Neural stem cell engineering: directed differentiation of adult and embryonic stem cells into neurons

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1. ABSTRACT

Both adult neural stem cells and embryonic stem cells have shown the capacity to differentiation into multiple cell types of the adult nervous system. They will therefore serve as valuable systems for basic investigations of cell fate choice mechanisms, as well as play important future roles in applications ranging from regenerative medicine to drug screening. However, there are significant challenges remaining, including the identification of signaling factors that specify cell fate in the stem cell niche, the analysis of intracellular targets and mechanisms of these extracellular signals, and the development of *ex vivo* culture systems that can exert efficient control over cell function. This review will discuss progress in the identification of signaling mechanisms and culture systems that regulate neural differentiation, neuronal differentiation, and neuronal subtype specification.

2. INTRODUCTION

Stem cells are defined by their ability to undergo “multipotent” or “pluripotent” differentiation into one or more cell types, as well as the capacity to expand in an undifferentiated, multi/pluripotent state. As this review will discuss, there has been significant interest in understanding and harnessing the mechanisms by which stem cells give rise to mature neurons.

Scientifically, there are basic biological motivations for understanding the role that stem cells play in the development of the nervous system, and stem cell culture systems that are readily manipulable can serve as useful tools for understanding the regulation of these processes. Biomedically, stem cells can serve as valuable sources of neural cells for the regeneration and repair of tissue from the devastating effects of neurodegenerative disorders including Alzheimer's Disease, Parkinson's Disease, Lou Gehrig's Disease, and many others.

Accordingly, the overall objective of this review will be to discuss what is known about the process of neural differentiation from neural stem cells and embryonic stem cells, including neural specification, pan-neuronal differentiation, and neuronal-subtype differentiation. While cell and developmental biological aspects of these processes will be mentioned, the emphasis will be on approaches to direct cell differentiation into therapeutically valuable cell types, as well as on their subsequent implantation into animal models. Furthermore, the role of signals from the stem cell niche or microenvironment in controlling cell behavior will be discussed, in particular soluble signals that have been the best studied to date.

Finally, it is clear that stem cells have tremendous potential for generating many types of neural cells and neurons, but significant progress will still be necessary to provide the precise control over cell function needed for therapeutic application.

3. ADULT NEURAL STEM CELLS AND NEUROGENESIS

Until recently it was generally believed in the field of neuroscience that neurogenesis does not occur in the adult mammalian brain, and it took decades for sufficient evidence to accumulate to overturn this paradigm. Autoradiography studies by Altman in the 1960s provided the earliest evidence for adult neurogenesis by showing colocalization of tritiated thymidine uptake with cells that morphologically appeared to be neurons. However, these studies relied solely on light microscopy to identify neurons and were thus not generally accepted (1-3). A decade later Kaplan provided further evidence using higher resolution electron microscopy analysis of cell morphology in conjunction with autoradigraphy experiments (4). Not until the nineties, however, was the concept of neurogenesis in the adult mammalian brain firmly established, aided by immunostaining techniques that could mark cells that had recently undergone mitosis and then differentiated into neurons (5, 6).

In the healthy adult mammalian brain, the hippocampus and subventricular zone to olfactory bulb pathway are the only two constitutively active neurogenic regions, and neurogenesis within these structures shares similar characteristics. In both, the putative stem cell divides slowly, has astrocytic characteristics, and gives rise to a transiently amplifying population of progenitors that differentiate into a mature neuronal phenotype. There is also evidence for injury or disease dependent neurogenesis in other regions, though it is possible that the new cells may originate from the subventricular zone or hippocampus (7). However, as will be discussed, neural stem like cells can be isolated and cultured from many regions that do not normally exhibit adult neurogenesis. The natural ability of neural stem cells isolated from numerous regions of the central nervous system to generate different neuronal phenotypes *in vivo* underscores the promise of stem cell based therapies for treating neurodegenerative diseases.

Throughout this review, the term neurogenesis will refer to the generation of neurons from neural stem cells. However, this simple term masks the set of complex processes that, in combination, regulate levels of neurogenesis in tissue: stem cell survival, stem cell proliferation, transient amplifying cell survival and proliferation, neuronal differentiation, and the subsequent survival of differentiated neurons. Each of these is likely a key regulatory point in the overall process of adult neurogenesis.

3.1. Hippocampal stem cells

The putative hippocampal neural stem cell exists in the subgranular zone (SGZ) of the dentate gyrus and is referred to as a type-1 cell (8). These cells (Figure 1) exhibit a radial glia like morphology, with a projection into the granule cell layer (GCL) and express the astrocytic marker GFAP and the immature neural stem cell marker nestin. These cells generate type-2 and type-3 cells, which are a transiently amplifying population of progenitors thought to be progressively lineage committed. Type-1, type-2, and type-3 cells may exhibit unique responses towards external stimuli. For example, it has been known that voluntary exercise increases neurogenesis (9), and Kronenberg *et al* reported that such exercise increased the proliferation of type-2 but not type-3 or type-1 cells (10). However, these cell types may also show similar responses, such as increases in proliferation in an animal stroke model, though type-1 cells did exhibit the greatest increases in proliferation (11). Furthermore, type-2 and type-3 cells are identified by the different cell markers they express. Type-2 cells are GFAP negative and nestin positive, and a fraction of these cells express the early neuronal marker doublecortin (DCX), whereas type-3 cells are nestin negative but express DCX (8). As these neural stem cells differentiate, they migrate a short distance into the granule cell layer (GCL), where they mature into granule cells (GC) that extend dendrites into the molecular cell layer and axons into the CA3 region of the hippocampus (12). The resulting mature granule cells are either functionally integrated into the hippocampal neuronal circuitry (13) or undergo programmed cell death. In addition to these findings with granule cells, it has been discovered that functional inhibitory interneurons are generated in the adult mammalian hippocampus (14). Besides generating new neurons, there is also evidence for limited astrocytic differentiation of hippocampal neural stem cells, but no conclusive evidence for the oligodendrocytic differentiation. However, it remains to be seen whether newborn astrocytes and neurons ultimately arise from the same neural stem cell or from two unique precursor populations (15). Despite the lack of evidence to date for a common precursor *in vivo*, cells with stem cell properties can be isolated from hippocampal tissue when cultured in the presence of fibroblast growth factor-

2 (FGF-2). These cells can undergo long term propagation *in vitro* while maintaining the capacity for multipotent differentiation into astrocytes, neurons, oligodendrocytes (16), and even endothelial cells (17). However, a very important and general caveat is that FGF-2 can deregulate bipotent neural precursors from the developing nervous system and convert them into tripotent cells (18). Therefore, it is unclear whether growth factors in culture function to support the proliferation of an endogenous stem cell population, or to convert non-stem cells into multipotent cells. This distinction has implications for the basic study of stem cells, but downstream therapeutic applications stand to benefit from either case.

One technical but important aspect of stem cell isolation is the subsequent system utilized for their culture. Hippocampal stem cells have typically been grown in monolayer culture on a layer of extracellular matrix protein (e.g. laminin) adsorbed to tissue culture plastic (16). By contrast, cells isolated from the subventricular zone, described in the subsequent section, have typically been cultured as non-adherent cell aggregates, or "neurospheres." Neurospheres have an advantage that they can theoretically arise from a single stem cell, and they are thus a useful assay to analyze the potency of stem cells within a population. However, cells across the diameter of the sphere experience varying or heterogeneous microenvironments, such that cell fate can be challenging to control within a sphere. By contrast, monolayer cultures expose cells to a more uniform microenvironment.

3.2. Subventricular zone (SVZ) and olfactory bulb stem cells

The other neurogenic niche in the adult nervous system is the SVZ, where stem cells reside just underneath the ependymal cell layer of the lateral ventricles (figure 1). The native stem-like cells of this niche have many characteristics of mature astrocytes, designated B cells, which are adjacent to the ependymal cell layer and give rise to a transit amplifying population of cells designated C cells. These C cells in turn give rise to a population of neuroblasts, termed A cells, that migrate along the well defined the rostral migratory stream (RMS) or pathway to the olfactory bulb (6, 19). Upon their arrival in the olfactory bulb, cells migrate radially and differentiate into interneurons in the granule and glomerular cell layers (20).

Humans, however, have a unique SVZ and olfactory bulb structure compared to other mammals. For instance, there is a hypocellular gap between the ependymal wall of the lateral ventricle and the inner layer of astrocytes that is filled with astrocytic cell processes. Importantly, *in vivo* some astrocytes within the SVZ express proliferative markers, indicating that the human SVZ is also a neurogenic region that contains actively dividing cells (21-23). Similar to rodents, humans have a RMS containing cells that are positive for the proliferative marker PCNA and neuroblast makers common to the rodent RMS such as PSA-NCAM and beta-tubulin III. However unlike the rodent RMS, due to the larger size of the frontal cortex in humans compared to rodents, the human RMS first follows a caudal path before entering the olfactory tract (24).

The olfactory bulb, the final destination of the RMS, contains cells that express markers consistent with the presence of a migrating neural stem cell population, i.e. proliferative markers that costain with immature neuronal markers such as doublecortin (DCX) and PSA-NCAM. Importantly, there is a cell population in the olfactory bulb that expresses markers consistent with neuronal differentiation: the early committed neuronal marker Tuj1 along with glutamic acid decarboxylase (GAD) for GABAergic neurons and tyrosine hydroxylase (TH) for dopaminergic neurons (25). Therefore, despite the structural differences between the rodent and human brain, there are similar SVZ to olfactory bulb pathways in humans and rodents with cells that expresses markers of proliferation and neuronal differentiation.

Similar to other species, a population of cells capable of extended proliferation and multipotent differentiation can be isolated and grown in culture from the human SVZ. In addition, they can be differentiated into astrocytes, oligodendrocytes, and neurons in neurosphere culture (23). Furthermore, Pagano *et al* isolated neural stem-like cells from the human olfactory bulb after invasive neurosurgery. These cells could proliferate in neurosphere culture under FGF-2 stimulation and were capable of differentiating into astrocytes, oligodendrocytes, and neurons much like the SVZ derived neural stem cells from Sanai *et al* (23, 26).

A population of neural stem cells can also be isolated from the non-CNS, human olfactory neuroepithelium, adjacent to the putative neurogenic olfactory bulb, and propagated as neurospheres *in vitro*. Unlike SVZ and olfactory bulb-derived neural stem cells, these neuroepithelial neurospheres contain cells that express astrocytic, neuronal, and epithelial markers but no oligodendrocytic markers. Olfactory neuroepithelium derived neural stem cells are a promising source for future cell based therapies as they can be easily isolated from the nasal cavity of a patient for potential use in autologous transplantation. Consequentially, they avoid the problems of difficult accessibility, limited availability, and histocompatibility inherent to other neural stem cell populations within the adult brain (27).

3.3 Progenitors derived from non-neurogenic regions

While it is generally accepted that the SGZ and SVZ are the only two active regions of CNS neurogenesis, progenitors can be derived from numerous non-neurogenic regions of the central nervous system, with the caveat that signals in culture have the potential to reprogram cell potency (18). Horner *et al* demonstrated that when spinal cords were examined one hour after BrdU administration, proliferating cells were detected in the parenchyma and near the ependymal cell layer. When similarly-treated spinal cords were harvested after several weeks, BrdU co-labeled newly generated astrocytes and oligodendrocytes, though without any measurable neuronal differentiation (28). In addition, after injury there was an increase in proliferating cells, but there is still debate to whether these newly generated cells arise solely from the epedymal region and undergo migration, or

are generated throughout the spinal cord (29-32). Furthermore, it remains to be determined if these proliferating cells are a unique bipotent population of progenitors, or a heterogeneous population of proliferating progenitors already committed to either an astrocytic or oligodendrocytic lineage. However, a population of cells with progenitor like properties can be isolated from cord tissue and cultured with growth factors as either neurospheres or in a cell monolayer. *In vitro*, these progenitor cells exhibit self-renewal and can differentiate into astrocytes, oligodendrocytes, and neurons (33, 34).

There is debated evidence for neurogenesis in the adult neorcortex (35, 36). Regardless, Palmer *et al* demonstrated that in the presence of FGF-2, one can isolate a proliferative glial precursor population from the rat neocortex. Additionally, these cells were able to generate neurons *in vitro* after long term expansion in FGF-2 (37). Laywell *et al* demonstrated that a monolayer of astrocytes generated from cortical tissue can form neurospheres when the medium is supplemented with epidermal growth factor (EGF) and FGF-2. In addition, they observed a loss of the ability of these astrocytes to form neurospheres from a monolayer cell culture when they were isolated from older animals. Furthermore, these astrocytic cells were multipotent, as they generated both neurons and glia after being cultured in EGF and FGF-2. Based on these findings they hypothesized that the isolated cells were immature astrocytes from the postnatal mice cortex (38).

Using adult human temporal cortex tissue, Walton *et al*, derived a monolayer of multipotent astrocytic progenitors, similar to the work of Laywell *et al.* These cells were capable of long term expansion *in vitro* in EGF and FGF-2 and avoided immortalization. Also, they expressed a mix of immature and mature astrocytic markers along with the immature neural stem cell marker nestin. Engraftment studies revealed these cells to be multipotent and capable of integration into the adult brain (39). However, more work is required to determine their capacity to differentiate into the different neural phenotypes of the adult brain.

Neural stem-like cells can also be isolated from other regions of the adult brain. Lie *et al* demonstrated that in the adult substantia nigra there is a proliferating population of glial progenitors that *in vivo* stains for the marker NG2, a glial progenitor marker. When marked with BrdU and examined weeks later, these cells had differentiated into astrocytes and oligodendrocytes but not neurons. However, when these cells were isolated and cultured in FGF-2 or FGF-8, they were capable of differentiating into neurons as well as astrocytes and oligodendrocytes (40). Markakis *et al* demonstrated that stem-like cells can also be isolated from the adult hypothalamus, though they did not report evidence for proliferation or neurogenesis *in vivo*. Again, following culture in FGF-2, these cells could differentiate into neurons, astrocytes, and oligodendrocytes (41). The capacity to isolate stem-like cells from non-neurogenic regions of the adult brain indicates either than signals within the local microenvironment limit an endogenous progenitor cell's capacity for neurogenesis, or that culture conditions convert a population of cells into ones with stem-like properties.

