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Final Narrative Report

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1. ORIGINAL AIMS:

Embryonic stem (ES) cells are a multipotent source that can give rise to various types of cells including neurons and glia, and their transplantation into the injured rat spinal cord may promote recovery. Our hypothesis is that neural stem cells (NSC) can be derived from ES cells and after transplantation will improve recovery from spinal cord injury (SCI). We will pursue two Specific Aims to analyze the use of ES cells to promote recovery from SCI. The first aim is to isolate radial glial NSC derived from ES cells for transplantation in SCI. We will analyze this transition in culture using radial glial (RG) markers including BLBP and GLAST. This will yield ES-derived RG/NSC with defined fates in vitro that will be transplanted into the injured spinal cord; their effects on secondary damage and their ability to promote recovery from SCI will be determined. Based on our preliminary results we expect to develop conditions for isolating RG/NSC from ES cells that will improve recovery after acute transplantation following SCI. The second aim is to enrich for oligodendrocyte progenitors from ES-derived NSC and test whether they protect the spinal cord acutely following SCI and later promote myelination. The first aim is the main thrust of this project while the second will explore the feasibility of using ES-derived RG/NSC to protect acutely against secondary damage and later to promote myelination.

2-3. PROJECT SUCCESSES AND CHALLENGES, IMPLICATIONS FOR FUTURE RESEARCH

Aim I: To enrich and isolate RG/NSC from ES cells for transplantation in SCI

1a. Analysis of the Transition from ES cells to RG/NSC in Culture

After submission of our proposal, Smith's group published that the combination of EGF and FGF2 are sufficient for long term maintenance of NSC (Conti et al. 2005). We have independently derived NSC from ES cells by very similar procedures using limited growth in N2/B27 medium containing FGF2. We then proceeded to determine their fates upon differentiation with various factors as proposed. We have analyzed specific growth factors that promote (e.g. LIF) or suppress (e.g. BMP2) expression of A2B5 immunogenicity in neural precursors (Li and Grumet 2007). However, these studies were performed on NSC that express myc and resist differentiation and we developed an alternative approach (described below) to stabilize them as NSC for transplantation to avoid rapid differentiation in the spinal cord.

In response to the reviewers comments, we decided to omit experiments originally described in section 1b1 (see below) using low vs. high passage RG3.6 cells. Instead, we performed experiments using activated Notch1 (actNotch), which we had proposed as a method of inhibiting differentiation of radial glia in vitro as described in our responses to the reviewers comments (correspondence 11/21/05). We introduced actNotch into a radial glial-like clone called L2.3 (Li et al. 2004) and the resulting cells (NL2.3) expressing actNotch maintained radial-like morphology both in vitro and in vivo (Li et al. 2006). Given that Notch1 promotes radial glial identity in cortical cells (Yoon et al. 2004) and NSC derived from ES cells (Lowell et al. 2006), we tested whether a similar approach would yield radial glia from ES-derived NSC. Although actNotch-GFP (a fusion protein with GFP to facilitate localization by fluorescence imaging) was detected in the nucleus as expected and appeared to promote the radial glia morphology, this approach was complex since high levels of actNotch either inhibits cell proliferation or promotes NSC death, which made it difficult to isolate clones expressing actNotch from ES-derived NSC.

To circumvent this problem, we designed DNA constructs encoding for actNotch-GFP in a Tet-Off system that allows suppression of gene expression by doxycycline. Vectors were first constructed for packaging in 2SM2 lentiviral vectors and indeed we confirmed nuclear localization during the packaging in HEK293 cells. However, whereas high titers were obtained for GFP expression alone as a positive control, the large size of the actNotch-GFP yielded very poor titers, which resulted after transduction of ES-derived NSC in low numbers of GFP+ cells that grew slowly. Somewhat improved cell survival was observed when ES cells were transduced but the clones grew slowly making them difficult to isolate and work with. As an alternative strategy, we recloned the actNotch-GFP and TET-Off response elements into a two-

vector system that allowed isolation of transfected cells after transfection with amaxa and selection with antibiotics (hygromycin and zeocin for the two plasmids). This system has now been validated with control of actNotch-GFP expression by doxycycline in HEK293 and in NSC in ongoing experiments conducted by a graduate student Ms. Elina Tzatzalos, who is currently supported by a fellowship grant from the IGERT Stem Cell Grant at Rutgers (she also received a NJCSCR fellowship that has been placed on hold while she is supported by the IGERT grant).

