Efficient Conversion of Astrocytes to Functional Midbrain Dopaminergic Neurons Using a Single Polycistronic Vector

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Abstract

Direct cellular reprogramming is a powerful new tool for regenerative medicine. In efforts to understand and treat Parkinson’s Disease (PD), which is marked by the degeneration of dopaminergic neurons in the midbrain, direct reprogramming provides a valuable new source of these cells. Astrocytes, the most plentiful cells in the central nervous system, are an ideal starting population for the direct generation of dopaminergic neurons. In addition to their potential utility in cell replacement therapies for PD or in modeling the disease in vitro, astrocyte-derived dopaminergic neurons offer the prospect of direct in vivo reprogramming within the brain. As a first step toward this goal, we report the reprogramming of astrocytes to dopaminergic neurons using three transcription factors – ASCL1, LMX1B, and NURR1 – delivered in a single polycistronic lentiviral vector. The process is efficient, with 18.2 ± 1.5% of cells expressing markers of dopaminergic neurons after two weeks. The neurons exhibit expression profiles and electrophysiological characteristics consistent with midbrain dopaminergic neurons, notably including spontaneous pacemaking activity, stimulated release of dopamine, and calcium oscillations. The present study is the first demonstration that a single vector can mediate reprogramming to dopaminergic neurons, and indicates that astrocytes are an ideal starting population for the direct generation of dopaminergic neurons.

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Introduction

Parkinson’s Disease (PD) is marked by progressive loss of dopaminergic neurons in the ventral midbrain. Although the somata of these neurons are located in the substantia nigra, it is their projections to the striatum that release dopamine to mediate motor control. For this reason, the caudate and putamen regions of the striatum have been the primary targets for cell replacement strategies in PD [1]. Restoring dopaminergic tone to the striatum via the engraftment of dopaminergic neurons has long been a goal in the field of regenerative medicine, beginning with the transplantation of fetal mesencephalic tissue [2–6]. Given the conflicting results of these studies, as well as the difficulty in obtaining sufficient quantities of fetal tissue, alternative cell sources have been pursued (well-reviewed in [7]). Both neural stem cells [8] and embryonic stem cells [9] have shown great promise in their ability to differentiate into dopaminergic neurons, while the advent of induced pluripotent stem (iPS) cells made real the possibility of generating patient-specific stem cell lines [10]. More recently, direct reprogramming has demonstrated that stem cells may not be necessary at all – three groups have reported that ectopic expression of small sets of transcription factors can directly convert fibroblasts to dopaminergic neurons [11–13]. Astrocytes are an attractive alternative to fibroblasts as a starting population of cells for reprogramming to dopaminergic neurons. Previous studies have demonstrated that astrocytes can be directly reprogrammed to neurons that form functional synapses [14,15]. Conversion of astrocytes to dopaminergic neurons would not only provide a new source of neurons for use in cell-based therapies for PD, but this approach also raises the possibility of direct in vivo reprogramming as a novel treatment strategy [16]. Since this virus-based strategy would require no cellular transplantation, many of the concerns of immunological rejection in cell replacement therapies would be negated. Furthermore, there is now considerable evidence that reprogrammed cells retain an epigenetic memory of their original cell type [17–19], and iPS cells derived from astrocytes have a greater propensity for neuronal differentiation than those derived from fibroblasts [20]. Thus, the developmentally close relationship of astrocytes to neurons may prove advantageous to effective reprogramming. In the present study, we report the direct conversion of astrocytes to dopaminergic neurons via three transcription factors, with the development of a polycistronic lentiviral vector to facilitate future efforts at in vivo reprogramming.
Results

Transcription factor screen and polycistronic vector generation

To identify a combination of transcription factors that is sufficient to mediate reprogramming to dopaminergic neurons, twelve transcription factors known to play critical roles in midbrain dopaminergic neuron development and/or maintenance [21–23] were cloned into the doxycycline-inducible lentiviral vector FU-tetO-Gateway (Figure 1A). Seventy-four unique combinations of these vectors were used to transduce mouse embryonic fibroblasts for the initial factor screen. RNA of transduced cells was harvested after 7 days of doxycycline-induced factor expression and assayed via RT-PCR for expression of tyrosine hydroxylase (Th) and DOPA decarboxylase (Ddc), the enzymes of dopamine synthesis. All experiments were performed in triplicate. The three-factor combination of ASCL1, LMX1B, and NURR1 resulted in the most robust expression of Th and Ddc (Figure 1A–B). This combination was selected for further analysis.

