

RESEARCH PAPER

Efficient combined use of electroacupuncture with ordered collagen scaffolds enhances the therapeutic efficacy of human spinal cord-derived neural progenitor cells therapy in spinal cord injury rats

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Most spinal cord injury (SCI) patients experience severe morbidity and permanent disability. Currently, effective clinical treatments remain limited. Beyond direct nerve damage, spinal cord lesions result in atrophy of lower limb muscles, thereby impeding motor function recovery. Our therapeutic approach for repairing complete SCI combines traditional Chinese electroacupuncture stimulation of the Jiaji (EX-B2) and Zusanli (ST36) acupoints with the transplantation of human spinal cord-derived neural progenitor cells (hsc-NPCs) delivered on an ordered collagen scaffold. Results indicated that electroacupuncture was applied both to enhance the efficacy of hsc-NPCs therapy for nerve regeneration at the lesion site and to prevent muscle atrophy, contributing to motor function recovery. This innovative approach integrates Eastern and Western medical perspectives to pursue breakthroughs in SCI treatment.

ordered collagen scaffold | human spinal cord-derived neural progenitor cell | electroacupuncture | spinal cord injury

INTRODUCTION

Currently, the majority of spinal cord injury (SCI) patients face lifelong wheelchair dependence due to the devastating consequences of movement disability and autonomic dysfunction. Although numerous treatment strategies, including rehabilitation, pharmacotherapy, electrical stimulation, and cell transplantation, have been applied, functional recovery following SCI remains limited (Ahuja et al., 2017). Enhancing poor regenerative capacity and facilitating motor recovery in SCI patients represent major challenges. Our study introduces a novel dual-pathway regenerative strategy for SCI, combining electroacupuncture (EA)-mediated neuromodulation with bioengineered

neural reconstruction. This integrated method involves transplanting human spinal cord-derived neural progenitor cells (hsc-NPCs) onto topographically ordered collagen scaffolds to facilitate neural regeneration. Within this dual-strategy therapy, EA modulates the transplant microenvironment, thereby enhancing the regenerative capacity of the collagen scaffold-supported hsc-NPCs. Additionally, the approach confers systemic protection against denervated muscle atrophy and autonomic dysfunction.

Neural-regenerating scaffolds overcome the therapeutic bottlenecks of SCI through a tripartite strategy of structural substitution, functional reconstruction, and microenvironment modulation. Ordered collagen scaffolds used in this study demonstrated superior biocompatibility and controlled

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degradability, minimizing inflammatory responses while providing sustained structural support for cell survival and differentiation (Cui et al., 2021; Li et al., 2019; Zou et al., 2020a; Zou et al., 2020b). Their interconnected pore network facilitated precise cell delivery to the injury site, offered 3D guidance for axonal regrowth, and enhanced mechanical stabilization of damaged tissue. By combining these scaffolds with hsc-NPCs, we created a synergistic microenvironment that significantly enhanced axonal regeneration at the lesion site, as confirmed by post-transplantation analysis. This integrated cell-scaffold system presents a promising therapeutic strategy for structural and functional neural repair.

Accumulating evidence demonstrates that EA generates therapeutic electric fields to improve the injured microenvironment by promoting axonal regeneration, alleviating secondary injury cascades, and reducing post-SCI complications in both clinical and preclinical studies (Dorsher and McIntosh, 2011; Wang and Liu, 2008; Wu et al., 2002; Xie et al., 2006). Here, we first demonstrated that EA stimulation at Jiaji (EX-B2) not only enhances the survival and functional integration of transplanted progenitor cells but also reactivates endogenous neurorestorative programs, leading to cross-synaptic reinnervation and motor function recovery across the injury site. The oriented topography of collagen scaffolds guides axonal regeneration, while their porous structure synergizes with the EA-induced extracellular matrix to mitigate neuroinflammatory responses. This dual intervention potentiates the efficacy of the hsc-NPC neural scaffold by securing graft viability and establishing a reparative niche at the injury epicenter. In addition, concurrent EA stimulation at Zusanli (ST36) significantly enhances lower limb muscle recovery following SCI by improving neuromuscular function and mitigating disuse atrophy through activation of somatosensory-motor pathways.

Conventional progenitor cell therapies face fundamental limitations due to the hostile SCI microenvironment, which induces cell death and impedes functional integration (Bayleyegn Derso et al., 2025; Xia et al., 2023). The translational value of our study lies in overcoming the limitations of conventional progenitor cell therapies for SCI, providing a minimally invasive, cost-effective EA-scaffold combination therapy that enhances therapeutic outcomes for hSC-NPC treatment. EA treatment, applied post-transplantation, offers unique advantages including non-invasiveness, minimal side effects, high patient compliance, and cost-effectiveness (Alam and Chen, 2023; Tan et al., 2022; Wu, 2022). Our work combines acupuncture meridian theory with modern neuro-engineering principles to create a new framework for nerve regeneration. Given its clinical feasibility and scalability, EA holds considerable promise as an adjunctive therapy for SCI rehabilitation in global clinical practice.

RESULTS

The characteristics of the ordered collagen scaffold

The ordered collagen scaffold has a diameter comparable to that of the spinal cord and is thus suitable for *in vivo* transplantation to bridge lesion gaps. The morphology of the ordered collagen scaffolds was observed using scanning electron microscopy (SEM), which revealed an irregular, multi-porous structure. In addition, the scaffold exhibits favorable hydrophilicity, with a mean pore diameter of approximately 60 μm , which is optimal

for nutrient diffusion and cellular infiltration (Figure 1A). Porosimetry analysis demonstrated that the ordered collagen scaffold possesses a pore structure conducive to tissue engineering, with a dominant pore size range (8–200 μm) and high intrusion volume. This configuration promotes cell migration and homogeneous distribution, while the interconnected porosity facilitates vital nutrient and gas exchange, thereby laying a key physical foundation for tissue regeneration (Figure 1B and C). Calcein-AM/PI staining confirmed that cell viability remained consistently high at all time points examined (0, 1, 3, and 7 d post-seeding), indicating the scaffold's long-term biocompatibility and favorable cytocompatibility (Figure 1D). Immunofluorescence analysis revealed that the scaffold's flattened surface topography not only supported robust cell adhesion but also guided the seeded hsc-NPCs to extend extensive cytoplasmic processes along the porous network. The scaffold exhibits excellent hydrophilic properties, high water absorption, and strong retention capacity, allowing it to quickly absorb and retain substantial amounts of moisture, which is a critical characteristic for its intended applications (Figure S1A).

The survival and migration of the grafted hsc-NPCs in different treated groups

A significant proportion of transplanted cells often perish within weeks due to the hostile microenvironment at the injury site, characterized by inflammation, hypoxia, and glial scarring, all contributing to extensive cell death. The *in vivo* study included five experimental groups (Figure 2A): a Control group (no treatment), a CS group (implantation of ordered collagen scaffold only), a CS+EA group (ordered collagen scaffold implantation combined with EA), a CS+hsc-NPCs group (implantation of ordered collagen scaffold pre-loaded with hsc-NPCs), and a CS+hsc-NPCs+EA group (implantation of hsc-NPCs-loaded ordered collagen scaffold combined with EA therapy). Immunofluorescence staining showed that the EA treatment significantly enhanced the survival rate of grafted hsc-NPCs. Quantitative analysis at 8 weeks post-transplantation revealed substantially higher survival rates of hsc-NPCs in the EA-treated group compared to non-EA controls (* $P < 0.05$). This improvement was supported by histopathological cell counts, which revealed 2.12 and 1.61-fold increases in the number of viable grafted cells at the lesion sites of EA-treated rats at 2 weeks (* $P < 0.05$) and 8 weeks (** $P < 0.01$) post-transplantation, respectively. These findings indicate that EA intervention effectively promotes the survival of collagen scaffold-seeded hsc-NPCs following transplantation into transected rat spinal cords. When integrated with biomaterial scaffolds and EA stimulation, hsc-NPCs exhibit improved survival rates, which critically influence functional recovery and are essential for clinical translation (Figure 2B–D). This strategy maximizes therapeutic efficacy per transplantation by optimizing resource utilization.

Immunofluorescence at 8 weeks post-transplantation showed that grafted neural cells in the EA group migrated significantly farther from the lesion site than controls. This indicates that the transplanted hsc-NPCs not only survived the hostile injury microenvironment but also remained functional and motile. Since SCI affects both the lesion core and surrounding regions, cell migration enables broader repair coverage than static grafts, significantly enhancing therapeutic potential. The observed migration also implies a moderated pathological environment,

