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PRIMER

How to make a midbrain dopaminergic neuron

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ABSTRACT

Midbrain dopaminergic (mDA) neuron development has been an intense area of research during recent years. This is due in part to a growing interest in regenerative medicine and the hope that treatment for diseases affecting mDA neurons, such as Parkinson's disease (PD), might be facilitated by a better understanding of how these neurons are specified, differentiated and maintained *in vivo*. This knowledge might help to instruct efforts to generate mDA neurons *in vitro*, which holds promise not only for cell replacement therapy, but also for disease modeling and drug discovery. In this Primer, we will focus on recent developments in understanding the molecular mechanisms that regulate the development of mDA neurons *in vivo*, and how they have been used to generate human mDA neurons *in vitro* from pluripotent stem cells or from somatic cells via direct reprogramming. Current challenges and future avenues in the development of a regenerative medicine for PD will be identified and discussed.

KEY WORDS: Dopamine neurons, Midbrain, Parkinson's disease, Regeneration, Reprogramming, Stem cells

Introduction

Dopaminergic (DA) neurons are capable of releasing dopamine, a catecholaminergic neurotransmitter. They are characterized by the presence of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of catecholamines, and are found throughout the mammalian central nervous system, including the ventral midbrain (VM) (Björklund and Hökfelt, 1983). Midbrain DA (mDA) neurons are arranged in three distinct nuclei: the *substantia nigra pars compacta* (SNc, also known as the A9 group), the ventral tegmental area (VTA, or A10 group) and the retrorubral field (RrF, or A8 group) (Björklund and Hökfelt, 1983; Dahlstroem and Fuxe, 1964) (Fig. 1A). Different populations of mDA neurons project to distinct areas and control or modulate specific functions, according to their targets [reviewed by Roeper (2013)]. VTA and RrF DA neurons project to the ventromedial striatum (*nucleus accumbens*), parts of the limbic system and prefrontal cortex, forming the mesolimbic and mesocortical systems (Fig. 1B). These neurons regulate emotional behavior, natural motivation, reward and cognitive function, and are primarily implicated in a range of psychiatric disorders (Carlsson, 2001; Chao and Nestler, 2004; Hornykiewicz, 1978). By contrast, DA neurons located in the SNc primarily project to the caudate-putamen, the dorsolateral striatum, forming the so-called nigrostriatal pathway, which predominantly regulates motor function and degenerates in Parkinson's disease (PD) (Box 1; Fig. 1A and B, in pink). This disorder was first described clinically by the British physician James Parkinson in 'An Essay of the Shaking Palsy' published in 1817 (Horowski et al., 1995). However, it was only many years later that the pathological basis for PD was found to relate to the degeneration of SNc neurons (Foix and Nicolesco, 1925; Graham, 1979; Hassler, 1938) and the loss of dopaminergic innervation of the striatum (Lloyd and Hornykiewicz, 1970). We now know that PD involves the degeneration of multiple neuronal subtypes besides SNc neurons (Jellinger, 1991). However, the cells that are most affected and responsible for many of the motor features in PD are mDA neurons of the SNc, a cell type that has become a primary target for cell replacement therapy (Box 1). Our ability to generate subtype-specific human mDA neurons *in vitro* might therefore hold the key for the development of future regenerative medicine for PD.

Transplantation of human fetal midbrain tissue in open-label clinical trials, in which both the researcher and the test subject know what treatment is administered, has provided proof of concept for cell replacement therapy in PD (Arenas, 2010; Evans et al., 2012; Hallett et al., 2014; Lindvall and Björklund, 2004, 2011). However, logistical and ethical difficulties in performing such studies using human fetal tissue have led to the search for more amenable cell preparations that could be standardized for quality, safety and functionality, prior to clinical use in PD. Human mDA neurons have been generated from multiple cell types *in vitro*, including neural stem/progenitor cells (NS/PCs) (Maciaczyk et al., 2008; Riaz et al., 2002; Ribeiro et al., 2012; Sanchez-Pernaute et al., 2001), pluripotent stem cells (PSCs) (Denham et al., 2012; Grealish et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011; Xi et al., 2012) and by lineage reprogramming of somatic cells such as fibroblasts (Caiazzo et al., 2011; Kim et al., 2011; Pfisterer et al., 2011). The *in vivo* functional capacity of PSC-derived mDA cells has recently been found to match that of fetal tissue (Grealish et al., 2014). Achieving cell preparations enriched for fully functional human SNc DA neurons *in vitro* and capable of selectively re-innervating the dorsal striatum or reconstructing the nigrostriatal pathway is thus the next challenge. In order to take advantage of the therapeutic potential that stem cells and reprogramming technologies currently offer, we must improve our knowledge of the molecular mechanisms that control mDA neuron development and use this to guide strategies to generate SNc DA neurons and improve functionality *in vivo*.

In this Primer article, we discuss recent advances in understanding mDA neuron development *in vivo*, and how these developmental principles have and continue to influence the development of stem cell therapies. We also outline the challenges and opportunities that lie ahead in order to efficiently generate safe and functional human mDA neuron preparations for cell replacement therapy, as well as for disease modeling and drug discovery.

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Development (2015) 142, 1918-1936 doi:10.1242/dev.097394

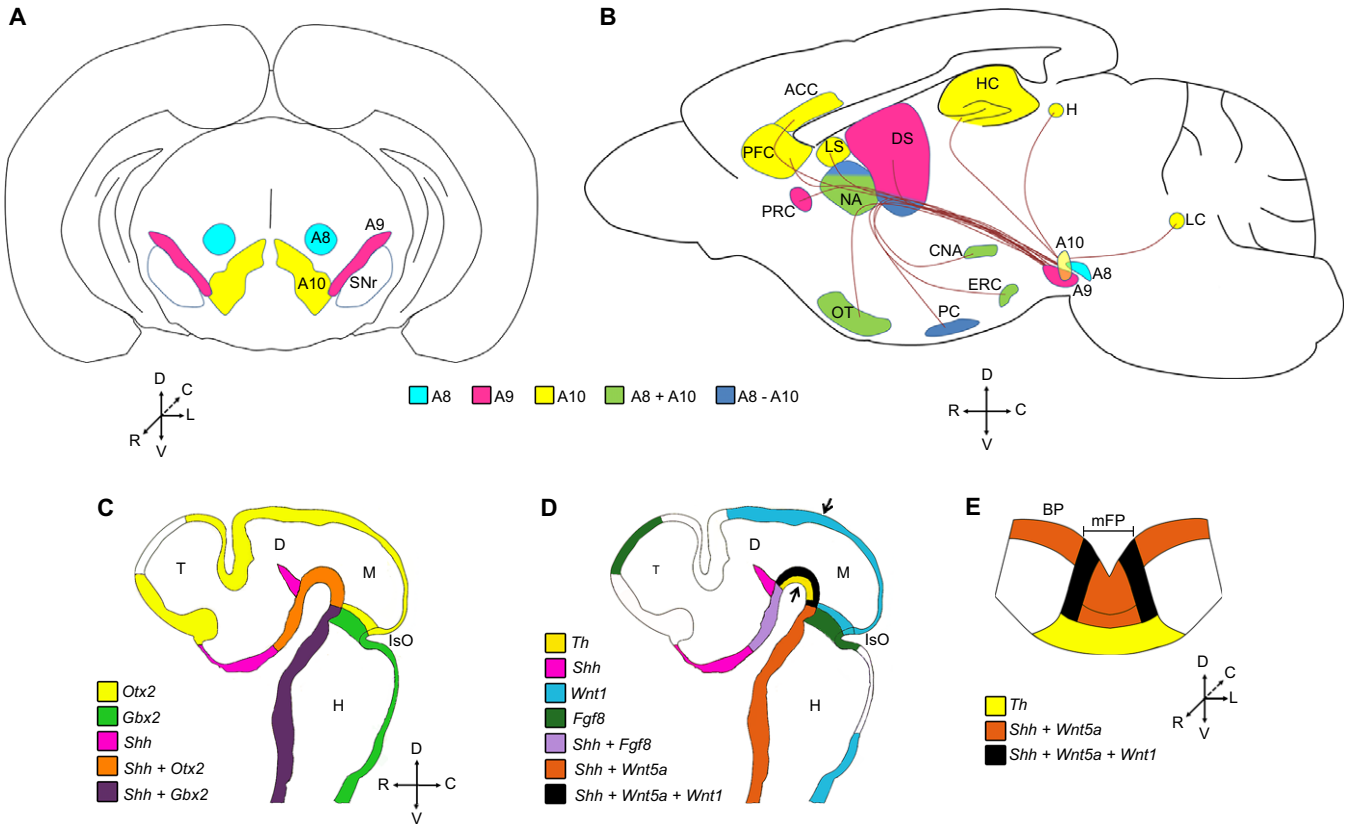

Fig. 1. Distribution of mDA neurons, their projections in the adult mouse brain and the expression of genes important for their development. (A) Coronal section of the adult brain at the midbrain level, showing the position of the three mDA nuclei: RrF/A8, SNc/A9 and VTA/A10. (B) Sagittal view of the adult brain with a schematic representation of mDA neurons and their projection areas. A8, retrorubral; A9, substantia nigra; A10, ventral tegmental area; A8+A10, structures innervated by A8 and A10; A8-A10, structures innervated by A8, A9 and A10; ACC, anterior cingulate cortex; CNA, central nucleus of the amygdala; DS, dorsal striatum; ERC, entorhinal cortex; H, habenula; HC, hippocampus; LC, locus coeruleus; LS, lateral septum; NA, nucleus accumbens; OT, olfactory tubercle; PFC, prefrontal cortex; PRC, perirhinal cortex; PC, pyriform cortex. (C,D) Sagittal view of an E11.5 mouse embryo, showing the expression of transcription factors and morphogens important for VM patterning in relation to *Th* expression. D, diencephalon; H, hindbrain; IsO, isthmic organizer; M, midbrain; T, telencephalon. (E) Coronal section through the VM at the level shown by arrows in D. BP, basal plate; mFP, midbrain floor plate.

mDA neuron development

During gastrulation, a posterior-to-anterior migration of cells takes place at the same time as the three germ layers (mesoderm, endoderm and ectoderm) are formed. At the rostral end of the embryo, the inhibitors DKK1 (dickkopf 1, a WNT inhibitor), NOG (noggin, a BMP inhibitor), LEFTY1 (left-right determination factor 1, a NODAL inhibitor) and CERBERUS (a multifunctional inhibitor of Wnt, BMP and Nodal), which suppress posterior signals and pattern the neural ectoderm, leading to the formation of the anterior neural tube (reviewed by Takaoka et al., 2007). As the development of the neural tube proceeds, two signaling centers are formed: the isthmic organizer (IsO), which defines the midbrain-hindbrain boundary (MHB) (Joyner et al., 2000; Rhinn et al., 1998; Wassarman et al., 1997), and the floor plate (FP), which controls ventral identities (Placzek and Briscoe, 2005) (Fig. 1C). During these and subsequent developmental stages, the combined action of transcription factors and morphogens from the IsO and the FP orchestrate multiple functions, including the regional identity of the VM, the specification and proliferation of mDA progenitors, mDA neurogenesis, as well as the differentiation and survival of mDA neurons.

