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Allotransplantation of adult spinal cord tissues after complete transected spinal cord injury: long-term survival and functional recovery in canines

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Spinal cord injury (SCI), especially complete transected SCI, leads to loss of cells and extracellular matrix and functional impairments. In a previous study, we transplanted adult spinal cord tissues (aSCTs) to replace lost tissues and facilitate recovery in a rat SCI model. However, rodents display considerable differences from human patients in the scale, anatomy and functions of spinal cord systems, and responses after injury. Thus, use of a large animal SCI model is required to examine the repair efficiency of potential therapeutic approaches. In this study, we transplanted allogenic aSCTs from adult dogs to the lesion area of canines after complete transection of the thoracic spinal cord, and investigated the long-term cell survival and functional recovery. To enhance repair efficiency, a growth factor cocktail was added during aSCT transplantation, providing a favorable microenvironment. The results showed that transplantation of aSCTs, in particular with the addition of growth factors, significantly improves locomotor function restoration and increases the number of neurofilament-, microtubule-associated protein 2-, 5-hydroxytryptamine-, choline acetyltransferase- and tyrosine hydroxylase-positive neurons in the lesion area at 6 months post-surgery. In addition, we demonstrated that donor neurons in aSCTs can survive for a long period after transplantation. This study showed for the first time that transplanting aSCTs combined with growth factor supplementation facilitates reconstruction of injured spinal cords, and consequently promotes long lasting motor function recovery in a large animal complete transected SCI model, and therefore could be considered as a possible therapeutic strategy in humans.

complete spinal cord injury, allotransplantation, adult spinal cord tissues (aSCTs), adult mammalian, long-term survival, functional restoration

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INTRODUCTION

The spinal cord consists of a bundle of nerves that facilitates communication between the brain and the rest of the body. Damage of the spinal cord caused by trauma or disease leads to cell and/or tissue death and axonal loss and interrupts the connections below the injured area, and thus can result in loss of sensation and voluntary motor functions and even paralysis (Ramer et al., 2014). Spinal cord injury (SCI) brings incalculable personal and care costs to both the patient's family and society, and is one of the most challenging medical problems. Despite the fact that several repair strategies have been developed to replace lost neurons and restore function, SCI treatment options are still limited.

A landmark investigation by Nygren et al. reported using embryonic brain tissue pieces to treat SCI for the first time in 1977 (Nygren et al., 1977). At 0.5–4 months after homologous transplantation of embryonic brain tissue pieces to the injured adult spinal cord, immature noradrenaline (NA)-containing cells from the locus coeruleus and 5-hydroxytryptamine (5-HT)-containing cells from the raphe nuclei were found to have survived, and the NA and 5-HT axons grew in the lesioned white matter. Reier et al. transplanted fetal spinal cord tissues (fSCTs) into the injured spinal cords of adult hosts and observed survival and differentiation of the donor grafts, generation of functional connections between the donor and recipient tissues and improved functional restoration (Reier, 1985; Reier et al., 1986; Reier et al., 1988; Reier et al., 1983). Although transplantation of embryonic central neural tissues could be potentially applied to SCI repair, the age of donor tissues is critical. It has been demonstrated that fSCTs isolated at embryonic day 12 or 14–15 (E12 or E14–15) exhibited high levels of survival and neural differentiation capacity after placement into the SCI lesion sites. But cells of embryonic day 17 (E17) fSCTs could not be detected 10 months after transplantation into adult rats with motoneuronal depletion caused by a neurotoxic lesion (Gulino et al., 2010). The differences in the survival and differentiation capacities of fSCTs from donors of different ages were probably due to the different developmental stages of the grafted fSCTs: motor neurons are generated at about E11–E12 and mature at about E17 (Barber et al., 1991; Sheard et al., 1984). Thus, for nearly 40 years researchers have only used embryonic day <14 tissues for acute and chronic SCI repair, as most of the cells in these fSCTs are neural stem/progenitor cells, which are the main cell types that survive and differentiate after fSCT transplantation (Jakeman and Reier, 2015). Spinal cord segments from adult donors have not been reported as candidate grafts for SCI treatment in decades.

