Cell. Mol. Life Sci. (2010) 67:3837–3847
DOI 10.1007/s00018-010-0463-y

Cellular and Molecular Life Sciences

REVIEW

Human motor neuron generation from embryonic stem cells and induced pluripotent stem cells

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Received: 26 March 2010 / Revised: 15 June 2010 / Accepted: 9 July 2010 / Published online: 29 July 2010

Abstract Motor neuron diseases (MNDs) are a group of neurological disorders that selectively affect motor neurons. There are currently no cures or efficacious treatments for these diseases. In recent years, significant developments in stem cell research have been applied to MNDs, particularly regarding neuroprotection and cell replacement. However, a consistent source of motor neurons for cell replacement is required. Human embryonic stem cells (hESCs) could provide an inexhaustible supply of differentiated cell types, including motor neurons that could be used for MND therapies. Recently, it has been demonstrated that induced pluripotent stem (iPS) cells may serve as an alternative source of motor neurons, since they share ES characteristics, self-renewal, and the potential to differentiate into any somatic cell type. In this review, we discuss several reproducible methods by which hESCs or iPS cells are efficiently isolated and differentiated into functional motor neurons, and possible clinical applications.

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Keywords Motor neuron · Embryonic stem cell · Induced pluripotent stem cell · Motor neuron diseases · Protocol

Abbreviations

MNDs	Motor neuron diseases
hESCs	Human embryonic stem cells
hiPSC	Human induced pluripotent stem cells
CNS	Central nervous system
ALS	Amyotrophic lateral sclerosis
SMA	Spinal muscular atrophy
NE	Neuroepithelial
EBs	Embryoid bodies
MEF	Mouse embryonic fibroblasts
hSCs	Human stem cells
bFGF	Fibroblast growth factor
RA	Retinoic acid
Shh	Sonic hedgehog
cAMP	Cyclic adenosine monophosphate
BDNF	Brain-derived neurotrophic factor
GDNF	Glial-derived neurotrophic factor
p75-NGFR	Nerve growth factor receptor

Introduction

Motor neurons are cells located in specific areas of the central nervous system (CNS), in the brain cortex (upper motor neuron, UMN), brain stem, and spinal cord (lower motor neuron, LMN), which control voluntary actions such as breathing and walking. Motor neuron diseases (MNDs) are neurodegenerative disorders that selectively affect motor neurons, clinically manifesting with progressive paralysis and, often, precocious death. MNDs can be

considered to be a spectrum of disorders that have a complex multifactorial etiology and a large clinical variability. They can be divided into three categories: those with combined UMN and LMN involvement (amyotrophic lateral sclerosis (ALS) (sporadic and familial forms)), those with LMN involvement (spinal muscular atrophy (SMA) and spinobulbar muscular atrophy (SBMA) or Kennedy's disease; Hereditary Motor Neuropathies (HMN) and progressive spinal muscular atrophy (PMA)) and those with UMN involvement (primary lateral sclerosis (PLS) and the hereditary spastic paraplegia (HSP)) [1, 2]. The contribution of genetics to the etiopathogenesis of MNDs accounts for a high percentage of SMA, HMN, and SPG and for a more limited fraction (about 10%) of cases of ALS [1, 2]. The most common MND form is ALS that starts in adult life, and the ensuing progressive paralysis is typically fatal within a few years, usually for respiratory failure. Actually no successful therapy for ALS is available except for Riluzole, the only FDA-EMEA-approved compound that slows disease progression and extends survival only by 2–3 months [3]. The lack of effective therapy means that although the incidence is comparable to that of multiple sclerosis (4–13 per 100,000), the prevalence is low (1–2 per 100,000) [4]. The term SMA refers to a group of hereditary LMN diseases in which the most common form is linked to mutations into SMN1 gene (SMA 5q, SMA1 (OMIM: 600354). SMA1 is one of the most common genetic causes of infant mortality, affecting approximately 1/10,000 births, with a carrier frequency of approximately 1/50 [5]. Currently, there are no effective therapies available for all of these MNDs that have major impacts on the personal and socio-economic lives of thousands of patients and their caregivers.

