Video Article

Rapid Fibroblast Removal from High Density Human Embryonic Stem Cell Cultures

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Abstract

Mouse embryonic fibroblasts (MEFs) were used to establish human embryonic stem cells (hESCs) cultures after blastocyst isolation¹. This feeder system maintains hESCs from undergoing spontaneous differentiation during cell expansion. However, this co-culture method is labor intensive, requires highly trained personnel, and yields low hESC purity⁴. Many laboratories have attempted to minimize the number of feeder cells in hESC cultures (i.e. incorporating matrix-coated dishes or other feeder cell types⁵⁻⁸). These modified culture systems have shown some promise, but have not supplanted the standard method for culturing hESCs with mitomycin C-treated mouse embryonic fibroblasts in order to retard unwanted spontaneous differentiation of the hESC cultures. Therefore, the feeder cells used in hESC expansion should be removed during differentiation experiments. Although several techniques are available for purifying the hESC colonies (FACS, MACS, or use of drug resistant vectors) from feeders, these techniques are labor intensive, costly and/or destructive to the hESC. The aim of this project was to invent a method of purification that enables the harvesting of a purer population of hESCs. We have observed that in a confluent hESC culture, the MEF population can be removed using a simple and rapid aspiration of the MEF sheet. This removal is dependent on several factors, including lateral cell-to-cell binding of MEFs that have a lower binding affinity to the styrene culture dish, and the ability of the stem cell colonies to push the fibroblasts outward during the generation of their own "niche". The hESC were then examined for SSEA-4, Oct3/4 and Tra 1-81 expression up to 10 days after MEF removal to ensure maintenance of pluripotency. Moreover, hESC colonies were able to continue growing from into larger formations after MEF removal, providing an additional level of hESC expansion.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3951/>

Protocol

1. Preparation of Mouse Embryonic Feeders

1. The MEFs used in the co-culture of hESCs should be previously treated by irradiation or Mitomycin-C to inhibit the cells from undergoing division.
2. Two hours prior to MEF seeding, coat each 60 mm plasma treated culture dish with 2 ml of 0.05% Gelatin.
3. Thaw a vial of treated MEFs and plate at $\sim 20,000$ cells/cm².
4. Allow the cells to adhere overnight.

2. Co-seeding hESCs onto MEFs

1. New colonies may be initiated from cryopreserved cultures or passaging current cultures onto new MEF-coated dishes, as below.
2. Wash the plate containing the large hESC colonies to be passaged 1 time with 3 ml of warmed 1x PBS.
3. Add 3 ml of Collagenase IV/PBS solution (1 mg/ml), and incubate for 5-10 min at 37 °C. (Note: The cells will not become distinct or ball up as seen with trypsin).
4. While the cells are still in the collagenase, use an edge of a 5 ml pipet tip, create a grid pattern on the colonies, breaking them into small cell clumps.
5. Use the end of the pipet tip (held in a perpendicular manner to the culture plate) to scrape the bottom of the culture plate, dislodging all the cells from the surface.
6. Collect the cells into a Falcon tube and spin at (1000 rpm) for 5 min.
7. After centrifugation, aspirate the medium, leaving the cell pellet in the Falcon tube.
8. Aspirate the MEF medium from the MEFs plated the day before.
9. Re-plate the cells at a 1:3 ratio from the original plate, and feed with 3 ml of hESC medium

3. Depleting MEFs from the Co-culture

Note: Human ESC cultures must be at high density, usually between 10 and 14 days of culture.

1. Place the tip of an aspirating pipet at the edge of the plate and allow the suction to gently pull up the edge of the MEF sheet.
2. Remove the media from the dish, and allow the tip to catch the cell sheet and slowly move the tip in an arced manner above the plate. Note: The sheet may clog in the tip of the pipet, but this is not problematic as it should not prevent the user from manually pulling the sheet from the surface of the culture plate. If the cells are stuck to the end of the aspirator, you can use the top of the culture dish to break up the cell sheet for complete aspiration.
3. Replace hESC medium immediately, and put the plate back into the incubator.

4. Representative Results

The end result of the MEF removal process produces small undisturbed hESC colonies (**Figure 1D**) able to undergo significant expansion into very large colonies (**Figure 2**) while maintaining expression of pluripotency makers SSEA-4, Oct 3/4, and Tra 1-81 (**Figure 3**).

Figure 1. ESC Colony Morphology Before and After MEF Removal. Human Embryonic Stem Cell Colonies at **A)** day 1 and **B)** day 2 after seeding onto culture dishes. **C)** High Density Human Embryonic Stem Cell Colonies (H7) surrounded by MEFs. The two hESC colonies are outlined in red dashes. **D)** After MEF removal using the aspiration technique described, we are left with an isolated hESC colony with very few MEFs surrounding the intact colony.

Figure 2. ESC Colony Expansion 10 Days After MEF Removal. The original hESC colony immediately after MEF depletion (**left**) is allowed to expand into a very large colony (**right**) approximately 800 μ m, 10x. Note that the original colony is imaged at the same magnification (10x) as the composite colony.

Figure 3. Immunostaining of MEF-depleted hESC colonies. Immunofluorescent identification of colonies up to 10 days after the MEF depletion shows that colonies maintain their expression of pluripotency markers as evident by Oct 3/4, SSEA-4, and Tra-1-81 staining. Moreover, these hESC colonies do not exhibit differentiation towards a mesodermal fate, indicated by the absence of Flk-1. **A, E, I, and M**) are transmission light images of the immunofluorescently stained colonies, 20, 10, 10 and 2x, respectively. **B, F, J, and N**) show the nuclei - DAPI stained cells of the immunofluorescently stained colonies, 20, 10, 10 and 2x, respectively. **C-D**) show the expression of human stem cell marker SSEA-4 only, and composite image, 20x. **G-H**) show the expression of human stem cell marker Oct 3/4 only, and composite image, 10x. **K-L**) show the expression of human stem cell marker Tra 1-81 only, and composite image, 10x. **M-P**) The last row of images depict a colony portraying the typical smooth borders seen at this magnification, 2x, and these colonies did not express **O**) Flk-1, an early marker of mesoderm differentiation, nor did they **P**) contain any fibroblasts as shown by absence of DDR2 staining.

Supplemental Video. Removing Fibroblasts by Sheet Aspiration. [Click here to view movie.](#)

Discussion

The method presented in this manuscript offers a rapid and less costly alternative to eliminating fibroblast feeder cells from human embryonic cell cultures. The successful removal of fibroblasts is dependent on the existence of a tightly confluent monolayer of these cells that develops in longer stem cell cultures. After 7-10 days, the growing hESC colonies will push the feeder fibroblasts in an outward direction, generating an increasingly dense fibroblast monolayer between colonies. The strong cell-to-cell attachments within the fibroblast monolayer allow its rapid removal as a cell sheet. It should be carefully noted that this purification technique is suggested for use as a method of MEF removal from hESC prior to experimentation only, and not utilized for routine expansion culture.

It is also possible to utilize this purification technique to rescue poor quality stem cell colonies. A high quality hESC colony should exhibit distinct borders between themselves and the feeder cells. If, however, the stem cell colonies have undergone partial differentiation, exhibiting less well-defined colony borders, then this method may also be used to remove the partially differentiated hESC colonies along with the MEF cell sheet, however, physical separation of hESC colonies with a pipet tip might be necessary to segregate any hESC connections with the unwanted differentiated cells or MEF.

Disclosures

No conflicts of interest declared.

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