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REGULAR ARTICLE

Sox11-modified mesenchymal stem cells accelerate cartilage defect repair in SD rats

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Abstract

Cartilage has a limited capacity to heal. Previously, we have shown that overexpression of Sox11 in rMSCs (Rat Mesenchymal Stem Cells) by lentivirus-mediated gene transfer leads to enhanced tri-lineage differentiation and accelerated bone formation in fracture model of rats. We observed that the fracture repair in the rats that received Sox11-modified rMSCs injection proceeded through an endochondral ossification process much faster than those in the control groups. However, the detailed role of Sox11 in rMSCs chondrogenic differentiation, as well as cartilage defect, is still not clearly clarified. Therefore, this study tests the hypothesis that Sox11 promotes chondrogenesis and cartilage defect repair by regulating β-catenin. Sox11 was transduced into rMSCs using lentiviruses. The expression levels of β-catenin and its downstream genes were evaluated by quantitative RT-PCR. The transcriptional activation of β-catenin was proved by dual-luciferase reporter assay and co-immunoprecipitation was performed to evaluate Sox11-β-catenin interaction. In addition, a cartilage defect model in SD rats was used to evaluate the cartilage regeneration ability of Sox11-modified rMSCs in vivo. We found that Sox11 transcriptionally activated β-catenin expression and discovered the core promoter region (from −242 to −1414) of β-catenin gene for Sox11 binding. In addition, Sox11 might regulate β-catenin at the post-transcriptional level by protein-protein interaction. Finally, using a cartilage defect model in rats, we found Sox11-modified rMSCs could improve cartilage regeneration. Taken together, our study shows that Sox11 is an important regulator of chondrogenesis and Sox11-modified rMSCs may have clinical implication for accelerating cartilage defect healing.

Keywords rMSCs · Sox11 · Cartilage · Chondrocyte · Chondrogenesis · β-Catenin

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Introduction

Osteoarthritis is a complex degenerative joint disease result from breakdown of joint cartilage and underlying bone (Pereira et al. 2015). It is characterized by the progressive loss of articular cartilage and chondrocytes within synovial joints. Not only are the elderly but also young people commonly injured by this disease (Gelber et al. 2000). As to the biological properties of avascularity and hypocellularity of cartilage, cartilage has little capacity to repair itself (Johnstone et al. 2013). The management of cartilage lesions is still a big challenge for surgeons due to its limited regenerative ability. Generally, several treatments have been attempted to restore joint surface defects, such as osteochondral autograft transplant system (OATS) and autologous chondrocyte implantation (ACI) and microfracture (Robert et al. 2007). Unfortunately, the cartilage formed by these procedures is frequently fibrocartilage instead of functional hyaline cartilage. The emergence of MSCs (mesenchymal stem cells) has provided a great promising prospective for cartilage tissue engineering (Mithoefer et al. 2009; Richter 2009). These MSCs from bone marrow, as well as other tissues, all have multipotent capacity to differentiate into a variety of other cell types, including osteoblasts, adipocytes, chondrocytes, myoblasts and neurons (Jiang et al. 2002; Pittenger et al. 1999). And in response to stimuli, MSCs have the ability of homing to the target damage tissue. Also, MSCs have been shown to be immunosuppressive and anti-inflammatory, they do not express MHC-II, CD80, CD86 and CD40 and minimally express MHC-I on the cell surface (Deans and Moseley 2000; Pittenger et al. 1999; Tse et al. 2000). These characteristics make MSCs a promising cell source for tissue engineering, particularly for cartilage and bone regeneration.

Sox11 belongs to the SoxC group of Sox transcription factors. The Sox (Sry-related HMG box) proteins are characterized by the HMG box, which is a highly conserved DNA binding domain. The main function of Sox11 is involved in neural development and organogenesis during fetal development (Azuma et al. 1999; Kuhlbrodt et al. 1998). It has been demonstrated that Sox11 was highly expressed in developing sensory neurons and ablating Sox11 caused an arrest of axonal outgrowth in vivo and in vitro (Lei et al. 2011). The Sox11 knockout mice showed craniofacial and skeletal malformations, asplenia and hypoplasia of the lung, stomach and pancreas (Sock et al. 2004), implying that Sox11 may be associated with bone development. Recently, the role of Sox11 in MSCs has been studied. It has been identified as one of the MSC-characteristic transcription factors involved in MSCs stemness regulation by DNA microarray analysis (Kubo et al. 2009) and Sox11 knockdown suppressed MSCs' self-renewal capacities and reduced their osteogenic and adipogenic differentiation potential. Our recent study also demonstrated that Sox11 could regulate trilineage differentiation of MSCs by activating the BMPs/Smad signaling pathway (Xu et al. 2015). However, the detailed role of Sox11 in MSCs chondrogenic differentiation, as well as cartilage defect, is still not clearly clarified.

