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# 1 *In vitro* and *in vivo* evaluation of an electrospun-aligned microfibrous 2 implant for *Annulus fibrosus* repair

3 Maude Gluais<sup>1,2</sup>, Johann Clouet<sup>1,2,3,4</sup>, Marion Fusellier<sup>1,5</sup>, Cyrille Decante<sup>1,6</sup>, Constantin Moraru<sup>1,7</sup>,  
4 Maeva Dutilleul<sup>1,2,8</sup>, Joëlle Veziers<sup>1,2,8,9</sup>, Julie Lesoeur<sup>1,2,8</sup>, Dominique Dumas<sup>10,11</sup>, Jérôme Abadie<sup>12</sup>,  
5 Antoine Hamel<sup>6</sup>, Eric Bord<sup>7</sup>, Sing Yian Chew<sup>13,14</sup>, Jérôme Guicheux<sup>1,2,9</sup>, and Catherine Le Visage<sup>1,2, \*</sup>

6 <sup>1</sup>Inserm, UMR 1229, RMeS, Regenerative Medicine and Skeleton, Université de Nantes, ONIRIS, Nantes, F-  
7 44042, France

8 <sup>2</sup>Université de Nantes, UFR Odontologie, Nantes, F-44042, France

9 <sup>3</sup>CHU Nantes, Pharmacie Centrale, PHU 11, Nantes, F-44093, France

10 <sup>4</sup>Université de Nantes, UFR Sciences Biologiques et Pharmaceutiques, Nantes, F-44035, France

11 <sup>5</sup>Department of Diagnostic Imaging, CRIP, ONIRIS, College of Veterinary Medicine, Food Science and  
12 Engineering, Nantes F-44307, France

13 <sup>6</sup>CHU Nantes, service de chirurgie infantile, PHU5, Nantes, F-44093, France

14 <sup>7</sup>CHU Nantes, service de neurotraumatologie, PHU4 OTONN, Nantes, F-44093, France

15 <sup>8</sup>INSERM, UMS 016, CNRS 3556, Structure Fédérative de Recherche François Bonamy, SC3M facility, CHU  
16 Nantes, Université de Nantes, Nantes, F-44042 France

17 <sup>9</sup>CHU Nantes, PHU4 OTONN, Nantes, F-44093, France

18 <sup>10</sup>Ingénierie Moléculaire et Physiopathologie Articulaire (IMoPA), UMR 7365 CNRS - Université de Lorraine,  
19 Vandœuvre-lès-Nancy, F54505, France

20 <sup>11</sup>Plateforme d'Imagerie et de Biophysique Cellulaire PTIBC-IBISA, FR3209 CNRS, Vandœuvre-lès-Nancy,  
21 F54505, France.

22 <sup>12</sup>Animal cancers as Models for Research in Comparative Oncology (AMaROC), ONIRIS, College of Veterinary  
23 Medicine, Food Science and Engineering, Nantes F-44307, France

24 <sup>13</sup>School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459

25 <sup>14</sup>Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 308232

26

## 27 **Abstract**

28 *Annulus fibrosus* (AF) impairment is associated with reherniation, discogenic pain, and disc  
29 degeneration after surgical partial discectomy. Due to a limited intrinsic healing capacity,  
30 defects in the AF persist over time and it is hence necessary to adopt an appropriate strategy to  
31 close and repair the damaged AF. In this study, a cell-free biodegradable scaffold made of  
32 polycaprolactone (PCL), electrospun, aligned microfibrils exhibited high levels of cell  
33 colonization, alignment, and AF-like extracellular matrix deposition when evaluated in an  
34 explant culture model. The biomimetic multilayer fibrous scaffold was then assessed in an ovine  
35 model of AF impairment. After 4 weeks, no dislocation of the implants was detected, **and only**  
36 **one sample out of six showed a partial delamination**. Histological and immunohistochemical  
37 analyses revealed integration of the implant with the surrounding tissue as well as  
38 homogeneously aligned collagen fiber organization within each lamella compared to the  
39 disorganized and scarcer fibrous tissue in a randomly organized control fibrous scaffold. In  
40 conclusion, this biomimetic electrospun implant exhibited promising properties in terms of AF  
41 defect closure, with AF-like neotissue formation that fully integrated with the surrounding  
42 ovine tissue.

## 1 **Keywords**

2 Intervertebral disc, herniation, electrospinning, polycaprolactone, multilayer scaffold

## 3 **1. Introduction**

4 Low back pain (LBP) is a major health issue that causes disability as well as a very substantial  
5 socioeconomic burden (as much as \$253 billion dollars annually in the United States alone)<sup>1,2</sup>.  
6 Impairment of the *Annulus fibrosus* (AF) structural integrity, arising either by biological  
7 remodeling or by repetitive/traumatic events, is believed to be one of the main causes of LBP<sup>3-</sup>  
8 <sup>7</sup>. Indeed, neovascularization and neoinnervation, infiltrating the intervertebral disc (IVD)  
9 through peripheral AF lesions, can give rise to discogenic pain<sup>8,9</sup>, while extensive AF radial  
10 tears may lead to IVD herniation and subsequently radicular pain<sup>10</sup>. The most common surgical  
11 procedure to treat IVD herniation is called partial discectomy or herniectomy and it consists of  
12 removal of the herniated tissue to relieve radicular pain. However, despite successful pain relief  
13 and improved function, 25% of patients experience long-term unsatisfactory outcomes<sup>11,12</sup>.  
14 Indeed, untreated defects in the AF have been shown to persist over time due to the limited  
15 intrinsic healing capacity of the AF. Such defects are associated with up to 20% of postoperative  
16 reherniations and they increase the prevalence of IVD degeneration by 20% compared to the  
17 general population<sup>11-20</sup>.

18 A limited number of procedures have been used for AF closure, including suturing and gluing  
19 techniques, which are considered to be relatively straightforward methods. However, neither  
20 simple suturing<sup>21</sup> nor more complex suture systems, such as the Dines knot<sup>22</sup> or Modified purse-  
21 string sutures<sup>23,24</sup>, significantly reduce the reherniation rate or the degenerative processes in *ex*  
22 and *in vivo* ovine and porcine models. In addition, a two-year follow-up multicenter randomized  
23 controlled clinical study (NCT00760799) investigating the effect of an FDA-approved  
24 commercial suture technique, the Xclose<sup>TM</sup> tissue repair system (Annulex Technologies,  
25 Minnetonka, Minnesota, USA), found that there was no statistical reduction of postoperative  
26 pain or the reherniation rate in the treated group<sup>25</sup>. It is also important to point out that suturing  
27 techniques exhibit a number of major limitations, such as the creation of puncture holes and  
28 permanent stretching of the AF margins, which could induce additional tears and accelerate the  
29 degeneration process<sup>26,27</sup>. Likewise, few studies have investigated gluing techniques using  
30 either genipin-crosslinked fibrin gels<sup>28-30</sup>, riboflavin-crosslinked high-density collagen  
31 gels<sup>31,32</sup>, or isocyanate-terminated polyethylene glycol-trimethylene carbonate (PEG-TMC)  
32 gels<sup>33</sup> in *ex vivo* and *in vivo* models. Although there was strong adhesion to the surrounding  
33 tissue, with possible prevention of *Nucleus pulposus* (NP) herniation, only partial

1 biomechanical restoration was achieved, with a disorganized fibrous tissue repair and no  
2 prevention of IVD degeneration. A combination of suturing and gluing techniques improved  
3 closure efficiency in a calf *ex vivo* model compared to the use of adhesive or sutures alone. This  
4 combined fixation technique has been shown to fail, however, after 100,000 cycles of load,  
5 thereby demonstrating its unsuitability for long-term AF sealing<sup>34</sup>.

6 In this context, the use of implant devices appears to be a promising alternative strategy since  
7 they can fill AF defects without damaging the surrounding tissue and they can induce tissue  
8 repair. The commercial implant Barricaid™ (Intrinsic Therapeutics, Woburn, Massachusetts,  
9 USA) has exhibited promising short-term outcomes (2 years) in clinical evaluations, with a  
10 reduction in leg and back pain, preserved disc height, and a reduced risk of symptomatic  
11 reherniation (NCT01283438)<sup>35,36</sup>. This device is currently undergoing evaluation in two post-  
12 market surveillance registries (NCT03366779 and NCT03180749). However, its invasive  
13 implantation method using anchorage to the adjacent vertebrae with a titanium insert is reason  
14 for concern due to possible endplate lesions<sup>37</sup> and osteophyte formation at the anchorage site,  
15 as has already been reported for other experimental vertebral-anchored annular devices<sup>38</sup>. To  
16 overcome the lack of clinically effective therapies, a large number of natural and/or synthetic  
17 implants for AF closure have been investigated *in vivo* such as a synthetic poly(lactic-co-  
18 glycolic acid) sponge<sup>39</sup>, a small intestinal submucosa implant<sup>38</sup>, and a textile polyglycolic  
19 acid/polyvinylidene fluoride implant<sup>40</sup>. Cell recruitment and collagen deposition have been  
20 shown to take place within these implants. In addition, numerous *in vitro* studies have  
21 highlighted that the scaffold topography guides the induction of hierarchical organization of  
22 bone marrow mesenchymal stem cells (BMSCs), AF cells, and extracellular matrix (ECM)  
23 deposition<sup>41-43</sup>.

