Video Article

A Rapid Filter Insert-based 3D Culture System for Primary Prostate Cell Differentiation

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Abstract

Conditionally reprogrammed cells (CRCs) provide a sustainable method for primary cell culture and the ability to develop extensive "living biobanks" of patient derived cell lines. For many types of epithelial cells, various three dimensional (3D) culture approaches have been described that support an improved differentiated state. While CRCs retain their lineage commitment to the tissue from which they are isolated, they fail to express many of the differentiation markers associated with the tissue of origin when grown under normal two dimensional (2D) culture conditions. To enhance the application of patient-derived CRCs for prostate cancer research, a 3D culture format has been defined that enables a rapid (2 weeks total) luminal cell differentiation in both normal and tumor-derived prostate epithelial cells. Herein, *a* filter insert-based format is described for the culturing and differentiation of both normal and malignant prostate CRCs. A detailed description of the procedures required for cell collection and processing for immunohistochemical and immunofluorescent staining are provided. Collectively the 3D culture format described, combined with the primary CRC lines, provides an important medium- to high- throughput model system for biospecimen-based prostate research.

Video Link

The video component of this article can be found at https://www.jove.com/video/55279/

Introduction

The identification and use of cancer therapies that are personalized to individuals are a primary goal in cancer research. Recently, new approaches have been developed that allow for greater ease in the establishment of primary cell cultures, potentially providing ways of both identifying and testing personalized therapies. For example, the R-spondin-based prostate organoid approach[1] allows for three-dimensional (3D) culturing of normal and metastatic prostate cancer cells in a commercial extracellular matrix (*e.g.*, Matrigel), while the Conditionally Reprogramming of Cells (CRC) method developed at Georgetown[2,3] utilizes more standard 2D culture conditions. Specifically, the combination of a Rho kinase inhibitor (Y-27632) and irradiated J2 murine fibroblast feeder cells lead to the indefinite culturing of keratinocyte CRCs[2]. The CRC methodology is extremely robust, with primary cell lines successfully established and maintained indefinitely from the prostate and many other normal and malignant epithelial tissues[3]. Importantly, our CRC technology allowed for the rapid identification of the etiological basis for recurrent respiratory papillomatosis in a patient who had failed a number of previous drug treatments. In addition, using normal and tumor-derived CRCs, the successful identification of an FDA approved drug, vorinostat, was made within two weeks of initial tissue biopsy. The patient was placed on vorinostat, resulting in the successful treatment of their disease[4].

The normal prostate gland is comprised of luminal, basal and the rare neuroendocrine cells[5]. Luminal cells form the columnar epithelial layer of the gland and express the androgen receptor (AR), as well as other luminal markers such as cytokeratins 8 and 18 and prostate-specific antigen (PSA)[6]. Conversely, basal cells are localized beneath the luminal layer and express cytokeratin 5 and p63, but low levels of the AR[5]. We[7,8,9] and others[10] have successfully used prostate CRCs in preclinical mechanistic drug sensitivity investigations. However, when grown under standard 2D tissue culture conditions, these cells fail to fully engage AR signaling[10]. Importantly, when placed under the renal capsule of immunodeficient mice, the CRCs regained normal prostate glandular architecture and function indicating that prostate CRCs retain their lineage commitment when placed in a permissive environment. The development of the filter insert-based cell culture system described here allows for the rapid (2 weeks) *in vitro* differentiation of prostate CRCs as evidenced by the increased expression of the AR and AR target genes as well as decreased levels of p63.

The filter inserts used contain polycarbonate membranes (pore size, 0.4 µm) that can support the culturing of mammalian cells. The system, as developed, makes use of normal and malignant prostate CRCs, 6 well culture dishes and the filter inserts. Cell culture media conditioned by J2 cells[11] is placed in the bottom chamber and prostate differentiating media in the top chamber. The techniques described herein support prostate luminal cell differentiation within a 2 week timeframe, consistent with the goals of personalized medicine. It was also imperative to develop the methodologies that allow for the comprehensive molecular, genetic and cellular profiling of the cultures. Approaches for isolating DNA, RNA and protein from cells released from the filter surface have been developed and streamlined for accurate repeat sample processing. Finally,

the methodology required for removing the filter to enable embedding, sectioning and for H&E, immunohistochemical and immunofluorescent staining, is fully described.

