Neural Differentiation from Pluripotent Stem Cells

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Introduction

Significant progress has been made over the last 2 decades in understanding the molecular basis for neural development. This accrued knowledge has been translated into improved methodologies for generating subtypes of central nervous system (CNS) and peripheral nervous system (PNS) neurons and glia from pluripotent stem cells (PSCs). PSCs are thought to be equivalent to cells of the inner cell mass (ICM) of the early mammalian embryo which gives rise to the three germ layers and the entire embryo. All of the cells of the CNS and PNS originate from the neural tube, which is derived from the ectodermal germ layer. CNS development follows an orchestrated chain of events controlled by signaling cascades and region-specific transcription factor codes that convey spatial and identity to multipotent neural stem cells (NSCs). The PNS is more spatially diffuse than the CNS and, therefore, its development is not as tightly orchestrated. All PNS neurons and glia arise from neural crest stem cells (NCSCs) migrating out of the neural tube. Because embryonic stem cells (ESCs) and induced PSCs (iPSCs) are equivalent to the ICM, it stands to reason that PSCs could be differentiated into the various neural lineages under the proper culture conditions. Years of research in stem cell biology and neural development have buttressed this reasoning and yielded experimental protocols for the generation of neural cells from PSCs. As is the case in vivo, the protocols involve the serial diminution of differentiation potential beginning with PSCs being differentiated to multipotent NSCs, which in turn are differentiated to region-specific progenitors that are matured to terminally differentiated neural cells (Fig. 1). Positional information and maturity is governed by culturing cells with compounds that can activate or repress key signaling pathways that are active (or inactive) during neural development. The availability of markers specific to a particular neural lineage allows one to track what cell types are being produced during the in vitro differentiation protocols.

Generating Neural Stem Cells from Pluripotent Stem Cells

Several methods may effectively generate NSCs from PSCs with the first step involving dissociation of PSCs and growth in media lacking either fibroblast growth factor 2 (FGF2) for human PSCs or leukemia inhibitory factor (LIF) for mouse PSCs. The most common step following disaggregation involves either growing the cells in suspension culture as embryoid bodies (EBs) or in adherent monoculture with some protocols including the addition of bone morphogenetic protein (BMP) inhibitors to inhibit mesodermal differentiation while guiding PSCs along the neural default pathway [1–5]. Each of these procedures requires culture on substrates and in media formulations that will favor differentiation to the neural lineage. Once human or mouse NSCs form, culture in the presence of FGF2 allows for their proliferation and expansion [2, 3]. The ability to expand NSCs, freeze and thaw them, and grow them for 10–20 passages makes these cells very powerful tools in neural differentiation protocols.

Differentiation of Pluripotent Stem Cells to Generic Central Nervous System Neural Lineages

Early studies differentiating embryonic carcinoma cells to neural lineages suggested that retinoic acid (RA) induced posterior CNS markers [6]. The availability of more specific markers for neuronal subtypes revealed that this is indeed the

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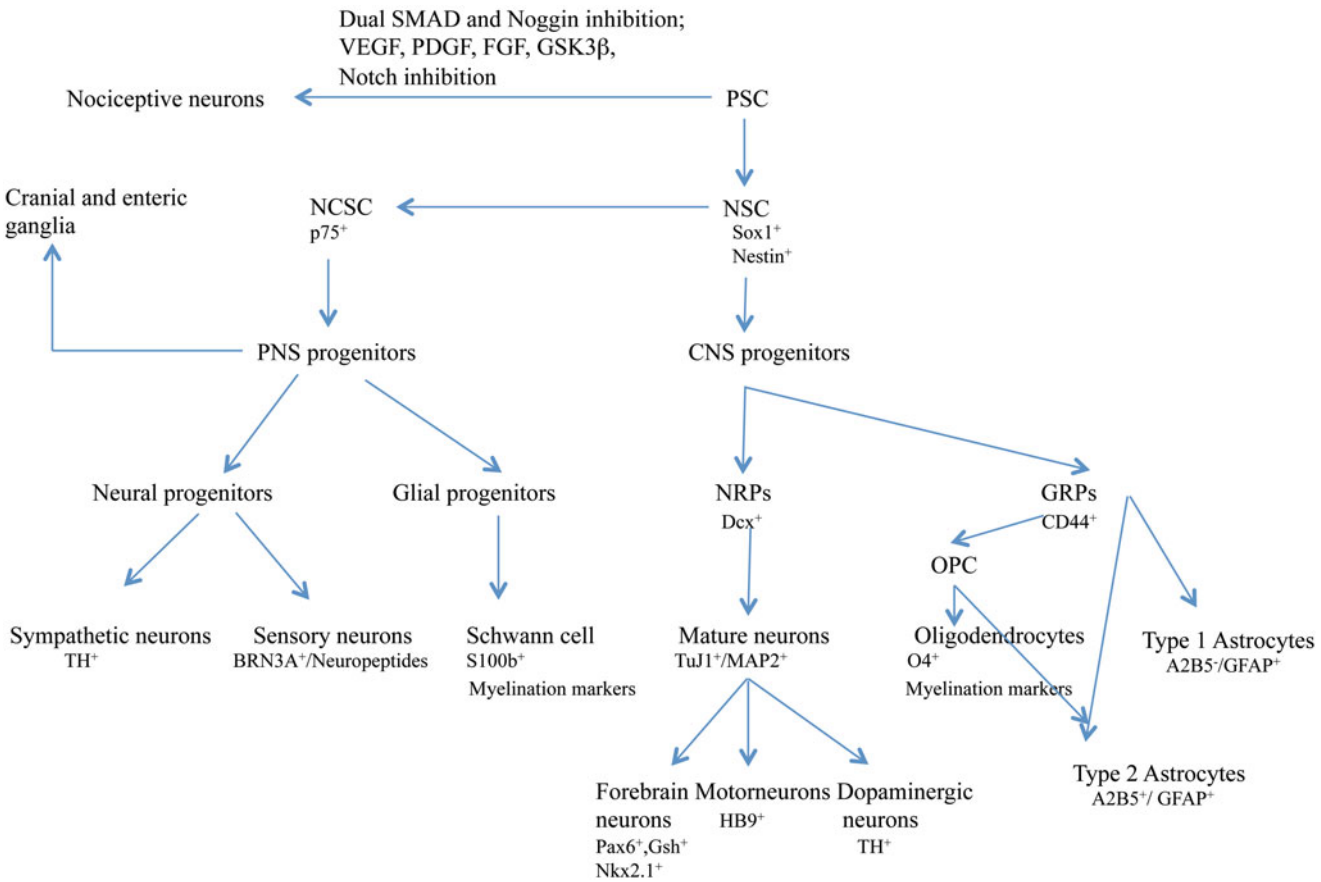

