Transcription Factor Hb9 Is Expressed in Glial Cell Lineages in the Developing Mouse Spinal Cord

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Abstract

Hb9 (Mnx1) is a transcription factor described as a spinal cord motor neuron (MN)-specific marker and critical factor for the postmitotic specification of these cells. To date, expression of Hb9 in other cell types has not been reported. We performed a fate-mapping approach to examine distributions of Hb9-expressing cells and their progeny (“Hb9-lineage cells”) within the embryonic and adult spinal cord of Hb9cre;Ai14 mice. We found that Hb9-lineage cells are distributed in a gradient of increasing abundance throughout the rostrocaudal spinal cord axis during embryonic and postnatal stages. Furthermore, although the majority of Hb9-lineage cells at cervical spinal cord levels are MNs, at more caudal levels, Hb9-lineage cells include small-diameter dorsal horn neurons, astrocytes, and oligodendrocytes. In the peripheral nervous system, we observed a similar phenomenon with more abundant Hb9-lineage Schwann cells in muscles of the lower body versus upper body muscles. We cultured spinal cord progenitors in vitro and found that gliogenesis was increased by treatment with the caudalizing factor FGF-8B, while glial tdTomato expression was increased by treatment with both FGF-8B and GDF-11. Together, these observations suggest that early and transient expression of Hb9 in spinal cord neural progenitors may be induced by caudalizing factors such as FGF and GDF signaling. Furthermore, our work raises the possibility that early Hb9 expression may influence the development of spinal cord macroglia and Schwann cells, especially at caudal regions. Together, these findings highlight the importance of using caution when designing experiments using Hb9cre mice to perform spinal cord MN-specific manipulations.

Key words: astrocytes; Cre recombinase; Hb9; oligodendrocytes; spinal motor neurons

Significance Statement

The transcription factor Hb9 is key in postmitotic specification of spinal cord motor neurons (MNs), and thought to be expressed specifically in this cell population. We performed fate-mapping experiments and found that Hb9-lineage cells are not restricted to motor neurons, but also include spinal cord macroglia and Schwann cells. Strikingly, Hb9-lineage cells are present in an increasing rostrocaudal gradient in the CNS. Proportions of Hb9-lineage astrocytes in cultures of spinal cord progenitors could be manipulated by treatment with caudalizing factors FGF-8B and GDF-11. These findings highlight an interesting developmental phenomenon in which there is an increasing rostrocaudal gradient of molecularly-defined cell types. Further, these findings urge caution for researchers using Hb9cre mice to study spinal motor neurons in isolation.
Introduction

Hb9 (Mnx1) is a well-established marker for motor neurons (MNs) within the developing CNS (Tanabe et al., 1998; SK Lee et al., 2004; Nakano et al., 2005). Its role in the consolidation of MN identity includes maintaining normal MN migratory patterns and proper muscle innervation (Pfaff et al., 1996; Arber et al., 1999). In mice, Hb9 expression has first been detected at embryonic day (E)9.5, which coincides with the emergence of the first postmitotic MNs in the spinal cord (Arber et al., 1999; Caldeira et al., 2017). In Hb9-mutant mice, migratory patterns of MNs are disrupted, MN differentiation is disturbed, motor axons project abnormally, and the phrenic nerve fails to form—resulting in stillbirth (Thaler et al., 1999; Yang et al., 2001). However, differentiation of MN progenitors within the spinal cord into somatic and visceral MNs remains constant (Arber et al., 1999; Stefani, 2014). Mutations in the human HB9 gene cause disruptions in dorsal-ventral patterning resulting in a congenital malformation, characterized by significant sacral defects, called Currarino syndrome (Dworschak et al., 2021).

Expression of Hb9 in the spinal cord of the embryonic chick has been shown to induce MN differentiation while suppressing the differentiation of V2 interneurons, which emerge from an adjacent progenitor domain (Thaler et al., 1999). Researchers studying avian embryonic development have found that Hb9 expression in MNs is largely regulated by the expression of the morphogen sonic hedgehog (Shh; Kahane and Kalcheim, 2020). Hence, Hb9 is widely considered to be a major regulatory switch that induces spinal cord MN cell fate. However, to date there have not been any published reports of Hb9 expression in other cell types within the spinal cord, except for a population of excitatory Shox2 non-V2a interneurons involved in rhythm generation (Caldeira et al., 2017).

To investigate Hb9 expression in early embryonic development, we performed fate mapping experiments using Hb9cre; Ai14 mice. Heterozygous Hb9cre mice, which express Cre recombinase in place of one copy of the Hb9 gene (Arber et al., 1999; Yang et al., 2001), were crossed with Ai14 (B6.Cg-Gt(Rosa)26Sortm14(CAG-tdTomato)Hze/J, #007914, The Jackson Laboratory), and Hb9 cre (B6.129S1-Mnx1tm4(cre)Tmj/J, #006600, The Jackson Laboratory) mice. Animals ranging from age postnatal day (P)0 to 12 weeks old were used for experiments. Animals had free access to food and water throughout the study and were group-housed (up to five littermates per cage) in standard Plexiglas cages on a 12/12 h light/dark cycle (light cycle = 6 A.M. to 6 P.M.).

