Characterization, differentiation and therapeutic application of NG2+ progenitor cells

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CHARACTERIZATION, DIFFERENTIATION AND THERAPEUTIC APPLICATION OF NG2+ PROGENITOR CELLS

JU PEIJUN

SCHOOL OF BIOLOGICAL SCIENCES

2013

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
ATCC	American Type Culture Collection
A2B5	Neuronal cell surface antigen
BAC	Bacterial artificial chromosome
BBB	Basso, Beattie, and Bresnahan scale
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CGT	Ceramidegalactosyltransferase
CG4 cell line	Central glial 4 cell line
ChAT	Choline acetyltransferase
CNS	Central nervous system
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CSPG-4	Chondroitin sulfate proteoglycan-4
DAPI	4', 6'-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's medium
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
(p)EGFR	(Phosphorylated) epidermal growth factor receptor
FACS	Fluorescence-activated cell sorting

FBS	Fetal bovine serum
FCM	Flow cytometry
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GalC	Galactocerebroside C
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GS	Glutamine synthetase
GSK-3β	Glycogen synthase kinase-3β
H&E	Hematoxylin & Eosin
HG	High glucose
hTERT	human Telomerase Reverse Transcriptase
IGF-1	Insulin-like growth factor 1
i.p.	Intraperitoneal
JNK	c-Jun N-terminal kinases
LCM	Laser capture microdissection
MAP2	Microtubule-associated protein 2
MBP	Myelin basic protein
MKK7	Mitogen-activated protein kinase kinase 7
MOG	Myelin oligodendrocyte glycoprotein
NF200	Neurofilament heavy 200
NPCs	Neural progenitor cells

NPCs	Neuronal precursor cells
NSCs	Neural stem cells
NSE	Neuron specific enolase
NT3	Neurotrophin-3
N2A cell line	Neuro-2a cell line
Olig2	Oligodendrocyte transcription factor 2
OPCs	Oligodendrocyte progenitor cells
OX42	CD11b/c equivalent
O-2A	Oligodendrocyte- type2- astrocyte
PBS	Phosphate buffered saline
PDGF-AA	Platelet derived growth factor-AA
PDGFαR	Platelet-derived growth factor-α receptor
p-ERK1/2	Phosphorylated extracellular signal-regulated kinase 1/2
PF	Paraformaldehyde
PKB, AKT	Protein kinase B
PLCγ	Phospholipase Cγ
PLL	poly-L-lysine
PLP	Proteolipid protein
p-MEK	Phosphorylated mitogen-activated protein kinase
P/S	Penicillin-Streptomycin
PVDF	Polyvinylidene difluoride membrane
RT	Room temperature

RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SCI	Spinal cord injury
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGZ	Subgranular zone
SHC	Src homology and collagen
siRNAs	Short interfering RNAs
SMI32	Neurofilament H Non-Phosphorylated
SPF	Specific pathogen free
SVZ	Subventricular zone
S100 β	S100 calcium-binding protein β
TAE	Tris-acetate-EDTA
β-tubulin ш	Neuronal specific class ш β-tubulin
T3	Triiodothyronine
WB	Western blot

SUMMARY

The widespread NG2-expressing cells in the central nervous system (CNS) are considered as heterogeneous populations with plastic lineage potential. They rapidly proliferate and differentiate in response to CNS injuries, thereby possessing a therapeutic potential.

In order to elucidate the lineage potential of NG2+ cells, twenty-four NG2+ clones were generated and induced for neuronal-glial lineage differentiation. *In vitro* differentiation analysis revealed that all the clones could differentiate into oligodendrocytes, and seven of them were bipotent of differentiating into both oligodendrocytes and astrocytes. One clone exhibited a multipotent capacity of differentiating into not only neuronal-glial lineages, but also chondrocytes. These distinct subtypes of NG2+ cells were further found to exhibit phenotypic heterogeneity based on the examination of a spectrum of neural progenitor markers (NG2, PDGFαR, Nestin, and A2B5). These results collectively suggest that the NG2+ cells contain heterogeneous progenitors with distinct differentiation capacities.

Based on the clonal analysis of NG2+ cells, we verified that NG2+ cells could be induced to acquire neuronal phenotypes by inhibiting epidermal growth factor receptor (EGFR) signaling pathway under the gliogenic conditions. We further confirmed this phenomenon with an independent glial cell line, Central Glial 4 (CG4) cells, and found that the EGFR downstream Ras-ERK axis played a key role during neuronal

differentiation of NG2+ cells. To explore the therapeutic significance of neurogenesis from NG2+ cells by EGFR inhibition, we set up the experimental contusive spinal cord injury (SCI) model. We found that antagonizing EGFR functions with a specific EGFR inhibitor (PD168393) caused significant numbers of NG2+ cells (either *in situ* or through *ex-vivo* transplantation) to acquire neuronal phenotypes in mouse SCI, which presumably led to accumulation of newly-generated neurons and contributed to the improved neural behavioral performance of these animals. Additionally, by attenuating EGFR signaling in the injured niche, improved histological recovery, reduced astrogliosis and microglia/macrophages post SCI were also observed, probably due to the synergetic benefit from EGFR inhibition after injury.

Taken together, the whole study suggests that NG2+ cells, well-known as glial progenitors, are heterogeneous populations with distinct lineage potentials at clonal level. Intriguingly, they could be manipulated for repairing neuronal loss post SCI by EGFR inhibition. These findings support the possibility of evoking endogenous neuronal replacement from NG2+ cells and suggest that EGFR inhibition may be beneficial for treating CNS trauma.

CHAPTER 1 Introduction

Observations made over the past decades have revealed one new glial cell population in the mammalian CNS, which has NG2 chondroitin sulfate proteoglycan expressed on the cell surface. These cells have been known as NG2 expressing cells (NG2+ cells) [1-3]. In addition to the four cell types in CNS, known as oligodendrocytes, astrocytes, microglia and neurons, the NG2+ cells come to be regarded as a novel "fifth neural cell type". They are found to be widely distributed throughout the developing and mature CNS, eventually accounting for about 8-9% of the total cells in adult white matter and 2-3% of the total cells in adult grey matter [4]. Although NG2+ cells have been initially referred to as oligodendrocyte progenitor cells (OPCs), they are demonstrated to give rise to other neural progenies besides oligodendrocytes and exhibit diverse functions [2, 5-7]. However, their detailed cellular characteristics, especially the differentiation capacity, remain to be characterized. Additionally, it is worth investigating whether their plastic nature could be harnessed for mediating repairment in injured adult CNS, due to the striking enrichment and activation of NG2+ cell populations after various CNS injuries.

1.1 NG2 proteoglycan and NG2+ cells

NG2 proteoglycan, a mammalian protein of 330 kDa (also named chondroitin sulfate proteoglycan-4, CSPG-4), is a single membrane-spanning chondroitin sulphate

proteoglycan with a large extracellular domain and a short cytoplasmic tail [8]. It is encoded by a single gene with multiple exons coding for 2327 amino acids, and no alternatively spliced variants have been reported so far. NG2 molecule is first characterized as a high-molecular-weight type 1 membrane proteoglycan in rat [1]. Independent NG2 homologue is discovered in mouse by immunoaffinity purification of the AN2 protein from early postnatal mouse brain [9], and the human homologue of NG2 proteoglycan is subsequently identified from human melanoma cell lines, termed melanoma chondroitin sulfate proteoglycan [10]. Generally, “AN2” and “NG2” are synonyms, and AN2/NG2 proteoglycan specify the same cell population in rodent CNS [11]. The term “NG2” was used more often in the studies.

The NG2 proteoglycan has been mainly identified as having co-receptor and /or modulary roles in a variety of signaling pathways underlying cell migration, proliferation and process outgrowth in CNS. For example, a cooperative relationship between NG2 proteoglycan and platelet-derived growth factor-α receptor (PDGFαR), the putative marker of OPCs, was demonstrated years ago [12]. NG2 proteoglycan has also been shown to promote the proliferation and motility of oligodendrocyte progenitors in developmental CNS [13]. During the glioma progression, NG2 proteoglycan is verified to contribute to the critical processes such as glioma proliferation, motility and survival [14].

NG2 proteoglycan is also found to regulate neurite outgrowth. Previous studies reveal that it could inhibit neurite outgrowth from several classes of cultured neurons *in vitro*

[15]. In particular, its N-terminal globular domain and juxtamembrane domain could inhibit neurite growth independently [16]. The *in vivo* experiments suggest that the accumulation of NG2 molecule at sites of CNS injury might also inhibit the re-growth of injured axons since the regeneration of these axons usually end in the areas rich in the NG2 proteoglycan [17, 18]. On the other hand, neutralizing NG2 molecule with anti-NG2 monoclonal antibody could promote the axonal re-growth [19]. Thus, these results indicate the inhibitory function of NG2 proteoglycan on axon regeneration in the injured CNS.

NG2 proteoglycan has been widely accepted as one of the hallmarks of glial progenitors in CNS. This cell type can be identified by virtue of expressing several neural markers, in particular, the NG2 proteoglycan. Nonetheless, NG2 proteoglycan is also expressed by some macrophages [20, 21], microglia [22] in pathological CNS as well as some immature schwann cells, perineural fibroblasts and pericytes [9, 23, 24]. These cell types can be clearly distinguished from NG2+ cells (glial progenitors) by their morphology and other specific lineage markers. Therefore, the NG2+ cells are specifically referred to as glial progenitors in this study.

The NG2+ cell population has been endowed with several names along with the progressive understanding of them. They are originally defined as oligodendrocyte-type2- astrocyte (O-2A) progenitors, because they are found to differentiate into both oligodendrocytes and type2-astrocytes *in vitro* [25-27]. “OPCs” [28] and “Synantocytes” [29] have been used to name these cells subsequently. It is not until

recently that “Polydendrocyte” has been proposed to define the NG2 expressing neural cells, which could reflect their morphological and functional heterogeneity [30].

1.2 Morphological features of NG2+ cells

The morphologies of NG2+ cells have been observed to be multiplex at distinct developmental stages as well as in different regions of CNS. During embryogenesis, the morphologies of NG2+ cells are revealed to be simple and oval [31]. In the neonatal phase, NG2+ cells extend multiple processes with a radial orientation in the grey matter, while those in the white matter usually have few short processes emanating from opposing poles of the cell body and aligning with the nerve fibres [32, 33]. As the CNS matures, the morphologies of these cells become more complex with greater arborizations, so that each cell within the grey matter occupies a distinct domain, while those in the white matter lie between myelinated axons [34]. Generally, the morphologies of NG2+ cells become more complex during the development of CNS. The complex morphology of NG2+ cells does not negate their role as oligodendrocyte progenitors but further assumes their potential functions in adult CNS.

1.3 Progenies of NG2+ cells

NG2+ cells are found to generate several neural progenies since they were identified in CNS. Though the oligodendrocyte fate of NG2+ cells in both white and gray matter is consistently reported, the findings related to astrocyte and neuronal fate of NG2+ cells vary. Therefore, there has been much debate regarding how heterogeneous the NG2+

cells are in terms of their ability to differentiate (Fig. 1). To clarify the progenies of NG2+ cells, especially in neonatal and adult rat (mouse) CNS, double immunofluorescence staining of NG2 proteoglycan with markers of neuron-glia lineages and *in vivo* fate mapping technologies have been mainly used to determine the lineage of NG2+ cells.

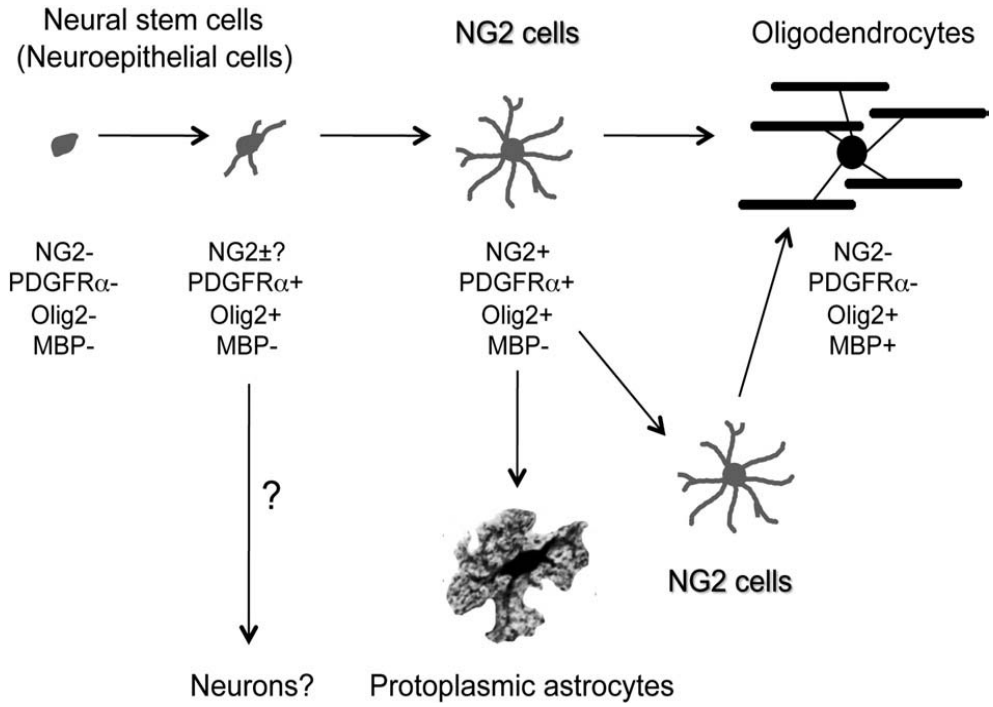


Figure 1 A schematic diagram showed the plastic lineages of NG2+ cells.

NG2+ cells that derived from the NG2-/PDGFαR- neural stem cells (NSCs) have the ability to self-renew in CNS. Majority of NG2+ cells express oligodendrocyte transcription factor 2 (Olig2) which is important for the development of NG2+ cells. NG2+ cells mostly undergo terminal differentiation into myelin basic protein (MBP) + oligodendrocytes, in which they lose the expression of NG2 proteoglycan and PDGFαR. MBP, which is expressed by the mature oligodendrocytes, is the major myelin sheath constituent in CNS, and plays an important role in the process of myelination of nerves. NG2+ cells could also give rise to protoplasmic astrocytes.

Currently, evidence accumulates to support the notion that NG2+ cells might acquire neuronal fate [7].

1.3.1 Oligodendrocyte lineage of NG2+ cells

1.3.1.1 NG2+ cells generate oligodendrocytes in embryonic and neonatal CNS

Oligodendrocytes belong to the macro-neuroglia pool in CNS, and function to insulate the axons of neurons by creating myelin sheath. In the developmental course of OPCs and oligodendrocytes, they are initially found to acquire the expression of PDGFαR at embryonic day 12-14 [35]. Subsequently, these cells migrate quickly to occupy almost all regions of CNS [36, 37].

As the PDGFαR+ cells migrate away from germinal zones in the forebrain or from the ventricular zone in the spinal cord of developmental CNS, they acquire NG2 expression gradually. At embryonic day 16, all the PDGFαR+ cells are found to be immunopositive for NG2 expression, which could reach the peak intensity during the first postnatal week [28]. Before losing the expression of NG2 proteoglycan and fully differentiating into oligodendroglia, they could enter an intermediate stage characterized by co-expressing NG2 proteoglycan and earliest oligodendroglial marker O4, usually in the first two postnatal weeks [38]. During the active myelination period, it has also been shown that NG2 proteoglycan and some mature oligodendrocyte antigens, such as DM-20/proteolipid protein (PLP), galactocerebroside C (GalC) and MBP, are co-expressed in a small fraction of NG2+ cells [25, 28, 39]. Such double labeled cells are suggested to represent cells in transit from NG2+ cells to mature

oligodendrocytes. These findings are further confirmed with fate-mapping techniques. For example, the enhanced green fluorescent protein (EGFP) driven by the 2'-3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) or the myelin PLP promoter are found to be expressed in NG2+ cells of neonatal mouse [40, 41]. The co-expression of NG2 proteoglycan with oligodendrocyte lineage markers not only reveal the intermediate stage of NG2+ cells differentiating into oligodendrocytes but also indicate the phenotypic heterogeneity of NG2+ cell populations. In this way, NG2+ cells at early developmental stages may become divergent in the course of CNS maturation. Additionally, more than 90% of embryonic NG2+ cells express Olig2, which is a basic helix-loop-helix transcriptional factor required for oligodendrocyte lineage specification [42]. Conclusively, specified by oligodendrocyte lineage markers and transcription factors, NG2+ cells are thought to have the highest possibility of committing lineal progression to myelinating oligodendrocytes in developmental CNS.

1.3.1.2 NG2+ cells gave rise to oligodendrocytes in adult normal CNS

Whether NG2+ cells in the adult CNS also function as oligodendrocyte progenitors attracts a lot of attention. NG2+ cells have been found to express mRNAs for PLP, MBP, CNPase, which are detected by laser capture microdissection (LCM) in both neonatal and adult mouse [43]. Moreover, double immunofluorescence staining experiment also shows the co-localization of NG2 proteoglycan and myelin oligodendrocyte glycoprotein (MOG) on cells in normal adult rat spinal cord. This phenomenon reveals that some NG2+ cells are MOG positive in the adult CNS [44], indicating the oligodendrocyte lineage potential of NG2+ subpopulations in adulthood.

Several lineage tracing methods have been further used to study the oligodendrocyte lineage potential of NG2+ cells. Retrovirally-labeling studies demonstrate that the retrovirus infected NG2+ cells likely represent the cycling NG2+ populations, and they would be responsible for the accumulation of oligodendroglia in adult rat neocortex or the forebrain [45, 46]. Additionally, pulse chase labeling with 5-bromo-2'-deoxyuridine (BrdU) has been used to trace the lineage of the proliferating NG2+ cells. In some regions of CNS, the majority of the BrdU labeling cells are NG2+ cells, and BrdU+ progenies are found to acquire mature oligodendrocyte markers. These results indicate that these BrdU+ oligodendrocytes may be derived from NG2+ cells over time in mature CNS [4, 47]. In the recent years, the Cre-lox fate mapping techniques which employ bacterial artificial chromosome (BAC) modification approach are used for generating NG2+ transgenic mice. In these transgenic mice, DsRed is specifically expressed in NG2+ cells. For example, NG2creER^{TM}BAC transgenic mice are generated, with the tamoxifen-inducible expression of Cre recombinase. In this way, NG2+ cells are directly and convincingly verified to give rise to oligodendrocytes progenies in rodent neonatal and adult CNS. These studies demonstrate that oligodendrocyte differentiation from NG2+ cells continues to occur in the adult CNS [48, 49]. Further studies reveal that the ability of NG2+ cells generating oligodendrocytes declines with age [50]. Meanwhile, the technique of homologous recombination in ES cells (knock-in) has also been used to confirm that NG2+ cells are closely associated with oligodendrocyte lineages [51]. Therefore, these studies have successfully shown that NG2+ cells in the normal adulthood could give

rise to oligodendrocyte progenies. The presence of NG2+ cells in adult CNS and their potential for giving rise to oligodendrocytes could be promisingly exploited for myelin repair in treating the demyelinating diseases of CNS.

1.3.2 Astrocyte lineage of NG2+ cells

The characteristic star-shaped glial cells in the brain and spinal cord are named as astrocytes. They mainly function to support endothelial cells, provide nutrients to the nervous tissue, and maintain the extracellular ion balance. They also play a role in the repair and scarring in the traumatic injured CNS [52-54].

The NG2+ cells are also found to differentiate into type2-astrocytes *in vitro* besides the oligodendrocytes, and share the similar bushy morphology of smooth protoplasmic astrocytes in the adult CNS at first sight. However, the investigation for the astrocyte fate of NG2+ cells *in vivo* has been obstructed for a long time, because few NG2+ cells are found to express astrocyte lineage markers. Although Zhou et al. describe that transcription of glial fibrillary acidic protein (GFAP) mRNA appears to be active in NG2+ cells [55], GFAP mRNA and protein have not been reliably detected in NG2+ cells in other reports [56]. Apart from antigenical discrepancy, NG2+ cells *in vivo* are also considered to be functionally segregated from astrocytes with respect to ion channels and glutamate responsiveness [30]. However, the transplanted NG2+ cells are found to generate astrocytes in the glia free environment and the injured CNS [57, 58]. These results indicate the astroglial lineage potential of NG2+ cells, especially in

the injured conditions of CNS. Moreover, these findings provide some clues that NG2+ cells probably possess the potential of generating astrocyte progenies.

During the development of CNS, some NG2+ cells in NG2creER^{TM} BAC transgenic mice have been recently found to generate protoplasmic astrocytes in the grey matter [48, 49]. Further evidence reveals that NG2+ cells in the ventral forebrain could generate astrocytes as early as in embryonic phase [50]. However, there is no proof that the fibrous astrocytes in the white matter are derived from NG2+ cells. These results demonstrate that some NG2+ cells could generate protoplasmic astrocytes. In other transgenic mice models which label different subpopulations of NG2+ cells, the discrepant astrocyte lineage potential of NG2+ cells have been demonstrated. In the PLP-CreERTM transgenic mice, NG2+/PLP+ cells are found to generate the protoplasmic astrocytes in most regions of CNS during normal postnatal development (since P7), exclusive of the white matter and dorsal forebrain [59]. However, other subpopulations of NG2+ cells, such as adult NG2+/PDGFαR+ or NG2+/Olig2+ cells have not been found to give rise to astrocyte progenies [60, 61]. Collectively, the above studies suggest that NG2+ cells may contain subpopulations with different astrocyte lineage potential. In other words, the inconsistent results from different labs may be due to the NG2+ subpopulations in distinct CNS regions and ages of mice used for the studies. This indicates that a systematic analysis for early embryonic NG2+ cell populations would be needed for elucidating the intrinsic astrocyte differentiation capacity of NG2+ populations.

1.3.3 Neuronal lineage of NG2+ cells

The neurogenesis is a process of generating functional neurons from neuronal specific precursors and is traditionally considered to be occurring mostly at embryonic and perinatal stages in mammalian CNS [62]. A little evidence accumulates that NG2+ cells might have the potential of generating neurons in adult CNS, but not in developmental CNS. Therefore, we mainly focus on discussing the neuronal lineage potential of NG2+ cells in normal adult CNS as follows.

1.3.3.1 Some NG2+ cells are distinguished from conventional glial progenitors in adult CNS

The NG2+ cells are well-known as adult OPCs, which are mitotically active and could differentiate into oligodendrocytes. However, it has been suggested that subpopulations of perinatal and adult NG2+ cells are not conventional oligodendrocyte progenitors [63]. They are revealed to be quiescent and have undefined roles in CNS [41, 64, 65]. It is not until recently that electrophysiological studies reveal one new subtype of NG2+ cells in the white matter. These NG2+ cells could become activated by reacting to the excitatory synaptic input from axons or receive GABAergic inputs. Thereafter, they modulate special functions of neurons via close contacts and generate trains of action potentials (Fig. 2) [29, 66, 67]. Thus, these results reveal that some NG2+ cells, unlike the conventional oligodendrocyte progenitor cells or glial progenitors, behave much like neurons. In this regard, NG2+ cells in the adult CNS are thought to be heterogeneous, comprising of common OPCs and the ones with neuron-like functions.

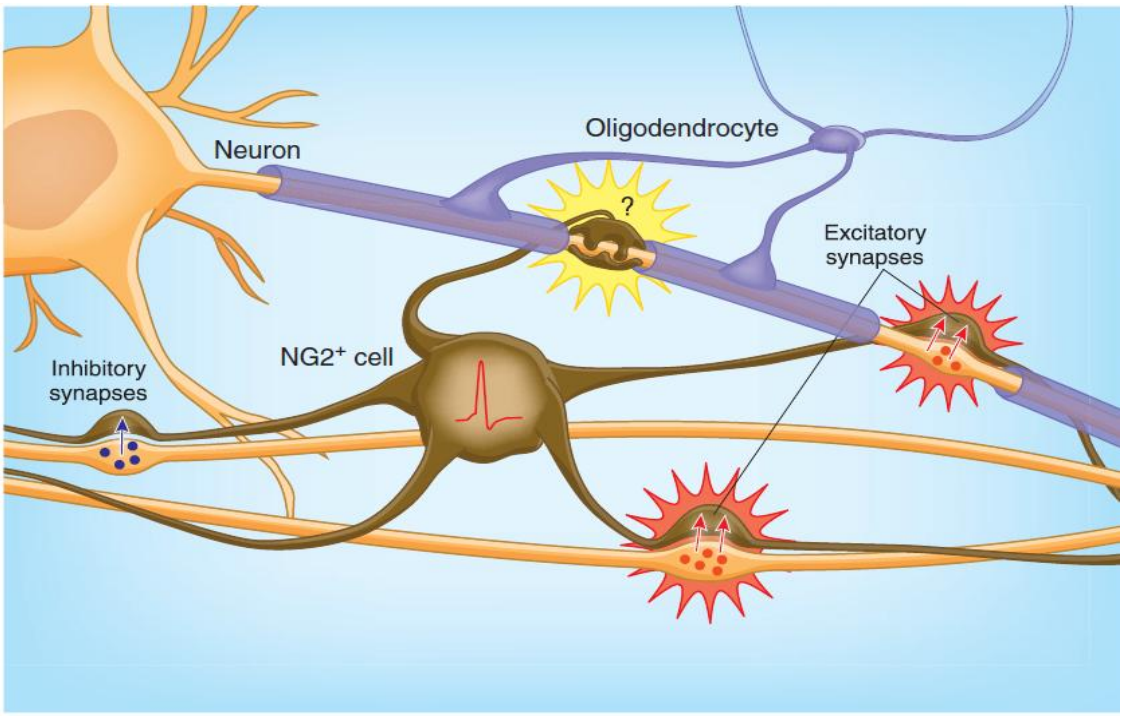


Figure 2 Some NG2+ glias in the cerebellar white matter directly contact the axon of neurons to fire action potentials via induction of the excitatory synaptic input.

Some wrapped the unmyelinated axons and others wrapped the nodes of Ranvier [68].

Additionally, the distribution of NG2+ cells has not been shown to be completely accordant with the distribution of conventional OPCs. During CNS development, the distribution of NG2+ cells is found to overlap with areas where multipotent NSCs reside [69]. Further studies reveal that NG2+ cells are distributed evenly throughout the gray and white matters of the mature CNS. Their abundance does not solely match the abundance of oligodendrocytes or myelin. To be exact, their distribution is relatively independent of the density of myelinated fibres [70]. Furthermore, some of the NG2+ cells are found to be associated with neuronal soma and dendrites, which

lack myelination [71]. Apart from the universal persistence in the mature CNS, NG2+ cells also proliferate abundantly even after the peak period of neonatal myelination [4]. This observation also contributes to the idea that NG2+ cells might not be just glial progenitors. If they are pure oligodendrocyte progenitors, their proliferative capacity is expected to decline or remain unchanged once the generation of mature myelinating cells is completed.

1.3.3.2 Evidence for neurogenesis from NG2+ cells in adult CNS

Adult neurogenesis continues mainly in some areas of postnatal vertebrate CNS, including the subventricular zone (SVZ) of the lateral ventricles, and subgranular zone (SGZ) in the dentate gyrus of the hippocampus [72, 73]. In these regions, several types of neural progenitors have been identified, according to their specific morphologies and expression of unique sets of molecular markers [74]. NG2+ cells are also found to reside in these regions, and are likely to participate in neurogenesis. For example, NG2+ cells in SVZ are characterized as a population of transit-amplifying type C-like multipotent progenitors [75] and are shown to generate hippocampal GABAergic interneurons [75, 76].

Evidence shows that NG2+ cells might be able to generate neuronal phenotypes in several regions of the brain. Subpopulations of NG2+ cells are shown to contribute to adult cortical neurongenesis, giving rise to piriform projection neurons and glutamatergic pyramidal neurons in the cortex [61, 77]. Additionally, NG2+

subpopulations are also found to generate GABAergic interneurons or TUC4 neurons in the neocortex and striatum [78, 79].

More direct evidence reveals that NG2+ cells could generate intermediate neuronal phenotypes, such as NG2+/Doublecortin+ immature neurons in the neocortex of adult rats [59, 78, 80], NG2+/ neuronal specific class ш β-tubulin (β-tubulin ш) + cells in the SVZ [75], NG2+/TOAD-64+ cells in the dentate gyrus of adult hippocampus and NG2+/NeuN+ interneurons in the adult neocortex, striatum and hippocampus zone of brain [76, 79]. The co-expression of those neuronal lineage markers and NG2 proteoglycan delineate the possible neuronal descendants of NG2+ cells in the adult CNS. Although the neurogenesis ability of NG2+ cells have been demonstrated by independent studies through the examination of neuronal markers and NG2 proteoglycan, further confirmation of the neuronal potential of NG2+ cells as well as the identification of NG2+ subpopulations which are capable of giving rise to neurons need to be determined.

