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BMP12 and BMP13 gene transfer induce ligamentogenic differentiation in mesenchymal progenitor and anterior cruciate ligament cells

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Abstract

Background aims—To date there are only very few data available on the ligamentogenic differentiation capacity of mesenchymal stromal/progenitor cells (MSC) and anterior cruciate ligament (ACL) fibroblasts.

Methods—We describe the *in vitro* potential of MSC and ACL cells to undergo ligamentogenic differentiation upon transduction with adenoviral vectors encoding the human cDNA for bone morphogenetic protein (BMP) 12 and BMP13, also known as growth and differentiation factors (GDF) 6 and 7, respectively.

Results—Transgene expression for at least 14 days was confirmed by Western blot analyzes. After 21 days of cell culture within collagen type I hydrogels, histochemical (hematoxylin/eosin (H&E), Azan and van Gieson), immunohistochemical and polymerase chain reaction (PCR) analyzes of the genetically modified constructs of both cell types revealed elongated, viable fibroblast-like cells embedded in a ligament-like matrix rich in collagens, vimentin, fibronectin, decorin, elastin, scleraxis, tenascin, and tenomodulin.

Conclusions—It appears that both MSC and ACL fibroblasts are capable of ligamentogenic differentiation with these factors. This information may aid in the development of biologic approaches to repair and restore ACL after injury.

Keywords

adenovirus; anterior cruciate ligament; BMP12; BMP13; collagen hydrogel; fibroblasts; gene transfer; mesenchymal stromal cells

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Introduction

The incidence of anterior cruciate ligament (ACL) ruptures is approximately 1/1000, and reconstruction with autologous tendon grafts is currently the gold standard of treatment (1,2). However, in large numbers of patients with this injury, ACL reconstruction still has complications of donor site morbidity, loss of knee function, recurrent instability and, in particular, premature osteoarthritis (3–6).

In an effort to improve the care of patients with ACL injuries, tissue-engineering approaches have been explored using different scaffold materials, including collagen, silk, biodegradable polymers and composite materials seeded with fibroblasts and mesenchymal progenitor cells (7–10). Remarkably, it has been shown that cells seeded in collagen hydrogels can degrade and reorganize the surrounding extracellular matrix (10,11), while specific culture conditions, including biomechanical stimulation (10,12,13) and matrix cross-linking (14), can be used to elicit a longitudinal orientation of the cells in a contracted collagen network.

For further enhancement of ligament tissue regeneration, the use of growth factors, including transforming growth factor-β1 (TGF-β1), insulin-like growth factor-I and -II (IGF-I/II), platelet-derived growth factor-B (PDGF-B) and basic fibroblast growth factor (bFGF), have been described (15–20). However, most of these factors have been described as increasing cell proliferation and ligament matrix synthesis of fibroblasts, but only a few have been found to induce ligamentogenic differentiation of mesenchymal progenitor cells. Recombinant growth and differentiation factor (GDF) 6 [bone morphogenetic protein (BMP) 13] and GDF7 (BMP12), members of the TGF-β superfamily, have been found to induce tendon and ligament formation following ectopic (21,22) or orthotopic (23–26) implantation. Therefore, the purpose of this work was to study the influence of BMP12 and BMP13 gene transfer on ligamentogenic differentiation of mesenchymal stromal cells (MSC) and ACL fibroblasts seeded into collagen hydrogel constructs.

Methods

Generation and amplification of recombinant adenoviral vectors

First-generation (E1 E3) serotype 5 adenoviral vectors, carrying cDNA of the human BMP12 or BMP13 gene, were kindly provided by Wyeth Inc. (Madison, NJ, USA). The resulting adenoviral vectors were designated Ad.BMP12 and Ad.BMP13, respectively. Vectors encoding luciferase (Ad.luc) and green fluorescent protein (Ad.GFP) were prepared as described previously (27). For generation of high-titer preparations, adenoviral vectors were amplified in 293 cells (ATCC, Manassas, VA, USA), purified on three rounds of CsCl density gradients, and dialyzed with viral titers, determined by optical density and standard plaque assay as described previously (27).

