Tescalcin knockdown inhibits osteogenic
differentiation of rat bone mesenchymal stem cells
by Wnt/β-catenin signaling pathway

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Research Article

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Abstract

Background

Bone marrow-derived mesenchymal stem cells (BMSCs) have the functions of self-renew and differentiating into osteoblasts, adipocytes and chondrocytes, which are regarded as one of the greatest bioscientific achievements in the regenerative medicine field. Tesalcin (TESC), an EF-hand Ca$^{2+}$ binding protein, plays a vital role in cell proliferation and differentiation. However, what the role of TESC in BMSCs still is unknown. The purpose of this study was to explore the functions.

Methods

Adenovirus was constructed to decrease the expression of TESC. BML-284 was used to active Wnt/β-catenin signaling pathway. qRT-PCR and western blot was used to detect the expression of mRNA and protein levels. ALP staining and activity were used to detect the change of ALP. ARS staining and quantitative analysis were used to determine the mineralization capacity. Immunofluorescence was used to show the expression of protein.

Results

Firstly, we found that the mRNA and protein levels of TESC was increased during the osteogenic differentiation. Next, we determined that TESC knockdown inhibited the expression of osteogenic-related genes and decreased the capacity of mineralization. Then, we found that Wnt/β-catenin signaling was inhibited after TESC Knockdown by detecting the Wnt/β-catenin signaling pathway-related protein expression. Afterwards, BML-284 was demonstrated to active Wnt/β-catenin signaling successfully and utilized to rescue the negative osteogenic differentiation of TESC knockdown.

Conclusion

In summary, our study indicated that TESC knockdown inhibited osteogenic differentiation of BMSCs by Wnt/β-catenin signaling pathway. We supposed that TESC acted in the progress of osteogenic differentiation as a key regulator. We provided a new target for the application of BMSCs in regenerative medicine.

1. Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs), firstly identified by Frieden[1], are pluripotent stem cells having functions of self-renew and multi-lineage differentiation, BMSCs can be differentiated into osteoblasts, adipocytes and chondrocytes by certain inductive methods[1, 2]. Preclinical studies
provided evidences that BMSCs can enhance osteogenic differentiation, increase bone mineral density, and prevent further deterioration of osteoporosis[3]. At the same time, the latest technologies such as gene modification, targeted modification and combined transplantation are promising methods to improve the therapeutic effect and efficacy of BMSCs[3, 4].

Tescalcin (TESC, CHP3) belongs to Ca\(^{2+}\) binding protein family including EF-hand and the family was first discovered and reported by Kretsinger and Nockolds in 1973[5, 6]. It has been demonstrated that TESC played an important role in various of cancers. For instance, It is reported that long noncoding RNA-TUG1 binding to miR-187-3p affects the development of pituitary adenomas by upregulating TESC[7]. Another study showed that TESC was detected to be highly expressed in hepatocellular carcinoma patients[8]. In renal cell carcinoma tissues, TESC was showed higher expression than in the normal tissues.[9]. At the same time, TESC has been demonstrated that are crucial for cell differentiation and proliferation[10–12].

Previous study showed that inhibition of glycogen synthase kinase 3 (GSK-3) phosphorylation by TESC negatively regulates cardiomyocyte hypertrophy [13]. As a key member of Wnt/β-catenin signaling pathway and the destruction complex, GSK-3 binds and phosphorylates β-catenin without Wnt, then phospho-β-catenin was ubiquitinated by b-TrCP. Proteasome acts on phospho-β-catenin and phospho-β-catenin are degraded. When Wnt is abundant, the complex is disrupted and phosphorylation of β-catenin caused by GSK-3 is blocked so that β-catenin is stabilized. Many previous studies have demonstrated that either drugs or genes can affect BMSCs in the osteogenesis by altering the Wnt/β-catenin pathway[14–16]. Therefore, we hypothesized that TESC could affect β-catenin protein expression to make changes to the Wnt/signaling pathway by binding to GSK-3β, thus changing process of osteogenic differentiation of BMSCs.

To demonstrate the hypothesis, we used the BMSCs from Sprague Dawley (SD) rats to conduct the experiments. The results demonstrated that TESC knockdown inhibited osteogenic differentiation of BMSCs in vitro by Wnt/β-catenin signaling pathway.

