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Directed Differentiation and Characterization of Spinal V3 Interneurons from Mouse Embryonic Stem Cells

Hao Xu

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Directed Differentiation and Characterization of Spinal V3 Interneurons from Mouse Embryonic Stem Cells

By

Hao Xu

A dissertation presented to the Graduate School of Arts & Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2015

St. Louis, Missouri
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Hao Xu

Washington University in St. Louis

August 2015
Dedicated to my parents.
ABSTRACT OF THE DISSERTATION

Directed Differentiation and Characterization of V3 Spinal Interneurons from Mouse Embryonic Stem Cells

by

Hao Xu

Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, 2015

Professor Shelly Sakiyama-Elbert, Chair

Neuronal populations involved in locomotion controlling central pattern generators within the spinal cord hold great potential for spinal cord injury therapy. Spontaneous recovery in rodent models suggests that a population that can reorganize around an injury site could be useful for functional recovery therapeutics after spinal cord injury. The glutamatergic, commissural, long-extending V3 interneurons shown to balance locomotor rhythm regularity and robustness within central pattern generators in vivo are both an ideal population for spinal cord injury therapeutics and a vital population to study as a part of locomotor circuitry. Unfortunately, due to the scarcity of these cells in the spinal cord, in vitro studies of dissociated V3 interneurons are technically challenging. Embryonic stem cells provide a bountiful cell source for the study of different cell types and regenerative medicine. While there are extensive reports on mouse embryonic stem cell derived spinal motoneurons, many other spinal neuronal populations have
not been derived. This dissertation focuses on the induction and characterization of V3 INs from embryonic stem cells. In the first study, an induction protocol for V3 interneurons from mouse embryonic stem cells was established. A motoneuron protocol was driven towards a more ventral fate by lowering retinoic acid concentration during induction and increasing the induction duration of morphogen sonic hedgehog signaling. In the second study, a selectable V3 interneuron cell line was generated by knocking the puromycin resistance enzyme, puromycin N-acetyltransferase, into the Sim1 locus on one allele within the mouse genome, allowing native Sim1 gene regulatory elements to drive puromycin N-acetyltransferase expression. Puromycin selection highly enriched for the V3 interneuron population, allowing the cultures to be characterized by electrophysiology and immunocytochemistry. Selected cells survived for four weeks and exhibited synaptic function as well as glutamatergic marker expression. This work establishes a method and a tool for high throughput, low labor acquisition of V3 interneurons for future studies.
Chapter 1: Introduction

1.1 Overview

The purpose of this thesis is to develop methods to better study spinal V3 interneurons (INs) for basic research and developing cell-based therapeutics. In murine models of spinal cord injury (SCI), spontaneous functional recovery had been observed and traditionally attributed to axonal sprouting through the injury site. However, recent studies suggest that this recovery may actually be due to new relay networks of propriospinal neurons formed around the injury[1-3]. This reorganization suggests that for designing more effective cell-based SCI treatments, a propriospinal relay population, particularly long extending propriospinal populations may yield better functional recovery than employing a single end target neuronal population (e.g. motoneurons (MNs)) [2]. V3 INs are excitatory glutamatergic, long extending spinal neurons which can cross the midline (commissural) and synapse onto multiple cell types (including motorneurons and other INs) [4]. They are part of the central pattern generator (CPG) circuitry
and are involved in locomotor rhythm generation, making them attractive candidates for cell based neuro-regenerative therapies [4]. To explore this hypothesis, it is necessary to obtain a sizable and relatively pure population of V3 INs for study. However, V3 INs only comprise ~10% of the ventral spinal population[5], making their isolation tedious and inefficient. Therefore, this thesis addresses that need by providing a high throughput, low labor method to obtain V3 INs from mouse embryonic stem cells (mESCs).

The first aim of this thesis introduces a differentiation protocol for the induction of V3 INs from mESCs. Using a previously established MN induction protocol as a starting point [6], Shh and RA exposure was varied in a six and eight day induction protocol to identify the best conditions for V3 IN induction. The induced cultures up-regulated Sim1 and Nkx2.2 mRNA expression and stained positive for post-mitotic V3 marker Uncx and progenitor markers Nkx2.2 and Ngn3. This work provided a scalable and simple way to generate V3 INs from mESCs.

The second aim of this thesis is to generate and characterize a puromycin selectable V3 cell line for better identification and study. Using recombineering techniques [7], a puromycin resistance gene was knocked into the Sim1, a V3 marker, locus of a mESC line. This allowed Sim1’s gene regulatory elements to control puromycin resistance gene expression thus making the cells drug selectable. This cell line yielded a highly enriched V3 population after puromycin selection as shown by Sim1 expression. The Sim1 expressing cells matured into VGluT2+ neurons that expressed synaptic markers.

The following introduction will provide necessary information and history to better understand this project and its significance. Stem cells, the populations of the ventral spinal cord, and various genomic engineering techniques will be discussed.
1.2 Stem Cells

Pluripotent stem cells hold great potential for regenerative medicine, drug discovery, and basic developmental research. While they exist only transiently during development in the native blastocyst, these cells can be isolated and cultured indefinitely under proper laboratory conditions [8,9]. Their potential to renew indefinitely and become any cell type within the body makes them a limitless cell source for research and cell-based therapeutics. Numerous differentiation protocols have been established for different types of pluripotent stem cells. This section overviews the history and types of pluripotent stem cells.

1.2.1 Mouse Embryonic Stem Cells

Early attempts at isolating pluripotent embryonic stem cells from the mouse embryo for long term culture were unsuccessful [9]. Prior to 1981, mouse embryonic carcinoma cells were the only pluripotent cells available [8]. Unfortunately, as cancer cells, they did not have a normal karyotype. In 1981, mESC culture was first reported by Evans and Kaufman [9]. By comparing the protein expression of embryonic carcinomas and cells isolated during different stages of early development, the timing for mESC appearance was determined. The resulting cells were cultured in embryonic carcinoma cell conditioned media and resembled embryonic carcinoma cells as they also formed teratomas but, unlike embryonic carcinoma cells, had a completely normal karyotype [9]. Originally, mESCs were cultured on mitomycin-C inactivated fibroblast feeder layers. However, regulatory factors differentiation inhibitory activity (DIA) and leukemia
inhibition factor (LIF) were identified for the maintenance of mESCs [10,11], such that feeder layers were no longer needed.

**1.2.2 Human Embryonic Stem Cells**

Following the success of mESCs, primate ESC cultures [12] and human embryonic stem cell cultures using cells from discarded *in vitro* fertilization embryos [13] were established. These human stem cells, like mESCs, were derived from early stage embryos and had the ability to differentiate into cells from all three germ layers. These cells could grow for 3-4 months without losing their undifferentiated morphology. Unfortunately, LIF, which was sufficient to maintain murine lines, was not sufficient to suppress spontaneous differentiation of human ESCs [14]. A combination of either Activin or Nodal and FGF2 was necessary for maintaining hESC pluripotency [15]. This was an important move because an animal-derived feeder layer or even a animal feeder layer based conditioned media for culturing cells were not animal-product free and chemically defined and were thus not acceptable for clinical applications [15]. While the establishment of hESC culture was a step forward in stem cell research, ethical concerns about their origin and use have resulted in a limitation of federal funding on hESC research in 2001 [16]. While that policy has since been overturned[17], the moral controversy surrounding hESCs still remain.
1.2.3 Induced Pluripotent Stem Cells (iPSC)

Due to the ethical and immunogenic concerns of hESCs, a different pluripotent cell source was needed. Early frog cloning and the successful cloning of Dolly the sheep showed that reprogramming of somatic nuclei could be achieved and, specifically, in mammals [18,19]. Additionally, Davis et al.’s conversion of fibroblasts into muscle lineage cells by MyoD transcription factor transduction showed that cell fates could be changed by transcription factor expression [20]. These observed somatic and transcription factor based reprogramming capacities were the beginning for achieving a new pluripotent cell source [21]. These efforts culminated in Shinya Yamanaka’s 2006 discovery that overexpression of 4 factors (Oct3/4, Sox2, Klf4, c-Myc, collectively called the Yamanaka factors) can convert fully differentiated murine cells back into an induced pluripotent state [22]. The resulting stem cells formed teratomas and had similar morphology, gene expression, and DNA methylation profiles as ESCs. Two years after the first iPSCs were reported, the same process was successfully repeated with human fibroblasts [23].

Many methods can be used to derive iPSCs. The original method delivered the Yamanaka factor by retroviral transduction [22,23]. This resulted in integration of the Yamanaka factor carrying vector into the genome. Currently, multiple integration-free methods exist, such as the use of plasmids[24], RNA-based Sendai virus [25], synthesized mRNAs[26] and even the direct delivery of proteins[27]. This wide variety of methods for obtaining integration free iPSCs provided the next step towards translational work.

Great expectations were placed on iPSCs because they could be generated from patients’ fibroblasts and thus bypass immunogenicity concerns for transplantation. Unfortunately, there
are still concerns over the safety of iPSCs for therapeutics. Epigenetic abnormalities[28,29], potential tumorigenicity[30,31], and immunogenicity[32] are all causes for concern. These issues provide significant hurdles for transplantation.

1.2.4 Heterogeneity after stem cell differentiation

The establishment of pluripotent cells opened up the possibility of deriving populations from a self-renewing cell population. While stem cell differentiation protocols are able to generate desired populations, these processes also result in heterogeneity of the final population. During any differentiation process, the proportion of cells that fully undergo differentiation is never 100%. The end population may contain some cells that are post-mitotic and cells that are still undergoing active mitosis. In some cases, the mitotic cells (e.g. undifferentiated pluripotent cells) can overtake the desired post-mitotic population. For example, during transplantation therapies using animal models, undifferentiated cells can result in the over proliferation of cells and even result in teratomas [33]. In spinal cord therapy studies, undifferentiated ESCs have been observed to over-proliferate and abolish initial function recovery [34]. Undesired proliferating populations could also result in cell masses in undesired locations [35]. This issue of heterogeneity needs to be addressed before any form of pluripotent stem cells can be used for transplantation therapeutics.
1.3 Ventral Spinal Neuron Populations

Developmental analysis of the spinal cord has been helpful for understanding locomotion circuitry. Eleven progenitor populations have been designated within the developing spinal cord: six dorsal and five ventral populations [36]. Many studies have focused on how different populations of spinal cord neurons arise, migrate, mature, and integrate themselves into the mature spinal cord. In particular, the ventral populations have been implicated in the function of major locomotor pathways.

During spinal cord development, the ventral neural tube is patterned by Shh released from the notochord, which is situated ventral to the neural tube [37-41]. Shh diffuses dorsally such that the more ventral the population, the greater the required Shh signal magnitude and duration[38]. Two classes of homeodomain proteins (class I and II) respond to the graded Shh signaling. Class I proteins are repressed by a specific Shh threshold and define the ventral boundaries of their progenitor domains while class II proteins require certain Shh thresholds to activate and define the dorsal boundaries of their progenitor domains [42]. These two classes of proteins interact to form the sharp progenitor domain boundaries. This morphogen gradient based determination of distinct cell types is often referred to as the French flag model [43]. While there is new evidence that the French-flag-like pattern is established partially by cell migration and sorting [44], the resulting banded pattern nonetheless characterizes the five ventral spinal progenitor populations: V0-V3 and MNs.

Two driving motivations for understanding ventral populations are understanding CPGs and deriving cell populations for spinal cord injury therapeutics. CPGs have been studied from
both a development based molecular marker perspective, with the use of genetically engineered mice [4,45], and from an anatomic perspective where the function of different neuronal types are assessed in the context of their connectivity [46-48]. Unfortunately, not all cells identified by their molecular markers have their roles characterized in the mature spinal cord. Likewise, the origin of every spinal neuronal population within the mature spinal cord is not well established. However, by studying CPGs from both anatomic and molecular marker perspectives, understanding of the development and function of CPGs can be achieved.

This section will be an overview of all five neuron classes in the ventral spinal cord. Background including progenitor and post-mitotic markers will be discussed as well as their roles within CPG. While this section is organized by the five neuronal classes, it should be emphasized that this is not the only way to understand and organize populations involved in CPGs and locomotion control. The choice in presenting the ventral spinal populations in this way is to provide appropriate context for the understanding the V3 INs from the perspective of this thesis, where V3 INs are generated and targeted by looking at molecular markers.

1.3.1 V0 INs

V0 INs arise in the middle of the neural tube and are the dorsal most population belonging to the ventral spinal cord[36]. The progenitor p0 population is marked by the transcription factor Dbx1[49]. As the V0 population matures, Evx1 is turned on in a subset of the V0 population, known as the V0v population [49,50]. The Dbx1+ cells that do not express Evx1 are termed V0ds. Almost all V0v are have been reported to be excitatory while V0ds are
approximately 50% GABAergic and 70% glycine-expressing. This greater than 100% total indicates a significant overlap of GABAergic and glycine expressing V0\textsubscript{D}s.

V0\textsubscript{V}s and V0\textsubscript{D}s have been studied for their role in locomotion. Both populations receive primary afferent excitatory signaling, innervation from serotonergic fibers, and synapse onto contralateral MNs [51]. Ablation of both populations resulted in synchronized locomotion activity [52,53]. The V0\textsubscript{D}s are responsible for slow locomotion alternation while V0\textsubscript{V}s are responsible for alternation at medium and fast speeds [53]. The current understanding of the V0 IN population is that V0\textsubscript{V}s are involved in left-right locomotion by activating contralateral inhibitory INs that inhibit MNs while V0\textsubscript{D}s modify locomotion based on sensory inputs.

Two other V0 populations, cholinergic V0\textsubscript{C}s and glutamatergic V0\textsubscript{G}s both arise around e11.5 from a small neuronal population that expresses Pitx2 [54]. This population is predominantly projects ipsilaterally While Zagoraiou et al. mention V0\textsubscript{G}s and identify their glutamatergic nature, their primary focus was on the significance of V0\textsubscript{C}s as the source of cholinergic input to MNs. V0\textsubscript{C}s been shown to fire in phase with MNs, indicating their recruitment within the same circuitry as MN activation. In mice where V0\textsubscript{C}s do not express the acetylcholine synthetic enzyme, choline acetyl transferase, mice are unable to enhance their muscle activation, indicating that V0\textsubscript{C}s modulate MN control of muscles [54]. The discovery of V0\textsubscript{C}s elucidated the role of cholinergic input onto MNs.

A diverse set of INs arise from the p0 progenitor domain. This diversity indicates that molecular and lineage based ways of characterizing INs may overgeneralize a population. However, the identification of different V0 subpopulation specific molecular markers also indicates how powerful this method is in understanding the signals involved in IN specification.
1.3.2 V1 INs

V1 INs are located just ventral to V0s in the ventral spinal population. V1 INs arise from the p1 progenitor domain which expresses Pax6, Dbx2, and Nkx6.2 [36]. Post-mitotic V1 INs most notably express En1, FoxD3, and Lhx2, with a sub population expressing Foxp2 [55]. V1 INs have been shown to be required for faster locomotion frequencies. In animals without V1 INs, the amount of time needed for a cycle of steps was lengthened from 3-4s to about 8s [56].

Even though not all populations derived from V1 INs have been identified, two notable inhibitory sub-populations arise from V1 INs: Renshaw cells and IaINs. Renshaw cells, first identified in 1946, mediate inhibition of MNs coupled to either extensor or flexor motor pools [57]. IaINs, identified in 1956, contribute to inhibition of motor pools due to input from Ia sensory inputs from the muscle [58]. These two cell types work together to regulate ipsilateral muscle activity during locomotion. While these two populations can be identified by physiological function alone, mature V1 INs can also be distinguished by their expression of calcium buffering proteins. Renshaw cells tend to display strong calbindin expression and weakly express parvalbumin while mature V1 IaINs only express parvalbumin. Furthermore, all Renshaw cells arise from the V1 domain [59,60] but not all IaINs are V1 IaINs [59,61], indicating that there is more than one developmental source of IaINs.

Renshaw cells and V1 IaINs enter the post-mitotic state at different points in time. Renshaw cells arise from an earlier V1 population (e10.5). They are identified by MafB (note that MafB is also present in MNs and other dorsal populations) after entering the post-mitotic stage and during migration. V1 IaINs arise from a later born V1 population which is positive for FoxP2 (e11.5) [55]. Although different V1 populations express MafB and FoxP2, neither is
unique to V1 INs and thus neither control the differentiation process into V1 population [55]. It has been shown that Pax6 and En1 regulate Renshaw cell development [60], but what is responsible for differentiating these cell types and other V1 INs is still unknown.

