5 Locomotor Recovery After Spinal Cord Transection: Transplantation of Oligodendrocytes and Motoneuron Progenitors from Human Embryonic Stem Cells

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Abstract

During the last few years, human embryonic stem cells have begun to take a place in the stem cell therapy panorama, especially in respect to the nervous system. The extensive experimental research efforts have focused on translating in vitro cellular regeneration to in vivo transplantation and survival of the transplants, in order to improve clinical outcomes. For spinal cord injury recovery, two major types of cells are in focus: the oligodendrocytes and motor neurons. In this chapter, we will discuss the progressive development of the cellular generation protocols and the locomotor outcome of their transplantation at sites on spinal cord injury. The newly advanced method of motor neurons and oligodendrocytes generation form human induced pluripotent stem cells will be also discussed.

Introduction

In 1908, the Russian histologist Alexander Maksimov proposed that all the blood cells and the process of hematopoiesis are derived from hematopoietic stem cells. Fifty-five years later, in 1963, the Canadian scientists James E. Till and Ernest A. McCulloch demonstrated the presence

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of these stem cells for the first time in mice bone marrow. Since then, research on the characteristics and therapeutic applications of these cells has initiated a new era of medicine.

Stem Cells

The presence of more than 200 types of cells in the human body gives a clue of the high differentiation potential of the stem cells. Stem cells are non-differentiated cells that have the capability of proliferation, self-renewal, formation of large numbers of different types of cells, and regeneration of the already differentiated tissues. The potency of the stem cells can be determined depending on how many types of differentiated cells, and of what germ layers, the stem cells are capable to produce. This is defined as the 'differentiation potential'. The totipotential stem cells constitute the first line of cells during fetal development following fertilization, and may give rise to extraembryonic and embryonic cells. Following the separation of inner and outer cell masses, the cells of the inner cell mass (embryonic cells) are defined as pluripotential stem cells, and may give rise to any type of cells from the yet-to-be formed three germ layers: ectoderm, mesoderm, and endoderm. When these layers are separated, cells of each layer are classified as multipotent, oligopotent, and unipotent, depending on their differentiation potentials (Cherian et al. 2011).

Of the above mentioned types of the stem cells, two have received most attention in regard to stem cells therapy: embryonic stem cells (ESC) and adult stem cells (ASC). The ESC are pluripotent cells that present during early stages of human development, and have a high proliferative capacity, and may differentiate into any type of cells. After the formation of the tissues and organs, most of them retain a population of stem cells during childhood and adulthood. These cells are defined as the ASC, which, under normal conditions, give rise to that specific line of cells of the retaining organ. However, other tissues, for unknown reason, including brain, spinal cord, heart, and kidneys, with minor exceptions, do not maintain their stem cells, which limit the use of ASC as a therapeutic choice in these tissues (Kiessling 2003). Recently, novel studies were capable of reprogramming these somatic cells into pluripotent stem cells, known as induced pluripotent stem cells (iPSC), as is discussed later in this chapter.

More than two decades of intense research on mouse ESC has provided insight into human ESC (hESC) research despite the differences between the two types of cells (Kang et al. 2007). They have also provided the proper methods of differentiating mouse ESC into several clinically relevant neural and non-neural cell types (Keirstead et al. 2005). In 1998, Thomson et al. were the first to isolate the hESC, using 14 inner cell masses of in vitro fertilization (IVF)-produced embryos as a source. Since then, the blastocysts of IVF- embryos constituted the major source of hESC. Other sources of ESC include nuclear transfer and therapeutic cloning. The former is achieved by transferring the nucleus of an adult differentiated egg into an enucleated egg, which is then stimulated to form blastocysts in a backward fashion. Therapeutic cloning is so called due to use of cloning to create ESC for therapeutic purposes (Cherian et al. 2011). Nevertheless, the directed generation and isolation to purity of specific clinically important neuronal phenotypes from human ES cells has yet to be accomplished.

A comparison between experimental generation of oligodendrocytes and motor neurons is summarized in Table 5.1.

hESC as a Source of Oligodendrocytes

Oligodendrocytes (OL) are one type of glial cells that provide support to the central nervous system (CNS), mainly by the formation of the myelin sheath. They extend into high numbers of branches and sub-branches expanding into sheets of myelin membranes that wrap around multiple neural axons. This myelin sheath facilitates the rapid salutatory conduction and insulation of the nerve cells (Hatch et al. 2007; Izrael et al. 2007).

Table 5.1 Characteristics and comparison between oligodendrocytes and motor neurons linages

	Oligodendrocytes linage	Motor neurons linage
First use	Nistor et al. (2005)	Li et al. (2005)
Inducers	RA, Noggin, SHH, AA, EGF, PDGF, FGF, CNTF, IGF, HGF, T3, puromorphamine	RA, Noggin, SHH, puromorphamine, SAGA, dorsomorphin, BDNF, CNTF, GDNF, FGF, IGF1, AA, cAMP, NT-3, ROCK
Inhibitors	BMP	BMP
Expressed genetic markers	Sox8, Sox9, Sox10, Olig1, Olig2, Nkx2.2, Nkx6.2, A2B5, NG2, PDGF-R, PLP, Ngn3, Gli1, Gli2, OMG, MBP, GalC, RIP, O4, O1	Pax6, Sox1, Sox2, Sox3, Nestin, Otx2, NG2, HOXB1, HOXB4, HOXB6, HOXC5, HOXC8, HOXC10, HLBX9, Olig2, Nkx2.2, Nkx6.1, Irx3, GFP, HB9, Islet1, islet2, ChAT, MAP2, β III-tubulin, Musashi1, PTCH, Tuj1
Secreted factors	TGF-β1, TGF-β2, activin A, VEGF, BDNF, midkine, SCF	–
Integration and maturation after transplantation	Yes, at early and late stages	Yes
Locomotor improvement	Yes, only at early stage	Yes
Studies on humans	No	No

However, this is not the limit of their function, which far exceeds their insulating role and extends to promoting neuronal and axonal survival by secreting different types of neurotrophic factors. Loss of the OL function and consequent demyelination and axonal degeneration can be seen in a number of neurological pathologies, including multiple sclerosis, Pelizaeus-Merzbacher, Alzheimer's disease, stroke, progressive multifocal leukoencephalopathy, multisystem degeneration, oligodendrogliomas, and spinal cord injury (Keirstead et al. 2005; Nistor et al. 2005).