3.4. Adult neural stem cell engraftment in vivo

Neural stem cells have promise for tissue regeneration from disease or injury; however, such therapies will require precise control over cell function to create the necessary cell type(s). There is not yet a complete understanding of the mechanisms that regulate cell proliferation and differentiation, and it is thus difficult to fully explore the plasticity of a neural stem cell population derived from any given region of the adult brain. One approach to evaluate cell potency is to engraft a cell population into different regions of the brain and analyze whether regional microenvironments can present signals to permit or guide cell differentiation. Such studies can serve as a basis for identifying key endogenous neural stem cell regulatory signal, as well as provide further insights into why most regions or microenvironments of the adult brain are not neurogenic. Furthermore, regulatory signals from niches that do support cell differentiation can be harnessed for future efforts in regenerative medicine. Finally, such efforts provide insights into the future therapeutic potential of cell engraftment.

3.4.1. Hippocampal derived neural stem cells

Hippocampus-derived neural stem cell cultures have exhibited substantial plasticity during engraftment studies. After homotypic (into regions of the brain from which the neural stem cell used in the experiment originated) and heterotypic (into regions of the brain other than where the neural stem cell originated) engraftment, these cells differentiate in a manner characteristic of the injection site. For example, early studies demonstrated that when these cells were engrafted into the hippocampus, they exhibited the capacity to undergo neuronal differentiation. In addition, when they differentiated and integrated into the dentate gyrus, they assumed characteristics indicative of the native granule cells. Furthermore, they differentiated into glia when incorporating into the CA fields of the hippocampus (42, 43). Interestingly, when the engraftment of hippocampal neural stem cells into the hippocampus is accompanied by tissue damage, there is an increase in astrocytic differentiation even within the normally neurogenic dentate gyrus (42), a shift that indicates an alteration in microenvironmental signals to selectively promote glial differentiation. When hippocampal neural stem cells are engrafted into the RMS, the majority migrates into the olfactory bulb and populates the granule and glomerular cell layers. They express mature neuronal markers, and a small percentage of cells that engraft into the glomerular cell layer express the dopaminergic marker TH. The cells also populate the granule and glomerular layers in the same proportions as endogenous SVZ neural stem cells, where the granule cell layer receives more new neurons than the glomerular layer. Lastly, cells injected into the non-neruogenic cerebellum did not differentiate into neurons, underscoring the lack of key pro-neuronal differentiation signals in this environment (43).

Engraftment into the retina further illustrates the capacity for hippocampal neural stem cells to respond to signals from non-native micrenvironments. When engrafted into healthy adult retina, they did not attain characteristics of cells commonly found in the retina. However, when implanted into the neonatal eye, they migrated, integrated, and adopted morphologies of retinal cell types such as Müller, amacrine, bipolar, horizontal, and photoreceptor cells. However, while they did express neuronal and glial markers, they did not express mature retinal cell markers (44). The injured eye, like the developing eye, thus apparently expresses cues that induce cell migration and differentiation into retinal cells. Hippocampal neural stem cells engrafted after injury, be it ischemic, mechanical, or neurodegenerative in nature, all exhibited incorporation into the retina where they expressed immature neuronal and astrocytic markers and adopted site-appropriate morphologies(45-49). However, even in disease models, similar to the developing eye, the resulting cells did not express mature markers associated with retinal cells. Furthermore, using electroretinogram and pupil light reflex studies, Grozdanic *et al* showed that hippocampal engraftments conferred no functional benefit compared to ischemic control animals (49). Investigation of the changes within the retinal microenvironment induced by injury has revealed modulation of signaling molecules known to regulate the behavior of hippocampal neural stem cells, which may underlie the limited neuronal differentiation observed in engrafted cells. For example, after ischemic injury FGF-2 expression increased in the retina, and there was elevated neurotrophic factor expression by microglia and Müller cells after light induced retinal degeneration (50, 51). These results indicate that expression of these and potentially other factors could alter the retinal microenvironment and thereby render it permissive to neural stem cell integration and differentiation. However, an insufficient duration of permissive signals, an absence of necessary signals, or the presence of inhibitory signals may be responsible for incomplete cell differentiation.

3.4.2. SVZ derived neural stem cells

Lois *et al* demonstrated that SVZ cells are capable of homotypic differentiation when engrafted in the adult SVZ, i.e. they exhibit migration and differentiation similar to endogenous neural stem cells (6). In a later study, Herrera *et al* engrafted cells isolated from the SVZ directly into different regions of the adult brain of healthy animals without any prior *in vitro* propagation of the cells. The SVZ derived cells generally differentiated into GFAP positive astrocytes when engrafted in the cortex, hippocampus, and striatum. The olfactory bulb was the only region where cells exhibited neuronal differentiation (52). Zhang *et al* demonstrated that when SVZ neural stem cells were propagated in the presence of FGF-2 for 8 days after isolation and then injected into the striatum, they expressed neuronal markers such as the intermediate neuronal marker Map2ab and the mature neuronal marker NeuN (53). Also, unlike the earlier results of Herrera *et al*, few cells expressed the astrocytic marker GFAP. This result raises the question of whether these engrafted cells responded to cues within the striatum or whether they experienced changes in potency or began to differentiate in culture prior to engraftment, which would imply that the striatum did not provide pro-neurogenic signals but that the FGF-2 effectively reprogrammed the engrafted cells. When propagated in the presence of FGF-2, 70% of the cells in the neurosphere culture expressed Tuj1 but not later markers of neuronal differentiation such as NeuN or Map2ab. It is possible that FGF-2 primed them to undergo neuronal commitment but not full differentiation, or alternatively that with additional culture they could differentiate further (53).

Injury models reveal that permissive signals that allow engrafted SVZ neurospheres to undergo neuronal differentiation can exist in the striatum. In a 6-hydroxydopamine (6-ODHA) chemical induced lesion model of Parkinson's disease, SVZ neurospheres were engrafted into the striatum, and their subsequent differentiation was analyzed. Prior to engraftment, neurospheres were propagated in media supplemented with either EGF and FGF-2 or FGF-2 alone. Unlike the results of Zhang *et al* (53), in the study of Richardson *et al*, neurospheres cultured in FGF-2 were predominately populated by GFAP positive cells with only a small population of Tuj1 positive cells (54). When these cells were engrafted into the striatum of lesioned animals, 2% of them differentiated into neurons. Furthermore, inducing neuronal differentiation with retinoic acid prior to engraftment did not elevate the neuronal differentiation of engrafted cells (54). Unlike Richardson *et al*'s work, when Meissner *et al* expanded cells under FGF-2 plus EGF, the cultured neurospheres exhibited a larger population of neuronal cells, evident from staining for neurofilaments, but again with a significant fraction of GFAP positive cells. However, after engraftment into lesioned animals, 2% of the cells differentiated into neurons that stained positive for the dopaminergic marker tyrosine hydroxylase (TH) (55).

Unlike such Parkinson's disease model experiments, which showed a slight but measurable increase in the neuronal differentiation of engrafted SVZ neurospheres compared to healthy control animals, a quinolinic acid model of Huntington's disease revealed a greater potential for neuronal differentiation in the striatum. Prior to engraftment, neurospheres were cultured in growth factor (EGF and FGF-2) supplemented media, and when characterized *in vitro* they expressed GFAP but not the neuronal marker Map2ab. However, cells engrafted in this disease model exhibited a robust differentiation of 35% of the cells into NeuN positive neurons. Furthermore, 15% of these cells expressed markers associated with medium spiny neurons (56). These models reveal that in key situations the striatal microenvironment expresses factors that support and induce spiny neuron and TH positive neuronal differentiation. It will thus be informative to identify the signals present in the quinolinic acid model of Huntington's disease, but not in the Parkinson's model, that induce increased neuronal differentiation of injected cells.

The plasticity of SVZ derived neural stem cells is further illustrated with heterotypic engraftment, i.e. into regions of the adult brain other than the striatum. Unlike the Herrera *et al* study in which SVZ progenitors where engrafted without *in vitro* propagation (52), when SVZ derived neurospheres were propagated *in vitro* and then engrafted into the hippocampus, they more readily underwent neuronal differentiation (57, 58). Richardson *et al* reported that 35% of cells integrated into the granule cell

layer and expressed neuronal markers. Furthermore, retinoic acid pretreatment did not further increase the neuronal differentiation after implantation (58). Aguirre *et al* used flow cytometry to select for a population of cells from the early postnatal SVZ that expressed the chondroitin sulfate proteoglycan NG2. When isolated cells were injected into the hippocampus, they expressed neuronal markers and integrated into the pyramidal layers of CA1 and CA3, as well as the hilar region of the dentate gyrus. Furthermore, Aquirre *et al* demonstrated that a subset of engrafted cells generated functional GABAergic interneurons (57). These studies reveal that SVZ neural stem cells can respond to signals within the hippocampal microenvironment and thereby differentiate into GABAergic neurons and granule cells following engraftment.

3.4.3. Spinal cord progenitors

Shihabuddin *et al* engrafted progenitor cells isolated from the spinal cord of adult rats. They saw that when transplanted back into the rat spinal cord after *in vitro* propagation in FGF-2, cells differentiated only into astrocytes and oligodendrocytes. However, when introduced into the hippocampus, they differentiated into NeuN positive neurons. Furthermore, they exhibited site-specific differentiation in the hippocampus, where the majority of the NeuN positive cells were observed in the granule cell layer, and the majority of the cells that integrated into the CA regions expressed astrocytic and oligodendrocytic cell markers (59). The hippocampal microenvironment, which apparently provides signals for neuronal differentiation not present in the spinal cord, reveals the neurogenic potential of spinal cord progenitors not observed in homotypic engraftments into the spinal cord.

3.4.4. Cortical derived progenitors

There are comparatively fewer studies that have analyzed the *in vivo* plasticity of astrocytic progenitors derived from the cortical tissue. In two studies, Zheng *et al* examined the behavior of neonatal mouse derived astrocytic progenitors engrafted directly into the RMS or the lateral ventricle of mice. They found that when injected into the RMS, some cells migrated to the olfactory bulb but did not express markers of mature olfactory bulb interneurons after their arrival (60). However, when engrafted into the lateral ventricle, a fraction of the cells migrated to the olfactory bulb and differentiated into mature olfactory interneurons (61). These studies provide evidence that although the RMS presents signals to direct migration, it lacks necessary signals that the SVZ utilizes to direct olfactory bulb interneuron differentiation. Walton *et al* examined the ability of adult human derived astrocytic progenitors to integrate, survive, and acquire a mature phenotype after injection into adult mice. When cells were engrafted into the lateral ventricle, the majority were detected in the ependymal wall. These human cells did not express mature neuronal markers or migrate into the olfactory bulb. Instead, the majority appeared to differentiate into astrocytes. By contrast, cells engrafted into the cortex differentiated primarily into neurons, with a rare few cells staining positive for astrocytic or oligodendrocytic markers. Furthermore, a few cells migrated and integrated into the CA1 and CA3 regions of the hippocampus, where they expressed neuronal markers and extended process characteristic of pyramidal neurons (39). These results indicate that the regulation of cell potency and differentiation dramatically differs between these murine and human cells.

3.4.5. Substantia nigra (SN) derived neural progenitors

Lie *et al* isolated a multipotent population of neural progenitors from the SN of adult rats. As mentioned in Section 3.3, these cells were capable of differentiating into astrocytes, oligodendryocytes, and neurons *in vitro*. Furthermore, cells propagated *in vitro* in either FGF-2 or FGF-8 did not express neuronal markers after a homotypic engraftment into the SN. Instead, 30% of engrafted cells expressed the glial progenitor marker NG2. However, when Lie *et al* performed a heterotypic engraftment into the hippocampus using either FGF-2 or FGF-8 propagated progenitors, 20% of the cells subsequently expressed beta-tubulin III and NeuN (40).

3.5. Inducing neuronal differentiation with microenvironmental signals and genetic manipulation

Engraftment studies reveal that neural stem cells have great potential for differentiation and integration into the nervous system. To treat neurodegenerative diseases and trauma to the nervous system, one could envision inducing adult neural stem cells to expand and generate new neurons to replace ones lost due to disease or injury. This approach can be achieved either *in vivo* by manipulating the endogenous stem cell population, or by an *ex vivo* approach involving the expansion and programming of cells outside the body. In either case, cells can be manipulated through the addition of regulatory protein and/or small molecule signals or by the direct introduction of new genetic information to the cells to modulate their behavior (62). However, the efficacy of a neural stem cell based therapy will likely rely upon the selective generation of a specific cell type or types, such as a specific neuronal subtype. To achieve this goal, the proteins, small molecules, and regulatory events that control cell differentiation must be investigated then harnessed to control cell function for therapeutic application. Through studying the signaling molecules present in the microenvironment during development and in the adult, and which key regulatory transcription factors the cells express, methodologies for controlling neuronal differentiation either *in vivo* or *ex vivo* can thus be achieved.

3.5.1. Retinoic acid

Retinoic acid, a small molecule signal known to regulate neuronal differentiation during development (63), is commonly utilized to induce neuronal differentiation *in vitro*. Furthermore, recent work in adult vertebrates reveals that retinoic acid plays a crucial role in the neurogenic regions of the adult nervous system. In the mouse subventricular zone-olfactory bulb pathway, the addition of retinoic acid or its precursor retinol to tissue explants increases the number of migrating cells in the RMS and proliferating cells in the SVZ. When a dominant negative retinoic acid receptor was expressed in explants, SVZ cell migration and differentiation was inhibited. Lastly, inhibition of retinoic acid synthesis decreased SVZ cell proliferation. These results indicate that retinoic acid in the SVZ regulates not only differentiation but also proliferation and migration (64). In the

adult hippocampus, retinoic acid depletion experiments revealed a slightly different function. In this study, mice were fed either a normal or retinol-deficient diet. The resulting decrease in retinoic acid signaling apparently did not affect the proliferation of SGZ cells; however, it did inhibit the generation and survival of new hippocampal neurons (65). Retinoic acid's pro-survival effect on progenitor cells is also observed in the spinal cord. Decreased retinoic acid signaling, again due to dietary depletion, induced motorneuron cell death in adult rats (66). Furthermore, retinoic acid in the spinal cord is apparently involved in neurite outgrowth after injury (67). These findings, coupled with the crucial role that retinoic acid plays during embryogenesis, underscores its importance in regulating and inducing neuronal differentiation.

Further work will be required to determine what role if any retinoic acid plays in specifying neuronal phenotype during adult neural stem cell differentiation. In culture, adult and embryonic derived SVZ neural stem cells exhibit a retinoic acid dose-dependent increase in the expression of the early neuronal marker beta-tubulin III. However, the acquisition of mature or phenotype specific markers for SVZ neural stem cells upon retinoic acid treatment remains to be addressed (64, 68).

