Toxoplasma gondii excretory/secretory proteins promotes osteogenic differentiation of bone marrow mesenchymal stem cells via aerobic glycolysis mediated by Wnt/β-catenin signaling pathway

WEIQIANG ZHAN[1,2*], JUANHUA QUAN[3*], ZHUMING CHEN[4*], TIANFENG LIU[1], MINGZHU DENG[1,2], ZIQUAN ZHAO[1,2], XUYANG WU[1,2], ZHUOLAN ZHONG[1,2], FEIFEI GAO[2] and JIAQI CHU[1,2]

[1]Orthopaedic Center; [2]Stem Cell Research and Cellular Therapy Center; [3]Laboratory of Gastroenterology, Affiliated Hospital of Guangdong Medical University; [4]Department of Orthopaedic Surgery, The Second Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong 524001, P.R. China

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Abstract. *Toxoplasma gondii* excretory/secretory proteins (TgESPs) are a group of proteins secreted by the parasite and have an important role in the interaction between the host and *Toxoplasma gondii* (*T. gondii*). They can participate in various biological processes in different cells and regulate cellular energy metabolism. However, the effect of TgESPs on energy metabolism and osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) has remained elusive. In the present study, TgESPs were extracted from the *T. gondii* RH strain and used to treat BMSCs to observe the effect of TgESPs on energy metabolism and osteogenic differentiation of BMSCs and to explore the molecular mechanisms involved. The osteogenic differentiation and energy metabolism of BMSCs were evaluated using Alizarin Red S staining, qRT-PCR, western blot, immunofluorescence and Seahorse extracellular flux assays. The results indicated that TgESPs activated the Wnt/β-catenin signaling pathway to enhance glycolysis and lactate production in BMSCs, and promoted cell mineralization and expression of osteogenic markers. In conclusion, the present study uncovered the potential mechanism by which TgESPs regulate BMSCs, which will provide a theoretical reference for the study of the function of TgESPs in the future.

Correspondence to: Dr Jiaqi Chu, Stem Cell Research and Cellular Therapy Center, Affiliated Hospital of Guangdong Medical University, 57 Renmin Avenue South, Zhanjiang, Guangdong 524001, P.R. China
E-mail: chujiaqi@gdmu.edu.cn

*Contributed equally

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Introduction

The incidence of segmental bone defects, slow bone healing and bone nonunion due to trauma, inflammation or tumors is increasing (1). The current treatment for such patients is bone grafting using autografts or allografts; however, both types have significant disadvantages and limitations (2). With the development of modern molecular biology and regenerative medicine, stem cell therapy has advanced the treatments for these diseases. Mesenchymal stem cells (MSCs) are multipotent cells of mesodermal and neural crest origin with stromal properties (3). MSCs are currently the most frequently utilized stem cells in preclinical and clinical studies of skeletal diseases (4). These cells can be extracted from a range of sources, including the bone marrow, adipose tissue, urine, placenta and umbilical cord. In addition, they exhibit three different biological properties, namely differentiation potential, secretion of growth-promoting factors and immunomodulation, which render them highly suitable as seed cells for cell therapy (5). Therefore, promoting osteogenic differentiation of MSCs may help accelerate fracture healing and treat bone defect and nonunion.

Bone formation requires the generation of numerous osteoblasts, which are mainly derived from bone marrow MSCs (BMSCs). Numerous regulatory factors can affect the osteogenic differentiation of BMSCs and several studies have indicated that the Wnt/β-catenin signaling pathway has an instrumental role in bone production (6-8). The binding of Wnt ligands to Frizzled and lipoprotein receptor-related protein 5/6 receptors activates Dishevelled (Dvl), which promotes β-catenin stabilization. Subsequently, β-catenin binds to T-cell factor/lymphoid enhancing factor (LEF) DNA-binding proteins to regulate the transcription of osteogenic-related target genes (9). Furthermore, several studies have demonstrated that the activation of the Wnt/β-catenin pathway

through the use of small molecule drugs or growth factors can promote systemic and local bone formation (10,11). This suggests that targeting the Wnt/β-catenin signaling pathway is an effective strategy for bone regeneration.

Genetic and functional studies have shown that the metabolism of glucose, fatty acids, and amino acids is an essential regulator of BMSCs differentiation. This is because BMSCs require large amounts of energy to maintain bone homeostasis. The balance between bone formation and resorption is disrupted when the energy metabolism of BMSCs is dysregulated (12). Glucose metabolism has a significant role in the differentiation of BMSCs and is primarily divided into glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). Recent studies have revealed that differentiated osteoblasts have higher levels of glycolysis and OXPHOS than undifferentiated cells. As cells differentiate, their rate of oxygen consumption decreases, while their rate of glycolysis increases, indicated by a decrease in the ratio of oxygen consumption rate (OCR) to extracellular acidification rate (ECAR) (13). This phenomenon is known as aerobic glycolysis and is similar to the Warburg effect observed in tumor cells. Aerobic glycolysis is a significant source of energy that promotes the differentiation of MSCs, and inhibition of glycolysis inhibits osteogenic differentiation of BMSCs (14,15). It was indicated that Wnt can enhance the levels of glucose transporter isoform 1 and hexokinase-2, leading to an increased consumption of glucose (16). In addition, Wnt promotes aerobic glycolysis by upregulating L-lactate dehydrogenase A (LDHA) and 3-phosphoinositide-dependent protein kinase 1 to increase lactate production rather than pyruvate production (17). Furthermore, knockdown of mitofusin-2 enhances the Wnt/β-catenin signaling pathway in induced pluripotent stem cell-derived MSCs, thereby enhancing aerobic glycolysis and osteogenic differentiation (18). All of the above-mentioned studies suggest that glycolysis may have a key role in the future treatment of different diseases using BMSCs and that the Wnt/β-catenin signaling pathway stimulates aerobic glycolysis in MSCs.

Toxoplasma gondii excretory/secretory proteins (TgESPs) are a class of proteins secreted by *Toxoplasma gondii* (*T. gondii*) that can circulate and remain on the surface of cells (19). In previous studies, a proteomics study detected ~512 proteins in TgESPs with various functions, such as extracellular proteases, hormones, neurotransmitters and antimicrobial peptides (20). They also participate in cell adhesion, migration and intercellular communication. TgESPs are therefore not only critical for the immune response of *T. gondii* but also enter the cells more readily (20,21). Recent studies have found that TgESPs can alter the metabolism of host cells, most of which involve the tricarboxylic acid (TCA) cycle, purine metabolism and tryptophan metabolism (22). Various metabolism-related proteins have also been identified in TgESPs (23). This indicates that TgESPs may affect the energy metabolism of cells after entering them. However, it is unclear whether TgESPs can affect the energy metabolism and osteogenic differentiation of BMSCs.

The present study aimed to reveal the molecular mechanisms through which TgESPs affect the metabolism and osteogenic differentiation of BMSCs. The study may help in developing a new therapeutic approach for bone repair.

Materials and methods

Isolation and culture of human BMSCs (hBMSCs). The experimental protocol conforms to the ethical standards of the Declaration of Helsinki and was approved by the ethics committee of the Affiliated Hospital of Guangdong Medical University (Zhanjiang, Guangdong, China). Bone marrow samples were obtained from the femoral cavity during the operation of five female patients aged 35 to 45 years with traumatic femoral head fractures. Each sample was treated individually and used for subsequent experiments. All participants provided written informed consent. The bone marrow samples were collected from each participant and hBMSCs were isolated using Ficoll density gradient centrifugation. Subsequently, the bone marrow samples were transferred to a 15-ml centrifuge tube and diluted with PBS at a ratio of 1:1. An equal volume of Histopaque®-1077 (Sigma-Aldrich; Merck KGaA) was then added to the diluted bone marrow sample and centrifuged at 400 x g and room temperature for 20 min without accelerating or braking. Cells in the middle layer were extracted and further resuspended in Modified Eagle's Medium α (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 10 U/ml penicillin G and 10 mg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.). The obtained cells were incubated at 37˚C in an incubator containing air with 5% CO_2 and saturated humidity. The medium was changed every three days; when the cells reached ~90% confluence, they were digested with 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.) and passaged at a 1:3 ratio. The cells from passages five to six were used in all subsequent experiments.

Identification of cell phenotype. The cells from passage three were used to identify phenotypes. When the cells had reached 80% confluence, they were digested with 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.), centrifuged at 800 x g for 3 min at room temperature and resuspended in PBS. The cell surface antigens CD90, CD73, CD105 and CD45 were detected via flow cytometry using the Human MSC Analysis Kit (cat. no. 562245; BD Biosciences). Data were analyzed using FlowJo 10.0.7 software (FlowJo LLC).

