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Canonical Wnt signaling differently modulates osteogenic differentiation of mesenchymal stem cells derived from bone marrow and from periodontal ligament under inflammatory conditions

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ABSTRACT

Background: Cellular plasticity and complex functional requirements of the periodontal ligament (PDL) assume a local stem cell (SC) niche to maintain tissue homeostasis and repair. Here, pathological alterations caused by inflammatory insults might impact the regenerative capacities of these cells. As bone homeostasis is fundamentally controlled by Wnt-mediated signals, it was the aim of this study to characterize the SC-like capacities of cells derived from PDL and to investigate their involvement in bone pathophysiology especially regarding the canonical Wnt pathway.
Methods: PDLSCs were investigated for their SC characteristics via analysis of cell surface marker expression, colony forming unit efficiency, proliferation, osteogenic differentiation and adipogenic differentiation, and compared to bone marrow derived mesenchymal SCs (BMMSCs). To determine the impact of both inflammation and the canonical Wnt pathway on osteogenic differentiation, cells were challenged with TNF-α, maintained with or without Wnt3a or DKK-1 under osteogenic induction conditions and investigated for p-IκBα, p-NF-κB, p-Akt, β-catenin, p-GSK-3β, ALP and Runx2.
Results: PDLSCs exhibit weaker adipogenic and osteogenic differentiation capacities compared to BMMSCs. TNF-α inhibited osteogenic differentiation of PDLSCs more than BMMSCs mainly through regulating canonical Wnt pathway. Blocking the canonical Wnt pathway by DKK-1 reconstituted osteogenic differentiation of PDLSCs under inflammatory conditions, whereas activation by Wnt3a increased osteogenic differentiation of BMMSCs.
Conclusions: Our results suggest a diverse regulation of the inhibitory effect of TNF-α in BMMSCs and PDLSCs via canonical Wnt pathway modulation.
General significance: These findings provide novel insights on PDLSC SC-like capacities and their involvement in bone pathophysiology under the impact of the canonical Wnt pathway.

1. Introduction

The periodontal ligament (PDL) is a complex soft connective tissue located at the interface of the alveolar bone and cementum, which maintains tooth attachment, nutrition and homeostasis as well as structural repair of damaged tissues [1]. Embedding diverse cell types [2], PDL cells represent the predominant cellular component of the PDL and are characterized by phenotypic heterogeneity as much as the capability to transform into neighboring cell types like osteoblasts or cementoblasts [3]. This cellular plasticity, attended by the fact that the PDL exhibits one of the upmost turnover rates in the body assumes the existence of a local stem cell (SC) niche for the maintenance of tissue homeostasis and repair [2,4]. However, the complex structural composition comprising both hard and soft tissues and the diverseness of the elements composing the PDL aggravate the detection of potential SC-like elements responsible for regeneration. Considering the osteoblast-like properties and the mesenchymal origin of PDL cells [5], a possible interconnection with the characteristics of bone marrow derived mesenchymal SCs (BMMSCs) can be assumed. Furthermore, MSC markers like STRO-1 and CD146 have already been identified on a subpopulation of PDL cells that potentially disclose them as periodontal ligament stem

Abbreviations: BMMSCs, bone marrow derived mesenchymal stem cells; PDLSCs, periodontal ligament mesenchymal stem cells; PCR, polymerase chain reaction; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; PPARγ, peroxisome proliferator-activated receptor-γ; LPL, lipoprotein lipase; p-IκBα, phosphonated inhibitor of nuclear factor kappa B; p-NF-κB, phosphonated nuclear factor kappa B; p-Akt, phosphonated v-Akt murine thymoma viral oncogene; p-GSK-3β, phosphonated glycogen synthase kinase 3 beta

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cells (PDLSCs) [2]. BMMSCs represent one of the best described multipotent MSC type that provided the basis for the establishment of gold standard criteria characterizing SCs, namely colony-forming unit (CFU) capacity, persistent self-renewal reflected by a high proliferation potency and developmental plasticity in terms of differentiation into multiple cell lineages [6]. However, the features of PDLSCs may differ from BMMSCs, as the mesenchymal origin of dental structures is designated by its developmental interdependency with the neural crest and therefore denoted as ectomesenchyme [6].

One of the central questions in PDL pathophysiology is the identification of the factors determining its turnover quality and regenerative efficiency, particularly regarding its involvement in pathological alterations of the alveolar bone caused by inflammatory insults. Investigations revealed that PDL cells are required for bone remodeling and regeneration, as they are involved in both the regulation of osteogenic differentiation and osteoclastic differentiation [7]. Furthermore, inflammatory processes in periodontal tissues seem to be modulated by resident PDL cells [8,9], correlating with data on MSC that have been shown to feature immunomodulatory capacities as well [10]. Consequently, a subpopulation of PDL cells with SC-like capacities might play a particular role in the regulation of bone pathophysiology.

Bone homeostasis is known to be fundamentally controlled by Wnt-mediated signals that comprise two main molecular pathways, namely the β-catenin-dependent canonical and the β-catenin-independent noncanonical Wnt pathway [11]. The canonical Wnt/β-catenin pathway enhances bone formation and entails osteogenic lineage differentiation of progenitor cell as well as PDL cells [7], thus capacitating these cells to implement hard tissue repair. Furthermore, Wnt/β-catenin signals suppress bone resorption by stimulating OPG expression but simultaneously suppressing RANKL expression in osteoblasts, and by inhibition of osteoclastogenesis [11]. The canonical Wnt/β-catenin pathway is mainly driven by Wnt1 and Wnt3a and leads to activation of β-catenin and in turn inhibition of GSK-3β kinase [12]. This cascade can be blocked by antagonists like Dickkopf-1 (DKK-1) through binding to one of the two Wnt receptor complexes, low-density lipoprotein receptor-related protein 5/6 (LRP5/6) [11]. β-catenin signals are not only required by osteoclast precursors for the process of osteoclastogenesis, but also inhibit their differentiation upon prolonged stimulation [13].

Bennett et al. reported that canonical Wnt pathway promotes the osteogenesis of murine BMMSCs and osteoprogenitor cells through up-regulation osteoblast-related genes [14,15]. However, our group observed that canonical Wnt signaling promoted osteogenesis of PDLSCs in full culture medium while inhibited it in osteogenic differentiation medium [16]. In MSCs, both an osteoinhibitory and osteoinductive impact of Wnt/β-catenin signals could be observed depending on the environmental conditions [17]. Knowing that periodontitis has a significantly higher prevalence than osteomyelitis, and that the regenerative capacities of periodontal tissues are significantly constrained under inflammatory conditions [18–21], inflammation may differently affect osteogenic commitment of PDLSCs and BMMSCs. However, the role of the canonical Wnt pathway in regulating osteogenic differentiation of BMMSCs and PDLSCs in an inflammatory microenvironment remains to be elucidated.

