Cell. Mol. Life Sci.
DOI 10.1007/s00018-010-0557-6

Cellular and Molecular Life Sciences

REVIEW

Using human pluripotent stem cells to untangle neurodegenerative disease mechanisms

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Received: 9 July 2010 / Revised: 14 September 2010 / Accepted: 4 October 2010

Abstract Human pluripotent stem cells, including embryonic (hES) and induced pluripotent stem cells (hiPS), retain the ability to self-renew indefinitely, while maintaining the capacity to differentiate into all cell types of the nervous system. While human pluripotent cell-based therapies are unlikely to arise soon, these cells can currently be used as an inexhaustible source of committed neurons to perform high-throughput screening and safety testing of new candidate drugs. Here, we describe critically the available methods and molecular factors that are used to direct the differentiation of hES or hiPS into specific neurons. In addition, we discuss how the availability of patient-specific hiPS offers a unique opportunity to model inheritable neurodegenerative diseases and untangle their pathological mechanisms, or to validate drugs that would prevent the onset or the progression of these neurological disorders.

Keywords Human embryonic stem cells · Induced pluripotent stem cells · Central nervous system · Brain · Peripheral nervous system · Cell culture

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Published online: 26 October 2010

Introduction

Much of our understanding of human brain dysfunction has come from the use of complementary in vitro and in vivo animal models. These models have not only been used to unravel intricate molecular signalling pathways that drive brain dysfunction, but also to discover new therapeutic molecules. However, numerous candidate drugs that had shown promise in animal model screens were inefficient when taken to human clinical trials—a failure of translation (reviewed in [1]). The lack of translation to the neurological clinic in particular results from specific factors including brain complexity and disease-specific phenotypes that cannot easily be modelled in non-human systems. Therefore, there is an urgent need for simple in vitro models using human-derived material to perform efficient screens and validate potent drugs to treat or delay the onset of neurodegenerative diseases. In this review, we discuss the use of neurons differentiated from human embryonic stem cells (hESCs) and human induced pluripotent stem (hiPS) cells to further advance our knowledge of the normal and diseased brain. We focus on the major signalling mechanisms that promote cell reprogramming, pluripotency or neuronal fate acquisition.

Pluripotent stem cells as a source of human neurons

hESCs are pluripotent stem cells that retain the ability to self-renew and to differentiate into all body cell types. These cells are characterised by the expression of a set of specific molecular markers that largely overlaps with these described for human embryonic carcinoma cells, mouse ES, and mouse hematopoietic stem cells [2]. Recently, the differentiation of neural cells from somatic human cells has been described. These reprogrammed human adult cells

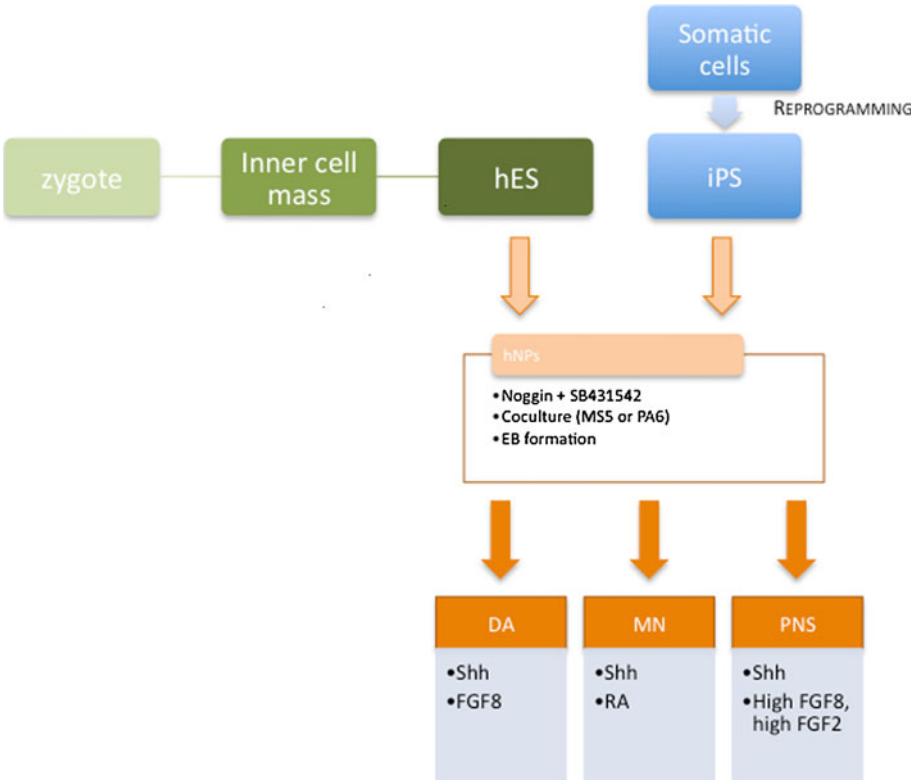

Fig. 1 Scheme illustrating the principal steps for differentiating hESCs or iPS cells into neurons. *hESCs* human embryonic stem cells, *hiPS* human induced pluripotent stem cells, *hNPs* human neural precursor cells, *DA* dopaminergic neurons, *MN* motor neurons, *PNS* peripheral nervous system derivatives, *EB* embryoid body

have been called hiPS cells [3, 4]. They have been derived from various sources (e.g. skin, blood) following transfection of specific set of genes, initially using viral infection but more recently plasmid- and protein-based methods. These cells show similar proliferative and differentiation potential to hESCs and have been subsequently differentiated into many cell types, including neurons (Fig. 1). Neural cells differentiated from hiPS cells derived from diseased patients will be critical to better understand the pathological mechanisms of brain diseases and to perform large scale drug screenings.