Protocol

1. Establishment of the 3D Cell Culture Insert System

1. Place 2 polycarbonate cell culture inserts in an inverted orientation (filter side up) down in a 6 well plate (**Figure 1A**).
2. Apply a thin layer of 0.1% gelatin in water to the bottom side of the insert and allow to dry (10-20 min) in a biological safety cabinet to maintain sterility.
3. Repeat step 1.2 two more times for a total of 3 applications of 0.1% gelatin on the inserts.
   NOTE: The gelatin coating will help limit diffusion between the chambers.
4. To collect the primary CRCs from standard culture conditions, add 5 mL of PBS to wash the culture flask.
   1. Trypsinize the cells using (1 mL for a T-25 and 2 mL for a T-75) 0.25% trypsin-EDTA for 5 min at 37 °C. Gently tap the side of the flask to aid in dislodging the cells. Then proceed to stop the trypsin reaction and collect the cells by adding 5 mL of complete DMEM.
   2. Collect the cells in a 15 mL tube. Centrifuge the tube at 4 °C and 188 x g and count using an automated cell counter or a hemocytometer.
   3. Re-suspend 600,000 CRCs in 250 µL conditioned media (CM) and add to the properly oriented (filter side down) culture insert (**Figure 1B**). This is referred to as the inner chamber.
      Note: CM is F-media conditioned by irradiated J2 cells and contains 10 µm Y-27632 as previously described[7,8,9,11].
5. Apply 2 mL of CM to the outer chamber of the 6 well plate and incubate at 37 °C overnight (for at least 18 h).
6. Carefully remove the CM on the inner chamber with a 1 mL or 200 µL pipette and slowly add differentiation media (DM) to the inner chamber with a 1 mL pipette until full. Be careful to not disturb the cell layer.

2. Maintenance of 3D Cultures

1. Check the cultures every day. Healthy cells appear as shown in **Figure 2**.
2. Change the CM in the outer chamber every day or every other day depending on the metabolism of the cells.
3. When the media of the inner chamber changes color (yellow), carefully remove the media on the inner filter chamber with a 1 mL or 200 µL pipette.
4. Aspirate the media in the outer 6 well plate chamber with an aspirating tip.
5. Using a 1 mL pipette, slowly apply fresh DM to the inner chamber until full. Be careful not to scrape or otherwise dislodge the cell layer.
6. Replace the media in the outer chamber with 2 mL of fresh CM.
7. Maintain the cultures for 2 weeks, and then proceed to processing for the desired bimolecular isolation.
   NOTE: Each row in the 6 well plate, a total of six inserts (**Figure 1B**), should be processed per experiment to acquire enough cells for DNA, RNA or protein for analysis.
8. Aspirate media in the outer chamber and rinse with 2 mL phosphate buffered saline (PBS).
9. Carefully remove media from the inner chamber with a 1 mL or 200 µL pipette and add 500 µL PBS. Avoid scraping or otherwise disturbing the cells on the membrane.
10. Aspirate PBS from the outer chamber and carefully remove PBS from the inner chamber with a 1 mL or 200 µL pipette. Once the PBS has been removed, proceed directly to the appropriate application detailed below. Do not allow the membrane to dry out. The culture can be kept briefly in the PBS until the application is ready to begin.

3. Processing the Filters for Pellet Banking

1. Add 100 µL 0.25% trypsin-EDTA to each inner chamber and incubate for 5 min at 37 °C.
2. Briefly and carefully agitate/scrape the cells on the membrane surface with a 200 µL pipette while pipetting up and down (avoid puncturing the membrane). Incubate for 1 min at 37 °C.
3. Add 250 µL of CM to the first inner chamber and pipette up and down gently to rinse the filter.
4. Transfer resuspended cells to the adjacent filter chamber that contains the same media conditions and repeat pipetting up and down.
5. Repeat this procedure for each of the inserts of a single condition. Collect and transfer cells to a 1.5 mL centrifuge tube.
6. Rinse each inner chamber with an additional 100-200 µL of CM to collect any remaining cells. Add to the 1.5 mL centrifuge tube in step 3.5.
7. Spin the cells at 423 x g in a microcentrifuge at 4 °C for 5 min.
8. Aspirate the supernatant and wash the cell pellet once with 1000 µL PBS.
9. Spin again at 423 x g in a microcentrifuge at 4 °C for 5 min.
10. Aspirate PBS and freeze at -80 °C for later use.

