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Original Article

Effect of TEAD4 on multilineage differentiation of muscle-derived stem cells

Jinze Wang[1*], Feixu Zhang[1*], Huidi Yang[1,2*], Huikuan Wu[1], Rong Cui[1], Yunjie Zhao[1], Cuihua Jiao[1], Xianxin Wang[1], Xin Liu[1], Liqiong Wu[1], Guangpeng Li[1], Xia Wu[1]

[1]*State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, Inner Mongolia University, Hohhot 010070, Inner Mongolia, People's Republic of China;* [2]*School of Basic Medical Sciences, Inner Mongolia Medical College, Hohhot, 010110, China.* [*]*Equal contributors.*

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Abstract: TEAD4 is a member of transcriptional enhancer factor (TEF) family of transcription factors and plays a pivotal role in regulating embryonic development and muscle regeneration. Known previously, dysfunction of TEAD4 in mouse myoblasts impairs myotube development. However, the effects of TEAD4 on multipotency of muscle-derived stem cells (MDSCs) have not been clearly understood. Recently, bovine MDSCs (bMDSCs) were successfully isolated from adult bovine muscle. Our derived bMDSCs could differentiate into mesodermal cells, including myotubes, adipocytes, and osteoid cells. Our results also revealed that bMDSCs had the capacity to develop into ectodermal and endodermal lineages including neuron-like cells and insulin-secreting cells. After TEAD4 knock-down (TEAD4-KD), bMDSCs still kept the original capacity to differentiate into neuron-like cells and insulin-secreting cells, as shown by acquisition of both neuronal and pancreatic markers normally expressed in differentiated cells. However, up-regulation of CAV3 and βMHC failed during myogenesis of bMDSCs with TEAD4-KD, although TEAD4-KD in bMDSCs did not affect osteoid cells and myotube formation. More interestingly, adipogenic differentiation of TEAD4-KD bMDSCs was significantly suppressed. During adipogenic differentiation, TEAD4-KD systematically impaired upregulation of TEAD1, TEAD2, and TEAD3, as well as the activation of C/EBP2, ADD1, and PPARγ as the key transcription factors for adipogenic differentiation. Finally, TEAD4-KD led to the failure of adipogenesis from bMDSCs. Together, our results support that TEAD4 is essential during adipogenic differentiation of bMDSCs. It has little effect on myogenesis of bMDSCs, and does not affect ostegenesis, neurogenesis, or pancreatic differentiation of bMDSCs. Our findings will be helpful for future study on the roles of the TEAD family during differentiation of MDSCs, and for controlling MDSC differentiation for stem cell applications.

Keywords: Muscle-derived stem cells, TEAD4, multilineage differentiation

Introduction

TEAD factors (TEADs), including TEAD1, TEAD2, TEAD3, and TEAD4, act as mediators of the Hippo signaling pathway. They regulate the processes of cellular proliferation, developmental oncogenesis, stem cell maintenance, and differentiation through either Hippo-dependent or Hippo-independent patterns [1-6]. TEAD members bind to their co-activators and regulate blastocyst and organ formation, like heart and muscle. However, different individual members of the TEAD family play specific roles during organogenesis [7]. Skeletal muscle is comprised of multinucleated muscle fibers that specialize in contractile movement. During muscle development, muscle precursor cells and myoblasts migrated from somites into nascent muscles, and fuse to form post-mitotic muscle fibers. As a member of the TEAD family, TEAD4 is highly expressed in developing skeletal muscle and somites [8, 9]. As the upstream factors of TEAD4, MYOD1 and MYOG bind to the TEAD4 promoter to activate expression during myogenic differentiation [10]. Knock down of TEAD4 in C2C12 myoblasts resulted in the formation of shortened myotubes. TEAD4 activates several muscle genes, including Myogenin, CDKN1A, and Caveolin 3 during myogenic differentiation, as determined by ChIP-chip and RNA-seq [11]. However, a recent study showed that TEAD1 compensates for the function of TEAD4, and supports TEAD4-KD in primary myoblasts resulting in differentiation into long multinucleate myotubes [12]. These previous findings reveal that TEADs play pivotal and complicated roles

in myogenesis of muscle cells. However, how TEAD4 acts as a particular member to affect the regenerative capacity and to regulate multilineage differentiation of muscle stem cells, has not yet been explored.

Satellite cells and muscle-derived stem cells (MDSCs) are the resident adult stem cells in muscle tissues that maintain the remarkable regenerative potency of skeletal muscles. As myogenic precursors, satellite cells are committed to the myogenic lineage by differentiating into myoblasts [13]. On the other hand, MDSCs represent a predecessor of the satellite cells that possess a higher regeneration capacity with a broader range of multilineage capabilities [14]. Particularly, MDSCs are mainly responsible for muscle growth and regeneration as the multipotent stem cells in skeletal muscle [15]. Recently, several MDSCs with different morphologies were isolated from muscle by sequential pre-plating, which proved the multilineage capacities for successful differentiation into chondrogenic, osteal, endothelial, adipose, muscle, and neural cell lineages *in vitro* and *in vivo* [16-18]. However, how TEAD4 effects the regenerative capacity and multilineage of muscle stem cells is not known.

In this study, MDSCs were derived from bovine muscle tissues. First, these bovine MDSCs were proven to have the capacity to differentiate into cells of all germ layers, including neuron-like cells, myotubes, adipocytes, osteoid cells, and insulin-secreting cells. Next, TEAD4 was knocked down in bMDSCs to investigate the role of TEAD4 on multilineage differentiation. TEAD4-KD bMDSCs were studied during induced germ layer differentiation to explore it's potential role.

Materials and methods

Unless otherwise mentioned, all reagents used were purchased from Life Technologies Company (USA). All the procedures involving the care and use of animals were approved by Inner Mongolia University's Animal Care and Use Committee.

Isolation and cultivation of bovine muscle-derived stem cells

MDSCs were isolated from the hind limb muscle tissues of newborn LuXi Yellow cattle. Minced skeletal muscle tissues were digested by 1 mg/mL collagenase IV for 2-3 h. The muscle cells were cultured into 1% gelatin coated dishes with MDSC medium at 37°C in a humidified atmosphere containing 5% CO_2 for 1 h, and then suspended cells were transferred to fresh gelatin dishes. After 24 h, unattached cells were collected and replated, and the pre-plates were repeated every 24 h for 5 days to eliminate fibroblasts and endothelial cells. Isolated MDSCs were passaged until 90% confluency. MDSC medium: Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 20% (v/v) horse serum (HS, Gibco).

Differentiation and trans-differentiation assay

For MDSCs differentiated into myotubes, the cells were passaged into 12 well plates. After 24 h of passage, MDSC medium was replaced with myogenic medium (DMEM supplemented with 2% HS), and the medium was renewed every two days. After 8 to 10 days, multinuclear myotubes could be observed, and myosin heavy chain (hereafter MYH) was detected by immunostaining. Fusion percentages of myotubes were assay as described [19] with a little modification. After immunostaining, differentiated cells were analyzed using red and blue emission filters, respectively, of Zeiss Observision A1 fluorescence microscope. In 10 visual fields, the number of myotubes, nuclei within myotubes, and the total number of nuclei per well were counted using the Image-J software from the National Institutes of Health (http://rsb.info.nih.gov/ij/). Fusion percentage of myotubes was determined as the number of myotube nuclei in relation to the total number of nuclei.

For MDSC transdifferentiated into neuron-like cells, adipocytes, osteoid cells, and insulin-secreting cells (IS cells), MDSC medium was replaced with neurogenic medium, containing DMEM, 10% FBS, and 2% Dimethyl sulfoxide (DMSO); osteogenic medium, containing DMEM, 15% FBS, 10 mM β-glycerophosphate sodium, 20 mM dexamethasone and 50 μg/mL Vitamin C; adipogenic medium, containing DMEM, 15% FBS, 10 μg/mL insulin, 10 μM dexamethasone and 200 mM Indometacin; pancreatic medium, containing DMEM, 10% KSR, 0.1 μM retinoic acid, 10 ng/ml bFGF, 1% ITS, 0.4

mM Monothioglycerol, 2 mM L-glutamine and 1 mM sodium pyruvate. Culture medium was replaced every 3 days. After 9 to 15 days, the differentiated cells were detected by immunocytochemistry and q-PCR.

Flow cytometry, immunohistochemical and histological staining

For flow cytometry, MDSCs were trypsinized, and incubated with CD90 (BD Bioscience, USA), CD34 (BD Bioscience, USA) and CD45 (Sino Biological, China) antibody for 20 min. After washing three times in PBS, the cells were analyzed in FACSAria (BD Biosciences).

