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Original Article

Exosomes derived from placental trophoblast cells regulate endometrial epithelial receptivity in dairy cows during pregnancy

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Abstract. Inadequate fetomaternal interactions could directly lead to pregnancy failure in dairy cows. Exosomes are widely involved in endometrial matrix remodeling, immune function changes, placental development, and other processes of embryo implantation and pregnancy in dairy cows. However, the role of exosomes derived from placental trophoblast cells in regulating the receptivity of endometrial cells and facilitating fetomaternal interaction remains unclear. In this study, bovine trophoblast cells (BTCs) were obtained from bovine placenta and immortalized by transfection with telomerase reverse transcriptase (TERT). Immortalized BTCs still possess the basic and key properties of primary BTCs without exhibiting any neoplastic transformation signs. Subsequently, the effect of trophoblast-derived exosomes (TDEs) on endometrial receptivity in endometrial epithelial cells (EECs) was determined, and the mechanism whereby TDEs and their proteins participate in the fetomaternal interaction during bovine pregnancy were explored. EECs were co-cultured with the exosomes derived from BTCs treated with progesterone (P4). Such treatment enhanced the expression of the endometrial receptivity factors, integrin αv, β3, Wnt7a, and MUC1 by changing the extracellular environment, metabolism, and redox balance in EECs via proteome alignment, compared with no treatment according to the DIA quantitation analysis. Our study demonstrated that trophoblast-derived exosome proteins are one of the most critical elements in fetomaternal interaction, and their changes may act as a key signal in altering endometrial receptivity and provide a potential target for improving fertility.

Key words: Dairy cows, Early pregnancy, Endometrial receptivity, Exosomes, Trophoblast

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Early pregnancy failures exist in as high as 50% of high-producing dairy cows and may lead to low reproductive efficiency and poor profitability for cattle production systems. This high pregnancy failure rate is generally considered to be because of insufficient communication between bovine fetuses and their environment [1]. In mammalian fetuses, the placenta, a transient organ, plays a critical role in improving fetomaternal interaction by ensuring adequate fetal oxygen and nutrient supply throughout the entire pregnancy, and determining the success of embryo implantation and pregnancy outcomes [2–4]. In bovine placenta, trophoblasts form a barrier between the mother and the fetus [5, 6]. Thus, understanding how obstetric diseases can be avoided and how the pregnancy rate can be increased during the first trimester and pregnancy is necessary for assessing the functions of trophoblasts. Only three bovine trophoblast cell lines, namely, F3 [7], CT-1 [8], and BT-1 [9], exist. F3 is separated from the bovine placenta in the first trimester; however, whether F3 is immortal has not been reported. Isolated primary trophoblast cells of the first trimester are known to have a finite lifespan [7]. BT-1 and CT-1, which are trophoblast stem cells derived from blastocysts, differ from those derived from first trimester placenta and can be cultured for more than 75 passages. However, no model has been built to study the functions of placenta, and samples of placenta are difficult to collect [8, 9]. Therefore, the establishment of a stable bovine trophoblast model derived from the first trimester placenta is helpful for better understanding the new mechanism of fetomaternal dialog.

Fetomaternal interactive activities comprise signal transmission between placental trophoblast cells and EECs to jointly advance embryonic development. In maternal uteruses, the success of fetomaternal interaction is determined by endometrial receptivity in EECs, which is related to uterine factors, such as integrin αv, integrin β3, Wnt7a, and mucin 1 (MUC1). Among them, integrins αv and β3 are transmembrane receptors that can bind to specific ligands of the extracellular matrix, and promote early embryo-maternal interaction by impacting the opening of the endometrium implantation window and the invasion of trophoblasts into EECs [10–12]. MUC1, an anti-adhesive factor, plays a negative role during early pregnancy by repressing the adhesion between trophoblast and uterine epithelial cells [13]. Wnt7a can establish a signal connection between EECs and mesenchyme, and mediate the initial adhesion process between the embryo and luminal epithelial cells by activating the Wnt/β-catenin signaling pathway [14, 15].

Exosomes, which are double-layered extracellular vesicles (EVs) with a diameter of 30–150 nm, can release vesicles into the extracellular space to mediate information exchange between the cells by fusing with the plasma membrane [16, 17]. Trophoblast cells can affect maternal physiological functions by sending specific biologically active molecules into maternal blood circulation through the villi space and packing them into exosomes [18, 19]. The orderly expression of exosomes in time and space can enable the smooth completion of the pregnancy [20]. Many studies have evaluated the role of the endometrium and exosomes secreted by embryos in

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the attachment of mammalian embryos; however, only few have examined the role of trophoblast-derived exosomes (TDEs) in the attachment of embryos. This study aimed to detect the receptivity changes in EECs caused by exosomes secreted by bovine trophoblast cell lines, which were established by introducing exogenous human telomerase during pregnancy.

Materials and Methods

Isolation, purification, and culture of bovine trophoblast cells

Placenta of early pregnant Holsteins (45–60 days after pregnancy; fetal cow with a crown-to-rump length of approximately 7 cm) were obtained from cows killed by exsanguination. This study was performed in accordance with the guidelines of the Animal Ethics Committee of the Beijing University of Agriculture (Permit No.: SYXK (JING) 2015-0004). The cells were isolated as described previously [6]. In particular, the fetal cotyledons were aseptically separated from the maternal caruncles and divided into 1 mm^3 sections, which were then dispersed into a 10 cm^2 dish (430167; Corning Incorporated, Corning, NY, USA) and cultured in a 37°C incubator (Forma-371; Thermo Scientific, Waltham, MA, USA) for 30 min until the sections conglutinated on the dish. The pieces were then rinsed and cultured with complete DMEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% exosome-depleted fetal bovine serum (FBS) (EXO-FBS-50A-1; SBI, Palo Alto, CA, USA) at 37°C in an atmosphere of 5% CO_2. The medium was changed every 3 days until the cells were observed under a phase-contrast inverted microscope. The cells were purified using the differential-velocity adherent method [21]. Primary bovine trophoblast cells were identified using immunofluorescence detection.

