Original article

Effect of growth and differentiation factor 6 on the tenogenic differentiation of bone marrow-derived mesenchymal stem cells

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Background Recent studies showed that bone marrow-derived mesenchymal stem cells (BMSCs) had risk of ectopic bone formation. In this study, we aimed to investigate the effect of growth and differentiation factor 6 (GDF-6) on the tenogenic differentiation of BMSCs *in vitro*, and then combined with small intestine submucous (SIS) to promote tendon regeneration *in vivo*.

Methods The BMSCs were isolated from the green fluorescent protein (GFP) rats, and were characterized by multi-differentiation assays following our previous study protocol. BMSCs cultured with different concentrations of GDF-6, without growth factors served as control. After 2 weeks, mRNA expression and protein expression of tendon specific markers were examined by qRT-PCR and Western blotting to define an optimal concentration of GDF-6. Mann-Whitney *U*-test was used to compare the difference in relative mRNA expression among all groups; $P \leq 0.05$ was regarded as statistically significant. The GDF-6 treated BMSCs combined with SIS were implanted in nude mice and SD rat acute patellar tendon injury model, the BMSCs combined with SIS served as control. After 12 and 4 weeks in nude mice and tendon injury model, the samples were collected for histology.

Results After the BMSCs were treated with different concentration of GDF-6 for 2 weeks, the fold changes of the specific markers (Tenomodulin and Scleraxis) mRNA expression were significantly higher in GDF-6 (20 ng/ml) group ($P \leq 0.05$), which was also confirmed by Western blotting result. The BMSCs became parallel in orientation after GDF-6 (20 ng/ml) treatment, but the BMSCs in control group were randomly oriented. The GDF-6 (20 ng/ml) treated BMSCs were combined with SIS, and were implanted in nude mice for 12 weeks, the histology showed neo-tendon formation. In the SD rat patellar tendon window injury model, the histology also indicated the GDF-6 (20 ng/ml) treated BMSCs combined with SIS could promote tendon regeneration.

Conclusions GDF-6 has tenogenic effect on the tenogenic differentiation of BMSCs, and GDF-6 (20 ng/ml) has better tenogenic effect compared to other concentrations. The GDF-6 (20 ng/ml) treated BMSCs combined with SIS can form neo-tendons and promote tendon regeneration.

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Tendon injuries are very common in our daily life and also sports activities. In the United States, there are more than 32 million tendon injuries caused by trauma and repetitive motions,[1] which cost an estimated $30 billion every year.[2] The current treatments include drugs, physiotherapy, and surgery, but the tendon healing outcome is poor, because the tendon healed with poor tissue quality after tendon injury. The crimp pattern of collagen fibers and fibrils was smaller than that of the control and the regenerated fibrotic scar tissue could not regain its original mechanical strength a long time after injury,[3] causing significant dysfunction and disability. Because of high incidence and poor healing results of tendon injury, stem cell therapy becomes a promising research direction in the treatment of tendon injury.

Mesenchymal stem cells existing in various mesenchymal tissues had been proved with high self-renewal and multi-differentiation potential, which played an important role in tissue repair and regeneration.[4] Bone marrow derived mesenchymal stem cells (BMSCs) were considered as a promising cell source in musculoskeletal

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regeneration.[5] Many studies have proved that intratendinous cell therapy with BMSCs following primary tendon repair could improve early tendon healing both histologically and biomechanically.[6,7] However, some studies found that MSC-collagen gel constructs in rabbit tendon defects significantly improved repair biomechanics with ectopic bone formation in 28% of the MSC-treated rabbit tendons.[8,9] So tenogenic differentiation of BMSCs may promote tendon healing and alleviate the risk of ectopic bone formation after transplantation.

Recent studies showed that BMSCs had the potentials of tenogenic differentiation to tenocytes induced by growth and differentiation factor-6 (GDF-6).[10-13] GDF-6/BMP-13 has been shown to promote neo-tendon formation after subcutaneous and intramuscular implantation[10] and tendon repair *in vivo*.[11] GDF-6/BMP-13 also can stimulate proliferation and collagen production of tendon fibroblasts *in vitro*.[12] Recently, GDF-6/BMP-13 was reported to promote the production of ligament-related matrix genes in MSCs and ACL fibroblasts.[13]

