

Silencing Smad7 Potentiates BMP2-induced Chondrogenic Differentiation and Inhibits Endochondral Ossification in Human Synovial Derived Mesenchymal Stem Cells

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Research

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Abstract

Background: Cartilage injuries pose formidable challenges for effective clinical management. Autologous stem cell-based therapies and transgene-enhanced cartilage tissue engineering may open new avenues for the treatment of cartilage injuries. Bone morphogenetic protein 2 (BMP2) is a promising chondrogenic growth factors for transgene-enhanced cartilage tissue engineering. However the BMP2 is failed to maintain a stable chondrogenic phenotype as it also induces robust endochondral ossification. Recently, human synovial derived mesenchymal stem cells (hSMSCs) arouse interested through the poor differentiation potential into osteogenic lineage. Smad7, a protein to antagonizes TGF- β /BMP signaling pathway has been discovered significant in the endochondral ossification. In the present study ,we further explore the effect of downregulate Smad7 in BMP2-induced chondrogenic differentiation of hSMSCs.

Methods: hSMSCs were isolated from synovium of human knee joint through adherence growth. In vitro and in vivo chondrogenic differentiation models of hSMSCs were constructed . Transgenes of BMP2, silencing Smad7 and Smad7 were expressed by adenoviral vectors. The osteogenic differentiation was detected by alkaline phosphatase staining, alizarin red staining. The chondrogenic differentiation was detected by alcian blue staining. Gene expression was determined by reverse transcription and quantitative real-time PCR (RT-qPCR), Immunofluorescence and immunohistochemistry. The subcutaneous stem cell implantation model was established and evaluated by micro-CT , h \times e staining, alcian blue staining and immunohistochemistry assay.

Results: Compared to other MSCs, hSMSCs performed less of capability to osteogenic differentiation. But the occurrence of endochondral ossification is still **inevitable** during BMP2 induced cartilage formation. We found that silencing Smad7 enhanced the BMP2-induced chondrogenic differentiation of hSMSCs in vitro. Also, it leading to much less of hypertrophic differentiation. The subcutaneous stem cells implantation assays demonstrated silencing Smad7 potentiates BMP2-induced cartilage formation and inhibits endochondral ossification.

Conclusion: This study strongly suggests that application of hSMSCs , cell scaffolds and silencing Smad7 can potentiate BMP2-induced chondrogenic differentiation and inhibit endochondral ossification. Thus, inhibit the expression of Smad7 in BMP2-induced hSMSCs differentiation may be a new strategy for cartilage tissue engineering.

Introduction

Autologous repair capacity of articular cartilage is very limited, because of the tiny mitotic activity of articular chondrocytes and absence of blood vessels to deliver putative precursor cells.[1]Tissue-engineering based treatment strategies consist of growth factors, seed cells and implant scaffolds may open new avenues for the treatment of cartilage injuries.[2,3] Many different growth factors such as transforming growth factor beta (TGF- β), bone morphogenetic protein (BMPs) and fibroblast growth factor (FGFs) have been identified for their ability to direct stem cell towards the chondrogenic

differentiation [4,5]. Among them, Bone morphogenetic protein 2 (BMP2), a member of the TGF- β superfamily, has shown greater potential to induce chondrogenic differentiation, osteogenic differentiation and endochondral ossification of MSCs. [6,7] As the BMP2 had been approved by the United States Food and Drug Administration (FDA) and the widely used in spine surgery, its bio-safety have been proved.[8,9] In our previous study indicated that BMP2 is capable to promotes MSCs chondrogenic differentiation, chondrocyte proliferation and hypertrophic differentiation. [10,11] A recent study by Charles K. F. Chan, found that localized co-delivery of BMP2 and soluble VEGFR1 (sVEGFR1) skewed differentiation of resident skeletal stem-cell toward articular cartilage.[12] All of this study suggested that BMP2 induced cartilage formation can be a promising solution for cartilage defects repairing.

However, the use of BMP2 for bioengineered cartilage construction is still disputable, because BMP2 not only promote the chondrogenic differentiation but also endochondral ossification. Previous studies showed that the hypertrophic markers like COL10 and MMP13 was expressed in the BMP2-induced ectopic cartilage-like mass at 21 day.[13] And the process of vascular invasion, matrix calcification and endochondral ossification are also detected in BMP2-induced MSCs, which lead to the destruction of chondrogenic phenotypes. [11,14] So the endochondral ossification is the main stumbling block to BMP2-induced cartilage formation.[10,11] Thus, it is conceivable that BMP2 may be used for clinic cartilage repair if BMP2-induced chondrogenesis hypertrophic and endochondral ossification is inhibited. For obtaining a stable chondrogenic phenotype, lots of efforts have been done to diminish the hypertrophic differentiation and endochondral ossification induced by BMP2.

Recently, human knee joint synovial derived mesenchymal stem cells (hSMSCs) are reported as one of the most ideal MSCs for cartilage regeneration research, because it possess certain advantages, including the tissue-specific, abundant source, powerful regenerative capabilities and stable pluripotent differentiation potential.[15,16] Mesenchymal stem cells can easily be isolated from different tissues and stimulate by cytokines leads to an upregulation of molecules typical of articular cartilage.[17,18] Meanwhile, there has been reported that synovial derived stem cells have relative poor differentiation potential to osteogenic lineage in vitro. [19,20] The BMP2 has been demonstrated governs the divergence of chondrogenesis in hSMSCs. [17] While, hSMSCs is also reported capable to endochondral ossification differentiation in vivo and thus also can impair to chondrogenesis.[18]

To further block BMP2-induced endochondral ossification, we aim to adjust the expression of Smad7 during cartilage formation. In our previous study, we found that overexpression of BMP2 leads to the up-regulate of Smad7 expression.[9] Smad7, belongs to the inhibitory Smads family, antagonizes TGF- β /BMP signaling pathway through multiple mechanisms both the cytoplasm and the nucleus. [21,22] Previous studies showed the Smad7 was an inhibitor at BMP2 induced chondrogenic differentiation.[23] Moreover, Smad7 is also the essential of endochondral ossification.[24] In this study, we hypothesize that the specific and stable BMP2 based tissue-engineered cartilage could be obtained by using hSMSCs through Smad7 expression knockdown. Meanwhile, Polyethylene glycol citrate-co-N-isopropylacrylamide (PPCNg) a biocompatible scaffolds was used to sustain cell attachment and proliferation.[25] We used

hSMSCs through adenoviruses infect to investigated the effect of osteogenic and chondrogenic differentiation both in vitro and in vivo. We demonstrated that silencing Smad7 expression not only potentiates chondrogenic differentiation induced by BMP2 , but also inhibits BMP2 induced hSMSCs chondrogenesis hypertrophic. Therefore, our investigation provides another possible strategy for BMP2 based cartilage repair . These results will offer abundant practical evidence to BMP2-mediated bioengineered cartilage construction .

Materials And Methods

Isolation and cell culture of hSMSCs

This research was approved by the Research Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. All participants are the patients from the orthopaedic Department of the First Affiliated Hospital of Chongqing Medical University, aged from 25 to 40 years old, and undergoing Lower limbs amputations. We was obtained informed consent from all of the three patients prior to their participation .

The primary hSMSCs were grown from human synovial membrane samples of knee joint as reported.[26] Briefly, human synovial tissue was washed with phosphate buffered saline (PBS) containing 1% penicillin and streptomycin for 3 times in a sterile petri dish, and then minced with sterile scissors for 1–2 mm fragments. Two or three synovial fragments then plated into 25-cm²flasks in 5 mL Dulbecco Modified Eagle Medium (DMEM,Gibco, China) containing 20% fetal bovine serum (FBS, Gibco, Australia) and 1% antibiotic-antimycotic solution (Gibco, China) and cultured for 14 days. At this time cells approximately grew to 80% confluency, take out the synovial fragments and passaged to T25 flasks as Passage 0 (P0). In each experiment, medium was refreshed every 3 days. When cells were up to 90% confluency, the cells were divided at a 1:2 ratio. In the subsequent experiments, only cells between passages 1 and 5 were used.

