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Enhanced conversion of induced neuronal cells (iN cells) from human fibroblasts: utility in uncovering cellular deficits in mental illness-associated chromosomal abnormalities

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Abstract

The novel technology of induced neuronal cells (iN cells) is promising for translational neuroscience, as it allows the conversion of human fibroblasts into cells with postmitotic neuronal traits. However, a major technical barrier is the low conversion rate. To overcome this problem, we optimized the conversion media. Using our improved formulation, we studied how major mental illness-associated chromosomal abnormalities may impact the characteristics of iN cells. We demonstrated that our new iN cell culture protocol enabled us to obtain more precise measurement of neuronal cellular phenotypes than previous iN cell methods. Thus, this iN cell culture provides a platform to efficiently obtain possible cellular phenotypes caused by genetic differences, which can be more thoroughly studied in research using other human cell models such as induced pluripotent stem cells.

The difficulty of accessing the brain is a major barrier to studying mechanisms involved in brain disorders associated with cell pathology/dysfunction (Dolmetsch and Geschwind, 2011; Pasca et al., 2011). Various techniques have been developed in an attempt to overcome this limitation, however induced pluripotent stem (iPS) cells require months to prepare, while olfactory cells are not central nervous system cells (Brennand and Gage,

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2011; Brennand et al., 2013; Gamo and Sawa, 2014; Horiuchi et al., 2013). The novel technology of induced neuronal cells (iN cells), which directly converts human peripheral cells into cells with postmitotic neuronal traits, is particularly promising for translational neuroscience (Kano et al., 2015; Vierbuchen et al., 2010; Yang et al., 2011). However, a major technical barrier that has limited the utility of iN cells is their low conversion rate (Ladewig et al., 2012).

To overcome this problem, we have optimized a recipe for the conversion media. iPS cell studies have showed that epigenetic modifiers act on chromatin state to facilitate reprogramming (Hochedlinger and Plath, 2009; Huangfu et al., 2008). Thus, we compared the effect of epigenetic modifiers on the direct conversion of adult fibroblasts to neurons. The epigenetic modifiers assessed in our study were: valproic acid (VPA), pan-HDAC inhibitor [Trichostatin A (TSA)], and DNA methylation inhibitor [5-azacytidine (5AZA)] (Graff and Tsai, 2013; Huangfu et al., 2008). We first examined the effects of VPA, TSA, and 5AZA individually on the conversion of control fibroblasts (Demographic information shown in Table 1).

Human skin fibroblasts were obtained from several resources. Skin fibroblasts from adult normal controls were collected from volunteers at the Johns Hopkins Hospital. Skin fibroblasts from patients with childhood-onset schizophrenia carrying genetic variants (16p11.2 microduplication, 22q11.21 microdeletion, and 22q13.3 microduplication) were collected by the lab of Dr. JL Rapoport, at NIMH (Bethesda, MD) (Ahn et al., 2014) (Table 1). The chromosomal alterations in fibroblasts were confirmed by the CGH array analysis (Supplementary Fig. 1). These cells were maintained in MEM (11095, Invitrogen) supplemented with 10 % fetal bovine serum (26140-079, GIBCO), 1 % non-essential amino acids (11140-050, GIBCO), 1 % sodium pyruvate (11360-070, GIBCO), and 1% penicillin-streptomycin (1540-122, GIBCO) in 5 % CO₂ incubator at 37 °C.

Lentiviral constructs encoding human *ASCL1*, *POU3F2*, and *MYT1L* were prepared as described previously (Kano et al., 2015). To produce lentivirus, HEK293FT cells (R700-07; Invitrogen) were transfected with a pLenti6.3 vector along with a set of packaging vectors (Invitrogen) using lipofectamine 2000 (11668019, Invitrogen). Lentivirus-containing supernatants were collected 72 h later, concentrated by ultracentrifugation, and aliquots were stored at –80 °C until time of use. Infectious units were determined using Lenti-X p24 Rapid-Titer Kit (632200, Clontech).

Fibroblasts were plated onto four-well plastic chamber slides (Lab-Tek chamber slides, Permanox, C6932, Sigma) coated with extracellular matrigel (E1270, Sigma). The matrigel was diluted 1:40 in DMEM (11995073, GIBCO). The following day, cells were infected with *ASCL1*, *POU3F2*, *MYT1L* lentiviruses, using fibroblast culture media, at 4:1 MOI in the presence of polybrene or Hexadimethrine bromide (8 mg/mL, H9268, Sigma). Infected fibroblasts were maintained in fibroblasts media for 48 h, until the media was changed completely to neuronal media containing 1:1 ratio Neurobasal media (21103, GIBCO) and DMEM/F12 (11320, GIBCO), supplemented with B27 supplement (17504, Invitrogen) and N2 supplement (17502, Invitrogen). Further media changes for the generation of iN cells were half media changes. Where indicated, neuronal media contained the small molecule

