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Homogeneous generation of iDA neurons with high similarity to *bona fide* DA neurons using a drug inducible system

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ABSTRACT

Recent work generating induced dopaminergic (iDA) neurons using direct lineage reprogramming potentially provides a novel platform for the study and treatment Parkinson's disease (PD). However, one of the most important issues for iDA-based applications is the degree to which iDA neurons resemble the molecular and functional properties of their endogenous DA neuron counterparts. Here we report that the homogeneity of the reprogramming gene expression system is critical for the generation of iDA neuron cultures that are highly similar to endogenous DA neurons. We employed an inducible system that carries iDA-inducing factors as defined transgenes for direct lineage reprogramming to iDA neurons. This system circumvents the need for viral transduction, enabling a more efficient and reproducible reprogramming process for the generation of genetically homogenous iDA neurons. We showed that this inducible system generates iDA neurons with high similarity to their *bona fide in vivo* counterparts in comparison to direct infection methods. Thus, our results suggest that homogenous expression of exogenous genes in direct lineage reprogramming is critical for the generation of high quality iDA neuron cultures, making such culture systems a valuable resource for iDA-based drug screening and, ultimately, potential therapeutic intervention in PD.

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1. Introduction

Recently, we and others have reported that mouse and human fibroblasts can be directly reprogrammed into different subtypes of neurons, including dopamine (DA) neurons, by viral transduction of specific transcription factors [1–5]. Generation of induced dopamine (iDA) neurons from abundant somatic cells make this system attractive for autologous cell based therapies because this approach can eliminate the necessity of passing through the pluripotent state and subsequent directed differentiation inherent in ES or iPS cell-based approaches [6]. Furthermore, direct lineage conversion eliminates the potential for undifferentiated pluripotent cells to form teratomas upon transplantation. Thus, the generation of iDA neurons has significant implications for the potential direct therapeutic use in Parkinson's disease (PD) and, in the near-term, has immediate applicability as an *in vitro* screening platform for the identification of small molecules targeting PD.

Previous studies characterizing iDA neurons revealed a number of differences in their molecular features when compared to primary DA neurons, with iDA neurons showing close similarity and some shared functional features of mesencephalic DA neurons [3–5,7]. Similarly, the functional efficacy of iDA neurons upon *in vivo* transplantation is lower than those of primary midbrain DA neurons [3]. One possible reason for these differences is the degree

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of heterogeneity within iDA cultures with respect to the number of cells that receive the proper dosage or optimal stoichiometric ratios of reprogramming factors for precise lineage conversion. This heterogeneity largely results from viral site-of-integration effects and insertion frequency and likely contributes to incomplete reprogramming and/or low reprogramming efficiency. The precedent for this phenomenon is abundant in the field of induced pluripotency [8–10]. Thus, cellular and genetic heterogeneity associated with direct conversion complicates the potential application of iDA neurons in PD drug screening and, ultimately, in cell replacement therapy.

Much effort has been applied to the development of new methods to overcome the challenges associated with the generation of induced cells. For example, expression of Lmx1a/Fox2a in ES/iPSCs or fibroblasts has been shown to lead to the efficient generation of DA neurons [11], decreasing complications associated with heterogeneity for the therapeutic application of DA neurons. Additionally, Tian et al. recently showed the generation of directly converted DA precursors [12], which resulted in the specific DA neuronal lineage, possibly supplying a reasonable source of cells for PD treatment. Moreover, several small molecules or engineered substrates that can improve DA neurons efficiency and quality have been identified [13–15]. Nevertheless, the low efficiency and heterogeneity of DA neurons still remains as a significant barrier in the safe and efficient DA neuron-based cell replacement for PD.

Thus, in this study, to test whether the quality and efficiency of iDA neuron generation can be improved by a homogenous factor expression system, we established an inducible system to homogenously generate iDA neurons directly from somatic cells. Analogous strategies in inducible pluripotency reprogramming systems have proven to be more efficient and synchronous than reprogramming by direct infection [8,10,16]. We show that inducible reprogramming in genetically homogenous cultures creates iDA neurons that are highly similar to primary midbrain DA neurons, both in their molecular and functional characteristics. Additionally, we show the direct lineage reprogramming of iDA neurons from cells of the hematopoietic lineage using this system, demonstrating that abundant and readily accessible somatic cell types, such as peripheral blood cells, are amenable to direct lineage reprogramming and can give rise to functional iDA neurons. Taken together, our results suggest that inducible gene expression in a genetically homogeneous system enables the efficient production of high quality iDA neurons, which can greatly facilitate the application of these cells in PD disease modeling, drug screening, and, ultimately, cell replacement therapy.

