
By

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ABSTRACT

SPINAL CORD REGENERATION IN THE LEOPARD GECKO

(*Eublepharis macularius*): INVESTIGATING THE STEMNESS, ACTIVATION AND
HETEROGENIETY OF EPENDYMAL LAYER CELLS

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The leopard gecko (*Eublepharis macularius*) is an emerging model for the study of spinal
cord regeneration. As for many lizards, geckos are able to self-detach their tail to avoid predation
and then regenerate a replacement. The replacement tail includes a regenerated spinal cord with a
relatively simple morphology: a monolayer of ependymal layer cells (ELCs) surrounded by
nerve tracts. We hypothesized that ELCs of the original spinal cord include populations of neural
stem/progenitor cells (NSPCs) that contribute to the fully regenerated spinal cord.

To identify ELCs as NSPCs, we first used a bromodeoxyuridine pulse-chase experiment
to document label-retaining populations prior to injury. We found that populations of ELCs were
label-retaining, even after a 140-day chase period. Next, we conducted a detailed spatiotemporal
characterization of ELCs before, during, and after tail regeneration. We determined that ELCs
are activated in response to injury, as evidenced by changes in proliferation and protein
expression. Prior to injury, ELCs express the hallmark NSPC marker SOX2. Following tail loss,
ELCs in the original stump of the tail become highly proliferative and express an expanded panel
of NSPC and lineage-restricted progenitor cell markers, including Musashi-1, SOX9, β-III
tubulin and HuC/D. This panel of markers continues to be expressed by ELCs of the fully
regenerated tail, and indicates that the replacement spinal cord includes at least two distinct populations. Most ELCs are SOX2+, GFAP+, HuC/D-. We interpret these cells as ependymo-radial glia (ERG). A second subset of ELCs are SOX2+, GFAP-, HuC/D+, which we identify as a neuronal-like population of cerebrospinal fluid-contacting (CSF-c) cells.

Although we were unable to successfully track labeled ELCs into the regenerating tail following in vivo electroporation, our data provides compelling evidence that this cell population is crucial for restoring a functional spinal cord. Our in vitro experiments indicate that culturing methods common to mammalian neural tissues are not necessarily well-suited to reptiles. These studies serve as a foundation for future experimental work that promises to advance our understanding of the biology of spinal cord repair and regeneration, with obvious implications for biomedicine and translational science.
ACKNOWLEDGEMENTS

One of the most formative pieces of advice I have ever been given was simple—no matter what, no matter when, always surround yourself with incredible people. The truth is, I have never been more overwhelmed by the depth of my support system than I am right now. I have been, and continue to be, surrounded by some of the most incredible people imaginable. With that, I will attempt to put into words what you all mean to me, but please know that without my usual editor (MKV), this may be a bit lengthier, a bit less polished, and a bit more emotional than my usual literary style.

I’d like to start off by acknowledging my advisor, Dr. Matt Vickaryous. Matt, I’ve been agonizing over exactly how to thank you for the energy and enthusiasm that you have brought to the Eublephoshere every single day during the last 5 years. You have been the single greatest influence in my academic career. You have challenged me and simultaneously have been the ultimate cheerleader. You have shaped the way I think and influenced how I’ll conduct science for the rest of my career. You have taught me the finer points of scientific communication and also the finer points of whiskey consumption. Thank you for trusting me to mentor new members of the lab, chase my “hot in the street” science dreams and for not stifling my many “Gilbert-isms”. I am forever grateful and know that I will always look back on these five years as some of the best times of my life, but, I also know that this is only the beginning of a lifetime of collaborations and conference banquets for us.

To my advisory committee. Jim, thank you for your enthusiasm for all things gecko-related, for your sound advice and of course for the creation of sweet gecko juice during the early days of our in vitro studies. Jon, thank for your insightful words and encouragement especially in the last few months of thesis writing. Andrew, thank you for your knowledge and patience as we worked through optimizing electroporation protocols in a challenging model system.

To my exam committee. Thank you to Dr. Susan Bryant for agreeing to participate in my defence, and most significantly for being one of the pioneers of the lizard model as a means to unravel the biology of regeneration. Thank you as well to Dr. John Vessey for participating in my defence, but also for your advice as I navigate between the end of my PhD and the beginning of my post-Doctoral training.

I would also like to acknowledge the substantial technical help I have received throughout the last five years. To Helen Coates, Monica Antenos, Allison McKay, Kata Osz and Jodi Morrison for their knowledge, patience and time. Additionally, to Kim Best, Frances Graziotto and Sally Cherry for their friendship and also their commitment to the department of Biomedical Science.

To the previous members of the Vickaryous Lab. To Katie, there is no one else who could understand my paired love of Regeneration and Frye boots quite like you. To Steph, for teaching me the importance of beautiful histology and for your endless enthusiasm for both evolution and the Backstreet Boys. To Sam, for our unforgettable conference escapades including but not limited to the beachfront suite in Vancouver. To Jason, for our road-trip in France with Estephania, to your antics in the lab. I’ll be telling stories of our pranks for years to
come. To Hanna, my little sister and most reliable computer technician, you were my sounding board during so many experiments and I’m so grateful to have been a part of the beginning of your scientific career. I would also like to acknowledge the current Vickaryous lab members including Sarah Donato and Stefanie Bradley, thank you for your help in the preparations during the last few months of this degree.

To my cubs: Kathy Jacyniak (K1000), Noeline Subramaniam (Moe) and Rebecca McDonald (Rebecko), you have made the last two years of my doctorate more memorable than I could have ever imagined. Your passion for science and for the Eublephosphere is unparalleled. Mentoring and teaching the three of you has been one of the biggest privileges of this degree, and I look forward to (many) more conferences, camping trips and whiskey nights together. I love you all.

To Jalene: Thank you for welcoming me into your home and for being the most incredible friend, mentor and support system for me throughout this degree. To Isla and Piper: I love you both and am incredibly lucky to have been able to a part of your lives from the beginning. I know you won’t read this now, but when you’re thirteen and come to visit for the weekend to escape the late night curfew your Dad will no doubt insist on- I’ll make you read this, and remind you how much you both mean to me. You are my family, and I’ll have your back no matter what.

To my extended family at both Wolf Run Farm, Faraway Farm and Olde McDonald Farm, you have all been such an incredible support system for me. My nights at the farm have kept me grounded and motivated, especially in the last stages of this degree. Pat, your generosity, care for Phatima and constant friendship mean more to me than you know. Karl, thank you for holding me to such a high standard, supporting my stubborn streak and making fun of me when I needed it most. To Mary, Shandiss and Trish for making my face hurt from laughing so hard, for being the sisters I never had and for always being up for an adventure. You are simply the best!

Last, but certainly not least, to my family: Mom, Dad and Richard. Thank you from the bottom of my heart. To my favourite TGFβ-enthusiast, Richard, you are the ultimate partner in crime. I cannot imagine a sibling who could challenge me or understand me more than you. From our early graduate school white board chats, to our GATA3 project, to our diverging doctorate plans, you’ve always been a loving critic and a fierce supporter and know that this is just the beginning. Mom, you are my best friend. You have supported my love of science, and my love of learning for as long as I can remember. You have kept me grounded, made me laugh (even when experiments weren’t going quite as I planned), you have always been a listening ear and a positive force in my life. Your impact is immeasurable. Dad, it is impossible for me to put into words my admiration and love for you. You have inspired me to strive for a profession that is also my passion and have always taught me to go the extra mile, in whatever I do. From a young age, you and Mom allowed me to be just stubborn enough to never give-up. Thank you both for your boundless love and confidence in me. You have helped make so many of my dreams become a reality, and I am forever grateful.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CSF-c</td>
<td>cerebrospinal fluid-contacting cell</td>
</tr>
<tr>
<td>CSPGs</td>
<td>chondroitin sulphate proteoglycans</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DH₂0</td>
<td>deionized water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>electrogenerated chemiluminescence</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELC</td>
<td>ependymal layer cell</td>
</tr>
<tr>
<td>ERG</td>
<td>ependymo-radial glia</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase-conjugated streptavidin</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MS222</td>
<td>ethyl 3-aminobenzoate methanesulfonic acid</td>
</tr>
<tr>
<td>MSI1</td>
<td>musashi-1</td>
</tr>
<tr>
<td>MSX1+2</td>
<td>Msh (muscle segment homeodomain protein) homeobox 1-2</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NBF</td>
<td>neutral buffered formalin</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclei</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NSPC</td>
<td>neural stem progenitor cell</td>
</tr>
<tr>
<td>PAX7</td>
<td>paired box protein Pax-7</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PD</td>
<td>proximal-distal</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>SHH</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SOX2</td>
<td>SRY (sex determining region Y)-box2</td>
</tr>
<tr>
<td>SOX9</td>
<td>SRY (sex determining region Y)-box9</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>tris buffered saline with Tween20</td>
</tr>
<tr>
<td>TUJ1</td>
<td>βIII-tubulin</td>
</tr>
<tr>
<td>VIM</td>
<td>vimentin</td>
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</tbody>
</table>
DECLARATION OF WORK PERFORMED

I declare that I performed all of the work reported in this thesis with the exception of the following items: Dr. Matt Vickaryous and Hanna Peacock assisted with the Bromodeoxyuridine injections and anesthesia trial; Dr. Matt Vickaryous, Dr. Andrew Bendall, Noeline Subramaniam, Kathy Jacyniak and Rebecca McDonald assisted with the chick and gecko electroporations. Rebecca McDonald, Noeline Subramaniam, Kathy Jacyniak and Sarah Donato performed some of the immunofluorescence for Vimentin, Bromodeoxyuridine (BrdU) and Glial Fibrillary Acidic Protein (GFAP).
CHAPTER 1: A REVIEW OF SPINAL CORD INJURY AND REGENERATION ACROSS VERTEBRATES

1.1 The Spinal Cord

The spinal cord is a fundamental component of the central nervous system (CNS), serving as the primary conduit of neural tissue linking the brain with the peripheral nervous system (Nieuwenhuys, 1964; Antos and Tanaka, 2010, Nolte, 2010; Fitzgerald et al., 2012). During development, the spinal cord is first recognizable as the caudal end of the neural tube. Within the neural tube, embryonic neuroepithelial cells line the central canal. This neuroepithelial or ventricular layer includes most of the CNS precursor cells (Schoenwolf et al., 2009). As development continues, ventricular layer cells proliferate, differentiate and migrate to become neurons, support cells (oligodendrocytes, astrocytes and microglia) or a specialized epithelial cell type (Schoenwolf et al., 2009). This epithelial population, herein referred to as ependymal layer cells (ELCs), exclusively lines the central canal or lumen of the spinal cord. This tubular arrangement of ELCs occupies a near central location within the spinal cord and is surrounded in turn by neuronal-rich grey matter and axon-rich white matter (Nolte, 2010; Fitzgerald et al., 2012). Owing to the presence of cilia, ELCs were initially recognized as participating in the circulation of cerebrospinal fluid. However, more recent studies have identified a second, potentially more dramatic role – as the primary source of neural stem/progenitor cells (NSPCs) in the adult nervous system (Bruni, 1998; Mothe and Tator, 2005; Meletis et al., 2008; Barnabe-Heider, 2010; Alfaro-Cervello et al., 2012).
1.2 Spinal Cord Injury (SCI) and Repair

Traumatic injuries to the spinal cord result in damage to both sensory and motor functioning, leading to significant physical impairments, compromised quality of life and, in severe cases, death (McDonald, 1999; Nolte, 2010). Within North America more than 14,000 new cases of spinal cord injuries (SCIs) are reported each year and between 285,000 and 315,000 North Americans are currently living with some form of SCI (McDonald and Sadowsky, 2002; Farr and Baxter, 2010). Ongoing efforts to improve recovery following SCI include technological advancements, such as engineered exoskeletons, efforts to promote axonal regrowth through the delivery of neurotrophic factors or reduction of inhibitory signals, as well as the transplantation of exogenously amplified NSPCs (Hurlbert, 2000; Hornby et al., 2005; Maier and Schwab, 2006; Alper, 2009; Bretzner et al., 2011; Zhang et al., 2016). Unfortunately, to date only modest structural and functional improvements have been achieved, underscoring the need for alternative strategies and a better biological understanding of the events that promote or inhibit recovery.

1.2.1 Activation of Endogenous NSPCs in the Mammalian Spinal Cord

One of the emerging strategies for addressing SCI involves the activation and recruitment of endogenous NSPCs (Barnabe-Heider and Frisen, 2008). Since their initial identification in the early 1990’s (Reynolds and Weiss, 1992), numerous studies have sought to explore how resident or endogenous populations of NSPCs within the adult CNS might be induced to promote functional recovery following injury (Mothe and Tator, 2005; Barnabe-Heider and Frisen, 2008; Meletis et al., 2008; Barnabe-Heider et al., 2010). Despite the obvious potential, numerous challenges remain. For example, efforts to modulate endogenous NPSCs have been blunted by
the inhibitory or non-permissive wound microenvironment of the mammalian CNS (reviewed in Tanaka and Ferretti, 2009). Subsequent attempts to ameliorate the non-permissive state of the CNS with the use of growth factors, hormones or pharmacological agents, often resulted in significant and complex side effects (reviewed in Miller and Kaplan, 2012). Adding to the confusion, in many cases the normal response of endogenous stem/progenitor cells to injury, whether it be differentiating to create new cells or modulating the environment or both, remains poorly understood (Miller and Kaplan, 2012). As a result, mechanistic insight into the exact role of NSPCs during repair and regeneration remains a major obstacle.

1.3 Spinal Cord Response Following Injury

1.3.1 Primary response

In mammals, traumatic SCIs induce both primary mechanical damage, as well as a cascade of secondary events that often worsen the injury (Figure 1; McDonald and Sadowsky, 2002). The primary injury mainly involves physical damage to the spinal cord and adnexa, including the adjacent vertebra(e), as well as disruption of the blood-spinal cord barrier (Anderson and Hall, 1993; McDonald and Sadowsky, 2002; Rowland et al., 2008). Direct damage to the spinal cord is often the consequence of fragmentation of surrounding ligaments and bone (McDonald and Sadowsky, 2002). Rupture of associated blood vessels and peripheral nerves further exacerbates the effect and extent of the injury. As blood vessels within the grey matter hemorrhage, the spinal cord begins to swell producing localized neuronal disruption and neurogenic shock (Tator and Koyanagi, 1997; Mautes et al., 2000). Neurogenic shock can occur within minutes of an SCI, thus initiating a secondary injury cascade (Figure 1; Atkinson and Atkinson, 1996; McDonald and Sadowsky, 2002).
1.3.2 Secondary response

The secondary response to SCI includes continued vascular damage, inflammation, excitotoxicity, necrosis, apoptosis, and the activation of astrocytes (Figure 1; Frisen et al., 1995; Liu et al., 1997; Schnell et al., 1999; Mautes et al., 2000; Park et al., 2004; Rowland et al., 2008). Vascular damage that begins in the grey matter spreads into the white matter, causing hypoperfusion of the entire cord at the level of the injury (Tator and Koyanagi, 1997). Depending on the severity of the injury, hypo-perfusion can reduce or even completely inhibit action potential propagation. Simultaneously, inflammation results in the localized recruitment of activated microglia and macrophages (Blight, 1992; Carlson et al., 1998; Mautes et al., 2000). Although the exact role of inflammatory cells following SCI remains controversial (Donnelly and Popovich, 2008), they are associated with increased neuronal death and demyelination (Blight, 1985; Hirschberg et al., 1994). Paradoxically, there is also evidence indicating that inflammation also promotes axon regeneration by inducing the production of pro-regenerative cytokines (e.g., transforming growth factor β; Hirschberg et al., 1994; Streit et al., 1996). In addition, the experimental transplantation of microglia (macrophages of the CNS) following SCI has been shown to promote neurite outgrowth (Rabchevsky and Streit, 1997). Ultimately however, damage to cells and blood vessels within the cord results in the release of neurotoxic chemicals. In particular, the uncontrolled release of neurotransmitter glutamate from injured neurons, axons and astrocytes results in excitotoxicity (Pitt et al., 2000). This drives the over-excitation of surrounding neurons, which initiates an influx of calcium ions, generating the production of free radials capable of killing otherwise healthy neurons (Park et al., 2004). Excitotoxicity not only impacts neurons, but also oligodendrocytes that myelinate axons within the CNS. While the first wave of cell death post-SCI is necrotic, a second, more widespread, wave of apoptotic cell death
follows (Beattie et al., 2000; Beattie et al., 2002). In the days and weeks post-SCI, oligodendrocytes continue to undergo apoptosis, even in spinal cord segments distant to the original site of injury (Beattie et al., 2000). The secondary injury culminates in the formation of a glial scar (Fawcett and Asher, 1999; Silver and Miller, 2004). In response to a SCI, resident astrocytes become ‘reactive’, altering their morphology and molecular expression (Eddleston and Mucke, 1993; Ridet et al., 1997; Sofroniew, 2005). While the glial scar does serve to limit the spread of injury to additional areas of the parenchyma (and hence appears to be neuroprotective), it also contains chondroitin sulphate proteoglycans (CSPGs), which inhibit axonal growth (Yiu and He, 2006; Bartus et al., 2012).

1.4 Barriers to Spinal Cord Regeneration

1.4.1 The Glial Scar

Within the context of the secondary response, astrocyte activation and subsequent glial scarring appears to be the most important factor influencing the success of functional recovery (Yiu and He, 2006). The glial scarring process is initiated by blood-borne macrophages (Popovich et al., 1998; Fawcett and Asher, 1999), which in turn induces the activation of resident astrocytes. Once activated, astrocytes become hypertrophic and proliferative, and are characterized by up-regulation of the intermediate filament proteins glial fibrillary acidic protein (GFAP) and Vimentin (Eddleston and Mucke, 1993; Ridet et al., 1997). Astrocyte recruitment to the site of injury serves both pro-and anti-regenerative roles (Rudge and Silver, 1990; Faulkner et al., 2004). Pro-regenerative roles of astrocytes and the glial scar include the release of neurotrophic factors, the uptake of excess glutamate and the removal of free radicals (Rothstein et al., 1996; Sablestrom et al., 2013). In addition, the core of the glial scar provides neurotrophic
support to remaining axons near the lesion (Rothstein et al., 1996; Sablestrom et al., 2013). In contrast, the anti-regenerative effects of astrocytes and the glial scar include the release of inhibitory extracellular matrix (ECM) components that limit functional recovery through the establishment of a physical barrier (McKeon et al., 1999; Jones et al., 2003; Silver and Miller, 2004).

Although the role of the glial scar is primarily the result of reactive astrocytes, it also receives contributions from pericytes and ELCs (Meletis et al., 2008; Barnabe-Heider et al., 2010; Goritz et al., 2011; Sablestrom et al., 2013). Pericytes and reactive astrocytes secrete CSPGs (McKeon et al., 1991; Goritz et al., 2011), macromolecules consisting of a protein core with a series of chondroitin sulphate glycosaminoglycan side chains (Morgenstern et al., 2002). Chondroitin sulphate interacts with other proteins in the ECM to regulate a range of cellular events. For example, during development CSPGs are fundamental in path finding (Faissner and Steindler, 1995), while in adults they play a role in maintaining stability and limit plasticity (Hockfield et al., 1990; Corvetti and Rossi, 2005). CSPGs are also potent inhibitors of neurite growth (Snow et al., 1990), and some (e.g., neurocan and phosphacan; McKeon et al., 1999; Bolventa and Fernaud-Espinosa, 2000) are highly up-regulated by reactive astrocytes following CNS injury (Levine, 1994; Jones et al., 2003; laci et al., 2007). Several studies have shown that the glycosaminoglycan side chains are largely responsible for the inhibitory effects of CSPGs; when degraded, through the application of chondroitinase ABC, the inhibitory effects are minimized (Moon et al., 2001; Bradbury et al., 2002; Kowano et al., 2012). CSPGs are thought to exert their inhibitory effects by physically blocking neurons from accessing neurite-promoting components of the ECM (Bovolenta and Fernaud-Espinosa, 2000).

Interestingly, the glial scar includes two distinct populations of astrocytic-like cells:
Sox9+/Vimentin+ cells, which are derived from the proliferation and migration of ELCs and GFAP+/nestin+ cells, which represent a reactive astrocyte population (Meletis et al., 2008). Evidence suggests that these two cell types exist in non-overlapping domains and that progeny from ELCs do not contribute to the inhibitory environment (Meletis et al., 2008). Instead, ELC-derived astrocytic cells (which dominate the core of the scar) play neuroprotective roles, both limiting the expansion of the lesion and providing neurotrophic support to surviving neurons adjacent to the lesion (Meletis et al., 2008; Sablestrom et al., 2013). Modulating the balance of glial scar roles to favour ELC-mediated neuroprotection over reactive astrocytic-mediated inhibition remains a primary goal of ongoing efforts to restore functional recovery in mammals (Ferretti et al., 2003; Antos and Tanaka, 2010; Sablestrom et al., 2013).

1.4.2 Oligodendrocyte Response

A second mechanism that limits CNS regeneration in mammals involves oligodendrocytes. The aptly named oligodendrocyte response was first shown to limit regeneration of CNS axons following an optic nerve crush injury (David and Aguyao, 1981; Schwab, 2004). These studies demonstrated that CNS axons can successfully regenerate in the presence of a peripheral nerve graft, thus emphasizing a critical role for the microenvironment in determining regenerative success (David and Aguyao, 1981). Since this time, several oligodendrocyte-based myelin inhibitory factors have been shown to limit axon regrowth in the CNS. Examples include neurite outgrowth inhibitor (NOGO), myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (Mukhopadhyay et al., 1994; Wang et al., 2002). To date, the contribution and specific role of each inhibitory factor remains poorly understood. The use of a specific NOGO blocking antibody (IN-1) has been shown to reduce the
inhibitory effects of NOGO and promote axonal regeneration leading, in some cases, to a partial functional recovery (Schwab, 1990; Schwab and Bartholdi, 1993; Fawcett and Asher, 1999). MAG is known to have opposing roles, either promoting or inhibiting outgrowth (Fawcett and Asher, 1999). MAG knockout mice show marginally increased axonal regeneration potential, but MAG’s effect alone is not believed to be sufficient to completely block regeneration (Schafer et al., 1996; Fitch and Silver, 1997). OMgp has been shown to play a role in restricting collateral sprouting (Kim et al., 2003; Simonen et al., 2003; Huang et al., 2005).

In addition to myelin inhibitory factors, oligodendrocytes also express repulsive guidance cues such as semaphorin 4D and ephrin B3, which also limit axonal repair (Schwab, 2004; Duffy et al., 2012). Semaphorin 4D is expressed in adults by oligodendrocytes and triggers growth cone collapse (Moreau-Fauvaque et al., 2003), while ephrin B3 is a midline repellent during development of the cortico-spinal tract, one of the key spinal tracts involved in movement (Kullander et al., 2001; Benson et al., 2005). Continued expression of ephrin B3 in the adult spinal cord exerts an inhibitory effect on spinal cord regeneration (Kullander et al., 2001; Benson et al., 2005). Controlling oligodendrocyte-based inhibition following SCI remains challenging (Yiu and He, 2006), although one promising approach seeks to target the RhoA/ROCK signaling pathway (Dergham et al., 2002). RhoA/ROCK signaling activates both myelin-based and CSPG-based inhibition (Monnier et al., 2003; Forgione and Fehlings, 2014). A better understanding of these signaling cascades following SCI may lead to therapies that promote a more regeneration-permissive environment.

1.5 Regeneration

The reparative response following injury varies greatly across vertebrate species. Within
mammals, injuries most commonly resolve through the formation of scar tissue, a structurally imperfect form of wound healing (Ferguson and O’Kane, 2004; Gurtner et al., 2008). Following injury, a conserved series of overlapping events is initiated (hemostasis, inflammation, re-epithelialization and fibrosis) that ultimately serves to re-establish homeostasis. However, as this reparative response fails to replicate the original tissue architecture and cellular population, the result is a structurally and functionality imperfect restoration (Gurtner et al., 2008; Occleston et al., 2010). In stark contrast to scar-formation, many non-amniotes (e.g., teleosts and urodeles) are able to heal injuries in a scar-free manner (Carlson, 2007; Levesque et al., 2010; Seifert et al., 2012a; Godwin and Rosenthal, 2014). Scar-free wound healing, and the absence of fibrotic tissue, appears to be a prerequisite for successful regeneration (Ferguson and O’Kane, 2004; Occleston et al., 2010). Although tissue regeneration by adult mammals is often viewed as limited, various examples exist. One of the best understood is digit tip regeneration. For some species of rodents and primates (including humans), a portion of the distal-most (terminal) phalanx can be spontaneously replaced following amputation (Neufeld and Zhao, 1995; Han et al., 2008; Muneoka et al., 2008). In addition, species of African spiny mouse (Acomys kempi and A. percivali) can near perfectly (and thus scar-free) heal full-thickness traumatic wounds to the skin, complete with hair follicles and glands (Seifert et al., 2012b).

1.5.1 Epimorphic Regeneration

For most species capable of regeneration, the restoration of replacement tissue is preceded by the formation of a blastema, a population of proliferating mesenchymal-like cells that aggregates at the site of injury. Blastema-mediated regeneration, also called epimorphic regeneration, is common to most regeneration-competent species including zebrafish, urodeles
(salamanders and newts), pre-metamorphic anurans (i.e., tadpoles), and lizards (e.g. Dent, 1962; Anderson and Waxman, 1985; Bellairs and Bryant, 1985; Brockes, 1997; Endo et al., 2004; McLean and Vickaryous, 2011). Despite the near-uniform homogeneous appearance of blastema cells, recent studies have demonstrated that the blastema organ derives from multiple tissues, and that individual cells retain a memory of their tissue origin. Emerging data from *Xenopus* tadpoles (Lin et al., 2007; Gaete et al., 2013), axolotls (Kragl et al., 2009), and zebrafish (Poss et al., 2003) now demonstrate that blastema cells are not pluripotent, but rather represent a heterogeneous population of lineage-restricted cells (Lin et al., 2007; Kragl et al., 2009).

Another key feature of blastema-mediated regeneration is the formation of a wound epithelium. In order to restore barrier functions to the skin, the epidermis boundary must be replaced across the wound site. This new boundary or wound epithelium is a stratified squamous epithelium that typically thickens (becomes hyperplastic) as the blastema is formed, and the gradually decreases in thickness as the wound is resolved (Delorme et al., 2012; Gilbert et al., 2015). Removal of wound epithelium inhibits blastema outgrowth, suggesting a dynamic and reciprocal relationship between the two structures (Whimster, 1978) although the exact nature of these interactions remains unclear.

It is also widely accepted that blastema-mediated regeneration is reliant on a sufficient nerve supply, specifically, paired appendage regeneration requires peripheral nerve innervation (Satoh et al., 2007) while tail regeneration relies on the spinal cord (Simpson, 1970). In addition, the spinal cord has been shown as a critical initiator, patterning centre and neurotrophic source for the successful regeneration of surrounding tissue (Simpson, 1964; Egar and Singer, 1972; Whimster, 1978) This is consistent across all regeneration-competent taxa including: teleosts (Goss, 1954); urodeles (e.g. Goldfarb, 1909; Holtzer, 1959) and lizards (Kamrin and Singer,

1.6 Models of Spinal Cord Regeneration

The ability to regenerate the spinal cord varies greatly across species (Tanaka and Ferretti, 2009). While all vertebrates appear to be capable of some degree of spinal cord repair, especially during early embryonic and fetal development, in mammals and birds this competence is sharply diminished postnatally (Altman, 1962; Huslebosch and Bittner, 1980). Similarly, anurans (frogs) are capable of spinal cord regeneration as tadpoles, but lose this ability following metamorphosis (Gaete et al., 2012). In contrast, teleosts (bony fish), urodeles (newts, axolotls) and some lizards (tail only) are able to spontaneously regenerate the spinal cord, and thus represent powerful and promising models to unravel the basic biological mechanisms that underlie the reparative process.