The Wnt signaling pathway is best known for its roles in tissue development and cancer (Clevers 2006). The canonical β-catenin signaling pathway has been associated with chondrogenesis and cartilage development (Chun et al. 2008). On the one hand, forced expression of the ligands of the β-catenin pathway has been shown to inhibit embryonic mesenchymal cells condensation and transition to cartilage nodules (Hwang et al. 2005). On the other hand, activation of the β-catenin has been shown to promote chondrocyte differentiation in a Sox9-dependent manner (Yano et al. 2005). The crosstalk with other signaling pathways may influence the effect of the β-catenin pathway as chondrogenesis and cartilage development are complicated biological processes.

In the present study, following our previous study, we further demonstrate that Sox11 transcriptionally activated β-catenin expression. In addition, Sox11 and β-catenin may have direct protein-protein interaction in the nucleus to direct chondrogenesis. Finally, using a cartilage defect rat model, we demonstrate rMSCs stably overexpressing Sox11 could facilitate cartilage repair in vivo.

Material and methods

Production of lentivirus and infection

The lentiviral vector expressing Sox11 was constructed in our previous report (Xu et al. 2015). Pseudo-lentiviruses were produced by transient transfection of 293FT packaging cells (Invitrogen, USA) using the calcium phosphate method. Culture supernatants were harvested at 48 and 72 h after transfection and lentiviral particles were concentrated using PEG6000 (Kutner et al. 2009). For transduction, 1×10^5 cells were seeded into a 6-well plate and incubated with lentiviruses and 8 μg/mL polybrene in an incubator for 24 h. After 48 h, blasticidin (Invitrogen, USA) was added into medium to select rMSCs stably expressing Sox11 (Sox11-rMSCs) or empty vector (con-rMSCs).

Isolation and culture of rMSCs

All experiments were approved by the Animal Research Ethics Committee of the authors' institution. Bone marrow was flushed out from the bone cavity of the Sprague-Dawley rats and subject to density gradient centrifugation over Lymphoprep™ (1.077 g/ml; AXIS-SHIELD, Norway) to

obtain the mononuclear cells (MNCs). The MNCs were cultured in α-MEM, 10% fetal bovine serum, 2 mM L-glutamine (Invitrogen, USA) at 37 °C with 5% CO_2. The cells were trypsinized and subjected to flow cytometry examination to confirm the rMSCs' surface markers (CD90, CD44, CD73, CD31, CD34, CD45).

Luciferase reporter gene assay

Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega, USA) according to the instructions. 293T cells were co-transfected with 400 ng empty vector or pLL3.7-Sox11, pcatenin-luc and 10 ng of the pRL-CMV using Lipofactamine 3000. Luciferase activity was measured at 48 h after transfection using FluoStar Galaxy (BMG Labtechnologies).

RNA extraction and real-time PCR

Total cellular RNA was isolated with RNeasy (Qiagen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized with MMLV reverse transcriptase (Promega, USA). PCR amplification was performed using the ABI StepOne Plus system (Applied Biosystems, CA, USA) with primer sets outlined in Supplemental Table 1. Primer sequences were determined through established GenBank sequences. β-Actin was used as an internal control to evaluate the relative expression.

Chromatin immunoprecipitation (CHIP) assay

CHIP assay was conducted according to Millipore Magna CHIP kit (catalog number 17-10085). Briefly, all of the subsequent steps were performed at 0–4 °C. Sox11 plasmid was transfected into HEK293T cells for 72 h. Then cells (about 2×10^7) were washed with phosphate-buffered saline (pH 7.4) and lysed for 15 min with rotation 800 g at 4 °C for 5 min. Cell pellets were collected and resuspended in 0.5 mL of Nuclear Lysis Buffer. Then samples were sonicated on ice using ULTRASONIC PROCESSOR (Model: GE 130 PB). Afterward, IP was performed with anti-Sox11 antibody (Abcam, 1:100) or rabbit IgG as negative control. Washes and purification of the CHIP DNA were performed as suggested by the manufacturer. Real-time PCR was performed using 1 μl of CHIP DNA solution. ChIP PCR analysis primer sets are shown in Supplementary Table 2.

