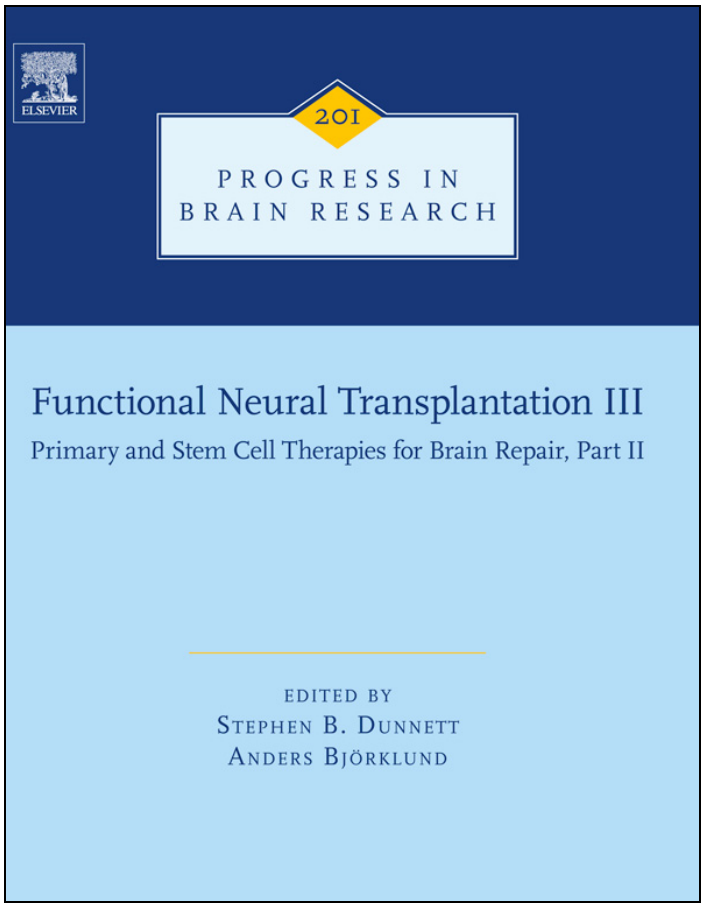

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CHAPTER

Generation of motor neurons from pluripotent stem cells

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Abstract

Alpha motor neurons (also known as lower or skeletal motor neurons) have been studied extensively for over 100 years. Motor neurons control the contraction of skeletal muscles and thus are the final common pathway in the nervous system responsible for motor behavior. Muscles become paralyzed when their innervating motor neurons die because of injury or disease. Motor neuron diseases (MNDs), such as Amyotrophic Lateral Sclerosis, progressively destroy motor neurons until those inflicted succumb to the illness due to respiratory failure. One strategy being explored to study and treat muscle paralysis due to motor neuron loss involves deriving surrogate motor neurons from pluripotent stem cells. Guided by decades of research on the development of the spinal cord, recent advances in neurobiology have shown that functional motor neurons can be derived from mouse and human embryonic stem (ES) cells. Furthermore, ES cell-derived motor neurons restore motor behavior when transplanted into animal models of motor dysfunction. The recent discovery that mouse and human motor neurons can be derived from induced pluripotent stem (iPS) cells (i.e., somatic cells converted to pluripotency) has set the stage for the development of patient-specific therapies designed to treat movement disorders. Indeed, there is now hope within the scientific community that motor neurons derived from pluripotent stem cells will be used to treat MNDs through cell transplantation and/or to screen molecules that will prevent motor neuron death. In this chapter, we review the journey that led to the generation of motor neurons from ES and iPS cells, how stem cell-derived motor neurons have been used to treat/study motor dysfunction, and where the technology will likely lead to in the future.

Keywords

embryonic stem cells, induced pluripotent stem cells, motor neuron disease, motor neurons, transplantation