Transcriptional regulation of VM patterning

One of the earliest and most crucial patterning events in the neural tube is the formation of the IsO at the MHB, which starts at E7.5 through the coordinated expression and mutual repression of the transcription factors, *Otx2* (orthodenticle homolog 2) in the midbrain and *Gbx2* (gastrulation brain homeobox 2) in the hindbrain, (Fig. 1C) (Broccoli et al., 1999; Millet et al., 1999; Wassarman et al., 1997). *Otx2* and *Gbx2* control the patterning of the MHB by regulating the expression of two morphogens, *Wnt1* (wingless-int1; wingless-type MMTV integration site family, member 1 – Mouse Genome Database) in the midbrain and *Fgf8* (fibroblast growth factor 8) in the hindbrain (Joyner et al., 2000; Rhinn et al., 1998) (Fig. 1D and Fig. 2, yellow area). Whereas *Otx2* is required for the expression of *Wnt1* (Puelles et al., 2004; Rhinn et al., 1999), the induction of *Fgf8* requires *Pax2* (paired homeobox 2) and is maintained by *Gbx2* (Ye et al., 1998). Importantly, *Fgf8*, but not *Wnt1*, is required (Chi et al., 2003) and sufficient (Martinez et al., 1999) for the induction of the IsO. Accordingly, cells at the MHB acquire anteroposterior information by interpreting the concentration gradient generated by the secretion of FGF8 in the IsO. Whereas high concentrations of FGF8 in the hindbrain drive a hindbrain cell fate, lower concentrations in the neighboring anterior tissue ensure that the cells adopt a midbrain identity (Basson et al., 2008; Sato and Nakamura, 2004; Suzuki-Hirano et al., 2005). In agreement with this, FGF8 can induce mDA neurons in brain explants, at a certain distance from the FGF8 source (Ye et al., 1998), by a process that requires *Wnt1* (Prakash et al., 2006)

Development (2015) 142, 1918-1936 doi:10.1242/dev.097394

Box 1. Parkinson's disease and its treatment

Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by the loss of mDA neurons of the SNc that project to the striatum (Fig. 1A,B). These neurons control motor behavior, and, as they degenerate, they result in several motor features of the disease, such as bradykinesia, rigidity, resting tremor, gait disturbances and postural instability (Lees et al., 2009). However, other cell types are also progressively affected in PD, such as *locus coeruleus* noradrenergic neurons (Hassler, 1938), basal forebrain cholinergic neurons (Tagliavini et al., 1984), peptidergic neurons (Agid et al., 1986) and serotonin neurons (Calabresi et al., 2013; Chaudhuri et al., 2006; Chaudhuri and Schapira, 2009; Fox et al., 2008; Halliday et al., 2011). The loss of these neurons results in non-motor features, including cognitive, affective, sleep, olfactory and intestinal alterations (Chaudhuri et al., 2006; Chaudhuri and Schapira, 2009).

The main treatment for PD is pharmacological. The most efficient drug is the dopamine precursor levodopa (Brichta et al., 2013), but other agents, including dopamine agonists, catechol-o-methyl-transferase (COMT) and monoamine oxidase type B (MAOB) inhibitors, as well as non-dopaminergic agents, such as antidepressants or cholinesterase inhibitors for dementia, are also prescribed (Connolly and Lang, 2014). The chronic use of levodopa is associated with motor complications, including fluctuations and dyskinesias (Stern et al., 2004; Weiner, 2004), whereas dopamine agonists can cause behavioral alterations (Jankovic and Aguilar, 2008). Deep brain stimulation (DBS), targeting the thalamus, subthalamic nucleus and globus pallidus, is currently used in PD patients whose motor symptoms cannot be adequately controlled by medication. The mechanism of action is not known, but DBS is thought to block depolarization, activate inhibitory neurons, desynchronize tremorogenic pacemakers and functionally disrupt neuronal networks (Jankovic and Poewe, 2012).

Although all these treatments relieve some symptoms of PD, they do not slow down disease progression or reverse the damage to mDA neurons, and the treatment loses efficacy. Cell replacement therapy has thus gained interest as a therapeutic option, as it has the potential to change the course of disease.

(Fig. 2, yellow and blue). Notably, *Wnt1* expression at the MHB is required for the development of both posterior midbrain and anterior hindbrain (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas and Capecchi, 1990), but its anterior expression in two paramedial bands within the midbrain FP (Fig. 1E) serves an additional, crucial role in the specification and differentiation of mDA progenitors [reviewed by Arenas (2014)].

A second major event is the patterning of the neural tube by the morphogen SHH (sonic hedgehog) (Roelink et al., 1995). SHH, initially secreted by the notochord specifies the most ventral region of the neural plate, the FP, by inducing the expression of *Foxa2* at E8 in mice (Ang et al., 1993; Sasaki et al., 1997). *Foxa2* plays a central role in the SHH network and is required for notochord and FP development as well as for ventral patterning (Ang and Rossant, 1994; Weinstein et al., 1994). By E8.5, the FP itself starts expressing *Shh* and becomes a secondary ventral organizer of the neural tube (Fig. 1C-E). A gradient of SHH from the FP regulates patterning in the ventro-dorsal axis: ventral progenitors exposed to higher concentrations of SHH respond by expressing different transcription factors compared with dorsal cells, exposed to lower concentrations, resulting in different ventro-dorsal identities (Briscoe and Ericson, 1999; Chiang et al., 1996; Ericson et al., 1996; Marti et al., 1995; Roelink et al., 1995). SHH regulates transcription by binding to its receptor PTCH1 (patched 1), which then releases its inhibition on a co-receptor, SMO (smoothened), that signals to prevent the processing of the GLI (glioma-associated oncogene) transcription factors GLI1 and GLI2 into repressors. Thus, *Shh* biases the balance of GLI processing towards an activated state, leading to the upregulation of ventrally expressed homeodomain transcription factors. In the midbrain FP, high SHH signaling upregulates *Foxa2* (Sasaki et al., 1997) (Fig. 2, pink area), whereas, in the basal plate (BP), a more lateral and dorsal position flanking the FP, lower levels of SHH upregulate *Nkx6-1* and *Otx2* (*Nkx* for NK homeobox protein) (Fig. 2, green area). FOXA2 also induces *Nkx6-1* in the BP (Nakatani et al., 2010) and suppresses *Nkx2-2* in the midbrain FP (mFP) (Ferri et al., 2007). Moreover, FOXA2 directly represses *Gli1-3* and upregulates *Shh* expression (Metzakopian et al., 2012). Overall, these studies indicate that ventral patterning involves both a temporal and spatial responses to SHH signaling.

Specification of mDA progenitors and suppression of lateral fates

Fate mapping experiments have indicated that mDA neurons originate from progenitors sorted for the FP marker CORIN (Ono et al., 2007) or expressing the radial glia marker *Glast/Slc1a* (glial high affinity glutamate transporter) (Bonilla et al., 2008). This shows that mFP radial glial cells, unlike FP radial glia in other brain regions, can undergo neurogenesis and are mDA progenitors. Additionally, mDA neurons have been fate-mapped from progenitors expressing *Shh* (Blaess et al., 2011; Hayes et al., 2011; Joksimovic et al., 2009a) or *Wnt1* (Brown et al., 2011; Yang et al., 2013a; Zervas et al., 2004), indicating that mFP radial glia are also constituents of the two main signaling centers, the mFP and the IsO. Moreover, information from both signaling centers is integrated in mDA progenitors, in which FOXA1/2 (Lin et al., 2009) and OTX2 (Ono et al., 2007) are present (Fig. 3) and regulate the expression of two LIM homeobox transcription factors, *Lmx1b* and *Lmx1a* (Fig. 2, blue area). Whereas *Lmx1b* is necessary for the differentiation of mDA progenitors (Smidt et al., 2000), *Lmx1a* is required for the specification of mDA neurons in the mFP (Andersson et al., 2006b; Deng et al., 2011) and, via *Msx1* (muscle segment homeobox homolog 1), to suppress the emergence of BP fates (Andersson et al., 2006a). Additionally, *Otx2* sustains the expression *Nkx6-1* in the BP and suppresses *Nkx2-2* in the mFP (Puelles et al., 2004). Thus, the concerted action of the Shh-Foxa2 and the Otx2-Wnt1-Lmx1a/Msx1 networks is essential not only for the specification of the mFP but also for the suppression of alternative neural fates (Fig. 2, green area).

In other parts of the neural tube, *Wnt1*/*β-catenin* (*Ctnnb*), *Lmx1a* and *Lmx1b* are typical dorsal roof plate genes (Millonig et al., 2000; Shimamura et al., 1994). However, in the mFP, they form a positive autoregulatory loop (Chung et al., 2009) (Fig. 2, red arrows) required for mDA specification: on one hand *β-catenin* directly upregulates *Lmx1a* and *Otx2*, and, on the other hand, *Lmx1a/b* directly upregulate each other as well as *Wnt1*, *Msx1* and two key genes involved in mDA neuronal differentiation and survival, *Nurr1* (*Nr4a2*, nuclear receptor 4a2) and *Pitx3* (pituitary homeobox 3, or paired-like homeodomain transcription factor 3). Accordingly, deletion of both *Lmx1a* and *Lmx1b* results in a near-complete loss of mDA neurons (Yan et al., 2011). Similarly, deletion of *Wnt1* results in the loss of *Lmx1a*, *Nurr1* and *Pitx3* in the mFP and a complete loss of mDA neurons (Andersson et al., 2013; Prakash et al., 2006). Combined, these results indicate that the specification of mDA neurons is controlled by the *Lmx1a/b-Wnt1/Ctnnb* autoregulatory loop together with *Otx1/2* and *Foxa1/2*.

mDA neurogenesis

mDA neurogenesis takes place in the ventricular zone (VZ) of the mFP, when mDA progenitors divide to generate postmitotic cells