The study of V1 IN maturation has revealed a complex maturation process. Early on in development, the reciprocal inhibitions of IaINs are present but weakly acting. Maturation of the circuitry most likely occur later corresponding to when an animal develops better locomotion control [61]. Studies looking at the establishment of CPGs before birth have indicated that Renshaw cells and V1 IaINs are organized before birth but only during maturation do specific synapses stabilize or weaken as stable and coordinated locomotion is established [61].

The study of V1 INs proved great insight into the diversification of an IN population. The fact that IaINs come from more than the V1 domain indicates a level of redundancy in IN maturation. Additionally, timing difference in Renshaw cell and V1 IaIN post-mitotic commitment indicates that in addition to Shh and RA gradients, a temporal factor is also play. These studies further expose the complexity of spinal and CPG development.

### 1.3.3 V2 INs

V2 INs are the third most ventral spinal population, located just dorsal to the MN domain [36,39]. Lhx-3 expressing progenitor p2 cells give rise to two main populations of V2 INs: V2a INs and V2b INs [62,63]. V2as are marked by the post-mitotic transcription factor marker Chx10 while V2b INs are marked by the transcription factor Gata3[63]. A third and fourth population, identified by Sox1 and Shox2 respectively, have been recently identified [64,65].
V2a INs are glutamatergic ipsilateral INs that are able to synapse onto MNs. They are involved in regulating breathing as well as left-right locomotion[66]. They have also recently been shown to be involved in forelimb reaching motions [67]. The precursor population to V2a INs also generates V2b INs. During differentiation, Notch activity contributes to V2a/ V2b cell fate determination [68,69]. Notch activation drives V2b formation while inhibition of notch results in V2a INs. Inhibitory V2b INs are involved in ipsilateral flexor-extensor coordination [70].

Sox1+ V2c INs are very similar to V2b INs and become V2b INs in the absence of Sox1. Shox2+ V2d INs have a marked overlap with Chx10+ V2a INs [64]. V2d INs have been shown to be ipsilaterally projecting onto ipsilaterally and commissurally extending cells and are most likely involved in the stabilization of locomotor frequency[65].

The different functions of V2a INs at different levels of the spinal cord indicate their roles in many aspects of motor function, making them another good target for better understanding CPGs and for spinal cord injury therapeutics. As seen by the V2b IN also being involved in flexor-extensor coordination, there may be some overlap in cell type function within the spinal cord. This could also be true for cell types involved in functional recovery. Thus, our lab is also investigating V2a INs in addition to the V3 INs reported in this thesis.

1.3.4 MNs

MNs are the second most ventral spinal population. As their name indicates, MNs are not considered interneurons; however, MNs are very important for locomotor function and are often
the direct synaptic targets of the interneuron populations. MNs arise from the Olig2 expressing pMN domain, which also gives rise to astrocytes and oligodendrocytes [71,72]. Post-mitotic MNs express Hb9 and Isl1 [73,74]. As MNs mature, they organize into rostral-caudal columns and cluster into pools that further specify their function within motor circuits. In fact, this highly predictable organization was first observed by George Romanes in 1941 and later elaborated upon in 1951[75]. This organization is positioned consistently along the rostral-caudal, dorsal-ventral, and medio-lateral axes of the spinal cord. The rostral-caudally organized columnar identities specify where they settle and which muscles they innervate [76-79]. Furthermore, within the columns, the neurons that innervate muscle groups with synergistic effects are also grouped together in pools [80,81]. This observed rostral-caudal patterning has been shown to be regulated by Hox genes which are patterned by a combination of Fgfs, Gdf11, and retinoid signaling [82]. Much work has been done to understand the molecular pathways involved in MN specification [83]. Lim homeodomain factors (Lhx3 and Lhx4), the Hox cofactor FoxP1, and many other molecular markers help specify MN subtype fate [84,85]. This further differentiation of MNs into subtypes is vital to motor control, especially the finer aspects of motor control due to their predictable and specific projections on to muscles.

One experiment found that MNs could be stripped of their columnar and pool organization by mutating FoxP1. In these mutants, there are only some MNs that project similarly to wildtype. Analysis of sensory input showed that appropriate sensory input was present only in the MNs that project in a similar manner as wildtype [86]. This indicates that sensory feedback synapse formation is independent of MN positioning but MN positioning is
vital for proper sensory feedback. This finding is important because it sheds light on the feedback process, without which, results in severely uncoordinated movements in animals [87].

1.3.5 V3 INs

V3 INs arise from the most ventral progenitor domain of the ventral spinal cord, the p3 domain. Marked by Ngn3 and Nkx2.2, p3 progenitor cells mature into Sim1 and Uncx expressing V3 INs [4,36,88,89]. A great majority of V3 INs project commissural axons with a few (<15%) projecting axons ipsilaterally [4]. There are even V3 INs that project to both halves of the spinal cord [4]. V3 INs have been shown to project onto many locomotor cell types, including MNs, IaINs, V2 INs, and Renshaw cells, indicating the importance of V3 excitatory input for many aspects of locomotion [4].

V3 INs are involved in locomotion rhythm bursting and symmetry during walking. Fictive locomotor studies have shown a decrease in the regularity of locomotor-like activity in spinal cords with blocked V3 neurotransmission. In the same spinal cords, motor outputs were observed to become asymmetrical across the left and right half of the spinal cord. Additionally, blocking V3 IN signaling also demonstrated a change in the duration of stepping during walking, generating asymmetrical and variable walking rhythms. [4] This elucidates the importance of V3 INs in establishing robust locomotion rhythm.

V3 migration and distribution have also been reported. V3 INs in the lumbar spinal cord separate into dorsal and ventral (V3DS and V3VS, respectively) populations after migration. These spatially separated populations are electrophysiologically distinct and can be distinguished by four main attributes: f-I slope, Cm, first spike frequency, and sag amplitude at -120 mV [90].
While some cells that were not spatially dorsal or ventral were found, the researchers were able to classify them based on these electrophysiological properties. Furthermore, these two populations were shown to be recruited differently during running or swimming in adult animals. Running recruited both populations while swimming tended to recruit V3vs [90]. This difference was present throughout post-natal locomotion maturation. At P0, both V3 IN subpopulations exhibit different properties than their P21 counterparts but the two subpopulations were still electrophysiological distinct [91].

While Sim1 is a marker for V3 INs, it is not required for V3 IN identity. In fact, V3 IN type (Uncx + and VGluT2 +) cells are present in Sim1 knockout mice. However, Sim1 plays an important role in migration and axon projection. Mice lacking Sim1 fail to display properly migrating V3 INs. Additionally, in Sim1 mutant mice, axons fail to extend as long as in control mice. [88]

V3 INs are one of the least well characterized cell types in the ventral spinal cord. While some aspects of their functions are known, subtype markers and the factors involved in subtype maturation are unknown. However, their role in rhythm generation and burst regulation make them an important target for understanding CPGs. Furthermore, their commissural projections make them a potential cell type for reorganization around an injury site and functional recovery after spinal cord injury. This work contributes to that understanding by offering a way to obtain V3 INs without tedious dissection and purification of cells.
1.3.6 Ventral Spinal Populations and Motor Circuits

Ventral spinal populations are a diverse population of neurons. While they have been broadly grouped into the aforementioned V0-V3 and MN populations, it should be evident that these methods of identification grossly understate the complexity of this system. Each population can be subdivided into more functionally specific categories. The diversity of these cells results in the complex system of feedback and signaling that is used to control locomotion activity in vertebrates. While using developmental molecular markers are important for lineage tracing and neuronal subpopulation identification, this method of identification is not the end all be all for understanding IN function.

The functional properties of ventral INs have some overlap and redundancy; silencing of a single population does not shut down all locomotion. While coordination has been shown to be decoupled (V0s) and robustness of rhythm disturbed (V3s), these populations are generally not the only INs involved in their respective aspect of locomotion. In fact, the role of many IN populations may overlap (both V2a and V0s are involved in left-right alternation) while other INs function to fine-tune motion (V0Cs’ cholinergic input onto MNs). Additionally, functionally specific populations may not arise from a single spinal population (e.g. not all IaINs are V1 in origin). All this taken together indicates a level of redundancy and attenuation in the overarching architecture of locomotion control.

Another caveat for understanding ventral spinal populations is the widely used fictive locomotion assay for accessing ablation induced functional perturbations. These preparations utilize drug cocktails to activate and record spinal circuitry activity. It was found that in VGluT2 mutant spinal cords, the drug cocktails were sufficient to induce “close-to-normal” patterns while
descending or ascending electric stimulation did not result in these patterns[92]. This surprising finding has direct implications for fictive locomotion assays that report more subtle changes in population ablated studies, suggesting that the chemical stimulation may bypass normal circuitry and directly affect recorded signals. This could also indicate that any IN population where drug cocktails were used could have a more severe phenotype than initially assumed[92].

Finally, sensory input participates in CPG circuitry feedback. As seen in the location based sensory input of MNs, spatial order is vital for appropriate sensory input. Furthermore, IaIN and dorsal V3 IN populations both relay sensory input as part of their function. To better understand sensory input processing in the CPG circuitry, the specific signal sources and synaptic targets of dorsal V3 INs, IaINs, and any cells involved in sensory processing should be studied.

1.3.7 Ventral Spinal Population Inductions from ESCs

Cells of the central nervous system have been obtained through the differentiation of ESCs. Cell types include, but are not limited to, MNs [6], V2a INs [93], dopaminergic neurons [94], cerebellar neurons [95], cortical pyramidal neurons [96], and even glia such as oligodendrocytes [97] and astrocytes [98].

MN differentiation was first reported by Wichterle et al. By utilizing RA, Wichterle et al. was able to drive caudalization of the induced cells into a spinal identity [6,99]. Furthermore, the strong Shh signaling was able to drive Olig2 and later Hb9 expression. The derived cells functionally matured into cholinergic neurons [100]. To look at how altering RA and FGF
signaling effects rostral-caudal spinal identity, the same group used FGF in their media to target more caudal identities, which they verified with Hox gene expression [101]. The resulting MNs were transplanted into chick neural tubes and observed to settle within columns and to innervate appropriate muscle groups [101]. Furthermore, the MN differentiation protocol has been applied to human ESCs and iPSCs [102,103]. The time scale for MN induction in human cells were much longer than in mouse ESCs. However, it’s been shown that a combination of Shh agonists can expedite the MN induction process [104].

Inductions have also been reported for other ventral spinal cell types. Brown et al reported the generation of V2a INs [15] from mESCs. Due to a large population of V2a INs in the brain stem and cervical spinal cord region, RA expression was dropped to 10 nM in the V2a induction [93,105]. Additionally, V2a relative location to MNs lead to SAG being replaced by a weaker Shh pathway agonist – purmorphamine[93]. Finally, to target the induction of V2as instead of V2bs, a notch inhibitor was used [93]. Additionally, V0 INs have been reported by Kim et al.’s induction of ~22% Evx1 expressing cell types by inducing with RA but not Shh signaling [106]. While there are many induction protocols to derive different CNS populations, many populations have not been covered. This dissertation provides a new method to derive a type of spinal interneurons not previously established.

1.4 Genomic Engineering

Since the creation of the first knock-out mouse in 1989 genomic editing has been a vital tool for studying and understanding cell types and protein function in animal models. The
culturing of mESCs paved the way for the development of genetically engineered animals, most notably, knockout mice. In the late 1980s, Mario Capecchi, created the first knock-out mouse where cultured and genetically altered stem cells were introduced into mice embryos to generate a chimeric animal [107-109]. Chimeric animals are genetically mosaic, containing cells with two different genotypes, some of which would be in the reproductive organs. The chimeric animal would then be bred so that some of the genetically altered cells would give rise to gametes and ultimately result in animals that are heterozygous for a mutation. The heterogeneous animals would then be bred to generate heterozygous mutant animals. In knockout mice, mutations to both alleles of a specific gene enable researchers to study the effects of removing a gene in an animal [108]. Since their invention, knock-out mice have become a widely used and powerful tool to understand the role of specific genes within an organism. In addition to knock-out mice, reporter and lineage tracing mice have also been generated [110]. Reporter mice express a specific protein (e.g. green fluorescent protein) within a specific gene locus such that the protein would be present when the gene is being expressed. This allows or the study of the time course of gene expression. Lineage racing mice are used to look for cells that at any point had expressed a certain gene [111,112]. The most common lineage tracing mechanism uses the target gene locus to control Cre-recombinase expression. After the expression of the target gene, Cre-recombinase would remove a stop sequence inserted before a ubiquitously expressed reporter protein so that the reporter protein would always be expressed [111,112]. Lineage tracing allows researchers to understand which cells at any given time expressed their gene of interest. These genetically engineered mice were vital for the identification and characterization of many
different cell populations, including V3 INs that are the focus of this thesis [4]. This section focuses on some of the history and current techniques for genomic editing.

1.4.1 Homologous recombination and early gene engineering

Genomic engineering has been sought after as early as the 1970s when Wigler et al. demonstrated that the herpes thymidine kinase gene could be transformed into mammalian cells [113]. Not long after, head-to-tail concatemers were observed in cells where DNA had been injected. The concatemers, a high copy number repetition in a single locus, revealed the homologous recombination mechanism in mammalian cells [114], which provided the basis for gene targeting in mice. Capecchi et al. demonstrated that two dysfunctional neomycin resistant gene could recombine to form a functional one [115]. Concurrently, Smithies et al. reported successful targeting of β-globin locus in cultured mammalian cells [116]. These demonstrations of editing and targeting a specific gene ultimately resulted in using ESCs to generate knock-out mice. Due to inefficiency of using electroporation to introduce a targeting vector, neomycin resistance was used as positive selection to isolate cells that had successfully incorporated the targeting vector [109]. Additionally, the occurrence of non-homologous recombination is about 1000 fold higher than homologous recombination in any given experiment [117]. Thus, the TK gene was used as a negative selection group to help eliminate non-homologous recombination[108]. These practices were widely adopted to study genes in mice. Over 7,000 genes’ in vivo functions have been reported by generating knockout mice, making homologous recombination a ubiquitous and influential technology [117]. There are even efforts to make a comprehensive mouse library for every protein-encoding gene [118].
Early genomic engineering work required PCR and enzyme based methods for amplifying and assembling long pieces of DNA [119]. These techniques were tedious and required a lot of verification to ensure a mutation-free PCR product. However, the use of artificial chromosomes for generating vectors via homologous recombination [120,121] and the advent of recombination technology, especially red-recombination, allowed large stretches of DNA to be incorporated into a backbone plasmid without the use of PCR [122,123]. This technology resulted in more efficient generation of targeting vectors for recombination. Unfortunately, the large homology arms were still unwieldy, necessitating better recombineering techniques.

1.4.2 Engineering Nucleases for Genome Editing

The use of genome targeting nucleases has greatly improved the efficacy of genomic engineering. Technology, such as clustered regulator interspaced sort palindromic repeats (CRISPR/CAS9), transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) has been able to generate specific double stranded breaks in DNA which have provided a 50,000-fold increase in the probability of homologous recombination [124]. These highly customizable and versatile techniques have been shown to more efficiently generate knock-out and knock-in organisms. In fact, genome editing with nucleases was named the “Method of the Year 2011” by Nature Methods [125]. This section will discuss three of the most common nuclease targeting systems.
Zinc Finger Nucleases (ZFNs)

First reported in 1996, ZFNs (Fig. 1.A) are a modular combination of multiple DNA-recognizing zinc finger proteins (ZFP) domains and the cleaving domain of endonuclease FokI [126]. The hybrid system allow for the creation of ZFNs that can bind to a wide variety of genetic targets. Each ZFN system is comprised of a set of heterodimer ZFN proteins; each with a set of ZFP to recognize a target and a cleavage domain to cut [127]. One ZFN monomer would bind upstream and the other would bind downstream of the cleavage site, with the FokI domains overlapping in the middle. This system takes advantage of the need for the FokI cleavage domain to dimerize prior to cleavage as the FokI cleavage domain cannot cut one side of a double strand by itself [128]. However, when both ZFN proteins recognize and bind to the correct targets, the cleavage domains can dimerize and cleave the DNA.

