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Original article

Telomerase enhances osteogenic ifferentiation of sheep bone marrow mesenchymal stem cells (BMSCs) by up-regulating PI3K/Akt pathway *in vitro*

X. Zhu[1], L. Zhou[1], Z. Liu[2], X. Chen[1], L. Wei[1], Z. Zhang[1], Y. Liu[1], Y. Zhu[1], Y. Wang[1], X. Yang[1], Y. Han[1]

[1] College of Animal Science and Technology,
Henan University of Science and Technology, Luoyang,471023, China
[2] Animal Husbandry and Veterinary Research Institute of Qingdao, 266000,Qingdao, China

Abstract

Telomerase reverse transcriptase (TERT) vectors were transfected into bone marrow mesenchymal stem cells (BMSCs) which were then cultured and selected to establish TERT-BMSC cell lines whilst sequencing BMSCs and TERT-BMSCs via transcriptome in this study to explore their regulatory mechanism and effect on osteogenic differentiation after TERT ectopic expression in sheep BMSCs. After sequencing and analysing differential genes, PI3K/Akt signalling pathway related to osteogenic differentiation was investigated. Western blot was used before and after applying the PI3K/Akt signalling pathway inhibitor LY294002 to detect protein expression levels of AKT and p-AKT. On the twenty-first day of osteogenic differentiation, RT-qPCR and Western blot were used to detect mRNA and protein expression levels of RUNX2 and OPN and alizarin red staining was utilised to analyse calcium salt deposition. Results showed that protein expression levels of AKT and p-AKT were significantly up-regulated, mRNA and protein expression levels of RUNX2 and OPN increased and calcium salt deposition increased after ectopic expression of TERT. After applying LY294002, the protein expression of AKT and p-AKT was down-regulated, mRNA and protein expression levels of RUNX2 and OPN were reduced and calcium salt deposition was reduced. These results confirmed the stable integration and expression of the exogenous TERT gene in BMSCs to promote the differentiation of BMSC osteoblasts, which may be mediated by the PI3K/Akt signalling pathway.

Key words: bone marrow mesenchymal stem cells, telomerase, proliferation, osteogenic differentiation, PI3K/Akt signalling pathway

Correspondence to: X. Zhu, e-mail: zhuxuemin7195@126.com
X. Zhu, L. Zhou contributed equally to this work

Introduction

Bone marrow mesenchymal stem cells (BMSCs) are stem cells with multi-directional differentiation potential isolated from bone marrow. Under the action of specific cytokines or physical and chemical factors, they can differentiate into diverse histiocytes, such as osteoblasts, chondroblasts, adipocytes and nerve cells (Czernik et al. 2013, Su et al. 2015). Clinical studies show that autotransplantation or allogeneic transplantation of BMSCs induces no immunological rejection, so they have been used as seed cells in bone tissue engineering and have become a research hotspot. Sheep has become one of the experimental animals commonly used in veterinary clinic due to their moderate size, relatively low feeding cost and economic value (Boos et al. 2014, Cuenca-López et al. 2014, Song et al. 2014, Kira et al. 2017). In this research, sheep were used as experimental animals. Sheep BMSCs were isolated from sheep bone marrow. During the passage process, it was found that there were obvious aging and differentiation phenomena when they were passed to the 7th and 8th generation, and lost the potential for multidirectional differentiation. Telomere is closely related to the control of cell life. The increasing number of cell divisions progressively shortens telomere. When telomere is shortened to a certain degree, the stability of the chromosome changes and the cell's loss of ability to divide leads to senescence and death (Meyerson et al. 1997). The maintenance of cell telomere length requires the activation of telomerase. The activity of telomerase is primarily determined by the expression level of telomerase reverse transcriptase (TERT), which provides a new idea for cell immortalisation (Daniel et al. 2012, Wang et al. 2019). Cell immortalisation involves the transfer of exogenous TERT gene to the target cell and induction of telomerase activity of the cell. Several studies have transferred the human TERT (hTERT) gene to human retinal epithelial cells and developed the first normal and immortalised cell line, which has been passed on for more than 150 generations (Hong et al. 2002). Abdallah et al. (Abdallah et al. 2005) enhanced the human mesenchymal stem cells through the ectopic expression of TERT and found that the spontaneous differentiation level of mesenchymal stem cells ectopically expressing TERT decreased (Tsai et al. 2010). Tang et al. (Tang et al. 2013) transfected the TERT gene into aging bone marrow mesenchymal stem cells (BMSCs) through lentiviral vectors and found that the proliferation and differentiation of transfected BMSCs are significantly enhanced after long-term cultivation. Therefore, ectopic expression of TERT (which is similar to increasing the telomerase activity) can produce somatic stem cells with strong differentiation ability and has potential application in clinical treatment. Our research group successfully transferred TERT into sheep BMSCs to obtain TERT-BMSC cell line. The experiment showed that the cell lines obtained by transferring TERT into sheep BMSCs to obtain TERT-BMSCs maintain the multidirectional differentiation potential (Zhu et al. 2017). Telomerase deficiency causes age-related bone formation damage in vivo (Wang et al. 2012). However, BMSCs of telomerase-deficient mice significantly reduce the differentiation ability of osteoblasts during in vitro culturing (Saeed et al. 2015). Overexpression of the hTERT gene in human BMSCs at the cellular level enhances telomerase activity and leads to the strengthened ability of osteoblast differentiation in vitro and ectopic bone formation in vivo (Li et al. 2015, Siqi et al. 2018), which provided ideas for the research. Then, what in vitro effect does TERT ectopically expressed in sheep BMSCs show on the osteogenic differentiation? What is its regulation mechanism? These are main problems explored in this study. Transcriptome sequencing, real-time fluorescence quantitative PCR and Western blot were used to investigate the biological characteristics and osteogenic differentiation under TERT ectopic expression in sheep BMSCs and explore the internal mechanism of osteogenic differentiation in detail. The outcome not only provides a rich and reliable cell source for veterinary clinical bone injury and bone defect treatment, but also provides a theoretical basis for the molecular mechanism of TERT ectopic expression of BMSCs in animals to treat bone diseases.

Materials and Methods

Cell culture

Sheep primary BMSCs were provided by the Animal Cell and Molecular Team from the College of Animal Science and Technology of Henan University of Science and Technology in Luoyang, China. Dulbecco's Modified Eagle Medium/F12 (DMEM/F12; GIBCO; NY, USA) supplemented with 10% foetal bovine serum (FBS; Solarbio; Beijing, China) and 100 U/ml of penicillin/streptomycin (Pen/strep, GIBCO) was used as culture medium of BMSCs, which was replaced every 3 days, and incubated at 37°C in 5% CO_2 atmosphere.

