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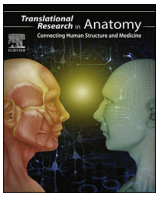

Review

# Stem cell therapy for spinal cord injury: The use of oligodendrocytes and motor neurons derived from human embryonic stem cells

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ABSTRACT

Over the past few years, the understanding of stem cells as a potential therapeutic source has significantly evolved, and the previous concept of irreparable neural injury is being reconsidered. Stem cells are pluripotent cells with high differentiation potential. Induced proliferation and differentiation of these cells under optimal *in vitro* conditions has been used to generate different transplantable cells of various types and stages of development. For spinal cord injury recovery, the human embryonic stem cells and, recently, the human induced pluripotent stem cells are used as a main source, and two major types of cells are the target: the oligodendrocytes and motor neurons. The extensive experimental research efforts have focused on translating *in vitro* cellular regeneration of these cells to *in vivo* transplantation and survival of the transplants, in order to improve clinical outcomes. In this review, we will discuss the progressive development of the cellular generation protocols and the locomotor outcome of their transplantation at sites on spinal cord injury.


**Contents**



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## 1. Introduction

In 1908, Alexander Maksimov, a Russian histologist, proposed that all the blood cells and the process of hematopoiesis are derived from hematopoietic stem cells. However, it was not until 1963, when the Canadian scientists, James E. Till and Ernest A. McCulloch, for the first time, could demonstrate the presence of these stem cells, in mice bone marrow. Since then, research on the characteristics and therapeutic applications of these cells has initiated a new era of medicine.

## 2. 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. The cells of the outer cell mass form the trophoblast supporting the growth of the embryo. When the germ layers are separated, cells of each layer are classified as multipotent, oligopotent, or unipotent, depending on their differentiation potentials [4]. These higher levels of stem cells are defined as the adult stem cells (ASC), which are retained in most tissues during childhood and adulthood, and, under normal conditions, give rise to that specific line of cells of the retaining organ. However, some tissues, for unknown reasons, including brain, spinal cord, heart, and kidneys, with minor exceptions, do not maintain their stem cells, which limits their regenerative ability following injury [17].

Pluripotent stem cells have been an ideal source for cellular transplantation, due to their extensive proliferation and differentiation potential. Of these cells, embryonic stem cells (ESC), which are present during early stage of development, have drawn most attention. 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 [13]. They have also provided the proper methods of differentiating mouse ESC into several clinically relevant neural and non-neural cell types [16]. Thomson et al. [33] were the first to isolate the hESC, using fourteen inner cell masses of *in vitro* fertilization (IVF)-produced embryos as a source. These blastocysts have since then constituted the major source of hESC. Other sources of ESC include nuclear transfer and therapeutic cloning. Nuclear transfer is achieved by transferring the nucleus of an adult differentiated egg (containing the DNA to be cloned) into an enucleated egg, which is then stimulated to form blastocysts, from which the ESC can be extracted. When nuclear transfer is performed for therapeutic purposes, using a nucleus of a somatic cell (e.g. skin cell), it is then called "therapeutic cloning" [4].

Following expansion, ESC are induced to form different cells of various stages of differentiation, including neuroepithelial cells, oligodendrocytes and their progenitors, as well as motor neurons and their progenitors. In this review, we will focus on the generation and transplantation of oligodendrocytes and motor neurons, and their progenitors.

A comparison between experimental generation of oligodendrocytes and motor neurons is summarized in Table 1 and Fig. 1.

## 3. 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 saltatory conduction and insulation of the nerve cells [9,12]. It also promotes neuronal and axonal survival by secreting different types of neurotrophic factors [16,22].

Zhang et al. [35] studied the ability of the oligodendrocytes progenitor cells (OPC) derived from hESC to secrete neurotrophic factors. Of all the genes tested, 49 growth factors were significantly expressed by OPC. Of these factors, transforming growth factor (TGF)-β1, TGF-β2, activin A, vascular endothelial (VEGF), brain-derived neurotrophic factor (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 [35].

Following spinal cord injury (SCI), significant loss of OL and OPC adds to the deleterious effect of direct trauma and subsequent inflammation and vascular disruption. Therefore, replacement of these cells has been one of the promising treatment options that may preserve the axonal function and suppress their progressive loss.