Embryo generation and neural progenitor cell (NPC) isolation

For generation of Hb9cre;Ai14 progeny, Hb9cre males were used as sires and Ai14 females were used as dams. Mouse embryos were generated through timed mating between heterozygous Hb9cre males and Ai14 (RCL-tdT)-D females. Adult female mice received intraperitoneal injections of luteinizing hormone-releasing hormone (5 I.U.; Sigma-Aldrich #L-4513) and 4 d later, females were paired with males overnight. When possible, pregnancy was confirmed by palpation of the abdomen 12 d later. Upon embryo collection, Cre+ embryos were visualized using a fluorescent flashlight with a green excitation filter (NIGHTSEA). Embryonic spinal cords were dissected in ice-cold HBSS. For in vitro studies, spinal cords were cut transversely at ~4.0–4.5

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mm from the rostral end to separate the anterior and posterior segments. In this way, the “anterior” segment included the cervical and upper half of the thoracic cord, and the “posterior” segment included the lumbar and lower half of the thoracic cord.

For neural progenitor cell (NPC) isolation, anterior and posterior spinal cord segments were digested separately in 0.125% trypsin at 37°C for 8–10 min (≤10 spinal cords were pooled for a single cell preparation). Fetal bovine serum (10% in DMEM) was then added at a 10:1 volume ratio to halt the trypsinization reaction, and spinal cords were centrifuged at 600 RCF for 2 min. Supernatant was removed and tissue was gently triturated in Neurobasal Medium + 2% B27 (NBM/B27) until cell suspension appeared milky and homogeneous (~20 passes through a P1000 pipette tip). Cell suspensions were then centrifuged at 600 RCF for 2 min. Supernatant was removed and cells were resuspended in 2–3 ml of NBM/B27, then passed through a 40-μm cell strainer. Cell viability was assessed by trypan blue exclusion and confirmed to be >95% in all cases. Cells were then plated in 48-well culture plates at 1 million cells per well, and cultured for 10 d with once daily media changes. Media was removed, and cells were fixed with 2% PFA for 20 min at room temperature, then washed and stored in PBS until immunocytochemistry was performed.

Drug treatment
For drug treatment experiments, only anterior spinal cord neural progenitor cells were used. Either CHIR 99 021 (2 μM; Tocris #4423, batch #13), recombinant human/murine FGF-8b (200 ng/ml; Peprotech #100–25, lot #0220161), recombinant human/murine/rat GDF-11 (50 ng/ml; Peprotech #120–11, lot #0314295), or vehicle (0.1% DMSO) was added into 250 μl of media beginning immediately after plating and continuing once daily for 7 d. Media were removed, and cells were fixed with 2% paraformaldehyde (PFA) for 20 min at room temperature, then washed and stored in PBS until immunocytochemistry was performed.

Spinal cord immunohistochemistry
Animals were euthanized by anesthesia overdose and transcardially perfused with 30 ml of 0.1 M phosphate buffer + 0.9% NaCl (PB) followed by 30 ml of 4% PFA in 0.1 M PB. Spinal columns were removed and postfixed in 4% PFA in 0.1 M PB overnight at 4°C, then cryopreserved in 30% sucrose in 0.1 M PB at 4°C for at least 3 d before cryosectioning. For fixation of embryos, the pregnant mother was perfused as above, then embryos were removed and postfixed in 4% PFA for 30 min to 2 h at room temperature. Embryos were then cryopreserved in sucrose for at least 3 d before cryosectioning. Adult and embryonic spinal cord tissue was embedded in Tissue-Tek OCT compound (VWR) and frozen on dry ice. Spinal cord tissue was cryosectioned in the transverse or sagittal plane to a thickness of 10 μm. Sections were either collected into a 24-well plate filled with storage solution or directly mounted on slides and stored at −20°C.

Either a 1-in-6 or a 1-in-12 tissue series was used for immunohistochemistry experiments. Sections were washed in tris-buffered saline (TBS) three times for 10 min each, then blocked in TBS containing 5% donkey serum (Lampire Biological Laboratories, #7332100) and 0.25% Triton X-100 until cell suspension appeared milky and homogeneous (~20 passes through a P1000 pipette tip). Cell suspensions were then centrifuged at 600 RCF for 2 min. Supernatant was removed and cells were resuspended in 2–3 ml of NBM/B27, then passed through a 40-μm cell strainer. Cell viability was assessed by trypan blue exclusion and confirmed to be >95% in all cases. Cells were then plated in 48-well culture plates at 1 million cells per well, and cultured for 10 d with once daily media changes. Media was removed, and cells were fixed with 2% PFA for 20 min at room temperature, then washed and stored in PBS until immunocytochemistry was performed.

Fluorescent imaging of neuromuscular junctions
Muscles for whole-mount labeling of neuromuscular junctions (NMJs) were prepared and immunostained as described previously (Yi Lee et al., 2016; Yi Lee, 2019). Briefly, the animals were transcardially perfused with PBS, pH 7.4. The extensor digitorum longus, extensor hallucis longus, diaphragm, soleus, sternomastoid and triangularis sterni muscles were dissected and fixed in 4% phosphate-buffered PFA, pH 7.4 for 20 min at room temperature and rinsed in three changes, 5 min each, of PBS. To label surface nicotinic acetylcholine receptor (AChR) at NMJs, fixed muscles were incubated with fluorescently-labeled α-bungarotoxin (α-BTX; a snake toxin which
binds specifically and with high affinity to AChR; 1:500; Invitrogen) before permeabilization. The presynaptic nerve terminals were labeled with a mixture of monoclonal antibodies (mAbs) to neurofilament and synaptic vesicles (2H3 and SV2, respectively). Schwann cells were labeled with a rabbit polyclonal antibody against S100B.