Ib. Fate of ES-derived NSC in the injured spinal cord and their effects in SCI

We have performed experiments to fractionate neural progenitor cells on the basis of their expression of cell surface markers (Aim Ia3). We used A2B5, which along with 4D4, has been suggested to mark glial restricted precursors (Li et al. 2004; Noble et al. 2004). We have used magnetic bead sorting to separate different lineages of cells (Li et al. 2008a). Quantitation using RT-PCR confirmed the efficiency of selecting A2B5- cells, which are nestin+ and BLBP+ (Li et al. 2008b) and represent an enriched population of radial glial-like NSC that can be used to introduce genes such as actNotch, which retards their differentiation (Lowell et al. 2006). This method provides an alternative approach to isolate nestin+ and BLBP+ radial glial NSC by removing the major population of A2B5+ cells from ES-derived NSC, which appear spontaneously as these cells begin to differentiate.

To facilitate analyses of cell transplants on SCI, we proposed in Aim Ib to analyze histological parameters at 2 weeks following injury. We had previously shown statistically significant decreases in ED1 staining for macrophages in the injury site at 6 weeks following injury when rat spinal cord received RG3.6 cell transplants (Hasegawa et al. 2005). We now report that at 2 weeks following injury, ED1 staining is significantly lower in rats that received radial glial transplants compared to controls injected with media (Chang et al. in revision). This will facilitate analysis of treatment at 2 weeks after injury as predicted. Moreover, we have analyzed further some of the earliest signals generated by acute transplant of NSC within 30 min after spinal cord contusion and have found that several genes associated with tissue protection (Hsp70 and Hsp32) and neural cell development (Foxg1, Top2a, Sox11, Nkx2.2, Vimentin) were significantly up-regulated by comparison to control treatments within 12 hours (Chang et al. in revision). The results provide further support for the idea that enhancement of NSC-like activity by exogenously transplanted cells may also enhance endogenous spinal cord cell responses that may promote functional recovery acutely after SCI.

Aim II: Enrichment of oligodendrocyte progenitors (OP) in ES-derived NSC and SCI

As proposed we proceeded to develop vectors in collaboration with Dr. Rick Cohen to introduce olig1 and 2 genes into NSC but we have not pursued this approach because we heard and then read after publication that related neural precursors called glial restricted precursors (GRPs) expressing olig genes and A2B5 (after CNTF treatment) promoted allodynia whereas BMP-treated GRPs promoted functional recovery after spinal cord transection without inducing allodynia (Davies et al. 2008). This raised serious concerns that NSC expressing olig genes and A2B5 might not be appropriate cells for SCI transplantation. Therefore, we decided to concentrate our efforts on the approach (see above in Aim I) to introduce actNotch since it suppressed expression of A2B5 and oligo genes (Li et al. 2006). This further justifies the efforts to develop an appropriate system to introduce actNotch in a system that allows controlled expression by drug treatment (i.e. with doxycycline). While this approach has not been straightforward as described above, we now have an efficient amaxa transfection system that allows control of transgene expression and experiments to use it in ES-derived NSC are in progress. Proof of concept experiments demonstrating that NSC expressing actNotch persist for at least one month in the spinal cord have been performed (Li et al. 2006).

In summary, we have:

- 1) Analyzed responses in spinal cord contusion to transplants of NSC from 1-14 days acutely after injury and transplant; the results suggest activation of developmental programs in host cells occurs within the first 12 hours (Chang et al. in revision);
- 2) Isolated NSC from ES cells that have radial glial-like properties (Tzatzalos, et al., 2008b) and developed magnetic bead sorting methods to remove A2B5+ cells (Li et al. 2008b), which can induce allodynia;
- 3) Isolated NSC expressing actNotch-GFP, which localizes to the nucleus and suppresses cell differentiation in vitro (Tzatzalos, et al., 2008b) and in vivo (Li et al. 2008a);
- 4) Analyzed the effects of LIF and BMP on NSC and observed that A2B5 (4D4) antigen expression is suppressed by BMP and enhanced by LIF (Li and Grumet 2007).