Oligodendrocytes in Spinal Cord Injury

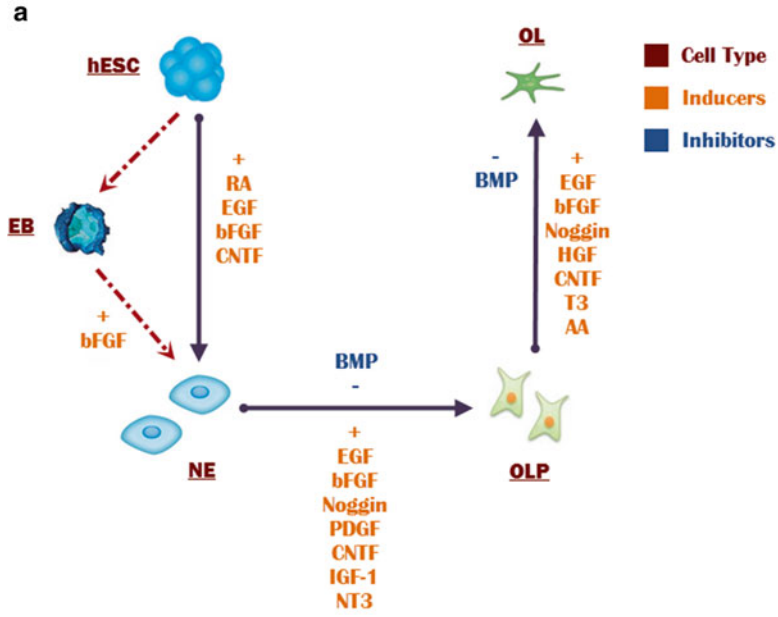
Following spinal cord injury (SCI), loss of OL and oligodendrocytes progenitor cells (OPC) occur via two main processes that are timed during the initial insult and the secondary degenerative changes: acute necrosis and subacute apoptosis. Autophagy may also occur for at least 21 days following injury. The acute necrotic phase starts as early as minutes to few hours after injury, and the subacute apoptotic phase extends up to weeks. During the early phase of injury, vascular rupture leads to release of toxic digestive proteolytic enzymes. Blood components may also induce apoptosis and necrosis of OPC, and inhibit their proliferation and migration. Other factors contributing to the acute injury include the free radical (reactive oxygen and nitrogen species) formation following ischemia and reperfusion, and excitotoxicity. The latter is mainly attributed to the release of glutamate and adenosine triphosphate (ATP) at the site of injury, which in turn activate the glutamate and P2X7 receptors, respectively. These receptors attract the OL and OPC to the site of injury and cause further cellular loss. They also lead to intracellular calcium release, which can trigger cellular apoptosis. Infiltration of the injury site by the early and late phase immune cells, including neutrophils, monocytes and their derived macrophages and microglia, and lymphocutes, and their various types of secreted inflammatory mediators lead to direct lyses and apoptosis of the OL and OPC, and inhibit their growth and proliferation. On the long term, Wallerian degeneration of the nerve axons induces sustained apoptosis of the OL, which is supported by the trophic factor released from these axons. Few weeks after injury, the above mentioned mechanisms lead to detected wide-spread demyelination of the nerve axons, and may continue to progress for 1–22 years following the injury. However, wide-range demyelination is prevented by concomitant remyelination, which may also start few weeks after the

insult. Although remyelination is not perfect, it is sufficient to preserve function of spared intact axons, and maintain their integrity. This acute phase remyelination is mediated by the proliferating myelogenic progenitor cells that present at the margins of injury, and identified by the expression of nerve/glial antigen 2 (NG2) or platelet derived growth factor receptor (PDGFR). Mature form of the OPC has less capacity to remyelinate, and needs prolonged exposure to growth factors to convert them into proliferating cells. The specification of OL from the progenitor cells is induced by the sonic hedgehog (SHH) and opposed by the bone morphogenetic factor (BMP). Both SHH and BMP are up-regulated at the site of injury. The presence of the astrocytes, often produced by the proliferating OPC, is essential. They play a role in maintaining the survival, proliferation and differentiation of the OPC and OL by secreting different typed of growth factors. These factors include PDGF, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and ciliary neurotrophic factor (CNTF). Other less important factors include neurotrophin (NT-3), brain-derived neurotrophic factor (BDNF), chemokines CXCL1, CXCL12, and CXCR4, and cytokines IL-1β, Leukemia inhibitory factor (LIF), transforming growth factor (TGF) β1. The increased expression of thyroid hormone receptor, the nuclear receptors peroxisome proliferator activated receptor (PPAR)-δ, and retinoic acid receptor also play a significant role in the OL differentiation. Most of these factors are secreted during the early phase of spinal cord injury. Thus, over long time, the capability of the OL and their progenitors decreases progressively in terms of maintaining the axonal function. Moreover, despite the essential early role of the astrocytes, at the 2nd to 4 weeks of injury, they begin to form a dense astrocytic scar surrounding the demyelinating axons, which prevent the OL and OPC from reaching the site of injury. They may also express other molecules (e.g. Jagged1) that inhibit maturation and differentiation of the OL and OPC. For these reasons, in addition to younger age, early treatment of spinal cord injury is significant in enhancing the locomotor function. This period is called the 'therapeutic window', and extends from the acute inflammation to before the scar formation (Faulkner and Keirstead 2005; Sharp and Keirstead 2007; Almad et al. 2011). Thus, one of the promising treatment options in the SCI is the OL and OPC transplantation, as replacement of the non-functioning cells may provide an ideal solution to preserve the axonal function and suppress their progressive loss.

