Retinoic Acid Receptor γ (Rarg) and Nuclear Receptor Subfamily 5, Group A, Member 2 (Nr5a2) Promote Conversion of Fibroblasts to Functional Neurons*

Zixiao Shi†§, Tianjin Shen‡, Yanli Liu†, Yuanyuan Huang§, and Jianwei Jiao††

From the †State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China, ‡University of Chinese Academy of Sciences, Beijing 100049, China, and *School of Life Sciences, Anhui University, Anhui 230601, China

Background: It is necessary to identify more factors to increase fibroblast to neuron conversion with non-integration adenovirus.

Results: Nuclear receptor Rarg and Nr5a2 or agonist greatly enhance the conversion efficiency.

Conclusion: Rarg and Nr5a2 both promote transdifferentiation and improve neuronal function.

Significance: It would be of potential advantage to obtain safe and functional neurons with high efficiency.

Somatic cells can be reprogrammed to neurons and various other cell types with retrovirus or lentivirus. The limitation of this technology is that these genome-integration viruses may increase the risk of gene mutation and cause insertion mutagenesis. We recently found that non-integration adenovirus carrying neuronal transcription factors can induce fibroblasts to neurons. However, the conversion efficiency by the adenovirus is lower than that of the retrovirus or lentivirus. Therefore, it is crucial to identify other factors or chemical compounds to obtain neurons with high efficiency. In this study we show that the combination of Rarg (retinoic acid receptor γ) and Nr5a2 (nuclear receptor subfamily 5, group A, member 2; also known as Lrh-1 (liver receptor homologue 1)) rapidly promote the iN cell maturation within 1 week and greatly facilitate the conversion with neuronal purities of ~50% and yields of >130%. They also improve neuronal pattern formation, electrophysiological characteristics, and functional integration in vivo. Moreover, the chemical compound agonists to Rarg and Nr5a2 function effectively as well. This approach may be used for the generation and application of IN cells in regenerative medicine.

The direct conversion of mouse and human fibroblasts (mesodermal lineage) to neurons (ectodermal lineage) (1, 2) skips the neuronal differentiation processing from embryonic stem cells or induced pluripotent stem cells, thereby providing the possibility of direct applications for clinical therapy. However, the direct transdifferentiation efficiency using a non-integrating system for induced pluripotent stem cell generation or other reprogramming is especially low (3, 4). We previously found that the combination of Asc1, Brn2, and Ngn2 (ABN)2 could convert fibroblasts to neurons (5). However, the efficiency was not high using the adenoviral delivery system either. Therefore, we set out to increase the conversion efficiency by screening other transcription factors or small molecules in combination with ABN.

Retinoic acid (RA) plays an important role during neurogenesis in the central nervous system of vertebrates (5–7), and RA is commonly used to promote neural stem cell differentiation into neurons in culture and in vivo (8, 9). RA triggers neuronal differentiation through activating RA receptors (RARs), and various RARs-related downstream molecules have been reported to be involved in the process (10, 11). RARs consist of three isoforms, RAR-α, RAR-β, and RAR-γ, that bind to both all-trans-RA and 9-cis-RA (12, 13). If RA signaling is disrupted, neurogenesis is affected, resulting in decreased numbers of newborn neurons (14, 15). As RAR is important for neuronal differentiation, it is possible that RAR signaling could be beneficial for fibroblast–neuron transdifferentiation and could enhance the conversion efficiency. To validate this hypothesis, we overexpressed RARs or used RA agonists to explore the function of RARs in the conversion of fibroblasts to neurons.

Because RARs belong to the family of nuclear receptors that are important for differentiation and neurogenesis (8), it will be of interest to uncover other nuclear receptor members that could be used to enhance neuronal conversion. Recent studies showed that one important orphan nuclear receptor, Nr5a2, can enhance the reprogramming efficiency in the derivation of induced pluripotent stem cells (16, 17). Therefore, we explored whether the nuclear receptors of Nr5a2 together with Rarg enable efficient conversion of fibroblasts to neurons. In this study we used non-integrating adenoviruses carrying a different combination of transcription factors to enhance neuronal conversion.

The data discussed in this publication have been deposited in NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE52993.

This work was supported by National Basic Research Program of China Grant 2014CBCB964903, National Science Foundation of China Grant 31371477, Strategic Priority Stem Cell Program Grant XDA01020301, and the Hundreds Talent Program.

The abbreviations used are: ABN, Ascl1 + Brn2 + Ngn2; RA, retinoic acid; RAR, RA receptor; Rarg, RA receptor-γ; Nr5a2, nuclear receptor subfamily 5, group A, member 2; IN, induced neuron; MEF, mouse embryonic fibroblast; CD437, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; DLPC, 1,2-dilauroylglycerol-3-phosphocholine; TTF, Tail-tip fibroblast; HEF, human embryonic fibroblast.
Rarg and Nr5a2 Enhance Fibroblasts Convert into Neurons

Factors of ABN (Ascl1, Brn2, Ngn2), Rarg, and Nr5a2 for the direct conversion of fibroblasts to functional neurons.

Here we show that overexpression of Rarg or Nr5a2 increases the fibroblast-to-neuron conversion efficiency. The combination of Rarg and Nr5a2 could not only significantly promote the conversion efficiency but also improve the neuronal pattern formation and physiological function of induced neuron (iN) cells both in vitro and in vivo. Similarly, the chemical compound agonists to Rarg and Nr5a2 work as effectively as the transcription factors. The strategy of using an adenoviral delivery system to obtain high iN cell conversion efficiency is promising and may be used as an improved tool for the application of iN cells in regenerative medicine.

EXPERIMENTAL PROCEDURES

Adenovirus Production and Infection—We used the Invitrogen Gateway Expression System to produce the adenovirus carrying the transcription factors Ascl1, Brn2, Ngn2, Rarg, and Nr5a2. The selected genes were amplified from a mouse cDNA library and inserted into the pENTR 3C vector (Invitrogen). According to the manufacturer’s instructions, the resulting plasmids were generated by homologous L/R recombination. Then, viral constructs were transduced into a 293A cell line, and high titer (10^8-10^10 IU/ml) viral particles were obtained through two rounds of amplification. The adenoviral titer was determined using the same method as that of the lentiviral titer. The selected adenoviral library and inserted into the pENTR 3C vector (Invitrogen).

