Video Article

Generation of Organotypic Raft Cultures from Primary Human Keratinocytes

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Abstract

The development of organotypic epithelial raft cultures has provided researchers with an efficient *in vitro* system that faithfully recapitulates epithelial differentiation. There are many uses for this system. For instance, the ability to grow three-dimensional organotypic raft cultures of keratinocytes has been an important milestone in the study of human papillomavirus (HPV)[1]. The life cycle of HPV is tightly linked to the differentiation of squamous epithelium[2]. Organotypic epithelial raft cultures as demonstrated here reproduce the entire papillomavirus life cycle, including virus production[3,4,5]. In addition, these raft cultures exhibit dysplastic lesions similar to those observed upon *in vivo* infection with HPV. Hence this system can also be used to study epithelial cell cancers, as well as the effect of drugs on epithelial cell differentiation in general. Originally developed by Asselineau and Prunieras[6] and modified by Kopan *et al.*[7], the organotypic epithelial raft culture system has matured into a general, relatively easy culture model, which involves the growth of cells on collagen plugs maintained at an air-liquid interface (Figure 1A). Over the course of 10-14 days, the cells stratify and differentiate, forming a full thickness epithelium that produces differentiation-specific cytokeratins. Harvested rafts can be examined histologically, as well as by standard molecular and biochemical techniques. In this article, we describe a method for the generation of raft cultures from primary human keratinocytes. The same technique can be used with established epithelial cell lines, and can easily be adapted for use with epithelial tissue from normal or diseased biopsies[8]. Many viruses target either the cutaneous or mucosal epithelium as part of their replicative life cycle. Over the past several years, the feasibility of using organotypic raft cultures as a method of studying virus-host cell interactions has been shown for several herpesviruses, as well as adenoviruses, parvoviruses, and poxviruses[9]. Organotypic raft cultures can thus be adapted to examine viral pathogenesis, and are the only means to test novel antiviral agents for those viruses that are not cultivable in permanent cell lines.

Video Link

The video component of this article can be found at http://www.jove.com/video/3668/

Protocol

1. Preparation for Organotypic Raft Cultures

1. The metal raft grids much first be treated with chromic sulfuric acid to remove any residue that could interfere with the differentiation process. Immerse metal grids in a glass beaker containing sulfuric acid for one hour, then continuously rinsed overnight with tap water. After the overnight rinse, raft grids should be rinsed for 3-5 hours in double distilled water.
2. To provide support for the rafts, bend three sides of the raft about 0.5 cm at equal distance from each other. The metal grids should then be autoclaved.
3. Prepare the 10X reconstitution buffer by adding 2.2 g NaHCO3 and 4.8 g Hepes. Dissolve in 100 ml of 0.05 M NaOH. Filter sterilize and store in 5 ml aliquots at -20°C.
4. Prepare the 10X DMEM without sodium bicarbonate. Filter sterilize and store in 5 ml aliquots at -20°C.
5. Prepare mouse epidermal growth factor: Dissolve 100 mg of EGF and 10 mg of BSA each in 10 ml deionized ddH_20. Combine EGF and BSA and add to 80 ml of deionized ddH_20 for a total volume of 100 ml. Filter-sterilize, aliquot (5 ml) and store in the -20°C. For each liter of E medium add 5 ml of 1 mg/ml EGF (final concentration 5 ng/ml).

2. Preparation of Collagen Gels

1. One collagen gel is required for each raft. The collagen should be kept on ice to prevent it from solidifying. Each collagen gel requires 3 ml of collagen mix consisting of: 2.4 ml of cold Rat-tail collagen type I (final concentration 3-4 mg/ml), 0.3 ml 10X reconstitution buffer, 0.3 ml 10X DMEM and1-2 X 10^6 mouse 3T3 J2 fibroblasts as feeder cells. For high concentration solutions of rat tail collagen, dilute in 0.02 M acetic acid to achieve the correct working concentration.
2. Determine the number of collagen gels (rafts) you will need and then calculate a master mix for the amount of collagen, 10X reconstitution buffer, 10X DMEM and J2 fibroblasts required. Include enough in each master mix for 3-4 extra gels due to the viscosity of the collagen.
3. To prepare the collagen gel, trypsinize the J2 fibroblasts and neutralize with medium. Combine all fibroblasts and place in a 50 ml conical vial. Count the cells to determine the number of rafts that can be made. Again, 1-2x10^6 fibroblasts are required per collagen gel and thus per raft. Spin down the cells at low speed.