For immunostaining, MDSCs and differentiated cells were washed with PBS, fixed in 4% paraformaldehyde for 20 min, then permeabilized with 1% Triton X-100 for 30 min, followed by blocking with 2% BSA (Sigma). The cells were incubated in primary antibody at 4°C overnight. After washing with PBS, the cells were incubated in secondary antibody at 37°C for 1 h, and then nuclei were stained by DAPI. SCA1, MYF5, PAX3/7, MYOG, NESTIN, and MYH antibodies were purchased from Cruz Biotechnology. NeuN, PDX1, and insulin antibodies were purchased from Abcam. Alkaline phosphatase (AKP) staining was performed with BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime) according to manufacturer's instructions.

For histology, after differentiation for 9 to 15 days, differentiated cells were washed with PBS, and then fixed in 4% paraformaldehyde for 20 min. After additional washes in PBS, the cells were incubated in 2% Alizarin red S for 30 s, in 0.3% Oil Red O solution for 10 min, and in 10 μg/mL dithizone for 2 h to detect mineralization, lipid droplets and IS cells, respectively.

For quantitative analysis of osteogenic and adipogenic differentiation, Alizarin red S stain was extracted with 10% cetylpyridinium chloride for estimating matrix mineralization at room temperature for 2 h. The absorbance of the extracted Alizarin red S stain was measured at 570 nm. Oil Red O stain was extracted with isopropanol for 15 min at room temperature, and the absorbance of the triplicate samples was measured at 510 nm.

Pancreatic differentiation of bMDSCs was quantitatively analyzed by detecting the concentration of insulin in the medium. After differentiation for 12 days, the cells were washed with DMEM medium, containing 0 mM, 15 mM and 25 mM glucose, respectively, and then cultured in these medium for 2 h. After incubation, insulin in medium was detected with insulin ELISA Kit (EIA06236, Wuhan Xinqidi Biologyical Technology. China) according to manufacturer's instructions.

Lentiviral production and infection

The lentiviral expressing and packaging vectors, pLL3.7, psPAX2 and pMD2.G, were purchased from Addgene (USA). Four functional shRNAs for silencing TEAD4 gene and a scrambled shRNA were synthesized, and cloning into pLL3.7 backbone, named pLL3.7-shRNA-528, pLL3.7-shRNA1097, pLL3.7-shRNA1331, pLL3.7-shRNA1448 and pLL3.7-shRNA-Control (Table S1). Expressing vectors together with packaging vectors at ratio of 3:2:1 (pLL3.7: psPAX2: pMD2.G) were transfected into 293FT cells using Lipofectamine 2000, following the instructions of the manufacturer. The virus-containing supernatant was collected after 2 days, and was concentrated by Lenti-X Concentrator (Clontech, USA). Virus supernatant supplemented with 8 μg/ml polybrene was used to infect bMDSCs. After two rounds of infection in two days, virus-containing medium was replaced with MDSC medium. The infected bMDSCs expressed green fluorescent protein (GFP) that was driven by CMV promoter in pLL3.7 vector.

SDS-PAGE and Western blot analysis

MDSCs were lysed in mammalian protein extraction reagent (CWBiotech, China) on ice for 20 min. Protein concentration was ascertained using Varioskan Flash (Thermo Fisher Scientific, USA). The lysate supernatant was boiled for 10 min, and incubated on ice for 5 min. Proteins were separated on 12% SDS-polyacrylamide gel, and transferred to PVDF membrane (Millipore, USA). After blocking in 5% skim milk in TBST (0.1% Tween-20, 500 mM NaCl, and 20 mM Tris-Cl (pH 7.5)) for 1 h, the membrane was incubated with TEAD1, TEAD2, TEAD3, and TEAD4 antibody (ab133535, ab92279, ab75192 and ab97460 Abcam, USA) at 4°C overnight, respectively. After washing with TBST, the membrane was incubated with HRP-conjugated goat anti-rabbit or donkey anti-goat IgG antibody (diluted 1:5000; Jackson Immunoresearch,

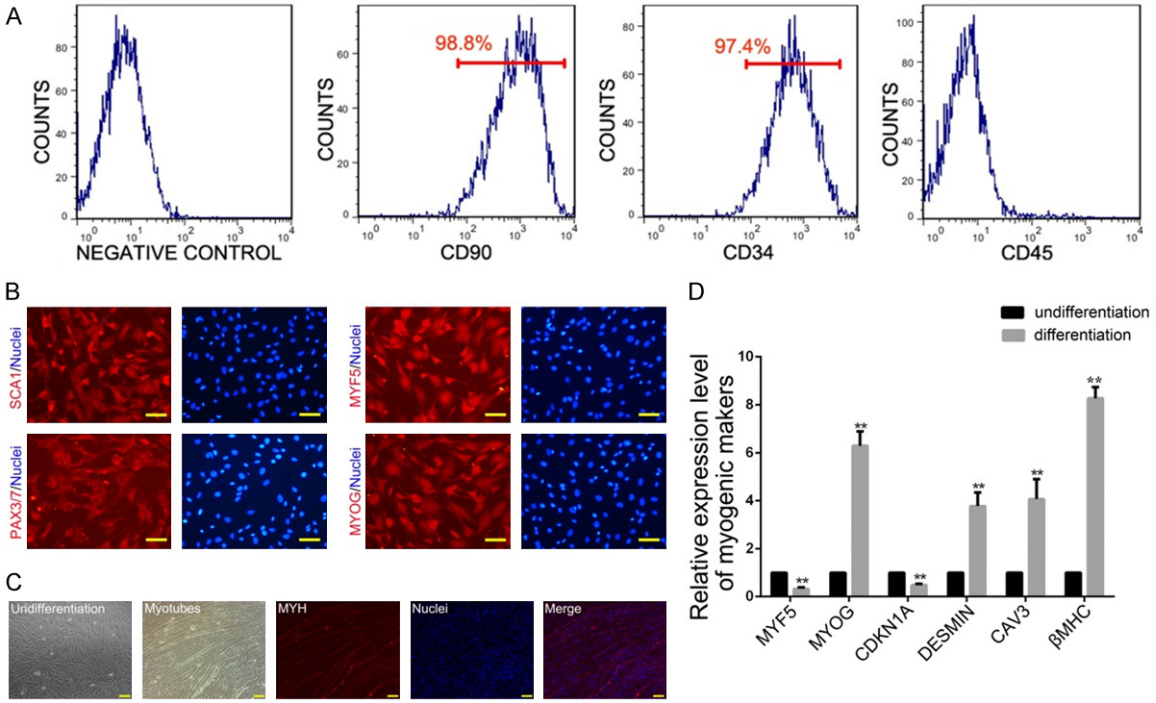

Figure 1. Isolation and characterization of bMDSCs. A. Flow cytometry analysis of surface markers in bMDSCs. B and C. Immunostaining of muscle stem cell specific markers and MYH in bMDSCs and bMDSC-derived myotubes, respectively, bar=100 μm. D. Expression of myogenic genes in differentiated and undifferentiated bMDSCs.

USA) for 3 h at room temperature. Detection was performed using Tanon-5200 chemoluminescence detector (YuanPingHao Biotech, China).

Quantitative RT-PCR

Total RNA was isolated from cells using MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan) according to manufacturer's instructions. RNA quality and quantity were examined using a NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific, USA). Concentration of total RNA was adjusted to 100 ng/μl, and reverse transcription was performed on MasterCycler Nexus (Eppendorf). Quantification of cDNA was performed by Applied Biosystems® 7500 Real-Time PCR System. The primer sequences are provided in Table S2. The mRNA levels were normalized by GAPDH mRNA level.

Statistical analysis

All values are presented as the mean ± SD for all experiments and controls. Statistically significant differences between groups were identified using t-test. *P* values of <0.05 were considered to significant, and $^{**/a/b/c}$Values with different superscripts differed significantly ($p< 0.05$). ND indicated no difference.

Results

Bovine muscle-derived stem cells have the capacity to differentiate into cells of three germ layers

After flow cytometry analysis, bMDSCs expressed surface markers of muscle stem cells, CD90 and CD34, and were negative for CD45 (**Figure 1A**). Other muscle stem cells markers including SCA1, PAX3/7, MYF5, and MYOG, were also positive staining in these cells (**Figure 1B**). Bovine MDSCs showed adherent character during cultivation. They were relatively long and thin and slightly concave, similar to mesenchymal stem cells (**Figure 1C**). After myogenic differentiation for 72 h, multinucleated myotubes with positive MYH staining were observed (**Figure 1C**). During myogenesis of MDSCs, Both MYF5 and cyclin-dependent kinase inhibitor 1A (CDKN1A) were significantly down-regulated, while MYOG, DESMIN, CAVEOLIN3 (CAV3), and βMHC were significantly up-regulated (**Figure 1D**). Together, the results clearly indicate that bMDSCs express markers of muscle stem cells and have myogenesis ability.