Cells culture

The immortalized mouse adipose-derived MSC (iMAD) cells and C3H10T1/2 cells lines were obtained from ATCC (Manassas, VA). As previously noted[27], MSCs were cultured in DMEM medium with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. Cells were grown in a monolayer culture under standard conditions (37 °C, 5% CO₂).

Crystal violet assay and CCK-8 assays

As reported [28], we seed 10⁴ cells in each well of 96-well culture plate and successively cultured for 11 days. At the indicated time-points ,cell were carefully washed with PBS and stained with 0.5% crystal violet/formalin solution at room temperature for 20-30 minutes. The stained cells were washed with tap water and air dried for taking macrographic images. Each time-point included six replicate wells. For

quantitative measurement, the stained cells were dissolved in 10% acetic acid and optical density was measured at 590 nm as described.[29]

To determine the change of cell proliferation ability by passage, we used Cell Counting Kit-8 (CCK-8, MedChemExpress) to test hSMSCs of passage 1 and 5. [30, 31]The results were recorded by microplate reader (Thermo Scientific™, USA) at absorbance of 450 nm. Growth curves were drawn, and the cell proliferation activity was analyzed.

Phenotypic identification of hSMSCs

The identification of MSCs requires the expression of corresponding surface antigens. [18] hSMSCs were harvested and resuspended in PBS containing 1% bovine serum albumin at approximately 1×10^6 cells/mL. 0.1 ml of the cell suspensions were incubated with CD73 (Biolegend), CD105 (Biolegend), CD90 (Biolegend), CD45 (BD Biosciences), CD14 (BD Biosciences), or CD34 (BD Biosciences) antibodies conjugated for 30 minutes at 4°C in the dark. After being washed three times with PBS, the labeled cells were resuspended in 0.2 mL of PBS and analyzed with the CytoFLEX system (Beckmancoulter). The acquired data were analyzed by using CytExpert software (Beckmancoulter).[26, 28]

Briefly, to identify the multiple differentiation potential [32], cells were respectively seeded in a 6-well plate and replaced differentiation medium when cells density reached 60%. Osteogenic medium was composed of DMEM with 10% FBS, 10 mM b-glycerophosphate (Sigma-Aldrich), 50 mg/mL ascorbate (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich). The osteogenic differentiation results were observed at 7 day by BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime) and at 21 day by Alzarin Red S staining (0.2%, pH = 8.3) (Solarbio). In term of chondrogenic differentiation, we used Synovial Mesenchymal Stem Cell Chondrogenic Differentiation Basal Medium(Cyagen)to induced hSMSCs for 21 day and assessed by Alcian Blue staining (1%) (Solarbio). Adipogenic differentiation medium solution A consisting of DMEM complemented with 10% FBS, 10 mM dexamethasone, 50 g/ml indomethacin (Sigma), 0.45 mM 3-isobutyl-1-methylxanthine (Sigma) and 10 g/ml insulin (Sigma). Adipogenic differentiation medium solution B consisting of DMEM complemented with 10% FBS and 10 g/ml insulin (Sigma). After solution A induced 3days medium is changed to solution B for another 1 day. After the appearance of morphologic features of differentiation, Oil Red O (0.5% in isopropanol) (Solarbio) staining was conducted to determine the intracellular lipid droplets formation.

Recombinant adenovirus construction

Recombinant adenoviruses were generated using AdEasy technology as describing previously [10, 11]. The coding regions of BMP2, Smad7, Red Fluorescent Protein (RFP) and Green Fluorescent Protein (GFP) were amplified with PCR, and cloned into adenovirus shuttle vectors. Then the vectors were utilized to generate recombinant adenoviruses in HEK 293 cells. [34]The siRNA target sites against human Smad7-coding region were cloned into the pSES adenovirus shuttle vector to create recombinant adenoviruses. [35] The resulting adenoviruses were designated as Ad-GFP, Ad-BMP2,Ad-Smad7 and Ad-siSmad7. Ad-GFP and Ad-RFP were used as a vector control.

RNA isolation and quantitative PCR (qPCR)

Total RNA was purified from cell in 60-mm dishes using Trizol (Invitrogen) according to the manufacturer's instructions. [35] Then the cDNA was obtained from total RNA extracted from cells through reverse transcription (RT) reaction kit (TAKARA, Japan). [36, 13] CDNA be completed with SYBR Premix Ex Taq™ (TaKaRa, Japan) and the program were carried out as follows: 95 °C 30 sec for one cycle; 95 °C 5 sec., 60 °C 30 sec., followed by plate read, for 40 cycles. PCR primers (Table S1) were designed using Primer3 plus in Supplementary Table 1. The relative expression levels of mRNAs in the groups were analyzed using the $2\Delta\Delta CT$ method. [37, 32]

ALP staining and activity

The ALP staining, cells were fixed with 4% paraformaldehyde for 30 min. Cells were then washed twice with PBS and stained using the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime). Staining was observed under a bright field microscope after 30 min. [32, 38]

The measurement of ALP activity, cells were washed twice with PBS and lysed with 150 μ L NP-40 lysis buffer (Beyotime). The cell lysates were quantified by an alkaline phosphatase assay kit (Beyotime) using p-nitrophenyl phosphate (pNPP) as the substrate. In the presence of magnesium ions, pNPP was hydrolysed by phosphatases to phosphate and p-nitrophenol. The rate of p-nitrophenol liberation is proportional to the ALP activity and can be measured photometrically. The ALP activity was measured by microplate reader (Thermo Scientific™, USA) at absorbance of 405 nm. [39]

Matrix mineralization assay (Alizarin Red S staining)

After infected with indicated adenoviruses, cells were cultured in the presence of ascorbic acid (50 mg/ml) and b-glycerophosphate (10 mM) for 14 day. The mineralization nodules were assessed by Alizarin Red S staining. [28, 32] Briefly, cells were fixed with paraformaldehyde at room temperature for 10 min. and washed with PBS (pH adjusted to 4.2), and fixed cells were left in 37 °C incubator with 2% Alizarin Red S for 10 min., followed by careful washing with distilled water. The calcium deposits were observed under microscope. [32] The results were performed in at least three independent experiments.

The chondrogenic and hypertrophic differentiation protocol

In the chondrogenic differentiation and the hypertrophic differentiation of hSMSCs, the micromass culture method was used as previous noted. [9, 10, 13] And then turn into the chondrogenic medium for 1 week, which was composed of DMEM with ITS (Sigma-Aldrich), 50 μ g/mL ascorbate (Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich) and 10 ng/mL transforming growth factor- β 3 (Sigma-Aldrich). Then, the cells were shifted in the hypertrophic medium for another week, which contained ITS supplement, 50 μ g/mL ascorbate, 1 nmol/L dexamethasone and 100 ng/mL triiodothyronine (T3, Sigma-Aldrich). [40]

Briefly, hSMSCs was washed with PBS, treated with 4% paraformaldehyde for 30 minutes, and again washed with PBS. Followed by stained with 0.5% Alcian blue in 0.1 M HCl (pH 1.0) for 12 hours. Then

photographed with microscope. [10, 11]

Immunohistochemical analysis

As the reported [11], cell samples were fixed in 4% paraformaldehyde for 10 min and washed with distilled water. Next, the cells were treated with 3% H₂O₂ for 15 min at room temperature to eliminate endogenous peroxidase activity and blocked with normal goat serum for 40 min at room temperature. Then, the cells were incubated with anti-Col2A1 (Abcam, UK; 1:200), anti-ColX (Santa Cruz Biotechnology; 1:200) and anti-MMP13 (Abcam, UK; 1:200) primary antibodies overnight at 4 °C. The sections were subsequently incubated with the secondary antibody IgG-HRP (CST; 1:200). The resulting sections were photographed under a microscope.

Stem Cell Implantation to Ectopic cartilage formation and Using polyethylene glycol citrate-co-N-isopropylacrylamide (PPCN) Scaffolds in Vivo.

