Expansion of Human Embryonic Stem Cells by Membrane Based Co-Culture

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Abstract

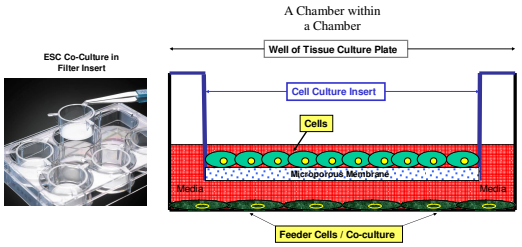
Human embryonic stem (hES) cells have revolutionized cell biology by allowing a better understanding of cellular differentiation and provide a potential cell source for cell therapy and tissue engineering. In addition to their therapeutic potential, ES cells provide an excellent *in vitro* system for the study of early development and human disease. These expanding roles for hES cells require novel techniques to facilitate the understanding of the mechanisms that govern their self-renewal and differentiation.

Typically, hES cells are maintained in direct co-culture with feeder cells and/or mixtures of exogenous factors. Here we demonstrate a hES culture system in which undifferentiated colonies can be maintained by an in-direct porous membrane based co-culture method. Porous membranes allow for the co-culture of cells from different origins in order to study how cells interact through indirect signaling or by providing a conditioned niche for the proper growth and identity of cell type. In this study, we examine the ability of murine and human embryonic fibroblast (EFs) to condition the media for the growth and undifferentiated expansion of hES cells in this indirect co-culture system.

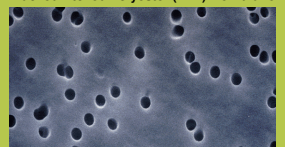
The configuration of this system involves growing the EFs in a feeder plate below membrane bottomed inserts containing hESCs. This arrangement allows for a physical separation between the two cell types eliminating the need for mitotically inactivating the EFs while continuing to allow the EFs to condition the media for maintenance of the hESCs pluripotence. In addition to improving the culturing and expansion of the hESC clones, the separation of the cell types during this filter based co-culture eliminates the requirement for removing the EFs before subsequent manipulations such as differentiation via embryoid body formation and allows for an easily removable growth substrate for electron and confocal microscopy.

Variations in this co-culture system can easily be adopted for the study of indirect cell-cell interactions to direct differentiation of stem cells and for screening of factors that may interfere with these processes.

Expansion of Embryonic Stem Cells by Porous Membrane Based Co-Culture

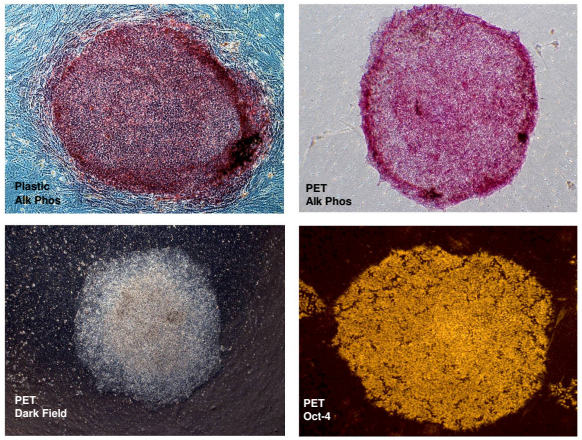


Tracked-Etched Polyester (PET) Membrane



1.0 µm pore size 2x10⁶pores/cm² Transparent

Human ES Cell Culture on Millicell® 1uM PET 6-well hanging inserts

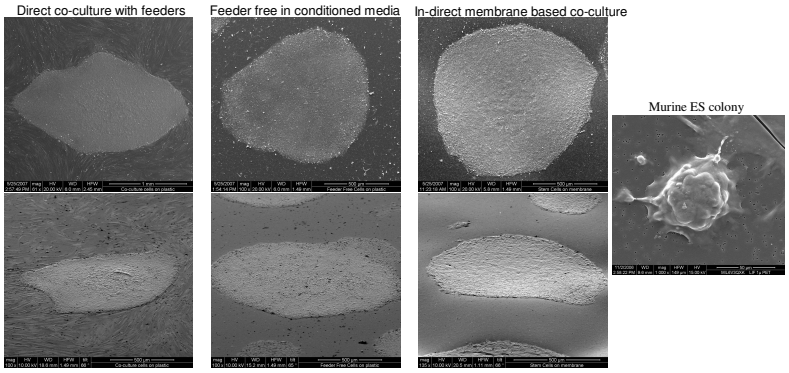


H9 Human embryonic stem cells were cultured in DMEM/F-12 media supplemented with bFGF either by direct co-culture on a mouse embryonic feeder layer in Matrigel® coated (1:30 dilution) 6 well TC plates or by indirect co-culture in Matrigel coated 6 well hanging 1um PET inserts. Indirect co-culture was performed by plating MEFs (strain CF-1) at approximately 40,000 cells/cm² onto 6 well tissue culture plates into which the inserts were placed. Alkaline phosphatase expression was determined by Napthol/Fast Red Violet staining of formaldehyde fixed colonies

Alkaline Phosphatase Staining: Colonies were fixed with 3.7% formaldehyde in PBS for 5 minutes. Napthol/Fast Red Violet solution (Millipore) was added to each well and incubated for 15 minutes before rinsing with PBS buffer. PBS was added to each well for cell visualization for phase contrast.