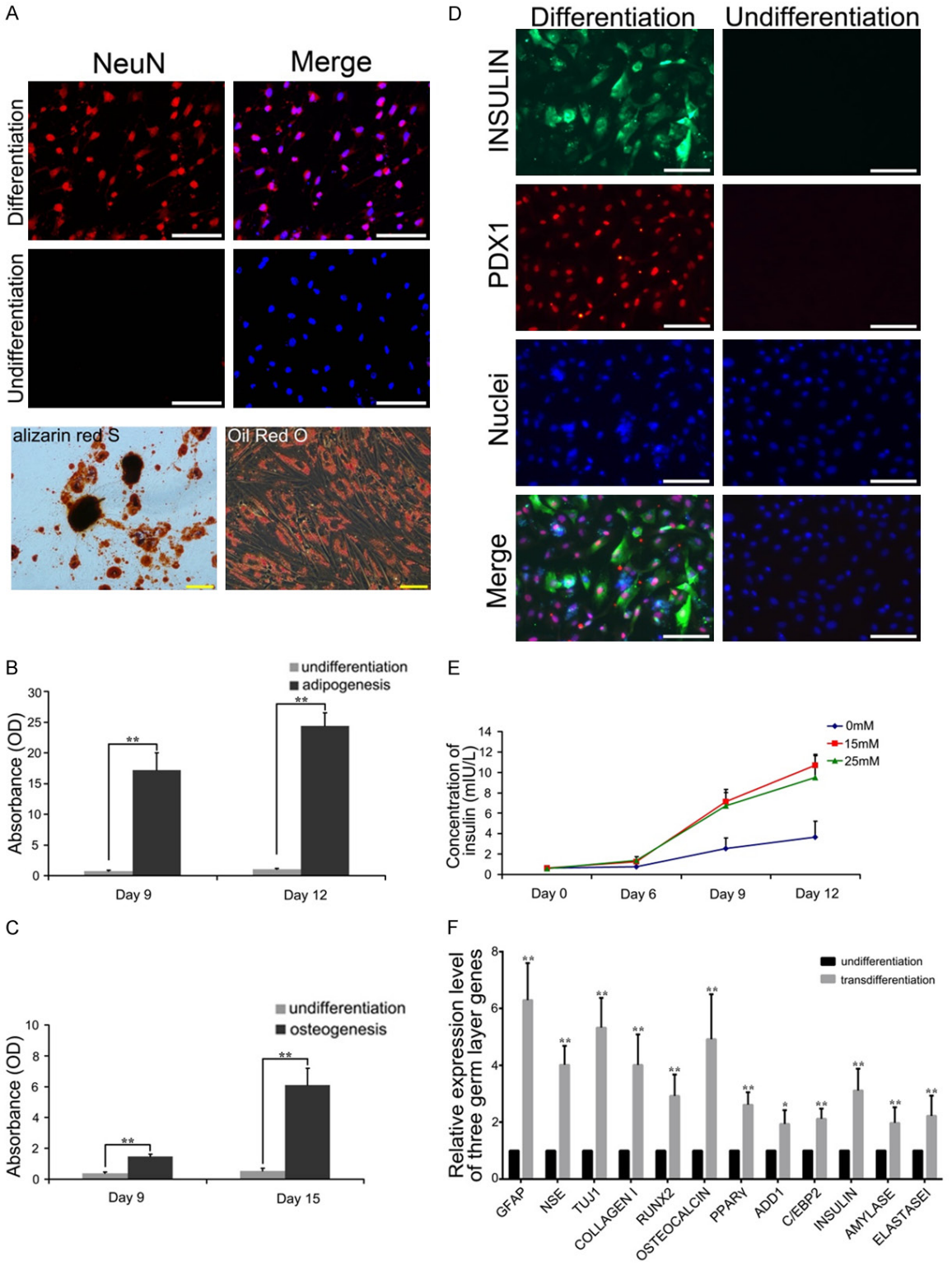

Figure 2. bMDSCs differentiate into cells of all three germ layers. A. Immunochemical and histological staining of NeuN, alizarin red S, and Oil Red O in bMDSC-derived neuron-like cells, osteoid cells, and adipocytes, bar=100 μm. B and C. Adipogenic and osteoblastic differentiation of bMDSCs were quantified using isopropanol and cetylpyridinium chloride monohydrate, respectively. D. Immunostaing of Insulin and Pdx1 in bMDSC-derived IS cells, bar=100 μm. E. Quantitative analysis of Insulin in medium during pancreatic differentiation of bMDSCs. F. Expression of three germ layers genes in differentiated and undifferentiated bMDSCs.

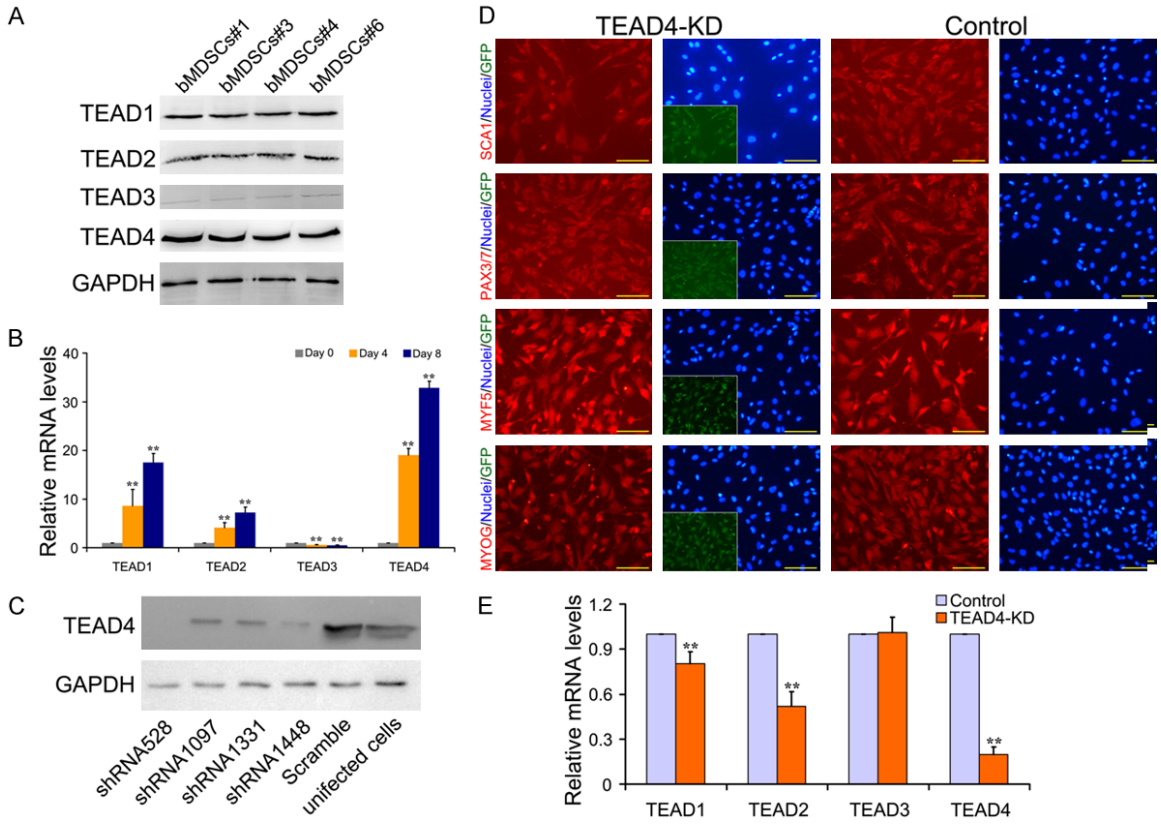

Figure 3. Expression of TEADs and knock down of TEAD4 in bMDSCs. A. Immunoblots showing TEADs protein levels in four bMDSC lines. B. Expression of TEADs during myogenic differentiation of bMDSCs. C. TEAD4 protein level in TEAD4-KD bMDSCs that expressed different shRNAs. D. Immunostaining of muscle stem cell specific markers in TEAD4-KD and control bMDSCs, GFP expression in these cells was showed in inserted picture, bar=100 μm. E. Expression of TEADs in TEAD4-KD and control bMDSCs.