Construction and identification of stable transfected TERT-BMSCs

TERT construct was generated according to the sequence published in GenBank (serial number: XM_027979954). Passage 2 of BMSCs was plated

in six-well plates, and 100 µl of lentivirus solution was added to each well. The medium was replaced after culturing in an incubator at 37°C for 24 h. After 48 h of infection, fluorescence of green fluorescent protein (GFP) expression was detected using fresh medium containing the optimal concentration of puromycin, which was added every 2-3 days. Cells were screened until resistant colonies were identified. Resistant cells were subcultured and their TERT expression was identified after overgrowing. Stably transfected cells were trypsinised to a single cell suspension, and then GFP was detected by flow cytometry.

Cell proliferation assay

Population doubling level (PDL), which refers to the number of doublings of the cell population from the start of culture to the present, was used to analyse the proliferation efficiency of BMSCs and TERT-BMSCs. The third passage of BMSCs and TERT-BMSCs was inoculated in a 96-well plate and cells were passaged at 80% confluency and subsequently cultured with a seeding density of 5×10^5 cells/ml in the PDL evaluation. The following formula was used to calculate the PDL level: $\log (N_H/N_0)/\log_2$, where N_0 refers to the number of cells that began to proliferate and N_H denotes the cumulative number of cells in the present.

Soft agar colony formation assay

Colony formation assay in soft agar was used to detect tumorigenicity of TERT-BMSCs *in vitro*. DMEM (3 ml) containing 0.9% agarose was added to the bottom of each well of a six-well plate, and the plate was placed in the incubator to solidify overnight. Logarithmically grown cells were digested and counted after 30 generations. Low-melting point agar (0.36%) containing 10% FBS-DMEM was prepared and mixed with 2×10^4 cells suspended in 2 ml of DMEM. Six-well plates were incubated overnight, and 2ml of DMEM containing 10% FBS medium was added to each well. The number of cloned cells was counted under an inverted microscope after 4-6 weeks, and C6 glioma cells were used as positive control. The C6 glioma cell line was obtained from Nanjing Kebai Biotechnology Co. Ltd.

Transcriptome sequencing

RNA was isolated from the third passage of BMSCs and TERT-BMSCs using RNAiso Plus reagent (Takara) for the sequencing and library preparation. RNA concentrations were determined using NanoDrop 2000 (Thermo Fisher; MA, USA). RNA integrity was detected using the RNA Nano 6000 Assay Kit of Agilent Bioanalyser 2100 system (Agilent Technologies; CA, USA). RNA (1 µg) per sample was utilised in the sample preparation. Sequencing libraries were established using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA) by following the manufacturer's instructions. Briefly, procedures used for establishing sequencing libraries include mRNA purification, cDNA synthesis, conversion of overhangs into blunt ends, size selection of adaptor-ligated cDNA, PCR and product purification. Sequencing was performed on an Illumina HiSeq Xten platform. For data analysis, 28,407,908 and 28,828,036 reads were synthesised from BMSC and TERT-BMSC cells, respectively. After performing the sequencing quality control, 17.13 Gb of clean data were obtained. The percentage of Q30 bases in each sample was at least 90.24%. The alignment of clean reads to reference genomes using TopHat showed that the efficiency of alignment between reads and reference genomes of each sample is between 75.32% and 75.73%. Gene expression levels were measured using fragments per kilobase of transcript per million fragments mapped (FPKM) (Patthy et al. 2019). Transcriptome data have high sensitivity in detecting gene expression and can detect FPKM values in the range of 10^{-2} to 10^4. Differentially expressed gene (DEG) analysis of the two groups was performed using DESeq (Ricci et al. 2016) by following the criteria of fold change (FC) ≥ 2 and false discovery rate (FDR) < 0.01. FDR is calibrated with a differentially significant p-value. Gene function analysis was performed using National Centre for Biotechnology Information (NCBI) nonredundant protein sequences (Nr), NCBI nonredundant nucleotide sequences (Nt), clusters of orthologous groups of proteins (COG), KEGG ortholog database (KEGG) and gene ontology (GO). Sequencing analyses were completed by Beijing Biomarker Technologies Co., Ltd. (Beijing, China).

PI3K/Akt signalling inhibition

LY294002 (Beyotime; Shanghai, China) is a PI3K inhibitor dissolved with DMSO. We seeded the passage 3 of BMSCs and TERT-BMSCs in six-well culture plates at a density of 6.0×10^3 cells/cm^2. Cells were pretreated with 10 µM of LY294002 for 1 h after the cells grew to 60% confluency and the control group was treated with an equivalent volume of DMSO.

Induction of osteogenic differentiation and alizarin red assay

After pretreatment of BMSCs and TERT-BMSCs with LY294002 and DMSO, the medium was replaced with an osteogenic induction culture medium (DMEM/

Table 5. PI3K/AKT signaling pathway related gene expression of BMSCs and TERT-BMSCs.

Genes	Definition	Reg	Log2 FC	FDR
FGF	fibroblast growth factor 10 precursor	up	4.57718628	0
VEGFC-D	vascular endothelial growth factor C, partial	up	1.214119149	4.66E^{-05}
NGFβ	beta-nerve growth factor isoform X2	up	2.667453434	0
EPHA2	ephrin type-A receptor 2	up	1.048316342	0.001646148
IL6	interleukin-6 precursor	up	1.527884787	1.60E^{-07}
IL6R	interleukin-6 receptor subunit alpha	up	1.426508445	0.002384737
COL1A	collagen alpha-2(XI) chain isoform X1	up	1.525846433	1.95E^{-04}
LAMC2	laminin subunit gamma-2 precursor	up	3.666543225	0
ITGA6	Integrin alpha-6	up	4.438056376	0
Gβγ	guanine nucleotide-binding protein G(I/S/O) subunit gamma	up	1.424990229	3.10E^{-07}
PI3K	phosphoinositide 3-kinase regulatory subunit 5	up	2.940205844	5.12E^{-06}
BCL2L1	bcl-2-like protein 1	up	1.31302329	8.80E^{-06}
BCL2	apoptosis regulator Bcl-2	up	1.654166692	5.22E^{-07}
PPP2R5	serine/threonine-protein phosphatase 2A regulatory subunit alpha serine/threonine-protein phosphatase 2A regulatory subunit B beta	down	-1.091152966	0.000605645
PPP2R2	fibroblast growth factor 10 precursor	down	-1.445903753	2.38E^{-05}

Abbreviations: Reg, regulate; FC, Fold Change; FDR, false discovery rate.

F12 + 10% FBS + 100 U/ml of Pen/strep + 10 nM of dexamethasone [Solarbio] + 50 mg/L of ascorbic acid [Solarbio] + 10 mM of β-glycerophosphate [Solarbio]). The induction medium was changed every 3 days for 21 days. Cells were stained with alizarin red (Solarbio) to illustrate calcified nodules after culturing for 21 days.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Primers were designed for RT-qPCR using Primer 5.0 software to refer to RNA sequences from the NCBI database (Table 1) and then synthesised by Shanghai Biotechnology Bioengineering Co., Ltd. The total RNA was reversely transcribed using a reverse transcription kit (Takara), followed by PCR on the Bio-Rad CFX96 thermocycler (Bio-Rad; CA, USA). The following PCR condition was applied: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The PCR sample composition includes 10 µl of SYBR green mix (Takara), 0.8 µl of forward primer, 0.8 µl of reverse primer, 0.4 µl of ROX reference dye or dye II (Takara), 2 µl of DNA template and ddH_2O up to 20 µl. Expression data were normalised to β-actin. The melting curve was used to evaluate the reliability of PCR results.

