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Transplantation of adult spinal cord grafts into spinal cord transected rats improves their locomotor function

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Grafted embryonic central neural tissue pieces can recover function of hemisected spinal cord in neonatal rats and promote axonal growth in adults. However, spinal cord segments from adults have not been used as donor segments for allogeneic transplantation. Here, we utilized adult spinal cord tissue grafts (aSCGs) as donor constructs for repairing complete spinal cord injury (SCI). Moreover, to provide a favourable microenvironment for SCI treatment, a growth factor cocktail containing three growth factors (brain-derived neurotrophic factor, neurotrophin-3 and vascular endothelial growth factor), was applied to the aSCG transplants. We found that the locomotor function was significantly improved 12 weeks after transplantation of aSCGs into the spinal cord lesion site in adult rats. Transplantation of aSCGs combined with these growth factors enhanced neuron and oligodendrocyte survival and functional restoration. These encouraging results indicate that treatment of complete SCI by transplanting aSCGs, especially in the presence of growth factors, has a positive effect on motor functional recovery, and therefore could be considered as a possible therapeutic strategy for SCI.

spinal cord injury (SCI), transplantation, adult spinal cord grafts (aSCGs), function recovery, adult host, transection

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INTRODUCTION

Spinal cord injury (SCI) caused by trauma or diseases affect millions of people worldwide, with limited capacity for spontaneous recovery (Lee et al., 2014). SCI leads to disruption of ascending and descending axons, damage of neurons and glia, a secondary phase of tissue loss, and chronic neurological deficits (Bramlett and Dietrich, 2007). Treatment is often hindered by the severe microenvironment of SCI, such as a lack of neurotrophic growth factors, inflammatory responses and scar formation (Han and Dai, 2016; Maier and Schwab, 2006; Xiao et al., 2016).

Methods for transplantation of solid pieces of embryonic neural tissue have been developed to replace lost spinal cord tissue and promote SCI repair (Horvat, 1991). Embryonic neural tissue was usually dissected and stripped of meninges and dorsal root ganglia, followed by transplantation into newborn or adult rats. Most of the transplanted embryonic neural grafts (>80%) survived in lesions in both the newborn and adult host rats for 1–16 months, and the donor tissue showed obvious differentiation. The transplants filled the

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lesion site, closely apposed to the injured surfaces of the recipient spinal cords, and therefore may have had some effect on suppressing dense glial scar formation (Reier et al., 1986). Investigation of axonal connectivity between the host and donor tissues showed that although the majority of host- and graft-derived axons were concentrated within the vicinity of the host-graft border, axons of some transplants (solid pieces of embryonic day 14 (E14) spinal cord) arose from the grafts and extended into the host spinal cord, and some host fibres extended into the transplants after implantation into hemisection aspiration cavities in the lumbar spinal cord (Jakeman and Reier, 1991). The growth and regrowth of axons between the donor segments and host tissues could be robust when the recipients were neonatal rats, and therefore more neural connections were observed (Iwashita et al., 1994). The lower thoracic transected rats with transplanted grafts could walk, run and climb with almost normal hind-forelimb coordination as the results of neural network reconstructing.

Another strategy for grafting is transplantation of autologous or allogeneic peripheral nerve (PN) segments as conduits for axons growing across the lesions. Further investigation found that the growth and regrowth of axons could be accelerated by growth factors through providing a favourable microenvironment (Tsai et al., 2005). PN grafts in combination with acidic fibroblast growth factor (aFGF) and chondroitinase ABC bridged complete thoracic spinal cord transections, as well as improved urinary function (Lee et al., 2013). A polylactic-co-glycolic acid microsphere-controlled release system showed that sustained delivery of three growth factors (brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and nerve growth factor (NGF)) at the injury site promoted regeneration of nerve fibres (Zhao et al., 2015). Our group has also carried out a series of studies using growth factors or antagonists combined with scaffolds to provide a supportive microenvironment for stem cell survival, migration and differentiation, which enhanced functional recovery (Chen et al., 2018; Li and Dai, 2018; Li et al., 2016b; Li et al., 2017b). For example, collagen scaffolds functionalized with three neurotropic factors (BDNF, NT-3 and bFGF) and two neutralizing proteins (EphA4LBD and PlexinB1LBD) promoted reconnection of injured neuronal circuits and locomotor functional recovery by providing a favourable microenvironment for neuroprotection and neuronal differentiation (Li et al., 2016b). Furthermore, administering vascular endothelial growth factor (VEGF) to the transection site of rats with SCI reduced the activation of microglial cells, facilitated neural survival, and improved functional recovery (des Rieux et al., 2014; Li et al., 2017a; Xu et al., 2017a).

