IFNT-independent effects of intrauterine extracellular vesicles (EVs) in cattle

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Abstract

Extracellular vesicles (EVs) present in uterine lumen are involved in conceptus-endometrial interactions during the pre-implantation period. Despite numerous studies conducted on interferon tau (IFNT), a major protein of maternal recognition of pregnancy, the effect of intrauterine EVs on the endometrium during pre-implantation periods has not been well-characterized. To characterize conceptus-derived intrauterine EVs independent of IFNT, transcripts found from RNA-seq analysis in RNAs extracted from primary bovine endometrial epithelial cells (EECs) treated with cyclic day 17 (C17) EVs, pregnant day 17 (P17) EVs or IFNT were analyzed. These analyses identified 82 transcripts uniquely induced by IFNT-independent P17 EVs, of which a large number of transcripts were associated with 'the TNF signaling pathway' and 'Inflammatory response'. Moreover, high expression of CD40L, a member of the TNF superfamily, and its receptor CD40 were found in P17 EVs and in EECs, respectively. Furthermore, the expression of TNF signaling pathway-related genes was up-regulated by the treatment with P17 EVs, but these increases were down-regulated by NF- κ B signaling inhibitor. These findings suggest that P17 EVs could induce a pro-inflammatory response in the endometrium, independent of IFNT, to regulate uterine receptivity, facilitating conceptus implantation.

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Introduction

Approximately half of embryonic losses are known to occur prior to the time of maternal recognition of pregnancy, which is around day 16 in cattle, during the critical period for corpus luteum maintenance or demise (Wiltbank *et al.* 2016). Over the past decade, various global analyses were implemented to study the expression of transcripts in conceptus or endometrium, identifying many molecules with potential importance during the implantation period (Mamo *et al.* 2011, Forde *et al.* 2014, 2015, Bai *et al.* 2018). Although data continue to accumulate, mechanisms by which these factors function during the conceptus attachment process remain unclear.

In cattle, endometrial gene expression between cyclic and pregnant animals did not differ until pregnant days 15–17 (Walker *et al.* 2010, Forde *et al.* 2011, Bauersachs *et al.* 2012), but transcriptomic differences between cyclic and pregnant animals become apparent at the time of pregnancy recognition. Interferon tau (IFNT) is the major factor essential for the process of maternal recognition of pregnancy (Bazer & Thatcher 2017, Ealy & Wooldridge 2017, Forde & Lonergan 2017, Roberts 2017). It has been reported that sufficient concentrations

of IFNT must be released by the elongating conceptus between pregnant days 15 and 17 (P15 and P17, respectively), inducing the expression of a large number of interferon-stimulated genes in the endometrium, which leads to the establishment of uterine receptivity to conceptus implantation in ruminant ungulates (Spencer *et al.* 2013, Wiltbank *et al.* 2016). In spite of extensive data accumulation, pregnancy rate in cattle remains relatively low due to early embryonic losses. These observations indicate that molecular mechanisms or factors in the endometrium which play a major role along with IFNT in pregnancy establishment periods need to be elucidated.

Different cell types generally interact with secretory soluble factors such as hormones and cytokines. In recent years, however, the role that extracellular vesicles (EVs) play in cell-cell interaction has been well-documented. EVs contain lipids, proteins and nucleic acids, and specific target cells have surface receptors, with which ligands from the originating cells have the potential to selectively interact (Burns *et al.* 2018). Evidence has been accumulated that EVs are present in most of bodily fluids, including uterine flushing fluids (UFs) (Piehl *et al.* 2013, Prunotto *et al.* 2013, Cleys *et al.* 2014, Nguyen *et al.* 2016). In ruminants, UFs components

have been reported to induce the conceptus elongation and attachment (Kusama *et al.* 2016, Simintiras *et al.* 2019a,b,c). It was also reported that EVs released by conceptuses and/or endometrium during the peri-implantation period are involved in biochemical and/or physical interactions between the conceptus and the endometrium (Burns *et al.* 2014, Ruiz-González *et al.* 2015, Burns *et al.* 2016, Nakamura *et al.* 2016, Kusama *et al.* 2018a).

