Common pitfalls of stem cell differentiation: a guide to improving protocols for neurodegenerative disease models and research

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Abstract Induced pluripotent stem cells and embryonic stem cells have revolutionized cellular neuroscience, providing the opportunity to model neurological diseases and test potential therapeutics in a pre-clinical setting. The power of these models has been widely discussed, but the potential pitfalls of stem cell differentiation in this research are less well described. We have analyzed the literature that describes differentiation of human pluripotent stem cells into three neural cell types that are commonly used to study diseases, including forebrain cholinergic neurons for Alzheimer’s disease, midbrain dopaminergic neurons for Parkinson’s disease and cortical astrocytes for neurodegenerative and psychiatric disorders. Published protocols for differentiation vary widely in the reported efficiency of target cell generation. Additionally, characterization of the cells by expression profile and functionality differs between studies and is often insufficient, leading to highly variable protocol outcomes. We have synthesized this information into a simple methodology that can be followed when performing or assessing differentiation techniques. Finally we propose three considerations for future research, including the use of physiological O₂ conditions, three-dimensional co-culture systems and microfluidics to control feeding cycles and growth factor gradients. Following these guidelines will help researchers to ensure that robust and meaningful data is generated, enabling the full potential of stem cell differentiation for disease modeling and regenerative medicine.

Keywords iPS cells · Differentiation · Neurodegeneration · Alzheimer’s disease · Dopaminergic neurons · Cholinergic neurons · Astrocytes

Introduction

Studying mechanisms and potential therapeutic targets of neurodegenerative diseases has historically been limited by the timely access to the affected tissue. Furthermore, the use of animal models for disorders without a clear genetic cause has shown to be of limited translational value to the clinical setting. The possibility of creating neuronal cultures from human stem cells, particularly from induced pluripotent stem cells (iPSC) of diagnosed individuals, has received wide attention for the potential of creating translatable disease-in-a-dish models [1]. Following the discovery of iPSCs, several high profile publications have fuelled the enthusiasm for their use in research into Parkinson’s disease [2], Alzheimer’s disease [3, 4], motor neurone disease [5] and mental disorders [6].

While optimized methods for reprogramming iPSCs have broadly been adopted (recently reviewed by Revilla et al. [7]), the protocols for differentiating iPSCs into neuronal cultures vary significantly for the same desired cell types. Strategies for converting stem cells into terminally differentiated cells predominantly follow observations from developmental mouse and rat studies, intending to model the in vivo progression of chemical signaling. However, variations in composition, concentration and timing of the signaling molecules can lead to marked differences in the resulting cultures and maturation...
stage of the desired cells. Furthermore, recent studies report variations in the differentiation efficiency between different stem cell lines, which is particularly relevant if the differences are diagnosis-specific [8, 9]. Finally, novel culturing methods, including three-dimensional cultures and hypoxic conditions, have been reported to influence the differentiation efficiency [10, 11]. To ensure the generation of meaningful results, stem cell derived cultures will therefore need to undergo thorough characterization to identify the composition and functionality of the differentiated cells.

The development and maturation of neuronal cells depends on different types of chemical signaling in vivo. Initially, growth factors and chemotactants released into the extracellular space by progenitor cells trigger location specific differentiation [12]. Upon neurite formation, local neurotransmitter release guides dendrite development and cell maturation to form the dynamic networks of the central nervous system [13, 14]. While growth factor signals can be recreated in vitro through time-dependent media supplementation, location and cell type-specific synaptic inputs cannot easily be modeled. It is therefore highly relevant to consider the developmental and maturation environment of the desired cell type when deciding to study disease processes in iPSC derived cultures.

With the present review, we provide an overview of existing differentiation strategies for neurodegenerative disease-relevant cell types of forebrain cholinergic neurons, midbrain dopaminergic neurons and cortical astrocytes. Advances in motor neuron differentiation have been extensively reviewed, including recently [15]. We address the variation in protocol efficiencies by providing a checklist that can be used to evaluate the quality and reproducibility of in vitro differentiation. Finally, we discuss recent culturing method developments aiming to improve the quality of stem cell derived neural cultures.

Human stem cell derived in vitro disease models have the potential to overcome the limitations of existing cell line work and can become a vital research stream next to animal modeling strategies. However, to progress in disease understanding and treatment development, the quality and biological relevance of stem cell-derived cultures need to be ensured.

From in vivo development to in vitro differentiation

Forebrain cholinergic neurons

Cholinergic neurons in the mammalian brain, including basal forebrain cholinergic neurons (BFCNs) and interneurons of the striatum, are defined by the production of acetylcholine (ACh) and its use as a neurotransmitter. BFCNs play an important role in cognitive functions, such as learning, memory and attention, and are implicated in the rapid eye movement sleep phase. The loss of BFCNs in neurodegenerative disorders, including Alzheimer’s disease, thus leads to severe cognitive impairments and memory deficits [16, 17].

Mammalian development of cholinergic neurons

During mammalian development, cholinergic neurons are derived from neural precursors of the ectoderm layer. After the formation of the neural tube, a selection of cells start to respond to high concentrations of sonic hedgehog (SHH), a morphogen that induces ventralization (differentiation towards the anterior) of the neural tube, and low concentrations of Wnt, a morphogen that induces caudalization (differentiation towards the posterior) [18]. The morphogens retinoic acid (RA), bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) are also known to play a key role in telencephalon development, the most anterior region of the developing brain [19]. In rodents and primates, BFCNs are formed in the medial ganglionic eminence (MGE), a ventral region of the telencephalon, with projections to the hippocampus and frontal cerebral cortex [20], areas implicated in the cognitive and psychological deficits of neurodegenerative diseases [21, 22].

The anterior/posterior patterning of the brain begins at embryonic day 8.5 (E8.5) in mice, when the telencephalic neuroepithelia expresses the transcription factors forkhead box G1 (FOXG1) and paired box 6 (PAX6) [23–25]. SHH together with FOXG1 induce the expression of FGFs, which activate downstream transcription factors characteristic of the ventral patterning. The expression of NK2 homeobox 1 (NKX2.1) at E9.5 defines the MGE region and by day E12.5 the expression of LIM homeobox 8 (LHX8) and insulin gene enhancer protein 1 (ISL1) determine a cholinergic fate. Choline acetyl transferase (ChAT) expression in neural precursor cells arises at postnatal day 7 (P7), with ChAT-positive cells increasing in number after P8 and remaining stable during adulthood [26].

While the full range of molecular drivers for BFCN development remains to be mapped, several essential factors for the differentiation, growth and survival of BFCNs have been identified. The cholinergic phenotype of embryonic BFCNs is induced and maintained by BMP9 [27], whilst FG2 and brain-derived neurotrophic factor (BDNF) stimulate cholinergic differentiation [28, 29]. The maturation, neurotransmitter phenotype, arborisation and survival processes are finally controlled through nerve growth factor (NGF) signaling [30]. These growth factors drive the gene expression profile of BFCNs through
specific transcription factors, such as LHX8 [31] and GBX1, a gastrulation box homeodomain protein, which can be used in the differentiation of BFCNs in vitro [32].

Characteristics of mature cholinergic neurons

Basal forebrain cholinergic neurons are characterized by their neuroanatomical location and the expression of ACh related genes. The key markers used to identify mature cholinergic neurons are involved in ACh synthesis (choline acetyltransferase, ChAT), transport (e.g., the vesicular ACh transporter, vAChT and the choline transporter, Cht1) and hydrolysis (e.g., acetylcholinesterase). Since NGF levels are directly related to the health and function of BFCNs [30], the high affinity neurotrophin receptor, TrkA, and the low affinity receptor, p75NTR, are also useful identifiers.

Due to their extraordinary complexity, the characterization of the complete morphology of cholinergic neurons has been difficult. In vitro studies from primary cultures showed that BFCNs have large cell bodies with two to four primary neurites [33]. However, a recent study in mice has demonstrated that individual cholinergic neurons from the basal forebrain region have axons that develop up to 50 cm in length, with approximately 1000 branches [34]. The electrophysiological profile of BFCNs has been characterized in rats, where BFCNs show regular spontaneous discharge patterns with mean spontaneous activity of 20 impulses/s [35]. Further key characteristics of the BFCNs are a slow spiking activity (4–10 Hz) and slow after potentials (400–700 ms) when compared with non-cholinergic neurons (3–60 ms) [36], which have also been shown in iPSC-derived BFCNs [37]. The addition of NGF can increase slow depolarization and enhance synaptic activity by upregulating ChAT activity [38].

Current differentiation strategies for cholinergic neurons

Cholinergic neurons derived from human embryonic stem cells (hESC) and iPSCs can become an important tool for modeling neurodegenerative diseases, such as Alzheimer’s disease. However, due to their complexity, few studies have successfully differentiated BFCNs. Most of these studies start the differentiation process with the generation of embryoid bodies, followed by the formation of neural rosettes and neurospheres. These neurospheres contain primordial cholinergic cells expressing specific transcription factors, such as LHX8 [31] and GBX1, a gastrulation box homeodomain protein, which can be used in the differentiation of BFCNs in vitro [32].

