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Combined transplantation of GDAs^{BMP} and hr-decorin in spinal cord contusion repair

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Research Highlights

(1) This study first verified that combined glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 and human recombinant decorin transplantation inhibited early inflammatory reactions and protected axons in rats with T₈ spinal cord contusion, inhibited astrocyte proliferation and glial scar formation, and promoted axonal regeneration and growth, all of which contributed to the recovery of motor and sensory functions below the level of spinal cord contusion. (2) This combined transplantation provides a potential new therapy for experimental research and clinical transformation for the repair of spinal cord injury.

Abstract

Following spinal cord injury, astrocyte proliferation and scar formation are the main factors inhibiting the regeneration and growth of spinal cord axons. Recombinant decorin suppresses inflammatory reactions, inhibits glial scar formation, and promotes axonal growth. Rat models of T₈ spinal cord contusion were created with the NYU impactor and these models were subjected to combined transplantation of bone morphogenetic protein-4-induced glial-restricted precursor-derived astrocytes and human recombinant decorin transplantation. At 28 days after spinal cord contusion, double-immunofluorescent histochemistry revealed that combined transplantation inhibited the early inflammatory response in injured rats. Furthermore, brain-derived neurotrophic factor, which was secreted by transplanted cells, protected injured axons. The combined transplantation promoted axonal regeneration and growth of injured motor and sensory neurons by inhibiting astrocyte proliferation and glial scar formation, with astrocytes forming a linear arrangement in the contused spinal cord, thus providing axonal regeneration channels.

Key Words

neural regeneration; spinal cord injury; astrocytes; glial scar; neural stem cells; combined transplantation; glial progenitor cells; glial cells; human recombinant decorin; brain-derived growth factor; glial fibrillary acidic protein; grants-supported paper; neuroregeneration

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INTRODUCTION

After central nervous system damage, injured axons are unable to produce functional connections because axons cannot effectively regenerate and an inhibitory microenvironment is formed^[1]. A variety of cells have been examined for transplantation after spinal cord injury, such as human umbilical cord blood stem cells^[2-4], marrow stromal cells^[5-6], Schwann cells^[7-8], astrocytes^[9-10], olfactory ensheathing glia^[11-14], and oligodendrocyte progenitor cells^[15-18]. Because of structural damage and scar formation in the white matter of the spinal cord^[19-25], as well as axonal growth inhibitors present in the glial scar tissue^[26-28] and myelin of the central nervous system^[29-31], insufficient endogenous regenerated axons traversed the lesion site after various cell transplantation into or around the injured spinal cord^[32-34].

After spinal cord injury, active astrocytes can induce migration of nerve cells. Mature astrocytes mainly maintain nerve morphology, but they also play an important role in providing nerve growth factor and communication between cells^[35]. However, mature astrocytes also affect nerve regeneration and hinder axonal extension through the secretion of harmful factors that form chemical glial barriers^[36]. Therefore, it is very important to favorably regulate astrocyte expression after spinal cord injury. Glial-restricted precursor cells from the embryonic spinal cord, which are the precursors of oligodendrocytes and astrocytes^[37-39], can improve the microenvironment, regulate astrocyte expression, inhibit glial scar formation and delay the expression of proteoglycans. Glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein (BMP)-4 (GDAs^{BMP}) not only have common characteristics of glial-restricted precursor cells, but can also fill the injured interface, rearrange tissues, protect injured axons, and promote axonal regeneration and functional recovery after acute spinal cord injury^[40]. Decorin decreases fibrosis, as the core protein of decorin, can inhibit collagen fiber proliferation

and neutralize transforming growth factor- β ^[40-42]. Decorin within the nervous system not only regulates the extracellular collagen matrix, it also promotes axon fibronectin adhesion, Schwann cell proliferation and neural cell survival^[43]. Human recombinant decorin (hr-decorin) has been shown to inhibit inflammation, glial scar formation and chondroitin sulfate proteoglycan expression, and may promote axonal growth across the injured interface after acute spinal cord injury^[44-46].

After spinal cord injury, astrocytes become activated and begin to proliferate. It is very difficult to effectively control the proliferation of astrocytes and inhibit the formation of the glial scar through a single method. To provide a favorable microenvironment for spinal cord regeneration, the stationary, activated and proliferative states of the astrocytes should be considered comprehensively after spinal cord injury. In this experiment, rats with T₈ spinal cord contusion were subjected to GDAs^{BMP} and hr-decorin transplantation. The response of a transection model of spinal cord injury could not objectively mimic clinical pathological changes of spinal cord injury as spinal cord contusion accounts for the vast majority of clinical cases. Hr-decorin inhibited astrocyte proliferation and glial scar formation, and even degraded preformed glial scar. GDAs^{BMP} transplantation to contusive spinal cord promoted astrocyte to form a linear arrangement that provided axonal growth channels, which promoted axonal connectivity and recovery of sensory and motor functions below the level of spinal cord contusion.

To confirm that combined GDAs^{BMP} and hr-decorin transplantation could effectively promote the recovery of sensory and motor functions below the level of spinal cord contusion, this study observed and analyzed the size of the cavity, the degree of spinal cord atrophy, the expression of astrocytes, and the extent of motor and sensory axonal regeneration in rats with T₈ spinal cord contusion after treatment with GDAs^{BMP} and hr-decorin transplantation.

RESULTS

Quantitative analysis of experimental animals

Before T₈ spinal cord contusion, 72 female Sprague-Dawley rats were randomly divided into hr-decorin injection group, GDAs^{BMP} transplantation group, combined GDAs^{BMP} and hr-decorin transplantation group, and spinal cord injury group (each group *n* = 18). The spinal cord injury group served as a control and did not receive any treatment. According to the time after spinal cord contusion, each group was randomly divided into 3-, 7- and 28-day subgroups (each subgroup *n* = 6). Five rats died of anesthetic accident and seven rats died of excessive bleeding during surgery. New rats were used to supplement the dead rats. Seventy-two rats were included in the final analysis.

Effects of combined GDAs^{BMP} and hr-decorin transplantation on the angles between astrocyte-lined axons in rats with spinal cord contusion

The angles between adjacent glial fibrillary acidic protein-positive astrocyte-lined axons were measured with Image Pro Plus 6.0 software at 28 days after spinal cord contusion, and the extent of linear arrangement was estimated by comparing the angles (0–90°; the smaller the degree, the more linear the arrangement). The angles in the hr-decorin treatment group and spinal cord injury group were far bigger than the GDAs^{BMP} treatment group and combined GDAs^{BMP} and hr-decorin transplantation group (*P* < 0.05; Figure 1), indicating that GDAs^{BMP} transplantation alone or combined transplantation could effectively promote linear arrangement of astrocyte-lined axons but spinal injection of hr-decorin alone had no such effect.