Isolation of bovine EECs

Bovine uteri were obtained from a slaughterhouse. EECs were separated and purified according to published procedures [16, 22], and cultured in complete DMEM/F12 medium supplemented with 10% exosome-depleted FBS at 37°C in an atmosphere of 5% CO_2. The purity of EECs was assessed using an immunofluorescence assay with the CK8 antibody (ab53280, 1:1,000 dilution, Abcam). The number of cells used in the follow-up experiments was less than that in Passage #5.

Giemsa stain assay

The cells were fixed with 4% paraformaldehyde (Solarbio; Beijing, China) for 40 min and washed with PBS. Thereafter, they were stained with Giemsa stain (Solarbio) for 20 min, and then observed under an inverted optical microscope (IX71; Olympus, Tokyo, Japan).

Cell viability assay

According to the manufacturer's instructions (Solarbio), the cells were seeded in a 96-well plate at a density of 10^4 cells per well and incubated with CCK-8 solution for 1 h. The absorbance of cells was measured at a wavelength of 450 nm with a microplate reader (Multiskan SkyHigh; Thermo Scientific). The relative cell viability was calculated using the following formula: Relative cell viability = (absorbance of test group/absorbance of control group) × 100%.

Enzyme-linked immunosorbent assay (ELISA)

The endocrine ability of hormones was analyzed according to the manufacturer's instructions (J&L Biological, Shanghai, China).

Western blot

The cells or exosomes were lysed in RIPA buffer (Solarbio) supplemented with phenylmethanesulfonyl fluoride (PMSF) (Solarbio) on ice for 30 min. The lysates were centrifuged at 12,000 rpm at 4°C for 20 min with an Allegra^{TM} 64R Centrifuge (Agilent, Santa Clara, CA, USA). The supernatants were collected, and the total protein concentration was measured using a BCA kit (Beyotime, Beijing, China). The lysates of the cells or exosomes were separated on SDS-polyacrylamide electrophoresis gels (4% stacking gel and 12% resolving gel) and transferred onto PVDF membranes (Millipore, Burlington, MA, USA) using an electroblotting apparatus. Thereafter, the membranes were blocked with 4% non-fat milk for 2 h and incubated with primary antibodies including CK7 (ab154334, 1:1,000 dilution, Abcam, Cambridge, UK), vimentin (ab45939, 1:1,000 dilution, Abcam), E-cadherin (ab40772, 1:1,000 dilution, Abcam), CD90 (ab92574, 1:1,000 dilution, Abcam), hTERT (ab32020, 1:1,000 dilution, Abcam), CD9 (orb94982, 1:1,000 dilution, Abcam), CD63 (ab134045, 1:1,000 dilution, Abcam), MUC 1 (ab45167, 1:1,000 dilution, Abcam), integrin αv (JX2361, 1:1,000 dilution, Beijing Jiaxuan Biotech Co., Ltd., Beijing, China), integrin β3 (JX5762, 1:3,000 dilution; Beijing Jiaxuan Biotech Co., Ltd.), Wnt7a (ab100792, 1:3,000 dilution, Abcam), CK8 (ab53280, 1:1,000 dilution, Abcam), and GAPDH (bsm-33033M, 1:2,000 dilution, Bioss, Beijing, China) at 4ºC overnight. Subsequently, the membranes were washed with PBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (bs-0295G-HRP; 1:3,000 dilution, Bioss) or goat anti-mouse (bs-0368G-HRP, 1:3,000, Bioss) secondary antibody for 2 h at 25°C. Proteins were visualized using an ECL chemiluminescence kit (Beyotime, Beijing, China). Densitometry of each immunoblot was performed using Image J software (National Institutes of Health, Stapleton, NY, USA). Total protein expression was normalized to that of GAPDH, which served as the control.

qRT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and quality of RNA were determined using an Agilent 2100 Analyzer and a NanoDrop 2000 microspectrophotometer. Complementary DNA was reverse transcribed from total RNA using a HiFi-MmlV cDNA kit (CWBIO, Jiangsu, China), and qRT-PCR for gene expression was performed on an ABI 7500 Sequencing Detection System (Applied Biosystems, Carlsbad, CA, USA) with the UltraSYBR One Step qRT-PCR Kit (CWBIO, Jiangsu, China). The specific primers were designed as follows: 5'-TATGCCGTGGTCCAGAAGG -3' (hTERT, sense), 5'-CAAGAAATCATCCACCAAACG -3' (hTERT, antisense), 5'-CGGCACAGTCAAGGCAGAGAAC -3' (GAPDH, sense), and 5'-CCACATACTCAGCACCAGCATCAC -3' (GAPDH, antisense).

Immunofluorescence assay

The cells were fixed with 4% paraformaldehyde for 40 min and washed with PBS. Thereafter, the cells were immersed in permeabilization buffer for 30 min (PBS, 0.1% Triton X-100 (Solarbio) and 1% BSA) and blocked with 1% BSA in PBS at room temperature for 1 h. The cells were incubated with primary antibodies, including CK7 (1:100 dilution), vimentin (1:100 dilution), E-cadherin (1:100 dilution), CD90 (1:100 dilution), and CK8 (1:100 dilution) at 4°C overnight, washed with PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (ab6881, 1:100 dilution, Abcam) in the dark at 37°C for 1 h. The cells were washed three times with PBS and incubated with DAPI (Solarbio) at room temperature for 5 min. Finally, the cells were observed with

a laser scanning confocal microscope (FV10i; Olympus) after three washes with PBS.

