Establishment of conditionally immortalized epithelial cell lines from the intestinal tissue of adult normal and transgenic mice.

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Running title:- Intestinal epithelial cell lines from knockout mice

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ABSTRACT.
It has proved to be impossible to culture epithelial cells from the gastrointestinal tract of adult animals. Researchers have had to use either cell lines derived from newborn rat small intestine or colon carcinoma cell lines that have retained some of the properties of the gastrointestinal mucosa. We have described a method for establishing conditionally immortalized cell lines from the stomach, small intestine, colon, pancreas and liver from tissue obtained from a transgenic mouse strain carrying a temperature sensitive mutant of the SV40 large T gene (the “Immortomouse”). This immortalizing gene has proved to be useful for establishing cell lines from a number of transgenic mice following crossbreeding of the “Immortomose” with the transgenic mouse of interest. These cell lines are being used in numerous studies. In this review we describe the methods for developing such lines and list the range of cell lines that have been developed from colon, small intestine, stomach, liver and pancreas of a number of transgenic mice.
INTRODUCTION.

Despite years of effort in many laboratories, it has proved to be impossible to establish epithelial cell lines from the normal adult colonic epithelium of rodents. The cell lines that are widely used have been derived from the small intestine of newborn rats (RIE (2); IEC-6 and IEC-18 (9)). The alternative approach taken by many researchers has been to use colon carcinoma cell lines that have retained properties of the colonic mucosa. There are hundreds of papers describing such studies using cell lines such as HT-29, Caco-2, T84, LoVo etc.

We have approached the problem of establishing epithelial cell lines from the adult murine intestinal mucosa by utilizing a transgenic mouse (the “Immortomouse”) which carries a temperature sensitive mutant of the SV40 large T gene (6). The SV40 large T gene is an immortalizing gene that binds to p53 and inhibits its role in senescence (3). At the permissive temperature (33°C), the gene product (SV40 large T protein) is in an active conformation and will bind to p53 thus facilitating immortalization of the cells whereas at the non-permissive temperature (37° -39°C) the conformation of the gene product changes and it will not now bind to p53 or immortalize cells.

The advantage of using a temperature sensitive form of this gene is that the mouse has an almost normal phenotype and can be reared in standard mouse facilities and breeds well although it does develop a benign thymic hyperplasia with age that shortens the breeding life of female mice and limits the number of litters.

We initially utilized this mouse as a source of intestinal tissues to develop conditionally immortalized cell lines from young adult mouse colonic epithelium (YAMC (Figure 1, (14)), mouse small intestinal epithelium (MSIE) (Figure 2, (14)) and Immortomouse stomach epithelium (ImSt, (Figure 3), Whitehead unpublished results). Subsequently, epithelial cell lines have been developed from normal liver (Figure 4) (1) and pancreas (Figure 5) (7).

The availability of this mouse has enabled us to introduce the immortalizing gene into other mouse strains by crossbreeding. Using this method we developed cell lines from the colonic epithelium of the MIN mouse (8) (Immortomouse x Min Colonic Epithelium – IMCE) (Figure 6) (12)) which carries one mutant Apc allele; the colon, stomach, liver
and pancreas of the GFP mouse (10) and the colon, stomach and liver of the ROSA26 mouse (15).

The IMCE cell line has proved to be useful for studying malignant progression because, although the IMCE cell line is phenotypically normal, it has proved to be susceptible to transformation. Transfection of the cells with an activated Ras gene (4) or an activated β-catenin gene (11) induced a malignant transformation in these cells but not in the parental Immortomouse (YAMC) cell line. In a recent review, this pair of cell lines was described as the best model system available for studying malignant progression in colon cancer (5).

In this review, we describe our method for developing epithelial cell lines from the intestinal tissue of transgenic mouse strains of interest that we have bred with the “Immortomouse”. This method has enabled us to establish cell lines from a number of transgenic mice (see Table). Using this system it should be possible to develop conditionally immortalized epithelial cell lines from the intestinal tissue of any transgenic mouse.

