A new co-culture method for identifying synaptic adhesion molecules involved in synapse formation

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Abstract The proper formation of synapses is essential for brain function. Synaptic cell adhesion molecules (CAMs) are thought to play essential roles in the initiation of the synapse formation process. The artificial synapse formation assay, in which non-neuronal cells and neurons are co-cultured, has been shown to be a powerful system for screening CAMs. However, controlling a large number of cell pools in co-culture is complicated, creating a potential barrier for high-throughput screening. This protocol describes a new co-culture method in which cDNA plasmid is transfected into human embryonic kidney 293T cells using polyetherimide 24 h after cells were mixed with neurons, and immunostaining and confocal imaging are employed for analyzing synaptogenesis. This optimized method is simpler and easier to perform than the traditional method for the examination of the synaptogenic activities of individual cell-surface proteins in isolation, and provides an unbiased screening platform for synaptogenic proteins.

Keywords Co-culture assay, Synapse formation, Synaptic cell adhesion molecules, Polyetherimide

INTRODUCTION

The brain is characterized by an enormous degree of complexity and diversity of neural networks, making it one of the most complicated organs, and plays essential roles in regulating body and mind, maintaining thinking and other important physiology actions. Chemical synapses are the elementary structure of neural networks and allow a neuron to communicate with other neurons through the release of neurotransmitters. The proper formation of a synapse is essential to the construction of neural circuits and cognitive functions, and alterations in this process lead to many neurological disorders, such as autism spectrum disorders and

mental retardation (Boda *et al.* 2010; McAllister 2007; Sudhof 2008; Zhang *et al.* 2009). Major efforts have been made to define the molecular composition of synapses (Harris and Weinberg 2012; Pereda 2014; Sudhof 2004; Sudhof and Malenka 2008), and now, our understanding of how synapses function in presynaptic terminals and postsynaptic spines has greatly expanded. However, much less is known about the molecular mechanism that determines the targeted initiation of synapse formation, partly due to the enormous variety of synapses in the brain.

Synaptic cell adhesion molecules (CAMs) were originally assumed to enable cell-cell recognition and play an important role in initiating the formation of synapses through trans-synaptic interactions (Sanes and Yamagata 2009; Yang *et al.* 2014). The artificial synapse

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formation (ASF) assay has been shown to be an efficient system for screening CAMs, having been tested by several families of known CAMs, including neurexins (Graf *et al.* 2004) and neuroligins (Varoqueaux *et al.* 2006), Synaptic cell adhesion molecules (SynCAMs) (Biederer *et al.* 2002), ephrinBs and EphBs (Aoto *et al.* 2007; Kayser *et al.* 2006), NGLs/LRRC4 s (Kim *et al.* 2006), LRRTMs (Linhoff *et al.* 2009), and PTPRO (Jiang *et al.* 2017). However, evidence from analyses of most known CAM knockout (KO) mice indicates that these CAMs mediate synapse maturation and synaptic plasticity rather than the initiation of synapse formation (Chubykin *et al.* 2007; Missler *et al.* 2003). Thus, the complete molecular mechanism for synapse formation remains elusive. Although the ASF assay is powerful for identifying CAMs, it is still inconvenient for high-throughput screening because non-neuronal cells have to be transfected and digested before they are mixed with neurons. Here, we describe a new co-culture method that is easier for screening CAMs.

MATERIALS AND EQUIPMENT

Materials and reagents

Mouse hippocampal neurons obtained using animal procedures performed in accordance with animal use rules and the requisite approvals of animal use committees of South-Central University for Nationalities, sterile cover glasses, HEK293T cells, Dulbecco's modified Eagle's medium (Gibco, 12800-017), 0.25% trypsin-EDTA phenol red (Gibco, 25200056), 0.05% trypsin-EDTA (Gibco, 25300054), DNA purification kits, Top10 strain, ampicillin plates, tissue culture flasks, 6- and 24-well tissue culture plates, and polyethylenimine (Polysciences, 24765-2).

Equipment

Laminar flow hood, tissue culture hood, cell culture incubator (37 °C, 100% humidity, 5% CO₂), hemocytometer, inverted epifluorescence microscope, a confocal microscope with a standard set of lasers and suitable image analysis software (Nikon N2 Confocal).

