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New Jersey Commission on Spinal Cord Research
FINAL REPORT

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SUMMARY OF PROGRESS:

Transplantation of radial glial clone RG3.6 in adult spinal cord improves outcome following SCI (Aim 1)
We have performed transplantation experiments to compare radial glia (i.e. RG3.6) to fibroblasts (both labeled with GFP). Histological analysis confirmed robust GFP fluorescence persisted in fibroblasts and RG3.6 cells but the fibroblasts remained almost exclusively in the spinal cord injury site. In contrast, the RG3.6 cells surrounded the injection site and were observed several mm rostral and caudal indicating they had migrated into spared white matter tracts where they were aligned along the rostral-caudal axis (Fig. 5F, 6C) as they did in the normal spinal cord (Fig. 3,4); figures refer to Hasegawa et al., 2005.

Walking function was analyzed using BBB scores, which were consistently higher for the rats that received RG3.6 cells than those that received fibroblasts (Hasegawa et al., 2005; Fig. 6D). The differences in BBB scores were modest but significant with the largest differences observed at 7 days (RG3.6 = 8.111 ± 0.491 vs. fibroblast = 6.000 ± 0.775), too early to result from regeneration or sprouting (Hasegawa et al., 2005), suggesting that the RG3.6 cells protected the spinal cord from secondary injury. These studies laid the foundation for recent experiments that provide additional evidence that signals associated with tissue protection (e.g. HSP70) and promotion of progenitor cells (e.g. vimentin) are produced specifically within 6-12 hours following spinal cord and contusion only when radial glia transplanted. We plan to submit a new grant on this subject soon.

Although implantation of various types of cells might improve recovery by simply replacing lost cells and preventing spinal cord shrinkage, the results indicated a statistically significant improvement of the RG3.6 cells vs. controls including fibroblasts (Hasegawa et al., 2005, Fig. 5D, 6D), suggesting that the RG3.6 cells had particular properties that were beneficial. Rats that received RG3.6 cell transplants exhibited preservation of some white matter both dorsal and ventral in the injury site that typically spanned across it (Hasegawa et al., 2005; Fig. 6F). In contrast, there was little or no white matter preservation in the injury site with fibroblast transplants (Hasegawa et al., 2005; Fig. 6G). To explore potential explanations for the ability of the RG3.6 to preserve spinal cord tissue, we describe below analyses of histological markers in tissues from the injured rats treated with RG3.6, fibroblasts or medium alone.

RG3.6 cells inhibited accumulation of CSPG and macrophages following spinal cord injury (Aim 1)
The early improvement in behavioral scores with RG3.6 cell transplants (Hasegawa et al., 2005; Fig. 5 and 6) suggested that the spinal cord was being protected from secondary damage. Various factors associated with neural tissue damage including chondroitin sulfate proteoglycans (CSPG) are believed to inhibit cell adhesion and axonal growth. Immunostaining with monoclonal antibody CS56 confirmed extensive deposits of CSPGs in the spinal cord following contusive injury (Hasegawa et al., 2005; Fig. 7A). CSPG staining was present in the injury site in a trabecular pattern as well as in the walls surrounding the cysts. In contrast, there was much less CSPG deposition following transplantation of RG3.6 cells or fibroblasts (Hasegawa et al., 2005; Fig. 7). Some weak CSPG staining was observed surrounding but not inside cysts that formed with RG3.6 transplants, suggesting that perhaps the radial glia prevented infiltration of other cells that may deposit CSPGs. Among these CSPGs, NG2 expression is best correlated with the injury site and may derive from several local sources including reactive astrocytes, oligodendrocyte precursors, and macrophages. We confirmed the enhanced accumulation of NG2 in and around the injury site of medium injected rats but NG2 was present at negligible levels in rats that received transplants of RG3.6 cells or fibroblasts (Hasegawa et al., 2005; Fig. 7).

Given that macrophages are a major source of CSPGs in the injured spinal cord, the inhibition of CSPG deposition in rats injected with RG3.6 cells raised the possibility that RG3.6 cells may interfere with

macrophages. Immunostaining for activated macrophages with monoclonal antibody ED1 revealed very few reactive macrophages in rats treated with RG3.6 or fibroblasts at 6 weeks (Hasegawa et al., 2005; Fig. 8) in contrast to the large numbers detected in contused rats treated only with medium (Hasegawa et al., 2005; Fig. 8). Additional studies at 2 weeks after contusion showed lower levels of ED1+ and IBA1 staining with the RG3.6 transplants. The combined results suggest that implantation of RG3.6 cells inhibit infiltration or accumulation of activated macrophages and this may explain the dramatic inhibition in CSPG levels and increased levels of white matter preservation.