In order to increase the efficiency of cells receiving all three transcription factors, as well as to reduce variability resulting from different ratios of the factors reaching individual cells, we constructed a polycistronic vector. The open reading frames of ASCL1, LMX1B, and NURR1 were linked via recombinant PCR such that viral 2A peptide sequences [24] separate the three genes, as shown in Figure 1C. A glycine-serine-glycine (GSG) linker was included upstream of each 2A sequence to facilitate protein cleavage. The ASCL1-P2A-LMX1B-T2A-NURR1 cassette (hereafter referred to as ALN) was inserted into FU-tetO-Gateway to produce the tetO-ALN vector. Cleavage at the 2A sites was validated by performing in vitro transcription and translation using the T7 promoter located at the start of the Gateway recombination in the presence of biotinylated lysine. A streptavidin-HRP western blot confirmed complete cleavage at both the T2A and P2A sites (Figure 1D). The tetO-ALN lentiviral vector delivered ALN to both astrocytes and fibroblasts at >99% efficiency, as determined by immunocytochemistry for the C-terminal V5 tag on NURR1 (data not shown).

Characterization of gene and protein expression in induced dopaminergic neurons

Astrocytes were transduced with FUdeltaGW-rtTA (the reverse tetracycline transactivator, [25]) and tetO-ALN. The following day, doxycycline was added to astrocyte medium to induce ALN expression (Day 0). After four days in astrocyte medium with doxycycline, transduced cells were switched to NB27G neuronal medium. Doxycycline was removed on Day 10. After 14 days from the initial ALN induction, 35.1 ± 1.5% of cells expressed type III beta-tubulin (clone TUJ1), a neuronal marker. 50.9 ± 3.3% of TUJ1+ cells were also positive for tyrosine hydroxylase, yielding an overall conversion rate of 18.2 ± 1.5% (Figure 2A). Quantification was performed by counting a total of 3357 cells in three independent reprogramming experiments.

We constructed a lentiviral reporter vector in which the cell surface marker, CD4, is expressed under the control of the neuron-specific MAP2 gene promoter. To further characterize the induced dopaminergic neurons, we sorted ALN-derived neurons

Figure 1. Transcription factor screen and polycistronic vector construction. (A–B) Evaluation of transcription factor combinations to induce reprogramming. RT-PCR results for a subset of the 74 transcription factor combinations tested for their ability to induce expression of DOPA decarboxylase (Ddc) and tyrosine hydroxylase (Th) in mouse embryonic fibroblasts after 7 days of factor expression. 0F: uninfected control; A: ASCL1; B: BRN2; L: LMX1B; N: NURR1; P: PITX3. The combination of ASCL1, LMX1B, and NURR1 (ALN) was the only combination to give robust expression of both Ddc and Th. (C) Polycistronic vector containing open reading frames of human ASCL1, LMX1B, and NURR1 linked by viral 2A sequences. (D) Complete cleavage at 2A peptide sites confirmed by in vitro transcription and translation (TnT) of lentiviral plasmids in the presence of biotinylated lysine. Streptavidin-HRP Western blot shows all newly synthesized protein. Lane 1: No DNA TnT control. Lanes 2–4: TnT performed on original single-factor plasmids. Lane 5: TnT for polycistronic ALN plasmid. Band intensity is proportional to the number of lysine residues present in each protein sequence.

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on Day 14 using the MAP2-CD4 reporter vector and magnetic beads conjugated to anti-CD4. RNA was isolated from sorted cells and assayed via RT-PCR for expression of a panel genes shown in Figure 3. Gene expression in sorted neurons was compared to astrocytes that did not receive ALN as well as mouse embryonic stem cells that had been differentiated to dopaminergic neurons via co-culture with PA6 stromal cells [26]. Sorted neurons displayed robust upregulation of genes expressed in midbrain dopaminergic neurons such as Pitx3, Lmx1a, Engrailed-1, aldehyde dehydrogenase, Foxa2, the vesicular monoamine transporter Vmat2, Msx1, and the dopamine transporter. Immunocytochemistry revealed expression of Girk2, a potassium channel widely expressed in A9/substantia nigra dopaminergic neurons [27], in virtually all (>99%) of the Th-immunoreactive cells (Figure 2B). Otx2, a protein expressed in mature A10/ventral tegmental area dopaminergic neurons [28,29], was not detected. Induced dopaminergic neurons exhibited robust synaptophysin expression (>99% of Th-immunoreactive cells, Figure 2C), suggesting the capacity to form synaptic connections.