1.6.1 Teleosts and Urodeles

Two of the most common models for the study of spinal cord regeneration are teleost (bony) fish and urodeles. Members of both groups can repair two major forms of SCI: (1) acute transectional ruptures to the body spinal cord, wherein the detached segments remain in place (e.g. Clemente, 1964; Chernoff, 2003; Dervan and Roberts, 2003); and (2) complete amputational injuries, including rupture and removal of the spinal cord and surrounding tail tissues (Anderson and Waxman, 1985). Following body spinal cord transection (but not amputation), the reparative process begins by sealing off both ruptured ends of the spinal cord with a clot of blood (sometimes referred to as a “scar” (Dervan and Roberts, 2003; Sirbulescu and Zupanc, 2011)) and then liberating ELCs (Butler and Ward, 1967; Sirbulescu and Zupanc,
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2011). Adjacent to both ends of the ruptured cord, the central canal initially closes off (Dervan and Roberts, 2003). The gap between the cranial and caudal segments (at the site of transactional injury) gradually becomes filled in as ELCs of the undamaged cord undergo an epithelial-mesenchymal transition (Chernoff, 2003). Within 10-14 days post-injury, proliferating ELCs begin re-establishing the central canal (Dervan and Roberts, 2003). Axonal outgrowth or axonogenesis occurs almost simultaneously and within 10 days there is evidence of axonal projections between or alongside the restored ELC population (Chernoff, 2003; Dervan and Roberts, 2003). Axonogenesis is followed by the return of support cells, namely astrocytes and oligodendrocytes (Doyle et al., 2001; Dervan and Roberts, 2003). Interestingly, while the regenerated portion of the spinal cord is structurally imperfect (in particular, it lacks well-defined grey and white matter), it is fully functional (Ward and Butler, 1967; Sirbulescu and Zupanc, 2011; Yu et al., 2011).

Whereas acute transectional wounds are healed with an interstitial-type reparative response, amputation injuries are healed via blastema-mediated (epimorphic) regeneration (Chernoff, 2003). Similar to transactional wounds, the site of amputation injury is initially sealed by a blood clot. Within hours/days, this temporary plug is discarded to reveal a newly formed epidermal boundary, the wound epithelium, deep to which lies the blastema (Waxman and Anderson, 1985; Stocum 2006). At the level of the spinal cord, the central canal initially collapses and then is capped by coagulated blood (Zhang et al., 2003). Rapidly, ELCs adjacent to the wound reassemble into a tubular form with a distal dilation, the terminal vesicle or ampulla (Waxman and Anderson, 1985; Egar and Singer, 1972; Clarke and Ferretti, 1998). This ependymal ampulla represents the leading edge of the regenerating spinal cord, and its outward growth into the blastema essentially matches regenerative outgrowth of the new tail (Poss et al.,
At least among urodeles, ELC proliferation occurs predominantly within the remaining stump of the original spinal cord, with only minor contributions from populations in the regenerated portion of the tail, including the ependymal ampulla (Egar and Singer, 1972; Norlander and Singer, 1978; Anderson et al., 1986; Zhang et al., 2000). In addition to ELCs, cell proliferation has also been observed in other cell types of the spinal cord (Dervan and Roberts, 2003; Reimer et al., 2008; Allen and Smith, 2012), suggesting that the source material for the replacement CNS is unexpectedly diverse (Benraiss et al., 1999; Chaar and Tsilfidis, 2006). The completion of regeneration is marked by the elongation of pre-existing axons into the regenerating spinal cord, matched by the generation of new neurons and glial cells (Chernoff et al., 2003; Tassava and Huang, 2005; Sirbulescu and Zupanc, 2011).

Interestingly, the replacement spinal cord often lacks some of the functionality of the original (Sirbulescu and Zupanc, 2011). Following spinal cord transection, ~16% of zebrafish failed to regain swimming ability after 15 weeks (Becker et al., 1997). Even when swimming behavior is recovered, individuals having sustained SCI are often unable to adapt to more challenging conditions, such as swimming against a current (Becker et al., 2004). In urodeles, the number of regenerating axons following transection rarely matches that seen prior to injury (Clarke et al., 1988), and altered swimming behaviors (characterized by more erratic body movements) have been observed (Butler and Ward, 1967; Davis et al., 1990).

### 1.6.2 Lizards

Among amniotes (vertebrates that develop an amnion), the most dramatic example of spontaneous multi-tissue regeneration comes from lizards. In order to escape predation, many species of lizard are able to voluntarily drop or autotomize a portion of their tails and then
regenerate a replacement. The replacement tail closely (but not perfectly) resembles the original, complete with skeletal support, musculature, pigmented skin and a functional spinal cord (Woodland, 1920; Bellairs and Bryant, 1985; McLean and Vickaryous, 2011; Alibardi, 2014). Curiously, one of the most recognizable differences between the original and fully regenerated tail is the morphology and cytoarchitecture of the spinal cord. Whereas the spinal cord of the original tail is identical to that of the body (meninges, white matter, grey matter organized into horns and a pseudostratified, tubular arrangement of ELCs), that of the replacement tail is markedly simpler: a monolayer layer of ELCs surrounded by several thousand regenerated, unmyelinated descending nerve fibers, and a connective tissue layer comparable with the dura matter (Kamrin and Singer, 1955; Simpson, 1968, 1970; Bellairs and Bryant, 1985; Alibardi and Meyer-Rochow, 1990). Although early descriptions indicated that no new neurons are present in the regenerate tail (Woodland, 1920; Simpson, 1970), later studies have reported the presence of GABA-ergic neuronal-like cells (Alibardi et al., 1991).

Emerging data points towards ELCs within the original lizard spinal cord as key contributors to, and regulators of, regeneration. Available data suggests that ELCs may represent a stem/progenitor cell population (Gilbert et al, 2015). For example, original spinal cord ELCs are relatively slow cycling (label retaining for up to 22 days following a bromodeoxyuridine (BrdU) pulse), and reportedly express the intermediate filament Nestin during early regeneration, and lineage-specific marker neuron-specific enolase (NSE) as differentiation begins (Alibardi, 2014). In addition, the spinal cord has been implicated as the source of a ‘trophic substance’ that drives regeneration (Kamrin and Singer, 1955; Alibardi and Lovicu, 2010) and their pial processes are known to serve as guides for descending nerve
tracts (Simpson, 1968; see also Bellairs and Bryant, 1985). The crucial role of ELCs during regeneration is further demonstrated by experimental manipulations. If segments of regenerate spinal cord and regenerate tail are exogenously implanted into dorsal tail wounds only those containing ELCs are able to successful initiate the tail regeneration program (Simpson, 1964). The ependymal layer also plays a key role in organizing and patterning the regenerate tail (Whimster, 1978; Wang et al., 2011). The length of the regenerate tail is directly proportional to how much of the original tail was lost, and its fusiform shape suggests that tail outgrowth is not simply a product of the diameter of the open wound (Whimster, 1978). Transecting the original spinal cord at the pelvic region prior to tail loss showed that without a continuous spinal cord, the length of the regenerate tail was ~2cm shorter than their intact spinal cord equivalents (Whimster, 1978). Additionally, proximal-distal (PD) positional identity marker, CD59 revealed a graded expression within the original spinal cord in *Gekko japonicas*, providing further evidence that the spinal cord is involved in controlling the size and pattern of the regenerate tail (Wang et al., 2011). While these data suggest that ELCs are responsible for successful spinal cord regeneration in lizards, the extent of their regenerative capacity, and whether this capacity is restricted to the tail, remains unclear.

1.7 Cells participating in spinal cord regeneration

We define neural stem/progenitor cells (NSPCs) as a specialized population of self-renewing CNS cells capable of generating neurons and glia. We use the term ependymal layer cell (ELC) as a location designator: a cell population lining the central canal of the spinal cord. As discussed below, ELCs include pools of NSPCs but the terms are not equivalent (i.e., not all
NSPCs are ELCs, and vice versa). Among teleosts and urodeles, NSPCs of the ependymal layer of the spinal cord are called ependymo-radial glia (ERG) (Becker and Becker, 2015) – hence ERG are NSPCs of the ependymal layer.

1.7.1 Neural Stem Progenitor Cells (NSPCs)

NSPCs are self-renewing and multi-potent cells that can differentiate along neuronal or glial lineages within the CNS (Reynolds and Weiss, 1992; Johansson et al., 1999; Temple, 2001; Graham et al., 2003; Seaberg and van der Kooy, 2003; Chojnacki et al., 2009; Magnusson and Frisen, 2016). Although normally quiescent, NSPCs are capable of proliferating and differentiating in response to the needs of homeostatic replenishment or injury. NSPCs can be isolated, expanded and differentiated in vitro (Weiss et al., 1996; Benraiss et al., 1996; Shihabuddin et al., 1997), but are more commonly characterized in vivo based on a relatively conserved pattern of marker expression. Of these, the transcription factor SOX2 (sex-determining region Y-box 2) is perhaps the best known (Ferri et al., 2004; Huangfu et al., 2008). SOX2 is one of four factors capable of inducing pluripotency in somatic cells (Takahashi and Yamanka, 2006), and plays a fundamental role in maintaining the stem/progenitor state and inhibiting differentiation (Bylund et al., 2003; Graham et al., 2003). Loss of SOX2 results in cells exiting the cell cycle and subsequent neuronal differentiation (Bylund et al., 2003; Graham et al., 2003; Hutton and Pevny, 2011). As a result of its association with NSPC populations, SOX2 is widely regarded as a predictor of regenerative capacity. A powerful example comes from the anuran *Xenopus*, which can regenerate the spinal cord as tadpoles (pre-metamorphic) but not as adults (post-metamorphic). In tadpoles, spinal cord cells up-regulate SOX2 in response to injury, whereas regeneration-incompetent adults do not upregulate SOX2 (Gaete et al., 2012).
Similarly, spinal cord regeneration fails when SOX2 is knocked-down in zebrafish (Hui et al., 2014; Ogai et al., 2014) or urodeles (Fei et al., 2014).

Along with SOX2, another widely used NSPC marker is the RNA-binding protein Musashi-1 (MSI-1). MSI-1 was originally discovered in Drosophila, where it was described as playing a role in regulating asymmetric divisions of sensory organ cells (Nakamura et al., 1994). Since then, its role has been expanded to include the mediation of NSPC self-renewal and regulation of Notch signalling, thus functioning to drive cell proliferation and inhibit differentiation (Sakakibara et al., 1996; Wang et al., 2008; Shibata et al., 2012). MSI-1 expression has been used to characterize NSPCs both in vivo and in vitro, across a wide range of regeneration competent (and incompetent) species (Barnabe-Heider et al., 2010; Ito et al., 2010; Mchedlishvili et al., 2012).

1.7.2 Ependymal Layer Cells (ELCs)

Adult NSPCs are known to reside within several regions of the brain, specifically the subventricular zone of the forebrain and the subgranular zone of the hippocampus and the spinal cord. Spinal cord NSPCs are located within the ependymal layer population lining the central canal. Although it remains unclear if all ELCs are NSPCs, there is abundant evidence that indicates members of this population play roles in CNS development, maintenance and regeneration (Bruni, 1998; Mothe and Tator, 2005; Meletis et al., 2008; Barnabe-Heider et al., 2010; Mothe et al., 2011). Given their location (bordering the lumen of the central canal) and the fact they are ciliated, ELCs are also involved in circulating cerebrospinal fluid (Anderson et al., 1986; Nolte, 2010; Alfara-Cervello et al., 2012). In support of their NSPC identity ELCs have the capacity to differentiate into different cells of the CNS, including neurons, both in vitro and
in vivo (Meletis et al., 2008, Barnabe-Heider et al., 2010). Furthermore, ELCs are known to express the hallmark stem/progenitor markers SOX2 and MSI-1 (Mchedlishvili et al., 2007; Barnabe-Heider et al., 2010; Gaete et al., 2012).

The use of cell tracking studies has begun to reveal the complexity of the ELC contribution during spinal cord regeneration. The main contributions come from ELCs located within ~ 500µm of the SCI and participating cells generally come from dorsoventrally restricted progenitor cell domains, similar to those described during CNS development (Schnapp et al., 2005; Mchedlishvili et al., 2007; Reimer et al., 2008; Reimer et al., 2009). Interestingly, at least some ELCs in urodeles are capable of spanning both dorsal and ventral domains and are predicted to represent a pool of multipotent NPSCs (Mchedlishvili et al., 2007).

1.7.3 Ependymo-Radial Glia (ERG)

Ependymo-radial glia (ERG; sometimes called tanyocytes (Reichenbach and Wolburg, 2005) or ependymoglia (Kirkham et al., 2014)) are NSPCs that persist into adulthood and reside within the ependymal layer of teleosts and urodeles (Chernoff et al., 2003; Becker and Becker, 2015). ERG have also been reported in the brain (but not the spinal cord) of turtles (Romero-Aleman et al., 2004; Trujillo-Cenoz et al., 2014).

ERG demonstrate a relatively conserved phenotype with the soma located along the ventricular lining of the brain or the ependymal layer of the spinal cord and have lengthy radial processes that extend to the pial surface (Rakic, 1995; Clinton et al., 2014). Similar to astrocytes, ERG express the intermediate filament GFAP (Noctor et al., 2001) and the end feet of the radial process are known to aid the integrity of the blood-spinal cord barrier (Grupp et al., 2010). ERG also express Vimentin, an intermediate filament protein otherwise common to
1.7.4 Cerebrospinal Fluid Contacting (CSF-c) Cells
Cerebrospinal fluid contacting (CSF-c) cells are a neuronal-like population that reside within the ependymal layer (Vigh et al., 1977). CSF-c cells are now known from a variety of vertebrates, including lampreys (Jalavand et al., 2014), zebrafish (Wyart et al., 2009), urodeles (Harper and Roberts, 1993), turtles (Russo et al., 2004), and mice (Petracca et al., 2016). The precise function of CSF-c cells remains poorly understood and they have been variably described as either neurons or glia (Russo et al., 2008). Based on morphology and electrophysiological properties, two distinct classes of CSF-c cells are known (Trujillo-Cenoz et al., 2007; Marichal et al., 2009; Jalavand et al., 2014). Type 1 CSF-c cells have a bulb-like ending that projects into the central canal and ventro-laterally oriented processes, while Type 2 CSF-c cells have a flattened cell body that projects into the central canal and have thin lateral processes that can be directed dorsally, ventrally or laterally (Russo et al., 2004; Jalavand et al., 2014). Type 1 and 2 cells also differ in aspects of function. Type 1 CSF-c cells express GABA, glutamate receptors and somatostatin, are capable of firing an action potential and display both spontaneous excitatory and inhibitory post-synaptic potentials (Christenson et al., 1991; Jalalvand et al., 2014). Type 2 CSF-c cells do not express GABA or somatostain and do not show any active neuronal properties (Jalalvand et al., 2014; Jalalvand et al., 2016a).

Immunohistochemical analysis of CSF-c cells reveals that Type 1 CSF-c cells also express the neuroblast markers, doublecortin (DCX) and polysialylated neural cell adhesion molecule (PSA-NCAM), as well as neuronal lineage markers including HuC/D and βIII-Tubulin, but not the mature neuronal marker NeuN (Russo et al., 2004; Russo et al., 2008; Marichal et al., 2009; Petracca et al., 2016). In contrast, type 2 cells do not express neuronal lineage markers, but express taurine (Jalalvand et al., 2014).
While the function of CSF-c cells across species remains unclear, several roles have been proposed. CSF-c cells appear to function as mechanoreceptors, sensing excessive neural activity and fluid movement and chemoreceptors, capable of sensing the pH of the CSF (Huang et al., 2006; Djenoune et al., 2014; Jalalvand et al., 2016a). Related to this, CSF-c cell support homeostasis within the spinal cord by stabilizing pH through decreasing motor activity by communicating with stretch receptor neurons (Jalalvand et al., 2016b). This is accomplished through the presence of both acid-sensing (ASIC3) and alkaline-sensing (PKD2L1) channels within a single CSF-c cell (Jalalvand et al., 2016b). CSF-c cells can also function as central pattern generators (Wyart et al., 2009) and excitation of these cells has been shown to induce a swim-like response. In addition, at least among zebrafish, CSF-c cells respond to both passive and active bending of the spinal cord (Bohm et al., 2016)

1.8 The Leopard Gecko: an emerging model for spinal cord regeneration

The leopard gecko (*Eublepharis macularius*; hereafter ‘gecko’) is an emerging model for the study of spinal cord regeneration (Szarek et al., 2016). As for many species of lizard (and tuatara), geckos are able to autotomize a portion of their tail to avoid predation and then spontaneously regenerate a replacement (Bellairs and Bryant, 1985; McLean and Vickaryous, 2011; Gilbert et al., 2013). Importantly, the spinal cord passes continuously from the body into the tail and there is no cauda equina. Therefore, the tail spinal cord can be accessed and manipulated without direct risk of injury to the body proper, drastically reducing the neurological impact of experimentation when compared to other SCI models (Szarek et al., 2016). Furthermore, the entire tail (including spinal cord) can be repeatedly lost and regenerated with functional recovery, without the need for clinical intervention (Delorme et al., 2012). Previous
work in our lab has generated a staging table of tail regeneration, recognizing seven distinct stages based on morphological characteristics (McLean and Vickaryous, 2011). These stages can be broadly clustered into three main phases: wound healing, early outgrowth, and late outgrowth. Wound healing begins immediately following tail loss and includes retraction of the newly ruptured spinal cord into the neural canal and the formation of an exudate clot to cap the site of tail loss. At the level of histology, wound healing includes a period of re-epithelialization and the formation of a wound epithelium, and the initiation of CNS regeneration (McLean and Vickaryous, 2011). Early outgrowth involves the proliferation of cells contributing to the formation of new tissues, while late outgrowth primarily involves the differentiation of tissues including skeletal muscle, adipose tissue, cartilage, and a replacement spinal cord. (McLean and Vickaryous, 2011). Although details of spinal cord regeneration remain poorly understood, the regenerate tail is innervated, and demonstrates both motor and sensory functionality (Szarek et al., 2016). While it has been suggested that ELCs are required for spinal cord regeneration, their identity and contribution to the new tail is unknown (Simpson, 1964; Whimster, 1978; Bellairs and Bryant, 1985; Singer and Duffy, 1994; McLean and Vickaryous, 2011).
Figure 1: Mammalian Response to Spinal Cord Injury. Spinal cord injuries involve a two-part response. The primary response occurs within the first 24 hours and involves mechanical damage, infiltration of blood products through a disruption of the blood-spinal cord barrier and the initiation of an inflammatory response. The primary response triggers a second, longer lasting inhibitory response that can span weeks to months. This secondary response includes excitotoxicity from uncontrolled glutamate release, cell death through both apoptosis and necrosis as well as the formation of an inhibitory glial scar.
RATIONALE

Spinal cord injuries (SCI) can lead to devastating functional impairments including paresis, paralysis and even death (Thuret et al., 2006). Although efforts to improve recovery following SCI are ongoing, results to date have produced only modest structural or functional improvements, underscoring the need for alternative strategies. One promising strategy to better understand spinal cord repair involves the adoption of a comparative approach, focusing on species capable of spontaneous spinal cord regeneration. The best-known examples of naturally occurring spinal cord regeneration comes from various non-amniotes, specifically teleosts and urodeles (e.g. Chernoff et al., 2003; Sirbulescu and Zupanc, 2011). In these models, spinal cord repair involves the recruitment of a population of endogenous neural stem/progenitor cells (NSPCs) that proliferate, migrate and differentiate into new astrocytes, oligodendrocytes and neurons. Ultimately, these cells contribute to the establishment of a functional replacement spinal cord. Among teleosts and urodeles, the primary source of NSPCs is the cell population surrounding the central canal – ependymal layer cells (ELCs) (Zhang et al., 2003; Schnapp et al., 2005; Mchedlishvili et al., 2007).

Among amniotes (lizards, bird and mammals), lizards are unique in their ability to regenerate the spinal cord following tail loss (Bellairs and Bryant, 1985). While previous investigations point towards ELCs as being necessary for successful spinal cord regeneration (Kamrin and Singer, 1955; Simpson, 1968; Whimster, 1978), it remains unclear if these cells represent NPSCs.

We hypothesized that in lizards, ELCs include populations of NSPCs that are activated in response to tail loss and contribute to spinal cord regeneration. To test this hypothesis, we explored three objectives:
1. To identify NSPCs in the spinal cord before, during and after tail regeneration.
2. To determine the source of the regenerated spinal cord using *in vivo* electroporation
3. To examine the in vitro potential of spinal cord cells to function as NSPCs
CHAPTER 2: CHARACTERIZATION OF EPENDYMAL LAYER CELLS 
IN THE REGENERATING SPINAL CORD OF THE LEOPARD GECKO 

(EUBLEPHARIS MACULARIUS)

2.1 INTRODUCTION

Spinal cord injuries (SCI) can lead to devastating functional and behavioural impairments including paresis, paralysis and in severe cases, death (Thuret et al., 2006). In mammals, the regenerative capacity of the central nervous system (CNS) is limited (Horner and Gage, 2000; McDonald and Sadowsky, 2002; Tanaka and Ferretti, 2009), and most SCI are resolved with the formation of a glial scar. Whereas glial scars are neuroprotective (against severe inflammation) they also inhibit axon regrowth (Frisen et al., 1995b; Liu et al., 1997; Rowland et al., 2008). Despite numerous efforts to reduce the negative impact of the glial scar, improvements to functional recovery remain limited (Zhao and Fawcett, 2013; Shen et al., 2014; Sekiya et al., 2015).

One of the main strategies to improve our understanding of spinal cord repair involves the adoption of a comparative approach (Tanaka and Reddien, 2011). In species capable of spinal cord regeneration, a permanent glial scar fails to form. Instead, SCI triggers the proliferation of a stem cell-like population of cells that is mobilized to replace missing tissue (Bellairs and Bryant, 1985; Benraiss et al., 1999; Monaghan et al., 2007). The most common examples of species known to regenerate the spinal cord following injury (including complete transection) include various teleost fish (Anderson et al., 1986; Hui et al., 2010) and urodele amphibians (Mchedlishvili et al., 2007; Dawley et al., 2012). Within amniotes, only lizards appear to be
capable of spinal cord regeneration, at least within the tail (Whimster, 1978, Alibardi and Miolo, 1990; McLean and Vickaryous, 2011; Szarek et al., 2016). Unique to lizards, spinal cord regeneration restores function but is not a perfect replica of the original organ. Whereas the original spinal cord consists of white matter, grey matter and a distinct organization of cells surrounding the central canal, herein referred to as ependymal layer cells (ELCs), the regenerate spinal cord includes only descending tracts and ELCs (Simpson, 1968; Egar et al., 1970; McLean and Vickaryous, 2011; Gilbert et al., 2015).

Among various species capable of spinal cord regeneration, ELCs are known to include populations of neural stem/progenitor cells (NSPCs) that are recruited in response to injury to replace lost or damaged neuronal cells (Reimer et al., 2008; Tanaka and Ferretti, 2009; Allen and Smith, 2012). In zebrafish, ELCs with stem/progenitor-like properties are known as ependymoradial glia (ERG). ERGs have a radial cell-like morphology (the cell body contributes to the lining of the central canal, while the lengthy radial process contacts the pial surface) and express markers otherwise characteristic of astrocytes, such as glial fibrillary acidic protein (GFAP) (Becker and Becker, 2015). Following injury, ERGs participate in replacing domain-specific cell types (Reimer et al., 2008; Reimer et al., 2009). For example, Olig2+ ERGs generate motor neurons from the ventro-lateral spinal cord, while serotonergic neurons are regenerated from a more ventrally located Nkx6.1+ ERG population (Reimer et al., 2008; Reimer et al., 2009). Comparable ERG-like cells have also been observed in urodeles and cell-tracking studies have shown that these populations contribute both new neurons and new glial cells to the regenerated spinal cord (Echeverri and Tanaka, 2003; Schnapp et al., 2005; Dawley et al., 2012).

Although less is known about the role of ELCs in lizards, they appear to be necessary for tail regeneration (Simpson, 1968, 1970; Egar et al., 1970, Whimster, 1978). When the spinal
cord is removed or blocked and ELCs are prevented from invading regenerating tail tissue, spinal cord regeneration (and new tail outgrowth) fails (Kamrin and Singer, 1955; Simpson, 1964, Whimster, 1978; Simpson and Duffy; 1994; Tanaka and Ferretti, 2009). Additionally, if isolated sections of spinal cord are transplanted to ectopic locations (e.g., into wounds created dorsal to the sacrum), supernumerary tails complete with descending tracts and ELCs are created (Whimster, 1978; Lozito and Tuan, 2015). Although ELCs of the original lizard spinal cord remain poorly characterized, they have been likened to NSPCs (Alibardi, 2014; see also Gilbert et al., 2015).

Here we provide the first detailed investigation of lizard ELCs prior to and following spinal cord injury. The model for our studies is the leopard gecko (*Eublepharis macularius*; hereafter ‘gecko’), a captive-bred and lab-amenable lizard (Whimster, 1978; McLean and Vickaryous, 2011; Delorme et al., 2012; Szarek et al., 2016). As for many lizards, geckos are able to self-detach the tail and then regenerate a replacement (McLean and Vickaryous, 2011; Delorme et al., 2012). Gecko tail regeneration is a relatively rapid (25+ days) injury-mediated, epimorphic (blastema-mediated) phenomenon (McLean and Vickaryous, 2011; Delorme et al., 2012; Gilbert et al., 2015). We determined that subsets of original tail ELCs are slow cycling, retaining a pulse-chase label for 140 days and that all ELCs express the hallmark NSPC protein SOX2. Additionally, we find that gecko ELCs are activated in response to tail loss, becoming increasingly proliferative and altering their pattern of protein expression. Finally, we show that ELCs of the regenerating/fully regenerate tail represent at least two distinct cell types: a large population of ERG-like cells and a less abundant group of cells comparable to cerebro-spinal fluid contacting cells (CSF-c cells). Taken together, our data underscores the complexity of the
regenerate spinal cord and provides compelling evidence in support of the predicted role of ELCs as endogenous stem/progenitor cells.

2.2 METHODS

2.2.1 ANIMAL CARE

Captive-bred leopard geckos (*Eublepharis macularius*) were obtained from a commercial supplier (Global Exotic Pets, Kitchener, Ontario, Canada). Geckos ranged in mass from 12-55 grams. Mass, snout-vent length and tail measurements were recorded each week to track growth and monitor health (Appendix 1). Animal Usage Protocol 1954 was approved by the University of Guelph Animal Care Committee, followed the procedures and policies of the Canadian Council on Animal Care, and closely adhered to the ARRIVE guidelines (Kilkenny et al., 2010). Our gecko colony was maintained at the Hagen Aqualab, University of Guelph, in a dedicated environmental chamber with an ambient temperature of 27.5°C and a 12:12 hour light/dark photoperiod. Husbandry protocols followed Vickaryous and McLean (2011). Briefly, geckos were housed individually in five gallon plastic (polycarbonate) enclosures. A subsurface heating cable (Hagen Inc., Baie d’Urfe, Quebec, Canada) was used to create a temperature gradient under one side of each enclosure. Geckos were fed three gut-loaded mealworms (larval *Tenebrio* spp.) daily, each dusted with powdered calcium and vitamin D3 (cholecalciferol) supplement (Zoo Med Laboratories Inc., San Luis Obispo, California, USA). Data for the histological and protein expression studies, was obtained from tissue samples representing 75 individuals and data for the long-duration (140 day) bromodeoxyuridine (BrdU) pulse-chase experiment represents tissue samples from 16 geckos.
2.2.2 BROMODEOXYURUDINE INJECTIONS

To identify slow-cycling cells and proliferating cells, we used bromodeoxyuridine (BrdU), a thymidine analog that incorporates into DNA during the synthesis phase of the cell cycle (Gratzner, 1982; Plickert and Kroicher, 1988). Our protocol is adopted from that of Wu et al. (2013). Briefly, BrdU solution was prepared by diluting 50mg of BrdU powder (Sigma-Aldrich, St. Louis, Missouri, USA) in 1mL dimethyl sulphoxide (DMSO) to make a 50 mg/mL stock solution. A working solution (5mg/mL) was then prepared by diluting 1mL of the stock in 9mL of sterile 1X phosphate buffered saline (PBS). The working BrdU solution was then injected twice daily (at 12 hour intervals) into the peritoneal cavity, just distal to the forelimbs. We alternated between left and right-sided injections at each injection interval.

2.2.3 TISSUE COLLECTION AND PREPARATION

Tail tissue was collected by inducing autotomy (self-detachment of the tail). Autotomy was accomplished by grasping the tail at the desired location of tail loss between the index finger and thumb and applying constant pressure until the tail self-detached. The first autotomy was always restricted to the distal third of the tail to permit tissue collection with a second (more proximal) autotomy. Tails were staged daily (following the morphological criteria of McLean and Vickaryous, 2011). Following the second autotomy event, the detached tail was injected with 10% neutral buffered formalin (NBF; Protocol Supplies, Kalamazoo, Michigan, USA) and placed in a centrifuge tube containing NBF for ~24 hours to establish proper fixation. Following fixation, tissue was transferred to 70% ethanol prior to processing.

