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Direct Cell Conversion of Somatic Cells into Dopamine Neurons: Achievements and Perspectives

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Abstract

In the last decade, direct reprogramming has emerged as a novel strategy to obtain mature and functional dopamine neurons from somatic cells. This approach could overcome issues linked to the use of human pluripotent stem cells such as ethical concerns and safety problems that can arise from the overgrowth of undifferentiated cells after transplantation. Several conversion methodologies have been developed to obtain induced DA neurons (iDANs) or induced DA neuron progenitors (iDPs). iDANs have also proved to successfully integrate in mice striatum, alleviating Parkinson's disease (PD) motor symptoms. In the next decade, human iDANs and/or iDPs could be translated to clinic to achieve a patient-tailored therapy, but current critical issues hinder this goal, such as the low conversion rate of adult human fibroblasts and the risks associated with lentiviral delivery of conversion factors. In this study, we summarize the strategies and recent improvements developed for the generation of mouse and human iDANs/iDPs. Furthermore, we discuss the more recent application of *in vivo* direct conversion, which may enable clinical therapies for PD by means of brain *in situ* delivery of dopaminergic reprogramming transcription factors.

Keywords: cell reprogramming, transdifferentiation, cell therapy, Parkinson's disease

Introduction

In the last 70 years, several studies demonstrated that the differentiation process is not unidirectional and irreversible (Graf, 2011). In 2006 and 2007, Shinya Yamanaka and colleagues showed that mouse and human somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) by overexpressing four transcription factors (TFs, Takahashi and Yamanaka 2006; Takahashi et al, 2007). Afterward, a new wave of cell reprogramming approaches was developed, especially in the field of direct cell conversion (or transdifferentiation). Direct cell conversion works by manipulating the expression of specific cell lineage TFs that are crucial during embryonic development (Graf et al, 2011) bypassing pluripotency, thus overcoming the concomitant erasure of epigenetic information (Silva and Smith, 2008) and the potential risks linked to iPSCs tumorigenicity (Wernig et al, 2008).

This new approach represents a promising alternative in the treatment of neurodegenerative diseases, in particular Parkinson's disease (PD), whose main pathological hallmark is the gradual loss of midbrain dopamine (DA) neurons. Consequently, the generation of pure or enriched cultures of midbrain DA neurons is of a special interest for PD treatment and modeling.

A successful direct conversion of induced DA neurons (iDANs) is often claimed on the basis of positive immunostaining for enzymes involved in the catecholamines synthesis (TH, tyrosine hydroxylase; DDC, DOPA decarboxylase), metabolism (VMAT2, vesicular monoamine

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transporter; DAT, DA transporter; DRD2, DA receptor D2), midbrain identity (NURR1, PITX3, FOXA1/2, CORIN, LMX1A/B), and on the proper electrophysiological functions, including the spontaneous action potential (AP) firing. However, the nonproliferative nature of iDANs limits their broad application. Alternative protocols focused on the production of induced DA neuron progenitors (iDPs), which can expand to the desired yield for transplantation purposes. These cells express the neuronal progenitor markers, including SOX1, PAX6, NESTIN, and ventral midbrain genes, but are not fully differentiated in mature DA neurons.

More than a decade of research has shown that different somatic cells can be directly reprogrammed to iDANs or iDPs (Caiazzo et al, 2011; Han et al, 2021; Kim et al, 2014; Kim et al, 2011; Lim et al, 2015; Liu et al, 2014; Ng et al, 2021; Oh et al, 2014; Tian et al, 2015; Yang et al, 2019) (Table 1).

Most of the approaches are based on the viral-mediated expression of TFs that are crucial for the development of ventral midbrain DA neurons. Subsequent investigations were made to further improve this conversion method by combining the expression of TFs with small molecules, biophysical cues, or noncoding RNAs.

In this study, we briefly outline the achievements and major hurdles met in direct conversion methods to generate iDANs and the subsequent perspectives for the treatment of PD.

TF-Mediated Direct Reprogramming of Fibroblasts into DA Neurons

In 2010, Marius Wernig's group identified three neurodevelopmental factors (ASCL1, BRN2, MYT1L; ABM) necessary to directly convert fibroblasts into induced neurons (iNs)(Vierbuchen et al, 2010).

Based on Wernig's ground-breaking study, several groups screened different reprogramming cocktails to generate iDANs by direct cell conversion. Malin Parmar's group showed that human iNs can be further patterned toward DA cell fate by overexpressing the TFs FOXA2 and LMX1A, which are able to convert human embryonic fibroblasts to DANs when combined with the ABM factors (Pfisterer et al, 2011). This study represents the first evidence of direct conversion from somatic cells toward a DAN phenotype. On the contrary, this study includes limited evidence of the functional properties of the transdifferentiated neurons, especially regarding the DA-specific features (*i.e.,* DA content and release, pacemaking activity) and reprogramming dynamics.

In an independent concomitant effort, Vania Broccoli's group (Caiazzo et al, 2011) selected several TFs for the direct reprogramming of mouse embryonic fibroblasts (MEFs) into iDANs based on DAN development literature. This screening found ASCL1, NURR1, and LMX1A (ANL) as the minimal combination of TFs required to directly reprogram both mouse and human fibroblasts into DA neurons. As expected, no intermediate progenitor stage was detected, whereas converted cells expressed many of the distinctive components of the DA machinery, such as TH, VMAT2, and DAT, and did not express markers associated with adrenergic or serotonergic neurons. Mouse and human iDANs express morphological and electrophysiological properties of mature DA neurons, including DA synthesis and release and pacemaking activity (Caiazzo et al, 2011).