4-5. IMPLICATIONS FOR FUTURE RESEARCH AND FUTURE PLANS

Our results with clones derived from primary brain NSC and using ES-derived NSC suggest that acute implantation of these cells can induce rapid signals in the contused spinal cord that activate genetic programs related to neural cell development. This is the first molecular evidence that rapid changes are generated in the contused spinal cord after transplantation of specific types of cells. The results suggest that endogenous spinal cord cells may be activated and may participate in recovery after SCI and future work is needed to explore this new facet of host responses in the injured spinal cord. Although the relationship between GRPs and radial glia NSC is still unclear (Li and Grumet 2007; Noble et al. 2004), the finding that BMP treatment in both populations suppresses expression of A2B5/4D4 antigens and oligo genes, suggests that this treatment may yield various types of cells that do not induce allodynia after transplant into SCI. Our recent observations that RG3.6 cells may induce allodynia (Li, Otsuka and Grumet, unpublished observations) may be explained by the fact that some of these cells express A2B5 and may resemble the CNTF-GRPs, which induced allodynia (Davies et al. 2008); thus, removal of the A2B5+ cells by magnetic bead sorting may be an important step in producing cells that do not promote allodynia. Alternatively, expression of actNotch, which promotes the radial glial NSC phenotype and suppresses expression of A2B5/4D4 antigens and oligo genes, may be an alternative approach to generate therapeutically applicable neural cells that promote functional recovery without promoting allodynia.

In conclusion, our plan is to continue to analyze the ES-derived NSC expressing ActNotch in a doxycycline-regulatable manner in vitro and in SCI. The ability to remove A2B5+ cells by magnetic bead sorting will ensure that cells resembling CNTF(LIF)-GRPs, which have been found to induce allodynia (Davies et al. 2008), will allow us to remove such cells if they persist and show these intolerable side effects that we can now assay for using Von Frey filaments for allodynia.

6. Publications emerging from this research (*reprints are enclosed for the published papers)

- Li, H.* and Grumet, M. (2007) BMP and LIF signaling coordinately regulate lineage restriction of radial glia in the developing forebrain. *Glia* 55: 24-35.
- Li H.*, Chang YW, Mohan K, Su HW, Ricupero CL, Baridi A, Hart RP, Grumet M. (2008a). Activated Notch1 maintains the phenotype of radial glial cells and promotes their adhesion to laminin by upregulating nidogen. *Glia* 56(6):646-58.
- Li H.*, Han YR, Bi C, Davila J, Goff LA, Thompson K, Swerdel M, Camarillo C, Ricupero CL, Hart RP and others. 2008b. Functional differentiation of a clone resembling embryonic cortical interneuron progenitors. *Dev Neurobiol* 68(14):1549-64.
- Tzatzalos, E., Thompson, K., Li, H., Moore, J., Cohen, R., and Grumet, M. (2008a) Control of Activated Notch1 Expression. Abstract to the Annual ISSCR Meeting, Philadelphia, PA.

- Tzatzalos, E., Thompson, K., Myung, Y., Li, H., Schachner, M. and Grumet, M. (2008b) Development of Binary DNA Vector System for Inducible Activated Notch Expression in Radial Glia. Abstract to the Annual 2008 NJ Stem Cell Research Symposium, 9/18-19/2008. Bridgewater, NJ
- Chang, Y.-W., Goff, L. A., H. L., Kane-Goldsmith, N., Tzatzalos, E., Hart, R. P., Young, W. and Grumet, M. Rapid induction of genes associated with tissue protection and neural development in contused adult spinal cord after radial glial cell transplantation. *J Neurotrauma* (in revision).

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