Dopaminergic Differentiation Potential of Neural Precursor Cells Derived from Embryonic Stem Cells

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1. Introduction

One major question in mammalian development is how the diverse cell types of the adult organism are generated from the initial population of undifferentiated cells in the preimplantation embryo. As part of this diversification process, the variety of neurons of the adult brain is generated from neural precursors specified during gastrulation and the posterior neurulation phase. The specification of neural precursors has a definitive influence on the fate of these cells and, consequently, in the neuron type derived after differentiation. Embryonic stem cells (ESCs), pluripotent cells derived from the inner cell mass of the blastocyt, can be used as a source of different neuron types in vitro, and the process of neuronal differentiation can thus be studied from the earliest stages starting from naïve non-neural undifferentiated cells. In particular, differentiation of ESCs into dopaminergic neurons has attracted a lot of attention for the relevance it may have in the design of cellular treatments for Parkinson’s illness, a neurodegenerative disease characterized by the specific death of these neurons. The midbrain dopaminergic (mDA) neurons constitute about 75% of all dopaminergic neurons in the adult brain (Wallen & Perlmann, 2003). They are located in the ventral region of the mesencephalon where they are organized laterally in the retrorubral field (RRF) and the substantia nigra pars compacta (SNC), and medially in the ventral tegmental area (VTA). The SNC neurons project to the dorsal striatum, forming the nigrostriatal pathway involved in the control of voluntary movements, and degeneration of this group of mDA neurons provokes the characteristic symptoms of Parkinson’s disease (von Bohlen und Halbach, 2004). The neurons of the VTA project to the ventromedial striatum and the subcortical and cortical areas, forming the mesocortical limbic system, which is involved in emotional behaviors and mechanisms of motivation and reward. Misregulation of mesocortical limbic system has been involved in the development of drug addiction and depression (Kelley & Berridge, 2002), and contributes to certain symptoms of schizophrenia (Egan & Weinberger, 1997). Unlike SNC and VTA groups, RRF neurons have not been widely studied but it is known they project to the dorsal striatum and also connect SNC and VTA neurons (Arts et al., 1996). There have been extensive efforts focused on the generation of mDA neurons from ESC, however, the limited success in this regard is largely due to our still only basic
understanding of the molecular mechanisms underlying mDA neuron development. Presently, it is known that the morphogens Sonic Hedgehog (Shh), Fibroblast growth factor 8 (Fgf8) and Wnt1 (Ye et al., 1998, Prakash et al., 2006) must act on early neural precursors at a particular dose and specific time window, as a failure to do so reduces or inhibits the correct differentiation process. It is known that the sequential and combinatorial action of the morphogens activate at least two transcriptional factors, Lmx1a and Foxa2 that interact directly or indirectly to regulate the expression of other transcription factors promoting dopaminergic differentiation or inhibiting alternative neuronal fates (Nakatani et al., 2010, Lin et al., 2009, Ferri et al., 2007, Andersson et al., 2006). The complete potential of ESCs to differentiate into dopaminergic neurons is revealed once embryoid body (EB) cells derived from ESCs, are transplanted to explants of embryonic mesencephalon, where they efficiently and specifically respond to morphogens by differentiating and expressing the genes encoding the relevant transcription factors (Baizabal & Covarrubias, 2009).

Despite recent advances in understanding mDA development, the molecular interaction networks between the extrinsic and intrinsic factors involved are far from being fully understood. In the present chapter we will review how knowledge of mDA development has aided the refinement of differentiation protocols to generate mDA neurons from ESC and, how the ESC-based studies have contributed to understanding the mechanisms of differentiation into mDA neurons. We will discuss these data in the context of the differentiation potential of neural stem cells from different sources, the mechanism of stem cell specification, and the relevance that this has in defining the stem cell population and the differentiation conditions useful for the improvement of protocols for producing mDA neurons for the treatment of Parkinson Disease.