1.3.3.3 Possible mechanisms regulating neurogenesis from NG2+ cells in adulthood

The neurogenesis from neural stem cells in adult CNS is well demonstrated to be regulated by both intrinsic and extrinsic cellular mechanisms, including the environmental influences, neurogenic niche factors inside the CNS, the cytoplasmic factors, transcriptional factors, epigenetic regulators, etc [62, 81]. However, the

underlying mechanism regulating the neuronal phenotypes generation from NG2+ cells remains largely unknown.

Recent evidence shows that NG2+ cells could be differentiated into neuronal lineages *in vitro*. When isolated from the complex autocrine and paracrine influences in the subcortical white matter and transferred into the low-density, serum free culture supplemented with (neurotrophin-3, NT3/ platelet derived growth factor AA, PDGF-AA)/bFGF, the adult NG2+ cells are demonstrated to give rise to neurons, oligodendrocytes and astrocytes [82]. Additionally, neonatal NG2+ cells could be reprogrammed into multipotent neural stem-like cells and neurons by manipulating extracellular signals [83]. These data suggest that the NG2+ cells might be the multipotential neural progenitors with the ability of differentiating into neuronal-glial lineages. They are in fact restricted but still-uncommitted progenitor lineages and most commonly generate glia by virtue of the parenchymal environment. These findings provide some hints that NG2+ cells have a more plastic developmental potential, though the *in vivo* environmental cues might specify their lineage potential only into glial lineages in most cases.

Epigenetic mechanism through inhibiting histone deacetylase activity plays a critical role in turning on and coordinating the neuronal gene expression in multipotent adult neural progenitors, while silencing the glial fate simultaneously [84]. Unexpectedly, oligodendrocyte progenitors could also gain multipotent potential and make a neuronal

fate choice instead of glial fate though the inhibition of histone deacetylase activity [85].

Taken together, these observations imply that NG2+ cells might be regulated by certain extracellular or intracellular signals, and exhibit cerain properties of NSCs *in vitro*. Due to the limited capacities of NSCs for compensating the neuronal loss in neuronal degenerative conditions of CNS, attention could be shifted towards probing into therapeutic potential of NG2+ cells. The underlying mechanisms for regulating neuronal differentiation from NG2+ cells needs to be investigated in details.

1.4 CNS diseases and spinal cord injury (SCI)

Diseases of any components of the CNS are defined as CNS diseases. Classified according to the etiology, the CNS diseases include trauma, infections, degeneration, structure defects, tumors, antoimmune disorders, and strokes [86-92]. Spinal cord injury mainly refers to the injury to the spinal cord, which is caused by physical wound. Four types of injures are categorized: solid cord, contusion, laceration and massive compression injuries [93]. According to the epidemiology study, each year approximately 130,000 individuals suffer an acute SCI in global, joining a recently estimated 2,500,000 who are living with chronic paralysis. The main etiologies of SCI are motor vehicle crashes, falls, acts of violence and explosive motor behavior [94]. Despite medical advances, the SCI patients still have to experience the neurological disability, with loss of motor, sensory and autonomic function. Thus a serious social

and economic burden on both individual and society would be unavoidable. Great efforts for finding a cure of SCI are needed to overcome this longstanding issue [95].

The pathophysiology of SCI has been so far investigated at molecular, cellular and gross anatomical levels. The primary mechanical impact results in neural tissue loss which includes the neural cell necrosis and/or apoptosis occurring at the injury center. And then, the damage spreads rostrally and caudally over time. Secondary degenerative events exacerbate the injury severeness over the next several weeks after the acute phase of injury, including infarction, reperfusion injury, excitotoxicity, apoptosis, the formation of glial scar and neuroinflammation. The complex cascade of these events leads to a greater loss of local neurons and glias, accompanied with the degeneration and demyelinatin of more axons [96]. Gradually, a chronic stage of injury follow up in the long term, where the cysts, cavities and scar tissue of spinal cord are formed [97]. Thereby, in most cases, permanent loss of sensory and motor functions of spinal cord as well as the structural destruction are generated post SCI.

Up to now, finding an effective treatment for SCI remains a challenge. As contusive injuries are the most common kind of spinal cord injuries happening to humans, thereby experimentally designed contusive injuries of mice were used in the current study for accurately mimicking those seen in humans [98].

1.4.1 Neuro-glial cell loss post SCI

SCI is demonstrated to affect all cell types populating the spinal cord, and the loss of function that follows SCI is largely attributable to the destruction of axonal tracts and demyelination of spared intact axons. There usually exists a temporal-spatial pattern of acute neuronal cell loss early after spinal cord contusion, especially the ventral horn motorneurons [99, 100]. Likewise, mature oligodendrocytes which are the source of myelin are extremely liable to the primary and secondary traumatic injuries and the loss of oligodendrocytes is a widely dispersed phenomenon during SCI that leads to persistent demyelination [101].

1.4.2 Immune reaction and glial scar formation post SCI

Repertoire of inflammatory microglia/macrophages also responds to SCI with an increment of number either through peripheral circulation or derivation from resident microglia [102]. The recruitment of neutrophil into the injured spinal cord also coincides with inflammatory macrophages infiltration into the cord [103]. Upon activation, the macrophages/microglia increases their mobility and mount an inflammatory response [104]. The exaggerated inflammatory response that follows SCI might be detrimental. These activated immune cells could release proinflammatory cytokines, oxidative stress, excessive nitric oxide, etc, which would induce the necrotic and apoptotic death of neuron-glias and lead to axonal damage and demyeliantion. [105, 106].

The injury of spinal cord results in an immediate loss of astrocytes, and soon afterwards a reaction of astrocytes is motorized. The ancestors of reactive astrocytes may be either the re-activated astrocytes *in situ* or the immature S100 calcium-binding protein β (S100 β) astrocytes and glial progenitors, assessed by measuring the up-regulation of GFAP [107]. The hypertrophic astrogliosis leads to the formation of the dense glial scar, which is a major inhibitory element in the post-injury environment for the abortive nature of axonal regeneration [108, 109].

1.5 NG2+ cells in pathological adult CNS

1.5.1 Proliferation of adult NG2+ cells in the demyelinated CNS, including the traumatic spinal cord

In normal adult CNS, NG2+ cells represent the major dividing population [4, 47, 110], although adult NG2+ cells down-regulate their proliferative capacity in comparison with the ones in neonatal phase [111]. In response to various demyelination conditions of CNS, NG2+ cells even exhibit enhanced proliferative capacity. This phenomenon has been consistently observed in the context of many CNS diseases, such as traumatic CNS injuries [33, 34], demyelinating diseases like multiple sclerosis [112], experimental autoimmune encephalomyelitis [113-115], neurodegenerative diseases, such as amyotrophoic lateral sclerosis [116], and focal cortical ischema [117]. Therefore, NG2+ cells could repopulate in various demyelinated conditions of CNS. The dynamic changes of NG2+ cell numbers indicate that they may play a role in demyelination diseases of CNS.

In the contusive injured spinal cord, a variety of cellular proliferation is remarkably stimulated therein. The immature populations of neural stem and/or progenitor cells are activated by injury and rapidly divide to replace the vulnerable populations. NG2+ cells predominate over the whole proliferative population post SCI, and they are demonstrated to be the major endogenous repertoire for compensating cell loss [118, 119]. NG2+ cells could almost restore their number as early as 3 days post spinal cord injury [120]. A sustained increase in the number of proliferative NG2+ cells could be observed in the following four weeks after injury (Fig. 3) [20, 118, 121, 122]. Numerous factors that regulate NG2+ cells proliferation post SCI have been identified, including growth factors, cytokines, chemokines, etc [122]. Therefore, NG2+ cells could proliferate and accumulate in response to SCI.

1.5.2 NG2+ cells give rise to oligodendrocytes for remyelination post SCI

Demyelination has been commonly observed after SCI, especially in the acute lesion environment. Despite the fact that adult NG2+ cells might have heterogeneous progenies, there is consensus that at least a portion of these cells acts as adult OPCs in response to SCI. The recruitment of endogenous NG2+ cells is deemed as the main source for remyelinating oligodendrocytes.

As stated before, there is a significant accumulation of NG2+ cells throughout the first few weeks after injury, but the number of NG2+ cells keeps stable over time. This suggests that some of them undergo differentiation or apoptosis. On the other hand, the number of oligodendrocytes is reduced by 93% at 7 days, but rise threefold at 14 days

after injury [65, 120, 123]. Presumably, the repopulated oligodendrocytes may be derived from the rapidly elevated NG2+ cells following SCI [124]. Moreover, the similar temporal-spatial dynamic changes between the total number of oligodendrocytes and NG2+ cells are also observed in the regions distal to the injury epicenter and lesion cavities, suggesting that these oligodendrocytes might also be produced by NG2+ cells [124, 125].

The generation of oligodendrocytes from the proliferative NG2+ cells for spontaneous remyelination after injury has also been supported by other evidence. When the spinal cords are x-irradiated, neither the proliferation of NG2+ cells nor remyelination occurs, suggesting that NG2+ cells are involved in the remyelination process [126]. Meanwhile, it is found that the surviving oligodendrocytes around the demyelinated lesions are not dividing and thus unable to improve remyelination [126-128]. These observations complementarily demonstrate that it is the dividing NG2+ cells that represent OPCs and differentiate into oligodendrocytes for remyelination post SCI.

Recently, more direct evidence is provided that NG2+ cells give rise to remyelinating oligodendrocytes post SCI. It is shown that the isolated NG2+ cells from traumatized spinal cord could differentiate into oligodendrocytes *in vitro* [129]. The chronic fate of the dividing NG2+ cells *in situ* is further determined through BrdU pulse chase labeling assay. More than half of cells which incorporate BrdU at 2-4 days post SCI are found to exhibit the oligodendrocyte phenotypes in 6 weeks after injury [107, 119]. In addition, the role of NG2+ cells in SCI is examined in CNP-EGFP transgenic mice.

The EGFP (CNPase)+/NG2+ cells are confirmed to undergo cellular and physiological changes in response to SCI for oligodendrocyte genesis [65]. The finding that at least subpopulation of NG2+ cells commits to the oligodendrocyte lineage has also been supported by the complementary transplantation experiments. NG2+ cells isolated from CNS or differentiated from embryonic stem cells have been shown to develop into mature oligodendrocytes when transplanted into the injured spinal cord [130-132]. Therefore, NG2+ cells are largely committed to the oligodendrocyte lineage for remyelination following SCI. Fostering remyelination from NG2+ cells throughout the injury region may be targeted for enhancing functional recovery from SCI.

1.5.3 The possibility of NG2+ cells generating reactive astrocytes after CNS injury

It is well known that primary physical force would induce the massive loss of astrocytes post SCI. However, the astrocyte density is found to restore normal level or become even higher in the lesion regions shortly post SCI [133]. The reactive astrogliosis, which may have either beneficial or detrimental effects in a context-dependent manner after CNS injury, could be the end point in the evolution of glial scar [134]. However, the source of newly divided scar-forming astrocytes is not well determined so far. It is also unclear whether the high re-occurrence of astrocytes is originated from NG2+ cells post SCI.

Studies on the lineage potential and electrophysiological properties of NG2+ cells have suggested that they are not the committed progenitor cells of oligodendrocyte

lineage. Thus, they are not with sole function of generating myelin either. Evidence accumulates that some reactive astrocytes originate from NG2+ cells after CNS injury. Populations of cells with a co-expression of GFAP and NG2 proteoglycan inside and around the lesion site of spinal cord or cortex are found to exhibit the similar morphological and antigenic characteristics to the type2-astrocytes [135, 136]. The similar transient phenomenon is also observed in the neocortical stab wound injury of NG2CreER transgenic mouse [137]. Additionally, studies using BrdU pulse chase labeling method also reveal that proliferative NG2+ cells in various lesion paradigms differentiate into GFAP+/BrdU+ reactive astrocytes, which might participate in scar formation and be detrimental for CNS repair [107, 136, 138-140]. Genetic fate mapping technique reveals that the major progenies of NG2+ cells (marked using Olig2-CreER*) are protoplasmic astrocytes in the cold-induced injury model of the cerebral cortex [141]. In parallel, another study with Nestin-CreER mice demonstrate that NG2+ cells could be the primary source of proliferative GFAP+ gliosis after adult cortical injury [142], suggesting that some NG2+ cells might give rise to astrocytes after CNS injury.

Moreover, NG2+ cells in spinal cord have been verified to initiate a programming event to become astrocytes post injury. One study shows that temporal shifts in progenitor fates are induced from 24 hours to 7 days by injury niches. NG2+ cells born 24-hours post injury generate gliotic astrocytes and participate in scar formation, whereas NG2+ cells born 7-days post injury produce oligodendrocytes (Sellers et al., 2009). Therefore, this finding suggests that the factors in microenvironment niches

would influence the astrocytes generation of NG2+ cells, apart from their major differentiation products, oligodendrocytes.

In contrast, some other studies also demonstrate that the great majority of the new-born reactive astrocytes are not from NG2+ cells in Olig2-CreER or NG2-creER transgenic mice with cortical stab injury [60, 137, 143]. Instead, they are considered as descended from parenchymal astrocytes that re-enter the cell cycle or from ependymal cells around the central canal [3, 144, 145]. The emerging consensus from these studies argues that NG2+ cells might not be the main ancestors of astrocytes after CNS injuries. Thus, the inconsistent opinions regarding the astrocyte genesis ability of NG2+ cells post injury might be due to different CNS injury models investigated in these studies. The capacity of generating astrocytes from NG2+ cells in injured CNS (spinal cord) is still in dispute.

1.5.4 The neurogenic potential of NG2+ cells post SCI

As stated above, NG2+ cells are found to dominate the proliferating cells in SCI, and generate the remyelinating oligodendrocytes. However, the contusive spinal cord injury induces remarkable antigenic and physiological changes of the adult NG2+ cells in comparison with the normal adult ones [146]. So far, it is still unclear whether NG2+ cells could be recruited as endogenous neuronal cell source for curing SCI.

Based on their inducible neurogenic potential *in vitro* and neuronal phenotyptes in some regions of brain [83], NG2+ cells may have the potential to promote extensive

neuronal replacement post SCI. Manipulation of these resident NG2+ cell populations would be therapeutic in terms of promoting neuronal repair and functional recovery after injury. There are several reasons for recruiting NG2+ cells to repair CNS injuries: (1) They occur ubiquitously throughout the nervous system, in contrast to neural stem cells only in defined CNS regions. (2) Subpopulations of NG2+ cells have been verified to possess neurogenic ability in many regions of CNS, suggesting their neurogenic potential in pathological CNS. (3) They can be reactivated and re-enter cell cycle quickly during injury, where they might acquire certain features of neural stem cell-like properties. These attributes make them the promising cells for endogenous cellular therapy of nervous diseases, as compared with the activity-limited neuronal precursor cells (NPCs). The investigation of neurogenic potential from the NG2+ cell populations might open a new perspective for treating CNS diseases.

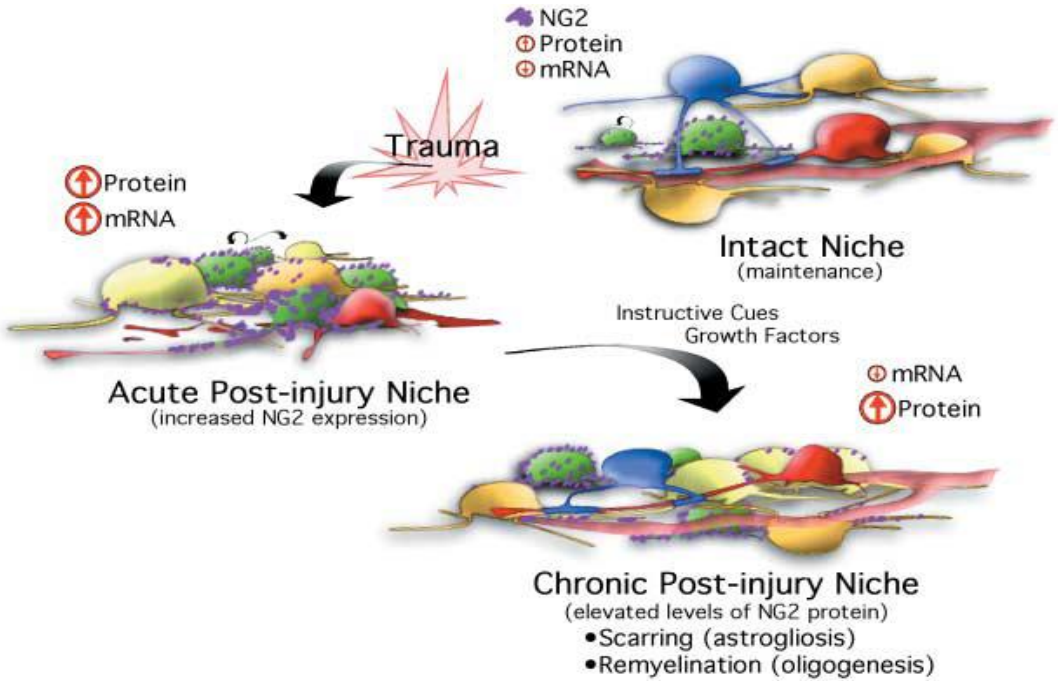


Figure 3 NG2+ cells and NG2 proteoglycan could be regulated according to the CNS microenvironment cues.

In the acute phase of CNS injury, NG2+ cells (green) resume an increment in numbers with a coincident increase in NG2 proteoglycan (magenta) expression. After the transition from the acute phase to chronic phase of injury, the proliferation of NG2+ cells slows down, whilst NG2 protein levels persist. As the tissue restoration continues, NG2+ cells are responsible for replenishing the oligodendrocytes that remyelinate axons or probably generate astrocytes for gliotic scar under the instructive cues [147].

Figure 1, 2 and 3 were adapted from reference 7, 65 and 137. I would like to thank these authors for sharing their work.

1.6 Aims of study

Though NG2+ cells have been identified as glial progenitors, accumulating evidence suggests that they are comprised of diverse subpopulations, whose cellular characteristics and roles in CNS are still not completely unraveled. The aims of my

project are to illuminate the lineage potential of NG2+ cells, and to disclose the therapeutic potential of NG2+ cells in the treatment of SCI.

NG2+ subpopulations have been found to give rise to oligodendrocyte, astrocyte and neuronal progenies in distinct regions of CNS. Their lineage heterogeneity has been elucidated separately at a population level. However, there is hitherto no exposition of these findings at a clonal level. Moreover, it is still unknown whether NG2+ cells are composed of multipotent neural progenitors or they are heterogeneous populations containing distinct neural lineage-committed progenitors. To address these issues, the first part of my project is to analyze the differentiation capacity of NG2+ cells at a clonal level and to examine the lineage hierarchy in these NG2+ clones.

Subpopulations of NG2+ cells are recently demonstrated to participate in adult neuronal genesis in the brain. It is still unknown how the NG2+ cells could be induced to exhibit neuronal phenotypes. Hence, the second aim of this study is to explore strategies for manipulating the neuronal fate of NG2+ cells. Moreover, the downstream signal transduction pathways are to be clarified.

NG2+ cells highly react to SCI for compensating the loss of glial cells. However, whether NG2+ cells could be recruited as endogenous neuronal cell source is still unknown. Based on the finding that the EGFR inhibitor (PD168393) induced NG2+ cells to obtain neuronal phenotypes, the third aim of my study is to evaluate the contribution of resident NG2+ cells to the regenerative neuronal repertoire in

PD168393 treated animals post SCI. Moreover, the therapeutic effect of PD168393 on improving neural functional recovery and the cellular changes of other cell types will also be investigated.

CHAPTER 2 Materials and Methods

2.1 Reagents

Antibiotics used in this study were from Invitrogen, and chemicals were from Invitrogen, Sigma-Aldrich or Merck, unless otherwise stated. Growth factors and hormones: PDGF-AA, I-DNA Biotechnology; triiodothyronine (T3, Sigma-Aldrich); insulin-like growth factor 1 (IGF-1, R and D systems, Minneapolis, MN). Cell culture medium and supplements: Dulbecco's Modified Eagle's medium (DMEM/F12, Gibco BRL); DMEM/High Glucose (DMEM/HG, Gibco BRL); HEPES (Sigma-Aldrich); Sodium Bicarbonate (Sigma-Aldrich); Fetal Bovine Serum (FBS, Hyclone); Fetal Calf Serum (FCS, GIBCO); Penicillin-Streptomycin (P/S, Sigma-Aldrich); Trypsin (Invitrogen); B27 supplement (PAA, GE healthcare); Neural Stem Cell supplement (PAA, GE healthcare); N1 supplement (Sigma-Aldrich); poly-L-lysine (PLL, Invitrogen); DMSO (Sigma-Aldrich); bovine serum albumin (BSA, Sigma-Aldrich). Disposable PCR reagents were from Promega and dNTPs (dTTP, dATP, dGTP, and dCTP) mixtures were obtained from Fermentas.

2.2 Medium

All medium were prepared in Milli-Q water and sterilized by autoclaving or filtering unless otherwise stated.

Table 1 Medium

Primary NG2+ cells and human telomerase reverse	DMEM/F12 supplemented with 2% FBS, 1% P/S, 1% HEPES buffer, PDGF-AA (10 ng/mL), B27

transcriptase (hTERT)-NG2+ clones culture medium	supplement (1:50), Neural Stem Cell supplement (1:50) and N1 supplement (1:100).
CG4 cells culture medium	DMEM/HG medium supplemented with 30% B104 conditioned medium, BSA (5%, 1:50), N1 supplement (1:100) and 1% P/S.
Neuro-2a (N2A) and B104 neuroblastoma culture medium	DMEM/HG medium with 10% FCS and 1% P/S.
Cells freezing medium	10 % (v/v) DMSO in heat-inactivated FBS.
CG4 cells freezing medium	10% (v/v) DMSO in DMEM/HG medium.

2.3 Buffer

Table 2 Buffer

10×phosphate buffered saline (PBS)	80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 1 L ddH_2O (distilled, de-ionized water), pH 7.4.
DEPC water	Add 1 mL of 0.1% Diethylpyrocarbonate (DEPC) to 1000 mL distilled water, mix well, and let set at room temperature (RT) for 1 hour before autoclaving
50×tris-acetate-ethylenediaminetetraacetic acid, EDTA (TAE)	242 g of Tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA (pH8.0), De-ionized water (topped up to 1 L)
0.8-1.2% agarose gel	Agarose was added in 1×TAE buffer (diluted from 50×TAE in de-ionized water) and microwaved till the agarose was dissolved
Lysis buffer	20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor, Roche Applied Science
2 × sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel-loading buffer	100 mM Tris-HCl (pH6.8), 200 mM dithiothreitol, 4% SDS, 0.2% Bromophenol blue and 20% glycerol
10% sodium dodecyl sulfate (SDS)	10 g of SDS was dissolved in 100 mL of de-ionized water, and heated to 68 ℃ for solubility, pH~6.6.
10% ammonium persulfate (APS)	1 g of ammonium persulphate was dissolved in 10 mL of de-ionized water

Lower resolution gel buffer	1.5 M Tris-HCl, pH 8.8
Upper stacking gel buffer	1 M Tris-HCl, pH 6.8
SDS-PAGE electrophoresis running buffer (10×)	Tris Base 60 g, Glycine 288 g, and SDS 20 g (topped up to 2 L)
10×transfer buffer	Tris Base 60 g and Glycine 288 g (topped up to 2 L)
1×transfer buffer	10% (v/v) 10× transfer buffer, 10% (v/v) methanol, (topped up to 1 L)
1×PBST washing solution (western blot, WB)	5 mL Tween-20, 500 mL 10×PBS (topped up to 5 L)
Blocking buffer	1×PBS-T containing 5 % (w/v) skim milk or BSA
Stripping buffer	16 mL 1 M Tris (pH6.8), 20 g SDS (For stripping, 12 mL stripping buffer, 105 μL β-metacapethanol, 50 ℃ for 45 minutes)
PBS/0.1%Triton X-100	1 mL Triton X-100 was added to 999 mL of 1× PBS

2.4 Cell culture

2.4.1 Isolation and culture of primary NG2+ cells

Adult female Spague-Dawley rats were purchased from the Laboratory Animal Center of National University of Singapore, housed in the specific pathogen free (SPF) microisolator cage system and fed with standard rodent diet *ad libitum*. All animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Nanyang Technological University.

Timed-pregnant rats were sacrificed through lethal exposure to CO_2 on the embryonic day 16. The embryos were removed out of the maternal uterus, and the cerebral cortex was dissected in cold sterile PBS, followed by removing the subcutaneous meanings and blood vessels carefully. The cerebral cortex was then mechanically dissociated in

cold PBS. The dissociated cells were passed through 40 μm mesh to remove the tissue debris, centrifuged (800 rpm, 5 minutes), and re-suspended in DMEM/F12 medium supplemented with 10% FBS, N1 supplement, and 1% P/S. The cells were incubated in uncoated cell-culture dish at 37 ℃ with 5% CO_2 in air overnight, and then transferred into the primary NG2 antibody coated dishes, incubated for 1 hour. They were then sequentially transferred to the secondary and third coated plate for another two hours.

After five times washing of the unbounded cells, the adherent NG2+ cells were trypsinized and maintained in the primary NG2+ cell culture medium in 37℃ humidified atmosphere with 5% CO_2 for further study. Collectively, isolation and culture of primary NG2+ cells followed previous protocols [148]. For all the cell cultures in this study, cells were always kept less than 100% (log phase of growth) but more than 10% confluency. Thereby, cultures were usually harvested when they reached 70-80% confluency and re-plated at a cell density of $1.5\text{-}2\times10^4$ cells /cm^2.

2.4.2 Culture of CG4 cells, N2A cells and B104 cells

CG4 cells, a permanent cell line of rat glial precursors, were generously provided by Dr. Liang Fengyi (National University of Singapore, Singapore). Briefly, CG4 cells were propagated in CG4 cells culture medium which is supplemented with the conditioned medium from B104 cells. The B104 conditioned medium was prepared as followed: B104 neuroblastoma cells were cultured in B104 growth medium until

100% confluency. The cells were washed with PBS and fed with serum-free DMEM/HG medium with N1 supplement. After 4 days, the medium was collected and centrifuged at 800 rpm for 5 minutes (4 ℃). The supernatant was then filtered (0.22 μm pore size). The shelf life of B104 conditioned medium is up to 6 months at −80 °C. For CG4 cell culture, PLL was used for coating the bottom of the flask in advance. PBS, instead of trypsin, was used to detach the CG4 cells from the bottom of the flask when they reached 80-90% confluency. After centrifuging the cells at 800 rpm for 5 minutes, the cell pellet was re-suspended by the growth medium, made up of half of the old growth medium and the same volume of fresh medium.

The neuroblastoma N2A cells, which are established from a spontaneous tumor of a strain A albino mouse, was kindly provided by Dr. Yu Weiping (National Neuroscience Institute, Singapore) and used as positive control for immunocytochemistry experiment. B104 rat neuroblastoma, which is a cell line of central nervous system origin, was also provided by Dr. Liang Fengyi. Both of them were maintained in DMEM/HG with 10% FBS and 1% P/S. They were trypsinized and split once every 2-3 days when they become 80-90% confluency.

2.4.3 Cell cloning with hTERT plasmid transfection

The telomerase over-expression is demonstrated to permit the generation of stable, nontransformed lines of neural progenitor cells, whose progeny are able to differentiate with the phenotypic stability [149]. 1×10^5 NG2+ cells were seeded on 60-mm tissue culture dish and cultured overnight. 1 μg hTERT plasmid (MBA-141,

American Type Culture Collection, ATCC) was transfected into primary NG2+ cells by electroporation. To generate stable cell lines harboring the desired plasmids, transfectants were selected with hygromycin at 250 μg/mL. Single clones were picked up and expanded in the NG2+ cell culture medium supplemented with hygromycin (100 μg/mL) [150].

For cell growth experiments, the cell outgrowths from these clones were assessed by cell counting. Briefly, all the NG2+ clones were seeded at the concentration of 10000 cell/ 100-mm dish. The cells were trypsinized, harvested and counted with 0.4% typan blue exclusion method from Day2 to Day6.

2.5 Multi-lineage differentiation of NG2+ cells

The NG2+ cells were analyzed for their capacity to differentiate into oligodendrocyte, astrocyte, neuronal lineages as well as chondrocytes. For neuronal- glial differentiation, cells were seeded at a density of 8000 cells/cm^2 onto PLL-coated coverslips in culture plates. The ones treated with DMSO, PBS or transfected with control vectors were used as control. The differentiation or control medium was changed every two days. The neuronal-glial phenotypes of the cells were examined by morphology, as well as phenotypic markers detection using reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemistry. All the experiments were repeated at least three times.

2.5.1 Glial differentiation

Oligodendrocyte differentiation was induced by culturing cells in DMEM/F12 medium supplemented with 0.5% FBS and 40 ng/mL T3. For astrocytes differentiation, cells were cultured in the DMEM/F12 medium supplemented with 10% FBS, 200 ng/mL IGF-1 and 40 ng/mL T3.

