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Induction of Neurogenesis and Angiogenesis in a Rat Hemisection Spinal Cord Injury Model With Combined Neural Stem Cell, Endothelial Progenitor Cell, and Biomimetic Hydrogel Matrix Therapy

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OBJECTIVES: Acute spinal cord injury is a devastating injury that may lead to loss of independent function. Stem-cell therapies have shown promise; however, a clinically efficacious stem-cell therapy has yet to be developed. Functionally, endothelial progenitor cells induce angiogenesis, and neural stem cells induce neurogenesis. In this study, we explored using a multimodal therapy combining endothelial progenitor cells with neural stem cells encapsulated in a bioactive biomimetic hydrogel matrix to facilitate stem cell–induced neurogenesis and angiogenesis in a rat hemisection spinal cord injury model.

DESIGN: Laboratory experimentation.

SETTING: University laboratory.

SUBJECTS: Female Fischer 344 rats.

INTERVENTIONS: Three groups of rats: 1) control, 2) biomimetic hydrogel therapy, and 3) combined neural stem cell, endothelial progenitor cell, biomimetic hydrogel therapy underwent right-sided spinal cord hemisection at T9–T10. The blinded Basso, Beattie, and Bresnahan motor score was obtained weekly; after 4 weeks, observational histologic analysis of the injured spinal cords was completed.

MEASUREMENTS AND MAIN RESULTS: Blinded Basso, Beattie, and Bresnahan motor score of the hind limb revealed significantly improved motor function in rats treated with combined neural stem cell, endothelial progenitor cell, and biomimetic hydrogel therapy ($p < 0.05$) compared with the control group. The acellular biomimetic hydrogel group did not demonstrate a significant improvement in motor function compared with the control group. Immunohistochemistry evaluation of the injured spinal cords demonstrated de novo neurogenesis and angiogenesis in the combined neural stem cell, endothelial progenitor cell, and biomimetic hydrogel therapy group, whereas, in the control group, a gap or scar was found in the injured spinal cord.

CONCLUSIONS: This study demonstrates proof of concept that multimodal therapy with endothelial progenitor cells and neural stem cells combined with a bioactive biomimetic hydrogel can be used to induce de novo CNS tissue in an injured rat spinal cord.

KEY WORDS: angiogenesis; biomedical engineering; biomimetic extracellular matrix; endothelial progenitor cells; hydrogel; neural stem cells; neurogenesis; polyethylene glycol; regenerative medicine; spinal cord injury

Each year in the United States, there are 12,000 to 20,000 new cases of acute spinal cord injury (SCI) (1, 2). SCI often leads to debilitating functional deficits that may impair patients' ability to perform activities of daily living and negatively impact quality of life. Furthermore, SCI may be associated with acute life-threatening complications such as respiratory failure, autonomic dysfunction, and embolism from deep-vein thrombosis, as well as subacute complications such as skin breakdown and infection (1, 2). The estimated lifetime cost for an individual with SCI is 1.1–4.6 million dollars (2).

Mammalian neural stem cells (NSCs), that have been shown to induce neurogenesis, were first propagated by Reynolds and Wiess (3) in 1992; endothelial progenitor cells (EPCs) that have been shown to therapeutically induce angiogenesis were first discovered by Asahara et al (4) in 1997. In order to reverse the motor deficit seen in acute SCI, several preclinical and clinical studies have focused on stem/progenitor cell therapy to induce spinal cord repair and reverse motor deficits (5–17). Unfortunately, despite extensive stem-cell therapy studies, there are currently no clinical effective treatments that have been proven to restore motor function in patients after an acute traumatic SCI (5–9).

Following spinal cord trauma, pathologic changes that occur to the CNS extracellular matrix (ECM) may inhibit spinal cord repair (10, 18). Functionally, in part, the ECM regulates stem/progenitor-cell ability to undergo differentiation through adhesion and integrin signaling (19, 20). After SCI, a glial scar results from activation of astrocytes and macrophages and modification of the native ECM, such as an increase in the concentration of chondroitin sulfate proteoglycan (CSPG) and keratan sulfate proteoglycans (10, 18, 21, 22). The net change in the molecular structure of ECM following CNS injury is induced proteolysis change to the ECM and scar and/or cyst formation; importantly, this change may inhibit stem cell–induced tissue regeneration (10, 21, 22). Therefore, a therapeutic approach in which the composition of the injured spinal cord tissue is altered to change the extracellular environment may render it more permissive for the induction of angiogenesis and neurogenesis with stem-cell therapy and, subsequently, enhance the recovery of motor function.

In the current study, we tested that the hypothesis that the interaction of NSCs and EPC's within a permissive extracellular environment created by a biomimetic hydrogel would improve functional and histological recovery after SCI in a rodent model. To provide an extracellular environment to potentially facilitate CNS tissue regeneration, we encapsulated NSCs and EPCs within a novel biomimetic hydrogel and implanted the encapsulated cells into a rat hemisection SCI. This biomimetic hydrogel has previously been shown to induce angiogenesis both in vitro and in vivo (23–25). The polyethylene glycol (PEG) biomimetic hydrogel used in this study contains a fibronectin-derived cell adhesion motif L-arginylglycyl-L-α-aspartyl-L-serine (RGDS) and a proteolytically degradable peptide sequence (Glycine-Glycine-Glycine-Glycine-Glycine-Proline-Glutamine-Isoleucine-Tryptophan-Glycine-Glutamine- Glycine-Glycine-Lysine-[alloc]-Glycine-Lysine (GGGGGPQGIWGQGG-Lys-[alloc]-GK)) that can be cleaved by matrix metalloproteinase (MMP)-2 and MMP-9 (23, 25). This biomimetic hydrogel was specifically engineered to have mechanical properties similar to CNS tissues, a cell adhesion sequence that allows for cell binding via targeted integrin receptors and a cleavable sequence to allow for cell migration throughout the biomimetic hydrogel and ultimately hydrogel degradation (23–26). In this study, we found that EPCs and NSCs encapsulated into a bioactive biomimetic hydrogel implanted into a rat hemisection SCI-induced de novo CNS tissue formation in vivo and improved motor function.

MATERIALS AND METHODS

Detailed materials and methods are presented in **Supplemental Digital Content 1** (http://links.lww.com/CCX/A638).

Rodent Experiments

All rodent experimental protocols were approved by the Duke University Institutional Animal Care and Use Committee (approval numbers: A222-14-09 and A201-17-08) and were in accordance with the National Institutes of Health guidelines for use of live animals.

Neural Stem-Cell Isolation and Characterization

Subventricular zone (SVZ) NSCs isolation was completed as previously described (27). F344 rats were euthanized, and brains were aseptically removed and

placed in 4°C modified Dulbecco's modified Eagle medium/nutrient mixture F-12. The SVZ was carefully dissected under a dissecting microscope, triturated, centrifuged, and then plated at 37°C, 5% CO_2 in N5 media. After 18 hours, nonadherent cells were removed from culture and replated in N5 media and allowed to proliferate. The cells were then used for implantation or immunostained with Nestin or cultured on Matrigel for characterization.