Western blot

The total cellular protein was by lysing and then heating at 95°C for 5 min. Protein (30 µg) was electrophoresed in 10% polyacrylamide gel at 120 V for 120 min and blotted onto polyvinylidene difluoride membranes for 90 min at 120 mA. Membranes were then blocked for 3 h with 5% nonfat dried milk in TBST (10 mM of Tris–HCl, pH 7.5, 150 mM of NaCl and 0.1% Tween 20). Membranes were subsequently incubated with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-labelled secondary antibodies (Bioss; Beijing, China) at room temperature for 1 h. Blots were then developed using ECL reagents. Primary antibodies, including anti-beta-actin (β-actin), anti-TERT, anti-AKT, anti-phospho-AKT (p-AKT), anti-osteopontin (OPN) and anti-RUNX2 polyclonal antibodies, were purchased from Bioss (Beijing, China). The results were normalised to the β-actin level.

Statistical analysis

Values were expressed as mean ± standard error (SD or SEM). Statistical analysis was performed via Student *t*-test by using SPSS 17.0 software (IBM; Chicago, IL). Differences were considered significant at $p<0.05$ and extremely significant at $p<0.01$.

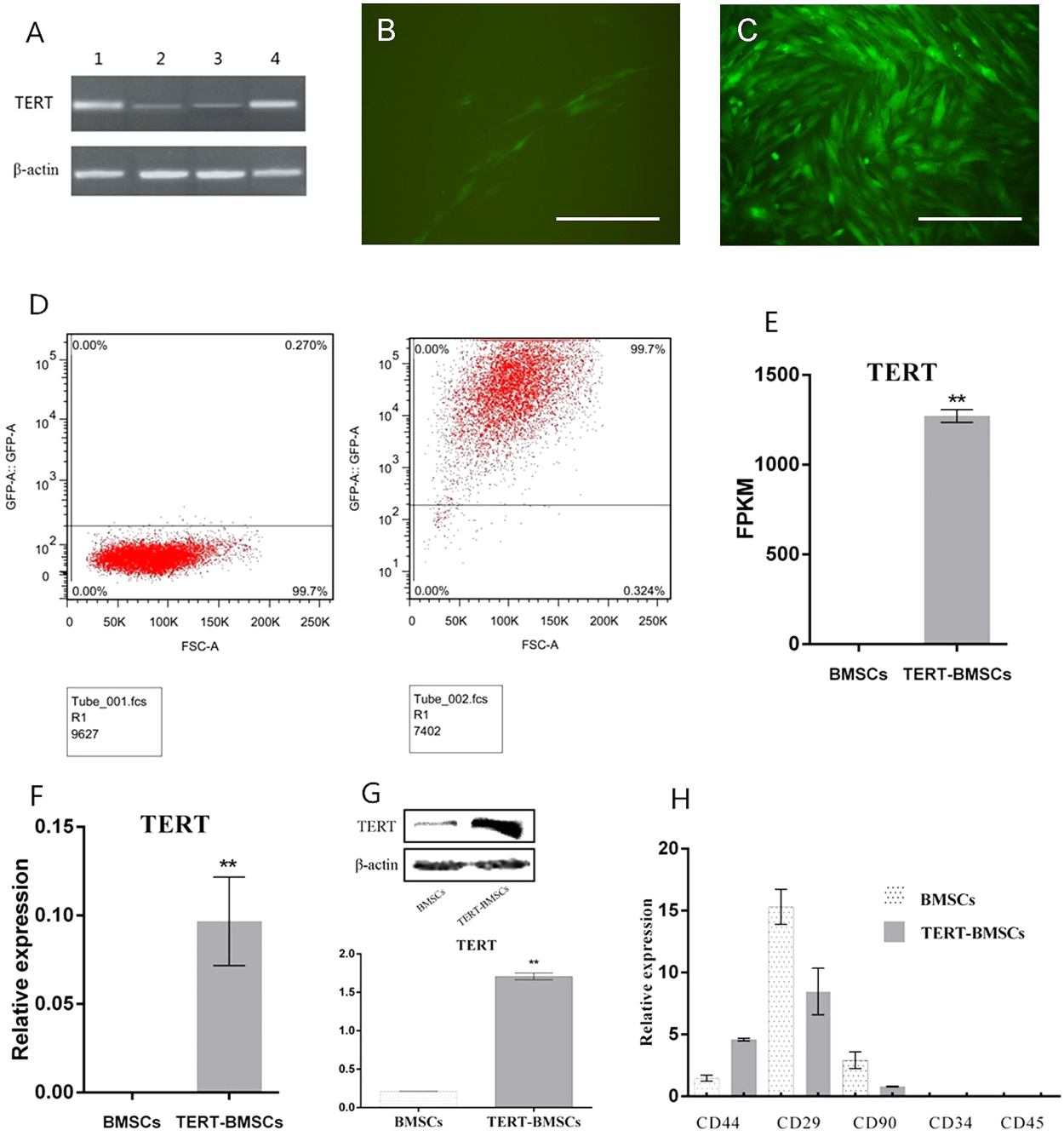

Fig 1. Construction and identification of TERT-BMSC stable cell lines. (A) TERT and β-actin mRNA expression by RT-PCR analysis. (B) Green fluorescence 48 h after lentivirus infection in BMSCs. (C) TERT-BMSC stable cell lines after the puromycin selection. (D) GFP positive rate. (E) TERT expression levels were detected by transcriptomic sequencing and qPCR (F). (G) TERT protein expression in TERT-BMSCs by western blot. (H) CD markers expression levels were detected by qPCR. Relevant gene expression levels were calculated using the $2^{-\Delta Ct}$ method. Abbreviations: FPKM, fragments per kilobase of transcript per million fragments mapped. Scale bars = 200 μm. * $p<0.05$, ** $p<0.01$ between TERT- BMSCs vs. BMSCs.