Adipogenic and chondrogenic differentiation assays. The hBMSCs were seeded in 6-well plates at a density of $1x10^5$ cells per well. The hBMSCs were cultured with adipogenic induction medium (α-MEM containing 10% FBS, 10 mg/ml insulin, 1 mM dexamethasone, 50 mM 3-isbutyl-1-methylxanthine and 50 μM indomethacin; all from Sigma-Aldrich; Merck KGaA) and chondrogenic induction medium (α-MEM containing 1% FBS, 1% insulin-transferrin-sodium selenite, 20 nM dexamethasone, 10 ng/ml TGF-β and 40 μg/ml proline; all from Sigma-Aldrich; Merck KGaA) and stained with Oil Red O or Alcian Blue staining solution (Cyagen Biosciences, Inc.). The images were acquired under a microscope and stored.

Extraction of TgESPs. The RH strain of *T. gondii* (kindly provided by Dr Young-Ha Lee, Department of Infection Biology, Chungnam National University School of Medicine, Republic of Korea) was allowed to proliferate in ARPE-19 cells (CRL-2302; American Type Culture Collection). *T. gondii* was collected and rinsed in PBS, centrifuged at 1,000 x g for 5 min

at room temperature, and this was repeated twice. Purified tachyzoites were collected in 1 ml Hank's balanced salt solution (Gibco; Thermo Fisher Scientific, Inc.) and oscillated at 3.57 x g in a constant temperature shaker at 37˚C for 4 h. After centrifugation at 14,000 x g thrice for 5 min each at 4˚C, the supernatant was stored as TgESPs.

Transmission electron microscopy. To visualize vesicle-like structures in TgESPs, the TgESPs were deposited on a nickel grid and stained with uranyl acetate and lead citrate, and images of the samples were acquired using a JEM-1400 transmission electron microscope (JEOL, Ltd.).

Cell viability assay. The effect of TgESPs on the proliferation of hBMSCs was evaluated using the Cell Counting Kit-8 (CCK-8; Zeta Life). In brief, hBMSCs were seeded in 96-well plates at a density of 3,000 cells per well and incubated with different concentrations of TgESPs (0, 1, 10, 20, 30, 40 and 50 μg/ml) for 24, 48 and 72 h. The wells were then filled with 10 μl CCK-8 solution and incubated in 5% CO_2 in a humidified environment for 2 h at 37˚C. The absorbance of the cells at 450 nm was measured using a microplate reader (BioTek Instruments, Inc.) and a cell proliferation curve was generated.

Osteogenic differentiation protocol and alizarin red S staining. The hBMSCs were seeded in 12-well plates at a density of $1x10^5$ cells per well. Once they reached >80% confluence, the medium was changed to the osteogenic induction medium (OIM), i.e. Dulbecco's modified Eagle's medium (DMEM) with low glucose (LG) content, supplemented with 100 nM dexamethasone (Sigma-Aldrich; Merck KGaA), 50 μM L-ascorbic acid-2-phosphate (Sigma-Aldrich; Merck KGaA), 10 mM β-glycerophosphate (Sigma-Aldrich; Merck KGaA) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The experimental group was incubated in OIM supplemented with 1 or 10 μg/ml TgESPs, whereas the control group was incubated in OIM without TgESPs, and the inhibitor group was treated with 25 μM Icg-001 (MCE) or 2 mM 2-deoxy-D-glucose (2-DG; Sigma-Aldrich; Merck KGaA) for three days to induce osteogenic differentiation. The osteogenic induction medium was changed every three days.

The cellular mineral deposition was evaluated using alizarin red staining. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Subsequently, they were washed thrice with PBS, treated with Alizarin Red S staining solution (Oricell) for 20 min at room temperature and washed thrice with PBS. The images were acquired and saved using a camera and microscope.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Cells were seeded in 6-well plates at a density of $2x10^5$ cells per well. Total RNA was extracted from the hBMSCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 7 days after osteogenic induction and reverse-transcribed into cDNA using HiScript III RT SuperMix (Vazyme), ChamQ Universal SYBR qPCR Master Mix (Vazyme) was used for qPCR, and both cDNA synthesis and qPCR were performed according to the manufacturer's protocol. The qPCR thermocycling conditions were as follows: 95˚C for 10 sec and 60˚C for 30 sec, performed for 40 cycles. The mRNA expression levels of target genes were normalized to those of the internal control (β-actin) and quantified using the $2^{-\Delta\Delta Cq}$ method (24). The primers used for PCR are listed in Table SI.

Western blot analysis. Cells were lysed using RIPA buffer (Invitrogen; Thermo Fisher Scientific, Inc.) 14 days after the induction of osteogenic differentiation. Protein concentrations were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). For each lane, 20 μg of protein were loaded on a 10 or 12% SDS-PAGE gel, resolved and electrotransferred to a polyvinylidene difluoride membrane (Merck Millipore). The membrane was then placed in 5% skimmed milk (0.5 g skimmed milk powder dissolved in 10 ml Tris-buffered saline), blocked at room temperature for 1-2 h, and incubated with a primary antibody at 4˚C overnight. After washing it thrice with Tris-buffered saline containing Tween 20 (TBS-T), the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing the membrane thrice with TBST (10 min each), immunoreactive bands were visualized using a chemiluminescence detection reagent (Merck Millipore). Finally, protein intensity was quantified using the ImageJ software version 1.8.0 (National Institutes of Health). All the antibodies used for western blot analysis are listed in Table SII.

Immunofluorescence (IF). Cells were induced to undergo osteogenic differentiation in OIM with or without TgESPs for 7 days. In addition, RUNX family transcription factor 2 (Runx2) expression was measured using IF. In brief, the cells were fixed in 4% paraformaldehyde for 20 min, permeabilized in 0.5% Triton X-100 (Sigma-Aldrich; Merck KGaA) for 30 min and blocked in 1% bovine serum albumin (Beijing Solarbio) for 1 h. The cells were then washed thrice with PBS (5 min each) and incubated overnight at 4˚C with a specific primary antibody against Runx2. The cells were subsequently incubated with AlexA-568-conjugated secondary antibody for 1 h at room temperature. The nuclei were stained with DAPI. Immunofluorescence was observed using a fluorescence microscope (Olympus Corporation). Details of all antibodies used for IF are provided in Table SII.

Seahorse extracellular flux assays. The hBMSCs were cultured in OIM for 7 days. Cells were harvested following trypsin digestion and re-seeded in Seahorse XF24 cell culture microplates (Seahorse Bioscience) at a density of $2x10^4$ cells per well. Cells were cultured overnight in OIM and the OCR and ECAR were measured using the Seahorse XF Cell Mito Stress Test kit (Seahorse Bioscience) and Seahorse XF Glycolysis Rate Assay kit (Seahorse Bioscience), respectively, according to the manufacturer's instructions. The Cell Mito Stress Test assesses ATP production and maximal mitochondrial respiration by successively adding specific inhibitors, such as oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, a potent mitochondrial oxidative phosphorylation uncoupling agent that promotes mitochondrial membrane depolarization. In this study, we employed FCCP to investigate the impact of TgESP on cellular energy metabolism.) and rotenone/antimycin A (Rot/AA). Afterwards, the glycolysis rate assay was carried out using Rot/AA and

2-DG to measure ECAR and intracellular glycolysis capacity. The OCR and ECAR were measured and analyzed using an XF24 Flux Analyser (Seahorse Bioscience).

Lactic acid measurements. For lactic acid measurement, a lactic acid detection kit (cat. no. BC2235; Beijing Solarbio) was used according to the manufacturer's protocol. The culture medium of the cells was collected, and mixed with working solution of the lactic acid detection kit. Subsequently, the mixture was incubated with the color reagent at 37˚C in the dark for 20 min. Finally, the absorbance at 570 nm was measured using a microplate reader (BioTek Instruments, Inc.) and the lactic acid concentration was calculated based a standard curve.

Cell wound healing assay. The cell wound healing assay was performed as previously described (25). In brief, hBMSCs were seeded at a density of $2x10^5$ cells per well in 6-well plates and treated with 1 or 10 μg/ml TgESPs for 24 h. After this treatment, a vertical scratch was made in the cell monolayer using a 20-μl micropipette tip. The cells were then washed once with PBS and incubated with serum-free basal medium. The migration of hBMSCs was observed and recorded using Leica DMI3000 microscope (Leica Microsystems GmbH) at 0 and 24 h. The cell migration distance was quantified by measuring the cell distance between the two sides using Photoshop 2021 (Adobe Inc.).

