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TGF-β1 activation in human hamstring cells through growth factor binding peptides on polycaprolactone surfaces

J. Crispim [a,b], H.A.M. Fernandes [c], S.C. Fu [d,e,1], Y.W. Lee [d,e,1], P. Jonkheijm [b,f,*], D.B.F. Saris [a,g,*]

[a] Department of Developmental Bioengineering, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Drienerlolaan 5, 7500 AE Enschede, The Netherlands
[b] Bioinspired Molecular Engineering Laboratory of the MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Hallenweg 15, 7522 NB Enschede, The Netherlands
[c] Center for Neuroscience and Cell Biology, Stem Cells and Drug Screening, University of Coimbra, Coimbra, Rua Larga, Faculty of Medicine, Pólo I, 1st floor, 3004-504 Coimbra, Portugal
[d] Department of Orthopaedics and Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China
[e] Lui Che Woo Institute of Innovative Medicine, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China
[f] Molecular Nanofabrication Group of the MESA+ Institute for Nanotechnology, University of Twente, Hallenweg 15, 7522 NB Enschede, The Netherlands
[g] Department of Orthopaedics, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

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ABSTRACT

The administration of soluble growth factors (GFs) to injured tendons and ligaments (T/L) is known to promote and enhance the healing process. However, the administration of GFs is a complex, expensive and heavily-regulated process and only achieved by employing supraphysiological GF concentrations. In addition, for proper healing, specific and spatial immobilization of the GFs (s) is critical. We hypothesized that biomaterials functionalized with GF-binding peptides can be employed to capture endogenous GFs in a spatially-controlled manner, thus overcoming the need for the exogenous administration of supraphysiological doses of GFs. Here we demonstrate that the modification of films of polycaprolactone (PCL) with transforming growth factor β1 (TGF-β1)-binding peptides allows GFs to be captured and presented to the target cells. Moreover, using a TGF-β reporter cell line and immunocytochemistry, we show that the GFs retained their biological activity. In human primary tendon cells, the immobilized TGF-β1 activated TGF-β target genes ultimately lead to a 2.5-fold increase in total collagen matrix production. *In vivo* implantation in rats clearly shows an accumulation of TGF-β1 on the polymer films functionalized with the TGF-β1-binding peptide when compared with the native films. This accumulation leads to an increase in the recruitment of inflammatory cells at day 3 and an increase in the fibrogenic response and vascularization around the implant at day 7. The results herein presented will endow current and future medical devices with novel biological properties and by doing so will accelerate T/L healing.

Statement of Significance

Our study describes the possibility to deliver hTGF-β1 to human derived hamstring cells using a non-covalent bioactive strategy. The significance of our results *in vivo* with our functionalized biomaterial with TGF-β1-binding peptides lies in the fact that these materials can now be employed to capture endogenous TGF-β1 in a spatially-controlled manner, overcoming the need for exogenous administration of supra-physiological TGF-β1 doses. Our method is different from current solutions that rely on global TGF-β1 administration, soaking the devices with TGF-β1, etc. Therefore we believe that our method is a significant change from current state-of-the-art in the types of devices that are used for ligament/tendon repair and that following our method can endow current and future medical devices with TGF-β1 binding properties.

* Corresponding authors at: Bioinspired Molecular Engineering Laboratory of the MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Hallenweg 15, 7522 NB Enschede, The Netherlands (P. Jonkheijm). Department of Orthopaedics, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands (D.B.F. Saris).

E-mail addresses: p.jonkheijm@utwente.nl (P. Jonkheijm), d.b.f.saris@utwente.nl (D.B.F. Saris).

[1] Rm 74034, 5/F/Lui Che Woo Clinical Science Building, Prince of Wales Hospital, Shatin, Hong Kong, China.

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1. Introduction

The anterior cruciate ligament (ACL) is one of the most commonly-injured ligaments in the knee, with a reconstruction rate of 42.7 per 100,000 capita in the United States in 2006 [1]. The highest reconstruction rate is reported in Australia with an incidence of 52.0 per 100,000 in Australia [2], while other countries had a reconstruction rate of 38 (Denmark), 37.8 (New Zealand), 34 (Norway) and 32 (Sweden) per 100,000 capita [3–6]. Due to its low self-regenerative capacity, surgical intervention is often needed in order to re-establish the biomechanical properties of the injured tissue. Auto- or allografts are used for ACL reconstruction; however, donor-site morbidity, pain, graft failure and risk of disease transmission are common problems associated with the procedure [7]. Moreover, in most cases, the biomechanical properties of the grafted tissue do not match the ACL's original properties, leading to maladaptive joint issues. Recent years have shown an increase in the number of publications exploring the potential of administrating GFs to promote tissue healing; therefore, the potential of GF administration to promote and enhance the healing process of damaged ACL is a realistic opportunity for improving the clinical outcome. GFs are key players in the wound healing cascade, orchestrating, in a temporal and spatial manner, cellular mechanisms crucial for the proper healing of the tissue, such as cell signalling, proliferation, migration, survival and differentiation [8,9]. The easiest and simplest mechanism to deliver GFs is in a soluble form. However, due to the low stability of GFs in the body and diffusion-related problems, supraphysiological concentrations and systematic administration are required in order to achieve the desired effect [9]. In order to overcome these problems, GFs have been immobilized onto biomaterials in order to avoid diffusion-related issues and achieve co-localization of the GFs in the wounded tissue. GFs can be immobilized in a covalent and non-covalent manner, via direct electrostatic means or by reactions between the GF and matrices, or interactions via other biological molecules such as heparin, gelatin or fibronectin [8,10]. The simplest strategy to immobilize GFs on biomaterials is through adsorption interactions, however, this is usually associated with an initial burst release of the GFs and therefore the requirement of high concentrations of GF in order to achieve long-term effects [10]. The immobilization of GFs through the formation of a covalent bond with the biomaterial or non-covalent interactions with affinity molecules (e.g., heparin) can achieve a sustained release of the GFs [10]. Nevertheless, the formation of this covalent bond often hinders the bioactivity of the GF, mostly due to conformational changes, ultimately affecting its biological activity [8]. Additionally, permanent immobilization of the GFs through a covalent bond will present the factor to the cells in a non-natural immobile way. In contrast, the non-covalent presentation of GFs is a more natural way to deliver these to the injured tissue. However, heparin-like structures do not offer any specificity towards the GFs and consequently other circulating GFs could be immobilized by these structures. One particular interesting GF is TGF-β1 since it is active during all stages of the T/L healing process [11]. Its expression levels increase during the inflammatory stage, immediately after the injury and thought to play an important role during this stage [12]. This GF is produced and released by platelets, macrophages, fibroblasts and other cell types [13–15]. TGF-β induces the recruitment of cells during the inflammatory stage, stimulates the synthesis of ECM components and type III collagen during the repair stage and, during the remodeling stage, is involved in the termination of cell proliferation and induction of type I collagen synthesis and secretion [16,17]. It has been shown in canines that the exogenous administration of transforming beta 1 (TGF-β1) significantly increases the bonding strength of the graft that was used to replace the original ACL [18]. In a rabbit model of patellar tendon injury, it was shown that the administration of TGF-β1 directly following wound closure increases the tangent modulus and the tensile strength of the regenerated fibrous tissue [19]. It has previously been shown that supramolecular nanofibers functionalized with a TGF-β1-binding peptide promote cartilage regeneration when compared with the non-functionalized nanofibers [20]. The use of short peptides to deliver GFs has several advantages over other GF delivery strategies, such as their easy and rapid synthesis using standard chemical peptide synthesis and purification using standard chromatography methods. GF-binding peptides overcome the disadvantages related to the previously-mentioned delivery strategies since the affinity towards the GFs is based on selective non-covalent interactions. In addition, the therapeutic use of synthetic peptides is currently a growing market with around 100 products on the market in the USA, Europe and Japan [21]. In fact, this sector has grown from 1 synthetic peptide per year entering clinical trials in 1970 to 20 candidates per year nowadays.

We hypothesized that functionalization of biomaterials with GF-binding peptides could capture the endogenous GFs, leading to their accumulation and consequently enhancing the healing process without having to administrate exogenous GFs. Here we present for the first time a non-covalent approach to specifically immobilize TGF-β1 (hTGF-β1) on polymer films and deliver hTGF-β1 to human-derived hamstring cells and thus promote T/L healing. By functionalizing PCL films with a TGF-β1-binding peptide, we were able to specifically immobilize hTGF-β1. Subsequently, we show *in vitro* that the functionalized films lead to an upregulation of ECM-related genes such as collagen type I and III, culminating in the enhanced production of collagen by human hamstring-derived cells. When implanted subcutaneously in rats, the films functionalized with TGF-β1-binding peptide capture more endogenous TGF-β1 than the control films, ultimately contributing to enhance the fibrogenic response and vascularization around the implant. These results demonstrate the potential of using this synthetic peptide sequence to capture and accumulate native TGF-β1 onto biomaterials in order to promote the healing of damaged tissues without the need to administer exogenous GFs.

2. Materials and methods

2.1. Materials

N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) was obtained from MultiSynTech. Chloroform and 1-methyl-2-pyrrolidinone (NMP) were purchased from WR Chemicals. NaOH was obtained from Riedel-de Haën. All other reagents or products were purchased from Sigma-Aldrich unless noted otherwise.

2.2. Peptide synthesis and purification

The synthesis of the peptide sequences was performed using standard Fmoc-solid phase peptide synthesis in a Syro II MultiSynTech automated peptide synthesizer. The TGF-β1-binding and nonbinding peptides with sequences KGLPLGNSH and KGHNLGLPS, respectively, were prepared on Fmoc-Rink 4-methylbenzhydrylamine (MBHA) resin (MultiSynTech GmBH, 50 mg scale, substitution 0.52 mmol/g), using 0.26 M HBTU, 0.52 M of *N,N*-diisopropylethylamine (DIPEA), 2 M of piperidine and 0.29 M of each amino acid. The N-termini of the final peptide sequences were manually acetylated in 16% acetic anhydride, 30% DIPEA and 54% NMP for one hour at room temperature.