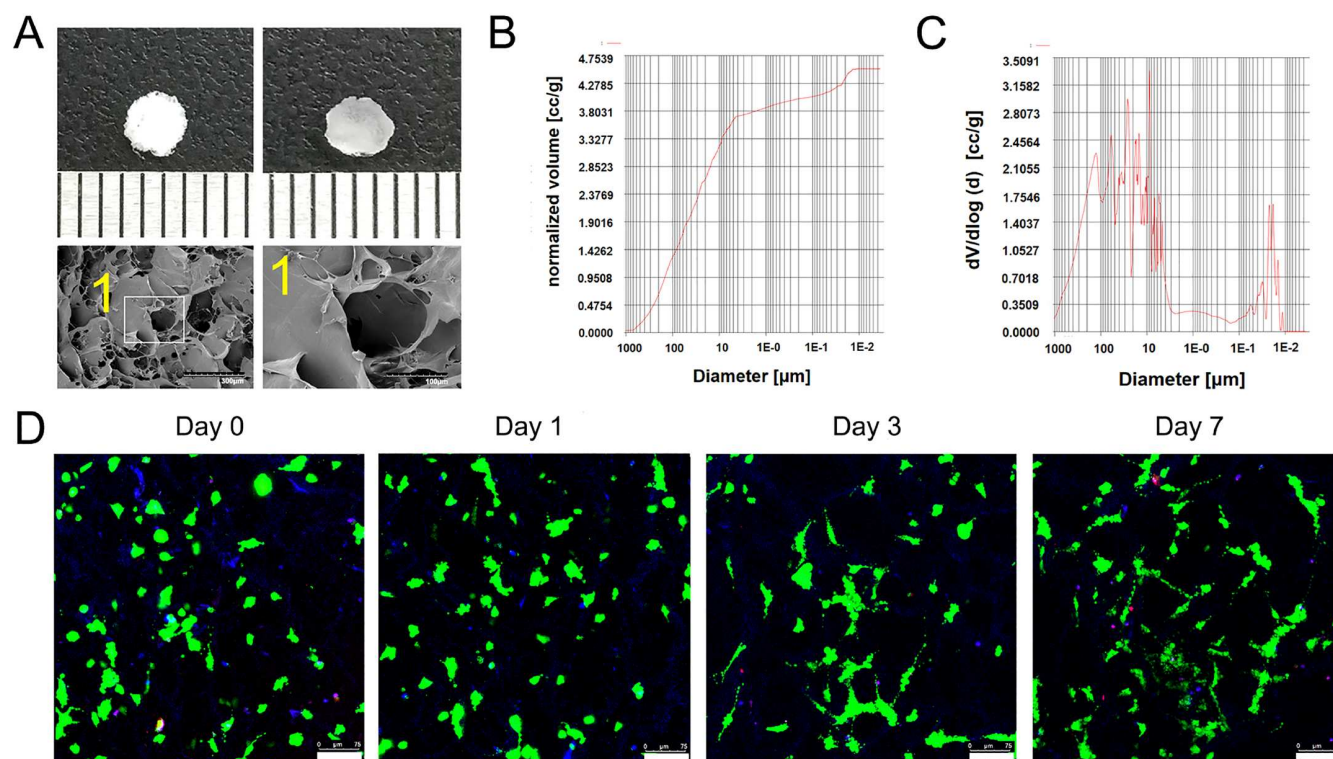


Figure 1 Schematic illustration and characterization of the CS. A, Macroscopic observation and SEM images revealing the porous microstructure of the ordered collagen scaffold. Scale bar, 300 μm (left), 100 μm (right). B, The cumulative intrusion curve shows an early onset pressure and a high saturation plateau, corresponding to a substantial total pore volume, suggesting the CS scaffolds possess a rich pore network. C, The pore volume distribution ($dV/d\log(d)$), which reflects the contribution of different pore sizes, exhibits a well-defined principal peak within the 8–200 μm range—a size favorable for cell migration and nutrient exchange. This pore structure promotes efficient cell seeding and uniform three-dimensional growth, which in turn establishes a key physical basis for successful tissue regeneration. D, Representative magnification (20 $\times$) view of Calcein-AM/PI staining confirmed the sustained high viability of the seeded hsc-NPCs on the CS scaffolds at all time points (0, 1, 3, and 7 d). Scale bars, 75 μm . CS, ordered collagen scaffold; hsc-NPCs, human spinal cord-derived neural progenitor cells.

with reduced inflammation and glial scarring likely due to EA facilitating cell movement. Taken together, these results show that the transplanted hsc-NPCs successfully engrafted, the host microenvironment improved, and the grafts actively participated in regeneration (Figure 2E).

Assessment of the proliferation and differentiation of transplanted hsc-NPCs in different treated groups at week 2 following SCI

The two-week post-injury time window represents the transition from acute inflammation to chronic pathology in SCI, during which transplanted hsc-NPCs typically encounter peak inhibitory signaling from glial scarring and chondroitin sulfate proteoglycans. Compared with non-stimulated controls, the upregulation of core pluripotency markers Sox2 and Nestin in the hsc-NPCs+CS+EA group was evidenced by immunofluorescence staining, indicating that the preserved self-renewal capacity was maintained under hostile microenvironments. EA stimulation was demonstrated to enhance the maintenance of stemness properties significantly. Furthermore, stimulation triggered concurrent activation of differentiation and proliferative programs, with β III-tubulin (Tuj-1) expression increasing 1.15-fold and the proliferation marker Ki67 showing 1.93-fold elevation at the 2-week post-implantation timepoint ($*P<0.05$). Concurrent preservation of stemness and proliferation under EA suggests that the regenerative-permissive temporal niche can be

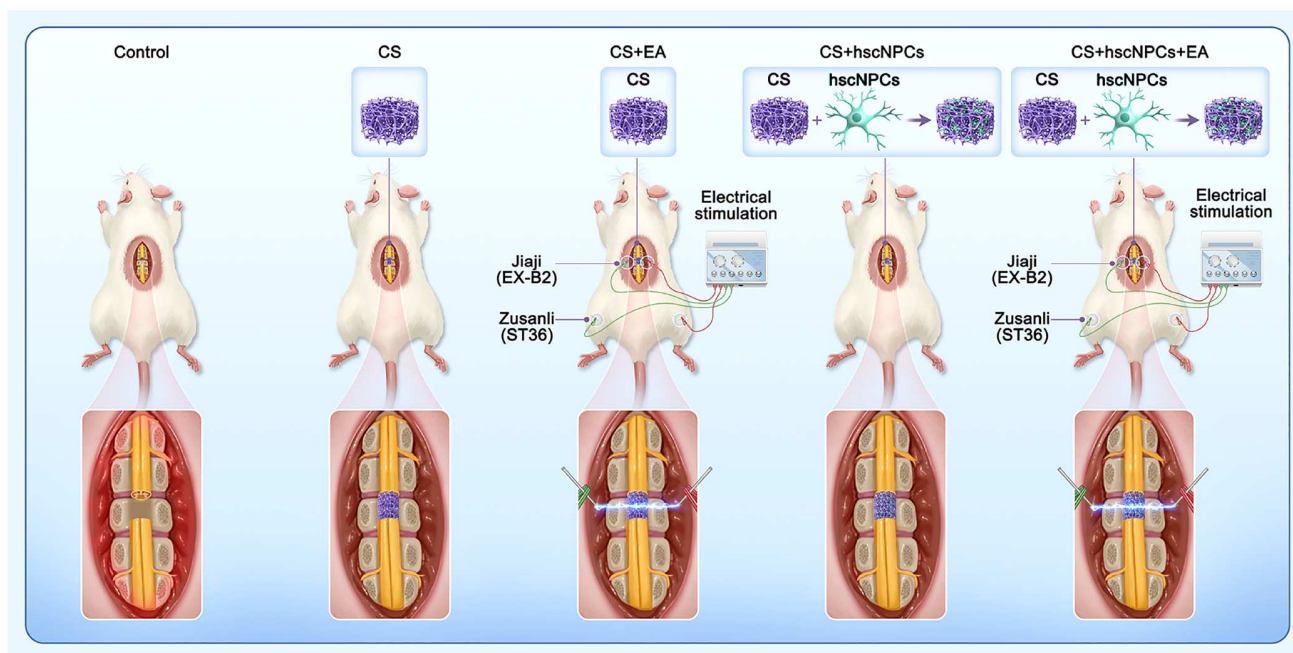
therapeutically modulated (Figure 3).

Collectively, these findings demonstrate that integrated EA stimulation significantly enhances the therapeutic efficacy of transplanted hsc-NPCs within the critical post-injury microenvironment. By simultaneously preserving core stemness properties, promoting robust proliferation, and initiating neuronal differentiation, EA uniquely drives complementary regenerative programs during the pivotal subacute phase. This dual capacity to maintain stemness while activating differentiation pathways indicates EA functionally modulates a regenerative-permissive temporal niche, overcoming microenvironmental barriers. This represents a targeted strategy to reprogram the hostile lesion environment during its transition from acute inflammation to chronic pathology, thereby enabling effective neural reconstruction at this therapeutically challenging juncture.

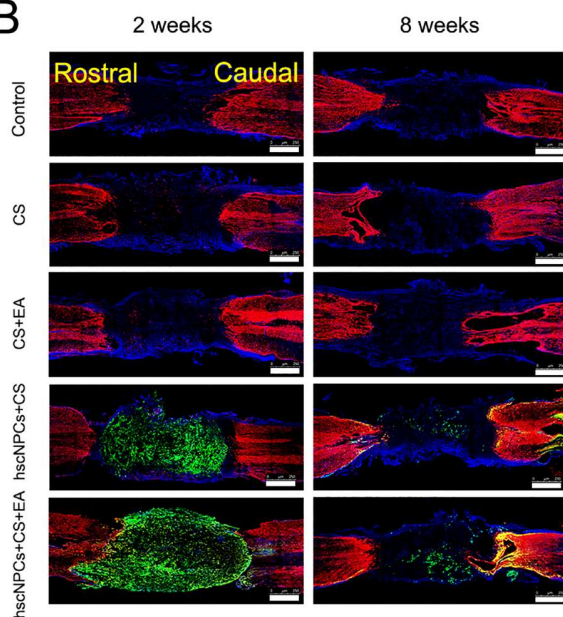
Assessment of *in vivo* differentiation status of transplanted hsc-NPCs at week 8 following SCI

While NPCs transplantation alone aims to replace lost cells, its efficacy is often limited by poor survival, uncontrolled differentiation, or poor functional integration. EA, as demonstrated by the enhanced marker expression profile, acts as a potent facilitator, guiding transplanted hsc-NPCs toward productive neuronal differentiation and functional maturation at the injury site. Immunofluorescence staining revealed that the neuronal

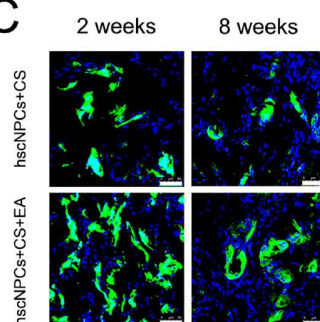
A



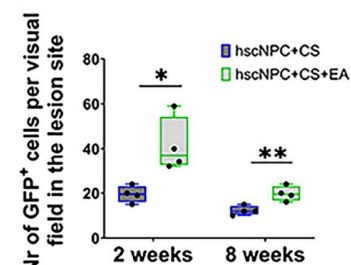
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D



E

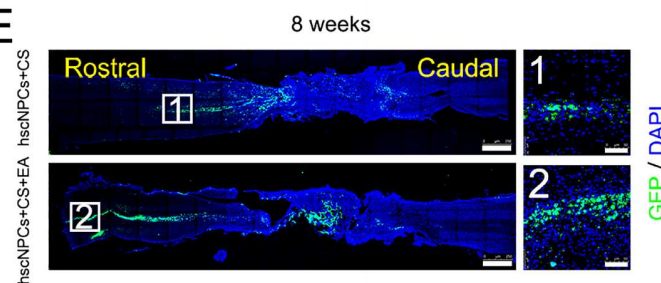


Figure 2 EA treatment significantly enhances the survival and migration of grafted hscNPCs within CS in a rat spinal cord transection model. A, Schematic illustration of the experimental groups and treatment protocols for the *in vivo* animal study. The groups are as follows: Control (untreated), CS (ordered collagen scaffold implantation alone), CS+EA (ordered collagen scaffold implantation with EA at Jiaji (EX-B2) and Zusanli (ST36) acupoints), CS+hscNPCs (ordered collagen scaffold loaded with hscNPCs), and CS+hscNPCs+EA (hscNPCs-loaded ordered collagen scaffold combined with EA). B, Representative immunofluorescence images show the survival of GFP⁺ hscNPCs (green) at the lesion epicenter 2 and 8 weeks after injury in hscNPCs+CS and hscNPCs+CS+EA groups. Cell nuclei are labeled with DAPI (blue) and the adjacent non-lesioned area outlined by GFAP⁺ reactive astrocytes (red). Scale bar, 250 μ m. C, High-magnification views (40 $\times$) of the survival and morphology of the GFP⁺ transplanted cells in hscNPCs+CS and hscNPCs+CS+EA groups at 2 and 8 weeks. Scale bar, 50 μ m. D, Quantitative analysis of the number of GFP⁺ hscNPCs per visual field within the lesion site at 2 and 8 weeks post-injury. Data are presented as mean $\pm$ SD ($n=4$ per group). *, $P<0.05$; **, $P<0.01$. E, Representative images illustrating the rostral and caudal migration of GFP⁺ hscNPCs (green) from the lesion site at 8 weeks post-injury. Cell nuclei are counterstained with DAPI (blue). Representative low-magnification (10 $\times$) view of the lesion site. Scale bar, 250 μ m. Representative high-magnification (40 $\times$) view of the lesion site. Scale bar, 50 μ m. CS, ordered collagen scaffold; EA, electroacupuncture; hscNPCs, human spinal cord-derived neural progenitor cells.

lineage markers Tuj-1 and microtubule-associated protein 2 (MAP2), along with the specific neurotransmitter-synthesizing enzymes choline acetyltransferase (CHAT) and 5-hydroxytryptamine (5-HT), showed significant upregulation in the lesion

epicenter of the EA treatment group compared to controls (Figure 4A).

