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LETTER TO THE EDITOR

Generation of dopaminergic neurons directly from mouse fibroblasts and fibroblast-derived neural progenitors

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Dear Editor,

Parkinson's disease (PD) is a neurodegenerative disease that afflicts around 1% of the population over age 65 [1]. One of the pathological hallmarks of PD is the degeneration of dopaminergic (DA) neurons at midbrain and the relatively focal lesion feature of PD makes cell replacement a promising approach for treating the disease [2].

We have previously shown that a sertoli cell, a mesoderm-derived terminally differentiated cell, can be reprogrammed directly to a neural stem cell-like cell by defined factors [3]. From the perspective of clinical applications, sertoli cells may not be an ideal cell source. Therefore, in the current study, we continued to examine whether fibroblasts can be converted to DA neurons through two separate routes. One is to directly convert fibroblasts to mature DA neurons (induced DA neurons) and the other is to induce fibroblasts first into neural progenitors (induced neural progenitor cells (iNPCs)) that possess the potential of DA neuron specification.

Mouse embryonic fibroblasts (MEFs) were isolated from E14.5 embryos. The mesodermal identity of MEFs was confirmed by immunostaining. In addition, no neural cell contamination was detected in MEF cultures (Supplementary information, Figure S1).

In a previous report, Vierbuchen *et al*. [4] have shown that mouse fibroblasts can be directly converted to mature neurons (induced neurons, iNs) by introduction of three factors (*Ascl1*, *Brn2*, and *Myt1l* or *Zic1*) without first going through a pluripotent state. Here, we showed that two iN factors – *Ascl1* and *Brn2* (AB) – can also convert MEFs to iNs that possess functional electrophysiological properties (Supplementary information, Figure S2).

Next, we tried to convert fibroblasts to DA subtype neurons with the addition of more specific factors on top of *Ascl1* and *Brn2*. We initially chose eight transcription factors (*Lmx1a*, *Lmx1b*, *Foxa2*, *Otx2*, *Nurr1*, *Ngn2*, *Pax6*, and *Sox2*). The above eight factors together with *Ascl1* and *Brn2* were introduced into fibroblasts (AB + 8F-induced cells), which were then subjected to an experimental paradigm that has been used to facilitate specification of DA neurons [5].

Three weeks after infection with the above 10 factors (AB + 8F), Tuj1-positive neurons emerged at an efficiency of 0.25% ± 0.07% (n = 6), and 3.86% ± 0.8% (n = 3) of these cells co-expressed TH (Supplementary information, Figures S3 and S4). When AB-iNs were subjected to the same culture condition in favor of DA neuron specification, no TH-positive neurons were detected (data not shown). To refine the pool of reprogramming factors, we tested different combinations of factors. We first combined *Ascl1* and *Brn2* with the five DA lineage-specific factors (*Lmx1a*, *Lmx1b*, *Foxa2*, *Otx2*, and *Nurr1*) for induction of DA neurons (AB + 5F). Three weeks after infection, 7.6% ± 2.38% (n = 5) of Tuj1-positive neurons were co-labeled with TH, suggesting that *Ngn2*, *Pax6*, and *Sox2* were dispensable for direct DA neuron conversion (Supplementary information, Figures S3 and S4). A recent study reported that *Lmx1a* and *Foxa2* together with the three iN factors (*Ascl1*, *Brn2*, and *Myt1l*) can generate DA neurons from human fibroblasts [6]. Therefore, we further separated the five DA lineage-specific factors into two groups, 2F (*Lmx1a* and *Foxa2*) and 3F (*Lmx1b*, *Nurr1*, and *Otx2*), and introduced each group together with *Ascl1* and *Brn2* into fibroblasts for DA neuron conversion.