The original ZFN design consisted of ZFP monomers recognizing a three base pair triplet. By fusing ZFP domains together, a longer and more specific sequence can be targeted for cleavage anywhere in a genome. Unfortunately, not all ZFPs for DNA triplets have been identified. Of the known ZFPs, many have been characterized only by in vitro binding. This limitation greatly lowers the predictability of ZFPs in live systems [129].

More recently, a method termed OPEN (oligomerized pool engineering) was reported. Using libraries of ZFP sequences, this method can identify combinations of ZFPs that can target 9 bp site with 70-80% efficiency [130]. However, this method requires high expertise and intense labor, which makes it non-ideal for widespread use. Another method for improving ZFP targeting is called CoDa (context-dependent assembly). This method uses previously established three ZFP arrays to target specific sites. By generating a library of N- and C- terminal ZFPs with
different centers, any N- and C- terminal ZFP can be joined if they share an identical middle ZFP. By accounting for the context of the adjacent ZFPs to design a desired ZF, this method can achieve comparable efficiency to OPEN while not requiring a comparable level of expertise or labor [131]. Despite improvements to designing de novo ZFNs, the efficiency is still not 100%. While ZFNs could be a very useful tool for cleaving DNA, affinity and specificity are problems that still must be addressed before widespread adoption. Furthermore, the design and assembly of ZFNs are still labor intensive and expensive which further limits the adoption of this method for genomic engineering[132].

**Transcription activator-like effector nucleases (TALENs)**

TALENs (Fig. 1.1B) also utilize the FokI endonuclease cleavage domain fused to a DNA binding domain. Instead of ZFPs, TALENs use TALEs (transcription activator-like effectors), which were originally isolated from pathogenic bacteria found in plants[133]. These TALEs are highly conserved amino acid sequence 33-35 in length [134]. The 12th and 13th residue of these repeat sequences are highly variable and function in DNA base recognition [134]. Understanding of this structure allowed for the engineering of TALE repeats to specifically bind to DNA sequences [135]. Currently the most common 12th and 13th variables for identifying guanine, adenine, cytosine, and thymine are, respectively, NN, NI, HD, and NG [135]. This simple mapping makes TALENS a more direct way to engineering specific nucleases than ZFPs.

While TALENs have been shown to function in a wide variety of genomic editing applications, the construction of TALENs is not a trivial matter. The highly repetitious nature of
the sequences makes assembly fairly challenging. While resources are available to aid in this, the manual intensiveness hinders TALENs from being readily adopted for genomic engineering[132].

![Diagram](image)

**Figure 1.1** Simple schematics of nucleases for genomic editing. A) ZFNs with various ZFs represented by circles and connected to a FokI. Two ZFNs binding to different stretches of the genomic DNA enables FokI to dimerize and form a double-stranded break. B) TALENS, like ZFNs, utilize the dimerization of FokI for DNA cleavage. Small rectangles represent TALEs that make up a complex TALEN DNA binding sequence. C) CRISPR/Cas9 system uses the guide RNA to specify the Cas9 protein’s binding and cleavage regions.

**Clustered regulator interspaced sort palindromic repeats (CRISPR/Cas9)**

CRISPRs (Fig. 1.1C) were first observed in bacteria and archaea in 1987[136] but their function as a bacterial immune system and evidence of adaptive CRISPR associated (Cas) related functions were not reported until 2007[137,138]. CRISPR provided immunity worked in three steps: insertion of invading DNA into the CRISPR array, generation of mature CRISPR RNAs from precursors, and the cleavage of foreign DNA by Cas proteins guided by the CRISPR RNAs [139]. While there are three types of CRISPR systems, only one requires a single Cas protein for guide RNA mediated cleavage [140]. The discovery of this single Cas protein, Cas9, as a double stranded break generator provided the basis for subsequent CRISPR/Cas9 system development.
Since its initial report in 2012, the CRISPR/Cas9 has been widely adopted due to its ease of use, efficiency, and versatility. In this system, to modify the DNA target site, one only needs to change the guide RNA sequence [141]. Furthermore, two mutations to the Cas9 protein were found that resulted in the Cas9 becoming a “nickase”, an enzyme that cut only one strand of DNA. When both mutations were introduced into the nuclease, Cas9 mutates into a DNA binding protein [140,141]. CRISPR/Cas9 system’s versatility lies in its ability to target multiple genes concurrently by introducing more than one guide RNA[142]. These possibilities have allowed CRISPR/Cas9 to have very broad uses in biology ranging from engineering cells to genetic studies.

However, CRISPR/Cas9s also have off target effects. The Cas9 protein can interact with off-target DNA and the guide RNA tolerates multiple mismatches [143,144]. While dosage titration of the Cas9 protein and guide RNA could help minimize these off target effects [143], a newer method for improving Cas9 specificity is combining the mutant DNA binding Cas9 protein with FokI monomer[145]. This new system couples the ease of Cas9/CRISPR manipulation with the improved specificity of a dimerizing system. Still, better control of the nuclease systems must be achieved before translation for human therapeutic uses.

1.5 Concluding Remarks

In this dissertation, I aimed to generate V3 INs from mESCs. Others’ work in understanding the developmental signaling pathways involved in ventral spinal specification as well as establishing MN/V2a induction protocols has provided a strong foundation and solid
starting point for this thesis. I considered the spatial and temporal expression patterns of different signaling molecules during development and accordingly altered previously differentiation protocols to yield the highest number of V3 INs. By doing so, I have established a novel induction protocol for generating V3 INs. Furthermore, to further purify V3 INs, I have developed a drug selectable cell line where a drug resistance gene is controlled by the post-mitotic V3 marker, Sim1. This selectable cell line provides a high throughput and simple method for enriching the V3 IN population after induction. Finally, characterization of the selected cells allowed for verification of V3 INs’ identity and function. In this thesis, I present the results from these two aims.
Chapter 2: Directed Differentiation of V3 Interneurons from Mouse Embryonic Stem Cells

2.1 Introduction

V3 interneurons (INs) are commissural glutamatergic neurons that have been shown to be involved in rhythm generation networks within the spinal cord known as central pattern generators (CPGs) [4,90,92]. They can be identified by the p3 progenitor domain markers, Nkx2.2 and Ngn3 and the post-mitotic IN marker, Sim1 [36,89]. More recently Uncx has been

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shown to be a mature V3 marker [88,146]. Initial fictive locomotor assays in Sim1 knockout mice suggest that V3 INs have a role in balancing locomotor outputs in the spinal cord to regulate left-right alternation in gait [4]. More recent studies have demonstrated that spatially separated and functionally distinct subpopulations of V3 INs (termed V3d and V3v) can be differentially recruited for running and/or swimming [90]. V3 INs also have the ability to cross the midline and synapse onto motoneurons (MNs) and other INs across multiple spinal segments [36]. These findings suggest that V3 INs play a critical role in locomotor coordination and may be involved in local reorganization after spinal cord injury (SCI). An efficient method to obtain V3 INs is necessary for enhanced understanding of CPGs and recovery after SCI.

ESCs can provide a plentiful cell source for cell replacement therapies and to study developmental biology. Protocols using RA and sonic hedgehog (Shh) signaling to direct differentiation of ESCs into various ventral spinal cell identities including MNs [6] and V2a INs [93] by mimicking conditions found in the developing ventral neural tube have been reported. During development, RA released from laterally positioned somites [147,148] and Shh released from the notochord and floor plate generate overlapping gradients on the neural tube [6,39-41]. Shh diffuses dorsally from the floor plate and notochord, generating a ventral to dorsal signaling gradient (Fig. 2.1A). RA is a caudalizing factor in neuronal differentiation [99,106,147,148] (Fig. 2.1D) and may help pattern the dorsal-ventral identity of embryoid body (EB)-derived neuronal populations in vitro [148]. These two overlapping gradients specify different progenitor domains (pMN and p0-p3) within the ventral cord, which give rise to MNs and ventral IN classes (V0-V3), and are identified by distinct transcription factors [36,37,39,149-
This complex interplay between Shh and RA serves as a basis for specifying ventral neural differentiation from ESCs.

Figure 2.1 (A) Diagram depicting the ventral developing neural tube. The gradient of Shh arising from the floorplate (FP) establishes the ventral progenitor domains (p1-3 and pMN). Specific transcription factors expressed in these domains drive the maturation of the committed domains (V1-V3 and MN), which are defined by specific transcription factors (shown on the far right). Adapted from Brown, et al.[39,93]. (B) A schematic depicting the effect of time and concentration on establishing progenitor domains. With increasing duration of high Shh exposure, more ventral domains are established. Adapted from Dessaud, et al. [37]. (C) Schematic depicting the distance from RA-expressing somites. (D) Illustration of the rostral-caudal axis and direction of increasing RA expression.
In this study, we establish a novel protocol to generate V3 INs from ESCs by assessing the effects of increasing Shh signaling, prolonging the duration of Shh exposure, and decreasing RA concentration on p3 progenitor and post-mitotic V3 IN marker expression in ESC-derived neural cultures. V2a INs, which lie dorsal to MNs, require a weaker Shh signaling agonist than do MNs. Since V3 INs lie ventral to both V2s and MNs [38,39], we hypothesized that further increasing the Shh signaling level would promote increased differentiation of V3 INs. Previous studies suggested that a more ventral spinal fate is specified by increasing the duration of Shh exposure, therefore we also chose to study the effects of varying the duration of Shh exposure of cell fate (Fig. 2.1B) [37,38,151]. Furthermore, because the progenitor p3 domain lies further from the RA-releasing somites than the pMNs, we hypothesized that a lower RA concentration could further improve V3 IN induction. Cells obtained with our protocol can be used for studying CPG connectivity and cell-based transplantation therapies.

2.2 Materials and Methods

2.2.1 Culture of ESCs

RW4 mouse ESCs (American Type Culture Collection, Manassas, VA) were cultured on T-25 flasks coated in 0.1% gelatin (Sigma, St. Louis, MO). Cells were cultured in complete media consisting of Dulbecco’s Modified Eagle Medium (DMEM 11965, Life Technologies, Carlsbad, CA) containing 10% newborn calf serum, 10% fetal bovine serum, and a 1:100 dilution of a 100x nucleosides mix (EMD Millipore, Bellerica, MA). Cells were routinely passaged every other day by washing with DMEM 11965 containing 25 mM HEPES (Life
Technologies) and dissociating with 1 mL 0.25% trypsin ethylene diamine tetra acetic acid (EDTA, Life Technologies), quenching with 4 mL complete media, and plating 1 mL into a new T-25 gelatin coated flask containing a final volume of 5 mL media with 1000U/mL leukemia inhibitory factor (EMD Millipore) and 100 μM β-mercaptoethanol (Life Technologies).

2.2.2 Formation and induction of EBs

RW4 ESCs were aggregated to form EBs on a non-adhesive agar-coated surface and induced to generate neural progenitors using our previously established 6 day induction protocol (2/4+)[6,99,153] or an 8 day induction protocol (2/6+). Cells were cultured in suspension for two days on 100 mm petri dishes pre-coated with 0.1% agar (Thermo Fisher Scientific, Waltham, MA) in DFK-5 media comprised of 1:1 DMEM/F12 (Life Technologies) with 5% knockout serum replacement (Life Technologies), 1x insulin transferrin selenium (Life Technologies), 100 μM β-mercaptoethanol, 50 μM nonessential amino acids (Life Technologies), and a 1:200 dilution of a 100x nucleosides mix. During the first two days, the cells are not exposed to RA or SAG (hence 2−), allowing the cells to aggregate into multi-cellular EBs. After the first two days of aggregation, EBs were plated on gelatin. 200 μL of EBs were removed from the 100 mm plate and replated onto one well of a gelatinized six well plate. DFK-5 media was added to a final volume of 2 mL and supplemented with RA (Sigma) (0 μM to 10 μM) and SAG (EMD Millipore) (0 μM to 1.25 μM) on day 3 through the end of the induction (either 4 days or 6 more days for 4+ or 6+, respectively). Media with RA and SAG was replaced every two days for four (2/4+) or six (2/6+) days (Fig. 2.2A).
2.2.3 Quantitative real-time PCR

Following induction, each well of a six well plate was lysed with 750 μL of buffer RLT from the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was extracted per the manufacturer’s instructions. High Capacity RNA-to-cDNA Kit (Life Technologies) generated cDNA from extracted RNA. TaqMan Gene Expression Assays (Life Technologies; Table 2.1) and TaqMan Fast Advanced Master Mix (Life Technologies) were subsequently combined with the purified cDNA for qRT-PCR. Reactions were then performed using a Step One Plus Applied Biosystems thermocycler with the default protocol: 95°C for 20s, 40 cycles of 95°C for 1s and 60°C for 20s. 

$C_t$ values, the number of cycles necessary for the fluorescent intensity to increase exponentially, were recorded and normalized to β-actin expression. The comparative $ΔC_t$ method [154] was used to analyze the mRNA expression levels in cultures post induction ($2^{-4}$ and $2^{-6}$). Fold differences in relative mRNA expression levels over the control cultures are reported for each gene (n ≥ 3 for all groups).

**Table 2.1**: TaqMan Gene Expression Assays for qRT-PCR

<table>
<thead>
<tr>
<th>Marker</th>
<th>Life Tech Identification</th>
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<tbody>
<tr>
<td>Beta-Actin</td>
<td>Mm00607939_S1</td>
</tr>
<tr>
<td>Sim1</td>
<td>Mm00441390_m1</td>
</tr>
<tr>
<td>Nkx2.2</td>
<td>Mm00839794_m1</td>
</tr>
<tr>
<td>HB9</td>
<td>Mm00658300_G1</td>
</tr>
<tr>
<td>RAX</td>
<td>Mm01258704_m1</td>
</tr>
</tbody>
</table>
2.2.4 Live/Dead Viability assay

Live/Dead reagent (Life Technologies) consisting of calcien-AM and ethidium homodimer was used to visualize live and dead cells, respectively. Cells were dissociated and incubated in Live/Dead reagents as per manufacturer instructions for flow cytometry. For each culture, 30,000 events were recorded using a BD LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data analysis was performed using FloJo software (FloJo, Ashland, OR).

2.2.5 Dissociation and plating of EBs

Standard tissue culture plates were pre-coated with 0.01% poly(ornithine) solution (Sigma) at 37°C for 1 hour. Poly(ornithine) was removed and plates were washed with a HEPES buffered saline solution 3 times. Plates were then coated with 0.01 mg/mL laminin (Life Technologies) in HEPES buffered saline solution overnight and washed 3 times before use as laminin coated plates. Induced 2^-/6^+ EBs were allowed to settle and supernatant was collected. Then 0.25% Trypsin-EDTA was used to dissociate the EBs. Dissociated cells were plated at a density of 100,000 cells/cm^2 in the collected DFK-5 supernatant.

2.2.6 Immunocytochemistry

For plated cultures, cells were fixed by adding 4% PFA in PBS to the cultures for 20 minutes at room temperature, permeabilized with 0.01% Triton X-100 (Sigma) for 20 minutes. Cells were then blocked with 5% normal goat serum (Sigma) in PBS for 1 hour and stained overnight in 2% normal goat serum in PBS with added primary antibody (mouse anti-Nkx2.2,
1:100, Developmental Studies Hybridoma Bank; mouse anti-Ngn3, 1:100, Developmental Studies Hybridoma bank; mouse anti- Uncx, 1:500, EMD Millipore) overnight at 4ºC. Cells were then stained for 1 hour in 2% normal goat serum in PBS with Alexa Fluor conjugated antibodies (goat anti-mouse IgG 488 Life Technologies) at 4ºC, then Hoechst 33258 (1:1000, Life Technologies) for 15 minutes at room temperature, and stored in PBS at 4ºC.

2.2.7 Probe synthesis for in situ hybridization

Plasmids for in situ hybridization probes were a generous gift from Dr. Paul Gray (Washington University) [155]. Gene fragments from verified plasmids were linearized by direct PCR amplification using ReadyMade Primers (SP6 Promoter and T7 Promoter, Integrated DNA Technologies, Coralville, IA). Digoxigenin (DIG)-labeled antisense and sense RNA probes were made using PCR products as template and T7 RNA polymerases (Roche, Indianapolis, IN). Probes were used at a concentration of 1–2 μg/ml. Sense counterparts of all probes were tested to ensure probe specificity.