Gu and Dai[14] reported that it was effective for porcine small-intestine submucosa (SIS) as an alternative of injured Achilles tendon, where 1 week after the operation the porcine SIS was already fused with the remaining part of the rabbit Achilles tendon, sixteen weeks after the operation all the Achilles tendons looked like the normal ones. A recent study showed that *in vitro* mechanical conditioning improved structural properties of SIS scaffolds seeded with tenocytes, which provided the basis for future studies in the use of *in vitro* conditioning (mechanical and chemical) of cell-seeded extra-cellular matrix (ECM) scaffolds for enhancing tendon repair *in vivo*.[15] Murphy et al reported that SIS could be a promising flexor tendon graft substitute as shown by earlier neovascularization, increased TGF-beta1 levels, and increased collagen deposition, along with greater intrinsic repair strength relative to both auto-graft and suture strength at week 4.[16]

In this study, we aimed to test the effect of GDF-6 at different concentrations on tenogenic differentiation of BMSCs using the defined specific markers in gene and protein levels. Then we aimed to investigate the tendon regeneration effect of tenogenic BMSCs combined with SIS in nude mice and rat patellar tendon acute injury model.

METHODS

Isolation of BMSCs

All animal experimental procedures were performed under the guidelines for animal scientific procedures approved by the host institution's ethical committee. The protocols of primary cell culture for tenocytes and BMSCs followed previous studies.[17,18] Immediately following sacrifice, all tibiae and femur of green fluorescent protein (GFP) rats were removed and dissected free of muscles. The bones were rinsed in sterilized phosphate buffered solution (PBS) before cutting into half, with the cut surface placed into the centrifuge tube and spun with 800 ×*g* for 15 minutes. The bones were then removed and the bone marrow tissues washed with PBS once and the mononuclear cells were isolated by density gradient centrifugation (850 ×*g*, 20 minutes) using Lymphoprep^{TM} (1.077 g/ml; AXIS-SHIELD PoC AS, Oslo, Norway), plated at a density of 1×10^5/cm^2 on T-75 flasks with basal culture media containing alpha modified Eagle medium (α-MEM), 10% fetal bovine serum (FBS), and 100 IU /ml penicillin-streptomycin (Invitrogen Life Technologies, Paisley, UK), and cultured in a humidified atmosphere at 37°C with 5% CO_2.

Multi-differentiation assays of BMSCs

Multi-differentiation assays of BMSCs were performed as previously described.[17] For osteogenic differentiation, isolated BMSCs at passage 3 were placed at 5000 cells/cm^2 in a 6-well plate and cultured in complete culture medium until the cells reached confluence. The cells were then cultured in the DMEM containing 10% fetal bovine serum (FBS) supplemented with 0.1 μmol/L dexamethasone, 0.2 mmol/L ascorbic acid-2 phosphate, and 10 mmol/L β-glycerophosphate. The medium was changed twice weekly. After 21 days, the cells were subject to Alizarin Red staining.

For chondrogenic differentiation, additional aliquots of 1×10^6 passage 3 BMSCs were pelleted down and cultured in 15 ml polypropylene tubes along with the chamber slide cultures. The chondrogenic induction medium consisted of serum free DMEM supplemented with 0.1 μmol/L dexamethasone, 0.2 mmol/L ascorbic acid-2 phosphate, 1 mmol/L sodium pyruvate, 1:100 diluted ITS+Premi, and TGF-β1 10 ng/ml. The medium was changed every other day. After 28 days the pellet cells were embedded in paraffin for Alcian Blue staining.

For adipogenic differentiation, isolated BMSCs at passage 3 were placed at 5000 cells/cm^2 in a 6-well plate and cultured in complete culture medium until the cells reached confluence. The cells were then cultured in the DMEM containing 10% FBS supplemented with 10^{-6} mol/L dexamethasone, 0.50 mmol/L methyl-isobutylxantine, and 50 μmol/L indomethacin. The medium was changed twice a week and after 14 days of culture, Oil Red O staining was performed to detect the accumulated lipid vacuoles in the cells.

