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Detailed Analysis of the Genetic and Epigenetic Signatures of iPSC-Derived Mesodiencephalic Dopaminergic Neurons

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SUMMARY

Induced pluripotent stem cells (iPSCs) hold great promise for in vitro generation of disease-relevant cell types, such as mesodiencephalic dopaminergic (mdDA) neurons involved in Parkinson's disease. Although iPSC-derived midbrain DA neurons have been generated, detailed genetic and epigenetic characterizations of such neurons are lacking. The goal of this study was to examine the authenticity of iPSC-derived DA neurons obtained by established protocols. We FACS purified mdDA (3 /+) neurons derived from mouse iPSCs and primary mdDA (3 /+) neurons to analyze and compare their genetic and epigenetic features. Although iPSC-derived DA neurons largely adopted characteristics of their in vivo counterparts, relevant deviations in global gene expression and DNA methylation were found. Hypermethylated genes, mainly involved in neurodevelopment and basic neuronal functions, consequently showed reduced expression levels. Such abnormalities should be addressed because they might affect unambiguous long-term functionality and hamper the potential of iPSC-derived DA neurons for in vitro disease modeling or cell-based therapy.

INTRODUCTION

The field of regenerative medicine experienced a powerful impetus after the groundbreaking discovery of induced pluripotency (Takahashi and Yamanaka, 2006). Numerous publications have shown that mouse as well as human induced pluripotent stem cells (iPSCs) have the potency to differentiate into various clinically relevant cell types, such as cardiomyocytes (Kuzmenkin et al., 2009; Ren et al., 2011), hepatocytes (Espejel et al., 2010), hematopoietic progenitors (Hanna et al., 2007), oligodendrocytes (Czepiel et al., 2011), and specific subtypes of neurons (Karumbayaram et al., 2009; Wernig et al., 2008). Such in vitro-generated iPSC-derived cell types provide new possibilities for disease modeling and cell replacement strategies. In particular, the generation of autologous iPSC-derived midbrain dopaminergic (DA) neurons provides a very interesting tool to study and treat Parkinson's disease (PD) (Roessler et al., 2013). However, future clinical application of iPSC-derived DA neurons can only be considered realistic if the desired cell population is strictly purified and completely defined.

Several groups have reported the generation of DA neu-

allowed us to strictly identify and purify DA neurons from either iPSCs or the ventral midbrain at specific developmental stages by fluorescence-activated cell sorting (FACS). We then subjected these mdDA neurons to genome-wide gene-expression analysis compng

between iPSC-derived mdDA neurons and primary DA neurons for transcription factors and axonal guidance factors, we found a high correlation for expression levels of ion channels, with the strongest overall correlation (r = 0.82) between E12.5 mdDA neurons and iPSC-derived DA neurons.

In view of the origin of iPSC-derived DA neurons, we also analyzed the transcript profile for pluripotency genes and fibroblast-related genes, visualized by dendrograms (Figure 2F). The gene expression of a set of pluripotency markers was subjected to cluster analysis, which showed similar transcript levels (e.g., , *4* (*5 1*), *42*, and *0 1* [also known as *1*]) in primary and iPSC-derived cell types (Figure 2F). This result not only

fibroblast-specific markers revealed differential expression in primary and iPSC-derived neurons, suggesting remnants of a still active fibroblast gene program in iPSC-derived DA neurons.

In summary, comparative gene-expression profiling of purified iPSC-derived DA neurons and embryonic mdDA neurons revealed a clear correlation, but less similarity was found between iPSC-derived DA neurons and P0 DA neurons. Downregulated genes in iPSC-derived DA neurons were mainly associated with biological functions such as nervous system development, neurogenesis, and neuron differentiation. These findings prompted us to investigate the nature of this downregulation and to extend our gene-expression analysis by performing in-depth epigenetic profiling focused on DNA methylation.