Experimental Generation of Oligodendrocytes from hESC

The first step in OL transplantation is to isolate a pure, functional lineage of these cells. Sources of human OL may include aborted fetuses, olfactory biopsies of the neuroepithelium, and differentiated hESC. The latter may provide the ideal source due to its high proliferation and differentiation potentials (Sundberg et al. 2010). The major challenge in this situation is the ability to produce high purity differentiated OL and OPC from directed differentiation of hESC, and reduced the of teratoma formation. The process of selective proliferation and differentiation of the hESC into fully mature OL passes through neural embryoid body (EB) cells, neural progenitor (NP) or neuroectodermal (NE) cells, glial-restricted precursor (GP) cells, and OPC formation (Fig. 5.1a).

In 2005, Nistor et al., Keirstead et al., and Faulkener et al. were the pioneers of this field, and were able to direct the hESC differentiation into high purity and functioning OL and OPC through yellow neurosphere or NE formation. They used a modified retinoic acid (RA) caudalizing protocol to induce restricted differentiation to a multipotent neural lineage, which could generate neurons, OL, and astrocytes by employing RA exposure, along with preferential selection of OL lineage cells by media components. The used glial restriction media (GRM) contained insulin and IGF, differentiation factor triiodothyroidin hormone, FGF, and epidermal growth factor (EGF), and it played important role in the proliferation and survival of the OL (Nistor et al. 2005). Their method generated about 80 %



b **OL Linage Markers**

hESC	NE	OLP	OL
SSEA4	Pax6	Olig1	PLP
OCT4	Nestin	Olig2	O1
TRA-1-60	Mash1	SOX8	O4
TRA-1-81	OPC	SOX9	MBP
		SOX10	GalC
		A2B5	RIP
		NG2	
		OMG	
		PDGF-R	
		Nkx2.2	
		Nkx6.2	
		Gli1	
		Gli2	

Fig. 5.1 (**a**) Generation of oligodendrocytes from human embryonic stem cells. *hESC* human embryonic stem cells, *EB* embryoid bodies, *NE* neuroepithelial cells, *OPC* oligodendrocytes progenitors, *OL* oligodendrocytes. (**b**) Cellular markers of the oligodendrocytes linage. *hESC* human embryonic stem cells, *NE* neuroepithelial cells, *OPC* oligodendrocytes progenitors, *OL* oligodendrocytes

population of OL capable of myelin formation in vivo, but no highly branched, ramified and mature OL were reported in vitro. Markers of the OL differentiation included Olig1, SOX10, A2B5, NG2, and PDGFR.

In 2007, Izrael et al. added Noggin, antagonist of BMP, following treatment with RA. The addition of Noggin turned out to be the key for the development of highly branched and mature OL in vitro, which also significantly enhanced their capacity to myelinate after transplantation. The RA functions through stimulation of the Nkx2.2 gene that is required for terminal differentiation of OL. However, for proper function, the Nkx2.2 needs the Sox10 gene activation, which is also an OL-specific gene, and is induced by the addition of Noggin at specific stages of development (Izrael et al. 2007). Olig2 transcription factor gene induction is also an important step in the differentiation of human OL and their

progenitors. Evidences of such role of Olig2 were provided following its induction using sonic hedgehog (SHH) (Hu et al. 2009a) and inhibition using BMP (Izrael et al. 2007). In 2007, Kang et al. used the growth factors EGF and PDGF to induce the formation of 81–91 % OPC from neural precursors after their isolation and expansion from the hESC using specific media. These progenitors were then treated with the removal of the growth factors and the addition of the thyroid hormone T3 to generate mature OL. The formed mature OL represented 81 % of the total cells number.

Hu et al. (2009b) described a simpler method that included the removal the G5 supplement media (containing insulin, transferrin, selenite, biotin, hydrocortisone, FGF, and EGF), which was applied for a certain period of time, and the addition of the hepatocyte growth factor (HGF) to enhances the proliferation of neural progenitors derived from hESC and promote the generation and maturation of OL. This method yielded OL with high purity (about 80 %).

In 2010, Sundberg et al. introduced a novel method for the generation of the OL from hESC using human recombinant growth factors and extracellular matrix (ECM) proteins. This was in contrast to the previous protocols that employed animal-derived (Matrigel) media, which is less suitable for clinical applications in humans. Their ECM media contained laminin, collagen IV, and nidogen-1 that together facilitate the OPC survival, maturation, and myelination. The growth factors used included; FGF, EGF, and CNTF for the initial neural differentiation; and PDGF-AA, EGF, bFGF, CNTF, and IGF-1 to enhance the survival, proliferation, and differentiation of the OL and their precursors. At the last stage of the cellular maturation, the CNTF, ascorbic acid (AA), and T3 were added. All these growth factors were associated, in variant degrees, with the expression of large number of genes, including PDGF-R, NG2, Nkx2.2, Sox10, Olig1/2, myelin basic protein (MBP), proteolipid protein (PLP), Ngn3, Sox9, Sox8, Sox10, Gli1, Gli2, Nkx6.2, oligodendrocyte-myelin glycoprotein (OMG), O4, and GalC. The expression of these genes is dependent on the stage of development and cellular differentiation from hESC to fully mature OL (Sundberg et al. 2010) (Fig. 5.1b).