Next, bMDSCs were differentiated into cells of three germ layers, like neuron-like cells, adipocytes, osteoid cells, and insulin-secreting cells. After culturing in neural induction medium, the bMDSC-derived cells became positive for NeuN and Nestin, markers typical for neural cells (**Figure 2A**, S1). In mRNA levels, additional neuronal markers, such as glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), and βIII-Tubulin (TUJ1) were significantly up-regulated during a time course of 9 days (**Figure 2F**). After culturing in either adipogenic or osteogenic medium, the bMDSC-derived cells became positive for Oil Red O, alizarin red S, and AKP staining (**Figure 2A**, S1). Compared to undifferentiated bMDSCs, the droplet and mineralization nodules significantly increased in the bMDSC-derived cells after adipogenic and osteogenic differentiation, respectively (**Figure 2B**, **2C**). Some adipogenic and osteogenic markers, including peroxisome proliferates activated receptor gamma (PPARγ), adipocyte determination and differentiation-dependent factor-1 (ADD1), CCATT enhancer-binding protein 2 (C/EBP2), Type I collagen, RUNX2, and OSTEOCALCIN were all up-regulated (**Figure 2F**). Interestingly, when the bMDSCs were cultured in pancreatic medium, dithizone positive cells could be achieved (Figure S1). After immunostaining, these cells were insulin and PDX1 positive (**Figure 2D**), which also had up-regulated expression of insulin, AMYLASE, and ELASTASE1 (**Figure 2F**). In addition, when stimulating differentiated cells by glucose, the secretion of insulin increased significantly during the time from day 0 to day 12 (**Figure 2E** and Table S3). These results showed that bMDSCs were multipotent cells that had the capacity to differentiate into cells of ectoderm, mesoderm and endoderm.

Knock down of TEAD4 has little effect on myogenesis of bMDSCs

A previous study showed that TEADs exist in the mouse primary and C2C12 myoblasts, and

have an important role in myogenesis [12]. However, the mechanism was still unclear for TEADs regulating myotube formation of muscle-derived stem cells. In bMDSCs, TEAD proteins could be detected, and expression of TEAD4 was highest while TEAD3 was lowest among four TEADs (**Figure 3A**). When bMDSCs were induced to undertake myogenic differentiation *in vitro* for 8 days, mRNA expressions of TEAD1, TEAD2, and TEAD4 were significantly induced, but expression of TEAD3 was significantly down-regulated (**Figure 3B**, $p<0.01$). Among the three up-regulated TEADs, TEAD4 was highly up-regulated from 19.1 to 32.8 times when compared the levels in undifferentiated cells at day 4 and day 8, respectively (Table S4). In order to investigate the effect of TEAD4 on differentiation of bMDSCs, its expression in bMDSCs was silenced by shRNA modifying knockdown. For obtaining the optimal effect of TEAD4 knockdown in bMDSCs, the candidates of shRNA were first screened from a pool of our constructed DNA plasmids, including sequence fragments of shRNA1097, shRNA1331, shRNA1448, shRNA528 and scrambled-RNA by real-time PCR and Western blotting. The short hairpin RNA528 plasmid was selected for its highest knockdown efficiency when comparing with others (Figure S2A). Western blotting and immunostaining assay further proved that TEAD4 protein was successfully reduced to undetected levels because of the significantly strong knockdown efficiency of shRNA528 (**Figures 3C**, S2B). TEAD4-KD did not affect the expression of muscle stem cell markers in undifferentiated bMDSCs (**Figure 3D**). Further, expression of TEAD1 and TEAD2 decreased significantly in TEAD4-KD bMDSCs, but knock down of TEAD4 did not affect expression of TEAD3 (**Figure 3E**, $p<0.01$).

After myogenesis, although TEAD4-KD bMDSC-derived myotubes lost the TEAD4 staining in the cytoplasm and nuclei, compared to controls (**Figure 4A**), long multinucleate myotubes were observed both in TEAD4-KD and control bMDSCs-derived cells (**Figure 4B**). The fusion rate of the muscle fibers was similar between all of differentiated cells that were derived from either TEAD4-KD or controls (**Figure 4C**, $p>0.05$). Results from these assays to determine the expression of myogenic genes revealed that TEAD4-KD in bMDSCs did not affect the expressions of MYOG, CDKN1A, and DESMIN during the induced differentiation. However, CAV3 as a well-defined TEAD4 target gene and βMHC were significantly down-regulated (**Figure 4D**). In addition, TEAD4-KD in bMDSCs did not affect the dynamic expression of mRNA for TEAD1, TEAD2, and TEAD3 during myogenic differentiation (**Figure 4E**). Together, knock down of TEAD4 in bMDSCs did not affect myotube formation, but the regulators of myotube fusion, CAV3 and βMHC failed to be up-regulated in the TEAD4-KD bMDSCs during myogenic differentiation. Therefore, these results confirm that TEAD4-KD has little effect on myogenesis of bMDSCs *in vitro*.

Knock down of TEAD4 suppresses adipogenic differentiation of bMDSCs

In order to determine whether TEAD4 has effects on regulating multipotency of bMDSCs, TEAD4-KD bMDSCs were differentiated into other cells of three-germ layer. When the cells were induced to adipogenesis, TEAD4-KD bMDSCs-derived adipocytes only had small triglyceride droplets assay with Oil Red O staining, in comparison with those differentiated from normal bMDSCs, showing the characteristics of mature adipocytes with large triglyceride droplets (**Figure 5A**). Quantitative analysis showed that knock down of TEAD4 in bMDSCs significantly suppressed bMDSCs ability to differentiate into adipocytes (**Figure 5B**, $p<0.01$). For mRNA expression, the mature adipocyte markers, LPL and AP2 were expressed significantly lower in TEAD4-KD bMDSC-derived cells than those in normal bMDSC-derived cells (**Figure 5C**). The expression of key transcriptional factors for adipogenic differentiation, C/EBP2, ADD1, and PPARγ were also lower in TEAD4-KD bMDSC-derived cells, compared to controls (**Figure 5C**). Different from myogenesis of bMDSCs, during adipogenesis of bMDSCs, the expression of TEAD1, TEAD2, and TEAD4 in controls were all up-regulated at day 6, but down-regulated at day 12 while expression of TEAD3 was only up-regulated at day 12 (**Figure 5D**). However, during adipogenesis of TEAD4-KD bMDSCs, up-regulation of TEAD1 and TEAD2 expression was inhibited at day 6 (**Figure 5D**, $p<0.01$), and the expression of TEAD3 was down-regulated from day 0 to day 12 (**Figure 5D**, $p<0.05$). These results suggest that TEAD4-KD significantly reduces the capacity of bMDSCs to commit to an adipogenic fate.