Nr5a2. The selected genes were amplified from a mouse cDNA library and inserted into the pENTR 3C vector (Invitrogen). According to the manufacturer’s instructions, the resulting plasmids were generated by homologous L/R recombination. Then, viral constructs were transduced into a 293A cell line, and high titer (10^8-10^10 IU/ml) viral particles were obtained through two rounds of amplification. The adenoviral titer was determined using the same method as that of the lentiviral titer, which takes advantage of 293T cells, except that the virus stock was diluted first because the high titer adenovirus could cause 293T cells to die quickly. Then, we used the adenovirus to infect mouse embryonic fibroblasts (MEFs) twice for 4 h per day at multiplicities of infection (number of viral particles per cell) of 10. Twenty-four hours post infection, half of the culture medium was changed into neural medium (Neural Basal/H11003/B-27, 5 ng/ml BDNF) every day for two successive days. Next, half of the medium was changed every 2 days until the cells were ready for immunostaining and electrophysiological experiments. Chemical compounds were freshly added in neural medium when culture medium was changed.

Fibroblast Isolation and Culture—We used E13.5 C57BL/6 mouse embryos to isolate primary MEFs, as described previously (1). The head, vertebral column, dorsal root ganglia, and visceral organs were removed, and then the remaining tissues were dissected into small pieces and digested for 10 min in 0.25% trypsin (Invitrogen). The dissociated cells were cultured in high glucose DMEM (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Biochrom), 0.1 mm non-essential amino acids, and 2 mm Glutamax in a 37 °C, and 5% CO2 incubator. The cells became confluent in ~2–3 days and were passaged in a 1:4 split. MEFs were used between passages 2 and 4.

Tail-tip fibroblasts (TTFs) were isolated from 6-week-old C57BL/6 mouse tail tips. The superficial dermis was peeled away, and the remaining tail tip was minced into 1-mm pieces. Two pieces were put into one gelatin-coated culture well of a 6-well plate; then, 2 ml of fibroblast culture medium was added, and the mixture was incubated at 37 °C for 5–7 days. Fibroblasts were collected until they migrated out of the tails and became fused; they were then passaged as MEFs.

Primary human embryonic fibroblasts (HEFs) isolated from the epidermal tissue of a 15-week-old fetus were obtained from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College. HEF were cultured until the fibroblasts became fused and digested by 0.25% trypsin and passed in a 1:3 split in fibroblast medium.

Conversion Efficiency—We counted the conversion efficiency by using the neuronal purity as the percentage of TuJ1 cells relative to the total final population (18). We randomly selected 8–10 visual fields for each well and calculated the total cell number visualized after DAPI staining and the total iN cell number indicated by TuJ1 staining. The efficiency was calculated by dividing the number of iN cells by the number of total cells in each visual field. We also calculated the neuronal yield as the percentage of TuJ1-positive cells relative to the initial population, as described previously (19).

Immunofluorescence—iN cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and blocked with 5% BSA, 4% donkey serum, and 0.1% Triton X-100 for 30 min. Primary antibodies were diluted in antibody dilution solution (ADS, PBS with 1% BSA, 0.1% Triton X-100) in the ratios specified below, and secondary antibodies were diluted 1:1000 in ADS. iN cells were incubated in primary antibodies overnight at 4 °C, and secondary antibodies were incubated for 60 min at room temperature. The following primary antibodies were used: rabbit anti-fibronectin (1:100, BOSTER), rabbit anti-S100A4 (fibroblast-specific protein 1 (FSPI)) (1:100, Proteintech), mouse anti-GFAP (1:1000, Sigma), mouse anti-Nestin (1:200, Millipore), rabbit anti-Tuj1 (1:1000, Sigma), mouse anti-Map2a (1:500, Millipore), mouse anti-NeuN (1:300, Millipore), mouse anti-synapsin (1:500, Synaptic Systems), rabbit anti-vGLUT1 (1:1000, Synaptic Systems), mouse anti-GAD67 (1:1000, Millipore), rabbit anti-TH (1:1000, Millipore), and DAPI (1:1000, Sigma). Alexa Fluor 488 and Alexa Fluor 546-conjugated secondary antibodies were obtained from Invitrogen. Cy2-, Cy3-, and Cy5-conjugated secondary antibodies were obtained from Jackson Immunoresearch.

Transplantation of iN Cells in Vivo—iN cells were labeled with GFP by lentivirus infection, and ~10^5 cells were transplanted into the cortices of P6–10 pups (C57BL/6 background, ice anesthetized) or the brain dentate gyrus area of 6-week-old C57BL/6 mice (anesthetized with 70 mg/kg pentobarbital sodium). The mice were perfused with 0.9% saline followed by 4% paraformaldehyde 1–4 weeks post-transplantation. The brains were dissected out and fixed in 4% paraformaldehyde overnight followed by dehydration in 0.1 M PBS containing 30% sucrose for 2 days at 4 °C. Consecutive coronal sections (30 μm) were sliced using a Leica SM 2000R Sliding Microtome and stored in tissue-collating solution (25% glyceral, 25% ethylene glycol in 0.1 M PBS) at −20 °C until use.

Electrophysiology—MEF-derived iN cells were placed on glass coverslips for electrophysiological detection 7–12 days post infection. Whole-cell patch clamp recordings in either voltage- or current-clamp mode were conducted to measure the voltage-activated sodium/potassium currents or action potentials, which were recorded using an Axopatch 200B or MultiClamp 700A amplifier (Molecular Devices). The electric
signals were filtered at 2–10 kHz, digitized at 20–100 kHz (Digidata 1322A; Molecular Devices), and further analyzed using pClamp version 9.2 software (Molecular Devices). The intracellular fluid contained 130 mM K⁺-gluconate, 20 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM Mg₂ATP, 0.3 mM Na₃GTP, and 10 mM sodium phosphocreatine (at pH 7.3, 310 mosmol), and the pipette ranged from 2.0 to 4.0 megahms. The extracellular fluid consisted of 124 mM NaCl, 3.3 mM KCl, 2.4 mM MgSO₄, 1.2 mM KH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose (at pH 7.4, 310 mosmol). The transmitter receptor blockers, tetrodotoxin (100 nM), AP5 (2(R)-amino-5-phosphonovaleric acid, 50 μM), and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 10 μM), were used in the bath solution for the detection of action potentials and spontaneous excitatory postsynaptic currents.