Results

Construction and identification of TERT-BMSC stable cell lines

RT-PCR results showed that positive clones exhibit a 203 bp band, which was consistent with TERT (Fig. 1A). GFP expression can be observed after infection at 48 h (Fig. 1B). TERT-BMSC stable cell lines were obtained through the puromycin selection (Fig. 1C). Flow cytometry was performed to identify the ratio of positive cells. The results showed that the expression of GFP in TERT-BMSCs and BMSCs is 99.7% and 0.270%, respectively (Fig. 1D). The TERT gene expression in TERT-BMSCs was significantly up-regulated compared with BMSCs via transcriptomic

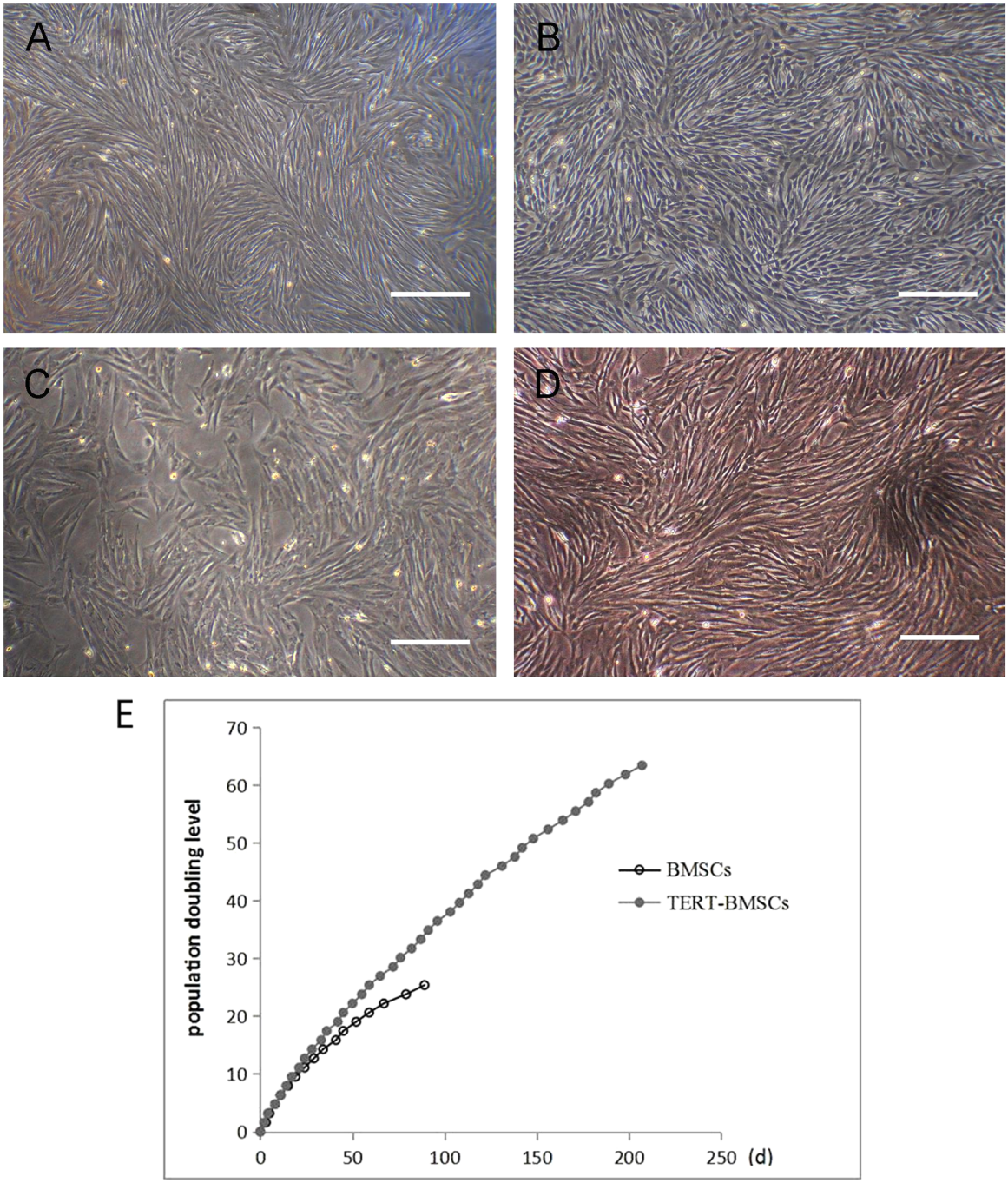

Fig 2. Comparison of morphology and proliferation between BMSCs and TERT-BMSCs. P3 (A) and P7 (C) of BMSCs; P3 (B) and P30 (D) of TERT-BMSCs. PDL of BMSCs and TERT-BMSCs (E). Abbreviations: d, day; P, passage. Scale bars = 200 μm.

sequencing (Fig. 1E) and RT-qPCR (Fig. 1F). Western blot analysis showed that TERT protein expression in TERT-BMSCs significantly increases (Fig. 1G). Soft agar colony formation assay showed the evident colony formation in C6 glioma positive control cells and absence of colony formation in BMSCs or TERT--BMSCs. This finding indicates the absence of tumorigenic capacity in TERT-BMSCs.

TERT construct was successfully transferred into sheep BMSCs. This study aims to determine whether BMSCs can maintain their original phenotype after the ectopic expression of TERT. The transcriptome sequencing results showed the increased expression of cell surface markers CD44, CD29, CD90/THY1, CD105/ENG and CD73 and decreased expression of cell surface markers CD34, CD45, CD11b, CD19 and HLA class II in BMSCs and TERT-BMSCs (Table 2). The RT-qPCR results showed that the increased expression of CD44, CD29 and CD90 and decreased expression of CD34 and CD45 in the two

Table 2. CD markers gene expression of BMSCs and TERT-BMSCs.

CD markers gene	Reg	Log2 FC	FDR
CD44	--	0.77598242	0.198792844
CD29	up	1.454517305	1.49E^{-06}
CD90	up	1.456087593	3.84E^{-07}
CD105	up	2.221594564	0
CD73	--	-0.13778012	0.996784435
CD166	--	-0.971014179	0.017408154
CD11b	--	-0.753706432	0.24035969
CD19	--	-0.808269491	0.07433533
CD14	up	4.003132946	0
CD34	--	--	--
CD45	--	0.93414879	0.630591571
HLA class II	down	-3.988123553	3.04E^{-13}

Abbreviations: Reg, regulate; FC, Fold Change; FDR, false discovery rate.

Table 3. Cell cycle related gene expression of BMSCs and TERT-BMSCs.

Genes	Definition	Reg	Log2 FC	FDR
CCNA1	cyclin-A1	--	0.8324	0
CCNA2	cyclin-A2	up	1.609176493	6.25E^{-07}
CCNB1	G2/mitotic-specific cyclin-B1	up	2.258053644	2.00E^{-14}
CCND1	G1/S-specific cyclin-D1	up	3.177745454	0
CCND2	G1/S-specific cyclin-D2	up	2.247713432	0
CCND3	G1/S-specific cyclin-D3 isoform X1	--	0.243134244	0.96886509
CCNE1	G1/S-specific cyclin-E1	up	1.665944103	6.98E^{-06}
CCNE2	G1/S-specific cyclin-E2	up	1.680171074	0.004950397
CDK1	cell division control protein 2 homolog	up	2.30509183	1.05E^{-08}
CDK2	cyclin-dependent kinase 2	--	-0.390968134	0.851826093
CDK4	cyclin-dependent kinase 4	--	-0.086185298	0.974388078
CDK6	cyclin-dependent kinase 6 isoform X1	up	1.133524195	0.000259403
RBL1	retinoblastoma-like protein 1	up	1.130783788	0.002481964
E2F1	transcription factor E2F1	up	1.955239141	1.01E^{-08}
E2F2	transcription factor E2F2	up	3.397395685	0.000709749
CDC25B	M-phase inducer phosphatase 2 isoform 1	up	1.131447614	0.000358508
CDC25C	M-phase inducer phosphatase 3 isoform 1	up	2.496740585	2.02E^{-06}
CDKN1A	cyclin-dependent kinase inhibitor 1/p21	up	2.750485876	0
CDKN2A	cyclin-dependent kinase inhibitor 2A isoform X1/p16	up	3.036987535	0
CDKN1B	cyclin-dependent kinase inhibitor 1B/p27	down	-1.80148724	2.60E^{-11}

Abbreviations: Reg, regulate; FC, Fold Change; FDR, false discovery rate.