4. Processing the Filters for RNA or DNA Isolation

1. Add 200 µL of guanidinium thiocyanate-phenol-chloroform or similar extraction reagent to the inner chamber and briefly agitate the cells on the membrane surface by pipetting up and down.
2. **Incubate in the extraction reagent for 5 min at room temperature with the outer chamber dry.**
   1. As the filter may dissociate from the insert, wash the disassociated filter membrane by pipetting the extraction solution up and down. Collect as much of the extraction reagent as possible by tilting the 6 well plate and aspirating any residual liquid.

3. Follow the manufacturer's protocol for purification and recovery of DNA, RNA or protein.

5. Processing the Filters for Protein Isolation

1. Place the filter insert in the 6 well plate on ice. To the inner chamber, add 10 µL of protein lysis buffer supplemented with 1 mM sodium fluoride (NaF), 1 mM sodium vanadate (NaV), 100 mM dithiothreitol (DTT) and 100 µL of anti-protease cocktail.
2. Agitate/scrape the cells on the membrane surface with a 20 µL pipette while pipetting up and down. Avoid generating bubbles in the lysis buffer.
3. Incubate the 6 well plate with filter inserts on ice for 10 min.
4. Repeat scraping and then rinse the membrane surface with the lysis buffer.
5. Collect the lysates from the 6 inserts and transfer to a 1.5 mL centrifuge tube on ice.
6. Rinse the first membrane with an additional 10 µL of lysis buffer and transfer to the next filter.
7. Repeat step 5.6 for all inserts, collecting any residual lysis buffer solution and add to the 1.5 mL tube (step 5.5).
8. Incubate the sample on ice for an additional 5 min.
9. Spin at maximum speed (16,873 x g in a table top centrifuge) for 15 min at 4 °C.
10. Pipette lysate supernatant into a fresh 1.5 mL tube.
11. Proceed to the analysis of protein concentration or store lysates at -80 °C.

6. Processing for H&E and Immuno-staining

1. Add 500 µL and 1 mL of 10% NBF (neutral buffered formalin) to the inner and outer chamber and let incubate overnight at 4 °C.
2. Aspirate the NBF from the outer chamber and add 1 mL hydroxyethyl agarose (HEA) processing gel to a 1.5 mL centrifuge. Slowly melt the agarose in a microwave using low power and repeated 10-20 s pulses until the agarose is melted.
3. Keep the HEA in a 37 °C warm bath to prevent solidification until ready to use.
4. Remove the NBF from the inner chamber and apply 25 µL of molten HEA to the inner chamber and allow the agarose to solidify for 2-5 min.
5. Wet 2 foam histology pads in 10% NBF and place one pad in an embedding cassette (see **Figure 3D**).
6. Use a #11 blade scalpel (which has a very sharp, fine-pointed blade) to score the filter from the bottom side of the insert chamber, partially releasing it from the plastic insert chamber.
7. Place a small amount (100-200 µL) of NBF in a Petri dish and immerse the partially dislodged filter in the NBF (**Figure 3A**).
8. With a #10 blade scalpel, gently press against the middle of the filter, from the inside of the insert chamber, to fully dislodge the filter from the insert barrel (**Figure 3B**). If there is any part of the filter that is still attached to the barrel, sever it with the scalpel.
9. Cut the HEA-coated filter in half with the #10 blade scalpel (**Figure 3C**).
10. Place each half of the filter onto the foam pad in the histology cassette prepared in step 6.4 (**Figure 3D**).
11. Add the second 10% NBF soaked sponge to the cassette, sandwiching the filter.
12. Snap seal the cassette, place in NBF, and incubate overnight.
13. Process filters into paraffin blocks for sectioning and staining as described previously[12].

Representative Results

The cell culture insert based system is a relatively simple and rapid procedure for producing 3D cultures of prostate CRCs that supports luminal cell differentiation. A schematic of the system is shown (**Figure 1A**) highlighting the application of the gelatin coating to the bottom surface of the filter insert. The inserts are only overturned for the application of the gelatin. In **Figure 1B** the filters are in the appropriate orientation for culturing. Using a transparent cell insert, a representative image of a healthy 3D prostate CRC culture is shown (10X) (**Figure 2**). The dense layering of healthy CRCs demonstrated is typical after establishment and can be visualized using standard light microscopy. During initial establishment, it is critical to visualize the layer of cells formed to ensure that the method is compatible with a given cell line and that appropriate attachment and layering of cells have occurred.

