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Original Article

Thrombospondin-1 inhibits ossification of tissue engineered cartilage constructed by ADSCs

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Abstract: Cartilage tissue engineering provides a new method in the treatment of cartilage defects, and adipose derived stem cells seem to be an ideal seed cell in cartilage tissue engineering because of its characteristics. However, ossification after in vivo implantation of tissue engineered cartilage remains a challenge. Thrombospondin-1 which has been reported to have an inhibitory effect on angiogenesis, may play an important role in inhibiting the ossification of tissue engineered cartilage constructed by adipose derived stem cells. Therefore, the effect of thrombospondin-1 in inhibiting the ossification of tissue engineered cartilage was evaluated in this study. Lentivirus vectors carrying thrombospondin-1 cDNA were transfected into adipose derived stem cells, and the transfected cells were used in the experiments. The expression of thrombospondin-1 was evaluated by quantitative reverse transcriptase-polymerase chain reaction and western blot, and the effects of thrombospondin-1 over-expression on angiogenesis were analyzed by angiogenesis assays. The quality of tissue engineered cartilage and the degree of ossification were assessed by biomechanical and molecular biology methods. The results showed that thrombospondin-1 infected cells have a high expression of thrombospondin-1 in mRNA and protein level, which inhibited the tube formation of endothelial cells, indicating the anti-angiogenic effects. Gene expression analyses in vitro showed that thrombospondin-1 inhibits the osteogenic differentiation of adipose derived stem cells significantly, and the results of in vivo study revealed that thrombospondin-1 significantly inhibits the expression of osteogenic genes. Compared to that in the control group, tissue engineered cartilage constructed by thrombospondin-1 transfected adipose derived stem cells in vivo showed a higher GAG content and lower compressive modulus, which indicating lower level of ossification. In conclusion, the current study indicated that the anti-angiogenic factor thrombospondin-1 suppresses the osteogenic differentiation of adipose derived stem cells in vitro, and inhibits ossification of tissue engineered cartilage constructed by adipose derived stem cells in vivo.

Keywords: Thrombospondin-1, ossification, tissue engineered cartilage, ADSC, lentivirus

Introduction

Since cartilage is an avascular tissue characterized by a low cell density and limited nutrient supply, it has limited intrinsic repair and regenerative capacity after defects. Stem cells based cartilage tissue engineering provides alternative therapy in the treatment of cartilage defects. Cartilage tissue engineering benefits a lot from autologous pluripotent stem cells, such as bone marrow mesenchymal stem cell, which is derived from the bone marrow stroma, they are capable of differentiating into osteogenic, chondrogenic and adipogenic cells. Bone marrow mesenchymal stem cells has limited source, while adipose derived stem cells (ADSCs) obtained in large quantities, with minimal discomfort, under local anesthesia would be more advantageous than bone marrow mesenchymal stem cells in tissue engineering [1], they can differentiate into chondrogenic, adipogenic, and osteogenic cells in vitro in some specific induction condition. Adipose derived stem cells may represent an alternative stem cell source to bone marrow mesenchymal stem cells in cartilage tissue engineering. However, the adipose

derived stem cells often fail to undergo complete chondrogenic differentiation, which leads to the formation of ossification after in vivo implantation, and may be caused by vascular in growth and endochondral ossification after in vivo transplantation. Hennig T et al reported that chondrogenesis of ADSCs was associated with hypertrophy according to premature collagen Type X expression, up-regulation of alkaline-phosphatase activity and in vivo calcification of spheroids after ectopic transplantation in SCID mice [2].

Most bones contribute to longitudinal growth and are gradually replaced by bone in the embryonic cartilaginous model via endochondral ossification [3]. During this process, chondrocytes proliferate, and undergo hypertrophy and death; then cartilage tissue was invaded by blood vessels and osteoblasts that first form spongy bone at the primary ossification center in the diaphysis [4]. Blood vessels invasion play an important role in endochondral ossification, and how to inhibit blood vessels invasion seems to be an alternative method in inhibiting ossification of tissue engineered cartilage.

Thrombospondin 1 (TSP-1), a protein in humans that is encoded by the THBS1 gene, is a subunit of a disulfide-linked homotrimeric protein [5]. The thrombospondin-1 protein is a member of the thrombospondin family, and it is a multidomain matrix glycoprotein that has been reported to be a natural inhibitor of neovascularization [6]. Thrombospondin-1 has been reported to be an endogenous inhibitor of angiogenesis and tumor growth [7]. Nör JE et al reported that thrombospondin-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway [8]. Castle VP et al reported thrombospondin-1 suppresses tumorigenesis and angiogenesis in serum- and anchorage-independent NIH 3T3 cells [9]. Streit M et al also showed that over-expression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas [10].

