Fibrin functionalization with synthetic adhesive ligands interacting with $\alpha 6\beta 1$ integrin receptor enhance neurite outgrowth of embryonic stem cell-derived neural stem/progenitors.

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ABSTRACT

To enhance fibrin hydrogel affinity towards pluripotent stem cell-derived neural stem/progenitor cells (NSPCs) and its capacity to support NSPC migration and neurite extension, we explored the tethering of synthetic peptides engaging integrin $\alpha 6\beta 1$, a cell receptor enriched in NSPCs. Six $\alpha 6\beta 1$ integrin ligands were tested for their ability to support integrin $\alpha 6\beta 1$ -mediated adhesion of embryonic stem cell-derived NSPCs (ES-NSPCs) and sustain ES-NSPC viability, migration, and neuronal differentiation. Due to their better performance, peptides T1, HYD1, and A5G81 were immobilized into fibrin and functionalized gels characterized in terms of peptide binding efficiency, structure and viscoelastic properties. Tethering of T1 or HYD1 successfully enhanced cell outgrowth from ES-NSPC neurospheres (up to 2.4-fold increase), which exhibited a biphasic response to peptide concentration. Inhibition assays evidenced the involvement of $\alpha 6\beta 1$ and $\alpha 3\beta 1$ integrins in mediating radial outgrowth on T1/HYD1-functionalized gels. Fibrin functionalization also promoted neurite extension of single ES-NSPCs in fibrin, without affecting cell proliferation and neuronal differentiation. Finally, HYD1-functionalized gels were found to provide a permissive environment for axonal regeneration, leading up to a 2.0-fold increase in neurite extension from rat dorsal root ganglia explants as compared to unmodified fibrin, and to significant improved locomotor function after spinal cord injury (complete transection), along with a trend toward a higher area positive for growth associated protein 43 (marker for axonal growth cone formation). Our results suggest that conjugation of $\alpha 6\beta 1$ integrin-binding motifs is of interest to increase the biofunctionality of hydrogels used in 3D platforms for ES-NSPC culture and potentially, in matrix-assisted ES-NSPC transplantation.

Statement of Significance Impact statement: The transplantation of NSPCs derived from pluripotent stem cells holds much promise for the treatment of central nervous system disorders. Moreover, the combinatorial use of biodegradable hydrogels with NSPCs was shown to contribute to the establishment of a more

permissive environment for survival and integration of transplanted cells. In this study, fibrin hydrogels functionalized with a synthetic peptide engaging integrin $\alpha 6 \beta 1$ (HYD1) were shown to promote neurite extension of ES-NSPCs, which is fundamental for the formation of functional neuronal relay circuits after NSPC transplantation. Notably, HYD1-functionalized fibrin per se led to enhanced axonal growth ex vivo and to an improvement in locomotor function after implantation in a rat model of spinal cord injury. Conjugation of $\alpha 6 \beta 1$ integrin-binding motifs may therefore be of interest to confer bioactivity to NSPC hydrogel vehicles.

Graphical abstract

Keywords: Hydrogel; Peptide immobilization; Neural stem/progenitor cells; Cell outgrowth; $\alpha 6 \beta 1$ integrin

1. INTRODUCTION

Progress in the establishment of protocols for the efficient generation of neural stem/progenitor cells (NSPCs) from pluripotent stem cells has fostered their use in experimental regenerative therapies for central nervous system (CNS) disorders, particularly those involving the loss of multiple neural subtypes [1]. Still, despite their ability to survive, differentiate and integrate into the host neural circuitry, often leading to functional benefits [2], [3], neural progenitors injected as suspensions directly into the lesion site of chronic injuries show low survival and/or poor integration [4], [5], [6]. To overcome these limitations, NSPCs are being combined with injectable biodegradable hydrogels which, besides allowing a higher retention and homogenous distribution of transplanted cells at the lesion site, create a supportive niche for cell survival, anchorage and differentiation, as well as for axonal regeneration. Due to its important role in hemostasis and wound repair, fibrin (Fb) has been successfully explored as a provisional matrix for cell delivery, including embryonic stem (ES)- and induced pluripotent stem (iPS)-derived NSPCs [3], [7]. In previous studies, we showed that ES-derived NSPCs embedded in a Fb hydrogel as single cells and cultured under neuronal differentiation conditions proliferate forming small-sized spheroids and differentiate establishing neuronal networks [8]. In the present investigation, we aimed at increasing Fb biospecificity towards ES-NSPCs through the incorporation of biophysical cues engaging cell receptors enriched in NSPCs involved in NSPC migration and neurite outgrowth.

The design of hydrogels targeted for the delivery of a particular cell type has to emulate the specific features of the cell microenvironment in native tissue [9]. In this sense, hydrogels should provide biophysical and/or biochemical cues present in the natural cell niche while presenting structural and mechanical properties matched to those of the native tissue [10]. On what concerns biophysical cues, while full length adhesive proteins provide hydrogels with multiple bioactive ligands for cell adhesion, ECM/growth factor binding, and self-assembly, short synthetic bioactive oligopeptides allow the incorporation of a higher density of bioactive domains and with higher control over their exposure [10]. Among the various cell surface adhesion receptors expressed by cells, integrins are interesting to target due to their linkage to the actomyosin cytoskeleton upon binding to ECM ligands. Integrin binding to ECM ligands is crucial for cell anchorage to the surrounding matrix, spreading, migration, and activation

of intracellular signaling pathways [11]. Additionally it is central for cell response to local mechanical stimuli, through force-induced conformational changes of proteins within focal adhesion complexes [12]. An attractive integrin candidate for NSPC targeting is integrin $\alpha 6 \beta 1$, an integrin highly expressed by human and mouse neural precursors that can potentially be used as a marker for human NSC isolation [13], [14]. Integrin $\alpha 6 \beta 1$ is mostly a receptor for laminins, which are present in the basement membrane of the embryonic neural tube [15] and in neurogenic niches [16]. Studies carried out with recombinant integrins showed that integrin $\alpha 6 \beta 1$ binds to several laminin (LN) isoforms, namely to LN 111, LN 322 and LN 511/521, the latter being its most preferred ligand [17]. Its role as a key mediator of NSPC migration was evidenced through antibody perturbation studies. Integrin $\alpha 6 \beta 1$ was found to be critical for chain migration of neural precursors *in vitro* [18] and *in vivo*, namely along the adult mouse rostral migratory stream [19]. In addition, integrin $\alpha 6 \beta 1$ has been shown to mediate outward migration of human ES-derived neural progenitors, as well as adhesion and outward migration of embryonic mouse neural progenitors, on laminin substrates [20], [21]. We therefore hypothesized that the covalent binding of synthetic ligands engaging $\alpha 6 \beta 1$ integrin to a hydrogel could enhance its ability to support ES-NSPC migration and neurite outgrowth. As in Fb, motifs interacting with $\alpha 6 \beta 1$ integrin receptors are absent [22], tethering of $\alpha 6 \beta 1$ integrin ligands to Fb was expected to enhance its capacity to support neurite extension from ES-NSPC-derived neurons, ultimately contributing to the integration of transplanted ES-NSPCs and neuronal relay formation.

In this study we investigated six peptides with reported ability to support $\alpha 6 \beta 1$ integrin-mediated cell adhesion and spreading: three peptides derived from native proteins (T1 from the angiogenic inducer CCN1 and AG10 and A5G81 from the C-terminus globular domain of human LN $\alpha 1$ chain and mouse LN $\alpha 5$ chain, respectively [23], [24], [25]) and two peptides with no sequence homology to any of the LN chains or to known proteins (HYD1 and P3), identified through the use of random peptide display libraries [26], [27], [28]. The N4 sequence presenting homology with the domain IV of netrin-4 was also tested, since netrin-4 forms a complex with the LN $\lambda 1$ chain which further binds to $\alpha 6 \beta 1$ integrin, leading to enhanced NSC migration and proliferation [29]. Peptides were initially examined for their ability to support ES-NSPC adhesion, viability, migration, and neuronal differentiation, when adsorbed to 2D surfaces. The peptides revealing the highest capability to promote NSPC adhesion mediated through $\alpha 6 \beta 1$ integrin were immobilized in Fb, and the resulting functionalized gels characterized in terms of peptide binding efficiency and ability to promote ES-NSPC migration, using a radial outgrowth assay. We report that Fb gels tethered with the synthetic peptide HYD1 are able to promote cell outgrowth from neurospheres and neurite extension of single ES-NSPCs. Moreover, we show that HYD1-functionalized Fb provides a permissive environment for axonal growth, leading to increased neurite extension from sensory neurons *in vitro* and to a trend for increased axonal growth along with improved functional recovery after spinal cord injury in adult rats, when compared to native Fb.

2. Materials and methods

2.1. Generation of neural stem/progenitor cells (NSPCs) from mouse ES cells

A modified mouse ES cell line (46C) established at the Institute for Stem Cell Research (Edinburgh University, Scotland, UK) expressing green fluorescent protein (GFP) under the promoter of the NSPC-specific *Sox1* gene, was used. Neural commitment of ES cells was attained in adherent monoculture and N2B27 medium [30]. At day 5 of the neural commitment protocol, SOX1-GFP expression was analyzed by flow cytometry, to assess the efficiency of neural conversion (Fig. S1), and cells used whenever more than 75% expressed SOX1-GFP. To obtain floating aggregates (neurospheres), ES-NSPCs were plated at 2×10^5 SOX1-GFP⁺ cells/mL into 35 mm non-tissue culture plastic Petri dishes (Ezi-Grip), and cultured in N2B27 medium supplemented with 10 ng/mL of epidermal growth factor and basic fibroblast growth factor (EGF and bFGF; both PeproTech) for three days under dynamic conditions (55 rpm) on a rotary orbital shaker.

2.2. Analysis of $\alpha 6$ and $\beta 1$ integrin expression in ES-NSPCs and floating aggregates of ES-NSPCs

The expression of $\alpha 6$ and $\beta 1$ integrin subunits in ES-NSPCs was assessed by immunocytochemistry at day 5 of the neural commitment protocol, after gentle dissociation through incubation with StemPro® Accutase® (Gibco; 2–

3 min; 37 °C). For analysis of integrin expression in floating aggregates of ES-NSPCs, neurospheres were incubated with StemPro® Accutase® (10 min; 37 °C) for cell dissociation, prior to be processed for immunocytochemistry. The distribution of $\alpha 6$ and $\beta 1$ integrin subunits in neurospheres was detected by *en bloc* immunohistochemistry, in neurospheres allowed to adhere for 2 h on poly-D-lysine (PDL, Sigma)-coated (10 $\mu\text{g/mL}$; 1 h; RT) glass coverslips. At this time point the PDL-coated coverslips were inverted and the upper part of the neurospheres analyzed by confocal laser scanning microscopy (CLSM, Leica TCS SP2).

2.3. Evaluation of $\alpha 6\beta 1$ synthetic ligands' ability to support ES-NSPC adhesion, viability, migration, and neuronal differentiation

2.3.1. Preparation of 2D substrates coated with $\alpha 6\beta 1$ ligands

Peptides T₁ (GTTSWSQCCKS), AG₁₀ (NPWHSIYITRFG), HYD₁ (KIKMVISWKG), P₃ (VSWFSRHRYSPPFAVS), N₄ (CGLPYSSVC), and A₅G₈₁ (AGQWHRVSVRWG) with a C-terminal amide were synthesized by GenScript (purity > 95%). Peptides were dissolved at 1.292 mM in Milli-Q ultrapure water (Millipore) or in acetic acid solutions, filter-sterilized, aliquoted, freeze-dried, and stored at -20 °C until further use. For adsorption onto 2D surfaces, peptides were diluted in Milli-Q ultrapure water and added to the wells of tissue-culture plates or to inserts in order to obtain peptide coating concentrations ranging from 5 to 100 nmoles/cm². Peptide adsorption was performed overnight at 37 °C. Non-adsorbed peptides were removed by rinsing twice the wells with PBS, and peptide surface density quantified. Briefly, physisorbed peptides were extracted with 1% (v/v) Triton (25 min; 4 °C; 200 rpm) and peptide concentration determined using the bicinchoninic acid method (Pierce BCA Protein Assay Kit; Thermo Scientific), according to the supplier instructions. Peptide concentration was extrapolated from a standard curve performed for each peptide, where absorbance was plotted against known concentrations of peptide.

