REGULATION OF NOTCH SIGNALING PATHWAY ON PROLIFERATION AND DIFFERENTIATION OF RAT'S NEONATAL BRAIN-DERIVED NEURAL STEM CELLS

By

ZHOU QINGZHONG

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Chairman : Associate Professor Wan Aliaa binti Wan Sulaiman, FRCP
Faculty : Medicine and Health Sciences

Spinal cord injury (SCI) is an important and disabling disease globally. It has been reported that regulation of Notch signaling can affect the proliferation and differentiation of neural stem cells (NSCs). However, when γ-secretase inhibitors and paralyzed spinal cord extracts regulate Notch signaling to better promote neuronal differentiation of NSCs, although its mechanisms remain unclear. The aim of this study were to establish a simplified and efficient method for culture and identification of neonatal rat brain-derived NSCs and to investigate the effects of spinal cord extracts (SCEs) from paralyzed rats at different time points on the proliferation and differentiation of neonatal rat brain NSCs.

First, primary rat brain NSCs were isolated and cultured. BrdU incorporation method was used to detect self-renew and proliferation capabilities of cells. Different NSCs specific antibodies (anti-nestin, NF200, NSE and GFAP antibodies) were used to identify NSCs specific surface markers and muti-differentiation capabilities by

immunofluorescence staining. A rat SCI model was constructed. HE staining was applied to measure the injury changes of spinal cord tissue in SCI rats. The extracts of rat spinal cords at 8, 24, 72h, 5, 7, 14 days were prepared and then co-treated with NSCs. The number of NSCs was measured by using MTT colorimetry to reflect the effect of different paralyzed SCEs on NSCs proliferative capacity. The total RNA of NSCs was respectively extracted to check the mRNA levels of Notch1 and Hes1 genes using RT-PCR. Finally, NSCs were cultured with different spinal cord extracts and DAPT and the total RNA of NSCs was respectively extracted to check the mRNA levels of Notch1 and Hes1 using RT-PCR. Brain derived cells from newborn rats (2 to 3 days) proliferate and aggregate into spherical-shaped clusters with sustained continuous and stable passaging. When BrdU was incorporated into the 5^{th} generation of passaged cells, positive BrdU cells and nestin cells were observed by immunofluorescence staining. After induction of dissociation using 5% fetal bovine serum, positive NF200, NSE and GFAP cells were observed by immunofluorescence staining. The extract of paralyzed spinal cord could upregulated Notch1 and Hes1 mRNA levels in NSCs to encourage NSCs proliferation, which can mimic the microenvironment after SCI. Among them, the SCE at 7 days had the strongest effect on promoting the proliferation of rat brain NSCs and the mRNA of Notch1 and Hes1, which increased with the raise of co-culture time. DAPT can down-regulate the expression level of NSCs Notch1 and Hes1 mRNA and inhibit the proliferation of NSCs, and blocking Notch signaling at 48-72 hours can better promote the differentiation of NSCs into neurons, with the highest ratio of differentiated neurons and more residual NSCs. This is a simplified and efficient method for neonatal rat brain-derived neural stem cell culture and identification, and it is speculated that

regulating the Notch/ Hes1 signal at 7days after SCI may be the best time to encourage the proliferation of endogenous NSCs.

Keywords: neural stem cells, rat spinal cord injury, spinal cord extracts, Notch, *Hes1*

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGAWALAN LALUAN INSYARAT NOTCH PADA PROLIFERASI DAN PEMBEZAAN SEL STEM SARAF DIPEROLEHI DARIPADA OTAK TIKUS NEONATAL

Oleh

ZHOU QINGZHONG

November 2023

Pengerusi : Profesor Madya Wan Aliaa binti Wan Sulaiman, FRCP
Fakulti : Perubatan dan Sains Kesihatan

Kecederaan saraf tunjang (KST) masih merupakan masalah perubatan global. Terdapat laporan bahawa peraturan isyarat Notch boleh menjejaskan percambahan dan pembezaan sel stem neural (SSN). Walau bagaimanapun, apabila perencat γ-secretase dan ekstrak saraf tunjang lumpuh mengawal isyarat Notch untuk menggalakkan pembezaan neuron sel stem neural dengan lebih baik, tetapi mekanismanya masih tidak jelas. Matlamat kajian ini adalah untuk mewujudkan kaedah yang mudah dan cekap untuk kultur dan pengenalpastian SSN yang diperolehi daripada otak tikus neonatal serta untuk menyiasat kesan ekstrak saraf tunjang (EST) daripada tikus lumpuh pada titik masa yang berbeza mengenai percambahan dan pembezaan SSN otak tikus neonatal. Pertama, sel stem saraf otak tikus primer SSN telah diasingkan dan dikulturkan. Kaedah penggabungan BRDU digunakan untuk mengesan keupayaan pembaharuan diri dan percambahan sel. Antibodi khusus SSN yang berbeza (antibodi anti-nestin, NF200, NSE dan GFAP) digunakan untuk

mengenal pasti penanda permukaan khusus NTCs dan keupayaan pembezaan muti dengan pewarnaan *imunofluorescence*. Kemudian, model kecederaan saraf tunjang tikus telah dihasilkan. Pewarnaan HE digunakan untuk mengukur perubahan kecederaan tisu saraf tunjang pada tikus lumpuh saraf tunjang. Seterusnya, ekstrak saraf tunjang tikus pada 8, 24, 72h, 5, 7, 14 hari telah disediakan dan kemudian dirawat bersama SSN. Bilangan SSN diukur dengan menggunakan colorimetri MTT untuk mencerminkan kesan EST lumpuh yang berbeza pada kapasiti percambahan SSN. Kemudian, jumlah RNA SSN masing-masing diekstrak untuk memeriksa tahap mRNA Notch1 dan Hes1 menggunakan RT-PCR. Akhirnya, sel stem neural dikulturkan dengan ekstrak saraf tunjang yang berbeza dan DAPT dan jumlah RNA SSN masing-masing diekstrak untuk memeriksa tahap mRNA Notch1 dan Hes1 menggunakan RT-PCR. Otak memperoleh sel-sel dari tikus yang baru lahir (2 hari hingga 3 hari) berkembang dan agregat ke dalam kelompok berbentuk sfera dengan lulus berterusan dan stabil. Apabila BrdU dimasukkan ke dalam generasi ke-5 sel-sel yang passaged, sel-sel BrdU positif dan sel-sel nestin diperhatikan oleh pewarnaan imunofluorescence. Selepas induksi penceraian menggunakan serum lembu janin 5%, sel NF200 positif, NSE dan GFAP diperhatikan oleh pewarnaan imunofluorescence. Ekstrak saraf tunjang lumpuh boleh meningkatkan tahap Notch1 dan Hes1 mRNA dalam NSCs untuk menggalakkan percambahan NSC, yang boleh meniru persekitaran mikro selepas SCI. Antaranya, SCE pada 7 hari mempunyai kesan terkuat dalam menggalakkan percambahan NSCs otak tikus dan mRNA Notch1 dan Hes1, yang meningkat dengan peningkatan masa budaya bersama. DAPT boleh mengawal tahap ekspresi NSC Notch1 dan Hes1 mRNA dan menghalang percambahan NSCs, dan menyekat isyarat Notch pada 48-72 jam dengan lebih baik boleh menggalakkan pembezaan NSC ke dalam neuron, dengan nisbah tertinggi neuron yang dibezakan dan

sel stem saraf yang lebih sisa. Ini adalah kaedah yang mudah dan cekap untuk kultur dan pengenalan sel stem saraf yang berasal dari otak tikus neonatal, dan spekulasi bahawa menyekat isyarat Notch / Hes1 pada 7 hari selepas kecederaan saraf tunjang mungkin masa terbaik untuk menggalakkan percambahan SSN secara dalaman.

Kata Kunci: sel stem neural, kecederaan saraf tunjang tikus, ekstrak saraf tunjang, Notch1, Hes1

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Wan Aliaa binti Wan Sulaiman, MBBCh BAO, MRES MRCP
Associate Professor (Medical)
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Nurul Huda binti Mohd Nor, BChBAO PhD
Senior Medical Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Mohd Hezery bin Harun, MD, Doc Ortho & Trauma, CMJA
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Feng Daxiong, PhD
Professor
Department of Orthopaedics
University
Southwest Medical University
(Member)

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 July 2024

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LIST OF ABBREVIATIONS

SCI	Spinal cord injury
NSCs/ENSCs	NSCs/endogenous NSCs
DAPT	N-[N-(3, 5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (γ-secretase inhibitor)
SVZ	subventricular region
SGZ	subgranular region
bHLH	helical loop-helix transcription factor
CSL	intracellular signaling molecule
EGF	epidermal growth factor
PS1/2	presenilin ½
NICD	Notch intra-cellular domain
ANK	ankyrin repeats protein
MAML-1	mastermind-like proteins-1
BrdU	5 '-bromine uracil
GFAP	glial acidic fibrin protein
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
IGF-1	insulin-like growth factor 1
TNF-α	tumor necrosis factor-α
il-1 β	interleukin-1 β
CNS	central nervous system
PDGF-R-α	platelet-derived growth factor receptor-α
BDNF	brain-derived neurotrophic factor
NGF	nerve growth factor
NT-3	neurotrophic factor 3

BMSCS	bone marrow mesenchymal stem cells
BBB	The Basso, Beattie and Bresnahan locomotor rating scale
SSEP	somatosensory evoked potential
DAPI	4′,6-diamidino-2-phenylindole
PBS	alkaline phosphate buffer without Ca and Mg
SFM	Serum-free neural stem cell culture medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
NF200	Neurofilin-200
NSE	neuron-specific enolase
HE	hematoxylin-eosin
RT-PCR	Reverse transcription-polymerase linked reaction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MCA	Melting curve analysis
SCE	Spinal cord extract

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Trauma has become the major cause of disability and death all over the world and shows a rising tendency in Asia (Joshipura et al., 2004). Spinal cord injury (SCI) is a common injury, mainly caused by traffic accident injury, violent injuries and work-related injuries (Hagen et al., 2012). It has a high incidence; high disability rate and high cost rate worldwide (Bárbara-Bataller et al., 2018). Moreover, its incidence is mainly concentrated in young and middle-aged patients under 30 years old, which brings a heavy burden to society, patients and their families (Hurbea et al., 2006; Chan et al., 2018; Geng et al., 2021). Lambrechts et al., 2021 reported that spinal cord injury with motor impairment is devastating, with each incident costing millions of dollars in medical and rehabilitation costs. According to relevant survey, the lifetime rehabilitation and treatment cost of SCI patients is more than 750,000 US dollars on average, and the annual cost of SCI patients in the United States is more than 6 billion US dollars (Tator, 2002). So far, the prevention, treatment and rehabilitation of SCI is still one of the global medical problems, so scholars at home and abroad are still making unremitting efforts to explore it.

Endogenous NSCs have unique advantages in the treatment of spinal cord injury. At present, the research of endogenous NSCs has also made breakthrough progress that endogenous NSCs can differentiate into more than 200 cell types in the body. How to regulate its differentiation direction in vivo, especially how to make it differentiate in the desired direction and how to avoid tumor-like differentiation after spinal cord

injury, is the direction of future efforts. On the other hand, all the current results have been obtained from animal experiments, while rodents have a strong ability to repair themselves after spinal cord injury.

Despite of a few limitations, the use of endogenous NSCs in the treatment of spinal cord injury is still a good approach (Hosseini et al., 2024). As long as the signal pathway of neuronal differentiation and its influencing factors are further studied, the application of endogenous NSCs in the treatment of spinal cord injury will be successful.

Many researchers have confirmed that NSCs (NSCs) are widely distributed in the spinal cord and other central nervous systems (CNS) of adult mammals. Reynods and Weiss, 1992 got NSCs NSCsfrom the striatum of adult mice aged 3 to 18 months. Later,Gage (2000), Suh (2007), and Weiss et al. (1996) confirmed the existence of NSCs in the lateral ventricular wall of the ventricular system, including epenymal and subventricular region (SVZ), hippocampal dentate gyrus subgranular region (SGZ), the ventricular zone of the third and fourth ventricle, and the thoracic and lumbosacral segments of the spinal cord. In the spinal cord, NSCs were mainly distributed in the periphery of the central canal of the spinal cord (more than 600um away from the central canal), and in the paraphenymal and subependymal areas of the white matter of the spinal cord (Ohori, 2006; Yamamoto, 2001; Martens et al., 2002). NSCs have the ability to expand, renew and differentiate into a variety of nerve cells such as neurons and glial cells (Gage, 2000). Dave et al. (2018), have proposed a method for generating stromal cells from human adipose tissue and differentiating them into nerve cells. Gronning et al. (2020) showed that cortical neurons derived from human long-

term neuroepithelioid stem (LT-NES) cells were generated by induced pluripotent stem cells (iPSCs) and transplanted into the cortex of stroke-injured adult rats, which improved neural function defects and established afferent nerves and cortical nerves. According to the above characteristics of NSCs, it is hoped that the lost neurons and oligodendrocytes can be replaced by promoting the differentiation of NSCs into neurons and oligodendrocytes, hence reconstructing axons and myelin sheath and further reshape the functional structure of injured spinal cord. Endogenous NSCsNSCshave great application prospects because they avoid immune rejection and ethical problems (Andres et al., 2008). Therefore, the alternative therapy of NSCs is promising to bring recovery to patients with SCI.

Activation of endogenous neural stem cells (ENSCs) and promotion of their proliferation, migration and differentiation to supplement the lost neurons and oligodendrocytes in injured spinal cord, thus promoting the repair of the structure and function of injured spinal cord. Autotransplantation of ENSCs has a wide application prospect because it avoids problems such as inconvenient sampling, invasive injury, rejection, tumorigenesis risk, immune reaction and ethics (Lu et al., 2006; Rasouli et al., 2006).

Direct transplantation of exogenous NSCs (embryonic or adult) into SCI sites: the transplanted NSCs could not only secrete nutritional factors to stimulate axon regeneration, but also supplement lost neurons and oligodendrocytes through proliferation and differentiation of NSCs to stimulate myelin regeneration (Cummings et al., 2005; Lepore and Fischer, 2005;). However, the application of exogenous NSCs transplantation is limited due to the above-mentioned problems. The

pathophysiological basis of dysfunctions of motor, sensory and extensor muscles below the injury level in SCI patients is the degeneration, necrosis and loss of a large number of neurons as well as the rupture and demyelination of spinal nerve conduction bundles caused by primary and secondary spinal cord injury after SCI (Oyinbo, 2011; Long, 2013). As a result, the focus of research on how to promote functional recovery after SCI lies in how to replenish lost neurons and how to promote regeneration of axons and myelin sheath. After SCI, the number of ENSCs (Okano et al., 2005) in the spinal cord is small and there is no microenvironment for nerve growth and development in the spinal cord, leading to the differentiation of endogenous NSCs in the direction of glial cells, and the formation of glial scar interferes with the repair of SCI. Zhou et al. (2014) and Feng et al. (2019) exhibited that the extract of injured spinal cord reflected the local microenvironment after SCI to some extent, and could better simulate the SCI microenvironment in vitro and promote the proliferation of NSCs. In conclusion, the local microenvironment after SCI can regulate the proliferation and differentiation of spinal cord endogenous NSCs. In addition, their own genes also participate in the regulation of endogenous neural stem cell proliferation and differentiation. The interaction between the regulation of their own genes and the local microenvironment as external signals constitutes a complex regulatory system of ENSCs proliferation and differentiation.

The regulation factors of autogenes include basic helical loop-helix transcription factor (bHLH; Notch signaling pathway (Yoon and Gaiano, 2005), Wnt/β-catenin signaling pathway (Zhao et al., 2023), Sonic hedgehog signaling pathway (Shimizu et al., 2005), and other regulation factors. Notch signaling pathway plays an important role in central nervous system development. It is composed of Notch, Notch ligand

and intracellular signaling molecule (CSL: CBF1, Suppressor of Hairless, Lag-1, a transcription factor that is responsible for activating the genes downstream of the Notch signalling pathway). Notch and notch ligand are single-order transmembrane proteins (Itoh et al., 2003). Notch is a 300 kDa single transmembrane protein, and its large extracellular domain consists of the three cysteine-rich Notch/Lin-12 repeats and 36 back-to-back EGF (epidermal growth factor) -like repeats. Currently, four homologous Notch proteins, namely Notch1-4, have been found in vertebrates. Among them, Notch1-3 genes were partially overlapped, and Notch1 were all expressed on the surface of NSCs (Lo et al., 2002). Notch ligand contains Jagged and Delta proteins in vertebrates (Okubo et al., 2021). After activation of the Notch signaling pathway (Yoon and Gaiano, 2005), the Notch receptor protein is first decomposed into heterodimer by one of the enzymes in the Furin family and binds to the ligand for conformational change. Then, the Notch receptor protein undergoes proteolysis twice. It was first hydrolyzed by tumor necrosis factor -α -invertase (TACE) and then hydrolyzed by γ -secretase on the envelope. Among them, presenilin 1/2 (PS1/2) participated in the hydrolysis of γ –secretase (Yoon and Gaiano, 2005). Notch receptors are enzymatically digested by γ -secretase, and Notch intra-cellular domain (NICD) is released from human cytoplasm and transferred into the nucleus (Hadland et al., 2001). NICD is the true activation form of Notch. It enters the nucleus and, through its RAM domain, binds to ankyrin repeats protein (ANK) and intracellular signaling molecules (CSL), and aggregates mastermind-like proteins-1 (MAML-1) to form co-excited complexes- NICD-CSL-MAML complex (Cante-Barrett et al., 2020).

1.2 Problem Statement

NICD-CSL-MAML complex continues to activate transcription of downstream Notch target genes- Hes genes (mainly Hes1 and Hes5), and increases the expression levels of Hes1 and Hes5 (Nandagopal et al., 2019). Activated Hes genes can maintain the state of NSCs and promote the proliferation of NSCs, and block the expression of downstream proneural genes such as Mash1, Ngn1 and Ngn2 to inhibit the neuronal differentiation of NSCs. Notch signaling pathway is primarily mediated by "lateral inhibition" (Wakanatsu et al., 2000).

Notch signaling pathway mediated lateral inhibition plays a key role in the proliferation and differentiation of ENSCs. When Notch signaling pathway is activated, NSCs proliferation can be promoted. When Notch signaling pathway activity is inhibited, NSCs enter the differentiation program. However, γ-secretase is the core of Notch signaling pathway regulation (Tagami et al., 2008). Gamma-secretase is involved in the enzymatic hydrolysis of Notch signaling pathway, and blocking γ-secretase can reduce NICD production. Due to the absence of NICD, downstream signaling molecules remain quiescent, thus allowing stem cells to enter the differentiation program. In addition, Yoshikawa et al. (2009) proposed in their study that blocking Notch signal can promote the differentiation of ENSCs into neurons in a hemorrhagic brain injury model in rats that early blocking Notch signal may lead to insufficient neural stem cell pool, while delayed blocking Notch signal may lead to delayed development and maturation of NSCs. It has also been suggested that the microenvironment exposed to ENSCs plays a decisive role in the regulation of ENSC proliferation and differentiation by Notch signaling pathway (Alexson et al., 2009; Shimojo et al., 2008).

Endogenous neural stem cells have unique advantages in the treatment of spinal cord injury. At present, the research of ENSCs has also made breakthrough progress that endogenous neural stem cells can differentiate into more than 200 cell types in the body. How to regulate its differentiation direction in vivo, especially how to make it differentiate in the desired direction and how to avoid tumor-like differentiation after spinal cord injury, is the direction of future efforts. On the other hand, all the current results have been obtained from animal experiments, while rodents have a strong ability to repair themselves after spinal cord injury.

Although there are many problems, the use of ENSCs in the treatment of spinal cord injury is still a good approach. As long as the signal pathway of neuronal differentiation and its influencing factors are further studied, the application of endogenous neural stem cells in the treatment of spinal cord injury will be successful.

1.3 Study Justification

Therefore, to study the effects of γ -secretase inhibitor-DAPT and local microenvironment after spinal cord injury on Notch signaling pathway is of great significance for exploring the mechanism of proliferation and differentiation of NSC. According to the above-mentioned content, we speculated that the local microenvironment after SCI, as an external regulatory signal, regulates ENSCs proliferation and differentiation through interaction with intracellular signals such as Notch signal. The study on the effect of local microenvironment on proliferation and differentiation of NSCs after SCI is of great significance for the repair of spinal cord injury. This study simulated the local microenvironment after spinal cord injury (SCI) by using paralyzed spinal cord extract in vitro. Therefore, in order to select the best

time to block Notch signaling pathway while maintaining the stability of stem cell pool, better promote the differentiation of NSCsinto neurons and the ultimately enhancing the potential of recovery of later functions. These findings and outcome of this study can be ideal to select the optimal timing for regulating the Notch signaling pathway to enhance NSCs proliferation effectively.

1.4 Hypothesis

The optimal time point method could promote NSC proliferation by regulating the Notch signaling pathway.

1.5 Objectives

1.5.1 Main Objective

To determine the simplified and efficient method for culture and identification of neonatal rat brain-derived NSCs.

1.5.2 Specific Objectives

1. Examining the temporal effects of paralyzed spinal cord extract on neural stem cell proliferation.
2. Assessing the impact of paralyzed spinal cord extract from different time points on the proliferation of NSCs.
3. To determine the stimulation of the local microenvironment after SCI with spinal cord injury extract in vitro
4. To proliferate and differentiate of NSCs following the inhibition of Notch signaling using the γ -secretase inhibitor-DAPT, applied at different time intervals.

5. To explore the impact of DAPT on both the proliferation and differentiation of NSC specifically targeting the Notch signaling pathway.

CHAPTER 2

LITERATURE REVIEW

2.1 Endogenous Neural Stem Cells and Spinal Cord Injury Repair

Spinal cord injury is mainly caused by trauma, which is characterized by high incidence, high disability rate, high cost rate, and the patients are mainly young and middle-aged (Bárbara-Bataller et al., 2018; Zhou et al., 2015; Hagen et al., 2012). Scholars at home and abroad have made unremitting exploration; so far it is still a global medical problem. The main current clinical treatment of SCI includes: reduction of secondary injury with high doses of methylprednisolone; Operation to relieve spinal cord compression, intensive nursing and rehabilitation treatment (Gerber et al., 2021; Roquilly et al., 2020). Although the above measures achieved a certain effect, but the effect of spinal cord injury is still not satisfactory. Cell transplantation therapy can supplement the lost nerve cells to connect the interrupted axon pathway and the transplanted cells can secrete neurotrophic factors to promote axon regeneration, which has become a hot spot in spinal cord injury research (Gong et al., 2020; Nori et al., 2017). At present, most of the research at home and abroad focuses on the transplantation of embryonic stem cells (ESCs), olfactory ensheathing cells, Schwann cells, induced Pluripotent Stem Cells (iPSCs), Mesenchymal Stem Cells (MSCs), Neural Progenitor Cells (NPCs), and NSCs (Reshamwala et al., 2022; Kawai et al., 2021; Gong et al., 2020; Nori et al., 2017). However, these cells have many disadvantages, such as inconvenient sampling, difficulty in in vitro culture and amplification, obvious immune rejection, ethical and legal restrictions, inaccurate localization of local transplantation, too few transplanted cells, small number of cells surviving after transplantation, and additional damage (Rasouli et al., 2006; Lu et al.,

2006). In theory, it is more suitable for clinical application and has broad research and application prospects by mobilizing NSCs in spinal cord tissue for alternative treatment and inducing endogenous neural stem cell proliferation and differentiation of damaged spinal cord (Qin et al., 2015). Now, this part reviews the progress of endogenous NSCs and spinal cord injury repair.

2.2 Distribution and Identification of Endogenous Neural Stem Cells in vivo

Neural stem cells are widely dispersed throughout the adult central nervous system. In an adult brain, Alvarez-Buylla et al. (2002) and Gage et al. (2000) have confirmed that endogenous NSCs exist in the subventricular region and the subdentate gyrus of the hippocampus. Horner et al. (2000) and Ohori et al. (2006) proposed that NSCs might exist in the white matter parenchyma of spinal cord. Meletis et al. (2008) proposed that NSCs might reside in the ependyma near the central canal. Also, Martens et al. (2002) further showed that NSCs may reside under ependyma. Yamamoto et al. (2001) isolated a kind of cell from the ependymal layer of spinal cord of female mice at 6-7 weeks. Added insulin into the medium after culturing the cell for 14 days in vitro, and the cells divided from the cell aggregates formed similar aggregates again. More than half of the cells in the progenitor cell aggregates were positive for Nestin expression, but the markers of neurons and glial cells (Yamamoto et al., 2001), such as MAP2, NG2, and GFAP, were negative. Adding fibroblast growth factor-2(FGF-2) and epidermal growth factor (EGF) to the medium reduced the number of nestin-positive cells in the progeny cell aggregates by 5-7% within 2-4d. Moreover, GFAP-positive astrocytes, TuJ1-positive neurons, and O4-positive

oligodendrocytes were detected in almost 100% of these cell aggregates after the plates were inoculated into single-cell layers 4d (Yamamoto et al., 2001).

2.2.1 Nestin

Lendah et al. (1999) identified a neuronal interfilament protein, called nestin, which is distributed in the cytoplasm and is a specific expression of neuroepithelial stem cells. It is involved in the formation and growth of neurites and growth cones and the establishment of connections between neurons and target cells. Its expression has a specific time sequence (Michalczyk et al., 2005). When NSCs differentiate into glial cells and neurons, nestin expression level is down-regulated and replaced by other special types of mesofilaments (Zhang et al., 2005). In addition, nestin can also be expressed to some extent in other tissues, such as in the central nervous system, in developing and adult brain endothelial stem cells (Morimoto et al., 2024, and in reactive astrocytes under pathological conditions such as trauma and glioma (Zhang et al., 2005).

2.2.2 Musash-1

Musash-1 is a neural RNA-binding protein expressed in fetal and adult NSCs (Siddall et al., 2006; Okano et al., 2005). The self-proliferative ability of NSCs (Hitoshi et al., 2002) was maintained by targeting mRNA and M-numb transmembrane receptor signals. In addition, Musash-1 is also expressed in neuronal and astroglial progenitors, and in mammalian intestinal, gastric and mammary epithelial stem cells (Sugiyama et al., 2006).

High mobility group (HMG-box) transcription factors in the SOX family play an important role in establishing and maintaining the multidirectional potential of NSCs (Jagga et al., 2021; Kim et al., 2003). Sox-1 may be the earliest neural marker expressed in embryonic stem cells (Aubert et al., 2003). It is expressed only in the nervous system and can identify the pool of NSCs proliferating in the nerve tube, so it is more specific than nestin in identifying NSCs (Liu et al., 2024; Aubert et al., 2003).

2.2.3 Cell Adhesion Factors

Some specific cell adhesion factors as cell surface antigens could be used for immunoscreening of NSCs. Corti et al. (2005) isolated NSCs from the brains of adult and embryonic mice and grew them into nerve spheres. Both Lewis X and CXCR4 antigens were detected by fluorescence activated cell sorting (Corti et al., 2007). Therefore, if these two surface proteins are detected simultaneously on the same cell surface, the cell can be preliminarily identified as NSCs. The immunofluorescence staining techniques for bromodeoxyuridine (BrdU) and nestin play crucial roles in cell biology research, particularly in studying cell proliferation and neural stem/progenitor cell identification, respectively.

2.2.4 5'-Bromine Uracil

5'-Bromine uracil (BrdU) homologous substitute for thymine that can be competitively incorporated into newly synthesized DNA during the S phase (Wang et al., 2022; Ngwenya et al., 2008). Stem cells can absorb labeled Brdu in the process of self-replication and proliferation (Ngwenya et al., 2008; Ross et al., 2008). Nestin immunofluorescence staining is used to detect nestin, an intermediate filament protein

expressed in neural stem/progenitor cells nestin immunofluorescence staining (Li et al., 2017; Cheng et al., 2016). Glial acidic fibrin protein (GFAP) is a specific marker molecule for astrocytes (Cobb et al., 2016; Tichy et al., 2013). The GFAP, or Glial Fibrillary Acidic Protein, is a widely used marker for identifying and visualizing astrocytes in immunofluorescence staining. Astrocytes are a type of glial cell in the central nervous system that provides support and maintenance functions for neurons.

In addition, NSCs have the potential to differentiate into neurons, oligodendrocytes, astrocytes, and other nerve cells. The terminal cells formed by NSC differentiation can be identified by the specific markers of nerve cells. NF200, neuron specific enolase and Beta-III-tubulin are widely used as neuron specific markers (Feng et al, 2019; Zhou et al., 2017; Long, 2013). By detecting these corresponding surface marker molecules, neural terminal cells differentiated from NSC can be identified, and thus NSCs can be identified from the differentiation potential function of NSCs. Furthermore, it has been found that NSCs selectively express surface markers of CD133+/CD34/CD45, forebrain embryonic antigens 1, A2B5, CD9, SSEA-1 (CD15), CD29, CD81, CD95, CD146, and P75 (CD271) (Uchida et al., 2000; Pruszak et al.,2007; Klassen et al.,2001). They are also seen as biomarkers for inscribing NSCs.

2.3 Mechanism of Proliferation and Differentiation of Endogenous Neural Stem Cells

NSCs are regulated by complex signaling pathways that control their self-renewal, proliferation, differentiation, and survival (Li and Fang, 2023). The directional differentiation of NSCs into neural cells can be regulated by their own genes and by external signals (Ye et al., 2022; Xu et al., 2021; He et al., 2020; Dong et al., 2019).

In vertebrates, genes with similar functions to drosophila achaete - Scute complex and atona-I encode basic helix-loop-helix (bHLH) transcription factors, called bHLH genes, which determine the fate of nerve cell differentiation (Dennis et al., 2019; Kageyama et al., 2005).

