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Direct Conversion of Human Pluripotent Stem Cells to Osteoblasts with a Small Molecule

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Abstract

Human pluripotent stem cells (hPSCs) that exhibit unlimited self-renewal ability and differentiate into all cell types in the human body are a promising cell source for cell based therapies and regenerative medicine. Small molecules hold great potential in deriving tissue-specific cells from hPSCs owing to their cost-effectiveness and scalability. Here, we describe a protocol of deriving osteoblasts from hPSCs by using a single, natural small molecule, adenosine. This protocol offers a simple experimental protocol to obtain large numbers of osteoblasts or osteoprogenitor cells from hPSCs with the ability to form functional bone tissues.

Keywords

human embryonic stem cells (hESCs); human induced pluripotent stem cells (hiPSCs); small molecule; adenosine; osteogenic differentiation

Introduction

This unit describes a protocol for deriving osteoblasts from hPSCs including human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) by using adenosine as a small molecule osteogenic inducer. The potential ability of adenosine to induce osteogenic differentiation of hPSCs stems from our studies examining the potential of biomaterials containing calcium phosphate and phosphate metabolism on osteogenic differentiation of stem cells (Kang et al., 2015; Shih et al., 2014).

NOTE: The following procedures are performed in Class II biological safety cabinet under aseptic conditions using sterile and fresh reagents.

NOTE: All cell cultures are performed in an incubator at 37 °C with 5% CO₂.

NOTE: Identical protocol is used for maintenance and differentiation of hiPSCs and hESCs.

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The authors declare no conflict of interest.

MAINTENANCE OF HUMAN PLURIPOTENT STEM CELLS

The cells were maintained and passaged as described elsewhere (Chang et al., 2013). The following materials were used for the *in vitro* expansion of hPSCs. Either MEF feeder layer (with gelatin/hPSC maintenance medium) or no feeder layer (with Matrigel/mTESR1) can be used to expand and maintain hPSCs.

Materials

Human induced pluripotent stem cells (hiPSCs, IMR90p18-iPS line, WiCell Research Institute) (Yu et al., 2007)

Human embryonic stem cells (hESCs, HUES9 line)

Mitotically inactivated (mitomycin C-treated) mouse embryonic fibroblasts (MEFs) (Conner, 2001)

hPSC maintenance medium (see Reagents and Solutions)

Accutase (Millipore, cat. no. SCR005)

6-well culture plate (9.5 cm² surface area per well)

Day before plating hPSCs

- 1 Add 2 ml of 0.1% (w/v) gelatin into each well of 6 well culture plate and incubate it at 37 °C for 1 h.
- 2 Aspirate off the incubated gelatin solution from the 6 well culture plate, plate mitotically inactivated MEFs at a density of 100,000 cells/cm² in 2 ml of hPSC maintenance medium, and culture for 1 day.

Plating hPSCs

- 3 After culturing the MEFs for 1 day, plate hPSCs (hiPSCs and hESCs) at a density of 20,000 cells/cm² in 2 ml of hPSC maintenance medium onto the MEF feeder layer.
- 4 Change the hPSC maintenance medium with 3 ml of fresh medium daily.
- 5 When the hPSCs reach approximately 80% confluence, passage with a split ratio of 1:6 by using Accutase until further use for differentiation.

OSTEOGENIC DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS

This protocol describes how to induce direct conversion of hPSCs into osteoblasts by using the small molecule adenosine.

Materials

Matrigel (BD Biosciences, cat. no. 354277)

Culture grade coverslip (15 mm in diameter; Fisherbrand, cat. no. 1254582)

12-well culture plate (3.8 cm² surface area per well)

Ca²⁺- and Mg²⁺-free phosphate buffered solution (PBS; Thermo Fisher Scientific, cat. no. SH30258.02)

Thiazovivin

Knockout DMEM

50 ml tubes

1.5 ml tubes

Parafilm

hPSC intermediate medium (see Reagents and Solutions)

hPSC differentiation medium (see Reagents and Solutions)

Hemocytometer

Coating of diluted Matrigel

- 1 On the day before plating hPSCs, thaw Matrigel on ice at 4°C overnight.
- 2 Dilute a volume of the Matrigel based on the lot number and according to manufacturer's suggestion with 25.6 ml of chilled DMEM in 50 ml tube on ice.
- 3 Add 1 ml of the diluted Matrigel solution into each well of 12-well culture plate.