24 Taken together, these results highlight the need for implants that function as a 3D scaffold that  
25 can mimic the complex native AF structure and that can guide cell migration, proliferation, and  
26 production of an AF-like tissue that can in turn fully integrate with the surrounding tissue.  
27 However, due to the inherent complexity of the AF tissue, designing such an implant remains  
28 a challenge. Specifically, the AF is an anisotropic structure that is arranged in concentric  
29 lamellae, of 200 to 400  $\mu\text{m}$  thickness in humans, and it is mainly composed of aligned and  
30 parallel collagen fibers and proteoglycans such as aggrecan. The collagen fibers are oriented  
31 obliquely at a precise angle of  $60^\circ$  to the vertical axis of the spine and this inclination alternates  
32 from left to right with each lamella. As a result, this confers a cross-ply structure that is essential  
33 for the biomechanical function of the AF<sup>44,45</sup>. Native AF cells are mostly elongated fibroblast-  
34 like cells that are aligned and parallel to each other and they produce mainly type I collagen<sup>46,47</sup>.

1 Numerous *in vitro* studies, using a variety of scaffolds, have demonstrated that 3D printing and  
2 electrospinning technologies are particularly suitable for designing highly organized micro- or  
3 nano-fibrous AF implants<sup>48-50</sup>. Polycaprolactone (PCL) in particular, which is a **biocompatible**  
4 **and slowly biodegradable** material with high mechanical properties that has already been used  
5 in numerous tissue engineering strategies and clinical applications, has been widely  
6 recommended for AF repair<sup>41,42,51-59</sup>. **To our knowledge, only two papers have described**  
7 **electrospun PCL implantation in a large animal model (pig lumbar IVD and goat cervical IVD).**  
8 In a recent elegant paper, a complex multilayer PCL scaffold, obtained by a combination of  
9 electrospinning and 3D printing technologies and sutures, was used successfully to repair an  
10 AF defect in pigs<sup>60</sup>. **In the second paper, agarose hydrogel for NP, electrospun PCL for AF and**  
11 **porous PCL for endplates were combined and implanted in a goat cervical model for up to 8**  
12 **weeks. Although this full disc replacement strategy aimed at treating patients who undergo total**  
13 **discectomy, where both damaged NP and AF are surgically removed, it confirmed the relevance**  
14 **of electrospun PCL for AF repair<sup>61</sup>.** In this scarce literature context, the present study aimed to  
15 develop an electrospinning technology to design an aligned electrospun PCL scaffold that  
16 mimics the singular structure of native AF tissue. The objective of this study was to determine  
17 whether this cell-free, aligned, electrospun PCL scaffold could induce spontaneous organized  
18 fibrous tissue formation *in vitro*, **in an original ex vivo model and in a sheep lumbar model.** The  
19 aligned scaffold was compared to a randomly organized electrospun scaffold as a control for  
20 AF-like tissue formation.

## 21 **2. Materials and Methods**

### 22 **2.1. Fibrous scaffold fabrication**

23 Random and aligned non-woven fibrous scaffolds of poly( $\epsilon$ -caprolactone) (PCL, **Mn 80,000**,  
24 Sigma-Aldrich, St. Quentin Fallavier, France) were produced by electrospinning. PCL was  
25 dissolved in 2,2,2-Trifluoroethanol (TFE, Sigma-Aldrich) at concentrations of 14 and 18 wt%  
26 for random and aligned scaffolds, respectively. One mL of PCL solution was loaded into a  
27 syringe that was attached to a 22-gauge blunt-end stainless steel needle and dispensed at a flow  
28 rate of 1 mL/h using a syringe pump (BSP-99M, Linari Engineering, Pisa, Italy). To collect the  
29 PCL fibers, a 13-cm diameter rotating wheel (kindly provided by Prof. SY Chew, Nanyang  
30 Technological University, Singapore) attached to a DC motor (07SP-9032, Xajong Co., Ltd.,  
31 Taichung, Taiwan) and regulated by a speed controller (XJC220-90C-B, Xajong Co.) was  
32 covered with aluminum foil **and placed 10 cm from the tip of the needle.** To obtain random  
33 fibers, which served as controls in this study, the needle was charged to +4.5 kV using a high-  
34 voltage positive generator (HVG-P60-WEB, Linari Engineering, Pisa, Italy), while the wheel,

1 rotating at ~320 rpm, was charged to -4 kV using a high-voltage negative generator (ES30N-  
2 10W, Gamma High Voltage Research Inc., Ormond Beach, Florida, USA). To obtain aligned  
3 oriented fibers, the needle was charged to +5 kV, while the wheel, rotating at ~2,400 rpm, was  
4 charged to -4 kV. **Electrospun materials were obtained in conditions with temperature ranging**  
5 **from 20.8°C to 25.0° and humidity ranging from 31% to 55%.**

## 6 **2.2. Fibrous scaffold characterization**

### 7 *2.1.1. Structural evaluation*

8 Random and aligned scaffolds were coated with gold-palladium using a Desk III Sputter Coater  
9 (Denton Vacuum, Moorestown, New Jersey, USA) and the surface morphology was examined  
10 by backscattering of electrons using a Scanning Electron Microscope (SEM, LEO 1450VP®,  
11 Carl Zeiss, Marly Le Roi, France) at an acceleration voltage of 5 kV and 25 mA. The  
12 directionality (relative to the main axis) and the average diameters of random and aligned  
13 electrospun fibers (n=3) were determined using ImageJ® (1.51s, National Institutes of Health,  
14 Bethesda, Maryland, USA).

15 To evaluate their thickness with ImageJ®, PCL scaffolds (n=3) were snap frozen in liquid  
16 nitrogen and cryosectioned perpendicular to the direction of the collective wheel rotation with  
17 a scalpel. The samples were prepared for SEM as described above.

18 The pore size distribution and the porosity of the scaffolds were measured by mercury  
19 porosimetry (Autopore IV 9500®, Micromeritics Instrument Corp., Norcross, Georgia, USA).  
20 Dry scaffolds (40-70 mg) were placed in the chamber and air was removed from the pores. The  
21 samples (n=3) were filled with mercury under reduced pressure (2 kPa), after which the pressure  
22 was incrementally increased to a maximum pressure of 30 kPa. The mercury intrusion volume  
23 was measured as the pressure increased, which allowed calculation of the pore size distribution  
24 within the samples and their overall porosity.

### 25 *2.1.2. Mechanical testing*

26 Uniaxial tensile testing was performed along the main fiber axis (the direction of the rotation  
27 during the electrospinning) using a texture analyzer (TA.HDplus, Stable Micro Systems®,  
28 Godalming, United Kingdom). The scaffold samples (n=3) were cut into 90 x 11 mm rectangles,  
29 which were clamped with serrated grips and subjected to the following testing protocol: (1)  
30 preload to 0.1 N for 5 min; (2) 10 preconditioning cycles to 0.1% strain applied at a rate of 2  
31 mm/s; and (3) elongation at a rate of 1 mm/s until failure. The strain was computed as the  
32 displacement normalized to the initial gauge length and the stress was calculated as the load  
33 normalized to the cross-sectional area. Young's Modulus was calculated as the slope of the

1 linear portion of the stress-strain plot. The final tensile strength and maximum elongation were  
2 determined from the stress and the strain, respectively, before sample failure.

### 3 **2.3. *In vitro* analysis**

#### 4 **2.3.1. *Sheep AF explant culture***

5 **In order to evaluate the spontaneous colonization of the implants when in contact with AF tissue**  
6 **after implantation, an *ex vivo* model was set-up.** The PCL scaffolds were first cut into circular  
7 samples (1.5 cm in diameter) and decontaminated in 70% ethanol for 30 min, after which both  
8 sides of the scaffolds were exposed to UV radiation for 30 min. Prior to culture, the samples  
9 were coated with fetal bovine serum (FBS; Dominique Dutscher, Brumath, France) overnight  
10 in a humidified incubator at 37 °C and 5% CO<sub>2</sub>.

11 Six female sheep (1-year-old) with an average body weight of 35 kg were used for the *in vitro*  
12 studies. The sheep were euthanized using an overdose of 140 mg/kg of pentobarbital  
13 (Dolethal<sup>®</sup>, Vetoquinol, France) and the IVDs were surgically isolated. AF tissues were  
14 carefully separated from NP tissues using a blade, diced into pieces (~ 0.5 x 1 cm), and rinsed  
15 in three consecutive baths of phosphate-buffered saline (PBS, Thermo Fisher Scientific,  
16 Illkirch, France) containing 2% penicillin-streptomycin solution (P/S, Invitrogen, Paisley, UK).  
17 The AF explants were subsequently placed on the FBS-coated PCL scaffolds and cultured in  
18 24-well ultra-low attachment plates in Dulbecco's Modified Eagle Medium (Invitrogen DMEM  
19 containing high glucose, GlutaMAX<sup>™</sup>, and pyruvate) supplemented with 10% FBS, 1% P/S,  
20 and 0.17 mM of ascorbic acid (2-phospho-L-ascorbic acid trisodium salt, Sigma-Aldrich). The  
21 medium was refreshed twice per week and the explants were cultured for 14 or 28 days.

#### 22 **2.3.2. *Scaffold surface colonization***

23 On day 14 and day 28, the AF explants were removed from the culture plates and the remaining  
24 scaffolds were fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were  
25 permeabilized with 0.5% Triton X-100 in PBS, for 10 min at room temperature and then stained  
26 with phalloidin-Alexa Fluor<sup>®</sup> 568 (1:200 in PBS, Thermo Fisher Scientific) for 1 h at room  
27 temperature to visualize the cytoskeletal F-actin using a fluorescence microscope (Axio  
28 Zoom.V16, Carl Zeiss). The cytoskeletal staining was used to approximate the cell area and to  
29 determine the surface area of the scaffolds covered by the cells after 14 and 28 days of culture.  
30 The quantitative analyses were performed using ImageJ<sup>®</sup> software (N=6, n=3).