Three weeks after infection, Tuj1-positive neurons were generated at an efficiency of 0.26% ± 0.1% (n = 4), and 8.77% ± 2.47% (n = 6) of Tuj1-positive neurons were co-labeled with TH in AB + 2F group (Supplementary information, Figures S3 and S4). The efficiencies of conversion to Tuj1- and TH-positive neurons were higher in AB + 3F group − 0.34% ± 0.08% (n = 4) for Tuj1-positive cells and 13.93% ± 4.25% (n = 6) of Tuj1-labeled neurons co-expressed TH (Supplementary information, Figure S4). In addition, the TH-positive neurons stained positive for Pitx3 and En1, two specific mesencephalic DA neuron markers [7, 8], in both AB + 2F and AB + 3F groups (Supplementary information, Figures S3 and S4), suggesting their midbrain identity. We also confirmed

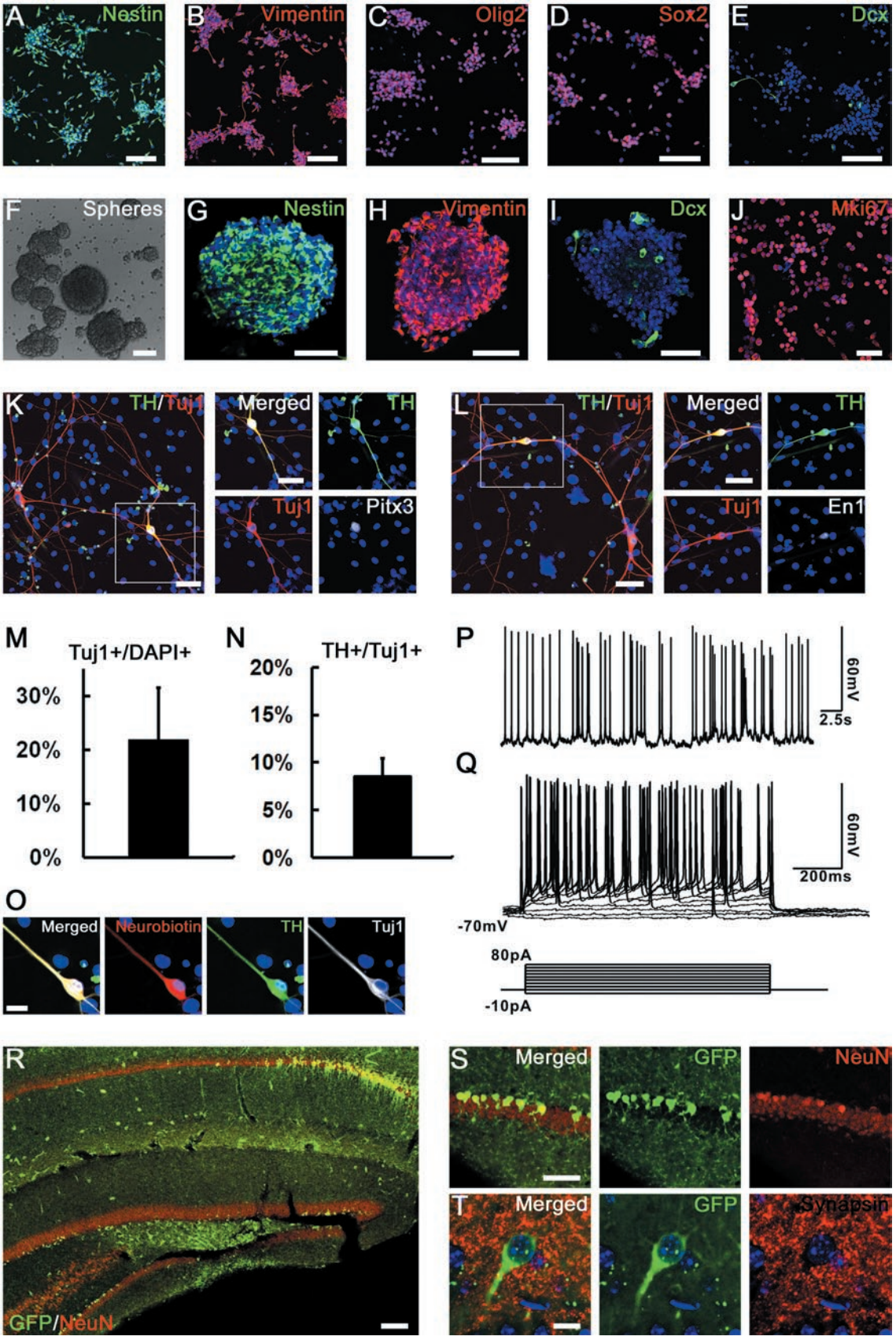
A Nestin
B Vimentin
C Olig2
D Sox2
E Dcx
F Spheres
G Nestin
H Vimentin
I Dcx
J Mki67
K TH/Tuj1
Merged
TH
Tuj1
Pitx3
L TH/Tuj1
Merged
TH
Tuj1
En1
M
Tuj1+/DAPI+
30%
20%
10%
0%
N
TH+/Tuj1+
20%
15%
10%
5%
0%
P
60mV
2.5s
Q
60mV
200ms
-70mV
80pA
-10pA
O
Merged
Neurobiotin
TH
Tuj1
R
GFP/NeuN
S Merged
GFP
NeuN
T Merged
GFP
Synapsin