Polyethylene glycol citrate-co-N-isopropylacrylamide (PPCN) was synthesized as previously described. [41] PPCN powder was dissolved in PBS (at 100 mg/mL), sterilized by syringe filtration with 0.22 µm filters, and kept at 4 °C. The subcutaneous ectopic bone formation was carried out as previously reported. [9, 10, 11] Briefly, sub-confluent hSMSC were infected with specific adenoviruses for 36 h, per injected set were resuspended 5×10^6 cells in 100 µL of PPCN scaffolds on ice. The athymic mice (4–6 week-old female) used to subcutaneously injection. At 4 weeks, the animals were euthanized for harvesting ectopic masses.

Micro-Computed Tomography (µCT) Analysis. Hematoxylin & Eosin (H&E) Staining and Alcian Blue Staining

The retrieved masses were fixed with 10% PBS-buffered formalin and imaged using Skyscan1174 X-Ray Microtomograph (Micro-CT) (Bruker Company, Belgian). NRecon software was used for 3D image reconstruction and all image data analysis was performed using the CT-AN software. BV/TV (%), Tb. N, Tb. Sp, Tb. Th, and BMD were measured as described.[9, 32]

After µCT imaging, the retrieved masses were decalcified, paraffin embedded, and sectioned. The sections were H&E and Alcian Blue stained as previously described. [9, 11]

Immunofluorescence stain assay

The paraffin-embedded tissue sections were deparaffinized, rehydrated, and subjected to staining with a MMP13 antibody (Abcam, UK; 1:200), Col10 antibody (Abcam, UK; 1:200).[32] Followed by incubation with the corresponding fluorophore-conjugated antibodies. The immunohistochemical staining results were observed by inverted fluorescence microscopy (Olympus), and the images were analyzed using an Olympus auxiliary system.

Statistical Analysis

Statistical analysis was conducted as described.[9, 10] Statistical significance was determined. All quantitative experiments were performed in triplicate and/or repeated through three independent batches of experiments. Statistical analyses were performed using the software package SPSS 14.0, and by one-way analysis of variance and the Student's t test. A p-value < 0.05 was defined as statistically significant.

Results

Isolated human synovial mesenchymal stem cells (hSMSCs) which exhibits a high proliferative activity in Passage 5

Human synovial-derived mesenchymal stem cells (hSMSCs) are considered to have the greatest potential for cartilage regeneration research due to their tissue-specific advantages. Although hSMSCs have been used rather extensively, their biological features are much differentiated due to age, arthritis or other joint conditions of donor.[18, 26] Here, we isolated the primary human synovial mesenchymal stem cell from the knee joint synovial membrane of three donors.

After 72 hours, fibroblast-like primary cells were observed migrating outwards from the minced fragments of synovial tissues.(Fig. 1A,a) Primary cells grew relatively slow, and by 14 days cultured in dish the cells were all over the field of vision. (Fig. 1A,b) Morphology of cultured passage 1 and 5 (P1, P5 respectively) cells showed a spindle-shaped appearance and plastic-adherent properties, and there was no distinct change at passage 5.(Fig. 1A,c,d) Crystal violet staining assay indicated that hSMSCs reached 70% confluence at day 5, while at day 9 cover the culture plate. (Fig. 1B) Quantitative assessment of the stained cells showed cell proliferation plateaued on day 5 and continued to increase after day 7.(Fig. 1C)

We used the Cell Counting Kit (CCK)-8 to detect the effect of passage on hSMSCs proliferative activity. And results showed similar growth capacities of P1 and P5 cell, and confirmed the crystal violet staining results that cell proliferation had a short plateau period after day 5.(Fig. 1D)

The hSMSCs express most of the MSCs markers and capable of multidirectional differentiation potential

According to the criteria for mesenchymal stem cell identification,we identify the surface marker of hSMSCs using flow cytometry. [29, 32] Flow cytometric results showed that P1 hSMSCs were positive for MSC markers CD73, CD90, and CD105 and weakly expressed hematopoietic markers CD34, CD14, CD45, which indicated that P1 hSMSCs express most of the consensus MSC markers, suggesting that these cells may possess MSC-like characteristics. (Fig. 1E)

In addition, we cultured in osteogenic, adipogenic or chondrogenic medium, the hSMSCs could readily be induced to differentiate into osteogenic, adipogenic and chondrogenic lineages, respectively. Osteogenic potential was confirmed by staining of alkaline phosphatase (ALP) and Alizarin Red (Fig. 1F). Chondrogenic potential was confirmed by staining sulphated glycosaminoglycans using Alcian blue(Fig. 1F), while adipogenic potential was evaluated by observation of small cytoplasmic lipid droplets stained using Oil Red O (Fig. 1F).

Recombinant adenovirus effectively overexpression transgenes in hSMSCs for a relatively long-term

We constructed recombinant adenovirus to stabilize overexpression of BMP2 and Smad7 using AdEasy technology, and another recombinant adenovirus that expressing small interfering RNA (siRNA) targeting the coding region of human Smad7 using the recently described established pSOS system.[9, 10, 13] In order to determine whether recombinant adenovirus can be effectively transduced in hSMSCs, we observed the expression of GFP and RFP by fluorescence microscopy 24 h after infection(Fig. 2,A). RT-PCR conformed that the transgenes were highly expressed in the hSMSCs that were infected with respective adenoviral vectors for 3 and 5 days.(Fig. 2,B) Meanwhile, we found that BMP2 effectively up-regulated Smad7 expression at 3 and 5 days after infection. (Fig. 2,C)

To determine whether these recombinant adenovirus could effective regulation Smad7 expressing level in hSMSCs, by using fluorescence microscopy and RT-qPCR. The effectively transduced by the adenoviral vectors at 48 h after infection. (Fig. 2,D) We found that Ad-Smad7 could greatly upregulate mRNA expression of Smad7 compared with control (Fig. 2,E). Meanwhile, Ad-siSmad7 efficaciously knock down Smad7 mRNA expression in hSMSCs from 5 to 14 day(Fig. 2,F).

The hSMSCs has less of osteogenic differentiation potential in vitro

To assess the BMP2-induced osteogenic differentiation potential of hSMSCs compared with other MSCs, we used C3H10T1/2 cells and immortalized mouse adipose-derived MSC (iMAD) cells. (Fig. 3,A) And infected with respective adenovirus vectors. First, we examined the ALP activities by ALP staining at day 5,which indicate early osteogenic differentiation activity(Fig. 3,B). The results showed that compared with the hSMSCs, the ALP activity of the C3H10T1/2 cells and iMads was considerably increased after BMP2 induced. Moreover ALP quantitative analysis on days 5 result exhibits that the ALP activity of the hSMSCs group was dramatically lower than other BMP2 induced group(Fig. 3,C). Second, Alizarin red S staining was used to examine the calcium deposition, which is one of the late osteogenic differentiation indicators. [6, 32] The results showed that the hSMSCs also had less calcium deposition compared to the C3H10T1/2 cells and iMads at 14 days. (Fig. 3D). Taken together, hSMSCs exhibit lower BMP2-induced osteogenic differentiation potential.

Smad7 reduce BMP2-induced Chondrogenic differentiation and dilate hypertrophic differentiation

To confirm the Smad7 function during BMP2-induced chondrogenic differentiation and hypertrophic differentiation in hSMSCs chondrogenesis, we harvested adenovirus infected hSMSCs and seeded as micromass, which were cultured in chondrogenic conditions for 7 days and then turn in hypertrophic medium conditions for 7 days.[10, 40, 44](Fig. 4.A) Alcian blue staining revealed that the level of sulfate glycosaminoglycans in the silence Smad7 group was remarkably higher than other group and the over express Smad7 group were relatively lower than the BMP2 group on day 7.(Fig. 4,B) Col2a1 is part of the most important molecular markers for chondrogenesis.[2] The Immunohistochemical analysis results revealed silence Smad7 could significantly promote Col2 expression, while over expressing Smad7 could inhibit BMP2-induced Col2 express.(Fig. 4,C) RT-PCR data revealed that successful transfected BMP2

groups were remarkably higher than the control group in the expression of chondrogenic marker genes, including Sox9, Col II and Aggrecan, while silence Smad7 group was most significantly increased during the chondrogenic differentiation process. Conversely, the over expressing Smad7 group was decreased compared with the BMP2 group. (Fig. 4,D)

To confirm the role of Smad7 in hypertrophic differentiation, Col X and MMP13 hypertrophic markers were detected by immunohistochemical staining. [45] The protein expression of Col X and MMP13 were clearly more unregulated in the BMP2 + Smad7 group at day 14 than in the other groups; the BMP2 + siSmad7 group showed lower protein expression level compared with the BMP2 group and the BMP2 + Smad7 group and but was higher than the GFP group.(Fig. 4,E,F) Similarly, the RT-qPCR results revealed that the mRNA levels of Runx2, Col X and MMP13 in the BMP2 + siSmad7 group were significantly up regulated at day 14 compared to the BMP2 group (Fig. 4,G).

