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Review Article

Neural commitment of embryonic stem cells: molecules, pathways and potential for cell therapy

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Abstract

The study of neuronal differentiation of embryonic stem cells has raised major interest over recent years. It allows a better understanding of fundamental aspects of neurogenesis and, at the same time, the generation of neurons as tools for various applications ranging from drug testing to cell therapy and regenerative medicine. Since the first report of human embryonic stem (ES) cells derivation, many studies have shown the possibility of directing their differentiation towards neurons. However, there are still many challenges ahead, including gaining a better understanding of the mechanisms involved and developing techniques to allow the generation of homogeneous neuronal and glial subtypes. We review the current state of knowledge of embryonic neurogenesis which has been acquired from animal models and discuss its translation into *in vitro* strategies of neuronal differentiation of ES cells. We also highlight several aspects of current protocols which need to be optimized to generate high-quality embryonic stem cell-derived neuronal precursors suitable for clinical applications. Finally, we discuss the potential of embryonic stem cell-derived neurons for cell replacement therapy in several central nervous system diseases.

Keywords: embryonic stem cells; neuronal differentiation; growth factors; small molecules; cell therapy

Introduction

During recent decades, important knowledge has been accumulated in the understanding of early neurogenesis. In parallel, the field of embryonic stem (ES) cell research has been expanding, and progress has been made in the generation of ES-cell derived mature tissue cells, including neurons. Human ES cells were first isolated in 1998 [1] and many human ES cell lines are now available. These cells allow the virtually unlimited generation of human neurons, therefore opening the way to the study of human neurogenesis, pharmacological screening and future cell therapy of neurodegenerative disorders. Many protocols aiming at the differentiation of ES cells along a specific cell lineage are based on the reproduction of events occurring during embryogenesis. However, these inductive events are only partially understood. In addition, spatial organization in a cell culture differs significantly from an embryo, rendering regional signalling very difficult to mimic. Despite these discrepancies, neuronal differentiation of ES cells towards neurons has been largely inspired by knowledge of early developmental events. Experimental data in ES cells largely confirmed the usefulness of extrapolating early neural specification events occurring in the embryo to cell culture.

In this review we focus on how ES cell differentiation can be driven along a neural lineage. Differentiation strategies are in part based on knowledge of inductive events during embryogenesis, but often also empirically derived. We will also examine the suitability of current protocols to generate clinical grade neurons for cell therapy and discuss challenges to be overcome in the future.

Neuronal commitment during embryogenesis

Studies on different species have contributed to the current knowledge of early neurogenesis. Work done on *Xenopus* has been particularly fruitful in elucidating many key steps of early neuronal commitment. Therefore, many studies discussed below were done on *Xenopus*. Generally these results can be extrapolated to mammalian neurogenesis [2].

During early embryogenesis and already before gastrulation, signals originating from extraembryonic and visceral endoderm govern the induction of the three germ layers [3]. In *Xenopus*, asymmetric distribution of key cell fate determinants result in the accumulation of nuclear β-catenin in the Nieuwkoop centre,

which generates signals, further patterning the formation of the three germ layers. Formation of mesoderm and endoderm is induced by nodal ligands of the transforming growth factor β (TGFβ) superfamily. Dorsally, nuclear β-catenin accumulates in the Nieuwkoop centre and initiates a set of genetic pathways to establish the organizer. The later secretes proneural molecules specifying a portion of the ectoderm to become neuroectoderm, whereas the remaining ectoderm gives rise to the epidermis [4]. Early neuroectoderm constitutes the neural plate, which folds and closes itself to constitute the neural tube. The latter will then give rise to the neural elements of the central nervous system, i.e. neurons, astrocytes and oligodendrocytes. These early specification events are orchestrated by multiple players, among which four signalling pathways play a major role during mammalian neurogenesis: the FGF2, bone morphogenetic protein (BMP), wnt–β-catenin and Notch pathways.

Fibroblast growth factor (FGF) signalling is one of the earliest known pathways involved in neural specification. It acts prior to and also synergistically with inhibition of BMP signalling [2] and activation of Notch signalling [5]. The FGF signalling cascade acts through activation of the extracellular signal-regulated kinase (ERK1/2) pathway, resulting in transcriptional activation of target genes. Blocking FGF signalling in ES cells virtually abolishes their neuroectodermal commitment [6]. During embryogenesis, BMP blocks neuroectoderm formation and favours alternative lineage choices. It acts through a Smad-dependent pathway and results in transcriptional activation of genes involved in alternative lineage choices [7]. BMP antagonists (noggin, chordin, follistatin) are produced by the notochord and the neighbouring mesoderm to block BMP signalling and thereby promote the formation of neuroectoderm [2]. Wnt–β-catenin signalling also plays an important role during early neurogenesis. When active, β-catenin allows LEF/TCF transcription factors to be activated and induce gene expression [8]. In *Xenopus*, this pathway is important at the blastula stage to allow the onset of neurogenesis to occur, but later it must also be antagonized to allow neural specification [9]. Finally, Notch signalling plays an important role in early neural induction, according to studies in the chick and the fly [2]; this is also corroborated by data in murine and human ES cells [5]. In mammals, four different types of Notch receptors are described [10]. Upon binding to the DSL (Delta, Serrate, Lag-2) family of ligands, Notch receptors are cleaved in their transmembranous portion. During early neurogenesis, this cleaved part of Notch, also called the Notch intracellular cytoplasmic domain, will activate expression of members of the Hes family of transcription factors, which will further regulate the fate of neuronal progenitors [10]. Therefore, a coordinated interplay between activation (FGF2, Notch, wnt-β-catenin) and inhibition (BMP and wnt–β-catenin) of several signalling pathways is crucial for proper neurogenesis to be initiated.

These different signalling pathways converge on the expression of early neural genes through mechanisms which are still poorly understood. One study has shown that Wnt and FGF signalling cooperate to activate the expression of Sox2, and this process seems to be independent of BMP signalling [11]. Sox2 is a member of the SoxB transcription factor family, which plays a particularly important role in the specification of neuroectoderm. Several of its members are expressed throughout the neural plate and are important for the maintenance of a neuroepithelial character [12]. In addition, their expression in ES cells has been shown to specify neuroectodermal lineage commitment [13].

In addition to the acquisition of a neural fate, cells further differentiate towards more restricted progenitors, which will differ according to their position along the rostro-caudal and dorso-ventral axes. Combinations of transcription factors will specify different progenitor types. For example, Olig transcription factors will specify oligodendrocytes [14], whereas Cerberus, Dickkopf (dkk) and Otx2 specify telencephalic precursors, giving rise to the cerebral cortex [15–17].

