Bioactive Tape With BMP-2 Binding Peptides Captures Endogenous Growth Factors and Accelerates Healing After Anterior Cruciate Ligament Reconstruction

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Investigation performed at the University of Twente, Enschede, the Netherlands

Background: The anterior cruciate ligament (ACL) has poor regenerative capacity, and an injury leads to loss of function, limiting quality of life and increasing the incidence of osteoarthritis. Surgical interventions can stabilize the joint and improve functional recovery. The delivery of growth factors (GFs) enhances the healing process; however, this is complex in its regulation, is high in costs, has side effects, and can only be accomplished with supraphysiological concentrations and thus is currently not clinically feasible. However, the immobilization of a patient's endogenous GFs in biomaterials can overcome these problems.

Purpose: To develop a method to capture endogenous bone morphogenetic protein–2 (BMP-2) and ultimately show enhanced ACL healing in vivo using this novel methodology.

Study Design: Controlled laboratory study.

Methods: BMP-2 binding peptides were synthetized, purified, and immobilized on polycaprolactone (PCL) films. The affinity between the peptide and human BMP-2 (hBMP-2) was confirmed with immunofluorescence and enzyme-linked immunosorbent assay. The C2C12 Luc reporter cell line was used to confirm the bioactivity of immobilized BMP-2. For in vivo experiments, the same functionalization technology was applied to the commercially available Polytape, and the functionalized tape was sutured together with the graft used for ACL reconstruction in rats. Each animal underwent reconstruction with either native Polytape (n = 3) or Polytape with BMP-2 binding peptides (n = 3). At 2 and 6 weeks after surgery, the graft was assessed by histology and micro–computed tomography.

Results: The covalent immobilization of the peptide in PCL was successful, allowing the peptide to capture hBMP-2, which remained bioactive and led to the osteogenic differentiation of C2C12. In vivo experiments confirmed the potential of the Polytape functionalized with the BMP-2 binding peptide to capture endogenous BMP-2, leading to enhanced bone formation inside the femoral and tibial tunnels and ultimately improving the graft's quality.

Conclusion: The incorporation of BMP-2 binding peptides into materials used for ACL reconstruction can capture endogenous hBMP-2, which enhances the healing process inside the bone tunnels.

Clinical Relevance: These results demonstrate the potential of using synthetic peptides to endow biomaterials with novel biological functions, namely to capture and immobilize endogenous GFs.

Keywords: BMP-2 (bone morphogenetic protein–2); growth factor binding peptide; anterior cruciate ligament; endogenous growth factor; bone regeneration

Owing to an aging population and increasing involvement in physical activities, musculoskeletal injuries are among the most common injuries worldwide. Of the 33 million injuries reported in the United States (US) per year, approximately 33% involve tendons and ligaments.[17]

The most frequently reported injury is an anterior cruciate ligament (ACL) tear or rupture, which accounts for more than 80,000 cases per year in the US alone, with an estimated cost of US$1.0 billion.[14] Because of improvements in quality of life and increasing participation of the population in physical activities, the incidence of these injuries is likely to rise, affecting patients' quality of life and increasing health care costs. Injuries to these tissues are always associated with pain, swelling, and disability, with the extent of damage dictating recovery time.[8,18]

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When tears or ruptures of the tissues occur, a surgical intervention is usually needed. The main goal of this surgical treatment is to stabilize and restore normal movement and homeostasis of the joint.[3] Because of the nature of these tissues and their inherent poor healing capacity, a surgical intervention is also needed to direct the natural healing process. However, even with the available treatments, complete healing of the damaged tissue is difficult to achieve, which can ultimately lead to scarring, restrictions to range of motion, stiffness/weakness of the joint, improper healing, and reinjuries.[22]

Several approaches using cells, biomaterials, and bioactive molecules have been previously investigated to accelerate ACL healing after surgery.[24,29,31,35] Of the bioactive molecules, growth factors (GFs) are extremely appealing because of their role in all the stages of the healing process.[1,4,5,19] After surgery, the weakest point in the femur-ligament-tibia complex is the interface between the ligament and bone.[7] Efficient osteointegration of the graft into the bone tunnels is therefore crucial to achieve proper joint stability and to start an earlier and more aggressive rehabilitation plan, which will shorten the time needed to return to normal daily life.[7] Therefore, GFs that enhance bone healing, such as bone morphogenetic proteins (BMPs), are becoming promising candidates to promote osteointegration of the graft, with special attention being given to the use of BMP-2.[6,9,16,26,28,30,32] However, the administration of soluble GFs is clinically challenging because of their low stability in the body and diffusion-related problems, which lead to the need for supraphysiological concentrations and systemic administration to achieve the desired result.[27] Additionally, Thomopoulos et al[34] showed that the addition of BMP-2 did not improve bone formation and mechanical properties when compared with a control in a canine flexor tendon.

We previously described the use of transforming growth factor–beta 1 (TGF-β1) binding peptides in polycaprolactone (PCL) films to capture and deliver TGF-β1 to human-derived hamstring cells. In vitro studies showed that captured TGF-β1 led to an upregulation of collagen at the mRNA and protein levels. When implanted subcutaneously in rats, the films functionalized with the peptide led to a higher recruitment of inflammatory cells after 3 days and a more robust fibrogenic response at day 7.

In the current study, we exploit a BMP-2 binding peptide to capture BMP-2 and induce bone healing.[23] We envision that the presence of BMP-2 binding peptides in biomaterials used in ACL reconstruction would capture endogenous BMP-2 and promote bone formation inside the tunnels, leading to a more robust healing process. The peptide was synthesized, purified, and covalently attached to PCL films. The chemical steps of the functionalization procedure were verified with water contact angle and X-ray photoelectron spectroscopy measurements. The interaction between the peptide and human BMP-2 (hBMP-2) was assessed in vitro with immunofluorescence and enzyme-linked immunosorbent assay (ELISA). The osteogenic differentiation of C2C12 cells was used as a model to confirm the bioactivity of captured hBMP-2 by the peptide. We hypothesized that functionalizing a tape intended to use with the graft in ACL reconstruction would allow the capture of endogenous hBMP-2 and promote bone formation inside the femoral and tibial tunnels. The capture of hBMP-2 by the peptide was assessed by immunohistochemistry and its effect assessed by histology and micro–computed tomography (μCT) in rats that underwent ACL reconstruction.