In summary, these results indicate that Smad7 has a negative effect on chondrogenic differentiation-related factors and a positive effect on hypertrophic differentiation-related factors at mRNA and protein expression levels.

Silencing Smad7 expression promotes chondrogenesis of ectopic PPCNg–hSMSCs composite and inhibited endochondral ossification in vivo

We further examined the effect of combined with PPCNg on BMP2-induced chondrogenic in vivo.[9, 10, 13] As a thermoresponsive macromolecule, PPCNg can provide a better in vivo microenvironment to hSMSCs. [25] The physical appearance of PPCNg remains liquid at 4°C and quickly gels to form a solid scaffold at 37°C.[40](Fig. 5,A) We mixed adenovirus infected hSMSCs and PPCNg at 4°C, and implanted on the subcutaneous in nude mice. (Fig. 5,B) We found that the cells transduced with Ad-GFP, Ad-Smad7 or Ad-siSmad7 alone failed to form any detectable masses (data not shown). The general observation and the micro-computed tomography (micro-CT) results showed that compared with the BMP2 group, the volume of ectopic mass in the BMP2 + siSmad7 group was increased.(Fig. 5,C,D) Quantitative analysis of bone histomorphology showed that compared with BMP2 + Smad7 group and BMP2 + siSmad7 group, bone volume/total volume (BV/TV) in BMP2 group were significantly increased. (Fig. 5,E)

In histological examination, the BMP2 + siSmad7 group have more mature chondrocyte were observed and a few of hypertrophic chondrocyte. (Fig. 6,A)In the BMP2 group the trabecular bone and vessel invasion was detected, this hint that endochondral ossification has already started. And the Smad7 group have observed that still large of undifferentiation hSMSCs. In immunohistochemical staining, we found that MMP13 and COL10 was also decreased in the BMP2 + siSmad7 group. (Fig. 6,B)These in vivo findings further confirm the results that the chondrogenesis-promoting effect of combined PPCNg and silenced Smad7 on BMP2-induced differentiation of hSMSCs.

Discussion

BMP2 induces not only cartilage formation but also endochondral ossification although the precise mechanisms remain to be fully understood.[9, 10, 13] Endochondral ossification is important for development, growth, and repair of long bones. [2]However, endochondral ossification is the main stumbling block for cartilage tissue engineering, because it damage the extracellular matrix of cartilage. [11, 12] BMP2-induced cartilage formation had been reported as a possible strategy for cartilage repairing. But the endochondral ossification during BMP2-induced differentiation still cannot be ignored, it must be inhibited for further application of BMP2-mediated cartilage repair.[9, 10, 13] In this study, we strive to obtain ideal chondrogenesis through the improvement of three elements: using human synovial derived MSCs, silencing Smad7 expression and implanting injectable thermo-sensitive cell scaffolds.

Mesenchymal stem cells can easily be isolated from different tissues, and common in vitro stimulate by cytokines leads to chondrogenic differentiation.[18] There has been reported that compared with bone marrow derived MSC (BMSC), synovial derived stem cells exhibits a relative poor potential of osteogenic differentiation.[19] Recent years, Cosimo De Bari et found that the adult synovial derived Gdf5-positive cells was failed to mineralize in vitro but still have potential to chondrogenic differentiation.[46] This result support that hSMSCs is difficult to endochondral ossification. Meanwhile, hSMSCs possess certain advantages, including the low immunogenicity in vivo and powerful regenerative capabilities in vitro.[7, 20, 26, 27]In the present study, the primary human synovial mesenchymal stem cell from the knee joint synovial membrane exhibit a high proliferative activity and multidirectional differentiation potential. Meanwhile, this primary cells identified by surface marker. However, we found that in BMP2 induced hSMSCs differentiation in vitro, the ALP activity were significantly increased. And it also mineralize when cultured in osteogenic differentiation medium at day 14. The results suggest that BMP2 induced osteogenesis ability still powerful in hSMSCs. In vivo, hSMSCs also capable to endochondral ossification differentiation by BMP2 induced. Even so ,compared with iMads and C3H10T1/2, hSMSCs have less osteogenic differentiation capacity. Our results support that, among the most studied MSCs, synovial stem cells are still one of the most chondrogenic specific .

Smad7 may be the key to inhibit endochondral ossification and affect chondrogenesis.[22, 23] Our results revealed that Smad7 upregulate in BMP2 overexpressed hSMSCs. As one of the two inhibitory Smads proteins, Smad7 is well known to inhibit TGF- β pathways through a variety of ways.[21, 47] Previous studies showed that overexpressing Smad7 disturbed mesenchymal condensation ,decreased chondrocyte proliferation and inhibited chondrocytes maturation.[22] In the endochondral ossification process of embryonic chondrogenesis, Smad7 knockout mice showed both skeletal defects and shortened hypertrophic zones in growth plates.[24] On the other hand, the exogenous expression of BMP2 and Smad7 by adenovirus transfection decreased the length of the hypertrophic zone of growth plate in fetal mouse tibial compared with transfected BMP2 only. [9]The effect of silencing Smad7 in BMP2-induced hSMSCs is still no reported. In this study, we used adenovirus transfection to significantly decreased the Smad7 expression in hSMSCs in order to obtain stable chondrocyte phenotype. In the chondrogenic differentiation experiment, silencing Smad7 expression significantly increased chondrogenic marker genes expression in hSMSCs like COL2a1 and SOX9. Further, in the hypertrophic differentiation experiment, the silencing Smad7 group have lower expression of hypertrophic chondrocyte

marker like Col X and MMP13, compared with the BMP2 induced only group. This indicates that silencing Smad7 is a feasible strategy to increased chondrogenesis and matrix accumulation while inhibited chondrocyte hypertrophic factor expression and inhibited endochondral ossification.

The cells attachment point and growth space is also indispensable to cartilage engineering.[2, 3] The subcutaneous transplantation of hSMSCs is limited by insufficient nutrient supply and irregular cell growth space, so we used PPCNg to improve this situation. This scaffold was mixed a biodegradable citrate-based thermosensitive macromolecule, poly (polyethyleneglycol citrate-co-N-isopropylacrylamide) (PPCN) with gelatin (PPCN-g).[25] The effectiveness of cell adhesion and survival properties of MSCs had been proved by previous experiment in BMP9 induced osteogenic differentiation.[41] These still no report about the PPCN-g apply in MSCs chondrogenic differentiation. We found that PPCN-g effective promoted the hSMSCs chondrogenic differentiation in vivo. In ectopic hSMSCs mass, the silencing Smad7 group not only has the increased number of chondrocytes, but also the reduced number of hypertrophy chondrocytes and the down regulate expression of hypertrophic factor COL10 and MMP13. While compared with the BMP2 group, these no obvious trabecular bone formation and new blood vessels invasion in the silencing Smad7 group. The standard endochondral ossification process is distinct from the in vitro experiment because lack of vascular invasion. Thus the in vivo results has more practical value to reference. The results strongly suggest that simultaneous application of hSMSCs, PPCN-g and interfering Smad7 expression could promote BMP2 induced chondrogenic differentiation and maintain chondrocytes phenotype.