Although most embryonic grafts survived in lesions of both neonatal and adult rat spinal cord and showed some positive effect on SCI recovery, the cell fate of grafts was still not clear. Moreover, in these previous studies, the donor neural tissue was all collected at E14. The role of donor segments harvested from spinal cords of adult rats on SCI repair has not been reported. In the present study, we hypothesized that transplantation of spinal cord tissue grafts from adult rats would improve locomotor recovery of adult rats with SCI. We also hypothesized that transplantation combined with neurotrophic growth factors would enhance cell survival, and consequently accelerate functional restoration. Using this strategy, donor spinal cord segments from green fluorescent protein (GFP) transgenic adult rats were transplanted into the completely injured spinal cord sites of adult rats (Figure 1). In addition, NT-3, BDNF, and VEGF were incorporated with the adult spinal cord tissue grafts (aSCGs) to boost their therapeutic effect. The number and type of surviving cells in the aSCGs, as well as locomotor function restoration were investigated. These studies represent the first application of adult spinal cord segments for repair of complete SCI in adult rats.

RESULTS

Characterization of GFP-positive donor cells in the aSCGs

We first examined the types of GFP-positive cells in the donor segments by immunofluorescence staining. Donor neurons were identified by double staining with GFP and the neuronal marker MAP2 (Li et al., 2016b). As shown in Figure 2A, some of the GFP-expressing cells in the aSCGs also expressed the mature neuron maker, MAP2. We also found that some GFP-positive donor cells were stained with astrocyte markers, such as ALDH1L1 and S100A4 (Figure 2B and C) (Sirko et al., 2015). Moreover, as shown in Figure 2D–F, a few donor cells were double stained with GFP and an oligodendrocyte precursor cell marker, NG2, or the mature oligodendrocyte markers, APC and MBP (Kucharova and Stallcup, 2010). RECA-1, an endothelial cell marker representing vessels (Palmer et al., 2000), was also observed in some of the GFP-positive donor cells (Figure 2G). These results indicated that the types of GFP-positive donor cells in the aSCGs included neurons, astrocytes, oligodendrocytes and endothelial cells.

Survival of the transplanted donor segments in spinal cord lesions

The spinal cord segments from adult GFP transgenic rats were implanted into the transection sites of the hosts immediately after SCI surgery, and the amount of cell survival was determined at 1, 4 and 12 weeks postinjury by histological analyses. The grafts could be easily distinguished from the recipient spinal cord by their GFP expression. No ob-

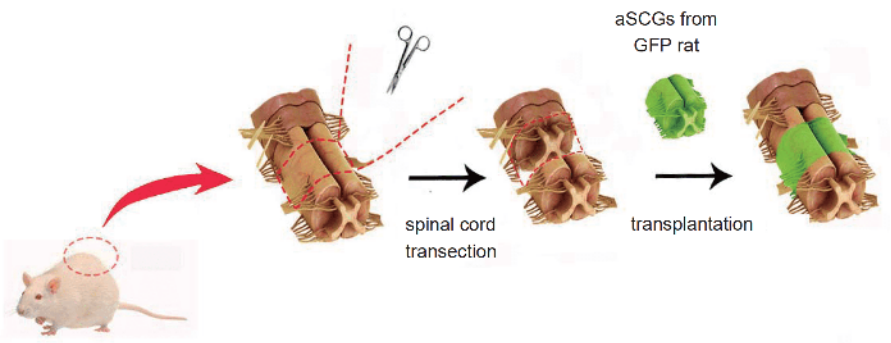

Figure 1 A schematic diagram illustrating transplantation of spinal cord tissue grafts from adult rats to a transected SCI model immediately after injury.