Fig. 1 The hierarchy of differentiation from a pluripotent stem cell (PSCs) to the neural lineages. Neural stem cells (NSCs) arising from a PSC can give rise to all of the neuronal and glial cell types of the peripheral nervous system (PNS) and central nervous system (CNS). The CNS progenitors can be subdivided to neuronal- and glial-restricted precursors (NRPs, GRPs). CNS GRPs also giving rise to an oligodendrocyte progenitor cell (OPC) that is bipotential in vitro. The PNS also has neuronal and glial progenitors that differentiate into mature neurons and glia. Markers used to identify a cell type are noted

case [7]. The original EB protocol enriches for the mesodermal lineage at the cost of ectoderm, but with the proper culture conditions this process can enrich for neural progenitors [8]. Bain et al. [9] added 500 nM RA during EB formation and found that it drove mouse ES cells towards a neuronal fate. Further characterization indicated that functional inhibitory and excitatory neurons were produced by RA induction [10, 11]. Some studies suggested that RA had the capacity to induce differentiation to both neurons and glia with an increased efficiency of glial differentiation upon the addition of 1 % fetal calf serum (FCS) and that the sequence in which the neural cell types appeared recapitulates in vivo development with neurons appearing first followed by glia [12–14]. RA appeared to act by inhibiting mesoderm specific genes and activating neuronal genes [15]. A detailed investigation of the effects of RA on ESC differentiation revealed that at 1–10 nM RA was permissive for mesodermal differentiation. However, at concentrations greater than 10 nM, more caudal progenitors started to be produced with spinal positional identity occurring at 1–10 nM RA [16]. As researchers gained new insights about cell culture medium additives and substrates for attachment that promoted neural differentiation of PSCs, RA was used only in those situations where caudal positional identity was desired [2]. All of the work mentioned above was performed in mouse PSCs, but RA has been found to have a similar affect in human cells.

Neural and Glial-Restricted Progenitors

Immunopanning was incorporated into the EB differentiation protocol to isolate neuronal-restricted precursors (NRPs) with the E-NCAM antibody [17]. These progenitors could be expanded with FGF2 and NRPs could be differentiated to neurons with FGF2 withdrawal and RA addition. Glial-restricted precursors (GRPs) were induced with the addition of platelet-derived growth factor (PDGF) and immunopanned with the A2B5 antibody. GRPs could be differentiated to mature glia with FGF2 withdrawal and addition of PDGF and triiodothyronine (T3). As is the case in vivo, the

differentiation potential of NRPs and GRPs was restricted to the neuronal and glial lineages, respectively. The ability to isolate NSCs and restrict their differentiation potential to neurons and glia opened up new opportunities for the directed differentiation of PSCs to specific neuronal and glial cell types.

Differentiation of NSCs to Specific CNS Neurons

Signaling pathways and transcription factors required for positional identity as well as many neuronal subtype-specific markers have been characterized to such an extent that it is possible to differentiate NSCs to many different neuronal types and then identify the subtypes that are present in the differentiated populations. CNS neurons develop from an NRP that has the capacity to generate a neuron at any dorsoventral or rostrocaudal position if cultured with the appropriate signaling molecule(s). The mature neurons can be identified with one or more markers that are unique to that subtype (schematized in Fig. 1). Differentiation of NSCs to forebrain, midbrain, and hindbrain/spinal cord neurons is described in the following sections.

Forebrain Neuron Differentiation

Dorsal/ventral patterning in the developing forebrain generates the dorsal-most pallium and the lateral, medial, and caudal ganglionic eminences (LGE, MGE, CGE) going from a dorsal to ventral direction. Wnts and BMPs are signaling molecules that dorsalize the forebrain while Sonic hedgehog (SHH) and FGF ventralize it [18]. Most neurons in the forebrain are excitatory pyramidal neurons derived from the pallial subventricular zone. Cortical inhibitory interneurons originate primarily from the MGE and CGE. Although neural progenitors derived from NSCs tend to be biased towards a rostral/cortical fate, additional knowledge about signaling molecules active in the neocortex has been used to develop differentiation protocols for more efficient production of specific forebrain neurons (Fig. 2).

