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Wnt signaling determines ventral spinal cord cell fates in a time-dependent manner

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The identity of distinct cell types in the ventral neural tube is generally believed to be specified by sonic hedgehog (Shh) in a concentration-dependent manner. However, recent studies have questioned whether Shh is the sole signaling molecule determining ventral neuronal cell fates. Here we provide evidence that canonical Wnt signaling is involved in the generation of different cell types in the ventral spinal cord. We show that Wnt signaling is active in the mouse ventral spinal cord at the time when ventral cell types are specified. Furthermore, using an approach that stabilizes β-catenin protein in small patches of ventral spinal cord cells at different stages, we show that Wnt signaling activates different subsets of target genes depending on the time when Wnt signaling is amplified. Moreover, disruption of Wnt signaling results in the expansion of ventrally located progenitors. Finally, we show genetically that Wnt signaling interacts with Hh signaling at least in part through regulating the transcription of *Gli3*. Our results reveal a novel mechanism by which ventral patterning is achieved through a coordination of Wnt and Shh signaling.

KEY WORDS: Canonical Wnt signaling, Activation, Mutation, Ventral patterning, Cell fate, *Gli2*, *Gli3*, *Shh*

INTRODUCTION

In the developing mammalian ventral spinal cord, five different neuronal cell types, comprising the motoneuron (MN) and four different interneurons (V0-V3), are generated from their respective neural progenitors (pMN, pV0-pV3) under the influence of signals from the notochord (Tanabe and Jessell, 1996). Shh, one of the Hedgehog (Hh) family of proteins, is strongly expressed in the notochord and later in the floor plate, and is able to mimic the ability of the notochord in inducing different ventral spinal cord cell types. In response to Shh signaling, neural progenitors activate or suppress the expression of several homeobox and basic helix-loop-helix (bHLH) transcription factors. The combinatorial expression of these transcription factors defines the fate of the neural progenitor cells (Briscoe et al., 2000).

Although Shh has been shown to be sufficient for the induction of distinct ventral spinal cord neurons, recent studies have suggested an alternative derepression mechanism whereby many neuronal cell types could be generated in the absence of Shh signaling. For example, although most of the ventral cell types are absent from *Shh* or *Smo* mutants because of the ectopic production of Gli3 repressor, most of the ventral neurons are generated in *Shh;Gli3* and *Smo;Gli3* double mutants (Litingtung and Chiang, 2000; Wijgerde et al., 2002). Similarly, in embryos that lack all Gli transcription factors and cannot respond to Hh signaling, many of the ventral neurons are also present (Bai et al., 2004; Lei et al., 2004). These results suggest that perhaps the primary role of Hh signaling is to repress excess Gli3 repressor in the ventral spinal cord so that progenitors can respond to other signals. An additional role for Shh signaling is to organize the formation of distinct progenitor domains, as different cell types intermix in the absence of Shh signaling (Bai et al., 2004; Fuccillo et al., 2006).

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In search of other signals that might influence the generation of ventral neurons, we focused on the Wnt pathway, as several Wnts, their receptors and inhibitors are expressed in the developing spinal cord (Hoang et al., 1998; Leimeister et al., 1998; Parr et al., 1993). Wnts signal through Frizzled and LRP factors, resulting in the stabilization of β-catenin, which then interacts with TCF/LEF transcription factors to control the expression of downstream target genes (Logan and Nusse, 2004). Previous studies have uncovered a prominent role for Wnt signaling in proliferation in the spinal cord. For example, ectopic expression of *Wnt1* or stabilized β-catenin caused overproliferation in the spinal cord, but did not cause patterning defects or ectopic expression of dorsal neuronal markers (Chenn and Walsh, 2002; Megason and McMahon, 2002). Furthermore, when Wnt signaling was disrupted or ectopically activated at E9.5 by *Brn4-Cre* (which is expressed throughout the spinal cord), the neuronal progenitors were either depleted or overproliferated (Zechner et al., 2003). Recent chick electroporation studies suggest that Wnt signaling might interact with Gli genes to regulate the expression of key transcription factors in the spinal cord (Alvarez-Medina et al., 2008; Lei et al., 2006). However, how Wnt signaling interacts with Shh signaling is a matter of controversy, with one study showing that Wnt signaling is required for Gli-mediated activation of *Nkx2.2* (Lei et al., 2006), whereas another study showed an antagonistic interaction between Wnt and Shh signaling (Alvarez-Medina et al., 2008). Furthermore, it is unclear whether Wnt signaling normally functions in early ventral progenitors at the time of neural tube closure to specify distinct neural progenitors, in addition to its role in establishing domain boundaries after neural tube closure as suggested by chick electroporation studies.

Here we use a genetic, inducible approach to perturb Wnt signaling in small domains of the mouse ventral spinal cord before and after the neural tube closes. We show that Wnt signaling activates downstream genes in a time-dependent manner, and that Wnt signaling regulates the ventral spinal cord cell fates in part through *Gli3*, but not *Gli2*. These data reveal a crucial role of Wnt signaling in regulating ventral cell fates during normal vertebrate development.

MATERIALS AND METHODS

Mouse breeding

The genotyping of *Gli1-CreER*, $Gli2^{zfd}$, $Gli3^{xt}$, conditional loss-of-function β-catenin ($Ctnnb1^{tm1Max}$), stabilized β-catenin ($Ctnnb1^{tm1Mmt}$) and *Olig1-Cre* mice has been described (Ahn and Joyner, 2004; Brault et al., 2001; Harada et al., 1999; Lu et al., 2002; Maynard et al., 2002; Mo et al., 1997). Double heterozygous mutant mice were maintained on a CD1 background. Tamoxifen (Sigma T-5648) was dissolved in corn oil at 20 mg/ml and was fed to pregnant females using a gavage feeding needle (FST, Foster City, CA) to activate CreER expressed from the *Gli1-CreER* allele.

Histology, immunohistochemistry and RNA in situ hybridization

Mouse embryos were dissected in cold PBS and fixed in 4% paraformaldehyde (PFA) for 1 hour at 4°C. Embryos were washed with PBS, cryoprotected in 30% sucrose and embedded in OCT (Tissue-Tek). Tissues were sectioned at 10-12 μm on a Leica Cryostat. For consistency, all analyses were performed at the forelimb level unless otherwise specified. Immunohistochemistry, X-Gal staining and RNA in situ hybridization were performed essentially as described (Bai et al., 2002; Bai and Joyner, 2001). RNA in situ probes have been described (Platt et al., 1997) with the exception of *Axin2* (Open Biosystems, clone 6827741). The primary antibodies used have been described (Pierani et al., 1999; Takebayashi et al., 2002), except for mouse anti-Lim3, Msx1/2 (DSHB), N-cadherin, Mash1 (BD Biosciences); rabbit anti-β-catenin (Sigma), Math1 (Helms and Johnson, 1998), Gsh1/2, Foxd3, Lbx1 (Gross et al., 2002), Pax2 (Zymed), Smad1/5/8 (Cell Signaling); and goat anti-β-galactosidase (Biogenesis). Secondary antibodies were purchased from Jackson ImmunoResearch or Molecular Probes. Nuclei were counterstained with Hoechst 33258 (Molecular Probes) or with 0.005% Nuclear Fast Red (Polyscientific).

Microscopy and statistical analysis

Pictures were taken with a Leica DMLB epifluorescence microscope fitted with a SPOT camera (Genetics Imaging Facility, supported by NIH-NCRR, RR-021228-01). Images were cropped and the brightness and contrast adjusted in Adobe Photoshop. For quantification purposes, three cross-sections at the forelimb level were examined from each embryo, and three embryos were used for each time point. Histograms show the average number of labeled cells in the section ± s.e.m. Student's *t*-test was used to calculate the *P*-value. To quantify the distribution of ectopic $Msx1/2^+$ or $Pax7^+$ cells, ventral spinal cord was divided into five equal domains and the number of ectopic cells within each domain was calculated.