For the long duration pulse-chase experiment, geckos were collected at 7, 30, 45 and 140 days following the pulse. Geckos were euthanized using an intra-abdominal injection of tricaine.
methansulfonate (MS222: dosage = 250-500mg) and then fixed using a transcardial perfusion of 1X phosphate buffered saline (PBS) followed by 10% neutral buffered formalin (NBF). Tissue was post-fixed by an additional 24-hour immersion in 10% NBF and then transferred to 70% ethanol prior to processing.

2.2.4 TISSUE PROCESSING

All tissue was prepared for serial histology by decalcifying tissue for 30 minutes in Cal-Ex ® (Fisher Scientific, Waltham, Massachusetts, USA). Tissue of interest was then dehydrated to 100% isopropanol, cleared in xylene and embedded in paraffin wax (Fisher Scientific, Waltham, Massachusetts, USA). Sections were cut at 5µm on a microtome, mounted on charged slides (Surgipath ® X-tra ®, Leica Microsystems, Concord, Ontario, Canada) and incubated at 37ºC overnight.

2.2.5 HISTOCHEMISTRY

To visualize tissue structure, representative sections at each stage of regeneration were stained using either hematoxylin and eosin or Masson’s trichrome. Slides were de-paraffinized and rehydrated to water through xylene (3 washes; 2 minutes), absolute isopropanol (3 washes; 2 minutes), 70% isopropanol (2 minutes) and deionized water (dH₂O) for 2 minutes. Hematoxylin and eosin staining was performed as follows: after being brought to water, slides were stained (10 minutes) with modified Harris hematoxylin (Fisher Scientific, Waltham, Massachusetts, USA) and then rinsed with deionized water. Slides were then dipped in 1% hydrochloric acid in 70% isopropanol (~6 times) and then rinsed again in deionized water. Slides were placed in ammonia water to differentiate the stain blue (~5 dips) and then rinsed in running
deionized water. Slides were dipped in 70% isopropanol (6 times) and then stained with eosin (1 minute). Briefly, slides were dehydrated with absolute isopropanol (4 washes; 2 minutes) and xylene (3 washes; 2 minutes) and were cover slipped using Cytoseal (Fisher Scientific, Waltham, Massachusetts, USA).

Masson’s trichrome staining was performed as described in McLean and Vickaryous (2011; see also Appendix 2). Briefly, once brought to water slides were stained with Mayer’s hematoxylin (10 minutes) and then rinsed in deionized water and placed in ammonia water to differentiate the stain blue (~5 dips). Slides were rinsed again in deionized water and then stained in 0.5% ponceau xylidine/0.5% acid fuchsin in 1% acetic acid solution and then rinsed again in deionized water. Slides were placed in 1% phosphomolydic acid (10 minutes) and rinsed in deionized water. Briefly, slides were placed in 2% light green (90 seconds) and rinsed again in deionized water. Slides were dehydrated through 95% isopropanol (2 minutes), absolute isopropanol (3 washes; 2 minutes and xylene (3 washes; 2 minutes) and cover slipped using Cytoseal (Fisher Scientific, Waltham, Massachusetts, USA).

2.2.6 ANTIBODY SPECIFICITY

The primary antibodies used are listed in Table 1. Specificity of the secondary antibody was confirmed with the use of an omission control (lacking the primary antibody); no immunostaining was detected on any of the omission controls.

α-tubulin (Sigma-Aldrich, USA) is a mouse monoclonal antibody raised against sarkosyl-resistant filaments from sea urchin sperm axonemes (manufacturer’s information). This antibody recognizes an epitope located at the C-terminal end of the α-tubulin isoform in a variety of organisms. A single ~50kDa band, the expected molecular weight (manufacturer’s information),
was detected on Western blots of gecko brain and spinal cord homogenates (Supplementary Figures 3-7). This antibody was used as a loading control for western blotting.

BrdU (Bromodeoxyuridine; Sigma-Aldrich, USA) is a mouse monoclonal antibody derived from the BU-33 hybridoma produced by fusion of murine myeloma cells and splenocytes from BALB/c mice immunized against BrdU (manufacturer’s information). BrdU expression was absent when geckos were not injected with the synthetic nucleoside (B5002, Sigma-Aldrich; Mahoney et al., 2009) (data not shown).

GFAP (Glial Fibrillary Acidic Protein; DAKO, Denmark) is a rabbit polyclonal antibody raised against cow spinal cord (manufacturer’s information). This antibody fails to immunostain tissue from GFAP−/− mice (Hanbury et al., 2003). The pattern of immunostaining observed in our gecko tissue matches that of previous descriptions of GFAP expression in other species, viz. astrocytes and radial glial cells (e.g., Lo et al., 2009).

HuC/D (Molecular Probes, USA) is a mouse monoclonal antibody raised against human HuC/D neuronal protein (manufacturer’s information). Specifically, it was raised against the human HuD peptide QAQRFRLDNLLN and binds to human Elav family members HuD, HuDpro (alternatively spliced form of HuD), and HuC (manufacturer’s information). Published work demonstrates a single ~40kDa band, the expected molecular weight (manufacturer’s information), using human cortical protein on a Western blot (Marusich et al., 1994). HuC/D immunostaining in the leopard gecko is exclusive to neuronal-like cells in the spinal cord and matches the staining pattern observed using the same antibody in chicks (Yang et al., 2010) and zebrafish (e.g. Lindsey et al., 2012), as well as the pattern of GFP staining in HuC:gfp transgenic zebrafish (Topp et al., 2008).
We used two Musashi-1 (MSI1) antibodies (both Millipore, USA). One (AB_92184) was a rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 5-21 of MSI1 and is known to react with human and mouse tissues (manufacturer’s information). We observed para-nuclear staining of SOX2+ NSPCs in the regenerating spinal cord of the leopard gecko consistent with a previous report documenting a comparable pattern expression by proliferating cells in the subventricular zone of mice (Maslov et al., 2004). The second (AB_2576205) was a mouse monoclonal antibody isolated from E17 mouse spinal cord tissue lysate (manufacturer’s information). Multiple bands between ~37 and 50kDa were stained on Western blots of brain and spinal cord homogenates (Supplementary Figure 3). Both antibodies demonstrated patterns of immunostaining identical with that of previous reports (Maslov et al., 2004; Nickerson et al., 2011) using the rabbit polyclonal MSI1 (AB_5977).

NeuN (Neuronal Nuclei; Millipore, USA) is a rabbit polyclonal antibody raised against a GST-tagged recombinant mouse NeuN N-terminal fragment (manufacturer’s information). A single ~42kDa band, the expected molecular weight (manufacturer’s information), was detected on Western blots of gecko brain and spinal cord homogenates (Supplementary Figure 4). An additional band was observed at 48kDa and two uncharacterized bands were also seen at 70 and 80kDa (also consistent with the manufacturer’s information). NeuN immunostaining is exclusive to neuronal-like cells of the spinal cord and is identical to the staining pattern observed (using the same antibody) in humans (Lo lacono et al., 2015) and mice (Wang et al., 2014).

PCNA (Proliferating Cell Nuclear Antigen; Santa Cruz Biotechnology, USA) is a rabbit polyclonal antibody raised against the epitope corresponding to amino acids 1-261 representing the full length human PCNA (manufacturer’s information). Cells expressing PCNA are most commonly observed among constitutively proliferating populations (e.g., basal cells of the
epidermis, hematopoietic tissues) and our staining in the leopard gecko matches that pattern of staining reported (using the same antibody) for other species including zebrafish (Simoes et al., 2014), geckos (McLean and Vickaryous, 2011), sengi (Slomianka et al., 2013) and humans (Low et al., 2011).

RT-97 (Developmental Studies Hybridoma Bank, USA) is a mouse monoclonal antibody raised against Wistar Rat neurofilaments (manufacturer’s information). Western blots from other studies reveal two bands: one at 200kDa, representing a phosphorylated subunit of the neurofilaments (Wood and Anderton, 1981), and one at 145kDa (e.g. Johnstone et al., 1997). RT-97 immunostaining in the gecko is exclusive to axons/neurites in the white matter and peripheral nerves and matches the staining pattern observed using the same antibody in other species (e.g. Marco-Gomariz et al., 2006 and Weigum et al., 2003).

SOX2 (SRY (Sex Determining Region-Y)-Box 2; Cell-Signalling, Canada) is a rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids surrounding Gly179 of human Sox2. A single ~35kDa band, the expected molecular weight (manufacturer’s information), was detected on Western blots of gecko brain and spinal cord homogenates (Supplementary Figure 5), consistent with studies on humans and mice (manufacturer’s information; Ohmine et al., 2012).

SOX9 (SRY (Sex Determining Region-Y)-Box 9; Abcam, Canada) is a rabbit polyclonal antibody raised against a synthetic non-phosphopeptide surrounding the epitope RKSVK from human SOX9. A single ~56kDa band, the expected molecular weight (manufacturer’s information), was detected on Western blots regenerating tail homogenates (stages IV–VI), as well as from homogenates of HH29-33 embryonic chick (Supplementary Figure 6) In regenerating tail samples an additional unknown band was detected at ~ 15kDa; in embryonic
chick a second unknown band was detected at ~75kDa. Using immunohistochemistry, SOX9 immunostaining was restricted to the nuclei of chondrocytes and ependymal cells of the spinal cord (McLean and Vickaryous, 2011).

TUJ1 (Beta-III-Tubulin; Millipore, USA) is a mouse monoclonal antibody raised against purified immunoglobulins (manufacturer’s information). A single ~50kDa band, the expected molecular weight (manufacturer’s information), was detected on Western blots of gecko brain and spinal cord homogenates (Supplementary Figure 7). TUJ1 immunostaining is exclusive to neuronal-like cells of the spinal cord and matches the staining pattern observed in mice (Hartman et al., 2015). This antibody also stains rat neuronal cells in vitro (Takaki et al., 2012).

Vimentin (Developmental Studies Hybridoma Bank, USA) is a mouse monoclonal antibody raised against vimentin isolated from chick optic tectum (manufacturer’s information). A single ~52kDa band, the expected molecular weight (manufacturer’s information), was detected on Western blots of gecko brain and spinal cord homogenates (Supplementary Figure 8). Neural stem progenitor cells in the lizard spinal cord as well as mesenchymal cells in the regenerating tail blastema express vimentin using both immunohistochemistry and immunofluorescence. Specificity has also been validated in embryonic chick both in vitro (Yan et al., 2010) and in vivo using immunohistochemistry where it labels Muller glia in the chick retina (Herman et al., 1993). It has also been tested in planarians using whole-mount immunofluorescence (Robb and Sanchez-Alvarado, 2002).

2.2.7 IMMUNOHISTOCHEMISTRY

To document the expression of PCNA (proliferation), RT-97 (neurofilaments), SOX2 (NSPCs), SOX9 (lineage-restricted cells) and Vimentin (mesenchymal-like cells), immunohistochemistry was performed (see Appendix 3). Antibody concentrations, blocking
solutions and DAB exposure time are summarized in Table 2. Slides were de-paraffinized and rehydrated to water through xylene (3 washes; 2 minutes), absolute isopropanol (3 washes; 2 minutes), 70% Isopropanol (2 minutes) and deionized water (dH$_2$O) for 2 minutes. Following rehydration slides were quenched for 30 minutes in 3% hydrogen peroxide (H$_2$O$_2$) (diluted in dH$_2$O) and rinsed with dH$_2$O (2min). Sections were then blocked with 5% normal goat serum (NGS) (Vector Laboratories, Burlingame, California, USA) diluted in sterile 1X PBS (Sigma-Aldrich, St. Louis, Missouri, USA) for one hour at room temperature. Primary antibodies were diluted in sterile, 1X PBS and applied to one section on the slide (PCNA [1:500] Santa Cruz Biotechnologies, Dallas, Texas, USA; RT-97 [1:100] Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA; SOX2 [1:500] Cell-Signalling, Whitby, Ontario, Canada; SOX9 [1:200] Abcam, Toronto, Ontario, Canada; Vimentin [1:100] Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) overnight at 4°C. The section closest to the label was reserved as the omission (negative) control (sterile PBS applied with omission of the primary antibody).

Following overnight incubation, slides were rinsed in PBS (3 washes; 2 minutes). Secondary antibodies were then diluted in sterile PBS and were applied to both sections on the slide for one hour at room temperature at an appropriate concentration (biotinylated goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for PCNA [1:1000], Sox9 [1:1000]; Sox2 [1:10000] and biotinylated goat anti-mouse (Vector Laboratories, Burlingame, California, USA) for RT-97 [1:200] and Vimentin [1:500]). Slides were then rinsed in PBS (3 washes; 2 minutes) and then incubated in Horseradish Peroxidase (HRP) [1:200] (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) diluted in sterile PBS for one hour at room temperature. Slides were rinsed again in PBS (3 washes; 2 minutes). Positive immuno-staining was visualized using a DAB staining kit (Vector Laboratories, Burlingame,
California, USA). Slides were then placed in deionized water to halt the DAB reaction, counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St. Louis, Missouri, USA) (for one minute, dehydrated with 70% isopropanol for 2 minutes, absolute isopropanol (3 washes; 2 minutes), cleared with xylene (3 washes; 2 minutes) and cover-slipped using Cytoseal (Fisher Scientific, Waltham, Massachusetts, USA).

2.2.8 IMMUNOFLUORESCENCE

2.2.8.1 Standard Two-Day Protocol

To document the expression of TUJ1, GFAP, MSI1, NeuN, PCNA, GFAP and Vimentin immunofluorescence was performed (see Appendix 4). Slides were de-paraffinized and rehydrated to water through xylene (3 washes; 2 minutes), absolute isopropanol (3 washes; 2 minutes), 70% isopropanol (2 minutes) and deionized water (dH\textsubscript{2}O) for 2 minutes. Slides were then rinsed in either phosphate buffered saline (PBS) or 1% Tween-20 in phosphate buffered saline (PBST) (3 washes; 5 minutes); see Table 3. They were then blocked for one hour in 10% normal goat serum (NGS) +0.3% triton-X-100 in sterile PBS or 5% NGS in sterile PBS at room temperature; see Table 3. Primary antibodies were diluted in either 10% NGS + 0.3% triton-x-100 in sterile PBS or sterile PBS and applied to one section on the slide (TUJ1 [1:100] Millipore, Temecula, California, USA; GFAP [1:1000] DAKO, Glostrup, Denmark; MSI1 [1:100] Millipore, Temecula, California, USA; NeuN [1:250] Millipore, Temecula, California, USA; PCNA [1:100] Santa Cruz Biotechnologies, Dallas, Texas, USA; Vimentin [1:50] Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA. Following overnight incubation at 4°C slides were rinsed in PBS or PBST (3 washes; 5 minutes) and then incubated in secondary antibody for one hour at room temperature (goat anti-mouse alexa 488 (Life
Technologies, Eugene, Oregon, USA) for TUJ1 [1:1000]; MSI1 [1:100] and Vimentin [1:500] and Cy3 goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for GFAP, NeuN and PCNA [1:1000]). Slides were rinsed again in PBST or PBS (3 washes; 5 minutes) and counterstained using a DAPI nuclear stain (Life Technologies, Eugene, Oregon, USA) diluted in sterile PBS [1:10000]. Finally, slides were rinsed in PBST or PBS (3 washes; 5 minutes) and cover slipped using fluorescent mounting media (DAKO, Glostrup, Denmark).

2.2.8.2 Modified Two-Day Protocol

To document expression and co-localization of HuC/D and NeuN we performed double immunofluorescence (see Appendix 5). Briefly, slides were de-paraffinized and rehydrated to water through xylene (3 washes; 2 minutes), absolute isopropanol (3 washes; 2 minutes), 70% isopropanol (2 minutes) and deionized water (dH₂O) (2 minutes). Slides were then rinsed for 40 minutes in phosphate buffered saline (PBS) and antigen retrieval was performed using a 50mM-Tris Base solution for 30 minutes at 95°C. Slides were cooled for 30 minutes and rinsed in 1% tween 20+ PBS for 10 minutes followed by PBS rinses (2 washes; 5 minutes). 10% normal goat serum (NGS) + 0.3% triton-x-100 in sterile PBS was used to block the tissue for 30 minutes prior. Primary antibodies diluted in 1% bovine serum albumin (BSA) + sterile PBS were applied to one section on the slide (HuC/D [1:10] Molecular Probes, Rockford, Illinois, USA; NeuN [1:250] Millipore, Temecula, California, USA); sterile PBS was applied to the other section as a negative control. Slides were incubated overnight at 4°C and then rinsed in 1% BSA +sterile PBS (3 washes; 10 minutes). Secondary antibody, diluted in sterile PBS was applied to both sections on the slide (goat anti-mouse alexa 488 [1:500]; goat anti-rabbit Cy3 [1:1000] for one
hour at room temperature. Slides were rinsed (2 washes; 10 minutes) in PBS and stained with nuclear markers DAPI [1:10000]. Slides were rinsed again with PBS (2 washes; 10 minutes) and cover slipped with fluorescent mounting media (DAKO, Glostrup, Denmark). For antibody concentrations and blocking details see Table 3.

### 2.2.8.3 One-Day Protocol

To label BrdU incorporation and to co-localize BrdU with SOX2, we used a one-day immunofluorescence protocol (see Appendix 6). Briefly, slides were de-paraffinized and rehydrated to water through xylene (3 washes; 2 minutes), absolute isopropanol (3 washes; 2 minutes), 70% isopropanol (2 minutes) and deionized water (dH2O) (2 minutes). Slides were then rinsed for 15 minutes in 1X phosphate buffered saline (PBS) and then one of two retrieval methods were used. To label BrdU immunopositive cells and stain all nuclei with DAPI, heat-mediated antigen retrieval was used. Specifically, we used citrate buffer retrieval for 12 minutes at 95°C. Slides were cooled for 20 minutes and then rinsed for 2 minutes in 1X PBS. To co-localize BrdU and SOX2, sections were incubated for 30 minutes in 2N hydrochloric acid at 37°C instead of citrate buffer and then rinsed for 2 minutes in 1X PBS. Methods for the remainder of this protocol were the same for both BrdU and SOX2 immunofluorescence. Slides were incubated for 20 minutes in 0.1% trypsin at 37°C (Sigma-Aldrich, St. Louis, Missouri, USA), rinsed again for 2 minutes in 1X PBS and then blocked for 30 minutes at 37°C in 5% normal goat serum in diluent (1% bovine serum albumin, 0.5% Tween 20, 0.1% sodium azide in 1X PBS). Next, primary antibody was diluted in diluent and slides were incubated for 2 hours at 37 °C (BrdU [1:100] Sigma-Aldrich, St. Louis, Missouri, USA; SOX2 [1:50] Cell-Signalling, Whitby, Ontario, Canada). Slides were then in 1X PBS (3 washes; 5 minutes), and then
incubated in secondary antibody at room temperature for 1 hour (goat anti-mouse alexa 488 [1:200] Life Technologies, Eugene, Oregon, USA; and Cy3 goat anti-rabbit [1:200] Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). Slides were rinsed in 1X PBS (3 washes; 5 minutes), and then stained with nuclear marker DAPI ([1:10000] Life Technologies, Eugene, Oregon, USA). Once more, slides were rinsed in 1X PBS (3 washes; 5 minutes), and then cover slipped with fluorescent mounting media (DAKO, Glostrup, Denmark). For antibody concentrations and blocking details see Table 3.

2.2.9 WESTERN BLOT ANALYSIS

2.2.9.1 Tissue Collection and Protein Quantification

To confirm the specificity of our antibodies, western blot analysis was performed. Fresh gecko tissue samples, including brain, spinal cord, original tail, regenerating tail, were collected following euthanasia using an intra-abdominal injection of tricaine methansulfonate (MS222: dosage= 250-500mg). Embryonic chick (stages 29-33) tissue was used in some instances as a positive control. Tissues were flash frozen using liquid nitrogen and stored at -80°C prior to use. Aliquots for western blot analysis were established by thawing tissue samples on ice and combining samples with a triple detergent RIPA lysis buffer (200µl/0.1g; 50mM Tris-HCl, 150mM NaCl, 0.2% NA Azide, 0.5% NA deoxycholate) and mechanically homogenized using either a sonicator (brain, spinal cord tissue) or a tissue homogenizer (original and regenerating tail, embryonic chick) until no particulate was visible. Samples were left on ice for 30 minutes prior to centrifugation. Samples were centrifuged at 6°C at 14000 rpm for 10 minutes. A 30µl sample was collected specifically for protein quantification using the DC BioRad assay (see Appendix 7). Following quantification, aliquots of each sample were made and stored at -80°C.
2.2.9.2 Western Blotting

To perform western blot analysis (see Appendix 8), aliquots of the required tissue were removed from the -80°C freezer, and thawed on ice. Volumes of tissue equivalent to 30 µg of protein were combined with 8X sample buffer and MilliQ water to reach a total volume of 30 µl, to be loaded into each lane of the gel. The protein, sample buffer, MilliQ solution was heated to 90°C for 5 minutes and then centrifuged briefly. SDS polyacrylamide (SDS-PAGE) gels were prepared. The stacking gel was a 5% gel and the separating gel was 12%. 5 µl of a precision plus protein standard dual colour molecular weight marker (Bio-Rad Laboratories, Mississauga, ON, Canada) and 30 µl of homogenized tissue sample was loaded into a 10 well gel with 1.5 mm thickness vertical electrophoresis plates (Bio-Rad Laboratories, Mississauga, ON, Canada). Proteins were separated by molecular weight through the application of an electrical current of 100 V as they moved through the stacking gel (~30 minutes) followed by an electrical current of 120 V as they moved through the separating gel (~100-120 minutes). After proteins had fully separated on the gel, they were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Mississauga, ON, Canada). Membranes were activated in methanol and then compressed adjacent to the gel and surrounded by two sheets of Whatman paper as well as two western blot sponges. Whatman paper and western blot sponges had been soaked previously for 20 minutes in transfer buffer. The sponge, Whatman paper, gel, PVDF membrane were combined in a transfer unit (Bio-Rad Laboratories, Mississauga, ON, Canada) and transferred for 90 minutes at 90V. Following transfer, PVDF membranes were dried and subsequently rehydrated in methanol and then rinsed on a belly-dancer in Tris-buffered saline with Tween 20 (TBST; 3 washes, 15 minutes each). Membranes were then blocked for 2 hours in a 5% skim milk in TBST block at room temperature (again on a belly dancer). Next, membranes were
incubated in primary antibody diluted in 5% skim milk in TBST overnight at 4°C on a rocker.

(TUJ1 [1:5000] Millipore, Temecula, California, USA; MSI1 [1:2500] Millipore, Temecula, California, USA; NeuN [1:2500] Millipore, Temecula, California, USA; SOX2 [1:1000] Cell-Signalling, Whitby, Ontario, Canada; Vimentin [1:100] Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA). On the following day, membranes were rinsed with TBST (3 washes, 15 minutes each) and then incubated in a horseradish peroxidase conjugated secondary antibody diluted in 5% skim milk block for 1 hour at room temperature on a belly dancer (anti-mouse IgG HRP-linked, Cell-Signalling, Whitby, Ontario, Canada, or anti-rabbit IgG HRP-linked, Cell-Signalling, Whitby, Ontario, Canada; see Table 4). The membranes were then rinsed again in TBST (3 washes, 15 minutes each) and then Tris-buffered saline (TBS; 5 minutes) and incubated with enhanced chemiluminescence (ECL Luminata Forte; Millipore, Temecula, California, USA) for 30 seconds. The membrane was placed in a ChemiDoc (Bio-Rad) to visualize protein bands. Molecular weights were confirmed by merging the ladder and with the blot following exposure. Following ECL, membranes were rinsed in TBS (10 minutes) and stripped (ReBlot Plus mild; Millipore, Temecula, California, USA) for 5 minutes. They were blocked again with 5% skim milk in TBST and then incubated in primary antibody (α-tubulin [1:100,000] Sigma-Aldrich St. Louis, Missouri, USA) and secondary antibody Anti-mouse IgG HRP-linked, Cell-Signalling, Whitby, Ontario, Canada) and visualized using ECL as described above to confirm that equal amounts of protein were transferred to the membranes. Blots were imaged on the chemiDoc. Brightness and contrast were adjusted in Adobe PhotoShop and images were merged using Adobe InDesign. See Table 4 for incubation times and concentrations.
2.2.10 STATISTICAL ANALYSIS

ELC and BrdU+ cell counts were completed for the long duration BrdU pulse-chase experiment. The sample size for ELC counts was determined using a power calculation (80% power, p=0.05) and established that 31 sections were required for analysis. ELC counts were completed manually using randomized Hematoxylin and Eosin sections from both pulse (n=31) and chase (n=31) tails on a Keyence VHX-1000 digital microscope. The mean number of ELCs per section was 39 (pulse) and 37 (chase) (see Appendix 9). BrdU+ cells from the pulse and chase were then counted manually using black and white images taken on a Zeiss Imager D.1 microscope (Table 5).

2.3 RESULTS

2.3.1 The fully regenerated spinal cord is a structurally imperfect replica of the original and lacks NeuN+ neurons

Unlike mammals, the spinal cord of lizards is uninterrupted from brainstem to the tip of the tail and there is no caudal equina (Figure 1A). As demonstrated by serial histology, the (original) spinal cord demonstrates a conserved cross-sectional organization along its entire length: an outer layer of nerve tracts (white matter) that surrounds aggregations of neuronal cell bodies organized into dorsal and ventral horns (grey matter), encircling a pseudostratified ependymal layer (Figure 1B,C; Supplementary Figures 1-2). Ependymal layer cells (ELCs) are continuous with cells from the ventricular zone of the brain, and enclose the central canal. Of the three meningeal layers, only the dura mater is readily identifiable (arrowheads: Figure 1C).

In stark contrast to the original organ, the fully regenerated spinal cord demonstrates a markedly simpler organization: bundles of descending tracts (representing the white matter)
surrounding a monolayer of ELCs (Figure 1D, E; Supplementary Figure 2). Descending tracts are enshrouded by connective tissue (reminiscent of meninges) and numerous blood vessels (Figure 1D,E). Conspicuously, neuronal cell bodies (i.e., grey matter) appear to be absent.

Matching the altered morphology of the regenerated spinal cord, the regenerated axial skeleton is also dissimilar to the original. Whereas the original tail is supported by a series of (bony) vertebrae, the regenerated tail is supported by a hollow cone of cartilage (Figure 1D, E).

To confirm the absence of neurons in regenerated tails, we immunostained with the neuronal markers NeuN and HuC/D (Figure 2). Whereas NeuN is considered to be a marker of mature neurons within the spinal cord, HuC/D is expressed by mature and immature neurons, as well as some neuronal-like cell types (e.g., cerebrospinal fluid contacting cells [CSF-c cells]; Marichal et al., 2009; Petracca et al., 2016). As expected, NeuN immunopositive (+) cells were present throughout the grey matter of the original spinal cord, but entirely absent from the fully regenerated spinal cord (Figure 2A,B; Supplementary Figure 2). Surprisingly, while HuC/D was detected in neuronal-like cells of the original grey matter (where it often co-localizes with NeuN; Figure 2A,B), it was also expressed by isolated ELCs of the fully regenerated spinal cord (Figure 2C, D) and throughout regeneration (Figure 2E-G). Characteristically, these HuC/D+ ELCs have broad apical surfaces that contact the central canal (e.g., Figure 2C-G).

2.3.2 The spinal cord invades the regenerate blastema within days of tail loss.

Previous work has determined that tail regeneration in geckos is a highly conserved process (McLean and Vickaryous, 2011). Seven morphological stages of regeneration are recognized, which broadly reflect three major events: wound healing (stages I-II); early outgrowth (stages III-IV); and late outgrowth (stages V-VII) (Figure 3). Prior to tail loss, the
spinal cord occupies a near central position within the tail as it passes through the vertebral canal of the tail vertebrae. Surrounding the tail vertebrae are bands of adipose tissue and then pairs of epaxial and hypaxial skeletal muscle. Parasagittal dorsal root ganglia flank each segment of the spinal cord.