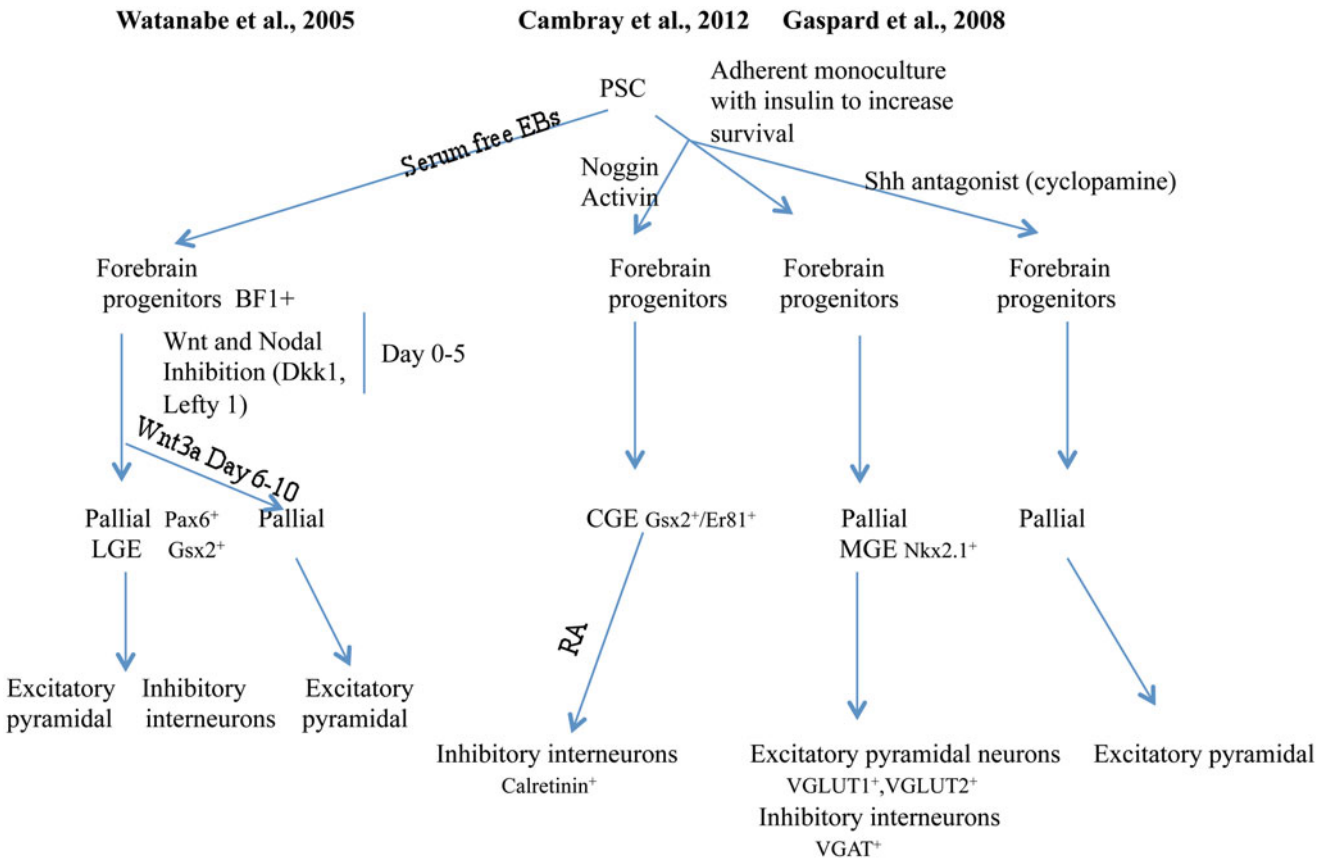

Fig. 2 Differentiation of forebrain neurons. Culture in serum-free medium as an embryoid body or in adherent monoculture can generate progenitors of the lateral, medial, and caudal ganglionic eminences (LGE, MGE, CGE). Upon further differentiation these precursors can become inhibitory interneurons or excitatory pyramidal based upon the factors in the culture medium. Publications which are summarized are listed at the *top* of the figure

Watanabe et al. [19] used a serum-free EB protocol to make NSCs from mouse ESCs that were enriched for forebrain progenitors. As Wnt and BMP signaling have an inhibitory effect during early neuralization, the Wnt inhibitor Dkk1 and the Nodal inhibitor Lefty1 were used to enrich for NSC. The resulting NSCs appeared to be biased towards forebrain progenitors. In contrast to its inhibitory role during the early stages of forebrain specification, Wnt signaling can promote pallial specification during later phases of this process [20]. Treatment with WNT3A at later stages of the differentiation protocol increased the number of pallial neurons (most likely pyramidal neurons) at the expense of LGE and MGE neurons. Conversely, SHH treatment at later stages of the differentiation protocol increased the number of MGE neurons while sharply diminishing pallial neurons. The temporal specification of neurons and effects of manipulating WNT, BMP, and SHH pathways were very similar to what occurs in vivo.

Adherent monocultures to produce NSCs from mouse ESCs yielded similar results [21]. As would be expected from developmental studies of the mouse neocortex, the number of excitatory pyramidal neurons could be increased in this protocol with the addition of the SHH inhibitor cyclopamine. The neurons were generated in a manner that faithfully reproduced the normal developmental profile in vivo and were functional as determined by transplantation studies in neonatal mice. A recent study with mouse and human ESCs found that in the monoculture protocol, addition of activin during progenitor differentiation and RA during neuron maturation resulted in the generation of calretinin$^+$ inhibitory interneurons that are derived from the CGE in vivo [22]. The authors claimed that activin promotes differentiation of NSCs by inhibiting an SHH-mediated mitogenic effect on forebrain precursors. Although RA caudalizes NSCs, its effects are context-dependent in progenitors derived from NSCs, and during the later stages of forebrain neuron development, it appears to synergize with activin to differentiate progenitors to specific mature forebrain neurons. In principle, forebrain neurons should be the easiest positional type to differentiate from NSCs and, as described above, much progress has been made in producing specific cortical subtypes from PSCs. However, because of the vast number of different cortical subtypes present, additional work remains to develop better methods for the controlled differentiation of PSCs to homogeneous subpopulations of these neurons.

