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Original Research Article

Bovine placental extracellular vesicles carry the fusogenic syncytin BERV-K1

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ABSTRACT

Syncytins are endogenous retroviral envelope proteins which induce the fusion of membranes. A human representative of this group, endogenous retrovirus group W member 1 envelope (ERVW-1) or syncytin-1 is present in trophoblast-derived extracellular vesicles and supports the incorporation of these extracellular vesicles into recipient cells. During pregnancy, placenta-derived extracellular vesicles participate in feto-maternal communication. Bovine fetal binucleate trophoblast cells express the syncytin, bovine endogenous retroviral envelope protein K1 (BERV-K1). These cells release extracellular vesicles into the maternal stroma, but it is unclear whether BERV-K1 is included in these extracellular vesicles. Here, extracellular vesicles were isolated from bovine placental tissue using collagenase digestion, ultracentrifugation, and size exclusion chromatography. They were characterized with transmission electron microscopy, nanoparticle tracking analysis, immunoblotting and mass spectrometry. Immunohistochemistry and immunoelectron microscopy were used to localize BERV-K1 within the bovine placental tissue. The isolated extracellular vesicles range between 50 and 300 nm, carrying multiple extracellular vesicle biomarkers. Proteomic analysis and immunoelectron microscopy confirmed BERV-K1 presence on the isolated extracellular vesicles. Further, BERV-K1 was localized on intraluminal vesicles in secretory granules of binucleate trophoblast cells. The presence of BERV-K1 on bovine placental extracellular vesicles suggests their role in feto-maternal communication and potential involvement of BERV-K1 in uptake of extracellular vesicles by target cells.

1. Introduction

In the bovine placenta, fetal cotyledons and maternal caruncles together form the placentomes [1]. Within these placentomes, fetal stem villi branch into smaller terminal villi [2], expanding into maternal crypts to ensure an efficient exchange. Fetal binucleate trophoblast cells (BNCs) fuse with maternal epithelial cells to form trinucleate feto-maternal hybrid cells [3] and express pregnancy associated glycoproteins (PAGs) [4] and placental lactogen [5], which are stored within their secretory granules. Lectins specifically binding to BNCs and their secretory granules are *Phaseolus vulgaris* leucoagglutinin (PHA-L) and *Dolichos biflorus* agglutinin (DBA) [6,7]. These secretory granules contain intraluminal vesicles [8] with a mean diameter of 50–60 nm [9]. The content of the granules is released by exocytosis into the maternal connective tissue after fusion of the BNC [10]. The vesicles are exocytosed along with the protein contents of the secretory granules and can be found as extracellular vesicles (EVs) in the maternal connective tissue close to trinucleate feto-maternal hybrid cells [8,9].

Bovine placental EVs isolated from primary trophoblast cells can influence the expression of uterine receptivity-related proteins in bovine endometrial cells [11]. Such observations indicate the participation of bovine placental EVs in feto-maternal communication and their potential to affect maternal cells locally and possibly systemically.

EVs are membrane-bound vesicles classified into three main subtypes among others [12,13]: microvesicles and exosomes [14], and apoptotic

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Abbreviations

BERV-K1	bovine endogenous retroviral envelope protein K1/ Fematrin-1
BNC	binucleate cell
CRL	crown rump length
DBA	*Dolichos biflorus* agglutinin
EV	extracellular vesicle
ERVW-1	endogenous retrovirus group W member 1, envelope/ syncytin-1
gd	gestational day
IEM	immunoelectron microscopy
IF	immunofluorescence
IHC	immunohistochemistry
MS	mass spectrometry
MVB	multivesicular body
NTA	nanoparticle tracking analysis
PAG	pregnancy associated glycoprotein
PHA-L	*Phaseolus vulgaris* leucoagglutinin
RUM1	syncytin-Rum1 envelope protein
TEM	transmission electron microscopy

bodies, which are released from dying cells [15]. Microvesicles (50–1000 nm), are shed by outward budding from the plasma membrane [16]. Exosomes (30–150 nm), are formed when multivesicular bodies (MVBs) fuse with the cell membrane and release their intraluminal vesicles into the extracellular space [17]. EVs transport various cargo molecules, such as proteins and nucleic acids, and thereby participate in cellular communication [18].

In the human placenta, EVs deriving from villous explant cultures carry ERVW-1 (syncytin-1), a human endogenous retroviral envelope protein [19]. ERVW-1 induces syncytialization and thus the formation of the syncytiotrophoblast through cell fusion [20]. It is found on EVs derived from villous cytotrophoblast explants and enhances the integration of these EVs into BeWo cells [21]. This phenomenon might be explained by the specific function of retroviral envelope proteins. The surface unit of retroviral envelope proteins interact with corresponding receptors, hASCT2 in the case of syncytin-1 [22], leading to conformational changes within the transmembrane unit and triggering membranal fusion [23]. Like ERVW-1, the bovine endogenous retroviral envelope protein K1 (BERV-K1) also has fusogenic capacities [24]. BERV-K1 is predominantly expressed in BNCs and was integrated into the genome of *Bovinae*, a subfamily of the *Bovidae* containing mostly larger-sized ungulates such as domestic cattle [24,25].

However, it is unknown if the EVs from BNCs carry BERV-K1 and which functions they might have. The migration of BNCs and their fusion with a maternal epithelial cell suggest its possible involvement in feto-maternal communication. The degranulation of the intraluminal vesicles contained in their secretory granules upon fusion, suggests a purpose of these vesicles at the feto-maternal interface. If present on the EV surface, fusogenic BERV-K1 might assist their function by facilitating their internalization into target cells, through its inherent ability to fuse membranes. In this study, we isolated EVs from bovine placental tissue and evaluated the presence of BERV-K1 on these EVs.

2. Materials & methods

2.1. Tissue collection

Bovine (*Bos taurus*) placental tissue samples were collected at the local abattoir (Schlachtbetrieb Zürich AG) approximately 30 min after bolt shot and exsanguination. The gestational stage was estimated based on the measured fetal crown-rump length (CRL) [26]. For EV-isolation, placentomes from 3 cows (gestational days (gd) 117, 133, 158, n = 3) were manually separated into cotyledons and caruncles. Caruncles were washed with ice-cold phosphate-buffered saline (PBS) and minced. For protein and RNA analysis, a caruncle (maternal tissue) and a cotyledon (fetal tissue) sample from each cow were acquired and frozen separately at −80 °C.

For transmission electron microscopy (TEM), placentomes were perfused with 0.1 M sodium potassium phosphate buffer with 4 % wt/vol paraformaldehyde and either 2.5 % wt/vol glutaraldehyde (gd 174) for conventional TEM or 0.05 % wt/vol glutaraldehyde (gd 145) for immunoelectron microscopy (IEM). A 1–2 mm thick slice was cut with a razor blade from the placentome and chopped into cubes of 1–2 mm side lengths.

Further, tissue from cows at midgestation (gd 203, 128, 94, n = 3) was prepared for immunohistochemistry (IHC), immunofluorescence (IF) microscopy and laser capture microdissection (LCM). Tissue was fixed 24 h in 4 % wt/vol neutral buffered formaldehyde (Formafix, Zurich, Switzerland), washed with ice-cold PBS for seven consecutive days, dehydrated, processed, and embedded in paraffin. Formalin-fixed paraffin embedded samples from three (n = 3) prepartal cows were available from a previous study [27]. In these animals, placentomes were collected during the prepartum progesterone decline, i.e., approximately 12–36 h before the onset of active labor, during elective cesarean sections. The animal experimental work was approved by the Regierungspräsidium Giessen (protocol II25.3–19c20/15cGI18/14). Additionally, midgestational placental tissue collected at gd 109 and used for confocal microscopy, was used in previous experiments [7].

2.2. Cell cultures

BT-H cells (RRID: CVCL_WK89, a kind gift from Dr. Rachael Tarlinton (University of Nottingham, UK) under the approval of Drs. Hashizume, Kizaki and Takahashi) are a bovine trophoblast cell model, originally isolated from *in vitro* fertilized bovine blastocysts [28]. They express BNC-specific proteins such as placental lactogen and PAG1 [29]. They were cultured in maintenance DMEM/Ham's F12 medium (Bio Concept, Allschwil, Switzerland) with 10 % v:v heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham MA, USA), 1 % v:v of 200 mM L-glutamine (Gibco, Life Technologies), 100 IU/ml penicillin and 100 µg/ml streptomycin (PANBiotech, Aidenbach, Germany). For collection of EVs, cells were seeded in 150 cm^2 cell culture flasks (Corning, New York, NY, USA) under standard culture conditions (i.e., 37 °C, 5 % CO_2 in air, in a humidified incubator). Before seeding, flasks were treated with collagen coating solution (Cell Applications Inc., Sigma-Aldrich, 125–50) according to the manufacturer's protocol. In short, 13 ml of the collagen coating solution was spread evenly in the flask and left to coat the plastic overnight. Before seeding the cells, the leftover solution was removed, and cell culture medium was added. At approximately 70 % confluency, medium containing FBS was removed and 25 ml medium without FBS was added, to avoid EV contamination from FBS. The cells were cultured under the same conditions for 48 h before conditioned medium collection for EV isolation. 50 ml of medium from two flasks of the same passage were pooled for the isolation of EVs. Three experiments from three different passages were performed (n = 3).