Development (2015) 142, 1918-1936 doi:10.1242/dev.097394

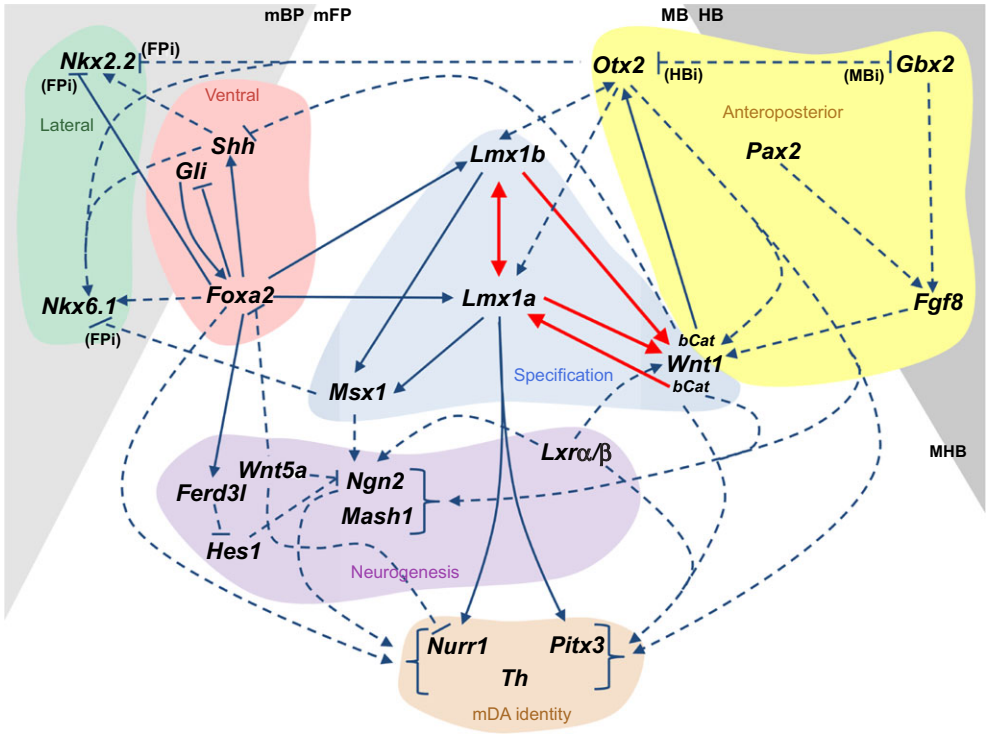

Fig. 2. Genetic networks controlling the development of the midbrain-hindbrain and mDA neurons in the mouse brain. Antero-posterior patterning (yellow area): The mutually repressive activities of *Otx2* in the midbrain (MB) and *Gbx2* in the hindbrain (HB) establish the midbrain-hindbrain boundary (MHB), where *Otx2* is inhibited in the hindbrain (HBi) and *Gbx2* in the midbrain (MBi). Midbrain floor plate (mFP) specification (blue area): LMX1B expression in the midbrain mFP directly regulates the expression of *Wnt1* and *Lmx1a*, which also regulate each other via CTNNB (*bCat*) or directly (LMX1A), forming an auto-regulatory loop (shown by red arrows). *Wnt1* regulates *Otx2* and *Lmx1a* via β-catenin, and *Lmx1a*/*b* regulate *Msx1* (Wnt1-Lmx1a/b-Msx1 network). Ventral patterning (pink area): FOXA2 regulates *Shh*, which feeds back onto *Foxa2* via GLI (Shh-Foxa2 network). FOXA2 also directly regulates *Lmx1a*/*b*, to coordinate the specification of mDA neurons. Lateral phenotypes (green area): Midbrain basal plate (mBP) markers (*Nkx2-2* and *Nkx6-1*) are inhibited in the mFP (FPi) by *Foxa2* and *Otx2* (*Nkx2-2*), and by *Msx1* (*Nkx6-1*). Neurogenesis (purple area): The expression of *Ngn2* is indirectly regulated by *Wnt5a*, *Foxa2* (via *Ferd3l* and *Hes1*), *Lmx1a*/*b* (via *Msx1*) and *Lxr alpha*/*beta* (*Nr1h3*/*Nr1h2*). *Wnt1*/*bCat* and *Otx2* regulate both *Ngn2* and *Mash1*. mDA neuroblasts and neurons (beige area): LMX1A directly regulates the expression of mDA postmitotic genes, such as *Nurr1* and *Pitx3*, which in turn regulate *Th*. These postmitotic genes are also regulated by *Foxa2*, *Ngn2*, *Wnt5a*, *Lxr alpha*/*beta*, *Wnt1*/β-*catenin* and *Otx2*. Solid lines indicate direct interactions as demonstrated by chromatin immunoprecipitation. All other interactions, whether direct or indirect, are shown by dashed lines. Arrowheads indicate activation and perpendicular lines denote inhibition.

which express the nuclear receptor *Nurr1*. These cells migrate through the intermediate zone (IZ) while they differentiate and become TH^+ mDA neurons on reaching the mantle zone (MZ) (Fig. 3A). In rodents, mDA cells appear at E10.5, whereas, in humans, mDA neurogenesis begins between 5.0 and 6.0 weeks post conception (PC), peaks at weeks 6-8 (Almqvist et al., 1996; Nelander et al., 2009) and ceases at weeks 10-11 (Freeman et al., 1991). mDA neurogenesis is controlled by two proneural genes of the basic-helix-loop-helix family, *Mash1* (mouse achaete-schute homolog 1) and *Ngn2* (*Neurog2*; neurogenin 2) (Fig. 2, purple area), expressed in the VZ of the mFP. *Ngn2* is required for mDA neurogenesis (Andersson et al., 2006a; Kele et al., 2006), whereas

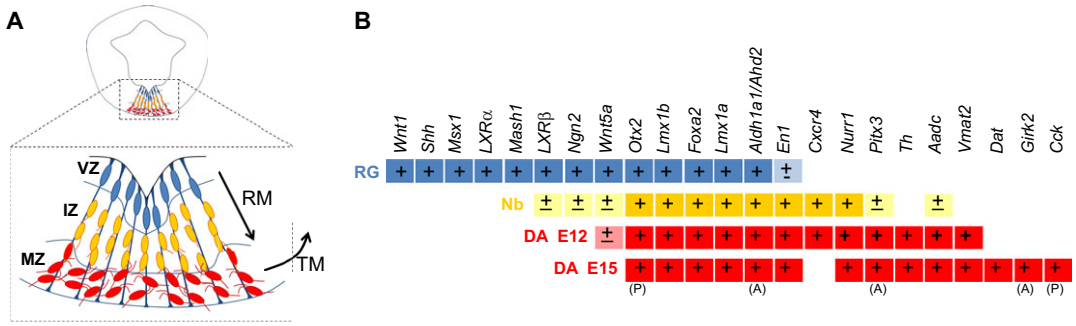

Fig. 3. Gene expression in the mDA lineage. (A) Schematic representation of a section through the mFP at E11.5. The ventricular zone (VZ) contains radial glia cells (RG, blue) that undergo neurogenesis to generate postmitotic neuroblasts (yellow) that migrate radially through the intermediate zone (IZ), over the processes of the RG and differentiate into mDA neurons (red) in the marginal zone (MZ). As cells become mDA neurons, they migrate tangentially towards the *substantia nigra pars compacta* (SNc). Arrows indicate radial migration (RM) of neuroblasts and tangential migration (TM) of mDA neurons. (B) RG (blue) cells express morphogens, proneural and early transcription factors, some of which are also expressed in neuroblasts (Nb, yellow) and mDA neurons, defining the entire mDA lineage. ±, low levels of expression; +, expressed; A, mainly anterior midbrain; P, mainly posterior midbrain.

Development (2015) 142, 1918-1936 doi:10.1242/dev.097394

Table 1. Late transcription factor genes, such as *Nurr1*, *En1* and *Pitx3*, regulate the expression of multiple genes in mDA neurons

	TFs			mDA						NFs		Post		
	Nurr1	*En1*	*Pitx3*	*Aadc/Ddc*	*Ahd2*	*TH*	*Vmat*	*Dat*	*Drd2*	*Bdnf*	*C-ret*	*Cck*	*Nts*	*Dlk1*
Nurr1		+	+	+	+	+	+	+	+	+	+			+
En1			+		+	+	+	+				+	+	
Pitx3	+	–			+	+	+	+	+	+		–	–	–

Note that *Pitx3* is highly expressed in the anterolateral mDA neurons, where it inhibits the expression of *en1* and posterior markers such as *cck*. +, positive regulation; –, negative regulation. TFs, transcription factors; NFs, neurotrophic factors; Post, posterior markers.

Mash1 is capable of partially compensating for the loss of *Ngn2* (Kele et al., 2006). These two proneural genes are directly or indirectly regulated by, and integrate information from, the Shh-Foxa2 and Lmx1a/b-Wnt1-Otx2 networks (Fig. 2, pink, blue and yellow areas), as well as nuclear receptors of the Liver X receptor family (*Lxrα/Nr1h3* and *Lxrβ/Nr1h2*) (Sacchetti et al., 2009) and the morphogen *Wnt5a* (Andersson et al., 2008) in order to control mDA neurogenesis (Fig. 2, purple area).

Repression of *Shh*, but not *Foxa2*, by *Wnt1/Ctnnb* in the mFP is required for mDA neurogenesis (Andersson et al., 2013; Joksimovic et al., 2009b; Tang et al., 2009). *Foxa2* then, together with *Foxa1*, dose-dependently controls mDA neurogenesis (Ferri et al., 2007; Stott et al., 2013) in two ways, by directly regulating the basic helix-loop-helix gene *Ferd3l* (Fer3-like, also known as *Nato3*) and *Lmx1a* (via *Msx1*) (Metzakopian et al., 2012). Whereas *Ferd3l* regulates mDA neurogenesis by repressing *Hes1* (hairy and enhancer of Split 1) (Ono et al., 2010), a suppressor of pro-neural genes, *Lmx1a* increases the expression of *Ngn2* via *Msx1* (Andersson et al., 2006b) (Fig. 2, purple area).

However, CTNNB also regulates mDA neurogenesis by directly regulating the expression of *Lmx1a* and *Otx2* (Chung et al., 2009), and *Otx2* is required for mDA neurogenesis by controlling the expression of *Ngn2* and *Mash1* (Omodei et al., 2008; Prakash et al., 2006). Similarly, *Wnt1* is required for the expression of *Ngn2* and *Mash1* in the mFP and for mDA neurogenesis (Andersson et al., 2008). Another important factor for mDA neurogenesis is the morphogen *Wnt5a*, which is expressed in the VM at E9.5 and becomes restricted to the BP and the mFP by E11.5-13.5 (Fig. 1D,E). *Wnt5a* is expressed in mFP radial glia, neuroblasts and partially in posterior mDA neurons (Fig. 3B), where it inhibits *Foxa2*, *Ngn2* and *Nurr1* expression (Andersson et al., 2008) (Fig. 2, purple area). Accordingly, deletion of *Wnt5a* does not impair mDA neurogenesis, but rather increases the number of postmitotic NURR1^{+}/TH^{-} cells (Andersson et al., 2008). Surprisingly, however, its deletion potentiates the neurogenesis defect in *Wnt1*$^{-/-}$ mice, indicating that there is cooperation between both Wnts in mDA neurogenesis (Andersson et al., 2013).