The presence of elevated Tuj-1, an early marker of neuronal commitment and neurite extension, indicates enhanced

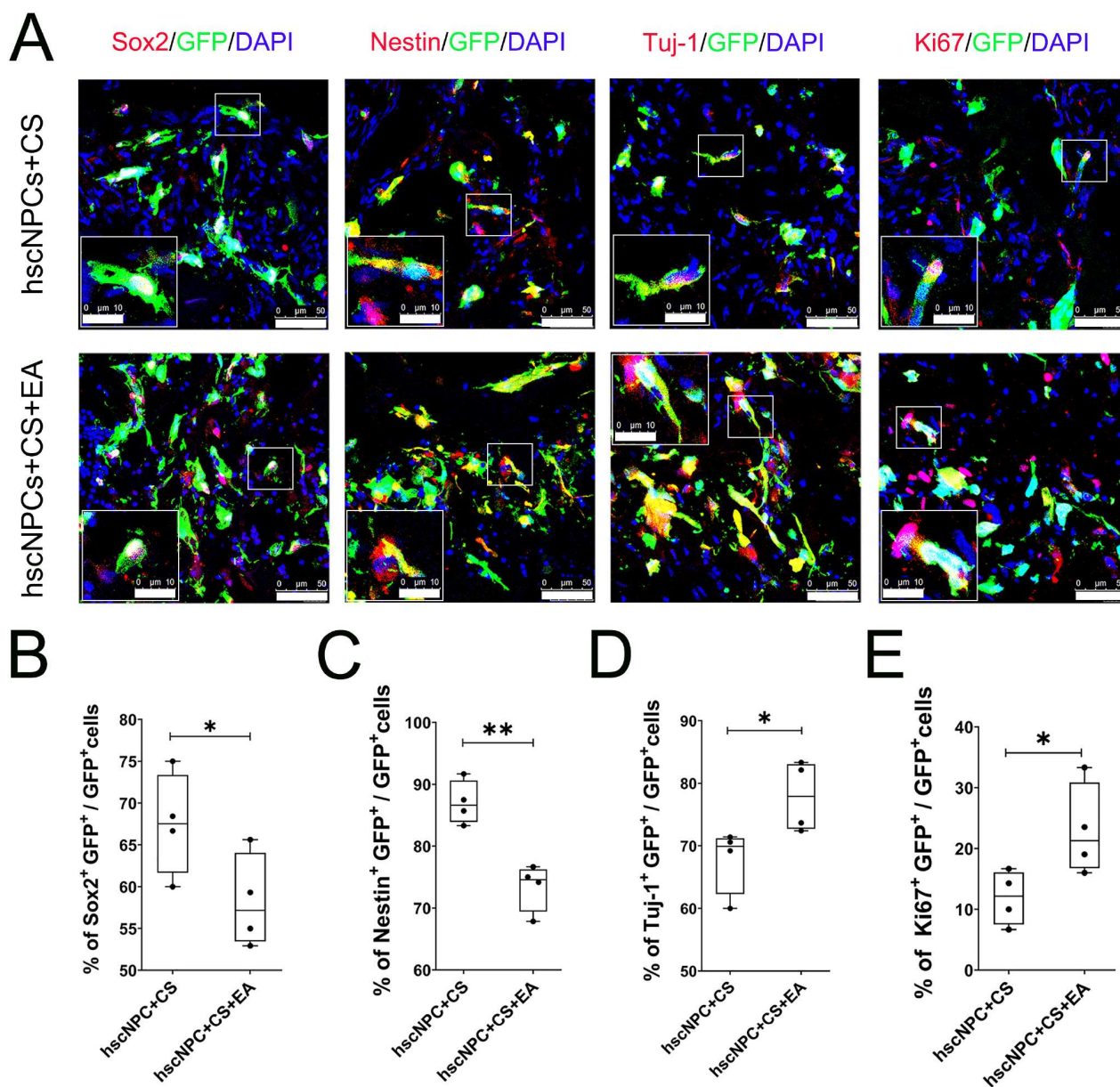


Figure 3 EA promotes stemness, proliferation, and differentiation of grafted hsc-NPCs at the 2-week post-injury timepoint. EA stimulation significantly upregulated pluripotency markers (Sox2 and Nestin) and concurrently enhanced neuronal differentiation (Tuj-1) and proliferation (Ki67) compared to controls. A, Representative immunofluorescence images of the lesion site showing the expression of stem/progenitor cell markers (Sox2 and Nestin), a neuronal marker (Tuj-1), and a proliferation marker (Ki67) in GFP⁺ hsc-NPCs (green). Nuclei are stained with DAPI (blue). Representative low-magnification (40 $\times$) view of the lesion site. Scale bar, 50 μ m. Representative high-magnification (40 $\times$) view of the lesion site. Scale bar, 10 μ m. B, Quantitative analysis of the percentage of Sox2⁺/GFP⁺ double-positive cells among the total GFP⁺ cell population, indicating the maintenance of a stem cell phenotype. C, Quantitative analysis of the percentage of Nestin⁺/GFP⁺ double-positive cells among the total GFP⁺ cell population, indicating a neural progenitor state. D, Quantitative analysis of the percentage of Tuj-1⁺/GFP⁺ double-positive cells among the total GFP⁺ cell population, indicating early neuronal differentiation. E, Quantitative analysis of the percentage of Ki67⁺/GFP⁺ double-positive cells among the total GFP⁺ cell population, indicating active proliferation of the transplanted cells. Data are presented as mean $\pm$ SD ($n=4$ per group). *, $P<0.05$; **, $P<0.01$. CS, ordered collagen scaffold; EA, electroacupuncture; hsc-NPCs, human spinal cord-derived neural progenitor cells.

initiation of neuronal differentiation at the injury site. Concurrent increases in MAP2, indicative of advanced neuronal maturation and dendritic arborization, suggest that cells are not merely committing to a neuronal fate but are developing complex structural characteristics essential for integration. Furthermore, the heightened expression of CHAT, the defining enzyme for cholinergic neurons critical for motor function and autonomic control, and 5-HT, representative of serotonergic pathways vital for locomotion modulation, mood, and autonomic regulation,

points towards the acquisition of specific, functionally relevant neurotransmitter identities.

The co-localization of myelin basic protein (MBP), neurofilament (NF), and synaptophysin (Syn) with GFP-labeled transplanted hsc-NPCs demonstrates that the grafted cells not only survived within the hostile injury microenvironment but also differentiated into functional neurons and oligodendrocytes. Specifically, MBP/GFP co-staining suggests remyelination, while NF/Syn/GFP co-staining may reflect synaptic integration and is