Soft agar assay

The soft agar assay was performed according to a published protocol [23]. Briefly, 1.2% agar (Solarbio) was added to a six-well plate (3516; Corning Incorporated) as a bottom layer, which was then kept at 4°C until the agar solidified. Subsequently, 5×10^4 cells were suspended in 1 ml of 0.6% noble agar (Solarbio) in DMEM/F12 medium and placed on the wells. The cells were cultured in an atmosphere of 5% CO_2 at 37°C overnight, and complete DMEM/F12 medium was added. Cells were treated with complete medium once per week, and colony growth was evaluated for the next two weeks. Cell clumps greater than 100 μm were considered colonies and were photographed using an inverted optical microscope.

Migration assay

The migration ability of BTCs was assessed using a 24-well plate in a BD Bio-Transwell chamber (BD Biosciences, Franklin Lake, NJ, USA). A total of 10^4 trypsinized cells were added to 1 ml of serum-free medium. Complete medium (500 μl) was added to the lower well, and 100 μl of the cell suspension was added to the upper well. The plate was incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Non-invading cells on the upper surface were removed with cotton swabs and the lower surface of the basement membrane was fixed in 4% paraformaldehyde for 30 min. Thereafter, the basement was washed three times with PBS and stained with crystal violet (Solarbio). Cells were observed and photographed using an inverted optical microscope.

Cell transfection

The plasmid was transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For transfection, the cells were seeded in 6-well culture plates and incubated with a plasmid-lipofectamine combination at 50 nM in serum-free OPTI-MEM medium (Gibco). The medium was replaced 4 h after transfection. The cells were selected with 400 mg/ml G418 (Solarbio) for the next two weeks. After selection, transfected cells were subjected to treatment for the follow-up experiment. The pCI-neo-hTERT plasmid was identified and donated by Prof. Jin from the Key Laboratory of Animal Biotechnology of the Ministry of Agriculture, Northwest A&F University [24].

Isolation of exosomes

To imitate the culture conditions for early embryos, the cells were treated with 2 ng/ml P4 (Sigma Aldrich) until their density reached approximately 80%. After 48 h of treatment, the medium was collected and centrifuged at 4°C ($300 \times g$) for 10 min to remove the cells. Thereafter, the supernatant was transferred to a sterile vessel. Exosomes were isolated from the supernatant according to the manufacturer's recommendations (BB-3901; Shanghai Bestbio Biotechnology Co., Ltd.).

PKH26 staining for exosomes

The PKH26 Red Fluorescent Cell Linker Kit (Sigma Aldrich) was used to label the exosomes isolated above. The exosomes were collected and resuspended in 100 μl of diluent C. In addition, 0.4 μl PKH26 ethanolic dye solution was mixed with 100 μl of diluent C to prepare a 2 × dye solution, which was added to the exosome suspension and incubated for 5 min. The reaction was stopped by adding 200 μl FBS. The exosomes were washed with PBS and collected for subsequent experiments.

Morphologic examination of exosomes with transmission electron microscopy

The exosomes were resuspended in PBS, and a 15-μl sample was added to a 300-mesh copper grid and dried for 90 sec. Each grid was washed five times in distilled H_2O, stained with 5 μl of 3% uranyl acetate (phosphotungstic acid) for 1 min, dried, and then placed on a filter paper at room temperature for 1 h. The samples were visualized using a transmission electron microscope (libra 120; Carl Zeiss AG, Germany) at 80 kV.

Sample preparation for proteomic analysis

Lysates were extracted from the exosomes with lysis buffer (500 mM Tris-HCl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, 2% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, pH 8.0) and the protein was solidified using a phenol-acetone assay. The precipitate was then solubilized using 4% SDS. Finally, the protein concentrations were calculated via the BCA assay, and the enzymatic protein concentrations were determined using the FASP assay.

DIA quantitation analysis

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the iProX partner repository with the dataset identifier PXD030586. Proteomic experiments were performed in triplicate. MS analyses were conducted using an Agilent 1100 high-performance liquid chromatograph (Agilent). Peptides were loaded and separated over a 120 min gradient run with a Waters XBridge C18 analytical column (5 μm 120 Å, 460 μm × 250 mm, Waters, China). Trapping was conducted for 3 min at a rate of 5 $\mu l \cdot min^{-1}$ with 97% buffer A (99% water, 2% ACN, pH 10.0), and 3% buffer B (98% ACN, 2% water, pH 10.0), before being eluted at 2–100% 0.1% FA in acetonitrile at a flow rate of 250 $nl \cdot min^{-1}$ (2–40% from 0 to 100 min, and 40–80% from 100 to 110 min). According to the requirements of the DDA library, analysis was completed to build the cross-merging, vacuum concentration, and mass spectrometry libraries after the flow-through peaks were removed. Proteome Discoverer 2.1 (Thermo Fisher Scientific, Rockford, IL, USA) was used to search and analyze the DDA data collected after classification. Biological samples were collected for the DIA data, and quantitative analysis was performed using the Skyline software (Department of Genome Sciences, University of Washington, Seattle, WA, USA). For pathway analysis, the Kyoto Encyclopedia of Genes and Genomes (KEGG) resources were used with the recommended analytical parameters. For gene ontology enrichment and network analysis, UniProt (www.uniprot.org) database resources (biological process, molecular function), Ingenuity Pathway Analysis, and Reactome Knowledgebase were utilized. Gplots were adopted for the thermal map of the proteins.

Statistical analysis

The values are presented as mean ± SD. Data from three independent experiments were analyzed. One-way analysis of variance (ANOVA) was used to compare the different groups involved in the experiments. When the comparison between the two groups was significantly different, * or + was used for $P < 0.05$, while ** or ++ was used for $P < 0.01$.

