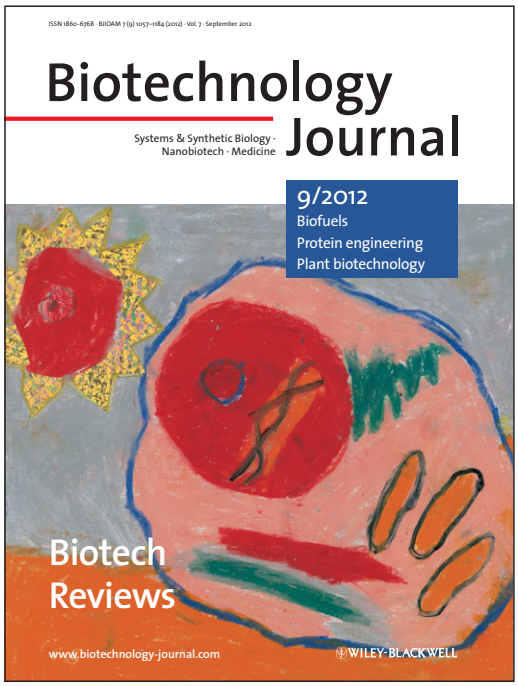

Cover illustration

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Review

Embryonic and induced pluripotent stem cell differentiation as a tool in neurobiology

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Cell lines with the ability to differentiate into all types of somatic and germ cells represent a pluripotent developmental stage that transiently exists in vivo in the epiblast cells of the pre-implantation embryo. Given the lack of access to human neurons, together with the limited numbers and heterogeneity of neurons obtainable from rodent primary cultures, the directed differentiation of pluripotent cell lines into defined cells of the neural lineage has provided a novel versatile tool in neurobiology. Offering a potentially unlimited source of material, directed differentiation of pluripotent cell lines has been particularly well combined with high-throughput transcriptomic and epigenetic analyses. Here, we first overview the potential of different pluripotent lines to give rise to different types of neurons. Then, we discuss the emerging use of neuronal differentiation systems as a tool for unravelling mechanisms that regulate neuronal development and specification, modelling complex neurological diseases and understanding neuronal dysfunction. Beyond providing original insights in many aspects of neuronal biology, these tools have greatly facilitated the development of novel therapeutic interventions for neurological disorders.

Keywords: Embryonic stem cells · Induced pluripotent stem cells · Neuronal differentiation · Pluripotency

1 Introduction to pluripotent cells

Unlike the totipotent fertilised egg, which is able to generate all embryonic and extra-embryonic tissues necessary for development, the cell lines that are discussed here are pluripotent. This term describes the fact that their differentiation potential is limited to generating all cells of the adult, including germ cells, but not typically cells of extra-embryonic lineages.

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Abbreviations: **AD**, Alzheimer's disease; **ALS**, amyotrophic lateral sclerosis; **ASD**, autism spectrum disorders; **EB**, embryoid body; **epiSCs**, epiblast stem cells; **FGF**, fibroblast growth factor; **FXS**, fragile X syndrome; **hES** cells, human embryonic stem cells; **iPS (cells)**, induced pluripotent stem (cells); **LIF**, leukaemia inhibitory factor; **mES (cells)**, mouse embryonic stem (cells); **MN**, motor neuron; **PD**, Parkinson's disease; **RA**, retinoic acid (all-trans); **SHH**, sonic hedgehog; **SOD**, superoxide dismutase

1.1 Rodent ES cells

The first pluripotent cell lines were isolated from mouse embryos in 1981 by two groups independently [1, 2]. They were directly derived from the epiblast of the pre-implantation blastocyst, a transient in vivo compartment that gives rise to all cell types of the embryo proper during development [3]. Mouse embryonic stem (mES) cells, as they are now called, have set stringent criteria for self-renewal and pluripotency for other cell types claiming these properties following their discovery. These can be summarised as indefinite self-renewal in vitro without undergoing transformation or senescence, the ability to be propagated from a single cell and retention of pluripotent differentiation potential throughout extending culturing. The primitive state of mES cells is ultimately demonstrated by their ability to contribute to chimeras when injected into host blastocysts, including contribution to the germline (Fig. 1). Strikingly, when combined in chimeras with tetraploid embryos, mES cells can form entire, viable foetuses [4].

