

# Placental Trophoblast Derived Exosomes Regulate Endometrial Epithelial Receptivity in Dairy Cows During Pregnancy

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## Research

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# Abstract

## Background

Inadequate feto-maternal interaction will directly lead to the failures of pregnancy and bring serious damage to dairy cows. Exosomes are widely involved in endometrial matrix remodeling, immune function changes, placental development, and other processes of embryo implantation and pregnancy of dairy cows. However, the role of placental trophoblast cells derived exosomes is still unclear in regulating the receptivity of endometrial cells and facilitating the interaction between mother and fetus.

## Methods

In this study, bovine trophoblast cells (BTCs) were obtained from bovine placenta and immortalized through the transfection of telomerase reverse transcriptase (TERT) gene. After that, the effect of trophoblast derived exosomes (TDEs) on endometrial receptivity in endometrial epithelial cells (EECs) was detected and the mechanism explored that TDEs and their proteins participated in feto-maternal interaction during bovine pregnancy. EECs were co-cultured with the exosomes derived from progesterone (P4) and treated with BTCs.

## Results

Immortalized BTCs still possessed the basic and key properties of primary BTCs without showing a neoplastic transformation sign. Exosomes derived from P4 and treated with BTCs enhanced the expression of endometrial receptivity factors in EECs by changing the extracellular environment, metabolism and redox balance in EECs with proteome alignment, compared with those untreated according to the DIA quantitation analysis.

## Conclusions

Our study found that trophoblast derived exosomal proteins are one of the most critical elements in feto-maternal interaction and their changes act as a key signal in altering endometrial receptivity and provided a potential target for improving fertility.

## Introduction

Early pregnancy failures are as high as 50% in high-producing dairy cows and lead to the low reproductive efficiency and poor profitability of the cattle production system, and such high pregnancy failure rate is generally considered to be the result of insufficient communication between the fetus and the environment<sup>[1]</sup>. In the mammalian fetus, placentae, a transient organ, play a critical role in feto-maternal interaction by ensuring adequate fetal oxygen and nutrient supply throughout the entire

pregnancy, and determining the success of embryo implantation and pregnancy outcomes<sup>[2-4]</sup>. In bovine placenta, the barrier between mother and fetus is formed by trophoblast<sup>[5, 6]</sup>. Thus, studying the function of trophoblast is vital to understand how to avoid obstetric diseases and increase pregnancy rate during first trimester and pregnancy. There were only three bovine trophoblast cell lines, namely, F3<sup>[7]</sup>, CT-1<sup>[8]</sup> and BT-1<sup>[9]</sup>. F3 is separated from bovine first trimester placenta, but there is no report on whether F3 are immortal while isolated primary first trimester trophoblast cells are known to have a finite lifespan<sup>[7, 10]</sup>. BT-1 and CT-1 can be cultured for more than 75 passages, but they are trophoblast stem cells derived from blastocysts which are different from trophoblast cells derived from first trimester placenta, so there is no a model to research the function of placenta and it's difficult to collect samples<sup>[8, 9]</sup>. Therefore, the establishment of a stable bovine trophoblast model derived from first trimester placenta is helpful for better revealing the new mechanism of mother-fetus dialogue.

Feto-maternal interactions are comprised of the signal transmission between placental trophoblast cells and EECs to jointly advance embryonic development. In the maternal uterus, the success of feto-maternal interactions is determined by endometrial receptivity in EECs which is related to uterine factors such as integrin  $\alpha$ v, integrin  $\beta$ 3, wnt7a and mucin 1 (MUC1). Among them, integrin  $\alpha$ v and  $\beta$ 3 are transmembrane receptors which can bind to specific ligands of the extracellular matrix. They can promote early embryo-maternal interactions by impacting the opening of the endometrium implantation window and invasion process of trophoblast into EECs<sup>[11-13]</sup>. MUC1, an anti-adhesive, plays a negative role during early pregnancy by repressing the adhesion between trophoblast and uterine epithelial cells<sup>[14]</sup>. Wnt7a can establish signal connection between endometrial epithelial cells and mesenchyme and mediate the initial adhesion process between embryo and luminal epithelial cells by activating Wnt /  $\beta$ -catenin signaling pathway<sup>[15, 16]</sup>.

Exosomes are double-layered extracellular vesicles with a diameter of 30–150 nm (extracellular vesicles, EVs) which release vesicles into the extracellular space to mediate the information exchange between cells by fusing with the plasma membrane<sup>[17, 18]</sup>. Trophoblast cells can affect the maternal physiological function by sending specific biologically active molecules to the maternal blood circulation through the villi space by packing them into exosomes<sup>[19, 20]</sup>. The orderly expression of exosomes in time and space will enable the entire pregnancy to complete smoothly<sup>[21]</sup>. There have been many studies on the role of endometrium and exosomes secreted by embryos in the attachment of mammalian embryos, but there are few studies on the attachment of embryos by TDEs. First trimester trophoblast cells have been successfully immortalized by importing exogenous TERT gene in goat<sup>[22]</sup>, porcine<sup>[23]</sup> and human<sup>[10]</sup>, but not bovine. Therefore, it is still unclear what's the role of placental trophoblast cells derived exosomes in regulating the receptivity of endometrial cells and facilitating the interaction between mother and fetus. The aim of this study was to detect the receptivity changes of EECs resulting from taking in the exosomes secreted by the bovine trophoblast cell line which is established by introducing exogenous human telomerase during pregnancy period.

