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THE STATE UNIVERSITY OF NEW JERSEY

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Final Narrative Report:

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Grant Title: Study of Radial Glia and Potential Application for Nerve Regeneration

Grant Number: 01-3007-SCR-S-0

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Date of Submission of the Report: September 23, 2003

Final Narrative Report for NJCSCR (Dr. Hedong Li)

ORIGINAL SPECIFIC AIMS OF THE PROJECT:

The C6-R cell line, generated in our lab from C6 glioma, has been shown to have unique properties that mimic authentic radial glia, e.g. radial morphology and capability of supporting neuronal migration. Whereas C6-R can be used as a model to study radial glia, it is still somewhat tumorigenic in vivo. In this proposal, we plan to identify and characterize genes that are selectively expressed in radial glia, and use this information to analyze new radial glial cell lines for transplantation.

I. Identification and characterization of genes selectively expressed in radial glia.
DNA microarray (genechip) techniques will be applied to search for genes that are selectively expressed in radial glia. We will engage two model systems to accomplish this specific aim, e.g. the C6-R cell line and primary radial glia. Genes that are identified in both systems are prime candidates for radial glial phenotype, and will provide a profile for analyzing new radial glial cell lines that will be obtained in the future.

II. Isolation of new radial glial cell lines using retrovirus:
Given limitations of the C6-R radial glial cells, we will create new radial glial cell lines derived from primary tissue that may be more suitable for in vivo transplantation studies. In addition, these new cell lines will provide homogeneous samples to search for genes that are selectively expressed in radial glia (Specific Aim I). We will create new radial glial cell lines from primary cultures using retrovirus, and characterize them using cellular markers and genechip analysis. Cell lines generated will be applied for transplantation studies in rat spinal cord injury models.

PROJECT SUCCESSES:

Radial glia not only guide immature migrating neurons during development but also direct the path of growing axons. Therefore, our hypothesis is that, when radial glial cells are transplanted into injured spinal cord, they may provide a permissive substrate for the regenerating axons to cross the injury site.

I. Identification and characterization of genes selectively expressed in radial glia.
As we proposed in the grant application, two systems were engaged in this gene-screening project. In primary radial glial culture sytem, we compared the mRNA expression of three states of treatment of neural stem cells: bFGF, LIF and BMP. Basic FGF is a growth factor that maintains and promotes proliferation of the cells as neural stem cells and radial glial cells. When primary radial glial cells isolated from E14 cortex are treated with bFGF + LIF, many cells develop bipolar processes and become more radial. The persistence of BLBP expression suggests that these cells are radial glia. In BMP, the cells differentiate and become mostly astrocytes. The GeneSpring program was used to analyze our gene chip data by organizing genes with similar patterns of change. The tree clustering result on the left (Figure 1) shows the expression level of all the genes on the RU34 genechip under the three conditions. The color of the bar shows the expression level of the gene. Red represents high expression while blue represents low expression. From these trees, we can select those genes that exhibit interesting patterns of change. One of the most interesting patterns we examined

was the *high-high-low* pattern (right, Figure 1). The genes on the right graph are those that are expressed when the cells are neural stem cells and radial glial cells, but are down-regulated when they differentiated into astrocytes. These genes are likely to include those that contribute to the radial glial phenotype.

Genechip comparisons of C6-R vs. C6 and neural stem cells/radial glia vs. astrocytes were combined to identify genes that show 2-fold higher expression in radial glia in both experiments. Among the 1200 genes on the Affymertix RU-34 chip, ~20 met these criteria with their relative levels of expression in the two types of experiments that were performed (Fig. 2). Among these, two have previously been identified in radial glia. Nestin is an intermediate filament protein found in stem cells and radial glia. Integrin-αv is expressed on radial glia and plays a key role in neuronal migration along radial glia. Growth factor receptors including FGFR were identified that are involved in survival and proliferation of stem cells. Cytoplasmic effector kinases in the MAP kinase pathway were also found and may act down stream of receptor tyrosine kinases, suggesting a role for this signaling pathway in radial glia. We then carried out quantitative-RT-PCR experiments to confirm the genechip data. The results showed that 16 out of 18 genes were confirmed in C6R system to be radial glial specific genes (Fig. 3).

