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A Conditionally Immortalized Colonic Epithelial Cell Line From A *Ptk*6 Null Mouse That Polarizes And Differentiates In Vitro

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Abstract

Background and aims—PTK6 is an intracellular src-related tyrosine kinase that regulates differentiation in the intestine, where knockout animals have increased proliferative activity and growth characteristics. To explore the phenotype further we attempted to establish epithelial cell lines from the intestinal mucosa.

Method—We mated *Ptk*6 null mice with a tsSV40 large T transgenic mouse (Immortomouse) to obtain null mice carrying the SV40 gene. Intestinal tissues from these mice were cultured.

Results—We established a *Ptk*6 null epithelial cell line from the colonic mucosa. Consistent with a role of *Ptk*6 in cell differentiation, these cells have the characteristics of a stable progenitor cell. In monolayer culture the cells form domes in the monolayer when confluent. When cultured on Transwell filters the cells polarize and formed an electrically resistant barrier. Formation of tight junctions was confirmed by demonstrating expression of ZO1 and occludin at the apical junctions whereas E-cadherin localized to the basolateral membrane. When cultured in collagen gel, the *Ptk6* null cells form complex organoids some of which resemble cups of cells. These organoids contain cells with differentiated phenotypes. Using immunohistochemistry and confocal microscopy we have been able to identify villin positive (absorptive cells) and a small percentage of mucin-containing cells (goblet cells) and chromogranin A positive cells (endocrine cells).

Discussion—This conditionally immortalized cell line represents an excellent cell culture model system for exploring the mechanisms of cell function and epithelial differentiation in the colonic mucosa.

Keywords

Mouse colon; epithelium; conditional immortalization; Tissue culture; differentiation

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Introduction

There are no normal colonic epithelial cell lines that differentiate in vitro. A number of human fetal small intestinal cell lines and culture methods have been developed that reiterate the crypt- villus axis in the small intestine in vitro[1,2,3] but no similar tissue culture systems have been developed for the colonic mucosa. Because of this, studies have relied on human colon carcinoma cell lines as models for studying the differentiation and function of the colonic mucosa[4-7]. These studies all suffer from the caveat that these cells are cancer cells and not karyotypically normal.

The development of the Immortomouse8 allowed the establishment of conditionally immortalized epithelial lines from colon and other intestinal tissues 9,[10]. We have now established a conditionally immortalized colonic epithelial cell line from the Protein Tyrosine Kinase 6 (PTK6 also known as the Sik or BRK) null mouse [11]. PTK6 is a soluble intracellular Src-related tyrosine kinase that is expressed in a variety of normal epithelia12, where its expression is associated with growth arrest and differentiation.

The *Ptk*6 null mouse colonic epithelial cell line is of particular interest because it has retained the ability to polarize and form tight junctions in vitro. This was demonstrated by the formation of domes or "henicysts" in monolayer cultures. The presence of domes is indicative of vectorial fluid transport and results in fluid accumulation under localized areas of the monolayer[13]. The cells also form a highly electrically-resistant monolayer when grown on filters. In addition, when cultured in a collagen gel the cells form organoids and have some cells that differentiate into absorptive cells, goblet cells and endocrine cells.

In our experience of using the Immortomouse culture system to establish more than 20 epithelial cell lines from the colonic mucosa of a number of different transgenic mice, we have never previously seen differentiation in cultures grown under permissive conditions [10,11].

Methods

Mouse breeding

The *Ptk*6 null mouse 11 was mated with an "Immortomouse" (tsA58SV40 large T transgenic, [8](Charles River Laboratories,). Mice heterozygous for both disruption of the *ptk6* gene and expression of the SV40 large T gene were mated and mice with both a homozygous deletion of the *ptk*6 gene and positive for the SV40 large T gene were euthanized at 12weeks of age. All animal care was carried out by trained technicians and all procedures were approved by the Vanderbilt University Institutional Animal Care Committee.

Genotyping

Two sets of primers were used to identify the genotype of the *Ptk*6 null cells. Use of the first set of primers: m*Ptk*6-WT.1 (5′-ATGGTGTCTTGGGACAAGGCTCACCTGG-3′) and m*Ptk*6-WT.2 (5′-CATCCAGCAGGGTGGCCCACCACCACAG-3′) results in the amplification of a 150bp fragment corresponding to the wild type *Ptk*6 allele. The second set of primers: m*Ptk*6 KO.3 (5′-CATACACTTCATTCTCAGTATTG-3′) and m*Ptk*6 KO.4 (5′-ACGTGGCTGTCCAGACATAG-3′) amplifies a 1.5 kb fragment that corresponds to the targeted allele.