Effect of combined GDAs^{BMP} and hr-decorin transplantation on the cavity size and cross-sectional area in the contusive spinal cord of rats

The cavities and cross-sectional area of spinal cord in glial fibrillary acidic protein-positive astrocyte fluorescence images (Figure 2) were measured and analyzed with Image Pro Plus 6.0 software. At 3 days after spinal cord contusion, hr-decorin injection or GDAs^{BMP} transplantation alone inhibited astrocyte proliferation, resulting in large cavities in the spinal cord center. GDAs^{BMP} alone or combined transplantation partially filled the injured spinal cord, so the cross-sectional areas of spinal cord were relatively larger than the other two groups. Seven days after treatment, hr-decorin significantly inhibited astrocyte proliferation (*P* < 0.05), resulting in the enlarged cavities in the hr-decorin injection group and thin

spinal cord in the combined transplantation group. Twenty-eight days later, the cavities were obviously reduced in the GDAs^{BMP} alone or combined transplantation groups, but were further enlarged in the spinal cord injury group (Figures 3–5).

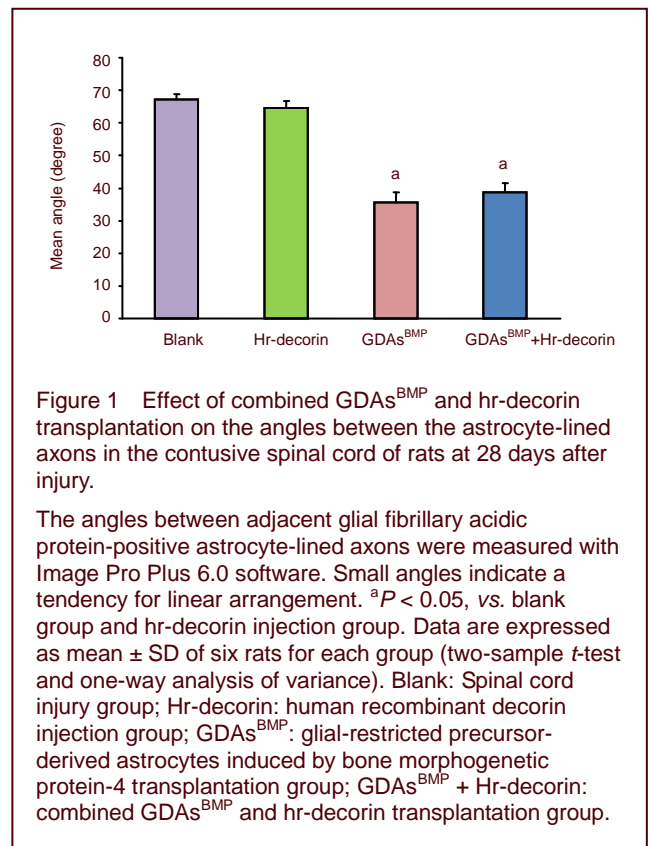


Figure 1 Effect of combined GDAs^{BMP} and hr-decorin transplantation on the angles between the astrocyte-lined axons in the contusive spinal cord of rats at 28 days after injury.

The angles between adjacent glial fibrillary acidic protein-positive astrocyte-lined axons were measured with Image Pro Plus 6.0 software. Small angles indicate a tendency for linear arrangement. ^a*P* < 0.05, vs. blank group and hr-decorin injection group. Data are expressed as mean ± SD of six rats for each group (two-sample *t*-test and one-way analysis of variance). Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and hr-decorin transplantation group.

Glial scar formation in the damaged region of rats undergoing combined GDAs^{BMP} and hr-decorin transplantation at 28 days after spinal cord contusion

The integrated absorbance values (area × average absorbance) of glial fibrillary acidic protein-positive astrocytes in the spinal cord injury group were significantly higher than the three treatment groups (*P* < 0.05). Hr-decorin inhibition of astrocyte proliferation and glial scar formation was slightly stronger than in the GDAs^{BMP} transplantation group, and combined transplantation enhanced this effect but it was not significant (*P* > 0.05) between the three treatment groups (Figure 6).

Effect of combined GDAs^{BMP} and hr-decorin transplantation on sensory and motor axons within contusive spinal cord of rats at 28 days after spinal cord contusion

A range of injured axons spread to 1 mm away from the injury center of the spinal cord. The injured axon terminals in the spinal cord injury group showed thinning, neuroma formation, bifurcation, germination, swelling,

winding or disorganized growth within the injured interface at 28 days after spinal cord contusion (Figure 2). The growth ability of sensory and motor axons was reduced, and passing rates of all axons in each point were significantly lower in the spinal cord injury group than the three treatment groups ($P < 0.05$). Hr-decorin injection, GDAs^{BMP} transplantation alone, or combined transplantation could effectively promote regeneration and growth of sensory and motor axons; nearly half of the sensory axons and some of the motor axons passed through the injury center; a small part of the sensory axons and motor axons reached a range of 5 mm from the injury center (Figures 7–9).

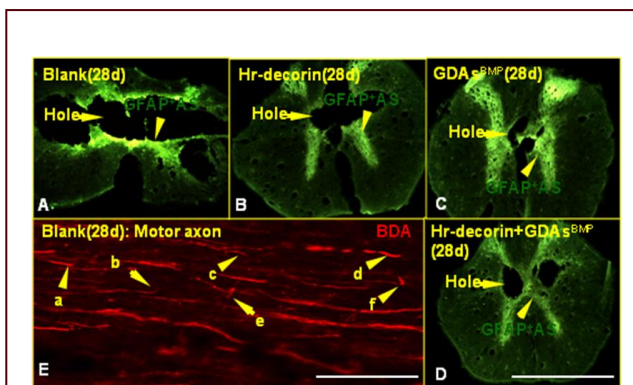


Figure 2 Expression of astrocytes, cavities and change of axonal terminal morphology in the center of the injured spinal cord at 28 days after injury.

Cross-sections (A–D) under a fluorescence microscope ($\times 50$) show the presence of astrocytes (fluorescein isothiocyanate, green) and cavities in the center of the injured spinal cord: 28 days after spinal cord contusion, the holes were further enlarged in the blank group, but were obviously reduced in the GDAs^{BMP} alone or combined transplantation groups. (A–D) Scale bar: 2 mm.

Longitudinal sections (scale bar: 125 μ m; E) under a fluorescence microscope ($\times 400$) showed axons (red) (avidin-CY3 fluorescence staining after biotinylated dextran amine axonal tracing) terminal morphology in the blank group: a shows axonal terminal neuroma formation; b shows a thinner axonal terminal; c shows axonal bifurcation and germination; d shows thicker axonal terminal; e shows axon whipping growth within the injured interface; f shows axonal flexion growth and terminal neuroma formation.

Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and hr-decorin transplantation group.

Effects of combined GDAs^{BMP} and hr-decorin transplantation on hindlimb motor function in rats with spinal cord contusion

At 1 day after spinal cord contusion, the Basso-Beattie-Bresnahan scores of rats in each group were 0. Three

days later, Basso-Beattie-Bresnahan scores of all rats were no more than 2 points, indicating that there was no significant recovery of hindlimb motor function. However, 7 days later, Basso-Beattie-Bresnahan scores in the treatment groups were significantly higher than the spinal cord injury group ($P < 0.05$). Twenty-eight days later, the recovery level of hindlimb motor function in the treatment groups increased significantly, especially in the combined GDAs^{BMP} and hr-decorin transplantation group (approaching 17 points; $P < 0.05$), while Basso-Beattie-Bresnahan scores in the spinal cord injury group were around 10 points (Figure 10).