Although a number of cell lines from a range of abdominal tissues are listed in the Table it should be noted that most of the cell lines from tissues other than the colon have not been characterized beyond demonstrating that the cells are epithelial. They are included to demonstrate the potential of the technique being described. The two exceptions to this are the original cell line derived from the liver of an Immortomouse (ImHep) which was shown to stain for both albumin and α-fetoprotein (1) and a cell line derived from the Immortomouse pancreas (IMPE) which was shown to produce insulin after transfection with pdx1 and culture in the presence of glucagon-like peptide 1 (7).

**MATERIALS.**

A. REAGENTS.

Cell-stripper; Catalog number 25-056-ci, Mediatech. Hendon, VA.

Collagenase Type 1; Catalog number 17100-017, Gibco, Grand Island, NY.

Dithiothreitol (DTT); Catalog number D0632, Sigma, St Louis, MO.

Ethylene diamine tetraacetic acid (EDTA); Catalog number ED455, Sigma.

Fetal calf serum (FCS); Catalog number sh30396.03, Hyclone, Logan, UT.

Hydrocortisone hemisuccinate; Catalog number h4881, Sigma).
Insulin (used for convenience as insulin, transferring, selenium stock solution, ITS); Catalog number I3146, Sigma.

LHC-9 medium; Catalog number 12680-013, Invitrogen, Carlsbad, CA.
Monoclonal anti-E Cadherin antibody; Catalog number 610181, BD, Bedford, MA.
Monoclonal anti-chromogranin A antibody; Catalog number MAB5268, Millipore, Danvers, MA.

Monoclonal anti-cytokeratin 18 antibody; Catalog number MAB3234, Millipore.
Monoclonal anti-MUC3 antibody; Catalog number RDI-MUC3abm, Research Diagnostics, Concord, MA.
Monoclonal anti-villin antibody; Catalog number MAB1639, Millipore.
Penicillin/Streptomycin; Catalog number 30-002-ci , Mediatech.
Phosphate buffered saline 0.1M pH 7.2; Catalog number p5368, Sigma.
Primocin; Catalog number VZA-1021, Amaxa Inc, Gaithersburg, MD. (This is an antibiotic mix designed for primary culture.)
Rat-tail collagen; Catalog number BD 354236. BD.
RPMI1640 tissue culture medium; Catalog number 10-040-cv, Mediatech.
Sodium hypochlorite; Catalog number 3248-1, VWR, Suwanee, GA.
α-thioglycerol (1-thioglycerol); Catalog number 88642, Sigma.
Trypsin/EDTA solution; Catalog number 23-053-ci -Mediatech.

B). EQUIPMENT
24 well tissue culture plates; Catalog number , tp92024, TPP, Midwest Scientific, Valley Park, MO.
6 well tissue culture plates ; Catalog number tp92006, TPP.
25cm² tissue culture flasks; Catalog number tp92026, TPP.
75cm² tissue culture flasks; Catalog number tp90076, TPP.
2ml Liquid N₂ storage vials; Catalog number 377224, Nunc, Roskilde, Denmark.
Lab-Tek 8-well chamber slides Catalog number 177402, Nunc.
Scienceware cloning disks; Catalog number Z374431, Sigma.
5% CO₂ incubator, 100% humidity.
Liquid N₂ storage freezer.
C). REAGENT SETUP


i). Fetal calf serum is aliquoted in 25ml volumes and stored at -20°C.

ii). Hydrocortisone hemisuccinate is a water-soluble form of hydrocortisone. A stock solution of 10^{-4}M is made in medium and filter sterilized. It is aliquoted in 5 ml amounts and stored at -80°C.

iii). ITS is diluted with medium to a stock solution of 100μg/ml insulin. It is filter sterilized and aliquoted in 5 ml volumes and stored at -80°C.

iv). Murine γ-interferon is diluted according to the manufacturer’s data sheet to a final concentration of 500 units per ml, filter sterilized and aliquoted in 5ml volumes. It is stored at -80°C. **NOTE.** Because there is no data on the stability of the interferon in tissue culture medium it is added fresh to the cultures with the medium.

v). α-Thioglycerol is made as a stock solution of 10^{-3}M in PBS, filter sterilized and stored in 5 ml aliquots at -80°C.

b). Tissue Culture Medium (final concentrations of ingredients are given).