#### 31 **2.3.3. *Cell proliferation***

32 On day 14 and day 28, the AF explants were removed from the culture plates and the remaining  
33 scaffolds were incubated with 10 μM 5-ethynyl-2'-deoxyuridine (EdU) solution for 24 h and  
34 then fixed with 4% paraformaldehyde for 15 min at room temperature. EdU labeling and

1 Hoechst staining were performed according to the manufacturer's specifications (ClickIT  
2 EdU<sup>®</sup>, Thermo Fisher Scientific). Visualization of the cell nuclei and cells displaying DNA-  
3 incorporated EdU was performed using a fluorescence microscope (Axio Zoom.V16). The  
4 quantitative analyses were performed using custom image-analysis software (N=6, n=3).

#### 5 *2.3.4. Cell morphology and phenotype*

6 On day 14 and day 28, the AF explants were removed from the culture plates and the remaining  
7 scaffolds were processed for SEM microscopy. The scaffolds were fixed with 4%  
8 paraformaldehyde for 15 min at room temperature and then dehydrated with an ethanol gradient  
9 followed by two successive incubations in hexamethyldisilazane for 15 min (HMDS, Sigma-  
10 Aldrich). The dried scaffolds were coated with gold-palladium and observed using a SEM as  
11 previously described (N=3, n=1).

12 In a separate experiment, the scaffolds were fixed with 4% paraformaldehyde on day 14 and  
13 day 28. The cells were permeabilized with 0.5% Triton X-100<sup>®</sup> and non-specific sites blocked  
14 with 3% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min. The cells were then stained  
15 with phalloidin-Alexa Fluor<sup>®</sup> 568 as previously described and then incubated overnight at 4 °C  
16 with anti-collagen type I primary antibody (1:250 in 1% BSA solution, Ab138492, rabbit  
17 monoclonal, Abcam, Cambridge, UK), or anti-aggrecan antibody (1:100 in 1% BSA, Ab1031,  
18 rabbit polyclonal, Merck Millipore, St. Quentin en Yvelines, France) and then with Alexa Fluor  
19 488 secondary antibody (goat anti-rabbit IgG (H+L), 1:1000 in 1% BSA solution, Thermo  
20 Fisher Scientific) for 1 h at room temperature. The cell nuclei were stained with Hoechst and  
21 all of the samples were imaged using a confocal microscope (A1RS, Nikon, Champigny sur  
22 Marne, France). In addition, the reflection mode of the confocal microscope allowed  
23 visualization of the non-labeled underlying PCL scaffold. Quantitative analyses of the PCL  
24 fiber, F-actin, and collagen type I fiber directionalities were performed using ImageJ<sup>®</sup> software  
25 (N=3, n=1).

### 26 **2.4. In vivo analysis**

#### 27 *2.4.1. Ethical aspects and animals*

28 Two female sheep (1-year-old; 29 and 36 kg, respectively, Vendée breed, GAEC HEAS farm,  
29 Ligné F-44850, France) were operated on in the accredited Centre of Research and Pre-clinical  
30 Investigations at the ONIRIS - National Veterinary School of Nantes with approval from the  
31 French Ministry of Agriculture and the ethics committee of the Région Pays de La Loire  
32 (Ethical number APAFIS 10248). All animal experiments were carried out in accordance with  
33 the EU Directive 2010/63/EU. Five lumbar IVDs (L1 - L2 to L5 - L6) per sheep were used for  
34 the experiments and the conditions were as follows: healthy group (2 IVD), unrepaired group

1 (2 IVD), repaired group including randomly organized scaffold (3 IVD), and aligned scaffold  
2 (3 IVD).

### 3 *2.4.2. Multilayer implant preparation*

4 The PCL scaffolds were first cut into rectangular samples (2 x 5 mm), with the aligned scaffolds  
5 sectioned in order to have the prevailing fiber direction oriented at 30° to the long axis of the  
6 rectangle. The samples were first incubated in 70% ethanol for 30 min and then both sides were  
7 decontaminated by exposure to UV light for 30 min.

8 The day before the implantation, 50 mL of blood was drawn from the jugular vein, transferred  
9 into a sterile 50 ml polypropylene centrifuge tube and allowed to coagulate for 30 min at room  
10 temperature. The tubes were then centrifuged at 10,000 x g for 15 minutes and the serum was  
11 recovered. The PCL scaffolds were immediately incubated overnight in the serum solution in  
12 an incubator at 37 °C and 5% CO<sub>2</sub>.

13 The serum-coated PCL samples were then stacked into multilayer constructs (2 mm height, ~ 8  
14 to 10 layers) to mimic the multilamellar organization of native AF tissue. For aligned fibers,  
15 successive layers were assembled running in alternate directions, mimicking the angle-ply  
16 structure of the AF.

### 17 *2.4.3. Surgery and tissue harvesting*

18 Sheep anesthesia was induced by intravenous injection of 2-5 mg/kg of ketamine (Imalgene  
19 1000<sup>®</sup>, Merial, Lyon, France) and 0.2 mg/kg of diazepam (Valium<sup>®</sup>, Roche, Boulogne-  
20 Billancourt, France), and maintained by inhalation of 1-3% isoflurane (Vetflurane<sup>®</sup>, Virbac,  
21 Carros, France) and intravenous injection of 1-3 mg/kg of propofol (PropoVet<sup>®</sup>; Abbott  
22 Laboratories Ltd., Maidenhead, UK). Analgesia was obtained by intravenous injection of 5  
23 µg/kg of fentanyl (Abbott Laboratories Ltd.) and then continuously perfused at a rate of 5-20  
24 µg/kg/h of fentanyl and 5-20 µg/kg/h of ketamine. The sheep were carefully bedded on their  
25 right side and their lumbar discs were exposed using a left retroperitoneal, transpoas approach.  
26 Once exposed, five lumbar IVDs were randomly assigned to one of the four conditions: either  
27 no defect induction (healthy control group), a box annulotomy without implant deposition  
28 (unrepaired control group), a box annulotomy with deposition of a random multilayer implant  
29 (random group), or a box annulotomy with deposition of an aligned multilayer implant (aligned  
30 group). For the annulotomy groups, a ventro-lateral scalpel-induced box defect (2 x 5 mm and  
31 2 mm depth) was created in the outer AF. All of the groups received an external  
32 polytetrafluoroethylene (PTFE) patch (1 x 1.25 cm, PTFE felt pledgets, 007976, Bard,  
33 Covington, Georgia, USA) glued on with cyanoacrylate adhesive (Leukosan<sup>®</sup> adhesive, 72541-

1 01, BSN Medical, Le Mans, France) to the adjacent vertebral bodies. Both sheep received 7  
2 mg/kg of amoxicillin and clavulanic acid during the surgery then 1.75 mg/kg/d for 5 days and  
3 0.5 mg/kg/d of meloxicam during the first 3 days.

4 After 4 weeks, the sheep were sacrificed under general anesthesia by intravenous injection of  
5 140 mg/kg of pentobarbital. Lumbar spines were harvested and immediately fixed with 4%  
6 paraformaldehyde for 3 days at room temperature.

#### 7 *2.4.4. X-ray imaging and MRI*

8 Pre-, post-operative, as well as pre-euthanasia X-ray imaging, was performed, as previously  
9 described<sup>62</sup>, using a radiography machine (Convix 80<sup>®</sup> generator and Universix 120 table) from  
10 Picker International (Uniontown, Ohio, USA). Coronal and sagittal plane radiographs of the  
11 sheep lumbar spines were taken with a collimator-to-film distance of 100 cm, exposure of 100  
12 mAs, and penetration power of 48 kVp.

13 Pre-, post-operative, as well as pre-euthanasia MRI of the entire lumbar sheep spines, were also  
14 performed as previously described<sup>63</sup> using a 1.5 Tesla MRI scanner (Magnetom Essenza<sup>®</sup>,  
15 Siemens Medical Solutions, Erlangen, Germany) with a standard spine coil to obtain T2-  
16 weighted images (TE: 86 ms, TR: 3000 ms; slice thickness: 3 mm) and T1-weighted images  
17 (TE: 12 ms, TR 322 ms; slice thickness: 3 mm).

18 The images were analyzed using Osirix software<sup>®</sup> (3.9, Osirix Foundation, Geneva,  
19 Switzerland). NP hydration was assessed by dividing the NP T2 signal in the middle axial  
20 position of the IVD by the T2 signal of the adjacent spinal cord in the same MRI slice. The disc  
21 height index (DHI) was measured as  $DHI = \text{disc height} / \text{adjacent vertebral height}$  on both the  
22 X-rays and the MRI.

#### 23 *2.4.5. Histological and immunohistochemical staining*

24 PFA-fixed lumbar discs were decalcified for 72 h in a decalcifier (Shandon TBD-2<sup>TM</sup>  
25 Decalcifier, 6764004, Thermo Fisher Scientific), frozen for 3 min in isopentane/dry ice and  
26 then embedded in Super Cryoembedding Medium (SCEM) (Section Lab, Hiroshima, Japan)  
27 and frozen again in isopentane/dry ice until the SCEM set. The frozen samples were sectioned  
28 at -30 °C in the axial orientation into 7 µm sections using a cryostat (CryoStar NX70<sup>®</sup>, Thermo  
29 Fisher Scientific, Waltham, Massachusetts, USA). Following standard protocols, the sections  
30 were stained with Hematoxylin Eosin Safran (HES), Masson's trichrome (MT), and Picrosirius  
31 red (PR).