In order to avoid predation, geckos (as for many lizard taxa) are able to autotomize a portion of the tail. This spontaneous process is associated with structural adaptations called fracture planes that facilitate tail detachment and minimize damage to adjacent tissues. Although fracture planes subdivide individual vertebrae, along with adjacent skeletal muscle, adipose tissue and skin, there are no obvious modifications to the original spinal cord (Gilbert et al., 2013). As a result, during autotomy the spinal cord is ruptured and often becomes frayed and ragged. Within minutes of autotomy (stage I: 0 to 12-24 hours post-autotomy; Figure 3A), the original spinal cord begins to passively retract into the vertebral canal. Once retracted, the ruptured margin of the spinal cord becomes capped by a blood clot (stage II: ~1-7 days post-autotomy; Figure 3B). While there is no outward evidence of regenerative outgrowth, serial histology reveals the first evidence of a blastema: a mass of proliferating mesenchymal-like cells (Gilbert et al., 2015). The blastema is located immediately distal to the site of spinal cord injury, deep to the clot (hatched black lines: Figure 3B). Over the coming days, the blastema continues to expand in size (the result of cell proliferation), gradually enveloping the entire distal margin of the autotomized tail. Expansion of the blastema is synchronous with the completion of re-epithelialization (stage III: ~8-12 days post-autotomy; Figure 3C). Once re-epithelialized, the blood clot is ablated and the neo-epidermis or wound epithelium is exposed. Deep to the wound epithelium, the blastema continues to enlarge resulting in an increasingly cone-like outgrowth. Although most of the blastema appears as a homogenous cell-rich aggregation, two organs are
easily recognizable: blood vessels and a tubular outgrowth of ELCs continuous with the original spinal cord. This so-called ‘ependymal tube’ represents the first evidence of the regenerating spinal cord. At its distal extremity, the regenerating spinal cord becomes dilated to form the ependymal ampulla. Characteristically, the regenerating spinal cord invades deep into the blastema to approach within ~200µm of the overlying wound epithelium. As early outgrowth continues (stage IV: ~13-18 days post-autotomy; Figure 3D), the regenerating spinal cord occupies a near central location within the blastema.

As outgrowth of the new tail continues, the ependymal ampulla becomes more pronounced. Otherwise the regenerating spinal cord does not appear to differ significantly in appearance from that of previous stages. In contrast, most other tissues of the regenerating tail are beginning to differentiate. By stage V (~19-25 days post autotomy; Figure 3E), cartilage and skeletal muscle are recognizable (first appearing in proximal locations adjacent to the original tail stump), occupying concentric locations around the regenerating spinal cord, while new scales first appear along the wound epithelium. At stage VI (~25-30 days post autotomy: Figure 3F) all the new tail tissues are essentially reconstituted, including cartilage, skeletal muscle, and adipose tissue. Regeneration concludes with the restoration of pigmentation (stage VII: ~30 days post-autotomy; Figure 3G). A summary of the key events associated with spinal cord regeneration are presented in Figure 3H.

2.3.3 The ependymal layer includes populations of neural stem/progenitor cells (NSPCs)

Previous investigations of various regeneration-competent species have shown that ELCs of the original spinal cord include populations of NSPCs (e.g., Schnapp et al., 2005; Reimer et al., 2008; Gaete et al., 2012). To determine if NSPCs were present within the ELC population of
the gecko (original) spinal cord, we first sought to identify slow-cycling cells using a long-duration bromodeoxyuridine (BrdU) pulse-chase experiment. During the seven-day pulse phase of the experiment we administered BrdU twice daily (intraperitoneal injections; 50mg/kg) (Figure 4A). Next we examined four chase time points: immediately following the pulse (0 days; n=4); 30 days post-pulse (n=2); 45 days post-pulse (n=2); and 140 days post-pulse (n=6) (Figure 4B). At the end of the pulse (0 days), 9.8% of ELCs were immunopositive for BrdU (Figure 4C; Table 5). In roughly half sections analyzed, 1-2 BrdU+ non-ELCs were also present within the grey matter. These findings indicate that less than one in ten ELCs cycled during the pulse period and that a very small proportion of cells within grey matter are also mitotically active. Although not quantified, we also observed isolated BrdU expressing ELCs at both 30 and 45 days post-pulse (Figure 4D, E). Following the 140-day chase, the number of BrdU labeled cells had diminished almost four-fold compared to the 0 day time point, with only 2.5% of ELCs being BrdU+ (Figure 4F; Table 5). Additionally, we also found that in less than a third of the sections analyzed, 1-3 label-retaining non-ELCs were present (Table 5).

Next, we asked if ELCs expressed the hallmark NSPC marker SOX2. SOX2 is a transcription factor involved in the maintenance of pluripotency and stem/progenitor cell renewal (Graham et al., 2003; Juuri et al., 2013). All ELCs, including all BrdU+ cells, expressed SOX2 (Figure 4G-J). In addition to ELCs from the original tail (Supplementary Figure 9A, B), SOX2 also labeled all ELCs in fully regenerated (Supplementary Figure 9C, D), and actively regenerating (Supplementary Figure 9E-G) tails. Beyond the ELCs in original tails, isolated SOX2+ cells were also observed within the grey matter of the spinal cord (Supplementary Figure 9A, B). Otherwise, all other cells of the original, regenerating and fully regenerated tail were SOX2-. 
To investigate whether ELCs in the original tail altered their protein expression and mitotic status in response to tail loss, we carried out a detailed immunohistochemical analysis at three key time points: (1) prior to tail loss (Figure 5A); (2) within 24 hours following tail loss (Figure 5B); and (3) once the new tail was fully regenerated (Figure 5C). Prior to tail loss, individual ELCs express the proliferation marker PCNA (Figure 5D) and the mesenchymal marker Vimentin (Figure 5D), in addition to SOX2 (Figure 5E). However, we did not detect expression of the NSPC marker MSI1 (Figure 5F), the neuronal lineage marker TUJ1 (Figure 5G), or glial lineage marker SOX9 (Figure 5H).

Following tail loss, we observed a marked change in protein expression by ELCs of the remaining original tail spinal cord stump. Isolated ELCs continued to express PCNA (Figure 5I), and SOX2 expression remained essentially ubiquitous (Figure 5J). However unlike original tails, within 24 hours of autotomy almost all ELCs are also labeled with Vimentin (Figure 5I) and MSI1 (Figure 5K). Furthermore, some ELCs alternatively immunostain for TUJ1 (Figure 5L) or SOX9 (Figure 5M), indicating a more lineage restricted phenotype. With the exception of MSI1, this altered pattern of protein expression persists throughout tail regeneration (data not shown), and is retained within the original (stump) spinal cord even once the tail is fully regenerated (Figure 5N, O, Q, R). MSI1 expression is present in the original tail stump during regeneration, but disappears once regeneration is complete (Figure 5P).

2.3.4 Cells of the blastema express NSPC markers

The earliest evidence of blastema formation occurs deep to the exudate clot, immediately adjacent to the spinal cord (stage II of regeneration; Figure 6A,B). In agreement with previous work (McLean and Vickaryous, 2011), this early-formed blastema includes populations of
PCNA+ cells (Figure 6C) indicating cell proliferation is occurring. Although the intermediate filament Vimentin was not detected (Figure 6C), we did observe isolated SOX2+ (Figure 6D) and MSI1+ (Figure 6E) cells, suggesting that NSPCs were present.

2.3.5 ELCs in the regenerating and fully regenerated spinal cord proliferate and express NSPC and lineage-restricted progenitor markers

Next we extended our immunostaining characterization to include ELCs of the regenerating spinal cord. Prior to tail loss, ELCs form a pseudostratified epithelium encircling the central canal (Figure 7A). In contrast, during regeneration (and upon its completion), ELCs are organized into a monolayer of cuboidal cells (Figure 7B-E). Similar to the original spinal cord, all ELCs of the regenerating and fully regenerated spinal cords are SOX2+ (Figure 7F-J). Additionally, while only modest expression of PCNA was observed in the original spinal cord (Figure 7K), during regeneration the majority of ELCs demonstrate evidence of proliferation (Figure 7L-O). Also unlike the original spinal cord, ELCs of the regenerating tail express Vimentin, MSI1 and TUJ1. Vimentin (Figure 7L-O) and MSI1 were detected throughout ELCs (Figure 7Q-T), whereas the neuronal lineage marker TUJ1 was typically located near the pial surface (or around the nerve tracts) although expression within the cell body was also observed (arrows; Figure 7Q-T). We also found that a subset of ELCs within the regenerating spinal cord were immuno-positive for the glial lineage marker SOX9 (data not shown; McLean and Vickaryous, 2011). Taken together, these results indicate that ELC protein expression is altered following injury and throughout regeneration.

Using our same panel of NSPC and lineage-restricted markers, we then characterized ELCs in the fully regenerated spinal cord (Figure 8). Similar to ELCs from the actively
regenerating spinal cord, individual ELCs from the fully regenerated spinal cord are PCNA+ (Figure 8B,D), while the expression of Vimentin (Figure 8B), SOX2 (Figure 8C), and MSI1 (Figure 8D) is essentially ubiquitous. Furthermore, isolated ELCs continue to express the lineage-restricted markers TUJ1 (Figure 8E) or SOX9 (Figure 8F). Taken together, our spatiotemporal characterization of original, regenerating and fully regenerated spinal cord (Figure 9) reveals that ELCs are activated in response to injury.

2.3.6 The regenerate spinal cord contains at least two distinct cell types

ERGs are a specialized CNS cell type capable of generating new neurons in the brain and spinal cord of teleosts and urodeles (Becker and Becker, 2015). Uniquely, ERGs exhibit characteristics common to both ependymal cells (e.g., they line the ventricles and central canal) and glial cells (i.e., they express astrocytic markers and their end feet help to seal the blood-spinal cord barrier). To determine if ERGs were present within the original and regenerating/fully regenerated spinal cords, we performed double immunofluorescence for two intermediate filaments commonly expressed by this cell type: GFAP and Vimentin. Prior to tail loss, GFAP expression is widespread among cells of the white and grey matter, as well as some isolated ELCs, while Vimentin expression is limited but visible surrounding the ependymal layer (Figure 10A, B). However, in the fully regenerated tail many ELCs robustly co-localize with both GFAP and Vimentin (Figure 10C, D). Next we investigated the pattern of expression during active tail regeneration. Interestingly, the onset of Vimentin and GFAP immunostaining by ELCs is not simultaneous. During the early outgrowth phase of regeneration, ELCs are labeled with Vimentin whereas GFAP expression was restricted to the descending tracts (Figure 10E). As outgrowth continues (Figure 10F,G), Vimentin and GFAP immunostaining becomes co-localized
among the majority of cells, in addition to descending tracts. Finally, we sought to determine the relationship between ELCs expressing GFAP and those expressing the neuronal marker HuC/D (Figure 11). Prior to tail loss HuC/D was detected among neurons in the grey matter, but conspicuously absent elsewhere while GFAP expression was relatively widespread (Figure 11A, B). Curiously, within ~12-24 hours following tail loss isolated ELCs of the original tail stump adopt a new protein expression phenotype: HuC/D+ but GFAP- (Figure 11A’, B’). HuC/D+, GFAP- ELCs were also present in the fully regenerated spinal cord (Figure 11 C, D), and at all the intermediate stages of regeneration (Figure 11E-G). Characteristically, these HuC/D+, GFAP- ELCs have wide apical (end bulb) surfaces that line the central canal, consistent with their identification as cerebrospinal fluid contacting cells (CSF-c cells; also known as central canal contacting cells; Marichal et al., 2009). In addition, similar to other species, gecko CSF-c cells express TUJ1 (a marker of neuronal cells; see Figure 7, 8), but not NeuN (see Figure 2). Combined, our data reveal that following spinal cord injury, ELCs includes populations of ERGs and CSF-c cells.

2.4 DISCUSSION

The evolved ability of some lizards to spontaneous regenerate the spinal cord provides a conceptually novel and less-invasive approach to investigate resident cell types involved in self-repair. We establish that ependymal layer cells (ELCs) within the spinal cord of the leopard gecko include populations of slow-cycling, SOX2+ neural stem/progenitor cells (NSPCs). ELCs are activated in response to injury (i.e., tail loss), and likely represent the reservoir recruited to form the replacement spinal cord. Unexpectedly, we found that following injury ELCs represent a heterogeneous population that includes at least two distinct cell types: ependymo-radial glia
(ERG: GFAP+, HuC/D-; Figure 13); and cerebrospinal fluid contacting cells (CSF-c cells: GFAP-, HuC/D+; Figure 13). Taken together, we conclude that the success of spinal cord (and possibly tail) regeneration relies (at least in part) on the activation of endogenous NSPCs within the ependymal mantle and that the regenerate spinal cord is a complex organ that consists of distinct glial and neuronal-like populations.

2.4.1 Ependymal layer cells in the tail spinal cord are neural stem/progenitor cells (NSPCs)

Three main lines of evidence support our interpretation of ELCs as NSPCs. ELCs are: (1) slow-cycling prior to injury; (2) ubiquitously express the stem maintenance marker SOX2; and (3) following injury, they become increasingly proliferative and change their pattern of protein expression to include several lineage restricted markers (e.g., TUJ1 and SOX9). Infrequent cell division or quiescence is commonly associated with dormant stemness potential throughout the lifespan of an organism (Momma et al., 2000; Magnusson and Frisen, 2016). Once induced to divide, these dormant cells then mediate repopulation via transiently amplifying precursor cells to participate in tissue repair and replacement (Momma et al., 2000; Becker and Becker, 2015; Richmond et al., 2016). As expected (given the timeframe of the pulse), only a small percent of original spinal cord ELCs (2.5%) were found to be slow-cycling at the end of a lengthy (140-day) chase period. Using the same 7-day pulse/140-day chase protocol, a near-identical proportion of slow-cycling stem-like cells (3%) have been reported from the dental epithelium of leopard gecko (Handrigan et al., 2010). Broadly comparable populations of slow-cycling cells have also been documented in adult rat neocortex (4.9% following a 168-day chase; Zhang et al., 2015) and in the invertebrate hydra endodermal epithelium (1.8%; Govindasamy et al., 2014),
although differences in organismal biology and experimental design make accurate comparisons between other models challenging.

One of our most striking findings is that all ELCs, including all slow-cycling cells, express SOX2. SOX2 is a hallmark transcription factor of NSPCs both in vitro (Takahashi and Yamanaka, 2006) and in vivo (Meletis et al., 2008; Gaete et al., 2012; Johnston et al., 2013; Fei et al., 2014). SOX2 plays a fundamental role in neuronal cell maintenance, proliferation and differentiation (Graham et al., 2003; Ferri et al., 2004). Although SOX2 expression has previously been observed in cells lining the ventricles within the brain of turtles (Trujilo-Cenóz et al., 2014), as well as the non-neuronal dental epithelia of snakes, geckos, alligators, and iguana (Gaete and Tucker, 2013; Juuri et al., 2013), this work marks the first report of SOX2 expression within the spinal cord of any reptilian species. Previous work investigating Xenopus tadpoles has established a strong correlation between SOX2 expression and regenerative-competence. The ELCs of tadpoles, which are capable of spinal cord regeneration, are SOX2+. However, following metamorphosis ELCs no longer express SOX2, and the spinal cord is no longer capable of regeneration (Gaete et al., 2012). The fundamental role of SOX2 in spinal cord regeneration is also supported by a study where CRISPR-mediated deletion of SOX2 inhibited cell proliferation and subsequent spinal cord regeneration in axolotls (Fei et al., 2014). One significant difference between our geckos and other regeneration-competent species was that we observed a constant global expression of SOX2 among ELCs, rather than a transient up-regulation following injury. In this respect, the gecko central nervous system appears to be more similar to that of embryonic mammals (Hutton and Pevny, 2011). In the developing mouse brain, constitutive SOX2 expression is limited to cell populations with the greatest stem potential, viz. radial glial cells (Hutton and Pevny, 2011). We predict that the ELC population in geckos is
fundamentally a more substantial pool of stem/progenitor cells than is observed in mammals, which may explain the remarkable regenerative abilities of this species.

We also found that gecko ELCs are activated following tail loss, expressing a distinctive panel of protein markers in addition to SOX2 and Vimentin. In particular, the NSPC marker MSI1, and the lineage-restricted markers SOX9 and TUJ1 were not detected until the spinal cord was ruptured. MSI1 is an RNA-binding protein involved in the maintenance of Notch-1 signalling by NSPCs (Nakamura et al., 1994) and is known to mediate asymmetric division (Okano et al., 2005). Interestingly, the *Xenopus* homolog of MSI1 (*nrp-1*) is widely expressed in the tadpole regenerative-competent spinal cord, but not in adults, where regenerative abilities are lost (Richter et al., 1990). The expression patterns of SOX9 and TUJ1 indicate that multiple cell types may be present among the ELC population. SOX9 is a transcription factor known to participate in the switch from neurogenesis to gliogenesis during embryonic development (Stolt et al., 2003), and hence marks glial populations. In contrast, TUJ1 is a neuron-specific lineage marker (e.g. Reubinoff et al., 2001; Toma et al., 2001). We hypothesize that SOX9+ ELCs in the tail following autotomy are ERGs, although the possibility that these cells are activated astrocytes cannot be excluded. A small number of ELCs within the regenerating spinal cord also expressed TUJ1 with a staining pattern similar to that observed in the developing mouse spinal cord (Petracca et al., 2016). To our knowledge this is the first report of its expression in ELCs during spinal cord regeneration in a lizard and provides compelling evidence that at least some cells within the regenerate spinal cord share neuronal-like properties. Taken together, our SOX9 and TUJ1 data strongly support the idea that ELCs within the regenerate spinal cord are a heterogeneous population.
Matching the obvious change in protein expression, ELCs also become increasingly proliferative following injury. Prior to injury, only a small subset of ELCs are PCNA+ and fewer than 10% of ELC population had cycled during a 7-day BrdU pulse. Following tail loss and throughout regeneration, we found that in the regenerating spinal cord almost all ELCs expressed PCNA. A similar proliferation response has been documented in the cerebral cortex of the lizard, Gallotia galloti, where a physical injury to the cortex produced an increase in mitotic cells between 1-30 days post-injury (Romero-Aleman et al., 2004). An increase in cell proliferation following injury has also been documented following spinal cord injury in a variety of teleosts including eels (Dervan and Roberts, 2003), zebrafish (Reimer et al., 2008), goldfish (Takeda et al., 2008), and various species of knifefish (e.g. Anderson and Waxman, 1985). Injury-mediated cell proliferation has also been observed in Xenopus (Gaete et al., 2012), axolotls (Zhang et al., 2000), and fresh-water turtles (Trachemys dorbignyi;Rehermann et al., 2011). It should be noted, however, that cell proliferation in response to central nervous system injury is not restricted to regeneration-competent species. In mice, ELCs were shown to be the most proliferative cell type in the post-injury spinal cord (demonstrating a 5.5-fold increase over uninjured controls; Barnabe-Heider et al., 2010). Taken together, our results show that normally quiescent ELCs demonstrate an obvious injury-mediated response, indicating involvement in the reparative response.

2.4.2 Heterogeneity in the regenerate spinal cord

Autotomy-activated ELCs are a heterogeneous population of cells. In response to tail loss, the once uniform ELC population is induced to yield (at least) two distinct cell types herein interpreted as ependymo-radial glia (ERG) and cerebrospinal fluid contacting cells (CSF-c cells).
(Figure 13). Both cell types are SOX2+ and NeuN-, and both occupy positions lining the central canal of the regenerate spinal cord. However, ERGs and CSF-c cells demonstrate inverted expression patterns for the astrocytic marker GFAP and the neuronal marker HuC/D (ERGs are GFAP+ and HuC/D−; CSF-c cells are GFAP− and HuC/D+). Consistent with their glial affinities, it also seems likely that ERGs also express Vimentin and SOX9, while CSF-c cells, as a special class of neuronal cell, also express TUJ1.

ERGs are best known from non-amniotes, including zebrafish and urodeles and uniquely characterized as having features common to both ependymal cells and astrocytes (Becker and Becker, 2015). Our interpretation of ERGs in geckos is drawn from various lines of evidence and is, to the best of our knowledge, the first time this cell type has been reported in the spinal cord of an adult amniote. In geckos (as for other species) the ERG somata contribute to the lining of the central canal, with a lengthy radial process making contact with the pial surface (Szaró and Gainer, 1988; Holder et al., 1990). Consistent with other studies, gecko ERGs express the NSPC protein SOX2, the classic glial marker GFAP, as well as Vimentin and (most likely) SOX9 (e.g., Sottile et al., 2006; Grupp et al., 2010; Stolt and Wegner, 2010; Becker and Becker, 2015).

Among regeneration-competent species, ERG are known for demonstrating astrocytic-like features including the ability to restore the blood-spinal cord barrier and being able to modulate glutamate and ion homeostasis (Grupp et al., 2010). Similar to previous studies involving the regeneration-competent urodele *Plethodon cinereus*, we observed that Vimentin expression was limited prior to injury but becomes more widespread following spinal cord rupture (Dawley et al., 2012). These authors also reported that Vimentin expression by ERGs co-localized within the intermediate filament Nestin following tail loss (Dawley et al., 2012). Although we did not investigate Nestin, previous work in a different tail-regenerating lizard (*Podarcis muralis*) has
reported its expression in both the regenerating spinal cord as well as regenerating peripheral nerve axons (Alibardi, 2014). Overall, our data show that gecko ERGs represent an NSPC population closely resembling those of other spinal cord regenerating species (Norlander and Singer, 1978; Holder et al., 1990; Mchedlishvili et al., 2007; Hutton and Pevny, 2011; Becker and Becker, 2015).

In addition to ERGs, we also found that the regenerating/fully regenerated spinal cord contains CSF-c cells. Consistent with this interpretation, our immunofluorescence reveals a subset of SOX2+ ELCs express HuC/D and TUJ1 but not NeuN or GFAP. Gecko CSF-c cells are also characterized by an apical nucleus that contacts the central canal and a basal process that extends into the spinal cord (Vigh et al., 1983; Jalavand et al., 2014). The presence of CSF-c cells in the lizard spinal cord has also been proposed by earlier authors (Alibardi and Meyer-Rochow, 1990; Alibardi and Miolo, 1990; Alibardi et al., 1993) and a similar population of HuC/D+ CSF-c cells contributing to the ventricular lining of the turtle brain are capable of firing action potentials (Russo et al., 2004).

Previous work in lamprey has identified two classes of CSF-c cells that differ on the basis of electrophysiological properties (type 1 evoke action potentials, type 2 do not), cell morphology (type 1 protrude into the lumen of the central canal, type 2 do not), and protein expression (Jalavand et al., 2014). Although we are unable to determine which, if either, of these classes is present in geckos, available evidence points towards type 1 CSF-c cells. Gecko CSF-c cells express the neuronal markers HuC/D+ and TUJ1+ and share a similar morphology with type 1 CSF-c cells, with bulb-like endings that project into the central canal and ventro-laterally oriented processes (Russo et al., 2004; Jalalvand et al., 2014). Additionally, previous work has
hypothesized that CSF-c cells in lizards are GABA-ergic neurons (Alibardi et al., 1993). A more definitive characterization of these cells awaits electrophysiological investigation.

2.4.3 Non-ependymal SOX2+ cells are present in the original spinal cord

In addition to SOX2+ ELCs, we also observed a second population of SOX2+ cells restricted to the grey matter of the original spinal cord. One possibility is that these cells represent oligodendrocyte progenitor cells (OPCs) within the lizard spinal cord. At least in mammals, lineage-tracing has revealed that OPCs respond to injury in a similar manner to ELCs, although they appear unable to differentiate into multiple cell types (Johansson et al., 1999; Horky et al., 2006; Ohori et al., 2006; Barnabe-Heider et al, 2010). Another possibility is that SOX2+ cells within the grey matter represent a population of astrocytes. Across vertebrates, astrocytes have been shown to sometimes behave as NSPCs and are known to give rise to new neurons in the subventricular zone and dentate gyrus (Alvarez-Buylla et al., 2002; Song et al., 2002). More commonly, however, the astrocytic response to injury involves hypertrophy and scar formation (Barnabe-Heider et al., 2010; Sabelstrom et al., 2013). Future studies should seek to co-localize the expression of one or more oligodendrocyte progenitor markers and astrocyte markers with SOX2-expressing cells in the grey matter of the lizard spinal cord to permit their identification.

2.4.4 Does neurogenesis occur during spinal cord regeneration?

Among teleosts and urodeles, replacement neurons in the regenerate spinal cord are derived from a population of ELCs within the original spinal cord (Egar and Singer, 1972; Benraiss et al., 1996; Zhang et al., 2003; Reimer et al., 2008; Tanaka and Ferretti, 2009; Becker
and Becker 2015). Our data supports the idea that a comparable, albeit truncated, neurogenic process is activated during spinal cord regeneration in geckos. Within the gecko spinal cord, ELCs include latent populations of NSPCs that are activated in response to injury, proliferate and differentiate into at least two cell types: stem-like ERGs and neuronal-like CSF-c cells that exist within the regenerate spinal cord. We suspect that while ERG may play a critical role in producing the majority of cells that make up the regenerate spinal cord, CSF-c cells may be fundamental in re-establishing function through their neuronal-like properties. While we cannot conclude that neurogenesis does occur during spinal cord regeneration in the leopard gecko, taken together, our findings strongly support the idea that at least a subset of cells within the regenerate spinal cord are progressing along a neurogenesis trajectory. We predict that, at least within lizards, the truncation of this program allows for the re-establishment of function, without the need to fully recapitulate the structural organization of the original spinal cord.

2.4.5 Spinal cord regeneration proceeds along a developmental trajectory

Regeneration is not a simple recapitulation of development. During spinal cord formation, neurons, glial cells and ELCs are produced by the proliferation of neural stem cells (neuroepithelial cells and radial glia) within the ventricular layer that surrounds the central canal (Gotz and Huttner, 2005; Schoenwolf, 2009). Post-mitotic neurons are born first, followed by glia (astrocytes and oligodendrocytes) and finally ciliated ELCs (Schoenwolf, 2009). Paired alar plates in the dorsal spinal cord give rise to sensory neurons, while paired basal plates give rise to motor neurons (Schoenwolf, 2009). These regions are specified by domain-specific dorso-ventral patterning cues, including Sonic Hedgehog (SHH) signaling from the floorplate (reviewed in Litingtung and Chiang, 2000) Additionally, neural crest cells contribute to the formation of
sensory (dorsal root) ganglia (Schoenwolf, 2009). Taken together, the sequence of events leading
to spinal cord formation can be summarized as: the proliferation of neural stem cells,
axonogenesis, neurogenesis, establishment of dorso-ventral domains, and the formation of dorsal
root ganglia. We propose that spinal cord regeneration proceeds along a comparable trajectory
as seen during spinal cord development, but that the completeness of the program varies across
species (Figure 14). Moreover, the degree to which a species is capable of progressing along the
trajectory determines the degree to which they are able to regenerate their spinal cord. At one
end of the spectrum are teleosts and urodeles, which are capable of regenerating a structurally
and functionally equivalent organ (Tanaka and Ferretti, 2009). In these species, ELCs lining the
central canal proliferate following tail loss and give rise to a terminal vesicle (reminiscent of the
neural tube). Ultimately, ELCs contribute to the formation of domain-specific sensory and motor
neurons, as well as support cells (Chernoff et al., 2003; Schnapp et al., 2005; Reimer et al.,
2009). Concurrently, axonogenesis re-establishes functional connections, and dorsal root ganglia
are re-formed, yet appear to be derived from the spinal cord (Geraudie et al., 1988; Mchedlishvili
et al., 2012)

Among other species, the developmental trajectory is truncated. For example, while tadpoles
are capable of regenerating a structurally and functionally equivalent spinal cord, dorsal root
ganglia fail to form (Lin et al., 2007). In lizards, the program is further truncated: the regenerate
spinal cord is functional, but not a structural equivalent. Tail loss in lizards triggers the
proliferation of ELCs that rapidly form a terminal vesicle, and the initiation of axonogenesis re-
establishes functional connections within the new tail (Bellairs and Bryant, 1985; McLean and
Vickaryous, 2011; Gilbert et al., 2015). However, while previous investigations reported that
spinal cord regeneration in lizards was strictly the result of ELC proliferation, ependymal tube
outgrowth and axonogenesis (Tanaka and Ferretti, 2009), our results suggest neurogenesis also occurs. During regeneration a subset of ELCs express the pan-neuronal marker HuC/D, and are otherwise consistent in appearance and protein expression with type 1 CSF-c cells, a functional, neuronal-like population in other species (Jalalvand et al., 2014).