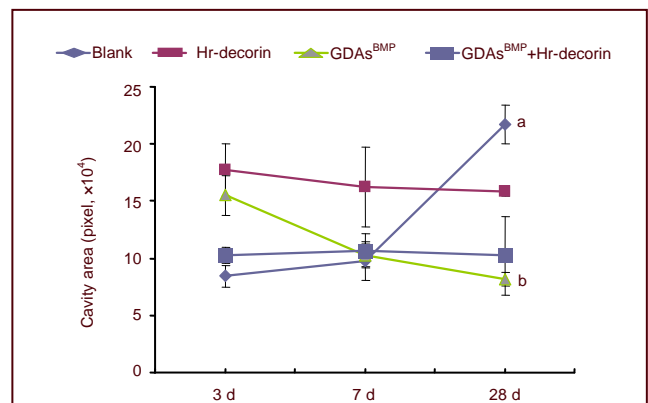


Figure 3 Effect of combined GDAs^{BMP} and hr-decorin transplantation on the cavity area in the contusive spinal cord of rats.

The cavity area of the spinal cord in the glial fibrillary acidic protein-positive astrocyte fluorescence image was measured and analyzed with Image Pro Plus 6.0 software, and calculated by pixels. The cavities of the blank group were smallest at 3 and 7 days, but largest at 28 days after spinal cord injury ($^a P < 0.05$, vs. blank group at 3 and 7 days). The hr-decorin injection group and combined GDAs^{BMP} and hr-decorin transplantation group had a decreasing trend, but there was little change. The GDAs^{BMP} transplantation group decreased significantly at 28 days after spinal cord injury compared with the other groups at the same time ($^b P < 0.05$). Data are expressed as mean \pm SD of six rats for each group (two-sample *t*-test and one-way analysis of variance).

Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and hr-decorin transplantation group.

DISCUSSION

GDAs^{BMP} transplantation after spinal cord contusion effectively filled the cavity in contusive spinal cord

Glial-restricted precursor cells can be directly isolated from embryonic spinal cord^[47], and neural stem cells and embryonic stem cells^[48-49] can also be separately

isolated from the central nervous system of mice and humans^[40, 50-54].

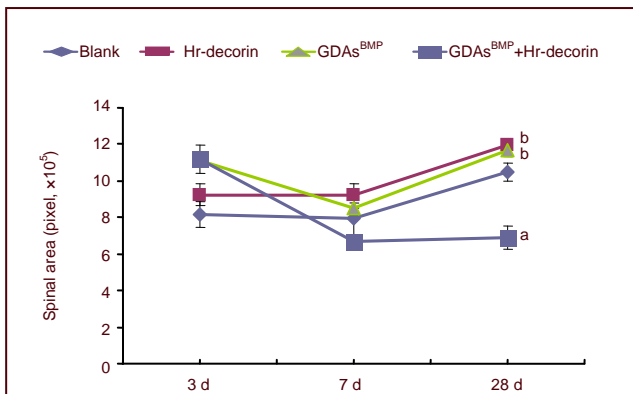


Figure 4 Effect of combined GDAs^{BMP} and hr-decorin transplantation on the cross-sectional area in the contused spinal cord of rats.

The cross-sectional area of the spinal cord in the glial fibrillary acidic protein-positive astrocyte fluorescence image was measured and analyzed with Image Pro Plus 6.0 software, and calculated by pixels. The cross-sectional area of combined GDAs^{BMP} and hr-decorin transplantation group was least at 7 and 28 days (^a $P < 0.05$, vs. the other groups at the same time); the cross-sectional area of hr-decorin group and GDAs^{BMP} transplantation group was always larger than the blank group (^b $P < 0.05$). Data are expressed as mean \pm SD of six rats for each group (two-sample *t*-test and one-way analysis of variance).

Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and hr-decorin transplantation group.

After severe spinal cord contusion, a large cavity gradually forms in the injury center that seriously affects regeneration and growth. Small cavities are initially formed because of an early serious inflammatory response, intramedullary hemorrhage, spinal cord edema, and glial cell activation and proliferation after severe spinal cord contusion. However, in the later stages, huge cavities are gradually formed in the spinal cord injury center due to gray and white matter necrosis; a large glial scar forms surrounding the cavity; most axonal paths are disconnected; and a very small number of motor axons cross the injury center. Many methods have been investigated for filling these cavities, but little success has been achieved. After treatment with GDAs^{BMP} from glial-restricted precursor cells induced with bone morphogenetic protein-4^[40, 55-57], although cavities formed in the first 7 days after spinal cord contusion, GDAs^{BMP} inhibited astrocyte proliferation and the inflammatory response, and drastically reduced cavity size after day 7, through the proliferation of GDAs^{BMP} and astrocytes, and regeneration of nerve tissue. At 3 days after spinal cord

contusion, the ability of hr-decorin to inhibit astrocyte proliferation and the inflammatory response reduced, resulting in obvious spinal cord edema. Subsequently, the spinal cord became tapered as acute inflammation subsided. Following this, the cross-sectional area of the spinal cord gradually recovered because most of the cavities were filled. The cavities decreased and the recovery of cross-sectional area of spinal cord provided a carrier for axonal regeneration and growth.

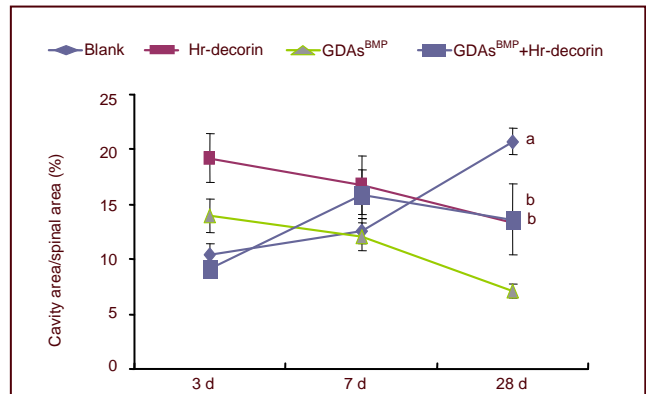


Figure 5 Effect of combined GDAs^{BMP} and hr-decorin transplantation on the ratio between the cavity and cross-sectional areas in the contusive spinal cord of rats.

The cavity and cross-sectional areas of spinal cord in the glial fibrillary acidic protein-positive astrocyte fluorescence image were measured and analyzed with Image Pro Plus 6.0 software, and calculated by pixels. The ratio between the cavity area and cross-sectional area of the blank group gradually increased to the maximum compared with the treatment groups at 28 days after spinal cord contusion (^a $P < 0.05$), while the hr-decorin treatment group and the GDAs^{BMP} transplantation group slowly decreased. The combined GDAs^{BMP} and hr-decorin transplantation group increased and then decreased compared with the blank and the GDAs^{BMP} transplantation groups at 28 days after spinal cord contusion (^b $P < 0.05$). Data are expressed as mean \pm SD of six rats for each group (two-sample *t*-test and one-way analysis of variance).

Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and hr-decorin transplantation group.

Formation of astrocyte linear arrangement and reduction in glial scar

The formation of a linear arrangement of astrocytes in the contusive spinal cord created the shortest path for axonal growth, which enhanced the efficiency of axons passing through the injury interface^[40]. After severe spinal cord contusion, a serious glial scar, characterized by an increased number and density of astrocytes, is formed within the injury interface. This blocks axonal growth, resulting in axon terminal atrophy, neuroma formation, and buckling or winding growth. Although

many methods have been examined previously, removal of the glial scar remains a difficult issue.

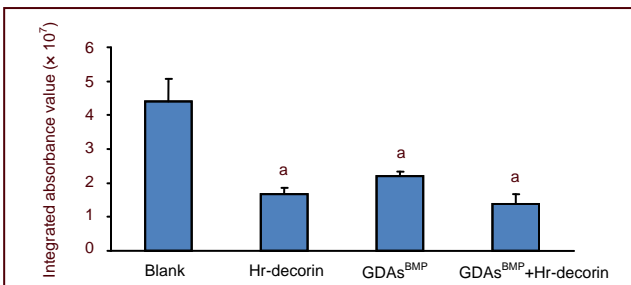


Figure 6 Effect of combined GDAs^{BMP} and hr-decorin transplantation on glial fibrillary acidic protein-positive astrocytes within the contusive spinal cord of rats at 28 days after injury.

Integrated absorbance values (area x average absorbance) of glial fibrillary acidic protein-positive astrocytes in immunofluorescence photographs were measured with Image Pro Plus 6.0 software. ^a $P < 0.05$, vs. blank group. Data are expressed as mean \pm SD of six rats for each group (two-sample t -test and one-way analysis of variance).

Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and hr-decorin transplantation group.

Thus, numerous studies have changed strategy to investigate mechanisms of preventing the formation of the glial scar in the early stages of spinal cord injury. After GDAs^{BMP} transplantation, the angle of astrocyte-lined axons significantly reduced, which denoted that the glial scar was significantly reduced and astrocyte-lined axons were evenly distributed. The results showed that GDAs^{BMP} transplanted into contusive spinal cord could effectively promote the linear arrangement of astrocyte-lined axons, while treatment with PBS or hr-decorin injection had no such effect.

Promotion of axonal regeneration and growth

GDAs^{BMP} derived from glial-restricted precursor cells are a particular type of astrocyte possessing numerous functions, such as support, nutrition, and signal contact. After GDAs^{BMP} transplantation into the spinal cord, the vast majority of the cavities were filled by astrocytes and GDAs^{BMP} promoted the linear arrangement of astrocyte-lined axons, which provided channels for axonal regeneration and growth in the host.

GDAs^{BMP} produce brain-derived neurotrophic factor, which can promote survival of neurons in the contusive spinal cord and prevent axonal atrophy^[40, 58]. GDAs^{BMP} can also delay the expression of chondroitin sulfate proteoglycans, thereby indirectly promoting axonal regene-

ration^[40]. At 28 days after GDAs^{BMP} transplantation, a number of motor axons crossed the injury center and reached the distal end of the spinal cord by growing along nerve channels established by astrocytes. The sensory axons in the spinal cord surface were increased, and moved around the cavity or through the injury center to reach the distal end of the spinal cord. In addition, GDAs^{BMP} may also have promoted the growth of other motor-related axons, such as the reticular tract and lateral corticospinal tract.

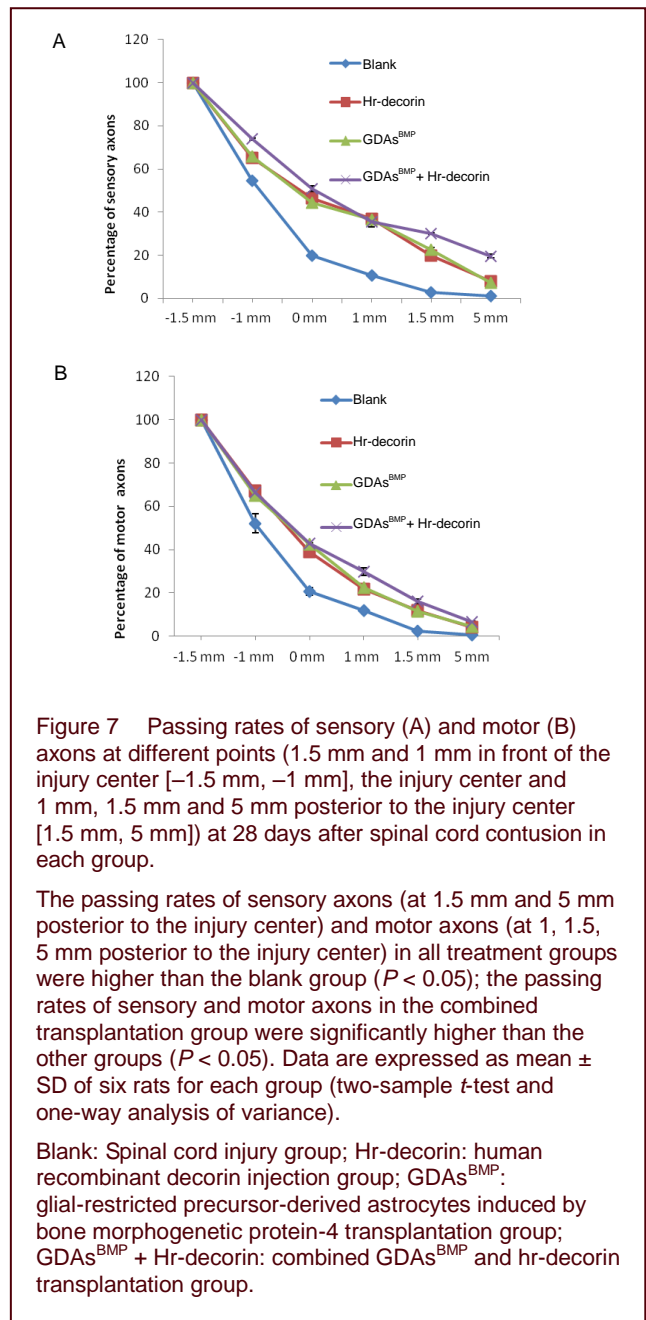


Figure 7 Passing rates of sensory (A) and motor (B) axons at different points (1.5 mm and 1 mm in front of the injury center [-1.5 mm, -1 mm], the injury center and 1 mm, 1.5 mm and 5 mm posterior to the injury center [1.5 mm, 5 mm]) at 28 days after spinal cord contusion in each group.

The passing rates of sensory axons (at 1.5 mm and 5 mm posterior to the injury center) and motor axons (at 1, 1.5, 5 mm posterior to the injury center) in all treatment groups were higher than the blank group ($P < 0.05$); the passing rates of sensory and motor axons in the combined transplantation group were significantly higher than the other groups ($P < 0.05$). Data are expressed as mean \pm SD of six rats for each group (two-sample t -test and one-way analysis of variance).

Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and hr-decorin transplantation group.

Hr-decorin injection after spinal cord contusion effectively inhibited glial scar formation and promoted glial scar degradation

Decorin is a small leucine-rich chondroitin sul-

fate/dermatan sulfate proteoglycan, which is very rich in the vascular border zone, the brain surface, the ependyma and mesh tissue, as well as being expressed in the central nervous system after injury. Hr-decorin can promote endothelial cell formation of blood vessels *in vitro* and the generation of capillaries *in vivo*, especially during the inflammatory stage. The degree of spinal cord blood stasis was significantly reduced at 7 days and disappeared at 28 days after hr-decorin injection by respectively comparing gross specimens of contusive spinal cord in rats, indicating that hr-decorin promoted the absorption of stagnant blood in the injured spinal cord. Hr-decorin has also been shown to inhibit inflammatory factors (transforming growth factor- β) and reduce the expression of I, III procollagen mRNA in cells, so it can effectively inhibit scar fibroblast proliferation.

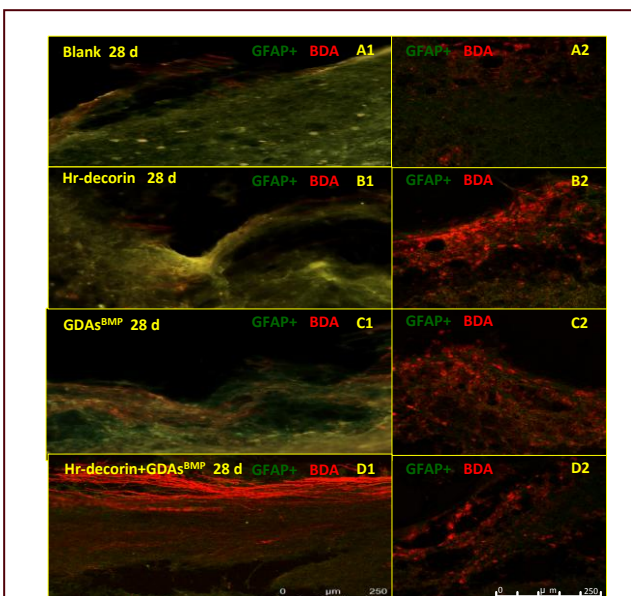


Figure 8 Effect of combined GDAs^{BMP} and hr-decorin transplantation on sensory axonal regeneration in the contusive dorsal spinal cord of rats at 28 days after injury.

Fluorescence microscopy (A1, B1, C1, $\times 200$) and confocal fluorescence microscopy (A2, B2, C2, D1, D2, $\times 200$) show the expression of astrocytes (fluoresceine isothiocyanate, green) and sensory axons (Cy3, red) passing through the injury center and interface at 28 days after spinal cord contusion. Longitudinal sections show that the sensory axonal pathway was damaged severely in the blank group and the Hr-decorin injection group, while the damage was less in the GDAs^{BMP} transplantation group and the combined transplantation group (A1, B1, C1, D1). Cross-sections show that the number of sensory axons in the three treatment groups was more than the blank group (A2, B2, C2, D2).

Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and hr-decorin transplantation group. Scale bar: 250 μm . d: Day.

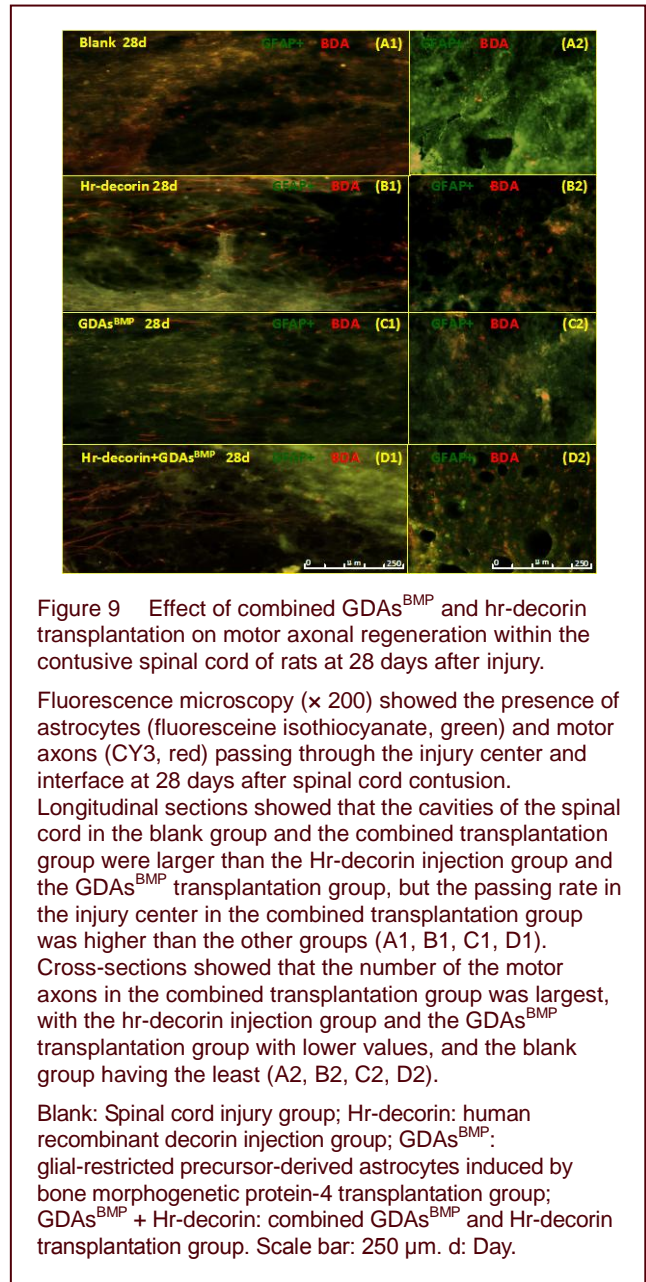


Figure 9 Effect of combined GDAs^{BMP} and hr-decorin transplantation on motor axonal regeneration within the contusive spinal cord of rats at 28 days after injury.

Fluorescence microscopy ($\times 200$) showed the presence of astrocytes (fluoresceine isothiocyanate, green) and motor axons (CY3, red) passing through the injury center and interface at 28 days after spinal cord contusion.

Longitudinal sections showed that the cavities of the spinal cord in the blank group and the combined transplantation group were larger than the Hr-decorin injection group and the GDAs^{BMP} transplantation group, but the passing rate in the injury center in the combined transplantation group was higher than the other groups (A1, B1, C1, D1). Cross-sections showed that the number of the motor axons in the combined transplantation group was largest, with the hr-decorin injection group and the GDAs^{BMP} transplantation group with lower values, and the blank group having the least (A2, B2, C2, D2).

Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and Hr-decorin transplantation group. Scale bar: 250 μm . d: Day.