This work also reports the first example of PD patient transdifferentiated DA neurons, even if with a low efficiency of conversion ($3\% \pm 1\%$). Concomitantly, Rudolph Jaenisch's group used a similar TF reprogramming cocktail (ASCL1, NURR1, PITX3, LMX1A, FOXA2, and EN1) together with the dopaminergic morphogens FGF8 and SHH (Kim et al, 2011). This work also shows the first example of a PD animal model treated with iDANs.

Oh and colleagues made a further adjustment to the reprogramming protocol to convert MEFs into iDANs with a higher efficiency (33% $TH^+/TUJ1^+$, Oh et al, 2014). MEFs were transduced with ASCL1 and NURR1 and cultured in a specific neural medium supplemented with a sequential cocktail of growth factors (bFGF and EGF) and morphogens (SHH and FGF8b) to allow the differentiation into mature DANs. They demonstrated that the number of TFs required for direct reprogramming can be reduced, thereby reducing the number of retroviral vectors needed to transduce the cells. At the same time, the conversion rate can be increased by fine tuning the culture conditions.

An alternative approach to generate iDANs was proposed by Broccoli group by overexpressing ANL factors in human embryonic stem cells (ESCs) to obtain TH-positive neurons in a fast (3 weeks) one-step protocol (Theka et al 2013). More recently, Marius Wernig's group started from this approach, but focused on the midbrain regional identity of ESC-iDANs. This represents a major aim for regenerative medicine, since iDANs could be eventually transplanted in PD patients and improperly specified cells may suffer from limited therapeutic potential. Eventually, they reported that the coexpression in human ESCs of ANL together with the additional midbrain-specific TFs EN1, FOXA2, and PITX3 can skew the lineage induction toward a midbrain phenotype (Ng et al, 2021). Anyways, the authors did not show a comparable effect on transdifferentiated MEFs, probably for the low coefficiency of infection using several viruses.

Generation of Induced DA Neuron Progenitors

Kim and colleagues hypothesized that DA progenitor cells may be a valuable alternative to terminally differentiated DA neurons for transplantation, since terminally differentiated cells have an increased chance to survive the engraftment procedure (Kim et al, 2014). They used a transient expression of the iPSC reprogramming factors (OCT4, SOX2, KLF4, and C-MYC) combined with the use of the dopaminergic morphogens FGF8 and SHH, to convert mouse fibroblasts into proliferating midbrain iDPs, which are significantly more potent than induced neural stem cells (iNSCs) in generating DANs.

Jialin Zheng's group used a different combination of TFs, BRN2, SOX2, and FOXA2 to convert mouse adult dermal fibroblasts to iDPs without using FGF8 and SHH. This indicates that these three factors are sufficient to induce cell precursors with dopaminergic-restricted differentiation potential and confirms the higher potential of iDPs in differentiating into DA neurons with respect to induced neuronal precursor cells (iNPCs)/iNSCs (Tian et al, 2015).

In a recent study, a similar protocol was used to generate human iDPs (He et al, 2019). However, generation of iDPs was inefficient when the conversion method was used on fibroblasts derived from a 39-year-old PD patient, posing the urge for the optimization of the protocol for future cell-based therapy of PD patients.

Table 1. Methods Used to Date, for Direct Reprogramming of Mouse and Human Fibroblasts into Dopaminergic Neurons or Dopaminergic Precursors

Authors	*Source cells*	*Induced cells*	*Conversion factors*	*Efficiency*	*Time conversion; iDAN markers*	*Transplantation; a. Functional integration; b. Relief of PD symptoms*
Pfisterer et al (2011)	Human fibroblasts	iDANs	ASCL1, BRN2, MYT11, LMX1A, FOXA2	4.3% $MAP2^{+}$[a]	24 days[b] TH	No
Caiazzo et al (2011)	Mouse and human fibroblasts	iDANs	ASCL1, NURR1, And LMX1A	$18 \pm 3\%TH^{+}/TUJ1^{+}$in mouse; $3 \pm 1\%$ $TH^{+}/TUJ1^{+}$ in adult human cells[c]	16 days[b] TH, VMAT2, ALDH1A1	No
Kim et al (2011)	Mouse embryonic fibroblasts	iDANs	ASCL1, PITX3, LMX1A, NURR1, FOXA2, EN1, SHH, FGF8	9.1% $Pitx3\text{-}eGFP^{+}$[c]	18 days[b] TH, TUJ1, DAT, AADC	Yes; a. TH staining in transplanted striatum; b. reduction in amphetamine-induced rotation scores
Addis et al (2011)	Mouse postnatal astrocytes	iDANs	ASCL1, NURR1, LMX1B	$18.2 \pm 1.5\%$ $TUJ1^{+}/TH^{+}$ neurons[a]	17 days[b] TH, TUJ1, GIRK2, SYNAPSIN	No
Liu et al (2012)	Human fetal lung fibroblasts	iDANs	ASCL1, NGN2, SOX2, NURR1, PITX3	Less than 4% iDANs[a]	12–21 days[b] TH, TUJ1, DDC, DAT	Yes a. HN (human nuclear protein), TH, DDC, DAT staining in the graft b. reduction in amphetamine-induced rotation scores
Theka et al (2013)	Human ESCs and iPSCs	iDANs	ASCL1, NURR1, LMX1A	$\sim$30% of $TUJ1^{+}/TH^{+}$ neurons[a]	21 days[b] TH, TUJJ1, DDC, VMAT2, ALDH1A1, Calbindin, GIRK2	No
Kim et al (2014)	Mouse embryonic fibroblasts	iDPs	OCT4, SOX2, KLF4, AND C-MYC, SHH, FGF8, JAK and GSK3b inhibitors	$57.2 \pm 7.2\%$ $TH^{+}/TUJ1^{+}$ Neurons[a]	13 days[b] PAX2, LMX1A, MSX1, NGN2, FOXA2, CORIN	No
Oh et al (2014)	Mouse fibroblasts	iDANs	ASCL1, NURR1 SHH, FGF8b	33% $TH^{+}/TUJ1^{+}$ neurons[a]	34 days[b] TH, LMX1A, AADC, VMAT2	No
Pereira et al (2014)	Human embryonic fibroblasts	iNs	ASCL1, BRN2, MYT1L, CHIR99021, SB431542, Noggin, LDN-193189	$61 \pm 4\%$ $MAP2^{+}$[c]	16–18 days[b] MAP2	Yes, <4% ability to generate TH neurons *in vivo*; a. Not assessed; b. Not assessed
Liu et al (2014)	Human lung fibroblasts	iDANs	MASH1, SOX2, NGN2, NURR1, PITX3 and dominant-negative P53	$35.53\% \pm 2.20\%$ $TUJ1^{+}$ neurons[a]	20 days[b] TH, DAT, DRD2, VMAT2	Yes, a. TH staining in coronal sections; b. reduction in amphetamine-induced rotational scores