Figure 1 TTFs are reprogrammed to neural progenitors that can differentiate to DA neurons and survive transplantation. **(A-E)** Characterization of iNPCs in monolayer culture. iNPCs readily expressed neural stem/progenitor cell markers, Nestin **(A)**, Vimentin **(B)**, and Olig2 **(C)**. **(D)** Endogenous expression of Sox2 was detected in an eight-factor (without exogenous *Sox2*)-induced iNPC line. **(E)** ~2.85% of iNPCs spontaneously differentiated to Dcx-positive neural precursors in monolayer culture. **(F-I)** iNPCs could form floating spheres in uncoated culture dishes **(F)**. Most of the sphere cells expressed Nestin **(G)** and Vimentin **(H)**, and ~4.67% of cells expressed Dcx **(I)**. **(J)** A majority of iNPCs stained positive for Mki67. **(K, L)** TTF-derived iNPCs were capable of differentiating to TH- and Tuj1-double-positive neurons 3 weeks after treatment with Shh and FGF-8. These TH-positive neurons co-expressed mesencephalic DA markers, Pitx3 **(K)** and En1 **(L)**. **(M, N)** The efficiency at which Tuj1-positive neurons were generated from TTF-derived iNPCs **(M)** and the proportion of Tuj1-positive neurons co-labeled with TH **(N)** 3 weeks after treatment with Shh and FGF-8. Error bars indicate SD values from at least three independent experiments. **(O)** A recorded iNPC-derived neuron stained positive for Neurobiotin, TH, and Tuj1. **(P,Q)** iNPC-derived DA neurons fired spontaneous action potentials **(P)** and mature evoked action potentials **(Q)** under the whole-cell current-clamp mode 3 weeks after differentiation. **(R)** A significant number of GFP-positive iNPCs were detected at DG 4 weeks after transplantation. Note that GFP-positive iNPC-derived neurons could also be detected at CA1 region. **(S)** Grafted GFP-positive iNPCs could migrate to the granular cell layer and give rise to NeuN-positive mature neurons. **(T)** A GFP-positive iNPC-derived neuron co-labeled with Synapsin. Blue, DAPI. Scale bars: 100 μm **(A-F, R)**, 50 μm **(G-L, S)**, 20 μm **(O)**, 10 μm **(T)**.

that cells from AB + 3F group possessed functional membrane properties 3 weeks after infection (Supplementary information, Figure S4).

Next, we tested the possibility of converting fibroblasts directly to neural progenitors. Here we chose postnatal tail tip fibroblasts (TTFs) as the originating materials, considering that postnatal tissue culture is more convenient from a clinical application perspective and that TTFs are less heterogeneous than MEF cultures. TTFs were isolated from the bottom third section of 3-day-old pup tails and nearly all the cells were positive for collagen I and α-SMA, and no expression of Pax6, Olig2, Sox2, Dcx, or Tuj1 was detected in the TTF cultures (Supplementary information, Figure S1).