The adult spinal cord tissues (aSCTs) are composed of neurons, astrocytes, oligodendrocytes and endothelial cells, and the retained natural tissue architecture and microenvironment with extracellular matrix (ECM) can be considered as an alternative tool to replace both the neurons and glia and support axonal growth. Initial proof-of-concept for the idea of transplanting aSCTs for SCI repair was conducted and reported by our group recently (Shen et al., 2019). Although others have reported that E17 fSCTs containing mature neurons fail to treat SCI as none of the donor cells survive either short- or long-term transplantation (Gulino et al., 2010), we found that aSCTs survived and motor function was improved after transplantation into adult transected SCI rats. Despite the fact that most of the surviving donor cells in aSCTs were oligodendrocytes, a few donor neurons remained at the lesion site 3 months after treatment with aSCTs.

To enhance the therapeutic effects of aSCTs and fSCTs in SCI repair, we and other research groups have applied exogenous neurotrophic factors such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and nerve growth factor (NGF) to these grafts for increasing survival levels of spinal cord tissues and promoting axonal growth, and thus improving functional recovery (Bregman et al., 1997; Coumans et al., 2001; Lynskey et al., 2006; Shen et al., 2019). Briefly, we reported that the addition of a growth factor cocktail containing BDNF, NT-3 and vascular endothelial growth factor (VEGF) to the aSCTs markedly increased survival levels of the donor grafts and cells, and Basso-Beattie-Bresnahan scores of the SCI rats (Shen et al., 2019).

The previous aSCT transplantation study was performed in a rodent complete SCI model with a 3 months treatment period. However, the spinal cord systems of rodents exhibit considerable differences from those of primates in scale, axonal number, location, termination patterns and function, and inflammatory response and secondary damage after SCI (Nout et al., 2012). We pioneered the use of a complete spinal cord transected canine model, which has comparable clinical signs to human SCI patients, to bridge the gap between rodent models and human patients. The canine model is more suitable than rodent models for development of candidate SCI treatment strategies (Han et al., 2018a). Moreover, aSCT survival, especially long-term survival, is an important consideration as it is closely correlated with the outcomes of SCI repair. In the current work, we investigated the repair effects of aSCTs in a large animal complete transected SCI model with a long treatment time. The aSCTs were isolated from adult canines and then placed into the lesion area of adult canines with complete transected SCI (Figure 1). In addition, a growth factor cocktail was applied to the grafts to enhance survival levels by providing a favorable microenvironment. Moreover, to test the long-term survival of the neurons in aSCTs, we used green fluorescent protein (GFP)-transgenic adult rats as donors to track the donor cells after transplantation into the injured spinal cord sites of adult rats. The donor cells in aSCTs were found to

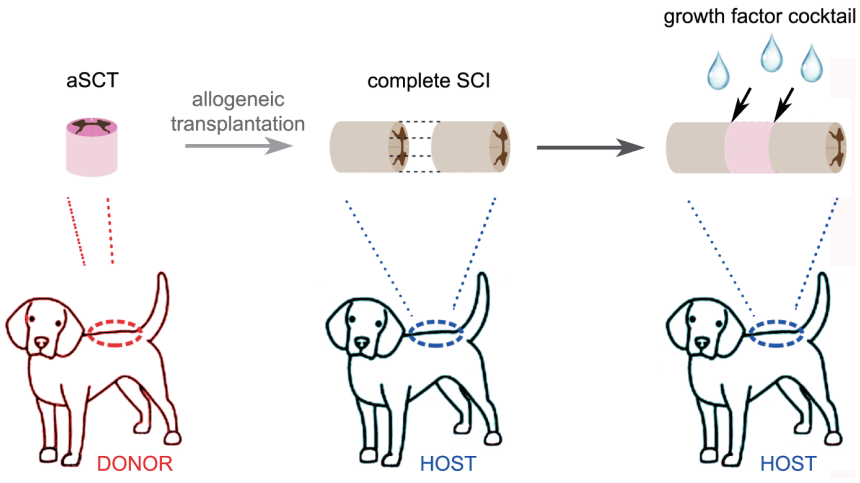

Figure 1 A diagram showing allogeneic transplantation of aSCTs from adult canines to the damaged thoracic spinal cord sites of adult canines with complete transected SCI with the addition of the growth factor cocktail.

survive for up to 6 months. We found that transplanting spinal cord tissues from adult dogs into the transected SCI site of adult dogs significantly improved the recovery of motor function and increased the number of neurons in the lesion area, particularly when combined with the addition of the growth factor cocktail supplement.

