CHARACTERIZING THE ROLE OF TRANSCRIPTION FACTOR HB9 IN GLIAL CELL DEVELOPMENT

An Undergraduate Research Scholars Thesis

by

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Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Jennifer Dulin

May 2020

Major: Business Honors, B.B.A

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ABSTRACT

Characterizing the Role of Transcription Factor Hb9 in Glial Cell Development

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Hb9 (*Mnx1*) is a transcription factor described as a spinal cord motor neuron-specific marker in embryonic development and a critical factor for the post-mitotic specification of these cells. As such, transgenic mice with mutations in the *Hb9* gene are commonly used for the study of spinal cord motor neurons. To date, the expression of Hb9 in other cell types has not previously been reported. We performed a fate-mapping approach to examine the localization of Hb9-expressing cells and their progeny ('Hb9-lineage cells') within the embryonic and adult spinal cord. We found that Hb9-lineage cells are distributed in a gradient of increasing abundance throughout the rostro-caudal spinal cord axis during developmental and postnatal stages. Furthermore, although the majority of Hb9-lineage cells at cervical spinal cord levels are motor neurons, at more caudal levels, Hb9-lineage cells include astrocytes and oligodendrocytes, the macroglial cells of the central nervous system. In the peripheral nervous system, we observed a similar phenomenon with Hb9-lineage Schwann cells present in an increasing rostro-caudal gradient throughout the body. These observations have several exciting implications. Hb9 may play an important role not only in astrocyte and oligodendrocyte development, but also in

development of Schwann cells, which are the glial cells associated with the peripheral nerves. Additionally, characterization of Hb9-lineage glial cells may reveal new functional roles for glia throughout the developing spinal cord. Through characterizing the role of Hb9 in glial cell development, describing the molecular pathways involved, and determining the differences in gene expression between Hb9+ and Hb9- spinal cord glial cells, the developmental function of Hb9 can be better understood.

DEDICATION

This research paper is dedicated to Dr. Jennifer Dulin, who has been far more than a research mentor throughout my time at Texas A&M. Dr. Dulin deeply cares about all her students, and she has helped me grow in aspects of life beyond science. Dr. Dulin is a very accepting, and she sets a high standard of inclusivity for those who work with her. Knowing that women in science are underrepresented, she has led by example by directly mentoring four female graduate and post-graduate students. In the lab, she is patient in teaching me and even more good-hearted when guiding me beyond honest mistakes. Dr. Dulin fosters a "teach, don't blame" culture in which a mistake becomes an opportunity to improve. She sets a high standard for herself and her lab members, and she prepares her undergraduate students to think critically like scientists. Dr. Dulin's passion for biomedical research is contagious and invigorating, and the work she is leading to ameliorate the suffering caused by spinal cord injury is truly selfless. She continues to show me how an ideal leader can create an effective, harmonious team, and I am excited about the many lessons I will discover from her leadership in the future.

ACKNOWLEDGMENTS

I would like to thank, most importantly, Dr. Jennifer Dulin for guiding me through this project and teaching me many of the challenging biology concepts I have not yet learned in class. I would also like to thank Dr. Miriam Aceves, Dr. Kim Loesch, and Ashley Tucker, who have guided me through animal breeding and experimental procedures. Additionally, I would like to thank Robert Adkins and Dr. Young il Lee for laying the groundwork for this project.

Thanks also go to the department faculty of both Biology and Business for making my time at Texas A&M University a great experience. I also want to extend my gratitude to the Craig H. Neilsen Foundation, the Paralyzed Veterans of America Research Foundation, and the TIRR Foundation for funding our research.

Finally, I would like to thank my beloved parents, Rama and Santhi Letchuman, for their encouragement and continued support, and I would like to show my appreciation for my dear friend Robert Steven Brown and his parents, Steve and Penny Brown, who have been at my side through all my endeavors.

CHAPTER I

INTRODUCTION

Spinal Cord Development

History regarding the understanding of the spinal cord goes back to Hippocrates, and even today, there are gaps in our understanding of the different populations of cells that make up the spinal cord and what factors affect the differentiation of those cells (Johal et al. 2018). After fertilization in humans, the nervous system begins to form after 16 days with the emergence of the neural plate (Borsani et al. 2019). This neural plate grows and then folds in on itself to become the neural tube, and the neuroepithelial cells lining this tube eventually give rise to neurons and glial cells (Greene and Copp 2009). The past few decades of research have demonstrated that there is a tremendous diversity of molecularly and functionally distinct cell types in the spinal cord (Jessell 2000; Lai, Seal, and Johnson 2016). However, the modulation of cellular differentiation in the context of early spinal cord development is not yet fully understood and is therefore a topic of active research. Understanding neural development is fundamental in realizing the complexities of diseases affecting cells within the spinal cord and the regenerative capacity of the spinal cord after injury.

Spinal Cord Injury

Spinal cord injury is a traumatic event that often results in a permanent loss of mobility and sensation, and it currently affects over 300,000 Americans. The injury causes a substantial burden on the physical, mental, and financial health of a patient and often ends with premature death ('Spinal Cord Injury (SCI) 2016 Facts and Figures at a Glance' 2016). Clinical studies focused on ameliorating the effects of spinal cord injury have posed significant challenges to

scientists as the struggle to achieve proper functional recovery remains evident. An increasingly common area of research embraces the transplantation of neural stem cells and their derivatives to restore locomotor function after spinal cord injury. However, as graft-host integration resulting in meaningful functional recovery remains moderate, exploration into how specific cell types within the spinal cord develop is crucial (Dulin et al. 2018). Research into cell transplantation after spinal cord injury could be improved by better tailoring graft tissue to the site of injury.

Transcription Factor Hb9

One transcription factor characterized for its role in neuronal cell specification is Hb9 (*Mnx1*). Previous work in the field of developmental neurobiology has identified a role for Hb9 as a critical factor in the development of spinal cord motor neurons, as shown in ***Figure 1***. In mice, Hb9 has been first detected at embryonic day 9.5 (E9.5), which coincides with when the first post-mitotic motor neurons emerge. In Hb9-mutant mice, migratory patterns of motor neurons are disrupted; however, differentiation of motor neuron progenitors within the spinal cord into somatic and visceral motor neurons remains constant (Arber et al. 1999; Stifani 2014).

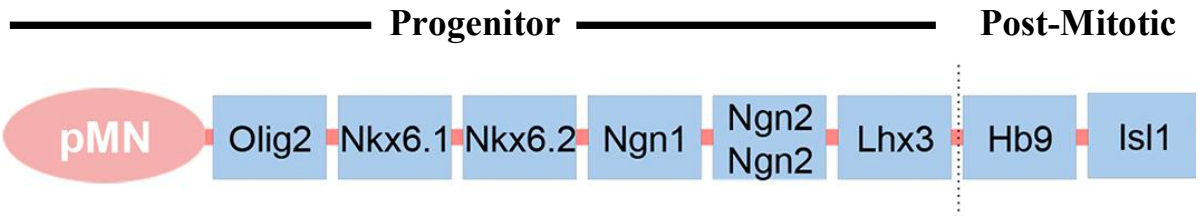


Figure 1. Transcription factors expressed in spinal cord motor neuron development, with Hb9 expression restricted to post-mitotic cells.