The peptides were cleaved from the resin and amino acid side groups were deprotected using 95% trifluoroacetic acid, 2.5% triisopropylsilane and 2.5% milliQ water. The peptides were then collected by precipitation in cold diethyl ether and the organic solvents were removed in a rotatory evaporator. The peptides were redissolved in milliQ water and lyophilized overnight. The resulting products were purified using standard preparative HPLC methods. MS (ESI): $m/z = 964.1$ [M+H] (calculated 963.1 for $C_{42}H_{70}N_{14}O_{12}$) for KGLPLGNSH. MS (ESI): $m/z = 964.6$ [M+H] (calculated 963.1 for $C_{42}H_{70}N_{14}O_{12}$) for KGHNLGLPS.

2.3. Preparation of peptide displaying PCL films

A 12.5% (w/v) solution of PCL (average M_n 45,000) in chloroform was prepared and homogenized by sonication. When the solution was completely homogeneous, PCL films were prepared by casting in a petri dish, pre-silanized with a PFDTS (1H,1H,2H,2H-perfluoro decyltrichlorosilane, ⩾97%, ABCR GmbH) anti-sticky layer. Upon solvent evaporation, the polymer was melted at 100 °C during 10 min and allowed to re-solidify. The polymer was then cut in circular films with a diameter of 21 mm in order to fit inside the wells of a 12-well plate. The individual circular films were extensively washed with demi-water and milliQ water and dried with a N_2 stream. The dried films were exposed to oxygen plasma for 5 min (at an oxygen pressure of 1.0 bar, a vacuum pressure of 200 mbar and a current of 40 A) and subsequently immersed in a 1 M NaOH solution for one hour with gentle agitation. PCL films were then washed and dried as mentioned above, and incubated with a solution of 50 mM 1:1 NHS/EDC in MES buffer for one hour with agitation. PCL films were washed and dried again as mentioned above and were incubated with 1 mM of the peptide in phosphate buffered saline (PBS) for 4 h with agitation. Films were then washed extensively with PBS and sterilized by incubating the films overnight in a solution of 10% penicillin/streptomycin (Life Technologies) in PBS prior to cell seeding

2.4. Water contact angle measurements

The wettability of the PCL films was determined by a drop contact angle system (Krüss Contact Angle Measuring System G10). The contact angle was measured and calculated using Drop Analysis software. All reported contact angles are the average of n = 6 measurements. MilliQ water was used to measure the contact angle of the films.

2.5. XPS measurements

XPS (X-ray photoelectron spectroscopy) spectra were measured using a Quantera scanning X-ray multiprobe instrument (Physical Electronics), equipped with a monochromatic Al Kα X-ray source operated at 1486.6 eV and 55 W. Spectra were referenced to the main aliphatic C 1 s peak set at 284.8 eV. The X-ray beam size was 200 μm and the data were collected from surface areas of 100 μm × 300 μm with a pass energy of 224 eV and a step energy of 0.8 eV for survey scans, at a detector input angle of 45°. Measurements were collected after three scanning cycles. Charge neutralization was achieved by low-energy electrons and low-energy argon ions.

2.6. Tensile testing

For tensile testing, samples with length of 41 mm and width of 4 mm were punched out and extracted from the PCL films. The mechanical properties of the PCL films were determined at room temperature using a Zwick Z020 tensile tester (Germany) with a 500 N load cell. The initial grip to grip separation was 30 mm and the crosshead speed used was 50 mm/min. All measurements were conducted in triplicate. The physical properties were extracted automatically from the stress-strain curves using the Instron software.

2.7. TGF-β1 binding and immunofluorescence

The PCL films were incubated with 1 μg/mL of hTGF-β1 (PeproTech) in 4 mM hydrochloric acid (HCl) containing 1 mg/mL bovine serum albumin (BSA) for one hour with gentle agitation. The films were then washed for 10 min three times with 1 mM phosphate buffered saline tween-20 (PBST) and then with PBS alone for a further 10 min. Next, the films were blocked for one hour with PBS containing 1% (w/v) BSA and subsequently washed as described above. Afterwards, the films were incubated with a 5 μg/mL solution of the primary antibody (mouse monoclonal anti-human TGF-β1, R&D systems) in blocking solution for one hour with agitation. The films were washed as mentioned above and then incubated with a 4 μg/mL solution of the secondary antibody (goat anti-mouse Alexa Fluor 546, Invitrogen) in PBS containing 1% w/v BSA for one hour with gentle agitation. Prior to fluorescence microscopy, the films were washed for 10 min three times with 1 mM PBST, rinsed three times with PBS and dried under a N_2 stream. For cell experiments, the sterile films were washed three times with PBS and incubated with hTGF-β1 in sterile 4 mM HCl containing 1 mg/mL BSA for one hour with gentle agitation. Subsequently, the films were extensively washed with PBST and PBS to remove any traces of the washing buffer prior to cell seeding.

2.8. In vitro quantification of bound TGF-β1

Bound hTGF-β1 was quantified by incubating the films with 1 μg/mL of TGF-β1 in 4 mM HCl containing 1 mg/mL BSA for one hour with gentle agitation. The supernatant was collected and the films were washed for 30 min with PBST (0.1% (v/v)). The buffer was then collected and mixed with the previously-collected supernatant. The collected solutions were analysed for unbound hTGF-β1 by an *anti*-human TGF-β1 ELISA kit (Abcam AB100647), according to the manufacturer's instructions. The amount of immobilized hTGF-β1 was calculated based on the difference between the incubation solution and the unbound hTGF-β1 quantified by the ELISA kit.

2.9. Cell culture

Mink Lung Epithelium Cells (MLEC – a kind gift from Daniel's Rifkin lab) were expanded in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Gaithersburg, MD) supplemented with 10% Fetal Bovine Serum (FBS, 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 sub-culturing or cryopreservation on reaching near confluence. This cell line, initially described by Abe et al. (1994), expresses luciferase under the control of a TGF target gene (Plasminogen activator inhibitor 1 – PAI-1 promoter) [22].

Hamstring cells (HT22, P3-4) were isolated using an outgrowth procedure, as previously described [23], and cultured in α-minimal essential medium (αMEM, Life Technologies) with 10% FBS (Gibco, Life Technologies), 100 U/mL penicillin, 100 μg/ml streptomycin and 0,2 mM L-ascorbic acid-2-phosphate magnesium salt (ascorbic acid, 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 sub-culturing or

cryopreservation on reaching near confluence. Experiments with hamstring cells were performed with cells until passage 4.

2.10. Luciferase assay

MLECs were seeded at 64,000 cells/cm^2 and allowed to attach overnight at 37 °C in a 5% CO_2 incubator. The medium was then replaced by DMEM without FBS and the cells were incubated for an additional period of 24 h. Cells were lysed and the luciferase quantified according to the manufacturer's protocol (Promega, E4530). Luciferase values were normalized for DNA content quantified by CyQUANT cell proliferation assay (Invitrogen).

2.11. Smad translocation assay

To assess the cellular localization of the Smad2/3 complex, hamstring cells were seeded at 10,000 cells/cm^2 and incubated for 24 h at 37 °C in a 5% CO_2 incubator. Samples were washed with PBS and cells were fixed with 4% (w/v) paraformaldehyde/PBS for 15 min at room temperature. Samples were washed with PBS and incubated with a filtered solution of 0,3% (w/v) Sudan Black in 70% ethanol for 30 min with gentle agitation. Films were washed three times for 5 min with PBS and the cell membrane was permeabilized with 0.1% Triton X-100/PBS for 15 min. After rinsing three times with PBS, films were blocked with a solution of 2% (w/v) BSA in 0.1% Triton X-100 in PBS at room temperature for one hour with gentle agitation. Monoclonal mouse anti-Smad2/3 (clone 18, BD Bioscience, 1:200) was incubated overnight at 4 °C in the blocking solution with gentle agitation. The secondary antibody goat anti-mouse Alexa Fluor 594 (DAKO, 1:200) was incubated at room temperature for one hour in the blocking solution. Nucleic acids were stained with DAPI (Life Technologies, 1:100) for 15 min at room temperature. Samples were washed three times for 5 min with 0.1% Triton X-100/PBS, rinsed with PBS and dried with a N_2 stream before mounting.

2.12. Gene expression analysis

For gene expression analysis, hamstring cells were seeded on films at 5000 cells/cm^2 and cultured for 3, 7 and 14 days in culturing medium. RNA was then isolated using TRIzol combined with a NucleoSpin RNA II kit (Bioke). Subsequently, 1 μg of RNA was used to synthesize cDNA using the SensiFast kit (Bioline). iQ SYBR Green Supermix (Bio-Rad) was used for quantitative polymerase chain reaction (qPCR) on a MJ Mini™ thermal cycler (Bio-Rad). Gene expression was normalized using the housekeeping gene B2M. The primer sequences used are as follows: collagen I forward: 5′-GTC ACC CAC CGA CCA AGA AAC C-3′, reverse: 5′-AAG TCC AGG CTG TCC AGG GAT G-3′; collagen III forward: 5′-GCC AAC GTC CAC ACC AAA TT-3′, reverse: 5′-AAC ACG CAA GGC TGT GAG ACT-3′; sox9 forward: 5′-ATC CGG TGG TCC TTC TTG TG-3′, reverse: 5′-TGG GCA AGC TCT GGA GAC TTC-3′; aggrecan forward: 5′-AGG CAG CGT GAT CCT TAC C-3′, reverse: 5′-GGC CTC TCC AGT CTC ATT CTC-3′; B2∗M forward: 5′-ACA AAG TCA CAT GGT TCA CA-3′, reverse: 5′-GAC TTG TCT TTC AGC AAG GA-3′.

2.13. Collagen quantification

For the analysis of the amount of collagen produced, hamstring cells were seeded on films at 5000 cells/cm^2 and cultured for 7 and 14 days in culture medium. Hydroxyproline quantification was used as a direct method for the determination of the collagen content in the samples. Cells were washed with PBS, lysed with 12 N HCl and scratched from the films. The lysate was transferred to a pressure-tight Teflon-capped vial and hydrolysed at 120 °C for three hours. After hydrolyzation, the amount of hydroxyproline was quantified using the Hydroxyproline Colorimetric Assay Kit (BioVision) according to manufacturer's instructions.

