FINAL NARRATIVE REPORT

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Role of Foxn4 in Spinal Cord Development and Regeneration

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A. Original Aims of the Project

The long-term objective of this proposal is to understand the molecular events that control the specification and differentiation of sensory and motor neurons during development and regeneration of the spinal cord. This application focuses on the role of Foxn4, a forkhead/winged helix transcription factor, in spinal neurogenesis. Foxn4 is expressed transiently by a subset of interneurons and their progenitors in the developing spinal cord and **has been hypothesized to play a key role in fate specification and/or early differentiation of a distinct spinal interneuron subtype.** The studies outlined in this proposal are designed to test this hypothesis using a comprehensive approach in the mouse and chick systems. Two specific aims will be pursued:

i) **To investigate the biological function of Foxn4 during spinal cord development by targeted gene disruption.** The proposed experiments aim to uncover the role of Foxn4 during neurogenesis of the spinal cord using a loss-of-function approach involving the production and characterization of *Foxn4* knockout mice.

ii) **To analyze the role of Foxn4 in the specification of interneurons by overexpression in spinal cord progenitor cells.** The planned experiments aim to understand the function of Foxn4 during spinal cord development using a gain-of-function approach involving retrovirus-mediated overexpression of Foxn4 in progenitors of the chick neural tube.

B. Project Successes

a. Foxn4 Is Expressed in a Subpopulation of p2 Progenitor Cells

As a first step to understand the role of Foxn4 during mouse spinal cord development, we characterized the types of cells that express Foxn4 by immunostaining. Starting from E9.5 and at E10.5-E11.5, Foxn4 is prominently expressed in a small cluster of cells located primarily within the ventral ventricular zone. These cells co-express Pax6, Mash1 and Lhx3 but not Gata3 or Chx10 even though they are positioned at the level of $Gata3^+$ or $Chx10^+$ cells. Because Chx10 marks the V2a interneurons and Gata3 is a marker of the V2b neurons, these results indicate that Foxn4 is expressed in a subset of cells within the p2 progenitor domain. Consistent with this, Foxn4-expressing cells are located ventral to $En1^+$ V1 interneurons but dorsal to $Nkx2.2^+$ V3 interneurons and $Hb9^+$ and $Isl1/2^+$ motor neurons. In addition, most $Foxn4^+$ cells are mitotically active as they can be labeled by short pulses of BrdU; only a small number do not incorporate BrdU in these experiments and thus may be postmitotic. A small number of $Lhx3^+$ or $Mash1^+$ cells are found to express Gata2. Similarly, Gata2 and Chx10 proteins are detected in some $Foxn4^+$ cells and/or their progeny marked by β-galactosidase (β-gal) expressed from the knock-in *lacZ* reporter in $Foxn4^{lacZ/+}$ mice, indicating that both Foxn4 and Mash1 may be expressed in a subset of p2 progenitors which can give rise to either V2a or V2b subtypes.

b. Loss of Foxn4 Function Causes a Complete Switch in Cell Fate of p2 Progenitors

To investigate the requirement of *Foxn4* for neurogenesis in the ventral spinal cord, we followed the fate of cells that would normally express Foxn4 in *Foxn4*$^{lacZ/lacZ}$ mutants. In heterozygotes, X-gal staining showed β-gal activity in the mesencephalon and rhombencephalon and throughout the spinal cord at E10.5-E11.5, in a pattern closely resembling that revealed by RNA in situ hybridization. In the spinal cord, β-gal expression, which is largely confined to the ventricular zone with some weak activity in the ventrolateral funiculus, begins to be downregulated at E12.5 and disappears by E13.5. *Foxn4* null mutants exhibited a similar pattern of *lacZ* expression in the developing CNS at E10.5-E11.5. In the spinal cord, however, *lacZ* is expressed by a much broader, more lateral group of cells that project their axons in the ventrolateral funiculus. Moreover, *lacZ* expression is not completely downregulated until E14.5, similar to a delay in the downregulation of *lacZ* expression in *Foxn4*$^{lacZ/lacZ}$ retinas.