cocktails (SMC) of SB-431542 (10 mM, S4317, Sigma), human recombinant noggin (500 ng/ml, 6057, R&D Systems), CHIR99021 (2 mM, 04-0004, Stegmet), and db-cAMP (200 mM, D0627, Sigma), for two weeks after infection. For further experiments, VPA (2 mM, P4543, Sigma), TSA (200 nM, 647926, CalBiochem), or 5AZA (20 mM, A2385, Sigma) were added to the neuronal media in combination with the SMC or alone, for one week after infection. For maintenance two weeks past infection, media was changed with neuronal medium containing BDNF (10 ng/ml, 248-BD, R&D Systems), GDNF (2 ng/ml, 21-GD, R&D Systems), NT3 (10 ng/ml, 267-N3, R&D Systems), db-cAMP (200 mM). Unoprostone (120373-24-2, gift from Sucampo Pharmaceuticals, Inc.) or 10 nM or DMSO (control) was added to the SMC medium on the 12th day of culture, for four days, and then it was added again to the neuronal medium containing BDNF (10 ng/ml), NT3 (10 ng/ml), GDNF (2 ng/ml), and db-cAMP (200 mM) for the last three days of culture. In all the experiments, cells were maintained at 37 °C in a 5 % CO₂ incubator.

To obtain quantification data, cells were fixed three weeks post-infection for MAP2 staining analysis. Fixation was done in 4 % paraformaldehyde (15713, Electron Microscopy Science) for 10 min at room temperature followed by incubation with 0.3 % Triton X-100 (9002-93-1, Sigma) in PBS (21-031, CORNING) for 10 minutes at room temperature. Cells were blocked in PBS containing 10 % normal goat serum (G6767, Sigma) and 1 % bovine serum albumin (A9647, Sigma) for 2 h at room temperature, followed by incubation with primary antibody diluted in blocking solution at 4 °C overnight. The primary antibody used was a mouse anti-MAP2 (1:500, M2320, Sigma), conjugated with the secondary antibody Alexa-488 (Life Technology), which was applied for 3 h at room temperature. Finally, cells were directly mounted with Prolong Gold anti-fade mounting media with DAPI (P-36931, Life Technology) onto cover glass. Cells were visualized using a Zeiss epifluorescence microscope. The calculations of conversion rate, and process length were analyzed based on three independent experiments using MAP2 immunostaining. iN cell conversion rate was defined by the ratio of MAP2 positive cells to DAPI positive cells with Image J program. The soma size and process length of iN cells were measured by using Image J program and Neuro J program, respectively. Statistical analyses were performed using t-test or ANOVA followed by Bonferroni adjustments.

Contrary to our expectation, we did not see any substantial increase in the conversion (fibroblasts to iN cells) rate by the addition of any of these epigenetic modifiers to the neuronal media (Fig. 1A and Supplementary Table 1). In contrast, we found a 1.93-fold increase in the conversion rate when the neuronal media was supplemented with the recently described small molecule cocktails (SMC) containing SMAD, bone morphogenic protein (BMP), and glycogen synthase kinase-3 β (GSK-3 β) signaling modifiers: SB431542, human recombinant Noggin, and CHIR99021, respectively (Ladewig et al., 2012), in the absence of epigenetic modifiers (Fig. 1A and Supplementary Table 1). When we tested the effects of epigenetic modifiers on neuronal conversion in the presence of SMC, we observed a significant increase in the conversion rate with VPA and TSA, but not 5AZA (Fig. 1A and Supplementary Table 1). Notably, we see a 4.7-fold increase in the conversion rate when the SMC is combined with VPA compared to the neuronal media only (Fig. 1A and Supplementary Table 1). A prostaglandin metabolite, later named prostone, is formed during early brain development, suggesting that it plays an important role in brain maturation

(Ueno et al., 1985). This motivated us to examine the effect of unoprostone isopropyl (unoprostone, UNO), an analog of prostone (Melamed, 2002; Thieme et al., 2001). Indeed, the direct conversion of fibroblasts into iN cells was 1.4-fold improved by the addition of UNO to the combination of SMC plus VPA (Fig. 1B, Supplementary Fig. 2, and Supplementary Table 1).