LXRα and LXRβ are two additional regulators of mDA neurogenesis. These nuclear receptors are ligand-dependent transcription factors that form obligate heterodimers with RXR (retinoid X receptors). LXRs are required to maintain the expression levels of *Lmx1b*, *Wnt1* and *Ngn2* in the developing midbrain, and deletion of both *Lxr* receptors decreased mDA neurogenesis, whereas their overexpression increased it (Sacchetti et al., 2009) (Fig. 2, purple area). Moreover, activation of LXRs by the endogenous midbrain LXR ligand 24,25-epoxycholesterol has been shown to selectively and specifically promote mDA neurogenesis, in an LXR-dependent manner (Theofilopoulos et al., 2013). These results indicate that LXRs are both required and sufficient for mDA neurogenesis during development and that LXR ligands constitute a new class of selective and cell type-specific regulators of neurogenesis (Theofilopoulos et al., 2013).

Migration of postmitotic mDA neuroblasts and neurons

After neurogenesis, postmitotic cells migrate through the IZ towards their final destination in the MZ (Hanaway et al., 1971; Kawano et al., 1995). mDA cells first migrate radially along the vimentin^{+} radial glial processes (Shults et al., 1990) (Fig. 3A) and then tangentially, to reach their final position in the SN, VTA and RrF (Hanaway et al., 1971; Marchand and Poirier, 1983) (Fig. 1A). *Cxcr4* (C-X-C motif chemokine receptor type 4) can be detected in the IZ and MZ (Fig. 3B) and is required for radial migration and fiber outgrowth of mDA neurons between E11.5 and E14.5 (Bodea et al., 2014; Yang et al., 2013b). Its ligand, *Cxcl12* (C-X-C motif chemokine 12) is expressed in the meninges and is sufficient to promote the migration of mDA neurons (Yang et al., 2013b). Whereas pharmacological inhibition or deletion of *Cxcr4* does not affect the number of mDA neurons, it affects their migration, as some cells remain in the IZ and do not reach the MZ (Yang et al., 2013b). Tangential migration of mDA neurons is regulated by the neural L1 cell adhesion molecule (L1CAM) (Demyanenko et al., 2001; Ohyama et al., 1998) and RELN (reelin) (Kang et al., 2010; Nishikawa et al., 2003). Mice lacking *Reln* or the cytoplasmic adaptor protein *Dab1* (disabled 1) have fewer PSA-NCAM^{+} tangential fibers and fewer mDA neurons reaching the SNc, despite normal numbers of mDA neurons being generated (Kang et al., 2010). Moreover, the RELN receptors LRP8 (low density lipoprotein receptor-related protein 8, also known as ApoER2) and VLDLR (very low density lipoprotein receptor) are also required for the migration and final positioning of mDA neurons (Sharaf et al., 2013). Finally, *Ntn1* (netrin 1) regulates mDA neuron migration in the mFP (Livesey and Hunt, 1997), and deletion of the NTN1 receptor *Dcc* (deleted in colorectal cancer), results in not only dorsally displaced mDA neurons, but also in a loss of mDA neurons and aberrant innervation of nigrostriatal and mesocortical targets (Flores et al., 2005; Riedl and Salvesen, 2007; Xu et al., 2010). Thus, multiple pathways regulate mDA neuron migration – most notably CXCL12/CXCR4 to control radial migration, and RELN signaling to control tangential migration.

Differentiation and survival of mDA neurons

After neurogenesis, migratory postmitotic neuroblasts in the IZ differentiate into mDA neurons in the MZ. This process is regulated by some of the early factors described above, such as *Otx2*, *Lmx1a/b*, *Foxa1/2* and the homeobox genes *En1/2* (engrailed 1/2), which remain expressed in postmitotic mDA cells (Fig. 3B). Early factors in turn regulate the activity of late transcription factors, such as *Nurr1* and *Pitx3* (Fig. 2, beige, Fig. 3B), which control the progressive acquisition of appropriate neurotrophic factor and DA neurotransmitter phenotype (Table 1).

The morphogen WNT5A also promotes the differentiation of rodent and human midbrain progenitors into functional mDA neurons (Andersson et al., 2008; Parish et al., 2008; Ribeiro et al., 2012). *Wnt5a*$^{-/-}$ mice show an excess of NURR1^{+};TH^{-} postmitotic DA neuroblasts but not mDA neurons, indicating a differentiation defect.

Wnt5a$^{-/-}$ mice display typical convergent-extension morphogenic defects, resulting in a rostro-caudal shortening and medial-lateral widening of the mDA domain (Andersson et al., 2008). Deletion of *Wnt1* does not affect conversion-extension and, unlike *Wnt5a*, decreases the number of NURR1$^+$ neuroblasts and TH$^+$ mDA neurons (Andersson et al., 2013). However, simultaneous deletion of *Wnt5a* and *Wnt1* further enhances the *Wnt1*$^{-/-}$phenotype and reduces the number of NURR1$^+$ and TH$^+$ mDA neurons to a greater extent, indicating a cooperation between these two *Wnts* in mDA differentiation (Andersson et al., 2013) (Fig. 1D,E). *Wnt1* is also required for the survival of mDA neurons and the expression of *Pitx3* (Prakash et al., 2006) as well as LMX1A in the mFP (Andersson et al., 2013), where LMX1A/B directly regulates *Nurr1* and *Pitx3* (Chung et al., 2009). However, FOXA1/2 are required for the expression of *Nurr1*, *En1* and *Ddc* (dopa decarboxylase, also known as aromatic L-amino acid decarboxylase, *Aadc*) in mDA neuroblasts and neurons, as well as the expression of *Th* in mDA neurons (Ferri et al., 2007; Stott et al., 2013). Thus, the two morphogen-controlled gene networks in the mFP, *Wnt1-Lmx1a* and *Shh-Foxa2*, cooperatively regulate not only mDA specification and neurogenesis, but also differentiation and survival (Fig. 2, beige area).

Transcription factors that are expressed in postmitotic mDA neurons from E10-10.5 to adult stages and control the acquisition of a mature mDA phenotype include *Nurr1*/*Nr4a2* (Zetterström et al., 1996) and *Pitx3* (Maxwell et al., 2005) (Fig. 3B). *Nurr1* is required for mDA neuroblast survival and differentiation into TH$^+$ mDA neurons (Zetterström et al., 1997). In *Nurr1*$^{-/-}$ mice, mDA neuroblasts become PITX3$^+$ but fail to survive and are gradually lost (Le et al., 1999; Saucedo-Cardenas et al., 1998). In agreement with this, NURR1 regulates the expression of several genes that define a mature mDA neuron, including *Th*, *Slc18a2/Vmat2* (solute carrier family-18 member-2/vesicular monoamine transporter-2), *Slc6a3/Dat* (solute carrier family-6 member-3/dopamine transporter), *Ddc/Aadc*, *Ret* (c-ret proto-oncogene), *Bdnf* (brain-derived neurotrophic factor) and *Cdkn1c* (cyclin-dependent kinase inhibitor 1C) (Gil et al., 2007; Jankovic et al., 2005; Joseph et al., 2003; Saucedo-Cardenas et al., 1998; Smits et al., 2003; Wallen et al., 2001; Volpicelli et al., 2007; Zetterström et al., 1997) (Fig. 3B; Table 1). *Pitx3* is one of the earliest markers of mDA neurons (Chung et al., 2005b; Jacobs et al., 2007; Nunes et al., 2003; Semina et al., 1997; Smidt et al., 1997). Unlike *Nurr1*, deletion of *Pitx3* does not change the number of VTA/A10 TH$^+$ mDA neurons. However the number of SNc/A9 neurons progressively decreases in *Pitx3*$^{-/-}$ mice and is dramatically reduced by birth, indicating that *Pitx3* is required for the survival of SNc/A9 mDA neurons (Hwang et al., 2003; Maxwell et al., 2005; Smidt et al., 2004; Smidt and Burbach, 2007; van den Munckhof et al., 2003; Yang et al., 2013b). One direct target of PITX3 is the *Aldh1a1* gene (aldehyde dehydrogenase-1a1, or *Ahd2*), which increases retinoic acid and upregulates *Th* and *Drd2* (dopamine receptor 2) but decreases *Dlk1* (delta-like 1 homolog) in anterior mDA cells (Jacobs et al., 2011). PITX3 also works independently of retinoic acid to upregulate *Vmat* and *DAT* and downregulate *Cck* (cholecystokinin) and *En1/2* (Jacobs et al., 2011; Veenvliet et al., 2013) (Table 1). Moreover, *Nurr1* and *Pitx3* regulate each other (Jacobs et al., 2009; Volpicelli et al., 2012) and are required for the maintenance of adult mDA neurons (Kadkhodaei et al., 2009; van den Munckhof et al., 2003). Interestingly, *Nurr1* also regulates *En1* (Sousa et al., 2007), which in turn regulates *Pitx3*, *Aldh1a1*, *Th*, *Slc18a2/Vmat2*, *Slc6a3*/*Dat*, *Cck* and *Nts* contributing to the proper induction of distinct mDA subsets (Veenvliet et al., 2013) (Table 1). Moreover, EN1/2 enhances the translation of mitochondrial complex I subunits and promotes the survival of adult mDA neurons in models of PD *in vivo* (Alvarez-Fischer et al., 2011). In sum, NURR1, PITX3 and EN1/2 are crucial regulators of terminal differentiation, as well as survival and maintenance of mDA neurons (Fig. 2, beige area).

The survival of mDA neurons *in vitro* and in animal models of PD is regulated by several families of neurotrophic factors, including the transforming growth factor family (TGFβ2/3) (Poulsen et al., 1994; Roussa et al., 2009); members of the neurotrophin family, such as brain-derived neurotrophic factor (BDNF) (Frim et al., 1994; Hyman et al., 1991); glial cell-line-derived neurotrophic factor (GDNF) (Akerud et al., 2001; Arenas et al., 1995; Beck et al., 1995; Choi-Lundberg et al., 1997; Gash et al., 1996; Kordower et al., 2000; Lin et al., 1993; Rosenblad et al., 1998; Tomac et al., 1995); other members of the GDNF family, such as neurturin or persephin (Akerud et al., 1999, 2002; Horger et al., 1998; Rosenblad et al., 1999, 2000); and a novel family formed by mesencephalic astrocyte-derived neurotrophic factor (MANF) and conserved dopamine neurotrophic factor (CDNF or Armetl1) (Lindholm et al., 2007; Petrova et al., 2003; Voutilainen et al., 2009).

Embryonic deletion of *Tgfβ3* (Zhang et al., 2007), but not *Bdnf* or *Gdnf* (Baquet et al., 2005; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), reduced the numbers of mDA neurons. However, conditional deletion of *Gdnf* in adult mice (*Gdnf*$^{f/f}$*;Cre-ER*^{TM}) causes mDA neuron loss (Pascual et al., 2008), indicating that GDNF is subsequently required to maintain the survival of mDA neurons. Interestingly, members of the TGFβ family are required for GDNF to exert its effects (Krieglstein et al., 1998), and the expression of the GDNF receptor *c-ret* (Trupp et al., 1996) is maintained by *Nurr1* (Decressac et al., 2012). Moreover, GDNF has been suggested to regulate *Bdnf* expression via a *Gdnf-Pitx3-Bdnf* trophic loop (Peng et al., 2011). Thus, multiple lines of evidence indicate that networks formed by morphogens, transcription factors and neurotrophic factors promote the differentiation and survival of mDA neurons.