The initial derivation and culturing conditions of mES cells were based on a feeder layer of mouse embryonic

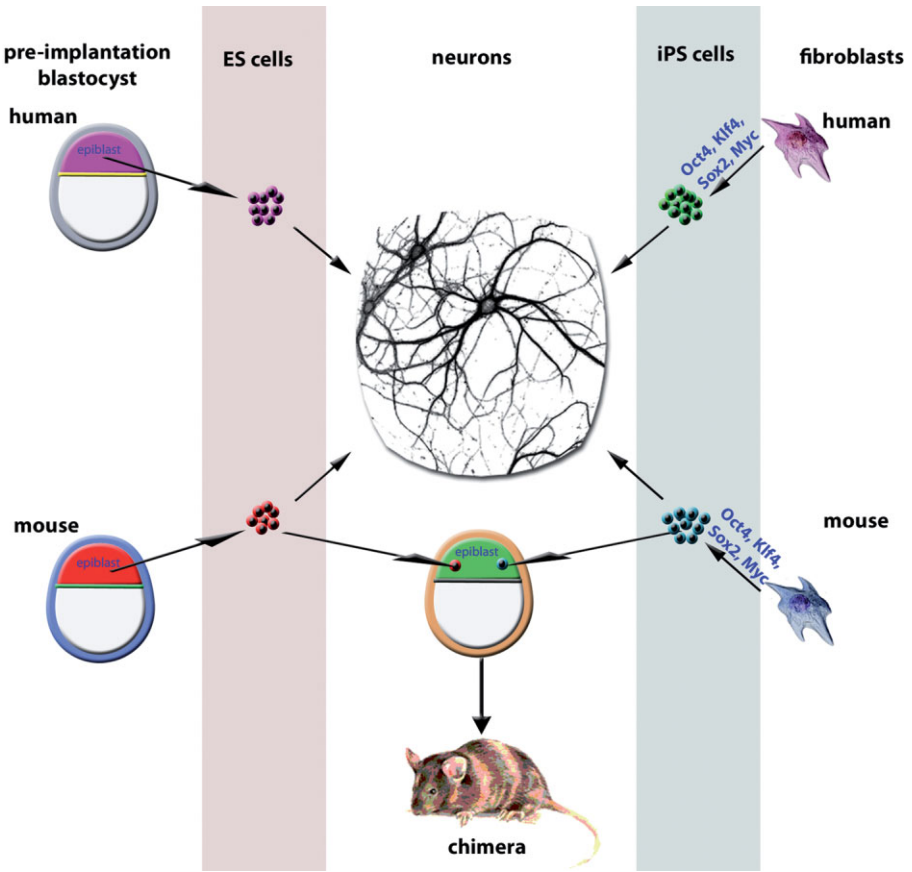

Figure 1. Differentiation of human and mouse ES and iPS pluripotent lines into neurons. ES lines are derived from the epiblast of pre-implantation blastocysts, while iPS cells are generated by reprogramming of somatic cells, such as fibroblasts, by defined factors. Both mouse ES and iPS cells contribute to chimeras and are germline competent.

fibroblasts and serum containing media. It was subsequently recognised that a key contribution of feeders was the secretion of the cytokine leukaemia inhibitory factor (LIF) [5]. Acting through activation of its receptor gp130, LIF initiates a cascade that involves activation of STAT3, ultimately leading to the suppression of differentiation programs [6]. This discovery initially suggested that pluripotency relies on external signals. However, LIF was found to be dispensable for normal development. Abrogation of LIF signalling [7, 8] revealed defects in only in diapause and embryo implantation [9, 10], inviting the speculation that there may be endogenous mechanisms controlling the pluripotent state and differentiation propensity of ES cells. Consistent with this notion, recent work has deciphered the endogenous signals necessary for the unique properties of mES cells. ES cells themselves secrete fibroblast growth factor (FGF) 4, which acts in an autocrine or paracrine fashion to drive ES cells into a general state of differentiation [11]. This mechanism accounts for the intrinsic propensity of ES cells to differentiate, reflecting the transient nature of their in vivo counterparts. Pharmacological or genetic inhibition of the FGF4 signalling cascade is sufficient to block differentiation of ES cells. Simultaneous inhibition of endogenous FGF4 and GSK3 signalling originally using three inhibitors [12], and more recently two inhibitors (2i) [13], is sufficient to maintain ES cells pluripotent in the absence of LIF. However LIF is recognised to further enhance the cloning efficiency of mES cells; therefore, the combined use of "2i+LIF" has allowed embryonic stem cell derivation from species and strains that were until now resistant.

mES cell lines were traditionally established from the Sv129 background, while other strains yielded low efficiencies that inhibited further experimentation. Likewise, derivation was not at all possible for other closely related rodents, such as the rat. The introduction of "2i+LIF" has overcome these difficulties, and germline-competent ES cell lines are now efficiently derived from many mouse strains such as CBA and MF1 [12] and NOD [14] as well as from two strains of the rat [15, 16]. Techniques facilitating homologous recombination in mES cells allowed the generation of an enormous array of mouse mutants that fundamentally changed the way gene function is addressed [17], as recognised by the award of the 2007 Nobel Prize in Medicine to Capecchi, Smithies and Evans [18]. Application of this targeted gene mutagenesis technology to other species will allow the wide manipulation of their genomes. This was recently achieved for rat ES cells, where the p53 and hprt loci were deleted by homologous recombination [19, 20]. The generation of rat mutants for

many genes of interest expected to follow this milestone will greatly enhance behavioural studies and in vivo modelling of human diseases.