We next analyzed the identity of *lacZ*$^{+}$ cells in E10.5-11.5 *Foxn4*$^{lacZ/lacZ}$ spinal cords using antibodies against a series of cell type-specific markers. There was a complete absence of cells expressing Mash1, Gata2 or Gata3 in the mutant (Fig. 1A-F and Fig. 2F), indicating a total failure in the genesis of V2b interneurons. This notion was further supported by the failure to detect *Gata2* RNA in situ signals in the *Foxn4*$^{lacZ/lacZ}$ spinal cord. In contrast, there was on average a 3-fold increase in the number of Chx10^{+}/Lhx3^{+} neurons that simultaneously expressed

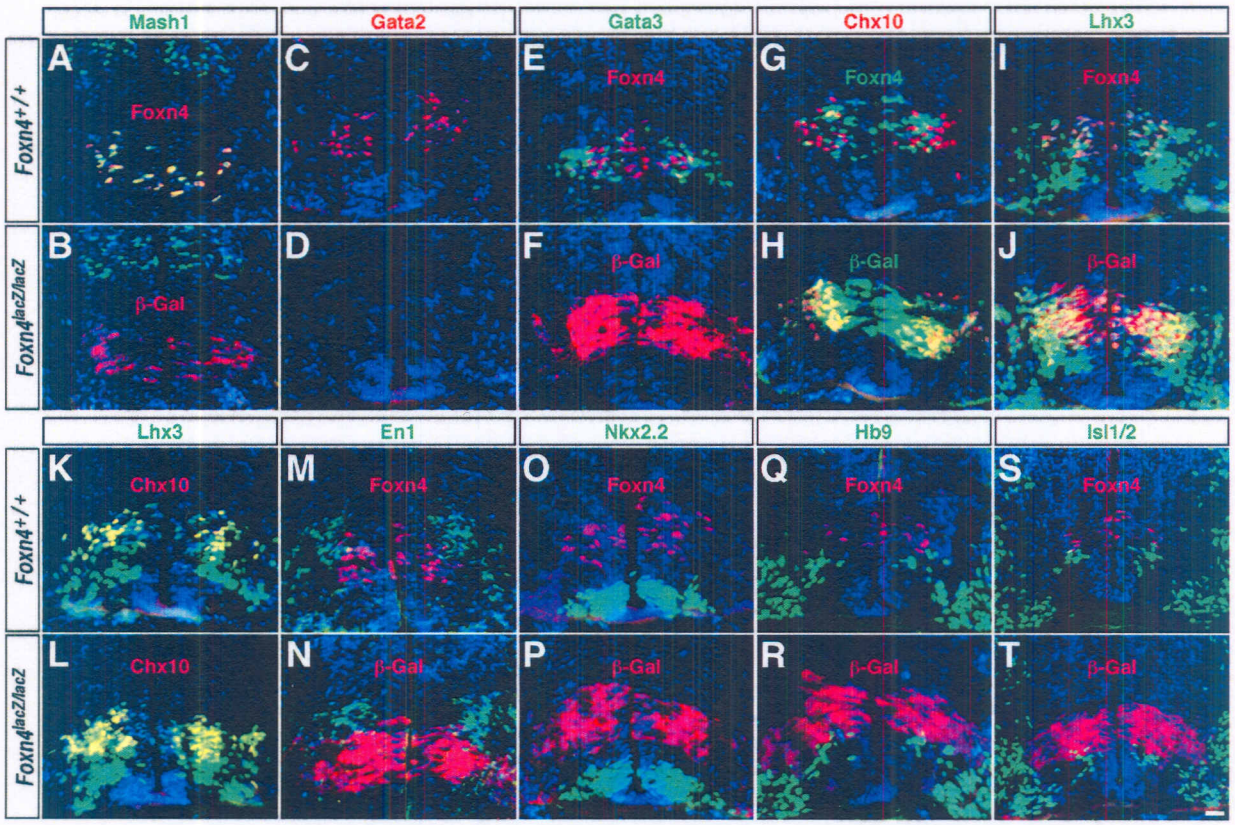

Fig. 1. Loss of V2b interneurons in the absence of *Foxn4*. (*A-T*) Matching spinal sections from *Foxn4*$^{+/+}$ and *Foxn4*$^{lacZ/lacZ}$ E10.5 (*A-P*) or E11.5 (*Q-T*) embryos were immunostained with the indicated antibodies along with weak counterstain by DAPI (blue). Loss of *Foxn4* function resulted in a complete loss of cells immunoreactive for Mash1, Gata2 or Gata3 but a dramatic increase of cells immunoreactive for Chx10, Lhx3 and β-galactosidase (*A-L*). There was no effect on the number of cells immunoreactive for En1, Nkx2.2, Hb9, or Isl1/2 (*M-T*). [Bar = 25 μm (*S* and *T*) and 18.8 μm (*A-R*).]