Isolation and cell culture of MSC and ACL fibroblasts

All chemicals were purchased from Sigma (Steinheim, Germany) unless stated otherwise. After approval of the institutional review board of the University of Würzburg (Würzburg, Germany), isolation of MSC was performed from the femoral heads of patients undergoing total hip arthroplasty (all because of osteoarthritis) using a protocol first described by Haynesworth *et al.* (28) and as modified by Noth *et al.* (29). MSC were isolated from four patients (a 35-year-old male, 65-year-old male, 58-year-old female and an 81-year-old female) who had no diseases other than osteoarthritis of the hip and no medication, except occasionally non-steroidal anti-inflammatory drugs for pain relief. The culture medium consisted of Dulbecco's modified Eagle medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Darmstadt, Germany), 1 U/mL penicillin, 100

μg/mL streptomycin (PAA, Linz, Austria) and 50 μg/mL ascorbate 2-phosphate (standard medium).

ACL tissue was retrieved from patients undergoing total knee arthroplasty after institutional review board (University of Würzburg) approval had been obtained. ACL fibroblasts were isolated by collagenase digestion as described elsewhere (30). For these experiments, ACL were harvested from four patients (three males, 61, 66 and 77 years old, respectively, and one 70-year-old female); the bony attachment sites and synovial sheath were removed, and the ligament fascicles were dissected, minced and digested with 0.1% (w/v) collagenase type I solution (Serva Electrophoresis GmbH, Heidelberg, Germany) for 18 h. ACL fibroblasts were recovered by filtration through a 70-μm nylon mesh cell strainer (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and centrifuged at 1500 r.p.m. for 10 min. Then the cell pellets were resuspended in standard medium, counted and seeded in 75-cm^2 tissue flasks (TPP). The medium was changed every 3–4 days and second-passage ACL fibroblast cultures were used in all experiments. When the cells reached confluence (after 10–14 days), they were detached with 0.25% trypsin containing 1 mm EDTA (PAA) and used for further experiments.

Adenoviral transduction and fabrication of collagen hydrogel constructs

For the experiments, MSC and ACL fibroblasts were seeded at 3.6×10^6 cells/175-cm^2 flask and transduced at 10 or 100 multiplicities of infection (MOI) of Ad.BMP12 or Ad.BMP13, in 5 mL serum-free medium, as indicated in the respective experiments. One day after transduction, the cells were trypsinized for the cell proliferation assay or production of the collagen hydrogel constructs, with marker gene [green fluorescent protein (GFP)/firefly luciferase (luc)] or untransduced cells serving as comparative controls.

For collagen hydrogel construct fabrication, 3×10^5 cells were suspended in 100 μL neutral buffer solution, followed by the addition of 100 μL collagen type I stock solution (Arthro Kinetics AG, Esslingen, Germany), as described elsewhere (10,31). After polymerization the constructs were transferred into a 48-well plate and 500 μL culture medium were added. The medium was changed every 2–3 days throughout the 21-day culture period. All constructs were evaluated biochemically, histologically and immunohistochemically, and by semi-quantitative reverse transcriptase–polymerase chain reaction (RT-PCR), after 21 days.

Transgene expression analyzes

At days 3, 7 and 14, cell lysates of the BMP12- and BMP13-transduced MSC in the hydrogel constructs were collected and frozen at –80°C. The protein content of each lysate was determined using a Rotiquant according to the instructions of the supplier (Carl Roth GmbH, Karlsruhe, Germany). Twenty micrograms of protein were boiled for 5 min in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis buffer (PAGE) (10 mm Tris, pH 6.8; 7.5% glycerol, 10% SDS, 0.025% bromphenol blue). Afterwards the protein was separated by 12% SDS-PAGE and electrotransferred to nitrocellulose membranes. To inhibit non-specific binding, the membranes were treated with buffer containing 0.1% Tween-20, 2% horse serum, 2.5% bovine serum albumin (BSA) and 2.5% milk powder in phosphate-buffered saline (PBS) for 2 h. Then the membranes were incubated overnight at 4°C in 0.1% Tween-20, 1% horse serum and 1% milk powder in PBS with primary antibody anti-BMP12 or anti-BMP13 (1:100; Acris Antibodies GmbH, Hiddenhausen, Germany). After three wash steps in washing solution (10 mm Tris, pH 7.5, 140 mm NaCl, 2 mm EDTA, 0.1% Triton X-100, 1% horse serum, 1% BSA and 1% milk powder), the membrane was incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000; Sigma-Aldrich, Munich, Germany) using a solution containing 0.1% Tween-20, 1% horse serum, 1% BSA and 1% milk powder in PBS. Then the membrane was washed with

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washing solution. Signals were detected by chemiluminescence using the ECL system (Amersham Biosciences, GE Healthcare Life Sciences, Freiburg, Germany).