In spinal cord derived progenitors, retinoic acid signaling induces neuronal over astrocytic differentiation, and it also appears to control the maturation of cells committed to a neuronal cell fate. In cells derived from rat E14 spinal neural tube ventricular zone, retinoic agonist treatment revealed a sequential role for the different retinoic acid receptor (RAR) subtypes. Specifically, when an agonist for RAR-alpha was added to the culture media first, the neural stem cells differentiated into glia, whereas a RAR-beta agonist instead induced immature motorneuron differentiation. However, mature motorneurons formed with the sequential addition of RAR-beta then RAR-alpha agonist, indicating that a specific developmental program of retinoic acid and RAR interaction controls the differentiation of spinal cord derived neural progenitors (69). It remains to be seen whether a similar paradigm of sequential retinoic acid receptor activation also functions in adult spinal cord neural progenitors, as well as neural stem cells derived from neurogenic regions of the adult brain.

All-trans retinoic acid, which can bind to both RAR subtypes, induced a pan-neuronal differentiation, and in particular the differentiation of a heterogeneous population of neuronal phenotypes that could be selectively altered by neurotrophic factors in cultured hippocampal neural stem cells (70). Takahashi *et al* showed that cells cultured with retinoic acid and fetal bovine serum (FBS) expressed markers for GABAergic, dopaminergic, and cholinergic neuronal phenotypes at low levels. They also discovered that different neurotrophic factors could modulate the fraction of cells that expressed markers for these different neuronal phenotypes, without increasing cell proliferation. For example, nerve growth factor (NGF) had only a modest effect on cholinergic neurons, while the other phenotypic populations remained unchanged. Neurotrophin-3 (NT-3) increased the total number of GABAergic and dopaminergic neurons while eliciting a similar modest increase in cholinergic neurons. Lastly, brain derived neurotrophic factor (BDNF) significantly increased the expression of cholinergic markers along with strong increases in GABAergic and dopaminergic expression (70). This varying phenotypic expression is presumably due to the differential activation of neurotrophin receptors by the different ligands. In particular, NGF preferentially binds TrkA, BDNF and NT-3 bind TrkB, and NT-3 binds TrkC. Despite the selective increases in neuronal phenotypes, these cultures were still dominated by glial cells, with only few neurons with a mixture of different phenotypes present. These findings also underscore the fact that generating homogeneous populations of neuronal phenotypes will require an improved understanding of mechanisms and signaling factors that regulate neuronal differentiation.

Finally, in addition to natural small molecules such as retinoic acid, novel, synthetic small molecules identified in high throughput assays for their ability to control stem cell function promise to greatly benefit the field (71, 72).

3.5.2. Morphogens and growth factors

Sonic hedgehog (Shh) is a morphogen involved in tissue patterning during development (73). It also plays a crucial role in the maintenance of neural stem cell niches in the adult brain. Lai *et al* showed that Shh increased hippocampal neural stem cell proliferation while maintaining their capacity to differentiate into neurons, astrocytes, and oligodendrocytes *in vitro*. Furthermore, viral vector mediated overexpression of Shh in the hippocampus also induced increased proliferation of endogenous hippocampal neural stem cells. This elevated proliferation *in vivo* correlated to a subsequent increase in the number of newborn neurons that incorporated into the GCL and expressed the mature neuronal marker NeuN (74). These results also indicated that Shh overexpression does not alter the lineage commitment of endogenous neural stem cells *in vivo*. Furthermore, *in vivo* administration of a pharmacological inhibitor of Shh signaling decreased cell proliferation in the hippocampus, implicating Shh as an endogenous signal that regulates adult neurogenesis. In a subsequent study, Ahn *et al* used Cre expression from a *gli1* promoter, which is a sensitive readout of Shh signaling activity, to genetically mark and trace the fate of stem cells in neurogenic regions of the adult brain. They observed that Shh signaling is active in proliferating cells of both the SVZ and hippocampus, particularly in the slowly dividing cells in both regions. Furthermore, by marking the cells and tracing their fate, they observed that although the majority of the cells that were stimulated by Shh differentiated into neurons, a small population differentiated into the other cell fates (75).

Wnt3a is another signaling molecule recently shown to play a key role in regulating neurogenesis in the adult brain. *In vivo* expression of a Wnt3a inhibitor reduced neurogenesis in the adult hippocampus, as evident from a decrease in the number of cells co-labeled with doublecortin and BrdU. By contrast, Wnt3 overexpression *in vivo* and *in vitro* increased neuronal differentiation without altering the proliferation rate of the cells, as the total number of BrdU positive cells did not change (76), in

contrast to Shh overexpression (74). However, Wnt3's overexpression *in vitro* resulted in a mixed culture of glia and neurons, indicating that there are most likely other signals found within the *in vivo* microenvironment that work in conjunction with Wnt signaling to ensure a neuronal cell fate commitment. Also, it is yet to be determined whether Wnt signaling induces a specific neuronal phenotype or serves as a nonspecific, pan-neuronal signal.

Studying the role of specific signals in culture can yield insights into their functions *in vivo*, as the roles of Shh and Wnt3a *in vitro* were effective predictors of effects observed *in vivo*. However, translating *in vitro* experiments to *in vivo* studies can reveal additional complexities, presumably due to additional signals found within the brain's microenvironment. In cell culture, for example, EGF and FGF-2 are commonly used to stimulate proliferation. However, an early study infused these proteins into the adult brain and found differential regulation of neurogenesis in the two neurogenic regions of the adult brain (77). Kuhn *et al* showed that both EGF and FGF-2 increased SVZ progenitor proliferation. However, only FGF-2 increased the generation of new neurons in the olfactory bulb. By contrast, EGF decreased the total number of newborn neurons and increased the number of newborn astrocytes in the olfactory bulb. Furthermore, EGF increased the net number of newborn cells in the striatum, though Kuhn *et al* did not determine whether this increase was due to migration from the SVZ or stimulation of an endogenous population of cells. However, it should be noted that although these EGF-induced cells in the striatum expressed proliferative markers, they did not express any neuronal markers. Interestingly, in the hippocampus EGF exerted a similar effect by increasing astrocytic differentiation while inhibiting neuronal differentiation, but neither FGF-2 nor EGF increased proliferation in the hippocampus above controls (77). Thus, *in vivo* FGF-2 and EGF exhibit divergent effects on neuronal differentiation, whereby FGF-2 selectively promoted olfactory neuronal differentiation, whereas EGF promoted astrocytic differentiation.

The use of growth factor infusion to elevate neurogenesis in the olfactory bulb has therapeutic implications. However, growth factor infusion did not elevate hippocampal neurogenesis or lead to newborn neurons in the striatum of a healthy brain. By contrast, damaged tissue presents microenvironments that are much more permissive to growth factor stimulation of neurogenesis. During ischemic injury, neurogenesis increases in both the SVZ and the hippocampus (78-80), indicating that this injury elicits pro-neurogenic signals. Furthermore, when a combination of EGF and FGF-2 was infused into the SVZ in an ischemic animal model, newborn cells that expressed neuronal cell markers were evident in the striatum (81). In the hippocampus, neurogenesis may or may not increase due to growth factor infusion after injury. Baldauf *et al* did not observe increased neurogenesis upon a combined infusion of FGF-2 and EGF, but Nakatomi *et al* saw elevated neurogenesis and a striking replacement of pyramidal neurons in the CA1 region of the hippocampus following a similar infusion (82). These studies differed in the duration of the growth factor administration, which may underlie the different conclusions and certainly stresses the importance of optimizing experimental (and potentially treatment) conditions.

The adult spinal cord is another microenvironment that demonstrates the potential for increased neurogenesis when injury is coupled with growth factor infusion. Ohori *et al* examined the effect of injecting FGF-2, EGF, and a GFP-encoding retrovirus into an injured spinal cord, where the retrovirus served to mark proliferating cells. In injured animals that were injected with the retrovirus only, the majority of the GFP marked cells expressed glial markers, with none expressing neuronal markers. However, in the FGF-2 and EGF infused animals, there was a marked increase in cell proliferation and the percentage of marked cells that expressed the neuronal marker Map2ab (83).

A combined effect of injury and growth factor infusion was also seen when transforming growth factor-alpha (TGF-alpha), which binds the same receptor as EGF, was infused into Parkinson's disease models. In 6-ODHA lesioned rats, there was an increase in proliferation in the SVZ and migration into the striatum in TGF-alpha infusions compared to lesioned controls (84, 85). Furthermore, without the lesion and consequent denervation, TGF-alpha did not induce proliferation or migration of SVZ neural stem cells (84). Unfortunately, Cooper *et al* did not observe any co-labeling of the newly generated cells in the striatum with neuronal markers such as beta-tubulin III, PSA-NCAM, and NeuN. Furthermore, they did not observe any behavioral effects due to the infusion. By contrast, Fallon *et al* observed increased neuronal differentiation in the striatum and consequentially a behavioral improvement in the lesioned rats in a similar study (85). It remains unclear whether TGF-alpha induces proliferation, enhances survival, or influences commitment to neuronal differentiation.

Neurotrophic factor infusion also modulates neurogenesis in the adult brain. Kobayashi *et al* examined cerebral ischemia recovery after glial cell line-derived neurotrophic factor (GDNF) infusion. They observed increased SVZ cell proliferation, improved survival, and an increase in the number of newborn neurons in the striatum that also exhibited medium spiny neuron characteristics. Furthermore, they showed that ischemic injury increased the expression of GDNF receptor (GFR-alpha) on proliferating cells in the SVZ, implying that GDNF worked directly on the neural stem cells found with the SVZ niche. These effects appeared to be selective for neurons since they did not observe an increase in newborn glia (86).

Trauma to the CNS is not always necessary for a signaling molecule to modulate neurogenesis in non-neurogenic regions of the brain. For example, BDNF, unlike EGF and FGF-2, increased adult neurogenesis in uninjured animals. BDNF infusion led to newborn BrdU positive cells that stained positive for neuronal markers in the olfactory bulb and importantly in the striatum as well (87, 88). Similar results were seen when a BDNF-encoding adenoviral vector was used to infect forebrain ependymal cells lining the SVZ. Viral-mediated BDNF overexpression again led to an increase in the number of neuronal marker

positive cells in the olfactory bulb and striatum (89). Furthermore, Chmielnicki *et al* combined BDNF overexpression with an increased expression of Noggin by the local ependymal cells to enhance the neurogenic effects of BDNF (90). This exogenous Noggin augmented endogenous ependymal Noggin expression, which is thought to mitigate the negative neurogenic effects of BMP signaling from ependymal cells in the SVZ microenvironment (91). The BDNF overexpression studies also confirmed that the newly generated striatal neurons expressed markers characteristic of medium spiny neurons (89). Consequentially, BDNF *in vivo* is sufficient to at least partially overcome negative environmental signals such as BMP and to selectively differentiate neuronal progenitors into striatal neurons, but it was insufficient to control the migration of cells into the striatum without also increasing migration into the olfactory bulb.

3.5.3. Neurotransmitters

There is accumulating evidence that neurotransmitters play a role in regulating adult neurogenesis. For example, in the hippocampus GABA stimulation activates calcium channels, which in turn increase the expression of the pro-neural transcription factor NeuroD1 in hippocampal neural stem cells to halt proliferation and lead to neuronal differentiation (92, 93). Ge *et al* further showed that ambient GABA induced depolarization of newborn granule cells is necessary for functional integration and synapse formation (94). *In vitro,* neural stem cells derived from the hippocampus also responded to glutamatergic stimulation, which again caused an activation of calcium channels and increased NeuroD1 expression. In these *in vitro* experiments, Deisseroth *et al* showed that excitation also decreased the expression of transcription factors Id2 and Hes1, which are known to negatively regulate neuronal differentiation (95). However, direct glutamatergic stimulation of hippocampal neural stem cells *in vivo* did not induce neuronal differentiation, though it appears that NMDA stimulation of the surrounding molecular cell layer may play a role in regulating the release of GABA in the microenvironment and ultimately in neuronal differentiation of the neural stem cells in the SGZ (96-98). However, more work will be required to elucidate the mechanisms underlying neurotransmitter regulation of neurogenesis in the hippocampus.

GABA plays a different role in the SVZ. GABA released by newly generated neuroblasts acts to inhibit the proliferation of the GFAP positive neural stem cells in the SVZ. Consequentially, GABA in this case functions as paracrine signal that negatively regulates neurogenesis (93, 99).

An intriguing series of conceptually similar studies examined dopamine and its effects on SVZ neural stem cell proliferation and differentiation. Dopamine receptors are expressed by SVZ neural stem cells. Furthermore, it has been shown that agonist stimulation of the dopamine receptor increased proliferation in the SVZ, while dopamine depletion caused by deafferentation in a chemical lesion model of Parkinson's disease led to decreased SVZ neural stem cell proliferation (100-102). This agonist driven stimulation of proliferation has been reported by Van Kampen *et al* to coincide with an increase in dopaminergic cell counts and striatum integrity, which ultimately alleviated symptoms in a Parkinson's disease animal model (103).

Dopamine also appears to play an important role in the neuronal phenotypic differentiation of SVZ neural stem cells. Normally the majority of migratory neuroblasts from the SVZ differentiate into granule cells in the olfactory bulb granule cell layer, while a few cells migrate to the glomerular cell layer and differentiate into periglomerular interneurons. Most granule cells are GABAergic, whereas periglomerular interneurons can express GABAergic or dopaminergic markers. In fact, a single periglomeral interneuron can co-express the GABAergic marker GABA and the dopaminergic marker TH (104). Neurogenesis of granule cells and interneurons is differentially affected by 6-ODHA induced deafferentation in a Parkinson's disease animal model. There is a marked reduction in the number of new neurons generated in the granule cell layer, but there is a corresponding increase in the number of new neurons generated in the glomerular cell layer. The majority of the newly generated periglomerular interneurons also expressed the dopaminergic marker TH, which indicates that dopamine depletion caused by the lesion increased dopaminergic periglomerular generation in the olfactory while inhibiting the generation of GABAergic granule cells (105), perhaps serving a compensatory role for the loss of dopamine due to the lesion. It will be interesting to see if other neurotransmitters can regulate the phenotypic differentiation of adult neural stem cells *in vivo* and *in vitro*.