2.3. EV isolation

To isolate EVs from the maternal part of the placenta, caruncle tissue (20 g), immediately after transport to the lab, was washed in PBS three times for 5 min at 37 °C on a shaker (60 rpm) and digested in 1 mg/ml collagenase I in 60 ml PBS for 15 min at 37 °C on a shaker (60 rpm). After filtration through a 70 µm cell strainer, the filtrate was subjected to several centrifugation steps at 4 °C discarding the pellet: 10 min at 50×*g*, 10 min at 300×*g*, 20 min at 2000×*g* and 70 min at 10,000×*g*. With this approach we tried to minimize risks of damaging cell membranes and

avoid intracellular contamination [30]. The supernatant was stored at −80 °C. After being thawed overnight at 4 °C the samples were ultracentrifuged (Sorvall WX80+ Ultracentrifuge, Thermo Fisher Scientific) in a fixed angle rotor (08322, T-647.5, Thermo Fisher Scientific) at 100,000×*g* for 2 h at 4 °C. The pellet was resuspended in 500 µl of sterile PBS and subjected to a size-exclusion chromatography (SEC) using iZON qEVs (35 original columns, IZON Science Europe Ltd, Oxford, UK). Fractions 1–16 (0.5 ml/fraction) were collected according to the manufacturer's instructions. Pooled fractions 7–9 (EV-enriched) were concentrated with centrifugal filters (Amicon Ultra-0.5 Centrifugal Filter Unit, 10 kDa MWCO, UFC5010, Sigma-Aldrich, Chemie GmbH, Buchs, Switzerland). EVs from BT-H cell culture medium were isolated using the same protocol.

2.4. Protein preparation and western blot analysis

Western blot analysis was performed according to a previously published protocol [31]. Briefly, tissue samples were sonicated in NET-2 lysis buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.05 % v:v NP-40) and protease inhibitor cocktail (10 µl/ml). After sonication and concentration adjustment, the proteins were solubilized in a reducing sample buffer (25 mM Tris-HCl, pH 6.8, 1 % wt/vol SDS, 5 % v:v ***β***-mercaptoethanol, 10 % v:v glycerol, 0.01 % v:v bromophenol blue). For the antibodies CD9 and CD81, a non-reducing sample buffer (lacking ***β***-mercaptoethanol) was used, as required for tetraspanins [32]. The antibodies against CD9 [33], CD81 [34,35], TSG101 [36,37] and calnexin [38] have been used before in bovine research. Protein from EV-samples were extracted by sonication in PBS. The protein concentration from EV samples was too low to be measured by Bradford assay, therefore 25 % v:v sample buffer was added. Electrophoresis was performed with 15 µg of the tissue proteins and with 18 µl (including the sample buffer) of the EV protein in 12 % v:v polyacrylamide gels (Bio-Rad Laboratories). After transfer onto PVDF membranes (Bio-Rad Laboratories), non-specific binding sites were blocked by 5 % wt/vol low-fat dry milk in PBS with 0.25 % v:v Tween20 (PBST) (Sigma-Aldrich). The membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies for 1 h at ambient temperature. Antibody incubation was performed in 2.5 % wt/vol low-fat dry milk in PBST. For washing steps in between the incubation steps PBST was used. Detailed information regarding the antibodies used can be found in Table 1. SuperSignal West Pico PLUS Chemiluminescent substrate (Thermo Fisher Scientific) was used to visualize the signals on a ChemiDoc XRS + System (Bio-Rad Laboratories) with Image Lab Software (Bio-Rad Laboratories).

2.5. Nanoparticle tracking analysis (NTA)

The concentration and size distribution of tissue EVs was measured with a NanoSight NS300 (Malvern Panalytical, Malvern, UK). The EV samples were diluted in 1 ml syringes between 1:1000 and 1:10000 and loaded using the Syringe Pump. Each sample was measured and video-recorded seven times for 1 min with a sCMOS camera at level 15. The videos were analyzed by the built-in NanoSight Software NTA3.1 Build 3.1.46 with a detection threshold of 3. Three replicates were analyzed by NTA and measurements of mean, and mode particle size and concentration particles/ml were performed.

2.6. Transmission electron microscopy and immunoelectron microscopy

Tissue blocks for conventional TEM were postfixed with 1 % wt/vol osmium tetroxide for 60 min, stained en bloc with 2 % wt/vol uranyl acetate overnight at 4 °C, dehydrated and embedded in epoxy resin. Ultrathin sections (70 nm) were cut with an ultramicrotome (Ultracut E, Reichert, Vienna, Austria). For cryosections, the blocks were plunge frozen in liquid nitrogen, cryosectioned at −110 °C (80 nm) and placed on hexagonal copper grids. The samples underwent postfixation in 1 % wt/vol glutaraldehyde for 5 min and were contrasted in 2 % wt/vol uranyl acetate in methylcellulose for 5 min at 4 °C. With a TEM (CM12, Philips, Eindhoven, Holland) sections were viewed and pictures were taken with a CCD camera (Orius SC1000, Gatan, Pleasanton, CA, USA).

EV samples on glow discharged grids coated with parlodion and carbon, washed 2 min with PBS, incubated for 1 h at 60 °C in citrate buffer for antigen retrieval and again washed twice in PBS for 2 min. After incubation with glycin for 10 min and a 3 min washing step in PBS with 1 % v:v bovine serum albumin (BSA), the EV samples were incubated for 1 h with the primary custom-made antibody against BERV-K1 (rabbit polyclonal, 1:50, peptides: YKSLHFKSPPKYPNC, IQDWSNPDPGQDPMI, Eurogentec, Seraing, Belgium) [24] in PBS with 1 % v:v BSA. Afterwards, four washing steps with PBS with 0.1 % v:v were performed each for 2 min. The secondary antibody (Table 1) was incubated for 45 min in PBS with 1 % v:v BSA and washing steps were performed first 4 × 2 min PBS and then 2 min water. Before negative contrast staining with 2 % wt/vol uranyl acetate for 1 min on ice, the samples were fixed in 1 % wt/vol glutaraldehyde in water for 5 min and again washed 4 × 2 min in water.

Immunogold labeling of tissue was performed the same way as for the EV negative contrasts without the antigen retrieval.

Table 1
List of all commercially available antibodies, control antibodies, lectins and streptavidin used for western blot analysis (WB), immunoelectron microscopy (IEM), immunohistochemical staining (IHC) and immunofluorescence staining (IF).

Antibody	Company	Reference Number	Host	Dilution
CD9	BioLegend	312102	Mouse monoclonal	WB 1:1000
CD81	Santa Cruz	sc-166029	Mouse monoclonal	WB 1:200
TSG101	Santa Cruz	sc-7964	Mouse monoclonal	WB 1:200
Calnexin	Novus Biologicals	NB100-1965	Rabbit polyclonal	WB 1:1000
Goat anti-rabbit IgG (H + L) Secondary Antibody, HRP	Thermo Fisher Scientific	31460	Goat anti-rabbit IgG	WB 1:15000
Goat anti-mouse IgG (H + L), Secondary Antibody, HRP	Thermo Fisher Scientific	W402B	Goat anti-mouse IgG	WB 1:15000
12 nm Colloidal Gold AffiniPure Goat Anti-Rabbit IgG (H + L) (EM Grade)	Jackson ImmunoResearch	111-205-144	Goat anti-rabbit IgG	IEM 1:30
Goat anti-rabbit IgG, antibody (H + L), biotinylated	Vector Laboratories	BA-1000-1.5	Goat anti-rabbit IgG	IHC 1:100
Goat anti-rabbit IgG (H + L) cross-adsorbed antibody, Alexa Fluor 594	Thermo Fisher Scientific	A-11012	Goat anti-rabbit IgG	IF 1:200
Rabbit IgG, Control Antibody	Vector Laboratories	I-1000-5	Rabbit	IF 1:200
Phaseolus Vulgaris Leucoagglutinin (PHA-L), Biotinylated	Vector Laboratories	B-1115-2		IF 1:100
Dolichos Biflorus Agglutinin (DBA), Biotinylated	Vector Laboratories	B-1035-5		IF 1:100
Streptavidin, Alexa Fluor 488 conjugate	Thermo Fisher Scientific	S11223		IF 1:1000

2.7. Mass spectrometry (MS) analysis

The EV isolates (with 4 % wt/vol SDS) were heated for 10 min at 95 °C followed by high-intensity focused ultrasound (VialTweeter Ultrasonic System, Hielscher Ultrasonics, Teltow, Germany). After precipitation and concentration with trichloroacetic acid (Sigma-Aldrich) at a final concentration of 5 % wt/vol, reduction and alkylation of the samples was performed. After digestion with trypsin, peptide separation was performed on an Orbitrap Fusion Lumos (Thermo Fisher Scientific) coupled with an ACQUITY ultra-performance liquid chromatography M-Class system (Waters, Milford, MA, USA). Analysis was carried out on an Iontrap mass spectrometer (Thermo Fisher Scientific). PEAKS Studio XPlus (Bioinformatic Solutions, Waterloo Ontario, Canada) was used to process acquired MS data for identification and the spectra were searched against the bovine UniProt database (including *Bos taurus*) resulting with accession numbers specific for UniProt. Results were imported into the Scaffold software (Scaffold_5.0.0, Proteome Software, Inc, Portland, OR, USA) and a protein FDR of 0.1 % with 2 unique peptides per identified protein and a peptide threshold of 99.0 % were applied and resulted in a total of 1151 proteins. Proteins not detected in all 3 samples were removed, resulting in a total of 711 proteins identified. The resulting list of proteins was compared to proteins datasets in Vesiclepedia [39]. Additionally, the protein dataset was analyzed for functional annotation using Enrichr [40] and FunRich [41].