Investigation on radial glial cytoskeleton:

Genechip comparison between C6 and C6-R has revealed several microtubule-associated proteins (MAPs) that are up- or down-regulated in C6-R cells. These results suggested that microtubules might be important for C6-R radial morphology. To test this idea, we have used C6-R, a radial glial like cell line and isolated perinatal cerebellar radial glia to ask what are the critical cytoskeletal elements in radial glial cells and how are they regulated. We treated C6-R cells for 2 h with drugs, which specifically disrupt either microtubules or actin filaments. Figure 4 shows that the microtubule-disrupting drug, nocodazole (NCD, 5μg/ml) turned C6-R cells into a flat, polygonal shape, which dramatically contrasts to the control treatment (DMSO). However, the actin-disrupting drug, Cytochalasin D (CytoD, 10 μM) collapses the cytoskeletal fibers around the nucleus, but leaves the radial processes of C6-R cells intact. This result indicates that, indeed, microtubule filaments are very important for C6-R radial morphology. Combining drug treatments and real time-RT-PCR techniques, our results showed that 1) microtubules, not actin, are critical to the polarized morphology of radial glial cells; 2) certain MAPs (e.g. MAP-1A, MAP-4 or MAP-7) may be responsible for organizing the microtubule filament into the radial pattern (Fig. 5); and 3) Microtubule affinity regulating kinases (MARKs) are present in radial glia and may be involved in regulating the phosphorylation state of MAPs for their functions (Fig. 5) [These observations have been published in GLIA 44: 37-46 (2003)]

II. Isolation of new radial glial cell lines using retrovirus:

Given limitations of the existing radial glial cell, C6-R, we proposed to create new radial glial cell lines derived from primary tissue that may be more suitable for in vivo transplantation studies. During this grant period, we generated new radial glial cell lines from rat embryonic cortex using a retrovirus containing an oncogene (v-myc) that would cause immortalization (Ryder et al., 1990). Thirteen candidate radial glial clones were identified by morphology and marker staining (BLBP+). L2.3, one of our best radial glial cell lines has been characterized in detail. L2.3 expresses several radial glial markers including BLBP,

vimentin, and nestin (Fig. 6); shows radial morphology both in vivo and in vitro (Fig. 6); and supports migration of granule neurons from cerebellum (Fig. 7). Characterization of L2.3 cells in culture has led to a manuscript (Li H et al, 2003) being submitted that clarifies the relationship between radial glia and restricted precursors during development.

Similar radial glial cell lines were also generated from GFP-rat embryonic cortex and characterized by criteria for radial glia. These include G3.6 and G4.7. G3.6 cells have been utilized for transplantation studies in both normal (Fig. 8) and injured (Fig. 9) spinal cord. Studies on G3.6 also led to a manuscript (Hasegawa K et al, 2003) describing the fact that immortalized radial glial cells tend to differentiate slower both in vivo and in vitro, when comparing to primary radial glia. These findings may provide reasons for using immortalized radial glia in treating the spinal cord injuries.

PROJECT CHALLENGES AND FUTURE DIRECTIONS:

We believe we fulfilled our initial aims for this grant. We identified certain genes that are specific to radial glial cells using our in vitro cell culture models, although the function of these genes need to be investigated further in terms of unique properties of radial glial cells. In situ expression of these genes in animal needs to be confirmed also.

