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Multiple molecular mechanisms control the development of the simple neural plate into neural tube that ultimately gives rise to diverse neural and glial cells. Bmp and Wnt signals emanating from the roof plate and overlaying ectoderm initiate patterning of the dorsal neural tube (Lee and Jessell, 1999), while ventral spinal cord patterning is controlled by Shh that is secreted from the notochord and floor plate (Chiang et al., 1996; Ericson et al., 1996). Absence of notochord leading to loss of Shh signaling ventrally, leads to dorsalization of the whole neural tube (Yamada et al., 1999; Liem et al., 1995). The present study however suggests an additional mechanism, where dorsal fates are kept off in the ventral spinal cord (SC) due to presence of inhibitors of dorsal signals. Specifically my analysis shows that Lbx1 is usually kept off in the ventral SC due to the presence of the Wnt inhibitors, the secreted frizzled related proteins (sFRP’s). Loss of Pax6 in Pax6<sup>Sey/Sey</sup> mutant’s leads to loss of sFRP’s in the ventral SC and results in activation of ectopic Lbx1 expression in cells normally fated to become V2 interneurons under the influence of Shh signaling. These results suggest that Pax6 and sFRP2 control ventral neuronal fates by restricting their response to Wnt signaling in the developing neural tube.

1. **Original aims of the project & project successes.**

1. **Characterization of the molecular changes in the post-mitotic V2 interneurons due to ectopic Lbx1 up-regulation in Pax6<sup>Sey/Sey</sup> mutant mice.**

   The first specific aim of the proposed project was to characterize the molecular changes in the post-mitotic V2 interneurons due to ectopic Lbx1 up regulation in the Pax6<sup>Sey/Sey</sup> mutant mice. This issue involved

   a). Determining the specific identity of ectopic Lbx1 cells.

   b). Determining which specific sub-populations of V2 interneurons are affected.

   **Both of these sub-aims have been accomplished as discussed in results section 2.**

2. **Characterization of the molecular changes in p2 progenitors leading to ectopic Lbx1 cells in V2 domain.**

   This aim had two specific sub-aims:

   a). Characterize the changes in progenitor factors that lead to ventral expansion of Lbx1.

   b). Determine the requirement of Mash1 in ventral Lbx1 up-regulation by generating Pax6<sup>Sey/Sey</sup>; Mash1<sup>−/−</sup> double mutants.

   a). The first part was to characterize the changes in the V2 progenitor domain of the Pax6<sup>Sey/Sey</sup> mutants that might suggest a possible mechanism of up-regulation of Lbx1 in this domain. We predicted that this might involve either loss of expression or ectopic gain of expression of a dorsal progenitor marker in this domain. **An extensive array of testing has been carried out for this analysis** based on expression patterns of genes that have already been identified (a “candidate-gene” approach). However, other unidentified genes might be involved in this up-regulation and these have not been included in the study. This part is addressed in **results section 1.**

   b). As proposed in this aim in our grant proposal, Mash1 might be one candidate gene since it is expressed in the Lbx1 progenitors both dorsally and in the V2 domain. To specifically address the role of Mash1 we have obtained the Mash1<sup>−/−</sup> mutant mice and analyzed the generation of V2 interneurons in these mice. This analysis has provided us with some valuable data of the genetic pathways involved in generation of the two sub-populations of the V2 domain: V2a and V2b. **We have successfully generated Mash1<sup>−/−</sup>; Sey<sup>−/−</sup> double mutants** to determine whether ectopic Lbx1 observed in Pax6<sup>Sey/Sey</sup> mutants require Mash1 activity. This part of the work is discussed in **results section 3.**
3. In-ovo electroporations to determine the ability of dorsal and ventral progenitor factors to regulate Lbx1 expression in the spinal cord.

The final aim was to employ in-ovo electroporations to determine the ability of dorsal and ventral progenitor factors to regulate Lbx1 expression in the SC. Several candidates have been tested that have led to a hypothesis elucidating the mechanism involved in the up-regulation of Lbx1 in the V2 domain. The details of this specific aim are discussed in results sections 4 and 5.