HEA is applied to the inside of the insert as outlined above. After application of the HEA, care must be taken when scoring the filter to remove it from the insert (**Figures 3A, 3B**). In all steps (**Figures 3A-3D**), care must also be exercised to minimize unnecessary movement of the HEA layer on the filter with cells. The CRC layer can be easily disrupted and damaged during the transfer to the H&E cassette (**Figure 3D**). After excision of the HEA-coated filter from the plastic barrel (**Figures 3A-3C**), the filter can be halved and processed for sectioning and staining using standard embedding cassettes (**Figure 3D**). Splitting the filter in half is important to maximize the available surface for cross sectioning on H&E and IF.

Immunofluorescent staining as well as bright field (BF) images of the cells cultured on the filter layer were collected using a microscope with DSU (Disk Scan Unit) spinning disc confocal capabilities. Representative results for histology and H&E staining are shown in a cross section of the filter insert and cells (20X) (**Figure 4**). With proper care, we demonstrate that a CRC layer on the filter inserts can be sectioned and stained, as is standard practice for tissue histology. During sectioning, it is possible for the CRCs to become detached from the filter as an intact layer of cells as demonstrated (**Figure 4**). The BF image shows multi-layered cell strata on top of the porous membrane (**Figure 5A**). The individual fluorescent images for nuclei (DAPI, **Figure 5B)**, p63 (**Figure 5C**) and the AR (**Figure 5D**) are shown, as is the merge of all three fluorescence markers (**Figure 5E**). Finally, a composite BF and IF overlay is shown (**Figure 5F**), establishing the localization of the fluorescence signal relative to the cells and the filter. The overlay of the DAPI stain with the p63 IF staining clearly demonstrates some cells still in a proliferative state. The localization of AR IF staining in the nucleus is indicative of functional reactivation of AR in prostate cells.

A comparison between the IF of our filter insert system and a sectioned prostate tissue is shown (**Figures 6A, 6B**) to demonstrate the similarity in development of our CRC insert layer to that of the prostate epithelium. The prostate duct shows expression of p63, consistent with prostate basal cells. p63 staining is also seen in the CRCs on the insert. A higher percentage p63 expressing cells was observed in our insert compared to the intact tissue section. In addition, both nuclear and cytoplasmic AR are seen in the prostate tissue section and while the CRCs on the filter insert show less overall AR expression, both nuclear AR expression and cytoplasmic staining is seen, similar to the intact prostate.