Important regulators of the genes expression during these stages are the MicroRNAs. These molecules are known as the "micromanagers" of gene expression, and they function by binding to the mRNA of protein coding genes. Full profile of the MicroRNA of human OL formation has been described for the first time by Letzen et al. in 2010. Identification of these molecules may provide key markers of OL maturation (Letzen et al. 2010).

In 2006, Zhang et al. studied the ability of the OPC derived from hESC to secret neurotrophic factors. Of all the genes tested, 49 growth factors were expressed by OPC at highly significant levels. Of these factors, TGF-β1, TGF-β2, activin A, vascular endothelial (VEGF), BDNF, midkine, and stem cell factor (SCF) proteins were of particular interests. These factors were found to play a remarkable role in neural regeneration and function restoration (Zhang et al. 2006).

hESC as a Source of Motor Neurons

Motor neurons (MN) are large nerve cells with extensive dendritic extension that are located in specific areas in the nervous system, including the brain cortex (upper motor neuron), brainstem, and spinal cord (lower motor neuron). By their specific location, the MN form connecting links between the CNS and skeletal muscles, and facilitate movement and breathing (Nizzardo et al. 2010; Takazawa et al. 2012).

Motor neuron diseases (MND) are group of neurodegenerative diseases that selectively affect the MN with consequent loss of function and motor paralysis. The MND can be divided into three categories: the first form involves both upper and lower MN, such as amyotrophic lateral sclerosis, which is the most common MND. The second form involves the lower MN, and includes spinal muscular atrophy and spinobulbar muscular atrophy or Kennedy's disease; hereditary motor neuropathies and progressive spinal muscular atrophy. The third form involves the upper MN and includes primary lateral sclerosis and

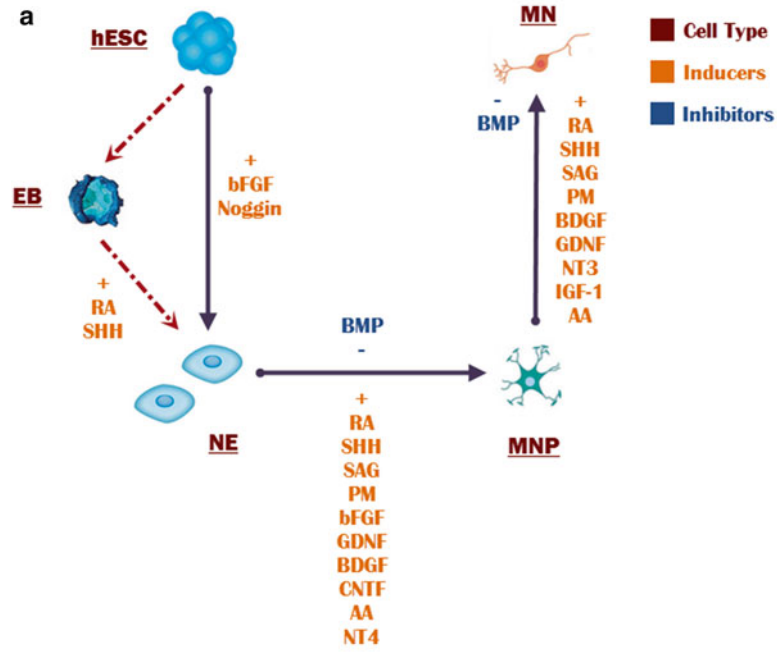
hereditary spastic paraplegia (Nizzardo et al. 2010). Beside these degenerative disorders, trauma (e.g. SCI) with its associated direct and delayed axonal loss remains one of the most common causes of permanent disability (Lopez-Gonzalez and Velasco 2012). Up to date, no effective therapy has been found to treat these conditions. However, because the degeneration of the MN is the main pathology in these conditions, their replacement has been proposed as a therapeutic potential. One major source for these cells is the hESC.

Experimental Generation of Motor Neurons from hESC

The process of MN derivation from the hESC can be achieved using different protocols. As in the case with the OL, the first step is the proper obtainment of the NE cell form the pluripotent hESC. This can be achieved either directly or with and intermediate process of EB formation. Then, these NE are used as a source of the motor neurons progenitors (MNP) and MN (Fig. 5.2a).

The first experiential generation of MN from hESC was done Li et al. (2005). After culturing the hESC in a feeder layer, differentiation into NE cells was induced using neural induction medium consisting of F12/DMEM, N2 supplement and heparin with or without bFGF. Two stages of NE cells could be identified: early and late stages. The early stage could be identified with the expression of Pax6 transcription factor gene but not Sox1. Thus, Pax6 is the earliest NE marker expressed during neural plate and tube formation. Both stages expressed Nestin. Even in the late stage, the NE cells were also positive for Otx2, a homeodomain protein expressed by forebrain and midbrain cells, but negative for HoxC8, a homeodomain protein produced by cells in the spinal cord, and engrailed 1 (En1), which is produced by midbrain. Thus, more selective differentiation was needed to produce caudal cells. Treating the NE cells, at early stage, with RA or bFGF was associated with caudalization of the NE cells. BFGF also induced rostral cells formation. The caudalization of the cells was marked by the expression of HOXB1, HOXB6, HOXC5, HOXC8, and HOXC10. All of these markers were induced by bFGF, whereas, HOXC10 was not induced by RA. BFGF, in contrast to RA, did not eliminate the expression of Otx2. Following caudalization, inducing ventral neural cell types was required for MNP formation. This was achieved by treating the culture with SHH, and was evident by the balanced expression of Class II (Olig2, Nkx2.2, Nkx6.1) and Class I (Irx3, Pax6) homeodomains. Continued exposure to bFGF suppressed the expression of SHH, class I and class II genes, and subsequently inhibited MN differentiation. The use of neuronal differentiation medium was needed at the differentiation stage. It consisted of the neurobasal medium, N2 supplement, and cAMP in the presence of RA and SHH for 1 week. Following the appearance of Olig2-MNP, BDNF, glial-derived neurotrophic factor (GDNF), IGF1 and a low concentration of SHH were added to the medium. The differentiated MN were characterized by the expression of HB9, Islet1/2, choline acetyltransferase (ChAT), MAP2, and β III-tubulin (Fig. 5.2b). The authors concluded that the cells with combined expression of Islet1/2 and HB9 are likely MN, whereas the cells with negative HB9 and positive Islet1/2 are likely interneurons. The differentiated cells represented almost 20 % of the culture, and the identity of the remaining 80 % of the cells remained unknown. The duration of the process ranged from 28 to 35 days.