Results.

1. Altered expression of sFRP’s in Pax6Sey/Sey mutant embryos.

Previous studies have shown that expression of the secreted Wnt antagonist sFRP2 is down regulated in the ventral spinal cords of Pax6Sey/Sey mutants (Kim et al., 2001), suggesting that Wnt signaling could be altered in these mice. To study this in more detail, we examined sFRP gene expression in Pax6Sey/Sey mutant embryos. In wild-type (WT) mouse embryos, both sFRP1 and sFRP2 expression is confined to dividing ventral ventricular zone (VZ) and we determined that sFRP1 is expressed in the p3, p2, p1 and p0 domains but only weakly if at all in the pMN domain at E10.5 (Fig. 1A,E), while sFRP2 expression is seen in all ventral progenitor domains except the p3 domain (Fig. 1C,E). In Pax6Sey/Sey mutant embryos, the expression of both sFRP1 and sFRP2 was altered in the thoracic spinal cord. The ventral domain of sFRP1 was absent; while the more dorsal domain was reduced in size and occupied an area within the expanded “p0” domain that generates Evx1+ cells Pax6Sey/Sey mutants (Fig. 1B,F). In contrast sFRP2 expression was down regulated in the VZ in Pax6Sey/Sey embryos, although weak expression was retained in the FP, a tissue that does not normally express Pax6 (Fig. 1D,F). Thus, loss of Pax6 results in down regulation of both sFRP1 and sFRP2 in ventral progenitors.

2. A dorsal interneuron marker is up regulated ventrally in Pax6Sey/Sey embryonic spinal cords.

Since sFRP proteins are negative regulators of the Wnt pathway, the loss of sFRP1 and sFRP2 expression from the ventral spinal cord of Pax6 mutants might be expected to result in aberrant activation of Wnt signaling in this region. As Wnt signaling is required for the proliferation and specification of some dorsal cell types, we analyzed dorsal interneuron development in Pax6Sey/Sey using cell-type specific markers. Consistent with this possibility, we found that Lbx1, a homeodomain (HD)- containing transcription factor normally restricted to post-mitotic dI4-6 dorsal interneurons (Gross et al., 2002; Muller et al., 2002), was up regulated in a specific and reproducible location in the ventral spinal cord of mutant embryos (Fig. 2A). Ectopic Lbx1+ cells were distinct from ventrally migrating Lbx1+ cells generated in the dorsal SC in wild-type embryos at these stages since and many co-expressed Lhx3 or Mash1 that specifically mark cells in the ventral V2 progenitor domain (Figs 2Aii, 3C). Ectopic Lbx1+ cells were intermingled with, but distinct from Chx10+ and Gata3+ cells that normally differentiate in the V2 domain (Fig. 2iii, iv). Very few if any ectopic Lbx1+ cells were found in the adjacent motoneuron (MN) or V0/V1 domains (Fig. 2Av-vii). Furthermore, ectopic expression of Lmx1b (dI5), or Tlx1/3 (dI3 & dI5) which are normally co-expressed with Lbx1 in the dorsal spinal cord, was not seen in Pax6Sey/Sey embryos, but some ectopic Lbx1 cells co-expressed Lim1/2, a LIM-HD containing factor found in both dorsal (dI2, dI4, dI6) and ventral (V0, V1 and V2) populations (Matise and Joyner, 1997). Thus, ectopic Lbx1 cells in the V2 domain in Pax6Sey/Sey embryos bear some of the molecular characteristics of dI4 or dI6 interneurons.