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1 INTRODUCTION

It seems fitting that alpha motor neurons (also known as lower or skeletal motor neurons) were among the first neuronal cell type to be intentionally derived from pluripotent stem cells (Wichterle et al., 2002). Alpha motor neurons have been studied for over 100 years, making them the most extensively researched cells in the mammalian central nervous system (CNS; Kernell, 2006). Our long-standing obsession with this small group of neurons is founded not only on their unique properties and attributes but also on our ability to analyze them. Motor neurons are the final common pathway in the induction of movement (Sherrington, 1906). This basic and easily quantifiable function is essential for the existence of all organisms possessing motor neurons (Arendt et al., 2008). In addition, motor neurons are relatively simple structures that are readily accessible for experimental analysis. In vertebrates, motor neurons are topographically mapped to the muscle targets they innervate (Hollyday et al., 1977; Landmesser, 1978a,b) making them ideal for the study of the assembly of neuronal circuits (Sharma and Izpisúa Belmonte, 2001). Motor neurons are also the target of diseases such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). These motor neuron diseases (MNDs) are devastating disorders that progressively destroy motor neurons, leading to increased muscle weakness and paralysis. There is no cure for MNDs, and even with advancements in supportive treatments most people with ALS die 3–5 years after diagnosis (Czaplinski et al., 2006). This grim reality has catalyzed stem cell biologists, MND specialists, and motor neuron scientists to work together in a collaborative effort to devise methodologies to derive motor neurons from pluripotent stem cells. There is great anticipation within the scientific community that motor neurons derived from pluripotent stem cells will be used to treat MNDs through cell transplantation therapies (Gowing and Svendsen, 2011) and to screen small molecules that will increase the survival/function of the diseased neurons (Vitale et al., 2011).

In the following sections, we review key developmental studies leading to the discovery of morphogens and transcription factors required for motor neuron differentiation and show how these discoveries were exploited to generate functional motor neurons from mouse and human embryonic stem (ES) cells. Second, we introduce induced pluripotent stem (iPS) cells and discuss how motor neurons derived from them can be used as a source of cells for transplantation therapy and as a model system to study MNDs. Finally, we consider current technologies that may circumvent some of the problems and concerns associated with the use of stem cell-derived neurons as a tool to treat CNS injuries and neurodegenerative diseases.

2 TOWARD THE GENESIS OF MOTOR NEURONS FROM STEM CELLS

One would be remiss to discuss the derivation of motor neurons from pluripotent cells without first reviewing some of the key developmental studies that provided the necessary insights, and tools, required for the task. Motor neurons are among

the first cell type to differentiate from neuronal precursor cells in the ventral spinal cord (known as the neural tube) during early embryogenesis (Hollyday and Hamburger, 1977). Prior to motoneurogenesis, the ventral region contains a specialized group of epithelial cells known as the floor plate that transiently occupies the ventral midline of the neural tube (Baker, 1927; Kingsbury, 1930; reviewed by Wilson and Maden, 2005). During its brief existence, the floor plate directs a multitude of functions, one of which is to induce motor neuron differentiation. Dodd and colleagues (Placzek et al., 1991) elegantly discovered this instructional role in chick embryos by inducing floor plates in ectopic regions in the dorsal half of the neural tube. The misplaced floor plates induced surrounding neuronal precursor cells to become motor neurons indicating that the floor plate was both necessary and sufficient to induce motor neuron differentiation. Although the regulatory molecules were not identified at the time, the authors postulated that the floor plate cells must release a diffusible factor that induces motoneurogenesis (Placzek et al., 1991; Yamada et al., 1991). A clue to the factor's identity came a few years later when McMahon and colleagues (Echelard et al., 1993) discovered three genes in mice related to the *Drosophila* segment polarity gene, hedgehog (Nüsslein-Volhard and Wieschaus, 1980). One of these genes, which they termed sonic hedgehog (Shh), was expressed in the floor plate, as well as the notochord (a transient mesenchymal structure underlying the floor plate) and zone of polarizing activity (ZPA; a medial posterior region of the developing limb bud). Because ectopic expression of Shh in the mouse CNS led to activation of floor plate genes in neighboring cells, they concluded that Shh is a morphogen that patterns the ventral neural tube (Echelard et al., 1993). Several investigators, including those working with Jessell, McMahon, and Beachy, went on to show that Shh not only patterns the ventral neural tube but also induces neuronal precursor cells to differentiate into motor neurons (Chiang et al., 1996; Martí et al., 1995; Roelink et al., 1994, 1995; Tanabe et al., 1995). The Shh saga culminated when Ericson et al. (1996) elegantly showed that Shh acts as a graded signal to ventralize the neural tube by inducing progenitor cells to acquire specific neuronal fates (motor neurons or discrete subclasses of interneurons) based on their concentration threshold for Shh (reviewed by Briscoe and Ericson, 2001). That is, progenitor cells near the floor plate acquire a motor neuron identity because they are exposed to a high concentration of Shh and have a high threshold for Shh-mediated induction. Conversely, progenitor cells near the sulcus limitans differentiate into discrete interneuron subtypes because they have a low threshold for Shh-mediated signaling and are exposed to a low level of Shh (Briscoe and Ericson, 2001).