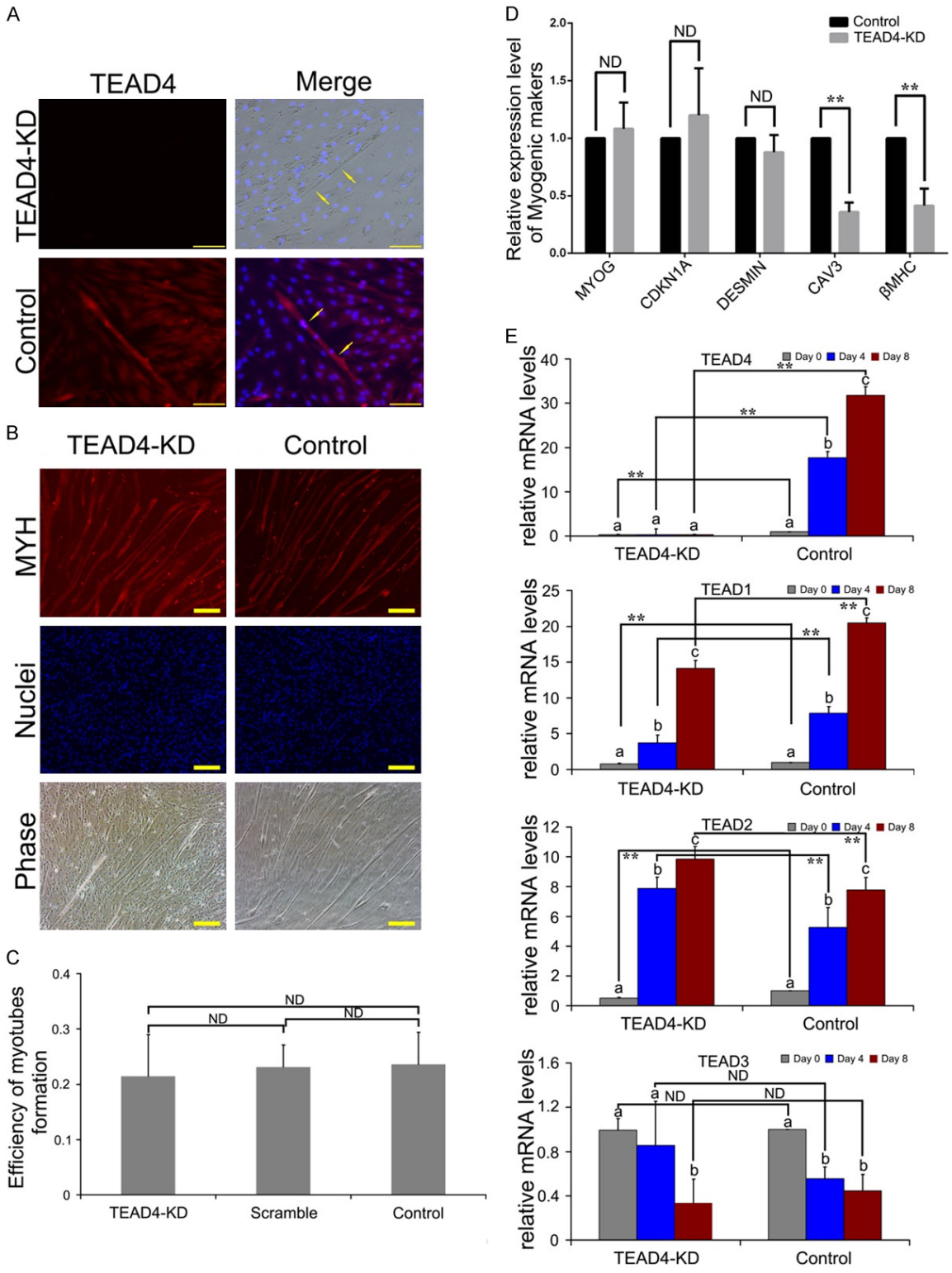

Figure 4. Analysis of myogenic differentiation in TEAD4-KD and control bMDSCs. A and B. Immunostaining of TEAD4 and MYH in TEAD4-KD and control bMDSC-derived myotubes, arrows showing the myotube, bar=100 μm. C. Fusion percentage of myotubes in differentiated TEAD4-KD and control bMDSCs. D. Expression of myogenic genes in differentiated TEAD4-KD and control bMDSCs. E. Expression of TEADs in TEAD4-KD and control bMDSCs during myogenesis.

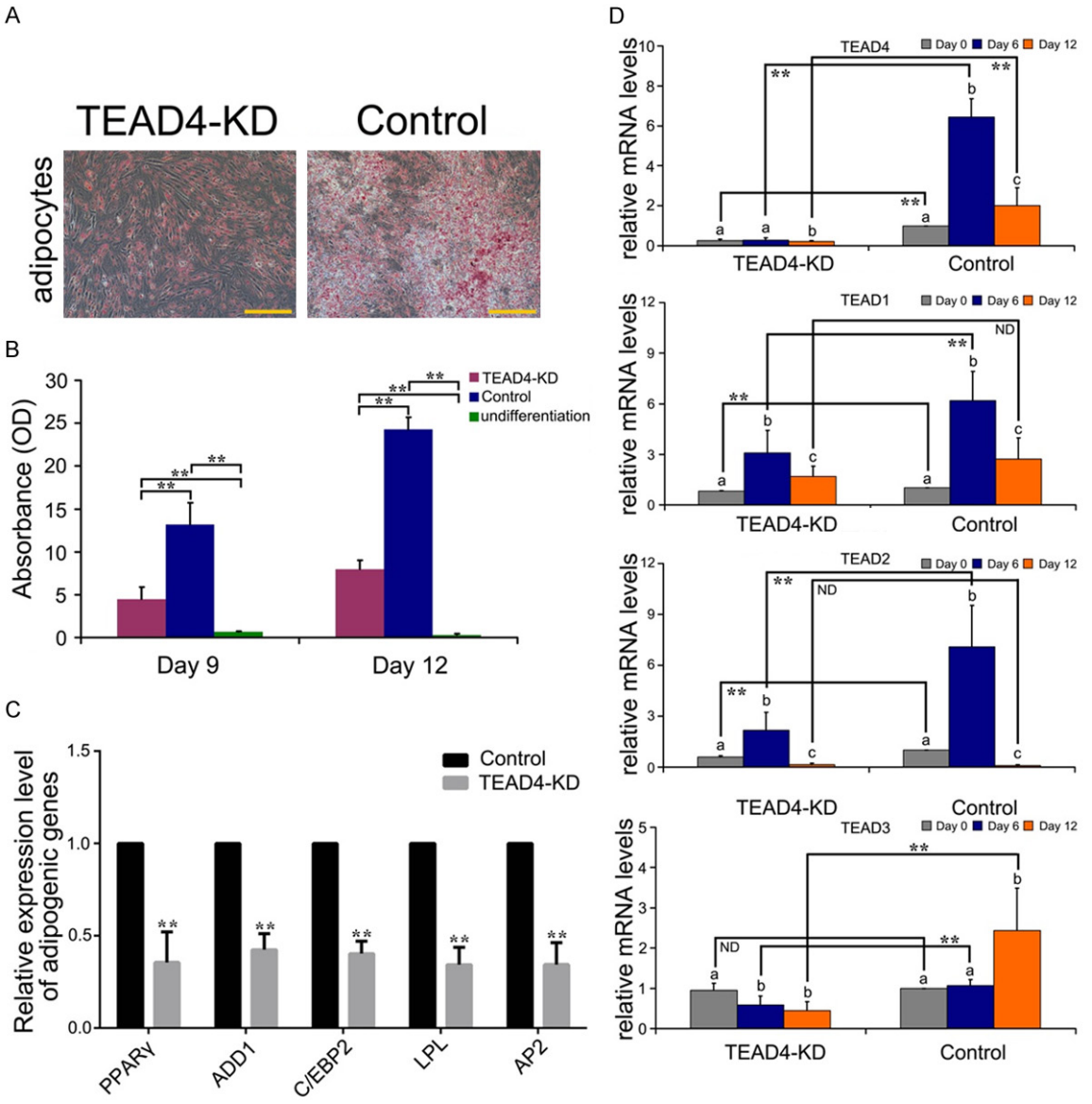

Figure 5. Analysis of adipogenic differentiation in TEAD4-KD and control bMDSCs. A. Oil red O staining detected TEAD4-KD and control bMDSC-derived adipocytes, bar=200 μm. B. Adipogenic differentiation of TEAD4-KD and control bMDSCs was quantified using isopropanol. C. Expression of adipogenic genes in TEAD4-KD and control bMDSC-derived adipocytes. D. Expression of TEADs genes in TEAD4-KD and control bMDSCs during adipogenesis.

Knock down of TEAD4 does not impair bMDSC differentiation into osteoid cells, neuron-like cells, and insulin-secreting cells

TEAD4-KD bMDSCs were differentiated into osteoid cells, neuron-like cells, and IS cells. When inducing bMDSCs to osteogenesis, mineralization nodules and AKP-positive cells were observed in both TEAD4-KD and control bMDSCs-derived cells (**Figures 6A**, S3). Quantitative mineralization showed that osteoid cells derived from TEAD4-KD bMDSCs and controls had similar levels of alizarin red S stain (**Figure 6B**). There was also no difference for mRNA levels of osteogenic markers, including Type I collagen, RUNX2 and OSTEOCALCIN between the differentiated cells from either TEAD4-KD or normal bMDSCs (**Figure 6C**). Dynamic expression of TEADs showed TEAD1, 2, and 3 were up-regulation, while TEAD4 was not significantly changed in control bMDCs from 0 to 9 days during osteogenesis. These factors also kept