Midbrain Dopaminergic Neuron Differentiation

Parkinson's disease (PD) is caused by a loss of midbrain dopaminergic (DA) neurons. Because of the clinical importance and interest in PD, there is a large body of work on differentiation of DA neurons from PSCs. DA neurons appear at the intersection of SHH and FGF8 signaling in rat neural plate explants [23]. The addition of ascorbic acid (AA) to rat midbrain cultures promotes the development of DA neurons [24]. This information along with general knowledge about neuronal differentiation was leveraged to design several protocols to differentiate PSCs to DA neurons (Fig. 3). Lee et al. [25] started with mouse ESCs, selected and expanded nestin positive NSCs from EBs, and induced differentiation through FGF2 withdrawal in a neuronal medium on polyornithine/laminin-coated plates. They observed the development of TH$^+$ DA neurons in 7–8 % of TuJ1$^+$ neurons. This frequency was doubled with the addition of SHH and FGF8 and further doubled if AA was also added. Kawasaki et al. [26] applied similar principles but with a different methodology to generate DA neurons. They grew mouse ESCs on PA6 cells, a stromal cell line derived from skull bone marrow. A very high proportion of the ESCs became NSCs and when differentiated further in culture medium supplemented with ascorbate ~30 % of the cells became TH$^+$ DA neurons. The stromal cell-derived inducing activity responsible for DA neuron differentiation has yet to be fully characterized. Growth on a PA6 stromal cell layer is also effective in generating DA neurons from human ESCs [27]. NSCs produced from human ESCs grown on a different stromal cell line, MS5, could be differentiated to DA neurons at very high efficiency with SHH and FGF8 treatment followed by culture in AA and brain-derived neurotrophic factor (BDNF) [28]. An EB method was also effective in generating dopaminergic neurons from human ESCs [29]. The one modification in this method from the similar mouse protocol was early addition of either FGF2 or FGF8 before FGF8/SHH combination treatment was used to generate midbrain DA progenitors. Similar protocols have been shown to be effective for scalable DA neuron production in defined xeno-free conditions from human ESCs and iPSCs [30, 31]. Importantly, a large proportion of the DA neurons generated in these studies were of the A9 subtype missing in PD patients. The ability to produce homogeneous populations of A9 DA neurons from human PSCs in defined xeno-free conditions will be critical for the effective development of cellular transplantation therapies for PD.

Spinal Cord Motor Neuron Differentiation

Pathways necessary for specification of spinal motor neurons (MNs) have been well defined for many years. MNs exhibit a columnar organization with subsets grouped according to the muscles they innervate. Diseases of MN dysfunction can differentially affect subtypes resulting in disease-specific spectra of movement disorders. The development of MNs can be divided into several steps including generation,

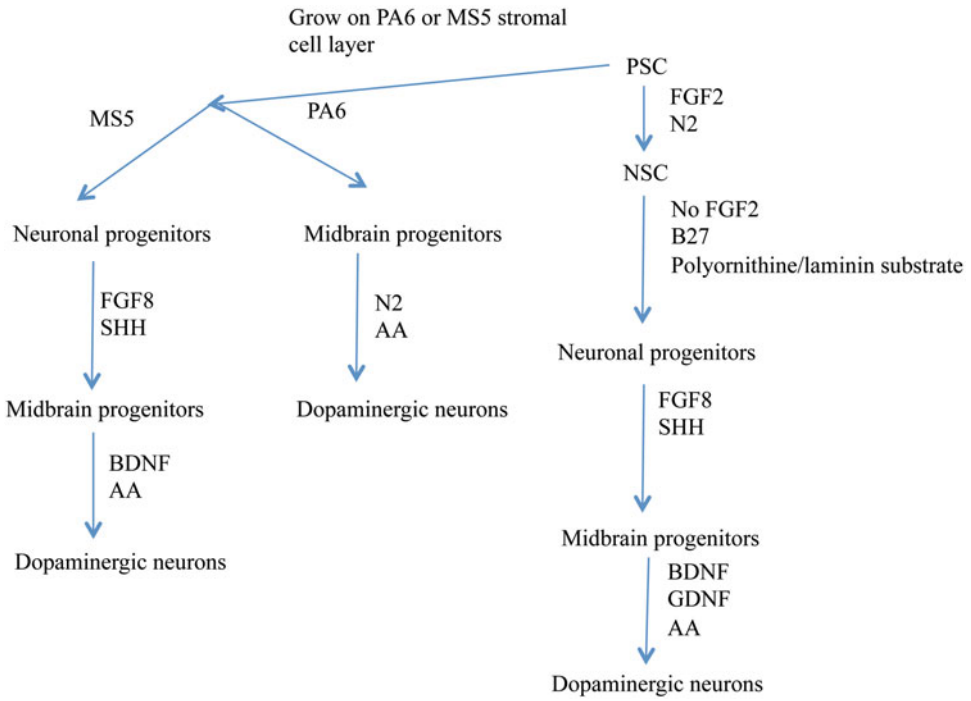

Fig. 3 Dopaminergic neuron differentiation from NSCs. The scheme for dopaminergic (DA) neuron differentiation from PSCs is shown with the two major protocols in which PSCs differentiate on a stromal cell layer or go through an embryoid body (EB) stage. The EB stage requires subsequent SHH/FGF8 treatment whereas one of the stromal co-culture protocols produces DA neurons without addition of FGF/SHH

subtype specification, apoptosis, and synaptogenesis. A dorsoventral SHH gradient specifies MNs in the ventral spinal cord early during vertebrate development. The gradient is translated into a transcription factor code that is required for the initiation of MN formation [32, 33]. Early pools of MNs pass through an apoptotic phase to fine-tune target neurons to the proper muscles. The final destination of the projections is under the control of a combinatorial LIM-homeodomain found within a particular MN. [34]. Protocols incorporating these principles have been utilized to successfully generate MNs from mouse and human PSCs.

PSCs are driven towards neuroectoderm by activation of the FGF pathway and inhibition of BMP signaling. These neuroectodermal cells are regionalized towards a caudal CNS position to become spinal progenitors, and these progenitors are ventralized by SHH to become MNs [32, 33]. This information has been utilized by several groups to differentiate mouse and human ESCs to MNs (Fig. 4). Wichterle et al. [35] treated EBs with RA and SHH to differentiate mouse ESCs to spinal motoneurons and ventral interneurons. The cells were directed towards MNs under high SHH concentrations and developed into ventral interneurons in moderate concentrations of SHH just as is the case in the developing embryo. The resulting MNs were restricted to a rostrocervical positional identity possibly because RA signaling pathways induced by the initial RA exposure continue to be active at later stages of differentiation and RA signaling is known to favor rostrocervical positions over thoracic and lumbar regions [36].