β-gal (Fig. 1G-L and Fig. 2F), indicating that p2 progenitors that would normally express Foxn4 now generate only V2a but not V2b neurons in the mutant spinal cord. Loss of *Foxn4* function has no effect on the generation of V1, V3 or motor neurons as no changes were detected in the number of neurons expressing En1, Nkx2.2, Hb9, or Isl1/2 in the null mutant (Fig. 1M-T and Fig. 2F). Similarly, it does not affect the expression of pre-pattern homeodomain factors Pax6 or Irx3 that are involved in the partitioning of p2 progenitor domain.

c. *Loss of Mash1 Function Causes an Incomplete Switch in Cell Fate of p2 Progenitors*

Given the co-expression of Foxn4 and Mash1 and the dependence of Mash1 expression on Foxn4 in p2 progenitors (Fig. 1), we examined the role of Mash1 in the specification of V2 neurons in *Mash1*$^{-/-}$ mice. Foxn4 expression did not change in *Mash1* null mutant (Fig. 2 *A* and *B*), indicating that *Foxn4* acts genetically upstream of *Mash1*. However, there was a significant reduction of Gata3^{+} and Gata2^{+} neurons, concomitant with a significant increase of Chx10^{+} or Lhx3^{+} neurons in *Mash1* null mutants (Fig. 2A-D). On average, the number of Gata3^{+} neurons was reduced by 52% while Chx10^{+} neurons were increased by 33% in mutant embryos (Fig. 2E). Thus, the neurogenesis defect in *Mash1*$^{-/-}$ spinal cords partially mimics the *Foxn4* mutant phenotype, suggesting that Foxn4 function is partly mediated by Mash1.

In summary, the subset of p2 progenitors (p2b) that give rise to V2b neurons require the expression of Foxn4 and Mash1 (Fig. 2G). Loss of *Foxn4* function abolishes the expression of Mash1, leading to