**Immunocytochemistry**

Fixed cells were washed with PBS and blocked with 5% donkey serum in TBS for 1 h at room temperature, followed by incubation with primary antibodies (Table 1) diluted in blocking solution for 1.5 h at room temperature. Cells were then washed in TBS three times for 5 min each, incubated in Alexa Fluor-conjugated secondary antibodies (Jackson ImmunoResearch) diluted 1:1000 in blocking solution for 1 h at room temperature, and then incubated in 5 µg/ml DAPI for 10 min. Cells were washed in TBS three times for 5 min each, and stored in TBS at 4°C before imaging.

**Image acquisition**

Samples labeled with fluorescent dyes were imaged in a dark room. Images were acquired using the same acquisition settings across all samples for each immunohistochemical label. Slides were imaged using a Nikon Eclipse upright fluorescence microscope equipped with a Prior Scientific XY motorized stage and a Zyla 4.2 PLUS monochrome camera (Andor), and cells were imaged using a Nikon Eclipse Ti2 inverted fluorescent microscope. Nikon NIS-Elements software was used for image acquisition and XY stitching. Images were captured with a 10× or 20× magnification objective. Confocal microscope was performed using an Olympus FV1000. To generate representative images (not used for quantification), the Extended Depth of Focus module in NIS-Elements was sometimes used to create focused TIFF files for analysis. For NMJ imaging, images were acquired using a Zeiss LSM 780 confocal system or a Leica DMR epifluorescence microscope equipped with a Hamamatsu cooled CCD camera controlled by a Macintosh computer with iVision software (BioVision Technologies).

**Image analysis**

All image analysis was performed using ImageJ software. Images of mCherry, NeuN, Olig2, and Sox9 immunoreactivity were thresholded using the ImageJ Auto Local Threshold function with Phansalkar’s thresholding method. Watershed was applied to binary images and the Analyze Particles function was used to count the total number of cells in the entire image [no regions of interest (ROIs) were drawn]. Automated cell counting methods were always validated by manual counts for two images within each batch; two independent experimenters hand-counted cells in a blinded fashion, then hand counts were compared with results generated with the automated cell counting macro. We found that interexperimenter counts were 99% accurate and >95% consistent with automated counts.

**Quantification of tdTomato+ cells in dorsal and ventral spinal cord**

ROIs were drawn for individual tdTomato+/NeuN+ cells (Fig. 1G,J) or tdTomato+/Sox9+ cells (Fig. 1H,J,K,L) in spinal cord tissue sections. A horizontal line was drawn onto each image through the central canal, such that the line separated the dorsal and ventral halves of the spinal cord. Individual cell ROIs were thereby determined to be either “dorsal” or “ventral.”

**Quantification of Schwann cells**

Within the confines of NMJs, the Hb9Cre-driven expression of tdTomato was deemed to be present in Schwann cells when its expression mirrors S100B fluorescence in addition to its accumulation in Schwann cell somata, but restricted to motor axon terminals when no Schwann cell labeling is observed.

**Quantification of cell cluster size**

Regions of interest were manually drawn around contiguous clusters of DAPI+ cells in which all cells were touching each other, and the total area of each cluster was measured in ImageJ. ROIs were drawn around every cluster in all images to avoid quantification bias.

**Statistical analysis**

GraphPad Prism 8 (GraphPad Software) was used to perform statistical analysis. The experimental design was random. All data are presented as mean ± SEM. Statistical significance was defined as \( p < 0.05 \). Detailed statistical methods are included in Table 2.

**Results**

tdTomato labels neurons and glial cells in increasing rostrocaudal abundance in the adult and embryonic Hb9cre;Ai14 mouse spinal cord