Stem cell-derived motor neurons show increasing promise as research tools in disease modeling and high-throughput molecular and drug screens [6, 7], as well as in devising therapeutic strategies for neurodegenerative disorders, for example as replacements for degenerating cells in the CNS [8–12]. The technical and ethical challenges of obtaining spinal motor neurons from fetal or post-mortem human tissues significantly complicate this area of research [13, 14]. Since the establishment of the human embryonic stem cells (hESCs) in the past decade [15], various protocols have been devised to differentiate hESCs into enriched populations of specialized differentiated cells, such as motor neurons [15, 16]. hESCs can be maintained in vitro for a prolonged period of time with a stable genetic background, and thus may provide a source of specialized human cells for biotechnological and clinical applications. Directed differentiation of specific lineages from hESCs is the first critical step toward the employment of hESCs in early human development investigation, as well as potential future clinical applications. The advance of cell replacement therapies with differentiated ESCs is, however, burdened with ethical concerns regarding the use of human embryos, as well as issues involving immune rejection of transplanted cells. Recently, several groups have demonstrated the feasibility of reprogramming various types of human adult somatic cells (such as skin fibroblasts) to an embryonic state by introducing pluripotency factors that yield a novel type of stem cells: induced pluripotent stem cells (iPSCs) [17–20]. The iPSCs meet the original defining criteria for ESCs; iPSCs give rise to all three germ layers, form teratomas, and contribute to chimeras and the germ line. However, these cells are not derived from embryos and would enable the generation of disease-specific cell types. Specific individual human iPS cells should provide the opportunity for cell replacement therapy without requiring immunosuppressive therapy to overcome immune rejection. Thus, hiPSCs become an appealing alternative source for motor neuron generation. These motor neurons might be a valuable research tool, creating a model for dissecting the cellular and molecular interactions underlying early human brain development, for investigating the pathogenesis of human diseases in vitro, and for drug screening. For example, two recent studies describe that patient-specific iPSCs can be successfully differentiated into motor neurons from ALS [21] and SMA patients [22] as proof of principle for the utility of this technology. This initial success encourages further in vitro studies in achieving the differentiation of motor neurons from patient-specific stem cells. The next milestone is to achieve a faithful reproduction of specific MNDs neuropathological features in these cells. Moreover, the primary objectives of hESC and hiPSC differentiation are to generate the relevant cell types to repair tissue damage, and to investigate gene regulation and proteomics in CNS neuronal development.

Protocols for motor neuron induction from hESCs and iPSCs

Several methods for motor neuron differentiation have been reported (Table 1); here we summarize the most important protocols (Fig. 1) to obtain a highly pure population of motor neurons from pluripotent stem cells (hESCs and hiPSCs).

Neuroepithelial cells achievement

The first step in hESC and hiPSC (collectively referred to below as human stem cells (hSCs)) differentiation is the obtainment of neuroepithelial (NE) cells with an appropriate medium. Three methods have been reported to obtain neural progenitors cells. The first method uses the

Table 1 Methods of motor neuron differentiation of hSC

Reference	Hu and Zhang [23]	Wada et al. [29]	Lee et al. [30]
NE cells achievement			
Starting cells	hESC	hESC	hESC on stromal feeder
Media	DMEM/F12 20% KRS	DMEM/F12 w/N2B27	DMEM/F12 w/N2
Factors	w/o FGF	noggin or dorsomorphin	noggin
Obtained cells	floating EB	Neural rosettes	Neural rosettes
MN differentiation			
Media	DMEM/F12 w/N2B27	DMEM/F12 w/N2B27	DMEM/F12 w/N2
Factors	Shh or puromorphamine, RA	Shh or SAG, RA	Shh, RA, BDNF, AA
Mn axon elongation			
Media	DMEM/F12 w/N2B27	DMEM/F12 w/N2B27	DMEM/F12 w/N2
Factors	GDNF, BDNF, IGF1, cAMP	GDNF, BDNF, NT3	GDNF, BDNF, Shh, AA, RA, noggin
Efficiency (%)	~50	~30	20
Duration (days)	35	38	>60
Reference	Marchetto et al. [34]	Karumayaram et al. [35]	Li et al. [36]
NE cells achievement			
Starting cells	hESC	hESC	hESC
Media	DMEM/F12 w/N2	DMEM/F12 20% KSR w/N2	DMEM/F12 w/N2
Factors	RA	RA, w/o FGF	Heparin, w/o FGF
Obtained cells	Neural rosettes	Neural rosettes	Neural rosettes
MN differentiation			
Media	DMEM/F12 w/N2	DMEM/F12 20% KSR w/N2	DMEM/F12 w/N2
Factors	Shh, RA, cAMP	Shh, RA	Shh, RA, cAMP
Mn axon elongation			
Media	DMEM/F12 w/N2	DMEM/F12 20% KSR w/N2	DMEM/F12 w/N2
Factors	GDNF, BDNF, IGF1, cAMP, RA, Shh	GDNF, BDNF, CNTF, Shh	GDNF, BDNF, Shh, IGF1, cAMP, RA
Efficiency	–	~39%	21%
Duration (days)	42–56	32–39	28–35
Reference	Li et al. [37]	Di Giorgio et al. [83]	Karumbayaram et al. [24]
NE cells achievement			
Starting cells	hESC	hESC	iPSC
Media	DMEM/F12 w/N2	DMEM/F12	hESC medium
Factors	Heparin, cAMP, RA	w/o FGF	w/o FGF
Obtained cells	Neural rosettes	EB	EB
MN differentiation			
Media	DMEM/F12 w/N2	DMEM/F12 w/N2	DMEM/F12 KSR w/FGF
Factors	Heparin, Shh or puromorphamine, RA, cAMP	Shh, RA, AA, glucose, BDNF	RA, puromorphamine
Mn axon elongation			
Media	DMEM/F12 w/N2	DMEM/F12 w/N2	hESC medium
Factors	GDNF, BDNF, IGF1, cAMP, RA, Shh or puromorphamine	GDNF	GDNF, BDNF, CNTF, Shh, RA
Efficiency (%)	~50	8	28–33
Duration (days)	>30	42	35–49
Reference	Dimos et al. [21]	Ebertet al. [22]	Erceg et al. [33]
NE cells achievement			
Starting cells	iPSC	iPSC	hESC
Media	DMEM/F12	Human neural progenitor growth medium w/B27	TeSR1 medium (Ludwig) voluven 6%