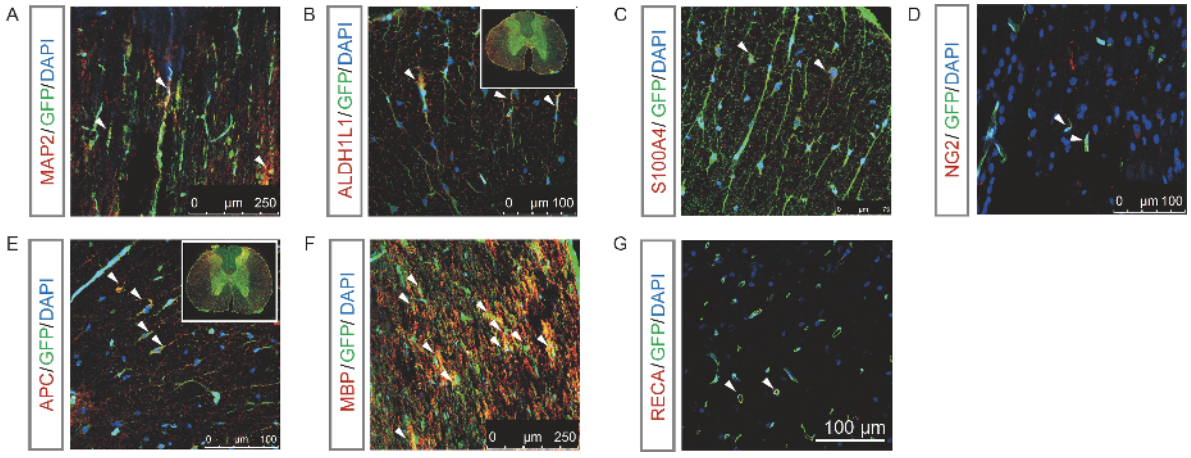

Figure 2 GFP labelled the spinal cord segments of the adult donors. Representative immunofluorescence images of double staining with GFP and (A) MAP2, (B) ALDH1L1, (C) S100A4, (D) NG2, (E) APC, (F) MBP, and (G) RECA in the aSCGs, respectively. The insets of GFP/ALDH1L1 and GFP/APC: lower magnification images.

vious differences were found in the morphology between the control group and the transplantation groups (T and T+G groups) (Figure S1 in Supporting Information), and all the donor tissues were fused with the hosts. As shown in Figure 3, after the 12-week treatment period, cells from the adult donor spinal cord tissues, as indicated by GFP fluorescence, were observed in the injury/graft sites. The size of the aSCGs and the number of cells in the aSCGs in both the T and T+G groups decreased with increasing treatment time. We further investigated the survival of neurons in the aSCGs. As shown in Figure 4, the number of β-III tubulin (Tuj-1) positive neurons in the T group was significantly higher than that in the control group at the injury sites.

With the presence of growth factors, more GFP-positive cells and larger donor segments remained in the lesions of the T+G group compared with the T group at the same time point following transplantation (Figure 3). As the growth factor cocktail provided a neuroprotective microenvironment for the grafts, neuron survival in the aSCGs in the T+G group was boosted; more β-III tubulin-positive cells were observed in the T+G group than in the T group at the same transplantation time period (Figure 4). These results suggested that the aSCGs survived in the adult recipients from 1 to 12 weeks post-transplantation, and a neuroprotective microenvironment increased the survival of the donor cells.

Surviving cell types in the engrafted aSCGs

As the above results demonstrated that the engrafted aSCGs with the addition of growth factors displayed the highest levels of cell survival, we further investigated the types of surviving cells in the engrafted aSCGs in the T+G group after 1, 4 and 12 weeks. As shown in Figure 5, all the double-stained cells in the aSCGs decreased with increasing post-transplantation time. A few GFP-positive neurons (<10%, stained with GFP/β-III tubulin, GFP/MAP2, and GFP/NF) and GFP-positive astrocytes (<10%, stained with GFP/GFAP, GFP/ALDH1L1, and GFP/S100A1) were observed. At one week after transplantation, more than half of the GFP-positive cells in the donor grafts were stained with oligodendrocyte markers (APC and NG2) and GFP-positive oligodendrocytes could still be found in the transplanted aSCGs at 12 weeks post-surgery. GFP/RECA double-positive cells could be detected at the first week but could hardly be found