RESULTS

Wnt signal transduction is active dorsally and ventrally in the early spinal cord

To address whether Wnt signaling is active at the time ventral progenitors are being specified, we examined the expression of a transgene reporter of Wnt signaling, *TCF/LEF-lacZ* (Mohamed et al., 2004), in the developing spinal cord at E8.5 and E9.5. *lacZ*-expressing cells were detected strongly in the mid/hindbrain (data not shown) and in the dorsal neural hinges (between neural and non-neural ectoderm) of the posterior hindbrain/anterior spinal cord where the neural tube is open (Fig. 1A). In the posterior regions of the embryo where the neural plate had not yet closed, *lacZ*-expressing cells were detected throughout the neural plate (Fig. 1C,D). In the intermediate region where the neural tube had already closed, *lacZ*-expressing cells were found both dorsally and ventrally in the spinal cord (Fig. 1B). To confirm that the pattern of reporter expression reflects endogenous Wnt signaling, we examined the expression of *Axin2*, a known target of Wnt signaling, using RNA in situ hybridization. We found a similar patterning of *Axin2* expression from anterior to posterior of the E8.5 embryo (Fig. 1E-H). At E9.5, *Axin2* expression was detected throughout the posterior neural tube (Fig. 1K), although the expression became dorsally restricted in more-anterior levels (Fig. 1I,J). These two lines of evidence indicating that Wnt signaling is active in the ventral neural tube led us to test whether Wnt signaling has a role in the determination of ventral progenitor cell fates.

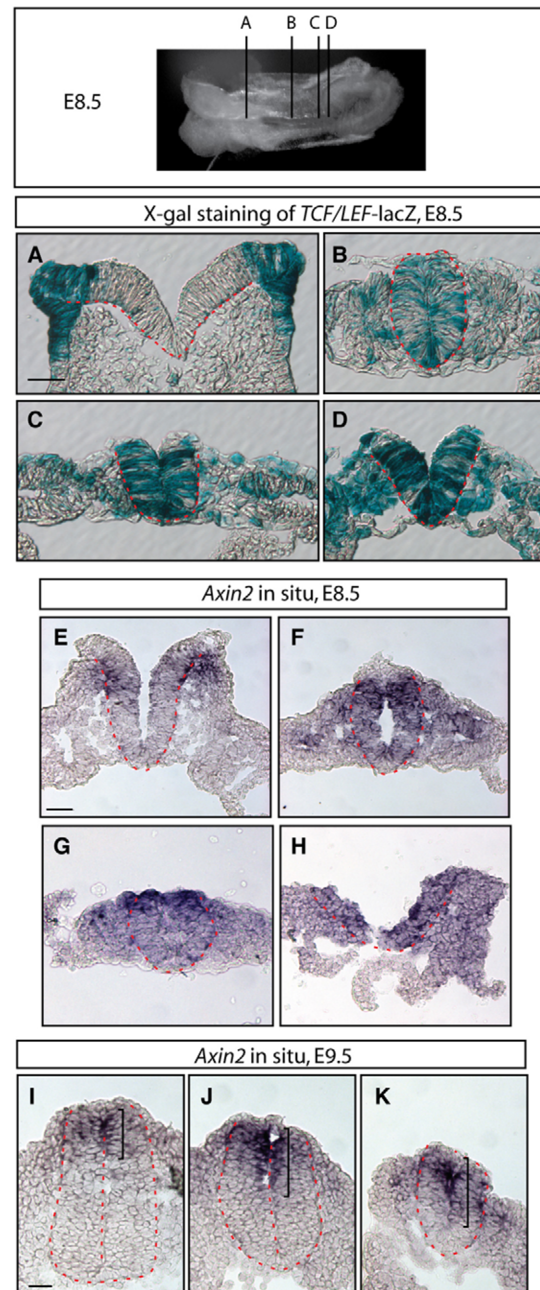

Fig. 1. Wnt signaling is active in the mouse ventral spinal cord at E8.5 but is downregulated by E9.5. (**A-D**) X-Gal staining of coronal sections of E8.5 *TCF/LEF-lacZ* embryos. Sections in A-D are shown from anterior to posterior, with the plane of section shown on an E8.5 embryo above the panels. (**E-K**) RNA in situ hybridization of *Axin2* in coronal sections of E8.5 and E9.5 embryos. At E8.5, *Axin2* is expressed in a similar pattern to *lacZ* in *TCF/LEF-lacZ* embryos (E-H). By E9.5, *Axin2* expression is more restricted in anterior (I) than intermediate (J) or posterior (K) sections (compare brackets). Red dashed line, margin of neural epithelium. Scale bars: 50 μm.

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Stabilized β-catenin in the ventral spinal cord promotes dorsal and inhibits ventral cell fates

To address whether Wnt signaling specifies ventral spinal cord neural progenitors, we created genetic mosaics of cells that express stabilized β-catenin. In this approach, Tamoxifen (TM) was used to activate an ER (estrogen receptor)-fused Cre recombinase expressed from the *Gli1* locus (*Gli1-CreER* knock-in), to induce the recombination of two conditional alleles: *Ctnnb1^{tm1Mmt}*, which has two loxP sites surrounding a β-catenin degradation signal in exon 3 and encodes stabilized β-catenin after recombination (Harada et al., 1999) (hereafter referred to as the *Ctnnb1^{gof}* allele); and a *Rosa26* reporter (*R26R*) that expresses β-galactosidase upon recombination (Soriano, 1999). Since β-galactosidase and stabilized β-catenin are expressed from two separate recombination events, they might not always coexpress in the same cells (Balordi and Fishell, 2007; Joyner and Zervas, 2006; Zong et al., 2005). Thus, we used β-galactosidase only to monitor the overall extent of recombination, and not to identify cells that expressed stabilized β-catenin.

We delivered TM (150 mg/kg body weight) at 09:00 and 18:00 h on E8 to activate Wnt signaling in early spinal cord progenitors. The number and distribution of cells expressing β-galactosidase were similar in control (*Gli1-CreER;R26R$^{+/-}$*) and experimental embryos (*Gli1-CreER;R26R$^{+/-}$;Ctnnb1^{gof}*) (Fig. 2A,D). We then examined whether dorsal markers were expressed in the ventral spinal cord as a result of stabilized β-catenin expression. We found that Pax7, which normally marks dorsal spinal cord progenitors, was ectopically expressed in patches of cells in the ventral spinal cord (Fig. 2E, white bracket). Dbx2, which marks intermediate spinal