2. Materials and Methods

2.1 Cell culture

SD rats BMSCs were obtained from Cyagen Biosciences (Guangzhou, China). DMEM/F12 medium (Gibco, USA) containing 15% FBS (Excell Bio, China) and 1% penicillin/streptomycin (Meilunbio, China) was used for BMSCs complete medium (CM). The incubator condition is 37°C, 5% CO\(_2\). Passage 3-4 cells were utilized in all studies. For osteogenic differentiation of BMSCs, cells were treated with osteogenic medium (OM) (CM supplemented with 10 nM dexamethasone (Solarbio, China), 10 mM-glycerophosphate (Sigma, USA), and 50 mM ascorbic acid (Sigma, USA)). BMSCs were cultured with BML-284(MCE,USA for at least 24h. BML-284 was added every 3 days during osteogenic differentiation.

2.2 Adenovirus construction and Transfection
The TESC knockdown adenovirus (Shanghai Obio Technology) were constructed. When the cell confluency was approximately 50%, adenovirus enhanced green fluorescent protein (EGFP) was added to each well at MOIs (multiplicity of infection) of 10, 20, 30 and 50 cultured in CM without FBS. The medium was changed to CM containing 15% FBS after 6 hours. Fluorescence was observed and recorded by a fluorescence microscope when the confluency was 80%-90%. The knockdown efficiency was demonstrated by qRT-PCR, western blot and IF after 48 hours with TESC knockdown adenovirus.

2.3 ARS (Alizarin Red S) Staining and Quantitative Analysis

The cells were washed by PBS and incubated with 4% paraformaldehyde (PFA) solution (Saint-bio, China) at room temperature for 30 min to be fixed. BMSCs were washed again and incubated with ARS solution (Solarbio, China) for 5-15 minutes. The images were taken by an electron microscope camera system (Nikon, Japan). Then, 10% cetylpyridinium chloride solution (Solarbio Life sciences, China) were added and incubated at room temperature for 30 minutes. The solution was then transferred in equal volumes to 96-well plates. A microplate reader (ThermoFisher, USA) was used to detect the optical density (OD) values at 570nm wavelength.

2.4 ALP (alkaline phosphatase) Staining and Quantitative Analysis

The cells after 7 days of osteogenesis were incubated with 4% PFA solution at room temperature for 30 min to be fixed. The ALP staining solution (Beyotime, China) were added in the plates for 30 min at room temperature. The images were taken by an electron microscope camera system (Nikon, Japan). The protein supernatants were adjusted to the same concentration and equal volumes were added into the 96-well plate according to The Alkaline Phosphatase Assay Kit (Beyotime, China). Then, the plated was placed into a 37°C incubator for 10-30 minutes. A microplate reader was used to detect the OD values at 405nm wavelength.

2.5 RNA Extraction and qRT-PCR

RNAiso Plus (TAKARA, Japan) was used for extracting total RNA following the product instructions. In a 20 μL reverse transcription reaction system, up to 1 μg of Total RNA can be used. cDNA was generated by a reverse transcription kit (TAKARA, Japan). qRT-PCR was performed with TB Green Premix Ex Taq II (TAKARA, Japan) on an ABI 7500fast System (Life Technologies, USA). The qRT-PCR reactions contain 10 μL TB Green Premix Ex Taq II, 6 μL RNase water, 0.5μL ROX Reference Dye 50X , 1.6 μL PCR primers (TAKARA, Japan), and a 2 μL cDNA. The cycling conditions were as follows: 1cycle at 95°C for 30s and 40 cycles at 95°C for 3s and 60°C for 30 s. Housekeeping gene (Actb) was used to perform the relative expression of each mRNA. \(2^{-\Delta\Delta CT} \) method was used to analyze the data and the results are presented in fold changes. The primer sequences were showed in Table 1.

Table 1. The sequences of Primes
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer-F (5’→3’)</th>
<th>Primer-R (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat-Runx2</td>
<td>CACAGGGTGACTCCCGTTACAA</td>
<td>TGTGACCCAGTGCAAATGAAGA</td>
</tr>
<tr>
<td>Rat-OPN</td>
<td>GCCGAGGTGATAGCTTGGCTTA</td>
<td>TTGATAGCCTCATCGGACTCCTG</td>
</tr>
<tr>
<td>Rat-OCN</td>
<td>GACCCTCTCTCTGTCACTC</td>
<td>GGGCTCAAAGTCCATTGGTG</td>
</tr>
<tr>
<td>Rat-TESC</td>
<td>GCGTGTGCGTGGGACAGATG</td>
<td>CTCCATGGTGAGGAAGCGGATGTG</td>
</tr>
<tr>
<td>Rat-β-Actin</td>
<td>CACCATGTACCCAGGCAGAGC</td>
<td>CCTGCTTGGCTGATCCACATC</td>
</tr>
</tbody>
</table>