Based on these findings, we hypothesized that in addition to IFNT, intrauterine EVs on P17 are essential for the process of maternal recognition of pregnancy, which regulates the uterine environment, facilitating subsequent conceptus implantation to the uterine epithelium. Using RNA-seq analysis in this study, we first evaluated effects of cyclic day 17 (C17) and P17 EVs on the primary bovine endometrial epithelial cells (EECs), from which pro-inflammatory response-related IFNT-independent factors were further evaluated.

Materials and methods

Collection of bovine uterine flushing fluids

All animal procedures in this study were performed in accordance with the guidelines of the Committee for Experimental Animals at Zen-noh Embryo Transfer Center, and approval was also obtained from the Ethics Committee of the University of Tokyo (IRB number 7A-6-605). Estrous synchronization, superovulation, and embryo transfer (ET) processes were performed as previously described (Ideta *et al.* 2007). Recipient heifers were given a single injection of 0.75 mg cloprostenol to synchronize their estrous cycles. The day of injection was considered day 0 of estrus. For ET processes, day 7 embryos were collected from super-ovulated and artificially inseminated (AI) Japanese black cattle. Two blastocysts each derived from the superovulation/AI procedure were then transferred non-surgically into the uterine horn of Holstein heifers, ipsilateral to the corpus luteum, on day 7 of the estrous cycle. Elongated conceptuses on day 17 were then collected non-surgically by uterine flushing with 500 ml sterile PBS (pH 7.2). Conceptuses in the UF media were obtained by centrifugation at 1000 g for 5 min and snap-frozen in liquid nitrogen. Following conceptus removal, the media and those from day 17 cyclic animals were centrifuged at 4000 g for 5 min to remove cell debris, supernatants were filtered through 0.22 µm membrane, and then samples were stored at -80°C until use.

Isolation of EVs from uterine flushing fluids

EVs were isolated from C17 and P17 UFs by adding exosome precipitation solution (Exo-Quick-TC, System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions. The UFs with Exo-Quick-TC were incubated overnight at 4°C and then centrifuged at 1500 g for 30 min at 4°C to pellet EVs. EVs were suspended and their protein concentrations adjusted either in PBS (1 mg/mL) for *in vitro* culture experimentations or in mammalian protein extraction

reagent (M-PER, Thermo Fisher Scientific) for Western blot analysis (Nakamura *et al.* 2016, Kusama *et al.* 2018a).

Transmission electron microscopy

The EVs in PBS were placed on a carbon-film grid and were partially dried. The staining solution of 2% uranyl acetate in water was added to the grids for 2 min and the excess liquid was blotted off with filter paper, followed by overnight drying at room temperature. The grids were analyzed through the use of a HITACHI H-7600 Transmission Electron Microscope (TEM, Hitachi High-Technologies Corporation, Tokyo, Japan) at Hanaichi UltraStructure Research Institute (Aichi, Japan).

Nanoparticle tracking analysis

The EVs isolated from C17 or P17 UFs and suspended in PBS ($2-6 \times 10^8$ particles/ml) were subjected to nanoparticle tracking analysis using a NanoSight NS300 (NanoSight Ltd, Amesbury, UK) instrument with 488 nm laser and a complementary metal-oxide-semiconductor camera (Andor Technology, Belfast, UK) and NanoSight NTA 3.2 software calibrated with 100 nm polystyrene beads (Thermo Fisher Scientific). Videos were recorded for 30 s during which the nanoparticle tracking analysis software (NanoSight Ltd) tracked each visible particle. The Stokes–Einstein equation was employed to determine the size distribution and number of particles within the sample (Nakamura *et al.* 2016).

Cell preparation and culture conditions

Isolation and culture of EECs were carried out as previously described (Sakurai *et al.* 2012). In brief, uteri of healthy Holstein cows were obtained from a local abattoir in accordance with protocols approved by the local Institutional Animal Care, Use and Ethics Committee at Okayama University, Okayama, Japan. Uteri of the early luteal phase (days 2–5) were excised and immediately transported to the laboratory. The uterine lumen was trypsinized (0.3% w/v), from which EECs were isolated. EECs were then cultured on collagen type IA-coated plates in Dulbecco modified Eagle medium/F12 (DMEM/F12) (1:1) medium (Wako Pure Chemical Industries) supplemented with 10% (v/v) newborn calf serum (Thermo Fisher Scientific), 2 mM glutamine (Thermo Fisher Scientific), and antibiotic/antimycotic solution (Thermo Fisher Scientific) at 37°C under 5% CO₂ in humidified air. EECs (5×10^4 cells/well) placed onto collagen type IA-coated 12-well plate were further incubated with or without C17 or P17 EVs (10 µg/well) in serum-free DMEM/F12 for 48 h. For the inhibition of NF-κB pathway, EECs (5×10^4 cells/well) were cultured in 12-well plate with or without P17 EVs (10 µg/well) and/or the selective IκB kinase inhibitor, NF-κB pathway inhibitor, 2-((aminocarbonyl)amino)-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA1) (20 nM, ab145522, Abcam) for 48 h.

