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Zebrafish as a Model to Investigate CNS Myelination

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Abstract

Myelin plays a critical role in proper neuronal function by providing trophic and metabolic support to axons and facilitating energy-efficient saltatory conduction. Myelination is influenced by numerous molecules including growth factors, hormones, transmembrane receptors and extracellular molecules, which activate signaling cascades that drive cellular maturation. Key signaling molecules and downstream signaling cascades controlling myelination have been identified in cell culture systems. However, *in vitro* systems are not able to faithfully replicate the complex *in vivo* signaling environment that occurs during development or following injury. Currently, it remains time-consuming and expensive to investigate myelination *in vivo* in rodents, the most widely used model for studying mammalian myelination. As such, there is a need for alternative *in vivo* myelination models, particularly ones that can test molecular mechanisms without removing oligodendrocyte lineage cells from their native signaling environment or disrupting intercellular interactions with other cell types present during myelination. Here, we review the ever-increasing role of zebrafish in studies uncovering novel mechanisms controlling vertebrate myelination. These innovative studies range from observations of the behavior of single cells during *in vivo* myelination as well as mutagenesis- and pharmacology-based screens in whole animals. Additionally, we discuss recent efforts to develop novel models of demyelination and oligodendrocyte cell death in adult zebrafish for the study of cellular behavior in real time during repair and regeneration of damaged nervous systems.

Keywords

oligodendrocytes; screens; live imaging; transgenic; mutation

Introduction

The speed and fidelity of signaling between neurons and their targets determines the efficiency of nervous system function. As animals have evolved, body sizes have grown, and axons similarly have had to elongate and increase in size to continue to innervate their targets. Evolution has adapted to the changing physical properties of these long, large caliber axons by wrapping axons in a thick insulating membrane termed myelin. During development, individual glial processes synthesize thick spirals of membrane around axons, concentrating the machinery necessary for action potential propagation into small

unmyelinated segments of the axon, the nodes of Ranvier. Saltatory conduction from one node to another increases conduction velocity more than ten fold, while myelin prevents excessive charge loss between the nodes, allowing electrical signals to flow further and faster (Buttermore et al., 2013). A growing body of evidence also supports roles for myelin in overall axon function well beyond its role in electrical conduction by providing nutrients and metabolic support at the glia/axon interface, maintaining the stability and health of axons (for extensive review, see Amaral et al., 2013; Saab et al., 2013).

Demyelination, or loss of the myelin sheath, has devastating consequences in both the central nervous system (CNS) and peripheral nervous system (PNS). It is a defining pathological feature of multiple sclerosis (MS) and also commonly occurs following traumatic brain injury (Richardson et al., 2010), spinal cord injury (Waxman, 1989), stroke (Zhang et al., 2013), and during normal aging (Bartzokis, 2004). A growing number of disorders have also been linked to dysmyelination including cerebral palsy (Azzarelli et al., 1980), schizophrenia (Walterfang et al., 2005), depression (Fields, 2008) and several devastating developmental disorders known collectively as the leukodystrophies (for extensive reviews, see Faust et al., 2010; Perlman and Mar, 2012). Thus, therapies aimed at replacing damaged myelin could minimize axonal loss and prevent the neurological decline associated with chronically demyelinated or dysmyelinated pathologies in a wide variety of developmental or adult disorders.

Myelin is supplied by specialized glia cells: oligodendrocytes in the CNS and Schwann cells in the PNS. While there are clearly unique elements of both cell types, many regulatory features of myelination are common between oligodendrocytes and Schwann cells. The current review focuses primarily on myelination in the CNS, but when appropriate, specific reference is made to PNS myelination.

Great strides have been made identifying molecular mechanisms that underlie the maturation of oligodendrocyte progenitor cells (OPCs) into mature myelinating oligodendrocytes (for extensive reviews, see Aggarwal et al., 2011; Emery, 2010). Nonetheless, how these signaling cascades are integrated to control the precise timing of differentiation of oligodendrocyte lineage cells and proper synthesis of myelin membranes remains poorly understood, particularly *in vivo*. Some aspects of myelination appear to be intrinsic to the oligodendrocyte, but it is likely that complex intercellular interactions between oligodendrocyte lineage cells, axons, astrocytes, microglia, and the vasculature are critical for directing the behavior of individual myelinating oligodendrocytes and precise timing of myelination. While cell culture models have been extremely useful in identifying key signaling molecules and downstream signaling cascades controlling oligodendrocyte maturation (Barateiro and Fernandes, 2014; Jarjour et al., 2012), *in vitro* systems are not able to faithfully replicate the complex *in vivo* signaling environment that occurs during development or following injury. *In vitro* systems are also limited in their ability to test toxicity or global side effects of potential therapeutic pro-myelinating molecules. Consequently, many promising molecules identified *in vitro* have failed to translate when moved to whole animal or clinical models. It remains labor intensive to test molecules *in vivo* in rodents due to small litter sizes, relatively long gestation times (~20 days), and the extended period of postnatal myelination. Furthermore, pharmacological manipulation to

target critical periods of prenatal oligodendrocyte development in pregnant and nursing dams often produces severe off target effects, which complicates interpretation of experimental outcomes. Thus, there is a great need for developing rapid, cost efficient *in vivo* models to investigate basic mechanisms of myelination and to test novel molecules in basic and translation research in myelin biology.

Zebrafish Myelination is Representative of Vertebrate Myelination

The zebrafish (*Danio rerio*), a member of the teleost family of ray-finned fish, has emerged as a powerful, rapid and inexpensive model to study vertebrate myelination *in vivo*. Robust myelination occurs within the first week of zebrafish life compared with the first month in rodents, dramatically shortening the time needed to investigate molecular aspects of myelination or to assay for positive and negative effects of molecules on glial development. Ultrastructure analysis has shown that myelin is fundamentally comparable between fish and mammals (see below), supporting the use of zebrafish as a relevant model system for discovery of conserved molecular mechanisms driving vertebrate myelination. Rapidly emerging technologies are also generating new ways of altering gene expression *in vivo*, making molecular manipulation of target genes and molecules in developing embryos relatively straightforward. For example, protein overexpression can be induced by cDNA/mRNA injection into early embryos and transient disruption of protein translation or function can be produced by injection of small oligonucleotides (Heasman, 2002) or constructs/mRNA encoding dominant-negative proteins (Nasevicius and Ekker, 2000). Because of the large number of offspring produced in a single mating, their small size and *ex utero* development, zebrafish embryos are particularly well suited for large-scale mutational and pharmacological screens to identify novel molecules and genes involved in vertebrate myelination (Pichler et al., 2003).