Cell proliferation assay

Proliferation of monolayer cells was determined using a CellTiter-Glo luminescent cell viability assay (ATP assay; Promega GmbH, Mannheim, Germany). Detection is based on using the luciferase reaction to measure the amount of ATP from viable cells. The ATP amount in the cells is a function of the viability of the cells. For this, BMP12- or BMP13-transduced cells were plated in 100 μL cell culture medium in 96-well plates (1000 cells/well) with clear bottoms, with untransduced or GFP-modified cultures serving as comparative controls. At the respective time-points, 100 μL CellTiter-Glo reagent were added to each well, followed by shaking for 2 min on a shaking plate and incubation for 10 min at room temperature in the dark. The luminescence was recorded with an Orion II Luminometer (Berthold Detection System, Pforzheim, Germany) for 0.25 s/well integration time. The proliferation of the cells was measured after 1, 2, 3, 4, 5, 6 and 7 days.

Cell viability and apoptosis assay

Cells within collagen hydrogel constructs were evaluated for cell viability and apoptosis after 21 days with the use of the apoptosis detection kit Annexin V-Cy3 (Sigma). Annexin V-Cy3 binds to phosphatidylserine present in the outer leaflet of the plasma membrane of early apoptotic cells. Additionally, 6-carboxyfluorescein diacetate (6-CFDA) was used to label viable cells (32). Collagen hydrogel constructs were incubated with 50 μL double labeling staining solution for 10 min at room temperature. After staining, gels were washed five times with 50 μL 1× binding buffer, rinsed twice with PBS, fixed overnight in PBS-buffered 4% paraformaldehyde, dehydrated through a series of ethanols, infiltrated with isoamyl acetate (Merck, Hohenbrunn, Germany) and embedded in paraffin. Sections of 1 μm were cut through the center of the gels. The apoptotic and living cells were observed on the respective mid-sections using a fluorescence microscope (Zeiss, Ulm, Germany).

Histologic and immunohistochemical analyzes

After 21 days, hydrogel constructs were washed twice with PBS, fixed overnight in PBS-buffered 4% para-formaldehyde, dehydrated through a series of ethanols, infiltrated with isoamyl acetate, and embedded in paraffin. Sections of 1 μm were cut through the center of the gels and stained with hematoxylin/eosin (H&E), Azan, Van Gieson and Masson-Goldner.

For immunohistochemistry, all sections were pre-treated by boiling in 10 mM citric acid (pH 5.5) for 10 min. Thereafter, the sections were incubated for 1 h at room temperature with Dako REAL™ antibody diluent (Dako), using primary antibodies for collagen type III (COL III; AB 747; Chemicon, Temecula, CA, USA), elastin (ELA; MAB 1681; Calbiochem, San Diego, CA, USA), vimentin (VIM; M0725; Dako, Hamburg, Germany), fibronectin (FN; F0916; Sigma) and tenascin (TN; CBL213; Chemicon). The slides were rinsed with PBS and visualized by treatment with Advance™ HRP link and Advance™ HRP enzyme for 20 min (Dako) followed by diaminobenzidine staining (DAB kit; Dako) for 10 min. The slides were finally counterstained with hemalaun (Merck, Darmstadt, Germany). For all immunohistochemical analyzes, controls without the primary antibody were performed.