32 Immunostaining for type I collagen was performed using anti-collagen type I primary antibody  
33 (1:250 in 0.1% Triton and 4% BSA, Ab138492, rabbit monoclonal, Abcam, Cambridge, UK)

1 and biotinylated goat anti-rabbit secondary antibodies (1:300 dilution, E0432, Dako, Agilent  
2 Technologies, Les Ulis, France). Briefly, sections were incubated with 0.1% trypsin (T9935,  
3 Sigma-Aldrich) solution for 30 min at 37 °C and then in freshly prepared 3% H<sub>2</sub>O<sub>2</sub> solution to  
4 inactivate internal peroxidases. The sections were then blocked with 10% goat serum in 4%  
5 BSA for 30 min and incubated with the primary antibody overnight at 4 °C. Incubation with the  
6 corresponding biotin-labeled secondary antibody for 1 h was followed by 45 min of incubation  
7 with horseradish peroxide-conjugated streptavidin (1:400, P0397, Agilent Technologies).  
8 Antibody binding was visualized with diaminobenzidine (DAB, K3468, Agilent Technologies)  
9 as the HRP substrate, and the sections were counterstained for 30 sec in hematoxylin. As a  
10 negative control, sections were processed using identical protocols but with omission of each  
11 primary antibody. All of the stained sections were observed using a slide scanner  
12 (Nanozoomer<sup>®</sup>, Hamamatsu Photonics, Hamamatsu, Japan) and all of the images were analyzed  
13 with NDP view2 software<sup>®</sup> (Hamamatsu Photonics).

#### 14 *2.4.6. Second harmonic generation*

15 The second harmonic generation signal measurements were performed using an APE  
16 picoEmerald laser that delivered synchronized 7 ps pulses at  $\lambda = 800$  nm to a scanning  
17 microscope (SP8-CARS, Leica Microsystems, Mannheim, Germany). In the SHG operation  
18 mode, the signal was filtered to detect collagen at 400 nm in the backward direction with a  
19 hybrid detector using a large dynamic range combined with low dark noise. The quantitative  
20 analyses of collagen fiber directionality were performed using ImageJ<sup>®</sup> software (n=3).

#### 21 **2.5. Statistical analysis**

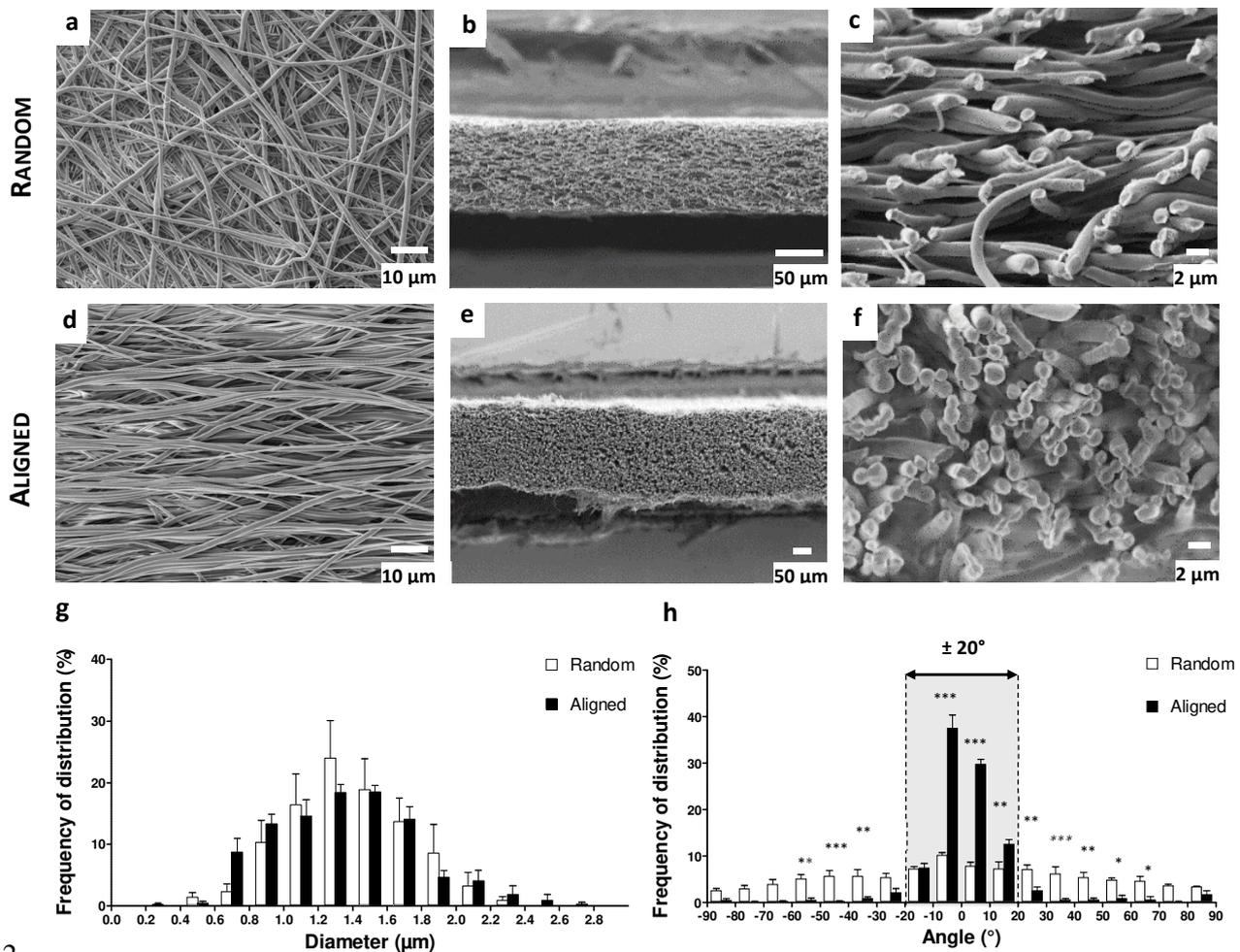
22 All of the data are presented as means  $\pm$  SEM. The statistical analyses were performed using  
23 Graph Pad Prism 5.0<sup>®</sup> software (GraphPad, San Diego, California, USA). Statistical  
24 significance was determined using two-way ANOVA followed by Bonferroni's post-test for  
25 multiple group comparisons with different variables (directionality), or a *t*-test for two-group  
26 comparisons (i.e., porosity, mechanical analysis, colonization, and proliferation). Statistical  
27 significance was set at  $p < 0.05$ . Unless otherwise stated, the experiments were repeated at least  
28 three times.

### 29 **3. Results**

#### 30 **3.1. Fibrous scaffold characterization**

31 Aligned and randomly organized fibrous scaffolds were successfully obtained by collecting  
32 electrospun fibers on a wheel rotating at high and low speed, respectively. SEM allowed  
33 visualization of both the surface and a cross-section of the scaffolds (Fig. 1). The cross-sectional

1 images of the fibrous scaffolds revealed a homogeneous distribution of non-hollow, random,  
 2 and aligned fibers throughout each scaffold (Fig. 1 b-c, e-f), with an average thickness of  $81 \pm$   
 3  $7 \mu\text{m}$  and  $127 \pm 8 \mu\text{m}$ , respectively. The SEM images allowed evaluation of both the diameter  
 4 and the orientation of the constitutive PCL fibers in both of the scaffolds. The random and the  
 5 aligned scaffolds displayed similar distributions of the fiber diameters (Fig. 1g), with average  
 6 fiber diameters of  $1.41 \pm 0.36 \mu\text{m}$  and  $1.33 \pm 0.40 \mu\text{m}$ , respectively. In the aligned scaffolds,  
 7  $88 \pm 3\%$  of the fibers (sum of the black boxes) were oriented at the same angle  $\pm 20^\circ$ , while  
 8 most of the fibers in the random scaffolds did not appear to have a preferential orientation (only  
 9  $33 \pm 2\%$ , sum of the white boxes) (Fig.1h). The random and the aligned scaffolds had similar  
 10 porosities of  $84 \pm 3\%$  and  $81 \pm 1\%$ , respectively, as determined by mercury porosimetry (data  
 11 not shown).



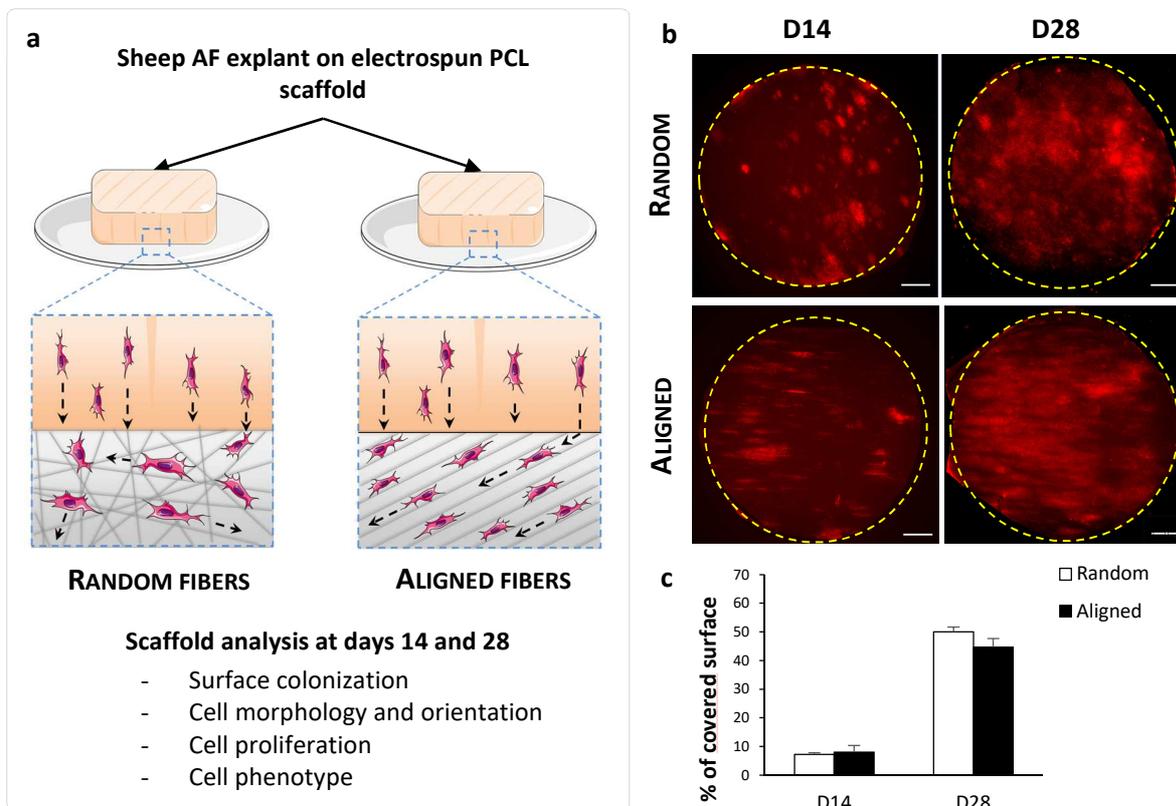
12  
 13 **Figure 1. Characterization of electrospun polycaprolactone (PCL) scaffolds.** (a-f) Scanning electron microscopy  
 14 (SEM) images of random and aligned scaffolds. Surfaces: (a, d) scale bar 10 μm. Cross-sections: (b, e) scale bar  
 15 50 μm, (c, f) scale bar 2 μm. (g) Diameter distribution and (h) angular distribution of the fibers in both of the  
 16 scaffolds. The results are expressed as means  $\pm$  SEM ( $n = 3$ ) (\*\*\*)  $p < 0.001$ , (\*\*)  $p < 0.01$ , statistical difference  
 17 between random and aligned scaffolds).