In vitro differentiation of embryonic stem cells

In 1981, Martin *et al* had already demonstrated the ability of ES cells to spontaneously differentiate towards many cell types *in vitro*, including neurons [18]. Differentiation of ES cells towards the neural lineage is characterized by progression through different stages resembling embryonic neurogenesis and results in the generation of different neuronal subtypes, astrocytes and oligodendrocytes (Figure 1). Whereas the generation of neurons among a mixed population of cells is easily obtainable, their directed differentiation towards the neural lineage remains more challenging. This can be achieved through modulation of the extracellular and/or intracellular environment (Figure 2). Extracellular factors, such as growth factors, extracellular matrix components or contact with other cell types, act on ES cells through interactions with cell membrane components. These interactions trigger intracellular signalling cascades and result in the modification of cell fate. Alternatively, the intracellular environment can be directly modified through transgenic expression of proteins or by the addition of cell-permeant proteins or small organic molecules to the culture medium. In this section we review currently used strategies to redirect ES cell fate towards neurons by modifying the extracellular and intracellular environment.

Extracellular environment

Expansion of embryonic stem cells in an undifferentiated state is generally performed in the presence of feeder cells and either serum or serum replacement. Differentiation protocols towards endodermal

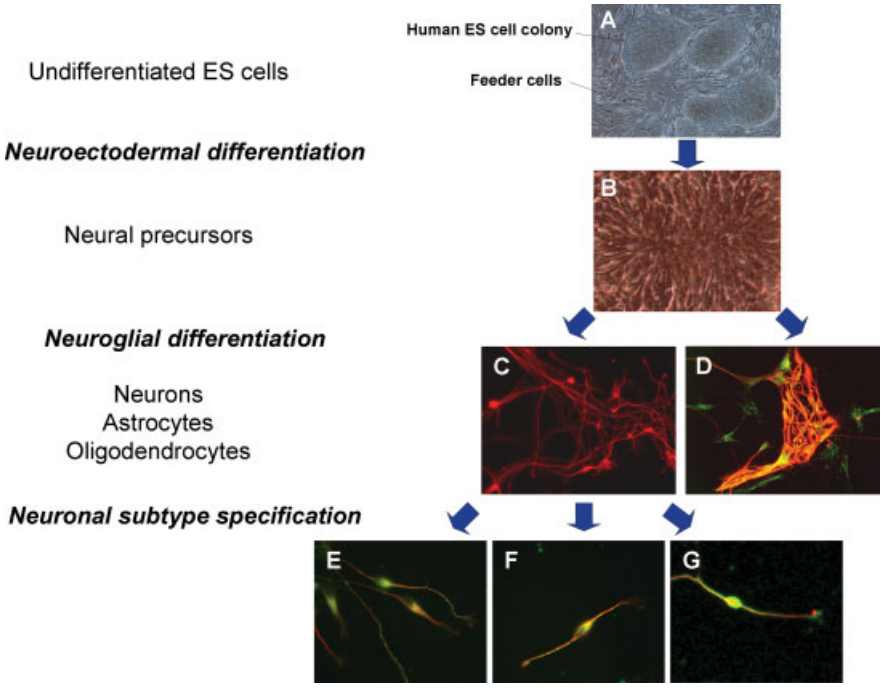

Figure 1. Neural differentiation steps of human ES cells. (A) For propagation, ES cells need to be maintained in an undifferentiated state, usually with the help of feeder cells. Differentiation of ES cells towards neurons involves two major steps. The first step is induction of neuroectodermal differentiation, achieved through serum removal, co-culture with stromal cells or other procedures described in the text. Neuroectoderm is characterized by a columnar shape and later by rosette-like structures resembling neural tubes (B). These neural progenitors will then further differentiate into neurons, which express markers such as β3-tubulin (C, red), but also into astrocytes expressing GFAP (D, red) and oligodendrocytes expressing CNPase (D, green). Mature neurons are further specified into diverse neuronal subtypes (E–G). They can be identified by co-staining for β3-tubulin (red) and subtype-specific markers (green) to identify, for example, glutamatergic (E), GABAergic (F) or dopaminergic (G) neurons

and mesodermal cell types maintain serum in the differentiation medium [19,20]. In contrast, ectodermal differentiation protocols are characterized by the omission of serum [6]. As these differentiation protocols have been derived empirically, the precise role of serum components in cell fate decisions is unclear.

However, as neuronal differentiation protocols are performed under serum-free conditions, it is necessary to add supplements. The N2 supplement, included in most neuronal differentiation protocols, consists of five molecules: transferrin, insulin, putrescine, progesterone and selenite. This supplement had been initially designed for the primary culture of neurons and has subsequently been used in ES cell differentiation protocols. These molecules might act as a 'minimal serum replacement', confer a growth advantage to neurons [21] or even affect neural cell fate decisions. Transferrin is essential for both proliferation of neuronal progenitors and survival of mature neurons [22]. Insulin is essentially required for progenitor proliferation [22]. Putrescine is a member of the polyamine family and is produced by decarboxylation of ornithine. Elevations of putrescine in response to axonal growth-promoting signals and to injury are crucial for axonal regeneration [23–26] and the addition of putrescine to the cell culture medium overcomes the inhibition of axonal regrowth caused by myelin [23]. In addition, putrescine has neuroprotective properties [27]. Sodium selenite is a trace metal and a component of selenoproteins. Selenite provides protection against excitotoxicity [28] and selenoprotein-P promotes neuronal survival in culture [29]. Progesterone is present at nanomolar concentrations in N2-containing medium; such concentrations of progesterone have previously been shown to confer neuroprotection *in vitro* [30].

Another widely used supplement allowing the growth of neurons in serum-free medium is referred to as B27 [31]. It is also commonly used in protocols for neuronal differentiation of ES cells [32–34]. B27 is more complex than N2 and contains over 20 components, among which are vitamins, hormones and growth factors (including insulin and transferrin), antioxidants (including catalase and superoxide dismutase) and fatty acids. Unfortunately, the patented composition of B27 has not been fully disclosed.