Total RNA extraction and semi-quantitative RT-PCR analyzes

After 21 days, five human hydrogel constructs per group were pooled, minced with scissors and homogenized using a pellet pestle. Total RNA was subsequently extracted with an additional purification step using an RNA isolation kit (Marcherey-Nagel, Düren, Germany)

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according to the manufacturer's instructions. The RNA was quantified spectrophotometrically and reverse-transcribed using random hexamer primers (GE Healthcare, Munich, Germany) and Bioscript RT (Bioline, Luckenwalde, Germany). Equal amounts of each cDNA synthesized (100 ng) were used as templates for PCR amplification in a 50-μL reaction volume using *Taq* DNA polymerase (Bioline) and 50 pmol ligament gene-specific primers (10). In Table I the sequences, annealing temperatures, number of cycles and product sizes of forward and reverse primers are listed for biglycan (BGN), COL III, decorin (DCN), ELA, FN, scleraxis (SCL), tenascin-C (TN-C), tenomodulin (TNMD) and VIM, with elongation factor 1α (EF1α) as an internal control. The RT-PCR products were separated electrophoretically on 1.5% agarose gels containing ethidium bromide and visualized using Bio Profile software (LTF, Wasserburg, Germany). The densities of the PCR bands were analyzed with Bio 1D/Capt MW software (LTF), and the mean ratio (fold change), normalized to EF1α housekeeping gene expression and related to controls, was calculated from three bands (one per patient).

Statistical analyzes

For each set of experiments described, $m = 4$ replicates with cells from different MSC or ACL donors were performed, with $n = 3$ pellets per group per replicate. Data from cell proliferation and RT-PCR analyzes were expressed as mean ± standard deviation (SD). Statistical significances between groups were determined by the Mann–Whitney test using PASW Statistic software (SPSS GmbH Software, Munich, Germany). A level of $P < 0.05$ was considered significant.

Results

BMP12 and BMP13 detection

By means of Western blot analyzes, BMP12 and BMP13 could be detected in Ad.BMP12- and Ad.BMP13-transduced MSC (Figure 1A) as well as ACL fibroblasts (data not shown) at an MOI of 100.

Effects of BMP12 and BMP13 gene transfer on cell proliferation, viability and apoptosis

No differences in proliferation rate could be observed for Ad.BMP12- and Ad.BMP13-transduced MSC (Figure 1B). No differences in proliferation were found for the ACL fibroblasts either (Figure 1B) at an MOI of 100. The transduction with Ad.BMP12 and Ad.BMP13 seemed to have no influence on the proliferation kinetics of MSC and ACL fibroblast compared with controls.

Double fluorescence staining with AnnV-Cy3 and 6-CFDA allowed differentiation between viable (only green), early apoptotic (green and red) and necrotic cells (only red). Three days after transduction, high levels of green fluorescence found in all groups revealed that the respective transductions of MSC (Figure 1C–E) and ACL fibroblasts (Figure 1I–K) did not affect cell viability compared with controls. Only very small proportions of cells appeared to be apoptotic or necrotic in all groups, as evidenced by red fluorescence (Figure 1F–H, L–N). For MSC and ACL fibroblasts in monolayer culture transduced with lower MOI, similar results were observed (data not shown).

Histochemical and immunohistochemical analyzes

After 21 days, staining with H&E showed a homogeneous cell distribution of both MSC and ACL fibroblasts within the hydrogel constructs (Figure 2A). Moreover, the BMP12- and BMP13-modified constructs revealed that the cells of both types were embedded in a dense collagenous matrix compared with controls, where less collagenous matrix was seen, as evidenced by matrix staining with Azan (Figure 2B) and Van Gieson (Figure 2C).

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Immunohistochemistry for COL III, FN, TN and VIM was performed after 21 days in culture (Figure 3). MSC revealed somewhat more intense stainings for COL III in the Ad.BMP12- and Ad.BMP13-transduced hydrogel constructs compared with corresponding control specimens or specimens with ACL fibroblasts, where no brown staining of the matrix was detectable (Figure 3A). FN could be observed in all MSC and ACL fibroblast collagen hydrogel constructs, with Ad.BMP12-transduced constructs showing most intense staining, followed by the Ad.BMP13-modified constructs. However, the marker gene-transduced controls of both cell types had only weak matrix staining (Figure 3B). Staining for TN in the MSC control and VIM in the MSC and ACL fibroblast controls was negative. Positive staining for TN (Figure 3C) and VIM (Figure 3D) was detected in the Ad.BMP12- and Ad.BMP13-transduced MSC and ACL fibroblast hydrogel constructs, while VIM staining was absent from the controls. TN was only weakly present in the ACL constructs. Major differences between groups of different vector doses could not be observed. For each stain, controls were performed without primary antibody, which were negative in all cases (data not shown).

