Human Skin Keratinocytes Can Be Reprogrammed to Express Neuronal Genes and Proteins After a Single Treatment with Decitabine

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Abstract

Patient-specific cell replacement therapy is fast becoming the future of medicine, requiring safe, effective methods for reprogramming a patient's own cells. Previously, we showed that a single transient transfection with a plasmid encoding Oct4 was sufficient to reprogram human skin keratinocytes (HSKs), and that this transfection resulted in a decrease in global DNA methylation. In more recent work we showed that decreasing global DNA methylation using the U.S. Food and Drug Administration–approved cancer treatment drug decitabine was sufficient to induce expression of endogenous Oct4. Here we report that a single treatment with decitabine, followed by 5 days in a defined neuronal transformation medium, then 7 days in a neuronal maintenance medium is sufficient to convert HSKs into cells that change their morphology substantially, gain expression of neuronal markers, and lose expression of keratinocyte markers. Within 1 week of treatment the cells express mRNA for β3-tubulin and doublecortin, and at the end of 2 weeks express mRNA for NeuN, FOXP2, and NCAM1. Additionally, at the end of this protocol, neurofilament-1, nestin, synapsin, FOXP2, and GluR1 proteins are detectable by immunostaining. Thus, we demonstrate a simple method that begins the process for producing cells for cell replacement therapies without using exogenously introduced DNA.

Key words: aging; regeneration; stem cells

Introduction

As the population ages, the need for developing a cell replacement therapy to treat neurodegenerative diseases increases.[1,2] Although induced pluripotent stem cells[3] reinvigorated the possibility that patients' cells could be used to create replacement neurons, many production methods used vectors that integrated exogenous DNA into the cellular genome. Our previous approach was to transiently express Oct4 in keratinocytes, then convert them into other cell types.[4,5] We found that Oct4 expression resulted in decreased global DNA methylation in the transfected keratinocytes.[5] We later determined that decitabine treatment produced global DNA demethylation in the keratinocytes, and that this alone was sufficient to induce Oct4 expression.[6] Combining our studies that Oct4 expression is sufficient to reprogram keratinocytes into neuronal cells,[4,5] and that treatment with decitabine is sufficient to induce expression of endogenous Oct4 protein,[6] we hypothesized that treatment of human skin keratinocytes (HSKs) with decitabine alone would be sufficient to produce HSKs with increased plasticity, and

that this process could be used to begin to convert cells into neurons.

In this article we show that a single treatment with the cancer treatment drug decitabine, followed by growth in neuronal transformation (NT) and neuronal maintenance (NM) media is sufficient to directly convert HSKs into neuron-like cells that express neuronal genes and proteins. These studies demonstrate that a recipient's own cells could be converted into a different cell type without the introduction of exogenous DNA.

Materials and Methods

Cell isolation, culture, and treatment

Primary adult HSKs were isolated from normal skin (14–51 years old) obtained from the Surgical Pathology Department at The University of Iowa Hospitals and Clinics with approval of The University of Iowa's Institutional Review Board using the method previously detailed.[6] Skin strips were soaked for 1 h in 10% antibiotic-antimycotic (Invitrogen, Carlsbad, CA) and incubated overnight in Dispase II (24 U/

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mL, Roche, Indianapolis, IN). Epidermal sheets were mechanically separated from the dermis, and HSKs were isolated using 0.25% trypsin (Invitrogen) for 30 min at 37°C; 8×10^5 cells/mL were plated in keratinocyte serum-free medium (KSFM, Invitrogen) + 100 µg/mL Primocin (Invitrogen) on dishes and slides coated with collagen type IV (BD Biosciences, Bedford, MA).

HSKs were subcultured, allowed to attach for 5 h, then treated with 25 mM decitabine for 2 days. Cells were then grown in NT medium (60 mL Dulbecco's modified Eagle's medium [DMEM] low glucose; Invitrogen), 40 mL of MCDB-201 (Sigma), 1 mL of linoleic acid albumin (Sigma), 100 µM dexamethasone (Sigma), 3 mg ascorbic acid 2-phos-

phate (Sigma), 100 ng/mL basic fibroblast growth factor (Sigma), 100 ng/mL sonic hedgehog (Sigma), 10 ng/mL fibroblast growth factor-8 (Cell Sciences, Canton, MA), and 10 ng/mL brain-derived neurotrophic factor (Cell Sciences) for 5 days, then maintained for 7 days in NM medium (Neurobasal Medium [Invitrogen] with B27 supplements and 2 mM glutamine).