Functional characterization of induced dopaminergic neurons

To evaluate the electrophysiological phenotype of induced dopaminergic neurons, patch clamping was performed on induced neurons between days 9 and 26 of initial ALN induction. Neurons were identified by screening for GFP driven by the MAP2 promoter. Current clamp recordings in Day 9 cells showed an immature spiking pattern – generally a single action potential per step current injection. By Day 14–21, neurons demonstrated repetitive firing of action potentials in individual step current injections (Figure 4A). Of 30 patched cells, 24 fired action potentials (80%). Recorded cells had an average resting membrane potential of \( -255.4 \) mV. In voltage clamp, large sodium and potassium currents were seen (Figure 4B), with an average maximum \( I_{Na} \) of \( 1346 \) pA. Electrophysiological properties of induced dopaminergic neurons are summarized in Table 1. Recordings were made on neurons generated in four independent reprogramming experiments.

It has been well established that midbrain dopaminergic neurons are pacemaker neurons, spontaneously firing action potentials at a rate between 1 and 9 Hz with an average of \( 4.5 \pm 1.7 \) Hz [30–33]. We observed spontaneous firing of action potentials in 43% of recorded MAP2-GFP+ induced neurons, a fraction that is consistent with the 50.9±3.3% of neurons that were immunoreactive for tyrosine hydroxylase (Figure 4C). The frequency of spontaneous firing ranged from 0.9 to 8.6 Hz, with an average of \( 5.6 \pm 1.2 \) Hz. The pacemaker activity of dopaminergic neurons is accompanied by rhythmic fluctuations in intracellular calcium ion concentration [34–37]. To determine whether induced dopaminergic neurons exhibit calcium oscillations...
tions, we transduced astrocytes (prior to reprogramming) with a lentiviral vector containing the genetically-encoded calcium indicator GCaMP3 [38] driven by the MAP2 promoter. We detected robust calcium oscillations with an average frequency of 0.49 ± 0.11 Hz (Figure 4E). A typical GCaMP3-expressing induced dopaminergic neuron is shown in Movie S1.

To confirm that induced dopaminergic neurons produce and release dopamine, we stimulated cells with 56 mM KCl and measured dopamine release via HPLC. Cells assayed at 17 days post-transduction with ALN released dopamine in response to membrane depolarization while un-transduced cells (0F) did not, as shown in Figure 4D. No significant levels of epinephrine, norepinephrine, or serotonin were detected.

Direct reprogramming of fibroblasts to dopaminergic neurons

Given the widespread use of fibroblasts as a starting cell population in reprogramming, we tested whether our polycistronic vector was effective on mouse embryonic fibroblasts. The ALN vector was able to reprogram mouse embryonic fibroblasts to Thv/TUJ1+ neurons, as shown in Figure S1, with an overall efficiency of 9.1 ± 0.9%. Fibroblast-derived dopaminergic neurons exhibited large sodium currents and spontaneous action potential firing (Figure S1B–D). Dopaminergic neurons generated from fibroblasts using a nearly identical set of transcription factors (ASCL1, LMX1A, and NURR1) were recently described and characterized in detail [12].

Discussion

Our study is the first to demonstrate direct reprogramming of astrocytes to dopaminergic neurons. These neurons exhibit gene and protein expression patterns that are consistent with A9 midbrain dopaminergic neurons. Given the mounting evidence that cells retain some epigenetic memory when driven to pluripotency and subsequent differentiation, such memory is almost certain to be maintained in direct reprogramming. Global epigenetic profiling that compares authentic dopaminergic neurons to those derived from astrocytes and fibroblasts will be informative in determining the relative completeness of these reprogramming processes. It will also be important to compare the ability of astrocyte- and fibroblast-derived dopaminergic neurons to engraft and function in animal models of PD.