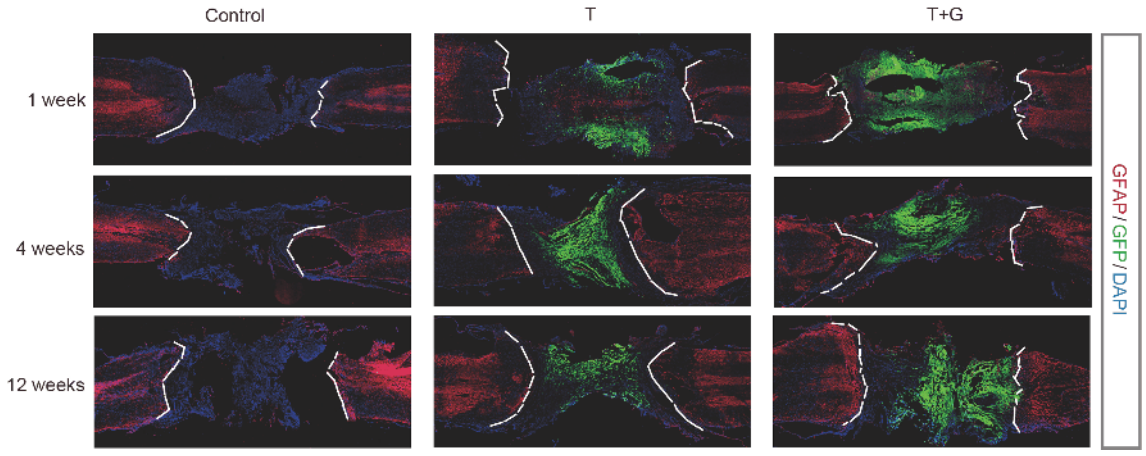

Figure 3 The survival of the transplanted aSCGs. Immunofluorescence images of the injured host spinal cord and transplanting aSCGs with or without addition of growth factors. GFP (green) represents the cells in donor segments, GFAP (red) represents the astrocytes in both donor and host tissues at 1, 4 and 12 weeks after surgery, respectively.

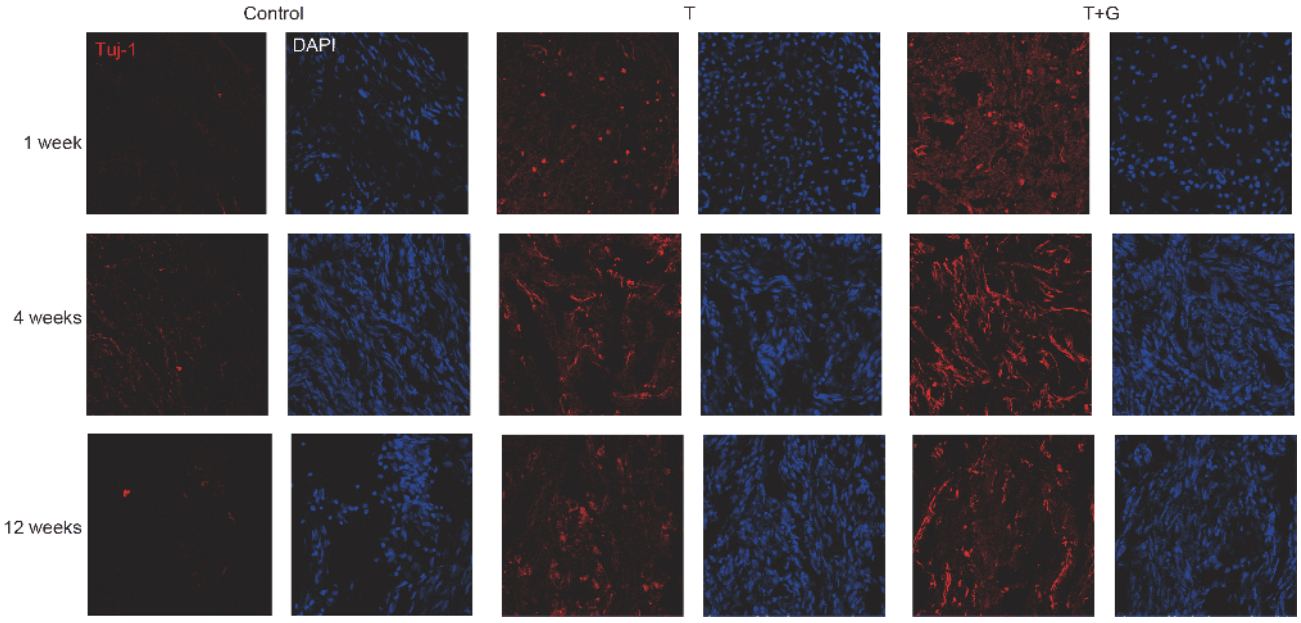

Figure 4 Immunofluorescence images of the lesion area in the control, T and T+G groups at 1, 4 and 12 weeks after transplantation, respectively. Tuj-1 (red) indicates the neurons at the lesion sites. Scale bar, 100 μm.

at 12 weeks post-implantation. After 12 weeks, most of the surviving cells in the aSCGs were oligodendrocytes, while only a few neurons and astrocytes from the donor existed in the lesion sites.