Ectodermal cells must first differentiate into neuroectoderm and form the neural tube prior to the ventralizing activity of Shh. Our knowledge of this process is limited compared to our understanding of the Shh signaling pathway. However, numerous studies indicate that ectodermal cells in the early embryo, as well as cultured ES cells, differentiate into neural progenitors cells by inhibiting BMP and regulating Wnt/FGF signaling (Chambers et al., 2009; Pera et al., 2004; Smith et al., 2008; Wilson et al., 2001). The first neural progenitor cells born during this initial phase of neurogenesis express genetic markers typical of forebrain cells (Wilson et al., 2000). Caudal neurons, such as those found in the spinal cord, therefore appear to

differentiate from the rostral cells through the action of additional morphogens (Wilson & Edlund, 2001). One morphogen believed to be involved in this caudalization is *all-trans*-retinoic acid (RA). This hypothesis is based on the fact that active retinoids are localized in the posterior region of early neurula stage embryos (Chen et al., 1994; Wagner et al., 1992), and their active retinoid receptors are found throughout the embryo during gastrulation. In addition, *Xenopus* embryos treated with RA during neural tube formation form enlarged spinal cords at the expense of forebrain tissue (Durston et al., 1989). Finally, while mutant mice lacking RA receptors have abnormal skeletal and CNS structures anterior to the cervical vertebrae, the structures posterior to this region are relatively normal (Lohnes et al., 1994). Taken together, it appears that a sequence of events occurs in the generation of motor neurons during development. First, ectodermal cells differentiate into rostral neuroectoderm through the actions of BMP, Wnt, and FGF. The posterior region of the neuroectoderm is then caudalized by RA. Finally, neural progenitors cells near the floor plate acquire a motor neuron identity through the ventralizing actions of Shh (Briscoe & Ericson, 2001; Wichterle et al., 2002).

3 DERIVATION OF MOTOR NEURONS FROM ES CELLS

In 2002, Hynek Wichterle in Tom Jessell's laboratory showed for the first time that mouse ES cells can be directed to differentiate into motor neurons by exposing them to RA and an agonist of the Shh signaling pathway (Wichterle et al., 2002). In brief, Wichterle et al. (2002) cultured ES cells as free-floating clusters, which he termed embryoid bodies, for 2 days before adding RA and the agonist. Five days later, the embryoid bodies expressed several transcription factors found in motor neurons including NeuN, Lhx3, Isl1, and Hb9 (the latter being unique to postmitotic motor neurons; Arber et al., 1999). To determine whether the neurons exhibited behavior typical of embryonic motor neurons, Wichterle et al. (2002) transplanted ES cell-derived motor neurons into the spinal cord of chick embryos at the time of motoneurogenesis. Remarkably, they found that the grafted motor neurons extended numerous axons out of the spinal cord through the ventral root where they continued to project to peripheral muscles. Intramuscular axons from the ES cell-derived motor neurons contained synaptic vesicles appropriate for neurotransmission and induced clustering of postsynaptic acetylcholine receptors on the developing chick muscle fibers. Taken together, this landmark study showed that mouse ES cells rapidly develop into neurons with the molecular identity and behavioral traits typical of fully differentiated motor neurons when exposed to only two morphogens. Surprisingly, exogenous manipulation of BMP, Wnt, and FGF signaling was not required to differentiate ES cells into neuroectodermal cells prior to motor neuron differentiation. Although the reasons for this are not known, it likely occurs because BMP, Wnt, and FGF signaling takes place spontaneously within the microenvironment of the free-floating embryoid bodies (Chambers et al., 2009).

Most studies deriving motor neurons from pluripotent cells are based on the original method developed by Wichterle et al. (2002). Two elements in his protocol have proven to be invaluable for studying motor neurons and thus are worthy of further discussion. First, Wichterle et al. (2002) used ES cells generated from the inner cell mass of blastocysts harvested from transgenic mice expressing enhanced green fluorescent protein (eGFP) under the control of the Hb9 promoter (Wichterle et al., 2002). The decision to develop this ES cell line was particularly insightful in light of the fact that only 20–40% of ES cells treated with RA and an activator of the Shh signaling pathway differentiate into motor neurons. The other 60–80% of the cells differentiate into glutamatergic neurons and glia (Miles et al., 2004). eGFP expression is therefore an ideal noninvasive biomarker to identify ES cell-derived motor neurons for anatomical and electrophysiological analysis. In addition, a pure population of $eGFP^+$ motor neurons can be obtained, if needed, using fluorescent activated cell-sorting techniques (e.g., Soundararajan et al., 2007; Wichterle et al., 2002).