2.2.8 In situ hybridization

Cell cultures were fixed and stained with modifications protocol from previously described [155-157]. Cell cultures were fixed in 4% paraformaldehyde (PFA) for 10 minutes and washed 3 times in diethylpyrocarbonate (DEPC, Sigma) treated PBS at room temperature. Next, cells were treated with RIPA buffer (150 mM NaCl (Thermo Fisher Scientific), 1% NP-40 (Thermo Fisher Scientific), 0.5% Na deoxycholate (Thermo Fisher Scientific), 0.1% Sodium
dodecyl sulfate (Sigma), 1 mM EDTA (Thermo Fisher Scientific), 50 mM Tris in diethylpyrocarbonate (Sigma) treated water at pH 8.0) and washed for twice with DEPC treated PBS. Then the cells were incubated in 0.1 M triethanolamine-HCl (1.3% triethanol amine (Sigma) and 0.4% HCl (Thermo Fisher Scientific)) with 0.25% acetic anhydride (Sigma) for 10 minutes. Cells were washed in 1x sodium citrate buffer for 5 minutes at room temperature. 3 additional washes with DEPC treated PBS were performed before cells were blocked in hybridization buffer (50% formaldehyde (Sigma), 5x sodium citrate buffer (SSC, Life Technologies), 0.3 mg/mL yeast RNA (Sigma), 0.1 mg/mL heparin (Sigma), 1x Denhardt’s solution (Life Technologies), 0.1% tween (Sigma) and 5 mM EDTA) for 4-6 hours at room temperature. Cells were incubated in hybridization buffer containing 1–2 μg/ml DIG-labeled antisense cRNA overnight at 65°C. Probed cells were washed in twice in 0.2× SSC at 62°C, and incubated in 0.2× SSC for 60 minutes at 65°C. Washed cells were adjusted to room temperature and blocked with 10% deactivated horse serum (Life Technologies) in phosphate buffered saline with 2 mg/mL bovine serum albumin and 0.1% Triton X-100 (PBT), and incubated in alkaline phosphatase-labeled anti-DIG antibody (1:2000 in 10% deactivated horse serum in PBT; Roche) overnight. Cells were further washed with PBT and color was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche). Staining was stopped with 4% PFA after visual inspection. Cell nuclei were stained with the DNA binding dye Hoechst (1:1000, Life Technologies) and by immunocytochemistry with Nkx2.2.

2.2.9 Flow cytometry
Immediately following the induction protocol, EBs were stained for flow cytometry. Cultures were dissociated with 0.25% trypsin-EDTA for 20 min at 37°C. An excess volume of complete media was added to quench the trypsin-EDTA, and cultures were triturated to obtain single cell suspensions. Cells were centrifuged at 360xg for 6 minutes, the media was removed, and the cells were fixed, permeabilized, and stained according to the Transcription Factor Buffer Set (BD Pharmingen 562725, Franklin Lakes, NJ) manufacturer’s instructions with mouse anti-Nkx2.2 (1:100), mouse anti-Isl1 (1:100; Developmental Studies Hybridoma Bank), and mouse anti-Hb9 (1:20; Developmental Studies Hybridoma Bank) primary antibodies and appropriate Alexa Fluor secondary antibodies (1:200, Life Technologies). Following the induction protocol, nuclei were stained with Hoechst (0.5 μg/ml, Life Technologies) for 15 min. For each culture, 10,000 events were recorded using a Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data analysis was performed using FloJo software (FloJo, Ashland, OR). Debris was removed by gating using the forward scatter versus side scatter and Hoechst fluorescence versus forward scatter plots. Control groups of cells stained with only secondary antibodies were used to determine gating parameters. Results of the flow cytometry are presented as percentage primary antibody+ cells out of the total Hoechst+ population. Sample gating is presented in Fig 2.5.

2.2.10 Statistics

For quantitative experiments, at least 3 replicates of each condition were analyzed. For cell counting, at least 3 images were analyzed of every replicate to provide a representative sample of the replicate. Statistica software (version 5.5, StatSoft, Inc., Tulsa, OK) was used for
statistical analysis. Significance was determined using Scheffé’s post hoc test for analysis of variance with 95% confidence. Average values are reported with error bars indicating the standard error of the mean.

2.3 Results

2.3.1 Effect of SAG concentration and duration of exposure on gene expression

The effect of increasing the concentration and duration of exposure of SAG on gene expression was examined using qRT-PCR to evaluate expression of V3 and pMN ventral neural markers (Sim1, Nkx2.2, and Hb9). Concentrations of SAG were varied from 0.5 to 1.25 µM with 2 µM RA present, and samples were collected after 2/4+ and 2/6+ inductions. In this notation, “2−” refers to the number of days ES cells are allowed to aggregate into EBs without (-) RA and SAG (middle section of Fig. 2.2A). The “4+” or “6+” refers to the number of days the EBs are exposed to (+) RA and SAG (right section of Fig. 2.2A). mRNA expression fold change was determined by comparing qRT-PCR C_t values of induced cells to uninduced cells that were not exposed to RA or SAG over the same culture period (n ≥3 for all conditions). Expression of the post-mitotic V3 marker, Sim1, increased across all concentrations of SAG when the SAG exposure time increased from 4 to 6 days (2/4+ to a 2/6+ induction) (Fig. 2.2B). After the 2/6+ induction, the group treated with 0.5 µM SAG exhibited significantly higher levels of Sim1 expression than the group treated with 1 µM SAG (22.5 ± 0.5 fold greater versus 15.0 ± 1.3 fold greater than uninduced control). Further reduction of the SAG concentration to as low as 0.05 µM
**Figure 2.2.** Effect of SAG concentration and duration of exposure on gene expression. (A) Schematic depicting the 2/6+ induction protocol where ESCs are aggregated in agar coated plates for 2 days and then exposed to 6 days of RA and Shh signaling. (B, D, E) qRT-PCR results (n=3) at the end of a 2/4+ and 2/6+ induction for mRNA levels compared to the uninduced (no RA, no SAG) control. (B) Sim1 gene expression after induction. All 2/6+ groups express significantly more Sim1 than their 2/4+ counterparts. 0.5 µM SAG at 2/6+ was significantly greater than 1 µM and 1.25 µM groups. * denotes p < 0.05 versus the same SAG concentration with the 2/4+ induction; # denotes p < 0.05 versus 1 µM SAG group at the same time point. (C) The effect of SAG concentration on cell death. # denotes p < 0.05 versus 1 µM SAG. ¤ denotes p < 0.05 versus 1.25 µM SAG. (D) Nkx2.2 gene expression after induction. At 0.5 µM SAG, Nkx2.2 expression at 2/6+ was significantly greater than at 0.75 µM, 1 µM, and 1.25 µM. ^ denotes p < 0.05 versus 0.75 µM, 1 µM, and 1.25 µM at the same time point (2/6+). (E) Hb9 expression after induction. No significance was observed across SAG concentration groups for Hb9 at either time points. * denotes p < 0.05 versus 2/4+ induction groups at the same SAG concentration.
μM did not alter Sim1 expression significantly. Additionally, after the 2/6+ induction, the group treated with 0.5 μM SAG exhibited significantly higher levels of Nkx2.2 mRNA (30.5 ± 4.4 fold greater than uninduced control) compared to other SAG concentrations tested (Fig. 2.2C). Furthermore, Hb9 expression did not change significantly with SAG concentration at a given time point, but it was lower for the 2/6+ induction compared to the 2/4+ induction in most groups (Fig. 2.2D). Hb9 and Nkx2.2 expression levels showed similar trends to the Sim1 results, indicating that prolonged exposure promotes the targeted gene expression for V3 INs. We observed, at higher SAG concentrations, more EBs detached in cultures on gelatin-coated plates. To understand why we were observing a decrease in Sim1 induction efficiency, we assessed the percentage of cells surviving after the 2/6+ induction with 0.5, 0.75, 1.0, and 1.25 μM SAG. We found that there was a significant increase in cell death at 1.25 and 1.0 μM SAG (Fig. 2.2C). Therefore, 0.5 μM SAG and the 2/6+ induction protocol were used for subsequent studies.

### 2.3.2 Effect of RA concentration on gene expression

To investigate the effect of RA concentration on ventral neural identity, qRT-PCR was used to evaluate changes in Sim1, Nkx2.2, and Hb9 expression. The concentration of RA was varied from 0.005 to 10 μM in combination with SAG (at a constant level of 0.5 μM), and samples were collected at the end of the 2/6+ induction protocol. Gene expression levels were compared to baseline concentration for the motor neuron induction protocol (2 μM RA), mRNA expression fold change was established by comparing qRT-PCR Ct values of all induced groups to the group treated with 2 μM RA and 0.5 μM SAG (n ≥ 3 for all conditions, highest Nkx2.2 and Sim1 levels in Fig. 2.2). When RA concentration decreased to 0.01 μM, Sim1 expression
**Figure 2.3.** The effect of RA concentration on ventral neural marker gene expression. (A) Sim1 and Nkx 2.2 expression after induction compared to 0.5 µM SAG and 2 µM RA. * denotes p < 0.05 versus 0.1 µM and 10 µM RA groups. Dotted line denotes upregulation threshold compared to 0.5 µM SAG and 2 µM RA. (B) Hb9 expression after induction compared to 0.5 µM SAG and 2 µM RA. *** denotes p < 0.001 versus all other groups. ** denotes p < 0.01 versus 0.0005 µM and 0.001 µM RA groups. # denotes p < 0.05 versus 0.0005 µM RA group. Dashed line denotes downregulation threshold compared to 0.5 µM SAG and 2 µM RA. (C) RAX expression after 2/6+ induction compared to 0 µM SAG and 0 nM RA. ^ denotes p < 0.05 versus 0 nM RA group. Dashed line denotes downregulation threshold compared to uninduced control.
was found to increase 11.4 ± 2.9 fold versus the 2 µM RA group (Fig. 2.3A). Similarly, decreasing RA concentration to 0.01 µM significantly increased Nkx2.2 mRNA levels (4.3 ± 0.4 fold) versus the 2 µM RA group. Hb9 expression levels decreased significantly when the RA concentration was lowered for all concentrations tested compared to the 2 µM RA group (Fig. 2.3B). Further lowering of RA concentration beyond 0.01 µM did not significantly change Sim1 or Nkx2.2 expression levels. The increase in Sim1 and Nkx2.2 combined with the decrease in Hb9 matched gene expression patterns expected for V3 INs, indicating lowering the concentration of RA did induce further ventralization of our culture.

To test for unwanted rostralization with lower RA levels, gene expression levels of retina and anterior neural fold homeobox (RAX, a hypothalamus marker) were examined using qRT-PCR. We tested the effect of varying the RA concentration from 0 to 10 µM with 0.5 µM SAG using a 2/6+ induction protocol. All data were compared to the uninduced condition at the same time point to investigate the extent of rostralization for each RA concentration. All conditions showed decreased RAX expression compared to uninduced controls (Fig. 2.3C, dashed line). Additionally, RAX expression was significantly lower for groups at 0.01 µM RA and 0.1 µM RA compared to the groups at 0 µM RA and 0.5 µM SAG. The down regulation of RAX suggests that Sim1 expression was not an indicator of hypothalamus induction in these cultures.

2.3.3 Immunocytochemistry and flow cytometry

To confirm the results observed by qRT-PCR, immunocytochemistry and flow cytometry were used to assess transcription factor expression at the protein level. Nkx2.2 immuno-
Figure 2.4. The effect of decreasing RA concentration on Nkx2.2 expression by immunocytochemistry. EBs fixed at the end of 2/6+ induction with 0.5 µM SAG and 2 µM RA (A-C), 0.1 µM RA (D-F), 0.01 µM RA (G-I). Hoechst nuclear staining (A,D,G), Nkx2.2 antibody staining (B, E, H), and overlay of the two stains (C, F, I) are shown. (J) Quantification of p3 (Nkx2.2 and Ngn3) and V3 (Uncx) marker immunocytochemistry on dissociated EBs plated onto laminin-coated plates. Values given as % cells positive for marker. * denotes significance (p < 0.05) compared to 2 µM RA. ^ denotes significance (p < 0.05) compared to 0.1 µM RA and 2 µM RA. Scale bar = 50 µm.
Figure 2.5. The effects of RA concentration on neuronal marker expression by flow cytometry. Flow cytometry performed on induced EBs after the 2/6+ induction with 0.5 µM SAG and varying RA concentrations (0.01, 0.1 and 2 µM). (A) Sample gating of flow cytometry for 2 µM RA and 10 nM RA for negative control (secondary antibody only, -ctrl), Nkx2.2, Hb9, and Isl1 stains. Region on left is negative while region on right is positive. (B) Quantification of percentage cells staining positive for Nkx2.2, Hb9, and Isl1 across various RA concentrations. * denotes significance (p < 0.05) versus compared to 2 µM RA groups.
cytochemistry on intact EBs (Fig. 2.4 A-I) qualitatively suggested an increase in Nkx2.2 mRNA levels with decreasing RA. To quantify this difference, immunocytochemistry was performed on dissociated EBs to see if p3 and V3 marker expression was increased in induced cultures. Total cell nuclei and nuclei positive for the markers were counted and the percentage of cells positive for the following markers: Nkx2.2, Ngn3, and Uncx was calculated. For all three markers, the percentage of cells positive for the given marker increased when RA concentration was decreased from 2.0 to 0.01 µM RA. Nkx2.2+ cells increased from 9% to 18%, Uncx+ cells increased from 1% to 8%, and Ngn3+ cells increased from 7% to 13% (Fig. 2.4J). Similarly, flow cytometry showed the percentage of Nkx2.2+ cells increased with decreasing RA concentration down to 0.01 µM RA (Fig. 2.5). Groups induced with 0.01 µM RA (and 0.5 µM SAG) using the 2'/6+ induction protocol generated significantly more Nkx2.2+ cells than those induced with 2 µM RA (and 0.5 µM SAG) (Fig. 2.5C). The percentage of cells positive for MN marker Isl1 also decreased significantly at 0.01 µM RA compared to 2 µM RA (Fig. 2.5D). The percentage of Hb9+ cells decreased significantly at both 0.01 µM RA and 0.1 µM RA compared to 2 µM RA, similar to the low Hb9 mRNA levels seen in Fig. 2.3B. The decrease in MN marker expression and increase in p3 marker expression corroborated that lowering RA further ventralized the induced neural population.

### 2.3.4 *In situ* hybridization of Sim1 on EBs

Due to the lack of a specific Sim1 antibody, *in situ* hybridization was used to confirm the presence of Sim1+ cells. EBs were dissociated after 2'/6+ induction (varying RA from 0.01 µM to 2 µM and constant 0.5 µM SAG). Performing *in situ* hybridization for Sim1 showed that
**Figure 2.6.** Sim1 and Nkx2.2 in dissociated EBs indicated by *in situ* hybridization and immunocytochemistry. Cultures induced with the 2/6+ protocol with 0.5 µM SAG and 2 µM RA (A-D), 0.1 µM RA (E-H), 0.01 µM RA (I-L) were dissociated and plated and then stained by *in situ* hybridization for Sim1 and immunocytochemistry for Nkx2.2. Sim1 *in situ* hybridization (A, E, I), Nkx2.2 antibody staining (B, F, J), Hoechst nuclear staining (C, G, K) and overlay of all three stains with Nkx2.2 false colored as red and Hoechst false colored as green (D, H, L) are shown. (M) Quantification of Scale bar = 50 µm.
lowering the RA concentration resulted in an increase in Sim1+ cells. At 0.01 μM RA, more Sim1+ cells were observed than at 0.1μM and 2μM RA (Fig. 2.6 M). When dissociated EBs were stained with Nkx2.2 antibody after in situ hybridization, Nkx2.2 and Sim1 expression was observed to co-localize (Fig. 2.6 I-L, arrows). This observation suggested that the induced cells are spinal V3 INs.

2.4 Discussion

Commissural glutamatergic V3 INs have been shown to be involved in rhythm regulation in the CPGs [92,158] by helping regulate left-right walking gait and generating robust rhythmic bursting [4,90]. Their role in regulating CPGs makes V3 INs an interesting target for understanding the development of the locomotor pathway and local rewiring after SCI. However, a readily available cell source for isolation of V3 INs in culture would allow greater understanding of their role at a cellular level. In this paper we present data on establishing a novel induction protocol for generating V3 INs from mouse ESCs.