Semi-quantitative RT-PCR analyzes

Semi-quantitative RT-PCR analyzes revealed the gene expression of BGN, COL III, DCN, ELA, FN, SCL, TN-C, TNMD and VIM in all samples after 21 days (Figure 4). The fold change calculated by densitometry was normalized to the EF1α housekeeping gene and related to the respective control cultures (Figure 4A–D). In the Ad.BMP12-modified MSC hydrogel constructs, the gene expression at doses of MOI 10 and 100 of the following ligament-associated matrix proteins was equal or less than the control (Figure 4A): BGN (1.02- and 0.94-fold), COL III (0.97- and 0.94-fold), DCN (0.84- and 0.93-fold), ELA (1.08- and 0.89-fold), FN (0.89- and 0.90-fold), TN-C (0.91- and 0.96-fold) and TNMD (1.01- and 0.97-fold). In contrast, VIM (1.05- and 1.18-fold) showed a slightly increased gene expression (Figure 4A). SCL was only higher at MOI 100 (0.94- and 1.10-fold).

The Ad.BMP13-modified MSC hydrogel constructs revealed distinct differences between MOI 10 and MOI 100. RT-PCR analyzes showed higher gene expression levels for the ligament-associated matrix proteins BGN (1.38-fold), DCN (1.18-fold), FN (1.31-fold) and VIM (1.04-fold) at a dose of MOI 100 (Figure 4C) compared with the untransduced control. At MOI 10 and 100, COL III (1.01- and 1.58-fold), ELA (1.00- and 1.58-fold), SCL (1.35- and 2.38-fold), TN-C (1.03- and 1.36-fold) and TNMD (1.26- and 1.41-fold) were equal or higher than the control.

In the Ad.BMP12-modified ACL fibroblast hydrogel constructs at a dose of MOI 100, the trans-gene expression of the ligamentogenic matrix proteins BGN (1.38-fold), COL III (1.66-fold), DCN (1.36-fold) and TN-C (2.16-fold) was higher than the control (Figure 4B). In contrast, TNMD (1.61-fold), ELA (1.24), SCL (1.04-fold) and VIM (1.10-fold) showed a higher gene expression at MOI 10 (Figure 4B). However, the matrix protein FN (0.65- and 0.92-fold) was always less compared with the control.

As shown in Figure 4D, the gene expression of the Ad.BMP13-modified ACL fibroblast hydrogel constructs revealed distinct differences between MOI 10 and MOI 100. The gene expression for BGN (1.58- and 2.18-fold), DCN (1.37- and 2.75-fold), COL III (0.93- and 1.46-fold), FN (1.22- and 1.45-fold) and TN-C (3.22- and 1.76-fold) was higher for MOI 10 and 100 than the control. SCL (1.54- and 1.51-fold) was clearly higher than the control but showed no differences between MOI 10 and 100. ELA (0.95- and 0.89-fold) showed also no differences between MOI 10 and 100 but was lower than the control. The gene expression of TNMD (1.65- and 0.87-fold) and VIM (1.41- and 0.76-fold) was highest for MOI 10 (Figure 4D).

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Discussion

For biologic ligament repair approaches, a well-balanced interplay between matrix material, cells, biologic and biomechanical factor(s) is of key importance. The ideal matrix material is not only suitable for promoting the presence of cells but also the bio-active factors involved. We chose to use a collagen type I hydrogel for two reasons. The hydrogel is well characterized, its use is standardized, and collagen type I is the primary matrix found in ligament and tendon (12). Additionally, it can be demonstrated that collagen type I hydrogels are effective carriers of adenoviral vectors and transduced cells (31). Different studies indicate that cells initiate a reparative response after an ACL rupture when a collagen hydrogel is placed between the ruptured ends of an ACL *in vitro* (33) or *in vivo* (34).

