Dopaminergic Differentiation of Human Embryonic Stem Cells on PA6-Derived Adipocytes.

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Dopaminergic differentiation of human embryonic stem cells on PA6-derived adipocytes.

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Dopaminergic differentiation of hESCs

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Abstract

Human embryonic stem cells (hESCs) are a promising source for cell replacement therapies. Parkinson's disease is one of the candidate diseases for the cell replacement therapy since the motor manifestations of the disease are associated with the loss of dopaminergic neurons in the substantia nigra pars compacta. Stromal cell-derived inducing activity (SDIA) is the most commonly used method for the dopaminergic differentiation of hESCs. This chapter describes a simple, reliable and scalable dopaminergic induction method of hESCs using PA6-derived adipocytes. Coculturing hESCs with PA6-derived adipocytes markedly reduces the variable outcomes among experiments. Moreover, the colony differentiation step of this method can also be used for the dopaminergic induction of mouse embryonic stem cells and NTERA2 cells as well.

Key words: Parkinson's disease, human embryonic stem cells, SDIA, adipocytes, dopaminergic differentiation

1 Introduction

With the recent findings of somatic cell nuclear transfer in humans, human embryonic stem cells (hESCs) become a promising "personalized" source of cells for regenerative medicine [1]. During the past decade, several groups have established various methods for differentiating hESCs into dopaminergic (DAergic) neurons [2-5]. The most commonly used method for the DAergic differentiation of hESCs is the stromal cell derived inducing activity (SDIA); however, there is a significant

variability for the SDIA of PA6 cells among and within the PA6 batches [6, 7]. We revealed that adipocytes within the heterogeneous PA6 population robustly express all previously claimed factors eliciting SDIA [6, 8-10]. Moreover, PA6-derived adipocytes also express GDNF, BDNF and PEDF that have been previously shown to promote dopaminergic neuron differentiation and survival. Therefore, it is reasonable to use the PA6-derived adipocytes as a more homogenous population of feeder cells to induce the DAergic differentiation of hESCs.

In this chapter, we describe a fast, simple, and reliable method for differentiating hESCs into dopaminergic neurons using PA6-derived adipocytes as feeder cells. The low variability in the outcomes of the DAergic differentiation in this method allows a more accurate platform for further studying the DAergic differentiation mechanisms. Our method can also be used for differentiating mouse embryonic stem cells (mESCs) and NTERA2 cells into dopaminergic neurons, which allows comparison studies in DAergic differentiation. Moreover, we describe a highly scalable method for generating neural progenitors (NPs). Our method is suitable for generating NPs for large-scale applications and monolayer differentiation. These hESC-derived NPs can also be frozen and stored for long times, which facilitates the planning and execution of large-scale assays. Our method includes three simple steps for DAergic differentiation: Coculturing hESCs on PA6-derived adipocytes, expansion of NPs and monolayer differentiation.

2 Materials

2.1 Equipment

1. Cell culture disposables.
2. Laminar flow hood with HEPA filter.
3. Fume hood.
4. CO_2 incubator with humidity and temperature control.
5. Refrigerated cell culture centrifuge.
6. Inverted phase constrast microscope.
7. Hemocytometer.
8. 37°C water bath.
9. Mr. Frosty 1°C freezing container (Thermoscientific/Nalgene).

2.2 Cell culture

1. PA6 maintenance medium: MEM-α medium (PAA, Austria) with 10% FBS (Invitrogen).
2. Adipogenic differentiation medium: MEM-α medium (PAA, Austria) with 10% FBS (Invitrogen) + 0.25 µM Dexamethasone (Thermofisher) + 0.5 mM 3-isobutyl-1-methylxanthine (Thermofisher).
3. Coculture (CC) medium: Glasgow's modified Eagle's medium (GMEM) + 8% knock out serum replacement (KSR) + 2mM Glutamax, 0.1mM non-essential amino acids (NEAA), 1mM pyruvate, 0.1mM 2-mercaptoethanol (2-ME) (All from Invitrogen).
4. NS medium : Neurobasal medium (Invitrogen) + 0.5mM Glutamax (Invitrogen)+ 0.01% heparin (Sigma-Aldrich).
5. Freezing medium for NPs: Conditioned NS medium + 20ng/ml bFGF + 20 ng/ml EGF + 10% DMSO
6. Accutase (Invitrogen).

