Appendix I

Microarray analysis of *Olig* mutant

The rationale for conducting a gene chip screen on FACS sorted GFP+ cells from *Olig1,2* mutant spinal cord has been discussed in Chapter 5. I will hereby focus on describing details of the procedure. Some preliminary results will also be presented.

Sample preparation for gene chip analysis

[1] Embryos from E13.0 or E14.5 *Olig1,2* het x het mating are phenotyped by X-gal staining of the head. Spinal cords from animals of different genotypes are dissected out separately.

[2] Dissociate spinal cords into a single cell suspension with Papain (Worthington), resuspend in FACS sorting buffer at 0.5-2 million cells/ml.

[3] FACS sorting with GFP auto-fluorescence; collect at least ~3,000 cells.

[4] Isolate RNA immediately with Absolute RNA Microprep Kit (CAT# 400805) from Stratagene.

[5] 1st and 2nd strand synthesis, amplify one round with MessageAmp aRNA Kit (CAT#1750) from Ambion, o/n rxt ~16 hr, yields ~3-6ug of aRNA per 10,000 cells.

[6] Amplify 2nd round with biotin-16-UTP and biotin-11-CTP with MessageAmp kit, starting with 1ug of 1st round aRNA, o/n rxt ~14 hr, yields up to 100ug of labeled aRNA.

[7] Fragment 30 ug of labeled aRNA at 0.5 ug/ul for 30’ as recommend by Affymatrix, average size~80-100 bp (3% non-denaturing gel against a RNA marker, sample denatured at 65C for 5’), this should be sufficient for one set of A, B, and C chips.

[8] Hybridize onto Affymetrix murine gene chips.

[9] Data analysis with Affymatrix Suite software. A gene is defined as differentially regulated if the average fold change between +/- and -/- samples is over 3 and the average difference is over 50 (the mean average of the whole chip is defined as 200).

To minimize cell death, a fast and reliable method is needed to determine the genotypes of the embryos. We decided to use X-gal staining of the head. Wild type embryos have no staining, while *Olig1,2 +/-* heterozygotes have weaker staining than *Olig1,2 -/-* homozygotes if they are developed in X-gal in the same amount of time. Due to variation among individuals, the distinction between heterozygotes and homozygotes was not always clear cut. In case of ambiguity, the embryos in question were either discarded, or processed individually. Their genotypes were confirmed later by PCR genotyping.

To reduce the system noise inherent in microarray analysis, at least two independent comparisons were performed with independent sample collection and preparation.

E14.5 screen

We conducted our first gene chip comparison with cells sorted from E14.5 mouse spinal cord (Figure 6). There are several reasons that this specific stage is chosen. First, we wish to examine the role of Olig in glial precursors. Due to persistence of the Histone-GFP protein, GFP+ motoneurons (in +/-) and interneurons (in -/-) are detectable 1-2 days after they have been produced. By E14.5, however, GFP protein is no longer detectable in these neurons. The GFP+ cells at this stage are therefore 100% glial precursors. Secondly, a preliminary FACS analysis of spinal cords from different stages suggests that at E14.5, GFP+ cells can be recovered at a reasonable percentage (~1% of total cells).

We also realized that there are potential drawbacks to use cells at this stage. The main concern is that by E14.5, GFP+ oligodendrocyte precursor cells have largely left the ventricular zone in heterozygotes, whereas in homozygotes, the majority of GFP+ cells (putative astrocyte precursors) are yet to migrate. Compared with cells inside the

ventricular zone, the ones outside exhibit many molecular differences in cell adhesion and cell cycle. Our comparison may therefore yield many candidates that reflect these non-specific differences. Most of which have nothing to do with *Olig* genes or glial specification.