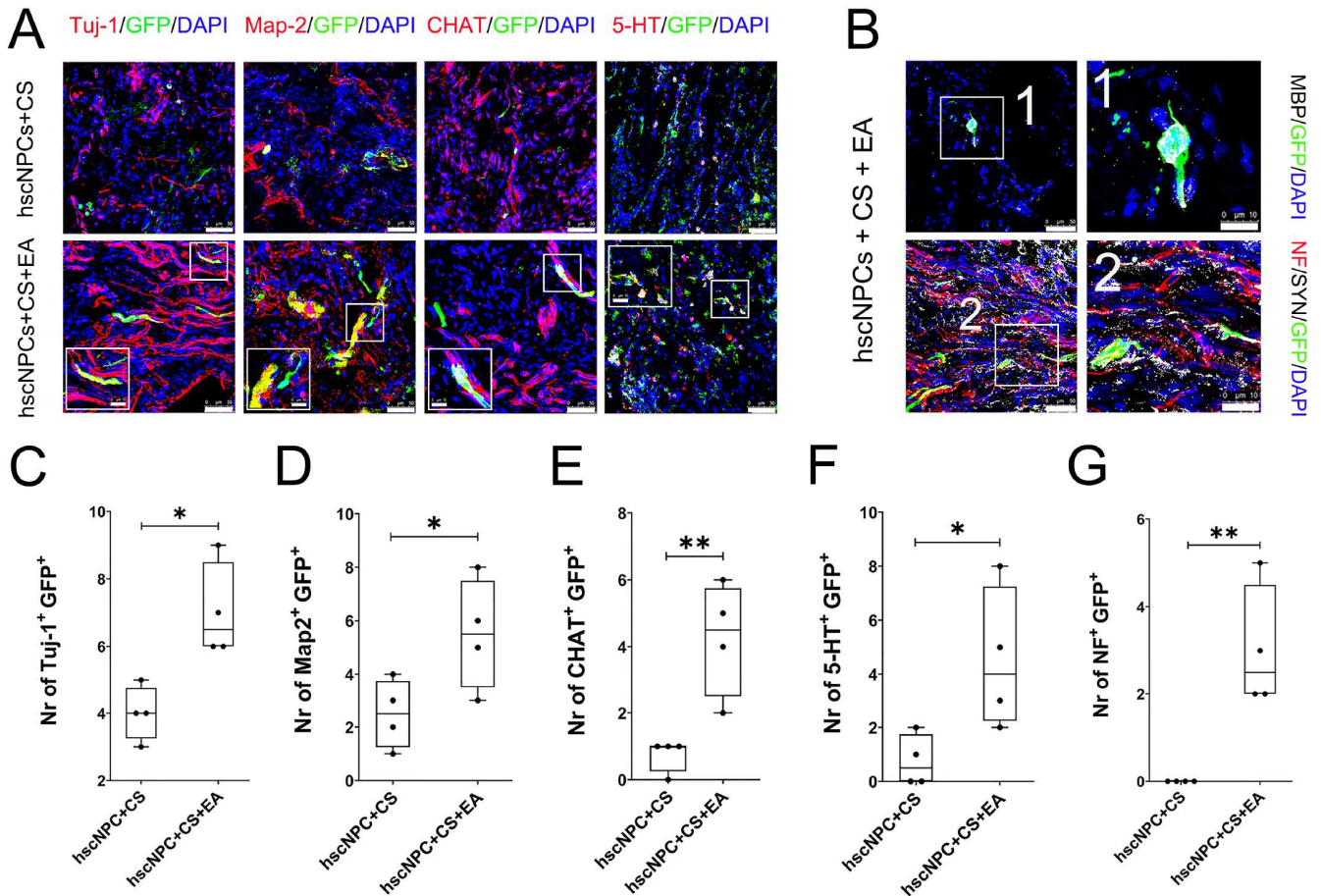


Figure 4 EA facilitates neuronal differentiation and functional maturation of grafted hsc-NPCs in the spinal cord lesion by 8 weeks post-SCI. A, Representative immunofluorescence images of the spinal cord lesion site at 8 weeks after injury, showing co-localization of GFP (green) with neuronal markers Tuj-1, MAP2, CHAT, and 5-HT (red), indicating the neuronal differentiation of the transplanted GFP⁺ cells. At the 8-week post-injury timepoint, immunofluorescence reveals markedly enhanced expression of neuronal markers (Tuj-1 and MAP2) and neurotransmitter-synthesizing enzymes (CHAT and 5-HT) in the EA group compared to controls, indicating promoted neuronal maturation and synaptic integration. Representative low-magnification (40 $\times$) view of the lesion site. Scale bar, 50 μ m. B, Grafted GFP⁺ hsc-NPCs differentiate into neurons and oligodendrocytes within the lesion site, as evidenced by co-expression of oligodendrocytic marker (MBP, top), neuronal marker (NF, bottom), and Synaptophysin marker (Syn, bottom). Representative low-magnification (40 $\times$) view of the lesion site. Scale bar, 50 μ m. C, Quantitative analysis of the number of Tuj-1⁺/GFP⁺ double-positive cells, indicating neuronal differentiation of the transplanted cells. D, Quantitative analysis of the number of MAP2⁺/GFP⁺ double-positive cells, indicating dendritic maturation of the transplanted cells. E, Quantitative analysis of the number of ChAT⁺/GFP⁺ double-positive cells, indicating cholinergic motor neuron differentiation of the transplanted cells. F, Quantitative analysis of the number of 5-HT⁺/GFP⁺ double-positive axons, indicating serotonergic innervation from the transplanted cells. G, Quantitative analysis of the number of NF⁺/GFP⁺ double-positive cells, indicating that the transplanted cells differentiated into neurons with mature axons. Data are presented as mean $\pm$ SD (n=4 per group). * P <0.05, ** P <0.01. CS, ordered collagen scaffold; EA, electroacupuncture; hsc-NPCs, human spinal cord-derived neural progenitor cells.

consistent with neural circuit reconstruction. These results suggest that the transplantation strategy has therapeutic potential and functional relevance. Importantly, these results raise the possibility that the grafted cells promote repair not solely through passive support, but by replacing lost neural elements, with the potential for functional integration (Figure 4B).

The quantitative analysis of upregulated differentiation and maturation markers offers compelling molecular evidence for the beneficial role of EA in promoting neural regeneration (Figure 4C–G). This synergistic approach holds considerable promise for reconstructing damaged neural circuits, particularly those involving cholinergic and serotonergic neurotransmission, which are essential for motor recovery and autonomic function, thereby offering a more effective avenue for achieving meaningful structural and functional repair following SCI than either intervention alone.

Assessment of the neural regeneration of endogenous NPCs within the lesion site at week 8 following SCI

Following injury, normally quiescent endogenous NPCs can be activated, proliferate, and migrate towards the lesion site. Under specific molecular cues within the injury microenvironment, they can differentiate into neurons, offering a promising therapeutic target. Immunofluorescence staining results indicated that the expression levels of Tuj-1, MAP2, CHAT, and GAP-43 proteins increased within the injury site in the EA treatment group. This suggests that EA of the SCI area can enhance the role of endogenous NPCs within the lesion site in promoting neural regeneration. The observed elevation of Tuj-1, MAP2, CHAT, and GAP-43 protein expression indicated that EA is a potent modulator of the intrinsic repair capacity of the injured spinal cord. The expression levels of these neuron-related proteins were

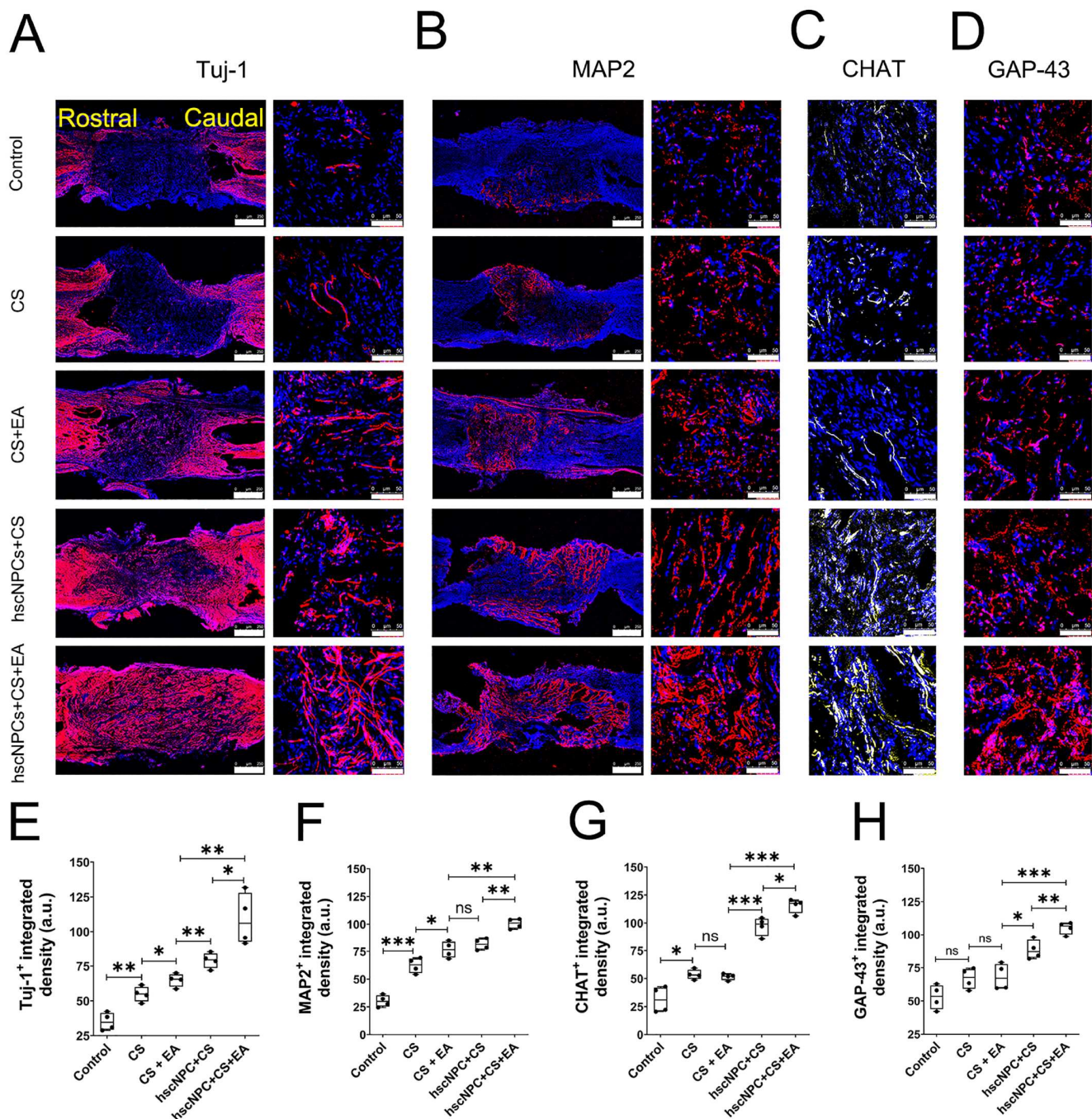


Figure 5 EA enhances endogenous neurogenesis and axon regeneration by 8 weeks post-injury. Immunofluorescence shows upregulation of neuronal markers (Tuj-1 and MAP2), cholinergic enzyme (CHAT), and axonal growth protein (GAP-43), indicating activation, differentiation, and neurite outgrowth of endogenous NPCs. A, Representative immunofluorescence images of the neuronal marker Tuj-1 in the spinal cord lesion site. Representative low-magnification (40 $\times$) view of the lesion site. Scale bar, 250 μ m. Representative high-magnification (40 $\times$) view of the lesion site. Scale bar, 50 μ m. B, Representative immunofluorescence images of the mature neuronal marker MAP2 in the spinal cord lesion site. Representative low-magnification (40 $\times$) view of the lesion site. Scale bar, 250 μ m. Representative high-magnification (40 $\times$) view of the lesion site. Scale bar, 50 μ m. C, Representative immunofluorescence images of the cholinergic neuron marker CHAT in the spinal cord lesion site. Representative high-magnification (40 $\times$) view of the lesion site. Scale bar, 50 μ m. D, Representative immunofluorescence images of the serotonergic fiber marker GAP-43 in the spinal cord lesion site. Representative high-magnification (40 $\times$) view of the lesion site. Scale bar, 50 μ m. E, Quantitative analysis of Tuj-1 $^{+}$ immunofluorescence intensity within the lesion area. F, Quantitative analysis of MAP2 $^{+}$ immunofluorescence intensity within the lesion area. G, Quantitative analysis of CHAT $^{+}$ immunofluorescence intensity within the lesion area at 8 weeks post-SCI. H, Quantitative analysis of the axonal growth marker GAP-43 $^{+}$ (growth associated protein-43) immunofluorescence intensity within the lesion area at 8 weeks post-SCI. Data are presented as mean $\pm$ SD ($n=4$ per group). ns, not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. Control group. CS, ordered collagen scaffold; EA, electroacupuncture; hsc-NPCs, human spinal cord-derived neural progenitor cells; NPCs, neural progenitor cells.

highest in the hsc-NPCs+CS+EA group (Figure 5A–D).

By promoting neurogenesis, neuronal maturation, and the

specific development of cholinergic and serotonergic phenotypes, EA significantly enhances the contribution of endogenous NPCs

towards neural regeneration and circuit restoration. The quantified upregulation of these markers underscores the immense therapeutic promise of electrical neuromodulation not only for symptom management but also as a direct catalyst for structural and, potentially, functional repair after SCI (Figure 5E–H). Further research is warranted to fully unravel the mechanisms and translate this promising approach into optimized clinical interventions.

