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Development of a 3D matrix for modeling mammalian spinal cord injury *in vitro*

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Abstract

Spinal cord injury affects millions of people around the world, however, limited therapies are available to improve the quality of life of these patients. Spinal cord injury is usually modeled in rats and mice using contusion or complete transection models and this has led to a deeper understanding of the molecular and cellular complexities of the injury. However, it has not to date led to development of successful novel therapies, this is in part due to the complexity of the injury and the difficulty of deciphering the exact roles and interactions of different cells within this complex environment. Here we developed a collagen matrix that can be molded into the 3D tubular shape with a lumen and can hence support cell interactions in a similar architecture to a spinal cord. We show that astrocytes can be successfully grown on this matrix *in vitro* and when injured, the cells respond as they do *in vivo* and undergo reactive gliosis, one of the steps that lead to formation of a glial scar, the main barrier to spinal cord regeneration. In the future, this system can be used to quickly assess the effect of drugs on glial scar protein activity or to perform live imaging of labeled cells after exposure to drugs.

Key Words: *nerve regeneration; spinal cord injury; 3D scaffold; in vitro models; astrocytes; reactive gliosis; neural regeneration*

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Introduction

In the past few decades, *in vitro* culture systems have become an attractive alternative for the study of complex biological systems. The simplicity of *in vitro* systems allows for more detailed studies at the cellular and subcellular levels using methods that are technically challenging in *in vivo* systems (*e.g.*, real time imaging). The oversimplification of complex biological systems could neglect important interactions of the cell with other cells and with the environment that in turn influences the cell response to particular stimuli. For example, the growth cones of *Xenopus* retinal neurons cultured *in vitro* are attracted by netrin, however, the same cue repels the growth cones when the substrate is coated with laminin-1 (Höpker et al., 1999; Ravi et al., 2015).

Although a great deal of information has been obtained from 2D cultures, it has become more evident that in these systems important input from the environment, in particular interactions between different cell types and with the proteins of the extracellular matrix (ECM), is not always reproducible *in vitro*. Interestingly, aside from cell-cell and cell-ECM protein interactions, the topography and the stiffness of the tissue itself can affect cellular behavior response to external stimuli. For example, mesenchymal stems cells can be induced into an osteogenic/adipogenic fate when cultured on stiff substrates, and into a neurogenic fate lineage when cultured on softer substrates (Aagaard et al., 1995; Thomas et al., 2012). 3D *in vitro* co-culture systems have emerged as way to introduce the input from cell, ECM and topography interactions to create models that better mimic *in vivo* conditions, without compromising the advantages of having a simpler system.

Different 3D model systems have been developed to study different cellular processes within the central nervous system. For example, dorsal root ganglion neurons have been seeded into collagen scaffolds to study growth cone migration (Dubey et al., 1999). Oligodendrocyte precursor cells (OPCs) have been seeded on polystyrene nanofibers to study myelination (Lee et al., 2012), and co-cultures of neurons and Schwann cells on poly-(caprolactone) (PCL) fibers have been achieved (Daud et al., 2012). To date there are several interesting advances in the development scaffolds for modeling spinal cord injury and/or for delivery of cells to the site on injury in mammals including the use of hydrogels and gelatin-electrospun poly (lactide-co-glycolide)/polyethylene glycol scaffolds (Bakshi et al., 2004; Kang et al., 2011; Donoghue et al., 2014; Shrestha et al., 2014; Liu et al., 2015). Although a great deal of effort has been put into designing 3D systems to improve recovery after spinal cord injury, limited

3D models have been developed to study the immediate response of cells in the spinal cord to injury.

There are two primary existent spinal cord injury models, both of which have limitations. The first, organotypic cultures, require the extraction of the spinal cord from the animals. This injury can trigger cellular repair processes and alter experimental results. The second, 2D *in vitro* co-cultures, is more easily manipulated, but the interactions of different cell types with the extracellular matrix composition and topography are missing. Therefore, there is a necessity for a 3D system designed specifically to study the response of astrocytes and neurons to traumatic injury in an *in vivo* like environment. We decided to develop a 3D *in vitro* model of the mammalian spinal cord that could mimic the *in vivo* architecture of the spinal cord, would allow different cell types to be grown on it and could be injured *in vitro*.