Although recombinant Shh protein activates Shh signaling in neuronal precursor cells *ex vivo* (Ericson et al., 1996), Wichterle et al. (2002) chose to use a synthetic small molecule, known as Hh-Ag, to activate the signaling pathway. This second important element in his protocol was critical because recombinant Shh protein is a very poor activator of Shh signaling in ES cells. This is likely due to the fact that recombinant Shh protein is not posttranslationally modified by cholesterol and fatty acids, both of which increase the potency of native Shh by 15- to 160-fold (Pepinsky et al., 1998; Taipale et al., 2000). Thus, while RA and native Shh differentiate ES cells into motor neurons, RA and recombinant Shh do not (Soundararajan et al., 2007). Hh-Ag is a potent activator of Shh signaling because it binds directly to the Shh pathway activator Smoothened (Smo; Frank-Kamenetsky et al., 2002), which is downstream of the hedgehog receptor Patched. This activation is sufficient to induce motor neuron differentiation when combined with RA. Smo agonists are now commercially available and are comparable to Hh-Ag in their capacity to promote motor neuron differentiation from ES cells.

3.1 Functional properties of mouse ES cell-derived motor neurons

In 2004, we sought to determine whether mouse ES cell-derived motor neurons acquire the same electrophysiological properties as young endogenous motor neurons (Miles et al., 2004). Using the same differentiation protocol developed by Wichterle et al. (2002), we found that cultured mouse ES cell-derived motor neurons expressed functionally appropriate GABA, glycine, and glutamate receptors as well as voltage-activated Na^+, K^+, and Ca^{2+} ion channels (Miles et al., 2004). Furthermore, when injected with depolarizing current, ES cell-derived motor neurons fired repetitive action potentials with a spike frequency adaption profile typical of endogenous motor neurons. Finally, we showed that ES cell-derived motor neurons make functional connections with muscle fibers when cocultured *in vitro* (Miles et al., 2004; see also Harper et al., 2004). Together with the chick embryo transplantation studies performed by Wichterle et al. (2002), these results indicate that mouse ES cell-derived

motor neurons acquire most, if not all, of the morphological, behavioral, and electrophysiological properties of their endogenous counterparts.

RA and Smo agonist-treated mouse ES cells appear to differentiate into a specific subclass of motor neurons whose molecular identity, behavior, and physiology resemble those of cells that reside in the medial aspect of the medial motor column (MMC_m). These motor neurons selectively project axons to epaxial muscles lining the vertebral column (Soundararajan et al., 2006, 2007, 2010; Wichterle et al., 2002). This conclusion is based on our observations that RA and Hh-Ag treated mouse ES cell-derived motor neurons migrate to the MMC_m, where they develop the same passive membrane properties as their endogenous MMC_m counterparts when transplanted into the neural tube of chick embryos at the time of motoneurogenesis (Soundararajan et al., 2006). Furthermore, we found that the grafted neurons selectively projected axons to epaxial muscles lining the vertebral column because they express guidance cues typical of MMC_m motor neurons (Soundararajan et al., 2006, 2007). Taken together, these results indicate that specific subclasses of neurons can be generated from ES cells and that additional morphogens are likely required to produce limb innervating motor neurons.

3.2 Human ES cell-derived motor neurons

Three years after motor neurons were generated from mouse ES cells, Zhang and colleagues (Li et al., 2005) and later Singh Roy et al. (2005) demonstrated that motor neurons could be derived from human ES cells. As one might expect, deriving motor neurons from human ES cells is more laborious, takes longer, and requires additional factors compared to the derivation of motor neurons from mouse ES cells (Li et al., 2005; Shin et al., 2005; Wada et al., 2009). Typically, human ES cells are cultured for 1 week on an adherent substrate to form rosettes of neuroectodermal cells that are later harvested/isolated and treated with RA and a Smo agonist to induce motor neuron differentiation. One to two weeks later, the cultures of differentiated motor neuron progenitor cells are treated with a cocktail of trophic factors (BDNF, GDNF, and CNTF, or IGF-1) known to potentiate motor neuron differentiation and survival (Zurn et al., 1996). Like motor neurons derived from mouse ES cells, human ES cell-derived motor neurons express motor neuron-specific transcription factors and receptors, acquire synaptic machinery appropriate for neurotransmission, fire spontaneous action potentials (Lee et al., 2007), and induce postsynaptic acetylcholine receptor clustering when cocultured with myotubes (Li et al., 2005; Singh Roy et al., 2005; Wada et al., 2009). Thus, while more expertise, patience, time, money, and perseverance are required to generate motor neurons from human ES cells, the final product is remarkably similar to endogenous motor neurons and those derived from mouse ES cells.