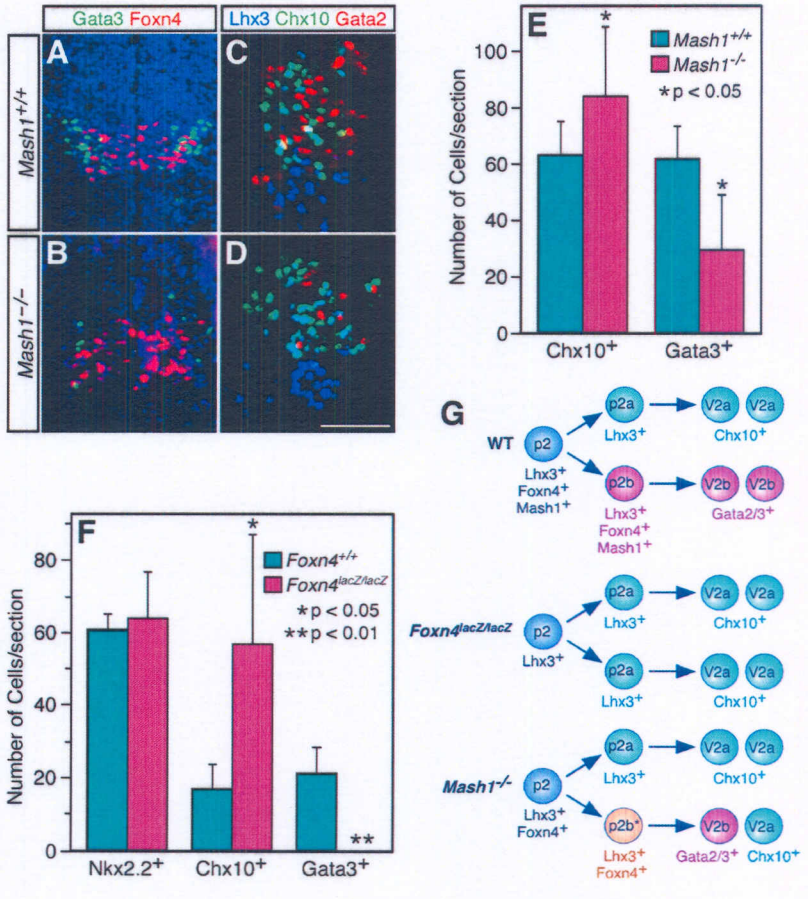

Fig. 2. Absence of *Mash1* or *Foxn4* both affect the generation of V2 neurons. (*A-D*) Matching spinal sections from *Mash1*$^{+/+}$ and *Mash1*$^{-/-}$ E10.5 embryos were immunostained with the indicated antibodies. Loss of *Mash1* function causes a significant decrease of neurons immunoreactive for Gata3 or Gata2, a significant increase of neurons immunoreactive for both Chx10 and Lhx3, but no change of Foxn4^{+} cells. (*E* and *F*) Quantitation of neurons that are immunoreactive for Chx10, Gata3 or Nkx2.2 in E10.5 *Mash1*$^{+/+}$ and *Mash1*$^{-/-}$ (*E*) or *Foxn4*$^{+/+}$ and *Foxn4*$^{lacZ/lacZ}$ (*F*) spinal cords. Each histogram represents the mean ± SD for 3-6 spinal cords. (*G*) Summary of changes in V2 cell fate in *Foxn4* and *Mash1* mutant spinal cords. [Bar = 50 μm (*A* and *B*) and 20 μm (*C* and *D*).]

a switch in progenitor fate from p2b to p2a, which expresses only Lhx3, a determinant of V2a neurons (Fig. 2G). Loss of *Mash1* function results in the formation of "p2b*" progenitors with a hybrid identity that express Lhx3 and Foxn4 and produce either V2a or V2b neurons (Fig. 2G).

d. Foxn4 Is Sufficient for V2b Lineage Specification

Given the necessity for Foxn4 in V2b neuron specification, we tested whether Foxn4 was also sufficient to promote a V2b fate by a gain-of-function analysis in the chick spinal cord using in ovo electroporation. A plasmid vector that expresses full-length mouse Foxn4 and a downstream GFP reporter was electroporated unilaterally in the st12-14 chick spinal cord (Fig. 3). Ectopic expression of Foxn4 mRNA was confirmed by in situ hybridization or by immunohistochemistry for GFP, which was expressed from the same mRNA. We found that V2b markers (Gata2, Gata3 and Scl) were induced ectopically in all parts of the spinal cord that expressed Foxn4. Gata2 transcription was induced robustly at 24 hours post-electroporation (50/50 embryos after 24 hours and 20/20 after 48 hours) (Fig. 3A,E,F). Scl and Gata3 were detected later, at 48 hours (0/5 embryos after 24 hours, 11/11 embryos after 48 hours for Scl and 0/5 embryos after 24 hours, 6/8 embryos after 48 hours for Gata3) (Fig. 3D,I,J). Electroporating a control vector that contained inverted Foxn4 coding sequences did not up-regulate Gata2 or Scl. Foxn4 was unable to induce V2a interneurons, judging by its failure to induce ectopic expression of Chx10 protein or mRNA (0/15 embryos) or Lim3 mRNA (0/7 embryos) after 24 or 48 hours (Fig. 3B,C,E,G,H). In contrast, the number of Chx10-expressing cells produced from the p2 domain was reduced by 62 ± 9% (mean ± standard error, n=9) on the electroporated side compared to the control side (Fig. 3G,H). It is noteworthy that the Chx10-positive V2a neurons that were spared in these

