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Immortalised mouse submandibular epithelial cell lines retain polarised structural and functional properties

Bríd M. Laoide[1,*], Yves Courty[2], Isabelle Gastinne[1], Cécile Thibaut[1], Odile Kellermann[3] and François Rougeon[1]

[1]Unité de Génétique et Biochimie du Développement, Institut Pasteur, 25, rue du Dr Roux, 75724 Paris Cédex 15, France
[2]Laboratoire d'Enzymologie et Chimie des Protéines, Faculté de Médicine, Université François Rabelais, 2 bis, Boulevard Tonnellé, 37032 Tours Cédex, France
[3]Laboratoire de Différenciation Cellulaire, Institut Pasteur, 25, rue du Dr Roux, 75724 Paris Cédex 15, France

*Author for correspondence (e-mail: blaoide@pasteur.fr)

SUMMARY

The mouse submandibular gland (SMG) is an excellent model for the study of many important biological phenomena such as hormonal regulation of differentiation, neurotransmitter control of secretion, epithelial transport, exocytosis and endocytosis as well as the regulation of mouse SMG specific gene expression, in particular, NGF, EGF and renin. The postnatal development and sexual dimorphism of the mouse gland permits the isolation of male SMGs of different ages, corresponding to different stages of differentiation, particularily with respect to the cytodifferentiation of ductal cell types. We have immortalized SMG epithelial cell lines using mice transgenic for the large T antigen of SV40 or polyoma viruses. Epithelial clusters from the dissected glands were placed in culture and cell lines were established from the immortalized population. Two cell lines, SIMS and SIMP, which retain structural and functional characteristics, are described here. The cell lines are immortalised but not transformed, as judged by the absence of anchorage independent growth potential and the lack of tumour formation in athymic nude mice. Confocal and electron microscopy examination demonstrate that SIMP and SIMS cells express E-cadherin and ZO-1 and have features of polarised epithelial cells. In addition, they form spherical cysts with a wide lumen when grown in type I collagen gels. When grown on a filter support SIMS cells form a tight monolayer, exhibit vectorial transport function and show exclusive Na^+,K^+-ATPase localisation to the basolateral domain. We determined the cell type restricted expression of cytokeratin markers in the mouse SMG in vivo and we demonstrate that SIMS and SIMP cell lines express duct-specific cytokeratins. Finally, the expression of a set of differentiation markers, including EGF, NGF and renin, was detected by RT-PCR and by indirect immunofluorescence staining in these lines. Thus, these polarised ductal cell lines, as well as having important intrinsic properties, represent well characterised mouse epithelial models which, until now, have not been readily available for cellular studies.

Key words: T antigen, Transgenic mouse, Differentiation, Na^+,K^+-ATPase, Renin, EGF, NGF

INTRODUCTION

The submandibular gland derives from evagination of the endodermal lining of the embryonic gut (Jacoby and Leeson, 1959; Leeson and Jacoby, 1959). The epithelial rudiment branches and the epithelial cells grow into the surrounding mesenchyme, which is responsible for overall gland morphogenesis (Banerjee et al., 1977). The development of the gland continues postnatally and shows a striking sexual dimorphism (Gresik, 1980). Inter- and intralobular duct cells are present neonatally and these then give rise to striated duct cells and to granular convoluted tubule (GCT) cells (Gresik, 1980). The differentiation of mouse GCT cells begins 12 to 15 days postnatally in both males and females and is regulated by tyroxine. However, from about three weeks onwards, when circulating levels of androgens are high, the differentiation in terms of both size and number of GCT cells, is extremely marked in male mice (Dunn and Wilson, 1974; Kaiho et al., 1975).

The mature cells have an important secretory function: among the proteins secreted are epidermal growth factor (EGF), nerve growth factor (NGF) and renin (reviewed by Gresik, 1994). The role of these biologically important peptides in the SMG and the regulation of their expression and synthesis remains unclear. Indeed, under certain physiological conditions, such as male aggressive behaviour, these peptides appear to be liberated into the bloodstream suggesting a possible endocrine role for the SMG. In addition, a number of authors have demonstrated that SMG derived EGF plays an important role in a variety of events including liver regeneration (Jones et al., 1995), hormonal responsiveness of the mammary gland (Sheffield and Welsch, 1987) and male reproductive function (Tsutsumi et al., 1986; Russell et al, 1990). However, the available in vivo data is conflicting and, therefore, a functional in vitro model is required in

order to facilitate the study of epithelial transport and the mechanism of neurotransmitter control of secretion, which, in vivo, directs SMG specific products to the saliva and may direct their systemic secretion under specific environmental conditions, either directly or by transcytosis.

