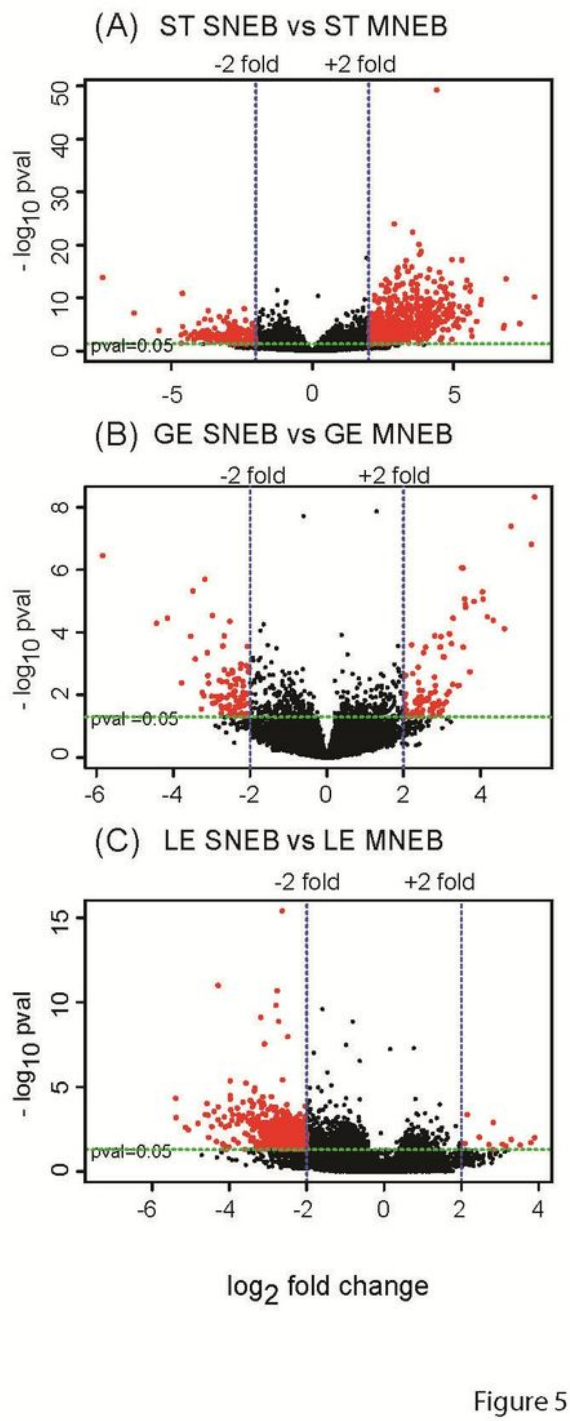

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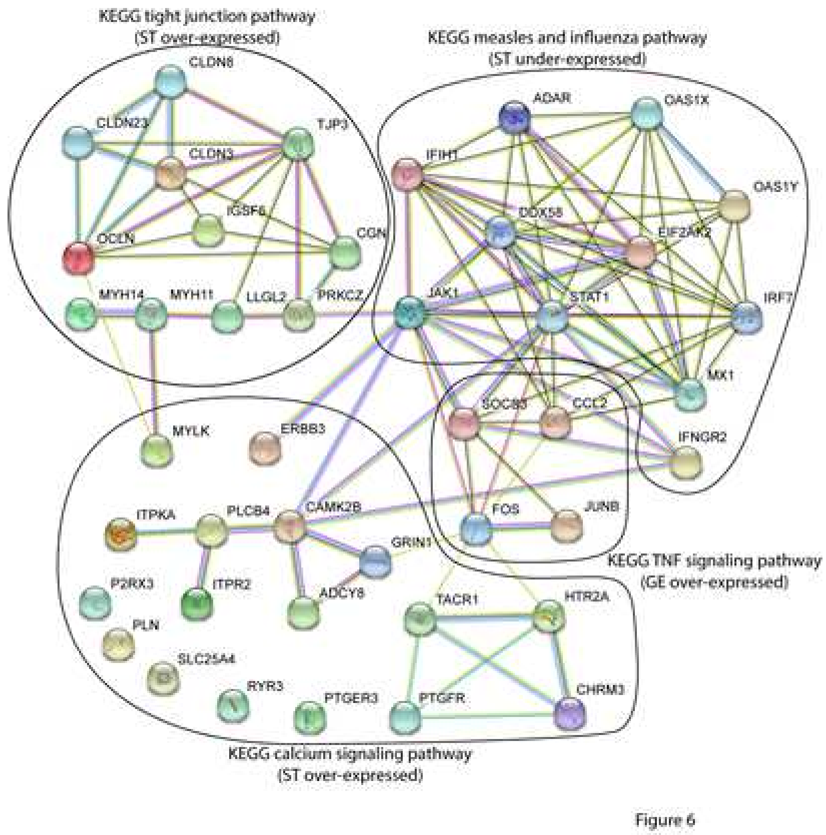