Tenogenic differentiation of BMSCs

BMSCs at passage 3 were plated at 5000 cells/cm^2 in 6-well plates and 24-well plates and cultured in complete culture medium until the cells reached confluence. The cells were then cultured with different concentrations of GDF-6 (5, 10, and 20 ng/ml, Recombinant Mouse GDF-6, Catalog No. 855-G6, R&D Systems, Inc.,

Minneapolis, MN, USA) at 37°C, 5% CO_2. Cells cultured without growth factors served as the control group. The medium was changed every 3 days. After 2 weeks, the mRNA expression of tendon specific markers was examined by quantitative real time RT-PCR (qRT-PCR), the protein expression of tenomodulin was also confirmed by Western blotting.

qRT-PCR

qRT-PCR was performed as previously described.[18-20] The cells were harvested and homogenized for RNA extraction with RNeasy mini kit (Qiagen, Germany). The mRNA was reversely transcribed to cDNA by the First Strand cDNA kit (Promega, Madison, WI, USA). A measured amount (5 μl) of total cDNA of each sample was amplified in a 25 μl reaction mix using the Platinum SYBR Green qPCR SuperMix-UDG with specific primers (Table 1) using the ABI StepOne Plus system (all from Applied Biosystems, CA, USA, Table 1). Cycling conditions were: denaturation at 95°C for 10 minutes, 45 cycles at 95°C for 20 seconds, optimal annealing temperature for 25 seconds, 72°C for 30 seconds, and finally at 60–95°C with a heating rate of 0.1°C/second. The expression of target gene was normalized to that of *β-actin* gene. Relative gene expression was calculated with the $2^{-\Delta CT}$ formula.

Western blotting

Western blotting was performed as previously described.[17,18,20] The cells were washed with cold PBS twice, and harvested by scraping in cold cell extraction buffer (No. FNN0011, Invitrogen Life Technologies, Paisley, UK). Protein concentration was determined by Bradford method (Bio-Rad, USA). Equal proteins were loaded onto 10% Tris/glycine gels for electrophoresis and then transferred to a PVDF membrane (Millipore, Bedford, MA, USA) and blocked in 5% nonfat milk (Bio-Rad) for 1 hour at room temperature with rocking. Then, the primary antibody, anti-tenomodulin (Tnmd) (1:200, SC-49325, Santa Cruz Biotechnology, USA), was added and incubated for 2 hours at room temperature. After washing in TBST three times (5 minutes for each time), the membrane was incubated with anti-goat horseradish peroxidase-linked secondary antibodies (SC-2020, Santa Cruz Biotechnology; 1:3000) for 1 hour at room temperature. Following three TBST washes, the protein was detected with the enhanced chemiluminescence (ECL) blotting reagents (Amersham Biosciences, USA) according to the manufacturer's instruction.

In vitro construction of cell-seeded SIS scaffold

GFP-BMSCs were plated at 5000 cells/cm^2 in a T75 flask and cultured in complete culture medium until the cells reached confluence. The cells were then incubated in α-MEM at normal serum concentration (10%) with (induction group) or without (control group) supplementation with ascorbic acid (25 μmol/L) (Catalog No. A-0278, Sigma, USA) and GDF-6 (5, 10, and 20 ng/ml, Recombinant Mouse GDF-6, Catalog No. 855-G6, R&D Systems, Inc., Minneapolis, MN, USA) at 37°C, 5% CO_2. The medium with or without GDF-6 and ascorbic acid was changed every 3 days. Interestingly, after treatment with GDF-6 and ascorbic acid for 2 weeks, the cells were seeded in 6 cm ×10 cm SIS scaffold for 2 hours at 37°C, 5% CO_2. Then the cell-seeded SIS scaffolds were folded to 0.5 cm × 10 cm size, which was rolled up and loaded on a 1-cm wide U-shaped spring, which was used in the following studies.

Implantation of cell-seeded SIS scaffold in nude mouse model

In order to demonstrate that cell-seeded SIS scaffold can form neo-tendon *in vivo*, six mice after anesthesia were taken, an incision was made on the dorsum and a subcutaneous pocket was created to expose posterior midline. The cell-seeded SIS scaffolds were sutured to posterior midline without further continuous tensile strength by Ethicon 6-0 suture, but these cell-seeded SIS scaffolds suffered intermittent tensile strength with the free movement of nude mice. At the end of 12 weeks (n=3), the implanted tissues were harvested for examination of cellularity and vascularity of the neo-tendon tissue by histology. The histology protocol is described below.