2. Development of midbrain dopaminergic neurons

Midbrain-DA neuronal differentiation initiates in the area rostral to the isthmus organizer and depends on the integration of anteroposterior and dorsoventral signals. These signals promote two related but distinct processes: specification of the general midbrain region, and specification of mDA precursor cells in particular (Fig.1). The distinction between these two processes is essential for the definition of the signaling cascade that leads to the generation of dopaminergic neurons.

The isthmus is formed at the mid-hindbrain border due to the complementary action of the Otx2 and Gbx2 genes in the anterior and posterior epiblast, respectively, at the end of gastrulation (E7.5) (Wurst & Bally-Cuif, 2001). Subsequently, Pax2 is expressed at the interface of the Otx2/Gbx2 domains, and Wnt1 in the Otx2-positive territories. The expression of Fgf8 starts at E8 at the caudal Gbx2 expression domain and later restricts to the isthmus at around E9, forming a mirror image of the Wnt1 expression domain. The activation of Fgf8 expression is controlled by Lmx1b which, directly or indirectly, is also important for maintaining expression of other isthmus genes such as Wnt1, En1/2, Pax2 and Gbx2 (Guo et al., 2007). The genes encoding the transcription factors En1/2 are expressed throughout the Otx2/Gbx2 expression domain interface; there, they are required for the maintenance of Fgf8 expression, though its early expression is independent of these two genes (Simon et al., 2004). Most of the morphogen activity of the isthmus is due to the products of Fgf8 and Wnt1 genes.

Crosstalk between the signals generated by Fgf8 and Wnt1 contributes to establish the identity and number of precursor cells committed to differentiate into mDA neurons (Lee et
al., 1997, Liu & Joyner, 2001, Matsunaga et al., 2002)(Fig. 1). In addition to their role in the formation of the midbrain, Fgf8 and Wnt1 seem to have a direct function in mDA neuron development. Fgf8 regulates Wnt1 expression in the isthmus (Chi et al., 2003) but an Fgf activity also controls Wnt1 expression in the ventral midbrain as revealed in double and triple mutant knockout mice for the genes encoding two or three of the Fgf receptors (Saarimaki-Vire et al., 2007). Importantly, Wnt1 is required for the ectopic induction of mDA neurons by Fgf8 in the forebrain and hindbrain (Prakash et al., 2006).

At the same time as the isthmus is forming, Shh from the notochord induces the establishment of the floor plate (FP) along the neural tube (Yamada et al., 1991) (Fig.1). Foxa2 expression in FP cells, via Shh activity-dependent Gli2 transcription factor binding sites in the Foxa2 promoter (Sasaki et al., 1997), is required for the formation of the FP (Matise et al., 1998). Foxa2, in turn, directly induces Shh expression in ventral domains of mesencephalon where is essential for the specification of ventral neural precursors (Jeong & Epstein, 2003). Fate-mapping experiments clearly establish that cells expressing Shh in the FP become mDA neurons (Ono et al., 2007). This is a a unique characteristic of mDA neurons, as at other regions along the ventral neural tube, FP cells are non-neurogenic (Joksimovic et al., 2009; Ono et al., 2007). As expected, lack of Foxa2 and the related transcription factor Foxa1 reduces the number of mDA neurons produced in the midbrain (Lin et al., 2009).

Lmx1a and Lmx1b are among the first markers that identify the mDA precursors and are key regulators of their differentiation. As with Fgf8 and Wnt1, Lmx1b has two separate roles; one is related to the formation and maintenance of the isthmus, as described above, and, consequently is essential for midbrain development; whereas the other seems to be directly involved in mDA neuron generation as Lmx1b expression is detected before emergence of mDA neurons (E7.5) and is maintained in the SNc and VTA until adulthood (Smidt et al., 2000). On the other hand, Lmx1a, whose gene is expressed in ventral midline of mesencephalon around E9, appears essential for mDA differentiation in the chick and its absence causes a reduction in dopaminergic neurons in the mouse (Ono et al., 2007, Andersson et al., 2006). Otx2 is necessary for the expression of Lmx1a in FP cells but, apparently, not for Lmx1b (Omodei et al., 2008, Ono et al., 2007). In addition, recent data suggest that Foxa2 is also required to induce and/or to maintain Lmx1a and Lmx1b expression (Lin et al., 2009). Therefore, Foxa2 and Otx2 may cooperatively function to define Lmx1a, and possibly also Lmx1b, expression in ventral midbrain precursors. Until now, it is not clear if Lmx1a and Lmx1b work in independent pathways or if they have redundant activities (see below).

