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Directed Conversion of Alzheimer's Disease Patient Skin Fibroblasts into Functional Neurons

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SUMMARY

Directed conversion of mature human cells, a[k]m fibroblasts to neurons, is of potential clinica[l] th[i]s for neurological disease modeling as well a[s] cell therapeutics. Here, we describe the effi[c]ient ge[n]eration of human-induced neuronal [(]hiN) cells fr[o]m adult skin fibroblasts of unaffe[c]ted indi[v]iduals a[n]d Alzheimer's patients, using viral tran[s]duction of transcription regulators and e[x]trinsic [s]upport factors. hiN cells from unaffected individuals display morphological, electrophysiol[o]gical, and gene expression profiles that typify glutamate[r]gic forebrain neurons and are competent to integrate functionally into the rodent CNS. hiN cells from familial Alzheimer disease (FAD) patients with presenilin-1 or -2 mutations exhibit altered processing and localization of amyloid precursor protein (APP) and increased production of Aβ42, relative to the same patient fibroblasts or hiN cells from unaffected individuals. Together, our findings demonstrate directed conversion of human fibroblasts to a neuronal phenotype and reveal cell type-selective pathology in hiN cells derived from FAD patients.

INTRODUCTION

Mature mammalian cells can be reprogrammed to alternative fates by introduction of lineage-specific transcription regulators. For instance, Myod1 expression has been shown to induce a myocyte phenotype in fibroblast cultures (Davis et al., 1987). Similarly, transduction of a set of pluripotency regulators is sufficient to convert skin fibroblasts to induced pluripotency stem (iPS) cells with embryonic stem cell characteristics (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). iPS cell technology has fueled much excitement in regenerative medicine, as these cells could be differentiated to generate "replacement" cell therapeutics. Patient iPS cell-derived neurons have also been proposed to serve as neurodegenerative disease models (Abeliovich and Doege, 2009).

A limitation to human iPS cell technology is that it remains inefficient (less than 1% of cells are typically reprogrammed) and time intensive; iPS cell generation and subsequent differentiation to a neuronal phenotype can take 1–2 months each. Furthermore, the pluripotent state is associated with tumorigenesis and genetic instability (Pera, 2011). Recently, the directed conversion of rodent skin fibroblasts to a neuronal fate was reported, utilizing a set of three forebrain transcription regulators and apparently circumventing the production of a pluripotent intermediate state (Vierbuchen et al., 2010). Here, we describe the directed conversion of adult human fibroblasts to a neuronal phenotype, termed human-induced neuronal (hiN) cells. To validate the approach, we show that hiN cells display electrophysiological properties of forebrain glutamatergic neurons and can integrate into mammalian CNS circuitry.

We further apply hiN cell technology to a panel of skin fibroblasts derived from patients with sporadic or familial forms of Alzheimer's disease (AD) and examine AD-associated neuronal pathologies. AD patients typically present with age-associated cognitive dysfunction, including reduced short-term (episodic) memory and spatial disorientation. These cognitive deficits are associated with neuronal and synaptic loss that is most prominent within the medial temporal lobe of the cerebral cortex and the hippocampus (Alzheimer, 1907). Additional pathological features of AD include extracellular amyloid plaques composed largely of Aβ fragments of amyloid precursor protein (APP) and intraneuronal tangles of Tau-paired helical filaments (Hardy and Selkoe, 2002). Rare, autosomal, dominantly inherited familial forms of AD (FAD) are caused by mutations in APP or in the two presenilin genes (presenlin-1 and –2, or PSEN1 and PSEN2) that encode components of the γ-secretase enzyme complex that is required for APP cleavage to Aβ (Hardy and Selkoe, 2002).

The amyloid hypothesis of AD, which is based on the aforementioned pathological and genetic findings, proposes that modified cleavage of APP by β-secretase and γ-secretase leads

to the generation of a pathogenic Aβ42 fragment. Consistent with this hypothesis, expression of disease-associated PSEN FAD mutations in cell and animal models leads to preferential accumulation of Aβ42 isoform relative to an Aβ40 isoform. Nonetheless, basic questions regarding the pathogenic mechanism of PSEN FAD mutations remain (De Strooper and Annaert, 2010; Shen and Kelleher, 2007). For instance, although PSEN FAD mutations increase relative Aβ42 production, they paradoxically reduce total γ-secretase activity, at least in cell-free and heterologous cell overexpression systems (Bentahir et al., 2006; Walker et al., 2005). The potential role of reduced γ-secretase activity in the disease process remains controversial. As the majority of studies to date rely on exogenous overexpression of PSEN FAD mutations in tumor cells, transgenic mice, or skin fibroblasts, the impact of endogenous PSEN FAD mutations on functional human patient neurons remains unclear. Moreover, why mutations in the broadly expressed PSEN-1 and -2 genes lead to a selective neuronal pathology is an open question.