Expression of Hb9 in the spinal cord of the embryonic chick has been shown to induce motor neuron differentiation while suppressing the differentiation of V2 interneurons, which emerge from an adjacent progenitor domain. Researchers studying avian embryonic development

have found that Hb9 expression in motor neurons is largely regulated by the expression of the morphogen sonic hedgehog (Shh) (Kahane and Kalcheim 2019). Hence, Hb9 is widely considered to be a major regulatory switch that induces spinal cord motor neuron cell fate. See ***Figure 2***. However, to date there have not been any published reports of Hb9 expression in other cell types within the nervous system.

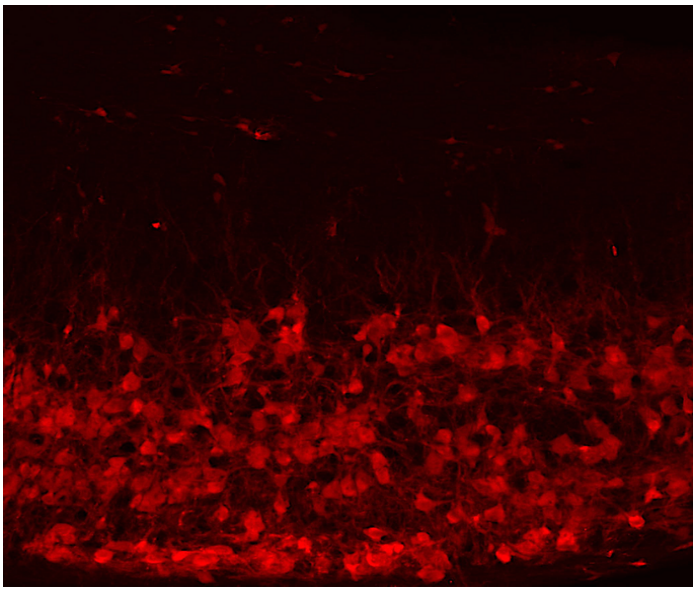


Figure 2. Motor neurons expressing tdTomato within postnatal mouse Hb9-Cre;Ai14 spinal cord tissue.

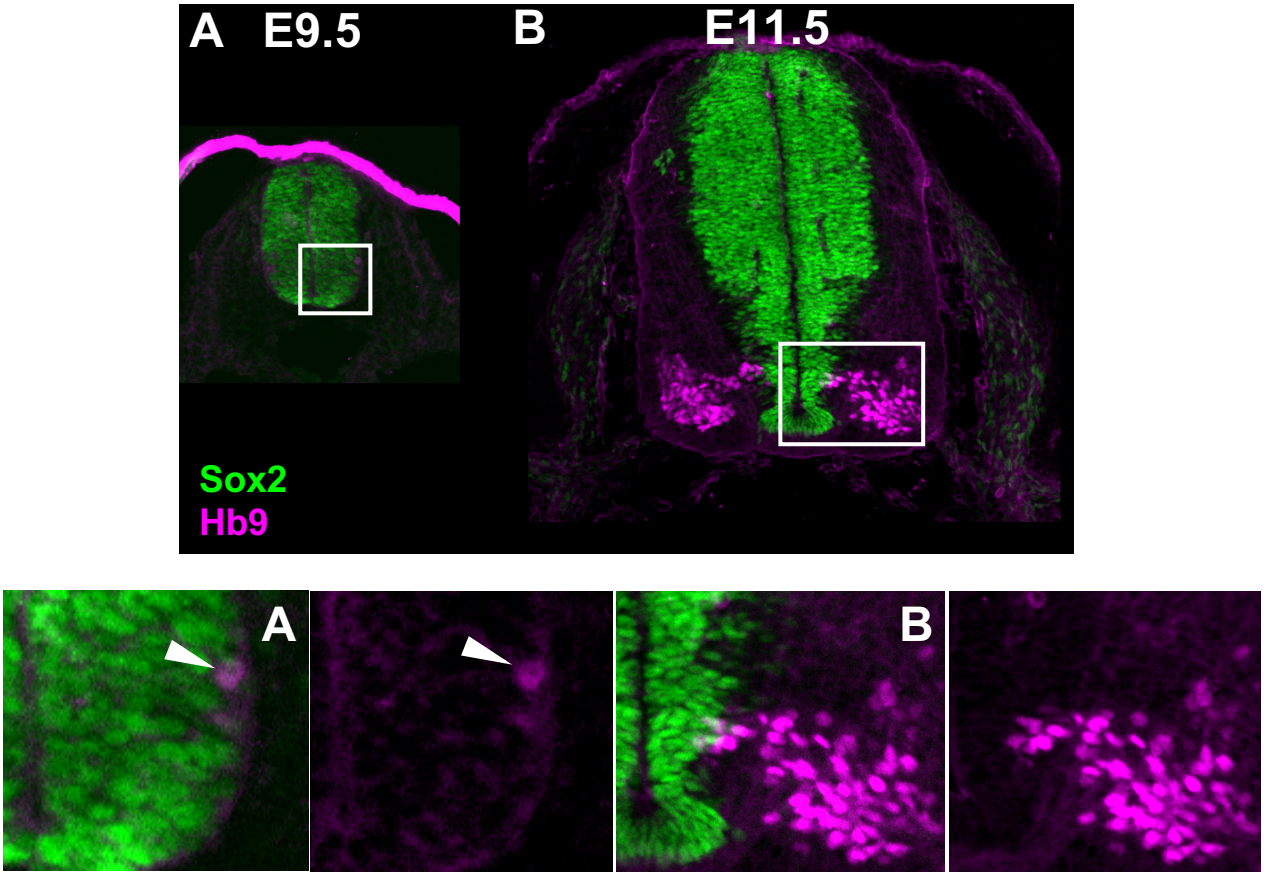


Figure 3. Hb9 expression in the embryonic neural tube. [A]- At E9.5, Hb9 is expressed in a small number of cells that do not co-express the neural progenitor cell marker Sox2. [B]- At E11.5, large numbers of post-mitotic Hb9+ motor neurons have migrated out of the progenitor motor neuron domain.

From E9.5 to E11.5, the migration of post-mitotic motor neurons out of the Sox2+ progenitor region of the neural tube can clearly be seen (***Figure 3***). This supports previous findings that Hb9 is expressed only in post-mitotic motor neurons. Our results indicate that Hb9 expression is likely to occur in early progenitors that give rise to glial cells, before the period of gliogenesis occurs.