To date, the currently available salivary cell lines have been derived from either neoplastic tissue or carcinogen-treated SMGs and have not been characterised in terms of epithelial properties (Barka et al. 1980; reviewed by Patton and Wellner, 1993). We report here the immortalization and characterisation of non-transformed epithelial cell lines from transgenic male mice SMGs of different ages. The mice used were transgenic either for the large T antigen of SV40 virus under the control of the adenovirus E1A promoter, or the PyLT antigen of the polyoma virus. This system allows the immortalisation of cell lines due to the low level constitutive expression of both transgenes in a wide variety of tissues. Male mice of different ages were chosen to represent distinct stages of SMG postnatal development. Two cell lines, SIMP, derived from a 12-day-old mouse and SIMS, derived from a 22-day-old mouse, were structurally and functionally characterised. After more than two years in culture they retain the differentiated properties of polarised ductal SMG epithelial cells.

MATERIALS AND METHODS

Production of transgenic mice

A 3.1 kb *Eco*RI-*Bam*HI fragment containing the adenovirus E1A promoter fused to the SV40 T gene was microinjected into the pronuclei of fertilized oocytes (Brinster et al., 1985) which were obtained from (C57/SJL/J) F_1 hybrid females mated to identical males. Transgenic mice were identified by Southern blot analysis using tail DNA. Two founder lines were bred by crossing to DBA/2 mice (a strain that has two renin genes, *Ren*-1 and *Ren*-2) and subsequent progeny generations by backcrossing to F_1 hybrids to obtain offspring which are homozygous for E1A-T and *Ren*-2. A homozygous PyLT mouse was a kind gift from Dr F. Cuzin (INSERM 273, University of Nice, France) and was crossed to DBA/2 mice to obtain *Ren*-2 hemizygotes.

Dissociation of SMGs and culture conditions

SMGs were removed from transgenic male mice of various ages under sterile conditions. For a given experiment three to four male sibling SMGs were pooled. The sublingual glands were finely chopped and then dissociated in the following buffer: 1× collagenase (0.16% collagenase type 2, 0.16% collagenase type 3 (Worthington) were dissolved in MEM, filtered and stored at −20°C), 0.5 U dispase (grade I, Boehringer)/ml, 0.002 U DNAse (Boehringer)/ml. Ductal cells were enriched for as previously described (Durban, 1990) by differential sedimentation of cell clusters of approximately 10 to 20 cells. These clusters were seeded on plastic coated with E-C-L matrix (commercialised matrix derived from Englebreth-Holm-Swarm mouse tumour and containing collagen IV, entactin and laminin), 100 μg/75 cm^2, allowed to adhere, and fed the following medium: MEM + non-essential amino acids + insulin (Sigma, 30 mg/ml), cholera toxin (ICN, 100 ng/ml) and EGF (Sigma, 20 ng/ml). After three to four passages the medium was changed to DMEM + 10% fetal calf serum (Gibco). Two types of filter supports were used: Nunc inorganic 0.2 μm pore membrane and Costar transwell Clear polycarbonate 0.4 μm pore size. For three-dimensional studies, Cellagen AC-3 (ICN, 0.3% solution of acid-solubilized type I collagen, pH3) was neutralised with 0.34 N NaOH and mixed with 10× RPMI. The collagen mixture was added to cells (2×10^5/ml gel) and the mixture allowed to gel. Cultures were then fed with standard medium.

Histological techniques and electron microscopy

SMG tissue was fixed using the AMeX method and was paraffin embedded; 5 μm sections were used for immunohistochemical staining. For electron microscopy, the cultures were fixed with 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide and embedded in Epon. Sections (70 nm) were prepared and contrasted with uranyl acetate and lead nitrate to visualise cell structures.

Immunohistochemistry

Cells were fixed in methanol/acetone (7/3) for indirect immunofluorescence staining with anticytokeratin antibodies and were fixed with paraformaldehyde (4%) and permeabilised with saponin for other immunofluorescence studies. The following primary antibodies were used: rabbit anti-mouse EGF (Promega), rabbit anti-mouse NGF (Sigma), rabbit anti-pig renin (generous gift from Dr Catherine Rougeot, Pasteur Institute, Paris, France), rat anti-polyoma T antigen (generous gift from Dr F. Cuzin, INSERM 273, University of Nice, France), mouse monoclonal anti-α1 subunit of the Na^+,K^+-ATPase protein (Pietrini et al., 1992), mouse monoclonal anti-SV40 T antigen (Hybridolab, Pasteur Institute, Paris, France), mouse monoclonal antibody TROMA-1 which reacts with parietal endoderm and specifically with cytokeratin 8 (Boller and Kremler, 1983), mouse monoclonal anti-cytokeratin 7 (clone RCK105: Monosan), mouse monoclonal anti-cytokeratin 19 (Boehringer Mannheim).