(*continued*)

Table 1. (Continued)

Authors	*Source cells*	*Induced cells*	*Conversion factors*	*Efficiency*	*Time conversion; iDAN markers*	*Transplantation; a. Functional integration; b. Relief of PD symptoms*
Lau et al (2014)	Human fetal lung fibroblasts	iNs	miR-124 regulated Ascl1, Brn2, and Myt1L	15% $NCAM^+$ cells[c]	24 days[b]	No
Tian et al (2015)	Mouse fibroblasts	iDPs	BRN2, SOX2 and FOXA2, SHH, and FGF8	TH 90%/$TUJ1^+$ Neurons[a]	14 days[b] NESTIN, ALDH1A1, CORIN, LMX1A, MSX1, NGN2, OTX2, MASH1, PITX3, NKX6.1	Yes; a. TH and TUJ1 double positive cells; b. increased rotarod performance but no significant recovery of motor deficits
Jiang et al (2015)	Human fetal lung fibroblasts	iDANs	ASCL1, NURR1, LMX1A P53 shRNA	$15.4 \pm 1.1\%$ TH^+ Cells[a]	10 days[b] TH, TUJ1	No
Park et al (2015)	Drug inducible mouse embryonic fibroblasts	iDANs	ASCL1, PITX3, NURR1, LMX1A	68% Pitx3-GFP^+ cells[c]	7 days[b] TH, DAT, TUJ1, AADC, VMAP2, GIRK2	Yes; a. not complete integration: no difference in spontaneous exploratory forelimb test a. reduction in amphetamine-induced rotational scores
Wang et al (2016)	Mouse embryonic fibroblasts	iDANs	SB431542, Noggin, RA, SHH, bFGF, EGF, GDNF, FGF8b.	$\sim$20% $TH^+/TUJ1^+$ cells[a]	3/4 weeks post chemical induction TH, EN1	No
Rivetti di Val Cervo et al (2017)	Mouse astrocytes *in vivo*	iDANs	NEUROD1, ASCL1 and LMX1A, and miR218	16% induced DA neuron[a]	18 days[b] *in vitro* 2 weeks *in vivo* TH, MAP2, SYN1, DDC, DAT, GIRK2, ALDH1A1 *in vitro*	No *In vivo* conversion; a. TH+ iDANs in the striatum b. reduction in apomorphine-induced circling behavior; no effect on amphetamine-induced rotations; complete rescue of spontaneous circling behavior; rescue of coordinated limb usage; restoration of complete gait cycle; rescue of axial symmetry and gait.
Yoo et al (2015)	Mouse embryonic fibroblasts	iDANs	ASCL1, PITX3, LMX1A, NURR1 and nanogrooved substrates	4.41% Pitx3-GFP^{+c}	10 days after plating on nanogrooved substrates[b] PITX3	No

(*continued*)

Table 1. (Continued)

Authors	*Source cells*	*Induced cells*	*Conversion factors*	*Efficiency*	*Time conversion; iDAN markers*	*Transplantation; a. Functional integration; b. Relief of PD symptoms*
Yoo et al (2017)	Mouse and human fibroblasts *in vitro;* mouse astrocytes *in vivo*	iDANs	ASCL1, PITX3, LMX1A, NURR1 and EMF exposure	~60% Pitx3-eGFP^{+c}	10 days[b] PITX3	No *In vivo* conversion a. TH^{+} cells in host striatum b. Restoration of movement in open field test; Increase in rearing behavior;
De Gregorio et al (2018)	Mouse embryonic fibroblasts	iDANs	ASCL1, NURR1, miR-34b/c cluster	19.5% ± 2.4% TH^{+c}	14 days[b] TH, DAT, VMAT2	No
He et al (2019)	Human fetal fibroblasts	iDPs	BRN2, SOX2 AND FOXA2	~80% of TH^{+}/TUJ1^{+} neurons	20 days[b] CORIN, LMX1A, OTX2, MASH1, PITX3 AND NKX6.1	No
Zhou et al (2020)	Mouse muller glia and astrocytes in striatum mouse	iDANs	*Ptbp1* CasRx-knockdown	32% TH^{+} Cells of the *in vivo* infected cells	3 months after injection TH, GFAP	Yes; a. TH, GFAP double-positive cells b. reduction in apomorphine-induced circling behavior; reduction in amphetamine-induced rotational scores; improvement in contralateral forelimb use and rescue of motor coordination
Qian et al (2020)	Mouse midbrain astrocytes *in vivo*	iDANs	*Ptbp1*-shRNA	22% of cell reprogrammed *in vivo* expressed TH and GIRK2 (A9 DA neurons)/GFAP^{+} cells	12 weeks after injection DAT, VMAT2, EN1, LMX1, PITX3	No *In vivo* conversion a. TH^{+} RFP^{+} fibers in the nigrostriatal pathway b. reduction in apomorphine-induced circling behavior; reduction in amphetamine-induced rotational score; time-dependent improvement in contralateral forelimb use
Ng et al (2021)	Mouse and human ESCs	iDANs	ASCL1, NURR1, LMX1A, PITX3, EN1, FOXA2	Not reported	21 days (for functional analysis)[b]	No