METHODS

Materials

N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), and the amino acids used for peptide synthesis were obtained from MultiSynTech. Chloroform and 1-methyl-2-pyrrolidinone (NMP) were purchased from VWR International. NaOH was obtained from Riedel-de Haën. All other reagents or products were purchased from Sigma-Aldrich unless noted otherwise.

Preparation of PCL Films Displaying BMP-2 Binding Peptides

A 12.5% (w/v) solution of PCL (average Mn = 45.000) in chloroform was prepared and homogenized by sonication. The homogenized solution was cast in a petri dish and presilanized with a 1H,1H,2H,2H-perfluorodecyltrichlorosilane (PFDTS [≥97%]; abcr) anti–sticky layer, and the solvent was allowed to evaporate overnight. The polymer was melted, allowed to again solidify, and then cut into circular films with a diameter of 21 mm to fit inside the wells

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of a 12-well plate. The individual circular films were extensively washed with demiwater and Milli-Q water and dried with a N_2 stream. The dried films were exposed to oxygen plasma for 5 minutes (at an oxygen pressure of 1.0 bar, vacuum pressure of 200 mbar, and current of 40 A) and subsequently immersed in a 1 M NaOH solution for 1 hour with gentle agitation. PCL films were then washed and dried as mentioned above and incubated with a solution of 50 mM 1:1 N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (NHS/EDC) in a 2-(N-morpholino) ethanesulfonic acid (MES) buffer for 1 hour with agitation. PCL films were washed and dried again as mentioned above and incubated with 1 mM of the peptide in phosphate-buffered saline (PBS) for 4 hours with agitation. Films were then extensively washed with PBS and sterilized by incubating the films overnight in a solution of 10% penicillin/streptomycin (Life Technologies) in PBS before cell seeding. Polytape (Neoligaments) was functionalized using the protocol described above.

BMP-2 Binding and Immunofluorescence

The PCL films were incubated with 1 μg/mL of hBMP-2 (Peprotech) in 0.5% (v/v) PBS Tween-20 (PBST) for 1 hour with gentle agitation. The films were then washed 3 times for 10 minutes with PBST and then with PBS for a further 10 minutes. Next, the films were blocked for 1 hour with PBS containing 1% (w/v) bovine serum albumin (BSA) and subsequently washed as described above. Afterwards, the films were incubated with a 2 μg/mL solution of the primary antibody (rabbit polyclonal anti-human BMP-2; PeproTech) in the blocking solution for 1 hour with agitation. The films were washed as mentioned above and then incubated with an 8 μg/mL solution of the secondary antibody (goat anti-rabbit Alexa Fluor 594; Invitrogen) in PBS containing 1% w/v BSA for 1 hour with gentle agitation. Before fluorescence microscopy, the films were washed 3 times for 10 minutes with PBST, rinsed 3 times with PBS, and dried under a N_2 stream. For cell experiments, the sterile films were washed 3 times with PBS and incubated with concentrated hBMP-2 in PBST for 1 hour with gentle agitation. Subsequently, the films were extensively washed with PBST (0.5%) and PBS to remove any traces of the washing buffer before cell seeding. Immunochemistry assays on the Polytape were performed using the procedure mentioned above.

Cell Culture

C2C12 Luc (a kind gift from Daniel Rifkin's laboratory) and C2C12 were expanded in Dulbecco's Modified Eagle Medium (Life Technologies) supplemented with 20% fetal bovine serum (Life Technologies), 100 U/mL penicillin (Life Technologies), 100 μg/mL streptomycin (Life Technologies), and 2 mM L-glutamine (Life Technologies). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. The medium was refreshed twice per week, and cells were used for further subculturing or cryopreservation on reaching near confluence. C2C12 Luc expresses luciferase under the control of a ID1 promoter, a BMP-2 target gene.[37]

Animal Surgery

This study was approved by the animal research ethics committee of the authors' institution (AEEC No. 16-172-MIS). Twelve male Sprague-Dawley rats (12 weeks old; mean weight, 395.5 ± 21.5 g) underwent unilateral ACL reconstruction with the ipsilateral flexor digitorum longus tendon according to our well-established protocol.[13] Animals were divided into 2 groups: native Polytape and Polytape functionalized with BMP-2 binding peptides. In brief, animals were anesthetized by an intraperitoneal injection of 10% ketamine/2% xylazine (0.3/0.2 mL; Ketalar [Alfasan Diergeneesmiddelen BV]) and maintained with an intramuscular injection of 10% ketamine. The ipsilateral flexor digitorum longus tendon was harvested through a longitudinal medial incision. Subsequently, the right knee was exposed, the ACL was excised, and 1.1 mm–diameter bone tunnels with around 7 mm in length were created from the footprint of the original ACL to the medial side of the tibia and anterolateral side of the femoral condyle with an angle of about 55° to the articular surface. The Polytape was sterilized with ethanol before it was put into the tunnel. The tendon graft and Polytape were fixed on the femoral and tibial tunnel exits with the suture tied over the neighboring periosteum with a constant tension of 4 N (provided by a freely suspended weight through a pulley system) with the knee fully extended. The animals were allowed to have free cage movement immediately after surgery. At week 2 or week 6, the animals were euthanatized by overdosing by 20% sodium phenobarbital with an intraperitoneal injection. The reconstructed ACL complex was harvested, and samples were used for μCT, followed by a histology assessment according to our previous validated protocols.[12,25]

Statistical Analysis

Data were analyzed using the Student paired t test, 1-way analysis of variance followed by the Tukey multiple comparison test ($P < .05$), or 2-way analysis of variance. The values represent the mean ± SD.