RNA extraction and quantitative RT-PCR

Using the ISOGEN reagent (Nippon Gene, Tokyo, Japan), total RNAs were extracted from cultured EECs according

to the manufacturer's protocols. The isolated RNA was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan), which was then subjected to qPCR amplification using PowerUP SYBR Green Master Mix (Thermo Fisher Scientific). All primers are listed in Table 1. The qPCR amplification was carried out on an Applied Biosystems STEP One Plus real-time PCR System (Applied Biosystems). Amplification efficiencies of all target genes and the reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin beta (ACTB), were examined through their calibration curves and found to be comparable. Average threshold (Ct) values for each target were determined by Sequence Detection System software v2.3 (Applied Biosystems) (Nakamura *et al.* 2017).

RNA sequencing, data, gene ontology, and pathway analyses

Total RNA for RNA-seq analysis was extracted from cultured EECs using Isogen (Nippon gene) according to the manufacturer's instructions. High-throughput sequencing libraries were prepared using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions, and the analysis was performed by MacroGen Japan (Kyoto, Japan). Primary sequencing data were deposited to the DDBJ (DNA Data Bank of Japan) Sequence Read Archive (<https://www.ddbj.nig.ac.jp/dra/index-e.html>) (accession numbers DRR174782 to DRR174790). Data analysis was performed as described previously (Kusama *et al.* 2017). Briefly, trimmed sequences were analyzed on the basis of the TopHat/Cufflinks pipeline based on the bovine genome (bosTau8) and reference annotations obtained from

UCSC genome browser (<https://genome.ucsc.edu>). Differential and significant gene expression analysis was performed with the use of FPKM (fragments per kilo-base of gene locus summarized mRNA per million reads). Genes were selected with the criteria of an absolute expression level >1 FPKM. The gene ontology (GO) and enriched signaling pathway analyses were performed with the Enrichr tool (<http://amp.pharm.mssm.edu/Enrichr/>). RNA-seq data sets obtained were compared with previously published data from culture endometrial explants treated with or without ovine IFNT or a day 15 AI-derived conceptus (Mathew *et al.* 2019, Sánchez *et al.* 2019) and those from IFNT-treated EECs (DRR083698 to DRR083699) (Kusama *et al.* 2017).

Western blot analysis

EVs and EECs lysed with M-PER (10 µg) were separated through SDS-PAGE and were then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking with Block Ace reagent (DS Pharma Biomedical, Osaka, Japan), membranes were incubated with rabbit polyclonal anti-human CD63 antibody (1:2000, EXOAB-CD63A-1, System Biosciences), rabbit polyclonal anti-human HSP70 antibody (1:2000, EXOAB-HSP70A-1, System Biosciences), rabbit polyclonal anti-human CD40L (1:100, ARP33831_P050, Aviva Systems Biology, San Diego, CA, USA), rabbit polyclonal anti-bovine TNFA (1:1000, GTX38917, GeneTex, Irvine, CA, USA), rabbit monoclonal anti-human NF-κB p65 (C22B4, 1:1000, Cell signaling Technology), rabbit polyclonal anti-human NF-κB p65 (acetyl K310) (1:1000, ab19870, Abcam), or rabbit polyclonal anti-human ACTB (1:5000, ab1801, Abcam). Immunoreactive bands were detected using enhanced

Table 1 Primers for real-time PCR analyses.