The bHLH gene family plays an important regulatory role in neurogenesis, neural development and neural differentiation (Dennis et al., 2019; Kageyama et al., 2005). The gene family can be divided into positive regulatory type (e.g., Mash1, Ngn1, Ngn2) and negative regulatory type (e.g., Hes1, Hes5), (Kageyama et al., 2005). Positive regulatory genes activate gene expression by forming heterodimer with bHLH factor -E47 and binding to E-box (CANNTG) (Ryoichiro et al., 2005). Positive regulatory genes promote the formation of different types of neurons. For example, Mash1 promotes γ -aminobutyric acid neurons in the ventral forebrain region, and Ngn2 promotes the generation of glutamate neurons in the dorsal region (Fode et al., 2000; Parras et al., 2000). Positive regulatory genes in the neural crest also promote the formation of different categories of neurons. Ngn1 and Ngn2 promote the generation of sensory neurons, while Mash1 promotes the generation of motor neurons (Kageyama et al., 2005; Lo et al., 2002). The positively regulated bHLH gene not only induces the expression of specific neuronal genes, but also inhibits the expression of specific glial genes (Lo et al., 2002). The mechanism is that glial fibrillary acidic protein is a specific astrocyte gene. Stat1/3 and Smad1 are bridled by the common activator p300 to increase the expression of regulated glial fibrillary acidic protein, while Ngn1 inhibits the expression of specific glial genes by dissociating the p300/Smad complex and glial promoter (Sun et al., 2001). Thus, the positively regulated bHLH gene promotes neuronal differentiation of NSCs by inhibiting glial

differentiation. Hes1, Hes3, and Hes5 are highly expressed in NSCs in the negatively regulated bHLH gene family. The Hes factor contains a conserved bHLH domain at the amino terminal, which forms a dimer and binds to DNA. Hes gene targets include positive regulatory genes such as Mash1, which inhibit Mash1 expression by directly binding to promoters (Kageyama, et al., 2005). Positive regulatory genes promote the differentiation of NSCs into neurons by forming heterodimers with bHLH factor E47. While Hes1 forms a non-functional heterodimer with E47 and inhibits the formation of MASH1-E47 heterodimer, thereby inhibiting the differentiation of NSCs (Kageyama et al., 2005). In addition, the Sox2 gene is an important gene that helps stem cells maintain their pluripotency and the apoptosis-related gene Bcl-XL can enhance the dopaminergic neuron formation of human NSCs (Lista et al., 2004).

Notch signaling is another pathway that controls the fate of NSCs differentiation, consisting of Notch receptor, CSL (DNA binding protein) and Notch ligand (DSL protein) (Del Bianco et al., 2010; Hansson et al., 2006). Notch receptor and its ligand are single-order transmembrane proteins. When the ligand (such as Delta) binds to the Notch receptor of the adjacent cell, the Notch receptor is cleaved by the proteasome, releasing the intracellular domain of Notch with nuclear localization signal (Del Bianco et al., 2010; Hansson et al., 2006; Yoon et al., 2005). It then goes into the nucleus and binds to CSL and regulates gene expression (Li et al., 2017; Yoon et al., 2005). The signal transmission of this pathway is mainly through protein interaction, which causes the change of transcriptional regulatory factors or binds transcriptional regulatory factors to target genes to achieve the regulation of specific gene transcription. Notch prevents NSCs from differentiating into neurons and maintains neural stem cell self-renewal (Kageyama et al., 2005. When Notch is activated, it

promotes the proliferation of NSCs by increasing the expression of Hes1 and Hes5 genes. When Notch activity is inhibited, stem cells enter the differentiation program (Figure 1) (Yoon and Gaiano, 2005). The Notch signaling pathway acts on NSCs by improving the expression of Hes1 and Hes5 genes. When inhibited, the expression of Hes1 and Hes5 genes decreased, and the competitive inhibition of Mash1 decreased, thus promoting NSCs to enter the differentiation process. However, when the role of Hes1 and Hes5 genes in promoting the proliferation and renewal of NSCs is weakened, the depletion of spinal cord NSCs could affect the recovery of injured spinal cord function.

The Notch signaling pathway regulates the differentiation of NSCs into glia with two different results: inhibiting the differentiation of NSCs into oligodendrocytes and promoting the differentiation of NSC into astrocytes (Venkatesh et al., 2017 and Ge et al., 2002). Ge et al. (2002) found that the number of astrocytes in the control group was only half of that in the treatment group after 4 days in the experiment of studying cerebral cortical cells of pregnant mice through the Fc segment of Delta protein. Cai et al. (2008) suggested that gamma-secretase is the core of Notch signaling pathway regulation. In their experiments, they used DAPT to block the action of gamma-secretase, thereby reducing the production of NICD. Due to lack of NICD- the activated form of Notch receptor, downstream signaling molecules are dormant to allow NSCs to differentiate toward neurons and oligodendrocytes. Ge et al. (2002) used DAPT to block the action of gamma-secretase in the follow-up study, and observed that the number of astrocytes decreased significantly after 4 days of DAPT blockade. These results suggest that activated Notch receptor can directly promote NSCs differentiation into astrocytes. Yoshikawa et al. (2009) proposed in their study

that blocking Notch signal can promote the differentiation of ENSCs into neurons in a hemorrhagic brain injury model in rats that early blocking Notch signal may lead to insufficient neural stem cell pool, while delayed blocking Notch signal may lead to delayed development and maturation of NSCs. In conclusion, Notch signaling pathway has different effects on different nerve cells, not only inhibiting, but also promoting cell growth, development and differentiation. Therefore, it can be speculated that appropriate timing of Notch signal blocking can better promote the differentiation of NSCs into neurons and later functional recovery. Finding the right way and time to block the Notch signaling pathway may solve this problem.

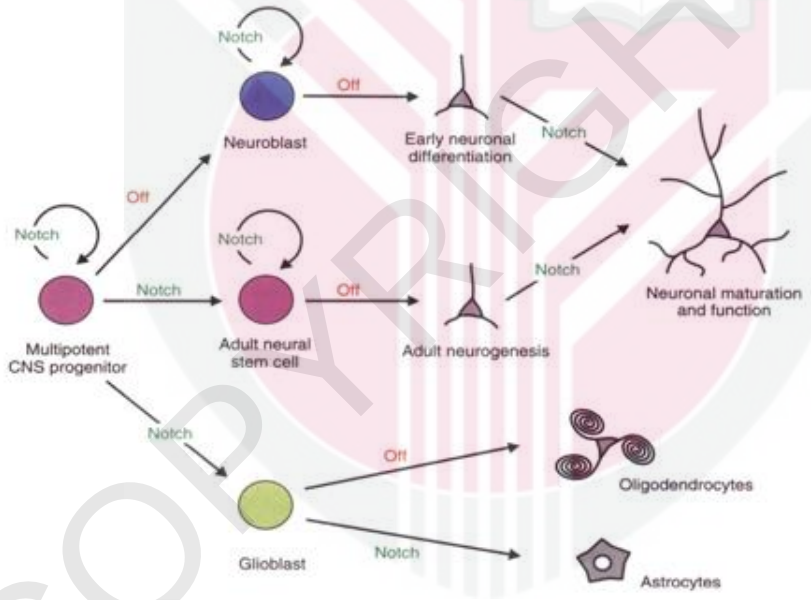

Figure 2.1 : Green (Notch) Represents Notch Activation and Red (off) Represents Notch Inhibition

2.4 Effects of Growth Factors on Proliferation and Differentiation of Endogenous Neural Stem Cells

Basic fibroblast growth factor (bFGF) plays a mitogenic role in the early stage of neural stem cell proliferation and enables NSCs to acquire the reactivity of epidermal

growth factor (EGF), while EGF plays a stronger mitogenic role in the late stage of neural stem cell proliferation (Tarasenko et al., 2004; Santa et al., 1999). Santa et al. (1999) sacrificed the female mice after mating 13.5 days by decapitation, took out the midbrain tissue containing substantia nigra from the embryos for culture, and added bFGF and EGF respectively. The cultured cells were labeled with rabbit anti-mouse nerve filament antibody or rabbit anti-mouse GFAP antibody. In the primary medium, a huge number of cell aggregates were formed with the addition of the two growth factors, and a small number of cell aggregates were formed with the addition of EGF alone, while no cell aggregates were formed with the addition of bFGF alone. Immunohistochemical analysis confirmed that these cell aggregates expressed neuronal and glial antibodies, and the second-generation cells, also expressed these antibodies. Thus, it can be determined that these cell aggregates are derived from NSCs. In addition, more neuronal antibodies were detected in the cell aggregates cultured with bFGF+EGF. After the second generation cells were cultured again using the above method, they found that bFGF alone did not form cell aggregates, and there was no statistical difference in the number of cell aggregates formed between EGF and bFGF+EGF. Therefore, they believed that bFGF was not needed for the later stage of neural stem cell proliferation.

Smad6, Smad7 and Noggin are antagonists of bone morphogenetic protein (BMP) (Hsieh et al., 2004). Insulin-like growth factor 1 (IGF-1) inhibits BMP by incrementally regulating Smad6, Smad7, and Noggin, thereby stimulating the differentiation of hippocamal stem cells into oligodendrocytes (Hsieh et al., 2004). Aberg et al. (2000) confirmed that the number of Brdu-labeled cells in the dentate gyrus of the hippocampus in the experimental group was significantly higher than that

in the control group in the experiment of continuous infusion of IGF-1 with micropump in the pituitary-resected rats.

Several researchers reported that adding BMP2 (20 ng/ml) into the medium significantly increased the number of GFAP-positive cells in the differentiated NSCs (Gomes et al., 2000). After Noggin inhibited BMP signaling, neural progenitors transplanted into spinal cord injured mice differentiated into neurons and oligodendrocytes (Setoguchi et al., 2004).

In addition, neurotransmitters have been found to play an important role in regulating the proliferation and differentiation of adult neural progenitor cells, synaptic integration, and functionally dependent adult neurogenesis（Platel et al., 2010).. For example different glutamate receptors allow glutamate to play different roles in the process of neuroblast production and migration (Gao et al., 2020; Neagoe et al., 2018). GABA secreted from peripheral astrocytes can reduce the migration rate of neuroblasts to olfactory vesicles through the GABAA receptor-mediated signaling pathway (Markwardtet al., 2009), and GABA-γ-2 signaling is an important factor involved in the activation and self-renewal mode of adult resting NSCs (Bao et al., 2017; Ge et al., 2006; Liu et al., 2005). In addition, Baser et al. (2019), Beaulieu et al. (2011), and Winner et al. (2006) found that dopaminergic afferent nerve fibers from the ventral tegmental region could stimulate the proliferation of neural precursor cells in the SGZ region in adult hippocampal tissues.

2.5 Endogenous Neural Stem Cells and Spinal Cord Injury

Pineau et al. (2007) demonstrated that pro-inflammatory factors such as interleukin-1 β (il-1 β) and Tumor necrosis factor-α (TNF-α) appeared rapidly in the injured area 5 min after spinal cord injury. TNF and IL-1β positive cells could be detected in the whole spinal cord after 30-45min. TNF, IL-1β, IL-6 and leukemia suppressor were significantly increased in the lesion area during 3-24h. These cytokines, especially TNF-α and IL-6, are expressed within minutes after spinal cord injury and affect NSCs (Pineau et al., 2007). TNF-α activation after CNS injury not only plays an important role in pathological development and inflammatory induction, but also regulating the proliferation of NSCs (Pineau et al., 2007). This effect may be achieved by increasing bFGF expression (Wu et al., 2000).TNF-α stimulated the proliferation of surviving oligodendrocytes and neuronal precursors, which was consistent with the results of TNF-RI and RII in knockout mice (Losif et al., 2006; Arnett et al., 2001; Wu et al., 2000).

Okada et al. (2004) used IL-6 receptor antagonist MRI6-1 to inhibit IL-6 signaling, and the function of mice with spinal cord injury was restored. In vitro experiments of the same study showed that adding IL-6 into the culture medium preferentially differentiated neural progenitor/stem cells into astrocytes, while adding MRI6-1 to block the IL-6 receptor inhibited the neural progenitor/stem cell differentiation into astrocytes. In addition, Okano et al., 2005 also found that the number of BrdU and GFAP double-labeled cells in the injured segment of mice was significantly reduced after intraperitoneal injection of MRI6-1 (100μg/g) immediately after spinal cord injury. Chen et al.（2021）demonstrated that all-trans retinoic acid treatment of NSCs

promotes neural stem cell differentiation by inhibiting presenin 1 and reducing Notch1 signaling pathway activity.

Il-1 β is significantly increased after spinal cord injury, which is thought to be a key factor in the initiation of an immune response and is responsible for neurological secondary injury (Pineau et al., 2007). Experiments have been conducted to protect nerves by inhibiting IL-1β. Vela et al. (2002) found in vitro that IL-1β inhibited the proliferation of oligodendrocyte precursors and promoted their differentiation into oligodendrocytes. This effect may be achieved by regulating the expression of platelet-derived growth factor receptor-α (PDGF-R-α) and p38 and P42 / P44 mitogen-activated protein kinase signaling pathways. It also promoted the maturation and survival of differentiated oligodendrocytes.

Vavrek et al. (2006) pointed out that residual neurons and glial cells after SCI can secrete brain-derived neurotrophic factor (BDNF), which could bind to corresponding receptors through target-derived, autocrine and paracrine modes to stimulate multiple signaling pathways to promote the differentiation, growth and survival of the CNS. Namiki et al. (2000) injected BDNF, nerve growth factor (NGF) and neurotrophic factor 3 (NT-3) into the sheath of adult rats with T3 level spinal cord injury. Four weeks later, a fluorescent tracer was injected into the tail of the spinal cord. Compared with the control group, the number of fluorescence-based cells in the red nucleus and sensorimotor cortex of the rats injected with BDNF was significantly higher, the spinal cord function was significantly recovered, and the formation of spinal cavity was significantly reduced. Kojima et al. (2002) confirmed in similar experiments that Nestin expression and BBB score of rats in the experimental group were significantly

higher than those in the control group. Mikami et al. (2004) found that co-culture of dendritic cells with NSCs in vitro could significantly stimulate the proliferation of NSCs. When dendritic cells were transplanted into the spinal cord of T8 injured adult mice, NT-3 was secreted in large quantities, and the spinal cord environment was significantly improved. ENSCs in spinal cord proliferated greatly and nerve genesis was active. After transplantation 14 days, BBB score indicated that the spinal cord function of the experimental group was significantly recovered. (16, 4± 4.3) of the spinal cord ENSC differentiated into immature neurons and (1.3 ± 1.0) differentiated into mature neurons. Yang and Fang (2021) found that bone marrow mesenchymal stem cells (BMSCS) exosomes can promote the differentiation of endogenous NSCs into neurons and promote the repair of spinal cord injury. Guo et al. (2021) found that bone-marrow derived NSCs could promote the secretion of neurotrophic factors in spinal cord tissue and promote the differentiation of endogenous NSCs to repair spinal cord nerve tissue and restore motor function in rats. As a member of the Shh family of secreting signal proteins, Shh plays a variety of roles in the development of vertebrates. In the nervous system, it organizes neural tube development by establishing regions of production of different homologous transcription factors along the dorsal ventral axis, with different regions developing into specific cells (Jarov et al., 2003). Hou et al. (2004) found that NSCs proliferated massively in Shh culture medium in vitro and showed a certain dose dependence. Bambakidis et al. (2004) found that in adult rats with T_8 / T_{10} spinal cord injury, the Shh signaling pathway was activated by intrasheath injection of Shh protein, which effectively counteracted negative signals in the microenvironment and stimulated the massive proliferation and differentiation of ENSCs into neurons. Importantly, BBB scores indicated some recovery of spinal cord function in the experimental group at 28 days postoperatively.

Wan (2019) found that the self-assembled nano-peptide Mir-29 sustained release system promoted the regeneration of endogenous neural stem cell axons and promoted the repair of spinal cord injury after transplantation. Shao (2020) fully demonstrated that the cationic carbon quantum dots with ultrafine particle size derived from lycium LBP oligosaccharides could significantly inhibit the differentiation of ENSCs into astrocytes, and hence guide the directed differentiation of NSCs into neurons in vitro and in vivo experiments. Li et al. (2020) found that transplantation of WNT5A-modified NSCs promoted tissue repair and recovery of motor function after spinal cord injury. Niu et al. (2019) demonstrated that conditioned culture of bone marrow mesenchymal stem cells inhibited the Notch1 signaling pathway, thereby attenuating oxidative stress injury of NSCs and promoting recovery of spinal cord function.Shin et al. (2018) verified that extracorporeal shock waves can induce the activation of endogenous NSC and improve spinal cord function. Hosseini et al. (2016) found that NSCs grafted on alginate scaffolds could achieve better clinical results in a rat model of spinal cord injury. The results of these animal experiments fully demonstrate the possibility of inducing endogenous NSCs to differentiate into neurons to promote spinal cord injury.

2.6 Animal Modelling of Spinal Cord Injury

The spinal cord is of great physiological significance to the human body. Many important nerve mediators pass through the spinal cord. Once spinal cord injury occurs, the pathophysiological changes of both primary spinal cord injury and secondary spinal cord injury are easy to cause neurological damage, resulting in organ and tissue damage of patients (Schef et al., 2003). However, these damages are often irreversible and not easy to treat, and will lead to high death and disability of patients,

and ultimately bring heavy burden to patients themselves, their families and society (Bilgen, 2005; Jakeman et al., 2000). At present, the treatment of spinal cord injury progresses slowly and the overall treatment effect of patients is not predictable in clinical work, which is caused by the complicated pathophysiological process after spinal cord injury. Therefore, in order to make a comprehensive and in-depth study of spinal cord injury, it is necessary to prepare an animal model of spinal cord injury that is close to clinical practice (Mountney et al., 2010; Zhang et al., 2010).

2.7 Animal Selection and Requirements for SCI Animal Models

Animal spinal cord injury models were used to analyze SCI in vivo. Models of a given human condition, disease or injury must not only be causally and functionally similar to human simulators, but must have advantages over simple clinical observations. Therefore, animal models of spinal cord injury should also allow for in-depth study of physiological and pathological events (Sharif et al., 2017). Zhang Qin et al. (2010) believed that the selection of animal models of spinal cord injury should follow the principles of economy, applicability, controllability and feasibility. Elliot et al. (2020) identified and discussed specific animal welfare issues and proposed feasible measures. The aim is to reduce animal use and suffering, reduce experimental variability and improve translatability in SCI research areas. So far, some reviews have focused on paradigms (Cheriyan et al., 2014; Akhtar et al., 2008) or outcomes (Filli et al., 2012; Battistuzzo et al., 2012; Nakae et al., 2011) in animal models of spinal cord injury. Sharif et al. (2017) found that rodents are the most commonly used species to model spinal cord injury. From a purely experimental point of view, using primates is the best choice (Simmons et al., 2008). However, primates are rarely used in actual experiments because they are expensive and rare, and are limited by expensive care

and housing, stringent regulatory and ethical requirements (Kundi et al., 2013). In the selection of experimental animals, not only the biological characteristics must be highly consistent with human beings, but also whether it is easy to obtain and the cost of acquiring animals must be considered. As a result, few people use orangutans, apes and monkeys as animal models, even though they are the closest species to humans in terms of biological characteristics and are large enough to be operated on. In actual basic experiments, the most commonly used animal models are mice, rats and guinea pigs. The advantage of using these small animals to make animal models is that the genetic background of these animals is clear, and the microbes in these animals are in an easier state to control (Li et al., 2010; Sekhon et al., 2001). The model obtained by using these small animals to make corresponding models is not only relatively stable, but also these small animals are relatively cheap, easy to manage, and easy to conduct large-scale breeding. For the animal model of spinal cord injury, guinea pigs and rats are the best animals to choose. Guinea pigs have the advantage of mild temperament, which is conducive to management and convenient in operation. Ma et al. (2010) believed that the advantage of rats was that once severe injury occurred, rats had the strongest tolerance to severe injury. The pathological changes in rats on the overall level are similar to those in humans. Especially, rats are easy to form cavities after surgery and carry out corresponding management after surgery, and there are few cases of postoperative infection in rats (Sheng et al., 2004). It is because of these advantages that rats are widely used in experiments. However, rats are quadrupeds rather than bipeds, and their corticospinal tracts are predominantly dorsal（Sharif et al., 2014）, and these differences need to be properly explained. On the other hand, more and more mouse models are being used to study the molecular biology and basic cells of SCI (Lee et al., 2013). Advantages of mouse models include genome similarity

to human, high reproduction rate, ease of handling and low cost (Jakeman et al., 2001). It is very difficult to use rats as knockout models for genetically modified animals (Pilcher, 2003), and so far mice are mainly used. However, mice are not suitable for building animal models of spinal cord injury, because they are not only small in size, but also tend to form dense connective tissue after surgery, which is still different from human beings in pathological changes to a certain extent (Lang et al., 2000). In addition, other vertebrates with unique regenerative abilities such as fish and lampreys have been studied to test new strategies for spinal cord injury in human (Doyle et al., 2001).

At present, none of a kind of specific animal model could not completely meet the clinical needs in the actual animal operation. An astute researcher should be able to select an appropriate model and result to evaluate the given specific objectives. All existing animal models of spinal cord injury and associated outcome assessments must be considered in order to select suitable models and test specific hypotheses. We believe that an ideal spinal cord injury model should include the following aspects: It is comparable in morphology; the established model must have absolute repeatability; there must be a closed local spinal cord injury; the changes in neurological function and neuropathology were consistent.

2.8 Prospects for Spinal Cord Injury Model Preparation

The purpose of animal models of SCI is to simulate a series of pathophysiological parameters of patients with spinal cord injury. A good, stable and reproducible animal model of spinal cord injury can provide a good idea for clinical treatment of patients with spinal cord injury (Zhou et al., 2013; Sampson et al., 2011). Although the spinal

cord injury models developed by scholars around the world can partially solve some problems, they still have some deficiencies. It still needs the persistent efforts of different researchers in different countries to make a model of spinal cord injury that is more closely related to clinical practice.

2.9 Embryology and the Diversity of NSCs

During certain type of tissue is developing, it starts as a sheet of cells that can turn into nerves. These cells are grouped into different areas based on which genes are using for development (Sen, 2023). Within these areas, there are special cells called neural stem cells (NSCs) that have unique genetic profiles and these NSCs can make different types of nerve cells (Kaminska et al., 2022; Kennea & Mehmet, 2002; Sen, 2023). The embryology of NSCs, also referred to as B1 cells, persist throughout adulthood in a specific region of the brain known as the ventricular-subventricular zone (Lopez-Virgen et al., 2023; Quaresima et al., 2022). Research on these NSCs has primarily focused on rodents, where they are found along the walls of the brain's lateral ventricles, near important areas like the cortex, hippocampus, striatum, and septum (Ming & Song, 2011). B1 cells share many characteristics with another type of brain cell called astrocytes, including the presence of specific proteins like BLBP, GLAST, and Sox2 (Giachino et al., 2014). These proteins are also seen in radial glia cells (RGs), which are NSCs during brain development. Studies have indicated that B1 cells originate from RGs (Merkle et al., 2004) and exhibit a structural organization similar to RGs, suggesting a sequential lineage from early neuroepithelial cells to RGs and eventually to adult B1 cells (Mirzadeh et al., 2008).

2.10 Neuroanatomy, Spinal Cord Morphology

The spinal cord is a cylindrical and well-structured part of the body. It starts at the foramen magnum, where it is a continuation of the medulla oblongata at the base of the skull, and it runs through the spinal canal within the vertebrae. In adults, it typically measures around 45 cm in men and 43 cm in women. It is divided into 31 segments: 8 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 1 coccygeal. Each segment has pairs of spinal nerves along with their ganglia. These nerves carry motor, sensory, and autonomic signals and exit through openings between the vertebrae (Ganapathy et al., 2019; Toossi et al., 2021).

There are two noticeable bulges in the spinal cord, one in the neck area (cervical) and the other in the lower back area (lumbar), where nerves branch out to form networks called plexuses. The width of the spinal cord varies in different regions, with the thoracic region typically ranging from 0.64 to 0.83 cm and the cervical and lumbar regions ranging from 1.27 to 1.33 cm. The diameter of the spinal cord segment also varies, with the widest being at C5 and gradually decreasing towards the middle of the back (T8), then increasing slightly at L3 (Diaz & Morales, 2016; Frostell et al., 2016).

2.11 Staining Techniques for SCI

Immunofluorescence staining techniques for BrdU and nestin are vital in cell biology research, particularly for studying cell proliferation and identifying neural stem/progenitor cells (Hashimoto et al., 2022; Hong et al., 2022). BrdU, a substitute for thymine, is incorporated into newly synthesized DNA during the S phase of cell replication (Wang et al., 2022). Stem cells absorb labeled BrdU during self-replication and proliferation.

Nestin immunofluorescence staining detects nestin, an intermediate filament protein expressed in neural stem/progenitor cells (Li et al., 2017; Cheng et al., 2016). Glial acidic fibrin protein (GFAP) serves as a specific marker for astrocytes, aiding in their identification and visualization through immunofluorescence staining (Liu et al., 2016).

Neural stem cells have the ability to differentiate into various types of nerve cells, including neurons, oligodendrocytes, and astrocytes. Specific markers such as NF200, neuron-specific enolase, and Beta-III-tubulin are commonly used to identify differentiated neurons (Feng et al., 2019). By detecting these markers, terminal cells formed through neural stem cell differentiation can be identified, thereby revealing the differentiation potential of neural stem cells. Moreover, NSCs express surface markers including CD133+/CD34/CD45, forebrain embryonic antigens 1, A2B5, CD9, SSEA-1 (CD15), CD29, CD81, CD95, CD146, and P75 (CD271) (Pruszak et al., 2007). These markers serve as biomarkers for characterizing neural stem cells.

2.12 SCI Health Complications

Medical complications associated with spinal cord injury (Hagen, 2015), along with brief explanations were mentioned below:

i. Autonomic Dysreflexia

Autonomic dysreflexia is a potentially life-threatening condition characterized by a sudden and dangerous increase in blood pressure, usually in response to a noxious stimulus below the level of the spinal cord injury. It primarily affects individuals with injuries at or above the T6 level (Biering-Sørensen et al., 2018).

ii. Deep Vein Thrombosis (DVT)

DVT refers to the formation of blood clots in the deep veins of the body, commonly occurring in the lower extremities of individuals with spinal cord injury due to reduced mobility and impaired circulation. If left untreated, DVT can lead to serious complications such as pulmonary embolism (Dhall et al., 2013).

iii. Neurogenic Bowel Dysfunction

Neurogenic bowel dysfunction refers to abnormalities in bowel function resulting from damage to the nerves controlling the gastrointestinal tract. It can manifest as constipation, fecal incontinence, or a combination of both, significantly impacting bowel management and quality of life (Ko, 2023; Rodriguez & Gater, 2022).

iv. Neurogenic Bladder

Neurogenic bladder is a dysfunction of the urinary bladder due to impaired nerve control, commonly seen in individuals with spinal cord injury. It can result in urinary retention, incontinence, urinary tract infections, and kidney complications if not properly managed (Cheng et al., 2024; Perez et al., 2022).

v. Orthostatic Hypotension

Orthostatic hypotension is a drop in blood pressure that occurs when transitioning from lying down to standing up, leading to symptoms such as dizziness, lightheadedness, and fainting. It is common in individuals with spinal cord injury due to autonomic nervous system dysfunction (Ong et al., 2020; Park et al., 2024).

vi. Pressure Ulcers (Decubitus Ulcers)

Pressure ulcers, also known as decubitus ulcers or bedsores, are localized areas of tissue damage caused by prolonged pressure on the skin and underlying tissues, typically over bony prominences. Individuals with spinal cord injury are at increased risk due to immobility and impaired sensation (Fryer et al., 2023; García-Rudolph et al., 2024).

vii. Respiratory Complications

Respiratory complications in spinal cord injury may include impaired breathing, reduced lung capacity, pneumonia, and respiratory muscle weakness. These complications can result from paralysis of respiratory muscles, impaired cough reflex, and altered respiratory mechanics (Hendershot & O'Phelan, 2022; Hirota et al., 2024).

viii. Spasticity

Spasticity is a condition characterized by involuntary muscle contractions and stiffness, commonly seen in individuals with spinal cord injury due to disrupted neural pathways. It can lead to muscle spasms, pain, and functional impairment (Yokota et al., 2023).

ix. Syringomyelia

Syringomyelia is a condition characterized by the formation of fluid-filled cavities (syrinxes) within the spinal cord, often resulting from trauma or structural abnormalities. It can lead to progressive neurological symptoms such as pain, weakness, and sensory deficits (Nadeem et al., 2022; Yuan et al., 2024).

These complications highlight the diverse and significant health challenges faced by individuals with spinal cord injury, emphasizing the importance of comprehensive management and multidisciplinary care to optimize outcomes and improve quality of life.

2.13 SCI Current Treatment Methods

Treatment for spinal cord injury (SCI) typically involves emergency care to stabilize the spine, potential surgery to remove obstructions or stabilize fractured vertebrae, traction to realign the spine and reduce pressure on the spinal cord, medications for

pain management and prevention of complications, physical and occupational therapy to improve mobility and independence, assistive devices such as wheelchairs and braces to aid in daily activities, functional electrical stimulation to activate paralyzed muscles, psychotherapy and counseling to address emotional and psychological challenges, and participation in experimental treatments through clinical trials to explore emerging therapies such as stem cell therapy and gene therapy (Cadotte & Fehlings, 2011; Failli et al., 2021; Silva et al., 2014). The specific treatment approach depends on factors such as the severity and location of the injury, overall health, and individual goals, with a multidisciplinary team of healthcare professionals collaborating to develop a personalized treatment plan for each individual with SCI.

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethical Approval

The animal ethics has been obtained on 15/11/2021 from the Southwest Medical University Animal Welfare, Luzhou City, Sichuan Province, China. The Ethical Approval number is: 20211115-005 (Appendix A).

3.2 Study Flowchart

This study mainly sued the cultured NSCs from the paralyzed spinal cord injury of rats. Fig. 3.1 shows the brief flowchart of the study.