RESULTS

Functionalization of PCL With BMP-2 Binding Peptides

To immobilize the peptide (Appendix Figure A1A; see Materials and Methods in the Appendix, available in the online version of this article), the PCL films were initially treated with oxygen plasma and hydrolyzed with 1 M of NaOH for 1 hour to introduce carboxylic acids at the surface of the films. The carboxylic acids were then reacted with NHS/EDC to form amine reactive esters at the surface of the film. The amine reactive esters were finally reacted with the free amine present in the lysine of the

BMP-2 binding peptide. Changes in surface wettability (Figures 1A and Appendix Figure A2; see Materials and Methods in the Appendix) and X-ray photoelectron spectroscopy measurements (Table 1 and Appendix Figure A3; see Materials and Methods in the Appendix) were used to evaluate the occurrence of chemical modifications. The fabricated native films consisted of 77.0% ± 0.1% carbon and 23.0% ± 0.1% oxygen and exhibited a wettability of 78.6° ± 2.0°. After oxygen plasma treatment, the hydrophobicity of the film decreased by 37.5° ± 1.1° ($P < .0001$), while the oxygen content increased by 3.6% ± 0.3% ($P < .0001$). Activation of the carboxylic acids with NHS/EDC led to an increase of 18.1° ± 0.7° ($P < .0001$) in wettability and the appearance of nitrogen at the surface of the film. The films were then incubated with the BMP-2 binding peptide, and an increase in the wettability of 6.3° ± 0.7° ($P < .0001$) and increase in nitrogen content of 1.6% ± 0.1% ($P < .0001$) were observed, indicating the covalent immobilization of the peptide on the PCL film.

BMP-2 Immobilization on Functionalized PCL Films

hBMP-2 solution at a concentration of 1 μg/mL was incubated with the films, and immunostaining against hBMP-2 was performed to confirm the binding of the GF to the functionalized films. Our results showed that the incubation of the native film with hBMP-2 led to a significant increase in the fluorescence signal when compared with the native film without hBMP-2 incubation. However, the maximum intensity was observed when the films with the BMP-2 binding peptide were incubated with hBMP-2 ($P < .0001$) (Figure 1, B and C). The amount of hBMP-2 captured by the films was quantified by ELISA (Figure 1D), and our results showed that more hBMP-2 was captured by the film when BMP-2 binding peptides were present. The surface concentration of hBMP-2 in the functionalized films was of 52.4 ± 9.5 ng/cm^2 while in the native films it was 32.8 ± 6.1 ng/cm^2 ($P < .05$). The release profile was also affected by the presence of the BMP-2 binding peptide (Figure 1E). During the first 5 days, the native films released 5.8 ± 1.4 ng, the films with the scrambled peptide released 2.4 ± 1.3 ng, and the films with the BMP-2 binding peptide released 0.6 ± 0.2 ng of hBMP-2, and after 5 days, the release slowed down for all the tested groups.

Immobilized hBMP-2 Retains Its Bioactivity

We used a BMP-2 luciferase reporter cell line to study the bioactivity of captured BMP-2. First, we incubated the films with different concentrations of hBMP-2 and subsequently seeded the C2C12 reporter cell line and analyzed luciferase activity (Appendix Figure A4A; see Materials and Methods in the Appendix). Our results showed that in the native films, regardless of the concentration of hBMP-2 used, no upregulation of luciferase was observed when compared with the native films without hBMP-2 incubation. When cultured on top of films functionalized with a BMP-2 binding peptide, C2C12 transfected with this luciferase construct exhibited a dose-dependent luciferase response to increasing incubation concentrations of hBMP-2 from 50 to 500 ng. The incubation of functionalized films with hBMP-2 amounts lower than 50 ng also led to an increase in the amount of luciferase activity; however, there was no significant difference in luciferase activity between 0.5 and 50 ng of hBMP-2. The maximum value of luciferase produced in response to immobilized hBMP-2 was observed when the functionalized films were incubated with 500 ng of hBMP-2. The reported value of 10.4 ± 1.6 for this concentration was statistically significant when compared with all the other amounts tested, except for the case of 250 ng, and thus, 500 ng was selected for the next experiment in which we included a control with films functionalized until the NHS/EDC step and with a scrambled peptide sequence (Figure 2A). Again, the incubation of films lacking a BMP-2 binding peptide with 500 ng of hBMP-2 before cell seeding did not lead to an upregulation of luciferase activity. In contrast, the presence of a BMP-2 binding peptide on the films allowed the capture of hBMP-2, which induced a 5.3 ± 2.0–fold increase in luciferase activity ($P < .05$). When the cells were seeded in the presence of the BMP inhibitor Noggin, the production of luciferase was completely suppressed ($P < .01$).

BMP-2 is known to induce the differentiation of C2C12 into the osteogenic lineage, with alkaline phosphatase (ALP) being one of the characteristic markers of osteoblast lineage cells.[20] To identify the amount of immobilized hBMP-2 needed to transdifferentiate C2C12 into the osteogenic lineage, a dose response study of immobilized hBMP-2 was performed (Appendix Figure A4B; see Materials and Methods in the Appendix). We observed a maximum response when the functionalized films were incubated with 5000 ng of hBMP-2, leading to a 12.7 ± 2.2–fold increase in the synthesis of ALP. This amount of BMP was then used for further experiments in which C2C12 was cultured on top of native and BMP-2 binding peptide functionalized films previously incubated with or without hBMP-2 before cell seeding (Figure 2B). In the films without hBMP-2 incubation before cell seeding, C2C12 was cultured either in the presence or absence of 200 ng/mL of soluble hBMP-2. Our results showed that the incubation of both native and functionalized films with hBMP-2 before cell seeding led to an increase in ALP activity. hBMP-2 captured by the BMP-2 binding peptide led to a 12.2 ± 1.2–fold increase in ALP production, whereas hBMP-2 nonspecifically adsorbed to the native films led to 5.6 ± 2.1 ($P < .05$). There was no statistical difference in ALP production between the groups treated with soluble hBMP-2 and the functionalized films preloaded with hBMP-2. The images of ALP staining (Figure 2C-E) are in agreement with the results obtained from quantification.