2.14. Subcutaneous implantation mouse model

All of the animal experiments performed were approved by the animal research ethics committee of the Chinese University of Hong Kong. Eight 12-week-old Sprague Dawley male rats were used in this study. The rats were anesthetized by intraperitoneal injection of 10% ketamine/2% xylazine (Kethalar, 0.3 ml: 0.2 ml); sedation was maintained by intramuscular injection of 10% ketamine (Sigma Chemical CO, St. Louis, MO). Subcutaneous implantation of PCL was performed. In brief, once the animals were anesthetized, shaved and washed, two incisions were made and native PCL and PCL functionalized with a TGF-β1 binging peptide were inserted into the pockets and fixed to the fascia. The skin wound was then closed using sutures. At day 3 and day 7 post implantation, animals were sacrificed and samples were harvested. Samples from the subcutaneous rat model were harvested at day 7 post implantation and rinsed with PBS. Harvested samples were fixed with 10% buffered formalin for 10 min and further permeabilized with PBST for 15 min. Samples were then washed with PBS and blocked with 1% (w/v) BSA for 1 h in a shaker. Next, the samples were washed three times for 10 min with PBST and incubated with a dilution of 1:100 of the primary antibody (rabbit polyclonal anti TGF-β1, Santa Cruz Biotechnology) overnight at 4 °C. After primary antibody incubation, samples were washed with PBST and incubated with a dilution of 1:100 of the secondary antibody (goat anti rabbit IgG-PE, Santa Cruz Biotechnology) for one hour at room temperature. Samples were washed with PBST before imaging.

2.15. Histology

At day 3 and day 7 post implantation, PCL implants and their surrounding tissues were harvested and rinsed with PBS. Harvested samples were fixed with 10% buffered formalin overnight and embedded in paraffin. Subsequently, 5 μm-thick paraffin sections along the transverse plane were collected. Haematoxylin and eosin (H&E) stained sections were examined under light microscopy (Leica Microsystems, Wetzlar, Germany).

2.16. Statistical analysis

The data were analysed using a Student's paired *t*-test, a one-way analysis of variance followed by a Tukey's multiple comparison test ($p < 0.05$) or a two-way analysis of variance. The values represent the mean and standard deviation of three independent measurements.

3. Results

3.1. Fabrication of PCL films presenting TGF-β1-binding peptides

In order to immobilize hTGF-β1 on the PCL film, a previously reported TGF-β1-binding peptide sequence LPLGNSH was synthesized and equipped with an extra lysine and a glycine at the N-termini (Fig. A1) [20]. The extra glycine functioned as a spacer between the film and the active sequence involved in the affinity for hTGF-β1, whereas the extra lysine provided an amine group to covalently immobilize the peptide to the film. The N-terminus of the peptide was acetylated, and consequently the covalent immobilization of the peptide to the film was solely achieved through the free amine from the lysine side group.

To covalently bind the peptide to the film, the native PCL films were initially treated with oxygen plasma to introduce carboxylic

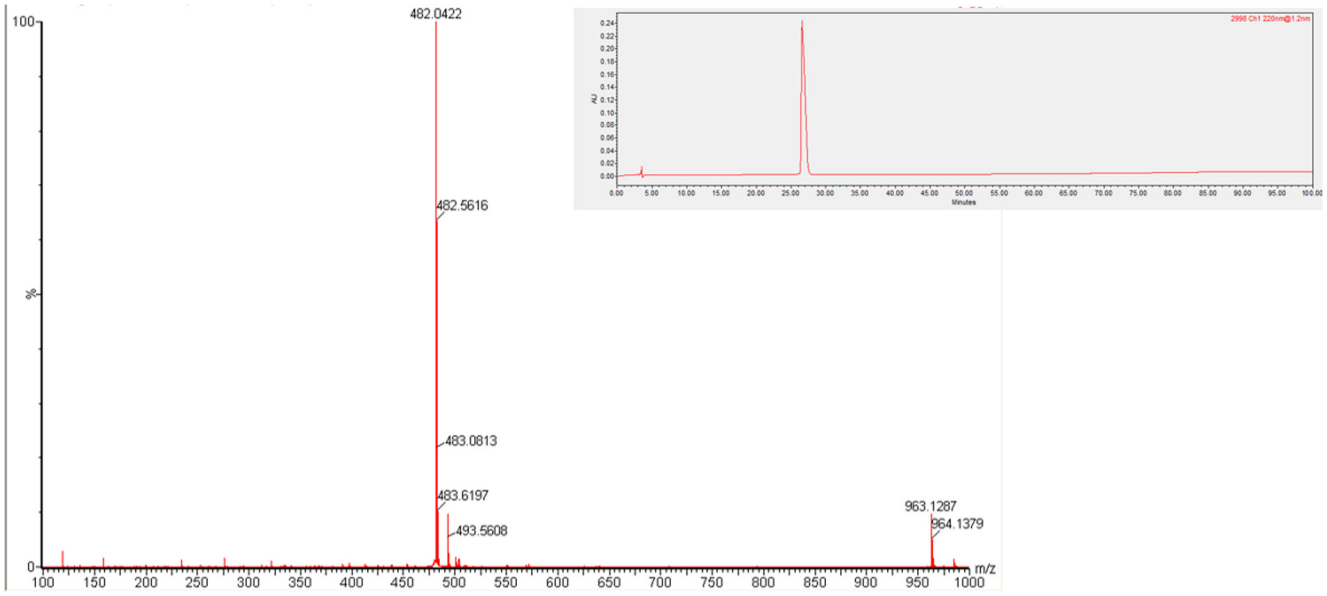

Fig. A1. Mass spectrometry of the TGF-β1-binding peptide after purification and respective analytical HPLC.

acid groups to the surface. Next, the carboxylic acids were activated with NHS/EDC treatment, leading to the formation of amine reactive esters that were used for the covalent immobilization of the TGF-β1 binding via the free amine present in the side chain of the lysine amino acid.

Surface wettability (Fig. 1) and XPS measurements (Table 1 and Fig. A3) were used to keep track of the chemical modifications during the immobilization procedure. XPS measurements of the native film showed an atomic composition close to the theoretical atomic composition for polycaprolactone. The native film also exhibited an 77 ± 2° that is consist with its hydrophobic character. After exposing the native PCL films to oxygen plasma for 5 min, the hydrophobicity of the surface decreased by 38 ± 2° ($p < 0.0001$) while the oxygen content increased by 3.6 ± 0.3% ($p < 0.0001$), whereas NHS/EDC treatment led to an increase of 19 ± 2° ($p < 0.0001$) and the appearance of nitrogen at the surface. Films with the amine reactive ester were then incubated with a PBS solution alone or with 1 mM of the TGF-β1-binding peptide in PBS. As expected, when the films with the NHS-activated acids were incubated with PBS, no significant change in the water contact angle was observed. In contrast, when the films were incubated with a solution of PBS containing the TGF-β1-binding peptide, a further increase of 10 ± 1° ($p < 0.0001$) in the contact angle and an increase of 2.1 ± 0.1% ($p < 0.0001$) in the nitrogen content of the surface was observed by XPS, indicating that the peptide was attached to the films. All films where characterized using a tensile test, and the elastic modulus, maximum force and the force at break were derived from the stress-strain curves (Fig. A2 and Table A1) and remained in the same order of magnitude.

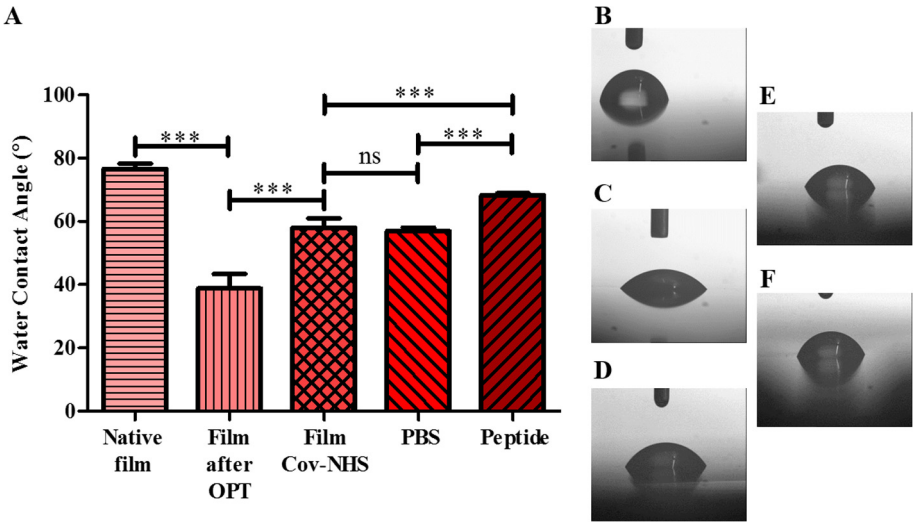

Fig. 1. (A) Water contact angle measurements of the films during the chemical functionalization procedure. (B) 'Native film' represents the PCL film without any chemical modification; (C) 'Film after OPT' is the native PCL film exposed for 5 min to oxygen plasma; (D) 'Film Cov-NHS' indicates the film with amine reactive esters after 1 h incubation with NHS/EDC; (E) 'PBS' is the film with reactive amine esters incubated for 4 h with PBS; (F) 'Peptide' is the films with reactive amine esters incubated during 4 h with a 1 mM of peptide in PBS. $^{***}p < 0.0001$ (ANOVA with Tukey's post hoc). The data represent the mean ± SD of two measurements per sample ($n = 3$).

Table 1
XPS results of carbon (C), oxygen (O), nitrogen (N) and other elements (silica and sodium) for the films during the chemical functionalization procedure and theoretical calculations with respective chemical formulas (excluding hydrogen) for PCL, NHS group and the peptide molecule. 'Native film' represents the PCL films without any chemical modification; 'Film after OPT' is the native PCL films exposed for 5 min to oxygen plasma; 'Film Cov-NHS' indicates the film with amine reactive esters after 1 h incubation with NHS/EDC; 'Peptide' is the film with reactive amine esters incubated during 4 h with a 1 mM of peptide in PBS. The data represent the mean ± SD of four measurements per sample.