2.3.2. ES-NSPC adhesion, viability, migration, and differentiation on 2D substrates coated with $\alpha 6\beta 1$ ligands

Peptide-coated wells/inserts were incubated with 1% (w/v) heat-inactivated bovine serum albumin (BSA, Sigma) to block non-specific adhesion, and used for assessment of ES-NSPC adhesion, viability, migration, and differentiation (details are provided in Supporting Information). Wells incubated in parallel with 1% (w/v) heat-inactivated BSA were used as negative control, while wells incubated with 100 $\mu\text{g/mL}$ of PDL, 20 $\mu\text{g/mL}$ of laminin 111 from Engelbreth-Holm-Swarm murine sarcoma (LN 111, Sigma), or 20 $\mu\text{g/mL}$ of human recombinant laminin 511 (LN 511, Biolamina), were used as positive controls. Glass coverslips previously coated with 10 $\mu\text{g/mL}$ of PDL and then with 5 $\mu\text{g/mL}$ of LN 111 (PDL-LN 111) were also prepared and used as an additional positive control in 24-h experiments or longer.

2.4. Preparation of functionalized fibrin (Fb) hydrogels

For the formation of Fb gels, a 6 mg/mL concentration of fibrinogen was selected, as this concentration was previously shown to yield gels permissive to ES-NSPC neurite extension and neuronal differentiation, and with storage modulus comparable to that of neural tissue in the adult brain [8], [31]. Synthetic $\alpha 6\beta 1$ ligands were covalently bound to Fb using the enzymatic cross-linking action of transglutaminase factor XIIIa [32]. For this purpose, bi-domain peptides containing the sequence of interest at the carboxyl terminus and a factor XIIIa substrate from the NH₂-terminal sequence of $\alpha 2$ -plasmin inhibitor (residues NQEQVSPL) at the amino terminus were synthesized at GenScript with a C-terminal amide (purity > 95%). A bi-domain peptide containing an inactive scrambled sequence of HYD₁ (HYDS – WIKSMKIVKG) [27] was also acquired for use in certain experiments. Peptides were dissolved at 1 mM in Milli-Q ultrapure water, filter-sterilized, aliquoted, and stored under nitrogen at -20 °C for use within a month. Fibrinogen solution was prepared dissolving plasminogen-free fibrinogen from pooled human plasma containing factor XIII (Sigma) in Milli-Q ultrapure water, followed by dialysis against tris-buffered saline (TBS, pH 7.4) for 24 h. The resulting fibrinogen solution was then sterile-filtered and its concentration determined spectrophotometrically at 280 nm, applying an extinction coefficient of 1.51 mL mg⁻¹ cm⁻¹ [33]. Fibrinogen solution was then diluted to 12 mg/mL with TBS. Functionalized Fb gels were formed applying equal volumes of the fibrinogen solution and a thrombin solution in TBS containing CaCl₂, aprotinin, and the bi-domain peptides [final concentration of Fb components: 6 mg/mL fibrinogen; 2 NIH U/mL

thrombin from human plasma; 2.5 mM CaCl₂; 10 µg/mL aprotinin (all Sigma-Aldrich); 1 to 320 µM of bi-domain peptides] into the wells of a 6-well non-tissue culture plate (Becton Dickinson). Polymerizing gels were then incubated at 37 °C for 30 min to allow cross-linking by factor XIIIa. Non-functionalized Fb gels and Fb gels containing 20 µg/mL LN 111 or LN 511 were also prepared and used as controls.

2.5. Characterization of functionalized Fb gels: Peptide incorporation, microstructure, and viscoelastic properties

The incorporation of bi-domain peptides into Fb was determined using ¹²⁵I-labeled bi-domain peptides, as iodination of α₂-plasmin inhibitor and that of peptides derived from its NH₂-terminus was previously shown not to alter their cross-linking to Fb by factor XIIIa [32], [34]. Functionalized Fb gels (50 µL) were prepared as described above using bi-domain peptides spiked with ¹²⁵I-labeled peptides, in order to achieve a final activity of $\cong 1.1 \times 10^4$ cpm/µg of bi-domain peptide. Fb gels containing ¹²⁵I-labeled soluble peptides or Na¹²⁵I (an amount leading to cpm values similar to those provided by ¹²⁵I-labeled bi-domains) were also prepared, and used as controls. Cross-linked Fb gels were individually transferred to radioimmunoassay (RIA) tubes, and gamma activity measured in a γ-counter (Wallac Wizard model 1470). The release of un-bound bi-domain peptides from Fb was followed over a 48-h period, incubating the gels with 125 µL of PBS buffer containing 2% (w/v) BSA. The buffer solution was transferred to new RIA tubes at 3, 6, 9, 12, 24, and 48 h for gamma activity counting, and replaced by fresh buffer. Peptide cumulative release was calculated and expressed as a percentage of Fb gel radioactivity prior to incubation in buffer, and the amount of bi-domain peptides incorporated into Fb determined when peptide diffusion reached an equilibrium plateau.

Changes in the structure of Fb network were detected using fluorescently-labeled fibrinogen. Functionalized Fb gels (50 µL) were prepared as described above, using a 1:100 ratio of Alexa Fluor® 488 human fibrinogen conjugate (Molecular Probes) to unlabeled fibrinogen. Cross-linked Fb gels were observed under CLSM using a Plan-Apochromat 63×/1.4NA Oil objective, and stacks of 10 optical sections covering a depth of 10 µm acquired. Average pore area was determined in 2D projections of CLSM stacks, using MATLAB® software for automatic segmentation of pores and area computation. For each condition, 6 images from 2 different Fb gels were examined.

The storage (G') and loss (G'') moduli of Fb hydrogels were determined by rheometry using a Kinexus Pro Rheometer (Malvern Instruments), as previously described [8].

2.6. Cell outgrowth from ES-NSPC neurospheres on functionalized Fb hydrogels

ES-NSPC migration on functionalized Fb gels was assessed using floating aggregates of ES-NSPCs (neurospheres) and the radial outgrowth assay. Functionalized Fb gels (10 µL) were formed as described above, in the lower wells of a 15-well µ-Slide Angiogenesis plate (Ibidi). Neurospheres with diameter in the range of 200–250 µm were isolated under the stereoscopic magnifier and seeded on functionalized cross-linked Fb gels (1 sphere per gel). Neurospheres were allowed to adhere for 2 h, and 40 µL of N2B27 medium supplemented with 10 ng/mL bFGF and 10 µg/mL aprotinin added to each well. Half of the medium was refreshed after 48 h of cell culture, and bFGF concentration reduced to 5 ng/mL. To assess the contribution of specific integrins to radial outgrowth, function blocking monoclonal antibodies against α₆ (clone NK1-GoH3, Serotec; 33.3 µg/mL), α₃ (clone P1B5, Millipore; 10 µg/mL), or β₁ (clone Ha2/5; BD Pharmingen; 10 µg/mL) integrin subunits, as well as isotype-matched controls for the α₆ (rat IgG2a clone 2A3, Millipore; 33.3 µg/mL), α₃ (mouse IgG1-k MOPC-21 clone, Millipore; 10 µg/mL), and β₁ antibodies (hamster IgM λ₁, Pharmingen; 10 µg/mL), were added to the culture medium. Radial outgrowth was determined in samples processed for F-actin/DNA staining at 72 h of cell culture and at 48 h for integrin blocking studies. Briefly, cells were fixed in 3.7% (w/v) paraformaldehyde (PFA) solution diluted 1:1 in culture media (30 min; 37 °C), permeabilized with 0.2% (v/v) Triton X-100 in PBS (20 min; RT), and incubated with 1% (w/v) BSA for 1 h, to minimize non-specific adsorption. F-actin was visualized incubating the samples with Alexa Fluor® 594-conjugated phalloidin (Molecular Probes; 1:100; 20 min; RT) while DNA was labeled with DAPI (Sigma; 0.1 µg/mL; 10 min; RT). Samples were finally mounted with Fluoromount™ (Sigma) and observed with the IN Cell Analyzer 2000 imaging system (GE Healthcare). Radial outgrowth was defined as the region comprised between the neurosphere edge

and the migration front. Radial outgrowth was determined in the 3D image stacks, by segmenting the volume of the neurosphere and that correspondent to cells cytoskeleton, using DNA and F-actin fluorescence images, respectively. This process was guided by an initial 2D maximal projection of the data in Z. The outgrowth area was given by the 2D maximal projection in Z of F-actin stack images subtracting the neurosphere area, given by the 2D maximal projection of DNA fluorescence images. Outgrowth area was subsequently normalized to the neurosphere area at time point zero, determined in phase contrast images of the neurospheres immediately before the addition of culture media. Maximal outgrowth distance was computed analyzing the two segmented volumes, namely the distance from the neurosphere volume boundary to the boundary of the volume correspondent to cells cytoskeleton (Fig. S2).

2.7. Neuronal differentiation of ES-NSPCs within functionalized Fb hydrogels

ES-NSPCs were embedded as single cells in functionalized Fb gels (final cell seeding density: 1×10^6 cells/mL) and cultured for periods up to 14 days under neuronal differentiation conditions. At different time points, the cell-matrix constructs were processed for analysis of neurite outgrowth, distribution of viable/dead cells, total cell number, and expression of characteristic phenotypic markers: nestin (NSPC marker), β III-tubulin (early neuronal marker), synapsin (synaptic vesicles marker), and glial fibrillary acidic protein (GFAP, astrocytic marker).

2.8. Neurite outgrowth from rat E18 dorsal root ganglia explants cultured within functionalized Fb hydrogels

The effect of immobilized $\alpha 6 \beta 1$ ligands on neurite outgrowth from primary neurons was assessed using dorsal root ganglia (DRGs) dissected from E18 Wistar rat embryos. DRGs were embedded in functionalized Fb gels (10 μ L) during Fb polymerization (1 DRG per gel), and average neurite length quantified after 48 h of culture in samples processed for β III-tubulin/DNA staining.

2.9. In vivo experiments

All procedures involving animals were approved by the local animal ethics committee and by the Portuguese official authority on animal welfare and experimentation (DGAV – Direção-Geral de Alimentação e Veterinária) in accordance with the EU Directive (2010/63/EU) and the Portuguese law (DL 113/2013). To assess the effect of functionalized Fb gels on axonal regeneration, an *in vivo* model of spinal cord injury (SCI; total transection) was used. Chitosan tubular scaffolds were used to bridge the cavity formed upon spinal cord transection and provide mechanical support to the hydrogel (Fig. S3). Tubular scaffolds were prepared from squid pen chitosan (France Chitine; degree of acetylation 3.55) after purified (endotoxin levels < 0.1 EU/mL), as previously described [35]. For each experimental group, 10 female Wistar rats (10–13 weeks old) from Charles River Laboratories were used. Briefly, the spinal cord was transected by removing a 4 mm region encompassing T8, and chitosan tubular scaffolds (4-mm-long) prefilled with functionalized Fb gel or unmodified Fb (control) inserted into the defect, aligned along the longitudinal axis of the spinal cord. Animals were allowed to recover for 9 weeks and their locomotor function assessed once a week, using the Basso, Beattie, and Bresnahan (BBB) score [36]. Ten weeks post-implantation, the animals were perfused and their spinal cords processed for cryostat sectioning. Longitudinal (coronal) cryostat sections (20 μ m thickness) were serially collected and immunofluorescence labeled for detection of growth associated protein 43 (GAP43, marker for axonal growth cone formation), β III-tubulin, GFAP, ionized calcium binding adaptor molecule 1 (Iba1, microglia and macrophage marker), nestin, and laminin. Sections were examined with the IN Cell Analyzer 2000 imaging system using a 20x/0.75 NA objective or using the CLSM. Axonal sprouting/regeneration in each animal was determined in 2D projections of the IN Cell Analyzer 3D stack images, analyzing the area of GAP43⁺ axons in the lesion area, expressed as a percentage of the total lesion area. For each animal, four cryostat sections were analyzed and GAP43⁺ area averaged. A further detailed description of the methodologies and analysis used in the *in vivo* experiments is provided in Supplementary Data.