[a]% converted cells/DAPI cells.
[b]Time conversion after reprogramming factors transduction/transfection.
[c]% converted cells/total cells plated.
DA, dopamine; eGFP, enhanced green fluorescent protein; EMF, electromagnetic field; ESCs, embryonic stem cells; iDANs, induced DA neurons; iDPs, induced DA neuron progenitors; iNs, induced neurons; iPSCs, induced pluripotent stem cells; PD, Parkinson's disease.

Enhancing TFs-Mediated Dopaminergic Reprogramming with Morphogens and Cell Cycle Inhibitors

Cell reprogramming can be accomplished by both transcriptional programming and activation of signaling pathways, such as WNT, FGF8, and SHH, which can synergize to induce iDANs (Kim et al, 2014; Kim et al, 2011; Oh et al, 2014).

Pereira and colleagues used a similar principle to optimize their conversion protocol from human fibroblasts. They described the highest conversion efficiency ($61\% \pm 4\%$ $MAP2^+$) to human iNs after combining small molecules that inhibit SMAD pathway and activate WNT signaling, with a delayed transgene activation after viral transduction of ABM factors (Pereira et al, 2014). Remarkably, adding small molecules does not affect the morphological complexity of iNs, and these neurons exhibit proper electrophysiological properties. The optimized protocol was next successfully applied to generate DANs by adding the dopaminergic fate determinants LMX1A, LMX1B, FOXA1, and OTX2.

The generation of a sufficient amount of human iDANs is a major concern in direct reprogramming. Human fibroblasts, in particular human adult fibroblasts, have shown to be less prone to transdifferentiation (Caiazzo et al, 2011; He et al, 2019; Pfisterer et al, 2011). The findings of Liu and colleagues shed light onto a mechanism that can represent a major hurdle for direct reprogramming, which is the activation of tumor suppressor gene p53 (Liu et al, 2014).

Based on previous studies (Hong et al, 2009; Marión et al, 2009; Menendez et al, 2010), Liu and colleagues tried to enhance TFs-mediated direct reprogramming by suppressing the cell cycle inhibitor p53 by means of a negative dominant p53. The strategy finally resulted in increased conversion efficiency ($35.53\% \pm 2.20\%$ $TUJ1^+$) of human lung fibroblasts by 5–20-fold (Liu et al, 2014). The inhibition of p53-p21 pathway increases the conversion rate by enhancing cell survival and not cellular proliferation, which would increase carcinogenic risk, suggesting the safety of the novel tool.

In a following study, Jiang and colleagues confirmed that p53 knockdown increases ANL-mediated reprogramming of human fibroblasts into iDANs (Jiang et al, 2015). Moreover, p53 knockdown in conjunction with proper culture conditions and cell cycle arrest at G1 leads to significantly increased transdifferentiation efficiency ($15.4\% \pm 1.1\%$ TH^+ cells) of human fibroblasts into functional DA neurons that exhibit DAN markers and correct dopaminergic transmission (Jiang et al, 2015). The three factors are crucial to attain a marked increase in direct reprogramming by synergistically inducing the epigenetic modifier TET1 (Jiang et al, 2015). This further demonstrates that the effects of p53 attenuation are independent of its actions on cell cycle, and involve the epigenetic remodeling (Jiang et al, 2015). Overall, these findings facilitate the understanding of molecular mechanisms associated with transdifferentiation and suggest the importance of epigenetic reprogramming in the transdifferentiation process.

Noncoding RNA Role in Dopaminergic Direct Reprogramming

MicroRNAs (miRNAs) are emerging as key regulators of self-renewal in DA neurons (Pascale et al, 2020) and their ability to enhance cell reprogramming has been well documented (Pascale et al, 2022).

miRNAs exhibit essential roles in brain development as they appear not only to be enriched in this organ (Petri et al, 2014) but also to be compartmentalized, in the neuronal contest, to control synaptic plasticity and local protein synthesis (Vo et al, 2010).

In the last few years, several groups used miRNAs in neuronal transdifferentiation approaches. The first evidence that miRNAs can be sufficient to generate transdifferentiated neurons was provided by Gerald Crabtree's group by using mir-124 and mir-9/9* (Yoo et al, 2011).

Soon after, Sheng Ding's group showed that by combining mir-124 and two TFs BRN2 and MYT1L, it is possible to obtain functional human hiNs starting from both dermal fibroblasts, which exhibit neuronal morphology, gene expression, and fire APs and produce functional synapses (Ambasudhan et al, 2011).