4. Aspirate the medium from the fibroblast pellet and resuspend in the appropriate amount of 10X reconstitution buffer, 10X DMEM and collagen for the number of rafts calculated. Add the collagen last to prevent the gel from solidifying too quickly. Once the collagen is added, mix quickly, but gently to prevent the introduction of bubbles into the gel. The gel should be a reddish orange color, indicative of the correct pH. If the gel mixture is too yellow, add a couple of drops of filter-sterilized 1N NaOH and mix gently to obtain the correct color.
5. Add 3 ml of the collagen:fibroblast mixture to the wells of a 6 well culture dish. The collagen mixture should be pipetted slowly down the side of each well to avoid generating bubbles. Place the plate in a 37°C tissue culture incubator and allow to solidify for 30 minutes.
6. After 30 minutes, add 3 ml of E medium with EGF to the top of the solidified collagen gel and return the plate to the incubator. The gels should be kept at 37°C for at least one day but used within four days. Optimal results occur after two days of incubation.

3. Preparing Keratinocytes for Differentiation

1. Low passage keratinocytes should be grown to approximately 70% confluency. Each raft requires 1-2x10^6 keratinocytes. Trypsinize the keratinocytes and neutralize with E medium with EGF. Count the cells to determine the number of rafts that can be made. Spin down the cells at low speed. Resuspend the pellet in 1 ml of E medium with EGF for each raft culture calculated.
2. Aspirate the medium from the collagen gels by tilting the 6 well plate. Add 2 ml of fresh E medium with EGF to each gel. For each gel, carefully add 1 ml of resuspended keratinocytes to the 2 ml of E medium already present in the well. Gently pipet the keratinocytes plus the 2 ml of media and then let fall drop-wise back onto the gel. Shaking or rocking the plate may result in uneven distribution of cells to one side of the collagen gel. Place the plate in the 37°C incubator.
3. The cells should be grown to confluency, replacing the E medium daily. The cells are confluent when the medium changes to yellow one day after a change in medium. This usually takes 2-4 days. If the medium has not changed by day four, proceed to the next step.

4. Making the Raft Cultures

1. Use sterile forceps to place a sterile metal raft grid, bent sides down, into a 10 cm dish. Aspirate the medium from collagen gel. To loosen the gel from the sides of the well, go around the perimeter of the gel with a sterile spatula using an up and down motion. To remove the collagen gel, tilt the plate slightly and lift by placing a spatula underneath. Lay the collagen gel on the metal grid without generating bubbles between the grid and the gel. Two gels can be placed on each grid.
2. In order to make an air-liquid interface, add E medium without EGF slowly to the bottom of the dish so the raft grid is touching the media but the collagen gel is not. The media should not come through the holes in the grid. Add the medium slowly to avoid generating bubbles under the grid, as this will prevent uniform differentiation. Incubate the rafts at 37°C and change the medium every other day maintaining the air-liquid interface. Harvest the rafts after 14 days.

5. Representative Results

When the protocol is performed correctly with primary keratinocytes, this procedure will result in a well-differentiated epithelium where the different layers of the skin are evident. This be achieved through histological examination by staining sections of raft cultures for hematoxylin and eosin (H&E), as shown in Figure 1B. H&E staining is also useful to examine the morphology of rafts and the effect of viruses or antiviral compounds on the ability of cells to stratify and differentiate. For example, rafts made from HPV-immortalized primary human keratinocytes (HFK-31) are notably thicker compared to rafts made from normal human foreskin keratinocytes (HFK), reflecting an increased rate of proliferation and the ability of HPV proteins to maintain differentiating cells active in the cell cycle (Figure 1B)[10]. This results in retention of nuclei throughout the epithelium, whereas normal keratinocytes exit the cell cycle upon differentiation, resulting in a breakdown of the nuclear envelope. To more closely examine differentiation, immunohistochemistry can be performed to examine specific differentiation markers, including cytokeratins, as well as involucrin, and filaggrin. Different cytokeratins are expressed at specific stages of differentiation[7]. As shown in Figure 2, cytokeratin 10 (K10) is not expressed in the basal layer, and is only found in the suprabasal layers that consist of cells progressively differentiating. In HPV positive rafts, the expression of K10 is delayed compared to that of normal keratinocytes, again reflecting the influence of HPV proteins on keratinocyte differentiation. Immunohistochemistry can also be used to examine the expression profile of viral genes. Shown in Figure 3 is a representative staining examining the expression of the HPV protein E1^E4, which is a late protein whose expression is restricted to the uppermost layers of the epithelium. As expected, no staining is observed in raft cultures from normal human foreskin keratinocytes.