The effect of EA stimulation on local inflammation at the injury site

Prior studies have shown that EA therapy activates endogenous anti-inflammatory mechanisms through acupoint stimulation, effectively suppressing the release of pro-inflammatory cytokines and inhibiting inflammatory cell infiltration. This dual modulation alleviates localized tissue inflammation, thereby promoting the resolution of inflammatory processes and tissue regeneration. Our immunofluorescence staining results showed a marked decrease in CD68-positive cells in the lesioned area in EA-treated groups compared with untreated controls. CD68 is considered a reliable marker of inflammatory cell infiltration and activation in damaged tissues. This observable reduction in CD68 immunoreactivity provides compelling visual and quantitative evidence that EA effectively mitigates the local inflammatory response associated with tissue injury. The diminished number of CD68⁺ cells suggests that EA likely interferes with key processes driving inflammation (Figure 6A and B). The ability of EA to substantially reduce inflammation, as directly visualized through this established macrophage marker, underscores its therapeutic potential. This anti-inflammatory effect is a crucial mechanism by which EA may promote a more favorable microenvironment for tissue repair and functional recovery following injury.

The mRNA sequencing data revealed that EA stimulation significantly attenuated neuroinflammation at the spinal lesion site (Figure 6C). High-throughput mRNA sequencing analysis of lesioned spinal cord tissue from animal models of SCI revealed that EA stimulation with specific parameters significantly attenuated neuroinflammation at the injury site, effectively inhibiting the neuroinflammatory cascade at the transcriptional level. This transcriptomic regulatory effect provides key molecular biological evidence that EA protects neurons and improves functional recovery after SCI by mitigating neuroinflammation. The results indicated that the key pro-inflammatory mediators were significantly downregulated following EA intervention, including the damage-associated molecular patterns S100b and HSP90aa1, the chemokine CXCL10, which mediates leukocyte recruitment and glial activation markers GFAP and VCAM1, and the interferon-induced GTPase MX2. This coordinated suppression spanned multiple neuroinflammatory axes: microglial reactivity (S100b and CXCL10), astrogliosis (GFAP and VCAM1), stress response (HSP90aa1), and antiviral defense pathways (MX2). The highly significant downregulation of key pro-inflammatory mediators reveals a concerted downregulation across multiple critical neuroinflammatory axes. This coordinated suppression strongly suggests that EA exerts its therapeutic benefits, at least in part, by simultaneously dampening microglial reactivity, astrogliosis, cellular stress responses, and associated innate immune signaling pathways at the spinal lesion site. This multi-targeted molecular mechanism provides compelling evi-

dence for EA's systemic anti-inflammatory action in the context of SCI.

The promotive effects of EA on the function of lower limb muscles in different treated groups

To get a good functional recovery following SCI, the lower limb muscle function is also required. Following the gastrocnemius and soleus muscles of the lower limb are separated and weighted, we calculate the mean ratio of both muscles. There was a statistically significant difference among the various groups. Weight loss in both the gastrocnemius and soleus muscles was reduced in all treatment groups compared with the control. The heaviest net weight of gastrocnemius and soleus muscles was the hsc-NPCs+CS+EA group (Figure 7A and B).

The percentage of collagen and muscle fibers in the gastrocnemius was detected by Masson trichrome staining. As shown in Figure 7C, the collagen fibers were stained blue, and the muscle fibers were stained red. Following SCI, the number of muscle fibers gradually decreased, accompanied by increased interstitial fibrosis. The percentages and distributions of muscle fibers and collagen fibers vary across the different therapeutic interventions in the CS, CS+EA, hsc-NPCs+CS, and hsc-NPCs+CS+EA groups. Muscle fibres of gastrocnemius were more abundant in hsc-NPCs+CS and hsc-NPCs+CS+EA group (Figure 7D).

Hematoxylin and eosin staining was employed to evaluate histopathological changes in denervated skeletal muscles across experimental groups. Morphological analysis revealed distinct tissue characteristics: skeletal muscle fibers exhibited characteristic eosinophilic staining, while interstitial fibrotic tissue appeared pale eosinophilic to basophilic. In control group specimens, extensive deposition of pale-staining collagenous fibrotic tissue was observed within the muscle interstitium, indicative of advanced degeneration. Strikingly, muscles from the hsc-NPCs+CS+EA treatment group demonstrated significant histological improvement: the relative area occupied by healthy, intensely eosinophilic muscle fibers was markedly increased, concurrent with a substantial reduction in interstitial collagen deposition (Figure 7E). This shift in tissue composition suggests attenuated fibrosis and enhanced structural preservation following combinatorial therapy.

We next evaluated the therapeutic effects of EA on neuromuscular junction (NMJ) regeneration and gastrocnemius muscle volume recovery at the 8-week post-injury time point. Transverse sections of gastrocnemius muscles were subjected to immunofluorescence costaining using α -bungarotoxin (BTX) conjugated to Alexa Fluor 555 for postsynaptic acetylcholine receptors (AChRs), alongside neuronal markers Tuj-1 and NF for presynaptic axons. BTX, a high-affinity neurotoxin derived from snake venom, selectively binds to nicotinic AChRs at NMJs, enabling precise visualization of postsynaptic structures.

Quantitative analysis revealed a significant potentiation of NMJ formation and motor reinnervation in combinatorial therapy groups (** $P < 0.01$ vs. monotherapy). Notably, the hsc-NPCs+CS+EA cohort exhibited the highest density of NMJ with robust colocalization of BTX⁺ endplates and Tuj-1⁺/NF⁺ nerve terminals (Figure 8A and B). Critically, EA intervention not only mitigates muscle atrophy but also actively enhances de novo synaptogenesis; these coordinated effects were fundamental to rebuilding functional neuromuscular units and achieving substantial functional recovery (Figure 8C and D).

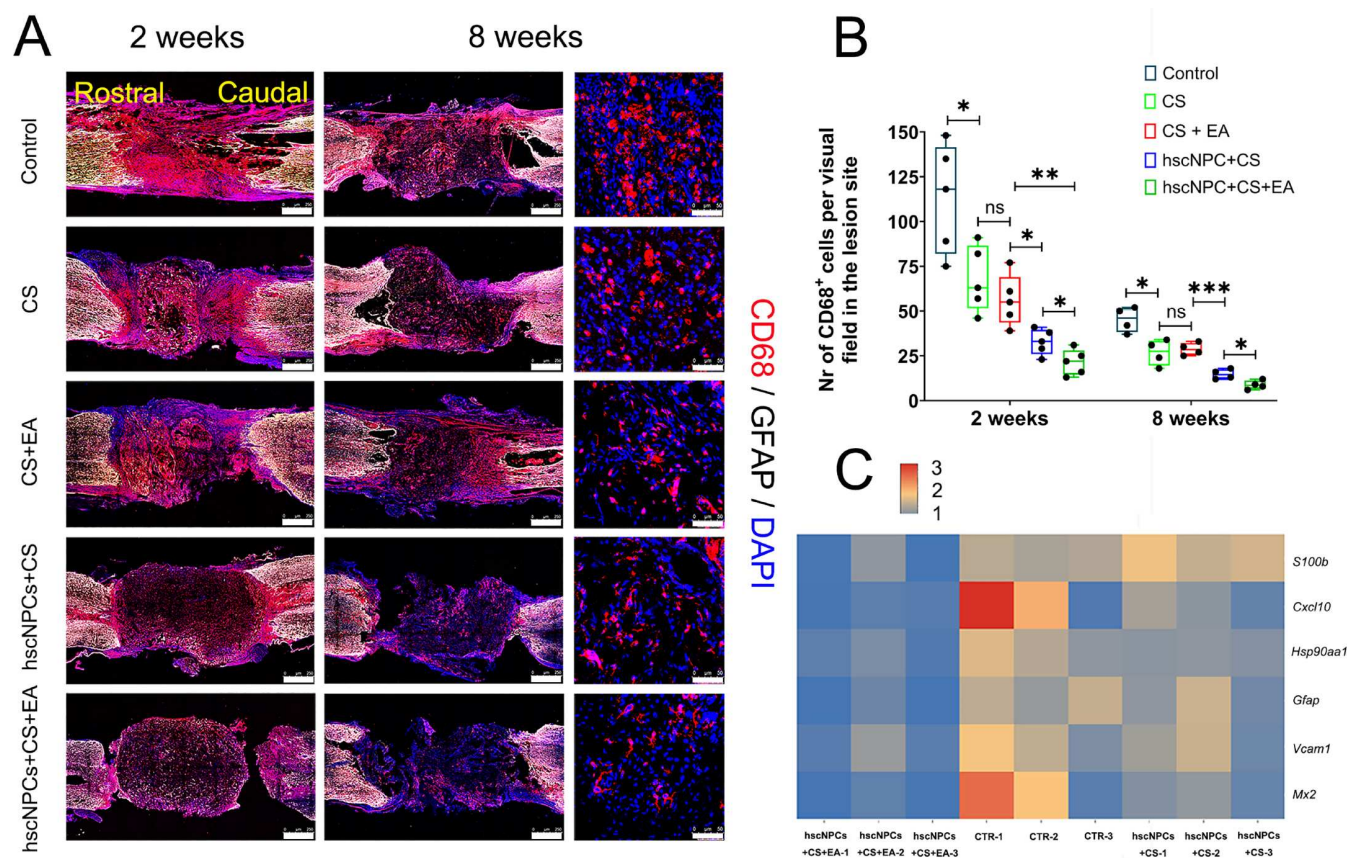


Figure 6 EA treatment markedly reduces inflammatory cell infiltration at the lesion site. **A**, Representative immunofluorescence images of CD68 in the spinal cord lesion site at 2 weeks (left) and 8 weeks (right) after injury. Nuclei are counterstained with DAPI (blue). The adjacent non-lesioned area is outlined by GFAP⁺ reactive astrocytes (white). Immunofluorescence shows a significant decrease in CD68⁺ cells, indicating effective suppression of local neuroinflammation and creation of a pro-regenerative microenvironment. Representative low-magnification (10×) view of the lesion site. Scale bar, 250 μm. Representative high-magnification (40×) view of the lesion site. Scale bar, 50 μm. **B**, Quantitative analysis of the number of CD68-positive cells within the lesion area at 8 weeks post-SCI. Data are presented as mean±SD (*n*=5 per group at 2 weeks, *n*=4 per group at 8 weeks). ns, not significant, **P*<0.05, ****P*<0.001 vs. Control group. **C**, Hierarchical clustering analysis of differentially expressed inflammation-related genes from mRNA sequencing of spinal cord tissue at the lesion site 2 weeks after injury. Each row represents a single gene, and each column represents an individual biological replicate. The color scale indicates the Z-score of gene expression levels (red, up-regulated; blue, down-regulated). RNA-seq analyses demonstrate coordinated downregulation of key pro-inflammatory mediators, indicating suppression of microglial activation, astrogliosis, and innate immune signaling. CS, ordered collagen scaffold; EA, electroacupuncture; hsc-NPCs, human spinal cord-derived neural progenitor cells.

Recovery of lower limb function in different treated groups

To achieve a thorough assessment of functional restoration in spinal cord-injured rats, a multifaceted evaluation strategy was implemented. Electrophysiological assessment of motor functional recovery following SCI demonstrated that the motor evoked potential (MEP) amplitude in the combination therapy group (hsc-NPCs+CS+EA) was significantly greater than that in monotherapy intervention groups, indicating enhanced neural signal transduction efficiency along the spinal motor pathways (Figure 9A and B). This increase suggests improved electrophysiological conduction, likely due to factors such as enhanced remyelination and superior synaptic integration of transplanted cells, which collectively contribute to the robust behavioral functional recovery.