3.3 ES cell-derived motor neurons: Preclinical studies

Cell replacement therapies, using ES cell-derived motor neurons, may restore motor function in individuals who have lost motor neurons due to injury or disease provided the transplanted cells integrate with the host's own circuitry. To determine whether

this can occur, Kerr and colleagues transplanted mouse ES cell-derived motor neurons into the spinal cord of adult rats paralyzed with a neuroadapted Sindbis virus (Deshpande et al., 2006; Harper et al., 2004). Neuroadapted Sindbis virus is a neuronotropic virus that specifically targets motor neurons in the spinal cord (Griffin et al., 1994; Jackson et al., 1987), causing hindlimb paralysis due to the loss of ~80% of the axons in the ventral root (Kerr et al., 2002, 2003). Although many transplantation strategies were used, the best outcomes occurred when GDNF secreting neural stem cells were injected into the ventral roots near the injection site and the rats were administered a phosphodiesterase 4 inhibitor and dibutyryl cyclic adenosine monophosphate (dbcAMP; Deshpande et al., 2006). The phosphodiesterase 4 inhibitor and dbcAMP were administered to improve axonal growth in the inhibitory, myelin-enriched environment of the spinal cord (Pearse et al., 2004), while GDNF-secreting cells were injected into the target nerve to entice axonal outgrowth into the periphery. Using this multitargeted approach, Deshpande et al. (2006) showed that ~15% of the ES cell-derived motor neurons were still alive 6 months after transplantation and many extended axons out through the ventral roots where they eventually formed neuromuscular junctions with denervated muscle fibers. Finally, rats receiving ES cell-derived motor neurons, GDNF secreting cells, and growth enhancers showed significantly better motor function based on hind limb grip strength and blinded locomotor assessments. Taken together, these preclinical studies suggest that cell replacement therapies using ES cell-derived motor neurons may be a viable treatment strategy for restoring meaningful movement in patients with motor neuron loss provided they extend axons out of the cord and innervate distal muscle fibers.

In 2007, Lee et al. (2007) showed that human ES cell-derived motor neurons survive transplantation and extend axons into the periphery via the ventral root when grafted into the developing spinal cord of chick embryos. These results indicate that human ES cell-derived motor neurons, like their mouse ES counterparts, respond to endogenous cues that guide their axons to appropriate muscle targets. To ascertain whether human ES cell-derived motor neurons survive and integrate in a postnatal spinal cord, Studer and colleagues transplanted 80,000 RA/Shh-treated human ES cells into the cervical spinal cord of adult rats (Lee et al., 2007). Several ChAT-expressing cells remained 6 weeks after transplantation, and extensive fiber outgrowth was observed outside the graft site. Outgrowth into the spinal nerves was not examined, but seems unlikely considering that GDNF-secreting cells were not transplanted into the spinal nerve, as described by Deshpande et al. (2006). Disease onset was delayed, and motor neuron survival improved, in rodent models of ALS (López-González et al., 2009) and SMA (Corti et al., 2010) when ES cell-derived motor neurons were transplanted into the spinal cords. However, these improvements were attributed to the release of neuroprotective factors from the transplanted cells rather than to functional integration with host tissue (Corti et al., 2010).

Transplanting motor neurons into the spinal cord is flushed with technical challenges including poor survival and growth. Even if the transplanted motor neurons innervate numerous muscles, it seems unlikely that meaningful motor function will be restored unless they receive synaptic input that is appropriate for the muscle

groups innervated (e.g., flexor vs. extensor muscle groups). These challenges can be avoided if ES cell-derived motor neurons are transplanted close to the target muscles. For example, inspired by transplantation studies using fetal spinal cord tissue (Thomas et al., 2000), we grafted ES cell-derived motor neurons into the distal end of transected peripheral mouse nerves and quantified muscle function 3–18 months later (Yohn et al., 2008; see also Craff et al., 2007; Kubo et al., 2009). We found that the ES cell-derived motor neurons grew axons into the denervated muscles where they formed functional synapses. Furthermore, when we electrically stimulated the grafted ES cell-derived motor neurons, the reinnervated muscles produced ~40% of their original prelesion force (Yohn et al., 2008). Surprisingly, we found that the number of slow muscle fibers increased, and denervation-associated muscle atrophy was reduced in the reinnervated muscles. These latter results indicated that transplanted ES cell-derived motor neurons integrated with the host tissue to the extent that they changed the phenotype of the muscle fibers they innervated (Craff et al., 2007; Yohn et al., 2008).