Generating human mDA neurons *in vitro*

Our ability to recreate human mDA neurons *in vitro* has built in large part on the growing knowledge of how these cells develop *in vivo* and has opened up unprecedented opportunities in disease modeling, drug discovery and cell replacement therapies for PD. Multiple candidate cell sources are now available to generate DA neurons that are potentially suitable for therapeutic applications (Fig. 4). These include endogenous fetal NS/PCs, PSCs and adult somatic cells, which can be used for reprogramming (Fig. 4A-D). However, not all protocols are equally efficient. Notably, NS/PCs expanded with mitogens as monolayers (Sanchez-Pernaute et al., 2001) or neurospheres (Maciaczyk et al., 2008; Riaz et al., 2002; Ribeiro et al., 2012), or when immortalized (Castelo-Branco et al., 2006; Courtois et al., 2010; Ramos-Moreno et al., 2012; Wagner et al., 1999), have shown variable numbers and quality of DA neurons (Fig. 5A-E), probably due to heterogeneity and/or late developmental stage of the cells and the protocols used. More recently, long-term-neuroepithelial stem (lt-NES) cells have emerged as a more homogeneous early stage population that can be derived from either human fetal hindbrain tissue (Tailor et al., 2013) or PSCs, which can be differentiated into TH$^+$ neurons (Falk et al., 2012; Koch et al., 2009) (Fig. 5F). However, it remains to be determined whether these cells could generate correctly specified mDA neurons with more advanced differentiation protocols. In this section, we will first focus on human PSCs, which can be correctly specified into mDA neurons that are functional *in vivo*, and are thus the closest to a potential clinical application; and second, on somatic cell

Development (2015) 142, 1918-1936 doi:10.1242/dev.097394

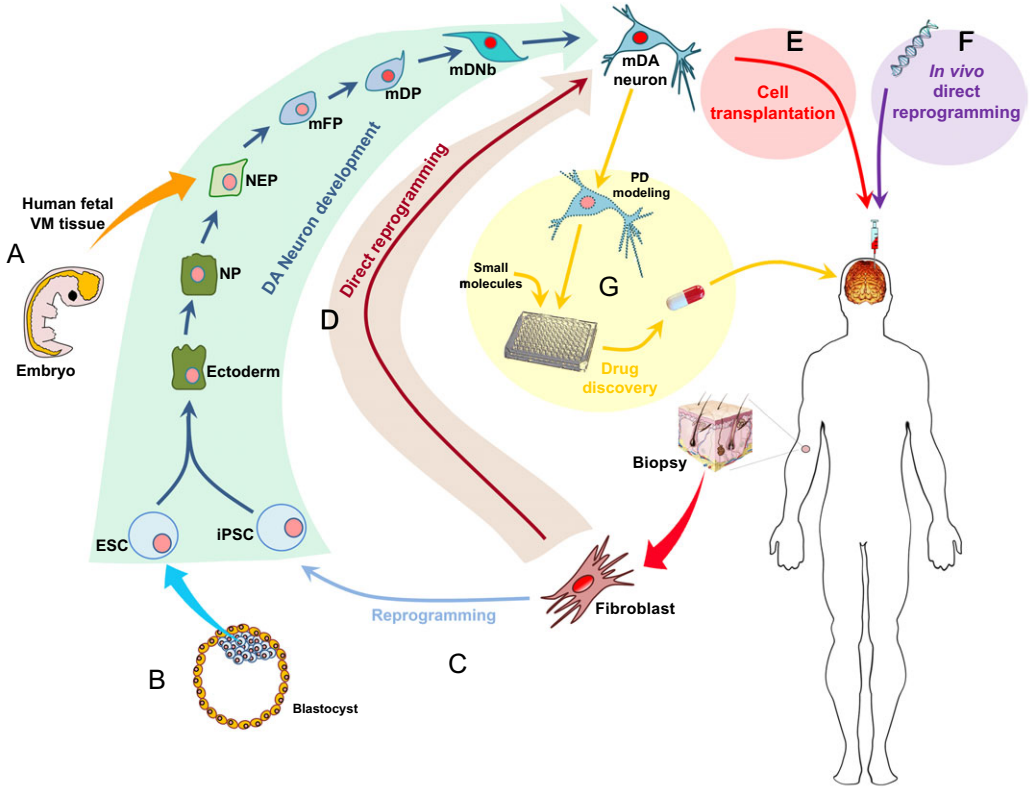

Fig. 4. Regenerative medicine-based strategies for the treatment of Parkinson's disease. (A) Human fetal ventral midbrain (VM) tissue has been the classical source of tissue for transplantation and has provided proof of principle for cell replacement therapy in Parkinson's disease (PD). (B,C) Midbrain dopaminergic neurons have also been generated from human PSCs, derived either from blastocysts (ESCs; B) or by reprogramming of fibroblasts (iPSCs; C). Protocols involve following the developmental steps to first generate ectoderm, neural plate (NP) and neuroepithelial cells (NEP), followed by midbrain floor plate (mFP), midbrain dopaminergic (mDA) progenitors (mDP), mDA postmitotic neuroblasts (mDNb) and mDA neurons. (D) An alternative way to create mDA neurons is by direct reprogramming of fibroblasts into induced mDA neurons. (E) mDA neurons and iDA cells can be used for transplantation. (F) Direct reprogramming could also be performed *in vivo*, to generate iDA neurons *in situ*, from host cells in the adult brain, without the need for cell transplantation. (G) mDA neurons derived from PD patients or with introduced PD mutations can be compared with controls or corrected mutations and used for disease modeling and drug discovery.

reprogramming, a novel and promising strategy to generate DA neurons.

Directed differentiation of PSCs into mDA neurons

Human PSCs (hPSCs), whether derived from the inner cell mass of an embryo at the blastocyst stage (embryonic stem cells, ESCs) (Thomson et al., 1998), or generated via reprogramming of differentiated somatic cells (induced pluripotent stem cells, iPSCs) (Takahashi et al., 2007), are an attractive cell source for regenerative medicine, given their capacity to self-renew and to generate all the cell types in an organism. Numerous protocols describing the differentiation of mDA neurons from PSCs have been developed during the last decade. Initial approaches to generate human DA neurons were based on adaptations of mouse NS/PC and ESC protocols, which relied on the generation of embryoid bodies, the use of stromal cells or astrocytes as feeders, and the activation of a few key signaling pathways (SHH, FGF8, NURR1) to recapitulate some aspects of mouse embryonic development (Kawasaki et al., 2000; Kim et al., 2002; Lee et al., 2000; Wagner et al., 1999). Early human ESC differentiation protocols produced high numbers of TH^+ neurons (Park et al., 2005; Perrier et al., 2004; Roy et al., 2006; Zeng et al., 2004), but none of them generated cells co-expressing two transcription factors required for proper mDA neuron specification, FOXA2 and LMX1A. Early protocols resulted in incorrectly specified TH^+ cells (Perrier et al., 2004) that, although capable of releasing dopamine, survived poorly after transplantation (Park et al., 2005), and could overgrow and generate undesirable progeny (Roy et al., 2006; Zeng et al., 2004).

One of the first protocols to induce correctly specified mDA neurons from human ESCs (hESCs) was based on the forced expression of *LMX1A* in hESCs treated with SHH and FGF8 (Friling et al., 2009). The resulting cultures contained 50% TH^+ neurons, most of which co-expressed mDA markers, such as LMX1B, NURR1, PITX3 and DAT (Fig. 5G). Moreover, very few serotonin or motor neurons were detected in the cultures, suggesting selective mDA specification. A similar protocol produced human mDA neurons that released dopamine, exhibited appropriate electrophysiological properties and survived intracerebral transplantation for up to 5 months (Sanchez-Danes et al., 2012a). However, the *in vivo* functionality of human *LMX1A*-overexpressing cells in animal models of PD was not examined.

A more rigorous temporal implementation and recapitulation of morphogenic signals important for mDA neuron development improved the differentiation protocols. The use of dual SMAD inhibitors at the beginning of the differentiation protocol, to inhibit BMP, nodal, activin and TGFβ signaling, improved neural induction and eliminated the need for feeder layers (Chambers et al., 2009). However, this protocol relied on patterning with SHH and FGF8 and did not result in correctly specified mDA neurons (Fig. 5H). Subsequently, it was found that administration of