For induction of neural progenitors, we initially chose nine transcription factors (*Pax6*, *Ngn2*, *Hes1*, *Id1*, *Ascl1*, *Brn2*, *Sox2*, *c-Myc*, and *Klf4*) as previously reported for converting sertoli cells [3]. After two rounds of retroviral infection/recovery, TTFs were replated in PDL/laminin-coated dishes in N2B27 medium with epidermal growth factor and basic fibroblast growth factor. About 5 to 10 actively proliferating colonies emerged per 1×10^5 cells 3 days after replating, but at most one or two of them were well-reprogrammed iNPC colonies (Supplementary information, Figure S5). Further characterization confirmed that the iNPC colonies expressed appropriate markers (Figure 1A-1J) and did not go through a pluripotent state (Supplementary information, Figure S5). Furthermore, eight factors (without *Sox2*) can also convert TTFs to iNPCs, as we have previously shown to convert sertoli cells [3]. The eight factors-induced iNPCs gave rise to different subtypes of neurons with functional membrane properties, following a pan-neuronal differentiation paradigm (Supplementary information, Figure S6). In addition, the iNPCs showed integration of exogenous factors (Supplementary information, Figure S7), a correct karyotype (Supplementary information, Figure S8), and a tripotency to differentiate to neurons, astrocytes, and oligodendrocytes (Supplementary information, Figure S9).

To specifically generate DA neurons from TTF-derived iNPCs, the iNPCs were subjected to a paradigm that facilitates the differentiation and maturation of DA neurons [5]. TTF-derived iNPCs readily gave rise to DA neurons after a 3-week differentiation (Figure 1K and 1L). Tuj1-positive neurons emerged at an efficiency of 21.8% ± 9.65% (n = 4), much higher than the efficiency (< 1%) at which iNs were generated in our system at the same time point (Figure 1M). Among the Tuj1-positive cells, 8.56% ± 1.84% (n = 3) were co-labeled with TH (Figure 1N). The TH-positive neurons also co-expressed specific midbrain DA markers, Pitx3 and En1 (Figure 1K and 1L), further suggesting their midbrain DA neuron identity. In addition, after a 3-week differentiation, the TH-positive neurons exhibited spontaneous action potentials and mature evoked action potentials by step current injection, suggesting a functional membrane property (Figure 1O-1Q).

To study the survival and differentiation of iNPCs *in vivo*, we bilaterally introduced GFP-labeled iNPCs into the dentate gyrus (DG) of hippocampus, a region of native neurogenesis in the adult brain of C57BL/6 mouse. Four weeks after transplantation, the mice were sacrificed for graft analysis. A significant number of GFP-positive cells were observed at the injection site (Figure 1R). Some grafted cells integrated into the granular cell layer at DG and stained positive for NeuN, verifying that TTF-derived iNPCs can survive transplantation and differentiate to mature neurons *in vivo* (Figure 1S). Furthermore, some GFP-positive cells were co-labeled with syn-

apsin (Figure 1T), suggesting that the grafted cells might have established synaptic connections with host neurons. Interestingly, we also observed grafted cells at the pyramidal layer at CA1. Those GFP-positive cells were co-labeled with NeuN and displayed complex neuronal morphologies with extensive neurite arborization. Further investigation is needed to elucidate how the grafted cells migrated and integrated to the pyramidal layer (Figure 1R). Up to now, we have examined 12 hippocampi, and have not detected any sign of tumorigenesis, suggesting that iNPCs may be a safe source material for replacement approaches.

Taken together, in the present study, we have successfully converted mouse fibroblasts to a specific neuronal subtype – DA neurons – through two different routes. One is to directly convert fibroblasts with DA lineage-specific factors combined with the two iN factors, *Ascl1* and *Brn2*, and the other is to first induce fibroblasts into neural progenitors and then differentiate them to DA neurons. With both methods, we have obtained DA neurons that possess functional membrane properties. The findings in this study may have important implications in disease modeling and regenerative medicine.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)