## Materials And Methods

# Isolation, purification and culture of bovine trophoblast cells

Placentae of early pregnant Holsteins (45–60 days of pregnancy, with a fetal cow with a crown-to-rump length of about 7 cm) were obtained from the cows sacrificed by exsanguination. This study was performed in accordance with the guidelines of the Animal Ethic Committee of Beijing University of Agriculture (Permit No.: SYXK(JING)2015-0004). The isolation of cells was performed according to the previous study<sup>[6]</sup>. Briefly speaking, the fetal cotyledons were separated from the maternal caruncles aseptically and minced into 1mm<sup>3</sup> pieces, which were dispersed into a 10 cm<sup>2</sup> dish and cultured in a 37 °C incubator for 30 min till the pieces conglutinated on the dish sturdily. The pieces were rinsed and cultured with complete DMEM/F12 medium (Gibco, USA) supplemented with 10% exosome-depleted fetal bovine serum (FBS) (EXO-FBS-50A-1, SBI, USA) at 37°C in an atmosphere of 5% CO<sub>2</sub>. The medium was changed every 3 days till the cells can be seen under a phase-contrast invert microscope. The cells were purified with the differential velocity adherent method<sup>[24]</sup>. Immunofluorescence detection was used to identify the primary bovine trophoblast cells (described below).

## Giemsa stain assay

The cells were fixed with 4% paraformaldehyde for 40 min and washed with PBS. After being dyed with Giemsa stain for 20 min, they were observed under a Nikon inverted optical microscope (Nikon, Japan).

## Cell viability assay

According to the manufacturer's instructions (Solarbio, Beijing, China), the cells were seeded in a 96-well plate at the density of 10<sup>4</sup> cells per well, incubated with CCK-8 solution for 1 h, and then measured for the absorbance using a wavelength of 450 nm. The relative cell viability was calculated with the following formula: Relative Cell Viability = (The Absorbance of Test Group / The Absorbance of Control Group) \* 100%.

## Enzyme-linked immunosorbent assay (ELISA)

Analysis of the endocrine ability of hormone was performed according to the manufacturer's instructions (JIL Biological, Shanghai, China).

## Western blot

The cells or exosomes were lysed with RIPA buffer supplemented with phenylmethanesulfonyl fluoride (PMSF) for 30 min on ice. The lysates were centrifugated at 12,000 rpm for 20 min at 4 °C. The supernatants were collected and measured for the total protein concentration with a BCA kit (Beyotime, Beijing, China). The lysates of cells or exosomes were separated on SDS-polyacrylamide electrophoresis gels (4% stacking gel and 12% resolving gel) and transferred onto PVDF membranes (Millipore, Massachusetts, USA) using an electroblotting apparatus. Then the membranes were blocked with 4% non-fat milk for 2 h and incubated with primary antibodies including CK7 (ab154334, 1:1,000, Abcam),

vimentin (ab45939, 1:1,000, Abcam), E-Cadherin (ab40772, 1:1,000, Abcam), CD90 (ab92574, 1:1,000, Abcam), hTERT (ab32020, 1:1,000, Abcam), CD9 (orb94982, 1:1,000, Abcam), CD63 (ab134045, 1:1,000, Abcam), MUC 1 (1:1,000, Beijing Jiaxuan Biotech Co., Ltd.), integrin  $\alpha$ v (1:1,000, Beijing Jiaxuan Biotech Co), integrin  $\beta$ 3 (1:3,000; Beijing Jiaxuan Biotech Co., Ltd.), Wnt7a (ab100792, 1:3,000, Abcam), CK8 (ab53280, 1:1,000, Abcam) and GAPDH (bsm-33033M, 1:2,000, Bioss) at 4°C overnight. They were washed with PBST and incubated with horseradish peroxidase (HRP) - conjugated goat anti-rabbit (bs-0295G-HRP, 1:3,000, Bioss) or goat anti-mouse (bs-0368G-HRP, 1:3,000, Bioss) secondary antibody for 2 h at room temperature. The proteins were visualized using an ECL chemiluminescence kit (Beyotime, Beijing, China). The densitometry of each immunoblot was performed using Image J software (National Institutes of Health, USA). With GAPDH as control, the expression of total protein was normalized.

## qRT-PCR

Total RNA was isolated from the cells with TRIzol reagent (Invitrogen, USA). The concentration and quality of RNA was determined using an Agilent 2100 Analyzer and an NanoDrop 2000 microspectrophotometer. Complementary DNA was reversely transcribed from total RNA using HiFi-MmlIV cDNA kit (CWBio, Jiangsu, China) and qRT-PCR for gene expression was performed on an ABI 7500 Sequencing Detection System (Applied Biosystems, 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. The washed cells were immersed with permeabilization buffer for 30 min (PBS, 0.1% Triton X-100 and 1% BSA) and blocked with 1% BSA-PBS for 1h at room temperature. The cells then were incubated with primary antibodies including CK7 (1:100), vimentin (1:100), E-Cadherin (1:100), CD90 (1:100), CK8 (1:100) at 4 °C overnight, washed with PBS and incubated with fluorescein isothiocyanate (FITC) - conjugated goat anti-rabbit secondary antibody (ab6881, 1:100, Abcam) in the dark for 1h at 37°C. The cells were washed with PBS three times and incubated with DAPI for 5min at room temperature. Next, the cells were observed with a laser scanning confocal microscopy (Olympus, Japan) after being washed with PBS three times.

## Soft agar assay

Soft agar assay was performed according to the published protocol<sup>[25]</sup>. The 1.2% agar was filled into a six-well plate as a bottom layer which then was placed still at 4 °C till the agar solidified.  $5 \times 10^4$  cells were suspended in 1 mL 0.6% noble agar in DMEM/F12 media and plated onto wells. The cells were cultured at 37 °C in an atmosphere of 5% CO<sub>2</sub> overnight and complete DMEM/F12 medium was added. They were fed with complete medium once a week and evaluated for the colony growth over the next two weeks. The cell clumps greater than 100  $\mu$ m were considered as colonies and photographed with an inverted optical microscope.