Cell adhesion assay. The cell adhesion assay was conducted following a previously reported protocol (26). In brief, hBMSCs were seeded in 6-well plates and treated with 1 and 10 μg/ml TgESPs for 24 h. After this treatment, the cells were digested, resuspended and seeded at a density of $1x10^4$ cells per well in 96-well plates coated with polylysine (Beijing Solarbio). After allowing the cells to attach for 1 h, non-adherent cells were washed off with PBS. Subsequently, 10 μl MTS assay solution was added to each well and incubated at 37˚C for 2 h. The absorbance was measured at 490 nm using a microplate reader (BioTek Instruments, Inc.). The relative cell adhesion rate was thus evaluated.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software; Dotmatics). Comparisons of data between two groups were performed using an unpaired two-tailed Student's t-test, while data were compared among three or more groups by one-way ANOVA followed by Tukey's post-hoc test. Values are expressed as the mean ± standard deviation and were based on results from at least three independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization and phenotypic identification of hBMSCs. hBMSCs were isolated and cultured from human bone marrow samples and passaged three to four times. After 10 days of primary culture, the attached hBMSCs appeared fibroblast-like and spindle-shaped and grew to 90% confluency (Fig. 1A). Alizarin Red S, Oil Red O and Alcian blue staining revealed that hBMSCs had the potential for osteogenic, adipogenic and chondrogenic differentiation, respectively (Fig. 1B). When the confluence of hBMSCs in passage three reached 90%, the surface markers assessed via flow cytometry showed that expression of the positive markers CD73 (99.9%), CD90 (99.1%) and CD105 (99.3%) was >95%, while that of the negative marker CD45 (0.2%) was <2% (Fig. 1C). These features suggest that the cells isolated from human bone marrow can be considered hBMSCs.

TgESPs contain secretory vesicles. Ramírez-Flores *et al* (23) observed that *T. gondii* can secrete tubular structures composed of vesicles *in vitro*, which disperse into a single exosomes-like vesicle after centrifugation. In the present study, TgESPs extracted by centrifugation also contained numerous vesicles (~100-160 nm in diameter) with the morphological characteristics of exosomes, as observed under the transmission electron microscope (Fig. 1A). The isolate also contained some fine particulate matter, which may be other soluble proteins.

TgESPs enhance hBMSC proliferation, adhesion and migration. To address the effect of TgESPs on hBMSC proliferation, the CCK-8 assay was performed. The results indicated that 1, 30, 40 and 50 μg/ml TgESPs had no inhibitory effect on the proliferation of hBMSCs, while 10 and 20 μg/ml TgESPs had a positive impact on the growth of hBMSCs after 24 and 48 h of treatment compared to that in the control group ($P<0.05$) (Fig. 2B). Further investigation of the effect of TgESPs on hBMSC behavior revealed that low concentrations of TgESPs promoted cell adhesion, and both 1 and 10 μg/ml concentrations enhanced cell migration in hBMSCs (Fig. S1). These results indicate that TgESPs not only promote the proliferation of hBMSCs at low concentrations, but also enhance cell adhesion and migration.

TgESPs enhance osteogenic differentiation of hBMSCs. To understand the effect of TgESPs on the osteogenic differentiation of hBMSCs, the effects of 0, 1, 10 and 20 μg/ml TgESPs on osteogenic differentiation of hBMSCs were examined using alizarin red staining. After 14 days of osteogenic differentiation induction, compared with that in the control group, TgESP treatment enhanced the osteogenic mineralization ability of hBMSCs. However, no obvious differences in mineralization were observed between 10 and 20 μg/ml (Fig. 2C); consequently, two concentrations, 1 and 10 μg/ml, were used in the subsequent experiments.

To elucidate the effect of TgESPs on osteogenic differentiation of hBMSCs *in vitro*, the expression levels of the osteogenesis-specific genes alkaline phosphatase (ALP), secreted phosphoprotein 1 (SPP1), RUNX2 and bone gamma-carboxyglutamate protein (BGLAP) were determined using RT-qPCR. The mRNA expression levels of ALP, SPP1, RUNX2 and BGLAP were significantly increased after seven days of TgESP treatment ($P<0.05$) (Fig. 2D). Next, the effect of TgESPs on the protein levels of Runx2, osteopontin (OPN) and osterix (OSX) was determined using western blotting and IF. The results showed that after ESP treatment, the protein expression of Runx2, OPN and OSX significantly increased in a concentration-dependent manner ($P<0.05$) (Fig. 2E-H). These findings indicate that TgESPs have a positive role in the osteogenic differentiation of hBMSCs.

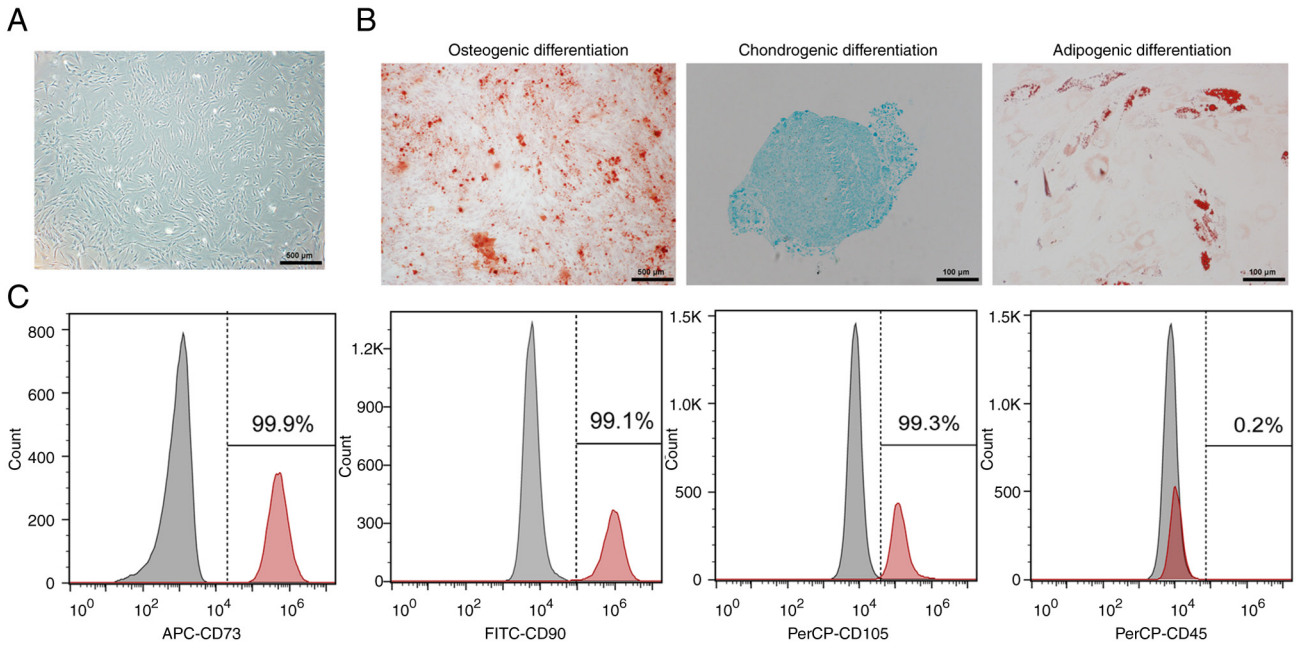

Figure 1. Identification of hBMSCs. (A) The morphology of hBMSCs was observed under a microscope (scale bar, 500 μm; magnification, x4). (B) Evaluation of the differentiation potential of hBMSCs. Osteogenic differentiation was assessed at 14 days using alizarin red S staining (scale bar, 500 μm). Chondrogenic differentiation was determined at 21 days using alizarin blue staining. Lipogenic differentiation was assessed at 14 days using Oil Red O staining (scale bars, 100 μm). (C) Flow cytometry-based detection of the surface marker antigens CD73, CD90, CD105 and CD45 in hBMSCs. TgESPs, *Toxoplasma gondii* excretory/secretory proteins; hBMSCs, human bone marrow mesenchymal stem cells.

TgESPs enhance osteogenic differentiation of hBMSCs through the β-catenin signaling pathway. The role of the Wnt/β-catenin signaling pathway in the osteogenic differentiation of BMSCs is crucial. To determine the mechanism by which TgESPs affect the osteogenic differentiation of hBMSCs, the expression of the Wnt/β-catenin signaling pathway was studied after TgESP treatment. First, the protein expression of LEF1, Dvl segment polarity protein 3 (DVL3), β-catenin and cyclin D1 was detected using western blotting. The results indicated that the protein expression of LEF1, DVL3, β-catenin and cyclin D1 was significantly increased after TgESP treatment ($P<0.05$) (Fig. 3A and B). This suggests that TgESPs may affect the Wnt/β-catenin signaling pathway during osteogenic differentiation of hBMSCs. After Icg-001 treatment, calcium deposition in hBMSCs was significantly reduced compared to that in the controls and addition of TgESPs restored the inhibitory effect of Icg-001 (Fig. 3C). In addition, the expression levels of osteogenic-specific genes and proteins, as well as β-catenin, were determined using RT-qPCR, western blotting and IF. The results suggested that the mRNA and protein expression of Runx2, OPN, OSX and β-catenin was significantly inhibited by Icg-001. The inhibition of these genes and proteins by Icg-001 was reversed due to the repressive effects of TgESP treatment (Fig. 3D-H).