Adult mice were sacrificed with approval by The University of Iowa Animal Use and Care Committee. Cortical tissue was rinsed in HEPES-buffered saline solution (HBSS) and minced. Cortical neurons isolated with papain (2 mg/mL in HBSS) for 15 min at 37°C.[7] Tissue was triturated 25 times in plating medium (DMEM high glucose supplemented with

FIG. 1. Phase contrast images illustrating that decitabine-treated human skin keratinocytes (HSKs) are induced to undergo neuronal morphologic transformation. **(a, b)** Untreated HSKs grown in keratinocyte serum-free medium (KSFM) for 14 days maintain typical morphology of keratinocytes cultured in low-calcium medium. **(c, d)** Untreated HSKs transferred to neuronal transformation (NT) medium for 5 days, followed by neuronal maintenance (NM) medium for 7 days exhibit morphological changes typical for keratinocyte differentiation. **(e–i)** Decitabine-treated HSKs grown 14 days in KSFM **(e, f)** show a great increase in cell number, but maintain a typical basal keratinocyte morphology, whereas decitabine-treated HSKs transferred to the NT medium for 5 days, followed by NM medium for 7 days **(g, h)**, alter their morphology to first form cell clumps, which lift off the dish, leaving behind cells that resemble cultured adult neuronal cells **(i,** higher magnification**)**.
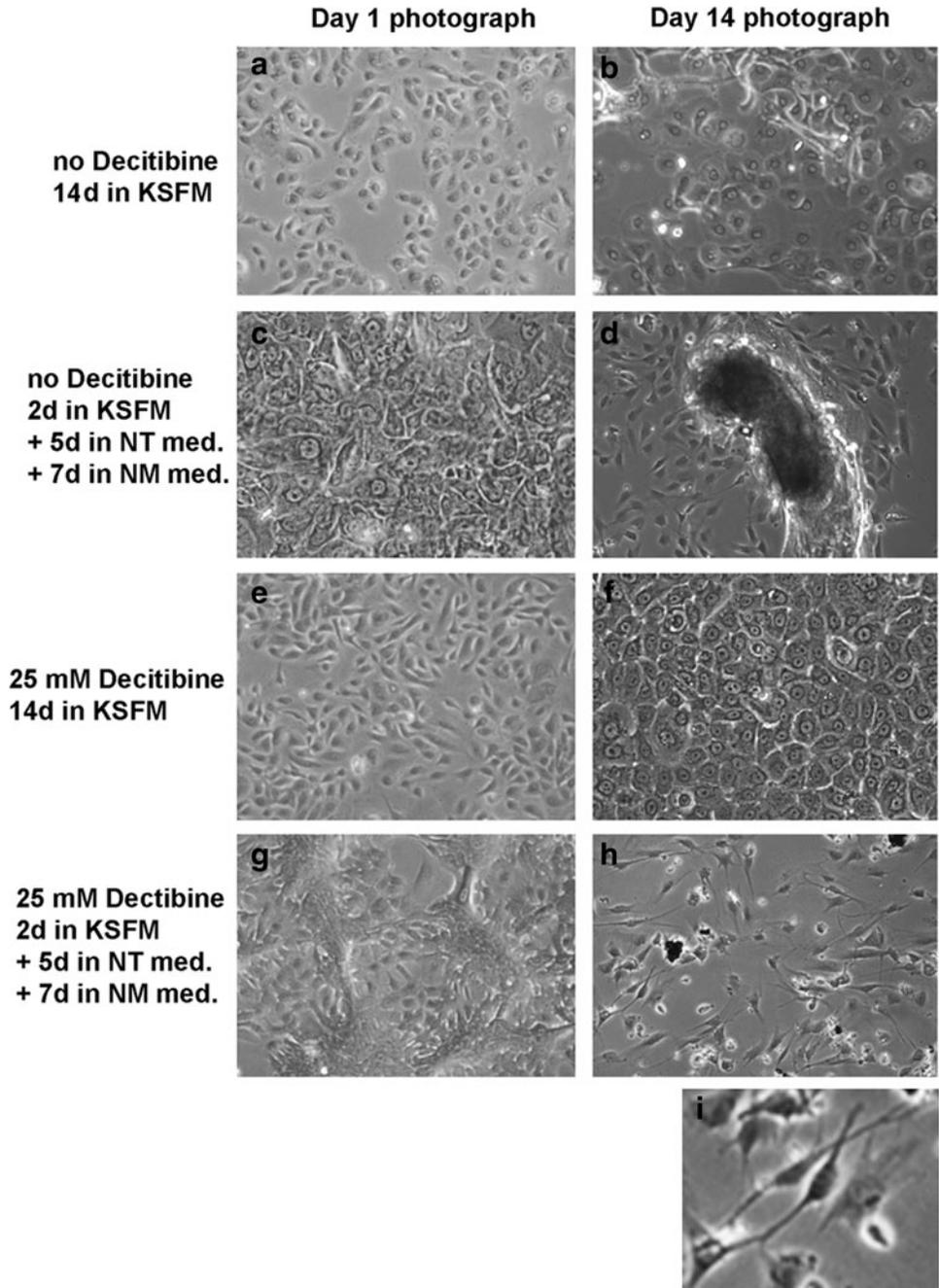
10% fetal bovine serum, 10% F-12, and 100 μg/mL Primocin), filtered through a 70-μm cell strainer (BD Falcon, Franklin Lakes, NJ), then plated on L-polyornithine coated dishes or slides for 20 min. Nonadherent cells (mostly glial cells) were rinsed off and fresh plating medium added. Individual adult mouse neurons were maintained for 10 days without feeding, then fed weekly with B27 Neurobasal Media with 100 μg/mL Primocin for 3–6 weeks.