Table 4. Osteogenic-related gene expression of BMSCs and TERT-BMSCs.

Genes	Definition	Reg	Log2 FC	FDR
BMP2	bone morphogenetic protein 4 precursor	--	-0.296233893	0.901803514
Smad4	mothers against decapentaplegic homolog 4	--	-0.944130575	0.008848362
OPN	osteopontin precursor	up	2.823623312	0
RUNX2	runt-related transcription factor 2 isoform X3	down	-1.701291308	$3.99E^{-10}$
SP	LOW QUALITY PROTEIN: transcription factor Sp7	up	1.59521019	0.000120472
OCN	BGLAP protein	up	4.878669736	0
Osteonectin	SPARC-like protein 1	up	2.060638353	0.017728203
COL1A	collagen alpha-2(XI) chain isoform X1	up	1.525846433	$1.95E^{-04}$

Abbreviations: Reg, regulate; FC, Fold Change; FDR, false discovery rate.

Table 5. PI3K/AKT signaling pathway related gene expression of BMSCs and TERT-BMSCs.

Genes	Definition	Reg	Log2 FC	FDR
FGF	fibroblast growth factor 10 precursor	up	4.57718628	0
VEGFC-D	vascular endothelial growth factor C, partial	up	1.214119149	$4.66E^{-05}$
NGFβ	beta-nerve growth factor isoform X2	up	2.667453434	0
EPHA2	ephrin type-A receptor 2	up	1.048316342	0.001646148
IL6	interleukin-6 precursor	up	1.527884787	$1.60E^{-07}$
IL6R	interleukin-6 receptor subunit alpha	up	1.426508445	0.002384737
COL1A	collagen alpha-2(XI) chain isoform X1	up	1.525846433	$1.95E^{-04}$
LAMC2	laminin subunit gamma-2 precursor	up	3.666543225	0
ITGA6	Integrin alpha-6	up	4.438056376	0
Gβγ	guanine nucleotide-binding protein G(I/S/O) subunit gamma	up	1.424990229	$3.10E^{-07}$
PI3K	phosphoinositide 3-kinase regulatory subunit 5	up	2.940205844	$5.12E^{-06}$
BCL2L1	bcl-2-like protein 1	up	1.31302329	$8.80E^{-06}$
BCL2	apoptosis regulator Bcl-2	up	1.654166692	$5.22E^{-07}$
PPP2R5	serine/threonine-protein phosphatase 2A regulatory subunit alpha serine/threonine-protein phosphatase 2A regulatory subunit B beta	down	-1.091152966	0.000605645
PPP2R2	fibroblast growth factor 10 precursor	down	-1.445903753	$2.38E^{-05}$

Abbreviations: Reg, regulate; FC, Fold Change; FDR, false discovery rate.

groups are consistent with the transcriptome sequencing results (Fig. 1H).

TERT expression enhances BMSC proliferation

Both BMSCs and TERT-BMSCs can maintain a spindle shape at the third passage (Figs. 2A and 2B). After passaging, passage 7 BMSCs appear flat and large with unclear cell edges (Fig. 2C) and fail to form a fusion whilst passage 30 TERT-BMSCs still maintain their original shapes and strong proliferation capacity (Fig. 2D). Although TERT-BMSCs were cultured for more than 7 months (>45 passages) (Fig. 2E), no remarkable senescence morphology was observed. Transcriptome sequencing was performed to analyse the expression of self-renewal regulatory factors related to the cell cycle. The results showed that cyclins (CCNA2, CCNB1, CCND1, CCND2, CCNE1 and CCNE2), cyclin-dependent kinases (CDK1 and CDK6), retinoblastoma-related pocket proteins (RBL1) and E2F family (E2F1 and E2F2) were significantly up-regulated in TERT-BMSCs compared with BMSCs. Expression levels of cyclins (CCNA1 and CCND3) and cyclin-dependent kinases (CDK2 and CDK4)