Downstream of Shh signaling, Foxa1/2 inhibit the expression of the gene encoding Nkx2.2 (Lin et al., 2009), a transcription factor present adjacent to the Lmx1a domain at early stages, and later between the dorsal and ventral Nkx6.1 domains in a narrow band that eventually give rise to GABAergic neurons (Nakatani et al., 2007)(Fig.1). On the other hand, Nkx6.1 is present in the mDA domain at the beginning of Lmx1a expression, but later its gene is repressed by Msx1; the Shh secreted by the FP activates Msx1 expression in the ventral midbrain (Andersson et al., 2006). Thus, Nkx6.1 expression only remains in the regions adjacent to the Lmx1a domain, where motoneurons are generated (Fedtsova & Turner, 2001).

The generation of mDA neurons must be coordinated with the conversion of FP cells into neuronal progenitors. This transition might be due to the action of the canonical Wnt signaling, activated by Otx2 in the midbrain (Ono et al., 2007, Omodei et al., 2008)(Fig. 1). Around E10.5, Wnt1 suppresses Shh expression levels, possibly via an Msx1-mediated
mechanism (Joksimovic et al., 2009), and consequently, induces the expression of \( Ngn2 \), encoding Neurogenin 2, a panneural basic helix-loop-helix protein (Ono et al., 2007). Foxa1/2 apparently also contribute to the activation of \( Ngn2 \) expression (Ferri et al., 2007), which reinforces this mDA differentiation regulatory pathway. The induction of \( Ngn2 \) expression together with that of \( Mash1 \) mark the initiation of neurogenesis itself (Kele et al., 2006) (Fig. 1). Neurogenesis is immediately follow by the expression of \( Nurr1 \) and \( Pitx3 \), genes encoding transcription factors that are involved in defining the dopaminergic phenotypic characteristics such as the presence of tyrosine hydroxylase (the rate-limiting enzyme in dopamine synthesis; TH), vesicular monoamine transporter 2 (Vmat2) and dopamine transporter (DAT) (Zetterstrom et al., 1997, Smits et al., 2003, Saucedo-Cardenas et al., 1998, Kim et al., 2003b, Ferri et al., 2007) (Fig. 1). Also, \( Nurr1 \) and \( Pitx3 \) appear to play a role in the maintenance of the mDA neurons (Smidt et al., 2004, Nunes et al., 2003). Foxa1/2 function cooperatively with Lmx1a to regulate \( Nurr1 \) expression in immature neurons, and also directly activate \( Th \) expression in mature neurons (Fig. 1). Therefore, it is possible that \( Nurr1 \) and Foxa2 cooperate to regulate \( Th \) expression during mDA differentiation. En1 and En2, in addition to their participation in the establishment of the mid-hindbrain border at early stages, also play an important role in the survival of mDA neurons (Alberi et al., 2004, Simon et al., 2001, Simon et al., 2004).

Fig. 1. Molecular network controlling mDA differentiation. (A) Frontal view representation of the embryonic mesencephalon in which the distinct regulatory events during mDA neuron development are shown. (B) Interactions between the distinct determinants of the key events that lead specific mDA differentiation during development (represented by the same colors as in A). The dashed arrows represent indirect or unconfirmed regulations. IsO, Isthmus organizer; FP, floor plate.
3. Neuralization and specification of ESC

The capacity of ESC to generate all cell types present in the embryo has made them a potential source of different neuron types \textit{in vitro}, and so the process of specific neural differentiation can be studied since its very first stage and manipulated in an easier and faster manner than in embryos.