3. A role for Mash1 in generating ectopic Lbx1 cells in the V2 domain of Pax6Sey/Sey embryos.

One striking observation is that Mash1 is common to two of the three dorsal progenitor domains from which Lbx1 cells normally arise and to the V2 domain where ectopic Lbx1 cells appear in Pax6Sey/Sey
mutants (Fig. 3B-D). It has been shown that Mash1 is required for the proper generation of V2 interneuron populations (Parras et al., 2002; Li et al., 2005); however, no ectopic Lbx1+ cells are seen in the ventral V2 domain in Mash1−/− mutants (Fig. 3G). To determine the potential role of Mash1 in the generation of ectopic Lbx1 in the V2 domain of Pax6Sey/Sey embryos, we generated Pax6Sey/Sey; Mash1−/− double mutants. In these double mutant mice at both E10.5 and 11.5, ~60% fewer Lbx1+ cells were detected in the V2 domain, compared to single Pax6Sey/Sey mutants (Fig. 3E-H, O). These results suggest that expression of Mash1 is partly required for the generation of ectopic Lbx1 cells in the V2 domain in Pax6Sey/Sey embryos.

Next we examined the effect of ectopic Lbx1 expression in the V2 domain of Pax6Sey/Sey embryos on Chx10 expressing V2a and Gata2/3 expressing V2b neurons that are normally generated in this domain in WT embryos. At E10.5, both V2a and V2b interneurons were reduced in the Pax6Sey/Sey embryos; however, by E11.5 the V2a (Chxl0+) population in the mutants recovered in Pax6Sey/Sey embryos to a level comparable to WT, while the reduction in V2b (Gata3+) interneurons persisted (Fig. 3M,N). We have recently shown that in Mash−/− mutants there is a selective reduction of V2b interneurons (Li et al., 2005). Therefore, Mash1 is required for the generation of V2b as well as most of the ectopic Lbx1+ cells in the V2 domain in Pax6Sey/Sey embryos.

4. Gshl and Mash1 synergize to induce Lbx1 interneuron cell fates.

It has recently been shown that overexpression of Mash1 using in ovo electroporation (EP) in chick embryos can induce dl3 interneurons (Fig. 4C,D). However, we found that EP of Mash1 repressed Lbx1 expression in the dl4-6 domains (Fig. 3B). Another closely related gene, Gshl, is expressed in dl4 and dl5 progenitors, but is not required on its own for dorsal cell patterning and specification in mice (Fig. 4A), so its role is less clear. To address this issue, we transfected these factors into progenitor cells in chick embryo spinal cords. Interestingly, Gshl induced ectopic Lbx1+ cells in the ventral spinal cord (Fig. 4E; Fig. S1) and repressed Brn3a+/Isll+ dl3 fates dorsally (Fig. 4F). However, co-EP of Gshl and Mash1 together induced even more widespread ectopic Lbx1 expression in ventral regions (Fig. 4G; Fig. S1). In addition, ventral cell types were reduced in transfected embryos, indicating that Gshl and Mash1 had redirected their fates to a more dorsal (Lbx1+) phenotype. Taken together, these results are consistent with the idea that Gshl and Mash1 synergize to activate Lbx1 expression in the dorsal spinal cord, with Gshl functioning to modify Mash1 activity, and Mash1 potentiating the ability of Gshl to induce Lbx1+ cells (Fig. 4J).

5. sFRP2 blocks induction of Lbx1.

To examine the effect of sFRP2 on Lbx1 in the dorsal neural tube, we transfected sFRP2 into the developing chick neural SC. In embryos transfected with sFRP2, neuronal differentiation was broadly inhibited, resulting in a smaller neural tube on the transfected side (Fig. S2), consistent with the previously demonstrated role for Wnt signaling in providing mitogenic cues in the dorsal spinal cord (Megason and McMahon, 2002). In addition, most dorsal cell fates, including Lbx1 and Lmx1b, which mark dl4-6 and dl5 cells, respectively, were reduced or absent from the transfected side (Fig. 6A, B). However, sFRP2 did not block Pax6 or Lhx3 expression in ventral progenitors (Fig. 6C,D), or the appearance of differentiated cell types that derive from ventral regions where sFRP2 is normally expressed, including MN (HB9+ or Isll+), V1 (En1+), or V2 (Gata3+ or Chx10+) cells (Fig. 6E,F). These results suggest that dorsal neuron subtypes are disproportionately sensitive to the inhibition of Wnt signaling by sFRP2.