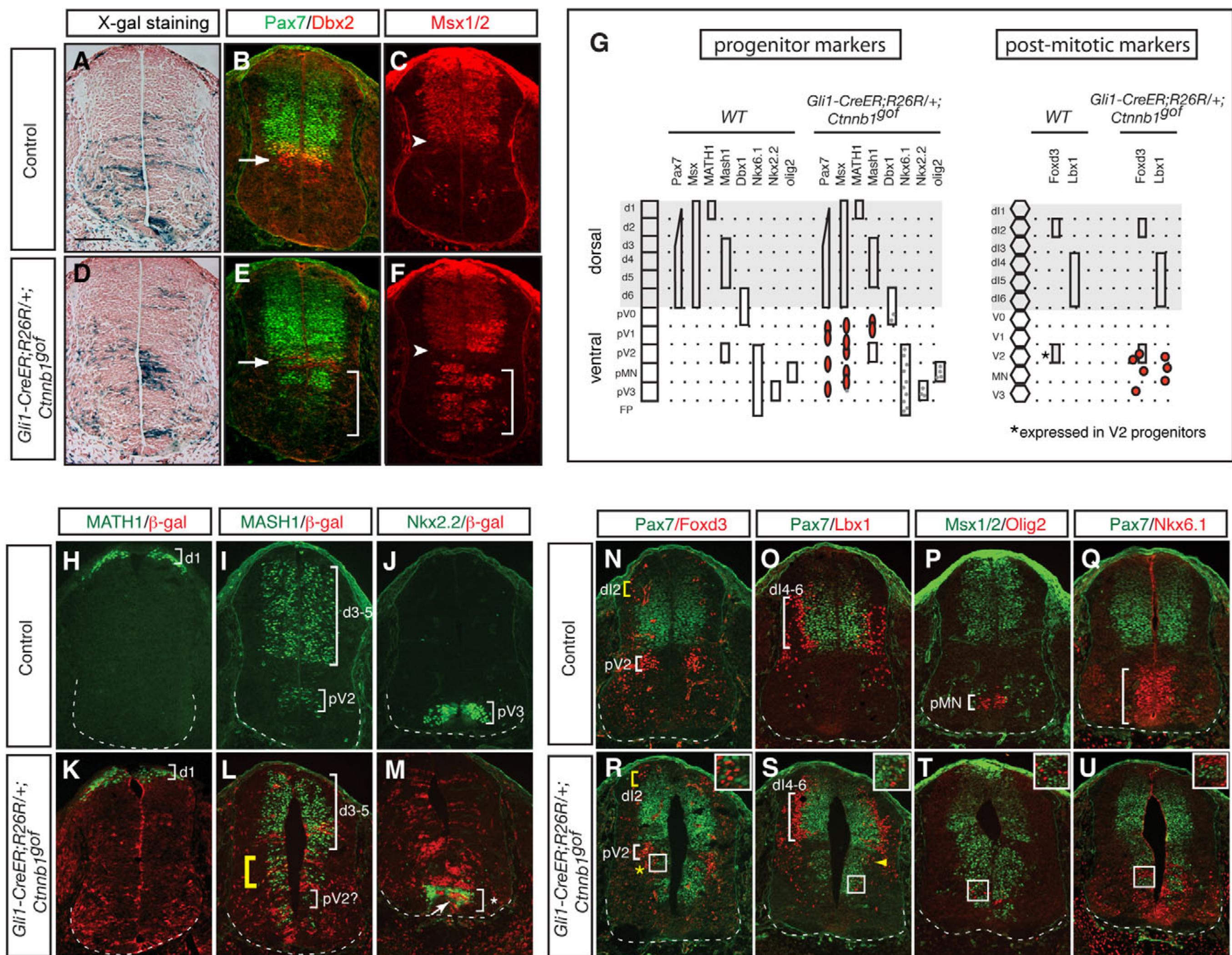

Fig. 2. Expression of stabilized β-catenin in the mouse ventral spinal cord inhibits ventral progenitor cell fates and promotes dorsal progenitor cell fates. Tamoxifen (TM) was given at 150 mg/kg body weight at 09:00 h on E8 and the embryos were analyzed at E11. (**A**,**D**) X-Gal staining of the *Rosa26* reporter. (**B**,**E**) The dorsal marker Pax7 was induced ventrally. (**C**,**F**) Msx1/2 proteins were also ectopically induced in the ventral spinal cord. (**G**) Schematics of the molecular markers used in this figure. White box indicates normal expression domains. Red ovals and circles indicate ectopic expression of dorsal markers in the ventral spinal cord. Stippled box indicates downregulation of ventral markers. (**H-U**) Expression of progenitor or neuronal markers. (N-U) Pax7 and Msx1/2 were used as markers for cells expressing stabilized β-catenin. Mash1 was ectopically expressed in the ventral spinal cord (yellow bracket in L). By contrast, Math1 was not ectopically induced (compare H with K). (N,O,R,S) Markers for dorsal neurons were ectopically induced. A small number of Foxd3^{+} (yellow asterisk in R) or Lbx1^{+} (arrowhead and white box in S) cells were also ectopically induced. White box indicates the area shown in the inset. Markers for ventral progenitors, such as Nkx2.2 (M), Olig2 (T) and Nkx6.1 (U), were inhibited. Note that the assignment of progenitor domains is based on residual wild-type (WT) pattern. Scale bar: 100 μm.

cord progenitors, was lost in some cells (Fig. 2E, arrow). Furthermore, we found that the Msx1/2 proteins (*Msx3* was not ectopically activated, data not shown), which are normally expressed in the dorsal progenitors (Fig. 2C), were ectopically expressed in patches of cells in the ventral ventricular zone (Fig. 2F, white bracket).

To determine which dorsal progenitors were induced as a result of persistent Wnt signaling in the ventral spinal cord, we examined the expression of different dorsal progenitor markers. We found that Math1 (Atoh1– Mouse Genome Informatics), a marker for d1 progenitors (Helms and Johnson, 1998), was not expressed ectopically (Fig. 2H,K). However, Mash1 (Ascl1), a marker for d3-d5 in the dorsal spinal cord (Gross et al., 2002) and for V2 progenitors (pV2) in the ventral spinal cord (Parras et al., 2002) (Fig. 2I), was ectopically expressed in the ventral spinal cord (Fig. 2L, yellow bracket).

We next determined whether dorsal interneurons were produced as a result of the switching of progenitor cell fates. In these experiments, we co-stained sections with antibodies to Pax7 or Msx1/2, two dorsal progenitor markers that were induced by stabilized β-catenin (see below), in order to identify ventral progenitors that had switched their cell fates. We first examined the expression of Foxd3, which marks post-mitotic dI2 dorsal neurons and pV1 progenitors (Dottori et al., 2001) (Fig. 2N), and of Lbx1, which marks dI4-6 interneurons (Gross et al., 2002) (Fig. 2O). We found a small number of ectopic Foxd3$^+$ cells (Fig. 2R, asterisk, next to green Pax7 staining) and Lbx1$^+$ cells (Fig. 2S, inset). The small number of ectopic dorsal neurons suggests that other factors are needed in addition to Wnt signaling for the generation of distinct dorsal neurons.

If stabilized β-catenin induces ventral cells to adopt dorsal fates, then a reduction in ventral cells expressing ventral fate markers would be predicted. We examined the expression of Nkx2.2 (a marker for pV3), Olig2 (a marker for pMN) and Nkx6.1 (a marker for pMN, pV2 and pV3), and found that Nkx2.2 (Fig. 2M), Olig2 (Fig. 2T) and Nkx6.1 (Fig. 2U) were all inhibited by the expression of stabilized β-catenin. In particular, Olig2 and Nkx6.1 were not expressed in cells that were Pax7$^+$ (Fig. 2T,U, insets). These results therefore suggest that expression of stabilized β-catenin in the ventral spinal cord inhibits ventral progenitor cell fates and, at the same time, promotes different dorsal progenitor cell fates (summarized in Fig. 2G).

Wnt signaling activates the expression of different subsets of targets cell-autonomously and in a time-dependent manner

One possible mechanism by which Wnt signaling regulates different cell fates is by activating different subsets of downstream targets in different developmental stages. If this is the case, then we would expect that early Wnt signaling in the ventral spinal cord induces one set of targets, whereas late Wnt signaling induces another set of targets. Alternatively, a reduction in the signaling strength in the ventral spinal cord might also contribute to the activation of different target genes. To distinguish between these two possibilities, we generated *Gli1-CreER;Ctnnb1^{gof};TCF/LEF-lacZ* embryos and activated Wnt signaling through stabilized β-catenin at different stages, using a low dose of TM (50 mg/kg body weight). Since Wnt signaling should be equally maximized, any differences in target gene expression should be due to the activation of Wnt signaling at different stages. In control embryos, a few β-galactosidase-positive cells were detected in the V2 progenitor region of the ventral spinal cord at E10.5 (Fig. 3A,E, white brackets). When TM was given at E7.5 (12:00 h), patches of progenitor cells expressing β-galactosidase were identified in the ventral spinal cord. These cells also expressed Pax7 and Msx1/2 (Fig. 3B,F, see also insets), suggesting a cell-autonomous activation