1.2 Human ES cells: Comparison to rodent ES lines

Thomson and colleagues [21–23] were the first to isolate ES cells from primates. Using pre-implantation blastocysts and culture conditions comprised of feeders and serum, they isolated pluripotent lines from the Rhesus monkey [21], from the marmoset (*Callithrix jacchus*) [22] and from the human [23]. Despite the apparent similarity of the conditions used to those traditionally employed for the derivation of mES cells, it is increasingly clear that the primate pluripotent lines differ significantly from those of rodents. One early observation was the fact that none of the primate lines responded to LIF, [24] and instead required FGF2 and Activin. Initially, these differences were attributed to species idiosyncrasies during early development, as the timing of embryonic genome expression varies significantly between rodents and primates [25], and the separation of the epiblast and extra-embryonic lineages is not well studied in primates.

Recent findings, however, have raised more doubts on the bona fide stem cell nature of the primate pluripotent lines isolated so far. In 2007 cell lines were derived from the epiblast of the post-implantation egg cylinder of the mouse, and termed epiblast stem cells (EpiSCs) to distinguish them from the more primitive mES cells [26, 27]. Reflecting the developmentally more restricted stage of their origin, EpiSCs demonstrated a poor ability to contribute to chimeras and were germline incompetent [27, 28]. Interestingly, the requirements of EpiSCs for self-renewal were found to be identical to those of primate ES lines, namely FGF2 and Activin. The striking similarity of EpiSCs and primate ES lines (particularly hES) is evident not only signalling components required for in vivo culture, but also in global gene expression as indicated by comparative analyses. This has challenged the view that primate ES lines represent a naïve embryonic state. The derivation of hES lines with genetic and epigenetic characteristics more similar to mES cells would greatly facilitate the transfer of knowledge, including differentiation protocols, between species, which to date remains problematic. However, significant progress has been achieved in genetically manipulating hES cells, using a variety of methods ranging from classical transfection [29] to lentiviral transduction [30, 31] and electroporation [32].

1.3 Induced pluripotent stem (iPS) cells from mouse and human

A ground-breaking advancement in the field was the demonstration by Takahashi and Yamanaka [33] in 2006 that mouse embryonic fibroblasts can be re-programmed to a pluripotent state by the forced expression of four factors (Oct4, Sox2, Klf4, and c-Myc) using retroviral vectors [33]. The resulting pluripotent cells were termed induced pluripotent stem (iPS) cells. Although the first generation of iPS cells were only partially reprogrammed, subsequent studies improved the criteria for selecting for reprogrammed cells [34-36] and eventually obtained iPS cells displaying genetic and epigenetic signatures characteristic of ES cells, such as transcriptional patterns, DNA demethylation of key promoter regions (such as Oct4 and Nanog), X chromosome re-activation in female lines and global patterns of histone methylation, in particular H3K4 and H3K27 tri-methylation [34–37]. In addition to these phenotypic similarities, iPS cells are able to contribute to chimeras when injected into blastocysts (Fig. 1), are germline competent and even give rise to entire embryos with the tetraploid complementation assay [36, 38, 39].

Subsequent studies have confirmed these findings using different combinations of transcription factors (such as Oct4, Sox2, LIN28 and Nanog) [40] and alternative ways for delivering the instructive genes, such as non-integrating adenoviruses [41] or transient plasmid transfection [35]. Moreover, reprogramming has been extended to use different mouse cell types as a starting material. These included hepatic cells [42] and pancreatic islet β-cells [41], blood cells [43], embryonic and adult neural stem cells [39, 44, 45], and intestinal and adrenal cells [36]. Importantly, successful reprogramming was accomplished across a number of species[46], and with several human cell types, including fibroblasts [47, 48], keratinocytes [34, 49] and neural progenitors [39, 50]. Two recent reviews provide in depth analysis on methods for iPS generation and the epigenetic state of iPS cells [51, 52].

2 Neuronal differentiation of pluripotent cell lines

The neuronal differentiation of pluripotent lines is a much studied and continuously advancing field. On-going characterisation of the central nervous system (CNS) is revealing an unprecedented degree of neuronal diversity. Directing pluripotent cells to neuronal fates has largely relied on the transferring knowledge gained from studying neurulation and the patterning of the early nervous system to derive the in vitro conditions required. Inspired by the "default" program of neural induction in amphibians [53, 54], early work on neuronal differentiation of mES cells focused on experiments in defined media and demonstrated that neural markers are expressed in spheroid, fru-floating cultures of ES cells (known as embryoid bodies (EBs), unless BMP and/or Activin proteins are present to block this differentiation [55, 56]. In search of neuralizing cues, differentiation strategies involving retinoic acid (RA) treatment were developed [57-59]. These methods produced cells with gross neuronal morphologies and expressing general neuronal markers such