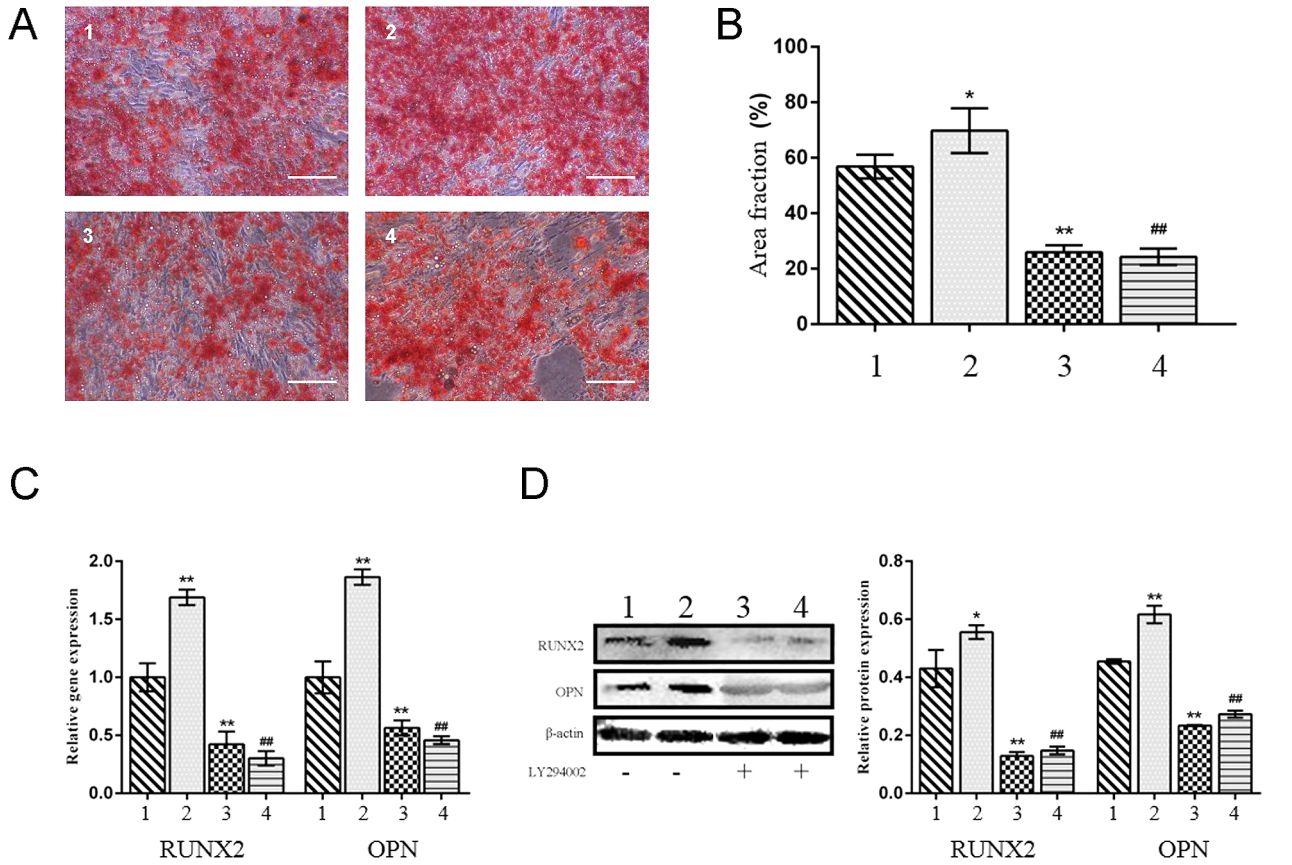

Fig 3. Osteogenic differentiation. (A) Alizarin red staining. (B) Alizarin red staining area fraction. (C) The expression of osteogenesis characteristic genes OPN and RUNX2 by qPCR. (D) The expression of osteogenesis characteristic proteins OPN and RUNX2 by western blot. Relevant gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Abbreviations: 1-4 represents BMSCs, TERT-BMSCs, BMSCs treated by LY294002 and TERT-BMSCs treated by LY294002, respectively. Scale bars = 200 μm. * $p<0.05$, ** $p<0.01$ between TERT-BMSCs vs. BMSCs or BMSCs treated by LY294002 vs. BMSCs; # $p<0.05$, ## $p<0.01$ between TERT-BMSCs treated by LY294002 vs. TERT-BMSCs.

did not significantly differ between TERT-BMSCs and BMSCs. Compared with BMSCs, the expression of cyclin-dependent kinase inhibitor, CDKN1A/p21 and CDKN2A/p16 significantly increased but that of CDKN1B/p27 significantly decreased in TERT--BMSCs (Table 3).

TERT expression promotes BMSC osteogenic differentiation

The transcriptome sequencing results showed that expression levels of typical osteogenic-related transcription factors OPN, Sp7, OCN, osteonectin and COL1A were significantly up-regulated in TERT-BMSCs compared with those in BMSCs and no significant difference was detected in expression levels of BMP2 and Smad4 whilst RUNX2 expression significantly decreased (Table 4). After 21 days of culturing in the osteogenic-induction medium, calcium content was determined via alizarin red staining. The dyeing-area fraction results showed that TERT-BMSCs demonstrated significantly higher calcium content than BMSCs (Figs. 3A and 3B). RT-qPCR and Western blot assays showed that the ectopic expression of TERT significantly up-regulated osteogenesis-characteristic factors OPN and RUNX2 at both mRNA and protein level after osteogenic induction of BMSCs (Figs. 3C and 3D).

PI3K/Akt signalling pathway is activated after TERT ectopic expression

The top-ten enriched pathways associated with TERT ectopic expression was identified using functional enrichment analysis (Fig. 4A). Amongst them, pathways in cancer, PI3K/Akt signalling and cell cycle were the top-three enriched pathways (enrichment score>6.0 and $p<0.05$). Furthermore, 73 PI3K/Akt signalling-related genes were significantly altered in TERT-BMSCs. Thirty-six genes, including FGF, VEGFC, NGF, EPHA2, IL6, IL6R, COL1A, LAMC2, ITGA6, Gβγ, PI3KR5, BCL2L1 and BCL2, positively regulate the PI3K/Akt signalling pathway and are significantly up-regulated (Table 5). By contrast, 37 genes, including PPP2R2/5 and p27, negatively regulate the