In 2005, Shin et al. used the mouse feeder layer for hESC culture, followed by the DMEM/F12 medium with N2, L-glutamine, penicillin, streptomycin, and bFGF. Following the removal of the feeder layer, the formed NE were suspended in neurobasal medium supplemented with L-glutamine, penicillin, streptomycin, B27, bFGF, and LIF. Differentiated NE cells were characterized by the expression of Nestin, Musashi1, or SOX 1, 2, and 3. Later on, exposure of the culture to SHH and RA, along with bFGF favored the differentiation into MN. The effect of SHH on the NE could be predicted by the expression of PTCH, the SHH receptor. However, it does not necessitate differentiation into MN. The effect of the bFGF was found to increase the MNP Olig2 gene expression,



b

MN Linage Markers

hESC	NE	MNP	MN
SSEA4	Pax6	β III-tubulin	β III-tubulin
OCT4	Sox1	MAP2	ChAT
TRA-1-60	Nestin	Islet1	HB9
TRA-1-81	Otx2	Tuj1	GFP
	BF1	HB9	Islet1
	PITCH	Lim3	Tuj1
		HOXB1	
		HOXB4	
		HOXB6	
		HOXC5	
		HOXC6	
		HOXC8	
		HLBX9	
		Olig2	
		Nkx2.2	
		Nkx6.1	

Fig. 5.2 (**a**) Generation of motor neurons from human embryonic stem cells. *hESC* human embryonic stem cells, *EB* embryoid bodies, *NE* neuroepithelial cells, *MNP* motor neurons progenitors, *MN* motor neurons. (**b**) Cellular markers of the motor neurons linage. *hESC* human embryonic stem cells, *NE* neuroepithelial cells, *MNP* motor neurons progenitors, *MN* motor neurons

which is further increased by combination of the three factors. Their combination also significantly increased the expression of HLBX9 gene. However, some of the cultured NE gave rise to MN even without SHH and RA exposure. This might be due to continuous exposure to low level of bFGF, but the exact mechanism was not defined. To detect the presence of MN within the culture, their phenotype markers were examined. These markers included Islet1, Tuj1, and ChAT, and based on this criterion, 20–30 % of the cultures were MN. The duration of the process was around 53 days.

Few months later, Singh Roy et al. (2005) induced differentiation of the hESC to MN through EB formation. The EB formation was induced using the mouse embryonic feeder cells and treated with collagenase type IV. It was also fed with the KO-medium (KO-DMEM supplemented with 20 % KO-Serum replacement) and bFGF. The EB were then treated with RA and SHH until the expression of neuronal marker β III-Tubulin. Then, the MN differentiation was induced by the use of DMEM/F12 media supplemented with N2, GDNF, BDNF or NT4, CNTF, B27, and fetal bovine serum (FBS), and with continuous application of RA and SHH. The differentiated MN were identified by the expression of Islet1, HB9, and ChAT genes. In their experiment, 37 % of β III-Tubulin cells were HB9 positive. The co-expression of the Green Florescent Protein (GFP) and the Hb9 gene in the differentiating MN allowed the use of fluorescence-activated cell sorting (FACS) for isolation of the MN from the culture to purity of more than 99 %. Their method, although faster to conduct and is associated with higher purity final selection, it resulted in less initial proportions of induced MN, and losing 76 % of all potential MN in the target population.

In 2007, Lee et al. used the F12/DMEM, N2 supplement medium to induce the neural differentiation of the hESC co-cultured with MS5 stromal cells, and with the addition of Noggin. The formation of the neural rosette was evident by the expression of Pax6 and Sox1, and, later, BF1 and Otx2, which are associated with anterior neural identity. Gbx2 and HoxB4, which marks the posterior neural identity, were not expressed during this default conditions. Isolated neural rosettes were then re-plated on polyornithine/laminin coated culture dishes and N2 medium supplemented with Noggin, AA, and BDNF in the presence of RA and SHH. The addition of RA and SHH was associated with induced caudalization and ventralization of the cells, respectively. This was marked by BF1 suppression and HOXB4 and HOXC8 (caudal identity markers), and Nkx6.1 and Nkx2.2 (ventral identity genes characteristic of the caudal CNS) up-regulation. With additional culture under the presence of RA and SSH, genetic expression indicated the formation of MNP (Nkx6.1, Olig2), early postmitotic motor neurons (NG2, Isl1), and more mature MN (ChAT and vesicular acetylcholine transporter). Many of the cells also expressed somatic MN marker HB9. In the third step, further differentiation was induced in the same medium with the absence of RA and SHH, but in the presence of GDNF, BDNF, and AA. This lead to expression of more mature MN markers, including ChAT, the gene required for Ach synthesis. Co-expression of ChAT and HB9 confirmed MN identity of the hESC progeny, and the additional expression of Lhx3 suggested MN of the medial motor column. According to the authors, a single hESC plated at day 0 on MS5 for neural induction yielded approximately 100 HB9 MN at day 50 with almost 20 % efficiency.