1 Using uniaxial tensile tests, typical stress-strain curves were obtained for the fibrous scaffolds  
 2 (data not shown) and the calculated mechanical properties were significantly different. The  
 3 Young's moduli of the random and the aligned scaffold were calculated to be  $16 \pm 0.5$  MPa vs.  
 4  $55 \pm 0.6$  MPa ( $p < 0.0001$ ), which corresponds to a significant 3.5-fold increase. The ultimate  
 5 tensile strengths were  $19 \pm 2.9$  MPa vs.  $11 \pm 0.6$  MPa, respectively, (no significant difference)  
 6 and the failure strains were  $545 \pm 33\%$  vs.  $55 \pm 6\%$  ( $p < 0.0001$ ), respectively.

### 7 3.2. *In vitro* analysis

#### 8 3.2.1. Scaffold surface colonization and cell proliferation

9 Sheep AF tissue explants were deposited onto random and aligned scaffolds for 14 and 28 days  
 10 (Fig. 2a) and cells colonizing the scaffold surfaces were identified by fluorescence microscopy  
 11 imaging of the F-actin staining (Fig. 2b). **Cell infiltration in the scaffolds was not evidenced.**  
 12 We then calculated the percentage of the scaffold surface covered by AF cells/cluster (Fig. 2c).  
 13 On day 14, the AF cells covered  $7.5 \pm 1.1\%$  and  $8.2 \pm 1.7\%$  of the surface of random and aligned  
 14 scaffolds, respectively (no statistical difference), while on day 28, there was an increase in the  
 15 percentage of surface covered by cells on both types of surface, with no statistical difference  
 16 between the two types of scaffold ( $50.0 \pm 3.8\%$  vs.  $44.7 \pm 3.2\%$ , respectively).

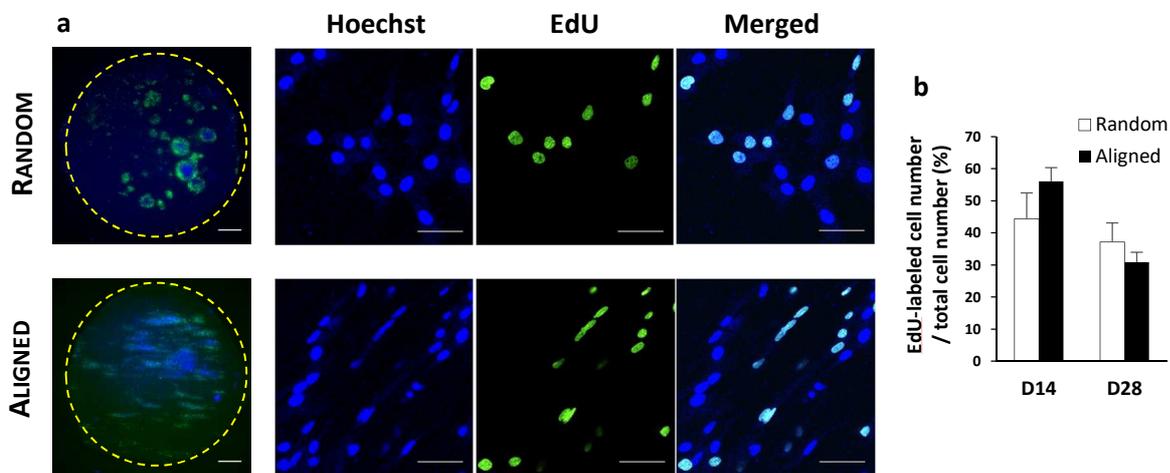


17

18 **Figure 2. *In vitro* culture on electrospun PCL scaffolds.** (a) Schematic representation of the sheep Annulus  
 19 fibrosus (AF) explant culture. Apposition of an AF tissue sample onto a random or an aligned scaffold. The cells  
 20 could spontaneously colonize the PCL surface and orient themselves according to the underlying scaffold  
 21 architecture. (b) On day 14 and day 28, the cells present on random and aligned surfaces (15 mm diameter, yellow  
 22 dashed circles) were stained for F-actin (red) and imaged by fluorescence microscopy (scale bar 2 mm). (c) The

1 percentage of PCL scaffold surface covered by the cells after 14 and 28 days of culture. The results are expressed  
2 as means  $\pm$  SEM ( $N = 6$ ,  $n = 3$ ).

3 The proliferation of AF cells on both types of scaffolds was investigated using a Click EdU  
4 assay, whereby incorporation of the modified thymidine analog EdU into newly synthesized  
5 DNA over a 24 h period was detected by fluorescence staining (Fig. 3). The percentage of cells  
6 with both Hoechst (nuclei) and EdU staining was found to be similar for the two types of  
7 scaffolds at both times (day 14:  $56 \pm 6\%$  and  $44 \pm 8\%$ , day 28:  $31 \pm 3\%$  and  $37 \pm 4\%$  for the  
8 aligned and the random scaffolds, respectively). Moreover, the total cell number on the scaffold  
9 surfaces was not statistically different for the random and the aligned scaffolds (data not  
10 shown), confirming the results obtained for the percentage of scaffold surface area covered by  
11 cells.



12

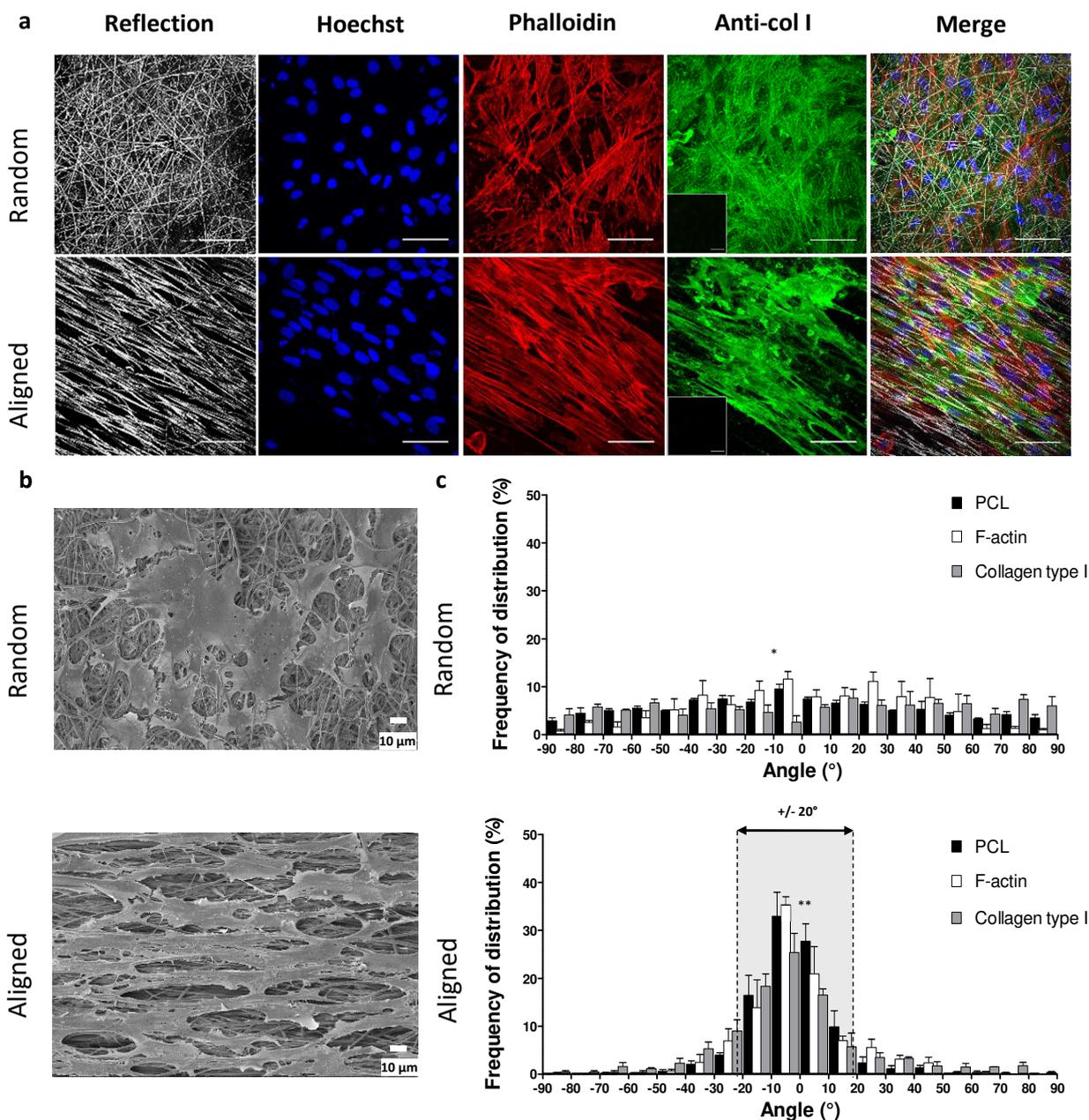
13 **Figure 3. AF cell proliferation on electrospun PCL scaffolds.** (a) At days 14 and 28, AF cells were incubated 24  
14 hours with Click-IT<sup>®</sup> EdU solution. The cell nuclei were stained and random and aligned surfaces (15 mm  
15 diameter, yellow dashed circles) were imaged by fluorescence microscopy (scale bar 2 mm). Positive cells were  
16 identified by both Hoechst nuclear staining (blue) and fluorescent EdU (green) signal (scale bar 50  $\mu$ m). (b) The  
17 percentage of positive EdU-labeled cells on both types of scaffolds on day 14 and day 28. The results are expressed  
18 as means  $\pm$  SEM ( $N = 6$ ,  $n = 3$ ).