Confocal microscopy

For confocal microscopy of cells embedded in collagen, the cells were fixed with 3% paraformaldehyde and permeabilised with saponin (0.5%), the nuclei were stained with propidium iodide (1:10 dilution; Boehringer Mannheim) and actin filaments were detected with FITC labelled phalloidin (Sigma). Samples were mounted in 50% glycerol and observed with a confocal microscope (Wild Leitz) at low magnification (×40 objective). For immunofluorescence confocal microscopy on 0.4 μM Transwell Clear (Costar) permeable filter supports, the cells were fixed for 1 minute with paraformaldehyde (4%) and then a 50% volume of cold ethanol was added successively until a final incubation at 4°C for 15 minutes in 100% ethanol. All subsequent processing of the cells was carried out in a 0.2% gelatin, 0.075% saponin, 0.5% BSA buffer. Filters were mounted in glycerol:PBS:phenylenediamine (1:1:0.01) and observed with a confocal microscope using a ×63 objective.

RT-PCR

A 10 μg sample of total cellular RNA was incubated with Superscript™ reverse trancriptase enzyme (GibcoBRL), following the suppliers instructions, and 1/10th of the reaction mixture was used for PCR analysis. The following primers were used:
EGF 912, 5′AGACGGGATCTCCTGTTTCG3′; EGF 1020, 5′GAGTCTGACATCGATCCCC3′; NGF 90, 5′CTAGTGAACATGCTGTGCC3′; NGF 313, 5′CATGGACATTACGCTATGC3′; RENIN EX7, 5′TCATGCAAGCCCTGGGAGCC3′; RENIN EX9, 5′CTTGTCTCTCCTGTTGGGAT3′.

PCR amplification was performed for 30 cycles and the products were analysed by Southern blot using the following oligonucleotides as probes and hybridizing overnight at 55°C using standard conditions. EGF 980, 5′CCAGGATGCCCATCCTCATA3′; NGF 221, 5′GCTCAACCTCAGTGTCTGG3′; RENIN EX8, 5′CGTAGTCCGTACTGCTGAGTGTGTAGGCCCTGCCTCCC3′.

The numbers refer to the position of the 5′ base in the cDNA sequence. EX 7, 8 and 9 refer to sequences from the corresponding exons of the *Ren* gene.

RESULTS

Transgenic mice used in this study

The approach used to obtain immortalized SMG epithelial cell

lines was to isolate SMGs from transgenic mice which express the large T antigen of either SV40 (T) or of polyoma (PyLT) viruses. The large T antigen alters the activity of *trans*-acting factors (e.g. AP-2 and Sph1) and can also bind to tumour supressor proteins such as Rb and p53, causing cells which are blocked in G_1 to enter S phase (Montenarh et al., 1986; Mitchell et al., 1987; De Caprio et al., 1988; Saffer et al., 1990). Transgenic mice carrying PyLT (PyLT mice) were a gift and have previously been described: no SMG tumours have been observed in these mice (Rassoulzadegan et al., 1983; Paquis-Flucklinger et al., 1993). To obtain mice transgenic for the SV40 large T antigen we used a vector construct, pK4 (Kellermann and Kelly, 1986; Kelly et al., 1986) which contains the SV40 T coding sequence downstream from the EIA adenovirus promoter-enhancer (see Materials and Methods). The E1A-T construct has been successfully used to immortalise cells at the stem cell stage while still allowing the expression of differentiation programs upon induction. In addition, the E1A promoter directs the low level expression of the T antigen gene in all three germ layer derivatives (Kellermann and Kelly, 1986; Kellermann et al., 1987, 1990; Buc-Caron et al., 1990; Poliard et al., 1993). We reasoned that a low level constitutive expression of the T antigen might be necessary and sufficient for the immortalisation of SMG cells but not their transformation. Indeed, none of the cell lines tested exhibit a transformed phenotype: they do not form colonies in soft agar and do not form tumours in athymic nude mice. The E1A-T region (see Materials and Methods) was used for injection into fertilized eggs. Two lines of E1A-T transgenic mice, each carrying a low copy number of the heterologous construct (Fig. 1A), were generated and used for further study. Families were established by crosses with DBA/2 (a mouse strain with two renin genes) partners. Both lines were tested for the presence of the *Ren*-2 gene, which is highly expressed in the SMG (Panthier et al., 1982; Gresik, 1994). Southern blot analysis of mouse genomic DNA confirmed that the PyLT mice are heterozygous for *Ren*-2 while the E1A-T transgenic mice are homozygous (Fig. 1B). The cell lines described below were derived from these mice, which, when killed, showed no visible tumours.