In the present study, PDLSCs were found to feature weaker adipogenic and osteogenic lineage commitment than BMMSCs, but exhibited to be more sensitive to the inhibitory effect of TNF-α on osteogenic differentiation. Blockage of the canonical Wnt pathway by DKK-1 reconstituted the process of osteogenic differentiation of PDLSCs under inflammatory conditions, whereas activation by Wnt3a increased osteogenic differentiation of BMMSCs, indicating the dual impact of the canonical Wnt pathway in regulating osteogenesis. Taken together, our findings provided novel insights in the SC-like capacities of PDLSCs and investigated their involvement in bone pathophysiology. Furthermore, our results suggest a diverse regulation of the inhibitory effect of TNF-α in PDLSCs and BMMSCs via modulation of the canonical Wnt pathway.

2. Material and methods

2.1. Cell culture and identification of stem cells

Primary cultures of human PDL cells (n = 6, aged from 16 to 18 years) were explanted from the middle third of the root surface of the teeth extracted for orthodontic reasons at the Dental Clinic of the Fourth Military Medical University, Xi'an, China. Adolescent donors were examined on defined variables for clinically healthy periodontal tissues with the absence of bleeding on probing, probing depth <4 mm and loss of attachment level <3 mm. Written informed consent was provided by all participants and the study was approved by the hospital's ethics committee. The PDL explants were enzymatically digested with type 1 collagenase (0.66 mg/ml; Sigma, St Louis, MO, USA) for 20 min. For the generation of single-cell suspensions, the tissues were filtrated through a 70-μm cell strainer (Millipore, Billerica, MA, USA), washed and incubated in a-MEM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS, 0.292 mg/ml glutamine (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco BRL) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

The explants were maintained in 6-well culture dishes (Costar, Cambridge, MA, USA) for 2 weeks until subconfluence was reached. To obtain homogeneous populations of PDLSCs, single-cell-derived colony cultures were generated by the limiting dilution technique [20,22]. Multiple colony-derived PDLSCs were used from passages 2 to 4.

To investigate the MSC phenotype, 5×10^5 PDLSCs were incubated with PE or FITC conjugated monoclonal antibodies for human CD14, CD31, CD90, CD105 (eBioscience, San Diego, CA, USA), CD146 and Stro-1 (R&D Systems, Inc., Minneapolis, MN, USA), or isotype-matched control IgGs. Cells were subjected to flow cytometric analysis using a Beckman Coulter Epics XL (Beckman Coulter, Fullerton, CA, USA).

Human BMMSCs (n = 6, aged from 16 to 25) were used as control cell line and cultured as previously described [23].

2.2. Colony forming unit (CFU) assay

To assess the CFU efficiency of PDLSCs and BMMSCs, day 14 cultures of single-cell suspensions (2×10^3 cells) were seeded in 10-cm-diameter culture dishes (Corning, Lowell, MA, USA). The newly formed colonies were visualized with 0.1% toluidine blue following 4% paraformaldehyde fixation. Aggregates of 50 or more cells were scored as colonies under the microscope (Leica Microsystems, Heerbrugg, Switzerland). CFU efficiency was determined by the number of colonies relative to the total number of seeded cells in each plate. Experiments were performed in triplicate.

2.3. Proliferation assays

For proliferation analyses of PDLSCs and BMMSCs, 5×10^3 cells/well were cultured in 96-well plates. The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out for 8 days according to the manufacturer's protocol (Sigma). Absorbance was determined at 490 nm with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Besides, cell proliferation was analyzed by an 5-ethynyl-2′-deoxyuridine (EdU) assay. 3×10^3 cells/well were seeded in 24-well culture plates overnight and cell proliferation was subsequently assessed by analysis of EdU incorporation into DNA with an EdU staining kit (RiboBio) according to the manufacturer's instruction. Furthermore, cell cycle analysis was performed on PDLSCs and BMMSCs. Single cell suspensions of both cells were harvested and fixed in ice-cold 75% ethanol 4 °C for 24–48 h, washed twice with PBS, stained with 100 mg/ml propidium iodide at 4 °C for 30 min and subjected to cell cycle analysis using an Elite ESP flow cytometer (Beckman Coulter, Fullerton, CA, USA). All experiments were performed in triplicate.

2.4. Osteogenic and adipogenic differentiation assays

To characterize and compare the lineage differentiation potential of PDLSCs and BMMSCs, cells were subjected to osteogenic induction and adipogenic induction.

3×10^5 cells/well were maintained in 6-well plates for 24 h and subsequently incubated with osteogenic medium (100 nM dexamethasone, 50 mg/ml ascorbic acid, and 5 mM b-glycerophosphate; Sigma) for 1 to 2 weeks according to the manufacturer's instructions. During this time, cells were challenged with or without tumor necrosis factor (TNF)-α (10 ng/ml; Pepro-Tech). To assess osteogenic differentiation, an ALP activity assay was performed after 7 d in culture with an ALP kit according to the manufacturer's protocol (Jiancheng, Nanjing, China). Cells were fixed with 70% ethanol and incubated with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue BB dissolved in 0.1 M Tris buffer (pH 9.3). Furthermore, cells were fixed with 75% ethanol and stained with 2% alizarin red (Sigma) after 14 d in culture to determine the osteogenic differentiation. For quantification of the alizarin red-stained nodules, calcium accumulation was detected by 2% alizarin red staining (pH 4.2), and calcium levels were measured using the Calcium Colorimetric Assay Kit (BioVision Co., USA).

5×10^5 cells/well were maintained in 6-well plates for 24 h and subsequently incubated with adipogenic medium (0.5 mM methylisobutylxanthine, 0.5 mM hydrocortisone, and 60 mM indomethacin; Sigma) for 4 weeks. Intracellular lipid accumulation was detected by staining with oil red O solution. All experiments were performed in triplicate.

2.5. Real-time polymerase chain reaction analyses

Total RNA of PDLSCs and BMMSCs was isolated (TRIzol; Invitrogen) and converted to cDNA (SuperScript First-Strand Synthesis Kit; Invitrogen). RT-PCR was performed using the QuantiTect SYBR Green PCR Kit (Toyobo, Osaka, Japan) and the Applied Biosystems 7500 Real-Time PCR Detection System with primers for osteogenic and adipogenic markers listed in Table 1. Experiments were performed in triplicate for each reaction. Primes are listed in Table 1.