Precise control over the phenotypic differentiation of adult neural stem cells will rely on elucidating key regulatory signals in their native microenvironment. Such basic studies can then lead to defined media conditions that manipulate neural stem cell behavior *in vitro* towards a specific neuronal phenotype. Furthermore, this understanding can be applied directly to regulating neurogenesis *in vivo* by infusing factors, or via delivery of genes encoding factors, to either augment or counteract endogenous signals.

3.5.4. Genetic manipulation

At this time, the addition of exogenous signals typically leads to a mixed population of cell types *in vitro* and *in vivo*, and at times to nonspecific migration *in vivo* into regions where neurogenesis would have unclear functional consequences. Furthermore, to effectively use this approach *in vitro* or *in vivo*, the dose, order of addition, and time of exposure to factors are all important parameters that must be optimized. This is a difficult task since many of the signals involved in regulating neuronal differentiation are only now being elucidated. However, by introducing key genes to a cell, such as important transcription factors, one can potentially increase the homogeneity of the cell population's response and direct the neuronal differentiation of adult neural stem cells to specific phenotypes while avoiding some of the aforementioned problems. This approach also

decouples the response of the cell to a certain extent from its microenvironment, an important consideration when cells are reintroduced back into the body into conditions that may not support their survival or differentiation. In the following sections most examples are drawn from studies of adult neural stem cells, but several illustrative examples are based on embryonic derived neural stem cells to enhance discussion of genetic manipulations to regulate neural stem cell behavior.

Parkinson's disease is characterized by the deterioration of dopaminergic neurons, and promising stem cell based therapies consequentially involve the generation and replacement of damaged tissue with dopamine producing cells. As mentioned in section 3.3.1, the addition of retinoic acid to hippocampal neural stem cells *in vitro* leads to the production of a small population of cells expressing TH, an enzyme essential to the production of dopamine (70).

To increase the yield of dopaminergic neurons, Sakurada *et al* first examined the effects of Shh or FGF-8, chosen because they regulate the generation of midbrain dopaminergic neurons during development (106, 107). FGF-8 did not increase TH expression in adult hippocampal neural stem cell culture. In addition, while Shh did increase TH expression, it only had an effect when the cells were grown in its presence for 10 days. When Shh was added simultaneously with retinoic acid induction of cell differentiation, with no Shh preincubation, TH expression did not change. Therefore, while they have both proven to be neural stem cell mitogens (74), Shh and FGF-2 signals were apparently sufficiently different that the neural stem cells required a recovery period from growth in FGF-2 in order for Shh to exert its effects on TH expression (108). The mechanism for the differences of these two mitogenic factors, however, is not yet known. In an attempt to enhance TH expression beyond the modest Shh effect, Sakurada *et al* used a retrovirus to overexpress Nurr1, a transcription factor belonging to the nuclear receptor super family that is expressed in midbrain dopaminergic neurons. Forced Nurr1 expression did not affect proliferation, but it did induce TH expression in nearly all infected cells *in vitro*. However, infected cells did not express detectable levels of DOPA, the dopamine precursor whose production is catalyzed by TH, and functional production of DOPA was only detected when the cells were differentiated with retinoic acid. Even though the resulting population uniformly expressed TH, the frequency of neuronal markers within these TH positive cells was still low (108). These results implicate a need for additional manipulations to increase neuronal maturation and the functional production of dopamine.

Similar results were observed in neural stem cells derived from E13/E14 rat fetal brain tissue, in which Nurr1 overexpression led to an increase in TH expression (109). However, infected cells again did not mature, similar to observations for hippocampal derived neural stem cells. Subsequent work by Park *et al* addressed this shortcoming by using retroviral vectors that co-expressed Nurr1 and a second transcription factor to force neuronal differentiation. They examined the bHLH transcription factors Mash1, Ngn1, Ngn2 and NeuroD1, all known to induce neuronal differentiation (110), and they reported that the induction of TH expression depended on which bHLH transcription was co-expressed with Nurr1. Ngn1, Ngn2 and NeuroD1 decreased the Nurr1-induced expression of TH, whereas Mash1 overexpression not only maintained expression but also increased the fraction of cells that expressed the neuronal marker Map2ab. Importantly, they showed that the combined expression of Mash1 and Nurr1 yielded neurons with electrophysiological properties similar to those of mature dopaminergic neurons (111). Furthermore, Park *et al* showed that when grafted into Parkinsonian rats, cells co-expressing Nurr1 and Mash1 reversed behavioral deficits. Intriguingly, a retrovirus encoding Shh, the anti-apoptotic protein Bcl-xl, and Nurr1 produced functionally mature dopaminergic neurons similar to the Mash1 vector studies (112). These studies demonstrate the potency of genetic manipulation and highlight the fact that significant additional work will be needed to explore whether extracellular signal combinations, added *in vitro* to culture media or infused *in vivo*, can achieve similar results. Also, they demonstrate the need to select the correct, specific combinations of genes to achieve the desired, mature neuronal subtype differentiation.

A similar paradigm was seen when Zhang *et al* examined the potential of adult human olfactory neuroepithelium derived progenitors to generate motorneurons. They examined the effects of transfecting the bHLH transcription factors Olig2 and Ngn2, and the homeobox transcription factor HB9, in different combinations. Olig2 and Ngn2 expression in the same cell did not yield motorneurons under any conditions tested. Motorneuron marker positive cells were observed only when Olig2 and HB9 or Ngn2 and HB9 were co-expressed in cells grown in medium supplemented with Shh, retinoic acid, and forskolin. Also, cells expressing these transcription factors and grown with the essential supplements generated more motorneurons than the medium supplement controls alone, but under the optimal conditions only fifty percent of the cells expressed motorneuron markers, clearly indicating a need to refine this method of motorneuron generation. Finally and importantly, they showed that when these media supplemented, transcription factor expressing cells were co-cultured with chicken skeletal muscle, they generated contacts characteristic of neuromuscular junctions, indicative of a functional motorneuron (113).

Controlling the *in vivo* phenotypic differentiation of adult neural stem cells may require not only the appropriate genes and extracellular signals that select for a specific lineage as seen the previous examples, but also genetic manipulation to prevent cells from responding to the microenvironment into which they are engrafted. The injured spinal cord represents a hostile microenvironment for neuronal differentiation of either endogenous or engrafted adult neural stem cells. Ideally, a stem cell based therapy would be capable of not only preventing the further deterioration of neurons but also generating new neurons that can functionally replace those lost during the injury. Hence, approaches may need to be developed to overcome such non-permissive microenvironments.

Vroemen *et al* engrafted GFP-expressing spinal cord progenitors into an acutely injured rat. The cells migrated and aligned along axonal pathways; however, they differentiated into astrocytes and oligodendrocytes only. This contrasts with *in vitro* observations where these cells are capable of differentiating into neurons when FGF-2 is removed and serum is added to induce differentiation (114). This difference demonstrates that the cells are capable of neuronal differentiation but that the injured spinal cord either lacks the permissive signals or actively inhibits neuronal differentiation. These results are not unique to spinal cord progenitors. When transplanted into the spinal cord, SVZ neural stem cells derived from either post-mortem or live rats survive but differentiate into mainly astrocytes with no measurable neuronal marker expression (115, 116).

In a similar study Hofstetter *et al* noted that when spinal cord progenitors were engrafted into an impact-injured spinal cord, they preferential differentiated into astrocytes (74%) and oligodendrocytes (17%) with only a small percentage of neurons (4%). Furthermore, these animals unfortunately exhibited allodynia-like hypersensitivity to temperature in their forepaws. They attempted to correct this undesired cell lineage distribution by using a retroviral vector to overexpress Ngn2 (117). Based on previous reports of the function of this bHLH transcription factor (110), engrafted cells would be predicted to shift to a neuronal cell fate. The Ngn2 expressing engrafted cells indeed exhibited a reduction in astrocytic differentiation (3%) and an increase in both neuronal and oligodendrocytic differentiation (32 and 37% respectfully). The increase in oligodendrocytic differentiation was an unexpected and compelling finding. Importantly, with the forced expression of Ngn2 by the engrafted cells, these animals did not exhibit any extreme forepaw temperature hypersensitivity. Furthermore, it was determined that increased myelination was the predominant contributor to functional recovery, but the possibility that the newly generated neurons functionally integrated cannot be ruled out. The exact neuronal phenotype of these engrafted cells has yet to be analyzed (117).

As discussed in section 3.5.1, FGF-2 and EGF infusion inhibited the astrocytic differentiation and induced the neuronal differentiation of the endogenous population of proliferating cells in the spinal cord after injury (83). Expanding on these results, Ohori *et al* combined growth factor infusion with genetic manipulation of the endogenous cells to selectively induce neuronal differentiation. Retroviruses (which selectively infect dividing cells) expressing either Ngn2 or Mash1, in combination with the aforementioned growth factors, were infused. Ohori *et al* saw that Ngn2 expression combined with the growth factor supplementation elicited an increase in the number of mature neurons in the injured spinal cord compared to growth factor alone. By contrast, in this context Mash1 induced an increase in the number of cells that expressed oligodendrocytic markers (83).

Signals that inhibit neuronal differentiation in the injured spinal cord must be identified in order to understand the preferential glial differentiation of spinal cord engrafted adult neural stem cells. It is known that Notch and BMP-2 signaling increase in the spinal cord during injury (118, 119). The *in vitro* culture of spinal cord progenitors revealed that these signals inhibit neuronal differentiation and selectively increase the expression of astrocytic and oligodendrocytic markers (118, 119). Yamamoto demonstrated that inhibiting Notch activity through a dominant negative Delta ligand *in vitro* yielded an increase in neuronal marker expression and a decrease in astrocytic marker expression. However, even with Notch signaling attenuation, only a small fraction of neurons (<5%) was generated. To further increase this *in vitro* yield, Ngn2 was overexpressed without modulating Notch signaling, resulting in a greater than tenfold increase in the total number of neurons, while the fraction of the cell population that expressed oligodendrocytic and astrocytic markers remained unchanged (119). These results differ slightly from Hofstetter *et al* in that Yamamoto *et al* did not observe decreased astrocytic differentiation (117). An inhibition in astrocytic differentiation is consistent with other observations where Ngn1, closely related to Ngn2, inhibited astrocytic differentiation in embryonic neural stem cells (120).

BMP is another inhibitory signal found within the spinal cord. Setoguchi *et al* demonstrated that the overexpression of the BMP antagonist Noggin increased neuronal and oligodendrocytic differentiation of adult spinal cord progenitors in neurosphere culture. In addition, fetal tissue stem cells genetically manipulated to overexpress Noggin exhibited a modest increase in neuronal and oligodendrocytic differentiation, with a slight decrease in astrocytic differentiation, in a spinal cord injury model (118). It is possible that other non-permissive signals underlie the modest effects seen by Noggin overexpression, prompting the possibility of a combinatorial approach to enhance neuronal differentiation. For example, inhibiting Notch and BMP-2 signaling may synergistically enhance neuronal differentiation more than suppressing either alone.

Controlling the phenotypic differentiation of adult neural stem cells is a complex and challenging problem. Through comparing embryonic and adult neural tissue, key signaling molecules that regulate neural stem cell behavior can be identified. However, additional effort is required to elucidate the underlying mechanism of their actions, to understand how these signals may act in concert to regulate cell behavior, and to harness this basic information to control cell behavior for therapeutic efforts. For example, extracellular signals can be supplied to cells in culture, infused *in vivo*, or overexpressed *in vivo* following gene delivery. Alternatively, intracellular factors such as transcription factors can be supplied via gene delivery, often resulting in more uniform cell differentiation into a particular lineage. An added benefit to genetic modification is that one can design neural stem cells that are impervious to detrimental signals expressed in damaged tissue while also supplying them with the necessary programming to efficiently control their behavior.

4 ADULT AND EMBRYONIC STEM CELLS

Adult stem cells reside throughout the body in niches within individual tissues and organs, such as the brain, and typically exhibit stereotypic differentiation into a limited number of phenotypes. By contrast, the unique feature that distinguishes embryonic stem cells from other types of organ-specific stem cells identified to date is their ability to differentiate into any cell type in the adult body. Mouse ES cells injected into a host blastocyst yield a chimeric mouse in which the ES cells contribute to all adult tissue types (121-123). *In vitro*, ES cells have the capacity to differentiate into a broad range of cells types, including neural stem cells and mature neurons. In the last several years since the isolation of the first human ES cell lines (124), progress has been made in establishing protocols for culturing human ES cells and directing their differentiation into both neural stem cells and neuronal subtypes. Transplantation of these ES cell-derived cell types has led to recovery of symptoms in animal models of PD, spinal injury and retinal degeneration after cell transplantation. Advancing our understanding various of aspects of stem cell biology will help to accelerate the optimization of protocols needed for cell replacement therapy.

5. EMBRYONIC STEM CELLS

Embryonic stem cells (ES cells) are truly immortal stem cells with the unique ability for unlimited self-renewal and pluripotent differentiation into any cell type in the adult body. The latter property has been proven for mice and is assumed for human cells. ES cells are derived directly from the inner cell mass of blastocysts from preimplantation embryos and can yield cells from any of the three embryonic germ layers: ectoderm, endoderm, and mesoderm (figure 1). The potential of using human ES cells for treating disease, drug discovery screens, and basic models for human development has elicited significant attention. Furthermore, the possibility to generate "personalized" or "designer" stem cells via somatic cell nuclear transfer (SCNT), otherwise known as cloning, further extends their potential utility (125, 126). In SCNT, genomic DNA from a somatic cell is injected into an enucleated oocyte and further developed into a blastocyst, from which embryonic stem cells containing the exogenous genome are extracted. The generation of autologous stem cell lines promises to help overcome the problem of immune-rejection. In addition, such "designer" stem cells will allow the creation of human ES cell lines with mutations conferring a predisposition to a disease, which will serve as a valuable research tool for studying disease mechanism and progression.

While there are several advantages in using human ES (hES) cells, in particular for biomedical applications, ethical issues surround them. Human embryos are most often destroyed as the stem cells are harvested. As a result, federal regulations limit funding of research to the initial set of derived hES cells, strictly withholding support for the study and derivation of new stem cells lines. There are approximately 10-20 federally-approved or "registry" cell lines that are used for research based on their consistent expression of surface markers, transcription factors as well as the capacity to proliferate and maintain pluripotency without changes in karotype. A recent study by Klimanskaya *et al* created new stem lines by deriving human ES cells from single blastomeres (127), which are routinely "picked" for genetic diagnosis without destroying the embryo. Such approaches may help resolve the controversy over the use and derivation of new human ES cell lines.