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Using a different strategy, Crompton et al. published a protocol for non-adherent differentiation of iPSCs into BFCNs [37]. In this procedure, neurospheres were treated with Nodal/transforming growth factor beta (TGF-β) inhibitor (small molecule inhibitor, SMI) to induce the endogenous expression of SHH, instead of its direct addition, resulting in a 90 % efficiency of β-III-tubulin/ChAT-expressing cells after 90 days [37].

Overall, only two of the mentioned protocols successfully reached >90 % ChAT-expressing cells. The main differences between the protocols are in their way of culturing (i.e., adherent by Bissonnette et al. [32] versus non-adherent by Crompton et al. [37]). This highlights the need for independent replication of both protocols to provide evidence for the use of either strategy. One potential advantage of the protocol developed by Bissonnette et al. involves using plasmid transfection via electroporation to trigger BFCN differentiation [32]. While this step allows fluorescently tagged cell sorting for purified cultures, transfection efficiency likely differs between each stem cell line and thus requires thorough optimization and counting of viable cells after sorting to produce replicable cultures.

In summary, the majority of published protocols for cholinergic differentiation are based on the initial addition of SHH or its endogenous induction to induce ventral forebrain fate and the expression of developmental markers of the MGE. While treatment with NGF has been shown to be highly important for the generation of mature ChAT-expressing BFCNs (Fig. 1; Table 1), the incomplete functional characterization of mature BFCNs limits us from recommending a particular protocol. This shortcoming can be addressed by transplanting BFCN precursors into rodents to compare the in vitro maturation with in vivo maturation of cells from the same origin. While three of the listed protocols show that engrafted BFCN precursors develop into integrated BFCNs [32, 37, 41], none of the studies compared the in vitro differentiated cells with their in vivo counterparts. We can, thus, not yet recommend a reliable BFCN differentiation protocol.
Midbrain dopaminergic neurons

Midbrain dopaminergic (mDA) neurons are predominantly expressed in the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) in rodents and primates [42–44]. SNc mDA neurons are required for initiation and control of motor functions, while VTA mDA neurons are important for reward behavior and cognition. Both nuclei are implicated in severe disorders, with degeneration of SNc mDA neurons being a hallmark of Parkinson’s disease, and impaired signaling of VTA mDA neurons being implicated in psychiatric disorders, such as schizophrenia and bipolar disorder. There is thus strong interest in differentiating human mDA neurons in vitro to study mechanisms contributing to the onset and progression of these disorders.

Mammalian development of mDA neurons

Midbrain DA neurons arise from NPCs of the ventral mesencephalon in mammals. The expression of aldehyde dehydrogenase 1 by progenitor cells at embryonic day 9.5

Table 1  Comparison of basal forebrain cholinergic neuron differentiation protocols

| Differentiation protocol | Nilbratt et al. [39] | Bissonnette et al. ([32], BMP9 treatment) | Bissonnette et al. ([32], nucleofection), Duan et al. [40] | Liu et al. [41] | Crompton et al. [37] |
|--------------------------|----------------------|------------------------------------------|-------------------------------------------------|-----------------|---------------------|
| Duration (days)          | ND                   | 34                                       | 34; 38 % after FACS purification                 | 45              | 90                  |
| Efficiency (ChAT<sup>+</sup> cells) | ND 69–78 % | 85 %                                     | EGF, FGF2, FGF8, NGF, RA, SHH                   |                 |
| Growth factors           | BDNF, CNTF, EGF, FGF2, NGF, NT-3 | BMP9, EGF, FGF2, FGF8, NGF, RA, SHH | BDNF, BMP9, cAMP, IGF-1, NGF, SHH               |                 |
| Developmental markers (protein, mRNA) | BF-1, DLX1, DLX2, GBX2, GSH2, ISL1, LHX8, MASH1, NKX2.1 | FORSE1, FOXG1, MASH1, NKX2.1 | FORX1, ISL1, MASH1, NKX2.1, OLIG2 |                 |
| Maturity markers (protein, mRNA) | ChAT, nAChRs, NMDAR, mAChRs, MAP2, p75NTR, TrkA, β-III-tubulin | AChE, Calbindin, ChAT, MAP2, p75NTR, TrkA, vAChT | ChAT, MAP2, p75NTR, vAChT | ChAT, MAP2, p75NTR, SYN-1, β-III-tubulin, vAChT |
| Physiological function | Ca<sup>2+</sup> response to ACh | ACh production and release | Spontaneous functional voltage-gated channels | ACh production and release | Function voltage-gated channels and cholinergic receptors |
(E9.5) in mice triggers the development of post-mitotic cells, which produce the dopamine synthesizing enzyme tyrosine hydroxylase (TH) [45]. These mDA neuron precursors express nuclear receptor related 1 protein (NURR1) at E10.5 and differentiate into dopamine producing, TH-expressing neurons at E11.5 in the mediobasal floor plate [45, 46]. Neurogenesis of mDA neurons peaks at E12.5 in mice and E80 in non-human primates [42, 47–49], with mDA neuronal development occurring earlier in the SNc than VTA [47]. While the timing of prenatal mDA neurogenesis is similar in mice and rats, neurite development and migration is completed in postnatal week one in mice and three in rats [46, 50]. This suggests a likely further extension to the developmental period in primates that has yet to be mapped in humans.

Neurogenesis of mDA neurons is driven by intrinsic and extrinsic signaling factors with precise temporal release patterns. The specific events have recently been reviewed in detail [51, 52]. Briefly, mDA progenitor cells receive target-derived neurotrophic factors from the mesencephalon floor plate of the doroventral neural tube axis (SHH and FGF8) [53–55] and later from the striatum (glial cell-derived neurotrophic factor, GDNF and neurturin, NRTN) [56–58]. Additional factors, such as BDNF, TGF3β, RA and ligands for members of the frizzled family of seven transmembrane receptors (Wnt1 and Wnt5a), promote mDA neuronal development and midbrain organization [59–63]. The variations in developmental timing of mDA neurons between species suggest an importance for the scheduling of the different factors between stem cells of different organisms.

**Characteristics of mature mDA neurons**

Midbrain DA neurons develop into a distinct cell type throughout their differentiation and maturation process which can be identified through functional, morphological and protein expression characteristics. The functional profile of mDA neurons requires the production of several key proteins, which have therefore been used as markers to identify mDA neurons within brain regions and in mixed primary cultures. The dopaminergic profile ultimately relies on the synthesis of dopamine from l-3,4-dihydroxyphenylalanine (l-DOPA) by TH, the re-uptake of dopamine from the synaptic cleft by dopamine transporters (DAT1), as well as the auto feedback loop, regulating dopamine production through activation of presynaptic dopamine receptor 2 (DR2).

Promoter-driven expression or antibody labeling of TH has consequently been used to identify DA neurons in post mortem tissue [64], primary cell cultures [65] and human stem cell derived differentiated neurons [66]. However, TH is required for the first synthesis step of catecholamines and therefore also present in noradrenergic cells [67]. In addition, TH expression is tightly regulated by neuronal activity via modulation of histone acetylation levels and is neither specific to DA neurons nor expressed at consistent levels in these cells [68]. Selection based on DAT expression has shown high specificity for mature mDA neurons from the VTA, with lower expression levels in the SNc [69]. Consequently, purifying embryonic mouse brain cultures for DAT resulted in a higher proportion of mature mDA neurons than selecting for TH [70, 71]. To address limitations of TH and DAT expression based selection, a recent study explored cell surface proteins with high specificity for mDA neurons. Ganat et al. identified the nicotinic ACh receptor subunit 3 and 6 to be highly colocalized with mDA neurons in the mouse [72], suggesting that these receptor subunits could be additional selection options for stem cell-derived mDA neurons [73], following confirmation in human samples.

The morphological profile of mDA neurons in vivo is dominated by their long projections, connecting the VTA with the neocortex via the mesocortical pathway and the SNc with the striatum via the nigrostriatal pathway. Limitations of the in vitro environment restrict this structural characteristic, moving the focus onto cell body size and shape. Midbrain DA neurons in the VTA are reportedly smaller and rounder than their elongated SNc relatives (13 vs. 19 μm Ø) [74]. Additionally, VTA mDA neurons are predominantly multipolar with radial projections while SNc mDA neurons have largely lateral and ventral projecting dendrites [75]. Although the reported characteristics are based on rodent in vitro and in vivo studies, they are expected to be observed in stem cell derived mDA neurons [76].