2.8. Immunohistochemistry, immunofluorescent staining and confocal microscopy

For IHC, paraffin sections (3 μm) were stained with an indirect immunoperoxidase method as reported previously [42]. Briefly, after deparaffination and rehydration, antigen retrieval was performed in 10 mM citrate buffer, pH 6 at microwave irradiation and the endogenous peroxidase activity was quenched with 0.3 % v:v hydrogen peroxide in methanol. Non-specific binding sites were blocked with 10 % v:v normal goat serum (LGC Seracare, Milford, MA, USA). Anti-BERV-K1 (1:2000) was incubated overnight at 4 °C. Preimmune serum of the same rabbit (1:2000) served as a negative control. After incubation with a secondary antibody, the signal was enhanced by streptavidin-peroxidase Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). DAB + substrate kit (Dako Schweiz AG, Baar, Switzerland) was used to detect peroxidase activity and the slides were counterstained with hematoxylin. Sections were observed with a Leica DMRXE microscope (Leica Microsystems, CMS GmbH, Wetzlar, Germany).

IF staining was performed on paraffin sections (3 μm) using the same first steps of the IHC protocol. BERV-K1 antibody (1:2000), PAG1-antibody (1:1000, rabbit polyclonal antibody, kindly gifted by Prof. Jean-François Beckers, University of Liège, Belgium), PHA-L and DBA were incubated overnight at 4 °C. PHA-L and DBA specifically stain the secretory granules of BNCs [7]. Preimmune serum from the same rabbit (1:1000) and a rabbit IgG control antibody served as negative controls for BERV-K1 and PAG1 respectively. DAPI (1:1000, Sigma-Aldrich), streptavidin conjugated to a fluorophore and a fluorochrome-conjugated secondary antibody were incubated for 1 h. The sections were fixed with 4 % wt/vol formalin and coverslipped. Images were taken with a fluorescent microscope (Leica DMI6000B fluorescence microscope, Leica Microsystems) equipped with a Leica K5 camera (Leica Microsystems).

Epoxy resin embedded sections 90 nm were treated 10 min with saturated ethanolic sodium ethoxide for epoxy resin removal and washed 3 times in 100 % ethanol [7] before undergoing the IF protocol. They were observed with a confocal laser scanning microscope (SP8, Leica Microsystems).

For immunocytochemistry, BT-H cells were seeded into Nunc Lab-Tek II Chamber Slides (Thermo Fisher Scientific), incubated until 90 % confluency, and fixed with 2 % wt/vol formalin at 37 °C for 10 min. After two 5 min washing steps with ice cold PBS (pH 7.25) with 0.3 % v:v Triton X-100 (Sigma Aldrich) (IHC-Buffer), antigen retrieval with citrate buffer at 60 °C for 10 min on a heating plate and blocking of non-specific binding sites with 10 % v:v goat serum, primary antibodies were incubated for 3 h and secondary antibodies for 1 h in IHC-Buffer with a set of three 10 min washes using IHC-Buffer in between. After another three 10 min washes with IHC-Buffer and a 10 min post-fixation with 2 % formalin, the slides were rinsed with tap water and coverslips were mounted. Information regarding the antibodies, the lectins and streptavidin can be found in Table 1.

2.9. Laser capture microdissection

Paraffin sections (12 μm) on PEN Membrane Glass Slides (Thermo Fisher Scientific) from three bovine midgestational and prepartal placentomal samples were stained with cresyl violet [43]. Areas from stem and terminal villi were identified using consecutive IHC slides stained for BERV-K1 and isolated using Laser-capture microdissection with the ArcturusXT Laser Capture Microdissection System (Thermo Fisher Scientific) and the Arcturus CapSure Macro LCM Caps (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Tissue from two caps per sample was detached by submersion in xylene. RNA isolation was performed immediately.

2.10. RNA isolation, reverse transcription and polymerase chain reaction (PCR)

RNA from LCM-tissue was isolated using the RNeasy FFPE Kit (Qiagen, Redwood City, Ca, USA) according to the manufacturer's protocol. In short, after removal of xylene, the samples were incubated with proteinase K first at 56 °C and later at 80 °C, for 15 min at each temperature. DNase treatment was performed for 15 min at ambient temperature and RNA was concentrated using the RNeasy MinElute Spin Columns provided in the isolation kit. RNA was eluted in RNase free water and the concentration was measured with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Due to the low mRNA concentration yield from paraffin embedded samples, the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to synthesize complementary DNA (cDNA) from 10 ng of total RNA per sample according to the manufacturer's protocol. RNA from cell culture samples was isolated using the TRIZOL® reagent, DNase treated with RQ1 RNase-free DNase (Promega, Dübendorf, Switzerland) reverse transcribed into complementary DNA using MultiScribe Reverse Transcriptase (Thermo Fisher Scientific). TaqMan PCRs were run in duplicates with the Fast Start Universal Probe Master (ROX) (Roche Diagnostics AG, Rotkreutz, Switzerland) as previously described [42]. Negative controls were run with autoclaved water. All reactions were performed in an automated fluorometer ABI PRISM 7500 Sequence Detection System (Thermo Fisher Scientific). Detailed information on primers and TaqMan probes are listed in Table 2. The expression of target genes was quantified using the comparative Ct method (ΔΔCt) and normalized to the expression of the reference gene *GAPDH*. Other reference genes (*ACTB* and *SDHA*) were not stably expressed in this tissue as revealed by the online RefFinder tool [44]. With GraphPad 3.06 Software (GraphPad Software, San Diego, CA, USA) an unpaired, two-tailed Student t-test was performed, to compare *BERV-K1* and *PAG1* transcript expression in stem and terminal villi. A P-value <0.05 was considered as statistically significant.

For the isolation of RNA from caruncles and cotyledons, frozen tissue samples (−80 °C) were crushed in the frozen state with a pestle. The ground tissue samples were then dispersed in 500 μl TRIZOL® reagent (Thermo Fisher Scientific), followed by homogenization. Afterwards, the RNA isolation and reverse transcription (RT) were performed following the protocol used for the RNA isolation from cell culture samples. For qualitative PCR, cDNA was amplified using the AmpliTaq Gold™ DNA Polymerase with Gold Buffer and $MgCl_2$ (Thermo Fisher Scientific) under the following conditions: 10 min at 95 °C, 39 cycles

Table 2
List of all TaqMan systems used for semi-quantitative RT-PCR.

Primer Gene Name	Company	Accession Number	Primer Sequence		Product Length (bp)
GAPDH	Microsynth	NM_001034034	Forward	5'-GCG ATA CTC ACT CTT CTA CCT TCG A-3'	82
			Reverse	5'-TCG TAC CAG GAA ATG AGC TTG AC-3'	
			Taq Man probe	5'-CTG GCA TTG CCC TCA ACG ACC ACT T-3'	
SDHA	Microsynth	NM_174178	Forward	5'-ATG GAA GGT CTC TGC GCT AT-3'	119
			Reverse	5'-ATG GAC CCG TTC TTC TAT GC-3'	
			Taq Man probe	5'-ACA GAG CGA TCA CAC CGC GG-3'	

Primer Gene Name	Company	Accession Number	Product Number	Product Length (bp)
BERV-K1	Thermo Fisher Scientific	NM_001245951.2	Bt04944388_s1	77
PAG1	Thermo Fisher Scientific	NM_174411.2	Bt03279187_m1	160
ACTB	Thermo Fisher Scientific	NM_173979.3	Bt03279175_g1	144

each with a 1 min step at 94 °C for denaturing and 1 min at 62 °C, and a final step for 10 min at 72 °C. The amplicons were visualized on an ethidium bromide stained 2 % wt/vol agarose gel [45,46].

3. Results

3.1. Characterization of bovine placental EVs

Round vesicles were found in EV isolates, that appeared cup-shaped, likely due to an artifact of the drying process for TEM [47], from all placental samples (Fig. 1A) as well as in EVs isolated from BT-H cells (Fig. 1B). The size range was analyzed using NTA resulting in a mean size of 151 nm, a median size of 131 nm, a mode size of 100 nm and a mean ± SEM concentration of $3.69 \times 10^{12} \pm 4.57 \times 10^{11}$ particles/ml for the three replicates of EVs isolated from tissue (Fig. 1C). With immunoblotting, the presence of EV enriched protein markers in both EV groups was detected. Thus, EVs from tissue were positive for known EV markers in the membrane (tetraspanins CD9 and CD81) and in the cytosol (TSG101). Calnexin, a marker for the endoplasmic reticulum, was observed in these isolates (Fig. 1D). EVs isolated from BT-H conditioned medium were also positive for CD9, CD81, TSG101, while calnexin was absent (Fig. 1E).