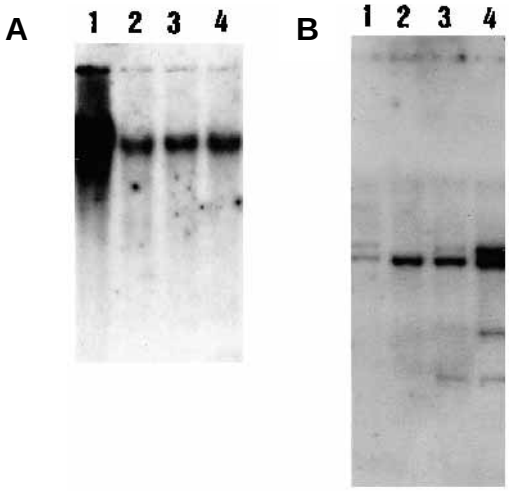

Fig. 1. Southern blot analysis of genomic DNA from transgenic mice from which SMG cell lines were derived. (A) Lanes 2-4, mouse tail DNA was digested with *Eco*RI and hybridized to a T antigen cDNA probe. There are few (1-2) copies of the transgene E1A-T. Lane 1 is a control DNA from a mouse transgenic for a vimentin promoter-T antigen construct (Hu-Vim-878-TsAT: Vicart et al., 1994) which has 10-20 copies of the transgene. (B) Southern blot using a 364 bp fragment of the 3′ end of the *Ren* gene as a probe. PyLT transgenic mice are heterozygous for the *Ren*-2 gene (lane 3) while E1A-T mice are homozygous (lane 4). The controls are DBA/2 mice which contain both *Ren*-1 and *Ren*-2 (lane 1) and Balb/c mice which possess only the *Ren*-1 gene (lane 2).

Culture conditions for the establishment of cell lines

The culture of epithelial cells from mouse SMGs has proven difficult. A number of reports have demonstrated successful short-term growth in primary culture (Wigley and Franks, 1976; Yang et al., 1982a,b; Durban, 1990; Takahashi and Nogawa, 1991; Nogawa and Takahashi, 1991). More recently, Durban (1990) has dissociated mouse SMGs and maintained growth of cells which express EGF for a period of two to three months in collagen gels. Using this method of dissociation and differential sedimentation (see Materials and Methods), SMGs of male transgenic mice, prepubertal and pubertal, were seeded on plastic coated with E-C-L matrix. The cells were fed standard medium containing growth inducers including insulin, EGF and cholera toxin (see Materials and Methods). Norleucine was also added which inhibits the growth of fibroblasts. Within twelve to fourteen days of seeding a number of morphologically distinct cell types were visible but only those clusters of cells which had a cobble-shaped appearance, typical of epithelial cells (Fig. 2A) were transferred to E-C-L-coated plastic, in an initial selection procedure. The clusters were allowed to adhere and grow for a minimum of ten days before passaging at high density. After nine to ten passages the growth inducers were removed and the cells were stained immunohistochemically for the presence of the T antigen. We were unable to detect the polyoma viral protein in any of the cultures derived from PyLT mice although these cultures have been maintained for more than two years in culture. Cells seeded under the same conditions from non-transgenic mice could only be maintained in culture for about one month. Immunochemical reaction to the SV40 T antigen is observed in the nuclei of cells derived from the E1A-T transgenic lines (Fig. 2B).

Cells with a cobble-shaped appearance which formed clusters were again selected from the immortalised polulation by trypsinizing clusters of approximately 10 to 12 cells and replating them in 24-well dishes coated with E-C-L matrix. In many cases these cells underwent a crisis; from those that survived and proliferated clones were isolated by limited dilution. Two cell lines are described in detail in the following sections; namely, SIMP derived from a 12-day-old PyLT transgenic SMG and SIMS derived from a 22-day-old E1A-T transgenic SMG. The 12-day-old gland has striated duct cells but not differentiated GCT cells while differentiation of GCT cells has begun in the 22-day-old SMG but the cells have not yet reached their adult morphology. These cell lines have a stable homogenous epithelial-like phenotype (Fig. 2C) and are routinely maintained in Dulbecco's modified minimal medium (DMEM) with 5% fetal calf serum.

