Review Article • DOI: 10.2478/s13380-012-0041-x • Translational Neuroscience • 3(4) • 2012 • 314-319

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Translational Neuroscience

BUILDING AUTHENTIC MIDBRAIN DOPAMINERGIC NEURONS FROM STEM CELLS - LESSONS FROM DEVELOPMENT

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Abstract

The challenge with controlling the differentiation of human pluripotent cells to generate functional dopaminergic neurons for the treatment of Parkinson's disease has undergone significant progress in recent years. Here, we summarize the differences between newer and older protocols for generating midbrain dopaminergic neurons from human pluripotent stem cells, and we highlight the importance of following developmental pathways during differentiation. The field has now developed to a point where it is timely to take human pluripotent stem cells one step closer to clinical use, and cell criteria to be fulfilled for such developments are outlined in this review.

Keywords

• Stem cells • Dopaminergic neurons • Differentiation • Development

Received 02 October 2012
accepted 15 October 2012

Cell replacement for neurodegenerative diseases

Cell replacement strategies, designed to substitute lost neurons by implantation of new functional cells, is a promising therapy for neurodegenerative diseases. For example, pioneering fetal cell transplantation trials in Parkinson's disease (PD) patients have been performed, and these studies provide proof-of-principle that neuronal replacement can also be effective in the disease-affected human brain. The grafted neurons were able to survive, reinnervate the striatum, and normalize striatal dopamine (DA) release leading to substantial clinical benefits, and in some patients even allowed for withdrawal of L-DOPA treatment [1-3]. The main roadblock identified in these clinical trials has been the lack of tissue availability and the heavy logistics associated with the need to access cells from fetal donors. This material is also difficult to standardize and quality control before implantation. Further development of this approach, therefore, will critically depend on the development of alternative sources of therapeutically effective cells derived from stem cells or other renewable cell sources.

Considerable progress has recently been made in the development of protocols that can generate transplantable and fully functional subtype-specific neurons from human pluripotent stem cells (hPSC). In this review we describe recent advancements in neuralization of hPSC with focus on the differentiation of human embryonic stem cells (hESC) into mesencephalic DA (mesDA) neurons. The achievements described here point towards developing hESC for an effective restorative therapy by neural transplantation in PD.

Previous and current strategies of hESC neuralization and patterning

Since the breakthrough of hESC derivation by Thomson and collaborators in 1998 [4], much research effort has been conducted towards developing strategies for directed differentiation of pluripotent stem cells into specific cell lineages and mature cell types. For neuronal differentiation, three main strategies for neuralization have been devised: Embryoid-body based differentiation, feeder-based differentiation and monolayer differentiation with dual SMAD inhibition [5-7]. As the protocols have gradually been refined, it became possible to generate almost pure cultures of proliferating neural stem cells (NSCs) through rosette isolation and/or neurosphere passaging [7,8]. Neural cells generated through these methods have almost exclusively consisted of progenitors positive for the marker PAX6, and it was hypothesized that the multipotent features of the PAX6$^+$ cells allowed for flexible patterning of the cells into various regional subtypes of neurons. For patterning into mesDA neurons, many protocols used sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8) [8], as these factors are expressed in the developing mouse midbrain and have been shown to hold an important role for midbrain DA formation in mouse explants [9]. However, despite these protocols giving rise to abundant numbers of neurons *in vitro* expressing the DA-producing enzyme tyrosine hydroxylase (TH), the cells did not behave as expected after grafting into the rodent brain. The grafts contained few or no TH$^+$ neurons, they had a propensity to form neural overgrowths or even teratomas, and they performed poorly in animal models of PD [10-15]. These studies hampered further clinical translation of the existing differentiation protocols, and they emphasized that evidence for functionality of DA neurons *in vitro* is not sufficient to predict their function *in vivo* (see also Box A).

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Building mesDA progenitors through developmental pathways

The lack of success in generating functional DA neurons of a mesencepahlic identity was explained when it was revealed that mesDA neurons have their cellular origin from the midbrain floor plate rather than from the neuroepithelium and that the floor plate transcription factor FOXA2 is an important gene necessary for correct mesDA specification and maintenance [16-19]. In rodents and humans, FOXA2 is expressed in the most ventral domain of the midbrain including the floor plate cells; in a region, which does not contain PAX6$^+$ progenitors [20], Figure 1.