Table 1. Summary of neuronal differentiation protocols[a)]

Neuronal type	Inductive signal	Reference
Mouse ES cells		
Inhibitory and excitatory neurons	RA	[58]
Glia and functional neurons, ND[b)]	RA	[59]
Dopaminergic and Serotonergic	ITSF defined medium, SHH, bFGF, FGF8	[66]
Dopaminergic and Serotonergic	SHH	[67]
Midbrain Dopaminergic	FGF8/SHH	[68]
Cervical MMC-like motor neurons	PA-6 co-culture	[74]
Caudal LMC-like motor neurons	No extrinsic cues	[75]
Telencephalic neurons	RA, monolayer	[85]
Telencephalic neurons	RA	[86]
Glutamatergic telencephalic neurons	DKK-1, Lefty, Wnt3a	[88]
Gabaergic telencephalic neurons	DKK-1, Lefty, SHH	[88]
Glutamatergic telencephalic neurons	RA, selection of rapidly dividing ES cells	[90]
Glutamatergic telencephalic neurons	Ngn2 expression	[91]
ND	Defined ITSF medium, FGF	[63]
ND	Default pathway	[54]
ND	Default pathway, chemically defined medium	[55]
ND	BMP-4 antogonists	[56]
ND	Default pathway	[57]
Human ES cells		
Some dopaminergic, serotonergic neurons	RA, NGF	[62]
Neurectoderm	BMP-2, Noggin	[131]
Midbrain dopaminergic	MS5 and S2 stromal cells, SHH, FGF8, BDNF	[71]
Forebrain and midbrain dopaminergic neurons	FGF2-FGF8, sHH	[132]
Dopaminergic neurons	Activators of Shh and Wnt signalling	[72]
Dopaminergic neurons	FGF2	[69]
Dopaminergic neurons	FGF2	[70]
Motor neurons	MS-5 stromal layer, plating in the presence of BDNF, SHH and RA	[80]
Motor neurons	RA, SHH	[81]
Glutamatergic telencephalic neurons	SMAD inhibition	[92]
Glutamatergic telencephalic neurons	RA	[93]
ND	RA, TGF-β, ActivinA, BMP4, HGF, EGF, NGF, bFGF	[61]
iPS cells		
Motor neurons	Ra, SHH	[82]
Glutamatergic telencephalic neurons	SMAD inhibition	[92]
Glutamatergic telencephalic neurons	RA	[93]
Dopaminergic	SHH, GSK3β inhibitor, FGF8	[73]

a) Abbreviations used: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; EGF, epidermal growth factor; GSK3β, glycogen synthase kinase-3-beta; HGF, hepatocyte growth factor; ITSF, insulin transferrin selenium fibronectin; NGF, nerve growth factor; TGF-β, transforming growth factor-beta
b) ND, identity not determined

a neurofilament light chain and synaptophysin. However, these cultures were contaminated by glial cells, mostly astrocytes, although oligodendrocytes were also generated and selectively expanded [60]. Similarly, RA was used to drive the differentiation of hES cells into a general neuronal state [61, 62].

In an alternative approach, EBs were not exposed to any inductive signal, but cultured in a selective, serum-free medium to eliminate non-neuronal cell types [63]. While this approach resulted in a dramatic decrease in cell numbers, the surviving population was homogeneously expressing the neural progenitor marker Nestin and differentiated into GABA-ergic neurons with high efficiency. In addition to treatment with inductive cues, a variety of scaffolds and biomaterials has been recently employed to promote neuronal differentiation. In one study, activated charcoal biosubstrate was used to pro-

mote neuronal differentiation of hES cells [64]. Similar results were obtained with Tussah silk fibroin scaffolds [65].

More recently, efforts have been concentrated on enhancing the efficiency of previous neural differentiation protocols and on directing the differentiation of pluripotent cells into defined types of neurons equivalent to in vivo cell populations. Some of these approaches are discussed below, and summarised in Table 1. Protocols for differentiating hES and human iPS cells into specific subtypes of neurons are under intensive development, with clues for successful strategies sought in mouse neural embryology studies and mES cell differentiation protocols.

2.1 Dopaminergic neurons

In one study [66], ES-derived neural precursors were treated with sonic hedgehog SHH and FGF8– associated with ventral midbrain and the hindbrain patterning respectively [67] - in order to obtain dopaminergic and serotonergic neurons. Dopaminergic neurons also have been obtained with relatively high efficiency by the direct co-culture of ES cells with a monolayer of bone marrow derived stromal cells (PA-6 cells) [68]. However, the molecular nature of the inductive signals secreted by the stromal cells remains elusive. Midbrain dopaminergic neurons have been differentiated from hES cells via several strategies: using FGF2 [69, 70], stromal cells along with growth factors [71] or activators of WNT and SHH signalling pathways [72]. Engraftment of the cells derived by WNT and SHH activation were found to improve several disease-associated behaviours in three different models of Parkinson's disease. Dopaminergic neurons were also recently derived from human iPS cells using a combination of SHH, GSK3β inhibitor and FGF8 [73].