We sought to generate transgenic mice with fluorescent reporter expression in spinal cord motor neurons. Because of the well-described role of Hb9 in consolidating MN identity and its use as a MN-specific marker (Arber et al., 1999; Nakano et al., 2005; Amoroso et al., 2013), we crossed male Hb9Cre mice with female Ai14 mice to generate F1 progeny with the Hb9cre;Ai14 genotype (Fig. 1A). We expected these mice to express the tdTomato (tdT) reporter in all Hb9+ MNs as well as Hb9+ interneurons (Wilson et al., 2005; Caldeira et al., 2017). Surprisingly, on inspection of spinal cord tissue of Hb9cre;Ai14 mice, we found that tdT expression was visible in an increasing rostrocaudal gradient, with low levels of fluorescence in the rostral spinal cord and high fluorescence levels in the caudal cord (Fig. 1B). We analyzed reporter expression in Ai14 mice (without Cre), and found a complete absence of tdTomato expression (Fig. 2), indicating that reporter expression is Cre-dependent, as expected. Transverse sections of spinal cord tissue were inspected at cervical, thoracic, lumbar, and sacral levels to examine patterns of tdT...
Figure 1. tdTomato is expressed in glial cells of the adult Hb9cre;Ai14 mouse in an increasing rostrocaudal gradient. A, Cartoon depicting the breeding scheme used to generate mice in this study. Hb9cre male sires were mated to Ai14 female dams to produce Hb9cre;Ai14 F1 offspring. B, Gross image of whole spinal cord from a P28 Hb9cre;Ai14 mouse. Black markings and arrowheads indicate the position of C1, T1, and L1 spinal levels. C, Transverse sections of cervical (C5), thoracic (T5), lumbar (L4), and sacral (S4) spinal cord labeled for choline acetyltransferase (ChAT) and tdTomato (tdT). Images are from a P28 Hb9cre;Ai14 mouse. C', Inset shows tdTomato+ axons in the fasciculus gracilis (fg) but not fasciculus cuneatus (fc) of the cervical spinal cord. C'', Inset shows small-diameter tdTomato+ neurons in the lumbar dorsal horn. D–F, High-magnification images of sagittal sections from the thoracic spinal cord of a P28 Hb9cre;Ai14 mouse. D, D', tdTomato+ cells with neuronal morphology. E, E', tdTomato+ cells with astroglial cell morphology that express glial cell marker Sox9 (arrowheads). F, tdTomato+ cells with oligodendrocyte-like morphology.
expression. We found that in the cervical spinal cord, tdTomato expression was largely restricted to choline acetyltransferase (ChAT)-expressing MNs, with the exception of very few tdT$^+$ cells with glial morphology as well as tdT$^+$ axons in the fasciculus gracilis (Fig. 1C'). Interestingly, we did not observe tdT$^+$ axons in the fasciculus cuneatus, which is composed of ascending axons derived from sensory neurons at cervical levels (Fig. 1C'). In contrast, at more caudal levels of the spinal cord, tdT$^+$ expression was not mostly restricted to MNs; abundant tdT$^+$ cells with glial morphology were distributed throughout the white and gray matter of the spinal cord (Fig. 1C). On closer inspection of tdTomato$^+$ cells, we found that they expressed markers of neurons, astrocytes, and oligodendrocytes (Fig. 1D–F). We therefore quantified the numbers of tdT$^+$ neurons and glial cells at cervical, thoracic, lumbar, and sacral spinal levels (Fig. 1G–I). Whereas only MNs were labeled in the cervical spinal cord gray matter (252 ± 101 tdT$^+$/NeuN$^+$ cells/mm$^3$), there were significantly more tdT$^+$/NeuN$^+$ neurons in the dorsal horn of the thoracic, lumbar, and sacral spinal cord (T: 2140 ± 269; L: 2380 ± 130; S: 441 ± 168 cells/mm$^3$; Fig. 1C'), (G). In the spinal cord gray matter, significantly fewer tdT$^+$ astrocytes were present in the cervical spinal cord (92.4 ± 46.8 tdT$^+$/Sox9$^+$ cells/mm$^3$) compared with all other levels (T: 2690 ± 337; L: 2770 ± 98.6; S: 5760 ± 371 cells/mm$^3$), with sacral levels containing more tdT$^+$ astrocytes than more rostral levels (Fig. 1H). In the spinal cord white matter, there were significantly more tdT$^+$ astrocytes in the sacral cord (1760 ± 255 tdT$^+$/Sox9$^+$ cells/mm$^3$) than all other levels (C: 71.4 ± 36.6; T: 679 ± 40.3; L: 797 ± 74.9 cells/mm$^3$; Fig. 1I).

We also compared the numbers of tdT$^+$ cells that were either located in the dorsal or the ventral halves of the spinal cord. We found that there were significantly greater numbers of tdT$^+$ neurons in the dorsal gray matter compared with the ventral gray matter in the sacral cord (Fig. 1J). Similarly, there were significantly greater numbers of tdT$^+$ astrocytes in the dorsal gray matter versus the ventral gray matter at these caudal spinal levels (Fig. 1K).

In contrast, white matter astrocytes showed the opposite effect; there were significantly greater numbers of tdT$^+$ astrocytes in the ventral white matter at sacral levels (Fig. 1L). We also quantified the percentages of Sox9$^+$ glial cells that expressed tdTomato at each spinal level, and found that the sacral spinal cord had the highest percentage of tdT$^+$ glia (83.2 ± 4.80%), significantly greater than all other levels (C: 0.818 ± 0.403%; T: 5.22 ± 0.884%; L: 17.7 ± 0.399%; Fig. 1M). Together, these data reveal that neurons and glial cells derived from Hb9$^+$ lineages, are distributed in an increasing gradient along the spinal cord rostrocaudal axis. We found that none of these cells had detectable Hb9 immunoreactivity in the adult spinal cord (data not shown), so we refer to these as “Hb9-lineage cells.”

The Hb9$^{cre}$ mouse has previously been used to target motor neurons, either for their deletion or gene inactivation, in examining innervation of postsynaptic skeletal muscle fibers (Yang et al., 2001; Chipman et al., 2014). In light of the above observation that glial populations within spinal cord are Hb9-lineage cells, we examined the possibility of similar glial expression in the peripheral nervous system. We examined patterns of tdTomato expression at NMJs within six different muscles along the rostrocaudal axis from postnatal Hb9$^{cre};$Ai14 mice. Although tdT$^+$ expression was restricted to motor axons at some NMJs (Fig. 3A), the reporter fluorescence was present also in S100B$^+$ Schwann cells at others and along the preterminal axons that innervate them (Fig. 3B). Schwann cells are derived from neural crest cells, which emerge from the dorsal most aspect of the neural tube and migrate to the periphery during development (Woodhoo and Sommer, 2008; Jessen and Mirsky, 2019). A majority of these help achieve salutatory conduction along peripheral axons by generating myelin sheaths. A subtype of Schwann cells found at NMJs, called terminal Schwann cells, do not form myelin but have been shown to help remodel neuromuscular synaptic morphology during development and after injury (Griffin and Thompson, 2008; Yi Lee et al., 2016, 2017). We quantified the percentage of NMJs that exhibited tdTomato expression in terminal Schwann cells, and found that muscles of the hindlimbs (e.g., extensor digitorum longus and soleus) contained greater proportions of labeled Schwann cells compared with muscles of the upper body (e.g., sternomastoid and triangularis sterni; Fig. 3C). This observation mirrors and is consistent with the greater abundance of tdT$^+$ glial cells in the caudal spinal cord (Fig. 1), suggesting that some caudalizing factor is potentially upstream of Hb9 activity early in neural tube development.