2.10. Statistical analysis

All *in vitro* and *ex vivo* experiments were performed at least three times and data treated using IBM® SPSS® Statistics Software (version 23). Statistically significant differences between two conditions were detected using the Student's *t*-test. To compare three or more conditions the one-way ANOVA was performed, followed by the Bonferroni correction for pairwise comparisons or the Dunnett's two-tailed test for comparisons with the control condition. Concerning the *in vivo* experiments, differences between BBB scores were assessed using a mixed model repeated measures ANOVA in which the *condition* tested was the between-subjects factor and *time* was the within-subjects factor (repeated measures), according to Scheff et al. [37]. A simple effect test and Bonferroni post hoc tests were then performed to detect specific differences between groups. Results were considered significant for $p < 0.05$.

A further detailed description of the methodologies followed in this study is provided in Supplementary Materials and Methods.

3. Results

3.1. Expression of $\alpha 6$ and $\beta 1$ integrin subunits by ES-NSPCs and floating aggregates of ES-NSPCs

The expression of $\alpha 6$ and $\beta 1$ integrin subunits in ES-NSPCs was examined by flow cytometry in neural progenitors from three different neural commitments. Representative fluorescence histograms are presented in Fig. 1. NSPCs derived from the 46C mouse ES cell line showed $\alpha 6$ integrin expression in nearly all cells examined ($95.7 \pm 0.5\%$; Fig. 1A), which is a percentage of $\alpha 6$ positive cells higher than that reported for NSPCs derived from human ES cells through the differentiation of embryoid bodies ($\sim 46\%$) [20]. This fact may be associated to the more homogeneous access of cells to oxygen/nutrients/morphogens in adherent culture conditions than in suspension cultures of differentiating embryoid bodies. Almost all 46C-derived NSPCs expressed the $\beta 1$ integrin subunit ($91 \pm 1.6\%$; Fig. 1A), similarly to human ES-NSPCs [20]. As in NSPCs $\alpha 6$ mostly dimerizes with $\beta 1$ integrin subunit (the percentage of cells expressing $\beta 4$, the other partner for $\alpha 6$, is either absent or lower than 15% [13], [20], [38]), we assumed that $\alpha 6\beta 1$ integrin was widely expressed by 46C-NSPCs. ES-NPCs subsequently expanded as floating aggregates retained the expression of $\beta 1$ integrin ($92.9 \pm 2.6\%$ $\beta 1$ integrin⁺ cells; Fig. 1B and D), but showed a small decrease in the percentage of $\alpha 6$ integrin positive cells ($88.3 \pm 0.5\%$; Fig. 1B and C). This reduction may be related to the presence of a small percentage of cells undergoing differentiation when grown as spheroids (13% of β III-tubulin⁺ cells, in average) [39], as NSC differentiation was reported to induce a decrease in $\alpha 6$ integrin expression without influencing that of $\beta 1$ integrin subunit [29].

3.2. ES-NSPC adhesion, viability, migration, and differentiation on 2D substrates coated with $\alpha 6\beta 1$ ligands

Six synthetic peptides with reported affinity to $\alpha 6\beta 1$ integrin were evaluated in terms of ability to support ES-NSPC adhesion mediated through $\alpha 6\beta 1$ integrin, viability, migration, and differentiation. For this purpose, ES-NSPCs were seeded on peptide-adsorbed wells and cultured for periods up to 6 days. Wells coated with PDL or LN 111, known to mediate nonspecific or integrin-dependent adhesion and migration of NSPCs, respectively [21], [38], [40], were used as positive controls. LN 511 coatings were used as an additional positive control, since LN 511 is described to be the most preferred LN isoform ligand for integrin $\alpha 6\beta 1$ [17]. Within the range of peptide coating concentrations initially tested (5 – 100 nmol/cm²), that of 10 nmol/cm² was found to allow the attainment of a plateau of adherent cell numbers on all peptide-adsorbed surfaces (Fig. S4). As a result, this peptide coating concentration was used in these studies. ES-NSPC adhesion to the different peptides was initially investigated using a centrifugation adhesion assay [41]. The application of a centrifugal force after 2 h of cell culture revealed for HYD1 higher percentages of adherent cells as compared to P3 and N4 ($p = 0.017$ and $p < 0.001$, respectively), and corresponding to 84% of those observed on LN 111 (Fig. 2A). Cell adhesion quantified by the crystal violet colorimetric assay did not show statistical significant differences among peptides though a similar trend was found. Specifically, HYD1 revealed numbers of adherent cells of approximately 80% of LN 111 whilst on P3- and N4-adsorbed surfaces cell numbers did not exceed 60% of those observed on LN 111 (Fig. 2B). To assess if cell adhesion to the different surfaces was integrin-dependent, we incubated ES-NSPCs with EDTA, as divalent cations such as Mn^{2+} modulate integrin ligand-binding affinity [42]. Incubation with EDTA resulted in a decrease in the

number of adherent cells on T1-, HYD1-, and A5G81-adsorbed surfaces of approx. 50% ($p \leq 0.013$, Fig. 2C), indicating that ES-NSPC adhesion to these peptides is divalent cation-dependent. Cell adhesion following ES-NSPC incubation with function-perturbing monoclonal antibodies against $\alpha 6$ and $\beta 1$ integrin subunits was subsequently performed, to assess the contribution of $\alpha 6 \beta 1$ integrin to cell adhesion. Antibody concentrations optimized to efficiently inhibit ES-NSPC cell adhesion were used (details are provided in Supplementary Data). In the presence of a function-perturbing antibody against $\alpha 6$ integrin subunit, cell adhesion to T1-, HYD1-, and A5G81-adsorbed surfaces was inhibited namely in the range of 41–56% ($p \leq 0.002$ vs. the isotype-matched control antibody or untreated cells), evidencing that ES-NSPC adhesion to these peptides is partially mediated through $\alpha 6 \beta 1$ integrin (Fig. 2D). Incubation with a function-perturbing antibody against $\beta 1$ integrin subunit led to similar or higher cell adhesion inhibition levels on these surfaces, further supporting this hypothesis ($p \leq 0.001$ vs. the isotype-matched control antibody or untreated cells). As expected, incubation with function-perturbing antibodies against $\alpha 6$ or $\beta 1$ integrin subunits resulted in cell adhesion inhibition levels on PDL similar to those induced by the isotype-matched control antibodies (one-way ANOVA followed by Bonferroni's test), pointing that cell adhesion to PDL is not mediated through $\alpha 6 \beta 1$ integrin. The effect of physisorbed peptides on ES-NSPC viability was assessed after 24 h of cell culture, using a resazurin-based assay. Results revealed for HYD1- and A5G81-adsorbed wells numbers of viable cells similar to LN 111, and higher cell numbers on HYD1-coated surfaces than on surfaces coated with T1, AG10, P3, or N4 ($p \leq 0.035$, Fig. 2E). Peptide effect on cell migration was evaluated plating ES-NSPCs into Transwell inserts with peptide-coated membranes (8- μ m pore). Amongst the tested peptides, HYD1 was the one supporting the highest cell migration ($p \leq 0.02$ vs. any other peptide, Fig. 2F). The reduced cell migration observed for peptide-coated membranes in comparison to Transwell inserts coated with LN 111/511 is possibly associated to the lack of sequences sensitive to cell-secreted proteases such as those present in LN, which have a direct effect on cell invasion/migration. Finally we assessed the ability of physisorbed peptides to support ES-NSPC differentiation along the neuronal lineage. At the end of 6 days of culture under neuronal differentiation conditions, NSPCs showed immunoreactivity against the early neuronal marker β III-tubulin on all peptide-adsorbed surfaces (60 to 84% of β III-tubulin⁺ cells, expressed as a function of LN 111), as well as long neuronal extensions which could not be observed on BSA-coated surfaces (Fig. S5). Importantly, none of the peptides induced astrocytic differentiation, as shown by the absence of GFAP⁺ cells. As HYD1, T1, and A5G8 were found to support both divalent cation- and integrin $\alpha 6 \beta 1$ -dependent NSPC cell adhesion, they were selected for immobilization in Fb. An additional information provided by these studies was the higher ability of physisorbed LN 511 (herein used as positive control) to promote ES-NSPC adhesion, viability and migration, when compared to LN 111. Specifically, coating with LN 511 resulted in a 1.4-fold increase in the number of adherent cells ($p < 0.001$), 1.5-fold increase in the number of viable cells ($p < 0.001$), and to a 1.8-fold increase in the number of migrated cells ($p < 0.001$), as compared to LN 111, (Fig. 2B, E-F).

3.3. Peptide incorporation into Fb hydrogels

The incorporation of bi-domain peptides into Fb was determined using ¹²⁵I-labeled bi-domain peptides. The release of un-bound peptides from Fb gels formed using a 20 μ M input concentration of bi-domain peptides in the polymerizing Fb gel is shown in Fig. 3A. The diffusion of un-bound bi-domain peptides reached equilibrium after 12 h of incubation in PBS containing BSA, while free iodine (Na¹²⁵I) diffused during the first 6 h. Peptide cumulative release determined after 24 h of incubation shows for all the peptides tested increased amounts of immobilized peptide with increasing input peptide concentrations. HYD1 and A5G81 bi-domain peptides revealed similar average binding efficiencies ($20.0 \pm 3.8\%$ and $19.2 \pm 3.7\%$, respectively), while T1 bi-domain peptide showed a higher average incorporation efficiency ($31.9 \pm 7.4\%$; $p < 0.001$, T1 vs. HYD1; $p < 0.001$, T1 vs. A5G81, one-way ANOVA) (Fig. 3B). To get insight into the fraction of peptide incorporated into Fb through non-covalent interactions (such as electrostatic interactions), Fb gels containing 20 μ M of soluble peptides (A5G81) were also prepared, and peptide diffusion compared to that of the correspondent bi-domain peptide. After 24 h of incubation, a reduced peptide retention was found for A5G81 as compared to the bi-domain peptide ($8.2 \pm 2.7\%$ vs. $21.9 \pm 3.6\%$; $p = 0.001$, Student's *t* test), as expected from the covalent immobilization of the peptide through the activity of factor XIIIa (Fig. S6).