REAGENT SETUP

Dissection solution

HEPES (1 mmol/L; Sigma, H4034) and NaHCO₃ (4 mmol/L; Sigma, S8875) prepared in Hank's balanced

salt solution (Sigma, H2387) with pH adjusted to 7.3 and 7.4 and osmotic pressure adjusted to 300 to 320 mOsm/kg, filtered through a 0.22-μm filter, and stored at 4 °C away from light for up to 1 month.

Stop digestion solution

FBS (5% (v/v); Invitrogen, 10099141; qualified, heat-inactivated), HEPES (1 mmol/L; Sigma, H4034) and NaHCO₃ (4 mmol/L; Sigma, S8875) prepared in Hank's balanced salt solution (Sigma, H2387) with pH adjusted to 7.3 to 7.4 and osmotic pressure adjusted to 300 to 320 mOsm/kg, filtered through a 0.22-μm filter, separated into 40 mL aliquots, and stored at −20 °C for up to 2 months.

Plating medium

Glucose (0.5% (w/v); Sigma, G6152), NaHCO₃ (2.2% (w/v); Sigma, S8875), FBS (10% (v/v); Invitrogen, 10099141; qualified, heat-inactivated), transferrin (0.1% (w/v); Calbiochem, 616420), and insulin (0.025% (w/v); Sigma, I6634), prepared in MEM (Gibco, 61100-061) with pH adjusted to 7.3 to 7.4 and osmotic pressure adjusted to 300 to 320 mOsm/kg, filtered through a 0.22-μm filter, separated into 40 mL aliquots, and stored at −20 °C for up to 2 months.

Neuronal growth medium

B-27 supplement (0.1% (w/v); Gibco, 17504-010), glucose (0.5% (w/v); Sigma, G6152), NaHCO₃ (2.2% (w/v); Sigma, S8875), FBS (5% (v/v); Invitrogen, 10099141; qualified, heat-inactivated), transferrin (0.1% (w/v); Calbiochem, 616420), and insulin (0.025% (w/v); Sigma, I6634), prepared in MEM (Gibco, 61100-061).

Neuronal growth medium plus 4-AraC

Neuronal medium adjusted to 4 μmol/L arabinocytidine hydrochloride (Ara-C; Sigma, C6645).

PROCEDURE

Coverslip preparation

- Place 300 coverslips in a 50 mL tube. Wash once with 30 mL xylene, twice with 30 mL acetone, once with 30 mL 100% ethanol, once with 30 mL 70% ethanol, and at least three times with 30 mL ddH₂O (Rotate at least 30 min for each step at room temperature).

- (2) Autoclave the coverslips in ddH₂O. Place all the coverslips in a new 50 mL tube.
- (3) Rotate the coverslips in 30 mL 0.1 mg/mL poly-L-lysine overnight at 4 °C to coat the coverslips and then store at 4 °C.
- (4) Place each 12 mm coverslip in individual plate wells and wash the coverslips three times with 0.5 mL ddH₂O.

Dissociated hippocampal culture preparation

- (5) Autoclave anatomy instruments before the experiment. To prepare the enzyme solution, incubate 0.25% trypsin + EDTA for approximately 30 min before use. Warm the plating medium before use (approximately 30 min).
- (6) Dissociate mouse hippocampal neurons on the day of birth (P0) to prepare mixed neuron/glia cultures. Dissect the hippocampus in a tissue culture hood, place the hippocampal tissue into a 15 mL tube (with HBS freshly from 4 °C) with dissection solution on ice and allow the mixture to settle on ice.
- (7) Wash these hippocampal preparations three times with ice-cold dissection solution and incubate with 1 mL 0.25% trypsin + EDTA for 12 min in the incubator.
- (8) Remove the enzyme solution, wash the tissue with HBS + 10% FBS three times, and place the tissue into 1.5 mL plating medium. Break down the tissue by trituration (using 800 µL pipette tip 15–20 times).
- (9) Centrifuge cells at 800 *g* for 5 min and resuspend them in 400 µL plating medium. Count cells and add plating medium to adjust the cell density to 80,000 cells/50 µL. Add 50 µL of cell-containing medium per well and place the plate in the cell culture incubator (Day 0).
- (10) Thirty minutes later, add 1 mL of plating medium per well and place the plate in the cell culture incubator.
- (11) On day 1, remove 500 µL medium from each well and replace it with 600 µL growth medium (0 µmol/L Ara-C).
- (12) On day 4, remove 500 µL medium from each well and replace it with 600 µL growth medium (4 µmol/L Ara-C).
- (13) On day 9, remove 500 µL medium from each well and replace it with 600 µL growth medium (4 µmol/L Ara-C).