In addition to providing a novel source of dopaminergic neurons for use in cell-based therapies for PD, our use of astrocytes as the starting population allows an approach that may obviate the need for grafting altogether – direct in vivo reprogramming to replace lost neurons. Such an approach is facilitated by the development of a polycistronic vector, especially since one of the three factors, ASCL1, has been shown to reprogram astrocytes to non-dopaminergic neurons in the absence of additional factors [14]. The fact that the polycistronic ALN vector reprograms astrocytes at such a high efficiency is also noteworthy, given the history of polycistronic vector use in generating iPS cells. A single vector delivering transcription factors that induce pluripotency was shown to be effective, but at a significantly lower rate than that which could be achieved when the factors were delivered individually [39]. This was presumed to indicate that a particular ratio of reprogramming factors is ideal for inducing pluripotency. In the case of reprogramming to dopaminergic neurons, we can conclude that either the ratio of factors is not as important or, less likely, that our polycistronic vector fortuitously delivers the factors in the appropriate proportions. The present study takes a crucial step toward the ultimate goal of in vivo reprogramming of

Figure 3. Transcriptional profile of astrocyte-derived dopaminergic neurons. Heat map of quantitative RT-PCR results comparing astrocyte-derived neurons magnetically sorted for a MAP2-CD4 reporter. Ast, uninfected astrocytes. ESN, mouse embryonic stem cell-derived neurons, generated via co-culture with PA6 stromal cells. Induced dopaminergic neurons express markers consistent with midbrain dopaminergic neurons. Color scale indicates change in Ct (threshold cycle) relative to the normalizing actin control. Higher delta Ct values correspond to lower relative gene expression, with every Ct decrease of 3.3 representing a ten-fold increase in relative expression.

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Figure 4. Functional characterization of astrocyte-derived dopaminergic neurons. (A) Action potential firing characteristics of induced dopaminergic neurons. Five overlapping traces are depicted derived from whole-cell current clamp recording of a representative induced dopaminergic neuron, elicited in response to hyperpolarizing and depolarizing current injection, increased from $-10$ to $+40$ pA in 10 pA increments. (B) Voltage-dependent sodium currents in induced dopaminergic neurons. Membrane potential was initially held at $-90$ mV and incrementally increased from $-60$ to $+20$ mV in 5 mV depolarizing steps. (C) Spontaneous action potential firing, consistent with a dopaminergic neuron pacemaker phenotype. The recording was conducted at resting membrane potential ($-51$ mV). (D) Dopamine release quantified by HPLC. Membrane depolarization was induced with 56 mM KCl at 17 days post-infection. 0F: uninfected astrocytes; ALN: induced dopaminergic neurons. (E) MAP2-GCaMP3 reveals rhythmic oscillations of intracellular calcium. Left panel displays a single frame of Movie S1, a recording of oscillating levels of intracellular calcium, which is presented as a histogram of GCaMP3 fluorescence intensity in the adjacent panel.

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Table 1. Electrophysiological properties of astrocyte-derived dopaminergic neurons.

| Capacitance (pF) | RMP (mV) | Spontaneous firing (%) | AP freq (Hz) | Threshold (mV) | $I_{Na}$-max (pA) | $I_{K}$-max (pA) | IR ($G\Omega$) |
|------------------|----------|------------------------|--------------|----------------|------------------|------------------|---------------|
| Mean             | 16.22    | 55.39                  | 43.00        | 5.60           | $-27.73$        | 1546.00         | 1381.91       | 1.48          |
| SEM              | 0.76     | 1.46                   | 11.00        | 1.17           | 2.27             | 175.21           | 151.51        | 0.23          |
| N                | 8        | 16                     | 21           | 7              | 11               | 11               | 11            | 16            |

RMP: resting membrane potential; AP freq: frequency of spontaneous action potentials; $I_{Na}$ and $I_{K}$: Maximum sodium and potassium currents; IR: input resistance.
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astrocytes to dopaminergic neurons. Future work will determine the feasibility of this approach in animal models of PD.