To further examine the role of sFRP2’s in generating ectopic Lbx1 interneurons, we made use of our transfection assay where Lbx1+ cells can be efficiently induced by co-transfection of Gshl and Mash1 (Fig. 4G). Transfection of a full-length mouse sFRP2 cDNA with Gshl and Mash1 completely blocked
the induction of ectopic Lbx1 cells (Fig. 5A,B; Fig. S1), while co-transfection of sFRP1 reduced, but did not block, induction of Lbx1 (Fig. 5C; Fig. S1). Since sFRP’s are secreted Wnt antagonists and can inhibit Lbx1 induction, this suggests requirement of Wnt signaling for generating Lbx1 by co-EP Gsh1 and Mash1. To test whether canonical Wnt signaling was required, we co-transfected three different factors which each selectively block canonical, but not non-canonical, Wnt signaling: an N-terminally deleted dominant-negative-Tcf4, dkk, or Axin (Peifer and Polakis, 2000; Megason and McMahon, 2002). Co-EP of each of these factors with Gsh1 and Mash1 failed to completely block the induction of Lbx1 like sFRP2 (Fig. 5D-F; Fig. S1). Because sFRP2 should be capable of blocking both canonical and non-canonical Wnt signaling extracellularly, these results raise the possibility that a non-canonical Wnt signal transduction pathway that is sensitive to inhibition by sFRP2 is involved in Lbx1 induction.

References:

II. Project challenges.
All the project goals have been largely addressed and have led to some very important conclusions, as addressed in the result section. One small challenge has been to equate the results observed in the two model systems employed: chick and the mouse.

III. Implications for future research.
Multiple molecular mechanisms control the development of the simple neural plate into neural tube that ultimately gives rise to diverse neural and glial cells. Bmp and Wnt signals emanating from the roof plate and overlying ectoderm initiate patterning of the dorsal neural tube (Lee and Jessell, 1999), while ventral spinal cord patterning is controlled by Shh that is secreted from the notochord and floor plate (Ericson et al., 1996). Absence of notochord leading to loss of Shh signaling ventrally, leads to dorsalization of the whole neural tube (Yamada et al., 1999; Liem et al., 1995). The present study however suggests an additional mechanism, where dorsal fates are kept off in the ventral spinal cord (SC) due to presence of inhibitors of dorsal signals.

IV. Plans to continue this research.
The analysis of Pax6<sup>Sev/Sev</sup> mutants showed a loss of sFRP’s in the ventral SC and led to the present hypothesis that sFRP’s normally prevent ventral expansion of Lbx1 in the developing SC. However, multiple sFRP’s are lost in the Pax6<sup>Sev/Sev</sup> mutants and it is difficult to infer whether all sFRP’s contribute equally to this function, or one specific sFRP is enough. To address this question, we have started examining the individual sFRP1 & sFRP2 mutant mice. This has led to several very interesting observations about regulation of Wnt signalling that I plan to pursue further.

V. List of publications emerging from this research.
   <sup>1</sup> These authors contributed equally to this work, <sup>2</sup> Corresponding authors.


Abstracts of Posters presented:

Figure 1. Down-regulation of sFRP1 and sFRP2 in the ventral spinal cord in Pax6 Sey/Sey mutants. (A,C) sFRP1 and sFRP2 are expressed in specific domains in the ventral VZ in the thoracic spinal cord of WT embryos. sFRP1 is expressed in the p3, p2, p1 and p0 domains, while sFRP2 is expressed in the FP, pMN, p2, p1, p0 and pdi6 progenitor domains (Q.L., M.P.M., unpublished). (B,D) In Pax6 Sey/Sey mutants, both sFRP1 and sFRP2 are down-regulated in the ventral spinal cord. sFRP1 expression is lost from the most ventral domain (red arrowheads), while sFRP2 is down-regulated everywhere except in the floorplate (arrowhead). (E,F) Summary of expression patterns of sFRP1 and sFRP2 in WT and Pax6 Sey/Sey mutant embryos. Transverse sections through the thoracic spinal cord at E11.5 shown in all figures.
Figure 2. Up-regulation of the dorsal interneuron marker Lbx1 in the ventral spinal cord of Pax6Sey/Sey mutants. (Ai-viii) The dorsal interneuron marker Lbx1 (d14-6 cells) is up-regulated in the ventral V2 interneuron domain in Pax6 S ey/Sey mutant embryos. Each pair of images shows left-right hemi-sections through WT (left panel) and Pax6 S ey/Sey mutant (right panel) spinal cords, stained with the same markers. (i, ii) Lbx1 expression arises within the dorsal aspect of the Lhx3 expression domain in Pax6Sey/Sey mutants, which demarcates MN and V2 progenitors. Arrowhead in (i) indicates ventrally migrating Lbx1 cells derived from the dorsal spinal cord. (iii, iv) Lbx1+ cells intermingle with, but are distinct from, Chx10+ and Gata3+ V2 interneurons in Pax6 S ey/Sey. (v-viii) Very few ectopic Lbx1 cells are found within the MN or V1/V0 generating domains in Pax6 S ey/Sey embryos. Note that En1+ V1 interneurons are absent in these mutants at this stage and Evx1+ V0 interneurons extend ventrally into the V1 domain (Burrill et al., 1997; Ericson et al., 1997). (B-G) Appearance of ectopic Lbx1 cells in the ventral lumbar spinal cord correlates with loss of sFRP2 expression in this region in Pax6 S ey/Sey mutants. At E10.5, no ectopic Lbx1 cells are detected in lumbar regions of Pax6 S ey/Sey mutants (D), and correspondingly sFRP2 expression is still detected (E). Later, at E11.5 when ectopic Lbx1+ cells appear in this region, sFRP2 expression is down-regulated (G). Transverse sections through the indicated regions of spinal cord at E10.5 (A-E) and E11.5 (F-G).
(A) Pax6 and Mash1 have overlapping expression patterns in the dl4, 5 as well as V2 progenitor domains in WT embryos. (B, C) Mash1 expression is common to Lbx1 progenitors in the dorsal spinal cord of WT embryos, as well as the V2 progenitor domain where ectopic Lbx1 appear in the Pax6 Sey/Sey mutant embryos. Arrowhead in (B) indicates ventrally migrating Lbx1 cells derived from the dorsal spinal cord, large bracket shows normal Lbx1 domain in the dorsal spinal cord, smaller bracket in (C) indicates V2 domain. (D) Schematic summarizing Mash1 expression patterns and changes observed in Lbx1 expression in Pax6Sey/Sey mutants. (E-G) Thoracic hemi-sections of the spinal cord show no ectopic Lbx1 in the V2 domain of WT (E) and Mash1/-/- mutant (G) embryos. The number of ectopic Lbx1 cells in the V2 domain is greatly reduced in Pax6Sey/Sey, Mash1/-/- double homozygotes (H) as
compared to Pax6Sey/Sey mutants (F,O). (I-P) Concomitant with the appearance of ectopic Lbx1 in the V2 domain, the number of Chx10+/Gata3+ V2 cells is reduced in Pax6\(^{Sey/Sey}\) and Pax6\(^{Sey/Sey}\); \(\text{Mash1}^+\) double mutant embryos. (I-N) In WT embryos, similar numbers of Chx10 and Gata3 expressing interneurons are generated in the V2 domain, while in \(\text{Mash}^+\) mutants (K, O) there is an increase in number of Chx10 cells at the expense of Gata3 cells (Li et al., 2005). (J,N) In Pax6\(^{Sey/Sey}\) \(\text{y}\) mutants, both the Chx10 and Gata3 cells are reduced