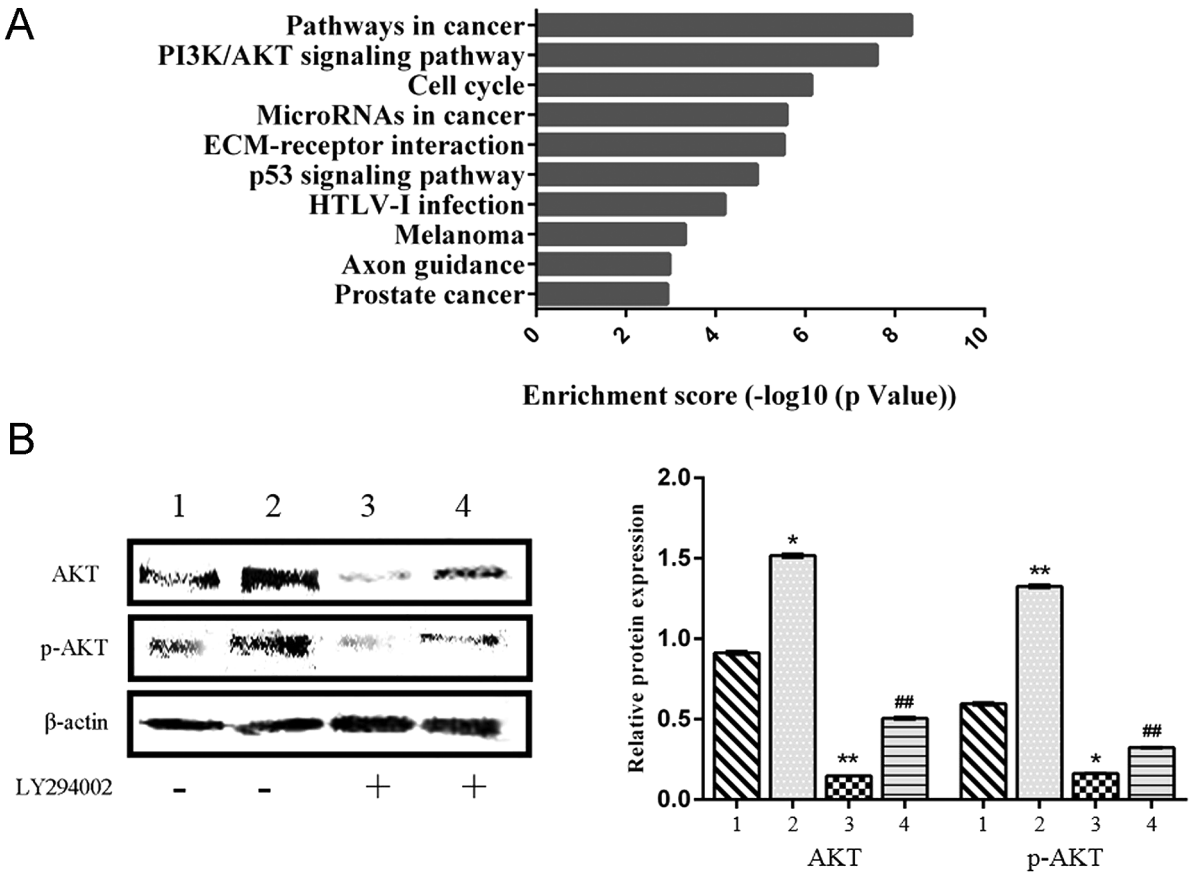

Fig 4. PI3K/Akt signaling pathway is activated after TERT ectopic expression. (A) The top 10 pathways that exhibited significant differences between the BMSCs and TERT-BMSCs. (B) The expression of AKT and p-AKT by Western blot. Abbreviations: 1 represents BMSCs; 2 represents TERT-BMSCs; 3 represents BMSCs treated by LY294002; 4 represents TERT-BMSCs treated by LY294002. * p<0.05, ** p<0.01 between TERT-BMSCs vs. BMSCs or BMSCs treated by LY294002 vs. BMSCs; # p<0.05, ## p<0.01 between TERT-BMSCs treated by LY294002 vs. TERT-BMSCs.

PI3K/Akt signalling pathway and are significantly down-regulated (Table 5). Western blot assays showed that the significantly up-regulated ectopic expression of TERT of both AKT and p-AKT proteins can be reversed by the PI3K inhibitor LY294002 (Fig. 4B). These results indicated that the PI3K/Akt signalling pathway in BMSCs is activated after the ectopic expression of TERT.

Inhibition of PI3K/Akt signalling pathway attenuates osteogenic differentiation of BMSCs

BMSC and TERT-BMSCs pretreated with LY294002 showed a significant reduction in calcification deposition after 21 days of osteogenic induction culturing (Figs. 3A and 3B) along with mRNA and protein levels of osteogenesis characteristic factors OPN and RUNX2 (Figs. 3C and 3D).

Expression of carcinogenic characteristic genes

The expression profiles of carcinogenic-related genes were analysed via transcriptome sequencing. The results showed that TERT-BMSCs demonstrate significantly increased transcript levels of CDKN1A/ /p21, CDKN2A/p16 and KLF4, significantly decreased transcript levels of c-myc, STAT-5, CDKN1B/p27, PPARγ, ARNT2, PDGF and PLAT and nearly unchanged transcript levels of NUPR1, p53, BMI-1, RXRA and GADD45 compared with BMSCs (Table 6).

Discussion

Telomerase is a ribonucleoprotein that can maintain the length of telomeres and control the proliferation and lifespan of cells. In addition to its function in telomere maintenance, telomerase has a pro-survival role by decreaseing apoptosis and increasing resistance against DNA damage (Tichon et al. 2009). It has been documented that high TERT expression can inhibit apoptosis and improve mitochondrial function via alleviating

Table 6. Carcinogenic characteristic genes expression of BMSCs and TERT-BMSCs.

Genes	Definition	Reg	Log2 FC	FDR
STAT5	signal transducer and activator of transcription 5B	down	-1.114567255	0.000313876
NUPR1	nuclear protein 1	--	-0.651009268	0.456178383
BMI-1	polycomb complex protein BMI-1	--	-0.049566309	0.950024986
PPARγ	peroxisome proliferator-activated receptor gamma X1	down	-5.56488693	0
RXRA	retinoic acid receptor RXR-alpha isoform X4	--	-0.750192268	0.14210803
ARNT2	aryl hydrocarbon receptor nuclear translocator 2 isoform X1	down	-1.016309257	0.006221212
PDGF	platelet-derived growth factor subunit A	down	-1.406704504	$1.78E^{-06}$
PLAT	tissue-type plasminogen activator	down	-1.244042821	$3.57E^{-05}$
GADD45	growth arrest and DNA damage-inducible protein GADD45	--	-0.253138423	0.96269035

Abbreviations: Reg, regulate; FC, Fold Change; FDR, false discovery rate.

intracellular ROS in osteosarcoma cells (Zhang et al. 2017). Short-term TERT inhibition may lead to cell cycle arrest in S-phase and ultimately induce apoptosis in B lymphoblastic cells (Celeghin et al. 2016), an accumulation of cells in the S-phase likely due to a decreased availability of proteins required for cell cycle progression (Giunco et al. 2013). The present study established TERT-BMSC cell lines by ectopically expressing TERT in BMSCs, and then detected the TERT expression at the mRNA and protein level by transcriptome sequencing, qPCR and Western blot, respectively. The results demonstrated that a stably transfected TERT-BMSC cell line was successfully established. The established TERT-BMSC stable cell lines possess an enhanced capacity for proliferation and therefore prolonged lifespan. TERT-BMSCs can still proliferate even at passage 30, while BMSCs showed some signs of aging at passage 7 or 8. It has been shown that the ability of TERT ectopic expression to extend the lifespan may be related to the site of integration and the levels of cell cycle-related proteins (Celeghin et al. 2016), but the internal mechanism still needs a further verification. The transcriptomic analyses showed that mRNA expression levels of cyclins, cyclin-dependent kinases, retinoblastoma-related pocket proteins and E2F proteins were significantly increased in TERT-BMSCs compared to those in BMSCs. CDKN1B/p27, a cyclin-dependent kinase inhibitor, was markedly reduced in the TERT-BMSCs, whereas CDKN1A/p21 and CDKN2A/p16 were significantly elevated, which may be associated with inhibition of excessive cell proliferation (Woo et al. 2017). Overall, it can be concluded from the results that ectopic expression of TERT promotes BMSCs proliferation by shortening the cell cycle.

While ectopic expression of TERT enhances the proliferation ability, it also has the risk of tumorigenesis. So, does ectopic expression of TERT in BMSCs pose a risk of carcinogenesis? Studies have confirmed that nonhuman primate ADSCs ectopically expressing TERT has no tumorigenicity in immune-deficient mice, and in chromosomal analyses no abnormal karyotypes or tumor-associated abnormal genes are observed. The present studies document that early passage human PrECs have sufficiently low p16 level to permit immortalization by ectopic expression of TERT. TERT-PrECs were non-tumorigenic when inoculated into intact male immunodeficient NSG mice (Graham et al. 2017). Ectopic expression of TERT in Stem cells from human exfoliated deciduous teeth (SHED) was studied by multilineage differentiation, karyotype, colony formation in soft agar, and tumor formation in nude mice of SHED and TERT-SHED. The result showed that immortalized SHED maintained the ability of mutilineage differentiation, at late passage showed normal karyotype, no soft agar colony formation, and no tumor formation in nude mice (Yin et al. 2016).