The unexpected finding of floor plate origin of DA neurons in the midbrain provided a new approach for generating authentic mesDA neurons via their developmental pathways. This led to the emergence of refined differentiation protocols aiming to generate progenitors of a floor plate origin rather than of a PAX6$^+$ origin. Although previous protocols had exposed cells to SHH, a ventralizing morphogen secreted by the notochord and floor plate [21], it has now become clear that the commonly used recombinant N-terminal domain of SHH is not sufficiently potent to ventralise cells into a floor plate fate *in vitro* [12,22,23]. Instead, complete ventralisation requires chemical SHH agonists and/or a modified form of SHH, in which the C-terminal cysteine of the peptide has been replaced by two isoleucines (SHH-C24II) to mimic the hydrophobicity of endogenous palmitoylation [12,22,23]. Additionally, early patterning is crucial, as efficient floor plate conversion requires SHH activation within the first 2-3 days of differentiation [23], Figure 2.

Although potent SHH activators have solved the problem of incomplete ventralisation, their use alone cannot induce midbrain floor plate fates from pluripotent cells, but rather result in anterior ventral fates of telencephalic or diencephalic origin in the absence of any posteriorizing agents [23,24]. Thus, the anterior-posterior positioning of cells to midbrain fates must be controlled by other means in order to yield progenitors expressing

BOX A. The deceit of TH expression

The enzyme tyrosine hydroxylase (TH) is a required enzyme for DA synthesis, and is often used as a key marker of DA neurons. Although absence of TH expression clearly excludes DA identity, its presence alone is not sufficient to determine a DA neuron phenotype. For example, noradrenergic (NA) neurons rely on TH expression [35], and the enzyme has also been found in subpopulations of GABAergic neurons of the striatum, olfactory bulb and cortex [36-39].

In addition to this, several studies have revealed that TH expression can be influenced by various external factors applied to neurons in vitro. Striatal cultures, which are normally devoid of DA neurons, can be induced to express TH in a high proportion of neurons when exposed to factors such as aFGF, FGF1, FGF2, BDNF, DA, activin and serum [40-43]. Importantly, the TH induction in these neurons is transient and it often occurs in cells, which also express markers of GABAergic neurons (i.e. GABA and Gad67).

The plasticity of TH expression in forebrain cultures *in vitro* may reflect the fact that some forebrain neurons transiently express TH during development [39,44,45]. It is possible that this transient neuronal state may be reinforced or captured by certain external factors, giving rise to apparent dopaminergic neurons in cell cultures.

Therefore, when aiming to produce neurons for cell replacement in PD, DA identity of TH expressing cells produced *in vitro* should always be confirmed using additional markers of mesencephalic cells and dopamine neurons coupled with the absence of GABA.