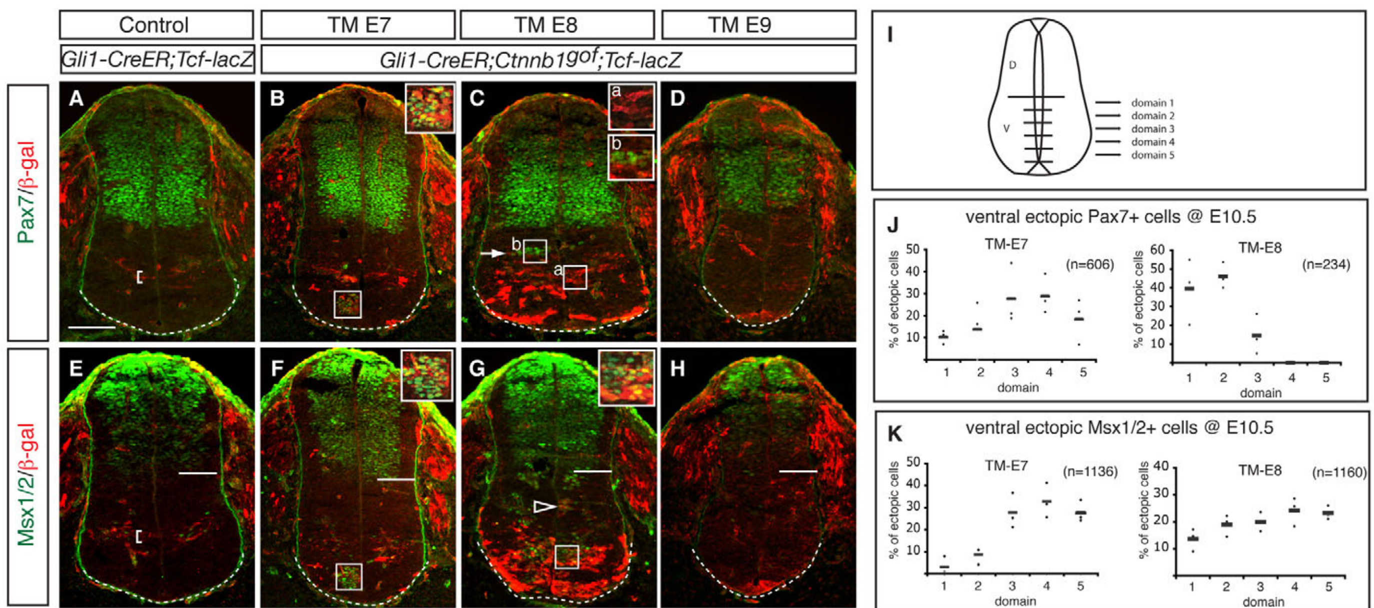

Fig. 3. Expression of stabilized β-catenin can induce different sets of targets in a time-dependent manner. (**A-H**) TM was used at 50 mg/kg body weight and embryos were analyzed at E10.5. Early induction (TM E7 at 12:00 h) induces Pax7 and Msx1/2 (B,F). Subsequent induction (TM E8 at 18:00 h) induces Msx1/2 but not Pax7 in the ventralmost cells, although dorsal cells still express both Pax7 and Msx1/2 (C,G). Late induction (TM E9 at 09:00 h) does not induce Pax7 or Msx1/2 (D,H). White lines indicate dorsoventral boundary. White box indicates the region shown in the insets. (**I**) Schematic of spinal cord divided into five ventral domains. (**J**,**K**) Distribution of ectopic cells within five ventral domains. Each dot in J and K represents the percentage of ectopic cells falling in one domain from one embryo (*n* represents the total number of ectopic cells counted). Thick lines represent the average from three different embryos. Scale bar: 100 μm.

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of dorsal markers. Since these patches of cells were located close to the floor plate, in the pV3 and pMN domains, this result suggests that induction of stabilized β-catenin respecifies the most ventral cells into dorsal cell fates. To quantify the distribution of the ectopic cells, we divided the ventral spinal cord into five equal domains that roughly correspond to the five progenitor domains (Fig. 3I), and found ectopic Pax7$^+$ and Msx1/2$^+$ cells distributed throughout these five ventral domains (Fig. 3J,K). We then examined whether expression of stabilized β-catenin at a later stage could activate these genes. We found that when TM was given at late E8.5 (12:00 h), progenitors located in the most ventral regions (domains 4 and 5) expressed Msx1/2 (Fig. 3G, inset), but not Pax7 (Fig. 3C, inset), suggesting a partial respecification of dorsal cell fates. Indeed, quantification revealed that Pax7$^+$ ectopic cells rarely occupied domains 4 and 5, although Pax7$^+$ cells were found in domains 1-3 (Fig. 3J). By contrast, Msx1/2$^+$ cells were distributed almost evenly throughout the ventral domains (Fig. 3K), similar to when TM was injected at E7.5. Finally, when TM was given at E9 (09:00 h), little, if any, ectopic expression of Msx1/2 or Pax7 was detected in the ventral spinal cord (Fig. 3D,H).

To exclude the possibility that ectopic Pax7$^+$ cells were not induced in embryos receiving TM injection at E8.5 because of a shorter exposure to Wnt signaling by the time of analysis at E10.5 (as compared with embryos receiving TM at E7.5), we let these embryos develop until E11.5. We found that, similar to when analyzed at E10.5, Msx1/2$^+$ cells were found throughout the ventral spinal cord, whereas Pax7$^+$ cells were detected mainly in domains 1-3 (see Fig. S1A-F in the supplementary material). These results suggest that differential activation of target genes is controlled by a time-dependent mechanism, rather than by the length of exposure to Wnt signaling.

In addition to Pax7, another dorsal marker, Gsh1/2 (Gsx1/2 – Mouse Genome Informatics), which normally marks dI3-5 in the dorsal spinal cord (Kriks et al., 2005), was found to be induced in a similar time-dependent manner in the ventral spinal cord (see Fig. S1G-J in the supplementary material). The observation that stabilized β-catenin induces different molecular markers in the ventral progenitors (TM7: Pax7, Gsh1/2 and Msx1/2; TM8: Msx1/2; TM9: no ectopic induction), coupled with the reduction of Wnt signaling in the ventral spinal cord during this period of time, raise the possibility that a gradual reduction in Wnt signaling in the ventral spinal cord allows for the activation of ventral-specific genes and the generation of distinct ventral progenitors.

Stabilization of β-catenin in MN progenitors promotes the generation of V2 neurons

The genetic mosaics, although powerful, do not permit the tracing of fate changes in a chosen population. To determine whether Wnt signaling is involved in specifying different ventral neuronal cell fates in specific groups of ventral progenitors, we used *Olig1-Cre* to induce recombination in discrete progenitor groups (pMN and pV3) in the ventral spinal cord starting from late E8 (Lu et al., 2002; Wu et al., 2006; Zhou and Anderson, 2002). Compared with *Gli1-CreER*-mediated recombination, *Olig1-Cre* is much stronger and can induce complete recombination of floxed alleles within cells expressing *Olig1* (Wu et al., 2006), allowing quantitative analysis of changes in cell fates. Furthermore, *Olig1-Cre* only induces a partial transformation of cell fates, as Msx1/2, but not Pax7, was induced in pMN/pV3 domains (see Fig. S2D-I in the supplementary material). The partially switched phenotype is similar to that found when TM was applied at E8.5 to *Gli1-CreER;Ctnnb1^{gof}* embryos to stabilize β-catenin (Fig. 3). We first examined the expression of two transcription factors, Pax6 and Irx3, that respectively define the dorsal limits of the pV3 and pMN domains. We found that the ventral limit of the Pax6 expression domain was extended ventrally towards the floor plate (Fig. 4A,E, arrowhead). Similarly, the ventral limit of the Irx3 domain was extended ventrally in the pMN domain (Fig. 4B,F, arrow) and the Mash1 expression domain was also expanded (Fig. 4C,G). As a result, there was a drastic reduction in the number of Olig2$^+$ pMNs [wild-type (WT), 56.4±2.5 versus 8.1±1.6; P<0.01] and Nkx2.2$^+$ pV3 neurons (WT, 87±1.7 versus 36.7±3.6; P<0.01) (Fig. 4D,H,M). The remaining pMNs did not form a tight cluster but were instead scattered around in the ventral spinal cord, which is likely to reflect disruption in the progenitor domains as a result of persistent Wnt signaling in the ventral spinal cord.