Endothelia Progenitor Cells Isolation and Characterization

EPCs were isolated previously described by Marrotte et al (28). F344 rats were euthanized, and the femur and tibia were aseptically removed. Isolated bone marrow-derived cells were plated on Vitronectin-coated six-well plates. For EPC characterization, after a 7-day culture, attached cells were labeled with 1,1′-dioctadecyl–3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-acetylated low density lipoprotein (Dil-acLDL) and fluorescein isothiocyanate labeled lectin. Cells demonstrating double-positive fluorescence of Dil-acLDL and lectin were identified as differentiating EPCs. To confirm EPC function, EPC in vitro tube formation assay was completed on Matrigel (28).

Biomimetic Hydrogel Matrix Synthesis

Biomimetic hydrogel matrix was synthesized as previously established by Schweller and West (25). Briefly, the peptide sequence GGGGGPQGIWGQGG-Lys-(alloc)-GK was synthesized with fluorenylmethyloxycarbonyl-protecting group chemistry and then conjugated to acrylate-PEG-succinimidyl valerate (Laysan Bio, Arab, AL). Similarly, the peptide sequence RGDS (GenScirpt, Piscataway, NJ) was conjugated with acryl-PEG-SVA in dimethyl sulfoxide with N,N-Diisopropylethylamine. Conjugated sequences were dialyzed and lyophilized and stored in inert argon gas at –80°C until needed.

Encapsulation of NSCs and EPCs in a Biomimetic Hydrogel Matrix

PEG-GGGGGPQGIWGQGG-Lys-(alloc)-GK-PEG and PEG-RGDS were dissolved in HEPES-buffered saline with triethanolamine containing eosin Y- and N-vinylpyrrolidone (Sigma, St. Louis, MO) with 2×10^4 EPCs/µL with 2×10^4 NSCs/µL (total 2×10^5 cells

in 5 µL) (25). Five microliters of the prepolymer solution mixed with cells was placed onto a glass slide and covered with a glass slide, and polymerized under a white light lamp, 195 mW/cm². The resultant EPC/NSC/biomimetic hydrogel was placed in EGM-2 at 4°C until implantation on the same day.

Spinal Cord Transection

Female Fischer F344 rats 120–130 g (Charles River, Wilmington, MA) were anesthetized with isoflurane through a face mask (29). Aseptically, a 1.5-cm midline skin incision was made over T9–T10, a bilateral laminectomy was completed, and the right side of the spinal cord was transected. Fascia was sewn over the spinal cord to keep the biomimetic hydrogel in place, and then, the incision site was sutured closed.

Behavioral Testing

The Basso, Beattie and Bresnahan (BBB) score is an ordinal scale from 0 to 21 and represents sequential recovery of joint movement, hind limb movement, stepping, limb coordination, trunk stability, paw placement, and tail movement. The rats were observed using a blinded observer for 5 minutes for toe, foot, ankle, knee, hip, and tail movement and evaluated with the BBB score every 7 days for 4 weeks (29).

Immunohistochemistry

Spinal cords were dissected, fixed overnight in 4% paraformaldehyde at 4°C, and then transferred to 30% sucrose for 72 hours. Longitudinal sections of spinal cords containing the lesion/graft site were sectioned. Blocking was done with 5% donkey serum, spinal cord sections were stained for vascular endothelial-Cadherin (1:200, Santa Curz), Laminin (1:200, Santa Cruz, Dallas, TX), Beta-III tubulin (1:200, R&D Systems, Minneapolis, MN), Nestin (1:200, Abcam, Cambridge, MA), glial fibrillary acidic protein (GFAP) (1:200 Abcam), CSPG (1:200, Sigma), and with secondary antibodies (1:200, Invitrogen, Carlsbad, CA).

Zymography

The conditioned media of cell-laden hydrogels (days 1–4) were collected, and gelatin zymography was performed, using 10% Ready Gel Zymogram Gel (Bio-Rad, Hercules, CA) to identify MMP secretion by

encapsulated cells. Protein standards (10–250 kDa; Bio-Rad) were used as ladders, and fresh medium was used as a reference. Gel images were captured, and band intensities were quantified using the BioRad Image Laboratory software ($n = 4$–5 hydrogels). Zymography was completed as previously described comparing conditioned media of EPCs and NSCs (30) (**Supplemental Digital Content 2**, http://links.lww.com/CCX/A639).

Statistical Analyses

The statistical analysis for the comparison of motor score between the groups was performed via SAS (Version 9.1, Cary, NC)-mixed procedure, followed with Tukey correction for multiple comparisons (28). Data are reported as mean ± SEM, and a two-tailed p value of less than 0.05 was taken as evidence of a statistically significant finding.

RESULTS

Endothelial Progenitor Cell and Neural Stem-Cell Characterization

EPCs isolated from the bone marrow were characterized as previously described by Marrotte et al (28), were positive for Dil-acLDL and isolectin labeling, and formed tubes when plated on Matrigel, an assay for in vitro angiogenic potential (**Fig. 1**). NSCs were isolated from the SVZ of adult rat brains and identified with NSC marker Nestin in cell culture (**Fig. 1A**) (27, 31). Isolated NSCs also showed the ability to differentiate into neurons (Beta III tubulin positive) and astrocytes (GFAP positive) on Matrigel (**Fig. 1B**) (32).

Combined NSC/EPC/Biomimetic Hydrogel Therapy Improved Motor Function in Rats With an Acute Hemisection Spinal Cord Injury

For therapeutic optimization, we studied cellular interactions of EPCs and NSCs encapsulated in the biomimetic hydrogel in vitro. In our preliminary in vitro data, we found that NSCs alone did not produce a significant level of MMP compared with EPCs. In addition, cell spreading of neurons coencapsulated with EPCs was greater than NSCs alone (Supplemental Digital Content 2, http://links.lww.com/CCX/A639). Therefore, for our in vivo studies, we proceeded with three groups: 1) control, 2) biomimetic hydrogel, and 3) NSCs/EPC/biomimetic hydrogel. Rats underwent

right-sided spinal cord incision to form a hemisection SCI. Fascia was sewn over the site of injury for all three groups, which held the biomimetic hydrogel in place for group 2 and group 3. Rats underwent blinded BBB scoring weekly for 4 weeks (0 = no observable hind limb movement, 21 = normal). BBB scores on week 4: 1) control 5.6 ± 2.7, 2) biomimetic hydrogel 8.6 ± 4.5, and 3) NSCs/EPC/biomimetic hydrogel 14.8 ± 0.8. Only rats that underwent combination therapy with NSC/EPC/biomimetic hydrogel showed significant improvement in motor function compared with control rats that only underwent hemisection ($p < 0.05$) (**Fig. 2**; and **Supplemental Video File 1**, http://links.lww.com/CCX/A641, **Supplemental Video File 2**, http://links.lww.com/CCX/A663, and **Supplemental Video File 3**, http://links.lww.com/CCX/A664).

Combined NSC/EPC/Biomimetic Hydrogel Therapy Improved Neuronal Spreading in the Area of Injury

Rats that underwent NSC/EPC/biomimetic hydrogel therapy demonstrated cell spreading and appeared to form interneuron connections within the biomimetic hydrogel and with neurons injured in the spinal cord (**Fig. 3A–D**). Conversely, in the untreated control group, in which no ECM deposition occurred, there was no cell integration in the injured area (**Fig. 3E–H**). In control spinal cords in which connective tissue did form within the injured area, no neural connections appeared to form within the injured area (**Fig. 3I–L**). Interestingly, in the acellular biomimetic hydrogel-only group, cell spreading interneuron connections were found within the biomimetic hydrogel (**Fig. 3M–P**), indicating that the biomaterial alone was capable of promoting a regenerative response.