**Gene Expression Microarray Analysis**—Mouse genome-wide gene expression analysis was performed using Gene chips HOA 5.1 (Phalanx Biotech Group, Inc.). Total RNA was extracted from MEFs and ABN + Rarg + Nr5a2-infected MEFs 12 days post infection and primary neurons from a hippocampus of postnatal day 0 C57BL/6 mouse (20); the total RNA was reverse-transcribed using an Amino AllylMessageAmp™ II aRNA Amplification kit (Ambion). For gene expression profiling analysis, mouse whole genome OneArray microarray v2 (MOA-002) chips were used with 26,423 mouse genome probes and 872 experimental control probes. The Rosetta Resolver (MOA-002) chips were used with 26,423 mouse genome probes and empirical Bayes methods were used to choose the differentially expressed genes for single channel experiment analyses. Hierarchical clustering analysis was applied using a Euclidean distance matrix and the complete-linkage clustering method. Linear models and empirical Bayes methods were used to choose the differentially expressed genes that showed >2-fold changes and an adjusted p value less than 0.05.

**PCR and Real-time PCR Analysis**—In cells and wild-type neurons were cultured in neuron medium. MEFs were cultured in DMEM +10% FBS medium. All cells were washed with serum-free medium before collection. TRIzol extraction of total RNA was performed according to the manufacturer’s instructions. Six hundred ng of total RNA was reverse-transcribed and then quantified using SYBR Green (Tiangen), and the pipette ranged from 2.0 to 4.0 megahms. The extracellular fluid consisted of 124 mM NaCl, 3.3 mM KCl, 2.4 mM MgSO₄, 1.2 mM KH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose (at pH 7.4, 310 mosmol). The transmitter receptor blockers, tetrodotoxin (100 nM), AP5 (2(R)-amino-5-phosphonovaleric acid, 50 μM), and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 10 μM), were used in the bath solution for the detection of action potentials and spontaneous excitatory postsynaptic currents.

**Flow Cytometry Analysis**—At 3 days post infection, cells of each group were dissociated into single cell suspensions for flow cytometry to analyze infection efficiency based on the ratio of GFP-positive cells. At 7 days post infection, cells were fixed with 4% paraformaldehyde and stained with Tuj1 primary antibody and Cy5 secondary antibody to analyze neuronal conversion efficiency. Stained cells were analyzed by FACS Caliber apparatus (BD Biosciences) with FlowJo software (Tomy Digital Biology). The data were illustrated as the percentage of total analyzed cells, which is the number of cells in each bin divided by the number of cells in the bin that contains the largest number of cells.

**Statistical Analysis**—Statistical analysis was evaluated by two-tailed Student’s t tests. Values were considered statistically significant at p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***) Data are presented as the mean ± S.D. or ± S.E. in different experiments, and all were described in the figure legends.

**RESULTS**

**Rarg and Nr5a2 Enhance Transdifferentiation**—MEFs were separated from E13.5 embryos and cultured as initial cells for neuronal conversion. MEFs showed classical fibroblasts morphology and were positive for fibroblast markers including fibronectin and fibroblast-specific protein 1 (FSP1, also called S100A4). No preexisting neurons, astrocytes, or neural progenitor cells were detected in the culture of the MEFs, as demonstrated by immunocytochemistry with specific markers such as TuJ1, NeuN, GFAP, and Nestin (data not shown). The mouse cDNAs of Ascl1, Brn2, Ngn2, Rarg, and Nr5a2 were carried by a modified commercial adenoviral vector (Invivogen, pAd/CMV/V5-DEST) under the cytomegalovirus promoter. The MEFs were infected once a day for two consecutive days (Fig. 1A) with several groups of adenoviruses carrying the genes for GFP, ABN, ABN + Rarg, ABN + Nr5a2, and ABN + Rarg + Nr5a2 (Fig. 1, B–G).

We counted the conversion efficiency by calculating the neuronal purity as the percentage of TuJ1 cells relative to the final population 7 days post infection. We also calculated the neuro-
Rarg and Nr5a2 Enhance Fibroblasts Convert into Neurons

A. Schematic diagram showing the experimental timeline:
- MEF plating
- 1st infection
- 2nd infection
- Change to neuron medium
- Immunostaining
- Functional test

B. Immunostaining images:
- ABN
- ABN+RARα
- ABN+RARα
- ABN+RARα

C. Bar chart showing neuronal purity (%):
- Control
- ABN
- ABN+RARα
- ABN+RARα
- ABN+RARα

D. Additional immunostaining images:
- ABN+Nr5a2
- ABN+Nr5a2+RARα
- ABN+RARα
- ABN+RARα

E. Bar chart showing neuronal purity (%):
- ABN+Nr5a2
- ABN+Nr5a2+RARα
- ABN+RARα
- ABN+RARα

F. Additional immunostaining images:
- ABN+DMISO
- ABN+DMISO+RARα
- ABN+CD437
- ABN+CD437+RARα

G. Bar chart showing neuronal purity (%):
- ABN+DMISO
- ABN+DMISO+RARα
- ABN+CD437
- ABN+CD437+RARα

H. Bar chart showing neuronal yield (%):
- Control
- ABN
- ABN+RARα
- ABN+RARα
- ABN+CD437+DLPC

I. Kinetics graph:
- Control
- ABN
- ABN+RARα
- ABN+CD437+DLPC
- ABN+Nr5a2+RARα

---

JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 289 • NUMBER 10 • MARCH 7, 2014
nal yield as the percentage of TuJ1-positive cells relative to the initial population, as described under “Experimental Procedures” and previously used by another laboratory (1). Because RA signaling through the RA receptor is necessary for neurogenesis and neural development, we applied Rarg into the three-factor combination of ABN. Three days post infection, the infected MEFs appeared to show neuronal morphology with thin processes due to the addition of Rarg into ABN. Seven days later, more mature neuronal cells with TuJ1-positive staining were clearly detected, and the neuronal purity ratio became 10.83 ± 2.67% (Fig. 1, B and C). Considering the important role of RA signaling, we subsequently studied whether RA could enhance the conversion. The results showed RA could intensively promote iN cell conversion when it was added to the culture medium, and we found that all-trans-RA at a concentration of 0.5 μM had the highest effect. Thus, neural medium used in the afterward experiments was all supplemented with all-trans-RA.