In the present study, we analyzed the expression of more than ten genes that may be involved in cancer transcriptional regulation. The results showed that the expression levels of the immortalized programming gene c-myc and oncogenic gene STAT-5 in the TERT--BMSCs were significantly downregulated. The cellular senescence tumor suppressor-related genes CDKN1A/ p21 and CDKN2A/p16 were significantly upregulated, but CDKN1B/p27 was significantly downregulated, which may be related to the enhanced proliferation of the BMSCs after TERT ectopic expression. The expressions of tumor angiogenesis-promoting genes ARNT2, PDGF and PLAT were significantly downregulated in the TERT-BMSCs and Klf4, as an inhibitor of tumor cell growth and migration, was found to be significantly upregulated. There was no significant difference in the expression between other cancer-

-related genes, such as p53 and NUPR1. In general, BMSCs ectopically expressing TERT showed a significantly increased proliferation capacity without the overexpression of the characteristic carcinogenicity--related genes and obvious malignant proliferation trend.

The ectopic expression of TERT in human endothelial cells does not affect their original phenotype and enhance their ability to form microvascular structures (Yang et al. 2001). Adipose mesenchymal stem cells of nonhuman primates can maintain their original phenotype after ectopic expression of TERT (Kang et al. 2004). Similarly, the ectopic expression of TERT in various cell types, including humans, mice, rats and other animals will maintain its characteristic phenotype (Yang et al. 2001, Kang et al. 2004, Saeed et al. 2015, Le et al. 2019). In this study, the constructed plasmid TERT expression vector was transfected into sheep BMSCs and then screened to obtain TERT-BMSC cell lines and transcriptome sequencing was performed on BMSC and TERT-BMSC cell lines. The analysis of surface marker genes of nonhaematopoietic stem cells showed that CD44, CD29, CD90/THY1, CD105/EN and CD73 are positively expressed whilst CD34, CD45, CD11b, CD19 and HLA class II are negatively expressed. Real-time fluorescence quantitative PCR was used to detect some nonhaematopoietic stem cell surface marker genes of BMSCs and TERT-BMSCs, including CD44, CD29, CD90, CD34 and CD45. The consistent related gene expression and transcriptome sequencing results confirmed that the characteristic phenotype of BMSCs remains unchanged after the ectopic expression of TERT.

BMSCs ectopically expressing TERT can maintain not only the original characteristic phenotype but also have a certain effect on the multidirectional differentiation potential of cells. Overexpression of TERT in human BMSCs shows high osteogenic and neural differentiation efficiency and reduced methylation levels (Tsai et al. 2010). However, the reproductive activity and osteogenic differentiation ability of BMSCs is reduced in TERT knockout mice likely due to the bone marrow inflammation microenvironment that promotes osteoclast formation (Saeed et al. 2011). The ectopic expression of TERT in primate adipose--derived mesenchymal stem cells can up-regulate the expression level of osteogenic markers, including sialyltransferase, osteoprotegerin, osteoblast-specific factor 2 and biglycan (Kang et al. 2004). Similarly, the overexpression of TERT in rat BMSCs also increases the expression of osteoblast-specific genes, including BSP and OCN. This study detected the significantly increased expression of osteogenesis-related transcription factors, including OPN, Sp7, OCN and osteonectin and decreased RUNX2 after the ectopic expression of TERT in sheep BMSCs. The mechanism of metastatic phenotype of RUNX2 in prostate cancer cells was induced not only by high bone turnover but also tissue invasion (Baniwal et al. 2010), thereby indicating that the ectopic expression of TERT may affect the role of RUNX2 in cell invasion. After osteogenic induction, the TERT-BMSC group demonstrated significantly higher formation of calcium salt deposition and expression of mRNA and protein of osteoblastic factors RUNX2 and OPN than the BMSC group, thereby indicating that the ectopic expression of TERT can enhance the osteogenic differentiation of BMSCs.

The ectopic expression of TERT can enhance the osteogenic differentiation of BMSCs, but its mechanism of action remains unclear. The PI3K/Akt signalling pathway is involved in the proliferation, migration and osteogenic differentiation of BMSCs (Fujita et al. 2004). The activation of PI3K/Akt signalling pathway mediates the enhanced effect of exosomes on the osteogenic capacity of human BMSCs (Zhang et al. 2016). This study revealed via functional enrichment analysis that the PI3K/Akt signalling pathway exhibits the maximum significant difference after the ectopic expression of TERT. Further analysis of gene differences involved in the PI3K/Akt signalling pathway demonstrated that typical positive regulatory genes, such as FGF, VEGFC, NGF, EPHA2, IL6, IL6R, COL1A, LAMC2, ITGA6, Gβγ, PI3KR5, BCL2L1 and BCL2, are significantly up-regulated whilst negative regulatory genes, including PPP2R2/5 and p27, are significantly down-regulated. The results of Western blot showed that the ectopic expression of TERT up-regulated the protein levels of AKT and p-AKT, thereby indicating that the ectopic expression of TERT enhances the PI3K/Akt signalling pathway. However, the treatment with PI3K/Akt signalling pathway inhibitor can significantly decrease the protein expression of AKT and p-AKT, calcium salt deposition and mRNA and protein expression of RUNX2 and OPN, thereby indicating that the PI3K/Akt signalling pathway plays a crucial role during the osteogenic differentiation promoted by TERT. The osteogenic differentiation of BMSCs involves multiple signalling pathways (Wang et al. 2017). Several studies have reported that the up-regulation of long noncoding RNA H19 can significantly promote the expression of osteogenic markers, β-catenin and target genes of Wnt/β-catenin signalling pathway, thereby indicating that H19 promotes osteogenic differentiation of rats ecto-mesenchymal stem cells by activating the Wnt/β-catenin signal (Gong et al. 2018). The overexpression of cell differentiation core transcription factor FOXA2 inhibits specific activities of osteogenesis, whereas FOXA2 gene knockdown

significantly increases the specific gene expression of osteoblast, sediment yield of calcium salt and alkaline phosphatase activities. Meanwhile, ERK also increases accordingly because its signalling pathway may be partially activated in the osteogenic differentiation of BMSC (Chen et al. 2018). Given that naproxen can reduce mineral deposits in the extracellular matrix through the hedgehog signalling pathway, the gene expression during osteogenic differentiation of BMSCs is down-regulated. Similarly, the activation of Notch signalling pathway inhibits the osteogenic differentiation of BMSCs (Kang et al. 2015). Other studies have analysed the effects of TGF-β signalling pathway activated by bone morphogenetic protein 2 and Smad protein and NF-KB signalling pathway activated by NF-KB receptor activator of nuclear factor-κ B ligand (RANKL) on osteogenic differentiation of BMSCs (Gu et al. 2016). Determining whether other signalling pathways are involved in the ectopic expression of TERT on the osteogenic differentiation of BMSCs requires further exploration.

TERT-BMSC cell lines established via the ectopic expression of TERT in this study maintain not only the normal phenotype of BMSCs but also maintain enhanced its osteogenic differentiation ability. The enhanced osteogenic differentiation ability of BMSCs caused by the ectopic expression of TERT may be mediated by the PI3K/Akt signalling pathway. This study provides a reliable cell source for research on clinical bone tissue engineering and bone tissue clinical regenerative medicine and a theoretical reference for future studies on the mechanism of multidirectional differentiation of somatic stem cells induced by the ectopic expression of TERT.

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