Results

Identification and telomerase transfection of BTCs

The cells derived from the bovine placenta were purified to passage #5 using the differential adhesion method. The cells grew as epithelial-like monolayers, which exhibited a cobblestone-like phenotype with some cells that displayed an outspread morphology at a high density, similar to the F3 trophoblast separated by Hambruch and hTERT-GTCs cells separated by Dong [7, 24]. The cells were found to display a clear epithelioid cell morphology under a phase-contrast inverted microscope (Fig. 1A). After staining with Giemsa stain, the cells were observed under a microscope. Minor binucleate cells were found among the uninucleate cells, which communicated with each other with their extended pseudopodia (Fig. 1B). BTCs were identified via the detection of the biomarkers of extravillous trophoblast cells using an immunofluorescence assay. Fetal bovine fibroblasts (FBFs) were used as negative controls. As expected, all of the purified primary BTCs were CK7 positive, while all of the fetal bovine fibroblast cells were negative. As 100% of the BTCs were CD90 negative, no fibroblast was present in the purified BTCs. Furthermore, the expression of two representative proteins in epithelial cells, vimentin and E-cadherin, suggested that BTCs were specialized epithelial cells (Fig. 1C). The pCI-neo-hTERT plasmid was transfected into primary BTCs at passage #5. Total RNA and protein were isolated from the primary BTCs, hTERT-BTCs (at passages #30 and #50), and HeLa cells, and the expression of telomerase was detected at the protein level using the western blot assay. The growth curve of BTCs, measured via the CCK-8 assay, showed a similar trend in the BTCs regardless of whether they were transfected with plasmid or not (Fig. 1D). However, the proliferation activity of the immortalized BTCs was higher than that of the primary BTCs. Further, passage 50 hTERT-BTCs entered the exponential growth phase after a 3-day latent phase, while the primary BTCs showed a 5-day latent phase. Telomerase protein was markedly and steadily expressed in hTERT-BTCs and HeLa cells; however, its expression level was significantly higher than that in primary cells (Fig. 1E–G, $P < 0.01$).

Immortalized BTCs maintain the functional characteristics of primary BTCs

Secretion of placental lactogen (PL) is one of the most important features of extravillous trophoblast cells [7, 25]. According to ELISA, both the primary BTCs and hTERT-BTCs manifested the ability of secreting PL (Fig. 2A). As confirmed by the transwell migration assay, both the primary BTCs and hTERT-BTCs had the same ability of cell migration (Fig. 2B). In addition, hTERT-BTCs at Passage #50 showed similar features of marker protein expression to those of primary BTCs (Fig. 2C, D). A soft agar assay was performed to confirm whether the hTERT-BTCs had anchorage-independent growth. Based on the results, hTERT-BTCs could not form colonies after 14 days of incubation; however, HeLa cells could form colonies and indicated that hTERT-BTCs could not transform into a malignant phenotype (Fig. 2E).

Isolation and identification of exosomes derived from BTCs

Exosomes were extracted from BTCs using an exosome extraction kit. Round vesicular membranes with a size of 30–150 nm were observed under a transmission electron microscope (Fig. 3A). CD63 and CD9, the tetraspanin family members localized to the internal vesicles of the exosomes, constituted the marker proteins of the exosomes. The expression of CD63 and CD9 was detected in whole cell lysates (WCL) and exosomes derived from the BTCs using an immunoblot assay. The lack of calnexin expression, an endoplasmic reticulum protein marker, suggested that the collected exosomes were free from cell debris contamination (Fig. 3B).

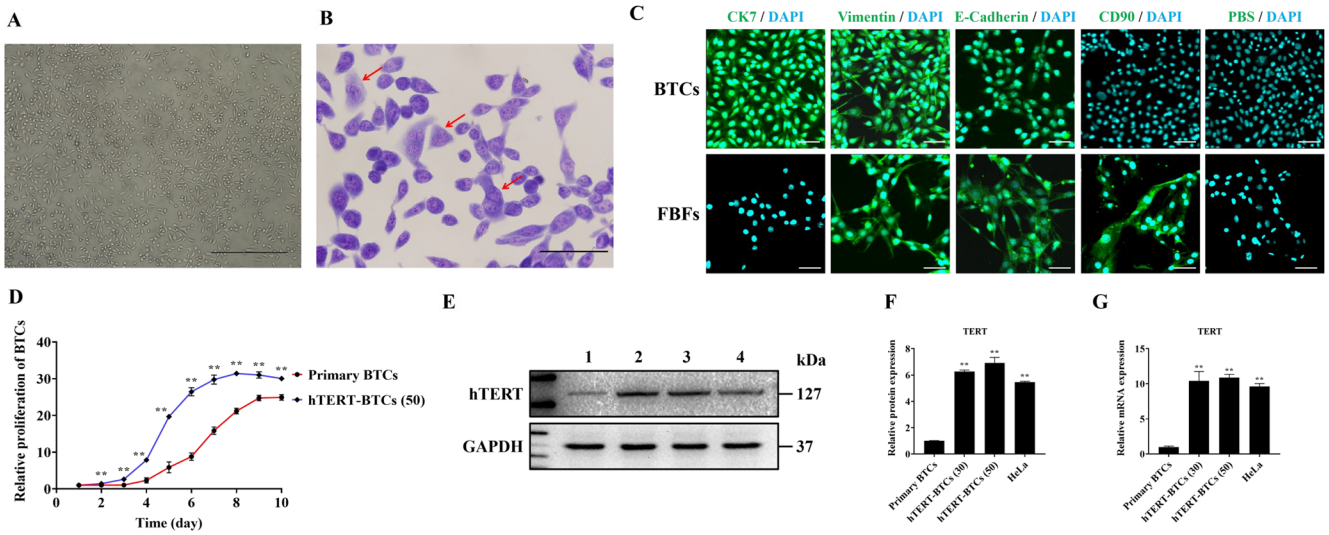

Fig. 1. Identification and hTERT-transfection of primary bovine trophoblast cells (BTCs) derived from bovine placenta. A, B) Morphological characteristics and Giemsa stain results of BTCs under a phase-contrast inverted microscope; bar = 1 mm in Fig. 1A, bar = 100 μm in Fig. 1B. C) Cytokeratin 7 (CK7), vimentin, E-cadherin, and CD90 expression in primary BTCs were evaluated by immunocytochemistry. The BTCs incubated with PBS instead of primary antibody and fetal bovine fibroblast cells served as the negative controls; bars = 100 μm. D) The growth and migration features of BTCs were analyzed by CCK-8. E, F) hTERT expression was detected by Western blot analysis. Lane 1, primary BTCs; lane 2, hTERT-BTCs at Passage #30; lane 3, hTERT-BTCs at Passage #50; lane 4, HeLa cells as positive control. G) Representative mRNA expression levels were measured by qRT-PCR and analyzed using the $2^{-\Delta\Delta Ct}$ method. The results are presented as mean ± SD of three independent experiments (* $P < 0.05$, ** $P < 0.01$ compared with primary BTCs).