The experimental comparison between the two supplements and the identification of elements required for ES cell neuronal differentiation has not been performed and is hampered by the non-disclosure of the composition of B27, but there is a need for such studies to be conducted in the future. It is, however, interesting to note that B27 contains abundant antioxidants, while N2 does not. Whether these antioxidants are simply neuroprotective or might impact on differentiation processes remains to be elucidated. Also, fatty acids might not simply serve as nutritional elements, but might be involved in differentiation signalling.

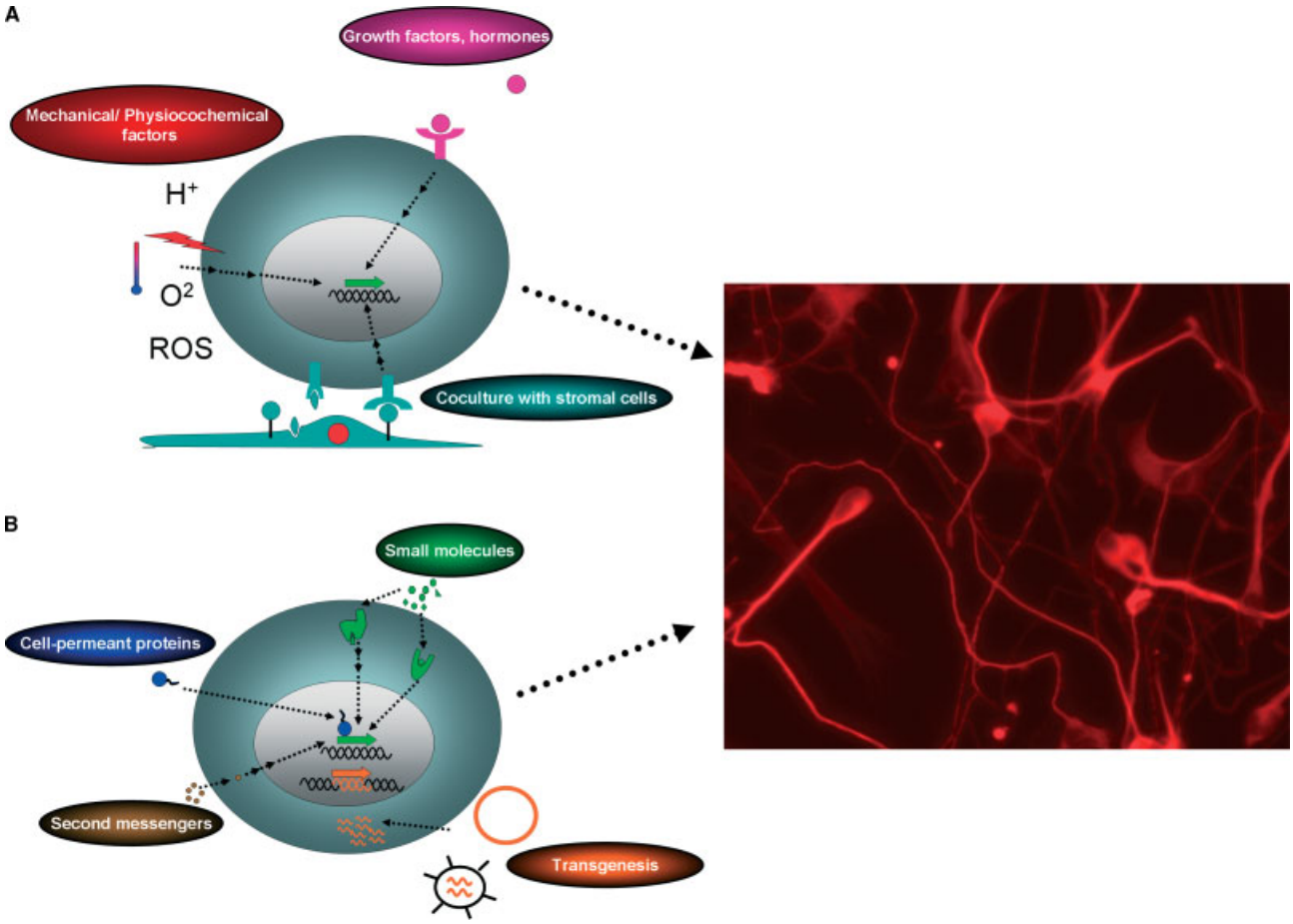

Figure 2. Strategies to direct neuronal differentiation. (A) Modulation of extracellular elements. Growth factors, hormones (pink) or factors derived from stromal cells (light blue) influence cell fate through interaction with cellular receptors. Mechanical, physical and chemical factors (red) also impact on cell differentiation. All those factors modulate intracellular signalling cascades that regulate gene transcription, thereby leading to cell differentiation. (B) Modulation of intracellular elements. Cell-permeant transcription factors (blue), second messengers (brown) or small molecules (green) are able to permeate cellular membranes. Cell-permeant transcription factors act directly at the transcriptional level, whereas second messengers or small molecules may act at different levels of signal transduction pathways. Plasmidic or viral vectors (orange) can transfer proneural genetic material to the host genome, resulting in a redirection of the ES cell differentiation programme

Growth factors and hormones

Neuronal differentiation was initially considered mainly as a default pathway, as suggested by studies performed in different animal models (reviewed in [2]). Along this line, work done in mouse ES cells demonstrated that neuronal differentiation was enhanced in serum-free medium [35], thus suggesting the presence of inhibitory signals in the serum, such as BMP family members. Low cell density was also found to be critical, suggesting the existence of paracrine factors inhibiting neuronal differentiation. Ying *et al* established a neuronal differentiation protocol of ES cells using a serum-free monolayer culture system [6]. This approach is based on the elimination of any inductive signals for alternative fates. However, under their conditions, basic FGF (bFGF) signalling was crucial for proper neuronal differentiation of ES cells. Interestingly, according to Smukler *et al* [36], mouse ES cells which are cultured in serum and growth factor-free medium acquire a primitive neural stem cell fate in the absence of exogenous or paracrine bFGF. However, under these conditions the cells were unable to survive further and proliferate without the addition of growth factors. Recently, it was shown that bFGF acts in synergy with Notch signalling during early neural specification of embryonic stem cells [37]. Therefore, the role of bFGF seems to be dispensable for the very initial step of neural fate specification but becomes essential later to sustain cell survival, proliferation and further differentiation.

There are only few growth factors used to commit ES cells towards neuroectoderm. bFGF is widely used and is present in most culture protocols, but it is generally not included for the initiation of neural induction; however, paracrine secretion of bFGF seems to play an important role in these events [6]. Noggin has been successfully used by several groups to direct embryonic stem cells towards the neuronal lineage [32,34,38]. A simple addition of noggin to the culture medium may result in the conversion of >90% of cells to neuroectoderm [32].