RESULTS

Because the spinal cord tissue segments contain multiple types of neural and non-neural cells and tissue architecture is maintained, aSCTs can be considered as a platform for both replacing the lost/damaged spinal cord and providing a three-dimensional structure for directing endogenous stem/progenitor cell migration and axon growth. In this study, we mainly focused on the long-term survival of aSCTs and functional improvement in different animal models. All dogs and rats survived the experimental procedures, and presented with no infections or pressure sores during the experimental period.

Surviving neurons in the transplanted aSCTs after transplantation

Previously, we have established the therapeutic potential of transplanting allogenic adult spinal cord tissue pieces for SCI repair, and investigated their survival levels in a rodent SCI model after a short period of time (Shen et al., 2019). To investigate the long-term survival behavior of aSCTs, we used GFP-transgenic rats as donors to trace donor neurons with GFP, and transplanted the GFP-labelled aSCTs from adult rats to the cavity of the damaged spinal cords of adult rats in the absence or presence of a growth factor cocktail. After treatment for 6 months, the samples were double stained with green fluorescent protein (GFP) and a neuronal marker, Tuj-1 (Shen et al., 2019). A double-stained GFP- and Tuj-1-positive neuron was observed in the visual field of the lesion site of a SCI rat following aSCT transplantation and growth factor cocktail supplementation (Figure 2A). This finding indicates that some of the neurons appearing at the damaged area may come from the aSCTs. In addition, GFP-positive cells of the aSCTs were detected in the transplantation (T) and transplantation+growth factor cocktail (T+G) groups, indicating the long-term survival of the transplanted adult spinal cord in the lesion gaps (Figure 2B). The addition of growth factors significantly enhanced the survival of the aSCTs.

Transplantation of aSCTs improved functional restoration in a transected SCI canine model

The Olby scoring system was used to evaluate the recovery of motor function in SCI dogs with or without transplantation or growth factor treatment. As shown in Figure 3, all dogs in the three experimental groups showed no limb movements and had Olby scores of 0 after surgery, while normal healthy dogs had scores of 14 (data not shown). The Olby scores of the injured dogs (control group) slightly increased and reached 4.50±0.96 at 6 months post-surgery, representing non-weight-bearing protraction of limbs, which indicates the limited self-repair capacity of injured spinal cords. Transplantation of aSCTs (T group) resulted in improved locomotor recovery of the SCI canines, with a score of 5.17±0.90 after treatment for 6 months. In comparison with the control group, aSCT transplantation showed benefit in functional restoration of SCI dogs, although >50% of the dogs still showed non-weight-bearing protraction of limbs. The dogs in the T+G group showed weight-bearing protraction of pelvic limbs and displayed Olby scores of 7.00±1.41, in-