Recently, microRNAs were shown to be involved in the conversion of fibroblasts to neurons (21, 22). Other nuclear receptors, such as Nr5a2, also participate in cell reprogramming (16, 17). Therefore, we performed studies to investigate whether these factors enhance the conversion in our system. After extensive screening, Nr5a2 was identified to be a good candidate to significantly enhance the conversion efficiency. The synergistic addition of Rarg and Nr5a2 with ABN greatly boosted the neuronal purity efficiency to 44.33 ± 4.25% and increased the neuronal yields to 131.48 ± 16.38% (Fig. 1H), showing a >10-fold enhancement compared with ABN alone (Fig. 1, D and E). The synergistic contribution of Rarg and Nr5a2 suggests that they could activate different signaling pathways to enhance the direct conversion of fibroblasts to neurons.

To check whether adenoviral integration occurred in the genome in this experiment, PCR analysis was performed using genomic DNA from uninfected MEFs, MEFs infected with control-GFP, and MEFs infected with the ABN+Rarg+Nr5a2 combination. No predicted PCR-amplified band was observed in any of the samples except in the positive control lane. GAPDH was simultaneously amplified as an internal control (data not shown).

We observed that the ABN+Rarg+Nr5a2 iN cells showed extremely abundant nerve neurites and synaptic connections at a very early stage (3–4 days post infection). To further analyze the fast and efficient effect of Rarg and Nr5a2, we stained cells with a marker for relatively mature neurons, Map2a, to trace the appearance of neuronal cells over time. ABN+Rarg+Nr5a2 only required 3 days to obtain Map2a-positive neuronal cells, and this conversion period was shortened 2-fold relative to ABN alone (Fig. 1I).

Conversion with Chemical Compound Agonists—Chemical approaches to manipulate biological systems have been proven to be powerful tools for studying reprogramming (23). It is advantageous to identify chemical compounds to replace transcription factors for converting fibroblasts to neurons. In this study, CD437 (6-[3-1-adamantyl]-4-hydroxyphenyl)-2-naphthlene carboxylic acid, a specific Rarg agonist (24), and DLPC (1,2-dilauroylglycerol-3-phosphocholine), an Nr5a2 agonist (25), were chosen to investigate whether these small molecules could substitute or partially substitute the corresponding transcription factors. We not only found that MEF expressed Nr5a2 but also detected DLPC increased endogenous Nr5a2 expression in our transdifferentiation system (data not shown). Applying the specific RA agonist CD437 to Rarg could enhance the conversion from MEFs and had a consistent effect as that of Rarg. Moreover, the combination of the DLPC and CD437 increased the conversion efficiency by ~2-fold (Fig. 1, F and G).

The conversion efficiency was also greatly enhanced when both the Rarg agonist CD437 and the Nr5a2 agonist DLPC were applied to the culture medium, and the result was quite favorable (Fig. 1, F and G). The effect of the agonists was dose-dependent on an effective concentration of CD437 at 0.1 μM and DLPC at 2 μM. The two chemical compound agonists synergistically functioned together to drive the neuronal purity efficiency to 37.83 ± 4.17 and the neuronal yields to 127.82 ± 21.64%; these values did not significantly differ from those for the transcription factors (Fig. 1, F and H).

To analyze the infection efficiency and conversion efficiency more accurately, we used flow cytometry to analyze the efficiencies. At 3 days post infection, the GFP-positive cell ratio of the three groups of ABN, ABN+CD437+DLPC, and ABN+Rarg+Nr5a2 was similar, which was among the range of 77.9–85.1%. At 7 days post infection, the ratio of TuJ1-positive cells in relative to total cells was calculated as conversion efficiency. The efficiencies were 1.94% for ABN, 40.8% for ABN+CD437+DLPC, and 46.2% for ABN+Rarg+Nr5a2 (data not shown). The iN cell conversion from MEFs by the chemical compound agonists was consistent with that by the transcription factors of Rarg and Nr5a2.

**Rarg and Nr5a2 or Chemical Compounds Facilitate Neuronal Maturity**—To further analyze the chemical compound effects, we examined the iN cell morphology and the expression of several specific neuronal markers in different stages of conversion. TuJ1-positive cells appeared quite early, and the mor-

---

**Figure 1. Generation of iN cells by the combination of the nuclear receptors Rarg and Nr5a2 as well as chemical compound agonists.** A, diagram depicting the procedures for transdifferentiation of MEFs to neurons by adenoviruses carrying ABN+Rarg+Nr5a2. B and C, RA signaling and Rarg enhance neuronal conversion, which was estimated by neuronal purity (TuJ1 cells to final total cells). TuJ1 staining was performed 7 days post-infection for MEFs infected with the corresponding adenovirus. D and E, nuclear receptor Nr5a2 promotes neuronal conversion. The combination of Rarg and Nr5a2 greatly enhance neuronal conversion from fibroblasts. ABN+Rarg+Nr5a2 iN cells show highly complex neuronal morphologies and higher efficiency. iN cells were stained with TuJ1 and quantified 7 days post infection. F and G, chemical compound agonists of Rarg and Nr5a2 effectively enhance conversion. The efficiencies were calculated for the conversion of MEFs to neurons with ABN, CD437 (Rarg agonist), and DLPC (Nr5a2 agonist) independently or in combination. H, quantification of neuronal yields (TuJ1 cells to initial plated cells; 100%: the number of TuJ1 cells equal to initial cells) 7 days post infection. I, the kinetics of transdifferentiation using different combinations. MEFs were treated with different combinations, and the Map2a fluorescence was detected every 12 h. The green bars indicate the emergence of GFP-positive cells, whereas the yellow bars indicate the presence of both GFP-positive and Map2a-positive cells. Two representative independent experimental sets are shown. GFP viruses were used as the control. The data are presented as the mean ± S.D. of cell counts (n = 10, 10 random yields were averaged in every experiment). *, p < 0.05; **, p < 0.01; ***, p < 0.001 (t test). Scale bars, 50 μm.
Rarg and Nr5a2 Enhance Fibroblasts Convert into Neurons