A similar differentiation paradigm was used to generate MNs from human ESCs except that FGF2 was used to derive NSCs from hESCs [37]. During differentiation of ESCs to NSCs, it was discovered that human NSCs, unlike mouse NSCs, have an early $Pax6^{+}/Sox1^{-}$ stage 8–10 days post neural induction protocol followed by a $Pax6^{+}/Sox1^{+}$ stage 14 days post-induction. The double positive cells were refractory to RA- and SHH-mediated MN induction. However, if $Pax6^{+}/Sox1^{-}$ NSCs were treated with RA/SHH, they developed into HOXC8 thoracic MNs and $ISL1^{+}/ISl2^{+}$ interneurons. This suggests that caudal identity is established at a very early stage in human NSCs and the possibility that FGF2 in combination with RA further caudalizes MNs. An enrichment protocol in which an HB9 enhancer drove GFP was used to purify MN progenitors with an RA/SHH differentiation protocol [38]. In addition to the enrichment step, this protocol differed from the others by growing human ESCs to confluence to form neuroepithelial rosettes. EBs were then made in the presence of RA and SHH and sorted for GFP^{+} MN progenitors which could be matured in the presence of neurotrophic factors. The result is a quicker protocol for the generation of functional, mature MNs. However, if MNs need to be generated from patient PSC lines, then routine use of reporters becomes cumbersome and expensive.

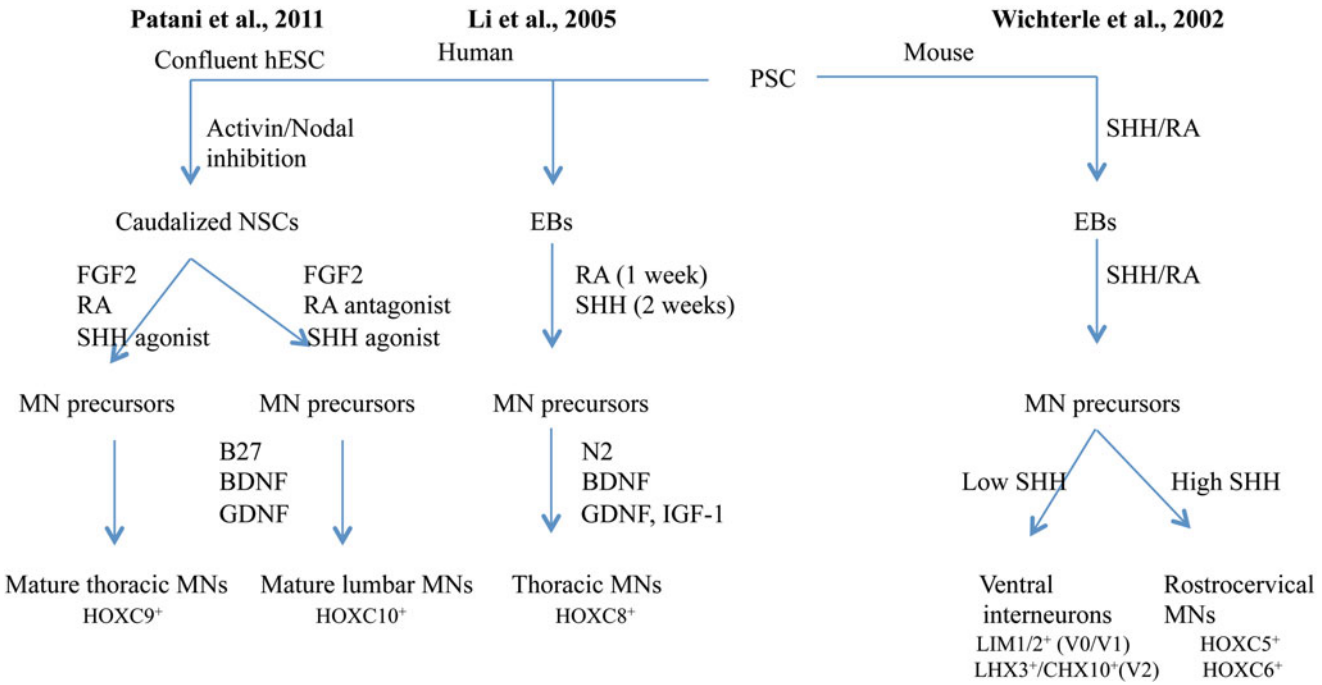

Fig. 4 Differentiation of motor neurons (MNs) from pluripotent stem cells (PSCs). Three standard protocols for generating MNs from mouse and human PSCs are outlined with a list of factors added to culture medium and markers used for analysis. Publications which are summarized are listed at the top of the figure

Several studies have shown that activin/nodal inhibition at the EB stage prevents differentiation to the mesendodermal germ layer and accelerates NSC formation with activin imparting caudal regionalization to the progenitor cells [39, 40]. Patani et al. [41] differentiated NSCs to MNs in the presence of the ALK4/5/7 inhibitor SB431542 with FGF2, the SHH agonist purmorphamine, and RA signaling antagonists. In terms of positional identity, there were significantly more HOXC10 MNs in the RA-independent group than in the RA treatment group, indicating that an RA-independent pathway leads to a more caudal lumbar positional identity. A significantly greater number of OLIG2$^+$ MN precursors and postmitotic MNs were found in the RA treatment group, indicating that work still needs to be done with additional small-molecule combinations to efficiently generate the entire suite of spinal MNs. Towards this end the overexpression of MN-specific transcription factors in the differentiation protocol has been used in an attempt to generate all spinal neurons. Adenoviral infection of human ESC- and iPSC-derived NSCs with the MN transcription factors LHX3 and ISL1 along with the neuronal specification factor NGN2 resulted in the generation of cervical and thoracic MNs 11 days post-infection [42]. The efficiency of MN neuron generation was similar to other protocols but the process occurred at a faster rate. Many research groups have now generated MNs from PSC with various modifications to the RA/SHH protocol, but improvements are still needed for production of the entire spectrum of hindbrain/spinal cord neurons.