5.1. Expanding and culturing embryonic stem cells

Since the first mouse ES cell line was derived in 1981 (128, 129), the properties and behavior of these cells have been extensively studied. Current protocols offer different ways of culturing ES cells to maintain their undifferentiated state, pluripotency, and genetic stability. Mouse ES cells are grown on mitotically inactivated feeders such as mouse embryonic fibroblasts (MEFs) or in media supplemented with leukemia inhibiting factor (LIF) without feeder cell support. Mouse ES cells are commonly grown on gelatin-coated plates in media supplemented with leukemia inhibiting factor (LIF). This is sufficient for maintaining their undifferentiated state (130).

In 1998, Thomson *et al* (1998) derived the first human ES cell lines from the inner cell mass of human blastocysts at approximately day 5 of development. They were grown on MEFs in media supplemented with fetal bovine serum. Because using MEFs and animal-derived products such as FBS present a risk of transmitting retroviruses and other pathogens, several protocols have been developed for culturing human ES cells in conditions that minimize exposure to animal-derived components. While MEFs are still used as a feeder layer for growing human ES cells, human-derived feeder layers have been successfully tested to replace MEFs. Human feeder layers are derived from several cell types such as fetal muscle and skin, adult fallopian epithelial cells, adult muscle cells (131), uterine endometrium (132), foreskin fibroblasts (133), and adult bone marrow cells (134). While feeder cells provide a robust method for growing ES cells, feeder-free systems also maintain their survival and undifferentiated state. Human ES cells have been grown on Matrigel coated plates with serum-replacement conditioned medium. Matrigel is composed of mouse sarcoma-derived extracellular matrix proteins and the conditioned medium consists of medium incubated with mouse or human feeder cells with a serum-replacer instead of FBS (135, 136). To further reduce the amounts of animal-derived components in culture, human serum (137), laminin (135), and fibronectin (138) have been used instead of Matrigel.

Another method for culturing human stem cells is using a feeder layer-free in a conditioned medium-free system. A combination of media supplements has been successfully identified and used for culturing undifferentiated human ES cells. These include: the combination of FGF-2, transforming growth factor-beta (TGF-beta), and LIF (138), activin A and FGF-2 (139), FGF-2 and TGF-beta (140), a high concentration of FGF-2 alone or at a reduced concentration in combination with the BMP antagonist Noggin (141, 142), and other combinations (143, 144). Richer chemically defined media, such as X-VIVO 10 which does not contain animal-derived products, have also been used (140, 145). Significant progress has thus been made toward

eliminating animal-derived components in human ES cell derivation and culture; however, completely animal and human protein-free culture system has not yet been established.

Both mouse and human ES cells grow in colonies, have high nucleus to cytoplasm volume ratios, and exhibit nucleoli at the single cell level. Several differences do exist between mouse and human ES cells in addition to their media requirements. For example, human ES cells grow slower with a doubling time of 24-48 h, compared to 15-20 hours for mouse ES cells. Mouse cells express cell surface antigens such as stage-specific antigen 1 (SSEA-1) and Forssman antigen, while human ES cells express ones not found on mouse ES cells such as SSEA-3, SSEA-4, tumor recognition antigens (TRA) TRA-1-60 and TRA-1-81, and GCTM-2. Despite such differences, protocols developed for mouse neural and neuronal differentiation have been adapted for differentiating human ES cells, as will be discussed in the following sections.

5.2. Embryoid body culture

Embryonic stem cells can be directed toward the neural lineage by first inducing them to form embryoid bodies (EBs). EBs are spontaneously generated when ES cells are cultured in suspension cultures without LIF or serum, in either non-adhesive dishes or hanging drops. The resulting EB cell aggregates form a tri-dimensional structure that, to a limited extent, simulates embryonic development *in vivo*. In particular, once cells start to aggregate, they begin to differentiate into a heterogenous population of progenitor cells that can form all cell types from the three germ layers, such as skeletal muscle, cardiac, hematopoietic and neuron-like cells. Neural progenitors are only a small fraction of the total cells in EBs. By using inductive stimuli during EB formation, a higher fraction of neural cells can be produced.

5.3. Inducing neural specification using media supplements and co-culturing with stromal cells

In the subsequent sections, methods of general neural induction will be discussed, followed by approaches for the generation of specific neuronal subtypes. Differentiation of ES cells into the neural and neuronal lineages in culture provides a basic tool for understanding the molecular and cellular mechanisms that control cell fate during development. And reciprocally, the characterization of proteins involved in developmental biological molecular programs has facilitated the engineering of stem cells toward a specific cell type.

Human ES cell-derived neural precursor and neuronal cell types are a potential source of cell-based therapies to treat conditions such as Parkinson's disease, spinal injury, and retinal degeneration. Significant progress has been made in developing efficient methods for directing neural, pan-neuronal, and neuronal-subtype specification from ES cells. Neural progenitors of ES cells are primarily defined by the expression of the intermediate filament marker nestin and transcription factors such as members of the Sox family ((146). It appears that most such Sox positive cells give rise to most cell types in the nervous system.

Numerous procedures have been developed to enrich neural precursors derived from mouse and human ES cells that can proliferate and differentiate into neurons, astrocytes, and oligodendrocytes. Mouse ES cells can be induced into neural progenitors by several methods, such as the use of retinoic acid (RA) treatment of EB (147, 148), a multistep-induction and selection culture (149), an adherent monoculture system in serum-free medium (150), and a co-culture with stromal cell types (151, 152). Human ES cells can be induced into the neural lineages using similar methodologies: through the formation of EBs in suspension (113), an adherent monoculture system (153), with co-culture (154) and through spontaneous differentiation of human ES cells (155, 156). The main disadvantage of these approaches is the nonspecific generation of cell populations derived from the three germ layers in the total cell population. Understanding the coordination and roles of intrinsic factors with extrinsic factors will be a critical step to channel or control ES cell differentiation and thereby enhance the derivation of neural precursors.

5.3.1. Retinoic acid

Retinoic acid is commonly used to direct neural specification. It has been well-established that retinoic acid has a role during in neural differentiation in embryos and later in development (157, 158). Bain *et al* were the first to show that retinoic acid treatment can induce neural differentiation in ES cells, i.e. a high proportion of the resulting cells expressed neuronal markers and had neuronal properties (147). In this work, EBs derived from mouse ES cells were cultured with retinoic acid for 4 days and then plated on laminin-coated dishes. The cells expressed neuronal markers beta-tubulin III and NF-M (M subunit of neurofilament) as well as neural-related genes such as transmitter synthesizing genes glutamic acid decarboxylase (GAD), TH, transmitter receptor subunits GluRs, and a cytoskeletal subunit, NF-L. This methodology is still commonly used, and in combination with other factors.

In a separate study, Okada *et al* tested the effects of different concentrations of retinoic acid on the neural differentiation of mouse ES cells (159). Lower retinoic acid levels (10^{-8} M) were found to induce neural progenitor cells from ES cells, indicated by the high protein expression of the neural precursor marker nestin and low expression of neuronal and glial markers beta-tubulin III and GFAP, respectively. In contrast, high levels of retinoic acid (2×10^{-6} M) reduced the expression of nestin while increasing beta-tubulin III and GFAP levels. Their findings are consistent with other studies demonstrating differentiation of neural progenitors at high RA concentrations (148, 158). Retinoic acid also directs neural differentiation in human ES cell derived-EB cultures (157). The addition of RA and nerve growth-beta (NGF) increased the proportion of neuronal cells that formed within human ES cell-derived EBs, as assessed by neural progenitor marker NF-L (neurofilament subunit L)

(160). While RA can serve as a neural and pan-neuronal differentiation signal, in subsequent sections it will be shown that combining RA with other exogenous molecules such as Shh can help direct ES cells into specific types of neurons.

5.3.2. Growth factors and morphogens in the induction of neural differentiation

Some molecular mechanisms known to direct neural differentiation of ES cells involve multiple classical pathways such as the activation of FGF and/or inhibition of BMPs and Wnts. The difficulty of directing stem cells into a homogenous population may be due to need to activate multiple parallel pathways to induce a specific cell response, some of which have yet to be identified. Numerous studies have analyzed the expression and roles of such developmentally important molecules in both mouse and human ES cells.

Recent studies have shown that Wnt signaling is involved in regulating the maintenance and differentiation of ES cells in culture (143, 161). Wnt has pro-neuronal activity in adult neural stem cells (76), yet the inhibition of Wnt proteins resulted in neural induction of ES cells. Aubert *et al* used a substractive cDNA hybridization screen to compare genes induced during the differentiation of mouse ES cells into embryoid bodies with and without retinoic acid (162). They found that overexpression of secreted frizzled related protein 2 (Sfrp2), an extracellular Wnt antagonist, promoted neural and neuronal differentiation in ES cells. The addition of Sfrp alone produced Sox 1 GFP+ cells at the same frequency (30-40%) as the addition of retinoic acid. Furthermore, in separate experiments, Wnt1 was overexpressed, and lithium ion (an inhibitor of the classical Wnt effector GSK3beta) was added to the culture. Both resulted in a significant reduction in the number of TuJ1 positive neurons, as well as in Sox-1 and Pax6 expression (162). Wnt inhibition through the overexpression of Sfrp enhanced neural induction. Conversely, Wnt activation through Wnt1 expression repressed neural induction of ES cells.

Recent work by Verani *et al* (163) corroborated these results investigating Wnt signal modulation by Dickkopf (Dkk)-1. Dkk-1 is a secreted glycoprotein that binds to Wnt co-receptors, causing them to be endocytosed and depleted from the plasma membrane. In this study, mouse ES cells were differentiated into neural cells, first through the formation of EBs and then by media supplemented with retinoic acid. Dkk-1 mRNA levels were found to be low in control cultures compared to high levels induced by retinoic acid exposure. This Dkk-1 upregulation correlated with reduced β-catenin levels in nuclear fractions, suggesting that Dkk-1 was associated with inhibition of the canonical Wnt pathway. Similar to the effects of retinoic acid, addition of exogenous Dkk-1 alone increased the expression of nestin and neural stem cell marker Dlx-2 (distal-less homeobox 2) in EBs. Dkk-1 knockdown, using antisense oligonucleotides or siRNAs targeting Dkk-1 mRNA, decreased the expression of Dlx-2 and beta-tubulin III in EBs exposed to retinoic acid. Results from the above studies suggest that in ES cells, inhibition of the Wnt pathway is involved in neural differentiation.

Evidence suggests that BMPs also negatively regulate the neural induction of ES cells. Exposure to BMP-4 and other TGFbeta-1 family members enhances the production of mesoderm during *in vitro* differentiation of mouse ES cells (164, 165). BMP-4 induces differentiation of human ES cells into trophoblast cells and to primitive endoderm (166). It has also been suggested that BMP-4 may induce the apoptotic death of neural precursors (167). BMP-2 also has a role in endoderm differentiation of mouse ES cells. The effect of BMPs in mouse ES cells may be mediated through the induction of Id gene expression, which in turn blocks neural lineage commitment while promoting differentiation into other fates (150). By antagonizing BMP-2 via adding soluble Noggin to human ES cells, neurospheres formed at a 10-fold greater frequency after 5 to 10 days after Noggin treatment, compared to those formed from spontaneous differentiation. It appears that Noggin can direct the differentiation of human ES cells towards neural progenitors. Since human ES cells were grown on a feeder layer in undefined culture conditions in the study, however, it is not entirely clear whether Noggin alone was responsible for the effect (168). Using a chemically defined serum-and feeder-free system, Itsykson *et al* demonstrated that Noggin promoted conversion of human ES cells into neural progenitors and inhibited differentiation into non-neural lineages (169). When BMP-4 was added, there was a reduction in neural progenitor differentiation, with the majority of the cells instead expressing cytokeratin-8 (Ck8), a non-specific epithelial marker. This effect was neutralized by the addition of Noggin. In further support of the role of Noggin, another study showed that its addition promoted formation of neural-tube like structures from human ES cells that were nestin- positive. When plated on Matrigel, these cells differentiated into neurons that stained for Map2 and beta-tubulin III after prolonged culture.

ES cells are commonly cultured in the presence of FGF-2, which is a known survival and proliferation factor for neural progenitors (37). Under the right conditions, FGF-2 has also been shown to induce the neural differentiation of human ES cells (153). In a recent study, Benzing *et al* devised a protocol to generate neural progenitors without formation of EBs or co-culture with stromal cells but instead in the presence of FGF-2. Human ES cells were transferred to DMEM/F12 medium supplemented with FGF-2 and subsequently plated onto Matrigel and propagated as an adherent culture. The colonies were then detached and cultivated in suspension culture as neurospheres. To induce neuronal differentiation, FGF-2 was removed from the culture. When the resulting cells were plated, they gave rise to neural progenitors that were able to differentiate into all three neural lineages.

Neural differentiation can also be induced by the overgrowth of undifferentiated human ES cells (155, 156). Reubinoff *et al* devised a protocol that maintained human ES cells in culture for 3-4 weeks without passage or replenishment of MEFs, which led to the spontaneous differentiation of human ES cells into neural progenitors (156). By manually transferring cells containing short processes presumptive of neural progenitors onto culture plates with defined medium supplemented with

FGF-2 and EGF, a highly enriched population of neural precursors was generated. Within these neurospheres, a high proportion of cells expressed neural markers nestin and NCAM, as well as the neuronal and glial marker A2B5. These neural precursors were able to differentiate into mature neurons, astrocytes, and oligodendrocytes. A similar method published concurrently induced neural differentiation through EB formation followed by culturing in the presence of FGF-2. Following EB formation, neural rosettes, structures that mimic neural tube formation *in vivo*, were generated. Rosettes were cultured in medium with FGF-2, harvested by selective dissociation and cultured as free-floating aggregates of neural precursors (155). These two methods generate and enrich expandable populations of neural progenitors from human ES cells.