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Culture

The cultures were established as described previously [10]. In brief, the colon was removed, opened with scissors and the contents washed out with PBS. The surface of the tissue was sterilized by incubation in 0.04% sodium hypochlorite in PBS for 15 minutes at room temperature. The colonic crypts were harvested from the tissue by incubation in sterile 3mM EDTA/0.5mM DTT in PBS for 90 minutes at room temperature in a screw capped tube. The tube was then shaken vigorously and the supernate containing the crypts was removed to 15 ml centrifuge tubes. The tubes were centrifuged at 400rpm for 5 minutes.

For culture we tested 2 media. The crypt suspension was divided into 2 parts and either resuspended in our standard medium - RPMI1640 with 5% fetal calf serum, 1μg/ml insulin, 10^{-5}M hydrocortisone, 10^{-6}M α-thioglycerol (all from Sigma, St Louis, MO), 10% LIM1863[14] conditioned medium, penicillin, streptomycin, gentamycin, Primocin (Amaxa AG, Cologne, Germany) and 10 units/ml murine γ-interferon (Peprotech, Rocky Hill, NJ) or in a serum-free medium LHC-9 (Invitrogen, Carlsbad, CA) plus antibiotics and murine gamma interferon as above. The crypts were plated at low density in 0.2 ml of medium into wells coated with rat tail collagen. The plates were then incubated at 33°C in a 5%CO2, humidified incubator. After 24 hours, the volume of the wells was increased to 1ml taking care not to detach any adherent cells.. The medium was changed twice weekly for 4 weeks then weekly.

Any wells with epithelial cell growth were passaged individually into a well of a 6-well plate when 50% confluent. Initially the cells could only be passaged using Cellstripper (Mediatech Inc., Herndon, VA). The cultures were incubated in 5 ml of this solution until the cells began to detach. They were then pipetted vigorously to detach more cells. The cell suspension was removed, centrifuged and the cells resuspended in LHC-9 medium. Fresh medium was also added to the cells remaining on the culture surface. The epithelial nature of the cells was shown by demonstrating the presence of epithelial markers. The cells were cultured on LabTek slides, fixed in 4% Paraformaldehyde and stained with antibodies to keratin 18 (a gift from Dr E.B. Lane, Dundee, Scotland) and E-cadherin (Zymed, San Francisco, CA).

Functional testing

The electrical resistance of the cell monolayer was tested by culturing 10^5 cells on collagen-coated 3μ pore Transwell filters (Corning Incorporated). The resistance of the monolayer was measured daily from day 4 (Millicell-ERS, Millipore Corporation, Billerica, MA). Once the resistance had increased the Transwell cultures were fixed in 4% Paraformaldehyde and stained with antibodies to villin 1 (Santa Cruz Biotechnology), β -catenin (eBioscience), E-cadherin (Zymed), mucin 2 (MUC-2; Abcam), chromogranin A (Abcam), occludin (Zymed) and ZO-1 (BD Biosciences). Specific antibody reactions were detected using secondary antibodies labeled with Cy-3 or alexa488. Dapi was used to stain nuclei to enhance detection of polarization. The Transwell filters were examined by confocal microscopy.

The cells were also cultured by embedding a single cell suspension in either a rat tail collagen type1 gel or in Extracellular Matrix Gel (ECM gel, Sigma) and cultured for 3 weeks at 33°C. The gels were fed weekly with growth medium and then fixed in Paraformaldehyde, washed well with PBS, stained by incubating with specific antibodies and then with appropriate fluorochrome-labeled second antibodies. The gels were then examined by confocal microscopy.

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Results

Colonic epithelial cells from the *Ptk*6 −/− mouse proved very difficult to culture. Colonic crypts from 12 mice were cultured in separate experiments using our standard tissue culture methods that had proved to be successful with more than 20 previous transgenic strains, without success. The crypts failed to adhere and proliferate and all cells in the culture died within a week when the standard medium was used. We were able to culture the crypts in LHC-9 medium and obtained both crypt adherence and slow growth. This culture was expanded and stored in liquid N_2 at an early passage level (p7). The cells grew slowly as a tight epithelial sheet (Fig 1). The cells could not be passaged using standard trypsin/EDTA methods as the cells failed to readhere and proliferate. We were able to passage the cells successfully using "Cellstripper". After 5 passages the cells could be transferred to our standard tissue culture medium (see above). By passage 20 the cells had adapted fully to culture and could be successfully passaged using trypsin/EDTA solution. When confluent, the cells form domes in the monolayer indicative of good tight junction formation and a fluid transport capacity (Fig 1).