RNA extraction and reverse transcription PCR

Total RNA was extracted from cultured cells using Trizol (Invitrogen) and reverse-transcribed using 1.5 μg of RNA, 50 μM Random Primer, 5×FS RT Buffer, 10 mM dNTP mix, 0.1 M DTT, 40 IU RNasin, 200 IU RTase superscript III, and nuclease-free DEPC water. RNA, Random Primer, and water were heated to 65°C for 5 min, then chilled. The reverse transcription (RT) reaction was incubated at 25°C for 5 min, 55°C for 1 h, and then 70°C for 15 min, using a GeneMate Genius (ISC Bioexpress, Kaysville, UT), and then chilled. For PCR, 1 μL of cDNA was added to 24 μL of the following: 10× PCR buffer; 25 mM MgCl$_2$, nuclease-free DEPC water; 10 mM dNTPs (Invitrogen); 5 IU *Taq* polymerase (Sigma); and 10 μM of each primer. PCR was performed for 35–40 cycles (denaturation at 94°C for 0.5–2 min, annealing at the specified Tm for 1 min, elongation at 72°C for 1–4 min). RNA from neuroepithelioma SK-N-MC cells was a positive control. Primers were K14 (GAA GGT GAA GGT CGG AGT C; GAA GAT GGT GAT GGG ATT TC; Tm = 57), SOX2 (GAC AGT TAC GCG CAC ATG AA; TAG GTC TGC GAG GTG GTC AT, Tm = 57), β3 Tubulin (CAT CCA GAG CAA GAA CAG CA; CTC GGG TGA ACT CCA TCT CGT, Tm = 57), NeuN (AGC GAC AGT TAC GGC AGA GT; ACT TGG ACT TGG TTG GAT GC, Tm = 57), FOXP2 (GCA GGC TGC CTT GGC AGA GA; GCT GAG GTG AGC AGC CCG GA, Tm = 58), Doublecortin (CTG TTC CCT GGA GGC TGT CCC TT; CAT CTG TTT CCT CAC ACA TGC CCA C, Tm = 58), and NCAM1 (GCC AGG AGA CAG AAA CGA AG; GGT GTT GGA AAT GCT CTG GT, Tm = 58).