RESULTS

Phenotypic Characterization of hiN Cells

We initially attempted to convert human adult skin fibroblasts (STC0022; see Table S1 available online) to hiN cells by viral cotransduction of a combination of three transcription factors—Ascl1, Brn2, and Myt1l—that were reported to be effective for reprogramming of rodent cells (Vierbuchen et al., 2010). These attempts were unsuccessful and led to prominent apoptotic cell death. Viral cotransduction of a set of brain transcription regulators—Brn2, Myt1l, Zic1, Nurr2, and Ascl1, in the presence of neuronal survival factors (including brain-derived neurotrophic factor [BDNF], neurotrophin-3 [NT3], and glial-conditioned media [GCM]), resulted in the generation of cells with a neuronal morphology (human-induced neuronal cells, or hiN cells) (Figures 1A–1N). Three weeks after viral transduction, hiN cells were positive for neuronal markers, including Tuj1, MAP2, Tau1, NeuN, NCAM, and neurofilament-160 kd (Figures 1B–1G, 1J–1N, and Figure S1). Such cells were never observed in fibroblasts transduced with control vector (Figures 1H and 1I). Cell staining with the astroglial marker glial fibrillary acidic protein (GFAP) was not detected in hiN cell cultures (Figure S1). More than 90% of MAP2-positive cells were positive for the neocortical glutamate neuron marker Tbr1 (Figure 1K), and theseTbr1-positive cells did not express the fibroblast marker, fibroblast-specific protein-1 (FSP1; Figure 1L). Approximately half of the MAP2-positive cells were positive for the mature glutamatergic neuron marker vesicular glutamate transporter-1 (vGLUT1) in a stereotypical punctate pattern (Figure 1M). Only rare MAP2-positive cells (less than 1%) displayed the GABAergic neuron marker, glutamic acid decarboxylase-65 (GAD65; Figure 1N).

We applied our hiN cell conversion protocol to a panel of nine adult human skin fibroblast lines in total (see Table S1). Quantitative analysis indicates that conversion efficiency of fibroblasts to MAP2-positive hiN cells across these lines ranged from 7.1% to 8.9% (as a percentage of input fibroblasts; n = 3 per group). After accounting for cell attrition during the 3 week culture, 28.4%–36.1% of the surviving cells were MAP2 positive (Figure 1O). Across these lines, 48.2%–60.9% of the MAP2-positive cells were also positive for vGLUT1 (Figure 1P).

Time course analysis indicated that MAP2- and vGLUT1-positive hiN cells first appear by day 7 after viral vector transduction and that maximal conversion occurs by (Figure 2A). After 21 days, hiN cell number decreases and this is accompanied by evidence of apoptosis (Figure and Figures C–S2G). Remaining cells displayed progressively elongated processes, as expected (Figure S2B). To determine minimal factors that are necessary and sufficient to generate cells, we removed individual transcription factors or extrinsic components from the conversion protocol. These data indicated that Ascl1 and Brn2 are essential for the process, whereas Zic1 and Myt1l modify efficiency, and Olig2 appears to be redundant (Figure 2B). After transduction with viral factor cocktails, converted cells maintained expression of the extrinsic virally encoded *Ascl1*, *Brn2*, and *Myt1l* transcription factors, as determined by RT-PCR analysis, whereas extrinsic *Zic1* expression was maintained only in a subset of cultures (Figure S2A). Maintenance of exogenous factor expression may have contributed to the apoptotic loss of hiN cells with extended culturing. Of the tested soluble extrinsic factors, only BDNF appeared essential for production of MAP2$^+$/vGLUT1$^+$ cells (Figure 2B and Figure S2D). A single polycistronic lentivirus vector harboring the genes *Ascl1*, *Brn2*, and *Zic1* (ABZ vector) was sufficient for the conversion process (Figure 2C and Figures S2K–S2N). ABZ vector-mediated conversion was highly efficient and could be further enhanced by adding *Myt1l* (Figure 2C and Figures S2O–S2V). Specifically, 62% ± 6% of the adult human fibroblasts that were transduced with the ABZ vector and 85% ± 15% of the cells that were transduced with the ABZ vector and *Myt1* acquired a MAP2-positive neuronal morphology phenotype (Figure 2C and Figures S2L–S2Q). These hiN cells expressed additional neuron markers, including Tau-1, Tuj1, Tbr1, and vGLUT1 (Figures S2R–S2V).

To further characterize the hiN cell phenotype, we performed whole-transcriptome gene expression profiling on neurons that were purified from hiN cell cultures. hiN cell cultures were subjected to fluorescence-activated cell sorting (FACS; Figures S2H–S2J) to select for neural cell adhesion molecule (NCAM, a marker for mature neurons as well as some neural progenitors)-positive cells. RNA preparations from FACS-purified hiN cells, total (“mixed”) cultures, and unconverted fibroblasts were then analyzed for genome-wide expression (Table S2). Hierarchical clustering analysis demonstrated that the transcriptome profiles of purified hiN cells were more similar to each other than to the originating fibroblasts (Figure 2D). Using gene ontology (GO) functional annotation, we identified genes that are most enriched within the purified hiN cell samples relative to the fibroblast samples (upregulated by at least 4-fold with a significance analyses of microarrays false discovery rate [FDR] cutoff of less than 25%). Consistent with a neuronal phenotype, the most highly enriched, functionally annotated gene sets in the purified hiN samples included “axonal projection” and “neuronal differentiation” genes (Figures 2E–2G and Table S2). Finally, we performed hierarchical clustering to broadly compare hiN cell gene expression profiles to those seen in human neurons (isolated from postmortem brain samples) and