If there was a change in progenitor cell fates, then we would expect alterations in the number of post-mitotic neurons. In WT embryos, MN and V2 interneurons are generated from ventral progenitors expressing Nkx6.1. As progenitor cells exit the cell cycle, Lim3 (Lhx3 – Mouse Genome Informatics) is expressed in both differentiating MNs and V2 interneurons (Thaler et al., 2002). When β-catenin was stabilized using *Olig1-Cre*, we found a significant reduction in the number of Isl$^+$ (Isl1) MNs (WT, 269.9±10.1 versus 146.4±4.5; P<0.01) and a significant increase in the number of Chx10$^+$ (Vsx2) V2 interneurons (WT, 38.4±1.8 versus 77.9±5.7; P<0.01) (Fig. 4I,K,M). Consistent with this, we also found a significant reduction in the number of Isl$^+$ Lim3$^+$ MNs (WT, 48±1.7 versus 21.1±2.7; P<0.01) (Fig. 4J,L,M). Together, these results show that sustained Wnt signaling in pMN inhibits the generation of MN while promoting V2 neurons.

Wnt signaling-mediated cell fate switching is not dependent on *Gli2*

Wnt signaling might act directly to specify cell fates, or it could interact with the Hh signaling pathway, as both Wnt and Shh signaling are active as early as E8.5 in the ventral neural tube. Indeed, a recent study using the chick electroporation system suggests that Wnt signaling cooperates with Gli2, the primary Gli activator, to regulate the transcriptional response of Shh targets and cell fates (Lei et al., 2006). To determine whether Wnt signaling-mediated cell fate switching functions through *Gli2*, we removed endogenous *Gli2* in embryos expressing stabilized β-catenin. Removal of *Gli2* results in the loss of V3 neurons and in the appearance of MNs in the ventral midline of the spinal cord (Fig. 5A,B) (Matise et al., 1998). When MNs and V2 interneurons were counted in *Olig1-Cre, Ctnnb1^{gof};Gli2$^{-/-}$* embryos, we found a significant reduction in the number of MNs (WT, 269.9±10 versus 155.8±13.2; P<0.01) and a significant increase in the number of V2 neurons (WT, 38.4±1.8 versus 67±8.2; P<0.01) (Fig. 5D,K). However, compared with *Olig1-Cre, Ctnnb1^{gof}* embryos, the number of MNs (P=0.53) or V2 interneurons (P=0.30) in *Olig1-Cre, Ctnnb1^{gof};Gli2$^{-/-}$* embryos was not significantly different (Fig. 5K). These results suggest that a drastic reduction in Shh signaling by removal of *Gli2* does not further alter the cell fate changes caused by activation of Wnt signaling.

Wnt signaling promotes V2 and inhibits MN cell fate in part through the activation of *Gli3*

To determine whether β-catenin-mediated cell fate specification involves activation of *Gli3*, we performed in situ hybridization. In WT embryos at E10.5, *Gli3* is expressed in a dorsal-to-ventral decreasing gradient in the spinal cord (Fig. 5E). When β-catenin was stabilized in the ventral spinal cord with *Olig1-Cre*, we found that the ventral expression domain of *Gli3* was extended towards the floor plate (Fig. 5F, red arrowhead).

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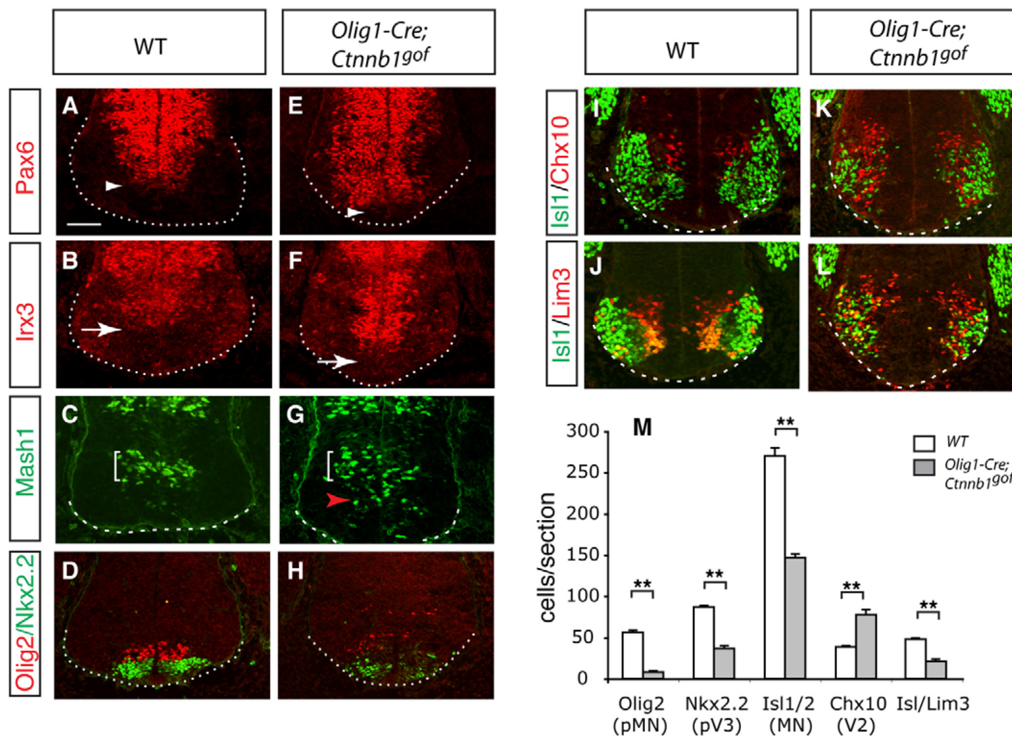

Fig. 4. Activation of Wnt signaling using *Olig1-Cre* promotes V2 neurons at the expense of MNs and V3 neurons at E10.5. (**A-L**) The ventral expression limits of Pax6 (A,E, arrowheads) and Irx3 (B,F, arrows) were extended ventrally, and ectopic Mash1$^+$ cells were also found (C,G red arrowhead). At the same time, there were reductions in the number of Olig2$^+$ pMN and Nkx2.2$^+$ pV3 (D,H). The number of Chx10$^+$ V2 interneurons was increased (I,K) and the number of Isl$^+$ MNs or Isl$^+$ Lim3$^+$ differentiating neurons reduced (J,L). (**M**) Quantification of different cell types in WT and *Olig1-Cre*;*Ctnnb1^{gof}* mouse embryos. Bar represents average ± s.e.m. **, P<0.01; *, P<0.05. Scar bar: 50 μm.