3.4. Cell outgrowth from ES-NSPC neurospheres on functionalized Fb hydrogels

To assess the effect of immobilized ligands on NSPC migration, floating aggregates of ES-NSPCs (neurospheres) were seeded on functionalized Fb gels and radial outgrowth (outgrowth area and maximal outgrowth distance) determined after 72 h of cell culture using automatic image analysis. As the quantification of the outgrowth distance relying on 2D maximal projections of image stacks may collapse or even ignore cell outgrowth in the Z axis, the maximal outgrowth distance was computed analyzing the cell outgrowth volume. Results are presented in Fig. 4A. The addition of soluble LN to Fb polymerizing gel was not able to promote cell outgrowth from neurospheres, as shown by the decrease in the radial outgrowth area observed for the two LN isoforms used ($p = 0.020$, Fb + LN 111 vs. unmodified Fb; $p = 0.008$, Fb + LN 511 vs. unmodified Fb). The analysis of the maximal outgrowth distance revealed the same trend, depicting a significant reduction in the maximal outgrowth in gels containing soluble LN 511 ($p = 0.008$ vs. unmodified Fb). Functionalization of Fb with A5G81 was also not effective in promoting cell outgrowth, independently of the input concentration tested. Yet, covalent binding of T1 or HYD1 bi-domain peptides showed a biphasic effect on radial outgrowth. T1 immobilization resulted in enhanced cell outgrowth area when incorporated at 20 or 40 μM in the polymerizing Fb gel, leading to a 1.9- and 2.2-fold increase relative to unmodified Fb, respectively ($p < 0.001$). In what concerns HYD1, input peptide concentrations of 10, 20, and 40 μM were efficient in promoting radial outgrowth ($p \leq 0.009$ vs. unmodified Fb), maximal enhancement being observed for an input concentration of 20 μM , which resulted in a 2.4- increase in the outgrowth area relative to native Fb. Still, when comparing radial outgrowth enhancement induced by T1 or HYD1, no statistically significant differences were found among them. With increasing input peptide concentrations a trend for a reduction in the outgrowth area was observed. This effect was more pronounced in the case of HYD1, which, at the maximal input concentration, led to cell outgrowth inhibition ($p = 0.006$, 320 μM HYD1 vs. unmodified Fb). Among the two peptides, HYD1 was the only peptide eliciting an increase in the maximal outgrowth distance besides leading to higher cell outgrowth areas. The maximal enhancement was observed for an input concentration of 20 μM , for which a 1.9-fold increase in the maximal outgrowth distance was observed ($p < 0.001$ vs. unmodified Fb). Maximal outgrowth distance revealed therefore a biphasic response to HYD1, and followed the same trend observed for the cell outgrowth area. We subsequently assessed if the addition of 20 μM of a bi-domain peptide containing a scrambled sequence of HYD1 (HYDS) was also able to induce an increase in radial outgrowth. As shown in Fig. 4B, radial outgrowth on HYDS-functionalized Fb gels was statistically similar to that of unmodified Fb (Student *t*-test), either considering the outgrowth area or the maximal outgrowth distance, evidencing the specificity of HYD1 to engage with ES-NSPCs. The T1-/HYD1-functionalized gels leading to maximal outgrowth area enhancement were then characterized in terms of ability to promote outward migration mediated through $\alpha 6 \beta 1$ integrin. The contribution of $\alpha 3 \beta 1$ integrin to radial outgrowth was also assessed, as HYD1, besides targeting the $\alpha 6 \beta 1$ heterodimer, also interacts with $\alpha 3 \beta 1$ integrin [27], an integrin which is also expressed by ES-NSPCs (Fig. S7). On unmodified Fb gels, incubation with function-perturbing monoclonal antibodies against $\alpha 6$ and $\alpha 3$ integrin subunits barely affected the cell outgrowth, reducing the outgrowth area by 18% and 31%, respectively ($p = 0.164$ and 0.041 respectively, vs. the correspondent isotype control antibody; Fig. 4C). In contrast, on T1-/HYD1-functionalized gels, blocking $\alpha 6$ and $\alpha 3$ integrin subunits decreased the outgrowth area by 71 to 88% ($p \leq 0.002$ vs. isotype-matched control antibodies or untreated cells), indicating that promotion of cell outgrowth by immobilized T1 and HYD1 is mediated through $\alpha 6 \beta 1$ and $\alpha 3 \beta 1$ integrins. The addition of a function blocking antibody to $\beta 1$ integrin subunit (which dimerizes with several α integrin subunits) completely inhibited cell outgrowth on both unmodified and T1-/HYD1-functionalized gels, reducing the outgrowth area by 94–96% ($p < 0.001$ vs. isotype-matched control antibodies or untreated cells). As the structural and viscoelastic properties of hydrogels can modulate cell behavior, we assessed whether the incorporation of T1 or HYD1 bi-domain peptides affected Fb network structure, specifically the average pore diameter, and the storage and loss modulus. As shown in Fig. 4D, for input peptide concentrations of 20 and 40 μM , T1 or HYD1 immobilization did not significantly impact the average pore area of Fb network, neither the storage modulus of the hydrogels (ANOVA followed by Dunnett's test). Representative graphs of frequency sweep tests of unmodified and functionalized Fb gels are provided in Fig. S8.

3.4.1. 3D culture of single ES-NSPCs in HYD1-functionalized Fb gels: Neurite extension, cell viability, proliferation, and neuronal differentiation

The impact of immobilized $\alpha 6 \beta 1$ integrin-binding ligands on ES-NSPCs when seeded as single cells in Fb was subsequently assessed. For this study Fb gels functionalized with 20 μM of HYD1 were used, due to their better performance in terms of ability to promote radial outgrowth from neurospheres (considering both the outgrowth

area and the maximal outgrowth distance). Results are presented in Fig. 5. ES-NSPCs entrapped within Fb as single cells rapidly proliferated forming spheroidal clone-like multicellular clusters, as previously reported [8]. Fibrin functionalization with HYD1 promoted neurite extension, as shown by the higher number of neuronal processes extending from cellular spheroids and higher length of the longest neurite found in HYD1-functionalized gels at day 6 of cell culture when compared to native Fb (1.5- and 2.0-fold higher, respectively, Fig. 5A). ES-NSPCs cultured in HYD1-functionalized Fb showed similar distribution of viable/dead cells at this time point of cell culture (Fig. 5B), and statistically similar cell numbers at days 7 and 14 of cell culture, as determined quantifying total DNA amount (Fig. 5C). Cell/Fb constructs processed for immunofluorescence microscopy after 14 days of cell culture reveal for both conditions cells expressing nestin (NSPC marker) and a dense network of neurites staining for β III-tubulin (early neuronal marker) with synaptic vesicles, radially sprouting from cellular spheroids and infiltrating the gel (Fig. 5D). Flow cytometry analysis of cells isolated from cell/Fb constructs at this same time point and subsequently processed for immunolabeling of β III-tubulin or GFAP (astrocytic marker), further suggests that immobilized HYD1 did not affect ES-NSPC differentiation in Fb (Fig. 5E).

3.5. Effect of HYD1-functionalized Fb hydrogel on neurite outgrowth from rat E18 dorsal root ganglia

To get insight into the effect of immobilized ligands on axonal growth, HYD1-functionalized Fb hydrogel was evaluated in terms of ability to support neurite outgrowth from rat E18 dorsal root ganglia (DRGs) explants. The average neurite length of DRGs cultured within Fb gel functionalized with 10, 20, 100, or 250 μ M of bi-domain peptide is presented in Fig. 6. Tethering of HYD1 resulted in enhanced neurite outgrowth for input concentrations ≥ 20 μ M, when compared to unmodified Fb ($p \leq 0.022$), maximal enhancement being attained at an input concentration of 20 μ M, for which a 2-fold increase in the average neurite length of DRGs was observed when compared to unmodified Fb ($p < 0.001$). Higher concentrations of bi-domain peptide resulted in lower enhancement in neurite outgrowth ($p < 0.01$, HYD1 20 μ M vs. HYD1 100 and 250 μ M). As T1- and A5G81-functionalized gels could also present ability to promote neurite extension of sensory neurons, these were subsequently evaluated. Similarly to HYD1-functionalized gels, neurite extension in both functionalized gels showed a biphasic response to peptide input concentration (Fig. S9), the highest increase in the average neurite length being observed in gels functionalized with 100 μ M of T1 bi-domain peptide (1.9-fold increase; $p \leq 0.001$ vs. unmodified Fb).

3.6. Effect of HYD1-functionalized Fb hydrogels on axonal regeneration, in a rat model of spinal cord injury

The effect of HYD1-functionalized Fb on axonal regeneration in an animal model of SCI was also evaluated. Fb gels functionalized with 20 μ M of HYD1 were selected for this purpose. Results from locomotor function assessment are presented in Fig. 7A. Repeated measures ANOVA showed a significant main effect for condition (Unmodified Fb versus HYD1-functionalized Fb; $F(1,16) = 5.03$, $p = 0.039$), a significant main effect for time ($F(3,5,56) = 21.27$, $p < 0.001$), and a significant condition $\times$ time interaction ($F(3,5,56) = 2.81$, $p = 0.040$), indicating that the effects observed on the BBB score over time differed depending on the condition. Simple effect tests further revealed significant differences between the two conditions ($p \leq 0.018$) from week 5 post-implantation until the end of the assay. Indeed, at the end of the experiment, 40% of the animals receiving HYD1-functionalized Fb attained a BBB score higher or equal to 8 (sweeping with no weight support or plantar placement of the paw with no weight support), while in animals receiving unmodified Fb, this score was only reached by 10% of the animals (Fig. S10A). After 10 weeks, spinal cords removed from operated animals showed the formation of a connective tissue bridge between the two stumps of the spinal cord (Fig. S10B). In both experimental groups, GAP43⁺ fibers were present at the rostral and caudal site of the lesion and inside the tubular scaffold (Fig. 7B). Still, animals treated with HYD1-functionalized Fb showed a trend to an increased area occupied by GAP43⁺ fibers ($p = 0.08$ vs. unmodified Fb; Fig. 7C). Both types of implants revealed infiltrating cells expressing β III-tubulin often associated to laminin in the central region of the implant/lesion site (Fig. 7D), as expected from regenerating neurons [43]. The tissue bridges also contained cells expressing the neural progenitor marker nestin (Fig. 7E), precursor cells that may derive from ependymal cells of the central canal or from subpial astrocytes, which have been reported to differentiate into radial glia cells upon activation by injury [44]. In animals treated with HYD1-functionalized Fb and similarly to the control group, cells expressing GFAP were mainly found at the borders of the lesion, whereas inside the tubular

scaffolds the astrocytic cell population was minimal (Fig. 7F). Finally, Iba-1 immunolabeling showed for the two experimental groups similar distribution of activated microglia and macrophages, both at the implant/spinal cord interface as well as within the implant (Fig. S10C).

4. Discussion

In this study we assessed if the grafting of synthetic peptides engaging the laminin cell receptor integrin $\alpha 6 \beta 1$ could be used as a strategy to enhance the ability of a hydrogel to support ES-NSPC migration and neurite outgrowth. For this purpose we used NSPCs derived from mouse ES cells in chemically-defined medium and adherent monoculture, after being shown to express $\alpha 6$ and $\beta 1$ integrin subunits.

Despite the attempts made to identify specific sequences mimicking the integrin binding activity of laminins, selection of the most effective $\alpha 6 \beta 1$ ligand is not straightforward. Conformation is important for ligand recognition and the optimal integrin $\alpha 6 \beta 1$ adhesive peptide might not necessarily correspond to an ECM sequence. For instance, studies by Ito and colleagues indicate that the binding site for integrin $\alpha 6 \beta 1$ in LN 511, located in the LG1 module of the globular domain of LN α chain, requires the tandem array of LG1–3 modules and the glutamic acid residue within the C-terminal region of LN γ chain for active conformation and full binding activity [45], [46]. For this study, we chose six synthetic peptides with reported ability to promote integrin $\alpha 6 \beta 1$ -mediated cell adhesion or spreading. Peptide capability to support ES-NSPC adhesion and migration was first evaluated using adsorbed peptides. Regardless of P3 and N4 reported affinity to the $\alpha 6 \beta 1$ receptor, both physisorbed peptides failed to promote divalent cation- or integrin $\alpha 6 \beta 1$ -dependent ES-NSPC adhesion. As these peptides have been used either conjugated with bovine serum albumin (P3) or in the soluble form (N4) [28], [29], these results suggest that peptides underwent conformational changes following physisorption which prevented ligand recognition. The other four peptides (T1, AG10, HYD1, and A5G81) showed ability to support integrin $\alpha 6 \beta 1$ -mediated ES-NSPC adhesion, while sustaining neuronal differentiation of ES-NSPCs. Of note, among all, HYD1 was the peptide eliciting the highest cell migration while leading to percentages of adherent cells and numbers of viable cells similar to those present on LN 111. All peptides were selected for immobilization into Fb except AG-10, since on AG10-adsorbed surfaces ES-NSPCs showed a tendency for reduced cell viability and migration. The fact that cell binding to AG10 was not affected by EDTA suggests that AG10 interaction with $\alpha 6 \beta 1$ integrin occurred distant from metal-ion-dependent adhesion sites (MIDAS), in which divalent cations such as Mn^{2+} modulate integrin ligand-binding affinity by inducing conformational changes [42], [47].