### 3.1. Experimental generation of oligodendrocytes from hESC

Potential sources of human OL include aborted fetuses, olfactory biopsies of the neuroepithelium, and hESC. The latter may provide

**Table 1**
Characteristics and comparison between oligodendrocytes and motor neurons linages.

| | Oligodendrocytes linage | Motor neurons linage |
|---|---|---|
| First use | [22] | [19] |
| 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 |

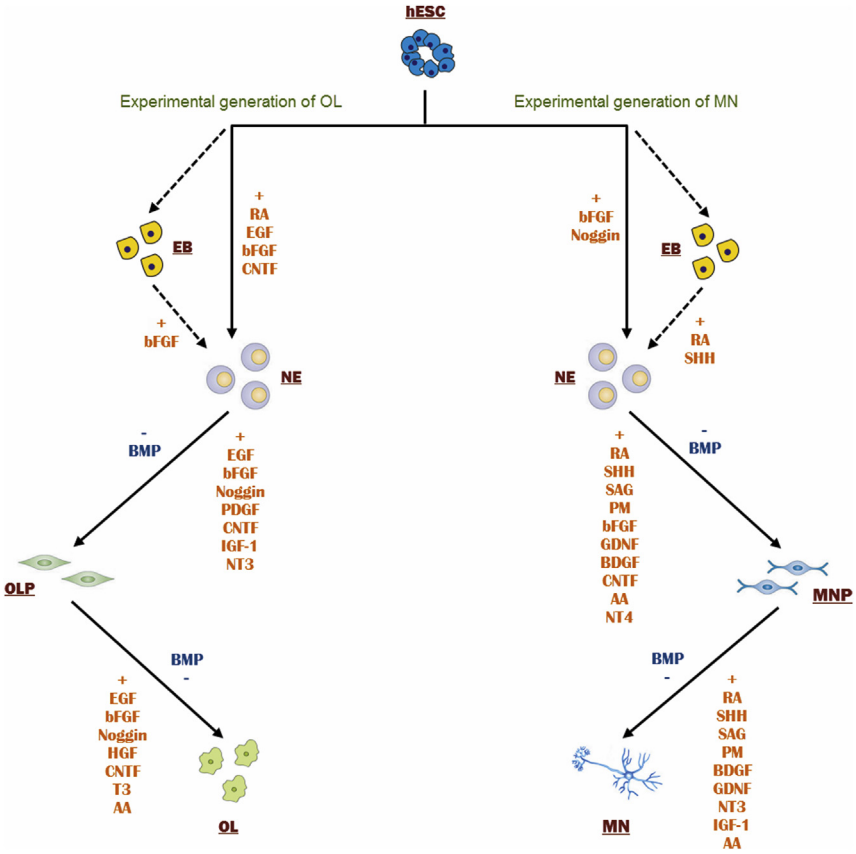


**Fig. 1.** Experimental generation of oligodendrocytes and motor neurons from human embryonic stem cells. hESC = Human Embryonic Stem Cells; EB = Embryoid Bodies; NE = Neuroepithelial cells; OPC = Oligodendrocytes progenitors; OL = Oligodendrocytes; MNP = Motor Neurons progenitors; MN = Motor Neurons.

the ideal source due to its high proliferation and differentiation potentials [30]. The major challenge in this situation is the ability to produce and isolate high purity OL and OPC from directed differentiation of hESC, and reduce the risk 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 neuroepithelial (NE) cells, glial-restricted precursor (GP) cells, and OPC formation. (See Fig. 1).

Nistor et al. [22] 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 can 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 insulin-like growth factor (IGF), differentiation factor triiodothyronin hormone, fibroblast growth factor (FGF), and epidermal growth factor (EGF), and it played important role in the proliferation and survival of the OL [22]. Their method generated about 80% 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, nerve/glial antigen 2 (NG2), and platelet derived growth factor receptor (PDGF-R).

Izrael et al. [12] added Noggin, antagonist of bone morphogenetic proteins (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, the Nkx2.2 needs the Sox10 gene activation for proper function, which is also an OL-specific gene, and is induced by the addition of Noggin at specific stages of development [12]. 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) [10] and inhibition using BMP [12].

Kang et al. [13] 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. [11] 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 enhance the proliferation of neural progenitors derived from hESC and to promote the generation and maturation of OL. This method yielded OL with high purity (about 80%).

