Final Narrative Report
Hyuk Wan Ko
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Aims:

The mammalian spinal cord is composed on many different types of neurons and glial cells that are organized along the dorsal-ventral and anterior-posterior axes and this organization is crucial for proper connectivity and thus motor and sensory functions. During embryonic development, key signals, such as the Sonic hedgehog (Shh) protein, are responsible for generating this pattern. I have been investigating how cells interpret Shh signals, which is useful for directing the differentiation of embryonic stem cells for transplantation therapy of spinal cord injury. The original aims of our project were to 1) characterize the role of primary cilia in Shh signaling in the context of neural differentiation of mouse ES cells and 2) to further characterize the effects of the cell cycle on Shh responses as it relates to transient phases of ciliogenesis. Several of the goals of the first aim were successful but technical problems prevented me from pursuing the work under the second aim. For this reason, I pursued two new lines of experiments that address the functional relationship between Shh signaling and primary cilia.

2. Project successes:

I examined the profile of ciliogenesis and Hedgehog responses during neural differentiation of ES cells. I found that the frequency of ciliogenesis increases dramatically in cells as they progress from the undifferentiated state (less than 10%) to the neural progenitor state (60-70%). In neural progenitors differentiated in vitro, cilia appear at a high frequency but this is still clearly below that we've seen on neural progenitors in vivo (90-95%) suggesting that important differences exist between neural progenitors in vitro and in vivo. I've stimulated the Hedgehog pathway during ES cell differentiation using the smoothened agonist SAG and found that a profound increase in Hedgehog pathway responsiveness correlates well with the profile of ciliogenesis during the differentiation process. In addition, I helped characterize the role of the Ift122, which is essential for proper assembly of primary cilia and Shh responses. We generated *Ift122* mutant ES cells and found that they inappropriately adopt ventralized fates when differentiated into neural progenitors in vitro and cell autonomously adopt ectopic ventral neural identities when differentiated in chimeric embryos (in vivo). Some of this work (on Ift122 ES cells) is included in a manuscript in preparation (Qin et al., in prep) and the remaining portion awaits characterization of ES cells completely lacking primary cilia (in progress). Because of technical difficulties associated with the second aim (see below), I pursued additional experiments to further study the relationship between primary cilia and Shh signaling. I am currently attempting to prevent disassembly of the primary cilium during the cell cycle of differentiating ES cells by disrupting the function of the tubulin deacetylase HDAC6, which has previously been shown to be required for this process in fibroblasts. My hypothesis is that preventing the disaasembly of the cilium in ES cells will increase overall fraction of ciliated ES cells and may thus allow for robust Shh responses during the differentiation process.

I generated high quality antisera against the transcription factor Gli2 (which mediates Shh signaling) that is suitable for immunolocalization, Immunoprecipiation,

and western blotting. My studies showed that Gli2 protein is localized at the tips of primary cilia in vitro and in vivo and, using this antibody, I was able to contribute to the characterization of three mutations in mouse genes studied by the lab, *FKBP8*, *Tulp3*, and *Ift122*. This resulted in my second authorship of three manuscripts, one which was published (Cho et al., 2008), another which is under revision for *Developmental Biology* (Norman et al.,), and a third which in preparation (Qin et al., in prep.).

In the process of Gli2 antibody characterization I tested Gli2 localization in *Gli2 (zfd)* mutant mice, which produce a nearly full-length forms of Gli2 lacking 3 zinc finger domains and a portion of the C-terminal flanking region. The expression level of mutant Gli2 protein is comparable with control wild type Gli2 yet this form is functionally null. Interestingly I found that this mutant form of Gli2 is unable to localize to cilia in vitro and in vivo. To further investigate the mechanism of Gli2 localization to primary cilia, I recapitulated the Gli2 localization of primary cilia in simple tissue culture system. I used commercially available engineered ligand mediated inducible system, which exogenously expresses 3HA tagged Gli2 (Gli2-3HA) at the level of 2-3 fold higher than endogenous Gli2 in NIH3T3 cells. Wile the wld-type version of Gli2 clearly localizes to tips of primary cilia, mutant versions failed to localize, consistent with my in vivo results. Using the in vitro system I set out to generate further small deletion to define the region responsible for Gli2 cilia localization. I mapped the zinc finger 5 (Znfn5) domain as an important region regulating cilia localization in Gli2. A small internal deletion of the Znfn5 domain blocked the cilia localization of Gli2. I am currently working to identify any element in Znfn5 domain regulating Gli2 cilia localization and transcription activity in Hedgehog signaling. Ultimately, I intend to test whether ciliary localization of Gli2 is required for its activity in mammalian cells by comparing and contrasting the activity of the mutant form in mammalian cells, which require cilia for Shh responses and Drosophila, which do not require cilia. This work should be submitted for publication in approximately one year.