2.6. Western blot analyses

Cytoplasmic and nuclear proteins were extracted from PDLSCs and BMMSCs with the Nuclear Extraction Kit according to the manufacturer's protocol (Millipore, Billerica, MA, USA). Total proteins were extracted from the cells by lysis in RIPA buffer (10 mM Tris–HCl, 1 mM EDTA, 1% sodium dodecyl sulfate, 1% Nonidet P-40, 1:100 proteinase inhibitor cocktail, 50 mM b-glycerophosphate, and 50 mM sodium fluoride). Protein concentrations of the extracted lysates were determined by measuring the absorbance at 595 nm using a protein assay solution (Bio-Rad, Hercules, CA, USA). Aliquots of 20–50 mg of the cell lysates per sample were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membranes were blocked with 5% milk for 2 h and incubated with the following primary antibodies: the osteogenic markers ALP (1:800, Abcam, Cambridge, England) and Runx2 (1:500, Abcam) and the adipogenic markers PPARγ (1:500, Abcam), LPL (1:1000, Cell Signaling Technology, Boston, MA), p-IκBα (Ser32/36) (1:800, Cell signaling Technology), p-NF-κB p65 (S536) (1:800, Epitomics, USA), p-Akt (Ser473) (1:800, Cell signaling Technology), p-GSK-3β (Ser9) (1:1000, Cell signaling Technology), β-catenin (1:800, Abcam), active-β-catenin (1:1000, Millipore, USA), and β-actin as housekeeping gene (1:800, Abcam) overnight. Immune complexes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (Boshide, Beijing, China). Immunodetection was performed using the Western-Light Chemiluminescent Detection System (Peiqing, Shanghai, China).

Table 1
Primer sequences for qRT-PCR and siRNA transfection.

Prime name	Sequence	Sequence
	Sense (5′ to 3′)	Antisense (5′ to 3′)
qRT-PCR		
ALP	GGACCATTCCCACGTCTTCAC	CCTTGTAGCCAGGCCCATTG
Runx2	CCCGTGGCCTTCAAGGT	CGTTACCCGCCATGACAGTA
PPARγ	CCACTTTGATTGCACTTT GGTACTCTTG	CTTCACTACTGTTGACTTCT CCAGCATTTC
LPL	AAGACTTTGTAGGGCATC TGAGAACGA	TGAGAAAGGGCTCTGCTT GAGTTGTAG
β-actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCT AGAAGCA
siRNA transfection		
Si-homo-β-catenin-425	GUCCUGUAUGAGUGGG AACTT	GUUCCCACUCAUACAGGACTT
Si-homo-β-catenin-1288	ACCCCAAGCUUUAGUAAATT	UUUACUAAAGCUUGGGGUTT
Si-homo-β-catenin-1743	CAGUUGUGGUUAAGCTCU UTT	AAGAGCUUAACCACAACUGTT
Negative control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Si: SiRNA.

2.7. Transfection assays

Small interfering RNA (siRNA) duplex oligonucleotides against human β-catenin and a negative control (Gene-Pharma Co., Shanghai, China) were chemically modified (2′-O-methyl) and transfected into the cells at a final concentration of 100 nM using the siPORT NeoFX (Ambion, TX, USA).

2.8. Wnt3a and DKK-1 treatment

PDLSCs and BMMSCs were maintained with or without human recombinant Wnt3a (25 ng/ml; R&D Systems) or the soluble Wnt inhibitor human recombinant DKK-1 (100 ng/ml; Pepro-Tech) under osteogenic differentiation culture conditions as previously described. At this, cells were inflammatory challenged or unchallenged with TNF-α (10 ng/ml). After 7 days in culture, cells were harvested and subjected to western blot analyses as previously described for the molecules β-catenin, p-GSK-3β, p-NF-κB p65, ALP, and Runx2. Besides, osteogenic differentiation was determined by ALP staining and alizarin red staining as much as by ALP and calcium concentration measurements according to the methods specified prior to this.

2.9. Statistical analyses

Results are presented as mean ± SD ($n = 6$) from at least three independent experiments and analyzed by a two-tailed unpaired Student's t test using SPSS software. P values < 0.05 were considered statistically significant. For analysis of multiple groups, the P values were adjusted using the Bonferroni method.

3. Results

3.1. PDLSCs and BMMSCs display connatural SC characteristics

Identification of CD markers via flow cytometry on PDLSCs and BMMSCs confirmed their stem cell like characteristics (Fig. 1A–B). Both cell types were positive for CD90, CD105, CD146 and STRO-1, but negative for CD14 and CD31. Regarding the fundamental characteristics of SCs to build colony-forming units, to proliferate in culture and to have the potential for multilineage differentiation, our analyses confirmed that the PDL exhibits a niche of SCs with features similar to BMMSCs. Comparison of the biological characteristics of PDLSCs and BMMSCs showed that the two cell types were capable of forming stem cell-like

