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Abstract

Obesity and its comorbidity incidence have increased worldwide during the past 10 years. In consequence, researchers have drawn their attention to the understanding of adipocyte differentiation. Several cellular model systems have been established; however no efficient protocol could be developed so far to differentiate the pluripotent embryonic stem cells to adipocytes. In this chapter, we describe a detailed protocol that is optimized for mouse embryonic stem cells. The result of this differentiation is a homogenous adipocyte monolayer culture that can be used for several applications including developmental and pharmacological research.

Keywords (separated by '-') Adipocyte differentiation - Mouse embryonic stem cell - Monolayer culture - Ascorbic acid

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Differentiation of Adipocytes in Monolayer from Mouse Embryonic Stem Cells

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Abstract

Obesity and its comorbidity incidence have increased worldwide during the past 10 years. In consequence, researchers have drawn their attention to the understanding of adipocyte differentiation. Several cellular model systems have been established; however no efficient protocol could be developed so far to differentiate the pluripotent embryonic stem cells to adipocytes. In this chapter, we describe a detailed protocol that is optimized for mouse embryonic stem cells. The result of this differentiation is a homogenous adipocyte monolayer culture that can be used for several applications including developmental and pharmacological research.

Keywords: Adipocyte differentiation, Mouse embryonic stem cell, Monolayer culture, Ascorbic acid

1 Introduction

Obesity has become a public health issue worldwide due to the increasing incidence and prevalence of overweight patients and related comorbidities. In the USA 39 % of the population is obese (1). These obesity-linked diseases affect quality of life and increase mortality rate in the affected population (2, 3). Therefore, understanding adipocyte differentiation regulation has drawn the interest of researchers. Two phases of fat cell differentiation process can be distinguished: (1) determination phase, which includes the stem cell differentiation to a preadipocyte stage, and (2) terminal differentiation phase that involves the events of the differentiation from preadipocyte to adipocytes (4). There are various models for adipocyte differentiation that focus in the terminal differentiation stage (4, 5). However, the ideal model system for adipocyte differentiation would encompass both the early and the late stages. In theory, somatic stem cells and pluripotent stem cells can be used for this purpose (6). It had been shown that these cells can differentiate into all three embryonic layers (7). The principal problem of this model system is the low efficiency of the adipocyte differentiation and heterogeneity of the culture (8–10). We recently described that with the addition of ascorbic acid one can overcomes these

issues (11) and here we present a step-by-step protocol with detailed instructions and notes. The adipocytes differentiated from embryonic stem cells using this protocol can be used for various aims including transcriptomic, proteomic studies and provide a novel model system for pharmacological research.

2 Materials

1. mESC medium: Dulbecco's modified essential medium (DMEM) with GlutaMAX (Gibco, 31966-021) supplemented with 15 % of Hyclone FBS (Thermo, SH30070.03), 100× Non-Essential Amino Acid solution (Sigma, M7145), 100× Penicillin/Streptomycin (PAA, P11-010), 0.1 mM 2-mercaptoethanol (Sigma, M3148), and ~~1,000 U~~ of Leukemia Inhibitory Factor (Millipore, ESG1107) (*see* **Note 1**).
2. Differentiation medium: DMEM with GlutaMAX (Gibco, 31966-021) supplemented with 10 % of Hyclone FBS (Thermo, SH30070.03), 100× Non-Essential Amino Acid solution (Sigma, M7145), 100× Penicillin/Streptomycin (PAA, P11-010) (*see* **Note 1**).
3. Feeder's medium: DMEM (Sigma, D5671) supplemented with 10 % of FBS, 100× Penicillin/Streptomycin (PAA, P11-010), 100× L-glutamine (PAA, M11-004).
4. Feeder's freezing medium: 1:1 ratio of feeder's medium and DMEM (Sigma, D5671) supplemented with 20 % of cell culture-tested FBS and ~~5 %~~ DMSO.
5. 0.05 % Trypsin-EDTA (Gibco, 25300-054) (*see* **Note 2**).
6. Primary mouse embryonic fibroblasts (PMEFs), mitomycin C treated: PMEFs are routinely isolated from 13.5-day-old mouse embryos (strain: C57BL/6 or CD-1). Isolated cells are cultured in 150 mm culture dishes and expanded by passaging them up to two times in a dilution 1:4. Before freezing, the cells are treated with 10 ~~ng/mL~~ mitomycin C (Sigma, M0503) for 3.5 h. After the mitomycin C treatment the PMEFs are washed twice with PBS and trypsinized. The cellular suspension is centrifuged for 325 × *g* and resuspended in feeder's freezing medium and aliquoted by adding 600,000 cells in each cryovial (*see* **Note 3**). PMEFs mitomycin C treated are also commercially available (Millipore, PMEF-CF).
7. Mouse embryonic stem cells: The described protocol has been optimized to E14 cells. Modified E14 cell lines are commercially available (ATCC® CRL-1821™).
8. Dimethyl-sulfoxide (DMSO) (Sigma, D5879).
9. Methanol (Analar NORMAPUR, 20847.295).