Using a previously established 2/4+ protocol for the induction of MNs from mouse ESCs as a starting point, we explored the effects of varying level and duration of exposure to Shh signaling on p3 and V3 marker expression [6]. Although we hypothesized that increasing both the magnitude and duration of Shh exposure would drive more ventral neural identities, qRT-PCR data indicated that increasing the duration rather than the concentration of Shh signaling agonist appeared to be more effective for induction of V3 INs. While the expectation that lower Shh exposure will result in more dorsal cell types has proven true for V2a and V0 differentiation,
it has been observed in our lab that higher concentrations of SAG do not improve MN induction and appear to have a negative effect on EB health. The decreased efficacy of SAG at concentrations higher than 1 µM has been reported at early time points (30 hrs) [29]. Gli, a downstream target of SAG, was coupled to a luciferase assay and luciferase activity was reported to peak between 0.1 µM and 1 µM SAG, indicating peak SAG-driven Gli activation occurs somewhere between these values [29]. We surmised the observed decrease in Sim1 expression in our studies at concentrations of 1 µM or greater could be due to toxic effects of high SAG levels. This was confirmed with live-dead quantification of our induced cultures (Fig. 2.2C). Since SAG efficacy was limited at higher concentrations, an increase in Sim1 expression levels was only observed with increasing SAG exposure time and not concentration.

Because V3 INs are further than MNs from RA-releasing somites along the neural tube [147], we hypothesized that lowering the RA concentration would increase V3 IN differentiation. Our qRT-PCR data showed that decreasing RA concentration not only increased Nkx2.2 and Sim1 gene expression but also decreased Hb9 expression, indicated that lowering RA allows differentiation of more ventral populations. This data was further supported by flow cytometry and immunocytochemistry for Nkx2.2 and Isl1, as well as in situ hybridization for Sim1. Others have also shown that lowering RA to the 0.001-0.01 µM range increases Nkx2.2 expression [148], which matches with our observations.

While we observed increases in Sim1 expression by qRT-PCR, validation of the induction protocol required the presence of cells with high levels of Sim1 expression. Since a specific Sim1 antibody is not available, we used in situ hybridization to confirm that the increase in overall Sim1 expression, as seen in qRT-PCR, can be attributed to some cells with high levels
of Sim1 expression and not low levels of Sim1 expression in most cells. When dissociated EBs were stained with Nkx2.2 antibody after in situ hybridization, Nkx2.2 and Sim1 expression was observed to co-localize (Fig. 2.6 I-L, arrows). This observation suggested that the induced cells are spinal V3 INs.

Because Sim1 is not uniquely expressed in V3 INs, it was necessary to ensure that the induction protocol did not give rise to other Sim1+ neural populations. One concern with decreasing RA concentration is potential rostralization of the resulting cells. Since Sim1 is also expressed in the hypothalamus [159], we wanted to ensure the cells we obtained were not hypothalamus cells. To this end, the level of gene expression of the hypothalamus marker RAX was measured in the Sim1+ cultures generated in the low RA, high SAG induction protocol. Due to the heterogeneity of induction protocols [6,106,148], if the induced Sim1+ EBs upregulate RAX, then they could have hypothalamic identity [160-162]. The drastic downregulation of RAX after induction (Fig. 2.5) suggests that the Sim1+ cells observed in the induced culture are not hypothalamic in nature. One caveat worth noting is that qRT-PCR experiments report population averaged data. Fold changes of mRNA expression levels are reflective of an average value across the whole experimental population in comparison to a control population. A large increase in expression level in qRT-PCR could represent a large increase in a few cells or a small increase in many cells. While we see a decrease in RAX expression across the whole population, the existence of a rare RAX+ cell cannot be ruled out. Unfortunately, a reliable antibody for RAX does not exist and thus population averaged experiments are the most direct way to quantify the extent of RAX expression.
This study establishes a novel induction protocol for inducing V3 INs from mouse ESCs by demonstrating successful differentiation of Sim1+, non-hypothalamic cells in cultures that also express the p3 progenitor marker Nkx2.2. A scarce population of V3 INs exists in the developing spinal cord, totaling ~10% of the four ventral spinal cell types, making dissection tedious and expensive. The duration and potentially scalable nature of this protocol make our method an easier, cheaper, and faster way to obtain V3 INs than dissection. Based on the Uncx and Sim1 staining, about 8% of the induced cells are V3 INs. One 100 mm Petri-dish of induced 2/6+ EBs has about 21 million cells. This means about 1.6 million V3 INs can be obtained from one induction culture on this small bench scale, which would generally be sufficient for a rodent transplantation study. This work paves the way for future transgenic drug-selectable or lineage tracing cell lines to better understand the role of V3 INs in the spinal cord. Additionally, this protocol for V3 IN induction could potentially be adapted for human ESCs or induced pluripotent stem cells, much as the MN differentiation protocols from mouse ESCs have been adapted for these human cell types [163,164]. This method to generate Sim1+ cells can serve as a cell source for future studies exploring the role of V3 INs in CPGs and SCI therapy.
Chapter 3: A Puromycin Selectable Cell Line for the Enrichment of Mouse Embryonic Stem Cell Derived V3 Interneurons

3.1 Introduction

Pluripotent stem cells have the potential to be a plentiful cell source for many different cell types. Differentiation protocols have been established to generate specific cell types from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). In the central nervous system, differentiation protocols for ESCs have been developed for several populations, including midbrain and hindbrain dopaminergic and serotonergic neurons [94,165], spinal motoneurons (MNs) [6,99], and V0, V2a, and V3 spinal interneurons (INs) [93,106,166]. These populations (MNs and INs) contribute to locomotion either by directly innervating muscle or by
playing a role in central pattern generator (CPG) circuitry [4,36,48,49,92]. By deriving ventral spinal populations from ESCs or iPSCs, we can better study these populations in culture and their role in locomotion and reorganization after spinal cord injury.

One ventral spinal population that may contribute to reorganization after spinal cord injury is the commissural, glutamatergic V3 IN population. V3 INs arise from the Nkx2.2+ p3 progenitor domain and express the transcription factor Sim1 upon reaching the post-mitotic stage [89]. They contribute to rhythm generation networks within the spinal cord by playing a role in regulating left-right alternation of gait and in balancing locomotor outputs [4,90]. This population has been shown to cross multiple spinal segments and synapse onto MNs and other INs [4,90]. During maturation, V3 INs separate spatially during post-mitotic development and become recruited during running or swimming behaviors [90]. Their importance in locomotion makes V3 INs an key target to better understand locomotor coordination and a potential cell therapy candidate for functional recovery and local reorganization after spinal cord injury.

Neurons of the ventral spinal cord have been generated from mouse ESCs by exposing embryoid bodies (EBs) to sonic hedgehog (Shh) pathway agonists and retinoic acid (RA) [6,99]. Shh forms a dorsal-ventral gradient in the neural tube during development and helps to establish the five ventral spinal progenitor domains, which then mature into post-mitotic spinal neurons [37,39,42,167]. RA from the lateral somites acts as a caudalizing factor to impart spinal identity [6,147,148]. The protocol to derive MNs from ESCs uses relatively high RA and Shh concentrations to induce spinal MNs [6,99,153]. To induce V3 INs, the p3 progenitor domain location was examined in relation to the progenitor MN domain location. Being a more ventral population, V3 INs sit closer to the floor plate and notochord, and therefore would be exposed to
increased Shh signal strength and duration [38]. Additionally, being further away from the lateral somites, V3 INs would be exposed to lower RA concentrations. While V3 INs can be generated via induction of mouse ESCs, the yield is relatively low (~8%) [166].

Unfortunately, ESC differentiation protocols generally yield heterogeneous populations, and this heterogeneity makes live cell identification and characterization difficult. Heterogeneity also negatively affects transplantation – often generating teratomas and causing regression after short-term improvements due to incomplete cell differentiation or maturation [33-35]. Some methods to reduce this heterogeneity include density-based centrifugation, fluorescence-activated cell sorting (FACS), and use of transgenic cell lines with antibiotic resistance. Density gradient-based MN separation protocols have been used to improve MN yield [168,169]. However, V3 INs are similar in size to many other IN populations, making isolation of V3 INs from other spinal cells difficult. FACS requires either a unique cell surface marker or genetic engineering of a fluorescent marker to isolate pure populations [170], and it is slow for purification of cells that make up a small percentage of the initial population (<10%) [171]. FACS also increases the risk for contaminated cultures and can result in low viability for post-mitotic neurons. Antibiotic resistance has a long history within biology for positive selection of desired traits and is much more scalable compared to FACS. Additionally once an antibiotic resistant cell line line is generated, no specialized equipment is required for cell isolation. Cell lines for antibiotic resistance have been previously utilized for the enrichment of neural lineage populations [172,173]. Recently, our lab generated mouse ESC lines expressing puromycin-N-acetyltransferase (PAC, a puromycin resistance gene) for the positive selection of induced neural progenitors and neuronal populations [153,174]. In these cell lines PAC was expressed either
under Olig2, a marker for progenitor MNs (pMNs), or Hb9, a marker for post-mitotic MNs. These cell lines utilized drug selection as a high throughput, low cost method to enrich cell populations for the desired cell type based on developmental marker expression.

Using similar methods, this paper details an approach to obtain high purity V3 IN cultures. Our V3 selectable (Sim1-Puro) ESC line incorporated PAC into the Sim1 locus to allow positive selection for V3 INs after induction. Recombineering was used to generate a vector that incorporated the PAC gene into the first exon of Sim1, between two Sim1 homology arms. The transgene was knocked into one allele of the Sim1 gene after a Cas9/CRISPR targeting system induced a double stranded break, generating a cell line that enabled the selection of Sim1+ cells. Resulting V3 INs were confirmed to express the post-mitotic marker Sim1, the glutamatergic marker VGluT2, and neuronal marker β-III tubulin. V3 INs exhibited neuronal maturation by electrophysiological measures, including decreasing resistance, increasing capacitance, and firing action potentials in response to current injections. After two weeks, they up regulated synaptic markers SV2, PSD95, and Bassoon, indicating their ability to form synapses. This cell line allows for an inexpensive and scalable method for isolating V3 INs for future study in vitro and for cell transplantation studies.
3.2 Materials and Methods

3.2.1 ESC culture

All ESCs were cultured on T-25 flasks coated in 0.1% gelatin (Sigma, St. Louis, MO). Cells were cultured in complete media consisting of Dulbecco’s Modified Eagle Medium (DMEM 11965, Life Technologies, Carlsbad, CA) containing 10% newborn calf serum (Life Technologies), 10% fetal bovine serum (Life Technologies), and a 1:100 dilution of a 100x nucleosides mix (EMD Millipore, Bellerica, MA). Cells were routinely passaged by washing with DMEM 11965 containing 25 mM HEPES (Life Technologies), dissociating with 1 mL 0.25% Trypsin EDTA (Life Technologies), quenching with complete media, and plating into a new T-25 gelatin-coated flask containing a final volume of 5 mL media with 1000U/mL leukemia inhibitory factor (LIF; EMD Millipore) and 100 µM β-mercaptoethanol (Life Technologies).

3.2.2 Generation of Sim1-Puro-pStartTK targeting vector

The Sim1-Puro-pStartTK targeting vector was constructed following a previously published protocol [7]. The backbone was a Gateway-compatible plasmid, pStartK (Addgene, Cambridge, MA). Sim1 homology arms were incorporated into pStartK from RP23-223M2 BAC ("BACPAC Resource Center", Children's Hospital Oakland Research Institute, Oakland, CA) using pstartK_Sim1_upstream and pstartK_Sim1_downstream primers (Table 3.1) by recombiner techniques with red recombinase competent bacteria (Sim1-pStartK, Fig. 3.1 A). A chloramphenicol resistance gene flanked by AscI cut sites from pkD3 (The E. Coli Genetic Stock Center, Yale University, New Haven, CT) was inserted into the open reading frame of the
Sim1 gene by recombineering with primers Sim1_CAT_Forward and Sim1_CAT_Reverse 900bp (Table 3.1). The chloramphenicol resistance gene was then replaced via restriction enzyme digestion and ligation by a dual resistance cassette consisting of, from 5’ to 3’: AscI cut site, Kozak sequence, PAC with bgh polyA signal, floxed phosphoglycerate kinase I promoter driving neomycin phosphotransferase (PGK-neo) with bgh polyA signal, and AscI site (gift from Dr. David Gottlieb, Washington University, St. Louis, MO) [153]. A negative selection thymidine kinase gene was incorporated into the finished vector (Sim1-Puro-pStartTK, Fig. 3.1 B) using pWS-TK3 plasmid (Addgene) and Gateway LR clonase II kit (Life Technologies).

### Table 3.1: Primers for Sim1-Puro Generation

<table>
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<th>Primer Name</th>
<th>Sequence</th>
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<td>AAAGTACTGTTTCTGGGGAAACTCTTAGTTTAGAGACCCTCTCTGTTCTA</td>
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<td></td>
<td>Acgactgaattggtctttaaagc</td>
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<tr>
<td>pstartK_Sim1_downstream</td>
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<td></td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
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<tr>
<td>Sim1_CAT_Reverse 900bp</td>
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<td></td>
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</tr>
<tr>
<td>Puro_Reverse Junction1</td>
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</table>

Capital letters indicate homology arms for red recombination.
3.2.3 Generation of Sim1-Puro ESCs

The Sim1-Puro cell line was generated from the RW4 mouse ESC line (American Type Culture Collection, Manassas, VA). Approximately $1 \times 10^7$ RW4 ESCs were resuspended in electroporation buffer with 10 μg of Sim1-Puro-pStartTK vector, and 200-300 ng of a Cas9 guide RNA vector (deemed gSim1.MS8.mSim1.g6a, with guide RNA (Fig. 3.1C, Cas9 Guide RNA) targeting 5' gtcctgctttccgg 3' near the Sim1 start codon (Fig. 3.1C, Cas9 Target)) in the MLM3636 plasmid (Addgene plasmid #43860) and 200-300 ng of the Cas9 nuclease expression plasmid p3s-Cas9HC (Addgene plasmid #43945). Both Cas9 vectors were from Genome Engineering Core, Washington University in St. Louis and originally gifted by Keith Joung and Jin-Soo Kim, respectively. Cells were electroporated using a Biorad Gene Pulser Xcell Eukaryotic System at 0.23kV and 975μF in a 0.4 cm cuvette (Bio-Rad, Hercules, CA). Following electroporation, cells were seeded on gelatin-coated 100 mm dishes for 24 hours then treated with G418 (200 μg/ml, Life Technologies) and 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU; 150 nM, Movarek Biochemicals, Brea, CA) for positive and negative selection, respectively. After 14 days, surviving clones were picked and seeded into individual wells of a gelatin-coated 96 well plate.

3.2.4 PCR screening on Sim1-Puro clones

Clones were screened for targeting events by junction polymerase chain reaction (JPCR, Fig. 3.1D). One primer binding outside of the left homology arm (5′ HA, Fig. 3.1D) and the other primer binding inside the PAC gene were used to screen for clones that properly incorporated the PAC gene. Reactions were performed using a Mastercycler Nexus Gradient
thermocycler (Eppendorf, Hauppauge, NY) with primers Sim1_Fwd_Junction1 and Puro_Reverse Junction1 (Table 3.1 and Fig. 3.1 D) at 95°C for 60s, followed by 35 cycles of 94°C for 20s, 60°C for 30s, and 72°C for 120s.

3.2.5 Copy number assay

Taqman Copy number assay (Life Technologies) was performed on cell lysates as per manufacturer instructions. Gapdh (Mm00186825_cn, Life Technologies) was normalized to RW4 ESCs, and PAC (custom ordered PAC assay, Life Technologies) was normalized to a previously published Hb9-Puro cell line [174]. Analysis was performed using Life Technologies CopyCaller v2.0.