Materials and Methods

Preparation of collagen sheets and 3D collagen tubes

Collagen tubes were prepared by mixing 24 mL of type I collagen (3.0 mg/mL, bovine hide, PureCol®, Advanced Biomatrix, Carlsbad, CA, USA) with 6 mL of 10× PBS, followed by adding 4 mL of 0.1 N NaOH to achieve pH8. The collagen mixture was ultrasonicated in a cooled water bath for 5 minutes and then placed in an incubator at 30°C for 3 days to form a gel (Li et al., 2009). The collagen gel was compressed into a thin sheet using the method developed by Brown et al. (2015). Briefly, a stainless steel mesh (~300 μm in mesh size) was placed on an absorbent paper, followed by a layer of nylon mesh (~50 μm in mesh size). The collagen gel was then placed on top of the nylon mesh and covered with another nylon mesh, a glass plate, and loaded with a 100 g flat weight for 8 hours. After compression, the collagen film was rinsed with deionized water to remove excess salts and then wrapped tightly around stainless steel rods with a 1.5 mm radius to form tubes. To fix these tubes, the chemical crosslinking was performed by using 9 mL of 50 mM 2-(N-morpholino)ethanesulfonic acid hydrate (MES, pH 7.0) containing 100 mM 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and 50 mM N-hydroxysuccinimide (NHS) overnight (Li et al., 2012).The reaction was quenched by immersing the collagen tubes in a solution containing 0.1 M Na_2HPO_4 and 2 M NaCl for 2 hours. After they were thoroughly rinsed with deionized water, the collagen tubes were removed from the stainless steel rods and kept at –20°C for cell culture study.

Collagen sheets were prepared by placing 350 μL of the aforementioned collagen mixture per well in a 24-well tissue culture plate. They were then incubated for 3 days as explained above. Cell culture was performed on collagen sheets that were not chemically crosslinked. The pore size of the collagen sheets was determined from SEM images using ImageJ (U. S. National Institutes of Health, Bethesda, MD, USA).

Scanning electron microscopy (SEM)

Low magnification SEM images of lyophilized collagen sheets were obtained with a tabletop scanning electron microscope (TM-300, Hitachi, Tokyo, Japan) operated at an accelerating voltage of 15 kV in combo mode. High magnification SEM images were obtained using a field emission SEM (FE-SEM) (JEOL6500, Jeol, Tokyo, Japan) operated at 5 kV. Collagen sheets were sputter coated with 5 nm Pt before FE-SEM visualization.

Rat primary cortical astrocytes

Rat primary cortical astrocytes were purchased from Invitrogen (Carlsbad, CA, USA) or Lonza (Basel, Switzerland). Cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) + 15% fetal calf serum (Sigma, St. Louis, MO, USA or Hyclone, Logan, UT, USA) + 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO_2 incubator. Media was changed every 3 days and cells would be passaged once they were approximately 80% confluent. For passage, cells were washed once with PBS and incubated for 5–15 minutes with an enzymatic cocktail (StemProAccutase, Live Technologies, Carlsbad, CA, USA) at 37°C. The enzymatic reaction was stopped by adding fresh media to the flask and cells were washed off the surface of the flask. Cells were then split 1:2. They were passaged until passage 5 (P5). P5 cells were used for the experiments. The cells were characterized by immunostaining against glial fibrillary acidic protein (rabbit-anti-GFAP, 1:800 dilutiond; Millipore, Billerica, MA, USA), mouse-anti-beta-III tubulin (1:1,000 dilution; Sigma), mouse anti-S100beta (1:50 dilution; AbDSerotec) and rabbit-Sox 2 (1:500 dilution; Millipore).

Culturing of cells on collagen sheets and tubes

Culturing cells on collagen sheets

Type I collagen sheets were cut to fit 96-well plates and coated with Fibronectin (2.5 μg/cm^2) and Laminin (1.5 μg/cm^2). A Fibronectin-Laminin solution in PBS was placed in the well covering the scaffold. The dish was incubated at 37°C on a rocking platform overnight. Afterwards, the scaffolds were washed once with PBS at 37°C overnight and incubated afterwards with astrocyte media (DMEM + 15% fetal bovine serum + 1% penicillin/streptomycin).