CHAPTER II

METHODS

Animals

Adult, postnatal, and embryonic Hb9-Cre mice (B6.129S1-*Mnx1*$^{tm4(cre)Tmj}$/J; Jax #006600) and Ai14 mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J; Jax #007914) were used for this study. Heterozygous Hb9-Cre mice, which express Cre recombinase in place of one copy of the *Hb9* gene, were crossed with Ai14(RCL-tdT)-D mice, which express the fluorescent reporter tdTomato in all cells containing Cre and their progeny. An Hb9+ cell and its progeny are permanently marked once activated by the Cre/loxP system. ***Figure 4*** graphically shows this mouse model used throughout all experiments.

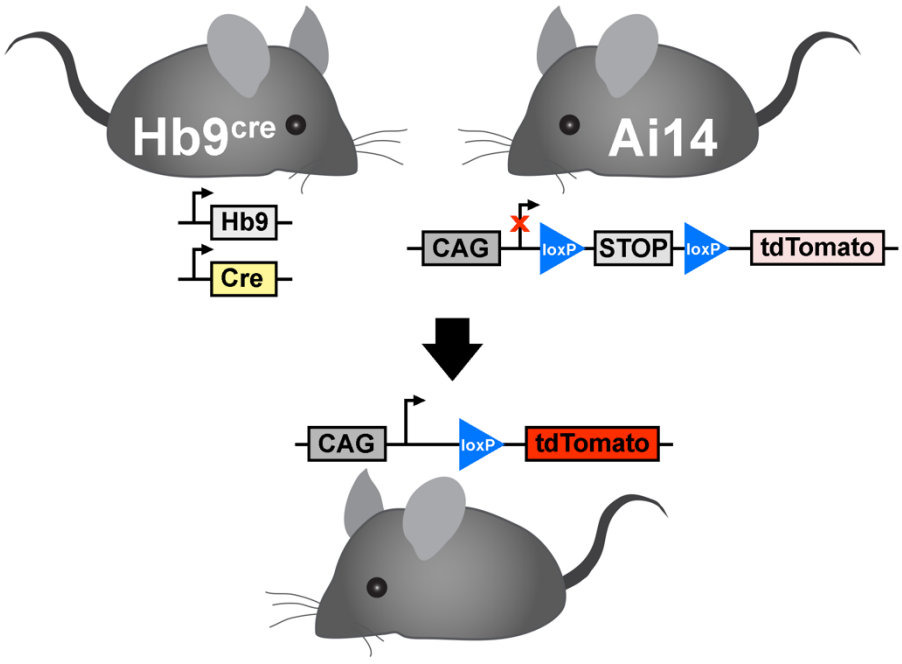


Figure 4. Cartoon describing Hb9-Cre;Ai14 cross. This genetic cross produces mice which express tdTomato in all Hb9+ cells and their progeny, allowing for a reliable way to study Hb9

expression throughout development. Abbreviations: Ai14 = Cre reporter allele; Cre = recombinase transgene in place of one copy of *Hb9*; CAG = gene promoter for tdTomato; loxP = DNA recognition sites; STOP = gene cassette preventing the expression of tdTomato until recombination by Cre occurs; tdTomato = red fluorescent protein used to mark Hb9+ cells and their progeny.

Embryos

Hb9-Cre;Ai14 embryos were generated through standard pairing protocols at time points ranging from embryonic day 9 (E9) to E18.5. Embryos were harvested for histology and tissue-clearing.

Clearing

Embryonic tissue was cleared using Sodium Dodecyl Sulfate (SDS) and placed in Refractive Index Matching Solution (RIMS) for imaging.

Immunohistochemistry

Tissue was cryosectioned and processed for immunohistochemistry. Both sagittal and transverse sections of embryonic tissue were direct mounted for staining process. Primary antibodies used are described in ***Table 1***.

Imaging and quantification

A Nikon Eclipse upright fluorescent microscope was used for image acquisition of samples processed for histology. A Light Sheet fluorescent microscope was used for image acquisition of samples processed for tissue clearing. Quantification was performed using ImageJ and statistical analysis was conducted using GraphPad Prism 7.

Table 1. Primary antibodies used for immunohistochemistry

Antibody	**Manufacturer**
Goat anti-mCherry	Sicgen #AB0040-200
Chicken anti-Glial Fibrillary Acidic Protein (GFAP)	Encor Bio #CPCA-GFAP
Rabbit anti-S100ß	Dako #Z0311
Rabbit anti-Pax6	Biolegend #901301
Goat anti-Sox9	Bio-Techne #AF3075
Mouse anti-SV2	DSHB Cat# SV2
Mouse anti-2H3	DSHB Cat# 2H3
Rabbit anti-ChAT	Genetex #GTX113164
Alpha-Bungarotoxin, Alexa Fluor 488 conjugate	ThermoFisher # B13422
Mouse anti-Olig2	DSHB #PCRP-OLIG2-1E9
Mouse anti-Pax7	DSHB #AB 528428
Rabbit anti-Sox2	Abcam # ab97959
Mouse anti-Hb9	DSHB #81.5C10-c

CHAPTER III

RESULTS AND DISCUSSION

For these studies, we used a fate-mapping strategy to permanently label all cells with Hb9 expression, and their progeny, with the fluorescent reporter protein tdTomato. This was achieved by crossing heterozygous Hb9-Cre mice, which express Cre recombinase in place of the endogenous Hb9 allele, to Ai14 mice that possess a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein tdTomato (***Figure 4***). In the F1 generation of the Hb9-Cre;Ai14 cross, the 50% of progeny inheriting the Cre allele will have the STOP cassette deleted in the Cre-expressing tissue(s), resulting in robust tdTomato fluorescence. Hence, the Hb9-Cre;Ai14 mouse serves as a robust reporter for Hb9 fate mapping because all of the cells with endogenous Hb9 expression, and their progeny, will be permanently labeled with tdTomato.

To characterize the lineage of Hb9-expressing cells in the spinal cord, we first analyzed patterns of tdTomato+ cells in the spinal cords of adult Hb9-Cre;Ai14 mice. Transverse spinal cord sections were taken and stained for choline acetyltransferase (ChAT), a well-established marker for mature spinal cord motor neurons.