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7. Trypsin (Invitrogen).
8. Poly-L-lysine (Sigma-Aldrich).
9. Laminin (R&D systems).
10. bFGF (Invitrogen).
11. Y-27632 dihydrochloride (Tocris)

2.3 Adipocyte staining

1. Stock solution: 300 mg Oil-Red-O powder (Fisher) in 100 ml 99% isopropyl alcohol.
2. Working solution: 3 parts Oil-Red-O stock solution mixed with 2 parts distilled water and filtered through Whatman paper (Fisher) (*see* **Note 1**).
3. Hematoxylin (Fisher).
4. 10% Formalin solution (Fisher).

3 Methods

3.1 Adipogenic differentiation of PA6 cells

1. Culture PA6 cells until total confluency.
2. Replace with fresh PA6 maintenance medium and continue culturing the cells in total confluency for an additional 2 days.
3. On the 3rd day, replace with fresh adipogenic differentiation medium to induce differentiation. Change this induction medium every other day, and culture the cells in this medium for 4 days.

4. On the 7th day, replace the adipogenic differentiation medium with PA6 maintenance medium and culture the cells for an additional 4 days (*see* **Note 2**).

3.2 Oil-red-O staining

All steps involving formalin and isopropanol should be performed in a fume hood

1- Remove the medium and rinse the cultures with DPBS.
2- Remove DPBS and fix the cells with 10% formalin for 30 minutes at room temperature.
3- Rinse the culture with distilled water and incubate in 60% isopropanol solution for 5 minutes.
4- Add freshly prepared Oil-red-O working solution and incubate for 5 minutes at room temperature.
5- Rinse the cultures with tap water at room temperature until the water rinses off clear.
6- (Optional) For counter staining, add hematoxylin and incubate for 1 minute at room temperature.
7- (Optional) Remove the hematoxylin and rinse the culture with room temperature tap water until the water rinses off clear.
8- Add tap water to cultures and observe under phase-contrast microscope.

3.3 Conditioning medium with PA6-derived adipocytes

1. Remove the PA6 medium and rinse the PA6-derived adipocyte culture with DPBS twice.
2. Add NS medium (12 ml for T75 flask) to the culture and incubate for 24 hours.
3. Collect the medium and centrifuge at 500 *x g* for 5 minutes to exclude the debris.
4. (Optional) By using a 0.22 μM hydrophilic PVDF syringe filter, sterilize the conditioned medium and remove any remaining cells.
5. If not used immediately, freeze and store the conditioned medium at -20°C (see **Note 3**).
6. Discard the culture (*see* **Note 4**).

3.4 Plating PA6-derived adipocytes as feeder cells

1. Coating the dishes. Coat culture dishes (or culture slides) with 0,1% gelatin and incubate at least for 30 minutes at room temperature before plating the feeder cells.
2. Single cell dissociation. Rinse the culture with DPBS twice. Add 0.05% trypsin-EDTA (3 ml for T75 flask) and incubate at 37°C for 5 minutes. Tap the flask few times from the sides and the bottom to detach the cells, and dissociate into single cells by pipetting several times. Add PA6 maintenance medium to stop the enzymatic reaction. Centrifuge at 300 *x g* for 5 minutes at 4°C. Carefully discard the PA6 medium and resuspend the pellet in CC medium (*see* above). Count the cells with a hemocytometer.

3. Plating cells. Aspirate the gelatin and wash the gelatin-coated dishes with DPBS once. Plate 80.000 cells/cm^2 (*see* **Note 5**). Incubate the feeder cells in the incubator for two days at 37°C and 5% CO_2.