A

B

C

D

E

F

G

[Graphs and images showing experimental results]

[Graphs and images showing experimental results]

[Graphs and images showing experimental results]
Rarg and Nr5a2 Enhance Fibroblasts Convert into Neurons

To further investigate the synaptic connections between cells, we detected the spontaneous postsynaptic current electrophysiological activity of Rarg- and Nr5a2-activated iN cells with neuronal morphology. After being cultured in neuron medium for 12–14 days, Rarg- and Nr5a2-activated iN cells continued to display full-blown and stable action potentials (Fig. 4, G and L) and Na+/K+ currents (Fig. 4, H and M) and frequently expressed synapsin (Fig. 4, J and O). Furthermore, iN cells could show spontaneous synaptic activity with each other without needing to be co-cultured with primary neurons (Fig. 4, I, K, N, and P).

The recorded iN cells (19 of 22 of the ABN+/Rarg+/Nr5a2 group and 16 of 18 of the ABN+/Rarg+/Nr5a2 group) showed postsynaptic currents, which indicated that iN cells were capable of forming synapses with surrounding cells. The majority spontaneous postsynaptic currents could be greatly blocked by the presence of a blocker combination of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, AMPA/kainate receptor antagonist), and AP5 ((2R)-(amino-5-phosphonovaleric acid, NMDA receptor antagonist) (data not shown), further indicating that the recorded postsynaptic currents were mainly excitatory spontaneous postsynaptic currents. The electrophysiological data also suggested that Rarg- and Nr5a2-activated iN cells not only had higher conversion efficiency but also showed better physiological function. The most important point is that Rarg and Nr5a2 intensely promoted rapid maturation of the induced neurons.

Other electrophysiological parameters were measured in voltage-clamp mode. Step depolarization induced the opening of voltage-dependent sodium and potassium ion channels, which correspond to the fast and inactivating inward sodium currents and the outward potassium currents, respectively, with a possible contribution from calcium currents to the whole-cell currents (Fig. 4). The action potentials and the fast and transient inward sodium currents were blocked by tetrodotoxin, a specific inhibitor of sodium ion channels (data not shown).

Rarg and Nr5a2 Also Promote Adult Mouse Fibroblast and Human Fibroblast Conversion—To determine whether Rarg and Nr5a2 could also promote iN cell conversion from adult mouse and human fibroblasts, TTFs were isolated from 6-week-old C57BL/6 mice, and HEFs were isolated from 15-week-old human foreskin tissue. TTFs and HEFs were detected to be fibroactin-positive and TuJ1/GFAP/nestin-

FIGURE 2. Rarg and Nr5a2 promote dendritic development of iN cells. A, confocal reconstruction and quantification of dendrites of Map2a-positive cells 7 days post infection. From left to right, the images represent global dendritic form of iN cells converted from ABN neurons, ABN+/CD437+/DLPC neurons, and ABN+/Rarg+/Nr5a2 neurons. B, quantification of the ratio of unipolar, bipolar, and multipolar cells in iN cells. The values represent the mean ± S.D. The numbers in the bars represent the total detected cell numbers. C, quantification of the total branch length per cell of iN cells. D, quantification of the total branch number per cell of iN cells. E, from left to right, the images show the single cell dendritic form of ABN neurons, ABN+/CD437+/DLPC neurons, and ABN+/Rarg+/Nr5a2 neurons 2 weeks post infection. F, quantification of the total branch length and number per cell of iN cells. G, analysis of dendritic complexity of GFP+ neurons. Cumulative distribution plots of the total dendrite length and branch numbers are shown. Each symbol represents a single iN cell infected with ABN, ABN+/CD437+/DLPC, and ABN+/Rarg+/Nr5a2. The numbers in the bars represent the numbers of examined cells. The values represent the mean ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (t test). Scale bars, 50 μm.
negative (data not shown). Tuj1-positive TTF-iN cells were observed 4 days post infection, and Tuj1-positive HEF-iN cells were observed 7 days post infection. The neuronal conversion efficiencies from TTFs and HEFs were lower than that from MEFs, which is consistent to previous report (2). TTF-iN cells and HEF-iN cells also expressed the pan-neuronal markers microtubule-associated protein 2a, neuronal nuclear protein, and synapsin (Fig. 5, A–F) 10–13 days post infection and demonstrated the electrophysiological signals of action potentials, sodium currents, and potassium currents (Fig. 5, G–J) 15 days post infection. However, iN cells converted from human fibroblasts using ABN alone required 20–30 days to become sufficiently mature to fire action potentials (data not shown). The data indicate that Rarg and Nr5a2 can promote iN cell conversion not only from mouse fibroblasts but also from human fibroblasts.

In Vivo Analysis of iN Cells after Transplantation—Furthermore, we investigated the iN cell conversion, survival, and function integration after transplanting infected MEF cells to the adult hippocampal area. To trace the transplanted cells in the brain, we first infected MEFs with a lentivirus that stably expressed GFP, then with adenoviral ABN + Rarg + Nr5a2.