## Migration and invasion assay

The migration ability of BTCs was assessed using a 24-well plate in the BD Bio-Coat Matrigel Invasion Chamber (BD Biosciences).  $10^4$  trypsinized cells were added into 1 mL serum-free media. 500  $\mu$ L complete media was added to the lower well while 100  $\mu$ L cell suspension to the upper well. The plate was incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere for 24 h. Those non-invading cells on the upper surface were removed using a cotton swabs and the lower surface of the basement membrane was fixed in 4% paraformaldehyde for 30 min, and then the basement was washed with PBS three times and stained with crystal violet. The cells were observed and photographed with an inverted optical microscope.

## Cell transfection

The plasmid was transfected into the cells using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. For transfection, the cells were seeded in 6-well culture plates and incubated with plasmid - lipofectamine complex at 50 nM in serum-free OPTI-MEM medium. The medium was replaced after 4 hours after transfection. The cells with 400mg / mL G418 (Solarbio, Beijing, China) were selected during two weeks. After selection, the transfected cells were subjected to the treatment for the follow-up experiment. pCI-neo-hTERT plasmid was identified and donated with Pro.Jin<sup>[22]</sup>.

## Exosomes isolation

To imitate the early embryo culture conditions, the cells were treated with 2 ng/mL P4 when their density was about 80%. After 48h, the medium was collected and centrifuged at 4 °C (300×g, 10 min to remove the cells), and then the supernatant was transferred to a sterile vessel. Exosome isolation were performed from the supernatant according to the manufacturer's recommendations (BB-3901, Shanghai Bestbio Biotechnology Co., Ltd.).

## Examination of exosomes morphology with transmission electron microscopy

The exosomes were resuspended in phosphate-buffered saline (PBS), and 15  $\mu$ L samples were added to a 300-mesh copper grid for 90 s and dried. Each grid was washed five times in distilled H<sub>2</sub>O, stained with 5 $\mu$ L of 3% uranyl acetate (phosphotungstic acid) for 1min and dried, and then placed on a filter paper for 1h at room temperature. The samples were visualized with a transmission electron microscope at 80 kV.

## Sample preparation for proteomic analysis

Lysates from the exosomes were extracted with lysis buffer (500 mM Tris-HCl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, 2%  $\beta$ -mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride, pH 8.0), the protein was solidified by phenol-acetone assay and the precipitation was solubilized in 4% SDS. The protein concentrations were calculated by BCA assay and the enzymolysis protein concentrations tested by FASP assay.

## DIA quantitation analysis

Proteomic experiments were performed in biological triplicate. MS analyses were performed on an Agilent 1100 high-performance liquid chromatography (Agilent, USA). Peptides were loaded and separated over a 120 min gradient run using a Waters XBridge C18 analytical column (5 $\mu$ m 120 Å, 460 $\mu$ m  $\times$  250 mm, Waters, China). Trapping was conducted for 3 min at 5 $\mu$ L min<sup>-1</sup>, 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 (2–40% from 0 to 100 min, 40–80% from 100 to 110 min (flow rate, 250 nL min<sup>-1</sup>). According to the building requirements of the DDA library, after the flow-through peaks were removed, cross-merging, vacuum concentration and mass spectrometry library building analysis were completed. 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 DIA data, and quantitative analysis was performed with Skyline software (Department of Genome Sciences, University of Washington, Ave. NE, Seattle, WA). For pathway analyses, Kyoto Encyclopedia of Genes and Genomes (KEGG) resources were utilized with recommended analytical parameters. For gene ontology enrichment and network analyses, UniProt ([www.uniprot.org](http://www.uniprot.org)) database resource (biological process, molecular function), Ingenuity Pathway Analysis, and Reactome knowledgebase were utilized. The heat map of proteins adopted gplots.

## Statistical analysis

The research values were presented as mean  $\pm$  SD. The data from three independent experiments were analyzed. One-way analysis of variance (ANOVA) was used to compare with the multiple time points and concentrations in the experiments. When the comparison between two groups means significant, \* or + was used for  $P < 0.05$ , and \*\* or ++ for  $P < 0.01$ .

## Results

### Identification and telomerase transfection of BTCs

The cells derived from the bovine placenta were purified to Passage #5 with the differential velocity adherent method. The cells, growing as epithelial-like monolayers, showed a clear epithelioid cell morphology under a phase-contrast inverted microscope (Fig. 1A). After being dyed with Giemsa stain, they were observed under a microscope. There were minor binucleate cells among the uninucleate cells, which communicated with each other by the extended pseudopodia (Fig. 1B). BTCs were identified by detecting the biomarkers of extravillous trophoblast cells via immunofluorescence assay. Fetal bovine fibroblast cells (FBFs) were used as a negative control. As expected, all of the purified primary BTCs were CK7 positive while all of fetal bovine fibroblast cells were negative. The result that 100% of BTCs were negative of CD90 exactly verified there were no fibroblast mixing in the purified BTCs. Furthermore, the expression of two representative proteins in the epithelial cells, vimentin and E-cadherin suggested that BTCs were epithelial-origin cells (Fig. 1C). pCI-neo-hTERT plasmid was transfected in 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 on the protein level by western blot assay. The growth curve of BTCs, measured by CCK-8 assay, showed a basically similar trend in

BTCs no matter whether they were transfected with plasmid or not (Fig. 1D). However, the proliferation activity of immortalized BTCs was higher than that of primary BTCs, and 50-passage 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 expressed greatly and steadily in the hTERT-BTCs and HeLa cells while the expression level was significantly higher than that in the primary cells (Fig. 1E-G,  $p < 0.01$ ).