Eventually, Andrew Yoo's group used miRNA-based approaches to generate directly reprogrammed GABA striatal neurons (Victor et al, 2014). More recently, direct reprogramming of fibroblasts to iDANs was achieved by combining the TFs ASCL1 and NURR1 along with miR-34b-5p and miR-34c-5p (miR-34b/c). These iDANs exhibited spontaneous and induced electrophysiological properties and synthesized DA, thus confirming that miRNAs can help dopaminergic transdifferentiation (de Gregorio et al, 2018).

Enhancing Dopaminergic Reprogramming by Modulation of Biophysical Parameters

Cell microenvironment entails not only biochemical signals but also specific biophysical parameters, which are mainly regulated by extracellular matrix proteins, as well as by the surrounding mechanical forces (Yoo et al, 2015).

The study conducted by Yoo and colleagues proved that nanoscale substrate topography can promote the direct conversion of MEFs into functional iDANs (Yoo et al, 2015). Nanogrooved substrates generate a greater number of cells with iDANs morphology, both before and after APNL (ASCL1, PITX3, NURR1, and LMX1A) induction. Also, these iDANs express a noticeable extent of dopaminergic markers and exhibit proper electrophysiological functions.

This effect was due, in part, to the induction of cytoskeletal reorganization, which in turn drives remarkable epigenetic changes, probably by changing nuclear shape (Yoo et al, 2015).

Therefore, nanotopographical cues exert a synergistic effect that can amplify the biochemical stimulation. These findings were further confirmed by a more recent study, which describes the safe and efficient conversion of mouse fibroblasts into expandable iNPCs and iDANs on nanoscale pattern substrates (Lim et al, 2019).

Another physical factor that has proved to potentiate epigenetic reprogramming is the electromagnetic field (EMF) (Yoo et al, 2017). Electromagnetized gold nanoparticles exposed to EMF generate a dramatic increase in direct reprogramming of mouse fibroblasts into iDANs after ectopic expression of DA lineage TFs (Yoo et al, 2017). Remarkably, electromagnetic stimulation results in a specific activation of histone modifiers, which increases the accessibility of neuronal loci to APLN factors to enhance the reprogramming into iDANs (Yoo et al, 2017).

Medical Perspectives Use of Dopaminergic Reprogramming for PD

PD is characterized by the selective loss of DA neurons in the substantia nigra pars compacta and decreased dopaminergic innervation in the striatum (Koch et al, 2009). The consequent loss of DA availability in striatal tissue is responsible for the motor impairments that severely affect PD patients. To date, there is no cure for PD, and cell replacement therapy could replenish the striatal tissue with new DA neurons. Therefore, transplantation of iDANs represents a promising strategy to treat PD patients and restore their neurological functions (Fig. 1). In first place, directly reprogrammed DA neurons do not have the potential tumorigenicity of iPSCs-/ESCs-derived DA neurons.

On the contrary, the methods used for direct conversion still carry safety risks linked to the use of viral transduction of reprogramming factors that may lead to genome integration and inactivation of tumor suppressor genes. In this regard, alternative protocols have been explored and will be further discussed in the following paragraphs. In contrast to iPSCs induction, which reverts cellular age to an embryonic stage that remains even after differentiation into neurons (Lapasset et al, 2011), transdifferentiated neurons maintain the aging signatures of the donor cells (Mertens et al, 2018).

The aging signatures of iDANs might represent a limitation for cell replacement but, on the contrary, provide an unparalleled opportunity for investigating age-related neurodegeneration, which characterizes PD (Mattson and Magnus, 2006), reproducing more accurate models of the disease. This aspect is relevant for the development of novel parkinsonian drugs and for personalized therapy design (Fig. 1). Therefore, future studies may use iDANs in place of iPSCs-derived DA neurons to develop drugs that modulate age-related processes, including the accumulation of damaged proteins. Modeling PD with iDANs could be particularly useful for idiopathic cases where the pathological phenotype of diseased DANs is mainly due to the overall epigenetic/aging profile rather than a genetic mutation.

Finally, iDANs must be analyzed for their *in vivo* phenotypic and functional properties, whereas the clinical efficacy must be assessed in PD models. A major hurdle in this field is represented by the low viability and the integration of transplanted cells in functional circuits (Zhou et al, 2020). Also, currently existing behavioral measures for motor impairments are based on rodent models with bilateral/unilateral DA depletion that have shown to be difficult to assess. More standardized tests must be included, which evaluate neurological, locomotor activities, body balance, and coordination to have a reliable measure of the effect of *in vitro*/*in vivo* reprogrammed iDANs on PD phenotype.

Overcoming the Flaws of Direct Reprogramming by Means of Viral Infection

The strategies previously described for direct reprogramming may not be optimal for clinical use, since they use integrating viral vectors that carry a risk of genomic modifications and positional mutagenesis with the insertion of transgenes (Nienhuis et al, 2006; Okita et al, 2008).

A further complication results from the variable number of copy numbers of the integrated provirus and random genomic integration which consequently lead to high genomic heterogeneity of the reprogrammed cell population (Dell'Anno et al, 2014; Nienhuis et al, 2006; Okita et al, 2008).