Myelin Structure and Composition is Conserved Between Fish and Mammals

Studies in trout, goldfish and zebrafish have established that the fundamental structure of myelin is conserved between fish and mammals. Homologs for myelin proteins such as myelin basic protein [Mbp; (Nawaz et al., 2013)], myelin protein zero [Mpz, P_0 in mammals, IP1/IP2 in the trout; (Bai et al., 2011)], and proteolipid proteins [Plp/DM20; (Schweitzer et al., 2006)] are all found in the fish. G-ratios, the relationship between axon diameter and myelin thickness, are known to be strikingly similar across a diverse set of vertebrates, including fish and mammals (Hildebrand and Hahn, 1978). Detailed studies have confirmed that myelin periodicity, membrane packing and lipid composition are consistent across multiple fish species (Avila et al., 2007), and comparable to mammalian myelin (Avila et al., 2007). Nevertheless, some unique features of fish myelin have been discovered. Myelin Protein zero (Mpz) appears to be expressed in both CNS and PNS myelin of fish, in contrast to the mammalian homolog P_0 , which is expressed only in PNS myelin (Bai et al., 2011). Additionally, several novel myelin proteins have been identified in fish such as Zwilling-A and Zwilling-B (Schaefer and Brosamle, 2009) and Claudin K (Claudin31), a member of the peripheral myelin protein 22 (PMP22) family (Munzel et al., 2012). It is thought that these membrane-associated proteins play a role in the adhesion of myelin membranes during development, but it is not clear if they are conserved or have been

replaced by other myelin proteins during mammalian evolution. For example, 36K protein, initially identified in trout myelin (Waehneldt and Jeserich, 1984), is thought to be a member of the short-chain dehydrogenase/reductase protein family that is expressed exclusively in oligodendrocytes in the CNS of teleosts, but not rodents (Morris et al., 2004). Nevertheless, a human homologue (FLJ13639) for 36K has been identified and transcripts for this gene are expressed in oligodendrocytes and neurons in the adult human brain (Morris et al., 2004). Currently, the function of 36K/ FLJ13639 in CNS myelin remains unknown.

Molecular Mechanisms Controlling Myelination are Conserved in the Fish

In mammals, individual oligodendrocytes are capable of producing upwards of 40 individual myelin segments (internodes) that wrap multiple axons within its distinct territory (Miller, 2002). Thus, myelination requires precise coordination of transcriptional, cytoskeletal, protein and lipid biosynthesis pathways to produce the exquisite cellular architecture of an individual mature myelinating oligodendrocyte. Even more complex is the coordination of the specific timing and alignment of myelinating oligodendrocytes within distinct brain nuclei and nerve tracks. Many of the intra- and extracellular signals driving generation, proliferation, migration and maturation of oligodendrocyte lineage cells during development and, to some extent, during remyelination following demyelinating injuries or diseases have been identified and extensively reviewed elsewhere (see Emery, 2010; Zuchero and Barres, 2013). It is striking that along with myelin structure, many of the molecular mechanisms driving myelination are also conserved in zebrafish (Raphael and Talbot, 2011). Transcription factors including Olig1, Olig2, Sox10, and Nkx2.2, known to control oligodendrocyte lineage progression in mammals, are found to function in a similar manner in fish (for review, see Monk and Talbot, 2009). Canonical signaling cascades such as Notch (Kim et al., 2008; Park et al., 2005), Wnt (Azim and Butt, 2011), Hedgehog (Chung et al., 2013; Park et al., 2004; Schebesta and Serluca, 2009) and ErbB/EGF (Lyons et al., 2005; Pruvot et al., 2014) also drive oligodendrocyte lineage progression and myelination comparably between fish and mammals. Finally, it is also noteworthy that some zebrafish genes share upwards of 70–90% homology with their human counterparts (Howe et al., 2013). Collectively, these data support the idea that molecular and cellular mechanisms that may be identified in the fish are likely conserved in other vertebrates, including humans.

Zebrafish as a Model For Studying Myelination

Transgenic Lines can be used to Analyze Myelination In Vivo

Perhaps one of the biggest strengths of zebrafish as a research tool is the ease of developing transgenic embryos to observe and manipulate cellular behavior *in vivo* in real time. Zebrafish embryos/larvae mature *ex utero* and remain optically transparent through the first week of life, allowing detailed imaging of tissue and cell development in the living animal. Generating transgenic animals has been streamlined by the adaptation of the Gateway modular cloning system for use in fish by the Chien Lab (Kwan et al., 2007), which is freely distributed in the fish community. This system takes advantage of conserved tandem repeats present throughout the zebrafish genome and high efficiency germ-line integration of transgenic cassettes can be achieved when constructs are co-injected with TOL2 transposase

mRNA (Kwan et al., 2007). As such, it has become standard practice to clone putative zebrafish promoters or use modified Bacterial Artificial Chromosomes (BACs) to develop transgenic animals with specific populations of fluorescently labelled cells (Bussmann and Schulte-Merker, 2011; Suster et al., 2011). Methods to conditionally activate transgenes have also been adapted to zebrafish, such as the yeast GAL4/UAS binary transcription system (Davison et al., 2007). Here, Gal4 protein is expressed under the control of cell specific promoter, which in turn activates a *UAS* cassette, (for example *UAS:EGFP*) driving expression of a desired protein only in a specific population of cells. Heat shock promoter-driven activation of transgenic constructs (Zhan and Gong, 2010) and Cre-mediated recombination are also being adapted to fish allowing for more precise cell-specific and temporal control of gene expression (Thummel et al., 2005), although it should be noted that development of Cre-mediated systems is still not optimized in zebrafish. Ongoing advances in genome annotation, next generation sequencing methodology, and genetic modification using Zinc Fingers (Urnov et al., 2010), TALENs (Campbell et al., 2013) and CRISPR methodology (Blackburn et al., 2013) are also rapidly speeding up the generation of site-specific nucleotide modifications and knockout animals to test the role of individual genes or even specific human SNPs *in vivo* (Gregory-Evans et al., 2007).

Live Imaging Studies have Yielded Novel Insights into Cellular Behavior of Myelinating Glia

Real time analysis of cellular behaviors such as migration, process extension and internode generation can give significant information on the function of individual genes or molecules in oligodendrogenesis and myelination. In rodents, live imaging studies have been used to track oligodendrocyte lineage cell behavior *in vivo*, but the instrumentation and technological challenges are still limiting factors. Access to the rodent CNS for live imaging is limited to the most superficial layers of the cortex and cerebellum (Hughes et al., 2013), which could represent distinct subsets of oligodendrocytes that may differ from oligodendrocyte populations in subcortical or deep white matter tracts. In comparison, the full zebrafish CNS is accessible for live imaging during oligodendrocyte specification, proliferation, and nascent myelination, especially with the generation of the Casper mutant line, which lacks both iridophores and melanocytes and remains transparent into both juvenile and adult stages (White et al., 2008). A variety of fluorescent reporter lines that label OPCs, oligodendrocyte cell bodies and/or their myelin sheaths are currently available (Table 1), and the developmental time course for OPC generation, migration and myelination in the spinal cord has been well characterized (Fig. 1A). Selection of specific transgenic lines should allow researchers to track behavior such as process extension and membrane synthesis in subpopulations of OPCs or maturing oligodendrocytes (Fig. 1B,C; respectively). It is also useful to label only subsets of oligodendrocytes because when the entire lineage is labeled it can be difficult to resolve complete cellular morphology. To overcome this problem, injection of DNA or mRNA into one to two cell embryos can also be used to induce mosaic expression of fluorescent constructs *in vivo* (Hyatt and Ekker, 1999). This mosaic approach isolates the full complement of processes and membrane extensions associated with single cells (see Fig. 2).