Peptides T1, HYD1, and A5G81, carrying a substrate for activated factor XIII from the $\alpha 2$ -plasmin inhibitor at the N-terminus, were bound to Fb through the enzymatic cross-linking action of factor XIIIa. This strategy allows the covalent incorporation of peptides into Fb to sites in the fibrin(ogen) α chain not used for intermolecular fibrinogen cross-linking [48], with retention of biological activity [49]. In the range of input peptide concentrations tested, a saturation plateau for covalent incorporation of the bi-domain peptides was not observed for any of the peptides. Peptides were incorporated at levels up to 6.27 ± 0.65 mol/mol fibrinogen (observed for a concentration of $320 \mu M$ of T1 bi-domain peptide in the polymerizing Fb solution), which is far below the saturating levels reported for peptide incorporation into Fb using this approach, namely of 17 mol of peptide per each of the two α chains of the fibrinogen molecule, attained when a large molar excess of peptide over fibrinogen is used [32]. The average peptide binding efficiencies found (19% through 32%), apparently low, are in fact slightly superior to the maximum cross-linking efficiency reported for factor XIIIa-catalyzed incorporation of bi-domain peptides containing the same factor XIIIa substrate (~14%) [49], a fact that may be associated with the different peptide labeling techniques used for peptide incorporation quantification.

To disclose the effect of immobilized T1, HYD1, and A5G81 on NSPC migration, the radial outgrowth assay was performed and Fb gels containing soluble LN 111/511 used as controls. Cell migration is a process requiring a sustained balance between adhesion and tractile forces governed by a signaling axis involving integrin-ligand interactions, RhoA signaling, and actomyosin contractility forces, ultimately dependent on matrix properties, soluble factors and proteolytic activity [50], [51]. Integrin-ligand interactions are key in this process, and depend on integrin expression levels, ligand levels, and integrin-ligand binding affinity [52], [53]. Studies have shown that the higher the integrin-ligand binding affinity, the lower the ligand concentration required to induce maximal cell migration or neurite extension [52], [53]. Tethering of T1 or HYD1 bi-domain peptides resulted in enhanced cell

outgrowth area when incorporated at 20 or 40 μM in the polymerizing Fb gel, and, in the case of HYD1, also in enhanced maximal outgrowth distance. The ability of HYD1 to induce radial outgrowth enhancement similar to that elicited by T1, but at lower levels of immobilized peptide in cross-linked gels relative to T1 ($p = 0.001$, HYD1 20 μM vs. T1 20 μM ; $p < 0.001$, HYD1 40 μM vs. T1 40 μM), suggests that HYD1 has a higher affinity for receptor binding than T1. Studies have evidenced that cell migration and neurite extension in 2D and in 3D microenvironments exhibit a biphasic dependence on ligand concentration, the fastest migration occurring at an intermediate level of receptor occupancy, behind which further increasing the cell-matrix adhesiveness results in reduced cell migration [52], [53]. The biphasic effect of immobilized T1 and HYD1 on radial outgrowth is consistent to these observations. Particularly the trend for cell migration inhibition observed in gels formed at input concentrations of HYD1 higher than 40 μM , suggests that over this input concentration of HYD1 Fb gels became too adhesive. The use of functional blocking antibodies evidenced that the migratory behavior elicited by T1 and HYD1 immobilized peptides was mediated by $\alpha 6 \beta 1$ and $\alpha 3 \beta 1$ integrins. In agreement to our results, the D-aminoacid sequence of HYD1 is described to interact with both $\alpha 6 \beta 1$ and $\alpha 3 \beta 1$ integrins and to support cell adhesion when immobilized [27]. T1 peptide from the angiogenic inducer CCN1 is described to support $\alpha 6 \beta 1$ -mediated cell adhesion, also in line with our findings, but, at the best of our knowledge, its interaction with $\alpha 3 \beta 1$ integrin has not been previously described [23]. Tethering of T1 or HYD1 at input peptide concentrations of 20 or 40 μM did not induce significant changes in the average pore diameter of Fb network or in Fb storage modulus. These results are in accordance with previous findings reporting minor disruption of Fb structure or alterations in Fb rheological properties due to peptide incorporation [49], [54]. Our results therefore indicate that the cell outgrowth enhancement observed in these gels was not associated to changes in Fb network structure or viscoelastic properties.

In our study, the incorporation of soluble LN 111/511 did not promote cell migration, despite of their multiple bioactive domains, resulting in partial inhibition of cell outgrowth, more evident in the case of LN 511. These findings may be related to the absence in Fb of binding motifs for LN and to the competition for receptor binding of exogenous soluble LN with cell-secreted LN, which is detected in 3D cultures of ES-NSPCs in Fb mostly near cellular aggregates [8]. As expected, blocking of $\alpha 6$ and $\alpha 3$ integrin subunits showed a minor impact on radial outgrowth on unmodified Fb, due to the absence in Fb of motifs interacting with these integrins [22]. The small levels of outgrowth inhibition registered may be related to the presence of cell-secreted LN, which, by being prevented to bind to $\alpha 6 \beta 1$ and $\alpha 3 \beta 1$ integrins was unable to provide adhesive cues for NSPC migration. In contrast, blocking of $\beta 1$ integrin subunit resulted in a high cell outgrowth inhibition on unmodified Fb ($\sim 96\%$, $p < 0.001$). Fb contains two endogenous RGD sequences that provide adhesiveness cues for neurite outgrowth [53]. Therefore, this result suggests that $\beta 1$ integrin subunit may be involved in ES-NSPC binding to Fb RGD domains, most possibly combined with $\alpha \nu$ integrin subunit [55]. Fibronectin, present as a contaminant in commercially-available fibrinogen and secreted by ES-NSPCs during Fb remodeling [8], may have also contributed to this effect. Fibronectin ability to support cell migration of neural progenitors is primarily mediated by $\alpha 5 \beta 1$ integrin [21] and, in contrast to LN, fibronectin is sequestered by Fb through high affinity binding domains [22]. Finally, the inability of A5G81 bi-domain peptide to promote radial outgrowth suggests that A5G81 may require a free N-terminus for its bioactivity.

The effect of immobilized $\alpha 6 \beta 1$ integrin-binding ligands on the behavior of ES-NSPCs when seeded as single cells in Fb was then assessed using Fb gels functionalized with HYD1 (20 μM in the polymerizing gel). HYD1-functionalized gels led to significantly higher neurite extension from ES-NSPCs in a 3D environment, further supporting the pro-migratory activity of immobilized HYD1. Alterations in ES-NSPC proliferation or in the percentage of cells undergoing neuronal differentiation were not detected. The lack of an effect of immobilized HYD1 on cell proliferation is not surprising given that $\alpha 6 \beta 1$ integrin is not involved in LN-induced NSPC proliferation, unlike in LN-induced NSPC migration [18]. Moreover, although ES-NSPC neuronal differentiation is induced on LN substrates [20], $\alpha 6 \beta 1$ integrin involvement in this process still remains to be examined.

Finally, as $\alpha 6$ integrin mRNA was reported to be up-regulated in regenerating DRG neurons and spinal motoneurons in adult rats after axonal injury [56], [57], HYD1-functionalized Fb gel was evaluated in terms of ability to promote neurite extension in an *ex vivo* model of axonal growth. Immobilized HYD1 revealed to be effective in promoting neurite extension of rat E18 DRG neurons inducing a biphasic response similar to that observed for ES-NSPC neurospheres. However, cell response of DRG neurons to $\alpha 6 \beta 1$ ligands was observed over a broader range of input peptide concentrations. This finding may be explained by the ability of sensory neurons

to post-translationally alter the amount of $\alpha 6 \beta 1$ integrin receptors expressed at the surface as a function of ligand availability, to maintain neurite outgrowth and neuronal growth-cone motility over a wide range of ligand densities [58]. Therefore, these results prompted us to assess the effect of HYD1-functionalized gel on axonal regeneration *in vivo*. To examine axonal regeneration we used a rodent model of SCI in which the spinal cord is completely transected. A 4-mm-long segment of the spinal cord was further removed to assure the absence of spared axons between the nerve stumps [43]. Even though being the most reliable model to study axonal regeneration, complete transections are extremely disabling to animals, and the BBB scores attained by untreated animals at 9 weeks post-surgery typically range from 2 to 3 [7], [59]. Previous studies report that polymeric conduits filled with Fb hydrogel lead to increased axonal regeneration as compared to unfilled conduits, when implanted in a completely transected spinal cord [60]. As the objective was to compare HYD1-functionalized Fb with unmodified Fb, and the presence of the chitosan porous conduit was transversal to the two conditions, animals receiving empty chitosan conduits were not included as a control group. We observed that rats treated with HYD1-functionalized Fb attained BBB scores higher than those observed for unmodified Fb, and notably, comparable to those reported for rats with complete spinal cord transection treated with much complex combinatorial therapies [59]. Furthermore, the presence of GAP43⁺ fibers both in the central, rostral and caudal sites of the implantation/lesion area in both experimental groups indicates that the tethering of HYD1 to Fb did not affect Fb ability to support axonal growth. While in partial spinal cord lesions, axonal growth may arise either from regeneration of transected axons or sprouting of neighboring intact axons, in complete transection lesions axonal growth can only be associated with regeneration, particularly if a spinal cord segment is also removed. As such, our results provide evidence that HYD1-functionalized Fb constitutes a permissive environment for axonal regeneration, either from axotomized neurons or from endogenous progenitors. Noteworthy, the trend for increased GAP43⁺ area in animals receiving HYD1-functionalized Fb as compared to those treated with unmodified Fb, correlated well with the improved locomotor function observed in this experimental group. Nevertheless, to further impact axonal regeneration HYD1 concentration might need to be adjusted, since previous investigations have shown an upregulation of $\alpha 6$ and $\beta 1$ integrin subunits after axonal injury in the adult rat [56]. Moreover, our results revealed that HYD1-functionalized Fb supports the deposition of laminin, a well-known axonal-growth-permissive molecule [61].

Taken together, these studies provide insight into the benefits of decorating hydrogels with synthetic adhesive ligands interacting with integrin $\alpha 6 \beta 1$, when envisaging their use in *in vitro* 3D platforms involving ES-NSPCs and in regenerative therapies of the injured CNS. Future *in vivo* studies are required to determine how HYD1-functionalized Fb gel will perform when used as vehicle for ES-NSPCs in an *in vivo* scenario of chronic CNS injury, namely in terms of ability to promote donor cell survival, migration and neurite outgrowth, as well as infiltration of resident neural progenitors.

5. Conclusion

In this study we have shown that functionalization of Fb hydrogels with the $\alpha 6 \beta 1$ integrin-binding peptides T1 and HYD1 provides adhesive cues promoting radial migration of ES-NSPC neurospheres on Fb. We found that this process is mediated by $\alpha 6 \beta 1$ and $\alpha 3 \beta 1$ integrin receptors and that ES-NSPC migration exhibits a biphasic response to immobilized T1 and HYD1 peptides, with maximum enhancement being attained at input peptide concentrations of 20 or 40 μ M. HYD1-functionalized Fb was also shown to support neuronal regeneration, leading to enhanced neurite extension of sensory neurons *in vitro* when compared to native Fb, and to a trend for increased axonal growth along with improved functional recovery after complete spinal cord transection. Our results point to the potential of HYD1-functionalized Fb for the 3D culture of ES-NSPCs and for application in regenerative approaches for the treatment of CNS injuries

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Fig. 1. (A, B) Representative flow cytometry histograms obtained for the analysis of $\alpha 6$ and $\beta 1$ integrin subunits expression in (A) ES-NSPCs and (B) floating aggregates of ES-NSPCs; cells incubated with the correspondent isotype control were used to determine the contribution of non-specific background staining (in black). (C, D) Expression of $\alpha 6$ and $\beta 1$ integrin subunits in floating aggregates of ES-NSPCs, as shown by immunofluorescence labeling of (C) $\alpha 6$ or (D) $\beta 1$ integrin subunit in whole neurospheres; both subunits are highly expressed at cell-cell boundaries (arrows); images show 2D projections of CLSM 3D stack images covering a depth of 2 μm .