The electrophysiological profile of mDA neurons has been characterized in vivo and in vitro, identifying specific action potential patterns. Midbrain DA neurons can express either high bursts (>15 Hz) or slow background (1–5 Hz) action potential discharges [64], with each discharge starting with a prominent hyperpolarizing pulse [77] and depend on the G-protein-regulated inward-rectifier potassium channel 2 (GIRK2). The burst pattern has been shown to depend on the morphology of the mDA neurons [78] and requires specific stimulation in vitro [79]. This suggests that the functional assessment of stem cell-derived mDA neurons by electrophysiological characterization is met with several challenges, as the full phenotype depends on the input of the surrounding network and extracellular concentration of dopamine [77]. However, as similar action potential patterns have been identified in iPSC-derived mDA neurons [73], the electrophysiological profile forms the third critical characteristic of in vitro differentiated mDA neurons after protein expression and dopamine handling.
Current differentiation strategies for mDA neurons

The strong disease relevance of mDA neurons and their distinct developmental pathway has driven the differentiation of mDA neurons in vitro. Following the availability of mouse NPCs, mouse and human ESCs and more recently iPSCs, different strategies have been developed to create in vitro cultures rich in mature mDA neurons (Table 2). The majority of differentiation protocols closely follow the in vivo developmental stages, progressing from stem cells to neural rosettes (sometimes via embryoid body formation) to create neural progenitor cells and begin the mDA neuron patterning (Fig. 2). Cells at each stage are supplemented with a cocktail of growth factors reportedly found during the relevant developmental stages, such as SHH, FGF8 and BDNF (Table 2). Despite extensive testing of variations in media compositions, including undefined commercial options, and differentiation timings, the reported efficiency ratios have remained low with 8–40% of generated cells expressing TH (Table 2). Higher yields are promised by the use of SMI, specifically targeting Wnt and GSK3β signaling [80] and factors involved in floor plate development [81]. However, since GSK3β signaling is tightly regulated during neurodevelopment [82], the risk of unintended consequences on mature mDA neurons remains to be assessed. To ensure the formation of physiologically relevant cells, the majority of protocols already go beyond TH expression analysis to identify mature mDA neurons. Critically, the thoroughness of each protocol in identifying the derived cultures still varies widely (Table 2), with two protocols presenting TH expression without stating their proportion within the differentiated cultures [80, 83]. By not reporting electrophysiological characteristics, for example, it remains unclear whether the created dopamine-producing cells are close representations of SNc, VTA or other dopamine neurons. The protocol by Kriks et al. [81] uses fully adherent differentiation to form a promising foundation for further development and optimization of mDA neuron differentiation, with the strong need for identifying the full spectrum of the generated cultures.

An important shortcoming across all mDA neuron differentiation protocols still remains: the direct comparison of in vitro differentiated neurons with engrafted and in vivo differentiated neurons. Two of the discussed protocols engrafted differentiated NPCs [84] or mDA neurons [81] into rodents, showing that these cells integrate and mature in their host environment. Neither of the protocols, however, compared the matured in vitro cultured cells with their in vivo relatives. The extent to which stem cell derived mDA neurons differ to their in vivo counterparts thus remains unclear. This information is vital to assess whether stem cell derived mDA neurons are appropriate for the range of questions they have been promised to answer.

Cortical astrocytes

Once defined as the “nerve glue” (neuroglia) for neurons, astrocytes have emerged as one of the key players in maintaining cellular homeostasis in the brain and spinal cord. Astrocytes regulate the ion concentration and remove excess neurotransmitter and cellular debris from the extracellular fluid surrounding neurons. In neurodegenerative diseases, however, this vast support network for neurons is often altered or dysregulated [88]. The dysregulation of astrocytes can lead to excessive neuroinflammation, a common pathology in neurodegenerative diseases, and contributes to neuronal deterioration [89]. It is, therefore, important to study the contribution of astrocytes to neuronal degeneration and disease.

Mammalian development of astrocytes

Astrocyte generation and maturation in vivo relies on precise temporal and positional stimuli provided by the cellular environment and neighboring cells [90]. In mice, the differentiation of astrocytes and other neural cells begins at E8.5 with neurulation of the neuroectoderm [91]. Neuroepithelial cells from the neuroectoderm firstly differentiate into radial glia, the precursors of astrocytes and NPCs [92]. Although radial glia and astrocytes are overlapping in their marker expression of proteins, such as vimentin and glial fibrillary acidic protein (GFAP) [93], the morphology, function and time of development differ significantly. In the mammalian brain, the main morphological characteristic of radial glia is their long processes, which extend from the ventricular zone all the way to the marginal zone of the pial surface [94]. Through asymmetric horizontal division, radial glia give rise to NPCs and initiate neurogenesis. The newly formed NPCs then migrate along the long processes of radial glia to form the different layers of the cortex [95]. In the late prenatal stage and early postnatal stage (E18-P7) in mice, after neurogenesis and neuronal migration are completed, radial glia differentiate into astrocytes, a process called astrogliogenesis.

The differentiation of radial glia and NPCs into astrocytes is dependent on the activation of several signaling pathways. Two of these pathways are the JAK/STAT and the BMP-SMAD pathway [96]. These pathways are extrinsically activated by growth factors, including CNTF, cardiotrophin 1 (CT-1) and leukemia inhibitory factor (LIF), which are released by early neurons and late NPCs in vivo. Activation of these mechanisms leads to
### Table 2 Comparison of a representative selection of midbrain dopaminergic neuron differentiation protocols

| Differentiation protocol | Brennand et al. [6] | Chambers et al. [80] | Fathi et al. [85] | Hartfield et al. [73] | Kriks et al. [81] | Perrier et al. [86] | Petit et al. [83] | Reinhardt et al. [84] | Tan et al. [87] |
|--------------------------|---------------------|---------------------|-------------------|---------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Duration (days) | 110 | 19 | 30 | 66 | 50 | 62 | 90 | 28 | 28 |
| Efficiency (TH^+ cells) | 8 % | ND (80 % PAX6^+) | 35 % | 20.31 % | 78 % | 40 % | ND (75 % β-III-tubulin^+) | 35 % | 33 % |
| Growth factors | AA, BDNF, DCAMP, FGF2, GDNF | AA, BDNF, cAMP, FGF2, FGFR, GDNF, Noggin, TGF3β | AA, BDNF, dCAMP, FGFR, GDNF, Noggin, SHH, SMI | AA, BDNF, CHIR, DATP, dBCAMP, FGFR, FGFR8, GDNF, Pulmorphamine, SHH, SMI, TGF3β | AA, BDNF, dCAMP, FGFR, FGFR8, GDNF, SHH, SMI | AA, BDNF, cAMP, FGF8, GDNF, SHH | AA, BDNF, cAMP, FGF8, GDNF, SHH, SMI | AA, BDNF, CHIR, dCAMP, FGF8, GDNF, SHH, SMI | FGF2, FGF8, SHH |
| Developmental markers | Nestin | CDX2, FGF5, Nestin, OTX2, PLZF, PAX6, SOX1, SOX17 | EN1, FOXA2, LMX1A, LMX1B, Nestin, PTX3, TAT, WNT1 | FOXA2, Nestin, NURR1, PAX6 | FOXA1, FOXA2, HES, LH2, LMX1A, NURR1, OTX2, PAX6 | CRIPTO, NCAM, Nestin, PAX6, SOX1 | DNMT3B, FOXA2, Nestin, PAX6 | Brachyury, FOXA2, Nestin, PAX6, PAX7, SOX1, SOX9, TAP2A | NURR1 |
| Maturity markers | GEPh, GluR1, MAP2, PSD95, SYN-1, TH, β-III-tubulin, VGAT, VGGLUT1 | TH, β-III-tubulin | MAP2, PTX3, TH | AADC, Calbindin, DAT1, GABA, GIRK2, IP3R, LRRK2, PTX3, α-synuclein, tau, TH | DAT, GIRK2, TH, β-III-tubulin | AADC, EN1, GAFA, MAP2, O4, PAX2, PAX5, SV2, SYN-1, TH, VMAT2 | TH, β-III-tubulin | MAP2, PAX3, PAX7, TH, β-III-tubulin | ALDH1A1, GFAP, MAP2, PTX3, SCN1A, TH, β-III-tubulin, VMAT2 |
| Physiological function | DA production, spontaneous Ca^{2+} oscillations, spontaneous EPSPs and IPSPs | ND | ND | DA production and uptake, spontaneous Ca^{2+} oscillations, mDA-specific mitochondrial responses, slow action potential trains | DA production, slow action potentials and spontaneous spikes | DA production, repetitive action potentials and spontaneous spikes | DA production | Spontaneous action potentials | ND |
downstream events including chromatin modification and induction of astrocyte specific gene expression [97]. Transcription factors, such as signal transducer and activator of transcription 3 (STAT3), mothers against decapentaplegic homolog 1 (SMAD1), SMAD4 and nuclear factor 1A (NF1A) are activated and initiate the expression of GFAP, S100 calcium binding protein B (S-100b) and glutamate aspartate transporter (GLAST), all of which are currently used as astrocyte specific markers [98–100]. Within the first week after birth, astrocytes develop branched processes and attain their star like structure in the postnatal brain and spinal cord in mice [101]. Moreover, expression of the mature astrocyte marker glutamate transporter 1 (GLT1), a membrane protein important for the protection of neurons from glutamate-mediated excitotoxicity, was observed 2–3 weeks after birth [102]. Mouse studies have further shown that developmental GLT1 expression is upregulated in vitro when astrocytes are co-cultured with neurons [103]. This indicates that neuronal signaling is important for astrocyte maturation.

To successfully differentiate stem cells into functionally and physiologically relevant astrocytes, the molecular pathways involved in the cell fate determination of astrocytes in vivo need to be replicated in vitro. However, as most of the developmental studies are based on mouse models, the translation of this information to human stem cell differentiation requires careful consideration.

**Characteristics of mature astrocytes**

In the pursuit of generating functional astrocytes, it is essential to assess the differentiated cells for the presence of astrocyte-specific characteristics that are generally observed in vivo. Morphologically, early astrocytes have large cell bodies with few processes. However, as astrocytes mature, more processes develop, elongate and branch out, giving the typical ‘star’ shape. In mice, this branching and elongation of processes takes place during the late postnatal stages (P14–P27) [104], suggesting that the astrocyte networks are formed after the maturation of neighboring neurons.