**Materials and Methods**

**Lentiviral expression vector construction**

To make a drug-inducible lentiviral vector that is compatible with Gateway recombination (Invitrogen), we removed the OCT4 open reading frame from FU-tetO-oCT4 [Addgene plasmid 19778, [25]] and replaced it with a Gateway cassette cloned from pEF-DEST51 (Invitrogen) to generate FU-tetO-Gateway, ORFs that lack stop codons can be cloned into this plasmid to express proteins with a C-terminal V5 epitope tag. ORFs and cDNAs were purchased from Open Biosystems (Table 2).

**Reporter vector construction**

The MAP2-GFP lentiviral reporter plasmid pGZ-hMAP2 was purchased from System Biosciences (SR10047PA-1). The MAP2 promoter sequence from this plasmid was then cloned along with the ubiquitin promoter-rtTA sequence to produce FU-tetO-Gateway. This destination vector was subsequently used to generate MAP2-CD4 (human CD4 sequence cloned from pMACS4-IRESII, Miltenyi Biotec) and to generate MAP2-GCaMP3 (GCaMP3 cloned from G-CaMP3, Addgene plasmid 19778, [25]) and replaced it with a Gateway cassette cloned from pEF-DEST51 into the backbone of the ubiquitin promoter-rtTA sequence to produce FU-tetO-Gateway. This destination vector was subsequently used to generate MAP2-CD4 (human CD4 sequence cloned from pMACS4-IRESII, Miltenyi Biotec) and to generate MAP2-GCaMP3 (GCaMP3 cloned from G-CaMP3, Addgene plasmid 22692). All lentiviruses were packaged in HEK-293T cells using the TnT T7 Coupled Reticulocyte Lysate System (Promega L4610) in the presence of biotinylated lysine (Promega L5061). 5 µL of each TnT-generated protein sample was resolved via PAGE, transferred to PVDF membrane, incubated with streptavidin-conjugated horseradish peroxidase (Cell Signaling Technology 3999, 1:2000), and detected with ECL Plus (GE Healthcare RPN2132).

**Polycistronic vector construction**

Open reading frames of human ASCL1, LMX1B, and NURR1 were cloned from the plasmids listed above using the following primers: B1-ASCL1-FWD: GGGGacaagtttgtacaaaaaagcaggctACCTGAGAGAGCTTGTACGAGG; NURR1-REV: GAAGTGTTAAAGCAAGCAGGAGA TGTTGAAGAAAACCCCGGCCCTATGCTTGTGTTCAGGCG; LMX1B-FWD: CTCTCTGCTTGCTTTAACAGAGAGAA GTTCGTGGCACCGGATTG; LMX1B-REV: ACGTGTTAAAGCAAGCAGGAGA TGTTGAAGAAAACCCCGGCCCTATGCTTGTGTTCAGGCG; B2-NURR1-REV: GGCAacactgtagcaagaagggtgGAAAAATAGGTGGTCCAG (extra Δ added to maintain reading frame with C-terminal V5-tag). The 3 ORFs were then joined via recombinant PCR using B1-ASCL1-FWD and B2-NURR1-REV, cloned into pDONR221, and subsequently cloned into FU-tetO-Gateway via Gateway recombination. Cleavage at 2A sites was verified by performing in vitro transcription and translation using the TnT T7 Coupled Reticulocyte Lysate System (Promega L4610) in the presence of biotinylated lysine (Promega L5061). 5 µL of each TnT-generated protein sample was resolved via PAGE, transferred to PVDF membrane, incubated with streptavidin-conjugated horseradish peroxidase (Cell Signaling Technology 3999, 1:2000), and detected with ECL Plus (GE Healthcare RPN2132).

**Cell culture**

Primary mouse embryonic fibroblasts (strain CF-1) were purchased from Millipore (PMEF-CFL) and cultured in DMEM (Gibco) containing 10% fetal bovine serum (Hyclone) and 1 x non-essential amino acids (Gibco). Primary postnatal mouse astrocytes (strain CD1) isolated from cerebral cortex were purchased from ScienCell (MA1800) and cultured in Astrocyte Growth Medium (AGM, Lonza). For reprogramming, astrocytes or fibroblasts were plated onto poly-L-lysine-coated glass coverslips or 6-well plates (BD Biocoat). Expression of transcription factors was induced by the addition of doxycycline (2 µg/mL). On Day 4, medium was switched to NB27G neuronal medium containing Neurobasal medium and 1 x B27 supplement (Gibco), with 20 ng/mL GDNF (R&D Systems) and doxycycline. Doxycycline was removed on Day 10. Mouse embryonic stem cells (line ESD3) were differentiated to dopaminergic neurons by co-culturing with PA6 stromal cells according to a published protocol [26].