2.5.2 Neuronal differentiation

To identify molecules which can induce neuronal differentiation from NG2+ cells, we tried out all the chemicals available in our lab, followed by the neuronal morphological evaluation. PD168393, which is a specific and irreversible inhibitor of EGFR tyrosine kinase activity [151], was identified as inducing the neuronal phenotypes in NG2+ (glial) cells. Briefly, the NG2+ (clonal) /CG4 cells were firstly treated with differentiation medium (1 μM PD168393 in DMEM/F12 medium supplemented with 5% FBS instead of the mitogens) for 3 days, followed by withdrawing FBS for another 3-4 days. In the mechanism study on neuronal phenotypes generation from NG2+ (glial) cells, the inhibitors of EGFR downstream signaling pathways were applied in the same differentiation protocol.

2.5.3 Chondrocyte differentiation

Cells were plated in a way of pelleted micromass culture (10 μL) at a density of 10^6 cells/mL. The cultures were maintained in the chondrocyte differentiation medium-

DMEM-Low Glucose with 1% FBS, supplemented with insulin (6.25 μg/mL), transforming growth factor β1 (10 ng/mL), and ascorbic acid-2- phosphate (50 nM) for 3 weeks. The medium was changed every two days. Cells that grew in the normal medium were used as control.

2.6 Plasmids transfection

The short interfering RNAs (siRNAs) targeting nucleotides of EGFR transcript were designed as follows: EGFR-3.1F siRNA, nucleotides 1167-1185, 5'-TGGCATAGGCATTGGTGAA-3'; EGFR-3.1R, 5'-TTCACCAATGCTATGCCA-3'; EGFR-4.1F siRNA, nucleotides 353-371, 5'-TCACCTATGTGCAAAGGAA-3'; EGFR-4.1R, 5'- TTCCTTTGCACATAGGTGA-3'. They were annealed and cloned into two different siRNA expression vectors, pSilencer 3.0-U6 and pSilencer 4.0-CMV (Ambion) respectively. The same vectors containing siRNA with scrambled sequence were used as control. The constitutively active Ras plasmid was generously provided by Dr. Sheng-Cai Lin (Xiamen University, China). pcDNA4A vector was used as the control vector. All the transfections in this study were conducted using electroporation and following manufacturer's instruction (Invitrogen).

2.7 Immunocytochemistry

2.7.1 Immunofluorescence staining

The oligodendrocyte, astrocyte, neuronal differentiated and control cells were fixed with 4% cold paraformaldehyde (PF) in PBS for 15 minutes on PLL-coated coverslips, and permeabilized with 0.1%Triton X-100 in PBS for 30 minutes. After two washes with PBS, cells were blocked with 4% BSA in PBS/0.1%Triton X-100 for 1 hour, followed by incubating with primary antibodies (diluted in PBS/0.1%Triton X-100/ 1% BSA) at 4 ℃ overnight. After removal of primary antibodies (Table 3), cells were washed with PBS twice and the appropriate secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or Rhodamine (diluted with PBS/0.1%Triton X-100) were added and incubated for 3 hours at RT. Cell nuclei were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI). CG4 cells and N2A cell line have been used as positive controls respectively. Negative controls included the omission of primary antibodies. For imaging, an Olympus IX71 fluorescent microscope (Nikon Kanagawa, Japan), or a confocal microscope LSM710 (Carl Zeiss) was used.

Table 3 Antibodies List

Primary antibody	Host	Dilution	Made in
Anti-phosphorylated EGFR (pEGFR)	Mouse	1:150 WB1:1000	Becton Dickinson
Anti-EGFR	Mouse	1:150 WB1:1000	Becton Dickinson
Anti-EGFR	Rat	1:500	Abcam
Anti-NeuN	Mouse	1:200	Chemicon
Anti-β-tubulin ш	Rabbit	1:1000	Covance
Anti-Microtubule-Associated Protein 2 (MAP2)	Rabbit	1:1000	Chemicon

Anti-GABA	Rabbit	1:1000	Chemicon
Anti-Neuron Specific Enolase (NSE)	Mouse	1:1000	Chemicon
Anti-Neurofilament Heavy (NF200)	Rabbit	1:2000	Sigma-Aldrich
Anti-Choline Acetyltransferase (ChAT)	Goat	1:500	Chemicon
Anti-Neurofilament H Non-Phosphorylated (SMI32)	Mouse	1:1000	Covance
Anti-myc	Mouse	1:5000	Invitrogen
Anti-Doublecortin	Goat	1:300	Santa Cruz Biotechnology
Anti- CD11b/c equivalent (OX42)	Mouse	1:200	Abcam
Anti-PDGF α R	Rabbit	1:300	Santa Cruz Biotechnology
Anti-Nestin	Mouse	1:200	Chemicon
Anti- Neuronal Cell Surface Antigen (A2B5)	Mouse	1:200	Chemicon
Anti-Galc	Goat	1:1000	Santa Cruz Biotechnology
Anti-oligodendrocyte marker (O1)	Mouse	1:1000	Sigma
Anti-CNPase	Rabbit	1:200	Santa Cruz Biotechnology
Anti-Nestin	Mouse	1:500	Millipore
Anti-S100 β	Mouse	1:1000	Abcam
Anti-Glutamine Synthetase (GS)	Goat	1:1000	Santa Cruz Biotechnology
Anti-GFAP	Rabbit	1:1000	Millipore
Anti-NG2	Mouse	1:250	Abcam
Anti-NG2	Rabbit	1:250	Millipore
Anti-BrdU	Sheep	1:200	Abcam
Anti-Green Fluorescent Protein (GFP, B-2)	Mouse	1:500	Santa Cruz Biotechnology
Anti-GFP	Rabbit	1:1000	Millipore
p-Extracellular Signal-Regulated Kinase 1/2, (p-ERK1/2)	Rabbit	1:1000	Cell signaling
ERK1	Rabbit	1:1000	Santa Cruz
p-Mitogen-Activated Protein Kinase, (p-MEK)	Rabbit	1:1000	Cell signaling
p-p90RSK	Rabbit	1:1000	Cell signaling
β-Actin	Mouse	1:1000	Chemicon
Secondary antibody	Dilution		Made in
Alexa Fluor 488 goat anti mouse	1:200		Invitrogen
Alexa Fluor 594 goat anti mouse IgG (H+L)	1:200		Invitrogen

Cy5 goat anti mouse IgG (H+L)	1:400	Invitrogen
Alexa Fluor 488 goat anti rabbit	1:400	Invitrogen
Alexa Fluor 568 goat anti rabbit IgG (H+L)	1:400	Invitrogen
Donkey anti-goat IgG-FITC	1:200	Santa Cruz Biotechnology
Donkey anti-goat IgG-R	1:200	Santa Cruz Biotechnology
Alexa Fluor 568 goat anti rat IgG (H+L)	1:400	Invitrogen
Rabbit polyclonal secondary antibody to sheep IgG (H+L)	1:200	Abcam

2.7.2 Alcian Blue staining

Micromass cultures were rinsed with PBS and fixed in 2% PF for 15 minutes. After washing twice with PBS, they were rinsed with 3% acetic acid for 5 minutes (pH 2.6), followed by staining with 1% Alcian Blue for 20 minutes. Cells were then washed twice with Milli Q water before being observed under the light microscopy. The highly sulfated proteoglycans of cartilage matrices were positively stained blue as a control.

2.7.3 Immunocytochemical data analysis

For immunocytochemical data analysis, all coverslips were scored with healthy nuclei being classified as positive or negative for marker protein expression. In this way, the quantification of oligodendrocytes, astrocytes and neuronal differentiation was determined by counting GalC and O1, S100 β and GS, NF200 and MAP2 positive cells. At least three fields were counted in each sample.

2.8 RT-PCR

2.8.1 mRNA extraction and RT-PCR

Total RNA was extracted with Trizol (Invitrogen). Briefly, cells were washed twice with ice cold PBS and lysed with Trizol (1 mL/6-cm dish). The cells were detached by pipetting and let stand for 5 minutes to ensure complete homogenization before transferring into 1.5 mL eppendorf tubes. 0.2 mL chloroform was added to each tube, followed by vigorous shakes for 15 seconds. The tubes were incubated at RT for 2 minutes before they were centrifuged at 12000 rpm for 15 minutes (4 ℃). The supernatant was lightly mixed up with 0.5 mL isopropanol. The mixture was incubated for 10 minutes at RT before centrifugalizing at 11800 rpm for 10 minutes (4 ℃). The supernatant was removed, and the RNA pellet was dissolved in 1 mL 75% ethanol. After spinning at 7400 rpm for 5 minutes, the RNA pellet was dissolved with DEPC water at 55 ℃. All the tubes and tips used for this experiment were RNase free.

An RT-PCR kit (Invitrogen) was subsequently used for reverse transcription of the isolated mRNA into cDNA. Briefly, up to 5 μg total RNA, 50 μM oligo $(dT)_{20}$ primers and 10 mM dNTP were mixed up, with DEPC water tapping up to 10 μL in total volume. The mixture was incubated at 65 ℃ for 5 minutes and immediately transferred onto ice for at least 1 minute. Thereafter, standard PCR procedures were performed to obtain the cDNA of interest. A 10 μL of cDNA synthesis mixture prepared as follows was added to each of the RNA/primer mixture. Reaction mixture: 2 μL of 10× RT reaction buffer, 2 μL of 0.1M DTT, 4 μL of 25 mM $MgCl_2$, 1 μL RNaseOUT^{TM} (40

U/μL), and 1 μL SuperScript™ III RT (200 U/μL). The total mixture was mixed gently and collected by brief centrifugation before incubation for 50 minutes at 50 ℃. The reaction was then heat inactivated at 85 ℃ for 5 minutes. The cDNAs were stored at -20 ℃ or used for PCR immediately.

2.8.2 Standard PCR protocol

PCR was routinely performed in a 20 μL reaction volume containing 1 μL template DNA, 0.5 μL of each oligonucleotide primer (10 μM), 0.5 μL of dNTP mix (10 mM), 0.5 μL of $MgCl_2$ (25 mM), 0.5 μL DNA polymerase (500U, 5U/μL), 5 μL of 5x reaction buffer, and MilliQ water (topped up to 20 μL). All PCR reactions were performed on a DNA Thermal Cycler (C1000™, Bio-RAD). The primer sequences (all from 1st base, Singapore), reaction conditions and the size of each product were listed in Table 4.

Table 4 Primers used in RT-PCR analysis

Gene	Primer sequence	Number of cycle	Annealing temperature (℃)	Product size (bp)
CNPase	5'-AGAGCTGCAGTTCCCTTTCCTTCA-3' 5'-TGTCATCGAGCACAAGAACCCTGA-3'	35	56	278
MAP2	5'-AACATGCTGCCTTAGTTCCTCA-3' 5'-CAGCTAAACCCCATTCATCCTT-3'	34	56	267
NF200	5'-ACCTATACCCGAATGCCTTCTT-3' 5'-AGAAGCACTTGGTTTTATTGCAC-3'	36	56.5	175
NeuroD	5'-GGAGTAGGGATGCACCGGGAA-3'	31	56	228

	5’-CTTGGCCAAGAACTATATCTGG-3’			
Reelin	5’-CAGGCCTCAGAAACACACAA-3’ 5’-AGCACCAGTAGAAATGGATGA-3’	33	56	241
Synapsin	5’-ACCACAAGAGGTGCAAGATAGG-3’ 5’-TAGTTCATGGTGGCAGCTTGGG-3’	31	56	212
β-Tubulin III	5’-GAACATGATGGCTGCCTGTGA-3’ 5’-CCCGTGTACCAGTGGAGGAA-3’	34	56.5	309
hTERT	5’-CTACAAGATCCTCCTGCTGC-3’ 5’-AGTCCAGGATGGTCTTGAAG-3’	39	56	395
Aggrecan	5’-CTGGAGACAGGACTGAAATC-3’ 5’-CTCCATTCAGACAAGGGCTT-3’	38	56.5	258
Collagen II	5’-GAACAACCAGATCGAGAGCA-3’ 5’-CTCTCCAAACCAGATGTGCT-3’	38	56.5	229
GAPDH	5’-TATCCGTTGTGGATCTGACAT-3’ 5’-CATGTAGGCCATGAGGTCCACCAC-3’	26	55	275

2.8.3 DNA electrophoresis

A 50 mL 0.8-1.2 % (w/v) agarose gel was used for analyzing 0.1-8 kb amplified DNA fragments. Ethidium bromide (EB) was added to a final concentration of 1 μg/mL, followed by casting gel in a mini-gel apparatus. Electrophoresis was carried out in a horizontal gel apparatus with the gel submerged in 1× TAE. DNA samples were loaded, and DNA fragments were visualized by fluorescence over a UV light (302 nm, UV transilluminator TM-20, UVP, San Gabriel, CA), under which DNA/EB

complexes fluoresced. The image was recorded with a Gel Doc 1000 imaging system GBox (Syngene).

2.9 Fluorescence-activated cell sorting (FACS) and phenotypic analysis

The expression levels of some antigens associated with neural progenitor cells (NPCs) in NG2+ clones were measured by FACS. 1×10^6 cells were re-suspended with 4% PF, and incubated at 4 ℃ for 15 minutes. 1% Saponin was used for permeabilizing cells for 1 hour at RT. Freshly diluted anti-NG2 (1:200), anti-PDGFαR (1:200), anti-Nestin (1:200), and anti-A2B5 (1:200) monoclonal antibodies were used for incubating cells for 1 hour at 4 ℃. Cells were then incubated in the FITC-conjugated anti-mouse or anti-rabbit secondary antibodies for 30 minutes at 4 ℃. After centrifuge, the pellets were then re-suspended with PBS for analysis. All the centrifuges were performed at 1000 rpm/min for 5 minutes, with at least two washes between every two separated steps. Samples stained with secondary antibodies only were used as negative controls. The flow cytometry (FCM) analyses were performed with fluorescence activated cell sorter (FACS Calibar, Becton-Dickinson).

2.10 Western blot

2.10.1 Protein extraction

Cells were washed twice with cold PBS, harvested by scraping at various time points (0 min, 2min, 10 min, 30 min, 60 min) after PD168393 treatment, lysed in lysis buffer, and spun at 14,000×g for 20 minutes at 4℃. The supernatant was collected and protein concentration was determined by protein assay kit.

2.10.2 Determination of protein concentration

The Bio-Rad protein assay was used for determining the protein concentration. The dye reagent was prepared by diluting at a ratio of 1:4 - stock dye reagent to MilliQ water. Five concentrations of BSA standard were prepared (the linear range of the assay for BSA is 0.1 to 1 mg/mL). 3 μL of each standard and sample solution were mixed with 1 mL diluted dye reagent respectively. After incubating at RT for 5 minutes, the absorbance of each solution was measured at 595 nm (Ultrospec 2100pro, Amersham/Biosciences). The standard curve was plotted based on the absorbance of standard protein solution at 595 nm. The concentration of the sample was determined from the plot.

2.10.3 SDS-PAGE

SDS-PAGE was performed as described by Laemmli [152] with modifications. Protein in samples (10 μg-30 μg) was mixed with an equal volume of 2× sample loading

buffer, resolved by loading the appropriate amounts in SDS-PAGE gel. Electrophoresis was carried out in a Mini Electrophoresis Set (Bio-RAD) at 120 V for 90 minutes. The gel was then processed for the western blot analysis. 8-12% SDS-PAGE gels were prepared as follows:

Table 5 SDS-PAGE gel preparation

Resolving Gel	8%	10%	12%
Milli Q Water (mL)	5.3	4.8	4.3
48% Acrylamide/Bis-acrylamide (mL)	2	2.5	3
1.5 M Tris-HCl, pH 8.8 (mL)	2.5	2.5	2.5
10% SDS (µl)	100	100	100
10% APS (µl)	100	100	100
TEMED (µl)	4	4	4

Stacking gel: Milli Q Water: 2 mL; 48% Acrylamide/Bis-acrylamide: 0.5 mL; 1.0 M Tris-HCl, pH 6.8: 0.38 mL; 10% SDS: 30 µl; 10% APS: 30 µl; TEMED: 3 µl

2.10.4 Transferring and immunoblotting

Proteins resolved in the SDS-PAGE gel were transferred onto the polyvinylidene difluoride membrane (PVDF, Millipore). The PVDF membrane and the gel were sandwiched between two filter papers, with one on each side and placed in a transfer cassette. The transfer cassette was submerged in the transfer buffer. Transfer of the proteins was carried out at 0.39 A for 90 minutes. After blocking with 5% skim milk

in PBST for 1 hour, membranes were incubated with the specific primary antibodies overnight at 4 ℃ (Table 3). Protein bands of interest were detected by Immobilon Western Chemiluminescent HRP Substrate system (Millipore) after incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hour at RT. To ensure equal protein loading, the developed membranes were stripped, followed by detecting with control antibodies.

2.11 Ras Activation Assay

Ras activity was detected using the Ras activation assay kit (Upstate Biotechnology) according to the manufacturer's specifications. Cell lysates were incubated with glutathione S-transferase-Raf-1 Ras binding domain-agarose beads and the GTP-bound Ras was precipitated, followed by immunoblotting with anti-pan Ras antibody.

2.12 Animal operation

2.12.1 Surgical procedures of experimental SCI

Swiss Albino female mice of 7-8 weeks old were obtained from Laboratory Animal Center of National University of Singapore (Singapore). They were housed in a SPF environment and fed with standard rodent diet *ad libitum*. All animals handling and experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanyang Technological University.

Mice were randomly selected for the PD168393-treated or control groups. The mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and laminectomies were performed at thoracic level 9-10. Standard New York University (NYU) weight-drop device was used, and contusion was produced by dropping a 10 g (1.0 mm diameter) rod from a height of 6.25 mm onto the exposed dura [153].

2.12.2 Drug delivery of EGFR inhibitor post SCI

3 μl of PD168393 (1 mM) or PBS solution were injected into the center of injured region with a 5 μl Hamiliton syringe immediately after the contusion. The needle was left in place for 5 minutes after injection to minimize liquid leakage. To achieve the sustained release, a gelatin matrix [154] containing 7 μL PD168393 (10 mM) or PBS was implanted into the damaged dorsal dura mater before suturing the muscles and skins [155, 156].

For transplantation experiment, 1 μl (0.5×10^5 cells/μl) NG2+ cells were injected twice into the lesion site of spinal cord immediately after the PD168393 or PBS injection. Subsequently, the gelatin matrix for sustained supply of PD168393 or PBS was embedded into the exposed spinal cord segment. No teratoma formation was observed in any transplanted animals. All transplant recipients received cyclosporine A (15 mg/kg, Sigma) daily, from 2 days before transplantation. To trace the cells, they were transfected with pEGFP-N1 vector (Clonetech) before transplantation.

2.12.3 Post-operative care of the experimental animals

After operation, animals were allowed to recover on a heating pad. The daily i.p. injections of ampicillin (1.5 mg/kg) and carprofen (5 mg/kg) were given for one week. Bladder expression was performed until the bladder reflection was successfully re-established.

2.12.4 Basso-Beattie-Bresnahan (BBB) locomotor scoring

The neural behaviour of animals post contusive spinal cord injury was examined with a 21-score system of the BBB scale [157]. At day 1, 3, 5, 7, 14, 21, 28 after injury, spontaneous open field locomotor activity of the hind limbs of each animal was monitored. Three examiners who were blind to the experimental groups performed the BBB test individually at each time point.

2.12.5 BrdU Administration

Animals were given the thymidine analogue BrdU labeling reagent (1 mL/100 g, Invitrogen) twice at 2-hour intervals on day 3 post injury, following the manufacturer's instruction. The phenotypes of BrdU+ cells were determined at 2 hours after injection, 1 week, 2 weeks and 3 weeks post SCI.

2.13 Tissue preparation and Immunohistochemistry

2.13.1 Tissue preparation

Mice were sacrificed on 3 days, 1, 2, 3 and 4 weeks after SCI. Overdose of xylazine and ketamine were given to the mice for deeply anaesthetizing. An incision was made at the base of the sternum, extended along the base of the rib cage and then up the side of the body through the ribs, allowing the rib cage to be lifted up. The connective tissue and diaphragm around the heart was dissected away and a small cut was made in the right atrium. This was followed by cardiac perfusion with cold heparinized saline and 4% PF in 0.1 M PBS (pH 7.4). Fixed spinal cord were dissected and immersed in PBS with 30% sucrose. The tissue was subsequently embedded in OCT, frozen in liquid nitrogen. Frozen sagittal sections (30 μm in thickness, 2 cm in length) were cut through the areas encompassing the injury site.

2.13.2 Immunofluorescence staining

For immunostaining, the slides were blocked with 4% BSA in PBS/0.1%Triton X-100 for 2 hours at RT and incubated overnight at 4℃ with the primary antibodies (Table 3) diluted in PBS/0.1%Triton X-100/1% BSA. In the double and triple counterstaining sections, relevant rabbit or mouse antibodies against NG2 proteoglycan and GFP were applied together with other primary antibodies. The sections were washed with PBS and incubated with corresponding secondary antibodies for 3 hours orderly at RT. Finally, the sections were counterstained with DAPI after PBS washing and mounted with Fluorosave® onto slides.

Staining for neuronal markers combined with BrdU was performed separately. The sections were firstly processed for marker staining (as stated above). The free-floating sections were then treated with 1 N HCl for 10 minutes on ice, followed by another 10 minutes of 2 N HCl treatment before incubation at 37 ℃ for 20 minutes. The sections were finally incubated in borate buffer (0.1 M) for 12 minutes at RT (pH7.4), followed by BrdU staining with routine immunostaining protocol.

2.13.3 Hematoxylin & Eosin (H&E) staining

To perform the H&E staining, the sagittal sections were mounted onto PLL-coated slides and dried overnight. Mounted slides were first submerged in PBS for 5 minutes, and then incubated in 4% PF for 2 minutes, followed by being rinsed in PBS and distilled water (for 1 minute each). They were subsequently transferred into Hematoxylin solution (for 2 minutes), and rinsed in distilled water twice before being dipped in differentiating solution (1% HCl in 100% ethanol). The slides were again rinsed in distilled water twice and incubated in Scott's tap water substitute (Magnesium sulfate buffered with sodium bicarbonate) for 5 minutes. After rinsed in distilled water for 1 minute, the slides were incubated in 95% ethanol for 30 seconds before being transferred to eosin Y solution for 20 seconds. Then, they were placed in 70% ethanol for 15 seconds to remove excessive Eosin staining. After staining, slides were air-dry before being covered with paramount (Innovex Biosciences, U.S.). Sections were viewed and analyzed under bright-field microscope (Olympus IX71).

2.14 Sampling and cell counting in sections

All the samples were imaged under a confocal microscope (Carl Zeiss LSM 510 and/or LSM 710), using the standard excitation and emission filters for visualizing DAPI, FITC (Alexa Fluor 488), Cyanine, Rhodamine (Alexa Fluor 568) or Far Red (Alexa Fluor 647). Confocal z-stacks were captured for each section with 0.3-1 μm increments. All the sections were processed simultaneously under identical light beam, wavelength in both control and PD168393-treated groups throughout the experiment. The photomicrographs were taken by 10×, 20× or 63× objective lenses and the Zen 2007 software (Carl Zeiss MicroImaging GmbH, Gottingen, German) were used for exporting the images. The generated panoramic images (by Adobe Photoshop 7.0) of sagittal sections in control and PD168393-treated groups were taken under the 10 × objective magnification.

Five animals per group were examined at each time point. Generally, the sagittal sections were investigated in the medial region between midline and peripheral rim of the spinal cord. These sections covered most areas of grey matter, the dorsal and ventrolateral white matters. They thus included regions as demonstrated by previous studies that the ventral areas of spinal cord were involved in hindlimb function, while the dorsal areas contributed to the sensory recovery [158, 159]. For individual quantification, at least three selected sections were examined from each slide.

2.14.1 Quantification of NG2+/pEGFR+, pEGFR+ cells, OX42+ cells in both PBS- and PD168393-treated groups

For the quantification of pEGFR+cells and NG2+/pEGFR+ cells, the counting was performed along the rostrocaudal axis until 3 mm rostral and -3 mm caudal to the injury epicentre. Within the 6 mm range, the sections were separated into twelve continuous 500 μm intervals. The cells with corresponding staining were measured within these specified areas. The OX42+ cells in both PBS- and PD168393-treated groups at 3 days and 21 days post SCI were determined similarly.

2.14.2 Quantification of proliferative cells in PBS- and PD168393-treated groups

For the quantification of proliferative cells at 2 hours after BrdU injection in 3 days post injury, total BrdU+ cells, NG2+/BrdU+ cells, OX42+/NG2-/BrdU+ cells, GFAP+/NG2-/BrdU+ cells, CNPase+/NG2-/BrdU+ cells, Nestin+/NG2-/BrdU+ cells were quantified in the 6 mm sections, which were separated into twelve continuous 500 μm intervals. The proliferative cells were counted as positive if the BrdU+ nucleus was unambiguously associated with the marker antigens.

2.14.3 Quantification of (NG2+)/BrdU-labeled neuronal cells in the spinal cord lesion site of PD168393-treated group

The proportion of 1-3 weeks-old (NG2+)/BrdU+/neuronal phenotypic cells was determined in the -1.5 mm~1.5 mm range of the injured region. Within the 3 mm range, the sections were separated into six continuous 500 μm intervals. In these areas,

the (NG2+)/BrdU+ neurons were counted as positive if the BrdU+ nucleus was unambiguously associated with the neuronal marker antigens.

2.15 Statistical analysis

Statistical analysis was done using a multiple-measurement ANOVA for comparison of PD168393-treated groups and control groups over time or a two-tailed Student's t test. In all cases, the data were expressed as mean ± standard deviation (SD). A P-value < 0.05 was considered statistically significant.

CHAPTER 3 Clonal Analysis for Elucidating the Lineage Potential of Embryonic NG2+ Cells

3.1 Introduction

NG2+ cells are mostly found to differentiate into oligodendrocytes [38, 42, 48, 49, 51], they are also demonstrated to generate the progenies of astrocytes and neurons [49, 61, 76, 82, 141]. Thus, they could commit to plastic lineages and exhibit multi-functional characteristics in both physiological and pathological CNS [2, 5, 6]. The inherent heterogeneity of this cell population has not been adequately addressed so far, especially their differentiation capacity remains to be characterized.

Independent studies of NG2+ cells at population levels have revealed that NG2+ cells could give rise to multiple neuronal-glial progenies. Several approaches are presently used to investigate the lineage potential of NG2+ cells, including the immunohistochemical methods and lineage tracing methods. The immunofluorescence staining of NG2 proteoglycan with neuron-glia markers illustrate the heterogeneous lineages of NG2+ cells. However, this method could not be used for characterizing lineage potential of whole NG2+ population, as NG2+ subpopulations with specific phenotypes are examined respectively. Lineage tracing methods employing nucleotide analogue BrdU and retrovirus have been used for tracing the lineages of proliferative NG2+ cells in adult CNS, and various labeled progenies are characterized thereafter. But they are ineffective for labeling the postmitotic NG2+ cells. Thus, these labeling approaches might not be used for evaluating the lineage potential of NG2+ cells either,

due to the heterogeneous proliferation capacity of NG2+ cells [65, 160]. Genetically modified mice with inducible Cre-ER recombination under different promoters have been used for *in vivo* fate mapping of adult NG2+ cells, but all these analyses are also performed on the population level. In brief, these results suggest the heterogeneous lineage potential of NG2+ cells, but leave some questions unanswered: Are NG2+ cells composed of mixed populations of uni-/bi-/multi-potent neural progenitors that are either neurogenic or gliogenic? Or are they a group of multipotent neural progenitors?

To understand the lineage heterogeneity of NG2+ cells, we assessed their differentiation ability in single (or clonal) NG2+ cells. The single clone based analysis could be useful in elucidating the lineage potential unique to the individual clones. Thus, it would allow us to characterize NG2+ cells via analyzing their lineage potential at a clonal level.

3.2 Results

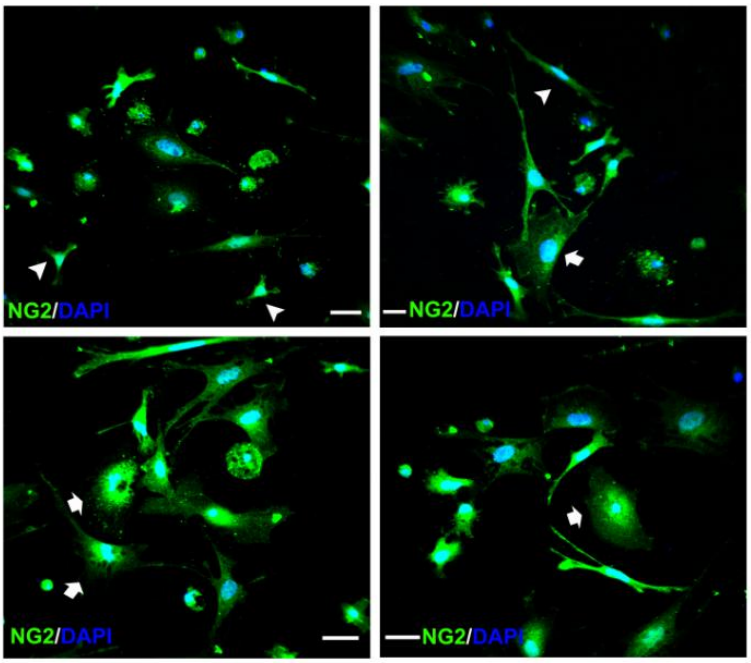
3.2.1 Isolation, characterization and immortalization of primary NG2+ cells

To explore the lineage potential of NG2+ cells, they were isolated from cerebral cortex of rat embryos (E16). The freshly-isolated embryonic NG2+ cells exhibited variable morphologies in culture. Most of them were morphologically identified as islands of flat cells with epitheloid soma, and few short thin or elongated irregular processes. A few of them were identified as bipolar or multipolar cells with smaller cell bodies. The

morphologies of primary cultured NG2+ cells in our study were consistent with the ones established in previous reports [129, 145, 146]. Immunostaining results of NG2 proteoglycan showed that the primary NG2+ cultures expressed predominant levels of NG2 proteoglycan, as shown in Fig. 4A. FACS result showed that around 99% cells expressed NG2 proteoglycan. The majority of the cells expressed PDGFαR. Neither the macrophage marker OX42 nor the specific neuronal progenitor marker Doublecortin was detectable in the cultures (Fig. 4B). Therefore, NG2+ cells have been reliably and successfully isolated.