Animal surgery

Twelve Sprague Dawley male adult rats (8 weeks, body weight of 250–300 g) were used in this study. To create the tendon defect, the central one-third of the patellar tendon (~1 mm in width) was removed from the distal apex of the patella to the insertion of the tibia tuberosity

Table 1. The primer sequence, product size, and annealing temperature of target genes for real time RT-PCR

Genes	Primer nucleotide sequence	Product size (bp)	Annealing temperature	Accession No.
β-actin	5'-ATC GTG GGC CGC CCT AGG CA-3' (forward) 5'-TGG CCT TAG GGT TCA GAG GGG-3' (reverse)	243	52	NM_031144
Type I Collagen (Col1a1)	5'-CCGGACTGTGAGGTTAGGAT-3' (forward) 5'-AACCCAAAGGACCCAAATAC-3' (reverse)	364	55	BT007205
Fibromodulin (Fmod)	5'-GCTCTGGGCTCCTACTCCTT-3' (forward) 5'-GTCCTGCCATTCTGAGGTGT-3' (reverse)	450	58	NM_080698.1
Scleraxis (Scx)	5'- AACACGGCCTTCACTGCGCTG-3' (forward) 5'- CAGTAGCACGTTGCCCAGGTG-3' (reverse)	123	58	NM_001130508.1
Tenomodulin (Tnmd)	5'-CCATGCTGGATGAGAGAGGTTAC-3'(forward) 5'-CACAGACCCTGCGGCAGTA-3' (reverse)	72	58	NM_022290.1
Tenascin C (TnC)	5'-CAGAAGCTGAACCGGAAGTTG-3' (forward) 5'-GGCTGTTGTTGCTATGGCQCT-3' (reverse)	278	55	NM_053861.1

with two stacked sharp blades according to our well-established protocol.[21-23] The operated rats were divided into three groups: (a) injury-only group (b) BMSCs seeded SIS scaffold group, and (c) GDF-6 treated BMSCs seeded SIS scaffold group. The scaffold was placed in the tendon defect and sutured to the patellar bone and tibia tuberosity using Ethicon 6-0 suture. Window injury only without scaffold served as control. The wound was then closed in layers. The animals were allowed to have free-cage activity until euthanasia. At 4 weeks after operation, all animals were killed and the patellar tendons were harvested for the examination of cellularity and vascularity of the regenerated tissue by histology.

Histology

The neo-tendon tissue and regenerated patellar tendon tissue were washed in PBS, fixed in buffered formalin and 100% ethanol, embedded in paraffin, cut longitudinally to 5-μm thick sections and mounted on 3-aminopropyl-triethoxy-silane (Sigma-Aldrich, St Louis, MO, USA) coated slides. After deparaffination, the sections were stained with haematoxylin and eosin (H&E). H&E staining was done as described previously.[21-23] The sections were examined under light microscopy (DMRXA2, Leica Microsystems Wetzlar GmbH, Germany).

Data analysis

Data were presented as mean ± SD and shown in boxplots. Mann-Whitney *U* test was used to compare the difference in relative mRNA expression among these groups. All the data analysis was done using SPSS (SPSS Inc, Chicago, IL, USA version 16.0). $P \leq 0.05$ was regarded as statistically significant.

RESULTS

mRNA expression of tendon specific markers for tenogenic differentiation of BMSCs treated by GDF-6

After tenogenic differentiation of BMSCs by GDF-6 (5, 10, and 20 ng/ml) for 2 weeks, mRNA expression of tenomodulin and scleraxis was significantly increased compared with control group (0 ng/ml, $P \leq 0.05$, Figure 1A–1C); the fold change of tenomodulin mRNA expression was increased by 1.43, 2.57, and 5.61 (P=0.043) in three concentrations, respectively (Figure 1A and B); the fold change of scleraxis mRNA expression was significantly increased by 1.62 (P=0.043), 2.96 (P=0.021), and 4.18 (P=0.021) in three concentrations, respectively (Figure 1A and C). The mRNA expression of tenascin C, fibromodulin and type I collagen had no significant difference among the three concentrations groups and control group (0 ng/ml, Figure 1A, D–F). The mRNA expression of tenomodulin and scleraxis was