If Wnt signaling inhibits the ventral cell fate specification through transcriptional activation of *Gli3*, then removal of the endogenous *Gli3* genes should rescue the MN defect phenotype caused by ectopic activation of Wnt signaling. Indeed, when *Gli3* was removed, we found a significant increase in the number of Olig2$^+$ pMNs (*Olig1-Cre;Ctnnb1^{gof}*, 8.11±1.64; *Olig1-Cre;Ctnnb1^{gof};Gli3$^{-/-}$*, 34.8±3.6; P<0.01) (Fig. 5I,J,M) and a recovery of Nkx6.1$^+$ progenitors (see Fig. S2L-N in the supplementary material). However, the number of pMNs was still significantly lower than in the WT embryos (WT, 56.4±2.5; P<0.01) (Fig. 5M). We then examined whether removal of *Gli3* increases the number of Nkx2.2$^+$ pV3 in embryos expressing stabilized β-catenin. We found many cells expressing variable levels of Nkx2.2 (Fig. 5J). When only cells expressing high (similar to WT) levels of Nkx2.2 were counted, we found a slight, and not significant, increase in Nkx2.2$^+$ cells (P=0.17). We also examined whether the number of post-mitotic neurons was changed. We found a significant decrease of Chx10$^+$ V2 interneurons (*Olig1-Cre;Ctnnb1^{gof}*, 77.9±5.7; *Olig1-Cre;Ctnnb1^{gof};Gli3$^{-/-}$*, 55.9±5.4; P=0.013), and a slight increase of post-mitotic MNs (*Olig1-Cre;Ctnnb1^{gof}*, 146.4±4.5; *Olig1-Cre;Ctnnb1^{gof};Gli3$^{-/-}$*, 166.5±11.7; P=0.12) (Fig. 5G,H,L). This result suggests that *Gli3* is only partially responsible for the Wnt-mediated repression of ventral cell fates.

Loss of Wnt signaling results in the ventral expansion and disruption of progenitor domains

To test whether Wnt signaling is required for the specification of ventral fates, we removed β-catenin function using *Olig1-Cre* and a conditional loss-of-function allele of β-catenin that has two loxP sites flanking exons 3-6 (*Ctnnb1^{tm1Max}*, hereafter referred to as *Ctnnb1^{lof}*) (Brault et al., 2001). Because expression of *Olig1* starts at ~E8.75, we examined whether the expression of β-catenin was affected at E9.5. We found a drastic reduction in the level of β-catenin in both pV3 (Fig. 6A,D, bracket) and pMN (Fig. 6E, bracket) domains. By E10.5, β-catenin was also clearly absent from ventral-lateral spinal cord that is normally occupied by MNs and V3 cells (Fig. 6F,I,J). Because β-catenin interacts with N-cadherin to regulate cell-cell adhesion, we investigated whether N-cadherin expression was affected. We found that the overall expression of N-cadherin was not affected (Fig. 6K,L), although in some sections cells were found to occupy the spinal cord lumen (Fig. 6I,J,L) and the floor plate appeared to be less compact (Fig. 6H), suggesting that deletion of β-catenin does affect cell adhesion at E10.5. To confirm that deletion of β-catenin does not affect the production or reception of Shh at E9.5, a time when neural progenitors are being specified, we examined the level of Shh protein (Fig. 6B,C), the number of floor plate cells expressing *Shh* (as estimated by mRNA in situ hybridization) and the expression of the Shh targets *Gli1* and *Ptch1* (see Fig. S3 in the supplementary material), and found no significant differences between the β-catenin mutant and WT embryos.

We next examined whether the generation of different cell types in the ventral spinal cord was affected at E10.5. We found a reduction in the ventral expression of Pax6 (Fig. 6M,P, bracket indicates the weak Pax6 expression domain) in β-catenin mutant embryos. This reduction in the ventral expression of Pax6 suggests a ventral-to-dorsal shift of the transcription codes in the ventral spinal cord, and predicts an expansion of ventral neurons. Indeed, we found a significant increase in the number of Nkx2.2$^+$ V3 interneurons (WT, 87±1.7; mutant, 107.7±5.1; P<0.01) (Fig. 6O,R,V). Furthermore, there was a significant increase in the

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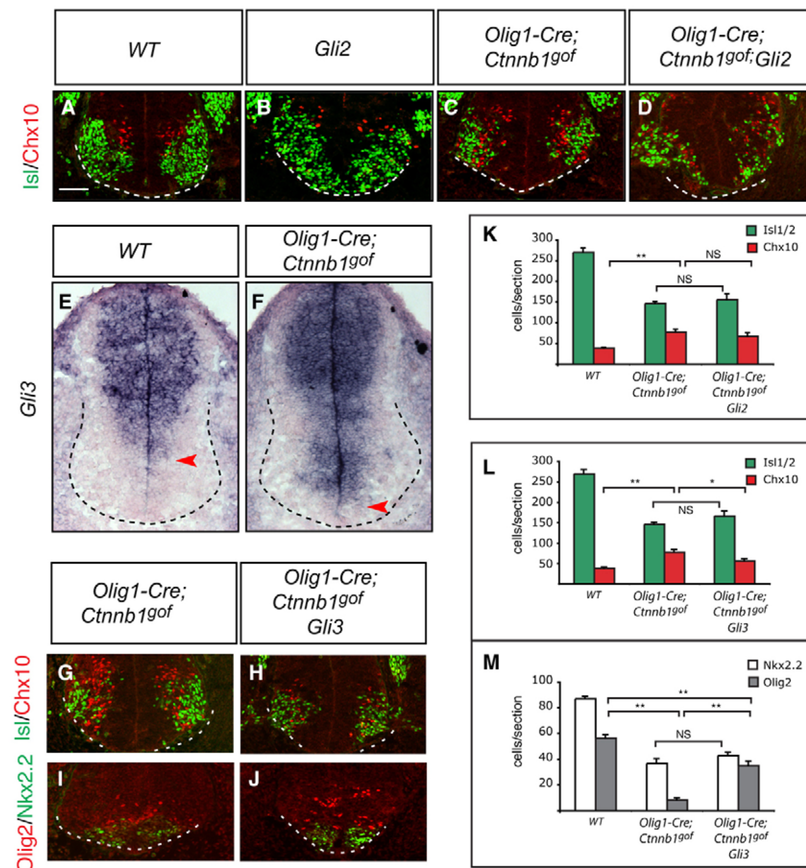

Fig. 5. Wnt signaling-mediated cell fate switching is partially dependent on *Gli3*, but not on *Gli2*. (**A-D**) Removal of endogenous *Gli2* did not significantly change the number of Isl+ MNs or Chx10+ V2 interneurons. Note that when *Gli2* is removed, some of the MNs tend to scatter around the ventral spinal cord. (**K**) Quantification of Isl1/2+ MNs and Chx10+ V2 interneurons. (**E**,**F**) Upregulation of *Gli3* transcription (red arrowheads) in mouse embryos with stabilized β-catenin. (**G-J**) Wnt signaling antagonizes Shh signaling partly through activation of *Gli3*. Further removal of *Gli3* partially releases the inhibition on pMN and pV3. Note the different levels of Nkx2.2 in J. (**L**,**M**) Quantification of different cell types. Embryos were analyzed at E10.5. Bars represent average number ± s.e.m. *, P<0.05; **, P<0.01; NS, not significant. Scale bar: 50 μm.

number of FoxA2+ cells, which include floor plate and some pV3 cells (WT, 24.2±0.5; mutant, 67.2±7.0; P<0.01) (Fig. 6O,R,V). The slight increase in floor plate cells is likely to be caused by changes in cell adhesion and is unlikely to affect neuronal specification as most of the ventral neurons have already been specified by E10.5 and, in addition, we did not detect any significant changes in the expression of *Gli1* or *Ptch1* (see Fig. S4 in the supplementary material). The numbers of pMNs and MNs in the β-catenin mutant and WT embryos were not significantly different (WT, 269.9±10.1; mutant, 263.9±15.7; P=0.75) (Fig. 6N,Q,V). One possibility is that the fate of pMN is partially fixed by the time β-catenin function is deleted. A second possibility is that the reduction in pMN is masked by an increase in cell proliferation, as we observed an increase in BrdU incorporation in the *Olig1-Cre* domain (Fig. 6V). Overall, there was no obvious change in the number of progenitors expressing Nkx6.1 (see Fig. S5A-D in the supplementary material). To exclude the possibility that dorsoventral patterning of the ventral spinal cord was affected, we examined the generation of other cell types that do not express *Olig1-Cre*. We found that the numbers of Chx10+ V2 interneurons and Evx1+ V0 interneurons were not affected (P=0.32 and P=0.75, respectively) (Fig. 6V).