RG3.6 cells preserve neurofilaments in spinal cord following injury (Aim 1)
Histological analyses indicated that functional recovery correlated with reductions in macrophages and CSPG but not with cyst formation, which was observed with RG3.6 but not with fibroblasts treated rats. To determine whether RG3.6 promoted axon sparing or regrowth, we immunostained for neurofilament proteins (NF). By comparison to implants of fibroblasts or medium alone, spinal cords that received RG3.6 cells showed improved tissue architecture and NF alignment (Hasegawa et al., 2005; Fig. 5, 6, 8 and 9). To analyze differences in NF staining among the different treatments, we measured areas occupied by NF in the remnants of the fiber tracts around the injury site. In sagittal cryosections, areas occupied by NF+ cells in dorsal fiber tracts were statistically greater for RG3.6 treated rats than rats treated with fibroblasts or medium alone (Hasegawa et al., 2005; Fig. 9). A similar trend was observed in ventral fiber tracts. The results indicate that NF staining was more organized and abundant in presumptive fiber tracts of rats implanted with RG3.6 than those treated with fibroblasts or medium alone.

The close co-alignment of NF+ fibers with GFP+ radial glia and the increase in the area of NF+ staining suggests that RG3.6 radial glia may interact with axons and promote axonal growth. To test this hypothesis, we cultured granule cell neurons on monolayers of RG3.6 cells and fibroblasts. Granule neurons extended long processes on monolayers of RG3.6 cells but not on fibroblasts where they tended to associate with each other. The close association of the neurites along radial glial fibers suggests an interaction that can guide neurite growth along the radial glia (Hasegawa et al., 2005; Fig. 10). These results suggest that RG3.6 cells provide a favorable substrate for neuronal interaction that can support axonal outgrowth.

These results confirm that stabilized a radial glial clone RG3.6 does improve functional recovery following injury and suggests that the transplants preserve and/or promote axons in and around the injury site. This sets a firm foundation to explore effects of other stabilized radial glia as described below.

Induction of activated receptors modulates radial glial phenotype (Aims 1 & 2)
In view of the evidence that Notch activation promotes radial glia, we tested the effects of introducing activated Notch (ActNotch) into radial glial clones L2.3 clone. We showed previously that ActNotch upregulated expression of the radial glial marker BLBP.

To determine the efficacy of expressing ActNotch in radial glial clones, we have isolated subclones of L2.3 cells that were induced to express ActNotch. Most of the clones (called NL2.3 cells) obtained were quite stable and homogenous in morphology with the cells exhibiting a striking polarization resembling radial glia. These result suggest that ActNotch expression can persist for long periods of time in culture and can stabilize the radial glial phenotype. Whereas L2.3 cells can differentiate into neurons and glia, the NL2.3 cells expressing activated Notch do not differentiate into neurons, only into astroglia. NL2.3 cells have been be transplanted into the spinal cord and they migrate extensively and maintain their radial morphology for at least 4 weeks in vivo. Future experiments will analyze their effects on behavioral recovery in vivo following spinal cord injury. This demonstrates the feasibility of introducing ActNotch into

neural stem cells where we expect it to promote a radial glial phenotype in those cells. A manuscript on this work is in preparation.

The earliest radial glia appear as neural stem cells that guide neural cell migration away from ventricular zones and subsequently radial glia become lineage restricted during development before they differentiate into more mature cell types in the CNS. We have previously shown that subpopulations of radial glial cells express markers for glial and neuronal restricted precursors (GRPs and NRPs) in expression patterns that are temporally and spatially regulated during CNS development. To characterize further the mechanism of this regulation in rat forebrain, we tested whether secreted factors that are present during development effect lineage restriction of radial glia. We found that in primary and clonal radial glial cultures LIF/CNTF up-regulates, whereas BMP2 down-regulates GRP antigens recognized by monoclonal antibodies A2B5/4D4, consistent with a graded distribution pattern in dorso-lateral ventricular regions in vivo. The regulation by LIF/CNTF of A2B5/4D4 is mediated through the JAK-STAT pathway. BMP2 promotes expression on radial glial cells of the NRP marker polysialic acid most likely by regulating N-CAM expression itself, as well as at least one polysialyl transferase responsible for synthesis of polysialic acid on N-CAM. Taken together, these results suggest that the generation of lineage-restricted precursors is coordinately regulated by gradients of the secreted factors BMPs and LIF/CNTF during development of dorsal forebrain (Li and Grumet, 2006).

To test additional affects of ActNotch on cell phenotype, we tested for the expression of cell-type specific markers. A2B5 has been recognized as a maker for GRPs and we found that expression of ActNotch in L2.3 suppressed its expression. Our previous results indicated that A2B5 expression increased in L2.3 cells with increased passage suggesting that these cells were acquiring characteristics of glial restricted precursors over time. The suppression of this effect by ActNotch suggests that it inhibits differentiation and prolongs the radial glial phenotype. In support of the latter idea, we found that expression of the radial glial marker BLBP remained high in L2.3 that expressed ActNotch. These results suggest that expression of ActNotch in radial glia retards their differentiation and stabilizes the radial glial phenotype. These results form a basis for future studies to test behavior of radial glia in vivo after being genetically modified in vitro.

PUBLICATIONS EMERGING FROM THIS RESEARCH

Hasegawa, K., Chang,Y.-W., Li, H., Berlin, Y., Kane-Goldsmith, N., Ikeda, O., and Grumet, M. Immortalization of radial glia cells delays their differentiation in vitro and in the adult spinal cord. Exp. Neurol 193: 394-410 (2005).

Li, H. and Grumet, M. BMP and LIF signaling coordinately regulate lineage restriction of radial glia in the developing forebrain. Glia 55: 24-35 (2006).