EA intervention targeting Jiaji (EX-B2) and Zusanli (ST36) acupoints profoundly enhanced postural stability in rats with complete spinal cord transection, evidenced by inclined plane testing wherein hsc-NPCs+CS+EA animals achieved a maximum stability angle of $38.05^{\circ} \pm 0.82^{\circ}$, surpassing the $34.02^{\circ} \pm 0.91^{\circ}$ recorded in hsc-NPCs+CS controls (***, *P*<0.001) (Figure 9C).

The maximum inclination angle represents the threshold at which an animal can maintain equilibrium against gravity without sliding or falling, directly reflecting the functional integrity of sensorimotor pathways. The core value of the inclination angle in the inclined plane test lies in its ability to quantify functional neuroplasticity.

Finally, spontaneous locomotor recovery in an open-field setting was quantitatively assessed using the BBB locomotor rating scale. The hsc-NPCs+CS+EA group achieved the highest BBB scores, showing a significant increase compared to the control group. These results indicate that animals receiving hsc-NPCs+CS+EA treatment exhibited improved hindlimb joint mobility, weight-supported plantar stepping, and coordinated gait patterns (Figure 9D). The significantly improved performance on the inclined plane test and BBB scale in hsc-NPCs+CS+EA-treated rats demonstrates functional recovery strongly correlating with restored electrophysiological sensorimotor conduction across the lesion. Mechanistically, EA enhanced neural differentiation, a critical step for reconstructing damaged circuits and facilitating graft-host integration.

The increased MEP amplitude induced by the combination

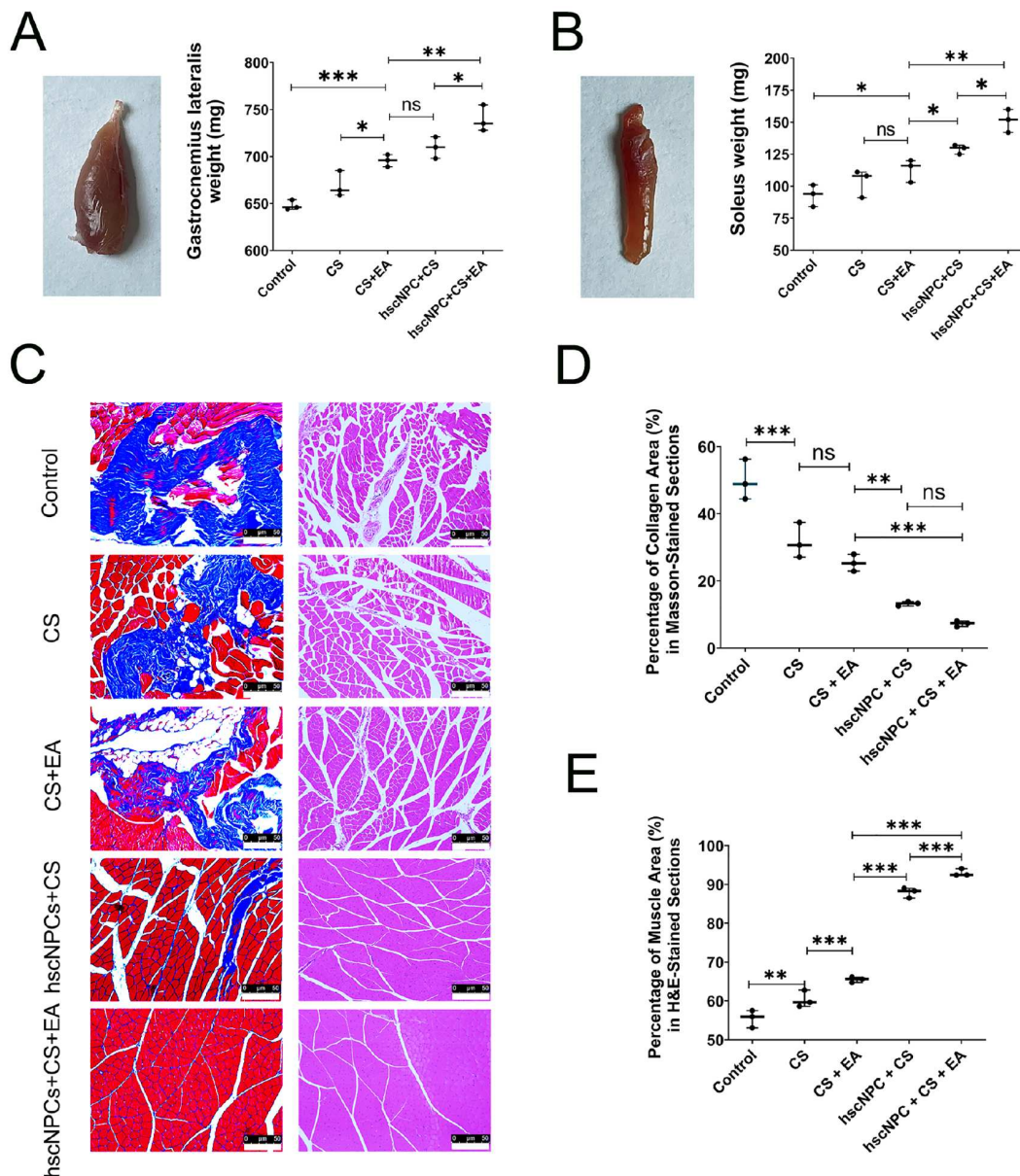


Figure 7 EA effectively counteracted muscle atrophy, promoted the maintenance of muscle fibers, and reduced collagen fiber deposition. A, Gross observation and quantitative analysis of the weight of the gastrocnemius muscle. B, Gross observation and quantitative analysis of the weight of the soleus muscle. C, Representative images of Masson's trichrome staining (left panel) and H&E staining (right panel) of gastrocnemius muscle sections. Scale bar, 50 μ m. D, Quantitative analysis of the collagen fiber area percentage in muscle sections based on Masson's trichrome staining. E, Quantitative analysis of the muscle fiber area percentage in muscle sections based on H&E staining. Data are presented as mean $\pm$ SD ($n=3$ per group). ns, not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. Control group. CS, ordered collagen scaffold; EA, electroacupuncture; hsc-NPCs, human spinal cord-derived neural progenitor cells.

therapy showed a significant positive correlation with behavioral recovery. These data collectively confirm that the functional improvement stems from a synergistic interaction between EA and cell transplantation, in which EA modulates a neuroplasticity-permissive milieu to maximize the integration and efficacy of transplanted hsc-NPCs, thereby promoting robust reconstruction of motor pathways.

The radiographically observed "joint space" on X-ray is a composite shadow of articular cartilage and synovial fluid. Its width primarily reflects articular cartilage thickness and integrity. Our findings demonstrated that all groups receiving EA therapy showed statistically significant improvements in radiographic joint space metrics compared with control cohorts

(Figure S2A). Notably, these positive alterations in joint space width were significantly increased compared with both untreated injury models and groups receiving alternative non-EA interventions (Figure S2B). Radiographic outcomes confirm that targeted EA at Zusanli (ST36) results in significant structural preservation of affected joints, as evidenced by improved joint space width, a key indicator of cartilage integrity.

The results demonstrate that EA at Jiaji (EX-B2) and Zusanli (ST36) improves joint structures and provides a modest improvement compared to both untreated and non-EA controls. This reproducible superiority suggests that EA contributes to joint pathophysiology by preserving structural integrity through chondroprotective effects and a pro-regenerative microenviron-

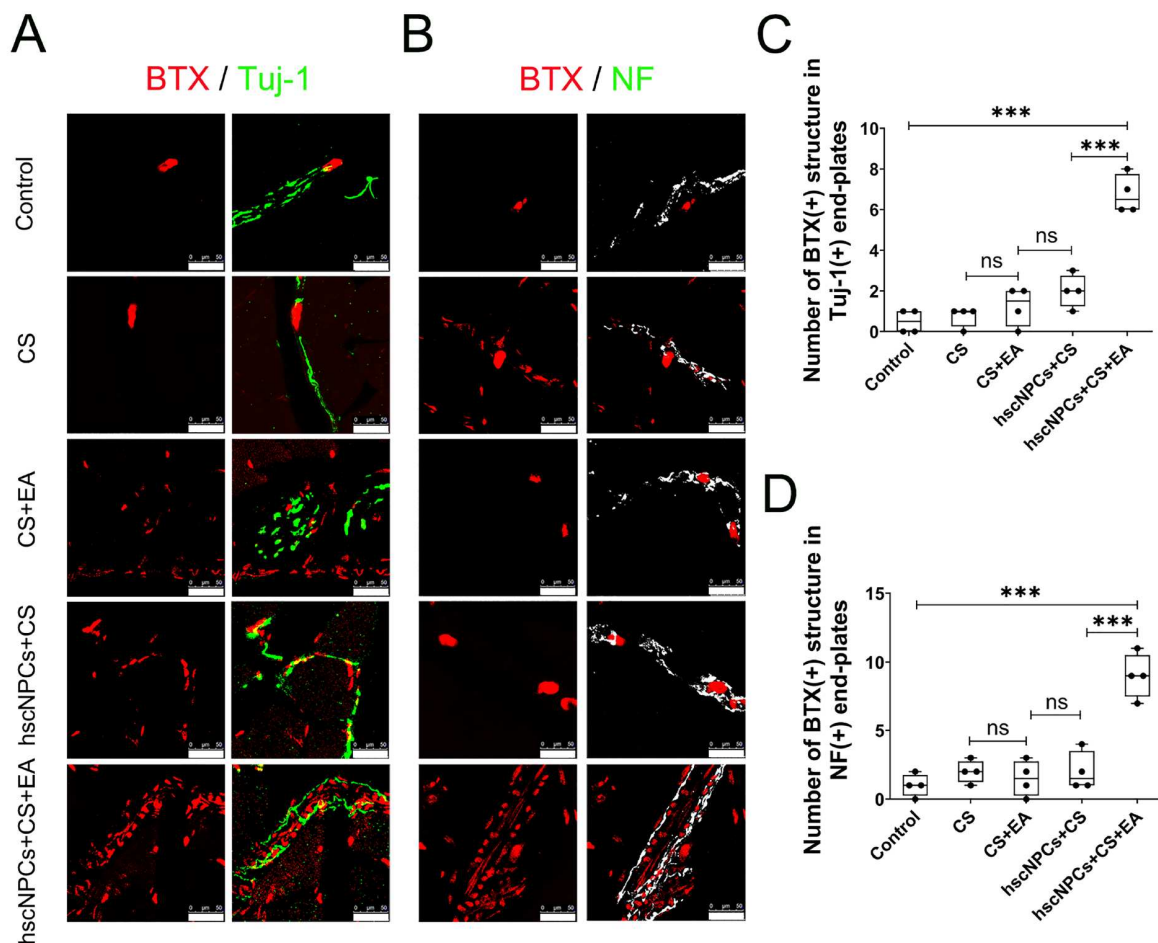


Figure 8 EA enhances NMJ regeneration and muscle recovery. A, Representative immunofluorescence images showing the morphology and reinnervation of NMJs, labeled with BTX (red) for postsynaptic AChRs, and antibodies against Tuj-1 (green) for presynaptic nerve terminals. Scale bar, 50 μ m. B, Representative immunofluorescence images showing the structure of NMJs, labeled for postsynaptic AChRs (BTX, red) and presynaptic axons (NF, green). Scale bar, 50 μ m. C, Quantitative analysis of the colocalization between BTX⁺ end-plates and Tuj-1⁺ nerve terminals, presented as the number of BTX⁺ structures colocalized with Tuj-1. D, Quantitative analysis of the colocalization between BTX⁺ end-plates and NF⁺ nerve terminals, presented as the number of BTX⁺ structures colocalized with NF. Data are presented as mean $\pm$ SD ($n=4$ per group). ns, not significant, *** $P<0.001$ vs. Control group. CS, ordered collagen scaffold; EA, electroacupuncture; hsc-NPCs, human spinal cord-derived neural progenitor cells. BTX, α -bungarotoxin.

ment, which may support comprehensive disease management and sustained symptom control.