Coimmunoprecipitation and western blot

The Sox11 plasmid was transfected into HEK293T cells for 72 h. The cells were washed with cold PBS twice and harvested by scraping in cold cell extraction buffer (Invitrogen, Cat. no. FNN0011). Protein concentration was determined by the Bradford method (Biorad, USA). Cell lysates were incubated with anti-β-catenin (1:100) or anti-Sox11 (1:100) antibody and protein A/G-magnetic beads under gentle agitation at 4 °C overnight. The beads were washed three times with buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 1% Triton X-100), resuspended in 10 μL of SDS–polyacrylamide gel electrophoresis sample loading buffer and boiled for 5 min. Western blot was then performed. Equal proteins were loaded onto 10% Tris/glycine gels for electrophoresis and then transferred to a PVDF membrane and blocked in 5% non-fat milk (Biorad, USA) for 1 h at room temperature with rocking. Then, the primary antibody, anti-β-catenin (1:1000, BD Company), anti-Sox11 (1:1000, Abcam) or anti-GAPDH (1:1000, Santa Cruz) was added and incubated for 2 h at room temperature or at 4 °C overnight. After washing in TBST for three times (5 min for each time), the membrane was incubated with horseradish peroxidase-linked secondary antibodies (anti-mouse or anti-goat) for 1 h at room temperature. Following three TBST washes, protein was detected with the enhanced chemiluminescence (ECL) blotting reagents (Amersham Biosciences, USA) according to the manufacturer's instructions.

Rat cartilage defect model

All rats were housed in a designated government approved animal facility at The Chinese University of Hong Kong according to The Chinese University of Hong Kong's animal experimental regulations. Animal surgery was approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong and carried out under the animal licenses issued by the Hong Kong SAR Government. All surgeries were performed under anesthesia and efforts were made to minimize the suffering of the animals. During surgery, a lateral parapatellar longitudinal incision was made to expose the knee joint. The synovial capsule was incised and the trochlear groove was exposed after medial luxation of the patella. With the knee maximally flexed, a defect (1.5 mm in diameter and 1.5 mm in depth) was created in the center of the groove, using a dental drill. All debris was removed from the defect with a curette and irrigation. Ten microliters of 2% hyaluronic acid hydrogel with 1×10^5 rMSCs or Sox11-rMSCs was immediately implanted into the defect cavity. The patella was physically relocated and the joint capsule and subcutaneous tissue was closed with Vicryl 5–0 sutures. The skin was closed with Vicryl 4–0 sutures. After 4 weeks, the animals were sacrificed. Samples were fixed in 4% neutral buffered formalin for 72 h at room temperature. Following fixation, samples were stored in 70% ethanol. Histological sections of the repaired tissue were analyzed using a grading system

Table 1 Histological scoring system for cartilage repair

Category	Points
Cell morphology	
Hyaline cartilage	0
Mostly hyaline cartilage	1
Mostly fibrocartilage	2
Mostly noncartilage	3
Noncartilage only	4
Matrix-staining (metachromasia)	
Normal (compared with host adjacent cartilage)	0
Slightly reduces	1
Markedly reduced	2
No metachromatic stain	3
Surface regularity[a]	
Smooth (> 3/4)	0
Moderate (1/2 to 3/4)	1
Irregular (1/4 to 1/2)	2
Severely irregular (< 1/4)	3
Thickness of cartilage[b]	
2/3 to 4/3	0
5/3 to 4/3	1
1/3 to 2/3 or > 5/3	2
< 1/3	3
Integration of donor with host adjacent cartilage	
Both edges integrated	0
One edge integrated	1
Neither edge integrated	2

[a] Total smooth area of the reparative cartilage compared with the entire area of the cartilage defect. [b] Average thickness of the reparative cartilage compared with that of the surrounding cartilage

reported by other researchers (Table 1) (Yamaguchi et al. 2016). The scoring was performed by three observers in a blinded manner.

Statistical analysis

All experiments were performed at least three times. The data were analyzed by independent two-tailed Student's *t* test using SPSS (version 16.0; SPSS Inc., Chicago, IL, USA). $p < 0.05$ was regarded as statistically significant.

Results

Sox11 overexpression augments the level of β-catenin in rMSCs

To detect the effect of Sox11 on β-catenin expression, Sox11 was transduced into rMSCs using lentivirus. The mRNA and protein levels of β-catenin were detected by real-time PCR and western blot respectively (Fig. 1a, b'). The result showed that β-catenin was significantly increased by Sox11 at both mRNA and protein levels.