On the molecular level, the expression of a selection of proteins is commonly used to characterize cells, in combination with cellular morphology. The most widely used marker in the characterisation of astrocytes is GFAP. In vivo mouse studies have shown that GFAP expression during astrocyte maturation peaks between E16-P1, slowly decreasing after this time point [105]. Mature astrocytes only express low levels of GFAP; however, GFAP expression in mature astrocytes may be upregulated following inflammatory activation [106]. Hence rather than being a marker for mature and functional astrocytes, GFAP expression is more indicative of either early immature astrocytes or reactive astrocytes. Another early developmental astrocyte marker is NF1A, an astrocyte specific transcription factor. This protein is highly expressed during mouse embryonic development (E10–E12.5), but its expression decreases as astrocytes mature [100]. Conversely to GFAP and NF1A, the expression of the astrocyte specific proteins aldolase C (ALDOC), GLAST and GLT1 increase as astrocytes mature [102, 107, 108]. These proteins therefore provide more appropriate markers of mature astrocytes than the commonly used GFAP. In humans, expression of the GLAST and GLT1 homologues, excitatory amino acid transporter 1 (EAAT1) and EAAT2, respectively, also increases with gestation time, based on studies of post mortem fetal brain tissue [109]. As such, increases in EAAT1 and EAAT2, coupled with a decrease

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**Fig. 2** Schematic diagram of midbrain dopaminergic neuron differentiation. The differentiation of midbrain dopaminergic neurons from pluripotent stem cell colonies is driven by the transition from spherical to adherent cultures and a staggered supplementation of growth factors, particularly SHH, FGF8 and later TGFβ3. The presence or absence of developmental and maturation markers are essential guides to monitor the differentiation progress at each culturing stage towards mature, functional midbrain dopaminergic neurons.
in the early astrocyte markers, GFAP and NF1A, could be used as a robust indication of astrocyte maturation.

Other astrocyte specific protein markers commonly chosen are S100β, aldehyde dehydrogenase 1 family member L1 (ALDH1L1) and the surface marker protein, CD44. The expression of these proteins increases at approximately E13 in mice and the expression profile does not significantly change throughout development [110–113]. Thus, while being astrocyte specific markers, these proteins are unsuitable indicators of astrocyte maturity.

Although the expression profile of astrocyte-specific proteins provides important information regarding their maturity, it does not offer information about their functionality. Astrocytes provide a support network for neurons by closely monitoring and responding to the extracellular environment. One of the main functions of astrocytes is protecting neurons from excess neurotransmitter stimulation, by taking up glutamate via GLT1 and GLAST [114]. Additionally, astrocytes are a key player of the innate immune system of the CNS. As such, they express toll-like receptors (TLRs), which are able to recognize foreign particles [115]. Activation of TLRs leads to the release of chemical signaling molecules, such as cytokines and chemokines [e.g., interleukin-6 (IL-6), interferon-β and fractalkine (CX3CL1)] [116]. These molecules recruit and activate other immune cells to the site of injury. Furthermore, activated astrocytes also release neurotrophic factors, such as NGF, CNTF and BDNF to aid neuronal survival and regeneration. The controlled release of these immune and neurotrophic factors is thus a critical aspect of astrocyte function, which needs to be confirmed during in vitro differentiation.

Overall, a panel of marker proteins should be used to identify the presence of mature astrocytes. Verifying their functional profile, particularly immune response and neurotransmitter handling, will ensure the relevance of in vitro astrocytes for modeling disease-relevant processes.

**Current differentiation strategies for astrocytes**

The generation of mature astrocytes for the purpose of studying neurological diseases has led to the development of several astrocyte differentiation protocols from pluripotent stem cells. However, these protocols vary in their duration, growth factor conditions and efficiency (Table 3).

The generation of neural rosettes and NPCs via embryoid body formation is similar across all protocols, lasting approximately 21 days. However major variations between protocols arise during the differentiation and maturation of NPCs to functional astrocytes. Once NPCs are present, the most common treatment in the generation of astrocytes is supplementation with epidermal growth factor (EGF) and FGF2 (Fig. 3), leading to the generation of early astrocytes, which has been confirmed by the expression of CD44, NF1A and vimentin [117–121]. Once

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**Table 3** Comparison of astrocyte differentiation protocols

| Differentiation protocol | Krencik et al. [117] | Emdad et al. [118] | Serio et al. [119] | Shaltouki et al. [120] | Roybon et al. [122] | Mormone et al. [121] |
|--------------------------|---------------------|------------------|------------------|----------------------|------------------|------------------|
| Duration (days)          | 120                 | 35               | 49               | 42                   | 90               | 35               |
| Efficiency (GFAP⁺ cells) | 90 %                | 70 %             | 90 %             | 70 %                 | 70 %             | 55 % (without sorting) |
| Growth factors           | CNTF (or LIF), EGF, FGF2 | CNTF, EGF, FGF2 | CNTF, EGF, FGF2 LIF | CNTF, FGF2, NRG1β1 AA, BDNF, CNTF, FGF2, GDNF, IGF, RA; maturation induced by withdrawal of growth factors | CNTF, EGF, FGF2 |
| Developmental markers (protein, mRNA) | CD44, GFAP, NF1A | GFAP, NF1A, vimentin | CD44, GFAP, NF1A | AQP4, CD4, GFAP, NF1A, S100β, vimentin | GFAP |
| Maturity markers (protein, mRNA) | S100β | AQP4, EAAT1 | EAAT1, S100β | ALDOC, EAAT1, S100β | ALDOC, EAAT2 |
| Physiological function | Propagation of Ca²⁺ waves, glutamate uptake | Migratory properties | Glutamate uptake, promotion of synaptogenesis in neuron co-cultures | Glutamate uptake, promotion of synaptogenesis in neuron co-cultures | Propagation of Ca²⁺, glutamate uptake, inflammatory response (IL-6 release) | Migratory properties |

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the presence of early astrocytes is confirmed, the matura-

Fig. 3 Schematic diagram of astrocyte differentiation. The differenti-

tion of astrocytes from pluripotent stem cell colonies follows the

eye neuronal developmental progress through spherical and adherent
culture stages. Gial progenitor formation is triggered by the
supplementation of EGF and FGF2, with CNTF being required for

the presence of early astrocytes is confirmed, the matura-

tion of these cells is generally triggered by the addition of

CNTF (Fig. 3). Although the growth factor conditions are

to very similar for most of the protocols (Table 3), a major

difference lies in the maturation duration between each

method. While Krenck et al. [117] matured the differen-
tiated astrocytes for 100 days, the maturation time

described by Emdad et al. [118], Serio et al. [119], Shal-
touki et al. [120] and Mormone et al. [121] vary between

14 and 21 days. The discrepancy in maturation time was

reflected in the proportion of GFAP-expressing cells,

which varied between 55 % [121] and 90 % [117, 119]. To

validate the functionality of the generated cells, some of

these studies also confirmed the ability of astrocytes to take

up glutamate from the cell medium [117, 119, 120].

Unlike the previously described astrocyte differentiation

protocols, Roybon et al. [122] used a different approach in
differentiating astrocytes. The generated NPCs were not

directly differentiated into astrocytes; instead the cells were

first caudalized using RA and ascorbic acid (AA), prior to

treatment with neurotrophic factors, including CNTF, to

generate neurons. The differentiation of astrocytes was

induced by the withdrawal of neurotrophic factors and the

introduction of foetal bovine serum. The generated astro-
cytes were matured for 50–90 days. While previous

protocols assessed the maturation of cells with the

expression of GFAP, Roybon et al. [122] used this protein

as a marker for early astrocytes and EAAT1, EAAT2 and

ALDH1L1 to identify mature astrocytes. Moreover, the

differentiated astrocytes took up glutamate and released IL-

6 upon stimulation with tumor necrosis factor-α and IL-1β

[122]. This study provides initial evidence that the differ-

entiated cells are able to mount an immune response; how-

ever, the complexity of this activation requires further

characterisation.

Further evaluations of astrocyte maturation and function

were conducted by transplanting pluripotent stem cell
derived astrocytes into mice brains [117, 120–122]. These

in vivo experiments primarily demonstrated that trans-

planted cells retained their astrocyte identity as indicated

by the expression of GFAP. Krenck et al. demonstrated

that transplanted differentiated astrocytes were able to form
direct contact with cerebral blood vessels after 6 months,
suggesting that in vitro derived astrocytes are able to mature

in vivo towards functional astrocytes [117]. Conversely,
however, Roybon et al. analyzed marker expression of

engrafted astrocytes in vivo and found high expression of

immature markers, such as NF1A [122]. Thus, even in vivo

transplantation of in vitro generated astrocytes may not be

able to promote full maturation of these cells. Further

characterisation of in vitro differentiated astrocytes is

therefore essential.