Conclusion

In summary, our findings suggested that simultaneous application of hSMSCs, PPCNg and silencing Smad7 can promote BMP2-induced chondrogenic differentiation and maintain chondrocytes phenotype stable. Thus, it is conceivable that this way may be exploited as novel strategies to treat cartilage injuries.

Abbreviations

hSMSCs: human synovial derived mesenchymal stem cells; BMP2:Bone morphogenetic protein 2; Smad7:SMAD Family Member 7; ITS:Insulin-transferrin-sodium; FBS:Fetal bovine serum; DMEM:dulbecco's modified eagle medium; iMads:Immortalized mouse adipose-derived mesenchymal cells; PPCNg:polyethyleneglycol citrate-co-N-isopropylacrylamide with gelatin; GFP:Green Fluorescent Protein; RFP:Red Fluorescent Protein; CCK8:Cell Counting Kit-8; SOX9:SRX-Box Transcription Factor 9; COL2:Collagen Type II; COL10:Collagen Type X; MMP13:Matrix Metalloproteinase 13; Agg:aggrecan; ALP:Alkaline phosphatase ;PBS:Phosphate-buffered saline; RUNX2:Runt-related transcription factor 2.

Declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by the Ethical Committee of The First Affiliated Hospital of Chongqing Medical University. The human study was approved by the Research Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. All participants signed informed consent on a voluntary basis.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study 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

WH and CZ conceived and designed the experiments. PX, ZZ, DC and YZ performed the experiments and collected the data. JL, QC and HC analyzed the data. CZ and WH contributed the reagents, materials, and analysis tools. PX, CZ, and WH wrote the manuscript. All authors read and approved the manuscript.

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Figures

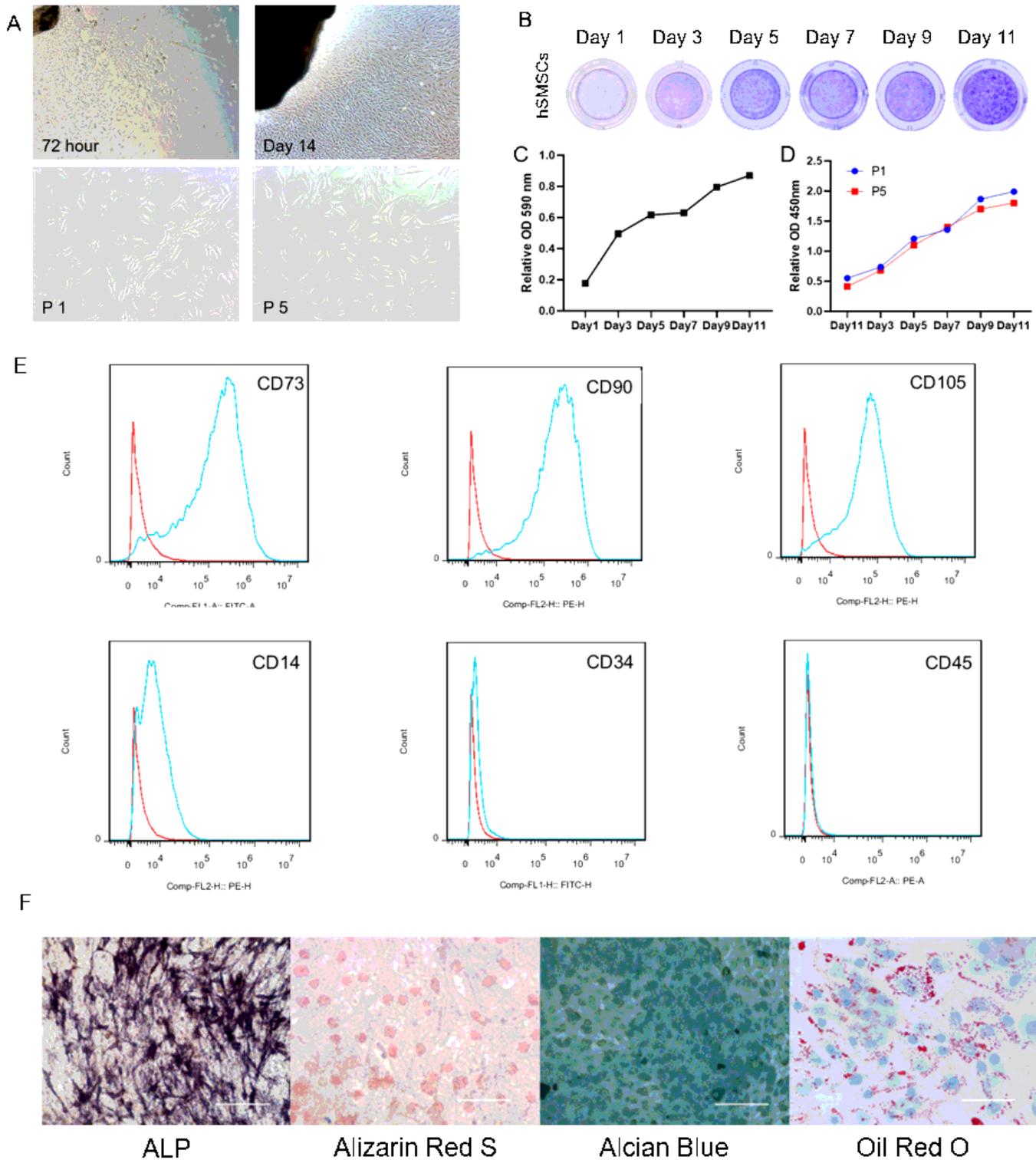


Figure 1

Morphology of cultured hSMSCs ; the proliferation potential and the identification of hSMSCs . (A). a1)hSMSCs were migrating outwards from the freshly harvested synovium at 72 hours; a2)Morphology of the confluence hSMSCs was recorded at day 14; a3) primary cultures (P0) and a4) the fifth passage (P5) with spindle shapes on cell culture dish (original magnification $\times 40$, scale bar =200 μm) (B). Cell proliferation assessed by crystal violet staining assay. General observation of the stained cells were

recorded at the indicated time-points. (C). The stained cells were dissolved for quantitatively OD reading at A590 nm. (D). Proliferation of P1 and P5 as determined by the CCK-8 method showed that no significant difference. (E). The MSC markers of hSMSCs at P1 by flow cytometry and hSMSCs highly expressed CD73, CD90, and CD105 and negatively expressed CD34, CD14 and CD45. (F). The potential for osteogenic, chondrogenic, and adipogenic differentiation of hSMSCs in vitro: osteogenic (alkaline phosphatase staining and Alzarin Red S staining), chondrogenic (Alcian Blue staining), adipogenic (Oil Red O staining) (original magnification $\times 200$, scale bar = 50 μm). The data are shown as mean \pm SD for three separate experiments.