Gene-Specific Methylation Profiles Correlate with Gene Expression for Specific DA Markers

First, in order to validate our genome-wide expression profiling data, we performed quantitative PCR (qPCR) experiments for a set of selected key DA genes (*3*, *1*, *1*, *1*, and *2*), *9*, and . The expression levels of these particular genes appeared to be in line with the results obtained by microarray analysis, and indicate that DA-specific marker expression in iPSC-derived DA neurons most closely resembles that of terminally differentiated-stage mdDA neurons (Figure 3A). As far as the expression of *3*, *1*, *1*, and *2* was concerned, the highest similarity was found between iPSC-derived DA and E16.5 mdDA neurons (Figure 3B).

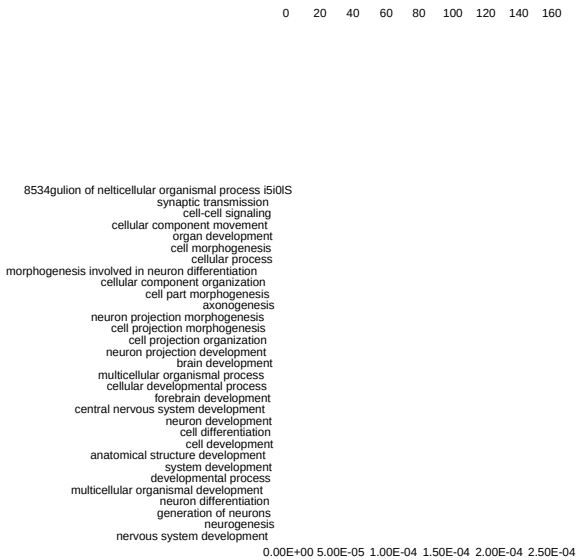
Next, we set out to compare the DNA methylation profiles associated with the selected key DA gene expression of iPSC-derived DA and primary mdDA neurons. DNA methylation is a major player in epigenetic regulation of gene expression, and strong methylation of promoters is generally associated with gene silencing. RRBS allowed us to analyze specific CpG methylation at nucleotide resolution on a genome-wide scale. RRBS provides coverage preferentially of CpG-rich regions, such as CGIs. CGIs are predominantly associated with promoter regions surrounding the transcription start site (TSS). We identified and analyzed CGIs and their methylation status for the aforementioned key mdDA factors and found a striking correlation between CGI methylation levels and gene expression (Figure 3C). *3*, the mdDA marker we used to purify both cell types, revealed equally low methylation in the CGI around exon 1. Active expression of *3* in both cell types might be due to this permissive DNA methylation state. CGIs in gene bodies of *1*, *1*, and *2* appeared to be more strongly methylated in iPSC-derived DA neurons than in primary mdDA neurons. This specific DNA methylation state in iPSC-derived DA neurons may401.63J/T.3(set)-321.70Td[(5d)-501.3(i64.1(iPSC-derive1[(,)-orrelation)-624(str[(,)-orrel52cd24(DA69.2(DNA(Nurr1)Ton)-320

A

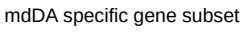
B

C

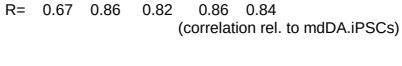
E


GO analysis of genes with reduced expression in mdDA.iPSCs
0 20 40 60 80 100 120 140 160
8534gulion of nelticellular organismal process i5i0lS
synaptic transmission
cell-cell signaling
cellular component movement
organ development
cell morphogenesis
cellular process
morphogenesis involved in neuron differentiation
cellular component organization
cell part morphogenesis
axonogenesis
neuron projection morphogenesis
cell projection morphogenesis
cell projection organization
neuron projection development
brain development
multicellular organismal process
cellular developmental process
forebrain development
central nervous system development
neuron development
cell differentiation
cell development
anatomical structure development
system development
developmental process
multicellular organismal development
neuron differentiation
generation of neurons
neurogenesis
nervous system development
0.00E+00 5.00E-05 1.00E-04 1.50E-04 2.00E-04 2.50E-04