Derivation of human embryonic stem cell-derived neurons

hESC lines are derived from the inner cell mass of the blastocyst and can self-renew in culture almost indefinitely. Their pluripotency status is validated by the ability to form teratomas after implantation into immunodeficient mice. Differentiation of hESCs into neural lineages both in vivo and in vitro is well established [5], and the key to generate large quantities of material and to ensure a stable differentiation is to keep a tight control of the initial undifferentiated state. Indeed, hESCs differentiate spontaneously when grown without appropriate supporting feeder cell layer or addition of exogenous trophic factors such as basic fibroblast growth factor (bFGF or FGF-2) [6, 7]. Because of the great promise of hESCs for tissue repair, accumulating efforts have been made to develop defined media and to culture hESCs lines without feeders [8] in animal-free product conditions (Good Manufacturing Practice, GMP) [9]. Undifferentiated hESCs harbour a typical morphology with tight junctions and large nuclei, they also express specific markers, including Oct4 transcription factor (POU5F1), the cell surface glycolipids SSEA-3 and SSEA-4, and the surface keratan sulphate antigens, TRA-1-60 and TRA-1-81 [10–12].

Modulators of somatic cell reprogramming

Core transcription factors

The core transcription factors that maintain hESCs in undifferentiated state include the homeodomain transcription factors, NANOG and Oct4, and the HMG-box transcription factor, Sox2 [13–15]. During development, expression of NANOG is restricted to cells of the inner cell mass, the pluripotent epiblast and primordial germ cells [16], and its inhibition in culture causes hESCs differentiation to extra-embryonic cell lineages [15, 17], while its overexpression promotes feeder-independent proliferation of hESCs [18]. Loss of Oct4 is associated with the loss of pluripotentiality and causes inappropriate differentiation

into trophectoderm, whereas overexpression of Oct4 results in differentiation into primitive endoderm and mesoderm, suggesting that precise Oct4 levels are necessary for pluripotency [19, 20]. NANOG, Oct4, and Sox2 co-occupy a substantial portion of their target genes and collaborate to form an extensive regulatory circuitry that includes autoregulatory and feed-forward loops to support the self-renewal of hESCs [21–23].

The reversion of somatic cells to pluripotent state is commonly referred to as cell reprogramming. The direct reprogramming of human somatic cells was initially accomplished by the combined transduction of genes encoding Oct4, Sox2, Klf4 and c-Myc [3, 4, 24]. While expression of Oct4 is highly specific to pluripotent stem cells, the other transcription factors are also expressed in other cells: Sox2 is endogenously expressed by neural stem and progenitor cells; Klf4 is detected in skin, stomach, intestine, and skeletal muscle; and c-Myc is ubiquitously expressed. Somatic cell reprogramming by a variation of the four-factor cocktail has been successfully achieved. Although Oct4 cannot be replaced by other Oct family members (Oct1 or Oct6) to generate iPS cells [25], Klf2 and Klf5 can replace Klf4, Sox1 and Sox3 can substitute for Sox2, and c-Myc can be swapped for N-Myc and L-Myc [25, 26]. In addition, Oct4 and Sox1–3 have been shown to be essential for reprogramming [27], while c-Myc and Klf4 enhanced the efficiency of hiPS cell colony formation. However, c-Myc is a controversial factor in somatic cell reprogramming for its oncogenicity and therefore has been replaced by Nanog and Lin28 to successfully generate hiPS cells [28–30]. A step further has been reached recently in mice, as Nr5a2 (an orphan nuclear receptor also known as Lrh-1) can replace Oct4 in the derivation of iPS from mouse somatic cells [31]. However, this remains to be demonstrated on human somatic cells.

Chemically defined molecules

Chemically defined molecules have also been shown to increase the efficiency of reprogramming, including valproic acid, an HDAC inhibitor that enhances this process by nearly 400-fold [32]. Recent studies further suggest that blocking the methylation of DNA or histones can facilitate cell dedifferentiation in mice. For example, the DNA methyltransferase inhibitor 5-azacytidine can improve the efficiency of iPS cell induction by approximately 100-fold [33]. In addition, experimental approaches that target specific signalling pathways, including the TGF-β pathway (with SB431542) in combination with a MEK inhibitor (PD0325901), can largely improve the efficiency and rate of human iPS cell induction [34].

Factors maintaining pluripotency

Undifferentitated hESCs can spontaneously differentiate into various cell types in vitro. Growth and expansion of these cells require a balance between survival, proliferation and self-renewal signals.

Factors involved in self-renewal

Feeder layers and extracellular matrices

One approach to maintain undifferentiated hESCs in culture is the use of specific culture environments that include either a feeder cell layer or a feeder cell conditioned medium. The majority of hESCs lines that have been isolated so far are routinely propagated on feeder layers of mouse embryonic fibroblasts, which provides signals required to maintain self-renewal [11, 35]. Feeder layer cells derived from human sources have also been developed and used for hESCs cultures in GMP conditions [36, 37].