Ectodermal differentiation of ESC through EB formation is commonly used to produce neural precursor cells (NPCs) \textit{in vitro}. The formation of EBs reflects the early developmental stages that lead to the formation of the epiblast and the primitive endoderm, immediately previous to gastrulation. EB cells can be neuralized with retinoic acid and produce about 40\% of neurons of several types (Fraichard et al., 1995, Bain et al., 1995). Alternatively, EB cells can be cultured in a neural-defined medium, and the NPC population be expanded with Fgf2; under these conditions, more than 70\% of the total cells are NPC which can be differentiated into neurons by removing Fgf2 (Okabe et al., 1996). Retinoic acid not only induces neuralization, but also instructs the cells to posterior fates (Okada et al., 2004), which limits the neuron types that can be derived from EBs; this potential problem can be overcome by treating the cells with low doses of RA. In fact, endogenous RA appears to be required for the neuralization in the absence of a direct RA addition to the medium (Engberg et al., 2010). Bmp signaling represses neural fates \textit{in vivo} (Hemmati-Brivanlou & Melton, 1997, Finley et al., 1999); in concordance, Noggin and Chordin, two Bmp inhibitors, induce neural differentiation of EB cells (Grtatsch & O’Shea, 2002). Another condition that promotes neural differentiation of EB cells is to block the Wnt/\(\beta\)-catenin pathway by adding its inhibitor Dkk-1 (Verani et al., 2007) or in the absence of Wnt and Nodal in cultures of EB cells in serum-free conditions (Watanabe et al., 2005).

Neuronal differentiation of ESC can also be induced without forming EBs. One protocol involves culturing the cells at low densities in a chemically defined, serum-free medium with LIF and Fgf2 (Tropepe et al., 2001). The cells of the spherical colonies formed in these conditions express the neural marker Nestin, and can differentiate further into neurons and glia; of note is that even though all cells in the colonies are NPC, very few of the cultured ESC generate colonies (0.2\%). It has been proposed that the colonies represent primitive neuroectodermal cells, as similar colonies can be obtained from the embryo (Hitoshi et al., 2004). A similar protocol to generate neural precursors without the formation of EBs is to grow the ESCs as a monolayer at low density in serum-free conditions with N2B27 medium (Ying et al., 2003). After 4 days, 60\% of the cells express Sox1, one of the first markers of neuroectodermal cells and these Sox1-positive cells, can later differentiate into mature neurons. This latter protocol could represent a simpler and direct system to assess neural differentiation and specification as exogenous growth factors and the formation of EB are not required.

In order to generate a particular type of neurons, it is necessary not only to neuralize the cells but also to expose them to other factors that promote specific neuronal differentiation. For instance, treating EBs with RA (with neuralizing and posteriorizing activities) and Shh (with ventralizing activity) oblige the cells to differentiate into motor neurons of the spinal cord (Wichterle et al., 2002). On the other hand, neural cells generated after inhibition of Wnt and Nodal signaling acquire a telencephalic identity (expression of Foxg1), and these neural cells can later be differentiated into subpopulations of telencephalic neurons depending on whether Wnt1 (forming pallial telencephalic neurons Foxg1\(^{-}\)Pax6\(^{+}\)) or Shh (basal telencephalic neurons Foxg1\(^{-}\)Nkx2.1\(^{+}\)) is added to the culture medium (Watanabe et al., 2005).
The fact that, in different culture conditions that promote ESC neuronal differentiation, NPCs emerge prior to neuron formation (as determined by the expression of Sox1, Sox2 and Nestin), and that at least some of these NPCs respond to morphogenetic cues, suggest that ESC neural differentiation in vitro follows a comparable program to that occurring in vivo. Therefore, ESCs are useful as a model to study early neural differentiation.