P5 astrocytes were seeded on top of Fibronectin-Laminin sheets pre-incubated with astrocytes conditioned media (filtered-sterilized media collected from 4-day-old astrocyte cultures) at a seeding density of 2.5 × 10^5 cells/cm^2 in 24-well plates. The next day the culture media was changed for fresh media and the cells were cultured for 4 days. In half of the sheets, compression injury was induced by holding the sheets between forceps for 30 seconds. The culture media was changed after injury. In cultures where cell proliferation was going to be quantified, the media contained 10 μM EdU (Invitrogen). 24, 48 or 96 hours after injury, injured and uninjured cultures were fixed with 4% paraformaldehyde at 4°C overnight for immunostaining against rabbit anti-GFAP (dilution 1:800), mouse anti-vimentin (dilution 1:100, Developmental Studies Hybridoma Center, Owa City, IA, USA). Fluorescent detection was carried out by incubating the samples for 1 hour at room temperature with Alexa-fluor-488 conjugated goat anti-rabbit and Alexa-fluor-568

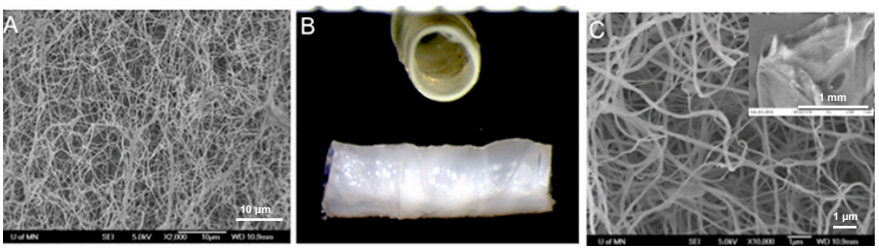

Figure 1 3D collagen tube to model spinal cord injury *in vitro*.
(A) Scanning electron microscope image of collagen sheet. (B) 3D collagen tube, diameter 1.2–1.77 mm and length is 5–7 mm. (C) Scanning electron microscopy image of fibrillar collagen scaffold with pores from 500 nm –10 µm. Insert is a low magnification picture of the scaffold film that shows the structural continuity of the collagen scaffold. Scale bars: 10 µm in A and 1 µm in C.

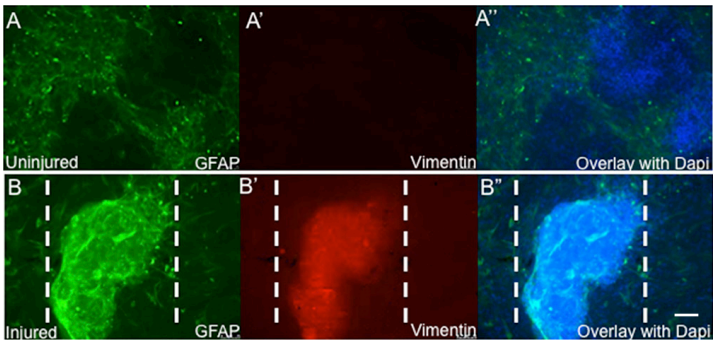

Figure 2 Astrocytes were grown and injured on collagen sheets.
(A) Astrocytes stained with anti-GFAP growing on 3D collagen sheet. (A') Uninjured astrocytes growing in the collagen sheets do not express vimentin. (A") Overlay of panel A and A'. (B–B") Injured astrocytes express higher levels of GFAP (green) and begin to express vimentin (red) (B'), the nuclei of the cells were stained with DAPI (blue). Dashed lines in panels B–B" indicate the borders of the scratch injury. Scale bar: 50 µm. GFAP: Glial fibrillary acidic protein; Dapi: 4′,6-diamidino-2-phenylindole.