Abbreviations OCN: osteocalcin; OPN: osteopontin; Runx2: Runt-related transcription factor 2 TESC: Tescalcin

2.6 Western Blot Analysis

Radioimmunoprecipitation (RIPA) buffer with 1% protease inhibitor and 1% phosphatase inhibitor (Biotime, China) was used to lyse the cells on ice for 30 minutes. Then, the cells were scraped off and centrifuged. The concentrations were detected by a BCA protein assay kit (Epizyme Biomedical Technology (EBT), China). The total protein was separated using 10% SDS-PAGE (EBT, China) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, United States) which was incubated with Blocking Buffer (1x) (EBT, China) for 30min at room temperature to be blocked. Then, the membrane was incubated with primary antibodies at 4°C overnight. The primary antibodies were as followed: Affinity Biosciences GAPDH antibody (AF7021), RUNX2 antibody (AF5186), Osteocalcin antibody (DF12303), Osteopontin antibody (AF0227), β-catenin antibody (AF6266), phospho-β-catenin antibody (DF2989), DKK-1 antibody (AF4600), GSK-3β antibody (AF5016), Proteintech TESC antibody (11125-1-AP), BAX antibody (50599-2-Ig) and BCL-2 antibody (12789-1-AP). The membrane was incubated with a secondary antibody goat anti-rabbit IgG (S0001, Affinity Biosciences) at room temperature for 2h. Omni-ECL™ Enhanced Pico Light Chemiluminescence Kit (EBT, China) was prepared to react with the membrane. Western Blotting Detection System (GE680, United States) and Image J software were used to detect and analyze the expression of protein.

2.7 Immunofluorescence

The slides with cells were incubated with 4% PFA solution for 15 min to be fixed. Afterwards, cells were permeabilized with 0.5% Triton X-100 at room temperature for 15min. Cells were blocked with 5% goat serum diluted in PBS at room temperature for 0.5h. Then, cells were incubated with primary antibodies as followed: Affinity Biosciences: Runx2 (1:100, AF5186), Osteocalcin (1:100, DF12303), GSK-3β (1:100, AF5016), β-catenin (1:100, AF6266), TESC (1:100, 11125-1-AP, Proteintech) at 4°C overnight. The fluorescence conjugated secondary antibody (Abcam, 150077) was diluted in PBS (1:500). Then the cells were incubated with the diluted secondary antibody at room temperature for 1 hr. DAPI(4′,6-diamidino-2-phenylindole) (Solarbio, China) diluted in PBS was used to stain the nucleus in the dark for 5 min at room
temperature. Finally, the cells were observed and images were recorded by an electron microscope camera system (Nikon, Japan) equipped with proper filters.

### 2.7 Statistical analysis

GraphPad Prism9 software (LaJolla, USA) was used for analyzing all of data. ImageJ (National Institutes of Health, USA) was used for images analysis. All data were performed as mean ± standard deviation (SD). Differences between two groups was analyzed by Student’s t test, and one-way ANOVA with post-hoc Tukey test was used to analyze the differences between multiple groups. P value < 0.05 was considered to be statistically significant.

### 3. Results

#### 3.1 TESC was elevated during osteogenic differentiation of BMSCs

To ensure the stemness and the ability of osteogenesis of BMSCs, we firstly performed the ALP staining and ARS staining on the 7th day and 14th day of osteogenesis. ALP staining showed that the insoluble dark blue to blue-purple NBT-formazan were observed (Figure1A). ARS staining showed the number of mineralized nodules were stained red (Figure1B). Then, we performed qRT-PCR and western blot assay to detect the expressions of osteogenic marker genes including runt-related transcription factor 2 (Runx2), Osteocalcin (OCN), Osteopontin (OPN) which were elevated at both mRNA and protein levels on 14th day of osteogenesis (Figure1C, D). The results above showed BMSCs have the capacity of osteogenic differentiation and stemness. Meanwhile, we found that TESC expression was increased on 14th day of osteogenesis by qRT-PCR and western blot assay (Figure1C, D, E).