3.2.6 V3 IN induction

RW4 ESCs and Sim1-Puro ESCs were aggregated to form EBs on a non-adhesive agar-coated surface and induced to generate neural progenitors using our previously established 8 day induction protocol (2/6+, where “2-” refers to the number of days ES cells are allowed to aggregate into EBs without (-) RA and smoothened agonist (SAG, a Shh pathway agonist) and “#” refers to the number of days the EBs are exposed to (+) RA and SAG; Fig. 3.2A) [166]. Cells were cultured in suspension for two days on 100 mm Petri dishes pre-coated with 0.1% agar (Thermo Fisher Scientific, Waltham, MA) in DFK-5 media comprised of 1:1 DMEM/F12 (Life Technologies) with 5% knockout serum replacement (Life Technologies), 1x insulin transferrin selenium (Life Technologies), 100 μM β-mercaptoethanol, 50 μM nonessential amino
acids (Life Technologies), and a 1:200 dilution of a 100x nucleosides mix. During the first two days, the cells aggregate into multi-cellular EBs. After aggregation, EBs were removed and allowed to settle. The supernatant was discarded and replaced with new DFK-5 media supplemented with 10 mM RA (Sigma) and 0.5 μM SAG (EMD, Millipore). Media was replaced every two days for six days.

3.2.7 Selection and differentiation of V3 INs

To test PAC expression in Sim1+ cells, RW4 and Sim1-Puro EBs were subjected to induction and selection protocols as illustrated in Fig. 3.2A. On 2/4+, standard tissue culture plates were pre-coated with 0.01% poly(ornithine) solution (Sigma) at 37°C for 1 hour. Poly(ornithine) was removed and plates were washed with a HEPES buffered saline solution 3 times. Plates were then coated with 0.01 mg/mL laminin (Life Technologies) in HEPES buffered saline solution overnight and washed 3 times before use as laminin coated plates. One day prior to the end of V3 IN induction (on 2/5+), EBs were allowed to settle, and the DFK-5 media supernatant was collected. EBs were dissociated with 0.25% trypsin-EDTA for 15 minutes at 37°C, quenched with complete media, pelleted and replated at 3.5x10^7 cells/cm^2 onto laminin-coated plates. Cells were incubated with 2 μg/mL puromycin (Sigma) in the collected DFK-5 supernatant for 1 day (designated as 1p, Fig. 3.2A). After one day, puromycin-containing media was removed and replaced with “P-Olig2-conditioned” media (see next section) with supplemental factors (1x Glutamax (Life Technologies), and 5 ng/mL each of NT-3, GDNF, and BDNF (all from Peprotech, Rock Hill, NJ). Cells were allowed to differentiate up to 14 days (designed as “+ #” where # is the number of days after selection, Fig. 3.2A).
3.2.8 Generation of P-Olig2-Conditioned V3 media for long term V3 cultures

After selection, cells were initially cultured in a 1:1 mix of DFK-5 and Neurobasal (Life Technologies) media with supplemental factors (listed above). However, due to the low cell density, extensive cell death was observed between 3 and 7 days post-selection (1p +3 and 1p +7). Thus, to improve cell survival, conditioned media generated by progenitor motor neuron cultures containing glia (oligodendrocyte and astrocytes) was used. A puromycin-selectable-pMN ESC line (P-Olig2), with PAC knocked into the Olig2 locus, underwent 2/4+ induction using DFK-5 media with 2 µM RA and 0.5 µM SAG (2 days without and 4 days with RA/SAG) as previously described [153]. On the last two days of pMN induction (2−/2+ - 2−/4+), cells were selected with 4 µg/mL puromycin. On 2−/4+, the selected EBs were dissociated and plated at a density of 100,000 cells/mL in a 1:1 mix of DFK-5 and Neurobasal media with 1x B27 (Life Technologies) in a 6 well laminin-coated plate. Conditioned media was collected and replaced with fresh media every two days. Selected P-Olig2 cells were cultured up to 14 days.

3.2.9 Live assay

Live reagent, calcien-AM (Life Technologies), was used to visualize live cells, respectively. Wells were washed with DMEM 11965 containing 25 mM HEPES and incubated with 0.325 µL/mL of 4 mM stock concentration calcien-AM (live) for 30 min at room temperature.
3.2.10 Probe synthesis for in situ hybridization

Plasmids for in situ hybridization probes were a generous gift from Dr. Paul Gray (Washington University in St. Louis) [155]. Gene fragments from verified plasmids were linearized by direct PCR amplification using ReadyMade Primers (SP6 Promoter and T7 Promoter, Integrated DNA Technologies, Coralville, IA). Digoxigenin (DIG)-labeled antisense and sense RNA probes were made using PCR products as template and T7 RNA polymerases (Roche, Indianapolis, IN). Probes were used at a concentration of 1–2 μg/ml. Sense counterparts of all probes were tested to ensure probe specificity.

3.2.11 In situ hybridization

To assess the purity of cells post selection, in situ hybridization was performed on dissociated cells. After 24 hours of selection, cultured cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes and washed 3 times in diethylpyrocarbonate (DEPC, Sigma) treated PBS at room temperature. Next, cells were incubated in 0.1 M triethanolamine-HCl (1.3% triethanol amine (Sigma) and 0.4% HCl (Thermo Fisher Scientific) with 0.25% acetic anhydride (Sigma)) for 10 minutes. Cells were washed in 1x sodium citrate buffer for 5 minutes at room temperature and permeabilized in 0.2M HCl in DEPC-water for 10 minutes. 3 additional washes with DEPC treated PBS were performed before cells were blocked in hybridization buffer (50% formaldehyde (Sigma), 5x sodium citrate buffer (SSC, Life Technologies), 0.3 mg/mL yeast RNA (Sigma), 0.1 mg/mL heparin (Sigma), 1x Denhardt’s solution (Life Technologies), 0.1% tween (Sigma) and 5 mM EDTA) for 4-6 hours at room temperature. Cells were incubated in hybridization buffer containing 1–2 μg/mL DIG-labeled antisense RNA probes (see previous
section) overnight at 65°C. Probed cells were washed in twice in 0.2× SSC at 62°C, and incubated in 0.2× SSC for 60 minutes at 65°C. Washed cells were adjusted to room temperature and blocked with 10% deactivated horse serum (Life Technologies) in phosphate buffered saline with 2 mg/mL bovine serum albumin and 0.1% Triton X-100 (PBT) and incubated in alkaline phosphatase-labeled anti-DIG antibody (1:2000 in 10% deactivated horse serum in PBT; Roche) overnight. Cells were further washed with PBT and color was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche). Staining was stopped with 4% PFA after visual inspection. Cell nuclei were stained with the nuclei binding dye Hoechst (1:1000 Life Technologies).

### 3.2.12 Immunocytochemistry

Neuronal cell identity was assessed in differentiated cultures using immunocytochemistry. Cell cultures were fixed with 4% PFA for 30 min then permeabilized in 0.01% Triton X (Sigma) for 15 min. The cells were blocked with 5% NGS for 1 hour at 4°C and incubated overnight at 4°C in 2% NGS solution with one or more of the following primary antibodies: rabbit anti-Tuj1 (for β-III tubulin, 1:200, Covance, Princeton, NJ), guinea pig anti-VGlut2 (1:3000, EMD Millipore), mouse anti-SV2 (1:100, Developmental Studies Hybridoma Bank), rabbit anti-MAP2 (1:1000, EMD Millipore), mouse anti-bassoon (1:600, Enzo Life Sciences, Farmingdale, NY). Primary antibody staining was followed by 3 washes in an excess volume of PBS for 15 min each. Each culture was then stained with appropriate Alexa Fluor secondary antibodies (1:1000; Life Technologies) for 1 hour at 4°C followed by an additional 3 washes in PBS. Cell nuclei were stained with Hoechst (1:1000).
3.2.13 Image capture and analysis

All images were captured using a MICROfire camera (Olympus, Center Valley, PA) attached to an Olympus IX70 inverted microscope using either a 10x or 20x objective. Images were merged and colored using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA). To remove human bias, ImageJ was used for the automated counting of Hoechst-labeled nuclei. Images underwent thresholding to remove background, then were converted to binary black and white images. The “Analyze Particles” function was used to count the nuclei, excluding any small (less than 600 pixels) punctate nuclei to prevent apoptotic Hoechst+ nuclear debris from being counted.

3.2.14 Quantitative real-time PCR (qRT-PCR)

Fourteen days after selection (1p +14), selected and unselected (control) cells on a 24-well plate were lysed with 350 μL of buffer RLT from the RNeasy Mini Kit (Qiagen, Valencia, CA). High Capacity RNA-to-cDNA Kit (Life Technologies) generated cDNA from RNA that was extracted per Qiagen’s instructions. TaqMan Fast Advanced Master Mix (Life Technologies) was combined with TaqMan Gene Expression Assays (Life Technologies; Table 3.2) and cDNA for qRT-PCR. Reactions were performed using a Step One Plus Applied Biosystems thermocycler (Life Technonologies) with the default protocol: 95°C for 20s, 40 cycles of 95°C for 1s and 60°C for 20s. The number of cycles necessary for the fluorescent intensity to increase exponentially, C_t values, were recorded and normalized to β-actin expression. The comparative
ΔC\textsubscript{i} method [154] was used to analyze the mRNA expression levels compared to undifferentiated Sim1-Puro ESCs. Fold differences in relative mRNA expression levels over the control cultures are reported for each gene (n ≥ 3 for all groups).

Table 3.2: TaqMan Gene Expression Assays for qRT-PCR

<table>
<thead>
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<th>Marker</th>
<th>Life Tech Identification</th>
</tr>
</thead>
<tbody>
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<td>Bassoon</td>
<td>Mm00464452_m1</td>
</tr>
<tr>
<td>Beta-Actin</td>
<td>Mm00607939_S1</td>
</tr>
<tr>
<td>Psd95</td>
<td>Mm00492193_m1</td>
</tr>
<tr>
<td>SV2a</td>
<td>Mm00491537_m1</td>
</tr>
<tr>
<td>VGluT2</td>
<td>Mm00499876_m1</td>
</tr>
</tbody>
</table>

3.2.15 Electrophysiology

Whole-cell electrodes had an open tip resistance of 2 to 6 MOhms when filled with K- or Cs-glucuronate internal solutions (in mM, all from Sigma): 140 K-glucuronate 10 NaCl, 5 MgCl\textsubscript{2}, 0.2 EGTA, 10 HEPES, pH adjusted to 7.4 with KOH; or, 130 Cs-glucuronate, 5 MgCl\textsubscript{2}, 0.2 EGTA, 10 HEPES, pH adjusted to 7.4 with CsOH. Both internal solutions were supplemented with 5 mM Na-ATP and 1 mM Na-GTP. Culture dishes were perfused at ~ 1 ml/min with Tyrode’s solution (in mM): 150 NaCl, 4 KCl, 2 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 10 glucose, 10 HEPES, pH adjusted to 7.4 with NaOH. Currents and membrane potentials were recorded with Axopatch 200 amplifiers (Molecular Devices, Sunnyvale, CA), filtered at 1 kHz and digitized at 10 kHz using pClamp 9.2 (Molecular Devices, Sunnyvale, CA). Tetrodotoxin (TTX, 0.5 µM), tetraethylammonium (TEA, 30 mM) and 4-aminopyridine (4-AP, 5 mM) were dissolved in
Tyrode’s. Agonists were dissolved at 100 µM in 160 mM NaCl, 2 mM CaCl₂, 10 mM HEPES, pH adjusted to 7.4 with NaOH. Drug solutions were applied by local perfusion from a multi-barreled delivery pipette [148].

3.2.16 Statistical analysis

Three biological replicates of each condition were performed. Three sample pictures were analyzed from each replicate for cell counting. Statistical analysis was performed in Statistica software (version 5.5; StatSoft, Tulsa, OK). Unless otherwise stated, multiple comparisons statistics were accomplished using Scheffe’s post hoc test for analysis of variance (ANOVA) with a 95% confidence level. Values are reported as the mean plus or minus standard deviation.

3.3 Results and Discussion

While ESCs provide a plentiful cell source to better study a variety of cell types, the heterogeneity of cells induced from ESC cultures by current protocols retains the problems of dissection and isolation. Recently, our lab reported a MN line where previously established Hb9 enhancer regions were used to drive expression PAC [174]. Unfortunately, a highly conserved efficient enhancer region for Sim1 has not been identified [175]. Thus, random insertion of a Sim1 enhancer-promoter driving PAC was not achievable. Homologous recombination has been used ubiquitously to generate knockout animals and cell lines [7]. Previously, our lab reported a Olig2-Puro cell line that has PAC knocked into the Olig2 locus, enabling the Olig2 gene regulatory elements to control PAC expression [153]. This method was adopted for the Sim1
locus, such that transgenic mouse ESCs were generated by the electroporation of a vector containing the PAC gene into the Sim1 locus. The use of Cas9/CRISPR technology greatly increased the efficiency of recombination by inducing a double stranded break in the first exon of Sim1. This paper describes a method to overcome the heterogeneity of ESC-derived V3 IN cultures using this Sim1-Puro ESC line.

### 3.3.1 Targeted PAC insertion into the Sim1 locus

The Sim1-Puro cell line was generated by using a targeting vector to insert a resistance cassette into the open reading frame of the Sim1 gene. Two homology arms ~ 2 kb and 10 kb in size were inserted into the targeting vector flanking the resistance cassette (Fig. 3.1A-B). RW4 ESCs were electroporated with the targeting vector and a set of Sim1 targeting Cas9/CRISPR plasmids. The expected homologous recombination event is illustrated in Fig. 3.1C. JPCR with one primer hybridizing to genomic DNA outside of the homology arms and one primer hybridizing within the resistance cassette was used to screen for insertion of the resistance cassette into the Sim1 locus (Fig. 3.1D-E).

While Sim1-Cre heterozygous animals have been reported with appropriate neuronal migration, in Sim1 knockout mice, the neurons fail to properly migrate [176]. Furthermore, Sim1−/− animals are not viable [176], further indicating the importance of keeping at least one allele of Sim1 intact. Thus, it was important that only one allele had the puromycin gene knocked
Figure 3.1: Generation and identification of Sim1-Puro cell line. A) Red recombineering was utilized to insert the region ~ 2 kb upstream to ~10 kb downstream of Exon 1 of the Sim1 gene from RP23-223M2 BAC into the pStartK backbone, generating the Sim1-pStartK plasmid. B) After AscI cut sites were introduced by another red recombination reaction, a PAC-PGK-Neo cassette was inserted into the open reading frame of Sim1 Exon1 in the Sim1-pStartK plasmid. Using gateway recombination, the pStartK backbone was replaced with the pWS-TK3 backbone to introduce the negative selection gene, TK. 5’ and 3’ homology arms are labeled. C) CRISPR/Cas9 targeting of the Sim1 gene was used to generate a double stranded break at the desired recombination location. Sim1-Puro-pStartTK recombined into the Sim1 locus as shown by the dotted lines. 5’ and 3’ homology arms are labeled. D) The Sim1-Puro cell line with PAC in Sim1 Exon 1. Junction PCR (JPCR) primers for approximately 2.6 kb was used to screen for the desired recombination event. E) Junction PCR bands of positive (+) and negative (-) clones with a 1kb ladder. F) Copy number assay shows Sim1 clones have one copy of PAC by comparison with RW4 ESCs and Hb9-Puro ESC controls. Abbreviations: AMP, ampicillin resistance gene; AscI, restriction enzyme site; attB1 & attB2, gateway recombination results; HA, homology arm; JPCR, junction PCR; ori, origin of replication; PAC, puromycin resistance gene; PGK, phosphoglycerate kinase promoter sequence; Neo, neomycin resistance gene; Sim1 ATG, translation start in Sim1 Exon1; TK, thymidine kinase.
into the Sim1 locus. Two clones (6E and 8E) that screened positive for insertion of PAC by JPCR were analyzed with a copy number assay, which reported values of ~ 1 for both clones (Fig. 3.1F), indicating that only one copy of the PAC gene was inserted into the cells, and that other Sim1 allele did not have PAC knocked in. The appropriate JPCR results coupled with the copy number assay results together indicate that one copy of PAC was successfully inserted into the Sim1 locus, resulting in the desired Sim1-Puro cell line. The resulting transgenic-ES cell line (Sim1-Puro Clone 6E) was used for all subsequent studies.