## **Immortalized BTCs maintain the functional characteristics of primary BTCs**

Secretion of PL and CG is one of the most important features of extravillous trophoblast cells<sup>[3]</sup>. Analyzed by ELISA assay, both primary BTCs and hTERT-BTCs manifest an ability of secreting PL and CG (Fig. 2A). Showing an invasive property confirmed by trans-well invasive assay, both primary BTCs and hTERT-BTCs have the same ability to migrate cells (Fig. 2B). In addition, hTERT-BTCs at Passage #50 showed the similar features of marker protein expression to those of primary BTCs (Fig. 2C, D). Soft agar assay was performed to confirm whether hTERT-BTCs have an ability of anchorage-independent growth. hTERT-BTCs were incapable of forming any colonies after 14-day incubation while HeLa cells formed, which indicated that hTERT-BTCs can't transform to a malignant phenotype (Fig. 2E).

## **Isolation and identification of exosomes derived from BTCs**

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

## **Effect of P4-treated BTCs derived exosomes on the endometrial receptivity of EECs**

All of the bovine EECs we cultured showed a typical "paving stone"-like appearance with the positive expression of CK8, a marker protein of EECs (Fig. 4A). In this study, the EECs co-incubated with the TDE stained with PKH26 (Sigma Aldrich, USA) showed a significant red fluorescence indicating that the TDE was fused with the EECs after 12 h of incubation (Fig. 4B). It has been reported that endometrial receptivity, as a key factor to determine the embryonic development process, can be affected by TDE and P4 stimulation during pregnancy. Exosomes derived from BTCs treated with or without 10 ng / mL P4 for 24 h were collected and co-cultured with EECs for 12 h. The expression changes of uterine receptivity factors related to embryo implantation were detected in the EECs by western blot assay. Compared with the BTCs which weren't treated, the P4-treated cells derived from exosomes increased the expression levels of uterine receptivity-related proteins in the EECs, such as integrin  $\alpha$ v, integrin  $\beta$ 3, and Wnt7a while decreasing the expression of MUC1 (Fig. 4C, D;  $p < 0.01$ ).

# Functional analysis of TDE proteome

Proteomic profiling was performed on the exosomes derived from the BTCs cultured with / without P4 by 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. The protein expression heatmap was shown for a total of 923 proteins identified in the proteomics analysis (Fig. S1). KEGG and GO classification showed that the proteins identified were concentrated on the immune system and endocrine system for antioxidant enzyme activity, binding, molecular function regulator, structural molecular activity, transporter activity, etc. (Fig. S1). Of these 923 proteins, 19 ones were differentially expressed in the cells under pregnancy situations, including 6 ones such as CTSD, PCOLCE and COPS6, that decreased expressions, and 13 ones such as GNPTG, EIF4A2 and FGB, that increased expression. (Fig. 5A, B). Functional annotation and pathway analysis of these differential expressed protein subsets associated with pregnancy group revealed the protein categories associated with collagen binding, catalytic activity and hydrolase activity (Fig. 5D, E).

## Discussion

Success of pregnancy relies on the differentiation, proliferation, and invasion of placental trophoblast cells during early pregnancy<sup>[26, 27]</sup>. The dysfunction of trophoblast may lead to severe complications threatening both the mother and the developing fetus and resulting in economic losses. Placenta trophoblast cells are consisted of two kinds, mononucleate trophoblast cells and binucleate trophoblast cells<sup>[28, 29]</sup>. In this study, the cells we separated and purified from placentae contained mononucleated cells mixing with minority multinucleated cells which had similar features to those of the first trimester trophoblast. The potential non-trophoblast components were mainly Hofbauer cells, blood leukocytes cells and fibroblast cells in this study. Hofbauer cells and blood leukocytes cells were easily deleted through the continuous passage<sup>[29]</sup>. We eliminated the fibroblast cells by continuous passage with the differential adhesion method. To confirm whether there was no fibroblast contamination, we detected the expression of CD90 / Thy1, the most iconic fibroblast biomarker, and found there were no fibroblast after the purification<sup>[10, 30]</sup>. Cytokeratin 7, one intermediate protein, is a foremost marker of trophoblast cells<sup>[31, 32]</sup>. The result that all of the cells were CK-7 positive confirmed that the cells we separated were derived by trophoblast and suitable for transfection.

To establish a normal bovine placental trophoblast cell line, we changed telomerase activity with the ectopic expression of exogenous hTERT gene. Based on western blot and RT-qPCR assay, the transfected hTERT-BTCs were positive for telomerase expression and have been cultured for over 50 passages without showing a sign of stopping the division. In addition, hTERT-BTCs showed a more aggressive growth rate compared to those without transfection, suggesting that exogenous telomerase expression accelerated the proliferation of BTCs. Thus, the cells can be considered to be immortalized. The secretory capability of pregnancy-related hormones such as chorionic gonadotropin (CG) and placental lactogen (PL) is one of the most critical features of trophoblast cells<sup>[10, 22]</sup>. The immortalized BTCs keep the same

characteristics of CG and PL secretion, and CK7, vimentin and E-cadherin expression till Passage #50. The trans-well Invasion assay indicated the immortalized BTCs inherited invasiveness. Thus, these results suggested that immortalized BTCs had epithelial-origin, endocrine and invasive features of extravillous trophoblast cells, which were maintained by these primary BTCs. Moreover, no CD90 expression in the BTCs suggested that there is no fibroblast contamination through the 50 passages of culturing process. In a word, the results showed that the immortalized bovine trophoblast cells possessed a prolonged lifespan and retained some properties of primary cells which are as same as the trophoblast cells previously reported<sup>[31]</sup>.