4. Specification and differentiation of ESCs into mDA neurons

4.1 Response of ESC-derived cells to extrinsic determinants

Shh and Fgf8 were the first morphogens identified that are sufficient to induce the generation of ectopic mDA neurons in explant cultures (Ye et al., 1998). Expectedly, these morphogens were among the first used to induce mDA differentiation of ESC in vitro (Fig. 2). EB cells cultured in the absence of growth factors, and the enriched NPC population subsequently expanded in the presence of Fgf8 and Shh, produce between 10-20% of mDA neurons after allowing neuronal differentiation, in comparison with the 1-2% of mDA neurons produced in their absence (expressed as a percentage of the total cells alive at the end of the culture) (Rodriguez-Gomez et al., 2007, Lee et al., 2000, Hedlund et al., 2008, Kim et al., 2002). Although the addition of Shh and Fgf8 after EB formation increases the number of mDA neurons produced, the proportion of mDA neurons generated is still generally low. A similar proportion of mDA neurons are obtained when ESC are grown on a monolayer of PA6 cells (stromal cells derived from skull marrow) (Kawasaki et al., 2000), or when ESC neuralized directly in monolayer culture are treated with Fgf8 and Shh (Ying et al., 2003) (Fig. 2). It is apparent from these data that the population of cells capable of responding to specific environmental cues in culture is limited and/or the culture conditions lack the relevant signals present in the embryonic mesencephalic niche necessary for the efficient differentiation. Alternatively, the time window of action of each growth factor needs to be reproduced as in vivo in order to get efficient specific differentiation.

Although ESC are pluripotent and have the full potential to derive into any neuron type, common neuralization protocols might limit the cells ability to differentiate into dopaminergic neurons. This is supported by the fact that most ESC-derived NPCs are unable to respond to cues present in the niche of endogenous differentiation dopaminergic neuron differentiation (Baizabal & Covarrubias, 2009). Fgf8 and Shh do not alter the differentiation potential of the neuralized ESC and only induce the specific dopaminergic differentiation of a small proportion of cells. However, when non-neuralized EB cells are exposed to the embryonic mesencephalic environment, efficient and specific differentiation results (Baizabal & Covarrubias, 2009) (Fig. 2). Therefore, the time window that allows proper dopaminergic specification appears to be around the time at which the cells neuralize.

Since there is no certainty that the adult brain has the proper environment for specific differentiation, transplantation of EB cells at this stage is not the proper way to evaluate their differentiation potential. Nonetheless, differentiation into dopaminergic neurons has been achieved when EB cells are transplanted to the striatum of the adult brain, the target region of dopaminergic neurons of substantia nigra. As expected only few dopaminergic neurons are found several weeks after implantation, and those neurons do not apparently result from induction processes occurring in the adult brain. In fact, the proportion of dopaminergic and serotoninergic neurons is similar to that obtained in vitro or when the cells are transplanted to the kidney capsule (Bjorklund et al., 2002, Deacon et al., 1998).
Transplantation to the embryonic brain could provide a more adequate milieu for specific differentiation of ESC or their derivatives. ESC-derived neural precursors transplanted to the embryonic and neonatal brain efficiently differentiate into neurons and glial cells (Brustle et al., 1997, Wernig et al., 2004, Zhang et al., 2001). However, some ESC-derived neurons that expressed Th and En1 can be found outside the midbrain (Wernig et al., 2004) suggesting that the cells are already committed before transplantation. In the previous type of experiments, cells are transplanted to embryos at stages long after specification of endogenous dopaminergic precursors has completed (E13), and therefore, the environment may not be the best suited to the induction of the dopaminergic differentiation. In contrast, robust specification and differentiation results when EB cells are transplanted to explants of E10.5 mesencephalon, just 1-2 days after specification of the endogenous mDA NPCs. In this condition, nearly 70% of the cells acquire neuronal markers and, of the total transplanted cells, 65% express Lmx1a and about 40% Th (Baizabal & Covarrubias, 2009). Therefore, at least up to E10.5, all the signals required to instruct EB cells into different mesencephalic phenotypes are present.