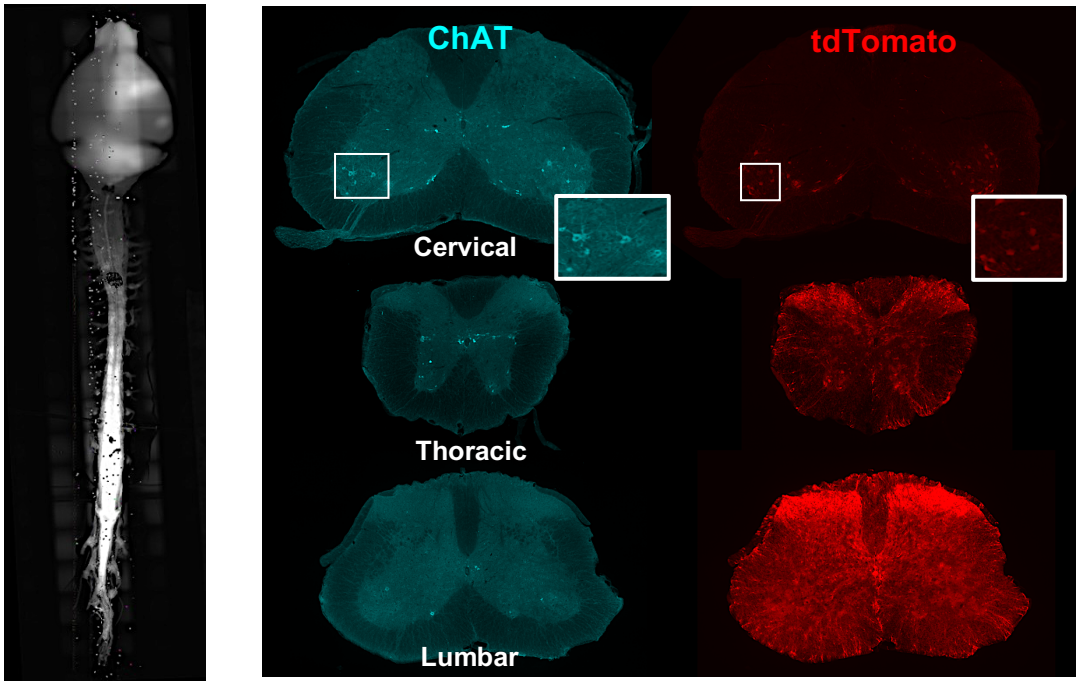


Figure 5. Left: Fluorescent image of tdTomato expression in the central nervous system of an adult Hb9-Cre;Ai14 mouse. Right: Expression of tdTomato in transverse spinal cord sections of adult Hb9-Cre;Ai14 mice at cervical, thoracic, and lumbar spinal levels. Choline acetyltransferase (ChAT) is labeled in cyan.

Motor neurons are located in the ventral horn of the spinal cord gray matter. Motor neurons are clearly seen in the ventral horns marked by ChAT as seen in ***Figure 5***. As expected, we observed tdTomato expression in the ventral horns of the spinal cord at the cervical level, corresponding with patterns of ChAT immunoreactivity. Unexpectedly, however, we observed that tdTomato expression dramatically increases from cervical to lumbar spinal cord levels. Observation of the whole spinal cord (left) reveals an increasing rostro-caudal gradient (from head to tail) of expression. Moreover, closer inspection of tdTomato expression patterns in the caudal spinal cord of these mice suggested that fluorescently labeled cells had a glial appearance. We

therefore hypothesized that during development, Hb9 must be expressed in cells other than post-mitotic motor neurons.

As the support cells of the nervous system, serving functions such as myelination and neurotransmitter uptake, glial cells are found across the entire nervous system. In characterizing the cells within the caudal spinal cord expressing tdTomato in ***Figure 5***, we used an antibody against glial fibrillary acidic protein (GFAP), which is an intermediate filament specifically expressed in astrocytes, a major class of glial cells in the central nervous system.

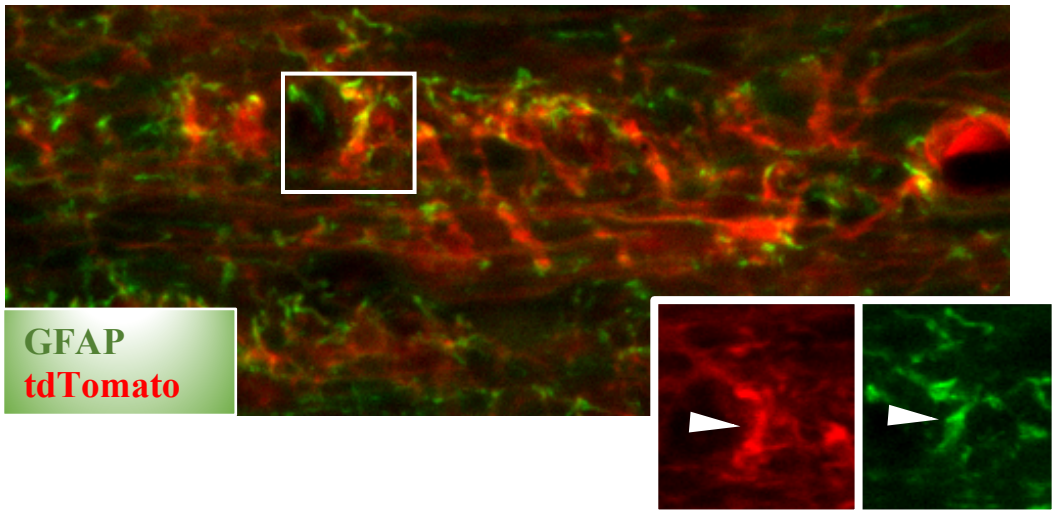


Figure 6. tdTomato expression in astrocytes in the lumbar region of the adult Hb9-Cre;Ai14 spinal cord. Glial fibrillary acidic protein (GFAP) is labeled in green and tdTomato is labeled in red. Inset shows expression of tdTomato in a GFAP+ cell (arrowhead).

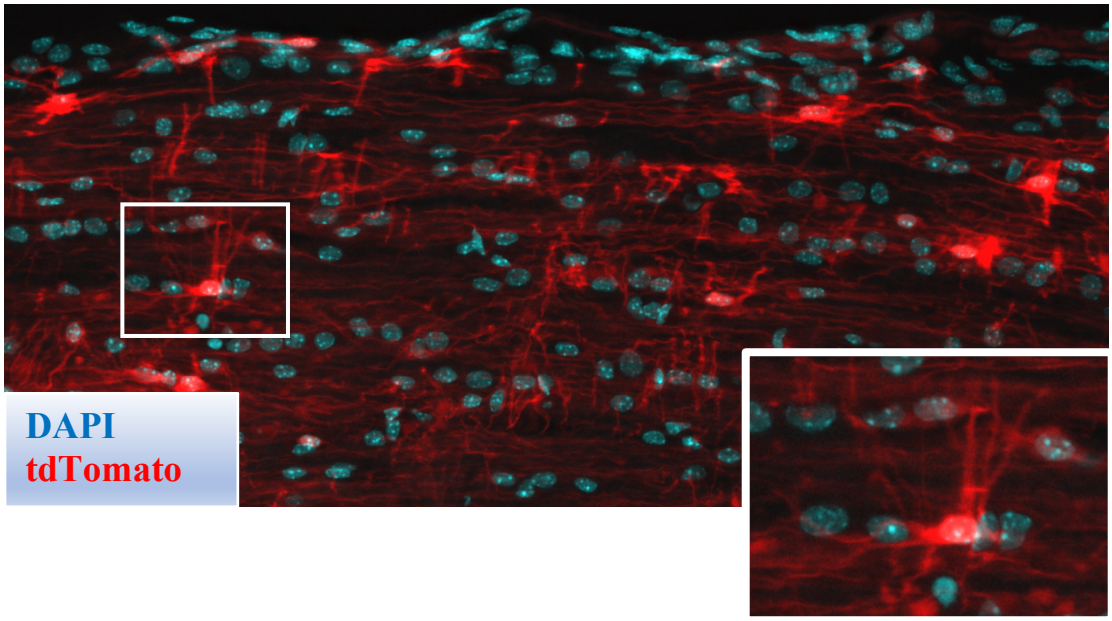


Figure 7. tdTomato expression in cells with typical oligodendrocyte morphology in the adult Hb9-Cre;Ai14 spinal cord. Nuclear stain DAPI is labeled in cyan; tdTomato is labeled in red.