Name (Accession No.)	Sequence	Product length (bp)
<i>BIRC3</i> (NM_001035293)	F: 5'-TCCAGATGTGGCCGTTGACT-3' R: 5'-CCTTCGGTTCCTCAATTGCTC-3'	129
<i>CD40</i> (NM_001105611)	F: 5'-CGGTAAAGGCGAATTCTTGTC-3' R: 5'-GCCTTCGACACATACACAAGTG-3'	139
<i>CSF2</i> (NM_174027)	F: 5'-GAATGACACAGAGTCGTCTCTG-3' R: 5'-AAGGAGCCCATGAGACTAGTG-3'	120
<i>CXCL2</i> (NM_174299)	F: 5'-AGAAGCTCTTGGATGGCTGTTCCA-3' R: 5'-AGATGGCCTTAGGAGGTGGTGATT-3'	91
<i>CXCL3</i> (NM_001046513)	F: 5'-ACTGTGGCCAAACCGAAGTC-3' R: 5'-TTGGTGCTGCCCTTGTTAG-3'	118
<i>CXCL5</i> (NM_174300)	F: 5'-TCGCCACTATGAGACTGCTATC-3' R: 5'-AACGCAGCTCTCTACAACG-3'	150
<i>IL6</i> (NM_173923)	F: 5'-ACCGAAGCTCTCATTAAAGCG-3' R: 5'-TTCTGCCAGTGTCTCCTTGC-3'	105
<i>MMP9</i> (NM_174744)	F: 5'-CCCGGATCAAGGATACAGCC-3' R: 5'-GGGCGAGGACCATACAGATG-3'	177
<i>NFKBIA</i> (NM_001045868)	F: 5'-TCCTGCACTAGCCATCATCC-3' R: 5'-TCTGGCTGGTTAGTGATCACAG-3'	145
<i>TNFAIP3</i> (NM_001192170)	F: 5'-TTGCAACATCCTCAGAAAGCC-3' R: 5'-AAATCCCACCCACCTTCAGAG-3'	101
<i>ACTB</i> (NM_173979)	F: 5'-ATATTGCTGCGCTCGTGGTTG-3' R: 5'-TAGGAGTCCTTCTGGCCCATG-3'	148
<i>GAPDH</i> (NM_001034034)	F: 5'-GCATCCCTGAGACAAGATGGTG-3' R: 5'-CATTGATGGCAACGATGTCCAC-3'	113

F: Forward; R: Reverse.

chemiluminescence (EMD Millipore) after incubation with horseradish peroxidase labeled goat anti-rabbit IgG (1:5000, Vector Laboratories, Burlingame, CA, USA). Signals were detected using C-DiGit Blot Scanner (LI-COR) and then band density was assessed with Image Studio DiGit software (version 5.2) (Kusama *et al.* 2018b).

Statistical analysis

All experimental data from qPCR analyses represent the results obtained from three or more independent experiments, each with triplicate assays. Data were expressed as the mean $\pm$ S.E.M. Significance was assessed using the Tukey–Kramer multiple comparisons test. A P-value of <0.05 was considered statistically significant.

Results

EVs present in bovine UFs on cyclic and pregnant days 17

To characterize EVs in bovine UFs based on the extracellular vesicles 2018 (MISEV2018) guidelines (Théry *et al.* 2018), EV markers, CD63 and HSP70, were detected in C17 and P17 UFs (Fig. 1A). Moreover, TEM detected vesicles of 50–150 nm in diameter in the isolated pellets from C17 and P17 UFs (Fig. 1B), and C17 and P17 EVs were subjected to nanoparticle tracking analysis, revealing 116.4 (mean) ± 50.2 nm (S.D.) and 108.9 ± 39.4 nm, respectively (Fig. 1C). These results indicated that EVs were secreted into the uterine lumen during pre-implantation periods.

Figure 1 Characterization of EVs isolated from UFs during the pre-implantation period. (A) Western blot analysis showed the presence of CD63 and HSP70 in pellets isolated from C17 or P17 bovine UFs. (B) Transmission electron microscopy analysis revealed the presence of 50–150 nm vesicles in UFs, consistent with those of EVs. Scale bar=400 nm (left) and 200 nm (right). (C) Nanoparticle tracking analysis of C17 or P17 revealed that the range of vesicles size is 50–200 nm, respectively.

Transcript changes in EECs treated with intrauterine EVs from day 17 cyclic animals

To examine effects of C17 EVs derived from bovine endometrium on gene expression in EECs, RNA-seq analysis was executed, identifying 9164 transcripts, from which eight genes were up-regulated and 11 genes were down-regulated (Fig. 2A). A pair plot comparison of C17 EVs treated and EECs without any treatment (control groups) showed that there were very few differentially expressed genes (DEGs) (Fig. 2B).