5.3.3. Stromal cell-derived inducing activity (SDIA)

Neural differentiation can be achieved at a higher efficiency by co-culture with stromal cell lines such as PA6 (151) and MS5 stromal cells (152). The neural inducing activity of these stromal cells, abbreviated SDIA (stromal cell-derived inducing activity), has not been identified. SDIA is able to support the growth and differentiation of ES cells into neural precursors, as well as their development into region-specific neuronal subtypes. Kawasaki *et al* developed the method for neural lineage induction by co-culturing ES cells with stromal cells without the addition of retinoic acid or forming EBs (151). After screening several primary cell lines, PA6 stromal cells derived from mouse skull bone marrow were found to efficiently induce the neural differentiation of mouse ES cells. ES cells were co-cultured without serum on PA6 cells for 8 days in differentiation medium and for an additional 6 days in G-MEM supplemented with N2 and other components. Ninety-two percent of the colonies were NCAM, nestin, and beta-tubulin III positive by day 12 of co-culture and did not express mesodermal markers. Further evaluation of the cells revealed that a high proportion of them were dopaminergic neurons. Specifically, 92% of the colonies contained cells positive for tyrosine hydroxylase, a dopaminergic neuron marker. At the cellular level, 52% of cells were beta-tubulin III postive neurons, and 47% were nestin positive. Of the beta-tubulin positive neurons, 30% were TH positive neurons. GABAergic, cholinergic and serotonergic neurons were also present (approximately 18%, 9% and 2% respectively).

A more recent study by Kitajima *et al* devised a co-culture method for producing neurospheres using PA6 stromal cells (170). To induce neural spheres, ES cells were differentiated on PA6 stroma for 7 days, detached, dissociated, and cultured in growth medium with FGF-2 and EGF. The resulting neurospheres expressed multiple neural markers such as nestin, MAP2 and GFAP, indicative of a heterogenous population of neural progenitors, mature neurons, and glial cells. Co-culture with PA6 cells for 0-13 days progressively increased the number of spheres generated in a time-dependent manner until day 11. The resulting neurospheres could be further propagated when switched to a serum-free culture and then differentiated into all three neural types. By changing the time of co-culture with PA6 cells, it was also possible to induce different proportions of neuronal and glial precursor cells. This system thus enabled the production of large numbers of spheres without utilizing EB formation. Also, using MS5 instead of PA6 stromal cells efficiently induced neural differentiation of mouse ES cells, and the resulting cells were able to differentiate into more neuronal types (GABAergic, sertonergic, dopaminergic and cholinergic neurons).

Co-culturing with stromal cell types with the addition of exogenous molecules to media has become a common method for neural induction of ES cells. It is a relatively fast and reproducible method for the generation of neural precursors and neuronal subtypes. Due to the concern that animal-derived components could contaminate human ES cell cultures (171), an alternative approach was recently developed using human amniotic membrane (154). The system is referred to as amniotic membrane matrix-based ES cell differentiation (AMED) and involves the co-culture of matrix layers of human amniotic membrane with human ES cells for neural induction. Fifteen days of AMED culture produced a population of human ES-derived cells that were greater than 85% nestin positive, and many of these formed rosette-like clusters. While this method eliminates the introduction of animal-derived products, identification of the factors involved in the regulation of neural differentiation, and overcoming inherent limitations in the scale up of processes involving cell co-cultures, need to be further addressed.

5.4. Neuronal-subtype specification

Using some of the same strategies for neural specification, several methods have been devised to direct mouse and human ES cells into specific neuronal subtypes, some of which have led to symptomatic recovery in animal models of PD, spinal injury, and retinal degeneration following cell transplantation. Directed differentiation of ES cells toward a specific lineage can be achieved by addition of a combination of growth factors and/or their antagonists, co-culture with cell types capable of inducing specific lineages, and transfection of ES cells with transcription factors (using conventional DNA delivery, lentiviral and adenoviral vectors, and homologous recombination).

The loss of specific types of neurons underlies many neurological disorders. An approach to treating these diseases is to elucidate the inductive signals and transcription factors that can direct differentiation into specific types of neurons such as dopaminergic (DA) and motor neurons (MN), then to induce stem cell differentiation into the desired lineages for cell replacement therapies. The field of developmental biology has identified numerous factors involved in positional specification and neural patterning during neural development, and several such factors have been found to control analogous processes of stem cell differentiation into specific neuronal phenotypes in culture. The resulting neuronal subtypes are commonly characterized by the expression of transcription factors, secretion of neurotransmitters, and the presence of transporters and synthesizing and metabolizing enzymes. Furthermore, electrophysiological characterization has demonstrated that the ES-derived neurons are functionally active *in vitro*, with the potential for use in cell replacement therapy *in vivo*.

5.4.1 Dopaminergic specification

Mesencephalic dopamine neurons (DA), which degenerate in patients with Parkinson's disease, are derived from progenitors located in the midbrain. The induction of these progenitors involves Shh and FGF-8 signaling, which have been shown to promote ventral midbrain fates in neural plate explants (106, 172). Likewise, Shh and FGF-8 in combination with ascorbic acid increase the yield of mouse and human ES cell differentiation into TH positive neurons *in vitro*. Ascorbic acid has been previously implicated in promoting DA neuronal differentiation from primary CNS cultures (173). Mechanistically, transcription factors such as Nurr1, Pitx3, En and Lmx1b are involved in the maturation of post-mitotic DA neurons. DA neurons from ES cells have been further functionally characterized by quantifying DA release upon depolarization with potassium chloride using reverse HPLC, as well as by their response to the addition of neurotransmitters such as GABA and glutamate.

Dopaminergic neurons have been efficiently generated from mouse ES cells by using at least two different methods. One is a multiple step method that causes differentiation through formation of EBs, followed by the combined addition of Shh and FGF-8 and at a later stage ascorbic acid (149). Thirty-four percent of the resulting TuJ1 positive neurons derived from mouse ES cells were TH positive (149). Lau *et al* devised a method to generate DA neurons from mouse ES cells through EB formation, but in a shortened protocol time (174). To enhance the neuronal fate of the ES cells, EBs were grown in KO DMEM supplemented with EGF, FGF-2, and ascorbic acid. After 3 days, EBs were plated on gelatin-coated dishes and cultured with DMEM/F12 containing EGF, FGF-8, Shh, and ascorbic acid. Cells were subsequently cultured in Neurobasal medium with ascorbic acid. This method generated approximately 40% DA neurons, characterized by their expression of dopamine transporter DAT in 14 days.

Another commonly used approach is a single-step method that involves co-culturing with PA6 cells, which produced approximately 30% TH positive cells (151). The co-culture method offers the advantage of more rapid differentiation and a more homogenous population of cells compared to EB-based techniques. A more efficient means to generate DA neurons from mouse ES cells is to combine the co-culture method with the overexpression of Nurr1. Nurr1 has been shown to be involved in the specification of DA neurons from progenitors in the ventral mesencephalic region and from ES cells (109, 175). When mouse ES cells overexpressing Nurr1 were grown on PA6 stromal cells and cultured in media with Shh, FGF-8, and ascorbic acid, approximately 90% of beta-tubulin III positive neurons were TH positive. This fraction was significantly higher than with control cells without transduction of Nurr1 and cells transduced with Nurr1 but not treated with Shh, FGF-8, and ascorbic acid. Nurr1 overexpression in mouse ES cells grown on PA6 stromal cells with the addition of the signaling molecules synergistically enhanced the generation of TH positive cells. Furthermore, the resulting cells expressed the dopamine synthesis enzyme, AADC (aromatic amino acid decarboxylase) and dopamine transporter, but did not express GABA or 5-HT, suggesting that most of the cells were dopaminergic (176).

In addition to its role in directing differentiation towards a DA phenotype, Nurr1 synergizes with Pitx3 to promote terminal maturation of midbrain DA neurons from both mouse and human ES cells (177). Lentiviral vectors carrying Nurr1, Pitx3, Lmx1b, or En1 were introduced at the neural precursor stage after induction into EBs from mouse ES cells. The combined transduction of Nurr1 and Pitx3 dramatically induced the expression of the late marker DAT (dopamine transporter), but not TH. Only Nurr1 alone induced expression of TH. In a mouse ES cell line expressing EYFP under the regulation of a Cre recombinase gene "knocked-in" to the DAT locus (derived from double transgenic blastocysts harboring Cre recombinase gene "knocked-in" to the DAT locus with Cre-inducible EYFP "knocked-in" at the ROSA26 locus (178)), the co-transduction of Nurr1 and Pitx3 increased the number of EYFP positive cells. Nurr1 or Pit3x alone induced TH and aldehyde dehydrogenase-2- (an earlier marker of dopamineric cells) positive cells, but the addition of both induced the expression of late midbrain DA neuron markers such as DAT and tyrosinase-related protein 1(Tyrp). GABAergic and sertonergic markers were not expressed.

In a recent effort to identify homeodomain proteins involved specifically in DA neuron cell fate, Andersson *et al* (179) used a cDNA library derived from mouse embryos as a template to screen for homeodomain encoding transcripts by PCR, as well as a large-scale *in situ* hybridization screen for CNS genes. Lmx1a was one of two transcription factors discovered. To determine whether Lmx1a can direct ES cells into DA neurons, mouse ES cells were transfected with a Lmx1a - EGFP fusion construct driven by a nestin enhancer. These cells were grown in media supplemented with FGF-2, FGF-8, and Shh. Only after 8 days in culture, cells transfected with Lmxla generated approximately 65% beta-tubulin III positive neurons that co-expressed TH. In addition, greater than 95% of Lmx1a-induced TH positive neurons expressed genes specific to midbrain DA neurons: Nurr1, Pitx3, En1. Lmx1a is required for the generation of DA neurons in chick embryo explants and can be used to engineer DA neurons from mouse ES cells. By using a combination of extrinsic and intrinsic factors, well developed DA neurons could thus be derived from ES cells, which may lead to a greater success in transplantation studies.

The two predominant methods developed on mouse cells, transfection of mouse ES cells with specific factors such as Nurr-1 and Lmx1a as well as co-cultures with stromal cells, have been translated to human ES cells to direct DA neuron differentiation. Similar results were found after human ES cells were transduced with lentiviral vectors carrying both Nurr1 and Pit3x at the neural precursor stage as well as co-cultured with stromal cells (177). Nurr1 and Pit3x together promoted the maturation of midbrain DA neurons and led to an increase in TH positive cells. In electrophysiological analysis, differentiated human ES cells displayed basic neuronal characteristics such as action potentials, burst firing, and miniature spontaneous excitatory postsynaptic currents. Transplantation of these cells into a mouse model of PD improved some motor ability 6 weeks

post-transplantation; however, there was limited maturation of the engrafted human ES-derived cells, as assessed by low expression of TH.

Human ES cells co-cultured with PA6 stromal cells generate a high frequency of DA neurons. After 3 weeks of culture with PA6s, 87% of the resulting colonies were TH positive. Using RT-PCR, it was found that numerous dopaminergic markers were expressed, such as the enzymes TH, DAT, AADC, and the transcription factors Ptx3, Lmx1b, and Nurr1. The total cell population also included other non-dopaminergic neurons subtypes expressing markers of cholinergic and glutaminergic neurons. When transplanted into the striatum of a rat model of PD (induced by injection of neurotoxin, 6-hydroxydopamine 6-OHDA, which selectively kills dopaminergic and noradrenergic neurons), some of the cells remained TH positive. However, only a small number of the transplanted cells survived, and some differentiated into non-neural cells that stained positive for smooth muscle actin marker (SMA) (180).

Co-culturing human ES cells with immortalized human midbrain astrocytes derived from developing ventral midbrain has also been used as a method to induce DA differentiation (181). Human ES cell lines were induced to form EBs and then differentiated in the presence of FGF-2, Shh, and FGF-8. Subsequent culture in BDNF, GDNF, and 0.5% FBS resulted in TH positive cells. However, when neural progenitors were cultured in the presence of FGF-2, Shh, and FGF-8 followed by co-culture with midbrain astrocytes, the proportion of TH positive cells increased from 11.8% to 39.6%. Furthermore, when the human ES cells were co-cultured with midbrain astrocytes from neural induction through differentiation, the percentage increased to 67.4%. Unidentified factors derived from midbrain astrocytes thus potentiated DA neuron differentiation. Small percentages (0.7%-4.1%) of serotonergic and GABAergic cells were also present. Most of the TH positive neurons generated using this method were positive for GIRK2 (G protein-coupled inwardly rectifying potassium channel), a marker specific for midbrain dopaminergic neurons. These DA cells were functional *in vitro,* as assessed by electrophysical studies, and expressed DAT. Six weeks after engraftment of these cells into a rat model of PA (6-OHDA), rats exhibited substantial motor improvement.

Human ES cells can also be differentiated into DA neurons by the use of Shh and FGF-8 under more defined conditions. Yan *et al* established a chemically defined system for directing human ES cells to neuroepithelial cells in a manner that emulates human neuroectodermal development in timing and in the formation of neural tube-like structures (182). Early exposure to FGF-8, before precursors become SOX1 expressing neuroepithelial cells, is necessary for midbrain DA neuronal differentiation *in vitro*. Human ES cells were grown in suspension and subsequently plated on adhesive culture dishes in chemically defined neural medium supplemented with FGF-2. FGF-8 was added to promote midbrain specification, later followed by the combination of FGF-8 with Shh. Early exposure to FGF-8 induced the expression of Wnt1 as well as midbrain-related transcription factors En1 and Pax2, and greater than 95% of the neural precursors expressed nestin. After three weeks of differentiation, approximately 31.8% of cells were TH positive, all TH positive neurons expressed beta-tubulin III and AADC, and most expressed VMAT2 (a protein involved in packaging DA). By using the combination of FGF-8 and Shh, human ES cells could thus be directed to the ventral midbrain fate to generate DA neurons. This chemically defined system for human ES derivation of DA neurons may be advantageous compared to the co-culturing methods, as it reduces exposure to animal-derived components and allows for more facile determination of factors that influence cell fate.

The protocols mentioned in this section successfully enhanced DA neuron generation and increased population homogeneity by using co-culturing and/or overexpression of transcription factors Nurr1, Pitx3, Lmxla. Addition of exogenous molecules such as Shh and FGF-8 also instructed DA differentiation. However, the stromal-derived factors (SDIA) that direct differentiation of ES cells into neural lineages and towards DA neurons must still be identified, which will both benefit basic knowledge of differentiation mechanisms and enhance the development of defined conditions to guide neuronal differentiation.