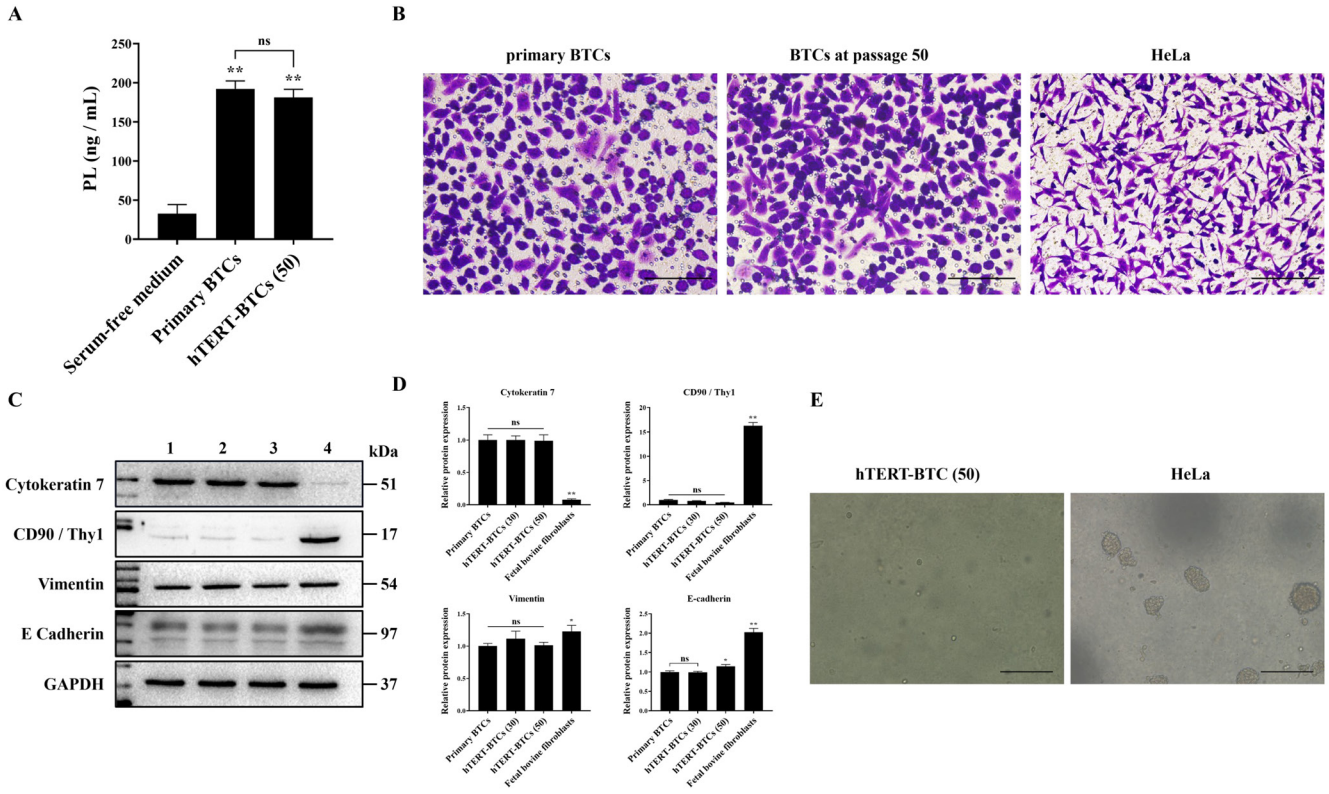

Fig. 2. Immortalized BTCs maintain the epithelial-origin and endocrine features of primary BTCs without exhibiting features of malignant transformation. A) The endocrine ability of PL was measured by ELISA. B) The migration ability of BTCs was measured by trans-well chamber migration assay; bars = 50 μm. C) CK7, CD90, vimentin, and E-cadherin expression levels were detected by Western blot analysis. Lane 1, primary BTCs; lane 2, hTERT-BTCs at Passage #30; lane 3, hTERT-BTCs at Passage #50; lane 4, fetal bovine fibroblast cells (FBFs) as the negative control. D) The protein expression levels were measured using ImageJ in the representative Western blot analysis. E) hTERT-BTCs did not form cell colonies while HeLa cells formed colonies larger than 100 mm in diameter after a 14-day culture. The results are presented as mean ± SD of three independent experiments (* $P < 0.05$, ** $P < 0.01$ compared with primary BTCs).