Sundberg et al. [30] introduced a novel method for the generation of the OL from hESC using human recombinant growth factors and extracellular matrix (ECM) proteins. This is 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

**Table 2**
Oligodendrocytes linage markers in experimental studies.

| Human embryonic stem cells | Neuroepithelial cells | Oligodendrocytes progenitor cells | Oligodendrocytes |
|---|---|---|---|
| SSEA4, OCT4, TRA-1-60, TRA-1-81 | Pax6, Nestin, Mash1, OPC | Olig1, Olig2, SOX8, SOX9, SOX10, A2B5, NG2, OMG, PDGF-R, Nkx2.2, Nkx6.2, Gli1, Gli2 | PLP, O1, O4, MBP, GalC, RIP |

facilitated the OPC survival, maturation, and myelination. The growth factors used included; FGF, EGF, and ciliary neurotrophic factor (CNTF) for the initial neural differentiation; PDGF-AA, EGF, basic FGF (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 was dependent on the stage of development and cellular differentiation from hESC to fully mature OL [30] (Table 2).

### 3.2. Locomotor outcome after 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. Transplantation of the OL-linage cells, generated in the experiment of [22], into the Shiverer mice, was associated with integration and differentiation into functional OL over a six-week 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 matter 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. and Faulkner and Keirstead [8,16] 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 latter 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 one month following injection. On the other hand, none of these microscopic and functional improvements were noticed in the late phase transplantation. The number of transplanted OL showed no difference in the ultimate outcome.

The limited improvement at the late phase transplantation is mainly related to the late pathologic changes at the site of SCI, which limits the OL and OPC migration to the site of injury, and suppresses their maturation and differentiation. Moreover, formation of OPC-directed immune response has also been suggested. These findings strongly support the therapeutic window theory for cellular transplantation [1,8,21,27].

Izrael et al. [12] transplanted the Noggin-treated OL into the brain of Shiverer mice. They were associated with significant local and distant integration and myelination within the brain tissue. In another study by Sharp et al. [26], they proclaimed that, besides 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. Functional improvement could be detected as early as 1 week after transplantation.

Cao et al. [3] described the use of neurotrpohic factors, i.e. CNTF, with the OL transplantation to improve their survival, integration, and differentiation [3]. Karimi-Abdolrezaee et al. [14] proved that the combined use of chondroitinase ABC (ChABC) and growth factors EGF, bFGF, and PDGF-AA with the transplant significantly improved 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 [14] (Table 3).

## 4. 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 [23,32].

SCI is associated with direct and delayed MN damage and axonal loss, which most significantly contributes to the functional

**Table 3**
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 |

deterioration following injury. Therefore, cellular replacement of MN and their progenitors has been proposed as a therapeutic option. One major source for these cells is the hESC.

### 4.1. 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 from 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. (See Fig. 1).

The first experiential generation of MN from hESC was done by Li et al. [19]. After culturing the hESC in a feeder layer, which allow their continuous growth in an undifferentiated state, 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 induced also 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, also did not eliminate the expression of Otx2. Following caudalization, inducing ventral neural cell types were 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 one week. Following the appearance of Olig2-MNP, BDNF, glial-derived neurotrophic factor (GDNF), IGF-1, 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. (Table 4) The authors concluded that the cells with combined expression of Islet1/2 and HB9 were likely MN, whereas the cells with negative HB9 and positive Islet1/2 were 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.

Shin et al. [28] 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 leukemia inhibitory factor (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 the differentiation into MN. The effect of the bFGF was found to increase the MNP Olig2 gene expression, which was 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 could not be 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.

Singh Roy et al. [29] 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 neurotrophin (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 was faster to conduct and was 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.

Lee et al. [18] 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 replaced 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 post-mitotic 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

**Table 4**
Motor neurons linage markers in experimental studies.

| Human embryonic stem cells | Neuroepithelial cells | Motor neurons progenitors | Motor neurons |
|---|---|---|---|
| SSEA4, OCT4, TRA-1-60, TRA-1-81 | Pax6, Sox1, Nestin, Otx2, BF1, PITCH | β III-tubulin, MAP2, Islet1, Tuj1, HB9, Lim3, HOXB1, HOXB4, HOXB6, HOXC5, HOXC6, HOXC8, HLBX9, Olig2, Nkx2.2, Nkx6.1 | β III-tubulin, ChAT, HB9, GFP, Islet1, Tuj1 |

**Table 5**
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/F12 N2 | RA, SHH, AA, cAMP, BDNF, GDNF | >30 | >30% | – | – | – |

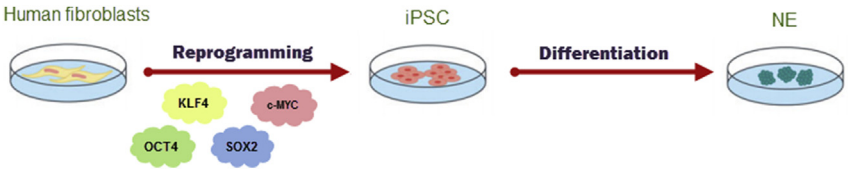


**Fig. 2.** Reprogramming of the human induced pluripotent stem cells from adult fibroblasts using pluripotency-related transcription factors (c-MYC, OCT4, KLF4, SOX2). iPSC = Induced Pluripotent Stem Cells; NE = Neuroepithelial cells.

with the absence of RA and SHH, but in the presence of GDNF, BDNF, and AA. This led to expression of more mature MN markers, including ChAT, the gene required for acetylcholine 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.