Capture of Endogenous BMP-2 in a Rat ACL Reconstruction Model

On the basis of the in vitro results, we hypothesized that biomaterials functionalized with GF binding peptides could capture endogenous GFs and accelerate the healing process. To test this hypothesis, we selected the commercially available Polytape, and upon functionalization with the BMP-2

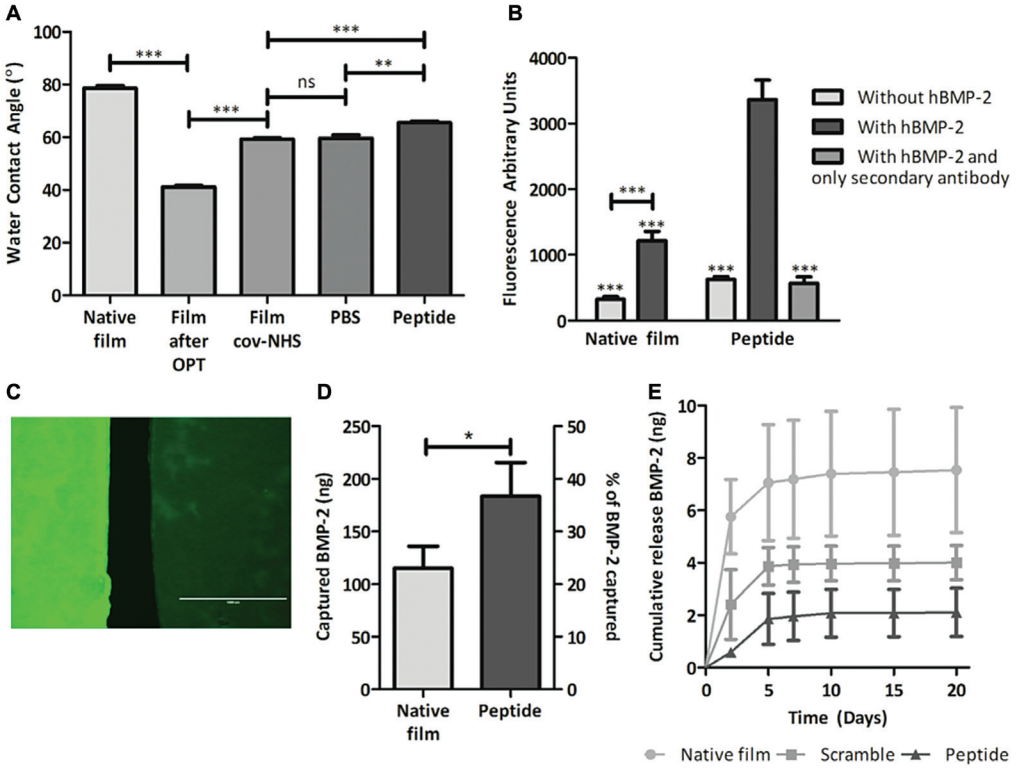

Figure 1. (A) Water contact angle measurements of the films during the chemical functionalization procedure. $^{**}P < .01$ and $^{***}P < .0001$ (2-tailed unpaired *t* test) between the stages before and after each chemical modification). The data represent the mean ± SD of 2 measurements per sample (n = 6). (B) Fluorescence quantification of the immunofluorescence assay against hBMP-2 immobilized on the films. The data represent the mean ± SD of 3 measurements per sample (n = 2). $^{***}P < .0001$ (2-tailed unpaired *t* test) with functionalized films incubated with hBMP-2 and immunofluorescence performed with primary and secondary antibodies. (C) Fluorescence image of the (left) functionalized film with hBMP-2 incubation versus (right) native film with hBMP-2 incubation (scale bar: 1000 μm). (D) Quantification of the amount of hBMP-2 captured by the films. $^{*}P < .005$ (2-tailed unpaired *t* test) between the native film and functionalized film. The data represent the mean ± SD of 2 measurements per sample (n = 3). (E) Release profile of captured hBMP-2 during 20 days at 37°C. The data represent the mean ± SD of 2 measurements per sample (n = 3). cov-NHS, covalent N-hydroxysuccinimide; ns, not significant; OPT, oxygen plasma treatment; PBS; phosphate buffered saline.