TgESPs enhance glycolysis during osteogenic differentiation of hBMSCs. Previous studies suggested that the cellular OCR and mitochondrial DNA content increase during osteogenic differentiation of hBMSCs (27,28), and that TgESPs can regulate host cell metabolism, particularly the TCA cycle (22). Therefore, it was hypothesized that TgESPs may also regulate the energy metabolism of hBMSCs during osteogenic differentiation. The results indicated that the OIM group with induced osteogenic differentiation had significantly higher OCR in hBMSCs than the LG-DMEM group without induced osteogenic differentiation. Upon addition of TgESPs, the basal, maximal, ATP production and respiration rates, as well as the spare respiratory capacity, were increased. However, no significant difference in OCR was observed among the induced osteogenic differentiation groups (Fig. 4A and B). This observation suggested that TgESPs had no significant effect on the mitochondrial OXPHOS of hBMSCs. Furthermore, the glycolysis rate of hBMSCs was measured and it was observed that the ECAR increased following hBMSC induction and that basal and compensatory glycolysis were enhanced after TgESP treatment (Fig. 4C and D). Subsequently, lactic acid production in cells was detected; the results indicated that hBMSCs produced significantly more lactic acid after 72 h of TgESP treatment (Fig. 4E). Next, to assess the biochemical basis of glycolysis, the gene and protein expression of several rate-limiting enzymes involved in critical steps of glycolysis was examined. The mRNA levels of PFKFB3, PFKFB4, enolase (ENO)3 and LDHA were significantly increased at three and seven days after osteogenic differentiation induction. In addition, TgESPs significantly increased the protein expression of PFKFB3 and LDHA in a concentration-dependent manner (Fig. 4F-H).

TgESPs enhance osteogenic differentiation of hBMSCs through glycolysis. According to the above-mentioned results, TgESPs can enhance glycolysis during the osteogenic differentiation of hBMSCs. Therefore, in the present study, the effect of glycolysis on the osteogenic differentiation of hBMSCs was investigated in further detail. After treatment with 2-DG (an inhibitor of glycolysis), the ECAR and lactate production in hBMSCs were significantly inhibited; however, the ECAR

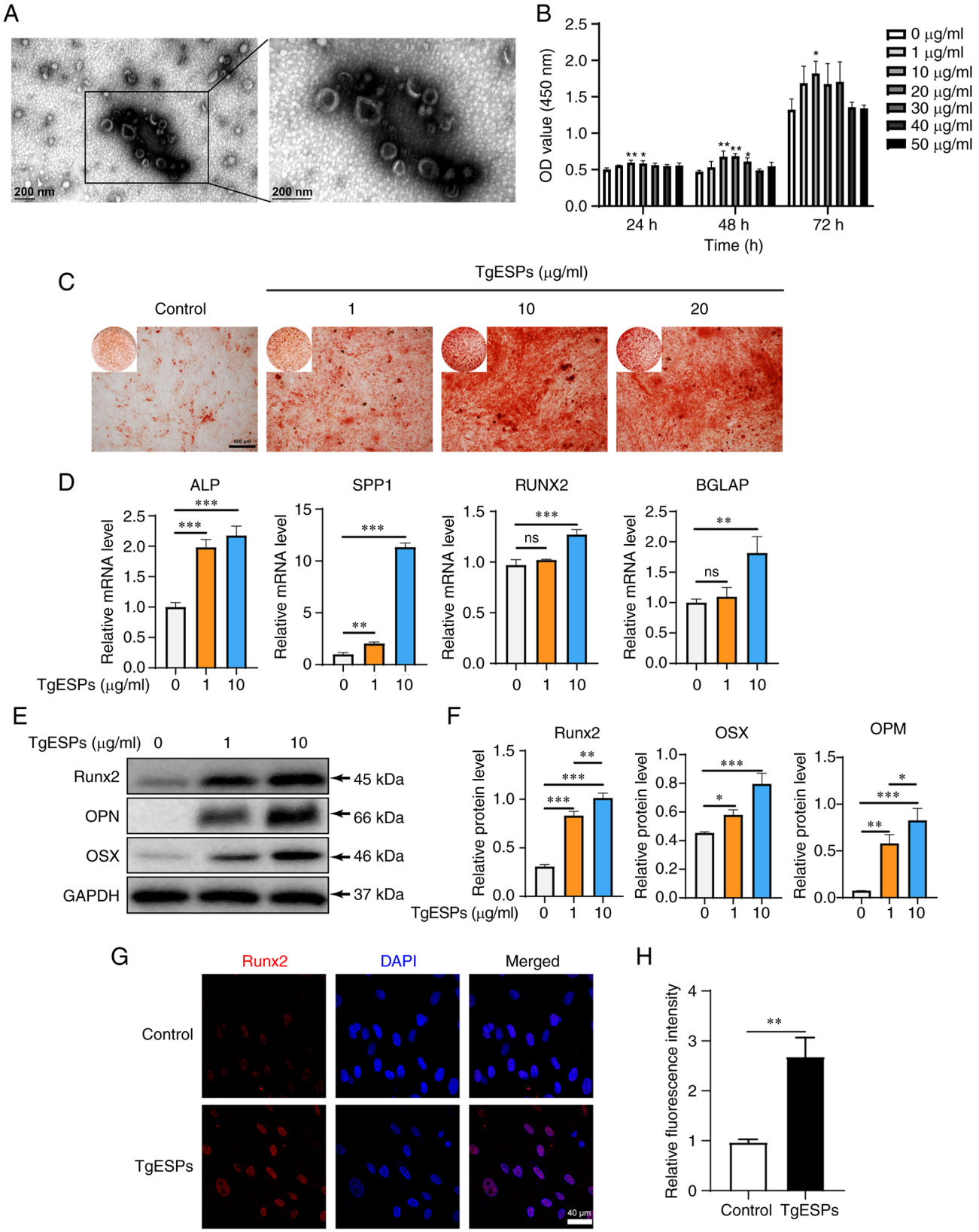

Figure 2. TgESPs promote the proliferation and osteogenic differentiation of hBMSCs. (A) TgESPs were observed by transmission electron microscopy (scale bar, 200 nm). (B) A Cell Counting Kit-8 assay was used to detect the effects of different concentrations of TgESPs (0, 1, 10, 20, 30, 40 and 50 μg/ml) on the proliferation of hBMSCs at 24, 48 and 72 h. (C) Alizarin red S staining was used to assess the TgESP-mediated osteogenic induction of hBMSCs for 14 days (scale bar, 500 μm). (D) Reverse transcription-quantitative PCR analysis of the mRNA levels of osteogenic markers in TgESP-stimulated hBMSCs after 7 days of osteogenic induction. (E) Western blot analysis of the protein levels of Runx2, OPN and OSX in TgESP-induced hBMSCs after 14 days of osteogenic induction. (F) Quantitative analysis of the expression of each protein using GAPDH as the internal control. (G) Immunofluorescence analysis of the protein levels of Runx2 after osteogenic induction for 7 days in hBMSCs treated with 10 μg/ml TgESPs or PBS (scale bar, 40 μm). (H) Quantification of immunofluorescence in G. *P<0.05; **P<0.01; ***P<0.001 vs. control group or as indicated; ns, no significance. TgESPs, *Toxoplasma gondii* excretory/secretory proteins; hBMSCs, human bone marrow mesenchymal stem cells; OD, optical density; Runx2, RUNX family transcription factor 2; OPN, osteopontin; OSX, osterix; ALP, alkaline phosphatase; SPP1, secreted phosphoprotein 1; BGLAP, bone gamma-carboxyglutamate protein.