Neural Stem Cells Were Found in the NSC/EPC/Biomimetic Hydrogel and Connective Tissue Within the Control Groups, but Not in Injured Areas Lacking ECM

Rats that underwent NSC/EPC/biomimetic hydrogel therapy for spinal cord hemisection demonstrated NSCs (nestin positive cells) in the surrounding spinal cord tissue, as well as in the biomimetic hydrogel (**Fig. 4 A–D**). Untreated control rats with connective tissue within the injured site, interestingly, demonstrated infiltration of native NSCs into injured area as well as the
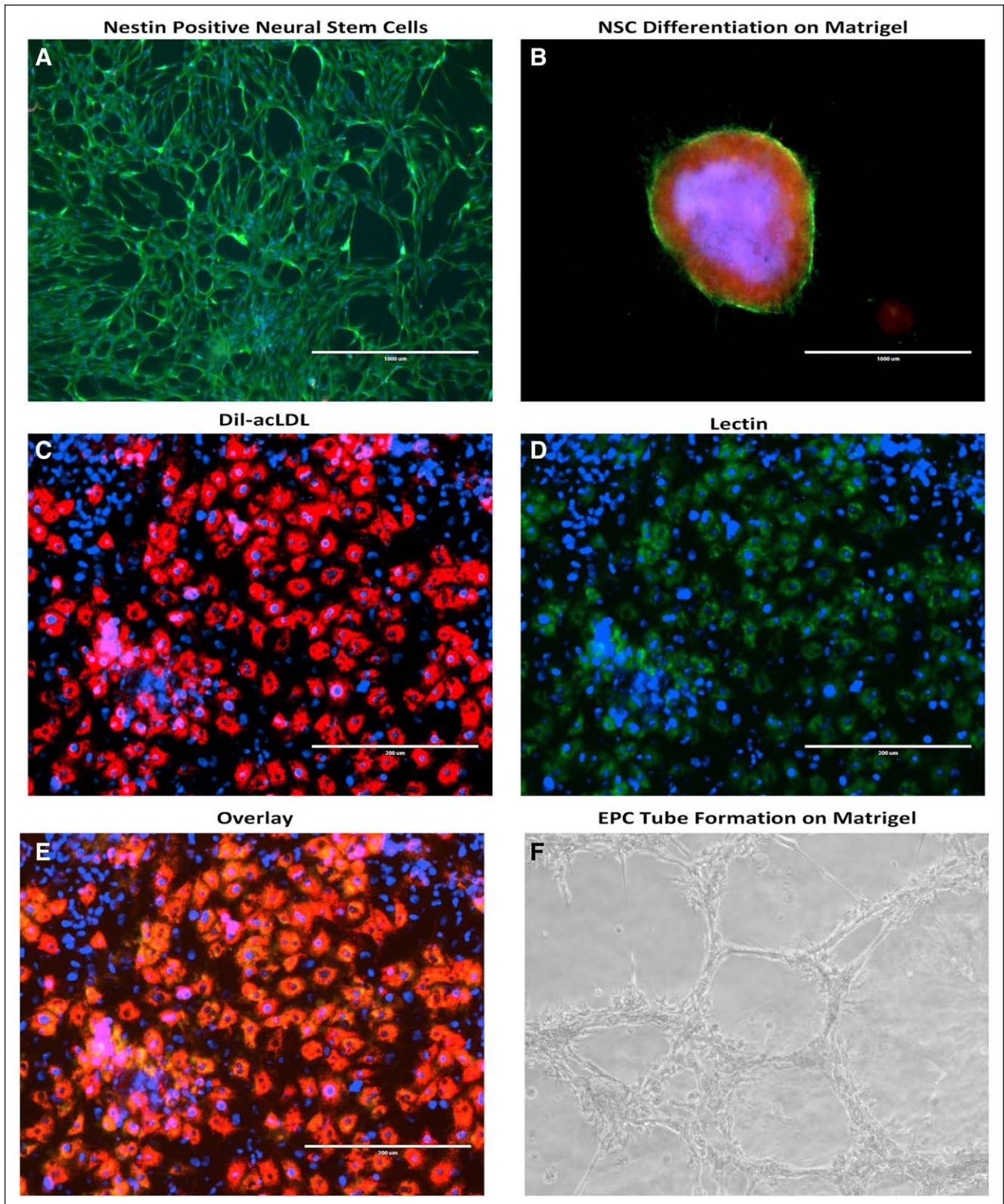
Figure 1. Characterization of rat subventricular zone (SVZ)-derived neural stem cells (NSCs) and endothelial progenitor cells (EPCs).
A, Nestin-positive NSCs (*green*) isolated from rat SVZ and nucleus (4′,6-diamidine-2′-phenylindole dihydrochloride [DAPI]: *blue*).
B, NSC differentiation on Matrigel, NSC differentiated into neurons (βIII tubulin: *red*), astrocytes (glial fibrillary acidic protein: *green*), and nucleus (DAPI: *blue*). Scale bar 1,000 μm. Characterization of rat bone marrow–derived EPCs (**C–F**), nucleus (DAPI: *blue*), uptake of 1,1′-dioctadecyl − 3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-acetylated low density lipoprotein [Dil-acLDL] (*red*) (**C**), and Lectin staining (*green*) (**D**), combined Dil-acLDL/Lectin (**E**), and EPC tube formation on Matrigel (10×) (**F**). Scale bar: 200 μm.
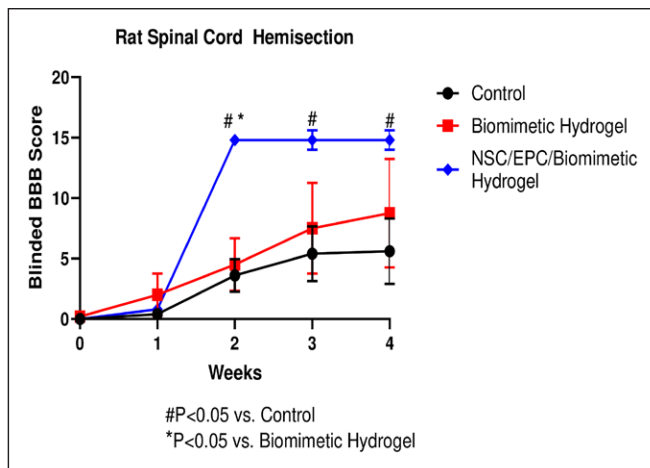
Figure 2. Blinded Basso, Beattie and Bresnahan (BBB) motor score. Rat right hind limb motor function improved significantly in the neural stem cell (NSC)/endothelial progenitor cell (EPC)/biomimetic hydrogel group (■) on weeks 2, 3, and 4, compared with the control group (●) and week 2 compared with the hydrogel-only group (◆) (# or *p < 0.05). No significant difference was found between the control group and the biomimetic hydrogel-only group (mean ± SEM, n = 4–5; of note, all groups started with 5 rats and one rat in the biomimetic hydrogel-only group died of unknown cause after week 2).

surrounding spinal cord tissue. Rats with no connective tissue or cyst formation in the injured area had NSCs in the surrounding spinal cord tissue but not in the area of hemisection. Infiltration of NSCs into the wounded area could also be found in the biomimetic hydrogel-only group.