SMG cells SIMS and SIMP polarise and express junction markers

Epithelia exhibit polarity, in keeping with their function in

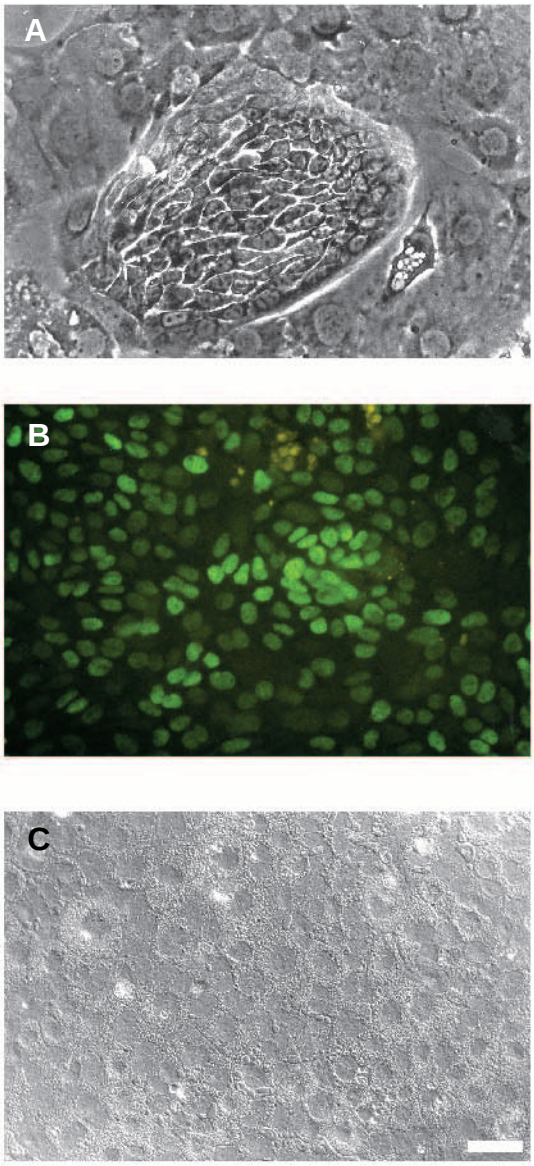

Fig. 2. (A) SMG epithelial-like clusters. The small cobble-shaped cells were isolated in an initial selection procedure.
(B) Immunochemical staining using an antibody directed against the large T antigen of SV40. The nuclei of all cells are stained and whorls of cells are evident. (C) Interference microscopy illustration of the cell line SIMS. Bar, 10 μM.

providing a boundary between the free surface and the underlying structures (reviewed by Handler, 1989). The development and maintenance of polarity is essential for the important secretory function of ductal cells of the SMG. We firstly determined whether cell lines SIMP and SIMS express E-cadherin (Nagafushi et al., 1987; Ringwald et al., 1987; Takeichi, 1988). E-cadherin proteins span the basolateral membrane and provide the initial adhesive forces between cells, forming the zonula adherens, an intermediate junction to which actin filaments are attached via catenin intermediates (reviewed by Takeichi, 1991). The cells were seeded on glass coated with E-C-L matrix for one week and stained for E-cadherin and F-actin (see Materials and Methods). Two distinct webs of actin were observed for SIMS cells, one at the basal surface and