Apart from bFGF and noggin, most other factors which have been used in this context have also a strong influence on the type of neuroectoderm induced, and

therefore limit the potential of the resulting cells. For example, retinoic acid was shown to have a caudalizing effect on neuroectoderm [39]. Therefore, only some subsets of neurons can be efficiently generated when retinoic acid is used to induce neuronal differentiation. Other growth factors, such as Sonic hedgehog (Shh), FGF8, brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) or vitamin C, have been used for the specification of neuronal subtypes or during later stages of differentiation [40,41]. Different types of neurons or glial cells can be generated using specific combinations of soluble molecules. Shh, FGF8 and vitamin C have been used to specify dopaminergic neurons from embryonic stem cells [40–44]. To specify motor neurons, factors such as Shh and retinoic acid (RA) are commonly used [45,46]. There is evidence that leukaemia inhibitory factor (LIF) is important for the maintenance of motor neuron phenotype in rodents [47]. However, it is not clear whether this is also true for human embryonic stem cell-derived motor neurons. Neural progenitors can also be specified towards glial cells; factors used to govern oligodendroglial cell fate include platelet-derived growth factor (PDGF) and the thyroid hormone T3 [40,48], whereas the addition of ciliary neurotrophic factor combined with epithelial growth factor (EGF) can induce an astrocytic phenotype [40].

Extracellular matrix

It has been shown that the contact of ES cells with different components of the extracellular matrix can alter their differentiation programme [49,50]. Laminin/polyornithin coating has been shown to enhance neuronal differentiation from ES cells as compared to plastic or other substrates [51]. In contrast, gelatin appears to favour glial commitment [51]. In addition, cells cultured on different substrates also show different phenotypic specifications [51]. The most convincing studies showing extracellular matrix-mediated differentiation of ES cells used a complex matrix isolated from human amniotic membrane [52]. Interestingly, this matrix not only induces neural differentiation but also favours forebrain and midbrain phenotypes. The nature of the neural-inducing signal present in this matrix remains unknown.

The use of extracellular matrix components to direct neuronal differentiation from ES cells remains largely unexplored. Such approaches could also contribute to redirect ES cell fate towards a neural phenotype. From a signalling point of view, soluble ligands derived from extracellular matrix components could be used to act in a similar manner, and would be much easier to handle. However, in addition to the binding of specific cell surface receptors, mechanical factors linked to cell attachment could also contribute to cell differentiation. For example, laminin has a certain degree of specificity for the attachment of neural cells [51]. It is used as a cell coating reagent in most culture protocols, and allows enrichment for neural cells by simple passaging.

Co-culture with stromal cells

Co-culture with stromal cells has proved efficient not only for the maintenance of the undifferentiated state of mouse and human ES cells but also for promoting neuronal differentiation. Several cell lines have been used for this purpose. The first one to be reported is the PA6 stromal cell line [53], isolated from skull bone marrow [54]. Strong neuronal induction of both mouse [53] and primate [55] ES cells was reported. Interestingly, this activity was maintained even if the stromal cells were fixed with paraformaldehyde, thus suggesting that the stromal cell-derived inducing activity (SDIA) is at least partly mediated through interaction with membrane-bound molecules. In addition to favouring neuroectodermal lineage commitment, dopaminergic differentiation was also markedly induced by PA6 SDIA. The MS5 stromal feeder cell line has also been successfully used to promote neuronal differentiation of mouse and human ES cells [40,41]. Efficient differentiation towards different neuronal subtypes as well as astrocytes and oligodendrocytes was achieved by combining co-culture with MS5 and the addition of soluble molecules. More recently, co-culture of primate ES cells with Sertoli cells has also shown potent neuronal induction and also induction towards a dopaminergic phenotype [44].

Therefore, many cell types seem to have the ability to direct neuronal differentiation from ES cells. Some of these lines appear to promote differentiation towards specific neuronal lineages [44,53,55], whereas others do not [40,41]. Of note, recent work by Lowell *et al*. [5] has shown that fibroblasts engineered to express Notch ligand Delta 1 are able to induce neuronal differentiation of both mouse and human ES cells. This could be a common mechanism for the SDIA of different cell lines, but it remains to be confirmed.

Mechanical and physicochemical factors

Factors such as pH, oxygen tension, osmolarity, temperature and mechanical stress could potentially modulate ES cell differentiation. However, modulation of these parameters has not been extensively explored in ES cell research. Studies by different groups reported a role for mechanical stress in vasculogenesis from ES cells [56–58]. Oxygen tension plays also a role in the differentiation of ES cells, with high oxygen concentrations favouring the maintenance of the undifferentiated state [59]. Interestingly, a recent study reported that differentiation of embryonic stem cells towards neurons can be enhanced by electrical stimuli [60]. An improved understanding of the role of mechanical and physicochemical factors in ES cell differentiation is necessary in order to fully exploit their potential in the redirection of ES cell fate.

Intracellular environment

Different strategies can be employed to directly modify the intracellular environment of ES cells. One option is to modify gene expression by the introduction of nucleic acids (e.g. small interfering RNAs, plasmids) into the target cell. Another possibility is to use small molecules or proteins crossing the plasma membrane and binding to intracellular targets to modify their activity.

Transgenesis

Introduction of transgenes in ES cells has been a technical challenge for a long time. To generate stable ES cell lines, electroporation of plasmidic DNA has been, and is still, widely used. However, this technique is cumbersome, time-consuming and hampered by clonal artifacts. Today, novel approaches allow the generation of stable ES cell lines expressing one or several transgenes. Viral vectors are particularly promising in these respects, and we and others have recently developed lentivectors allowing rapid generation of virtually pure transgenic ES cell lines [61–63]. These constructs have the ability to carry ubiquitous or tissue-specific expression modules [62,63], allowing action on ES cell fate at a given stage of differentiation. Different transgenesis strategies can be used to enhance the yield of ES cell-derived neurons.

