Higher BMP receptor expression and BMP-2-induced osteogenic differentiation in tendon-derived stem cells compared with bone-marrow-derived mesenchymal stem cells

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Abstract

Purpose Surgical reattachment of tendon to bone often fails due to regeneration failure of the specialised tendon–bone junction (TBJ). The use of mesenchymal stem cells for TBJ regeneration has been reported with promising results. Tendon-derived stem cells (TDSCs) with high proliferative and multi-lineage differentiation potential have been isolated. As stem

cells residing in tendons, TDSCs can be considered a new cell source for TBJ repair. Bone morphogenetic protein 2 (BMP-2) is a potent osteogenic factor with roles in normal bone healing and pathological ectopic bone formation in soft tissues. The use of BMP-2 to promote TBJ repair has been well reported. This study aimed to compare TDSCs to the gold standard bone-marrow-derived mesenchymal stem cells (BMSCs) with respect to osteogenic response to BMP-2 in vitro.

Method The clonogenicity and multi-differentiation potential of TDSCs and BMSCs were identified by colony-forming-unit assay, osteogenic, adipogenic and chondrogenic differentiation assays. Their osteogenic response to BMP-2 in vitro was examined by alkaline phosphatase (ALP) cytochemical staining, ALP activity assay and Alizarin red S staining of calcium nodule formation. Messenger RNA (mRNA) and BMP receptor (types IA, IB and II) protein expression were examined by quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) and Western blotting.

Results Our results showed that both TDSCs and BMSCs exhibited stem cell properties, including clonogenicity and multi-differentiation potential. TDSCs expressed higher mRNA and protein levels of BMP receptors IA, IB and II. They also exhibited higher osteogenic differentiation with and without BMP-2 stimulation compared with BMSCs.

Conclusions TDSCs with/without BMP-2 might be an attractive source for TBJ repair compared with BMSCs.

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Introduction

Tendon–bone junction (TBJ) injuries are very common in sports. Surgical reattachment of tendon to bone often fails and presents difficulty for tendon-to-bone healing due to the lack of regeneration of the specialised transitional tissue

called the entheses [1–3]. The use of tissue engineering to augment TBJ repair has been studied intensively in recent times. The use of an appropriate cell source and growth factor are critical to the success of tissue engineering [4]. In this regard, previous preclinical studies for TBJ repair used autologous bone-marrow-derived mesenchymal stem cells (BMSCs) [5, 6] to accelerate early tendon-to-bone tunnel healing, with promising results. Whereas stem cells isolated from different tissues share some common characteristics, including adherence to plastics, clonogenicity and multilineage differentiation potentials, they might also exhibit different degrees of proliferative and differentiation potentials. This has implications for selecting an appropriate cell source and conditions for engineering specific tissue types [7, 8]. Multipotent stem cells have been isolated from tendon tissues [9], and we also isolated stem cells called tendon-derived stem cells (TDSCs) from flexor tendon [10] and patellar tendon of rats [11].

Bone morphogenic protein 2 (BMP-2) is an osteogenic growth factor that has been used in many studies to augment bone and TBJ repair [12–18]. Osteogenic responses of stem cells from different sources to BMP-2 can be different. In a study to compare skeletal site-specific differences of BMSCs to BMP-2 stimulation, adult orofacial BMSCs were responsive to BMP-2 than were iliac-crest BMSCs, as indicated by higher gene expression of alkaline phosphatase (ALP), osteopontin, Msh homebox 2 (MSX-2) and osterix in serum-free insulin-containing medium [19]. Because of the role of BMP-2 in bone and cartilage formation, it is commonly found in bone and cartilage and is generally absent in tendons. However, ectopic expression of BMP-2 could occur in pathological conditions and might lead to tendon and ligament ossification [20, 21]. Because of the importance of BMP-2 on stem cells in TBJ engineering and tendon pathology, this study aimed to compare the osteogenic response of TDSCs and BMSCs to BMP-2 *in vitro*. The expression of BMP receptors (BMPR-IA, BMPR-IB, BMP-II) were also examined in these two cell types to explain any differences in BMP-2-induced osteogenic differentiation that might be observed, as these receptors have been reported to mediate BMP-2 osteogenic response [16, 22, 23]. This study investigated whether TDSCs would be an attractive new source for therapeutic stem-cell-based TBJ repair and offers new insights into the role of TDSCs in tendon physiology/pathology.