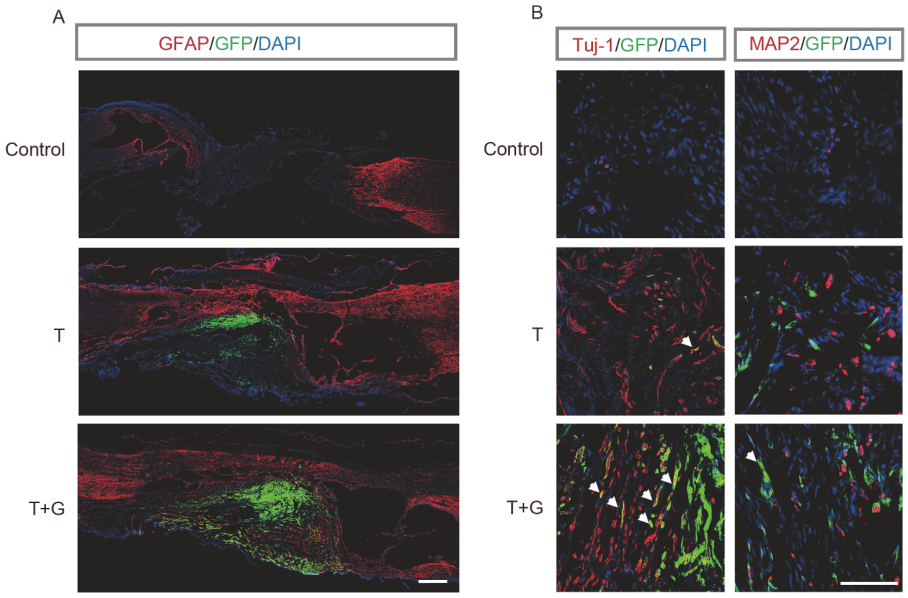

Figure 2 Survival of the transplants and neurons after transplanting aSCTs in SCI rat for 6 months. A, Immunofluorescence images of GFP (green) represent the cells in donor segments, GFAP (glial fibrillary acidic protein) (red) represent the astrocytes in both donor and host tissues (scale bar=500 μm). B, Double staining of neurons in the damaged areas with Tuj-1 (red)/GFP and MAP2 (red)/GFP, at 6 months after surgery in the control, T and T+G groups (scale bar=100 μm). Double stained cells are indicated by white arrows.

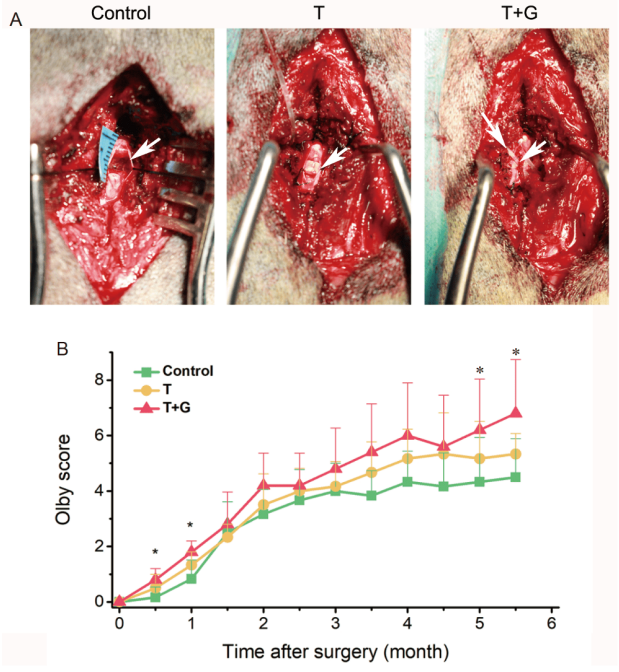

Figure 3 Surgery and functional recovery. A, Photographs of the procedure of transplanting aSCTs to the lesion sites of complete SCI canines, with or without the addition of the growth factor cocktail. Arrows are used to indicate the injured area in the Control group, transplanted aSCTs in the T group and transplanted aSCTs supplied with the growth factor cocktail in the T+G group, respectively. B, Olby scores of the dogs with SCI, aSCT transplantation after injury, and aSCT transplantation in combination with growth factors after transection, at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 months post-surgery. *, $P<0.05$.

dicating significant enhancement of functional recovery in the transected SCI canines.

Transplantation of aSCTs increased the numbers of neurons at the lesion sites

Based on the above Olby score results, we hypothesized that the improvement of weight-bearing protraction of the limbs in the T+G group may be due to the presence of neurons in the damaged area. Therefore, to assess the existence of neurons or axons in the lesion area, the spinal cord samples were harvested 6 months after transplantation of aSCTs into the SCI dogs and immunostained with neuronal markers including NF and MAP2 (Yin et al., 2018). No positive staining for NF, MAP2 or Tuj-1 was observed in the lesion site of the transected SCI dogs, indicating a lack of neurons and axons (Figure 4). After the transplantation of aSCTs, we could find small numbers of positive stained dendrites and axons. To increase the number of neurons in the damage zone, a growth factor cocktail was applied in situ during the treatment period. Because the addition of the growth factors provided a neurotrophic microenvironment, higher numbers of NF-, MAP2- and Tuj-1-positive cells or nerve fibers were observed in the lesion area in samples of the T+G group than in those in the T group.