3.2. Proteomic analysis of bovine placental EVs

Mass spectrometry identified a total of 711 proteins in all three analyzed samples. Among them were numerous proteins, listed in Table 3, that are expected to be enriched in EVs according to the EV guidelines 2018 [48]. Comparison with Vesiclepedia's protein database showed that 66 were among the '100 most identified proteins in EVs in Vesiclepedia (Supplementary Table 1) [39]. Another 44 proteins have not been detected in EVs before, according to Vesiclepedia [39], among them BERV-K1 and syncytin-rum1 envelope protein (RUM1) (Supplementary Table 2). All proteins found in the three samples can be viewed in the supplementary file. Enrichr [40] showed gene ontologies related to the lysosomal, secretory granule, endosomal and extracellular vesicle pathways (Fig. 2A). Jensen compartments [49] found enriched gene ontologies related to different aspects of the extracellular vesicle pathway (Fig. 2B). FunRich [41] also hinted at an enrichment in exosomal and lysosomal pathways (Fig. 2C).

3.3. BERV-K1 in bovine placental EVs

In IEM, a subpopulation of EVs, derived from bovine placental tissue, was immunogold-positive for BERV-K1 (Fig. 3A).

3.4. Distribution of BERV-K1 in the bovine placenta

3.4.1. Spatial and temporal distribution

IHC detected BERV-K1 predominantly in BNCs (Fig. 4A). In midgestational placental samples, expression of BERV-K1 was prominent in stem villi and weak in terminal villi (Fig. 4A). The same pattern was visible in IF, however, PHA-L displayed a more even distribution in BNCs of different localizations (Fig. 4B). In prepartal bovine placental tissue, BNCs from stem and terminal villi were stained evenly for BERV-K1 in IHC and IF (Fig. 4C). These findings were verified on a transcriptional level using a combination of LCM and RT-qPCR. *BERV-K1* mRNA in midgestational samples was significantly more abundant in stem villi than terminal villi (Fig. 4D), while mRNA-levels of *PAG1* were comparable to each other (Fig. 4E). Prepartal samples did not differ significantly in their *BERV-K1* mRNA-levels between stem and terminal villi (Fig. 4F). *PAG1* could not be used as reference on the amount of BNCs in prepartal samples as its expression decreases drastically shortly before parturition [4].

3.4.2. Secretory granular localization

For a more precise subcellular localization of BERV-K1 within BNCs, confocal microscopy was used on samples fixed with 0.5 % wt/vol glutaraldehyde for better tissue preservation. Double staining with anti-BERV-K1 and DBA, demonstrated their co-localization in the secretory granules of BNCs (Fig. 5A). BERV-K1 expression seemed ring-shaped whereas the signal for DBA was visible in the middle of BERV-K1 labeled rings (Fig. 5A). PAG1 was located more centrally and thus appeared to have a greater direct overlap with DBA than BERV-K1 (Fig. 5A). For an even more accurate localization, IEM with anti-BERV-K1 was performed on placental samples. To maintain antigenicity, a reduced amount of glutaraldehyde was used, leading to a decreased tissue conservation. Consequently, the membranes of secretory granules were partly broken. In the broken secretory granules several small vesicles were found with sizes matching those of exosomes, some of them being immunogold-positive for BERV-K1 (Fig. 5B). In tissue fixed with a higher concentration of glutaraldehyde without immunogold-labeling, the small vesicles were mainly found within the secretory vesicles (Fig. 5C).

3.5. BERV-K1 in BT-H cells

In immunocytochemistry, most BT-H cells did not show positive signals for BERV-K1 but do express PAG1 and bind PHA-L. However,

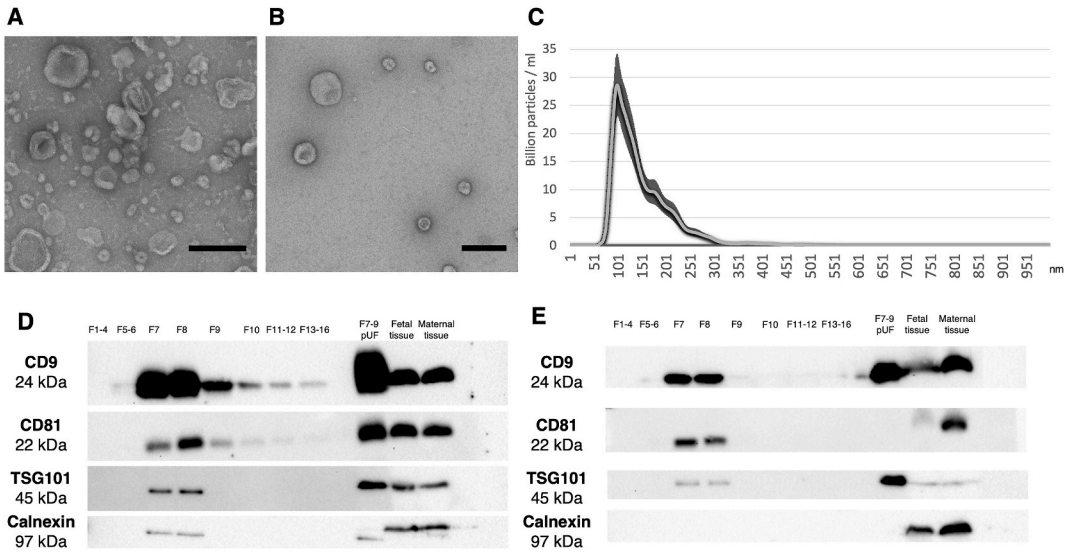

Fig. 1. Characterization of bovine placental EVs A) Transmission electron microscopy image of negatively contrasted EVs isolated from bovine placental tissue. Bar = 200 nm. B) Transmission electron microscopy image of negatively contrasted EVs isolated from BT-H cells. Bar = 200 nm. C) Nanoparticle tracking analysis of three independent EV samples isolated from bovine placental tissue. D) Immunoblotting of EV samples isolated from bovine placental tissue. F1 – F16 represent the fractions collected after size exclusion chromatography. F7 – 9 pUF represents the pooled fractions 7–9 after concentration with ultrafiltration. Fetal tissue samples were isolated from cotyledonary tissue and maternal tissue samples were isolated caruncular tissue. E) Immunoblotting of EV samples isolated from BT-H cells. F1 – F16 represent the fractions collected after size exclusion chromatography. F7 – 9 pUF represents the pooled fractions 7–9 after concentration with ultrafiltration. Due to a lack of sample material, no protein has been added in the F7-9 pUF well detecting for CD81. Fetal tissue samples were isolated from cotyledonal tissue and maternal tissue samples were isolated caruncular tissue.

Table 3
List of EV enriched proteins detected in bovine placental EVs.

Transmembranal EV biomarkers	
Name	Symbol
CD9 molecule	CD9
Heterotrimeric G proteins	GNA*
Integrins	ITGA*, ITGB*
Lysosomal-associated membrane protein 2	LAMP2
Heparan sulfate proteoglycan 2	HSPG2
Basigin	BSG
GPI-anchored 5'nucleotidase	NT5E
Epithelial cell adhesion molecule	EPCAM
Cytosolic EV biomarkers	
Name	Symbol
Tumor suppressor gene 101	TSG101
Programmed cell death 6 interacting protein, Alix	PDCD6IP
Charged multivesicular body proteins	CHMP*
Vacuolar protein sorting 4 homolog A	VPS4A
Flotillin 1	FLOT1
EH-domain containing	EHD*
Transforming protein RhoA	RHOA
Annexins	ANXA*
Heat Shock protein family A member 8	HSPA8
ADP-ribosylation factor 6	ARF6
Syndecan binding protein	SDCBP

singular cells were stained for BERV-K1 (Fig. 5D). In PCR, amplicons at an expected size of 77 bps were detected, suggesting the gene expression of *BERV-K1* mRNA in BT-H cells (Supplementary Fig. 1).

4. Discussion

Feto-maternal communication in the bovine placenta has been extensively studied regarding the importance of successful reproduction in cows. Recently, the role of EVs in feto-maternal communication became of interest to researchers. The present study elucidated some facets of bovine placental EVs to further our understanding of their potential implications in feto-maternal crosstalk. To the authors' knowledge, this is the first study showing successful isolation of EVs directly from bovine placental tissue and identifying BERV-K1 as a molecular component of these EVs. Due to the low number of samples and the widespread timepoints, which result from the difficulty of obtaining the samples, any quantitative statements would be preliminary. Accordingly, the study retains a preliminary character.

Bovine placental EVs from primary bovine trophoblast cells have been isolated before and were shown to participate in feto-maternal communication by affecting expression levels of uterine receptivity-related proteins in endometrial epithelial cells [11]. In the present study, the isolated samples displayed several characteristics of EVs, such as their size, shape, and protein composition. Nonetheless, the detection of certain intracellular markers (e.g. calnexin) in placental isolates suggested the potential presence of contamination from intracellular compartments. This could be attributed to the fact that EVs were isolated from tissue and not from cell culture medium or body fluids. EV samples deriving from murine brain tissue also retain cellular markers such as calnexin [50].