5.4.2. Motor neuron specification

By using extracellular signals and transcription factors that have been implicated in the development of the spinal cord, MNs have been derived from mouse and human ES cells. Wichterle *et al* were the first to show that retinoic acid and Shh (or a Shh agonist), which are known to be involved in the establishment of the caudal and ventral positional identity, respectively, can direct mouse ES cells into spinal progenitors to functional MNs (148). In this work, mouse ES cells were initially cultured into EBs in media supplemented with RA. The resulting neural progenitors expressed Hoxc5 and Hoxc6 (markers of spinal cord cells with rostral cervical character) and lacked markers of early midbrain identity. These ES-derived cells with initial midbrain-like character were subsequently caudalized by the addition of RA to yield spinal progenitors. The combination of Shh agonist with RA induced MN differentiation. The percentage of MNs expressing HB9, a homeodomain protein and MN marker, increased with higher Shh agonist concentrations. Twenty to 30% of cells in EBs were HB9 positive and expressed the neuronal markers NeuN and beta-tubulin III, as well as the MN markers Lsl1 and Lhx3. To identify and isolate a pure population of ES cell-derived MNs, mouse ES cells were derived from transgenic mice that express GFP under the HB9 promoter. Twenty to 30% of cells from EBs grown in media containing RA and Shh agonist were GFP and HB9 positive. When transplanted into the spinal cord of chick embryos, the differentiated cells were found in ventral regions of the spinal cord, and axons appeared to form synapses with target skeletal muscles. While the overall MN differentiation efficiency of this method was relatively low, this important work established the concept that knowledge of developmental neurobiology (183) could be applied to direct ES cells into MNs *in vitro.*

To obtain a more pure population of MNs from ES cells, Ikeda *et al* generated MNs by transfecting mouse ES cells with Mash1, a proneural transcription factor involved in the differentiation of numerous neuronal populations (184). Transfected cells expressed the neural progenitor marker nestin and the neuronal markers NeuroD, NFM, and beta-tubulin III, as well as the motor neuron markers Islet1 and HB9. Immunochemical staining showed that 50% of Mash1-transfected cells were positive for Islet1, though HB9 expression was not examined. The Mash1-transfected cell population was heterogenous and contained cells at various stages of differentiation. To test whether they could promote behavioral recovery, these cells were transplanted into a mouse hemiplegic model (developed by lesion of the motor cortex) and promoted partial behavioral recovery 28 days post-transplantation.

These approaches developed in mouse ES cells have been applied to human ES cells with success. RA addition before cells express SOX1 (i.e. prior to neuroepithelial fate), and subsequent addition of Shh agonist, efficiently induced caudalization and ventralization, respectively, to direct human ES cells into MNs (185). This earlier addition of RA was important for caudalization of the cells for efficient induction of ES cells to MNs. Based on immunochemical analyses, cells were beta-tubulin III, Map2, and Islet1/2 positive, and 50% of the Islet1/2 positive cells also stained for HB9. Cells were treated with RA, then Shh, and were subsequently propagated in brain derived neurotrophic factor (BDNF), glial cell line neurotrophic factor (GDNF), and insulin growth factor (IGF) to result in neuronal maturation. These factors have previously been shown to enhance motor neuron differentiation and survival of cells from embryonic rat ventral mesencephalon neuron cultures (186). The human ES cell derived MNs expressed choline acetyltransferase (an enzyme involved in the synthesis of acetylcholine), vesicular acetylcholine (involved in packaging acetylcholine into presynaptic vesicles), and beta-tubulin III. These MNs were functionally active *in vitro*, as assessed by electrophysiological analyses, as well as the development of synapses and functional neuromuscular transmission when co-cultured with myotubes (185). Using a similar protocol to the one developed by Li *et al* (185), human ES cells were transfected with a HB9-driven GFP vector and selected via fluorescence activated cell sorting to obtain a more homogenous population of ES-derived MNs (187).

In summary, MNs can be generated from both mouse and human ES cells using a combination of RA and Shh. ES cell-derived MNs were electrophysiologically functional *in vitro* and could restore partial motor function when engrafted into animals with spinal cord injury (184). These differentiation protocols, however, were still inefficient at yielding a large numbers of MNs.

5.4.3. Other neuronal subtypes

While there has been a strong focus on developing methods to generate dopaminergic and motor neurons, numerous studies have also been directed towards glutamatergic, serotoninergic, peripheral sensory and sympathetic neurons, and retinal precursors. These efforts may enable drug discovery efforts or direct treatment for various diseases such as schizophrenia, peripheral neuropathies, and retinal degeneration.

In one recent study, murine ES cells were differentiated into glutamatergic neurons (188). The ES cells were first cultured as EBs, which were then grown in the presence of RA and serum. After the EBs were dissociated and plated in N2 medium, cells were subsequently grown in differentiation media (DMEM with additional supplements) to generate precursors that stained for nestin, Pax-6, and radial glial cells markers RC2 and brain lipid binding protein (BLBP). After 4 days of differentiation, 85% of the cells expressed beta-tubulin III. Following seven days of differentiation, 93% cells were positive for glutamate vesicular transporter 1 (vGlut 1). The cells formed glutamatergic synapses, and their electrophysical activity was increased with the addition of glutamate. Importantly, less than 1% of the cells were positive for other neurotransmitters. With additional time, the neurons expressed vesicular GABA transporter (vGAT), indicative of mature GABAergic differentiation. These cells were only 5% of the population, however, and 1-2% of the population expressed glia marker GFAP. Importantly, this protocol produced a fairly homogenous population of neuronal precursor cells that generated neurons with a glutamateric phenotype.

Serotoninergic neurons can also be generated from mouse ES cells. To optimize neuronal differentiation, mouse ES cells were first dissociated into single cells prior to culture as EBs. HEPES was removed from media during neural progenitor expansion and neuronal differentiation, as it was previously found to inhibit differentiation of TH positive neurons (189). After 4 days in suspension cultures as EBs, cells were plated and grown in serum-free ITSF medium (DMEM with insulin, transferrin, selenium, and fibronectin), which selects for growth of nestin positive cells (189). Nestin positive cells were then grown in N2 medium with FGF-2. When this medium was supplemented with either the combination of FGF-8 and Shh or Shh alone, approximately 11% of TuJ1 positive neurons were identified as serotoninergic neurons (149).

By modifying the co-culturing method with PA6 stromal cells, Pomp *et al* generated peripheral neurons and neural crest cells from hES cells (190). Cells were co-cultured with PA6 stromal cells without the addition of exogenous factors. After 4 weeks in culture, 51% of the colonies were TuJ1 positive, and 34% were positive for both TuJ1 and peripherin (a marker of peripheral neurons), indicative of peripheral sensory neurons. Some colonies also expressed TH and peripherin, suggesting that these cells were sympathetic neurons. In a separate experiment, cells co-cultured with stromal cells for 4 weeks expressed peripheral sensory neuron markers peripherin, transcription factor Brn3a (brain specific homeobox/POU domain protein 3A), and genes characteristic of peripheral sensory neurons. Because peripheral sensory and sympathetic neurons develop from the neural

crest, neural crest markers were investigated and also found to be expressed and increased early in co-culture. This study emphasizes the need to completely characterize cell phenotypes that arise from different differentiation conditions. In this case, PA6 stromal cell co-culture, commonly used for dopaminergic differentiation of ES cells, can also give rise to numerous other types of neurons.

Retinal cells have also been generated and tested *in vivo*. Retinal pigment epithelial cells have been identified in cell populations after ES cells were co-cultured with PA6 stromal cells (151). Using a modified culture system, Ikeda *et al* (191) generated neural retinal precursors from mouse ES cells at a higher efficiency than co-culture with stromal cells (which produced fewer than 3% of colonies that were positive for Rx and Pax6, transcription factors that are markers of neural retinal progenitors (151, 192)). To achieve the higher efficiency, mouse embryonic stem cells were grown as serum-free floating culture to form embryoid body-like aggregates (SFEB). The ES cell aggregates were then plated and treated with leftyA (left-right determination factor 2) and activin (members of the transforming growth factor-beta superfamily), Dkk1, and serum. This combination preferentially induced retinal progenitor differentiation, such that 6.4% of cells of the culture were Rx and Pax6 positive. Six3, a transcription factor that is expressed in developing retina, was also expressed in the cultures. To further characterize the differentiated cells, Rx positive cells were co-stained for the expression of Otx2, a homoedomain protein involved in early and late stages of eye and retinal development (193). Approximately 96% of Rx positive cells also expressed Otx2. To test whether this culture system could generate photoreceptors, cells were cultured for a longer duration and then stained for markers. The early photoreceptor precursor marker Crx was present in a small number of cells that also stained with Otx2. Five days later, some of the colonies expressed late photoreceptor marker rhodopsin, but these represented fewer than 5% of the total number of cells. To determine the cause for the low efficiency observed, the competence of the progenitors was tested. Cells were either infected with a lentivirus to overexpress Crx or co-cultured with mouse retinal cells. Both methods increased the number of cells expressing rhodopsin. Overexpression of Crx induced 21% of infected cells to express rhopdopsin, while 36% of the cells in co-cultured aggregates expressed both photoreceptor markers rhodopsin and recoverin. This work demonstrates that defined medium conditions can generate retinal precursors that have the capacity to differentiate into photoreceptor cells. With the identification of inductive factors presented in co-cultures, this method could further improve the cell differentiation efficiency.

5.5. Therapeutic potential of embryonic stem cells in animal models

In vitro, ES cells can be differentiated into neurons that are electrophysiological active, exhibiting voltage-gated currents, expression of neurotransmitters, and formation of synaptic contacts. But whether ES-cell derived neurons can functionally incorporate into brain regions, differentiate into the desired cell type and survive for an extended period of time is still being investigated. In addition, formation of teratomas is still a concern in transplantation studies (194, 195). The following section reviews studies in which the engraftment of cells derived from mouse and human ES cells shows promise for potential cell replacement therapy, particularly when their presence is correlated with symptomatic improvements in animal models of Parkinson's disease, spinal cord or motor cortex injury, and retinal degeneration.

5.5.1. ES cell-derived dopaminergic cells

The differentiation and subsequent engraftment of mouse cells have shown promise in improving behavorial deficits in animal Parkinson's disease (PD) model (109, 194, 196). Recent studies have tested whether human ES cell-derived neural precursors (197) or DA neurons (195) can effectively treat animal models of PA.

Ben-Hur *et al* differentiated human ES cells via the addition of Noggin (197). These cells were then cultured as neurospheres in suspension using the protocol of Reubinoff *et al* (156). Greater than 90% of the cells expressed neural markers PSA-NCAM and nestin, as well as NCAM and A2B5. These neurospheres also had the potential to differentiate into DA neurons after replating in media with mitogens to allow spontaneous differentiation. Genes involved in midbrain development and DA markers were detected by RT-PCR. Furthermore, immunocytochemical studies showed that 0.56% of the cells were beta-tubulin III and TH positive. 400,000 of these human ES cell-derived neural progenitors were injected into a rat model of Parkinson's disease, followed by evaluation of cell differentiation, survival, and function. Twelve weeks post-transplantation, the average number of human cells detected using human-specific anti-mitochondria and anti-RNP (cytoplasmic ribonuclear protein complex) antibodies was 73.5% of the initial number of cells injected. It was unclear whether the counts were elevated by the proliferation of the transplanted cells. Within the grafts, there was a heterogenous distribution of cells that expressed nestin, mushashi 1, NCAM, the astroglial progenitor marker CD44, GFAP, NF, and NeuN. There was a small fraction of TH positive cells (0.18% of the total number of cells in the graft) detected. Some of these cells expressed DAT and transcripts of midbrain and dopaminergic neurons. Only 0.2% of the cells expressed proliferating cell nuclear antigen (PCNA), and teratomas were not detected. Twelve weeks after transplantation, improvements in rotational behavior and stepping and placing movements were observed. Importantly, the degree of behavioral improvement assessed by amphetamine-induced rotational behavior correlated with the number of TH positive neurons. This study showed that neurospheres derived from human ES cells have the potential to improve behavioral symptoms in PA models despite the low number of DA neurons. Differentiation of an even larger number of TH positive cells during engraftment may provide greater behavioral improvement.

Results from a recent study, however, highlight the need for caution in using human ES cell-derived DA neurons for the treatment of PA (195). In this work, human ES cells were differentiated into DA neurons using a PA6 stromal cell co-culture method. ES cells were differentiated for 16, 20, or 23 days, dissociated into single cell suspensions, and injected into the right

striatum of a rat model of PD (6-OHDA). The percentage of beta-tubulin III and TH positive cells in suspensions prepared for transplantation varied depending upon the duration of differentiation. After 23 days of differentiation, the co-expression of beta-tubulin III and TH positive cells was greatest, representing approximately 37% of all cells. At 2 weeks after transplantation, all grafts contained cells that co-expressed BrdU and H-nuc (human nuclear antigen). Co-localization of the two markers was greatest in cells differentiated for 16 days prior to implantation (30% of H-nuc positive cells). Importantly, detection of BrdU uptake suggests that many of the implanted cells proliferated. At 13 weeks post-transplantation, the latest time point analyzed, only 2% of the cells (median value from cells pre-differentiated for 20 and 23 days) co-expressed the two markers. This may correlate with the observation that teratomas were absent in animals engrafted with cells pre-differentiated for 23 days. However, teratomas were found in all animals injected with cells pre-differentiated for 16 days. In addition, neuronal differentiation was limited. Only 10 to 50 TH positive cells were found in grafts in all samples. Not surprisingly, treated animals did not exhibit behavioral improvement, assessed by amphetamine-induced rotation test.

Zeng *et al* performed a similar study in which they pre-differentiated human ES cells with PA6 stromal cells for 8 and 22 days prior to transplantation into the same rat model of PD (6-OHDA) (180). They observed a higher percentage of TH positive cells (3.8 to 8.8%) 5 weeks after post-transplantation. In the graft sites, however, they also identified a significant number of non-neural cells expressing smooth muscle actin (SMA). The results from these studies strongly support the need for additional optimization and improvement in cell selection prior to transplantation, as well as methods to direct differentiation *in vitro* to provide functional cells that can integrate into the host nervous system. Furthermore, improving the survival of differentiated cells and eliminating undifferentiated cells to prevent teratomas are significant concerns.