[CRITICAL STEP] Medium change is performed by replacing only half of the old medium with

fresh medium. Changing the medium completely will result in neuronal cell death.

HEK293T cell-hippocampal neuron mixed culture

- (14) On day *in vitro* (DIV) 7 of neuronal culture, place HEK293T cells atop the dissociated hippocampal neurons.
[CRITICAL STEP] Resuspend HEK293T cells in growth medium (2 µmol/L Ara-C).

HEK293T cell transfection

- (15) One day later, on DIV 8 of the mixed culture, transfect the mixed cells to express the membrane protein to be tested for its role in synapse induction. Polyetherimide (PEI) transfects cDNA plasmids into the HEK293T cells specially but not neurons in HEK293T-neuron mixed culture.

Synapse induction analysis by synaptic marker immunostaining

- (16) Perform analysis 48 h after the transfection of the HEK293T-neuron mixed cultures. Fix and stain mixed cultures with synapsin (synaptic marker).
- (17) Observe the induction of presynaptic specializations mainly along the cell boundary and atop these co-cultured HEK293T cells by confocal microscopy imaging.

RESULTS

The efficacy of the optimized co-culture assay to determine the synaptogenesis activity of CAMs by transfection of hippocampal neurons and HEK293T mixed cells was examined. The HEK293T cells were digested and seeded atop dissociated hippocampal neurons on DIV 7. After 24 h, both neurons and HEK293T cells were transfected with FUGW-GFP together with PCMV5-neurologin 2 (NL2) plasmids by PEI methods, differing from the most commonly used co-culture assay (Fig. 1A). Because cultured neurons failed to be transfected by PEI, only HEK293T cells successfully expressed GFP and NL2 protein. The co-culture activity of the optimized co-culture assay was measured by immunostaining and confocal microscopy (Fig. 1B). We transfected NL2 in HEK 293T cells for 24 h and then co-cultured the HEK293T cells with hippocampal neurons on DIV 9 for an

Fig. 1 Schematic illustrating the procedures of the original and optimized co-culture assays. **A** Schematic drawing of the original co-culture assay of hippocampal neurons mixed with HEK293T cells expressing target DNA. **B** Schematic drawing of the optimized co-culture assay of hippocampal neurons mixed with HEK293T cells expressing target DNA. Scale bars, 20 μ m

additional 48 h as a positive control, and an empty pCMV5 vector was used as the negative control. (Fig. 2A). Synapse staining with an antibody against synapsin was performed, and a program based on the co-culture index measurement (Jiang *et al.* 2017) was executed to identify positive cells (Fig. 2B). In our optimized co-culture assay, the activity of

HEK293T cells expressing GFP and NL2 was significantly greater than that in the control in which HEK293T cells expressed FUGW-GFP and the pCMV-5 vector (Fig. 3A, B). The data showed that the optimized co-culture system achieved similar synapse formation as the system using pre-existing protocols for co-culture assay employing NL2.

Fig. 2 NL2 expression in HEK293T cells induces synapse formation in a co-culture assay. **A** Representative images of hippocampal neurons co-cultured with HEK293T cells expressing FUGW-GFP together with pCMV5 vector (control) or pCMV5-NL2 (NL2) stained by synapsin. Scale bars, 20 μ m. **B** Summary graph of the co-culture index of cells from the control or pCMV5-NL2-transfected HEK293T cells co-cultured with hippocampal neurons. Summary graph shows the mean $\pm$ SEM; statistical comparison was made with Student's *t*-test (***P* < 0.001)

Fig. 3 NL2 expression in HEK293T cells induces synapse formation in the optimized co-culture assay. **A** Representative fluorescence images of hippocampal neurons co-cultured with HEK293T cells expressing FUGW-GFP together with pCMV5 vector (control) or pCMV5-NL2 (NL2) stained by synapsin in the optimized co-culture assay. Scale bars, 20 μ m. **B** Summary graph of the co-culture index of cells from the control or pCMV5-NL2-transfected HEK293T cells co-cultured with hippocampal neurons. Summary graph shows the mean $\pm$ SEM; statistical comparison was made with Student's *t*-test (***P* < 0.001)