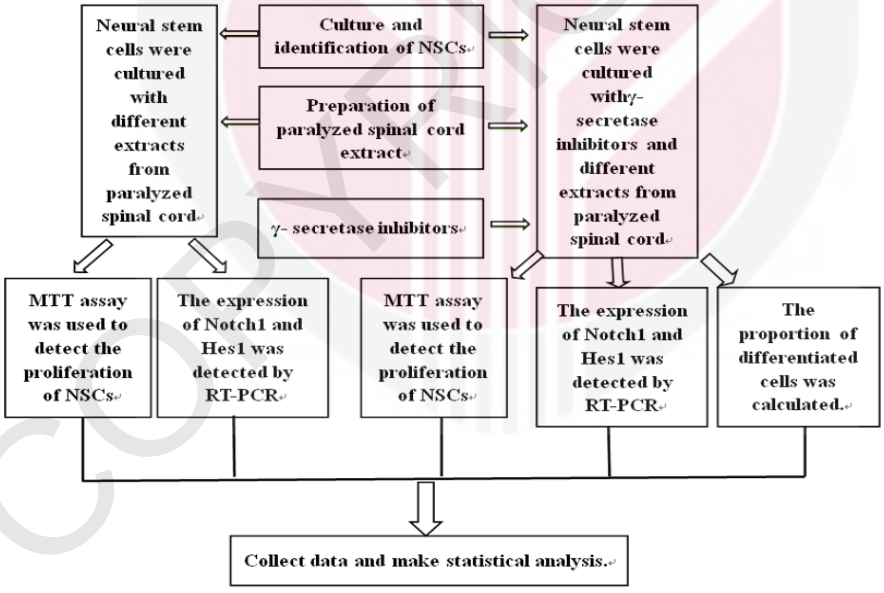

Figure 3.1 : A Brief Flowchart of the Study

3.3 Experimental Animals

Initially, a total of fifty-five healthy adult female SD rats (body weight: 220-250g, clean grade, provided by Animal Experiment Center of Southwest Medical University). Animal models of paraplegia were developed after one week of feeding under standard conditions. Then, twelve healthy adult female SD rats and four healthy adult male SD rats in estrus were selected (body weight: 220-250g, clean grade, provided by Animal Experiment Center of Southwest Medical University). The ratio of males to females was 3:1. When white emboli appeared in the vagina of female mice in the morning of the next day, it was recorded as 1 day of pregnancy. After female rats gave birth, a number of newborn SD rats (within 2-3D) were collected for the extraction of brain-derived NSCs from newborn SD rats.

3.4 Solutions for the Experimental Study

3.4.1 Fibroblast Growth Factor (bFGF) Solution

Dissolve each 10 ug freeze-dried powder in 500ul DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) (1:1) medium. The concentration of the solution was 20ng/ul and divided into sterile EP tubes (100ul per EP tube), and then stored at -20℃. Dilute when used. The final concentration is 20ng/ mL.

3.4.2 Epidermal Growth Factor (EGF) Solution

The lyophilized powder was dissolved in 1000ul DMEM/F12 (1:1) medium with a final concentration of 20ng/ul. The powder was divided into sterile EP tubes with a concentration of 100ul per tube and stored at -20 ℃. When used, the final concentration is 20ng/mL.

3.4.3 PBS Solution (Alkaline Phosphate Buffer without Ca and Mg)

0.01m PBS dry powder (2L dosage) which has been configured by Beijing Solebo Technology Co., LTD to dissolve in 2000ml double steamed water, adjust the PH value in the range of 7.2-7.4, and sterilize by high temperature steam after separate packaging for later use.

3.4.4 Serum-Free Culture Medium Solution for NSCs

Serum-free neural stem cell culture medium (SFM) should be kept fresh. It is usually prepared once a week, about 100ml at a time. The specific preparation method was as follows: bFGF and EGF (both 20ng/ml), glutamine (2mM), B27 additive (2%) and penicillin-streptomycin double antibody solution (100U/ mL) were added to 100mL DMEM/F12 (1:1). Filter it through microporous filter (0.22um), then adjust PH to 7.2-7.4 with NaOH, and store at 4℃ for later use.

3.4.5 Mixed Enzyme Solution

0.04g trypsin, 0.02 hyaluronidase and 0.004g canine organic acid were weighed and dissolved in the prepared PBS solution in a fixed volume of 30ml. Filtered through a micro-porous filter (0.22um), and then divided into sterile EP tubes, 1ml for each EP tube, and stored at -20℃ for later use.

3.4.6 Thiazolam MTT Solution

MTT0.5g was dissolved in 100ml phosphate buffer (PBS) medium. Remove bacteria from solution with a 0.22μm filter and store at 4 ° C, dark. During configuration, containers must be wrapped in aluminum foil to protect them from the bright light.

3.4.7 DAPT Solution

Each 10mg lyophilized powder was dissolved in 500ul dimethyl sulfoxide (DMSO) at a final concentration of 20ug/ul. The solution was packed in sterile EP tubes (100ul per EP tube) and stored at -20℃. When used, it also needs to be diluted so that its final concentration is 50umol/L.

All the reagents and the instruments used for this study are listed in the Appendix B and Appendix C, respectively.

3.5 Culture Methods

3.5.1 Sampling of NSCs and Primary Neural Stem Cell Culture

Newborn SD rats were selected (within 2-3 days), soaked in 75% alcohol for disinfection for 10min, and then placed in a super-clean table. The cranial cavity of newborn SD rats was exposed surgically, and the brain tissue was isolated and placed in a sterile incubator (PBS solution of 0.01M, PH 7.2, and 37℃ water bath for 10min beforehand, the same as below). The pia meningeal and choroid plexus tissues were removed under anatomic microscope after the blood stains on the surface of the brain tissue were washed. Wash it again and transfer it to a second sterile incubator. The brain tissue of the isolated newborn SD rats (within 2-3 days) was then cut into small fragments of about 1 mm3 with a curved scalpel and transferred to a centrifugal tube(10ml, the same as below) with a sterile pipette . After centrifugation (1000rpm, 1min), the clear liquid above the centrifuge tube is discarded. Each cortex was divided into three test tubes, and 1ml of the enzyme mixture was added to each tube. They were then gently oscillated in a 37℃ water bath for 5min, centrifuged at 1000rpm for

5min, and the supernatant was discarded. 0.2 volume of 5% fetal bovine serum was added, and the pipette was used to blow the serum gently for about 10 times, then it was gently shaken under 37℃ water bath for 5min, centrifuged at 1800rpm for 5min, and the supernatant was discarded. Add DMEM/F12 (1:1) medium and gently blow with pipette 20 times. The cells were then filtered through a stainless steel cell sieve (200 mesh), centrifuged for 5min at 1000rpm, and the supernatant was discarded. The cells were then suspended with SFM0.5ml and gently blown several times to form a single cell suspension. The cells were inoculated with a density of 1×10^6/ mL (counting cell density of Typan blue, excluding dead cells) in 25mL disposable culture bottles or glass culture bottles and then cultured in a constant temperature incubator with saturation humidity and 5% CO2 at 37℃. Centrifugation was performed at intervals of 1 to 2 days (1000rpm, 5min), and then half of the liquid was changed, and subculture was carried out at about 4 to 5 days depending on the situation. Cell growth was observed daily under an inverted phase contrast microscope. The contents were observed and recorded, including culture medium color, impurities in culture medium, cell refraction, density, neuroglobule formation size and refraction, etc.

3.5.2 Subculture of NSCs

After 4-5 days of primary culture (determined by the growth of cells and nerve balls), all nerve balls and culture medium were transferred and collected into 15mL conical tubes. Centrifuge the conical tube (1000rpm, 5min). After discarding the clear liquid above, add 1ml TrypLETM. Then gently blow with the pipette. Then，they were gently shaken under 37℃ water bath for 5min, centrifuged at 1800rpm for 5min, and the supernatant was discarded. Add SFM 0.5ml to resuspend the cells, and gently blow

the cells about 10 times with 1000ul (about 20 times with 200ul). The cells were inoculated in 25mL disposable culture flask or glass culture flask with a density of 1×10^6/ mL (counting cell density of Trypan blue, excluding dead cells), and then cultured in the above constant temperature incubator (saturated humidity, 37℃, 5% CO2 constant temperature incubator).

3.6 Cryopreservation and Recovery of NSCs

Single cell suspension was obtained by passage of NSCs. 1ml of freshly prepared freezed-storage solution (DMEM/F12 base medium: Fetal bovine serum: DMSO= 7:2:1) was added, and the cell density was adjusted to 5×106/ mL. Place the tube in a 4℃ refrigerator for 3 hours, and then put it in a plastic foam box. Then immediately placed in the liquid nitrogen gas phase (-80℃) overnight, and finally frozen in the liquid phase of liquid nitrogen. When the cells were resuscitated, the cryotube was removed from the liquid phase of liquid nitrogen and immediately placed in a 37℃ water bath for rapid melting. The cells were transferred into the centrifuge tube and slowly added into 4ml base culture medium. After centrifugation (1000rpm, 5min), the supernatant was discarded. SFM was added to resuspended cells, and the cells were inoculated in 25mL disposable or glass culture bottles at a density of 1×106/ mL (counting cell density of Trypan blue, excluding dead cells) and then cultured in the above constant temperature incubator.

3.7 Identification of NSCs

The NSCs transmitted to the fifth generation and those recovered after cryopreservation were identified. The self-proliferation and renewal ability of the cells

was evaluated by BrdU incorporation method. This technique is particularly useful for studying cell proliferation rates, identifying cells in different phases of the cell cycle, and assessing tissue turnover in various biological contexts. If Brdu is positive by immunocytochemical test with specific antibody, it indicates that the cell is in the period of division and proliferation. Therefore, the division, proliferation and renewal potential of NSC can be identified by detecting Brdu. Specific surface markers and multidirectional differentiation of NSCs were identified by immunofluorescence staining with different neural specific antibodies (anti-nestin, NF200, NSE and GFAP antibodies).

3.8 Different Immunofluorescence Staining Techniques

The proliferation and renewal ability of NSC was identified by Immunofluorescence staining of BrdU**,** Nestin, DAPI, GFAP and NSE techniques.

3.8.1 Immunofluorescence Staining of BrdU

In the case of BrdU, the NSCs of the fifth generation were taken and cultured, and BrdU was added into SFM culture medium 24h after passage. The final concentration of BrdU is 10umol/L. After further culture for 5 days, the suspension cultured NSCs were inoculated with 1×10^6,500 ul into a 24-well plate with poly-lysine-coated cover glass, and then put in a constant temperature incubator with saturated humidity, 37℃ and 5% CO2 for 4h. The following steps are used for the Immunofluorescence staining method.

- Step 1: The culture medium was sucked up and the cells were washed with PBS solution (0.01mol/L, PH7.2) 3 times, 5min each time (cleaning gently).

- Step 2: 4% paraformaldehyde was added and fixed at room temperature for 15min.
- Step 3: The fixative solution was sucked and washed with PBS solution (0.01mol/L, PH7.2) for 3 times, 5min each.
- Step 4: The sealing solution (containing 10% sheep serum and 0.3%Triton for sealing) was incubated at 37℃ for 1h (150ul per well, covered with glass slides). After removing the sealing solution, directly add primary-anti-mouse anti-BrdU monoclonal antibody (diluted with PBS solution to the final concentration of 1:200) 150ul and spread the cover glass. PBS solution (0.01mol/L, PH7.2) was used instead of primary antibody as negative control. It was placed in a constant temperature incubator and incubated at 37℃ for 2h.
- Step 5: After suck up the liquid, the specimens were washed with PBS (0.01mol/L, PH7.2) for 3 times, 5min each.
- Step 6: Fitc-labeled sheep anti-mouse IgG secondary antibody (diluted with PBS to a final concentration of 1:40). Then it was placed in a constant temperature incubator (37℃) for incubation for 1h.
- The light was avoided in each of the following steps.
- Step 7: The second antibody was aspirated and the specimen was washed with PBS (0.01mol/L, PH7.2) 3 times, 5min each.
- Step 8: Drop 20ul of anti-fluorescence quenching agent on the slide.
- Step 9: The home-made right-angle tip lifted the cover glass and then loaded the cover glass with the cell side. Avoid bubbles.
- Step 10: Finally, it was observed, photographed and recorded under fluorescence microscope.

3.8.2 Immunofluorescence Staining of Nestin

In the case of nestin, a small number of NSCs, which were passed to the fifth generation and cultured into nerve spheres, were directly inoculated into a 24-well plate of polylysine-coated cover glass. Then it was placed in the incubator with saturated humidity, 37℃ and 5% CO2 constant temperature for further cultivation for 4h. The following steps are used for the immunofluorescence staining method.

- Step 1: The culture medium was sucked up and the cells were washed with PBS solution (0.01mol/L, PH7.2) 3 times, 5min each time (cleaning gently).
- Step 2: 4% paraformaldehyde was added and fixed at room temperature for 15min.
- Step 3: The fixative solution was sucked and washed with PBS solution (0.01mol/L, PH7.2) for 3 times, 5min each.
- Step 4: The sealing solution (containing 10% sheep serum and 0.3%Triton for sealing) was incubated at 37℃ for 1h (150ul per well, covered with glass slides). After removing the sealing solution, directly add primary-anti-rabbit anti-Nestin polyclonal antibody (diluted with PBS solution to the final concentration of 1:200) 150ul and spread the cover glass. PBS solution (0.01mol/L, PH7.2) was used instead of primary antibody as negative control. It was placed in a constant temperature incubator and incubated at 37℃ for 2h.
- Step 5: Suck up the liquid in step 5, then the specimens were washed with PBS (0.01mol/L, PH7.2) for 3 times, 5min each.
- Step 6: RBITC-labeled Sheep anti-rabbit IgG secondary antibody (diluted with PBS to a final concentration of 1:40). Then it was placed in a constant temperature incubator (37℃) for incubation for 1h.

The light was avoided in each of the following steps.

- Step 7: The second antibody in ⑦ was aspirated and the specimen was washed with PBS (0.01mol/L, PH7.2) 3 times, 5min each.
- Step 8: DAPI stained nuclei were added (150ul per hole, covered with cover glass) and DAPI dye was removed after 3min reaction at normal room temperature. And then the specimen was washed with PBS (0.01mol/L, PH7.2) 3 times, 5min each. Drop 20ul of anti-fluorescence quenching agent on the slide.
- Step 9: The home-made right-angle tip lifted the cover glass and then loaded the cover glass with the cell side. Avoid bubbles.
- Step 10: Finally, it was observed, photographed and recorded under fluorescence microscope.

3.9 Immunofluorescence Staining of GFAP (Astrocytes)

In the case of GFAP the appropriate amount of the 5th generation NSC was inoculated into a 24-well plate with polylysine-coated cover glass by using SFM containing 5% FBS. Then put in a constant temperature incubator with saturated humidity, 37℃ and 5% CO2 for 5 days. The following steps are used for the Immunofluorescence staining method.

- Step 1: The culture medium was sucked up and the cells were washed with PBS solution (0.01mol/L, PH7.2) 3 times, 5min each time (cleaning gently).
- Step 2: 4% paraformaldehyde was added and fixed at room temperature for 15min.
- Step 3: The fixative solution was sucked and washed with PBS solution (0.01mol/L, PH7.2) for 3 times, 5min each.

- Step 4: The sealing solution (containing 10% sheep serum and 0.3%Triton for sealing) was incubated at 37℃ for 1h (150ul per well, covered with glass slides).

- Step 5: After removing the sealing solution, directly add Primary-anti-mouse anti-GFAP monoclonal antibody (diluted with PBS solution to the final concentration of 1:200) 150ul and spread the cover glass. PBS solution (0.01mol/L, PH7.2) was used instead of primary antibody as negative control. It was placed in a constant temperature incubator and incubated at 37℃ for 2h.

- Step 6: The liquid were drained and then the specimens were washed with PBS (0.01mol/L, PH7.2) for 3 times, 5min each.

- Step 7: RBITC-labeled sheep-anti-mouse IgG secondary antibody (diluted with PBS to a final concentration of 1:40). Then it was placed in a constant temperature incubator (37℃) for incubation for 1h.

- The light was avoided in each of the following steps.Step 8: The second antibody in was aspirated and the specimen was washed with PBS (0.01mol/L, PH7.2) 3 times, 5 min each.

- Step 9: Drop 20ul of anti-fluorescence quenching agent on the slide.

- Step 10: The home-made right-angle tip lifted the cover glass and then loaded the cover glass with the cell side. Avoid bubbles.

Finally, it was observed, photographed and recorded under fluorescence microscope.

3.10 Immunofluorescence Staining of NF200 (Neurons)

The following steps are used for the Immunofluorescence staining method.

- Step 1: The appropriate amount of the 5th generation NSC was inoculated into a 24-well plate with polylysine-coated cover glass by using SFM containing 5% FBS. Then put in a constant temperature incubator with saturated humidity, 37℃ and 5% CO2 for 5 days.

- Step 2: The culture medium was sucked up and the cells were washed with PBS solution (0.01mol/L, PH7.2) 3 times, 5min each time (cleaning gently).
- Step 34% paraformaldehyde was added and fixed at room temperature for 15min.
- Step 4The fixative solution was sucked and washed with PBS solution (0.01mol/L, PH7.2) for 3 times, 5min each.
- Step 5: The sealing solution (containing 10% sheep serum and 0.3%Triton for sealing) was incubated at 37℃ for 1h (150ul per well, covered with glass slides).
- After removing the sealing solution, directly add Primary-anti-mouse anti-NF200 monoclonal antibody (diluted with PBS solution to the final concentration of 1:200) 150ul and spread the cover glass. PBS solution (0.01mol/L, PH7.2) was used instead of primary antibody as negative control. It was placed in a constant temperature incubator and incubated at 37℃ for 2h.
- Step 6: The liquid were drained and then the specimens were washed with PBS (0.01mol/L, PH7.2) for 3 times, 5min each.
- Step 7: RBITC-labeled sheep-anti-mouse IgG secondary antibody (diluted with PBS to a final concentration of 1:40). Then it was placed in a constant temperature incubator (37℃) for incubation for 1h.
- Step 8: The second antibody in step 7 was aspirated and the specimen was washed with PBS (0.01mol/L, PH7.2) 3 times, 5min each.
- Step 9: Drop 20ul of anti-fluorescence quenching agent on the slide.
- Step 10: The home-made right-angle tip lifted the cover glass and then loaded the cover glass with the cell side. Avoid bubbles.
- Step 11: Finally, it was observed, photographed and recorded under fluorescence microscope.

3.11 Immunofluorescence Staining of NSE (Neurons)

The following steps are used for the Immunofluorescence staining method.

- Step 1: The appropriate amount of the 5th generation NSC was inoculated into a 24-well plate with polylysine-coated cover glass by using SFM containing 5% FBS. Then put in a constant temperature incubator with saturated humidity, 37℃ and 5% CO2 for 5 days.

- Step 2: The culture medium was sucked up and the cells were washed with PBS solution (0.01mol/L, PH7.2) 3 times, 5min each time (cleaning gently).

- Step 3: 4% paraformaldehyde was added and fixed at room temperature for 15min.

- Step 4The fixative solution was sucked and washed with PBS solution (0.01mol/L, PH7.2) for 3 times, 5min each.

- Step 5: The sealing solution (containing 10% sheep serum and 0.3%Triton for sealing) was incubated at 37℃ for 1h (150μl per well, covered with glass slides). After removing the sealing solution, directly add Primary-anti-mouse anti-NSE monoclonal antibody (diluted with PBS solution to the final concentration of 1:200). 150 ul and spread the cover glass. PBS solution (0.01mol/L, PH7.2) was used instead of primary antibody as negative control. It was placed in a constant temperature incubator and incubated at 37℃ for 2h.

- Step 6: The liquid were drained and,then the specimens were washed with PBS (0.01mol/L, PH7.2) for 3 times, 5min each.

- Step 7: RBITC-labeled sheep-anti-mouse IgG secondary antibody (diluted with PBS to a final concentration of 1:40). Then it was placed in a constant temperature incubator (37℃) for incubation for 1h.

- Step 8: The second antibody as aspirated and the specimen was washed with PBS (0.01mol/L, PH7.2) 3 times, 5min each.

- Step 9: Drop 20μl of anti-fluorescence quenching agent on the slide.Step 10The home-made right-angle tip lifted the cover glass and then loaded the cover glass with the cell side. Avoid bubbles.

Finally, it was observed, photographed and recorded under fluorescence microscope.

3.12 Immunofluorescence Staining Protocol with DAPI

The following steps are used for the Immunofluorescence staining method.

- Step 1: Remove the culture medium and wash the cells with PBS solution (0.01 mol/L, pH 7.2) three times for 5 minutes each, gently cleaning the cells.
- Step 2: Fix the cells by adding 4% paraformaldehyde at room temperature for 15 minutes.
- Step 3: Remove the fixative solution and wash the cells with PBS solution (0.01 mol/L, pH 7.2) three times for 5 minutes each.
- Step 4: Incubate the cells with a sealing solution containing 10% sheep serum and 0.3% Triton X-100 at 37°C for 1 hour (150 μl per well, covered with glass slides).
- Step 5: Remove the sealing solution and add DAPI solution (diluted in PBS to a final concentration of 1:1000) to each well (150 μl per well, covered with glass slides). Ensure the cell culture is protected from light during this step.
- Step 6: Incubate the cells at 37°C for 30 minutes.
- Step 7: Remove the DAPI solution and wash the cells with PBS (0.01 mol/L, pH 7.2) three times for 5 minutes each.
- Step 8: Mount the cover glass with anti-fade mounting medium on the slide, ensuring the cells are facing downward to avoid bubbles.

- Step 9: Finally, observe, photograph, and record the stained cells under a fluorescence microscope, using appropriate filters for DAPI fluorescence.

All steps were performed in a dark environment or avoid exposure to light to preserve fluorescence integrity. Adjust the DAPI concentration and incubation time based on specific experimental requirements and cell types.

This protocol outlines the steps for immunofluorescence staining using DAPI to visualize cell nuclei, providing a clear and structured approach for conducting the experiment. Adjustments can be made based on individual experimental conditions and requirements.

3.13 Preparation of Spinal Cord Model of SD Rats

Forty-eight healthy adult female SD rats (weighing 220-250g) were randomly divided into sham operation group (n = 6), operation paralysis group (n = 36), and normal group (n = 6). The rats were kept in cages for 2 weeks under the same environment. The sham operation group and the operation paralysis group were anesthetized with 2% pentobarbital sodium (0.25-0.3ml/100g) by intraperitoneal injection. After the effect of anesthesia, penicillin (5000U/100g) was injected into the thigh muscle. The rats were fixed in prone position, the surgical area (back shaving) was prepared, and a conventional iodophor disinfection towel was applied. An incision of about 6cm in length was made in the middle of the back. The skin and muscle were incised to expose the $T_{8\text{-}10}$ spinous process and lamina. T_9 and part of T_8 and T_{10} spinous processes and lamina were removed by the bonseur, and about 3mm×4mm spinal cord was exposed in this segment. In the surgical paralysis group, a homemade-NYU (New York

University) Impactor -10g impactor was used to strike the T_9 spinal cord from a height of 25mm. The symbol of successful attack is paralysis of lower limbs-rat tail spasmodic swing, lower limbs and body retraction flapping (Basso et al., 1995). Then, close the incision layer by layer. 21-point functional evaluation scale was used from the Basso, Beattie and Bresnahan locomotor rating scale to measure it (Appendix D).

The model animals were isolated and routinely fed with standard feed. Ambient temperature 22~25℃, and regular ventilation. Keep padding soft and absorbable. Intramuscular injection of penicillin (5000U/100g) twice a day in the first 3 days after surgery. Manual discharge 3 times a day, and iodophor was used to disinfect the urethral opening after each manual squeezing of urine. The rats with urethral hemorrhage were given intramuscular penicillin (5000U/100g) 3 times a day and urinated 5 times a day. After the urine was normal, it was restored to artificial timed urination 3 times a day after surgery until the bladder function was restored. The padding should be changed every 2 days after surgery. Weigh the rats once a day or every two days after surgery. If the preoperative weight loss of more than 20% was classified as unqualified, it was not used and executed. In the sham operation group, only lamina fenestration was performed at the same segment. The normal group was inoperable.

3.14 Preparation of Extracts from Paralyzed Spinal Cord

According to the surgical procedure described in 3.2.3.1, the injured spinal cord of the rats in the surgical paralysis group was extracted 8h,24h,72h,5d,7d and 14d after the establishment of the injury model (6 rat models were used at each time point) to prepare spinal cord injury fluid. After the $T_{8\text{-}10}$ spinal cord was fully exposed, the

nerve roots adjacent to the spinal cord were cut with small knife, and then the spinal cord of the segment was removed completely (each specimen weighed about 100~120mg). The removed spinal cord was immediately placed in PBS solution prepared at 4℃. The bloodstains and impurities on the surface were washed, and the capsule and part of nerve roots were pruned off. Each sample was placed in a 2ml grinder, and 1ml homogenized PBS solution was added to each sample at 4℃. After centrifugation (3000rpm, 10min), the supernatant was filtered twice through a needle filter (0.22μm). The supernatant was divided into sterile EP tubes, 1mL for each EP tube, and stored at -20℃ for later use. The T_8-T_{10} segment of the spinal cord was removed in the sham group and the normal group in the same way to prepare spinal cord extract.

3.15 Changes of Spinal Cord Tissue Injury by Hematoxylin-Eosin (HE) Staining

According to the procedures in 3.2.3.1, seven healthy adult female SD rats (weighing 220-250g) were selected to make spinal cord injury models (one of them was a normal control group without surgical treatment). Normal control group rats were intubated to the ascending aorta via left ventricle, and the right atrial appendage was cut open and then injected with about 200 mL normal saline. When the outflow fluid was cleared, 40 g/L paraformaldehyde 250 mL was reinjected. After the rats had stiff limbs, the infusion was stopped. The $T_{8\text{-}10}$ segment of the spinal cord was removed. After the spinal cord capsule and nerve roots were removed under a microscope, the surface blood stains were washed with normal saline. Then soak in 40 g/L paraformaldehyde for a fixed time of 6-8 h. Dehydrated and fixed in a mixture of 20% sucrose and 40 /L paraformaldehyde. The specimens were embedded in paraffin and

made into wax blocks. The samples were stained with hematoxylin-eosin and stored in a refrigerator at 4℃ for future use. The other 6 model rats received the above HE staining at 8 h, 24 h, 3 d, 5 d, 7 d and 14 d of spinal cord injury, respectively. The changes of the normal control group and the paralyzed spinal cord were observed under the microscope, including the differences of red blood cells, inflammatory cells, neurons, edema and cavity formation.

3.16 Measurement of the Proliferation of NSCs by Cell Counting Method

According to the method of subculture of NSCs mentioned above, NSC single cell suspension was obtained by transferring to the fifth generation NSCs. The cell density of single cell suspension was adjusted to 1×10^6/ mL by SFM and inoculated into 9 24-well plates with 500μl per well. Divided into 9 groups（each group of 24 wells，Including:

- Group A - blank control group (PBS solution was added into the medium for co-culture),
- Group B -- normal group (normal spinal cord extract was added into the medium for co-culture),
- Group C - sham operation group (sham operation spinal cord extract was added into the culture medium for co-culture),
- Group D -- paralyzed group 1 (8h paralyzed spinal cord extract was added to the culture medium for co-culture),
- Group E -- paralytic group 2 (24h paralytic spinal cord extract was added to the medium for co-culture),
- Group F-- paralytic group 3 (72h paralytic spinal cord extract was added into the culture medium for co-culture),

- Group G -- paralytic group 4 (5 days paralytic spinal cord extract was added into the medium for co-culture),
- Group H -- paralytic group 5 (7 days paralytic spinal cord extract was added into the medium for co-culture), and
- Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added to the culture medium for co-culture).

Every group was incubated in an incubator with saturated humidity, 37℃ and 5% CO2 constant temperature for further cultivation. Three Wells were taken from each of the 9 groups every 24h. Single cell suspension was obtained by mechanical blowing and the number of cells in every well was counted. The average value of the 3-well count in each group was recorded. The growth curves were plotted with time as abscissa and cell number as ordinate.

3.17 Detection of the Effects of Paralyzed Spinal Cord Extracts at Different Times on NSC Proliferation by Thiazoline (MTT) Colorimetric Assay

3.17.1 Cell Inoculation

The NSCs transmitted to the fifth generation were prepared into single cell suspension and inoculated into 96-well plates at a density of 1×10^6/ml, with a volume of 100ul per well. The grouping and cultivation conditions were the same as before. The volume of spinal cord extract added in each well was changed to 5ul (equivalent to 5% of the volume of culture medium, and the volume of PBS added in blank control group A was changed to 5ul accordingly). 9 reholes were made for each treatment group, and blank group was made at the same time. Each group was incubated in a incubator with saturated humidity, 37℃ and 5% CO2 constant temperature for further cultivation (all treatment factors in each group were the same except for spinal cord

extract and PBS solution in blank control group). Each group was tested at 24, 48, 72, 96h and 120h, respectively.

3.17.2 Colour

10ul MTT solution (5mg/ml) was added to each well during detection. Each group was incubated in a incubator with saturated humidity, 37℃ and 5% CO2 constant temperature for 4h. Add 100ulFormanzan solution to each well, and continue to incubate in the incubator until Formanzan is completely dissolved under an ordinary light microscope. In general, all the purple crystals will dissolve after incubation at 37 ℃ for about 4h.

3.17.3 Colorimetry

The wavelength of 570nm was selected to measure the light absorption value of each hole on the full spectral spectrophotometer, and the results of each group were recorded and compared.

3.18 Detection of Gene Expression of Notch1 and Hes1 by Reverse Transcription-Polymerase Linked Reaction (RT-PCR)

3.18.1 Design and Synthesis of Primers

All primers were designed and synthesized using Primer6 primer design software according to primer design principles, and were synthesized and inspected by Shenggong Bioengineering (Shanghai) Co., LTD. The primer was centrifuged at 3000rpm for 1min before opening the cover. The concentration of primer was 100uM

by adding rnA-free enzyme water dilution and it was stored at -20℃ for later use. When used, dilute to the working liquid concentration of 10uM.