10. Absolute Ethanol (Analar NORMAPUR, 20821.296).
11. Sterile nuclease-free water (NFW).
12. All-trans retinoic acid (ATRA) (Sigma, R2625): Prepare a 1 mM stock in DMSO, keep in the dark, and store it at −20 °C.
13. L-Ascorbic acid (AsA) (Sigma, A4403) 12.5 and 25 mg/mL stock in NFW: Store it at −20 °C up to 1 month, keeping it in dark conditions (*see* **Note 4**).
14. Rosiglitazone (Selleckchem, S2505): Prepare 1 mM stock in DMSO:ethanol.
15. 3-Isobutyl-1-methylxanthine (IBMX) (Sigma, I5879) 0.5 M stock in DMSO:ethanol (*see* **Note 5**).
16. Insulin solution 10 mg/mL (Sigma, I9278): Dilute it in differentiation medium to 1 mg/mL.
17. Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.46 mM KH_2PO_4).
18. 3,3′,5-Triiodo-L-thyronine sodium salt (T3) (Sigma, T6397): Prepare a 3 μM stock in differentiation medium, and aliquot it to avoid freeze-thaw cycles.
19. Dexamethasone (Sigma, D4902) 0.1 mM stock in ethanol: It is very stable at −20 °C.
20. Indomethacin (Sigma, I7378) 30 mM stock in methanol: Can be stored at 4 °C up to 1 month.
21. Accutase (PAA, L11-007).
22. Sterile gelatin, 0.1 %.
23. Cell culture dishes 100 mm, 150 mm (Greiner Bio-One, 664160 and 639160, respectively) and 6-well plates (TPP, TPPA92006).
24. Cell Strainer, 40 μm (BD Bioscience, 352340).
25. Bacteriological grade dishes 150 mm (SARSTEDT, 82.1184).
26. 15 and 50 mL conical sterile tubes.
27. 10 mL pipette tips.

3 Methods

All procedures should be done under sterile conditions using aseptic techniques. Appropriated cell culture hood should be used. All centrifugations are carried out at 325 × *g* during 5 min. Standard 37 °C humidified 5 % CO_2 incubator is needed.

3.1 mES Cell Culture

1. Prepare gelatinized plates for PMEFs. Add 1 mL 0.1 % gelatin to 2 wells of a 6-well plate. Incubate at least for 30 min at 37 °C in humidified incubator (*see* **Note 6**). Thaw one cryovial of

PMEFs, and immediately remove the freezing medium containing DMSO by centrifugation. Resuspend the cell pellet in feeder's medium and plate them into the previously gelatinized wells after removing the gelatine (*see* **Note 7**).

2. 12 or 24 h after preparing the feeder's monolayer thaw 1,000,000 mES cells. To remove DMSO freezing medium transfer the content of the cryovial into a 15 mL centrifuge tube containing 4.5 mL mESC medium and centrifuge the cells. Resuspend gently the cell pellet in 4 mL fresh mESC medium and plate the cells in the 2 wells containing mitomycin C-treated PMEFs (*see* **Note 8**). Change medium daily using mESC medium.
3. Under these conditions the cells should be ready to use after 3 days in culture. The mES cells should reach 70–80 % confluency (Fig. 1a).

3.2 mES Cell Feeder Depletion

1. As a first step of differentiation split the culture in 1:5 ratio in feeder-free condition. First, wash once the cells with PBS, add trypsin, and incubate for 3–5 min in the incubator. Add mESC medium to inactivate the trypsin and dissociate the stem cell clumps until single-cell suspension is achieved. Then split in 1:5 ratio (*see* **Note 9**). Change mESC medium daily. The feeder-free stem cells change their morphology (Fig. 1b) but they keep their high capacity of cell proliferation.
2. After 2 days of culture the feeder-free mES cells can be passaged. Repeat the feeder-free passage two times more. As the last two feeder-free passages can be done in 1:10 or 1:15 dilution, here cells can be expanded to the needed amount (*see* **Note 10**).