CNS Glia Differentiation

Analogous to the way in which neurons are derived from NRPs, GRPs give rise to all the glia of the CNS [43]. The GRP can differentiate into either Type 1 or Type 2 astrocytes or to an oligodendrocyte progenitor cell (OPC). The OPC in turn has the capacity to differentiate into either Type 2 astrocytes or oligodendrocytes in vitro [44]. However, it is not clear if OPCs are bipotential or can only differentiate into oligodendrocytes in vivo [45, 46]. Studies with rodent fetal NSCs and cultures of rodent glial cells have yielded insights into how glia are formed. Knowledge gained from these studies has been transferred to protocols for the differentiation of CNS glia from PSCs.

CNS Astrocyte Differentiation

Astrocytes are the most abundant cell type in the CNS and fall into two classes. A2B5$^-$ Type 1 astrocytes arise only from tripotential GRPs, whereas in vitro A2B5$^+$ Type 2 astrocytes are derived from either GRPs or bipotential oligodendrocyte precursor cells (OPCs) [43, 44]. Immunopurified A2B5$^+$ cells from the rat spinal cord generated mature non-process bearing astrocytes if grown in medium with FCS [43, 47]. Exposure of GRPs to ciliary neurotrophic factor (CNTF) resulted in less mature process

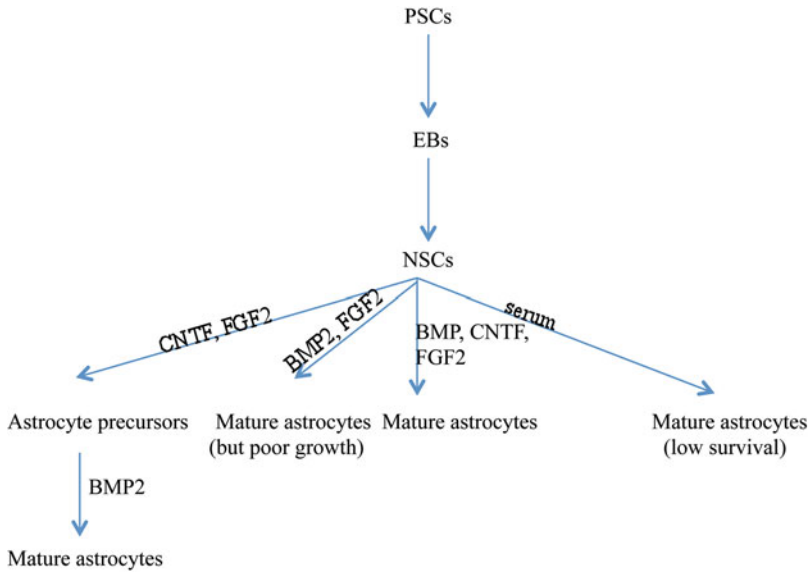

Fig. 5 Differentiation of PSCs to astrocytes. Once NSCs are produced from PSCs, the addition of various factors can lead to the generation of either astrocyte precursors or mature astrocytes. CNTF addition produces astrocyte precursors but mature astrocytes can be made if BMP2 is also added to NSCs

bearing astrocytes. Treatment of primary cells from mouse astrocyte precursors or GRPs from rat spinal cord with BMPs also resulted in the generation of a mature astrocyte population [47, 48]. Cardiotrophin-1 promotes astrocyte differentiation of fetal mouse neuroepithelial cells through the signal transducer and activator of transcription 3 (STAT3) which is also downstream of CNTF signaling [49]. CT-1 synergizes with BMP2 in the differentiation of astrocytes from neuroepithelial precursors [50]. STAT3/CNTF-independent induction of astrocyte fate occurs when the Notch pathway is activated in adult rat multipotent progenitor neurons [51].

The wealth of information regarding factors promoting commitment to the astroglial fate has resulted in the development of several protocols for the differentiation of PSCs to astrocytes (Fig. 5). Krencik and Zhang [52] developed a method in which NSCs were generated from PSCs and then grown in serum-free medium with supplements until the progenitor stage. At this point EGF, FGF2, and CNTF were added until the cells were committed to the astrocyte fate, at which point the cells were matured with CNTF. This method requires ~4 months at the end of which most of the cells are mature, functional $GFAP^+$ astrocytes. Emdad et al. [53] were able to differentiate PSCs to astrocytes with similar culture conditions using only CNTF over a 5-week period. Approximately 80 % of the astrocytes were $GFAP^+$ and there were no additional benefits to including CT-1 or Notch activators in the culture medium. Newer protocols incorporating CNTF, FCS, BMPs, and other factors shown to be important for astrocyte development need to be tested so that pure populations of mature astrocytes can be generated in a timely manner.

Oligodendrocyte Differentiation

Oligodendrocytes are one of the last CNS lineages to develop in vivo. Oligodendrocyte precursor cells (OPCs) arise from the caudal portion of the neural tube with ventralization by SHH driving OPC production [54]. In vivo OPCs rapidly migrate throughout the brain and initiate myelination. PDGF and T3 are important factors in OPC proliferation and differentiation. PDGF is required to maintain OPCs in a proliferative state and T3 promotes differentiation towards mature oligodendrocytes [55–58]. OPCs isolated from rat optic nerve differentiate to oligodendrocytes at the expense of astrocytes if grown in serum-free medium [44]. Generation of oligodendrocytes from PSCs generally involves making NSCs and then transiting NSCs through an OPC phase by addition of the appropriate growth factors. Growth factor removal allows progenitors to develop into mature oligodendrocytes (Fig. 6).

The first report of the differentiation of CNS glia from mouse ESCs found that growth in serum-free medium with PDGF resulted in the production of a mixed population of glial cells after short-term withdrawal of PDGF although prolonged withdrawal promoted greater differentiation to oligodendrocytes [59]. Oligodendrocytes were also derived from mouse ESCs as “oligospheres” in a suspension protocol using serum in the culture medium with RA to caudalize NSCs and T3 to differentiate towards the oligodendrocyte lineage [60]. Both studies showed that when transplanted into rats or mice that were deficient in myelination, the transplanted cells could myelinate host axons. Another oligodendrocyte differentiation protocol from mouse ESCs used SHH and serum-free medium with both PDGF and T3 to induce differentiation to oligodendrocytes [61].