Materials and methods

Isolation and culture of rat TDSCs and BMSCs

All experiments were approved by the Animal Research Ethics Committee, the Chinese University of Hong Kong.

Male green fluorescent protein (GFP) Sprague–Dawley rats [SD-Tg (CAG-EGFP) Cz-004OsB four to six weeks old, body weight 150–220 g] were used in this study. Both TDSCs and BMSCs were isolated from the same rats. The procedure for isolating TDSCs was established [10, 11, 24]. The midsubstance of patellar tendons was excised from healthy rats overdosed with 2.5% sodium phenobarbital. Care was taken that only the midsubstance and not tissue in the TBJ was collected. Peritendinous connective tissue was carefully removed, and the tissue was stored in sterile phosphate-buffered saline (PBS). The tissue was minced, digested with type I collagenase (3 mg/ml; Sigma-Aldrich, St Louis, MO, USA) for two hours and then passed through a 70- μ m cell strainer (Becton Dickinson, Franklin Lakes, USA) to yield single-cell suspension. The released cells were washed in PBS and resuspended in culture medium containing low-glucose Dulbecco's modified Eagle's medium (LG-DMEM) (Gibco), 10% foetal bovine serum (FBS), 100U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine (complete DMEM culture medium) (all from Invitrogen Corporation, Carlsbad, CA, USA). The isolated nucleated cells were plated at the optimal low-plating cell density (500 cells/cm²) for stem cell isolation and cultured at 37°C, 5% carbon dioxide (CO₂), to form colonies. At day two after initial plating, cells were washed twice with PBS to remove the nonadherent cells. At days seven to ten, these colonies were trypsinised and mixed together as passage 0.

For BMSC isolation, tibiae and femurs were removed from healthy rats and dissected free of muscle. The bones were rinsed in sterilised PBS before being cut into halves. With the cut surface facing the bottom of the centrifuge tube, the tube was spun at 800 g for 15 minutes. The bones were removed and bone marrow tissues washed with PBS. Mononuclear cells were then isolated by density-gradient centrifugation (850 g, 30 min) using Lymphoprep™ and resuspended in culture medium containing alpha minimum essential medium (α -MEM) (Gibco), 10% FBS, 100U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine. These mononuclear cells were plated at an optimal low cell density (10⁵ cells/cm²) to isolate stem cells and cultured at 37°C, 5% CO₂ to form colonies. At days seven to ten, these colonies were trypsinised and mixed together as passage 0. The optimal initial seeding density for TDSC and BMSC isolation was determined using the colony-forming-unit (CFU) assay based on the same criteria: (1) that the colony size was not affected by colony-to-colony contact inhibition; colonies <2 mm in diameter and faintly stained were ignored, and (2) that the greatest number of colonies per nucleated cells was obtained. The optimal initial cell density thus determined was 500/cm² for TDSCs isolated from the patellar tendon and 10⁵/cm² for BMSCs isolated from the bone marrow. Both TDSCs and BMSCs were subcultured when they reached 80–90% confluence. The

medium was changed every three days. Cells at passage three were used for all experiments. The clonogenicity and multi-lineage differentiation potential of TDSCs and BMSCs were characterised before experiments by using CFU assay, osteogenic, adipogenic and chondrogenic differentiation assays, as described in previous studies [10]. TDSCs and BMSCs from GFP rats were used because we would trace the fate to these cells in future tissue engineering studies. There is no evidence that GFP expression would materially change cell biological characteristics other than provide a label for cell tracing.