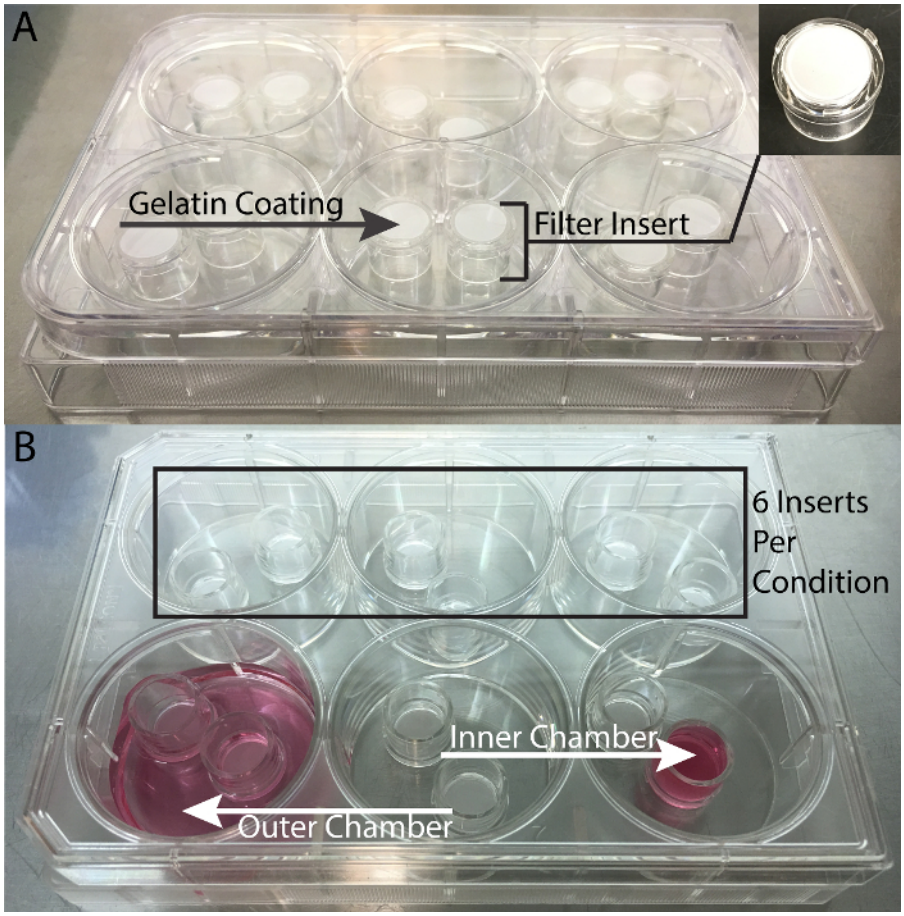

Figure 1: Representative Images of the 6-well Culture Dish and Insert that Comprise the 3D Culture System. (**A**) Inverted inserts for applying the gelatin coating to the underside of the filter (arrow). A larger image of the insert and filter is show (inset). (**B**) Properly placed inserts. The inner and outer chambers of the system are shown (arrows). The boxed row in B shows how multiple samples are obtained for a given experimental condition. At the conclusion of the 2 week growth period these inserts can be pooled to yield sufficient material for analysis. Please click here to view a larger version of this figure.

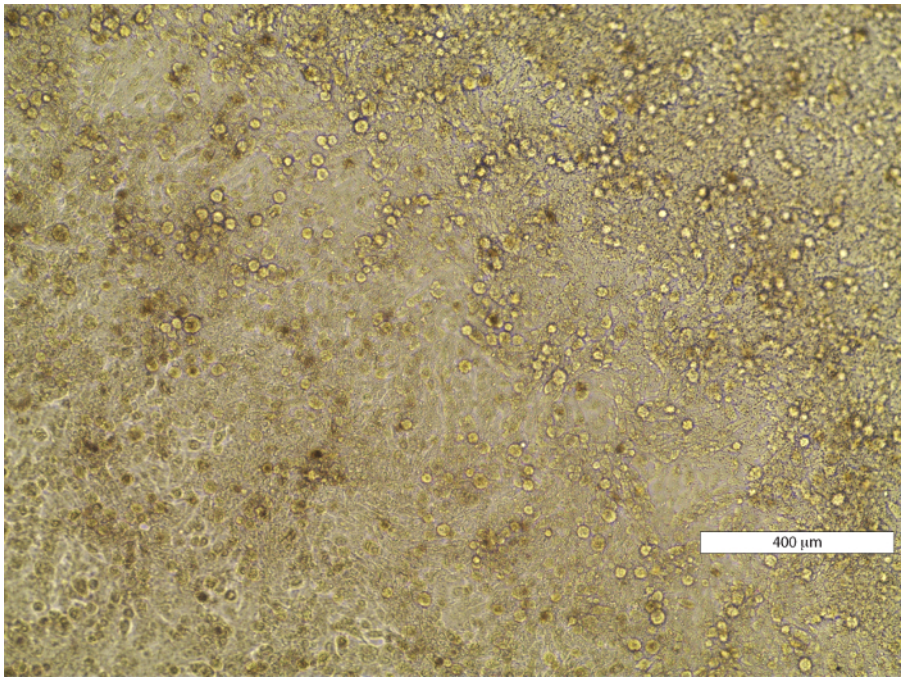

Figure 2: A Representative Image of a Layer of Healthy CRCs Seeded onto a Transparent Filter Membrane is Shown. Both inner and outer chambers contained conditioned media. Please click here to view a larger version of this figure.