In addition to the work discussed above, I have further pursued the relationship between cilia and Shh signaling through my studies of the *Broadminded* (*Bromi*) gene. Specifically, I studied the *Bromi* mouse mutation, which specifically disrupts responses of neural progenitors to the highest levels of Shh signaling in vivo. I found that the *Bromi* gene product is required at a step in the Shh pathway downstream of the Patched1 receptor and the Shh pathway inhibitor Rab23. In addition, genetic removal of the Gli3 repressor proves sufficient to restore appropriate activity of the Shh pathway in *Bromi* mutants. *Bromi* mutants generate primary cilia but these cilia exhibit a curled or branched morphology and show mislocalization of the transcription factor Gli2. I identified the *Bromi* gene through positional cloning/complementation and found that it encodes a novel protein with TBC and Armadillo repeat domains. Interestingly, I found through affinity purification/mass spectroscopy that the Bromi protein interacts with the cell cycle-related kinase (CCRK). This kinase has not been extensively characterized but its homolog in *Chlamydomonas*, called lf2 or long flagella 2, has been shown to regulate the length and morphology of the flagellum (which is functionally analogous to the mammalian primary cilium). I am currently investigating the relationship between

Bromi, CCRK, and a target of CCRK phosphorylation, MRK, in mammalian ciliogenesis and Shh responsiveness. I am also in the process of generating *Bromi* mutant embryonic stem cells to assay their responses to Shh pathway agonists in vitro. We are now beginning to prepare the first part of this work for publication (in which support from NJCSCR will be prominently acknowledged).

3. Project challenges.

The major challenge I faced related to the work proposed under Aim 2. This aim depended on developing and using a visualization system to determine the relationship between cell cycle phase with Hedgehog responses with high temporal resolution. Unfortunately, although I generated a Gli binding site-destabilized Venus construct for this purpose, I was not able to generate transgenic lines expressing Venus in a hedgehog-dependent manner. It seems that expression of the construct is very sensitive to integration site as other investigators in the field have told us they encountered similar problems with using Gli binding site constructs in stable cell lines. Because this prevented me from pursuing this aim, some of the data I obtained from Aim1 on ES cells and ciliogenesis must await further complementary studies before it is ready for publication. My findings are consistent with those of another study was recently published making a strong case for the role of cilia in Shh signaling in human ES cells (Kiprilov et al., *J. Cell Biol.*, 2008) but publication of that study has meant that I must obtain more compelling functional data before this portion of my work can be submitted.

4. Implications for future research

A significant body of research has shown that Sonic hedgehog is an extremely useful factor for directing the differentiation of embryonic stem (ES) cells into key cell types in the spinal cord such as motorneurons. Indeed, ongoing research is exploiting the use of Shh and other factors to generate large numbers of differentiated neurons from human ES cells for the treatment of spinal cord injury through transplantation. Nevertheless, our understanding of how this potent factor signals in mammalian cells is far from clear preventing us from precisely controlling the level of signaling activity to generate the full range of neural cell types from differentiating ES cells.
For example, methods using retinoic acid, aimed at differentiating ES cells into spinal rather than rostral neural character, greatly reduce the efficiency of generating ventral neural cell types such as floor plate cells, V3 interneurons and motorneuron subtypes. We propose that a better understanding the cellular context of embryonic stem cells, specifically with respect to the generation of primary cilia, will allow us to efficiently and precisely control the Shh signaling pathway so that the full spectrum of spinal neural cell types can be generated in large numbers from differentiating ES cells in vitro.

5. Plans to continue this research

We are currently pursuing many aspects of the work described above. First and foremost, we are working to assemble a manuscript based on the role of Bromi in Shh signaling and ciliogenesis that should be submitted within the next two months. This

work will lay the groundwork for submitting a new proposal aimed at understanding the precise relationship between Bromi, and the CCRK and MRK kinases with respect to ciliagenesis as well as further investigation of how appropriate ciliary structure affects the behavior (function, post-translational modification, and localization) of Shh pathway signaling components within the developing cilium. In addition, we are continuing to pursue the importance of Gli2 localization to the cilium in the context of an Individual Research Grant awarded to Dr. Eggenschwiler. Finally, I am now attempting to increase Shh responses in differentiating ES cells by inhibiting (through shRNA and pharmacological approaches) the activity of the tubulin deacetylase HDAC6 so that cilia may persist longer within the cell cycle.

6. Publications emerging from this work:

a) Cho A, Ko HW, Eggenschwiler JT. (2008). FKBP8 cell-autonomously controls neural tube patterning through a Gli2- and Kif3a-dependent mechanism. *Dev Biol.* Sep 1;**321**(1):27-39. PMID: 18590716

b) Norman, R.X., Ko, HW, Huang, V., Eun, C.M., Naggert, J. and Eggenschwiler, J.T. Tulp3 controls neural patterning in the mouse through inhibition of Shh signaling. (draft—to be submitted 11/8/08 to *Hum. Molec. Genet.*)

c) Qin, J., Ko, HW, Lin, Y., Paxour, G, and Eggenschwiler, J.T. Mouse intraflagellar transport Protein 122 is required for Shh pathway inhibition by promoting retrograde transport within primary cilia. (in preparation)

d) Ko, HW, Tran, J., and Eggenschwiler, JT. High-level Hedgehog responses in the mouse rely on Broadminded, a factor controlling ciliary structure and CCRK activity. (for submission in Dec. 08).