DISCUSSION

Despite the significant clinical potential of transplanting functional biomaterials integrated with NPCs for SCI repair, the hostile lesion microenvironment severely compromises grafted tissue survival and neural circuit reconstruction. To address this limitation, our study innovatively combines EA at Jiaji (EX-B2) and Zusanli (ST36) acupoints with transplantation of hsc-NPCs seeded on ordered collagen scaffolds. Results demonstrate that this synergistic strategy significantly enhances therapeutic outcomes by overcoming microenvironmental barriers to promote neural regeneration and functional recovery. While the bioengineered scaffold ensures structural support for graft integration, EA stimulation further promotes neuronal survival, axonal regrowth, and tissue repair, leading to faster restoration of lower limb motor function post-injury. The combined intervention of EA and scaffold-supported hsc-NPC transplantation emerges as a highly promising translational approach for comprehensive SCI rehabilitation. Furthermore, the therapeutic efficacy of cell

transplantation is also governed by the intrinsic properties of the cells. For instance, in osteoarthritis, engineered exosomes delivering miR140 alleviate disease progression by promoting mitophagy in chondrocytes (Liu et al., 2025b). Recent evidence indicates that for thoracic SCI, thoracic-derived neural stem cells possess superior survival, neuronal differentiation, and microenvironment-modulating capacities compared to non-matched segments, leading to better functional recovery (Zhang et al., 2026). This highlights segmental matching as a potential key factor for further optimizing our combinatorial strategy.

In SCI treatment, simultaneous EA stimulation at both Jiaji (EX-B2) and Zusanli (ST36) acupoints demonstrates significant therapeutic efficacy and complementary effects. Modern studies note their ability to modulate inflammation and neurotrophic factors when stimulated via EA. Jiaji (EX-B2) points deliver targeted paraspinal neuromodulation at injury segments by suppressing pro-inflammatory cytokines, enhancing neurotrophic factor expression, and improving spinal microcirculation through sympathetic ganglion stimulation. Distal Zusanli (ST36) stimulation potentiates systemic neurorestoration by activating the vagal-adrenal axis, attenuating secondary injury, and upregulating endogenous neural growth factors, thereby

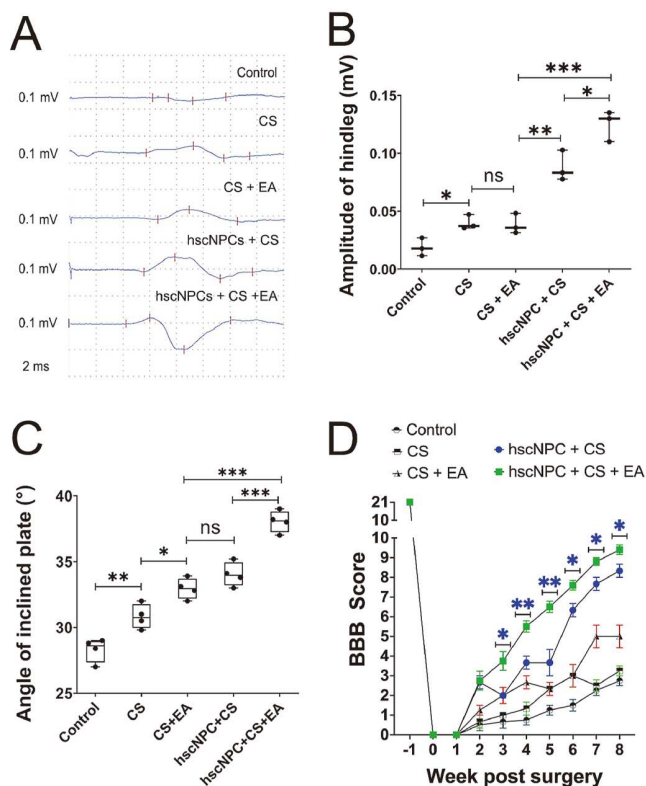


Figure 9 EA promotes electrophysiological and motor functions in rats with SCI. A, Representative MEP waveforms recorded from the hindlimb muscles in different treatment groups at 8 weeks post-injury. B, Quantitative analysis of the MEP amplitude. Enhanced MEP amplitude strongly correlated with behavioral recovery, confirming that EA facilitates functional reinnervation and graft-host integration through modulation of a plasticity-permissive microenvironment ($n=3$ per group). C, Functional recovery assessed by the inclined plane test. The maximum angle at which an animal could maintain its position for 5 s was recorded ($n=4$ per group). D, Locomotor recovery was evaluated using the BBB locomotor rating scale over 8 weeks post-injury. EA improved inclined plane performance, with a higher maximum angle and higher BBB scores, indicating robust restoration of spinal conductivity, postural stability, and locomotor function ($n=4$ per group). Data are presented as mean $\pm$ SD. ns, not significant, $*P<0.05$, $**P<0.01$, $***P<0.001$ vs. Control group. CS, ordered collagen scaffold; EA, electroacupuncture; hsc-NPCs, human spinal cord-derived neural progenitor cells; BBB, Basso, Beattie & Bresnahan locomotor rating scale; MEP, motor evoked potential.

accelerating IGF-1-mediated motor recovery. The advantages of EA over pharmacological interventions have been demonstrated in reducing motor neuron death, promoting myelin regeneration, reducing glial scar formation, ameliorating tissue hypoxia and blood deficiency (Liu et al., 2025a; Liu et al., 2024; Wan et al., 2025), and significantly improving functional recovery in preclinical SCI models. Our research further reveals that EA applied at the Jiaji (EX-B2) acupoint substantially enhances the survival rate of transplanted engineered hsc-NPC tissues. Historically, NPC transplantation for SCI has been critically limited by poor graft survival at lesion sites (Ngo and Harley, 2020; Zhang et al., 2023). Our novel combinatorial therapy effectively overcomes these inherent limitations of NPC transplantation.

In addition to EA, the ordered collagen scaffold engineered in our group enhances microcirculation at the spinal cord lesion site while providing exceptional cytocompatibility, optimizing nutrient delivery and metabolic waste exchange for grafted hsc-NPCs. Following transplantation, this scaffold architecture

prevents infiltration of hsc-NPCs by inflammatory cells and anchors the grafts against displacement by vascular forces and cerebrospinal fluid flow. Crucially, its aligned porous structure directionally guides regenerating nerve tissue growth through orderly channels, facilitating targeted reconnection between severed neural stumps.

The finding that EA overcomes these barriers to increase the expression of key neuronal proteins strongly suggests that it creates a more permissive and conducive niche for the transplanted hsc-NPCs. EA likely achieves this by modulating multiple facets of the local microenvironment: it may enhance the survival of grafted cells by reducing pro-apoptotic signals; stimulate their proliferation or direct migration towards the lesion core; critically, it appears to drive their differentiation potentially down defined neuronal pathways, accelerating the progression from progenitor states to mature, phenotypically specified neurons. Moreover, EA could promote the structural integration of these newly derived neurons by encouraging neurite outgrowth and synaptogenesis, and potentially mitigate the inhibitory effects of glial scars or chondroitin sulfate proteoglycans. This ability of electrical neuromodulation to significantly amplify the therapeutic impact of exogenously transplanted hsc-NPCs represents a promising combinatorial strategy for SCI repair. Future studies could achieve even greater synergy by combining EA-modulated niches with transplanted cells that have intrinsic advantages for the specific injury segment, such as the documented enhanced anti-scarring and anti-inflammatory properties of thoracic NPCs in thoracic SCI (Zhang et al., 2026).

SCI frequently precipitates severe motor dysfunction through two interrelated pathological processes: progressive neurogenic muscle atrophy and secondary joint deformation (Elbasiouny et al., 2010; Marwaha et al., 2020). These degenerative changes critically impair musculoskeletal integrity, ultimately leading to functional disability. To address this, we implemented EA stimulation at the Zusanli (ST36) acupoint, a well-established therapeutic target in neuromotor rehabilitation. Historically, Zusanli (ST36) acupoint has been clinically employed to treat lower extremity weakness, gait abnormalities, pain syndromes, and sensory impairments (Huang et al., 2019; Lai et al., 2020; Li et al., 2011). In the present study, we demonstrate that targeted EA intervention at the Zusanli (ST36) acupoint significantly mitigates post-SCI muscular degeneration and enhances joint range of motion. This novel finding reveals the previously unrecognized therapeutic potential of Zusanli (ST36) acupuncture in preserving musculoskeletal architecture following neural trauma, thereby offering a promising neuromodulatory strategy for functional recovery.

Taken together, our findings demonstrate that EA stimulation brings remarkably robust therapeutic effects in the challenging context of complete spinal cord transection injury. The significant alleviation of neurogenic muscle atrophy and the restoration of joint range of motion underscore EA's potent capacity to mitigate critical secondary pathologies following severe neural trauma. As a modality deeply rooted in traditional Chinese medicine, EA has not only validated ancient empirical knowledge regarding acupoints' influence on lower limb function but has now revealed its novel, specific efficacy in counteracting the debilitating musculoskeletal sequelae of complete SCI. Given this compelling evidence of EA's neuroprotective and restorative potential, EA emerges as a highly promising

therapeutic strategy with substantial clinical translation value. Incorporating EA into future neurorehabilitation strategies offers considerable potential to improve both functional recovery and quality of life in SCI patients.