Table 3.1 : The Primer Sequences of Notch1, Hes1 and GAPDH

Gene	**Upstream Primer** (5'-3')	**Downstream Primer** (5'-3')	**Amplification length (bp)**
Notch1	CCAGTACAACCCGCTAAGGC	GGGACAAGGTATTGGTGGAGA	119
Hes1	GCGCCGGGCAAGAATAAATG	TCGGTGTTAACGCCCTCACAC	267
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	450

3.18.2 RNA Extraction

The fifth-generation NSCs at the logarithmic growth stage were inoculated into 6-well plates at a density of 1×10^6/ mL. Six multiple holes were made for each group, with the volume of each hole being 2ml. 100ul of spinal cord extract was added to each well (Group A-blank control group was prepared with 100ul of PBS solution, equivalent to 1/20 volume of medium). NSCs in each group were cultured in the above constant temperature incubator, and total NSC RNA was extracted from each group 24 and 48h after culture, respectively. Total RNA of cells was extracted according to the instructions of the kit. Specific methods were as follows:

NSCs from each treatment group were transferred into a 15ml centrifuge tube (without RNase), centrifuged at 1500rpm for 5min. After discarding supernatant, 1ml lysate RZ was added. After 5min at 15-30℃, the tube was transferred to a new EP tube (1.5ml, RNase free, all the following). 200μl of chloroform was added. The tubes were covered and shook vigorously for 15s, then placed them at room temperature for 3min. Centrifugation was performed at 12000rpm for 10min at 4℃. Transfer the upper water

phase to the new EP tube, approx. 400ul. 0.5 times volume (about 200μl) of anhydrous ethanol was added slowly and mixed. The solution was transferred into adsorption column CR3 and centrifuged at 12,000 rpm for 30s at 4℃. Discard the waste liquid in the collection pipe. 500ul deproteinizing solution RD was added into adsorption column CR_3. Centrifuge at 12,000rpm for 30s at 4℃. Discard the waste liquid in the collection pipe. 600ul rinse solution RW was added to adsorption column CR_3 and stood at room temperature for 2min. Then, centrifuge at 12,000 rpm for 30s at 4℃. Discard the waste liquid in the collection pipe. Repeat this step several times. The adsorption column CR_3 was placed in a 2ml collection tube. Centrifugation at 12,000 RPM, 4℃, 2min. The adsorption column CR_3 was dried in the ultra-clean table. The adsorption column CR_3 was transferred into the new EP tube and 30 ul RNA-free H_2O was added. After standing at room temperature for 2min, centrifugation was performed at 12,000 RPM at 4℃ for 2min. The resulting fluid is the cell's total RNA. The obtained RNA was stored at -70℃ or reverse transcription reaction was performed immediately.

3.18.3 Concentration and Purity of Total RNA

RNA concentration and purity in 2.4.3.2 (general dosage: 2μl) were measured by UV spectrophotometer. The $A_{260/280}$ values of total RNA in each group were all in the range of 1.80-2.0 (Fig.3.2), which indicates that RNA purity was good.

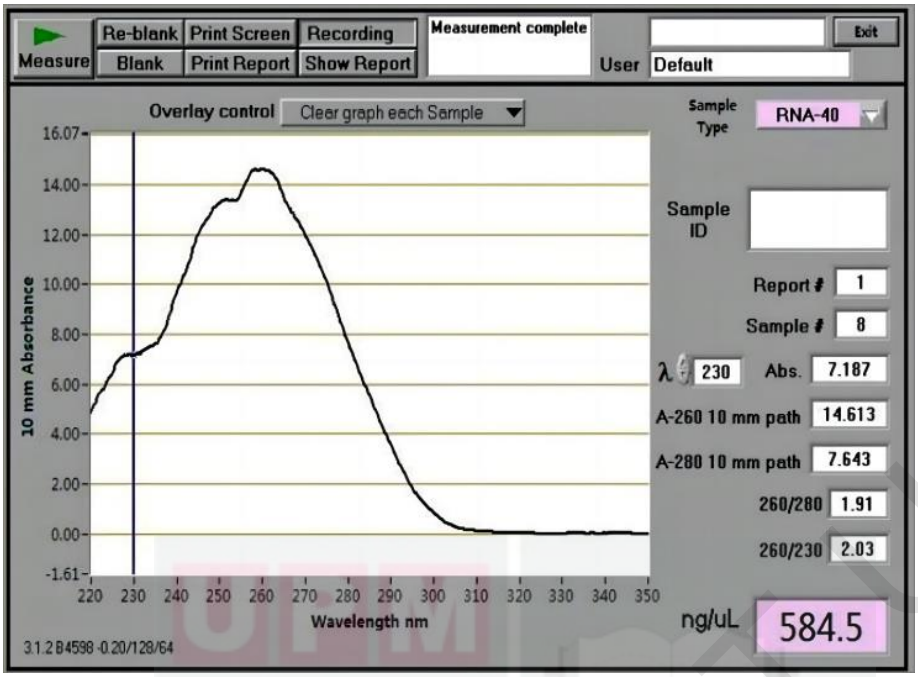

Figure 3.2 : Determination of RNA Concentration and Purity

3.19 Synthesize cDNA by Reverse Transcription

Follow instructions for the BioBRK RT-PCR Kit. First, the reverse transcription reaction fluid was prepared, and the specific reaction system was as follows in the table 3.2:

Table 3.2 : Components Used in PCR

No.	PCR Reaction Mixtures	Volume
1.	RNase-Free H_2O	10µl
2.	5×RT Buffer	4µl
3.	dNTP Mixture	2µl
4.	Super-RI	0.5µl
5.	RT-Enhancer	0.5µl
6.	Oligo（dt）18	1µl
7.	Experimental RNA sample	1µl
8.	ReverTra Ace	1µl
9.	**Total reaction volume**	**20µl**

After the mixture was evenly mixed, the centrifugation was instantaneous at 3000rpm. Then, reverse transcription reaction was carried out on the PCR apparatus according to the following conditions: 42ºC for 35min, 99ºC for 5min followed by 4 ºC for 5 mins for one cycle. After reverse transcription, the cDNA was stored at -20℃ for future use or PCR was performed immediately.

3.20 Reverse Transcription-Polymerase Linked Reaction (RT-PCR)

PCR reaction solution (per sample) was prepared according to the table 3.3. The mixture was mixed well, centrifuge instantly at 3000rpm, and PCR amplification reaction was performed on the PCR instrument. The PCR products obtained were stored at -20℃ or analyzed by agarose gel electrophoresis immediately.

Table 3.3 : PCR Components and the PCR Cycling Conditions Used

No.	PCR Reaction Mixtures	Volume	PCR Cycling Conditions	Time and No. of Cycles
1.	Reverse transcription reaction stock	2 μl	Initial Denaturation: 94ºC	2 mins/1
2.	RNA - Free H_2O	6 μl	Denaturation: 94ºC	
3.	Upstream specific PCR primers	1 μl	The annealing temperature was 58℃ for *Notch1*, 56℃ for *Hes1* and 59℃ for *GAPDH*.	30 seconds/ 30
4.	Downstream specific PCR primers	1 μl	Extension: 72ºC	
5.	2x Taq PCR MasterMix	10μl	Final Extension: 72ºC	10 mins
6.	**Total reaction volume**	**20μl**	Final step at 4ºC	infinity

3.21 Agarose Gel Electrophoresis and Analysis of Electrophoresis Results

The PCR reaction products were electrophoretic on 2% agarose gel at 110 volt for 30-50min with 8μl volume, and then photographed by UV digital gel imaging analysis

system. Grayscale analysis was carried out on the electrophoretic bands of the product by gel image analysis system, and optical density quantitative analysis was carried out with GAPDH as internal reference. The optical density ratio Notch1/GAPDH represented the expression level of Notch1 mRNA, and Hes1/GAPDH represented the expression level of Hes1 mRNA. The experiment was repeated for three times and the average value was taken.

3.22 Measurement of the Proliferation of NSCs by Cell Counting Method

According to the method of subculture of NSCs mentioned above, NSC single cell suspension was obtained by transferring to the fifth generation NSCs. The cell density of single cell suspension was adjusted to 1×10^6/ mL by SFM and inoculated into 7 24-well plates with 500ul per well. Divided into 7 groups (each group of 24 Wells)，including: Group I - blank control group (PBS solution was added into the medium for co-culture), Group II - Normal group (normal SCE was added into the medium for co-culture), Group III - sham operation group (sham operation SCE was added into the medium for co-culture), Group IV - paralysis group (injured SCE was added into the medium for co-culture), Group V - normal + DAPT group (DAPT was added after normal SCE was added to the culture medium for 24h). Group VI- sham operation +DAPT group (DAPT was added after sham operation SCE was added to the culture medium for 24h)， and group VII - paralyzed +DAPT group (DAPT was added after injured SCE was added to the culture medium for 24h) . DAPT concentration of V, VI and VII groups were all 50μmol/L. Each group was incubated in a incubator with saturated humidity, 37℃ and 5% CO2 constant temperature for further cultivation. Three Wells were taken from each of the 7 groups every 24h. Single cell suspension

was obtained by mechanical blowing and the number of cells in every well was counted. The average value of the 3-well count in each group was recorded. The growth curves were plotted with time as abscissa and cell number as ordinate.

3.23 Synthesize cDNA by Reverse Transcription

The extracted and quantified RNA was used for the cDNA synthesis. The instructions were followed as per the guidelines given in the BioBRK RT-PCR Kit. First, the reverse transcription reaction fluid was prepared, and the specific reaction system was as mentioned in table 3.4.

Table 3.4 : Components of the PCR Used for cDNA Synthesis

No.	PCR Reaction Mixtures	Volume
1.	RNase-Free H_2O	10μl
2.	5×RT Buffer	4μl
3.	dNTP Mixture	2μl
4.	Super-RI	0.5μl
5.	RT-Enhancer	0.5μl
6.	Oligo（dt）18	1μl
7.	Experimental RNA sample	1μl
8.	ReverTra Ace	1μl
9.	**Total reaction volume**	**20μl**

After the mixture was evenly mixed, the centrifugation was instantaneous at 3000rpm. Then, reverse transcription reaction was carried out on the PCR apparatus according to the following conditions: 42ºC for 35 mins and 99ºC for 5 mins for a cycle followed by 4ºC for 5 mins. After reverse transcription, the cDNA was stored at -20℃ for future use or PCR was performed immediately.

3.24 Reverse Transcription-Polymerase Linked Reaction (RT-PCR)

PCR reaction solution (per sample) was prepared as per mentioned in the table 3.5. Mixed well, centrifuge instantly at 3000rpm, and PCR amplification reaction was performed on the PCR instrument according to the following conditions mentioned in table 3.6:

Table 3.5 : Components Used in the RT-PCR

Component	Volume (µl)
Reverse Transcription Reaction Stock	2
RNA-Free H2O	6
Upstream Specific PCR Primers	1
Downstream Specific PCR Primers	1
2x Taq PCR MasterMix	10
Total Reaction Volume	**20**

Table 3.6 : PCR Cycling Conditions Used for PCR Amplification

Temperature (℃)	Time	Action	Number of Cycles
94	2 min	Initial Denaturation (1 cycle)	1
94	30 sec	Denaturation	
AT*	30 sec	Annealing	30
72	30 sec	Extension	
4	Untimed	Heat Preservation	N/A

AT- annealing temperature

AT equals annealing temperature*： The annealing temperature was 58℃ for Notch1, 56℃ for Hes1 and 59℃ for GAPDH. The PCR products obtained were stored at -20 ℃ or analyzed by agarose gel electrophoresis immediately.

3.25 Agarose Gel Electrophoresis and Analysis of Electrophoresis

The PCR reaction products were electrophoretic on 2% agarose gel at 110Vd for 30-50min with 8ul volume, and then photographed by UV digital gel imaging analysis

system. Grayscale analysis was carried out on the electrophoretic bands of the product by gel image analysis system, and optical density quantitative analysis was carried out with GAPDH as internal reference. The optical density ratio Notch1/GAPDH represented the expression level of Notch1 mRNA, and Hes1/GAPDH represented the expression level of Hes1 mRNA. The experiment was repeated for 3 times and the average value was taken.

3.26 Influence of DAPT on NSCs Differentiation

According to the method of subculture of NSCs mentioned above, NSC single cell suspension was obtained by transferring to the fifth generation NSCs. The cell density of single cell suspension was adjusted to 1×10^5/ mL by SFM and inoculated into 3 6-well plates with 100μl per well. Divided into 2 groups (The control group was set with 3 multiple holes, the treatment group with 15 multiple holes, and the treatment group with 3 multiple holes at each time point), including: Group i-control group (NSCs culture medium was added with paralytic spinal cord extract for co-culture) and Group ii- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture, DAPT was added to block Notch signal at 1d, 2d, 3d, 5d and 7d, respectively, and the final concentration of DAPT was 50μmol/L). The control group was added with equal doses of DMSO. The cells were incubated for 24 hours in the above constant temperature incubator, and differentiated for 48 hours after induced by 5% fetal bovine serum. The remaining stem cells (excluding nerve spheres) in 30 fields were counted sequentially under an inverted phase contrast microscope with a 40x objective lens. Then immunofluorescence staining was performed. Calculate the average value of 3 holes.

3.27 Fluorescent Staining of Neurons and Astrocytes

Differentiated neurons and astrocytes were rinsed gently with PBS solution prepared at 37℃ for 3 times, 2min each. NSE and GFAP were used for immunostaining identification. The specific methods were the same as before.

Specific antibodies of neurons and astrocytes in this experiment are: Neuron: Primary antibody is mouse anti-NSE monoclonal antibody 1:200. Secondary antibody is RBITC-labeled Sheep anti-mouse IgG 1:40. Astrocytes: Primary antibody is mouse anti-GFAP monoclonal antibody 1:200. Secondary antibody is RBITC-labeled Sheep anti-mouse IgG 1:40.

3.28 Counting and Data Statistics of Neurons and Astrocytes

Under fluorescence microscope, the total number of differentiated cells in 30 fields was sequenced with a 40-power objective lens. The percentage of NSE positive cells (neurons) and GFAP positive cells (astrocytes) at each sealing time point in each group was calculated (the sum of the two was 100%). Calculate the average value of 3 holes.

3.29 Sample Size and Statistical Methods

55 rats initially and then newborns 9 groups X triplicates = 27 rats were used. For all the time points these same mice/cells were used for further studies in this research work.

For qualitative research, descriptive methods were used. For quantitative research, all data were expressed as mean ± standard deviation with normal distribution and

homogeneity of variance ($\bar{X}$ ±s). Comparison between multiple groups was performed by one-way ANOVA. SNK method was used for pairwise comparison. Note: The above detection data were collected and analyzed by SPSS27.0 statistical software. P < 0.05 was considered statistically significant.

CHAPTER 4

RESULTS

4.1 Development of NSCs

The first-generation cells derived from newborn SD rats appear small, equal sized, glossy spheres. At 24 h after the primary culture, larger amounts of cells aggregate in the middle of potion of the growth medium. These single floating cells can differentiated and renewed (Figure 4.1A). There are structured in glossy, spherical-shaped, adherent single cells at the bottom of the culture bottle. Moreover, many structural looseness, highly refractile, spherical-shaped clusters and membraniform flocculation also exist in the culture medium, which are considered as clumps of dead cells and uneliminated tissues such as blood vessels and pia mater, respectively. At 2^{nd} to 3^{rd} days after the primary culture, several and even dozens of translucent, highly refractile, spherical-shaped or thyrsiform cell clusters are discovered (Figure 4.2B). Further, only a small amount dead cell clusters were left in the culture medium. At 4^{th} to 5^{th} days after the primary culture (Figure 4.3C), the cell mass bulks up to a spherical shape when large numbers of cell clusters aggregate and form translucent slippery spheres under an inverted phase-contrast microscope. However, refractivity at the center of the sphere decreases and few aggregated dead cells are observed. Compared with that of neurospheres at five days after the primary culture, the volume of neurospheres at eight days after the primary culture is obviously big and the refractivity is poor and it shows dark brown in most parts of the sphere center (Figure 4.1D). Passaging conducted at that time only dissociates neurospheres into smaller cell clumps and few single floating cells rather than single-cell suspensions. It is speculated that this is associated with tight cell-cell junction around the neurospheres.

Moreover, many small sized adherent cells are observed at two days after the passaging with small quantity of low density single floating cells. Adherent differentiation occurs in plenty of large volume neurospheres during the continuous culture.

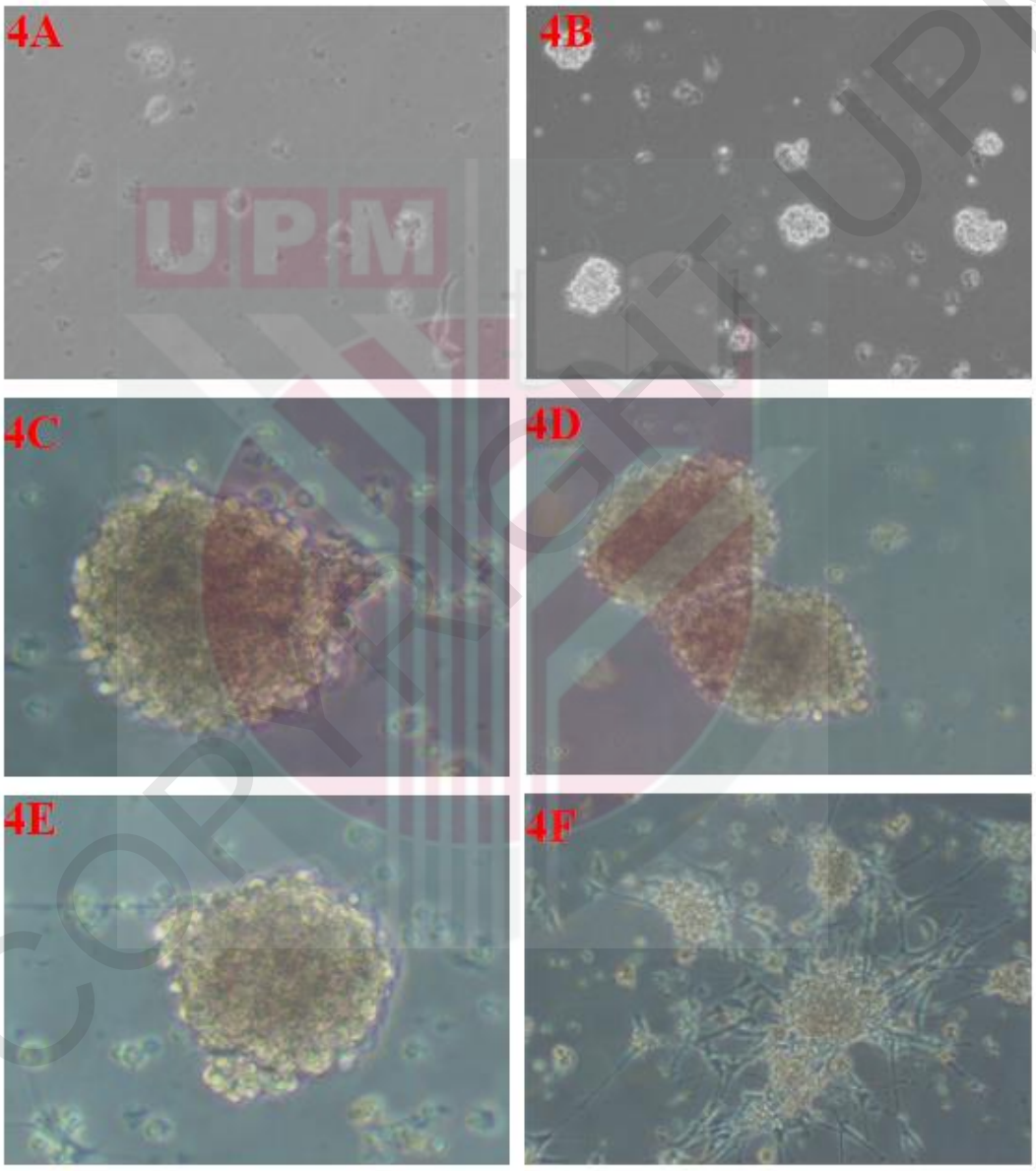

Figure 4.1 : General Character of Newborn Rat's NSC (Inverted Phase Contrast Microscope). Development of NSCs derived from newborn rats (inverted phase-contrast microscope) at 24 h after the primary culture (100×) (A), 3 d after the primary culture (100×) (B), 5 d after the primary culture (100×) (C), 8 d after the primary culture (100×) (D), 4 d after 5 passages (×200) (E) and 5 d after induction of dissociation (×200) (F)

Generally, there are two methods of cell dissociation: mechanical and enzymatic. It is discovered that it is hard to control enzyme concentration and time, which easily lead to cell death. Comparatively, TrypLE™ Express digestion method combined with mechanical disassociation method can harvest sufficient normomorph, uniform size single floating cells with glossy cytoplasm and there are relatively few cell debris and impurities in the culture medium. In addition, medium-sized cloned neurospheres in good shape can be observed in the passaged NSC culturation (Figure 4.1E). At four hours after the induction of dissociation of 5th generation of passaged NSCs using 5% fetal bovine serum, adherent neurospheres and gradual migration of cells away from neurospheres are observed. At approximately five days after the induction of dissociation, spheres become flattened and form various shaped cells surrounding the neurospheres. At approximately five days after the induction of dissociation, the spheres flatten and make up lots of various shapes of cells. These cells connect to each other into a mesh-structure (Figure 4.1F).

4.2 Identification of NCSs

The incorporation of BrdU into the 5th generation of passaged cells (oval shaped clustered cells) showed positive after BrdU immunofluorescence staining (green fluorescence, Figure 4.2A) and nestin immunofluorescence staining (red fluorescence, Figure 4.2B). At five days after the induction of dissociation of 5th generation of passaged cells using 5% fetal bovine serum, NF200 (green fluorescence, DAPI nucleic acid stain revealed the blue fluorescent cell nucleus (oval shape), Figure 4.2C), NSE (red fluorescence, blue fluorescent obtained with DAPI nucleic acid stain, Figure 4.2D) and GFAP (red fluorescence, DAPI nucleic acid stain revealed the blue fluorescent cell nucleus, Figure 4.2E) positive immunoreactivities were found.

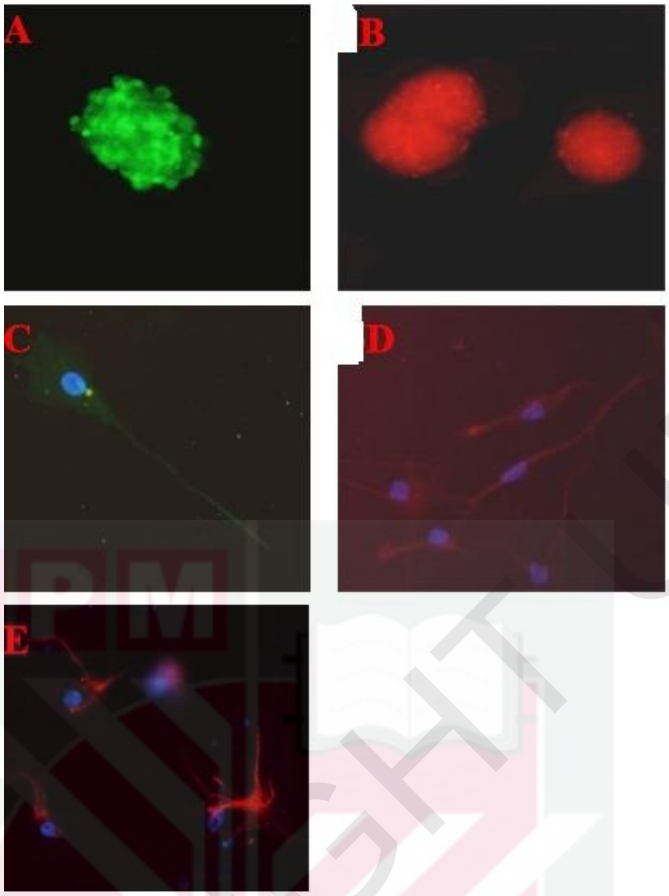

Figure 4.2 : Immunofluorescence Staining and Identification of NSCs. Note: Fig. A: BrdU immunofluorescence staining (green fluorescence, ×100). Fig. 4.2B: Nestin immunofluorescence staining (red fluorescence, ×100). Fig. 4.2C: NF200+DAPI immunofluorescence staining (green fluorescence refers to NF200, blue fluorescence refers to DAPI, ×200). Fig. 4.2D: NSE+DAPI immunofluorescence staining (green fluorescence refers to NSE, blue fluorescence refers to DAPI, ×200). Fig. 4.2F: GFAP+DAPI immunofluorescence staining (green fluorescence refers to GFAP, blue fluorescence refers to DAPI, ×200)

- Red Fluorescence: Indicates specific cellular structures or organelles; changes reflect alterations in cellular organization or dynamics.
- Green Fluorescence: Represents gene expression, protein localization, or cellular processes; changes signify variations in protein abundance or localization.
- Blue Fluorescence: Stains cell nuclei; used for assessing cell proliferation, nuclear morphology, or cell cycle progression.

4.3 Preparation of Spinal Cord Model of SD Rats

The methods of preparation of spinal cord model of SD rats were described in the methodology section.

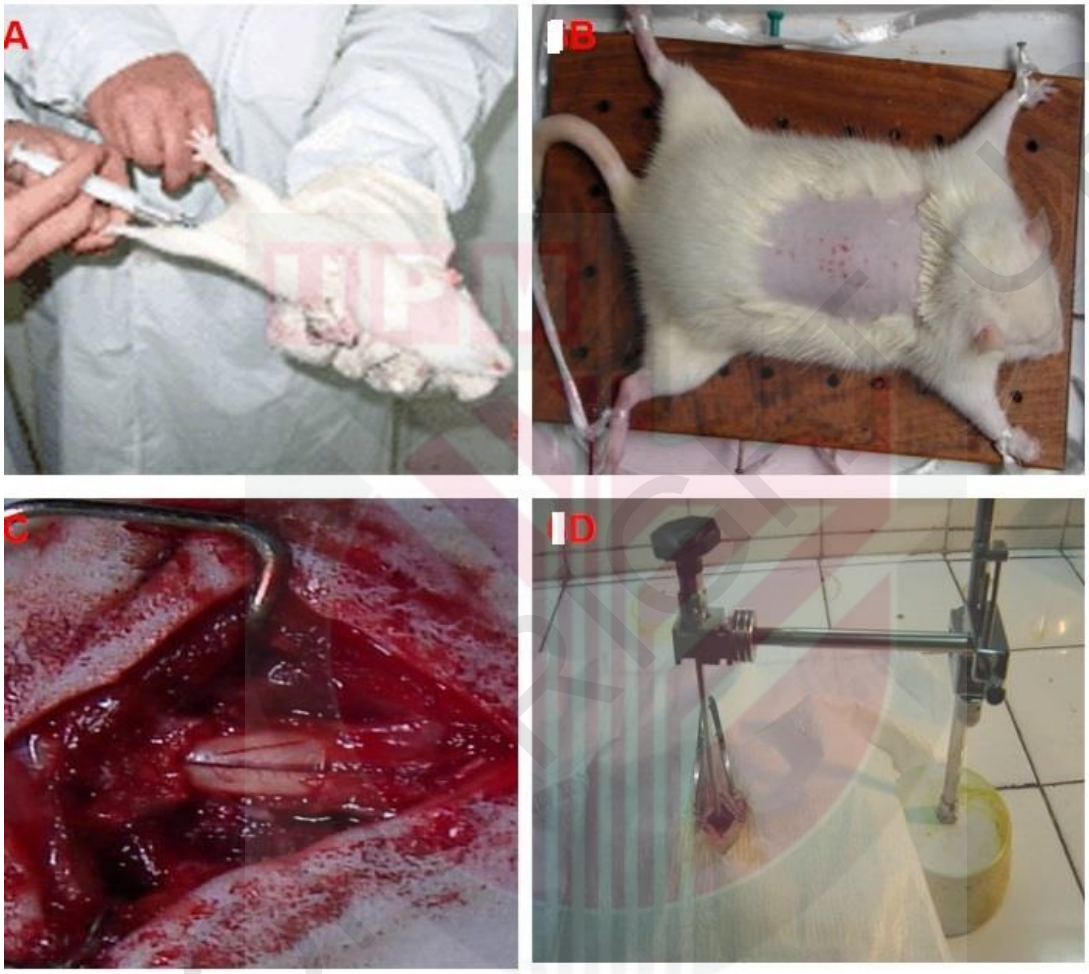

Figure 4.3 : Development of Spinal Cord Injury Model in Rats. Fig. 4.3A: The rats were anesthetized. Fig. 4.3B: The rats were immobilized and skinned before operation. Fig. 4.3C: The exposed spinal cord during the operation. Fig. 4.3D: Rat model of spinal cord injury by Weight-Drop method

Healthy adult female SD rats (weighing 220-250g) were chosen. After the effect of anesthesia, the rats were fixed in prone position. An incision of about 6cm in length was made in the middle of the back. T9 and part of T8 and T10 spinous processes and lamina were removed by the bonseur, and about 3mm×4mm spinal cord was exposed

in this segment. In the surgical paralysis group, a homemade-NYU (New York University) Impactor -10g impactor was used to strike the T9 spinal cord from a height of 25mm. The symbol of successful attack is paralysis of lower limbs-rat tail spasmodic swing, lower limbs and body retraction flapping (Basso et al., 1995). The model of spinal cord injury in rats is shown in Figure 4.3A-E. Totally, 3 rats per treatment of group were used for the study.

4.4 Tissue Changes of Injured Spinal Cord by Using Hematoxylin-Eosin Staining (HE Staining)

HE staining can reflect the pathological changes after SCI. The pathophysiological changes of SCI are the basis of our study. Through HE staining, we found the pathological changes of the spinal cord at different injured time points were different, which may also indicate that the paralyzed spinal cord extract at different injured time points is also different.