Fig. 2. Evaluation of six synthetic peptides in terms of ability to support ES-NSPC adhesion, viability, and migration. ES-NSPCs were seeded on TCPS wells coated with physisorbed peptides or with BSA, PDL, LN 111, or LN 511 (controls). (A) Percentage of adherent cells after the application of a centrifugal force (mean $\pm$ SD, $n = 5-6$; * $p < 0.05$ vs. LN 111 one-way ANOVA followed by Dunnett's test, † $p < 0.05$ vs. HYD1, § $p < 0.05$ vs. AG10 and HYD1, one-way ANOVA followed by Bonferroni's test). (B) Number of adherent cells as a function of LN 111, determined using the crystal violet colorimetric assay (mean $\pm$ SD, $n = 4$; * $p < 0.05$ vs. LN 111, one-way ANOVA followed by Dunnett's test). (C) Number of adherent cells in the presence of EDTA, expressed as a percentage of untreated cells – None (mean $\pm$ SD, $n = 4$; * $p < 0.05$ vs. None, Student's t test). (D) Number of adherent cells in the presence of function-perturbing antibodies against $\alpha 6$ and $\beta 1$ integrin subunits, expressed as a percentage of untreated cells – None (mean $\pm$ SD, $n = 4-5$; * $p < 0.05$ vs. None; † $p < 0.05$ vs. None and the correspondent isotype control antibody, one-way ANOVA followed by Bonferroni's test). (E) Number of viable cells after 24 h of cell culture expressed as a function of LN 111, as determined using a resazurin-based assay (mean $\pm$ SD, $n = 4-6$; * $p < 0.05$ vs. LN 111, one-way ANOVA followed by Dunnett's test; † $p < 0.05$ HYD1 vs. T1, AG10, P3, and N4, one-way ANOVA followed by Bonferroni's test). (F) Cell migration through peptide-coated membrane inserts, as a function of LN 111 (mean $\pm$ SD, $n = 4$; * $p < 0.05$ HYD1 vs. any other peptide, one-way ANOVA followed by Bonferroni's test).

Fig. 3. Peptide incorporation into Fb gels, as determined using ¹²⁵I-labeled peptides. (A) Peptide release profile from Fb gels formed using a 20 μM input concentration of bi-domain peptides in the polymerizing Fb gel (mean $\pm$ SD, $n = 4$); (B) Peptide incorporation into Fb gels as a function of input peptide concentration (mean $\pm$ SD, $n = 4$).

Fig. 4. ES-NSPC migration on T1-, HYD1-, and A5G81-functionalized Fb hydrogels, as assessed using floating aggregates of ES-NSPCs and the radial outgrowth assay. (A) Outgrowth area and maximal outgrowth distance as a function of input peptide concentration; cell outgrowth was normalized to outgrowth on unmodified Fb (Unm Fb), which corresponds to an input peptide concentration of 0 μM; the cell outgrowth on Fb gels containing soluble LN (LN 111 or LN 511) is also shown in the bar charts at left (mean ± SD, $n = 6$; $*p < 0.05$ vs. Unm Fb, one-way ANOVA followed by Dunnett's test); binding of T1 or HYD1 bi-domain peptides promoted increase in cell outgrowth area, the highest enhancement being observed for Fb gels functionalized with 20 μM of HYD1 or 40 μM of T1 (2.4-fold increase vs. native Fb); representative 2D projections of CLSM stack images of neurospheres on Fb hydrogels are shown at right, where radially migrating cells can be observed; scale bars = 300 μm. (B) The outgrowth area and the maximal outgrowth distance were not significantly altered when a scrambled sequence of HYD1 (HYDS, 20 μM) was used to functionalize Fb (mean ± SD, $n = 5$). (C) Incubation with function blocking antibodies against α6, α3, and β1 integrin subunits evidenced that promotion of cell outgrowth by immobilized T1 and HYD1 was mediated through α6β1 and α3β1 integrins (mean ± SD, $n = \geq 4$; $*p < 0.05$ vs. None; $\dagger p < 0.05$ vs. the correspondent isotype control antibody, one-way ANOVA followed by Bonferroni's test). (D) Average pore size (mean ± SD, $n = 6$) and storage and loss moduli (G' and G'', mean ± SD, $n = 3$) of T1-/HYD1-functionalized gels, as determined using fluorescently-labeled fibrinogen and rheology, respectively; representative 2D projections of CLSM stack images of T1-/HYD1-functionalized gels are shown; scale bars = 20 μm.

Fig. 5. ES-NSPC behavior within Fb hydrogels functionalized with 20 μM of HYD1 when seeded as single cells and cultured under neuronal differentiation conditions. At day 6 of cell culture, higher extension of neuronal processes from cellular spheroids was found in HYD1-functionalized gels (A; mean $\pm$ SD, $n = 15\text{--}17$ spheroids). When compared to Unm Fb, HYD1-functionalized Fb gels showed similar distribution of viable/dead cells at this time point of cell culture (B; viable cells are in green and dead cells in red), as well as similar cell numbers at both day 7 and 14 of cell culture (C; mean $\pm$ SD, $n = 5$ Fb drop cultures). Immobilized HYD1 did not hinder ES-NSPC differentiation in Fb, as suggested by immunofluorescence labeling of βIII -tubulin and synapsin in cell/constructs at day 14 of cell culture (D), and by flow cytometry analysis of βIII -tubulin and GFAP expression in cells isolated from cell/Fb constructs at the same time point (E; mean $\pm$ SD, $n = 2$, each correspondent to a pool of 6 Fb drop cultures); representative 2D projections of CLSM stack images are shown. Statistically significant differences were detected using Student's t test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Neurite outgrowth from rat E18 DRG explants in HYD1-functionalized Fb hydrogels, as a function of input peptide concentration. DRGs were embedded in functionalized Fb gels and neurite outgrowth determined after 48 h of culture in samples processed for β III-tubulin/DNA staining. Average neurite length was normalized to neurite extension in Unm Fb (mean $\pm$ SD, $n = 6-9$; * $p < 0.05$ vs. Unm Fb, ANOVA followed by Dunnett's test). Representative 2D projections of CLSM stack images are shown. Scale bar = 200 μ m.

Fig. 7. Evaluation of HYD1-functionalized Fb gels in a rat model of SCI. Chitosan tubular porous scaffolds filled with Fb gels functionalized with 20 μ M of HYD1 were inserted into a 4-mm-long defect, aligned along the longitudinal axis of the spinal cord. (A) From week 5 post-implantation until the end of the experiment, animals treated with functionalized Fb consistently exhibited a significantly higher locomotor capacity compared to control (Unm Fb), as determined using the BBB locomotor rating scale (mean $\pm$ SEM, $n = 9-10$, $*p < 0.05$ vs. Unm Fb, mixed repeated measures ANOVA followed by simple effect test). Ten weeks after implantation, histological analysis was performed in longitudinal (coronal) cryostat sections of the spinal cord. (B) Immunofluorescence labeling of GAP43 revealed the presence of growing axons inside the scaffold in both conditions; dashed lines delimit the lesion area. (C) Area fraction of fibers expressing GAP43; the dot plot depicts the values found for each animal, the mean, and the standard error of the mean ($n = 9-10$). Both types of implants revealed (D) infiltrating cells expressing β III-tubulin often associated to laminin (arrows), (E) Nestin $^{+}$ cells inside the implanted scaffolds, and (F) GFAP $^{+}$ cells surrounding the lesion area (glial scar). R – C, rostral to caudal orientation of sections.

SUPPLEMENTARY DATA

Fibrin functionalization with synthetic adhesive ligands interacting with $\alpha 6 \beta 1$ integrin receptor enhance neurite outgrowth of embryonic stem cell-derived neural stem/progenitors

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SUPPLEMENTARY TABLES

Table S1 – Solutions and primary antibodies used in immunohistochemistry

Marker	Permeabilization	Blocking buffer	Primary antibody			
			Name	Reference	Company	Dilution
$\alpha 3$ integrin subunit	None	5% (v/v) NGS in PBS	Anti-integrin $\alpha 3$ (clone P1B5)	MAB1952P	Millipore	1:50
$\alpha 6$ integrin subunit			Rat anti-human CD49f (clone NKI-GoH3)	MCA699 GA	Serotec	1:100
$\beta 1$ integrin subunit			Anti-mouse CD29 (clone KMI6)	14-0292-82	eBiosciences	1:25
Nestin	0.2% (v/v) Triton X-100 in PBS	5% (v/v) NGS in PBS containing 0.05% (v/v) Tween-20	Mouse anti-nestin antibody (clone rat-401)	MAB353	Chemicon	1:100
β III-tubulin			Rabbit anti- β III-tubulin antibody	ab18207	Abcam	1:500
Synapsin			Rabbit anti-synapsin antibody	ab1543P	Abcam	1:1000
GFAP		M.O.M. TM mouse IgG blocking reagent (Vector)	Purified mouse anti-GFAP antibody	556327	BD Pharmingen	1:500

Abbreviations: FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; NGS, normal goat serum.

Table S2 – Solutions and antibodies used in immunocytochemistry

Marker	Permeabilization	Blocking buffer	Primary antibody/isotype control				Secondary antibody			
			Name	Reference	Company	Dilution	Name	Reference	Company	Dilution
α3 integrin subunit	None	5% (v/v) NGS in PBS	Anti-integrin alpha3 (clone P1B5)	MAB1952P	Millipore	1:50	APC-conjugated	sc-3818	Santa Cruz Biotech.	1:100
							goat anti-mouse IgG			
Mouse IgG1 negative Control			CBL610	Millipore	1:50	F(ab')2				
α6 integrin subunit			Rat anti-human CD49f (clone NKI-GoH3)	MCA699GA	Serotec	1:50				
			Rat IgG2a negative control	MCA1124R	Serotec	1:4	APC-conjugated	sc-3832	Santa Cruz Biotech.	1:100
						goat anti-rat IgG				
β1 integrin subunit			Anti-mouse CD29 (clone KMI6)	14-0292-82	eBiosciences	1:20	F(ab')2			
			Rat IgG2a negative control	MCA1124R	Serotec	1:4				
Nestin		5% (v/v) NGS in PBS containing	Mouse anti-nestin (clone rat-401)	MAB353	Chemicon	1:100	PE-conjugated goat anti-mouse IgG1	sc-3764	Santa Cruz Biotech.	1:100

βIII-tubulin	0.2% (v/v) Triton X-100 in PBS	0.1% (w/v) Saponin	Mouse anti-βIII-tubulin	MMS-435P	Covance	1:500	APC-conjugated goat anti-mouse IgG F(ab') ₂	sc-3818	Santa Cruz Biotech.	1:100
GFAP			Alexa Fluor® 647 mouse anti-GFAP	561470	BD Pharmigen	1:100	None			
			Alexa Fluor® 647 mouse IgG2b isotype control	558713	BD Pharmigen	1:100				

Abbreviations: NGS, normal goat serum; GFAP, glial fibrillary acidic protein.

SUPPLEMENTARY MATERIALS AND METHODS

Embryonic stem cell culture

A mouse ES cell line (46C) established at the Institute for Stem Cell Research (Edinburgh University, Scotland, UK) expressing green fluorescent protein (GFP) under the promoter of the neural-specific *Sox1* gene was used. ES cells were propagated in KnockOut™ ESC/iPSC medium (Gibco) supplemented with 1% (v/v) penicillin/streptomycin (Pen/Strep, Gibco), 2 μ M GSK-3 inhibitor IX (Calbiochem), and 1000 U/mL leukemia inhibitory factor (Chemicon) in 6-well tissue culture plates pre-adsorbed with 0.1% (w/v) gelatin (Sigma).