Thence, the current study was to determine whether over-expression of thrombospondin-1 in ADSCs could prevent ossification of tissue engineered cartilage constructed by ADSCs. Lentivirus vectors carrying thrombospondin-1 DNA was transfected into adipose derived stem cells, and the expression of thrombospondin-1 in mRNA and protein level was evaluated by Q-PCR and western blot, and the effects of thrombospondin-1 over-expression on angiogenesis were analyzed by angiogenesis assays in vitro. The quality of tissue engineered cartilage in vivo and the degree of ossification were assessed by biomechanical and molecular biology methods.

Materials and methods

The experimental protocol adhered to the rules of the animal protection act of China was approved by the university's laboratory animal care and use committee.

Isolation and expansion of human adipose derived stem cells

Informed consent was obtained from each patient prior to surgery, and the institution alethics committee approved the study protocol.

Human ADSCs were isolated as previously described [11]. Briefly, human adipose tissues were taken from healthy donors during liposuction. Then, the adipose tissue was dissociated enzymatically for 1 hour at 37°C using 0.1% collagenase type I (Gibco, Grand Island, NY, USA). After that, the mixture was neutralized by adding DMEM containing 20% FBS and centrifuged at 2,000 rpm for 5 minutes. The cell pellet was re-suspended in growth medium and re-centrifuged to better isolate floating mature adipocytes from the pelleted cells of stromal vascular fraction (SVF). Finally, SVF was suspended in growth medium and cultured in culture plate in a 37°C incubator with 5% CO_2. Cells were subpassaged using 0.25% trypsin containing 0.1% EDTA after reaching 80%-90% confluent. Cells at passage 3 were used in the subsequent experiment.

ADSCs transfected with lentivirus thrombospondin-1 vectors

To generate Lv-TSP-1, human TSP-1 sense complementary DNA was constructed in a lentivirus plasmid, as previously described [12, 13]. The resulting plasmid was used for transfection studies and was further used for lentivirus TSP-1 plasmid construction. Lentivirus expression vector and lentivirus package vectors pHelper1.0, pHelper2.0, and pVSVG (Invitrogen) were co-transfected into 293T cells. Lentivirus

Table 1. Primer sequences for RT-PCR

Gene	Primers	Product
Osteocalcin	Forward primer 5'-CATCTTCGTGCTCCTCACAGG-3'	194 bp
	Reverse primer 5'-AAACGCCAAAAGCCAAAGCC-3'	
RunX2	Forward primer 5'-CGCCTCACAAACAACCACAG-3'	225 bp
	Reverse primer 5'-TCACTGTGCTGAAGAGGCTG-3'	
COL1A2	Forward primer 5'-CTGCGACACAAGGAGTCTGC-3'	140 bp
	Reverse primer 5'-CCAGTCTCCCCCAAACAAGC-3'	
Osteopontin	Forward primer 5'-TCCAACGAAAGCCATGACCA -3'	355 bp
	Reverse primer 5'-TTGAGCGCTAGTCAGAACCA-3'	
Thrombospondin 1	Forward primer 5'-TGTTCTCTACTGGCTTTATGTCA-3'	348 bp
	Reverse primer 5'-GTTGCGTGTGCTTCAGTGTT-3'	
Aggrecan	Forward primer 5'-GTTTCCACAAGGGAGAGAGGG-3'	109 bp
	Reverse primer 5'-GTAGGTGGTGGCTAGGACGA-3'	
COL2A1	Forward primer 5'-CAAAGAGGACATGGGGCACT-3'	452 bp
	Reverse primer 5'-ACCTTTGTCACCACGATCCC-3'	
Sox-9	Forward primer 5'-AGGAAGTCGGTGAAGAACGG-3'	275 bp
	Reverse primer 5'-AAGTCGATAGGGGGCTGTCT-3'	
GAPDH	Forward primer 5'-GAGAAGGCTGGGGCTCATTT-3'	231 bp
	Reverse primer 5'-AGTGATGGCATGGACTGTGG-3'	

supernatant was collected and the titer of lentivirus was determined, then lentivirus vector with optimal target sequence infection of ADSCs cells [14]. The cells were derived into three groups: Lentivirus thrombospondin-1 vectors (Lv-TSP-1); Lentivirus GFP vectors (Lv-GFP); Control group (no transfection).

Fluorescence-activated cell sorting analysis

To assess the effect of TSP-1 transfection on the characteristics of cells, transfected cells and non-transfected cells were used for fluorescence-activated cell sorting analysis. 10^6 cells were washed in PBS and incubated for 1 h at 4°C with fluorescence-conjugated mouse antihuman mAb (CD73, CD44, CD105 and CD90). Cells were centrifuged at 2000 g, supernatants removed and cells washed thrice in PBS. Finally, labeled cells were resuspended in 1 ml PBS and subjected to analyze with Beckman Coulter FC 500 [15].

Gene expression analysis

In order to evaluate the gene expression, RNA was isolated for quantitative reverse transcriptase-PCR (RT-PCR) as described previously [16]. The expression levels of TSP-1, collagen II (col2A1), runx2, OCN, OPN, aggrecan, sox9, collagen I (col1A2) and were quantified by real-time RT-PCR using the ABI Prism 7900 sequence detection system (Applied Biosystems) and thermos script SYBR Green qRT-PCR Kit. The relative gene expression was performed by the standard curve method. The primers and probe sets are shown in the **Table 1**.