In summary, current astrocyte differentiation protocols

use growth factors aligned with reported in vivo develop-

ment. The heavy reliance on GFAP as an indicator for

differentiation success and the limited reporting of func-
tional characteristics prevent a reliable assessment of the

maturity of the created cells. Furthermore, the differentia-
tion periods vary vastly between the protocols, highlighting

the uncertainty about the required phenotype of the gen-
erated astrocytes for relevant experimentation. To date, the

protocol described by Krenck et al. [117] (and Serio et al.
[119]) has reported the highest proportion of GFAP⁺
cells (90 %) with the confirmation of physiological functions,
such as the propagation of Ca²⁺ waves and glutamate

handling, following a labor-intensive maturation duration

of 4–6 months. Roybon et al. reduced the maturation time

of pluripotent stem cell derived astrocytes to 90 days,
while retaining a similar efficiency [122]. This protocol
[122] relies on several markers to track the maturity of the
generated astrocytes and reports the most extensive physiological confirmation by showing the propagation of Ca^{2+}, glutamate handling and inflammatory response upon activation. For the purpose of accurate disease modeling, the protocol by Roybon et al. shows the essential maturity confirmation necessary, consisting of a thorough expression analysis of maturity markers (EAAT1, EAAT2 and ALDOC), in conjunction with functional characterisation (neurotransmitter processing and inflammation response) relevant to the studied disease.

**Assessing differentiation techniques**

**Checklist for high quality and reproducible differentiated cultures in vitro**

Induced pluripotent stem cells offer much promise in developing in vitro models to understand neurodegenerative disease mechanisms and for testing potential therapeutics. However, it is essential to generate a meaningful cell population that is both physiologically and clinically relevant. Evidently, an understanding of the desired cell type and its key characteristics is necessary to deliver high quality and reproducible cultures.

The following are common experimental considerations to promote cell culture purity and consistency when performing or assessing differentiation:

1. **Adjust culture environment for each differentiation stage.** The extracellular matrix (ECM) directly and indirectly affects the maintenance and differentiation of stem cell and neural cultures [123]. Human ESCs and iPSCs have been historically cultured on living feeder cells for mechanical and chemical stimulation, but recent research has shown that complex ECM protein combinations are superior in promoting culture stability and proliferation [124, 125]. The ECM dynamically evolves during neurodevelopment to form separate compartments in the CNS [126], comprised of different ECM protein combinations [127, 128]. Recapitulating in vivo neurodevelopment in culture thus requires adjustment of the ECM environment for the desired cell type and developmental stage. Differentiation of neocortical cells has shown to benefit from combining ECM proteins such as collagen I and fibronectin [129], but new differentiation protocols might require performing an ECM microarray [130]. Advanced three-dimensional culture surface modification can additionally be used to further the maturation of the desired cell type [87].

2. **Optimize cell counts and the maturation duration of the protocol.** Cell plating density affects differentiation, potentially due to alterations in effective local growth factor concentrations. Careful assessment of the appropriate cell density for plating needs to be optimized since different cell lines and different cell types proliferate at different rates. Longer protocols may be required for mature phenotypes but can lead to greater differences in the development of the required cell type compared to contaminating (unwanted) cell types. For example, developing neurons exit the cell cycle, whereas contaminating cells may continue to divide unless removed or their growth is inhibited.

3. **Identify checkpoints along the differentiation pathway and use a panel of cell markers.** Expression of appropriate markers at checkpoints should be confirmed; also consider the use of positive or negative selection of cells, based on cell surface marker expression. At the final stage of differentiation the expression of a robust panel of cell-specific markers should be assessed for each line.

4. **Develop reporters to assess differentiated cell types.** Reporters using fluorescent protein expression driven by an appropriate promoter can be used to identify cell types of interest. For example, motor neurons differentiated from ESCs have been identified in mixed cultures via lentiviral delivery of a construct bearing a GFP driven by the homeobox-9 promoter [131] and stable GFAP-driven TagRFP has been used to select iPSC-derived astrocytes [132]. Similar methods can be employed for other cell types using the promoter sequence of an appropriate cell-specific marker. Automated imaging options, such as the Incucyte live cell imaging system, can be used to provide images that are free from operator bias. Furthermore, fluorescent protein expression can also be used to sort the cells following differentiation, increasing the purity of the cell type of interest.

5. **Monitor the quality of cultures by assessing cellular functionality.** The expression of a panel of marker proteins provides some evidence that the appropriate cell type has been generated. However, there are very few proteins that are expressed in one cell type alone; many commonly used (and supposedly cell-specific) markers are often expressed in multiple cell types. Further tests are therefore required to confirm that functional cells have been generated. Functionality can be assessed using in vitro live cell assays and should focus on non-biased assessments, such as receptor ligand quantification in culture medium, prior to experimental use.

The steps involved in developing and optimizing a differentiation protocol are outlined in Box 1.
Increasing the quality of cultures—suggestons for improvement

Cell culture experiments need to mimic the physiological environment as closely as possible, in order to yield biologically relevant conclusions. The following developments will allow the experimental set-up to more closely represent the in vivo cellular background:

1. **Use of a hypoxic chamber to prevent chronic oxidative stress.** The majority of cell culture experiments are performed under atmospheric oxygen conditions, i.e. 21% O₂. However, cells in the brain and much of the body experience far lower levels, thought to range between 1 and 11% O₂ [133]. Recent data suggests that the process of reprogramming somatic cells to iPSCs is more efficient when performed under hypoxic conditions [134, 135]. Reprogramming requires a shift in cellular metabolism from oxidative to glycolytic conditions [10] and the hypoxia-inducible factors play a role in the coordination of these metabolic changes [136]. Comparisons of differentiation protocols under atmospheric vs. physiological O₂ conditions are still limited. However, in the case of differentiation of cardiomyocytes, hypoxic culture increased differentiation yields by up to 1000-fold [137]. Measurements throughout the brain suggest that local oxygen tensions are heterogeneous in nature, exhibiting spatial and temporal differences depending on the microenvironment [138]. For example, neurons of the cerebral cortex experience a low oxygen field compared to venous O₂ [138]. Neurons in such an oxygen environment are more sensitive to changes in cerebral blood flow, in the provision of oxygen and nutrients and are potentially more sensitive to oxidative stress. Investigations using in vivo imaging, for example by two-photon microscopy, allow the mapping of the partial pressure of oxygen at the μm/s resolution [139]. A detailed understanding of the local oxygen environment will allow us to provide in vivo conditions during in vitro experiments, an important consideration for further development. Future studies need to consider the implications of performing differentiation, and experiments, at atmospheric O₂.

2. **Problems with feeding cycles could be partially overcome with microfluidics or bioreactors.** A consideration in cell differentiation is the consistent and appropriate provision of nutrients and growth factors. Traditional cell feeding cycles of every 24 or 48 h can lead to dramatic variations in nutrient delivery and gradients of trophic factors. During the course of a culture, the concentrations of glucose, growth factors and micronutrients can vary widely in the growth medium, along with the pH. The proliferation and differentiation of stem cells is drastically affected by growth medium supplements [140], with the outcome that under different nutrient conditions the same cell line could yield different phenotypes. Microfluidics can be used to manipulate the delivery of medium on the micrometer scale, allowing for more controlled temporal and spatial supply of nutrients [141]. Alternatively, larger cell preparations could be cultured in bioreactors that stir the growth medium; agitation has led to higher yields of differentiated cells in some protocols [137]. The use of bioreactors that are engineered to cultivate three-dimensional cultures, under hypoxic conditions, provides a further promising development [142].

3. **Development of accurate co-culture systems and three-dimensional cultures.** The architecture of the brain is clearly a complex multicellular environment. The presence of diverse cell types in cultures can be detrimental when studying cell-specific effects. However, a mixture of cell types may be required to understand a particular cellular process, such as

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**Box 1: Checklist for differentiating meaningful neural cell cultures from pluripotent stem cells**

A. Select appropriate differentiation protocol
   1. Method creates suitable cell type composition for hypothesis testing
   2. Cell phenotype characteristics can be developed in vitro
   3. Differentiation process follows in vivo developmental pathway

B. Optimize culturing method
   1. Adjust cell seeding density and media supplement concentrations to each stem cell line
   2. Adjust O₂ concentration for optimal differentiation and hypothesis testing
   3. Modify extracellular environment for each differentiation stage

C. Confirm culture quality
   1. Monitor differentiation progress via marker expression consistency at relevant check points
   2. Record culture composition via expression ratios of relevant markers for each line
   3. Perform functional assays to confirm culture suitability for hypothesis testing
neuronal:glial interactions or neuroinflammation. In addition, other cell types may be required for appropriate maturation. For example, astrocytes are required for the maturation of functional synapses [143] and in organizing the neuronal extracellular matrix [144]. As differentiation protocols improve so does our capacity for generating the appropriate mix of cells that function together. A key aspect of many neurodegenerative disorders is that multiple cell types are affected. Being able to accurately model the system holistically would be a huge leap forward in understanding the role of cellular interactions in disease pathogenesis. The generation of specific neuronal and glial subtypes remains under-developed. To accurately model the brain for neurodegenerative disease research, future studies will need to address the issue of subtype specification, potentially incorporating single cell analysis. In the case of excitabile cells, cell function can be analyzed by electrophysiological assessment, coupled with single cell reverse transcription PCR to characterize the molecular signature of the cells generated. Faithful recapitulation of the molecular profile of specific cell subtypes is an important goal for future research.