Transplantation of aSCGs improved electrophysiological and locomotor function recovery of rats with spinal cord transection

We evaluated whether transplantation of aSCGs promoted motor functional recovery after transection SCI by measurement of motor-evoked potentials (MEPs) and Basso-Beattie-Bresnahan (BBB) scoring, which assessed electrophysiological and hindlimb locomotor recovery, respectively. Rats with transplantation showed notable improvement of MEPs in both latency and amplitudes, in comparison with the control group with spinal cord transection alone (Figure 6A). Twelve weeks after aSCG transplantation, the latency and amplitude of MEPs in the transplants with growth factor supplementation (T+G group) were restored from ~6.8 and ~0.01, to ~4.3 and ~0.02, respectively, although the results were worse than the levels in normal healthy rats (Figure 6B and C). The shortened latent periods and enlarged amplitudes of both the T and the T+G group suggested enhanced electrophysiological recovery with aSCG transplantation. No significant differences were

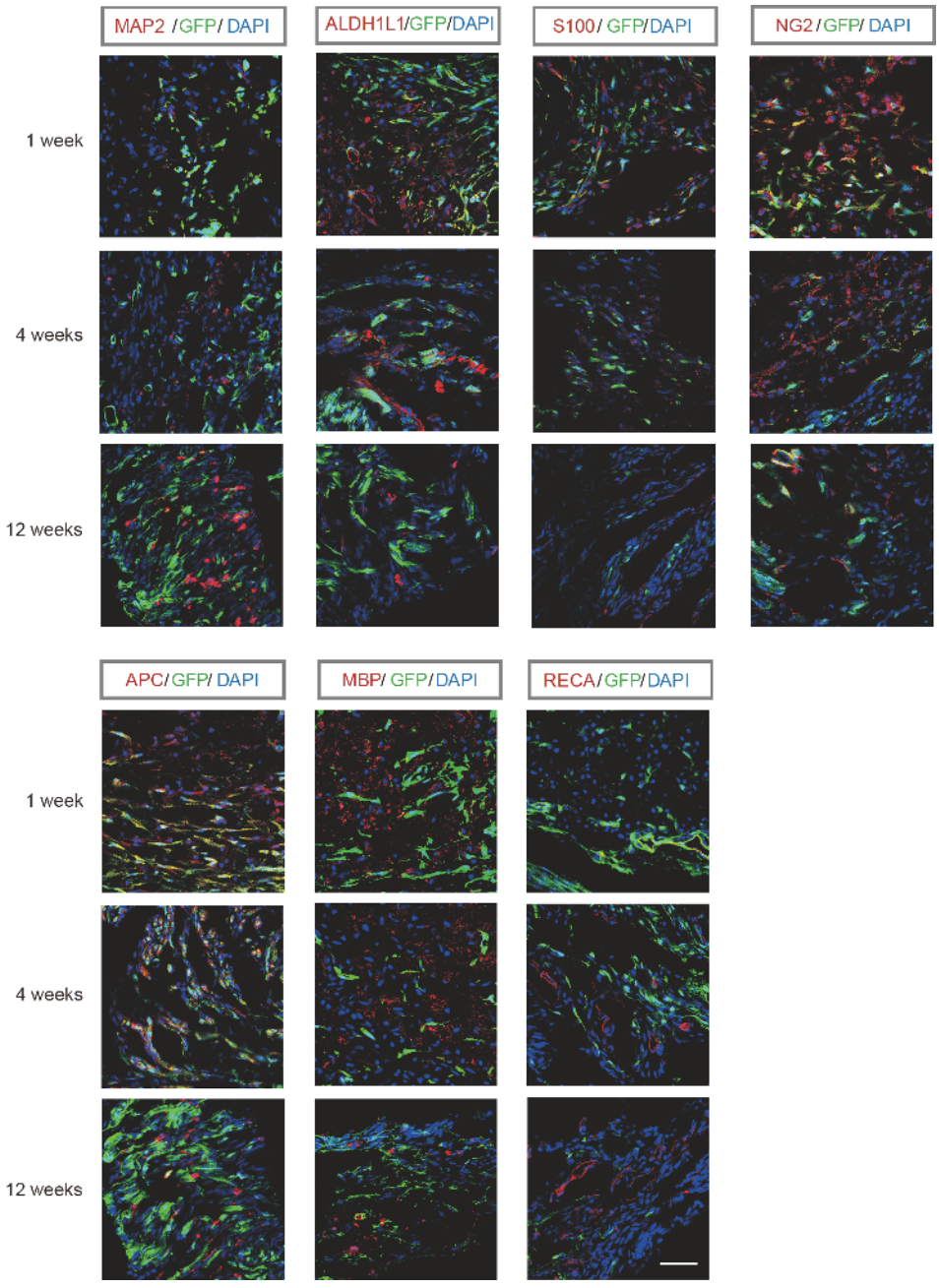

Figure 5 Surviving cell types in the engrafted aSCGs in the T+G group. Representative immunofluorescence images of double staining with MAP2/GFP, ALDH1L1/GFP, S100A4/GFP, NG2/GFP, APC/GFP, MBP/GFP, and RECA/GFP in the aSCGs at 1-, 4-, and 12-week post-treatment, respectively. Scale bar, 100 μm.

observed between the T group and the T+G group.