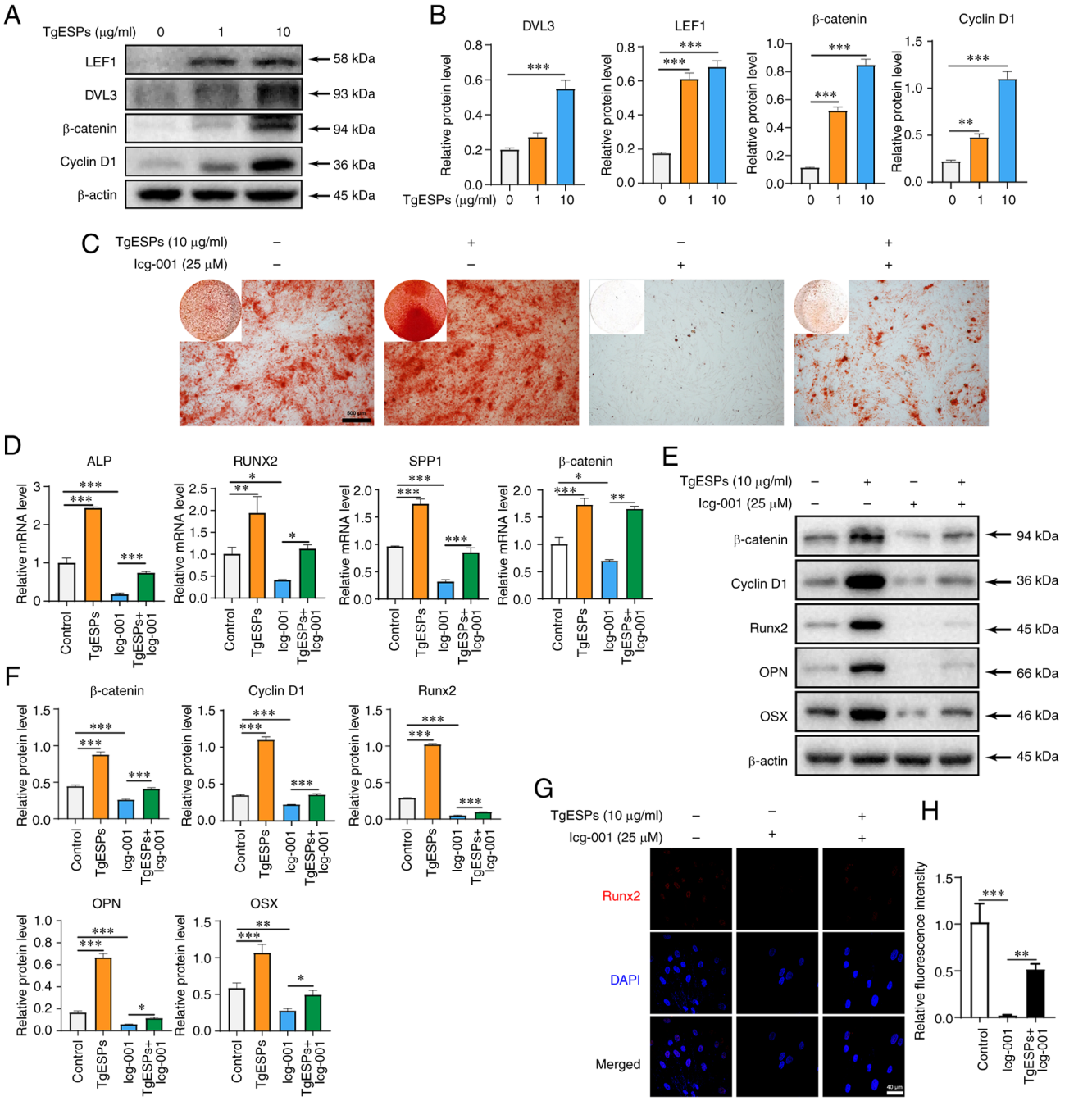

Figure 3. TgESPs positively regulate the Wnt/β-catenin signaling pathway during osteogenic differentiation of hBMSCs. (A) Western blot analysis of the levels of LEF1, DVL3, β-catenin and cyclin D1 proteins in TgESP-induced hBMSCs after 14 days of osteogenic induction. (B) Quantitative analysis of the expression of each protein. (C) Alizarin red S staining was used to assess the ability of hBMSCs cells to mineralize with TgESPs (10 μg/ml) in the presence or absence of Icg-001 (25 μM; scale bar, 500 μm). (D) Reverse transcription-quantitative PCR analysis of mRNA levels of ALP, RUNX2, SPP1 and β-catenin in hBMSCs after 7 days of osteogenic induction by TgESPs in the presence or absence of Icg-001. (E) Western blot analysis of protein levels of Runx2, OPN, OSX, β-catenin and cyclin D1 in hBMSCs after 14 days of osteogenic induction by TgESPs in the presence or absence of Icg-001. (F) Quantitative analysis of the expression of each protein using β-actin as the internal control. (G) Immunofluorescence analysis of protein levels of Runx2 in hBMSCs after 7 days of osteogenic induction by Icg-001 in the presence or absence of TgESPs (scale bar, 40 μm). (H) Quantification of immunofluorescence in G. *P<0.05; **P<0.01; ***P<0.001. TgESPs, *Toxoplasma gondii* excretory/secretory proteins; hBMSCs, human bone marrow mesenchymal stem cells; Runx2, RUNX family transcription factor 2; OPN, osteopontin; OSX, osterix; ALP, alkaline phosphatase; SPP1, secreted phosphoprotein 1; BGLAP, bone gamma-carboxyglutamate protein; LEF1, lymphoid enhancer binding factor 1; DVL3, dishevelled segment polarity protein 3.

and lactate secretion in hBMSCs were restored after simultaneous treatment with TgESPs and 2-DG (Fig. 5A and C). Quantification of the ECAR data showed that basic and compensatory glycolysis was able to reverse the inhibition effect of 2-DG after TgESP treatment (Fig. 5B). Alizarin red staining indicated that calcium deposition in the 2-DG group was much lower than that in the control group and TgESP group, but it increased again in the TgESPs+2-DG group as compared with that in the 2-DG group (Fig. 5D). Furthermore, RT-qPCR and western blotting results indicated that 2-DG inhibited the rate-limiting enzymes of glycolysis, while TgESPs reversed this inhibition (Fig. 5E, F and H). In addition, it was found that