Real time studies of myelination in the zebrafish have provided tremendous insight into the behavior of myelinating glia in their native environment (summarized in Table 2). For example, 36 h time lapse imaging of the developing spinal cord in a membrane-tethered *sox10* reporter fish has shown that OPCs behave stochastically, moving rostrally and caudally through the spinal cord, jumping on and off axon tracks as they migrate away from their specification in the primary motoneuron domain (pMN) (Kirby et al., 2006). These experiments also show that, in contrast, to the classic bi- and tripolar OPC cell morphology observed in tissue culture, OPCs *in vivo* have many processes that are constantly moving and likely encountering a wide array of growth cues in the environment around them. Migrating OPCs extend and retract a dynamic array of processes, and surprisingly continue to divide even after developing a complex multiprocessed morphology, previously suggested to be an indicator of a post mitotic OPC that is beginning to differentiate (Hardy and Reynolds, 1991). Intriguingly, Kirby et al. (2006) also observed that individual processes were very sensitive to neighboring OPC processes, collapsing and retracting on contact, similar to the contact-mediated repulsion of axon growth cones upon encountering repulsive cues. This avoidance behavior has also been observed in adult mice among fluorescently labeled cortical NG2 cells, the presumptive adult OPC population (Richardson et al., 2011). These cells appear to form individual territories separating them from neighboring NG2 cells (Hughes et al., 2013). In both the fish and mouse, laser ablation of individual OPCs induces proliferation and differentiation (Hughes et al., 2013; Kirby et al., 2006). Neighboring OPCs rapidly colonize the empty territory, filling the space vacated by the ablated cell, as though an inhibitory cue has been removed. This suggests that there is a defined optimal density of OPCs. It has been hypothesized that a mechanosensory mechanism might control this density, and thereby regulates the timing of differentiation of OPCs into myelinating oligodendrocytes (Rosenberg et al., 2008). This would ensure that a sufficient number of OPCs have dispersed along axons and established their independent territories before initiation of myelination (Chong et al., 2012; Kawai et al., 2009). The tractability of the zebrafish nervous system may allow some of these population dynamics in myelinating glia to be studied in real time *in vivo*.

Important questions about the adaptability of the oligodendrocyte in response to intrinsic and extrinsic cues are also being studied in zebrafish. In Lee et al. (2010), live imaging of 3dpf *tg(olig2:EGFP)* larvae demonstrated that OPC migration is defined by bursts of progenitor movement interspersed with periods of pausing and exploratory process extension, with cells often reversing or abruptly changing trajectory when they resumed migration. In this study, loss of function of both alleles of *neurofibromin 1 (NF1)* gene in zebrafish larvae resulted in aberrant proliferation and migration of OPCs, with cells traveling greater distances than in control animals. Interestingly, the increase migratory distances did not result from increased migration velocity, but rather decreases in periods of OPC pausing. Not only are these studies relevant to understanding malignant transformation in gliomas, as mutations in NF1 are known to give rise to peripheral nerve sheath tumors in Neurofibromatosis type 1 patients, but they also support the idea that proper migration and dispersal of OPCs may be dependent on these periods of pausing to perhaps integrate extracellular developmental cues.

Following migration, differentiating OPCs extend processes, contact their target axons and begin generating nascent myelin membranes that eventually generate compact myelin. To investigate whether each individual oligodendrocyte myelinates only one size of axon or produces a predetermined amount of myelin, a fish line was generated that produced supernumerary neurons, placing vastly different caliber axons within the territory of an individual oligodendrocyte (Almeida et al., 2011). Here, ectopic Mauthner axons, large caliber sensory neurons normally found in the ventral spinal cord, were generated in the dorsal spinal cord of the fish, and the capacity of individual oligodendrocytes to myelinate these extra axons was measured. This study demonstrated that individual oligodendrocytes can simultaneously myelinate a mixture of large and small caliber axons, significantly increasing the total amount of myelin that a single cell normally makes. All together, these findings support the idea that individual oligodendrocytes have an intrinsic plasticity and respond to local environmental cues to determine the amount of myelin an oligodendrocyte must generate for each individual axon.

Another study focused on the time frame over which individual spinal cord oligodendrocytes differentiate and generate their myelinating processes (Czopka et al., 2013). Using mosaic analysis to isolate individual cells, myelination was followed over multiple days and in multiple regions of the spinal cord. This study established that the full differentiation program of a cell occurred in a defined period of time. Specifically, time lapse imaging of individual oligodendrocytes showed that within 5 h of forming their first nascent internode, oligodendrocytes extended processes, contacted axons and generated their full complement of internodes. Strikingly, this 5-h window was consistent across the entire spinal cord, regardless of when or where in the spinal cord the OPC was differentiating. Furthermore, during this critical window, altering Fyn kinase signaling, a known regulator of oligodendrocyte process extension (Kramer-Albers and White, 2011), increased the rate of internode generation, but it did not result in formation of any additional internodes. Thus, while the speed of myelination can be increased, once a cell has formed its full complement of nodes, it does not produce any additional myelinating processes. Czopka et al. (2013) also noted that myelinating processes were sometimes retracted, a process termed “myelin pruning” by Liu et al. (2013), who observed that ~28% of early myelinating processes were lost between 2 and 7 dpf, and that this pruning is dependent on erbB signaling. Furthermore, pruning is correlated to axon diameter, with smaller axons more likely to lose nascent myelin nodes than larger axons.

Finally, live imaging studies have identified distinct subpopulations of OPC progenitors in the zebrafish, an ongoing debate in mammalian models. A transgenic line generated with the Nkx2.2a promoter, a transcription factor expressed by a subset of pMN-derived progenitors, clearly separates myelinating OPCs from non-myelinating OPCs in the developing spinal cord (Kucenas et al., 2008). Subsequent studies with the Nkx2.2a line showed that Sox10, thought to be important for OPC lineage progression, is required for oligodendrocyte survival once myelination has begun, as cells lacking Sox10 die despite contacting axons (Takada et al., 2010). Importantly, the non-myelinating OPC population (Nkx2.2 negative) expanded to maintain normal numbers of total OPCs and initiated wrapping, yet ultimately failed to express normal levels of critical myelin genes. Interestingly, despite also lacking Sox10, the newly generated myelinating cells survived much longer than the initial wave of

myelinating oligodendrocytes. This observation supports the hypothesis that multiple populations of OPCs exist that have intrinsically different roles, but remain plastic to environmental cues.

Overall, it remains unknown if intrinsic developmental programs control the sequential generation of distinct populations of OPCs in vertebrates, or if a common progenitor pool exists, which responds to extrinsic factors (such as competition for instructional cues from axons) to drive the selection and differentiation of subsets of OPCs into myelinating glia. The specification of transcriptionally distinct myelinating vs non-myelinating populations of OPCs could be unique to the zebrafish. However, even in mammals, the idea that all myelinating oligodendrocytes are derived from a single homogeneous pool of OPCs, or that all OPCs share equivalent myelinating potential, is a growing area of debate. It seems reasonable to speculate that heterogeneous populations of OPCs may also be present in mammals. It will be interesting to see if distinguishing features of subpopulations of OPCs such as their electrical properties (Karadottir et al. 2008) or their developmental origin (e.g., the so-called dorsal vs. ventral progenitor populations; for an extensive review see Richardson et al., 2006) influences their myelinating potential during development or in response to injury.