In our approach, the bioactive factors were delivered via cells transduced by adenoviral vectors encoding BMP12 or BMP13, leading to ligament matrix formation in the respective constructs (Figures 2–4). Notably, transductions even at high viral doses (MOI 100) were not detrimental for cell viability and proliferation (Figure 1). Our study is consistent with the findings of others using recombinant human BMP12 (16) and BMP13 (35) protein, in that we also report the induced formation of a ligament tissue-like matrix when these factors are added to undifferentiated precursor cells *in vitro*.

Remarkably, several studies have shown somewhat stronger ligament/tendon formation in response to ectopic (21,22,36,37) and orthotopic (23–26) implantation of BMP12 and BMP13 *in vivo*. In this respect, it is important to note that the *in vitro* study presented here is limited to static cultures, and that stronger effects might have been observed in an environment where additional biomechanical stimulation was present, such as in a bioreactor or *in vivo*.

Nevertheless, it could be shown that BMP12 and BMP13 gene transfer into MSC and ACL fibroblasts induces ligamentogenesis. Although there is no single specific marker for ligamentogenesis, we evaluated the expression of SCL, a basic helix–loop–helix (bHLH) transcription factor, as one indicator of ligament and tendon development (38). Despite its multiple functions during early embryonic development, SCL marks the tendon progenitor population that forms the fourth somitic compartment, the syndectome, and is expressed continuously through differentiation into the mature tenocytes and ligament cells (39,40). Schweitzer *et al.* (40) could show that the later expression of SCL is specific to the developing connective tissue that mediates the attachment of muscle to bone, including tendons, as well as ligaments mediating the connection between bones. Murchison *et al.* (39) revealed that SCL function is required for the normal force-transmitting tendons. In our study, PCR results revealed distinct SCL expression in Ad.BMP12- and Ad.BMP13-transduced MSC and ACL fibroblasts at MOI 100 compared with the control.

Another important marker for ligament and tendon development is TNMD, a member of a new family of type II transmembrane glycoproteins (41). A distinctly higher TNMD expression in Ad.BMP13-transduced MSC at MOI 100 and in Ad.BMP12- and Ad.BMP13-transduced ACL fibroblasts at MOI 10 was detected in comparison with the controls. Furthermore, BMP12 transduction resulted in an increase of SCL and VIM expression in MSC, and BGL, COL III, DCN and TN-C expression in ACL fibroblasts (Figure 4A, C). Genetic modification with BMP13 at MOI 100 also enhanced the expression of BGL, COL III, DCN, ELA, FN, TN-C and VIM for MSC, and BGL, COL III, DCN, FN and TN-C for ACL fibroblasts (Figure 4B, D).

These findings confirm that expression of BMP12 and, particularly, BMP13 enhanced certain aspects of the ligament and tendon healing response when high doses of vectors were used. In general, ACL fibro-blasts offered a higher expression of specific markers of

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ligament and tendon in comparison with controls (Figure 4). This was consistent with the immunohistochemical analyzes, which revealed the presence of FN, TN and VIM, but not COL III, in the BMP12- and BMP13-transduced cells (Figure 3), with positive staining in the ACL fibroblast cultures being more intense than the MSC. Therefore, it might be that BMP12 and BMP13 are more effective in mediating ligamentogenesis in more committed cell types, such as ACL cells, but might be less powerful inducers of ligamentogenesis in uncommitted precursors.

In summary, we have shown that BMP12 and BMP13 gene transfer into MSC and ACL fibroblasts induces ligamentogenic differentiation, with the ACL fibroblasts being more powerful responders. Therefore, ACL cell populations might be better candidates for ligamentogenic induction using BMP12 and BMP13 compared with undifferentiated precursors obtained from bone marrow.