Element	C	O	N	Other elements
Native film	77.0 ± 0.1	23.0 ± 0.1	–	–
Film after OPT	71.3 ± 1.7	26.6 ± 0.5	–	2.1
Film Cov-NHS	75.5 ± 0.3	23.2 ± 0.3	0.8 ± 0.2	0.5
Peptide	73.8 ± 0.5	22.6 ± 0.2	2.9 ± 0.1	0.7
Calculated for PCL (C_6O_2)	75.0	25.0	–	–
Calculated for –NHS group (C_4NO_3)	50.0	37.5	12.5	–
Calculated for peptide molecule ($C_{42}N_{14}O_{12}$)	61.8	17.6	20.6	–

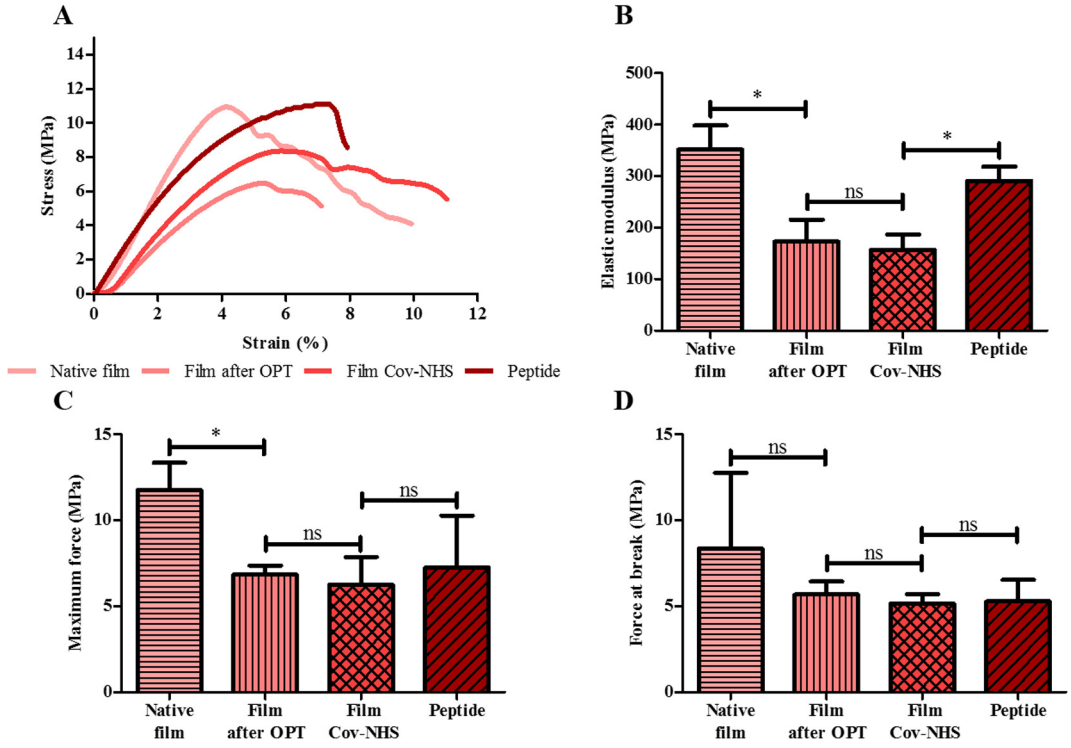

Fig. A2. (A) Stress-strain curve, (B) Elastic modulus, (C) Maximum force and (D) Force at break of different films. 'Native film' represents the PCL film without any chemical modification; 'Film after OPT' is the native PCL film exposed for 5 min to oxygen plasma; 'Film Cov-NHS' indicates the film with amine reactive esters after 1 h incubation with NHS/EDC; 'Peptide' is the film with reactive amine esters incubated during 4 h with a 1 mM of peptide in PBS. $^{*}p < 0.05$ (two-tailed unpaired *t*-test). The data is represented as the mean ± SD of triplicates (n = 3).

Table A1
Elastic modulus, maximum force and force at break of the different films. 'Native film' represents the PCL film without any chemical modification; 'Film after OPT' is the native PCL film exposed for 5 min to oxygen plasma; 'Films Cov-NHS' indicates the film with amine reactive esters after 1 h incubation with NHS/EDC; 'Peptide' is the film with reactive amine esters incubated during 4 h with a 1 mM of peptide in PBS. $^{*}p < 0.05$, $^{**}p < 0.01$ (two-tailed unpaired *t*-test). The data is represented as the mean ± SD of triplicates (n = 3).

Properties	Elastic modulus (MPa)	Maximum force (MPa)	Force at break (MPa)
Native film	351.7 ± 38.0	11.8 ± 1.3	8.4 ± 3.6
Film after OPT	172.5 ± 30.5	6.8 ± 0.4	5.7 ± 0.5
Film Cov-NHS	156.5 ± 21.5	6.3 ± 1.1	5.2 ± 0.4
Film with Peptide	291 ± 22.4	7.3 ± 2.5	5.3 ± 1.0

3.2. TGF-β1 immobilization on the functionalized PCL films

To confirm the attachment of the peptide to the films and the subsequent binding of the GF to the functionalized films, immunostaining was performed on both native and functionalized films incubated either with or without hTGF-β1. These results showed a statistically significant increase in functionalized films incubated with the hTGF-β1 (Fig. 2A; $p < 0.0001$). In addition, immunostaining demonstrated a homogenous distribution of the GF within the film (Fig. 2B and C). In addition to showing a specific interaction of hTGF-β1 with the peptide, the absence of unspecific interactions of the primary or secondary antibody with the functionalized film was also demonstrated. When native PCL films were incubated with hTGF-β1, no significant increase in the fluorescence intensity was observed, indicating that the interactions occurred between hTGF-β1 and the native films were negligible. To summarize, the fluorescence signal reported for the functionalized films is the result of the specific binding of hTGF-β1 to the TGF-β1-binding peptide attached to the films.

In order to estimate the amount of hTGF-β1 bound to the TGF-β1-binding peptide functionalized film, an ELISA assay against hTGF-β1 was performed (Fig. 3). Our results show that no GF remained on the native film after a one-hour incubation, which

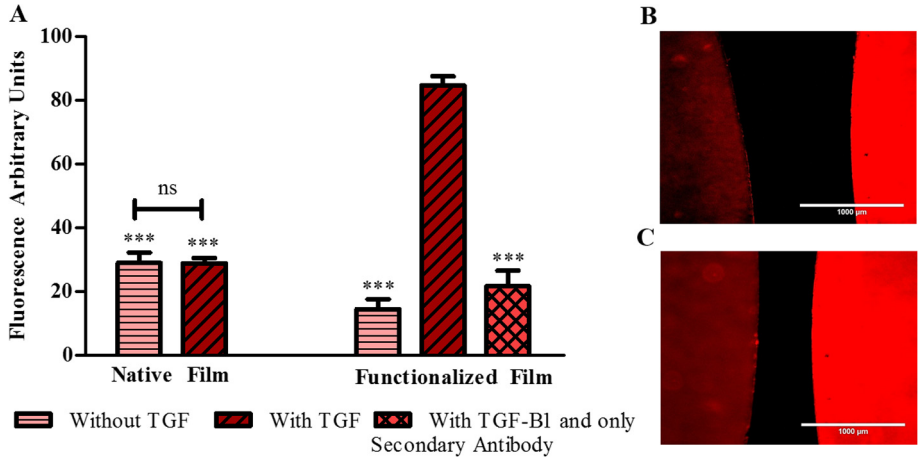

Fig. 2. (A) Fluorescence quantification of the immunochemistry assay against hTGF-β1 immobilized on the films. The data represent mean ± SD of two measurements per sample (n = 3). $^{***}p < 0.0001$ (ANOVA with Tukey's post hoc test) with functionalized films incubated with hTGF-β1 and immunochemistry performed with primary and secondary antibodies. (B) Fluorescence image of the functionalized film without hTGF-β1 incubation (left) vs functionalized film with hTGF-β1 incubation (right) (bar: 1000 µm). (C) Fluorescence image of the native film with hTGF-β1 incubation (left) vs functionalized film with hTGF-β1 incubation (right) (bar: 1000 µm).

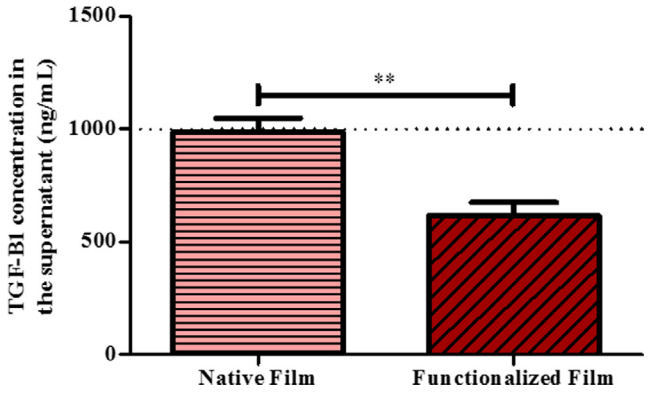

Fig. 3. Quantification of the amount of immobilized hTGF-β1 by the TGF-β1-binding peptide was analysed using an ELISA assay against hTGF-β1. $^{**}p < 0.005$ (two-tailed unpaired *t*-test) between native film and functionalized film. The dashed line represents the hTGF-β1 concentration of the incubation solution. The data are represented as mean ± SD of two measurements per sample (n = 3).