In the absence of feeders, hESCs require physical interactions with the extracellular matrix (ECM). The ECM as a major niche element provides not only a scaffold for cellular support but also an immediate microenvironment that triggers regulatory signals to support stem cell proliferation, migration and cell fate decision [2]. The ECM is composed of a mixture of matrix molecules which are typically large glycoproteins. Several defined matrices reproducing the ECM environment have been shown to ensure hESCs self-renewal. Amongst them, Matrigel^{TM} has been widely used [8, 38]. It is a soluble basement membrane extract of the Engelbreth–Holm–Swarm mouse tumor that contains laminin, collagen IV, entactin and heparan sulphate proteoglycan, as well as various growth factors [FGF-2, epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), nerve growth factor (NGF) and transforming growth factor-β1 (TGF-β1)] [39–42]. Matrigel^{TM} is a complex mixture of poorly defined elements and its quality varies from batch to batch. Thus, some authors have replaced it with defined purified proteins, such as type IV collagen, fibronectin, laminin, and vitronectin, alone or in combination [8, 43–45]. Each of these factors efficiently supports attachment and proliferation of hESCs [40]; however, a recent comparative study found that these substrates, along with human or fetal bovine serum coatings, are inferior to Matrigel^{TM} as culture substrates [46]. A recent promising approach consists in the use of defined culture environments that are not based on proteins derived from the ECM but on synthetic biomaterials. Indeed, hESCs have been successfully cultured on polymer hydrogels functionalised with cell adhesion peptides [47] or encapsulated in hyaluronic acid (HA)-based hydrogels

[48]. hESCs have also been maintained encapsulated in calcium alginate hydrogels for several months without passaging [49].

In addition to the culture substratum, the composition of the medium is another critical parameter for efficient long-term maintenance of hESCs cell pluripotency. In an effort to remove foetal bovine serum from the culture medium, KnockOut^{TM} serum replacement (KO–SR) has been successfully developed but contains albumax, a poorly defined lipid-rich bovine albumin [50]. More recently, a fully defined medium named TeSR1^{TM} has been designed and was able to maintain the pluripotency of different human cell lines [42]. The ultimate goal for translational research is to culture hESCs in GMP conditions using a highly standardised and chemically defined medium in combination with the appropriate supportive matrix.

Trophic factors

Several intrinsic and extrinsic factors, which include growth factors and secreted proteins, have been described as maintaining hESCs self-renewal and pluripotency while preventing spontaneous differentiation. The undifferentiated state of hESCs is regulated by the combined action of these factors.

Wnt signalling pathway Critical transducers of the canonical Wnt signalling pathway have been shown to play an important role for the maintenance of hESCs pluripotency [51, 52]. Activation of the canonical Wnt pathway controls the stability of β-catenin by preventing its phosphorylation by the glycogen synthase kinase-3β (GSK-3β) that targets it for ubiquitin-mediated destruction. Particularly, the binding of Wnt to Frizzled receptors activates Dishevelled, which results in inhibition of GSK-3β, and subsequent stabilisation of β-catenin (Fig. 2). Free β-catenin forms nuclear complexes with members of the TCF/LEF transcription factor family to promote expression of numerous genes. Inactivation of GSK-3β by using specific pharmacological inhibitors such as 6-bromoindirubin-3′-oxime (BIO) maintained hESCs in an undifferentiated state in feeder-free conditions [53]. In contrast, the addition of recombinant Wnt1 or Wnt3a to the culture medium can stimulate hESCs proliferation [52, 54], but is not sufficient to maintain pluripotency [55]. This apparent discrepancy may be explained by the fact that Wnt signalling on stem cells is dependent on cell-intrinsic as well as other extrinsic signals [56]. Indeed, there are major differences among these experiments especially in tissue culture methods or hESCs lines.

Fibroblast growth factor-2 FGF-2 plays a major role maintaining the self-renewal ability of hESCs cultured on feeder layers [7, 50] by activating receptors on both feeder and hESCs cells and thus promoting gene transcription and regulation of cell cycle progression [57]. FGF-2 likely promotes self-renewal of hESCs in several ways. It directly activates the mitogen-activated protein kinase (MAPK) pathway [58, 59], while it indirectly acts on fibroblast feeder cells to activate transforming growth factor-β1 (TGF-β1) and activin A signalling which together support hESCs self-renewal [57, 60]. Treatment with FGF-2 also inhibits the expression of differentiation-promoting factors such as bone morphogenetic protein 4 (BMP4). In combination with Noggin, an inhibitor of BMP signalling, FGF-2 stimulates feeder-free growth of hESCs [61]. In addition, heparin, a soluble derivative of heparan sulphate that acts as cofactor for FGF-2, also promotes the growth of hESCs probably by stabilising FGF-2 [44]. Although, FGF-2 is required for the renewal of hESCs, it is not sufficient to stimulate long-term proliferation of hESCs cells [42].

Transforming growth factor β family TGF-β family ligands have also been implicated in the control of hESCs proliferation and maintenance (Fig. 2). Ligands that activate SMAD2/3, including TGF-β1, Activin A, and Nodal, enhance hESCs self-renewal while ligands that activate SMAD1/5/8, such as BMPs, promote differentiation of these cells [43, 62, 63]. Thus, addition of TGF-β1, Activin A or Nodal, or repression of BMPs by addition of Noggin or dorsomorphin to the culture medium, promote the expansion hESCs [62–64]. TGF-β directly regulate the expression of *Nanog* via SMAD binding elements in the *Nanog* promoter [65]. Noggin and FGF-2 signals synergise to inhibit BMP signalling, sustain expression of pluripotency-associated genes such as *Nanog*, *Oct4* or *Sox2*, and promote long-term proliferation of naive hESCs [61, 66]. In addition, treatment with FGF-2 stimulates key factors of the TGF-β pathway such as Activin A and TGF-β1 while differentiation-promoting factors such as BMP4 are inhibited [67]. Pigment epithelium-derived factor (PEDF) can substitute FGF-2 or TGF-β/Activin/nodal ligand supplementation for long-term pluripotent growth of hESCs through MEK/ERK signalling pathway [68].