The increasing level of P4 was accompanied by early embryonic development process, so we suppose that P4 secreted by corpus luteum may stimulate trophoblast and change placenta derived exosomes to enhance the endometrial receptivity. In the present study, the exosomes derived from BTCs were stimulated with P4 in a concentration during the early pregnancy and co-cultured with EECs. We found these exosomes exert a positive effect on the expressions of endometrial receptivity-related proteins in EECs manifested by the upregulation of Integrin  $\alpha$ v,  $\beta$ 3 and Wnt7a while the downregulation of MUC1.

To detect if there were different proteins loaded in trophoblast derived exosomes, mass-spectrometry-based proteomics were performed and we found whether treated with P4, TDE proteins both focused on the immune system and endocrine system, while TDE could adjust the functions of EECs and regulate endometrial receptivity through immune and endocrine system. For example, TDE can give assistance to fetus escaping from maternal immune system and improve the secretory function of endometrium<sup>[16, 33]</sup>. What's more, our results are also consistent with the existing literature that trophoblast can regulate endometrial receptivity<sup>[34]</sup>. From the further KEGG and GO classification research of differentially expressed proteins, we found that the expressions of these proteins including CTSD (cathepsin D), PCOLCE (procollagen C-endopeptidase enhancer), COPS6 (COP9 signalosome complex subunit 6) which can decompose extracellular matrix were all decreased with a P4 stimulation while the expressions of these antioxidant and hydrolase related proteins, such as ATIC (bifunctional purine biosynthesis protein ATIC), NANS (N-acetylneuraminase synthase) and ESD (S-formylglutathione hydrolase), were increased. Dysregulation of endometrial extracellular matrix remodeling can impair receptivity which is linked to pregnancy outcomes<sup>[35]</sup>. Redox homeostasis and energy metabolism plays an important role on maternal receptivity by affecting endometrial response to conceptus-derived pregnancy signals<sup>[36]</sup>. In combination with the result that the cells derived from exosomes and treated with P4 can upregulated the expression of endometrial receptivity related proteins, the results of differentially expressed proteins between exosomes implied that endometrial receptivity was increased with a P4 stimulation under a pregnant situation through TDE by maintaining a steadier state of oxidation balance and extracellular environment. The expression levels of some transcription factors mediating cellular response, such as EIF4A2 (Eukaryotic initiation factor 4A-II), were also affected by P4 stimulation. In brief, TDE which aims on immune and endocrine ways, may regulate endometrial receptivity by ameliorating the extracellular environment, regulating cell redox balance and changing some of cell signal transduction during pregnancy.

## Conclusion

In summary, we established an immortal placental trophoblast cell line by transferring exogenous hTERT. With this cell line, we found P4 regulates the function of exosomes derived from trophoblast cells, reversely regulates the receptivity of EEC, and ensures normal embryo development during pregnancy.

## Abbreviations

BTCs  
Bovine trophoblast cells; TERT:Telomerase reverse transcriptase; TDEs:Trophoblast derived exosomes; EECs:Endometrial epithelial cells; P4:Progesterone; MUC1:Mucin 1; FBFs:Fetal bovine fibroblast cells; qRT-PCR:Quantitative real-time PCR; CCK-8:Cell counting kit-8; GAPDH:Glyceraldehyde-3-phosphate dehydrogenase; HRP:Horseradish peroxidase; FITC:Fluorescein isothiocyanate; DAPI:4',6-diamidino-2-phenylindole; CD9:Cluster of differentiation 9; CD63:Cluster of differentiation 63; CD90:Cluster of differentiation 90; CK7:Cytokeratin 7; CK8:Cytokeratin 8; CG:chorionic gonadotropin; PL:placental lactogen; DIA:Data independent acquisition; KEGG:Kyoto encyclopedia of genes and genomes; GO:Gene ontology; CTSD:cathepsin D; PCOLCE:Procollagen C-endopeptidase enhancer; COPS6:COP9 signalosome complex subunit 6; ATIC:bifunctional purine biosynthesis protein ATIC; NANS:N-acetylneuraminase synthase; ESD:S-formylglutathione hydrolase; EIF4A2:Eukaryotic initiation factor 4A-II.

## Declarations

### Ethical Approval and Consent to participate

Not applicable.

### Consent for publication

Written informed consent for publication was obtained from all participants.

### Acknowledgments

Not applicable.

### Competing interests

The authors declare no conflict of interest.

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## Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

Y. S was a major contributor in writing the manuscript. Q. L and Q. Z analyzed and interpreted the experimental data. Z. L and X. Y helped to collect placenta samples. Y. G and L. X contributed a lot to revise the draft. X. W and H. N conceived and designed the study. All authors read and approved the final manuscript.