Table 1 continued

Factors	w/o FGF	w/FGF, EGF, heparin	–
Obtained cells	EB	EB	Neural rosettes
MN differentiation			
Media	DMEM/F12 w/N2	DMEM/F12 w/N2	GRM medium
Factors	Shh agonist, RA	Shh, RA	RA
Mn axon elongation			
Media	DMEM/F12 w/N2B27	DMEM/F12 w/N2	–
Factors	GDNF, BDNF, CNTF	GDNF, BDNF, Shh, RA, cAMP, ascorbic acid	–
Efficiency (%)	~20	~15	70–80
Duration (days)	38	56–100	42–44

generation of embryoid bodies (EBs), the second method differentiates hSCs directly into neural rosettes without the formation of EBs, and the third method is based on the progressive differentiation of EBs into neural rosettes.

The first protocol begins the neural differentiation process with the lifting of hSC colonies from mouse embryonic feeder fibroblasts. The detachment of hSCs from MEFs and the formation of aggregates in suspension (EBs) initiate the differentiation process. The hSCs aggregates are usually grown as free-floating spheres in regular cell culture dishes or flasks; any contaminating MEFs remain attached to the flask. The cell culture medium is identical to that used for hSC culture without fibroblast growth factor (bFGF), except from Ebert et al. [22] that used human neural progenitor growth medium supplemented with B27, bFGF, EGF, and heparin. The differentiation process does not require exogenous growth factors, but it may be favored by the addition of noggin [23–25].

The second protocol generates neural rosettes from hSC colonies under adhesion conditions. Neural rosettes are unique clusters of small, elongated (or columnar) cells surrounding a central, small, cell-free zone [26, 27]. Cells are plated on poly-L-lysine/laminin (PLL/LM)-coated culture dishes in N2B27 neural differentiation medium, a 1:1 mix of DMEM/F12 and Neurobasal media supplemented with N2 and B27 for the growth and long-term viability of post-mitotic neurons. The medium is additionally supplemented for the first 10 days with mouse or human recombinant noggin or dorsomorphin as an inhibitor of bone morphogenetic protein (BMP)-signaling. Noggin, a secreted polypeptide that binds and inactivates members of the transforming growth factor-b (TGF-b) superfamily of signaling proteins, is critical for neural tube fusion during embryogenesis [28]. In most culture methods, noggin is thought to act as a suppressor of non-neural differentiation pathways, and as an enhancer of the directed differentiation of pluripotent stem cells into neuroectoderm through antagonistic action on BMP signaling pathways [28]. Primary colonies are plated onto new PLL/LM-coated culture dishes and cultured for another 7 days with N2B27 supplemented with BMP inhibitors supplemented with the BMP inhibitor dorsomorphin, resulting in neural rosettes [29]. As an alternative method, the generation of neural rosettes via co-culture of hESCs on stromal feeder in serum replacement medium has also been described [30]. At day 16, cultures were switched to modified N2 medium [31], and noggin was added to the culture in a subset of the experiments [30]. In the literature, another protocol to achieve neural rosettes is described: hESC colonies were transferred in modified TeSR1 medium [32] until the cells attached and appeared rosette structures [33].