IFNT-independent effects of P17 EVs in EECs

Along with others, our group previously indicated that conceptus-derived EVs were present in UFs, which could function to modify endometrial response

Figure 2 Transcript changes in EECs treated with intrauterine EVs on cyclic day 17. RNAs were extracted from primary bovine endometrial epithelial cells (EECs) incubated without (control) or with C17 EVs (10 μ g) for 48 h and then were subjected to RNA-seq analysis. Data were from three independent *in vitro* culture experiments. (A) Fold changes in 19 differentially expressed genes (DEGs) with q-values less than 0.05 and 2-fold changes identified by RNA-seq were shown. (B) These diagrams show pair plots comparison between control and C17 EVs-treatment groups and density plots in each group. Figure shows the correlation coefficient between control and C17 EVs.

(Burns *et al.* 2016, Kusama *et al.* 2017). To characterize the transcripts induced by conceptus-derived molecules in detail, RNA-seq analysis of EECs treated with P17 EVs was executed and the DEGs with 2.0-fold changes were then compared among C17 EVs, P17 EVs, and previously reported conceptuses treatment groups (Mathew *et al.* 2019, Sánchez *et al.* 2019). Forty-one DEGs found in common between P17 EVs and conceptuses treatment groups indicated that these DEGs were regulated by conceptus-derived EVs (Fig. 3A). Pathway and GO analyses were then executed to identify conceptus-derived DEGs' significantly enriched pathways and biological process terms, from which the most enriched were associated with 'Interferon signaling' and 'Cellular response to type I interferon' respectively (Fig. 3B).

Although it was reported that bovine conceptus induces the IFNT-independent expression of endometrial transcriptomes during the pre-attachment period (Mathew *et al.* 2019), their molecular mechanisms have not been characterized. To test the hypothesis that IFNT-independent endometrial transcriptome was regulated by intrauterine EVs, in addition to C17 and P17 EVs-treatment groups, the effect of IFNT on the gene expression in EECs previously demonstrated (Kusama *et al.* 2017) was compared. The venn diagram shows the number of DEGs with 2.0-fold changes among C17 EVs, P17 EVs, and IFNT treatment groups compared with those of the control group. Of these 114 DEGs, 82 transcripts were expressed in only the P17 EVs-treatment group, but not the C17 EVs or the IFNT-treated group (Fig. 3C). IFNT-independent DEGs induced by P17 EVs were then subjected to enrichment pathway and GO analyses. These analyses revealed significantly enriched pathways and biological process terms, from which the most enriched were 'The tumor necrosis factor (TNF) signaling pathway' and 'Inflammatory response' (Fig. 3D). The details of these analyses are summarized in Tables 2 and 3. Furthermore, qPCR analysis ascertained that changes in TNF signaling pathway-related genes, *CXCL2*, *CXCL3*, *CXCL5*, *CSF2*, *NFKBIA*, *TNFAIP3*, *IL6*, *BIRC3* and *MMP9*, were similar to those of the RNA-seq analysis (Fig. 3E).

Effect of exosomal CD40L on the activation of NF- κ B in EECs

RNA-seq analysis and qPCR in this study revealed that along with TNF signaling pathway-related genes, *CD40*, a member of the TNF receptor superfamily, was up-regulated by the treatment of P17 EVs (Fig. 4A). Furthermore, *CD40* ligand (*CD40L*) protein expression was higher in P17 intrauterine EVs than that of C17 (Fig. 4B), whereas *TNFA* was not detected (result not shown). A previous study reported that the binding *CD40L* to *CD40* leads to activation of NF- κ B signaling (Hostager & Bishop 2013). We therefore investigated whether P17 EVs induced the activation of NF- κ B in EECs, resulting

Figure 3 IFNT-independent effects of P17 EVs in EECs. (A) Venn diagram shows the number of genes with 2.0-fold changes among C17 EVs, P17 EVs, and conceptuses treatment groups compared with control group, from which 41 transcripts (red rectangle) were identified in common between P17 EVs and conceptuses treatment groups. (B) Up-regulated conceptus-derived DEGs in EECs treated with P17 EVs were functionally classified by the biological process in GO terms and enriched pathway analyses. (C) Venn diagram shows the number of genes with 2.0-fold changes among C17 EVs, P17 EVs, and IFNT treatment groups compared with control group, from which 82 transcripts (red rectangle) were identified in only P17 EVs-treatment group. (D) Up-regulated IFNT-independent DEGs in EECs treated with P17 EVs were functionally classified by the biological process in GO terms and enriched pathway analyses. (E) Fold changes in TNF signaling pathway-related genes in EECs incubated without (control) or with P17 EVs (10 μ g) for 48 h ($n=3$ each) were examined by qPCR or RNA-seq. *ACTB* and *GAPDH* mRNA served as internal controls for RNA integrity.