Can motor neurons transplanted into a peripheral nerve be used clinically? In mice, ES cell-derived motor neurons survive for at least 18 months if they make functional connections with previously denervated muscle fibers (Yohn et al., 2008). If ES cell-derived motor neurons survive and function equally well when transplanted into human peripheral nerves, they could be used to restore meaningful function to paralyzed muscles through exogenous electrical stimulation. This concept is based, in part, on studies restoring hand function in persons with tetraplegia using neuroprosthesis (Gan et al., 2012; Peckham et al., 2002). This technology uses externally controlled electrical stimulation of endogenous nerves through implanted electrodes to activate paralyzed flexor and extensor muscles to open and close the hand. This rehabilitation strategy is only feasible, however, if meaningful force is generated upon electrical nerve stimulation. Unfortunately, many spinal cord injuries induce motor neuron cell death around the trauma site. As a result, many muscles in individuals with a spinal cord injury become permanently denervated and incapable of producing force even when the peripheral nerve is electrically stimulated (Thomas et al., 1997). Under such conditions, ES cell-derived motor neurons transplanted into the peripheral nerve could become surrogate motor neurons that would produce meaningful contractions upon electrical stimulation. A similar cell replacement strategy could be used in instances where motor nerves were lost due to a peripheral nerve injury or were surgically excised due to a peripheral neuroma.

4 iPS CELL-DERIVED MOTOR NEURONS

Developing clinical therapies involving neurons derived from human ES cells is burdened with social and logistical concerns because it requires embryonic tissue that has, or at the very least had, the potential to generate life. Furthermore, allografts usually require ongoing immunosuppression to prevent tissue rejection, although this may be less of an issue when cells are transplanted into the CNS (e.g., Mendez et al.,

2008). Many of these societal concerns and technical challenges would likely be resolved if transplanted neurons were derived from the recipient's own somatic cells rather than embryonic tissue.

In a landmark study, designed to define the smallest number of factors needed to bestow the pluripotency of ES cells onto terminally differentiated somatic cells, Takahashi and Yamanaka (2006) showed that mouse embryonic and adult fibroblasts become pluripotent when forced to express four ES cell-specific genes. These four genes, transcription factors octamer 3/4 (*Oct4*), SRY box–containing gene 2 (*Sox2*), Kruppel-like factor 4 (*Klf4*), and *c-Myc* are now commonly known as "Yamanaka" factors (Liu et al., 2008), and the pluripotent cells derived from their forced expression are termed iPS cells (Takahashi and Yamanaka, 2006; Fig. 1). In their initial study, Takahashi and Yamanaka (2006) showed that mouse iPS cells were truly pluripotent because they formed teratomas (differentiated into cell types found in all three embryonic germ layers) when transplanted into immunodeficient mice. However, they differed from ES cells in their DNA methylation pattern. By refining their reprogramming protocol further, Yamanaka and colleagues overcame these issues and showed that adult mouse fibroblasts can be converted into iPS cells that contribute to adult chimeras (Okita et al., 2007). Shortly afterward, Yamanaka and two other groups generated iPS cells from human fibroblasts (Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007), setting the stage for the generation of patient-specific pluripotent stem cells that can be used for autologous tissue transplantation or for modeling neurodegenerative diseases (Kiskinis and Eggan, 2010).

In 2008, Kevin Eggan and colleagues showed that iPS cells could be generated from a skin biopsy collected from an individual diagnosed with a familial form of ALS (Dimos et al., 2008). Using the same differentiation protocol developed for human ES cells, Dimos et al. (2008) went on to show that human iPS cells could be directed to differentiate into neurons expressing motor neuron restricted transcription factors. A year later, Clive Svendsen and colleagues generated cells with the morphological and genetic signature of motor neurons from iPS cells derived from a patient with SMA (Ebert et al., 2008). Furthermore, they found that SMA protein was increased in iPS cell-derived motor neurons when treated with the SMA-inducing compounds, valproic acid and tobramycin. These latter results suggest that iPS cell-derived motor neurons retain biochemical traits similar to those found in the host's own nervous system. More recently, Bilican et al. (2012) derived motor neurons from iPS cells generated from fibroblasts collected from an individual with a TDP-43 M337V mutation (another familial form of ALS; Sreedharan et al., 2008). The iPS cell-derived motor neurons fired TTX-sensitive repetitive action potentials when depolarized (see also Karumbayaram et al., 2009) and, like their endogenous counterparts, displayed biochemical features of TDP-43 misaccumulation (Bilican et al., 2012). Thus, iPS cell-derived motor neurons, derived from biopsies taken from individuals with SMA or a familial form of ALS, acquire key pathological characteristics associated with the disease presumably because they were genetically imprinted by the somatic cells from which they were derived.