**Immunocytochemistry**

Cells on glass coverslips were fixed in 4% paraformaldehyde with 0.15% picric acid, blocked in 10% chicken serum, 1% bovine serum albumin (w/v), 0.3% Triton X-100 in PBS and stained with antibodies to tyrosine hydroxylase (Millipore MAB318 or Santa Cruz sc-14007, both 1:200), type III beta-tubulin (clone TUJ1, Covance MMS-435P, 1:400), synaptophysin (Santa Cruz sc-9116, 1:200), Girk2 (Santa Cruz sc-14007, both 1:200), type III beta-tubulin (clone TUJ1, Covance MMS-435P, 1:400), and V5 (Invitrogen R96025, 1:400). Detection was performed with alexa-fluor labeled secondary antibodies (Molecular Probes) and coverslips were mounted in ProLong Gold Antifade Reagent with DAPI (Invitrogen). Imaging was performed on an Olympus IX-81 fluorescent microscope (Olympus, Japan) with FITC, Texas Red, and Rhodamine filters. ImageJ (National Institutes of Health, Bethesda, Maryland) was used for all image processing. For quantification, astrocytes were counted using a 10x objective on glass coverslips or 6-well plates.
**RT-PCR**

RNA was collected using the RNeasy Mini Kit (Qiagen 74104) and first-strand cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen 18080051) with random hexamer primers. Quantitative real-time PCR was performed on an ABI 7900HT system (Applied Biosystems) using the TaqMan gene expression assays listed in Table 3 and normalized to beta actin (Mm00607939_s1). Analysis of gene expression data was performed with DataAssist version 3.0 (Applied Biosystems).

**Electrophysiology**

Voltage- and current-clamp whole cell patch clamp recordings were performed on MAP2-GFP+ cells with neuronal morphology, visualized using an upright fixed stage microscope (Olympus) and a 40 x water immersion objective. All recordings were performed at room temperature (20–24°C) using glass patch pipettes, fabricated on a Flaming-Brown micropipette puller (P-97, Sutter Instruments). The electrodes were 6–8 MΩ in resistance when filled with potassium gluconate-based intracellular solution (in mM: 145 K-gluc, 2 MgCl₂, 2.5 KCl, 2.5 NaCl, 0.5 GTP-Tris, 0.1 BAPTA, 2 ATP-Mg and 10 HEPES). The recording chamber was perfused with aCSF solution with the following composition: 155 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 3 mM CaCl₂, 25 mM glucose and 10 mM HEPES, pH 7.35. Neurons were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), sampled at 10 kHz and digitized using Digitizer 1320A (Molecular Devices), with data stored for off-line analysis (Clampfit 10; Axon Instruments, Inc., Foster City, CA). In voltage clamp mode, cells were held at a membrane potential of −60 mV to record the potential pacemaker current fluctuations. To evoke voltage-gated currents, the command potential was held initially at −90 mV, then stepped to potentials ranging from −60 mV to +20 mV in 5 mV increments. In current clamp mode, cells were recorded for 2–10 min to examine spontaneous fluctuations in membrane potential and possible spontaneous firing. In order to calculate input resistance, all cells were injected with at least one hyperpolarized command current.

**HPLC for dopamine quantification**

Membrane depolarization was evoked by incubating cells in a well of a 6-well dish with 1 mL aCSF containing 56 mM KCl for 15 minutes at 37°C. The aCSF solution was then collected and catecholamines were extracted onto 30 mg activated alumina (Wako 018-09561). The alumina was washed with ultrapure water and dried on Ultrafree-MC GV filters (Millipore UFC30GV00). Catecholamines were eluted into 100 μL with 100 μM EDTA. 20 μL samples were analyzed on an HTEC-500 HPLC system with electrochemical detection (Eicom) using a reversed phase C18 separation column (Eicompak CA-5ODS). Mobile phase consisted of 88% 0.1 M phosphate buffer pH 6.0, 12% methanol, 600 mg/L sodium octanesulfonate, and 50 mg/L EDTA-2Na. Analysis was performed at 25°C with a flow rate of 230 μL/min and dopamine quantities were calculated by comparison of area under curve measurements to known standard dilutions of dopamine hydrochloride (Sigma H8502).