After these technical improvements, we validated the usefulness of the media containing the SMC, VPA, and UNO to study how major mental illness-related chromosomal abnormalities might impact the morphological phenotypes of iN cells. Specifically, we focused copy number variations (CNVs) associated with neurodevelopmental disorders including childhood-onset schizophrenia (Addington and Rapoport, 2009; Ahn et al., 2014; Dunham et al., 1999; Golzio et al., 2012; McCarthy et al., 2009; Nicolson and Rapoport, 1999; Toritsuka et al., 2013). iN cells obtained from age-, gender-, and race-matched healthy subjects were used as controls. We compared the conversion rate and morphological characteristics of iN cells among individuals [2 controls and patients with childhood-onset schizophrenia carrying the 22q11.2 microdeletion (2 cases), 16p11.2 microduplication (1 case), or 16p11.2 microduplication together with 22q13.3 microduplication (1 case)] (Table 1). The conversion rate of iN cells from a patient with 16p11.2 duplication was significantly higher than those of other subjects (Fig. 2 and Supplementary Fig. 2). However, all the individuals showed similar morphological characteristics of iN cells, such as cell soma size, the number of process, longest process length, or ratio of longest process length to cell soma size (Fig. 3 and Supplementary Fig. 3). Of note, small soma and long process are representative morphological characteristics of converted neuronal cells (Kano et al., 2015).

In the present study, we established a new protocol to improve the direct conversion of fibroblasts into iN cells by combining SMC, VPA, and UNO. Using this improved media, we have successfully observed detailed morphological phenotypes in iN cells derived from controls and childhood-onset schizophrenia patients. Our study facilitates future research to address functional phenotypes associated with genetic variations both under basal and stress conditions that model environmental risk factors for major mental disorders in human neuronal cells.

This study also raises a new biological question: How does the new recipe combining the SMC, VPA, and UNO increase the conversion rate of iN cells? Representative roles of the SMC, VPA, and UNO are in signal cascades, epigenetic modifications, and activation of Big Potassium (BK) channels, respectively (Cuppoletti et al., 2007; Huangfu et al., 2008). The improved conversion rate may be due to the synergistic effects of them. It would be worthwhile to investigate these effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We improved a recipe for the direct conversion of fibroblasts into iN cells
- We characterized iN cells affected by childhood-onset schizophrenia-associated CNVs
- This iN cell culture provides cellular phenotypes caused by genetic differences

Fig. 1.

Media containing small molecule cocktails (SMC), valproic acid (VPA), and unoprostone (UNO) increases the iN cells conversion rate. (A) The SMC in combination with a single epigenetic modifier (VPA, 2 mM or TSA, 200 nM) in neuronal media promotes a general increase in the production of iN cells derived from fibroblasts of normal control subjects following 3 weeks in culture. These iN cells are functional and mostly glutamatergic in subtype (Kano et al., 2015). White bars indicate iN cells produced in neuronal media only or with the addition of one of the epigenetic modifiers. The combination of SMC with VPA induced a 4-fold increase in iN cells compared to the neuronal media plus VPA alone. (B) Cells from normal control subjects, cultured for 3 weeks in the SMC plus VPA (2 mM) and

UNO (10 nM) (striped bar) media showed a further 1.4-fold increase in the iN cell conversion rate compared to cells in the SMC plus VPA media (black bar). The conversion rate is expressed as a fold change of MAP2 positive cells over DAPI positive cells in a 200x field. Means $\pm$ s.e.m. (* $p < 0.05$, *** $p < 0.001$). TSA, Trichostatin A; 5AZA, 5-azacytidine. The number of analyzed fields are shown in Supplementary Methods.

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Fig. 2.

iN cells from a patient with 16p11.2 duplication showed significantly higher conversion rate than other subjects. All cells were grown in the neuronal media containing small molecule cocktails (SMC), valproic acid (VPA) and unoprostone (UNO). Means $\pm$ s.e.m.

(*** $p < 0.001$, **** $p < 0.0001$). The number of analyzed fields are shown in Supplementary Methods.

Fig. 3.

Similar morphological phenotypes were observed in iN calls from controls and patients with childhood-onset schizophrenia carrying different copy number variations. All cells were grown in the neuronal media containing SMC, VPA, and UNO. The soma size, process length, and process number were assessed in a 200x field. Means $\pm$ s.e.m. The numbers of analyzed cells are shown in Supplementary Methods. (A) Cell soma size (μm^2). (B) The number of process. (C) Length of the longest process (μm). (D) Ratio of longest process length to cell soma size.

Table 1

Demographic information of normal control and childhood-onset schizophrenia subjects used for this study. Cauc., Caucasian; AA, African American.

	CNVs	ID	Age	Gender	Race	Age of onset	Note
Control	N/A	C3	46	Male	AA	N/A	Figure 1
		C4	51	Female	AA		
		C5	48	Female	AA		Figures 2,3
		C1	25	Female	Caus.		
		C2	27	Female	Caus.		
Child-onset schizophrenia*	22q11.2 deletion	1804	23	Female	Caus.	9	Figures 2,3
		1275	24	Female	Caus.	10	
	16p11.2 duplication	2011	16	Female	Caus.	8	
	16p11.2 duplication +22q13.3 duplication	676	28	Female	Caus.	10	

* reported in Ahn *et al.*, 2014.