Forward Screens and Microarray Analysis Have Identified Several Novel Genes in Myelination

While imaging studies have yielded great insight into the behavior of oligodendrocyte lineage cells in intact nervous systems, labs continue to search for unique markers for oligodendrocyte lineage cells, and novel transcription factors and signaling molecules that drive oligodendrogenesis and myelination (Dugas et al., 2006). Given their small size and high fecundity, zebrafish are readily adaptable to large scale screens to identify genetic mechanisms driving myelination, and in fact such screens have identified genes often suggested to be relevant in earlier rodent studies.

In a typical forward screen, *N*-ethyl-*N*-nitrosourea (ENU) treatment is used to induce mutations during spermatogenesis in male zebrafish (Grunwald and Streisinger, 1992; Solnica-Krezel et al., 1994). Mutagenized males are then outcrossed, in some cases to a reporter line such as tg(*olig2*:EGFP) for OPCs (Table 1), and the resulting offspring are raised to adulthood. This F1 generation is then backcrossed to stabilize any mutations and subsequent generations are screened for phenotypic changes in OPC proliferation, migration or maturation. Linkage mapping and alignment with the most recent genome assembly is then used to map the point mutation and identify the gene and specific mutation underlying the mutant phenotype (Beier and Herron, 2004).

Using ENU methodology, two independent forward screens (Pogoda et al., 2006; Snyder et al., 2012) identified both overlapping signaling pathways and several novel genes critical for vertebrate myelination. First, by analyzing changes in the movement of *mbp* transcripts out into myelin, a screen in the Talbot laboratory identified thirteen mutants with defective myelination uniquely in the CNS, PNS or in both CNS and PNS (Pogoda et al., 2006). Some of these mutants were mutations in well established signaling cascades, such as in the *erbB* genes, which regulate neuregulin signaling and which had already been implicated in

regulating PNS myelination in rodents (Michailov et al., 2004). However, the usefulness of such forward genetic screens by phenotype is clear from the fact that this screen also identified several unexpected molecules that impact CNS and/or PNS myelination. For example, this screen identified a novel role for Kif1b, a kinesin motor protein (Lyons et al., 2009), which had previously been implicated in axonal trafficking of synaptic vesicles and mitochondria. Analysis of mutant st43 (*kif1b*) embryos showed that *mbp* and *36k* transcripts failed to traffic into myelin sheaths, and were instead mislocalized to oligodendrocyte cell bodies, where they generated ectopic myelin-like membranes. Thus, this zebrafish screen identified a specific mutation in a motor protein that rodent studies had suggested likely serves to localize specific mRNAs to myelinating membranes, perhaps preventing inappropriate expression of myelin proteins in non-myelinating compartments of the oligodendrocyte (Carson et al., 2001).

Next, a second independent ENU screen in the Appel laboratory, established that the motor protein Dynein cytoplasmic 1 heavy chain 1 (*Dync1h1*) is also necessary for proper myelination in zebrafish PNS (Langworthy and Appel, 2012). In *Dync1h1* mutants, appropriate numbers of Schwann cells colonized the lateral line (a PNS sensory structure in fish) but failed to initiate wrapping of axons. Here, rather than transporting *mbp* transcripts directly, *Dync1h1* appeared to mediate activation of myelination programs via cyclic adenosine monophosphate (cAMP) signaling in Schwann cells. This is a unique connection to earlier rodent studies demonstrating that elevation of cAMP can mimic axonal contact *in vitro* by activating Oct6 and *krox20*, transcription factors that control PNS myelination (Monuki et al., 1989). The role of cAMP in myelination is also supported by findings in a separate mutant uncovered in the Talbot screen, showing that the orphan G-coupled protein receptor, Gpr126, which regulates cAMP levels in Schwann cells, is critical for initiation and maintenance of membrane wrapping in the PNS (Monk et al., 2009).

Both of these forward screens demonstrate that novel intracellular mechanisms critical for myelination can be uncovered in the fish. However, performing screens requires multiple generations of fish to isolate stable mutants; thus large scale screens are labor intensive and not well suited for small laboratories or fish facilities with limited space. Early screens also required a major investment in time-consuming linkage mapping studies to identify the specific gene disrupted during ENU mutagenesis. To speed the process of identifying potentially valuable mutations, more recent screens are combining chemical mutagenesis with TILLING (Targeting Induced Local Lesions In Genomes) to identify point mutations in specific genes, a technique initially developed in plants, which has been adapted to zebrafish, and can now be automated for high throughput analysis (Amsterdam and Hopkins, 2006; Henikoff et al., 2004). Identification of new mutations is also being enhanced with the rapid expansion of next generation sequencing technology as the method of choice to define point mutations in mutants isolated from traditional forward ENU screens (Henke et al., 2013; Voz et al., 2012).

Several groups have also taken a microarray approach to discover novel genes in oligodendrocyte development (Chung et al., 2011; Takada and Appel, 2010). One microarray screen (Takada and Appel, 2010) looked at transcripts from cells purified by fluorescent activated cell (FAC) sorting from tg(*olig2:EGFP*) zebrafish. Because *Olig2* is

expressed in other cell types during the time periods examined, embryos were treated with Trichostatin A (TSA), an HDAC inhibitor known to block oligodendrocyte development (Sun and Taneja, 2000). EGFP-positive cells were then sorted from embryos during time periods associated with OPC migration or nascent myelination (see Fig. 1), and mRNA from control embryos or TSA-inhibited “oligodendrocyte-deficient” embryos was compared. This microarray looked specifically for changes in transcripts during the transition from migrating OPCs (36hpf) to myelinating oligodendrocytes (3–4 dpf), and validated multiple candidate molecules that may be necessary for myelination, several of which are expressed in oligodendrocytes but not Schwann cells. These include *TLC domain containing 1 (tlcd1)*, which may be involved in production and trafficking of ceramide, a lipid required for proper myelin membrane assembly (Jackman et al., 2009) and *Claudin k*, noted above, which was first identified in this screen. Zebrafish Claudin K may be related to the tight junction protein claudin11/oligodendrocyte-specific glycoprotein (OSP) in mammals, a tetra-spanin protein that appears to be critical for proliferation and migration of OPCs via integrin mediated signaling cascades (Tiwari-Woodruff et al., 2001) and in the compaction and maintenance of myelin membranes (Chow et al., 2005). The specific role of Claudin K in zebrafish myelination remains unknown, but it appears to be highly expressed during oligodendrocyte development and following demyelinating injury (Münzel et al., 2012). Finally, this screen also identified *swap70* and *SH2 domain containing 3Cb (sh2d3cb)*, which is expressed in oligodendrocytes but not Schwann cells. Swap70, a regulator of F-actin dynamics in process extension (Chacon-Martinez et al., 2013), has since been shown to play a previously unknown role in controlling neural progenitor cell exit from the cell cycle during OPC specification (Takada and Appel, 2011). The role of *sh2d3cb* in oligodendrogenesis remains unknown.