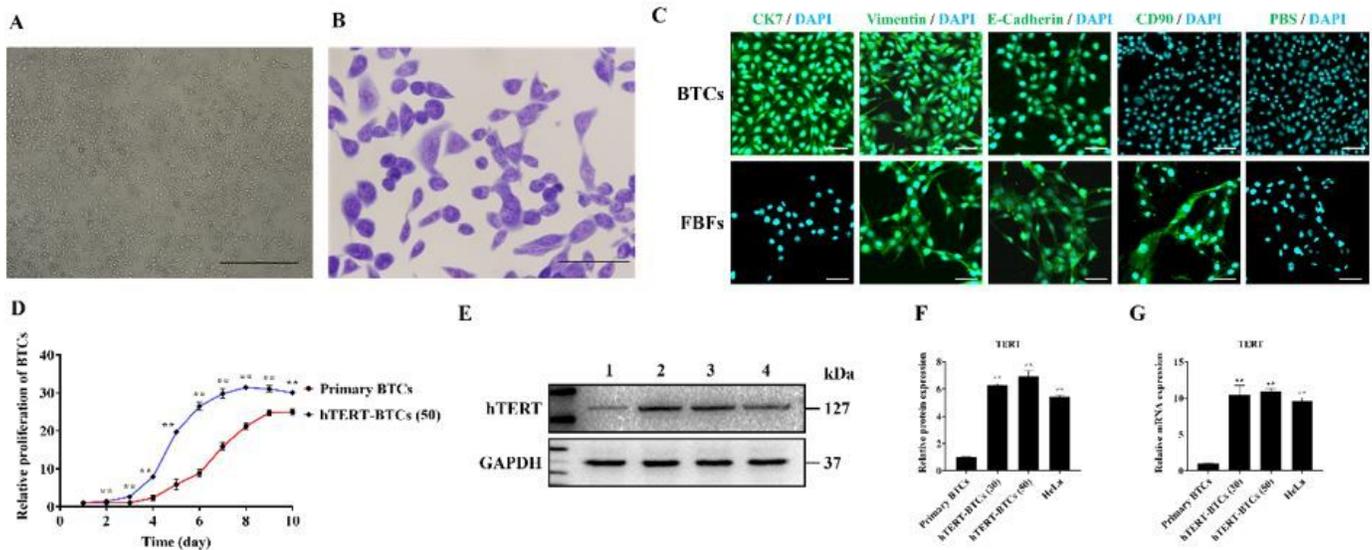
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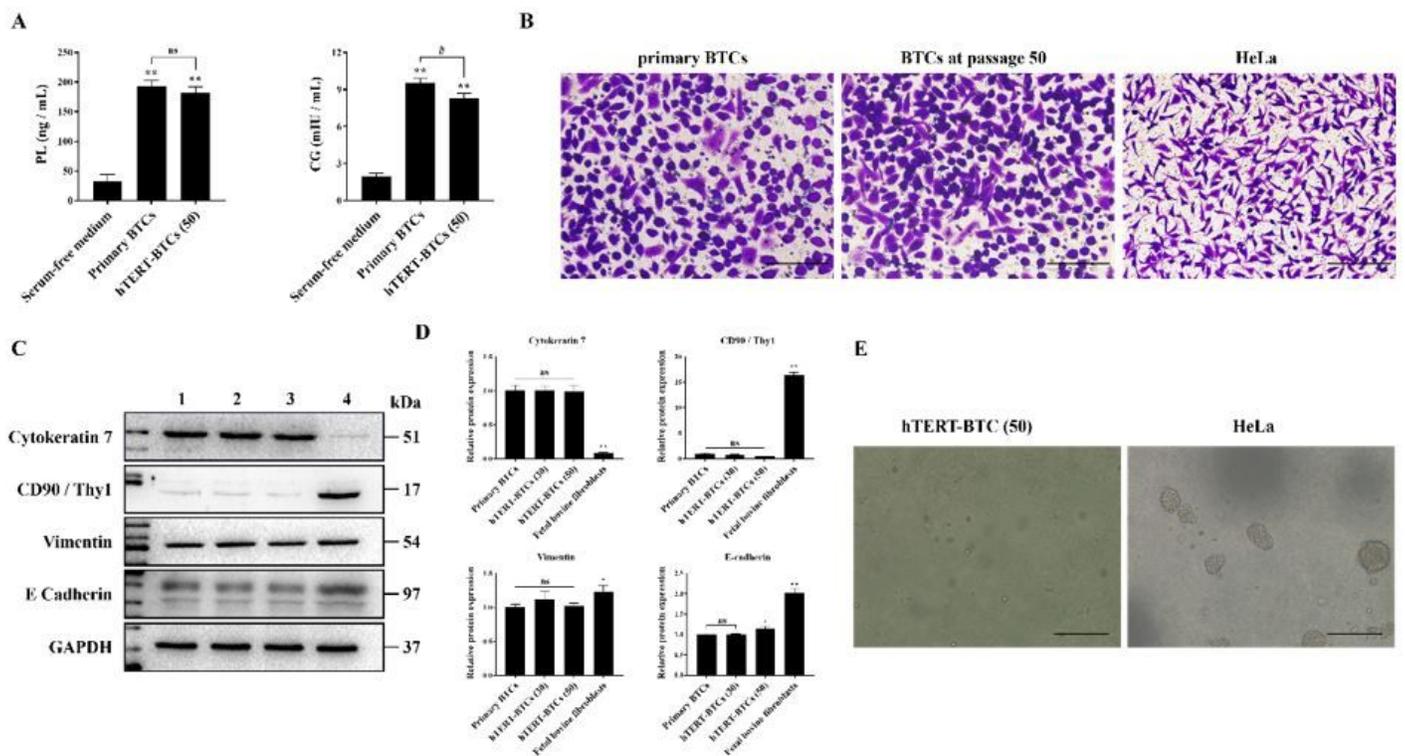
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## Figures