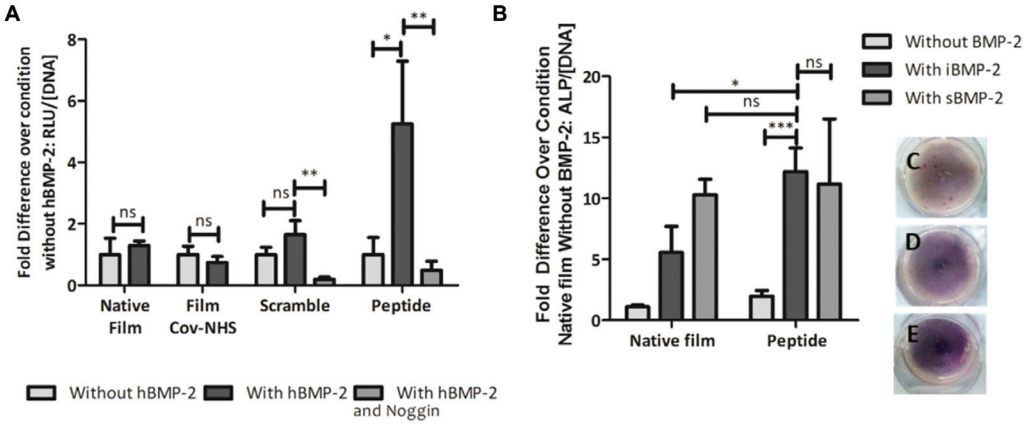

Figure 2. (A) Effect of immobilized hBMP-2 on the expression of luciferase by a BMP-2 reporter cell line. Luciferase values were corrected for the amount of DNA and normalized to the condition without the incubation of BMP-2 before cell seeding. $^{*}P < .05$ and $^{**}P < .01$ (2-tailed unpaired *t* test). The data represent the mean ± SD of 3 samples. (B) Quantification of ALP activity in response to immobilized hBMP-2 (ihBMP-2) and soluble hBMP-2 (shBMP-2) in native and functionalized films. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .0001$ (2-tailed unpaired *t* test). The data represent the mean ± SD of 2 measurements per sample (n = 3). ALP staining in (C) functionalized films without hBMP-2, (D) functionalized films with ihBMP-2, and (E) functionalized films with shBMP-2. Cov-NHS, covalent N-hydroxysuccinimide; ns, not significant.

TABLE 1
X-ray Photoelectron Spectroscopy Results During the Chemical Functionalization Procedure and Theoretical Calculations With Respective Chemical Formulas[a]

	Carbon	Oxygen	Nitrogen	Other (Silica and Sodium)
Native film	77.0 ± 0.1	23.0 ± 0.1	—	—
Film after oxygen plasma treatment	71.3 ± 1.7	26.6 ± 0.5	—	2.1
Film after NHS/EDC	75.5 ± 0.3	23.2 ± 0.3	0.8 ± 0.2	0.5
Peptide film	74.5 ± 0.0	22.3 ± 0.0	2.4 ± 0.1	—
Calculated for native PCL (C_6O_2)	75.0	25.0	—	—
Calculated for NHS/EDC (C_4NO_3)	50.0	37.5	12.5	—
Calculated for peptide ($C_{47}N_{13}O_{13}$)	64.4	17.8	17.8	—

[a]Data represent the mean ± SD of 4 measurements per sample. "Native film" represents the polycaprolactone (PCL) films without any chemical modification. "Film after oxygen plasma treatment" characterizes the native PCL films exposed for 5 minutes to oxygen plasma. "Film after NHS/EDC" indicates the films with amine reactive esters after 1-hour incubation with N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (NHS/EDC). "Peptide film" signifies PCL films with reactive amine esters incubated for 4 hours with 1 mM of peptide in phosphate buffered saline.

binding peptide, we used it in combination with a graft for the reconstruction of a rat ACL. The Polytape, like PCL, is a polyester and allowed us to use the same functionalization procedure. The chemical modifications performed during the functionalization protocol were confirmed with X-ray photoelectron spectroscopy (Appendix Figure A5 and Table A1).

Upon confirming the covalent immobilization of the peptide in the Polytape, we checked with immunofluorescence whether the functionalized Polytape could capture and immobilize hBMP-2 (Appendix Figure A6). For the in vivo reconstruction of a rat ACL, the Polytape was sutured together with the graft and both used for reconstruction. After 2 and 6 weeks, the samples were harvested and assessed with μCT and immunohistochemistry. At the time of harvest, no significant body weight loss was observed (Appendix Table A2). Additionally, neither inflammation nor differences in gross morphology were observed (Appendix Figure A7). Our μCT results showed that at week 2, there was no difference in bone mineral density (BMD) and bone volume/total volume (BV/TV) inside the femoral and tibial tunnels between the groups. However, at week 6, there was a statistically significant improvement in BMD and BV/TV inside the femoral tunnel ($P < .01$ and $P < .001$, respectively) (Figure 3, A and B) as well as an improvement in BMD and BV/TV in the tibial tunnel ($P < .05$ and $P < .001$, respectively) (Figure 3, C and D) for the group with the BMP-2 binding peptide. The control group did not show a significant improvement in BMD and BV/TV inside the femoral or tibial tunnels from week 2 to week 6. At week 6, the BMD inside the femoral tunnel for the BMP-2 binding peptide group was significantly higher when compared with the native group ($P < .01$) (Figure 3A). The BV/TV at week 6 inside the femoral tunnel was significantly higher as well in the BMP-2 binding peptide group when compared with the native group ($P < .001$) (Figure 3B). Inside the tibial tunnel at week 6, the BMP-2 binding peptide group had a significantly higher BMD ($P < .05$) (Figure 3C) and BV/TV ($P < .05$) (Figure 3D) when compared with the control group. Representative μCT-reconstructed 3-dimensional images of the bone tunnels are shown in Appendix Figure A8. These results showed that bone formation was enhanced inside the bone tunnels when the graft was sutured together with the Polytape functionalized with the BMP-2 binding peptide.

To better understand this observation, we analyzed the tissue response and the capture of endogenous BMP-2 by immunohistochemistry in the BMP-2 binding peptide group and compared it with the native group. The results of histological scoring are shown in Appendix Table A3. At week 2, the total score of the BMP-2 binding peptide group (score: 14-16) was better than that of the native group (score: 17-18). Less graft degeneration was observed in the BMP-2 binding peptide group (Figure 4I). Similar observations were found at week 6 with better graft corporation (Figure 4N), graft-bone healing (Figure 4L), and total score in the BMP-2 binding peptide group (score: 12-13) when compared with the native group (score: 15-17). Similar observations were found in the femoral and tibial tunnels. At week 2, higher numbers of Sharpey fibers were observed at the interface of the BMP-2 binding peptide group (Figure 4O) when compared with the native group (Figure 4G). The graft was also less degenerated in the BMP-2 binding peptide group (Figure 4I), and the cells were aligned in the interface between the bone and graft in contrast with the native group (Figure 4A). At week 6, new bone formation was observed in both groups (Figure 4, B and D and J and L). Chondrocyte-like cells could be observed in the graft next to the Polytape in one sample in the BMP-2 binding peptide group but not in the native control group (Figure 4J). Overall better healing was observed in the BMP-2 binding peptide group. The intra-articular midsubstance was also analyzed, and no difference in cellularity and graft degeneration was observed between the groups at week 2 and week 6 (Appendix Figure A9).