Effect of BMP-2 on the osteogenic differentiation of TDSCs and BMSCs

TDSCs and BMSCs were plated at 4×10^3 cells/cm² in 24-well plates and cultured in complete DMEM culture medium until the cells reached confluence. They were then incubated in complete DMEM culture medium with or without recombinant human (rh)BMP-2 (100 ng/ml) (Wyeth, Cambridge, MA, USA) at 37°C, 5% CO₂. The dose was chosen based on our previous study that tested the effect of different BMP-2 concentrations (0, 50, 100, 250, 500 and 1,000 ng/ml), and 100 ng/ml was the lowest dose that induced the TDSC osteogenic differentiation. This same dose was also used in a previous study investigating the effect of BMP-2 on the osteogenic response of tendon stem/progenitor cells (TSPC) [9]. At day three, ALP activity of TDSCs and BMSCs was assessed by ALP activity kit and ALP cytochemical staining assay. At day ten, the calcium nodule formation in TDSCs and BMSCs was assessed by Alizarin red S staining.

ALP cytochemical staining assay

Cells were washed and fixed with 70% ethanol (EtOH) for 30 minutes at 4°C. After cell equilibration with ALP buffer [0.1 M sodium chloride (NaCl), 0.1 M Tris–hydrochloride (HCl) pH 9.5, 50 mM magnesium chloride (MgCl₂) and 0.1% Tween-20], ALP substrate solution [0.5 mg nitroblue tetrazolium chloride (NBT) and 0.25 mg 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (BCIP) in 1 ml ALP buffer] was added to the cells for 20 minutes at 37°C. The colour reaction was stopped by washing the cells with distilled water, and the positive stain was viewed under the microscope.

ALP activity assay

Cells were washed and lysed with lysis buffer with protease inhibitor cocktail (Thermo Fisher Scientific, Bremen, Germany). The supernatant was assayed for ALP activity with an ALP assay kit (BioSystems, Barcelona, Spain) using 4-

nitrophenylphosphate as substrate. P-nitrophenol production was measured at OD 405 nm for 30 minutes at 37°C based on the standard curve prepared with different p-nitrophenol concentrations. The linear portion of the product curve was used to calculate enzyme activity by linear regression. ALP activity was expressed as nanomoles p-nitrophenol per minute per milligram protein in the ALP activity assay.

Alizarin red S staining and quantification assay

Cell/matrix layer was washed with PBS, fixed with 70% EtOH and stained with 0.5% Alizarin red S (pH 4.1, Sigma, St. Louis, MO, USA). To quantify the amount of Alizarin red S bound to the mineralised nodules, cells were rinsed with water and extracted with 10% (w/v) cetylpyridinium chloride (CPC) in 10 mM sodium phosphate, pH 7.0, for 15 minutes at room temperature. The dye concentration in the extract was determined at OD 562 nm.

Quantitative real-time RT-PCR (qPCR)

Quantitative real-time reverse-transcriptase polymerase chain reaction (qPCR) was performed, as previously described [25]. Cells were harvested and homogenised for RNA extraction with Rneasy mini kit (Qiagen, Hilden, Germany). Messenger RNA was reverse-transcribed to complementary DNA (cDNA) by the First Strand cDNA kit (Promega, Madison, WI, USA). Then, 5 µl of total cDNA of each sample was amplified in final volume of 25 µl of reaction mixture containing Platinum SYBR Green qPCR SuperMix-UDG ready-to-use reaction cocktail and specific primers for *BMPR-1A, 1B, 1I* or *β-actin* using the ABI StepOne Plus system (all from Applied Biosystems, CA, USA) (Table 1). Cycling conditions were denaturation at 95°C for ten minutes, 45 cycles at 95°C for 20 seconds, optimal annealing temperature (Table 1) for 20 seconds, 72°C for 30 seconds and at 60–95°C with a heating rate of 0.1°C/s. Target gene expression was normalised to that of *β-actin* gene. Relative gene expression was calculated with the $2^{-\Delta CT}$ formula.