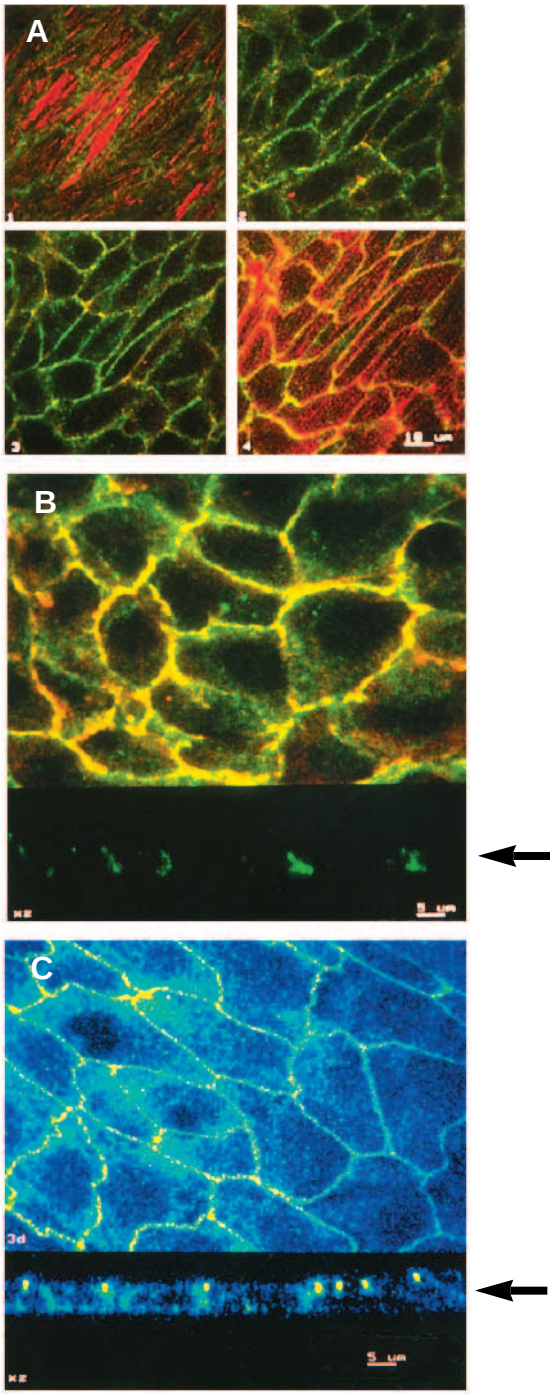

Fig. 3. Confocal microscopy images of cell lines SIMP and SIMS showing features of polarised epithelial cells. (A) Double immunofluorescence using an antibody to E-cadherin and FITC-labelled phalloidin to detect actin filaments. The four sequential images show SIMS cells from the basal surface (1) through the basolateral domain (2,3) to the apical surface (4). Actin filaments are found at the basal and apical surfaces of SIMS (in red), while E-cadherin is found at the basolateral membrane (green).
(B) Immunostaining as in A, the cell line is SIMP and the *xz* image demonstrates the localisation of E-cadherin (green) at the lateral membrane (arrow). The amount of actin filaments is less substantial in these cells and is almost absent from the apical surface. (C) ZO-1 expression in the cell line SIMS. A three-dimensional image is shown at the top and an *xz* image at the bottom. The localisation of the ZO-1 protein is at the apical-basolateral boundary (arrow).

one at the apical surface (Fig. 3A) while actin staining was predominant at the basal surface in the case of SIMP. Both cell lines stain strongly for E-cadherin and an *xz* confocal image of E-cadherin localisation on the lateral surface (Fig. 3B, arrow), demonstrates that the protein is correctly localised. The regulated expression and localisation of E-cadherin is essential for the control of polarity (McNeill et al., 1990; Takeichi, 1991; Cerijido et al., 1993; Gumbiner, 1996; Drubin and Nelson, 1996) and its presence is therefore an important feature of SIMS and SIMP cells. We then determined whether the cells form tight junctions by testing for the presence of the ZO-1 protein, one of the cytoplasmic plaque proteins which interacts with the membrane protein, occludin, to form the zona occludens or tight junction. The tight junction serves as an occluding barrier between the apical and basolateral regions of the cell as well as playing an important role in regulating the permeability between cells (Cerijido et al., 1993). SIMS and SIMP cells, seeded on glass coated with E-C-L matrix for one week, were immunohistochemically stained using anti-Z0-1 antibodies. As shown by confocal microscopy (Fig. 3C) the ZO-1 protein is correctly localised demonstrating that both cell types are functionally polarised and thus capable of a secretory function.

Ion transport in SIMS cells: restriction of Na^+,K^+-ATPase to the basolateral domain

The apical surface of cells grown on plastic faces the culture medium while the basolateral surface, instead of facing the extracellular fluid, in culture faces the Petri dish. When tight junctions form they effectively limit the access of medium to the basolateral surface (Rodriguez-Boulan and Nelson, 1989). However, when epithelial cells are cultured on a