500ml RPMI 1640.

5% FCS.

1μg/ml insulin (ITS suitably diluted can be used.)

10^{-5}M α-thioglycerol.

10^{-6}M hydrocortisone.

100 units/ml Penicillin.

100ug/ml Streptomycin.

100μg/ml Primocin.

10 units/ml mouse gamma interferon (reduced to 5 units/ml once the culture is established). This is added as needed. It is not added to the stock medium, as the stability of the interferon in medium is unknown.

The medium is stored at 4°C and is not used for more than 1 week.
PROCEDURE.

A).  MICE AND BREEDING

Immortomice are obtained from Charles River Laboratories (Wilmington, MA). When purchased, the mice are on a CBA/CA x C57/Bl10 background. The mice were transferred to a C57/Bl6 background as most of the transgenic mice received for cell line development are on this background. Breeding of mice with both the homozygous gene deletion and the temperature-sensitive SV40 large T gene requires two rounds of breeding, first creating a heterozygous knockout mouse carrying the tsSV40 large T gene then mating two of these heterozygous mice to obtain mice with the homozygous transgene and carrying the SV40 large T gene. The presence of the immortalizing gene is demonstrated by PCR as described previously (14). The presence of the transgene is demonstrated according to the protocol provided by the laboratory supplying the mouse. With patience, multiple mutations can be added (we have established a small intestinal cell line that is p53 -/- x MIN x Immortomouse using this method (Whitehead, unpublished results)).

B).  TISSUE PROCESSING AND CULTURE METHOD

a)  Colon, Small Intestine and Stomach.

For the establishment of cell lines from these tissues, it is advantageous if the mice can be raised until they are at least 3 weeks old and preferably 6 to 8 weeks old as this allows the isolation of pure epithelial cells from the stomach and colon using a non-enzymatic method using EDTA and DTT (14).

After euthanizing the mouse according to the protocol of the Institution, the required organs are removed. The colon, small intestine and glandular part of the stomach are opened lengthwise and the contents washed out with multiple changes of PBS. The tissues are incubated in individual screw-capped tubes in 20 ml of PBS plus 0.04% sodium hypochlorite for 15 minutes at room temperature in a 50 ml screw capped centrifuge tube. The dilute hypochlorite solution is then discarded and the tissue is washed twice in sterile PBS. From this point the tissue should be considered to be sterile and all procedures and solutions should be sterile.
The PBS is removed and replaced with 25ml of 3mM EDTA/0.5mM DTT in PBS (sterile filtered). This is incubated for 60 minutes at 4° C. The EDTA/DTT solution is removed and 20ml of PBS added. The top of the screw-capped centrifuge tube is tightened and the tube is shaken as vigorously as possible for 30 seconds. The released crypts (Figure 7) are removed to 15ml centrifuge tubes and centrifuged at 400RPM for 5 minutes. The shaking process can be repeated with fresh PBS but this can lead to fibroblast contamination.

The crypt pellet is resuspended in growth medium (with freshly added γ-interferon) and plated at low density (approximately 100-200 crypts per well) using a minimal volume (0.1 to 0.2ml) of medium into the wells of 24 well plates that have been coated with rat-tail collagen. The plates are incubated at 33°C in 5% CO₂ in a fully humidified incubator. After 24 hours, 1ml of medium is added gently to every well. The growth medium is replaced twice weekly.

Within 24-48 hours, some crypts will attach to the bottom of the wells and cell patches will begin to form (Figure 8). Most of these cells will die out in 1-2 weeks but small patches of cells will persist in occasional wells and then start to expand. The cell patches are passaged into the well of a 6-well plate when the well is >50% confluent. Re-feed the original well as some cells remain.