Angiogenesis Occurred in the Area of Spinal Cord Hemisection for All Three Groups, Except for the Cystic/Non-ECM Area of the Control Group

Rats that underwent NSC/EPC/biomimetic hydrogel therapy for spinal cord hemisection demonstrated blood vessel formation and laminin staining within the wounded area and surrounding tissue (**Fig. 4D–F**). The control rats that developed connective tissue within the injured area also demonstrated the formation of blood vessels and laminin staining within the injured area (**Fig. 4J–L**). Control rats that formed cysts or lacked ECM deposition showed blood vessel formation and laminin staining within the spinal cord tissue near the injured area but not in the area of injury (**Fig. 4G–I**). Biomimetic hydrogel rats showed blood vessel formation and laminin staining within the injured area and within the surrounding spinal cord tissue (**Fig. 4M–O**).

Astrocytes Migrate to the Site of Injury and Expression of CSPG Increased in the Spinal Cord for All Three Groups

Astrocytes migrated to the site of injury for all three groups (**Fig. 5A–L**). However, there was minimal infiltration of astrocytes and minimal positive staining for CSPG within the injury site of the three groups.

DISCUSSION

In the current study, we demonstrate proof of concept that modification the CNS environment via replacing the ECM with a novel biomimetic hydrogel containing EPCs and NSCs in a transected area allows for initiation of tissue repair in an injured spinal cord. Within the 4-week period, we found that control rats developed either a scar or did not form ECM within the injured site, leaving an acellular cavity (Fig. 3E–L). In control rats with an acellular cavity, there was no infiltration of neurons or formation of a neural network. In control rat spinal cords that developed ECM within the injured site, we unexpectedly found infiltration of neurons (Fig. 3I–L) and NSCs (Fig. 4C). Within this native ECM/scar tissue, neurons (βIII tubulin-positive cells) remained circular and did not form neural connections (Fig. 3I–L). In rats that were implanted with NSC/EPC/biomimetic hydrogel or biomimetic hydrogel alone, cell spreading occurred and neural networks formed (Fig. 3A–D). Interestingly in all rats, control and experimental, we found the migration of NSCs to the area of injury (Fig. 4A–D). The observable difference in the formation of a neural network in the rats treated with NSC/EPC/biomimetic hydrogel or biomimetic hydrogel alone demonstrates that this biomimetic hydrogel with a fibronectin motif (RGDS) and an MMP cleavable sequence allows the initiation of tissue repair in an injured spinal cord. Consistent with the finding of increased neural