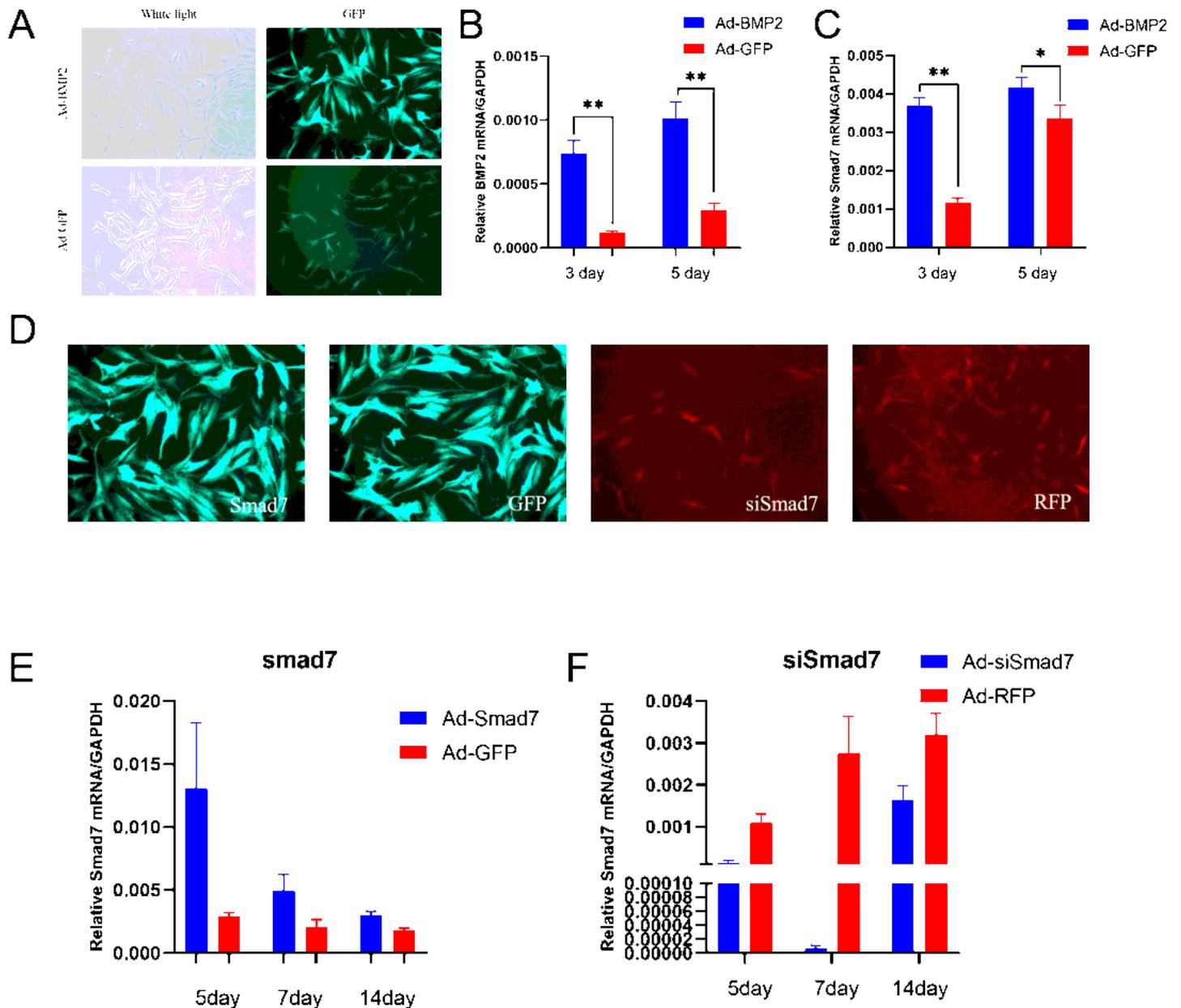


Figure 2

Adenovirus-mediated effective transduction of BMP2, Smad7, siSmad7, RFP and GFP into hSMSCs. (A). Representative bright field and GFP fluorescence fields were recorded at 24 h after infection (scale bar =

100 μ m). (B). RT-PCR analysis of adenovirus-mediated transgene expression. Total RNA was isolated at 3 and 5 days after infection and subjected to RT-PCR using BMP2 primers. (C). RT-PCR analysis of Smad7 expression at mRNA level. BMP2 upregulated Smad7 mRNA levels at days 3 and 5 compared to the GFP group ($p < 0.01$). (D). The recombinant adenovirus Ad-GFP (green), Ad-Smad7 (green), Ad-RFP (red) and Ad-siSmad7 (red) were shown to effectively transfect hSMSCs for 48 h (scale bar = 100 μ m). (E). Ad-Smad7 upregulated the expression of Smad7 at 5, 7, and 14 day. (F). Ad-siSmad7 silences the expression of Smad7 from 5 to 14 day. All samples were normalized with the house-keeping gene GAPDH. Each experiment was done in triplicate. The data are shown as mean \pm SD for triplicate. * $P < 0.05$. *** $P < 0.001$.

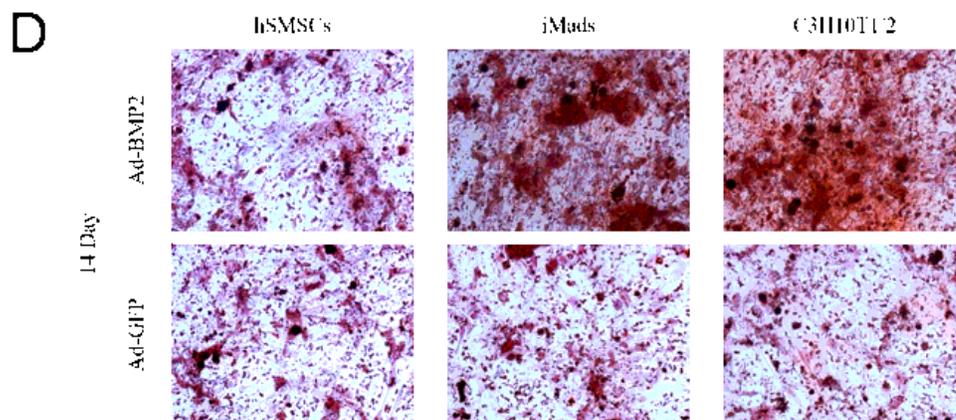
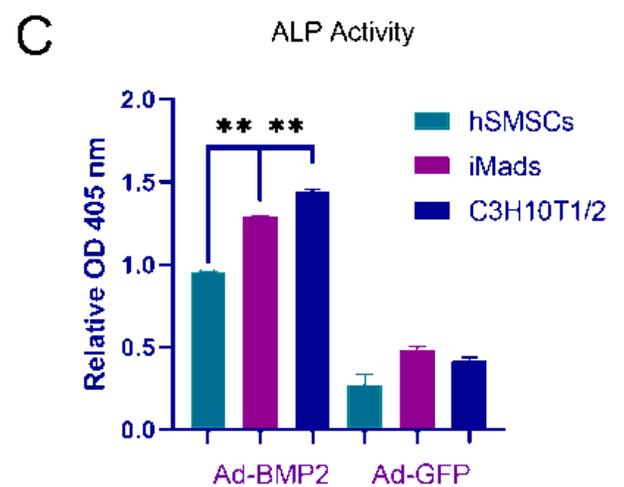
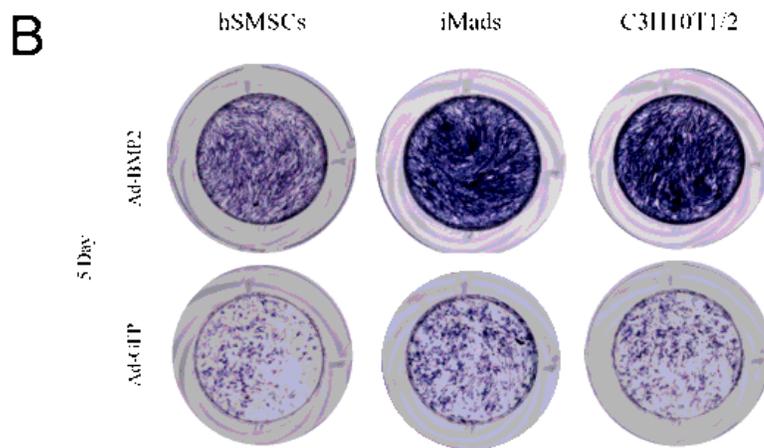
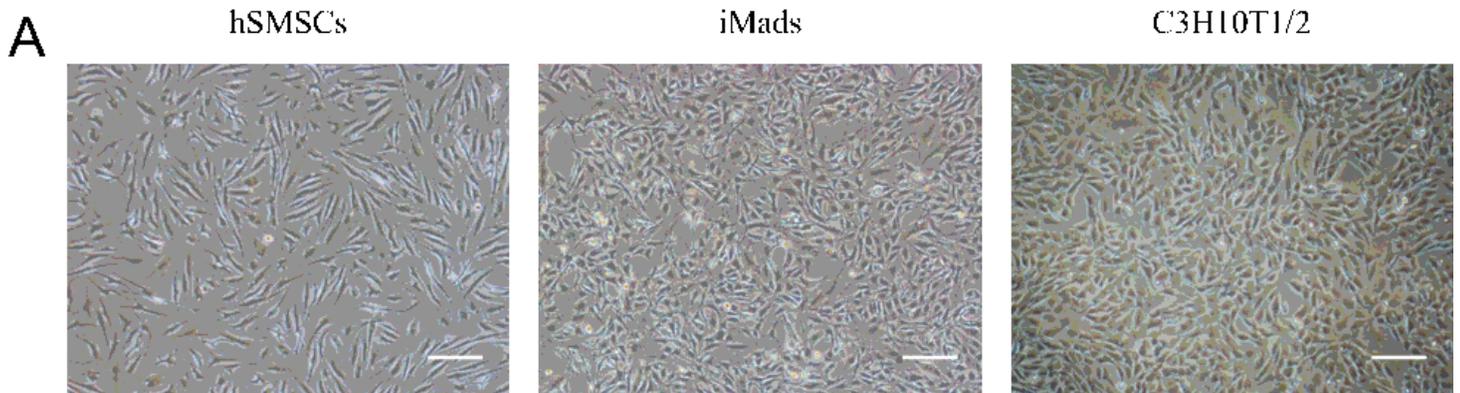