To confirm that the culture had not been contaminated with another cell line the cells were retested after passage 20 and PCR studies showed that the cells were still *ptk6* −/− (Figure 2). As controls, PCR was also performed with DNA from wild type and *ptk*6 null mice

The cells were cultured on collagen-coated transwells at passage 23 and the electrical resistance of the cells was measured. The electrical resistance of the monolayer increased suddenly once the culture became confluent and on day 7 the resistance had increased from an initial value of 140 milliOhms to > 1000milliOhms (Table 1).

Transwell cultures were stained for the presence of, β-catenin and E-cadherin. When examined by confocal microscopy E-cadherin (Fig 3A) and β-catenin (not shown) were distributed on the basolateral surfaces. The cells also stained with antibodies to occludin (Fig. 3B) and ZO-1 (Fig 3C).

Single cells were suspended in both a collagen gel and in ECM. After 3 weeks the *ptk*6 null cells had formed both spheroids (Fig. 3E and 4A) and cup-shaped structures (Figs 3F-I) in the collagen. Some of the cup-shaped structures also extended processes from the organoid into the collagen (Fig. 4A). The cup-shaped organoids predominated in the ECM gel cultures (Fig 4B). Within these structures we were able to find cells that stained for chromogranin A (Fig. 3D), villin1 (Fig. 3E) or mucin (Fig 3F) indicating differentiation along these pathways.

Collagen and ECM gel cultures were also processed for ultrastructural studies. Stained sections showed organoid structures with lumens (Fig. 5C). Polarized cells with microvilli were found in many of the spheroids (Fig 5A and B).

Discussion

There are currently no tissue culture models of the normal colonic epithelium that both demonstrate its barrier function, fluid transport and differentiation capacity. Because of this most in vitro studies have depended on the use of colon carcinoma cell lines. While these cell lines can be useful for barrier function and transport studies [15] and differentiation studies[5,16] they are based on the use of malignant rather than normal cells.

The difficulty encountered in establishing a cell line from the *Ptk*6 null mouse is not easily explained by the phenotype of the adult *Ptk*6 null mouse because defects in proliferation or attachment were not detected in the normal intestines of these animals[11]. However studies in

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cell lines have indicated that paxillin, a multi-domain adaptor protein involved in cell spreading and motility, is a substrate of *Ptk*6[18]. It is possible that the absence of *Ptk*6-mediated regulation of paxillin in *Ptk6* null cells had an impact on epithelial cell attachment.

In addition to being expressed in normal gastrointestinal epithelial cell linings [17], PTK6 expression is induced in human breast tumors [12] where it enhances growth, promotes anchorage independence and migration [18,19]. Thus, PTK6 appears to have different functions in different contexts.

Although PTK6 expression is normally restricted to non-dividing differentiated epithelial cells in the healthy adult mouse where it promotes differentiation of enterocytes[11], recent data indicate that PTK6 is induced in proliferating crypt epithelial cells after stress, where it has distinct functions. Expression of PTK6 in epithelial progenitor cells in vivo promotes apoptosis through inhibition of prosurvival signaling pathways (Tyner and Haegebarth, in preparation). A role for PTK6 in promoting apoptosis was previously demonstrated in Rat 1a cells, which were sensitized to apoptotic stimuli (serum starvation and UV irradiation) after the reintroduction of PTK6 [20]. The ability of PTK6 to negatively regulate pathways that promote cell survival such as the AKT and MAPK pathways, may also influence the differentiation process in cultured cells. To determine if disruption of *Ptk*6 contributes to the progenitor cell phenotype of these cells and their ability to differentiate in vitro, we have attempted to reintroduce PTK6 into the *Ptk*6 null cell line. However, reintroduction of CMV-PTK6 cDNA expression constructs into *Ptk*6 null cells resulted in apoptosis. Since it has not been possible to isolate cells with constitutive reintroduction of PTK6, cells with inducible expression constructs are now being generated.