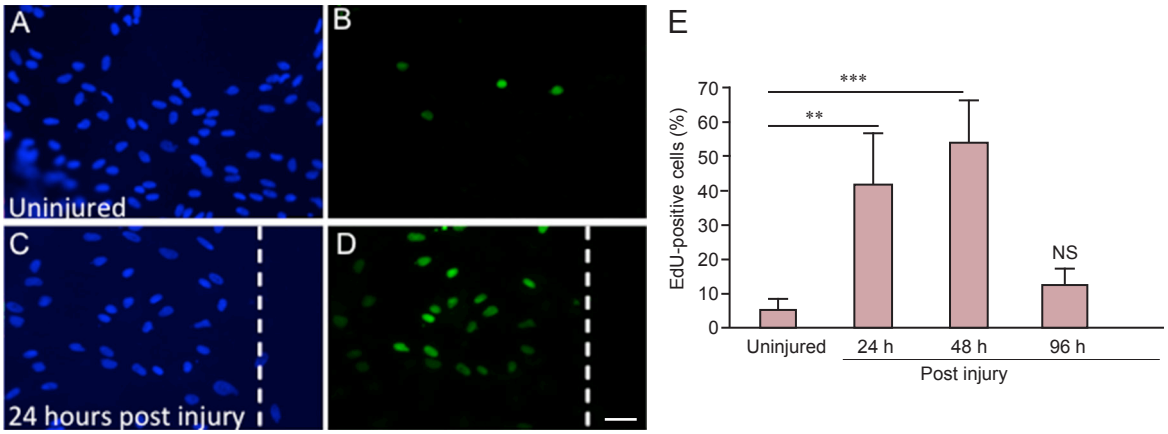

Figure 3 Astrocytes proliferated in response to injury.
(A, B) Astrocytes growing in the collagen sheets were actively proliferating as determined by EdU incorporation (green) and all nuclei were stained with DAPI (blue). (C, D) Cell proliferation was increased in astrocytes 24 hours (h) after the collagen sheets were injured by compression. Dashed lines in C, D indicate the border of the injury site. (E) Maximum cell proliferation after injury was observed 24 and 48 hours post injury, and by 96 hours post injury cell proliferation returned to basal homeostatic levels. Significance was determined by one-way analysis of variance with Dunnett's multiple comparison test. Error bars are SD. $^{**}P < 0.01$, $^{***}P < 0.001$, NS: Not significant. Scale bar: 50 µm.

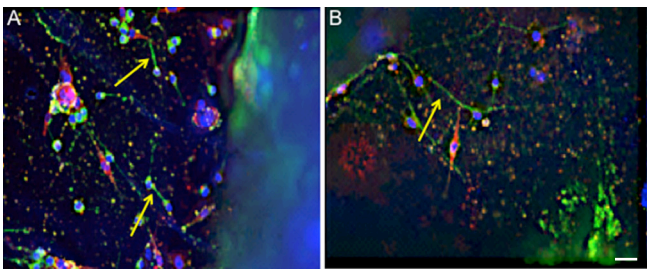

Figure 4 Astrocytes and neurons were co-cultured in the 3D tubes.
(A) B35 derived neurons can grow in the 3D collagen tubes that were previously seeded with astrocytes, astrocytes were stained red with anti-glial fibrillary acidic protein and the neurons were stained green with anti-β-III tubulin (yellow arrows). (B) After 6 days in culture, neurons stained for β-III tubulin extended longer axons along the collagen surface (yellow arrow). Nuclei of all cells were stained with 4′,6-diamidino-2-phenylindole (blue). Scale bar: 50 µm.

conjugated goat anti-mouse antibodies (Invitrogen, dilution 1:200). Detection of EdU incorporation was done following the instructions of the "Click-it-EdU" kit from Invitrogen. Images were taken using an inverted Leica DMI 6000B fluorescent microscope (Leica, Wetzlar, Germany) at 10× and 20× magnification ($n = 3$).

Culturing cells in 3D collagen tubes

Collagen tubes were coated with fibronectin and laminin as described before. After coating, the tubes were washed once with PBS at 4°C overnight. The tubes were then incubated in astrocytes conditioned media at 37°C overnight. P5 astrocytes were seeded in the tubes at a density of 2.5×10^5 cells/cm^2 using a hanging drop system made in polydimethylsiloxan (PDMS) (Doshi, 2009). On the next day, the tubes were placed in 24-