D


mdDA specific gene subset

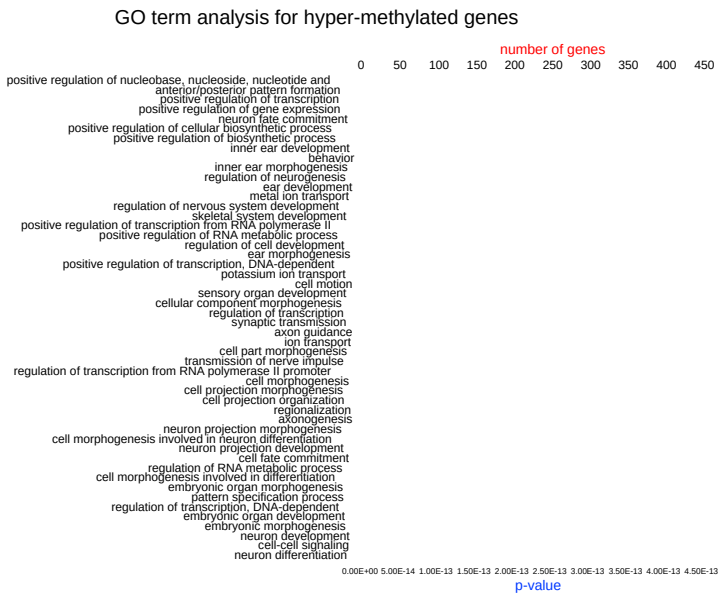
F


R= 0.67 0.86 0.82 0.86 0.84
(correlation rel. to mdDA.iPSCs)

mdDA-specific genes. However, we identified a subset of

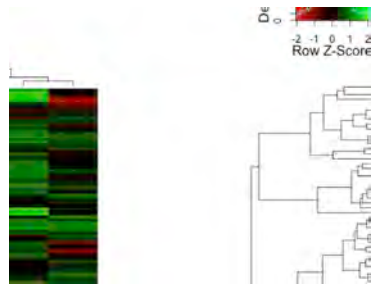
Although transcript analysis indicated that proviral gene expression introduced during reprogramming was silenced in iPSC-derived mdDA neurons, we still found residual expression of fibroblast markers. It was previously shown that iPSCs are prone to differentiate along their somatic parental lineages because they maintain a parental epigenetic memory (Bar-Nur et al., 2011; Kim et al., 2010; Sullivan et al., 2010). Although some studies suggested that such epigenetic memory is restricted to early-passage iPSCs (Polo et al., 2010), others reported that parental epigenetic states also persist in late-passage iPSCs (Kim et al., 2011).

A


GO term analysis for hyper-methylated genes
number of genes
0
50
100
150
200
250
300
350
400
450
positive regulation of nucleobase, nucleoside, nucleotide and
anterior/posterior pattern formation
positive regulation of transcription
positive regulation of gene expression
neuron fate commitment
positive regulation of cellular biosynthetic process
positive regulation of biosynthetic process
inner ear development
behavior
inner ear morphogenesis
regulation of neurogenesis
ear development
metal ion transport
regulation of nervous system development
skeletal system development
positive regulation of transcription from RNA polymerase II
positive regulation of RNA metabolic process
regulation of cell development
ear morphogenesis
positive regulation of transcription, DNA-dependent
potassium ion transport
cell motion
sensory organ development
cellular component morphogenesis
regulation of transcription
synaptic transmission
axon guidance
ion transport
cell part morphogenesis
transmission of nerve impulse
regulation of transcription from RNA polymerase II promoter
cell morphogenesis
cell projection morphogenesis
cell projection organization
regionalization
axonogenesis
neuron projection morphogenesis
cell morphogenesis involved in neuron differentiation
neuron projection development
cell fate commitment
regulation of RNA metabolic process
cell morphogenesis involved in differentiation
embryonic organ morphogenesis
pattern specification process
regulation of transcription, DNA-dependent
embryonic organ development
embryonic morphogenesis
neuron development
cell-cell signaling
neuron differentiation
0.00E+00
5.00E-14
1.00E-13
1.50E-13
2.00E-13
2.50E-13
3.00E-13
3.50E-13
4.00E-13
4.50E-13
p-value