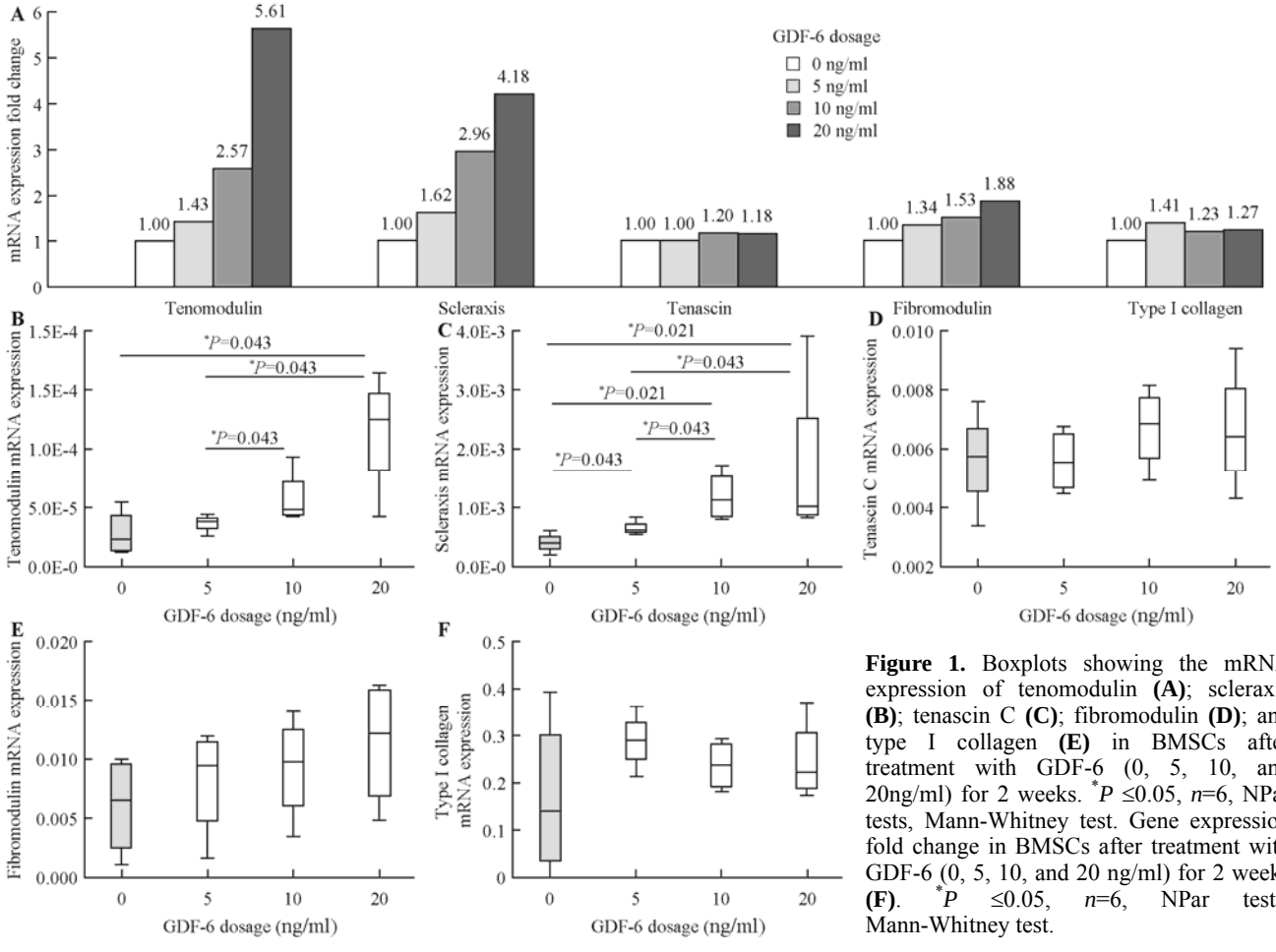

Figure 1. Boxplots showing the mRNA expression of tenomodulin **(A)**; scleraxis **(B)**; tenascin C **(C)**; fibromodulin **(D)**; and type I collagen **(E)** in BMSCs after treatment with GDF-6 (0, 5, 10, and 20ng/ml) for 2 weeks. $^{*}P \leq 0.05$, n=6, NPar tests, Mann-Whitney test. Gene expression fold change in BMSCs after treatment with GDF-6 (0, 5, 10, and 20 ng/ml) for 2 weeks **(F)**. $^{*}P \leq 0.05$, n=6, NPar tests, Mann-Whitney test.

higher in GDF-6 (20 ng/ml) than the other two concentrations (5 and 10 ng/ml) (Figure 1A–C). So 20 ng/ml was considered as optimal condition of GDF-6 in tenogenic differentiation of BMSCs.

Western blotting of tenomodulin

After tenogenic differentiation of BMSCs by GDF-6 (20 ng/ml) for 2 weeks, the protein expression of tenomodulin was increased, and it was higher in GDF-6 (20 ng/ml) group than in the control group (Figure 2).

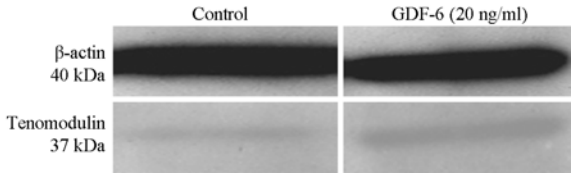

Figure 2. Photomicrographs showing Western blotting result of tenomodulin protein expression in BMSCs at P3 with control (0 ng/ml) for 2 weeks, BMSCs at P3 with GDF-6 (20 ng/ml) for 2 weeks. Beta-actin was the reference gene.