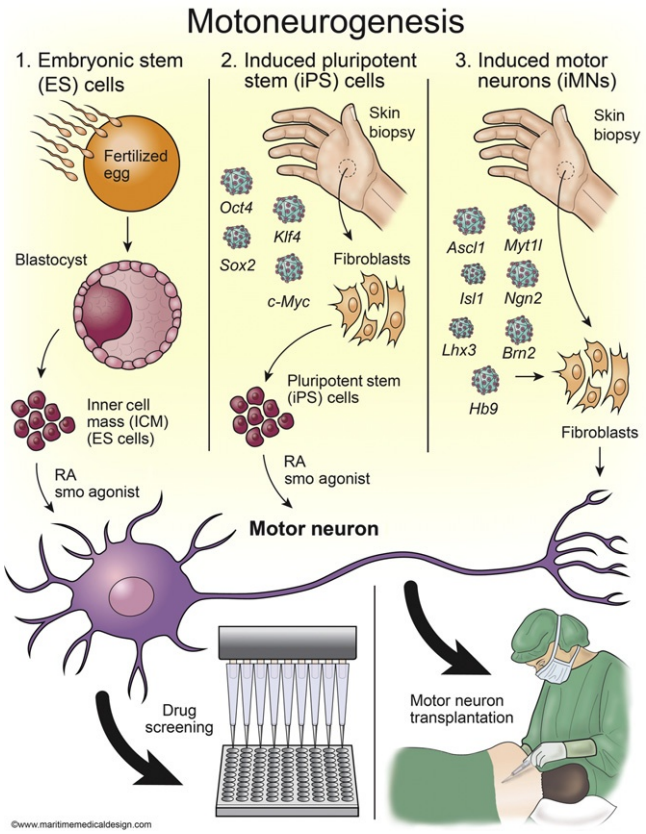

FIGURE 1

Illustration showing the derivation of motor neurons using three distinct techniques. (1) ES cells isolated from the inner cell mass of a blastocyst differentiate into motor neurons when treated with RA and an Smo agonist. (2) Skin cells are converted into iPS cells by forced expression of specific genes (typically Oct4, Klf4, Sox2, and c-Myc). iPS cells later differentiate into motor neurons when exposed to RA and an Smo agonist. (3) Skin cells are directly converted into motor neurons by forced expression of seven genes.

Nonhereditary neurodegenerative disorders such as sporadic ALS may be attributed to a combination of oligogenic inheritance and environmental signals (Andersen and Al-Chalabi, 2011). If true, motor neurons derived from iPS cells generated from individuals with sporadic ALS should be genetically predisposed to manifest the disease. This would make iPS cell-derived neurons an ideal model system to screen compounds to treat ALS and other sporadic neurodegenerative disorders such as Parkinson's disease. This view is substantiated by recent reports demonstrating that iPS cell-derived neurons recapitulate several disease phenotypes (Carvajal-Vergara et al., 2010; Lee et al., 2009; Urbach et al., 2010; reviewed by Grskovic et al., 2011).

5 DIRECT CONVERSION OF FIBROBLASTS INTO INDUCED MOTOR NEURONS

Inspired by Yamanaka and colleagues pioneering studies on iPS cells (Takahashi and Yamanaka, 2006), several groups have shown that it is possible to reprogram fibroblasts into postmitotic neurons using distinct sets of transcriptions factors (Caiazzo et al., 2011; Pfisterer et al., 2011; Vierbuchen et al., 2010). More recently, Kevin Eggan and colleagues used a similar approach to directly convert mouse and human fibroblasts into a type of motor neuron they termed induced motor neurons (iMNs; Son et al., 2011). They found that seven factors (Ascl1, Brn2, Myt1l, Lhx3, Hb9, Isl1, and Ngn2) were sufficient to induce mouse fibroblasts to become motor neurons while an additional eighth factor (NEUROD1) was required for successful induction of human fibroblasts (Son et al., 2011). iMNs displayed electrophysiological characteristics and acquired gene expression patterns typical of endogenous motor neurons. In addition, they formed functional synapses with muscle fibers in culture and extended axons into the periphery when transplanted into the developing chick spinal cord. Like endogenous (Haidet-Phillips et al., 2011) and ES cell-derived motor neurons, iMNs degenerated when cocultured with astrocytes expressing a mutated form of superoxide dismutase 1 (Son et al., 2011). Finally, Son et al. (2011) demonstrated that fibroblasts converted into iMNs do not transit through a proliferative state. This latter observation is particularly important with respect to cell replacement therapies because grafts containing iMNs will not contain proliferative-competent cells that could become tumorigenic. The same is not true for transplants containing ES and iPS cell-derived motor neurons (Victor Rafuse, unpublished results). While this approach for generating motor neurons is exciting, it remains to be determined whether iMNs have the capacity to form functional connections with denervated muscle fibers when transplanted into an adult host (e.g., Yohn et al., 2008).