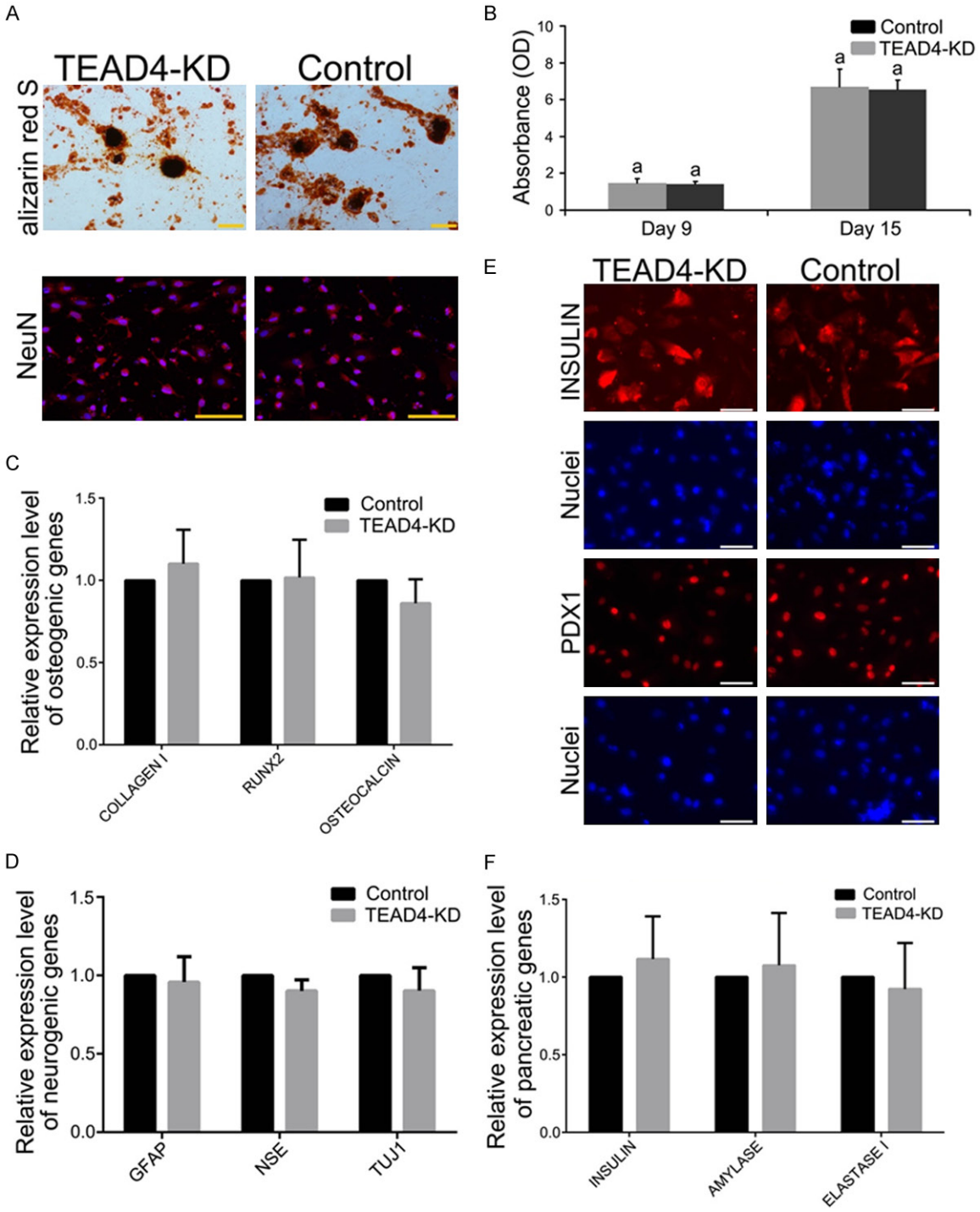

Figure 6. Analysis of neurogenic, osteoblastic, and pancreatic differentiation in TEAD4-KD and control bMDSCs. A. Immuno-cytochemical and histological staining of NeuN and alizarin red S in TEAD4-KD and control bMDSC-derived neuron-like cells and osteoid cells, bar=100 μm. B. Osteoblastic differentiation in TEAD4-KD and control bMDSCs was quantified using cetylpyridinium chloride monohydrate. C and D. Expression of osteogenic and neurogenic genes in TEAD4-KD and control bMDSC-derived osteoid and neuron-like cells, respectively. E. Immunostaining of Insulin and Pdx1 in TEAD4-KD and control bMDSC-derived IS cells, bar=100 μm. F. Expression of pancreatic genes in TEAD4-KD and control bMDSC-derived IS cells.

up-regulation in TEAD4-KD cells that was similar to the dynamic expression of control cells during osteogenesis (**Figure 7**).

After neurogensis, NeuN and NESTIN positive neuron-like cells were derived from both TEAD4-KD and control bMDSCs (**Figures 6A**, S3),

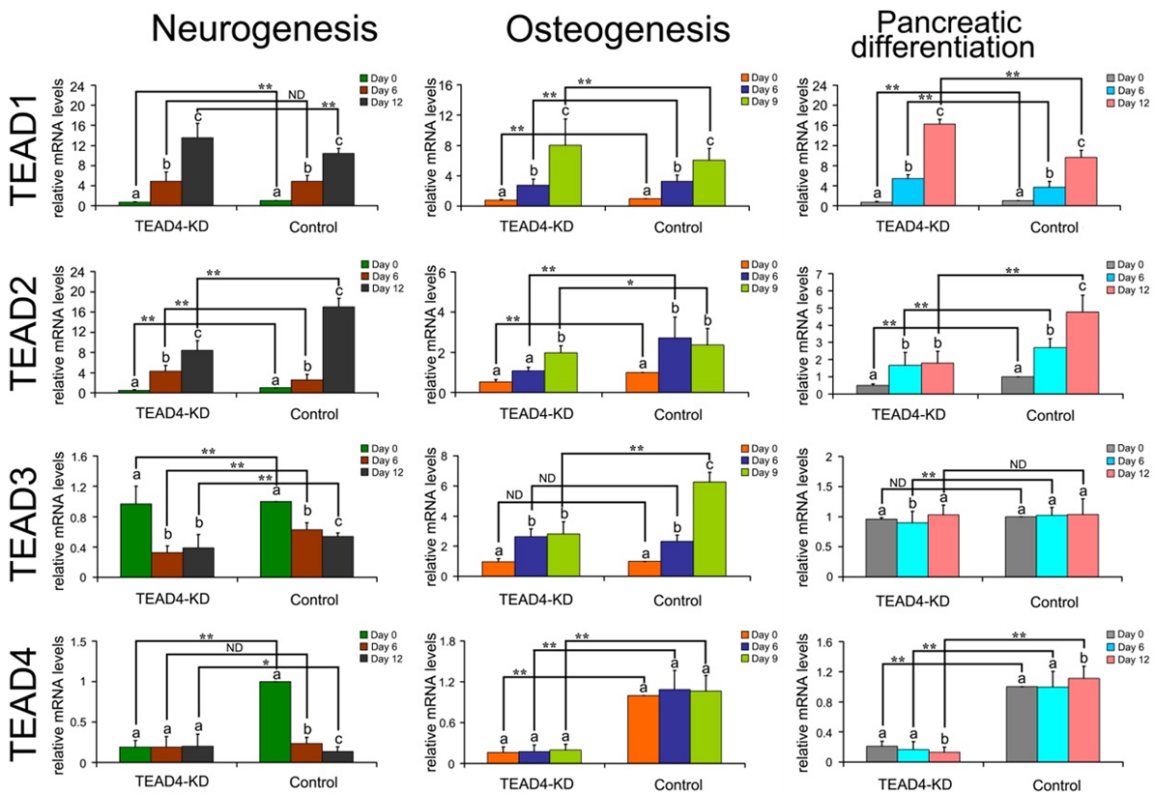

Figure 7. Expression of TEADs genes during neurogenic, osteogenic, and pancreatic differentiation of TEAD4-KD and control bMDSCs.

and expression of neuronal markers showed no difference between differentiated cells from either TEAD4-KD or normal bMDSCs (**Figure 6D**). For neurogenesis from 0 to 12 days, up-regulation of TEAD1, 2, and down-regulation of TEAD3, 4 were detected in control cells, and the similar dynamic expression of TEAD1, 2, and 3 in TEAD4-KD cells was also observed (**Figure 7**).

TEAD4-KD bMDSCs also maintained the capacity to differentiate into IS cells that were dithizone, insulin, and PDX1 positive (**Figures 6E**, S3). During pancreatic differentiation, secretion of insulin was comparable in IS cells differentiated from TEAD4-KD and normal bMDSCs (Figure S4 and Table S5, $p>0.05$), the expression of many pancreatic markers had no significant difference between these two groups of differentiated cells (**Figure 6F**). Expression of TEAD1 and TEAD2 was up-regulated in TEAD4-KD and control cells from 0 to 12 days, while TEAD3 and TEAD4 were not significantly changed during this process (**Figure 7**).

Together, TEAD4 was not necessary for bMDSCs to differentiate into many cell lineages of ectoderm, mesoderm and endoderm, like neuron-like cells, osteoid cells, and IS cells.