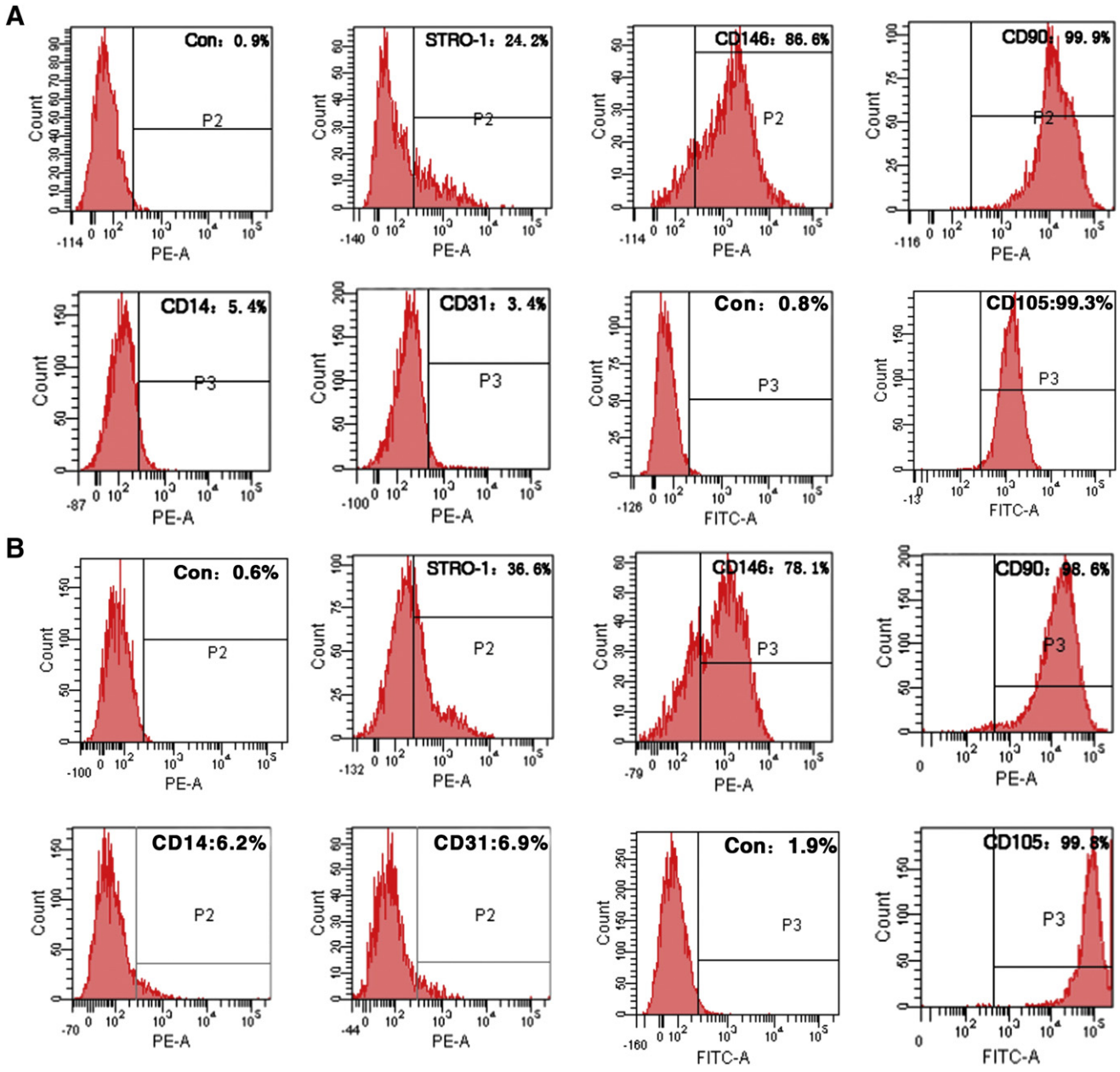

Fig. 1. Identification of stem cell related cell surface markers on PDLSCs and BMMSCs. (A, B) Detection of cell surface marker characteristic of mesenchymal stem cells on PDLSCs and BMMSCs. Analyses were performed via flow cytometry detecting PE or FITC conjugated monoclonal antibodies for human CD14, CD31, CD90, CD105, CD146, Stro-1, or isotype-matched control IgGs. Con: control.

adherent clonogenic cell clusters, which were visualized with 0.1% toluidine blue in the CFU assay (Fig. 2A).

Assessing the proliferative capacities of PDLSCs and BMMSCs, MTT assay, EdU staining and flow cytometric analysis of cell cycle showed that the proliferation rates of the two cell types did not statistically differ. The results of the experiments are presented in Fig. 2B–D.

In order to compare the lineage differentiation potential of PDLSCs with BMMSCs, osteogenic and adipogenic lineage commitment was induced by defined culture conditions. Osteogenic differentiation was identified by alizarin red staining as much as by gene and protein expression analyses of ALP and Runx2. Adipogenic differentiation was determined by oil red O staining as much as by gene and protein expression analyses of PPARγ and LPL. The results show that both the adipogenic and osteogenic differentiation capacities were more distinct in BMMSCs than in PDLSCs, which is illustrated in Fig. 2E–H.

3.2. TNF-α differentially affects osteogenic differentiation of PDLSCs and BMMSCs

The inflammatory cytokine TNF-α had different effects on the osteogenic differentiation of PDLSCs and BMMSCs. Osteogenic differentiation was determined by ALP staining and ALP activity at day 7 and by alizarin red staining at day 14. Furthermore, calcium level analysis was performed at day 14. The expression of the osteogenic markers ALP and Runx2 was analyzed by RT-PCR and by Western blot after 7 days of osteogenic lineage commitment due to appropriate culture conditions. The results (Fig. 3A–F) consistently suggest an inhibitory effect of TNF-α on osteogenic differentiation of both PDLSCs and BMMSCs. PDLSCs were more sensitive to the inflammatory cytokine compared to BMMSCs in terms of a stronger repression of ALP, Runx2 and calcium expressions just as the formation of alizarin red-stained nodules.