#### 3.2 TESC adenovirus can effectively knock down the expression of TESC

Firstly, BMSCs were transfected with TESC adenovirus EGFP to verify the optimal MOI. The MOIs of 10, 20, 30 and 50 were selected, respectively. The results showed the fluorescence brightness is the brightest in the field of 50. This concentration can meet the needs of subsequent experiments (Figure2A). Next, we transinfected the cells with the adenovirus concentration of 50. The results of qRT-PCR and western blot assay showed that TESC adenovirus can knockdown down the expression of TESC effectively at both mRNA and protein levels (Figure2B, C). Among them, Ad-TESC-01 has the best effect (Figure2B, C). The IF results showed that Ad-TESC-01 can reduce the expression of TESC (Figure2D).

#### 3.3 TESC knockdown inhibited osteogenesis of BMSCs

The qRT-PCR and western blot assay results showed that the expression of Runx2, OCN and OPN was decreased after osteogenic differentiation of 14 days at both protein and mRNA levels (Figure3A, B) in the ad-TESC groups. The ALP staining showed that BMSCs transfected with Ad-shTESC-01 were stained slightly. We concluded that TESC knockdown reduced the production of ALP and ALP activity (Figure3C, D). Meanwhile, the ARS staining showed that TESC knockdown inhibited the mineralized nodules
formations (Figure 3E, F). Finally, IF results showed TESC knockdown inhibited the expression of osteogenic marker genes (Figure 3G).

### 3.4 TESC knockdown inactivated Wnt/β-catenin signaling pathway and BML-284 activated it

Western blot assay was performed to detect changes of β-catenin, phospho-β-catenin (p-β-catenin), Dickkopf 1 (DKK-1) and GSK-3β. The results showed that DKK-1, phospho-β-catenin and GSK-3β in Ad-shTESC-01 group were expressed higher than control group, but β-catenin expression was lower, conversely (Figure 4A, B). BML-284 chemical structure was shown in Figure 4C. The results of western blot assay showed β-catenin was expressed higher in 1.0uM groups than other concentration groups (Figure 4D, E). The results indicated that the expression of β-catenin was higher in BML-284 groups than control groups after osteogenesis (Figure 4F, G).

### 3.5 Inhibition of osteogenic differentiation of BMSCs caused by TESC knockdown was reversed partially by BML-284 activating Wnt/β-catenin signaling pathway

The results of western blot assay showed that the decreased β-catenin proteins expression and increased GSK-3β and p-β-catenin proteins expression due to TESC knockdown were partially rescued by BML-284 (Figure 5A, B). IF showed β-catenin and GSK-3β had the same results with western blot assay after osteogenic differentiation for 3 days (Figure 5C, D). At the same time, Runx2, OCN and OPN expression were partly rescued by BML-284 (Figure 5E, F). IF showed Runx2 and OCN had the same results as western blot (Figure 5G, H). Finally, the reduced ALP activity and mineralized nodules formations caused by TESC knockdown were rescued by BML-284 (Figure 5I-L).

### 4. Discussion

It has been reported that TESC should play a role in cell growth and differentiation[12]. Levaya and Slepak[11] found that TESC knockdown was required for HL-60 differentiation into granulocytes. Additionally, they found that TESC was critical for the primary stem cells and hematopoietic precursor cell lines differentiating into megakaryocytes[10]. Kobayashi[13] found that TESC interacted with GSK3β and inhibited GSK3α/β phosphorylation. As GSK3β phosphorylation leads to β-catenin phosphorylation, and the proteasome can degrade phospho-β-catenin leading to β-catenin instability. β-catenin nucleus translocation is inhibited, so is Wnt/β-catenin signaling which is known for involving BMSCs differentiation[17]. Hence, the above evidences support the hypothesis that TESC knockdown enhances GSK3-β phosphorylation leading to inhibition of Wnt/β-catenin signaling.