Immunostaining

Cells were rinsed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min, permeabilized for 25 min with 0.2% Triton-X in PBS, blocked with 4% normal rabbit serum, then incubated for 2 h at room temperature with primary antibodies: K14 (1:500, Covance, Princeton, NJ), Nestin (1:50, Santa Cruz Biotechnology, Dallas, TX), FOXP2 (1:100, Santa Cruz), GLUR1 (1:200, Millipore, Billerica, MA), or NF-1, synapsin, SV2 (1:5, University of Iowa Developmental Studies Hybridoma Bank). Cells were rinsed and secondary antibody conjugated with Alexa 594 or Alexa 488 (Invitrogen) was added (1:1000 in 12% bovine serum albumin) for 30 min. Nuclei were stained in 4′,6-diamidino-2-phenylindole. Slides were stored at 4°C until photographed using a Nikon Eclipse E600 (Nikon, Melville, NY) epifluorescent microscope.

Results and Discussion

Previously, we made three discoveries that impacted how we thought about cellular reprogramming. First, Oct4 expression in a few mouse keratinocytes, followed by growth in neuronal medium produced cells similar to mouse neurons.[4]

Second, transient transfection with OCT4 reprogrammed HSKs and demethylated HSK DNA.[5] Third, DNA demethylation by decitabine reactivated and maintained Oct4 expression in 96% of HSKs for 2 days.[6] Thus, we hypothesized that DNA demethylation together with the elicited Oct4 expression would be sufficient to begin conversion of HSKs into neuron-like cells. Here we report that after a 2-day treatment with decitabine, then 5 days in NT medium, followed by 7 days in NM medium, HSKs substantially changed their morphology (Fig. 1g–i). Untreated HSKs kept their keratinocyte morphology if grown in KSFM (Fig. 1a, b), and underwent typical keratinocyte differentiation if grown in the NT and NM media (Fig. 1c, d), likely due to high calcium in the media.[8] HSKs treated with decitabine but maintained in KSFM increased their cell number, as we had previously found,[6] but maintained the typical appearance of cultured basal keratinocytes (Fig. 1e, f), indicating that the decitabine treatment alone did not produce neuronal conversion. Thus, only the full treatment began the cellular conversion. Since these cells are not fully converted, only beginning to convert, we call them KNeurons.