First, transgenesis may allow redirection of ES cell differentiation through expression of transcription factors involved in cell fate decisions. Zhao *et al* [13] expressed SoxB proteins in mouse ES cells and observed the promotion of ectodermal differentiation at the expense of mesodermal and endodermal differentiation. Over-expression of transcription factors can also direct ES cells towards a specific neuronal phenotype. Chen *et al*. [64] have shown that over-expression of Nurr-1 promotes dopaminergic differentiation of ES cells. The limitation of such approaches is that most transcription factors should be present only during a defined developmental window. One option would be the use of promoters active only at a particular stage of differentiation. Another possibility is to use inducible gene expression systems [65–67] to allow the temporal control of transgene expression. Such strategies should allow the physiological expression pattern of transcription factors to be mimicked more closely and *in vitro* differentiation protocols to be improved.

Another approach to enrich for a specific cell population is to express a selection marker under the control of a cell type-specific promoter. Green fluorescent protein (GFP) expression controlled by the Sox-1 [68] or the Tα1–α-tubulin [69] promoter allows sorting for early neuroectodermal cells or young neurons, respectively. The same approach can also be used to select for more differentiated cells, such as motor neurons derived from human ES cells [70]. A major limitation of this technique is the poor survival of some cell types after cell sorting. An alternative approach consists in the expression of an antibiotic resistance using a tissue-specific promoter, to allow the selective killing of undesired cells. Kolossov *et al* achieved high-quality purification of cardiomyocytes from mouse ES cells using targeted puromycin resistance expression, and demonstrated the absence of teratoma formation upon injection of these cells in the animal [71]. Such strategies could also allow purification of neuronal progenitors at a defined stage of differentiation.

Second messengers

Second messengers are interesting candidates to modulate embryonic stem cell differentiation. For example, the cAMP analogue dibutyryl cAMP enhances dopaminergic differentiation from ES cells [72]. Calcium and reactive oxygen species (ROS) also play important roles during ES cell differentiation. A decrease in extracellular calcium impairs differentiation towards cardiomyocytes [73]. Antioxidants also decrease cardiomyogenesis, whereas the simple addition of hydrogen peroxide increases differentiation towards cardiomyocytes [74]. It is also probable that NO–cGMP signalling plays a role during the differentiation of embryonic stem cells (reviewed in [75]), although no experimental data are available to show the effect of modulating this pathway. A better comprehension of the role of second messengers during neuronal differentiation is necessary to allow their controlled modulation for stem cell engineering.

Cell-permeant proteins

Recombinant proteins can be engineered to cross the plasma membrane and therefore directly reach the intracellular space. They consist of a protein transduction domain fused to the protein of interest [76]. Krosl *et al*. showed that a recombinant Tat-HoxB4 protein could promote the self-renewal of haematopoietic stem cells [77]. If applied to embryonic stem cells, this approach could allow the sequential intracellular delivery of specific transcription factors at given developmental stages to guide cells through differentiation. For example, the Tat–Pdx1 fusion protein has been successfully used to redirect human ES cells towards a pancreatic lineage [78], although with limited efficiency. Unfortunately, the success rate of this approach has a poor predictive value, with an important variation in the efficiency of intracellular delivery of different cell-permeant proteins [76].

Small molecules

Small organic molecules can reach the intracellular space very easily, provided that they have the required chemical properties (Lipinski's rule of five [79]). Many of these molecules can modulate intracellular pathways and a large number are already validated for clinical use, thereby being ideal candidates for future cell therapy. Their availability in large libraries allows

the performance of high-throughput screening assays on ES cells to discover differentiation modulators and has led to the discovery of compounds modulating ES cell fate [80–83]. Ding *et al* discovered a highly specific glycogen synthase kinase 3β (GSK-3β) inhibitor promoting neuronal differentiation of ES cells [83]. More recently, another molecule was shown to maintain ES cells in an undifferentiated state [82]. All these discoveries represent promising avenues of research into targeting different intracellular pathways for the optimization of cell differentiation.

Generation of clinical grade neurons

One of the most challenging issues in human ES cell research is the generation of neurons which could be used for therapy in humans. First, cells have to be free of any potentially harmful substances. Many differentiation protocols include material of animal origin, which is of concern for several reasons. Pathogens might be transmitted to human cells, even though such events have not been found to occur so far [84]. In addition, feeder cells can transfer xenogenous glycans to human ES cells and induce an immune response [85]. The second major challenge is to predifferentiate ES cells homogeneously to the adequate developmental stage. Neuronal precursors must be mature enough to avoid the emergence of tumours. On the other hand, they must still have the potential to engraft and develop functional connections with the host tissue. In this section, we discuss how the differentiation strategies discussed above can be applied to the generation of clinical grade neurons.

Addition of soluble factors or the use of extracellular matrix components can be adapted to the generation of clinical grade cells, provided that they are synthesized under good manufacturing conditions. Recently, several research teams have explored this approach and have developed chemically defined media whose components could potentially be replaced by clinical grade equivalents [33,86,87]. Co-culture systems with stromal cells are more difficult to use in this context, as most of these lines are of animal origin [41,44]. One possibility is to replace these by human cell lines, but such lines have not been described as yet. The use of human-derived matrix components with neural-inducing properties is also an alternative [52]. Although mechanical and physicochemical factors have not yet shown their use in neuronal differentiation of ES cells, these factors remain to be investigated, as they are simple and should be easy to adapt to good manufacturing practice conditions.

It is not clear at this point whether vector-mediated transgenesis of ES cells will be suitable for clinical applications. Indeed, insertion of transgenes into regions involved in cell cycle regulation carries some risk of tumourigenesis. This is illustrated by cases of leukaemia in patients treated by gene therapy, in which the expression of an oncogene was enhanced by the insertion of a retroviral vector [88]. These transforming events are less frequent when using lentivectors compared to traditional retroviral vectors [89]. However, a study in primate cells has shown that lentivectors often integrate close to genes involved in cell cycle regulation [90] and may therefore also confer the risk of malignant transformation. One way to control this problem could be the establishment of clonal transgenic ES cell lines, for which the copy number and insertion site(s) of the transgene(s) are well characterized. Another promising approach is the use of vectors which do not integrate into the host genome and are therefore devoid of any risk of insertional mutagenesis [91].

Intracellular-acting molecules, such as cell-permeant transcription factors or second messengers, are also promising and their synthesis could be performed under good manufacturing practice conditions. However, their use in redirecting stem cell fate has not yet been extensively investigated. In contrast, small organic molecules have already shown their ability to modulate stem cell fate and to enhance the neuronal differentiation of ES cells [82,83]. Their low cost and the validation of many of these compounds for use in humans makes them ideal candidates for large-scale production of clinical grade ES cell-derived neurons.