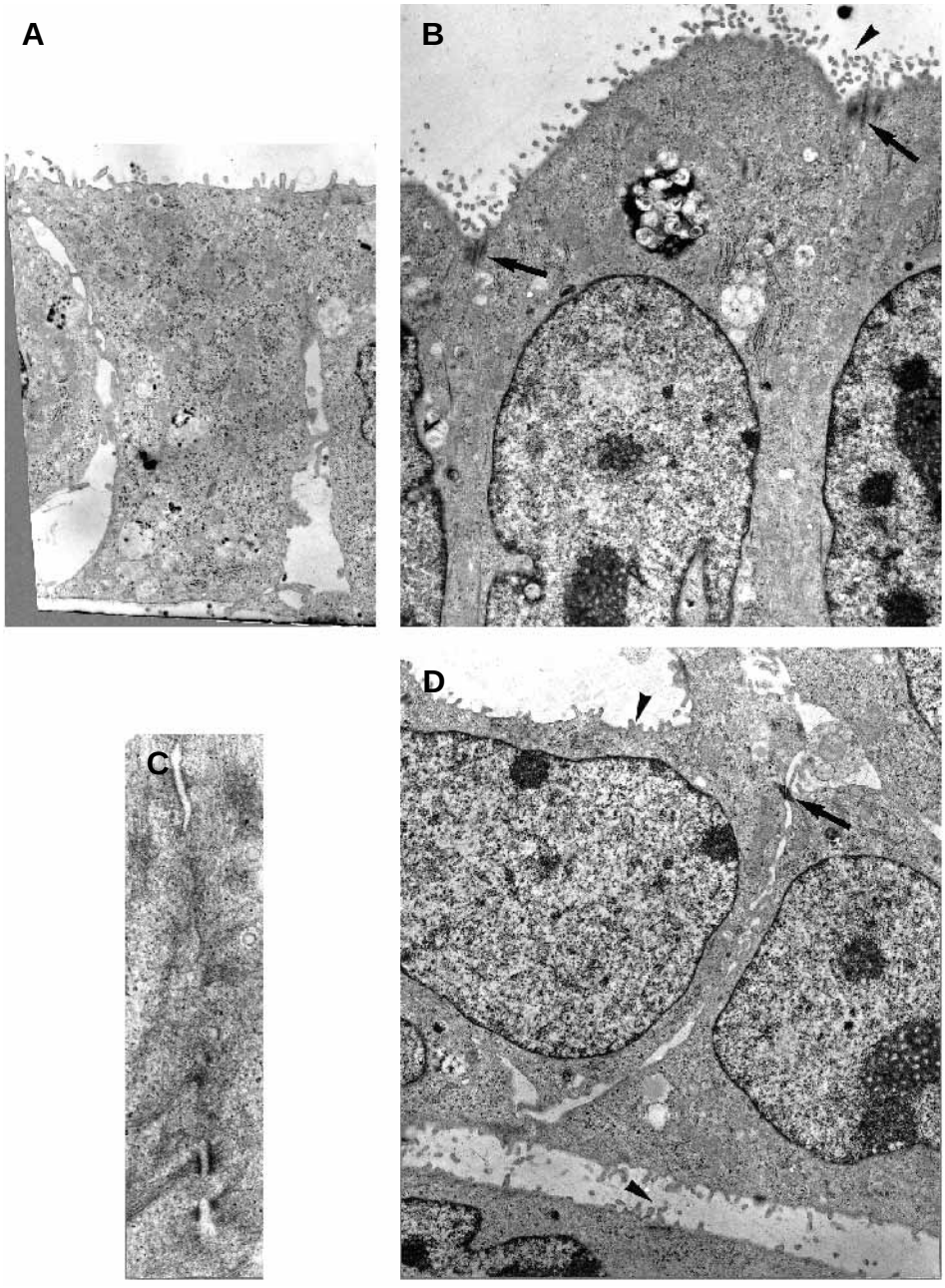

Fig. 4. Electron microscopy of SIMS cells grown on filter supports. The sections were cut at a slight angle to the filter support due to the difference in rigidity between the filter and the resin and thus the apical surface appears slightly out of focus. SIMS cells have a columnar appearance (A). Microvilli (B,D, arrowheads) at the apical surfaces facing the lumen, desmosomes at the basolateral membrane (C) and tight junctions at the apical-basolateral junction (B,D, arrows) are present. There are numerous rough endoplasmic reticula (B). A, ×3,000; B, ×4,400; D, ×4,000; C, ×20,000.

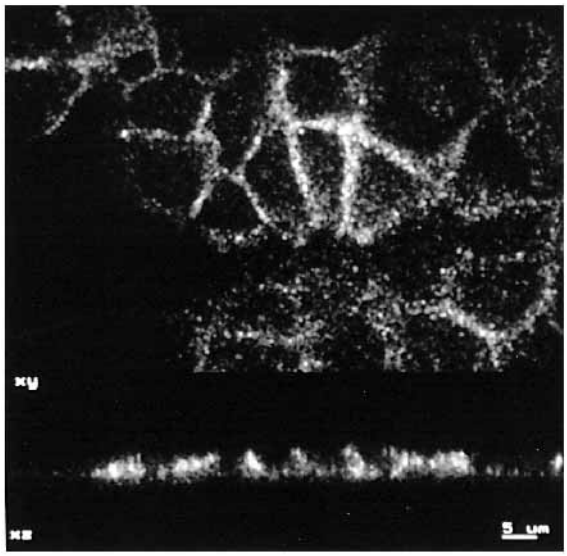

Fig. 5. Confocal immunofluorescence images demonstrating the basolateral distribution of the Na^+,K^+-ATPase protein in polarised SIMS cells. SIMS cells, grown to confluency on 0.4 μM Transwell (Clear) permeable supports for 5 days, were processed for immunofluorescence using an α1 subunit specific antibody and a fluorescein isothiocyanate (FITC)-coupled secondary antibody. The top panel is a confocal *xy* image while the bottom panel is an *xz* image demonstrating the exclusive localisation of the enzyme within the basolateral domain.