MATERIALS AND METHODS

Cell culture and ethics statement

hsc-NPCs were obtained from aborted fetuses as previously reported (Xu et al., 2023). The primary isolated hsc-NPCs were cultured in the proliferation medium. The recipe of the proliferation medium was prepared according to the published protocol. Medium is changed every other day. When cells reached 80% confluence, they were dissociated into single-cell suspensions using Accutase (Cellco, Shanghai, China) and were passaged 1:3. Passages 4–6 cells were used in the experiment. Before grafting *in vivo*, the cells were transfected with a lentivirus expressing green fluorescent protein (GFP; Miga Technology Co., Ltd., Shenzhen, China) to facilitate convenient tracking of the grafted cells. And then the transfected GFP-labelled cells were seeded on the ordered collagen scaffolds. The use of hsc-NPCs was approved by the Institutional Review Board of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School, and informed consent was obtained from all donors or their legal guardians. All experimental procedures involving these cells were conducted in strict compliance with the relevant ethical guidelines and regulations.

Preparation and characterization of ordered collagen scaffolds

The ordered collagen scaffolds were prepared according to a previously published protocol (Zou et al., 2020a; Zou et al., 2020b). The surface morphology of the ordered collagen scaffold was observed under a SEM (S-3000N; Hitachi, Japan). Water absorption capacity was determined according to the formula $(W_w - W_d)/W_d \times 100\%$, where W_w and W_d represent the wet and dry weights of the scaffold, respectively (Koçyiğit, 2024). Furthermore, the porous architecture of the scaffolds was characterized by mercury intrusion porosimetry. This technique operates on the principle that the pressure required to intrude mercury into the pores is inversely proportional to their size, thereby enabling the calculation of pore size distribution. In this method, a dried scaffold sample is placed in a chamber, evacuated, and then immersed in mercury. The pressure is then gradually raised, and the volume of mercury intruded at each pressure step is recorded.

Calcein-AM/PI staining for cell viability assessment

Cell viability and distribution on ordered collagen scaffolds were assessed at 0, 1, 3, and 7 d using a calcein-AM/propidium iodide (PI) double-staining assay. Human spinal cord-derived neural progenitor cells were cultured on the scaffolds. Prior to staining, the cell-scaffold constructs were washed with PBS and incubated with calcein-AM working solution at 37°C for 30 min in the dark. Subsequently, the samples were stained with PI for 5 min, then gently rinsed with PBS. Viable cells fluoresced green due to calcein-AM esterase activity, whereas dead cells exhibited red fluorescence from PI binding to nucleic acids. The stained

constructs were immediately visualized and imaged using fluorescence microscopy. All procedures were conducted according to the manufacturer's protocol.

Animal surgery

All animal experiments were performed in accordance with the guidelines and standards of the Chinese Ministry of Public Health Guide for the Care and Use of Laboratory Animals. Adult SD rats, 8-week-old, which do not differentiate between genders and weigh 230–250 g, were purchased from SPF Biotechnology Co., Ltd. (Beijing, China). To construct a complete transection of the SCI model, microsurgery scissors exposed the T10 lamina, and then 1 mm of the spinal cord tissue was completely removed. All the rats ($n=35$) were randomly assigned to five groups: Control group (untreated following SCI), CS group (implanted with the ordered collagen scaffold following SCI), CS+EA group (implanted with the ordered collagen scaffold accompanied with EA following SCI), hsc-NPCs+CS (implanted with the ordered collagen scaffold seeded with hsc-NPCs following SCI), hsc-NPCs+CS+EA group (implanted with the ordered collagen scaffold seeded with hsc-NPCs accompanied with EA following SCI). The ordered collagen scaffold, seeded with or without hsc-NPCs, was inserted into the gap in the injured spinal cord immediately after removal of the spinal cord tissue. All groups in need of EA treatment received regular EA treatment once a week at the Jiaji (EX-B2) acupoint and twice a week at the Zusanli (ST36) acupoint after the operation, lasting for a total of 8 weeks (Figure 2A).

EA treatment

One day after grafting, EA treatment was initiated in the hsc-NPCs+CS+EA group. The EA stimulation parameters were a dilatational wave, with current frequencies of 60 Hz for 1.05 s and 2 Hz for 2.85 s, a stimulation duration of 20 min per treatment, and a frequency of treatment once a day.

Electrophysiology analysis

As a non-invasive test, an electrophysiological test was performed to evaluate MEPs at week 8 after the operation (Zou et al., 2020a). During the test, the positive electrode was placed in the motor area of the cerebral cortex, and the negative electrode was placed 0.5 cm away from it. In the meantime, the recording electrode was inserted into one side of the gastrocnemius muscle, and the reference electrode was placed 2 cm away from it (Zhao et al., 2017). The peak-to-peak amplitudes and the start time of the first reaction to the stimulus were calculated as amplitude and latency values.

Animal behavior test analysis

To comprehensively evaluate functional recovery in rats with SCI, two established behavioral tests, the Inclined Plane Test and the Basso, Beattie & Bresnahan (BBB) Locomotor rating scale, were administered during the 8-week postoperative period (Yang et al., 2016). At the 8-week endpoint, each rat underwent testing. Starting from a horizontal position, the plane's angle of inclination was gradually increased in small increments (typically 2°–5°). For each angle, the rat was carefully positioned

perpendicular to the slope's axis, with its head facing upwards. The angle was held constant for each trial, and the maximum tilt angle at which the rat stayed on for 5 s without slipping off was recorded. Each trial was measured three times.

The animals' hindlimb movements were also assessed with the BBB scale. Rats were placed in an open field with sufficient space for unrestricted movement. Points are awarded based on defined criteria for joint movement, weight support, stepping ability, coordination, and paw placement. The weekly BBB scores provided a temporal profile of locomotor recovery dynamics throughout the entire 8-week observation period.

RNA sequencing

Total RNA was extracted using TRIzol reagent. RNA purity and concentration were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Sequencing libraries were prepared with the VAHTS Universal V10 RNA-seq Library Prep Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions and sequenced on an Illumina NovaSeq X Plus platform. All library preparation, sequencing, and primary bioinformatic analyses were performed by Shanghai OE Biotech Co., Ltd (Shanghai, China). After sequencing, reads were quality-controlled, mapped to the reference genome, and gene expression levels were quantified and normalized. Differential expression analysis was performed using established pipelines, with genes meeting the thresholds of an adjusted *P* value (*q*-value) < 0.05 and |fold change| > 2 defined as differentially expressed genes (DEGs). Sample group metadata was used for comparative analysis. Results were visualized using volcano plots, and significant DEGs were subjected to functional enrichment and pathway analysis.

Immunofluorescence staining

The rats were sacrificed, and the spinal cords were cut and fixed in 4% paraformaldehyde for 24 h, followed by a 20%, 30% sucrose gradient dehydration until the spinal cords sank to the bottom. The immunofluorescence staining was performed according to previously published methods (Zou et al., 2020a). The samples were embedded in OCT and cryosectioned at 15 μ m. For immunofluorescence staining, the sections were permeabilized and blocked with buffer containing 1% Triton X-100 and 5% BSA for 1 h at 37°C, then incubated with primary antibodies overnight at 4°C. Subsequently, the sections were washed 3 times with PBS for 5 min each. The corresponding secondary antibody was incubated for 1 h at 37°C. Finally, sections were sealed with fluorescent mounting medium and then observed using a Leica confocal microscope.

Muscle mass analysis and histological analyses

Hindlimb muscle atrophy was assessed through gastrocnemius and soleus weight ratios. Muscles were dissected, weighed using an electronic balance, and relative weight calculated as: (ipsilateral muscle weight/contralateral muscle weight) × 100%. Gastrocnemius muscles from different groups were fixed with 4% paraformaldehyde and then embedded in paraffin. The paraffin-embedded tissue blocks were sectioned at a thickness of 5 μ m.

Haematoxylin and eosin staining and Masson staining were performed as previously described (Cui et al., 2018). Following deparaffinization in xylene and rehydration through graded ethanols to distilled water, sections were stained in Harris's Haematoxylin for 5 min. After differentiation and bluing to enhance nuclear contrast, slides were counterstained in 0.5%–1% Eosin Y for 1 min. Finally, sections were dehydrated through graded ethanols, cleared in xylene, and coverslipped with a resinous mounting medium. For Masson staining, deparaffinized and rehydrated sections were stained with Weigert's iron hematoxylin for 10 min, rinsed in water, and subsequently treated with Biebrich scarlet-acid fuchsin for 10 min. Following rinsing, slides were differentiated in phosphomolybdic-phosphotungstic acid for 10 min, then transferred directly to aniline blue solution for 5 min. Sections were briefly rinsed in acetic acid, dehydrated through graded ethanol, cleared in xylene, and resin-mounted.

Evaluation of NMJ

Immunofluorescence was adopted to quantify the number of innervated NMJs in gastrocnemius muscle sections in blind tests. The innervated NMJs were labelled by colocalization between BTX and NF or neuron-specific Tuj-1 (Hsieh and Chen, 2024). The frozen sections of muscle tissue were incubated with 5% BSA, 1% normal goat serum (NGS), and 0.1% Triton for 4 h, and then incubated with primary antibody Tuj-1 and NF overnight at 4°C. Following washing 3 times with TBS, slides were incubated with Alexa Fluor TM 488 goat anti-rabbit IgG (1:500) and BTX (1:500) (Thermo Fisher Scientific, Cat#T1175) for 2 h at room temperature. After three additional TBS rinses, the slides were mounted in mounting medium containing DAPI. An Observer confocal microscope (Leica Microsystems, Germany) with a 20× objective was used to obtain high-resolution images of NMJs.

Radiographic evaluation of knee and ankle joints in rats

Rats are anesthetized to ensure complete immobility throughout image acquisition. For both joint regions, the hind limbs are stabilized in a reproducible neutral position using positioning devices. X-ray imaging was performed with a high-resolution digital X-ray system (Softex M-60, Softex, Japan). All examinations were conducted under standardized conditions. Images are acquired at a fixed source-to-detector distance, with typical exposure parameters set to 40–45 kVp and 100–200 mA for 0.1–0.2 s, optimized to enhance contrast in both soft tissue and bone. A calibration step-wedge may be incorporated to standardize grayscale values. Common measurements include joint space width, bone density, osteophyte formation, and limb alignment. All analyses are performed using dedicated image analysis software to ensure objectivity and reproducibility in quantitative evaluations.

Statistical analysis

Differences among the five groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test in SPSS 20.0, which compares each experimental group to a single control group. All data were presented as mean ± SD, and *P* values < 0.05 were considered statistically significant.

Data availability

The sequence data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the accession number GSE309136.

Compliance and ethics

The authors declare that they have no conflict of interest. Animals were used with the approval of the Animal Care and Use Committee of the Institute of Genetics and Developmental Biology, the Chinese Academy of Sciences (AP2023027). Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The use of hsc-NPCs was approved by the Institutional Review Board of Nanjing Drum Tower Hospital, with donor-informed consent, and all experiments complied with ethical guidelines.

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Supporting information

The supporting information is available online at <https://doi.org/10.1007/s11427-025-3321-9>. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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