2.2 Motor neurons (MN)

Initial methods for differentiating ES cells into MNs used RA and SHH to guide neural progenitors towards a ventral fate [74]. The resulting neurons expressed characteristic MN markers such as the transcription factors Hb9, Isl1/2, and Lhx3, the enzyme choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT). Moreover, they developed MN-specific functional properties such as the ability to cluster acetylcholine receptors when synapsing onto cultured myotubes. Under these culturing conditions of RA/Shh, a specific class of MNs was generated with a gene expression profile and functionality that is similar to MNs of the cervical level of the spinal cord occupying the medial motor column [74-78].

Recently, additional subtypes of MNs were obtained in the absence of extrinsic cues. The resulting neurons have expression profiles characteristic of more caudally positioned MNs of the branchial and thoracic levels, and a subset of them acquire molecular identities typical of neurons in the lateral motor column. [75, 77, 79]. Given the relevance of motor neurons in many human disorders, it will be critical to transfer this knowledge to hES and human iPS cells. To this end, two recent studies have demonstrated that motor neurons can be generated from different lines of human and monkey ES cells. In one study, human and monkey ES lines were co-cultured on a stromal layer of MS5 cells in a neural medium. Subsequently arising neural rosettes were dissociated and plated in the presence of BDNF, SHH and RA. Under these conditions, 20% of neurons acquired a motor neuron identity, many of which displayed markers characteristic of caudal brachial motor neurons [80]. Similar identities were obtained by a second study [81] using a slightly different induction protocol. In addition, it was recently demonstrated that functionally mature motor neurons could be derived from human iPS cells using previously described protocols based on RA and SHH [82]. However, the molecular identity of this population was not examined in any detail.

2.3 Cortical neurons

Work from the laboratories of David Price, Magdalena Gotz and Francois Guillemot, amongst others, has revealed the basic mechanisms of corticogenesis in the mouse brain. It is now well established that excitatory glutamatergic neurons, constituting the majority of neurons in the cortex, are derived from radial glial progenitors of the dorsal telencephalon expressing the transcription factor Pax6 [83]. By contrast, the smaller population of GABAergic inhibitory neurons also present in the cortex are not induced locally. They are derived from Pax6-negative radial glial cells of the ventral telencephalon and migrate tangentially along established routes to enter the cortex and integrate in the laminar structure of the cortical plate [84].

Using RA in a monolayer differentiation protocol of mES cells [85], Conti and colleagues reported the generation of radial glial progenitors [86], which could be maintained in a self-renewing proliferative state by the combined action of bFGF and EGF. These conditions are very similar to those used for the culture of neurospheres generated from the mouse brain [87]. Though convenient, the expansion of cortical progenitors using these growth factors diversifies the progenitor population, resulting in a heterogeneous progeny of excitatory and inhibitory telencephalic neurons, along with a high percentage of glial cells. Another protocol directing mES cells into neural progenitors of telencephalic identity, reported over 90% of cells positive for the telencephalic marker BF1 [88]. This was achieved by treatment with DKK1 and LEFTYA, known antagonists of Wnt and Nodal signalling respectively. Further treatment of these progenitors with WNT3a induced a dorsal pallial identity, generating up to 75%

Pax6 positive radial glial progenitors, whereas SHH treatment promoted a ventral telencephalic identity at the cost of pallial telencephalic differentiation.

Bibel and colleagues successfully directed mES cells into dorsal telencephalic radial glial cells with an unmatched level of homogeneity. This RA-based protocol relied on selecting for the most rapidly dividing mES cells, in order to enrich for naïve ES cells as a starting material [89, 90]. Over 90% of cells represented Pax6-positive radial glial, which after one or two divisions were terminally differentiated into virtually pure populations of glutamatergic pyramidal neurons. This protocol closely recapitulates the events of cortical development during mouse development. In addition, because of its homogeneity it has been useful in many applications, including the epigenetic analysis of neural specification as discussed later. Interestingly, it was recently shown that overexpression of Neurogenin-2 (Ngn2) in mES cells is sufficient to drive their differentiation into glutamatergic telencephalic neurons in the absence of other external cues [91]. This study opens the possibility that more neuronal identities could be induced by forced expression of key transcription factors that coordinate their fate.

Recapitulation of human corticogenesis in vitro has been a difficult challenge. Chambers and colleagues demonstrated that both hES and human iPS cells can be directed to a cortical fate upon dual inhibition of the SMAD signalling pathway. Relatively homogeneous populations of Pax6-positive cortical progenitors were obtained with this method, while there was also some residual differentiation towards a neural crest type of progenitor cell [92]. More recently, Shi and colleagues developed an RA-based multistep protocol for differentiating hES and human iPS cells into cortical progenitor cells [93]. This method closely recapitulates in vivo development, as all classes of cortical projection neurons are generated in the expected temporal order. In the future, this promising system will enable researchers to gain new insights on the molecular regulation of human brain development.