that the P17 EVs increased acetylated NF- κ B, an active NF- κ B, in EECs (Fig. 4C). To further examine whether P17 EVs containing *CD40L* up-regulated the TNF signaling pathway-related gene expression through the activation of NF- κ B signaling, EECs were treated with the P17 EVs in combination with or without NF- κ B pathway inhibitor *TCPA1*. Treatment of *TCPA1* decreased the expression of *CXCL2*, *CXCL3*, *CXCL5*, *CSF2*, *NFKBIA*, *TNFAIP3*, *IL6*, *BIRC3*, and *MMP9*, all of which were induced by P17 EVs (Fig. 4D).

Table 2 Gene expression related to P17 EVs-induced enriched pathways, which are independent of IFNT.

Enrichment pathway	P-value	Genes
Up-regulation		
TNF signaling pathway	1.08E-10	<i>NFKBIA, IL6, CSF2, TNFAIP3, CXCL3, MMP9, CXCL2, CXCL5, BIRC3</i>
NOD-like receptor signaling pathway	3.57E-08	<i>NFKBIA, IL6, CXCL8, TNFAIP3, CXCL2, BIRC3</i>
Cytokine-cytokine receptor interaction	2.40E-07	<i>IL22RA1, CD40, IL6, CSF2, CXCL8, CXCR4, CXCL3, CXCL2, CXCL5</i>
Transcriptional misregulation in cancer	1.53E-07	<i>CD40, IL6, CSF2, CXCL8, NFKBIZ, PLAT, MMP9, BIRC3</i>
NF-kappa B signaling pathway	6.82E-07	<i>NFKBIA, CD40, CXCL8, TNFAIP3, CXCL2, BIRC3</i>
Legionellosis	1.11E-06	<i>NFKBIA, IL6, CXCL8, CXCL3, CXCL2</i>
Chemokine signaling pathway	3.02E-06	<i>NFKBIA, CXCL8, CXCR4, ARRB1, CXCL3, CXCL2, CXCL5</i>
Salmonella infection	1.02E-05	<i>IL6, CSF2, CXCL8, CXCL3, CXCL2</i>
Rheumatoid arthritis	1.28E-05	<i>IL6, CSF2, CXCL8, MMP1, CXCL5</i>
Intestinal immune network for IgA production	2.00E-05	<i>PIGR, CD40, IL6, CXCR4</i>

Discussion

This study provides the first evidence that intrauterine EVs-induced IFNT-independent modifications to the bovine endometrial transcriptome. Although several studies have shown the presence and potential function of the EVs and EVs containing IFNT during the implantation period in ruminants (Burns *et al.* 2016, Mellisho *et al.* 2017), their IFNT-independent effects on EECs have not been characterized. In this study, IFNT-independent P17 EVs, but not C17 EVs, induced 82 transcripts, and a large number of transcripts were associated with 'the TNF signaling pathway' and 'Inflammatory response'. Moreover, high expression of CD40L, a member of TNF superfamily, and its receptor CD40 were found in P17 EVs and in EECs, respectively. Furthermore, up-regulation of TNF signaling pathway-related genes by treatment with P17 EVs was down-regulated by NF- κ B signaling inhibitor, suggesting that P17 EVs, independent of IFNT, induced TNF signaling pathway-related genes via CD40L/CD40/NF- κ B pathway in primary bovine endometrial epithelial cells.