Fig. 2. Schematic representation of diverse strategies used to generate mDA neurons from ESCs.
4.2 Intrinsic determinants

As more is discovered about the transcription factors that control dopaminergic differentiation, the possibilities to induce specific differentiation through the overexpression of one or more transcription factors have increased. It might be predicted that the ectopic expression of a master gene(s) will be sufficient to induce specific neuronal differentiation programmes. However, the success in the searching for such “master genes” in general has been rather low, perhaps due to the requirement for a specific combination of transcription factors to be expressed in a competent recipient stem cell. The ‘strength’ of a master gene can be defined as a function of its ability to promote specific differentiation of precursor cells from the earliest stages possible. In this sense, the ‘strongest’ master gene will be that which individually promotes the specific differentiation of undifferentiated ESCs. It is apparent that none of the transcription factors of the dopaminergic network have the ability to induce specific differentiation following expression in undifferentiated ESCs.

Overproduction of transcription factors acting at late stages of dopaminergic differentiation (dopaminergic determinants) have been tested in different cell culture systems. Overexpression of Nurr1 in embryonic NPC from distinct regions of the brain cultured as a monolayer or after forming neurospheres, induces TH expression (Kim et al., 2003a, Kim et al., 2007); however, other characteristics of dopaminergic neurons have not been reported. When overexpression of Nurr1 is induced in NPC generated from ESCs, TH and other dopaminergic neuronal markers, such as DAT, are detected, however, Nurr1 has no influence over transcription factors important for mDA specification (Martinat et al., 2006, Sonntag et al., 2004). Overexpression of Pitx3 in undifferentiated ESC or in ESC-derived neural precursors is not sufficient to induce the complete mDA phenotype, but promotes expression of a subset of markers, particularly Aldh2 (Martinat et al., 2006, Chung et al., 2005). Even Nurr1 and Pitx3 together are not sufficient to induce the complete mDA phenotype, and again, they only appear to influence terminal dopaminergic maturation (Martinat et al., 2006). The previous observations are in agreement with a direct role of these transcription factors in the expression of the \(\text{Th}^+\) gene and other genes that contribute to the dopaminergic phenotype. Nonetheless, an effect of Nurr1 on dopaminergic differentiation appears to occur when is constitutively overexpressed during the differentiation of ESC in the presence of Fgf8 and Shh as it enhances approximately four-fold the quantity of dopaminergic neurons generated (Kim et al., 2002).

Several factors relevant for the neuralization and specification of mDA NPCs have been overexpressed. Overexpression of Msx1 alone in ESC does not induce the generation of mDA neurons, though it is sufficient to induce Ngn2 expression as it appears to occur \textit{in vivo} (Andersson et al., 2006). In contrast, the overexpression of Lmx1a under the control of the Nestin promoter in ESC treated with Fgf8 and Shh causes an extensive increase in cells expressing \(\text{Msx1}\) and a reduction in those expressing \(\text{Nkx6.1}\) (Fig. 2). Also, there is a robust generation of immature neurons that express \(\text{Th}\), many of which also express additional genes encoding mDA neuronal markers, including \(\text{Lmx1a, Lmx1b, En1/2, Nurr1, Pitx3, Foxa2, DAT and Vmat2}\) (Friling et al., 2009, Andersson et al., 2006). This highly specific differentiation is observed under two distinct \textit{in vitro} protocols suggesting that the effects are due to the action of \(\text{Lmx1a}\) and not to particular culture conditions. In agreement with a key role for Lmx1a also in human mDA neuron development, overexpression of Lmx1a under the control of the nestin promoter in human ESC generated abundant TH+ cells (Friling et al., 2009). It is worth mentioning that Lmx1a and other transcription factors within the dopaminergic specification network are not able to promote specific
differentiation when overexpressed in neurospheres cells, suggesting that these NPCs are not competent to respond to these specification factors (Roybon et al., 2008).

In the previous protocols, the use of the nestin promoter to drive Lmx1a expression appears key for the efficient mDA differentiation of ESC-derived neural precursors. The exogenous Lmx1a initiates its expression at the time Nestin+ neural precursors first emerge in culture (Andersson et al., 2006, Friling et al., 2009), which may provide the right time window for the action of Lmx1a in which ESC-derived neural precursors are competent to specifically respond and generate mDA neurons. These data reinforces the idea mentioned above proposing that specification and neuralization are interdependent process that must occur simultaneously or within a narrow window during the differentiation process.

