Final Narrative Report
Cho, Ahryon
Grant Number: 05-2911-SCR-E-0

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The Role of FK506-Binding Protein 8 in Mammalian Neural Patterning and Axon Guidance

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1. Original aims of the project

This project was aimed to discover the action of FK506-binding protein 8 (FKBP8) in Sonic hedgehog signal transduction which is crucial for the appropriate neural patterning in mammals. Understanding the regulation of cell identity specification in the developing spinal cord is important for therapeutic applications aimed at differentiating Embryonic Stem (ES) cells for transplantation therapy in the treatment of spinal cord injury.

Cells in developing neural tube determine their neuronal identities in response to extracellular instructive signals. One of those signals is Sonic hedgehog, a mammalian homolog of *Drosophila* hedgehog. Neural tube cells receive and interpret Shh signals through transmembrane proteins such as Patched and Smoothened, then transmit the information to the cell's nucleus. This results in the expression of many target genes controlling cell identities driven by the Gli transcription factors. Between signaling events at the plasma membrane and transcriptional events in the nucleus, many proteins are involved in the tightly regulated relay of Shh signaling information, some of which belong to intraflagellar transport system (IFT). However, most Shh signaling events inside the cell are still unclear with respect to the players and their functions. Surprisingly, the targeted mutagenesis in Fkbp8, which encodes a member of immunophilin FK506-binding protein family, leads to the expansion of Shh-dependent ventral cell types at the expense of dorsal neural subtypes in the neural tube. This result suggest that FKBP8 functions as a negative regulator (or "brake") for the Shh pathway. Thus, given the ventralized neural patterning in Fkbp8 mutants, I have carried out experiments to satisfy these purposes under this fellowship.

(1) to investigate at which step in Shh signaling pathway FKBP8 functions
(2) to understand the biochemical mechanism by which FKBP8 fulfills its antagonistic role in Shh pathway
(3) to address the role of FKBP in guiding the differentiation of embryonic stem cells into specific neuronal cell types found in the spinal cord.
(4) to investgate the role of FKBP8 in axon guidance in the spinal neural tube.

2. Project successes

(1) FKBP8 regulates Shh pathway at a step downstream of Smoothened (Smo) and upstream of IFT and Gli2.

a) I found that in *Smo/Fkbp8* double mutants, even though Shh is not expressed, ventral, Shh- and Smo-dependent cell fates are abundantly specified and dorsal cell types are reduced/restricted. This patterning phenotype resembles that of *Fkbp8* single mutants and directly contrasts with that seen in *Smo* mutants. . This indicates that FKBP8 has an important role in repressing the activity of the Shh pathway independently of upstream stems in the Shh pathway involving Shh, Patched, and Smoothened.

b) The neural patterning phenotype of *Gli2/Fkbp8* double mutants is almost identical to that of *Gli2* single mutants, opposite to that seen in *Fkbp8* single mutants. Thus, disruption of

Gli2 activity prevented the ventralizing effets caused by mutation of *Fkbp8*, demonstrating that FKBP8 antagonizes Shh signaling by a mechanism dependent upon Gli2.

c) Kif3a is a subunit of Kinesin II, an antrograde IFT motor that is required for generating primary cilia and activation of the Shh pathway. To test whether FKBP8 requires IFT and cilia function to regulate Gli2 protein, we generated *Kif3a/Fkbp8* double mutants. I found that *Kif3a* single mutants and *Kif3a/Fkbp8* double mutants have the identical dorsal-ventral neural patterning phenoypes. This suggests that Fkbp8 regulates the Shh pathway at a step that id dependent on intraflagellar transport and the generation of primary cilia.

These data are part included in a manuscript (Cho et al., 2008) listed below

(2) FKBP8 is unlikely to regulate post-translational modification, stability, and nuclear transport of Gli2 in a direct manner.

Because the epistasis studies clearly showed that FKBP8 regulates the Shh pathway in a Gli2-dependent manner, I assayed the status of endogenous Gli2 protein in wild-type and *Fkbp8* mutant embryos. Although I was successful in monitoring several aspects of Gli2, such as protein levels and mRNA expression, electrophoretic mobility (indicative of post-translational modification), and nuclear localization, *Fkbp8* mutants showed no changes in any of these. In collaboration with Hyuk Wan Ko in the lab, I also monitored Gli2 subcellular localization in *Fkbp8* mutants. We found that Gli2 clearly localizes to the tips of primary in vivo and in vitro but this aspect does not appear to be perturbed in *Fkbp8* mutants. Moreover, co-imnunoprecipitation experiments showed that Gli2 and FKBP8 do not physically associate in the cell.