In 2008, Li et al. induced the NE cells differentiation using the DMEM/F12 medium supplemented with N2, Heparin, and cAMP. RA and SHH were then added to the culture. Purmorphamine was occasionally used to replace the variable activity and high cost of SHH, with the same level of action. In contrast to the first protocol, exposure to SHH was maintained during the MNP Olig2-cells phase. This exposure increased proliferation of these cells, but if sustained until the differentiation phase it will suppress MN formation and induce OL formation. Thus, after maximal proliferation of the Olig2-cells, the SHH may be reduced. BDNF, GDNF, and IGF1 were also added to the culture. The differentiated MN produced this way represented more than 96 % of the total hESC-differentiated progenies. Which is the largest percentage produced so far.

In 2009, Wada et al. used a 1:1 mix of DMEM/F12 supplemented with N2 and Neurobasal medium supplemented with B27, with addition of Noggin or Dorsomorphin, to induce hESC differentiation. Following the neural rosette formation, differentiation into MNP and MN was induced by the addition of RA and either SHH or SAGA. Higher concentration of RA had no effect on raising the MN cell numbers. The addition of RA and SHH was also associated with caudalization of the cells, marked by increased expression

of HOXB4 and the suppression of the BF1. However, no significant difference in the expression level of β III-tubulin was noticed, suggesting no difference in neurogenesis itself. Similar results were obtained using SAGA (SHH agonist) as a substitute to SHH. For further maturation, the culture was supplemented with BDNF, GDNF, and NT-3. These mature MN expressed both HB9 and ChAT gene markers. The authors proclaimed that the use of the FGF neurospheres following the first stage of NE cells formation and before the second stage (neural rosette) may expand the numbers of the NE cells up to more than 30-fold while preserving the potency of motor neuron differentiation. They also added that this effect was more significant than using a EGF/FGF neurosphere. That is due to the EGF effect on suppressing the caudalization of the cells. Their last step included purification of the MN from the culture by gradient centrifugation, which raised the purity of the isolated MN from 30 to 80 %.

In 2012, Takazawa et al. induced the EB differentiation from hESC using the DMEM:F12 medium with 20 % Knockout Serum Replacer (KSR), betamercaptoethanol (BME), L-Glutamine and non essential Amino Acids (NEAA). Rho-associated kinase (ROCK) inhibitor, Noggin, and bFGF were added to the culture. The formed EB were then re-suspended on DMEM F:12 medium with N2 supplement, NEAA, L-Glutamine, Heparin, bFGF, Noggin, and ROCK inhibitor. The ROCK inhibitor, and Noggin and bFGF were discontinued at days 5 and 10, respectively, and a diluted Wnt3a-L-cell conditioned medium, RA, AA, db-cAMP, and SHH protein were added regularly until day 18. Then Wnt3a-conditioned medium was discontinued, SHH was increased, and the BDNF was added. At day 25, the medium was switched to the neural differentiation medium (Neurobasal medium with N2 and B27, L-Glutamine, NEAA, AA, db-cAMP), with BDNF, GDNF, IGF-1, CNF, SHH, and RA. The GFP and Hb9 expression were the marker for differentiated MN appeared around the day 31. Morphological changes, including soma size, branching and neurite outgrowth, were also monitored from day 31 to 40.

Locomotor Outcomes

Oligodendrocytes Transplantation

In almost all the experimental studies, induced SCI in animal models, including mice and rats, was the target of the hESC-derived OL and OPC transplantation. Shiverer mice are genetically modified with an autosomal trait mutation in the MBP-gene located on chromosome 18. The absence of this gene and its coded MBP protein causes severe myelin deficiency throughout the CNS. These mice have an average life span of 8 week, which give a relatively short but sufficient window for follow-up. Transplantation of the OL-linage cells, generated in the experiment of Nistor et al. (2005), into these mice was associated with integration and differentiation into functional OL over a 6 weeks follow-up period. This was evident by the formation of compacted, multilayered myelin sheath under microscope, and the expression of MBP, with exclusive distribution within the white mater of the spinal cord surrounding the site of injection. No data on the locomotor improvement were included and only early transplantation was examined. Keirstead et al. (2005), and Faulkner and Keirstead, in the same year, transplanted the OPC into adult rats with induced SCI at early and late phases, 1 and 10 weeks post-injury, respectively. Eight weeks following the injection at the early phase, at least 55 % of the axons around the site of injury were remyelinated. This is almost 136 % more than the endogenous remyelination in control group. To assess the functional improvements, the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) and the four-parameter kinematic analyses were used. The later measures the rear paw stride length, rear paw stride width, rear paw toe spread, and rear paw rotation. On both scales significant gradual improvement was noticed with the early phase transplantation compared to the control group, and persisted for almost 1 month following injection. On the other hand, none of these microscopic and functional improvements were noticed in the late phase transplantation. In addition to the previously mentioned limitation of later transplantation, the formation of OPC-directed

immune response has been suggested. These findings strongly support the therapeutic window theory. In their experiments also, the number of transplanted OL showed no difference in the ultimate outcome.

Izrael et al. (2007) transplanted the Noggin-treated OL into the brain of Shiverer mice. These were associated with significant local and distant integration and myelination within the brain tissue. In another study by Sharp et al. (2010), they proclaimed that, beside the final result, the progressive improvement in the symptoms was faster as compared to control group. Their results were recorded using Forelimb-movement scores, which measures the forelimb stride length, proximal forelimb step range, and passed-perpendicular step frequency. These could be detected as early as 1 week after transplantation. In the same year, Cao et al. described the use of neurotrpohic factors, i.e. CNTF with the OL transplantation to improve their survival, integration and differentiation (Cao et al. 2010). Karimi-Abdolrezaee et al. also proved that the combined use of chondroitinase ABC (ChABC) and growth factors EGF, bFGF, and PDGF-AA with the transplant significantly improve the outcome of OL differentiation, myelination, and functional outcome of chronic spinal cord injury. The ChABC antagonizes the chondroitin sulfate proteoglycans (CSPG) of the glial scar, which negatively influence the long-term survival, migration and differentiation of the transplanted cells (Karimi-Abdolrezaee et al. 2010) (Table 5.2).