networks in the NSC/EPC/biomimetic hydrogel group, we found that the BBB behavioral score, NSC/EPC/biomimetic hydrogel demonstrated significantly improved motor function compared with the control rats (Fig. 2). Our in vitro findings that NSCs did not produce a significant level of MMPs compared with EPCs (Supplemental Digital Content 2, http://links.lww.com/CCX/A639) lead us to coencapsulate NSC with EPC to allow for the MMP degradation of the biomimetic hydrogel in vivo.
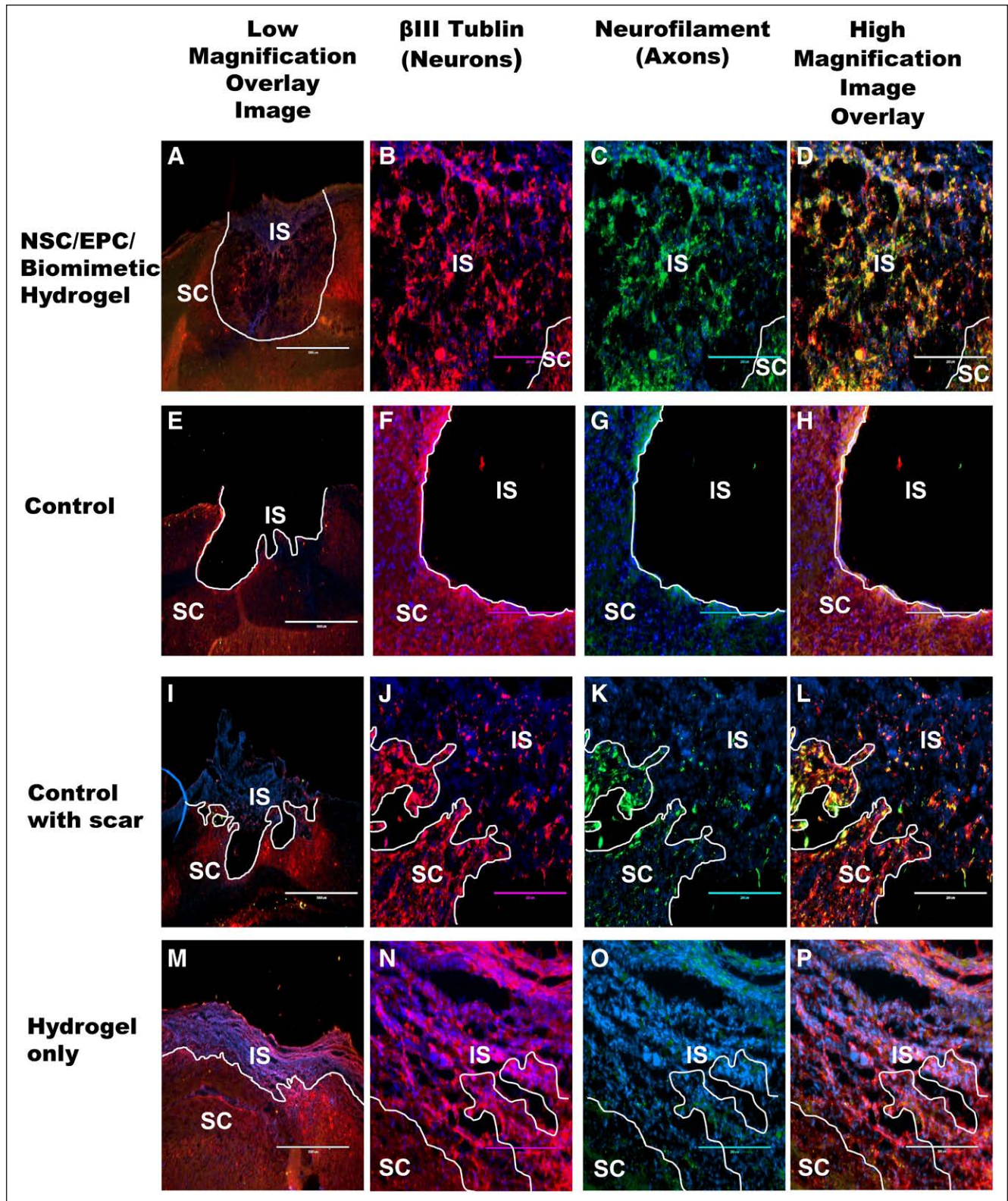
Figure 3. De novo CNS tissue formation occurs in the experimental group neural stem cell (NSC)/endothelial progenitor cell (EPC)/ biomimetic hydrogel therapy but not in the control group. Neuron staining (βIII tubulin staining: *red*) of rat spinal cords (SCs) 4 wk after hemisection, with (**A–D**) NSC/EPC/biomimetic hydrogel therapy, (**E–H**) control/no therapy demonstrating cyst formation, (**I–L**) control/ no therapy demonstrating scar formation, and (**M–P**) biomimetic hydrogel-only therapy. (**A**), (**E**), (**I**), and (**M**) scale bar = 1,000 μm. Remainder scale bar = 200 μm. IS = injury site.
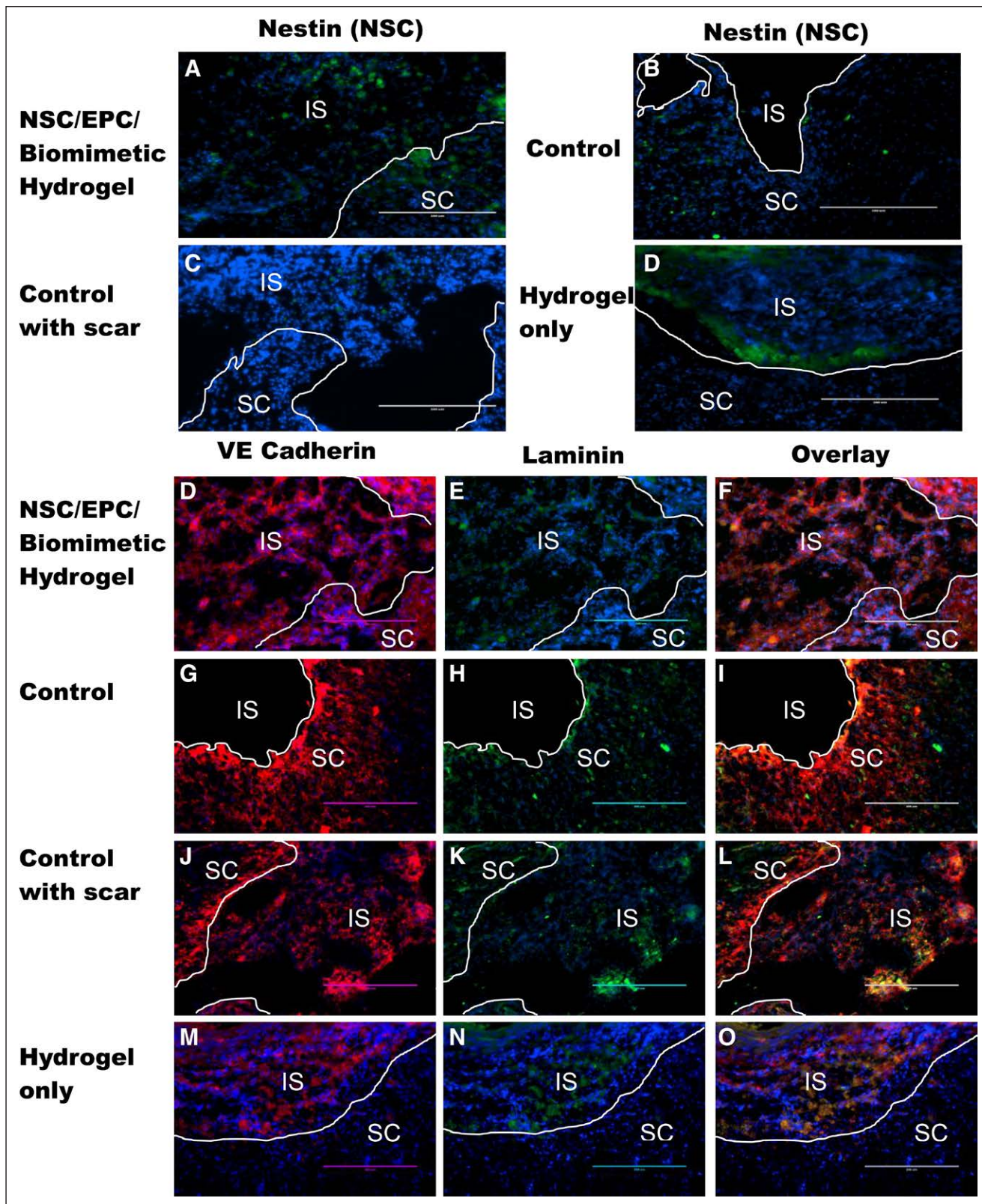
Figure 4. Neural stem cells (NSCs) migrate to the site of injury in all groups. NSC (Nestin: *green*) of rat spinal cords (SCs) 4 wk after hemisection, with (**A**) NSC/endothelial progenitor cell (EPC)/biomimetic hydrogel therapy, (**B**) control/no therapy demonstrating cyst formation, (**C**) control/no therapy demonstrating scar formation, and (**D**) biomimetic hydrogel-only therapy. Angiogenesis occurs in the experimental group NSC/EPC/biomimetic hydrogel therapy, and endothelial cell migration occurs to the site of injury in all groups. Endothelial cells (vascular endothelial [VE] cadherin: *red*) and Laminin (*green*) of rat SCs 4 wk after hemisection, with (**D–F**) NSC/EPC/biomimetic hydrogel therapy, (**G–I**) control/no therapy demonstrating cyst formation, (**J–L**) control/no therapy demonstrating scar formation, and (**M–O**) biomimetic hydrogel-only therapy. Scale bar = 200 µm. IS = injury site.
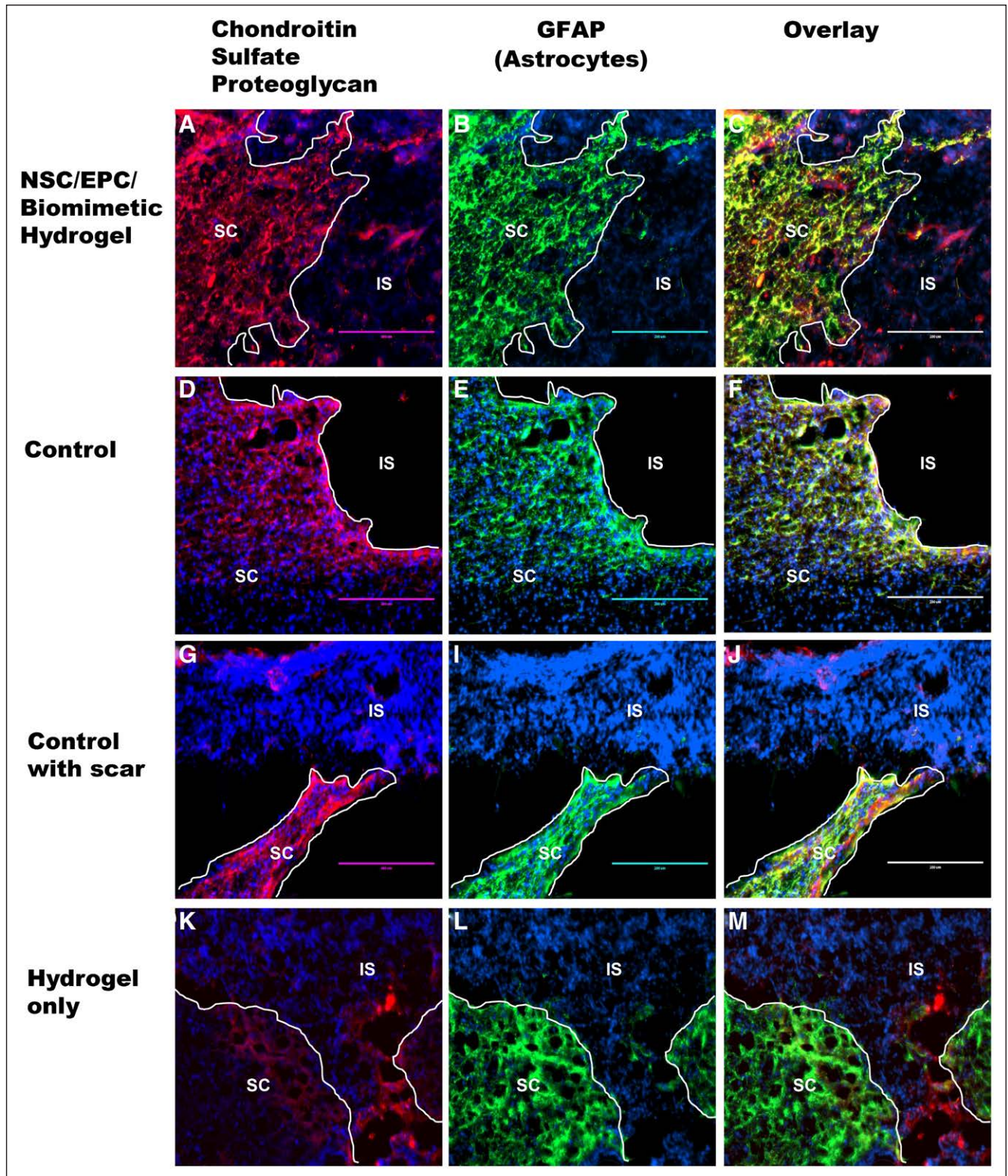
Figure 5. Astrocytes migrate to the site of injury in all groups and have limited infiltration into the biomimetic hydrogel. Astrocyte (glial fibrillary acidic protein [GFAP]: *green*) and chondroitin sulfate proteoglycan (*red*) of rat spinal cords 4 wk after hemisection, with (**A–C**) neural stem cell (NSC)/endothelial progenitor cell (EPC)/biomimetic hydrogel therapy, (**D–F**) control/no therapy demonstrating cyst formation, (**G–I**) control/no therapy demonstrating scar formation, and (**J–L**) biomimetic hydrogel-only therapy. Scale bar = 200 μm. IS = injury site.