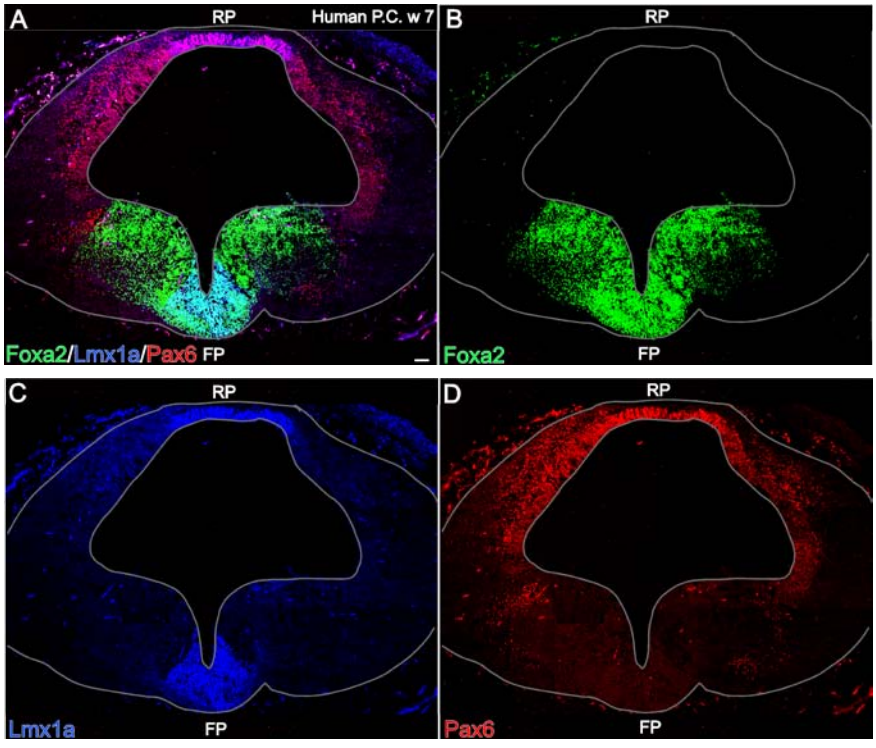

Figure 1. Expression profiles of the developing human midbrain. Images show a cross section of the human fetal midbrain, post-conception (P.C.) week 7, stained for PAX6 (red), LMX1A (blue) and FOXA2 (green). Dopaminergic neurons are born in the most ventral part of the midbrain expressing FOXA2 and LMX1A, whereas PAX6 is expressed only in the lateral and dorsal domains. LMX1A is also expressed in the roof plate, but here it does not co-label with FOXA2. RP: roof plate, FP: floor plate.