We studied the distribution of different types of neurons in the lesion sites by staining with antibodies against 5-hydroxytryptamine (5-HT), tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) (Li et al., 2017). As shown in Figure 5A, more positive stained 5-HT-, TH- and ChAT-expressing neurons were observed in the lesions of the dogs in the T+G group than in the control and T groups, which is consistent with the above immunofluorescence results. The

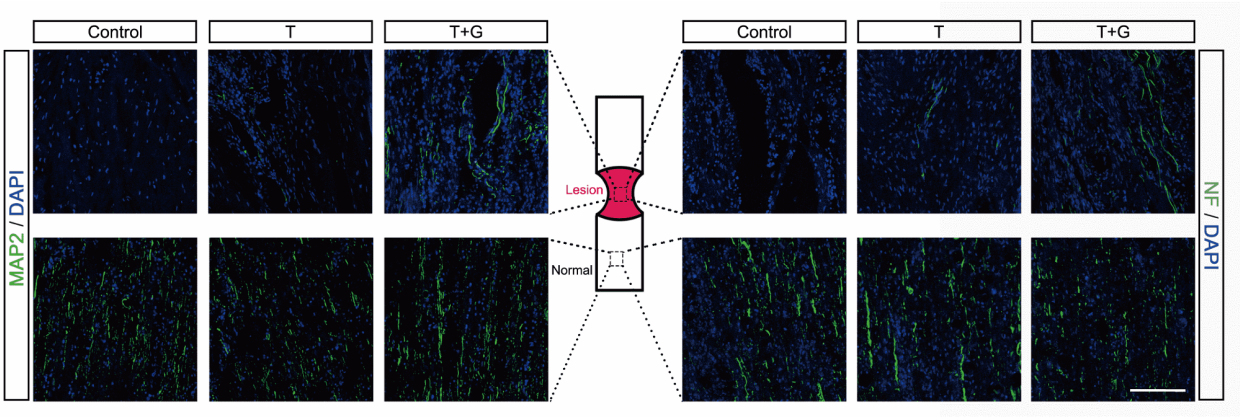

Figure 4 Immunofluorescence images of NF and MAP2 in the SCI lesion sites and host normal uninjured area of the control, T and T+G groups. Scale bar=100 μm.

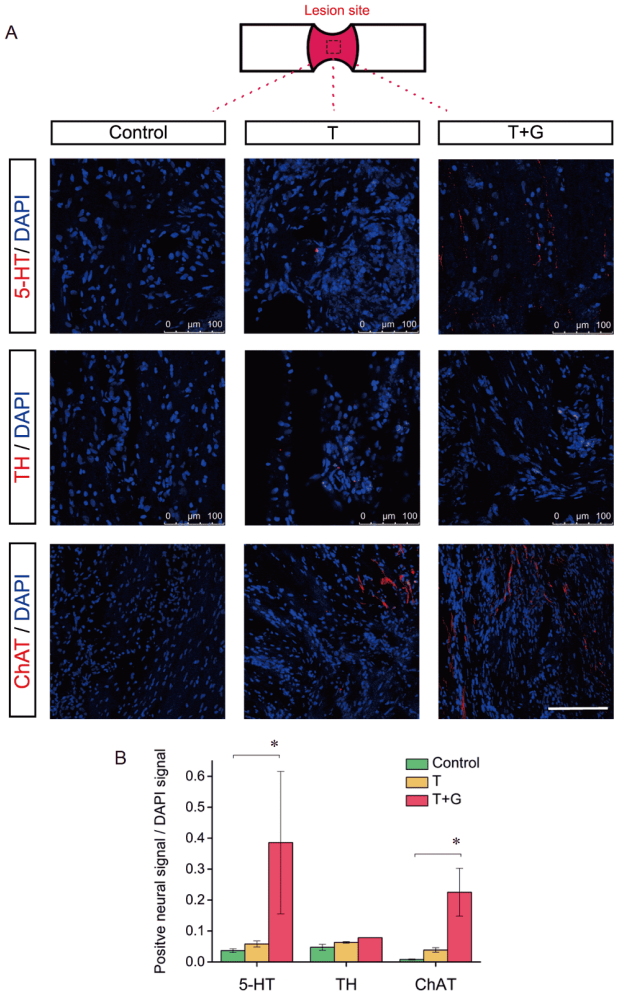

Figure 5 Different types of neurons in the lesion sites. (A) Immunofluorescence images and (B) semi-quantification of 5-HT-, TH- and ChAT-positive (red) neurons in the SCI lesion sites of the control, T and T+G groups. Scale bar=100 μm. *, P<0.05.