3.5 Colony differentiation

1. Dissociation of the cells. The day before dissociation (Day -1) add 10 μM ROCK-inhibitor (Y-27632 dihydrochloride) to the hESC culture to increase cell survival during the dissociation process. Depending on the hESC line used, dissociate hESCs either enzymatically or mechanically (*see* **Note 6**). If the hESCs will be dissociated mechanically, replace the hESC maintenance medium with CC medium just before cutting the colonies into small clumps. Use a 23 Gauge needle or commercially available stem cell cutting tools to cut hESC colonies into small clumps (10-20 cells per clump) (Fig. 1C). Plate the dissociated small clumps on feeder cells. Only at the first day of coculture, add 4 ng/ml bFGF to the medium to improve cell survival.
2. Maintenance. Culture the cells in the incubator at 37°C and 5% CO_2. Replace half of the medium carefully every other day without exposing the colonies to air. For neural progenitor generation and monolayer differentiation, transfer the "neural colonies" on day 14 as described below. For full colony differentiation continue until day 21 (*see* **Note 7**).

3.6 Monolayer neural progenitor generation and differentiation

1. Detach the “neural colonies” on day 14 (*see* **Note 8**) using a 23 Gauge needle under a microscope.
2. Plating. Collect the detached colonies using a 100 ml micropipette and plate on a gelatin coated 35 mm dish.
3. Maintenance. Use NS medium conditioned with PA6-derived adipocytes for culturing. Freshly add 10 ng/ml of bFGF and 2% B27 (without retinoic acid) to the medium. Replace half the medium every other day.
4. Passaging. Within few days, cells from the attached colonies spread outward (Fig. 1H). Any colonies displaying morphological difference than the neural colonies at this stage should be removed using a micropipette. When the cells become confluent (typically in 10-14 days), aspirate the medium and wash the cells once with PBS. Add 0.4 ml Accutase and incubate for 5 minutes at 37°C and 5% CO_2. Gently triturate to single cells using a 1 ml pipette. Add NS medium and centrifuge at 300 *x g* for 5 minutes at 4°C. After centrifugation resuspend the cells in conditioned NS medium with 10 ng/ml of bFGF and 2% B27 (without retinoic acid). Plate the cells (5×10^4 cells/cm^2) onto a new gelatin coated dish. From the second passage and on, the neural progenitors can be split at a 1:3 ratio using the same procedure (*see* **Note 9**). The neural progenitors at this stage can be frozen, used for further analyses or differentiated (*see* **Note 10**).
5. Monolayer DAergic differentiation. Plate NPs on glass chamber slides (or plastic culture dishes) coated with poly-l-lysine and laminin (*see below*) at the density of 100,000 cells/cm^2. Use CC medium at this stage. To increase cell survival, add 4 ng/ml of bFGF to the medium just for the first day. To induce

terminal differentiation, culture the cells in the absence of bFGF afterwards. Perform half medium change every other day. Culture the cells until day 9.

3.7 Poly-L-lysine and laminin coating

1. Dilute Poly-l-lysine (PLL) with DPBS at 1:2 ratio.
2. Cover the culture dish or slide with this diluted PLL and incubate at 37°C overnight.
3. Remove the PLL and wash the culture slide with DPBS once.
4. Cover the culture slide with 10µg/ml laminin and incubate at 37°C overnight.
5. Wash the culture slide with DPBS once before use.

3.8 Freezing and storing neural progenitors

1. Follow the steps described above for passaging.
2. After centrifugation suspend the cells in freezing medium .
3. Count the cell number using a hematocytometer.
4. Distribute the cells into cryotubes.
5. Put the cells into -80°C freezer overnight in a 1°C freezing container.
6. Transfer the cells into -150°C next day.

3.9 Thawing neural progenitors

1. Warm up 9 ml NS medium to rinse.

2. Thaw the vial in 37°C water.
3. Add 9 ml pre-warmed NS medium and spin down at 500 *x g* for 5 minutes.
4. Suspend the pellet in conditioned NS medium and plate on 0.1% gelatin-coated dishes.