3.3 Embryonic Body (EB) Formation in "Hanging Drop" Method

1. The feeder-free stem cells are trypsinized. The cellular suspension should be counted and diluted for EB formation. Briefly, before adding trypsin to the cell culture wash once with PBS. The trypsin is inactivated by 4 mL differentiation medium per 1 well of 6-well plate (*see* **Note 11**). Count the total cell number and prepare a cell suspension by diluting the cells in differentiation medium to 1×10^5 cells/mL. Prepare the bottom of 150 mm bacteriological Petri dish by adding 15 mL PBS and 100 μL differentiation medium. The latter step will decrease the surface tension of the liquid. Finally, place 20 μL drops of the 1×10^5 cells/mL suspension onto the inner surface of the lids of bacteriological grade dishes with a multichannel pipette. This step is referred as "Adipocyte differentiation Day 0" (Fig. 1c). Invert the lid over the bottom of the bacteriological Petri dish and place it very carefully in the incubator.

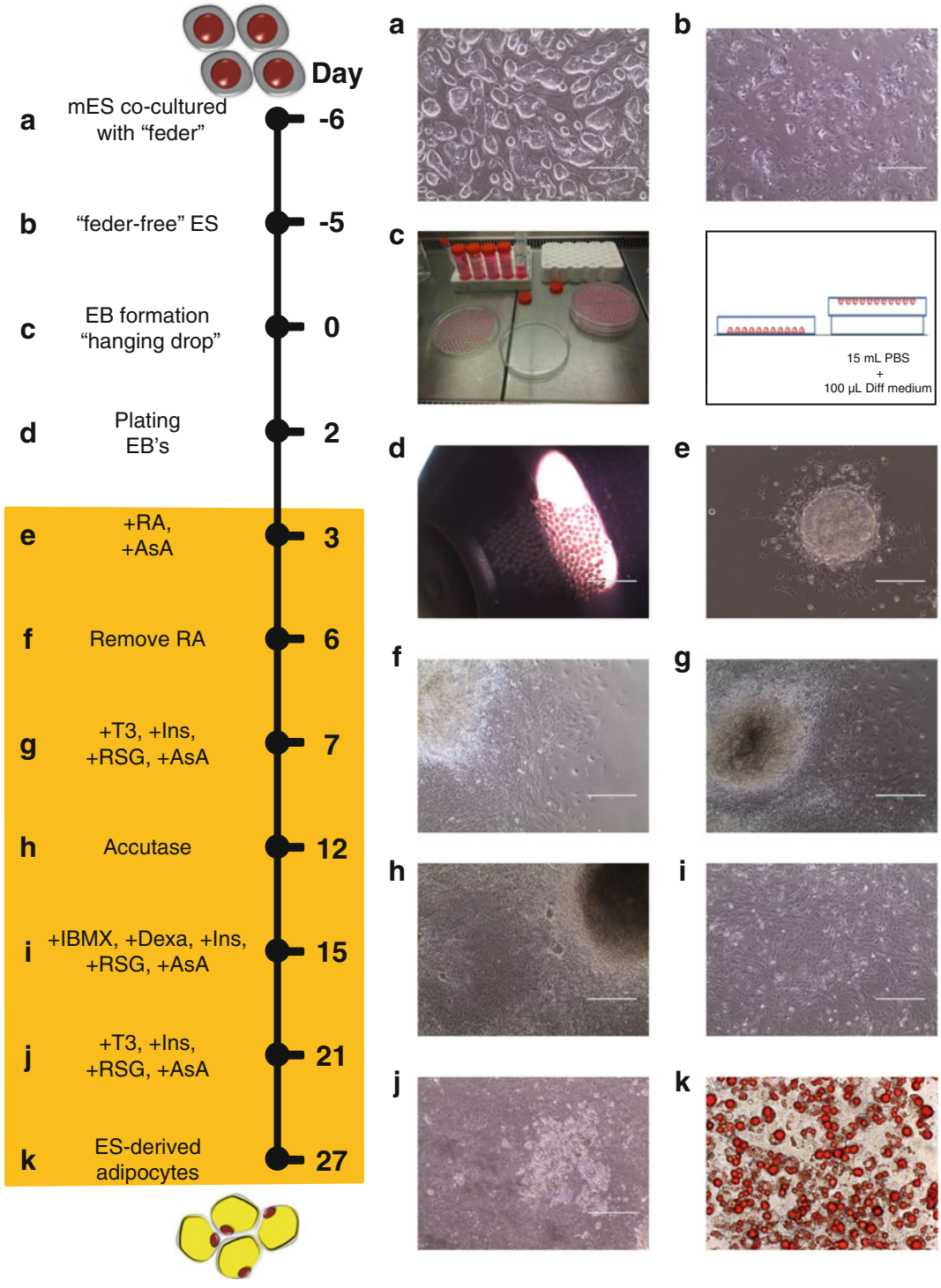

Fig. 1 Stepwise adipocyte differentiation protocol from mouse embryonic stem cells. (**a**–**k**) is a representative picture of the steps mentioned in the text

2. After 2 days the EBs are collected. Slowly remove the lid of the bacteriological plates, invert it, and wash the lids with 10 mL PBS to collect the EBs into a 50 mL conical sterile tube. Remove the medium by allowing EBs to sediment for 10–20 min at room temperature (day 2, Fig. 1d). Aspirate the PBS and resuspend the EBs in differentiation medium. The EBs obtained from one lid can be plated in 1 well of gelatin-precoated 6-well plate in 1 mL differentiation medium (*see* **Note 12**). Place back in the incubator.

3.4 Induction of Adipocyte Differentiation of Plated EBs

1. One day after plating the EBs (at day 3), change differentiation medium containing only 1 μM ATRA and 12.5 μg/mL AsA in final concentration and change medium every day (Fig. 1e) (*see* **Note 13**).
2. Three days after starting the ATRA treatment (day 6, Fig. 1f) remove ATRA from the medium. Table 1 contains a summary of medium and supplements used along the differentiation.
3. Next day (day 7, Fig. 1g), change the differentiation medium by adding the freshly prepared hormonal cocktail: AsA (12.5 μg/mL), T3 (3 nM), insulin (0.5 μg/mL), and