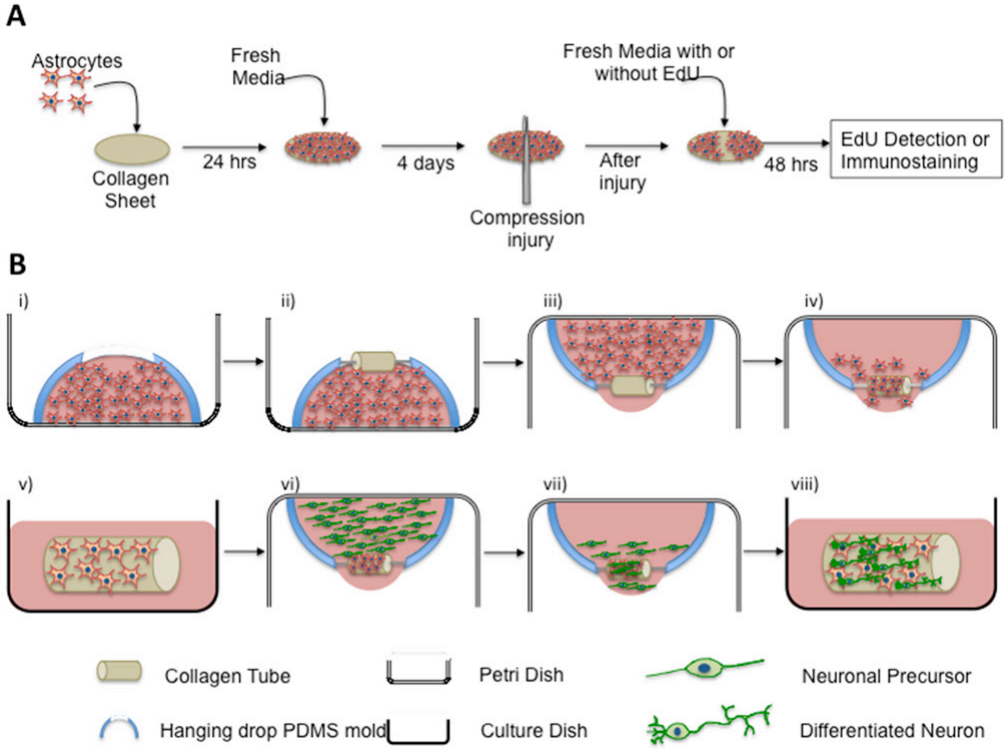

Figure 5 Schematic diagram of the procedure used to seed cells in the collagen matrix.

(A) Fibronectin/laminin-coated type I collagen sheets were seeded with astrocytes. Twenty-four hours after seeding, the media was changed and the cultures were incubated for 4 days. Compression injury was induced by holding the sheets between forceps for 30 seconds. The culture media (with or without 10 μM EdU) was changed after injury. Cultures were fixed 48 hours after injury for immunostaining or EdU detection. (B) Astrocytes and neurons were seeded on fibronectin/laminin-coated type I collagen tubes using a hanging drop system (i). An astrocyte cell suspension was put inside the polydimethylsiloxan (PDMS) hanging drop mold in a Petri dish (ii). Fibronectin/laminin-coated type I collagen tubes were placed on top of the PDMS hanging drop mold (iii). The Petri dish was inverted to induce a drop of cell suspension to cover the collagen tube (iv). The system was kept at 37°C overnight to allow the even seeding over the tube's surface (v). On the next day, the tubes were placed in culture dishes with fresh media and incubated for 4 days (vi). B35 cells were seeded on astrocytes-laden collagen tubes using the hanging drop system (vii). The system was kept at 37°C overnight to assure an even seeding over the tube surface. The next day the tubes were placed in culture dishes with fresh media and incubated for 4 days (viii). The cells were then serum starved (1% FBS) to induce neuronal differentiation. The media was changed daily and the tubes were fixed for immunostaining after 6 days of serum starvation.

well culture dishes with fresh media. The tubes were incubated for 4 days. Rat neuronal neuroblasts (B35 cell line) were seeded on astrocytes-seeded-collagen tubes at a density of 2.5×10^5 cells/cm^2 using the hanging drop system. The next day the tubes were placed in culture dishes with fresh media and incubated for 4 days. The cells were then serum starved (1% fetal bovine serum) to induce differentiation and neurite outgrowth. The media was changed daily for 6 days and then the tubes were fixed with 4% paraformaldehyde at 4°C overnight for immunostaining. Staining against rabbit anti-glial fibrillary acidic protein (GFAP) (dilution 1:800) and mouse anti-β-III-tubulin (dilution 1:1,000; Raleigh, NC, USA). Fluorescent detection was carried out by incubating the samples 1 hour at room temperature with Alexa-fluor-488 conjugated goat anti-rabbit and Alexa-fluor-568 conjugated goat anti-mouse antibodies (dilution 1:200; Invitrogen). Images were taken using an inverted Leica DMI 6000B fluorescent microscope at 10× and 20× magnification.