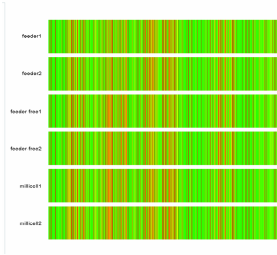
Immunocytochemistry Analysis: Colonies were fixed with 90% methanol and permeablized with 0.1% saponin. Primary antibodies, mouse anti-Oct-4 (Millipore # MAB4401) and rabbit anti-Nanog (Millipore # AB5731) were followed by the secondary antibodies anti-mouse Cy3 and anti-rabbit Cy2, respectively. DAPI containing mounting fluid was used to visualize cell nuclei (Data not shown)

In-direct Co-Culture Promotes Typical Human ES Cell Colony Morphology



H9 cells co-cultured and passaged five times with human foreskin fibroblasts on plastic, feeder free on plastic or by indirect co-culture on 1um PET membrane. SEM samples were prepared by fixation in 4% gluteraldehyde followed by successive drying by incubations in increasing percentage of ethanol (to 100%). Cells were OsO4 treated before drying for image acquisition. SEM images were acquired by coating with ~ 150Å gold for contrast enhancement and electrical continuity. Representative images were collected in the FEI Quanta FEG ESEM 200 under high vacuum at 15 keV.

In-Direct Co-culture Maintains Undifferentiated Phenotype: Global and Pluripotency Marker Gene Expression Profiling



There are no identified significant differences by ANOVA on the whole dataset at an FDR 0.1

Dendogram indicates feeder-free and Millicell conditions were more similar than the direct feeder condition

Selected Pluripotency Markers

Systematic Name	Name	Direct feeder 1	Direct feeder 2	Feeder-free 1	Feeder-free 2	Millicell Indirect 1	Millicell Indirect 2
ILMN_25184	Alk Phos	2,016	1,900	3,858	4,004	1054	1064
ILMN_29401	Cadherin 1	100.3	111.9	100.5	83.92	107	103.4
ILMN_15987	GATA6	84.32	87.55	87.9	83.25	91.95	94.2
ILMN_4778	NANOG	345	372	1,007	1,016	1,029	1,081
ILMN_27111	Oct-4	4,349	4,475	5,663	6,012	7,326	6,958
ILMN_26895	Oct-4 (duplicate)	3,922	4,099	5,516	5,216	6,112	6,094
ILMN_13292	SOX2	110.3	108.8	114.5	110.8	114.1	98.28

*FDR p-value=0.0487

Gene expression data of ES colonies after five passages in each specified condition was analyzed on illumina Human WG-6 Expression BeadChips (v2) using the manufacturers BeadArray Reader and collected primary data using the supplied Scanner software. Data analysis was done in three stages. First, expression intensities were calculated for each gene probed on the array for all hybridizations (6 in total) using illumina's Beadstudio V2 software. Second, intensity values were quality controlled and normalized: quality control was carried out by using the illumina Beadstudio detection P-value set to < 0.05 as a cutoff. This removed genes which were effectively absent from the array (that is, were not detected). After this step, the initial ~46000 genes were reduced to ~20,000 (~60% reduction). All the arrays were then normalized using the /normalize.quantiles/ routine from the Affy package in Bioconductor. This procedure accounted for any variation in hybridization intensity between the individual arrays.

These normalized data were imported into GeneSpring® and analyzed for differentially expressed genes. The groups of biological replicates were described to the software and significantly differentially expressed genes determined on the basis of ANOVA tests. Using parametric tests, not assuming variances equal, there were no significant differences between any of the three groups at FDRs between 0.05 and 0.25.

Summary

- Human embryonic stem cell colonies can be grown and passaged on membrane filters via indirect co-culture with embryonic fibroblasts (EFs) without the need for direct, cell to cell contact.
- Co-cultured ESC colonies show alkaline phosphatase activity and expression of the pluripotency markers Oct-4 and Nanog
- Gene expression analysis indicates indirect membrane co-culture and feeder-free with conditioned media were more similar than the direct feeder co-culture condition, however no significant overall differences were identified between the three conditions
- The filter based co-culture expansion of ESC colonies has several advantages, including
  - Indirect co-culture method does not require mitotic inactivation of EFs grown in feeder tray since ESC colonies are not in direct contact with the EF feeder layer and allows for continual media conditioning.
  - Ease of EF-free ESC isolation for down stream applications such as differentiation, embryoid body formation or molecular analysis.
  - Indirect co-culture enables the study of indirect cell-cell interactions with other cell types to direct differentiation of stem cells and for screening of factors that may interfere with these processes.

References

Thomson, J.A., J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, and J.M. Jones. **Embryonic stem cell lines derived from human blastocysts.** Science 282 (1998): 1145-1147.

Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA. **Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of Human ES cells.** Nat Methods. 2005 Mar;2(3):185-190.

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. **Feeder-free growth of undifferentiated human embryonic stem cells.** Nat Biotechnol. 2001 Oct;19(10):971-4.