Culture grade coverslip surfaces can also be used for coating and differentiation.

- 4 Wrap the plate with parafilm and coat at 4°C overnight.

hPSC plating

- 5 Wash cells with Ca²⁺- and Mg²⁺-free PBS. Treat and dissociate hPSCs (hiPSCs and hESCs) with Accutase in a 37°C incubator for 10 min.
- 6 Wash with knockout DMEM and centrifuge at 200 g for 5 min.
- 7 Aspirate off the overnight-incubated Matrigel solution from the 12-well culture plate.
- 8 Count hPSCs with hemocytometer and seed cells into Matrigel-coated plates at a density of 10,000 cells/cm² in 2 ml of *hPSC intermediate medium* supplemented with 2 µM Thiazovivin.

After 1 day (D1) of plating, adhesion of evenly spaced single or small colony of cells is observed (Figure 1).

- 9 Gently replace half volume of *hPSC intermediate medium* with fresh medium daily for another 2 days (D1-D2).

This procedure should be performed gently so as not to disturb adhesion and growth of hPSCs.

After 3 days (D3) of post-plating of hPSCs, the adhered cells proliferate into small colonies (Figure 1).

Differentiation of hPSCs (D3-D21)

- 10 After 3 days of culture, gradually replace the *hPSC intermediate medium* to *hPSC differentiation medium* (growth medium containing adenosine) using the mixtures of two media with decreasing concentrations of the intermediate medium (80%, 60%, 40%, 20%, and 0% at 3, 3.5, 4, 4.5, and 5 days of culture, respectively).
- 11 After 5 days of culture, change the *hPSC differentiation medium* with fresh medium every two days.

After 10 days of culture, the cells gradually grow to confluence (Figure 1).

Examine the cells for osteoblastic markers such as osteocalcin expression, and mineralization with Alizarin Red S staining (Figure 2).

The differentiated cells can be collected by using trypsin to analyze for their bone-forming potential in vivo.

REAGENTS AND SOLUTIONS

Gelatin stock

Dissolve 0.1% (w/v) gelatin from porcine skin (Sigma-Aldrich, cat. no. G1890) in PBS.

Store up to 1 year at room temperature.

hPSC maintenance medium

Knockout DMEM (Life Technologies, cat. no. 10829-018)

10% (v/v) Knockout serum replacement (Life Technologies, cat. no. 10828028)

10% (v/v) human plasmanate (Talecris Biotherapeutics)

1% (v/v) non-essential amino acids

1% (v/v) Gluta-MAX

1% (v/v) penicillin/streptomycin

55 μ M 2-mercaptoethanol

30 ng/ml of recombinant human basic fibroblast growth factor (bFGF)

Store up to 4 weeks at 4°C

The hPSC maintenance medium was used with fresh supplementation of 30 ng/ml of bFGF daily.

Thiazovivin stock

Thiazovivin (Selleckchem, cat. no. S1459) was prepared as 2 mM stock concentration, aliquoted, and stored at -20°C until use.

bFGF stock

bFGF (Thermo Scientific, cat. no. 13256029) was prepared as 30 µg/ml stock concentration, aliquoted, and stored at −20°C until use.

hPSC intermediate medium

Knockout DMEM (Life Technologies, cat. no. 10829-018)

10% (v/v) Knockout serum replacement (Life Technologies, cat. no. 10828028)

10% (v/v) human plasmanate (Talecris Biotherapeutics)

1% (v/v) non-essential amino acids

1% (v/v) Gluta-MAX

1% (v/v) penicillin/streptomycin

55 µM 2-mercaptoethanol

Store up to 4 weeks at 4°C

hPSC differentiation medium

High glucose DMEM

10% (v/v) fetal bovine serum (Thermo Fisher, cat. no. 16000044)

4 mM L-glutamine

1% (v/v) penicillin/streptomycin

30 µg/ml of adenosine

Store up to 4 weeks at 4°C

The differentiation medium was supplemented with 30 µg/mL of fresh adenosine every two days.