After hr-decorin treatment for 3 days, the inflammatory response and the proliferation of astrocytes were effectively suppressed and the product of the inflammatory response was degraded, which resulted in larger cavities than the spinal cord injury group. Following this, hr-decorin promoted plasminogen/plasmin synthesis, which not only inhibited synthesis of chondroitin sulfate proteoglycans, it also promoted degradation of chondroitin sulfate proteoglycans. Hr-decorin injection again at 14 days helped to inhibit scar formation and also degrade the glial scar that had formed in the earlier stages.

Protection of axons and promotion of axonal regeneration

Hr-decorin within the nervous system regulates collagen

extracellular matrix, and promotes the fibronectin adhesion of axons, Schwann cell proliferation and neural cell survival^[43]. Hr-decorin effectively inhibited the early inflammatory response and promoted the formation of tiny blood vessels, prevented further secondary damage to injured axons, and provided a better microenvironment for axonal regeneration and growth. Hr-decorin inhibited astrocyte proliferation (early) and degraded the glial scar (late), resulting in a reduction in the inhibition of axonal regeneration and growth. Hr-decorin could not effectively induce a linear arrangement of astrocytes within the spinal cord, but could reduce the density of astrocytes, allowing axons to extend into the cavity. Hr-decorin promoted the synthesis of plasmin, which activated neurotrophic factors, degraded a variety of axonal growth inhibitors, and promoted reconstruction of the central nervous system^[44]. The experimental results demonstrate that hr-decorin not only effectively protected the injured spinal cord, it also promoted axonal regeneration to a certain extent.

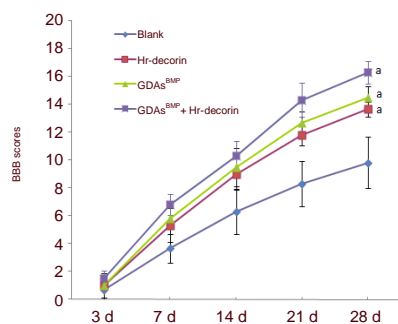


Figure 10 Effect of combined GDAs^{BMP} and hr-decorin transplantation on the Basso-Beattie-Bresnahan (BBB) scores in rats with contusive spinal cord.

Motor function of the hindlimbs in rats was divided into 22 grades (BBB scores: 0 to 21 points). 0 points meant complete paralysis of hindlimbs and 21 points meant complete normal motor function of the hindlimbs. Three days later, BBB scores of all rats were no more than 2 points. At 7, 14, 21, 28 days after spinal cord contusion, ^a $P < 0.05$, vs. blank group. Data are expressed as mean \pm SD of six rats for each group (two-sample *t*-test and one-way analysis of variance).

Blank: Spinal cord injury group; Hr-decorin: human recombinant decorin injection group; GDAs^{BMP}: glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 transplantation group; GDAs^{BMP} + Hr-decorin: combined GDAs^{BMP} and hr-decorin transplantation group.

Combined GDAs^{BMP} and hr-decorin transplantation after spinal cord contusion more effectively inhibited the inflammatory response and reduced secondary axonal injury

Three days after spinal cord contusion, hr-decorin and

GDAs^{BMP} transplantation inhibited the inflammatory response and may have decreased astrocyte proliferation, with no obvious thinning of the spinal cord; the remaining spinal cord tissue significantly increased as transplanted GDAs^{BMP} filled the cavity and both hr-decorin and GDAs^{BMP} effectively protected the spinal cord. Seven days later, because the inflammatory response and spinal cord edema decreased along with the inhibition of astrocyte proliferation by hr-decorin and GDAs^{BMP} treatment, the spinal cord had obviously thinned. At 28 days after spinal cord contusion, the inflammatory response had disappeared; GDAs^{BMP} and hr-decorin had constantly inhibited astrocyte proliferation and glial scar formation, while hr-decorin alone also possibly inhibited GDAs^{BMP} proliferation. Nevertheless, because of GDAs^{BMP} proliferation, there were no significant changes in the areas of residual spinal cords and cavities, and the spinal cord tended to be stable.

Decreased glial scar and promotion of astrocyte linear arrangement

Combined GDAs^{BMP} and hr-decorin transplantation for contusive spinal cord constantly inhibited the early inflammatory response and astrocyte proliferation, which substantially reduced glial scar formation. At 28 days after spinal cord contusion, treatment with either hr-decorin injection, GDAs^{BMP} transplantation or combined GDAs^{BMP} and hr-decorin transplantation effectively suppressed astrocyte proliferation and scar formation. Although there was no statistically significant difference between the treatment groups, the decreasing trend of glial scar appeared in the combined transplantation group. The angles between astrocyte-lined axons after combined transplantation and GDAs^{BMP} transplantation were smaller, suggesting that both GDAs^{BMP} transplantation alone or combined transplantation effectively promoted the linear arrangement of astrocyte-lined axons in the host.

Significantly improved abilities of axonal regeneration and growth

The results suggest that combined transplantation promoted regeneration and growth of sensory and motor axons more effectively than hr-decorin injection or GDAs^{BMP} transplantation. First, combined transplantation further strengthened inhibition of the inflammatory response, promoted the formation of spinal cord capillaries and reduced chondroitin sulfate proteoglycan expression. Second, GDAs^{BMP} promoted the linear arrangement of astrocytes to provide channels for axonal growth. Third, GDAs^{BMP} secreted brain-derived neurotrophic factor to protect axons and neurons from atrophy in the early

stages, and promoted axonal growth in the later stages. Finally, hr-decorin and GDAs^{BMP} provided a favorable microenvironment for axonal regeneration and growth at an early stage, which extended the time window for axonal regeneration in the acute stage.

Enhanced hindlimb motor function

Previous studies demonstrated that GDAs^{BMP} transplantation at 3 days after spinal cord promoted axonal regeneration and plasticity of axonal connections, and resulted in recovery of motor function^[40]. At 28 days after spinal cord contusion, GDAs^{BMP} transplantation or hr-decorin injection significantly promoted recovery of hindlimb motor function in rats. However, the mechanisms of functional recovery for both treatments were different. The inhibitory effects of hr-decorin on the early inflammatory response and glial scar formation were stronger than GDAs^{BMP}, whereas GDAs^{BMP} transplantation provided brain-derived neurotrophic factor, which in turn prevented axonal and neuronal atrophy. GDAs^{BMP} transplantation induced astrocytes to form a linear arrangement within the host that provided growth channels for axons passing through the injury center and interfaces, whereas hr-decorin alone could not change astrocyte arrangement. Because hr-decorin injection and GDAs^{BMP} transplantation had different mechanisms for the treatment of spinal cord contusion, a combined transplantation was adopted. Hr-decorin inhibited the inflammatory response in the early stages, and protected axons and neurons together with GDAs^{BMP}, thus creating a better microenvironment for enhancing axonal regeneration and growth and significantly promoting the recovery of hindlimb function. Furthermore, the recovery of hindlimb motor function was more obvious with time in the combined treatment group, indicating that the speed and extent of motor axonal regeneration were stronger in the later stages. Together, these results indicate that combined transplantation established axonal regeneration channels, reduced the glial scar and provided a favorable microenvironment for axonal regeneration and growth, which obviously enhanced the speed and degree of motor axon regeneration.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiment was performed at the Laboratory of Academy of Military Medical Sciences, Beijing Institute of

Neuroscience, Capital Medical University, China from 2009 to 2011.