### 19 3.2.2. Cell morphology, phenotype, and orientation

20 The cell morphology on the random and the aligned scaffolds was investigated by SEM and  
21 fluorescence microscopy to analyze the influence of scaffold architecture (Fig. 4a and 4b). The  
22 AF cells had an extensively spread morphology on both types of scaffold, with noticeable  
23 differences, however. On the random scaffolds, the cells were spread in all directions in an  
24 irregular manner, while on the aligned scaffolds, the cells were elongated with a spindle-shaped  
25 morphology typical of fibroblast-like cells and they were mostly oriented along the same  
26 direction of the underlying fibers. The orientation of the colonizing cells on both of the patterns  
27 was assessed by measurement of the direction of their actin filaments, while the reflection mode  
28 of the confocal microscope allowed visualization of the non-labeled PCL fibers. In the random

1 group, similar to the underlying PCL fibers, the angular distribution of the F-actin exhibited no  
2 preferential fiber orientation (Fig 4c). In the aligned group, for convenience, the major axis of  
3 the PCL fibers was chosen as a reference and assigned a  $0^\circ$  angle. Similar to the angular  
4 distribution measured on the SEM images (Fig. 1g), the reflection mode showed that  $87 \pm 3\%$   
5 of the PCL fibers were oriented along the major axis  $\pm 20^\circ$ , while the phalloidin staining showed  
6 that  $77 \pm 2\%$  of the F-actin was oriented in the same direction in the colonizing cells. This  
7 strongly suggests that there was a close correlation between the angular distribution of the actin  
8 filaments and that of the underlying PCL fibers.

9 Aggrecan and type I collagen, which are typically synthesized by AF cells, were investigated  
10 by immunostaining. On both types of scaffold, the cell cytoplasms were positively stained for  
11 aggrecan and type I collagen after 14 and 28 days of culture (data not shown). Moreover,  
12 fibrillar structures stained by anti-col I antibody were observed (Fig. 4a). Some stained fibers  
13 were found in areas devoid of F-actin, which strongly suggests that these fibers were  
14 extracellular (data not shown). While on random scaffolds, the angular distribution of these  
15 extracellular fibers revealed no preferential fiber orientation (Fig. 4c),  $66 \pm 5\%$  of the positively  
16 stained extracellular fibers were oriented along the major axis of the underlying aligned scaffold  
17  $\pm 20^\circ$ .



1  
2 **Figure 4. Sheep AF cell morphology, phenotype, and orientation on electrospun PCL scaffolds.** (a) Random  
3 and aligned scaffolds were processed and stained to visualize the PCL fibers (gray), nuclei (blue), F-actin (red),  
4 and type-I collagen (green) using confocal microscopy (day 28, scale bar 50  $\mu\text{m}$ ). *Inserts are negative controls of*  
5 *collagen type I antibody (day 28, scale bar 50  $\mu\text{m}$ ).* (b) Random and aligned scaffolds were processed and analyzed  
6 by SEM (scale bar 10  $\mu\text{m}$ ). (c) The angular distributions of the PCL fibers, F-actin, and type I collagen fibers at  
7 day 28 on random and aligned scaffolds. The results are expressed as means  $\pm$  SEM ( $N = 3$ ,  $n = 1$ ) (\*\*  $p < 0.01$ ,  
8 \*  $p < 0.05$ , statistical difference between PCL and collagen type I, no difference between PCL and F-actin).  
9

### 10 3.3. *In vivo* implantation

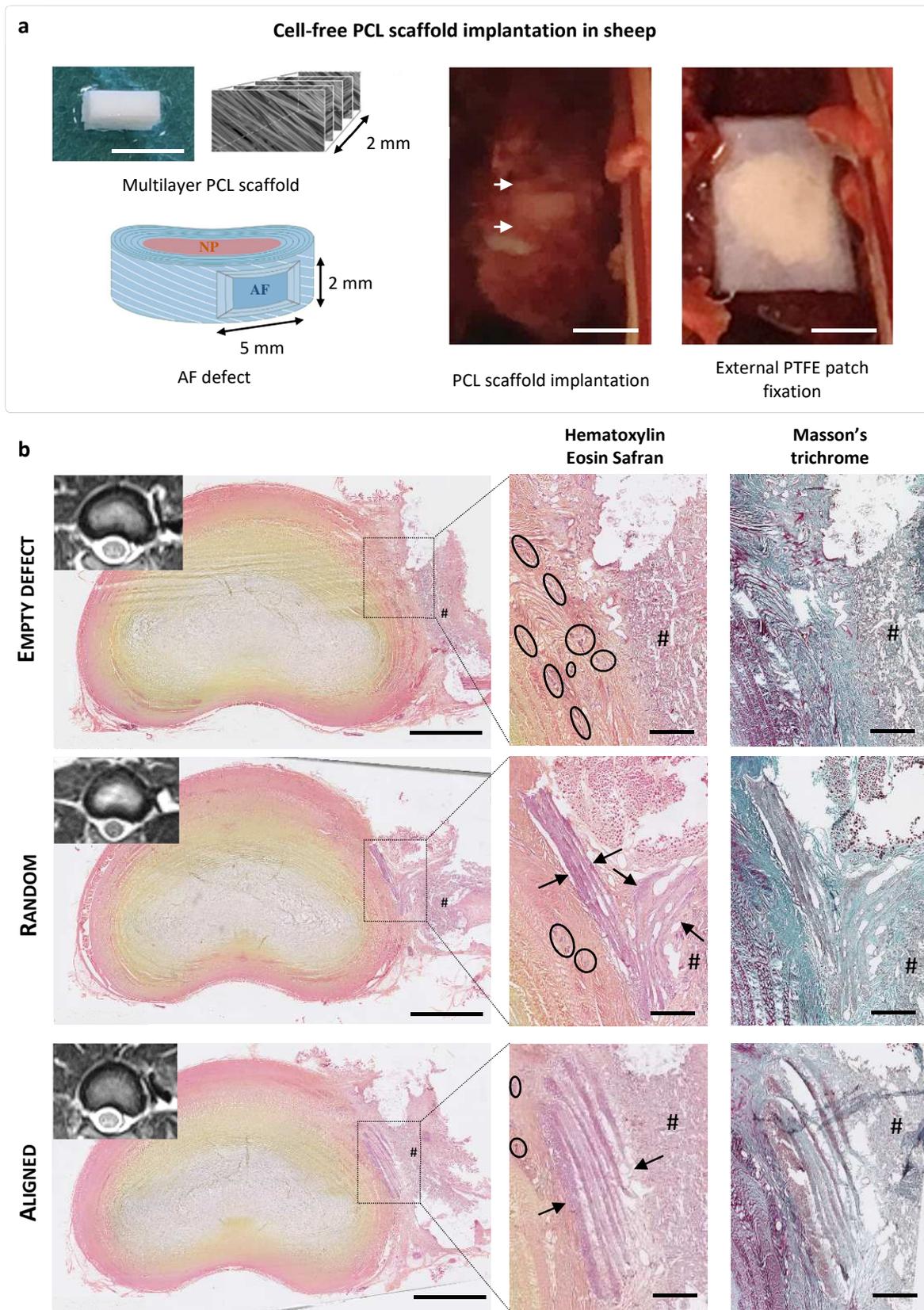
11 To assess the ability of our multilayer PCL scaffold to promote AF regeneration, an *in vivo*  
12 study was conducted in two sheep for 4 weeks. In lumbar discs, a defect was successfully  
13 created in the outer AF followed by implantation of random or aligned multilayer scaffolds with  
14 external PTFE patches to secure them (Fig. 5a).

### 1           3.3.1. *X-ray imaging and MRI*

2     There were no signs of surgery-related complications in the animals. X-rays and MRI taken  
3     preoperatively, postoperatively, and at one month were used to monitor lumbar disc heights and  
4     NP hydration. Quantitative analysis showed preservation of disc height at 4 weeks in both of  
5     the implanted groups as well as in the control groups (empty defect and uninjured disc) (data  
6     not shown). NP hydration was assessed using T2-weighted signal intensity measurements and  
7     there was no statistical difference between the various groups (data not shown). Moreover, 4  
8     weeks after implantation, external PTFE patches were identified by MRI, which indicated that  
9     there had been no displacement compared to the postoperative images.