Neural commitment of ES cells

Neural commitment of ES cells was attained in chemically-defined medium and adherent monoculture on gelatin-coated wells [1]. Prior to initiating neural commitment, cells were cultured at a high cell seeding density (1×10^5 cells/cm²) during 24 h. To start monolayer differentiation, cells were dissociated with StemPro® Accutase® (Gibco), resuspended in N2B27 medium, and plated onto gelatin-coated wells at 2×10^4 cells/cm², the medium being renewed every 2 days. N2B27 is a 1:1 mixture of DMEM/F12 medium without phenol red, supplemented with 1% (v/v) modified N2 [1] and Neurobasal™ medium (supplemented with 2% (v/v) B27 and 1% (v/v) L-glutamine) supplemented with 1% (v/v) Pen/Strep (all Gibco). At day 5 of the monolayer differentiation protocol, SOX1-GFP expression was analyzed by flow cytometry, to assess the efficiency of neural conversion. For this purpose, cells were dissociated, resuspended in PBS buffer containing 2% (v/v) fetal bovine serum, and run on a flow cytometer (FACSCalibur™, Becton Dickinson). Live cells were gated based on forward scatter (size) and side scatter (cell complexity) criteria, and fluorescence gates set using undifferentiated 46C ES cells as negative control. A minimum of 10 000 events was captured inside the gate, and data analyzed using FlowJo™ software. A representative fluorescence histogram is shown in Figure S1.

Immunohistochemistry

Samples were washed twice with PBS, fixed in 3.7% (w/v) paraformaldehyde (PFA) solution diluted 1:1 in culture media (30 min; 37°C), and, when staining for intracellular markers, permeabilized with 0.2% (v/v) Triton X-100 in PBS (45 min; RT). Samples were then incubated with blocking buffer (60 min) and then with primary antibodies (overnight; 4°C). To detect primary antibodies, samples were incubated with the appropriate species-specific Alexa Fluor® conjugated secondary antibodies (Molecular Probes; 1:500) for 4 h. When necessary, nuclei were counterstained with DAPI (Sigma; 0.1 μ g/mL) for 10 min. Samples were finally mounted with Fluoromount™ (Sigma) and observed under confocal laser scanning microscopy (CLSM, Leica TCS SP5II). All primary antibodies and solutions are listed in Table S1.

Immunocytochemistry

Cells were fixed in 1% (w/v) PFA in PBS buffer (20 min; 4°C) and, when staining for intracellular markers, permeabilized with 0.2% (v/v) Triton X-100 in PBS (10 min; 4°C). Cells were subsequently incubated in blocking buffer (20 min; 4°C) and then with primary antibodies or with isotype controls (30 min; RT). Primary antibodies were detected by applying isotype-specific or species-specific secondary antibodies (30 min; RT). Cells were finally washed three times with FACS buffer [2% (v/v) fetal bovine serum and 0.1 % sodium azide (w/v) in PBS], and analyzed by flow cytometry. Cell debris and dead cells were excluded from the analysis based on electronic gates using forward scatter and side scatter criteria and unlabeled cells used to set fluorescence gates. Cells stained with secondary antibody only or with the correspondent isotype control were used to eliminate nonspecific background secondary antibody staining. All antibodies and solutions are listed in Table S2.

Evaluation of $\alpha 6 \beta 1$ synthetic ligands' ability to support ES-NSPC adhesion, viability, migration, and differentiation

Peptides were physisorbed to the wells of tissue-culture plates or to inserts (overnight; 37°C) as described in methods, rinsed twice with PBS, and incubated with 1% (w/v) heat-inactivated BSA (1 h; 4°C) to block non-specific adhesion.

Cell adhesion centrifugation assay: to compare cell adhesion of ES-NSPCs to the various physisorbed peptides, a centrifugation cell adhesion assay that applies uniform detachment forces was used [2]. ES-NSPCs were fluorescently-labelled with 2 μ M of Calcein AM (Molecular Probes; 10 min; 37°C), resuspended in N2B27 medium, and transferred to peptide-adsorbed surfaces at 3×10^5 cells/cm². Cells were allowed to adhere for 2 h, and, before subjecting the cells to centrifugation, pre-spin fluorescence readings measured using a microwell plate reader (BioTek SynergyMx). After overfilling the wells with PBS until a positive meniscus was observed, the plates sealed with transparent tape, inverted, and spun at 100 RCF (relative centrifugal force) for 5 min. Following cell detachment, the media was gently aspirated from the wells, and the wells refilled with medium for measurement of post-spin fluorescence readings. For each well, the adherent cell fraction was determined as the ratio of post-spin to pre-spin fluorescence readings (λ_{ex} = 485 nm; λ_{em} = 535 nm).

Cell adhesion inhibition assay: the contribution of divalent cations and $\alpha 6/\beta 1$ integrin subunits to cell adhesion was assessed quantifying cell adhesion in the absence or presence of EDTA or function-blocking antibodies, respectively. Due to the high number of experimental conditions involved, the crystal violet method was used as an alternative to the cell adhesion centrifugation assay, to avoid delay in centrifugation and, as a result, different incubation periods between multi-well plates. For this purpose, ES-NSPCs were incubated for 15 min at 37°C in N2B27 medium in the absence or presence of 5 mM EDTA or monoclonal antibodies against $\alpha 6$ (clone NKI-GoH3, Serotec; 20 μ g/mL) or $\beta 1$ (clone Haz/5, BD Pharmingen; 10 μ g/mL) integrin subunits. To evaluate the contribution of non-specific antibody interactions to cell adhesion inhibition, incubation with isotype-matched controls for $\alpha 6$ (rat IgG2a, Millipore; 20 μ g/mL) and $\beta 1$ antibodies (hamster IgM $\lambda 1$, Pharmingen; 10 μ g/mL) was also performed. Preliminary experiments were performed to optimize the concentration of both monoclonal antibodies in terms of cell adhesion inhibition. Concentrations of 20 and 10 μ g/mL were selected for incubation with $\alpha 6$ and $\beta 1$ monoclonal antibodies, respectively, since the use of higher concentrations did not result in statistical significant higher cell adhesion inhibition levels (data not shown). Cell suspension was then transferred to peptide-adsorbed surfaces at 3×10^5 cells/cm², and cells were allowed to adhere for 2 h in the absence or presence of inhibitors. At the end of this period, the wells were washed twice in order to remove non-adherent cells, fixed in 3.7% (w/v) PFA solution diluted 1:1 in culture media (30 min; 37°C), and stained with 0.1% (w/v) crystal violet in 20% methanol for 30 min. Excess dye was washed off with distilled water and the plate was left to dry overnight. For crystal violet extraction, the cells were incubated with 10% (v/v) CH₃COOH, and adherent cells quantified by measuring the optical density at 570 nm (BioTek SynergyMx microwell plate reader).

Cell viability: ES-NSPCs were resuspended in N2B27 medium supplemented with 10 ng/mL of bFGF, and plated on peptide-adsorbed surfaces at 2×10^4 cells/cm². After 24 h of cell culture, the number of viable cells was quantified using a resazurin-based assay [3]. Briefly, the cell layers were incubated with culture medium containing resazurin (Sigma) at a final concentration of 10 μ g/mL (4 h; 37°C). At the end of this period, 100 μ L of the culture medium was transferred to a black-walled 96-well plate, fluorescence measured (λ_{ex} = 530 nm; λ_{em} = 590 nm), and the fluorescence values correspondent to unseeded wells subtracted. Cell numbers were extrapolated from a standard curve where fluorescence was plotted against known number of cells seeded on PDL-LN 111 coated coverslips in parallel.

Cell migration: the migratory capacity of ES-NSPCs was evaluated using a transwell system with 8- μ m pore membrane (Falcon®) and vascular endothelial growth factor (VEGF) as chemoattractant. Both sides of the transwell membrane were coated (overnight; 37°C). Peptide-adsorbed membranes were rinsed and blocked as described for the preparation of peptide-adsorbed wells, and the inserts placed in culture wells of companion plates containing 700 μ L of N2B27 medium supplemented with 50 ng/mL of VEGF. ES-NSPCs were resuspended in N2B27 at 10×10^5 cells/mL and 200 μ L of cell suspension added to the upper chamber. Cells were allowed to migrate towards VEGF during 4 h (37°C; 5% CO₂). After incubation, cells were fixed in 3.7% (w/v) PFA solution diluted 1:1 in culture media (30 min; 37°C), and the cells remaining in the upper side of the insert removed by cotton swabs. The membrane was incubated with 0.1 μ g/mL DAPI for DNA labeling, and the nuclei of migrated cells counted under an inverted optical fluorescence microscope (Axiovert 200, Carl Zeiss) in 10 randomly selected fields/membrane using a 20 $\times$ magnification objective.

Neuronal differentiation: to assess the ability of physisorbed peptides to support ES-NSPC differentiation along the neuronal lineage, ES-NSPCs were resuspended in N2B27 and plated on peptide-adsorbed surfaces at 2×10^4 cells/cm². Cells were cultured for 6 days following a protocol for neuronal differentiation [4]. Initially, cells were cultured in N2B27 supplemented with 10 ng/mL bFGF and, at day 2 of culture, the medium was switched to the mix N2B27:Neurobasal/B27 (1:1) without growth factor, half of the medium being changed every other day. At day 6 of cell culture, the samples were processed for double immunofluorescence staining of β III-tubulin (early neuronal marker) and glial fibrillary acid protein (GFAP, astrocytic marker), and incubated with DAPI for DNA labelling. Samples were finally observed under fluorescence microscopy and the percentage of β III-tubulin⁺ cells quantified using an automatic image analysis tool developed using Matlab[®]. The image analysis approach comprised the application of a low pass filter, applied to both nuclei and β III-tubulin image channels followed by an Otsu automatic segmentation to obtain binary images. Subsequently the β III-tubulin image was compared with the nuclei image. The nuclei overlapping the β III-tubulin objects were quantified, and expressed as a percentage of the total number of nuclei. For each condition, 20 randomly selected fields from two replicate cultures were analyzed, corresponding to a minimum of 2000 cells on peptide-adsorbed surfaces.

¹²⁵I-labeling of bi-domain peptides

To facilitate radioiodination, bi-domain peptides lacking a tyrosine residue (such as T1, HYD1, and A5G81 bi-domain peptides) were reacted with sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate (Sulfo-SHPP, Pierce-Thermo Scientific[™]), to conjugate a tyrosine-like residue to the N-terminal amine. Briefly, bi-domain peptides (2 mg) were dissolved in 100 mM borate buffer and modified with Sulfo-SHPP according to the supplier at a lower pH (8.5), to increase selectivity towards the N-terminal amine. Bi-domain peptides were separated from non-reacted Sulfo-SHPP by gel filtration (PD MidiTrap G-10 columns, GE Healthcare) using PBS as elution buffer, and further detected by UV-vis spectrophotometry at 280 nm. Peptide concentration was determined by the bicinchoninic acid method (Pierce[®] BCA Protein Assay, Thermo Scientific[™]), using standard curves prepared from known amounts of the target peptides, freshly resuspended. Bi-domain peptides (5 μ g) were subsequently labeled with 0.5 mCi of Na¹²⁵I (Perkin Elmer) using the Iodogen method [5], and further purified using a PD MidiTrap G-10 column, to remove free ¹²⁵I ions. Labeled peptides were stored at -20°C and used the following day.