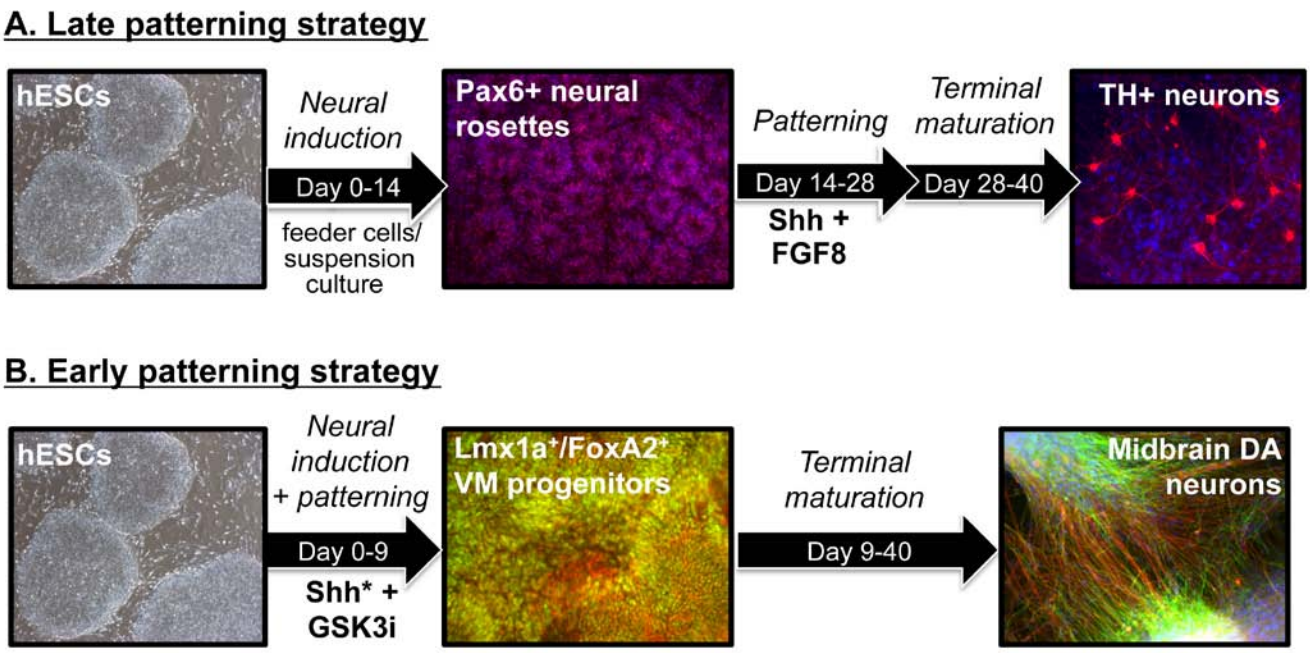

Figure 2. Overview of new and previous differentiation strategies: Late patterning versus early patterning. A. Previous neural differentiation strategies involved an initial neuralisation of the pluripotent cells to generate pure cultures of PAX6$^+$ neural progenitor cells. Once these cultures were established, the cells were patterned with growth factors relevant for the neurons in question, in the case of DA neurons, SHH and FGF8 were applied. After a patterning phase of about 14 days, the cells were terminally differentiated in the presence of maturation factors to yield TH$^+$ neurons [8]. B. Newer differentiation strategies involve early patterning of the cells, beginning already at the stage of pluripotency, in order to control neural progenitor fates. Patterning towards ventral midbrain is efficiently achieved with a high-potency form of SHH (SHH*) or SHH agonists together with GSK3 inhibition [12,24]. Once the correct midbrain progenitor cells are obtained – already after 9-10 days, the cells will spontaneously form mesDA neurons after grafting or when kept under neuronal maturation conditions.

not only FOXA2, but also midbrain markers such as LMX1A and Engrailed 1 (EN1). Previous protocols have used FGF8 as a caudalising agent, however, newer differentiation protocols have shown that FGF8 only poorly induces midbrain phenotype from ESC, and that it is in fact dispensable for patterning of mesDA neurons [12,24]. Although initial chick studies showed a potent midbrain-inducing effect of FGF8 [25], later studies in the chick and mouse have clarified that FGF8 is an activator of hindbrain genes and not of midbrain genes [26,27]. FGF8 thus plays an indirect role in midbrain specification through suppression of OTX2 and activation of GBX2, which consequently leads to midbrain patterning though formation of the midbrain-hindbrain boundary and WNT1 production from the isthmic organizer (IsO). An additional pitfall of FGF8 usage is the fact that FGF8 also plays a role in induction of TH$^+$ neurons from the forebrain [9], and this is not desirable in mesDA differentiation paradigms (see also Box A).

Recent alternative strategies have obtained efficient midbrain patterning by early chemical inhibition of glycogen synthase kinase 3 (GSK3) during differentiation [12,24]. Although the molecular mechanisms behind this effect remain to be resolved, GSK3 inhibition is known to activate canonical WNT signaling [28], and this likely constitutes an important component of the midbrain patterning effect. WNT signaling is involved both in very early anterior-posterior patterning of the neural plate and also in later midbrain maturation, mediated by WNT1 originating from the IsO [29]. The fact that increasing levels of GSK3 inhibition causes increasing caudalisation of hESC-derived neural cells [24], indicates that GSK3 inhibition mimics the early gradient of WNTs secreted from the paraxial mesoderm, which functions to induce progressive and gradual caudalisation of the neural plate [30], Figure 3.

The combination of potent SHH activation and early GSK3 inhibition allowed for efficient derivation of ventral midbrain progenitors, which were PAX6-negative, and instead expressed a unique combination of markers only found in the midbrain floor plate: FOXA2, LMX1A, EN1 and CORIN. These progenitors have been transplanted in two separate studies, in both cases yielding high numbers of mesDA neurons in the grafts and producing efficient reversal of motor symptoms in animal models of PD [12,24]. Although previous studies have verified the expression of Engrailed and LMX1A in the patterned neural cultures, it is important to keep in mind that these two markers are also expressed in roof plate cells [31,32], Figure 1. The expression of FOXA2 and the survival and function of the cells *in vivo* distinguishes these ventral midbrain-derived DA neurons from their PAX6-derived counterparts. The latter have since been shown to express islet 1 (ISL1) or PAX6 in the mature neurons [22], indicating a ventral thalamic DA phenotype rather than a ventral midbrain phenotype [33], which may explain their poor function *in vivo* [12,34].

