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## Functionalized collagen scaffold implantation and cAMP administration collectively facilitate spinal cord regeneration



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### ABSTRACT

Previous studies have demonstrated that several mechanisms, including numerous inhibitory molecules, weak neurotrophic stimulation and deficient intrinsic regenerative responses, collectively contribute to the failure of mature spinal cord axon regeneration. Thus, combinatorial therapies targeting multiple mechanisms have attracted much attention. In the present study, a porous collagen scaffold was used to support neuronal attachment and bridge axonal regeneration. The scaffold was specifically functionalized using neutralizing proteins (CBD-EphA4LBD, CBD-PlexinB1LBD and NEP1-40) and collagen-binding neurotrophic factors (CBD-BDNF and CBD-NT3) to simultaneously antagonize myelin inhibitory molecules (ephrinB3, Sema4D and Nogo) and exert neurotrophic protection and stimulation. Cerebellar granular neurons cultured on the functionalized collagen scaffold promoted neurite outgrowth in the presence of myelin. Furthermore, a full combinatorial treatment comprising functionalized scaffold implantation and cAMP administration was developed to evaluate the synergistic repair ability in a rat T10 complete removal spinal cord injury model. The results showed that full combinatorial therapy exhibited the greatest advantage in reducing the volume of cavitation, facilitating axonal regeneration, and promoting neuronal generation. The newborn neurons generated in the lesion area could form the neuronal relay and enhance the locomotion recovery after severe spinal cord injury.

#### Statement of Significance

A porous collagen scaffold was specifically functionalized with neutralizing proteins and neurotrophic factors to antagonize the myelin inhibitory molecules and exert neurotrophic protection and stimulation for spinal cord regeneration. Cerebellar granular neurons seeded on the functionalized collagen scaffold showed enhanced neurite outgrowth ability *in vitro*. The functionalized scaffold implantation combined with cAMP administration exhibited synergistic repair ability for rat T10 complete spinal cord transection injury.

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## 1. Introduction

Spinal cord injury (SCI) is typically followed by loss of sensation and voluntary movements below the level of the lesion [1]. Unlike neonatal or peripheral nervous system neurons, which do exhibit substantial regeneration capabilities after injury, the adult mammalian central nervous system (CNS) neurons are generally

incapable of spontaneously regenerating into or beyond the lesion site [2]. Decades of research has demonstrated that the poor regeneration of mature CNS axons is attributed to several intrinsic and extrinsic mechanisms, including weak or deficient neuronal regrowth-related gene responses to support axonal elongation and regeneration, the absence of neurotrophic stimulation and neuroprotection effects of growth factors [3], the lack of permissive matrices to support axonal attachment and extension through lesion sites [4], the presence of various extracellular matrix and myelin-associated inhibitors [2,5,6], and the existence of secondary damage comprising vascular changes, inflammation and scarring [1].

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Myelin-associated inhibitory ligands have been identified among the various negative factors regarded as impediments to the regeneration of adult CNS neurons, and the receptors and signaling pathways associated with these factors have been intensively investigated [7]. Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) are candidate myelin-associated inhibitory molecules, and studies on the inhibitory effects of these molecules for spinal cord regeneration have been fairly thorough and specific [8–10]. Furthermore, some axon guidance molecules (e.g., ephrinB3 and sema4D) expressed in mature oligodendrocytes have also been implicated as inhibitory molecules that restrict the regeneration of injured CNS axons [11] and inhibit the outgrowth of postnatal cerebellar and sensory neurites *in vitro* [12]. Recently, by utilizing CBD-EphA4LBD and CBD-PlexinB1LBD to neutralize ephrinB3 and sema4D, respectively, we have also proven the therapeutic potential of that treatment on promoting axonal regeneration and functional recovery after adult rat SCI [13]. In the past decade, research efforts have been focused on the therapeutic effects of the transplantation of bio-scaffolds in combination with neurotrophic factors or stem cells to the injured spinal cord. These strategies for treating SCI effectively promote the regeneration of many types of neurons and enhance locomotion recovery [4,14–18]. Additionally, many studies have revealed that increasing the intracellular cAMP levels through the administration of dibutyryl cAMP (dbcAMP) to activate the intrinsic regrowth-related genes in uninjured residual neurons after SCI could also effectively promote axon regeneration [4,19,20]. Although many strategies independently targeting each of the mechanisms contributing to the failure of axonal regeneration after SCI have shown potential to recover the motor function of animals, the repair effect was consistently modest or limited [18,21–23]. Therefore, current studies are now focused on investigating the effects of combination therapies that target multiple mechanisms to achieve the best curative effect and provide optimal clinical reference.

Collagen is a popular biomaterial commonly used for spinal cord injury repair, reflecting the low antigenicity, excellent biocompatibility and good biodegradability of this material when implanted *in vivo* [14,22,24]. In our previous studies, we examined either neurotrophic factors or neutralizing proteins fused with a collagen-binding domain (CBD) could further enable collagen scaffolds as excellent drug sustained-releasing carriers [14,22–24]. In the present study, a well-organized linear porous collagen scaffold was developed to support neuronal attachment and bridge axonal regeneration. In addition, the collagen scaffold was functionalized with neutralizing proteins CBD-EphA4LBD, CBD-PlexinB1LBD and NEP1-40 to neutralize the known myelin inhibitory molecules ephrinB3, sema4D and Nogo-66 [25], respectively. Moreover, two neurotrophic factors, CBD-BDNF and CBD-NT3, were also administered into the collagen scaffold to provide neurotrophic stimulation and neuroprotection effects. In addition to the implantation of the functionalized collagen scaffold into a rat T10 complete removal SCI model, cAMP was also injected into the uninjured spinal cord to activate intrinsic neuronal regenerative mechanisms (Fig. 1). The findings indicated that the use of combinatorial therapy to manage intrinsic and extrinsic regenerative mechanisms could effectively facilitate axonal regeneration and revascularization after spinal cord injury.