Neuronal differentiation of ES-NSPCs cultured within functionalized Fb hydrogels

ES-NSPCs were dissociated with StemPro[®] Accutase[®] into single cells and embedded in functionalized Fb gels [6]. Briefly, ES-NSPCs were resuspended in fibrinogen solution, and functionalized Fb gels (50 μ L) formed in the wells of a 6-well non-tissue culture plate (6 gels per well) as described in methods (final cell seeding density: 1×10^6 cells/mL). Polymerizing gels were incubated at 37°C for 30 min before the addition of 3 mL of culture medium. Cells were cultured within the gels for periods up to 14 days following a protocol for neuronal differentiation [4]. Initially cells were cultured in N2B27 supplemented with 10 ng/mL bFGF and, at day 2 of culture, the medium was switched to the mix N2B27:Neurobasal/B27 (1:1) without growth factor. At day 8, half of the medium was replaced by the mix N2B27:Neurobasal/B27 (1:3) supplemented with 20 ng/mL brain-derived neurotrophic factor and 50 ng/mL nerve growth factor (BDNF and NGF; PeproTech), half of the medium being changed every other day. To delay Fb degradation, 5 μ g/mL of aprotinin were added to the culture medium.

Neurite outgrowth: neurite outgrowth from cellular spheroids was quantified at day 6 of cell culture in samples processed for β III-tubulin/DNA staining. This time point was selected to avoid overlapping of cell processes extending between neighboring spheroids (which could compromise image analysis of neurite outgrowth). Processed samples were analyzed by CLSM. Spheroid- and neurite-containing regions were obtained by automatic segmentation of 2D maximum intensity projections of DNA and β III-tubulin CLSM image stacks, respectively. Given the segmented regions, the number of neuronal processes protruding from the edge of cellular spheroids was manually annotated, and the length of the longest neurite automatically computed using MATLAB[®] software.

Distribution of viable/dead cells: the cell/matrix constructs were processed for analysis of cell viability at day 6 of cell culture. Samples were rinsed with warm PBS, and sequentially incubated with 4 μ M calcein AM (30 min; 37°C) and 6 μ M propidium iodide (Sigma; 10 min; 37°C) for detection of viable and dead cells, respectively. After

incubation, samples were washed twice with PBS, transferred to culture media, and immediately observed under CLSM.

Total cell number: total cell number was estimated by quantifying total DNA amount in each gel at days 7 and 14 of cell culture, after cell isolation from the cell-matrix constructs. Briefly, at the end of each culture period the constructs were washed twice with warm PBS, and sequentially incubated with 1.25 mg/mL of collagenase type II (Gibco; 1 h; 37°C) and 1× trypsin-EDTA (Gibco; 20 min; 37°C) in the orbital shaker (70 rpm). After trypsin inactivation with serum-containing media cells were gently dissociated, centrifuged, and incubated with 1% (v/v) Triton X-100 (60 min; 4°C) in the orbital shaker (200 rpm) for cell lysis. The resultant cell lysate was centrifuged (10 000×g; 10 min) to remove cell debris and total DNA quantified using the Quant-iT™PicoGreen® dsDNA assay kit (Invitrogen), according to the manufacturer's instructions. Cell numbers were inferred from the mean DNA content of a known number of ES-NSPCs ($n = 3$) frozen at the beginning of the experiment.

Expression of characteristic phenotypic markers: after 14 days of cell culture, cell phenotype was analyzed by immunohistochemistry and by flow cytometry. Immunohistochemistry analysis was performed in whole-mount cultures as described above, using antibodies against nestin (NSPC marker), β III-tubulin (early neuronal marker), synapsin (synaptic vesicles marker), and glial fibrillary acidic protein (GFAP, astrocytic marker). For flow cytometry analysis, cells from six-pooled gels were isolated as described above, and the resultant single cell suspensions processed for immunocytometry (see above), using antibodies against nestin, β III-tubulin, and GFAP. Representative fluorescence histograms are shown in Figures S11 and S12.

Neurite extension from rat E18 dorsal root ganglia cultured within functionalized Fb hydrogels

Dorsal root ganglia (DRGs) were dissected from E18 Wistar rat embryos. Functionalized Fb gels (10 μ L) were formed in the lower wells of a 15-well μ -Slide Angiogenesis plate from Ibidi as described for the radial outgrowth assay, and DRG explants transferred to the center of polymerizing Fb gels (1 DRG/well) with the help of Dumont forceps. After incubation at 37°C for 30 min, 40 μ L of DMEM/F12 medium supplemented with 2% (v/v) B27, 30 ng/mL nerve growth factor (NGF, Calbiochem), 1% (v/v) P/S, 1.25 μ g/mL amphotericin B (Gibco), and 10 μ g/mL aprotinin were added to each well. Neurite outgrowth was quantified after 48 h of cell culture. Samples were processed for β III-tubulin/DNA staining, and average neurite length quantified in images acquired by inverted optical fluorescence microscopy (Axiovert 200, Carl Zeiss). Average neurite length was computed as the width of an annulus of area equal to the area of the neurite zone: $L = (1/\pi)^{1/2} [(A_{\text{inner}} + A_{\text{outer}})^{1/2} - (A_{\text{inner}})^{1/2}]$, where A_{inner} represents the area covered by the cell bodies and A_{outer} corresponds to the area between the cell bodies and the neurites ends [7].

In vivo experiments

To assess the effect of functionalized Fb gels on axonal regeneration, an *in vivo* model of spinal cord injury (SCI; total transection) was used. Chitosan tubular scaffolds prefilled with functionalized Fb gel or unmodified Fb (control) were used to bridge the cavity formed upon spinal cord transection.

Preparation of chitosan porous conduits filled with functionalized Fb gel: chitosan cylindrical hollowed porous scaffolds (4 mm long and inner diameter of 1.8 mm; Fig. S3A) were prepared as previously described [8], from squid pen chitosan (France Chitine; degree of acetylation 3.55), after purified (endotoxin levels < 0.1 EU/mL). The scaffolds were sterilized in 70% (v/v) ethanol, equilibrated in sterile PBS (2×; 10 min), and 30 μ L of HYD1-functionalized polymerizing Fb gel (6 mg/mL fibrinogen; 2 NIH U/mL thrombin; 2.5 mM CaCl_2 ; 25 μ g/mL aprotinin; 20 μ M of HYD1 bi-domain peptide) were added to the inner channel of the porous conduits, under the stereoscopic magnifier. The Fb-filled scaffolds were incubated at 37°C for 30 min to allow cross-linking, and then placed in 500 μ L of N2B27 medium containing 5 μ g/mL of aprotinin. Chitosan porous conduits filled with unmodified Fb were prepared in parallel to be used as controls.

Animal surgery, implantation of Fb-filled porous conduits, and post-operative care: a total of 20 female Wistar rats (Charles River Laboratories) 10-13 weeks old and weighting 210-240 g were used. Animals were given proper bedding material (corn cob) and shredded paper and cardboard rolls as nesting material. Food and water were provided *ad libitum*. Animals were randomized into 2 experimental groups, to be implanted with either HYD1-

functionalized Fb or unmodified Fb control. Surgeries were done in parallel over multiple days. Prior to surgery, buprenorphine (0.04 mg/kg) and glycosylated serum were administered subcutaneously. Animals were anesthetized through intraperitoneal administration of a saline solution containing ketamine (37.5 mg/kg) and medetomidine (0.25 mg/kg). Surgical procedures started when deep anesthesia was observed (loss of postural, caudal, pedal and palpebral reflexes). The animal fur was removed right above the T10 vertebrae, and the surgical area disinfected with 70% (v/v) ethanol and povidone-iodine solution (3x). During surgery, the animals were kept on a heating pad kept at 37°C, a saline solution was applied to the animal's eyes every 5 min, and deep anesthesia maintained through a continuous supply of 1% isoflurane mixed with oxygen. The spinal cord was exposed by laminectomy made at the T7-T9 level and the cord was fully transected by removing a 4 mm region encompassing T8 using a straight spring scissor (Fine Science Tools). The porous conduit filled with HYD1-functionalized Fb/unmodified Fb was then inserted into the defect, aligned along the longitudinal axis of the spinal cord (Fig. S10B), assuring that the severed ends of the cord fit tightly. Tisseel Lyo® (Baxter; 100 µL/animal) was applied to the top of the lesion to hold the tubular scaffold in place. After suturing the muscle and the skin layers, atipamezole (1 mg/kg) was administered subcutaneously to reverse medetomidine-induced anesthesia. After surgery, the animal was placed in a box containing humid food and an electric heating blanket to maintain the animal body temperature. During the first 3 days post-surgery, animals received buprenorphine (0.04 mg/kg) twice a day, as well as Duphalyte® (injectable solution with vitamins and aminoacids), subcutaneously, to relief pain and prevent animal dehydration, respectively. To prevent urinary infections Bactrim™ was added to the water (4.4 mL/500 mL of water) during the first week post-surgery. Throughout all the post-operative period, the bladders were manually voided twice a day. Animals were monitored every day and weighted weekly.

Locomotor recovery and histological analysis: locomotor function was assessed during training (pre-injury) and post-injury, weekly during 9 weeks, using the Basso, Beattie, and Bresnahan (BBB) open-field locomotion rating scale [9]. Before surgery, all the animals were healthy with a maximum BBB score, and animals were evenly distributed among both groups. The test was conducted in a blinded manner by two independent operators. Ten weeks post-implantation, the animals were anesthetized with ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) and transcardially perfused with 150 mL of ice-cold sodium phosphate buffer 0.2 M (pH = 7.4) followed by 4% (w/v) PFA in sodium phosphate buffer. The spinal cord was carefully dissected, post-fixed in 4% (w/v) PFA (overnight at 4°C), washed with PBS (3x), and transferred to 30% (w/v) sucrose in PBS at 4°C for 24 h, for cryoprotection. Spinal cords were then transferred to fresh 30% sucrose solution in PBS and stored at -20°C until use. For tissue sectioning, spinal cords were embedded in Richard-Allan Scientific™ Neg-50™ Frozen Section Medium (Thermo Scientific). Longitudinal (coronal) cryostat sections were cut at 20 µm increments and serially collected in SuperFrost® slides for immunohistochemical staining, with every 10th section mounted on the same slide. Sections were rinsed in PBS, blocked with 5% donkey serum or normal goat serum in 0.3% (v/v) Triton-X 100 in PBS (1 h; RT), and incubated with the following primary antibodies in blocking buffer (overnight; 4°C): rabbit anti-GAP43 (AB5220, Millipore; 1:500), rabbit anti-GFAP (Z0334, Dako; 1:400), mouse anti-βIII-tubulin (801201, Biolegend; 1:500), mouse anti-nestin (MAB353, Chemicon; 1:200), rabbit anti-laminin (L9393, Sigma; 1:100) and rabbit anti-IBA1 (019-19741, Wako; 1:500). Sections were washed with PBS and then incubated with Alexa-fluorophore-conjugated donkey or goat secondary antibodies (Molecular Probes) in blocking buffer (1:1000; 1 h; RT). Following incubation, the samples were rinsed with PBS and nuclei stained using Hoechst 33342 (Sigma; 1 µg/mL; 10 min; RT). The sections were finally mounted in Fluoromount™ and examined with the IN Cell Analyzer 2000 imaging system (GE Healthcare) using a 20x/0.75 NA objective or using the CLSM. Axonal sprouting/regeneration in each animal was determined in 2D projections of the IN Cell Analyzer 3D stack images, analyzing the area occupied by GAP43⁺ axons in the lesion area, expressed as a percentage of the total lesion area (see dashed lines; Fig. 7B). Similar and sequential sections were stained for GFAP to help to identify the lesion margins, and matched to lesion margins defined in the GAP43 stained sections. Sections processed in parallel in the absence of primary antibodies were used to establish the threshold pixel intensity. For each animal, four sections from two different slides were analyzed, and the GAP43⁺ area averaged. All analyses were conducted in a blinded manner.

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Supplementary Figure 1.

Supplementary Figure 2.

Supplementary Figure 3.

Supplementary Figure 4.

Supplementary Figure 5.

Supplementary Figure 6.

Supplementary Figure 7.

Supplementary Figure 8.

Supplementary Figure 9.

Supplementary Figure 10.

Supplementary Figure 11.

Supplementary Figure 12.