Western blot analysis

Immunoblotting was performed according to aprotocol described previously [17]. Briefly, the samples were lysed in lysis buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin and 1% Triton X-100. After centrifugation at 12,000 rpm, 50 mg of total protein of each sample was loaded into a 12% sodium dodecylsulfate polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membrane (Amersham Biosciences Inc., Piscataway, NJ, USA). The blocked membranes were then incubated with the indicated antibody (thrombospondin-1 (TSP-1) Ab-2, mouse monoclonal antibody, thermo fisher scientific Inc), and the immunoreactive bands were visualized using chemiluminescent reagent as recommended by the super signal west dura extended duration substrate kit (Pierce Chemical, Rockford, IL, USA). The signals of the bands were quantified. The results were expressed as relative-density [18].

Tube formation assay

Human umbilical vein endothelial cells (HUVEC) were cultured in 12-wellplates. The supernatants of TSP-1 transfected ADSCs or with those of uninfected ADSCs were harvested. The endothelial cells were incubated in the above supernatants for a total of 48 hours to allow the formation of tube-like structures. Tube formation was quantified as described previously [19]. The angiogenesis index was determined or

each field as the total length of connected tubes/surface of analysis [20].

Osteogenic induction and alizarin red staining

The ADSCs were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in DMEM containing 10% FBS. To induce osteogenic differentiation the media was added bone morphogenetic protein 2 (BMP-2, 300 ng/mL, R&D Systems, Minneapolis, MN, USA), ascorbic acid (0.05 mM), dexamethasone (10^{-8} M) and glycerophosphate (10 mM) [21]. After 16 days of osteogenic induction, the plate was sent for Alizarin Red staining as previously described [22].

ALP activity

Enzyme activity assay was performed according to the enzyme assay kit manual (No. 104-LL, Sigma). Substrate solution (4 mg p-nitrophenyl phosphate/mL) was mixed homogeneously with equal volume of alkaline buffer solution (1.5 M 2-amino-2 -methyl-1-propanol, pH 10.3) at 37°C in a water bath for 1 min. Then, 0.1 mL supernatant was added to 1 mL of the mixture, and reacted at 37°C for 15 min, at which point the enzyme-substrate reaction was stopped by the addition of 10 mL 0.05 N NaOH. The absorbance at 405 nm was monitored with a spectrophotometer and results were calculated as U/mg and data were expressed as ALP activity normalized by the protein concentration [23].

Scaffold construct and chondrogenic differentiation

ADSCs at the third passage were harvested with Trypsin-EDTA and seeded at 3×10^6 cells/mL of PGA scaffolds (5 mm diameter and 2 mm thickness). Cell-Scaffolds constructs were cultured with DMEM with 10% fetal bovine serum, glutamine 2 mM, penicillin 100 IU/mL, streptomycin 100 μg/mL and amphotericin B 2.5 μg/mL, and incubated at 37°C under a humidified atmosphere with 5% CO_2 for 48 hour. After 48 h, the medium was exchanged with chondrogenic medium (high glucose-DMEM supplemented with 10% fetal bovine serum (Gibco), 10 ng/ml TGF-β (R&D), 40 ng/ml IGF (R&D), 40 μg/mL L-Proline (Sigma), 50 μg/mL L-acid ascorbic (Sigma), 100 nM dexamethasone (Sigma) glutamine 2 mM (Sigma), penicillin 100 U/mL (Sigma), streptomycin 100 μg/mL (Sigma), amphotericin B 2.5 μg/mL (Sigma), 100 μg/mL sodium pyruvate (Sigma) [24].

Subcutaneous implantation and histological staining

After 8 weeks of chondrogenic induction in vitro, the cell-scaffolds were transplanted into nude mice subcutaneously for 8 weeks, and then the specimens were fixed in 4% paraformaldehyde overnight, and then embedded in paraffin. Serial 5-μm sections of the specimens were cut and stained with HE to evaluate the cartilage tissue.

Collagen I and II content

Collagen I and II content assay were performed as previously described [25, 26]. The harvested specimen were rinsed with dd-H_2O, lyophilized for 12 h followed by adding 1 mL cold H_2O and incubated overnight at 4°C in a micro centrifuge tube. After lyses with repeated freeze thawing and sonication cycles, samples were centrifuged at 10,000 rpm for 3 min. The precipitates obtained was sent for collagen I assay (type I collagen detection kit, chondrex, catalog # 6008) and collagen II assay (type II collagen detection Kit, chondrex, catalog # 6018) using according to the manufacturer's instructions.