immunofluorescence densities of 5-HT-, TH- and ChAT-positive neurons (normalized to DAPI density) in the T+G group were significantly higher than those in the control group (Figure 5B). These findings suggested that transplanting aSCTs provided exogenous neurons in the damaged cavities, and the addition of growth factors to the implanted tissue pieces increased the numbers of the existing neurons.

DISCUSSION

In our previous study of transplanting aSCTs for SCI repair, the positive effects of the growth factor cocktail on donor and host cell survival, neural differentiation and functional outcomes, and the influences of donor and recipient age on axonal growth and regrowth into the grafts and hosts were fully discussed (Shen et al., 2019). Although utilizing a rat SCI model can replicate the majority of responses and mechanisms of human SCIs, it may not be the most appropriate model for developing and investigating the SCI repair strategies for potential clinical application, as there are significant neuroanatomical and functional differences between rodents and primates. Thus, a complete SCI large animal model might be more suitable than rodents for the study of SCI repair.

Our laboratory has conducted a series of independent investigations of using tissue engineering strategies to treat canines with completely transected SCI, and had some great successes (Han et al., 2018a; Han et al., 2014; Han et al., 2015; Han et al., 2018b; Li et al., 2017; Yin et al., 2018). It has been demonstrated that implanting the NeuroRegen scaffold (a linear-ordered collagen scaffold) into the lesion area after the complete removal of 5 mm spinal cord tissue restored the Olby scores from 4.5 (control group: SCI dogs) to 5.5 after 4 months (Li et al., 2017). NeuroRegen scaffolds modified with drugs or growth factors promoted neurogenesis, and slightly increased the outcomes of Olby scores (~6.5 at 4 months (Li et al., 2017) and 6 months (Yin et al., 2018), and less than 7.0 at 9.5 months (Han et al., 2015)). In addition, implanting a NeuroRegen scaffold loaded with

human placenta-derived mesenchymal stem cells increased the scores from 5.0 (control group) to ~7.0 after treating the SCI canines for 9 months (Han et al., 2018b). Another group has also reported that implantation of a gelatin sponge scaffold loaded with neurotrophin-3-overexpressing Schwann cells and TrkC-overexpressing mesenchymal stem cells to complete SCI dogs increased their Olby scores to 7.6 at 6.5 months post-surgery (Wu et al., 2018). Based on these discoveries, we speculate that the aSCTs may have acted as a "scaffold" filled with mature neural cells and neural networks, which therefore improved locomotor recovery, resulting in a score of 7.0 at 6 months (T+G group).

A possible mechanism of functional recovery through aSCT transplantation may be via the reconstruction of neuronal circuits in the damaged gap and restoration of connections at the broken ends of the spinal cord. Transplanting fetal CNS tissues was the first attempt to build a neuronal relay for SCI repair (Reier et al., 1986). The fetal CNS tissues may extend axons to the host spinal cords, while the axons of the adult host can grow into these transplanted CNS tissues (Bonner and Steward, 2015). The axonal growth and regrowth form new neural connections, resulting in functional recovery. In this study, the aSCTs probably served as a neuronal relay by providing neurons to replace the lost cells and acting as a substrate to support axon growth (Bonner and Steward, 2015; Li et al., 2017; Lin, 2019).

We speculate that the observed NF-, MAP2-, Tuj-1-, ChAT-, 5-HT- and TH-positive neurons in the damaged area of the complete SCI canines with aSCT transplantation may be attributed to two sources: one is the surviving neurons of the aSCTs, the other is the neurogenic differentiated endogenous stem/progenitor cells. The neurons were present in aSCTs 6 months after transplantation, and may contribute to the formation of a neural relay in the transected injury site. Some of the neurons without GFP co-staining appearing in the visual field of the SCI damage area may come from the adult hosts. SCI induces activation, migration and differentiation of endogenous neural stem/progenitor cells (NSPCs) (Meletis et al., 2008; Mothe and Tator, 2005). Thus, some non-GFP-expressing neurons/axons might indicate the growth of host axons or be generated from the host NSPCs.