Another microarray similarly screened FAC-sorted EGFP-positive OPCs or myelinating oligodendrocytes (2 dpf and 5 dpf; respectively) from either *tg(olig2:EGFP)* or *tg(sox10:EGFP)* zebrafish (Chung, et al., 2011). As in the Tanaka and Appel (2010) microarray analysis, larvae were treated with TSA to block oligodendrocyte development and RNA from either control embryos or TSA-inhibited, oligodendrocyte-deficient embryos were compared using a zebrafish specific microarray that contained ~20,000 genes. Seven candidate genes were found to be highly upregulated in oligodendrocytes, including *bhlhe40* (basic helix-loop-helix family, member e40), *rbp2a* (retinol binding protein 2a, cellular), *rock2b* (rho-associated, coiled-coil containing protein kinase 2b), and *sepp1a* (selenoprotein P, plasma, 1a). *bhlhe40*, is a cAMP inducible protein in the bHLH family of transcription factors, which links back to the observation in rodent cells that cAMP signaling may be a key modulator of axon-induced myelin membrane production (Monuki et al., 1989). Interestingly, transcription of *bhlhe40* is regulated by Vitamin D signaling (Seuter et al., 2013), and *bhlhe40* has also recently been shown to influence memory T cell development (Hu and Chen, 2013) and cytokine production by reactive T cells during neuroinflammation (Lin et al., 2014). Altogether, these data support the concept that *bhlhe40* could play multiple roles in the pathology and repair of demyelinating lesions, and its conservation in the fish may present a novel *in vivo* model for functional studies of its role in oligodendrocyte development or during oligodendrocyte injury or inflammation (see below). Also identified in the screen were *rbp2a* and *rock2b*, genes linked to Retinoic Acid and Rho-

GTPase signaling, respectively, which are signaling cascades also known to affect the timing of OPC differentiation and myelin membrane extension (reviewed in Huang and Franklin, 2011). An intriguing protein identified by this microarray screen is *sepp1a*, a protein that binds the nutrient selenium, which has been shown to influence oligodendrocyte morphogenesis, survival and myelin gene upregulation in rodent cells *in vitro* (Espinosa de los Monteros et al., 1997; Gu et al., 1997). The mechanism underlying selenium signaling in oligodendrogenesis remains unknown, but selenium is a common media supplement for maintaining rodent OPCs in culture (Pedraza et al., 2008) and selenium deficiency may be linked to MS (Foster, 1993; Johnson, 2000; Warren, 1982). Conservation of selenium signaling in the fish could open a new avenue of research on the role of this and other micronutrients in oligodendrocyte development *in vivo*.

Several molecules have been identified in rodents as being critical for oligodendrogenesis but due to the complexity of the underlying synthesis or signaling cascades, or the number of potential sites of action, it has been difficult to determine the precise role these molecules are playing in cellular maturation *in vivo*. One prime example of this is determining what role, or roles, cholesterol synthesis plays in myelination. Cholesterol influences fluidity of most cellular membranes and is highly concentrated in oligodendrocytes where it interacts with a variety of myelin proteins (Muse et al., 2001) and can constitute 25 percent or more of the lipid content of myelinating membranes (Morell and Jurevics, 1996). Previous work in mice has shown that depleting cholesterol in oligodendrocyte lineage cells by conditionally inactivating squalene synthase, an enzyme critical for cholesterol synthesis, produces hypomyelination (Saher et al., 2005). However, determining the specific way in which cholesterol deficiency leads to dysmyelination in rodents has been challenging, due to the inability to completely deplete cholesterol in oligodendrocyte lineage cells, which can derive cholesterol from other cells in the nervous system (Pfriege and Ungerer, 2011), and the lack of precise temporal control of cholesterol synthesis during distinct stages of maturation such as process generation or membrane synthesis.

A recent study in zebrafish has shed light on how cholesterol synthesis affects myelination and identified a novel role for the cholesterol biosynthesis pathway in oligodendrogenesis beyond its potential role in myelin membrane synthesis. Mathews et al. (2014) describe a zebrafish mutant, named overthrow (*ovt*), in which OPCs migrate past their axonal targets, and subsequently fail to express transcripts for myelin proteins. The *ovt* phenotype was mapped to a point mutation of the Hmg-CoA synthase 1 gene (*hmgcs1*), an enzyme that controls an early, rate-limiting step in the cholesterol biosynthesis pathway, *hmgcs1* activity influences synthesis of farnesyl pyrophosphate, a precursor for both cholesterol biosynthesis and protein prenylation, a lipid modification that localizes proteins to the cell membrane. Using an elegant combination of pharmacological manipulation, genetic rescue of *hmgcs1* expression and live imaging experiments, they were able to demonstrate these different arms of the cholesterol biosynthesis pathway had distinct and non-overlapping functions in oligodendrocyte lineage progression. Specifically, they provide the first evidence that prenylation is necessary for migrating OPCs to recognize and stop at their target axons, while the cholesterol biosynthesis branch is required for nascent membrane ensheathment and myelin gene expression. While prenylation of specific myelin proteins has been noted in

earlier studies (De Angelis and Braun, 1994; Sepp-Lorenzino et al., 1994), this mutant identifies a new part of the cholesterol pathway that is selectively important for the ability of OPCs to migrate effectively. Overall, this study demonstrates how exploiting the strengths of the zebrafish model, including forward genetic screens, live *in vivo* imaging and precise temporal manipulation of complex signaling cascades is yielding new insights into fundamental mechanisms underlying myelination of vertebrate nervous systems.

Pro-myelinating Drug Screening in Zebrafish

While genetic screens and *in vivo* imaging have contributed a deeper understanding to the molecules and cellular behaviors that drive myelination of the nervous system in vertebrates, another benefit of the zebrafish model for myelination studies is that they are highly amenable to large-scale pharmacological screens to test small molecules *in vivo*. Each mating typically produces hundreds of offspring. Thus large numbers of embryos are available for bath treatment with drugs to pharmacologically test the *in vivo* activity of molecules at specific developmental time points. Combined with appropriate transgenic reporter lines, pharmacological screens in zebrafish embryos could also identify when in the lineage a potential pro-myelinating compound affects myelination by assaying for specific cellular behaviors, e.g. OPC migration or proliferation, or oligodendrocyte process generation and membrane synthesis. Extensive drug library screens have been performed in cell culture models (Merrill, 2009), but *in vitro* systems are not able to predict how cells will respond when these signaling cascades are activated *in vivo*, particularly when activating signaling cascades in the entire body simultaneously. To test a large number of compounds effectively, it will be necessary to develop a medium-throughput platform that accurately and rapidly assays oligodendrocyte lineage development or myelination, while at the same time being cost efficient and highly reproducible.