Our analysis at E14.5 yielded over 1000 candidate genes that were either up- or down-regulated in the absence of *Olig1,2* using A, B and C chips. Large numbers of these are cell-cycle related and adhesion molecules, potentially false positives as discussed above. Nevertheless, several genes known to be expressed by oligodendrocyte progenitor cells, such as PDGFRa, Sox10 and Ng2 were down-regulated in the -/- sample. In contrast, glutathione transferase, an astroglial marker, was up-regulated in the -/- sample.

We next used in situ hybridization to assess which of the differentially expressed genes are truly regulated by *Olig*, focusing on transcription factors and molecules with demonstrated functions in the nervous system. Our preliminary results suggest that most of the candidate genes have real mRNA level differences between the two samples compared, but only a small number is regulated by *Olig*. These putative *Olig* target genes are being further studied.

E13.0 screen

To avoid the ventricular versus non-ventricular problem, ideally cells should be taken at a stage when they are all inside the ventricular zone in both *Olig1,2 +/-* and *-/-* animals. This calls for isolating cells from younger embryos. The younger the embryos are, however, the more likely that neurogenesis in the ventral spinal cord has not finished completely so that the isolated GFP+ cells may be contaminated with neurons. Moreover, younger embryos yield less cells and the percentage of GFP+ cells drops as well. After balancing all these factors, we decided to use embryos at E13.0. At this stage, neurogenesis in the ventral spinal cord has mostly finished. In addition, all GFP+ cells in homozygotes and most GFP+ cells in heterozygotes are inside the ventricular zone. What about the small number of GFP+ cells that have already migrated in

heterozygotes at E13.0? To eliminate these cells, we took advantage of the fact that PDGFRa is expressed in all migrating oligodendrocyte precursor cells (OPC), but not in most of the OPCs in the ventricular zone. By FACS sorting with both GFP and PDGFRa, the GFP+PDGFRa- ventricular population of OPC can be specifically isolated (Figure 6).

Microarray analysis of GFP+PDGFRa- populations at E13.0 revealed that the total number of differentially expressed genes is much less than that from the E14.5 screen. For example, large numbers of cell-cycle related genes found in the E14.5 screen are not present in the E13.0 comparison. This suggests that the two sorted populations of cells at E13.0 are much more similar to each other that the ones from E14.5, thus validating our optimization efforts.

Genes involved in motoneuron-interneuron development were found in the E13.0 comparison, for example, Irx3 and chx10 are up-regulated in the -/- sample. This result suggests that V2 interneuron generation may still be ongoing at E13.0 in the ventral spinal cord. Alternatively, interneuron generation may have completed by E13.0, but GFP protein persists in these interneurons. Most of the putative *Olig* target genes uncovered in the E14.5 screen are conserved in the E13.0 screen. Thus, despite various caveats for both screens, similar results are obtained.

Functional assays

Different functional assays can be devised for different candidate *Olig* downstream target genes according to their putative functions. For example, genes up-regulated in the absence of *Olig* could be astrocyte genes, or repressors of oligodendrocyte fate. Putative astrocyte genes can be recognized by their co-labeling with established astroglial markers such as GFAP and S100b *in vivo* and *in vitro*; a repressor of oligodendrocyte can be revealed by expressing this gene in purified neural stem cells in culture and allow the NSCs to differentiate. As NSCs can give rise to neurons, oligodendrocytes and astrocytes in culture, ectopic expression of an oligodendrocyte repressor should abolish the generation of this glial type from NSC cultures without affecting neuronal and astroglial fates.

Similarly, genes down-regulated in *Olig* null mutants could function as activators of oligodendrocyte fate. In this case, a good assay is to express these candidate activators in neurospheres established from *Olig1,2 -/-* double mutants. As *Olig1,2 -/-* mutant neurospheres lose the ability to generate oligodendrocytes, if this ability is restored by the ectopic expression of any of the candidate activators, this is strong evidence that this particular gene acts down stream of *Olig* in promoting oligodendroglia specification.