Mass Spectrometry revealed 711 proteins present in all three placental samples used for the isolation of EVs, some of them potentially lead to functional implications. Among them was PTGFRN, the inhibitor of the prostaglandin F2α receptor. It inhibits prostaglandin F2α effects, which include myometrial contractions and luteolysis [51,52]. The presence of multiple integrins in EVs might mediate their organotropic uptake into specific target organs [53]. Additionally, different steroid-hormone-related factors were identified, among them steroid sulfatase (STS). In the bovine placenta, STS is expressed in caruncular epithelium and some BNCs [54]. STS converts estrogen sulfates into free, biologically active estrogens, which may support caruncular differentiation [55].

Nevertheless, our attention was drawn to the fact that BERV-K1 was found in bovine placental EVs. IEM revealed a subpopulation of EVs being positively labeled, possibly deriving from BNCs/trinucleate cells, as these cells seem to be the main source of BERV-K1 in the bovine placenta [56]. The BERV-K1-negative EVs might originate from other cells or present another subpopulation of EVs from BNCs. The elevated

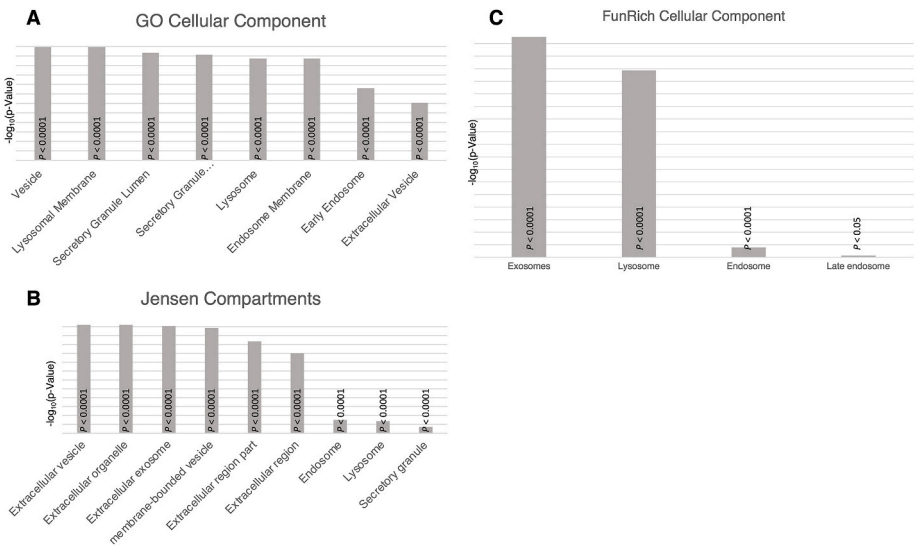

Fig. 2. Proteomic analysis of bovine placental EVs A) Enriched Cellular Component Gene Ontologies using Enrichr. B) Enriched Compartments using Enrichr. C) Enriched Cellular Components using FunRich.

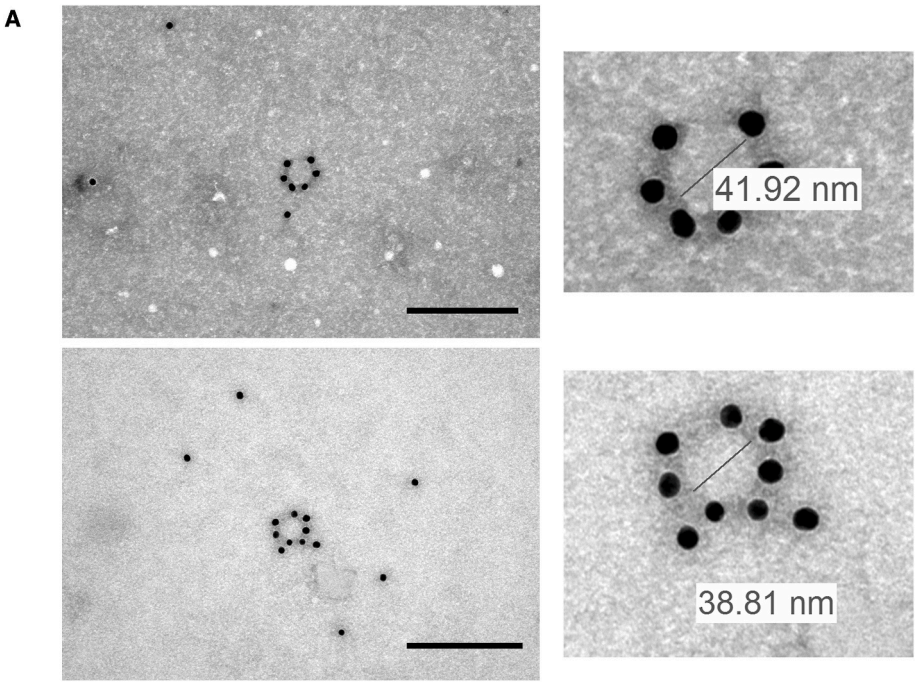

Fig. 3. BERV-K1 in bovine placental EVs A) Immunoelectron microscopy of EVs labeled with anti BERV-K1 isolated from bovine placental tissue. Insets on the right show a close-up of the EVs labeled with anti-BERV-K1. Bars = 200 nm.

expression of BERV-K1 in stem villi compared to terminal villi in midgestational placentae may explain the differential presence of BERV-K1 on isolated EVs. Fetal stem villi branch into smaller intermediate villi which again branch into even smaller terminal villi to enlarge the fetal absorptive surface and enhance placental substance exchange [2]. This villus architecture is built around the vasculature network with a single artery in the stem villus branching into arterioles in the intermediate villi and into capillary convolutions in the terminal villi [2]. EVs from stem villi might carry more BERV-K1 and exert a different function than EVs from terminal villi.

A murine endogenous retroviral envelope protein syncytin-a leads to the formation of the first syncytiotrophoblast layer in the mouse placenta [57] and therefore has a similar function to the human ERVW-1. The occurrence of analogous characteristics in different species, which are not due to common ancestry, is considered convergent evolution [58]. ERVW-1 eases the incorporation of human placental EVs into BeWo cells [21]. Considering the concept of convergent evolution, BERV-K1, present on bovine placental EVs, might have an analogous function and could facilitate their internalization into target cells, thereby delivering their cargo to the cytoplasm.

In most cases, cells take up EVs by endocytosis, introducing the EVs into the endosomal pathway of the recipient cell, risking degradation upon fusion with a lysosome [59]. Fusion with the superficial cell membrane, occurs less often, but leads to a direct delivery of the EV cargo into the cytoplasm [59]. It has been hypothesized, that EVs and retroviruses share pathways for vesicle biogenesis [60] such as the ESCRT complex and tetraspanins [61] possibly allowing retroviruses to exploit EVs for entering host cells and avoiding immune responses [62].

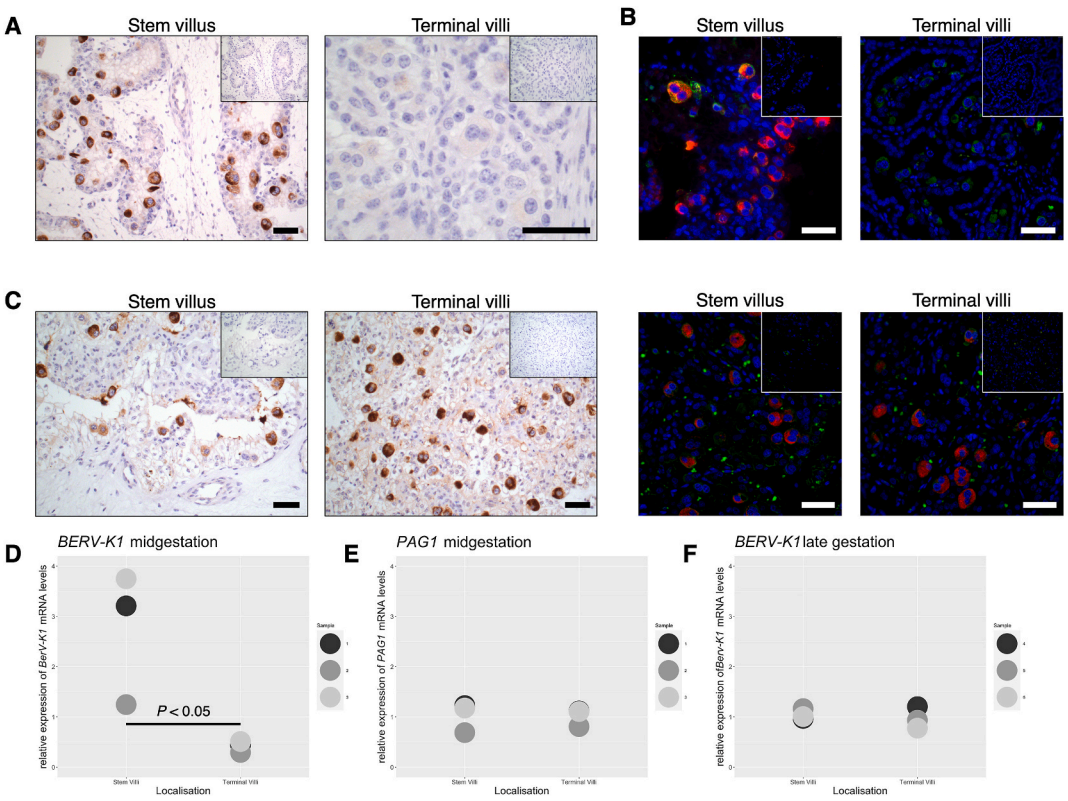

Fig. 4. Distribution of BERV-K1 in the bovine placenta: Spatial and temporal distribution A) Immunohistochemistry in the left pictures shows the distribution of BERV-K1 around a stem villus and the right picture shows its distribution in the terminal villi in a midgestational pregnancy. The insets at the upper right corners represent the negative controls. B) Immunofluorescence of midgestational bovine placental tissue showing the distribution of BERV-K1 (red), PHA-L (green) and DAPI (blue) in a stem villus and in terminal villi. The insets at the upper right corners represent the negative controls. C) Immunohistochemistry and immunofluorescence of prepartal bovine placental tissue showing the distribution of BERV-K1 (brown/red, respectively), PHA-L (green) and DAPI (blue). The insets at the upper right corners represent the negative controls. Bar = 50 μm in all figures. D) Relative gene expression of *BERV-K1* isolated by laser capture microdissection from stem villi compared to terminal villi in midgestational bovine placental tissue. E) Relative gene expression of *PAG1* isolated by laser capture microdissection from stem villi compared to terminal villi in midgestational bovine placental tissue. F) Relative gene expression of *BERV-K1* isolated by laser capture microdissection from stem villi compared to terminal villi in prepartal bovine placental tissue.