A major limitation of current differentiation protocols is the lack of synchronicity of the neuronal cell population generated. Bibel *et al* have been able to differentiate mouse ES cells towards a pure population of radial glial cells [92]; however, this has not yet been achieved in human ES cells. The production of a homogeneous cell population is crucial for cell therapy in humans, for the following reasons. First, the presence of poorly differentiated ES cells must be avoided, given that the latter cause teratoma formation upon *in vivo* injection [1]. Strategies allowing selective killing of undifferentiated cells [93] or selective survival of the population of interest [71] are promising, but carry the drawbacks of transgenesis. Tumours may also arise from predifferentiated cells. It was shown that ES cells predifferentiated towards the dopaminergic neuronal lineage can give rise to slow-growing tumours following implantation into the rat brain [94]. Second, cells that are too advanced in their differentiation may be unable to integrate properly into the host tissue. As illustrated by the implantation of retinal progenitors [74], cells must fit into a certain developmental window to allow their functional integration into the host tissue. Therefore, synchronization of the cell population remains one of the most important challenges for the generation of ES cell-derived clinical grade neurons.

Neuropathology and cell replacement therapy

Cell replacement therapy is an emerging concept in regenerative medicine and is targeted at the treatment

of pathological conditions caused by the loss of a particular cell type. Potential applications include a broad range of diseases, such as myocardial infarct, retinal degeneration, neurodegenerative diseases, etc. A common feature of these diseases is the limited efficiency of the presently available treatments which generally remain symptomatic. A curative treatment should restore the function which was lost as a consequence of cell death. Human embryonic stem cells are very interesting candidates for such a purpose, as they represent a virtually unlimited source of any cell type of the body. There are two key conditions to be fulfilled in order to achieve these goals. First, ES cells have to be differentiated to the cell type lacking in a particular pathological condition. Second, the host environment must be permissive enough to allow proper integration of the grafted cells. This will be particularly challenging when inflammation and immune reactions play an important role in the course of the disease, such as in multiple sclerosis. Of note, stem cells have been reported to have immunomodulatory properties after implantation in the mouse brain [95]. These properties could be particularly useful in the context of therapeutic applications. We discuss here some neuropathological conditions which could benefit from cell replacement therapy.

Parkinson's disease

Parkinson's disease is mainly characterized by motor symptoms, but patients also develop later cognitive decline. The principal affected region is the *substantia nigra* of the mesencephalon. Normally, this region appears black upon macroscopic examination because it contains high amounts of neuromelanin. In Parkinsonian patients this black colour is almost completely lost (Figure 3A), due to the degeneration of dopaminergic neurons of the *substantia nigra*. The role of these neurons is to release dopamine within the striatum to regulate motor coordination.

The implantation of ES cell-derived dopaminergic neurons directly into the substantia nigra would be a very attractive option and could allow the original nigro-striatal pathway to be restored. However, experiments performed with fetal neurons implanted in the mesencephalon have shown that these cells were unable to extend axons towards the denervated striatum [96–98]. For this reason, the most widely used approach has been the implantation of dopaminergic neurons directly into the striatum. Previously, fetal mesencephalic dopaminergic neurons have been used [99], but this approach requires several fetuses to treat one patient and raises important immunological and ethical considerations. More recently, many

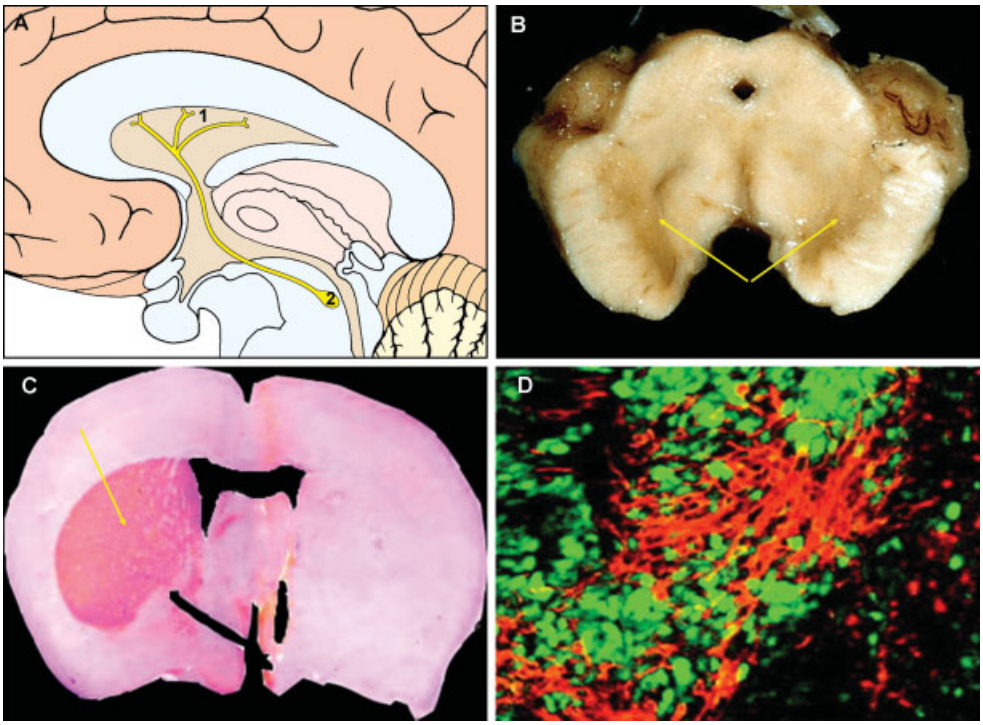

Figure 3. Cell therapy of Parkinson's disease. (A) The nigrostriatal pathway (yellow) is constituted by projections of dopaminergic neurons originating from the substantia nigra and projecting to the striatum. ES cell-derived neurons are generally injected into the striatum (1); given the potential of ES cell-derived neurons to extend axons, injection into the substantia nigra is an attractive possibility (2). (B) Macroscopic aspect of the mesencephalon of a Parkinsonian patient (adapted from http://www.neuropathologyweb.org/, with permission). Arrows show the substantia nigra which has lost is dark colour due to degeneration of dopaminergic neurons. (C) Implantation of dopaminergic neurons in the rat striatum. The brain was stained against the dopaminergic-specific protein tyrosine hydroxylase (arrow). (D) Higher magnification of implanted cells, showing the presence of numerous dopaminergic neurons (red) among non-dopaminergic neurons (green). (C, D) Reproduced with permission from AlphaMedPress

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research groups have aimed at targeting differentiation of human ES cells towards dopaminergic neurons [41,42,94,100–102]. ES cell-derived dopaminergic neurons have been implanted into the striatum of animal models of Parkinson's disease and several studies reported significant clinical improvement [26,41,43,103]. An example of implantation of human ES cell-derived dopaminergic neurons in the rat striatum is illustrated in Figure 3B [44]. It is important to note that even if many neurons are dopaminergic (Figure 3C), the majority of cells are non-dopaminergic neurons (Figure 3C). This is a common limitation of current protocols, which do not allow phenotypically pure cell populations to be generated. However, recent encouraging improvements in differentiation protocols [41,44,53,94] suggest that the generation of pure populations of dopaminergic neurons will be feasible in the near future. In addition, clinical improvements reported by several studies upon implantation of ES cell-derived dopaminergic neurons are very encouraging and suggest that Parkinson's disease is a good candidate for future cell replacement therapy.