3 Neuronal differentiation as a discovery tool in neurobiology

Neuronal differentiation from pluripotent cells has contributed to our understanding of mechanisms relating to neural specification and has facilitated the discovery of novel roles for genesmissed using classical genetic approaches. This has been possible for several reasons. Importantly, one can analyse the effect of mutations in neurons of genes that are embryonic lethal and would require the generation of conditional knockouts in order to unravel a defect in the nervous system. In addition, neurons derived from pluripotent cell lines differentiate simultaneously and mature in a controlled environment that is devoid of the developmental gradients present in vivo. As a result, the effects of genomic manipulations can be readily observed in the absence of internal or external parameters that would alter the neuronal response. Moreover, neuronal differentiation can provide unlimited numbers of neurons. The quantity and homogeneity of the cell product cannot be matched by isolating neurons from rodent brains. These features make pluripotent cell derived neurons ideal for performing high throughput type of analyses that provide novel perspectives on mechanisms regulating basic processes, such as of neuronal induction and differentiation.

A differentiation protocol that has proved extremely useful in this respect is that developed by Bibel and colleagues [89, 90], which closely recapitulates the events of corticogenesis during development. Using this system, it was demonstrated that the long known differences of neurons of the central or peripheral nervous systems with regards to their dependence on neurotrophic support for survival during development arises from the kind of neurotrophin receptor they express. More specifically, it was shown that TrkA and TrkC, the two neurotrophin tyrosine kinase receptors mainly expressed in the PNS, instruct neurons to die in the absence of their respective neurotrophin ligands, whereas the highly similar TrkB, widely expressed in the central nervous system (CNS), does not have this function [94].

Epigenetic analyses have demonstrated that ES cell pluripotency and developmental potential are restricted by de novo DNA methylation and dynamic switches in Polycomb targets [95]. Hundreds of promoter regions, including pluripotency regulating genes, become methylated in neural-committed progenitor cells, while loss and acquisition of H3K27me3 at additional targets characterised both progenitors and mature neurons. Surprisingly, many neuron-specific genes that become activated upon terminal differentiation are Polycomb targets only in progenitor cells. Moreover, promoters marked by H3K27me3 in stem cells frequently become DNA methylated during differentiation, suggesting context-dependent crosstalk between Polycomb and DNA methylation. These results provide novel insights into the epigenetic mechanisms that control pluripotency and neuronal differentiation potential.More recently, Tiwari and colleagues [96] employed this neuronal differentiation protocol to reveal a new role for the topoisomerase isoenzyme Topo2β in the survival of cortical neurons. Previously thought as a house keeping enzyme whose function was to resolve topological problems arising from DNA-templated processes, Topo2β was found to be upregulated in post-mitotic neurons and to bind preferentially to active chromatin regions containing H3K4 methylation. Functionally, in the absence of Topo2β activity, the neurotrophin receptor p75, a Top2β target, was upregulated, causing degeneration of neurons.In another study and using a similar protocol, it was demonstrated that the Polycomb group protein Suz12 is required for the neuronal dif-

ferentiation of ES cells [97], as Suz12 null ES cells were incapable of commitment to the neural lineage and displayed an aberrant epigenetic landscape compared to wild type cells in the course of differentiation.

4 Modelling human diseases and developing new therapies using human pluripotent lines

To date, studies on human brain dysfunction have been restricted to analysis of post-mortem tissues of patients. In addition to often being poorly preserved, these tissues usually represent the end-stage of the disease. While mouse models have contributed greatly to understanding human neuronal conditions, they are limited and usually do not recapitulate the full spectrum of the human phenotype. The recent development of techniques allowing the culture and differentiation of human pluripotent lines has provided a novel powerful tool, the applications of which just begin to be grasped. For example, it is the first time that neurodegenerative and neurodevelopmental diseases can be examined living human neurons and in a controlled environment.

Most relevant to advancing current strategies for disease modelling is the possibility to generate iPS lines from somatic cells of patients. Upon differentiation of these patient pluripotent lines into the neuronal subpopulation of interest, it is feasible to perform comparative analyses and to detect early disease-specific molecular signatures in living human neurons of relevant identity. Most importantly, one can generate neurons that capture the genetic material from the patient, which includes not only the mutated gene(s), but also all the genetic and epigenetic modifiers that play an important but yet largely unknown role in the pathology of most neurological diseases. Lastly, once the relevant neural defects are determined in vitro, the so-called "disease-in-a-dish" approach allows for the screening of drugs that can ameliorate the disease-specific phenotype

4.1 Neurodevelopmental disorders

Neurodevelopmental disorders include a wide range of diseases characterised by impaired neuronal function during brain development. They commonly have a strong genetic component that often arises in gametogenesis, and although they can result from a single mutation, they are often multigenic [98]. Neuronal differentiation of iPS cells is particularly well suited for modelling complex neurodevelopmental disorders, as it recapitulates the early steps of neuronal commitment and synaptic maturation. It is increasingly appreciated that the study of iPS cells isolated from genetic backgrounds known to result in neurodevelopmental disorders is a promising approach for dissecting the cellular defects underlining these disorders. Three specific examples are discussed below.