## 2. Experimental methods

### 2.1. Protein expression and purification

The cDNA sequence for collagen binding domain (CBD) was fused at the N-terminus of mature human BDNF or NT3 DNA fragment with a His-linker and inserted into pET-28a (Novagen,

Germany) to generate pET-CBD-BDNF and pET-CBD-NT3. The recombinant proteins CBD-NT3 and CBD-BDNF were prepared as previously described [22,23]. The cDNA sequences for EphA4-LBD and PlexinB1-LBD were separately cloned into pET-28a in a manner similar to BDNF to generate pET-CBD-EphA4 LBD and pET-CBD-PlexinB1 LBD. The recombinant proteins CBD-EphA4 LBD and CBD-PlexinB1 LBD were prepared as previously described [13].

### 2.2. Myelin preparation

Myelin was isolated from adult rat as previously described [13]. To isolate myelin, the tissue was homogenized in 0.3 M sucrose and layered onto a gradient of 1.23 M and 0.85 M sucrose. The samples were centrifuged for 45 min at 75,000 g, and the crude myelin fraction was collected at the 0.85/1.23 M interface. The crude myelin was washed twice with cold water, resuspended in 0.32 M sucrose, layered onto 0.85 M sucrose, centrifuged, and collected from the 0.32/0.85 M interface. After the removal of excess sucrose, myelin was resuspended 1:1 in DMEM, homogenized and stored at  $-80^{\circ}\text{C}$  until further use.

### 2.3. Preparation of the collagen scaffold

The collagen scaffolds were generated as previously described [13]. The collagen membranes were immersed in 0.5 M acetic acid solution for 8 h at  $4^{\circ}\text{C}$ , and subsequently mixed in a blender for 15 min to obtain a homogeneous collagen solution, followed by neutralization with 4 M NaOH. The homogeneous solution was dialyzed in deionized water for 5 days and subsequently lyophilized. The obtained porous collagen scaffolds were cut into  $0.2 \times 0.5 \times 0.5$  cm pellets, and the solid pellets were immersed in 40 mL MES (pH 6.5) containing 1 mg/ml 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 0.6 mg/ml N-hydroxysuccinimide (NHS) at  $37^{\circ}\text{C}$  for 4 h. After crosslinking, the pellets were successively washed with 4 M NaCl and ddH<sub>2</sub>O at  $37^{\circ}\text{C}$  for 1 h and subsequently lyophilized again. The pellets were sterilized using Co<sup>60</sup> and stored at  $4^{\circ}\text{C}$  until further use.

### 2.4. CGN isolation, culture, and neurite outgrowth assay

The CGNs from the post-natal rat cerebellum were isolated and cultured as previously described [26]. For neurite outgrowth assays, 10  $\mu\text{L}$  of PBS containing 200 ng myelin was pretreated in 48-well plates coated with 100 g/ml of poly-L-lysine. Subsequently, 3  $\mu\text{g}$  CBD-EphA4 LBD, 3  $\mu\text{g}$  CBD-PlexinB1 LBD and 0.7  $\mu\text{g}$  NEP1-40 (or 3  $\mu\text{g}$  CBD-EphA4 LBD, 3  $\mu\text{g}$  CBD-PlexinB1 LBD, 0.7  $\mu\text{g}$  NEP1-40, 5  $\mu\text{g}$  CBD-BDNF and 5  $\mu\text{g}$  CBD-NT3) were added to the 48-well plate, followed by the addition of the dissociated CGNs. The cells were incubated for 20 h, and neurite outgrowth was subsequently assessed.

For the CGN neurite outgrowth assay on a 3D scaffold, 10  $\mu\text{L}$  of PBS containing 3  $\mu\text{g}$  CBD-EphA4 LBD, 3  $\mu\text{g}$  CBD-PlexinB1 LBD and 0.7  $\mu\text{g}$  NEP1-40 (or 3  $\mu\text{g}$  CBD-EphA4 LBD, 3  $\mu\text{g}$  CBD-PlexinB1 LBD, 0.7  $\mu\text{g}$  NEP1-40, 5  $\mu\text{g}$  CBD-BDNF and 5  $\mu\text{g}$  CBD-NT3) was initially absorbed onto the scaffold. After incubation for 30 min at RT, 10  $\mu\text{L}$  of dissociated CGNs was mixed with 200 ng myelin, loaded onto the scaffold and further incubated for 3 h. Subsequently, 300  $\mu\text{L}$  of medium was added, and the cells were cultured for 20 h, followed by an assessment of neurite outgrowth.

After fixing with 4% formaldehyde (Merck), the cells were immunostained for the neuronal marker  $\beta$ III-tubulin (1:500, Invitrogen) overnight at  $4^{\circ}\text{C}$  and subsequently incubated with the Alexa Fluor 488-conjugated secondary antibody (Invitrogen) for 1 h at room temperature. Hoechst 33342 (1 mg/ml) dye was used to stain nuclear DNA. The respective images were captured using a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss). Six

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