IFNT is the main trophoblastic factor which acts on uterine endometrium and attenuates luteolytic mechanisms, leading to the continued production of progesterone. Our previous study showed that P17, P20, and P22 EVs contained IFNT and that the expression of IFN-stimulated genes (ISGs) such as *ISG15*, *MX1*, *MX2*, *STAT1*, and *STAT2* in EECs was up-regulated in response to the these EVs (Kusama *et al.* 2018a). To investigate

effects of conceptus-derived EVs, the DEGs in uterine endometrium were compared among C17 EVs, P17 EVs, and conceptuses treatment groups (Mathew *et al.* 2019, Sánchez *et al.* 2019). 41 DEGs were found as common factors between P17 EVs and conceptuses treatment, which were associated with 'interferon signaling' and 'Cellular response to type I interferon' in significantly enriched pathways and biological process terms, respectively. These findings were consistent with those from our previous ovine and bovine studies (Nakamura *et al.* 2016, Kusama *et al.* 2018a) and supported the notion that EVs containing IFNT secreted from elongated conceptus acts on the endometrial epithelium during the peri-implantation period.

RNA-seq analysis identified 9164 transcripts in EECs treated with C17 EVs, from which eight genes, *MMP2*, *CPNE8*, *KRT17*, *COL1A2*, *MAGE4B*, *COL1A1*, *CXCL5*, and *LY6E*, were up-regulated and 11 genes, *PLAC8*, *RTKN2*, *LIMD1*, *MIR2309*, *PHOSPHO1*, *HSD3B1*, *CTHRC1*, *TNFAIP2*, *FRMD4A*, *SLCO2A1*, and *TRRAP*, were down-regulated compared with the control. There were very few DEGs in C17 EVs-treated EECs: the correlation coefficient with control EECs was 0.99; however, of these DEGs, factors that may govern uterine environment required for conceptus implantation were observed. For example, one of the new IFNT-regulated genes with various potential roles such as immunity, differentiation, and proliferation was up-regulated in pregnant cows (Mansouri-Attia *et al.* 2009,

Table 3 Gene expression related to P17 EVs-induced GO terms, which are independent of IFNT.

GO_BP Term	P-value	Genes
Up-regulation		
Inflammatory response	1.16E-08	<i>PTGFR, CD40, IL6, NCR3, CXCL8, CXCR4, CXCL3, CXCL2, CXCL5, APOL3</i>
Response to molecule of bacterial origin	4.01E-08	<i>CD40, IL6, CXCL8, TNFAIP3, CXCL3, CXCL2, CXCL5</i>
Response to lipopolysaccharide	5.05E-08	<i>CD40, IL6, CXCL8, ZC3H12A, TNFAIP3, CXCL3, CXCL2, CXCL5</i>
Regulation of leukocyte chemotaxis	5.76E-08	<i>CD40, IL6, CXCL8, ZC3H12A, TNFAIP3, CXCL3, CXCL2, CXCL5</i>
Positive regulation of leukocyte migration	1.09E-07	<i>CXCL5, CXCL3, CXCL2, NFKBIA, CXCL8, CD40</i>
Regulation of I-kappaB kinase/NF-kappaB signaling	4.13E-07	<i>NFKBIA, CD40, ECM1, ZC3H12A, TNFAIP3, APOL3, BIRC3, LTF</i>
Positive regulation of chemotaxis	6.17E-07	<i>IL6, CXCL8, CXCL3, CXCL2, CXCL5</i>
Cytokine-mediated signaling pathway	1.13E-06	<i>IL22RA1, NFKBIA, CD40, IL6, CSF2, CXCL8, MMP1, CXCL3, MMP9, CXCL2, CXCL5, BIRC3</i>
Positive regulation of neutrophil chemotaxis	1.38E-06	<i>CXCL8, CXCL3, CXCL2, CXCL5</i>
Positive regulation of neutrophil migration	1.63E-06	<i>CXCL8, CXCL3, CXCL2, CXCL5</i>

Figure 4 Effect of exosomal CD40L on the activation of NF-κB in EECs. (A) Fold changes in *CD40* in EECs incubated without (control) or with P17 EVs (10 μg) for 48 h ($n=3$ each), which were subjected to qPCR or RNA-seq analysis. *ACTB* and *GAPDH* mRNA served as internal controls for RNA integrity. (B) The lysates of C17 and P17 EVs were subjected to Western blotting. The bar graphs show CD40L levels normalized by HSP70 levels ($n=3$ each). $*P<0.05$ vs C17 EVs. (C) EECs were treated without (control) or with P17 EVs (10 μg) for 48 h. Cell lysates were subjected to Western blotting. The bar graphs show acetylated NF-κB p65 levels normalized by total NF-κB p65 levels ($n=3$ each). $*P<0.05$ vs control. (D) EECs were treated without or with P17 EVs (10 μg) and/or TCPA1 (20 nM, NF-κB pathway inhibitor) for 48 h. RNAs were subjected to qPCR to examine changes in TNF signaling pathway-related gene expression. *ACTB* and *GAPDH* mRNAs served as internal controls for RNA integrity. Letters over the bars indicate significant differences between treatment means ($P<0.05$).