Firstly, ALP and ARS staining were performed at 7th and 14th day osteogenesis of BMSCs. The insoluble dark blue to blue-purple NBT-formazan of ALP staining and the red mineralized nodules of ARS staining showed that the cells we used have the ability of mineralization and ALP production. Then, Runx2, OCN and OPN was increased at 14th day of osteogenesis detected by qRT-PCR and western blot. The above results demonstrated that the cells have the stemness and meet the needs of further experiments. At the same time, we determined that TESC expression is increased during osteogenic differentiation using qRT-
qPCR and western blot assay, respectively (Fig. 1). The results showed that TESC may function in the progress of BMSCs differentiation. However, what role of TESC plays in osteogenic differentiation of BMSCs still remains unknown. Hence, we constructed the TESC knockdown adenovirus and assure the knockdown efficiency (Fig. 2). The knockdown efficiency of TESC was reliable so that we picked up the most efficient adenovirus-Ad-TESC-01 for the ARS, ALP and IF. Then, we found that Runx2, OCN and OPN expression were decreased after BMSCs being transfected TESC knockdown adenovirus after osteogenic differentiation for 14 days, so the IF results. As we know, runx2 is vital for the proliferation and differentiation of BMSCs and has the capacity of inducing the major bone matrix protein genes expression in vitro, and runx2 is an important transcriptional regulator in the process of targeted osteogenic differentiation of BMSCs[18]. Osteocalcin (OCN), which is the most abundant non-collagenous protein in bone and exclusively generated by osteoblasts, considered as one of the markers of bone formation[19]. Osteopontin (OPN) is a secreted protein involved in many biological activities and is commonly identified as an important factor in neuron-mediated and bone mass production[20]. So, we concluded that TESC knockdown can affect the osteogenesis. Next, we demonstrated that TESC knockdown inhibited ALP activity and mineralization capacity. It was reported that alkaline phosphatase (ALP) is highly expressed in the cells of mineralized tissues, which is considered as one of the most important enzyme for BMSCs osteogenic differentiation[21]. Finally, we found that TESC knockdown can reduce the mineralized nodules. The ability of mineralization is considered as one of the most important markers of osteogenesis. In summary, the above evidences (Fig. 3) demonstrated that TESC knockdown inhibits osteogenic differentiation of BMSCs. However, the mechanism still needs to be investigated.

As we mentioned before, TESC has the capacity of inhibiting GSK3-β phosphorylation[12]. Therefore, Wnt/β-catenin signaling pathway comes to mind naturally. So we selected four Wnt/β-catenin signaling pathway-related proteins which were β-catenin, phospho-β-catenin, Dickkopf 1 (DKK-1) and GSK-3β. DKK-1 is widely identified as an inhibitor of canonical Wnt/β-catenin signaling[22]. We determined that TESC knockdown inhibited Wnt/β-catenin signaling by up regulating DKK-1 and GSK-3β. β-catenin, as a core protein of Wnt/β-catenin signaling[17], was down-regulated and phospho-β-catenin expression was increased. These results showed that Wnt/β-catenin signaling was inhibited after TESC knockdown which was consistent with the hypothesis. BML-284 was an activator of Wnt/β-catenin signaling[23]. Although previous experiments[23, 24] have demonstrated its effectiveness, we still need to verify its functionality through experiments. Firstly, we selected five different concentrations ranging from 0.5uM to 10uM, and verified the highest expression in the concentration of 1.0uM. Next, we verified that β-catenin was up-regulated after BML-284 being induced for 14 days. In summary, BML-284 is a reliable Wnt/β-catenin signaling pathway activator (Fig. 4).

Finally, we validated that BML-284 could partially rescue TESC-mediated osteogenic differentiation by Wnt/β-catenin signaling pathway. The expression changes caused by TESC knockdown were partially rescued by BML-284 activating the Wnt/β-catenin signaling. In addition, we demonstrated the decreased ALP activity and mineralization capacity due to TESC knockdown was rescued by BML-284. Above all, we concluded that Wnt/β-catenin signaling was an important pathway for TESC-mediated osteogenesis of
BMSCs. The results demonstrated that TESC knockdown can inhibited Wnt/β-catenin signaling pathway to inhibited the progress of osteogenesis. (Fig. 5).

Although the above conclusion was firstly reported, the present research has its limitations as well. First, we did not perform experiments to explore the functions of TESC in vivo. Second, we did not check the results of TESC over-expression. Therefore, more experiments are needed in the future to support this hypothesis.

5. Conclusion

We firstly demonstrated that TESC knockdown inhibits osteogenic differentiation rats BMSCs. Although many factors are involved and play important roles in the process of osteogenic differentiation, our experiments increased the understanding of the role of TESC in BMSCs and also provide a new target for the application of BMSCs.

Abbreviations

BMSC Bone marrow-derived mesenchymal stem cells
TESC Tescalcin
ALP Alkaline phosphatase
ARS Alizarin red staining
OPN Osteopontin
OCN Osteocalcin
Runx2 runt-related transcription factor 2
DKK-1 Dickkopf-1
GSK-3β Glycogen synthase kinase 3β

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable
Vailability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the shortage of public datasets. but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Dong Wu: Conceptualization, Writing - Original Draft, Writing - Review & Editing; Longhuan Piao: Methodology, Software; Sen Qin: Validation, Data Curation; Shuai Liu: Resources, Investigation ,Visualization and Formal analysis; Guangbin Wang: Supervision, Project administration, Funding acquisition. All authors read and approved the final manuscript.