Western blotting

Cells were lysed, and the concentration of total soluble protein was measured by bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA). Then, 50 µg of protein was denatured, fractionated by electrophoresis on 12% (w/v) sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) and electrophoretically transferred onto nitrocellulose membranes (Pall, Ann Arbor, MI, USA). The membrane was blocked with 5% (w/v) nonfat dry milk in TBST solution (25 mM Trizma base (3.025 g), 125 mM NaCl (7.3 g) and 1 ml Tween-20, pH 7.6) incubated with

Table 1 Primer sequence, product size and annealing temperature of target genes for quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

Gene	Primer nucleotide sequence	Product size (bp)	Annealing temperature	Accession no.
<i>β-actin</i>	5'-ATCGTGGGCCGCCCTAGGCA-3' (forward) 5'-TGGCCTTAGGGTTCAGAGGGG-3' (reverse)	243	52	NM_031144
<i>BMPR-IA</i>	5'-GCCACCTGGACACCAGAGC-3' (forward) 5'-GCAGGCTTGCCCTGCGTG-3' (reverse)	101	60	S75359
<i>BMPR-IB</i>	5'-CACCACGGAGGAAGCCAGC-3' (forward) 5'-GGCACAGGCCGCTGACAGAC-3' (reverse)	239	60	NM_001024259
<i>BMPR-II</i>	5'-GGTGCTGGTCTCACATTG-3' (forward) 5'-GAGGCGGACTGAGTGGTG-3' (reverse)	180	58	NM_080407

primary antibody against BMPR-IA (1:1,000), BMPR-IB (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or BMPR-II (1:1,000) (BD BioSciences, San Jose, California, USA), followed by horseradish peroxidase-conjugated secondary antibody (1:1,000; Dako, Glostrup, Denmark). Immunoreactive bands were detected by enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, Little Chalfont, UK). The membrane was stripped with stripping

buffer (62.5 mM Tris-HCl, 2% SDS, 100 mM 2-mercaptoethanol, pH 6.7) and reprobed with β -actin antibody (1:3,000, Santa Cruz) as a loading reference. Semiquantitative image analyses of receptor protein expression were performed using the Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA, USA, version 4.6.3), and the mean expression level of the target protein relative to β -actin was presented.

Fig. 1 Clonogenicity and multi-lineage differentiation potential of tendon-derived and bone-marrow-derived mesenchymal stem cells (TDSCs and BMSCs). **a, c** Formation of cell colonies as indicated by crystal violet staining. Magnification $\times 1$. **b, d** Chondrogenic differentiation of TDSCs and BMSCs: cell pellet of TDSCs (**b**) and BMSCs (**d**) expressed proteoglycan, as indicated by positive Safranin O/fast green staining. Magnification $\times 200$. **e–h** Osteogenic differentiation of TDSCs and BMSCs: calcium nodules formed only when TDSCs (**e, f**)

and BMSCs (**g, h**) were cultured in osteogenic medium (**e, g**) compared with basal medium (**f, h**), as indicated by Alizarin red S staining. Magnification $\times 100$. **i–l** Adipogenic differentiation of TDSCs and BMSCs. Lipid droplets formed only when TDSCs (**i, j**) and BMSCs (**k, l**) were cultured in adipogenic medium (**i, k**) compared with basal medium (**j, l**), as shown by Oil red O staining. Magnification $\times 100$

Data analysis

Data is shown in boxplots. Comparison between groups was done using Mann–Whitney *U* test. All data analysis was done using SPSS (SPSS Inc, Chicago, IL, version 16.0). $P \leq 0.050$ was regarded as statistically significant.

Results

TDSC and BMSC stem cell properties

Our results showed that the nucleated cells isolated from rat patellar tendon and bone marrow formed colonies after being cultured for seven to ten days in vitro (Fig. 1a,c). Both TDSCs and BMSCs at passage three formed pellets that expressed proteoglycan (Fig. 1b,d) when cultured in chondrogenic medium for 21 days. Alizarin-red-positive calcium nodules were observed only when TDSCs and BMSCs were cultured in osteogenic induction media for 21 days (Fig. 1e,g) but not in basal media (Fig. 1f,h). Oil red O-positive lipid droplets were observed when TDSCs and BMSCs were cultured in adipogenic induction media for 21 days (Fig. 1i,k) but not in basal media (Fig. 1j,l).