Screening Small Molecule Libraries in Zebrafish for Pro-myelinating Molecules

The Franklin laboratory successfully developed a screening assay using tg(*olig2*:EGFP) embryos (Buckley et al., 2010). Here, embryonic fish were bath exposed to 1,120 compounds, and potential pro-myelinating compounds were identified by assaying changes in the total numbers of dorsally migrating OPCs in the spinal cord and quantitative increases in *mbp* transcripts. Overall, the screen identified compounds that increased migration of OPCs *in vivo*, including an agonist for the peroxisome proliferator-activated receptor (PPAR) and a novel biogenic amine that targets adenylate cyclase and cAMP signaling. However, subsequent analysis by quantitative PCR showed that while PPAR increased OPC migration, it had no effect on *mbp* mRNA expression. In contrast, the biogenic amine also increased OPC migration, but only transiently decreased *mbp* mRNA expression. Conversely, this screen found other molecules that had little effect on migration but actually impacted *mbp* mRNA expression. Perhaps the most important result of this study is that it demonstrates that pharmacological screens in zebrafish embryos can rapidly identify distinct effects of novel compounds on specific stages of oligodendrogenesis and myelination. The Buckley et al. (2010) screen also took advantage of a known library of compounds that had already been tested for safety in humans. Such drug reprofiling platforms could vastly speed

up the process of small molecule discovery to clinical trials, hopefully speeding up the process of FDA approval of potential pro-myelinating therapies.

Development of High Throughput Screening Platforms in Zebrafish

Drug discovery also uses structure activity relationship (SAR) analysis, in which small libraries are synthesized based on subtle modification of a known agonist or antagonist to a specific signaling cascade or a novel small molecule (Wawer and Bajorath, 2009). Small changes in chemical structure can have a large impact on efficacy of receptor binding, ability to cross the blood brain barrier (BBB) and/or drug half-life. Here again, an appropriate medium-throughput screening platform in zebrafish could greatly enhance the optimization of bioactive synthetic agonists and antagonists for pathways such as WNT, EGF/ErbB, or AKT/mTOR, well-described pathways impacting myelination [for more information see Fancy et al. (2010)]. Recently, Pruvot et al. (2014) tested a selection of EGF pathway inhibitors on zebrafish for specific developmental effects, as well as potential off target effects and toxicity. Embryos were treated from 4 hpf to 4 dpf with different synthetic EGFR antagonists (known as tyrphostatins), then assayed for changes in *mbp* mRNA expression by *in situ* hybridization or quantitative PCR. Perhaps unsurprisingly, the different tyrphostatins had a large range of affinities (EC₅₀'s from 1.1 to 45 μ M) for blocking EGF receptors, different efficacies for blocking *mbp* expression in whole animals, and produced a range of morphological defects. This study only tested a small number of drugs (<10) but supports the idea that a medium-throughput zebrafish screen could be used to narrow down thousands of compounds into a few promising lead compounds, which would be moved up the pipeline based on enhanced specificity and reduced general toxicity. Additionally, screening of therapeutic compounds for myelination in zebrafish may also be able to identify orally bioactive compounds, since drugs delivered after 3 dpf must cross the blood brain barrier (BBB), which begins forming in zebrafish at that stage, and rapidly becomes impermeable to large molecules (>1,000 kD; Jeong et al., 2008). While this could complicate interpretation of screens and produce false negatives (e.g., misclassifying bioactive molecules as having no effect on myelination), it could also serve to optimize for potential small molecules that are capable of crossing the BBB *in vivo*, an important criterion for potential therapeutic compounds.

Ultimately, rapid and highly reproducible pharmacological screening platforms need to be developed in order to take full advantage of the zebrafish model for drug discovery. The Buckley et al. (2010) and Pruvot et al. (2014) studies relied on transcriptional analysis of *mbp* expression using *in situ* hybridization and quantitative PCR, both time consuming processes, particularly in small labs lacking the robotic automation available in industry. Given the plethora of candidate molecules implicated in oligodendrocyte lineage progression and myelination and the ability to generate synthetic compound libraries, the zebrafish could prove very useful in identifying potential pro-myelinating drugs, particularly if transgenic animals could be developed as a fluorescent readout for oligodendrocyte maturation or myelination *per se*, for automated imaging analysis. For example, a fluorescent reporter line is being developed that expresses a prenylated form of EGFP under the control of a 12 kb *myelin protein zero (mpz)* promoter (Preston and Macklin, in preparation), which is one of the most highly expressed proteins in the myelin membranes of

zebrafish (Bai et al., 2011). This line expresses a membrane bound EGFP in the processes of myelinating oligodendrocytes in the spinal cord and brain, starting with the large Mauthner axons at 2.5 dpf. Expression of EGFP is restricted to cells with the morphology of myelinating oligodendrocytes by 6 dpf (Fig. 1C). We are currently validating this line as an *in vivo* fluorescent readout for myelination.

Studying Demyelination/Remyelination in the Fish

Developmental Myelination Versus Remyelination of the Adult Nervous System

It should be noted that all the zebrafish studies discussed here were undertaken in the developing embryo/larvae, which uses the optimal elements of the zebrafish, i.e., analysis of large clutches of transparent fluorescently tagged embryos *ex utero*. Remyelination does recapitulate many aspects of developmental myelination. For example adult OPCs must respond to damage and activate myelination programs to replace damaged and dying cells. Nonetheless, analysis of developmental myelination may be quite different from remyelination in the adult nervous system, particularly in an injury context. Numerous studies have shown that OPCs are commonly found in and around large demyelinated plaques (Chari and Blakemore, 2002; Kang et al., 2010). Yet these recruited OPCs often fail to mature and replace damaged or dying cells, supporting the notion that dysregulation of differentiation and maturation programs accounts for some of the remyelination failure seen in MS and other demyelinating injuries (Hagemeyer et al., 2012).

In other vertebrates, there is a large population of OPCs in the adult CNS, which are available to remyelinate axons after injury. While similar developmental signaling cascades are likely activated in adult OPCs to induce proliferation and maturation into myelinating cells, it remains unclear if novel molecules or mechanisms are also necessary in the adult to enhance replacement of damaged oligodendrocytes following injury or disease. Spontaneous remyelination after injury commonly occurs in humans and rodents, but the capacity for neural repair appears to decline with age in both mammals and fish (reviewed in van Wijngaarden and Franklin, 2013). This loss of remyelination capacity could result in chronic demyelination or a declining rate of repair following repeated insults (reviewed in Fancy et al., 2010). As a result, large chronically demyelinated plaques are commonly seen in progressive forms of MS. Given that demyelinated axons display conduction deficits (Felts et al., 1997) and may be more susceptible to atrophy and death (Rasminsky and Sears, 1972), chronic demyelination is likely involved in the axon loss seen in late stages or severe forms of MS (for more information see Miller and Leary, 2007; Trojano et al., 2003). Therefore, therapeutic strategies that protect axons or promote remyelination may prevent escalating loss of motor, sensory and cognitive function common in MS patients or those with other demyelinating injuries.

Modeling Demyelinating Injuries in Zebrafish

The injured environment of the adult brain may provide unique challenges to efficient reactivation of myelination programs. It is now becoming clear that a variety of molecules produced in response to inflammation and demyelination such as chondroitin sulfate proteoglycans (CSPGs) and glycosaminoglycans (GAGs) may accumulate in the glial scar

and actively inhibit OPC maturation, even after inflammation has subsided. Detailed discussion of the nature of inhibitory signals at sites of demyelination has been reviewed elsewhere (Harlow and Macklin, 2014; Sherman and Back, 2008), and is beyond the scope of this review. However, it is clear that zebrafish have a large regenerative capacity and it is thought that one of the reasons is directly due to their lack of glial scar formation in the nervous system following injury (Goldshmit et al., 2012). It is tempting to speculate that the adult zebrafish could be useful for testing cellular responses *in vivo* to some of these extracellular matrix signals found in chronically demyelinated lesions. For example, following demyelination in the adult zebrafish nervous system, do the processes of newly generated OPCs collapse or avoid contact with certain CSPGs or combinations of ECM materials, such as those found in chronically demyelinated lesions?