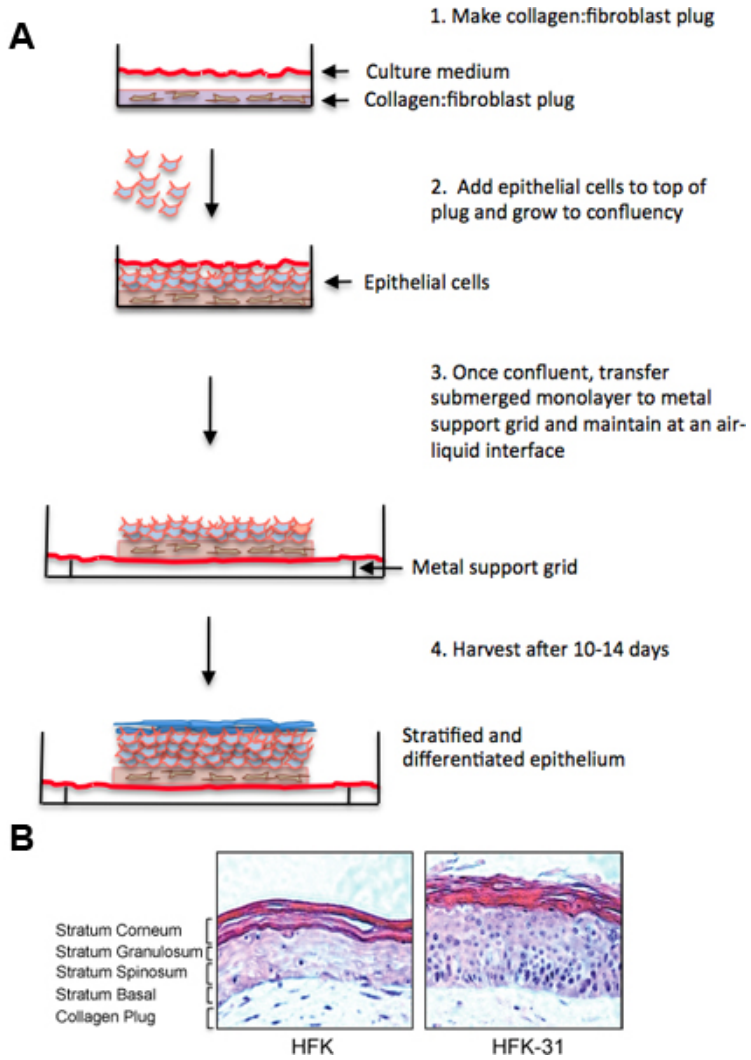

Figure 1. A) Outline of method for preparing organotypic raft cultures from primary keratinocytes or epithelial cell lines. B) Hematoxylin and eosin staining of organotypic raft cultures generated from human foreskin keratinocytes stably maintaining HPV-31 (HFK-31) episomes, or from normal human foreskin keratinocytes (HFK). The individual epithelial layers are identified, as well as the collagen plug.