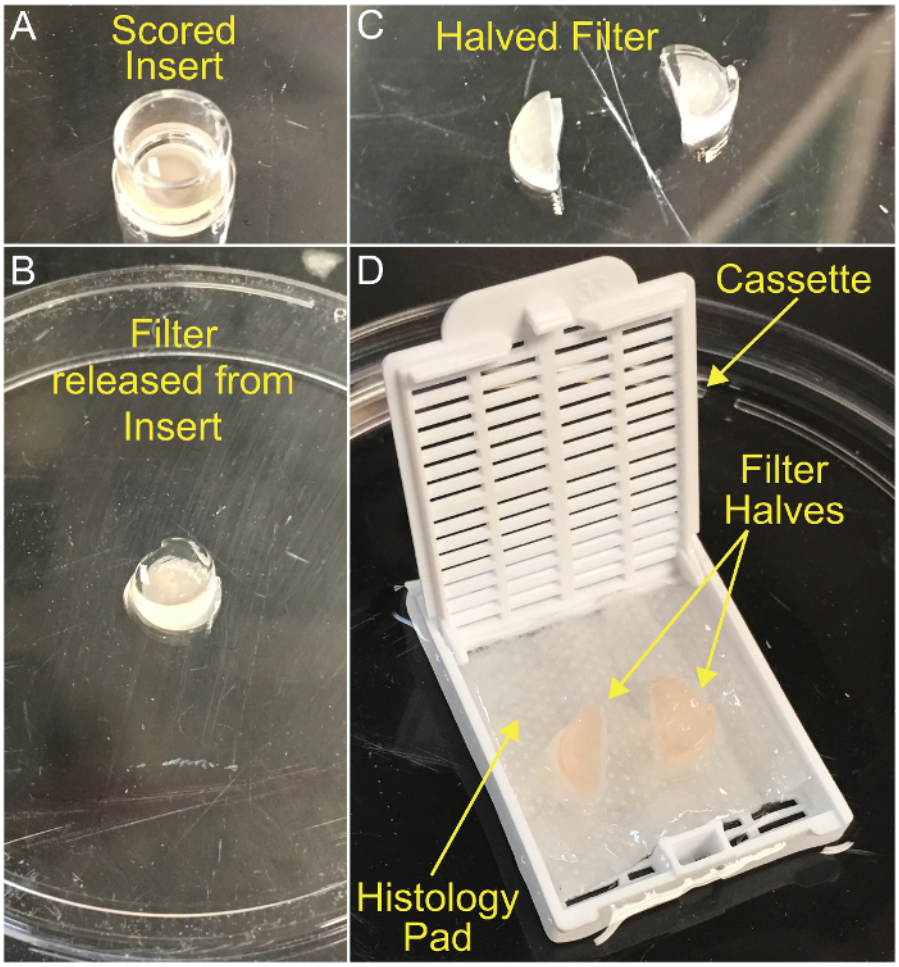

Figure 3: A Representative Image of a Filter Halved into Two Sections and Placed into a Paraffin Cassette for Embedding. (**A**) Filter insert with HEA coating after scoring and before removal. (**B**) The released filter insert from the polycarbonate barrel. (**C**) The correctly halved filter. (**D**) Proper placement and orientation of the two halves inside the embedding cassette. Please click here to view a larger version of this figure.

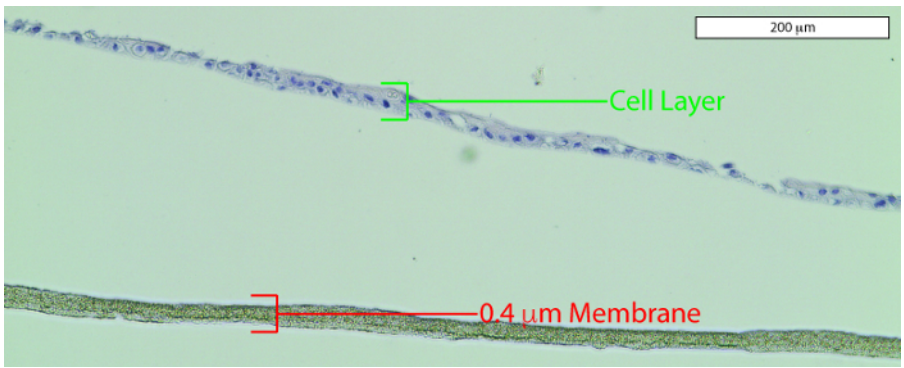

Figure 4: A Representative H&E Stained Cross Section of the Filter Insert and Cells (20X). Both the membrane (bottom) and cell layer (top) are shown. Please click here to view a larger version of this figure.