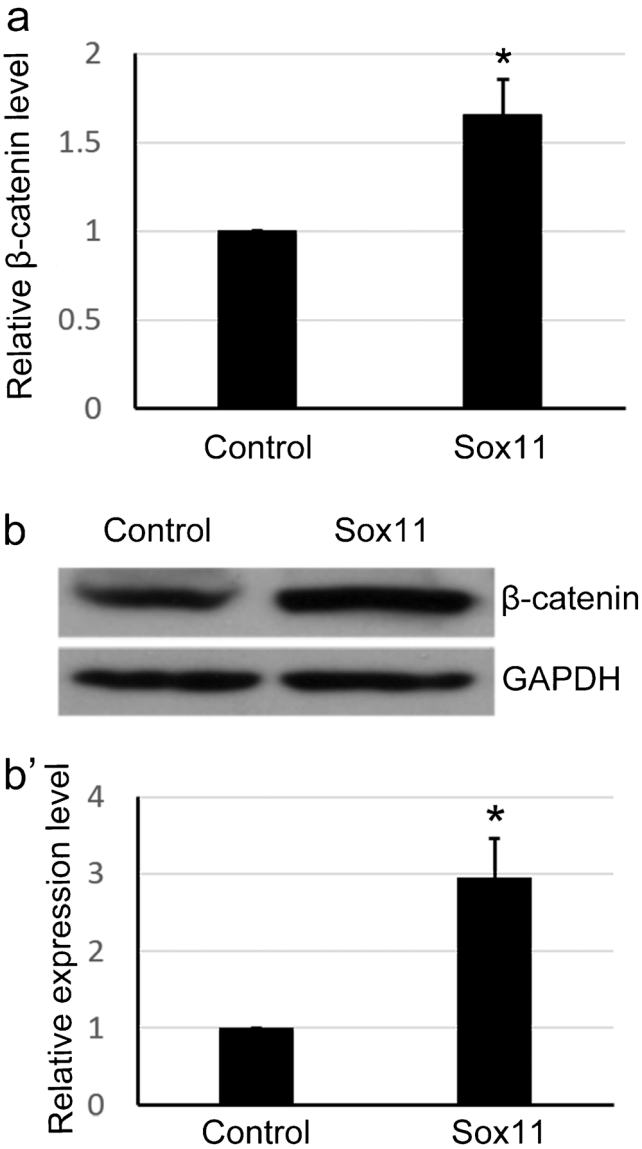

Fig. 1 Sox11 overexpression increases β-catenin at both mRNA and protein levels. (**a**) Total RNA used for real-time PCR is extracted from MSCs (control) or MSCs transduced with Sox11 (Sox11-MSC). The mRNA level of β-catenin is detected using real-time PCR. β-Actin is used as an internal control. The mRNA level of β-catenin in control is arbitrarily given as 1.0. The data are expressed as mean ± SD ($n = 3$). (**b**) Total proteins extracted from control and Sox11-MSC are analyzed by western blot using anti-β-catenin antibody. β-Actin is used as loading control. (**b'**) The intensity of the bands is quantified using Image J. *$p < 0.05$

Sox11 overexpression activates downstream targets of β-catenin in rMSCs

C-myc and cyclin D1 are known as downstream targets of the Wnt/β-catenin signaling pathway. So we also checked their

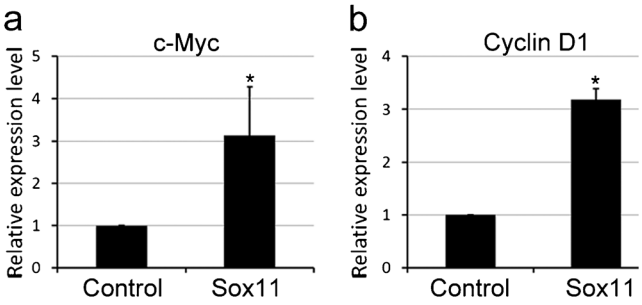

Fig. 2 Sox11 activates c-myc and cyclin D1 expression in MSCs. Total RNA is extracted from MSCs (control) or MSCs transduced with Sox11 (Sox11-MSC). The mRNA levels of c-myc and cyclin D1 are detected using real-time PCR. β-Actin is used as an internal control. The mRNA level of β-catenin in control is arbitrarily given as 1.0. The data are expressed as mean ± SD ($n = 3$, $^*p < 0.05$)

mRNA expression levels by real-time PCR. The result showed that levels of C-myc and cyclin D1 were significantly increased by Sox11overexpression (Fig. 2a, b).