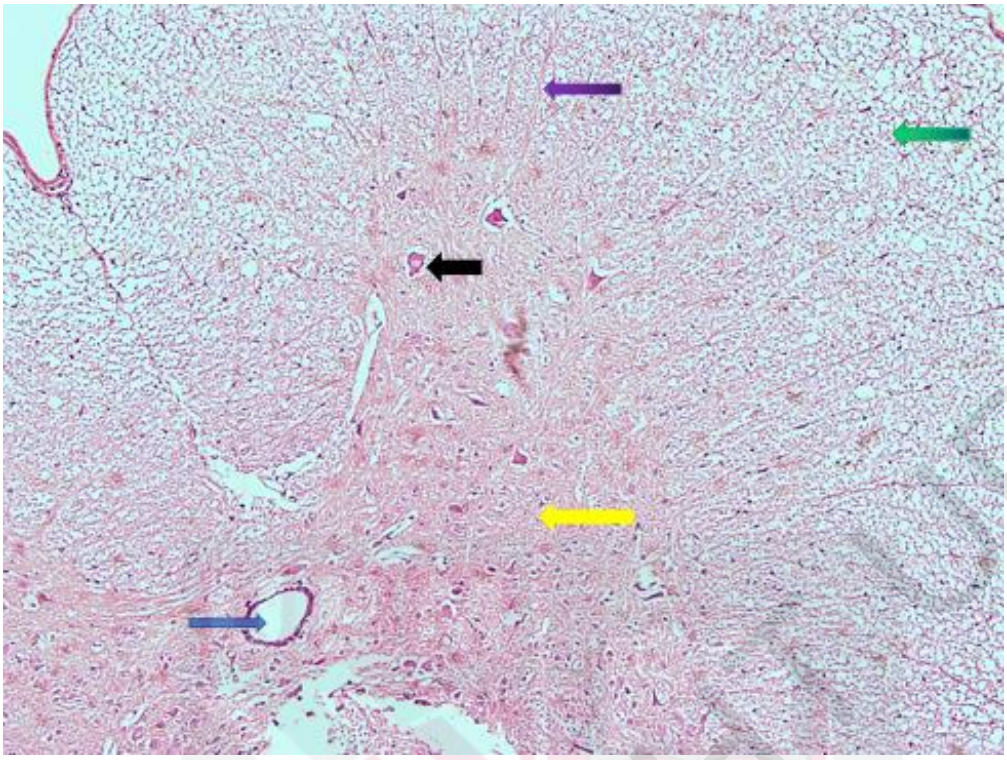

Figure 4.4 : Histopathology of Normal Rat Spinal Cord. Blue arrow: central canal of spinal cord. Green arrow: white matter. Yellow arrow: gray matter. Black arrow: Normal neurons. Purple arrow: Nerve fibers

In the normal spinal cord (Figure 4.4), the tissue structure is regular and the fibers are arranged neatly. The boundary between gray matter and white matter is clear, the gray matter cells are evenly distributed, the cell bodies are large, the nucleoli are obvious, and the neurons are abundant. The nerve fibers in the white matter are arranged along the longitudinal axis of the spinal cord.

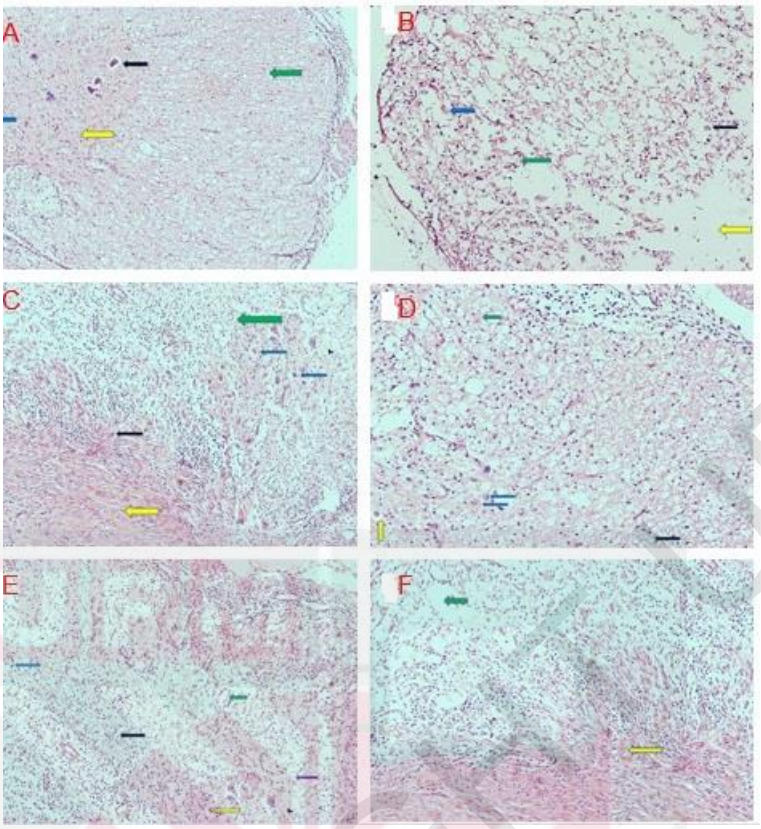

Figure 4.5 : A-F. The Histopathology of Rats with Spinal Cord Injury

- 8 hours after SCI (Figure 4.5A): Neurons and glial cells were abundant in the gray matter, the gray matter boundary could be distinguished, and a few red blood cells exudated. Note：Blue arrow: red blood cells. Green arrow: white matter. Yellow arrow: gray matter. Black arrow: Normal neurons.

- 24 hours after SCI (Figure 4.5B): the spinal cord tissue structure was disturbed, the gray matter was damaged, the central duct was ruptured, a large number of red blood cells infiltrated, the number of neurons decreased, and the residual neuron swelling with vacuole formation. Note：Blue arrow: red blood cells. Green arrow: the destruction of white matter. Yellow arrow: the central duct was ruptured. Black arrow: the residual neuron swelling with vacuole formation.

- 3 days after SCI (Figure 4.5C): The boundary between gray and white matter is not clear, and nerve cells are reduced. In gray matter, a large number of erythrocytes exudated, some glial cells and neurons showed cell body

shrinkage, nuclear shrinkage, nuclear fragmentation, apoptotic corpuscles formation and other apoptotic characteristics. Note: Blue arrow: neurons showed cell body shrinkage, nuclear shrinkage, and nuclear fragmentation. Green arrow: the destruction of white matter. Yellow arrow: the destruction of gray matter. Black arrow: red blood cells.

- 5 days after SCI (Figure 4.5D): Reduction and even necrosis of nerve cell bodies and glial cells of neurons can be seen in the anterior and posterior corners of gray matter. Inflammatory exudation increased and erythrocyte distribution area expanded. The posterior structure of the white matter was damaged with a small amount of inflammatory cell infiltration and spot bleeding. Note: Blue arrow: red blood cells. Green arrow: the destruction of white matter. Yellow arrow: neurons showed cell body shrinkage, nuclear shrinkage, and nuclear fragmentation. Black arrow: Inflammatory exudation increased.

- 7 days after SCI (Figure 4.5E): Edema and bleeding improved, significantly less than before. Neuronal cells continue to decrease and necrotic tissue fragments appear. Voids form in the gray and white matter areas. In some areas, glial cell proliferation, capillary formation, inflammatory cell infiltration is obvious. Note: Blue arrow: glial cell proliferation. Green arrow: capillary formation. Yellow arrow: neurons showed cell body shrinkage, nuclear shrinkage, and nuclear fragmentation. Black arrow: Inflammatory exudation increased .Purple arrow: necrotic area.

- 14 days after SCI (Figure 4.5F): The arrangement of the spinal cord tissue is loosened and the number of neurons is further reduced. Cavity and glial scar formation. Note: Green arrow: cavity formation. Yellow arrow: glial scar formation.Overall the histological changes post-spinal cord injury progress from initial abundant neurons and glial cells with red blood cell exudation, to tissue disruption, gray matter damage, neuronal loss, and inflammation, culminating in necrosis, glial scar formation, and tissue remodeling over time, gradually 8 hours to 14 days as shown in the figure 4.5.

4.5 Effect of Spinal Cord Extracts on Growth Curves of NSCs

9 groups (Group A-I) were cultured for 5 days, and the number of NSC cells in each group was measured at each time points on day 1, 2, 3, 4 and 5 (Table 4.1). After statistical analysis, the number of cells in the paralysis group was significantly higher than that in groups A, B and C at each time point ($P<0.05$), the number of cells in group H (adding 7d paralyzed spinal cord extract in the medium for co-culture) was the highest, which was higher than that in other paralyzed groups, while there was no significant difference in the number of cells in groups A, B and C at each time point ($p> 0.05$). The growth curves of NSCs in each group were shown in Figure 4.6.

Table 4.1 : Cell Numbers of NSCs of Each Groups at Different Time Point (Mean ± SD, $\times 10^6$, n =3)

Group	1d	2d	3d	4d	5d
A	0.663±0.031	0.920±0.026	1.767±0.025	2.363±0.050	2.707±0.050
B	0.667±0.031	0.907±0.032	1.783±0.031	2.280±0.046	2.697±0.021
C	0.677±0.153	0.873±0.021	1.780±0.020	2.313±0.055	2.683±0.012
D	0.797±0.153	1.213±0.025	2.003±0.055	2.563±0.031	2.903±0.025
E	0.827±0.125	1.287±0.031	2.167±0.055	2.667±0.035	2.997±0.025
F	0.887±0.115	1.310±0.026	2.223±0.021	2.723±0.021	3.007±0.026
G	0.937±0.145	1.403±0.025	2.410±0.031	3.057±0.029	3.233±0.025
H	0.997±0.125	1.493±0.025	2.511±0.030	3.007±0.027	3.433±0.015
I	0.897±0.153	1.313±0.025	2.103±0.055	2.663±0.031	2.993±0.025

Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added).

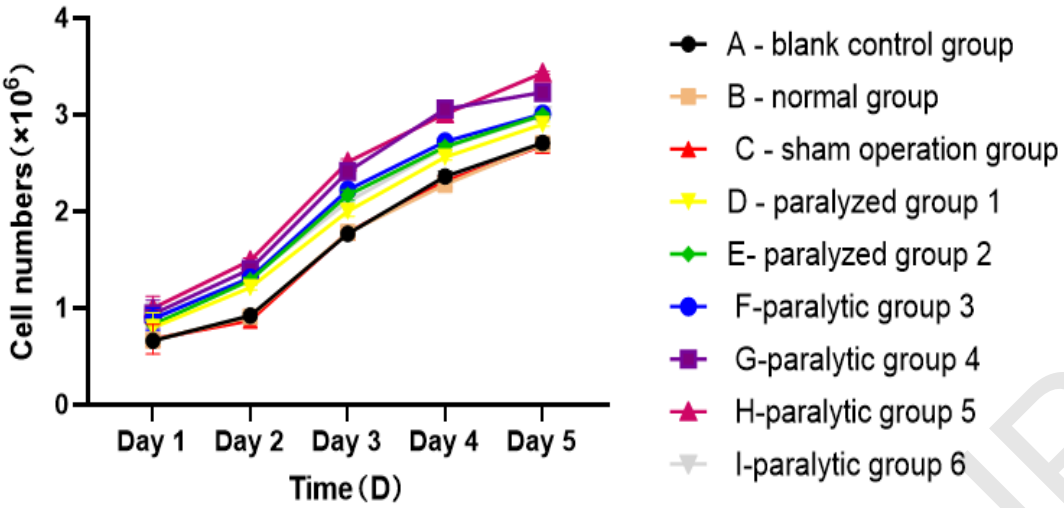

Figure 4.6 : Curve of Growth of NSCs in Each Group. Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added)

4.6 The Results of MTT Assay on the Proliferative Ability of NSCs in Each Group

9 groups (Group A-I) were cultured for 5 days, and MTT values of NSCs in each group were measured at each time point on day 1, 2, 3, 4 and 5 (Table 4.2). Statistical analysis showed that there was no significant difference in cell MTT values among groups A, B and C at each time point ($P > 0.05$). The MTT value in the paralysis group was significantly higher than that in groups A, B and C ($P<0.05$), and the MTT value of cells in group H (that is, 7 days paralyzed spinal cord extract was added to the medium for co-culture) was significantly higher than that in other 8 groups at each time point ($P<0.05$). The results of proliferation ability of NSCs in each group are shown in Figure 4.7. All the raw data values are mentioned in the Appendix F.

Table 4.2 : The MTT Values of NSCs of Each Groups at Different Time Point (Mean ± SD, n=10)

Group	1d	2d	3d	4d	5d
A	0.1317±0.0250	0.1860±0.0187	0.2378±0.0288	0.2865±0.0238	0.3224±0.0175
B	0.1424±0.0331	0.1813±0.0324	0.2452±0.0310	0.2806±0.0337	0.3094±0.0273
C	0.1487±0.0303	0.1955±0.0311	0.2276±0.0273	0.3070±0.0197	0.3149±0.0173
D	0.1939±0.1613	0.2384±0.0353	0.2911±0.3306	0.3533±0.0274	0.3831±0.0166
E	0.1839±0.1513	0.2284±0.0313	0.2951±0.3106	0.3733±0.0284	0.3931±0.0216
F	0.1919±0.1523	0.2287±0.0313	0.2851±0.3106	0.3633±0.0287	0.3911±0.0197
G	0.2039±0.1516	0.2784±0.0343	0.3116±0.3376	0.3733±0.0268	0.4331±0.0182
H	0.2139±0.1733	0.2864±0.0253	0.3217±0.3276	0.3812±0.0297	0.4376±0.0232
I	0.1899±0.1589	0.2384±0.0373	0.2811±0.2913	0.3511±0.0274	0.3831±0.0147

Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added).

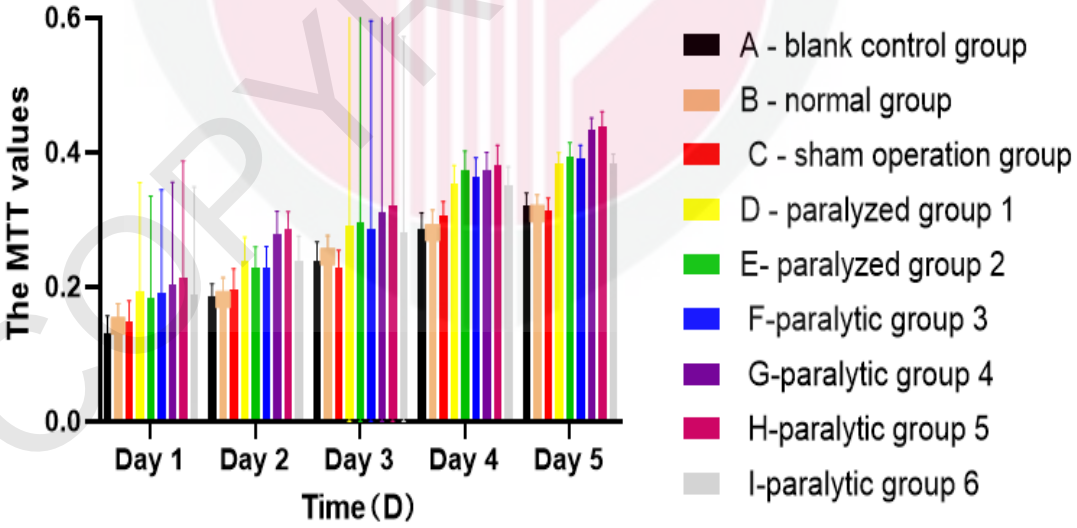

Figure 4.7 : The MTT Values of NSCs of Each Groups at Different Time Point. Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic

spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added)

4.7 RNA Integrity Detection

The results of agarose gel electrophoresis showed that three bands (5s, 18s, 28s) were visible in the extracted RNA. The rRNA band brightness ratio at 28s and 18s is greater than 1, indicating that the RNA has not been degraded and has good integrity (Figure 4.8).

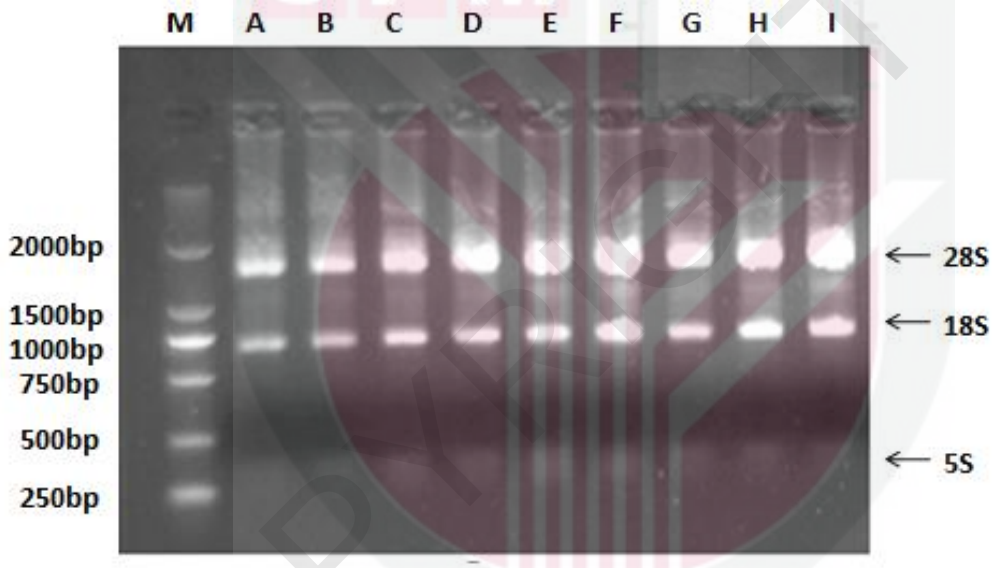

Figure 4.8 : RNA Integrity Detection of Each Group. Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added). M: DNA Marker

4.8 Detection of Gene of the Expression of Notch1 and Hes1 by Reverse Transcription-Polymerase Linked Reaction (RT-PCR)

The gray scale analysis and comparison of electrophoresis bands of PCR products in each group were conducted, and the relative copy number of mRNA of Notch1 and Hes1 genes in each group was calculated using internal reference as the standard. The expression of mRNA in each group after co-cultured for 24 hours and 48h were exhibited in Table 4.3, 4.4 and Figure 4.9, 4.10. Melting curve analysis of Notch1 and Hes1 mRNA of each group after co-cultured for 24h and 48h (Appendix E).

Table 4.3 : The Expression of mRNA in Each Group after Co-Cultured for 24 Hours (Mean ± SD, n =3)

Group	Notch1	Hes1
A	1.003±0.097	1.016±0.212
B	1.175±0.162	1.146±0.213
C	1.076±0.212	1.156±0.352
D	1.312±0.333	1.540±0.386
E	1.879±0.148	1.875±0.084
F	2.325±0.427	2.207±0.199
G	2.712±0.183	3.960±0.569
H	3.800±0.495	4.659±0.422
I	3.107±0.278	3.602±0.495

Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added).

Table 4.4 : The Expression of mRNA in Each Group after Co-Cultured for 48 Hours (Mean ± SD, n =3)

Group	Notch1	Hes1
A	1.004±0.099	1.010±0.176
B	1.203±0.170	1.177±0.119
C	1.063±0.067	1.282±0.462
D	1.629±0.249	2.213±0.756
E	2.108±0.136	2.735±0.599
F	3.035±0.128	3.725±0.294
G	4.132±0.574	5.302±1.192
H	7.217±0.279	7.675±0.838
I	4.191±0.291	6.302±0.637

Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added).

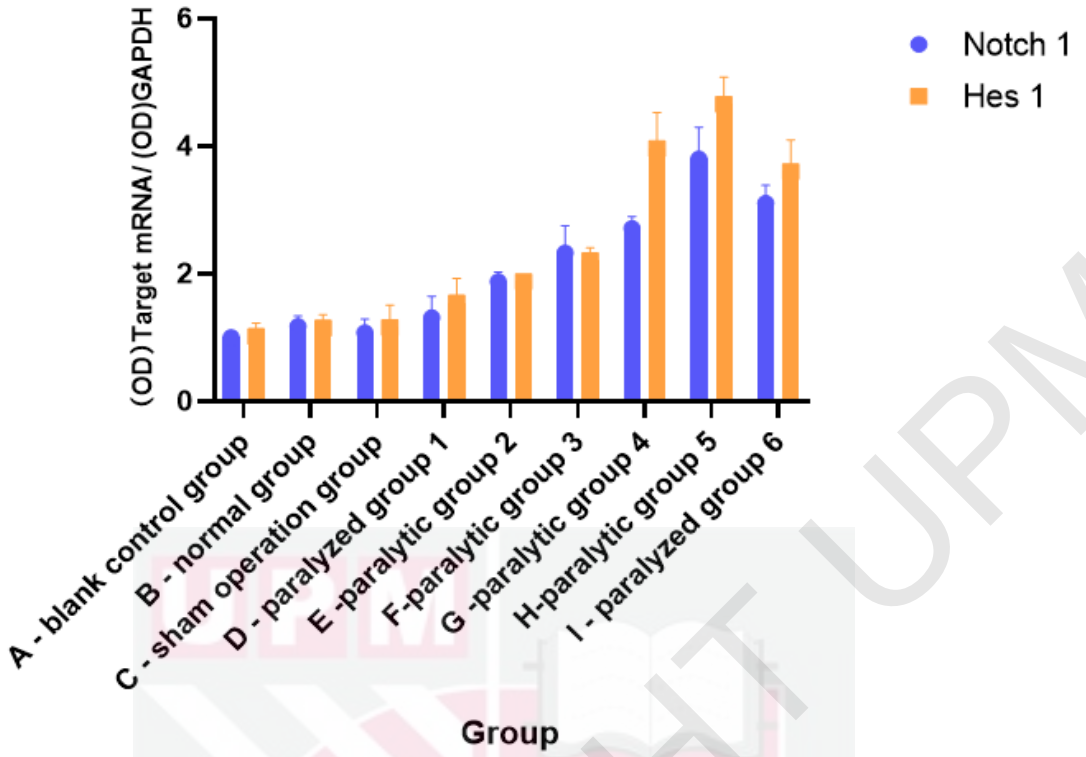

Figure 4.9 : The Expression of Target mRNA in NSC after Co-Cultured for 24 Hours. Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added)

By analyzing the PCR amplification results of Notch1 and Hes1 genes co-cultured for 24 h and 48 h, the mRNA expression of Notch1 and Hes1 in the whole paralysis group was stronger than that in groups A, B and C (< 0.05), and there was no significant difference in Notch1 and Hes1 mRNA expression in groups A, B and C (P> 0.05). In the paralytic group, the mRNA expressions of Notch1 and Hes1 in the Group H-paralytic group 5 (i.e., the culture medium was co-cultured with 7d paralytic spinal cord extract) were significantly higher than those in the other 8 groups (P<0.05). The expression levels of Notch1 and Hes1 genes in group D, E, F, G, H and I measured at

48 hours and the values measured at 24 hours were statistically analyzed, and it could be found that: The mean expressions of Notch1 and Hes1 genes in groups D, E, F, G, H and I at 48 h were higher than those at 24 h, but the differences were not statistically significant (P>0.05).

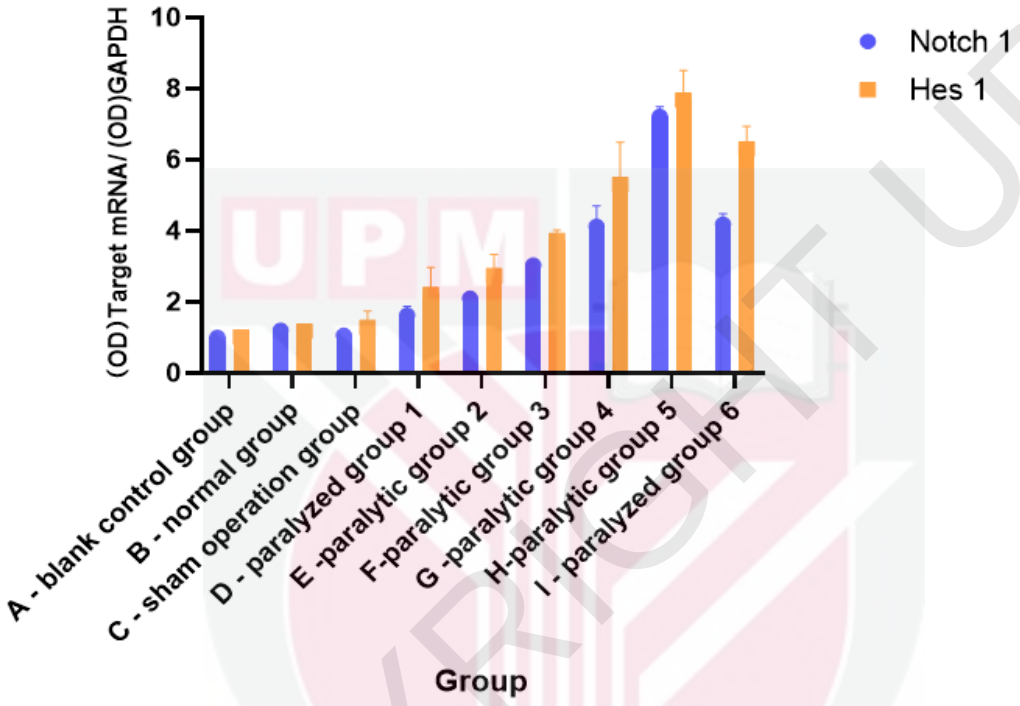

Figure 4.10 : The Expression of Target mRNA in NSC after Co-Cultured for 48 Hours. Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added)

4.9 Effects of DAPT and Spinal Cord Extract on the Growth Curve of NSCs

7 groups (Group I-VII) were cultured for 5 days, and the number of NSC cells in each group was measured at each time point on days 1, 2, 3, 4 and 5 (Table 5). The growth curve of NSC in each group was shown in Figure 14.

Table 4.5 : The Numbers of NSCs of Each Groups at Each Time Point (Mean ± SD, $\times 10^6$, n =3)

Group	1d	2d	3d	4d	5d
I	0.654±0.042	0.932±0.016	1.856±0.032	2.298±0.153	2.697±0.050
II	0.707±0.053	0.912±0.047	1.793±0.031	2.248±0.015	2.717±0.031
III	0.699±0.165	0.893±0.031	1.789±0.025	2.301±0.046	2.679±0.021
IV	0.817±0.032	1.194±0.046	2.112±0.153	2.656±0.025	2.897±0.035
V	0.537±0.031	0.821±0.015	0.876±0.050	1.416±0.025	1.518±0.153
VI	0.541±0.025	0.814±0.021	0.832±0.015	1.342±0.046	1.493±0.055
VII	0.673±0.026	1.099±0.031	1.209±0.029	1.576±0.031	1.876±0.015

Group I- blank control group (PBS solution was added into the medium for co-culture). Group II- Normal group (normal SCE was added). Group III- sham operation group (sham operation SCE was added). Group IV - paralysis group (injured SCE was added). Group V - normal +DAPT group (DAPT and normal SCE was added). Group VI- sham operation +DAPT group (DAPT and sham operation SCE was added). Group VII - paralyzed +DAPT group (DAPT and injured SCE was added)

According to statistical analysis, the number of cells in groups V and VI decreased significantly at each time point compared with groups I, II and III after notch signaling pathway was blocked ($P < 0.05$). Compared with group IV, the number of cells in group VII decreased significantly at each time point ($P < 0.05$). Compared with groups V and VI, the number of cells in group VII was higher at each time point ($P < 0.05$). There was no significant difference in cell number among groups I, II and III at each time point (>0.05), and there was no significant difference in cell number between groups V and VI at each time point (> 0.05).

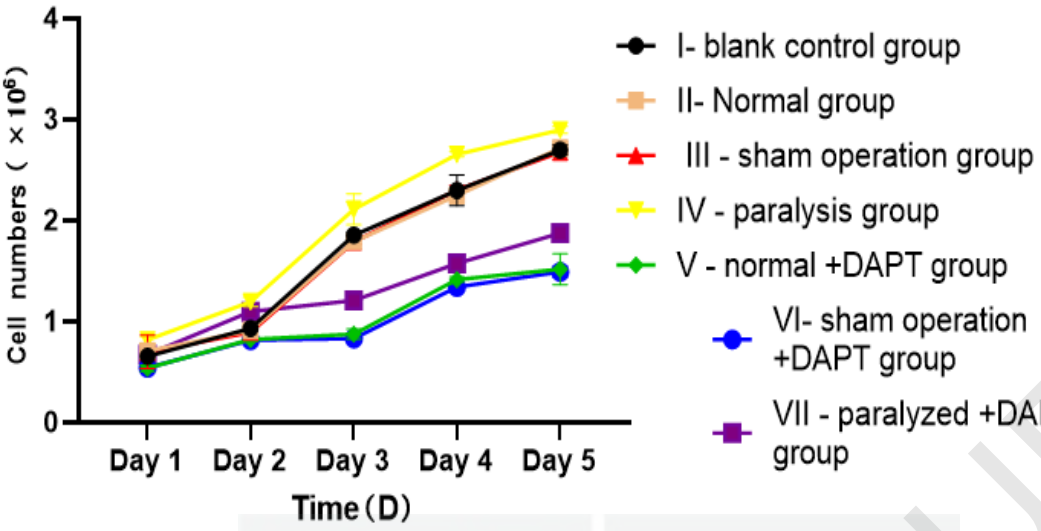

Figure 4.11 : The Growth Curve of NSCs of Each Group. Group I- blank control group (PBS solution was added into the medium for co-culture). Group II- Normal group (normal SCE was added). Group III- sham operation group (sham operation SCE was added). Group IV - paralysis group (injured SCE was added). Group V - normal +DAPT group (DAPT and normal SCE was added). Group VI- sham operation +DAPT group (DAPT and sham operation SCE was added). Group VII - paralyzed +DAPT group (DAPT and injured SCE was added)

4.10 Notch1 and Hes1 Gene Expression

The electrophoretic bands of products of Notch1 gene, Hes1 gene and internal reference GAPDH were within the range of 100-200bp, 200-300bp and 100-200bp of DNA Marker, respectively (Figure 4.12 and 4.13). The gray scale analysis and comparison of electrophoresis bands of PCR products in each group were conducted, and the relative copy number of mRNA of Notch1 and Hes1 genes in each group was calculated using internal reference as the standard (Tables 4.6 and 4.7 and Figure 4.14 and 4.15).