At the opposite end of the spectrum are birds and mammals, with an incomplete regenerative response. More specifically, the regenerative trajectory is almost immediately truncated and the spinal cord is neither functionally nor structurally replaced. ELCs respond to injury by proliferating (Barnabe-Heider et al., 2010), but instead of forming a terminal vesicle and supporting axonogenesis, these cells simply isolate the injured area and a glial scar is formed. As a result, axonogenesis fails to re-establish functional connections (Fawcett and Asher, 1999; Edgerton et al., 2004).
### TABLE 2 TABLES

**TABLE 1: Antibody Information**

<table>
<thead>
<tr>
<th>Name of Antibody</th>
<th>Immunogen and MW</th>
<th>Manufacturer, host species, mono-vs. polyclonal, Cat. No., antibody ID</th>
<th>Dilution used in IHC, IF and WB, approximate MW in the <em>E. macularius</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tubulin (loading control)</td>
<td>Sarkosyl-resistant filaments from sea urchin sperm axonemes MW: 50kDa</td>
<td>Sigma-Aldrich (St. Louis, USA) Mouse Monoclonal (IgG1) Cat#T5168, AB_477579</td>
<td>WB: 1:250,000 MW: 50kDa</td>
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<tr>
<td>β-actin (loading control)</td>
<td>Synthetic peptide corresponding to the amino-terminal residues of human β-actin MW: 45kDa</td>
<td>Cell Signalling (Whitby, Canada) Rabbit Polyclonal Cat# 4967, AB_330288</td>
<td>WB: 1:5000 MW: 45kDa</td>
</tr>
<tr>
<td>BrdU (Anti-Bromodeoxyuridine)</td>
<td>Purified immunoglobulin</td>
<td>Sigma-Aldrich (St. Louis, USA) Mouse Monoclonal (IgG) Cat#B8434, AB_476311</td>
<td>IF: 1:100</td>
</tr>
<tr>
<td>GFAP (Glial Fibrillary Acidic Protein)</td>
<td>Cow spinal cord MW: 50kDa</td>
<td>DAKO (Denmark) Rabbit Polyclonal (Ig fraction) Cat#Z0334, AB_60013382</td>
<td>IF: 1:200</td>
</tr>
<tr>
<td>HuC/D (Anti-human neuronal protein HuC/HuD)</td>
<td>Human HuC/HuD neuronal protein MW: ~40kDa (Marusich et al., 1994)</td>
<td>Molecular Probes (Rockford, USA) Mouse monoclonal (IgG2bk) Cat#16A11, AB_221448</td>
<td>IF: 1:10</td>
</tr>
<tr>
<td>MSI1 (Anti-Musashi-1)</td>
<td>GST-tagged recombinant protein MW: 39kDa</td>
<td>Millipore (Temecula, USA) Mouse Monoclonal (IgG2ak) Cat#MABE268, AB_2576205</td>
<td>IF: 1:100 WB: 1:2500 MW: ~37-50kDa</td>
</tr>
<tr>
<td>MSI1 (Anti-Musashi-1)</td>
<td>Synthetic peptide, amino acids 5-21 of Musashi MW: 39kDa</td>
<td>Millipore (Temecula, USA) Rabbit Polyclonal Cat#AB5977, AB_92184</td>
<td>IF: 1:100</td>
</tr>
<tr>
<td>Antibody Name</td>
<td>Description</td>
<td>Supplier</td>
<td>Cat#/AB#</td>
</tr>
<tr>
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<td>------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>NeuN (Anti Neuronal-Nuclei)</td>
<td>GST-tagged recombinant protein MW: 42/48 kDa</td>
<td>Millipore (Temecula, USA)</td>
<td>Rabbit Polyclonal Cat# ABN78, AB_10807945</td>
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<tr>
<td>PCNA (Proliferating Cell Nuclear Antigen)</td>
<td>Full length PCNA (human origin) MW:36kDa</td>
<td>Santa Cruz Biotechnology (Texas, USA)</td>
<td>Rabbit Polyclonal (IgG) Cat#sc-7907,AB_2160375</td>
</tr>
<tr>
<td>RT-97 (neurofilaments)</td>
<td>Wistar rat neurofilaments MW: 200kDa, 145kDa</td>
<td>DSHB (Iowa, USA)</td>
<td>Mouse Monoclonal (IgG1) Cat#RT97, AB_528399</td>
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<tr>
<td>SOX2 (sex-determining box region Y)-Box 2</td>
<td>Synthetic peptide MW: 35kDa</td>
<td>Cell Signalling (Whitby, Canada)</td>
<td>Rabbit Polyclonal (IgG) Cat#2748, AB_823640</td>
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<tr>
<td>SOX9 (SRY (sex-determining box region Y)-Box 9)</td>
<td>Synthetic non-phosphopeptide MW: 56kDa</td>
<td>Abcam (Toronto, Canada)</td>
<td>Rabbit Polyclonal (IgG) Cat#ab71762, AB_1270870</td>
</tr>
<tr>
<td>TUJ1 (Anti-Tubulin, Beta III)</td>
<td>Purified immunoglobulin MW: 50kDa</td>
<td>Millipore (Temecula, USA)</td>
<td>Mouse Monoclonal (IgG) Cat#MAB5564, AB_11212768</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Chick optic tectum MW:52kDa</td>
<td>DSHB (Iowa, USA)</td>
<td>Mouse Monoclonal (IgG1) Cat# H5, AB_528506</td>
</tr>
</tbody>
</table>
### TABLE 2: Summary Table for Optimized Immunohistochemistry Protocols for Proteins of Interest (PCNA, RT-97, SOX2, SOX9 and Vimentin)

<table>
<thead>
<tr>
<th></th>
<th>Quench (minutes)</th>
<th>Antigen Retrieval</th>
<th>Block</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>HRP</th>
<th>DAB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCNA</strong></td>
<td>30</td>
<td>None</td>
<td>1 hour at room temp, 5% NGS in 1X PBS</td>
<td><strong>1:500</strong>, overnight at 4°C (Santa Cruz Biotech, Cat# sc-7907)</td>
<td><strong>1:1000</strong>, 1 hour at room temp (Biotinylated goat anti-rabbit, Jackson Immuno-Research Laboratories, Cat# 111-066-003)</td>
<td>1:200 (Jackson Immuno-Research Laboratories, Cat# 016-030-084)</td>
<td>30 seconds, diluted in 5mL dH₂O (Peroxidase Substrate Kit, Vector Laboratories, SK-4100)</td>
</tr>
<tr>
<td><strong>RT-97</strong></td>
<td>20</td>
<td>None</td>
<td>1 hour at room temp, 5% NGS in 1X PBS</td>
<td><strong>1:100</strong>, overnight at 4°C (Developmental Studies Hybridoma Bank, Cat# RT-97)</td>
<td><strong>1:200</strong>, 1 hour at room temp (Biotinylated goat anti-mouse IgG, Vector Laboratories, Cat# BA-9200)</td>
<td>1:200 (Jackson Immuno-Research Laboratories, Cat# 016-030-084)</td>
<td>20 seconds, diluted in 5mL dH₂O (Peroxidase Substrate Kit, Vector Laboratories, SK-4100)</td>
</tr>
<tr>
<td><strong>SOX2</strong></td>
<td>30</td>
<td>None</td>
<td>1 hour at room temp, 5% NGS in 1X PBS</td>
<td><strong>1:500</strong>, overnight at 4°C (Cell- Signalling, Cat# 2748)</td>
<td><strong>1:10,000</strong>, 1 hour at room temp (Biotinylated goat anti-rabbit, Jackson Immuno-Research Laboratories, Cat# 111-066-003)</td>
<td>1:200 (Jackson Immuno-Research Laboratories, Cat# 016-030-084)</td>
<td>15 seconds, diluted in 5mL dH₂O (Peroxidase Substrate Kit, Vector Laboratories, SK-4100)</td>
</tr>
<tr>
<td>Protein</td>
<td>Time (min)</td>
<td>Blocking Buffer</td>
<td>Primary Antibody Dilution</td>
<td>Primary Antibody Incubation</td>
<td>Secondary Antibody Dilution</td>
<td>Secondary Antibody Incubation</td>
<td>Staining Solution</td>
</tr>
<tr>
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</tr>
<tr>
<td>SOX9</td>
<td>30</td>
<td>None</td>
<td>1:200, overnight at 4°C (Abcam, Cat# ab71762)</td>
<td>1:1000, 1 hour at room temp (Biotinylated goat anti-rabbit, Jackson Immuno-Research Laboratories, Cat# 111-066-003)</td>
<td>1:200 (Jackson Immuno-Research Laboratories, Cat# 016-030-084)</td>
<td>1 minute, diluted in 5mL dH2O (Peroxidase Substrate Kit, Vector Laboratories, SK-4100)</td>
<td>1 hour at room temp, 5% NGS in 1X PBS</td>
</tr>
<tr>
<td>Vimentin</td>
<td>30</td>
<td>None</td>
<td>1:100, overnight at 4°C (Developmental Studies Hybridoma Bank, Cat# H5)</td>
<td>1:500, 1 hour at room temp (Biotinylated goat anti-mouse IgG, Vector Laboratories, Cat# BA-9200)</td>
<td>1:200 (Jackson Immuno-Research Laboratories, Cat# 016-030-084)</td>
<td>30 seconds, diluted in 5mL dH2O (Peroxidase Substrate Kit, Vector Laboratories, SK-4100)</td>
<td>1 hour at room temp, 5% NGS in 1X PBS</td>
</tr>
<tr>
<td>Protein</td>
<td>Protocol Type</td>
<td>Retrieval Method</td>
<td>Rinse Type</td>
<td>Block</td>
<td>Primary Antibody</td>
<td>Secondary Antibody</td>
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<td></td>
</tr>
</tbody>
</table>
| BrdU    | One day       | 1) 2N HCL at 37°C for 30 min  
2) 0.1% Trypsin at 37°C for 20 min | 1X PBS | 5% NGS in diluent for 30 min at 37°C (see appendix X for recipe) | 1:100, 2 hours at 37°C in diluent (Sigma-Aldrich, Cat# B8434) | 1:200, 1 hour at room temp (Goat anti-mouse IgG Alexa Fluor 488, Life Technologies, Cat# A11001) |
<p>| GFAP    | One day       | none             | 1X PBS | 5% NGS diluted in 1X PBS for 1 hour at room temp | 1:1000, overnight at 4°C diluted in 1X PBS (DAKO, Cat # Z0334) | 1:1000, 1 hour at room temp (Cy3-conjugated Goat anti-Rabbit IgG, Cat# 111-165-144) |
| HuC/D   | Modified two day | Tris buffer retrieval at 95°C for 30 min | 1X PBS | 10% NGS in 0.3% triton-x-100 diluted in 1X PBS for 30 minutes at room temp | 1:10, overnight at 4°C diluted in 1% BSA in sterile 1X PBS. (Molecular Probes, Cat# 16A11) | 1:500, 1 hour at room temp (Goat anti-mouse IgG Alexa Fluor 488, Life Technologies, Cat# A11001) |
| MSI1    | Two day       | none             | 1X PBST | 10% NGS in 0.3% triton-x-100 diluted in 1X PBS for 1 hour at room temp | 1:100, overnight at 4°C diluted in 10% NGS in 1X PBS. (Millipore, Cat# MABE268) | 1:100, 1 hour at room temp (Goat anti-mouse IgG Alexa Fluor 488, Life Technologies, Cat# A11001) |</p>
<table>
<thead>
<tr>
<th>Protein</th>
<th>Day(s)</th>
<th>Diluent</th>
<th>Concentration 1:1</th>
<th>Temperature 1:1</th>
<th>Concentration 1:2</th>
<th>Temperature 1:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuN</td>
<td>Two day</td>
<td>none</td>
<td>1X PBST</td>
<td>10% NGS in 0.3% triton-x-100 diluted in 1X PBS for 1 hour at room temp</td>
<td>1:250, overnight at 4°C diluted in 1X PBS (Millipore, Cat# ABN78)</td>
<td>1:1000, 1 hour at room temp (Cy3-conjugated Goat anti-Rabbit IgG, Cat# 111-165-144)</td>
</tr>
<tr>
<td>PCNA</td>
<td>Two day</td>
<td>none</td>
<td>1X PBST</td>
<td>5% NGS diluted in 1X PBS for 1 hour at room temp</td>
<td>1:100, overnight at 4°C diluted in 1X PBS (Santa Cruz Biotech, Cat# sc-7907)</td>
<td>1:1000, 1 hour at room temp (Cy3-conjugated Goat anti-Rabbit IgG, Cat# 111-165-144)</td>
</tr>
<tr>
<td>RT-97</td>
<td>Two day</td>
<td>none</td>
<td>1X PBST</td>
<td>10% NGS in 0.3% triton-x-100 diluted in 1X PBS for 1 hour at room temp</td>
<td>1:400, overnight at 4°C diluted in 1X PBS (DSHB, Cat# RT97)</td>
<td>1:500, 1 hour at room temp (Goat anti-mouse IgG Alexa Fluor 488, Life Technologies, Cat# A11001)</td>
</tr>
<tr>
<td>SOX2</td>
<td>One day</td>
<td>1) Sodium citrate buffer at 95°C for 12 min 2) 0.1% Trypsin at 37°C for 20 min</td>
<td>1X PBS</td>
<td>5% NGS in diluent for 30 min at 37 °C (see appendix X for recipe)</td>
<td>1:50 (diluted in diluent. (Cell- Signalling, Cat# 2748)</td>
<td>1:100, 1 hour at room temp (Cy3-conjugated Goat anti-Rabbit IgG, Cat# 111-165-144)</td>
</tr>
<tr>
<td>TUJ1</td>
<td>Two day</td>
<td>none</td>
<td>1X PBST</td>
<td>10% NGS in 0.3% triton-x-100 diluted in 1X PBS for 1 hour at room temp</td>
<td>1:100, overnight at 4°C diluted in 10% NGS in 1X PBS. (Millipore, Cat# MAB5564)</td>
<td>1:1000, 1 hour at room temp (Goat anti-mouse IgG Alexa Fluor 488, Life Technologies, Cat# A11001)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Two day</td>
<td>none</td>
<td>1X PBS</td>
<td>5% NGS diluted in 1X PBS for 1 hour</td>
<td>1:50, overnight at 4°C diluted in 1X PBS (Goat anti-mouse IgG Alexa Fluor 488, Life Technologies, Cat# A11001)</td>
<td>1:500, 1 hour at room temp (Goat anti-mouse IgG Alexa Fluor 488, Life Technologies, Cat# A11001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>at room temp</td>
<td>PBS (DSHB, Cat# H5)</td>
<td>IgG Alexa Fluor 488, Life Technologies, Cat# A11001)</td>
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**TABLE 4:** Summary of Optimized Western Blotting Protocols for Proteins of Interest Requiring Validation (*TUJ1, MSX1+2, MSI-1, NeuN, Shh, SOX2, SOX9 and Vimentin*)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight (kDa)</th>
<th>Gel Percentage</th>
<th>Amount of Protein Loaded (µg)</th>
<th>Transfer Type, Voltage (V) and Time</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Exposure Method and Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUJ1</td>
<td>50</td>
<td>12%</td>
<td>30</td>
<td>Wet; 90 V for 90 minutes</td>
<td>1:5000 dilution (Millipore, Cat# MAB5564)</td>
<td>1:1000 dilution (Anti-mouse IgG HRP-linked, Cell-Signalling, cat# 7076)</td>
<td>ECL (Millipore Luminata Forte HRP substrate); 10 seconds</td>
</tr>
<tr>
<td>MSX1+2</td>
<td>28.2 and 30.5</td>
<td>12%</td>
<td>30</td>
<td>Wet; 90 V for 90 minutes</td>
<td>1:50 (DSHB, Cat#4G1)</td>
<td>1:2000 dilution Anti-mouse IgG HRP-linked, Cell-Signalling, cat# 7076)</td>
<td>ECL (Millipore Luminata Crescendo HRP substrate); 60 seconds</td>
</tr>
<tr>
<td>MSI1</td>
<td>39</td>
<td>12%</td>
<td>30</td>
<td>Wet; 90 V for 90 minutes</td>
<td>1:2500 dilution Millipore, Cat# MABE268)</td>
<td>1:2500 dilution (Anti-mouse IgG HRP-linked, Cell-Signalling, cat# 7076)</td>
<td>ECL (Millipore Luminata Forte HRP substrate); 60 seconds</td>
</tr>
<tr>
<td>Protein</td>
<td>Nucleus</td>
<td>Dilution</td>
<td>Voltage</td>
<td>Time</td>
<td>Primary Antibody</td>
<td>Secondary Antibody</td>
<td>Detection System</td>
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<td>-----------------</td>
</tr>
<tr>
<td>NeuN</td>
<td>42/48</td>
<td>12%</td>
<td>30</td>
<td>90 V for 90 minutes</td>
<td>1:2500 dilution (Millipore, Cat# ABN78)</td>
<td>1:2500 dilution (Anti-rabbit IgG HRP-linked, Cell-Signalling, cat# 7074)</td>
<td>ECL (Millipore Luminata Forte HRP substrate); 30 seconds</td>
</tr>
<tr>
<td>Shh</td>
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(Shh see appendix 14)
| Vimentin | 52 | 12% | 30 | Wet; 90 V for 90 minutes | 1:100 dilution (DSHB, Cat# H5) | 1:500 dilution (Anti-mouse IgG HRP-linked, Cell-Signalling, cat# 7076) | ECL (Millipore Luminata Forte HRP substrate); 30 seconds |
### TABLE 5: BrdU Cell Counts for Long Duration Pulse-Chase Experiment

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Figure 1: Anatomy of the spinal cord. (A) Schematic of the leopard gecko (*Eublepharis macularius*) in lateral view. The central nervous system (brain and spinal cord) are highlighted in white. Hatched lines indicate the location and plane of section of panels B-E. Note that the spinal cord passes continuously from the body into the tail. The original tail spinal cord in transverse (B) and longitudinal (C) view. The histological organization of the white matter, grey matter and ependymal layer closely resembles that of a
mammalian spinal cord. In contrast, the organization of the regenerate spinal cord, shown in transverse (D) and longitudinal (E), consists strictly of descending nerve tracts and a prominent ependymal layer surrounding the central canal; no grey matter is present. The regenerate spinal cord is surrounded by a cartilaginous cone. (B-E) Masson’s Trichrome. ∗= central canal; cc= cartilaginous cone; ct= connective tissue; dm= dura mater; el=ependymal layer; gm= grey matter; wm= white matter. Scale bar= 50µm.
Figure 2: NeuN expression is restricted to the original spinal cord while HuC/D is expressed by cells within both the original and regenerate spinal cords. The neuronal markers HuC/D and NeuN are expressed by cells throughout the grey matter of the original spinal cord (A,B); white hatched ellipses are examples. HuC/D+ axons are also present in the white matter (A, B). In the regenerate spinal cord (C,D) NeuN expression is
absent. However, isolated ELCs are HuC/D+ in the fully regenerated spinal cord (C-D), as well as throughout tail regeneration including stage IV (E), V (F) and VI (G); white arrows. *= central canal; el=ependymal layer; gm= grey matter; wm=white matter. Scale bar =20µm.
Figure 3: Histological overview of wound healing and tail regeneration following tail autotomy. Previous work (McLean and Vickaryous, 2011) has organized wound healing and tail regeneration into seven discrete morphological stages. (A) Stage I (0-24 hours post-autotomy) follows tail loss and is characterized by the ruptured spinal cord initially being exposed to the external environment and then passively retracting into the vertebral canal. (B) At stage II (1-7 days post-autotomy), the site of tail loss has developed a temporary exudate clot. Although there is no outward evidence of regeneration at this time, cell proliferation (and hence the earliest formation of the blastema) begins distal to the torn margin of the original spinal cord (hatched area). (C) By stage III (8-12 days post-autotomy) the clot is lost to reveal a newly completed wound epithelium. Deep to the wound epithelium the blastema begins to expand both distally and laterally (hatched area). A continuous outgrowth of the ependymal layer of cells from the original spinal cord begins to invade the blastema, giving rise to the regenerating spinal cord (rsc). At this stage the regenerating spinal cord is centrally positioned within the blastema, and has started to form a distal dilation, the ependymal ampulla. (D) Stage IV (13-18 days post-autotomy) is characterized by a dome-like blastema that continues to expand distally (hatched area). Concurrent with blastema outgrowth, the regenerating spinal cord continues to pass into the newly forming tail tissue. (E) Stage V (19-25 days post-autotomy) begins as the dome–like blastema continues to expand distally and marks the first phase of late outgrowth. As regeneration proceeds, specific tissues within the tail begin to differentiate in a proximal to distal fashion. Early evidence of musculature, adipose tissue and cartilage are evident proximally, while the spinal cord persists as the only differentiated structure in the distal blastema. (F) Stage VI (25-30 days post-
autotomy) is characterized by continued outgrowth and differentiation of specific tissue types within the tail. Cartilaginous support begins to surround the regenerate spinal cord during this late outgrowth phase. (G) Stage VII (~30 days post-autotomy) marks the completion of tail regeneration. Clearly defined integumentary structures, connective tissue, cartilaginous support and a regenerate spinal cord are visible. (H) Schematic summary of regenerative events related to the spinal cord. All histological sections are stained with Masson’s trichrome. *= central canal; at= adipose tissue; bl=blastema; cc= cartilaginous cone; cl=clot; de=dermis; em=epaxial muscle; hm=hypaxial muscle; no=notochord; rsc=regenerate spinal cord; sc= spinal cord; we=wound epithelium. Scale bar =500µm.
Figure 4: A subset of ELCs are slow-cycling. (A) Schematic representation of the injection sites (black arrows) and dosage of bromodeoxyuridine (BrdU) administered (50mg/kg) during the pulse phase. (B) Timeline of injections and tissue collection. BrdU was injected twice daily for 7 days and tissue was collected at four timepoints (Pulse: Day 7, Chase: Days 30, 45 and 140). At each time point BrdU immuno-positive cells were present within the ependymal layer (C-F); white arrows, although the number of
BrdU positive cells appeared to diminish at 140 days (F). BrdU expression co-localized with SOX2 at all (G-J) time points. * = central canal; el = ependymal layer. Scale bar = 10
Figure 5: ELCs of the original spinal cord are activated following tail loss. Longitudinal sections of original spinal cord prior to tail loss (A), immediately following tail loss (less than 24 hours) (B) and from a fully regenerated tail (C). Prior to tail loss, a subset of
ependymal cells express PCNA (D) while SOX2 is expression is ubiquitous (E). However, ELCs are immuno-negative for MSI1 (F), TUJ1 (G) and SOX9 (H). Following tail loss, protein expression changes. ELCs continue to express the proliferation marker PCNA (I), along with Vimentin (I) and SOX2 (J). In addition, ELCs begin to express the NSPC marker MSI1 (K) as well as the lineage-restricted markers TUJ1 and SOX9 (M); inset in (M) is the omission control. Following regeneration a subset of ELCs from the original stump continue to express PCNA (N), as well as NSPC markers Vimentin (N), SOX2 (O). However, MSI1 is no longer expressed (P). Expression of lineage-restricted markers TUJ1 (Q) and SOX9 (R) persists. *= central canal; el=ependymal layer. Scale bar = 10µm.
Figure 6: Cells of the early blastema proliferate and express NSPC markers. Caudodorsal view of a stage II regenerating tail (A). As revealed by serial histology the spinal cord has retracted into the vertebral canal, and a clot has formed at the distal end of the cord. This stage also marks the first evidence of the blastema, juxtaposed between the ruptured end of the spinal cord and the clot (B). Cells within the early blastema (hatched box in B) express the proliferation marker PCNA (C), and the NSPC markers SOX2 (D) and MSI1 (E). Scale bar = 10µm.
Figure 7: ELCs are activated following tail loss. Histological sections in transverse view of ELCs prior to (A) and throughout regeneration (B-E) stained with Masson’s trichrome. All ELCs in the original (F), regenerating (G-I) and fully regenerate spinal cord
(J) express the NSPC marker SOX2. Prior to injury (K), cell proliferation (as evidenced by PCNA expression) is limited to isolated cells and the intermediate filament Vimentin was not detected. During tail regeneration, the majority of ELCs are immunoreactive for PCNA and Vimentin is expressed by all (L-O). Expression of NSPC marker MSI1 and lineage-restricted marker TUJ1 is also absent from the original ependymal layer (P). During the outgrowth phases, MSI1 expression is near ubiquitous in ELCs (Q-T) and TUJ1 expression is peri-nuclear in a subset of ELCs (Q-T). *= central canal; el=ependymal layer. Scale bar = 10 µm.
Figure 8: ELCs of the regenerate spinal cord continue to express proliferation and neural stem/progenitor cell (NSPC) markers following regeneration. Longitudinal sections of regenerated spinal cord (A) were immuno-stained for a panel of markers following the completion of regeneration. A subset of ELCs in the regenerate spinal cord are PCNA immuno-positive (B, C) while mesenchymal marker Vimentin (B) and NSPC markers SOX2 (C) and MSI1 (D) are expressed in all ELCs. Several cells of the ependymal layer also expressed the neuronal lineage-restricted TUJ1 (white arrows) but do not express mature neuronal marker NeuN (E). A subset of ELCs were also positive
for the glial lineage-restricted marker SOX9 (F) inset in (F) is the negative control. *=
central canal; el=ependymal layer. *Scale bar = 10µm.*
Figure 9: Summary of protein expression data for ELC activation. Within the original ependymal layer prior to tail loss, a subset of ELCs express proliferation marker PCNA and NSPC markers SOX2 and Vimentin are also expressed (A). Following tail loss, protein expression changes, ELCs almost all express PCNA and the NSPC panel expands to include not only expression of SOX2 and Vimentin, but also MSI1 as well as lineage-restricted progenitor markers, TUJ1 and SOX9 (B). During regeneration all markers are expressed (C). This expression pattern persists in the original spinal cord following regeneration with the exception of MSI1, which is no longer expressed (D). Within the completed regenerated ependymal layer (E), all markers are expressed.
Figure 10: ELCs express the radial glial markers Vimentin and GFAP following tail loss. Prior to tail loss (A, B) ELCs are GFAP+ and weakly Vimentin + (arrow in B).