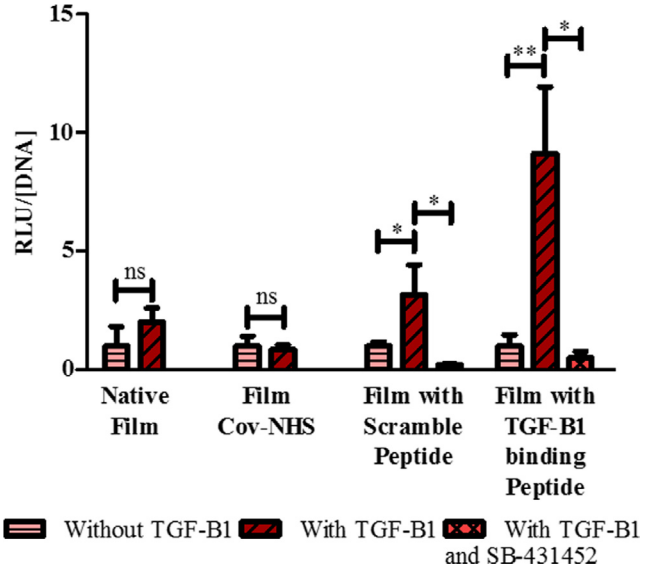

Fig. 4. Effect of immobilized hTGF-β1 on the expression of luciferase by a TGF-β reporter cell line. Relative luminescence units (RLU) were corrected for the amount of DNA and normalized to the condition without incubation of hTGF-β1 prior to cell seeding. $^{*}p < 0.05$ and $^{**}p < 0.01$ (two-tailed unpaired *t*-test). The data are represented as mean ± SD of two measurements per sample (n = 3).

is in agreement with the results from the immunostaining (Fig. 2). In contrast, 37% of the initial amount of hTGF-β1 (1 µg/mL) remained on the films functionalized with the TGF-β1-binding peptide, giving a surface concentration of 123 ± 16 ng/cm^2 of hTGF-β1 ($p < 0.005$). The ELISA assay further confirmed the results obtained in the immunostaining, indicating that it is possible to specifically immobilize hTGF-β1 on the film functionalized with a TGF-β1-binding peptide sequence.

3.3. Bioactivity of the immobilized TGF-β1 in a TGF-β1 reporter cell line

A TGF-β1 reporter cell line was used in order to assess whether the immobilized GF retained its bioactivity. The films were first incubated with a solution containing 0 or 50 ng of hTGF-β1, the cells were seeded in the absence or presence of 10 µM of a specific TGF-β1 inhibitor (SB-431452) and the amount of luciferase was quantified and normalized to the amount of DNA (Fig. 4).

When both native and Cov-NHS films were incubated with hTGF-β1 before cell seeding, no increase in the luciferase activity was observed when compared with the values obtained for the same films without hTGF-β1 incubation. However, when the films functionalized with a TGF-β1-binding peptide were incubated with hTGF-β1 before cell seeding, a 9 ± 3-fold increase in luciferase activity was observed ($p < 0.01$). In order to demonstrate that the interaction of the GF was specifically mediated by the TGF-binding peptide, a nonbinding, scrambled peptide (Fig. A4) sequence was included as a control. When these films were incubated with hTGF-β1 before cell seeding, an increase of 3 ± 1 in luciferase activity was also observed ($p < 0.05$). The inclusion of a TGF-β1 inhibitor completely suppressed the observed luciferase activity ($p < 0.05$).

3.4. Immobilized hTGF-β1 activates the TGF pathway via Smad2/3 in human hamstring cells

The bioactivity of the immobilized hTGF-β1 was evaluated in human hamstring-derived cells, which were isolated from the hamstring graft – the most commonly used graft for ACL

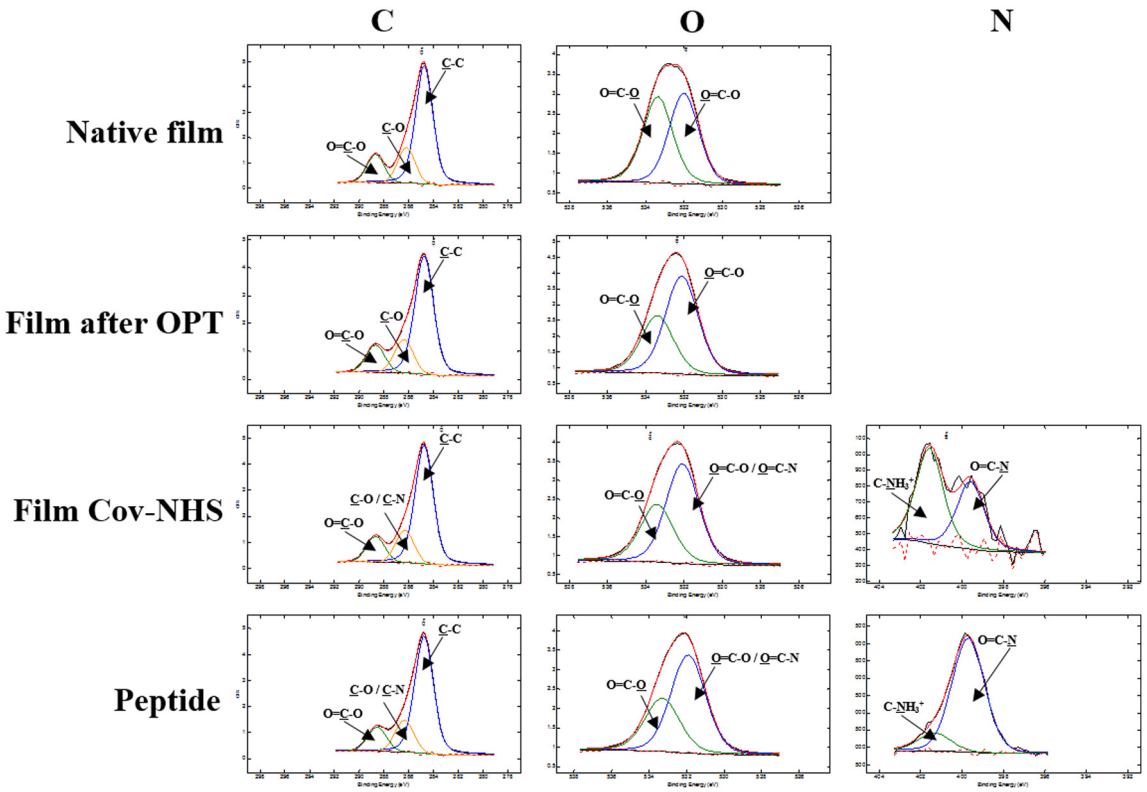

Fig. A3. XPS spectrums of carbon (C), oxygen (O) and nitrogen (N) for the films after each chemical modification. 'Native film' represents the PCL films without any chemical modification; 'Film after OPT' is the native PCL films exposed for 5 min to oxygen plasma; 'Film Cov-NHS' indicates the films with amine reactive esters after 1 h incubation with NHS/EDC; 'Peptide' is the films with reactive amine esters incubated during 4 h with a 1 mM of peptide in PBS.

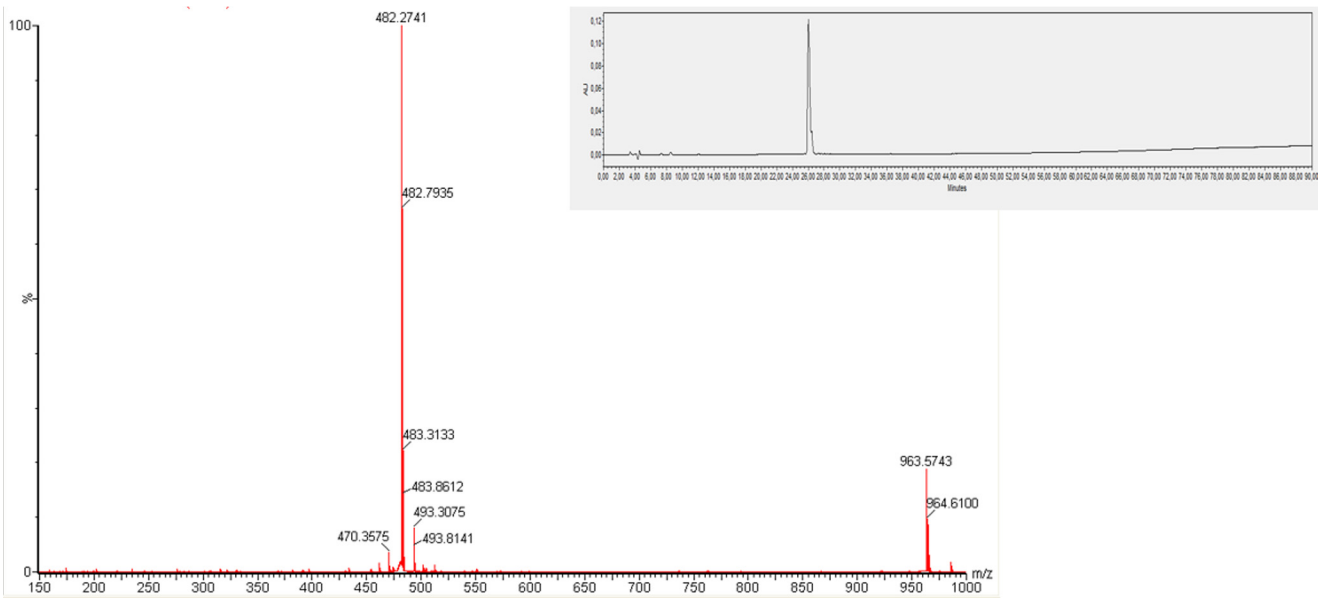

Fig. A4. Mass spectroscopy of the TGF-β1 scramble peptide after purification and respective analytical HPLC.

reconstruction. To that end, the intracellular localization of the SMAD2/3 complex, a key player in the TGF-β1 signalling pathway, was monitored (Fig. 5). Upon phosphorylation, this complex binds to SMAD4 and translocates from the cytoplasm into the nucleus, activating the transcription of TGF-β1 target genes [24]. When the cells were seeded on functionalized films without pre-incubation with hTGF-β1, the SMAD2/3 complex was found both in the cytoplasm and in the nucleus. In contrast, when the cells were seeded on the functionalized films that were previously incubated with hTGF-β1, the SMAD2/3 complex was mostly detected in the nucleus, clearly demonstrating a TGFb1-mediated translocation mechanism.