We successfully generated numerous cell lines with radial glial properties, including G3.6 that was generated with green florescent marker. G3.6 radial glial cells are currently being studied in injured spinal cord following transplantation. The radial glial cell lines that were generated by immortalization in the grant showed increased stability both in vitro and in vivo, e.g. immortalization slowed down the differentiation of these cells by comparison to primary radial glia. However, whether the increased stability is sufficient to bridge the injury site is still an open question that needs to be studied further. Transplantation studies showed these radial glial cell lines did differentiate in animal after long period of time. Another concern is the tumorogenesity of these immortalized cells. However, our recent results indicated these radial glial cell lines did not generate tumors in contrast to C6R, where mass formation did occur . In the future, we will test genes that have been demonstrated to maintain radial glial properties (such as activated Notch) by introducing them into primary radial glial cells along with green florescent marker. These cells are expected to keep radial glial properties and should not have problems with tumor formation.

PUBLICATIONS:

Hedong Li, Yana Berlin, Ronald P. Hart, and Martin Grumet. "Microtubules are Critical for Radial Glial Morphology: Possible Regulation by MAPs and MARKs" Glia, 2003. GLIA 44: 37-46 (2003).

H. Li ; J. Babiarz; J. Woodbury; N. Kane-Goldsmith; M. Grumet. "Cortical Radial Glial Cells Precede Expression of Markers for Restricted Precursors During Embryonic Development". Submitted.

Hasegawa K, **Li H**, Chang Y, Berlin Y, Grumet M. "A study on differentiation of neural stem cells and immortalized stem cell line both in vitro and in vivo". In preparation.

H. Li *;J. Babiarz ;M. Grumet. "A Lineage Study on Radial Glia: Relationship with neuronal-restricted precursors (NRPs) and glial-restricted precursors (GRPs)" Abstract #564.4, Annual Conference of Society for Neuroscience, 2003, submitted.

H. Li, J. Babiarz, Y. Berlin, R. Hart, M. Grumet. "Microtubules are important for radial glial morphology: involvement of MAPs and MARKs". Abstract #424.3, Annual Conference of Society for Neuroscience, 2002.

H.- Li ;N.- Kane-Goldsmith ;R.-P.- Hart ;M.- Grumet. "Genes that are differentially expressed in radial glial cells". Abstract #899.2, Annual Conference of Society for Neuroscience, 2001.

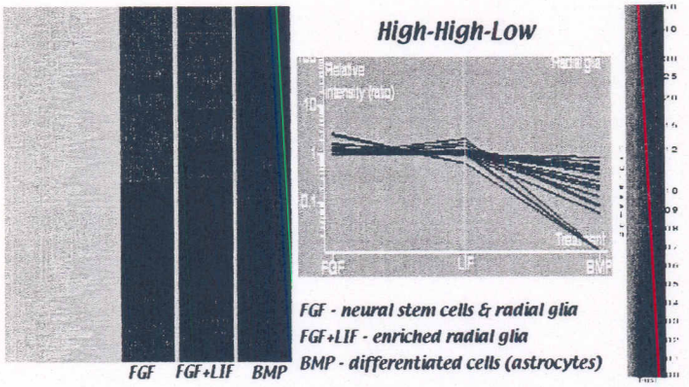


Fig 1. Genechip analysis using GeneSpring. The GeneSpring program was used to analyze our gene chip data by organizing genes with similar patterns of change. In the primary radial glial culture system, we compared the mRNA expression of three states of treatment of neural stem cells: bFGF, LIF and BMP. The tree clustering result on the left shows the expression level of all the genes on the RU34 genechip under the three conditions labeled. The color of the bar shows the expression level of the gene. Red represents high expression while blue represents low expression. From these trees, we can select those genes that exhibit interesting patterns of change. One of the most interesting patterns we examined was the *high-high-low* pattern (right). The genes on the right graph are those that are expressed when the cells are neural stem cells and radial glial cells, but are down-regulated when they differentiated into astrocytes