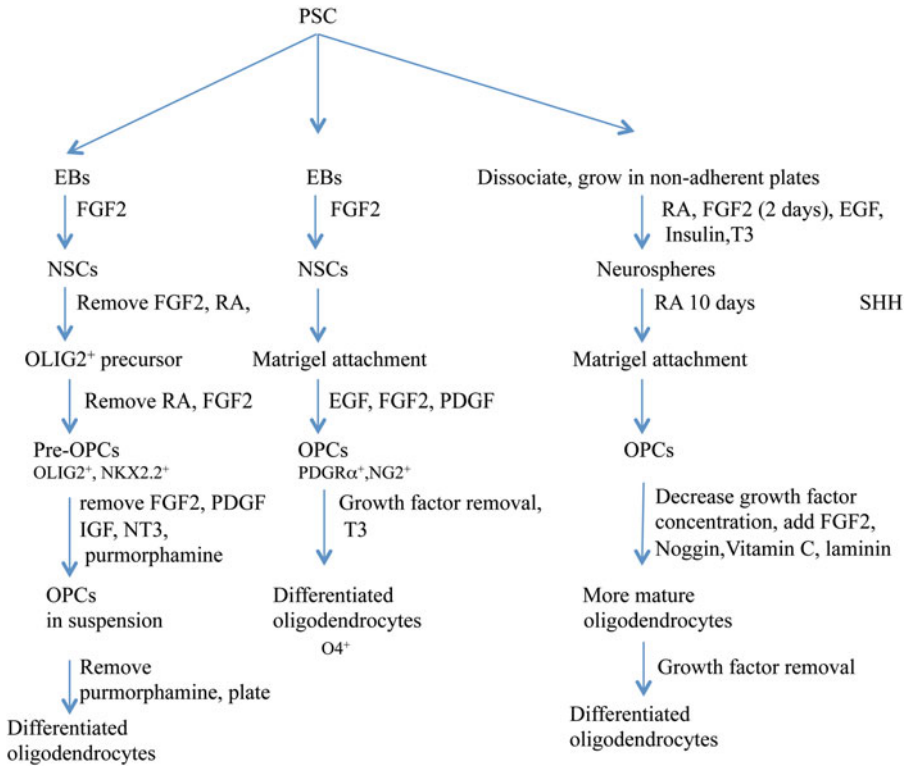

Fig. 6 Oligodendrocyte differentiation from PSCs. Three oligodendrocyte differentiation protocols are summarized with key steps, compound and growth factor additions to cell culture medium, and markers at key stages on route to mature oligodendrocytes identified

One of the first reports demonstrating differentiation of human ESCs to oligodendrocytes also made "oligospheres" in serum-free culture conditions with RA as a caudalizing factor and T3 to differentiate to mature cells over a ~40-day time period [62]. The resulting oligodendrocytes had the capacity to myelinate axons when transplanted into a myelination-deficient mouse model. Although the oligodendrocytes generated in this study matured when transplanted into a myelination-deficient mouse model, the authors found very few mature cells in vitro. Izrael et al. [63] used a similar sphere-based protocol but added noggin after RA treatment to induce expression of Sox10, which is required for terminal differentiation of oligodendrocytes. Exposure of OPCs to noggin at the appropriate time frame increased the yield of mature oligodendrocytes. Because OPCs are derived from OLIG2 precursors, a more recent protocol first generated these precursors from NSCs with RA and SHH treatment and then followed the in vivo development profile for oligodendrocytes by using previously described growth factor combinations to produce differentiated oligodendrocytes over a ~100-day differentiation protocol [64]. One of the critical steps in this protocol is the removal of FGF2 when transitioning from the pre-OPC to OPC stages. The inconsistent results seen from growing OPCs in suspension and the length of time needed to produce oligodendrocytes from PSCs highlights the need to develop new methods to differentiate human PSCs to mature oligodendrocytes.

Differentiation of Neural Stem Cells to PNS Neurons and Glia

PNS neurons mediate communication between organs and the CNS. The PNS is derived from NCSCs which themselves originate in the dorsal neural tube before undergoing an epithelial-mesenchymal transition and migrating throughout the embryo. As is the case for the CNS, there is some rostrocaudal positional identity linking the region from which the NCSCs migrate (cranial, vagal, trunk) to the types of cells that they can become [65]. Trunk NCSCs generate autonomic sensory neurons and Schwann cells and nearly all of the work performed to date has been on differentiating PSCs to these PNS lineages.

Several labs have developed methods to isolate NCSCs from PSCs and differentiate them to peripheral neurons and glia (Fig. 7). p75 has been established as a good marker for NCSCs and Lee et al. [66] observed extensive p75 staining at clusters surrounding the central NSC rosettes of cells differentiated from human PSCs. p75 FACS sorting isolated cells that were positive for multiple NCSC markers and could be