Li et al. [20] 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 previous protocols, 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 would suppress MN formation and induce OL formation. Thus, after maximal proliferation of the Olig2-cells, the SHH may be reduced. BDNF, GDNF, and IGF-1 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.

Wada et al. [34] used a 1:1 mix of DMEM/F12 supplemented with N2 and Neurobasal medium supplemented with B27, with the 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 (SHH agonist). 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 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 an 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%.

Takazawa et al. [32] 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 resuspended 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 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, CNTF, SHH, and RA. The GFP and Hb9 expression was the marker for differentiated MN appeared around the day 31. Morphological changes, including soma size, branching, and neurite outgrowth, were also monitored from day 31–40.

### 4.2. Measurement of motor neurons maturation

Following isolation, functional maturation of the MN, including their electrical properties, actions potential generation and conduction, and the receptive and terminal synaptic function, can be 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 can be proven 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 can also be measured, and are more consistent with spinal MN [32]. The neurotransmitter sensitivity of the MN can be assessed by applying the desired neurotransmitter, e.g. GABA, glutamate, dopamine and acetylcholine to the MN culture, and measuring the resultant current formation [6]. The synaptic function can also be studied with neighboring neurons [19] and skeletal muscle cells [34]. This can detect 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 has been reported to be essential for electrophysiological maturation [18].

### 4.3. Locomotor outcome after motor neurons transplantation

Lee et al. [18] tested the survival capability of MN via transplantation into the spinal cord of a chick embryo. They found that these cells 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 corresponded with the physiological MN maturation. There was also evidence of extensive fiber outgrowth and cell migration toward the ventral surface of the spinal cord. However, over the six weeks period, no signs of axonal outgrowth outside the CNS were addressed [18].

In the study of Erceg et al. [7], 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, where the samples showed partial recovery of the hindlimb movements, and scored 6 out of 21 on the BBB scale. This was significantly higher than the 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 and functionally active motor units was seen [7] (Table 5).

In the above experiment, the first application of combined cellular transplantation was documented. Erceg et al. [7] 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.

*Lastly*, transplantation of OL, MN, and their progenitors proved that the generation of differentiated and highly purified cell line from the hESC was associated with decreased risk of tumor formation, i.e. teratoma. It also decreased the risk of undesired differentiation, including astrocyte and scar formation, graft-induced sprouting, and allodynia [27].

## 5. 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. For a long time, this process was considered unidirectional. In 2006, Takahashi and Yamanaka [31] 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. 2) 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 is still 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 may create a situation that favors incomplete reprogramming vectors.

The use of these cells is 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 as they represent an autologous transplant. 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.

The major disadvantages of these cells include their genetic instability and high teratogenic potential associated with the process of reprogramming and culture, and also endogenous within the cells of origin. These findings and others suggest that the iPSC are more carcinogenic than the ESC [2]. However, despite these findings, the functional, morphological, and oncogenic manifestations of these cells and their impact *in vivo* are still unclear [25].

### 5.1. Neuronal regeneration

As in the case of hESC, human iPSC (hiPSC) are capable of differentiating towards all neural cell types, including neurons and glia, 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 [5,15] and OL [24] generation from fibroblast-derived human iPSC were capable of inducing functional cells through an intermediate EB, in a process exactly similar to the hESC. However, none of these cells were tested *in vivo*.

## 6. Conclusion

Loss of MN, OL, and their progenitors at the site of SCI, represent a major contributing factor to the ensuing functional deterioration. Therefore, replacement of these cells has become a topic of extensive spinal cord research. In early studies, these cells were derived from the ESC, but with the breakthrough development of iPSC, the latter have become an alternative source. Up to date, and despite the promising outcomes of MN and OL transplantation in animal models of SCI, the popularity of this type of cellular transplantation has decreased, as we can see with the lack of experimental trials in the last two years. Moreover, there were no studies done on humans. The reason is mostly related to the complexity of hESC isolation, and the risk associated with the use of iPSC.

## Conflict of interest statement

The authors declare that they do not have any conflict of interest.

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