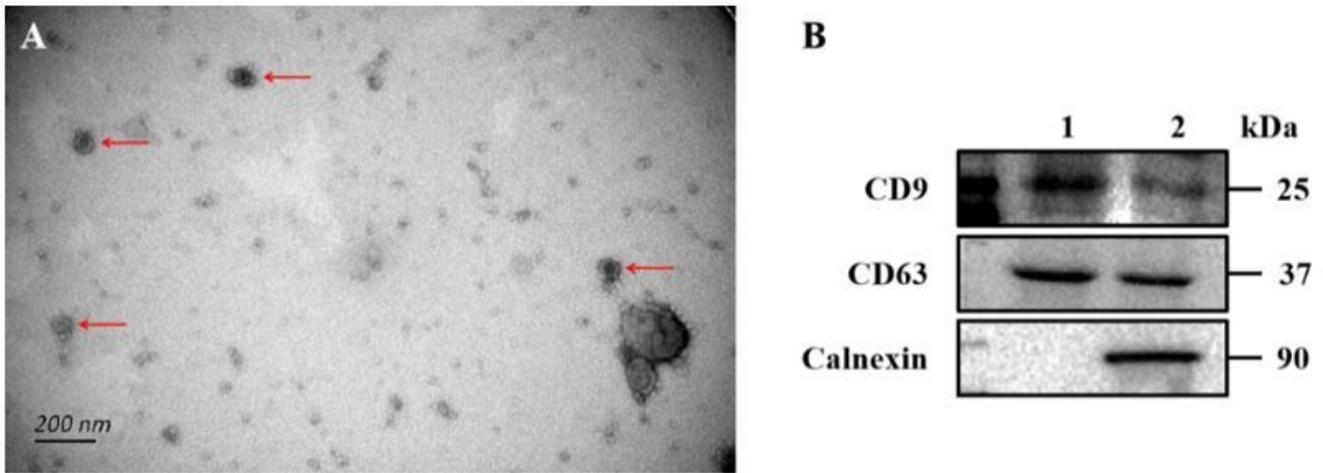
**Figure 1**

Identification and hTERT-transfection of primary bovine trophoblast cells (BTCs) derived from bovine placenta. A, B) Morphological characteristics and Giemsa stain result of BTCs under the phase-contrast inverted microscope; bars = 1 mm. C) Cytokeratin 7 (CK7), vimentin, E-Cadherin and CD90 expressions in the 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  $\mu$ m. 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 was primary BTCs; lane 2 was hTERT-BTCs at Passage #30; lane 3 was hTERT-BTCs at Passage #50; lane 4 was HeLa cells for a positive control. G) Representative mRNA expression levels were measured by qRT-PCR assay and analyzed with  $2^{-\Delta\Delta C_t}$  method. The results were represented as the means value  $\pm$  SD obtained from three independent experiments (\* $p$  < 0.05, \*\* $p$  < 0.01 compared with primary BTCs).