Following tail loss, ELCs (as well as descending tracts) express both GFAP and Vimentin+ (C-G). *= central canal; el=ependymal layer; gm= grey matter; wm=white matter. Scale bars in D and G= 20µm.
Figure 11: ELCs only express the neuronal marker HuC/D following tail loss. Prior to tail loss (A,B) neuronal cells of the grey matter and axons in the white matter, but not ELCs, express the neuronal marker HuC/D. Following tail loss, HuC/D is expressed by ELCs within the original tail stump (A’,B’), as well as by ELCs in the regenerated spinal cord (C, D). Double immunofluorescence reveals two populations of ELCs: HuC/D+, GFAP- cells; and HuC/D-, GFAP+ cells (C-G). GFAP expression is also seen in the
cartilage surrounding the regenerated spinal cord. * = central canal; cc = cartilaginous cone; el = ependymal layer; gm = grey matter; wm = white matter. Scale bars in F and I = 20 μm.
**Figure 12: ELCs of the regenerate spinal cord represent two distinct populations.**

All ELCs express the neural stem/progenitor markers SOX2 and MSI1 (white border-black shading), and most also express the intermediate filament markers GFAP and Vimentin. Combined, these data suggest that most ELCs represent ependymo-radial glia. A second, smaller population of ELCs are HuC/D+, GFAP-. We identify these cells as neuronal-like central canal-contacting cells, and argue that they are unique to the regenerating spinal cord.
Figure 13: Schematic representation of the cellular composition of the regenerate spinal cord. Within the regenerate spinal cord, at least two distinct populations of cells are present. The majority of ELCs are ependymo-radial glia (ERG: pink cells) that provide guidance for the regeneration of descending tracts (yellow circles) and may provide astrocytic-like support within the regenerate cord. A second, less abundant population of cells are cerebrospinal fluid contacting cells (CSF-c: green cell). Within the
leopard gecko, CSF-c are consistent with a Type-1 (neuronal-like CSF-c) and may play a fundamental role in the re-establishment of spinal cord function.
Figure 14: Spinal cord regeneration proceeds along a developmental-like trajectory. Across vertebrates, the ability of the spinal cord to regenerate varies dramatically. In urodeles and teleosts, spinal cord regeneration results from an ependymal response, axonogenesis, neurogenesis, re-establishment of dorso-ventral domains and the reconstitution of dorsal root ganglia to restore both structure and function of the spinal cord. In anuran tadpoles, this process is truncated and while dorsal root ganglia do not re-form, the regenerate spinal cord appears structurally and functionally similar to the original. In lizards, the process is truncated further.
Regeneration includes an ependymal response, axonogenesis and some degree of neurogenesis to restore functionality, however, the structure of the regenerate spinal cord is markedly different. On the far end of the spectrum are birds and mammals, in these species, an ependymal response isolates the injury however neither structural nor functional regeneration of the spinal cord occurs.
Supplementary Figure 1: Anatomy of the original tail spinal cord resembles that of the body spinal cord. (A) Dorsolateral view of the leopard gecko (*Eublepharis macularius*). Hatched lines indicate the locations of transverse sections (B), representing the body spinal cord, and (C), representing the original tail spinal cord. (B-C) Hematoxylin and eosin. *= central canal; el=ependymal layer; gm= grey matter; wm=white matter. *Scale bar = 100µm.*
Supplementary Figure 2: Variation in cross-sectional anatomy of the spinal cord along the proximal-distal axis of the original and regenerate spinal cord. Dorsal view of the original (A) and regenerate (B) tail (hatched lines=transverse sections in the proximal (C, F), medial (D, G) and distal (E, H) spinal cord). RT-97+ neurofilaments are
common throughout the white matter of the original spinal cord, whereas the grey matter is characterized by NeuN+ neuronal cell bodies (C-E). Although the distinction between grey and white matter is obvious in proximal locations, it becomes blurred in the distal-most third of the spinal cord (E). Interestingly, the outer diameter of the ependymal tube remains relatively constant across the proximal (C), medial (D) and distal (E) spinal cord (hatched lines), whereas the diameter of the central canal appears to increase. Although RT-97+ neurofilaments are present surrounding the ependymal layer of the regenerate spinal cord (F-H), NeuN expression is absent. Nuclei are stained with DAPI (C-H). *= central canal ; cc=cartilaginous cone; el=ependymal layer; gm=grey matter; wm=white matter. Scale bar= 20µm.
Supplementary Figure 3: Western blot analysis for Musashi-1 an ~39kDa protein expressed by neural stem/progenitor cells. *E. macularius* brain and spinal cord homogenates were used to analyze protein specificity in the tissue of interest. 30 µg of protein was loaded, incubated with anti Musashi-1 antibody and visualized using HRP substrate and chemi-luminescence. Multiple bands were detected between ~35-50kDa in all three biological replicates (A-C). α-Tubulin was used as a loading control for each sample and was observed at the predicted molecular weight: 50kDa (D-F)
**Supplementary Figure 4: Western blot analysis for NeuN an ~42kDa protein expressed by mature neurons.** *E. macularius* brain and spinal cord homogenates were used to analyze protein specificity in the tissue of interest. 30 µg of protein was loaded, incubated with anti NeuN antibody and visualized using HRP substrate and chemiluminescence. Bands were detected at ~42kDa in all three biological replicates (A-C). A faint band was also observed at ~48kDa (as predicted by the manufacturer’s information), and uncharacterized bands were also observed at ~70 and 80kDa (also predicted by the manufacturer’s information). α-Tubulin was used as a loading control for each sample and was observed at the predicted molecular weight: 50kDa (D-F)
Supplementary Figure 5: Western blot analysis for SOX2, a 35 kDa protein required for the maintenance of self-renewal. *E. macularius* brain and spinal cord homogenate were used to analyze protein specificity in the tissue of interest. 30 µg of protein was loaded, incubated with SOX2 antibody and visualized using HRP substrate and chemi-luminescence. Bands were detected at 35kDa in all three biological replicates (A-C). α-Tubulin was used as a loading control for each sample and was observed at the predicted molecular weight: 50kDa (D-F)
Supplementary Figure 6: Western blot analysis for SOX9 an ~56kDa protein expressed by radial glial cells and during gliogenesis. *E. macularius* tail tissue homogenates including: original tail and stages IV-VI as well as Hamilton and Hamburger staged 29, 31 and 33 embryonic chick embryo homogenates were used to analyze protein specificity in the tissue of interest. 30 µg of protein was loaded, incubated with anti SOX9 antibody and visualized using HRP ECL substrate and exposed to X-ray film for 10 seconds. Bands were detected at ~56kDa in (A). A faint band was also observed at ~15kDa (as predicted by the manufacturer’s information) in the tail samples, and uncharacterized bands were also observed at ~75kDa in the embryonic chick samples. α-Tubulin was used as a loading control for each sample and was observed at the predicted molecular weight: 50kDa (B)
Supplementary Figure 7: Western blot analysis for Beta-III-Tubulin, a 50kDa protein expressed by neurons and neural progenitor cells. *E. macularius* brain and spinal cord homogenates were used to analyze protein specificity in the tissue of interest. 30 µg of protein was loaded, incubated with anti Beta-III-Tubulin antibody and visualized using HRP substrate and chemi-luminescence. Bands were detected at ~50kDa in all three biological replicates (A-C). α-Tubulin was used as a loading control for each sample and was observed at the predicted molecular weight: 50kDa (D-F)
Supplementary Figure 8: Western blot analysis for Vimentin, a 52kDa protein expressed by neural stem/progenitor and mesenchymal cells. *E. macularius* brain and spinal cord homogenates were used to analyze protein specificity in the tissue of interest. 30 µg of protein was loaded, incubated with anti Beta-III-Tubulin antibody and visualized using HRP substrate and chemi-luminescence. Bands were detected at ~56kDa in all three biological replicates (A-C). α-Tubulin was used as a loading control for each sample and was observed at the predicted molecular weight: 50kDa (D-F)
Supplementary Figure 9: ELCs express neural stem/progenitor cell marker SOX2.

Cells within the ependymal layer of the spinal cord in original, regenerate and regenerating spinal cord express NSPC marker SOX2. In the original spinal cord, shown here in transverse (A) and longitudinal view (B), ELCs along with a small subset of cells within the grey matter are immunopositive for SOX2. In the regenerate spinal cord, SOX2 expression is restricted to the ependymal layer (C). Cells in the surrounding connective
tissue and cartilaginous cone are immuno-negative for SOX2 (D). During regeneration SOX2 expression is consistent with that of the original and regenerate spinal cord, in stage IV (E) and stages V(F) and VI (G). cells within the ependymal layer ubiquitously express SOX2. *= central canal; el=ependymal layer; gm= grey matter; wm=white matter. *Scale bars in D and G= 20μm.
CHAPTER 3:

CELL TRACKING DURING THE INTIATION OF SPINAL CORD REGENERATION IN THE LEOPARD GECKO (*EUBLEPHARIS MACULARIUS*)

3.1 INTRODUCTION

Many lizards are able to voluntarily detach (or autotomize) a portion of their tail to escape predation and then regenerate a replacement. Tail regeneration begins with scar-free wound healing and the formation of a blastema, an aggregation of proliferating, mesenchymal-like cells (Hughes and New, 1959; Moffat and Bellairs, 1964; Bellairs and Bryant, 1985; McLean and Vickaryous, 2011; Delorme et al., 2012; Gilbert et al., 2015). Although it is widely understood that the blastema represents the primary source of new, lineage-restricted cells within the replacement tail (Gilbert et al., 2015), the origin of the contributing cells – at least in lizards – remains unclear.

Emerging evidence from limb-regenerating axolotls (Kragl et al., 2009) and digit tip regenerating mice (Rinkevich et al., 2011) indicates that the blastema is a heterogeneous pool recruited from multiple sources. More specifically, the blastema is derived from multiple resident (i.e., non-circulating) tissue-specific populations (reviewed in Poss et al., 2003; Kragl et al., 2009; Rinkevich et al., 2011). For the spinal cord, these resident stem/progenitor populations are referred to as ependymal layer cells (ELCs). ELCs line the central canal and appear to be essential for spinal cord regenerative success across species (Norlander and Singer, 1978; Benraiss et al., 1999; Zhang et al., 2003, Garcia et al., 2012). In response to spinal cord injury
(SCI), ELCs change their proliferative status and protein expression and at least in some teleosts and urodèles, serve as the primary (if not exclusive) source of cells restoring the spinal cord (O’Hara et al., 1992; Chernoff et al., 1998; Zhang et al., 2000; Mchedlishvili et al., 2007; see also Chapter 2). For taxa such as lizards however, less is known.

One strategy to identify the cellular origin of new tissues is to label original populations \textit{in situ} and then track these cells into the regenerated organ. Here we use microelectrode-mediated electroporation to visualize resident populations of ELCs. Electroporation uses weak electrical pulses to temporarily displace lipid molecules, thus increasing the permeability of the cell membrane and allowing large, charged molecules to enter the cell (Neumann et al., 1982; Potter, 1988). Previous work has successfully employed electroporation to transf ect resident cells of the central nervous system with plasmids encoding fluorescent proteins in various species including \textit{Xenopus} tadpoles (Haas et al., 2001; Lin et al., 2007), axolotls (Mchedlishvili et al., 2007) and embryonic (Saito, 2006) and adult mice (Barnabe-Heider et al., 2008). Once transfected, migrating cells can then be visualized in the regenerated tissue, providing evidence of tissue origin (Potter, 1988; Echeverri and Tanaka, 2003).

Using \textit{in vivo} electroporation, we sought to label and track cells from the spinal cord of the representative tail-regenerating lizard, the leopard gecko (\textit{Eublepharis macularius}). Our primary targets for transfection were ELCs from the original spinal cord. Although our results were mixed, we did manage to label cells of the original spinal cord. Based on our findings we provide recommendations for future efforts to visualize cell tracking in lizards, specifically, the use of viral-based vector cell tracking.

\textbf{3.2 METHODS}
3.2.1 ANIMAL CARE

Captive bred leopard geckos (*Eublepharis macularius*) were obtained from a commercial supplier (Global Exotic Pets, Kitchener, Ontario, Canada). Geckos ranged in mass from 12-55 grams. Mass, snout-vent length and tail measurements were recorded each week to track growth and monitor the health of experimental geckos (e.g. Appendix 1). Animal Usage Protocols 1954 (tissue collection and electroporation); 2493 (cutaneous biopsies); and 1847 (anesthesia) were approved by the University of Guelph Animal Care Committee. All Animal Usage Protocols followed the procedures and policies of the Canadian Council on Animal Care and closely adhered to the ARRIVE guidelines (Kilkenny et al., 2010). All gecko husbandry practices were consistent with the protocols laid out in Vickaryous and McLean (2011). Leopard geckos were housed at the Hagen Aqualab at the University of Guelph in an environmental chamber with an ambient temperature of 27.5°C and a 12 hour light: 12 hour dark photoperiod. Geckos were housed individually in five gallon plastic (polycarbonate) tanks. A temperature gradient was created in each of the tanks using a subsurface heating cable (Hagen Inc., Baie d’Urfe, Quebec, Canada) under one side of each enclosure. Geckos were fed a diet of gut-loaded mealworms (larval *Tenebrio* spp.) dusted with powdered calcium and vitamin D3 (cholecalciferol) supplement (Zoo Med Laboratories Inc., San Luis Obispo, California, USA). Each gecko received three mealworms per day. For eGFP optimizations in embryonic chick n=12 and for eGFP trials in leopard geckos n=10 (for all trials combined).

3.2.2 ANESTHESIA

Leopard geckos were anesthetized by intramuscular injection of 30mg/kg Alfaxan (Alfaxalone) (Table 1; diluted 2mg/mL in sterile, injectable 0.9% sodium chloride) in a 0.5cc
insulin syringe (Abbott Laboratories, Saint-Laurent, Quebec, Canada). Alfaxan is a synthetic 
neuroactive steroid that has been successfully used in clinics to achieve a surgical anesthetic 
plane in reptiles including tortoises (Agrionemys horsfieldii; Hansen and Bertelsen, 2013), turtles 
(Trachemys scripta elegans) and green iguanas (Iguana iguana; Bertelsen and Sauer, 2011). To 
our knowledge, this is the first time Alfaxan has been used in leopard geckos (hereafter 
‘geckos’). Alfaxan induces anesthesia through activity on GABA<sub>A</sub> receptors within the central 
nervous system. It works by enhancing the effects of GABA at this receptor resulting in a 
hyperpolarization of cells and the inhibition of action potential production. Injections were 
performed bilaterally in a divided dose on either side of the spinal cord in geckos (into the 
epaxial muscles). The optimal dose of Alfaxan was determined by evaluating induction, plateau 
and recovery time of each individual at two different doses (20mg/kg and 30mg/kg; see 
Appendix 12).

3.2.3 AUTOTOMY

To expose the original spinal cord for central canal electroporation we performed tail 
autotomy (Chapter 2). Briefly, autotomy was accomplished by grasping the distal third of the tail 
(between the index finger and thumb) and applying constant pressure until the tail self-detached. 
However, since autotomy was immediately followed by electroporation, geckos were first 
anesthetized and then, once the surgical plane of anesthesia was reached, autotomized. Following 
electroporation, geckos were allowed to recover and the tails started to regenerate. Tails were 
staged daily (following the morphological criteria of McLean and Vickaryous, 2011). After a 
predetermined length of time (Table 1) a second autotomy was performed to collect the 
transfected tissue.
3.2.4 BIOPSIES

To optimize electroporation parameters, we sought to transflect cells in two superficial locations following the creation of a full-thickness biopsy excisional wound (Figure 1A). These locations included a dorsal tail biopsy (Figure 1B) and a biopsy to an early formed (stage III; McLean and Vickaryous, 2011) blastema (Figure 1C). To perform biopsies, geckos were anesthetized with Alfaxan. Once a surgical plane was reached, full-thickness excisional biopsies were created using a 3mm disposable biopsy tool (Integra Miltex, Burlington, Ontario, Canada) following the protocol of Peacock et al. (2015). Briefly, biopsies to the original tail skin were centered on one large, prominent tubercle scales. Depth of the biopsy was adjusted to include the epidermis, dermis and a portion of the hypodermis. Biopsies to the blastema were taken from the center of the outgrowing tissue, and the depth was modified to include the overlying wound epithelium and a small portion of the underlying blastema. Excised tissue was removed using forceps and a scalpel.

3.2.5 PLASMID PREPARATION

We used an enhanced green fluorescent protein (Figure 2; eGFP: generously provided by Dr. Andrew Bendall, University of Guelph, Guelph, ON). Plasmid preparation was performed using either the PureLink™ Quick Plasmid DNA Miniprep Kit (Invitrogen, Lohne, Germany) or the QIAGEN® Plasmid Maxi Kit (Hilden, Germany) following manufacturer’s protocols. DNA concentrations were quantified using a NanoDrop spectrophotometer. Plasmids were aliquoted and stored at -20°C until used.
3.2.6 PREPARATION OF EMBRYONIC CHICKS FOR ELECTROPORATION

To confirm the expression of eGFP by our expression vector, we electroporated embryonic chicks (≈48 hours-3.5 days: Hamilton and Hamburger (HH) stages 12-22; Hamilton and Hamburger, 1951) with our DNA solution. Prior to electroporation, 3mL of albumin was removed from each egg using a 5mL needle/syringe into the side of the egg. The injection location was then resealed using clear adhesive tape. Next, eggs were windowed using scissors and flat forceps, and the embryo was relocated to the centre of the window, allowing for ease of access during electroporation. The surrounding vitelline membrane was removed and a drop of fast green was added to visualize the underlying embryo. Using the micromanipulator/Hamilton syringe apparatus, eGFP was injected into the neural tube and then the embryo was electroporated (details below). Following electroporation, eggs were sealed with clear adhesive tape and placed back at 37°C in an incubator.

3.2.7 eGFP INJECTION AND ELECTROPORATION

We targeted four locations for eGFP injection. In Trial 1, eGFP was injected into the neural tube of embryonic chicks. In Trials 2-6, eGFP was either injected into the central canal of the spinal cord (Figure 1A; Trials 2,4-6) or into a full-thickness biopsy wound (Figure 1B, C; Trials 3, 5). Prior to injection, 10µL eGFP plasmid was combined with 1µL fast green. A Hamilton syringe, attached to a micromanipulator, was first filled with mineral oil and then a drawn glass capillary tip and connective tubing were mounted onto the syringe. The eGFP plasmid was then back-loaded into glass capillary to ensure precise and controlled injection of the plasmid. The plasmid was slowly injected into the site of interest and then the tip was retracted prior to electroporation. Electroporation was accomplished through the use of
electrodes spaced 1-2mm apart, connected to a BTX ECM 830 electroporator (Harvard Apparatus, Inc. Holliston, MA, USA; Figure 3). 100\(\mu\)L of sterile, 1X phosphate buffered saline (PBS) was added to the site of electroporation prior to pulsing to facilitate conductance. The number of pulses, pulse length, voltage and distance between pulses are summarized in Table 3. Following transfection, animals were left between 24 hours and 8 days before tissue was collected for analysis.

3.2.8 TISSUE COLLECTION AND PREPARATION

Embryonic chick tissue was collected by immersing embryos in 10% neutral buffered formalin (NBF; Protocol Supplies, Kalamazoo, Michigan, USA) for 24 hours, transferred to 70% ethanol and then processed. Transfected gecko tail tissues were collected by inducing a second autotomy event. Following this second autotomy, the detached tail was injected with 10% NBF and placed in a centrifuge tube containing NBF for ~24 hours to establish proper fixation. Following fixation, tissue was transferred to 70% ethanol prior to processing (see Chapter 2, Methods).

3.2.9 IMMUNOHISTOCHEMISTRY

Refer to Chapter 2, Methods for immunohistochemistry protocols. eGFP and GFP antibodies tested are summarized in Table 2.

3.2.10 IMMUNOFLUORESCENCE

Refer to Chapter 2, Methods for immunofluorescence protocols. eGFP and GFP antibodies tested are summarized in Table 2.
3.3 RESULTS

To label and track gecko (and embryonic chick) cells in vivo, we conducted a series of trials using microelectrode-mediated electroporation (Table 3). To confirm the efficacy of our eGFP plasmid for transfection and detection, we first electroporated embryonic chicks (HH 12-22). Only one of the 12 embryos electroporated survived and demonstrated successful eGFP transfection into the neural tube (a HH stage 22 embryo; Figure 4A). Transfected spinal cord tissue was confirmed ~24 hours following electroporation on a Leica stereo epi-fluorescent microscope (Figure 4A). Next, the embryo was fixed, embedded in paraffin and serial sectioned. On representative section we alternatively performed immunohistochemistry or immunofluorescence staining to visualize eGFP+ cells (see Chapter 2). Unexpectedly, we failed to identify eGFP+ cells within the neural tube (Figure 4B), although our immunofluorescence data did reveal a small number of immunoreactive cells in a somite located lateral to the neural tube (Figure 4B’). It should be noted however, that our immunofluorescence protocol yielded widespread auto-fluorescence (Figure 4B). Consistent with our immunofluorescence data, our immunohistochemistry failed to detect eGFP in the neural tube (Figure 4C,C’).

The remaining electroporation trials focused on gecko tissues. All geckos survived tail loss/biopsy and electroporation and showed no obvious signs of distress post-anesthesia. In trial 2 (n=1), we autotomized the tail and then targeted the central canal of the spinal cord for electroporation. We collected tail tissue 48 hours post-transfection. In trial 3 (n=1), we created a 3mm biopsy punch to the blastema of a regenerating tail (stage III; McLean and Vickaryous, 2011), followed by electroporation. We collected the tail tissue, including the site of biopsy, 48 hours post-transfection. Although tissues from trials 2 and 3 were fixed and prepared for paraffin
embedding, both were rendered unusable for histological analysis as a result of a power failure during tissue processing. In trial 4 (n=3), we autotomized the tail and then targeted the central canal of the spinal cord for electroporation. Tail tissues were collected tissue at two different time points following transfection: 48 hours (n=2) and 8 days (n=1). We failed to detect eGFP+ cells in tissues from both time points (Figure 5A, B). In trial 5 we made two important modifications to our protocol. First, we changed the electrodes from paddles to small needles (30.5 gauge PrecisionGlide Needles; Beckton Dickinson and Co, Franklin Lakes, NJ, USA). Second, we changed our plane of serial section from transverse (cross-section) to longitudinal, enabling us to capture more of the spinal cord in each section analyzed. For trial 5 (n=4) we both autotomized the tail and then targeted the central canal of the spinal cord (n=3) and we created a 3mm biopsy punch to a stage III blastema of a regenerating tail (n=1). Following transfection, tail tissue was collected at two different time points: 3 days post-electroporation (n=1, tail autotomy) and 5 days (n=2 tail autotomy, n=1 blastema biopsy). Once embedded in paraffin, all tissue was sectioned in the longitudinal plane. Although we did not detect any expression of eGFP in the blastema biopsy tissue, eGFP+ cells were present within the distal end of the the original spinal cord (Figure 6A, B). In an effort to increase our sample size and confirm results, we performed a sixth trial (n=3) employing the same procedure as the tail autotomy transfections conducted during trial 5. Somewhat surprisingly, we failed to detect any eGFP+ cells in any tissues collected at 3 days (n=2) and 9 days (n=1) post-transfection.

3.4 DISCUSSION

To track cells from the original spinal cord into the regenerating tissue of the tail, we used eGFP plasmid injection and transfection via electroporation. While we did detect a small
population of eGFP+ cells within the original spinal cord 3 days post-transfection (in one out of six trials), no labeled cells were observed in the newly formed blastema. At this time, the exact contribution of the original spinal cord to the regenerated tail/spinal cord remains unclear.

Among other species capable of spinal cord regeneration, it is well documented that cells of the regenerate spinal cord come from the original (Echeverri and Tanaka, 2002; Zhang et al., 2003; Mchedlishvili et al., 2007). Indeed, as demonstrated by tissue grafting experiments in axolotls (between wildtype and GFP expressing individuals), cells located within 500µm from the site of amputation are the primary source of cells required for spinal cord regeneration (Mchedlishvili et al., 2007). Whereas our immunohistochemistry and immunofluorescence data (see Chapter 2) lead us to predict an identical spinal cord source in geckos, confirmation of this hypothesis awaits future cell tracking investigations.

The use of electroporation to track cells in vivo is reported to have several advantages including speed of delivery, low cytotoxicity and that it typically does not result in high mortality rates when used on post-natal animals (Barnabe-Heider et al., 2008). While all our experimental geckos readily tolerated the electroporation and transfection procedure, we did encounter a number of technical challenges. In particular, we experienced difficulties with electrode placement, especially with respect to repeatability, issues with auto-fluorescence using AlexaFluor488-tagged antibodies and developing the protocol with access to only a small sample size (n=10).

While injecting the eGFP plasmid into the central canal of the spinal cord was technically feasible, positioning the electrodes and keeping them in place (on either side of the spinal cord) during the electroporation phase was both challenging and not easily replicated, due to the close association between the spinal cord and surrounding vertebrae. To address this issue, we
investigated different shaped electrodes comparing paddle-shaped with pin-shaped. Unfortunately, neither of these options were small enough for placement alongside the ruptured spinal cord. As an alternative, we propose that future cell-tracking studies should employ a viral-based vector that can be injected into the central canal. A viral-based vector is likely to achieve a higher transfection efficiency and because different viruses have different tropisms, might demonstrate a greater degree of specificity to target ELCs (Karra and Dahm, 2010). The most obvious disadvantages of this approach are the need to address biosafety concerns and limited access to isolation housing facilities. We propose that future experiments should utilize either an adenovirus or a lentivirus-based vector. Adenoviruses have the advantage of preferentially transfecting glial cells, although they are reported to induce substantial immune responses when used in vivo (Buning et al., 2008). Alternatively, lentiviruses are capable of infecting non-dividing cells and have high transfection efficiencies, making them an ideal mode for transfection of an expression vector for cell-tracking studies (Karra and Dahm, 2010). Lentiviral vectors have been used successfully in mammalian models of spinal cord injury, specifically to introduce neurotropic factors such as brain-derived neurotrophic factor (BDNF) to the site of injury, thus promoting axonal outgrowth (Tuinstra et al., 2012). Additionally, eGFP lentiviral vectors have been previously used to successfully label neural stem/progenitor cells in vitro (Mothe et al., 2005; Okada et al., 2005).

A second issue we encountered was non-specific staining and auto-fluorescence, particularly from blood cells. We suggest that our problems with immunostaining were primarily the result of our use of AlexaFluor488-tagged secondary antibodies. Auto-fluorescence of gecko blood cells appears to closely match the excitation of this bright green fluorescent dye. To address this limitation, we recommend that future studies employ an alternatively labelled
secondary antibody, such as Cy3. Related to this, we further recommend that future viral-based transfection or electroporation studies should employ mCherry expression vector or a comparable fluorophore.

Lastly, we acknowledge that the number of geckos used was insufficient to optimize the electroporation procedure. Thus 10 experimental geckos, was likely an insufficient sample size to document in vivo eGFP transfection. Previous in vivo studies have shown that the transfection efficiency of electroporation can be comparatively low. For example, electroporation studies of axolotls use sample sizes ranging from 25-100 animals, with transfection success rates ranging from 1% to 30%, with an average of three labelled-cells per axolotl (depending on electroporation parameters such as pulse length and voltage; Echeverri and Tanaka, 2003). While it is possible to increase our sample size, unlike other common electroporation models (i.e. embryonic chicks, embryonic mice and axolotls), geckos are not translucent, and therefore epifluorescence alone cannot be used to detect eGFP+ cells. Increasing the sample size would necessitate substantial increases in workload and most significantly, unnecessary animal usage. The use of viral-based techniques hold greater promise for higher rates of transfection and hence reduced animal usage, consistent with the ARRIVE guidelines (Kilkenny et al., 2010).