Figure 3

The hSMSCs has lower osteogenic differentiation potential in vitro. (A). Morphology of the confluence cells was shown (scale bar = 200 μ m). (B). hSMSCs , iMads and C3H10T1/2 cells were infected with AdGFP and AdBMP2. The ALP activity were measured at 5 days after transfection using ALP histochemical staining. (Gross observation) (C). And the ALP quantification activity were detect at OD 405nm. (D). The mineralization and calcium deposition at the late osteogenic differentiation were observed by Alizarin Red S staining assay. (scale bar = 100 μ m) Each experiment was done in triplicate. $^{**}P < 0.05$. $^{***}P < 0.001$.

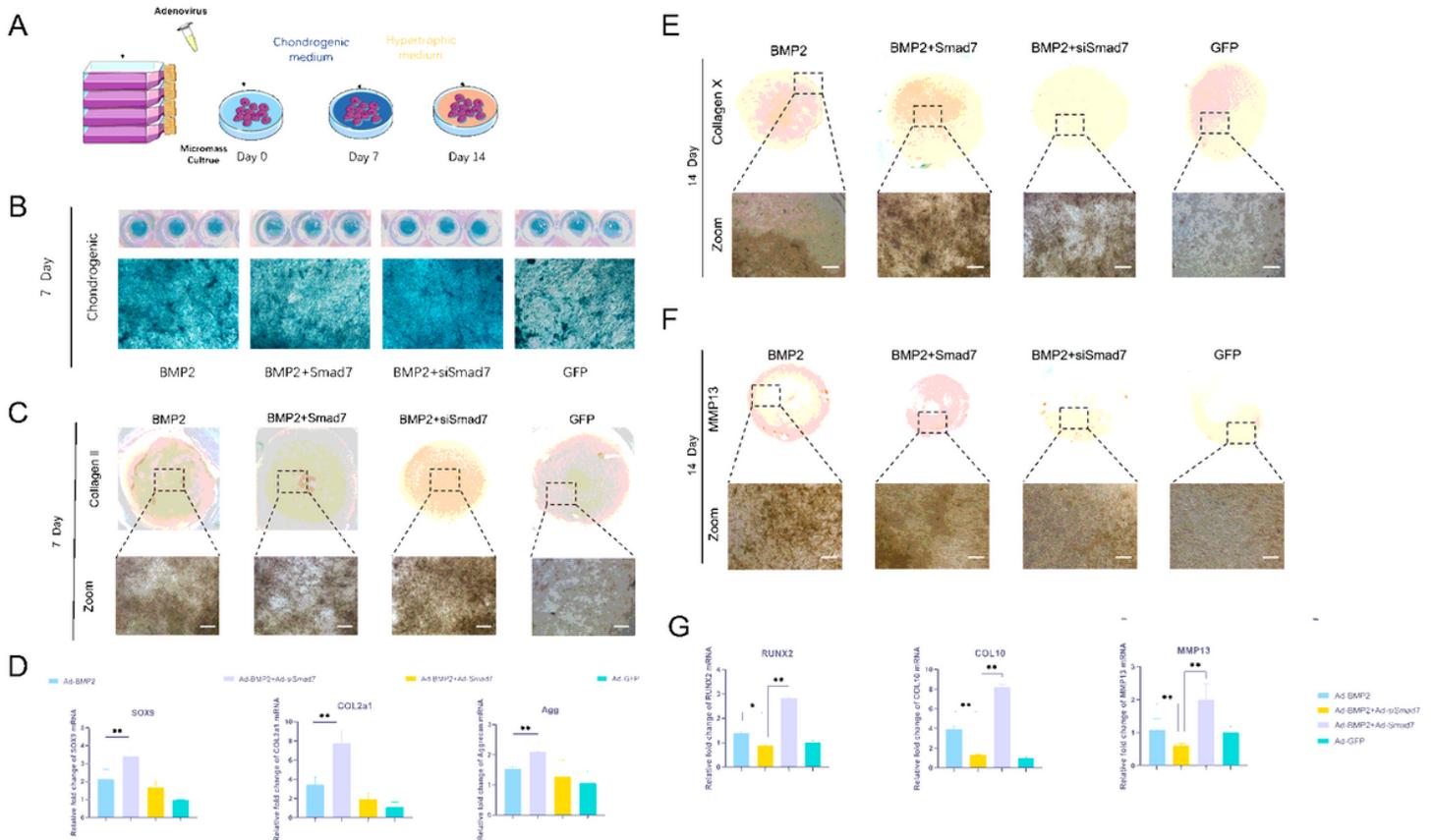


Figure 4

Smad7 reduce BMP2-induced Chondrogenic differentiation and dilate hypertrophic differentiation. (A).The experimental design of Smad7 regulation during chondrogenic differentiation and hypertrophic differentiation. (B). Alcian blue staining for sulfated glycosaminoglycans in micromass cultures of hSMSCs on day 7 after transduction of indicated recombinant adenoviruses, gross observation and microscope examination (40X) are shown. (C). Type II collagen (Col2) protein level was measured after 7 days by immunohistochemical analysis. Microscope bars was 100 μ m. (D). RT-qPCR assay determined that Smad7 inhibits the expression of chondrogenic relative factors, including SOX9 , Aggrecan and COL2 at BMP2-induced chondrogenic differentiation. (E). Col10A1 protein level was measured after 7 days chondrogenic cultured and 7 days hypertrophic cultured by immunohistochemical analysis and both gross observation and microscope examination exhibits Smad7 promote its expression level. Bars 100

µm. (F). MMP13 protein level also measured by immunohistochemical staining. (G). Smad7 potentiates the expression of hypertrophic relative factors of hSMSCs :RT-qPCR assay was determined that hypertrophic differentiation relative factors, including Runx2, Col10 and MMP13. All of the quantification data are done in triplicate and shown as mean ± SD. **P* < 0.05. ****P* < 0.001.