Materials

A total of 72 female Sprague-Dawley rats weighing 200 ± 10 g and one embryonic day 13.5 rat were obtained from the Academy of Military Medical Sciences, Beijing, China (license No. SCXK (Military) 2007-004).

Methods

Glial-restricted precursor cell isolation and GDAs^{BMP} generation

After intramuscular injection with 1% pentobarbital sodium solution (45 mg/kg), spinal cords of embryonic day 13.5 rat embryos were isolated under an anatomical microscope^[40, 48]. After the embryonic spinal cords were cut into 1 mm × 1 mm pieces and digested with 0.25% trypsin/ethylene diamine tetraacetic acid (Invitrogen, Carlsbad, CA, USA) solution, residual trypsin/ethylene diamine tetraacetic acid solution was neutralized by Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) with a standard 10% fetal bovine serum (Hyclone Co.). The digested spinal cord tissues were gently pipetted with a Pasteur pipette, and the cells were then collected and diluted to 1 × 10⁶ cells/mL. After adding anti-A2B5 (Sigma-Aldrich Co., St. Louis, MO, USA) (0.1 mg/mL) and phycoerythrin anti-mouse IgM (eBioscience Co., San Diego, CA, USA; 1:100) in sequence, A2B5- positive cells accounted for 33.18% by flow cytometry analysis, then the primary A2B-positive glial-restricted precursor cells (90.65%) were ultimately sorted by fluorescence-activated cell sorting. The primary glial-restricted precursor cells were maintained on a fibronectin/laminin substrate (Sigma-Aldrich) in DMEM/ F12 (Hyclone Co.) Sato-medium with 20 ng/mL rat basic fibroblast growth factor (Sigma-Aldrich) *in vitro*. Culture media containing DMEM/F12 and rat basic fibroblast growth factor were changed every 2 days. After serially passaging for no more than three generations, more glial-restricted precursor cells were obtained. To differentiate glial-restricted precursor cells into GDAs^{BMP} (A2B5-negative/glial fibrillary acidic protein-positive), glial-restricted precursor cells were seeded on a fibronectin/ laminin substrate in DMEM/F12 Sato-medium with 20 ng/mL basic fibroblast growth factor and 10 ng/mL of human recombinant bone morphogenetic protein-4 (R&D Systems Co., Ltd., Minneapolis, MN, USA) for 7 days. GDAs^{BMP} were collected by centrifugation and diluted by adding an appropriate volume of PBS. Subsequently, the anti-A2B5 and rabbit anti-rat glial fibrillary acidic protein polyclonal antibody (Sigma-Aldrich) was added. After incubation for 1 hour in a 37°C CO₂ incubator, the sec-

ondary antibody including phycoerythrin anti-mouse IgM and goat anti-rabbit IgG-fluoresceine isothiocyanate (FITC) fluorescent antibody (Sigma-Aldrich) were added for 1 hour. A2-positive cells were removed by fluorescence-activated cell sorting, and purified A2B-negative glial fibrillary acidic protein-positive GDAs^{BMP} were obtained. The purified GDAs^{BMP} were collected by centrifugation, and diluted into cell suspensions (30 000 cells/ μ L) with Dulbecco's modified Eagle's medium/F12 for transplantation.

Preparation of T₈ spinal cord contusion model

After intraperitoneal injection with 1% pentobarbital sodium (Merck Co., Darmstadt, Hessen, Germany; 35 mg/kg), female rats (body weight 200 \pm 10 g) were fixed on the operating table in the prone position. The T₈₋₉ spinous process and lamina were exposed along the posterior midline of the spine (T₇₋₁₀), T₈ and the upper part of T₉ spinous process and lamina were carefully removed to fully expose the dura. Subsequently, the T₈ spinal cord was bruised with an NYU device (New York University, New York, NY, USA; 10 g \times 50 mm). Contusive spinal cord suffered from swelling and congestion, and motor and sensory function in rats was lost completely^[59]. The Basso-Beattie-Bresnahan scores of all rats were 0 the next day and no more than 2 points at 3 days after spinal cord contusion, indicating that the combat strength fully met the requirements of the experiment. Later, rats with spinal cord contusion were injected with penicillin (50 000 U/kg per day) for 3 days, and urinated manually at least twice a day until autonomic micturition reflex appeared.

Drug injection or GDAs^{BMP} transplantation

The strategies of drug injection or GDAs^{BMP} transplantation to rat spinal cord were divided into three cross-sections (Figure 11A), two injection points on each cross-section (Figure 11A, B) and two levels on each point (Figure 11A). The rats with T₈ spinal cord contusion were fixed in the prone position under rat brain stereotaxic apparatus with a micropipette installed with a 100 μ m tiny glass needle. According to the design, the needle with drug or GDAs^{BMP} was slowly injected to 1.5 mm depth from the spinal cord dorsal surface directly for 1 minute, then withdrawn to 0.5 mm depth for 10 minutes.

Treatment of T₈ spinal cord contusion in rats

Rats in the hr-decorin injection group, GDAs^{BMP} transplantation group, combined hr-decorin and GDAs^{BMP} transplantation group and spinal cord injury group were respectively injected with hr-decorin (R&D Systems Co., Ltd.; 1 μ g/ μ L), GDAs^{BMP} suspension (30 000 cells/ μ L), hr-decorin and GDAs^{BMP} suspension (the same concentra-

tion and density as above), and PBS solution (0.1 mol/L) into the 12 injection sites within the damaged area (each site 0.5 μ L; a total of 6 μ L). Two weeks later, rats in the 28-day subgroup with hr-decorin injection received intraperitoneal injection of 0.5 mL hr-decorin solution (12 μ g/mL). The rats undergoing GDAs^{BMP} transplantation received intramuscular injection of cyclosporine A (Novartis AG, Basel, Switzerland; 10 mg/kg) every day.

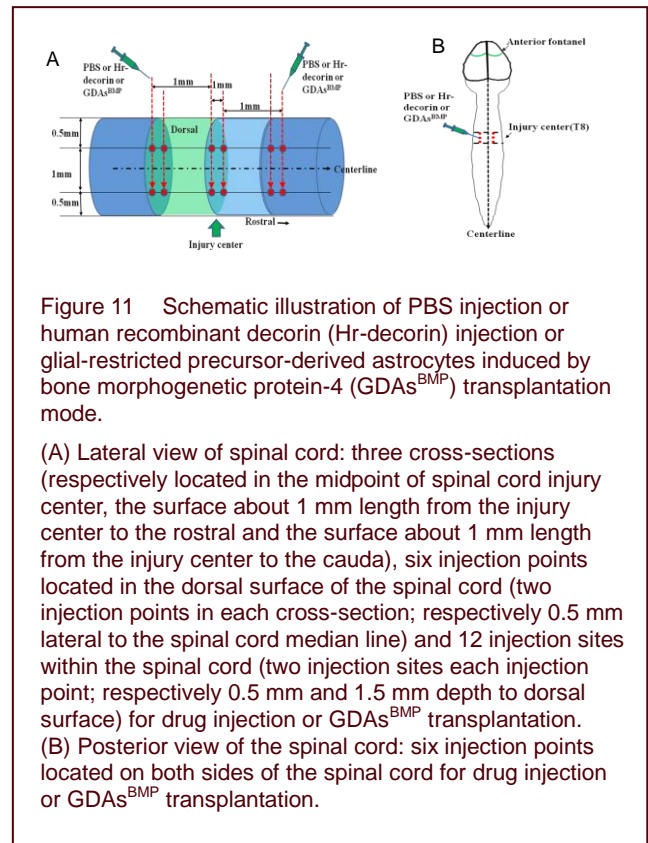


Figure 11 Schematic illustration of PBS injection or human recombinant decorin (Hr-decorin) injection or glial-restricted precursor-derived astrocytes induced by bone morphogenetic protein-4 (GDAs^{BMP}) transplantation mode.