Alternately, viral factors are incorporated into EVs and support fusion of the EV membrane with the plasma membrane or the endosomal membrane upon endocytosis [62]. EVs from ovine uterine luminal fluid carry envelope mRNA of endogenous Jaagsiekte sheep retrovirus [63], which is essential for conceptus elongation [64]. Collectively, these studies show that retroviral elements are carried by some EV subpopulations and have important functions via these EVs.

The present study described a distinct spatial and temporal distribution of BERV-K1 in bovine placentomes and its presence in secretory granules of BNCs. BERV-K1 is mainly expressed in BNCs around stem villi throughout midgestation. Before parturition however, BERV-K1 expression seems to be equivalent in both, stem and terminal villi. Other factors, such as the prostaglandin-endoperoxide synthase 2 (PTGS2/COX2) [27], estrogen sulfotransferase (SULT1E1) [65] and CYP17A1 [66] have a similar spatial and temporal distribution even though they are localized in uninucleate trophoblast cells [27]. By releasing more EVs containing BERV-K1 from stem villous derived BNCs, these EVs are potentially better internalized into target cells and possibly deliver different cargo molecules than EVs from terminal villi derived BNCs. Accordingly, the target cells might be better equipped in developing a more mature placenta or they are possibly enriched with BERV-K1 to prime them for fusion events with BNCs. Within the secretory granules, BERV-K1 was located more in the periphery, surrounding the more centrally located DBA-staining. This pattern raises the question if BERV-K1 is either located on the membranes of secretory granules or it is distributed in the periphery of the secretory granules. The contents of secretory granules, containing BERV-K1, are released from the cell only after the fusion of a BNC with a maternal epithelial cell. The function of BERV-K1 to fuse trophoblast cells with maternal epithelial cells therefore cannot be attributed to the BERV-K1 contained within secretory granules and it possibly performs other functions within the target cells. BERV-K1 is predominantly expressed in the BNCs of placentomes as described in previous studies [24,67]. Recently, single-nuclei RNA sequencing found RUM1 being upregulated within intercotyledonary BNCs [68]. RUM1 is another bovine endogenous retroviral envelope protein and was first located in BNCs of placentomal tissue [25]. Our study demonstrated the presence of BERV-K1 on bovine placental EVs and proteomic analysis suggested the presence of RUM-1. Unlike BERV-K1, which exhibits fusogenic activity at a physiological pH 7 [24], the fusogenicity of RUM1 is increased at a lower pH 5 [25]. The participation of RUM1 in cell fusion has been questioned due to its functionality at an unphysiological pH [24]. On EVs it might facilitate the fusion of the EV membrane with the endosomal membrane after endocytosis, since the endosomal lumen is acidified by an ATP-driven proton pump [69]. Our study, demonstrating the presence of RUM1 on the placental EVs, suggests a possible alternative function of RUM1 in the placenta. Thus, it might facilitate the uptake of EVs under acidic conditions in the target cell's endosomal compartment. Syncytins carried by EVs and facilitating their uptake into target cells may provide an explanation for why species, that do not present cell fusion events in the placenta, such as the horses, nevertheless express endogenous retroviral envelope proteins in their placentae [70].

Additionally, the presence of BERV-K1 in bovine trophoblast was verified in the BT-H cell culture model. BT-H cells were isolated from *in*

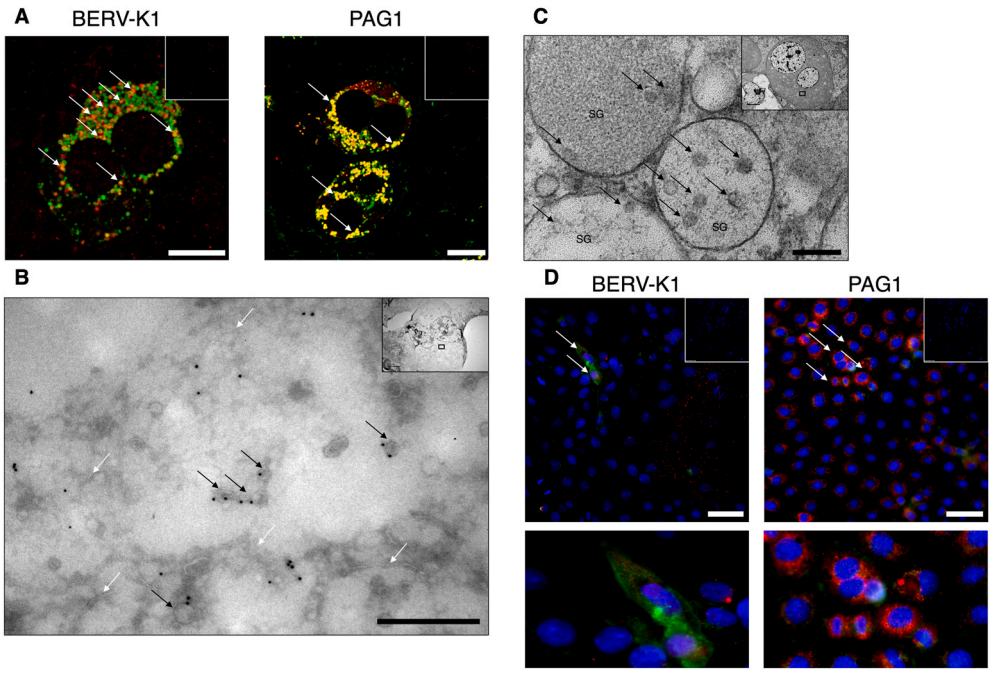

Fig. 5. Distribution of BERV-K1 in the bovine placenta: Localization in BNC and BT-H cells A) Immunocytochemistry of bovine placental tissue with DBA (green) and anti-BERV-K1 (red) on the left and DBA (green) and anti-PAG1 (red) on the right. Left: anti-BERV-K1 signal appears to be surrounding DBA signal (white arrows). Right: Co-localization of anti-PAG1 and DBA signal (white arrows). Inset in the upper right corner represents the negative control. Bar = 10 μm B) Immunoelectron microscopy of bovine placental tissue fixed with 0.05 % wt/vol glutaraldehyde to localize anti-BERV-K1. Intraluminal vesicles with BERV-K1 are marked (black arrows). Fragments of secretory granule membranes are marked (white arrows). Upper right corner represents the whole BNC, marked in black, the localization of the close-up picture. Bar = 500 nm. C) Transmission electron microscopy of bovine placental tissue, fixed with 2.5 % wt/vol glutaraldehyde. The image shows a part of a BNC. Black arrows mark intraluminal vesicles within the secretory granules of this BNC. Upper right corner represents the whole BNC, marked in black, the localization of the close-up picture. SG: secretory granules. Bar = 200 nm. D) Immunocytochemistry of BT-H cells localizing BERV-K1 (red) and PAG1 (red) respectively, DBA (green) and DAPI (blue). BERV-K1 positive cells are marked (white arrows). Images at the bottom show close-ups of the cells expressing BERV-K1 and PAG1. Insets in the upper right corners represent the negative controls. Bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

vitro fertilized bovine blastocysts and serve as an *in vitro* model of bovine trophoblast [28]. In the present study, IHC and PCR revealed the presence of BERV-K1 in this cell line under our culture conditions. Further, the EVs which were isolated from the cultured cells, contain EV-markers CD9, CD81 and TSG101. Calnexin was absent in the EVs from cells, pointing towards reduced contamination compared to EVs isolated from the tissue. However, the cells were cultured under FBS-free conditions, which might induce cell stress and could affect EV secretion [71].