Three days post infection, the cells were transplanted into the cortices of postnatal day 6–10 pups (C57BL/6 background) (Fig. 6, A–D) or the dentate gyrus (a native neurogenesis area in the adult mouse brain) of the hippocampus of 6-week-old C57BL/6 mice (Fig. 6, E–H). The mice, which received grafts bilaterally, were euthanized 1–4 weeks after transplantation, and the brain sections were collected for analysis. The donor cells that showed GFP fluorescence were restricted to the injection site within the cortex or dentate gyrus (Fig. 6, A and E). The transplanted cells also swiftly converted into neurons in vivo. Within 1 week after transplantation, doublecortin-positive cells labeled with GFP were detected (Fig. 6, B and F), suggesting that the MEF cells were successfully converted into neurons in vivo. Furthermore, the iN cells matured soon in vivo, and NeuN-positive cells were detected 2 weeks after transplantation (Fig. 6, C and G). To explore whether the grafted cells could establish functional connections with host neurons, we stained the sections with synapsin 2–4 weeks post-transplantation, and the data indicated that some grafted cells had received extensive presynaptic innervation from other neurons within 2 weeks (Fig. 6, D and H). The iN cells were postmitotic; therefore, they theoretically did not possess oncogenicity. For 4–6 months

FIGURE 3. Characterization of Rarg and Nr5a2-activated IN cells by neuron-specific staining and electrophysiological detection. A–C, at 7–12 days post-infection, ABN + Rarg + Nr5a2 IN cells express the pan-neuronal markers Map2a (A), synapsin (B), and NeuN (C) along with Tuj1. D–F, after a longer culture period of 12–15 days, MEF-derived IN cells express a specific neuronal marker for excitatory neurons, vGLUT1 (D), a marker for inhibitory neurons, GAD67 (E), and a marker for dopaminergic neurons, tyrosine hydroxylase (F). G–K, the IN cells were electrophysiologically recorded 12 days post infection ABN + CD437 + DLPC. G, representative traces of action potentials evoked by step-depolarization of the membrane in current-clamp mode. The membrane potential was current-clamped at approximately −65 mV. H, representative traces of whole-cell currents in voltage-clamp mode. The lower panels show that IN cells were held at −55 mV, and step depolarization at 10-mV intervals was applied from −80 to +60 mV. The insets show sodium currents. I, IN cells show spontaneous action potentials. J, Map2a-positive IN cell coexpressed synapsin 12 days post infection. K, representative spontaneous postsynaptic currents (PSCs) recorded from ABN + CD437 + DLPC IN cells. L–P, the IN cells were electrophysiologically recorded 12 days post infection. The IN cells were converted by ABN + Rarg + Nr5a2 IN cells. Scale bars, 20 μm.

FIGURE 4. Characterization of Rarg and Nr5a2-activated IN cells in vivo. A–C, at 7–12 days post-infection, ABN + Rarg + Nr5a2 IN cells express the pan-neuronal markers Map2a (A), synapsin (B), and NeuN (C) along with Tuj1. D–F, after a longer culture period of 12–15 days, MEF-derived IN cells express a specific neuronal marker for excitatory neurons, vGLUT1 (D), a marker for inhibitory neurons, GAD67 (E), and a marker for dopaminergic neurons, tyrosine hydroxylase (F). G–K, the IN cells were electrophysiologically recorded 12 days post infection ABN + CD437 + DLPC. G, representative traces of action potentials evoked by step-depolarization of the membrane in current-clamp mode. The membrane potential was current-clamped at approximately −65 mV. H, representative traces of whole-cell currents in voltage-clamp mode. The lower panels show that IN cells were held at −55 mV, and step depolarization at 10-mV intervals was applied from −80 to +60 mV. The insets show sodium currents. I, IN cells show spontaneous action potentials. J, Map2a-positive IN cell coexpressed synapsin 12 days post infection. K, representative spontaneous postsynaptic currents (PSCs) recorded from ABN + CD437 + DLPC IN cells. L–P, the IN cells were electrophysiologically recorded 12 days post infection. The IN cells were converted by ABN + Rarg + Nr5a2 IN cells. Scale bars, 20 μm.
Rarg and Nr5a2 Enhance Fibroblasts Convert into Neurons

A

B

C

D

E

F

G

H

I

J

-65mV

-50mV

-60mV

-80mV

ABN+Rarg+Nr5a2

Map2a

Map2a/Tuj1

TTF

NeuN

NeuN/Tuj1

synapsin

synapsin/Tuj1

Map2a

Map2a/Tuj1

HEF

ABN+Rarg+Nr5a2

NeuN

NeuN/Tuj1

synapsin

synapsin/Tuj1

70pA

1000pA

200ms

20ms

20mV

20mV

60mV

60mV

200ms

-65mV

-50mV

200ms

60mV

60mV

200ms
Rarg and Nr5a2 Enhance Fibroblasts Convert into Neurons

MARCH 7, 2014 • VOLUME 289 • NUMBER 10
JOURNAL OF BIOLOGICAL CHEMISTRY

FIGURE 5. Rarg and Nr5a2 also promote the conversion of TTF to neurons. A–C, TTF-derived iN cells co-expressed the pan-neuronal markers microtubule-associated protein 2a (A), neuronal nuclear protein (B), and synapsin (C) 10 days post infection. D, action potentials in response to step current injections of TTF-derived iN cells. E, whole-cell currents recorded by step depolarization from −80 to 60 mV in TTF-derived iN cells. F–H, HEF-derived iN cells co-expressed the pan-neuronal markers Map2a (F), NeuN (G), and synapsin (H) 12 days post infection. I, action potentials in response to step current injections of HEF-derived iN cells. J, whole-cell currents recorded by step depolarization from −80 to 60 mV in HEF-derived iN cells.

FIGURE 6. Transplantation of ABN + Rarg + Nr5a2 iN cells in vivo. A and E, schematic representation of iN cell transplantation and overview of grafted GFP + cells in the brain cortex or dentate gyrus 2 weeks after transplantation. Green, GFP; blue, DAPI. B, F, iN cells express the immature neuronal marker doublecortin (DCX) 1 week after transplantation. C and G, iN cells express the mature neuronal marker NeuN 2 weeks after transplantation. D and H, iN cells express synapsin 2 weeks after transplantation. Scale bars, 50 μm (A and E), 10 μm (B–D and F–H).
Rarg and Nr5a2 Enhance Fibroblasts Convert into Neurons

after transplantation of iN cells in >30 mice, we did not observe tumor formation, indicating the safety of using iN cells in vivo and the potential of these cells as a source for cell replacement therapy.