**Hb9 is expressed in neural progenitors in the embryonic mouse spinal cord**

The observation that Hb9-lineage cells include glial cells as well as neurons in the adult mouse spinal cord suggests that Hb9 might be expressed in a subset of neural progenitor cells in the developing spinal cord. We assessed native patterns of Hb9 expression in the embryonic cervical spinal cord and found that Hb9 immunoreactivity is first detectable around E9.5, is expressed in greater numbers of cells by E10.5–E11.5, and does not

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**continued**

F$^+$, Some tdTomato$^+$ cells exhibit nuclear expression of Olig2. G–I, Quantification of the number of tdTomato$^+$ (G) neurons, (H) gray matter (GM) astrocytes, and (I) white matter (WM) astrocytes at cervical (C5), thoracic (T5), lumbar (L4), and sacral (S4) spinal segments. J–L, Quantification of the numbers of tdTomato$^+$ (J) neurons, (K) gray matter astrocytes, and (L) white matter astrocytes in the dorsal or ventral halves of each spinal cord tissue section. M, Quantification of the percent of total Sox9$^+$ glial cells that express tdTomato at each spinal level. Detailed descriptions of statistical analyses are provided in Table 1. **p < 0.05, ***p < 0.01, ****p < 0.001, *****p < 0.0001. All data are mean ± SEM N = 3 per group. Scale bars = 2 mm (B), 250 μm (C), 100 μm (D, F), 50 μm (C$'$), and 25 μm (C', D', E', F').
Table 2: Detailed description of statistical analyses used in this study

| Figure | Parameter | Sample size | Statistical test | Significance level |
|--------|-----------|-------------|------------------|--------------------|
| 1G | tdTomato+ neurons in P28 spinal cord | N=3 mice per group | Ordinary one-way ANOVA with Tukey’s multiple comparisons test | F(2,8) = 90.22, p < 0.0001 (C vs T: p = 0.0003, C vs L: p = 0.0001, C vs S: p < 0.0001, T vs S: p < 0.0001, L vs S: p = 0.0002) |
| 1H | tdTomato+ gray matter astrocytes in P28 spinal cord | N=3 mice per group | Ordinary one-way ANOVA with Tukey’s multiple comparisons test | F(2,8) = 81.54, p < 0.0001 (C vs T: p = 0.0004, C vs L: p = 0.0004, C vs S: p < 0.0001, T vs S: p = 0.0001, L vs S: p = 0.0002) |
| 1I | tdTomato+ white matter astrocytes in P28 spinal cord | N=3 mice per group | Ordinary one-way ANOVA with Tukey’s multiple comparisons test | F(2,8) = 26.84, p = 0.0002 (C vs T: p = 0.0486, C vs L: p = 0.0206, C vs S: p < 0.0001, T vs S: p = 0.0022, L vs S: p = 0.0045) |
| 1J | tdTomato+ neurons in P28 spinal cord (dorsal vs ventral) | N=3 mice per group | Two-way ANOVA with Sidak’s multiple comparisons test (source of variation: dorsal vs ventral) | F(1,16) = 133.7, p < 0.0001 (T dorsal vs T ventral: p < 0.0001, L dorsal vs L ventral: p < 0.0001, S dorsal vs S ventral: p < 0.0001) |
| 1K | tdTomato+ gray matter astrocytes in P28 spinal cord (dorsal vs ventral) | N=3 mice per group | Two-way ANOVA with Sidak’s multiple comparisons test (source of variation: dorsal vs ventral) | F(1,16) = 51.32, p < 0.0001 (T dorsal vs T ventral: p = 0.0004, L dorsal vs L ventral: p = 0.0294, S dorsal vs S ventral: p = 0.0001) |
| 1L | tdTomato+ white matter astrocytes in P28 spinal cord (dorsal vs ventral) | N=2–3 mice per group | Two-way ANOVA with Sidak’s multiple comparisons test (source of variation: dorsal vs ventral) | F(1,14) = 25.83, p = 0.0002 (S dorsal vs S ventral: p < 0.0001) |
| 1M | Percent of all Sox9+ cells that express tdTomato in P28 spinal cord | N=3 mice per group | Ordinary one-way ANOVA with Tukey’s multiple comparisons test | F(2,8) = 242.9, p < 0.0001 (C vs T: p = 0.0055, C vs L: p < 0.0001, T vs S: p = 0.0289, T vs S: p < 0.0001, L vs S: p < 0.0001) |
| 2C | tdTomato+ Schwann cells at NMJs in P28 mice | N=3–4 per group (between 110 and 344 NMJs quantified per individual muscle) | Ordinal one-way ANOVA with Bonferroni’s multiple comparisons test | F(5,14) = 3.392, p = 0.0324 (TS vs SOL: p = 0.0306, TS vs EDL: p = 0.0539) |
| 7D | tdTomato+ neurons in vitro | N=12 wells per group | Unpaired t test | t = 25.15, df = 22, p < 0.0001 |
| 7E | tdTomato+ Sox9+ cells in vitro | N=6 wells per group | Unpaired t test | t = 8.772, df = 10, p < 0.0001 |
| 7F | tdTomato+ Olig2+ cells in vitro | N=6 wells per group | Unpaired t test | t = 8.287, df = 10, p < 0.0001 |
| 8C | Cluster size in vitro + drug treatment | N=6 wells per group (between 153 and 319 clusters quantified per well) | Ordinary one-way ANOVA with Bonferroni’s multiple comparisons test | F(4,1096) = 363.7, p < 0.0001 (VEH vs CHIR: p < 0.0001, VEH vs FGF: p < 0.0001, VEH vs FGF+GDF: p < 0.0001) |