So far, ES cell-derived dopaminergic neurons have been implanted into the striatum of animal models. As discussed above, this is based on the limited capacity of implanted fetal neurons to extend axons. However, given the important plasticity of ES cell-derived neurons, it is possible that a physiological sprouting of dopaminergic axons might occur. Thus, it is conceivable that implantation of ES cell-derived neurons in the substantia nigra might allow them to reinnervate the striatum.

Ischaemic stroke

Ischaemic stroke is one of the leading causes of death in developed countries, and is most frequently caused by the rupture of an atherosclerosis plaque. It is characterized by the loss of neurons in a vascular territory of the brain and often affects several functionally distinct areas and different neuronal subtypes. Consequently, the symptomatology is very variable and consists most often of sensorimotor deficits and less often of cognitive impairments.

In these conditions, replacement of lost neurons is extremely challenging. Indeed, implanted cells should be able to replace a variety of neuronal subtypes and to restore cortical networks as well as subcortical and corticospinal projections. Figure 4B illustrates an example of the macroscopic aspect of a brain infarct causing swelling and compression of adjacent brain structures. Several studies suggest that ES cells can integrate and differentiate into the damaged brain [104–106]. One example is illustrated on Figure 4C [105] and shows the implantation of ES cell-derived neurons marked with GFP in infarcted regions of the rat brain. The implanted cells can survive over prolonged periods and many of them develop long neuritic extensions (Figure 4D). Clinical improvements of animals have been observed after implantation of cells, but it remains unclear whether this benefit can

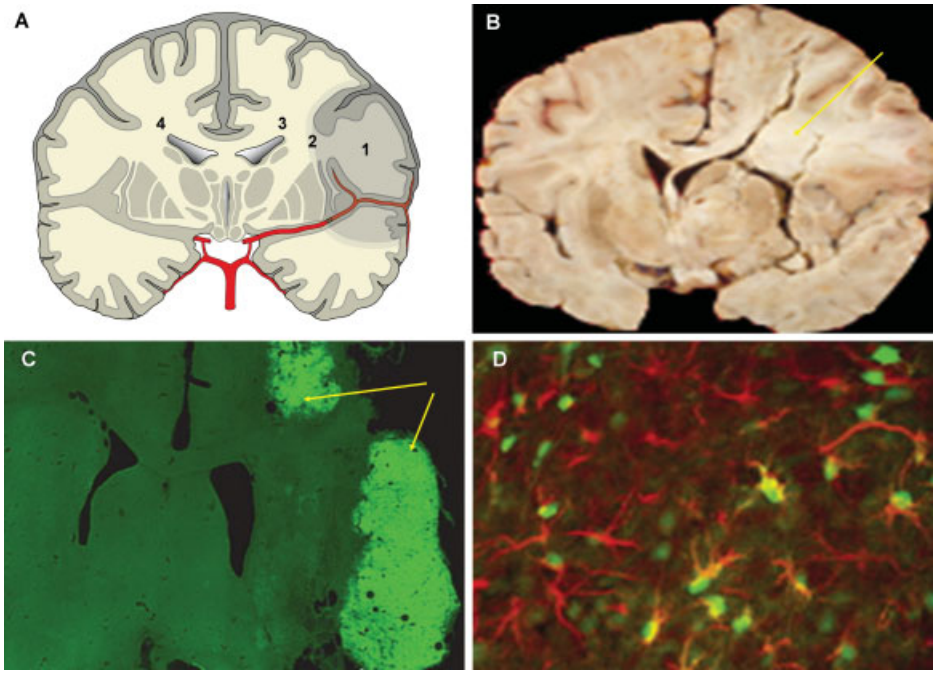

Figure 4. Cell therapy of ischaemic stroke. (A) Ischaemic stroke is caused by the occlusion of a brain artery and results in lesions in the respective vascular territory. Different sites could be envisaged for the injection of ES cell-derived neurons: 1, infarct zone; 2, penumbra zone; 3, ipsilateral healthy tissue; 4, controlateral tissue. (B) Macroscopic aspect of the whole brain of a patient with ischaemic stroke (adapted from http://www.neuropathologyweb.org/, with permission). The arrow indicates the affected region. (C) Embryonic stem cell-derived neurons expressing green fluorescent protein (GFP) were implanted in the mouse brain after cerebral infarction (arrows). (D) Higher magnification shows GFP-positive (green) cells stained against a dopaminergic neuron-specific protein (red). (C, D) Reproduced with permission from Oxford Journals

be attributed to the functionality of implanted cells. The optimal site for implantation of ES cell-derived neurons in the case of stroke remains unclear. It is likely that cells injected directly into the lesioned area during the acute phase of cerebral stroke will poorly survive due to necrosis, matrix degradation and disrupted local blood supply. However, the important migratory capacity of ES cell-derived neuronal precursors [107] suggests the possibility of implanting them at the margin of the infarcted zone to repopulate the damaged area. It remains to be determined whether cells should be injected in the penumbra zone surrounding the infarct [Figure 4A(2)], which is characterized by delayed neuronal cell death caused by apoptosis, or rather into healthy neighbouring tissue [Figure 4A(3), (4)]. Interestingly, one study reported that clinical benefits from implanted progenitors were identical when these were implanted ipsilateral or controlateral to the lesion; however, the cells survived better when implanted on the controlateral side [108].

Multiple sclerosis

Multiple sclerosis is a degenerative disease characterized by the destruction of myelin sheets produced by oligodendrocytes, resulting in the impairment of neuronal connections throughout the central nervous system. During the later course of the disease, oligodendrocytes and neurons are lost. Lesions are usually circumscribed to demyelinating plaques, and often a few individual lesions are responsible for the symptomatology.