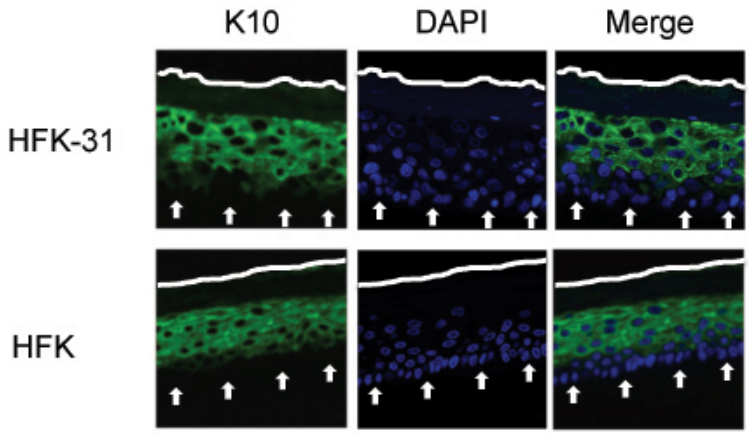

Figure 2. Cytokeratin 10 (K10) expression is restricted to the suprabasal layers of the epithelium. Immunohistochemistry was performed on cross sections of organotypic raft cultures generated from HFK-31 cells, as well as normal HFKs using an antibody to K10. Cellular DNA was counterstained with DAPI. Images were captured using confocal fluorescence microscopy. Arrows indicate the basal layer of the epithelium.

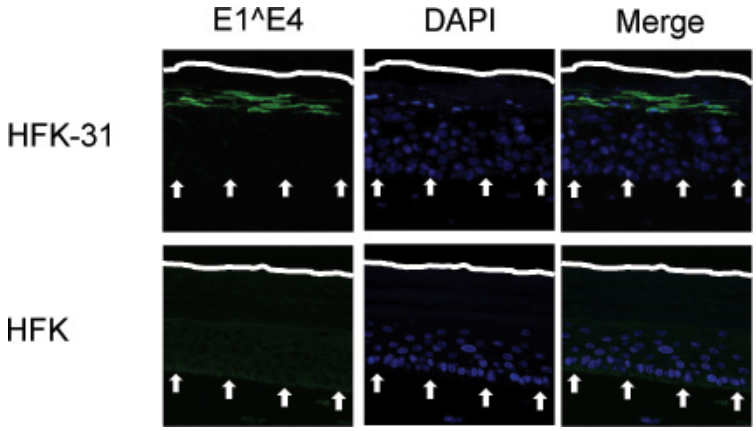

Figure 3. The HPV E1^E4 protein is produced late in the productive phase of the viral life cycle. Immunohistochemistry was performed on cross sections of organotypic raft cultures generated from HFK-31 cells, as well as normal HFKs using antibodies to E1^E4. Cellular DNA was counterstained with DAPI. Images were captured using confocal fluorescence microscopy. Arrows indicate the basal layer of the epithelium.

Discussion

We describe here a method that can be used to study epithelial differentiation in general, but can also easily be adapted to study viral pathogenesis, as well as the efficacy of potential therapeutics. Many viruses target epithelial cells either as the primary site of infection, as in the case of HPV, or at some point in the viral life cycle, as with herpesviruses. Although growing organotypic raft cultures is time consuming, the ability to faithfully recapitulate *in vivo* epithelial differentiation provides an extremely useful method to examine virus:host cell interactions. To successfully grow a fully differentiated epithelium there are some critical steps that must be acknowledged. In order to retain the ability to differentiate in raft cultures, one must have a sufficient number of fibroblast feeders in the collagen gel to maintain the keratinocyte monolayer. Poor differentiation in raft cultures can also be due to too low a density of keratinocytes on the collagen:fibroblast gel, improper raft construction, or failure to change the media everyday. One additional parameter that must be considered to ensure the quality of epithelial differentiation is the type of fibroblast feeder used. For the procedure described here, mouse 3T3 J2 fibroblasts were used. While other fibroblasts can be used as feeders, it is recommended that fibroblasts that divide rapidly, or could potentially migrate up to the dermal surface not be used7. Although this protocol calls for harvesting of the rafts after 14 days, rafts can be harvested before this time point, as well as after. However, after 14 days the rafts will progressively become thinner. In addition to sectioning raft cultures for immunohistochemical analysis, rafts can also be harvested for RNA and DNA, as well as virus production.

Disclosures

We have nothing to disclose.

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