(Continued)
overlap with the neural progenitor marker, Sox2 (Fig. 4). These findings are consistent with previous reports of Hb9 protein expression in the embryonic spinal cord as early as E9.5 in mice (Arber et al., 1999; Thaler et al., 1999). We examined tdT expression in embryonic Hb9cre;Ai14 mice and detected an increasing rostro-caudal gradient of tdT cells in the spinal cord (Fig. 5A), similar to the adult (Fig. 1B). This gradient was apparent as early as E9.5, with very few tdT cells present at rostral spinal cord levels compared with caudal levels (Fig. 5B). To determine whether tdTomato expression in the early Hb9cre;Ai14 spinal cord was potentially because of off-target Hb9 expression, we therefore assessed Hb9 immunoreactivity at E9.5. Hb9 was detected in the cytoplasm, but not nuclei, of tdTomato-negative cells in the E9.5 spinal cord (Fig. 5C), suggesting that Hb9 is expressed, but not transcriptionally active in these cells (Leotta et al., 2014). While most tdTomato-expressing cells in the spinal cord did not have detectable Hb9 immunoreactivity at this stage, we detected a few tdT cells (<1%) that had nuclear Hb9 immunoreactivity in the lumbar spinal cord (Fig. 5C). Together, these findings suggest that Hb9 activity is present in progenitor cells of the neural tube at stages earlier than E9.5, before MNs are born (Arber et al., 1999).

We next sought to better characterize the populations of Hb9-lineage cells present in the embryonic lumbar spinal cord. At E15.5, neurogenesis in the spinal cord is largely complete and gliogenesis is underway (Lu et al., 2015; Lai et al., 2016). We detected tdTomato expression in large numbers of cells expressing Sox9, indicating glial (astrocyte and oligodendrocyte) identity (Stolt et al., 2003;

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**Table 2: Continued**

| Figure | Parameter | Sample size | Statistical test | Significance level |
|--------|-----------|-------------|------------------|-------------------|
| 8E     | Percent neurons *in vitro* + drug treatment | \( N = 6 \) wells per group | Ordinary one-way ANOVA with Dunnett’s multiple comparisons test | \( F_{(4,25)} = 8.967, p = 0.0001 \) |
| 8F     | Percent astrocytes *in vitro* + drug treatment | \( N = 6 \) wells per group | Ordinary one-way ANOVA with Dunnett’s multiple comparisons test | \( F_{(4,25)} = 36.21, p < 0.0001 \) |
| 8G     | Percent tdTomato *in vitro* + drug treatment | \( N = 6 \) wells per group | Ordinary one-way ANOVA with Dunnett’s multiple comparisons test | \( F_{(4,25)} = 2.502, p = 0.0680 \) |
| 8H     | Percent tdTomato *neurons* in *vitro* + drug treatment | \( N = 6 \) wells per group | Ordinary one-way ANOVA with Dunnett’s multiple comparisons test | \( F_{(4,25)} = 3.596, p = 0.0190 \) |
| 8I     | Percent tdTomato *astrocytes* in *vitro* + drug treatment | \( N = 6 \) wells per group | Ordinary one-way ANOVA with Dunnett’s multiple comparisons test | \( F_{(4,25)} = 6.630, p = 0.0009 \) |

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**Figure 2.** tdTomato (tdT) is not expressed in Ai14 spinal cords in the absence of Cre recombinase. Images of sagittal spinal cord sections from cervical (C4–C6; top row) and lumbar (L2–L6; bottom row) spinal cords of adult Ai14 mice. Neurons are labeled with NeuN and astrocyte nuclei are labeled with Sox9. Scale bars = 100 \( \mu \text{m} \).
In addition, we also identified tdTomato expression in a subset of cells expressing Pax7, a definitive marker of dorsal spinal cord NPCs including those that give rise to neural crest (Mansouri and Gruss, 1998; Murdoch et al., 2012; Roellig et al., 2017; Fig. 5D,E). We only identified a very small proportion of tdT cells that also expressed Olig2, a marker of pMN progenitors that give rise to MNs and oligodendrocytes (Novitch et al., 2001; SK Lee et al., 2005; Fig. 5G). Hence, in the Hb9cre; Ai14 mouse, Hb9-lineage cells include dorsal spinal cord cells and glial cells, consistent with our observations in the adult mouse (Fig. 1). This is further confirmed by immunostaining performed in the neonate (Fig. 6).

Fig. 5D,E). In addition, we also identified tdTomato expression in a subset of cells expressing Pax7, a definitive marker of dorsal spinal cord NPCs including those that give rise to neural crest (Mansouri and Gruss, 1998; Murdoch et al., 2012; Roellig et al., 2017; Fig. 5f). We only identified a very small proportion of tdT cells that also expressed Olig2, a marker of pMN progenitors that give rise to MNs and oligodendrocytes (Novitch et al., 2001; SK Lee et al., 2005; Fig. 5G). Hence, in the Hb9cre; Ai14 mouse, Hb9-lineage cells include dorsal spinal cord cells and glial cells, consistent with our observations in the adult mouse (Fig. 1). This is further confirmed by immunostaining performed in the neonate (Fig. 6).