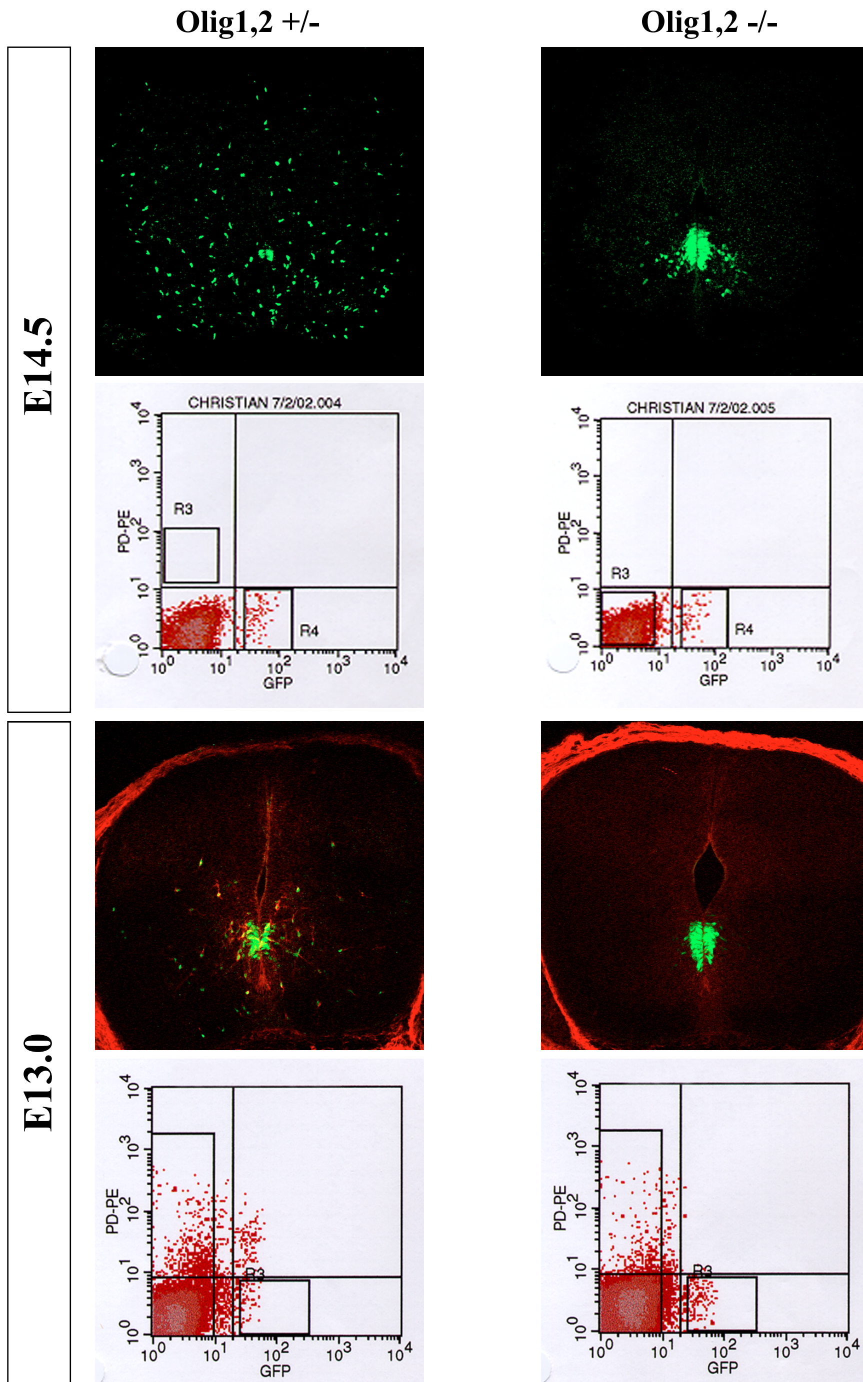

Figure 6. FACS sorting for microarray analysis