Cell morphology of GDF-6 treated BMSCs

After treatment with GDF-6 (20 ng/ml) for 2 weeks, the BMSCs became slim and parallelly aligned at the cell morphology (Figure 3B); however, the BMSCs without GDF-6 treatment were still randomly aligned with spindle or fibro-like shape in the control group (Figure 3A). The fluorescent images also showed this difference between control group and GDF-6 (20 ng/ml) group (Figure 3C and D).

Neo-tendon formation of cell-seeded SIS scaffold in nude mouse model

The cell-seeded SIS scaffolds (BMSCs and GDF-6 treated BMSCs) were folded to 0.5 cm × 10 cm size, which was rolled up and loaded on a 1 cm wide U-shaped spring (Figure 4A), which was used in the following studies. The cell-seeded SIS scaffolds were divided into two groups: (a) BMSCs seeded SIS scaffold group (b) GDF-6 (20 ng/ml) treated BMSCs seeded SIS scaffold group. The cell-seeded SIS scaffolds were sutured to

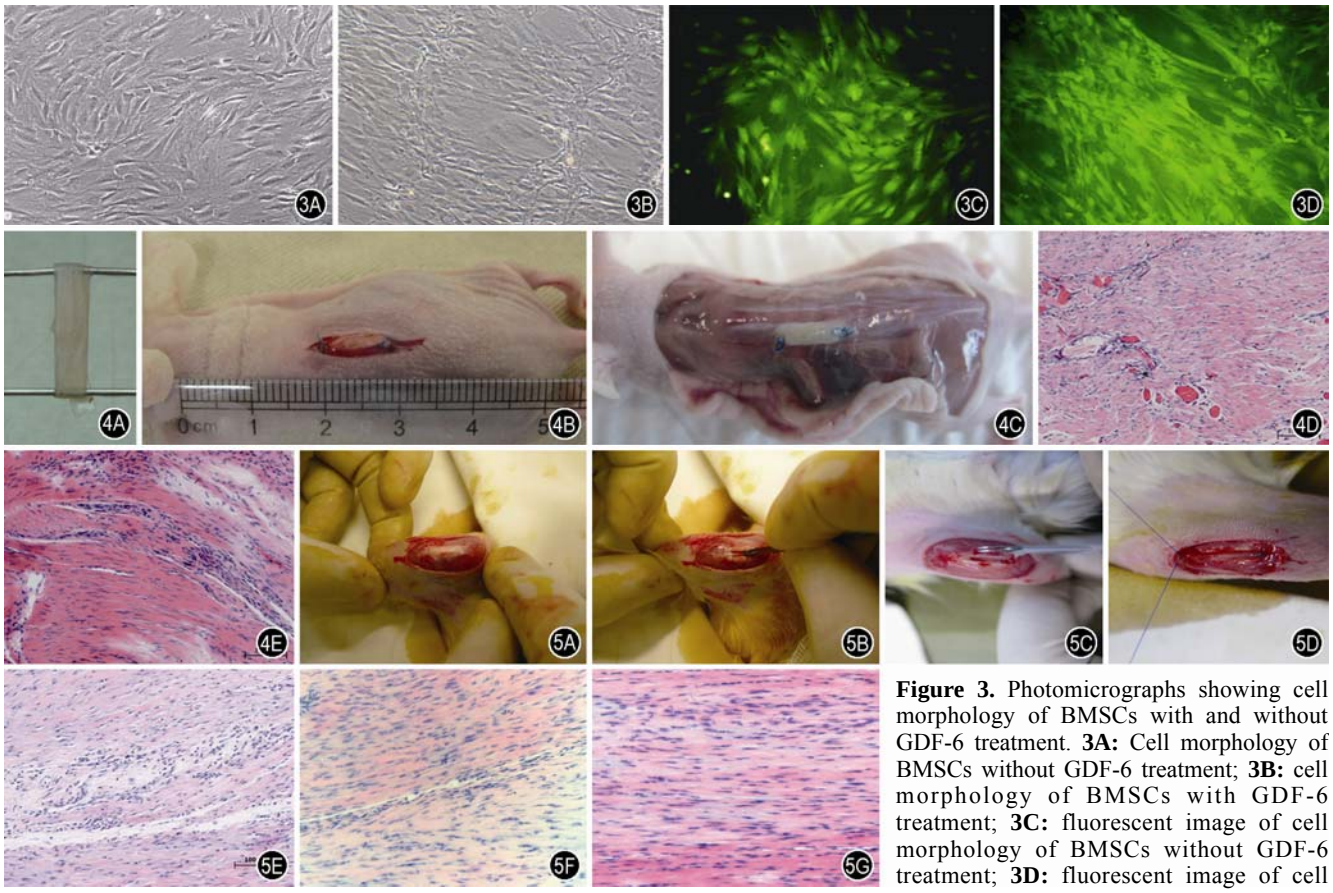

Figure 3. Photomicrographs showing cell morphology of BMSCs with and without GDF-6 treatment. **3A:** Cell morphology of BMSCs without GDF-6 treatment; **3B:** cell morphology of BMSCs with GDF-6 treatment; **3C:** fluorescent image of cell morphology of BMSCs without GDF-6 treatment; **3D:** fluorescent image of cell morphology of BMSCs with GDF-6 treatment. Original magnification ×100.

Figure 4. Photomicrographs showing neo-tendon formation of cell-seeded SIS scaffold in nude mouse model. **4A:** The cell-seeded SIS scaffolds were rolled up and loaded on a 1 cm wide U-shaped spring; **4B:** The cell-seeded SIS scaffolds were sutured to posterior midline by Ethicon 6-0 suture; **4C:** the regenerated tissues by cell-seeded SIS scaffolds at 12 weeks post-operation; **4D:** H&E staining of BMSCs seeded SIS scaffold in nude mice for 12 weeks; scale bar: 100 μm; **4E:** H&E staining of GDF-6 treated BMSCs seeded SIS scaffold in nude mice for 12 weeks; scale bar: 100 μm.

Figure 5. Photomicrographs showing tendon regeneration by cell-seeded SIS scaffold in SD rat patellar tendon acute injury model. **5A:** intact patellar tendon of SD rat; **5B:** the central tendon defect was removed with two stacked sharp blades; **5C:** the central tendon defect and two stacked sharp blades; **5D:** the scaffold was placed in the tendon defect and sutured to the patellar bone and tibia tuberosity using Ethicon 6-0 suture; **5E:** H&E staining of injury-only control group at 4 weeks post-operation; scale bar: 100 μm; **5F:** H&E staining of BMSCs seeded SIS scaffold group at 4 weeks post-operation; scale bar: 100 μm; **5G:** H&E staining of GDF-6 treated BMSCs seeded SIS scaffold group at 4 weeks post-operation; scale bar: 100 μm.