The findings that NSCs and endothelial cell migrate to the site of injury for all groups may indicate that the CNS, at least in part, may have the ability to regenerate, and lack of "regenerative ECM" may be a significant limitation for spontaneous CNS repair after injury. Therefore, modification of the CNS ECM to an optimized environment may make the environment conducive to stem/progenitor-cell adhesion and activation allow for repair and return of functional tissue (parenchyma) in the CNS. Interestingly, in the biomimetic hydrogel-only group, we found induced formation of a neural network (Fig. 3 *M–P*); however, the motor function of biomimetic hydrogel-only group did not improve as well as the NSC/EPC/biomimetic hydrogel group. This finding may indicate that ECM modification alone is not sufficient to induce significant improvement in motor function.

In this study, we found that astrocytes will migrate to the peri-injury site area; however, there was minimal astrocyte migration/integration into the biomimetic hydrogel (**Fig. 5**). Consistent with other studies, we also found expression of CSGP in the tissue surrounding the wound (33–35). Although astrocytes are in part responsible for the formation of the glial scar (which can inhibit CNS regeneration), astrocytic cells are necessary for maintenance of normal CNS homeostatic function. Astrocytes are functionally important in the CNS to maintain glycogen storage for neurons, supply of lactate for metabolic support for neurons, regulation of extracellular ions, and neural transmitter processing (such as glutamate processing). Astrocytes are also an integral part of the blood-brain barrier (36–38). Additionally, in noninjury conditions, astrocytes will produce ECM components such as CSPG, which will stabilize axon and dendrite synapses (39). Therefore, future development of biomimetic or modified ECM for SCI will likely need to include the appropriate adhesion molecules and modified stiffness, which would allow for astrocyte infiltration and subsequent glial support of neurons and structural reformation of the blood-brain barrier.

EPCs, the primary cells responsible for the induction of angiogenesis and vascular repair, once implanted into injured tissue are known to induce upregulation of several growth factors (28, 40). The novel biomimetic hydrogel developed by Schweller and West (25) contains a fibronectin-derived motif RGDS, which allow EPCs and endothelial cell to adhere to the biomimetic hydrogel and promotes angiogenesis both in vivo and in vitro (23). Angiogenesis and subsequent perfusion of an injured site is sine qua non for wound healing and tissue repair (28, 41). Interestingly, a recent study published by Boldrini et al (42) revealed a possible link between angiogenesis and neuroplasticity. In the NSC/EPC/biomimetic hydrogel group and the biomimetic hydrogel-only group, we found de novo blood vessel formation (Fig. 4 *D–L*), whereas in the control groups in which there was no ECM formation and a gap remained at the site of injury, angiogenesis did not occur (phase contrast images: **Supplemental Digital Content 3**, http://links.lww.com/CCX/A640). The development and optimization of biomimetic hydrogels will need to include molecular mechanisms that induce angiogenesis and promote the reformation of the blood CNS barrier.

There are wide ranges of biomaterials that can be used to modify the CNS environment after injury (43). Transformation of the injured CNS tissue into an environment conducive to repair with biomaterials may promote cellular-induced repair, augment the natural course of wound healing, and promote de novo tissue formation and the return of clinically significant motor function after SCI. Hydrogels can be modified to incorporate growth factors, support cell adhesion, proliferation, and differentiation and modify stiffness to resemble surrounding ECM (23, 25, 33, 43–45). However, the optimal hydrogel configuration/chemical composition needed to improve motor function is currently unknown (43). In a study by Hakim et al (33), a nondegradable hydrogel oligo[poly(ethylene glycol) fumarate] (OPF+) was embedded with Schwann cells and implanted into rats with a full spinal cord transection. Despite decreased scar formation in the group implanted with OPF+, few axons transversed the lesion, and there was no significant improvement in motor function (33). A multipolymer hydrogel made of poly(ethylene glycol), poly(L-lysine) hydrobromide and poly(lactic-co-glycolic acid) was combined with neural progenitor cells and endothelial cells and used by Rauch et al (41), to treat hemisection SCI in a rat model. In the Rauch et al (41) study, they found an improvement in angiogenesis and decreased scar formation when compared with the control groups; however, no improvement in motor function was seen. In a previous study by Hong et al (46), an imidazole-poly(organophosphazenes) hydrogel (I-5) was used to promote generation of function CNS tissue after a

contusional SCI in a rat model. The I-5 hydrogel had a stiffness of approximately 600 pascals at 37°C and contained a histamine moiety imidazole group, which allowed macrophage to infiltration into the area of injury. The authors found therapy of a contusion SCI with I-5 hydrogel: promoted increased fibronectin expression, decreased the size of the cystic cavities that were formed, induced macrophage remodeling, improved motor neuron survival, and improved the BBB functional outcome score by 2 points at 6 weeks postinjury (46). In both the study by Hong et al (46) and the biomimetic hydrogel-only group in the current study, no improvement in functional outcomes was found at week 4. Indicating that when determining efficacy, the required time frame for monitoring functional outcomes in hydrogel-based treatments needs to be established. Also of note, the functional recovery in the NSC/EPC/biomimetic hydrogel in the current study demonstrated a more robust average improvement of the BBB score (9.6), at week 4.