Motor Neurons Transplantation

Following the *in vitro* culture, differentiation, and isolation of the MN, functional maturation, including the electrical properties, actions potential generation and conduction, and the receptive and terminal synaptic function, is analyzed. Measurement of the intrinsic membrane properties of the MN shows more hyperpolarized resting membrane potential and decreased input resistance with maturation. The generation and repetitive firing of action potential was also proved using the whole-cell patch clamp and/or the voltage-clamp configuration, which measure the function of the ion-channels within the cells, especially the voltage-gated Na and K-channels. Other electrical characteristics including spike frequency adaptation and rebound action potential firing are also measured, and are more consistent with spinal MN (Takazawa et al. 2012). The neurotransmitter sensitivity of the MN can be also assessed by applying the desired neurotransmitter, e.g. GABA, glutamate, dopamine and acetylcholine to the MN culture, and measuring the resultant current formation (Erceg et al. 2008). The synaptic function are being also studied with neighboring neurons (Li et al. 2005) and skeletal muscle cells (Wada et al. 2009). This detects the maturation of the synaptic vesicles at the axonal terminal, and the consequent up-regulation of the neurotransmitter, i.e. acetylcholine, receptor at the post-synaptic membrane. Co-culture of the MN with astrocytes derived from the respective hESC line was reported to be essential for electrophysiological maturation (Lee et al. 2007).

Following the MN differentiation and isolation, Lee et al. tested the survival capability of the cells via transplanting them into the spinal cord of a chick embryo. They found that these MN were able to survive and extend axonal fibers outside the CNS. The next step was to test their fate in adult CNS. Thus, they injected the HB9-cells into the ventral spinal cords of 3-month-old Sprague-Dawley rats. Observations at 1 day, 2 weeks, and 6 weeks after transplantation revealed progressive loss of the HB9 expression and increase in ChAT expression, which corresponds to physiological MN maturation. There were also evident of extensive fiber outgrowth and cell migration toward the ventral surface of the spinal cord. However, over the 6 weeks period, no signs of axonal outgrowth outside the CNS were addressed (Lee et al. 2007).

In the study of Erceg et al. (2010), the MNP transplantation into rats with transected spinal cord showed significant locomotor improvement in a 4 months follow-up period. The locomotor improvement was assessed clinically, were the samples showed partial recovery of the hindlimb movements, and on the BBB score with 6 out of 21. This is noticeably higher compared to the

Table 5.2 Experimental generation of oligodendrocytes from hESC and their transplantation

Study	Year	Media	Factors	Duration (days)	Purity (%)	Recipient	Integration and differentiation	Locomotor Improvement
Nistor et al.	2005	DMEM/F12, B27	RA, bFGF, EGF, IGF-1, T3	42	>95	Shiverer mice	Yes	–
Keirstead et al.	2005	DMEM/F12, B27	RA, bFGF, EGF, IGF-1, T3	42	>95	Rats	Yes	Significant, only at early stage transplantation
Faulkner and Keirstead	2005	DMEM/F12, B27	RA, bFGF, EGF, IGF-1, T3	42	>95	Shiverer mice	Yes	Significant, only at early stage transplantation
Izrael et al.	2007	ITTSPP/B27	RA, Noggin, SHH, bFGF, EGF, T3	>70	94	Shiverer mice	Yes	–
Kang et al.	2007	DMEM/F12, N2	bFGF, EGF, PDGF, T3	<50	81	–	–	–
Hu et al.	2009	DMEM/F12, N2	RA, SHH, purmorphamine, Noggin, bFGF, EGF, IGF-1, PDGF	>50	84	–	–	–
Hu et al.	2009	DMEM/F12	HGF	>50	>80	–	–	–
Sundberg et al.	2010	DMEM/F12, N2	EGF, bFGF, IGF-1, PDGF, CNTF, AA, T3	>80	>90	–	–	–
Sharp et al.	2010	DMEM/F12, B27	RA, EGF, FGF	42	<98	Rats	Yes	Significant

control group. On the electrophysiological study 4 months after the transplantation, partial conduction at the site of injury was present, compared to blind conduction in the controls. Immunohistological examination at the injection site showed clear evidence that these progenitors have the capacity to differentiate into mature OL and neurons in the lesion site. Nevertheless, no evidence of anatomically, physiologically, and functionally active motor units was seen (Erceg et al. 2010) (Table 5.3).

In the above experiment, the first application of combined cellular transplantation was documented. Erceg et al. (2010) used both the OL and MNP in rats with transected spinal cord. Over a 4 months follow-up, the functional locomotor recovery showed a better hindlimb recovery, and significantly higher BBB score and electrophysiological function compared to single-cell transplantation.

Lastly, transplantation of these cells proved that the generation of differentiated and highly pure cell line from the hESC is associated with decreased risk of tumor formation, i.e. teratoma. It also decreases the risk of undesired differentiation, including astorocyte and scar formation, graft-induced sprouting, and allodynia (Sharp and Keirstead 2007).