The results of the present study suggest a subpopulation of EVs derive from secretory granules of BNCs. This is an interesting finding, as literature mostly discusses two origins of EVs, the shedding from the plasma membrane as microvesicles [16] or the release from MVBs as exosomes [17]. Intraluminal vesicles with 50–60 nm are found in the secretory granules of BNCs and around trinucleate cells [9]. In the present study, EV samples isolated from bovine caruncular tissue also contained similarly sized vesicles, marked with anti-BERV-K1, suggesting their origin from BNCs. It is likely that these EVs derive from secretory granules of BNCs and are degranulated into the maternal stroma. Some reports suggest the alternative formation of EVs in other cellular organelles and not only in MVBs. Thus, activated blood platelets release exosomes from either MVBs or ***α***-granules [14]. The latter, i.e. ***α***-granules belong to a group of cell type specific subcellular compartments that share features with endosomes and lysosomes, termed lysosome-related organelles [72]. Weibel-Palade bodies, another type of lysosome-related organelles formed at the trans-Golgi network [73], contain intraluminal vesicles that can be immunolocalized with CD63 [74], a common EV marker [48]. Cytolytic granules of natural killer (NK) cells, another example of lysosome-related organelles, also carry intraluminal vesicles that are released from the cell upon target cell contact [75]. It has been proposed that some of these lysosome-related organelles emerge by the fusion of secretory granules and multivesicular endosomes to form these hybrid organelles [72]. Collectively, there seem to be crossovers and merging events between the endosomal and the secretory pathways. The specific secretory granules containing intraluminal vesicles in the BNCs might be another example of these pathways intertwining to produce EVs. Proteomic analysis of the bovine placental EVs showed enriched gene ontologies related to the lysosomal, secretory granule, endosomal and extracellular vesicle system, another hint that several cellular pathways might be involved in the production of these vesicles. However, to verify this hypothesis, more studies are needed to determine the molecular composition and definite origin of these specific secretory granules.

5. Conclusion

In conclusion, EVs from bovine caruncular tissue could be isolated with the method presented in this study. The isolated EVs were round and of an expected size for EVs and their protein composition include several proteins typically found in EVs. Additionally, a subpopulation of EVs appeared to carry BERV-K1. By immunohistochemistry we showed that the expression of BERV-K1 differed between BNCs of specific locations in the placentome, and this difference appeared diminished towards parturition. This study suggests the presence of BERV-K1 within secretory granules of BNCs. BERV-K1 has a fusogenic capacity and is likely to induce the fusion of BNCs with maternal epithelial cells [24]. However, intraluminal vesicles in secretory granules carrying BERV-K1 are released only after the fusion, indicate an additional function of BERV-K1 present on EVs in the bovine placenta. ERVW-1 on human trophoblast derived EVs ease the incorporation of these EVs into target cells [21]. It appears plausible that BERV-K1, present on BNC-derived

EVs, could serve a similar function in the bovine placenta by facilitating the uptake of these EVs by target cells.

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CRediT authorship contribution statement

Jasmin Galli: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carmen Almiñana:** Writing – review & editing, Methodology, Formal analysis. **Mahesa Wiesendanger:** Methodology. **Gerhard Schuler:** Writing – review & editing, Methodology. **Mariusz Pawel Kowalewski:** Writing – review & editing, Supervision, Resources, Methodology. **Karl Klisch:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial or personal interests influencing the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2024.04.012.

References

[1] Strahl H. Die Embryonalhüllen der Säuger und die Placenta. G Fischer Jena: Anonymous Hertwig's Handbuch der vergleichenden und experimentellen Entwicklungslehre der Wirbeltiere; 1906. p. 235–368.
[2] Leiser R, Krebs C, Klisch K, Ebert B, Dantzer V, Schuler G, et al. Fetal villosity and microvasculature of the bovine placentome in the second half of gestation. J Anat 1997;191(Pt 4):517–27.
[3] Wooding FBP, Wathes DC. Binucleate cell migration in the bovine placentome. J Reprod Fertil 1980;59:425–30.
[4] Green JA, Xie S, Quan X, Bao B, Gan X, Mathialagan N, et al. Pregnancy-associated bovine and ovine glycoproteins exhibit spatially and temporally distinct expression patterns during pregnancy. Biol Reprod 2000;62:1624–31.
[5] Duello TM, Byatt JC, Bremel RD. Immunohistochemical localization of placental lactogen in binucleate cells of bovine placentomes. Endocrinology 1986;119:1351–5.
[6] Munson L, Kao JJ, Schlafer DH. Characterization of glycoconjugates in the bovine endometrium and chorion by lectin histochemistry. J Reprod Fertil 1989;87:509–17.
[7] Klisch K, Leiser R. In bovine binucleate trophoblast giant cells, pregnancy-associated glycoproteins and placental prolactin-related protein-I are conjugated to asparagine-linked N-acetylgalactosaminyl glycans. Histochem Cell Biol 2003;119:211–7.
[8] Lee CS, Wooding FB, Morgan G. Quantitative analysis throughout pregnancy of intraepithelial large granular and non-granular lymphocyte distributions in the synepitheliochorial placenta of the cow. Placenta 1997;18:675–81.
[9] Klisch K, Schraner EM. Intraluminal vesicles of binucleate trophoblast cell granules are a possible source of placental exosomes in ruminants. Placenta 2020;90:58–61.
[10] Wooding FB, Beckers JF. Trinucleate cells and the ultrastructural localisation of bovine placental lactogen. Cell Tissue Res 1987;247:667–73.
[11] Su Y, Li Q, Zhang Q, Li Z, Yao X, Guo Y, et al. Exosomes derived from placental trophoblast cells regulate endometrial epithelial receptivity in dairy cows during pregnancy. J Reprod Dev 2022;68:21–9.
[12] Doyle LM, Wang MZ. Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. Cells 2019;8:727.
[13] van Niel G, Carter DRF, Clayton A, Lambert DW, Raposo G, Vader P. Challenges and directions in studying cell-cell communication by extracellular vesicles. Nat Rev Mol Cell Biol 2022;23:369–82.
[14] Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. Blood 1999;94:3791–9.
[15] Wickman G, Julian L, Olson MF. How apoptotic cells aid in the removal of their own cold dead bodies. Cell Death Differ 2012;19:735–42.
[16] Trams EG, Lauter CJ, Salem N, Jr HU. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochim Biophys Acta 1981;645:63–70.
[17] Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem 1987;262:9412–20.
[18] Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654–9.
[19] Tolosa JM, Schjenken JE, Clifton VL, Vargas A, Barbeau B, Lowry P, et al. The endogenous retroviral envelope protein syncytin-1 inhibits LPS/PHA-stimulated cytokine responses in human blood and is sorted into placental exosomes. Placenta 2012;33:933–41.
[20] Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L, et al. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. Nature 2000;403:785–9.
[21] Vargas A, Zhou S, Ethier-Chiasson M, Flipo D, Lafond J, Gilbert C, et al. Syncytin proteins incorporated in placenta exosomes are important for cell uptake and show variation in abundance in serum exosomes from patients with preeclampsia. FASEB J 2014;28:3703–19.
[22] Blond JL, Lavillette D, Cheynet V, Bouton O, Oriol G, Chapel-Fernandes S, et al. An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor. J Virol 2000;74:3321–9.
[23] Gong R, Peng X, Kang S, Feng H, Huang J, Zhang W, et al. Structural characterization of the fusion core in syncytin, envelope protein of human endogenous retrovirus family W. Biochem Biophys Res Commun 2005;331:1193–200.
[24] Nakaya Y, Koshi K, Nakagawa S, Hashizume K, Miyazawa T. Fematrin-1 is involved in fetomaternal cell-to-cell fusion in Bovinae placenta and has contributed to diversity of ruminant placentation. J Virol 2013;87:10563–72.
[25] Cornelis G, Heidmann O, Degrelle SA, Vernochet C, Lavialle C, Letzelter C, et al. Captured retroviral envelope syncytin gene associated with the unique placental structure of higher ruminants. Proc Natl Acad Sci USA 2013;110:E828–37.
[26] Schnorr B, Kressin M. Embryologie der Haustiere: 14 Tabellen. Enke; 2011.
[27] Schuler G, Teichmann U, Kowalewski MP, Hoffmann B, Madore E, Fortier MA, et al. Expression of cyclooxygenase-II (COX-II) and 20alpha-hydroxysteroid dehydrogenase (20alpha-HSD)/prostaglandin F-synthase (PGFS) in bovine placentomes: implications for the initiation of parturition in cattle. Placenta 2006;27:1022–9.
[28] Shimada A, Nakano H, Takahashi T, Imai K, Hashizume K. Isolation and characterization of a bovine blastocyst-derived trophoblastic cell line, BT-1: development of a culture system in the absence of feeder cell. Placenta 2001;22:652–62.
[29] Koshi K, Suzuki Y, Nakaya Y, Imai K, Hosoe M, Takahashi T, et al. Bovine trophoblastic cell differentiation and binucleation involves enhanced endogenous retrovirus element expression. Reprod Biol Endocrinol 2012;25.
[30] Crescitelli R, Lasser C, Lotvall J. Isolation and characterization of extracellular vesicle subpopulations from tissues. Nat Protoc 2021;16:1548–80.
[31] Gram A, Buchler U, Boos A, Hoffmann B, Kowalewski MP. Biosynthesis and degradation of canine placental prostaglandins: prepartum changes in expression and function of prostaglandin F2alpha-synthase (PGFS, AKR1C3) and 15-hydroxyprostaglandin dehydrogenase (HPGD). Biol Reprod 2013;89:2.
[32] Kowal EJK, Ter-Ovanesyan D, Regev A, Church GM. Extracellular vesicle isolation and analysis by western blotting. In: Kuo WP, Jia S, editors. Extracellular vesicles: methods and protocols. New York, NY: Springer New York; 2017. p. 143–52.
[33] Roerig J, Schiller L, Kalwa H, Hause G, Vissiennon C, Hacker MC, et al. A focus on critical aspects of uptake and transport of milk-derived extracellular vesicles across the Caco-2 intestinal barrier model. Eur J Pharm Biopharm 2021;166:61–74.
[34] Saenz-de-Juano MD, Silvestrelli G, Bauersachs S, Ulbrich SE. Determining extracellular vesicles properties and miRNA cargo variability in bovine milk from healthy cows and cows undergoing subclinical mastitis. BMC Genom 2022;23:189.
[35] Go G, Jeon J, Lee G, Lee JH, Lee SH. Bovine milk extracellular vesicles induce the proliferation and differentiation of osteoblasts and promote osteogenesis in rats. J Food Biochem 2021;45:e13705.
[36] Deng S, Essandoh K, Wang X, Li Y, Huang W, Chen J, et al. Tsg101 positively regulates P62-Keap1-Nrf2 pathway to protect hearts against oxidative damage. Redox Biol 2020;32:101453.
[37] Ogunnaike M, Wang H, Zempleni J. Bovine mammary alveolar MAC-T cells afford a tool for studies of bovine milk exosomes in drug delivery. Int J Pharm 2021;610:121263.