Caudalizing morphogens promote gliogenesis and glial tdTomato expression in cultured spinal cord neural progenitor cells

In normal development, morphogen gradients in the environment including Wnts, FGFs, and Gdf11 activate differential gene expression in spinal cord NPCs along the rostrocaudal axis (Liu et al., 2001; Nordström et al., 2002; Chanut, 2006; Liu, 2006). In order to identify putative factors that could modulate Hb9 expression in the spinal cord, we first established an in vitro assay conducive to drug treatment. The anterior or posterior halves of E12.5 Hb9cre;Ai14 spinal cord were isolated, dissociated, then cultured for 10 d (Fig. 7A). As expected, cultures of NPCs derived from the anterior spinal cord contained relatively few tdTomato+ cells compared with posterior NPCs (anterior: 3.00 ± 0.137%, posterior: 19.0 ± 1.13%, p < 0.0001; Fig. 7B). We analyzed the
numbers of NeuN\(^+\) (neuron), Sox9\(^+\) (astrocyte/oligodendrocyte), and Olig2\(^+\) (MN/oligodendrocyte) cells expressing tdTomato and found that for each cell type, there were greater numbers of cells expressing tdTomato in posterior cultures versus anterior cultures (Fig. 7C–F). Thus, the rostrocaudal gradient of tdT\(^+\) cells is maintained even after removing neural progenitors from the environment of the developing spinal cord and culturing \textit{in vitro}.

Using this \textit{in vitro} assay, we next tested whether tdT expression in NPCs derived from the anterior E12.5 spinal cord could be induced by treatment with caudalizing factors (Fig. 8A). Beginning immediately after plating, and continuing once daily for 7 d, cells were treated either with the Wnt activator CHIR 99 021 (CHIR), FGF-8B, GDF-11, or both FGF-8B \& GDF-11. Interestingly, we found that treatment with FGF alone or FGF+GDF significantly increased the size of cell clusters after 7 d \textit{in vitro} (Fig. 8B, C). Cell density more than doubled in FGF-treated conditions (vehicle: 12,400 ± 763 cells/mm\(^2\), FGF-8B: 27,900 ± 2150 cells/mm\(^2\), \(p<0.0001\) by Student’s \(t\) test), suggesting that addition of FGF-8B promoted cell survival and/or increased proliferation. GDF-11 treatment had no effect on cell density or cluster size, whereas CHIR treatment significantly reduced cluster size (Fig. 8B, C) and cell density (CHIR: 9140 ± 1030 cells/mm\(^2\), \(p=0.031\) vs vehicle). We next evaluated the effects of treatment on neurogenesis and gliogenesis of cultured NPCs. Notably, we found that FGF treatment significantly reduced neuronal differentiation (Fig. 8E), and markedly increased glial differentiation (Fig. 8F). This suggests a progliogenic effect of FGF on spinal cord NPCs, similar to previous work showing that activation of FGF signaling induced astrocyte cell fates in the cerebral cortex (Dinh Duong et al., 2019). Despite the increased cell survival and/or proliferation on FGF treatment, we did not observe any differences in the percentage of total cells expressing tdTomato in any treatment group (Fig. 8G), nor did we observe any differences in the percentage of neurons that express tdT (Fig. 8H).

However, we found that both CHIR and FGF+GDF treatment significantly increased the percentage of total astrocytes that expressed tdT (Fig. 8I). This observation that caudalizing factors increased reporter expression in glial cells suggests that combined FGF and GDF signaling may be implicated in the increased abundance of Hb9-lineage glial cells in the caudal spinal cord.

**Discussion**

The Hb9\(^{cre}\) mouse has been used in hallmark studies to characterize the role of this transcription factor in differentiation of spinal MNs and consolidation of their identity (Arber et al., 1999). Recently, one study reported that YFP expression in Hb9\(^{cre}\):Rosa26-YFP mice was not only...
restricted to MNs, but also dorsal horn populations (Caldeira et al., 2017). However, the authors did not describe expression of reporter protein in glial cells of the spinal cord. Here, we present the surprising finding that Hb9-lineage cells include astrocytes and oligodendrocytes of the adult spinal cord, and Schwann cells in the peripheral nervous system. Our data suggest that Hb9 is expressed transiently in a population of cells, possibly neural progenitors, which give rise to neural crest and glial cells. These unexpected findings have implications for the design of experiments using the Hb9 cre mouse line. Indeed, genetic crosses made between the Hb9 cre mouse and mice with Cre-dependent alleles should be carefully monitored so that off-target effects are avoided. The use of an inducible Hb9 cre line (Koronfel et al., 2021), or viral vector-mediated gene expression instead of genetic crosses, might be better ways to achieve MN-specific manipulations in the mouse nervous system.