Next, we analyzed by immunohistochemistry whether the improvement in healing and bone formation observed in the BMP-2 binding peptide group was caused by the capture of endogenous BMP-2 by the functionalized Polytape (Figure 5). BMP-2 was detected in the femoral and tibial tunnels in both groups at week 2 and week 6 after ACL

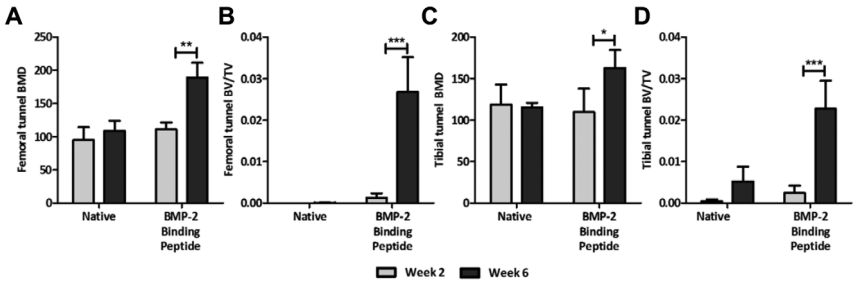

Figure 3. Mineralized tissue formation inside bone tunnels after anterior cruciate ligament reconstruction. Bone mineral density (BMD) (mgHA/cm^3) of newly formed mineralized tissue inside the (A) femoral tunnel and (C) tibial tunnel. Bone volume/total volume (BV/TV) of newly formed tissue inside the (B) femoral tunnel and (D) tibial tunnel. *$P < .05$, **$P < .01$, and ***$P < .0001$ (2-way analysis of variance). The data represent the mean ± SD (n = 3).

reconstruction. However, the amount of BMP-2 was much higher in the BMP-2 binding peptide group (Figure 5, E-H) when compared with the native group (Figure 5, A-D). These results show that the functionalization of biomaterials with a BMP-2 binding peptide can enhance the capture of endogenous BMP-2, thus enhancing bone formation and ultimately accelerating graft-to-bone healing after ACL reconstruction.

DISCUSSION

In this work, we described a strategy to immobilize endogenous hBMP-2 via the interaction with a BMP-2 binding peptide. To that end, a previously reported sequence, YPVHPST, was synthesized and equipped with an extra lysine and a glycine at the N-termini.[33] The affinity between the peptide and hBMP-2 was confirmed using immunostaining and ELISA. Both assays showed that the capture of hBMP-2 was maximum when the BMP-2 binding peptides were present at the surface of the PCL film.

To further investigate whether immobilized hBMP-2 retained its bioactivity, a cell line was used that was stably transfected with a construct consisting of a BMP/Smad enhancer from the Id1 promoter that was fused to a luciferase reporter gene.[37] We hypothesized that if immobilized BMP-2 retained its bioactivity, it would activate the Id1 promoter and induce the production of luciferase. When the cells were seeded on the top of films lacking the BMP-2 binding peptide, which were incubated with 500 ng of hBMP-2, no production of luciferase was observed. This can be explained by the fact that these films lack an affinity for hBMP-2; therefore, there is no or insufficient hBMP-2 on the surface to trigger the BMP signaling pathway and consequently luciferase production by these cells. In contrast, the presence of a BMP-2 binding peptide on the films allowed the immobilization of hBMP-2, which induced a 5.3 ± 2.0–fold increase in the amount of luciferase produced ($P < .05$). When the cells were seeded in the presence of Noggin, which is known to bind to BMP and therefore block its interaction with the cell membrane receptors, the luciferase produced was abolished ($P < .01$).[15] These results show that hBMP-2 captured by the BMP-2 binding peptide remains bioactive and activates the BMP signaling cascade by the Smad-dependent pathway.

The production of ALP, a hallmark of osteogenic differentiation, was quantified to study the effect that immobilized hBMP-2 has on the differentiation of C2C12.[2] The incubation of both native and functionalized films with hBMP-2 led to an increase in the amount of ALP produced. However, ALP production in response to hBMP-2 immobilized by the BMP-2 binding peptides was 2.4 times higher when compared with the response to hBMP-2 nonspecifically adsorbed in the native films. This can be explained by a higher surface concentration of hBMP-2 in the functionalized films and because of the longer presentation and availability of hBMP-2 due to the affinity interaction with the BMP-2 binding peptide. There was no difference in ALP production between the soluble treatment in the native films and the functionalized films preloaded with hBMP-2. This highlights the advantages of delivering hBMP-2 to cells via the interaction with the BMP-2 binding peptide over nonspecifically adsorbing the GF to the biomaterial.