Discussion

TEADs play a comprehensive role in mammalian development. TEAD4, as one of four known members of TEADs, has a highly conserved DNA-binding domain and binds the same cis-acting sequence [20]. During muscle development, TEAD4 binds several muscle regulators, and plays an important role for muscle differentiation in mouse primary and C2C12 myoblasts [11, 12]. Muscle-derived stem cells as a predecessor of satellite cells and myoblasts in muscle tissue of adults are considered to possess a high regeneration capacity. Until now, mesenchyme-like, sphere-like, and non-adherent MDSCs have been isolated from muscle tissue, and these cells have different multipotency *in vivo* and *in vitro* [21-23]. Recently, only non-adherent mouse MDSCs have had the capacity to differentiate into endodermal, mesodermal,

and ectodermal cell types [18, 23]. In this study, the mesenchyme-like bovine MDSCs were derived from hind limb muscle tissues of newborn cattle. They were similar with human and mouse MDSCs as proven by the expression of muscle stem cell markers [21]. Bovine MDSCs were multipotent and could thus differentiate into various cells of all three-germ layers, including myotubes, neuron-like cells, osteoid cells, adipocytes, and insulin-secreting cells. Their mutlipotency was very similar to mouse MDSCs [16-18, 23]. Bovine MDSCs expressed all four TEADs, and most high-level expression of TEAD4 was detected among these TEADs. In addition, TEAD4 was also robustly up-regulated, and revealed important roles during bMDSC differentiation into myotubes. However, when TEAD4 expression was silenced in bMDSCs, formation of long multinuleate myotubes did not occur. These results were similar to that previously reported for TEAD4-KD primary myoblasts [12]. Expression analysis of other TEADs revealed that TEAD1 and TEAD2 may compensate for TEAD4 function to regulate formation of myotubes in primary myoblasts [12] and can be normally up-regulated during myogenesis of TEAD4-KD bMDSCs. In addition, TEAD4-KD in bMDSCs impaired expression of CAV3 and βMHC, which were consistent with the previous findings when TEAD4 was silenced in primary myoblasts during myogenic differentiation [12]. These results suggest that TEADs have similar roles in regulating MDSCs and myoblasts differentiate into myotubes.

For investigating regulation of TEAD4 in multipotency of MDSCs, TEAD4-KD bMDSCs were transdifferentiated into adipocytes, osteoid cells, neuron-like cells, and IS cells. Interestingly, knockdown of TEAD4 inhibited bMDSC differentiation into adipocytes, and few triglyceride droplets were observed in the TEAD4-KD bMDSC-derived adipocytes. During adipogenesis, it was found that C/EBP2, a key transcriptional factor for launching adipogenic differentiation, was activated. It triggered transcription of PPARγ and C/EBP1, which regulated genes whose expression helped to establish the adipocyte phenotype. Proteomic analysis confirmed that C/EBP2 interacted with YAP/TAZ/TEAD complex, promoting YAP/TAZ TEAD-dependent transcription [24]. In this study, TEAD4-KD in bMDSCs significantly suppressed activation of C/EBP2 and PPARγ during adipogenic differentiation. Up-regulation of ADD1, AP2, and LPL was inhibited, which led to the failure of adipogenesis. The results suggested that TEAD4 is involved the regulation and activation of adipogenesis. In addition, knock down of TEAD4 in bMDSCs also significantly suppressed up-regulation of TEAD3 at later stages of adipogenesis. A previous study confirmed that TEAD3 was necessary to regulate pre-adipocyte proliferation and differentiation into adipocytes [25]. Therefore, our results suggest that TEAD4 affects expression of both adipogenic genes and other TEADs to regulate adipogenic differentiation.

When differentiating TEAD4-KD bMDSCs into nerve cells, osteoid cells, and IS cells, derivation of these cells was not affected. Previously, expression of TEAD1, 2, and TEAD4 were detected in nerve cells, but TEAD1 and TEAD2 were important to regulated neuronal development and number by the Hippo signaling pathway [3, 26]. In this study, we found that knock down of TEAD4 did not affect up-regulation of TEAD1 and TEAD2 during neurogenesis, thus revealing that nerve cells are equally derived from TEAD-KD and control bMDSCs. During osteogenesis, TEADs regulated bone-marrow-derived skeletal stem/stromal cells and mesenchymal stem cells (MSCs) homeostasis and proliferation by the Snail/Slug-YAP/TAZ axis [27, 28], while Runx2, as a master factor of bone and cartilage development, regulated these cells differentiate into osteoid cells [29, 30]. Knock down of TEAD4 in bMDSCs did not impair dynamic expression of other TEADs, and expression of Runx2 was normally activated during osteogenesis, and then did not affect formation of osteoid cells. For pancreas development, TEAD1 and its coactivator YAP activated key pancreatic signaling mediators and transcription factors [4]. Knock down of TEAD4 in bMDSCs also did not impair reactivation of TEAD1 during pancreatic differentiation. Therefore, insulin-secreting cells were equally derived from TEAD4-KD and control bMDSCs.

In summary, bMDSCs as multipotent stem cells have the capacity to differentiate into the cells of three germ layers. Furthermore, TEAD4-KD did not affect bMDSC differentiation into neuron-like cells, osteoid cells, and insulin-secreting cells, but had little effect on myogenesis by impairing up-regulation of CAV3 and βMHC.

However, knockdown of TEAD4 in bMDSCs significantly inhibited adipogenic differentiation, as shown by the significantly reduced levels of droplets, and significantly suppressed expression of key transcriptional factors for adipogenic differentiation and markers for mature adipocytes. Therefore, TEAD4 activity is essential for adipogenic differentiation of MDSCs. Our results will be helpful to further investigate the mechanism of TEAD4 actions during adipogenic differentiation and to control the induction of MDSC differentiation for stem cell applications.

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Disclosure of conflict of interest

None.

Address correspondence to: Xia Wu and Guangpeng Li, State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, Inner Mongolia University No. 24 Zhao Jun Road, Hohhot 010070, Inner Mongolia, People's Republic of China. Tel: 86-471-5227683; Fax: 86-471-5227683; E-mail: wuxia@imu.edu.cn (XW); gpengli@imu.edu.cn (GPL)

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Table S1. shRNA for silencing TEAD4

Functional Sites in TEAD4 (bp)	Forward Sequence of shRNA	Reverse complementary sequence of shRNA
528-547	5'AACGGACAAGCCCATCGACAATGATTCAAGAGATCATT-GTCGATGGGCTTGTCCTTTTTTC3'	5'TCGAGAAAAAAGGACAAGCCCATCGACAATGATCTCTT-GAATCATTGTCGATGGGCTTGTCCGTT3
1097-1116	5'AACGCTCCAAGCTCTGGATGTTGGTTCAAGAGACCAA-CATCCAGAGCTTGGAGCTTTTTTC3'	5'TCGAGAAAAAAGCTCCAAGCTCTGGATGTTGGTCTCTT-GAACCAACATCCAGAGCTTGGAGCGTT3'
1331-1350	5'AACGGGCAGACCTCAACACCAACATTCAAGAGATGTTG-GTGTTGAGGTCTGCCCTTTTTTC3'	5'TCGAGAAAAAAGGGCAGACCTCAACACCAACATCTCTT-GAATGTTGGTGTTGAGGTCTGCCCGTT3'
1448-1467	5'AACGCAAGCAGGTGGTGGAGAAAGTTCAAGAGA-CTTTCTCCACCACCTGCTTGCTTTTTTC3'	5'TCGAGAAAAAAGCAAGCAGGTGGTGGAGAAAGTCTCTT-GAACTTTCTCCACCACCTGCTTGCGTT3'
Control	5'AACGAGGTGGTTCGATGACTCATTCAAGAGATGAGTCATC-GAACCACCTCTTTTTTC3'	5'TCGAGAAAAAAGAGGTGGTTCGATGACTCATCTCTT-GAATGAGTCATCGAACCACCTCGTT3'

Table S2. real-time PCR primers

Primers	Forward sequence	Reverse sequence
MYF5	GGCCTGCAAAGCATGCAAAA	CCTCTGGTTAGGGTTGGTCG
MYOG	GCGCAGACTCAAGAAGGTGA	GCAGGCGCTCTATGTACTGG
DESMIN	AAGAACAATGACGCTCTGCG	AGCAAAGCGGTCCTCTAGC
CAVEOLIN 3	TTGAAGACGTGATCGCGGAG	AGAAGGAGATGCAGGCGAAC
βMHC	CCTACCTGCGCAAGTCAGAGA	CTGTCTCGGCGGTGACTTT
CDKN1A	ACGTCTCAGGAGGACCACTT	GGAGCAGATCAGTCTGCGTT
GFAP	CCAGGATCTGCTCAACGTCA	CAGGCTGGTTTCTCGGATCT
NSE	CAGCTCAGGTCTGTCTGTGG	TATGTCGGTAGAGGGGCAGT
βIII-tubulin	ACAATGCCACACTGTCCATCC	CCCCACTCATGGTGGCTGAC
Type I collagen	CATGACCGAGACGTGTGGAA	TCGACTCCGGTGGTTTCTTG
RUNX2	GATGACTCTAAACCTAGTTTGTTCTCTGAT	TAATCTGACTCTGTCCTTGTGGATTAAAAG
OSTEOCALCIN	CTTCTGGAATCAGGCAGGGAT	GAGGAACTGGGGCTGTGATT
INSULIN	TGTACCTGGTGTGCGGAGAG	CTGGTAGAGCGAGCAGACGC
ELASTASE1	CAAATCCTGCAGCAGCTCAACGT	CCGGATGATAGAGTTGATCCAGTT
AMYLASE	GATTGTCGTCTCGTTAGTCTTCTT	CACTTGGTGTTTAGATTATGGAGTT
PPARγ	CCGGGTCTGTGGGGATAAAG	GCAAGGCACTTCTGAAACCG
ADD1	TCAAGGCAGACTCGTTGCTC	TTGATGGGCAGCTTGTCAGT
C/EBP2	AGCGACGAGTACAAGATCCG	TTGAACAAGTTCCGCAGGGT
LPL	TCAGGACTCCCGAAGACACA	TACAAGGCAGCCACGAGTTT
AP2	TTTGAATGGGGGTGTGGTCA	ACGATGCTCTTGACTTTCCTGT
TEAD1	GGCACGCCAACCATTCTTAC	CATAAAAAGCCCCGGCATCG
TEAD2	AGCCAGTATGAGAGCCTGGA	GCGGTACACGAACCTTCCAT
TEAD3	TTCAGTCCAAGCTGAAGGACCA	AAACCGTGAGGAAGCCGAGA
TEAD4	AAGTTCTGGGCAGACCTCAA	GTGCTTCAGCTTGTGGATGA