Recently, studies with ESC have revealed novel interactions among the different factors participating in mDA differentiation. Based on the transcription factor network determined during mDA development, different combinations of Foxa2, Lmx1a and Otx2 have been introduced into ESC-derived neural precursors and the effect on mDA differentiation determined. When genes encoding the three factors are ectopically expressed in the absence of Shh in the culture medium, robust synergistic induction of mDA markers is observed with the majority of TH+ neurons also coexpressing Lmx1b and Nurr1 (Chung et al., 2009). This highly efficient generation of mDA neurons is not observed when Foxa2 or Otx2 are introduced alone. Therefore, it is apparent that Lmx1a is the key specification determinant of mDA differentiation in ESCs that, in concert with Otx2, could give a midbrain identity to neural precursor cells. Foxa2 might be required to activate the Shh pathway relevant for efficient mDA differentiation.

Studies with ESCs have identified a Wnt1-Lmx1a/b loop relevant for the specification of mDA neurons. In ESC Wnt1 induces the activation of Lmx1a independent of both Shh and Otx2, whereas, Lmx1a and Lmx1b upregulate Wnt1 by binding directly to its promoter (Chung et al., 2009). This autoregulatory loop, in turn, directly regulates Otx2 expression, through the canonical Wnt signaling pathway, and also Nurr1 and Pitx3 expression through Lmx1a and Lmx1b. The Wnt1-Lmx1a/b loop also functions in the embryonic midbrain (Chung et al., 2009), demonstrating that this is not a culture artifact and that ESCs is a suitable system to study mDA differentiation. In this system, Lmx1a and Lmx1b appear to regulate each other, and the absence of one compensates the function of the other in the regulation of Wnt1, Nurr1, Pitx3 and Th expression (Chung et al., 2009).

5. Conclusions

Taking together the data from in vivo and in vitro studies, at least two major pathways appear to regulate mDA specification, which may be referred to as “Shh-Foxa2” and “Wnt1-Lmx1a/b” (Fig. 1). These pathways, to some extent, are independent, as Wnt1 or Lmx1a do not affect Shh or Foxa2 expression; although maintenance of Lmx1a expression is dependent on Foxa2 activity. At a later stage, these two pathways converge on Nurr1 and dopaminergic differentiation itself initiates.

Most stem cell-based differentiation protocols are focused on the addition of growth factors to the culture medium, in part because genetic manipulations are generally not desirable in the generation of cells for therapeutic purposes (Fig. 2). However, finding the right combination of growth factors and the time of action, as well as the competent stem cell population responsive to those factors is a difficult task. On the other hand, as we learn more about the transcriptional network that control specific fates, the use of “master genes”
becomes more attractive. Therefore, engineering ESC lines that overproduce transcription factor(s) required for mDA development, in addition to the relevant basic information provided for the understanding of the molecular mechanisms involved in dopaminergic differentiation, it presents a promising alternative to obtain authentic mDA neurons for treating Parkinson’s disease (Fig. 3).

It does not appear to be far the time at which there are one or several protocols to produce efficiently mDA neurons in vitro. However, the development of a cell therapy adequate for the treatment of patients suffering Parkinson’s disease will require more than a protocol to produce mDA neurons, considering that it is unlikely that the adult brain possesses the optimal environment for mDA NPC differentiation and neuron maturation. For instance, it will be relevant to determine the differentiation stage at which implantation and survival of the transplanted cells is more efficient, as well as to identify additional factors needed to reconstitute the nigrostral pathway.

Fig. 3. ESC-based therapies for Parkinson’s disease. ESCs are a potential source of mDA neurons useful in cell therapies for Parkinson’s disease. The efficiency to generate mDA neurons from ESCs is increasing as we learn more about the factors controlling mDA neuronal differentiation from studies with both embryos and ESCs.

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7. References


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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

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