Conclusions

Creating in vitro models of central nervous system disorders with human stem cells provides the medical research community with a powerful new research tool. To ensure the use of this option to its full potential, differentiation strategies need to be carefully planned and executed depending on the cell type desired and the experimental read-out. Confirmation of a robust panel of cell-specific markers, coupled with functional assays, is further required to provide evidence that the appropriate developmental pathway has been effectively recapitulated. Future studies need to focus on cultivating cells in more physiologically relevant environments by manipulating oxygen tension, overcoming issues with growth factor and nutrient gradients and developing multicellular and three-dimensional culture systems. Employing these quality improvement and control measures will lead to more reliable and reproducible results with strong clinical relevance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Csete M (2010) Translational prospects for human induced pluripotent stem cells. Regen Med 5:509–519. doi:10.2217/rme.10.39
2. Devine MJ, Rytten M, Vodicka P et al (2011) Parkinson’s disease induced pluripotent stem cells with triplication of the α-synuclein locus. Nat Commun 2:440. doi:10.1038/ncomms1453
3. Israel MA, Yuan SH, Bardy C et al (2012) Probing sporadic and familial Alzheimer’s disease using induced pluripotent stem cells. Nature 482:216–220. doi:10.1038/nature10821
4. Ooi L, Sidhu K, Poljak A et al (2013) Induced pluripotent stem cells as tools for disease modelling and drug discovery in Alzheimer’s disease. J Neural Transm 120:103–111. doi:10.1007/s00702-012-0839-2
5. Egawa N, Kitaoka S, Tsukita K et al (2012) Drug screening for ALS using patient-specific induced pluripotent stem cells. Sci Transl Med 4:145ra104. doi:10.1126/scitranslmed.3004052
6. Brennand KJ, Simone A, Jou J et al (2013) Modelling schizophrenia using human induced pluripotent stem cells. Nature 473:221–225. doi:10.1038/nature09915
7. Revilla A, González C, Iriondo A et al (2015) Current advances in the generation of human iPSC cells: implications in cell-based regenerative medicine. J Tissue Eng Regen Med. doi:10.1002/term.2021
8. Brennand K, Savas JN, Kim Y et al (2014) Phenotypic differences in hiPSC NPCs derived from patients with schizophrenia. Mol Psychiatry. doi:10.1038/mp.2014.22
9. Hartley BJ, Tran N, Ladrán I et al (2015) Dopaminergic differentiation of schizophrenia hiPSCs. Mol Psychiatry 20:549–550. doi:10.1038/mp.2014.194
10. López-Iglesias P, Alcaina Y, Tapia N et al (2015) Hypoxia induces pluripotency in primordial germ cells by HIF1α stabilization and Oct4 deregulation. Antioxid Redox Signal 22:205–223. doi:10.1089/ars.2014.5871
11. Lu HF, Lim S-X, Leong MF et al (2012) Efficient neuronal differentiation and maturation of human pluripotent stem cells encapsulated in 3D microfibrous scaffolds. Biomaterials 33:9179–9187. doi:10.1016/j.biomaterials.2012.09.006
12. Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24:677–736. doi:10.1146/annurev.neuro.24.1.677
13. Hua JY, Smith SJ (2004) Neuronal activity and the dynamics of central nervous system development. Nat Neurosci 7:327–332. doi:10.1038/nn1218
14. Lujañ R, Shigemoto R, López-Bendito G (2005) Glutamate and GABA receptor signalling in the developing brain. Neuroscience 130:567–580. doi:10.1016/j.neuroscience.2004.09.042
15. Lee S, Huang EJ (2015) Modeling ALS and FTD with iPSC-derived neurons. Brain Res. doi:10.1016/j.brainres.2015.10.003
16. Mufson EJ, Counts SE, Perez SE, Ginsberg SD (2008) Cholinergic system during the progression of Alzheimer’s disease: therapeutic implications. Expert Rev Neurother 8:1703–1718. doi:10.1586/14737175.8.11.1703
17. Whitehouse PJ, Price DL, Struble RG et al (1982) Alzheimer’s disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237–1239
30. Kawano H, Ohyama K, Kawamura K, Nagatsu I (1995) Growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. J Neurosci 10:558–570.

31. Consomni S, Leone S, Becchetti A, Amadeo A (2009) Developmental and neurochemical features of cholinergic neurons in the murine cerebral cortex. BMC Neurosci 10:18. doi: 10.1186/1471-2202-10-18

32. Tao W, Lai E (1992) Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. Neurosci 24:1217–1281. doi:10.1146/annurev.neuro.24.11.1217

33. Matsumoto K, Tanaka T, Furuyama T et al (1996) L3, a novel murine LIM-homeodomain transcription factor expressed in the ventral telencephalon and the mesencephyme surrounding the oral cavity. Neurosci Lett 204:113–116

34. Bissonnette CJ, Lyass L, Bhattacharyya BJ et al (2011) The controlled generation of functional basal forebrain cholinergic neurons from human embryonic stem cells. Stem Cells 29:802–811. doi:10.1002/stem.626

35. Wu H, Williams J, Nathans J (2014) Complete morphologies of basal forebrain cholinergic neurons in the mouse. Elife 3:e02444

36. Markram H, Segal M (1990) Electrophysiological characteristics of cholinergic and non-cholinergic neurons in the rat medial septum-diagonal band complex. Brain Res 513:171–174

37. Crompton LA, Byrne ML, Taylor H et al (2013) Stepwise, non-adherent differentiation of human pluripotent stem cells to generate basal forebrain cholinergic neurons via hedgehog signaling. Stem Cell Res 11:1206–1221. doi:10.1016/j.scr.2013.08.002

38. Moises HC, Womble MD, Washburn MS, Williams LR (1995) Nerve growth factor facilitates cholinergic neurotransmission between nucleus basalis and the amygdala in rat: an electrophysiological analysis. J Neurosci 15:8131–8142

39. Nilbratt M, Porras O, Marutle A et al (2010) Neurotrophic factors promote cholinergic differentiation in human embryonic stem cell-derived neurons. J Cell Mol Med 14:1476–1484. doi:10.1111/j.1750-1326.2009.00916.x

40. Duan L, Bhattacharyya BJ, Belmadani A et al (2014) Stem cell derived basal forebrain cholinergic neurons from Alzheimer’s disease patients are more susceptible to cell death. Mol Neurodegener 9:3. doi:10.1186/1750-1326-9-3

41. Liu Y, Weick JP, Liu H et al (2013) Medial ganglionic eminence-like cells derived from human embryonic stem cells correct learning and memory deficits. Nat Biotechnol 31:440–447. doi:10.1038/nbt.2565

42. Morrow BA, Redmond DE, Roth RH, Elsworth JD (2005) Development of A9/A10 dopamine neurons during the second and third trimesters in the African green monkey. J Comp Neurol 488:215–223. doi:10.1002/cne.20599

43. Prakash N, Wurst W (2006) Development of dopaminergic neurons in the mammalian brain. Cell Mol Life Sci 63:187–206. doi:10.1007/s00401-005-5387-6

44. Vernier P, Moret F, Callier S et al (2004) The degeneration of dopamine neurons in Parkinson’s disease: insights from embryology and evolution of the mesostriatocortical system. Ann N Y Acad Sci 1035:231–249. doi:10.1111/j.1749-6632.2004.9199.x

45. Walleén A, Zetterström RH, Solomin L et al (1999) Fate of neuronal cells originated by ES cell differentiation: factors and fates. Dev Biol 214:122–131

46. Bayer SA, Wills KV, Triarhou LC, Ghetti B (1995) Time of neuron origin and gradients of neurogenesis in midbrain dopaminergic neurons in the mouse. Exp Brain Res 105:191–199

47. Marti J, Wills KV, Ghetti B, Bayer SA (2002) A combined immunohistochemical and autoradiographic method to detect midbrain dopaminergic neurons and determine their time of origin. Brain Res Brain Res Protoc 9:197–205

48. Morrow BA, Roth RH, Redmond DE et al (2007) Apoptotic natural cell death in developing primate dopamine midbrain neurons occurs during a restricted period in the second trimester of gestation. Exp Neurol 204:802–807. doi:10.1016/j.expneurol.2007.01.009

49. Voorn P, Kalsbeek A, Jorritsma-Byham B, Groenewegen HJ (1988) The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. Neuroscience 25:857–887

50. Abeliovich A, Hammond R (2007) Midbrain dopamine neuron differentiation: factors and fates. Dev Biol 304:447–454. doi:10.1016/j.ydbio.2007.01.032

51. Roussa E, Kriegstein K (2004) Induction and specification of midbrain dopaminergic cells: focus on SHH, FGF8, and TGF-β. Cell Tissue Res 318:23–33. doi:10.1007/s00441-004-0916-4

52. Springer
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53. Hayes L, Ralls S, Wang H, Ahn S (2013) Duration of Shh signaling contributes to mDA neuron diversity. Dev Biol 374:115–126. doi: 10.1016/j.ydbio.2012.11.016

54. Joksimovic M, Anderegg A, Roy A et al (2009) Spatiotemporally separable Shh domains in the midbrain define distinct dopaminergic progenitor pools. Proc Natl Acad Sci USA 106:19185–19190. doi: 10.1073/pnas.0904285106

55. Ye W, Shimamura K, Rubenstein JL et al (1998) FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. Cell 93:755–766

56. Kholodilov N, Yarygina O, Oo TF et al (2004) Regulation of the

57. Oo TF, Kholodilov N, Burke RE (2003) Regulation of natural
dopaminergic neurons of the substantia nigra by striatal glial cell-line-derived neurotrophic factor in vivo. J Neurosci 23:5141–5148