In spite of our inconclusive cell tracking experiments, available data from geckos (see Chapter 2) and mounting evidence drawn from comparative investigations (e.g., axolotls, mice) continues to support the prediction that ELCs from the original spinal cord are the primary cellular source for the regenerated spinal cord in geckos. Although our recommended approach for future investigations is the use of a viral-based vector, some limitations of this technique are worth acknowledging. Specifically, viral-based cell tracking could introduce immune or inflammatory responses, which could change the environmental conditions within the gecko.
spinal cord following injury (Karra and Dahm, 2010). Additionally, the use of this technique does not guarantee the exclusive cell tracking of ELCs within the spinal cord, making it difficult to confirm their identity as the source of cells that make up the regenerate spinal cord.
CHAPTER 3 TABLES

**TABLE 1: Alfaxalone Doses and Dilutions**

<table>
<thead>
<tr>
<th>Size of Gecko</th>
<th>Dilution</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
<th>Alfaxalone Volume</th>
<th>0.9%NaCl Volume</th>
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</thead>
<tbody>
<tr>
<td>10-15g</td>
<td>1:4</td>
<td>10mg/mL</td>
<td>2mg/mL</td>
<td>300µL</td>
<td>1200µL</td>
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<tr>
<td>16-30g</td>
<td>1:3</td>
<td>10mg/mL</td>
<td>2.5mg/mL</td>
<td>500µL</td>
<td>1500µL</td>
</tr>
<tr>
<td>31-65g</td>
<td>1:2</td>
<td>10mg/mL</td>
<td>3.3mg/mL</td>
<td>600µL</td>
<td>1200µL</td>
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</table>
TABLE 2: Antibody Information

<table>
<thead>
<tr>
<th>Name of Antibody</th>
<th>Immunogen and MW</th>
<th>Manufacturer, host species, mono-vs. polyclonal, Cat. No., antibody ID</th>
<th>Dilution used in IHC, IF and WB, approximate MW in the <em>E. macularius</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>DSHB-GFP-12A6</td>
<td><em>Aequorea victoria</em> MW: 27kDa</td>
<td>DSBH (Iowa, USA) Mouse Monoclonal (IgG1, kappa light chain) Cat#DSHB-GFP-12A6, AB_2617417</td>
<td>IHC: 1:10-1:100 IF: 1:10-1:100</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>Highly purified recombinant full length GFP made in <em>Escherichia coli</em></td>
<td>Abcam (Toronto, Canada) Rabbit Polyclonal (IgG) Cat#ab6556, AB_305564</td>
<td>IHC: 1:100-1:1000 IF: 1:100-1:1000</td>
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</table>
### TABLE 3: Summarized results from all electroporation experiments

<table>
<thead>
<tr>
<th>TRIAL INFORMATION</th>
<th>PARAMETERS</th>
<th>COLLECTION TIMEPOINT</th>
<th>RESULTS</th>
</tr>
</thead>
</table>
| **Trial 1**<br>
*Plasmid Trial with Embryonic Chicks (n=12)* | Pulse number=3  
Pulse length= 5ms  
Internal = 1.0s  
Voltage= 15V  
Electrode distance= 2mm | 48 hours | 1) GFP plasmid successfully transfected into the neural tube of HH29 chick  
2) No specific staining using immunohistochemistry or immunofluorescence (see Fig. 4) |
| **Trial 2**<br>
*Gecko Trial #1 (n=1)*  
Gecko ID=15-July-01, Mass= 40.8g  
Location: spinal cord (central canal) | Pulse number=5  
Pulse length= 16ms  
Internal = 1.0s  
Voltage= 35V  
Electrode distance= 2 mm | 48 hours | Processor power failure. Re-processed, but not usable for analysis. |
| **Trial 3**<br>
*Gecko Trial #2 (n=1)*  
Gecko ID=15-July-01, Mass= 36.9g  
Location: blastema biopsy | Pulse number=5  
Pulse length= 16ms  
Internal = 1.0s  
Voltage= 35V  
Electrode distance= 2 mm | 48 hours | Processor power failure. Re-processed, but not usable for analysis. |
| **Trial 4**<br>
*Gecko Trial #3 (n=3)*  
Gecko ID= 1-April-03, Mass= 52.6g  
Gecko ID= 14-JA-08, Mass= 45.1g  
Gecko ID= 14-JA-02, Mass= 54.6g  
Location= spinal cord (central canal) | Pulse number=5  
Pulse length= 16ms  
Internal = 1.0s  
Voltage= 35V  
Electrode distance= 2 mm | 48 hours (n=2)  
14-JA-02  
14-JA-08  
8 days (n=1)  
1-April-03 | 1) No specific GFP positive staining in the original or regenerating spinal cord (see Fig. 5)  
Optimization  
-try different electrodes  
-section longitudinally to capture more of the spinal cord |
### Trial 5
**Gecko Trial #4 (n=4)**
- Gecko ID= 23-Oct-05, Mass= 36.0g
- Gecko ID= 23-Oct-06, Mass= 33.0g
- Gecko ID= 23-Oct-03, Mass =40.5g
- Location= spinal cord (central canal)
- Gecko ID= 15-June-14, Mass= 13.6
  - Location= blastema biopsy

<table>
<thead>
<tr>
<th>Pulse number</th>
<th>Pulse length</th>
<th>Internal</th>
<th>Voltage</th>
<th>Electrode distance</th>
<th>Days (n)</th>
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<tr>
<td>5</td>
<td>16ms</td>
<td>1.0s</td>
<td>35V</td>
<td>2 mm</td>
<td>3 days (n=1)</td>
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<td>5 days (n=2)</td>
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<td></td>
<td></td>
<td></td>
<td>15-June-14</td>
</tr>
</tbody>
</table>

1) GFP+ cells visible and located distal to the original spinal cord (see Fig. 6)

### Trial 6
**Gecko Trial #5 (n=3)**
- Gecko ID= 23-Oct-04, Mass= 52.1g
- Gecko ID= 23-Oct-07, Mass= 46.4g
- Gecko ID= 23-Oct-08, Mass= 47.8g
- Location= spinal cord (central canal)

<table>
<thead>
<tr>
<th>Pulse number</th>
<th>Pulse length</th>
<th>Internal</th>
<th>Voltage</th>
<th>Electrode distance</th>
<th>Days (n)</th>
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<tr>
<td>5</td>
<td>16ms</td>
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CHAPTER 3 FIGURES

Figure 1: Injection locations for electroporation optimizations in the leopard gecko. We chose three discrete locations for electroporation. (A) The distal end of the spinal cord (white hatched ellipse in A). We also used 3mm biopsy punches of the (B) blastema (white hatched ellipse in B) and (C) a 3mm dorsal tail biopsy punch (white hatched ellipse in C). Note: we used the vital dye, fast green to visualize the injection of the eGFP plasmid solution. See text for details.
Figure 2: Enhanced green fluorescent protein (eGFP) plasmid map
Figure 3: Electroporation apparatus. To support the gecko following anaesthesia and to make the regions of interest accessible, we used an obliquely cut plastic graduated cylinder attached to an adjustable clamp on a stand (1). EGFP plasmid was injected through a glass pipette attached to Hamilton syringe (2) and electroporations were carried out using a BTX Electro Square Porator, ECM 830 and 5mm electrodes (3).
Figure 4: eGFP expression in Hamilton and Hamburger stage 22 chick embryo. (A) eGFP transfection of an HH22 chick as viewed in wholemount using an epifluorescent dissection microscope. Transfected cells were localized to the distal half of the neural tube (hatched lines=B, C). (B) Immunofluorescence for eGFP revealed no specific staining within the neural tube, although a subset of cells within a somite lateral to the neural tube appeared to be labelled with eGFP (B’:white arrows). (C) Immunohistochemistry for eGFP, no positive DAB staining was detected. Additionally, no difference was detected between the positive and omission control (C’). scale bar= 10µm.
Figure 5: Results from Trial 4. No evidence of eGFP transfection in the spinal cord was visible in transverse sections at (A) 48 hours (negative control, A’) or at (B) 8 days (negative control, B’). scale bar = 10µm
Figure 6: eGFP positive cells are present in the original spinal cord 3 days following in vivo transfection via electroporation. 3 days following eGFP transfection in the spinal cord, a small subset of eGFP+ cells (white hatched box in A), were seen at the distal end of the original spinal cord, shown here in longitudinal view (hatched line in A=ruptured end of the spinal cord). eGFP+ cells shown at a higher magnification (white arrows in B). **scale bars =10µm**
4.1 INTRODUCTION

Across vertebrate species, the cell population surrounding the central canal of the spinal cord – ependymal layer cells (ELCs) – includes pools of endogenous neural stem/progenitor cells (NSPCs) that participate in neuroregeneration (Stensaas, 1983; Anderson et al., 1986; Chernoff et al., 2003; Benraiss et al., 1999; Reimer et al., 2008; Dawley et al., 2012). In teleosts and urodeles, injury to the spinal cord induces NSPCs of the ependymal layer to proliferate and ultimately differentiate into new neurons, astrocytes and oligodendrocytes (Echeverri and Tanaka, 2002; Schnapp et al., 2005; Dawley et al., 2012). Curiously, resident NSPCs of the ependymal layer also share features in common with astrocytes, including a lengthy radial process and the expression of markers such as glial fibrillary acidic protein (GFAP). As a result, NSPCs within the ependymal layer are sometimes referred to as ependymo-radial glia (ERG). Within the original spinal cord of zebrafish, various ERG domains have been recognized, with each domain capable of regenerating one or more neuronal subtypes and glial support cells (Reimer et al., 2008; Reimer et al., 2009). For example, Olig2+ ERGs from the ventro-lateral domain of the spinal cord generate motor neurons while a more ventral population of Nkx6.1+ ERG restore serotonergic neurons (Reimer et al., 2008; Reimer et al., 2009).

In mammals, NSPCs are known to reside within the subventricular zone of the cerebral
hemispheres, the subgranular zone of the hippocampus and among ELCs of the spinal cord (Doetsch and Alvarez-Buylla, 1996; Kuhn et al., 1996; Goldman et al., 1997). Similar to teleosts and urodeles, these cell populations are also induced to proliferate in response to injury (Mothe and Tator, 2005; Meletis et al., 2008; Barnabe-Heider et al., 2010). Isolation and propagation of NSPCs involves the use of a free-floating NSPC-cluster culture system, or neurosphere assay (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996). Neurospheres are created by using non-adherent plates and disassociated brain (and spinal cord) cells are cultured in media with mitogenic growth factors, including epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Reynolds and Weiss, 1992). Once formed, neurospheres can be induced to differentiate by removing the mitogenic growth factors (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Weiss et al., 1996). Although the ability to culture neurospheres is well documented in humans, rats and mice, this technique remains largely unexplored in other amniotes.

Among amniotes, one of the most striking examples of spontaneous multi-tissue regeneration is observed in lizards. Following tail loss, many species of lizard are able to regenerate a functional replacement appendage complete with muscle, skeletal tissue and a new spinal cord (Bellairs and Bryant, 1985; McLean and Vickaryous, 2011; Szarek et al., 2016). Evidence to date clearly indicates that the spinal cord of the original tail plays a crucial role in tail regeneration. When the spinal cord is removed, regeneration fails to proceed and when ELCs are ectopically transplanted, supernumery tails are formed (Simpson, 1964, Whimster, 1978; Simpson and Duffy; 1994; Tanaka and Ferretti, 2009). However, the in vitro potential of ELCs remains unexplored.

Here we seek to isolate, propagate and characterize cells from the central nervous system
(CNS; brain and spinal cord) from the representative tail-regenerating lizard, the leopard gecko (Eublepharis macularius). More specifically, we investigate conditions for the primary culture of original and regenerating spinal cord cells, as well as blastema using both tissue dissociation and tissue explant culture techniques. In addition, we employ the neurosphere assay to determine if lizard CNS cells are neurosphere-forming (and hence include populations of NSPCs) and if they can be driven to differentiate along both neuronal and glial lineages.

4.2 METHODS

4.2.1 ANIMAL CARE

Captive bred leopard geckos (Eublepharis macularius) were obtained from a commercial supplier (Global Exotic Pets, Kitchener, Ontario, Canada). Geckos ranged in mass from 12-55 grams. Mass, snout-vent length and tail measurements were recorded each week to track growth and monitor the health of experimental geckos (e.g. Appendix 1). Animal Usage Protocol 1954 was approved by the University of Guelph Animal Care Committee. All Animal Usage Protocols followed the procedures and policies of the Canadian Council on Animal Care and closely adhered to the ARRIVE guidelines (Kilkenny et al., 2010). All gecko husbandry practices were consistent with the protocols laid out in Vickaryous and McLean (2011). Leopard geckos were housed at the Hagen Aqualab at the University of Guelph in an environmental chamber with an ambient temperature of 27.5°C and a 12 hour light: 12 hour dark photoperiod. Geckos were housed individually in five gallon plastic (polycarbonate) tanks. A temperature gradient was created in each of the tanks using a subsurface heating cable (Hagen Inc., Baie d’Urfe, Quebec, Canada) under one side of each enclosure. Geckos were fed a diet of gut-loaded mealworms (larval Tenebrio spp.) dusted with powdered calcium and vitamin D3 (cholecalciferol)
supplement (Zoo Med Laboratories Inc., San Luis Obispo, California, USA). Each gecko received three mealworms per day. The total number of geckos used during cell culture trials was 9.

4.2.2 TISSUE COLLECTION

For blastema primary cell culture for collagenase dissociation and explant culture the tail was sterilized using 70% ethanol and was collected by inducing autotomy (self-detachment of the tail). Autotomy was accomplished by grasping the tail at the desired location of tail loss between the index finger and thumb and applying constant pressure until the tail self-detaches. The first autotomy was always restricted to the distal third of the tail to permit tissue collection with a second (more proximal) autotomy. Tails were staged daily (following the morphological criteria of McLean and Vickaryous, 2011). Blastema tissue was always taken from a tail at stage III of regeneration. For spinal cord and brain cell culture trials geckos were euthanized using an intra-abdominal injection of tricaine methansulfonate (MS222; dosage= 250-500mg). Following autotomy or euthanasia, tissue to be cultured was carefully dissected out (i.e. the blastema was excised from remaining original tail stump; the spinal cord was removed from the vertebral canal) and placed in a 70% ethanol in a 35mm plates. Tissue was then prepared for either: collagenase dissociation, explant culture, or the neurosphere assay.

4.2.3 COLLAGENASE DISSOCIATION

For collagenase dissociation, tissue was rinsed in 70% ethanol for 30 seconds, followed by four rinses in 1X phosphate buffered saline (PBS) with 2% antibiotic/antimycotic. Each tissue sample was then minced into smaller pieces and placed into petri dishes. Tissue pieces were rinsed again using 1X PBS until cleared of blood, after which the PBS was pipetted off and
tissue was transferred into collagenase using the side of a scalpel blade. Tissue pieces were incubated in collagenase for 30 minutes, and lightly agitated every 10 minutes. Culture dishes containing 3mL of media (Dulbecco’s Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 1% antibiotic/antimyotic, 20ng/mL EGF, 5µg/mL insulin) were prepared. For each dissociated tissue piece, 1mL was placed in each dish along with the media. Dishes were checked under the microscope to confirm the presence of cells and the media was changed the following day to eliminate any dead cells. Subsequently, media was changed every third day until cells reached 80% confluence. We investigated two incubation temperatures (31°C and 37°C), both with a constant CO₂ concentration of 5%.

4.2.4 EXPLANT PREPARATION

For explant culture, tissue was rinsed (3 times) in 70% ethanol and (3 times) in 1X PBS +2% antibiotic/antimycotic, and then sliced into at least eight small segments. We used 35mm culture dish scored with a straight blade to allow for better explant adhesion. Explants were placed onto the scored regions and allowed to dry for 5 minutes with the dish cover on, after which 2.5mL of media (DMEM with 10% Fetal Bovine Serum, 1% antibiotic/antimycotic, 20ng/mL EGF, 5µg/mL insulin) was added. We investigated three coating substrates: gelatin, laminin and Poly-D-Lysine. Media was changed every three days and cells were imaged using an Olympus IX81 spinning disk microscope. We investigated two incubation temperatures (31°C and 37°C), both with a constant CO₂ concentration of 5%.
4.2.5 PASSAGING CELLS

Cells were passaged once they had reached 80% confluence. The media was removed and 1X PBS was added to rinse any remaining serum components, followed by 2.5mL of 0.25% trypsin, 0.04% EDTA. Cells were incubated with trypsin for 3-5 minutes at 37°C. Once the majority had detached, the remaining solution was collected in a 15mL centrifuge tube and an equal volume of media was added (to inactivate the trypsin). The solution was then centrifuged and the resulting pellet was re-suspended in 5mL of culture media (DMEM with 10% Fetal Bovine Serum, 1% antibiotic/antimycotic, 20ng/mL EGF, 5µg/mL insulin).

4.2.6 NEUROSPHERE ASSAY

To determine if cells from the brain and spinal cord were NPSCs, we used a neurosphere assay (STEMCELL Technologies™). Brain and spinal cord tissue was collected, sectioned using a straight blade, and then transferred into the NeuroCult ® tissue collection solution for enzymatic dissociated (as per manufacturer’s instructions). Once dissociation was complete, the pellet was re-suspended in 1mL of complete NeuroCult ® NSC Proliferation Medium containing 20ng/mL recombinant human Epidermal Growth Factor (rhEGF), 10ng/mL recombinant human basic Fibroblast Growth Factor (rhFGF-b) and 2µL/mL Heparin. Media was changed every 2 days. Temperature of the incubator was set at 37°C with a constant CO₂ concentration of 5%.

4.2.7 IMMUNOFLUORESCENCE

To characterize cells in culture, immunofluorescence for Vimentin (an intermediate filament common to NSPCs and mesenchymal-like cells), GFAP (a marker of astrocytes) and RT-97 (a neurofilament present in neurons) was performed. Briefly, media was removed from
flasks and cells were rinsed with 1X PBS (2 minutes; 2 rinses) before being fixed with 10% neutral buffered formalin (NBF) for 1 hour. Cells were placed back in 1X PBS overnight. Next, cells were rinsed with 0.1% Triton-X-100 in 1X PBS for 15 minutes and then washed with 1X PBS (2 minutes; 2 rinses). Cells were blocked with 5% bovine serum albumin (BSA) for 10 minutes and incubated in primary antibody diluted in sterile 1X PBS overnight at 4°C. (Vimentin [1:50] Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA; GFAP [1:200] DAKO, Glostrup, Denmark; RT-97 [1:50] Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA). Following overnight incubation, cells were rinsed with 1X PBS (2 minutes; 2 rinses) and then incubated with fluorescently tagged secondary antibodies for 1 hour at room temperature: goat anti-mouse alexa 488 (Life Technologies, Eugene, Oregon, USA) for Vimentin and RT-97 [1:200]; and Cy3 goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for GFAP [1:1000]. Cells were then counterstained with DAPI nuclear stain (Life Technologies, Eugene, Oregon, USA) diluted in sterile 1XPBS [1:500], rinsed again in 1XPBS and 2 drops of ProLong™ gold (Vector Laboratories, Burlingame, CA, USA) was added to each flask. Flasks were imaged using Olympus IX71 inverted fluorescence microscope and stored at 4°C.

4.3 RESULTS

Cell culture conditions in lizards have been reported for several taxa including skinks (Stephenson, 1966), anoles (Simpson and Cox, 1967) and Australian dragon lizards (Ezaz et al., 2008). Although these studies served as useful guidelines for establishing primary cell cultures from leopard gecko CNS tissues, our results were mixed (Table 1). Blastema cells were collected tissue from a regenerating tail immediately following re-epithelialization and loss of
the clot (morphological stage III of tail regeneration; McLean and Vickaryous, 2011; see Chapter 2). While we were unable to dissociate the blastema using a collagenase approach, we did observe migrating blastema cells when we used explant cultures (Figure 1). Explant-derived blastema cells adhered well to both scratched and laminin coated plates. In contrast, cells derived from spinal cord explants did not adhere, nor did they survive on any surface (data not shown).

Next we used a neurosphere assay to isolate NSPCs from brain and spinal cord tissue samples. In mammals, NSPCs are known to form spheres in culture using Neurocult™ media supplemented with EGF and FGF in low-adherence plates (e.g. Deleyrolle and Reynolds, 2009). Unexpectedly, we did not detect any evidence of neurosphere formation, although we did observe the adherence of dissociated cells from both brain and spinal cord tissue. While these cells did reach confluence, attempts at passaging proved unsuccessful (Table 1). We then used immunostaining to characterize these primary CNS cell populations. The majority of CNS-derived cells expressed the intermediate filament Vimentin (Figure 2A). Subsets of the cultured cells also expressed the astrocyte marker GFAP (Figure 2B) and neuron marker RT-97 (Figure 2C). We did co-stain cells with GFAP and RT-97 but not observe any evidence that any cell co-expressed these markers.

### 4.4 DISCUSSION

Results from our in vitro study were inconclusive. While we were able to culture blastema and spinal cord tissue explants and propagate the cells to confluence, we were unable to passage the resulting population. Despite the use of non-adherent culture plates and other conditions associated with the formation of neurospheres (free-floating cultures), we found that dissociated cells from the brain and spinal cord of the gecko failed to form spheres and did
adhere to plates. Our immunostaining data indicates that astrocytes and neurons were likely present, along with large numbers of Vimentin+ cells. Although Vimentin expression is common to NSPCs (Szaro and Gainer, 1988; O’Hara et al., 1992) it is also detected in fibroblasts and other mesenchymal-like cells (Mendez et al., 2010; Gilbert et al., 2015)

We experimented with two different culture temperatures, 31°C and 37°C, both of which have been used to culture lizard cells (Clark et al., 1970; Ezaz et al., 2008; Polazzi and Alibardi, 2011; Lozito and Tuan, 2015) and generate neurospheres in mammals (STEMCELL™; manufacturer information). Although previous work in urodeles has successfully generated ependymal cell explant cultures using fibronectin-coated plate at 21°C with no added CO2, adherence was limited to cells collected between 1-2 weeks post-lesion (O’Hara et al., 1992). We attempted to dissociate cells from the blastema, spinal cord and brain using collagenase and the NeuroCult ® tissue collection solution (as per manufacturer’s instructions). While the collagenase did not dissociate our tissues of interest, the NeuroCult ® tissue collection solution did. Future trials should include alternative dissociation approaches such as trypsin and papain in parallel with the NeuroCult ® tissue collection solution to determine which provides the largest number of viable primary cells.

Establishing cell lines from reptiles is challenging and efforts to date have been largely unsuccessful (Simpson and Cox, 1976). However, the use of an organ culture approach to investigate the regenerate cartilage of the replacement tail has proven to be an amenable method for in vitro investigations of anole lizards (Anolis sagrei and A. carolinensis; Lozito and Tuan, 2015; Lozito and Tuan, 2016). These and other studies (e.g., Ezaz et al., 2008) hold promise for future in vitro investigations particularly those that are focused on short-term cultured
populations. The next step in generating longer term cultures awaits a more fundamental understanding of reptilian cell biology.
### CHAPTER 4 TABLES

**TABLE 1:** Summarized results from all cell culture experiments

<table>
<thead>
<tr>
<th>TRIAL NUMBER</th>
<th>DISSOCIATION METHOD</th>
<th>TEMP/ %CO₂</th>
<th>MEDIA DETAILS</th>
<th>RESULTS</th>
<th>MODIFICATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2</td>
<td>Collagenase digestion of blastema</td>
<td>37°C (1) 31°C (2) 5% CO₂</td>
<td>DMEM with -10%FBS -1% antibiotic/antimycotic -20ng/mL EGF -5ug/mL insulin</td>
<td>Cells did not adhere to plates</td>
<td>Use gelatin coated dishes</td>
</tr>
<tr>
<td>3, 4</td>
<td>Collagenase digestion and blastema explants</td>
<td>37°C (3) 31°C (4) 5% CO₂</td>
<td>DMEM with -10%FBS -1% antibiotic/antimycotic -20ng/mL EGF -5ug/mL insulin</td>
<td>Cells not reliably adhering to plates</td>
<td>Try different ways to coat dishes</td>
</tr>
<tr>
<td>5, 6</td>
<td>Explant culture of spinal cord and blastema</td>
<td>31°C 5% CO₂</td>
<td>DMEM with -10%FBS -1% antibiotic/antimycotic -20ng/mL EGF -5ug/mL insulin</td>
<td>Explants adhered to laminin coated plates, cells were growing, reached confluence, but not passagable</td>
<td>4 new types of surfaces 1) scratch plates 2) Laminin 3) poly-D-lysine 4) Fibronectin</td>
</tr>
<tr>
<td>7, 8, 9</td>
<td>Neurosphere assay for gecko brain and spinal cord</td>
<td>37°C 5% CO₂</td>
<td>Neurocult ® NS-A Proliferation Medium</td>
<td>Cells survived, no evidence of neurospheres, but evidence of differentiated CNS cell types.</td>
<td>Re-ran with same parameters 3X. Results consistent across trials.</td>
</tr>
</tbody>
</table>
Figure 1: **Explant cell culture from a stage III blastema.** Tissue was diced and plated onto 35mm petri dishes as explants (A). Plates were coated with either laminin or the surface directly below the explanted tissue was scratched to assist in adherence. Cells reached confluence (B), yet, we were unable to passage them. Cells were grown at 37°C with 5%CO₂.
Figure 2: *In vitro* characterization of cells dissociated using the Neurosphere Assay.

Gecko spinal cord cells were dissociated and cultured using Neurosphere conditions and cells reached confluence. Using immunofluorescence we found that cells expressed mesenchymal marker Vimentin (A); a subset of cells expressed astrocyte marker GFAP (B) and a second subset expressed neuronal marker RT-97 (C).
CHAPTER 5: CONCLUDING REMARKS AND FUTURE DIRECTIONS

5.1 CONCLUSIONS

This study sought to explore spinal cord regeneration in the tail-regenerating lizard *Eublepharis macularius*, the leopard gecko. Tail-regenerating lizards such as geckos provide several important advantages in the study of spinal cord regeneration. Lizards are amniotes and the closest living relatives of mammals capable of spontaneously regenerating a functional spinal cord. The original spinal cord of lizards is structurally similar to that of mammals and passes continuously throughout the length of the tail without the formation of a cauda equina. As a result, the spinal cord can be easily accessed without direct injury to the body proper. Furthermore, the tail (including spinal cord) can be regenerated within a relatively short timeframe, ~30 days (McLean and Vickaryous, 2011) and entire segments can be detached without the need for anesthesia or analgesics.

Emerging evidence points towards cells lining the central canal of the spinal cord – ependymal layer cells (ELCs) – as playing a key role in spinal cord and complete tail regeneration (Kamrin and Singer, 1955; Simpson, 1968, Whimster, 1978). Although ELCs in lizards are generally acknowledged as including populations of neural stem/progenitor cells (NSPCs; Alibardi, 2014b), to date these cells remain poorly characterized. We hypothesized that ELCs of the original spinal cord include populations of NSPCs that are activated in response to tail loss. In order to test our hypothesis, we investigated three objectives. First, we conducted an *in vivo* characterization of the gecko spinal cord before, during and after tail regeneration. Specifically, we sought to determine if slow cycling (label-retaining) cells were present in the spinal cord using a 7 day pulse-140 day chase paradigm. Next we examined the expression of a
panel of proliferation, NSPC and lineage-restricted progenitor markers in original, regenerating and completed regenerated tails. We then attempted to use in vivo electroporation to track cells from the original spinal cord into the regenerate tail and we used the Neurosphere Assay to document ELCs as a putative population of NSPCs in vitro.

5.1.1 Are ependymal layer cells (ELCs) neural stem/progenitor cells (NSPCs)?

We determined that ELCs within the original spinal cord are a population of NSPCs. Our results reveal that ELCs of the original spinal cord share a number of characteristics common to NSPCs. More specifically populations of ELCs are slow-cycling, constitutively express the hallmark NSPC protein SOX2 and are activated in response to injury. Stem/progenitor cells are generally viewed as a relatively dormant reserve that can be recruited in response to trauma or the need for tissue replenishment (Richmond et al., 2016) Under homeostatic conditions, NSPCs are predicted to be quiescent, we found that within the original spinal cord, that 2.5% of ELCs within the original spinal cord are slow-cycling, as evidenced by their ability to retain a BrdU label 140 days after the pulse.

One of our most compelling findings in support of ELCs including a population of NPSCs was our SOX2 data. We determined, for the first time in a reptile, that SOX2 is expressed by essentially all ELCs, including those of the original, regenerating and completely regenerate spinal cords. SOX2 is required for spinal cord regeneration among amphibians (tadpoles and urodeles) (Gaete et al., 2012; Fei et al., 2014). Curiously, in contrast to other regeneration-competent models, we observed that gecko ELCs constitutively express SOX2; in other taxa SOX2 is transiently upregulated following injury. A similar pattern of constant SOX2 expression is also observed in embryonic mammals during neurogenesis (Hutton and Pevny, 2011), leading
us to suspect that geckos ELCs represent a less differentiated/more embryonic-like stem/progenitor population. We also determined that ELCs from the original tail also express the intermediate filament and NSPC marker, Vimentin. Furthermore, following injury and during regeneration, ELCs express the NSPC marker and RNA-binding protein MSI1.

Finally, we also determined that ELCs are activated in response to injury. Activation, defined here as a change in mitotic status and protein expression, was observed by ELCs following tail loss. Prior to injury, isolated ELCs of the original spinal cord demonstrate evidence of proliferation (i.e., express PCNA) and the only NSPC markers detected were SOX2 and Vimentin. Following injury this pattern of expression changed. In the original tail stump and throughout the regenerating spinal cord proliferating ELCs were abundant. And while SOX2 and Vimentin expression was sustained, populations of ELCs newly began to express MSI1 and two lineage-restricted progenitor markers, TUJ1 (neuronal lineage) and SOX9 (glial lineage). With the exception of MSI1, expression of these markers persisted within the original spinal cord, even once the regenerate tail was fully formed. A similar activation response has been reported for ELCs from other regeneration-competent species (Allen and Smith, 2012; Zhang et al., 2000) and appears to be a key characteristic of NSPCs during successful spinal cord regeneration.

5.1.2 Are ELCs a homogeneous population?

Prior to injury ELCs appear as a homogeneous population of cells lining the central canal of the spinal cord. More specifically they are SOX2+, MSI1-, HuC/D-, TUJ1-, SOX9- and we propose that they are a reserve pool of NSPCs. Curiously, we determined that, following injury, gecko ELCs represent at least two distinct cell populations. Following tail loss, we observed that some ELCs stop expressing GFAP and start expressing the neural marker HuC/D, whereas other
populations are distinctly GFAP+, HuC/D-. We identify the GFAP+, HuC/D- populations as ependymo radial glia (ERG), and the GFAP-, HuC/D+ cells as cerebrospinal fluid contacting (CSF-c) cells. ERG are a specialized cell type demonstrating both ependymal and astrocytic features and are a known population of multipotent NSPCs in non-amniotes that are activated during neuroregeneration (Mchedlishvili et al., 2007; Chapouton et al., 2010). While previous work has reported the presence of ERG in the brain of turtles (Romero-Aleman et al., 2004; Trujillo-Cenoz et al., 2014), to our knowledge this investigation marks the first report of their presence within the lizard spinal cord.