Experiments such as these would necessitate developing novel models for studying demyelination in the adult zebrafish, since OPC or oligodendrocyte cell death during development cannot be considered *de facto* demyelination. Multiple models of demyelination are available in rodents and have been extensively studied for decades. Injection of the detergent lyssolecithin into white matter tracks or addition of the copper chelating molecule cuprizone to rodent chow have proven to be excellent paradigms for inducing demyelination and studying spontaneous remyelination *in vivo*. However, these models often lack the immune component associated with immune-mediated demyelination such as seen in MS patients. Immune-mediated demyelination models in rodents such as experimental autoimmune encephalomyelitis (EAE) induced by myelin peptides or infection by Theiler's murine encephalomyelitis virus (TMEV) result in demyelination. However, studying the immune component of the model separately from the ability of the tissue to remyelinate is difficult. While many of these models could be readily adapted to the adult fish, the crucial question is what information could adapting these models to the fish provide?

One advantage to developing a bon fide demyelination/ remyelination model in the fish is the potential to again observe complex cellular responses to injury, oligodendrocyte death and/or demyelination in real time. For example, based on rodent studies, it remains debatable whether oligodendrocyte ablation leads to immediate changes in myelin structure and myelin loss (Caprariello et al., 2012; Oluich et al., 2012; Pohl et al., 2011). To answer these kinds of questions, it would be necessary to focally control myelin damage or oligodendrocyte death and then image cellular responses in real time. Recently it has been shown that expression of the bacterial enzyme nitroreductase (NTR) can be driven selectively in oligodendrocyte lineage cells using an *mbp* promoter driven Gal4/UAS system in adult fish (Chung et al., 2013). When these fish are treated with metronidazole, NTR converts the drug into a cytotoxic metabolite, allowing for controlled temporal ablation of myelinating oligodendrocytes *in vivo* with-out harming neighboring NTR-negative cells (Chung et al., 2003; Curado et al., 2007). This results in significant demyelination in adult fish, which can partially recover over 2 weeks. If this demyelination system could be developed in the transparent Casper line (White et al., 2008) and coupled with minimally invasive confocal imaging, it might be possible to watch behaviors of different populations of cells, such as macrophages and microglia (Peri and Nusslein-Volhard, 2008; Rosenberg

et al., 2012) in response to injury *in vivo* in real time. This would certainly open a new avenue to study cellular behavior in response to injury and could identify fundamental mechanisms driving clearance of myelin debris, particularly given the tractability of the zebrafish nervous system as compared with mammals.

Importantly, zebrafish appear to possess a full complement of immune cells such as neutrophils (Gray et al., 2011), and antigen presenting T cells (Langenau et al., 2004). Furthermore, an adaptive immune response similar to rodent EAE models can be generated by inoculating adult fish with a homogenate of the CNS (zCNS) and Complete Freund's Adjuvant (CFA) (Quintana et al., 2010). This triggers invasion of FoxP3-positive regulatory T cells into the CNS of adult fish (Quintana et al., 2010). Could studies in the adult fish begin to resolve some of the controversies over which class of immune cells trigger demyelination in the CNS or how cells activated in the periphery target to the brain and either cause or exacerbate autoimmune-mediated damage? Much has been learned from analyzing these questions in rodents, but being able to identify cells, their behavior and their impact in real time may provide new insight into fundamental mechanisms driving repair of white matter. While all these questions remain purely speculative at this time, it will be interesting to see if the zebrafish model will have a significant contribution to our understanding of demyelinating diseases such as MS and spinal cord injury, and if its utility will extend beyond its now well established role in identifying novel mechanisms in developmental myelination *in vivo*.

Conclusions and Perspectives

Many fundamental questions remain in oligodendrocyte biology and the usefulness of the zebrafish model for identifying completely novel mechanisms common to all of vertebrate myelination is still being discovered. Many of these questions have already been highlighted in this review: How are extrinsic and intrinsic cues integrated in single cells to control oligodendrocyte lineage progression? Are there distinct subpopulations of OPCs? If so, how are they specified, and how much does such heterogeneity contribute to myelination during development and following oligodendrocyte injury? What is the myelinating potential of a single cell and how plastic is that potential? What regulates the production of the correct number of OPCs that ensures sufficient myelinating cells and sufficient myelin for efficient speed and fidelity of axonal conduction?

While zebrafish have helped answer numerous questions also being tested in rodents, many questions still remain, and some of these may be more easily addressed in the tractable nervous system of the fish. For example, *in vitro* studies certainly support the notion that the initiation of myelination (contact with axons and nascent membrane extension) can occur in the absence of axonal signals, even on inert plastic fibers (Lee et al., 2013). However, the signals driving elaboration of these early myelin wrappings into bona fide compact myelin, or the maintenance of healthy myelin in adult animals, are very poorly understood. Despite years of research, a major unanswered question remains what are the signals that define the g ratio of myelin, i.e., the constant ratio of myelin thickness to axon thickness? How does the oligodendrocyte know when to stop adding new membrane? Additionally, given that a recent study shows that even single axons can have myelinated and unmyelinated segments

(Tomassy et al., 2014), what are the signaling mechanisms that determine whether a particular axon (or axon segment) will be myelinated? The ability to image cellular behavior *in vivo*, both at the level of single cells and in populations of cells, may provide important insight into these questions.

Similarly, another significant area of investigation is how critical axonal activity (i.e., electrical signaling) is for determining which axons are selected for myelination? With the zebrafish, it should be possible to monitor in real time the entire period of process extension and node generation, but it will be necessary to precisely and reproducibly control the electrical activity of individual axons in a gradable manner. The advent of optogenetics and more precise temporal- and lineage-specific gene expression control should make experiments such as these realistic. Critically, we will also need to know how the plethora of extrinsic signaling cascades are integrated in an individual cell to drive myelination. Massive cellular remodeling is required for transitioning from a migratory OPC into an extensively multi processed cell capable of generating dozens of internodes simultaneously. This coordination occurs both at the level of kinase activity networks and at the level of transcription to activate gene networks. Work from our laboratory and others supports the concept that cross talk between signaling pathways ensures that multiple pathways are available to activate myelination programs (Chew et al., 2010; Dai et al., 2014; He and Lu, 2013; Wu et al., 2012). Will the fish allow us to begin testing this type of cross talk *in vivo* by combining pharmacology with genetic manipulation in real time?