2. Results

2.1. Generation of an expression system for the generation of genetically homogenous iDA neurons

Previously, we and others have shown that both mouse and human fibroblasts can be directly reprogrammed into iDA neurons through the viral transduction of several transcription factors, such as Ascl1, Pitx3, Nurr1, Lmx1a, Foxa2 and EN1 [3–5,7]. Among these factors, the combined activity of Ascl1 and Pitx3 is sufficient to facilitate conversion into a cell with DA neuronal identity (and activation of an endogenous *Pitx3-eGFP* knock-in reporter allele), while ectopic expression of additional factors is thought to be involved in the further maturation of 2 factor-induced *Pitx3-eGFP*$^{+}$ iDA neurons.

We asked if the efficiency and quality of iDA neuron generation could be improved using a genetically homogenous inducible reprogramming system. We first generated mouse embryonic stem (ES) cells containing both a reverse tetracycline transactivator (M2rtTA) and a *phosphoglycerate kinase 1* (*pgk1*) promoter-driven puromycin resistance gene targeted to the *ROSA26* locus (*ROSA26-M2rtTA*) in addition to an enhanced green fluorescent protein (*eGFP*) (in the Web version)gene targeted to the endogenous *Pitx3* locus. These ES cells were next infected with doxycycline (dox)-inducible lentiviruses encoding the transcription factors (Fig. 1A). Previously, we reported that the viral transduction of a 6-factor combination (Ascl1, Pitx3, Nurr1, Lmx1a, EN1, and Foxa2) could generate iDA neurons that closely resemble midbrain DA neurons [6]. However, when we prepared ES cells harboring all 6 factors, they exhibited promiscuous differentiation even in the absence of dox, precluding their maintenance over multiple passages (data not shown). Thus, we removed the non-requisite factors EN1 and Foxa2 from the 6-factor combination, generating *Pitx3-eGFP* ES cells harboring a 4-factor combination (Ascl1, Pitx3, Nurr1 and Lmx1a). We generated several unique clones of these 4-factor ES cells (J1, J2, J3), all of which were stable and could be maintained in culture in the absence of dox for more than 20 passages (Fig. 1B). Hereafter, we refer to these cells as *Pitx3-eGFP-TRE-4F*.

Southern blot analysis probing for proviral integration of Ascl1, Pitx3, Lmx1a and Nurr1 vectors showed that each of the three ES cell lines were independent clones carrying approximately 10 proviral integrations for each gene (Fig. 1D). These *Pitx3-eGFP-TRE-4F* ES cells were GFP negative in the absence of dox (not shown), and all of the ES cell colonies showed morphology characteristic of mouse ES cells (Fig. 1B). The pluripotency of these ES cells was confirmed by the expression of marker genes, including *Oct4*, *Nanog*, and *Sox2*, and neuronal genes, including *TH*, *Tuj1 and Brn2/4*, were not expressed. Additionally, no differences in the expression of these genes were observed between the ES cells (Fig. 1C), demonstrating the inducible factors has no detrimental consequences for the ability of the ES cells to maintain pluripotency, nor does it result in any aberrant expression of neuronal-lineage genes in the absence of Dox stimulation. To generate inducible somatic cells, we injected these ES cell lines into blastocysts and generated chimeras composed of *Pitx3-eGFP-TRE-4F* somatic cells as well as host blastocyst-derived cells. Mouse embryonic fibroblasts (MEF) were prepared from these chimeras and treated with puromycin to select against cells derived from the host blastocyst by virtue of the constitutively active puromycin resistance cassette at the *ROSA26* locus.

To assess inducible expression of the reprogramming factors in *Pitx3-eGFP-TRE-4F* MEFs, we performed transgene-specific quantitative RT-PCR. We detected robust dox-dependent factor expression in all three *Pitx3-eGFP-TRE-4F* MEF lines, with J1 giving the most robust expression (Sup. Fig. 1 A–D). Therefore, we used inducible fibroblasts from *Pitx3-eGFP-TRE-4F* J1 for subsequent experiments. Taken together, these results indicated that transgene expression was strictly dependent on the presence of the drug, with little to no leaky expression as no viral transcripts were detected in the absence of dox.

2.2. Efficient direct lineage reprogramming of Pitx3-eGFP-TRE-4F fibroblasts into iDA neurons

We next assessed the reprogramming activity of *Pitx3-eGFP-TRE-4F* MEFS in response to dox treatment. The addition of dox led to dramatic morphological changes and efficient generation of cells resembling DA neurons (Fig. 2A and B). Seven days after dox induction, most fibroblasts in the culture appeared to be TH + iDA neurons, and immunofluorescence demonstrated that iDA neurons had reactivated the DA neuronal markers TH, DAT, Tuj1, AADC, VMAP2, and Girk2 (Fig. 2C and Sup. Fig. 2C). We observed the highest induction of TH + iDA neurons in *Pitx3-eGFP-TRE-4F* J1 fibroblasts (Fig. 2D). The reprogramming efficiencies of the different

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