Acknowledgements

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References


**Figures**
The expression of TESC was increased during osteogenic differentiation. (A-B) ALP staining and ARS of BMSCs staining after osteogenic differentiation 7 days and 14 days, respectively. Scale bar, 250μm. (C-E) qRT-PCR and western blot assay were used to detect the mRNA level and protein level expression of TESC and the osteogenesis-related genes OCN, OPN, Runx2 and TESC after osteogenic differentiation 14 day.
Mean ± SD. Groups (14d) vs. Control groups (0d). p < 0.05; **, p < 0.01; All experiments are repeated three times.

Figure 2

**TESC knockdown adenovirus efficiency was verified.** (A) The optimal MOI was demonstrated by observing under a fluorescent microscope. scale bar, 100 μm. (B-C) qRT-PCR and western blot assay were performed
to demonstrate the efficiency of TESC knockdown at both mRNA and protein level, respectively. Mean ± SD, All groups vs. Control groups. (D) IF was used to illustrate the TESC protein expression after TESC knockdown. Scale bar, 50μm. ns, not significant *, p < 0.05; **, p < 0.01; All experiments are repeated three times.

Figure 3

TESC knockdown inhibited osteogenic differentiation. (A-B) After TESC knockdown, the osteogenic-related genes OCN, OPN and Runx2 were detected after osteogenic differentiation for 14 days by western blot assay and qRT-PCR at protein and mRNA level. Mean ± SD. All groups vs. control. (C-F) ALP staining and
activity were detected after osteogenic differentiation 7 days. ARS staining and quantitative statistical analysis were demonstrated after osteogenic differentiation 14 days. Mean ± SD. scale bar, 250μm. All groups vs. control. (G) IF was performed to illustrated Runx2 and OCN protein expression after knockdown after osteogenic differentiation for 3 days. Scale bar, 50μm. ns, not significant; *, p<0.05; **, p<0.01; ***, P<0.001; ****, P<0.0001. All of experiments are repeated three times.

Figure 4
TESC knockdown inactivated the Wnt/β-catenin signaling pathway and BML-284 activated it. (A-B) After TESC knockdown, the protein level expression of Wnt/β-catenin signaling pathway-related genes including β-catenin, p-β-catenin, GSK-3β and DKK-1 were detected by western blot assay and quantitative statistical analysis after osteogenic differentiation for 14 days. Mean ± SD. Vs. control. (C) The chemical structure of BML-284. (D-E) The optimal concentration of BML-283 acted on BMSCs was demonstrated by western blot assay and quantitative statistical analysis. Mean ± SD, BML-284 group vs. PBS group. (F-G) The effect of BML-284 on Wnt/β-catenin signaling on osteogenic differentiation for 14 days was verified by western blot assay and quantitative statistical analysis. Mean ± SD, vs. PBS group. * p < 0.05, **, p < 0.01, *** p < 0.001. All experiments are repeated three times.
Figure 5

The osteogenic differentiation capacity of BMSCs induced by TESC knockout could be partially rescued by BML-284. (A-B) After TESC knockdown and adding BML-284, Wnt/β-catenin signaling pathway-related genes including β-catenin, p-β-catenin and GSK-3β were detected by western blot assay and quantitative statistical analysis after osteogenic differentiation for 14 days, Mean ± SD. Compared with groups. (C-D) IF and statistical analysis were performed to verified the expression of β-catenin and GSK-3β after
osteogenic differentiation 3 days. Mean ± SD. Scale bar, 50μm. Compared with groups. (E-F) After TESC knockdown and adding BML-284, osteogenic-related genes OCN, OPN and Runx2 were detected after osteogenic differentiation 14 days by western blot assay and statistical analysis. Mean ± SD. Compared with groups. (G-H) Runx2 and OCN protein expression was verified by IF and statistical analysis after osteogenic differentiation 3 days. Mean ± SD. Scale bar, 50μm. Compared with groups. (I-L) ALP staining was observed and recorded under a microscope and ALP activity were performed at 7 days of osteogenesis. ARS staining were observed and recorded under a microscope and quantitative statistical analysis were demonstrated after osteogenic differentiation 14 days. Mean ± SD. Scale bar, 250μm. Compared with groups. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. All the experiments are repeated three times.