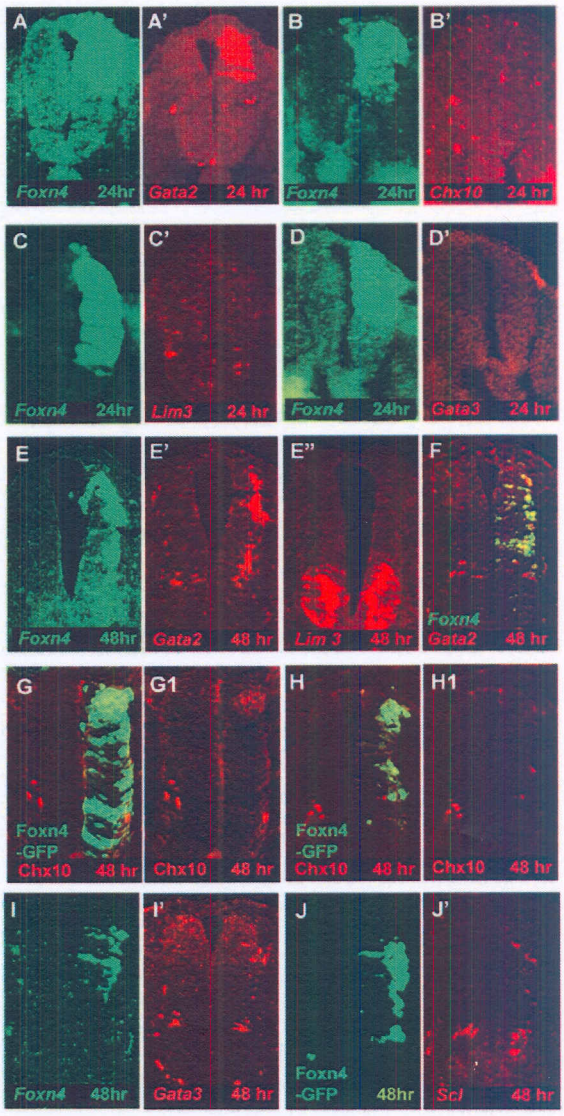

Fig. 3. Foxn4 is sufficient to induce V2b and suppress V2a interneurons. Chick embryos were electroporated at st12-14 with Foxn4-GFP and harvested after 24 h or 48 h. Expression of the vector was confirmed by in situ hybridization for Foxn4 or immuno-labelling for GFP (panels marked "Foxn4-GFP"). Consecutive sections are labelled (A,A',A'' etc.). Different fluorescence channels of the same micrograph are labeled (A,A1,A2 etc.). Foxn4 induces robust ectopic expression of Gata2 at either 24 h or 48 h postelectroporation (A,E,F). Foxn4 induced Gata3 (D, I) and Scl (J) only after 48 h. Foxn4 does not induce ectopic expression of Chx10 (B, G,H) or Lim3 (C,E"). On the contrary, Foxn4 represses endogenous Chx10 in the p2 domain (G,H)

electroporation experiments did not co-express Foxn4 (e.g. Fig. 3G,H), consistent with the idea that Foxn4 acts cell-autonomously to repress the V2a program while promoting V2b neuron specification.

Further analyses showed that mRNA encoding the Notch ligand Delta-like 4 (Dll4) was expressed in scattered cells within the p2 progenitor domain. Some of the Dll4-positive cells in the p2 domain co-expressed Foxn4. Many of these Foxn4/ Dll4 double-positive cells were found at the ventricular surface, where mitosis occurs. Forced Foxn4 expression was able to activate Dll4 expression in the chick spinal cord and Dll4 overexpression suppressed V2a neuron differentiation. Thus, the inhibitive effect of Foxn4 on V2a fate appears to be mediated by Delta-Notch signalling.

C. Project Challenges

This project has been carried out smoothly as proposed and we have accomplished both of the specific aims in the original application.

D. Implications

We have identified Foxn4 as a key determinant of one subtype of sensory interneurons. These studies have thus provided an important insight into the genetic regulatory hierarchy that governs the development of distinct sensory neuron subtypes and may provide the foundation for significant improvement of stem cell-based therapies for spinal cord injury. For instance, we may use Foxn4 to achieve controlled differentiation of V2b neurons from stem cells when necessary.

E. Future Plans

Future studies will be focused on understanding the molecular mechanism by which Foxn4 activates Mash1 and Dll4 expression. In addition, we will use microarray to profile gene expression in wildtype and mutant spinal cords to identify Foxn4 downstream genes that may mediate the essential role of Foxn4 in the specification of sensory interneurons.

F. List of Publications

Li, S., Misra, K., Matise, M. and **Xiang, M.** (2005) Foxn4 acts synergistically with Mash1 to specify subtype identity of V2 interneurons in the spinal cord. *Proc. Natl. Acad. Sci. USA* **102**:10688-10693.

Del Barrio, M. G., Taveira-Marques, R., Muroyama, Y., Yuk, D.-I., Li, S., Wines-Samuelson, M., Shen, J., Smith, H. K., **Xiang, M.**, Rowitch, D., and Richardson, W. D (2007) A regulatory network involving Foxn4, Mash-1 and Delta-like 4/Notch-1 generates V2a and V2b spinal interneurons from a common progenitor pool. *Development* (submitted).