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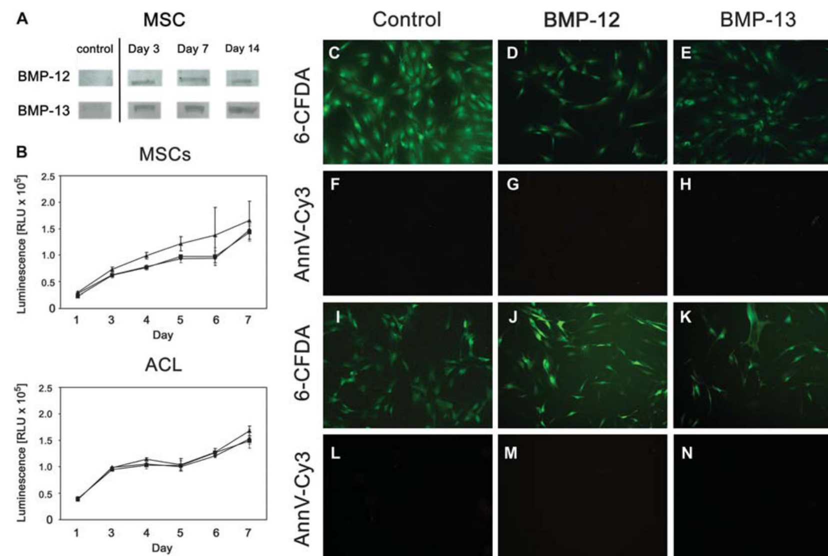

Figure 1.

Cell proliferation and viability in BMP12- and BMP13-modified ligament constructs. (A) Western blot analyzes to detect BMP12 and BMP13 in Ad.BMP12- and Ad.BMP13-transduced MSC at MOI 100 after 3, 7 and 14 days. (B) Time–course of cell proliferation by MSC and ACL fibroblasts following transduction with adenovirus encoding BMP12 and BMP13. Analyzes of cell vitality and apoptosis in MSC (C–H) and ACL fibroblasts (I–N) 3 days after transduction with Ad.BMP12 (D,G) and Ad.BMP13 (E,H) compared with the control (C,F). The cells were double-stained with 6-CFDA and Annexin V-Cy3, allowing for discrimination between living cells stained green with 6-CFDA, necrotic cells stained red with Annexin V-Cy3, and apoptotic cells stained for both. Bar = 20 μm. Original magnifications ×100.

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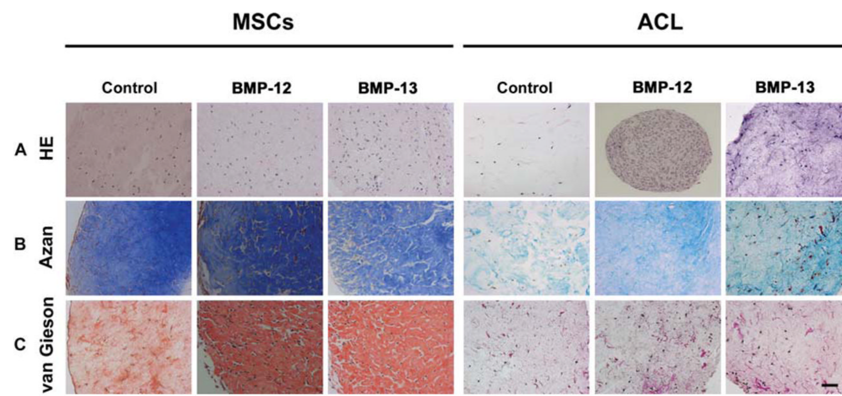

Figure 2.
Histochemical analyzes of BMP12- and BMP13-modified MSC and ACL hydrogel constructs. (A) H&E staining, (B) Azan staining and (C) Van Gieson staining. Bar = 100 μm. Original magnifications ×200.

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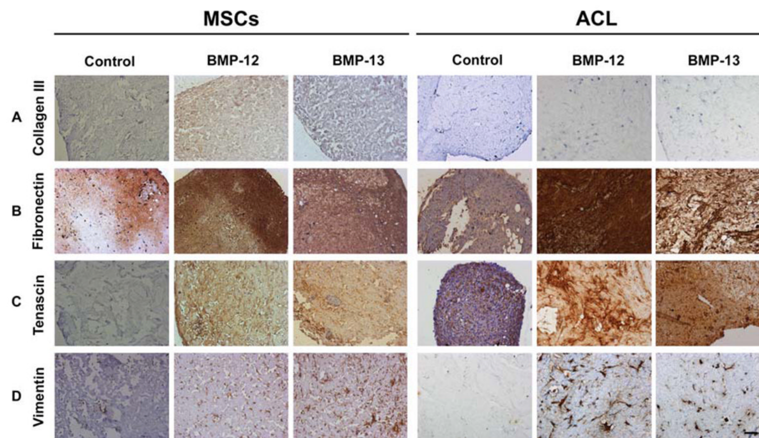

Figure 3.