To further confirm that Wnt signaling is indeed involved in early cell fate determination, we removed β-catenin function in early embryos using *Gli1-CreER*-mediated recombination. Because the delivery of high dosages of TM (225 mg/kg) at early E7.5 resulted in embryonic lethality (data not shown), we generated mosaic embryos using a lower dosage of TM (150 mg/kg) at late E7.5 (18:00 h) and recovered the embryos at E10.5. We found an increase in the numbers of FoxA2+ or Nkx2.2+ pV3 cells and Isl1/2+ MNs (Fig. 6T,U), a reduction in the number of Chx10+ V2 cells (Fig. 6T) and a dorsal shift in the Pax6 expression domain (n≥3 embryos) (Fig. 6S). A similar change was observed when TM was delivered at early E8.5 (09:00 h) (see Fig. S5 in the supplementary material). Together, these results demonstrate that the inhibition of canonical Wnt signaling in specific domains of the ventral spinal cord promotes an expansion of ventral cell types.

DISCUSSION

A time-dependent and cell-autonomous mechanism regulating the action of Wnt signaling in the ventral spinal cord

At least three different mechanisms could account for the switching of ventral progenitor cell fates in response to Wnt signaling. First, the switching of cell fate is dependent on Wnt signal strength. In this scenario, a strong Wnt signal induces dorsal cell types, whereas a weak signal induces ventral cell types. However, because different cell types were induced by stabilized β-catenin, Wnt signal strength, although possible, is unlikely to play a crucial role in cell fate determination. The second possibility is that cell fate switching is dependent on the duration of the Wnt signal. For example, the longer that Wnt signaling is maintained in a cell, the more likely it is that the cell will adopt a dorsal cell fate, similar to a mechanism that has been proposed for Shh action (Ahn and Joyner, 2004; Dessaud et al., 2007). In this scenario, the dorsal-most cell type, d1, is specified because these cells receive the longest exposure to Wnt signaling (from E8.5 to E10.5), whereas other cells adopt more-ventral cell fates because they receive shorter exposure to Wnt signaling. However, extending the length of Wnt signaling does not appear to

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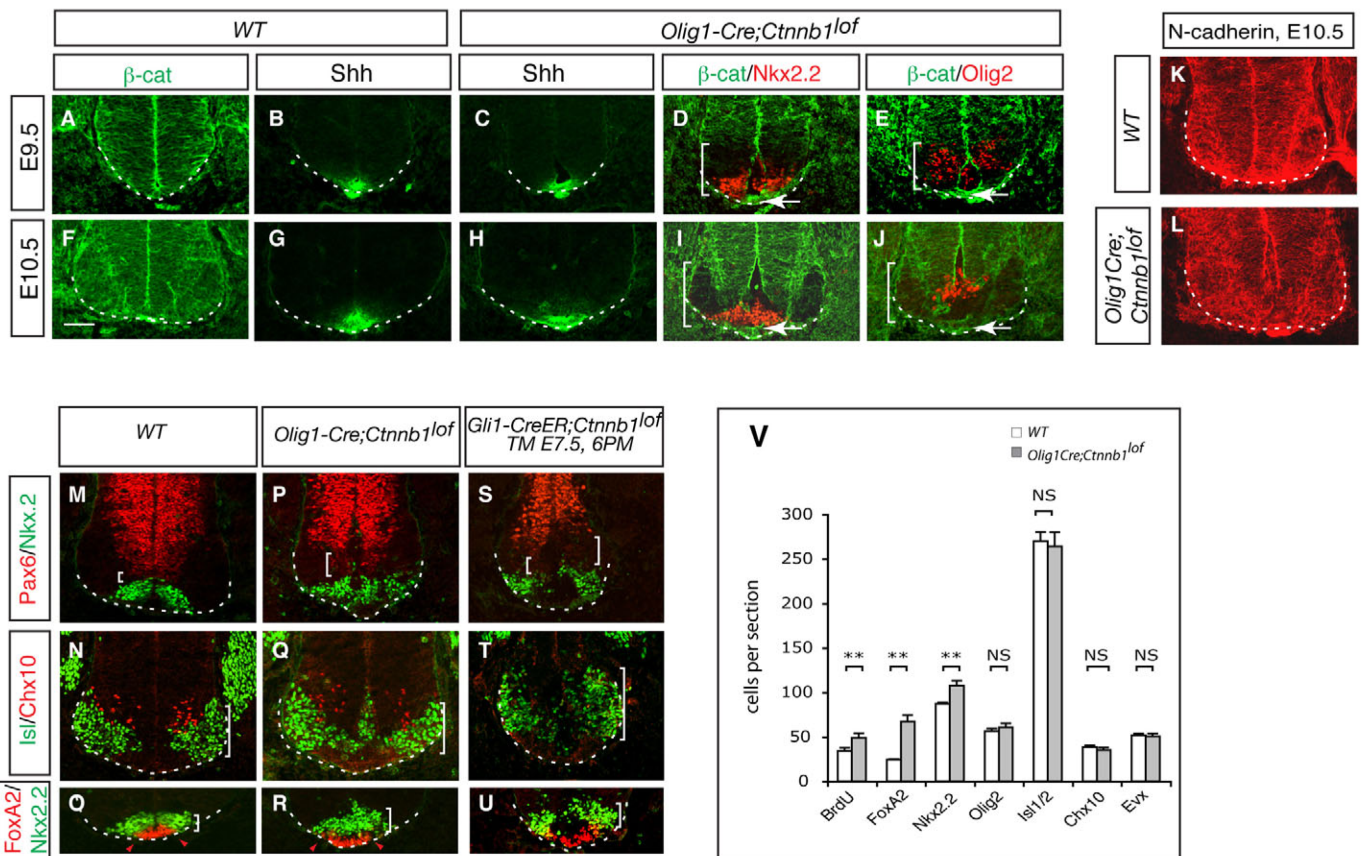

Fig. 6. Disruption of canonical Wnt signaling in the ventral spinal cord promotes ventral cell fates. (**A-J**) Although β-catenin was depleted by E9.5 in both pMN and pV3 domains in mutants (white brackets in D,E), the level of β-catenin in the floor plate (arrows in D,E,I,J) and the expression of Shh protein (C,H) were not significantly altered. (**K**,**L**) No drastic changes were seen in the expression of N-cadherin in mutants. (**M-R**) Expansion of ventral markers in *Olig1-Cre;Ctnnb1^lof^* mutants at E10.5. Loss of β-catenin results in a dorsal shift of the Pax6 domain (M,P, white brackets indicate weak Pax6 domains) and in an increase in the numbers of $Nkx2.2^+$ and $FoxA2^+$ cells (O,R, white brackets indicate $Nkx2.2^+$ domains), although the numbers of Isl^+ MNs or $Chx10^+$ V2 interneurons were not affected (N,Q, white brackets indicate Isl^+ MN domains). (**V**) Quantification of the changes in cell number as a result of disruption of Wnt signaling using *Olig1-Cre*. **, $P<0.01$; NS, not significant ($P>0.05$). (**S-U**) Disruption of β-catenin using *Gli1-CreER* resulted in expansion of Isl^+ (T), $Nkx2.2^+$ or $FoxA2^+$ cells (U), and a reduction of $Chx10^+$ V2 interneurons (T). TM was given at 18:00 h on E7.5. Embryos were analyzed at E10.5. Scale bar: 70 μm in A-E; 50 μm in F-U.

alter the expression of dorsal markers. Lastly, it is possible that different cell fates are specified depending on when Wnt signaling is active. In this scenario, Wnt signaling is capable of activating different genes at different time points, based on the changing competence of the cells. Indeed, we found that early Wnt signaling (induced with TM at E7.5) activated the expression of several dorsal markers, Pax7, Gsh1/2 and Msx1/2. By contrast, Wnt signaling induced with TM at E8.5 could only induce the expression of Msx1/2, and Wnt signaling induced subsequently did not activate dorsal markers. Our results therefore strongly support the time-dependent mechanism of Wnt signaling in the ventral spinal cord.