The third protocol forms neural rosettes from EBs, which are plated in laminin/poliornithin-coated plates in the presence of a neural induction medium consisting of F12/DMEM, N2 supplement, and retinoic acid (RA). The cells organize into neural tube-like rosettes and, after 7–8 days in culture, sonic hedgehog (Shh) and cyclic adenosine monophosphate (cAMP) are added to the culture media for one additional week [34]. Alternatively, the human EBs can be held as suspension cultures in the absence of bFGF for 6 days, followed by cell plating on laminin-coated dishes in N2 medium [24, 35]. After 6 days, RA is added and the cells differentiate for 3 days until columnar cells form [24, 35]. Medium consisting of F12/DMEM, N2 supplement with heparin (2 g/ml), Shh, RA and cAMP with or without FGF2 can be used for 15 days [36, 37].

NE cells obtained by all the preceding protocols show very early neural tube morphology in cross section, and express early neural markers such as nestin, Musashi-1, β-tubulin, OLIG2, Nkx6, and the NE transcription factors PAX6, SOX1, SOX2, and SOX3.

Motor neuron differentiation

The acquisition of a precise identity by the different spinal cord populations, including motor neurons, is created with

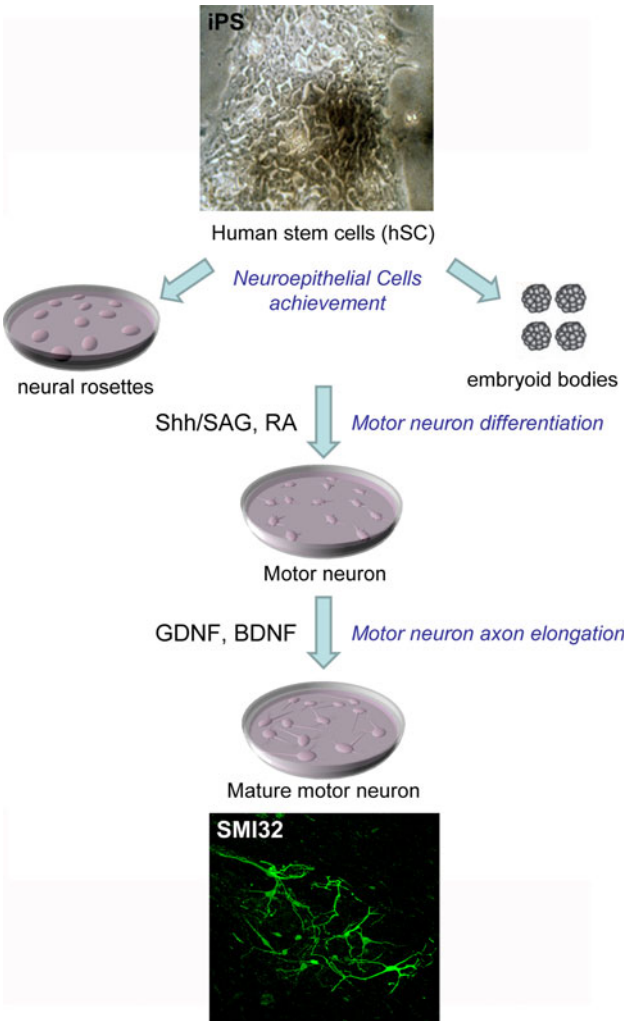

Fig. 1 Differentiation of hSCs into motor neurons. Schematic procedures for the in vitro differentiation of hSC (a picture of iPS cells is shown here). hSC are differentiated into neural rosettes or neural precursors were produced by neural selection and expansion procedures after embryoid bodies formation. Neural precursors are differentiated into motor neurons by treating the cells with signaling molecules, such as Shh or Shh agonists, and RA. The cells achieved further maturation in the presence of motor neuron growth factors like BDNF or GDNF. In the lower picture, the detection of motor neurons from differentiated iPS was performed here by immunocytochemistry for SMI32, a neurofilament protein expressed by motor neurons