6 SUMMARY AND FUTURE DIRECTIONS

There has been tremendous progress since Wichterle et al. (2002) showed that motor neurons could be derived from mouse ES cells using RA and an Smo agonist (Fig. 1, left column #1). Since then, ES cell-derived motor neurons have been shown to resemble their endogenous counterparts anatomically, physiologically, biochemically, and behaviorally. Furthermore, ES cell-derived motor neurons partially restore motor behavior when transplanted into animal models of motor dysfunction. More recently, iPS cells have been shown to readily differentiate into motor neurons when treated with RA and an Smo agonist (Fig. 1, middle column #2). Although not as well studied, iPS cell-derived motor neurons have many of the same anatomical, biochemical, and physiological properties as their ES cell-derived equivalents. Motor neurons can also be derived directly from somatic cells (Fig. 1, right column #3). Unlike those derived from ES and iPS cells, motor neurons derived by this latter technique do not require

morphogens or the generation of pluripotent progenitor cells. Despite their origin and methods of derivation, iMNs have anatomical and electrophysiological properties that are typical of young endogenous motor neurons.

There are now three novel ways to generate motor neurons using techniques that were virtually unheard of just over a decade ago (Fig. 1). Future research must now focus on their application for studying and treating motor neuron disorders due to injury and/or disease. For example, transplantation studies will need to pioneer better ways to prolong the survival of the transplants and promote better outgrowth from the graft. In addition, meaningful movement will only be restored if the transplanted motor neurons are activated in a manner that is appropriate for the muscles they reinnervate. Consequently, future *in vivo* studies will have to evaluate the type of synaptic inputs the transplants receive and determine whether they are suitable for regulating voluntary and involuntary movements. Because denervated muscle fibers progressively lose their ability to be reinnervated over time (Fu and Gordon, 1995), future transplantation studies will have to be performed to determine when stem cell-derived motor neurons should be transplanted for best functional outcomes.

In addition to cell replacement therapies, stem cell-derived motor neurons and iMNs are also well suited for studying the pathophysiology causing MNDs. Both iMNs and iPS cell-derived motor neurons can be derived from somatic cells collected from mice models of MNDs and from individuals with a MND. These motor neurons exhibit much of the same pathophysiology that their endogenous counterparts do (e.g., Bilican et al., 2012; Son et al., 2011) making them ideal for rapidly screening thousands of compounds that may promote better survival. However, motor neurons become dysfunctional long before they perish in MNDs (Balice-Gordon et al., 2000). Consequently, novel *in vitro* model systems will have to be developed in order to rapidly screen motor neuron dysfunction and not simply cell death. Finally, it is well established that the largest motor neurons die first in MNDs (Fischer et al., 2004; Theys et al., 1999) while other motor neurons such as those of the Onuf nucleus are relatively resistant to the disease (Carvalho et al., 1995). ES and iPS cells typically differentiate into smaller postural motor neurons within the MMC_m when treated with RA and an Smo agonist (Soundararajan et al., 2006; Wichterle et al., 2002). Consequently, future research should examine whether pluripotent cells, or iMNs, can be directed to differentiate into larger motor neurons, and if so, whether they exhibit different pathophysiology compared to ES cell-derived MMC_m motor neurons. It is likely that important insights into MNDs will be gained by systematically comparing motor neurons that are vulnerable to the disease with those that are not. In summary, the last 25 years have been witness to a remarkable journey of discoveries in the field of motoneurogenesis. In less than a quarter of a century, we have gone from determining that the floor plate regulates dorsoventral patterning of the neural tube to identifying a small set of genes that can convert adult skin cells into functional motor neurons. With thisamazing progress in science, it is hard to imagine what the next 25 years will have in store. Hopefully, they will include the discovery of effective strategies to treat, or even cure, MNDs as well as motor dysfunction due to injury.

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