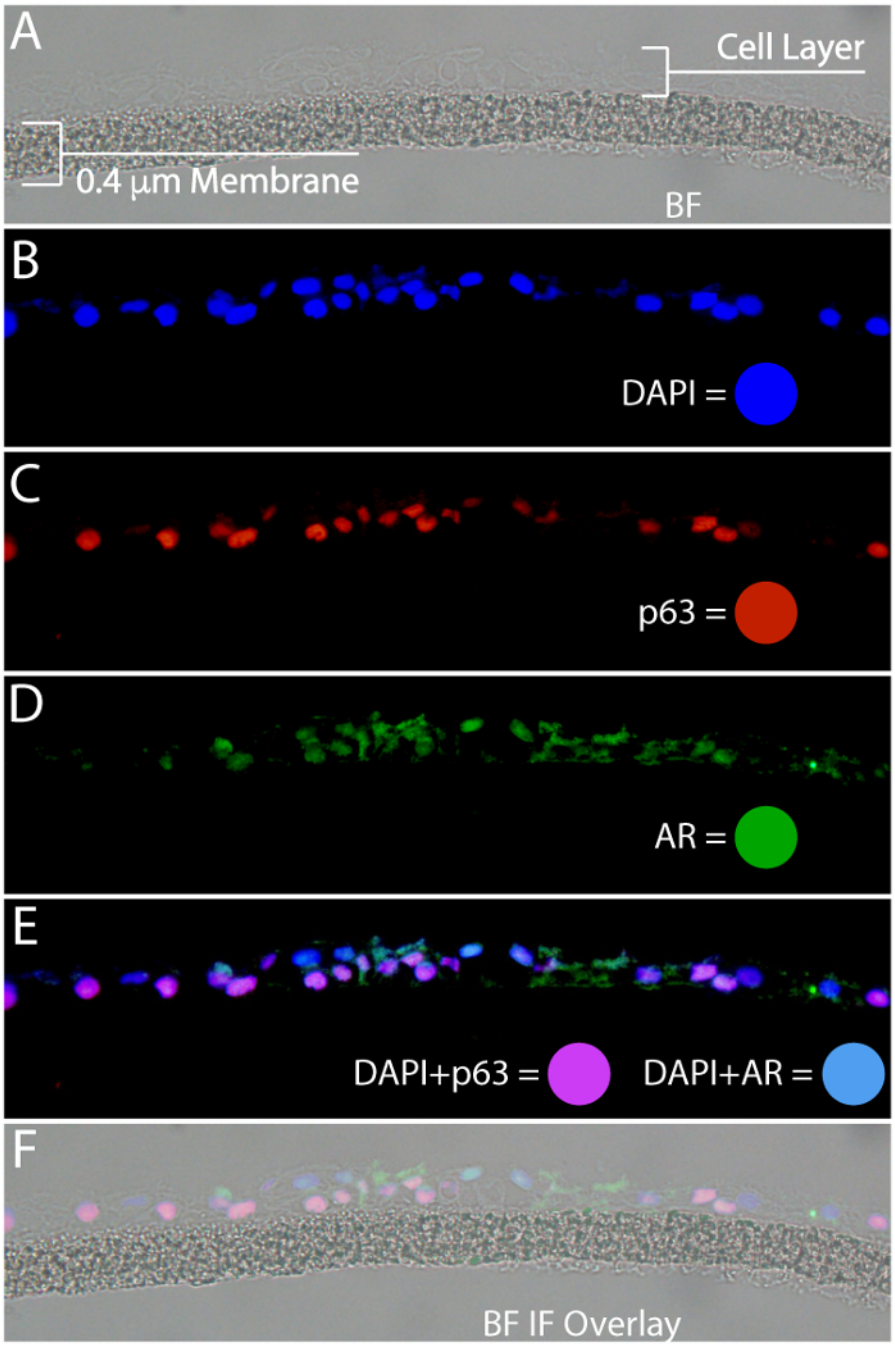

Figure 5: A Series of Representative Bright Field (BF) and Immunofluorescence (IF) Images of Cells Sectioned on the Filter (40X). Cross sections of the filter and cells are shown. An unstained BF image of the cells on the filter surface **(5A)** is accompanied by images for the DAPI stained nucleus **(5B)** and immunofluorescent staining for two different proteins, p63 **(5C)** and the androgen receptor **(AR, 5D)**. A composite overlay of the cell sections **(5E, F)** defines localization of the IF signals in the context of the cells and filter. Please click here to view a larger version of this figure.