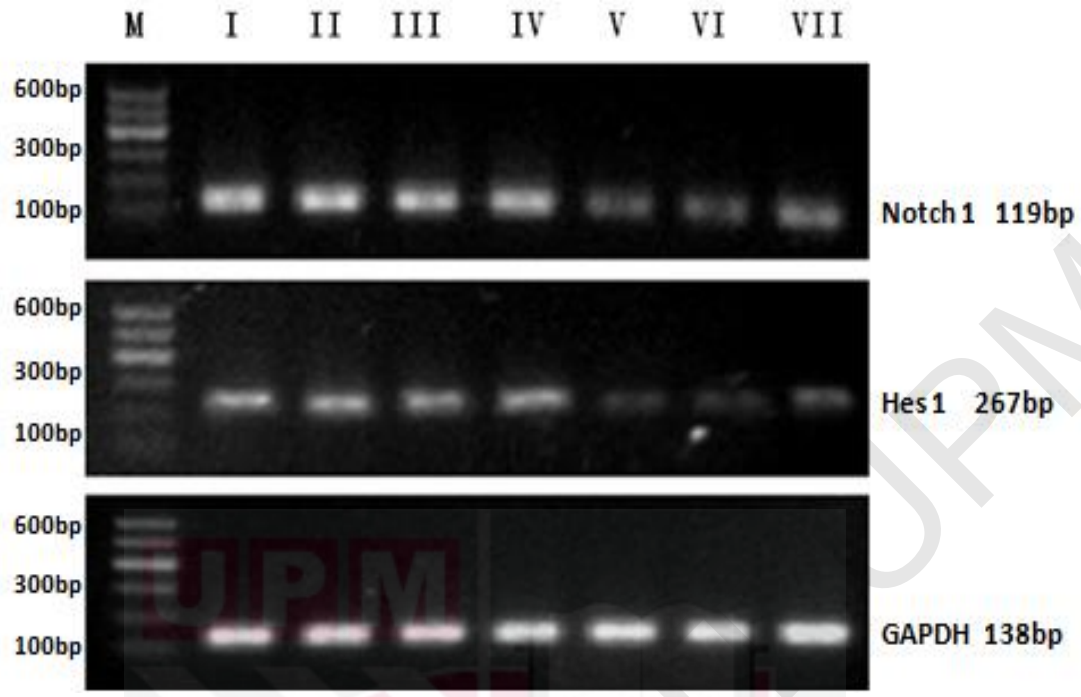

Figure 4.12 : The Electrophoresis of PCR Products of Notch1 and Hes1 mRNA of Each Group after Co-Cultured for 24h. Group I- blank control group (PBS solution was added into the medium for co-culture). Group II- Normal group (normal SCE was added). Group III- sham operation group (sham operation SCE was added). Group IV - paralysis group (injured SCE was added). Group V - normal +DAPT group (DAPT and normal SCE was added). Group VI- sham operation +DAPT group (DAPT and sham operation SCE was added). Group VII - paralyzed +DAPT group (DAPT and injured SCE was added)

Table 4.6 : The Expression of mRNA in Each Group after Co-Cultured for 24 Hours (Mean ± SD, n =3)

Group	Notch1	Hes1
I	0.986±0.035	0.942±0.023
II	0.989±0.051	0.987±0.031
III	0.945±0.031	0.918±0.036
IV	1.162±0.108	1.129±0.035
V	0.854±0.036	0.840±0.024
VI	0.849±0.064	0.836±0.052
VII	1.061±0.109	1.020±0.036

Group I- blank control group (PBS solution was added into the medium for co-culture). Group II- Normal group (normal SCE was added). Group III- sham operation group (sham operation SCE was added). Group IV - paralysis group (injured SCE was added). Group V - normal +DAPT group (DAPT and normal SCE was added). Group VI- sham operation +DAPT group (DAPT and sham operation SCE was added). Group VII - paralyzed +DAPT group (DAPT and injured SCE was added).

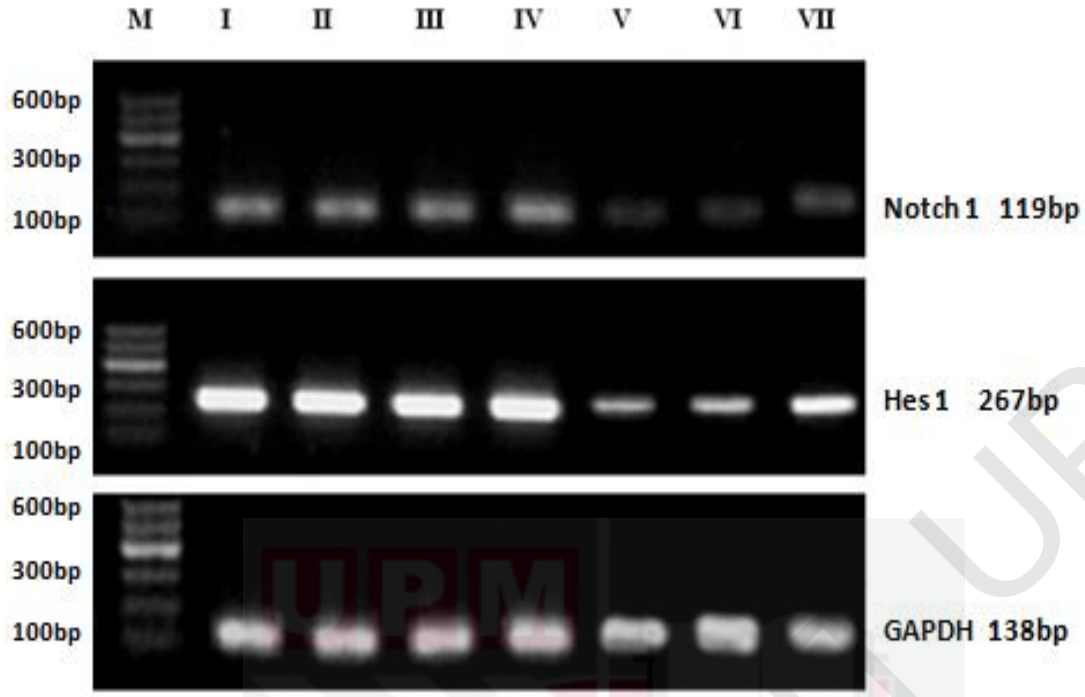

Figure 4.13 : The Electrophoresis of PCR Products of Notch1 and Hes1mRNA of Each Group after Co-Cultured for 48h. Group I- blank control group (PBS solution was added into the medium for co-culture). Group II- Normal group (normal SCE was added). Group III- sham operation group (sham operation SCE was added). Group IV - paralysis group (injured SCE was added). Group V - normal +DAPT group (DAPT and normal SCE was added). Group VI- sham operation +DAPT group (DAPT and sham operation SCE was added). Group VII - paralyzed +DAPT group (DAPT and injured SCE was added)

Table 4.7 : The Expression of mRNA in Each Group after Co-Cultured for 48 Hours (Mean ± SD, n =3)

Group	Notch1	Hes1
I	0.924±0.016	0.895±0.003
II	0.940±0.013	0.942±0.019
III	0.933±0.032	0.935±0.156
IV	1.176±0.045	1.189±0.032
V	0.893±0.136	0.887±0.031
VI	0.891±0.033	0.876±0.155
VII	1.081±0.031	1.052±0.041

Group I- blank control group (PBS solution was added into the medium for co-culture). Group II- Normal group (normal SCE was added). Group III- sham operation group (sham operation SCE was added). Group IV - paralysis group (injured SCE was added). Group V - normal +DAPT group (DAPT and normal SCE was added). Group VI- sham operation +DAPT group (DAPT and sham operation SCE was added). Group VII - paralyzed +DAPT group (DAPT and injured SCE was added).

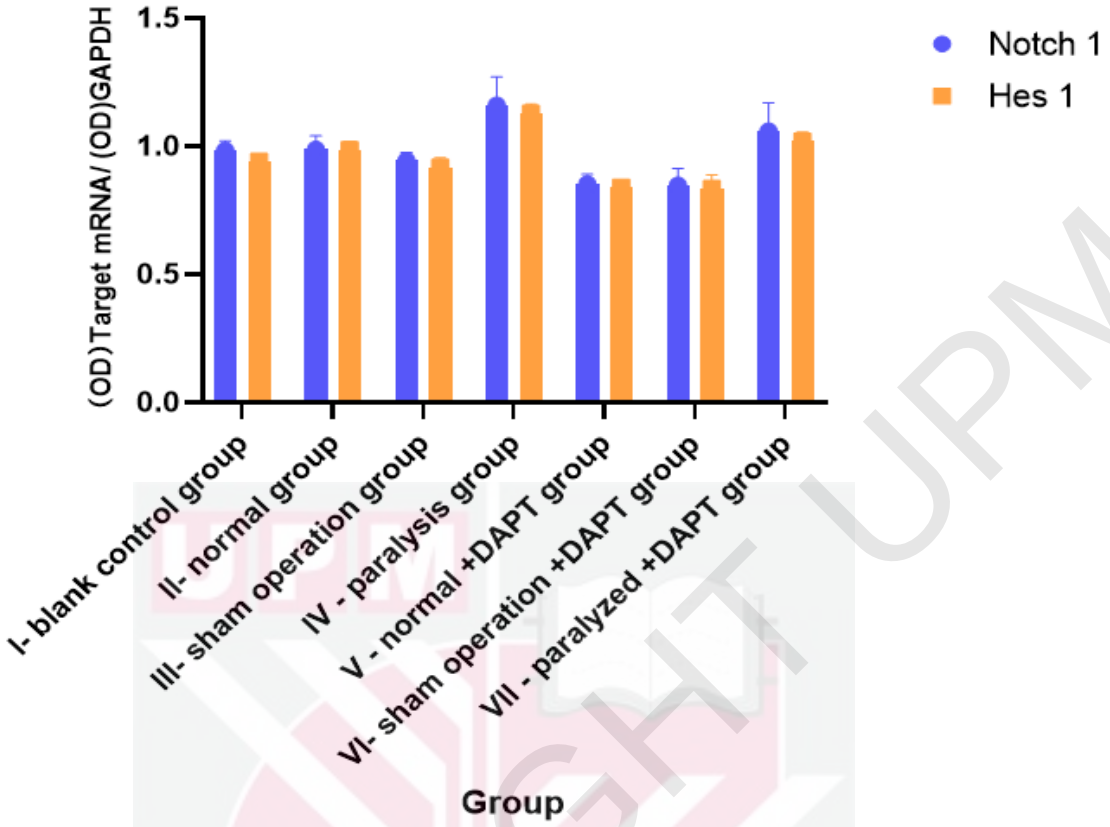

Figure 4.14 : The Expression of Target mRNA in NSC after Co-Cultured for 24 Hour. Group I- blank control group (PBS solution was added into the medium for co-culture). Group II- Normal group (normal SCE was added). Group III- sham operation group (sham operation SCE was added). Group IV - paralysis group (injured SCE was added). Group V - normal +DAPT group (DAPT and normal SCE was added). Group VI- sham operation +DAPT group (DAPT and sham operation SCE was added). Group VII - paralyzed +DAPT group (DAPT and injured SCE was added)

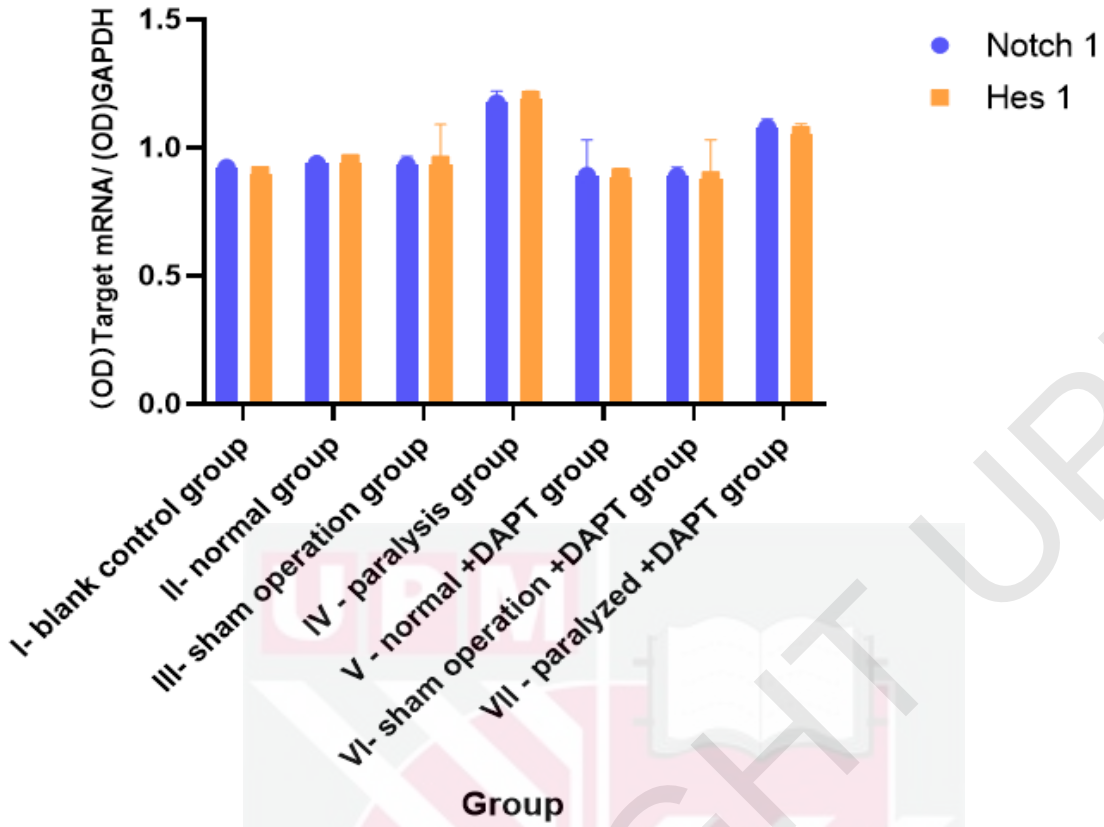

Figure 4.15 : The Expression of Target mRNA in NSC after Co-Cultured for 48 Hour. Group I- blank control group (PBS solution was added into the medium for co-culture). Group II- Normal group (normal SCE was added). Group III- sham operation group (sham operation SCE was added). Group IV - paralysis group (injured SCE was added). Group V - normal +DAPT group (DAPT and normal SCE was added). Group VI- sham operation +DAPT group (DAPT and sham operation SCE was added). Group VII - paralyzed +DAPT group (DAPT and injured SCE was added)

Statistical analysis showed that after notch signal blocking, the expression levels of Notch1 mRNA and Hes1 mRNA in groups V and VI were significantly decreased compared with those in groups I, II and III ($P < 0.05$). The expression levels of Notch1 mRNA and Hes1 mRNA in group VII were significantly lower than those in group IV ($P < 0.05$). The expression levels of Notch1 mRNA and Hes1 mRNA in group VII were higher than those in groups V and VI ($P < 0.05$). There were no significant differences in Notch1 mRNA and Hes1 mRNA expression levels among groups I, II and III ($P> 0.05$). The expression levels of Notch1 mRNA and Hes1 mRNA in groups V and VI were not significantly different ($P> 0.05$).

4.11 The Effect of DAPT Blocking Notch Signal on NSCs Differentiation

Differentiated cells in each Group (I and II) at each closure time point: the percentage of NSE-positive cells (neurons) and GFAP-positive cells (astrocytes) and the number of remaining stem cells were counted (Table 4.8, 4.9, 4.10, and Figure 4.16, 4.17, 4.18).

After blocking the notch signaling pathway, the number of stem cells in the Group II was smaller than that in the Group I at each time point ($P<0.05$), and the percentage of NSE-positive cells (neurons) was higher and the percentage of GFAP-positive cells (astrocytes) was lower in the Group ii when DAPT was added for 2d and 3d ($P<0.05$).

Table 4.8 : The Proportion of Neuron after Blocked Notch Signal at Each Time Point (Mean ± SD, n=3)

Group	1d	2d	3d	5d	7d
I	8.93±0.036	9.79±0.814	9.87±0.618	10.21±0.411	10.87±0.365
II	8.99±0.147	15.49±0.207	16.76±1.211	12.27±0.681	12.29±0.898

Group I-control group (NSCs culture medium was added with paralytic spinal cord extract for co-culture). Group II- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture, DAPT was added to block Notch signal at 1d, 2d, 3d, 5d and 7d, respectively, and the final concentration of DAPT was 50umol/L). The control group was added with equal doses of DMSO.

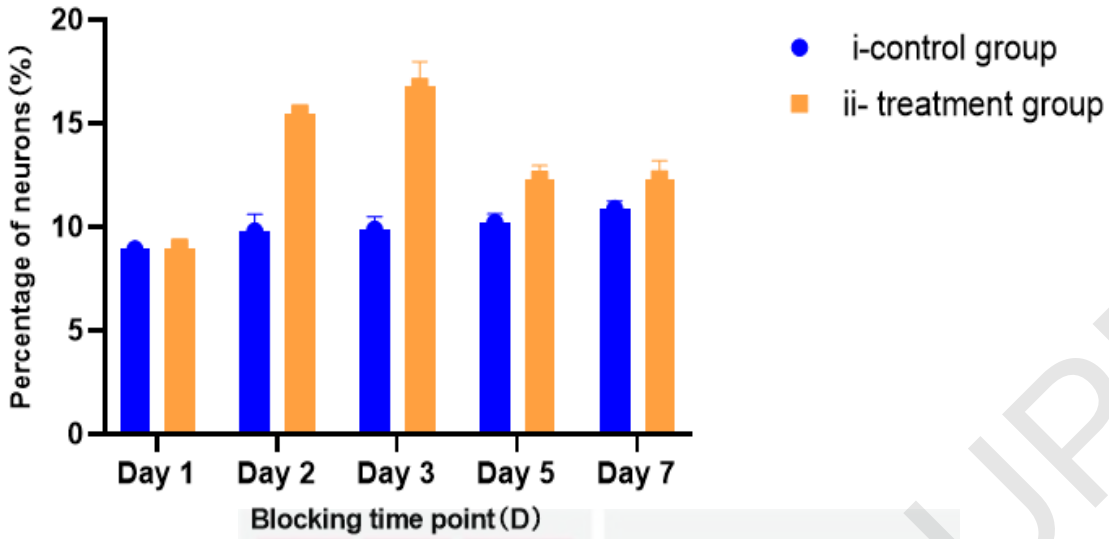

Figure 4.16 : The Proportion of Neuron at Each Blocking Time Point. Group I-control group (NSCs culture medium was added with paralytic spinal cord extract for co-culture). Group II- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture, DAPT was added to block Notch signal at 1d, 2d, 3d, 5d and 7d, respectively, and the final concentration of DAPT was 50umol/L). The control group was added with equal doses of DMSO

Table 4.9 : The Proportion of Astrocytes after Blocked Notch Signal at Each Time Point (Mean ± SD, n=3)

Group	1d	2d	3d	5d	7d
I	91.07±0.036	90.21±0.814	90.13±0.618	89.79±0.411	89.13±0.365
II	91.01±0.147	84.51±0.207	83.24±1.211	87.73±0.681	87.71±0.898

Group I-control group (NSCs culture medium was added with paralytic spinal cord extract for co-culture). Group II- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture, DAPT was added to block Notch signal at 1d, 2d, 3d, 5d and 7d, respectively, and the final concentration of DAPT was 50umol/L). The control group was added with equal doses of DMSO.

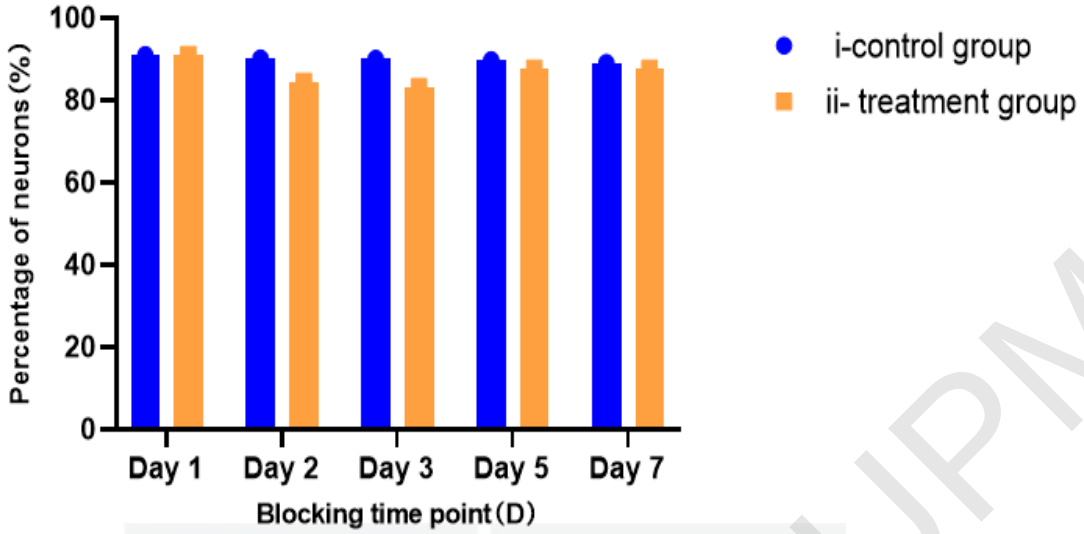

Figure 4.17 : The Proportion of Astrocytes at Each Blocking Time Point. Group I-control group (NSCs culture medium was added with paralytic spinal cord extract for co-culture). Group II- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture, DAPT was added to block Notch signal at 1d, 2d, 3d, 5d and 7d, respectively, and the final concentration of DAPT was 50umol/L). The control group was added with equal doses of DMSO

Table 4.10 : The Remaining Number of NSCs after Blocking Notch Signal at Different Time Point (Mean ± SD, $\times 10^3$, n=3)

Group	1day	2d	3d	5d	7d
I	0.476±0.025	0.856±0.031	0.987±0.025	1.124±0.036	1.176±0.015
II	0.363±0.036	0.783±0.050	0.854±0.035	0.936±0.015	0.973±0.021

Group I-control group (NSCs culture medium was added with paralytic spinal cord extract for co-culture). Group II- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture, DAPT was added to block Notch signal at 1d, 2d, 3d, 5d and 7d, respectively, and the final concentration of DAPT was 50umol/L). The control group was added with equal doses of DMSO.

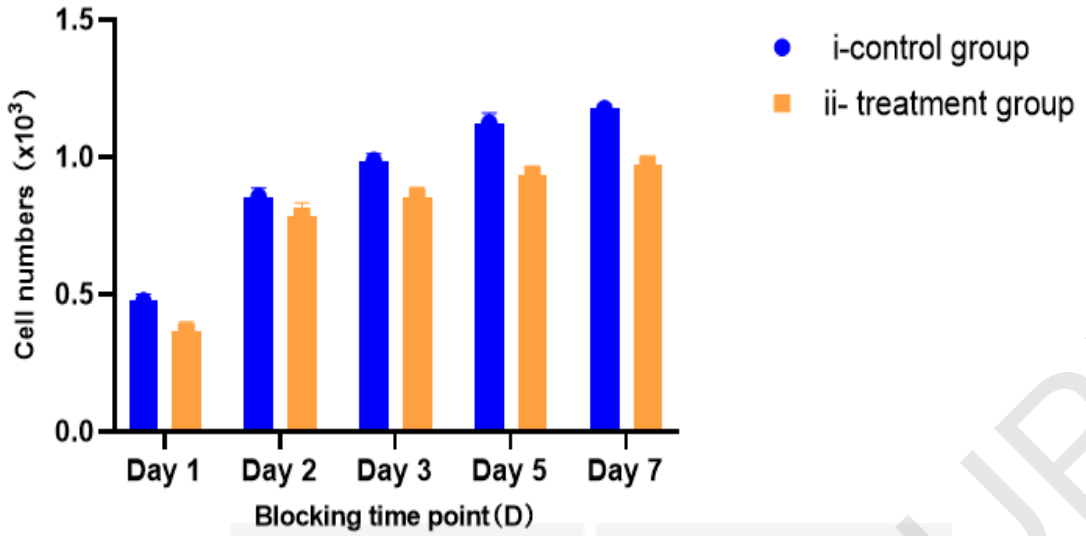

Figure 4.18 : The Remaining Number of NSCs at Each Blocking Time Point. Group I-control group (NSCs culture medium was added with paralytic spinal cord extract for co-culture). Group II- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture, DAPT was added to block Notch signal at 1d, 2d, 3d, 5d and 7d, respectively, and the final concentration of DAPT was 50umol/L). The control group was added with equal doses of DMSO

Data analysis in the Group II (Table 11, Figure 22): the number of differentiated cells added with DAPT at 1d was significantly less than that added with DAPT at 2d, 3d, 5d and 7d ($P<0.05$), while the number of differentiated cells added with DAPT at 5d and 7d was less than that added with DAPT at 2d and 3d ($P<0.05$).

Table 4.11 : The Number of Differentiated Cells in Group ii at Each Blocking Time Point ($\bar{x}$ ±s, ×10³, n=3)

Group	1d	2d	3d	5d	7d
Group II	0.278±0.036	0.541±0.015	0.563±0.021	0.439±0.025	0.427±0.015

Group II- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture, DAPT was added to block Notch signal at 1d, 2d, 3d, 5d and 7d, respectively, and the final concentration of DAPT was 50umol/L). The control group was added with equal doses of DMSO.

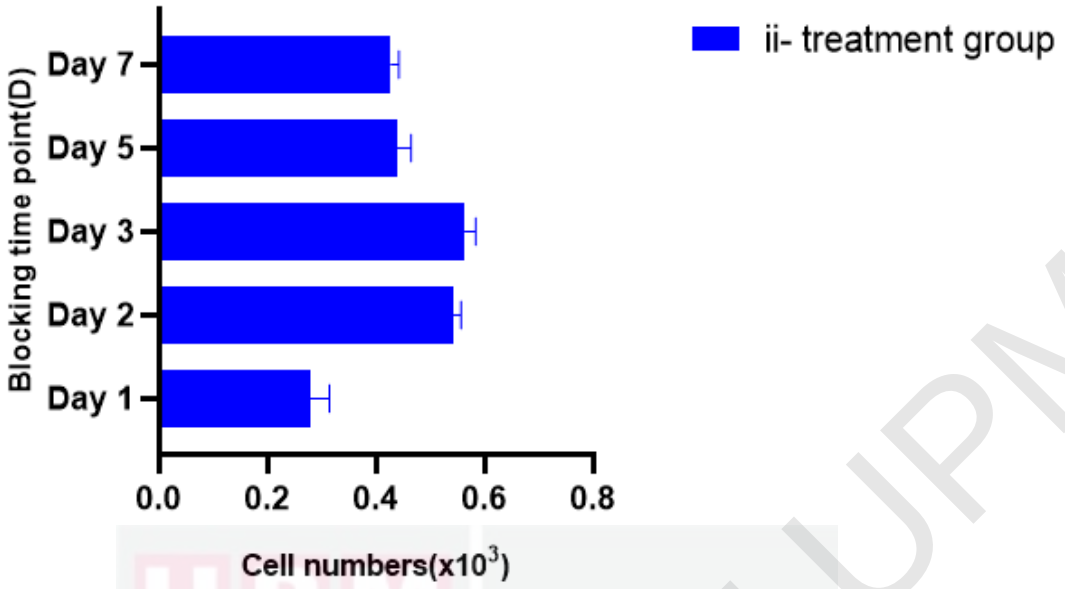

Figure 4.19 : The Number of Differentiated Cells at Each Blocking Time Point. Group II- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture, DAPT was added to block Notch signal at 1d, 2d, 3d, 5d and 7d, respectively, and the final concentration of DAPT was 50umol/L). The control group was added with equal doses of DMSO

CHAPTER 5

DISCUSSION

The findings from this study provide valuable insights into the potential therapeutic strategies for enhancing NSCs proliferation and differentiation following spinal cord injury. By simulating the local microenvironment post-SCI using paralyzed spinal cord extract and inhibiting the Notch signaling pathway with γ-secretase inhibitor (DAPT), the study examined temporal effects on NSC behavior. The observed temporal effects of paralyzed spinal cord extract on NSC proliferation underscore the importance of the microenvironment in modulating NSC responses after injury. Additionally, the study highlights the role of Notch signaling in regulating NSC fate decisions, suggesting that targeted inhibition of this pathway with DAPT can promote NSC differentiation into neurons. Selecting the optimal timing for Notch pathway modulation could enhance NSC proliferation while promoting neuronal differentiation, potentially improving functional recovery post-SCI. Moreover, the identification of various NSC niches within the central nervous system (CNS), including the spinal cord, underscores the potential for endogenous repair mechanisms. The multidirectional differentiation capacity and low immunogenicity of NSCs further support their therapeutic potential for SCI repair. These findings contribute to the development of targeted strategies aimed at harnessing NSC plasticity and regenerative potential for SCI treatment**.**

5.1 Culture and Identification of Neonatal Rat Brain-Derived NSCs

Since 1990s, many researchers have demonstrated that NSCs are widely present in CNS including spinal cord. In the adult brain, Alvarez-Buylla et al. (2002) and Gage

et al. (2000) have confirmed that ENSCs exist in the subventricular region and the subdentate gyrus of the hippocampus. Horner et al. (2000) and Ohori et al. (2006) proposed that NSCs might exist in the white matter parenchyma of spinal cord, Meletis et al. (2008) proposed that NSCs might reside in the ependyma near the central canal, and Martens et al. (2002) further proposed that NSCs may reside under ependyma. Yamamoto et al. (2001) isolated a kind of cell from the ependymal layer of spinal cord of female mice at 6-7 weeks. Also NSCs not only have the probabilities of division, regeneration proliferation and multidirectional differentiation (differentiate into nearly all types of CNS nerve cells such as neuron and glial cell), but also exhibit low immunogenicity and good histocompatibility (Suh et al., 2007; Ohori et al., 2006; Martens et al., 2001; Yamamoto et al., 2001; Weiss et al., 1996;). This refutes the theory (Gage, 2000) that the damaged cells are unable to be regenerated and repaired following the injuries to the nerve and the spinal cord, and provides new hope to the reconstruction of structure and function in CNS disorders such as SCIs and cerebral injuries. NSCs, as the seed cells enhancing CNS regeneration, spontaneously become cores and foundation of neurological research. Efficient approaches for enhancing pure NSCs yield in the research thus becomes crucial. The present study established a method for NSC amplification, culture, purification and identification *in vitro* to provide sufficient-necessary condition for further investigation of NSCs. Cell culture technology is the basic premise for the investigation of NSCs. Mature techniques for the isolation and culture of NSCs have been developed. In addition to proving the presence of NSCs and its potential of self-renewal and multidirectional differentiation, Reynolds and Weiss et al. (1992) also demonstrated that NSCs could proliferate massively *in vitro*, which establish crucial conditions for future investigation of NSCs. However, application of methods for *in vitro* amplification of stem cells is currently

restricted to CNS. By contrast, cloning of monolayer cells (Shimojo *et al.*, 2008; Gage, 2000) offers an alternative approach for NSC culture. This approach is widely used because using this approach we can not only derive an abundant amount of pure NSCs in a short time but also determine the differentiation capacity for a single NSC or its precursor cells.