Once the well of the 6 well plate is confluent, the cells are passaged into a 25cm² flask and then into a 75cm² flask. The cells are stored in liquid N₂ at as early a passage level as possible. The cultures are never split more than 1:2.

NOTE. Although this method yields fibroblast-free crypt suspensions from colon and stomach on most occasions, it is impossible to obtain similar cell preparations from small intestine as villi are also isolated by this procedure. The stromal cells in the villi can therefore lead to fibroblast contamination of these cultures. Although this is a potential problem, we have cultured a number of epithelial cell lines from small intestinal tissue.

b) Liver and pancreas.

To culture the liver and pancreas, the tissue is removed, incubated in 0.04% sodium hypochlorite for 15 minutes at room temperature to sterilize the surface, washed with PBS
and minced finely with sterile scissors. The tissue is incubated in a mixture of 3 units/ml collagenase and 1 unit per ml neutral protease in tissue culture medium for 90 minutes at 4°C with gentle shaking. The mixture is allowed to settle and the supernate removed to centrifuge tubes and centrifuged at 1000rpm for 5 minutes. The cell pellet is washed once with medium and resuspended in culture medium to which fresh γ-interferon has been added and plated in a small volume of medium in collagen-coated wells. The plates are incubated in a fully humidified 5% CO₂ atmosphere at 33°C. One ml of medium is added after 24 hours. The plates are observed regularly and medium is changed twice weekly. Any cell growth is passaged with trypsin/EDTA when the well in >50% confluent. Using this method we have established pure cultures of both hepatocytes and pancreatic epithelial cells by cloning epithelial cell patches early in the culture. For cloning, the plate was marked in the area of the epithelial patch, the medium was removed and a sterile cloning disk that has been dipped in Trypsin/EDTA solution was placed on the epithelial patch. After 5 minutes the disk is removed to the well of a 24 well plate and growth medium is added.

C). TRYPsinIZATION PROCEDURE.
The growth medium is removed and Trypsin/EDTA solution is added to each well. This is then removed and the plates or flasks are incubated at room temperature until the cells begin to detach. This can be monitored using an inverted microscope. Medium is then added to the well or plate and the cells are detached by pipetting. Initially the cells from a well of a 24-well plate will be transferred to a well of a 6-well plate. Once this is confluent the cells are transferred to a 25cm² flask, then to a 75cm² flask. After this the cells are split 1:2.

D). COLLAGEN COATING
The wells are coated with a solution made by diluting 1ml of the stock collagen solution in 100ml of sterile PBS. One ml of solution is pipetted into each well of multiple 24-well plates. The plates are incubated overnight at room temperature, the solution is removed and the wells are washed once in PBS and drained well before use. The plates can be air-dried in the tissue culture hood and stored at 4°C. Alternatively collagen-coated plates can be purchased commercially.

E). STORAGE IN LIQUID N₂
Confluent flasks are trypsinized and resuspended in culture medium containing 20% FCS and 10% DMSO at a concentration of $2 \times 10^6$ cells per ml. One ml aliquots are pipetted into storage ampoules and frozen at 1°C per minute for 2 hours before being transferred to the liquid N$_2$ storage facility. To recover the cells from storage, an ampoule of cells is thawed rapidly at 37°C and the cells are resuspended in 15ml of culture medium and added to a 75cm$^2$ culture flask.

F). CELL CHARACTERIZATION

The cells must be checked for the genotype of interest by PCR or other applicable genotyping technique.

The cells should be checked for mycoplasmal contamination on a regular basis using one of the commercially available testing kits. Contamination with mycoplasma can be treated with Plasmocin (Amaxa) or Mycoplasma Removal Agent (Invitrogen).

The epithelial nature of the cells must be demonstrated. Cells that have been cultured on coverslips or Lab-Tek slides are fixed in 4% paraformaldehyde for 2 hours at 4°C, washed well with PBS and stained with antibodies to keratin 18 and E-cadherin using standard immunohistochemical techniques.