To generate the single-cell derived NG2+ cells for the long term culture, we transfected NG2+ cells with pGRN145 hTERT plasmid. The mixed hTERT-NG2+ cells were examined for hTERT expression by RT-PCR (Fig. 4C). In total, twenty-four single-cell derived NG2+ clones (named by G+Arabic Numbers) were subsequently generated from the immortalized embryonic NG2+ cells, and further studies were performed to analyze the cellular properties based on the individual hTERT-NG2+ cell lines.

A



B **C**

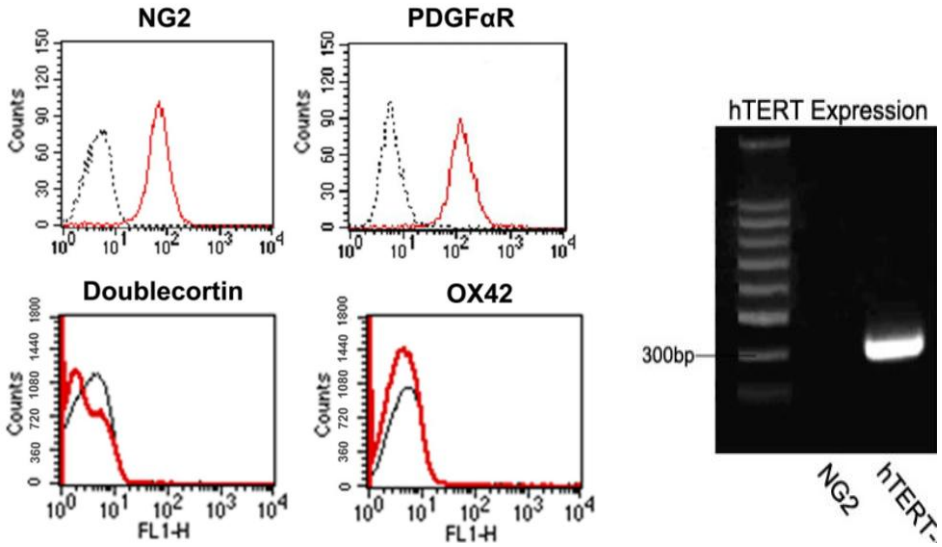


Figure 4 The identification and hTERT plasmid transfection of primary NG2+ cells.

(**A**) Immunocytochemical characterization of primary NG2+ cultures. Representative morphologies in separate fields under microscope showed NG2 green fluorescence in

the cytoplasm of cells. Nuclei were counterstained with DAPI in all images. Examples of flat cells with epitheloid soma in all the images were indicated by arrow. They were either with few short thin or elongated irregular processes. Examples of bipolar or multipolar cells with smaller cell bodies in all the images were indicated by arrowhead. Bars, 30 μm; (**B**) Characterization of NG2+ cells by FACS. The four panels showed the representative FACS analysis of NG2 proteoglycan (99±0.48% in 10^4 cells), PDGFαR (94±1.78% in 10^4 cells), Doublecortin and OX42 negative (3.4×10^5 cells) in primary NG2+ cell cultures. Black (dash) line: negative control, red line: NG2 (upper left panel), PDGFαR (upper right panel), Doublecortin (lower left panel), OX42 (lower right panel) expression. (**C**) Immortalization of the NG2+ cells with hTERT gene. The NG2+ cells were transfected with hTERT plasmid. RT-PCR was performed to detect the presence of hTERT (395bp) in hTERT-NG2+ cells.

3.2.2 Multilineage differentiation potential of clonal NG2+ cells *in vitro*

Subpopulations of NG2+ cells have been proven to be able to acquire oligodendrocyte, astrocyte and neuronal fates both *in vitro* and *in vivo* [2]. To determine whether NG2+ cells were multipotent at a clonal level or were heterogeneous populations containing various committed progenitor cells, we performed the oligodendrocyte, astrocyte, neuron differentiation assay to the established 24 NG2+ clones.

All of the clonal NG2+ cell lines were subjected to oligodendrocyte differentiation. Most clones could acquire typical oligodendrocyte morphology, with small soma and multiple finely interlaced arbors. The oligodendrocyte phenotypes were confirmed by the expression of oligodendrocyte-specific lineage markers GalC and O1, which were commonly found to be expressed in cultured oligodendrocytes [161]. We found that some clones could be well differentiated into oligodendrocytes. Take the clone shown

in Fig. 5A and 5B for example, 91.21 ± 1.3% of the clonal cells expressed GalC, and 82.75 ± 5.3% of them expressed O1. Some other clones poorly differentiated into GalC+ oligodendrocytes at a percentage of 38.16 ± 1.4% (Fig. 5C) and 45.12 ± 2.1% of them could differentiate into O1+ oligodendrocytes (Fig. 5D). These results demonstrated that the embryonic NG2+ clones possessed the oligodendrocyte differentiation potential. However, some of the clones showed retarded morphologic differentiation, and their process arbors appeared relatively fewer (Fig. 5C, 5D), suggesting that variance existed in the oligodendrocyte differentiation capacity of these clones.

Previous studies have shown that NG2+ cells generate astrocyte progenies in certain areas and pathologic conditions of the CNS [48, 49, 136, 140]. In our experiment of astrocyte differentiation, 8/24 of the clones were able to exhibit a type-2 astrocyte like morphology, with triple or multiple branching processes after induction. In the presence of 10% FBS plus IGF-1 and T3, those clones with obvious morphologic changes expressed S100 β and GS in 1 week (Fig. 5E, 5F). Both S100 β and GS are expressed primarily by astrocytes and function in astrocytosis. These results demonstrated that some NG2+ clones could also differentiate into astrocytes, as well as the oligodendrocytes *in vitro*.

To investigate the neuronal lineage potential of NG2+ cells, we tried several strategies to initiate the neuronal differentiation from the NG2+ clones and EGFR inhibitor PD168393 was selected [162]. The neuronal morphologic changes could be observed

in one of the clones within 4 days of induction, exhibiting obviously elongated neurites. After 1 week of induction, large numbers of bipolar cells with conspicuous soma could be observed. The neuron-like cells mostly exhibited long extending processes with spines and varicosities. Seldom were morphologies found to be multipolar. The neuronal differentiated cells were further found to express neuronal cytoskeleton component, NF200 (83.01 ± 1.6% in total NG2+ cells) or neuronal microtubules stabilizing protein, MAP2 (24.38 ± 1.1% in total NG2+ cells) (Fig. 5G, 5H). These results indicated that clonal NG2+ cells possessed the neuronal-generating properties.

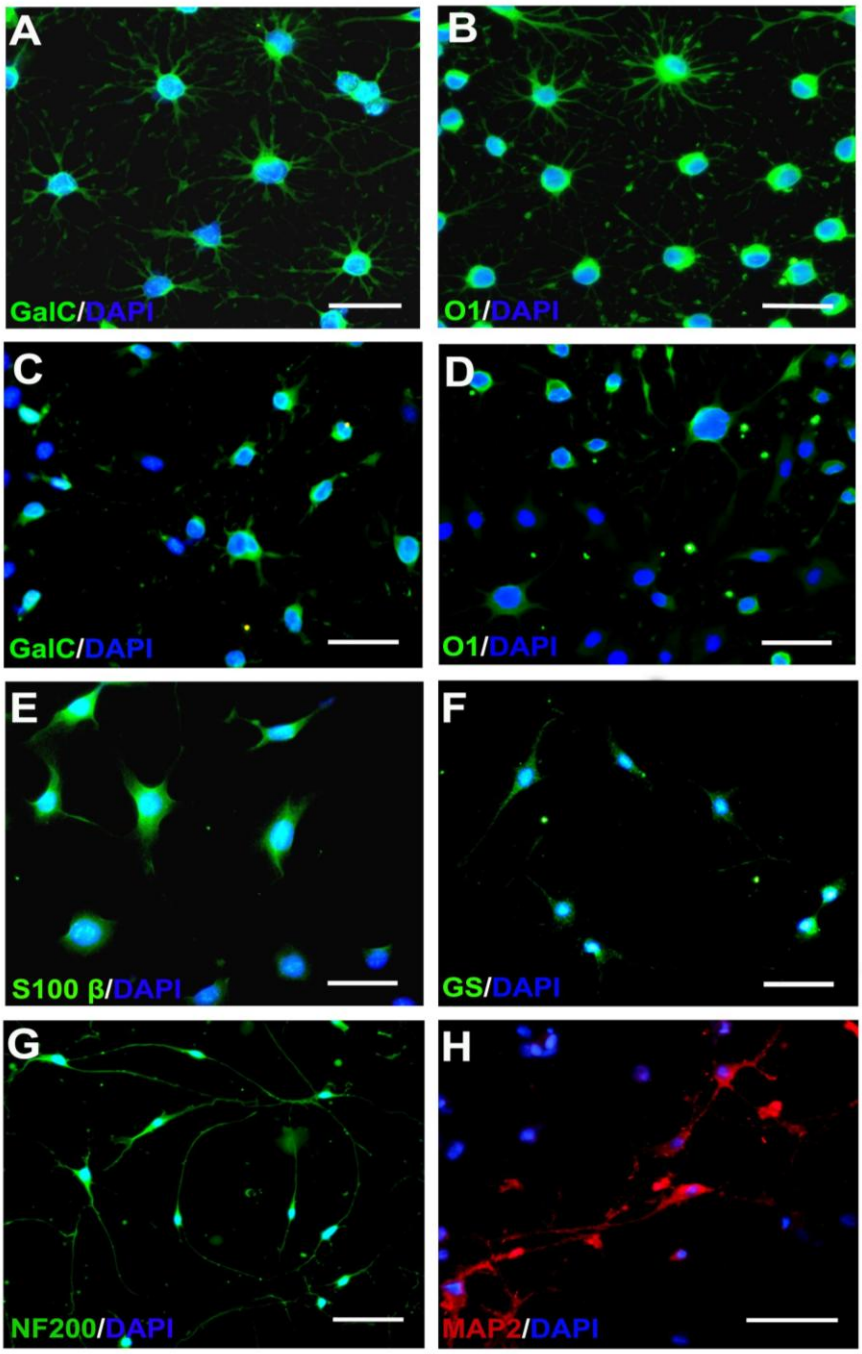


Figure 5 Oligodendrocyte, astrocyte and neuronal differentiation of clonal NG2+ cells.

(**A**, **B**) Some of the clones could differentiate into high percentage of GalC and O1 positive oligodendrocytes. (**C**, **D**) Some gave rise to less oligodendrocytes with fewer process arbors. (**E**, **F**) After astrocytes' induction, one third of the clones could

differentiate into astrocytes. The differentiated clonal cells were assayed for S100 β (**E**) and GS (**F**) to confirm the astrocyte differentiation. (**G**, **H**) Neuronal differentiation of clonal NG2+ cells. One clone could be induced into NF200 (**G**) and MAP2 (**H**) positive neuronal phenotypes. Bars, 30 μm.

As shown in Fig. 6A, 6C, 6E, the NG2+ clone that was able to differentiate into neuronal cells could give rise to oligodendrocyte and astrocyte lineages as well. The immunostaining results further confirmed the expression of relative lineage markers, such as O1 for oligodendrocytes and S100 β for astrocytes, apart from NF200 for neuronal differentiation (Fig. 6B, 6D, 6F). Therefore, there existed one clone that had the tri-lineage potential (multipotent clone; MP clone, 1/24, Table 6). The rest of NG2+ clones were not able to acquire neuronal phenotypes. It was worth noting that all the clones could differentiate into oligodendrocytes, although 19/24 had a higher capacity of oligodendrocyte differentiation (+***, Table 6), and the others had a relatively lower potential (+*, Table 6). When they were subjected to astrocyte differentiation, about 8 of them could yield astrocytes besides the oligodendrocytes (bi-potential clone; BP clone, 8/24, Table 6). Hence, all these clones showed neuronal-glial differentiation potential that had been tested. The results for oligodendrocyte, astrocyte and neuronal differentiation potential of all the clones are summarized in Table 6. Overall, these results demonstrated that the clonal NG2+ cells were composed of heterogeneous progenitors with distinct differentiation potential. The clonal analysis reflected the multiple lineage potential of NG2+ cells. It is therefore a straightforward way of interpreting the heterogeneous NG2+ cell populations.

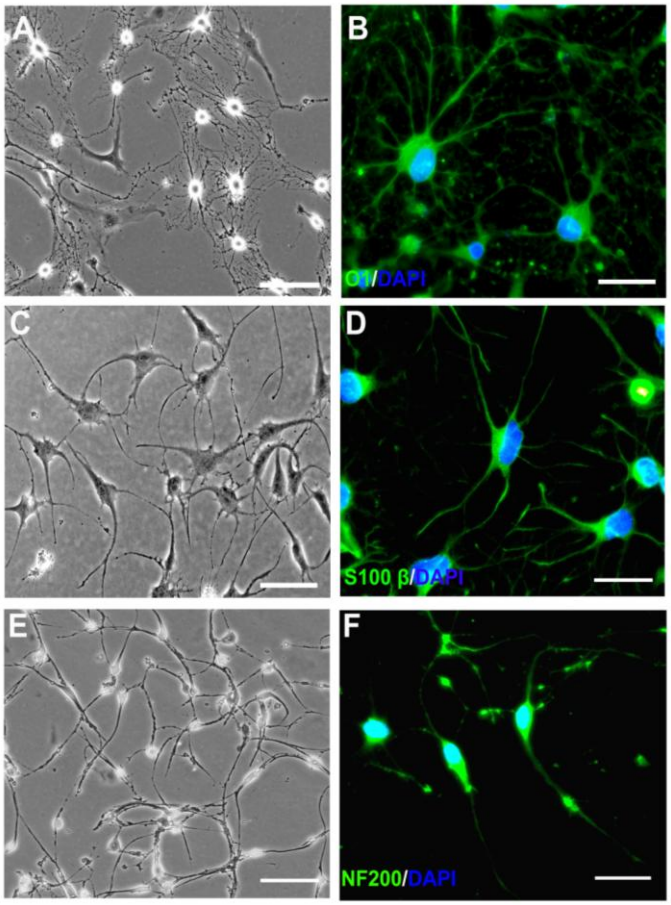


Figure 6 The multipotent NG2+ clone exhibited tri-lineage potential.

The multipotent NG2+ clone could differentiate into oligodendrocytes (**A**, **B**), astrocytes (**C**, **D**) and neuronal phenotypes (**E**, **F**) after induction. Bars of **A, C**, **E**, 30 μm; Bars of **B**, **D**, **F**, 15 μm.

Table 6 Differentiation potential of NG2+ clones

Phenotype	No. of Clones	% of total	Oligodendrocyte genesis	Astrogenesis	Neurogenesis
			GalC, O1	S100 β, GS	NF200, MAP2
+***/+/+	1	1/24	+***	+	+
+***/-/-	14	14/24	+***	-	-

+***/+/-	4	4/24	+***	+	-
+*/-/-	2	2/24	+*	-	-
+*/+/-	3	3/24	+*	+	-
-/-/-	0	0	NA	NA	NA

+ for positive, - for negative, +*** for highly oligodendrocyte genesis, +* for lowly oligodendrocyte genesis

Twenty-four NG2+ clones have been treated with differentiation medium as described in the Materials and Methods. The lineage potential of these cells were analyzed and characterized.

As NG2 proteoglycan has been demonstrated to play a role in the developing rat limb [163-166], these clones were also investigated for their chondrogenic differentiation potential. Only the MP clone was found to be able to differentiate into chondrocytes after 3 weeks of induction. As shown in Fig. 7A, the MP clone dramatically changed its morphology to a paving stone-like shape and grew in a compact cluster. Alcian Blue staining was performed to detect the expression of proteoglycans, the extracellular matrix synthesized by chondrocytes. An intensive staining was observed after 2 weeks of induction. The chondrogenic differentiation was also confirmed by RT-PCR analysis (Fig. 7B). The expression level of collagen II, a predominant collagenous protein of cartilage matrix, and another major non-collagenous protein in cartilage matrix, aggrecan, was up-regulated after 1 week of induction. This result showed that a subpopulation of NG2+ cells from embryonic cerebral cortex could differentiate into chondrocytes.

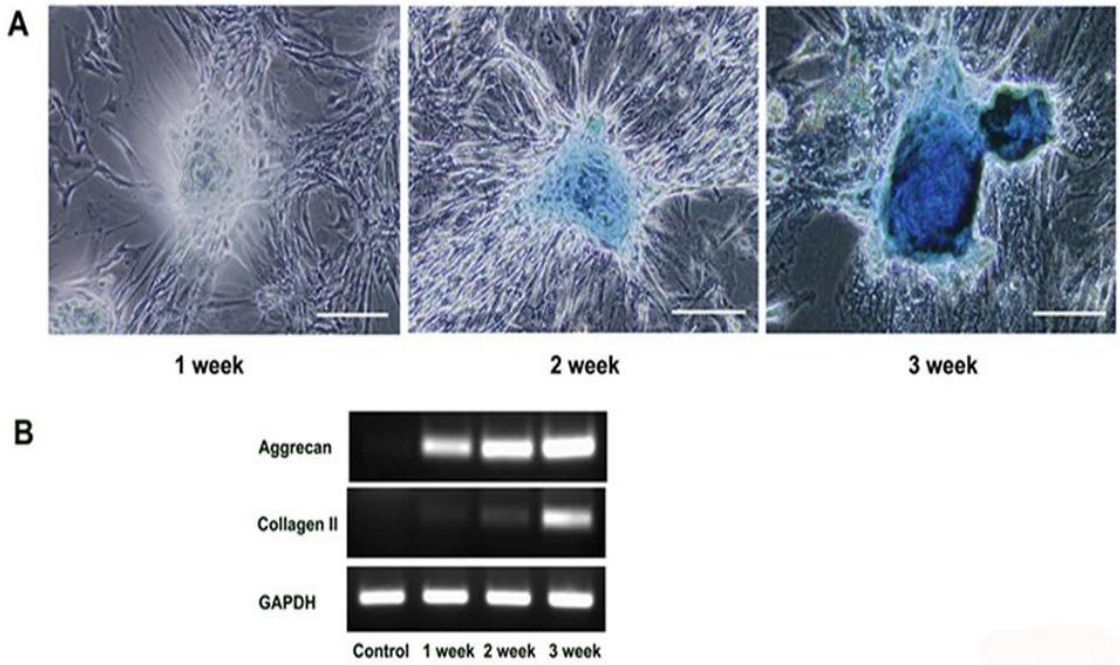


Figure 7 The multipotent clone possessed chondrogenic differentiation potential. (**A**) Cells gradually exhibited a pave stone-like shape, and were stained positively by Alcian Blue in 2 weeks after induction. (**B**) RT-PCR results showed that the chondrocyte markers collagen II and aggrecan were gradually increased during a 3-week induction (Lane 2, 3, and 4). Lane 1: Control NG2+ cells, which were not subjected to the chondrocyte differentiation. Bars, 150 μm

3.2.3 Phenotypic characterization of clonal NG2+ cells

To further characterize the clones with distinct lineage potential, the expression levels of neural progenitor markers NG2, PDGFαR, nestin and A2B5 were analyzed by FCM (Fig. 8A-8F). Cell samples from each group with distinct differentiation ability were chosen randomly.

G5, which was only prone to generate high percentage of oligodendrocytes, expressed NG2 at 76.63 ± 1.18%, PDGFαR at 97.16 ± 2.90%, nestin at 37.94 ± 1.94% and A2B5 at 11.45 ± 0.54%. G45, representing low percentage of oligodendrocyte genesis, expressed NG2 at 88.74 ± 2.78%, PDGFαR at 97.51 ± 2.48%, nestin at 92.95 ± 2.55% and A2B5 8.87 ± 0.19%. For the BP differentiation clones, G9, which was highly differentiated into oligodendrocytes and astrocytes, expressed NG2 at 86.23 ± 3.18%, PDGFαR at 98.97 ± 0.15%, nestin at 76.34 ± 1.78% and A2B5 at 4.35 ± 1.07%. G27, capable of giving rise to astrocytes and only a small portion of oligodendrocytes, expressed NG2 at 85.68 ± 3.06%, PDGFαR at 98.53 ± 2.28%, nestin at 8.29 ± 1.75% and A2B5 at 34.23 ± 1.37%. The MP clone, G23, expressed NG2 at 98.32 ± 1.04%, PDGFαR at 80.60 ±1.91%, nestin at 30.68 ± 2.45% and A2B5 at 0.25 ± 0.16%. In brief, these representative clones highly expressed progenitor markers NG2 and PDGFαR, which fluctuated within a narrow range, while the progenitor markers nestin and A2B5 were unevenly expressed at lower levels. A direct relationship between the levels of phenotypic markers in these clones and their differentiation ability could not be built up yet, although there did exist the distinct expression profiles among each group of NG2+ clones. Further studies will be needed to establish the link between the different phenotypes and heterogeneous functions in NG2+ subpopulations.

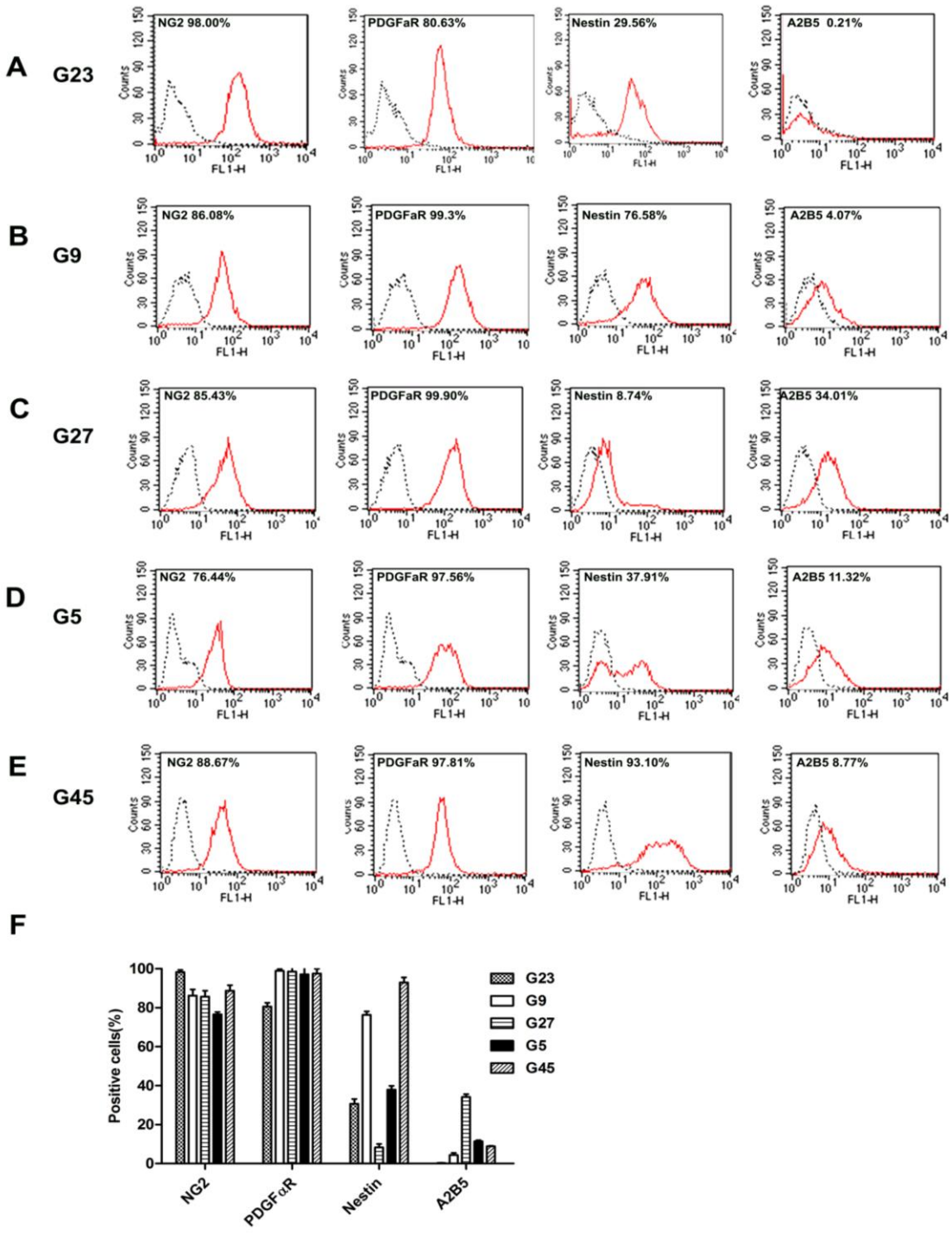


Figure 8 Neural phenotypes of clonal NG2+ cells were characterized using FCM (Passage three).

Four neural progenitor markers have been investigated: NG2, PDGFαR, Nestin, and A2B5. (A) G23, with multipotent differentiation potential. (B-C) $G9^{high\text{-}oligo}$ and

$G27^{low\text{-}oligo}$, representatives with bi-potential differentiation, oligodendrocytes and astrocytes. (D-E) $G5^{high\text{-}oligo}$ and $G45^{low\text{-}oligo}$, representatives for only committing to oligodendrocyte lineage. Black (dash) line: negative control; red line, from left to right: NG2 (column 1), PDGFαR (column 2), Nestin (column 3), A2B5 (column 4) expression. (F) The expression levels of neural progenitor markers of the representatives with distinct differentiation potential.

Apart from the distinct phenotypes and differentiation capacities of these clones, we also observed that different clonal NG2+ cells exhibited variation in cell morphology (Fig. 9). Their predominant morphology could be described as flattened epitheloid cells with or without irregular un-branched processes, as shown in G5, G9, and G45. Some other morphology could also be observed. In particular, G23 exhibited elongated or irregular soma, while G27 exhibited a large nucleus with polygonal soma. The distinct morphologies in NG2+ clones were consistent with what were observed in primary NG2+ cell culture. Moreover, it reflected the heterogeneous morphological properties of NG2+ cells [129, 145, 167].

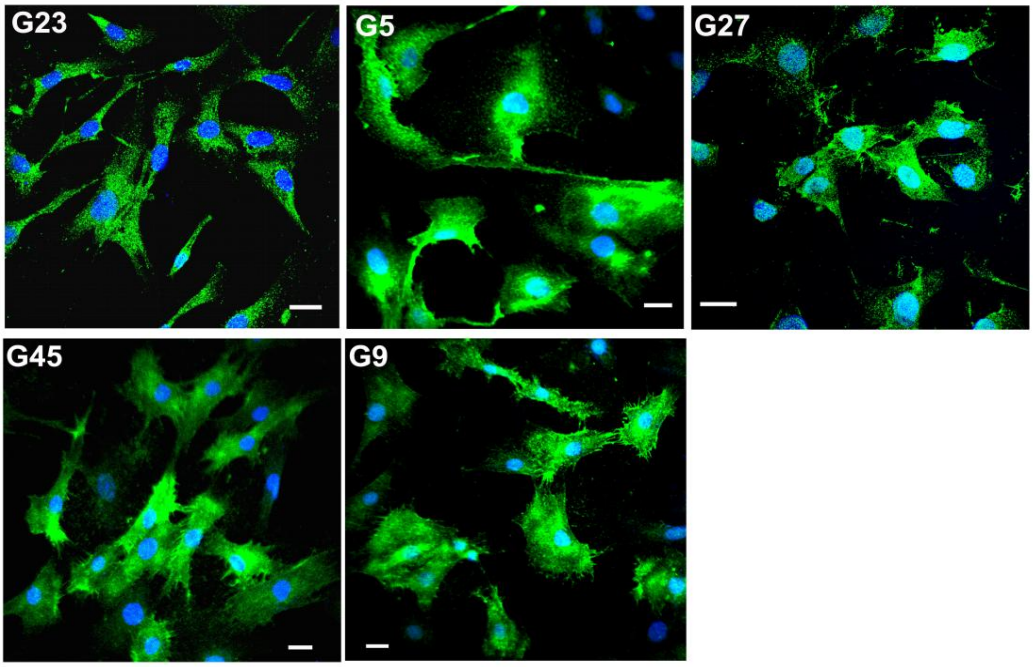


Figure 9 The great majority of cells, visualized with DAPI (blue), from individual NG2+ clones expressed NG2 proteolycan (green).