[38] Gao HN, Guo HY, Zhang H, Xie XL, Wen PC, Ren FZ. Yak-milk-derived exosomes promote proliferation of intestinal epithelial cells in an hypoxic environment. J Dairy Sci 2019;102:985–96.
[39] Pathan M, Fonseka P, Chitti SV, Kang T, Sanwlani R, Van Deun J, et al. Vesiclepedia 2019: a compendium of RNA, proteins, lipids and metabolites in extracellular vesicles. Nucleic Acids Res 2019;47:D516–9.
[40] Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinf 2013;14:128.
[41] Pathan M, Keerthikumar S, Chisanga D, Alessandro R, Ang CS, Askenase P, et al. A novel community driven software for functional enrichment analysis of extracellular vesicles data. J Extracell Vesicles 2017;6:1321455.
[42] Kowalewski MP, Mason JI, Howie AF, Morley SD, Schuler G, Hoffmann B. Characterization of the canine 3beta-hydroxysteroid dehydrogenase and its expression in the corpus luteum during diestrus. J Steroid Biochem Mol Biol 2006;101:254–62.
[43] Amini P, Ettlin J, Opitz L, Clementi E, Malbon A, Markkanen E. An optimised protocol for isolation of RNA from small sections of laser-capture microdissected FFPE tissue amenable for next-generation sequencing. BMC Mol Biol 2017;18:22.
[44] Xie F, Wang J, Zhang B. RefFinder: a web-based tool for comprehensively analyzing and identifying reference genes, vol. 23. Funct Integr Genomics; 2023. p. 125.
[45] Kowalewski MP, Schuler G, Taubert A, Engel E, Hoffmann B. Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. Theriogenology 2006;66:1423–30.
[46] Gysin T, Kowalewski MP. The involvement of hypoxia-inducible factor 1α (HIF1α)-stabilising factors in steroidogenic acute regulatory (STAR) protein-dependent steroidogenesis in murine KK1 granulosa cells in vitro. Reprod Fertil Dev 2021;33:865–80.
[47] Jung MK, Mun JY. Sample preparation and imaging of exosomes by transmission electron microscopy. J Vis Exp 2018;4:56482.
[48] Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 2018;7.
[49] Binder JX, Pletscher-Frankild S, Tsafou K, Stolte C, O'Donoghue SI, Schneider R, et al. COMPARTMENTS: unification and visualization of protein subcellular localization evidence. Database 2014;2014:bau012.
[50] Huang Y, Cheng L, Turchinovich A, Mahairaki V, Troncoso JC, Pletnikova O, et al. Influence of species and processing parameters on recovery and content of brain tissue-derived extracellular vesicles. J Extracell Vesicles 2020;9:1785746.
[51] Singh LP, Sadiku A, Verma OP. Prostaglandin F2 alpha-induced response of the bovine ovary, oviduct (uterine tube), and uterus. Am J Vet Res 1979;40:1789–91.
[52] Coulson A. Early termination of pregnancy in cattle with dinoprost. Vet Rec 1979;104:553–4.
[53] Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. Nature 2015;527:329–35.
[54] Greven H, Kowalewski MP, Hoffmann B, Geyer J, Rex-Haffner M, Ugele B, et al. Bovine placental steroid sulphatase: molecular cloning and expression pattern in placentomes during gestation and at parturition. Placenta 2007;28:889–97.
[55] Schuler G, Greven H, Kowalewski MP, Doring B, Ozalp GR, Hoffmann B. Placental steroids in cattle: hormones, placental growth factors or by-products of trophoblast giant cell differentiation? Exp Clin Endocrinol Diabetes 2008;116:429–36.
[56] Koshi K, Ushizawa K, Kizaki K, Takahashi T, Hashizume K. Expression of endogenous retrovirus-like transcripts in bovine trophoblastic cells. Placenta 2011;32:493–9.
[57] Dupressoir A, Vernochet C, Bawa O, Harper F, Pierron G, Opolon P, et al. Syncytin-A knockout mice demonstrate the critical role in placentation of a fusogenic, endogenous retrovirus-derived, envelope gene. Proc Natl Acad Sci USA 2009;106:12127–32.
[58] Maloy S, Hughes K. Brenner's encyclopedia of genetics. Elsevier Science; 2013.
[59] Mulcahy LA, Pink RC, Carter DR. Routes and mechanisms of extracellular vesicle uptake. J Extracell Vesicles 2014;3.
[60] Gould SJ, Booth AM, Hildreth JE. The Trojan exosome hypothesis. Proc Natl Acad Sci USA 2003;100:10592–7.
[61] Nolte-'t Hoen E, Cremer T, Gallo RC, Margolis LB. Extracellular vesicles and viruses: are they close relatives? Proc Natl Acad Sci U S A 2016;113:9155–61.
[62] van Dongen HM, Masoumi N, Witwer KW, Pegtel DM. Extracellular vesicles exploit viral entry routes for cargo delivery. Microbiol Mol Biol Rev 2016;80:369–86.
[63] Burns G, Brooks K, Wildung M, Navakanitworakul R, Christenson LK, Spencer TE. Extracellular vesicles in luminal fluid of the ovine uterus. PLoS One 2014;9:e90913.
[64] Dunlap KA, Palmarini M, Varela M, Burghardt RC, Hayashi K, Farmer JL, et al. Endogenous retroviruses regulate periimplantation placental growth and differentiation. Proc Natl Acad Sci USA 2006;103:14390–5.
[65] Khatri P, Frenette G, Sullivan R, Hoffmann B, Schuler G. Expression of SULT1E1 protein in bovine placentomes: evidence for localization in uninucleated trophoblast cells. Placenta 2011;32:431–40.
[66] Schuler G, Ozalp GR, Hoffmann B, Harada N, Browne P, Conley AJ. Reciprocal expression of 17alpha-hydroxylase-C17,20-lyase and aromatase cytochrome P450 during bovine trophoblast differentiation: a two-cell system drives placental oestrogen synthesis. Reproduction 2006;131:669–79.
[67] Polei M, Gunther J, Koczan D, Furbass R. Trophoblast cell differentiation in the bovine placenta: differentially expressed genes between uninucleate trophoblast cells and trophoblast giant cells are involved in the composition and remodeling of the extracellular matrix and O-glycan biosynthesis. BMC Mol Cell Biol 2020;21:1.
[68] Davenport KM, Ortega MS, Liu H, O'Neil EV, Kelleher AM, Warren WC, et al. Single-nuclei RNA sequencing (snRNA-seq) uncovers trophoblast cell types and lineages in the mature bovine placenta. Proc Natl Acad Sci USA 2023;120:e2221526120.
[69] Saermark T, Flint N, Evans WH. Hepatic endosome fractions contain an ATP-driven proton pump. Biochem J 1985;225:51–8.
[70] Stefanetti V, Marenzoni ML, Passamonti F, Cappelli K, Garcia-Etxebarria K, Coletti M, et al. High expression of endogenous retroviral envelope gene in the equine fetal part of the placenta. PLoS One 2016;11:e0155603.
[71] Lehrich BM, Liang Y, Fiandaca MS. Foetal bovine serum influence on in vitro extracellular vesicle analyses. J Extracell Vesicles 2021;10:e12061.
[72] Marks MS, Heijnen HF, Raposo G. Lysosome-related organelles: unusual compartments become mainstream. Curr Opin Cell Biol 2013;25:495–505.
[73] Wagner DD, Olmsted JB, Marder VJ. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. J Cell Biol 1982;95:355–60.
[74] Streetley J, Fonseca AV, Turner J, Kiskin NI, Knipe L, Rosenthal PB, et al. Stimulated release of intraluminal vesicles from Weibel-Palade bodies. Blood 2019;133:2707–17.
[75] Peters PJ, Borst J, Oorschot V, Fukuda M, Krähenbühl O, Tschopp J, et al. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. J Exp Med 1991;173:1099–109.