In 2014, Lau and colleagues first described an approach for iN generation that circumvents these issues. They used integration deficient lentiviral vectors, which carry miR-124

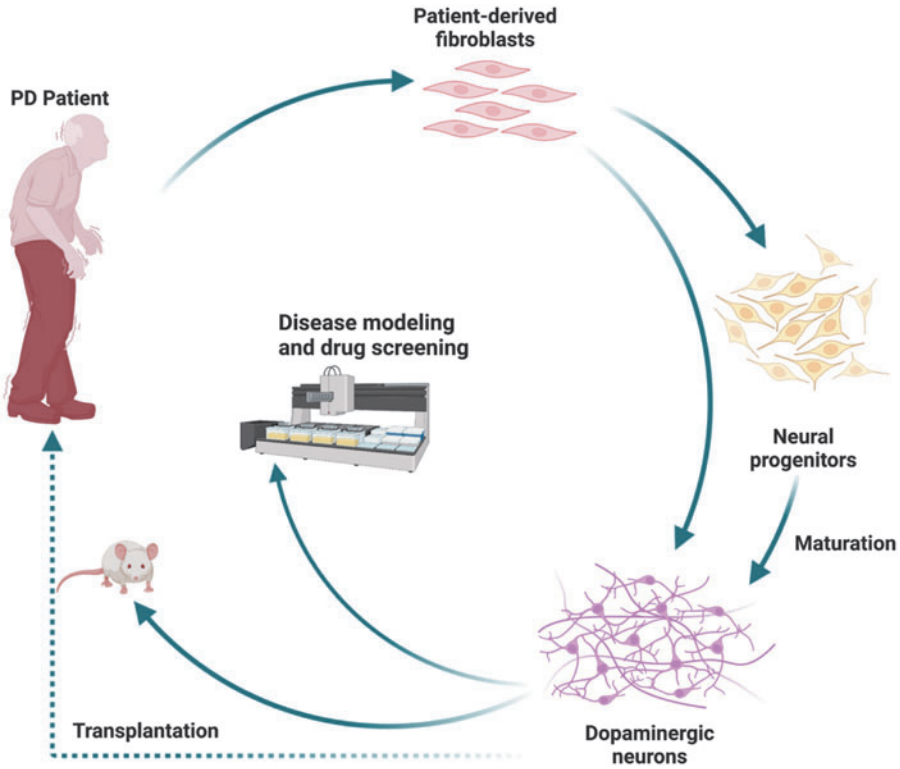

FIG. 1. Steps in direct reprogramming: fibroblasts are isolated from PD patients; they can then undergo direct reprogramming becoming either dopaminergic neurons or first converted into neural progenitors and then into DA neurons. The DA neurons are then transplanted for cell replacement therapy or used for disease modeling and drug screening. DA, dopamine; PD, Parkinson disease.

regulated conversion factors ABM (Lau et al, 2014). The resulting conversion efficiency (15% $NCAM^+$ cells) was lower respect to the integrative virus, since the non-integrative vectors are diluted upon proliferation, but this can be overcome by using a protocol with minimized proliferation after transduction, increased vector load, and/or number of vector transduction, which finally leads to a comparable conversion efficiency, purity, and morphology of iNs (Lau et al, 2014). Even so, superior electrophysiological properties were demonstrated for neurons induced by integrative and nonregulated viral vectors, demonstrating that this strategy needs further adjustments before it may be applied for the generation of human DA neurons.

Park and colleagues developed an alternative approach to efficiently generate genetically homogeneous iDANs, thus overcoming the heterogeneity and incomplete reprogramming associated with random viral integration (Park et al, 2015). They first generated mESCs engineered with a puromycin resistance gene, the enhanced green fluorescent protein (eGFP) gene targeted to the endogenous *Pitx3* locus, and the integrated proviruses *Ascl1*, *Pitx3*, *Nurr1*, and *Lmx1a* under the control of a dox-inducible promoter. So, they generated chimeras and, therefore, MEFs were prepared from these mice. In this way, they generated genetically homogenous and inducible MEFs that harbor a four TFs combination and only require dox administration for their expression (Park et al, 2015).

Mirakhori and colleagues conducted another viral-free protocol for induction of iDPs, which was based on a TAT-mediated protein transduction system that comprised a mixture of SOX2 and LMX1A, in combination with small molecules to generate iDPs (Mirakhori et al, 2015). Shortly after, Wang and colleagues described an improved protocol for direct conversion of MEFs into functional DA-like neurons, which uses both small molecules inhibiting TGF-β and BMP signaling and defined growth factors, without the necessity for viral transduction (Wang et al, 2016). This suggests that TFs can be replaced if signaling pathways are properly modulated in a precise timeframe with the addition of defined growth factors. Next, Playne and colleagues (Playne et al, 2018) reported for the first time that human adult fibroblasts can be reprogrammed to neural precursor cells by nonviral SOX2/PAX6 transfection and can give rise to DAN-like cells.

Nonetheless, these cells express late ventral midbrain dopaminergic fate markers such as NURR1 and PITX3, but critical early regional markers LMX1A, FOXA2, and EN1 were not expressed. Neither the exposure to patterning molecules during or after reprogramming nor the addition of LMX1A/FOXA2 to the transfection cocktail was sufficient to induce a sustained ventral midbrain dopaminergic iNP phenotype. This is probably due to the short term of transgene expression mediated by plasmid transfection. Alternative safe gene expression systems and more powerful cues rather than plasmid transfection are required for generating ventral midbrain dopaminergic progenitors from adult human fibroblasts.