Bars, 20 μm

The cell outgrowths from these clones were also assessed. Over 6 days in culture, these clones were found to exhibit heterogeneous growth capacities, with the numbers of cells ranging from 2.89 $\pm 10^4$ to 46.76 $\pm$ 10^4 (Fig. 10). These results further elucidated the cellular heterogeneity of embryonic clonal NG2+ cells.

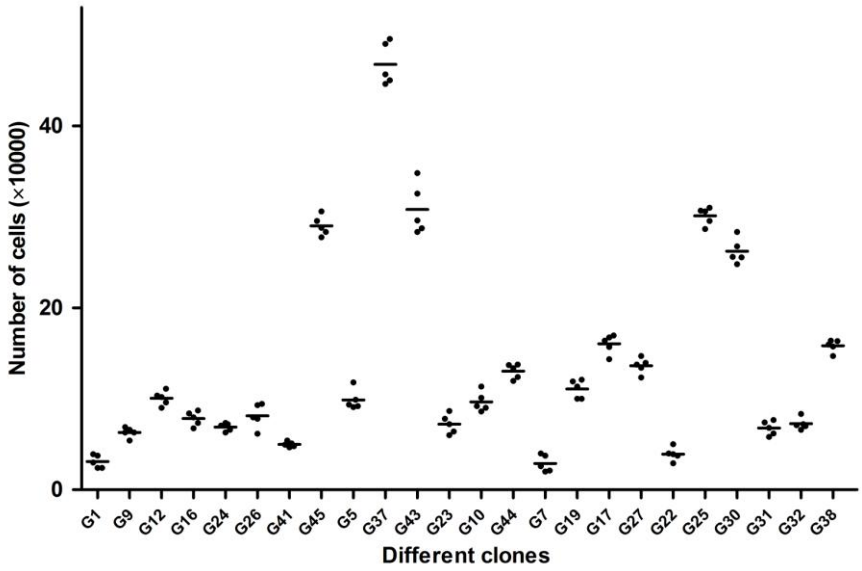


Figure 10 Measurement of cell growth from hTERT transfected NG2+ clones.
Cell numbers from individual NG2+ clones on the 6th day. Each result was the average of five measurements.

3.3 Discussion

It is well established that NG2+ cells are heterogeneous in their progenies, which might be determined by either the extrinsic environmental cues or their intrinsic properties, and yet confused the understanding of NG2+ cells [2]. To elucidate the lineage heterogeneity of NG2+ cells, a number of single cell derived NG2+ clones from embryonic cerebral cortex were investigated here. These clones expressed different levels of progenitor markers and differentiated into oligodendrocytes, astrocytes, neurons, or even chondrocytes with different lineage compositions. Together, the results provide the evidence that the NG2+ cells are not a mixture of homogeneous multipotent progenitors, but rather composed of subpopulations with distinct neural lineage potentials. Understanding the lineage heterogeneity of these

NG2+ clones would help explaining the distinct lineage potential observed in NG2+ populations.

In this study, we found that all the clones could commit to the oligodendrocyte lineage, possibly because the intrinsic properties of these clones allow them to acquire the oligodendrocyte phenotypes. Alternatively, it is likely that these clones might be efficiently induced to generate oligodendrocyte progenies in the current experimental conditions. These results indicated that the oligodendrocyte pathway should be the default route that the NG2+ clones could all progress through. This finding is consistent with the conclusions of previous studies, in which the oligodendrocyte potential of NG2+ cells have been demonstrated at the population level (as reviewed in the Chapter One).

Additionally, the NG2+ clones available in our lab were characterized as expressing the phenotypic markers of OPCs, as they were found to express PDGFαR as well as A2B5. This is consistent with the early studies which identified NG2+ cells as OPCs. The first indication that NG2+ cells are OPCs came from the observation that 95% OPCs were positive for NG2 by immnunopanning against A2B5 [28, 168], or NG2+ cells could generate A2B5 positive oligodendrocyte precursor cells [167]. NG2+ cells are also found to co-express with the PDGFαR, whose function has been shown to be specific to OPCs [12, 169, 170]. Taken together, our results demonstrated that all these NG2+ clones are OPCs, and verified the oligodendrocyte lineage potential of NG2+ cells at a clonal level *in vitro*. Moreover, our data also address that different NG2+

clones had distinct capacities of oligodendrocyte differentiation. This indicated that NG2+ subpopulations may have the distinct potential of generating oligodendrocytes. .

Although some studies confirmed that the NG2+ cells were developmentally related to the O-2A lineage [25, 31], it is still necessary to investigate whether NG2+ cells are bipotential cells that can generate both oligodendrocytes and astrocytes or they are heterogeneous populations that are progenitors of either oligodendrocytes or astrocytes. Astrocytes differentiation of NG2+ cells have been confirmed separately by several strategies, either *in vitro* or *in vivo* [48, 49, 51, 59, 171]. In our study, clonal analysis was used to determine the astrocyte potential of a single cell derived NG2+ population. We demonstrated that 8/24 of the NG2+ clones could differentiate along both oligodendrocyte and astrocyte lineage. Therefore, individual NG2+ clones possessed heterogeneity in generating astrocyte progenies. The heterogeneity of these NG2+ clones in generating astrocyte progenies may not simply depend on the bi-potency of single NG2+ clone, but rather on a mixture of cells committed to different lineages. This provided direct evidence for the existence of a hierarchy in the astrocyte differentiation of NG2+ cells, and help explaining the findings that NG2+ cells had the different capability of giving rise to astrocyte progenies when the CNS microenvironment changed.

In the current study, we demonstrated for the first time that single cell derived NG2+ clone, apart from owning the oligodendrocyte and astrocyte lineage potential, could also differentiate into neuronal lineages. Herein, we provided the immediate evidence

that the embryonic NG2+ populations could be induced to exhibit the multi-potentiality. This result could strongly support that NG2+ subpopulations are one source of neurogenesis *in vivo*. However, we found that only one of the twenty-four clones was induced to acquire neuronal phenotypes. Hence, there was low frequency of clone with neuronal potential in NG2+ populations. Consistently, previous studies reveals that there is limited neuronal lineage potential of NG2+ cells [61, 77]. In the future study, manipulation of neuronal potential from whole NG2+ cell population *in situ*, especially in the neuronal degenerative CNS, needs to be explored. Moreover, the underlying mechanism for the alternative neuronal fate decision by NG2+ cells remains to be figured out.

Traditionally, it was thought that NPCs could only commit to neuronal and glial fates. However, recent studies suggest that NPCs are capable of trans-differentiating into cell types of mesoderm, such as that they may differentiate into haematopoietic cells [172, 173]. In the current study, we found that the multipotent NG2+ clone could differentiate into chondrocytes as well. To the best of our knowledge, it is the first evidence that a subpopulation of neural NG2+ cells had the chondrogenic differentiation potential.

To sum up, we demonstrated that the heterogeneous NG2+ cells at a clonal level displayed a multi-differentiation potential *in vitro*. A scenario is evident in their differentiation potential, with oligodendrocyte lineage alone or the oligodendrocyte and astrocyte lineages together. What is more, NG2+ clone could be differentiated into

neurons and chondrocytes, besides glial lineages. Collectively, our results implicated the distinct differentiating properties of defined NG2+ populations *in vitro*. It enhanced the view that NG2+ cells could exhibit the multi-potentiality and have the potential of responding to CNS injury for remyeliantion and neuronal repair.

CHAPTER 4 Induction of Neuronal Phenotypes from NG2+ Cells by Inhibiting EGFR in Mouse Spinal Cord Injury

4.1 Introduction

In CNS trauma, there is a drastic loss of neuronal cells causing permanent neurological dysfunction [174, 175]. Compared with transplantation of exogenous neuronal cells which integrates less efficiently into the damaged tissue and generates predominantly undesirable progenies [176, 177] , the endogenous cell replacement is expected to be a more effective treatment [178, 179]. Though many studies reveal that neural stem cells persist in the adult CNS [74], their neurogenic ability in most regions of the CNS is very limited [180]. In the spinal cord, the production of new neurons is demonstrated to be almost arrested in the adulthood [181]. Therefore, seeking alternative cell population *in situ* with neuronal potential has great significance in the treatment of spinal cord injury.

The NG2+ cells are the most abundant cycling cells in adult CNS [47] and they rapidly proliferate in response to injury for glial cell replacement [118, 129, 182]. However, recent evidence reveals that they also contribute to adult neurogenesis in several regions of the brain [2, 61, 77, 78]. Moreover, results from *in vitro* studies reveal that glial progenitors could be induced to differentiate into neurons [83, 85, 183]. In our previous work, single-cell derived clonal NG2+ cells could be differentiated into neuronal phenotypic cells *in vitro,* indicating the multi-potentiality of whole NG2+

cell population. Thus, it is possible that in the injured adult CNS, the NG2+ cells might be a source of endogenous neurogenesis.

Recent studies demonstrate that large numbers of NG2+ cells acquire neural stem cell-like phenotype in the injured CNS [24, 120, 135-137, 184]. However, the neurogenic capacity of NG2+ cells has never been documented post SCI. This indicates that the factors presented in the injury environment might bias the generation of glial cells from NG2+ cells. EGFR, which belongs to the receptor tyrosine kinase (RTK) family, has a wide range of biological effects on the migration [185, 186], proliferation [187] of glial progenitors. Importantly, EGFR signaling is shown as one of the vital factors for promoting glial differentiation post injury. Over-expression of EGFR accelerates the remyelination process after CNS injury [188, 189]. Activation of EGFR is also known to trigger quiescent astrocytes into reactive astrocytes [140, 190, 191]. Therefore, EGFR signaling is a key factor for gliogenesis in traumatic CNS. Given that antagonizing signals for gliogenesis would benefit the neurogenesis from neural stem cells [192, 193], it could be of great significance to determine whether inhibition of EGFR signaling would induce neurogenesis from NG2+ cells in SCI.

4.2 Results

4.2.1 EGFR inhibition induces neuronal phenotypes of NG2+ cells *in vitro*

As stated before, the cultured clonal NG2+ cells could be induced to gain the neurogenic capacity in a two-phase differentiation strategy through EGFR inhibition.

These results not only demonstrated the multipotential of whole NG2+ cell population, but also implied the EGFR specific inhibitor-PD168393 as a potent neurogenic inducer from the commonly known NG2+ glial progenitors.

To ensure that the induction of neuronal phenotypes from NG2+ cells were mediated by EGFR inhibition, we knocked down the expression of EGFR in NG2+ cells by EGFR-specific siRNA based plasmid. The reduction of EGFR expression level was determined by western blot. As shown in Fig. 11C, the level of EGFR was remarkably reduced by more than 90% as compared to control cells. Both EGFR and control siRNA plasmids transfected cells were subjected to the two-phase neuronal differentiation protocol. As shown in Fig. 11A and 11B, the EGFR silencing cells exhibited small cell bodies with extending processes, and expressed distinct level of NF200 and β-tubulin ш. Consistently, RT-PCR results revealed that some more neuronal lineage-specific genes were strongly up-regulated (Fig. 11D), as compared with control cells. These results further verified that EGFR inhibition could induce NG2+ cells to acquire neuronal phenotypes.

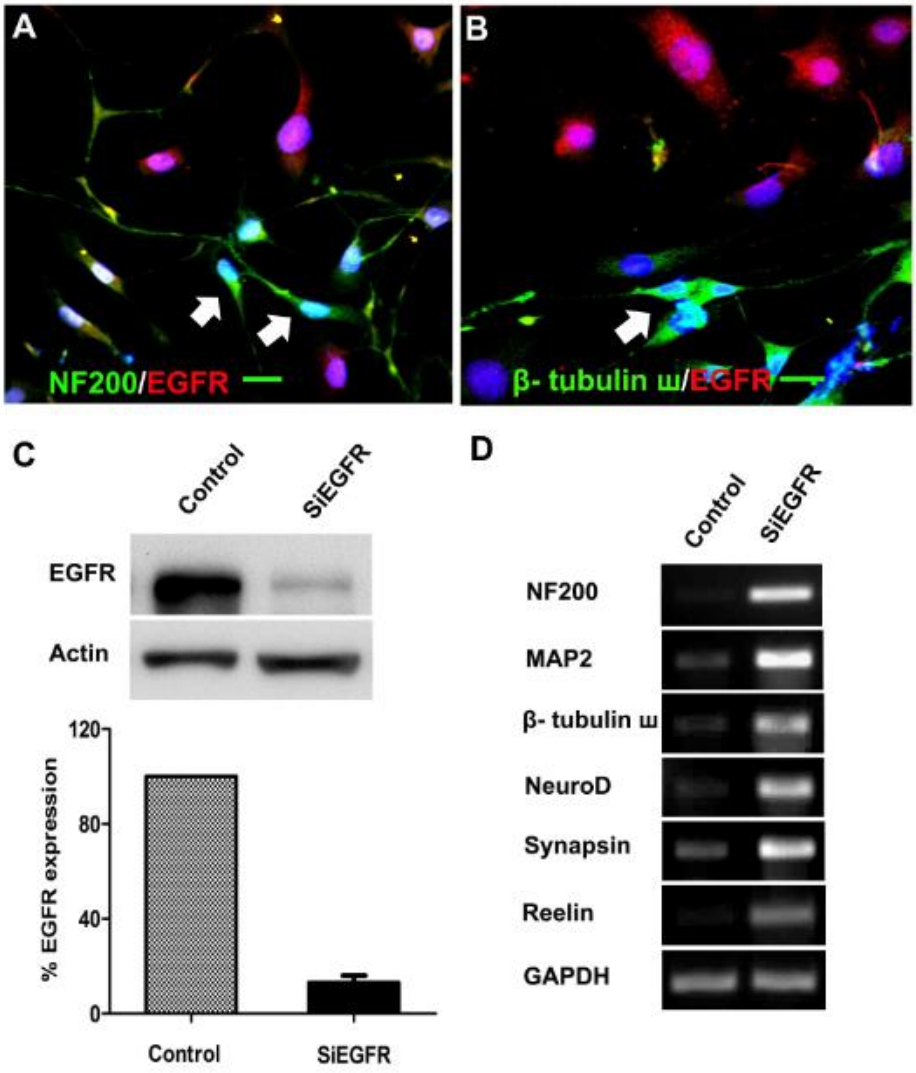


Figure 11 EGFR silencing promotes neuronal phenotypes from NG2+ cells.

(**A**-**B**): Immunocytochemistry revealed that the EGFR (red) silencing cells that underwent neuronal differentiation expressed NF200 (green) and β-tubulin ш (green). The EGFR silencing cells which exhibited neuronal phenotypes were indicated by arrows. Nuclei were counterstained with DAPI in all images. Scale bars: 20 μm. (**C**): NG2+ cells were transfected with either siEGFR or control siRNA plasmids, and analyzed by immunoblotting with anti-EGFR antibody. β-actin was detected as a loading control. Densitometry result showed that the reduced EGFR protein level was more than 90%. (**D**): RT-PCR results revealed that with two-phase differentiation

experiments, expression of several neuronal markers were up-regulated in siEGFR-transfected cells, as compared with control cells.

To further confirm the effect of PD168393 on neuronal phenotypes generation from glial progenitors, another well-established glial progenitor cell line, CG4 cells, was employed. With the treatment of PD168393, some CG4 cells acquired the neuron-like morphology with extending processes. To confirm the acquired neuronal phenotypes, we examined neuronal markers NF200 and MAP2 by immunostaining. As shown in Fig. 12, the immunostaining results demonstrated distinct expression of NF200, MAP2 in PD168393-treated cells. Quantitative analysis revealed that 25.39±3.55%-27.68±5.69% of CG4 cells was differentiated into NF200 and MAP2 positive neurons after 9 days of treatment (Fig. 12). Collectively, these results demonstrated that the EGFR inhibitor could promote the neuronal phenotypes of NG2+ (glial) cells *in vitro*.

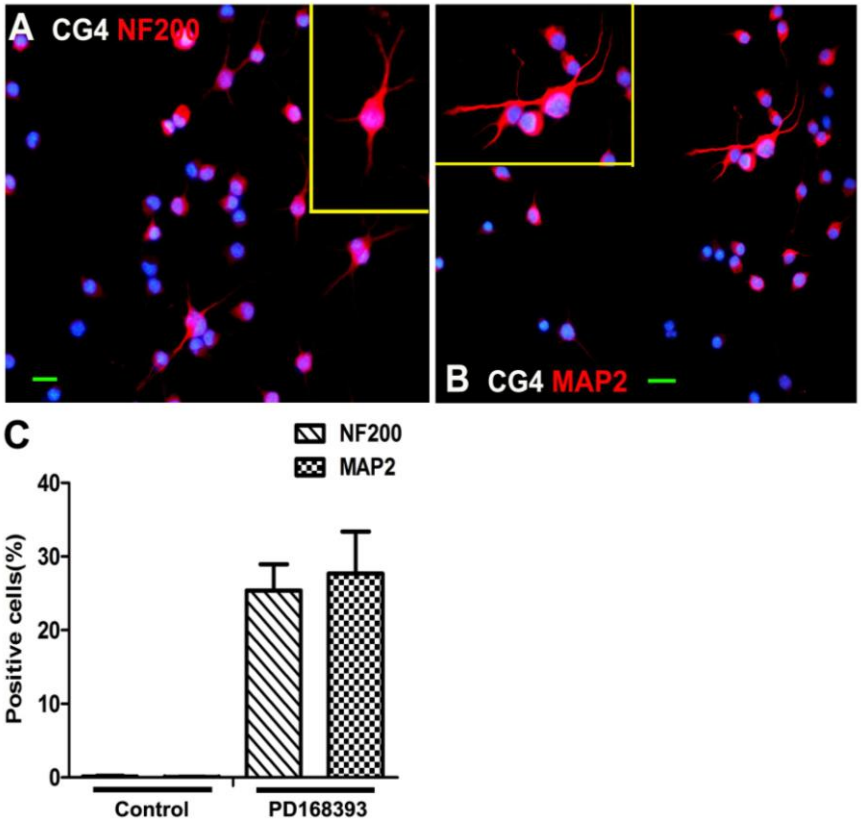


Figure 12 PD168393 induces neuronal phenotypes from CG4 cells *in vitro*.
Treatment with 1 μM PD168393 resulted in detectable levels of NF200 (**A**, red), MAP2 (**B**, red) in CG4 cells. Nuclei were counterstained with DAPI in all images (**C**): Quantification of NF200+ and MAP2+ cells in CG4 cells treated with PD168393. Scale bars: 20 μm

It has been shown that the oligodendrocytes differentiation from CG4 cells occurs after withdrawal of the mitogens in culture medium [194]. In our neuronal differentiation protocol, the mitogens in the culture medium were either substituted for 5%FBS or withdrawn. We therefore wondered if oligodendrocyte differentiation was induced besides the neuronal differentiation of CG4 cells. As shown in Fig. 13, significant CNPase+ oligodendrocytes (CNPase is a myelin associated enzyme and expressed exclusively by oligodendrocytes) were found to be induced in both 5% FBS

group and 5% FBS+PD169393 group, while the MAP2 phenotypic neurons could only be observed in the latter one. Therefore, with the treatment of PD168393, the neuronal phenotypes of CG4 glial progenitors could be induced without perturbing oligodendrocyte genesis.

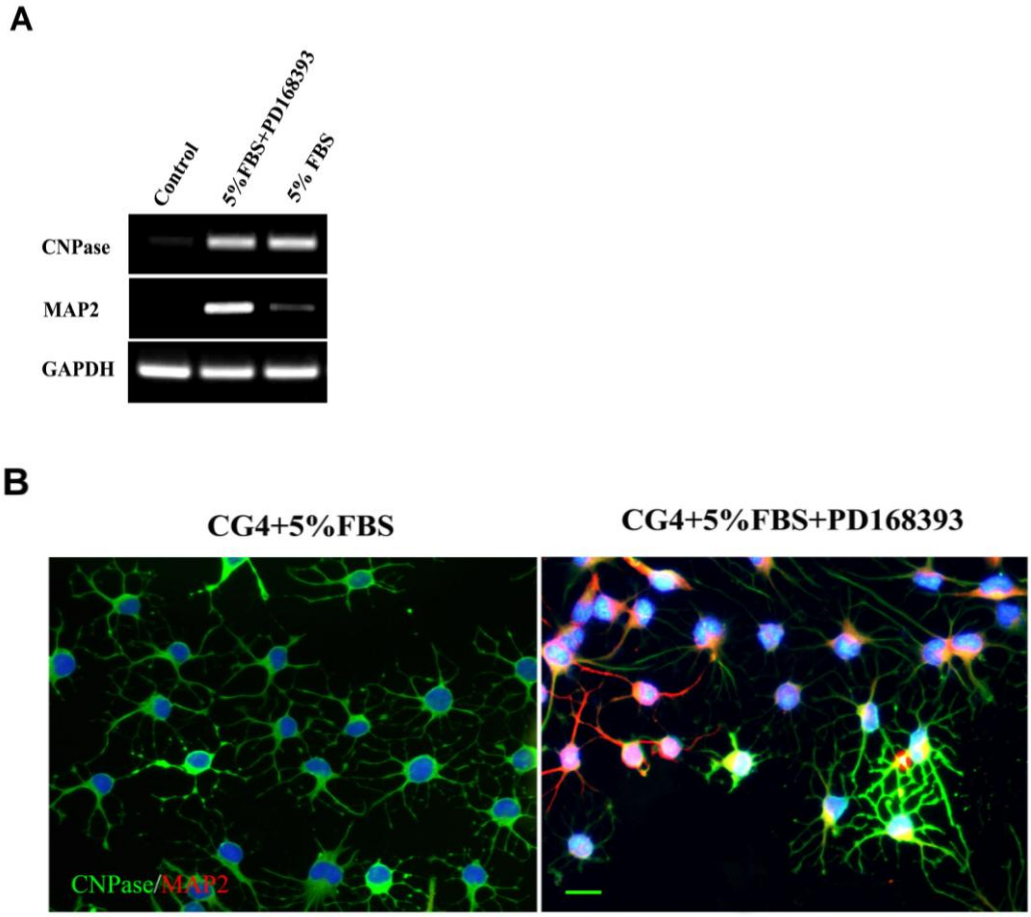


Figure 13 PD168393 promotes neuronal phenotype from CG4 cells without perturbing the oligodendrocyte differentiation.

(**A**): RT-PCR result showed that CNPase was up-regulated in both 5% FBS group and PD168393-supplemented group, while MAP2 was only induced in the latter one. (**B**): Immunocytochemistry results showed that applying PD168393 together with 5% FBS to CG4 cells induced both MAP2+ (red) neuronal phenotype and CNPase+ (green)

oligodendrocytes. Nuclei were counterstained with DAPI in all images. Scale bars: 17 μm.

4.2.2 EGFR inhibitor efficiently reduces the level of phosphorylated EGFR in injured spinal cords of mice and improves the locomotor functional recovery

To determine if EGFR inhibition could induce neurogenesis from endogenous NG2+ cells in CNS trauma and its related therapeutic potential, we established the mouse SCI model with standard contusion method [153]. The gelatin matrix containing PD168393 or PBS as control was implanted into the damaged region of spinal cord immediately post SCI. To evaluate neural functional improvement, we measured the locomotor activity of animals with the BBB scores for 4 weeks [157]. As shown in Fig. 14A, the locomotor activity of animals treated with PD168393 was remarkably improved as compared with the control mice ($P< 0.001$).

To determine the inhibitory efficiency of PD168393, we examined the level of pEGFR by quantifying the number of phosphorylated EGFR expressing cells over 14 days post injury. As shown in Fig. 14D of control group, the pEGFR positive cells increased markedly and peaked at 7 days post injury. However, they were significantly suppressed within the first 3 days after administration of PD168393 (Fig. 14E, Repeated measurement ANOVA, $P<0.001$). Similarly, the cells expressing both pEGFR and NG2 proteoglycan increased with the onset of SCI, which were effectively inhibited by PD168393 in the first week post SCI (Fig. 14B, 14F, 14G, Repeated measurement ANOVA; $P<0.001$ at 3days and $P<0.01$ at 7days). The inhibitory effect

decreased thereafter in PD168393-treated group. Interestingly, NG2+/pEGFR+ cells appeared mainly in the scar area and surroundings, and they exhibited two kinds of morphology (Fig. 14C, C1-C6). One was round with a thin rim of NG2+/pEGFR+ immunopositivity near the cell membrane, indicating a typical macrophage-like morphology. Such profiles were not included in cell counts. The second one had complex process arborizations, reflecting visibly active phenotypes. These results suggested that PD168393 could effectively inhibit the activity of EGFR post SCI, especially in NG2+ cells.

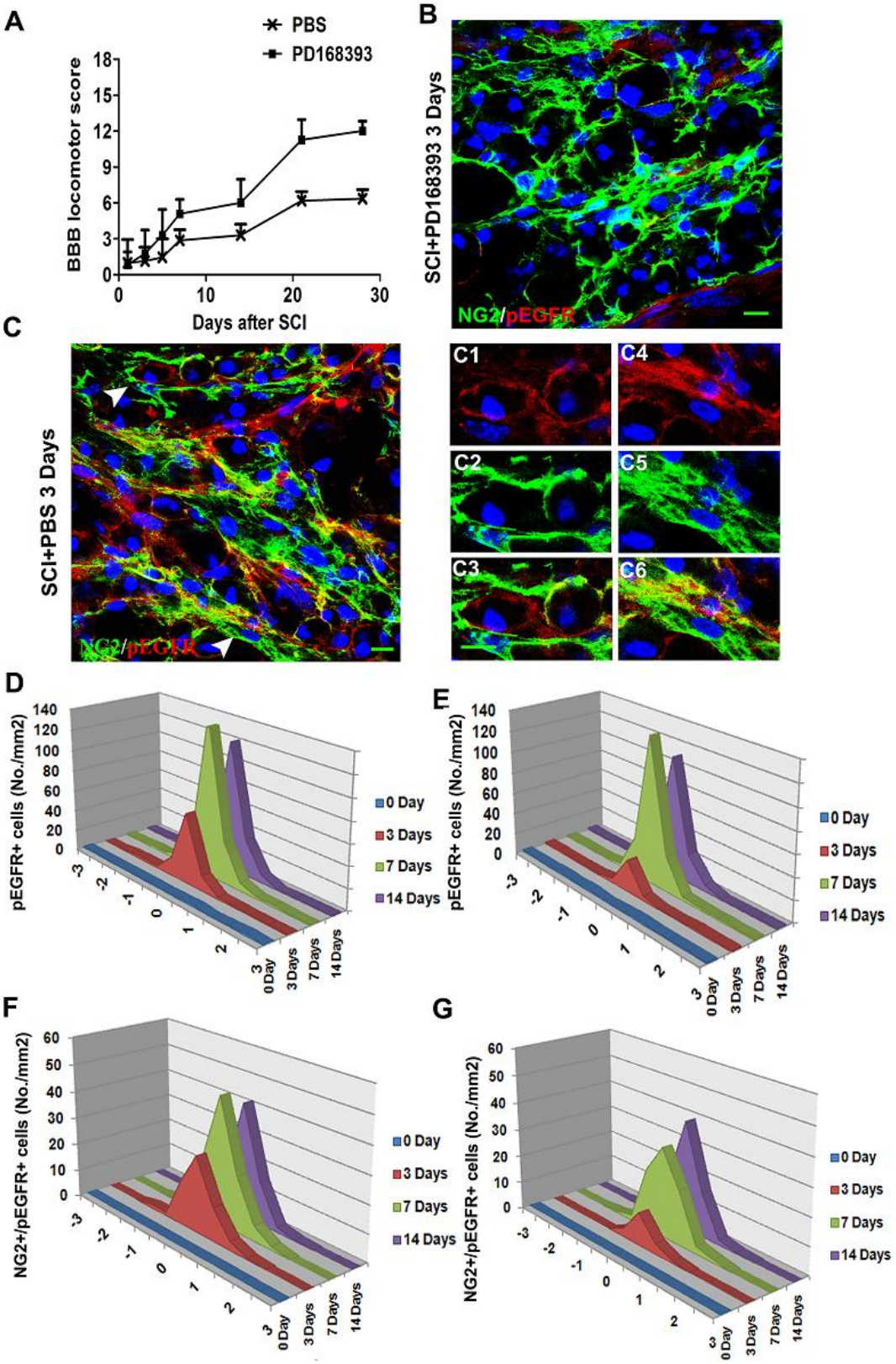

A
PBS
PD168393
BBB locomotor score
Days after SCI
B
SCI+PD168393 3 Days
NG2/pEGFR
C
SCI+PBS 3 Days
NG2/pEGFR
C1
C2
C3
C4
C5
C6
D
pEGFR+ cells (No./mm2)
0 Day
3 Days
7 Days
14 Days
E
pEGFR+ cells (No./mm2)
0 Day
3 Days
7 Days
14 Days
F
NG2+/pEGFR+ cells (No./mm2)
0 Day
3 Days
7 Days
14 Days
G
NG2+/pEGFR+ cells (No./mm2)
0 Day
3 Days
7 Days
14 Days

Figure 14 Effects of PD168393 on locomotor functional recovery and phosphorylated EGFR in injured spinal cords of mice.