### Table 3. TaqMan assays for RT-PCR.

| Gene Symbol | Gene Product         | TaqMan Assay       |
|-------------|----------------------|--------------------|
| Aldh1a7     | aldehyde dehydrogenase | Mm00496380_m1      |
| Cacna1g     | Cav3.1 calcium channel | Mm00486572_m1      |
| Calb1       | Calbindin-1          | Mm00486645_m1      |
| Ddc         | DOPA decarboxylase   | Mm00516688_m1      |
| En1         | Engrailed-1          | Mm00438709_m1      |
| Foxa2       | forkhead box A2      | Mm0176565_s1       |
| Gabra1      | GABA A receptor subunit alpha-1 | Mm00439046_m1 |
| Gad2        | glutamic acid decarboxylase 2 | Mm00484623_m1 |
| Grin1       | NMDA receptor 1      | Mm00433800_m1      |
| Kcnm3       | KCa2.3 potassium channel | Mm00446516_m1      |
| Lmx1a       | LIM homeobox transcription factor 1 alpha | Mm00473947_m1 |
| Mapt        | microtubule-associated protein tau | Mm00521988_m1   |
| Mx1         | msh-like homeobox 1   | Mm00440330_m1      |
| Ngn2        | neogenin 2           | Mm00437603_g1      |
| Pax2        | paired box gene 2     | Mm01217939_m1      |
| Pax5        | paired box gene 5     | Mm00435501_m1      |
| Pitx3       | paired-like homeodomain transcription factor 3 | Mm0194166_g1 |
| Pouf3f2     | POU domain, class 3, transcription factor 2 (Bm2) | Mm0043777_s1 |
| Scn3a       | Nav1.3 sodium channel, alpha subunit | Mm00658167_m1 |
| Slc5a3      | dopamine transporter  | Mm00438388_m1      |
| Slc17a7     | vesicular glutamate transporter 1 (vGlut1) | Mm00812886_m1   |
| Slc18a2     | vesicular monoamine transporter 2 (Vmat2) | Mm0053038_m1 |
| Syn2        | synapsin II          | Mm00449780_m1      |
| Th          | tyrosine hydroxylase  | Mm0047557_m1       |

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Supporting Information

Figure S1 Fibroblast-derived dopaminergic neurons. (A) The ALN polytopic vector is effective in converting fibroblasts to dopaminergic neurons, demonstrated by immunocytochemistry for the neuronal marker TUJ1 (14.9±2.3% positive) and the dopaminergic marker tyrosine hydroxylase (9.1±0.9% positive); the pan-nuclear marker DAPI is also shown. Scale bars 50 μm. (B) Action potential firing characteristics of fibroblast-derived dopaminergic neurons. Four overlapping traces are depicted derived from whole-cell current clamp recording of a representative induced dopaminergic neuron, elicited in response to hyperpolarizing and depolarizing current injection, increased from −20 to +10 pA in 10 pA increments. (C) Voltage-dependent sodium currents in fibroblast-derived dopaminergic neurons. Membrane potential was initially held at −90 mV and incrementally increased from −60 to +40 mV in 5 mV depolarizing steps. (D) Spontaneous action potential firing, consistent with a dopaminergic neuron pacemaker phenotype. The recording was conducted at resting membrane potential (−57 mV). (TIF)

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Movie S1 Calcium oscillations in an astrocyte-derived dopaminergic neuron. The genetically-encoded calcium indicator GCaMP3 is expressed under the control of the neuron-specific MAP2 promoter. Fluorescence level increases with increased levels of intracellular calcium. Rhythmic oscillations of calcium levels are seen, consistent with the midbrain dopaminergic phenotype. (WMV)

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Author Contributions

Conceived and designed the experiments: RCA MAD DAC JDG. Performed the experiments: RCA F-CH RLW. Analyzed the data: RCA F-CH MAD DAC JDG. Contributed reagents/materials/analysis tools: RCA F-CH RLW. Wrote the paper: RCA.