(A) Lateral view of spinal cord: three cross-sections (respectively located in the midpoint of spinal cord injury center, the surface about 1 mm length from the injury center to the rostral and the surface about 1 mm length from the injury center to the cauda), six injection points located in the dorsal surface of the spinal cord (two injection points in each cross-section; respectively 0.5 mm lateral to the spinal cord median line) and 12 injection sites within the spinal cord (two injection sites each injection point; respectively 0.5 mm and 1.5 mm depth to dorsal surface) for drug injection or GDAs^{BMP} transplantation. (B) Posterior view of the spinal cord: six injection points located on both sides of the spinal cord for drug injection or GDAs^{BMP} transplantation.

Motor function of rats scored by Basso-Beattie-Bresnahan scoring

At 3, 7, 14, 21 and 28 days after spinal cord contusion, behavioral changes and hindlimb motor function of rats were observed to obtain Basso-Beattie-Bresnahan function scores^[60]. These included the number and scope of hindlimb joint activities, load level of hindlimbs, coordination of forelimbs and hindlimbs, claws and tail activities. Basso-Beattie-Bresnahan function scores of rats were divided into 22 grades (0 to 21 grades): zero indicated complete paralysis and 21 indicated normal function.

Tracking of biotinylated dextran amine anterograde motor or sensory axons

The ability of axonal regeneration or growth after spinal cord contusion was observed with biotinylated dextran amine (Molecular Probes Co., Ltd., Junction, OR, USA) anterograde motor or sensory axonal tracking. At 2 weeks before rats were executed, 10% biotinylated dex-

tran amine 10 000 was injected into both sides of the cerebral motor cortex in rats for anterograde corticospinal tract tracking^[61]. At 8 days before rats were executed, ascending endogenous axons were tracked by injecting 10% biotinylated dextran amine 10 000 into cuneate and gracile white matter at the T₁₀ spinal level in both sides of the spinal cord^[40].

For biotinylated dextran amine anterograde motor axonal tracking, the rats were fixed under a stereotaxic apparatus (Stoelting, Chicago, IL, USA) in the prone position. A total of 16 holes (eight holes in each side; diameter of 0.5 mm) located in the 16 points were respectively drilled. Biotinylated dextran amine was slowly (0.1 μ L/min) injected into the 16 points (Figure 12A, 1.0 mm depth to the cerebral motor cortex surface; each point 0.5 μ L) for 5 minutes. For biotinylated dextran amine anterograde sensory axonal tracking, T₁₀ dura mater was fully exposed, and then the rats were fixed under a stereotaxic apparatus in prone position. Biotinylated dextran amine was slowly (0.1 μ L/min) injected into two points (Figure 12B); 0.5 mm depth to the spinal cord surface; each point 0.5 μ L) for 10 minutes.

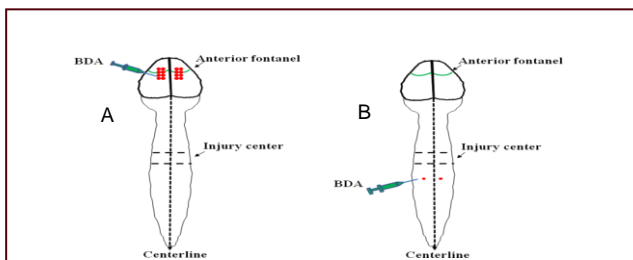


Figure 12 Biotinylated dextran amine (BDA) anterograde motor or sensory axonal tracking in rats.

(A) BDA anterograde motor axonal tracing: BDA was slowly (0.1 μ L/min) injected into the 16 injection points (front bregma 1.0 mm, bregma center, behind bregma 1.0 mm and 2.0 mm as the center, as well as 1.5 mm and 2.5 mm lateral to the spinal cord median line) within the cerebral cortical motor area; (B) BDA anterograde sensory axons tracing: BDA was slowly (0.1 μ L/min) injected into the two injection points in the distal dorsal spinal cord (locating T₁₀ spinal level in both sides) about 5 mm from the spinal cord injury center.

Double immunofluorescence histochemistry

A 3, 7 and 28 days after spinal cord contusion, six rats from each group were executed for double immunofluorescence histochemistry to observe the ability of motor and dorsal ascending sensory axonal regeneration (biotinylated dextran amine marked), and astrocyte expression within the contusive spinal cord (glial fibrillary acidic protein expression). After cardiac perfusion, the spine was completely removed and post-fixed in 4% paraformaldehyde solution for 2 hours. The osseous structure of

the spine was stripped off; the spinal cord was completely removed and protected in 30% sucrose/PBS solution (4°C) overnight. The spinal cord was embedded in optimal cutting temperature medium (SAKURA, Tokyo, Japan) for serial frozen sectioning. Serial longitudinal sections (50 μ m) were obtained from the dorsal to spinal medullary canal. Cross-sections (50 μ m) were respectively obtained at -1.5 mm, -1 mm, 0 mm, +1 mm, +1.5 mm and +5 mm points ("0", "-" and "+" indicating the spinal cord injury center, approaching and withdrawing from the biotinylated dextran amine injection point). After antigen repairing, sections were immersed in sodium citrate buffer with 0.1% Triton X-100 for 5 minutes, treated with 5% standard goat serum and closed for 30 minutes at room temperature. Subsequently, rabbit glial fibrillary acidic protein polyclonal antibody (Sigma-Aldrich; 1:100) was added at 4°C overnight. The next day, sections were incubated with Avidin-Cy3 (Sigma-Aldrich; 1:50; marked biotinylated dextran amine) and goat anti-rabbit IgG-FITC (Zymed Laboratories Co., San Diego, CA, USA) (1:50) fluorescent antibody for 2 to 3 hours at room temperature, and then immersed in PBS with 0.1% Tween-20. Finally, biotinylated dextran amine (red) and glial fibrillary acidic protein (green) expression of the spinal cord sections were respectively observed with DMLA 4000B fluorescence microscope (Leica, Wetzlar, Germany) and TCS SP5 confocal microscope (Leica), and measured with the Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Data were expressed as mean \pm SEM and analyzed using a two-sample *t*-test and one-way analysis of variance with SPSS 11.5 software (SPSS, Chicago, IL, USA). A *P* value < 0.05 was considered statistically significant.

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