Transplanted E14 central neural tissues become well integrated with the host and well vascularized (Horner et al., 1996; Reier et al., 1986). In the process of aSCT transplantation, blood vessel formation would be a critical factor for possibly enhancing integration and increasing repair efficiency, as it is required to provide a nutrient- and oxygen-rich microenvironment for the grafts (Pellegata et al., 2018). It is impossible to connect blood vessels in the aSCTs to those in the adult hosts, therefore, stimulating vascularization is of fundamental importance for increasing the survival level of the grafts. To encourage angiogenesis we added VEGF, which can induce proliferation and migration of vascular endothelial cells, to the aSCTs. In a previous study, we discovered that although the number of endothelial cells from the donor grafts was lower at 3 months after transplantation, the blood vessels were detectable with RECA (an endothelial cell marker) positive staining in the lesion sites (Shen et al., 2019). Therefore, we speculate that some of the RECA-positive staining endothelial cells might be from the hosts. Vascularization will be the key challenge in aSCT transplantation, and strategies of either stimulating vascularization of the grafts or enhancing angiogenesis from existing vascular networks should be explored in future spinal cord tissue transplantation studies.

In this study, we transplanted aSCTs to the damaged cavity of the canine complete spinal cord transection model, and measured their long-term effects on SCI repair. We found that aSCT transplantation promoted the functional restoration of the complete transected SCI canines, and the therapeutic efficiency of the aSCTs was significantly improved by adding a growth factor cocktail with exhibiting higher number of neurons at the site of injury. We also reported that the neurons in the aSCTs survived for 6 months. Taken together, the current study explored the feasibility of using aSCTs for SCI treatment. We believe that with further development of spinal cord tissue protection technology, aSCT transplantation may be applied in the clinic.

MATERIAL AND METHODS

Animals

Twelve adult female Beagle canines, designated as recipients, and eighteen adult male Beagle canines, designated as donors, were purchased from Animal Department of Xiangya Medical University (Changsha), and were housed in temperature- and humidity-controlled animal quarters for at least 7 d before surgery. Adult female athymic nude rats (T cell-deficient; 200–300 g), and adult male transgenic Sprague-Dawley rats expressing green fluorescent protein (GFP; 170–200 g), were purchased from Beijing Vital River Laboratory Animal Technology Co, Ltd (Beijing). Animal housing and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH Publications No. 8023, revised 1978).

Surgery procedures

After 8–10 h of preoperative fasting and water deprivation, the canines were anesthetized by subcutaneous administration of xylazine hydrochloride (8 mg kg^{-1}) and ketamine (2.5 mg kg^{-1}). The hair on the back of the canine was shaved and the skin was cleaned with povidone-iodine. All surgeries were performed under sterile conditions. Physiological sal-