To examine the extent of conversion, we first assessed expression of neuronal genes by RT-PCR. Untreated HSKs expressed keratin K14 (data not shown), but none of the neuronal markers even if grown in NT and NM media (Fig. 2, lanes 1–2). Decitabine treatment alone induced HSKs to express the embryonic transcription factor SOX2, but no neuronal markers (Fig. 2, lane 3). HSKs treated 2 days with
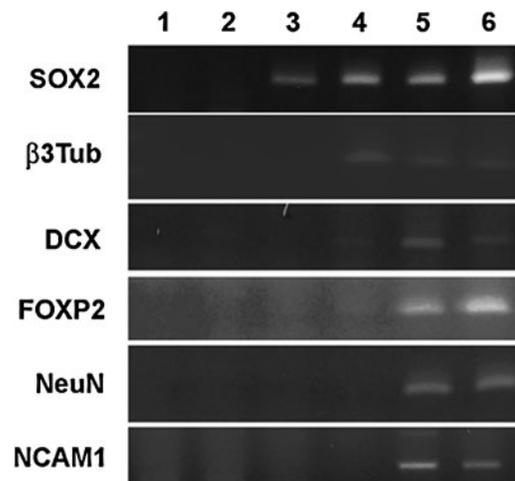
FIG. 2. Reverse-transcription polymerase chain reaction (RT-PCR) showing that decitabine treated HSKs cultured in NT and NM media produce cells that express neuronal genes. Lane 1, untreated HSKs sampled after 14 days in KSFM; lane 2, untreated HSKs sampled after 2 days in KSFM, then 5 days in NT medium, followed by 7 days in NM medium; lane 3, HSKs sampled after 2 days with 25 mM decitabine; lane 4, HSKs sampled after 2 days with decitabine, then grown 5 days in NT medium; lane 5, HSKs sampled after 2 days with decitabine, then grown 5 days in NT medium, followed by 7 days in NM medium; lane 6, positive control SK-N-MC neuroepithelioma cell line. SOX2, sex determining region Y box 2; β3Tub, tubulin β3 chain; DCX, doublecortin; FOXP2, forkhead box protein P2 (a neural transcription factor); NeuN, neuronal nuclear antigen; NCAM1, neuronal cell adhesion molecule 1.
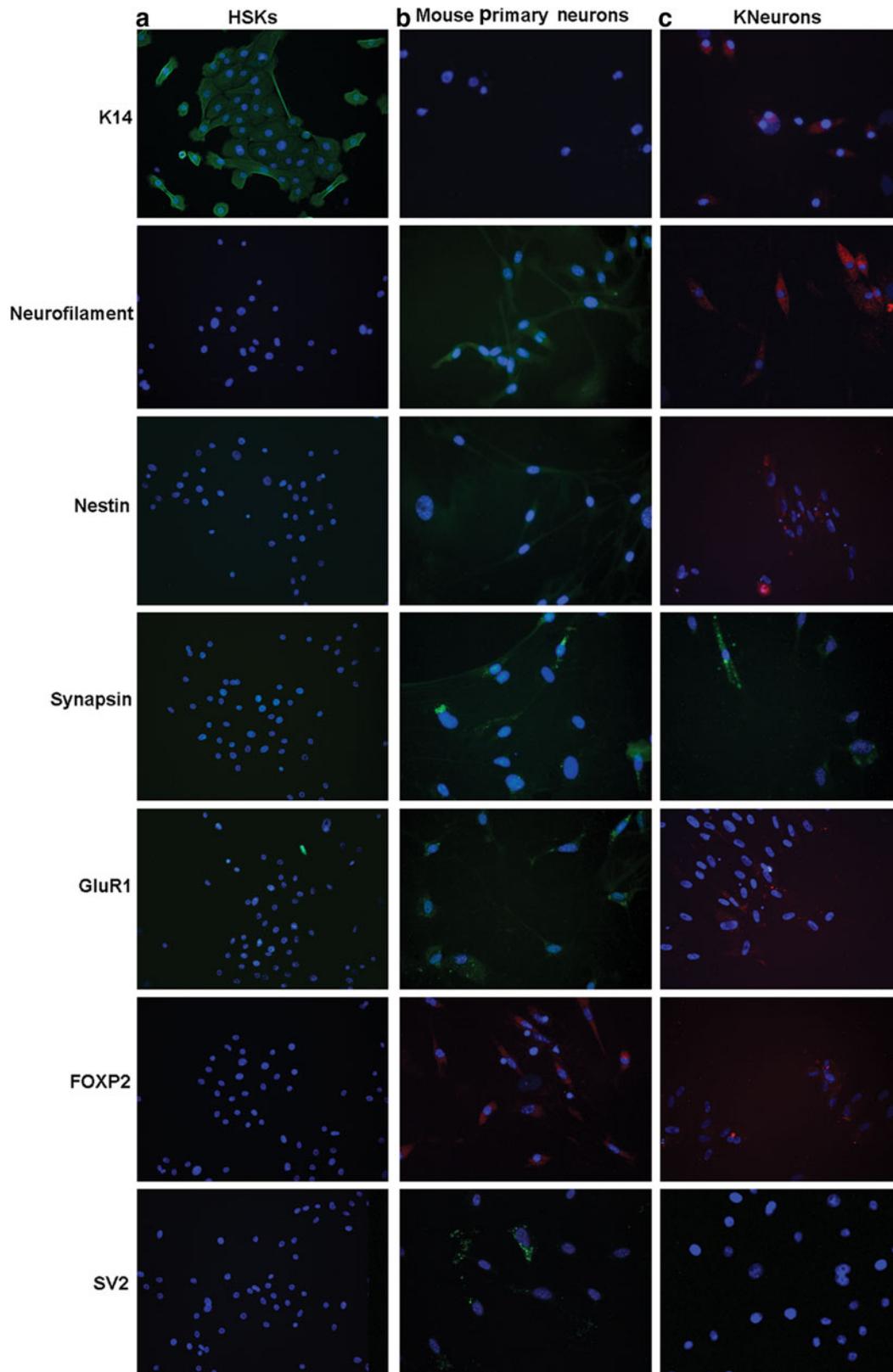
FIG. 3. Immunofluorescent images demonstrating that decitabine-treated HSKs cultured in NT and NM media produce cells that express neuronal proteins. Control HSKs grown in KSFM **(a)** express K14, but no neuronal proteins, whereas the control mouse cortical neurons **(b)** express all the neuronal proteins, but not K14. KNeurons **(c)**—cells treated 2 days with decitabine, then transferred to NT medium for 5 days, followed by NM medium for 7 days—express the neuronal proteins NF-1, nestin, synapsin, GluR1, and FOXP2, but not the synaptic vesicle protein SV2. They only show patchy expression of K14. Primary antibodies are denoted on the left side of the figure. Red, Alexa 594 secondary antibody; green, Alexa 488 secondary antibody; blue, 4′,6-diamidino-2-phenylindole. K14, keratin 14; NF-1, neurofilament-1; GluR1, glutamate receptor-1 found in glutamate-gated ion channels in excitatory neurotransmitters; SV2, synaptic vesicle protein SV2.