ALP activity in TDSCs and BMSCs

Some ALP cytochemical staining was observed in TDSCs without the addition of BMP-2 but not in BMSCs at day three (Fig. 2a–d). Addition of BMP-2 increased ALP cytochemical staining in TDSCs but not in BMSCs (Fig. 2e–h). Similar results were observed in the ALP activity assay at day 3 (Fig. 2i). Basal ALP activity in BMSCs was low and was significantly higher in TDSCs compared with that in BMSCs ($p=0.050$). Addition of BMP-2 significantly increased ALP activity in TDSCs ($p=0.050$) and was also significantly higher than that in BMSCs ($p=0.050$) (Fig. 2i).

TDSCs and BMSCs calcium nodule formation

Spontaneous calcium nodule formation, as indicated by Alizarin red S staining, was observed in TDSCs but not in BMSCs at day ten under our culturing conditions (Fig. 3a–d). Addition of BMP-2 significantly increased the amount of calcium nodules in TDSCs and slightly in BMSCs (Fig. 3e–h). Quantification of the Alizarin red S stain bound to the calcium nodules showed that there was significantly higher spontaneous calcification in TDSCs compared with that in BMSCs ($p=0.050$) (Fig. 3i). Whereas BMP-2 significantly increased calcium nodule formation in both TDSCs and BMSCs compared with that

Fig. 2 Alkaline phosphatase (ALP) cytochemical staining in tendon-derived and bone-marrow-derived mesenchymal stem cells (TDSCs and BMSCs) at day 3 treated with and without bone morphogenic protein 2 (BMP-2) stimulation. TDSCs treated a–b without and e–f with BMP-2 showed higher ALP cytochemical activity staining than BMSCs treated c–d without and g–h with BMP-2. a, c, e, g magnification $\times 1$; b, d, f, h magnification $\times 100$. Scale bar = 100 μ m. i ALP activity in TDSCs and BMSCs at day 3 with and without BMP-2 induction. $*p \leq 0.050$

Fig. 3 Alizarin red S staining and quantification of Alizarin red S bound to calcium nodules in tendon-derived and bone-marrow-derived mesenchymal stem cells (TDSCs and BMSCs) treated with and without bone morphogenetic protein 2 (BMP-2) for 10 days. **a–h** Alizarin red S staining of calcium nodules. **i** Intensity of Alizarin red S stain bound to calcium nodules. TDSCs treated **a–b** without and **e–f** with BMP-2 showed higher Alizarin-red-S-bound calcium nodules than BMSCs treated **c–d** without and **g–h** with BMP-2. **a, c, e, g** magnification $\times 1$; **b, d, f, h**) magnification $\times 100$. Scale bar = 100 μm . * $p \leq 0.050$

in controls, it was significantly higher in TDSCs than in BMSCs ($p=0.050$) (Fig. 3i).

BMP-receptor mRNA expression in TDSCs and BMSCs

There was significantly higher mRNA expression of *BMPR-IA* ($p=0.028$) and *BMPR-II* ($p=0.006$) in TDSCs compared with that in BMSCs (Fig. 4a, c). The mRNA expression of *BMPR-IB* was higher in TDSCs compared with that in BMSCs, but there was no significant difference between the two cell types ($p=0.361$) (Fig. 4b).

BMP receptor protein expression in TDSCs and BMSCs

The expression of proteins BMPR-IA, BMPR-IB and BMPR-II was detected in both TDSCs and BMSCs (Fig. 5a–c). There was significantly higher expression in TDSCs compared with that in BMSCs (all $p=0.050$) (Fig. 5d–f).