Sox11 augments β-catenin promoter activity

Four consecutive 5′-end deletion mutant plasmid constructs of the DNA sequence region from − 2000 to + 20 of β-catenin promoter were employed to find out the core binding sites of Sox11 (Fig. 3a). Equal amounts of each construct, as well as empty vector, were transfected into HEK293 cells respectively, followed by the measurement of promoter activity by dual luciferase assay. We found that β-catenin promoter activity was significantly higher in HEK293 cells harboring constructs

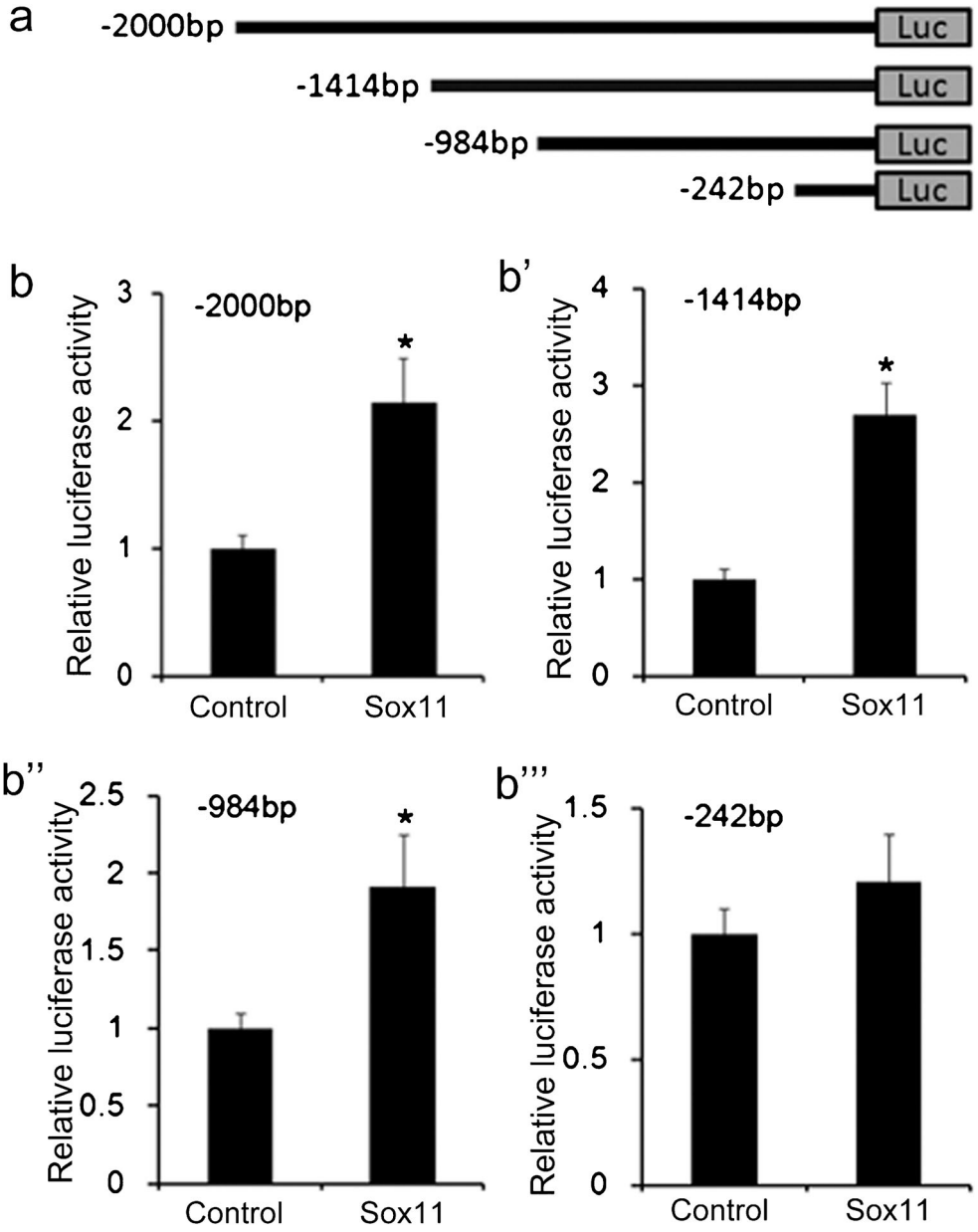

Fig. 3 Sox11 transcriptionally activates the expression of β-catenin. Luciferase activity assay is conducted in 293 T cells as described in the Methods. (**a**) Different constructs of β-catenin promoter are established to find the main binding sites of Sox11. (**b, b**”) The relative luciferase activity is quantified as normalized luciferase activity of control ($n = 3$). The data represent mean ± SD. $^*p < 0.05$