58. Horger BA, Nishimura MC, Armanini MP et al (1998) Neurturin
exterts potent actions on survival and function of midbrain
dopaminergic neurons. J Neurosci 18:4929–4937

59. Farkas LM, Dünker N, Roussa E et al (2003) Transforming
growth factor-beta(s) are essential for the development of mid-
brain dopaminergic neurons in vitro and in vivo. J Neurosci 23:5178–5186

60. Castelo-Branco G, Wagner J, Rodriguez FJ et al (2003) Differe-
tional regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. Proc Natl Acad Sci USA 100:12747–12752. doi: 10.1073/pnas.030120

61. Schulte G, Bryja V, Rawal N et al (2005) Purified Wnt-5a increases differentiation of midbrain dopaminergic cells and dishevelled phosphorylation. J Neurochem 92:1550–1553. doi:10.1111/j.1471-4159.2003.03022.x

62. Avantaggiato V, Acampora D, Tuorto F, Simeone A (1996) Retinoic acid induces stage-specific repatterning of the rostral central nervous system. Dev Biol 175:347–357. doi:10.1006/dbio.1996.0120

63. Baquet ZC, Bickford PC, Jones KR (2005) Brain-derived neu-
trotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. J Neurosci 25:6251–6259. doi:10.1523/JNEUROSCI.4601-04.2005

64. Roep E (2013) Dissecting the diversity of midbrain dopamine neurons. Trends Neurosci 36:336–342. doi:10.1016/j.tins.2013.03.003

65. Gaven F, Marin P, Claeyssen S (2014) Primary culture of mouse
dopaminergic neurons. J Vis Exp 91:e51751. doi:10.3791/51751

66. Smidt MP, Burbach JPH (2007) How to make a meso-
dopaminergic neuron. Nat Rev Neurosci 8:21–32. doi:10.1038/nrn2039

67. Pau KY, Ma YJ, Yu JH et al (1997) Topographic comparison of the expression of norepinephrine transporter, tyrosine hydroxylase and neuropeptide Y mRNA in association with dopamine-beta-hydroxylase neurons in the rabbit brainstem. Brain Res Mol Brain Res 48:367–381

68. White RB, Thomas MG (2012) Moving beyond tyrosine
hydroxylase to define dopaminergic neurons for use in cell replacement therapies for Parkinson’s disease. CNS Neurol Disord Drug Targets 11:340–349

69. Haber SN, Ryoo H, Cox C, Lu W (1995) Subsets of midbrain
dopaminergic neurons in monkeys are distinguished by different levels of mRNA for the dopamine transporter: comparison with the mRNA for the D2 receptor, tyrosine hydroxylase and calbindin immunoactivity. J Comp Neurol 362:400–410. doi:10.1002/cne.903620308

70. Kessler MA, Yang M, Gollomp KL et al (2003) The human

71. Zhou W, Lee YM, Guy VC, Freed CR (2009) Embryonic stem cells with GFP knocked into the dopamine transporter yield purified dopamine neurons in vitro and from knock-in mice. Stem Cells 27:2952–2961. doi:10.1002/stem.216

72. Ganat YM, Calder EL, Kriks S et al (2012) Identification of embryonic stem cell-derived midbrain dopaminergic neurons for engragment. J Clin Invest 122:2928–2939. doi:10.1172/JCI58767

73. Hartfield EM, Yamasaki-Mann M, Ribeiro Fernandes HJ et al (2014) Physiological characterisation of human iPSC-derived dopaminergic neurons. PLoS One 9:e87388. doi:10.1371/journal.pone.0087388

74. Thompson L, Barraud P, Andresson E et al (2005) Identification of dopaminergic neurons of nigral and ventral tegmental area subtypes in grafts of fetal ventral mesencephalon based on cell morphology, protein expression, and efferent projections. J Neurosci 25:6467–6477. doi:10.1523/JNEUROSCI.1676-05.2005

75. Grace AA, Onn SP (1989) Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded in vitro. J Neurosci 9:463–481

76. Yan Y, Yang D, Zarnowska ED et al (2005) Directed differen-
tiation of dopaminergic neuronal subtypes from human embryonic stem cells. Stem Cells 23:781–790. doi:10.1634/stemcells.2004-0365

77. Ungless MA, Grace AA (2012) Are you or aren’t you? Challenges associated with physiologically identifying dopamine neurons. Trends Neurosci 35:422–430. doi:10.1016/j.tins.2012.02.003

78. Köttner R, Feizelmeier M (1998) Species-dependence and relationship of morphological and electrophysiological properties in nigral compacta cells. Prog Neurobiol 54:619–632

79. Kuznetsov AS, Kopell NJ, Wilson CJ (2006) Transient high-
frequency firing in a coupled-oscillator model of the meso-
cerebral dopaminergic neuron. J Neurophysiol 95:932–947. doi:10.1152/jn.00691.2004

80. Chambers SM, Fasano CA, Papapetrou EP et al (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol 27:275–280. doi:10.1038/nbt.1529

81. Kriks S, Shim J-W, Piao J et al (2011) Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson’s disease. Nature 480:547–551. doi:10.1038/nature10648

82. Hur E-M, Zhou F-Q (2010) GSK3 signalling in neural develop-
ment. Nat Rev Neurosci 11:539–551. doi:10.1038/nrn2870

83. Petit I, Kesner NS, Karry R et al (2012) Induced pluripotent stem cells from hair follicles as a cellular model for neurode-
velopmental disorders. Stem Cell Res 8:134–140. doi:10.1016/j.

84. Reinhardt P, Glatza M, Hemmer K et al (2013) Derivation and expansion using only small molecules of human neural pros
cengers for neurodegenerative disease modeling. PLoS One 8:e59252. doi:10.1371/journal.pone.0059252

85. Fathi A, Rasouli H, Yeganeh M et al (2015) Efficient differen-
tiation of human embryonic stem cells toward dopaminergic neurons using recombinant LMX1A factor. Mol Biotechnol 57:184–194. doi:10.1007/s12033-014-9814-5

86. Perrier AL, Tabar V, Barberi T et al (2004) Derivation of midbrain dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci USA 101:12543–12548. doi:10.1073/pnas.0404700101