Autism spectrum disorders (ASD) are heritable neurodevelopmental diseases, characterised by impaired social interaction and repetitive behaviour. Although there is an alarmingly increasing prevalence in the population, there is currently a lack of early diagnostic markers and efficient treatments. Moreover, because of their complex nature, there are no reliable mouse mutants that closely model autistic disorders. Taken together, these limitations make iPS cells the basis of an attractive new alternative model. Among ASDs, Rett Syndrome is a rare disorder caused by mutations on the methyl CpG-binding protein (MeCP2) gene. Two groups recently analyzed neurons derived from iPS cells of Rett patients [99, 100]. Consistent with Rett animal models [101], they have detected a decrease in the size of the neuronal somata compared to wild type controls. Moreover, there was a marked decrease in the connectivity of glutamatergic neurons that was confirmed by electrophysiology, suggesting aberrant network formation. Importantly, treatment with insulin growth factor 1, a growth factor known to ameliorate the phenotype of Rett mice, improved the Rett phenotypes of iPS cell derived neurons, suggesting that synaptic defects can be rescued at the cellular level [102, 103]. In another study, Rett iPS cell-derived neural progenitors were used to demonstrate the regulation of mobile elements in the genome by MeCP2, putting forward a new potential molecular mechanism underlying Rett Syndrome [104]. As this field is at its nascence, systematic approaches are necessary in order to determine whether neurons derived from iPS cells of patients with other forms of ASD share common cellular phenotypes with those of Rett patients. Secondly, it will be important to determine which phenotypes or biological markers are robust enough to be useful in clinically relevant drug screenings.

Fragile X syndrome (FXS) is an X-linked condition with variable penetrance among patients, caused by triplet repeat induced silencing of the fragile X mental retardation (FMR1) gene. It is thought that, during neuronal differentiation, expansion of the triplet repeats results in hypermethylation of FMR1 and chromatin modifications such as histone H3 deacetylation, histone H3K9 methylation, and histone H3K4 demethylation [105, 106]. Using early human blastomeres isolated from FXS blastocysts in the context of preimplantation genetic diagnosis, it was found that FMR1 silencing occurs only upon differentiation [107]. Inconsistent with this finding, when skin and lung fibroblasts from three patients with FXS were used to create iPS cells [108], the reprogramming process failed to reverse the methylation of FMR1. Consequently, the authors concluded that iPS cells are not ideally suited to model FXS, although they did not discuss whether defects nonetheless occurred in FXS iPS cell-derived neurons. It remains to be determined whether this lack of activation of FMR1 in the iPS cells is not due to partial re-

programming of the somatic cells and could be overcome by more thorough selection of the reprogrammed cells.

Schizophrenia is a common psychiatric disorder characterised by positive (hallucinations and delusions), negative (loss of affect), and cognitive symptoms. Though the overt symptoms of schizophrenia are manifested during adolescence, it is increasingly accepted that aberrant cognition precedes the disease onset [109]. The first report of iPS cells was from patients with mutations in DISC1, a gene responsible for development of schizophrenia in a small portion of cases. These iPS cells were derived using an integration-free method but have not yet been differentiated into neurons [110]. Another group generated neurons from iPS of patients with complex genetic backgrounds. It was demonstrated that these neurons have reduced neuronal connectivity, reduced neurite length, reduced PSD95 dendritic protein levels and altered gene expression profiles relative to controls. Interestingly, defects in neuronal connectivity and gene expression were ameliorated following antipsychotic treatment [111].

4.2 Neurodegenerative disorders: A focus on iPS cells

Neurodegenerative disorders include a variety of hereditary or sporadic diseases that involve the chronic, progressive loss of neuronal structure and function. Since aging is the most consistent risk factor for neurodegenerative diseases, and the aging population is rising in developed countries, it is of great importance that we unravel the causes of cell death that are characteristic of these diseases.

The first neurodegenerative disease modelled using human iPS cells was smooth muscle atrophy (SMA). iPS cells were derived from a child with a mutation in SMN (SMA type 1) and from his unaffected mother as a control [112]. Both the fibroblasts and the iPS cells from the child showed reduced levels of full length SMN, while motor neurons derived from these cells were unable to survive in culture past six weeks [113]. Two compounds known to increase SMN levels, valproic acid and tobramycin, could partially restore the reduction in SMN protein, though their effects on neuronal survival were not investigated.