CSF-c cells have been described for various vertebrate species (e.g. Harper and Roberts, 1993; Jalavand et al., 2014). Two classes are recognized, one of which (type-1 CSF-C cells) has been shown to have neuronal-like properties (i.e. they express GABA and glutamate receptors and are capable of firing an action potential) and may in fact represent a population of immature neurons (Petracca et al., 2016). We hypothesize that this neuronal type of CSF-C cell is also present among the ELC population of geckos. In order to confirm a neuronal-type function, electrophysiological studies will be needed to determine whether these cells are capable of generating an action potential.

5.1.3 Are ELCs the source of the regenerate spinal cord?

While we were unable to confirm that ELCs are a source of cells that contribute to the regenerate spinal cord using in vivo electroporation, this hypothesis is supported by our characterization of ELCs as populations of NSPCs (likely ERG). Involvement of ELCs in successful spinal cord regeneration has been demonstrated in teleost fish (reviewed in Becker and Becker, 2015) and urodèles (Echeverri and Tanaka, 2002; Mchedlishvilli et al., 2007), and is
consistent with previous studies conducted on lizards (Simpson, 1968; Whimster, 1978; see also Bellairs and Bryant, 1985). Because we were unable to exclusively label ELCs, we are unable to comment on the relative contribution of these cells to the regenerate spinal cord. However, our findings taken together with previous work support a hypothesis that ELCs within the original spinal cord are multipotent stem/progenitor cells that are activated following tail loss and produce all cell types within the regenerate spinal cord (although the possibility that ELCs are a mixed population of multipotent and lineage restricted cells cannot be excluded at this time).

5.1.4 Is spinal cord regeneration limited to the tail?

Although many lizards can unambiguously regenerate the spinal cord of the tail, whether this ability is also shared by the spinal cord of the trunk remains unclear. In teleosts and urodeles, spinal cord regeneration does extend to more rostral areas (Chernoff et al., 2002; Tanaka and Ferretti, 2009; Allen and Smith, 2012; Gaete et al., 2012), but evidence from lizards is mixed. While some reports suggest that lizards do not recover from trunk spinal cord injuries (Simpson, 1983; Simpson and Duffy, 1994), others suggest they can within a one month timeframe (e.g Raffaelli and Palladini, 1969; Alibardi, 2010). In one study, the author performed a lumbar transection of the spinal cord in the lizard *Podarcis muralis* and observed that a ‘bridge’ of regenerating axons re-joined the ruptured ends (Alibardi, 2014). Whereas functional recovery was reported, the role (if any) of ELCs was not described. The injury-mediated response and regenerative potential of ELCs within the body spinal cord of the geckos awaits future investigation.

5.2 FUTURE DIRECTIONS
While this work serves as a biological foundation for future studies aimed at understanding the process of spontaneous spinal cord regeneration in amniotes, many questions remain unanswered. Are ELCs the source of the cells for the regenerate spinal cord and do other cell populations also contribute? Are developmental programs redeployed during spinal cord regeneration and, if so, does spinal cord regeneration in lizards essentially represent a truncation of the fully differentiated process? What governs the permissiveness of the microenvironment that ultimately facilitates success of spinal cord regeneration? Finally, can ELCs be propagated in vitro and be employed as a therapeutic strategy to diminish or even resolve central nervous system injuries?
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**APPENDIX 1: WEIGH-MEASURE DETAILS FOR LONG-TERM LABEL RETENTION STUDY**

### Week 1

<table>
<thead>
<tr>
<th>Gecko</th>
<th>Mass (g)</th>
<th>S-V (mm)</th>
<th>Tail Length (mm)</th>
<th>R. Tail Length (mm)</th>
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<tr>
<td>Control-1</td>
<td>19.3</td>
<td>102</td>
<td>37</td>
<td>25</td>
</tr>
<tr>
<td>Control-2</td>
<td>7.7</td>
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</tr>
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<td>50</td>
<td></td>
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<tr>
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<td>85</td>
<td>61</td>
<td><em><strong>now 5-20</strong></em></td>
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<td></td>
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APPENDIX 2: DETAILED HISTOCHEMISTRY PROTOCOLS

Hematoxylin and Eosin

Solutions
Acid Alcohol
1% HCL in 70% isopropanol

Ammonia Water
5 drops ammonium hydroxide
250mL dH2O

Eosin Stock Solution
10g Eosin Y
1g Phloxine B
dissolve in 1000 mL of 80% ethanol

Eosin Working Solution
200mL Eosin stock solution
200mL dH2O
600mL absolute ethanol
5mL glacial acetic acid

Protocol
1. Absolute xylene (3 washes; 2 minutes each)
2. Absolute isopropanol (3 washes; 2 minutes each)
3. 70% isopropanol (2 minutes)
4. Deionized water (2 minutes)
5. Modified Harris Hematoxylin (10 minutes)
6. Rinse slides to remove excess Hematoxylin in running deionized water
7. 1% Acid alcohol (6-10 dips)
8. Rinse in deionized water
9. Ammonia water (6 dips; or until blue)
10. Rinse in deionized water
11. 70% isopropanol (6 dips)
12. Eosin (1 minute)
13. Absolute isopropanol (3 washes; 2 minutes each)
14. Absolute xylene (3 washes; 2 minutes each)
15. Coverslip

**Modified Masson’s Trichrome**

**Solutions**

0.5% Ponceau Xylidine-0.5% Acid Fuchsin in 1% acetic acid
1.25g Ponceau Xylidine 2R in 250mL 1% acetic acid solution
1.25g Acid Fuchsin in 250mL 1% acetic acid solution
combine together

1% Phosphomolybdic Acid
10g phosphomolybdic acid
1000mL dH2O

2% Light Green
2g light green yellowish SF
100mL 2% citric acid solution
mix 1:10 in dH2O

Ammonia Water
5 drops ammonium hydroxide
250mL dH2O

**Protocol**

1. Absolute xylene (3 washes; 2 minutes each)
2. Absolute isopropanol (3 washes; 2 minutes each)
3. 70% isopropanol (2 minutes)
4. Deionized water (2 minutes)
5. Mayer’s Hematoxylin (10 minutes)
6. Rinse slides to remove excess Hematoxylin in running deionized water
7. Ammonia water (6 dips; or until blue)
8. Rinse in deionized water
9. Ponceau Xylidine/Acid Fuchsin (2 minutes)
10. Rinse in deionized water
11. 1% phosphomolybdic acid (10 minutes)
12. Rinse slides to remove excess phosphomolybdic acid in running deionized water
13. 2% Light Green (90 seconds)
14. Rinse in deionized water
15. 95% isopropanol (2 minutes)
16. Absolute xylene (3 washes; 2 minutes each)
17. Absolute isopropanol (3 washes; 2 minutes each)
18. Coverslip
APPENDIX 3: TWO-DAY IMMUNOHISTOCHEMISTY SOLUTIONS AND PROTOCOL

Solutions
Citrate Buffer Stock Solution A (0.1M Citric Acid)
1.92g citric acid powder
100mL dH₂O

Citrate Buffer Stock Solution B (0.1M Sodium Citrate dihydrate)
14.7g sodium citrate dehydrate powder
500mL dH₂O

Working Citrate Buffer
9mL Solution A
41mL Solution B
450mL dH₂O
adjust pH to 6.0

DAB
1 drop buffer
1 drop H₂O₂
2 drops DAB
5-10mL dH₂O

Protocol
1. Absolute xylene (3 washes; 2 minutes each)
2. Absolute isopropanol (3 washes; 2 minutes each)
3. 70% isopropanol (2 minutes)
4. Deionized water (2 minutes)
5. Quench endogenous peroxidases in 3% H₂O₂ (20-30 minutes)
6. 1X PBS rinses (3 washes; 2 minutes each)
7. Citrate buffer retrieval if necessary (if not, skip to step 9). 12 minutes at 90°C, cool for 20 minutes in solution.
8. 1X PBS rinses (3 washes; 2 minutes each)
9. Block in 3-5% normal goat serum (150µL/section; 1 hour, room temperature in a humidity chamber)
10. Tip off block from slides
11. Apply primary antibody at optimized concentration (150µL/section; overnight, 4°C in a humidity chamber)
12. 1X PBS rinses (3 washes; 2 minutes each)
13. Apply biotinylated secondary antibody at optimized concentration (150µL/section; 1 hour, room temperature in a humidity chamber)
14. 1X PBS rinses (3 washes; 2 minutes each)
15. Apply streptavidin conjugated horseradish peroxidase (150µL/section; 1 hour, room temperature in a humidity chamber)
16. 1X PBS rinses (3 washes; 2 minutes each)
17. DAB for optimized time
18. Stop reaction by rinsing slide in dH2O
19. Mayer’s Hematoxylin (1 minute)
20. Rinse in dH2O
21. Ammonia water (6 dips, or until blue)
22. Rinse in dH2O
23. Absolute isopropanol (3 washes; 2 minutes each)
24. Absolute xylene (3 washes; 2 minutes each)
25. Coverslip
APPENDIX 4: TWO-DAY IMMUNOFLUORESCENCE PROTOCOL AND SOLUTIONS

Solutions
Phosphate Buffered Saline (PBS) with 0.1% Tween 20
1mL Tween 20
1000mL PBS

0.3% Triton-X-100 in PBS
10mL 10X PBS
90mL dH₂O
300µL Triton-X-100

Protocol
1. Absolute xylene (3 washes; 2 minutes each)
2. Absolute isopropanol (3 washes; 2 minutes each)
3. 70% isopropanol (2 minutes)
4. Deionized water (2 minutes)
5. 1X PBS (or PBST) (3 washes; 5 minutes each)
6. Block tissue in either 10% NGS in 0.3% Triton-X-100 in PBS or 5% NGS in 1X sterile PBS (150µL/section; 1 hour, room temperature in a humidity chamber)
7. Apply primary antibody at optimized concentration diluted in either 10% NGS in 0.3% Triton-X-100 in PBS or in 1X sterile PBS (150µL/section; overnight, 4°C in a humidity chamber)
8. 1X PBS (or PBST) (3 washes; 5 minutes each)
9. Apply fluorescent-conjugated secondary antibody at optimized concentration diluted in 1X sterile PBS (150µL/section; 1 hour, room temperature in a humidity chamber)
10. 1X PBS (or PBST) (3 washes; 5 minutes each)
11. Counterstain with DAPI (1:10,000; 5 minutes)
12. 1X PBS (or PBST) (3 washes; 5 minutes each)
13. Coverslip with fluorescent mounting media
APPENDIX 5: MODIFIED TWO DAY IMMUNOFLUORESCENCE SOLUTIONS AND PROTOCOL

Solutions
50mM TBS with 0.05% Tween 20 (for retrieval)
6.1g Tris Base
8.8g NaCl
Q.S. to 1000mL with dH$_2$O
Adjust pH to 9.0
Add 0.5mL Tween 20

1% Bovine Serum Albumin (BSA) in 1X PBS
1g BSA
100mL 1XPBS

Protocol
1. Absolute xylene (3 washes; 2 minutes each)
2. Absolute isopropanol (3 washes; 2 minutes each)
3. 70% isopropanol (2 minutes)
4. Deionized water (2 minutes)
5. 1X PBS (40 minutes)
6. Tris retrieval (30 minutes at 95-100°C; cool for 30 minutes in solution)
7. 1X PBST (10 minutes)
8. 1X PBS (2 washes; 5 minutes each)
9. Block tissue in 10% NGS in 0.3% Triton-X-100 in PBS (150µL/section; 30 minutes, room temperature in a humidity chamber)
10. Apply primary antibody at optimized concentration diluted in 1% BSA in sterile 1X PBS (150µL/section; overnight, 4°C in a humidity chamber)
11. 1% BSA in 1XPBS (3 washes; 10 minutes each)
12. Apply fluorescent-conjugated secondary antibody at optimized concentration diluted in 1X sterile PBS (150µL/section; 1 hour, room temperature in a humidity chamber)
13. 1X PBS (2 washes; 10 minutes each)
14. Counterstain with DAPI (1:10,000; 5 minutes)
15. 1X PBS (2 washes; 10 minutes each)
16. Coverslip with fluorescent mounting media
APPENDIX 6: ONE-DAY IMMUNOFLUORESCENCE SOLUTIONS AND PROTOCOL

Solutions
0.1% Trypsin
0.1g Trypsin
100mL 1X sterile PBS

1% BSA-0.5% Tween 20-0.1% Sodium Azide Diluent
1g Bovine Serum Albumin (BSA)
0.5mL Tween 20
0.1mL Sodium Azide
100mL 1X sterile PBS

Blocking Solution
0.5mL Normal Goat Serum (NGS)
10mL diluent

Protocol
1. Absolute xylene (3 washes; 2 minutes each)
2. Absolute isopropanol (3 washes; 2 minutes each)
3. 70% isopropanol (2 minutes)
4. Deionized water (2 minutes)
5. 1X PBS rinse (15 minutes)
6. 2N HCL (30 minutes at 37°C)
   OR
   Citrate buffer retrieval (12 minutes at 95°C and 20 minutes to cool)
7. 1X PBS rinse (2 minutes)
8. 0.1% Trypsin (20 minutes at 37°C)
9. 1X PBS rinse (2 minutes)
10. 5% NGS block in diluent (30 minutes at 37°C)
11. Primary antibody incubation in diluent (2 hours at 37°C)
12. 1X PBS rinse (3 washes; 2 minutes each)
13. Secondary antibody incubation in sterile 1X PBS (1 hour at room temperature)
14. 1X PBS rinse (3 washes; 2 minutes each)
15. DAPI counterstain (1:10,000; 5 minutes)
16. 1X PBS rinse (3 washes; 2 minutes each)
17. Coverslip with fluorescent mounting media
APPENDIX 7: PROTEIN QUANTIFICATION SOLUTIONS AND PROTOCOL

Solutions
RIPA lysis buffer stock (100mL)
10mM Tris-HCL pH 7.6 (0.1576g in 90mL water before adding the rest of the reagents)
5mM EDTA (0.14612g)
50mM NaCl (0.2922g)
30mM tetraysodium pyrophosphate (0.7976g)
1% Triton-X-100 (1mL)

Protease Inhibitor (per mL of RIPA buffer)
2uL Aprotinin stock (2.5mg/mL)
20uL Phenylmethanesulfony fluoride (0.0871gm/10mL)
2uL Sodium Orthovanadate (0.1mM)
50uL NaF stock (50mM)
Pepstatin A stock (1mg/mL)
Leupeptin stock (2mg/mL)

Protocol
1. Combine appropriate amount of RIPA lysis buffer with tissue (for brain and spinal cord 200µL is a useful starting volume) NOTE: These values may need to be adjusted depending on the amount of tissue
2. Add protease inhibitors in the appropriate proportions to the RIPA lysis buffer
3. Place tissue sample in RIPA lysis buffer and mechanically homogenize using a homogenizer or a sonicator (for central nervous system tissue)
4. Place tissue on ice for 30 minutes to let bubbles settle
5. Centrifuge tubes at 140000 rpm in the 6°C microcentrifuge for 10 minutes
6. Remove supernatant and discard the pellet. Aliquot appropriate amounts and store aliquots at -80°C
7. Keep one aliquot from each tissue sample from protein quantification
8. In a 96 well plate add the following solutions from the DC Bradford Assay Kit
   • 5µL sample or BSA standard
   • 25µL working reagent A
   • 200µL reagent B
   NOTE: if the sample contains detergents (as with lysis buffers) working reagent A requires 20uL reagent S for each 1mL of reagent A
9. BSA standards have concentrations of 4mg/mL, 2mg/mL, 1mg/mL, 0.5mg/mL and blank
10. Once all standard and samples (and reagents) have been added to the plate, place plate on a rocker for 15 minutes
11. Place in spectrophotometer and set to 630nm
12. Calculate protein concentration based on absorbance values
13. If the protein concentration is above the highest standard (4mg/mL), samples can be diluted in the plate. For a 50% dilution, add 2.5µL of water to each well and start again at step 8.
APPENDIX 8: WESTERN BLOTTING RECIPES AND DETAILED PROTOCOL

Solutions

8X Sample Buffer
2.0mL 2M Tris HCL (pH 6.8)
4.0mL 20% Sodium Dodecyl Sulphate (SDS)
0.617g DDT
4.0ml glycerol
0.035g Bromophenol blue

10% Ammonium Persulphate (APS)
0.1g AP
1mL dH2O

1.0M Tris Base
12.12g Tris Base
80mL dH2O
pH to 6.8
Q.S to 100mL with MilliQ water

1.5M Tris Base
18.16g Tris Base
80mL dH2O
pH to 8.8
Q.S to 100mL with MilliQ water

5X Tris-Glycine (Electrophoresis) Buffer

15.1g Tris Base
72.1g Glycine
10mL of 10% SDS
Q.S. to 1000mL with MilliQ water

Transfer Buffer
3.02g Tris Base
14.0g Glycine
200mL Methanol
Q.S. to 1000mL with MilliQ water

10X Tris-Buffered Saline
24.2g Tris Base
80.0g NaCl
pH to 7.6
10mL Tween 20
Q.S. to 1000mL with dH2O
5% Skim Milk Block
5g skim milk powder
100mL 1X TBST

5% Loading Gel
5.5mL MilliQ water
1.3mL 30% Acrylamide
1.0mL 1.0M Tris (pH 8.8)
0.08mL 10% SDS
0.08mL 10% APS
0.008mL TEMED

12% Running Gel
9.3mL MilliQ water
5.3mL 30% Acrylamide
5.0mL 1.5M Tris (pH 8.8)
0.2mL 10% SDS
0.2mL 10% APS
0.012mL TEMED

Protocol
1. Aliquot calculated volume of 8X sample buffer, milliQ water and protein into a microcentrifuge tube
2. Heat at 90°C for 5 minutes
3. Load samples and ladder into lanes on the gel
4. Run electrophoresis unit at 120V until samples reach the bottom of the gel (approximately 1.5-2 hours)
5. Transfer to membrane using “transfer sandwich”- from bottom to top (1 sponge, 1 thick Whatman paper, gel, 1 PVDF membrane, 1 thick Whatman paper, 1 sponge)
6. Transfer at 90V for 90 minutes surrounded by ice
7. Dry PVDF membrane following transfer
8. Rehydrate with methanol
9. Rinse membrane in TBST (3 washes; 10 minutes each)
10. Block in 5% skim milk in TBST, 1 hour, on shaker at room temperature
11. Dilute primary antibody in 5% skim milk in TBST
12. Apply antibody in 5% skim milk to membrane, leave overnight on a shaker at 4°C
13. Rinse membrane in TBST (3 washes; 10 minutes each)
14. Dilute HRP conjugated secondary antibody in 5% skim milk in TBST
15. Apply antibody in 5% skim milk to membrane, leave for 1 hour on a shaker
16. Rinse membrane in TBST (3 washes; 10 minutes each)
17. Rinse membrane in TBS (5 minutes)
18. Apply electrochemiluminescent substrate to membrane for 1 minute, remove excess
19. Place in ChemiDoc for appropriate exposure time
   OR
   Expose to x-ray film for varying amounts of time (touch, 15 seconds, 30 seconds, 1 minute)
20. Develop film
   OR
   Save images and merge with ladder
## APPENDIX 9: EPENDYMAL CELL COUNTS

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*P= Pulse  
C=Chase*
Ependymal layer cells of the regenerating spinal cord are a heterogeneous population that includes ependymo-radial glial and neuronal-like central canal contacting cells. (A) Dorsal views of the tail during stage IV (A), stage V (B), stage VI (C) and stage VII (D) (hatched line
indicates the plane of section in panels E-P). Throughout regeneration (E-H), virtually all ependymal layer cells are SOX2+, and many co-express GFAP and Vimentin, intermediate filaments that are otherwise characteristic of ependyo-radial glia. Throughout regeneration (M-P), a subset of ependymal layer cells in close proximity to the central canal express the pan-neuronal marker HuC/D, consistent with their identification as neuronal-like central canal-contacting cells. Scale bar = 20µm
APPENDIX 11: ANAESTHESIA TRIAL USING ALFAXALONE (ALFAXAN)

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<th>Trial 1</th>
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1= no resistance/muscle tone  
2= partially reduced muscle tone  
3= muscle tone of a conscious individual

TRIAL 1:

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Induction (5.4-3.9) 3 minutes  
Plateau (22.1-39.1) 12 minutes  
Recovery (17.5-25.1) 20 minutes

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19F (CONTROL) no alfaxalone

198
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<th>0</th>
<th>1= no resistance/muscle tone</th>
<th>2= partially reduced muscle tone</th>
<th>3= muscle tone of a conscious individual</th>
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TRIAL 1:

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Induction (5.4-3.9) 3 minutes
Plateau (22.1-39.1) 14 minutes
Recovery (17.5-25.1) 15 minutes

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Induction (5.4-3.9) 4 minutes
Plateau (22.1-39.1) 12 minutes
Recovery (17.5-25.1) 15 minutes

19F (CONTROL) No alfaxalone
APPENDIX 12: THE IDENTIFICATION OF PATTERNING MOLECULES DURING SPINAL CORD REGENERATION IN THE LEOPARD GECKO (EUBLEPHARIS MACULARIUS)

12.1 INTRODUCTION

Many lizards are able to voluntarily drop their tail (=autotomy) as a predation response and subsequently regenerate a replacement appendage (Bellairs and Bryant, 1985; McLean and Vickaryous, 2011). While the regenerate tail is not structurally identical to the original, it is a highly organized, multi-tissue structure, strongly suggesting that tail regeneration in lizards involves a tightly-coordinated patterning and growth process (Simpson, 1964; Bellairs and Bryant, 1985; Wang et al., 2011).

Among lizards, it is well known that the spinal cord plays a critical role in the success of regeneration (Kamrin and Singer, 1955; Whimster, 1978). Rapidly following tail loss, the regenerating spinal cord projects into the blastema, the cellular source of most of the regenerating tail tissues. Previous investigations of teleosts (e.g. Reimer et al., 2009) and urodeles (e.g Schnapp et al., 2005) have shown that the spinal cord of these species plays a fundamental role in dorsoventral (D-V) patterning of the regenerate tail. Although the near central position of the regenerate spinal cord in lizards shares a similar location to those of other regeneration-competent species, to date little is known about its possible role in patterning the new tail.

Two candidate molecules involved in this process are the morphogen, sonic hedgehog (SHH) and the homeobox genes, Msh homeobox 1+2 (MSX1+2). SHH is best known for its patterning role during embryogenesis, where it is expressed in the notochord, floor plate of the
neural tube to induce cell differentiation in a concentration-dependent fashion (Tanabe and Jessel, 1996; Briscoe et al., 1999; Litingtung and Chiang, 2000). In contrast, MSX1+2 is expressed dorsally within the neural tube, following activated by secreted bone morphogenetic proteins (BMPs), specifically BMP4 and BMP7 (Liem et al., 1995; Tanabe and Jessel, 1996; Duval et al., 2014). Previous work has documented SHH and MSX1+2 in both the original and regenerating spinal cords of urodeles and teleosts, with a pattern of expression that closely resembles that observed during development (Schnapp et al., 2005; Reimer et al., 2009).

Here we sought to document the presence and location of Shh and MSX1+2 during tail regeneration in the leopard gecko, *Eublepharis macularius*. While we were successful in identifying SHH and MSX1+2 during regeneration using western blot analysis, but we were unable to localize expression of either transcription factor within the original or regenerating spinal cord.

### 12.2 METHODS

#### 12.2.1 ANIMAL CARE

Please refer to Chapter 2, Methods for animal care guidelines and animal utilization protocols.

#### 12.2.2 IMMUNOHISTOCHEMISTRY

Please refer to Chapter 2, Methods for immunohistochemistry protocols. MSX1-2 and Shh antibodies are summarized in Appendix 12: Table 1.

#### 12.2.3 WESTERN BLOT ANALYSIS
Please refer to Chapter 2, Methods for tissue collection, protein quantification and western blotting protocols. MSX1-2 and Shh antibodies are summarized in Appendix 12: Table 1.

12.3 RESULTS

To document the expression of patterning markers SHH and MSX1+2 in original and regenerating spinal cords, we used immunohistochemistry and western blot analysis. Using western blot analysis, we detected bands for both SHH and MSX1-2 in regenerating tail samples, but not in original tail samples (see Appendix 12; Figure 1 for SHH western blot and Appendix 12; Figure 2 for MSX1-2 western blot). More specifically, we found a ~26kDa band in all regenerate and control (whole chick embryo lysates) samples and we also observed a faint band at ~20kDa in the regenerating tail samples consistent with the molecular weight of SHH (Ericson et al., 1996). We did not detect any expression of SHH in the original tail. In mid-late stage regenerating tail tissue (stages IV-VI), we also observed a band at ~28kDa, with a second faint band at ~34kDa in stage IV samples, consistent with the molecular weight of MSX1-2. We were unable to localize SHH and MSX1-2 performing immunohistochemistry on tissues samples representing original and regenerating spinal cord prior to and during regeneration.

12.4 DISCUSSION

Our results indicate that both SHH and MSX1+2 are expressed during tail regeneration. However, we were unable to localize either transcription factor using immunohistochemistry. We suspect that differences in how the tissues were treated prior to processing could explain the discrepancy in results. For example, prior to immunohistochemical analysis tissue is fixed and processed, while for western blot analysis it is not.
Our findings are consistent with the prediction that developmental programs are reactivated during regeneration in lizards, however future studies should seek to localize their expression. Interestingly, within the regenerate tail there is limited evidence of D-V polarity and we suspect that this might be the result of a more diffuse pattern of SHH and MSX1+2 expression. Alternatively, expression during regeneration could reflect that seen during development where SHH expression is expressed ventrally and MSX1+2 is expressed dorsally within the spinal cord. We propose that future studies using in situ hybridization would be beneficial in further characterizing expression of patterning molecules during lizard tail regeneration.
APPENDIX 12 TABLES

**TABLE 1: Antibody Information**

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<tr>
<th>Name of Antibody</th>
<th>Immunogen and MW</th>
<th>Manufacturer, host species, mono-vs. polyclonal, Cat. No., antibody ID</th>
<th>Dilution used in WB and approx. molecular weight in <em>E. macularius</em></th>
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</thead>
<tbody>
<tr>
<td>MSX1+2 (Msh homeobox 1+2)</td>
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<td>WB: 1:50 MW:28kDa and 34kDa</td>
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<tr>
<td>SHH (Sonic Hedgehog)</td>
<td>Synthetic peptide MW: 20kDa</td>
<td>DSHB (Iowa, USA) Mouse Monoclonal (IgG1), Cat#5E1, AB_2188307</td>
<td>WB: 1:50 MW: 26 and 20 kDa</td>
</tr>
</tbody>
</table>
Western blot analysis for Sonic Hedgehog (SHH) an ~20kDa protein involved in patterning during development. *E. macularius* tail tissue homogenates including: original tail and stages IV-VI as well as Hamilton and Hamburger staged 29, 31 and 33 embryonic chick embryo homogenates were used to analyze protein specificity in the tissue of interest. 30 µg of protein was loaded, incubated with anti-SHH antibody and visualized using HRP ECL substrate and exposed to X-ray film for 10 seconds. Bands were detected at ~26kDa in all regenerate and control samples, no bands were detected in the original tail homogenate (A). A faint band was also observed at ~20kDa (as predicted by the manufacturer’s information) in the regenerating tail samples. α-Tubulin was used as a loading control for each sample and was observed at the predicted molecular weight: 50kDa (B)
Western blot analysis for MSX1+2, 30.5kDa (MSX1-derived protein) and 28.2kDa (MSX2-derived protein) involved in patterning during development. *E. macularius* tail tissue homogenates including: original tail and stages IV-VI homogenates were used to analyze protein specificity in the tissue of interest. 30 µg of protein was loaded, incubated with anti-MSX1+2 antibody and visualized using HRP ECL substrate and exposed to X-ray film for 10 seconds. Bands were detected at ~28kDa in stage IV-VI samples (A). A band was also observed at ~34kDa in stage IV sample. β-actin was used as a loading control for each sample and was observed at the predicted molecular weight: 42kDa (B)
REFERENCES


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