Insulin-like growth factors Insulin-like growth factors (IGFs) also promote pluripotency. Specific blocking of the IGF1 receptor reduces hESCs self-renewal while addition of an IGF1 analogue in conjunction with FGF-2 and Activin A supports long-term growth of hESCs [69]. IGFII also promotes feeder-mediated expansion of embryonic stem cells. Indeed, FGF-2 stimulates hESCs-derived fibroblast feeder cells to secrete IGFII, which in turn enhances hESCs self-renewal [67].

Chemically defined molecules

High basal MEK/ERK activity is required for maintaining hESCs in an undifferentiated state. Therefore, inhibition of

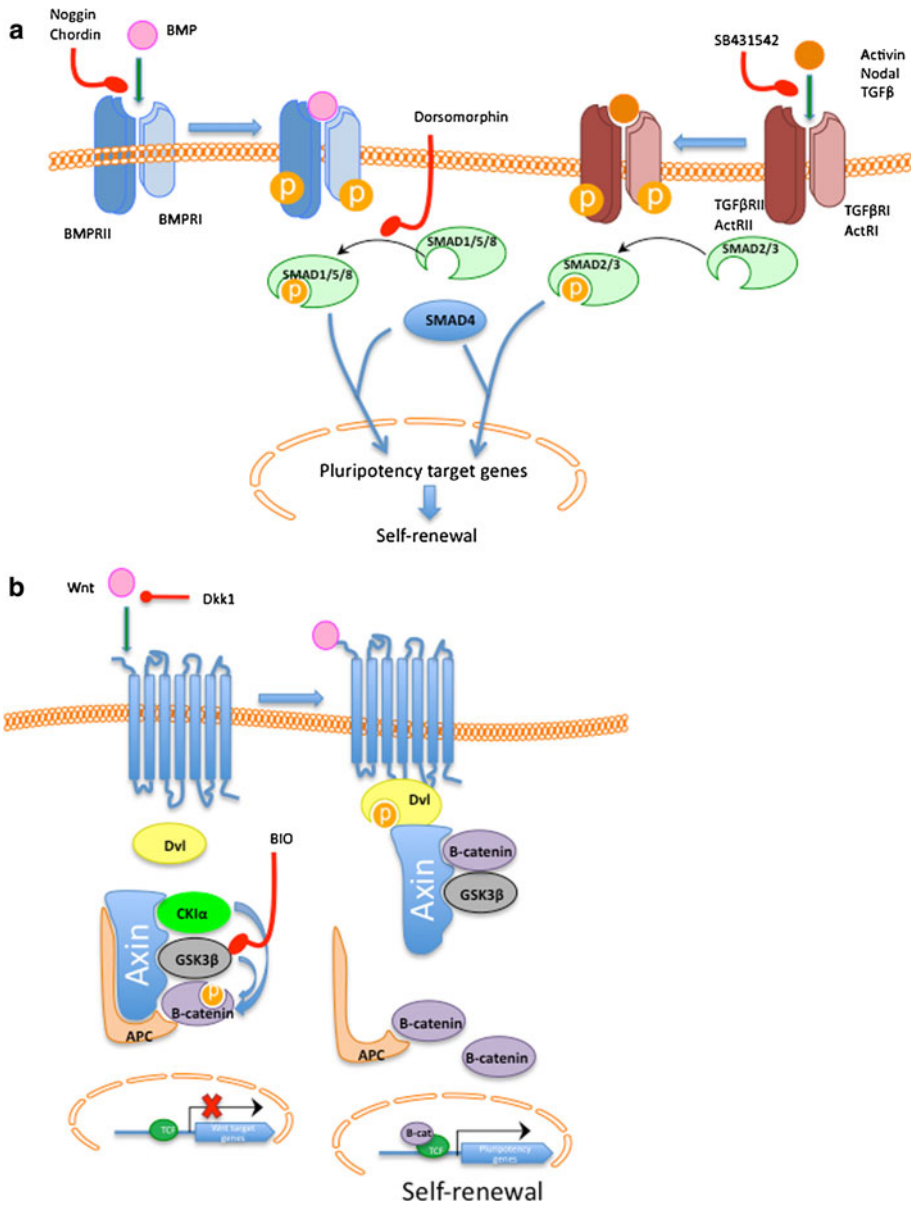

Fig. 2 TGF-β superfamily and Wnt signalling pathways showing signalling proteins associated with TGF-β (**a**) and Wnt (**b**) pathway regulation. **a** Two major signalling pathways of the TGF-β superfamily. *Right* the signalling pathway of BMPs, while signalling pathways of TGF-β, activin, and nodal are shown on the *left*. Ligand binding promotes the assembly of heteromeric complexes, which activate Smads after phosphorylation. Activated Smad complexes translocate into the nucleus, where they regulate transcription of target genes involved in puripotency. **b** When Wnt is present, the axin complex is inhibited by Dishevelled (*Dvl*), leading to cytosolic accumulation of β-catenin, which is transported into the nucleus where it bind to Tcf and activates target genes involved in self-renewal. *Blunted red arrows* represent inhibitory pathways