5.5.2. ES cell-derived motor neurons

There has been success in using ES cell-derived MNs to enhance recovery from spinal cord injury and motor neuron disease models (184, 198-201). Behavior improvements following cell transplantation, however, have not been directly correlated with the formation of neuromusclular junctions between transplanted cells and host muscle. The latter are challenging in part due to the well-known the inhibitory effects of myelin on axon growth. In an important recent study by Deshpande *et al* (201), sixty-thousand mouse ES cells transduced with a HB9-GFP reporter were transplanted into the ventral gray matter of the lumbar spinal cord in adult rats that were paralyzed. Three and six months after grafting, transplanted cells expressed HB9 and appeared morphologically similar to the host motor neurons. The cells also expressed the presynaptic marker synaptophysin. Approximately 17-20% of the engrafted cells survived, and 35-39% of those cells were motor neurons. GDNF, which has been shown to provide trophic and tropic support for MNs, appeared to act as an attractive cue for ES cell-derived motor axons. In addition, dcAMP (dibutyryl cyclin adenosine monophosphate), which is known to inhibit myelin, and rolipram, a phosphodiesterase 4 inhibitor that helps to facilitate neuromuscular junctions, aided the formation of neuromuscular junctions between transplanted cells and host cells and thereby subsequently improved physiological and behavioral recovery. Specifically, the combined administration of the phosphodiesterase type 4 inhibitor and GDNF to animals, in concert with engraftment of mouse ES cell-derived MNs preincubated with dbCAMP, enhanced grafted cell survival, integratation, and neuromuscular junction formation with target skeletal muscle. The study did not report formation of teratomas, though there was evidence that other cell types were derived from the transplanted cells. Results from this important study suggest that administration of factors that influence host cellular function *in vivo* may be needed for the integration of ES cell-derived MNs.

Ikeda *et al* was previously discussed in reference to the differentiation of MN neurons from mouse ES cells transfected with a Mash1-GFP reporter construct (184). The resulting cells were transplanted underneath the injured motor cortex of a mouse hemiplegic model where lesions were localized in the motor cortex of the left hemisphere. Cells were characterized 28 days post-grafting. Transplanted cells were observed in the injured motor cortex, as detected by the expression of a histidine protein tag (which was incorporated in the Mash1-GFP construct) and GFP. The expression of beta-tubulin III, NFM, and motor neuron marker Islet 1 were also observed in the injured motor cortex. Because some of the transplanted cells located near host cells were also positive for synaptophysin, it is possible that the new cells formed synapses. When treated hemiplegic mice were anayzed in motor function tests, there was significant behavioral recovery; this effect was observed after 10 days but was greatest 17 days post-transplantation. In a recent study, ES cells transfected with Mash1 were transplanted into a mouse model of spinal cord injury, characterized by complete transection of the spinal cord causing complete paralysis of the tail and hind limbs (202). Transplanted cells expressed the MN specific markers Islet1 and Lim1/2 4 weeks post-transplantation. Cells were also NFM positive, and synaptophysin was expressed in a part of the cells. There were slight behavorial improvements observed 1 week post-transplantation, but at 4 weeks, more than 50% of the mice were able to coordinate their hind limbs with their fore limbs, and in a vertical grid test some of the mice were able to grasp the grid with their hind limbs. This study supports a role for Mash1 in the development of MNs from ES cells that can improve motor deficits *in vivo*. Mash1-transfected ES cells also reduced gliogenesis in the mice with spinal cord injury, possibly by inhibiting the expression of myelin-derived proteins, such as Nogos, which limit axonal regeneration. Also, the Mash1-transfected ES cells themselves did not express Nogo receptors, which may present an advantage in using them for cell replacement therapy.

5.5.3. ES cell-derived retinal progenitors

Retinal engraftment of ES-derived cells has also been tested for the restoration of retinal function. It has been shown that grafted ES cell-derived neural progenitors survive, differentiate into mature neurons, and can develop limited connectivity with the host retina (203). Recently, Banin *et al* derived neural precursors from human ES cells that expressed eGFP under the

human elongation factor -α promoter (204). After culture on MEFs, precursors were mechanically isolated and replated in serum-free media with FGF-2 and EGF for 4 weeks. In addition to expressing neural precursor markers such as PSA-NCAM and nestin, the cell population expressed Chx 10 and CRX (cone-rod homeobox protein), a retinal progenitor and photoreceptor marker, respectively. The neural precursors also expressed retinal progenitor markers Six6, Six3, Pax6, Rx, Lhx2, Chx10 and photoreceptor markers CRX, recoverin, Nr1 (N-methyl-D-aspartate receptor), and blue opsin. Single-cell suspensions of neural progenitors were engrafted into the subretinal and/or vitreal space of adult and neonatal rats. Staining with anti-GFP, anti-human mitochondria and anti-human Ki67 antibodies identified transplanted cells in 42% of adult and 44% of neonatal eyes. No teratomas were observed. In addition to differentiating into cells expressing NF70 and beta-tubulin III, neural progenitors yielded some GFAP positive cells. In addition, in 7 out of 11 sub-retinal grafts, transplanted cells expressed photoreceptor markers NRL, blue cone opsin, and rhodopsin, but these markers were not present in the intra-vitreal or inner retinal grafts. Apparently, only the subretinal compartment provided signals and cues that promoted photoreceptor differentiation. This study showed that when transplanted into the subretinal space of rat eyes, human ES cell-derived neural precursors can differentiate into retinal cells, although the functional connectivity of these cells was not tested. Human ES cells have the potential to replace degenerating retinal and retinal pigment epithelial cells. Identification of retinal factors that regulate the migration of transplanted cells and play a role in cellular differentiation is important.

These collective results on implantation of ES-derived cells in animal models highlight the significant promise of this approach, as well as the need for substantial additional studies. For example, comparative analysis of engraftment studies is difficult given the large number of variables between studies, such as differences in media and culture conditions, animal disease models, markers and methods for detection of engrafted cells, and differences in the time after transplantation that analysis is conducted. Progress in functional cellular integration of ES-derived cells, leading to and tissue recovery, will rely on understanding the relative contribution of exogenous and endogenous signals in directing cell fate. This knowledge must then be applied to develop robust differentiation protocols in fully defined procedures that will be safe for transplantation. With further advances in understanding stem cell biology, human ES cells will be a renewable source for cell replacement therapy.

6 CONCLUSION

This collective work demonstrates that both adult neural stem cells and embryonic stem cells have the capacity to differentiate into numerous, valuable cell types of the adult nervous system both *in vitro* and *in vivo*, as demonstrated by marker expression, electrophysiological, and behavioral data. Despite these major advances, however, in most cases the differentiation into a target cell type was of limited efficiency. Therefore, it is clear that significant further investigation is necessary to identify crucial signals, or likely combinations of signals, that specify cell fate. However, as is already evident, the roles of signals are highly context-dependent, and the same signals can specify distinct responses between adult and embryonic cells, and even between different types of embryonic cells, and these efforts will thus benefit from combinatorial approaches (205). The identification of such signals will also benefit from the elucidation of differences between neurogenic and non-neurogenic microenvironments *in vivo* (206). In addition, significant progress has been made in identifying soluble signals, but with few exceptions (207) the role of extracellular matrix in the stem cell niche remains to be explored.

There are numerous basic biological questions that remain. In the area of adult neurogenesis, it is still unclear whether different cell lineages *in vivo* arise from a common stem cell or separate unipotent precursors. A related question is whether growth factors support the self-renewal of the stem cell *in vitro*, or whether alternatively they convert progenitors into stem cells (18). Likewise, cells with stem properties have been isolated and cultured from essentially every region of the central nervous system, but do these correspond to a quiescent stem cell population *in vivo*, or do they result from culture in growth factors? Also, what role do new neurons serve in the adult brain? In addition, both adult and embryonic stem cell cultures can be readily manipulated, including genetically, and they will increasingly serve as a valuable system for the studying basic cell and developmental biology. For example, studying the roles of epigenetics and RNA biology in stem cell behavior will benefit each of these fields (208). In addition, human embryonic stem cells will greatly enable studies of human genetics.

A number of challenges also remain in the biomedical application of stem cells. It is unclear at this early point whether adult or embryonic cells will be more suitable for specific applications. Also, the benefits of cell implantation vs. manipulation of endogenous progenitors should be explored (62). In addition, in animal models that exhibit behavioral improvement upon cell implantation, future work will determine whether the benefit is derived from functional integration of new neurons vs. secretion of protective factors or other protective roles by the implanted cells. Furthermore, while stem cells have long term direct therapeutic potential, the fields of pharmacology, toxicology, and drug discovery will benefit from them in the short term (209).

Basic studies of stem cell control will aid in the development of synthetic microenvironments (210) that present a repertoire of regulatory signals to the cells and thereby potentially control cell function very precisely. Besides providing scaleable systems for culturing stem cells for numerous applications, these synthetic platforms also offer the potential to systematically dissect components of the microenvironment, which will benefit basic knowledge of cell behavioral control.

In conclusion, while the roots of the neural stem cell field date back to over four decades ago (2), it is a relatively young field. The current pace of activity promises to yield significant advances in the ability to precisely and efficiently control function, which will enable applications in regenerative medicine, pharmacology and toxicology, and drug discovery

7. ACKNOWLEDGEMENT

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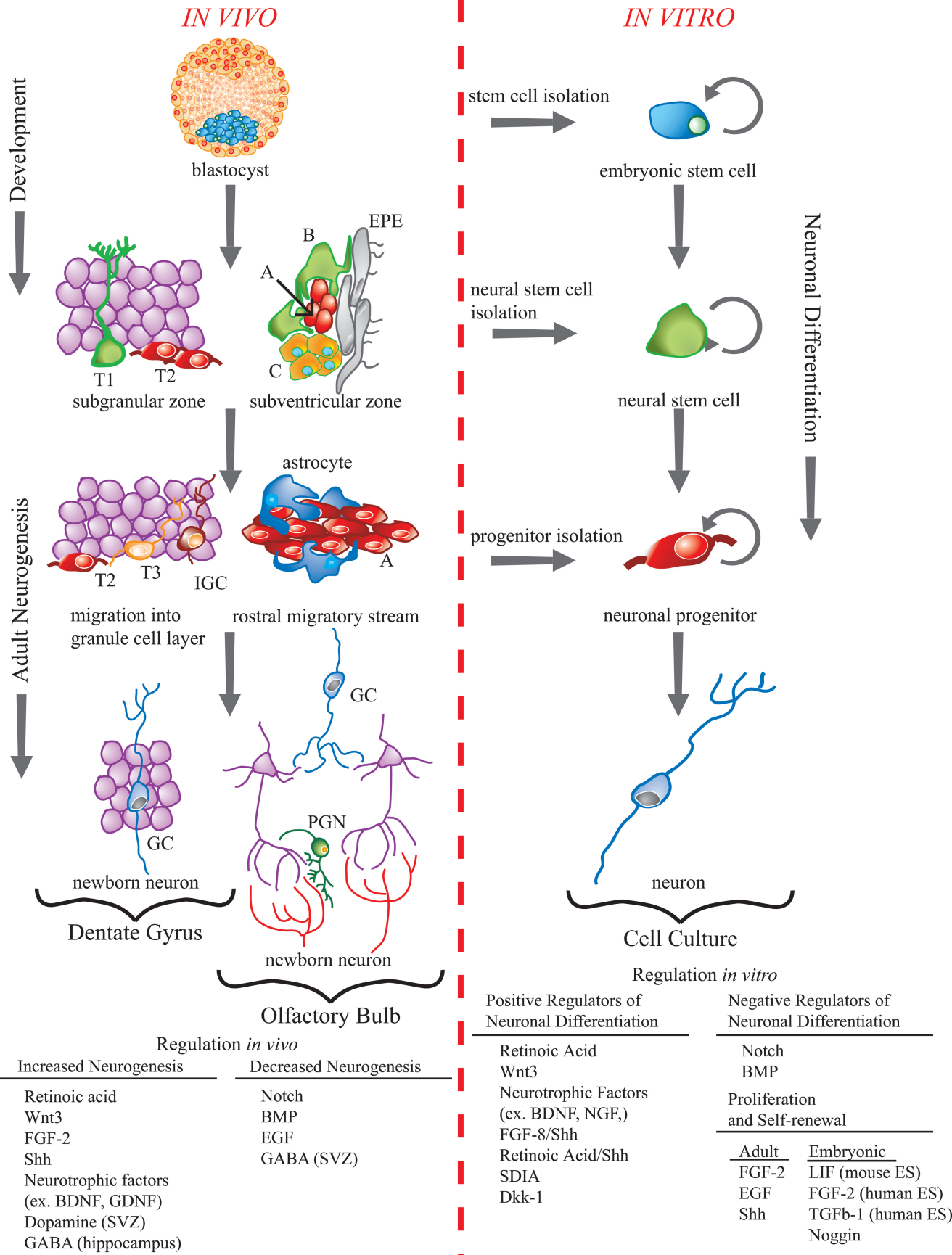
Figure 1. Schematic representation of stem cells *in vivo* and *in vitro*. *In vivo*, embryonic stem cells can be isolated from the inner mass cells of the developing blastocyst. These cells are capable of self-renewal and can differentiate into all of the three primary germ layers: endoderm, mesoderm and ectoderm. Within the ectodermal lineage, they can generate neural stem cells, which during differentiation become progenitor cells that are characterized by more limited proliferation. These cells with the appropriate cues can ultimately generate neurons. Neural stem cells and progenitors can also be isolated directly from later stage embryos or from the adult organism, and they show parallel proliferative capabilities and plasticity *in vitro*. *In vivo* there are two neurogenic regions in the adult brain. The hippocampus contains type-1 (T1) cells the putative neural stem cells that are located in the subgranular zone of the dentate gyrus and have astrocytic properties. As they differentiate, these cells become type-2 (T2) and type-3 (T3) cells. Eventually they become immature granule cells (IGC), which mature into granule cells in the granule cell layer of the dentate gyrus. In the subventricular zone the putative neural stem cell known as B cells (B) exist near the ependymal cell (EPE) wall of the lateral ventricle. They differentiate into transiently amplifying C cells (C), which become migrating

neuroblasts or A cells (A). While in contact with astrocytes, A cells migrate along the rostral migratory stream into the olfactory bulb where they differentiate into granule cells (GC) and periglomerular neurons (PGN).



Regulation *in vivo*

Increased Neurogenesis	Decreased Neurogenesis
Retinoic acid	Notch
Wnt3	BMP
FGF-2	EGF
Shh	GABA (SVZ)
Neurotrophic factors (ex. BDNF, GDNF)	
Dopamine (SVZ)	
GABA (hippocampus)	

Regulation *in vitro*

Positive Regulators of Neuronal Differentiation	Negative Regulators of Neuronal Differentiation
Retinoic Acid	Notch
Wnt3	BMP
Neurotrophic Factors (ex. BDNF, NGF,)	
FGF-8/Shh	
Retinoic Acid/Shh	
SDIA	
Dkk-1	

Proliferation and Self-renewal

Adult	Embryonic
FGF-2	LIF (mouse ES)
EGF	FGF-2 (human ES)
Shh	TGFb-1 (human ES)
	Noggin

FIGURE 1