Given the complexity of neural development and the serious neurologic consequences of demyelinating and dysmyelinating disorders, having a variety of model systems will certainly provide greater insight into vertebrate myelination and repair processes. The valuable genetics and imaging capabilities of the zebrafish model system are already enhancing the discovery and validation of novel proteins, lipids or signaling pathways driving myelination *in vivo*. The zebrafish model could also aid in the development of a cost-efficient and rapid platform for validation of candidate pro-myelinating molecules identified in high throughput cell culture systems before moving onto the more labor intensive and expensive rodent models of *in vivo* myelination. This will speed up the validation of desperately needed therapies for promoting remyelination following demyelinating or dysmyelinating events. While zebrafish studies are becoming a natural extension of rodent studies, novel approaches in the fish are likely to be developed, which will expand this model beyond its niche as an outstanding vertebrate developmental system, and also provide insight into *in vivo* aspects of myelination and remyelination in juvenile and adult vertebrates.

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FIGURE 1.

Transgenic zebrafish lines can be used to study specific stages of oligodendrogenesis in developing embryos. (A) Time line for the specification, migration and maturation of oligodendrocyte lineage cells. (B) Lateral view of developing spinal cord in ~3 day post fertilization (dpf) *tg(olig2:EGFP) × tg(sox10:mRFP)* transgenic embryos. Co-expression of EGFP and RFP is used to identify dorsally migrated OPCs (arrowheads). Ventral OPCs are also present, dispersed among olig2+ progenitors and motoneurons in the primary motoneuron (pMN) domain. (C) Lateral view of developing spinal cord in ~6 dpf *tg(mpz:mEGFP) × tg(sox10:T-RFP)* transgenic embryos. Nascent myelinating internodes are visualized by expression of a membrane tethered EGFP, which localizes to the growing membrane of oligodendrocytes in both dorsal and ventral columns of the spinal cord. Nuclear localized RFP identifies the nuclei of OPCs and oligodendrocytes, as well as some interneurons.

FIGURE 2.

Transient expression of reporter constructs reveals detailed single cell morphology of oligodendrocyte lineage cells *in vivo*. Mosaic expression of a $mpz^{12kb};EGFP.PA.Tol2$ DNA construct in $tg(sox10:mRFP)$ larvae highlight the complex morphology of a single myelinating oligodendrocyte (A; green and B; white) in the dorsal spinal cord of 4 dpf live zebrafish. Similarly, a $mpz^{12kb};Kaede.PA.Tol2$ DNA construct labels several oligodendrocyte lineage cells (C; green) in the spinal cord of a 4dpf $tg(mbp:Gal4;UAS:mCherry.CAAX)$ larva (C; red).

TABLE 1

Multiple Transgenic Lines have been Generated to Study Oligodendrogenesis in the Fish

Transgenic line	Expression	Comments	References
<i>olig2:EGFP</i>	pMN derived progenitors, motor neurons, OPCs, oligodendrocytes, some neurons in brain and retina	Cytosolic EGFP, dorsal migration of OPCs, cell body counts	Shin, et al., 2003
<i>olig2:dsRED</i>	Similar to <i>olig2:EGFP</i> , some interneurons	Cytosolic dsRED, similar to <i>olig2:EGFP</i>	Kucenas, et al., 2008
<i>olig1:EGFP</i>	Subset of OPC and oligodendrocytes, reported in hindbrain and spinal cord	Cytosolic EGFP, subset of <i>olig2</i> + OPCs, late OPCs/premyelinating OLs	Schebesta and Serluca, 2009
<i>sox10:mRFP</i>	OPCs, oligodendrocytes, Schwann cells, neural crest cells	Membrane tethered RFP, OPC processes and nascent myelin internodes	Carney, et al., 2006
<i>sox10:T-RFP</i>	Similar to <i>sox10:mRFP</i> , some interneurons	Nuclear localized RFP, counts of OPCs & Schwann cells	Hines and Appel, unpublished
<i>nkx2.2:mEGFP</i>	Subset OPCs, Myelinating oligodendrocytes, ventral spinal cord derivatives, gut and intestinal neurons	Membrane tethered RFP, OPC processes and nascent myelin internodes	Ng, et al., 2005
<i>plp:EGFP</i>	Oligodendrocytes, possibly in early neural progenitors	Cell body and most processes of maturing OPCs and oligodendrocytes	Yoshida and Macklin, 2005
<i>mpz:mEGFP</i>	Early hindbrain and spinal cord progenitors (24 hfp), OPCs and oligodendrocytes, Schwann cells	Processes and nascent internodes of OPCs and myelinating oligodendrocytes	Preston and Macklin, in preparation
<i>mbp:EGFP</i>	Myelinating oligodendrocytes and Schwann cells	Myelinating oligodendrocytes and subset of Schwann cells, embryonic and adult fish	Jung, et al., 2010
<i>mbp:Gal4;UAS: NTRmCherry</i>	Similar to <i>mbp:EGFP</i> , Gal4/UAS driven expression of mCherry fused Nitroreductase in myelinating oligodendrocytes and Schwann cells	Genetic ablation of NTR-mCherry positive myelinating glia in fish exposed to Metronidazole (MET)	Jung, et al., 2010
<i>claudinK:Gal4; UAS:mEGFP</i>	Gal4/UAS driven expression of mEGFP in Oligodendrocytes, adult retinal and optic nerve	Membrane EGFP in processes and internodes of myelinating oligodendrocytes, embryos and adults	Munzel, et al., 2012

TABLE 2**Live Imaging Experiments in Zebrafish Reveal Oligodendrocyte Lineage Behavior *In Vivo***

Study	General summary	Key findings
Kirby et al. 2006	First view of OPC migration in an intact nervous system	• Cells with complex morphology rapidly extend and retract exploratory processes during migration in the spinal cord; Multi-processed cells are still capable of dividing; OPCs can jump on and off axonal tracts during migration; Laser ablation of individual OPCs induces neighboring progenitors to divide.
Kucenas et al. 2008; Takada et al. 2010	Transcription factors Nkx2.2a and Sox10 regulate progenitor specification and maturation	• Nkx2.2a expression identifies distinct myelinating vs. non myelinating populations of OPCs; Sox10 is required for survival of myelinating oligodendrocytes; Loss of myelinating cells induces proliferation of non-myelinating OPC.
Lee et al. 2010	Mutation of known tumor suppressor NF1 in zebrafish embryos recapitulates many aspects of human disease	• NF1 regulates proliferation and differentiation of myelinating glia; OPC migration is interspersed with periods of pausing; Migration distance can be controlled by time spent pausing; NF1 negatively controls Ras signaling to limit OPC proliferation and migration.
Almeida et al. 2011	Artificially changed the number and size of axons during myelination	• Myelinating oligodendrocytes are plastic: producing more myelin if needed; Single oligodendrocytes can appropriately myelinate different caliber axons simultaneously.
Czopka et al. 2013	Used mosaic analysis to follow process extension and myelination of individual oligodendrocytes	• Oligodendrocytes generate their full complement of internodes during a 5 h window; After this critical window, existing nodes can be retracted, but new nodes are never formed; Fyn Kinase signaling modulates the rate but not the number of nodes formed by a cell.
Lui et al. 2013	Used mosaic analysis and time lapse imaging to follow single OPC and oligodendrocytes over multiple days	• ~28% of nascent internodes are retracted during the developmental window studied; Internode retraction is more likely to occur on small caliber axons than large caliber axons; ErbB/Neuregulin signaling regulates this so-called "myelin pruning".