Statistical analyses

Quatitative results are presented as the mean ± SD. Unless otherwise stated, analyses were performed using Microsoft Excel or GraphPad Prism. Data set means were compared using one-way analysis of variance for three or more tests. The Dunnett's multiple-comparison test was used to determine the significance of differences between any two groups. Differences between groups were considered significant at three different levels (P values of < 0.05, < 0.01 and < 0.001).

Results

3D collagen tubes and sheets

We have previously tested several different substrates including agarose and PCL nanofiber tubes for the co-culture of astrocytes and neurons in a 3D format. We found that in each case only one cell type was viable on that particular substrate (data not shown). We then tried type I collagen as it is the most abundant protein in the ECM of human tissues and it is therefore the most common material used for 3D *in vitro* culture systems (Bakshi et al., 2004; Madigan et al., 2009). Collagen is versatile and can be used in the form of hydrogel, sponge, or fiber mesh. The mechanical properties of collagen scaffolds can be adjusted by varying collagen concentrations and crosslinking densities (Gee, 2012).Therefore, type I collagen has been used for the construction of artificial tissue for different systems such as skin, oral mucosa, bone,

cartilage, and bladder (Yannas et al., 2010; Abou Neel et al., 2013).

Tubular and sheet collagen scaffolds, composed of nano-sized fibrils, were obtained by a fibrillogenesis process triggered by a temperature increase. They exhibited a mesh-like porous structure (**Figure 1A**, **C**). Plastic compression and the crosslinking reaction were conducted to obtain densified and stable collagen tubes. The plastic compression squeezed out more than 99.9 % of fluid as the thickness of the collagen gel was reduced from ~10 cm to ~100 μm. The chemical crosslinking used EDC and NHS and lead to the formation of peptide-like crosslinks between free amino and carboxylic acid groups of the collagen molecules. As found in our previous study, 82% of free amino groups of collagen were reacted and the average width of the fibrils was 142 nm (Li et al., 2012). Furthermore, the collagen sheets could be moulded into a tube structure, the internal diameter of which can be specified depending on the user needs (**Figure 1B**).

Collagen sheets support the growth of primary cortical astrocytes

We then tested the capacity of collagen sheets to support astrocyte attachment and growth. We observed that astrocytes were able to grow on fibronectin and laminin coated collagen sheets. Primary rat cortical astrocytes showed normal morphology and normal expression of GFAP (**Figure 2A**). The initial collagen sheets did not support the proliferation and migration of astrocytes; however, collagen sheets with a structure providing a pore size of ~1.6 μm allowed for normal growth of the astrocytes as determined by monitoring cell proliferation using BrdU incorporation and antibody staining.

***In vitro* injury model**

To test whether cells grown in the collagen scaffolds could induce an *in vivo* like response in astrocytes, we decided to use a compression injury model, which is an injury model commonly used for *in vivo* spinal cord injury studies in mice and rats (LaPlaca et al., 2007; Lee and Lee, 2013). Astrocytes seeded collagen sheets were compressed by holding them tightly with forceps. The compression injuries were done along the diameter of the sheet. After injury, the sheets were put into fresh media and incubated for 48 hours. Immunostaining showed increased expression of GFAP in astrocytes close to the injury site (**Figure 2B**) in comparison to uninjured cultures (**Figure 2A**). Increased expression of GFAP is one of the main hallmarks of reactive gliosis, which is the response of the astrocytes to spinal cord injury *in vivo* (Pekny and Nilsson, 2005).