GAG and compressive modulus

GAG assay was performed following the method previously described [27, 28]. Briefly, the samples were frozen at -20°C overnight and then lyophilized for 48 hours. Dry weights were recorded and then the samples were digested in papain, 125 μg/mL papain (Sigma) in 50 mmol phosphate buffer (pH 6.5) containing 2 mmol N-acetyl cysteine, for 18 hours at 60°C. After digestion, sulfated GAG content was measured using the blyscan sulfated GAG assay kit, a 1,9-dimethyl-methylene blue colorimetric assay (Accurate Chemical and Scientific Corp., Westbury, NY).

The biomechanical test was performed by using a biomechanical analyzer (Instron, Canton, MA, USA) following the previous method, a constant compressive strain rate of 1 mm/min was applied until a maximal force of 100 N was achieved to obtain a force-displacement curve. The

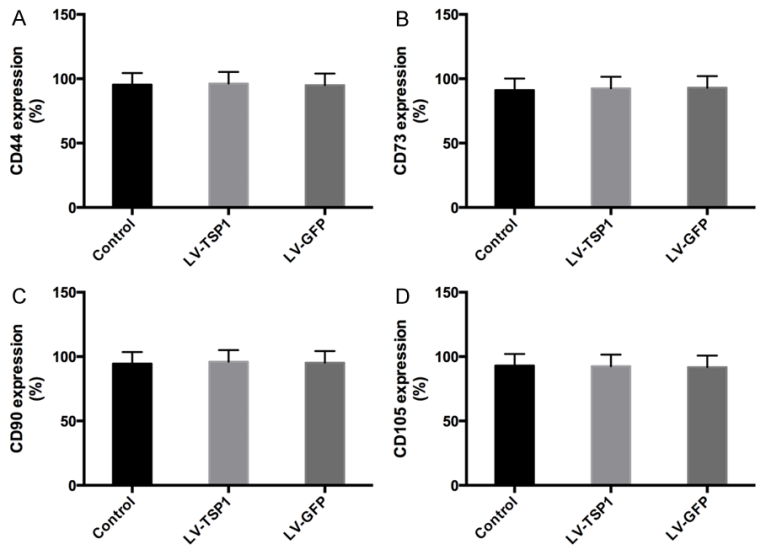

Figure 1. Flow cytometry analysis. TSP-1 transfected ADSCs and non-transfected ADSCs revealed very similar strongly positive expression for CD44 and CD73, CD90 and CD105 expression.

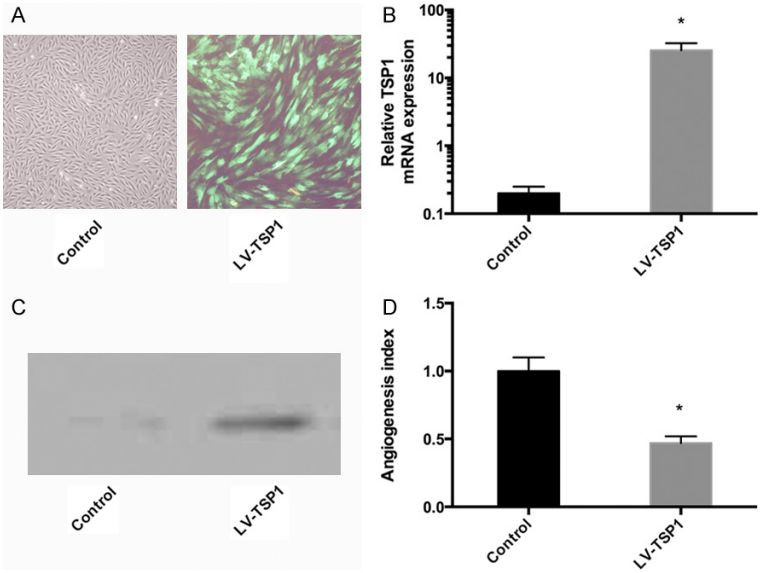

Figure 2. Analysis of TSP-1 transfection in vitro. After 48 h of transfection, TSP-1 transfected cells showed fluorescence expression. The mRNA and western blot showed the higher expression of tsp-1 in experimental group than that in control group (P<0.05). The experimental group revealed a significantly reduced angiogenesis index of theHUVEC than that in control group (P<0.05).

compressive modulus was calculated according to the force-displacement curve [29].

Statistic analysis

All data are presented as the mean ± SD. differences between groups two-way ANOVA followed by Sidak's or Tukey's multiple comparisons test. *P* values less than 0.05 were considered significant.

Results

Flow cytometry analysis

Cell surface antigen phenotyping was performed on transfected ADSCs and non-transfected ADSCs by flow cytometry. Notably, Lentivirus transfection does not effect positive expression of stem cell surface antigen. The transfected ADSCs and non-transfected ADSCs revealed very similar strongly positive expression for CD44 and CD73, CD90 and CD105 expression (**Figure 1**).