decitabine followed by growth in NT medium expressed SOX2 and the early stage neurogenesis markers tubulin β3 chain and the microtubule-associated protein doublecortin (Fig. 2, lane 4). After 7 days in the NM medium, KNeurons also expressed the later stage neuronal transcription factors, FOXP2, NeuN, and the neuronal cell adhesion molecule, NCAM1 (Fig. 2, lane 5). The control SK-N-MC cell line expressed all neuronal markers (Fig. 2, lane 6). That SOX2 was expressed first is consistent with its role with Oct4 in reprogramming cells[4,5,9] and suggests that HSKs need to be "primed" before they can begin the conversion into KNeurons.

Cellular distribution of neuronal proteins in KNeurons showed some similarities to primary cultured neurons as assessed by immunocytochemistry. As expected, untreated HSKs exhibited filamentous K14 immunostaining and no staining for the neuronal proteins (Fig. 3a), while primary mouse cortical neurons stained for all neuronal proteins, but did not show K14 staining (Fig. 3b). Although some cells in KNeuron cultures showed patchy, nonfilamentous K14 staining, most had no K14 (Fig. 3c), suggesting that the treatment may begin the conversion of some HSKs earlier or to a greater degree. Staining for neuronal proteins was similar between KNeurons and mouse cortical neurons with three exceptions: (1) neurofilament and nestin were slightly clumped in KNeurons; (2) staining was lighter in KNeurons for synapsin (a neuron-specific phosphoprotein that coats and binds synaptic vesicles), FOXP2 (a neural transcription factor), and GluR1 (a membrane protein in the glutamate-gated ion channel in excitatory neurotransmitters); and (3) SV2 (a synaptic vesicle protein) found in the mouse cortical neurons, was not in the KNeurons, indicating that KNeurons had only begun to convert but were not fully converted into neurons.

Even though donors ranged from 14 to 51 years old, we saw no differences in responses in younger keratinocytes versus older keratinocytes. The timeline of neuronal marker expression followed that seen in adult neurogenesis,[10] with doublecortin and β3-tubulin appearing first and NeuN and NCAM1 appearing later. However, unlike typical adult neurogenesis, KNeuron cultures expressed both doublecortin and β3-tubulin together, suggesting that the cells had not fully differentiated or that there was a mixed population of cells in these cultures. Full differentiation may not be desirable prior to cell therapy. Neurons mature differently in the developing brain than in adult neurogenesis, in which maturing neurons likely require stimulation from already present neurons prior to establishing downstream synapses and final maturation.[11,12] This is thought to prevent newly formed adult neurons from disrupting established neuronal pathways. Thus, using cells that have only begun to differentiate into neurons may be preferable for adult cell therapy.

Acknowledgment

Author Disclosure Statement

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