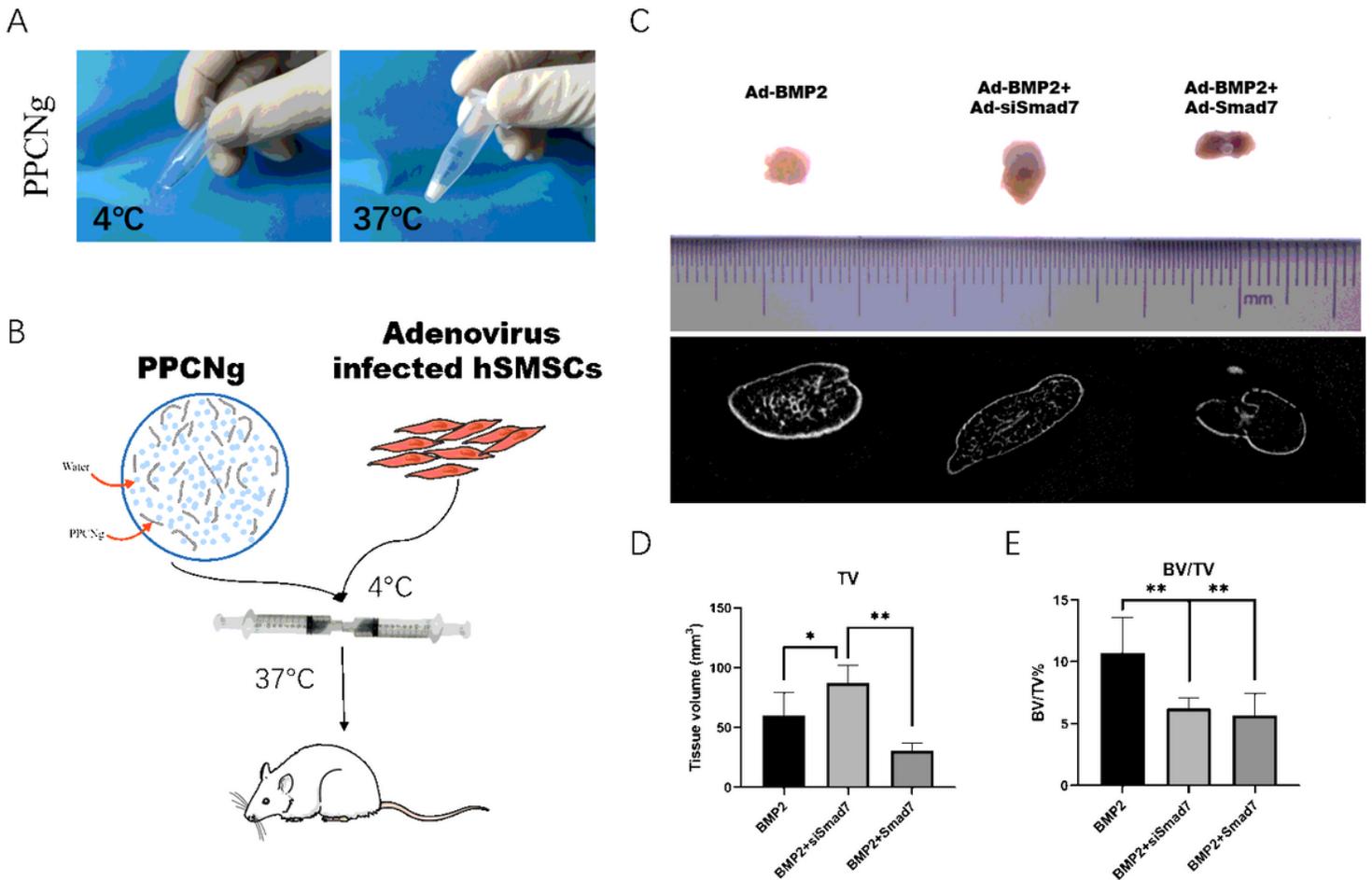


Figure 5

Silencing Smad7 enhance the BMP2 induced subcutaneous ectopic cartilage formation (A). Before and after thermal induced gelation of PPCNg alone, respectively. (B). Illustrative diagram showing that hSMSCs treated as experimental design seeded on the scaffolds were implanted into the subcutaneous space of the nude mice and harvested for analysis after 4 weeks . (C). Macrographic images of ectopic masses. The micro-CT scanning to determine the surface and the volume of mass. (D). The retrieved masses were subjected to microCT analysis: Quantification results of the relative values of BV/TV were analyzed. These data are shown as mean ± SD repeat triplicate. **P* < 0.05. ****P* < 0.001.

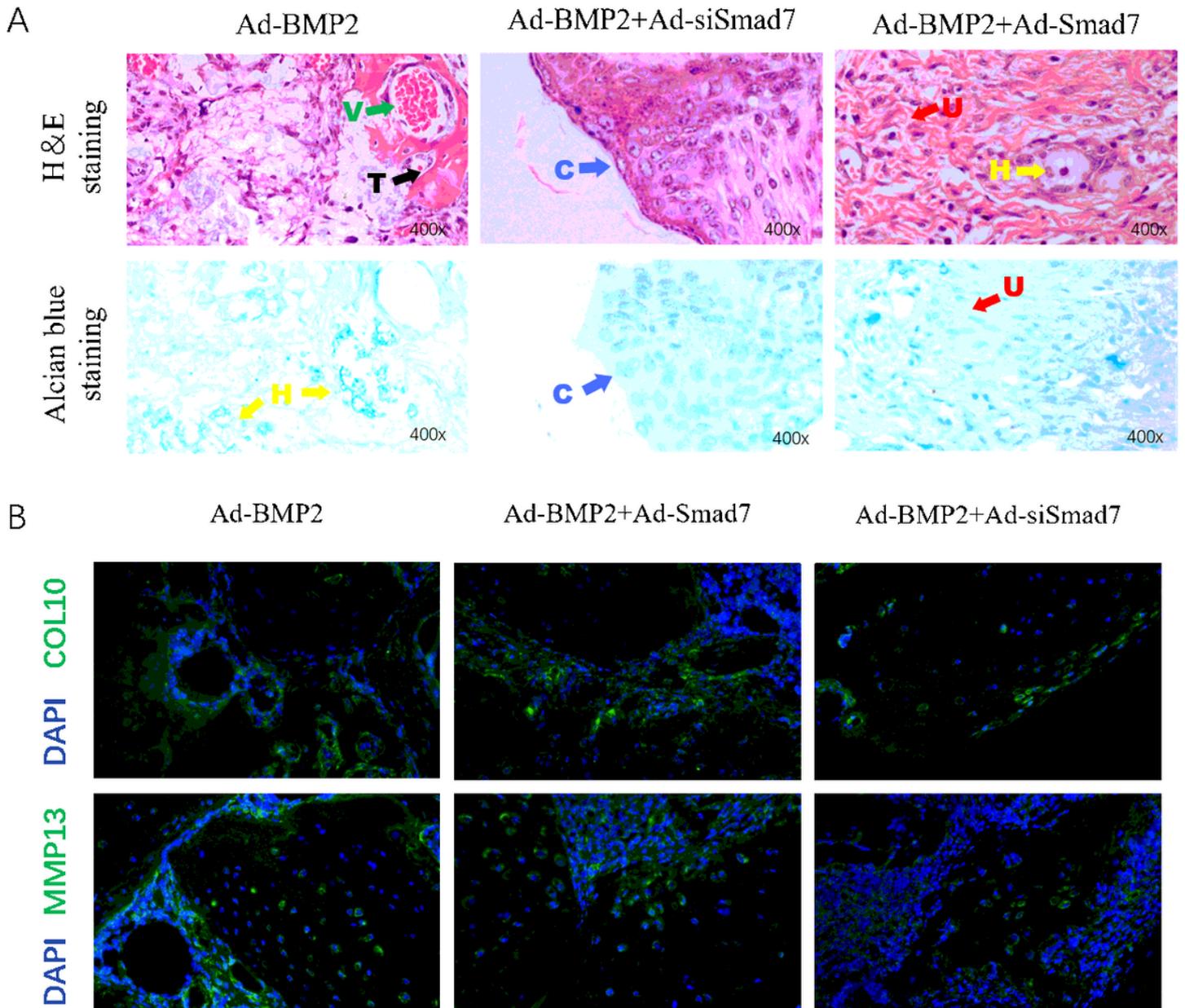


Figure 6

Histological analysis of the ectopic chondrogenesis. (A). The samples of ectopic masses were subjected to H&E staining and Alcian Blue staining to determine the formation of chondrocyte like cells and hypertrophic chondrocyte like cells (magnification $\times 400$, scale bar = $50 \mu\text{m}$). Representative images are shown. U, undifferentiated MSCs; C, chondrocytes; H, hypertrophic chondrocytes; TB, trabecular bone ;V, vessel. (B). Immunofluorescence staining were used to determine the effects of Smad7 on the expression of hypertrophic and angiogenesis marker, including MMP13 (Green) and VEGF (Green) and DAPI (blue) (magnification of up images = $\times 400$, scale bar = $50 \mu\text{m}$).

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