Table 1
Summary of medium and adipogenic cocktails in mES adipocyte differentiation

Days of differentiation	Medium	Supplemented with
Until day 0	mESC medium	LIF
Day 0 to day 3	Differentiation medium	
Day 3 to day 6	Differentiation medium	ATRA 1 μM AsA 12.5 μg/mL
Day 6 to day 7	Differentiation medium	AsA 12.5 μg/mL
Day 7 to day 15	Differentiation medium	Rosiglitazone 0.5 μM[a] Insulin 0.5 μg/mL T3 3 nM AsA 12.5 μg/mL
Day 15 to day 21	Differentiation medium	IBMX 0.5 μM Dexamethasone 0.1 μM Insulin 20 μg/mL Rosiglitazone 0.5 μM[a] Indomethacin 0.06 mM AsA 25 μg/mL
Day 21 to day 27	Differentiation medium	Insulin 20 μg/mL Rosiglitazone 0.5 μM[a] AsA 25 μg/mL T3 3 nM

Leukemia inhibitory factor (LIF), All-trans retinoic acid (ATRA), Ascorbic acid (AsA)
[a]The addition of rosiglitazone is optional; *see* **Note 14** for details

rosiglitazone (0.5 μM). Change this medium daily; prepare every day freshly from stock solutions. The addition of rosiglitazone is optional (*see* **Note 14**).

4. At day 12 of adipocyte differentiation (Fig. 1h) disperse the cells by 1 mL accutase. Before the enzymatic digestion wash the culture with PBS once. Incubate the cells with accutase for 20–25 min in the incubator. Add 2 mL differentiation medium, resuspend thoroughly, and place the cellular suspension in a 50 mL sterile conical tube. Finally wash each well with 2 mL differentiation medium and collect it on the same tube (*see* **Note 15**). Plate the cells in differentiation medium supplemented with AsA (12.5 μg/mL), rosiglitazone (0.5 μM), insulin (0.5 μg/mL), and T3 (3 nM) (*see* **Note 16**).
5. Do not change the medium between days 12 and 15 of the differentiation.
6. After day 15 (Fig. 1i) change medium every 3 days supplemented with AsA (25 μg/mL), dexamethasone (0.1 μM), insulin (20 μg/mL), rosiglitazone (0.5 μM), indomethacin (0.06 mM), and IBMX (0.5 mM). The addition of rosiglitazone is optional (*see* **Note 14**).
7. At day 21 of adipocyte differentiation (Fig. 1j) and day 24 change differentiation medium containing AsA (25 μg/mL), insulin (20 μg/mL), rosiglitazone (0.5 μM), and T3 (3 nM). The addition of rosiglitazone is optional (*see* **Note 14**).
8. Assess the cultures for the presence of lipid droplet containing adipocytes at day 27 (Fig. 1k) (*see* **Note 17**).