For the timing of NSCs passaging, the present study found (Zhou et al., 2014) that although passaging at two-to-three-day primary culture could facilitate the dissociation of neurospheres and formation of single-cell suspensions, the number of single cells derived was unsatisfactory at low densities which was not conductive to amplification and proliferation of NSCs after passage. Passaging performed when primary NSCs are cultured for seven to eight days could only dissociate neurospheres into small clusters of cells with a small amount of single floating cells instead of single-cell suspensions, which was presumably related to the tight junctions between peripheral neurosphere cells (Lobo et al., 2003). Moreover, small clusters of adherent cells with small portion of low density single floating cells were discovered after only two days of passage. Differentiation of adherent large neurospheres occurred after a continued cultivation. The observation of nine-to-ten-day primary NSC culture revealed (Zhou et al., 2017) that differentiation occurred in plenty of adherent large neurospheres with lots of dead cells, which also inhibits passaging. Comparatively, when passaging was performed after four to five days of primary culture, it not only promotes easy generation of single-cell suspensions dissociated from neurospheres but also harvests sufficient single floating cells with fewer dead cells and improved amplification and proliferation capabilities of NSCs. It follows that the optimal timing for NSC passage is after four-to-five-day primary or passage NSC culture. During the

progress of *in vitro* culture, junctions can be formed between NSCs, which assemble to form neurospheres, through cytoskeletal protein and calnexin induced cell-cell adhesion mediation (Lobo et al., 2003). For neurospheres formed through primary culture or passage culture carried out for two to three days, the junction between NSCs is relatively loose making dissociation and formation for single floating cells easy. However, an analysis of the grow curve over this period displayed that passaging which is carried out when NSCs entered the logarithmic growth phase from the incubation phase might destroy the rapid amplification of NSCs resulting in passaged NSCs' repeated incubating and missing the optimal timing for amplification and at last failure to yielding sufficient NSCs in a short time. On the fourth to fifth day of primary culture or subculture, the number of NSCs increased significantly, the volume of neurospheres grew bigger and cell-cell junctions became tight when the junctions were still able to be dismantled by some means to obtain single cells comfortably. Notably, cell culture gradually reached a plateau period with anchoring cell-cell junctions over time. Oppositely, when the primary or passage culture last for seven to eight days, cell-cell junctions would become so tight that it was difficult to cleave or dissociate. Forced disconnection might end up with NSC damage, dissolution, death, adherence and differentiation. Furthermore, it was observed that the number of neurospheres decreased with their increasing volume suggesting the bulked volume of neurospheres might arise from neurosphere fusion instead of large amplification of NSCs. This can be interpreted as with the increasing volume of neurospheres, it is difficult for the cells within the spheres to contact the culture medium, which then prevents cells within the spheres from absorbing nutrition and affecting the process of metabolism, causing decline in the proliferation abilities of cells within the neurospheres, death of a large number of cells and generation of necrotic cores (Mori

et al., 2006). From this view, balance should be reached between easy dissociation of neurospheres into single cells and rapid amplification of NSCs without damaging the proliferation abilities of cells within spheres by the excessive volume of neurospheres or causing death during the choice of timing for NSC passage. After summarizing the results of experiments, it is believed that the optimal timing for NSC passage is on the fourth to fifth day of primary or passage NSC culture.

Currently, the commonly used methods for passaging are enzymatic digestion method and mechanical method. After analyzing the investigations, the present study discovered (Zhou et al., 2022; Feng et al., 2019; Zhou et al., 2014) that (i) although mechanical method alone is easy to operate and needs a very few of centrifugation, the force and times of tapping and pipetting is difficult to control and it increases the probability of mechanical damage to cells, which is not conducive to the experimental operation; (ii) for enzymatic digestion method, trypsin is mainly used. However, the effect of trypsin in digestion is likely to cause chemical damage to NSCs. Moreover, usage of serum or enzyme inhibitor to counteract the digestive effect of trypsin may reduce the vitality and proliferation rate of NSCs and then influence the experimental results; (iii) concerning the combination of enzymatic digestion method and mechanical tapping and pipetting method, in spite of a very few of blasting is needed and small scale of blasting can dissociate neurospheres into single-cell suspensions, it does not elevate the number of single cells while the amplification capability of NSCs and number of neurospheres decrease significantly; (iv) in terms of TrypL^{TM} Express digestion method combined with mechanical tapping and pipetting method, TrypL^{TM} Express, a high purity recombinant enzyme, has trypsin-like property, which cleaves peptide bonds on the C-terminal sides ofarginine and lysine. TrypL^{TM} Express has

high thermostability compared with trypsin. There is no need to use serum or enzyme inhibitor to compromise its digestive effect. Instead, the effect of TrypL^{TM} Express is mild with less toxicity. However, the action of TrypL^{TM} Express should not last long. This study used TrypL^{TM} Express combined with mechanical tapping and pipetting methods as the passaging method. Using this passaging method, sufficient regular shaped, even sized sheen floating single cells were harvested. What's more, cell culture medium is clear with few fragments and elemental impurities, and NSCs holds vigorous growth and proliferation abilities. After observing the process of NSC culture post passaging, it discovered proper sized and good shaped colony-like neurospheres. Based on this, this study recommend using TrypL^{TM} Express combined with mechanical tapping and pipetting method as the method for NSCs passaging. Using this passage method, a large number of suspended single cells with regular shape, uniform size and good light transmission were obtained, and the culture medium was clear, with less cell debris and impurities in the medium, and the growth and proliferation ability of NSC was strong. By observing the culture process of NSC after passage, a large number of clonoid nerve balls with appropriate size and good shape could be found. Therefore, we believe that the TrypLTM Express digestion combined with mechanical blowing method can be selected as the NSCs passage method.

Additionally, NSCs has no obvious morphological characteristics to distinguish other types of cells, and the identification of the two cultured and differentiated cells by morphology alone will often be confused by some cells with similar appearance. Therefore, the key to clarifying cell properties lies in cell identification methods(Feng et al., 2019; Zhou et al., 2017; Zhou et al., 2014). In order to identify the cells that we've grown，immunofluorescence staining was used to identify whether the cultured

cells derived from newborn rats' brains were NSCs. The 5th generation of passaged cells showed positive after both bromodeoxyuridine/5′-bromo-2′-deoxyuridine (BrdU) immunofluorescence staining (Hijroudi et al., 2022; Qiu et al., 2021) and nestin immunofluorescence staining (Li et al., 2017; Cheng et al., 2016). BrdU, a thymidine analog, can incorporate into newly synthesized DNA during the S-phase of the cell cycle (Yu et al., 2022; Ngwenya et al., 2008). Its property of continuous self-replication and self-reproduction by stem cells makes it take in labeled Brdu during replication. Thus, when immunocytochemical detection is carried out, BrdU positive suggests the cells are in the division and proliferation phrase. Accordingly, NSCs' potential of division and proliferation can be identified by Brdu detention. Nestin, also called NES, is a major cytoskeletal protein in stem cells in the mammalian CNS and its expression is inversely associated with cell differentiation (Rietza et al., 2001). Nestin is a specific biomarker for NSCs and functions as one of factors to identify NSCs. However, nestin is also expressed in pancreatic progenitor cells (Wang et al., 2009; Wang et al., 2005), mammary Glands cells (Richter et al., 2013) and endodermal cells (Wiese et al., 2004). Therefore, nestin positive cells are not necessarily NSCs. The identification of NSCs cannot only based on a certain feature. Apart from nestin immunofluorescence staining, specific biomarkers for NSCs differentiation should be considered. NSCs have the potential to differentiate into oligodendrocytes, astrocytes, neurons, and other nerve cells (Santos et al., 2022; Shi et al., 2019; Shi et al., 2018). The terminal cells formed by NSCs differentiation can be identified by the specific markers of nerve cells. Neurofilin-200 (NF200) and neuron-specific enolase (NSE) are widely used as neuron-specific markers (Zhang et al., 2018). Glial acid fibrin (GFAP) is a specific marker of astrocytes (Mamber et al., 2012). By detecting these corresponding surface marker molecules, the nerve terminal cells differentiated from

NSCs can be identified, and NSCs can be functionally identified by their differentiation potential. The present study selected neuron-specific biomarkers neurofilament protein 200 (NF200) and neuron specific enolase (NSE) and astrocyte-specific biomarker glial fibrillary acidic protein (GFAP) respectively for the identification of muti-differentiation of NSCs. This study used immunofluorescence staining for the detection of cultured cells derived from newborn rat brains in respect of the above described some characteristics of NSCs. It was observed that the 5^{th} generation of passaged culture cells showed BrdU positive after BrdU immunofluorescence staining and nestin positive after nestin immunofluorescence staining. NF-200, NSE, GFAP positive cells were discovered following immunofluorescence staining at five days after induction of dissociation was performed in 5% fetal bovine serum. This implied that the cultured cells fulfil the criteria for NSC identification—expression of nestin, abilities of differentiation, renewal and proliferation and moreover the capabilities of dissociation. The composition of all these factors can demonstrate the cultured cells are NSCs. Above all, the approaches used in this study yields sufficient purified NSCs, which then are employed for the further study of NSCs.

5.2 Effect of Paralyzed Spinal Cord Extract on Proliferation of NSCs

Spinal cord injury is a severely devastating disease to the nervous system, with an incidence population mainly concentrated in young adults, which not only brings tremendous physical trauma and emotional cost to the patients themselves, but also brings a serious economic burden to families and society, and is an urgent public health problems (de Almeida et al., 2023). Current clinical treatments for SCI are mainly based on early surgical decompression, stabilization of spinal structure,

dehydration anti inflammation, being supported by neurotrophic and targeted rehabilitation training (Zipser et al., 2022). However, patients remain with significant neurological disability after treatment. Therefore, since the disruption of neural circuits after SCI is restricted by many unfavorable conditions for nerve regeneration and repair, such as ischemia hypoxia, inflammatory response, demyelinating lesions, and glial scar formation (Havelikova et al., 2022), it has become one of the most intractable disorders in the modern field of spine surgery. NSCs, a class of multipotent cells with self-renewal and differentiation capacities, are present in the lateral subventricular zone (SVZ) and sub granular zone (SGZ) of the dentate gyrus in the adult mammalian brain and play crucial roles in mammalian life-long neurodevelopment and repair(Curtis et al., 2018). When NSCs in the SVZ region after injury occur in the central nervous system (CNS) proliferate massively after activation and can migrate to the injured region to differentiate into functional neurons, integrate in the damaged neural circuits and thereby promote neurological recovery (Xue et al., 2021). Studies have found (Zhang et al., 2020) that transplanted NSCs can exert neuroprotective and immunomodulatory effects and promote tissue repair by preventing tissue damage, interfering with pathogenic processes or by rescuing endogenous neural cells. Although previous studies（Feng et al., 2019; Zhou et al., 2014) have confirmed the role of NSCs for nerve repair and regeneration, the ability of NSCs to proliferate and differentiate is limited, and the poor external environment in the injury area limits their nerve repair effect. Thus, targeting this cell population of NSCs, investigating the methods and underlying mechanisms that promote their proliferation is of great fundamental and clinical interest.

Ferrucci et al. (2017) confirm the widespread distribution of endogenous NSCs within the central nervous system such as the adult mammalian spinal cord. NSC differentiation into neurons and oligodendrocytes is the most desirable method for SCI repair by cell transplantation, which overcomes the associated issues of in vitro expansion, cell number, transplantation injury, immune rejection and ethics brought by exogenous cell transplantation (Jinnou et al., 2021; Sugaya et al., 2018; Trueman et al., 2013). Studies (Nicaise et al., 2022; Kahroba et al., 2021) have discovered that the secondary response after SCI leads to the release of a variety of inflammatory mediators and growth factors, which constitute a complex local microenvironment of the injured spinal cord with both favorable factors for NSC proliferation and differentiation and unfavorable factors for NSC survival. Mothe et al. (2005) demonstrated that NSCs in the ependymal layer after SCI began to proliferate in an in vivo test, reached a proliferation peak at 7 days, and NSCs after SCI migrated from the central canal to the periphery. Ma et al. (2020) found that ependymal cells were able to significantly proliferate and migrate toward the injury area after SCI. The above experiments confirmed that the local microenvironment could promote the proliferation of endogenous NSCs after SCI. Also, the formation of a glial scar hinders the repair of SCI due to the low number of NSCs in the spinal cord and the differentiation of endogenous NSCs that do not proliferate appropriately to neurons and oligodendrocytes in the local microenvironment after SCI. Feng et al. (2019) suggested that spinal cord extracts (SCEs) after SCI can promote the proliferation of NSCs. Niu et al. (2019) have confirmed that Notch1/Hes1 signaling pathway can be used as a novel target for SCI treatment. So, selecting the optimal method to promote endogenous NSCs proliferation as well as differentiation into neuronal cells in the spinal cord after SCI is a challenge faced by endogenous NSCs for the repair of SCI.

This study investigated the optimal time point for promoting NSCs proliferation by preparing SCEs for different time periods after SCI in rats. In addition, the underlying mechanism was further explored to provide a theoretical basis for promoting endogenous NSCs proliferation for the optimal time after SCI.

SCI often leads to permanent impairment of sensation, movement as well as sphincter function below the injury plane in patients (Tsehay et al., 2022). The main causes are necrotic, degenerative, apoptotic, loss of neuronal cells and formation of a glial scar locally after SCI, disruption of nerve conduction tracts, demyelinating changes, and the presence of numerous factors in the microenvironment after SCI that inhibit axonal regeneration and elongation (Harkema et al., 2022). Therefore, compensating lost neurons after SCI become a research hotspot for SCI repair. NSCs can self-divide, proliferate, and differentiate into all categorizes of neural cells in the

CNS, including neuronal cells, astrocytes, and oligodendrocytes, among others (Yu et al., 2019). Studies have found (de Freria et al., 2021) NSCs to be indispensable for re-establishing myelin entry and remodeling the structure and function of the spinal cord after injury. Therefore, alternative therapeutic strategies for NSCs become a research hotspot for cell transplantation to treat SCI.

Currently, an alternative therapeutic approach to NSC for the treatment of SCI is to replenish lost neurons and oligodendrocytes within the injured spinal cord by activating ENSCs, and encouraging their proliferation, migration, and differentiation to facilitate the repair of injured spinal cord structure and function (Stenudd et al., 2015). Further, mechanisms of ENSCs differentiation include directed differentiation

of NSC into neural cells by two mechanisms, self-gene regulation and regulation by extrinsic signals (Zhang et al., 2016). Vertebrate bodies encode genes that produce bHLH transcripts, termed bHLH genes, which can determine the fate of neural cell differentiation (Imayoshi et al., 2015). The bHLH gene family (Kageyama et al., 2019) plays an integral regulatory role in neurogenesis, neurodevelopment, and neural differentiation and is divided into two categories: positively regulated (e.g., Mash1, Ngn1, Ngn2) and negatively regulated (e.g., Hes1, Hes3, Hes5). Among the negatively regulated bHLH genes, the Hes gene family, including Hes1, Hes3, and Hes5, has been confirmed to be highly expressed in NSCs (Ochi et al., 2020). The targets of Hes include positively regulated genes such as Mash1, which inhibits the differentiation of NSCs by forming nonfunctional heterodimers with E47 (Ito et al., 2003). Besides, another pathway that controls the differentiation of NSCs is the Notch signaling pathway, which consists of Notch receptor, CSL (DNA binding protein) and Notch ligand (DSL protein), among others (Wissel et al., 2018; Del Bianco et al., 2010; Hansson et al., 2006). Notch and its ligands are both single pass transmembrane proteins, and when Notch and ligands of adjacent cells are bound, they are cleaved by the proteasome to release the notch intramembrane domain of Notch with a nuclear localization signal into the nucleus to bind to CSL, which in turn regulates gene expression (Ge et al., 2002). Signaling by the Notch pathway primarily achieves regulation of specific gene transcription by causing changes in transcriptional regulators through protein interactions or binding transcriptional regulators to target genes (Wang et al., 2015). Notch has been demonstrated to prevent the differentiation of NSCs into neurons and maintain NSCs self-renewal (Wang et al., 2020). When notch is activated, it promotes neural stem cell proliferation by up regulating Hes1 and Hes5 gene expression. When Notch activity is inhibited, Hes1 and Hes5 gene

expression is downregulated, and then the competitive inhibition of Mash1 is diminished, which in turn promotes NSCs into the differentiation progress (Harada et al., 2021; Venkatesh et al., 2017). Yan et al. (2016) discovered that prematurely inhibiting Notch signaling would result in the inhibition of neural stem cell self-proliferation. And inhibiting Notch signaling too late will result in many NSCs converting into glia and forming a glial scar locally. Zhang et al. (2008) showed that folic acid treatment was involved in the proliferation and differentiation of neonatal murine NSCs by increasing the expression of Notch1 / Hes1 pathway. Besides, safflower seed oil, containing oleic acid and palmitic acid, could enhance the stemness of cultured embryonic NSCs through Notch1 and induces neuronal differentiation (Ghareghani et al., 2017). Therefore, the effect of local microenvironment on the proliferation and differentiation of NSCs after SCI is of great significance to guide the choice of time to block Notch signaling for better promoting recovery of SCI. Thus, from the perspective of the whole microenvironment after SCI, this experiment observed the effect of paralyzed spinal cord extract at different times on the proliferation of NSCs by simulating the local microenvironment after SCI with paralyzed spinal cord extract in vitro to better guide the timing of blocking Notch signaling pathway and promote the differentiation of NSCs into neurons.

In this session of our experiment, paralyzed SCEs at different time periods was used to co culture with NSCs after passage and culture in vitro to observe the time-dependent change of the effect of paralyzed SCEs on the proliferation of NSCs to guide the selection of the best time to block Notch signal. The results displayed that the paralyzed SCEs encouraged NSCs proliferation and the Notch/Hes1 pathway expression, with the most significant effect at 7 days of paralysis. Liu et al. (2019)

confirmed that SCI as a stimulus can lead to massive proliferation and migration of endogenous NSCs in the spinal cord to the injury area. Moreover, Feng et al. (2019) exhibited that SCI-SCEs treatment upregulated the mRNA expression levels of Notch1 and Hes1, but repressed the differentiation of NSCs into neurons. Zhou et al. (2014) demonstrated that SCI-SCEs could promote the proliferation of NSCs by up regulating the expression of Notch1 and Hes1 mRNA, which indicated that the microenvironment of SCI may encourage the proliferation of NSCs, and activation of Notch signaling pathway is one of the mechanisms by which the local injury microenvironment after SCI promotes ENSCs proliferation. Above studies provide a theoretical basis for inducing ENSCs to differentiate into neurons to treat SCI in vivo, so it is feasible to facilitate SCI repair by inducing endogenous NSCs to differentiate into neurons and restore conduction pathways.

In summary, our experiments investigated the effect on the proliferative capacity of NSCs by co-culturing the extracts from paralyzed spinal cords, which illustrated that the extracts from 7d paralyzed spinal cords had the greatest effect on NSCs proliferation, and indirectly demonstrated that regulating Notch signaling selected at 7d of SCI may provide an optimal time for NSCs proliferation and developmental regeneration. This study may supply a usable reference for the therapy of clinical SCI. However, the present study also had some limitations. First, this study was only investigated at the cellular level and lacked animal experiments. Besides, we also need to further explore its therapeutic sensitivity for SCI-SCEs by using Notch1/Hes1 pathway inhibitors. The above will be the next issue to be addressed in the further study.

5.3 Effects of DAPT and Paralyzed Spinal Cord Extract on Notch Signaling Pathway

Spinal cord injury is a serious, complex and irreversible neurological disease (Courtine et al., 2019; Eckert et al., 2017; Singh et al., 2014). SCI is usually caused by traumatic events, including accidental traffic accidents, falls, sports and violence, leading to serious sensory, motor and permanent neurological dysfunction, which seriously affects the patient's work and quality of life, placing a huge burden on society and the patient's family (Oyinbo et al., 2011).With the confirmed distribution of NSCs in the spinal cord, the expansion and differentiation of ENSCs in the spinal cord are used to supplement neurons, repair spinal cord conduction tracts and reshape myelin sheath to restore the structure and function of the spinal cord, bringing new hope for the rehabilitation of SCI patients. Current research directions include(Li et al., 2017; Stenudd et al., 2015; Cai et al., 2008): (1) Inducing NSCs in the spinal cord to differentiate into injured or lost cells; (2) NSCs cultured in vitro were transplanted to the site of spinal cord injury; (3) NSCs were used as vectors for gene therapy. These research directions not only make full use of the characteristics of NSCs to differentiate them into damaged or lost nerve cells, but also take full advantage of the role of NSCs in expressing foreign genes. Through these ways, it is hoped to achieve the purpose of relieving symptoms and curing the disease. However, domestic and foreign scholars have encountered many problems in the process of using spinal ENSC to achieve the above purpose. The regulatory mechanism of SCI local microenvironment as a foreign regulatory signal to regulate the proliferation and differentiation of ENSCs through the interaction with Notch signaling pathway has not been clearly studied. Therefore, in order to better regulate the proliferation and differentiation of ENSCs and repair the damage, we need to further understand the

influence of Notch signaling pathway in SCI microenvironment on endogenous NSCs (ENSCs) proliferation and differentiation. In this study, the paralyzed spinal cord extract was used to simulate the post-SCI microenvironment in vitro, and the effect of Notch signal on NSCs was understood by blocking Notch signal with γ-secretase inhibitor DAPT, so as to select the appropriate time to block Notch signal and promote NSCs to differentiate into neurons, so as to promote the repair of SCI.

Current studies (Kang et al., 2008; Pineau et al., 2007; Aberg et al., 2003; Vela et al., 2002; Mason et al., 2001; Arnett et al., 2001; Aberg et al., 2000) on the influence mechanism of SCI microenvironment on NSCs suggest that the secondary reaction after SCI leads to the release of tumor necrosis factor-a (TNF-a), IL-6, IL-1a, IL-1b, EGF, bFGF, insulin-like growth factor 1 and other growth factors and inflammatory mediators. These factors constitute the complex local microenvironment of SCI, in which there are both favorable factors for proliferation and differentiation of NSCs and unfavorable factors for survival of NSCs. Fu et al. (2019) confirmed that differentiation inhibitor 1 of ep300 interaction (EID1) plays a crucial role in the proliferation of NSCs. Yu et al. (2019) found that melatonin promoted the proliferation of spinal NSCs in adult mice through PI3K/AKT signaling pathway. Studies by Zhao et al. (2019) showed that the absence of chromatin regulator Dpy30 would affect the proliferation and differentiation of NSCs. Gao et al. (2017) exhibited that microRNA-342-5p can act as a downstream effect factor of Notch signaling pathway to regulate the differentiation of NSCs into intermediate neural precursors and astrocytes. Meletis et al. (2008) confirmed that ependymal cells proliferated significantly and migrated to the injured area after SCI. Horky et al. (2006) also proved through experiments that the proliferation of ENSCs in the parapyendymal central

duct of the spinal cord, the dorsal horn of the spinal gray matter and the injured area reached a peak within 24 hours after SCI. Mothe et al. (2005) used DIL markers in the spinal cord microinjury model in the in vivo experiment, and found that the ependymal cells expressed Nestin 1 day after injury, and the expression reached a peak on day 7. The number of ependymal cells expressing Brdu+ at the injury site at 3 days after injury was 8.6 times that at 1 day after injury, and the number of ependymal cells migrated from the central duct increased significantly at 3 days after injury. It can be seen that the proliferation of NSCS in the ependymal layer began immediately after SCI and reached its peak on day 7, and the NSCs migrated from the central canal to the periphery after SCI.These in vivo experiments demonstrated rapid activation and proliferation of ENSC after SCI.In this study, various spinal cord extracts were co-cultured with brain derived NSCs of newborn SD rats purified, amplified and identified in vitro.We observed that the number of newborn rat NSCs detected in group IV (paralyzed SCE co-cultured in NSC medium) and the expression of NSCs' Notch1 and Hes1 genes after 24h and 48h culture were higher than that in group I - blank control group (PBS co-cultured in NSC medium), group II - normal group (normal SCE co-cultured in NSC medium), and group III - sham operation group (NSC culture medium with sham operation SCE co-culture) ($P< 0.05$), indicating that the injured spinal cord extract can promote the proliferation of NSC in neonatal rats and up-regulate the expression levels of NSCs' Notch1 and Hes1 mRNA in neonatal rats. This is consistent with the above results in vivo. It can be seen that the paralyzed spinal cord extract used in this experiment can reflect the local microenvironment of SCI to some extent, and the local microenvironment after SCI can promote the proliferation of NSCs.

Notch is a fate signal integrator widely used in stem cell development to keep cells self-renewing (Sun et al., 2010). Notch signaling has been shown to play multiple roles in the development of the CNS (Yoon et al., 2005). The Notch signaling pathway is believed to maintain "Stem-ness" and prevent cell cycle exit and maturation (Gomi et al., 2016). Numerous studies provide ample evidence. For instance, Alexson et al. (2006) found that targets of the Notch signaling pathway work together to block terminal differentiation and preserve stem cell pools. Yoneyama et al. (2017) exhabited Notch inhibitor extracted from *Calotropis gigantea* induces neuronal differentiation in NSCs. Notch signaling pathway is key to the formation, self-renewal, and tri-differentiation of human NSCs of in vitro origin (Venkatesh et al., 2017). MicroRNA-9 stimulation improves NSCs with zoanthamine differentiation by modulating Notch signaling(Li et al., 2019).As an effective inhibitor of Notch signaling pathway, DAPT transmits cell signals through receptors and ligands of neighboring cells, affecting the morphology and neural differentiation of NSCs(Wang et al., 2016).In this experiment, by blocking the notch signaling pathway, we observed: (1) Compared with groups I, II and III, the number of neonatal rats NSCs detected at each time point and the expression levels of NSCs' Notch1 and Hes1 genes measured 24h and 48h after culture in groups V and VI were significantly decreased ($P< 0.05$).(2) Compared with group IV, the number of newborn rats NSCs detected in group VII at each time point and the expression levels of NSCs' Notch1 and Hes1 genes measured 24h and 48h after culture were significantly decreased ($P <0.05$). (3) Compared with groups V and VI, the number of neonatal rats' NSCs detected at each time point and the mRNA expression levels of neonatal rats NSCs' Notch1 and Hes1 detected at 24h and 48h after culture in group VII were higher ($P <0.05$).(4) There was no significant difference in cell number among groups I, II and III at each time point

(P>0.05), and there was no significant difference in cell number between V and VI groups at each time point (P>0.05).It can be seen that γ-secretase inhibitor DAPT can down-regulate the expression levels of NSCs' Notch1 and Hes1 mRNA. In addition, the experimental results show that DAPT may inhibit the proliferation of NSCs by regulating Notch signaling pathway, and in addition to the microenvironment after SCI, the self-renewal, and differentiation of NSCs are also regulated by Notch signaling pathway. This is consistent with the important role that Notch signaling pathway plays in the regulation of self-maintenance, proliferation and differentiation of NSCs, which has been confirmed by many researchers (Piccin et al., 2013; Dong et al., 2012; Hiromi et al., 2011; Pierfelice et al., 2011; Cai et al., 2008; Lubman et al., 2007; Rasouli et al., 2006;). Cai et al. (2008) also found in their study that DAPT may inhibit the proliferation of NSCs by regulating Notch signaling pathway and promote the differentiation of NSCs into neurons. The differentiation of NSCs is regulated by a variety of factors, such as multiple coding genes (Winokurow et al., 2019), miR-NAs (Gao et al., 2017), traditional Chinese medicine (Son et al., 2014), hormones (Yu et al., 2019), etc. However, the exact mechanism by which effectively activating endogenous NSCs promotes functional recovery after SCI remains unclear. Then, how does DAPT inhibit the proliferation of NSCs and promote the differentiation of NSCs into neurons by regulating Notch signaling pathway? This is determined by the genetic component of the Notch signaling pathway. Notch signaling pathway is composed of notch, notch ligand and intracellular signaling molecules (CSL), in which Notch and its ligand are single transmembrane proteins (Itoh et al., 2003). The typical Notch signaling pathway begins with the combination of the Notch receptor and the adjacent Notch ligand (Kopan et al., 2009; Schweisguth et al., 2004). After binding to the ligand, conformational changes occur in the extracellular domain of the Notch

receptor. Then, the Notch receptor is first hydrolyzed by tumor necrosis factor a-converting Enzyme near the capsule in the extracellular domain (Brou et al., 2000).The Notch receptor continues to be enzymolized by gamma-secretase (gamma-secretase) and releases notch intra-cellular domain (NICD)-activated form of Notch receptor(Ma et al., 2008).After NICD enters the nucleus, its RAM domain and ankyrin (ANK) repeat sequence bind to intracellular signaling molecules (CSL) and aggregate Mastermind-like protein-1(MAML-1) to form co-excitatory complex--NICD-CSL-MAML complex(Hiromi et al., 2011).The complex continued to activate the transcription of Hes genes (mainly Hes1 and Hes5), and increased the expression levels of Hes1 and Hes5 (Lai et al., 2004; Ohtsuka et al., 1999). Activated Hes genes can maintain the state of NSCs and promote the proliferation of NSCs (Ohtsuka et al., 2001). In addition, HES genes can also block the expression of downstream Mash1, Ngn1, Ngn2 and other protonerve genes to inhibit the differentiation of NSCs into neurons (Kageyama et al., 2008; Fischer et al., 2007; Fryer et al., 2004). Gamma-secretase is the core of Notch signaling pathway regulation (Tagami et al., 2008). DAPT inhibits the action of gamma-secretase by acting on presenilin (Morohashi et al., 2006), thereby reducing the production of NICD. The downstream signaling molecules are dormant due to the absence of NICD-the activated form of Notch receptor (Geling et al., 2002), allowing NSCs to differentiate into neurons. Furthermore, Yoshikawa et al. (2009) have found that blocking Notch signaling pathway with DAPT at different times will have different effects on the differentiation and development of NSCs: premature blocking of Notch signaling may lead to insufficient NSC pool, while delayed blocking of Notch signaling may lead to delayed development and maturation of NSC. Therefore, we believe that it should choose an optimal time to block Notch signal by using DAPT. We observed group 1: control

group and group 2: treatment group in the experiment and found that: (1) compared with group i, the number of the remaining stem cells in group ii was lower at various time points ($P < 0.05$), and when DAPT was added for 2d and 3d co-culture, the percentage of NSE-positive cells (neurons) was higher and the percentage of GFAP-positive (astrocytes) was lower in group ii($P < 0.05$) (2).Intra-group analysis of Group ii: the number of stem cells added with DAPT at 1d was significantly less than that added with DAPT at 2d, 3d, 5d and 7d ($P < 0.05$), while the number of differentiated cells added with DAPT at 5d and 7d was less than that added with DAPT at 2d and 3d ($P < 0.05$).It can be seen that DAPT can inhibit the proliferation of NSCs. Premature blocking of Notch signaling (before 48 hours) may lead to the insufficiency of neural stem cell pool, while delayed blocking of Notch signaling after 72 hours may lead to delayed development and maturation of NSCs. Blocking Notch signaling at 48-72 hours can better promote the differentiation of NSC into neurons, with the highest ratio of differentiated neurons and more residual NSCs. This time point may be the best time to block Notch signaling pathway.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

The study explores using paralyzed spinal cord extract to influence neural stem cell (NSC) behavior post-SCI, revealing temporal effects on NSC proliferation. Inhibiting the Notch signaling pathway with DAPT enhances NSC differentiation into neurons, suggesting a promising strategy for promoting neural regeneration after spinal cord injury. Understanding NSC niches and their potential for multidirectional differentiation underscores their therapeutic relevance in CNS repair.