3.3.2 Increased purity of Sim1+ cells after puromycin selection

To test for PAC expression in Sim1+ cells, RW4 and Sim1-Puro EBs were subjected to induction and selection (2/6+/1p) protocols as illustrated in Fig. 3.2A. Visual assessment of calcein-AM staining of selected and unselected cultures showed that 2 µg/mL puromycin was sufficient to kill all RW4 cells. The unselected RW4 culture (Fig. 3.2B) looked healthy and confluent, while the selected RW4s had no remaining live cells (Fig. 3.2C). In the Sim1-Puro selected and unselected cultures, the unselected cultures contained many cells of non-neuronal morphology, while the selected culture contained fewer cells with non-neuronal morphology (Fig. 3.2D-E). The efficacy of RW4 selection indicated that the 2 µg/mL puromycin concentration had sufficient potency to remove all non-PAC-expressing cells but allowed survival of PAC expressing cells, whereas 4 µg/mL puromycin resulted in very low cell viability (data not shown). Thus, 2 µg/mL puromycin was used for subsequent studies.
Figure 3.2: The effect of selection on survival of RW4 and Sim1-Puro ES cells. A) Diagram depicting induction and selection process. Induction and differentiation nomenclature is indicated by parenthesis while the time course (in days) is denoted by the time line. Briefly, ES cells are allowed to aggregate for 2 days (2⁻) to form EBs. EBs are induced with RA and Shh for 5 days (5⁺) and selected with puromycin in the presence of RA and Shh for 1 day (6⁺/1p). Selected cells are allowed to differentiate after selection (1p + #). B & D) Live (calcein-AM) staining on unselected cells. C & E) Live staining of selected cells. Scale bar = 50 µm.
Figure 3.3: Puromycin selection increases the percentage of cells expressing Sim1 in Sim1-Puro cultures. A-C) Sim1 in situ hybridization (dark cell bodies) and nuclear staining (blue) on unselected cells. D-F) Sim1 in situ hybridization and nuclear staining on selected cells. A&D) Sim1 in situ hybridization. B&E) Nuclear marker Hoechst (blue). C&F) Overlaid images. G) Selected cultures enriches for percentage of Sim1+ cells. * denotes p < 0.05 compared to unselected group.
Due to the lack of a specific Sim1 antibody, the purity of cultures post-selection was assessed by performing *in situ* hybridization on dissociated cultures. The percentage of Sim1$^+$ cells increased significantly from 11% in unselected cultures to 83% after selection (Fig. 3.3). This increase indicated that puromycin selection of the Sim1-Puro line successfully enriched for Sim1$^+$ cells. Furthermore, the presence of Sim1$^+$ cells via *in situ* hybridization corroborates the copy number assay data and suggests that the Sim1 gene on the non-altered allele should be functional, at least to the properly spliced mRNA stage.

Based on the *in situ* hybridization results, the Sim1-Puro cell line is able to enrich a culture for Sim1$^+$ cells, such that most of the cells in a selected culture are Sim1$^+$. The purity of the selected Sim1-Puro line is lower compared to the 99% purity of MNs reported from the Hb9-Puro line previously generated in our lab. One reason for lower purity is that the Hb9-Puro line was assessed with an antibody while the Sim1-Puro line was assessed with *in situ* hybridization – a mRNA based technique. Because mRNA is transient and expressed prior to protein synthesis, a portion of the surviving cells could be Sim1 mRNA negative but have recently expressed Sim1 mRNA and thus still have Sim1 and PAC protein present. They would not be Sim1$^+$ by *in situ* hybridization, but would still be V3 INs and might stain positive with a Sim1 antibody, if one existed. Another reason for having a lower percentage of Sim1$^+$ cells is that, compared to the Hb9-Puro study, a lower puromycin concentration was used in this study for selection. Previously, 4 µg/mL puromycin was used in for the Hb9-Puro cell line and 2 µg/mL was used here with the Sim1-Puro cell line because less than 100 cells/cm$^2$ was observed after 4 µg/mL puromycin selection. Hb9 mRNA levels increase ~400 fold versus uninduced controls (no RA and no SAG) after MN induction, whereas Sim1 mRNA levels only increase ~100 fold after
induction [153,166], suggesting that Sim1 is not as strongly expressed as Hb9. This fold change difference can be partially attributed to the lower induction protocol efficiency (~60% for MNs vs ~10% for V3 INs), but it could also be due to Sim1 gene regulatory elements driving less robust expression than those for Hb9. Lower levels of PAC expression in each cell could result in increased sensitivity at a lower puromycin concentration. While the resulting cultures are not 100% Sim1+ cells, the observed enrichment is sufficient to allow for further studies of ESC-induced V3 INs.

3.3.4 Selected cultures exhibit neuronal markers and achieve functional maturity

Glial-conditioned media was needed for the long-term survival of selected Sim1-Puro V3 INs. Initially, without conditioned media, we observed axonal degeneration by 7 days in culture and ultimately cell death. We hypothesized that providing glial signaling cues would aid in V3 IN survival. Thus, the P-Olig2 ESC line, where the PAC gene was incorporated into the Olig2 locus was used to obtain glia and to produce conditioned media. Selection of P-Olig2 cells results in both MNs and glia (oligodendrocytes and astrocytes)[153]. While this was not ideal for generating a high purity MN population, the resulting pMN population generated glial-conditioned media suitable for long-term cultures of V3 INs. Using this conditioned media, V3 IN cultures survived for more than 14 days, which was necessary for maturation and synapse formation to be observed.
Figure 3.4: Sim1-Puro cultures exhibit glutamatergic and neuronal markers through the first week post-selection. A-D) Staining 3 days post selection. E-H) Staining 7 days post selection. Immunocytochemistry marker labeled on far left. A&E) β-III Tubulin (red in combined); B&F) VGluT2 (green in combined); C&G) Combined image; D&H) Zoomed in combined image. Scale bar = 100 µm.
To assess the phenotype selected cultures, immunocytochemistry was performed. β-III tubulin was used to verify the neuronal identity of the selected cells. As seen in Fig. 3.4 A and E, selected cells express β-III Tubulin with axonal morphology at both 3 and 7 days post selection (1p +3 and 1p +7). VGluT2, a vesicular glutamate transporter found in synaptic vesicles at presynaptic nerve terminals of excitatory neurons, was used as a marker of glutamatergic neurons. As seen in Fig. 3.4 B and F, selected cells exhibited punctate VGluT2 staining along their axons at 3 and 7 days post selection. The VGluT2 and β-III tubulin staining aligned well (Fig. 3.4 C&D), and the VGluT2 puncta are clearly visible in the zoomed in insets (Fig. 3.4 G&H). These two antibodies verify that the selected cells are indeed glutamatergic neurons.

Because these images were taken 4 days apart, some disparities between the left and right columns of Fig. 3.4 were expected. We expected to observe maturation of axonal processes (longer axons and more connectivity between each other), which can be observed in Fig. 3.4G. Furthermore, at 7 days post-selection, the cells are more sparsely distributed. This is not unexpected due to pruning observed in neuronal development. While some debate is present in the literature over whether INs undergo apoptosis as observed in MNs, it has been shown that ventral spinal INs do undergo apoptosis after they become post-mitotic [176,177]. This occurs generally between e14 and P0 [176]. Our induction ends (1p +0) when Sim1 is strongly expressed, roughly equivalent to e11.5 [88], so apoptosis would be expected in the ensuing week. Taken together, the differences between the left and right columns of Fig. 3.4 are consistent with expected neuronal maturation.

To determine whether selected V3 INs are maturing, immunocytochemistry was performed on cultures 2 weeks post selection. MAP2, a dendritic marker, was used to identify
Figure 3.5: Sim1-Puro cultures exhibit synaptic marker protein and mRNA expression after 2 weeks. A-F) 2 week selected cultures stained with dendritic marker MAP2 and synaptic markers, SV2 and Bassoon. Synaptic markers (B and E, red) exhibit puncta that overlap with MAP2 (C and F, white arrows) suggesting synapse formation. Scale bar = 50 µm. G) 2 week selected cultures express increased synaptic marker (Bassoon, PSD95, and SV2) and VGluT2 (glutamatergic neuron) mRNA levels compared to unselected cultures (normalization control - Sim1-Puro ESCs). * denotes p <0.05 compared to the unselected group.
neurons in culture. SV2 and Bassoon staining shows positive, punctate, synaptic marker staining along axons, indicating potential synapse formation (Fig. 3.5 A-F). The presence of positive synaptic marker staining within the cell body was not expected. We hypothesized that if the synaptic marker mRNA was still being expressed, protein translation could be in progress and result in positive staining of the cell body. Thus, qRT-PCR was performed on 1p +14 (2 week post-selection) to assess expression levels of synaptic markers. Synaptic markers SV2, Bassoon, and PSD95, as well as glutamatergic neuron marker, VGluT2 were assessed in selected and unselected cultures. The resulting qRT-PCR data shows an upregulation of all markers in all conditions compared to the ESC controls and an increase in all marker expression between selected and unselected cells (Fig. 3.5 G). These results must be considered within the context of a population-averaged assay. While the results suggest that there are more cells expressing synaptic markers in selected versus unselected cultures, the data only indicate that in selected cultures there is a greater percentage of neurons than in unselected cultures. This is due to normalization of the data to the internal control β-actin, effectively normalizing expression levels to cell count. Additionally, the relative low levels of synaptic marker expression suggest that neurons within the cultures at 2 weeks are working towards but have not yet formed mature synapses.

3.3.5 Electrophysiology

In addition to using immunocytochemistry to characterize the Sim1-Puro line, electrophysiological recordings of the selected cells were also used to assess maturation. Whole-
Table 3.3: Electrophysiological properties of Sim1-Puro selected cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P + 2-5</th>
<th>P + 6-13</th>
<th>MN Properties^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacitance (pF)</td>
<td>18.3 ± 1.3 (51)</td>
<td>24.2 ± 0.9 (68) *</td>
<td>31.3 ± 1.1</td>
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<tr>
<td>Input Resistance (GOhm)</td>
<td>1.64 ± 0.49 (51)</td>
<td>0.96 ± 0.17 (68) *</td>
<td>0.570 ± 0.028</td>
</tr>
<tr>
<td>V rest (mV)</td>
<td>-28.5 ± 2.1 (43)</td>
<td>-43.7 ± 1.2 (64) *</td>
<td>-53 ± 1 mV</td>
</tr>
<tr>
<td>% V rest &lt; -50 mV</td>
<td>5%</td>
<td>28% *</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Rheobase (pA)</td>
<td>10.6 ± 2.3 (14)</td>
<td>18.3 ± 2.7 (34)</td>
<td>--</td>
</tr>
<tr>
<td>1st spike latency (msec)</td>
<td>214 ± 43 (14)</td>
<td>153 ± 19 (24)</td>
<td>--</td>
</tr>
<tr>
<td>1st spike amplitude (mV)</td>
<td>84 ± 4.5 (14)</td>
<td>95 ± 3.5 (24)</td>
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<td>absolute amplitude (mV)</td>
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<td>34 ± 3.5 (24)</td>
<td>--</td>
</tr>
<tr>
<td>1st spike threshold (mv)</td>
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<td>-37.4 ± 0.9 (24)</td>
<td>--</td>
</tr>
<tr>
<td>1st spike width (msec)</td>
<td>4.4 ± 0.5 (14)</td>
<td>2.9 ± 0.3 (24) *</td>
<td>--</td>
</tr>
<tr>
<td>10 Hz 1st latency (msec)</td>
<td>44 ± 3.2 (14)</td>
<td>37 ± 2.0 (24)</td>
<td>--</td>
</tr>
<tr>
<td>10 Hz 1st frequency (Hz)</td>
<td>11 ± 0.3 (14)</td>
<td>12 ± 0.3 (24) *</td>
<td>--</td>
</tr>
<tr>
<td>10 Hz frequency adaptation</td>
<td>1.2 ± 0.04 (14)</td>
<td>1.3 ± 0.05 (24)</td>
<td>--</td>
</tr>
<tr>
<td>10 Hz after potential (mv)</td>
<td>0.9 ± 0.5 (14)</td>
<td>-0.9 ± 0.7 (24)</td>
<td>--</td>
</tr>
<tr>
<td>f-I slope 1st interval</td>
<td>0.59 ± 0.07 (14)</td>
<td>0.44 ± 0.03 (24)</td>
<td>--</td>
</tr>
<tr>
<td>f-I slope average frequency</td>
<td>0.54 ± 0.07 (14)</td>
<td>0.36 ± 0.03 (24)</td>
<td>--</td>
</tr>
<tr>
<td>Sag at -90 mV (mV)</td>
<td>3.3 ± 1.1 (14)</td>
<td>3.3 ± 0.7 (24)</td>
<td>--</td>
</tr>
<tr>
<td>Peak I Na (nA)</td>
<td>-1.06 ± 0.12 (40)</td>
<td>-2.09 ± 0.16 (58) *</td>
<td>--</td>
</tr>
<tr>
<td>Vm for peak I Na (mV)</td>
<td>-21.0 ± 1.6 (40)</td>
<td>-26.1 ± 1.4 (58) *</td>
<td>--</td>
</tr>
</tbody>
</table>

Values presented as mean ± SEM (number of cells). * denotes significantly different from P + 2-5d by t-test, Mann-Whitney rank sum test, or Z-test. Cell capacitance and input resistance were determined from 10 mV voltage clamp steps from a holding potential of -80 mV. First spike latency, amplitude, absolute amplitude, threshold and half-width were determined for the first spike recorded at threshold depolarization. In addition, 800 msec depolarizations that elicited spiking with an average frequency of 10 Hz were used to measure 1st latency, instantaneous frequency from the first inter-spike interval (ISI), frequency adaptation (ratio of last to first ISI), after potential as well as the initial slope of frequency versus current (f-I) plots of instantaneous and average frequency. Sag in voltage responses was determined for 800 msec hyperpolarizing current injections from -60 mV. ^ Select properties acquired from ESC derived MNs, reported by Miles et al. 2004 [100] and McCreedy et al. 2014 [174].
**Figure 3.6:** Sim1-Puro selected cultures exhibit action potential firing and voltage gated currents consistent with neuronal maturation. (A) Sub- and supra-threshold voltage responses recorded under current clamp in a 1p +2 cell with 50 msec square pulse injection (6 and 8 pA). (B) Examples of cells firing single (1p +11), adapting (1p +11) and multiple (1p +10) action potentials during an 800 msec depolarizing pulse (44 pA). (C) Currents mediated by tetrodotoxin-sensitive sodium channels evoked by steps from -100 to +60 mV from a holding potential of -80 mV. (below) Peak inward current plotted as a function of step potential (1p +13; Cs-glucuronate internal solution). (D) Outward currents mediated by voltage-gated potassium channels for steps from -100 to +60 mV from a holding potential of -80 mV recorded in 0.5 µM TTX alone and in TTX plus 5 mM 4AP. (below) 4AP-sensitive currents revealed by subtracting current in TTX and 4AP from current in TTX alone (1p +8, K-glucuronate internal solution). (E) Current-voltage relation for steady-state (SS) current recorded in TTX plus 4AP (open symbols) and for peak 4AP-sensitive current (filled symbols).
than 60 additional cells between 6 and 13 day post-selection (1p +6 to 1p +13; late). Cell capacitance increased and input resistance decreased with time after selection consistent with an increase in cell size (Table 3.3). Resting membrane potential ($V_{\text{rest}}$) became more negative with time as did the proportion of cells that maintained $V_{\text{rest}}$ less than -50 mV without the need for DC hyperpolarization. As shown in Fig. 3.6, depolarizing current injection elicited action potentials in cells as early as 2d after puromycin selection. With DC hyperpolarization to -60 mV and long duration (800 msec) depolarizing current pulses, all 14 of the early (1p +2 to 1p +5) cells tested were able to fire multiple action potentials, 57% showed prominent spike adaptation, and the remaining 43% fired repetitively with little adaptation. Most late (1p +6 to 1p +13) cells (70%) also fired multiple spikes, however 30% only fired single action potentials. For cells that produced multiple spikes the firing properties (Table 3.3) were more similar to the ventral than the dorsal V3 interneuron population as characterized in mouse spinal cord slices [90,91].