MEK/ERK activity by specific MEK inhibitors PD98059 and U0126, or by RNA interference, rapidly caused the loss of self-renewal capacity [59]. MEK/ERK signalling co-operated with phosphoinositide 3-kinase (PI3K)/AKT signalling in maintaining hESC pluripotency. However, MEK/ERK signalling had little or no effect on regulating hESC proliferation and survival, in contrast to PI3K/AKT signalling [70].

microRNA

In parallel to protein-coding genes, microRNAs (miRNAs), which constitute a class of small non-coding RNAs with central roles in gene silencing [71], are also connected to the transcriptional regulatory circuitry of hES cells which expressed a unique set of miRNA signature [72, 73]. Some of the identified miRNAs, including mir-200c, -302d, -372, -199a, -19a and -217, are markers of pluripotency and are expressed in the undifferentiated state [74]. These miRNAs have been shown to be upregulated by ActivinA, an important factor in maintaining pluripotency [75]. Contrarily, some miRNAs represse pluripotent genes and act to regulate the differentiation of hES cells into one of the three different embryonic lineages [76–78]. For example, increased expression of miR-145 inhibits hESCs self-renewal by repressing the expression of the pluripotency genes Oct4, Sox2 and Klf4 and favours differentiation into mesoderm and ectoderm lineages [79].

Factors involved in hESCs survival

hESCs cells show low survival rate in single cell suspension ($\sim$1%) [35, 50]. This has been a hurdle for the development of cell culture maintenance techniques and gene transfer studies where clonal selection is usually required. Undifferentiated hES undergo apoptosis upon detachment from the ECM, a self-initiated process termed "anoikis" [80]. This phenomenon is substantially decreased by the addition to the culture medium of Y-27632, a synthetic compound that inhibits Rho-associated kinase (ROCK) [81]. A role for FGF-2 and neurotrophins in hESCs survival has also been demonstrated. Indeed, anoikis of hES or hiPS cells is inhibited by FGF-2 via repression of caspase activities through the triggering of the ERK and AKT signalling pathways [82], while addition of BDNF or NT-3 to hESCs cultures improves the clonal survival and may facilitate their manipulation [83].

Neuroectodermal differentiation of hESCs

There are three major approaches differentiate hESCs into human neural precursor cells (hNPs): promoting the direct neural differentiation of hESCs colonies; co-culturing hESCs cells with a feeder layer of stromal cells; or applying a multistep procedure which involves the formation of embryoid bodies (EBs).

Direct differentiation

During embryogenesis, neurulation is the first step in organogenesis. Therefore, hESCs are expected to differentiate spontaneously and directly into neural cells. The formation of ectodermal derivatives can be induced by prolonged culture of hESCs without changing the feeder cells [11]. Indeed, under serum-free conditions and without addition of morphogens, hESCs differentiate into a homogenous population of neuroepithelial cells that organise into "rosettes"-like structures, which are shared cytoarchitectural and molecular features with the neural tube [84, 85]. This differentiation process occurs in approximatively 2 weeks, a timing corresponding to the development of the neural plate/tube in a human embryo [86]. However, cells at the rosette stage can acquire both central (CNS) and peripheral (PNS) nervous system fates [84, 87], a broad differentiation potential lost upon extended periods of proliferation in vitro. Therefore, the cell population obtained, after application of this differentiation strategy, is largely heterogeneous [88–90]. Thus, there is an urgent need to improve culture conditions to promote the conversion of these cells into a homogeneous population of hNPs. Consistently, efficient differentiation of hESCs into NPs has been achieved using high concentrations of BMP inhibitors (Noggin or dorsomorphin) [91–93]. More recently, a well-defined feeder-free hESCs neural induction system employing simultaneously BMP pathway inhibitors and an activin/nodal/TGF-β inhibitor (i.e. SB431542) efficiently yields homogeneous hNPs [94, 95]. This dual inhibition of SMAD signalling leading to a controlled conversion into a homogeneous population of neural progenitors would be a more convenient approach for both basic and applied scientific research.

Stromal cell lines

Stromal cells are loose connective tissue cells found in number of organs, such as gonads and bone marrow. They provide matrix support for other cells in organs. In order to promote neural differentiation of hESCs, they have been co-cultured with stromal cell lines such as the mouse PA6 cell line isolated from mouse skull bone [96–98] or the MS5 line derived from the aorta–gonad–mesonephron [99–102]. Such cell lines secrete, or at least express, factors, not yet fully identified, that promote the formation of neural rosettes and that are collectively called "stromal cell-derived inducing activity" (SDIA) [97, 99, 103]. This co-culture method was based on the fact that mesodermal signalling contributes to neural induction as demonstrated for the differentiation of both mouse and primate ESCs into neurons [103, 104]. Although this technique efficiently promotes differentiation of hESCs into neurons, such a model is not suitable to dissect the molecular mechanisms that drive neuronal differentiation as factors secreted by such cells varied from one stromal cell line to another.

Embryoid body formation

hESCs can be directed towards the neural lineage after generation of embryoid bodies (EBs). When hESCs differentiate in suspension culture, they form a three-dimensional aggregate of cells known as an EB [105]. After extensive differentiation, EBs form a multilayered structure that contains a mixed population of cells including neural cells. To increase neural differentiation and improve survival of desired cell types, growth factors or morphogens have been commonly added to the culture medium [85, 87, 89, 106, 107]. There are main disadvantages associated with EB culture including: (1) the variability of their size (due to different initial cell numbers or duration of differentiation), which affects the yield of hNPs generation; (2) the heterogeneity of morphogen concentrations present in the different layers of the EBs forming a concentration gradient that leads to the generation of cells at different developmental stages belonging to tissues of different germ layer; and (3) the aggregation of

cells in EBs prevent a clear monitoring of cell morphology during differentiation. Recently, Koch et al. [108] develop a new protocol using both hESC-derived EBs in short-term culture and direct differentiation in adherent culture conditions, which permit a controlled, stepwise differentiation of hESCs into hNPs. In addition, an alternative air–liquid interface culture system has been recently developed and gives rise to a homogenous population of hNPs [109]. This method allows three-dimensional organisation and the formation of densely interconnected neural tissues.