The transfection of lentivirusTSP-1 into ADSCs

As shown in **Figure 1**, non-transfected ADSCs (using a light microscope) and non-transfected cells (using a fluorescence microscope) was observed after 48 h of transfection, most of cells in experimental group contained fluorescence expression, indicating the successful transfection of TSP-1 into ADSCs (**Figure 2A**). The results of mRNA and western blot showed there was a higher expression of TSP-1 in experimental group than that in control group (P<0.05) (**Figure 2B**, **2C**).

HUVEC was cultured inthesupernatants of ADSCs after transfection with TSP-1 and in the supernatants of ADSCs without transfection respectably. The angiogenesis index of the HUVEC was significantly reduced in experimental group than that in control group, which indicated that secreted transgenic TSP-1 in the supernatants of TSP-1 transfected cells inhibited the tube formation of HUVEC (**Figure 2D**).

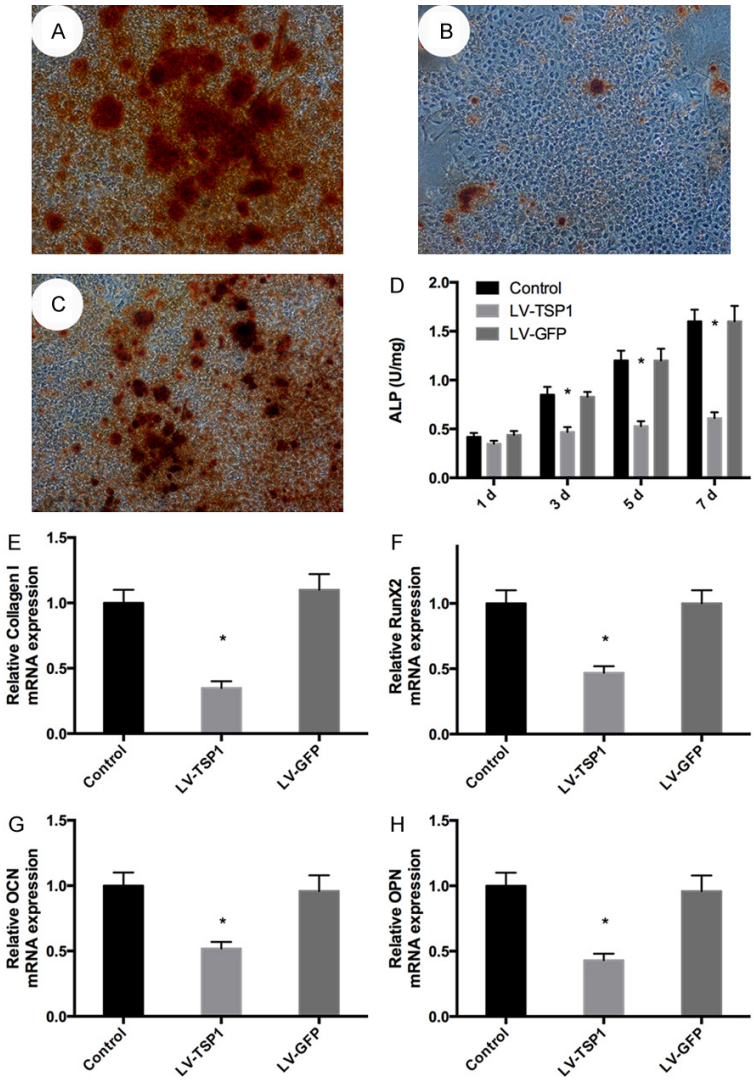

Figure 3. Osteogenic differentiation assay. TSP-1 transfected cells groups showed lower positive expression for alizarin red S Staining after 16 days osteogenic induction differentiation. The relative expression of osteogenic genes (Collagen I, RunX2, OCN, and OPN) was lower than that in experimental group ($P<0.05$).

Osteogenic differentiation

Osteogenic differentiation was performed by using ADSCs with TSP-1 transfection and cells without transfection. After 16 days, some plates were sent for alizarin red S staining, the other for Q-PCR. Alizarin red is used to identify calcium deposits, there was lower positive expression in TSP-1 transfected group than that in control groups, meaning TSP-1 inhibit osteogenic differentiation of TSP-1 transfected ADSCs. In addition, ALP activity was also lower in TSP-1 transfected group than that in control groups. The relative expression of osteogenic genes (collagen I, runX2, OCN, and OPN) reduced significantly in experimental group (**Figure 3**).

In vivo constructed tissue

After 8 weeks of in vivo transplantation, the constructed tissue in vitro formed cartilage or bone-like tissue. The histological staining showed cartilage lacunas were observed in 3 groups, meaning TSP-1 transfected cells and non-transfected cells differentiate into chondrocytes (**Figure 4**).

Biochemical and biomechanical evaluation

The collagen I content, collagen II content and GAG deposition of in vivo tissue was further quantified by biochemical analysis. Collagen I content is lower in TSP-1 transfected group, while collagenII content is higher in TSP-1 transfected group. Compared with the other two groups, it indicated TSP-1 may enhance chondrogenic differentiation (**Figure 5A**, **5B**).