BBB scoring was used to further quantitatively estimate functional improvement. As shown in Figure 6D, during the 12 weeks post-treatment, the mean scores of rats with spinal cord transection alone (control group) were less than 6, meaning that they regained spontaneous movement of two joints. Six weeks after transplantation with growth factor supplementation, the mean scores were increased to approximately 9, representing plantar placement of the paws. At 12 weeks post-treatment, the mean scores of the T and T+G groups were approximately 8. The mean scores of the rats in T+G group improved rapidly in the first 6 weeks, and were then maintained at a high level over the following 6 weeks, while in the T group, the mean scores showed a sustained increase throughout the entire course of recovery, achieving the same level as the T+G group. These results suggested that transplantation of aSCGs restored the motor function of hindlimbs of rats with spinal cord transection, and that growth factor supplementation accelerated motor function recovery at the early phase after transplantation.

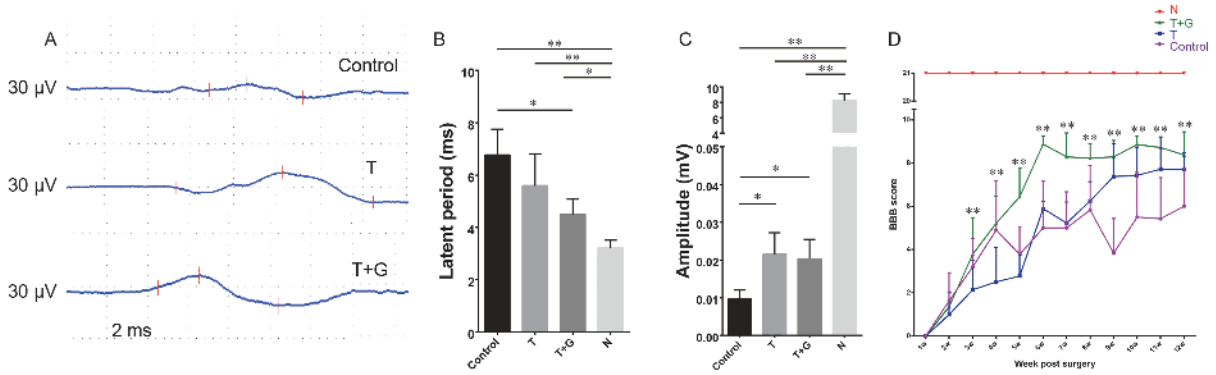

Figure 6 Transplantation of the aSCGs enhanced electrophysiological recovery and locomotor function improvement. A, MEP (motor evoked potential) recording of rats in SCI, T and T+G groups at x post-surgery, respectively. (B) Latent periods and (C) amplitude of MEP results of different groups. Values are mean±SD. *, $P<0.05$; **, $P<0.01$. D, Locomotor function test curves in BBB scoring system of normal, SCI, T and T+G groups from surgery to 12 weeks postinjury. Values are mean±SD. **, $P<0.01$ comparing T+G group with SCI (control) group.

DISCUSSION

Utilizing allogeneic foetal central neural tissues for newborn and adult SCI repair has made some progress in the research and clinical field over the past decades. Most studies have shown that donor grafts from embryonic central neural tissues replaced the injured spinal cord structures, and consequently suppressed glial scar formation, improved axon regrowth, and restored motor function in newborns. However, to the best of our knowledge, utilizing adult spinal cord tissue as donor segments has not been reported. In addition, there are still some concerns that need to be addressed before utilizing spinal cord tissue for SCI repair. In this study, we first applied allogeneic aSCGs for the treatment of transected spinal cord. Unlike foetal central neural grafts, aSCGs contain more mature neurons, astrocytes and oligodendrocytes, and fewer stem/progenitor cells, and thus would face more challenges than embryonic tissue grafts. In this study, we found that all the GFP-labelled donor spinal cord segments survived in the lesions for 12 weeks post-transplantation, similar to the survival of transplanted embryonic neural grafts (Reier et al., 1986).