The stiffness of ECM can in part regulate cell structure, motility, proliferation, and differentiation (47–49). Previous studies have shown that softer ECM promotes NSC differentiation into neurons, whereas stiffer ECM promotes NSC differentiation into glia cells, such as astrocytes (50–52). Interestingly, stiffer ECM has been shown to be more consistent with a glial scar (34, 51, 52). The stiffness of the I-5 hydrogel in the study by Hong et al (46) and the biomimetic hydrogel used in the current study was in the range of 600 Pascals (25). The data from the current study and the study by Hong et al (46) suggest that modifying the extracellular environment to a stiffness in the range of 600 Pascals may provide a change in the extracellular environment, which allows for the induction of spinal cord repair with return of motor function. Whereas in studies in which no functional recovery was found, the stiffness of the hydrogel was approximately 130,000 Pascals in the study by Hakim et al (33) and greater than 2,500 Pascals in the study by Rauch et al (41) (53). Although the optimal extracellular environment stiffness modification needed for functional recovery is not known, combined, these studies provide initial evidence that high stiffness hydrogel therapy may not be conducive for the induction of functional recovery in SCI.

The biomimetic hydrogel used in this study differs significantly from previously developed scaffolds. First, this is a biomimetic hydrogel that was developed to have low stiffness (25), which, in this case, could promote NSC differentiation to neurons. Additionally, lower stiffness can allow for cell spreading within the biomimetic hydrogel (23, 25). Second, this biomimetic hydrogel contains an adhesion sequence that is related to ECM fibronectin (RGDS). Fibronectin has been shown to be important in the induction of angiogenesis, stabilization of the blood-brain barrier, NSC differentiation into neurons, as well as neuron adhesion to connective tissue (54, 55). Third, this biomimetic hydrogel contains an enzymatically cleavable novel peptide sequence (GGGGGPQGIWGQGG-Lys-[alloc]-GK) (23, 25). This enzymatically cleavable sequence allows for degradation of the biomimetic hydrogel with MMP2 and MMP9 in the injured area, and replacement with native ECM after cell infiltration has already occurred (23). Extensive research into the how the CNS responds to different changes of the extracellular environment will be important to optimize not only the cellular response with the formation of neuronal connections and blood-brain astrocyte response but most importantly improvement in functional outcomes. Given there are multiple potential therapeutic candidate hydrogels that can undergo several modifications, a significant number of further studies are required in order to determine which biomaterial modifications will provide the optimal environment to illicit functional recovery in patients with SCI.

CONCLUSIONS

Santiago Ramón y Cajal, one of the pioneers of the field of Neuroscience, previously developed the hypothesis that the inability of axons to elongate within the injured CNS is not related to intrinsic properties of the axons, but instead, axon elongation is restricted by the environment in which the axons are located (56, 57). Since that time, many studies have shown that the CNS lacks the innate mechanistic ability to regenerate autonomously (21, 58). Our current work provides evidence that modification of the tissue environment after SCI may be one important step in the induction of CNS regeneration. In this study, we demonstrate that environment modification of the spinal cord site of injury, via implantation of NSCs and EPCs incorporated within a novel biomimetic hydrogel, allows for formation of neural and vascular networks. We observed an improvement in hind limb motor function in these rats compared with the control group. In order to

facilitate tissue repair of injured CNS tissue, multifactorial optimization will be required including: modification of ECM to foster adhesion, migration and differentiation of stem/progenitor cells, as well as the induction of angiogenesis and determination of the optimal type(s) of therapeutic stem/progenitor cells. Future research should focus on optimizing biomimetic hydrogel stiffness, the degradation molecules, and adhesion molecules that will allow for astrocyte integration, cell types and cell number for implantation, and cell tracking to gain a better understanding of how neural and blood vessel networks are formed in a biomimetic hydrogel. Overall, in this study, our data demonstrate proof of principle that, following SCI, changing the environment of the injured area via cell therapy combined with a bioactive biomimetic hydrogel can induce formation of de novo CNS tissue and improve functional recovery.

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REFERENCES

1. Selvarajah S, Hammond ER, Haider AH, et al: The burden of acute traumatic spinal cord injury among adults in the united states: An update. *J Neurotrauma* 2014; 31:228–238

2. Stein DM, Sheth KN: Management of acute spinal cord injury. *Continuum (Minneap Minn)* 2015; 21:159–187

3. Reynolds BA, Weiss S: Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992; 255:1707–1710

4. Asahara T, Murohara T, Sullivan A, et al: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; 275:964–967

5. Livecchi MA: Spinal cord injury. *Continuum (Minneap Minn)* 2011; 17:568–583

6. Guercio JR, Kralic JE, Marrotte EJ, et al: Spinal cord injury pharmacotherapy: Current research & development and competitive commercial landscape as of 2015. *J Spinal Cord Med* 2019;42:102–122

7. Kumar AA, Kumar SR, Narayanan R, et al: Autologous bone marrow derived mononuclear cell therapy for spinal cord injury: A phase I/II clinical safety and primary efficacy data. *Exp Clin Transplant* 2009; 7:241–248

8. Frolov AA, Bryukhovetskiy AS: Effects of hematopoietic autologous stem cell transplantation to the chronically injured human spinal cord evaluated by motor and somatosensory evoked potentials methods. *Cell Transplant* 2012; 21(Suppl 1):S49–S55

9. Sarmento CA, Rodrigues MN, Bocabello RZ, et al: Pilot study: Bone marrow stem cells as a treatment for dogs with chronic spinal cord injury. *Regen Med Res* 2014; 2:9

10. Silver J, Miller JH: Regeneration beyond the glial scar. *Nat Rev Neurosci* 2004; 5:146–156

11. Grégoire CA, Goldenstein BL, Floriddia EM, et al: Endogenous neural stem cell responses to stroke and spinal cord injury. *Glia* 2015; 63:1469–1482

12. Cusimano M, Biziato D, Brambilla E, et al: Transplanted neural stem/precursor cells instruct phagocytes and reduce secondary tissue damage in the injured spinal cord. *Brain* 2012; 135:447–460

13. Kadoya K, Lu P, Nguyen K, et al: Spinal cord reconstitution with homologous neural grafts enables robust corticospinal regeneration. *Nat Med* 2016; 22:479–487

14. Cheng Z, Zhu W, Cao K, et al: Anti-inflammatory mechanism of neural stem cell transplantation in spinal cord injury. *Int J Mol Sci* 2016; 17:E1380

15. Nguyen HX, Hooshmand MJ, Saiwai H, et al: Systemic neutrophil depletion modulates the migration and fate of transplanted human neural stem cells to rescue functional repair. *J Neurosci* 2017; 37:9269–9287

16. Amemori T, Ruzicka J, Romanyuk N, et al: Comparison of intraspinal and intrathecal implantation of induced pluripotent stem cell-derived neural precursors for the treatment of spinal cord injury in rats. *Stem Cell Res Ther* 2015; 6:257

17. Abematsu M, Tsujimura K, Yamano M, et al: Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. *J Clin Invest* 2010; 120:3255–3266

18. Norenberg MD, Smith J, Marcillo A: The pathology of human spinal cord injury: Defining the problems. *J Neurotrauma* 2004; 21:429–440