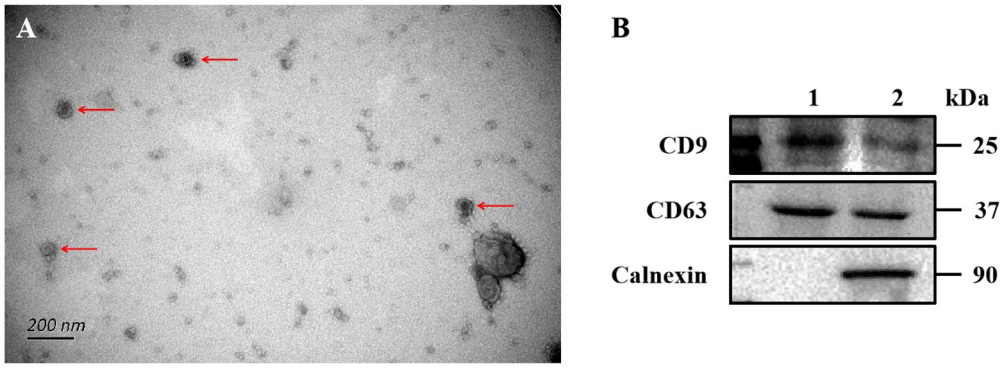

Fig. 3. Identification of BTC-derived exosomes. A) Morphological observation of the BTC-derived exosomes under a transmission electron microscope; bars = 200 nm. B) Verification of exosomes by Western blot assay. Lane 1, BTC-derived exosomes; and lane 2, BTCs.

Effect of P4-treated BTC-derived exosomes on the endometrial receptivity of EECs

All of the bovine EECs showed a typical "paving stone"-like appearance with positive expression of CK8, a marker protein of EECs (Fig. 4A). Using immunofluorescence identification, the purity of EECs was found to be greater than 95%. In this study, the EECs co-incubated with the TDE stained with PKH26 (Sigma Aldrich) exhibited significant red fluorescence, indicating that the TDE was fused with the EECs after 12 h of incubation (Fig. 4B). Endometrial receptivity, a key factor in determining the embryonic development process, is reported to be affected by TDE and P4 stimulation during pregnancy. The exosomes derived from the BTCs treated with or without 10 ng/mL P4 for 24 h were collected and co-cultured with EECs for 12 h. Changes in the expression of uterine receptivity factors related to embryo implantation were detected in EECs by western blotting. Compared with the BTCs that were not treated, the P4-treated cells derived from the exosomes increased the expression levels of uterine receptivity-related proteins in the EECs, such as

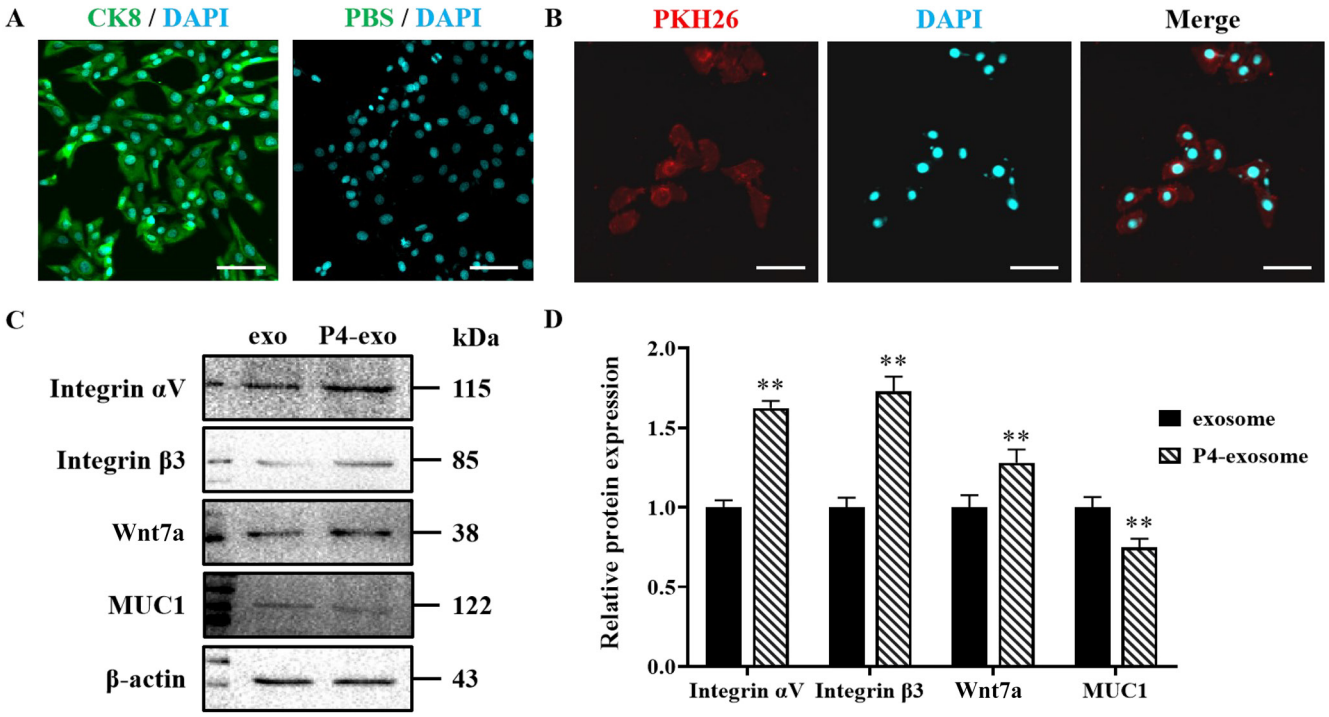

Fig. 4. P4-treated BTCs-derived exosomes enhance the endometrial receptivity of EECs. A) CK8 expression in EECs was detected using immunofluorescence assay; bars = 100 µm. B) Confocal microscopy of the internalization of fluorescently-labeled exosomes in EECs after 12 h of incubation; bars = 50 µm. C) Expression levels of integrin αv, integrin β3, Wnt7a, and MUC1 were detected using the Western blot assay. D) The protein expression levels were measured using ImageJ in representative Western blot analysis.

integrin αv, integrin β3, and Wnt7a, and decreased the expression of MUC1 (Fig. 4C, D; $P < 0.01$).