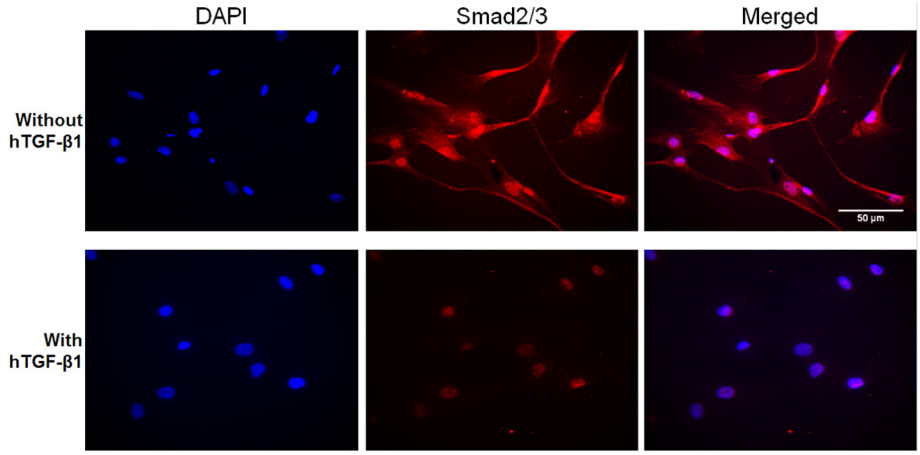

Fig. 5. Immobilized hTGF-β1 induces the translocation of Smad2/3 into the nucleus in human-derived hamstring cells. Scale bar: 50 μm.

3.5. Immobilized hTGF-β1 specifically activates TGF-β1 target genes

In tissue healing, various components of the extracellular matrix (ECM), such as collagens and proteoglycans, are responsible for the restoration of tissue homeostasis. Given that the immobilized hTGF-β1 promotes SMAD2/3 translocation to the nucleus, the expression of TGF-β1 target genes in human-derived hamstring cells was evaluated. The expression of collagen types I and III, which are the major components of T/L, and aggrecan (Acan), which is a component of ECM and Sox9 and a chondrogenic differentiation marker, were studied by qPCR (Fig. 6). Our results show that the expression of collagen type I, Acan and Sox9 are similar over time. A statistically significant upregulation was observed at day 3 for the three genes on the functionalized films with immobilized hTGF-β1. For later time points (days 7 and 14), a downregulation was observed in the expression of these genes for all conditions tested. In the case of collagen type III, an upregulation was observed in the case of functionalized films with immobilized hTGF-β1, which, in contrast to the previously-mentioned genes, was maintained over time. These data show that the immobilized hTGF-β1 is regulating the transcription of TGF-β1-target genes, as previously reported [25–28]. Interestingly, in the case of collagen type III, this activation is sustained without needing to add exogenous hTGF-β1 to the cells.

3.6. Effects of immobilized hTGF-β1 on endogenous collagen production

In order to ascertain whether the upregulation observed for collagen at the gene level was effectively leading to protein upregulation, the total amount of hydroxyproline was quantified as a direct measure of total collagen protein production (Fig. 7). No differences were observed in the amount of collagen between day 7 and day 14 for the films where no immobilized hTGF-β1 was present. In contrast, when the cells were seeded on functionalized films with immobilized hTGF-β1, a 2.5 ± 0.4-fold increase was observed after 14 days for the amount of collagen ($p < 0.01$).

3.7. TGF-β1-binding peptide captures native circulating TGF-β1 leading to its accumulation on the implanted functionalized polymer

We hypothesized that once implanted *in vivo*, the TGF-β1-binding peptide would capture and accumulate the native circulating TGF-β1 GFs that are released during the inflammatory stage and induce a more pronounced tissue response around the implant. Both native and functionalized PCL films were subcutaneously implanted in the backs of rats, facing the fascia. Each animal, with a total of three animals per time point, was implanted with both native and functionalized film. At day 3 and day 7 after implantation, samples were harvested and used for immunofluorescence against TGF-β1 and histology. Only the samples from day 7 were used for immunofluorescence against TGF-β1. Immunostaining against TGF-β1 showed a higher fluorescence signal in the films functionalized with the TGF-β1-binding peptide when compared with the native films after 7 days of implantation in all three animals (Fig. 8). This accumulation of the native TGF-β1 in the films functionalized with the TGF-β1-binding peptide led to a higher cellularity around the implant (mostly inflammatory cells) at day 3 when compared with the native films (Fig. 9A–D). At day 7, much higher amounts of inflammatory and fibroblast-like cells (indicated with yellow arrows) were observed around the functionalized films when compared with the control (Fig. 9E–H). Additionally, an increased number of blood vessels (indicated with v) were found around the functionalized PCL implant after 7 days (Fig. 9H). Overall, these results show that the TGF-β1 captured by the peptide led to a more pronounced inflammatory response, ultimately leading to the increased recruitment of inflammatory cells, the fibrogenic response and vascularization around the implant.

4. Discussion

In this work, we hypothesized that the immobilization of hTGF-β1 via the interaction with a TGF-β1-binding peptide covalently bound to PCL would allow the presentation of hTGF-β1 over time and induce a cellular response without needing to add exogenous hTGF-β1. Our study shows that it is possible to non-covalently immobilize hTGF-β1 on PCL functionalized with a TGF-β1-binding peptide, and that the immobilized hTGF-β1 retains its bioactivity by activating target genes and inducing collagen synthesis on human-derived hamstring cells *in vitro*, and promotes tissue healing *in vivo*.

TGF-β1 is known to play a key role in several cellular mechanisms during wound healing [29]. It is known that TGF-β1 is active during all stages of T/L healing, effecting the regulation of cell migration, cell proliferation, stimulation of cell-matrix interactions, collagen synthesis and the termination of cell proliferation [11]. Several studies have indicated that the administration of TGF-β1 promotes and accelerates T/L healing. Klein et al. (2002) showed that the administration of TGF-β1 significantly increased collagen I and III production in sheath fibroblasts, epitenon and endotenon tenocytes isolated from rabbit flexor tendons [30]. DesRosiers et al. (1996) demonstrated that TGF-β1 increased the

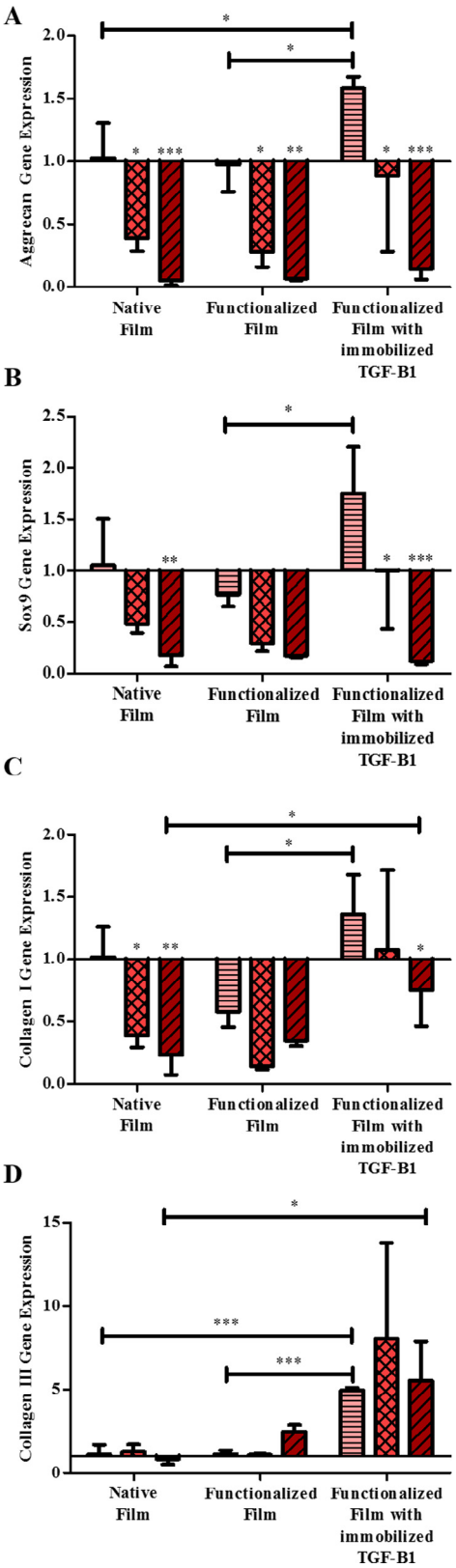

Fig. 6. Immobilized hTGF-β1 induces the expression of TGF-β1 target genes in human-derived hamstring cells. qPCR analysis of (A) aggrecan, (B) Sox9, (C) collagen type I and (D) collagen type III. All genes were normalized to B2M gene and the conditions normalized to the native film at day 3. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.0001$ with a two-way ANOVA for the analysis of gene expression over time and a one-way ANOVA with Tukey's post hoc test for the analysis of gene expression between all conditions at a particular time point. The data are represented as mean ± SD of triplicates.

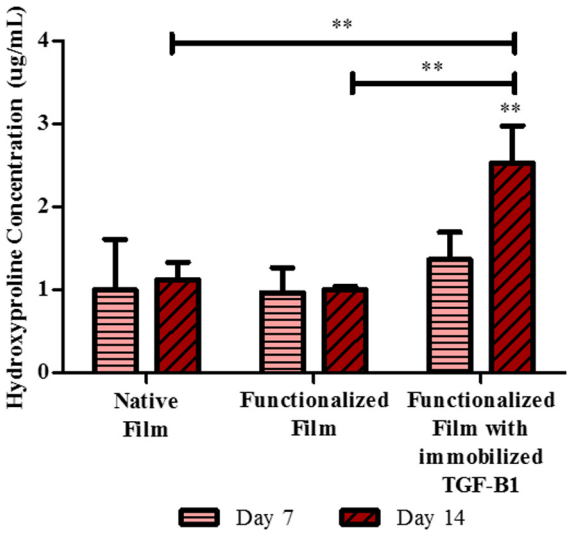

Fig. 7. Immobilized hTGF-β1 induces the synthesis of collagen in human-derived hamstring cells. The data was normalized to the condition of native film at day 7. $^{**}p < 0.01$ with a two-way ANOVA for the analysis of collagen production over time and a one-way ANOVA with Tukey's post hoc test for the analysis of collagen production between all conditions at a particular time point. The data are represented as mean ± SD of triplicates.

production of both collagen and non-collagen proteins in canine ACL fibroblasts [31]. Despite the importance of GF during wound healing, soluble administration is not an efficient strategy because it is associated with diffusion problems, low stability of the GF and, to achieve long-term effects, supraphysiological concentrations and systematic administration are often needed. In order to overcome these problems, GF incorporation onto biomaterials can be an alternative to locally delivering the desired GFs in a sustained approach. However, no study has yet been performed assessing the effects of immobilized TGF-β1 in T/L repair. Herein, we described a system to non-covalently deliver hTGF-β1 and thus accelerate T/L healing. We chose PCL, a biodegradable and FDA-approved polymer, and functionalized it with a previously reported TGF-β1-binding peptide in order to immobilize hTGF-β1 in the polymer.