In addition, foetal grafts produce neurotrophic factors, and thus might play a role in the reactivation of host neuron populations and in donor cell survival (Gaillard and Sauve, 1995). Therefore, to provide a suitable transplantation microenvironment after SCI, we supplied a growth factor cocktail containing BDNF, VEGF, and NT-3 to boost survival of donor cells and stimulate the host cells. Utilizing BDNF and NT-3 to promote axonal regrowth, neurite extension, neurogenesis, and consequently, functional recovery, has been systematically investigated in our laboratory (Han et al., 2014; Li et al., 2016a; Li et al., 2016b; Shi et al., 2014). Addition of VEGF to this growth factor cocktail also enhances cell survival, axonal outgrowth, neural stem cell activation, and functional outcome, and decreases secondary degeneration (Liu et al., 2019; Sondell et al., 1999; Widenfalk et al., 2003). In the current study, we found that neurons, astrocytes, and oligodendrocytes in the grafts survived during the 1–12 week post-transplantation period. Most of the surviving donor cells were oligodendrocytes, which might be contributed to by NT-3 and BDNF, which induce oligodendrocyte proliferation and myelination of regenerating axons in contused adult rat spinal cords (McTigue et al., 1998). Furthermore, VEGF is an inducer of angiogenesis and is also involved in endothelial cell proliferation, migration, and survival. Therefore, with the addition of VEGF, neovascularization could be enhanced and consequently, functional restoration accelerated (Wang et al., 2018; Xu et al., 2017b). However, the number of the endothelial cell in the transplants decreased in a transplantation time-dependent manner; few functional blood vessels in the aSCGs could be found after long-term transplantation, even in the presence of VEGF. The lack of a newly formed blood vessel network might be one of the obstacles for applying aSCGs in SCI repair.

The environment of the host spinal cord also affects the restorative capacities of grafts. Embryonic central neural tissues transplanted into the injured spinal cord of neonatal rats exhibited significant motor function recovery and axonal outgrowth, in comparison with those transplanted into adult rats (Iwashita et al., 1994; Reier et al., 1986). Host age influences both the density and the topology of foetal tissue graft afferents in the central nervous system (Gaillard and Sauve, 1995). The mature environment of the adult recipients not only influences the number of surviving neurons within a graft and the size of the graft itself, but also presents a poorly permissive environment for axonal regrowth (Collier et al., 1999; Hallas et al., 1980; Spencer et al., 2003). Based on these studies, we assumed that the transplantation environment of the adult recipients would influence the number of surviving cells in the aSCGs, and thus affect the

functional recovery capacity of aSCGs. In addition, in our study, a SCI transection model was used, which generates a severe environment for aSGCs survival and consequently might also suppress the restorative capacity of aSCGs.

When utilizing aSCGs for functional restoration after spinal cord transection, one of the concerns is whether the adult grafts can send afferents to adult host targets. Mature aSCGs might have less potential for axon regrowth and circuitry reconstruction than immature foetal tissues (Mobley and Greengard, 1985); however, cells in aSCGs might lack functional connections with the host tissue and form a boundary at the donor-recipient interface. Conversely, endogenous neural progenitor cell migration and neurogenesis in the lesion sites of adult hosts might contribute to generation of new functional connections. Our group and others have demonstrated that endogenous neurogenesis improves functional recovery after SCI, especially with a combination of growth factors (e.g. NT3 and BDNF) (Li et al., 2016b). To enhance functional recovery after transplanting aSCGs in lesion sites, efficient rebuilding of neuronal connections by both the donor neural cells and host endogenous neural progenitor cells is required. Thus, in future we may incorporate aSCGs with modified biomaterials for SCI treatment to improve the survival of the grafts, accelerate neovascularization, and promote formation of neural connections between the donor tissues and recipients.

In this study, we transplanted spinal cord pieces from adult rats to treat rats with spinal cord transection, and tested their effects on functional recovery. With the supplementation of growth factors, the number of surviving cells (neurons and oligodendrocytes) in the aSCGs, as well as graft size, increased, and hence resulted in enhancement of locomotor function restoration. These results support the potential utility of aSCGs for SCI repair.

MATERIALS AND METHODS

Animals

Seventy-two adult female athymic nude rats (T cell-deficient), weighing 200–300 g each, were used as recipients, and 48 adult female transgenic Sprague-Dawley rats expressing GFP ubiquitously, weighing 170–200 g, were used as donors. All the rats were purchased from Beijing Vital River Laboratory Animal Technology Co, Ltd (Beijing, China). Animal housing and experiments were performed in accordance with Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