a remarkable defined program during mammalian embryonic development [38]. The allocation of cells in the spinal cord depends on two signaling systems that intersect along the rostrocaudal and dorsoventral axes of the neural tube. The first step in the development of motor neurons is the acquisition by the neural cells of a "spinal cord" character by a series of extrinsic signals that include in particular RA (caudalization). Then, cell specification proceeds along the dorsoventral axis in response to graded extrinsic that comprise in particular sonic hedgehog (Shh) (ventralization) [39–41]. In brief, Shh and RA, with FGF signaling induce the expression of distinct combinations of transcription factors in neural progenitors. These initial profiles are subsequently refined by a cross-repressive interaction between transcription factors expressed. Progenitors that give rise to motor neurons depend on the activation of the bHLH protein Olig2 and the homeodomain factors Pax6, Nkx6.1, and Nkx6.2 [42–44]. Thus the first major result of the dorsoventral signaling network is to define the identity of motor neurons in contrast to a ventral interneuronal phenotype. Then, motor neurons became positive for a specific set of homeodomain transcription factors (like Hb9, Lhx3, Isl1, and Isl2), that control some common features of all spinal motor neurons, such as an axon that project outside the spinal cord and the cholinergic transmission [45–49]. The additional signaling pathways during the development are probably necessary for the successive diversification of motor neurons into distinct subtypes. In fact, within the spinal cord, the motor neurons that innervate these diverse muscle targets are organized into discrete clusters defined as motor columns, divisions, and pools that are relatively fixed. Like the previous embryonic developmental processes, motor neurons acquire positional information in response to graded signals which promote specific transcriptional programs within progenitor and postmitotic cells. In this regional specification process, a critical step, along the rostro-caudal axis, is the establishment of selective patterns of Hox gene expression within specific motor neuron subtypes. These genes are key factors that control motor neuron subtype identity and target muscle specificity. Systematic analysis of the expression of Hox gene expression in chick spinal cord demonstrated that nearly two dozen are expressed by motor neurons, in a manner consistent with a role in motor pool specification [50]. Finally, the final picture of network signaling in motor neuron development is even most complicated given that, although Hox protein activities appear to be essential in the generation of diverse motor neuron subtypes, several lines of experiments support the hypothesis that additional factors are necessary to restrict their functions [51]. Motor neuron differentiation in vitro seems to recapitulate the molecular events involved in normal spinal cord development.

It has been described that murine ES cells differentiate preferentially into medial motor column neurons that project preferentially to axial musculature [52]. Indeed, experiments with mouse cells suggest a propensity for the rostral spinal cord identity as supported by the HoxC5 and HoxC6 expression [53]. On the other hand, experiments with hESC-derived motor neurons demonstrated the derivation of motoneuron progeny with more caudal phenotypes as assessed by the expression of HoxC8 [36]. However, the work by Lee et al. [30] showed that gene expression analysis of hESC-motor neurons indicated a biased expression toward class 4–6 Hox genes similar to the profile reported for mouse ESC-derived motor neurons.

Immunocytochemistry demonstrated that hESC motor neurons are positive for both rostral (HoxC6) and more caudal (HoxC8) markers. Overlap of HoxC6 and HoxC8 supports a posterior type brachial motoneuron identity.

However, the propensity of human stem cells to differentiate into rostral or caudal subtypes as well as to generate medial or lateral motor column as well as cranial motor neurons has to be thoroughly investigated. Indeed, the specific differentiation signal that promotes the generation of specific neuronal subtypes to reproduce the vast spectrum of spinal cord motor neurons for in vitro and cell replacement applications must be investigated. The modulation of the network of Hox genes that control columnar and pool motor neuron identity may be crucial to precisely control the differentiation of precursor cells into specific motor neuron subtypes.

Most researchers use a neurobasal medium containing N2, RA, and Shh to induce motor neuron differentiation from NE cells. RA plays important roles in the development, regeneration, and maintenance of nervous system cells [10, 54]; in particular, RA promotes the induction of caudally committed NE cells [55], and its effectiveness in promoting differentiation is enhanced by the presence of additional signaling molecules, such as Shh, that are critical for patterning the early embryo, particularly the ventral neural tube. Treatment of human SC-derived neuroectodermal cells with RA and Shh has resulted in the generation of spinal motor neurons [30, 56, 57]. Various effective concentrations have been reported, from 1 nM to 1 μM for RA, and from 50 to 500 ng/ml for Shh [17, 21–23, 25, 29, 30, 34–37, 58]. Other differentiation cocktails have been described, supplementing RA and Shh with B27 [23, 29], cAMP [34, 37], heparin [37], brain-derived neurotrophic factor (BDNF), and ascorbic acid [25, 30]. Purmorphamine or SAG, small-molecule activators of the Shh pathway, can substitute for Shh and, in association with RA, promote the formation of motor neurons at various concentrations (0.5, 1, 2, and 5 μM) [23, 24, 29, 37]. However Erceg et al. changed the medium in GRM (DMEM:F-12, B27 supplement, human insulin, progesterone, putrescin, sodium selenite, human holotransferrin) supplemented with 10 μM RA and maintained obtained motor neurons in the presence of bFGF [33].

RT-PCR and immunocytochemistry of obtained motor neurons demonstrate the expression of specific motoneurons markers such as Islet1, ChAT, HB9 [29, 34, 35] and HOX [35, 36] genes.