Cheng et al. 2017). In this study, down-regulation of *PLAC8* was observed in C17 EVs-treated EECs, which may be caused by the autocrine effects of C17 EVs. A previous study in which EVs are secreted from uterine epithelia as well as conceptus trophoblast in ruminants (Burns et al. 2016) supports this finding. These results support that EVs, released from uterine endometrium and/or elongated conceptus, are involved in intercellular communications in an autocrine and/or paracrine manner.

In previous studies, transcriptome profiles, obtained from bovine endometrial explants treated with or without ovine IFNT, AI-derived conceptus, and

IVF-derived conceptuses, also demonstrated IFNT-independent endometrial response (Mathew et al. 2019, Sánchez et al. 2019). Among IFNT-independent DEGs found in those endometrial explants, only *BIRC3* and *ECM1* were found as common transcripts between theirs and our DEGs of P17 EVs-treated EECs. Finding only two transcripts as the common transcripts was not surprising because the experimental conditions of the previous studies and ours such as co-cultured time, the origin of IFNT, or the day of pregnant stage differed.

It is known that CD40 is expressed in a wide range of immune cells including monocytes, dendritic cells, and B cells and that upon CD40L binding to CD40, elicitation of immune and inflammatory processes is mediated by the activation of an intracellular NF-κB pathway signaling (Henn et al. 1998, Qian et al. 2002). In the present study, CD40L and its receptor CD40 was found in P17 EVs and EECs treated with P17 EVs, respectively. Treatment with P17 EVs induced the activation of NF-κB, and treatment with NF-κB signaling inhibitor down-regulated P17 EVs-induced TNF signaling pathway-related gene expression in EECs. In addition, our previous study indicated that higher expression of CD40 was found in EECs treated with intrauterine P17 EVs compared with those of intrauterine EVs on day 20 of pregnancy (unpublished observation). These findings suggest that P17 EVs containing CD40L could bind to CD40 displayed by endometrial epithelium, as well as immune cells, which induces inflammatory response mediated by the activation of NF-κB in endometrial epithelium during pre-attachment period.

A delicate interplay of pro- and anti-inflammatory signaling takes place within the endometrium during early pregnancy in mammals (Mor et al. 2011). Our work provides evidence that P17 EVs, independent of IFNT, induced significantly enriched 'TNF signaling pathway' and 'Inflammatory response' in EECs. During initial crosstalk with the endometrium, conceptuses released cytokines, many of which were characterized as pro-inflammatory mediators (Kelly et al. 2001, Mathew et al. 2016). The pro-inflammatory response enhances the expression of other cytokines and/or chemokines, which in turn recruit immune cells such as macrophages and dendritic cells to the site of inflammation (Granot et al. 2012). These immune cells secrete different factors that may affect uterine natural killer cell differentiation and stimulate the luminal endometrial cells to produce adhesion molecules enabling the attachment of conceptus to the endometrium. In humans, the success of implantation is apparently attributed to an injury-induced inflammatory response that leads to the development of an adequate decidua supporting implantation (Gnainsky et al. 2010). Taken together, these findings suggest that the inflammatory response in the endometrium induced by P17 EVs is required for the acquisition of uterine receptivity for successful implantation and the subsequent pregnancy outcome.

In conclusion, the comparison of global transcriptome analyses among C17 EVs-, P17 EVs-, and IFNT-treated EECs revealed that induction of TNF signaling pathway-related genes via CD40L/CD40/NF- κ B pathway was independent of IFNT effects in P17 EVs-treated EECs. Therefore, P17 EVs independent of IFNT, some of which secreted from conceptus, could induce a pro-inflammatory response in the endometrium to regulate the uterine receptivity, facilitating conceptus implantation to the uterine epithelium.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K N and K K performed experiments and analyses. K N, K K, and K I wrote the main manuscript text, and K N and K K prepared all figures and tables. A I and M H were involved in the planning of the entire experimentation and provided uterine flushing and bovine tissues samples. K N, K K, A I, K I, and M H reviewed the manuscript.

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