Cell replacement therapy should aim at the implantation of *in vitro*-generated oligodendrocytes to reconstitute myelin sheets around axons. Implanted ES cell-derived oligodendrocytes precursors could be implanted directly into a lesioned area. Previous studies have shown the possibility of differentiating ES cells towards oligodendrocytes and their ability to remyelinate axons *in vivo* [48,109–112], but symptomatic recovery from diffuse demyelinating conditions upon oligodendrocyte implantation has not been established.

Spinal cord lesions

Spinal cord lesions are mostly of traumatic origin and often affect young patients. In general, the most pronounced defects consist in the dramatic loss of motor functions in specific parts of the body, depending on the site of lesion. No treatment is available and spontaneous recovery is generally very poor.

Different strategies of cell replacement therapy can be envisaged. One possibility consists in the implantation of *in vitro*-generated motor neurons into the spinal cord. These neurons should be able to regenerate axons contacting peripheral muscles and establish functional connections with afferents originating from upper motor pathways. Several research groups have shown the possibility to direct human ES cell differentiation towards motor neurons. These cells were able to integrate into the chick spinal cord and reinnervate distant targets [45,46,113]. However, functional recovery after grafting remains unclear. A second possibility is to implant oligodendrocytes into the damaged spinal cord to remyelinate axons in order to restore connectivity. One study reported the implantation of human ES cell-derived oligodendrocytes in the injured rat spinal cord [114]. Oligodendrocytes were able to remyelinate host axons, but the symptomatology remained unchanged. Finally, a very attractive possibility is a combined cell replacement therapy using both motor neurons and oligodendrocytes, which should allow both reconnection of nervous tracts and their myelinization.

Other neuropathological disorders

Amyotrophic lateral sclerosis (ALS) is characterized by a selective loss of motor neurons in the spinal cord and subsequently in the cerebral cortex. Similarly to spinal cord lesions, implantation of ES cell-derived motor neurons could potentially promote recovery from motor defects.

Alzheimer's disease is the most frequent cause of dementia in developed countries. There is currently no treatment allowing reverse the course of the disease to be reversed. It is characterized by widespread neuronal cell death, touching almost every area of the brain. Therefore, a cell replacement therapy approach for this disease is very difficult to envisage.

Huntington's disease is a genetically inheritable disease caused by the amplification of triplets, resulting in polyglutamine repeats in the huntingtin protein. This form of the protein is neurotoxic and causes neurodegeneration, which is most pronounced in the striatum. Patients develop chorea, and secondarily also dementia. Recently, cell replacement therapy of Huntington's disease using fetal transplants has been performed in humans [115]. The improvement of the symptomatology was only transitory in these studies and it remains unclear to what extent cell replacement therapy could provide a benefit for these patients. However, there is no study about the use of ES cell-derived neurons to treat Huntington's disease, and it is possible that longer-term effects could be achieved.

Schizophrenia is a psychiatric condition characterized by psychosis and deep impairment in social and occupational functioning. Recently, it was shown that a specific subtype of GABAergic neurons are lost in the dorso-lateral prefrontal cortex of affected individuals (reviewed in [116]). It is therefore conceivable that the implantation of specific GABAergic neurons directly into affected areas could help to restore normal brain functions in these patients.

Severe epilepsies. Epilepsy is characterized by the repeated occurrence of seizures, which can be very frequent in some individuals and severely perturb daily life. In many cases of generalized epilepsy, a focal source is propagating excitatory waves to the rest of the brain. This source can often be

identified and is characterized by its high excitability. An attractive possibility would be to implant ES cell-derived inhibitory neurons into epileptic foci to lower their excitability threshold and consequently the frequency of seizures. One study reported the control of epilepsy in the rat using adenosine-producing ES cell-derived neural precursors [117]. Treatment of epilepsy by implantation of ES cell-derived neurons is still in its infancy but should be particularly promising in cases of severe drug-resistant generalized epilepsy.

Conclusions and future perspectives

Currently available protocols allow the directed differentiation of ES cells into neural precursors and mature neurons. However, there is still a long way to go to obtain highly pure and clinical grade neuronal cell populations. The acquisition of a more thorough knowledge of early neural specification events is a prerequisite to improve differentiation protocols. In addition, experimental knowledge acquired from *in vitro* differentiation studies should allow the range of tools for the generation of ES cell-derived neurons to be broadened. The development of high-throughput screening assays testing molecules compatible with good manufacturing practice should be particularly fruitful for this purpose. Such assays could also greatly contribute to the discovery of unknown pathways involved in early neural specification events.

The engineering of a new type of pluripotent cell from human fibroblasts has recently attracted wide attention [118,119]. These cells have been generated through the expression of four genes involved in transcription and chromatin remodelling (*Oct-4, Sox-2, myc* and *Klf4*); *myc*, which is a protooncogene, is probably dispensable [120]. Similar to embryonic stem cells, induced pluripotent cells (iPS) have a very broad differentiation potential. As they can theoretically be generated from primary fibroblasts from the patient to be treated, they could be used to perform autologous cell replacement therapy and therefore avoid problems of rejection. However, at present such cells are generated through lentiviral vector-mediated integration of the respective transgenes into the host cell genome, which potentially might present a risk of malignant transformation. Therefore, it is important to develop other strategies, such as non-integrating transgenesis or cell-permeant proteins, to introduce these transcription factors into the host cells. This will be a difficult challenge, as it remains unclear whether the short-term transgene expression achieved by such methods will be sufficient to generate and maintain iPS cells. Another concern is the extended period of time between the isolation of fibroblasts and the implantation of iPS-derived cells, which is incompatible with cell therapy in cases of emergency, such as myocardial or brain infarction.

In general, the iPS approach should not be considered distinct from the ES cell approach. Both approaches allow pluripotent stem cells to be obtained that can be differentiated into neurons using comparable protocols. At present, high-quality ES cells are already available for use. However. in the long run, iPS might replace ES cells. In the near future, several pathologies of the central nervous system could benefit from cell replacement therapy. Considerable progress has been made in the targeted differentiation of ES cells towards cell types such as dopaminergic neurons and oligodendrocytes. Given that there is currently no efficient treatment for most neurodegenerative diseases, it is likely that that cell replacement therapy based on neuronally differentiated pluripotent stem cells will soon be tested in clinical trials.

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