Next, to check the high-throughput CAM screening ability of the optimized co-culture assay in synapse formation, NL1–4 and PTPRO were employed as a test set of synaptic adhesion molecules. Transfected HEK 293T cells were co-cultured with hippocampal neurons by co-culture assay or optimized co-culture assay (Fig. 4A, B). Then, the co-culture index and the required time and tips, including subculture of HEK293T cells, transfection and co-culture steps of the two assays, were quantified. Compared with the co-culture assay, the optimized co-culture assay achieved similar co-culture activity based on the co-culture index measurement (Fig. 4C, D). However, the time and tips required for the optimized co-culture assay were significantly decreased (Fig. 4E). Therefore, the optimized co-culture assay represents a new method for high-throughput CAM screening in synapse formation. Taken together, the data obtained using this assay have implications for synapse formation, and this optimized assay can be used not only for the identification of novel synaptogenic molecules but also as a more convenient method for high-throughput CAM screening with an abbreviated operating procedure.

DISCUSSION

The identification of CAMs involved in synapse formation is critical for understanding synaptogenesis and

neural circuits. Although other identification methods involving single-cell RNA sequencing (RNAseq) or high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been reported (Foldy *et al.* 2016; Frese *et al.* 2017), co-culture assays are still the most widely used methods to identify CAMs; however, the complicated control of high-throughput screening may represent a barrier in the screening process. Here, the data demonstrated that this optimized co-culture assay can identify CAMs through the transfection of NL2 plasmids into HEK293T cells by PEI after HEK293T-neurons were mixed (Fig. 3). Furthermore, using NL1–4 and PTPRO plasmids as a test set of CAMs for screening, the optimized co-culture assay achieved similar co-culture activity based on co-culture index measurement. Moreover, the data showed that the time and tips required for the optimized co-culture assay were significantly decreased (Fig. 4).

Several alternative methods bypassing the co-culture assay have been reported to identify CAMs (Foldy *et al.* 2016; Frese *et al.* 2017). Single-cell RNAseq was used to identify CAMs; neurons were first electrophysiologically characterized, and their transcriptome was subsequently analyzed by aspiration of the cytosol followed by single-cell RNAseq. This method has the advantage of analyzing gene expression in specific cell types in a defined circuit. LC-MS/MS using a systematic and in-depth proteome analysis of cultured hippocampal neurons, which can establish a quantitative map of the

Fig. 4 Co-culture assay and optimized co-culture assay for synaptic adhesion molecule screening. **A, B** Representative image of hippocampal neurons co-cultured with HEK293T cells expressing a test set of synaptic adhesion molecules using the co-culture assay (**A**) or optimized co-culture assay (**B**). **C, D** Summary graphs of the co-culture index of cells from the control, NL1, NL2, NL3, NL4 or PTPRO-transfected HEK293T cells co-cultured with hippocampal neurons using the co-culture assay (**C**) or optimized co-culture assay (**D**). **E** The time (left) and tips (right) required for the co-culture assay compared with those for the optimized co-culture system. Scale bars, 20 μ m; scale bars apply to all panels in a set. All summary graphs show the mean $\pm$ SEM; statistical comparisons were made with Student's *t*-test (***P* < 0.01, ****P* < 0.001; *n.s.* not significant)

neuron-specific proteome during developmental stages, was also reported to identify CAMs. Overexpression of CAMs in cultured neurons is another simple and efficient way to identify specific CAMs or CAM families (Kim *et al.* 2006). Recently, a novel fluorescence-activated synaptosome sorting (FASS) method with substantially improved conventional synaptosome enrichment protocols enabling high-resolution biochemical analyses of specific synapse subpopulations was reported (Biesemann *et al.* 2014). Employing primary cultured cells from knock-in mice with fluorescent glutamatergic synapses together with this optimized co-culture assay could be a simpler and more powerful system to identify special excitatory postsynaptic CAMs. This method is relatively time-effective and especially suitable for handling a large variety of CAM screens.

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Compliance with Ethical Standards

Conflict of interest All the authors declare that they have no conflict of interest.

Animal rights and informed consent All institutional and national guidelines for the care and use of laboratory animals were followed. All experimental procedures involving mice were performed under a protocol approved by the animal research ethics committee of South-Central University for Nationalities.

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