New Jersey Commission on Spinal Cord Research
INDIVIDUAL GRANT FINAL REPORT

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REPORT

1. Original aims of the project

1) Determine the requirement for p57^{Kip2} in spinal cord neuronal differentiation. Targeted knockout mutations of the *p57^{Kip2}* gene have demonstrated a diverse requirement for this factor in various tissues throughout the mouse embryo. We have recently found that p57^{Kip2} expression is restricted to subsets of spinal interneurons, and based on this we hypothesize that p57^{Kip2} is required for proper interneuron differentiation in the spinal cord. To test this we will examine the phenotype in existing *p57^{Kip2}* mutant mice to ascertain its requirement in controlling differentiation in spinal interneurons.

2) Determine whether p57^{Kip2} and p27^{Kip1} have overlapping functions in spinal cord neuronal differentiation. The p27^{Kip1} protein is closely related to p57^{Kip2}, sharing important conserved protein domains. p27^{Kip1} expression is detected in most or all post-mitotic neurons in the spinal cord. Together, these findings suggest the possibility that p27^{Kip1} and p57^{Kip2} could have overlapping roles in spinal cord neuronal differentiation. To test this, we will generate *p27^{Kip1} /p57^{Kip2}* double mutant mice and examine the spinal cord phenotype using approaches similar to those in Specific Aim 1.

3) Determine the ability of the p57^{Kip2} protein to direct interneuron-specific cell differentiation. The p57^{Kip2} protein is expressed in many classes of spinal cord interneurons but is specifically excluded from motor neurons. Our hypothesis is that p57^{Kip2} may play an instructive role in interneuron differentiation. To test this, we will mis-express p57^{Kip2} specifically in post-mitotic motor neurons in cultured mouse spinal cords, and assay whether transfected cells adopt characteristics of differentiating interneurons. This experiment will also allow us to test whether in vitro transfection will be a useful approach for specifically modulating the cell cycle in differentiating neurons in the spinal cord.

2. Project successes

We have been successful in fully completing the first two aims of our original proposal, and have adopted a new strategy to help us overcome problems encountered in implementing the experiments in Aim 3 (discussed below).

In Aim 1, we proposed to undertake a detailed analysis of spinal neuronal differentiation abnormalities that we identified in *p57^{Kip2}* mutant mice. The p57 protein is an important cell cycle regulator that we hypothesized plays a critical role in mediating the transition of dividing neural progenitor ("stem") cells into newly born neurons. Our analysis has led to two major findings: 1) *p57^{Kip2}* does not function alone in regulating neural progenitor cell cycle withdrawal, but is necessary for neurons to exit at the correct time during development, and 2) The p57 protein must be down-regulated in order for newly born neurons to differentiate properly into mature neurons. Follow-up preliminary data from this latter finding indicates that a specific part of the p57 protein is responsible for inhibiting neural differentiation and has been incorporated into a recently-submitted grant to the NJCSCR.

In Aim 2, we proposed to determine whether p57 and the closely related p27 cell cycle regulator that is co-expressed with p57 in many newly born neurons play redundant roles in mediating cell cycle withdrawal and/or differentiation. Our analysis has revealed the neither p27 nor p57 are required for neuronal precursor cell cycle exit in the spinal cord. These results indicate that the decision to exit the cell cycle and differentiate occurs prior to the expression of these factors in progenitor cells, an insight that will now allow a more refined focus on identifying the factors and mechanisms responsible for mediating this critical step in spinal cord neuronal production.

In Aim 3, we proposed to determine the function of the p57 protein in regulating spinal neuron differentiation by developing a transfection assay in cultured mouse spinal cord explants. While we have not been entirely successful at culturing mouse spinal cord tissue as planned, we have developed an alternative approach to addressing this issue using chick embryos and in ovo electroporation that will be described below.

In addition, results from the first two aims and from our alternative approach to aim 3 have been incorporated into a manuscript that is in preparation for submission to a major Neuroscience-related journal in the coming months (see attached).

3. Project challenges

The only significant challenge we encountered during the course of the proposed experiments was in culturing mouse spinal cord explants. We were able to cleanly excise segments of spinal cord tissue from early neurogenic-stage (E9.5) mouse embryos and to transfect these in vitro using an electroporation strategy similar to that used for transfecting chick embryos in ovo. However, upon culturing for 18-24 hours, we found that significant tissue distortion occurred in the tissue culture dish, even when a hydrated collagen matrix gel was used to provide additional 3-dimensional support to the explanted tissue. Even though transfected cells could be detected after this time, as judged by strong GFP reporter expression, our analysis was not informative since tissue distortion over the culture period prevented us from identifying specific cytoarchitectural features that distinguish progenitors from differentiated neurons. These problems prompted us to seek out an alternative strategy that should prove fruitful using chicken embryos in ovo to deliver ectopic p57 protein by transfection, preliminary data from which has been incorporated into an application that is pending at the NJCSCR.

4. Implications for future research and clinical treatment

The long-term goal of our work is to improve the success of stem cell replacement therapies to treating spinal cord injury (SCI) by characterizing the activity of an important regulator of neurogenesis in the embryonic spinal cord. Some of the most exciting strategies for restoring function following SCI involve therapies that seek to employ immature "stem cells" to replace the function of cells that are lost as a consequence of the trauma. Much attention has been focused on isolating and characterizing stem cells that can, when transplanted into the CNS, develop into fully functioning neurons. While promising, to date the success of these approaches has been limited, perhaps due to the fact that the adult spinal cord does not present a conducive environment for the formation of neurons from immature cells. Furthermore, most current studies focus primarily on the ability of transplanted cells to adopt a "generic" neuronal identity.

However, the mature spinal cord is composed of hundreds of distinct neuronal cell classes that are generated exclusively during embryonic development, Therefore, in order to restore even partial function, transplanted stem cells must do much more than simply differentiate into a neuron with an unspecified identity but rather must generate at a minimum the types of neurons that comprise the circuitry controlling movement and sensation

Neuronal stem cells possess the potential to generate the wide array of cell types that normally exist in the adult spinal cord. These cells are essentially specialized versions of normal spinal cord progenitor cells found in the developing embryo. Their utility as a therapeutic tool depends on preserving their full potential in vivo in adult injury sites; and understanding the steps that normal progenitor cells must undergo to generate neurons is critical to the success of this approach. The cell-cycle regulators being investigated in this proposal play a central role in regulating the transition of multi-potent progenitors into newly-born neurons with distinct phenotypic properties in the developing spinal cord, and are likely to be critically involved in this same process in transplanted stem cells. Therefore, understanding their functions could be crucial to the success of such SCI treatment strategies.

5. Plans to continue this research, including applications submitted to other sources for ongoing research

Our experiments have raised a number of interesting questions that we plan to pursue in the future and that will further our understanding of the important cell cycle/differentiation regulators that are under investigation. In fact we have recently submitted a 2-year grant proposal to the NJCSCR to continue this research as well as to extend it into several new and promising directions.

6. Publications emerging from this research

We are currently preparing a manuscript for submission within the next few months, and have attached a draft copy of this to the current final report.

PUBLICATIONS AND PRESENTATIONS

1. Poster presentation at the 2003 Society for Developmental Biology Mid-Atlantic Regional Meeting, "Cyclin-dependent kinase inhibitors regulate cell proliferation and neural differentiation in the developing vertebrate spinal cord"

2. Gui, H., Li, S. and Matise, M.P. (2004) $p57^{kip2}$ regulates timely cell cycle exit and differentiation in the developing vertebrate spinal cord (in preparation).