In our experience, in almost all cases the cultured cells are undifferentiated and cannot be induced to differentiate under standard conditions. However the phenotype of cells derived from colon or small intestine can be tested by staining cells that have been cultured on Lab-Tek slides with antibodies to villin (absorptive cells), mucin (goblet cells) and chromogranin A (endocrine cells) using standard immunohistochemical techniques.

NOTE. Although we have established cultures from stomach, liver and pancreas, we have not had the resources to characterize these cells but have cultured the tissues to demonstrate the usefulness of the technique. As referred to above, the original hepatocyte culture and a pancreatic culture have both been phenotyped and shown to retain characteristics of the tissue of origin. Cultures from the stomach, liver and pancreas should also be stained with tissue and cell type specific markers before use.
DISCUSSION.

Once established, the conditionally immortalized epithelial cells grow readily at the permissive temperature in vitro and provide a novel means of studying the function of the specific gene of interest in vitro. Because the cells are only conditionally immortalized, they will die over a period of 7 to 10 days if cultured at the non-permissive temperature however there is little evidence of differentiation during this period. The most differentiation that we have achieved during growth of YAMC and MSIE cells at the non-permissive temperature is a 2-3-fold increase in the level of di-peptidyl peptidase and sucrase-isomaltase (brush border enzymes) with no evidence of morphological differentiation (Whitehead, unpublished results). In the majority of our intestinal cell lines there are no morphological changes such as the appearance of microvilli or mucin granules that would suggest that differentiation is occurring.

The one exception to this is a cell line that we established from a Ptk6 null mouse (13). The colonic epithelial cells from this mouse form hemicysts (domes) in monolayer culture indicative of vectorial fluid transport. When cultured on filters the cells form a polarized electrically resistant monolayer with good tight junction formation. When cultured in a collagen gel the cells differentiate into absorptive cells, goblet cells and endocrine cells (13). This line should prove to be useful in both physiologic studies and studies of the factors controlling differentiation in the colon.

It should be noted that the establishment of this cell line required several changes to our standard culture technique. After more than a dozen attempts to culture cells from this transgenic mouse using our standard methods we used a serum-free medium (LHC-9) and obtained both cell adhesion and slow growth. We then found that the cells did not survive standard trypsinization and had to use a non-enzymatic cell removal solution (Cell-stripper). After 20 passages, the cell line that we established had adapted to both our normal tissue culture medium and our routine trypsinization procedures without losing its novel phenotype. We have not experienced these problems with any other transgenic mouse and have not failed to establish cell lines from any other mouse using
our standard techniques although it sometimes requires culture attempts from more than one mouse to obtain a cell line.

We have tested many media formulations and many additives in our attempts to establish cell lines from these tissues. Apart from our experience with the Ptk6-/- cell line described above, the medium formulation described in the Methods section has been suitable for all of our studies.

We believe that this method based on the use of the Immortomouse offers the potential to develop epithelial cell lines from intestinal tissue of most if not all transgenic mice. The isolation technique described here yields a purified crypt population from the colon and stomach and a mixed villus and crypt population from the small intestine. Any fibroblast contamination in the cell preparation can lead to problems as the fibroblasts are also immortalized and readily overgrow the epithelial cells leading to a long and difficult cloning to establish a pure epithelial culture. This is also the problem with the culture of embryonic tissue and tissue from very young mice where separation of the epithelial cells from the underlying stromal tissue before culture is not possible.

Of all the intestinal organs, small intestinal epithelium has proved to be the most difficult from which to establish epithelial cell lines. This is partly due to the fact that villi are always isolated with the crypt suspension. This leads to contamination of the culture with stromal cells, which can be very difficult to remove as these cells are also immortalized. Also, in our experience, small intestinal crypts do not establish as readily in culture as colonic crypts and stomach pits using these culture conditions.

There are many advantages to the use of a cell line for studies compared to the use of the whole animal. This method provides a constantly renewable source of conditionally immortalized cells that have been derived from the tissue of interest and which express the genetic phenotype of interest. This can provide the source material for studies of the function of the gene of interest in a simple controlled environment.