Relative Expression Level		L ▸H		H ▸H		▸L
Genbank	Product	C6	C6R	FGF	LIF	BMP
M34384	[illegible]	0.45	1.30	1.37	1.00	0.18
S58528	[illegible]	0.39	1.48	1.34	0.97	0.14
D12498	FGF receptor-1	0.19	1.73	1.12	1.04	0.43
S54008	fibroblast growth factor receptor 1 beta-isoform	0.01	2.09	1.19	1.02	0.45
U57715	FGF receptor activating protein FRAG1	0.50	1.28	1.04	1.02	0.53
Z14113	platelet-derived growth factor receptor alpha	0.23	1.65	1.51	1.03	0.10
U48596	MAP kinase kinase kinase 1 (MEKK1)	0.38	1.34	1.48	0.99	0.23
U91847	p38 mitogen activated protein kinase	0.51	1.17	1.37	0.98	0.35
X74227	IP3 3-kinase	0.39	1.19	1.19	1.00	0.47
X16709	insulin-like growth factor II gene	0.47	1.88	1.13	1.22	0.10
U44979	kinesin-related protein 2	0.48	1.41	1.00	1.06	0.01
AF021985	myotonic dystrophy kinase-related Cdc42-binding kinase	0.09	1.28	1.98	0.86	0.04
U68562	chaperonin 10 (hsp60)	0.50	1.30	1.19	0.99	0.40
U01022	Huntington's disease protein	0.11	1.31	1.25	0.84	0.09
U78090	potassium channel regulator 1	0.50	1.13	1.11	1.14	0.45
X17607	adrenergic receptor, beta-2 adrenergic receptor	0.16	2.06	0.99	1.22	0.38
AF065431	Bcl-2 related ovarian death gene product BOD	0.20	1.86	1.71	0.95	0.06
U64889	zyxin	0.34	1.27	1.16	1.00	0.21

Fig 2. Genes that are selectively expressed in Radial Glia. Genechip comparisons of C6-R vs. C6 and neural stem cells/radial glia vs. astrocytes were combined to identify genes that show 2-fold higher expression in radial glia in both experiments. Among the 1200 genes on the Affymertix RU-34 chip, 18 met these criteria and are listed with their relative levels of expression in the two types of experiments that were performed. Among these, two have previously been identified in radial glia and are highlighted in red.

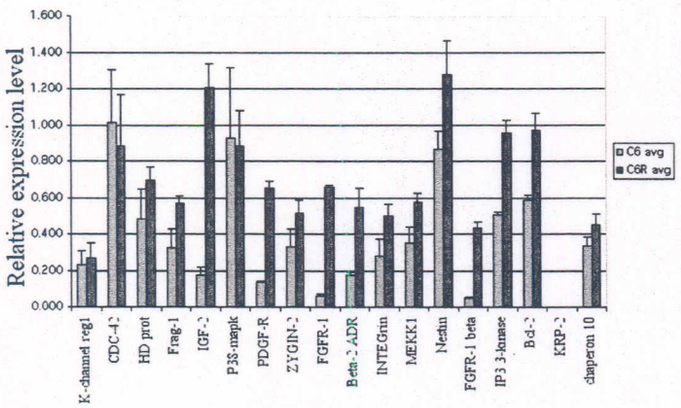


Fig 3. Quantitative-RT-PCR confirmation on C6R vs C6 comparison. Among the 18 genes selected in Fig 2, primer pairs were designed by using Primer Express software. Quantitative-RT-PCR reactions were carried out by triplicates for each and relative expression levels were plotted on Y axis. A house keeping gene, GAPDH was used to normalize expression levels. 14 out of 18 genes showed significantly higher expression level in C6R than in C6 ($p<0.05$), but the magnitude of differences varies between genechip and RT-PCR result.