We first synthetized the TGF-β1-binding peptide with sequence KGLPLGNSH and acetylated the N-termini. The fact that the covalent immobilization of the TGF-β1-binding peptide only occurs through the amine group from the side chain of the lysine amino acid allowed us to achieve a homogeneous orientation of the peptide on the film and consequently of the GFs. This overcame problems related to the heterogeneous presentation of the GFs to the cells, which is a major disadvantage of the methods commonly employed for the immobilization of GF. The wettability and XPS measurements of the films were used to assess the chemical modifications during the immobilization procedure. The water contact angle for native PCL films was 77 ± 2°, consistent with the hydrophobic character of the polymer due to the presence of hydrophobic aliphatic hexane units [32]. XPS measurements of this film showed an atomic composition of 77 ± 0.1% of carbon and 23 ± 0.1% of oxygen which is similar to the theoretical atomic composition for PCL. After 5 min of oxygen plasma treatment, the oxygen content of the film increased to 26.6 ± 0.3% presumably due to the appearance of carboxylic and hydroxyl groups on the film. This change on the oxygen content at the surface led to a decrease of the hydrophobicity to 39 ± 5°, which is in agreement with results reported in the literature [33]. Treatment with NHS/EDC led to the appearance for the first time of nitrogen at the surface due to the immobilization of NHS groups on the films. Replacement of the hydrophilic carboxylic groups by a more hydrophobic ester

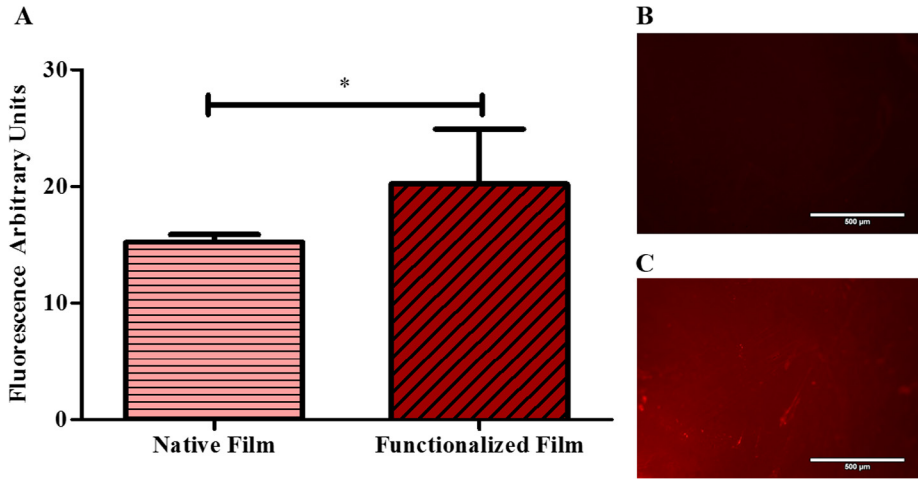

Fig. 8. (A) Fluorescence quantification of the immunochemistry assay against TGF-β1 immobilized on implanted films. The data represent mean ± SD of five measurements per sample (n = 3). $^{*}p < 0.05$ (two-tailed unpaired *t*-test). (B) Fluorescence image of the native film implanted subcutaneously in rats. (C) Fluorescence image of the film functionalized with TGF-β1-binding peptide implanted subcutaneously in rats.

group led to an increase to 58 ± 3° in the contact angle of the film, consistent with work previously reported [34]. After incubation of the films containing the amine reactive esters with PBS, no change in the wettability of the film was reported. This was expected since no chemical modification of the film occurred. In contrast, when the films were incubated with the TGF-β1-binding peptide in PBS for 4 h, an increase in the hydrophobicity of the film was reported. This increase in hydrophobicity could be explained by the presence of hydrophobic amino acids such as proline and leucine in the peptide sequence. In addition, reaction of the NHS groups with the peptide led to an increase in the nitrogen content at the surface. This change in the nitrogen content was expected due to the higher atomic fraction of nitrogen in the peptide molecule when compared with the NHS group. The chemical modifications performed at the surface of the polymer did not drastically affect the mechanical properties. The oxygen plasma treatment was the step with bigger impact in the mechanical properties, leading to a significant decrease in the elastic modulus and the maximum force. The following chemical modifications did not significantly change the mechanical properties, except in the step to covalently attach the peptide which led to a significant increase in the elastic modulus.

The TGF-β1-binding affinity of the peptide was further confirmed by immunochemistry and ELISA quantification. From the immunochemistry and ELISA, we concluded that negligible hTGF-β1 remained non-specifically immobilized on the native PCL. In contrast, incubation of the PCL functionalized with a TGF-β1-binding peptide with hTGF-β1 led to an increase in the fluorescence intensity, which was in agreement with the ELISA quantification data. These results show that the immobilization of the hTGF-β1 in the films is solely mediated by interaction with the TGF-β1-binding peptide.

The majority of the strategies used to immobilize GFs are associated with a loss of GF bioactivity. In order to confirm that the immobilized hTGF-β1 retained its bioactivity, the response of a TGF-β reporter cell line to the immobilized hTGF-β1 was studied and the intracellular localization of the SMAD2/3 complex in human-derived hamstring cells was assessed. Abe et al. (1994) described for the first time a cell line efficiently transfected with an expression construct containing a PAI-1 promoter fused to the firefly luciferase reporter gene. PAI-1 is involved in the regulation of ECM homeostasis and cell mobility, and is a target gene of TGF-β1 [35]. We hypothesized that if the immobilized TGF-β1 retained its bioactivity, it would activate the PAI-1 promoter and induce the production of luciferase, which could then be easily quantified using a luciferase assay. No production of luciferase was observed when films without a TGF-β1-binding peptide were incubated with hTGF-β1 before cell seeding. This can be explained by the fact that these films lack affinity for TGF-β1, and therefore no hTGF-β1 is available to activate the PAI-1 promotor. In contrast, when the films functionalized with a TGF-β1-binding peptide and a scramble peptide were incubated with hTGF-β1 before the cell seeding, an increase in luciferase activity was observed, reaching a maximum for the film functionalized with a TGF-β1-binding peptide. The scramble peptide may display some residual affinity towards hTGF-β1 and consequently immobilize it on the film, leading to the activation of the PAI-1 promotor. However, luciferase activity was maximal in the film functionalized with the TGF-β1-binding peptide, which can be explained by a higher affinity for hTGF-β1 when compared with the scramble peptide and consequently a higher surface concentration of TGF-β1 available to the cells. When the cells were seeded in the presence of SB-431452, a specific inhibitor of TGF-β type I receptors [36], the luciferase produced in response to the immobilized hTGF-β1 was completely suppressed. Upon binding to cell surface receptors type I and II, phosphorylation of the type I receptor by the type II receptor kinases occurs [24]. The activated type I receptor phosphorylates Smad2 and Smad3, which then form a complex with Smad [24]. The entire complex translocates into the nucleus where they regulate gene expression [24]. Since the hamstring tendon is one of the most common grafts used for ACL reconstruction, we assessed whether the immobilized TGF-β1 was activating the TGF-β pathway by following the intracellular localization of the SMAD2/3 complex in human-derived hamstring cells. In fact, when these cells were cultured on functionalized films with immobilized hTGF-β1, the SMAD2/3 complex was found in the nucleus, clearly showing activation of the pathway. These results are in agreement with the luciferase assays, showing that the immobilized GFs are bioactive and capable of activating the TGF-β1 pathway through the Smad signalling mechanism.

Upon confirmation of the bioactivity of the immobilized TGF-β1, we assessed the expression of known TGF-β1 target genes in human-derived hamstring cells. Our qPCR results showed an upregulation of both aggrecan and Sox9 in the presence of immobilized hTGF-β1 at day 3, with a downregulation at later time points reaching the values of the films without immobilized hTGF-β1. Lorda-Diez et al. (2009) analysed the effects of TGF-β supplementation in developing limb mesenchymal micromass cultures to study the role of TGF-β signalling in chondrogenesis and