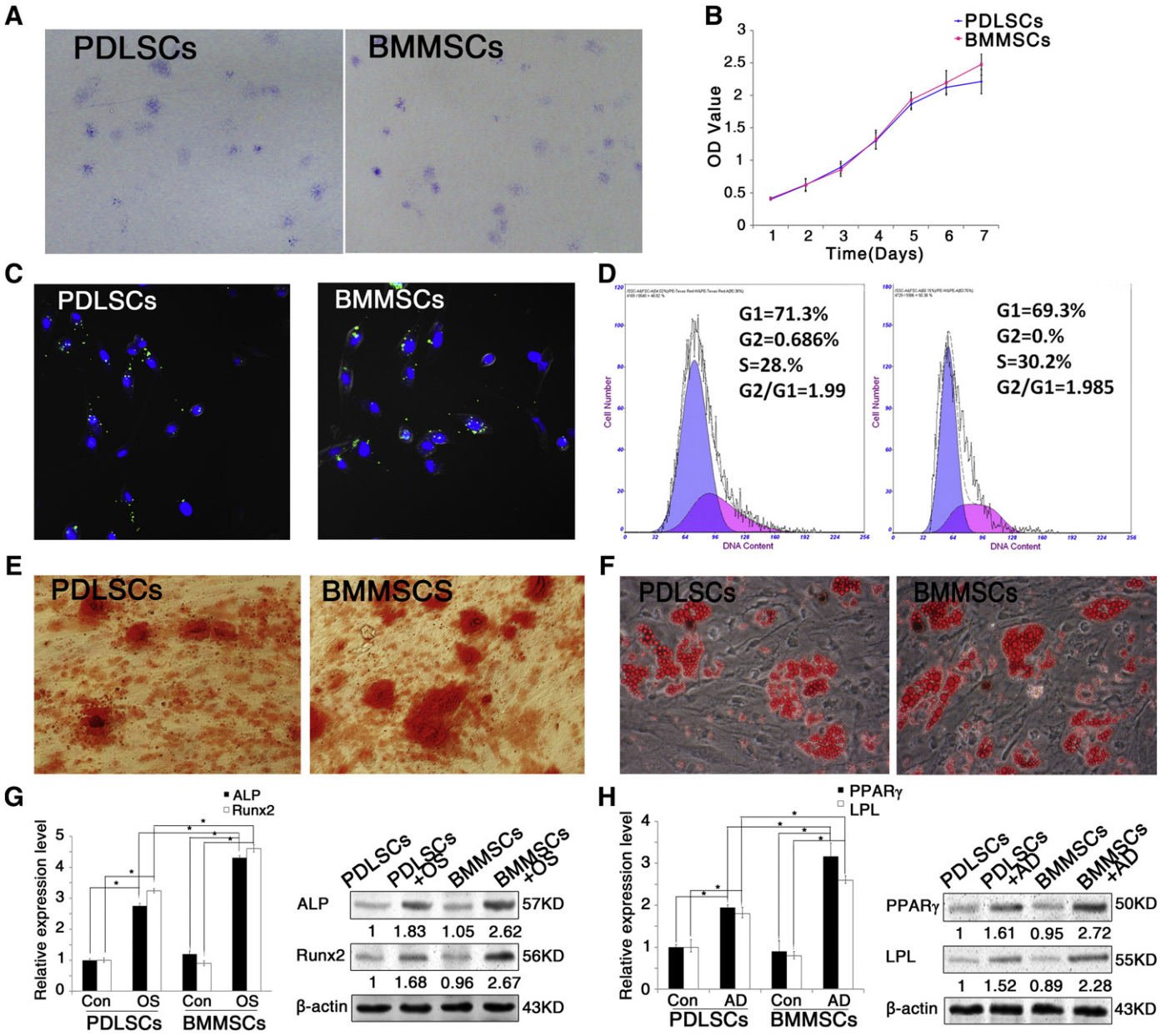

Fig. 2. Verification of related stem cell characteristics in PDLSCs and BMMSCs. (A) Analysis of the single-colony cluster formation capacity via single-colony cluster stained with 0.1% toluidine blue in both PDLSCs and BMMSCs. (B–C) Identification of similar proliferation rates of PDLSCs and BMMSCs by means of MTT assay, EdU staining and cell cycle assay by flow cytometry. (E, F) Osteogenic differentiation and adipogenic differentiation of PDLSCs and BMMSCs assessed by alizarin red staining and oil red O staining (400×). (G, H) Expression of osteoblast-related genes and adipogenic-specific genes investigated by real-time PCR and Western blot. The data are shown as mean ± SD. *$P < 0.05$, $n = 3$. OS: osteogenic induction; AD: adipogenic induction.

3.3. Canonical Wnt pathway exerts opposite effect on regulating osteogenic differentiation of PDLSCs and BMMSCs in inflammatory conditions

Western blot analyses revealed that TNF-α activated NF-κB pathway significantly in PDLSCs while it almost exerted no effect on BMMSCs during osteogenic induction (Fig. 4A). Results presented in Fig. 4B showed that TNF-α exposure opposingly modulated p-Akt, p-GSK-3β and active β-catenin expressions and promoted p-GSK-3β and β-catenin translocation into the nucleus more than 1.8-fold and 1.6-fold in PDLSCs. Contrarily, the expression levels of β-catenin and p-GSK-3β in BMMSCs were slightly decreased by the impact of TNF-α.

To further elucidate the different effects of TNF-α on inhibiting osteogenic differentiation capacity of the two cells. We modulated the canonical Wnt pathway by Wnt3a and DKK-1. Our data illustrated that Wnt3a activated while DKK-1 blocked the canonical Wnt pathway in PDLSCs and BMMSCs both to the same extent but did not affect the NF-κB pathway (Fig. 4C). Western blot analysis showed that the expressions of ALP and Runx2 were decreased by Wnt3a and increased by DKK-1 in PDLSCs, while inducing the opposite expression patterns in BMMSCs (Fig. 4D), suggesting that the canonical Wnt pathway exerts an opposite effect on regulating osteogenic differentiation of PDLSCs and BMMSCs.

The chemically modified (2′-O-methyl) Si-β-catenin oligonucleotides (Si-β-catenin-1743) decreased β-catenin expression nearly 4-fold compared with the control (Fig. 5A). After transfection with Si-β-catenin for 1 day, PDLSCs and BMMSCs were cultured in osteogenic differentiation medium with 10 ng/ml TNF-α for additional 7 or 14 days. ALP staining and alizarin red staining, ALP activity, calcium level analyses, and results of ALP and Runx2 expressions indicated

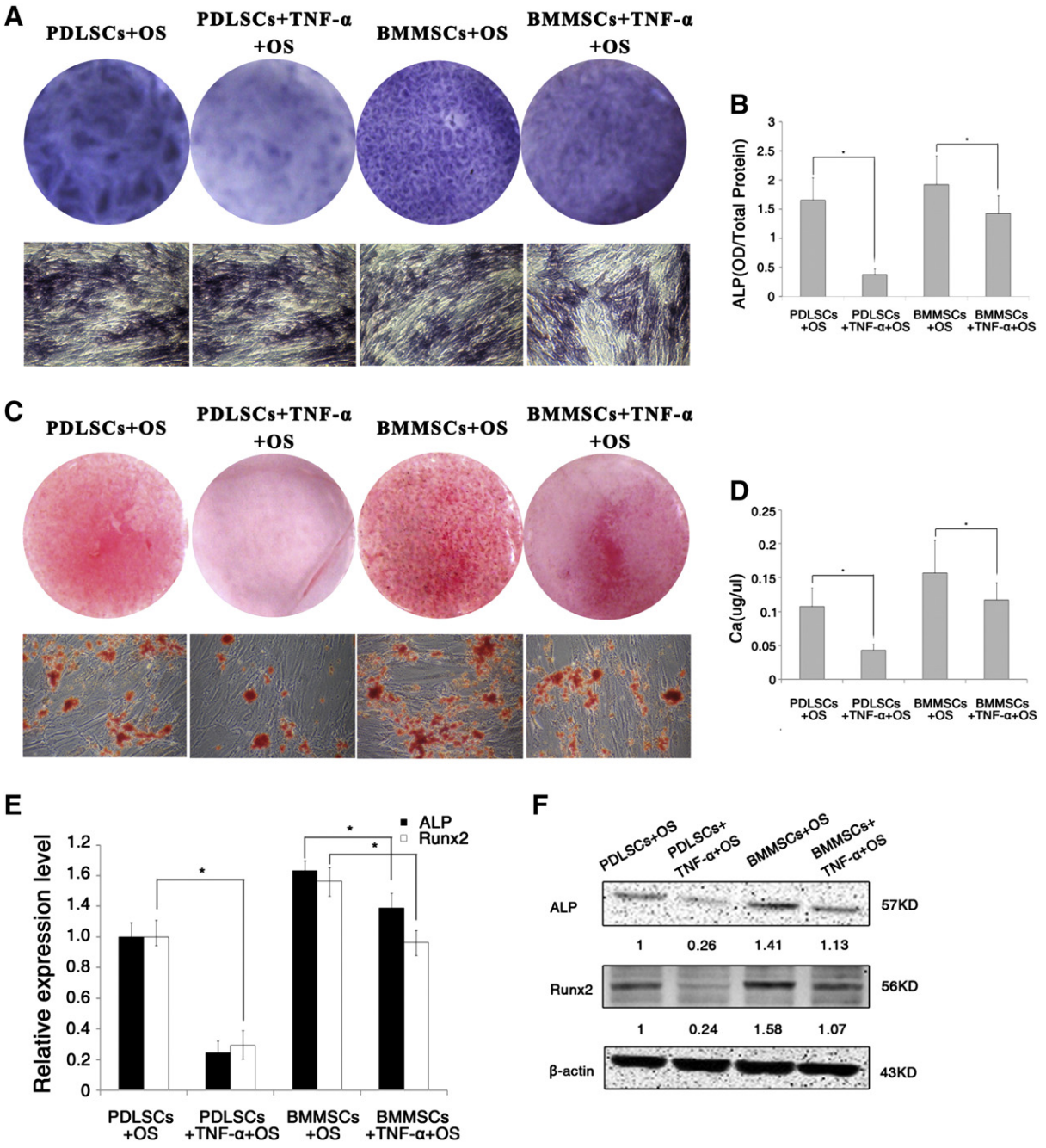

Fig. 3. Discriminative influence of TNF-α on osteogenic differentiation of PDLSCs and BMMSCs. PDLSCs and BMMSCs were cultured in osteogenic differentiation medium with or without TNF-α for additional 7 or 14 days. (A, B) Osteogenic differentiation was determined by ALP staining and ALP activity at day 7. (C, D) Osteogenic differentiation was analyzed by alizarin red staining and calcium level analyses at day 14. (E, F) The expressions of ALP and Runx2 were measured by real-time PCR and Western blot at day 7. The expression levels of mRNA and protein were normalized to β-actin. The data are shown as mean ± SD. *$P < 0.05$, $n = 3$. OS: osteogenic induction.

concordantly that knockdown of β-catenin promoted the osteogenic differentiation in PDLSCs while it inhibited in BMMSCs under inflammatory conditions (Fig. 5B, C).