4 Notes

1. The quality of the medium and serum used during the stem cell culture is a crucial factor for the differentiation process. We recommend using the mentioned medium and serum for culturing the stem cells and for the differentiation. If another medium or serum would be used it is very important not only to assess the morphology of the stem cells during expansion but also the ability to form EBs and differentiate. LIF should be added to the medium freshly or the mESC medium containing LIF should be used preferably within 3 days.
2. Either for expansion or EB formation, after chemical disruption of the stem cell culture the cells should be in a single-cell suspension. In our experience not all the brands of trypsin achieve this and we recommend the 0.05 % trypsin mentioned in Section 2.
3. The freezing medium for the mitomycin-treated PMEFs is important for their survival. Also the freezed mitomycin-treated PMEFs should be shortly collocated in liquid nitrogen (within 1

or 2 days after freezing) for longer term storage. PMEFs should be tested for mycoplasma in case of each new preparation.

4. Ascorbic acid stock should not be used after 1 month of storage. It is a light-sensitive compound. It is recommended to prepare a new stock for each differentiation process. Similar results were obtained using sodium L-ascorbate (A4034) and L-ascorbic acid 2-phosphate sesquimagnesium salt (Sigma, A8960).
5. DMSO can inhibit the adipocyte differentiation; therefore concentrated stocks are recommended. IBMX 0.5 M stock can be difficult to resuspend; it is advisable to heat it in the 37 °C water bath until the solution is completely homogeneous and store it in aliquots to avoid repeated freeze-thaw cycles.
6. Gelatin 0.1 % incubation times less than 30 min before plating PMEFs can lead to suboptimal attaching ratio of mitomycin-treated PMEFs.
7. In our experience 600,000 PMEFs mitomycin C treated can perfectly be used in 2 wells of a 6-well plate or in one T-25 flask. The cells can be used from 12 h up to 1 week after thawed.
8. Thawing 1,000,000 mES cells can be done in 2 wells of 6-well plate or in one T-25 flask.
9. The cell culture should be split 1:5 or 1:7 ratio for the first passage without feeders. mES cells are sensitive to feeder depletion; thus higher dilutions could result in stem cell spontaneous differentiation.
10. Other mES cell strains might be sensitive to feeder-free conditions as for example induced pluripotent stem cells. In such case feeder-free passages are not recommended. These stem cells should be feeder depleted the day 0 of the differentiation. Briefly, after trypsinizing the stem cell culture, the cells are incubated in a gelatinized wells or flask for 20–30 min. Pipette the cell suspension to a 15 mL centrifuge tube and count the cell number. After this step follow the EB formation step.
11. Single-cell suspension is needed for a reproducible and adequate EB formation.
12. EBs are very sensitive to mechanical manipulation. Use 10 mL pipette tips when collecting and plating the EBs. Avoid pipetting EBs several times.
13. One-day plated EBs could detach easily; therefore, remove the medium with pipette instead of water pump/vacuum suction at least in the first 3 days.
14. Addition of rosiglitazone is optional; it does not significantly increase the adipocyte differentiation in stem cells, but can produce increased differentiation of Ucp-1+ adipocytes in the terminally differentiated cultures (11).

15. If the extracellular matrix cannot be disrupted, use a 40 μm mesh to remove the clumps.
16. This protocol offers the advantage that can be used for several applications. The cells can be repleted in different dishes and well-plate formats: 100,000 cells per well of a 6-well plate, 50,000 cells per well of a 24-well plate, 1,000,000 cells per 100 mm dish, and 1,800,000 cells per 150 mm cell culture dish (11).
17. The adipocyte cultures, due to their high content of adipocytes, and the monolayer culture can be evaluated using standard techniques including transcriptome, epigenetic, and immunostaining analysis.

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