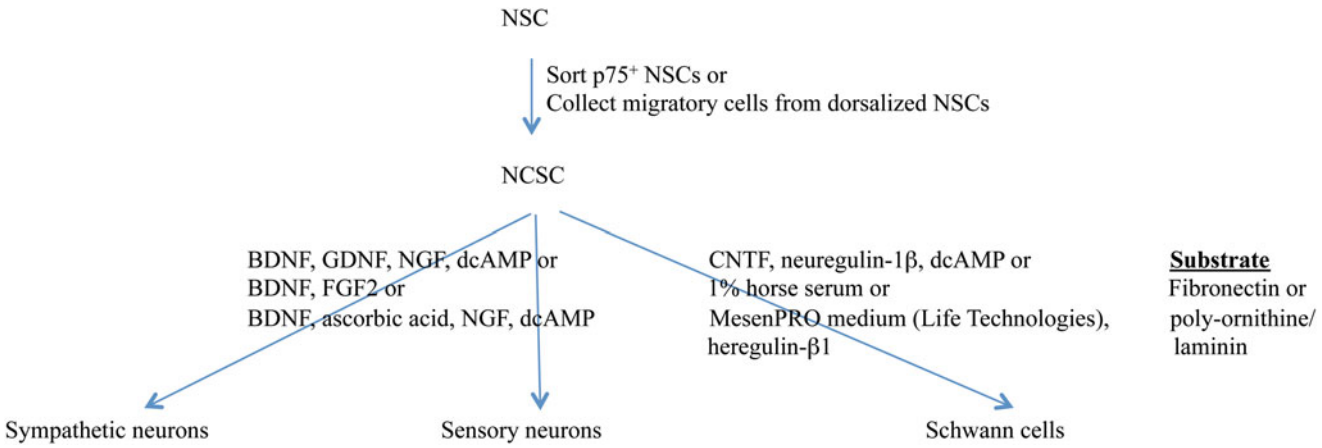

Fig. 7 Differentiation of neural crest stem cells (NCSCs) to peripheral nervous system (PNS) neurons and glia. The methods to isolate NCSCs from NSCs and factors used to differentiate them to PNS neurons and glia from three studies are summarized with substrates for neuronal and glial differentiation listed on the *right side*

differentiated to various neural crest lineages including PNS neurons and glia. The number of $p75^+$ cells increased significantly if rosettes were cultured in medium with FGF2 or BMP2. Withdrawal of FGF2 and EGF and addition of BDNF, GDNF, NGF, and dibutyryl cAMP (dbcAMP) resulted in the generation of PNS sympathetic and sensory neurons. Alternatively, if CNTF, neuregulin, and dbcAMP were added to the medium, Schwann cells developed. A similar p75 sorting strategy was used to isolate NCSCs except that EBs were grown in medium with FGF2 and AA and 50 % medium conditioned by the PA6 stromal cell lines to enrich for NCSCs prior to sorting [67]. PNS neuron differentiation was achieved with BDNF, AA, NGF, and dbcAMP and Schwann cells were generated when MesenPRO medium (commercially available from Invitrogen) and heregulin-β1 were used.

Another study enriched for NCSCs by culturing PSCs in FGF2, EGF, insulin, and nicotinamide and subsequently plating the cells on a fibronectin matrix [68]. This dorsalized the resulting NSCs with a migratory population of cells that were positive for NCSC markers. The migratory cells could be propagated on a Matrigel substrate and replated on a fibronectin substrate for differentiation to PNS neurons with FGF2 and BDNF and Schwann cells with 1 % horse serum [69]. PNS nociceptive neurons could be produced directly from NSCs grown in adherent monolayer culture with dual SMAD inhibition and three additional inhibitors [70]. These three compounds inhibited vascular endothelial growth factor, PDGF, and FGF (SU5402), glycogen synthase-3β (CHIR99021), and Notch signaling (DAPT). The differentiation occurred at a much faster rate than what happens in vivo with the neurons still exhibiting the functional properties of their in vivo counterparts. The results of this work suggest that small-molecule screens offer great promise in facilitating quick, high efficiency neural differentiation of PSCs.

Very little work has been done in differentiating to other PNS ganglia of cervical or vagal origin. Several studies have shown that enteric ganglia (vagal origin) can be differentiated from mouse or human PSCs. Treatment of EBs with BDNF resulted in the generation of cells that were positive for receptors and ligands expressed by enteric ganglia [71]. These neurons had a calcium signaling profile reminiscent of in vivo enteric ganglia. There have been no reports of differentiating PSCs to cranial ganglia, although differentiation to non-PNS cells from NCSCs migrating from cranial and vagal regions has been shown [68, 69]. These will not be discussed in any detail as they are beyond the scope of this chapter.

Differentiation to Retinal Pigmented Epithelium: Organoids from PSCs

Diseases of the visual system affect a significant percentage of the population. Because these diseases are often the result of degeneration of retinal pigmented epithelium (RPE) cells, the ability to generate RPE from PSCs offers hope for the treatment of these disorders. The earliest reports of generating RPEs from PSCs found that ~8 % of primate ESCs differentiated to RPE [72]. In the first paper describing RPE differentiation from human ESCs, the ESCs were grown to confluence in the absence of FGF2, and RPE appeared spontaneously if the cells were continuously grown for 6–9 months [73]. Generation of EBs from mouse ESCs with WNT and Nodal inhibitors and subsequent addition of activin and serum resulted in the appearance of retinal progenitors [74]. However, these progenitors did not differentiate into more mature RPE cells unless co-cultured on retinal cells. A defined differentiation medium was developed in which the addition of RA and taurine resulted in more efficient conversion of mouse, monkey, and human PSCs to

mature RPE [75]. WNT and nodal inhibitors are critical requirements for RPE differentiation as NSCs generated without these inhibitors failed to generate RPE. An exciting new development has been the ability to produce optic cups from both mouse and human PSCs using the medium described above in a three-dimensional cell culture system [76, 77]. The capacity to generate complex tissue structures with PSCs as a starting material provides hope for the development of a new class of regenerative therapies.

Conclusions

In this chapter we present a large database of knowledge demonstrating that differentiation of PSCs to neural lineages is technically feasible. Researchers can now generate CNS neurons found throughout the brain and spinal cord, and many studies show that these neurons are functional electrophysiologically in vitro and when transplanted into animal models in vivo. Great advances have also been made in differentiating CNS astrocytes, but further improvements need to be made in producing mature oligodendrocytes in vitro. Although differentiation to PNS neurons has not progressed as far as that in CNS, advances are being made. In contrast, it appears the protocols for making PNS glia (Schwann glia) are near full optimization. The capacity to generate such a wide array of neural cell types offers hope that cellular transplantation may be an achievable therapeutic objective for many nervous system disorders. One of the most promising tissue types under investigation is the eye, with the possibility that both cells and tissues can be produced from PSCs. Future work needs to focus on fine-tuning existing methods to increase purity and yields of the neural populations of interest, mining the available knowledge on neural development, and utilizing chemical screens to develop protocols for the differentiation of neural cell types and subtypes which have not been successfully derived from PSCs.

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