Factors involved in differentiation of hESCs into hNPs

In the absence of define morphogens, hESCs-differentiated hNPs is not clear. It has been shown that these progenitors exhibited a dorsal telencephalic trait [110], or a ventral anterior fate as attested by the expression of highly restricted transcription factors such as irx3, pax6 and Nkx6.1 (ventral markers) or Gbx2, Nkx6.1, HoxA2 and HoxB2 (anterior markers) [108, 111]. Recently, Pax6 has been identified as necessary and sufficient to induce neuroepithelial cells specification of cultured hESC [112].

All cells in neural rosettes remain responsive to instructive cues enabling their differentiation into a broad range of cell type in the presence of the appropriate set of morphogens. For example, inhibition of Wnt proteins or activation of Shh signalling almost completely converts the primitive dorsal telencephalic precursors to ventral progenitors [110]. However, this potential is subsequently lost in the presence of growth factors such as FGF-2 and EGF [84]. Maintenance of hNPs phenotype is ensured by activation of Shh and Notch pathways. Exposure to *N*-[(3, 5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1, 1-dimethylethyl ester (DAPT), a specific inhibitor of γ-secretase, leads to inhibition of Notch signalling and is sufficient to induce premature neuronal differentiation of neural rosettes [84, 113]. On the other hand, addition of Shh to the culture medium of neural rosettes prevent their neuronal differentiation.

Regional specification of neural cells

During the process of neural tube closure, the neuroepithelia is patterned to generate distinct classes of neural progenitors that contribute later to the formation of the forebrain, the midbrain, the hindbrain, and the spinal cord. Neuronal phenotypes are determined by a complex interaction between extrinsic signalling molecules and cell-intrinsic transcription factors. Manipulation of signalling cues (FGF-2, Wnt, Noggin, and BMP) allowed the development of feeder-free culture conditions for differentiation of hESCs towards neural lineages [114]. Indeed, pathways important for in vivo neuronal development can be recapitulated in culture to direct hESCs differentiation into specific neuronal subtypes in vitro [99]. The major difficulty arises from the identification and attribution of differentiated neuron to specific neuronal classes, as this is solely based on expression of set of specific markers and electrophysiological properties that are not easy to analyse in vitro. Moreover, neurons derived through different protocols can express similar markers without necessarily demonstrating equal functionality.

Differentiation of hESCs towards DA neurons

Mesencephalic dopaminergic (DA) neurons, which die in patients suffering from Parkinson's disease, are derived from embryonic progenitors located at the ventral midline of the midbrain [115]. During development, the generation of DA neurons, i.e. functional tyrosine hydroxylase (TH)-positive neurons, depends on Sonic hedgehog (Shh) signalling by ventral midline cells and on the activity of the FGF family member 8 (FGF-8), secreted by the isthmic organiser (a signalling centre localised at the isthmic constriction) [116, 117]. The primary role of these signalling molecules is to establish a ventral midbrain identity at initial stages of neural development. The intrinsic determinants that act downstream Shh and upstream of transcription factors influencing the maturation of DA neurons have not yet been identified.

Shh and FGF-8 have been successfully used to induce DA differentiation of hNPs (see "Neuroectodermal differentiation of hESCs"), i.e. co-culture of hESCs with stromal feeder cells or the EB-based multistage method. Indeed, the sequential addition of Shh and FGF-8 initiates the differentiation of hNPs into midbrain precursors and eventually leads to a substantial increase in the number of neurons expressing TH and transcription factors typical for midbrain neurons (Park et al. [96]). It is important to notice that early exposure of Pax6 expressing neuroepithelial to FGF-8 is critical for dopaminergic differentiation [118]. Transforming growth factor-α (TGF-α), which is secreted in early embryonic structures where midbrain dopaminergic neurons are generated, might be important for the differentiation of DA neurons in vitro and in vivo [119]. After culturing hESCs-derived cells for 21 days in a medium containing TGF-α, about 15% of them become TH-positive and release dopamine [106]. More recently, a set of candidate genes, including stromal cell-derived factor 1 (SDF-1/CXCL12), pleiotrophin (PTN), insulin-like growth factor 2 (IGF2), and ephrin B1, have been shown to induce the differentiation of hESCs into DA neurons [120]. In addition, liver X receptors ligands (i.e. oxysterols) treatment of hNPs increased neurogenesis and the net number of mature DA neurons, while reducing proliferating progenitors [121].