Moving towards clinical translation

With the new protocols generating a large number of authentic mesDA neurons that survive well and function *in vivo* without forming tumours or generating neural overgrowths, it is now both realistic and timely to generate hESC-derived progenitors that are suitable for clinical use. Such cells need to fulfill basic safety criteria as outlined in Cooper et al. *Functional Neural Transplantation III. Primary and Stem Cell Transplantation for Brain Repair, Part 1* (Progress in Brain Research, Volume 200, in press). Additionally, the cell preparation needs to meet a set of well considered and stringent cell-specific criteria *in vitro,* as well as fulfill functional potency criteria in pre-clinical tests using animal models of PD (see Box B).

In vitro, the cell preparation should contain a high number of mesDA progenitors as determined by expression of FOXA2, LMX1A, OTX2, EN1 and CORIN expression, as we know that hESC-derived progenitors with these characteristics will differentiate into functional mesDA neurons after grafting into DA deinnervated striatum, whereas progenitors of a forebrain or hindbrain phenotype will not [24]. The cell preparation should not contain cells expressing markers of pluripotent cells or non-neural cells, and only a low number of non-DA progenitors, such as cells expressing PAX6, FOXG1 or GBX2. *In vivo*, there should be a complete absence of ES cell markers and a near complete lack of markers for cell proliferation once the graft has matured in the host brain. The cell preparation should result in good survival of fully mature DA neurons with a sufficient degree of efficiency. The grafted DA neurons should express markers of mature DA neurons (TH, DAT, VMAT2) as well as key A9 midbrain markers (PITX3, GIRK2), while not expressing markers of GABAergic neurons (see Box A and B). Additionally, extensive innervation of host striatum by TH-expressing fibers in rat or mouse models of PD should be observed and functional potency confirmed by the grafts' ability to release DA and to improve performance in behavioral tests at comparable levels to fetal midbrain cells.