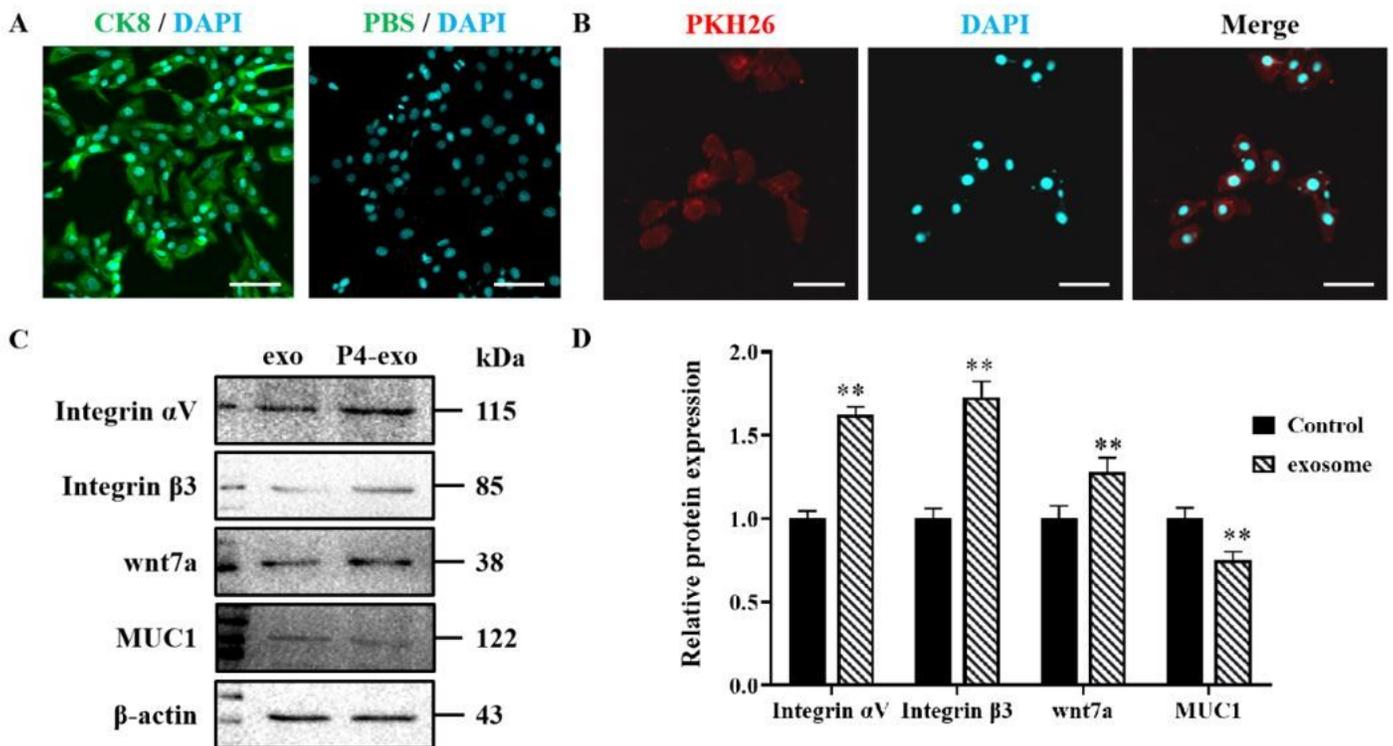
**Figure 2**

Immortalized BTCs maintain the epithelial-origin and endocrine features of primary BTCs without showing the features of malignant transformation. A) The endocrine ability of CG and PL was measured by ELISA assay. B) The migration ability of BTCs was measured by trans-well chamber migration assay; bars = 50  $\mu$ m. C) CK7, CD90, vimentin and E-Cadherin expressions were detected by Western blot analysis. Lane 1 was primary BTCs; lane 2 was hTERT-BTCs at Passage #30; lane 3 was hTERT-BTCs at Passage #50; lane 4 was fetal bovine fibroblast cells (FBFs) for a negative control. D) The protein expression levels were measured by image J in the representative Western blot analysis. E) hTERT-BTCs did not form cell colonies while HeLa cells formed those larger than 100  $\mu$ m in diameter after 14-day culture. The results were represented as the means value  $\pm$  SD obtained from three independent experiments (\* $p$  < 0.05, \*\* $p$  < 0.01 compared with primary BTCs).



**Figure 3**

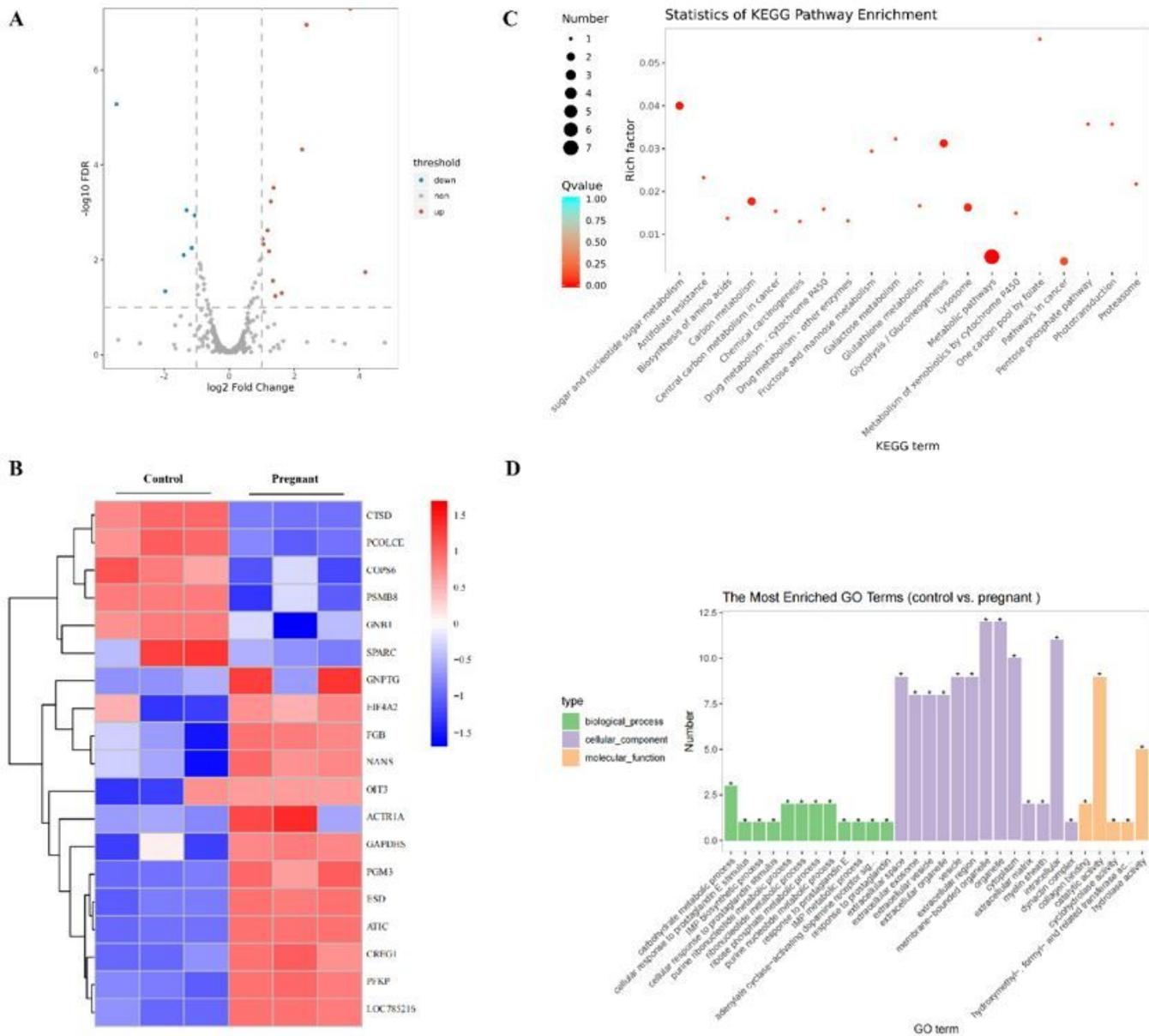
Identification of BTC-derived exosomes. A) Morphological observation of the BTC-derived exosomes under a transmission electron microscope; bars = 200 nm. B) Verification of exosomes by Western blot assay. Lane 1 was BTC-derived exosomes; lane 2 was BTCs.



**Figure 4**

P4-treated BTCs-derived exosomes enhance the endometrial receptivity of EECs. A) CK8 expression in EECs was detected by Immunofluorescence assay; bars = 100  $\mu$ m. B) Confocal microscopy of the internalization of fluorescently-labeled exosomes in EECs after 12 h incubation; bars = 50  $\mu$ m. C)

Expressions of integrin  $\alpha$ v, integrin  $\beta$ 3, Wnt7a and MUC1 were detected by western blot assay. D) The protein expression levels were measured by image J in representative Western blot analysis.



**Figure 5**

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

## Supplementary Files

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