6.1 Summary and Conclusion

Firstly, some characteristics of NSCs were identified by immunofluorescence staining in this study. After the addition of BrdU, the BrdU immunofluorescence staining was positive and Nestin immunofluorescence staining was positive in the 5th generation, and NF-200, NSE and GFAP immunofluorescence staining were positive after 5 days of differentiation induced by 5% fetal bovine serum, indicating that the cultured cells were in line with the key points of identification of NSCs: It can not only express Nestin, divide, renew and proliferate, but also has the ability of multidirectional differentiation. It confirmed that the cultured cells are NSCs. Therefore, the present study developed a simplified and efficient method for neonatal rat brain-derived neural stem cell culture and identification, which may be applied to the further experimental study of NSCs.

Secondly, our experiments investigated the effect on the proliferative capacity of NSCs by co-culturing the extracts from paralyzed spinal cords, which illustrated that

the extracts from 7d paralyzed spinal cords had the greatest effect on NSCs proliferation, and indirectly demonstrated that regulating Notch signaling selected at 7d of SCI may provide an optimal time for NSCs proliferation and developmental regeneration. Thus，it is speculated that regulating the Notch/Hes1 signal at 7d after SCI may be the best time to encourage the proliferation of endogenous NSCs, and this study may supply a usable reference for the therapy of clinical SCI.

Finally, the experimental study found that paralyzed spinal cord extract can up-regulate the mRNA expression levels of NSCs' Notch1 and Hes1 and promote the proliferation of NSCs, which can better simulate the local microenvironment after SCI ， DAPT can down-regulate the expression level of NSCs' Notch1 and Hes1 mRNA and inhibit the proliferation of NSCs, and blocking Notch signaling at 48-72 hours can better promote the differentiation of NSCs into neurons, with the highest ratio of differentiated neurons and more residual NSCs. Consequently this time point (in the present experimental condition, blocking at 48-72 hours)may be the best time to block Notch signaling pathway，which may provide new ideas for clinical treatment of SCI.

6.2 Recommendations for Future Research

From the perspective from the current study, there are still some limitations in this study. Primarily, the limitations are in the development of methods for effective isolation, culture, and identification of NSCs in vitro. Secondly, the evaluation of the safety and effectiveness of NSCs obtained is the premise of clinical application. Besides, we also need to further explore its therapeutic sensitivity for SCI-SCE by using Notch1/Hes1pathway inhibitors. Finally, this study was only conducted at the cellular level in vitro, lacking animal experiments in vivo. Further research should try

to identify the relevant influencing factors behind these problems and plan possible research proposals.

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APPENDICES

Appendix A

Ethical Approval

西南医科大学实验动物福利伦理审查批件

Approval Letter for Southwest Medical University Experimental animal welfare ethical review

编号 NO.:20211115-005

项目名称 ***Project***	γ-分泌酶抑制剂和瘫痪脊髓提取物调节 Notch 信号通路对新生大鼠脑源性神经干细胞增殖和分化的影响 ***Effects of γ- secretase inhibitors and paralyzed spinal Cord extracts on the proliferation and differentiation of brain-derived neural stem cells in newborn rats by regulating Notch signaling pathway***		
项目类别 ***Project Categories***	基础研究 ***Basic research***		
审查类别 ***Review Categories***	初始审查 ***Initial review***	审查方式 ***Review Pattern***	快速审查 ***Rapid review***
项目负责人/***PI***	周庆忠 ***Zhou Qingzhong***	职称/***Title***	中级 ***Attending physician***
审评材料 ***Form be handed***			
项目申报书 ***Research proposal***			
审查结果 ***Decision***			
结论：同意 ***Conclusion: APPROVED***			
审评意见 ***Comments***			
经伦理委员会对该研究议案等资料进行评审后，认为该提案的实验设计及实验方案符合实验动物福利伦理要求，能有效保障研究对象的权益。 伦理委员会批准同意这个研究提案进行。 ***After reviewing the research proposal and other materials, the Ethics Committee found that the experimental design and scheme of the proposal met the welfare and ethical requirements of experimental animals and could effectively protect the rights and interests of research subjects. The ethics committee approved the proposed study.*** 伦理委员会（盖章） 日期/***Date***： 2021.11.15			

Appendix B

List of Reagents Used

No.	Name of the reagent	Origin and Article Number
1.	DEME/F12 (1:1)	Hyclone Corporation (SH30023.01b)
2.	Standard Fetal Bovine Serum	Hyclone Corporation (SV30087.01)
3.	bFGF(10 ug)	Peprotech Inc. (100-18B)
4.	EGF (20ug)	PEPROTECH Inc. (AF-100-15A)
5.	B27 additive (50×)	Gibco Corporation (1216255)
6.	TrypLTM Express (1×)	Gibco Corporation (12604)
7.	Hyaluronidase (100mg)	Sigma Corporation (H3506)
8.	Canine Uroquinolinic acid (250mg)	Sigma Corporation (K3375)
9.	Trypsin (500mg)	Sigma Corporation (T5266)
10.	Polylysine	Biyuntian Biotechnology Co., LTD. (ST508)
11.	Penicillin - streptomycin solvent	Biyuntian Biotechnology Co., LTD. (ST488)
12.	DAPI staining fluid	Biyuntian Biotechnology Co., LTD. (C1005)
13.	Determined by MTT kits	Biyuntian Biotechnology Co., LTD. (C0009)
14.	Anti-fluorescence quenching agent	Biyuntian Biotechnology Co., LTD. (P0126)
15.	Seal with sheep serum	Solebo Technology Co., LTD. (S8080)
16.	Rabbit polyclonal antibody against Nestin	Baishi Biological Engineering Co., LTD. (BA1289)
17.	Mouse anti-BRDU monoclonal antibody	Baishi Biological Engineering Co., LTD. (BM0201)
18.	Mouse anti-NF200 monoclonal antibody	Baishi Biological Engineering Co., LTD. (BM0100)
19.	Mouse monoclonal antibody against NSE	Baishi Biological Engineering Co., LTD. (BM0201)
20.	Rabbit polyclonal antibody against GFAP	Baide Biological Engineering Co., LTD. (BA0056)
21.	FITC labeled Sheep Anti-Rabbit IgG	Baishi Biological Engineering Co., LTD. (BA1105)
22.	FITC labeled Sheep Anti-mouse IgG	Baishi Biological Engineering Co., LTD. (BA1101)
23.	RBITC labeled Sheep anti-Rabbit IgG	Boolsen Biotechnology Co., LTD. (BS-0295G-RBITC)
24.	Total RNA Extraction Kit	Tiagen Biochemical Technology Co., LTD
25.	DNA Marker	Tiagen Biochemical Technology Co., LTD. (DM080530)
26.	Pyrolysis liquid RZ	Tiagen Biochemical Technology Co., LTD. (RK145)
27.	RT-PCR Kit	Borick Biotechnology Co., LTD. (BRK100T)
28.	PCR Primers	Sangong Bioengineering (Shanghai) Co., LTD
29.	DAPT	Gene Operation

Appendix C

List of the Instruments Used

No.	Name of main instrument	Actual origin
1.	PowerShot digital camera	Cannon, Japan
2.	Inverted phase contrast microscope photographic system	Leica, Germany
3.	Ultra clean table	Shanghai Lishen Scientific Instrument Co., LTD
4.	LDZ5-2 centrifuge	Beijing Medical Centrifugal Airport
5.	High speed multi-function refrigerated centrifuge	Thermo, Inc
6.	Fluorescence microscope	OLYMPUS Corp. of Japan
7.	Ultra-low temperature refrigerator (-80℃)	Thermo, Inc
8.	Constant temperature incubator for CO2 gas	Thermo, Inc
9.	Constant temperature incubator	Shanghai Ketong Test Instrument Factory
10.	Electronic balance	Shanghai Shangping Instrument Co., LTD
11.	Anatomical microscope	OLYMPUS Corp. of Japan
12.	Full spectrum spectrophotometer	Thermo, Inc
13.	Ultraviolet spectrophotometer	Beckman Company, USA
14.	The PCR instrument	BioRad Inc
15.	Electrophoresis apparatus	Beijing 61 Factory
16.	Uv digital gel imaging analysis system	BioRad Inc
17.	Electric blast drying oven	Tianjin Tester Instrument Co., LTD
18.	Vertical pressure steam sterilizer	Shanghai Shenan Medical Instrument Factory
19.	Homemade Allen rack	Animal Experiment Center, Southwest Medical University
20.	Somatosensory evoked potentiometer	Dantec Corporation, USA
21.	Microsampler	Gilson
22.	Microporous membrane filter (0.22um)	GelmenScience
23.	Culture flask and culture plate	Corning Corporation
24.	EP tube and centrifugal tube	Corning Corporation
25.	Surgical instruments	Animal Experiment Center, Southwest Medical University

Appendix D

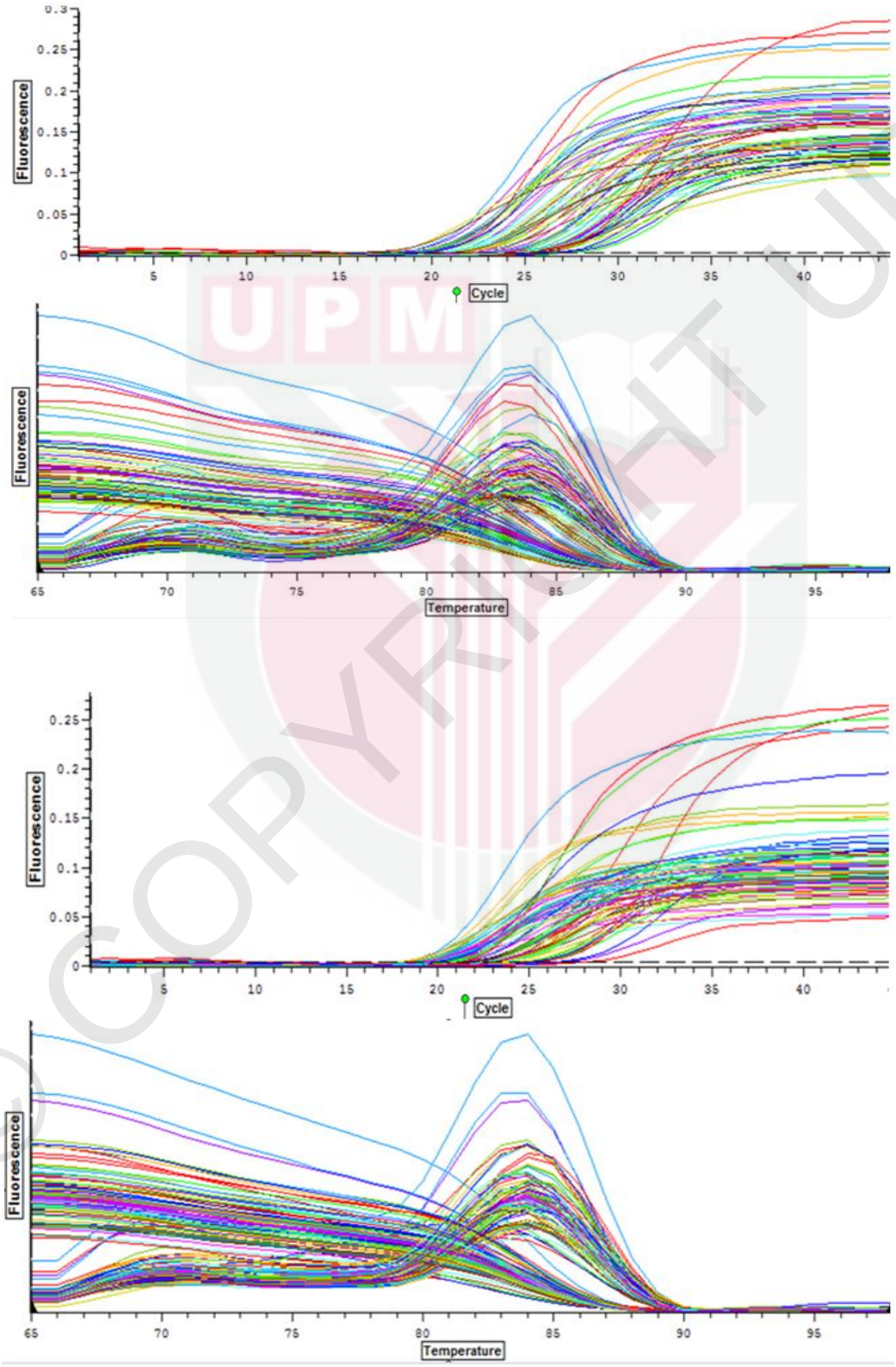
21-Point Functional Evaluation Scale

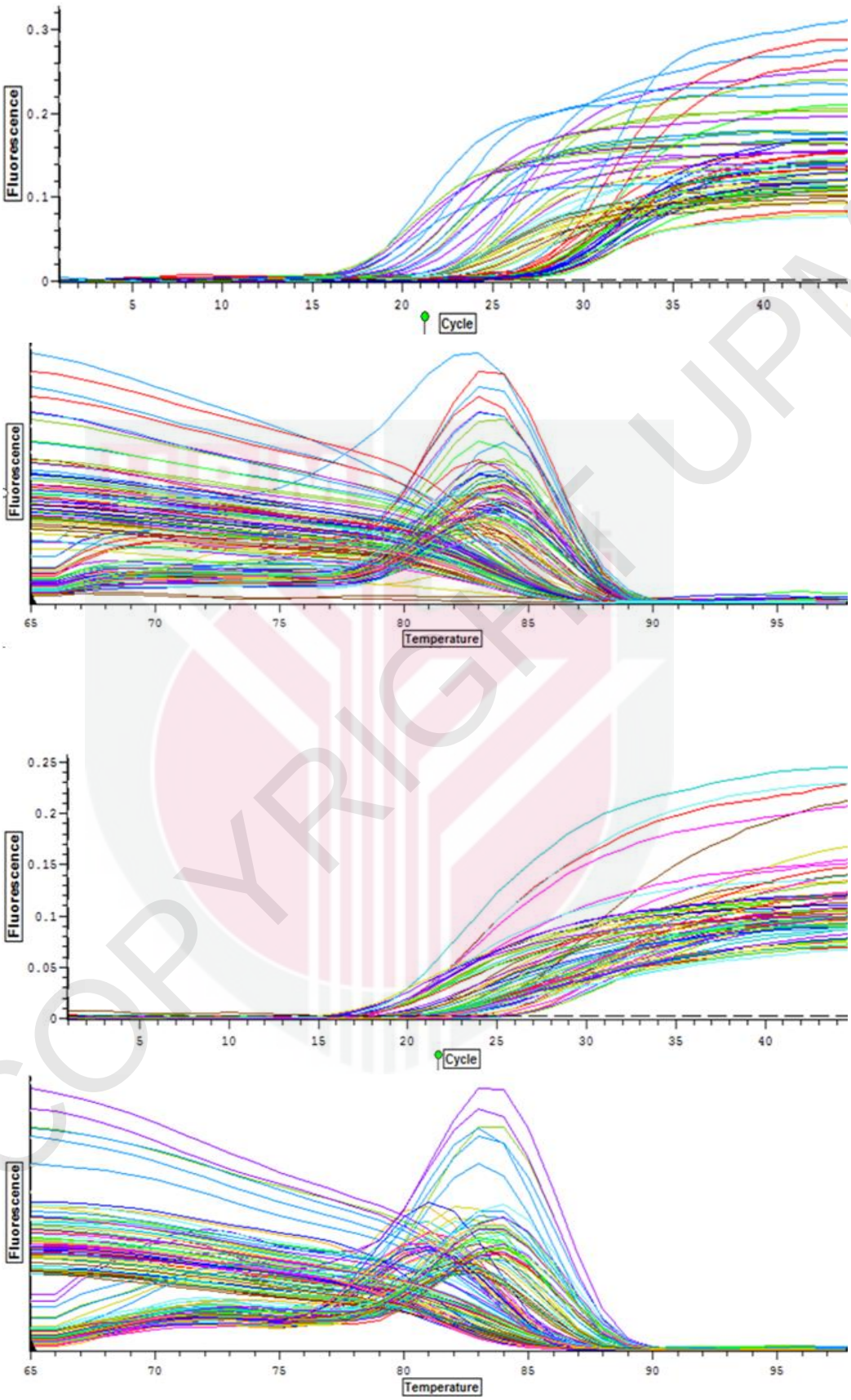
Score	Operational definitions of categories and attributes
0	No observable movement of the hindlimbs.
1	Slight (limited) movement of one or two joints, usually hip and/or knee.
2	Extensive movement of one joint or extensive movement of one joint and slight movement of the other.
3	Extensive movement of two joints.
4	Slight movement of all three joints of the hindlimbs.
5	Slight movement of two joints and extensive movement of the third joint.
6	Extensive movement of two joints and slight movement of the third joint.
7	Extensive movement of the three joints in the hindlimbs.
8	Sweeping without weight bearing or plantar support of the paw without weight bearing.
9	Plantar support of the paw with weight bearing only in the support stage (i.e., when static) or occasional, frequent or inconsistent dorsal stepping with weight bearing and no plantar stepping.
10	Plantar stepping with occasional weight bearing and no forelimb-hindlimb coordination.
11	Plantar stepping with frequent to consistent weight bearing and occasional forelimb-hindlimb coordination.
12	Plantar stepping with frequent to consistent weight bearing and occasional forelimb-hindlimb coordination.
13	Plantar stepping with frequent to consistent weight bearing and frequent forelimb-hindlimb coordination.
14	Plantar stepping with consistent weight support, consistent forelimbhindlimb coordination and predominantly rotated paw position (internally or externally) during locomotion both at the instant of initial contact with the surface as well as before moving the toes at the end of the support stage or frequent plantar stepping, consistent forelimb-hindlimb coordination and occasional dorsal stepping.
15	Consistent plantar stepping, consistent forelimb-hindlimb coordination and no movement of the toes or occasional movement during forward movement of limb; predominant paw position is parallel to the body at the time of initial contact.
16	Consistent plantar stepping and forelimb-hindlimb coordination during gait and movement of the toes occurs frequently during forward movement of the limb; the predominant paw position is parallel to the body at the time of initial contact and curved at the instant of movement.
17	Consistent plantar stepping and forelimb-hindlimb coordination during gait and movement of the toes occurs frequently during forward movement of limb; the predominant paw position is parallel to the body at the time of initial contact and at the instant of movement of the toes.
18	Consistent plantar stepping and forelimb-hindlimb coordination during gait and movement of the toes occurs consistently during forward movement of limb; the predominant paw position is parallel to the body at the time of initial contact and curved during movement of the toes.
19	Consistent plantar stepping and forelimb-hindlimb coordination during gait and movement of the toes occurs consistently during forward movement of limb; the predominant paw position is parallel to the body at the instant of

	contact and at the time of movement of the toes, and the animal presents a downward tail some or all of the time.
20	Consistent plantar stepping and forelimb-hindlimb coordination during gait and movement of the toes occurs consistently during forward movement of limb; the predominant paw position is parallel to the body at the instant of contact and at the time of movement of toes, and the animal presents consistent elevation of the tail and trunk instability.
21	Consistent plantar stepping and coordinated gait, consistent movement of the toes; paw position is predominantly parallel to the body during the whole support stage; consistent trunk stability; consistent tail elevation

Appendix E

Melting Curve Analysis of Notch1 and Hes1 mRNA of Each Group after Co-Cultured for 24h and 48h





Appendix F

Raw Data

The MTT Values of NSC of Each Groups at Different Time Point (Mean ± SD, n=10)

Group	1d	2d	3d	4d	5d
A	0.1317±0.0250	0.1860±0.0187	0.2378±0.0288	0.2865±0.0238	0.3224±0.0175
B	0.1424±0.0331	0.1813±0.0324	0.2452±0.0310	0.2806±0.0337	0.3094±0.0273
C	0.1487±0.0303	0.1955±0.0311	0.2276±0.0273	0.3070±0.0197	0.3149±0.0173
D	0.1939±0.1613	0.2384±0.0353	0.2911±0.3306	0.3533±0.0274	0.3831±0.0166
E	0.1839±0.1513	0.2284±0.0313	0.2951±0.3106	0.3733±0.0284	0.3931±0.0216
F	0.1919±0.1523	0.2287±0.0313	0.2851±0.3106	0.3633±0.0287	0.3911±0.0197
G	0.2039±0.1516	0.2784±0.0343	0.3116±0.3376	0.3733±0.0268	0.4331±0.0182
H	0.2139±0.1733	0.2864±0.0253	0.3217±0.3276	0.3812±0.0297	0.4376±0.0232
I	0.1899±0.1589	0.2384±0.0373	0.2811±0.2913	0.3511±0.0274	0.3831±0.0147

The Remaining Number of NSC after Blocking Notch Signal at Different Time Point (Mean ± SD, ×10 3, n=3)

Group	1d	2d	3d	5d	7d
I	0.476±0.025	0.856±0.031	0.987±0.025	1.124±0.036	1.176±0.015
II	0.363±0.036	0.783±0.050	0.854±0.035	0.936±0.015	0.973±0.021

The Cell Numbers of NSC of Each Groups at Different Time Point in Each Group (Mean ± SD, $\times 10^6$, n =3)

Group	1d	2d	3d	4d	5d
			Part 1		
A	0.663±0.031	0.920±0.026	1.767±0.025	2.363±0.050	2.707±0.050
B	0.667±0.031	0.907±0.032	1.783±0.031	2.280±0.046	2.697±0.021
C	0.677±0.153	0.873±0.021	1.780±0.020	2.313±0.055	2.683±0.012
D	0.797±0.153	1.213±0.025	2.003±0.055	2.563±0.031	2.903±0.025
E	0.827±0.125	1.287±0.031	2..167±0.055	2.667±0.035	2.997±0.025
F	0.887±0.115	1.310±0.026	2.223±0.021	2.723±0.021	3.007±0.026
G	0.937±0.145	1.403±0.025	2.410±0.031	3.057±0.029	3.233±0.025
H	0.997±0.125	1.493±0.025	2.511±0.030	3.007±0.027	3.433±0.015
I	0.897±0.153	1.313±0.025	2.103±0.055	2.663±0.031	2.993±0.025
			Part 2		
I	0.654±0.042	0.932±0.016	1.856±0.032	2.298±0.153	2.697±0.050
II	0.707±0.053	0.912±0.047	1.793±0.031	2.248±0.015	2.717±0.031
III	0.699±0.165	0.893±0.031	1.789±0.025	2.301±0.046	2.679±0.021
IV	0.817±0.032	1.194±0.046	2.112±0.153	2.656±0.025	2.897±0.035
V	0.537±0.031	0.821±0.015	0.876±0.050	1.416±0.025	1.518±0.153
VI	0.541±0.025	0.814±0.021	0.832±0.015	1.342±0.046	1.493±0.055
VII	0.673±0.026	1.099±0.031	1.209±0.029	1.576±0.031	1.876±0.015

The Expression of Target mRNA in Each Group after Co-Cultured for 24 Hours (Mean ± SD, n =3)

Group	Notch1	Hes1
	Part 1	
A	1.003±0.097	1.016±0.212
B	1.175±0.162	1.146±0.213
C	1.076±0.212	1.156±0.352
D	1.312±0.333	1.540±0.386
E	1.879±0.148	1.875±0.084
F	2.325±0.427	2.207±0.199
G	2.712±0.183	3.960±0.569
H	3.800±0.495	4.659±0.422
I	3.107±0.278	3.602±0.495
	Part 2	
I	0.986±0.035	0.942±0.023
II	0.989±0.051	0.987±0.031
III	0.945±0.031	0.918±0.036
IV	1.162±0.108	1.129±0.035
V	0.854±0.036	0.840±0.024
VI	0.849±0.064	0.836±0.052
VII	1.061±0.109	1.020±0.036

The Expression of Target mRNA in Each Group after Co-Cultured for 48 Hours (Mean ± SD, n =3)

Group	Notch1	Hes1
	Part 1	
A	1.004±0.099	1.010±0.176
B	1.203±0.170	1.177±0.119
C	1.063±0.067	1.282±0.462
D	1.629±0.249	2.213±0.756
E	2.108±0.136	2.735±0.599
F	3.035±0.128	3.725±0.294
G	4.132±0.574	5.302±1.192
H	7.217±0.279	7.675±0.838
I	4.191±0.291	6,302±0.637
	Part 2	
I	0.924±0.016	0.895±0.003
II	0.940±0.013	0.942±0.019
III	0.933±0.032	0.935±0.156
IV	1.176±0.045	1.189±0.032
V	0.893±0.136	0.887±0.031
VI	0.891±0.033	0.876±0.155
VII	1.081±0.031	1.052±0.041

The Proportion of Neuron and Astrocytes after Blocked Notch Signal at Each Time Point (Mean ± SD, n=3)

Group	1 d	2 d	3d	5d	7d
			The proportion of neuron		
I	8.93±0.036	9.79±0.814	9.87±0.618	10.21±0.411	10.87±0.365
II	8.99±0.147	15.49±0.207	16.76±1.211	12.27±0.681	12.29±0.898
			The proportion of astrocytes		
I	91.07±0.036	90.21±0.814	90.13±0.618	89.19±0.411	89.13±0.365
II	91.01±0.147	84.51±0.207	83.24±1.211	87.73±0.681	87.71±0.898

The Number of Differentiated Cell in Group ii at Each Blocking Time Point (Mean ± SD ×10 3, n=3)

	1d	2d	3d	5d	7d
Group II	0.278±0.036	0.541±0.015	0.563±0.021	0.439±0.025	0.427±0.015

Note: Part 1: Group A - blank control group (PBS solution was added into the medium for co-culture). Group B - normal group (normal spinal cord extract was added). Group C - sham operation group (sham operation spinal cord extract was added). Group D - paralyzed group 1 (8h paralyzed spinal cord extract was added). Group E -paralytic group 2 (24h paralytic spinal cord extract was added). Group F-paralytic group 3 (72h paralytic spinal cord extract was added). Group G -paralytic group 4 (5d paralytic spinal cord extract was added). Group H-paralytic group 5 (7d paralytic spinal cord extract was added). Group I - paralyzed group 6 (14-day paralyzed spinal cord extract was added).

Part 2: Group I- blank control group (PBS solution was added into the medium for co-culture). Group II- Normal group (normal SCE was added). Group III- sham operation group (sham operation SCE was added). Group IV - paralysis group (injured SCE was added). Group V - normal +DAPT group (DAPT and normal SCE was added).Group VI- sham operation +DAPT group (DAPT and sham operation SCE was added). Group VII - paralyzed +DAPT group (DAPT and injured SCE was added). Group I- control group (NSCs culture medium was added with paralytic spinal cord extract for co-culture). Group II- treatment group (NSCs culture medium was added with paralytic spinal cord extract for co-culture)

LIST OF PUBLICATIONS

Zhou Q, Tian L, Jia X, Ling KH, Mohd Nor NH, Harun MH, Feng D, Wan Sulaiman WA. Extract from the paralyzed spinal cord of rats enhances neural stem cell proliferation in the neonatal rat brain by upregulating the Notch1/Hes1 pathway. Minerva Pediatr (Torino).2023 Oct; 75(5): 780-783. doi: 10.23736/S2724-5276.23. 07322-6. Epub 2023 Jun 7. PMID: 37284814.

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Zhou Q, Yang Y, Zhang G, Nor N H B M, Harun M H B, Sulaiman W A W, Feng D. Investigation of γ-secretase Inhibitor DAPT as a Potential Regulator of Notch Pathway in Rat's NSCs after Spinal Cord Injury. J Nat Sc Biol Med. 2023; 14:176-188. DOI: https://jnsbm.org/menuscript/index.php/submissions/article/view/241