Exogenous Transplantation of Directly Converted Neurons

The therapeutic potential of *in vitro* reprogrammed iDANs has been assessed in several studies. Rudolph Jaenisch's group showed the functional integration of mouse iDANs induced through ectopic expression of TFs in combination with neurotrophic factors in a mouse model of PD (Kim et al, 2011). Eight weeks after transplantation, the implanted *Pitx3* -eGFP+ cells led to a significant reduction in amphetamine-induced rotation scores in 6-hydroxydopamine lesioned mice. Then, Liu et al provided the first evidence for the clinical efficacy of human DAN-like induced by the combined expression of five TFs MASH1, NGN2, SOX2, NURR1, and PITX3. These cells are able to attenuate rotational behavior at 8 weeks postinjection in PD rats (Liu et al, 2014). Although these tests provide promising readouts, they do not include assays that measure a clinical phenotype of PD.

Motor functions should be evaluated for drug-independent motor dysfunctions, including the forelimb-use asymmetry and motor coordination. The application of iDPs for PD cell therapy could be advantageous in handling and obtaining the cells *in vitro* as well as in proper integration *in vivo*. At the same time, they should be more appropriate than NSCs, because they are further fate specified and can give rise to a greater proportion of DANs (Kim et al, 2014). This strategy was improved by Jialin Zheng's group (Tian et al, 2015), who generated iDPs, which are more DAN-restricted *in vitro* and *in vivo*, and have higher self-renewal capacity thanks to the inducible expression of *L-Myc*. These cells can alleviate the motor symptoms of transplanted PD mice.

Notably, no tumor formation was detected, confirming the safety of *L-Myc* conditional expression and the superior clinical functionality of engineered iDPs over NPCs (Lim et al, 2015; Tian et al, 2015). The protocol developed by Pereira and colleagues, which uses small molecules and delay in transgene activation, resulted in the highest conversion efficiency ($61\% \pm 4\%$ $MAP2^+$) of fetal human fibroblasts into iNs that survive and mature after transplantation in adult rat brain (Pereira et al, 2014). Nevertheless, the optimized and highly efficient protocol developed *in vitro* did not translate to an equal high performance *in vivo*.

Exposure to small molecules *in vitro* significantly increased conversion efficiency and purity, whereas exposure to small molecules *in vitro* before transplantation did not result in a higher number of neurons after grafting. This suggests that additional factors are present *in vitro* but are lacking *in vivo* (Pereira et al, 2014), as reported by ensuing investigations (Lim et al, 2015), and strategies for increasing TH neuron yield content in the grafts must be devised. Also, phenotypic and functional stability of reprogrammed cells were not assessed *in vivo* (Pereira et al, 2014). In contrast, Vania Broccoli's group demonstrated that mouse iDANs reprogrammed with ANL factor cocktail preserve the proper neuronal and electrophysiological functions even after long-term engraftment in the brain tissue of Parkinsonian rats, without showing any proliferation nor reversion to a progenitor neuronal state (Dell'Anno et al, 2014).

They also provided the first proof of principle that iDANs establish functional synapses with resident neurons and can integrate into the host synaptic circuitry. Transplanted iDANs led to a significant recovery not only in drug-induced motor asymmetry in PD rats, but also in the stepping test, which is a drug-independent behavioral assay. Notwithstanding, the grafted iDANs resulted in a less effective recovery with respect to primary embryonic DANs. This result could be explained with the high genomic heterogeneity in

the reprogrammed cell population, related to casual integration of the transgenes and consequent expression levels, which finally result in biochemical and functional variability among reprogrammed iDANs (Dell'Anno et al, 2014).

In Vivo Direct Dopaminergic Reprogramming

Despite the increasing evidence that iDANs share all the features of primary DANs, it is possible that, after transplantation, those exogenous neurons face immune system-mediated rejection. A valid alternative is represented by *in situ* direct reprogramming of brain resident astrocytes. Indeed, astrocytes offer several advantages for *in vivo* reprogramming, being abundant, highly proliferative upon injuries and plastic in the regard to cell fate (Yu et al, 2020).

In vitro conversion of primary mouse astrocytes into iDANs has been initially proved using the TFs ASCL1, NURR1, and LMX1B (Addis et al, 2011). Ernest Arenas's group managed to obtain *in vivo* direct reprogramming of astrocytes to functional iDANs in mouse models. They administered *NEUROD1*, *ASCL1*, and *LMX1A*, and miR218, cloned into dox-inducible lentiviral vectors, obtaining an even more efficient conversion when chromatin remodeling cofactors were added. The iDANs obtained helped to restore motor behavior in a PD animal model, reducing both apomorphine-induced circling behavior and spontaneous circling behavior (Rivetti Di Val Cervo et al, 2017). At the same time, Yoo et al (2017) provided the proof of principle for EMF-based *in vivo* conversion as a noninvasive strategy to enhance *in vivo* reprogramming of iDANs.

Along with APLN lentiviral particles, gold nanoparticles were implanted in solution into the striatum of PD mice and then they were exposed to EMF for the next 4 weeks. EMF exposure increased the number of TH+ cells, which also expressed functional characteristics of midbrain DANs, including functional synapses and pacemaking activity.

This finally resulted in restoration of movements in open field tests and improvement in rearing behavior, although tests evaluating other PD motor dysfunctions were not included (Yoo et al, 2017). Zhou et al proposed an alternative approach to convert mouse glia into DA neurons *in vivo*, which is based on the injection of AAVs that express CRISPR-CasRx targeting a *Ptbp1* (polypyrimidine tract-binding protein 1), a negative regulator of mir-124 and mir-9/9*. After 3 months of injection, the system generated a high percentage of TH/GFAP-positive cells that were found to restore motor functions in both drug-induced (apomorphine and amphetamine-induced motor activities) and drug-free tests (including the rotarod and cylinder tests). This study provides a more reliable assessment of the effects of converted cells on PD motor symptoms (Zhou et al, 2020).