3.4. Canonical Wnt pathway and TNF-α diversely interact in osteogenic differentiation of PDLSCs and BMMSCs

Intervention in the regulation of canonical Wnt pathway partially reversed the inhibitory effect of TNF-α on osteogenic differentiation of PDLSCs. Fig. 6A presents the results of ALP staining and alizarin red staining, demonstrating that inhibition of the canonical Wnt pathway by DKK-1 rescued the osteogenic differentiation capacity of PDLSCs compared to the exclusive TNF-α constituent group. These findings were quantitatively confirmed by ALP and calcium concentration measurements stated in Fig. 6B. Western blot analyses corroborated the effect of DKK-1, as ALP and Runx2 expressions were increased and demonstrated a reduction of β-catenin (Fig. 6C).

When using Wnt3a to activate the canonical Wnt pathway, the osteogenic differentiation capacity of BMMSCs was slightly increased, while the effect was significantly weaker than in PDLSCs. The results suggest that the inhibitory effect of TNF-α is differently regulated in BMMSCs compared to PDLSCs. When TNF-α was added, ALP and alizarin red staining patterns slightly changed by the presence or absence of Wnt3a (Fig. 6D). Again, ALP and calcium concentration measurements

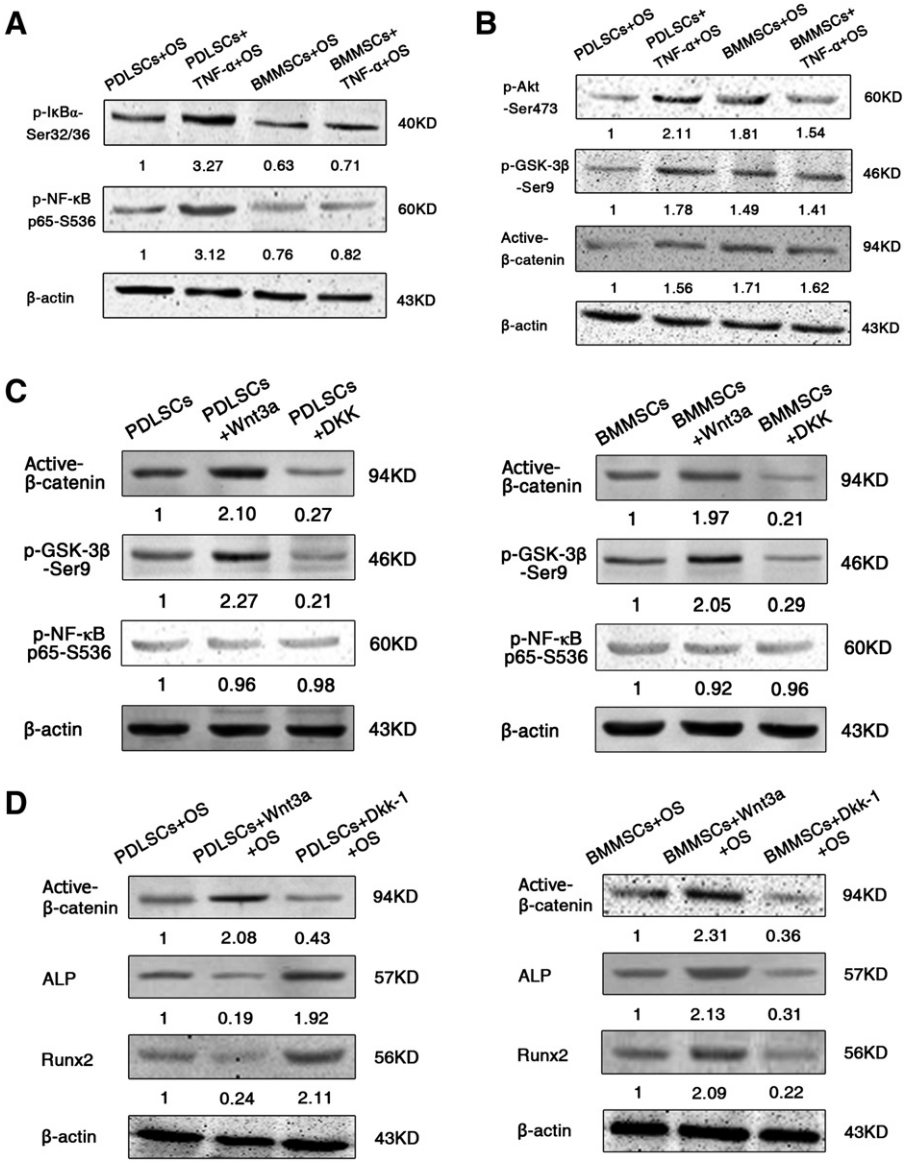

Fig. 4. Impact of canonical Wnt pathway on the inhibitory effect of TNF-α on osteogenic differentiation in PDLSCs and BMMSCs. (A, B) PDLSCs and BMMSCs were cultured in osteogenic differentiation medium with or without TNF-α for 7 days, the expressions of p-IκBα, p-NF-κB p65 (A), p-Akt, p-GSK3β and active-β-catenin (B) were measured by Western blot. (C) After treatment with Wnt3a and DKK-1, the expressions of active-β-catenin, p-GSK3β and p-NF-κB p65 were measured by Western blot. (D) Western blot analysis showed the expressions of active-β-catenin, ALP and Runx2 after PDLSCs and BMMSCs were cultured in osteogenic differentiation medium with Wnt3a and DKK-1 for 7 days. The expression levels of mRNA and protein were normalized to β-actin. The data are shown as mean ± SD. *$P < 0.05$, $n = 3$. OS: osteogenic induction.

(Fig. 6E) as much as Western blot analyses of ALP, Runx2 and β-catenin underlined these results (Fig. 6F).

4. Discussion

The results of our study revealed that PDLSCs and BMMSCs, although of the same mesenchymal origin, possess diverse stem cell characteristics, especially under the influence of a microenvironment conditioned with TNF-α. Presumably due to their tissue specific character, PDLSCs exhibit a weaker adipogenic and osteogenic differentiation capacities compared to BMMSCs.