One important consideration of our study is whether the observed reporter expression patterns might be attributed to mosaicism. Mosaic patterns of recombination of Cre-dependent alleles can be a significant confounding factor in studies that use Cre driver mice for fate mapping. Indeed, the majority of Cre mouse lines have been shown to exhibit some degree of unreported recombinase activity, including mosaicism (Heffner et al., 2012). There are several lines of evidence suggesting that tdTomato expression in glial cells of Hb9 cre;Ai14 mice is not because of mosaicism. First, we observed that this pattern of reporter expression is reproducible across multiple animals from different litters and consistently observed in all Hb9 cre;Ai14 mice we analyzed. In addition, we identified identical patterns of gene expression in Hb9 cre;Gq-DREADD mice (data not shown), suggesting that this is not an artifact related to the Ai14 mouse. In contrast, patterns of recombination because of mosaicism are unpredictable and inconsistent between littermates (Heffner et al., 2012). For this study, we only used male Hb9 cre sires, not female Hb9 cre dams, because maternal Cre expression has been shown to affect Cre excision patterns (Eckardt et al., 2004).

Furthermore, hb9:GFP zebrafish embryos have been shown to exhibit a similar pattern of graded GFP expression that increases rostrocaudally in the developing spinal cord (Arkhipova et al., 2012).

Our descriptive study has several limitations. The most major limitation is that we were unable to detect Hb9 immunoreactivity in tdTomato + cells, except for in a few cells in E9.5 embryos. We also failed to detect Hb9 transcript levels in these embryos (data not shown). Our results indicate that Hb9 expression occurs earlier than E9.5; however, because of technical limitations we did not assess earlier stages of development. Further work is needed to determine exactly when Hb9 is expressed in spinal cord progenitors, and these progenitors’ identities. Additionally, the exact mechanism by which Hb9-lineage cells become distributed in a gradually increasing rostrocaudal gradient is not clear. It is apparent that this gradient exists from as early as E9.5 in the spinal cord, which coincides with the very beginning of neurogenesis. This indicates that the graded distribution of Hb9-lineage cells is probably not because of migration, but potentially to gradients in environmental factors such as morphogens. Our in vitro results showed that addition of the caudalizing morphogen FGF-8B to cultured spinal cord neural progenitors was sufficient to promote massive gliogenesis at the expense of neurogenesis; moreover, addition of FGF-8B + GDF-11 increased the numbers of tdTomato + glial cells in these cultures. It is interesting to note that the effects of FGF-8B on cell cluster size were partially mitigated by addition of GDF-11 to cell culture (Fig. 8B, C). GDF-11 is a member of the TGFβ superfamily, which includes BMPs and GDFs as well as other family members such as Nodal (Simoni-Nieves et al., 2019). Because BMPs can sometimes exert antagonistic effects on FGF signaling during normal development (Schliermann and Nickel, 2018), we speculate that GDF-11 may likewise antagonize proliferative effects of FGF in developing neural progenitor cells. Although we did not mechanistically demonstrate a link between FGF signaling and...
Hb9 transcriptional activity, our results collectively suggest that caudalizing morphogens may be upstream of glial Hb9 expression. Indeed, in studies describing the directed differentiation of embryonic stem cells in vitro, it has long been appreciated that caudalizing morphogens such as Sonic hedgehog are required for generation of motor neurons (Wichterle et al., 2002). Future work is needed to determine whether morphogen gradients in early development may induce rostrocaudal Hb9 expression in a concentration-dependent manner.

Our results also raise new, unanswered questions. First, it is unclear whether there are transcriptional and/or functional differences between Hb9-lineage glial cells and non-Hb9-lineage glial cells. Glial heterogeneity is an understudied but important topic in neurobiology, and transcriptionally distinct astrocyte populations have been shown to exist in different regions of the CNS (Tsai et al., 2012; Batiuk et al., 2020; Huang et al., 2020; Clarke et al., 2021). Future work could perform fluorescent-activated cell sorting on spinal cord tissue of Hb9cre;Ai14 mice and compare transcriptional profiles of Hb9-lineage versus non-Hb9-lineage astrocytes to determine these populations are transcriptionally diverse. Characterization of the differences between these cell types may reveal new functional roles for molecularly-distinct glial subtypes throughout the spinal cord. In addition, it would be interesting to test whether loss of Hb9 expression affected development of spinal cord glial cells. Finally, the distribution of molecularly-defined cell types in a gradually
Figure 8. Treatment with caudalizing factors promotes gliogenesis and glial tdTomato expression in cultured NPCs. A, Cartoon shows experimental design. Anterior portions of E12.5 Hb9\textsuperscript{Cre};Ai14 spinal cords were dissociated, plated, and treated in culture for 7 d with either vehicle, CHIR 99 021, FGF-8B, GDF-11, or both FGF-8B + GDF-11. B, Images of DAPI staining in cultured cells at 7 DIV. C, Quantification of DAPI\textsuperscript{+} cell cluster size (\textmu m\textsuperscript{2}). D, Representative images of cells in each treatment group immunolabeled for tdTomato, NeuN, and Sox9. E-I, Quantification of (E) the percentage of all cells that are neurons, (F) the percentage of all cells that are astrocytes, (G) the percentage of all cells that are tdTomato\textsuperscript{+}, (H) the percentage of neurons that express tdTomato, and (I) the percentage of astrocytes that express tdTomato. All data are mean $\pm$ SEM N=6 per group. $^*$p $<$ 0.05, $^{**}$p $<$ 0.01, $^{***}$p $<$ 0.001, $^{****}$p $<$ 0.0001. Scale bars = 100 \mu m.
increasing rostrocaudal gradient is an intriguing and uncommon observation, and it is unclear exactly how this occurs. More work is needed to understand the mechanisms by which caudalizing morphogens induce transient Hb9 expression in the developing spinal cord.

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