4 Notes

1. Although the stock Oil-Red-O solution can be stored up to one year at room temperature, working solution is only stable for 2 hours. Therefore, working solution should be freshly prepared just before staining.
2. Adipogenic differentiation protocol for PA6 cells slightly differs from the protocols for other preadipocyte cell lines. It does not require the addition of insulin or PPARγ [11]. In our hands, we could differentiate PA6 cells into adipocytes with 81% efficiency; however, there might exist variations between batches. It should also be noted that PA6 cells in the culture do not differentiate homogeneously at the same pace. Thus, not only mature adipocytes with big lipid vesicles, but also "less-mature" adipocytes with smaller lipid vesicles exist in the culture at the end of the differentiation (Fig. 1D). To determine the differentiation efficiency accurately, oil-red-O staining is recommended.
3. The conditioned medium can be stored at -20 for up to 1 year.
4. Each PA6-derived adipocyte culture can only be used once for conditioning medium .
5. Not all the PA6 cells become adipocytes at the end of the differentiation. If the differentiated PA6 culture is plated with low numbers of cells, undifferentiated

PA6 cells start to proliferate, increasing their percentage in the culture. Therefore, high numbers of cells are plated in order to stop proliferation of the undifferentiated PA6 cells by contact inhibition (Fig 1D).

6. If the hESCs are enzymatically dissociated, plate 250 hESCs/cm^2 suspended in CC medium. When calculating the number of hESCs after enzymatic dissociation, be aware of the number of the feeder cells in the suspension if hESCs are maintained on feeder cells. Subtract the number of feeder cells when calculating the exact number of hESCs to be plated.
7. Our results revealed that not only hESCs but also mESCs and NTERA2 cells can also be differentiated into DAergic neurons on PA6-derived adipocyte coculture (Fig 2). Similar to hESCs, 21 days of differentiation is required for NTERA2 cells whereas 14 days of culturing is sufficient for mESCs.
8. Similar to PA6 coculture, morphologically different types of colonies are observed on the 14^{th} day of PA6-derived adipocyte coculture [7, 12]. Among those colonies, “neural colonies" that are rich in neural cells are identified according to a series of morphological criteria: They have a round shape and brownish color, often extend short processes outside the core of the colony, and are easily detached from the feeder cells using a 23 Gauge needle (Fig 1 E-G). Other colony types such as the ones having flat, amorphous or cystic morphology do not have the required neural identities (Fig 1F).
9. After each passage, two morphologically distinct cell types emerge in the NP culture dish. Large, flat-like cells resembling astrocytes and small, round-shaped, dark colored cells with high nucleus-to-cytoplasm ratio. Our ICC results revealed that the small cells are the neural progenitors. The large, flat cells initially form a monolayer occupying a large proportion of the culture

dish; however, this is not the time point to passage cells. On top of this monolayer, small round-shaped cells (NPs) start to emerge as clusters (Fig 1H). When those neural progenitors, cover more than half of the culture dish, the cells can be passaged. It should also be noted that coculturing hESCs with PA6-derived adipocytes significantly reduces the percent of Ki67-positive NPs generated compared to NPs generated with PA6-coculture (25 % vs. 59%, $p<0.002$). Therefore, in this protocol NPs have low proliferation rate and reach to passaging confluency in 14-18 days.

10. The NPs generated can be expanded several passages without loosing their differentiation capacity. They can also be frozen and stored for long periods. Following cryopreservation, typically ½ to 1/3 of the plated cells survive. We usually plate 1M cells on 35mm culture dish, 2.5M cells on T25 flasks and 6M cells on T75 flasks after thawing.