This suggests that FKBP8 may regulate Gli2 activity through other regulators of Gli proteins. I began to test this possibility by analyzing the Gli antagonist Suppressor of Fused (Sufu). However, *Fkbp8* mutants showed normal levels of Sufu protein and the physical association between Gli2 and Sufu (assayed by coimmunoprecipitation) was intact in these mutants. Collectively, these data suggest that FKBP8 does not control Gli2 function through the currently known modes of regulation. However, as more data accumulate, we in the field recognize that endogenous control of Gli2 activity by Shh signals must involve modes that have yet to be discovered. As more of these modes are revealed, such as the recent identification of Dyrk2-mediated Gli phosphorylation, we are confident that FKBP8 function can be fit into the mechanism.

(3) Establishing homozygous *Fkbp8* mutant embryonic stem cells.

For neural differentiation experiments, we established marked homozygous *Fkbp8* mutant embryonic stem cells, as well as control cell lines. We then assayed the effects of the Fkbp8 mutation on differentiation of ESCs both in vitro and in vivo. We found that loss of Fkbp8 has only modest effects on cell fate specification during in vitro differentiation in comparison to the clear effects seen in Fkbp8 mutant animals. We believe this is due to the

fact that loss of Fkbp8 results in potent ventralization in vivo by two modes – a cell autonomous and a non-cell autonomous mode (see below), and that the non-cell autonomous mode is not applicable in vitro.

(4) Loss of FKBP8 in vivo results in neural ventralization by two modes.

With the marked *Fbkp8* mutant ESCs, we generated the chimeric embryos consisting of a mix of mutant and wild-type cells. The neural patterning of these chimeric embryos was assayed with various neural tube markers and we found that the gene product of Fkbp8 changes neural cell identity by a cell-autonomous and a non-cell-autonomous mechanism. Subsequent experiments indicated that while the cell autonomous mechanism involves the Shh pathway directly, the non-cell autonomous mechanism involves suppression of Bone Morphogenetic Protein production through Gli2, resulting in the loss of dorsalizing signals. These data are included in Cho et al., 2008.

(5) Model for the action of FKBP8 in neural patterning (Figure 1)

Based on our data, we propose a model to explain how Fkbp8 controls cell fate in the neural tube (figure 1). FKBP8 is required primarily for restricting the activity of Gli2 at a step downstream of Smo. This, in turn, prevents activity of the Shh pathway outside of the normal domains. In addition, keeping Gli2 in check also allows for the normal expression of the BMPs, which act to counter the effects of Shh and allow for dorsal neural fates to be specified.

(6) A role for Shh signaling in the balance between neural progenitor proliferation and differentiation.
Shh signaling is traditionally thought to promote progenitor proliferation and to inhibit differentiation in the neural tube. However, I found that the Fkbp8 mutant neural tube shows excessive neural differentiation and reduction of proliferating progenitors despite showing potent upregulation of the Shh pathway. These effects appear to result from increased Shh pathway activity because they are suppressed in Fkbp8/Gli2 double mutants. Moreover, a similar shift in favor of differentiation is seen in mutants lacking other Shh pathway antagonists (*Rab23* and *Tulp3* mutants). This suggests that although moderate levels of Shh are required to promote proliferation (via factors such as the Cyclin D proteins), very high levels of activity can promote terminal differentiation at the expense of continued proliferation. These data are included in Cho et al (2008).

3. Project challenges

In my original proposal, I proposed several experiments based on the relationship between FKBP8 and calcineurin phosphatase. Since then, there have been studies showing that FKBP8 does not interact with calcineurin. Consistent with these studies, I was unable to observe any significant results which support the hypothesis FKBP8 regulates Shh

signaling through the inhibition on calcineurin. Although these studies occupied a good amount of effort, we have been able to move away from this hypothesis and concentrate on other potential modes of regulation. While my studies to identify the mechanism by which FKBP88 regulates Gli2-dependent target gene expression have resulted in generally negative findings, they have served to focus our attention on other (new) modes of regulation.

I also investigated the effects of the *Fkbp8* mutation on axon guidance in the spinal neural tube. While I found a number of pronounced defects in the projections of both motor neuron (efferent) axons and sensory (afferent) axons, those data indicate that the effects are likely to be indirect results of Shh-dependent changes in Shh-dependent neural cell identity rather than a separate mechanistic role for FKBP8 in axon guidance because 1) the patterns of axon trajectory defects were rescued in *Fkbp8/Gli2* double mutants (in which the ventralization of neural cell fates was suppressed) and 2) we observed qualitatively similar defects in axon guidance in other mouse mutants showing ventralized patterning phenotypes due to up-regulation of Shh signaling.