posterior midline without further continuous tensile strength by Ethicon 6-0 suture (Figure 4B). At 12 weeks post-operation, the implanted tissues became relatively tight tendon-like tissue with some angiogenesis and vascularity (Figure 4C). HE staining showed more mature and tight collagen fibers and less cellularity was observed in the GDF-6 (20 ng/ml) treated BMSCs seeded SIS scaffold group (Figure 4E) than that of BMSCs seeded SIS scaffold group (Figure 4D). The spindle shaped cells longitudinally aligned along the collagen fibers in GDF-6 (20 ng/ml) treated BMSCs seeded SIS scaffold group (Figure 4E), but the cells in the BMSCs seeded SIS scaffold group were relatively randomly aligned (Figure 4D).

Promote tendon regeneration by cell-seeded SIS scaffold

To create the tendon defect in intact patellar tendon (Figure 5A), the central one-third of the patellar tendon (~1 mm in width) was removed from the distal apex of the patella to the insertion of the tibia tuberosity with two stacked sharp blades (Figure 5B and C). The scaffold was placed in the tendon defect and sutured to the patellar bone and tibia tuberosity using Ethicon 6-0 suture (Figure 5D).

In order to investigate the effect of cell-seeded SIS scaffolds on tendon regeneration, the patellar tendon samples at 4 weeks post-operation were collected for histology. Both the BMSCs group and the GDF-6 treated BMSCs group have lower cellularity (Figure 5F and G) compared to that of the control group (Figure 5E), but the BMSCs group seemed to have higher cellularity than that of GDF-6 treated BMSCs group (Figure 5F and G). The extracellular matrix production in GDF-6 treated BMSCs group was obviously more than the other two groups, and the BMSCs group was more than that of the control group as shown by H&E staining ability of the extracellular matrix (Figure 5E–G). The healing tendon cells in the control group and BMSCs group were round and randomly oriented (Figure 5E and F), but most cells became more elongated and longitudinally-arranged along with parallel collagen fibers (Figure 5G).