19. Cheah M, Andrews MR: Integrin activation: Implications for axon regeneration. *Cells* 2018; 7:E20

20. Frantz C, Stewart KM, Weaver VM: The extracellular matrix at a glance. *J Cell Sci* 2010; 123:4195–4200

21. Yuan YM, He C: The glial scar in spinal cord injury and repair. *Neurosci Bull* 2013; 29:421–435

22. Leal-Filho MB: Spinal cord injury: From inflammation to glial scar. *Surg Neurol Int* 2011; 2:112

23. Schweller RM, Wu ZJ, Klitzman B, et al: Stiffness of protease sensitive and cell adhesive PEG hydrogels promotes neovascularization in vivo. *Ann Biomed Eng* 2017; 45: 1387–1398

24. Moon JJ, Saik JE, Poché RA, et al: Biomimetic hydrogels with pro-angiogenic properties. *Biomaterials* 2010; 31:3840–3847

25. Schweller RM, West JL: Encoding hydrogel mechanics via network cross-linking structure. *ACS Biomater Sci Eng* 2015; 1:335–344

26. Zhu J: Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering. *Biomaterials* 2010; 31:4639–4656

27. Paez-Gonzalez P, Asrican B, Rodriguez E, et al: Identification of distinct ChAT+ neurons and activity-dependent control of postnatal SVZ neurogenesis. *Nat Neurosci* 2014; 17:934–942

28. Marrotte EJ, Chen DD, Hakim JS, et al: Manganese superoxide dismutase expression in endothelial progenitor cells accelerates wound healing in diabetic mice. *J Clin Invest* 2010; 120:4207–4219

29. Basso DM, Beattie MS, Bresnahan JC, et al: MASCIS evaluation of open field locomotor scores: Effects of experience and teamwork on reliability. Multicenter Animal Spinal Cord Injury Study. *J Neurotrauma* 1996; 13:343–359

30. Wu Y, Puperi DS, Grande-Allen KJ, et al: Ascorbic acid promotes extracellular matrix deposition while preserving valve interstitial cell quiescence within 3D hydrogel scaffolds. *J Tissue Eng Regen Med* 2017; 11:1963–1973

31. Scheffler B, Walton NM, Lin DD, et al: Phenotypic and functional characterization of adult brain neuropoiesis. *Proc Natl Acad Sci U S A* 2005; 102:9353–9358

32. Gelain F, Bottai D, Vescovi A, et al: Designer self-assembling peptide nanofiber scaffolds for adult mouse neural stem cell 3-dimensional cultures. *PLoS One* 2006; 1:e119

33. Hakim JS, Esmaeili Rad M, Grahn PJ, et al: Positively charged oligo[poly(ethylene glycol) fumarate] scaffold implantation results in a permissive lesion environment after spinal cord injury in rat. *Tissue Eng Part A* 2015; 21:2099–2114

34. Moshayedi P, Ng G, Kwok JC, et al: The relationship between glial cell mechanosensitivity and foreign body reactions in the central nervous system. *Biomaterials* 2014; 35:3919–3925

35. Ohtake Y, Li S: Molecular mechanisms of scar-sourced axon growth inhibitors. *Brain Res* 2015; 1619:22–35

36. Hillen AEJ, Burbach JPH, Hol EM: Cell adhesion and matricellular support by astrocytes of the tripartite synapse. *Prog Neurobiol* 2018; 165–167:66–86

37. Magistretti PJ, Allaman I: Lactate in the brain: From metabolic end-product to signalling molecule. *Nat Rev Neurosci* 2018; 19:235–249

38. Giannoni P, Badaut J, Dargazanli C, et al: The pericyte-glia interface at the blood-brain barrier. *Clin Sci (Lond)* 2018; 132:361–374

39. Dyck SM, Karimi-Abdolrezaee S: Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system. *Exp Neurol* 2015; 269:169–187

40. Caplice NM, Doyle B: Vascular progenitor cells: Origin and mechanisms of mobilization, differentiation, integration, and vasculogenesis. *Stem Cells Dev* 2005; 14:122–139

41. Rauch MF, Hynes SR, Bertram J, et al: Engineering angiogenesis following spinal cord injury: A coculture of neural progenitor and endothelial cells in a degradable polymer implant leads to an increase in vessel density and formation of the blood-spinal cord barrier. *Eur J Neurosci* 2009; 29:132–145

42. Boldrini M, Fulmore CA, Tartt AN, et al: Human hippocampal neurogenesis persists throughout aging. *Cell Stem Cell* 2018; 22:589–599.e5

43. Mukherjee N, Adak A, Ghosh S: Recent trends in the development of peptide and protein-based hydrogel therapeutics for the healing of CNS injury. *Soft Matter* 2020; 16:10046–10064

44. Gros T, Sakamoto JS, Blesch A, et al: Regeneration of long-tract axons through sites of spinal cord injury using templated agarose scaffolds. *Biomaterials* 2010; 31:6719–6729

45. Shrestha B, Coykendall K, Li Y, et al: Repair of injured spinal cord using biomaterial scaffolds and stem cells. *Stem Cell Res Ther* 2014; 5:91

46. Hong LTA, Kim YM, Park HH, et al: An injectable hydrogel enhances tissue repair after spinal cord injury by promoting extracellular matrix remodeling. *Nat Commun* 2017; 8:533

47. Hastings JF, Skhinas JN, Fey D, et al: The extracellular matrix as a key regulator of intracellular signalling networks. *Br J Pharmacol* 2019; 176:82–92

48. Ahmed M, Ffrench-Constant C: Extracellular matrix regulation of stem cell behavior. *Curr Stem Cell Rep* 2016; 2:197–206

49. Lane SW, Williams DA, Watt FM: Modulating the stem cell niche for tissue regeneration. *Nat Biotechnol* 2014; 32:795–803

50. Georges PC, Miller WJ, Meaney DF, et al: Matrices with compliance comparable to that of brain tissue select neuronal over glial growth in mixed cortical cultures. *Biophys J* 2006; 90:3012–3018

51. Bartlett RD, Choi D, Phillips JB: Biomechanical properties of the spinal cord: Implications for tissue engineering and clinical translation. *Regen Med* 2016; 11:659–673

52. Discher DE, Mooney DJ, Zandstra PW: Growth factors, matrices, and forces combine and control stem cells. *Science* 2009; 324:1673–1677

53. Hynes SR, Rauch MF, Bertram JP, et al: A library of tunable poly(ethylene glycol)/poly(L-lysine) hydrogels to investigate the material cues that influence neural stem cell differentiation. *J Biomed Mater Res A* 2009; 89:499–509

54. Harris GM, Madigan NN, Lancaster KZ, et al: Nerve guidance by a decellularized fibroblast extracellular matrix. *Matrix Biol* 2017; 60–61:176–189

55. Ma W, Tavakoli T, Derby E, et al: Cell-extracellular matrix interactions regulate neural differentiation of human embryonic stem cells. *BMC Dev Biol* 2008; 8:90

56. David S, Aguayo AJ: Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. *Science* 1981; 214:931–933

57. Ramón y Cajal S, DeFelipe J, Jones EG: Cajal's Degeneration and Regeneration of the Nervous System. New York, NY, Oxford University Press, 1991

58. Silver J, Schwab ME, Popovich PG: Central nervous system regenerative failure: Role of oligodendrocytes, astrocytes, and microglia. *Cold Spring Harb Perspect Biol* 2014; 7:a020602