To further confirm the induction of reactive gliosis in response to injury *in vitro*, we decided to look at other markers of reactive gliosis and glia scar formation. Vimentinis also overexpressed in response to injury (Pekny and Nilsson, 2005). Immunostaining showed a clear upregulation of vimentin expression in cells close to the injury site (**Figure 2B**) in comparison to uninjured cultures where vimentin expression is not detected (**Figure 2B'**). Interestingly, the observed vimentin expression is restricted to the cells that are also overexpressing GFAP in the injured cultures (**Figure 2B', B"**). In the uninjured cultures, vimentin expression was not detected even in the regions with higher GFAP expression (**Figure 2A'**).

Astrocyte proliferation is another marker of reactive gliosis in response to spinal cord injury. Cell proliferation is commonly evaluated by quantification of 5-ethynyl-2′-deoxyuridine (EdU) incorporation. EdU is a synthetic nucleoside that is incorporated specifically into the DNA of actively dividing cells. We evaluated EdU incorporation after injury, as a marker of injury-induced proliferation. We observed that EdU incorporation was higher in cells close to the injury site (**Figure 3C**, **D**) in comparison to uninjured cultures (**Figure 3A**, **B**). Maximum astrocyte proliferation occurred in cells 3–4 cell diameters away from injury site, and by 96 hours post injury, the proliferation levels return to homeostatic levels (**Figure 3E**). Increased GFAP expression and cell proliferation are both indicators that astrocytes are behaving in an *in vivo* like manner in response to injury in our *in vitro* model.

To determine if different cell types can be grown together in 3D collagen tubes, we co-cultured astrocytes with B35 cells. B35a neuroblast cell line, differentiated into neurons in the tubes that already contained astrocytes (**Figure 4**). Initially the cells had very short axons (**Figure 4A**), however, after 6 days in culture, the cells extended longer axons (**Figure 4B**).

Overall this data indicates that astrocytes grown on fibronectin and laminin coated type I collagen matrix proliferate, upregulate GFAP and vimentin, in response to injury, thus mimicking the *in vivo* response of astrocytes after spinal cord injury. In addition, we have determined that the 3D collagen tubes can also support the co-culture of neurons and astrocytes. Therefore, this *in vitro* system could be potentially used for studying the response to injury and interactions of many different cell types in the spinal cord. This collagen matrix is easily and relatively cheaply made and is a natural extracellular matrix component, we have outlined the process by which we have prepared both the seeding of the collagen sheets and the tubes in **Figure 5**.

Discussion

Astrocytes are major players in the response to spinal cord injury; they become hypertrophic, up-regulate the expression of GFAP, proliferate and deposit components of the extracellular matrix, in particular collagen type IV and CSPG (Burda and Sofroniew, 2014). A common cause of spinal cord injury is the compression of the spine, usually as a consequence of car crash (Boido et al., 2011). However, one of the most common models to study astrocyte response to injury is scratch assay in 2D *in vitro* monocultures. This injury model presents little relevance to how actual injuries occur *in vivo*. In scratch assays, proliferation of astrocytes and migration towards the injury is observed; however, GFAP up regulation or deposition of components of the extracellular

matrix is very poor (Környei et al., 2000; Zhu et al., 2007; Yang et al., 2009). When we performed compression injuries on 3D collagen scaffolds, we observed that astrocytes started to proliferate, revealed by the incorporation of EdU, but also showed other markers of reactive gliosis, such as GFAP and vimentin upregulation. Since the response to injury in our 3D culture system seems to mimic the response observed in mammals *in vivo*, the fibronectin and laminin coated collagen tubes seem to be a proper substrate to study the molecular and cellular mechanisms that govern spinal cord injury in mammals.

In conclusion, we showed the development of an *in vitro* system that can properly recapitulate some key aspects of astrocyte behavior in response to injury. The composition, architecture, chemical and mechanical properties of the material used for the collagen scaffolds are critical parameters. Such parameters can have a profound influence in cell-cell interactions and the response of cells to external stimuli, therefore, affecting the relevance of the *in vitro* model. We also showed that a 3D system composed by type I collagen tubes coated with fibronectin and laminin and seeded with primary rat astrocytes, is a favorable approach to develop a functional *in vitro* mammalian spinal cord where the molecular and cellular mechanisms of repair after spinal cord injury can be studied.

Author contributions: *JFDQ and KE conceived the study. YL and CA designed and manufactured the scaffolds. JFDQ carried out the experiments. All authors analyzed data, contributed to writing of the paper, and approved the final version of this paper for publication.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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