Table S3. Quantitative analysis of insulin stimulating by 0 mM, 15 mM and 25 mM glucose during pancreatic differentiation of bMDSCs

	Glucose concentration	Day 0	Day 6	Day 9	Day 12
Pancreatic differentiation	0 mM	0.63±0.20[a]	0.75±0.37[a]	2.55±1[a]	3.63±1.59[a]
	15 mM	0.61±0.23[a]	1.25±0.17[b]	7.12±1.2[b]	10.70±1.07[b]
	25 mM	0.594±0.20[a]	1.36±0.35[b]	6.72±1.29[b]	9.47±2.15[b]
Undifferentiation	0 mM	0.45±0.09[a]	0.60±0.17[a]	0.38±0.19[a]	0.25±0.12[a]
	15 mM	0.60±0.18[a]	0.60±0.23[a]	0.44±0.24[a]	0.27±0.08[a]
	25 mM	0.55±0.13[a]	0.64±0.15[a]	0.44±0.24[a]	0.35±0.15[a]

[a,b]Values in columns with different superscripts differ significantly ($p<0.05$).

Table S4. Expression of TEADs during myogenic differentiation of bMDSCs

	TEAD1	TEAD2	TEAD3	TEAD4
Day 4	8.61±3.35[a]	4.13±1.0[a]	0.55±0.11[a]	19.06±1.36[a]
Day 8	17.51±1.81[b]	7.21±1.11[b]	0.44±0.15[b]	32.84±1.39[b]

[a,b]Values in columns with different superscripts differ significantly ($p<0.05$).

Table S5. Quantitative analysis of insulin stimulating by 15 mM glucose during pancreatic differentiation of TEAD4-KD and control bMDSCs

	Day 0	Day 6	Day 9	Day 12
TEAD4-KD	0.51±0.16[a]	0.53±0.10[a]	7.59±1.92[a]	9.87±1.16[a]
Control	0.50±0.12[a]	0.517±0.11[a]	7.19±1.77[a]	10.59±1.51[a]

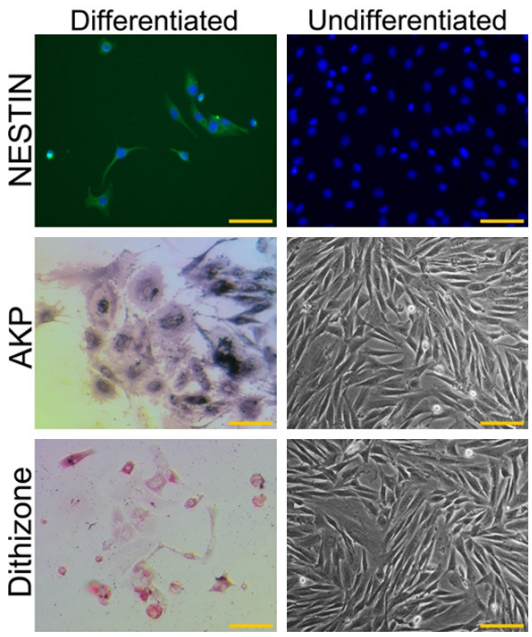

Figure S1. Differentiate bMDSCs into neuron-like cells, osteoid cells, and insulin-secreting cells. Immunochemical and histological staining of Nestin, AKP and dithizone in bMDSC-derived neuron-like cells, osteoid cells and IS cells, bar=100 μm.

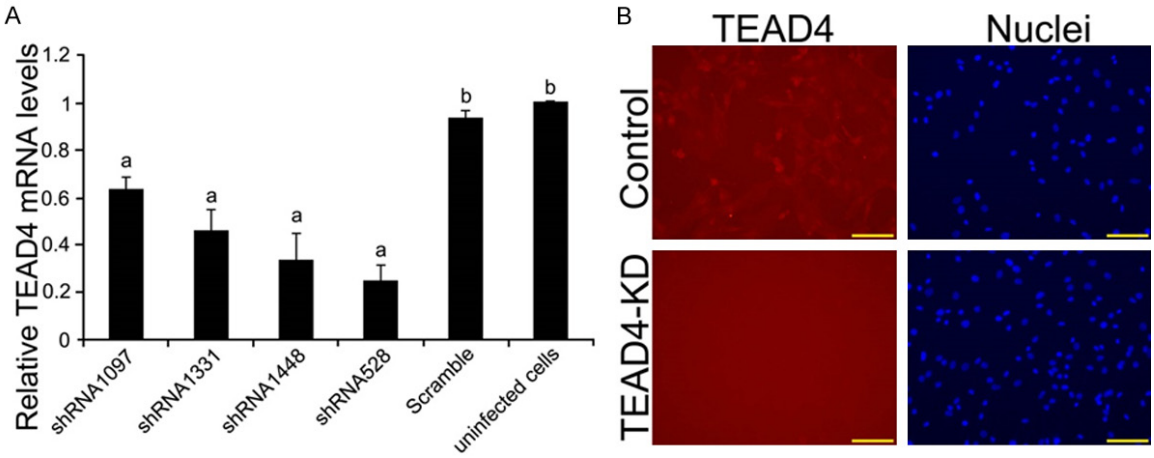

Figure S2. Expression of TEAD4 in TEAD4-KD bMDSCs. A. Expression of TEAD4 in bMDSCs after infection with lentivirus containing TEAD4 knockdown plasmids. B. Immunostaining of TEAD4 in TEAD4-KD and control bMDSCs, bar=200 μm.

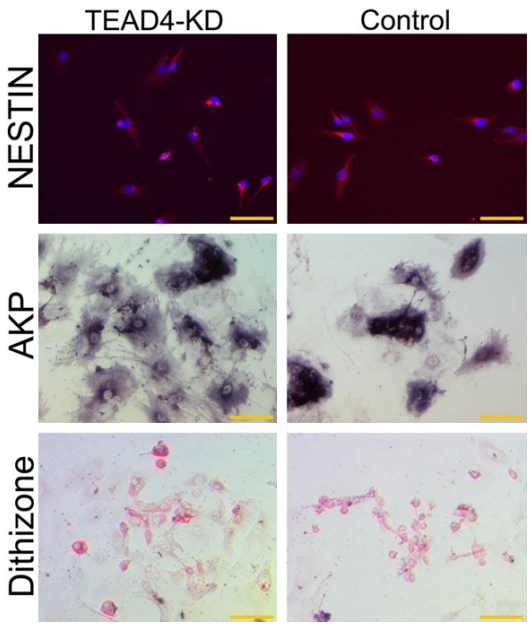

Figure S3. Differentiation of TEAD4-KD and control bMDSCs into neuron-like cells, osteoid cells, and IS cells. Immunochemical and histological staining of NESTIN, AKP and dithizone in TEAD4-KD and control bMDSC-derived neuron-like cells, osteoid cells and IS cells, bar=100 μm.

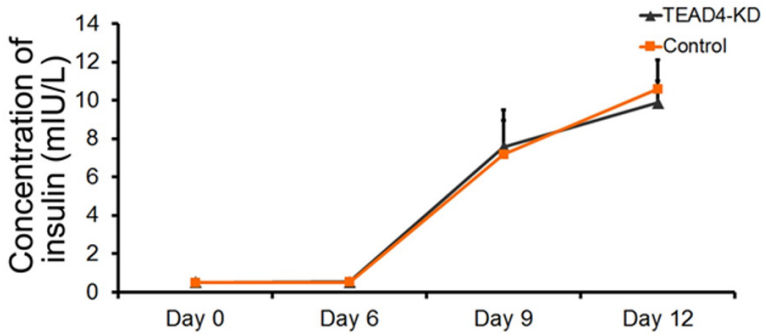

Figure S4. Quantitative analysis of Insulin in medium during pancreatic differentiation of TEAD4-KD and control bMDSCs.