Several characterised transcription factors, including Nurr1, Pitx3, Engrailed-1, Lmx1a and b, and Msx1, control the maturation of postmitotic DA neurons but not in the initial specification of these cells [122–125]. Several attempts have been made to increase the efficiency of dopaminergic differentiation by genetically modifying hESCs. Lentiviral-mediated delivery of Pitx3 and Nurr1 genes, encoding transcription factors implicated in early DA neuronal specification, has been successfully used [126]. More recently, it has been shown that only Lmx1a-expressing hNPs develop into midbrain DA neurons after transplantation into 6-hydroxydopamine-treated rat model of Parkinson's disease [127]. However, unlike mouse ESCs [128], exogenous expression of Lmx1a gene is not sufficient to induce or increase the number of DA neurons from hESCs [127]. Other factors that promote the survival of DA neurons are also necessary to induce a high yield of TH-positive neurons. Glial cell line-derived neurotrophic factor (GDNF), which has been reported to stimulate the differentiation and survival of mesencephalic DA neurons, has been successfully used for regulating and/or maintaining a differentiated DA phenotype from hNPs [98, 129]. By acting in co-operation with Shh, TGF-β is also required for the induction and determination of DA neurons [130]. In addition, TGF-β3, at the terminal stage of hNPs cell differentiation, significantly enhances mRNA expression bcl-2, a well-known anti-apoptotic gene [131, 132]. Dibutyryl-cAMP (db-cAMP) is an additional essential mediator for the maintenance of hNP-derived DA neurons. db-cAMP has been shown to increase the yield of differentiated DA neuron from CNS cultures and to promote cell survival by promoting the phosphorylation of specific genes or more specifically by reactivating Akt/protein kinase B [133]. BDNF has also been found to support the survival and differentiation of DA neurons [134, 135]. Its neurotrophic functions are likely to complement and overlap with those of other secreted factors. Similarly, ascorbic acid has been reported to increase the survival and differentiation of human DA neurons [136, 137].

Differentiation of hESCs towards spinal cord motoneurons

Motor neurons (MNs) are lost in many conditions, including spinal cord injury, amyotrophic lateral sclerosis (ALS), and spinal muscular atrophy (SMA). Therefore, hESCs could be used to provide a source of differentiated human MNs for the study of the disease mechanisms. In the ventral part of the neural tube, there are five different progenitor domains: p3, pMN, p2, p1, and p0 progenitors (from ventral to dorsal). pMN progenitors later give rise to MNs while all other progenitors give rise to different types of ventral interneurons [138]. These progenitor domains generate different neuron subtypes according to a positional identification code in the spinal cord, which in turn is the result of the exposure to different concentrations of morphogens and growth factors [88]. Expression of Olig2, a basic helix–loop–helix transcriptional factor, is a determinant factor in establishing the pMN domain [139]. The daughters of pMN cells will then select whether they differentiate into motor neurons or become oligodendrocytes. This process is under the control of unique combinations of bHLH and homeodomain transcription factors such as LIM-homeodomain transcription factors (LHX3, LHX4), Hox genes (Nkx2.2. Phox, HB9) and Pax6 [140–142]. The conversion of unspecified neuroepithelial cells in the ventral neural tube to pMN precursor cells is driven by Shh from the notochord and floor plate, followed by the expression of Pax transcription factors to ventralise the hindbrain [143]. Neural tissue then develops anterior character unless exposed to caudalising signals. FGFs, Wnts, RA, Nodals, and Bmps are among the signalling molecules that have been proposed as caudalising factors [144]. Among these, RA signalling causes a very strong level of caudalisation in the spinal cord and induces differentiation of caudal CNS specification, i.e. MNs.

On the basis of these developmental principles, several protocols have been developed combining different morphogens and various concentrations of growth factors to promote the differentiation of spinal cord MNs. hESCs are converted into hNPs (see "Neuroectodermal differentiation of hESCs"), then specified to Olig2-expressing pMN cells in the presence of RA and Shh. Shh can be efficiently replaced by small molecules such as purmorphamine [145, 146] or SAG (a chlorobenzothiophene-containing Hh pathway agonist) [93], which directly bind and activate smoothened receptor [147]. It has been suggested that the ability to undergo MN specification under these conditions (RA + Shh) is temporally restricted to the earliest stages of neural induction [85, 148]. Indeed, addition of RA before expression of SOX1 (i.e. prior to neuroepithelial fate) is required for the caudalisation of hESCs and their differentiation into pMN progenitors. These progenitor cells are subsequently propagated in BDNF, GDNF, NT-3 and IGF to further generate post-mitotic, HB9-expressing MNs and mature to functional motor neurons thereafter [93, 145]. These factors have been previously shown to enhance motor neuron differentiation and survival of cells from embryonic rat ventral mesencephalon neurons cultures [149] or on MN in rodent ALS models [150].

Differentiation of hESCs towards peripheral nervous system derivatives

To understand the physiopathology and discovering new treatments for peripheral nervous system pathologies, such

as familial dysautonomia, neurocristopathies, Charcot–Marie tooth disease or hearing loss, there is a need to differentiate peripheral nervous system cells from hESCs. The sensory nervous system of vertebrates has a dual origin, arising from neural crest and placode cells both originating from ectoderm. Patterning events during gastrula and early neurula stages lead to the subdivision of the ectoderm into at least four distinct domains: neural plate, neural crest, pre-placodal ectoderm (PPE) and future epidermis [151]. While large concentrations of BMP antagonists are required to promote neural plate formation, several studies indicate that intermediate concentrations of these molecules induce neural crest and PPE formation [152, 153]. In addition, signalling pathways known to establish the posterior axis of the neural plate (e.g. wnt, FGF, RA) are also required for neural crest and PPE induction. However, different concentrations of BMP antagonists favour one fate over the other, and caudalising factors promote neural crest over placodal fate. Indeed, activation of the Wnt pathway promotes the generation of neural crest at the expenses of placodes [153] while FGF-3 and FGF-10 are necessary for placode induction [154].