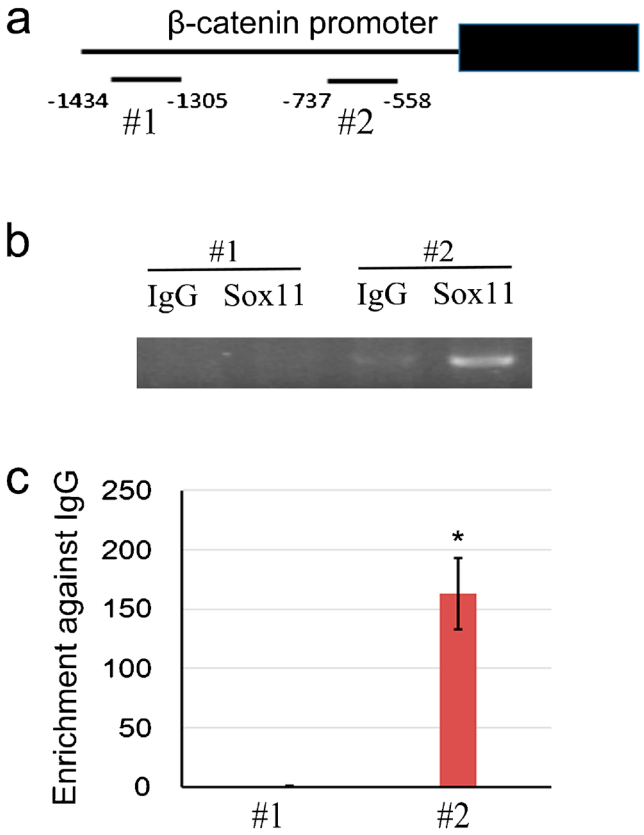

Fig. 4 Sox11 bound to regions of β-catenin promoter. (**a**) Schematically shows the different promoter regions detected by qPCR. (**b**) Electrophoresis of the products of qPCR. (**c**) qPCR shows that Sox11 bound to different regions of β-catenin promoter against IgG enrichment. #1 and #2 represent three primers located within β-catenin promoter region

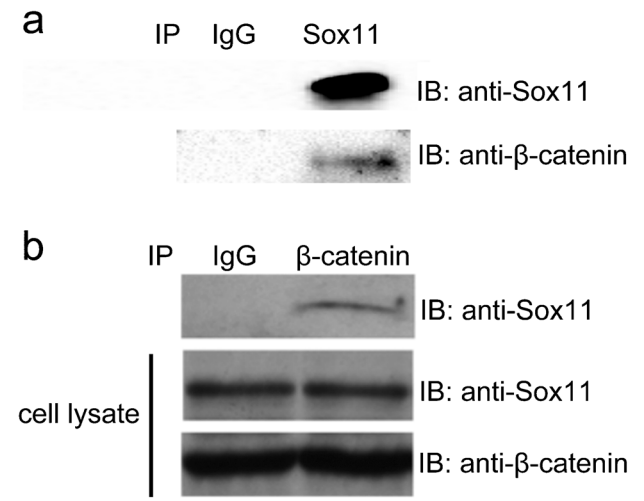

Fig. 5 Sox11 interacts with β-catenin in vitro. HEK293T cells are transfected with Sox11 by lipofactemine 2000, then total proteins are collected using RIPA buffer at 72 h after transfection. The cell lysates are immnunoprecipitated with Sox11 antibody (**a**), or β-catenin antibody (**b**) and magnetic beads. β-Catenin antibody and Sox11 antibody are used to detect the precipitated proteins by western blot, respectively

with 5′-ends from − 242 to − 2000, while there was no difference in cells harboring the construct with 5′-ends from − 242 to + 20 (Fig. 3b, b”’). These data suggested that binding sites of Sox11 in β-catenin promoter may lie in the region from − 242 to − 2000.

Then, we further conducted the chromatin immunoprecipitation (ChIP) assay to detect the binding of Sox11 with β-catenin promoter. Both the qPCR and electrophosis result showed that Sox11 could bind with β-catenin promoter (Fig. 4a–c). As a comparison, the immunoprecipitated products using normal IgG did not contain the β-catenin promoter DNA sequences.

Sox11 interacts with β-catenin in vitro

We next studied by coimmunoprecipitation whether Sox11 was able to interact with β-catenin in vitro. HEK293T cells were transfected with Sox11; total proteins were collected at 72 h after transfection. The cell lysates were immnunoprecipitated with Sox11 or β-catenin antibody. The interaction between β-catenin and Sox11 was detected by western blot (Fig. 5a, b). The result demonstrated that Sox11 and β-catenin did have physical interaction in vitro, which may inhibit the degradation of β-catenin and lead to augmented Wnt signaling.

Sox11 overexpressing rMSCs promotes cartilage regeneration

Finally, in order to determine the effect of Sox11 overexpressing rMSCs on cartilage regeneration, 10 μl of 2% hyaluronic acid hydrogel with 1×10^5 rMSCs or Sox11-rMSCs was immediately implanted into the defect cavity. By 6 weeks, the osteochondral defects were filled with newly formed cancellous bone covered by a superficial layer of hyaline cartilage or fibrocartilage tissue in the Sox11-rMSCs group, while the control group still showed big gaps as indicated by Safranin O staining and IHC staining using Sox9 or Aggrecan antibody (Fig. 6a, a’, c–e’). The histologic grading scale for cartilage repair showed that the score associated with the Sox11-rMSCs group was significantly improved compared with that from the control group (Fig. 6b).