Figure 6 shows colocalization of tdTomato expression with GFAP+ astrocytes, demonstrating that Hb9 is expressed in lineages of cells that give rise to astrocytes. Furthermore, ***Figure 7*** shows tdTomato-expressing cells that exhibit morphology similar to oligodendrocytes, the class of glia within the central nervous system which function to myelinate axons. Together, these results indicate that Hb9 has some role in the development of glial cells or their progenitors within the adult spinal cord. Utilizing the current breeding model described in ***Figure 4***, tdTomato expression within a cell does not confirm that Hb9 is expressed in that particular cell; rather, tdTomato expression tells us that a cell or one of its progenitors once expressed Hb9. To determine the time frame in which Hb9 might be expressed during glial cell development, a postnatal day 0 (P0) Hb9-Cre;Ai14 mouse was produced. The results corroborate with those found in the adult mouse and are shown in ***Figure 8***.

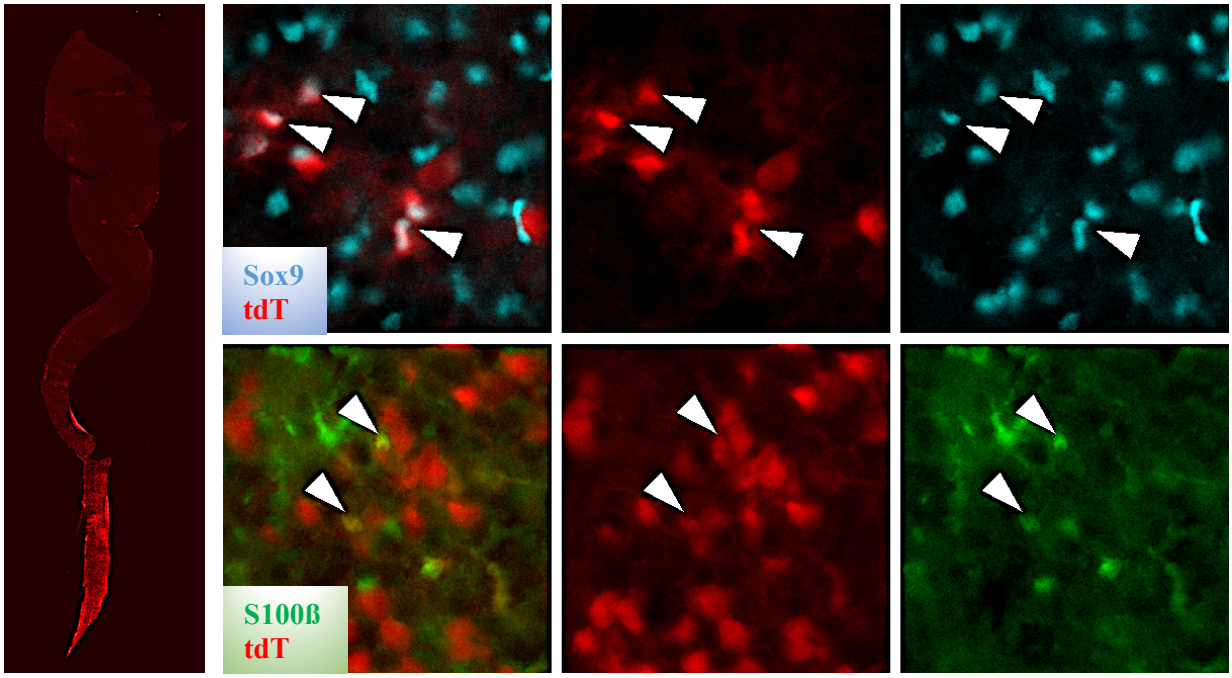


Figure 8. tdTomato expression within a newborn Hb9-Cre;Ai14 mouse, colocalized with Sox9+ (cyan) and S100ß+ (green) cells.

In ***Figure 8***, we see that tdTomato expression is present in an increasing rostro-caudal gradient in the newborn (P0) mouse, similar to the adult spinal cord. Furthermore, tdTomato is expressed in Sox9+ cells. Sox9 is a transcription factor that is highly involved with the neurogenic to gliogenic fate-switch, and its expression specifies a cell within the central nervous system towards a glial fate. S100ß is a marker for astrocytes within the central nervous system, and the colocalization apparent within ***Figure 8*** demonstrates that Hb9 is expressed in cell lineages giving rise to glial cells.

The gestation period for a mouse is approximately 20 days. During this time, rapid development occurs, and several morphogenic factors, such as Wnt and Fibroblast Growth Factors (FGF), dictate anterior-posterior patterning. To determine whether Hb9-lineage cells were present in early neural development during the period of anterior-posterior patterning, Hb9-Cre;Ai14

embryos were produced. During several stages of embryonic development, we observed the same increasing rostro-caudal gradient observed in postnatal tissue. This is seen in ***Figure 9*****,** which shows embryonic day 15.5, 12.5, and 10.5.