Motor neuron axon elongation

Motor neurons must extend their axons to the body periphery and connect with their appropriate target in order to obtain complete functionality in vivo. This crucial step has been recently demonstrated to be feasible, although is difficult to achieve in vitro [10–12, 53]. After isolation from tissue culture, motor neurons maintain their polarity and grow processes with the characteristic features of dendrites and axons. Neuron survival and neurite growth are stimulated by neurotrophic factors, added to the culture medium, that mediate their effects through specific receptors. The classic supplementary molecules are BDNF and glial-derived neurotrophic factor (GDNF), both of which promote axonal elongation. Other than BDNF and GDNF, insulin-like growth factor-1 [23, 34, 36], ciliary neurotrophic factor [21, 24, 35], neurotrophin-3 [29], and cAMP [22, 23] may support axon growth. On the other hand, use of a single neurotrophic factor—GDNF—has also been reported [25]. Reduced concentrations of Shh and RA are recommended at this stage, as high concentrations inhibit motor neuron differentiation [23, 35]. At the end of the differentiation stage, cells are positive for synapsin and can incorporate α-bungarotoxin when cocultured with myotubes, indicating that they can form functional neuromuscular junctions [23, 34, 36].

The establishment of the phenotypic maturation and the ability of differentiated motor neurons to generate action potentials are crucial step that are determined by studying their electrophysiological properties. It is well established that the firing of repetitive action potentials in response to current injection is typical behavior of adult vertebrate motor neurons [59] and that this repetitive firing develops as a function of their maturation [60]. All of the authors that performed electrophysiological analysis used the standard whole-cell patch clamp technique, in particular current clamp [24, 29, 30, 34–36] and/or voltage clamp [29, 33, 34, 36] recording. In all of these experiments, a correct functional activity specific of bona fide motor neuron was demonstrated.

A detailed understanding of the naturally occurring variations among ESC, and likely iPS cell lines, linked to donor characteristics, method of derivation and quality of cells, will also be important for evaluating specific differentiative potential of different cell lines into motor neurons. Hu et al. [61] compared the capacity of iPSCs versus hESCs to differentiate into neural cells (neurons and glia). They showed that both hESCs and iPSCs follow the same events and time course during neuroectodermal differentiation. However, although hESCs differentiate into neural cells with a similar efficiency no matter of the cell line used, iPSC-derived neural cells show greater variation and lower efficiency in neuronal differentiation [61]. The same difference was observed by Karambayaram et al. [24]. However, once human iPS cells are specified to a neural lineage, they could be differentiated to form motor neurons with a similar efficiency as hESCs. Even if, up to now, no specific direct comparison in the motor neuron

differentiation capacity between the different ES or iPS cell lines were performed, no specific differences are evident. Even if each research group used its own protocol for the generation of motor neurons, a consensus can be found in the use of retinoic acid and sonic hedgehog pathway agonist as necessary step in the differentiation process into motor neurons.

As we described above, hES and iPS cells can be differentiated into motor neurons through the formation of embryoid bodies (EB) or omitting the EB step by direct differentiation into neural rosettes. Given that each hES can differentiate into multiple cell types and that the formation of EBs leads to the formation of heterogeneous cell types, further development of new methods that avoid the EB formation is useful to obtain the goal of a more homogenous cell population. It has been recently described that hESC lines can be derived from floating inner cell masses in suspension culture conditions that do not involve feeder cells or microcarriers. This culture system allows the propagation of the pluripotent stem cells as floating clusters without their differentiation into EBs. These hESC clusters can be differentiated in suspension into neural spheres. These results open the pathway for large-scale expansion and controlled differentiation of hSC [62].

The majority of the hESC and iPS cell derivation and culture protocol involve the use of animal-derived products at several steps, making them unsuitable for the generation of clinical-grade cells.

However, the generation of hESC and iPSC under xeno-free condition has been achieved [63, 64]. Even if the generation of clinical grade stem-cell derived motor neuron has not yet been achieved, it is possible, with the present technical knowledge, to substitute the animal-reagents of most of described protocols with xeno-free and clinical-grade products.

Isolation of highly pure motor neuron populations

For subsequent experiments, such as therapeutic transplantations for MND treatment and the creation of in vitro models, it is important to purify or enrich motor neurons from an hSC-derived mixed cell population. At present, there are no absolutely specific cell-surface markers for human motor neurons, but several reports have described primary motor neuron purification from spinal cords of embryonic chicks [65–67], rats [67–69], and mice [70–72] using protocols based on gradient cell centrifugation, surface markers (p75), and the expression of motor neuron-specific reporter genes.

Gradient centrifugation has been applied to purify hESC-derived spinal motor neurons [22]. A discontinuous gradient was prepared by overlaying two different densities of Biocoll, and then cells were placed in the gradient. Following centrifugation, two interfaces were carefully collected and immunochemistry was performed, indicating that 78% of the cells in the second interface were HB9-positive [29].