Discussion

Cartilage damage resulting from trauma or pathologic factors is very common and often reduces the patient’s quality of life. Chondrocyte implantation is not an ideal choice for cartilage repair due to its poor expansion capacity (Phull et al. 2016). rMSCs have chondrogenic differentiation potential and have become a promising cell source for tissue engineering. Interestingly, mobilization of endogenous rMSCs has been

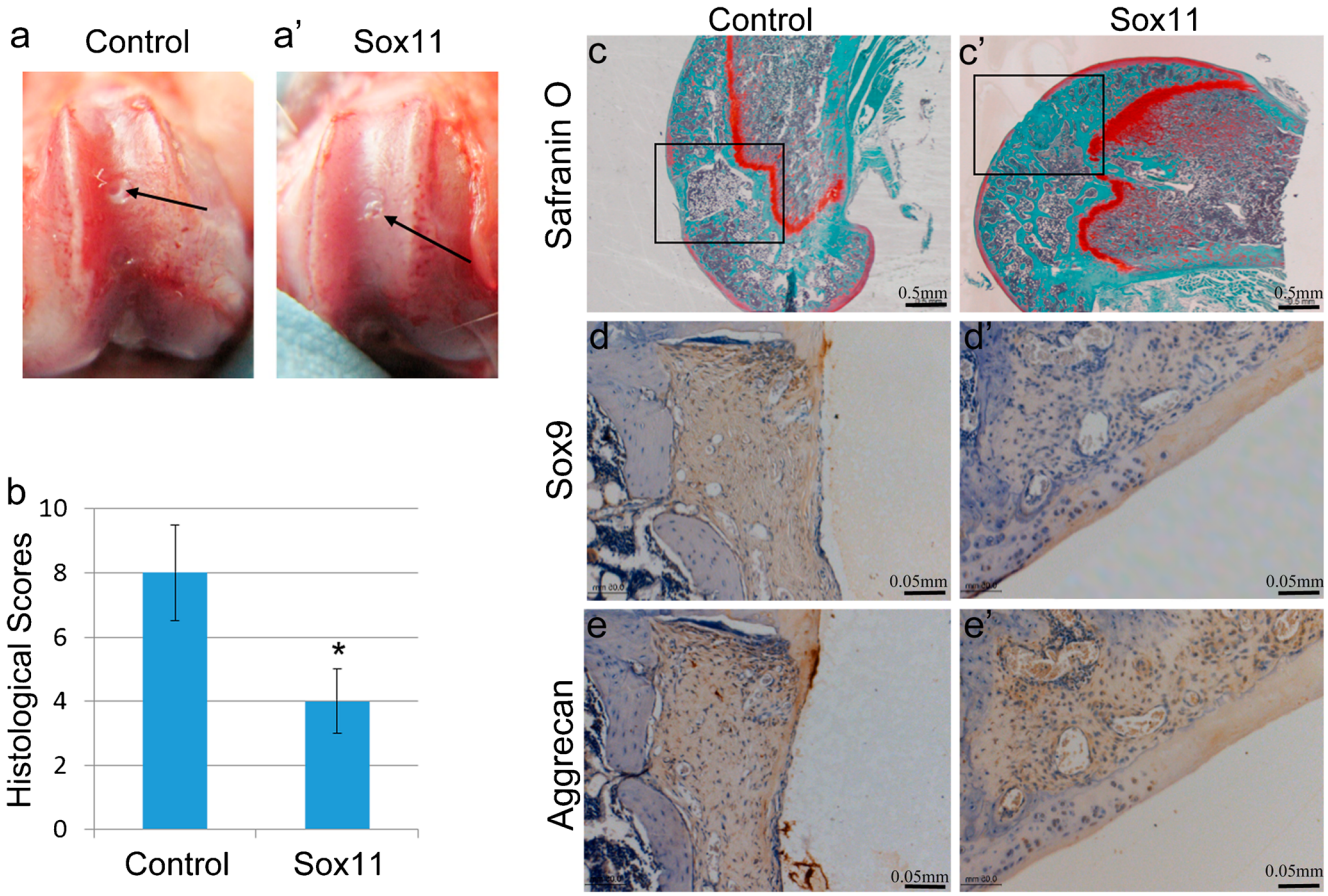

Fig. 6 Cartilage defect repair by Sox11 overexpressing MSCs in SD rats. The osteochondral defect model is established and MSCs or Sox11-MSCs were transplanted as described in the methods. (**a**, **a'**) Macroscopic observation of cartilage defect at 4w after cell transplantation. For the control group, the defect area is not completely filled. (**b**) Histological score for the cartilage defect after cell transplantation. The histological staining findings are quantified by the scoring system (Table 1). Data are expressed as the mean ± SD ($n = 3$; $p < 0.05$). (c–e') Histological observation of the cartilage defect area, including Safranin O staining and IHC staining using Sox9 or Aggrecan antibody