Discussion

Our results showed higher osteogenic differentiation of TDSCs compared with BMSCs with and without BMP-2 induction in vitro. We found that some ALP cytochemical staining was observed in TDSCs without the addition of BMP-2 at day three and spontaneous formation of calcium nodules as indicated by Alizarin red S staining at day ten after TDSCs reached confluence in the monolayer cell culture. These findings suggested higher osteogenic differentiation potential of TDSCs compared with BMSCs in complete DMEM culture medium in vitro. Spontaneous osteogenesis was also reported in other mesenchymal stem cells (MSCs), such as BMSCs [26, 27], which might be due to endogenous BMP expression in MSCs [28]. Our previous study showed that TDSCs also exhibited higher osteogenic differentiation potential in osteogenic medium supplemented with dexamethosone, β -glycerolphosphate and ascorbic acid compared with BMSCs isolated from the same animal [29]. Our results thus suggest that TDSCs might be an attractive source for TBJ repair upon endogenous and/or exogenous BMP-2 stimulation, as bone formation is one of the key determinants of successful TBJ regeneration. Further studies will be performed to confirm

the higher osteogenic differentiation potential of TDSCs compared with BMSCs in vivo.

We further observed higher protein and mRNA expression of BMP receptors IA, IB and II in TDSCs compared with BMSCs. BMPR-IB expression was significantly higher in TDSCs compared with that in BMSCs, whereas

Fig. 4 Messenger RNA expressions of **a** *BMPR-IA*, **b** *BMPR-IB* and **c** *BMPR-II* in tendon-derived and bone-morphogenic-derived mesenchymal stem cells (TDSCs and BMSCs). $*p \leq 0.050$

BMPR-IB mRNA expression of—while higher in TDSCs compared with that in BMSCs—was not statistically significant ($p=0.361$) (Fig. 4b). We believe the discrepancy between mRNA and protein data was more likely due to small sample size ($n=5$) and different sensitivity of Western blotting and qPCR, which led to a statistically insignificant difference in mRNA data. However, we cannot completely rule out the possibility of a post-transcriptional level of receptor regulation. Further study is required. We are not sure whether the observed higher osteogenic response in TDSCs compared with BMSCs is due to the higher BMPR-IA, BMPR-IB and BMPR-II expression. However, the roles of these receptors in mediating BMP-2-induced osteogenic differentiation of stem cells have been reported in the literature [16, 22, 23]. Overexpression of dominant-negative BMPR-1A, but not BMPR-1B, in mesenchymal progenitors C3H10T1/2 reduced osteogenesis and osteogenic marker expression [osteocalcin, osteopontin, parathyroid hormone/PTH-related peptide (PTH/PTHr) Preceptor, Runx2] induced by BMP-2 overexpression [22]. Further study is required to confirm the functional activity of BMP receptors and their

downstream targets in mediating the higher BMP-2-induced osteogenic response observed in TDSCs compared with that in BMSCs.

Of note was the fact that the receptors we studied were not solely activated by BMP-2, and the signals of other members of the BMP family were also mediated through these common receptors. BMP-12 and BMP-13, which are tenogenic, activated the same sets of receptors as BMP-2 [16, 23]. Besides BMP-2, BMPs such as BMP-4 and BMP-7 are osteochondrogenic and have been reported to play important roles in bone and TBJ regeneration [30, 31]. Whether TDSCs is also responsive to BMP-4 and BMP-7 and showed differential response compared with BMSCs requires further research.

TDSC origin is still not clear. As we observed differential osteogenic responses of TDSCs and BMSCs to BMP-2 stimulation and differential expression of BMP receptors between the two cell types, TDSC is probably a unique cell type, distinct from BMSC. This is further supported by tenogenic and some chondrogenic marker expression in TDSCs but not in BMSCs, as shown in our

Fig. 5 **a–c** BMPR-IA, BMPR-IB, and BMPR-II protein expression in tendon-derived and bone-morphogenic-derived mesenchymal stem cells (TDSCs and BMSCs). **d–f** Semiquantitative image analysis results. $*p \leq 0.050$

previous study [10]. Bi et al. reported that mouse tendon progenitor/stem cells (TPSCs) expressed higher mRNA levels of scleraxis (*Scx*), *Comp*, *Sox9* and *Runx2* compared with mouse BMSCs, whereas human TPSCs expressed higher levels of tenomodulin (*TNMD*) than did human BMSCs [9].