Motor neuron immunomagnetic isolation is rapid and quantitative, essential technical details when dealing with fragile neuronal cells. A panning immunological technique was developed in the 1990s and successfully applied to neuronal cell isolation, including motor neurons [42, 47, 48]. The expression of a motor neuron surface-specific antigen was immunologically targeted in order to separate motor neurons from dispersed cell suspensions [68, 73, 74]. The transiently expressed nerve growth factor receptor p75-NGFR was chosen for motor neuron isolation from embryonic day 15 to postnatal day 15 rat spinal cord [75] and mouse spinal cord [72]. p75-NGFR is a highly expressed receptor [76–78], and antibodies against p75-NGFR have been successfully used to enrich motor neurons from spinal cord cell suspensions [68]. Human developing spinal motor neurons also express p75-NGFR [79], and a simple, reproducible, quantitative immunomagnetic method has been developed to isolate motor neurons [6, 80]. This method represents a modification of the original panning technique previously described for rodents [67]. However, p75 antigen is also expressed in ganglia cells [81], and thus can be used with relative specificity when motor neuron are isolated from tissue after mechanical isolation of ventral horns [6]. However, p75 antigen can be less useful when obtained from hSCs that generate several neuronal populations in vitro.

Recently, researchers have isolated motor neurons through Hb9-driven GFP expression. hSCs have been transfected with plasmids encoding GFP, placed under the control of a motor neuron-specific enhancer inside the gene encoding the transcription factor Hb9. As motor neuron differentiation is induced under the influence of Shh and RA activate Hb9-driven GFP expression, permitting isolation of GFP-expressing cells by fluorescence-activated cell sorting (FACS). This approach allows high-efficiency induction and isolation of functional motor neurons from hSCs [58] and has been employed by multiple groups [24, 25, 35]. As an alternative, post-mitotic human motor neurons can be visualized after transduction with a lentivirus expressing GFP under the control of the Hb9 promoter [34].

Clinical relevance

Human-derived motor neurons as an in vitro model of human motor neuron disease

In vitro models of human pathologies offer new insights into MNDs. For example, in an in vitro model of

astrocyte-dependent motor neuron damage, human Hb9-GFP ESC-derived motor neurons were co-cultured with wild-type glial cells or glial cells harboring a mutant allele of the superoxide dismutase 1 gene (SOD1G93A) [25]. This study demonstrated that human motor neurons are selectively sensitive to the glial toxic effect, while interneuron populations are unaffected. Specific toxicity to motor neurons was associated with several significant changes in the expression of pro-inflammatory glial genes [25]. Motor neuron numbers decreased by approximately 50% in the presence of mutant SOD1-expressing astrocytes, with no detectable effect on other neuron subtypes [34]. The activation of mutated astrocytes was observed, as were the production of oxygen radicals and overexpression of pro-inflammatory genes [34].

The possibility of obtaining motor neurons from hSCs has opened the door to the direct modeling of MNDs in the cell culture using human motor neurons. Stem cells can now be manipulated to express mutant genes linked to MNDs. Decreased smn expression in neuroblastoma hybrid (NSC-34) cell lines was achieved through the use of small interfering RNAs, reproducing an in vitro model of SMA [82]. Decreased smn levels increase apoptosis, but increased smn can confer neural protection [82].

The expression of a mutant version of SOD1 has been linked to familial ALS in hESC-derived motor neurons [34]. Three different ALS-associated SOD1 mutations (G93A, A4V, and I113T) have been expressed in hESC-derived motor neurons, resulting in motor neurons with characteristics of ALS-related degeneration such as reduced neurite extension and enhanced cell death. Previous co-culture studies performed in vitro with murine cells have shown that astrocytes carrying mutant SOD1 have a toxic effect on wild-type mouse motor neurons [83, 84] and that this event is more severe in mouse motor neurons expressing the SOD1 mutation [83]. The use of hESC allowed reproducing these results demonstrating that also human motor neurons, but not interneurons, are selectively sensitive to the toxic effect of glial cells carrying an ALS-causing mutation in the SOD1 gene [34, 83]. Overall, these studies potentially serve as a platform for the in vitro study of motor neuron pathology and potential treatments for motor neuron degeneration [35]. The previously reported strategy of expressing disease-inducing mutations in hESC-derived neurons may also be useful for studying genetic forms of other neurodegenerative diseases with patient subpopulations that carry known genetic defects.

All data obtained with hES are confirmed and supported by results initially generated with murine ES [83–85].