The GAG amount of experimental group is higher in experimental than that in the other two groups, while the compressive modulus in experimental group is lower in experimental than that in the other two groups (**Figure 5C**, **5D**).

mRNA expression

After 8 weeks of in vivo culture, the harvested tissues were sent for Q-PCR assay. These results suggested that the experimental group expressed chondrogenic mRNA (aggrecan, collagen II, and Sox-9) at significantly higher level than those in control groups (**Figure 6**). Meanwhile, the expression of collagen I, OCN, and runX2 was significantly reduced in TSP-1 transfected group (**Figure 6**). These results sug-

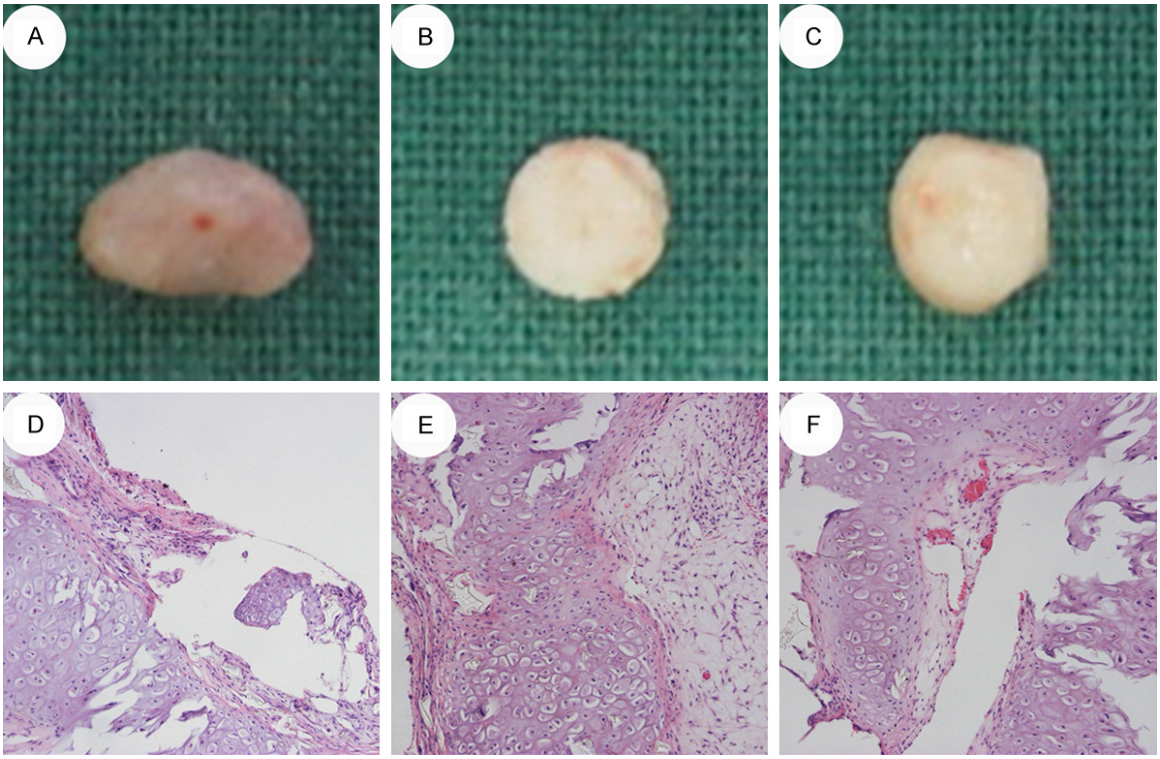

Figure 4. Gross view and HE staining of in vivo tissue. After 8 weeks of in vivo transplantation, the constructed tissue in vitro formed cartilage or bone-like tissue. The HE staining showed cartilage lacuna were observed in 3 groups, means TSP-1 transfected cells and non-transfected cells differentiate into chondrocytes.

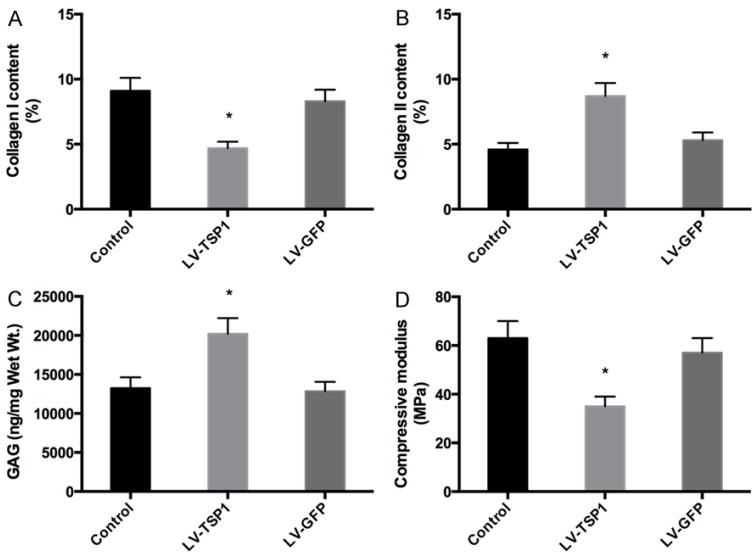

Figure 5. Biochemical and biomechanical evaluation. Collagen I content reduced significantly in TSP-1 transfected group ($P<0.05$), while Collagen II content increased in TSP-1 transfected group, compared with the other two groups. The GAG amount of experimental group is higher in experimental than that in the other two groups, while the compressive modulus in experimental group is lower in experimental than that in the other two groups ($P<0.05$).

gested the over-expression of TSP-1 strongly promoted chondrogenic differentiation and inhibited the osteogenic differentiation.