ine was used to compensate for body fluid loss during the operation and anesthesia was maintained by propofol. An 8 cm long midline incision was made on the back of the female canines to expose the T8–T10 vertebrae. Laminectomy was performed at the T9 vertebral level. A transection and removal of a 5 mm long section of T9 spinal cord was performed and the spinal meninges were reserved. The bleeding at the SCI site was controlled with gelatin sponge. The female canines were randomly divided into three groups: 1) Control group (n=7), 5 mm long transected SCI without treatment. 2) T group (n=9), a 5 mm long transection of the T9 spinal cord was performed in the male dogs using the procedures described above. Then the 5 mm long T9 spinal cord segments of male canines were removed and carefully put into normal saline. After being dried with gelatin sponge, the donor aSCTs of the male canines were transplanted into the lesion sites of the female canines in the correct rostral and caudal orientation. 3) T+G group (n=9), the same aSCT transplantation procedure was conducted, followed by grafting of Alzet® osmotic minipumps (model 2ML4; DURECT Corporation, USA) filled with the growth factor cocktail (Fan et al., 2018; Shen et al., 2019; Yin et al., 2018) (containing neurotrophin-3 (NT3, 15 μg), brain-derived neurotrophic factor (BDNF, 15 μg), vascular endothelial growth factor (VEGF, 3 μg), basic fibroblast growth factor (bFGF, 7.5 μg) and Taxol (7.7 μg, liposome form, Nanjing Luye Sike Pharmaceutical Co., Ltd) in rostral and caudal positions, and fixed to the dorsal muscle nearest the lesion site of the female canines. Then two catheters connecting the minipumps were carefully put into the rostral and caudal donor-host interfaces. The minipumps continuously delivered the growth factor cocktail at a pumping rate of 2.5 μL h^{-1}. At 2 weeks post-surgery, the initial minipumps filled with the growth factor cocktail were replaced with new ones, which were removed after a further 2 weeks. Previous studies have demonstrated that Taxol administration promoted locomotion recovery, enhanced axonal regeneration, increased neurogenesis and reduced scar formation in both rodent and canine SCI models (Hellal et al., 2011; Fan et al., 2018; Yin et al., 2018). In this study, we aim to further enhance the positive therapeutic effects by adding Taxol into the growth factor cocktail during aSCT transplantation. After surgery, the dissected spinal meninges, muscle and skin layers of the host SCI canines were sutured carefully. All the canines were treated with cyclosporine A (12 mg kg^{-1}) and methylprednisolone (4 mg kg^{-1}) daily during the whole experimental time period. The surgery procedure of the complete transected SCI rat model was conducted as described in our previous study (Shen et al., 2019).

Behavioral assessment

The Olby scoring system was used to quantitatively estimate the functional outcomes after transplanting aSCTs into the lesion area of spinal cord-transected dogs (Olby et al., 2004). Each dog was put in an open field and allowed to walk freely and was independently scored for spontaneous or voluntary hindlimb movements over 6 months by two individuals who were unaware of the experimental conditions. The results were expressed as mean±standard deviation. Statistical analyses were performed using t-tests and P<0.05 was considered statistically significant (compared with the control group).

Histological analysis

At 6 months after surgery, the animals were sacrificed and their spinal cords from T5–T11 were harvested and fixed with 4% (v/v) formaldehyde for 48 h then 25% sucrose for 48 h. The spinal cord samples were embedded in Tissue-Tek® O.C.T. (Sakura Finetek USA, Inc., USA), and then frozen sectioned using a Leica CM1950 cryostat (Leica Camera Inc., Germany). The spinal cord sections were blocked with phosphate-buffered saline (PBS) containing 5% serum, incubated with primary antibodies overnight at 4° C, followed by rinsing three times with PBS, and then incubated with secondary antibodies. Cell nuclei were stained with DAPI (VECTASHIELD® antifade mounting medium with DAPI; VECTOR Laboratories; USA). The primary and secondary antibodies used in this study were: mouse anti-microtubule-associated protein 2 (MAP2; ab11267, Abcam, UK) 1:500; rabbit anti-neurofilament (NF; ab8135, Abcam); rabbit anti-5-hydroxytryptamine (5-HT; 20080, Immunostar, USA) 1:500, rabbit anti-choline acetyltransferase (ChAT; ab6168, Abcam) 1:500; rabbit anti-tyrosine hydroxylase (TH; ab112, Abcam) 1:500; goat anti-GFP (ab5450, Abcam) 1:1000; chicken anti-GFP (ab13970, Abcam) 1:500; rabbit anti-GFAP (ab7260, Abcam) 1:500; mouse anti-beta-III tubulin (Tuj-1; 05-559, Merck KGaA, Germany) and Invitrogen AlexaFluor® 488 and 594 donkey IgG (H+L) (Thermo Fisher Scientific, USA) 1:500. Images were acquired with a Leica TCS SP8 confocal microscope.

Compliance and ethics *The author(s) declare that they have no conflict of interest. Animals were used with the approval of Animal Care and Use Committee of Institute of Genetics and Developmental Biology, the Chinese Academy of Sciences and Xiangya Hospital. Animal experiments were conducted in accordance with Guide for the Care and Use of Laboratory Animals from National Institutes of Health.*

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