Under voltage clamp, Sim1-Puro selected cells exhibited fast transient inward current and slow rising transient and sustained outward currents with depolarizing voltage steps from a holding potential of -80 mV (Fig. 3.6). Inward current was blocked by the selective sodium channel antagonist, tetrodotoxin (TTX, 0.5 µM). Outward current was reduced in cells filled with the potassium channel blocker cesium, or by extracellular exposure to the organic potassium channel blockers, tetraethylammonium (TEA, 30 mM) and 4-aminopyrididine (4-AP, 5 mM). As observed in other cell types [178], TEA inhibited sustained outward currents, while 4-AP reduced transient outward current. These results indicate the Sim1-Puro cells are expressing functional ion channels and behave in an appropriate manner for glutamatergic neurons.
Figure 3.7: Excitatory and inhibitory agonists activate appropriate whole-cell currents in Sim1-Puro selected cultures. A) Whole-cell currents evoked by kainate (K), GABA, glycine (Gly) and NMDA (N; with 1 µM added glycine). Holding potential, -80 mV. B) Agonist-evoked currents recorded during voltage ramps from -100 to +50 mV at 1.2 mV/msec. GABA and glycine evoked current that reversed polarity at -48.6 +/- 2.0 mV (n=21) and -47.7 +/- 2.2 mV (n=10), respectively, consistent with activation of channels selective for chloride. Kainate and NMDA evoked currents reversed at -1.4 +/- 5.4 mV (n=24) and -5.3 +/- 7.2 mV (n=4), respectively, consistent with activation of cation selective channels. C) Currents increased with time in culture after puromycin selection (n=30 to 45 cells per bar). *denotes p<0.01 (Mann-Whitney rank sum test).
Figure 3.8: Sim1-Puro selected cultures present synaptic currents consistent with glutamatergic neurons. (A) Whole-cell current recorded at -80 mV during exposure to Tyrode’s solution containing elevated potassium (10 mM; KCl alone) as indicated by the open box, or 10 mM potassium Tyrode’s that also included 30 µM NBQX and 50 µM APV to block AMPA/kainate and NMDA receptors, respectively, as indicated by the filled box. Short segments during each exposure are shown on a 10 fold expanded time scale. (B) Plots of mean +/- sem amplitude (Amp, pA), frequency (freq, Hz) and width at half amplitude (half-width, msec) of synaptic events evoked during exposure to elevated KCl alone (open bars, 4 cells) or with 200 µM of the GABA antagonist bicuculline methiodide (grey bars, 3 cells).
Selected Sim1-Puro cells expressed a number of neurotransmitter-gated channels, as determined by exposure to the inhibitory transmitters γ-aminobutyric acid (GABA) and glycine, as well as excitatory agonists for AMPA/kainate and NMDA receptors. Agonists were applied at 100 µM, and the NMDA solution was supplemented with 1 µM glycine, which is required as a co-agonist. At a fixed holding potential of -80 mV, all four agonists evoked inward currents (Fig. 3.7A). When the holding voltage was ramped from negative to positive potentials, the currents reversed polarity consistent with the ionic selectivity of their underlying ion channels. Current evoked by GABA or glycine reversed near -50 mV as expected for chloride selective channels, whereas kainate and NMDA evoked currents that reversed near zero mV, consistent with selective permeability to cations. These results indicate the Sim1-Puro cells are expressing appropriate neurotransmitter receptors.

The data presented indicates that selected cells exhibited maturation by decreasing input resistance, increasing membrane capacitance, and acquiring more negative resting potentials. Inward and outward currents, appropriate agonist and blocker responses, and spike firing and spike adaptation also point to the in vitro maturation of the V3 INs. Additionally, the data presented within Table 3.3 suggests similar trends as the values reported in Borowska et al. [14, 37] with the more mature Sim1-Puro V3 INs having capacitance and input resistance values that fall fairly close to the error range previously reported for the ventral V3 IN population [91]. A few differences in the cells recorded in our study deserve mention. Firstly, the ESC-derived V3 population is likely to exhibit more rostral positional identity than the lumbar populations studied by Borowska et al. [91]. The use of low RA to derive V3 INs will result in a more rostral phenotype [166] than the lumbar population analyzed in earlier studies [14, 37]. Secondly, the
values previously reported for V3 INs were determined by recordings from slices and not from neurons in dissociated cell culture. The difference in environment may underlie modest differences between our results and previously reported data. Not only do the ESC-derived V3 INs in vitro lack appropriate cues for migration, they are also deprived of physiologically normal pre- and post-synaptic connections. Thus, the slightly different than slice recording measurements observed in isolated ESC-derived V3 INs are not unreasonable.

In addition to currents evoked by exposure to exogenous agonists, some of the selected Sim1-Puro cells displayed spontaneous inward currents that resembled excitatory postsynaptic currents observed in primary neuronal culture (Fig. 3.8). The frequency of spontaneous events increased substantially during local perfusion with elevated KCl (10-20 mM) to depolarize presynaptic terminals. Consistent with the glutamatergic phenotype of V3 interneurons, spontaneous currents in selected Sim1-Puro cultures were unaffected by the GABA_A receptor antagonist, bicuculline (200 µM) but were eliminated during superfusion with a combination of NBQX (30 µM) and APV (50 µM), glutamate receptor antagonists that block AMPA/kainate and NMDA receptors, respectively [179]. These observations of spontaneous inward currents resembling postsynaptic currents functionally confirms the glutamatergic phenotype expected for V3 INs. Furthermore, these recordings at 1p +10 confirms the qRT-PCR data and indicates that selected Sim1-Puro V3 INs are able to mature into synapsing glutamatergic neurons.
3.6 Conclusion

In this study, we have demonstrated that PAC was successfully knocked into the Sim1 locus of a mouse ESC line. The resulting cell line allows for the enrichment of Sim1+ cells post induction. The selected population exhibits characteristics consistent with what is expected of V3 INs at this stage of development. This novel cell line allows for the further understanding of an understudied population of spinal INs. The Sim1-Puro cell line could be useful in understanding IN maturation and CPG formation. Co-culturing Hb9-Puro MNs and Sim1-Puro V3 INs could be a starting point for a bottom-up approach to understanding CPG circuitry and ultimately designing novel therapeutics for spinal cord injury.
Chapter 4: Summary and Future Direction

4.1 Summary of Findings

The overall goal of this thesis was to develop scalable, high throughput methods and tools to lay the groundwork for future studies of excitatory and commissural V3 INs. V3 INs have great potential for better understanding the role of CPGs in the spinal cord and ultimately improvements in spinal cord injury therapy. This thesis accomplished two goals: the development of an induction protocol to generate V3 INs from mESCs, and the generation of a drug-selectable V3 IN line for the purification of ESC induced V3 INs. The successful establishment of a V3 IN induction protocol allows for ESCs to become a limitless V3 IN cell source while the generation of a selected post-mitotic V3 IN cell line allows for a highly purified cell culture that opens up many possibilities for future studies.

In Chapter 2, a method to induce V3 INs from mESCs was established. A 2/6+ V3 IN induction protocol used longer SAG durations and lower RA concentrations than a previously established 2/4+ MN induction protocol to significantly increase expression of Sim1, a V3 IN
marker. The induced cultures were also positive for p3 markers Ngn3 and Nkx2.2 and post-mitotic V3 marker Uncx. After induction, cultures had about 8% V3 INs based on Sim1 in situ hybridization and Uncx immunocytochemistry data. A decrease in RAX expression after V3 IN induction indicated that the Sim1+ identity of the induced cultures were likely not hypothalamus-like. The results of this chapter provided insight into how to alter RA and SAG signaling to manipulate induction protocols towards a more ventral spinal identity.

In Chapter 3, the issue of low V3 IN yield from the induction protocol was addressed by developing a selectable cell line, Sim1-Puro, in which the PAC gene was expressed under Sim1 gene regulatory elements. Homologous recombination was used to incorporate the PAC gene into the open reading frame of the Sim1 gene, effectively knocking out Sim1 and knocking in PAC on one allele. The resulting cell line utilized the Sim1 regulatory elements to control PAC expression and provided puromycin resistance to post-mitotic V3 INs. Puromycin was then used to kill off non-Sim1 expressing cells, resulting in the improvement of V3 IN purity. The selected cultures matured and exhibited physiological properties and molecular markers expected from V3 INs. The results of this chapter demonstrate a high throughput method to purify V3 INs from mESC inductions.

Overall, the research presented in this thesis establishes a set of tools for future studies of spinal INs. The establishment of the 2/6+ V3 IN induction protocol and the puromycin-selectable Sim1-Puro cell line provides a way to obtain V3 INs without tedious dissection from embryos. This thesis offers a high throughput, scalable, high purity method for obtaining V3 INs in vitro. Future studies can use the generated cells for better understanding the role of V3 INs in CPGs and reorganization after spinal cord injury.
4.2 Recommendation for Future Directions

The establishment of a V3 IN induction protocol and a selectable V3 IN cell line is the first step towards understanding V3 INs. The electrophysiological characterization of the Sim1-Puro cell line shows it can functionally mature, indicating in its usefulness in further V3 IN studies. Additionally, the understanding of how RA and Shh work together to generate V3 INs compared to MNs provides insight on how to further manipulate induction protocols to generate other cell types or apply the V3 IN induction reported in this thesis to other cells. Finally, the methods used to generate the Sim1-Puro cell lines can be applied to many other molecular markers to generate a wide variety of cell lines to better study V3 INs.

4.2.1 Further V3 Studies

The generated Sim1-Puro cell line allows for the purification of V3 INs from a heterogeneous induction. This enables better understanding of V3 INs by more precise controlling of the culture environment. Recording cellular activity on multi-electrode arrays can provide information on V3 INs’ ability to synapse onto themselves [180]. Culturing V3 INs on islands would allow for autosynapsing [181]. Using calcium channel dyes can also allow for visualization of signal propagation [182,183]. These studies would be the first steps in understanding V3 IN function in vitro.

Similar studies can be performed on cocultures. Coculturing V3 INs with previously reported Hb9-Puro MNs and other INs could culminate in modeling CPGs in vitro. A well-mixed coculture of INs and MNs could allow for recordings on multi-electrode arrays to get a sense of clustering spikes and signal propagation. Additionally, coculturing selected V3 INs and neuronal
cell types within multi-chamber microdevices would allow for quantification of axonal growth[184-186] and even allow for unidirectional synapsing between two cell types[187]. Applying calcium dyes to any of the previously mentioned culture conditions could be useful for visually following CPG activity and further understanding the roles of different INs in CPGs from a bottom up in vitro approach.

The generated V3 INs were not used for in vivo studies. The scarcity of the V3 INs after induction makes EBs less structurally sound than the EBs used for studying Olig2-Puro cells in vivo[174]. However, transplantation studies could show how efficient the induction and subsequent selection process is for removing teratoma causing mESCs. These assessments would be useful for understanding what steps and precautions may be needed for translation. Finally, in vivo studies could be useful to understand how and if the generated V3 INs could be beneficial to functional recovery after spinal cord injury.

4.2.2 Additional Induction Protocols

While V3 INs are an interesting cell population for study of CPGs and functional recovery after SCI, they are not the only IN population of interest. All the ventral spinal populations play a role in CPGs and locomotor control. MNs, V2as, and now V3s have been generated from mESCs. Additionally, V0s have been shown to be generated with a high RA only induction. However, a more comprehensive set of protocols for the generation of these populations would be useful. V1s as well as a variety of ventral IN subtypes are still needed. Additionally, as more markers for ventral IN subtypes are identified and more developmental pathways are elucidated, differentiation protocols for specific IN subtypes could be established.
For instance, DAPT was used to drive V2a differentiation preferentially over V2b differentiation [68,93]. This work could then be used for bottom up methods of in vitro modeling of CPG in addition to the mathematical models that have been previously reported[188].

The induction method established in this work could be translated to human cell cultures. Due to the difference in cell types, the duration of induction would need to be extended appropriately to mimic the extension seen when translating the mESC MN protocol to human cell types [102,103]. As MN protocols in human iPSCs have demonstrated [104], a combination of multiple Shh pathway agonists may be useful to speed up the induction of MNs. This combinatorial exposure to different agonists (purmorphamine, SAG, etc) could be useful for generating V3 INs in human cell cultures, potentially even increasing the efficiency of the protocol without toxicity issues.

4.2.3 Other Cell Lines

While a drug-selectable cell line is effective for the isolation of V3 INs, knocking other genes into the Sim1 locus could also be useful. A lineage tracing line where Cre recombinase is knocked into the Sim1 locus and a flox-stopped fluorescent marker in the safe harbor ROSA 26 site would allow for easy identification of all cells that expressed Sim1 at any point, even after Sim1 is turned off [111,112]. This line could be useful to see how Sim1+ cells mature within the context of supportive glia that arises from the induction, as opposed to the use of glia conditioned media in this thesis. Furthermore, inserting a ubiquitously expressed gene for fluorescent proteins into the Sim1-Puro line could be useful to quickly visually differentiate V3 INs from other cell types within mixed cultures[189]. Other V3 and p3 markers, such as Uncox
and Nkx2.2, respectively, could be used to generate alternative cell lines for studying V3 INs with Sim1 fully intact or the p3 population as a whole. These possibilities can, of course, be applied to any IN population.
References


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Education

Washington University in St. Louis: Saint Louis, MO

- Ph.D. Biomedical Engineering, Aug 2015
- Advisor: Dr. Shelly Sakiyama-Elbert
- Thesis Title: “Directed Differentiation and Characterization of V3 Spinal Interneurons from Mouse Embryonic Stem Cells”
- 2013 Summer: Mentored Undergraduate Summer Student Robin Harland in designing and working towards construction a puromycin N-acetylytransferase and eGFP cassette for a transgenic drug selectable-reporter cell line.
- 2011 Spring: Teaching Assistant for Biotechnology Laboratory Course in which students were in lab 8 hours a week and were taught how to manipulate plasmids, to culture E. coli, Cho and PC12 cells, and to mutate, heterologously express, purify, and evaluate proteins

Yale University: New Haven, CT

- B.S. Biomedical Engineering, Distinction in Major, May 2009
- Accepted into the STARS II program to do research in Biomedical Engineering
- Received Yale College Dean’s Research Fellowship in the Sciences to research during summer 2007
- Accepted as a participant in the 2006 Summer Perspectives on Science Program

Laboratory Experience

Washington University in St. Louis: Saint Louis, MO

- Lab Rotation and Graduate Student Member, Fall 2009 and Summer 2010-Present

  I am continuing my research training in biomedical engineering in Dr. Shelly Sakiyama-Elbert’s lab. My project consists of two parts: the establishment of an induction protocol for obtaining V3 interneurons from mouse embryonic stem cells and the generation of a transgenic drug selectable V3 interneuron mouse embryonic stem cell line for the future study of V3 interneurons.

- Lab Rotation, Spring 2010

  My spring rotation was in Dr. Donald Elbert’s lab. I worked on making protein gradients by using poly(ethylene glycol) (PEG) microspheres of different densities. My portion of the project involved making microspheres containing heparin and analyzing protein loading due to electrostatic interactions with heparin by using fluorescently labeled proteins.
Yale University: New Haven, CT

- Research Assistant, Fall 2006 - 2009

I worked in Dr. Erin Lavik’s lab in Biomedical Engineering under the direction of Millicent Ford Rauch and Dr. Lavik. Projects: The integration of microvasculature and neural progenitor cells for a novel spinal cord implant and Examining neural stem cell and endothelial cell seeded hydrogels in angiopoietin-1 enhanced media

- Participant in the Perspectives on Science Program, Summer 2006

I worked in Dr. Erin Lavik’s lab under Millicent Ford Rauch in the development of a tissue engineered spinal cord implant.

Publications and Presentations

Xu H and Sakiyama-Elbert S. A puromycin selectable cell line for the enrichment of V3 interneurons after induction. In Preparation.


Xu H and Sakiyama-Elbert S. The Directed Differentiation of Spinal V3 Interneurons from Mouse Embryonic Stem Cells. Poster session presented at: MSDBM 2014. 52nd Annual Midwest Society for Developmental Biology Meeting; 2014 ; St. Louis, MO.


**Laboratory Skills**

Stem cell culture, motoneuron and interneuron induction, recombineering, flow cytometry, plasmid manipulation, bacteria cloning, immunocytochemistry, immunohistochemistry, ELISA, electroporation, fibrin gel fabrication, western blotting, SDS-page, agarose gel electrophoresis, DNA precipitation, fluorescent imaging, qRT-PCR, *E. coli* culture, tissue sectioning, tissue fixation, heterologous protein expression in *E. Coli*

With some experience in: hematoxylin and eosin staining, eriochrome cyanide staining, polymer scaffold production (salt leeched sponges and hydrogels), primary neural stem cell isolation, rodent behavior and care, and rodent perfusion

**Additional Skills**

Bilingual Chinese/English, Native Speaker and Literate in both Word Processing, Excel, Powerpoint, Photoshop, and Illustrator, ApE (a Plasmid Editor)