Development (2015) 142, 1918-1936 doi:10.1242/dev.097394

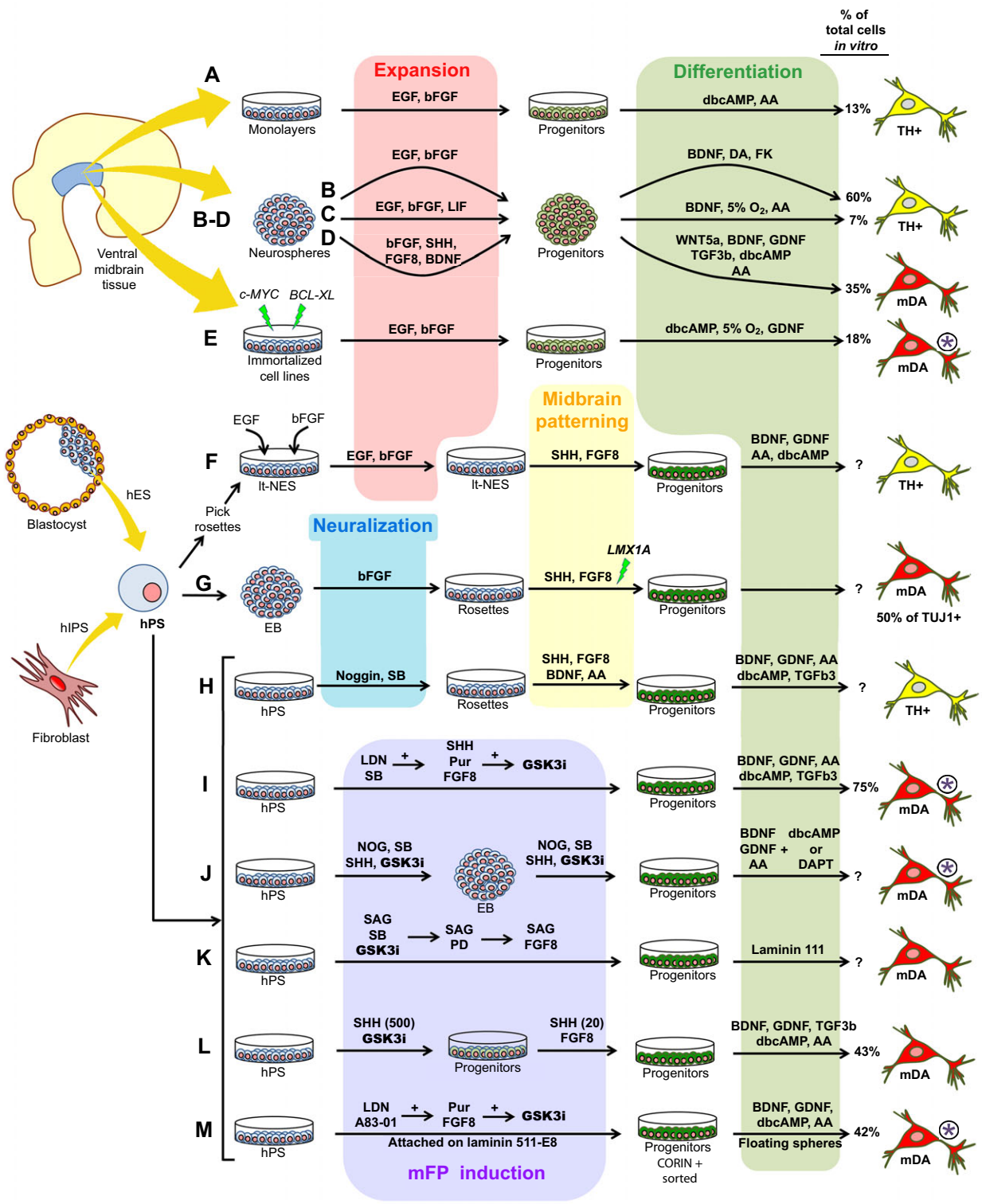

Fig. 5. Protocols for the differentiation of human NS/PCs (A-E) and PSCs (F-M) into mDA neurons. Human fetal VM tissue has been used for the expansion and differentiation of mDA progenitors/stem cells into TH^+ cells (yellow) or correctly specified mDA neurons (red). These cells have been grown as monolayers (A), neurospheres (B-D) or immortalized cell lines (E). Human PSCs (hPSCs) derived from blastocysts (ESCs) or reprogrammed fibroblasts (iPSCs) have been used to generate stable long-term neuroepithelial stem (lt-NES) cells (F), embryoid bodies (EB, G), or for direct differentiation into mDA neurons (H-M). References: A, Sanchez-Pernaute et al. (2001); B, Riaz et al. (2002); C, Maciaczyk et al. (2008); D, Ribeiro et al. (2012); E, Courtois et al. (2010); F, Falk et al. (2012); Koch et al. (2009); G, Friling et al. (2009); H, Chambers et al. (2009); I, Kriks et al. (2011); J, Kirkeby et al. (2012); K, Denham et al. (2012); L, Xi et al. (2012); M, Doi et al. (2014). Small molecules, such as GSK3β inhibitors (GSK3i), have allowed mFP induction and mDA specification. SMAD inhibitors (LDN, SB, A83-01) have substituted noggin (NOG). Shh has been substituted by the smoothened agonist (SAG) and/or purmorphamine (Pur). AA, ascorbic acid; DAPT, γ-secretase inhibitor; dbcAMP, dibutyryl cyclic adenosine monophosphate; FK, forskolin. Green lightning symbols in E and G indicate overexpression of the indicated factors. * Circled asterisk indicates cells that have shown functionality in animal models of PD.

Development (2015) 142, 1918-1936 doi:10.1242/dev.097394

high levels of SHH during neural induction was crucial for FP specification (Fasano et al., 2010). However, correct midbrain specification was not achieved until WNT/β-catenin signaling, an essential pathway in mDA neuron development (Andersson et al., 2013; Castelo-Branco et al., 2004, 2003; Chung et al., 2009; Joksimovic et al., 2009b; Prakash et al., 2006; Tang et al., 2010; Yang et al., 2013a), was implemented. Indeed, addition of GSK3β inhibitors allowed for the generation of correctly specified hPSC-derived mDA neurons in a reliable and efficient manner. This was first clearly demonstrated by the Studer group (Kriks et al., 2011), who produced cultures with 80% $FOXA2^+$ cells and 75% TH^+ cells (Fig. 5I). Further characterization of these cultures revealed abundant expression of genes involved in mDA neuron development and high levels of co-localization of LMX1A and FOXA2 in DA progenitors and neurons, indicating correct mDA specification. These cells exhibited biochemical and electrophysiological properties of mature mDA neurons, and, when transplanted in rodent models of PD, they survived, retained marker expression, did not form overgrowths and induced functional recovery in a battery of drug-induced and spontaneous motor-behavioral tests. Subsequently, the Parmar group used a similar protocol, but involving the formation of embryoid bodies (Kirkeby et al., 2012). This also led to correctly specified and functional mDA cells capable of long-term survival without overgrowth formation, which innervated target structures and improved both drug-induced and spontaneous motor behavior in a rat unilateral 6-OHDA model of PD, similar to fetal human midbrain tissue (Grealish et al., 2014). GSK3β inhibitors have been also successfully used in other protocols to produce correctly specified mDA neurons (Denham et al., 2012; Xi et al., 2012) (Fig. 5K,L). These protocols varied somewhat in the doses and the starting day of GSK3β inhibitor treatment, with day 3 mimicking closer midbrain development. Finally, a more recent study used laminin 511-E8 to promote adhesion of hPS-derived cells during mFP induction, followed by differentiation as floating spheres (Fig. 5M) (Doi et al., 2014). These protocols lead to correctly specified mDA neurons that survived transplantation and improved drug-induced circling behavior.

These recent reports emphasize the importance of implementing development-based protocols to recapitulate the *in vivo* exposure to morphogens such as WNT1 and SHH. In this context, it should be noted that GSK3β inhibitors have off-target effects (Bain et al., 2007) and that GSK3β regulates pathways other than WNT/β-catenin (Hur and Zhou, 2010). Moreover, activating WNT/β-catenin signaling in dopaminergic cells does not always lead to mDA differentiation, as found in the case of WNT3A (Andersson et al., 2013; Castelo-Branco et al., 2003). Future experiments should thus focus on examining whether other pathways are activated and the extent to which these are required for the observed effects. Furthermore, the need for other factors in WNT/β-catenin-activated cultures should be reassessed. For instance, in the absence of GSK3β inhibitors, a period of FGF/ERK signaling blockade followed by FGF8 treatment upregulated *Wnt1* and promoted mDA differentiation of ESCs (Jaeger et al., 2011). However, in the presence of GSK3β inhibitors, FGF8 was not required for mDA differentiation of ESCs (Kirkeby et al., 2012). In sum, understanding the precise effect of and requirement for different signaling molecules is essential in order to further refine current protocols, to develop cell replacement therapies and to selectively generate mDA neurons of the SNc/A9 subtype (Box 2), the cells that improve functional outcomes in animal models of PD (Grealish et al., 2010).

Direct reprogramming of somatic cells into mDA neurons

The discovery that mature somatic cells such as fibroblasts can be reprogrammed into iPSCs (Takahashi and Yamanaka, 2006) or other somatic cell types (Davis et al., 1987) by forced expression of transcription factors has revolutionized regenerative medicine, as it indicates that we might be able to generate any cell type from a mature somatic cell. Direct reprogramming relies on our capacity to directly (by forced expression) or indirectly (i.e. via microRNAs and small molecules) regulate the activity of lineage-specific transcription factors that reassign cell fate (Ladewig et al., 2013). To date, numerous somatic cell types have been generated via direct reprogramming, including induced DA (iDA) neurons that have been used for transplantation (Caiazzo et al., 2011; Dell'Anno et al., 2014; Kim et al., 2011; Liu et al., 2012b; Pfisterer et al., 2011; Torper et al., 2013) (Fig. 4D,E).

In vitro, induced neurons (iNs) have been obtained by forced expression of pan-neuronal factors in cells derived from the three germinal layers of the embryo (Fig. 6A). The most common factors used to reprogram fibroblasts (Vierbuchen et al., 2010), hepatocytes (Marro et al., 2011) or astrocytes (Torper et al., 2013) into iNs are *Brn1*, *Ascl1* and *Myt1l* (*BAM*). By contrast, the reprogramming of iDA cells (Fig. 6B) was achieved using midbrain-specific factors, such as *Lmx1a*, *Ascl1* and *Nurr1* (*LAN*) alone (Caiazzo et al., 2011) or together with additional factors, such as *FoxA2*, *En1* and *Pitx3* (Kim et al., 2011). In both cases, the resulting iDA cells showed expression profiles resembling more closely those of mDA neurons than of fibroblasts. iDA cells expressed endogenous *Lmx1a* (Caiazzo et al., 2011) or endogenous *Nurr1* and *Pitx3* as well as *eGFP* from the endogenous *Pitx3* locus (Kim et al., 2011). Furthermore, the rodent-

Box 2. DA neuron subpopulations

mDA neurons are traditionally classified by their anatomical position, projection field, function and marker expression. SNc/A9 DA neurons primarily express *Aldh1a1* and *Girk2*/*KCNJ6* (G protein-regulated inward rectifier K^+ channel subfamily-J member-6), whereas VTA/A10 neurons predominantly express the calcium-binding proteins *Calb1*, *Calb2* (calbindin 1 and 2) and *Cck* (cholecystokinin) (Veenvliet et al., 2013).

Recent studies have shown that *Otx2* is predominantly expressed in VTA/A10 neurons in the developing and adult brain (Chung et al., 2005a; Di Salvio et al., 2010a,b) and is selectively required for the specification (Di Salvio et al., 2010a) and neurogenesis (Di Giovannantonio et al., 2013) of VTA/A10 neurons. Similarly, *Wnt1* hypomorphic mice (swaying, $Wnt1^{SW/SW}$) exhibit a complete loss of VTA, but not SNc mDA neurons, a phenotype transiently phenocopied by *Fgf8* conditional knockout ($En1^{Cre}$) mice, at E12.5 (Ellisor et al., 2012). On the contrary, conditional deletion of *Foxa1* and *Foxa2* ($DAT^{cre/+}$;*Foxa1*/*2*) showed a greater loss of *Girk2* and SNc/A9 than *Calb1* and VTA/A10 neurons (Stott et al., 2013). Similarly, deletion of *Sox6*, a factor predominantly expressed in SNc/A9 neurons, reduced the number of TH^+/$Aldh1a1^+$ SNc/A9 neurons, while increasing that of TH^+/Cb^+ VTA/A10 neurons, indicating that *Sox6* is required for the specification of A9 neurons (Panman et al., 2014). Thus, whereas *Otx2* and *Wnt1* are predominantly required for the specification of VTA/A10 neurons, *Foxa1*/*2* and *Sox6* are mainly necessary for the specification of SNc/A9 neurons. Combined, these results suggest that the balance between the Otx2-Wnt1-Lmx1a/b and the Shh-Foxa1/2 pathways (Fig. 2) control mDA neuron subtype specification.

However, our knowledge of the molecular differences between these cells is still limited, and it is possible that additional genes are ultimately responsible for subtype identity. Interestingly, five molecularly distinct mDA neuron subtypes in mice have been suggested very recently (Poulin et al., 2014). Studies examining unbiased global gene expression in single human mDA neurons are required to confirm and identify novel, molecularly defined mDA neuron subtypes.