Global Gene Expression and Real-time PCR Detection of ABN + Rarg + Nr5a2 iN Cells—To explore more details in the similarities and differences between ABN + Rarg + Nr5a2 iN cells and primary neurons, we compared the global gene expression pattern of mature iN cells 12 days post infection with mouse primary neurons and MEFs by microarray analysis. Cells for the array experiment were the total final cells population including converted iN cells and non-converted cells and formed a mixed population. The mixed population might reflect the actual changes in gene expression levels compared with those in MEFs because TuJ1-negative cells could be partially converted cells and present some neuron-specific genes in addition to those of TuJ1-positive iN cells. Hierarchical clustering revealed that the global gene expression profile of iN cells showed a higher degree of similarity to primary neurons than to MEF cells. Among 4384 differentially regulated genes with a >2-fold change between primary neurons and MEFs, and 2587 genes were down-regulated or up-regulated in the exact same manner between iN cells and MEFs. The others were almost classified in the same family and possessed analogous function between the two groups (Fig. 7A).

In iN cells, functionally categorized genes associated with neurogenesis, synaptic transmission, and axonogenesis were up-regulated; examples include NCAM, doublecortin, NeuroD, Sox2, Syap1, Snca, Ncadl, Ngr1, Npy, and Myt11 (Fig. 7B). Functionally categorized genes associated with fibroblast activity and mitosis were down-regulated (Fig. 7B). For instance, fibroblast growth factor 5 was completely undetected in iN cells and neurons, which indicated that even the mixture of conversion cells had lost their initial nature and turned to the other state. We also highlighted the genes listed under the GO (Gene Ontology) biological process category neurogenesis during neural development and differentiation. Additionally, indicated by the microarray results, Rarg activated retinoic acid signaling pathway members, such as CRABP, peroxisome proliferator-activated receptor-γ, and CYP2D22, which participate in neuronal differentiation or survival. Nr5a2 also up-regulated many neural metabolism process or transition-associated molecules, such as NSMCE2, RANBP9, and CHEK. The data discussed in this publication have been deposited in NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE52993 (www.ncbi.nlm.nih.gov).

Subsequently, we selected some neuron specificity genes to examine their relative expression level using real-time fluorescence quantitative PCR in MEFs, ABN + Rarg + Nr5a2 iN cells, and primary neurons. The data show that those neuron-specific genes were up-regulated in iN cells compared with the expression in MEFs, and the most changed gene was escalated >50-fold (Fig. 7C). The up-regulated tendency was identical for primary neurons and MEFs, which is consistent to the change from microarray analysis.

DISCUSSION

Research on transdifferentiation or transdetermination can be traced back to 1980s, when transient expression of DNA prepared from specific primary cells or cell lines could transform or induce differentiation of the recipient cells (26). Since then scientists began to explore the effects of ectopic specific gene expression or re-activation of endogenous genes during transdifferentiation process.

Virus-mediated gene transfer offers more advantages over DNA/RNA-mediated gene delivery, especially in the comparatively long term expression of the introduced genes. Therefore, retrovirus-, lentivirus-, or adenovirus-mediated gene delivery system has been widely used in the scientific field of committed differentiation and embryonic development.

It was first reported that functional neurons could be directly generated from mouse primary fibroblasts through ectopic expression of three transcriptional factors (1). Subsequently, functional specific neurons such as dopaminergic neurons were also successfully reprogrammed from both mouse and human fibroblasts (27). However, all these breakthroughs depend on the integrating lenti/retroviral system, which is known to increase the risk of insertional mutagenesis. We previous found that adenovirus transiently expressing Ascl1, Brn2, and Ngn2 can convert fibroblasts to neurons (18). However the induction efficiency is low; therefore, it is ideal to identify other factors to obtain high conversion efficiency.

In this study we used adenoviruses carrying a different combination of transcription factors of Rarg and Nr5a2 with ABN for the conversion of mouse embryonic and adult fibroblasts to neurons as well as human fibroblasts. We successfully rapidly converted fibroblasts to neurons with ABN + Rarg + Nr5a2 factors, and chemical compounds agonists could replace some of those factors. Our data showed that the combination of Rarg and Nr5a2 could produce an enhanced effect during conversion, indicating that RA signaling functioned synergistically with Nr5a2 to mediate the transdifferentiation. It has been demonstrated that the factors of Rarg and Nr5a2 could enhance reprogram fibroblasts to induced pluripotent stem cells (17). The possible mechanism as they explained is that these two factors may bind to key pluripotency genomic loci and promote activation of these genes. In our study Rarg and Nr5a2 may act differently as a general modulator of neural related genes, as at least RA signaling with important roles in brain has been widely studied in neural regulation (5) and Nr5a2 is also expressed in the brain (28). However, we could not exclude the other possibility that these two factors partially reprogram fibroblasts after
activate pluripotency genes. Then, the neural-specific transcription factors and neuronal medium further promote fibroblasts to neurons.

The iN cells showed neuronal morphology and neuronal gene expression patterns, generated action potentials, and formed synaptic connections. The data indicated that the iN cells mature much more rapidly and are functionally homoplasmic to primary neurons. The iN cells could survive >1 month after the transgenes were silenced in vitro. The adenoviral integration free system and the small molecule protocol for neuronal conversion would broaden the application of iN cells. Future studies are required to study the molecular mechanism of converting fibroblasts to neurons and to study the functions of iN cells in vivo. Moreover, it will be of interest to obtain iN cells of specific neuronal subtypes from fibroblasts. The data from the small molecule treatment indicated that RA signaling and Nr5a2 had important roles during the neuronal conversion. The strategy of including chemical compounds would ultimately be beneficial and promising for cell therapy and clinical applications. In conclusion, safe and functional iN cells that require less time to mature and gain higher efficiency would have potential in regenerative clinical applications. Adenoviral transduction may be used as an improved tool for the application of iN cells in regenerative medicine.

Acknowledgments—We are grateful to Dangsheng Li, Qi Zhou, and Baoyang Hu for critical comments and discussions, members of the Jiao laboratory for discussions, and Shiwen Li for technical assistance.