### 10           3.3.2. *Histological and immunohistochemical analyses*

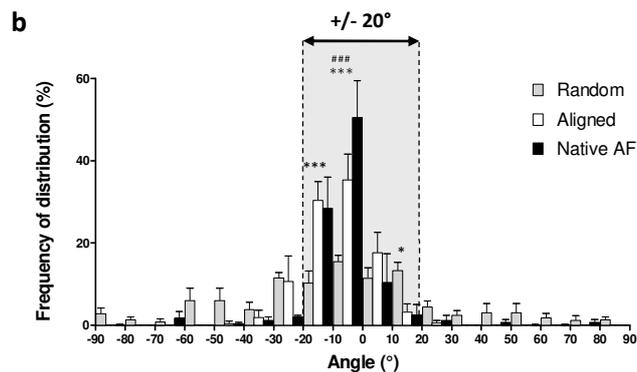
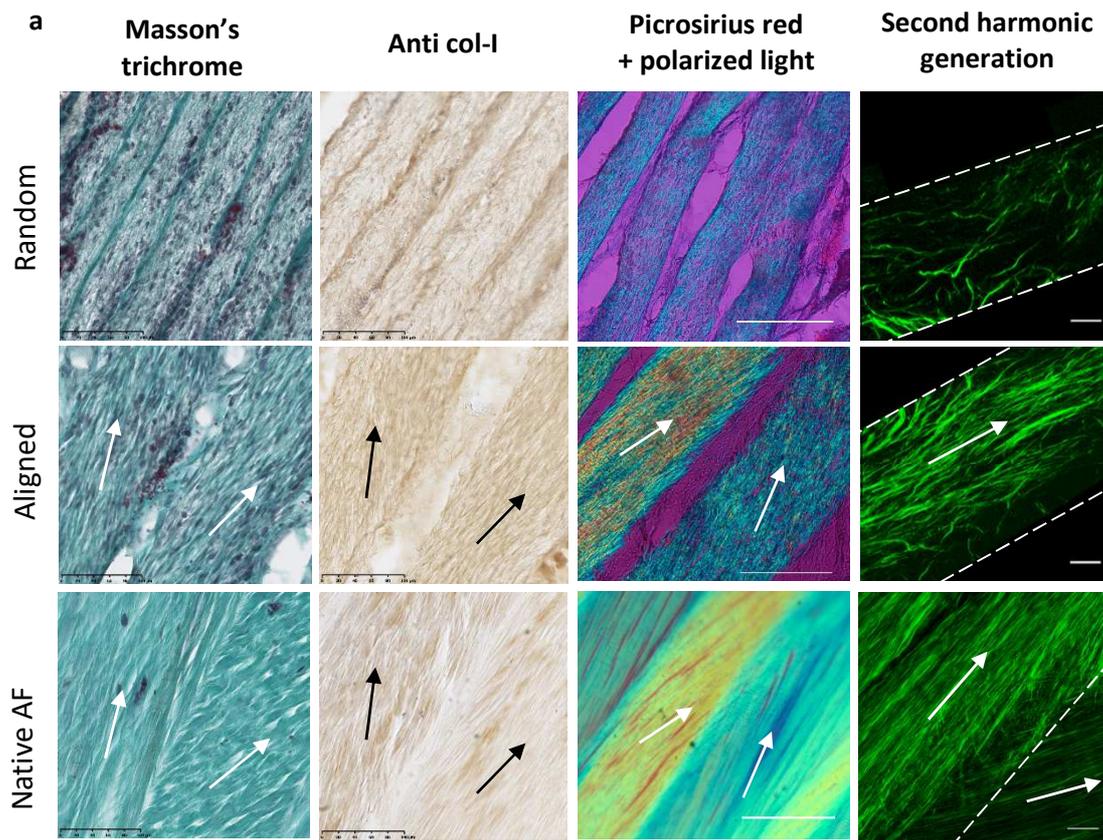
11     Histological staining of decalcified cryosectioned IVDs with HES and TM demonstrated  
12     retention of NP, with regular and intact inner AF lamellae (Fig. 5b). The location of the defect  
13     was confirmed to be in the outer AF. The empty defect exhibited irregular fibrous reparative  
14     tissue with numerous vascular ingrowths at 4 weeks. This tissue was highly cellularized with  
15     fibroblast-like cells displaying elongated nuclei. This collagen-rich tissue was disorganized  
16     with no preferential orientation of the neosynthesized collagen fibers. In both of the repaired  
17     groups (i.e., the random and the aligned group), the multilayer PCL scaffolds were well  
18     positioned within the outer AF defects without any sign of dislocation after 4 weeks of  
19     implantation. Only one randomly organized scaffold exhibited delamination of the superficial  
20     PCL layer. Both implants were well integrated into the surrounding AF, with continuous  
21     collagen fibers at the implant margins. **There was no sign of pronounced foreign body reaction**  
22     **in the implant and in the region surrounding the implant.** Homogeneous cell infiltration  
23     throughout the implants was observed and the cells had elongated nuclei, suggesting  
24     colonization by fibroblast-like cells. A regular fibrous tissue was observed inside the implants,  
25     between the PCL layers, and within each PCL layer, with very little vascular ingrowth.



1  
 2 **Figure 5. In vivo study using an ovine model.** (a) Experimental procedure. Using a left retroperitoneal approach,  
 3 a scalpel-induced box defect (2 x 5 mm and 2 mm depth) was created in the outer annulus of lumbar discs (between  
 4 the white arrows), and multilayer PCL implants (random or aligned) were implanted and secured in the defect by  
 5 gluing an external polytetrafluoroethylene (PTFE) patch to the adjacent vertebral bodies (scale bar 5 mm). (b)  
 6 MRI was performed 4 weeks after the surgery and intervertebral discs were explanted, cryo-sectioned, and  
 7 histochemically stained with hematoxylin/eosin/safran (HES) and Masson's Trichrome (MT) (scale bar: 5 mm for

1 *full disc sections, 1 mm for high magnification sections; PTFE patch identified by #, vascular ingrowth identified*  
2 *in black circles).*

3  
4 Masson's trichrome and immunohistochemical staining of type I collagen revealed positively  
5 stained fibrous tissue in both the random and the aligned scaffolds, with noticeable differences  
6 between the two (Fig. 6a). The collagen fiber bundles in the aligned scaffolds appeared to be  
7 denser and more oriented compared to the ones in the random scaffolds. In addition, observation  
8 of picrosirius red staining under crossed polarizer revealed a strong birefringent intensity in the  
9 aligned scaffolds. The birefringence confirmed that the collagen fibers were aligned, which was  
10 not observed in the random scaffolds. Moreover, the collagen fibers within adjacent layers of  
11 the PCL were aligned along two opposing directions (Fig. 6a, white arrows), which closely  
12 replicates the cross-ply structure of native AF tissue. Consistent with the immunohistochemical  
13 staining, second harmonic generation microscopy revealed alignment of the collagen fibers  
14 within a single aligned PCL layer compared to a more disorganized and less dense collagen  
15 network in the random group. Quantitative analysis of the collagen orientation within a single  
16 PCL layer was performed on the SHG images of both scaffolds and was compared to that of  
17 native sheep AF tissue (Fig. 6b). For convenience, the major axis of the collagen fibers was  
18 systematically chosen as a reference and assigned a  $90^\circ$  angle. In native AF tissue,  $92 \pm 6\%$  of  
19 the collagen fibers were oriented along the major axis  $\pm 20^\circ$ , while  $87 \pm 4\%$  and only  $50 \pm 3\%$   
20 of the collagen fibers followed the same direction within a single layer of the aligned and the  
21 random scaffolds, respectively.



1 **Figure 6. Identification of neocollagen synthesis after 4 weeks of implantation.** (a) Immunohistological staining  
 2 with Masson's trichrome and anti-type I collagen of random and aligned implanted scaffolds (scale bar 100  $\mu$ m).  
 3 The picosirius red staining in association with polarized microscopy allowed visualization of the collagen  
 4 orientation (dashed arrow, scale bar 100  $\mu$ m). The second harmonic generation microscopy allowed visualization  
 5 of the collagen deposition within a single membrane (dashed line) (scale bar 20  $\mu$ m). (b) The angular distributions  
 6 of the collagen fibers within a single membrane of random and aligned scaffolds. The results are expressed as  
 7 means  $\pm$  SD (N = 1, n = 3) (### p < 0.001, statistical difference between native AF and aligned scaffold, \*\*\*  
 8 p < 0.001, \* p < 0.05 statistical difference between native AF and random scaffold).

## 9 4. Discussion

10 The IVD has a pivotal role in spine kinematics. It provides stability during motion such as  
 11 compression, distraction, sliding/twisting, and bending. IVD function results from the  
 12 cooperative action of the NP and the AF to appropriately respond to a wide range of loads by  
 13 contributing to the distribution and transmission of loads between the vertebral bodies. Any

1 disruption in one of the IVD regions has a pronounced effect on the overall function of the IVD  
2 and hence on the stability of the spine.

3 AF damage is known to induce IVD degeneration and is one of the most extensively used  
4 methods for induction of IVD degeneration in animal models, including mice, rats, rabbits,  
5 dogs, pigs, and sheep<sup>64</sup>. These data showed that structural alteration of the AF is likely to induce  
6 leakage of the NP outside the IVD and to activate a cascade of cell-mediated responses, thereby  
7 leading to IVD degeneration<sup>24,26,31,65-67</sup>. AF lesions persist over time when left unrepaired and  
8 they may promote herniation of the NP tissue towards the spinal canal as well as a neural and  
9 vascular ingrowth within the defect<sup>3-7,19</sup>.