All of the cell lines listed in the table are available for research studies. As noted above, most of these cell lines have not been characterized beyond demonstrating that they are epithelial and are listed to demonstrate the usefulness of the technique. Anyone wishing to study these cells should undertake the characterization to demonstrate the retention of organ-specific properties.
It should be noted the Ludwig Institute for Cancer Research claims intellectual property rights to the Immortomouse and to cell lines derived using this mouse and a Materials Transfer Agreement has to be signed before these cells can be shipped. We can facilitate this once a request for a cell lines is received.
REFERENCES


Table. Epithelial Cell Lines Derived from the Immortomouse and from Transgenic Mice Crossed with the Immortomouse.

<table>
<thead>
<tr>
<th>Immortomouse</th>
<th>Colon (YAMC), Small Intestine (MSIE), Stomach (InSt), Liver (ImHep).</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-expressing</td>
<td>Colon, Stomach, Liver, Pancreas (ImPan),</td>
</tr>
<tr>
<td>MIN</td>
<td>Colon (IMCE)</td>
</tr>
<tr>
<td>ROSA 26</td>
<td>Stomach, Colonic stroma, Liver</td>
</tr>
<tr>
<td>ADAM9 -/-</td>
<td>Colon, Stomach, Liver</td>
</tr>
<tr>
<td>ADAM17 -/-</td>
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</tr>
<tr>
<td>Amphiregulin -/-</td>
<td>Colon</td>
</tr>
<tr>
<td>COX-1 -/-</td>
<td>Colon, Stomach, Liver, Pancreas</td>
</tr>
<tr>
<td>COX-2 -/-</td>
<td>Colon, Stomach, S.I., Liver, Pancreas</td>
</tr>
<tr>
<td>Cyclin D1 Overexpressing</td>
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</tr>
<tr>
<td>EGF-R -/-</td>
<td>Colon, Liver</td>
</tr>
<tr>
<td>KSR -/-</td>
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<tr>
<td>MTGR1 -/-</td>
<td>Small Intestine, Liver</td>
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<td>MYO1a -/-</td>
<td>Colon, Stomach</td>
</tr>
<tr>
<td>P55 -/-</td>
<td>Colon, Stomach, S.I., Pancreas</td>
</tr>
<tr>
<td>P75 -/-</td>
<td>Colon, Stomach, Liver</td>
</tr>
<tr>
<td>P53 -/- x MIN</td>
<td>S.I.</td>
</tr>
<tr>
<td>P120 -/-</td>
<td>Stomach</td>
</tr>
<tr>
<td>Ptk6 -/-</td>
<td>Colon, Stomach</td>
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<tr>
<td>Raf -/- floxed</td>
<td>Colon</td>
</tr>
<tr>
<td>SMAD 3/-</td>
<td>Colon, Liver, Pancreas</td>
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<tr>
<td>TGFb RII (floxed)</td>
<td>Colon, Stomach, S.I., Liver, embryonic fibroblasts</td>
</tr>
<tr>
<td>Waved 2</td>
<td>Colon</td>
</tr>
</tbody>
</table>

- All of these cell lines are currently stored in liquid N\textsubscript{2} at the DDRC Novel Cell Line Development Core Facility, Vanderbilt University and are available by contacting:

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FIGURE LEGENDS.

Figure 1. YAMC (colon) cells in monolayer culture, passage 23. Microscope magnification x100.

Figure 2. MSIE (small intestine) cells in monolayer culture, passage 20. x100.

Figure 3. ImSt (stomach) cells in monolayer culture, passage 16. x100.

Figure 4. ImHep (hepatocyte) cells in culture, passage 21. x100

Figure 5. ImPan (pancreas) cells in culture, passage 16. x100.

Figure 6. IMCE (Immortomouse x MIN) colon cells in culture, passage 21. x100.

Figure 7. Crypts liberated from adult mouse colon after EDTA/DTT extraction. x100.

Figure 8. Patch of cells from an attached crypt after 24 hours in culture. x100.