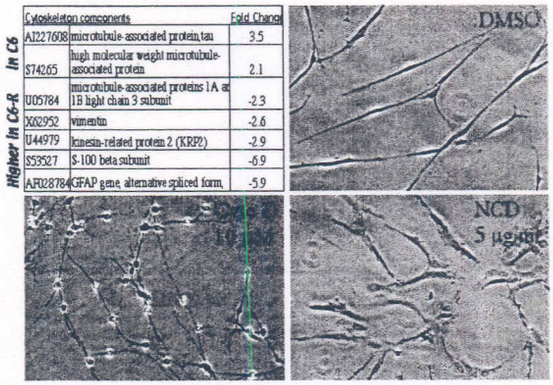


Fig 4. Microtubules are important for radial morphology of C6R cells. The microtubule-disrupting drug, nocodazole (NCD, 5μg/ml), upon 2 hours treatment, turned C6-R cells into a flat, polygonal shape, which dramatically contrasts to the control treatment (DMSO). However, the actin-disrupting drug, Cytochalasin D (CytoD, 10 μM) collapses the cytoskeletal fibers around the nucleus, but leaves the radial processes of C6-R cells intact. The increase in microtubule-associated protein (MAP) 1A and 1B, revealed by genechip comparison might be critical for stabilizing the cytoskeleton in radial glial cells (top left).

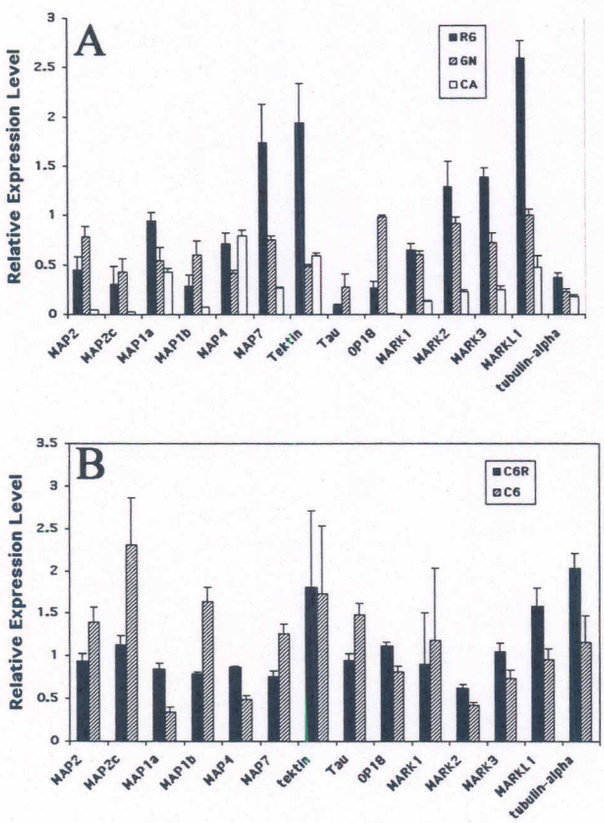


Fig 5. Real-time RT-PCR analysis of MAPs and MARKs on mRNAs from cerebellar radial glia. Cerebellar radial glia and granule neurons were isolated following percoll gradient centrifugation (Hatten, 1985) and differential adhesion, and the resulting cultures were characterized for purity by immunostaining with markers to identify radial glia (BLBP) and neurons (NeuN). RT-PCR analyses indicated that MAP-1a, MAP-4, MAP-7 and tektin are present and expressed at higher levels in cerebellar radial glia by comparison to granule neurons. Cerebellar radial glia (RG), granule cells neurons (GN), and cerebellar astrocytes (CA). Samples were analyzed in triplicate and the standard deviations are indicated.