REFERENCES

1. Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Südholz, T. C., and Wernig, M. (2010) Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463, 1035–1041
2. Pang, Z. P., Yang, N., Vierbuchen, T., Ostermeier, A., Fuentes, D. R., Yang, T. Q., Citri, A., Sebastiano, V., Marro, S., Südholz, T. C., and Wernig, M. (2011) Induction of human neuronal cells by defined transcription factors. Nature 476, 220–223
3. Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., and Hochedlinger, K. (2008) Induced pluripotent stem cells generated without viral integration. Science 322, 945–949
4. Zhou, W., and Freed, C. R. (2009) Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells 27, 2667–2674
5. Maden, M. (2002) Retinoid signalling in the development of the central nervous system. Nat Rev. Neurosci. 3, 843–853
6. Lane, M. A., and Bailey, S. J. (2005) Role of retinoid signalling in the adult brain. Prog. Neurobiol. 75, 275–293
7. Zechel, C. (2005) Requirement of retinoic acid receptor isotypes α, β, and γ during the initial steps of neural differentiation of PCC7 cells. Mol. Endocrinol. 19, 1629–1645
8. Jacobs, S., Lie, D. C., DeCicco, K. L., Shi, Y., DeLuca, L. M., Gage, F. H., and Evans, R. M. (2006) Retinoic acid is required early during adult neurogenesis in the dentate gyrus. Proc. Natl. Acad. Sci. U.S.A. 103, 3902–3907
9. Rhinn, M., and Døllé, P. (2012) Retinoic acid signalling during development. Development 139, 843–858
10. Lu, J., Tan, L., Li, P., Gao, H., Fang, B., Ye, S., Geng, Z., Zheng, P., and Song, H. (2009) All-trans retinoic acid promotes neural lineage entry by pluripotent embryonic stem cells via multiple pathways. BMC Cell Biol. 10, 57
11. McCaffery, P., Zhang, I., and Crandall, J. E. (2006) Retinoic acid signaling and function in the adult hippocampus. J. Neurobiol. 66, 780–791
12. Giguère, V., Ong, E. S., Segui, P., and Evans, R. M. (1987) Identification of a receptor for the morphogen retinoic acid. Nature 330, 624–629
13. Dräger, U. C. (2006) Retinoic acid signaling in the functioning brain. Science’s STKE 2006, pe10
14. Boylan, J. F., Luftin, T., Achkar, C. C., Taneja, R., Chambon, P., and Gudas, L. J. (1995) Targeted disruption of retinoic acid receptor α (RAR α) and RARγ results in receptor-specific alterations in retinoic acid-mediated differentiation and retinoic acid metabolism. Mol. Cell. Biol. 15, 843–851
15. Luo, T., Wagner, E., Crandall, J. E., and Dräger, U. C. (2004) A retinoic acid critical period in the early postnatal mouse brain. Biol. Psychiatry 56, 971–980
16. Heng, J. C., Feng, B., Han, J., Jiang, J., Kraus, P., Ng, J. H., Orlov, Y. L., Huss, M., Yang, L., Luftin, T., Lim, B., and Ng, H. H. (2010) The nuclear receptor Nsr3a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. Cell Stem Cell 6, 167–174
17. Wang, W., Yang, J., Liu, H., Lu, D., Chen, X., Zenonos, Z., Campos, L. S., Rad, R., Guo, G., Zhang, S., Bradley, A., and Liu, P. (2011) Rapid and efficient reprogramming of somatic cells to induced pluripotent stem cells by retinoic acid receptor γ and liver receptor homolog 1. Proc. Natl. Acad. Sci. U.S.A. 108, 18283–18288
18. Meng, F., Chen, S., Miao, Q., Zhou, K., Lao, Q., Zhang, X., Guo, W., and Jiao, J. (2012) Induction of fibroblasts to neurons through adenoviral gene delivery. Cell Res. 22, 436–440
19. Ladewig, J., Mertens, J., Kesavan, J., Doerr, J., Poppe, D., Gläwe, F., Herms, S., Wernet, P., Kögli, G., Müller, F. J., Koch, P., and Brüstle, O. (2012) Small molecules enable highly efficient neuronal conversion of human fibroblasts. Nat. Methods 9, 575–578
20. Banker, G., and Goslin, K. (1998) Culturing Nerve Cells, 2nd Ed., pp. 339–370, MIT Press, Cambridge, MA
21. Yoo, A. S., Sun, A. X., Li, L., Stachkova, A., Portmann, T., Li, Y., Lee-Messer, C., Dolmetsch, R. E., Tsien, R. W., and Crabtree, G. R. (2011) MicroRNA-mediated conversion of human fibroblasts to neurons. Nature 476, 228–231
22. Ambasudhan, R., Talantova, M., Coleman, R., Yuan, X., Zhu, S., Lipton, S. A., and Ding, S. (2011) Direct reprogramming of adult human fibroblasts to functional neurons under defined conditions. Cell Stem Cell 9, 113–118
23. Li, W., Jiang, K., Wei, W., Shi, Y., and Ding, S. (2013) Chemical approaches to studying stem cell biology. Cell Res. 23, 81–91
24. Gianni, M., Zanotta, S., Terao, M., Garattini, S., and Garattini, E. (1993) Effects of synthetic retinoids and retinoic acid isomers on the expression of alkaline phosphatase in F9 teratocarcinoma cells. Biochem. Biophys. Res. Commun. 196, 252–259
25. Lee, J. M., Lee, Y. K., Mamrosh, J. L., Busby, S. A., Griffin, P. R., Pathak, M. C., Orlund, E. A., and Moore, D. D. (2011) A nuclear-receptor-dependent phosphatidylcholine pathway with antidiabetic effects. Nature 474, 506–510
26. Davis, R. L., Weintraub, H., and Lassar, A. B. (1987) Expression of a single transacted cDNA converts fibroblasts to myoblasts. Cell 51, 987–1000
27. Caiasso, M., Dell’Anno, M. T., Dvoretskova, E., Lazarevic, D., Taverna, S., Leo, D., Sotnikova, T. D., Menegon, A., Roncaglia, P., Colciago, G., Russo, G., Carninci, P., Pezzi, G., Gainedinov, R. R., Gustinich, S., Dityatev, A., and Broccoli, V. (2011) Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature 476, 224–227
28. Giguerev, N., Tobet, S., and Majdic, G. (2005) Widespread expression of liver receptor homolog 1 in mouse brain. Neuroendocrinol. Lett. 26, 541–547