Our investigations evidenced that TNF-α could activate both NF-κB and the canonical Wnt pathway more distinctly in PDLSCs during the osteogenic induction process. Importantly, our group has previously demonstrated that NF-κB pathway and canonical Wnt pathway cross-talk through modulating GSK3β [24], and that the canonical Wnt pathway plays an important role in regulating osteogenic differentiation of PDLSCs in different microenvironments [16]. Our analyses evidenced that the osteogenic differentiation of PDLSCs was inhibited by Wnt3a and enhanced via DKK-1, while inducing the opposite expression patterns in BMMSCs. Furthermore, Wnt3a activated the canonical Wnt pathway and DKK-1 inhibited it in both cell types. However, the NF-κB pathway was not susceptible to either Wnt3a or DKK-1 challenge. On the basis of these findings we reasoned that the different effects of TNF-α seen in PDLSCs and BMMSCs might be caused by the canonical Wnt pathway. Then, we used siRNA to knockdown β-catenin, observing that the osteogenic differentiation capacity was not affected by TNF-α after downregulation of β-catenin. Finally, after adjunct of DKK-1 or Wnt3a, the osteogenic differentiation in PDLSCs and BMMSCs could be perpetuated despite the exposure to TNF-α. These results reinforced the hypothesis that TNF-α has different effects on PDLSCs and BMMSCs mainly through targeting the canonical Wnt pathway.

Collectively, the effect of the canonical Wnt pathway on osteogenic differentiation of stem cells has two sides, promotion and inhibition,

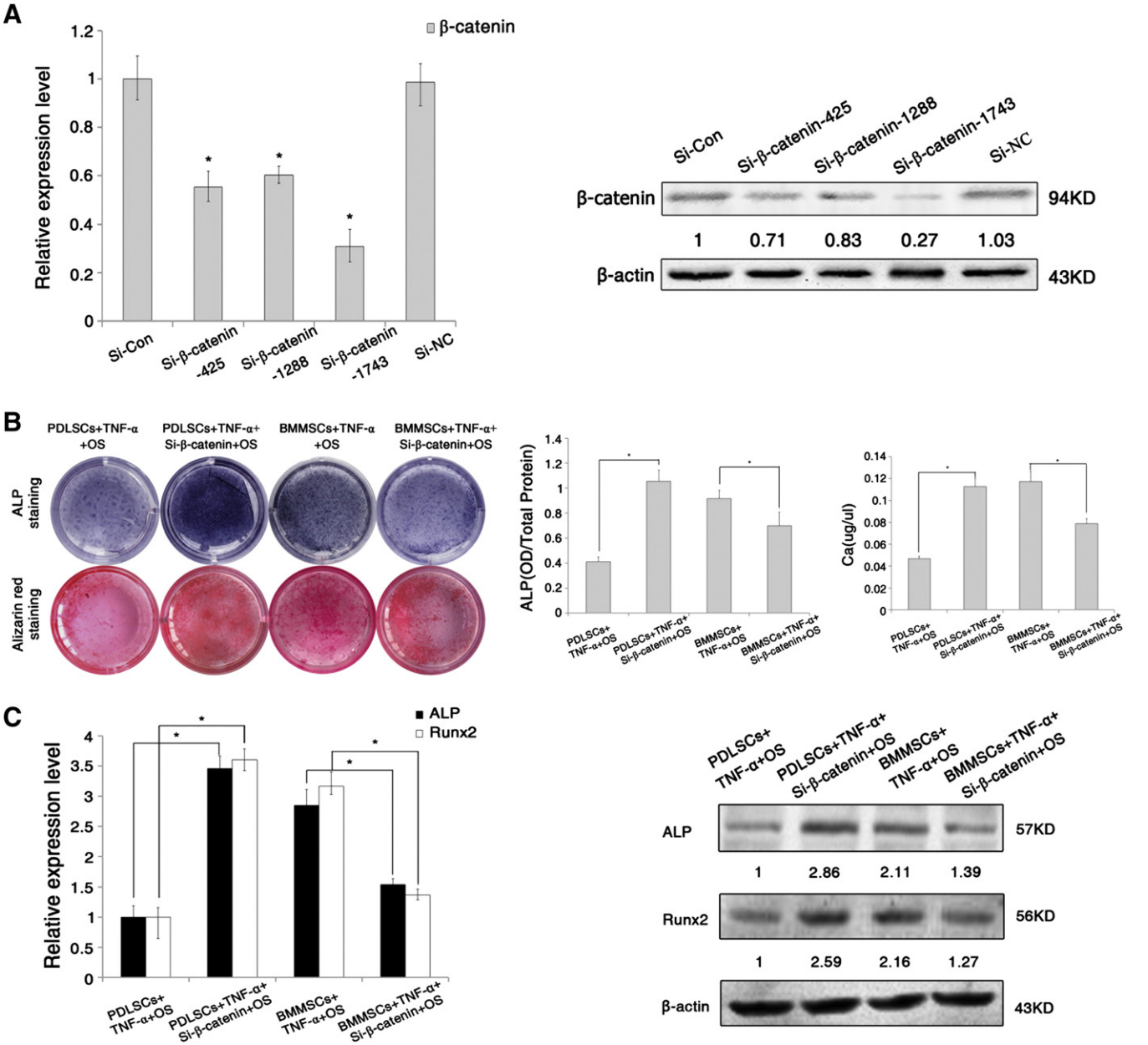

Fig. 5. Down-regulation of β-catenin has diverse effects on osteogenic differentiation of PDLSCs and BMMSCs under inflammatory conditions. To confirm the effect of β-catenin on osteogenic differentiation, PDLSCs and BMMSCs were transfected with chemically modified siRNA oligonucleotides for 1 day and then cultured in osteogenic differentiation medium with TNF-α for additional 7 or 14 days. (A) The expression levels of endogenous β-catenin after transfection were determined by real-time PCR and Western blot. (B) Osteogenic differentiation was determined by ALP staining at day 7 and alizarin red staining at day 14. ALP activity and calcium level analyses were performed at days 7 and 14. (C) The expressions of ALP and Runx2 were confirmed by real-time PCR and Western blot analyses at day 7. *P < 0.05. Si-Con: siPORT reagent alone; Si-β-catenin: chemically modified siRNA oligonucleotides; Si-NC: siRNA negative control; OS: osteogenic induction.

depending on the cell type, the microenvironment and other co-factors, as demonstrated by our previous research [20].

Knowledge about the differentiation qualities of PDLSCs in view of the signaling pathways and environmental factors involved is of substantial importance for their application in regenerative therapy, for example regarding the mechanisms of intravenously administered BMMSCs that are supposed to migrate towards dental organs [6]. Thus, attainments on the modulation of PDLSC characteristics would be beneficial for clinical regenerative therapies of dental structures.