As an alternative motor neuron source, recent studies have reported the use of iPS technology to create in vitro models of MNDs. iPS cells were generated from the primary fibroblasts of an 82-year-old woman diagnosed with a familial form of ALS; these cells exhibited ESC properties and were able to differentiate into motor neurons and glia [21]. In another study, iPS cells were derived from a type-1 SMA patient and his unaffected mother, showing that these cells can generate differentiated neural tissue and motor neurons while maintaining the lack of SMN1 expression and the disease phenotype of selective motor neuron death [22]. iPS-SMA cells initially produced similar numbers of neurons and motor neurons to wild-type cells, but at later time points, motor neuron production was compromised and/or motor neuron degeneration increased. Moreover, iPS-SMA cells responded to compounds known to increase SMN protein in a similar fashion to fibroblast-SMA cells [22]. This is the first report describing disease-specific effects on human motor neuron survival in an iPS model, as well as the benefit of the drug-induced increase of protective proteins.

HSC-derived motor neurons provide the opportunity to study early disease pathways in living human tissue; traditionally, human CNS tissue had to be obtained post-mortem. Such human cell-specific disease models will facilitate the investigation of disease mechanisms, a crucial step in identifying new therapeutic targets. This cell model is especially useful for studying diseases lacking rodent models that accurately reflect the human pathology.

Therapeutic development based on hSC-derived motor neurons

ESCs and iPSCs have great potential for cell therapy against MNDs, representing a potential source of motor neurons for cell replacement. However, one limit using ES-derived cells is host immunorejection of the transplanted cells. Conversely, iPS cells can be generated from the tissues of the same patient enabling the generation of autologous motor neurons. Indeed, in case of a MNDs linked to a known genetic defect, ex vivo gene therapy correction of the patient stem cells could be used before the cells are differentiated in motor neuron and then transplanted. Furthermore, the complete genetic correction of iPS cells from Duchenne muscular dystrophy as initial proof of concept of this strategy has been recently described [86].

Although the road to the clinical application of hESC-derived motor neurons remains fraught with obstacles, important first steps in this journey are the ability to generate unlimited numbers of motor neuron progeny and the capacity for in vivo survival and integration of these cells into the developing and adult spinal cord. To date, only one study has been performed regarding in vivo hSC-derived motor neuron survival and engraftment [30]. Transplantation into the developing chick embryo of hESC-derived motoneuron progeny resulted in robust engraftment, maintenance of motoneuron phenotype and markers, and long-distance axon growth outside the CNS, reaching peripheral targets in the

trunk musculature [30]. Transplantation into the adult rat spinal cord yielded neural grafts comprising a large number of human motor neurons with outgrowth of choline acetyltransferase-positive fibers [30]. This observation suggests that hESC-derived motor neurons may be able to project toward the ventral root in the adult spinal cord, similar to the embryonic chick spinal cord, albeit at a different time scale. No signs of tumorigenicity in any of the grafted animals were noted [30]. These data provide evidence for in vivo survival of hESC-derived motor neurons, a key requirement in the development of hESC-based cell therapy and future preclinical development. Additional studies are required regarding the capacity for long-distance axonal growth and the ability to innervate appropriate peripheral targets in the adult spinal cord.

Translation of such therapeutic approaches to human patients has been relatively slow. However, there have been remarkable changes towards hSC-derived motor neuron clinical applications since the first clinical trial using human spinal cord-derived neural stem cells. The use of neural stem cells derived from the spinal cord of an aborted fetus was approved by the US Food and Drug Administration for the treatment of ALS in September 2009. This is the first time that these cells have been transplanted into humans, and this study is designed to ascertain whether the procedure and the cells are safe (http://www.neuralstem.com).

Conclusions

MNDs such as ALS and SMA are characterized by the progressive loss of motor neurons over time. Current therapies, which are ineffective for several of these disorders, focus on symptomatic treatment by orally administered drugs.

Recent progress in stem cell research has opened new avenues for the in vitro generation of large numbers of various neural cell types, and for their use in the repair of the nervous system. A thorough understanding of MND-specific pathways must grow in parallel with progress in fundamental stem cell biology, with the aim of obtaining reliable experimental information and adequate cell sources for in vivo neuroprotection and neurorepair. We have presented protocols and results from studies regarding the use of ESCs and iPSCs for motor neuron generation, both as in vitro models and potential cell-based MND therapies. Translation of hSC-derived motor neurons to the clinic is possible in the future.

Acknowledgments The financial support of the following research grants to S.C. and G.P.C. is gratefully acknowledged: FSMA and SMA Europe Grant, Telethon grant: GGP09107, "Neuroprotection in Spinal Muscular Atrophy (SMA) using neural stem cells as a therapeutic approach." We wish to thank the Associazione Amici del Centro Dino Ferrari for their support. We wish to thank Dr. Serena Ghezzi for her help in graphic illustration.

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