ACL reconstruction requires the drilling of bone tunnels in the femur and tibia to pass the graft that is going to replace the injured ACL. To stabilize the joint, it is compulsory to have proper bone healing inside the tunnels to promote bone-tendon and bone-bone integration. Accelerated bone healing will promote stabilization of the joint and consequently allow the use of a more aggressive rehabilitation approach. Moreover, because the problem of tunnel widening is common after ACL reconstruction, strategies to prevent tunnel widening are highly desirable. Previous work in humans showed that the BMP-2 levels in synovial fluid are significantly higher 7 days after ACL surgery than preoperatively.[38] This is likely caused by drilling of the tunnels and the role that BMP-2 has in bone healing.[31] Recently, bone marrow–derived mesenchymal stem cells genetically modified with BMP-2 were applied on the bone-graft interface and improved ACL reconstruction healing in a rabbit model.[6] However, the transplantation of genetically modified stem cells requires a Good Manufacturing Practice facility for the operation theater, a long preparation time to obtain enough stem cells, and genetic modification, which limit its clinical application. Therefore, technologies that are

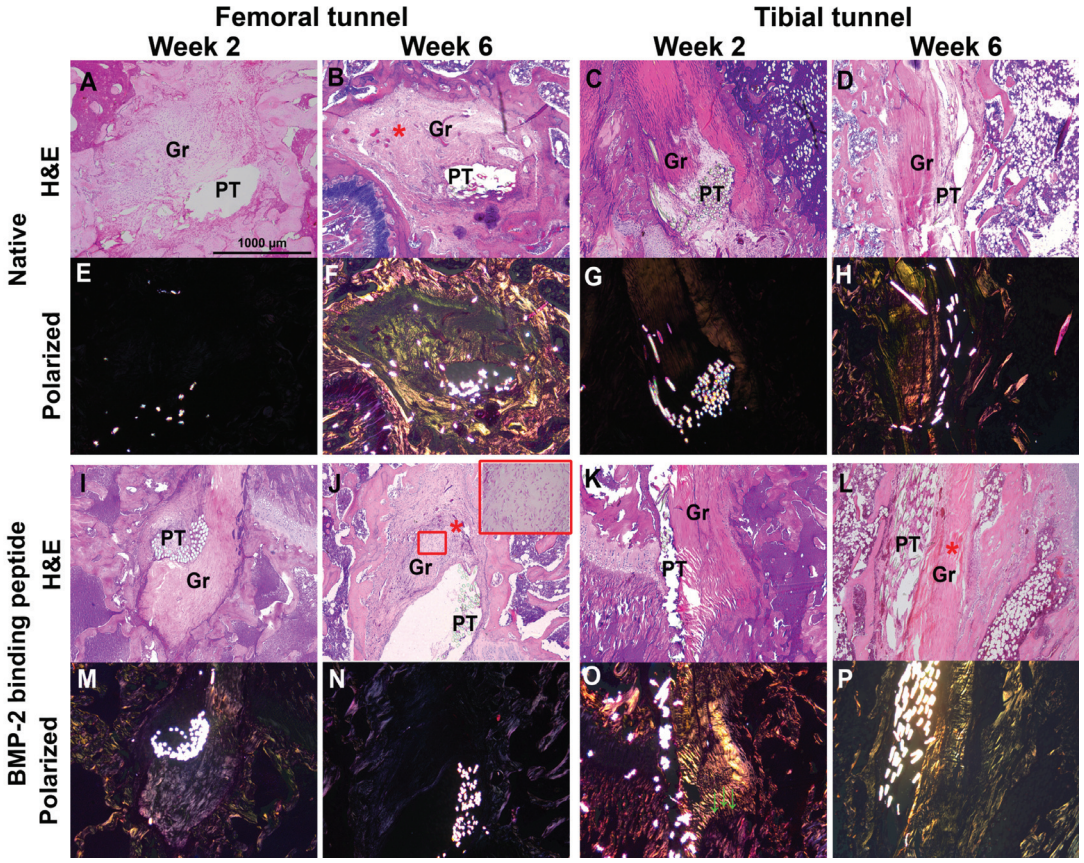

Figure 4. Microscopy of femoral and tibial tunnels after anterior cruciate ligament reconstruction. Hematoxylin and eosin (H&E) staining and polarized images of the native Polytape at week 2 inside the (A, E) femoral tunnel and (C, G) tibial tunnel and at week 6 inside the (B, F) femoral tunnel and (D, F) tibial tunnel. H&E staining and polarized images of the Polytape functionalized with BMP-2 binding peptides at week 2 inside the (I, M) femoral tunnel and (K, O) tibial tunnel and at week 6 inside the (J, N) femoral tunnel and (L, P) tibial tunnel. Gr, graft; PT, Polytape. The asterisk denotes newly mineralized tissue, the red squares in J denote chondrocyte-like cells, and the green arrows in O denote Sharpey fibers. Scale bar: 1000 μm.