87. Tan KKB, Tann JY, Sathe SR et al (2015) Enhanced differen-
tiation of neural progenitor cells into neurons of the...
mesencephalic dopaminergic subtype on topographical patterns. Biomaterials 43:32–43. doi:10.1016/j.biomaterials.2014.11.036
88. Lobisger CS, Cleveland DW (2007) Gial cells as intrinsic components of non-cell-autonomous neurodegenerative disease. Nat Neurosci 10:1355–1360. doi:10.1038/nn1988
89. Hennessy E, Griffin ÉW, Cunningham C (2015) Astrocytes are primed by chronic neurodegeneration to produce exaggerated chemokine and cell infiltration responses to acute stimulation with the cytokines IL-1β and TNF-α. J Neurosci 35:8411–8422. doi:10.1523/JNEUROSCI.2745-14.2015
90. Kessaris N, Pringle N, Richardson WD (2008) Specification of CNS glia from neural stem cells in the embryonic neuroepithelium. Philos Trans R Soc Lond B Biol Sci 363:71–85. doi:10.1098/rstb.2006.2013
91. Ybott-Gonzalez P, Gaston-Massuet C, Girdler G et al (2007) Neural plate morphogenesis during mouse neurulation is regulated by antagonism of Bmp signalling. Development 134:3203–3211. doi:10.1242/dev.008177
92. Anthony TE, Klein C, Fishell G, Heintz N (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 41:881–890. doi:10.1016/S0896-6273(04)00140-0
93. Sancho-Tello M, Vallès S, Montoliu C et al (1995) Developmental pattern of GFAP and vimentin gene expression in rat brain and in radial glial cultures. Glia 15:157–166. doi:10.1002/glia.440150208
94. Sild M, Ruthazer ES (2011) Radial glia: progenitor, pathway, and partner. Neuroscience 17:288–302. doi:10.1177/0096985310385870
95. Noctor SC, Flint AC, Weissman TA et al (2001) Neurons promote neurogenesis and inhibit glial differentiation by independent mechanisms. Cell 104:365–376. doi:10.1016/S0092-8674(01)00224-0
96. Lyden B, Higashimori H, Morel L (2013) Developmental maturation of astrocytes and pathogenesis of neurodevelopmental disorders. J Neurodev Disord 5:22. doi:10.1186/1866-1955-5:22
97. Nakashima K, Wiese S, Yanagisawa M et al (1999) Developmental requirement of gpl30 signaling in neuronal survival and astrocyte differentiation. J Neurosci 19:5429–5434
98. Hong S, Song M-R (2014) STAT3 but not STAT1 is required for astrocyte differentiation. PLoS One 9:e86851. doi:10.1371/journal.pone.0086851
99. Sun Y, Nadal-Vicens M, Misono S et al (2001) Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell 104:365–376. doi:10.1016/S0092-8674(01)00224-0
100. Deneen B, Ho R, Lukaszewicz A et al (2006) The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. Neuron 52:953–968. doi:10.1016/j.neuron.2006.11.019
101. Takahashi T, Misson J-P, Caviness VS (1990) Glial process formation and branching in the developing murine neocortex: a qualitative and quantitative immunohistochemical analysis. J Comp Neurol 302:15–28. doi:10.1002/cne.903020103
102. Kugler P, Schleyer V (2004) Developmental expression of glutamate transporters and glutamate dehydrogenase in astrocytes of the postnatal rat hippocampus. Hipocampus 14:975–985. doi:10.1002/hipo.20015
103. Yang Y, Gozen O, Watkins A et al (2009) Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. Neuron 61:880–894. doi:10.1016/j.neuron.2009.02.010
104. Bushong EA, Martone ME, Ellisman MH (2004) Maturation of astrocyte morphology and the establishment of astrocyte domains during postnatal hippocampal development. Int J Dev Neurosci 22:73–86. doi:10.1016/j.jdevelneu.2003.12.008
105. Agius E, Decker Y, Soukkarieh C et al (2010) Role of BMPs in controlling the spatial and temporal origin of GFAP astrocytes in the embryonic spinal cord. Dev Biol 344:611–620. doi:10.1016/j.ydbio.2010.05.017
106. Brahmacari S, Fung YK, Pahan K (2006) Induction of glial fibrillary acidic protein expression in astrocytes by nitric oxide. J Neurosci 26:4930–4939. doi:10.1523/JNEUROSCI.5480-05.2006
107. Regan MR, Huang YH, Kim YS et al (2007) Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS. J Neurosci 27:6607–6619. doi:10.1523/JNEUROSCI.0790-07.2007
108. Cahoy JD, Emery B, Kaushal A et al (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci 28:264–278. doi:10.1523/JNEUROSCI.1478-07.2008
109. Bar-Peled O, Ben-Hur H, Biegon A et al (1997) Distribution of glutamate transporter subtypes during human brain development. J Neurochem 69:2571–2580. doi:10.1046/j.1471-4159.1997.69062571.x
110. Vives V, Alonso G, Solal AC et al (2003) Visualization of S100B-positive neurons and glia in the central nervous system of EGFP transgenic mice. J Comp Neurol 457:404–419. doi:10.1002/cne.10552
111. Yang Y, Vidensky S, Jin L et al (2011) Molecular comparison of GLT1+ and ALDH1L1+ astrocytes in vivo in astroglial reporter mice. Glia 59:200–207. doi:10.1002/glia.21089
112. Haidet-Phillips AM, Hester ME, Miranda CJ et al (2008) A transcriptome resource for understanding brain development and function. J Neurosci 28:264–278. doi:10.1523/JNEUROSCI.2745-14.2015
113. Zamanian JL, Xu L, Foo LC et al (2012) Genomic analysis of reactive astrogliosis. J Neurosci 32:6391–6410. doi:10.1523/JNEUROSCI.6221-11.2012
114. Perez C, Vanoni C, Bossi M et al (2000) The GLT-1 and GLAST glutamate transporters are expressed on morphologically distinct astrocytes and regulated by neuronal activity in primary hippocampal cocultures. J Neurochem 75:1076–1084
115. Farina C, Krumholz M, Giese T et al (2005) Preferential expression and function of Toll-like receptor 3 in human astrocytes. J Neuroimmunol 159:12–19. doi:10.1016/j.jneuroim.2004.09.009
116. Farina C, Aloisi F, Meinl E (2007) Astrocytes are active players in cerebral innate immunity. Trends Immunol 28:138–145. doi:10.1016/j.it.2007.01.005
117. Krencik R, Weick JP, Liu Y et al (2011) Specification of transplanted astroglial subtypes from human pluripotent stem cells. Nat Biotechnol 29:528–534. doi:10.1038/nbt.1877
118. Emdad L, D’Souza SL, Kothari HP et al (2011) Efficient differentiation of human embryonic and induced pluripotent stem cells into functional astrocytes. Stem Cells Dev 21:404–410. doi:10.1089/scd.2010.0560
119. Serio A, Bilican B, Barnarda SJ et al (2013) Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of TDP-43 proteinopathy. Proc Natl Acad Sci USA 110:4697–4702. doi:10.1073/pnas.1300398110
120. Shaltouki A, Peng J, Liu Q et al (2013) Efficient generation of astrocytes from human pluripotent stem cells in defined conditions. Stem Cells 31:941–952. doi:10.1002/stem.1334
121. Mormone E, D’Sousa S, Alexeeva V et al (2014) “Footprint-free” human induced pluripotent stem cell-derived astrocytes for in vivo cell-based therapy. Stem Cells Dev 23:2626–2636. doi:10.1089/scd.2014.0151
122. Roybon L, Lamas NJ, Garcia-Diaz A et al (2013) Human stem cell-derived spinal cord astrocytes with defined mature or reactive phenotypes. Cell Rep 4:1035–1048. doi:10.1016/j.celrep.2013.06.021
123.Gattazzo F, Urciuolo A, Bonaldo P (2014) Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochim Biophys Acta 1840:2506–2519. doi:10.1016/j.bbagen.2014.01.010

124. Fukusumi H, Shofuda T, Kanematsu D et al (2013) Feeder-free generation and long-term culture of human induced pluripotent stem cells using pericellular matrix of decidua derived mesenchymal cells. PLoS One 8:e55226. doi:10.1371/journal.pone.0055226

125. Lam MT, Longaker MT (2012) Comparison of several attachment methods for human iPS, embryonic and adipose-derived stem cells for tissue engineering. J Tissue Eng Regen Med 6(Suppl 3):s80–s86. doi:10.1002/tterm.1499

126. Bandtlow CE, Zimmermann DR (2000) Proteoglycans in the developing brain: new conceptual insights for old proteins. Physiol Rev 80:1267–1290

127. Kwok JCF, Dick G, Wang D, Fawcett JW (2011) Extracellular matrix and perineuronal nets in CNS repair. Dev Neurobiol 71:1073–1089. doi:10.1002/dneu.20974

128. Rauch U (2007) Brain matrix: structure, turnover and necessity. Biochem Soc Trans 35:656–660. doi:10.1042/BST0350656

129. Cooke MJ, Zahir T, Phillips SR et al (2010) Neural differentiation regulated by biomimetic surfaces presenting motifs of extracellular matrix proteins. J Biomed Mater Res A 93:824–832. doi:10.1002/jbm.a.32585

130. Flaim CJ, Dick G, Wang D, Fawcett JW (2011) Extracellular matrix microarray for probing cellular differentiation. Nat Methods 2:119–125. doi:10.1038/nmeth736

131. Marchetto MCN, Mustri AR, Mu Y et al (2008) Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells. Cell Stem Cell 3:649–657. doi:10.1016/j.stem.2008.10.001

132. Holmqvist S, Brouwer M, Djelloul M et al (2011) Simultaneous two-photon imaging of oxygen and blood flow in deep cerebral vessels. Nat Med 17:893–898. doi:10.1038/nm.2394

133. Carreau A, El Hafny-Rahbi B, Matejuk A et al (2011) Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. J Cell Mol Med 15:1239–1253. doi:10.1111/j.1582-4934.2011.01258.x

134. Chen G, Guilbranson DR, Hou Z et al (2011) Chemically defined conditions for human iPS cell derivation and culture. Nat Methods 8:424–429. doi:10.1038/nmeth.1593

135. Liu Y, Mukhopadhyay P, Pisano MM et al (2013) Repression of Zeb1 and hypoxia cause sequential mesenchymal-to-epithelial transition and induction of aid, Oct4, and Dnmt1, leading to immortalization and multipotential reprogramming of fibroblasts in spheres. Stem Cells 31:1350–1362. doi:10.1002/stem.1382

136. Mathieu J, Zhou W, Xing Y et al (2014) Hypoxia-inducible factors have distinct and stage-specific roles during reprogramming of human cells to pluripotency. Cell Stem Cell 14:592–605. doi:10.1016/j.stem.2014.02.012

137. Correia C, Serra M, Espinha N et al (2014) Combining hypoxia and biomechanics boosts induced pluripotent stem cell differentiation to cardiomyocytes. Stem Cell Rev 10:786–801. doi:10.1007/s12015-014-9533-0

138. Holmqvist S, Brouwer M, Djelloul M et al (2015) Generation of human pluripotent stem cell reporter lines for the isolation and reporting on astrocytes generated from ventral midbrain and ventral spinal cord neural progenitors. Stem Cell Rev 11:786–801. doi:10.1007/s12015-014-9533-0

139. Halldorsson S, Lucumi E, Gómez-Sjöberg R, Fleming RMT (2015) Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. Biosens Bioelectron 63:218–231. doi:10.1016/j.bios.2014.07.029

140. Silva MM, Rodrigues AF, Correia C et al (2015) Robust expansion of human pluripotent stem cells: integration of bioprocess design with transcriptomic and metabolomic characterization. Stem Cells Transl Med 4:731–742. doi:10.5966/stemc.2014.0270

141. Ullian EM, Sapperstein SK, Christopherson KS, Barres BA (2001) Control of synapse number by glia. Science 291:657–661. doi:10.1126/science.291.5504.657

142. Faissner A, Pyka M, Geissler M et al (2010) Contributions of astrocytes to synapse formation and maturation—potential functions of the perisynaptic extracellular matrix. Brain Res Rev 63:26–38. doi:10.1016/j.brainresrev.2010.01.001