(**A**): PD168393 improved locomotor function of mice with SCI. The BBB scores were obtained from 1 day to 4 weeks after injury. Repeated-measurement ANOVA showed the difference between control and treated animals to be significant ($P<0.001$). Immunohistostaining of pEGFR (red) and NG2 proteoglycan (green) at 3 days after SCI of both PD168393-treated (**B**) and control (**C**) groups. Examples of two types of NG2+/pEGFR+ cells were denoted with arrowheads respectively, and shown in panels **C1-C6** of higher magnification. Nuclei were counterstained with DAPI in all images. (**D**)-(**G**): Quantitative analysis of pEGFR immunoreactivity between control (**D**) and PD168393-treated (**E**) groups in 3 days, 1 week and 2 weeks post injury. The number of NG2+/pEGFR+ cells was quantitatively compared between control (**F**) and PD168393-treated (**G**) groups in 3 days, 1 week, and 2 weeks post injury. X-axis: 0 represented the injury epicenter. -3,-2,-1 represented the distance (mm) caudal to the injury epicenter, while 1, 2, 3 represented the distance (mm) rostral to the injury epicenter. Scale bars: 10 μm.

4.2.3 Subpopulations of NG2+ cells acquire neuronal phenotypes via EGFR inhibition

Since we have demonstrated that cultured NG2+ cells could be induced to acquire neuronal phenotypes *in vitro*, we further sought to determine if NG2+ cells *in situ* could acquire neuronal phenotypes by EGFR inhibition. To monitor the new-born neurons, the S-phase marker, BrdU, was used to trace the proliferative cells post injury. Previous studies have shown that BrdU was maximally incorporated on the third day after SCI [119, 120]. Therefore, the BrdU injection was performed on the third day post injury, and the phenotypes of BrdU+ cells in the parenchymal region of spinal cord were analyzed at 2 hours after the BrdU injection. According to our observation,

more than half of proliferative cells were NG2+ cells (NG2+/BrdU+ cells). Other cell types, such as microglias/macrophages (OX42+/NG2-/BrdU+ cells), astrocytes (GFAP+/NG2-/BrdU+ cells), oligodendrocytes (CNPase+/NG2-/BrdU+ cells), as well as few neural stem cells (Nestin+/NG2-/BrdU+ cells) were also observed (Fig. 15). To exclude the possibility that BrdU labeled the existing neurons, we performed immunostaining of spinal cord sections with the neuronal markers of NeuN, β-tubulin ш, MAP2, GABA, SMI32, ChAT and NSE at 2 hours after BrdU injection. None of the BrdU+ cells scanned was found to be immunopositive for any of the above neuronal markers.

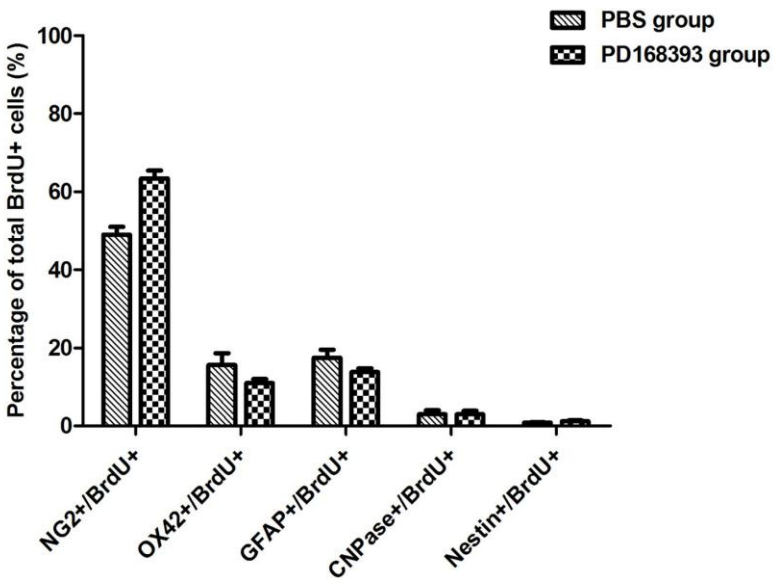


Figure 15 Quantification of proliferative cells in PBS- and PD168393-treated groups at 2 hours after BrdU injection in 3 days post injury.

Numbers of proliferative NG2+ cells, OX42+/NG2- cells, GFAP+/NG2- cells, CNPase+/NG2- cells and Nestin+/NG2- cells were quantified in both groups. Data are expressed as the percentage of classified proliferative cells relative to total proliferative cells.

To determine if proliferative NG2+ cells in the PD168393-treated group could give rise to neuronal progenies, we labelled the sections with antibodies against NeuN, the neuron-specific nuclear protein [195], BrdU and NG2 proteoglycan. Two weeks after injury, we found that some NG2+/BrdU+ cells in PD168393-treated group expressed distinct levels of NeuN in the nucleus (Fig. 16A). This result demonstrated that PD168393 could induce the expression of NeuN in proliferative NG2+ cells. Quantitative analysis further showed that the number of NeuN+/NG2+/BrdU+ cells was 11.95±3.50% in 3054.4 BrdU+ cells at one week post injury. This number decreased but was still significant at three weeks post injury (Table 7). Some BrdU+/NeuN+ neurons with faint NG2 proteoglycan expression or without NG2 proteoglycan expression were also found (Fig. 16A2-A3). Quantitative analysis of these cells showed that the number of NeuN+/NG2-/BrdU+ cells increased to 19.80±4.24% of 1744.2 BrdU+ cells at three weeks post SCI (Table 7). Therefore, the significant number of NeuN+(NG2+)/BrdU+ cells could contribute to the increased new-born neurons in the injured spinal cord of PD168393-treated group. To further verify the neuronal progenies of NG2+ cells, we examined the expression of other neuronal markers β-tubulin ш and MAP2 in NG2+/BrdU+ cells for visualizing neuronal structures. As seen in Fig. 16B, some β-tubulin ш+/NG2+/BrdU+ cells were found in the lesion epicentre at 1-2 weeks post injury, accounting for 4.30±0.63% in 3853.2 BrdU+ cells (Table 7). Occasionally, some β-tubulin ш+/BrdU+ neurons with long axon-like processes (Fig. 16C) were found in the ventral white matter around the lesion center at one week post injury. The number of these cells increased over 3

weeks, accounting for 7.14±0.25% of 2353.2 BrdU+ cells (Table 7). Additionally, a small number of MAP2+/NG2+/BrdU+ cells with faint NG2 staining were also observed at the lesion site (Fig. 16D).

Table 7 Quantification of (NG2+)/BrdU+ neuronal cells in the spinal cord lesion site

Immunoreactivity(-1.5 mm~1.5 mm)	Age of BrdU+ cells		
	2h	1 week-2 weeks	3 weeks
NeuN/NG2	-	11.95±3.50% (3054.4), n=5, 1 week	3.76±1.35% (1702.8), n=5
NeuN	-	8.25±2.67% (3369.6), n=5, 2 weeks	19.80±4.24% (1744.2), n=5
β-tubulin ш/NG2	-	4.30±0.63% (3853.2), n=5, 1 week	+/-
β-tubulin ш	-	+/-	7.14±0.25% (2353.2), n=5
MAP2	-	+, with faint NG2 expression	+/-
NSE/NG2	-	+/-	+/-
NSE	-	+	13.30±1.11% (3366.6), n=5
GABA/NG2	-	7.11±1.76% (2853.2), n=5, 1 week	+/-
GABA	-	+/-	14.64±1.42% (1562.2), n=5
ChAT/NG2	-	+/-	+/-
ChAT	-	-	+/-
SMI32/NG2	-	5.19±1.81% (3409.6), n=5, 1 week	+/-
SMI32	-	-	6.19±1.22% (1824.2), n=5

Mean % BrdU+ cells ±SD, the number of cells in category divided by number of BrdU+ cells analyzed;

n, the number of analyzed spinal cord samples;

+, some cells of this type observed; -, not observed; +/-, very few observed

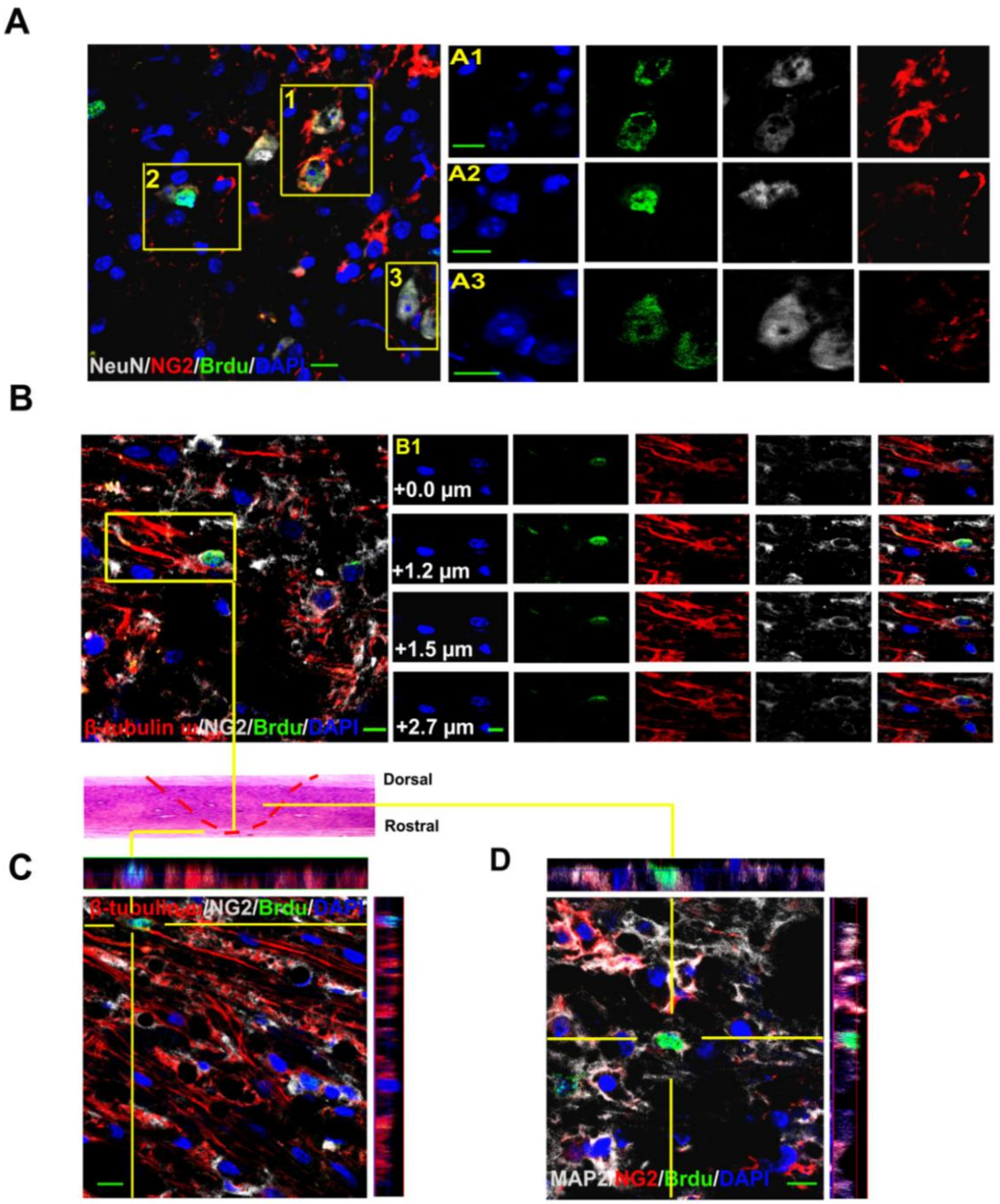


Figure 16 PD168393 induces new-born (NG2+) cells to express neuronal specific makers post SCI.

(**A**): 2-week-old NeuN+/(NG2+)/BrdU+ cells were induced in PD168393-treated group. (**A1**): NeuN immunoreactivity was detected in the nucleus of NG2+/BrdU+ cells. (**A2**): A 2-week-old BrdU+ cell labeled with NeuN and faint NG2 proteoglycan (**A3**): 2-week-old BrdU+ cells labeled with NeuN. (**B**): 1-2-week-old NG2+/BrdU+

cells were immunoreactive for β-tubulin ш. Color separation in higher magnification views of (**B1**) demonstrated the co-localization of BrdU, β-tubulin ш and NG2 proteoglycan. Note that the BrdU, β-tubulin ш and NG2+ proteoglycan expression peaked in brightness at the same level (z=1.2 μm). (**C**): 1-2-week-old (NG2-) BrdU+ cell labeled with β-tubulin ш was found in the white matter nearby the lesion center, as shown in the orthogonal view. (**D**): MAP2+/NG2+/BrdU+ cells were shown in the orthogonal view. Nuclei were counterstained with DAPI in all images. The cell locations shown in Fig. 16 were directed in the small diagram of a sagittal section, and the analyzed region included the injured area circled with a red dashed curve. Images showed the single focal planes (**A**) or z-axis projections of 20×0.3 μm (**B**), i.e., 20 planes, 0.3 μm apart (**B1**), 14×0.46 μm (**C**), 16×0.38 μm (**D**). Scale bars: 10 μm.

We observed some BrdU+/GFAP+ cells post injury (Fig. 17). Since GFAP+ cells have recently been shown to produce neuronal cells [196-198], we wondered if these cells contributed to the proliferative neuronal cell pool in our experiment. With the immunostaining of antibodies against GFAP, BrdU and NeuN, we did not observe any GFAP+/BrdU+/NeuN+ cells in both control and PD168393-treated groups (Fig. 17), suggesting that PD168393 treatment may not affect the neuronal differentiation from GFAP+ cells in SCI.

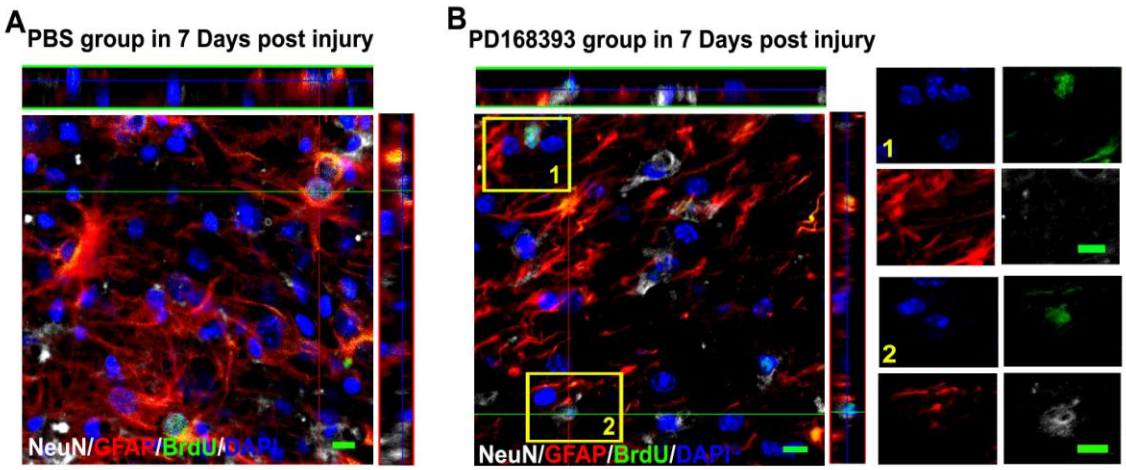


Figure 17 GFAP+/BrdU+ cells do not generate NeuN+ neuronal phenotype in both PBS- and PD168393-treated groups.

(**A**): GFAP+/BrdU+ cells in the PBS group were not immunopositive for NeuN. (**B**): GFAP+/BrdU+ cells in the PD168393-treated groups were not immunopositive for NeuN either. Examples of the GFAP+/BrdU+, NeuN+/BrdU+ cells were boxed, and shown in the higher magnification of (**1**) and (**2**). Nuclei were conterstained with DAPI in all images. Images showed z-axis projections of 20×0.48 μm (**A**), 17×0.56 μm (**B**), Scale bars: 10 μm.

The contusive spinal cord injury led to the cellular loss that destructed the descending motor and ascending sensory tracts. Since the PD168393 treatment benefits the locomotor recovery, we sought to investigate if NG2+ cells could give rise to motor neuronal phenotypes. The mature motor neuronal markers: SMI32 and ChAT were examined. As shown in Fig. 18C and 18D, we detected some SMI32+/NG2+/BrdU+ cells and few ChAT/+NG2+/BrdU+ cells in the ventral horn of injured region at one week time point. About 5.19±1.81% SMI32+/NG2+/BrdU+ cells in 3409.6 BrdU+ cells and a few ChAT+/NG2+/BrdU+ cells were observed therein (Table 7). With EGFR inhibition, sensory functions are also greatly improved from experimental SCI

[199]. However, the cellular contribution for this recovery was not clear. Studies provide evidence indicating that changes in the spinal GABAergic system contribute to the chronic pain after SCI [200]. As the restoration of GABAergic system through transplantation is demonstrated to attenuate the tactile hypersensitivity following SCI [201], we therefore examined if the NG2+/BrdU+ cells could generate GABAergic neurons *in situ* with the treatment of PD168393. As shown in Fig. 18A and 18B, we visualized that some NG2+/BrdU+ cells in the lesion area expressed GABA. Quantitative analysis in Table 7 revealed that there were 7.11±1.76% GABA+/NG2+/BrdU+ cells in 2853.2 BrdU+ cells at one week post SCI. These results indicated that NG2+ cells post injury could express both motor and sensory neuronal markers by EGFR inhibition. Additionally, we observed that plenty of BrdU+ cells with faint or without NG2 proteoglycan expression were immunopositive for each of the following functional neuronal markers: GABA, NSE, SMI32, ChAT (Fig. 19A-19D) in the lesion region of PD168393-treated group at three weeks post injury. However, in the parallel experiment, we did not observe any BrdU+ neuronal phenotypic cells in PBS group (Fig. 20, Fig. 21). Taken together, these results demonstrated that inhibition of EGFR could induce neurogenesis post SCI, which was probably from the proliferative NG2+ cells.

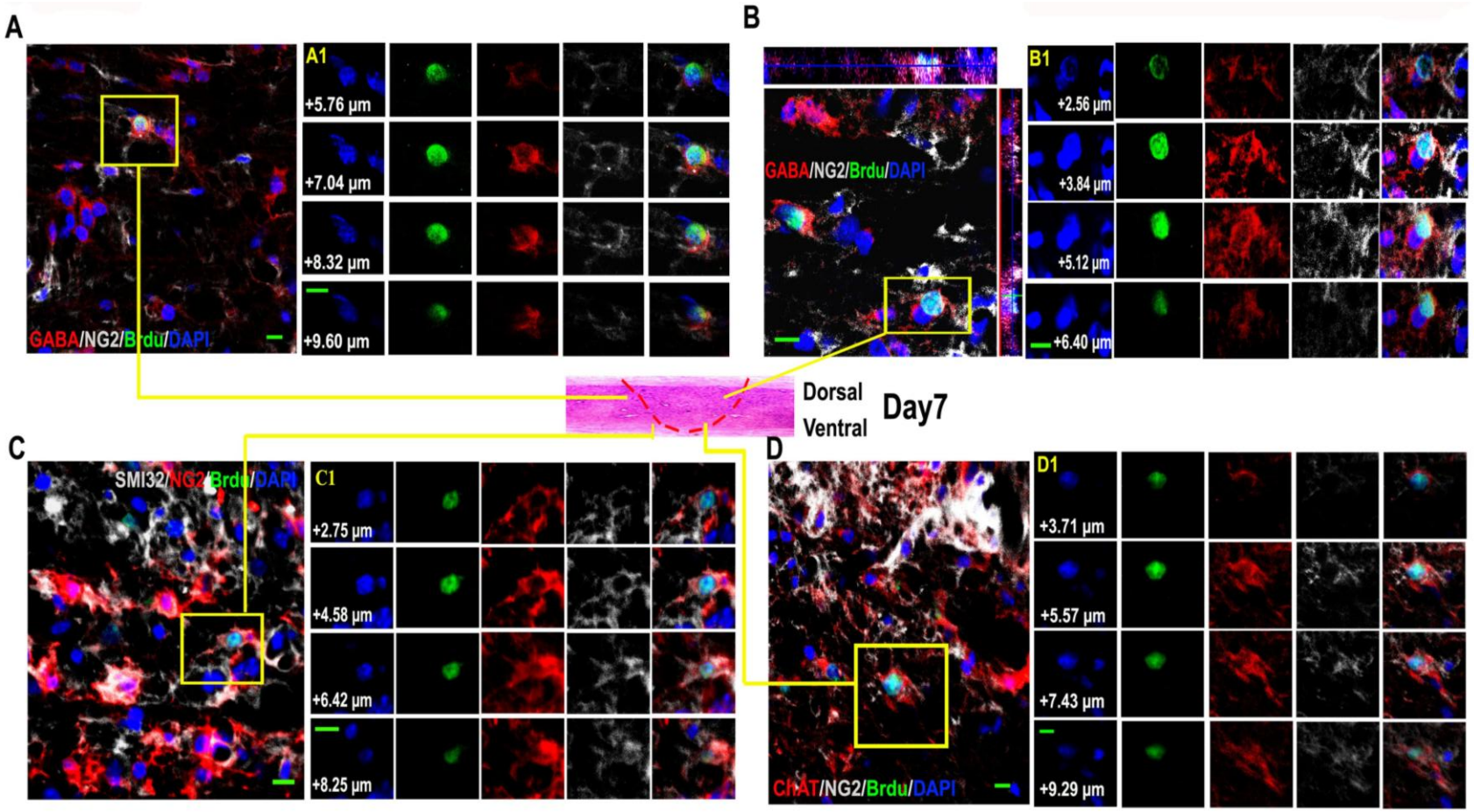

A
A1
+5.76 μm
+7.04 μm
+8.32 μm
+9.60 μm
GABA/NG2/Brdu/DAPI
B
B1
+2.56 μm
+3.84 μm
+5.12 μm
+6.40 μm
GABA/NG2/Brdu/DAPI
Dorsal
Ventral
Day7
C
SMI32/NG2/Brdu/DAPI
C1
+2.75 μm
+4.58 μm
+6.42 μm
+8.25 μm
D
D1
+3.71 μm
+5.57 μm
+7.43 μm
+9.29 μm
ChAT/NG2/Brdu/DAPI

Figure 18 PD168393 induces new-born NG2+ cells to express functional neuronal markers post SCI.

1-week-old GABA+/NG2+/BrdU+ cells were found in the surroundings (**A**) and lesion center (**B**). Higher magnification views of z-stacks were shown in (**A1**) and (**B1**), depicting that GABA staining was observed in cell bodies and processes of NG2+/BrdU+ cells, and the intensity of GABA peaked together with NG2 proteoglycan and BrdU. In the ventral grey matter, 1-week-old SMI32+/NG2+/BrdU+ (**C**) and ChAT+/NG2+/BrdU+ cells (**D**) were observed. NG2 immunoreactivity in the cell body of BrdU+/SMI32+ and BrdU+/ChAT+ cells appeared to be correlated with the intensity of SMI32 or ChAT staining, as revealed in color separation (**C1**, **D1**). Nuclei were counterstained with DAPI in all images. The cell locations in Fig. 18 were directed in the small diagram of a sagittal section, and the analyzed region included the injured area circled with a red dashed curve. Images showed z-axis projections of 17×0.60 μm (**A1**), 16×0.60 μm (**B1**), 12×0.92 μm (**C1**), 14×0.93 μm (**D1**). Scale bars: 10 μm.

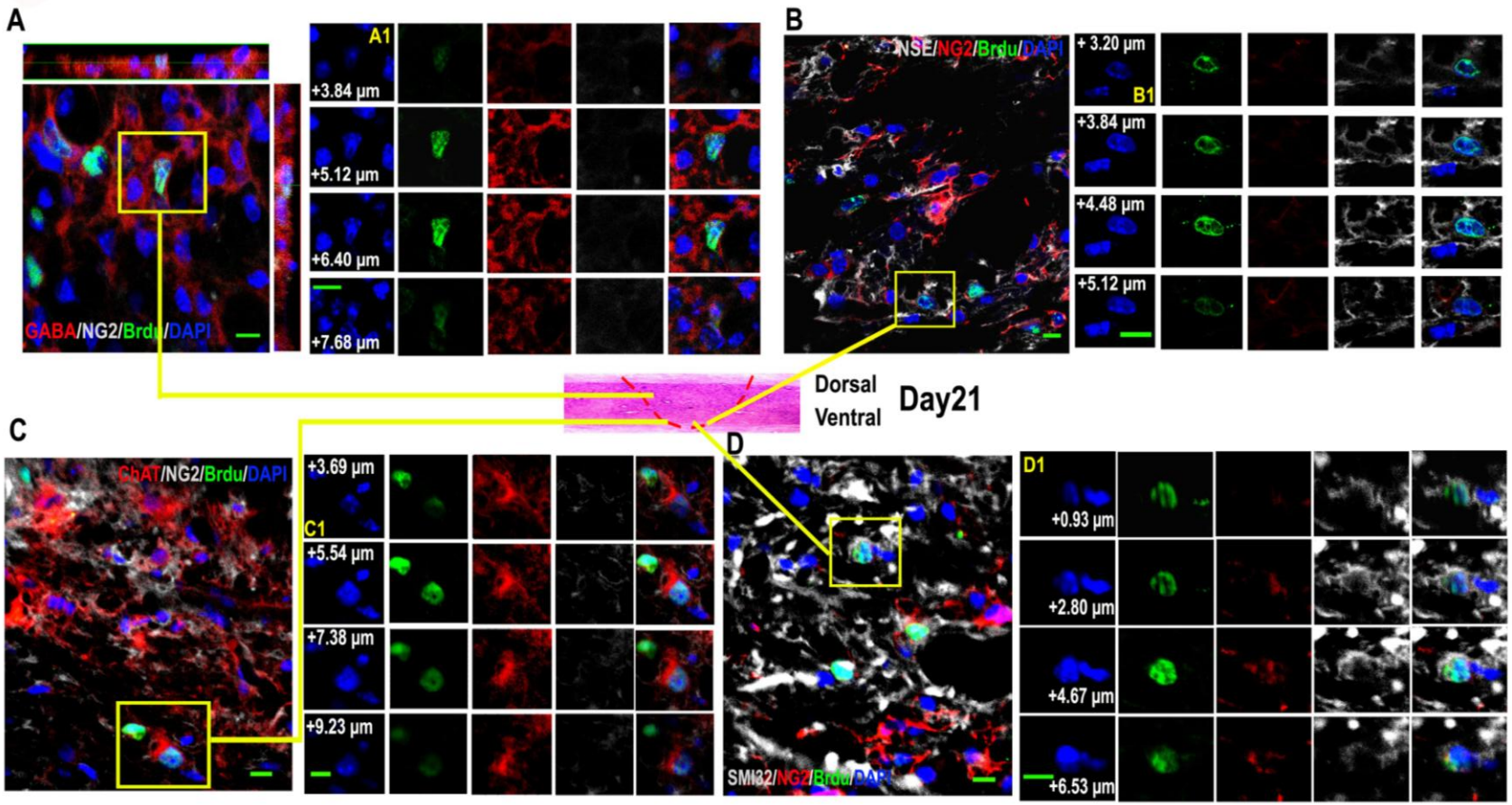

A
A1
+3.84 μm
+5.12 μm
+6.40 μm
+7.68 μm
GABA/NG2/Brdu/DAPI
B
NSE/NG2/Brdu/DAPI
+ 3.20 μm
B1
+3.84 μm
+4.48 μm
+5.12 μm
Dorsal
Ventral
Day21
C
ChAT/NG2/Brdu/DAPI
+3.69 μm
C1
+5.54 μm
+7.38 μm
+9.23 μm
D
SMI32/NG2/Brdu/DAPI
D1
+0.93 μm
+2.80 μm
+4.67 μm
+6.53 μm

Figure 19 PD168393 induces new-born functional neuronal cells in 3 weeks post SCI.

As shown in orthogonal view of panel (**A**) and color separation in (**A1**), 3-week-old GABA+/BrdU+ cells were found inside the lesion region. 3-week-old NSE+/BrdU+ cells were found within the box in the multi-channel view of (**B**) and analyzed in higher magnification images of color separation view (**B1**). Sample z-planes through the box area in (**C**) were shown in color separation of **C1**, demonstrating the co-expression of ChAT and BrdU. Note that the BrdU and ChAT expression both peaked in brightness at the same level (z=5.54 μm). A SMI32+/BrdU+ neuron was seen near the border of grey matter (**D**), as the boxed cell shown in **D1**. Nuclei were counterstained with DAPI in all images. The cell locations in Fig. 19 were directed in the small diagram of a sagittal section, and the analyzed region included the injured area circled with a red dashed curve. Images showed z-axis projections of 19×0.61 μm (**A1**), 13×0.59 μm (**B1**), 13×0.92 μm (**C1**), 15×0.93 μm (**D1**). Scale bars: 10 μm.