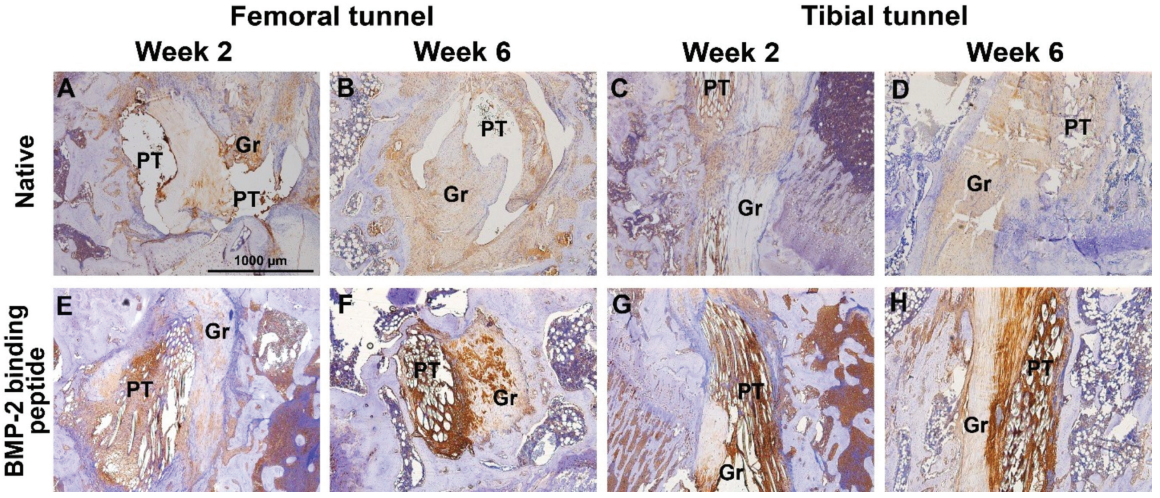

Figure 5. Immunohistochemistry staining of BMP-2 of the native Polytape at (A, C) week 2 and (B, D) week 6 inside the (A, B) femoral tunnel and (C, D) tibial tunnel. Immunohistochemistry staining of BMP-2 of the Polytape functionalized with BMP-2 binding peptides at (E, G) week 2 and (F, H) week 6 inside the (E, F) femoral tunnel and (G, H) tibial tunnel. Gr, graft; PT, Polytape. Scale bar: 1000 μm.

easily translated into clinics are more appealing. After the promising in vitro results, we tested whether the incorporation of BMP-2 binding peptides into biomaterials would allow the capture of endogenous BMP-2 and accelerate bone healing in an ACL reconstruction model. Micro-CT data showed an improvement in bone tunnel mineralization in the BMP-2 binding peptide group when compared with the other group. The increased mineralization implied that the problem of tunnel widening can be solved. In addition, better histological scores, less graft degeneration, and better graft-bone healing were also found in the BMP-2 binding peptide group. Finally, a higher amount of BMP-2 was found in the BMP-2 binding peptide group inside both tunnels at both time points. These results show that the BMP-2 binding peptide group captured and localized endogenous BMP-2 at the healing site during healing and elicited the biological function of BMP-2. Of all the GFs known to be involved in the healing and regeneration process, only BMP-2 and BMP-7 are clinically approved by the US Food and Drug Admnistration (FDA) to be used in bone therapy. Infuse (recombinant hBMP-2; Medtronic) and OP-1 (recombinant hBMP-7; Olympus) are the 2 authorized products that make use of BMPs. Both are composed of a collagen sponge carrier that localizes recombinant hBMP at the site of implantation, slowly releasing the GF over time. Infuse is clinically used in the treatment of degenerative disc diseases, while OP-1 is used for the treatment of long bone nonunion fractures and for revision posterolateral lumbar spine fusion.[10,11] Because the BMPs were simply adsorbed to the collagen sponge through weak interactions (eg, electrostatic and van der Waals forces) and the short-life of this GF in the body, the localization and presentation of BMPs in the implantation site for a sufficient time to induce human osteogenesis were only achieved through the use of supraphysiological amounts of the GF by these 2 medical devices.[21] The use of such large amounts of BMPs is known to be associated with several adverse effects and also raises new risks. Zara et al[36] showed that the use of high BMP-2 concentrations leads to the formation of cyst-like bony shells filled with adipose tissue in a rat femoral segmental defect model. In the same study, the use of high BMP-2 concentrations also resulted in significant tissue inflammatory infiltrates in a rat traumatic femoral onlay model. Several other studies have also reported that the "off-label" use of BMPs/Infuse has led to severe adverse effects, such as heterotopic ossification, osteolysis, infections, neurological complications, and cancer.[11] In our study, the Polytape functionalized with the BMP-2 binding peptide led to the capture of endogenous BMP-2, which promotes bone healing inside the tunnels in an ACL reconstruction model, and this may in turn lead to better graft-to-bone incorporation. The problem of tunnel widening may be solved as well. This was achieved without the need to add exogenous BMP-2. Our findings highlight the potential of using this peptide sequence in the design/improvement of medical devices to deliver and/or capture hBMP-2 to/from the surrounding tissue. This development would allow a longer retention time of the GF in the biomaterial, which could potentially reduce the amount of exogenous GF required to successfully repair the damaged tissue. The use of synthetic peptides in medicine and tissue engineering offers several advantages when compared with the use of proteins. The biggest advantage is the production and purification method. Synthetic peptides are mainly produced through chemical synthesis, while recombinant molecules are produced by biological systems such as bacteria, yeast, cells, and/or animals.[37] The use of chemical synthesis, such as solid-phase peptide synthesis, offers higher productivity, reproducibility, decreased costs, and no concern regarding virus/disease transmission when compared with biological systems to produce proteins.[4,16,37] In terms of regulation, the FDA defines that synthetic peptides, with a length up to 100 amino acids, are treated as a conventional chemical medicinal product. The European Medicines Agency (EMA) does not make such a classification, and peptides are only treated as biological products if they are produced/extracted from natural sources or recombinantly produced.[36] A BMP-2 binding peptide can nowadays be purchased for US$4.6/mg, while the costs of BMP-2 and an anti-human BMP-2 antibody are US$3070/mg and US$1350/mg, respectively. The use of a synthetic BMP-2 binding peptide over BMP-2 would minimize the risk of adverse effects associated with the use of BMP-2, leading to more efficient, robust, and safer therapy for the patient. Also, the risk associated with virus/disease transmission would be minimized by using the synthetic peptide, and the overall costs of associated therapy are drastically decreased.

CONCLUSION

Here, we describe a strategy to immobilize hBMP-2 via the interaction with a BMP-2 binding peptide. Our in vitro studies showed that immobilized hBMP-2 activated the BMP signaling cascade through the Smad-dependent pathway and supported the osteogenic differentiation of C2C12. The subsequent in vivo results showed that the BMP-2 binding peptide captured endogenous BMP-2, which led to an improvement in bone healing in a rat ACL reconstruction model. Further studies can be performed in larger animal models to confirm the results. This opens realistic possibilities for improved outcomes using current surgical techniques and biomaterial-based innovative GF capture technology that uses patients' own factors to improve healing after ACL reconstruction.

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