4. Implications for future research and/or clinical treatment

These experiments have brought to light several important aspects of the Shh pathway relevant for Shh-mediated differentiation of embryonic stem cells. First, we recognize that there appear to be many modes of regulating the activity of the Gli transcription factors downstream of Smoothened extending beyond simple control of Gli stability and nuclear localization. This highlights the need to explore these other modes so that tight control is feasible and factors such as FKBP8 serve as a gateway into understanding these still mysterious mechanisms. Second, we recognize that activity of the Shh pathway can alter neural fates in vivo by indirect mechanisms, such as control of *Bmp* gene expression, in addition to the more direct mechanisms previously studied. Third, we recognize that Shh signaling has a more complex relationship between neural proliferation and differentiation than previously thought. Thus, we now need to consider the possibility that methods of achieving very high levels of Shh pathway activity during the in vitro differentiation of ES cells may facilitate, rather than inhibit, efficient terminal differentiation. Thus, our previous concerns that manipulation of the pathway to produce the most ventral neural cell fates (floor plate and V3 interneurons) would result in potent cell fate change but lack of terminal differentiation may, in fact, be unwarranted.

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

I have finished my studies and defended my Ph.D. thesis at Princeton University and I have since moved backed to my home in South Korea. However, my advisor, Jonathan Eggenschwiler, would like to proceed with this work focusing on the mechanism of FKBP8 control of Gli2 activity as well as investigating the mechanistic basis for how high levels of

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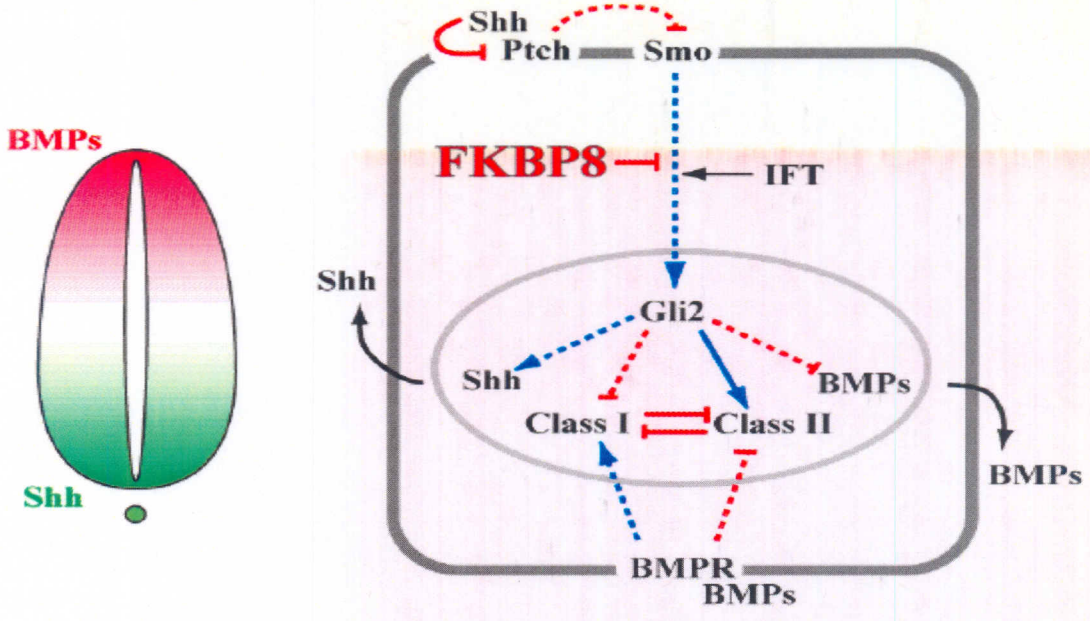
Shh signaling promote neural differentiation. The lab is now investigating how Shh signaling acts to recruit Gli2 to the promoters of Shh target genes and hopes to use this information to investigate how FKBP8 fits into this story.

6. List and include a copy of all publications emerging from this research, including those in preparation

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

Figure 1.



Neural tube patterning is governed by two major instructive signals. BMPs from the roof plate act as dorsalizing factors, whereas Shh produced in the floor plate functions as a ventralizing factor. BMPs and Shh form reciprocal gradients of the activity along dorsal-ventral axis of the neural tube. In the neural tube cell, FKBP8 antagonizes the Shh signaling pathway at a step that is independent of Smoothened but dependent on kinesin II-mediated intraflagellar transport and Gli2. As a result of FKBP8 function, Gli2 is prevented from directly activating Shh target genes (class II genes) outside of their normal domains. In addition, inhibition of Gli2 activity also allows for proper expression of the *Bmp* genes, which, in turn, is required to counteract the effects of Shh on neural pattern via an indirect (non-cell autonomous) pathway.