As stem cells residing inside the tendon tissue, the higher responsiveness of TDSCs to BMP-2 signal—and hence osteogenic differentiation—might seem to be difficult to explain. However, as tendon functions to connect bone to muscle, the resident stem cells in tendons should possess high osteogenic, chondrogenic and myogenic differentiation potential as well as responsiveness to induction signals of these tissues, in order to regenerate the tendon-fibrocartilage-bone and musculotendinous junctions. However, the myogenic differentiation potential of TDSCs has not been formally investigated and requires further study. Despite this, the potential of TDSCs to establish and maintain the TBJ and musculotendinous junction is plausible based on a previous study [30]. It has been reported that bone ridge formation was initiated by tendons, whereas the subsequent growth phase was muscle dependent [30]. During initiation of bone ridge formation, scleraxis bound to the *Bmp4* promoter and regulated its transcription in tendon cells at the insertion site. BMP4 expression then bound to its receptor ALK3 in tuberosity-forming chondrocytes, leading to BMP signaling activation and initiation of bone ridge formation [30]. A regulatory mechanism to prevent erroneous TDSCs differentiation to junctional cell types (bone, chondrocytes, muscles) in tendon midsubstances other than tenocytes therefore may be in place in order to maintain tendon homeostasis. In this regard, MSX2 was reported to act as a molecular defence mechanism for preventing ossification in ligament fibroblasts [32]. An activated form of Smad8 protein inhibited the osteogenic pathway in MSCs known to be induced by BMP-2 while promoting tenogenic differentiation [33]. The Smad-dependent pathway was also reported to regulate myogenic differentiation inhibition and osteoblastic differentiation induction of myoblasts induced by BMPs [34]. Constitutively activated Smad1 overexpression was reported to induce myoblast osteogenic differentiation, whereas nuclear translocation of Smad4 inhibited myoblast myogenic differentiation [34]. It is not known whether Smad proteins and MSX2 also function to inhibit osteogenic differentiation and promote TDSC tenogenic differentiation, as reported in previous studies [30, 32, 34]; further study is required. Other factors, such as mechanical loading [35] and local extracellular matrix environment [9], may also function to suppress TDSC osteogenesis inside the tendon midsubstance. For instance, Zhang et al. reported that low mechanical stretching promoted tendon stem cell differentiation into tenocytes, whereas large stretching induced differentiation of some

tendon stem cells into adipogenic, chondrogenic and osteogenic lineages [35]. Biglycan and fibromodulin depletion in a double knockout animal model has shown increased TPSC sensitivity to BMP-2 signalling and tendon ossification, suggesting that normal biglycan and fibromodulin levels might have roles in regulating the BMP-2 signalling pathway in TPSCs [9]. We have also shown disturbance in extracellular matrix expression, with sustained proteoglycan expression, including biglycan and fibromodulin, as well as high collagen type III/type I ratio in our ossifying tendinopathy animal model [25]. During pathological conditions with ectopic BMP-2, 4 and 7 expression, TDSC differentiation to osteoblasts/chondrocytes might lead to ossification inside the tendon midsubstances [36]. For instance, we reported ectopic TBJ formation with high BMP-2, 4 and 7 expression inside the patellar tendon in our ossifying tendinopathy animal model [37, 38] and clinical samples of patellar tendinopathy [39]. A change of tendon loading caused by mechanical overload, compression or disuse has been implicated as the possible aetiologies of tendinopathy [40], and repeated cyclic stretching increased BMP-2 expression, which promoted osteogenesis in TDSCs in vitro [11]. Ectopic overexpression of chondro-osteogenic BMPs was also suggested to account for the chondrogenic transformation and ectopic mineralisation of rotator cuff tendon in patients [21].

In conclusion, our results show that TDSCs express higher BMP receptor levels and exhibited higher osteogenic differentiation with and without BMP-2 stimulation. TDSCs may be a unique cell type, distinct from BMSCs. TDSCs with or without BMP-2 might be an attractive cell source for TBJ engineering.

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