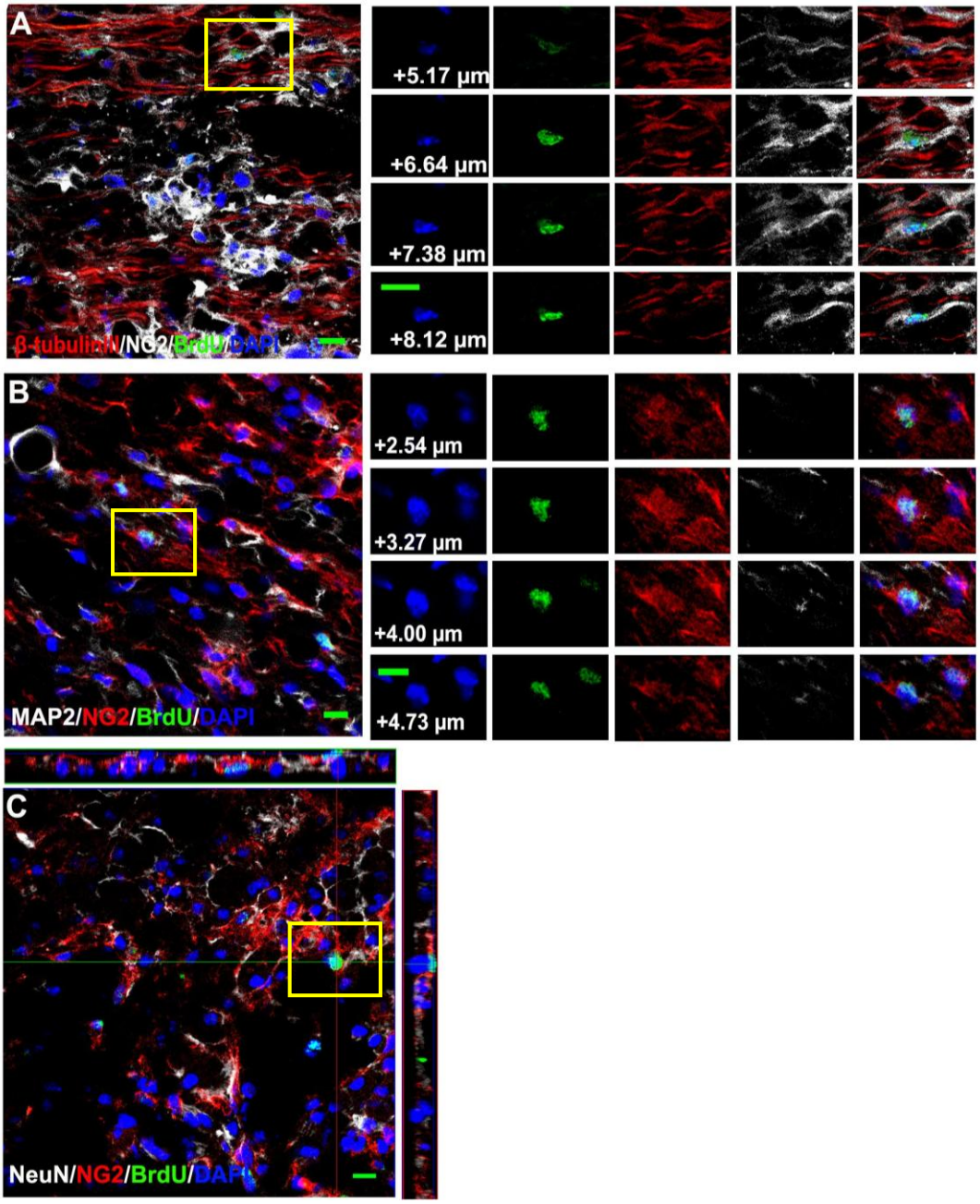


Figure 20 BrdU+ cells in PBS group were not immunopositive for β-Tubulin ш (A), MAP2 (B), NeuN (C).

Nuclei were counterstained with DAPI in all images. Images showed z-axis projections of 35×0.37 μm (**A**), 23×0.36 μm (**B**), 32×0.37 μm (**C**). Scale bars: 10 μm.

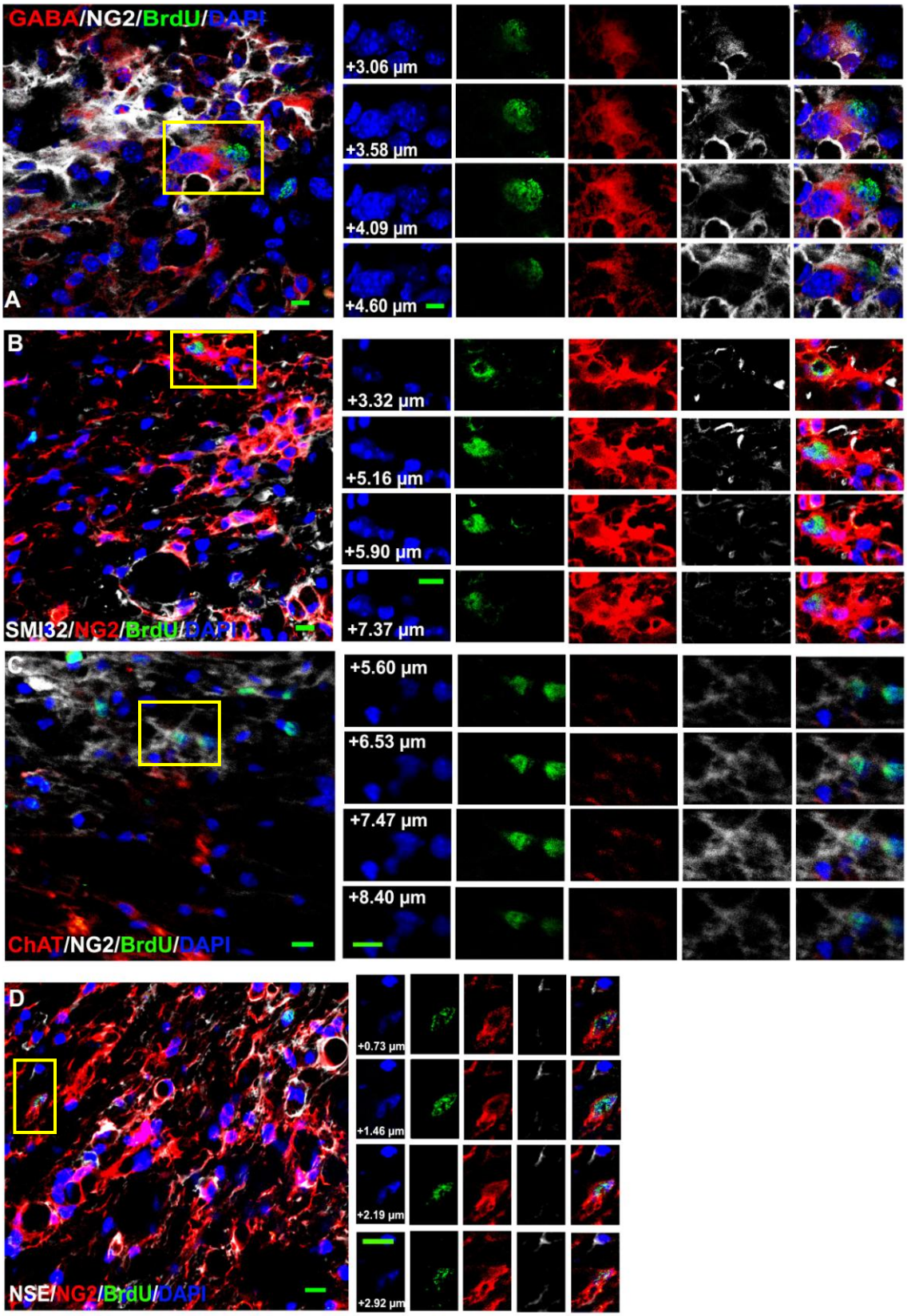

GABA/NG2/BrdU/DAPI
A
+3.06 µm
+3.58 µm
+4.09 µm
+4.60 µm
B
SMI32/NG2/BrdU/DAPI
+3.32 µm
+5.16 µm
+5.90 µm
+7.37 µm
C
ChAT/NG2/BrdU/DAPI
+5.60 µm
+6.53 µm
+7.47 µm
+8.40 µm
D
NSE/NG2/BrdU/DAPI
+0.73 µm
+1.46 µm
+2.19 µm
+2.92 µm

Figure 21 BrdU+ cells in PBS group were not immunopositive for GABA (A), SMI32 (B), ChAT (C), and NSE (D).

Nuclei were counterstained with DAPI in all images. Images showed z-axis projections of 14×0.51 μm (**A**), 33×0.37 μm (**B**), 15×0.93 μm (**C**), 26×0.37 μm (**D**). Scale bars: 10 μm.

4.2.4 EGFR inhibition promotes neuronal phenotypes from transplanted NG2+ cells in the post-injury niche

We observed the significant numbers of NeuN+-, β-tubulin ш+-, GABA+- and SMI32+- NG2+/BrdU+ cells in PD168393-treated groups (Table 7), which suggests that these new-born neurons might differentiate from NG2+/BrdU+ progenitors. To explore the direct evidence that EGFR inhibition could induce the NG2+ cells to adopt neuronal phenotypes in the injured niche, we performed transplantation experiments of NG2+ cells in the acute phase of SCI. Four weeks post SCI, some transplanted NG2+ (GFP+) cells were found to express MAP2, β-tubulin ш and NF200 (Fig. 22A-22D) in both the rim region and center of injury site in PD168393-treated group. Moreover, there were numbers of GFP+ cells co-expressing functional neuronal markers such as GABA and SMI32 in the surroundings of lesion site by EGFR inhibition (Fig. 22E, 22F). The *ex vivo* transplantation results complementarily demonstrated that the NG2+ cells were able to differentiate into neuronal cells in the injured niche by local delivery of EGFR inhibitor PD168393.

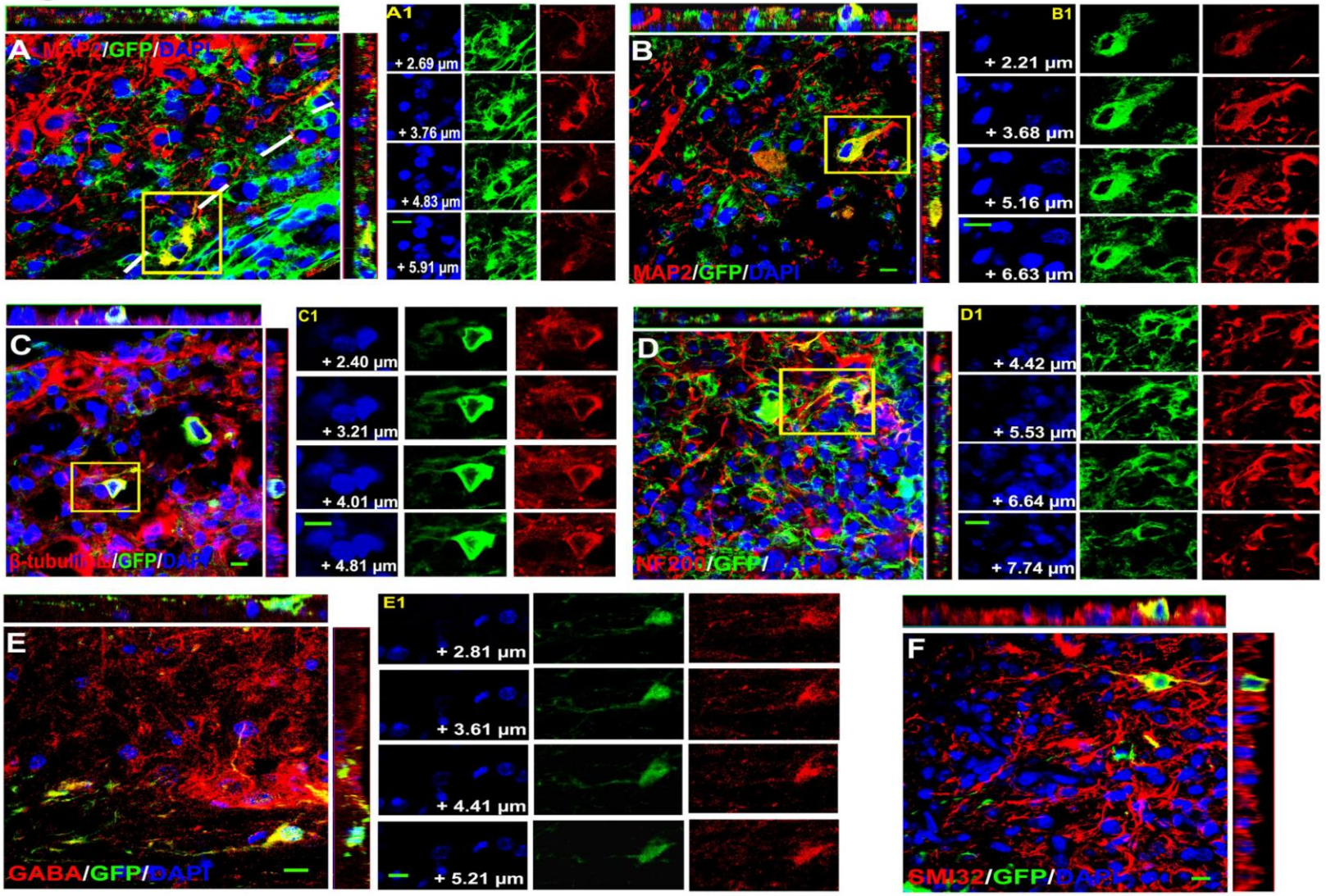

A
MAP2/GFP/DAPI
A1
+ 2.69 μm
+ 3.76 μm
+ 4.83 μm
+ 5.91 μm
B
MAP2/GFP/DAPI
B1
+ 2.21 μm
+ 3.68 μm
+ 5.16 μm
+ 6.63 μm
C
β-tubulin III/GFP/DAPI
C1
+ 2.40 μm
+ 3.21 μm
+ 4.01 μm
+ 4.81 μm
D
NF200/GFP/DAPI
D1
+ 4.42 μm
+ 5.53 μm
+ 6.64 μm
+ 7.74 μm
E
GABA/GFP/DAPI
E1
+ 2.81 μm
+ 3.61 μm
+ 4.41 μm
+ 5.21 μm
F
SMI32/GFP/DAPI

Figure 22 PD168393 promotes transplanted NG2+ cells to acquire neuronal phenotypes in the post-injury niche.

Four weeks post injury, neuronal phenotypes of transplanted NG2+ cells by EGFR inhibition were assessed by co-localization of EGFP (green) and neuronal markers (red). (**A**-**B**)**:** Immunoreactivity of MAP2. Color separation in higher magnification showed that the expression intensity of GFP and MAP2 correlated and peaked simultaneously, z=3.76 μm in **A1** and z=3.68 μm in **B1**. (**C**): Immunoreactivity of β-tubulin ш. High magnification view of selected individual cell z-planes was shown in **C1**. (**D**): Immunoreactivity of NF200. Higher magnification views demonstrated that co-localization of GFP and NF200 peaked at z=6.64 μm (**D1**). (**E**): Some GFP+/GABA+ neurons were also found. In the higher magnification of color and z-plane separations, the intensity of GFP and GABA immunoreactivity resembled each other in individual z-planes, **E1**. (**F**): Immunoreactivity of GFP and SMI32 in orthogonal view. (**A**): white dashes bordered the area of injury site. (**B**, **D**): Injury center and (**C**, **E**, **F**): nearby region and surroundings. Nuclei were counterstained with DAPI in all images. Images showed z-axis projections of 25×0.54 μm (**A**), 20×0.74 μm (**B**), 22×0.40 μm (**C**), 23×0.55 μm (**D**), 26×0.40 μm (**E**), 23×0.40 μm (**F**). Scale bars: 10 μm.

We further examined the double staining of MAP2/GFP, β-Tubulin ш /GFP, NF200/GFP, GABA/GFP and SMI32/GFP in control group in four weeks post SCI. In contrast, none of the GFP+ cells were found to express any of the above neuronal markers (Fig. 23). Some GFP+ cells in the control group have a greater number of tertiary branches and the extensive arborization of processes. Additionally, the presence of spines gave them a bushy, fuzzy appearance (Fig. 23A). Others showed close spatial relationships with neurons, surrounding or wrapping the neuronal cell bodies (Fig. 23C, 23E, 23G) and axons (Fig. 23H).

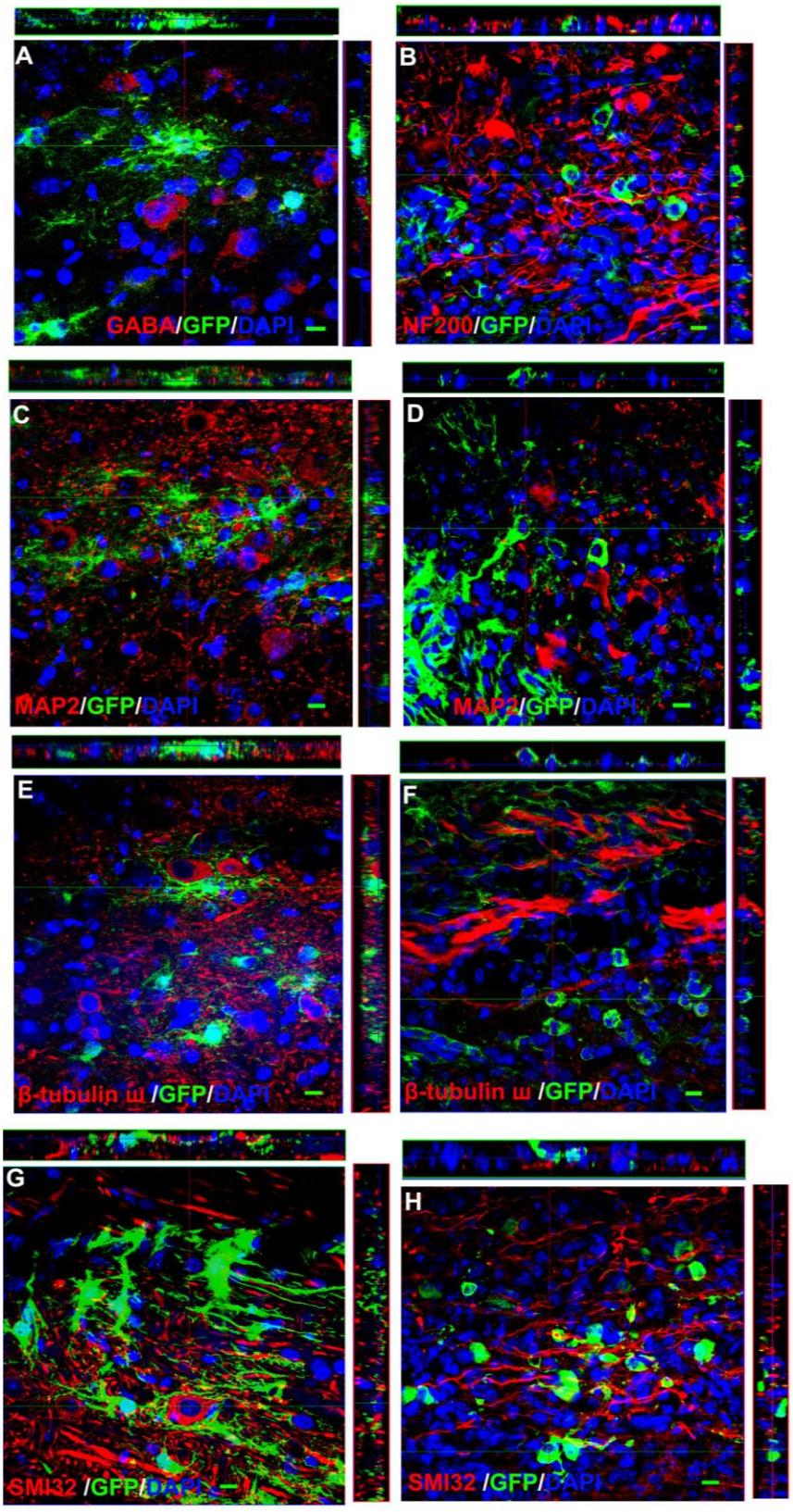

A
GABA/GFP/DAPI
B
NF200/GFP/DAPI
C
MAP2/GFP/DAPI
D
MAP2/GFP/DAPI
E
β-tubulin ш /GFP/DAPI
F
β-tubulin ш /GFP/DAPI
G
SMI32 /GFP/DAPI
H
SMI32 /GFP/DAPI

Figure 23 Transplanted NG2+ (GFP+) cells in PBS group were not immunopositive for GABA (A), NF200 (B), MAP2 (C, D), β-Tubulin ш (E, F), SMI32 (G, H) in the orthogonal view.

Nuclei were counterstained with DAPI in all images. Images showed z-axis projections of 27×0.35 μm (**A**), 31×0.35 μm (**B**), 30×0.34 μm (**C**), 33×0.34 μm (**D**), 29×0.35 μm (**E**), 28×0.35 μm (**F**), 34×0.39 μm (**G**), 35×0.34 μm (**H**). Scale bars: 10 μm.

4.2.5 The Ras/ERK pathway contributes to the induced neuronal phenotypes of NG2+ cells by EGFR inhibition

In order to determine the mechanism underlying EGFR inhibition-induced neuronal phenotypes, we examined the activities of the major kinases in EGFR downstream pathways *in vitro*. As shown in Fig. 24A, we found the phosphorylation of Ras and MEK being significantly attenuated by PD168393. A similar inhibition pattern was also observed on kinases immediate downstream of MEK, including ERK1/2 and p90RSK. However, other examined downstream kinases, such as phospholipase Cγ (PLCγ), mitogen-activated protein kinase kinase 7 (MKK7), c-Jun N-terminal kinases (JNK), Src homology and collagen (SHC), protein kinase B (PKB, AKT) and glycogen synthase kinase-3β (GSK-3β), were not obviously affected. To determine if PD168393-mediated inhibition of Ras/ERK pathway is involved in neuronal differentiation from NG2+ cells, we applied Ras inhibitor (FTI277) or MEK inhibitor (U0126) in the two-phase differentiation experiment respectively. After 6-7 days, the treated cells exhibited neuron-like morphology, similar to those treated by PD168393. NF200 expression was induced, as shown by RT-PCR (Fig. 24B-1) and immunostaining (Fig. 24B-2). Quantitative analysis showed that the numbers of NF200+ cells in FTI 277- and

U0126-treated group were 76.86% and 57.67% respectively of those in PD168393-treated group (Fig. 24D). To further validate the crucial role of Ras/ERK molecules in the neuronal differentiation of NG2+ cells, we transfected them with a constitutively active form of Ras, followed by treatment with PD168393. As shown in Fig. 24C, the expression of NF200 was decreased by transfection of active Ras, as compared with the control vector. Immunostaining experiments further revealed that none of the cells harboring the plasmid (Fig. 24C lower panel, myc, green color) acquired neuronal morphology and expressed NF200. Together, these findings demonstrated that Ras/ERK pathway played a crucial role in the PD168393-mediated neuronal phenotypes from NG2+ cells.

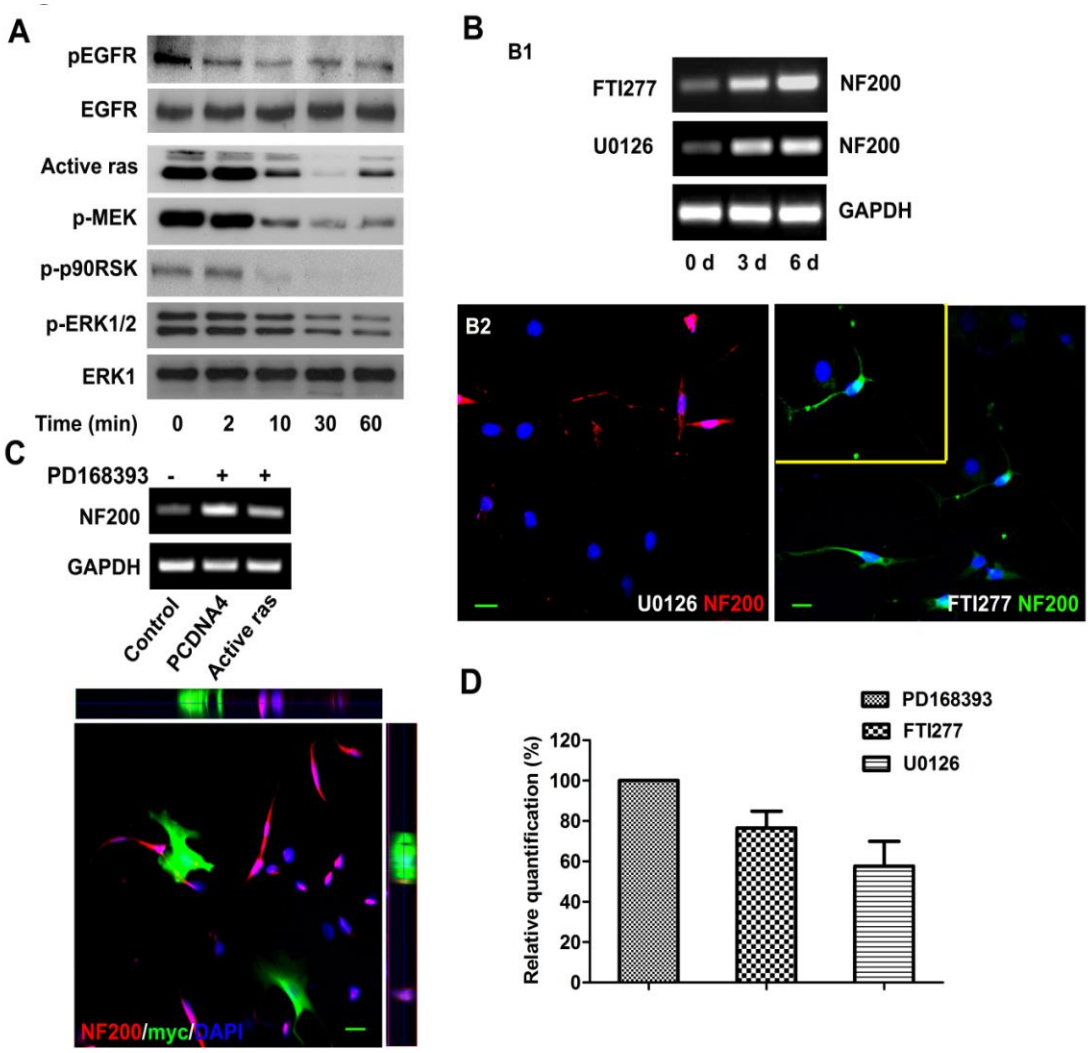


Figure 24 The induced neuronal phenotypes of NG2+ cells by PD168393 are associated with reduction of Ras/ERK signaling.

(**A**): Reduced phosphorylation of EGFR downstream molecules in PD168393-treated NG2+ cells at different time points were screened by immunoblotting. (**B**): Ras-MEK inhibition induced neuronal phenotype of NG2+ cells. RT-PCR (**B1**) and immunostaining (**B2**) results showed the induced expression of NF200 in NG2+ cells by Ras inhibitor FTI-277 and MEK inhibitor U0126. (**C**): Over-expression of active Ras blocked the induction of neuronal phenotype in NG2+ cells by PD168393. The expression of transfected active Ras was indicated by myc-fusion protein (Green) and NF200 immunoreactivity (red) was only found in some non-transfected cells. Nuclei

were counterstained with DAPI in all images. (**D**): Numbers of NF200+ cells in glial cultures with the treatment of Ras or MEK inhibitor were quantified. Scale bars: 20 μm.

4.3 Discussion

Glial cells are recently demonstrated to be able to differentiate into neuronal lineages [202] and participate in the neuronal replacement in CNS injuries [203]. These findings shed new light on the endogenous neurogenesis from glial cells for the treatment of CNS injury. Here we provided evidence for the first time that in mouse spinal cord injury, NG2+ cells could be manipulated to acquire neuronal phenotypes by inhibiting EGFR signaling, which correlated with remarkable locomotor improvement. We further found that Ras-ERK signaling pathway played an important role in EGFR inhibition-mediated neurogenesis of NG2+ cells.

The glial versus neuronal determinant function of EGFR on neural stem cells is previously demonstrated in developmental CNS, as its up-regulation during embryogenesis pushes neural stem cells into glial lineage at the expense of neurogenesis [186, 204]. In our study, the highly activated EGFR signaling post SCI [199, 205], which is considered as one of gliogenic signalings for NG2+ cells [189], was significantly suppressed by PD168393. Under the circumstance, some NG2+ cells were found to acquire neuronal phenotypes with the inhibition of EGFR activity. This finding is consistent with several previous studies that suppression of the gliogenic determinants allows the progression of neural stem cells toward a neurogenic fate in

the CNS injury [203, 206, 207]. It further augments the therapeutic application of NG2+ cells for endogenous neuronal replacement.