Only ~70-90% of ectopic $Msx1/2^+$ or $Pax7^+$ cells coexpressed *TCF/LEF-lacZ* (see Fig. S6C in the supplementary material). However, this is likely to be an underestimate because the *TCF/LEF-lacZ* transgenic reporter was not expressed in all E10.5 progenitors that expressed stabilized β-catenin (see Fig. S6B-B″ in the supplementary material). The action of Wnt signaling on cell fate changes is likely to be cell-autonomous. However, we cannot completely exclude non-autonomous effects, particularly in light of the reduction in the Irx3 ventral expression domain, which lies outside of the *Olig1-Cre* expression domain, in β-catenin mutant embryos (see Fig. S6D,E in the supplementary material). Nevertheless, we did not observe upregulation of phosphorylated Smad1/5/8 in embryos expressing stabilized β-catenin (see Fig. S2J,K in the supplementary material), suggesting that BMP pathways were not activated in response to stabilized β-catenin.

Although the removal of β-catenin using *Olig1-Cre* affects the morphology of the floor plate at E10.5, the action of Wnt signaling on cell type switching appears to be direct, based on the following observations. First, there was no significant change in the level of Shh protein, the number of Shh-expressing cells or in the response to Shh at E9.5, when cell fate is being specified (see Fig. 6C; see Fig. S3 in the supplementary material). Even at E10.5, when most of the cells have been specified, no significant changes in the expression of *Gli2*, Shh, *Ptch1* or *Gli1* were observed (see Fig. 6G,H; see Fig. S4 in the supplementary material), although the floor plate appeared to be less compact. In fact, deletion of floor plate does not affect the generation of most ventral neurons, except for V3 cells, as has been demonstrated in *Gli2* mutants (Ding et al., 1998; Matise et al., 1998). Lastly, activation of Wnt signaling in small patches of cells using *Gli1-CreER* reveals that activation of Wnt signaling directly affects cell fate (Fig. 3).

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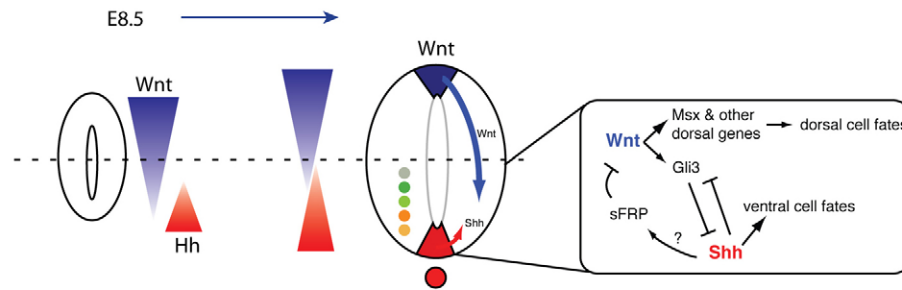

Fig. 7. A model for how Wnt signaling influences the specification of cell fate in the mouse ventral neural tube. At E8.5, Wnt signaling is active throughout the neural tube when Shh signaling is initiating in the floor plate. As development proceeds, there is a shift of Wnt signaling in the ventral spinal cord. From ventral to dorsal, progenitor cells are released from Wnt signaling gradually. The release from Wnt signaling creates a permissive environment for cell fates to be specified. Wnt signaling promotes dorsal cell fates through the activation of dorsal genes (in particular *Msx1/2*) and of *Gli3*, which encodes a major repressor of Shh signaling. Shh signaling, in turn, may induce Wnt inhibitors, such as sFRPs (secreted frizzled-related proteins), to antagonize Wnt signaling. Hh, Hedgehog.

Wnt signaling has multiple roles in spinal cord development

Previous studies have suggested that Wnt signaling plays multiple roles in neural development. For example, Wnt signaling controls the rostrocaudal patterning of the motor column (Nordstrom et al., 2006), the proliferation of neural progenitors (Chenn and Walsh, 2002; Megason and McMahon, 2002; Zechner et al., 2003) and the specification of dorsal spinal cord cell types, either directly or through interactions with the BMP pathway or *Olig3* (Chesnutt et al., 2004; Ille et al., 2007; Muroyama et al., 2002; Zechner et al., 2007). Wnt signaling also appears to prevent the expansion of Shh signaling into the dorsal spinal cord, an effect that is mediated through the regulation of the *Gli3* gene (Alvarez-Medina et al., 2008).

Whether Wnt signaling is required to regulate different progenitor cell fates during normal ventral spinal cord development has been unclear. We identified a novel function of Wnt signaling in regulating cell fates in the ventral spinal cord. We found that Wnt signaling was active in ventral progenitors at the time when the neural tube closes at E8.5. Later, there is a ventral-to-dorsal shift in the domain of Wnt signaling such that by E9.5, the most ventral progenitors are no longer responsive to Wnt signaling (Fig. 1). This shift in Wnt signaling correlates with the appearance of ventral cell types and suggests that Wnt signaling might be involved in cell fate determination in the ventral spinal cord. Indeed, we found that activation of Wnt signaling in pMN and pV3 inhibited these two cell types and, at the same time, promoted a more dorsal V2 cell fate. Furthermore, disruption of Wnt signaling at early E8.5 using *Gli1-CreER* resulted in an expansion of ventral cell fates (Fig. 6S-U; see Fig. S4 in the supplementary material). Together, these results provide strong evidence that Wnt signaling is required for normal patterning in the early neural tube.

So if Wnt signaling promotes dorsal character, how can it be involved in regulating distinct cell types in the ventral spinal cord? One likely mechanism is through a combination of restricting the extent of Wnt signaling and restricting the ability of Wnt signaling to regulate downstream targets in the ventral spinal cord. The ventral-to-dorsal shift in Wnt signaling, together with a restricted ability to regulate downstream targets, is likely to create a permissive molecular environment that enables different ventral cell fates to emerge. Such a release-of-inhibition mechanism has been described in the development of other neural tissues. For example, the attenuation of FGF signaling emanating from the posterior mesoderm is necessary for the emergence of neuronal cell types in the ventral spinal cord (Diez del Corral et al., 2003). Similarly, Shh expression, which is initially detected in the ventral hypothalamus, needs to be inhibited in those tissues in order for different cell types to develop (Manning et al., 2006). In all these cases, regulated inhibition of these signaling molecules is required for the specification of normal cell fates.

In addition to activating dorsal markers, Wnt signaling appears to interact directly with Shh signaling through the regulation of Gli genes. We show that removal of endogenous *Gli2*, which encodes the major Gli activator, does not affect the Wnt-mediated switching of cell fates (Fig. 5B). Furthermore, we independently found that Wnt signaling activates the transcription of *Gli3*, which encodes the primary transcriptional repressor of the Shh signaling pathway. By activating the transcription of *Gli3*, the extent of Shh signaling can be restricted. However, *Gli3* is unlikely to be the sole effector of Wnt signaling in the ventral spinal cord because removal of endogenous *Gli3* in embryos with stabilized β-catenin only partially rescues the inhibitory effect of Wnt signaling on pV3 and pMN (Fig. 5). Furthermore, expression of *Gli3* alone is not sufficient to activate the expression of dorsal genes, such as *Pax7* or Msx. A second likely effector of Wnt signaling are the Msx genes, as these were ectopically induced in embryos with stabilized β-catenin (Fig. 3) and have previously been shown to activate the expression of dorsal spinal cord genes in response to BMP signaling (Liu et al., 2004; Timmer et al., 2002). Therefore, Wnt signaling, through activating both dorsal genes and *Gli3*, ensures that ventral genes are inhibited and dorsal genes are activated, and that different ventral cell types emerge in coordination with Shh signaling (Fig. 7).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/22/3687/DC1

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