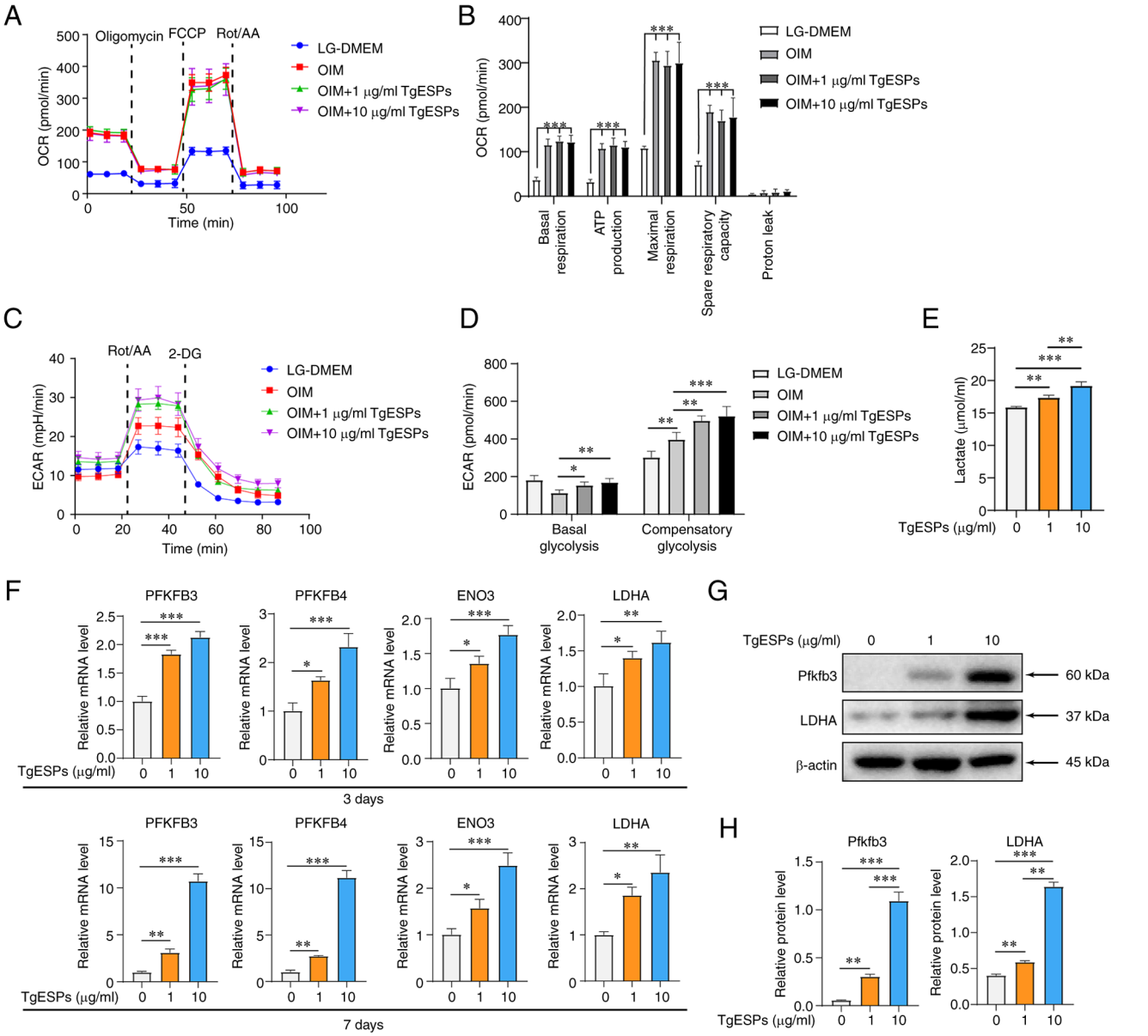

Figure 4. TgESPs enhance aerobic glycolysis during hBMSC osteogenic differentiation induction. (A) Seahorse analysis of the OCR of hBMSCs, in which osteogenic differentiation was induced with TgESPs for 7 days. (B) Basal respiration, ATP production, maximum respiratory capacity and proton leak in the OCR assay. (C) Seahorse analysis of the ECAR of hBMSCs, in which osteogenic differentiation was induced with TgESPs for 7 days. (D) Basal and compensatory glycolysis in the ECAR assay. (E) Lactate production by hBMSCs after 3 days of TgESP treatment. (F) Reverse transcription-quantitative PCR analysis of the mRNA levels of PFKFB3, PFKFB4, ENO3 and LDHA in TgESP-induced hBMSCs after 3 and 7 days of osteogenic induction. (G) Western blot analysis of the protein levels of PFKFB3 and LDHA in TgESP-induced hBMSCs after 14 days of osteogenic induction. (H) Quantitative analysis of the expression of each protein using β-actin as the internal control. *P<0.05; **P<0.01; ***P<0.001. TgESPs, *Toxoplasma gondii* excretory/secretory proteins; hBMSCs, human bone marrow mesenchymal stem cells; LDHA, lactate dehydrogenase A; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; ENO3, enolase 3; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; LG, low glucose; Rot/AA, rotenone/antimycin A; 2-DG, 2-deoxy-D-glucose; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone.

2-DG inhibited the mRNA and protein expression of Runx2, OPN and OSX, while TgESPs reversed the inhibitory effect of 2-DG on osteogenic differentiation (Fig. 5E-I). These results suggest that TgESPs can affect glycolysis in hBMSCs and enhance their osteogenic differentiation ability.

TgESPs regulate glycolysis of hBMSCs through β-catenin. The regulation of glycolysis in MSCs has been reported to be regulated by the Wnt/β-catenin signaling pathway (29). Therefore, it was hypothesized in the present study that TgESPs can affect glycolysis through the Wnt/β-catenin signaling pathway during the osteogenic differentiation of hBMSCs. Upon assessing the glycolytic rate, both basal and compensatory glycolysis were found to be significantly lower in the OIM+Icg-001 group than those in the OIM group (P<0.05). In addition, TgESP treatment was observed to reverse the inhibitory effect of Icg-001 on glycolysis (Fig. 6A and B). Furthermore, cellular lactate production showed the same trend as that described above (Fig. 6C). Next, the gene and protein expression of rate-limiting enzymes in the critical step of glycolysis were examined. The mRNA expression of PFKFB3, PFKFB4, ENO3 and LDHA was significantly reduced in the Icg-001 group (P<0.05), but

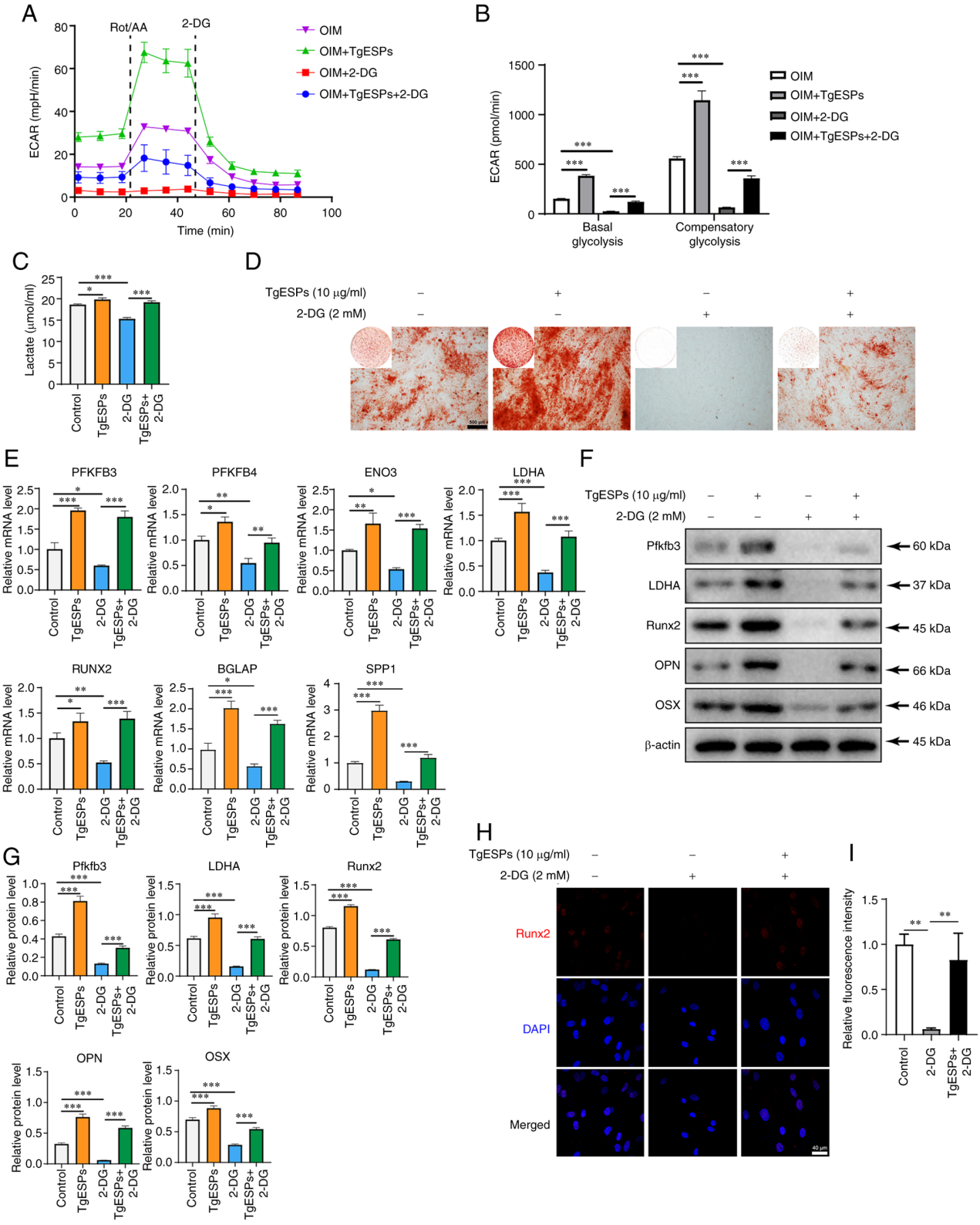

Figure 5. TgESPs promote the osteogenic differentiation of hBMSCs via aerobic glycolysis. (A) Seahorse analysis of ECAR showed that in the presence or absence of 2-DG (2 mM), the addition of TgESPs (10 μg/ml) hBMSCs induced osteogenic differentiation over 7 days. (B) Basal and compensatory glycolysis in the ECAR assay. (C) Lactate production in hBMSCs after three days of TgESP treatment in the presence or absence of 2-DG. (D) Alizarin red S staining was used to assess the ability of hBMSCs to mineralize with TgESPs in the presence or absence of 2-DG (scale bar, 500 μm). (E) Reverse transcription-quantitative PCR analysis of mRNA levels of PFKFB3, PFKFB4, ENO3, LDHA, RUNX2, BGLAP and SPP1 in hBMSCs after 7 days of osteogenic induction by TgESPs in the presence or absence of 2-DG. (F) Western blot analysis of protein levels of PFKFB3, LDHA, RUNX2, OPN and OSX in hBMSCs after 14 days of osteogenic induction by TgESPs in the presence or absence of 2-DG. (G) Quantitative analysis of the expression of each protein using β-actin as the internal control. (H) Immunofluorescence analysis of protein levels of Runx2 in hBMSCs after 7 days of osteogenic induction by 2-DG in the presence or absence of TgESPs (scale bar, 40 μm). (I) Quantitative analysis of the protein levels in H. *P<0.05; **P<0.01; ***P<0.001. TgESPs, *Toxoplasma gondii* excretory/secretory proteins; hBMSCs, human bone marrow mesenchymal stem cells; LDHA, lactate dehydrogenase A; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; Runx2, RUNX family transcription factor 2; OPN, osteopontin; OSX, osterix; ALP, alkaline phosphatase; ENO3, enolase 3; ECAR, extracellular acidification rate; Rot/AA, rotenone/antimycin A; 2-DG, 2-deoxy-D-glucose.