Surgery procedures

For the rat T8 complete transection SCI model, the procedure was performed as we previously described (Han et al., 2018a; Han et al., 2018b; Li et al., 2017b), with slight modification. Briefly, the host rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1}). A midline incision was made on the back of a nude rat to expose the T8–T10 vertebrae. Laminectomy was performed at the T8–T9 vertebral level using a #11 surgical blade. A transection and removal of T8–T9 spinal cord was performed, and bleeding at the SCI site was controlled with gelatine sponges. All SCI rats were randomly divided into three groups. Group C: control group, without treatment after transection injury. Group T: aSCG transplantation group, with transplantation of allogeneic adult spinal cord tissues. A transection of T8–T9 spinal cord was first made in GFP-transgenic rats using the same procedure as described above. Then the T8–T9 spinal cord segments from GFP-transgenic rats were harvested and carefully placed into normal saline. After careful drying with a gelatine sponge, they were grafted into lesion sites in nude rats in a normal orientation. The grafted aSCGs united with the recipient spinal cord at both the rostral and caudal stumps, approximately forming a continuous spinal cord, and 40 μL of prefabricated collagen gel biomaterial (prepared as described in our previous work (Li et al., 2016b)) was gently and slowly injected into each host-graft interface using a microsyringe. Group T+G: aSCG transplantation combined with growth factors, whereby spinal cord segments from GFP transgenic rats were grafted into spinal cord lesion sites in nude rats, and then 40 μL of prefabricated collagen gel biomaterial mixed with NT-3 (1 μg), BDNF (1 μg), and VEGF (0.5 μg) was directly injected into each host-graft interface. The growth factors used in this study were prepared as in our previous manuscripts (Fan et al., 2010; Han et al., 2009; Zhang et al., 2009). The muscle and skin layers were then sutured.

Electrophysiology

After implantation for 12 weeks, rats were anesthetized by intraperitoneal administration of sodium pentobarbital, and cortical motor-evoked potentials (cMEPs) were measured using the Keypoint bichannel-evoked potential/electromyography system (9033A07, Dantec Company, Copenhagen, Denmark) as previously described (Chen et al., 2018). Briefly, two steel stimulating electrodes were inserted into the cranial surface of the motor cortex behind the anterior fontanel. A reference electrode and two recording electrodes were inserted into dorsal skin at a depth of 3 mm and into the contralateral gastrocnemius muscle at a depth of 2 mm. Then, cMEPs were recorded following a single stimulation at an intensity of 45 mA.

Behavioural assessment

The Basso-Beattie-Bresnahan (BBB) scale was applied to

estimate functional locomotion recovery in rats (Chen et al., 2018; Fan et al., 2018). Briefly, two independent observers unaware of experimental conditions recorded the BBB score for each rat at the different time points after surgery. During assessment, the rats moved freely in an open field and their hindlimb motion was observed.

Immunofluorescence staining

Spinal cord samples from T5 to T11 were harvested 12 weeks after surgery and fixed in 4% paraformaldehyde. The samples were embedded in Tissue-Tek® O.C.T, and then sectioned using a Leica CM1950 cryostat (Leica Camera Inc, Wetzlar, Germany). The sections were first blocked using serum/phosphate buffered saline (PBS; 1:20) for 1 h at room temperature (RT) and then incubated with primary antibodies overnight at 4°C. After rinsing three times with PBS, the sections were then incubated with secondary antibodies for 1 h at RT. Cell nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, USA). Primary antibodies used were goat anti-GFP (ab5450, Abcam, Cambridge, UK) 1:1,000; mouse anti-MAP2 (ab11267, Abcam) 1:500; rabbit anti-NG2 (ab129051, Abcam) 1:200; rabbit anti-GFAP (ab7260, Abcam) 1:500; mouse anti-endothelial cell antibody (RECA; ab9774, Abcam) 1:25; mouse anti-β-III tubulin (Tuj-1; 05-559, Merck KGaA, Darmstadt, Germany) 1:500; rabbit anti-APC (ab15270, Abcam) 1:100; rabbit anti-S100A4 (ab124805, Abcam) 1:100; mouse anti-myelin basic protein (MBP; SMI-99P, BioLegend, San Diego, USA), 1:500; and rabbit anti-ALDH1L1 (GTX131047, GeneTex, Irvine, USA) 1:500. Secondary antibodies, used at 1:500, were Alexa-Fluor® 488 and 594 donkey IgG (H+L) (Invitrogen, Carlsbad, USA). Images were acquired with a Leica TCS SP8 Confocal Microscope.

Statistical analysis

All data were expressed as mean±standard deviation. Statistical analyses were performed using ANOVA and $P<0.05$ was considered statistically significant.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Photo images of the transection injured spinal cords with or without aSCGs transplantation at 1-, 4- and 12-week post treatment, respectively.

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