hESCs-derived NPs, obtained indifferently from co-culture with MS5 or directly using dual inhibition of SMAD signalling (cf "Neuroectodermal differentiation of hESCs"), can be derived into neural crest progenitors in the presence of different cytokines such as Wnt3a [155], NGF [156, 157], BMP4 [158–160], FGF-8 [155], FGF-2 [5, 161, 162] or RA [155]. Importantly, all these molecules act in a temporal and concentration-dependent manner. In contrast, treatment with FGF and BMP antagonists (SU5402 and Noggin, respectively) prevent the induction of neural crest progenitors, i.e. p75-positive cells [163]. Peripheral neuronal differentiation is induced by withdrawal of FGF-2/EGF and exposure to BDNF, GDNF, NGF and dbcAMP, yielding peripherin-positive neurons. Schwann cell differentiation requires the presence of CNTF, neuregulin-1β, FGF-2 and dbcAMP [155, 163].

While obtaining neural crest-derived neurons and Schwann cells is now well established, some key steps remain to be discovered such as the precise identification and isolation of neural crest precursors (i.e. cranial versus sacral lineage) or the key factors required for the isolation of placode derivatives.

Pathological cell lines

hESCs

In recent years, numerous hESCs cell lines have been generated, most of them genetically normal. Nevertheless, through donation of embryos affected by genetic disorders, as revealed by preimplantation genetic diagnosis, many hESC lines that carry mutations for disorders, such as Myotonic dystrophy or Huntington's disease have been established [164]. hESC lines that carry monogenic diseases can be used in vitro to model the disease, bypassing the need for animal models and providing new tools for analysing and understanding the pathological mechanisms of the disease.

iPS cells derived from patients

The isolation of hiPS cells offers a new approach to model some human diseases. The reprogramming of somatic cells into hiPS cells goes through several steps including the integration of recombinant viruses carrying the expression of pluripotent genes (Oct4, Sox2, Klf4 and Myc) into the host genome. After transcription of these viral encoded genes, the corresponding proteins accumulate in the cytoplasm and are then imported back into the nucleus where they activate the transcription of the first wave of host genes whose promoters are accessible. This activity then triggers epigenetic mechanisms that contribute to remodel the chromatin in order to switch on endogenous genes critical for pluripotency and switch off these responsible for cell differentiation. Importantly, the full conversion of somatic cells into hiPS cells also requires a definitive silencing of the integrated viral genomes carrying the reprogramming initiators. It is now accepted that hiPS cells share major morphological, molecular and developmental features with blastocyst-derived ES cells. Thus, hiPS cells represent a critical tool to shed some light on disease mechanisms such as murine models of human congenital disorders which often provide a limited representation of human pathophysiology. Consistently, disease-specific hiPS cell lines have recently been engineered to untangle the pathological events of neurodegenerative disorders, including Parkinson's or Huntington's diseases [165, 166]. An important next step has come from studies that established the culture conditions to differentiate motor neurons from hiPS cells derived from patients suffering from ALS [167] or SMA [30].

SMA is a common inherited neuromuscular disease that often leads to infant mortality. It results from the degeneration of motor neurons and muscle weakness and atrophy. The most common form of SMA is caused by mutation in the survival motor neuron (SMN) gene and manifests over a wide range of severity affecting infants through adults. Importantly, motor neurons can be differentiated from SMA-derived hiPS cells; they have appropriate morphology and they maintained the disease genotype but showed molecular defects that recapitulate some aspects of this genetically inherited disorder. Indeed, hiPS derived from SMA patients initially produce the same

number of motor neurons as compared to control hips in culture, but the disease phenotype selectively impedes motor neuron production or increases their degeneration at later time points. A selective death of these neurons may result from the loss of SMN protein accumulation in the nucleus, a defect that has been partially rescued with valproic acid or tobramycin treatments of hiPS and neurons derived from SMA patients. This cell model represents a promising tool to study the disease mechanisms of SMA and to discover new drugs that could prevent or delay the specific degeneration of motor neurons.

Familial dysautonomia (FD), another fatal neurodegenerative disorder that results from an aberrant splicing of *IKBKAP*, has recently been modelled using patient-specific hiPS cells. Neural crest progenitors (NCP) differentiated from FD hiPS cells showed a very low level of *IKAP* transcripts that was compatible with the tissue-specific human pathology observed in patients suffering from this disease. In addition, comparative transcriptome analyses revealed the significant reduced expression of several transcripts in FD-derived hiPS cells compared to control hiPS cells. Among the candidates validated by RT–qPCR, many genes were involved in peripheral neurogenesis and neuronal differentiation. In addition, wound healing assays showed the reduced ability of FD-derived NCP to migrate, a feature previously described for other IKAP-depleted tissues by us and other laboratories [168, 169]. Finally, this study validated kinetin as an active compound that rescued both the splicing defect and the downstream expression of key peripheral neuron genes in NCP derived from FD's hiPS cells [170]. According to this elegant study, FD patient-specific hiPS cells represent a valuable resource to better understand the pathological mechanisms of familial dysautonomia.

Although the terminal differentiation of hiPS into mature and functional neurons remains to be achieved, these encouraging studies are the first to establish the hiPS as a reliable in vitro model to better understand the pathological mechanisms of neurological disorders. They also demonstrate that hiPS and their differentiated progenies can be used as a platform to perform high-throughput screenings aimed at discovering new molecules that would interfere with the identified pathological signalling pathways and would thus contribute to developing new treatments to cure or prevent the onset of these devastating diseases.

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