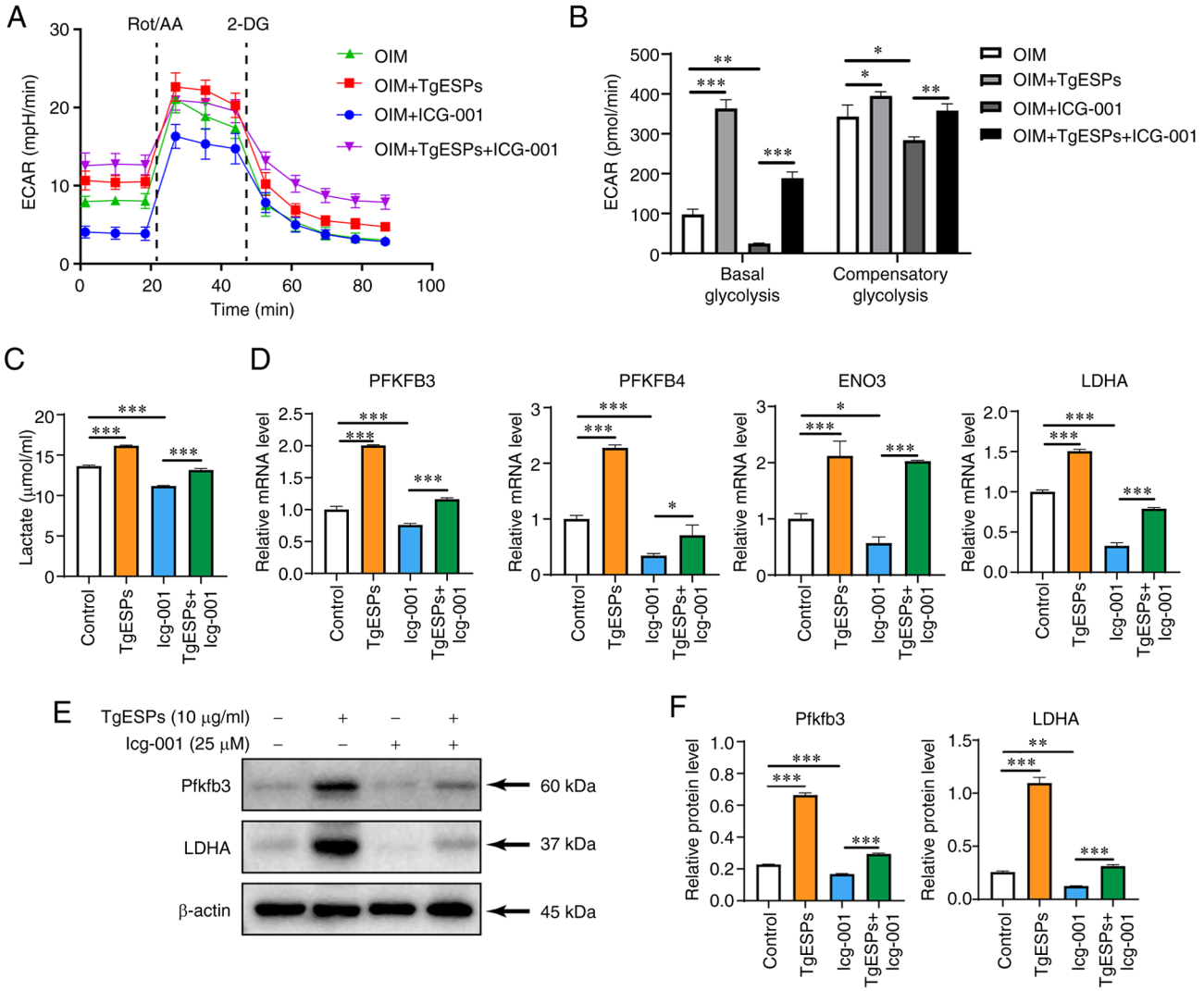

Figure 6. TgESPs regulate aerobic glycolysis through the Wnt/β-catenin signaling pathway. (A) Seahorse analysis of ECAR in the presence or absence of Icg-001 with the addition of TgESPs to hBMSCs for inducing osteogenic differentiation for 7 days. (B) Basal and compensatory glycolysis in the ECAR assay. (C) Lactate production in hBMSCs after three days of TgESP treatment in the presence or absence of Icg-001. (D) Reverse transcription-quantitative PCR analysis of mRNA levels of PFKFB3, PFKFB4, ENO3 and LDHA in hBMSCs after 7 days of osteogenic induction by TgESPs in the presence or absence of Icg-001. (E) Western blot analysis of protein levels of PFKFB3 and LDHA in hBMSCs after 14 days of osteogenic induction by TgESPs in the presence or absence of Icg-001. (F) Quantitative analysis of the expression of each protein using β-actin as the internal control. *P<0.05; **P<0.01; ***P<0.001. TgESPs, *Toxoplasma gondii* excretory/secretory proteins; hBMSCs, human bone marrow mesenchymal stem cells; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; ENO3, enolase 3; LDHA, lactate dehydrogenase A; ECAR, extracellular acidification rate; Rot/AA, rotenone/antimycin A; 2-DG, 2-deoxy-D-glucose.

addition of TgESPs increased the expression of these genes again (Fig. 6D). Furthermore, the inhibition of the PFKFB3 and LDHA proteins caused by Icg-001 was partially reversed by TgESPs (Fig. 6E and F). These results suggest that β-catenin is crucial in regulating downstream glycolysis and promoting the osteogenic differentiation of hBMSCs.

Discussion

In the present study, it was observed that TgESPs enhanced the expression of osteogenesis-specific marker genes and proteins, thereby promoting the osteogenic differentiation of hBMSCs. In addition, activation of the Wnt/β-catenin signaling pathway during osteogenic differentiation and enhanced cellular glycolysis were observed. To further validate the relationship between Wnt/β-catenin signaling and glycolysis, the inhibitory effects of β-catenin were evaluated. It was observed that Icg-001, an inhibitor of β-catenin, and 2-DG, an inhibitor of glycolysis, partially inhibited the increase in osteogenic differentiation of hBMSCs caused by TgESPs. These results suggest that TgESPs can promote osteogenic differentiation of hBMSCs through activation of the Wnt/β-catenin signaling pathway and glycolysis.

As an abundant tissue in the body, the bone has numerous different functions and has an instrumental role in human movement, hematopoiesis and the provision of calcium and phosphorus (30). However, bone regeneration is a complex and carefully orchestrated process involving various cells, including vascular endothelial cells, MSCs, inflammatory cells and fibroblasts (31). Accordingly, stem cells have a role in the healing of fractures and bone defects have a crucial role in fracture and bone defect healing. Osteoblasts are directly differentiated from BMSCs and are particularly critical during early bone formation and fracture repair. They synthesize