DISCUSSION

BMSCs, from bone marrow tissues, have been demonstrated to have self-renewal and multi-differentiation potentials many years ago,[5] which means the BMSCs can form colonies and proliferate quickly, it can also differentiate into many tissue cells such as bone, cartilage, fat, muscle, neuro, myocardium et al under specific conditions, respectively.[5,24-27] In our study, the BMSCs, isolated from the bone marrow tissue of GFP rat, showed multi-differentiation potentials, self-renewal ability and positive expression of MSC surface markers, which suggested that the isolated BMSCs were the expected cell type for our future study according to our established protocol.[17]

So far, there are no standard assessments for assessing the successful tenogenic differentiation of BMSCs. The osteogenic, chondrogenic, and adipogenic differentiation of stem cells all have well-known assessments and specific markers, but the tenogenic differentiation of stem cells into tenocytes is the lack of clearly defined tenogenic molecular biomarkers. Recently, tenomodulin,[28,29] scleraxis,[30,31] *Smad8*,[32-34] and *Epha4*,[35-37] were reported to be more specific markers for tendon tissue. Besides, tenascin C, type I collagen, type III collagen, fibromodulin, decorin, and biglycan were reported to be tendon extracellular matrix components and had been used as biomarkers for differentiation of stem cells into tenocytes in different studies.[38-43] So first in our previous study, the mRNA expression of these tendon related markers (scleraxis, tenomodulin, smad8, Epha4, tenascin C, type I collagen, type III collagen, fibromodulin, decorin, and biglycan) were compared between BMSCs and tenocytes to define specific markers for tenogenic differentiation of BMSCs. Among tendon specific markers, tenomodulin, scleraxis, tenascin C, fibromodulin, and type I collagen were chosen as the specific markers for tenogenic differentiation of BMSCs in our next study (unpublished data).

GDF-6/BMP-13 belongs to the transforming growth factor beta superfamily, which is a group of proteins involved in early regulation of cell growth and development. GDF6 has been shown to play an important role in the patterning of the bone and joint formation.[44] GDF-6/BMP-13 has been reported to promote tendon healing *in vivo* in many studies before.[11,45-48] The ectopically formed neo-tendon/ligament by GDF-6 expressed elastin, decorin, type I collagen, sine oculishomeobox homolog 1/2 (six 1/six 2), and aggrecan but not alkaline phosphatase and osteocalcin mRNA.[10] MSCs supplemented with BMP-13 strongly inhibited matrix mineraliziation and ALP activity during osteogenic differentiation of MSCs *in vitro*.[49] BMP-13 gene transfer also induced ligamentogenic differentiation in mesenchymal progenitor and anterior cruciate ligament cells.[13] BMP-13 could stimulate cell proliferation and collagen production of human patellar tendon fibroblasts.[12]

These studies about tenogenic effect of GDF-6 on stem cells only used few tendon specific or related markers as assessment parameters, and were not systematically investigated on the tenogenic effect in gene, protein, and function levels.

In our study, we first chose tenomodulin, scleraxis, tenascin C, fibromodulin and type I collagen as assessment parameters to investigate the tenogenic effect of GDF-6 on BMSCs in gene and protein levels according to our previous results. GDF-6 (20 ng/ml) showed better tenogenic effect on the tenogenic differentiation of BMSCs compared to other concentrations in gene and protein level according to our

in vitro study.

For the functional assessment, we combined the tenogenic BMSCs with SIS scaffold and implanted it into nude mice model and SD rat patellar tendon acute injury model to demonstrate that the tenogenic BMSCs by GDF-6 had function for neo-tendon formation and tendon regeneration. Our result showed that the tenogenic BMSCs seeded SIS scaffold was better than BMSCs seeded SIS scaffold in neo-tendon formation and tendon regeneration, which means our tenogenic condition (GDF-6 20 ng/ml) was effective and functional *in vivo*.

In conclusion, GDF-6 has tenogenic effect on the tenogenic differentiation of BMSCs, and GDF-6 (20 ng/ml) has better tenogenic effect compared to other concentrations by our successful and overall tenogenic differentiation assessment in gene, protein, and function level by *in vitro* and *in vivo* study. So this tenogenic condition (GDF-6 20 ng/ml) on BMSCs will have excellent prospects for tendon regeneration.

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