Discussion

Cartilage tissue engineering provides a promising therapy for the repair of cartilage defects, and ADSCs seems to be an ideal seeding cells for tissue engineering. However, the clinical application of tissue engineered cartilage is still challenged by one problem-ossification, which may be explained by that: For one hand, incomplete chondrogenic differentiation leads to the unstable chondrocytes phenotype after in vivo transplantation; for the other hand, vessel invasion promotes ter-

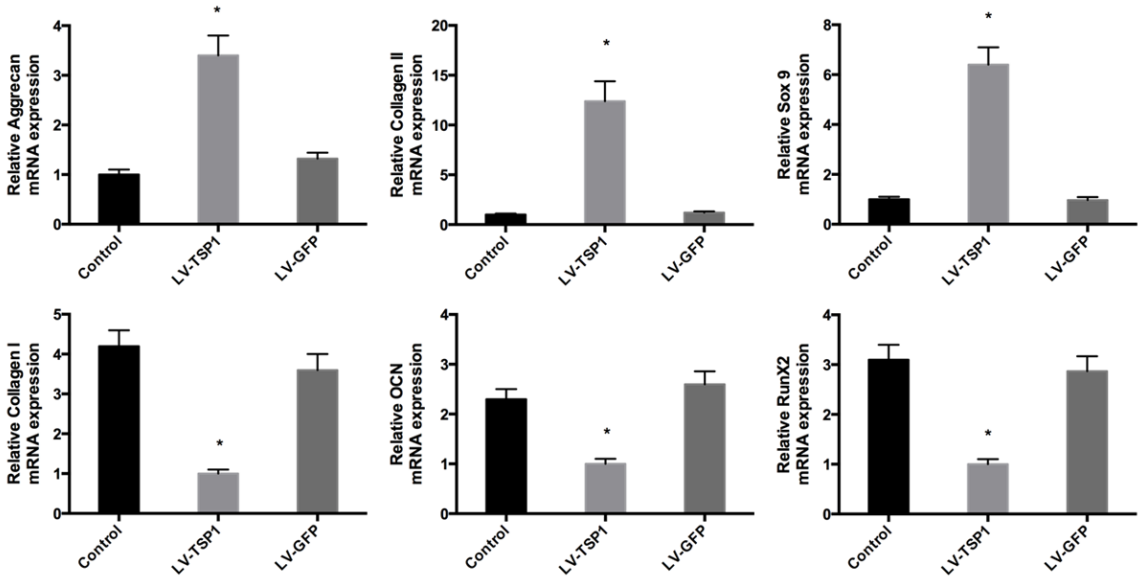

Figure 6. mRNA expression. The chondrogenicm RNA (Aggrecan, Collagen II, and SOX-9) in the experimental group were significantly higher levels than those in control groups ($P<0.05$), and the expression of collagen I, OCN, and RunX2 was significantly lower inTSP-1 transfected group ($P<0.05$).

minal chondrogenic differentiation and inadvertent endochondral ossification [20]. It is well known that cartilage tissue is significantly different from other tissues for its rich highly sulfated extra cellular matrix (ECM), and is peculiar in its avascularity, hence materials, such as nutrition and oxygen is transported from connected tissue or eriosteum mainly by diffusion [30]. Cartilage tissue retained the avascularity in the presence of anti-angiogenic proteins, such as thrombospondins or chondromodulin 1 under physiologic conditions [31, 32]. Thence the present study was aimed to determine if over-expression of thrombospondin-1 could prevent vascular invasion and ossification of tissue engineered cartilage constructed by ADSC.