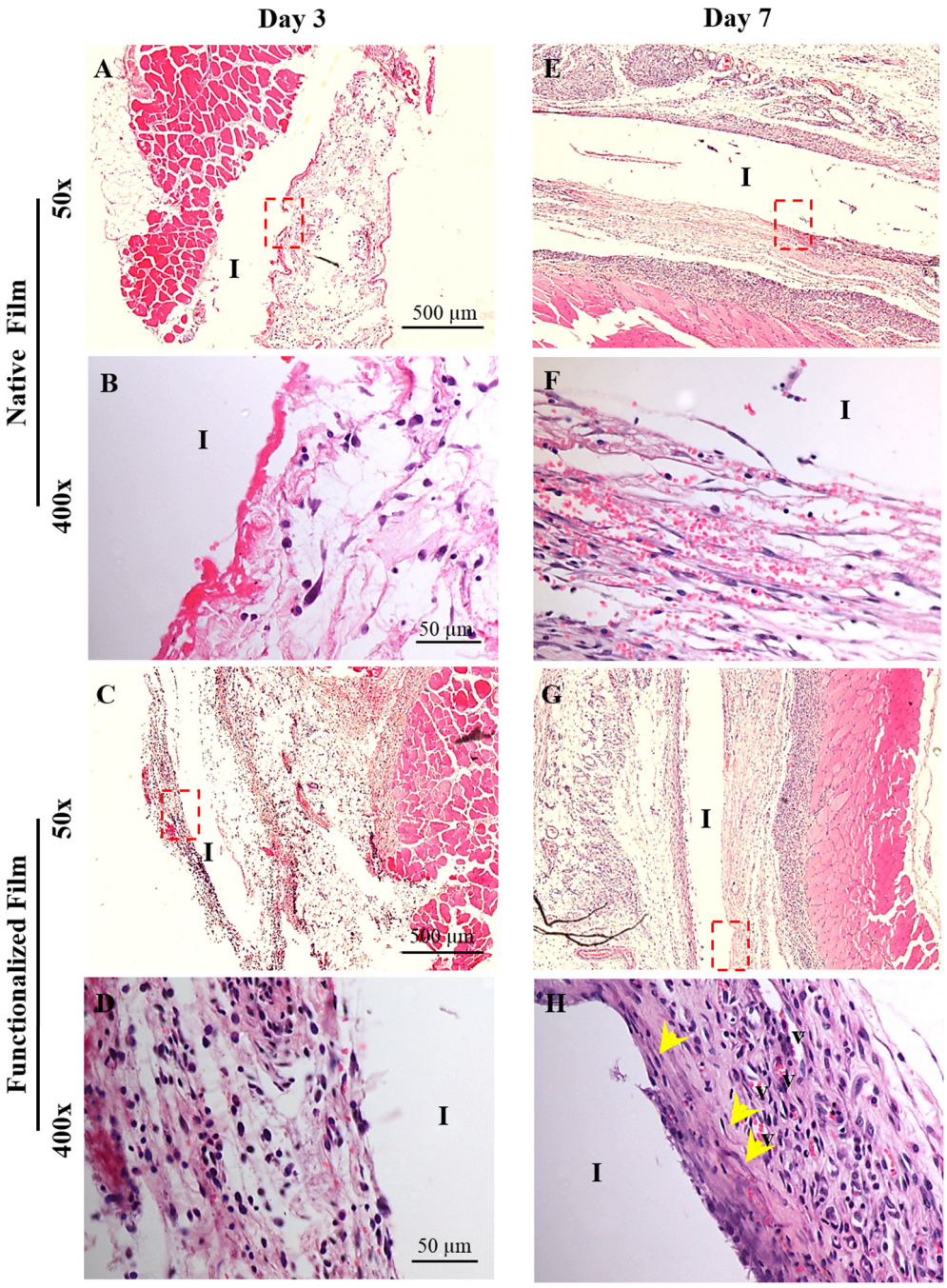

Fig. 9. Histological evaluation of sample sections after 3 (A–D) and 7 (E–H) days of implantation. Haematoxylin and eosin staining on native films (A, B, E and F) and functionalized films (C, G, D and H). Implant (I), blood vessels (v), fibroblast-like cells (yellow arrows), magnified area (red dashed box) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

fibrogenesis [37]. It was shown that the addition of TGF-β to the cultures downregulated the expression of aggrecan and Sox9 while upregulating the expression of scleraxis and tenomodulin. It was also shown that blocking Smad with SB-431542 restores the chondrogenic potential of the cultures treated with TGF-βs. Our results show that the immobilized hTGF-β1 does not induce chondrogenic differentiation in hamstring cells, which could lead to the formation of undesired ectopic cartilage in T/L reconstruction. For collagen types I and III, an upregulation at day 3 was also observed in the presence of immobilized hTGF-β1. However, for collagen type I, a downregulation was observed at later time points, while in the case of collagen type III, the expression was sustained over time. Klein et al. (2002) reported an upregulation in the expression of type I and III collagen at the protein level in rabbit tendon cells upon exposure to any TGF-β isoform [30]. Although collagen type I is the major constituent of T/L, the downregulation observed at later is formed from collagen type I [38]. While not being a major component of T/L, collagen type III is crucial for tissue time points for collagen type I in the presence of immobilized hTGF-β1 may be beneficial for the wound healing, since mature scar tissue healing due to its ability to rapidly crosslink and stabilize the repair site [39]. One study showed that subcutaneous wounds in collagen type III-deficient mice had significantly more scar tissue area at 21 days post wound compared to wild-type mice [40].

In conclusion, the downregulation of collagen type I observed over time in the presence of immobilized TGF-β1 may reduce the formation of scar tissue, which would compromise the biomechanical properties of the tissue, whereas the sustained expression of collagen type III could help in stabilizing the wound site and promote graft-to-bone integration. From the hydroxyproline quantification, we confirmed that the upregulation of collagen gene levels in response to the immobilized hTGF-β1 was being translated to a higher content of collagen protein.

Despite the known role of GFs in the healing process, only BMP-2, BMP-7 and platelet derived growth factor BB (PDGF-BB) are currently FDA approved to be used in clinical situations [41,42]. The unknown effect of the administration of GFs, a lack of suitable delivery vehicles that lower the amount of GFs needed and the costs associated with these therapies are all drawbacks that must be optimized in order to implement an efficient and safe therapy based on these proteins and therefore get approval from the medical authorities. Several groups tried to engineer biomaterials to sequester endogenous GFs and promote cellular responses, without the need to administer exogenous GFs. Hudalla et al. (2011) modified self-assembly monolayers (SAMs) with a heparin-binding peptide [43,44]. These modified (SAMs) captured the heparin and soluble GFs present in serum. These substrates enhanced human mesenchymal stem cell (hMSC) proliferation by amplifying the signalling of endogenous basic fibroblast growth factor (bFGF) and osteogenic differentiation of hMSC by enhancing endogenous BMP signalling. These cellular responses were observed in media supplemented only with serum, suggesting that capture of endogenous GFs may overcome the need of supraphysiological concentrations of GFs. Another study developed poly(ethylene glycol) (PEG) gels functionalized with fluvastatin-releasing grafts, to promote BMP-2 and ALP production by encapsulled hMSC, and growth factor binding heparin domains, to sequester the BMP-2 produced by the hMSC [45]. This design allowed the authors to locally enhance the production of BMP-2 and control its retention. Ghanaati et al. (2009) showed that mixing heparin binding peptide amphiphile (HBPA) nanofiber gels with heparan sulfate (HS), promoted vascularization when injected subcutaneously [46]. The authors justified the observations by claiming that the HS, captured by the HBPA, bound and retained endogenous GFs such as vascular endothelial growth factor (VEGF) and FGF-2. Shah et al. (2010), developed peptide amphiphiles (PA) nanofibers containing a TGF-β1 binding peptide to promote cartilage regeneration in a articular cartilage defect rabbit model [20]. They observed no difference between the groups treated with the TGF-binding PA with or without additional GF. The authors suggested that enhancement in cartilage observed in defects without addition of exogenous TGF-β1 was due to capture of endogenous TGF-β1 by the TGF-β1 binding peptide present in the PA nanofibers. Strategies that use GF-binding molecules to sequester endogenous GFs, can overcome the risks associated with the administration of high doses of exogenous GFs and consequently increase the safety and decrease the associated costs. Everything together can potentially accelerate the translation into pre-clinical and clinical studies. In this study, we hypothesized that functionalizing biomaterials with a TGF-β1-binding peptide would turn the implanted biomaterial into an anchor point for the endogenous TGF-β1, leading to its accumulation, consequently enhancing the healing response of the surrounding tissue without needing to administer exogenous TGF-β1. Upon cutaneous injury, a blood clot forms to stop the blood leaking from the damaged blood vessels. Platelets within this clot release several cytokines, including TGF-β1 [47]. In turn, this TGF-β1 will attract inflammatory cells, such as leukocytes and macrophages, which will release more TGF-β1 and initiate the inflammatory phase. To test this hypothesis, films of native and functionalized PCL were subcutaneously implanted in the backs of rats. After 3 and 7 days, the implants were harvested and histologically stained. The implants harvested 7 days after implantation were also immunostained against TGF-β1. From our immunochemistry against TGF-β1, a higher fluorescence signal was reported in the functionalized PCL, showing that the TGF-β1-binding peptide captured the TGF-β1 released during the inflammation, which led to its accumulation on the functionalized film. This accumulation eventually led to a more pronounced healing response at the injury site. There was a higher cellularity around the functionalized films in all the animals at day 3, which was largely composed of inflammatory cells. After 7 days, the cellularity was once again higher around the functionalized films. In addition to inflammatory cells, fibroblast-like cells and a denser blood vessel network were, after 7 days, solely found around the functionalized PCL. In conclusion, these results show that the TGF-β1-binding peptide captured the endogenous TGF-β1, leading to its accumulation on the PCL. This higher local accumulation of TGF-β1 induced a more pronounced inflammatory response due to the recruitment of more inflammatory cells to the injury site, which consequently led to a more robust tissue formation phase, characterized by the recruitment of fibroblast cells and neovascularization. These results show the potential of using the TGF-β1-binding peptide in the design and improvement of biomedical devices, giving them the capacity to function as an anchor point for the patient's natural circulating TGF-β1. This would allow the TGF-β1 that is naturally circulating or that is produced and released during the inflammatory stage to be captured and consequently to locally enhance its concentration, leading to a more robust healing process without needing to add exogenous GFs. This strategy could be potentially easier to translate into pre-clinical and clinical studies since it avoids the use of supraphysiological concentrations of exogenous GFs, making a safer, easier and cheaper approach when compared with strategies that rely in the administration of exogenous GFs.

5. Conclusions

A strategy to immobilize hTGF-β1 in PCL has been detailed through the interaction with a TGF-β1-binding peptide. The immobilized hTGF-β1 presented to the cells is bioactive and capable of activating the TGF-β pathway in primary human-derived hamstring cells. This is the first time that a method for the immobilization and delivery of hTGF-β1 for T/L healing has been described. Our *in vitro* studies showed that the immobilized hTGF-β1 induced the expression of collagen types I and III in human-derived hamstring cells without effecting chondrogenic related genes (Sox9 and aggrecan). *In vivo* studies showed that there was a higher accumulation of native TGF-β1 on the films functionalized with a TGF-β1-binding peptide, which led to a more pronounced recruitment of inflammatory cells, a fibrotic response and neovascularization around the implant. This highlights the potential of using this peptide sequence in the design of improved medical devices through adding the capacity to capture the patient's native TGF-β1 to the device and therefore locally enhance the effect that this GF has in tissue repair.

Disclosures

The authors have no conflicts of interest.

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