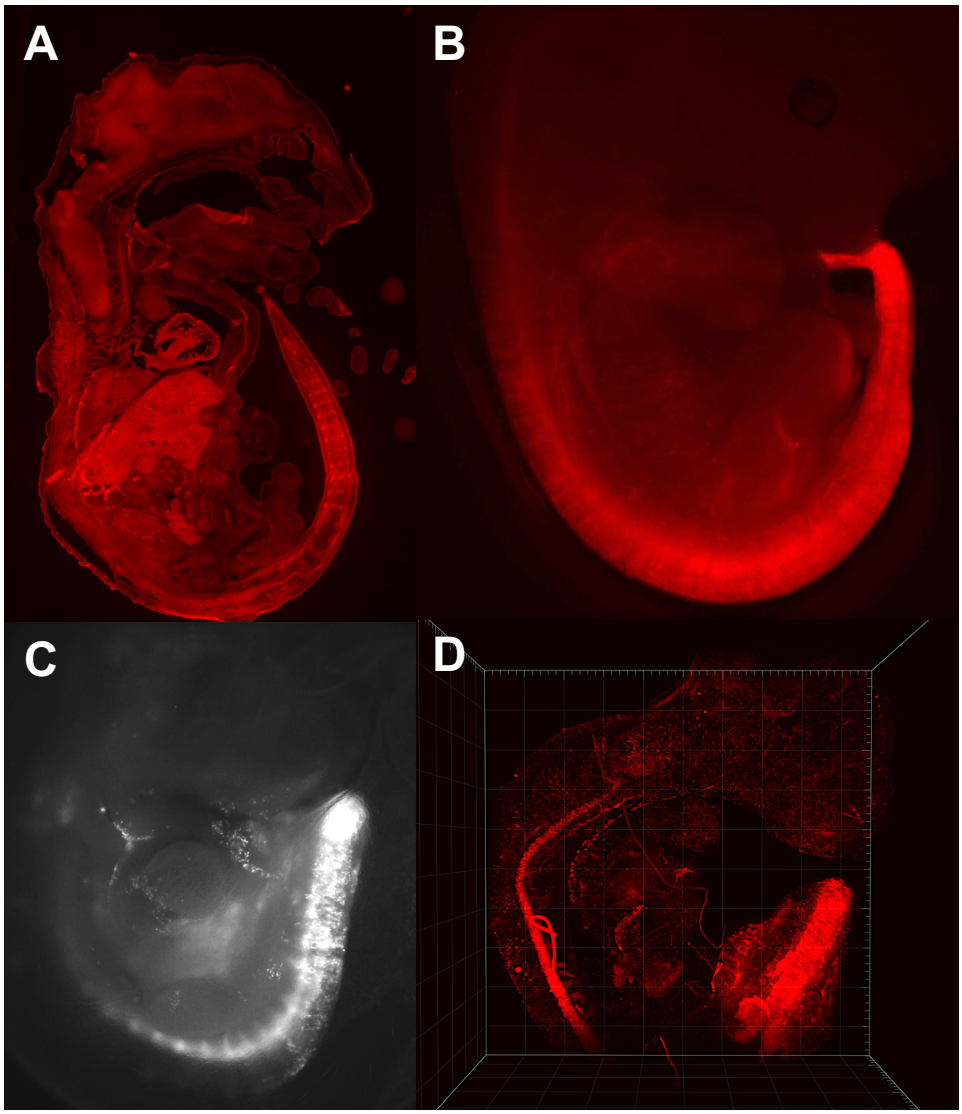


Figure 9. Expression of tdTomato in Hb9-lineage cells across embryonic development of Hb9-Cre;Ai14 mice. [A]- E15.5 sagittal section; [B]-E12.5 full embryo; [C]-E10.5 full embryo; [D]-E10.5 cleared embryo

Figure 9 shows that the increasing rostro-caudal gradient of tdTomato expression is not confined to postnatal tissue. ***Figure 9*** **[A]** shows that tdTomato expression is found far beyond the spinal cord. ***Figure 9*** **[B,C,D]** shows that this expression pattern must be induced early in development, suggesting potential induction by morphogenic factors expressed in anterior/posterior gradients, such as Wnt, FGFs, or retinoic acid.

In characterizing the tdTomato+ cells within these embryonic time points, we used an antibody against GFAP, which is an intermediate filament protein expressed by astrocytes (***Figure 10***); Olig2, which is a transcription factor expressed in oligodendrocyte progenitor cells (***Figure 11***); Pax7, which is a transcription factor expressed in neural crest cells, which give rise to numerous cell lineages including glia (***Figure 12***); and Sox9, which is a transcription factor that determines glial fate (***Figure 13***).

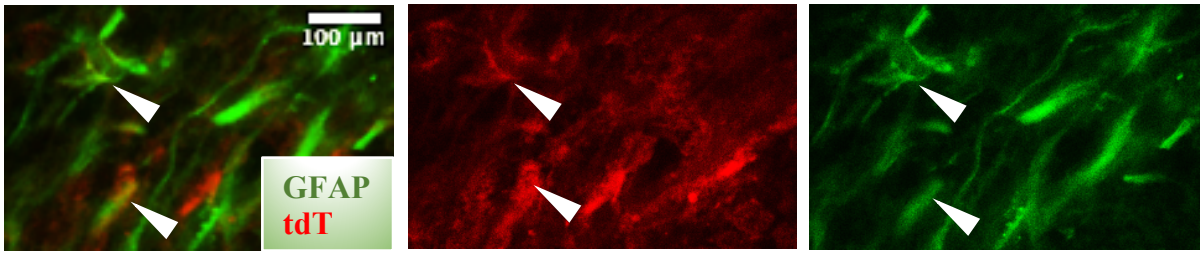


Figure 10. tdTomato expression within a E18.5 spinal cord, colocalized with GFAP+ astrocytes

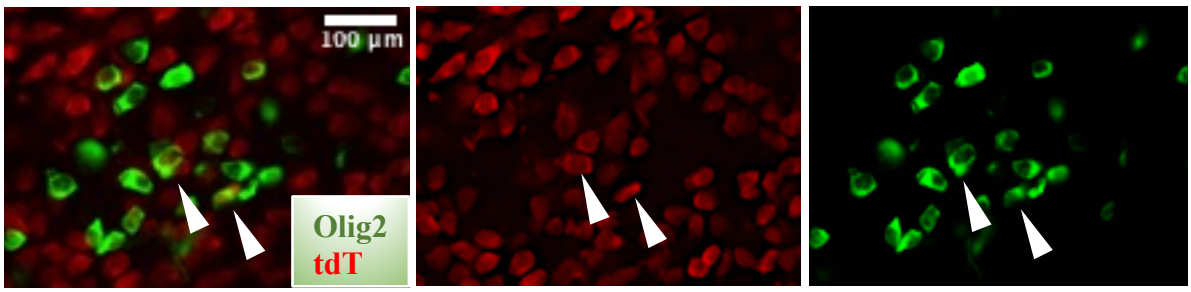


Figure 11. tdTomato expression within a E15.5 spinal cord, colocalized with Olig2+ cells

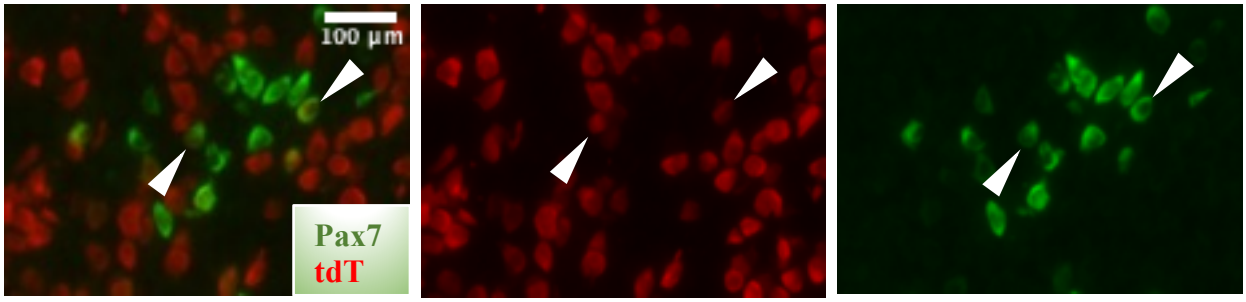


Figure 12. tdTomato expression within a E15.5 spinal cord, colocalized with Pax7+ cells