Ptk6 null colon cell cultures are multipotential as absorptive cells, and a small number of goblet cells and endocrine cells have all been identified in both the cultures grown on filters and in the collagen gels (Fig. 3). In addition the cells form a polarized monolayer with good tight junction formation and develop a high resistance across the monolayer when grown on filters. These findings suggest that either a stem cell or an early, uncommitted progenitor cell has been immortalized by the SV40 large T gene. The *Ptk*6 null cell line has the most potential to differentiate reported to date, with more differentiation potential than any Immortomouse colonic epithelial cell line previously cultured in this laboratory. [9,10]

The reason that the *Ptk*6 −/− mouse colonic epithelium yielded an epithelial cell line with the capacity to differentiate in vitro is unknown. It is possible that the lack of the Ptk6 gene led to an increase in the pool of uncommitted or partially committed progenitor cells and that the immortalization of one of these cells has led to the establishment of this cell line.

When tested after 20 passages the cells had lost their sensitivity to trypsin. This has allowed the cells to be passaged much more easily. The cells were tested for the absence of PTK6 to demonstrate that the change in behavior was not due to cross-contamination with another cell line although this was considered unlikely as this line has a number of unique properties. The PCR result demonstrated that the cells were still lacking the PTK6 gene (Fig. 2).

Now that the cells are more easily passaged they should prove useful in studies of polarization, differentiation and transport.

Acknowledgments

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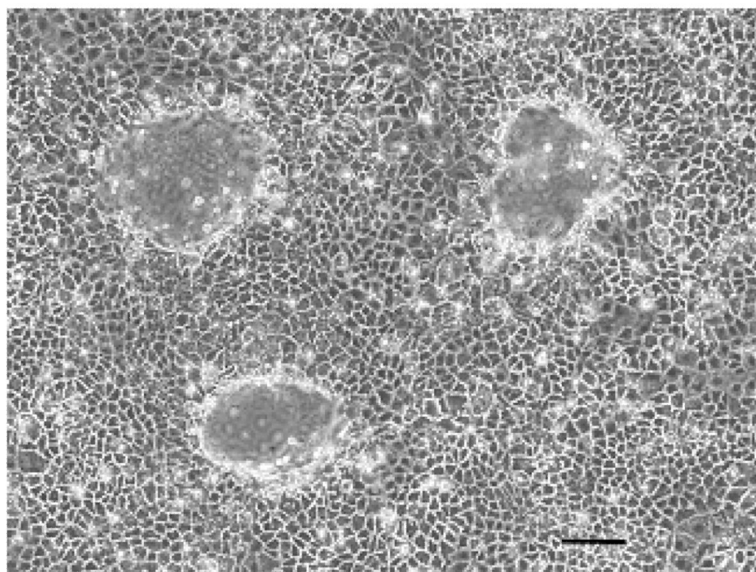

Figure 1.
Ptk6 cells growing in monolayer culture at passage 23. Numerous domes are present in all cultures.

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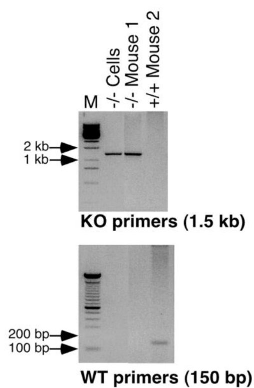

Figure 2.

PCR of cultured cells harvested at passage 25 to demonstrate that the cells have retained their null phenotype. When using mPTK6 KO primers, a ~1.5 kb PCR product expected in −/− cells. When using mPTK6 WT primers, a ~150 bp PCR product expected in +/+ cells.

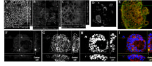

Figure 3.
*Ptk*6 null cells polarize and differentiate on filters and form organoids in collagen.
PTK6 cells were plated on Transwell filters (A-C) or were plated in collagen as a single cell suspension (E-I) and were allowed to grow into organoids. Shown are confocal horizontal (XY; D-I) and vertical sections (XZ and YZ; A-C, F-I) through the filters and an organoid. A, Cells on filters stained for E-cadherin, 40X objective 2X zoom. B, Cells on filters stained for occludin, 40X objective. C, Cells on filters stained for ZO1, 40X objective. D, Cells in an organoid stained for chromogranin, 60X objective 4X zoom. E, Organoid stained for villin (red), and β-catenin (green), 60X objective 1.5X zoom. F, Organoid stained for mucin2,), G, β-catenin (red), H, DAPI, blue) or I, overlay of all three 60X objective.