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FIGURES

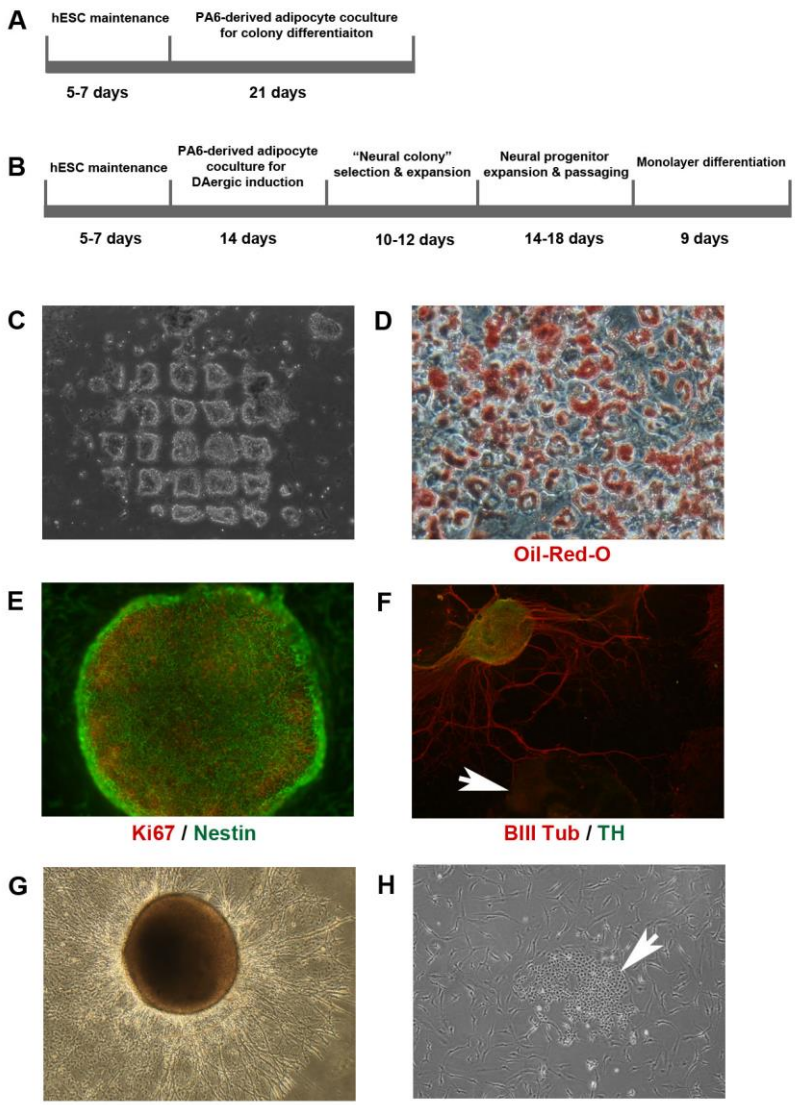

Figure 1. Dopaminergic differentiation of hESCs. (A) Coculturing hESCs on PA6-derived adipocytes give rise to dopaminergic colonies in 3 weeks (B) Three-step protocol requires the selection of neural colonies on day 14 of coculture, expansion of neural progenitors, and monolayer differentiation (C) Undifferentiated hESC colonies are mechanically cut into small clusters for

coculturing (D) Oil-red-O staining of PA6-derived-adipocytes confirms the successful adipogenic differentiation (E) “Neural colonies” have a round, dome shape in the culture. Immunostaining results of the “neural colonies” on day 14 demonstrate that those colonies are rich in nestin expressing cells. Some cells also express the proliferation marker Ki67 (F) On day 21 of coculture, neural colonies with round shape robustly express TH and BIII-tubulin markers whereas other types of colonies (arrowhead) poorly express those markers (G) When the “neural colonies” are selected on day 14 and plated on gelatin coated dishes, neural progenitors start to spread outward (H) During NP expansion, morphologically distinct two types of cells emerge. First, flat shaped cells emerge and start to cover the surface. Later, neural progenitors start to emerge as clusters (arrowhead) on top of these cells.

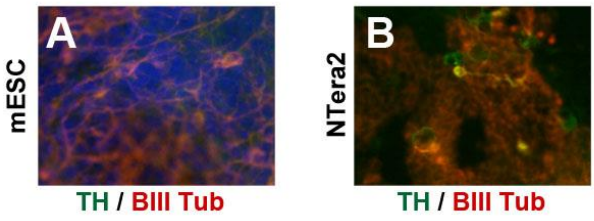

Figure 2. DAergic inducing activity of PA6-derived adipocytes on different cell types. Coculturing on PA6-derived adipocytes promotes the DAergic differentiation of (A) mESCs and (B) NTERA2 cells.