Parkinson's disease (PD) is the most prevalent neurodegenerative disorder, commonly characterised by deficits in initiation and control of motor skills. These deficits are traditionally attributed to the death of dopaminergic neurons in the substantia nigra and subsequent loss of dopamine in the striatum. Many mouse models and post-mortem tissue studies have provided insight into the pathogenesis of PD; however, the former consistently failed to recapitulate the features of PD and the latter are end-stage representations [114]. The generation of iPS cells from PD patients has been reported, however the focus of these studies remains largely methodological [48, 115, 116]. Nguyen and colleagues described the first biologically relevant cellular phenotype from iPS cell-derived neurons from Parkinson's patients carrying a point mutation in LRRK2, the most common genetic cause of PD [117]. Dopaminergic neurons derived from these PD iPS cells expressed increased levels of alpha-synuclein, the protein whose dysfunction is a common feature of all PD cases, and showed increased sensitivity to cellular stressors including hydrogen peroxide, MG-132, and 6-hydroxydopamine [118]. However, inhibiting LRRK2 activity with a kinase inhibitor did not prevent this phenotype. Another study, which included three patients with mutations in the mitochondrial protein PINK1, reported that the resulting iPS cell- derived neurons exhibited decreased mitochondrial recruitment of lentivirally expressed PARKIN; this phenotype was rescued by the forced expression of wild type PINK1 [119]. Further studies, with larger cohorts, will be necessary to confirm the phenotype observed and characterise downstream, potentially therapeutic, molecular targets.

Alzheimer's disease (AD) is the most common late onset neurodegenerative disease, characterised by a severe and progressive dementia. Neuropathology consists of neurofibrillary plaques and tangles, composed primarily of amyloid-beta (Aβ) peptide and hyperphosphorylated Tau, respectively, in the cerebral cortex and some subcortical regions including the hippocampus. Similar to PD, before reprogramming technology, the study of AD was severely limited by the lack of relevant mouse models. Therefore, the generation of iPS cells from patients with sporadic forms of the disease, which represent the majority of the cases, should provide insight into forms of neurodegeneration that were previously impossible to model. Aside from proof of concept experiments demonstrating that it is possible to derive iPS cells from AD patients, there have been surprisingly no following studies reporting on the analysis of AD iPS cell-derived neurons. This may reflect a limitation for using differentiation protocols that recapitulate neuronal development to model late onset diseases [120]. However, human iPS cell-derived forebrain neurons were recently employed as a cellular model to screen three different drugs that interfere with the production of Aβ [121]. Using these cells as a platform, important differences could be detected in the way the proteolytic machinery operates in younger as compared to older neurons, in line with the disease's late onset.

Amyotrophic lateral sclerosis (ALS) is the most common adult onset motor neuron disease, mainly characterised by muscular atrophy and weakness accompanied by a fast and progressive loss of motor neurons in the cortex, brainstem and spinal cord. Life expectancy is usually two to five years after disease onset and there is currently no cure or effective therapy. Clinical trials based on ALS mouse models have largely failed, suggesting a need for the exploration of better approaches. More than ten different genes have been implicated in ALS, including

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superoxide dismutase 1 (SOD1, ALS1) [122] and vamp-associated protein B/C (VAPB, ALS8) [123].Two groups have succeeded in deriving iPS cells from patients carrying two different mutations, previously discovered to account for familial forms of the disease. Dimos and colleagues [124] derived iPS cells from two sisters with mutations in the SOD1 gene (SOD1L144F). Motor neurons were generated from one of the ALS patients, but analyses were not performed to describe their phenotype. In another study, Mitne-Neto and colleagues [125] generated iPS cells from four patients with mutations on VAPB gene as well as from three unaffected siblings as controls. They detected significant reduction in the levels of VAPB protein, particularly in a hES cell-derived motor neuron-enriched population, suggesting that reduction of VAPB could be involved in the initial steps of ALS degeneration [125]. These observations may be relevant to other forms of ALS, as reduction of VAPB protein was recently reported in sporadic ALS patients with no identified genetic mutations [126]. Because other cells belonging to the motor neuron niche (i.e. astrocytes and microglia) have been shown to play a role in the pathology of ALS, it remains to be seen if the iPS cell-derived cells can also recapitulate the non-cell autonomous aspects of the disease [127–130].

5 Concluding remarks

Pluripotent cell lines represent a unique cellular state that is characterised by the amazing ability to differentiate into each and every cell type in the body. These cells represent an exciting tool to neurobiology, helping us comprehend the epigenetic and molecular events underlying the specification of an impressive array of diverse neuronal subtypes. Moreover, the possibility to study pluripotent lines generated from specific patients of neurological disorders introduces unprecedented possibilities for improving the diagnosis, modelling and treatment of these disorders that are just beginning to be exploited.

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