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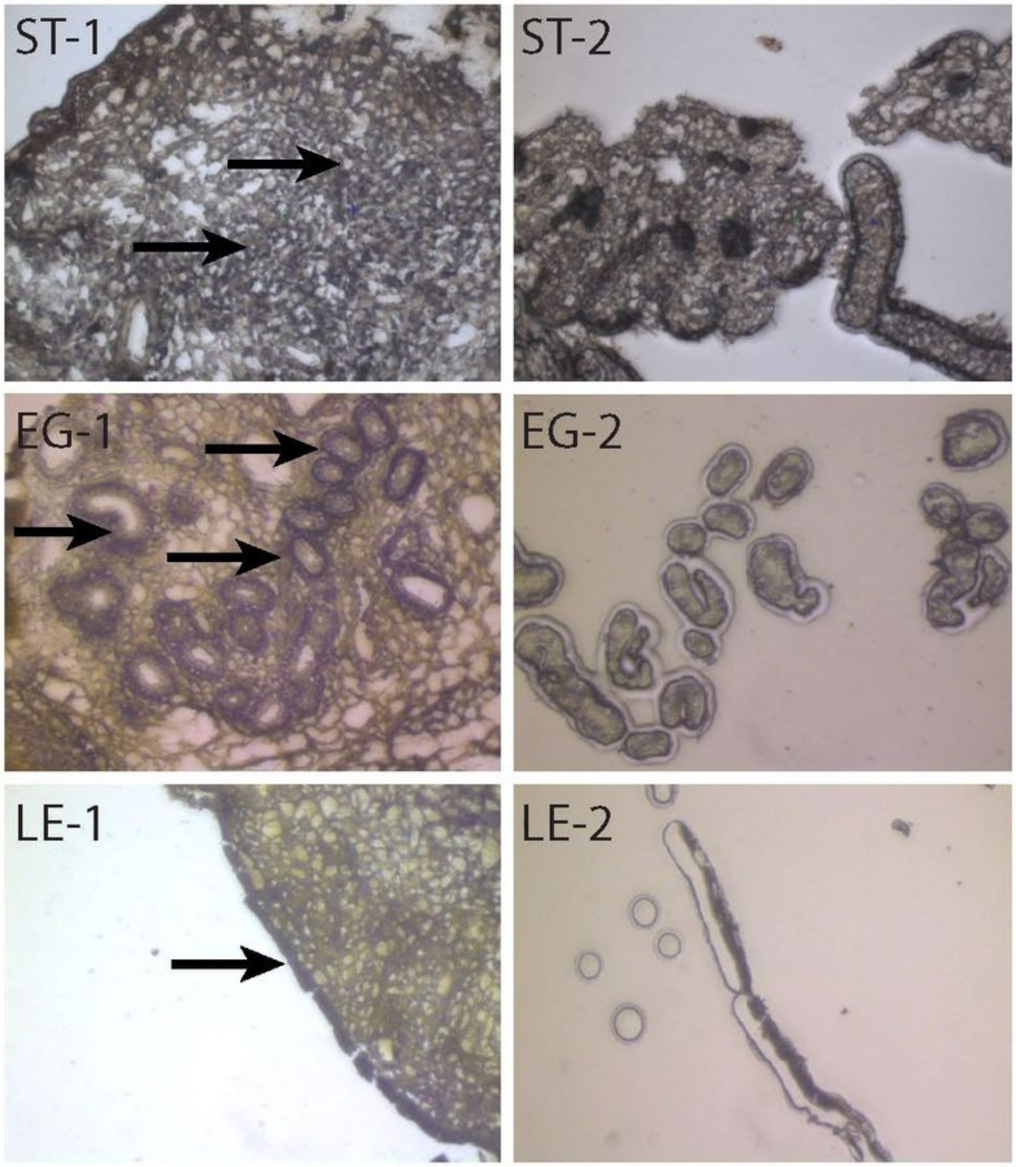

figure 7

Figure 7

Isolation of the three bovine endometrial cell types by LCM: stromal cells (ST), glandular epithelial cells (GE) and luminal epithelial cells (LE), before [(1): left column and arrows)] and after [(2): right column] capture by LCM. (400x magnification)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.pdf
- TableS1TS1LETS2GETS3ST.xlsx
- TableS5davidSTunderexpressed.pdf
- TableS2GOREVIGO05TS1TS2TS3.xlsx
- TableS6davidSToverexpressed.pdf
- TableS3PCAtables.xlsx
- TableS7davidGEoverexpressed.pdf
- TableS4TS8TS9TS10DEGSNEBvsMNEB.xlsx
- TableS8davidLEunderexpressed.pdf
- TableS9.pdf