Using the same rationale, Qian et al (2020) attempted to convert mouse astrocytes into iDANs *in vivo* by means of AAV carrying a shRNA against PTB. They also made a wide characterization of the converted cells, demonstrating the proper regional identity, functional integration, and restoration of disease-relevant motor phenotypes.

Converted neurons expressed multiple DAN markers, such as DAT, VMAT2, EN1, LMX1A, and PITX3. Also, cultured midbrain-derived astrocytes produced a fivefold higher proportion of TH-positive neurons compared to cultured cortical astrocytes, confirming a strong regional specificity of neuronal conversion. These *in vitro* studies strongly suggest that midbrain astrocytes have a higher propensity to generate iDANs, thanks to higher basal levels and more robust induction of lineage-specific TFs. Finally, mice showed improvements in each behavioral test performed, two based on drug-induced rotation and one based on spontaneous motor activities, demonstrating full correction of motor phenotype in the chemical-induced PD mouse model.

Future Directions

Viral transduction of TFs has proved to be the most successful approach for direct reprogramming of DANs so far (Caiazzo et al, 2011; Han et al, 2021; He et al, 2019; Kim et al, 2014; Kim et al, 2011; Lim et al, 2015; Liu et al, 2014; Oh et al, 2014; Pfisterer et al, 2011; Tian et al, 2015). Important enhancements were attained by combining TFs-mediated reprogramming with other molecular determinants intervening in the differentiation process (Kim et al, 2014; Pereira et al, 2014) and miRNAs (de Gregorio et al, 2018; Jiang et al, 2015). Most of these strategies use lentiviral mediated delivery of the conversion factors, which results in higher conversion efficiencies than viral-free methods, such plasmid transfection and protein-based induction systems (Mirakhori et al, 2015).

However, lentiviral vectors can casually integrate in the genome, leading to insertional mutagenesis and carcinogenic risk, which makes them less suitable for cell-based therapy. Moreover, the variable site of integration and copy number of integrated viruses causes genomic heterogeneity at the single-cell level, which implies a variable expression of transgenes and consequent variable phenotype, which then reduces the clinical performance *in vivo* (Dell'Anno et al, 2014). To date, reducing the number of required lentiviruses by means of synergistic effect of biochemical and biophysical stimulation may represent a feasible strategy to limit the drawbacks of lentiviral transduction. Also, biophysical stimulation could increase the reproducibility of the induction protocol and provide factors that are still lacking in *in vitro* culture systems (Lim et al, 2015; Pereira et al, 2014) by mimicking *in vivo* cues (Yoo et al, 2015; Yoo et al, 2017).

Some studies investigated alternative induction strategies that include nonintegrative and self-regulating viral vectors (Lau et al, 2014), optimized protein-based technology (Robinson et al, 2018), and a drug inducible and genetically homogeneous system (Park et al, 2015). In particular, the findings of Robinson et al may be exploited for *in vivo* generation of induced DANs from inflammatory astrocytes.

Another relevant aspect for direct reprogramming of iDANs is the proper regional identity of reprogrammed cells, which has been considered in a recent report for reprogramming from mouse and human ESCs (Han et al, 2021), and needs to be also validated for direct conversion of iDANs from differentiated cells.

As previously reported, iDANs maintain the age signatures of donor cells, which offer the chance to model age-related neurodegenerative diseases. However, this appears a double-edged sword due to the age-related decrease in neuronal reprogramming (Zhou et al, 2020), an issue that has to be further investigated.

Recently, researchers have been focusing on *in vivo* direct reprogramming of iDANs, which avoids the immune rejection risks related to transplantation (Qian et al, 2020; Rivetti Di Val Cervo et al, 2017; Robinson et al, 2018) and offers a more powerful and clinically feasible approach to treating neurodegeneration by replacing lost neurons. On the contrary, recent results from the group of Chun-Li Zhang indicated that endogenous neurons are the main source for the *in vivo* reprogrammed neurons as assessed by more accurate genetic tracing methods. This work poses serious needs to reconsider the potential of brain *in vivo* reprogramming and the urge to use more strict tracing systems to ensure the real outcome of *in vivo* reprogramming (Wang et al, 2021).

Moreover, the evaluation of the clinical efficacy of *in vivo* reprogramming may benefit from more standardized studies that assess the *in vivo* functional integration in the host circuitry and the restoration of both drug-induced and spontaneous motor impairments in PD models.

Finally, to optimize direct cell reprogramming-based technologies and their applications, it might be helpful to explore molecular mechanisms underlying direct reprogramming, such as chromatin remodeling dynamics. In this regard, Della Valle and colleagues found that L1 retrotransposition correlates with chromatin opening and long noncoding RNA production that accompanies direct somatic cell reprogramming, paving the way for future studies that may shed light on the mechanistic link between L1 reactivation and activation of lineage-specific genetic programs (Della Valle et al, 2020).

In summary, direct cell conversion provides several new tools to generate iDANs both *in vitro* and *in vivo*, but additional issues need to be addressed to generate efficient gene and cell therapy approaches for PD (Man et al, 2018).

Authors' Contributions

Conceptualization: S.A., R.P., M.C.; writing—original draft preparation: S.A., R.P., and M.C; writing—review and editing: S.A., R.P., and M.C. All authors have read and agreed to the published version of the article.

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