Development (2015) 142, 1918-1936 doi:10.1242/dev.097394

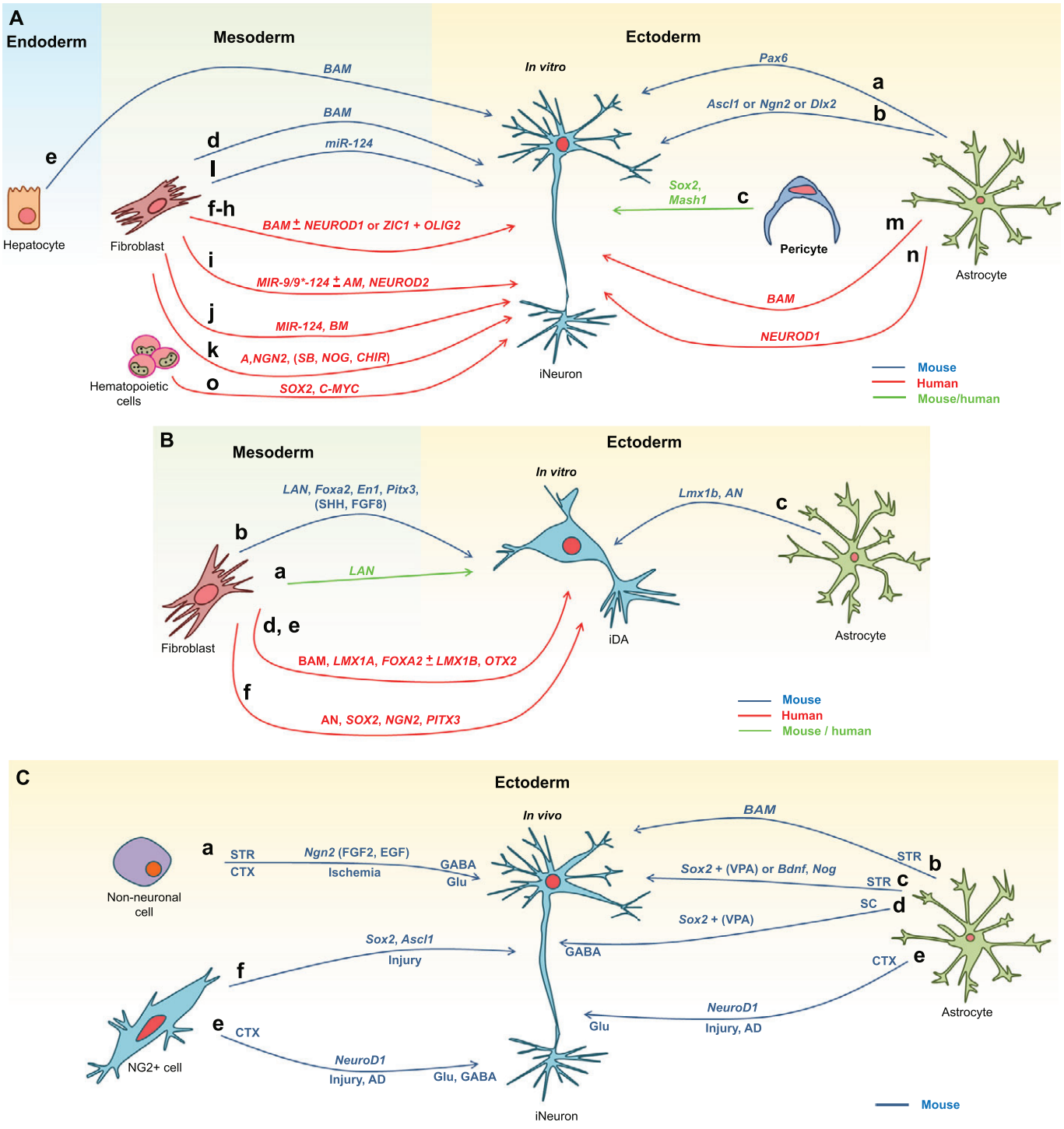

Fig. 6. Direct reprogramming of somatic cells into induced neurons and dopamine neurons. Cells from different species (mouse, blue arrows; human, red arrows; both, green arrows) and germ layers (endoderm, blue area; mesoderm, green area; ectoderm, yellow area) have been reprogrammed into neurons. (A) Schematic representation of the protocols used to obtain induced neurons (iNs) *in vitro*, from hepatocytes, fibroblasts, hematopoietic cells, pericytes and astrocytes. Proteins and small molecules are in parentheses. Factor combinations: *BAM* (*Brn1*, *Ascl1* and *Myt1l*); *LAN* (*Lmx1a*, *Ascl1* and *Nurr1*). References: a, Heins et al. (2002); b, Berninger et al. (2007); Heinrich et al. (2010, 2011); c, Karow et al. (2012); d, Vierbuchen et al. (2010); e, Marro et al. (2011); f, Pfisterer et al. (2011); g, Pang et al. (2011); h, Qiang et al. (2011); i, Yoo et al. (2011); j, Ambasudhan et al. (2011); k, Ladewig et al. (2012); l, Xue et al. (2013); m, Torper et al. (2013); n, Guo et al. (2014); o, Castaño et al. (2014). (B) Induced dopamine neurons (iDAs) have been generated *in vitro* from fibroblasts and astrocytes by different protocols. References: a, Caiazzo et al. (2011); b, Kim et al. (2011); c, Addis et al. (2011); d, Pfisterer et al. (2011); e, Torper et al. (2013); f, Liu et al. (2012b). (C) Induced neurons produced *in vivo* by reprogramming of endogenous mouse brain cells, such as astrocytes and NG2^{+} cells in the cerebral cortex (CTX), spinal cord (SC) or striatum (STR) *in situ*. Whereas cells with glutamatergic (Glu) or GABAergic traits (GABA) have been observed, iDA neurons have not yet been obtained from adult brain cells *in vivo*. References: a, Grande et al. (2013); b, Torper et al. (2013); c, Niu et al. (2013); d, Su et al. (2014); e, Guo et al. (2014); f, Heinrich et al. (2014). AD, Alzheimer's disease transgenic model; CHIR, GSK3b inhibitor; NOG, noggin; SB, Smad inhibitor; VPA, valproic acid.

derived cells exhibited mature DA neuronal properties as defined electrophysiologically and through dopamine release. Lastly, these two studies showed integration of murine-derived iDA cells into the mouse brain. The iDA neurons produced by Kim and colleagues were able to partially alleviate motor deficits in a mouse model of PD, but required tenfold more cells than fetal DA tissue grafts, indicating that their level of functionality is low (Kim et al., 2011). A slight variation of this protocol, using *Lmx1b* instead of *Lmx1a*, also reprogrammed primary mouse postnatal cortex astrocytes into functional iDA cells, although these have not been tested *in vivo* (Addis et al., 2011). Notably, the fact that somatic cell types derived from different germ layers, such as fibroblasts and astrocytes, can be reprogrammed into iDA cells by a similar combination of factors highlights the inherent robustness of such factors in the reprogramming process.

In an alternative strategy, a pan-neuronal identity was first induced with *BAM*, before further mDA neuron factors were added, including *Lmx1a* and *Foxa2* alone (Pfisterer et al., 2011) or in combination with *Lmx1b* and *Otx2* (Torper et al., 2013). These cells successfully engrafted in the striatum of a 6-OHDA rat model of PD, but very few cells survived after 6 weeks (Pfisterer et al., 2011), indicating that they do not behave like human fetal or hESC-derived mDA neurons. In another study, *Ascl1*, *Nurr1*, *Pitx3* and two additional factors, *Sox2* and *Ngn2*, but not *Lmx1a*, were used (Liu et al., 2012b), although, again, the resulting iDA cells engrafted in the striatum of 6-OHDA lesioned rats but survived poorly and did not exhibit a neuronal morphology.

More recently, the Broccoli group (Dell'Anno et al., 2014) demonstrated pan-DA marker expression, functional integration *in vivo* without tumor formation or overgrowths, and partial reduction of motor deficits in an *in vivo* model of PD by using mouse iDA cells reprogrammed with LAN. Expression of DREADDs (designer receptors exclusively activated by designer drugs) in iDA cells and treatment with the corresponding ligand further improved firing activity, dopamine release and behavioral recovery in a model of PD. These results represent an important milestone in the field as the efficiency of iDA cells was comparable to that of embryonic mDA neuron grafts. In the future, it will be important to perform similar studies with human iDA cells and examine their *in vivo* long-term expression of midbrain-specific markers.

Direct *in vivo* reprogramming of *in situ* adult mouse brain cells into neurons has become a reality in recent years (Fig. 4F), although iDA cells have not yet been obtained *in vivo*. Neurons have been reprogrammed from other types of neurons (De la Rossa et al., 2013; Rouaux and Arlotta, 2013), unidentified non-neuronal cells (Grande et al., 2013) (Fig. 6Ca), astrocytes (Guo et al., 2014; Niu et al., 2013; Su et al., 2014; Torper et al., 2013) (Fig. 6Cb-e), and $NG2^+$ cells (Guo et al., 2014; Heinrich et al., 2014) (Fig. 6Ce,f) have been reprogrammed into neurons *in vivo*. These neurons have shown mature electrophysiological properties, but their neuronal phenotypes are often incomplete and their numbers are low. Further developments are thus necessary to develop this method for cell replacement therapy for PD. Notably, injury promoted (Grande et al., 2013; Guo et al., 2014) or was required (Heinrich et al., 2014) for neuronal reprogramming *in vivo*, indicating that this strategy is particularly well suited for neural repair. Indeed, the reprogramming of glial cells into neurons would allow neurons lost by disease to be replaced, while reducing the excess of reactive glia, and regaining balance between glia and neurons in neurodegenerative diseases.

The induction of iDA cells: a regulatory logic?

The various combinations of reprogramming factors used to generate iDA neurons *in vitro* (Fig. 6B) all combine *Ascl1* with additional midbrain- and DA neuron-specific transcription factor genes. As *Ascl1* alone is sufficient to convert mouse fibroblasts into iN cells (Chanda et al., 2014; Vierbuchen et al., 2010), but is not required for mDA neuron development (Kele et al., 2006), *Ascl1* is likely to play a generic neurogenic or neuronal reprogramming role and has been proposed to act as a pioneer factor (Wapinski et al., 2013). Another gene widely used for the induction of mDA fate is *Lmx1a*, a known fate determinant required for mDA neuron development (Andersson et al., 2006b; Deng et al., 2011), which is essential for mDA neuron programming and a core component in direct iDA reprogramming. Other midbrain transcription factor genes, such as *Nurr1*, *Foxa2* and *Pitx3*, appear dispensable as single genes in some of the protocols, but in combination with *Ascl1*, and at least one other mDA transcription factor, contribute to improve iDA reprogramming. Thus, currently available data suggest a model in which the acquisition of an iDA phenotype requires the combination of the pioneer factor *Ascl1*, *Lmx1a* as a core iDA reprogramming factor, and at least one or more mDA neuron transcription factor, as helpers in iDA reprogramming. Combined, these results indicate that iDA reprogramming follows basic developmental principles and emphasizes the importance of understanding the mechanism of action of developmental factors in the context of reprogramming. In the future, it will be important to characterize the transcriptional networks and phenotype of iDA cells in order to determine whether a mature mDA-like iDA cell can be generated, whether different protocols lead to similar or distinct iDA cells and whether different cells correspond to separate routes and/or stages of reprogramming.