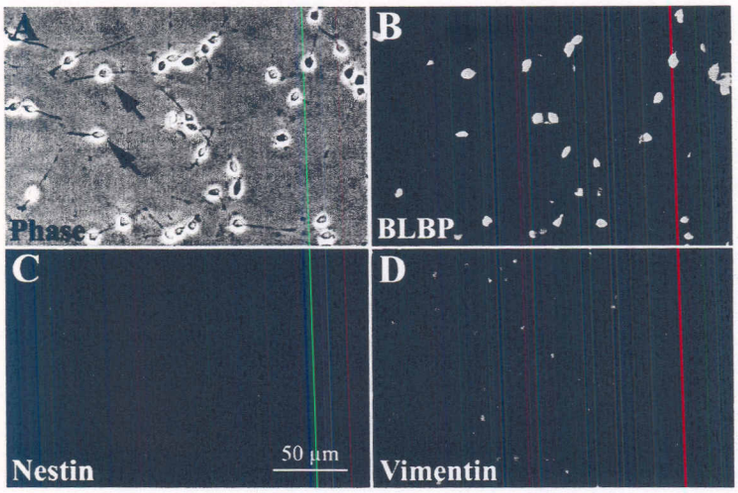


Fig 6. Characterization of radial glial clone, L2.3. Radial glial clone L2.3 was derived from E14 rat embryonic cortex following immortalization. (A) These cells exhibit bipolar morphology on laminin-coated substrate and have relatively long processes, which resemble radial glia in vivo. L2.3 cells express radial glial markers including BLBP (B, corresponding to phase), nestin (C) and vimentin (D).

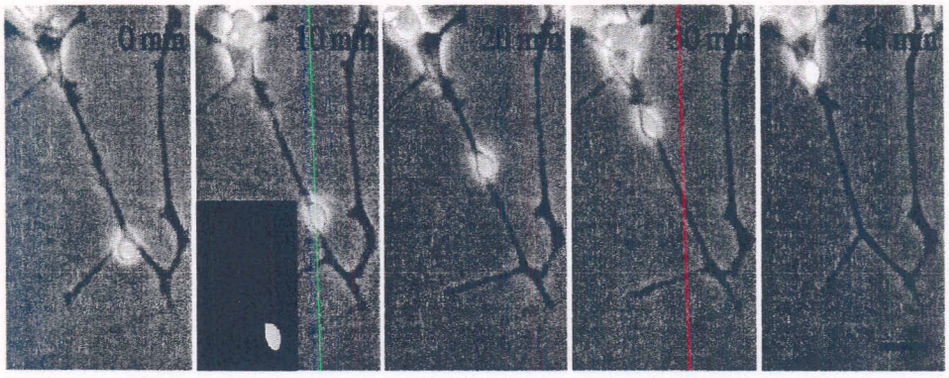
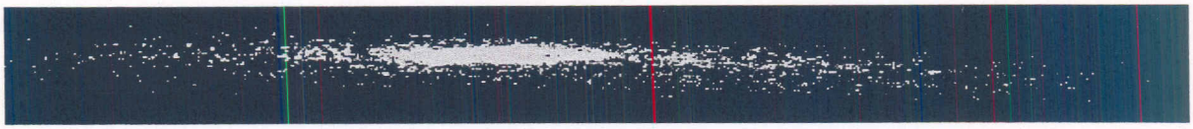


Fig 7. Radial glial clone (L2.3) support migration of granule neurons from cerebellum. GFP-labeled granule neurons were isolated from GFP-rat (P4) and plated onto cultured L2.3 cells. After overnight in co-culture, timelapse images were recorded. This figure is one of 10 migrating neurons recorded. The flourescent insert shows that this neuron has typical morphology of migrating neurons, that is, a tear-drop shaped cell body and a leading process. Average speed=107 um/hour; bar=20 um.