Motor Neurons and Oligodendrocytes Derived Human Induced Pluripotent Stem Cells

During fetal development, the pluripotent stem cells start to acquire a restricted and specific potency that gradually becomes directed towards one cell line generation. This process was always considered unidirectional. In 2006, Takahashi and Yamanaka were able to go back in cellular time by reprogramming adult somatic cells into pluripotent cells similar to the ESC using defined pluripotency-related transcription factors (i.e. Oct3/4, Sox2, c-Myc and Klf4) (Fig. 5.3). Yamanaka was honored with the Nobel Prize for this remarkable discovery. These novel types of stem cells were named "Induced pluripotent stem cells" or iPSC. Since this first description, various studies have been conducted using different protocols and cells of origin from different species. However, the main cellular origin stills the skin fibroblast. These protocols also tried to decrease the risks and health concerns and obstacles associated with the use of iPSC which include the integration of transcription factors with oncogenic properties, mutagenesis from insertion of the genes, the use of viral vectors and, lastly, the slow and relatively inefficient reprogramming process that may creates a situation that may favor incomplete reprogramming vectors. Detailed review of these methods is beyond the scope of this chapter.

The use of these cells is still considered an extensive, rapid, and important advancement that may solve the ethical issue related to the use of ESC. They may also be used for cell replacement therapy without requiring immunosuppressive therapy. Moreover, the fact that these cells are derived from individual patients makes it possible to develop customized stem cell therapies, generate disease-specific stem cell lines, and perform genetic corrections.

Genetic Variations

Despite the reported close genetic similarities between ESC and iPSC, genetic variations have been found. The use of single-nucleotide polymorphism (SNP) analysis on the pluripotent (i.e. ESC and iPSC) and non-pluripotent cells by Laurent et al. (2011) showed that the former have an increased overall frequency of sub-chromosomal copy number variations (CNV), with variations enriched in specific genomic regions. Comparing the ESC and iPSC showed that the ESC exhibit large number of duplications, compared to moderate amount of deletions found throughout the iPSC lines. These deletions were mainly present during reprogramming at the sites of tumor suppressor genes. Moreover, the oncogenic genes were duplicated with extended culture of the iPSC. Similar results were obtained by other authors (Gore et al. 2011; Hussein et al. 2011; Lister et al. 2011) who found

Table 5.3 Experimental generation of motor neurons from hESC and their transplantation

Study	Year	Media	Factors	Duration (days)	Purity (%)	Recipient	Integration and differentiation	Locomotor Improvement
Li et al.	2005	DMEM/F12, N2	RA, SHH, GDNF, BDNF, IGF1, cAMP	28–35	20	–	–	–
Shin et al.	2005	DMEM/F12, N2	RA, SHH, bFGF	53	20–30	–	–	–
Singh Roy et al.	2005	DMEM/F12, B27	RA, SHH	>40	20	–	–	–
Lee et al.	2007	DMEM/F12, N2	RA, Noggin, SHH, BDNF, GDNF, AA	50	20	Chick embryo Rats	Yes	–
Li et al.	2008	DMEM/F12, N2	Heparin, SHH, puromorphamine, RA, cAMP, GDNF, BDNF, IGF1	<30	50	–	–	–
Wada et al.	2009	DMEM/F12, N2, B27	SHH, SAG, RA, GDNF, BDNF	38	30	–	–	–
Erceg et al.	2010	DMEM/F12, N2	RA, SHH, GDNF, BDNF, CNTF	38	20	Rats	Yes	Significant
Takazawa et al.	2012	DMEM/F12N2	RA, SHH, AA, cAMP, BDNF, GDNF	>30	>30	–	–	–

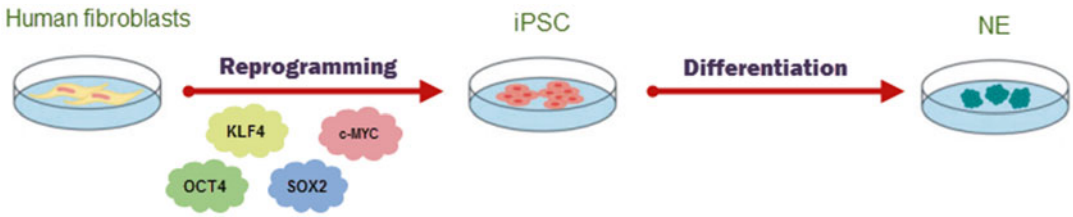


Fig. 5.3 Reprogramming of the human induced pluripotent stem cells from adult fibroblasts using pluripotency-related transcription factors (c-MYC, OCT4, KLF4, SOX2). *iPSC* induced pluripotent stem cells, *NE* neuroepithelial cells

that half of these mutations arise during the reprogramming process, despite the method used, and the other half is present in the original cells with lower expression. An average of five protein-coding point mutations per region was found by Gore et al. (2011) independent of the used method. Although these genes are cancer-related genes, gene ontology analysis did not reveal any pathway enrichments, which suggest a random rather than selective process (Gore et al. 2011; Panopoulos et al. 2011). In addition to these genetic mutations, epigenetic mutations were also reported by Lister et al. (2011) who detected more than a thousand differentially methylated regions or hotspots between iPSC and ESC lines.

All of these findings and others (Ben-David and Benvenisty 2011) suggest that the iPSC are more carcinogenic than the ESC, with chromosomal aberration acquired during three stages: from the original cells, during reprogramming, and during culture. However, despite these findings, the functional, morphological, and oncogenic manifestations and their in vivo variations and impact are still unclear (Panopoulos et al. 2011).

Neuronal Regeneration

As in the case of hESC, human iPSC (hiPSC) are capable of differentiating toward all neural cell types, including neurons, glia, NE, and MN, which in turn are used to treat various kinds of neurological pathologies. Moreover, due to their beneficial characteristics and despite their potential risks, the hiPSC is becoming an appealing alternative source for neuronal generation.

Experimental studies on MN (Ebert et al. 2009; Karumbayaram et al. 2009) and OL (Ogawa et al. 2011) generation from fibroblast-derived human iPSC were capable of inducing functional cells through and intermediate EB, in a process exactly similar to the hESC. However, none of these cells were tested in vivo. Other studies that involved direct transplantation of the human iPSC –derived neural spheres will be discussed in the next chapter.

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