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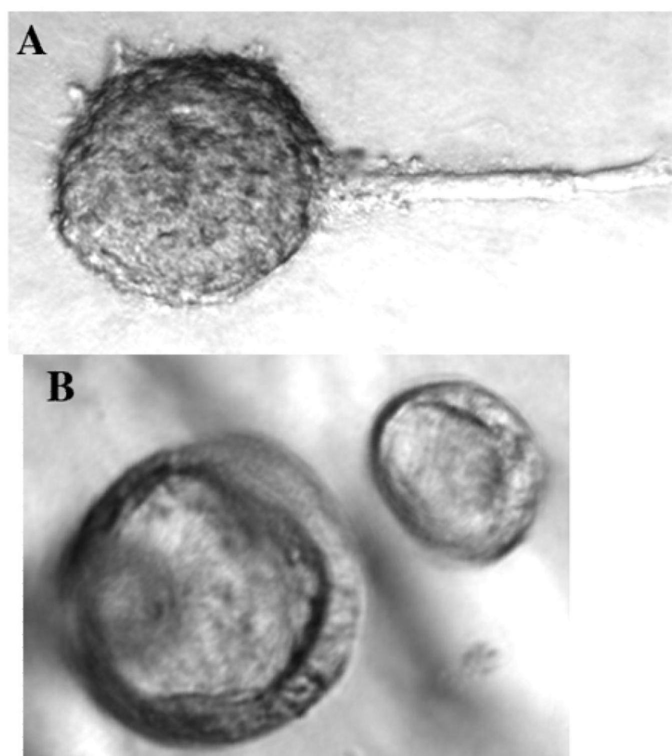

Figure 4.

Organoids formed in collagen and ECM. An organoid formed a ball of cells with a process coming off one side in collagen (Panel A) and formed cups of cells in ECM as seen by DIC confocal microscopy 40X objective 2X zoom.

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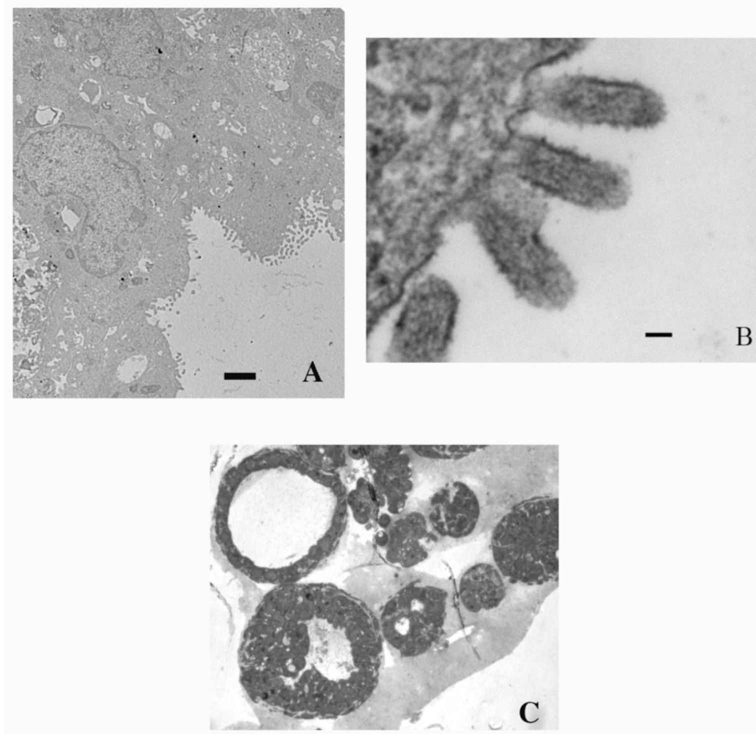

Figure 5.

A Electron micrograph of *Ptk*6 null cells grown in ECM (A) and collagen gel (B) showing surface microvilli. Magnification 5600×. Bar = 2μ. B Microvilli on polarized cell surface. Magnification 103000X. Bar = 0.01μ. C. Thick section through organoids showing lumen formation ×600.

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Table 1

Resistance of wild type YAMC and *Ptk6* null on Transwell cultures

Cell Line	Day 2	Day 10
YAMC p14 [*]	<100 [#]	<100
PTK6 null p24	150	>1000

[*] 10^5 cells of each cell line were plated on 21mm clear 3μ Transwells in 6 well plates and incubated at 33°C.

[#] Resistance in milliohm)