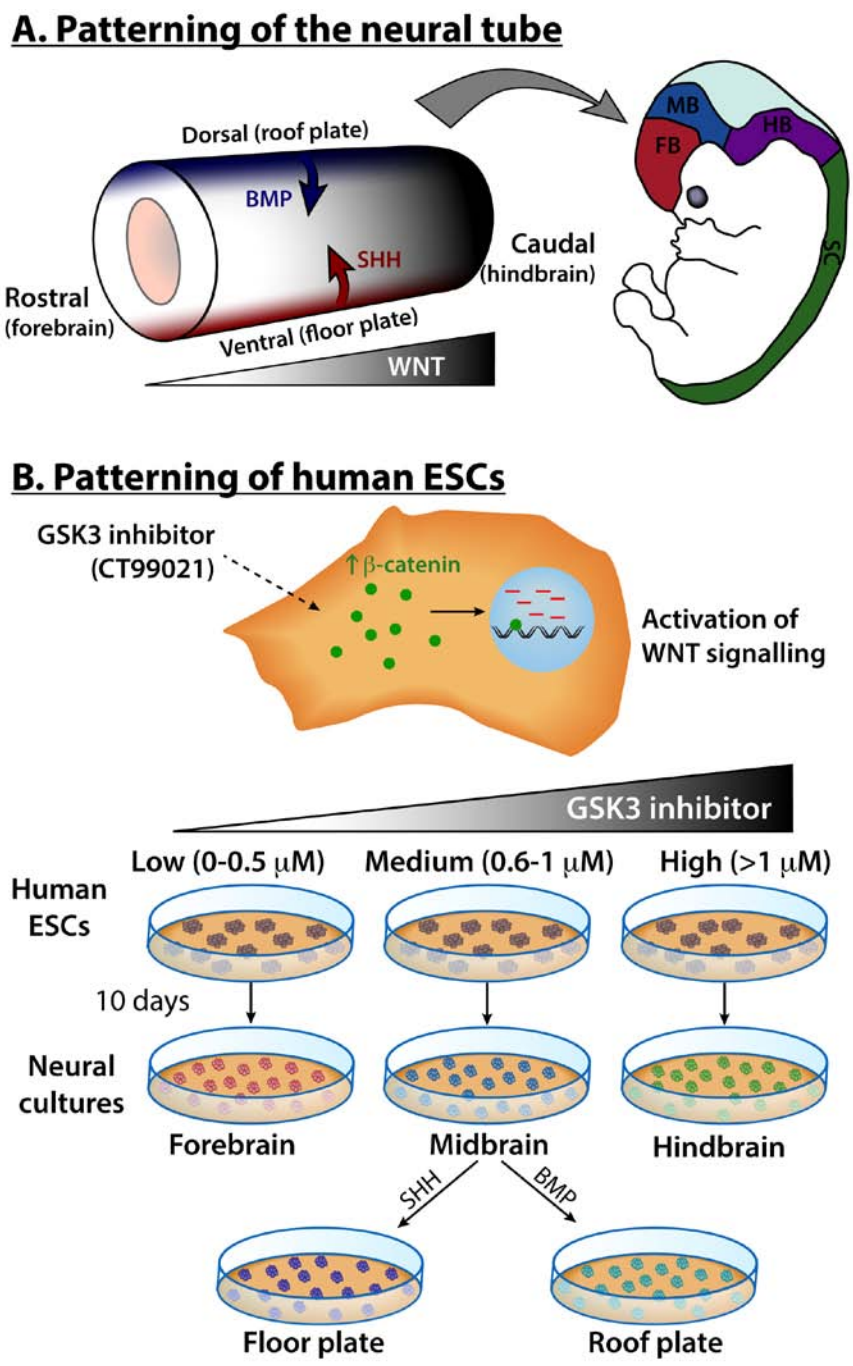

Figure 3. Hypothesis of dose-dependent WNT-mediated patterning of hESC. A. In the developing neural tube, rostro-caudal patterning is mediated through a WNT gradient secreted from the paraxial mesoderm, and dorso-ventral patterning is mediated through secretion of BMP from the roof plate and SHH from the floor plate. FB: forebrain, MB: midbrain, HB: hindbrain, SC: spinal cord. B. Chemical inhibition of GSK3 with the compound CT99021/CHIR99021 can dose-dependently activate WNT signalling in hESC, and thereby induce gradual rostro-caudal patterning of neural fates from forebrain to hindbrain; equivalent to the situation in the developing neural tube. The dorso-ventral identity of the neural fates can be further controlled by SHH and BMP signalling *in vitro* (summary of data shown in [24]).

Box B: Cell criteria for clinical translation

In vitro characteristics, progenitors:
- Expression of FOXA2, LMX1A, EN1 and CORIN
- Absence of pluripotency markers and low expression of non-neural markers
- Low numbers of rosettes and non-DA progenitors (i.e. PAX6$^+$, FOXG1$^+$, GBX2$^+$)

In vivo characteristics, grafts:
- Absence of pluripotency markers
- High proportion of neurons expressing TH, DAT and VMAT but not GABA/Gad67
- Expression of GIRK2 and PITX3
- Mature morphology and extensive innervation
- DA release and restoration of motor function

Conclusion

Forced patterning of the wrong progenitor cells may result in artifacts of unstable neuronal phenotypes that cannot survive transplantation or do not give rise to functional neurons *in vivo*. Recent differentiation protocols have shown the importance of following *bona fide* developmental pathways when directing the differentiation of ES cells towards specific subtypes of neurons in order to ensure that the neurons obtained posses an authentic and stable transcriptional profile, which will result in authentic and functional neurons after transplantation. It is now possible to generate authentic progenitors for DA neurons of a mesencephalic identity with high efficiency from hESC that function when transplanted into DA-deinnervated striatum. It is time to take the next step towards the clinical use of such cells by developing procedures allowing for large-scale production of cells under GMP conditions, while producing cells which fulfill the criteria outlined in this review to be used for pre-clinical potency and safety testing, and eventually for grafting in PD patients.

Acknowledgements

We would like to thank Jenny Nelander for supplying Figure 1 and our major funding agencies: Swedish Research Council, EU 7th framework funded project Neurostemcell and Lundbeck foundation.

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