Although the proliferation and migration of neural stem cells in the central canal of spinal cord during injuries are well studied [47, 208], new-born neurons from neural stem cells post SCI are very limited and vulnerable [209, 210]. Moreover, recent data reveal that these ependymal stem cells eventually lose their proliferative capacity during their migration to the lesion site [211, 212]. Therefore, their contribution to neurogenesis in various spinal cord disorders has been in doubt. On the other hand, the proliferative neural stem cells (BrdU+/Nestin+/NG2-) in the parenchymal region might rarely exist following SCI [133]. In our study, they only accounted for less than 1% of the total proliferating cells at 2 hours after BrdU injection at 3 days post injury (Fig. 15). Therefore, neural stem cells in the parenchymal regions of spinal cord were not labeled with BrdU efficiently, and were unlikely to contribute to the significant numbers of new-born neurons in the parenchymal spinal cord, which were shown to be NeuN+/BrdU+, β-tubulin ш+/BrdU+, GABA+/BrdU+, NSE+/BrdU+ and SMI32+/BrdU+ cells at 21 days post injury. This result suggested the endogenous neuronal replacement could be promoted by EGFR inhibition. However, it is next-to-impossible that these new-born neurons were generated from neural stem cells with limited proliferative capacity in parenchymal spinal cord.

Additionally, both *in vitro* and *in vivo* studies also reveal that the GFAP+ cells could differentiate into neurons [196-198], and GFAP+ astroglias can resume proliferation after SCI. Hence the reactive GFAP+ cells might be another cell source for the BrdU+ neurons promoted via EGFR inhibition in three weeks post injury. However, evidence appears to weaken this hypothesis: one was that we failed to detect any GFAP+/BrdU+ neuronal cells at one week post SCI, whilst quite a number of NG2+/BrdU+ neuronal cells were discovered in the PD168393-treated group. The other was that PD168393 treatment was found to suppress the number of (proliferative) GFAP+ cells after injury in this study and others [205]. This would greatly weaken the possibility that BrdU+ neurons were generated from GFAP+ cells. Although it can not be completely excluded that some new-born neurons were derived from GFAP+ cells, our results could be interpreted such that EGFR inhibition could mediate some neurogenic properties of glial cells upon injury, especially in NG2+ cells.

Furthermore, the co-expression of mature neuronal markers (NeuN, β-tubulin ш, GABA, SMI32, ChAT, and MAP2) and NG2 proteoglycan in some cells undergoing mitosis suggested that these intermediate neurons may be generated from NG2+ cells. In line with our study, the proliferative NG2+ cells in adult neocortex and striatum have also been demonstrated to display an intermediate neuronal phenotype [79]. In order to confirm the specificity of BrdU labelling the proliferative cells and to exclude the possibility of BrdU incorporation into the damaged neuronal cells, neurogenesis from NG2+ cells was demonstrated independently through our cell transplantation experiments. The transplanted NG2+ cells could acquire several neuronal phenotypes

by EGFR inhibition at four weeks post SCI. Overall, attenuating the EGFR signaling in injured spinal cord could differentiate NG2+ cells into neuronal cells, which survived and matured therein. Given that NG2+ cells are abundant in adult CNS and rapidly proliferate in response to CNS trauma, our findings of their neuronal replacement potential on site make them a promising endogenous cell source for SCI treatment.

Several studies have demonstrated that glial progenitors could differentiate into neurons *in vitro* via two main mechanisms: one is that glial progenitors are firstly induced to become multipotential by addition of gliogenic cues [83, 183] and the other is that neurons are directly differentiated from glial progenitors without dedifferentiation [76, 82]. In our *in vitro* experiments, we used PD168393 supplemented with FBS to promote the neuronal conversion from two types of glial progenitors. In this strategy, the EGFR inhibitor-mediated neuronal differentiation was initiated, when the cells were incubated with gliogenic cues from FBS [194]. Furthermore, reduced Ras/ERK signaling was found to be involved in this neuronal conversion. Blocking Ras or MEK activities in the same experimental condition could induce the neuronal phenotype from NG2+ cells, resembling the effect of EGFR inhibition. In contrast, forced expression of Ras activity blocked the neuronal differentiation process. Therefore, inhibition of Ras/ERK pathway was critical for neuronal trans-determination from NG2+ cells mediated by EGFR inhibition. Previous studies reveal that activating Ras pathway is an important promoter for glial lineage generation [181], and downstream ERK is the key factor for regulating neural stem

cells of differentiating into glial lineages [213, 214]. Our work demonstrated that suppression of pro-glial machinery of EGFR and its downstream Ras/ERK pathway in NG2+ cells could force them into neuronal phenotypes. These *in vitro* findings may indicate the similar mechanisms in neuronal differentiation of NG2+ cells post SCI.

EGFR-inhibition mediated neural functional improvement from SCI have been shown to benefit from axon regeneration [162, 215, 216], the decrease of secondary damage [205], and better remyelination. Our work demonstrated that EGFR inhibition could stimulate neurogenesis from glial cells, mainly the NG2+ cells, in SCI. This finding provides evidence that the on site proliferating NG2+ cells could be one of the promising cell sources for endogenous neuronal replacement in the treatment of various neuronal disorders.

CHAPTER 5 EGFR Inhibition Ameliorates Histological Pathology of Spinal Cord Injury

5.1 Introduction

Traumatic spinal cord injury causes motor and sensory neurological deficits. The neural dysfunctions are found to be associated with acute and chronic histological destruction of spinal cord [217]. By examining the morphologic changes, such as lesion volume, lesion length, and spared area of grey/white matter at the injury region, a significant linear relationship could be established between the dimensions of tissue at the lesion site and the behavioral deficit [218, 219]. Therefore, we determined to investigate the histopathological improvement of SCI with treatment of EGFR inhibitor, PD168393.

Glial scar formation is one of the most prominent cellular processes that occur post SCI, which is composed of not only astrocytes, but also infiltrated/activated microglia/macrophages, endothelial cells, fibroblasts and meningeal cells. Though the scar could be responsible for walling off damaged areas from further erosion, much of the attention has been paid to their detrimental effects. The scar may either serve as a physical barrier for the recovery of injured spinal cord or express inhibitory molecules, which chemically arrest the re-growth of injured axons across the lesion [220, 221]. The activated microglia on site/infiltrated circulating macrophages, which are the principal components of scar, are shown to produce inflammatory factors and cause the secondary tissue damage [102]. Thus, reduction of the astrocytes and

microglia/macrophages are considered as one of therapeutic strategies for ameliorating neural dysfunctions of SCI.

Multiple molecular mechanisms are involved in the reaction of astrocytes and microglia/macrophages after CNS injuries, which could be exploited as therapeutic targets. It has been found that the activated EGFR is involved in the activation of astrocytes and macrophages in SCI [190, 191, 199]. We therefore assumed that EGFR inhibition may also benefit the functional recovery of contusive SCI through attenuating of scar genesis and secondary damages [222].

5.2 Results

5.2.1 PD168393 treated animals have better histological recovery

To further substantiate our suggestion that the functional improvement of PD168393 treated animals was accompanied with better tissue preservation, HE staining was performed on the sections from sham-operated and the injured spinal segments at 3 weeks after injury. The representative images of sham-operated, PBS-treated, PD168393-treated groups were shown in Fig. 25, A1-A3. Spinal cord contusion resulted in obvious necrosis, reactive gliosis at the lesion epicenters as well as the adjacent rostral-caudal regions of the spinal cord parenchyma in both PBS- and PD168393-treated groups. PBS-treated spinal cords showed an enlarged site of injury and scar area. In contrast, the PD168393-treated groups showed a higher degree of neural preservation (Fig. 25, A2-A3). Consistently, stereological presentation of spinal

cord tissue in either dorsal-ventral or rostral-caudal direction showed that PD168393 treatment led to significant more tissue recovery (Fig. 25B). Taken together, these results demonstrated that scar size and lesion region were smaller in PD168393-treated groups.

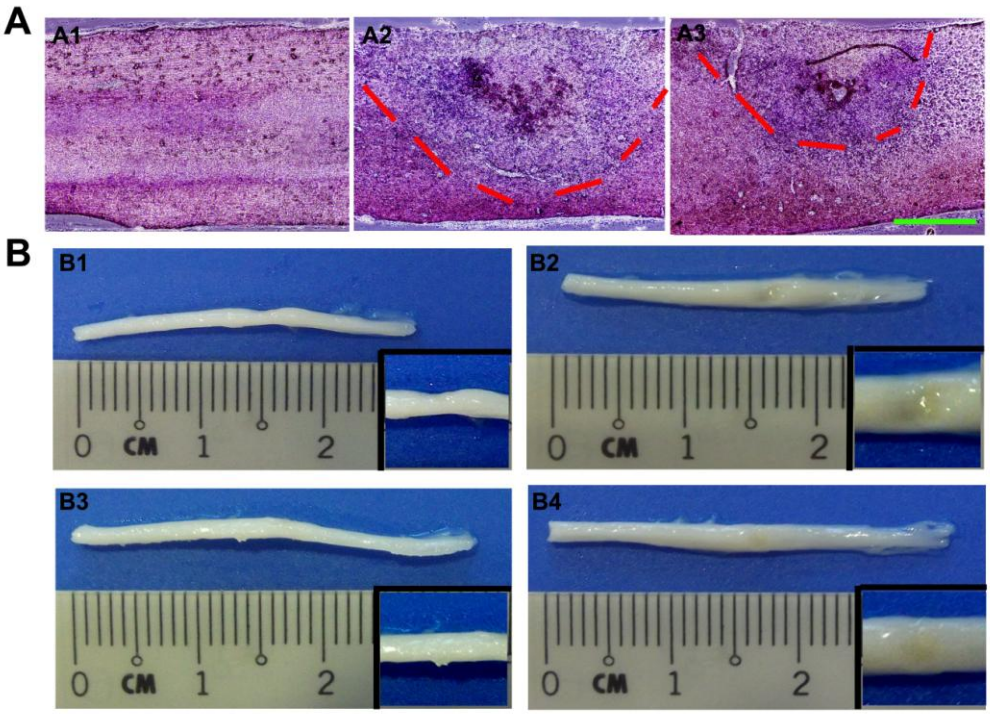


Figure 25 Effects of EGFR inhibition on general histological recovery post SCI. (**A**): In sham-operated (**A1**), PBS (**A2**), and PD168393 (**A3**) treated groups at 3 weeks post injury, histological changes were shown by HE staining. The injured spinal cords showed distinct cell loss, cell aggregation and scar formation at the injury epicenter in PBS- and PD168393-treated groups. (**B**) 3-cm spinal cords encompassing the injury site were dissected from PBS (**B1**, **B2**) and PD168393 treated (**B3**, **B4**) mice respectively at 21 days post SCI.

5.2.2 EGFR inhibition reduces the proliferative OX42+ macrophages and GFAP+ astrocytes in 3 days after injury

SCI results in an almost-immediate increase and activation of microglia/macrophages [223]. The microglia/macrophages release and respond to a multitude of cytokines, including the proinflammatory cytokines, which mainly feed into death signaling pathways of neuronal-glial cells [224]. It is possible that under certain conditions the recruitment and activity of microglia/macrophages need to be suppressed to avoid extended damage [225]. In order to investigate if PD168393 treatment could reduce the proliferation of microglia/macrophages, we performed the double immunofluorescence staining of BrdU and microglia/macrophage marker (OX42) in spinal cord sections of PBS and PD168393 treated mice with SCI. We found that in the 3-day experiment, SCI potently stimulated the proliferative OX42+ microglia/macrophages. However, PD168393 treatment significantly attenuated the number of BrdU+/OX42+ cells (Fig. 26A, 26B, 26E, two-tailed Student's t test, $P<0.05$).

The astrocytes are generally considered as a vital source for scar formation, and probably represent one minor cell source of proinflammtory cytokines after injury [226, 227]. With the immunostaining of BrdU and astrocyte marker GFAP, we found that in the PD168393-treated groups, proliferative GFAP+ astrocytes (GFAP+/BrdU+ cells) were greatly suppressed in 3 days after injury (Fig. 26C, 26D, 26F, two-tailed Student's t test, $P<0.01$). In summary, our results demonstrated that PD168393

treatment could attenuate the proliferation of microglia/macrophages and astrocytes in response to SCI, which might be detrimental to the recovery of SCI.

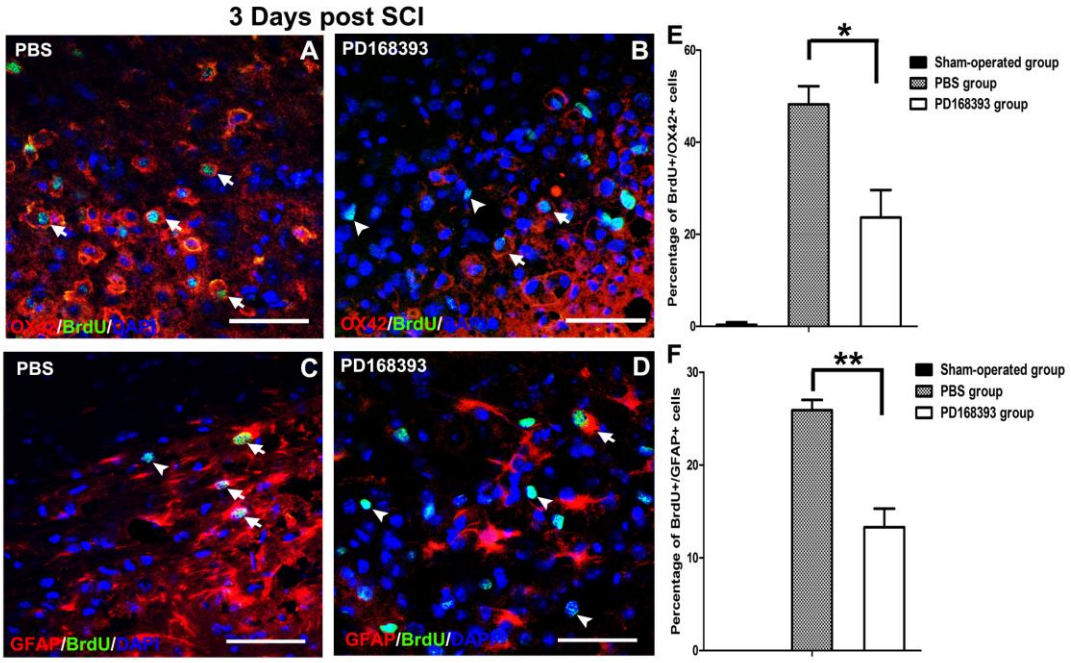


Figure 26 EGFR inhibition reduces the proliferative OX42+ macrophages and GFAP+ astrocytes in 3 days after injury, as compared with PBS groups.

Immunofluorescent images showed the BrdU+/OX42+ (**A**, **B**), BrdU/GFAP+ (**C**, **D**) cells at the injury epicenter in both PBS- and PD168393-treated groups. Nuclei were counterstained with DAPI in all images. Examples of double labeled OX42+/BrdU+ cells and GFAP+/BrdU+ cells in all the images were indicated by arrow. Examples of BrdU+ only cells were indicated by arrowhead. Quantification of OX42+/BrdU+ cells or GFAP+/BrdU+ cells in PBS (grey column) and PD168393 (white column) treated mice with SCI were shown in (**E**) and (**F**). Scale bars: 50 μm.

5.2.3 PD168393 treated mice have less OX42+ cells post SCI, as compared with PBS treated ones

It is well known that contusive spinal cord injury is also accompanied by a strong inflammatory reaction including immune infiltration. To further investigate the effects of PD168393 on immune responses of microglia/macrophages to contusive spinal cord injury, OX42 staining was performed in PBS- and PD168393-treated groups. We found that there was a significant increase of OX42 immunoreactivity in PBS groups at both 3 days and 21 days after injury (Fig. 27A, 27C). However, the increase was attenuated in the cords of PD168393-treated groups (Fig. 27B, 27D). We also quantitatively evaluated the expression of OX42 at 3 days post injury (three spinal cord samples from each group). Although an increment of OX42+ cells was detected in contusive injured spinal cords with PD168393 treatment, they were significantly less than the ones in PBS groups (Fig. 27E, $P<0.01$, multiple measurement ANOVA). Notably, PD168393 treatment reduced half of the OX42+ cells in the lesion center. Similarly, OX42+ cells in PD168393-treated groups were also significantly reduced in 21 days after injury, as compared to PBS groups. The significant reduction of OX42+ cells was observed throughout the whole lesion region ($P<0.01$, multiple measurement ANOVA). These findings demonstrated that PD168393 treatment could remarkably prevent the reaction of OX42+ microglia/macrophages post injury.

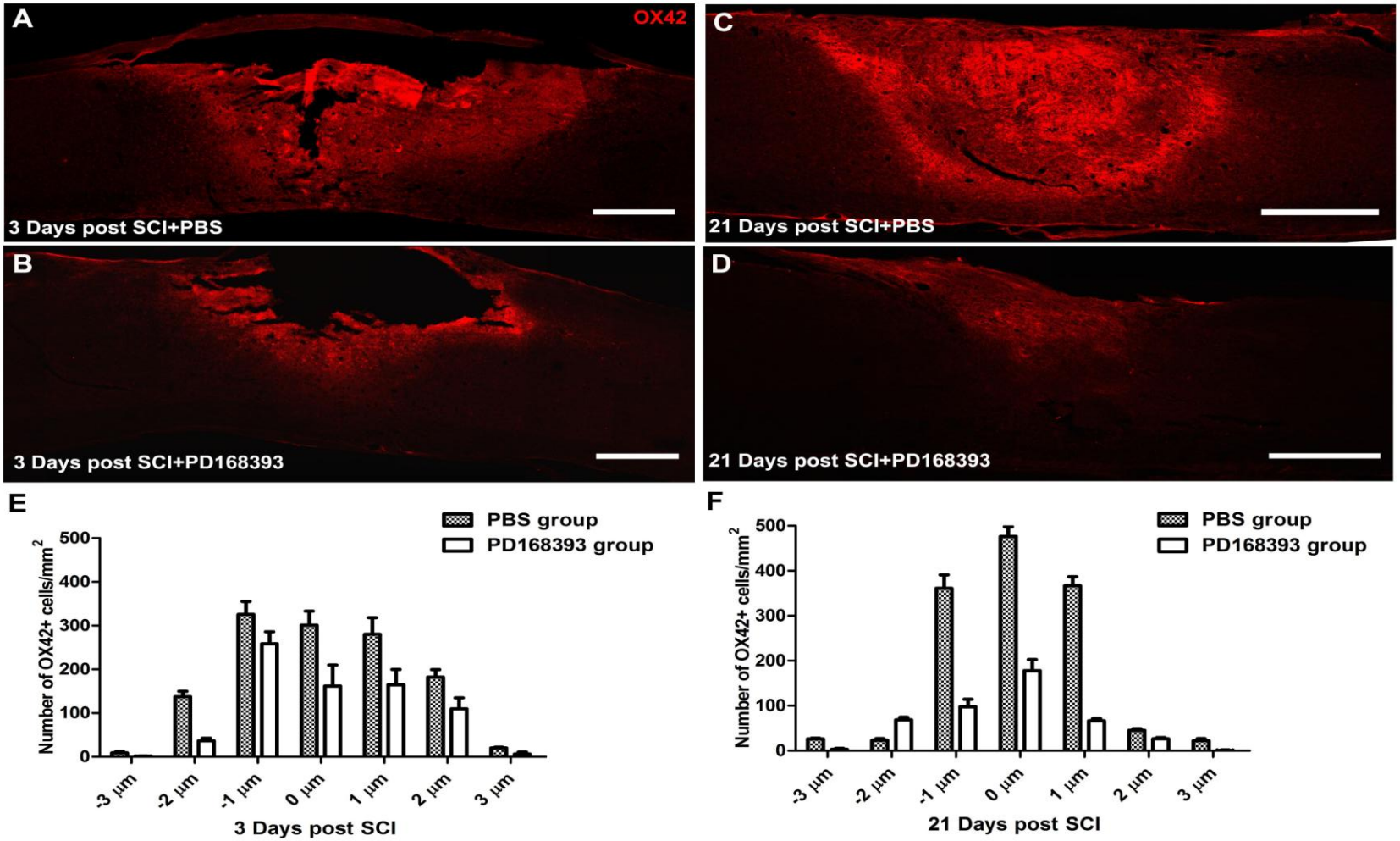

A
OX42
3 Days post SCI+PBS
B
3 Days post SCI+PD168393
C
21 Days post SCI+PBS
D
21 Days post SCI+PD168393
E
PBS group
PD168393 group
Number of OX42+ cells/mm2
500
400
300
200
100
0
-3 μm
-2 μm
-1 μm
0 μm
1 μm
2 μm
3 μm
3 Days post SCI
F
PBS group
PD168393 group
Number of OX42+ cells/mm2
500
400
300
200
100
0
-3 μm
-2 μm
-1 μm
0 μm
1 μm
2 μm
3 μm
21 Days post SCI

Figure 27 PD168393 treated mice have less OX42+ cells post SCI, as compared with PBS treated ones.

The generated panoramic images of spinal cord sections in 3 days and 21 days post SCI were shown in (**A**, **C**: PBS) and (**B**, **D**: PD168393). Quantification of OX42+ cells in PBS (grey columns) and PD168393 (white columns) treated mice with SCI were shown in (3 days: **E**) and (21 days: **F**). Scale bars: 500 μm. $p<0.01$, multiple-measurement ANOVA.

5.3 Discussion

5.3.1 EGFR inhibition improves tissue healing and attenuates astrocytes and microglia/macrophages post SCI

In this chapter, we mainly demonstrated the better tissue healing post SCI in EGFR inhibitor treated groups. This result supported the previous conclusion that EGFR-inhibition mediated neural functional improvement from SCI. Additionally, the inhibitory effects of PD168393 on the astrocytes and microglia/macrophages have also been suggested to contribute to the neural functional recovery of SCI.

Injury to the adult CNS has been demonstrated to lead to a complex series of cellular and molecular events, as cells respond to the trauma and try to initiate a spontaneous regeneration [228]. The processes of glial scar formation as well as the accumulation of inflammatory cells within the lesion have been revealed to contribute to the regeneration failure, what is more, the distruption of the local architecture of the CNS [103, 221, 228]. Numbers of molecular signalings have been found to be involved in those events. For example, induction of EGFR occurs in reactive astrocytes and

microglia/macrophages after CNS injury [229, 230], and is demonstrated to play a role in the process of astrocyte activation and following glial scar formation [191]. In our study, inhibition of EGFR signaling could greatly reduce the number of proliferative astrocytes in the acute phase after injury. This result is consistent with previous finding that astroglial scar formation could be greatly suppressed though EGFR inhibition [205]. Moreover, the microglia/macrophages, which are also the prominent cell populations within the glial scar, could be attenuated via EGFR inhibition post SCI. The repertoire of OX42+ inflammatory cells after CNS injury was demonstrated to produce inflammatory cytokines, protease and other factors that are cytotoxic [224]. If the microglia/macrophages are experimentally depleted or inactivated, the neuroprotection, increased regeneration, and improvements in motor, sensory, and autonomic function could be promoted after SCI [231, 232]. In view of that, the inhibition of EGFR signaling could help reducing the scar formation and the secondary damages after injury, both of which could be beneficial for rebuilding neural circuits at the recovery stage.

5.3.2 Neurogenesis and inhibition of gliosis/inflammation respectively contribute to functional recovery in PD168393 treated mice with SCI

Spinal cord injuries caused such a great loss of neural cells that they damaged the architecture of spinal cord and impaired neuronal circuits. As discussed in previous section, a significantly functional recovery was observed in PD168393 treated mice with SCI. One of the important benefits of EGFR inhibition was the neurogenesis induction from the NG2+ cells in SCI. These neurons produced following EGFR

inhibition might integrate into the architecture of cell-depleted regions after SCI, as they survived at least one month (Fig. 19 and 22). Hence, the neuronal function might be recovered through EGFR inhibition.

The functional recovery of SCI by EGFR inhibition could be attributed to the neuronal cells derived from NG2+ cells but not limited to. In this section, significant inhibition of gliosis/inflammation was observed in PD168393 treated group. Thus, the secondary damage was greatly attenuated. This might improve regional microenvironments so that tissue repair together with neurological functional recovery would be promoted in SCI. Taken together, EGFR inhibition may prove to be important in the development of therapies that optimize systemwide recovery following SCI.

CHAPTER 6 Conclusions

Besides oligodendrocytes, preponderance of evidence revealed that NG2+ cells could commit to other neural lineages, like astrocytes and neurons. Thus, they are known for having heterogeneous progenies. In this study, clonal analysis has been used to study the lineage potential of NG2+ subpopulations. We provided evidence that embryonic clonal NG2+ cells have different abilities of differentiating into oligodendrocytes, astrocytes, neurons and chondrocytes *in vitro*. Each type was found to express distinct levels of the neural progenitor markers (NG2, PDGFαR, Nestin, and A2B5), which may further specify the heterogeneity of NG2+ cells. Therefore, we conclude that NG2+ cells are composed of mixed populations of unipotent (generating oligodendrocytes only), bipotent (generating both astrocytes and oligodendrocytes), and multipotent (generating oligodendrocytes, astrocytes, neurons and chondrocytes) neural progenitors. The lineage potential analysis of NG2+ cells would have important ramifications for how we view their ability to repair the injured CNS, as NG2+ cells are highly reactive in CNS pathologies.

NG2+ cells proliferate at a significant rate for progenitor's expansion in CNS injuries and have been mainly revealed to play a role in the process of gliogenesis. In this study, we provided evidence that inhibition of EGFR signaling pathway could induce the NG2+ cells to acquire neuronal phenotypes post contusive SCI. We also found that the Ras/ERK signaling molecules played crucial roles in EGFR inhibition-induced neuronal phenotypes of NG2+ cells *in vitro*. Therefore, NG2+ cells have been

identified as one of cell sources for compensating neuronal loss post SCI in PD168393 treated animals. The findings supported the therapeutic potential of NG2+ cells for endogenous neuronal replacement in CNS injuries. It also suggested a beneficial effect of EGFR inhibitor, PD168393, in experimental SCI.

In SCI, a lot of players have been found to participate in motor and sensory dysfunctions, thus sufficient functional recovery could be achieved by regulating multiple cellular targets. Several mechanisms could be proposed for explaining the curative effect of PD168393 on SCI: (1) Promoting axon regeneration [162, 215], (2) Inducing neuronal replacement from the NG2+ cells, as discussed in Chapter Four. (3) The inhibitory effect of PD168393 on the astrogliosis and microglia/macrophages post SCI, resulting in reduced scar barriers and secondary damages. Therefore, it is possible that the EGFR inhibitor PD168393 has multi-effects and targets several cell populations in SCI, suggesting that it might be exploited as effective treatment candidate against SCI.

In summary, elucidating the differentiation capacities of clonal NG2+ cells will be essential for disclosing the heterogeneous lineage potential of whole NG2+ cell populations. Characterization of EGFR inhibition-mediated neuronal phenotypes generation from NG2+ cells will provide us with critical insight on manipulating the neuronal fate of endogenous NG2+ cells in pathologic CNS. It is thus possible that NG2+ cells might be manipulated to replace neurons lost to insults in the injured

spinal cord. Furthermore, the EGFR inhibitor-PD168393 might be suggested as the potential pharmacological approach for treating SCI.

CHAPTER 7 References

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CHAPTER 8 Publication List

Publications from this study

1. **Peijun Ju**, Si Zhang, Yeeshan Yeap and Zhiwei Feng. Inhibiting Epidermal Growth Factor Receptor (EGFR) in vitro and in Mouse spinal cord Injury. GLIA. Accepted.

2. **Pei-Jun Ju**, Rui Liu, Hai-Jie Yang, Yin-Yan Xia and Zhi-Wei Feng. Clonal analysis for elucidating the lineage potential of embryonic $NG2^+$ cells. Cytotherapy, 14 (5): 608-620，2012.

3. SI ZHANG*, **PEIJUN JU***, Editha Tjandra, YEESHAN YEAP, AND ZHIWEI FENG. EGFR inhibition modulates glial cells and ameliorates deficiency of spinal cord injury, *co-first author, manuscript in preparation.

Publications from other studies

4. A novel role for neural cell adhesion molecule in modulating insulin signaling and adipocyte differentiation of mouse mesenchymal stem cells. Yang HJ, Xia YY, Wang L, Liu R, Goh KJ, **Ju PJ**, Feng ZW. J Cell Sci, 2011, 124(15): 2552-2560.

5. Rui Liu, Si Zhang, Haijie Yang, **Peijun Ju**, Yinyan Xia, Yu Shi, Tse Hui Lim, Alvin St Lim, Fengyi Liang, Zhiwei Feng. Characterization and therapeutic evaluation of a $NG2^+$ cell population on mouse spinal cord injury. Neurobiology of Disease. In revision.

6. Lei Wang, Yu Shi, **Peijun Ju**, Rui Liu, Siok Ping Yeo, Yinyan Xia, Zhiwei Feng. A novel role of diphthamide synthesis 3: potentiating the metastasis of melanoma cells through Akt signaling pathway. PLoS ONE. Accepted.