10 Effective AF closure and repair would provide significant clinical benefits including the  
11 lessening of IVD degeneration, the prevention of IVD recurrent herniation associated with  
12 radicular pain, and prevention of neural and vascular ingrowth associated with discogenic pain.  
13 For these reasons, over the past decade, strategies for addressing IVD regeneration have  
14 increasingly focused on AF closure and repair. In this context, the objective of this study was  
15 to design an electrospun **PCL** biomaterial that closely mimics the singular structure of native  
16 AF tissue and to assess its ability to appropriately repair AF defects in an ovine model. **PCL is**  
17 **a biocompatible material with high mechanical properties that possess a very slow degradation**  
18 **rate (2 to 3 years depending on the starting molecular weight and form). The degradation of this**  
19 **semi-crystalline polyester has been reported in 2 steps, with first a hydrolytic cleavage of the**  
20 **ester bonds in the amorphous regions which releases a 6-hydroxycaproic acid that can be**  
21 **absorbed by the cells to be metabolized in the Krebs cycle and eliminated in the urine. The**  
22 **second stage, which is slower, concerns the intracellular degradation of crystalline domains by**  
23 **macrophages, giant cells and fibroblasts<sup>51</sup>. Therefore, PCL is particularly interesting for the**  
24 **preparation of implantable devices, where a low degradation rate is highly relevant for slow**  
25 **remodeling tissues such as AF.**

26 The AF is arranged in concentric lamellae composed of aligned collagen fibers, which allow  
27 NP confinement by resisting tensile stretching<sup>68</sup>. We hypothesized that an effective AF repair  
28 material should approximate the ability of the native tissue to withstand the IVD mechanical  
29 environment and guide the neoformation of an AF-like tissue. The use of electrospinning  
30 technology in association with a collector wheel allowed production of aligned and randomly  
31 organized fibrous scaffolds with similar micro-sized PCL fibers and porosity. The random  
32 scaffold was chosen as a control group to monitor the *in vitro* and *in vivo* effect of micropattern  
33 orientation on AF cells. The aligned scaffold exhibited **uniaxial** tensile mechanical properties  
34 similar to those of a single AF lamella Young's Modulus (ranging from 31 to 65 MPa)<sup>69</sup>. To

1 our knowledge, only two other studies to date have reported the development of materials with  
2 a similar tensile modulus, produced by electrospinning, alone or in association with 3D  
3 printing<sup>42,70</sup>. Thus, the use of electrospinning appears to be an appropriate strategy to design a  
4 scaffold with architectural and mechanical properties that match native AF tissue and that could  
5 sustain the complex IVD mechanical loads and provide stability at implantation.

6 In the current study, AF explant culture on electrospun scaffolds was used to assess the  
7 feasibility of developing an acellular electrospun scaffold for AF repair. To our knowledge, this  
8 is the first time that an explant culture model has been used to mimic the *in vitro* ability of AF  
9 cells present in the surrounding tissue to migrate, colonize, and proliferate on an implant. We  
10 demonstrated that the cells were able to colonize and proliferate on both types of scaffold  
11 surface, with increasing colonization of the surface area over time. No difference was seen  
12 between the random and the aligned scaffolds. This appears to indicate that once in contact with  
13 the PCL electrospun scaffold, the cells present in AF tissue can spontaneously populate and  
14 proliferate on the scaffold, irrespective of its architecture. The cell proliferation appeared to  
15 have a tendency to decrease between day 14 and day 28, which could be explained by the  
16 increase in the covered scaffold surface. **Observation of colonies on the surface of the scaffolds  
17 strongly suggests that, after an initial migration phase, cell proliferation could be a major factor  
18 for the increase of cell number and percentage of covered surface.**

19 We next determined that the scaffold architecture strongly influenced the cell morphology and  
20 that it allowed maintenance of the cellular phenotype. **As specific phenotypic markers of AF  
21 cells are still lacking, we analyzed the production of proteins such as type I collagen and  
22 aggrecan core protein.** Both of these were synthesized by the cells on the random and the aligned  
23 scaffolds. The quantitative analysis revealed a close match between the PCL fiber orientation  
24 and the cell cytoskeleton and subsequent extracellular type I collagen deposition. The cell  
25 morphology and the extracellular matrix (ECM) deposition pattern are in accordance with  
26 previous findings where micropatterned scaffolds were shown to exhibit contact guidance that  
27 induced cell and ECM alignment<sup>19,56,71-77</sup>. These results highlight the importance of mimicking  
28 the native AF architecture to first induce alignment of the cells and to then promote the  
29 deposition of an anisotropic ECM.

30 Random and aligned scaffolds were stacked into multilayer scaffolds to replicate the  
31 multilamellar structure of AF tissue. We then embarked on an ovine study to investigate  
32 whether the multilayer fibrous scaffolds could be tolerated by sheep and induce the formation  
33 of anisotropic repair tissue without eliciting an inflammatory response. Sheep have been used  
34 extensively as an experimental model to investigate both IVD diseases and treatment

1 strategies<sup>32,38,40,64,78</sup>. Indeed, sheep IVDs have numerous similarities with human IVDs  
2 including anatomy, size, cell population and, interestingly, biomechanical properties, which  
3 make this animal an ideal model to approximate the human IVD pathophysiology<sup>79</sup>. In our pilot  
4 study, a box-shaped defect was induced in the outer AF to allow the evaluation of our  
5 biomaterial in this specific region. Such a superficial defect was selected to avoid exposure to  
6 the NP pressure which often prevents maintenance of implants within the defect, thereby  
7 limiting the evaluation of the tissue repair within the implants<sup>80,81</sup>. After 4 weeks of  
8 implantation, all implant of the repaired groups (i.e., the random and the aligned groups) were  
9 successfully retained by use of external PTFE patches that were glued to the adjacent vertebrae,  
10 as indicated by the histological staining. However, it was noted that in one of the random  
11 groups, half of the PCL superficial layer appeared to have delaminated (Fig. 5b). MRI showed  
12 that the external patch was displaced in its ventral part. This was confirmed by histological  
13 staining, where muscle tissue was seen adjacent to the implant instead of the PTFE patch. It  
14 would appear that, upon implantation, the external patch was not properly fixed to the vertebral  
15 body, and its dislocation induced delamination of the multilayer scaffold underneath. This  
16 indicates the importance of a combined strategy to secure an AF repair implant within the  
17 defect. It is worth noting that although we aimed at replicating the AF cross-ply structure, PCL  
18 layers were stacked but not glued nor sutured to each other, thus facilitating the expected  
19 spontaneous cell infiltration. We also hypothesized that deposition of collagen fibers between  
20 the PCL layers might have induced the formation of a cohesive matrix that prevented a critical  
21 delamination of the layered implants. This anchoring strategy will need to be further evaluated  
22 in a full thickness defect, that corresponds to a herniated condition, where the implant will be  
23 exposed to the NP content.

24 To our knowledge, this *in vivo* study is the first to demonstrate, in a sheep lumbar model, a  
25 successful biological integration of electrospun PCL with the host's surrounding AF tissue, with  
26 no granuloma formation around the implants. Numerous cells were seen within each PCL layer  
27 of the random and the aligned scaffolds, suggesting that the implant porosities were sufficient  
28 to induce cell infiltration and tissue ingrowth throughout each layer. Newly formed collagen  
29 fibrous tissue was clearly observed within each implant, as evidenced by collagen type I  
30 staining. Observation by picrosirius red staining under polarized light and second harmonic  
31 generation allowed identification and quantification of the regular and the oriented collagen  
32 fibers within the aligned scaffold. Moreover, all of the histological analyses appear to show  
33 numerous and dense collagen fibers in the aligned scaffold, while fewer collagen fibers were  
34 deposited in the random scaffold *in vivo*. Dense fibrous repair tissue was also determined to be

1 present in the empty group, indicating spontaneous healing of the superficial defect. However,  
2 histological observation revealed that the tissue was highly disorganized due to the absence of  
3 a guiding implant. This is in accordance with previous studies in sheep and rabbits reporting  
4 superficial healing of AF defects by deposition of a disorganized collagen tissue with  
5 mechanical properties that cannot withstand the physical loads that arise during the subject's  
6 lifetime<sup>6,82-84</sup>. The present *in vivo* study confirms the results obtained *in vitro* and it once again  
7 demonstrates the importance of contact guidance of the scaffold architecture for deposition of  
8 a highly organized repair tissue. Moreover, these results are in accordance with data recently  
9 reported in an *in vivo* porcine AF defect model using a PCL nano- and micro-sized multilayer  
10 fibrous scaffold. Similar to our study, this complex implant induced the deposition of aligned  
11 and well-integrated collagen fibers on the fibrous layers and a reduction of the degenerative  
12 process after 12 weeks of recovery<sup>60</sup>. Despite the use of different *in vivo* models, both studies  
13 demonstrated the potential of PCL-mimicking fibrous scaffolds for AF repair.

14 Further investigations should be undertaken to compare the mechanical properties of this newly  
15 formed fibrous tissue with native AF tissue and to evaluate its long-term efficiency in  
16 maintaining IVD structural and mechanical integrity. **Finally, both the implant and the**  
17 **anchoring technique appear promising in this pilot study and could be translated into a herniated**  
18 **IVD model<sup>40</sup>. Indeed, implantation of a thicker multilayer aligned scaffold** will be required to  
19 assess whether the scaffold can promote full-thickness AF repair, prevent NP herniation, and  
20 limit disc degeneration.

## 21 **5. Conclusion**

22 In this study, we successfully developed a cell-free PCL fibrous scaffold with morphological  
23 and mechanical properties that mimicked those of the native AF lamellae and that was able to  
24 promote spontaneous *in vitro* cell colonization, proliferation, and organization. Using an ovine  
25 model, we demonstrated that the aligned multilayer scaffold provided an essential inductive  
26 microenvironment for the production of collagen fibrous tissue that mimics the outer AF region.  
27 This implant could potentially act as a 3D scaffold that induces the production of AF-like tissue  
28 and that could prevent IVD recurrent herniation, as well as neural and vascular ingrowth, and  
29 that might also slow the IVD degeneration process.

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## 7 **7. Conflict of interest**

8 The authors declare no financial conflicts of interest.

## 9 **8. Data availability**

10 The raw/processed data required to reproduce these findings cannot be shared at this time due  
11 to technical limitations.

## 12 **9. References**

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