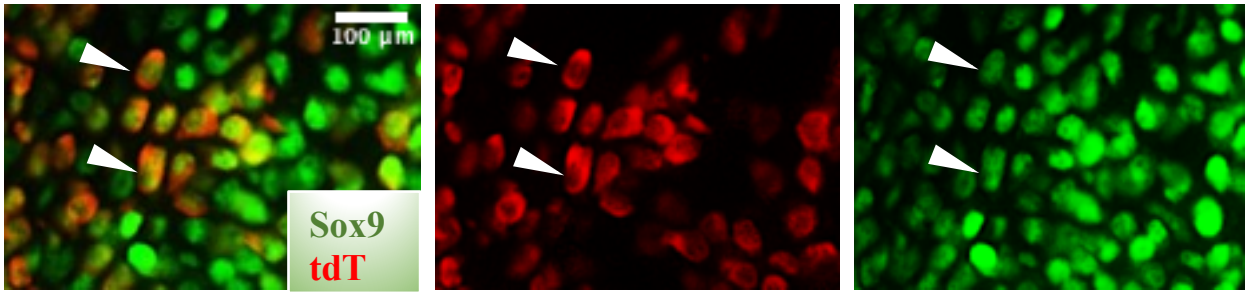


Figure 13. tdTomato expression within a E15.5 spinal cord, colocalized with Sox9+ glial progenitors

While the results described thus far have focused on the Hb9-lineage cells within the central nervous system, Dr. Young il Lee (Texas A&M Department of Biology) has shown that, within the peripheral nervous system (PNS), Hb9 is expressed in Schwann cell lineages.

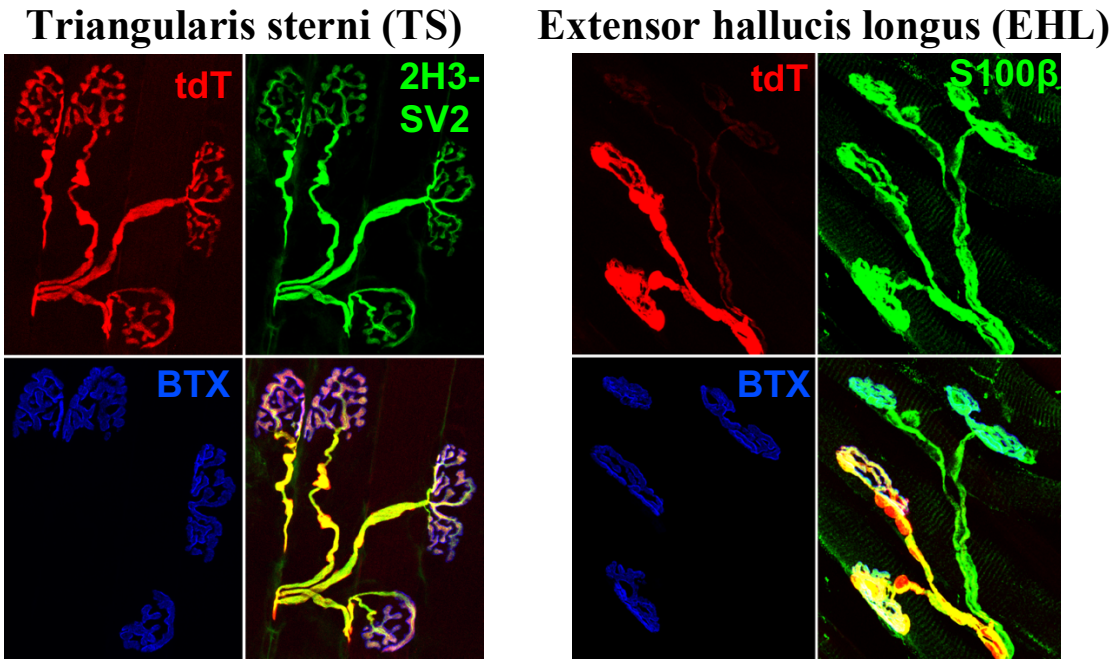


Figure 14. Hb9 expression in motor axons and Schwann cells. Abbreviations: tdT = tdTomato; 2H3-SV2 = motor axon and nerve terminal marker within the PNS; BTX = alpha bungarotoxin (marker for neuromuscular junctions); S100ß = Schwann cell marker in the PNS

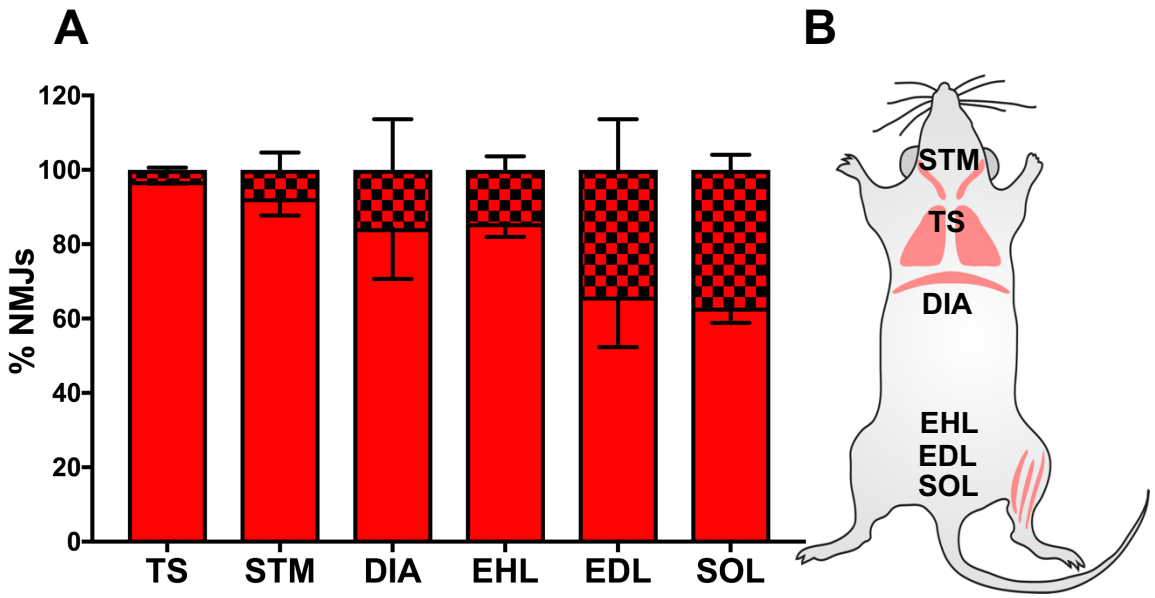
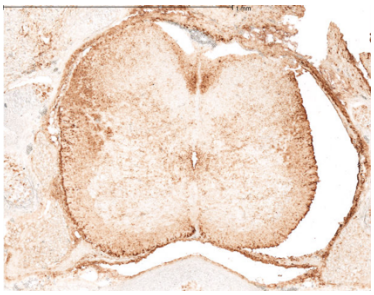
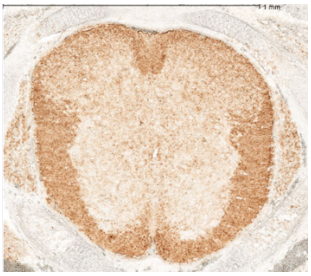
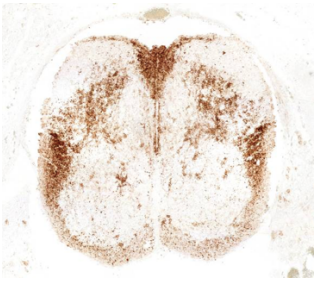
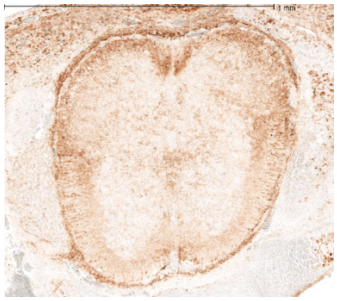
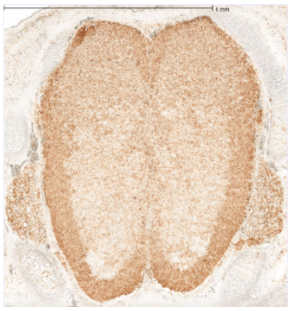
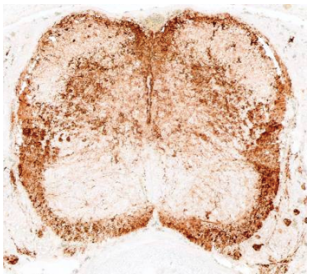
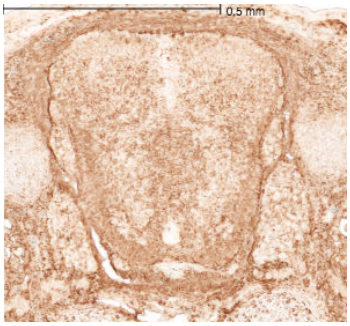
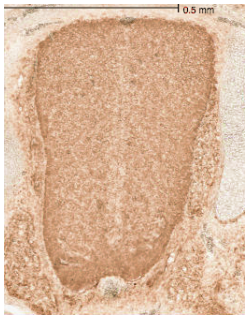