proved to benefit cartilage defect. For example, in situ recruitment of human bone marrow-derived rMSCs using IL-8 and MIP-3α have been shown to enhance tissue regeneration of an osteochondral defect site in beagle knee articular cartilage (Park et al. 2015).

Our previous study demonstrated that Sox11 could regulate trilineage differentiation of rMSCs by activating the BMPs/Smad signaling pathway and transplantation Sox11 overexpressing rMSCs could accelerate bone fracture healing (Xu et al. 2015). Interestingly, we found that at 7 days after rMSCs injection, the calluses from rats that received Sox11-rMSCs injection showed larger areas of cartilage, indicating that the fracture repair in those rats proceeded through an endochondral ossification process much faster than those in the control groups. However, the detailed role of Sox11 in rMSCs chondrogenic differentiation, as well as cartilage defect, is still not clearly clarified.

Sox11 belongs to the SoxC group of Sox transcription factors. The Sox11 knockout mice showed craniofacial and skeletal malformations, asplenia and hypoplasia of the lung, stomach and pancreas (Sock et al. 2004). It has been identified as one of the rMSC-characteristic transcription factors involved in rMSCs stemness regulation by DNA microarray analysis (Kubo et al. 2009) and Sox11 knockdown suppressed the self-renewal capacity and reduced the osteogenic and adipogenic differentiation potential (Gadi et al. 2013). β-Catenin is an important factor involved in the Wnt signaling pathway that plays crucial roles during embryogenesis and cancer and also many other physiological processes (Lie et al. 2005). The accumulated β-catenin protein can be translocated into the nucleus to transcriptionally activate the expression of a variety of genes, such as cyclin D1, c-myc and Runx2 (Ben-Ze'ev et al. 1999; Gaur et al. 2005a; Kinzler et al. 1998). In addition, the Wnt/β-catenin signaling pathway has been shown to promote osteogenesis by directly stimulating Runx2 gene expression, which is a master

transcriptional factor of osteoblast differentiation (Gaur et al. 2005b).

Chondrogenesis is a multistep process that starts with the recruitment of chondrogenic mesenchymal cells into condensations (Kozhemyakina et al. 2015). Then, these cells differentiate into chondrocytes that produce cartilage-specific extracellular matrix proteins including Aggrean and type II collagen. Sox9, generally regarded as the main transcription factor of chondrogenesis (Lefebvre and Dvir-Ginzberg 2017), has been reported to interact with the Wnt/β-catenin signaling pathway (Akiyama et al. 2004). They found that either overexpression of Sox9 or inactivation of β-catenin in chondrocytes of mouse embryos produces a similar phenotype of dwarfism with decreased chondrocyte proliferation, delayed hypertrophic chondrocyte differentiation and endochondral bone formation. However, previous studies have also shown that the Wnt/β-catenin signaling activity promotes bone formation and inhibits chondrocyte differentiation during skeletal development (Day et al. 2005). In our study, we found that Sox11 could transcriptionally activate β-catenin and there was protein-protein interaction between these two molecules. We believe that the activation of β-catenin signaling could partially explain our finding that Sox11 promotes chondrogenesis and cartilage defect repair. Apart from Sox11 and Sox9, some other Sox proteins have also been found to bind with β-catenin to regulate the Tcf/Lef-mediated signaling activity of β-catenin (Zorn et al. 1999). These results demonstrate that Sox proteins and the Wnt/β-catenin signaling pathway are involved in fine-tuning chondrogenesis.

In the present study, following our previous study, we further demonstrated that Sox11 transcriptionally activated β-catenin expression. In addition, Sox11 and β-catenin may have direct protein-protein interaction in the nucleus to direct chondrogenesis. Finally, using a cartilage defect rat model, we demonstrated rMSCs stably overexpressing Sox11 could facilitate cartilage repair in vivo.

Author contributions Study design: Gang, Haibin; Acquisition of data: Liangliang, Shunmei, Sien, Yonghui; Analysis and interpretation of data: Liangliang, Shunmei, Sien, Yonghui; Manuscript preparation: Liangliang, Shunmei; Statistical analysis: Weiping, Wei.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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