proportionately, while in Pax6\(^{Sey/Sey}\); \(\text{Mash1}^+\) double homozygotes the number of Chx10 cells falls between the numbers observed in \(\text{Mash}^+\) embryos and Pax6\(^{Sey/Sey}\) mutants (M,N). A compounding loss of Gata3 expressing V2b cells is observed in the double mutants. (M-P) Quantification of V2 interneurons in each genetic background, represented as the average number of stained cells per section ± s.e.m. (error bars) W=Wild-type, \(P=\text{Pax6}^{Sey/Sey}\), \(M=\text{Mash1}^+\), \(P;M=\text{double mutants}\) (P) Total number of cells within the V2 domain in each case (obtained by adding Chx10+/Gata3+/Lbx1+ cells). Transverse sections through the thoracic spinal cord at E10.5 (A-D) and E11.5 (E-P) are shown. Dashed line in E-L indicates approximate boundary between the VZ (to the left) and MZ (to the right). Arrows in E-H indicate stream of ventrally migrating Lbx1 cells derived from the dorsal spinal cord that migrate outside in the MZ. *=significant at \(p<0.001\); **=significant at \(p<0.01\), compared to WT (M-N) or Pax6Sey/Sey (O).
Figure 4. Efficient induction of ectopic Lbx1 by co-transfection of Gsh1 and Mash1.
(A) Gsh1 mRNA (purple) is expressed in the dl4-6 progenitor region from where cells expressing Lbx1 HD protein (brown) originate. (B-D) Transfection of a mouse Mash1 cDNA into chick embryos using in ovo electroporation (EP). (B) Mash1 suppresses Lbx1 expression (blue asterisk). The double headed dashed arrow indicates the ventral boundary of Lbx1 expression in the dorsal spinal cord. Panel at right shows GFP expression to indicate extent of transfection. The right side in this and all subsequent figures is the EP side. (C,D) Mash1 induces ectopic Brn3a (arrowhead in C), which is normally expressed in the...
dI1-3 and dI5 domains, and Isll/2 (arrowhead in D), which marks dI3 cells (brackets mark normal domains in each figure). (E,F) EP of Gsh1 alone induces Lbx1 (arrowhead) in ventral cells, and represses dI1-3 interneuron fates (blue asterisk in F). (G,H) Co-EP of Mash1 with Gsh1 results in induction of ectopic Lbx1+ cells in the ventral spinal cord (arrowhead) at much higher efficiency than EP of Gsh1 alone (see also Fig. S1). In addition, Brn3a+ dI3/5 cell fates are repressed (blue asterisks). (I) Co-EP of Gsh2 with Mash1 suppresses Lbx1 expression, unlike EP of Gsh1 + Mash1 (compare I with G). (J) Summary of individual and combined effects of Gsh1 and Mash1 on dorsal neuronal fate induction. Transfected side is indicated by yellow bolt; transverse sections through thoracic spinal cord region shown in all figures.
Figure 5. sFRP2 blocks the induction of Lbx1. (A,B) Overexpression of a full-length mouse sFRP2 cDNA blocks the induction of ectopic Lbx1 induction in the ventral spinal cord by co-transfection of Gsh1+Mash1. (C) sFRP1 does not antagonize the induction of ectopic Lbx1+ cells by Gsh1+Mash1 as efficiently as sFRP2. (D-F) Blockage of the canonical Wnt pathway signal transduction by co-electroporation of dominant-negative-Tcf4 transcription factor (D), dkk (E) or Axin (F) fail to block Lbx1 induction by Gsh1+Mash.

Figure 6. sFRP2 inhibits Lbx1 cell fate specification in the dorsal spinal cord. (A,B) Transfection of sFRP2 into dorsal progenitors inhibits neurogenesis and blocks dorsal Lbx1+ and Lmx1b cell fates, but has no effect on Pax6 (C) or Lhx3 (D) expression in progenitors. (E, F) Ventral cell fates such as Isl1/2 and Gata3 are reduced but not blocked by EP of sFRP2 in ventral regions.
Figure 7. Transfection of Gsh1 into ventral progenitors blocks sFRP1 and sFRP2 expression. (A,B) EP of Gsh1 represses both sFRP1 and sFRP2 expression in ventral progenitors. Panel at right shows GFP expression in near-adjacent sections to indicate extent of transfections. (C) Model for the regulation of Wnt signaling by Pax6 and sFRP2 in the spinal cord.