Figure 15. tdTomato expression within the peripheral nervous system shown to exist in an increasing rostro-caudal gradient. [A]- Percent of neuromuscular junctions containing tdTomato expression only in motor axons (solid red bar) or in both motor axons and Schwann cells (checkered bar). Data is mean ± s.e.m., N= 3 animals per group. [B]- Abbreviations: TS = triangularis sterni muscle; STM = sternocleidomastoid muscle; DIA = diaphragm muscle; EHL = extensor hallucis longus; EDL = extensor digitorum longus; SOL = soleus muscle

Dr. Lee studies neuromuscular junctions (marked by alpha-bungarotoxin or BTX), which are where spinal cord motor axon terminal branches contact muscle cells. In his results, shown in ***Figure 14*** and ***Figure 15***, he found Hb9-lineage cells, marked by tdTomato, to be expressed in an increasing rostro-caudal gradient within the muscles of the peripheral nervous system, similar to the pattern previously described within the central nervous system. ***Figure 14*** shows that within the triangularis sterni muscle, tdTomato expressed matches up perfectly with the motor axon marker 2H3-SV2, as expected. However, within the extensor hallucis longus muscle located in the

leg, tdTomato expression is colocalized with S100ß, which is expressed in Schwann cells, the glial cells of the peripheral nervous system.

Table 2. Genes expressed in an increasing rostro-caudal gradient within the E15.5 spinal cord (from the GENSAT project at Rockefeller University, www.gensat.org)

	Frizzled homolog 2 (Fzd2)	**Frizzled homolog 3 (Fzd3)**	**Fibroblast growth factor 15 (Fgf15)**
Cervical			
Thoracic			
Lumbar			

In hypothesizing what genes may be upstream of Hb9, causing the unique increasing rostro-caudal gradient observed, we searched the Gene Expression Nervous System Atlas (GENSAT) to examine the regional expression patterns of over 1,000 genes expressed within the E15.5 mouse spinal cord. As seen in ***Table 2***, members of the Frizzled gene family, Fzd2 and Fzd3, and a fibroblast growth factor (FGF) gene, Fgf15, were identified to have increasing rostro-caudal gradient expression patterns present within the spinal cord, similar to Hb9-lineage cells. The class of Frizzled genes is responsible for the encoding of Wnt receptors. The Wnt signaling pathway is involved with axial patterning of the neural tube during development (Huang and Klein 2004). FGF proteins are also involved with anterior-posterior patterning during neural development. This may indicate that the Wnt signaling pathway or FGF proteins are playing a role upstream of Hb9 expression. We are planning to conduct future *in vitro* studies to validate whether FGF and Wnt signaling can modulate expression of Hb9 in embryonic spinal cord neural progenitors.

CHAPTER IV

CONCLUSION

The data show that within the embryonic and postnatal spinal cord, Hb9-lineage cells are distributed in an increasing gradient along the rostro-caudal axis. This distinctive localization of molecularly-defined cells has yet to be described in the literature, and in fact an increasing rostro-caudal gradient of expression has only been described for a handful of genes. These findings suggest an early role for Hb9 activity in progenitors that give rise to not only neurons and glial cells, but also neural crest cells, which give rise to cells of the peripheral nervous system including Schwann cells. The observed rostro-caudal pattern of Hb9-lineage cellular localization suggests that Hb9 expression may be induced by morphogens that are normally expressed in anterior/posterior gradients during early development, such as Wnt, FGFs, or retinoic acid.

Furthermore, immunohistochemical analysis of both embryonic and postnatal mouse spinal cord tissue has revealed Hb9 expression in cell lineages giving rise to astrocytes and oligodendrocytes. In the peripheral nervous system, a similar phenomenon was observed with Hb9-lineage Schwann cells present in an increasing rostro-caudal gradient throughout neuromuscular junctions of the body. These observations have several exciting implications. Hb9 may play an important role not only in astrocyte and oligodendrocyte development, but also in the development of Schwann cells, which are the glial cells associated with the peripheral nerves. Characterization of the differences between Hb9+ and Hb9- glial cells may reveal new functional roles for molecularly-distinct glial subtypes throughout the spinal cord.

Experiments are currently being conducted to further characterize the time-course of Hb9 expression within the embryonic spinal cord. Experiments to harvest E7.5-E9.5 embryos are being

planned, and *in vitro* assays are being developed to determine the effects of morphogens such as FGF, Wnt, Growth/Differentiation Factor 11 (GDF11), and retinoic acid on Hb9 expression within cultured spinal cord neural progenitor cells. These morphogens may induce greater Hb9 expression. Other *in vitro* assays being planned include a cell sorting experiment to examine gene expression profiles of tdTomato+ versus tdTomato- glial cells. These experiments will further elucidate the role Hb9 plays in glial cell development.

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