Immunohistochemical analyzes of BMP12- and BMP13-modified MSC and ACL hydrogel constructs. Staining for (A) COL III, (B) FN, (C) TN and (D) VIM. Positive immunostainings are indicated by the brown-colored network in the vicinity of the blue-colored cells (stained with hematoxylin). Bar = 100 μm. Original magnifications ×200.

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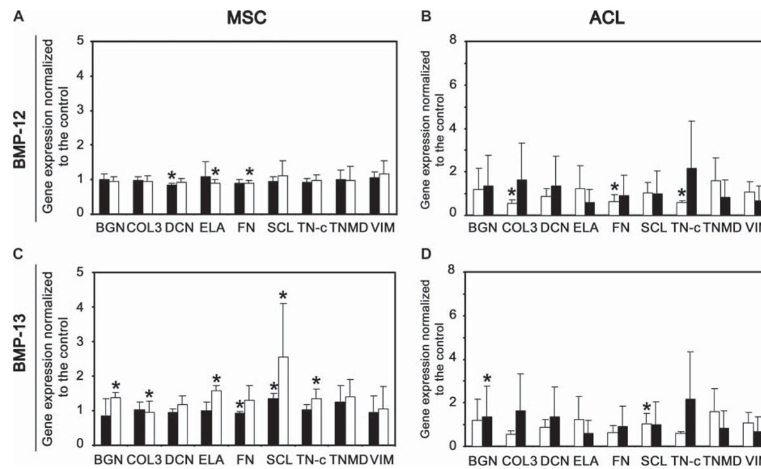

Figure 4.

RT-PCR analyzes of ligament-specific marker gene expression of BMP12- and BMP13-modified MSC (left) and ACL fibroblast (right) collagen hydrogel constructs. Densitometry of PCR products of Ad.BMP12- (A, B) and Ad.BMP13- (C, D) transduced MSC (A, C) and ACL fibroblast (B, D) collagen hydrogel constructs. Fold changes of BGN, COL III, COL V, DCN, ELA, FN, SCL, TN-C, TNMD and VIM are shown. Gene expression was normalized to the housekeeping gene EF1α and related to controls. Mean values ± SD are derived from three patients. Differences compared with the control were considered significant for $P < 0.05$ (*).

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Table I

RT-PCR primer sequences for semi-quantitative RT-PCR.

Gene	RT-PCR primer sequences (5′–3′)	Cycle number	Annealing temperature (°C)	Product size (bp)
EF1α	Sense: AGGTGATTATCCTGAACCATCC Antisense: AAAGGTGGATAGTCTGAGAAGC	27	54	234
BGN	Sense: GAGAGGCTTCTGGGACTTCA Antisense: AGGTGGGTGTGACAGAGTCC	27	58	113
COL III	Sense: GCGGAGTAGCAGTAGGAG Antisense: GTCATTACCCCGAGCACC	27	56	173
DCN	Sense: AATTGAAAATGGGGCTTTCC Antisense: GCCATTGTCAACAGCAGAGA	27	53	220
ELA	Sense: GCAGTGCCTGGGGTCCTTGGAG Antisense: GCTGCTTTAGCGGCTGCAGCTGG	35	58	211
FN	Sense: TGGAACTTCTACCAGTGCGAC Antisense: TGTCTTCCCATCATCGTAACAC	30	58	451
SCL	Sense: CCTGAACATCTGGGAAATTTAATTTTAC Antisense: CGCCAAGGCACCTCCTT	37	56	111
TN-C	Sense: TCAAGGCTGCTACGCCTTAT Antisense: TCAAGGCTGCTACGCCTTAT	40	57	230
TNMD	Sense: CCATGCTGGATGAGAGAGGT Antisense: CTCGTCCTCCTTGGTAGCAG	40	58	123
VIM	Sense: GACCGCTTCGCCAACTACATCGAC Antisense: GGTCATCGTGATGCTGAGAACTTCG	40	65	1060