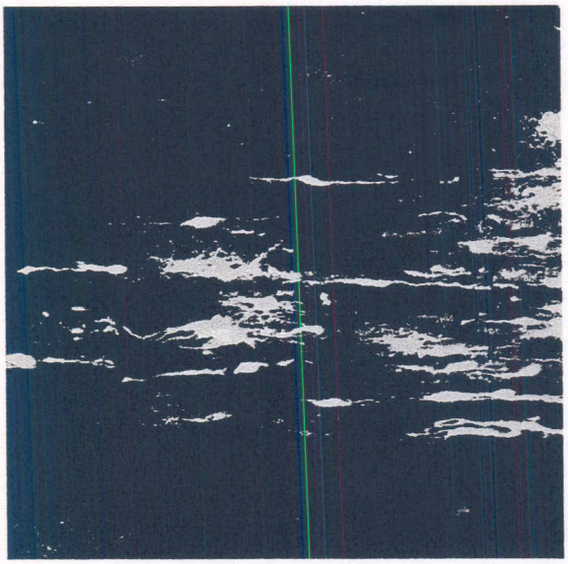
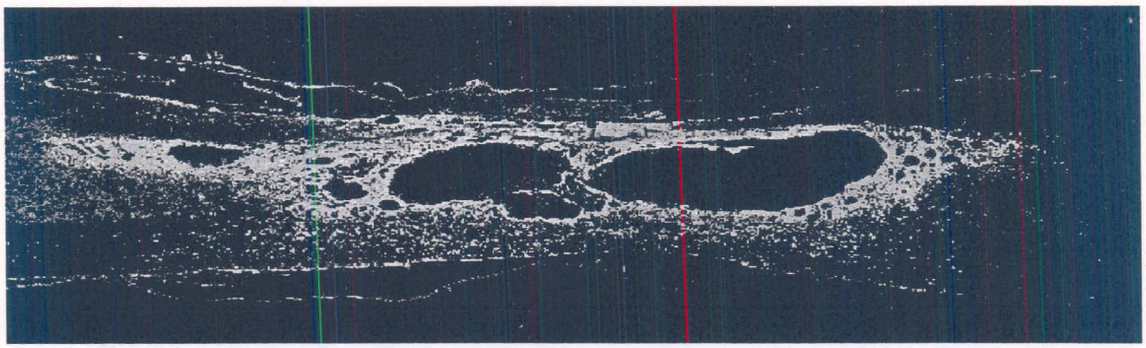


Fig. 8. Migration of GFP-labeled radial glial clone (G4.7) 2 weeks after transplantation in the normal adult spinal cord (Top). Higher magnification (bottom) on the tip of migrated cells. Bipolar, undifferentiated GFP-labeled cells were often seen. Green, GFP label; Blue, NeuN (neuronal marker) staining; Red, GFAP (astrocyte marker) staining.



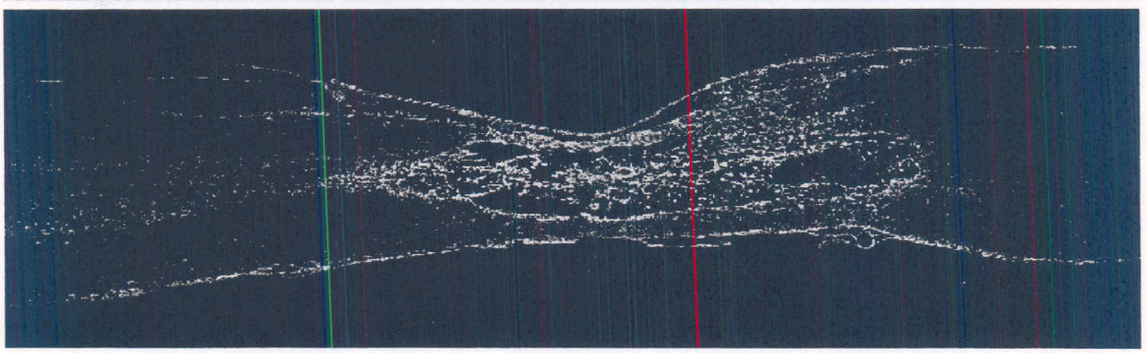


Fig. 9. 6 weeks after transplantation, G3.6 cells (green cells in image A) were located densely around the injured cavity, and migrated extensively in white matter. In control spinal cord (bottom), NG2 expression (showing by Cy5 in purple) is stronger than G3.6 treated spinal cord (top). Neurofilaments (showing by rhodamine in red) are concentrated rostral to the injury site both in G3.6 and control. Shrinkage is severe in control spinal cord (bottom).