Conclusions, perspectives and future directions

Our knowledge of the developmental mechanisms that regulate mDA neuron development, as well as the tools currently available for generating mDA neurons (cells, factors and protocols), have grown exponentially in the last few years. These developments have generated optimism in the regenerative medicine field with regard to the future development of cell replacement therapy and drug discovery for PD. However, issues important for mDA neuron development and phenotype stability, such as genetic and epigenetic regulatory mechanisms or the strength of signaling, are still poorly understood.

Reprogrammed iDA cells for cell transplantation

As discussed above, direct reprogramming is currently emerging as a possible alternative to stem cell-derived mDA neurons for cell replacement therapy. The advantages of such a method would be that it could avoid the generation of proliferative immature cells, reducing the risk of tumor/outgrowth formation, and autologous cells would not require immunosuppression. However, it remains to be determined whether the phenotype and functionality of directly reprogrammed human iDA cells is the same as human mDA neurons derived from other sources, and whether transplanted human iDA cells are sufficiently stable and safe to be used for long-term correction of functional and behavioral deficits in models of PD. Progress in the generation of iDA cells will thus require: (1) A better understanding and ability to optimize the iDA reprogramming mechanisms, potentially by exploiting novel microRNAs or small molecules; (2) a detailed characterization of the iDA phenotypes generated by distinct reprogramming factor combinations, and of phenotype stability compared with endogenous DA cells; (3) determining the safety of the method with regard to insertional mutagenesis, partial reprogramming, proliferation and host inflammatory/immune response; (4) for *in vivo* iDA reprogramming, the development of

Development (2015) 142, 1918-1936 doi:10.1242/dev.097394

cell type-specific efficient reprogramming methods to selectively target the desired cell type whilst avoiding possible damage to other cells. So far, no iDA cells have been produced from endogenous adult brain cells *in vivo*, and selective targeting has been achieved by using Cre-recombinase or tetracycline transactivator (tTA) driver mouse lines. Viruses or carriers capable of selectively, efficiently and safely targeting specific cell types will be necessary in the future.

Towards cell replacement therapies for PD with PSCs

Recent protocols for generating mDA neurons from hPSCs offer the possibility of generating an unlimited number of safe and functional cells *in vivo* (Kriks et al., 2011) that survive for up to 6 months. In addition, they are capable of innervating distant target structures (Grealish et al., 2014) and modulating the glutamatergic input to striatal medium spiny neurons (Steinbeck et al., 2015). This has been made possible because of an improved understanding of mDA neuron development and a focus on studying human cells, overcoming species differences and allowing the preclinical development of stem cell-based cell replacement therapy for PD. Future challenges include improving our understanding of mDA neuron subtype specification and developing more effective protocols to generate human SNc/A9 mDA neurons. Strategies to improve protocols might involve cell sorting (Box 3), the incorporation of novel developmental factors to current differentiation protocols, as well as achieving the right signaling level or balance between different factors and pathways (Box 2; Fig. 2). Shorter protocols, for example starting from hPSC-derived mDA progenitors, would also facilitate the development of clinical applications by saving time and costs. All these protocols will need to be upscalable, use defined human components and be thoroughly tested in order to be good manufacturing practice (GMP) compliant and thus useful for cell replacement therapy in the clinic. Moreover, the functionality of GMP-produced hPSC-derived mDA neurons should also be examined in animal models of PD. For use in replacement therapy, cells must meet a number of criteria, including the ability to (1) not only survive transplantation, but integrate without excessive growth; (2) acquire a stable mature phenotype *in vivo* with the loss of immature markers in long-term surviving grafts; (3) re-innervate the host striatum, but no other targets; (4) release dopamine and exhibit appropriate electrophysiological properties; (5) induce functional recovery in sensorimotor behavioral tests relevant to PD; and (6) attain an homogeneous population of highly efficient SNc/A9 cells in order to reduce the number of cells required for transplantation. Finally, transplantation in primates might be useful in order to prepare clinical trials and ascertain the degree of fiber outgrowth and functional capacity of the cells, as well as issues of immunogenicity in the case of allogeneic or autologous iPSC/iN work.

The challenges facing cell replacement therapy in PD are great because new therapies need to be competitive against existing therapies, such as L-DOPA or DBS. The experience from successful open label trials using fetal hVM tissue suggests that cell replacement can be a competitive therapy. However, in order to further develop this strategy, new double-blind fetal tissue clinical trials with improved structure are needed (Evans et al., 2012). Future trials will also probably involve the use of hPSCs, as they are more amenable and upscalable sources of mDA neurons. hESCs are currently considered safer than iPSCs, as they do not require genetic modification. However, iPSCs can be generated with safer non-integrative methods, such as RNA or protein transduction or small molecules (Hargus et al., 2010; Kiskinis and Eggan, 2010; Sundberg et al., 2013). The advantage of iPSCs is that they can be derived from readily accessible autologous somatic cells, which is ethically less controversial. Moreover, PD-iPSCs would not require immunosuppression, but they might carry genetic or environmental damage, predisposing them to the development of PD. Avoiding this problem would involve correction of the cells or the use of immunosuppression with hESCs or hiPSCs derived from healthy individuals. Either way, the creation of hPSC banks covering the main human leukocyte antigens (HLAs) might allow treatment of multiple patients.

Reprogrammed cells as tools for disease modeling and drug discovery

PD iPSC-derived mDA, but not PD iDA cells, have been so far used for PD modeling. The main advantage of human PD iPSC-derived mDA cells compared with non-reprogramming strategies is that

Box 3. Selection of mDA cells for transplantation

In many cases, differentiation and reprogramming protocols generate heterogeneous populations of cells. Fluorescence-activated cell sorting (FACS) can be used both to remove undesired cell types and to enrich for mDA neurons or subtypes thereof. This approach has been used to select for neural fate, by using markers such as the glycoprotein CD133 (prominin 1, PROM1) and NCAM1 (neural cell adhesion molecule 1, or CD56) (Pruszak et al., 2007). For transplantation studies (Fig. 4E), committed, but not fully differentiated mDA rodent cells (Jönsson et al., 2009; Villaescusa and Arenas, 2010) at a $NURR1^{+}$ neuroblast stage (Ganat et al., 2012), have been found to perform best.

More recently, $NCAM^{+}CD29^{low}$ (CD29 for integrin beta 1 or ITGB1) cells were sorted from a population of human PSCs differentiated with the midbrain floor plate protocol (Sundberg et al., 2013) to remove non-neural tumorigenic cells and enrich for *FOXA2*, *LMX1A* and TH^{+} neurons. The transplantation of these cells led to improved behavioral recovery in an animal model of PD (Sundberg et al., 2013). Similarly, human iPSCs sorted with CORIN and expressing the mDA markers FOXA2 and LMX1A survived grafting in 6-OHDA lesioned rats and induced behavioral recovery without tumor formation (Doi et al., 2014). However, sorting protocols that enrich for SNc/A9 cells have yet to be developed, and it is also important to eliminate cell types such as serotonergic cells that might cause side graft-induced dyskinesias (Carlsson et al., 2009, 2007; Politis et al., 2010).

Box 4. Generation of mDA cells for *in vitro* PD modeling

With the advent of reprogramming technologies, PD-specific iDA cells and iPSC-derived DA neurons have become very attractive options for PD modeling (Fig. 4G). Thus far, studies have focused on PD-iPSCs carrying *LRRK2* mutations (Cooper et al., 2012; Liu et al., 2012a; Nguyen et al., 2011; Reinhardt et al., 2013; Sanchez-Danes et al., 2012b; Sanders et al., 2014), *SNCA* triplication (Byers et al., 2011; Devine et al., 2011), $SNCA^{A53T}$ mutation (Ryan et al., 2013; Soldner et al., 2011), *PINK* mutations (Cooper et al., 2012; Seibler et al., 2011) or *GBA1* heterozygosity (Mazzulli et al., 2011; Schöndorf et al., 2014). Most of these studies relied on protocols with suboptimal WNT/β-catenin activation, which do not give rise to correctly specified mDA neurons. However, those studies using sufficient WNT/β-catenin activation led to cell preparations with variable percentages of mDA neurons, ranging from 20% (Schöndorf et al., 2014) to 70% (Ryan et al., 2013).

As alterations in PD are cell-type specific and context dependent, it is necessary to further improve current mDA differentiation protocols for PD-iPSCs. Key considerations include the generation of control and PD-iPS cells with identical genetic backgrounds (Hockemeyer et al., 2009; Maetzel et al., 2014; Wang et al., 2013; Zou et al., 2009), improving yield of correctly specified and functional SNc/A9 neurons, reducing the culture time by starting from stable intermediates (e.g. mDA progenitors) and developing high content assays, in which the cells of interest and their phenotypes are examined at a single-cell level at an appropriate spatial and temporal resolution (Jain and Heutink, 2010; Xia and Wong, 2012).

modeling is performed in human cells directly derived from PD patients (Box 4). This allows the study of features related to the genetic background of the patients, such as mutations, haplotypes or polymorphisms associated with the development of certain types and features of PD. Another advantage of reprogrammed cells is that, compared with postmortem samples, they can capture early stages of the disease, which might be crucial for the development of therapies aimed at preventing neurodegeneration. Reprogrammed cells can be used to generate other cell types affected in PD or even create neural organoids (Kadoshima et al., 2013; Lancaster et al., 2013), which have opened up the possibility of modeling features related to the anatomical or functional organization of cells in a tissue. So far, correctly specified PD-iPS-derived mDA neurons have been successfully used to model oxidative and nitrosative stress (Ryan et al., 2013), as well as autophagic defects in PD (Schöndorf et al., 2014). PD-iPSCs are thus considered the cell type of choice for high-throughput screening (HTPS) and drug development.

Gene editing technologies, such as zinc-finger nucleases, TALENS or the *CRISPR/Cas9* system, are currently being used to correct or introduce PD mutations and control for the genetic background of PD-iPSCs (Chung et al., 2013; Liu et al., 2012a; Reinhardt et al., 2013; Ryan et al., 2013; Sanders et al., 2014; Schöndorf et al., 2014; Soldner et al., 2011). These methods can be used to introduce/correct multiple mutations and examine epistatic interactions between PD genes, allowing more complete iPSC models in which several features of PD are simultaneously examined. In sum, multiple developments in this fascinating area of research are likely to improve *in vitro* modeling and drug discovery for PD, which, in turn, should contribute to novel therapeutic interventions.

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