Adenosine stock

Adenosine stock (Sigma-Aldrich, cat. no. A4036) was prepared at 1.5 mg/ml stock concentration in DMEM, aliquoted, and stored at −20°C until use.

COMMENTARY**Background Information**

hPSCs hold great potential as cell sources in regenerative medicine, disease models, and drug screening platforms, owing to their pluripotency and self-renewal (Amabile et al., 2009; Hwang et al., 2011; Murry et al., 2008). In addition, hiPSCs offer unique opportunities for patient-specificity. Deriving tissue-specific cells from hPSCs demands effective, simple, and low-cost differentiation protocol. Small molecules are appealing differentiation stimulators owing to their cost-effectiveness; however, their efficacy in inducing differentiation of hPSCs into desired phenotypes has yet to be established (Li et al., 2010). Recent studies have demonstrated that small molecules, such as 1α,25-OH vitamin D3 (Zur

Nieden et al., 2003), compactin (Phillips et al., 2001), or stepwise use of four small molecules consisting of CHIR99021, cyclopamine, smoothened agonist, and 4-(4-methoxyphenyl)pyrido[4',3':4,5]thieno[2,3-b]pyridine-2-carboxamide (Kanke et al., 2014) combined with conventional osteogenic induction medium potentiate osteogenic differentiation of hPSCs. In this unit, we describe the use of adenosine as a small molecule to induce direct conversion of hPSCs into osteoblasts. This protocol presents a simple and direct method to derive functional osteoblasts of hPSCs for their use to repair bone defects.

Critical Parameters and Troubleshooting

1. For differentiation culture, the adhesion of hPSCs depends on the quality of the Matrigel coating. If the Matrigel stock solution is inadequately diluted or is not maintained in a chilled state prior to coating, it could result in poor coating, thus leading to inconsistent adhesion of hPSCs.
2. For differentiation culture, during the initial 3 days of hPSC culture, changing the whole volume of culture medium to fresh medium could interfere with adhesion and growth of hPSCs. Changing half volume of culture medium to fresh medium daily can avoid such issue.
3. For differentiation culture, during 3–5 days post-plating of hPSCs, abrupt change of medium from hPSC maintenance medium to hPSC differentiation medium could result in substantial cell death. Gradual change of medium using mixtures of the two fresh media with decreasing concentrations of the maintenance medium can avoid such issue.

Anticipated Results

The hPSCs differentiated by following this protocol are expected to express osteoblastic marker, such as osteocalcin, and produce calcified bone that can contribute to bone repair without teratoma formation as described elsewhere (Kang et al., 2016).

Time Considerations

The differentiation protocol takes over 21 days of culture to convert hPSCs into functional osteoblasts.

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Significance Statement

This protocol describes a simple and effective method of using exogenous adenosine to generate bone-forming cells (osteoblasts) from human pluripotent stem cells (hPSCs), human embryonic stem cells and induced pluripotent stem cells. This protocol could potentially enable generation of a large number of osteoblasts from hPSCs for studies requiring large numbers of cells, including tissue regeneration and the study of skeletal diseases.

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Figure 1.

Adenosine-induced osteogenic differentiation culture of hPSCs. Bright-field images of hiPSCs and hESCs cultured for 1, 3, 10, 21 days in growth medium supplemented with 30 μg/ml of adenosine. hPSCs were attached after 1 day of culture and grew to form small colonies after 3 days of culture in hPSC intermediate medium. After 3 days of culture, the hPSC intermediate medium was gradually changed to hPSC differentiation medium (growth medium containing adenosine). The cells grew to confluence after 10 days of culture and remained confluent until differentiation protocol is completed after 21 days of culture. Scale bars represent 200 μm.

Figure 2. Adenosine-induced expression of osteogenic markers of hPSCs. Immunofluorescent staining of osteocalcin (green), F-actin (red), and nuclei (blue) as well as Alizarin Red S staining of hiPSCs and hESCs after 21 days of culture in hPSC differentiation medium (growth medium containing adenosine). Scale bars indicate 100 μm.