Functional analysis of the TDE proteome

Proteomic profiling was performed on the exosomes derived from the BTCs cultured with/without P4 via nanoLC-MS/MS data-dependent acquisition. The samples were analyzed in biological triplicate, with technical replicates and a stringent metric for protein and peptide identification. A thermal map for protein expression was displayed for a total of 923 proteins identified in the proteomics analysis (Supplementary Fig. 1). KEGG and GO classification showed that the identified proteins were concentrated on the immune and endocrine systems for antioxidant enzyme activity, binding, molecular function regulator, structural molecular activity, transporter activity, etc. (Supplementary Fig. 1). Of these 923 proteins, 19 were differentially expressed in the cells after P4-exo treatment, including 6, such as CTSD, PCOLCE, and COPS6, which decreased their expression, and 13 proteins, such as GNPTG, EIF4A2, and FGB, which increased their expression. (Fig. 5A, B). Functional annotation and pathway analysis of these differentially expressed protein subsets associated with the P4-exo treatment revealed the protein categories associated with collagen binding, catalytic activity, and hydrolase activity (Fig. 5D, E).

Discussion

The success of pregnancy relies on the differentiation, proliferation, and migration of placental trophoblast cells during early pregnancy [2, 26]. Placenta trophoblast cells are classified into two types: mononucleate trophoblast cells and binucleate trophoblast cells [27, 28]. Binuclear trophoblast giant cells, which are unique trophoblast giant cells in the placenta of ruminants [29], are the first layer of the maternal-fetal interface. Together with multiple mononuclear cells, trophoblast giant cells form the surface layer of cotyledon villi and then fuse with the carbonium of maternal uteruses to form the functional unit of the placenta. In ruminants, embryo implantation begins on day 16 of gestation, the binuclear trophoblast giant cells form on day 17 of embryo development, and the process of implantation is completed on day 22 [30, 31]. During the first 3 months after implantation, the binucleated trophoblast giant cells continued to fuse with the endometrial epithelial cells and played a placental regulatory role [6]. Therefore, trophoblast lines isolated from pregnant cows at days 45–60 of pregnancy can be used to study the regulation of the placenta from the embryo implantation stage to early pregnancy. In this study, the cells isolated and purified from the placenta contained mononucleated cells mixed with minority multinucleated cells that had similar features to those of the first trimester trophoblasts. In this stage, the trophoblast exhibits a phenotype with CK-7, vimentin, and pregnancy-associated glycoproteins (PAGs) expression and PL secretion [7, 25]. In this study, the potential non-trophoblast components were mainly Hofbauer cells, blood leukocytes, and fibroblast cells. Such Hofbauer cells and blood leukocytes are easily deleted through continuous passage [28]. Fibroblast cells were eliminated by continuous passage using the differential adhesion method. To confirm the absence of fibroblast contamination, we detected the expression of CD90/Thy1, the most iconic fibroblast biomarker, and found no fibroblasts after purification [24, 32]. Cytokeratin 7, an intermediate protein, is the foremost marker of trophoblast cells [24, 33]. As all cells were CK-7 positive, the isolated cells were confirmed to be derived from trophoblasts and suitable for transfection.

