Cell Growth Protocol for H9 and BG02 hESC Lines

From: Stanford University Baker lab/HudsonAlpha Myers lab
Date: 4/1/2009

H9 (WiCell) and BG02 (Bresagen) cell culture and differentiation protocols

H9 and BG02 are human embryonic stem cell lines. The cells are adherent in culture. The H9 karyotype is XX euploid. The BG02 karyotype is XY euploid.

Room/incubator conditions

The following protocols take place in a sterile mammalian tissue culture room. Growth is performed in 5% CO_2 at 37^{o}C.

Maintenance cell culture protocol:

Undifferentiated H9 and BG02 hESC lines are maintained on mouse embryo fibroblast (MEF) feeder layers. MEFs are obtained from mouse embryos. Embryos are trypsinized, separated into single cells, and frozen in a solution of 90% fetal bovine serum (FBS) and 10% DMSO. Prior to use, cells are snap thawed and expanded for 2-3 weeks, and then are irradiated with a cesium irradiator for 2.27 minutes. The irradiated mouse embryonic fibroblasts are plated onto 10 cm dishes at a density of 2 million cells per dish. Each dish is then plated with 2.5 million ES cells. Growth medium is Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12) supplemented with 20% knockout serum replacement (Gibco/Invitrogen), 1 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 8 ng/ml recombinant human fibroblast growth factor-basic (bFGF; Peprotech). Cultures are passaged with 200 U/ml type IV collagenase (Gibco/Invitrogen) at the split ratio of 1:3 to 1:4 every 4–5 days. hESCs used in experiments are low passage (<40 divisions) and are karyotyped prior to use.

Cell culture prior to differentiation

MEF-conditioned medium is produced by conditioning MEFs for at least 24 hours in W8 medium, which is composed of Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12) supplemented with 20% knockout serum replacement (Gibco/Invitrogen), 1 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 8 ng/ml recombinant human fibroblast growth factor-basic (bFGF; Peprotech).

Prior to differentiation, undifferentiated hESCs are transferred from MEFs onto Matrigel (1:20 dilution; BD Biosciences), and are cultured in MEF-conditioned medium. This transfer

is performed by adding collagenase to the ES cells and MEFs. Once all of the cells are in solution, they are transferred to a 15 mL conical tube, which we let sit at room temperature for 15-30 minutes. The ES cells settle in clumps at the bottom of the tube while MEFs remain in solution. The media is aspirated from the ES cells, and they are carefully resuspended in W8 medium. This rinse process is repeated two more times. ES cells are resuspended in MEF-conditioned medium, and 1.25 million ES cells are plated onto a 10 cm dish coated with Matrigel. Undifferentiated hESCs sit on Matrigel for two days prior to differentiation, receiving new MEF-conditioned medium daily. The final day of maintenance on Matrigel is considered Day 0 of differentiation experiments.

In vitro differentiation

For differentiation of hESCs into definitive endoderm, the MEF-conditioned medium is removed from the undifferentiated hESCs, and is replaced with RPMI-1640 medium supplemented with glutamax, 100 ng/ml recombinant human activin A (R&D Systems), penicillin/streptomycin, and defined fetal bovine serum (FBS; HyClone) at sequentially increasing concentrations up to 2% (0, 0.2 to 2%). Cells are kept on Matrigel and receive new media on Days 1and 3 of the 5-days differentiation. Day 1 medium lacks FBS, Day 2 medium receives 0.2% FBS, and the new media on Day 3 contains 2% FBS. Day 5 cells are considered definitive endoderm after FACs sorting with CXCR4 and characterization with RT-qPCR markers. CXCR4+ cells comprise 40-70% of Day 5 cells. Re-analysis of the cells after sorting shows >90% of cells are CXCR4+.

To generate EB-derived cells, clumps of undifferentiated hESCs (~5 million cells per plate) are plated in suspension onto ultra-low adhesion dishes (Corning) in DMEM containing 20% FBS (Gibco/Invitrogen). This promotes the formation of embryoid bodies (EBs). After 8 days, EBs are plated down onto standard tissue culture dishes coated with gelatin type A (Sigma) in DMEM supplemented with 20% FBS and 100 ng/ml FGF-acidic (aFGF; Peprotech). Plating promotes EBs to differentiate towards hepatocytes, and these EB-derived cells are cultured for two to three weeks. Roughly 50% of EBs stick and survive after plating.

Harvesting for Fluorescent Activated Cell Sorting (FACS)

Cells are harvested and dissociated by using 0.05% trypsin/EDTA (Invitrogen) followed by neutralization with phosphate buffered saline pH 7.4 (PBS) containing 10% FBS. Cells are strained with 40 µm strainer (BD Biosciences) and washed twice in PBS containing 0.2% bovine serum albumin and 0.09% sodium azide (Staining Buffer). Cells are labeled with CXCR4-PE (R&D Systems) at 10 µl per $2.5x10^5$ cells for 30-45 minutes on ice, washed twice, resuspended in the Staining Buffer and isolated using a FACS Aria (BD Bioscience) at the Stanford Shared FACS Facility.