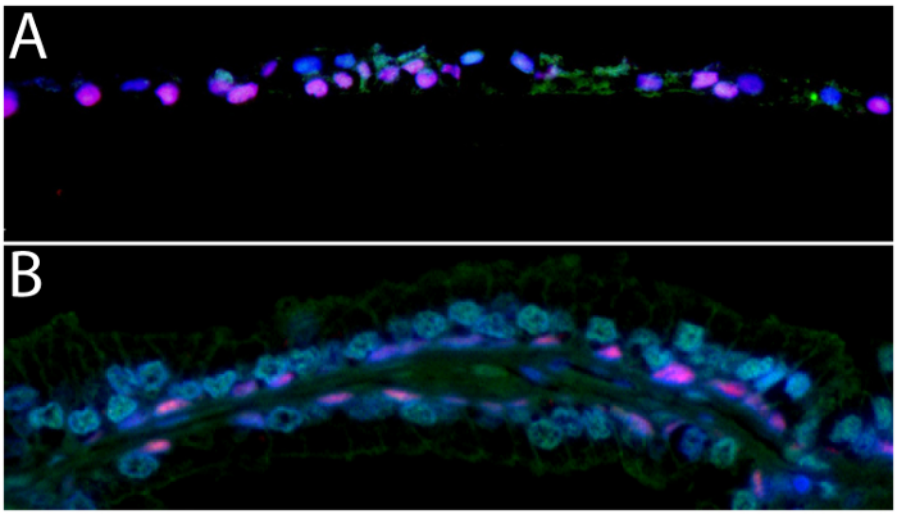

Figure 6: A Representative Immunofluorescence (IF) Image of Cells Sectioned on the Filter *versus* a Section of Prostate Epithelium from Tissue (20X). A cross sectional image of our filter insert is shown **(Figure 6A)** compared to the ductal epithelial layers of an intact prostate **(Figure 6B)**. The staining of p63, the AR and the nucleus was performed as in **Figure 5**. Please click here to view a larger version of this figure.

Discussion

Primary cell lines are an important and rapidly developing platform for cancer research. The culture insert-based 3D culture system supports the differentiation of primary prostate CRCs within a two-week timeframe. The CRC filter method represents a new, medium throughput method for prostate research. Existing mouse PDX models are time consuming and extremely expensive, and many of the PDX samples cannot be grown in culture, limiting investigator-initiated experimentation and their usefulness. In addition, while success rates of establishing PDX vary greatly[13], the CRC method has a high rate of success in establishing cell lines[3]. We consider our system to be complementary to the R-spondin-based prostate organoid approach; however, a lack of success with regards to establishment of organoids from primary prostate cancer has been reported[14]. Additionally, the media, methods of establishment, and maintenance of CRCs are significantly simpler than the R-spondin-based prostate organoid approach. As an added benefit, the CRCs can be established and used to test matched normal and tumor cultures for direct molecular comparisons.

Some technical issues remain to be addressed when establishing this system. First, the gelatin coating applied to the underside of the filter insert helps, but does not eliminate, diffusion of the medias across the membrane. Frequent media changes are used to maintain the respective gradient of differentiation (upper or apical cell layer) *vs* growth (lower or basal layer of cells) conditions within the chamber. Second, successful 3D CRC cultures required a relatively high cell seeding density. Lower seeding densities yielded poor results with regards to the integrity of the cell layer that develops on the filter surface. This is a common consideration when culturing CRCs, as they grow most vigorously when maintained at high cell densities. Finally, the proper removal and subsequent paraffin embedding of the intact cell layer was critical for defining the extent of cell differentiation. The addition of hydroxyethyl agarose (HEA) both decreased cell loss and improved the overall morphology of the cells compared to non-embedded filters (not shown). Once properly embedded, the filters were sectioned and processed in a manner indistinguishable from typical cell and tissue samples.

We are just beginning to emulate the architecture of the prostate epithelium using this filter method. Additional modifications to the culture conditions, to media and to the substrate are warranted, which can be easily addressed. For example, reformulated differentiation medias that may better promote luminal vs basal differentiation can be rapidly tested. Since lentiviral infection of CRCs has been performed as easily as with commercial cancer cells[7] and the 2D CRCs have also been transfected with CAS9/CRISPR gene editing technology vectors to permanently modify the cell genome (data not shown), the effect of mutated, deleted or repaired genes can be tested. Similarly, lineage tracking can be made possible by introducing lineage specific reporter genes.

In addition to altering the media or the cells, modifications to the filter surface can also be tested. Different filter membrane substrates are available commercially. In addition, we have found that the commercial extracellular matrix provides a suitable growth environment when applied to the inside of the filter. As one of the goals of the insert system is to better model the prostate microenvironment, perhaps the most interesting modifications could be the introduction of normal or tumor-derived stromal cells, either in co-culture with the CRCs within the confines of the insert or in the bottom chamber to condition the growth media. Modifying the insert well with sections of decellularized prostate tissue is also possible. In this approach, stromal/epithelial interactions could allow the primary cells to use the decellularized tissue as a scaffold for growth.

The ability to establish primary cells and to subsequently provide the conditions for their differentiation is an ideal format for more biologically relevant research into normal glandular development and for cancer research. The system described herein provides a platform by which an unlimited number of cell types and modifications can be combined to tailor the system to individual experimental needs and to reliably collect the samples for analyses.

Disclosures

The authors have nothing to disclose.

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