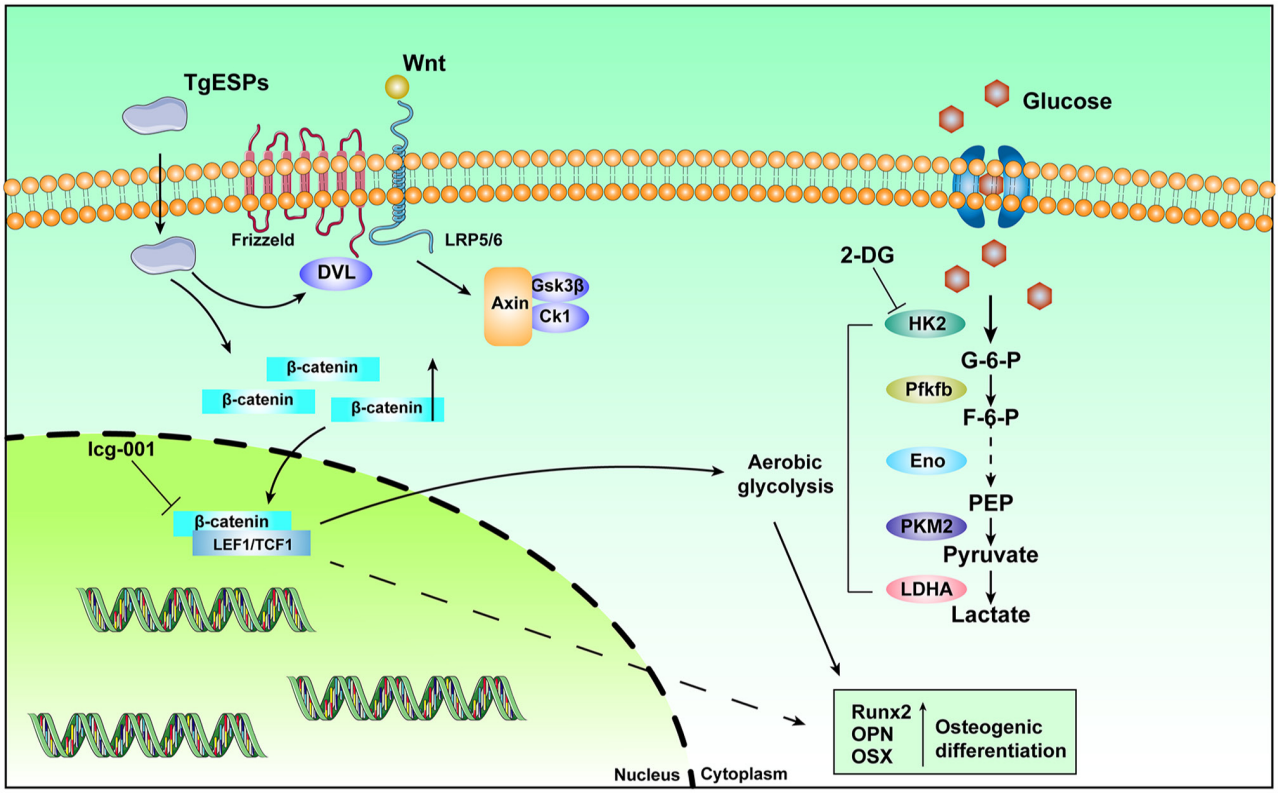

Figure 7. Illustration of a hypothetical model in which TgESPs regulate aerobic glycolysis to promote osteogenic differentiation through the Wnt/β-catenin signaling pathway. TgESPs, *Toxoplasma gondii* excretory/secretory proteins; 2-DG, 2-deoxy-D-glucose; HK2, hexokinase-2; LDHA, lactate dehydrogenase A; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PKM2, pyruvate kinase M2; G-6-P, glucose-6-phosphate; PEP, phospho-enol-pyruvate; Runx2, RUNX family transcription factor 2; OPN, osteopontin; OSX, osterix; ENO, enolase; LEF1, lymphoid enhancer binding factor 1; DVL3, dishevelled segment polarity protein 3; GSK3β, glycogen synthase kinase 3β; Ck1, casein kinase 1.

proteoglycans and type I collagen to form unmineralized osteoid tissues (32). Therefore, an increasing number of researchers are working on methods to differentiate BMSCs into osteoblasts to promote bone regeneration (33).

In translational medicine, BMSCs are widely used as promising seed cells for accelerating bone healing. According to a report, exosome transplantation from BMSCs promotes angiogenesis and bone healing in rats with femoral nonunion (34). Therefore, biologically active molecules that can provide an optimal osteogenic microenvironment for BMSCs are expected to accelerate bone formation in future clinical applications (35). In the present study, it was observed that TgESPs enhanced cell differentiation toward osteoblasts while promoting the proliferation of BMSCs. This observation indicated their potential use in bone tissue engineering for the treatment of bone defects.

Previous studies have identified TgESPs as a complex group of proteins secreted by *T. gondii* and antibodies against these secreted antigens have been used as diagnostic markers for toxoplasmosis infection (36). However, recent research has expanded the focus to investigate the components of TgESPs and their effects on cellular functions (23). Studies have revealed that TgESPs can influence the dissemination of *T. gondii* within tissues (37,38). The present study also investigated the effects of TgESPs on cellular behavior of hBMSCs, including cell adhesion and migration. Our results revealed that low concentrations of TgESPs promoted cell adhesion, and both 1 and 10 μg/ml concentrations enhanced cell migration in hBMSCs. These findings suggest that TgESPs may regulate cell-to-cell interactions and promote cell migration in BMSCs.

MSCs require a balance between bone formation and resorption to maintain bone homeostasis, and the integrity of energy metabolism is crucial in this process. According to most studies, stem cells rely mainly on glycolysis for metabolism (39). This may be because glycolysis provides MSCs with the major cofactors and substrates required for their biosynthesis and proliferation (40). Furthermore, MSCs are thought to be dependent on anaerobic energy metabolism, which may be explained by the long-term evolutionary adaptation of stem cells to their anoxic niche (41). However, during BMSC differentiation, there is a high demand for energy sources. Therefore, the energy acquisition pathway of BMSCs shifts from glycolysis to oxidative mitochondrial metabolism, generating large amounts of energy through OXPHOS in mitochondria to meet differentiation requirements (27). By contrast, the mode of energy metabolism in osteoblasts is dominated by aerobic glycolysis, wherein the overexpression of hypoxia-inducible factor-1α activates glycolysis, leading to increased osteoblast and bone formation in mice (42). Early osteogenic differentiation requires the synthesis of collagen to upregulate OXPHOS. In addition, with the increase in OXPHOS, reactive oxygen species levels tend to increase, which may shift cellular metabolism to glycolysis (13,43). Thus, both mitochondrial oxidative metabolism and glycolysis, which are both modes of energy metabolism, are essential for osteogenic differentiation. In the present study, it was found

that mitochondrial oxidative metabolism was upregulated and basal glycolysis was downregulated in BMSCs during early osteogenic differentiation. This result is consistent with previous findings (44). Furthermore, both basal and compensatory glycolysis were upregulated in BMSCs after TgESP treatment, whereas the mitochondrial oxidative metabolism remained unchanged. Subsequently, the glycolysis of BMSCs was inhibited, which resulted in a decrease in glycolysis in conjunction with a decrease in osteogenic differentiation. However, addition of TgESPs restored the glycolytic and osteogenic capacities of BMSCs. This observation indicates that TgESPs can regulate glycolysis in BMSCs and promote their osteogenic differentiation through glycolysis.

In addition, the signaling pathways that mediate the regulation of glycolysis by TgESPs were investigated. Previously, most developmental signaling pathways were shown to regulate the osteogenic differentiation of MSCs, but their effects on cellular energy metabolism need to be further investigated. The Wnt/β-catenin signaling pathway is critical for differentiation and development and has an instrumental role in bone homeostasis (45). A recent study indicated that activation of the Wnt/β-catenin signaling pathway can restore osteoblasts from a state of impaired glycolysis and osteogenic differentiation (46). Furthermore, the Wnt/β-catenin signaling pathway can regulate cellular glycolytic metabolism during osteogenic differentiation and elevate the levels of essential glycolytic enzymes to induce aerobic glycolysis, further enhancing bone formation *in vivo* (29). It was also noted that the levels of β-catenin expression were markedly increased during osteogenic differentiation in the TgESP group compared to the control group. In addition, TgESPs were able to partially reverse the inhibition of β-catenin after blocking β-catenin expression with Icg-001 and a similar phenomenon was observed for glycolysis in BMSCs. The present findings indicate that TgESPs are capable of elevating glycolysis via the Wnt/β-catenin signaling pathway, which subsequently stimulates the osteogenic differentiation of BMSCs. In the present investigation, it was noted that the increase in osteogenic differentiation in BMSCs was only partially suppressed by β-catenin inhibition, implying that TgESPs may also exert their effects through other mechanisms. To date, the role of TgESPs in the osteogenic differentiation of MSCs has remained elusive. As such, future research should aim to uncover the ways in which TgESPs influence the osteogenic differentiation process through additional signaling pathways.

There are certain limitations to the present study. First, the results were not validated *in vivo* due to the fact that cell proliferation and differentiation may vary under different cultural conditions, leading to potentially different results. Furthermore, there are three main ways of cellular energy metabolism, namely glucose, fatty acid and amino acid metabolism, although in the present study, only changes in glucose metabolism were detected, while the potential mechanisms by which TgESPs regulate fatty acid and amino acid metabolism were not explored further. Finally, although the composition of TgESPs has been determined (23), the specific proteins responsible for the effect were not clearly identified in the present study. Therefore, further studies need to clarify which specific components of TgESPs regulate BMSCs.

In conclusion, the present study indicated that TgESPs enhance the osteogenic differentiation of hBMSCs. They increase the expression of glycolytic enzymes and lactate production under adequate oxygen conditions, leading to an increase in aerobic glycolysis (Fig. 7). Furthermore, TgESPs regulate this process by modulating the Wnt/β-catenin signaling pathway, thus stimulating osteogenic differentiation.

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Availability of data and materials

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

Authors' contributions

JC and JQ designed the research. ZC and TL collected the bone marrow samples. WZ, MD, ZZ, XW, ZZ and FG performed the experiments and analyzed the data. JC, JQ and WZ wrote and revised the manuscript. WZ and JC checked and confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The hBMSCs were all derived from the bone marrow of patients with traumatic femur fractures who had provided written informed consent. The protocol was approved by the Ethics Committee of the Affiliated Hospital of Guangdong Medical University (Zhanjiang, China).

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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