B

C


Row Z-Score

Earlier studies reported that PSCs mostly contain methylation-free promoters, as well as methylation-free intergenic and orphan CGIs (Illingworth et al., 2010). De novo methylation was only found upon loss of pluripotency, suggesting that transcriptional repression in PSCs is established predominantly via other mechanisms (Fouse et al., 2008; Mohn et al., 2008). Indeed, we observed that most de novo methylation events occurred during differentiation from pluripotent cells to multipotent precursors isolated from an iPSC-derived NESTIN-GFP reporter line (data not shown).

General methylation states appeared to be comparable between iPSC-derived DA neurons and primary embryonic mdDA neurons ($r = 0.85$), indicating that our in-vitro-generated DA neurons widely adopted the epigenetic signature of their primary counterparts. Nonetheless, we found several thousand genes hypermethylated in iPSC-derived DA neurons. This hypermethylation was found predominantly at an intermediate level ranging from 40% to 60% DNA methylation. At this point, we can only speculate about whether hypermethylation is due to retained epigenetic memory or to heterogeneous differentiation/maturation stages within the PITX3-GFP FACS-sorted iPSC-derived population. To our knowledge, a specific neuronal subtype derived from iPSCs has not been characterized to such an extent, but similar differences in methylation profiles have been observed in reprogrammed mesenchymal stromal cells compared with ESCs (Shao et al., 2013). Interestingly, our gene-expression data show reduced expression of *1* and *3* in iPSC-derived DA neurons compared with E14.5 mdDA neurons. Tet proteins have been shown to be crucial for establishment of pluripotency, development, and neuronal activity (Koh et al., 2011; Rudenko et al., 2013; Zhang et al., 2013). Presently, very little is known about the activity of Tet proteins in specific subtypes of neurons (primary or PSC derived). It is becoming more and more clear, however, that their demethylation activity is crucial for functionally bona fide, healthy neurons (Gavin et al., 2013; Ma et al., 2009). Our DNA methylation profiling of iPSC-derived DA neurons versus primary mdDA neurons did not allow a distinction between 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC). Neuronal gene activation was recently shown to be mediated by specific MeCP2 binding to 5hmC (Li et al., 2013; Mellén et al., 2012). Therefore, we cannot rule out the possibility that elevated 5hmC levels in iPSC-derived mdDA neurons might contribute to gene activation rather than to silencing. However, within the population of hypermethylated genes, we did observe gene clusters with substantially lower transcript levels. Interestingly, we also found

EXPERIMENTAL PROCEDURES

Mice

3(/+) embryos at several developmental stages were obtained by intercrossing C57BL6/J with *3(/)* mice. *3(/+)* embryos are heterozygous for wild-type PITX3 and have normal mdDA system development (Maxwell et al., 2005). Overlap of endogenous PITX3 with GFP has been shown to be ~100% (Maxwell et al., 2005). All procedures were approved by and performed according to the guidelines of the Dutch ethics committees for animal experiments (UMCU and UvA).

iPSC Generation and Propagation

Mouse embryonic fibroblasts were isolated from E14.5 embryos of *3 /+* and - mice, both of which were previously described and characterized (Yamaguchi et al., 2000; Zhao et al., 2004). Fibroblasts were cultured until passage 5–8 and then retrovirally transfected with the four Yamanaka reprogramming factors. Separate vectors containing either *4*, *4*, *2*, or were used for pluripotency induction. Retroviruses were obtained from Phoenix Eco packaging cells transfected with the reprogramming factors (for vector information: Addgene, http://www.addgene.org). The detailed induction protocol was previ-

with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.03.001.

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andH3ed5-537.K4/K(2d)1104.tri2(metra)11.1(latial.)]TJ T*C(cell)-stem
pluripoten.
cel.(,)322623(Nurell)]TJ/T1_21T89.3710Td5-420

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