To establish a normal bovine placental trophoblast cell line, we

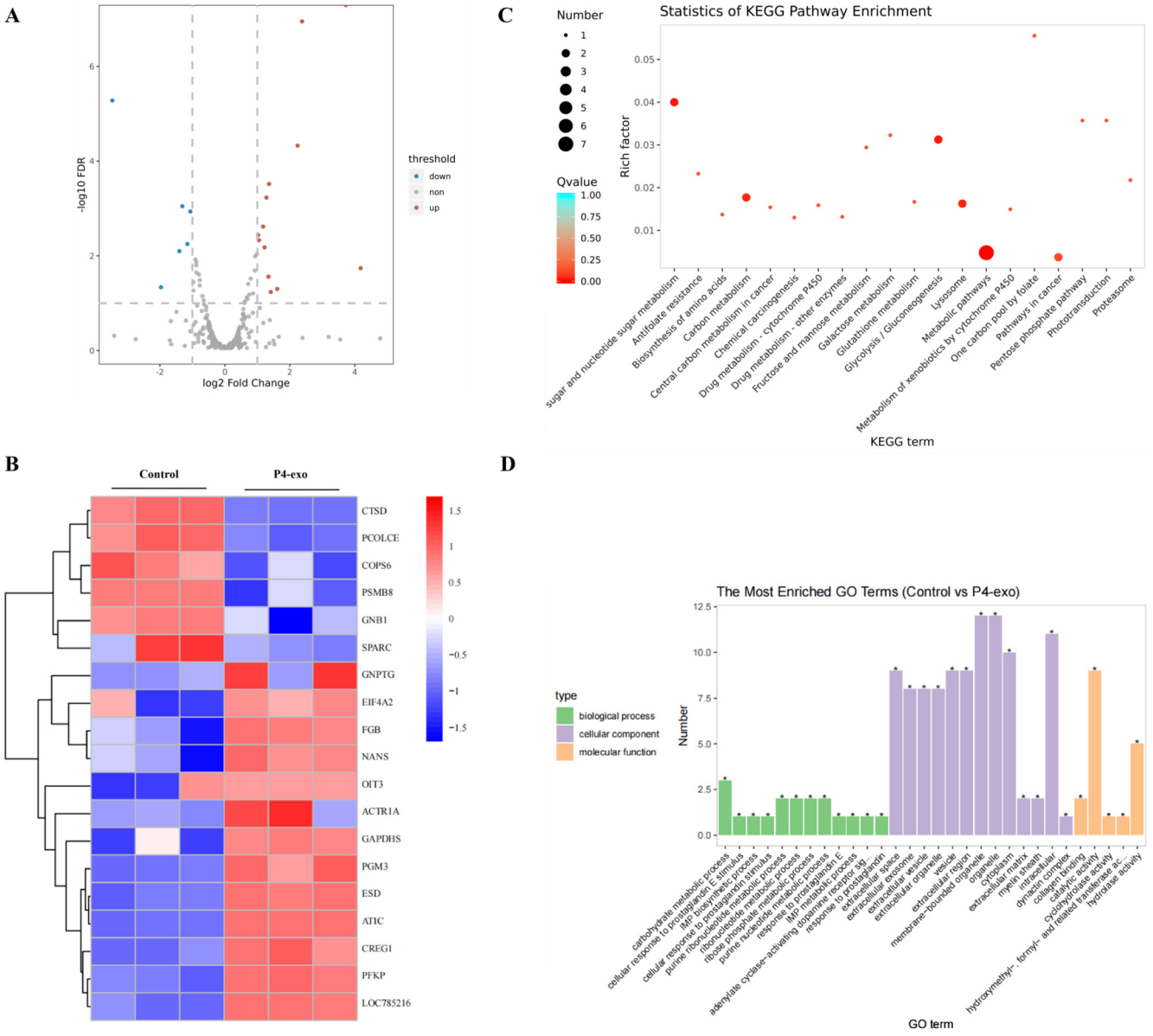

Fig. 5. Differentially expressed protein landscape of TDE before and after P4 stimulation. A) Differential protein volcano map. B) Clustering heat map of TDE differential proteins. Statistics of GO C) and KEGG D) enrichment classification of the differential proteins.

changed telomerase activity by ectopic expression of the exogenous *hTERT* gene. Based on western blot and RT-qPCR assays, the transfected hTERT-BTCs were positive for telomerase expression and were cultured for over 50 passages without showing signs of division termination. In addition, hTERT-BTCs showed a more aggressive growth rate than cells those without transfection, suggesting that exogenous telomerase expression accelerated the proliferation of BTCs. Accordingly, the cells could be considered immortalized. The secretory capability of pregnancy-related hormones, such as PL, is one of the most critical features of trophoblast cells [7, 25]. The immortalized BTCs maintained the same characteristics of PL secretion, and the expression of CK7, vimentin, and E-cadherin until passage #50. The trans-well migration assay indicated that immortalized BTC inherited migration. These results suggest that the immortalized BTCs were of epithelial origin and had endocrine and migration features of extravillous trophoblast cells, which were maintained by the primary BTCs. The lack of CD90 expression in the BTCs suggested that there was no fibroblast contamination during the 50 passages of the culturing process. In summary, the immortalized bovine trophoblast cells possessed a prolonged lifespan and retained some properties of the primary cells, which is consistent with the trophoblast cells previously reported [33]. To date, trophoblast cells in the first trimester have been successfully immortalized by importing exogenous TERT gene in goats [24], pigs [34], humans [32], and bovine cells.

Many studies have confirmed that exosomes play a crucial role in communication between the placenta and maternal body [35]. Progesterone (P4) concentration increases with the progression of pregnancy and maintains a high concentration throughout pregnancy. The decrease in P4 concentration often indicates miscarriage [36]. P4 has a certain regulatory effect on the differentiation and functional execution of placental trophoblast cells and can mediate uterine

receptivity in ruminants [27, 37, 38]. Therefore, BTCs are treated with P4 to simulate the placental environment during gestation. TDE has multiple adjustment functions for uteruses, such as regulating local immunity, suppressing inflammation of EECs, and adjusting endometrial receptivity [20, 29, 39]. In the present study, we found that exosomes derived from BTCs treated with P4 at 10 ng/ml exerted a positive effect on the expression of endometrial receptivity-related proteins in EECs, which was manifested by the upregulation of integrin αv, β3, and Wnt7a, and the downregulation of MUC1.

In ruminants, placenta-derived exosomes are mainly secreted by trophoblast cells [29]. In terms of promoting maternal-fetal communication, proteins carried by TDE can not only enhance endometrial receptivity and provide a necessary environment for the placenta to be implanted into the endometrium, but also participate in the regulation of the fusion of trophoblast cells and endometrial epithelial cells [20, 29, 35]. Therefore, mass-spectrometry-based proteomics was performed to detect different proteins loaded in exosomes derived from trophoblasts treated with P4. In this study, the TDE proteins both concentrated on the immune and endocrine systems, while TDE could adjust the functions of EECs and regulate endometrial receptivity through the immune and endocrine systems. For example, TDE can assist fetuses in escaping the maternal immune system and improve the secretory function of the endometrium [29, 40]. Our results are consistent with existing literature, indicating that trophoblasts can regulate endometrial receptivity [41]. Based on KEGG and GO classification analysis of differentially expressed proteins, we found that the expression of these proteins, including cathepsin D (CTSD), procollagen C-endopeptidase enhancer (PCOLCE), and COP9 signalosome complex subunit 6 (COPS6), which can decompose the extracellular matrix, decreased with P4 stimulation, while the expression of these antioxidant and hydrolase related proteins, such as bifunctional purine biosynthesis protein ATIC (ATIC), N-acetylneuraminate synthase (NANS), and S-formylglutathione hydrolase (ESD), increased. The dysregulation of endometrial ECM remodeling can impair receptivity, which is linked to pregnancy outcomes [42]. Redox homeostasis and energy metabolism play important roles in changing maternal receptivity by affecting the endometrial response to conceptus-derived pregnancy signals [43]. In addition to the result that exosomes derived from P4 treated cells can upregulate the expression of endometrial receptivity related proteins, the results of differentially expressed proteins between exosomes implied that endometrial receptivity increased with P4 stimulation during pregnancy through TDE by maintaining a steadier state of the oxidation balance and the extracellular environment. The expression levels of some transcription factors mediating the cellular response, such as eukaryotic initiation factor 4A-II (EIF4A2), were also affected by P4 stimulation. In brief, TDE targeting the immune and endocrine pathways may regulate endometrial receptivity by ameliorating the extracellular environment, regulating the cell redox balance, and changing some of the cell signal transduction pathways during pregnancy.

In summary, we established an immortal placental trophoblast cell line through the transfer of exogenous hTERT. Using this cell line, we found that P4 can regulate the functions of exosomes derived from trophoblast cells, reversely regulate the receptivity of EECs, and ensure normal embryo development during pregnancy.

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