The impact of pro-inflammatory cytokines on MSC behavior has only sparsely been elucidated yet and existing data are diverging with regard to promotion versus inhibition of lineage commitment [25,26]. Our investigations revealed that TNF-α inhibited the bone-regeneration ability of PDLSCs more than BMMSCs. This may be an effect of changes in their differentiation potential attributed to the multifunctional character of their original source, as PDL cells are the connecting alignment at the border between the alveolar bone and tooth surface that inter alia feature characteristics of non-professional antigen-presenting cells [8,9]. At this, a functional adjustment of PDLSCs to the environmental and immunological conditions as defense mechanism against inflammatory insults at the expense of osteogenic transformation can be supposed. Regarding the modulation of stem cell characteristics by an inflammatory microenvironment, the Wnt/β-catenin signaling pathway is critically involved in the transformation of PDLSCs according to

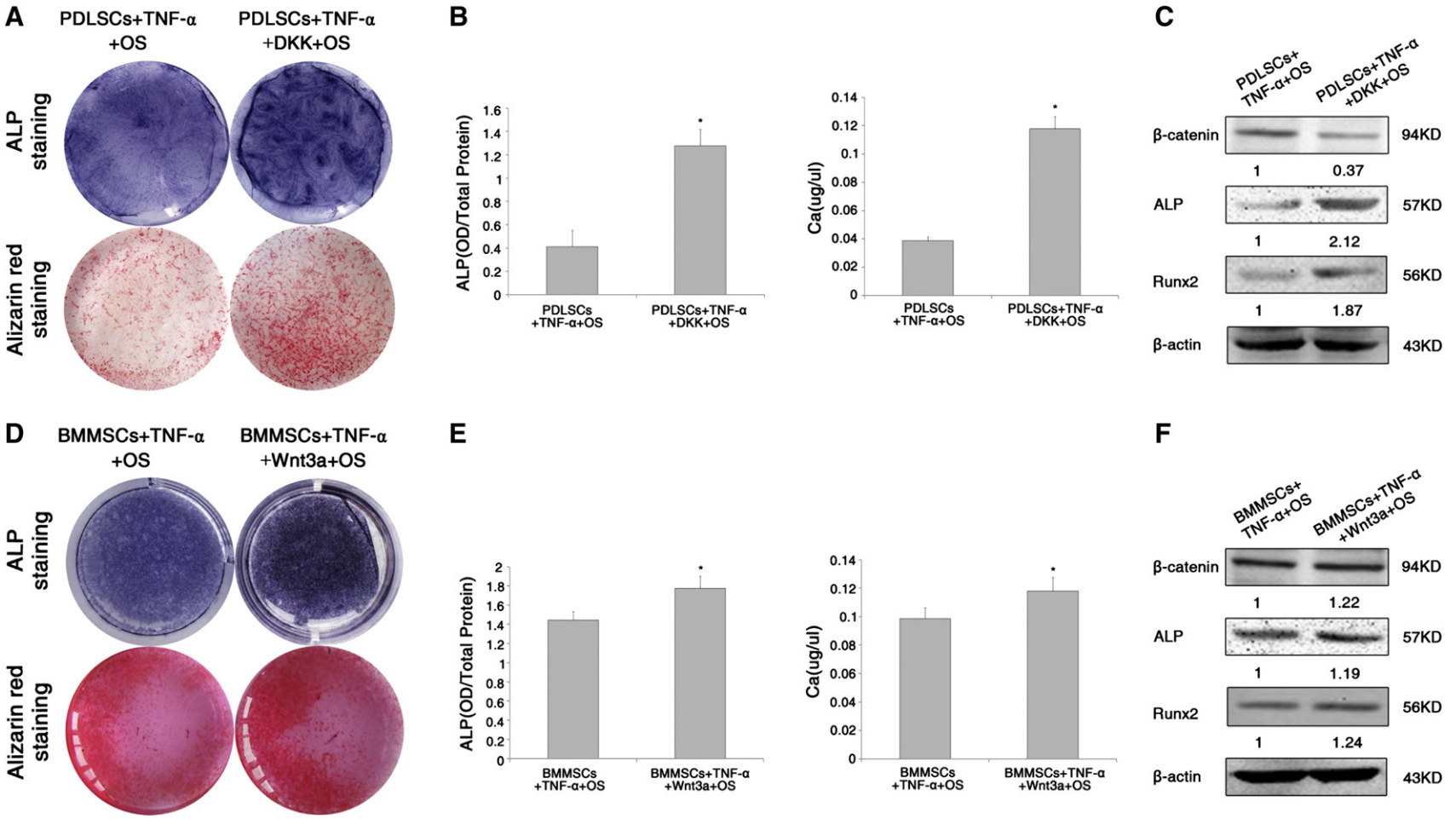

Fig. 6. Regulation of canonical Wnt pathway can partially reverse the inhibitory effect of TNF-α on osteogenic differentiation. PDLSCs and BMMSCs were cultured in osteogenic differentiation medium with TNF-α. Then, Wnt3a or DKK-1 was added for an additional 7 or 14 days. (A, D) Osteogenic differentiation was determined by ALP staining at day 7 and alizarin red staining at day 14. (B, E) ALP activity and calcium level analyses were performed at day 7 and day 14. (C, F) The expressions of β-catenin, ALP and Runx2 were measured by Western blot at day 7. The expression levels of protein were normalized to β-actin. The data are shown as mean ± SD. *$P < 0.05$, $n = 3$. OS: osteogenic induction.

our findings. Previous researches demonstrated that canonical Wnt pathway promotes osteogenic differentiation of BMMSCs [14,15]. Recently, investigations revealed that the Wnt/β-catenin signaling pathway qualifies to repress various stem cell differentiation processes, like odontoblast-like differentiation of dental pulp stem cells or osteogenic differentiation of adipose-derived MSCs [27,28], which is in accordance with the findings of our study. Against the background that the developmental plasticity of stem cells presumably accommodates the specific acquirements of the individual organ and its pathophysiological status, the modulation of PDLSC differentiation under the impact of the Wnt/β-catenin signaling pathway might reflect this incident. Various primary directions of human stem cell transformation pathways have been described [29], but up to date, differentiation processes towards a cell type with immunomodulatory characteristics, such as antigen presenting cell marker expression or phagocytotic activity, have been disclosed. Against the background that the Wnt pathway has been elucidated to impact immune cell performance of both innate and acquired immunity [30–33], a potential governance of PDLSC differentiation by this signaling network under the regulation of inflammatory cytokines towards a cell type with immune cell-like features as mentioned above is feasible and needs further investigation in the future.

Collectively, this study revealed that the periodontium incorporates a niche of resident PDLSCs with stem cell features, that are modified by inflammatory insults presumably by the major impact of the WNT/β-catenin signaling pathway. A deeper understanding of these underlying mechanisms will help to achieve considerable advances in periodontal tissue regeneration on the basis of stem cell application techniques.

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