In the current study, we used lentivirus vectors to carry thrombospondin-1 cDNA, lentivirus vectors provided along term and stable release of thrombospondin-1, comparing to the short half-life of recombinant proteins in vivo, and adenovirus vectors. Lentiviral vectors offered several attractive properties as gene-delivery vehicles, including: (i) sustained gene delivery through stable vector integration into host genome; (ii) the capability of infecting both dividing and non-dividing cells; (iii) broad tissue tropisms, including important gene- and cell-therapy-target cell types; (iv) no expression of viral proteins after vector transduction; (v) the ability to deliver complex genetic elements, such as polycistronic or intron-containing sequences; (vi) potentially safer integration site profile; and (vii) a relatively easy system for vector manipulation and production [33]. Lentivirus vector mediated transgene method provides an efficient tool for in vitro modification of MSCs that does not interfere with differentiation. Zhang et al reported that transgene expression via lentiviral vectors was maintained in culture for at least 5 months, lentiviral vectors were able to transduce clonogenicmesenchymal progenitor cells, which were capable of maintaining transgene expression by their MSC progeny, over several cell divisions and during differentiation into adipocytes, and the terminal adipocyte cell differentiation was unaffected by lentivirus-mediated reporter gene transfer [34]. We transfected thrombospondin-1 cDNA into ADSCs, and the results of mRNA and Western blot showed a higher expression of thrombospondin-1 in experimental group than that in control group. Subsequently, we performed the tube formation assay that was used to evaluate vascular activity of cells, the tube-forming capacity of HDMECs was reduced in a medium containing supernatants of TSP-1-transfected ADSCs, indicating TSP-1

inhibit angiogenic capability. Wang et al reported that overexpression of thrombospondin-1 domain containing 7A (THSD7A) carboxyl-terminal fragment in HUVECs inhibited cell migration and disrupted tube formation, while suppression of THSD7A expression enhanced HUVEC migration and tube formation [35].

In the in vitro study, we found that thrombospondin-1 suppressed the osteogenic differentiation of ADSCs. Transcription factors that play critical roles in regulation of chondrogenic and osteogenic gene expression under the control of these extracellular factors include runx2, sox9 and Collagen I. The ALP activity was decreased in the thrombospondin-1 transfected group, and the osteogenic gene (collagen I, runX2, OCN, and OPN) was also reduced in the experimental group. TSP-1 blocked osteoblast differentiation of ADSCs grown in osteogenic media as measured by decreased runx2 and alkaline phosphatase expression. Canfield et al reported in their report, the production of a mineralised matrix by vascular pericytes was promoted by the presence of antibodies to TSP-1 in the culture medium and was inhibited by exogenous TSP-1, which means high levels of TSP-1 inhibit pericytemineralisation, supporting the view that this protein plays a role in pericyte differentiation and bone formation [36].

Transforming growth factor-β (TGF-β) is a critical regulator in bone development and remodeling, and thrombospondin-1 is a major regulator of latent TGF-β activation and is critical for regulation of TGF-β activity in multiple diseases, which must be activated from its latent form in order to signal. Bailey Dubose showed thrombospondin-1 inhibits osteogenic differentiation of human mesenchymal stem cells through latent TGF-β activation [37]. It was speculated that TSP-1 exerts its inhibitory effect on osteoblast differentiation via activating latent TGF-β, since a peptide which blocks TSP-1 TGF-β activation reduced TGF-β activity and restored osteoblast differentiation as measured by increased Runx2 and alkaline phosphatase expression [37].

Besidesthein vitro experiments, thrombospondin-1 strongly prevented endochondral ossification in vivo study. After 8 weeks of in vivo implantation, we found the cartilage in thrombospondin-1 transfected group formed cartilage-like appearance, while vascular invasion or ossification was observed in part of the surface in some specimens. It is well known that in OA progresses, collagen orientation changes and collagen content decreases, collagen II content decreased, while collagen I content increased. In the current study, collagen I content was higher in control groups, while collagen II content was higher in thrombospondin-1 transfected group, indicated some tissue become ossified.

Cartilage has a zonal architecture with decreasing glycosaminoglycan (GAG) content through the depth, and GAG content in cartilage decreases with increasing age. GAG concentration decreases in the OA disease process, especially in the superficial layer [38]. GAG content assay was also performed, we found GAG content reduced in the control groups, implying cartilage may undergo endochondral ossification.

In addition, endochondral ossification is an important biological progress in mammalian skeletal development and tissue patterning, that is also involved in osteoarthritis pathogenesis. Multipotent mesenchymal cells differentiate into chondrocytes and further differentiate into hypertrophic chondrocytes, subsequently replaced with bone tissues [3, 39]. Among this progress, matrix metalloproteinase 13 (MMP13) and collagen X play a unique and important biological role. Riko et al reported that matrix metallopeptidase 13 (MMP13) is an important target of Osterix, that is essential to endochondral ossification, and the introduction of MMP13 stimulated the calcification of matrices in Osterix-deficient mouse limb bud cells. Col10a1 is high expression on the calcification and degradation of chondrogenic matrices [39, 40]. In the control groups, MMP13 and Col10a1 had higher expression than that in experimental groups, that may be because degradation of cartilage matrices, vascular invasion into cartilage tissues, and cartilage replacement with bone tissues.

In conclusion, thrombospondin-1 exerts inhibition of ossification of tissue engineered cartilage, the effect of thrombospondin-1 cannot only be ascribed to its anti-angiogenic properties, but also could inhibit osteogenic differentiation directly and other still unknown molecular mechanisms and pathways, which need to be further investigated.

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Disclosure of conflict of interest

None.

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