porous support the medium has equal access to both surfaces. We therefore used filters coated with E-C-L matrix to allow the cells to anchor and to orient the apical-basolateral axis. SIMP and SIMS were grown to confluency and maintained for one week in standard medium before fixation and preparation for electron microscopy examination. The cells show typical epithelial features including microvilli, desmosomes and tight junctions (Fig. 4A-D). Nuclei are large and occupy a significant portion of the cells, rough endoplasmic reticulum and mitochondria are abundant and there are few secretory granules. SIMS cells form tight monolayer sheets and are approximately 4 μM in height. In agreement with confocal microscopy data, abundant actin filaments are present in the apical region. SIMP cells are larger (approximately 7 μM) and are more loosely arranged in confluent cultures. Neither cell type show basal striations (Fig. 4A).

To determine whether polarised SIMS cells can actively transport ions, we examined the plasma membrane distribution of the Na^+,K^+-ATPase protein whose enzymatic activity is responsible for the active transport of Na^+ and K^+ in mammalian cells. Because polarised epithelial cells regulate the ion composition between biological compartments, the restriction of the pump to a specific plasma membrane domain is essential for vectorial transport function (Rodriguez-Boulan and Nelson, 1989). The epithelia of the SMG, in particular, have a crucial role in fluid and ion transport (Turner, 1993). SIMS

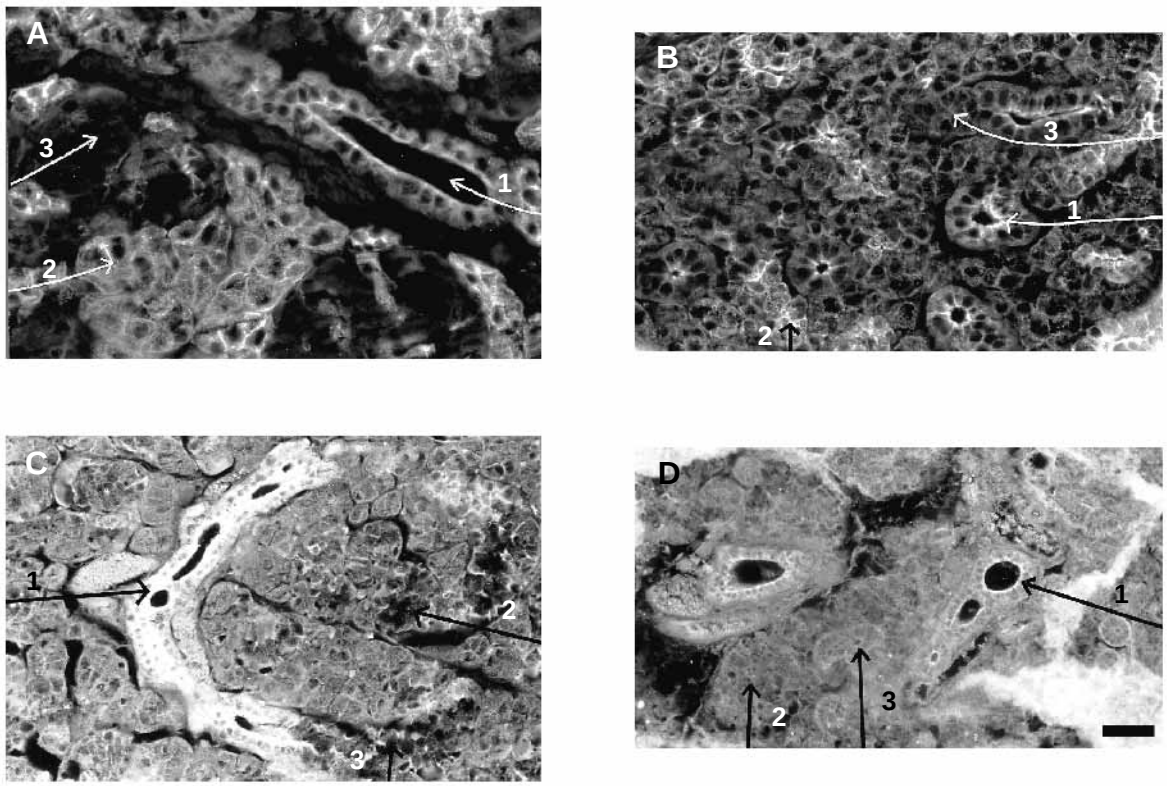

Fig. 6. Cell type specific cytokeratin expression in the mouse SMG. Immunohistochemical staining of adult and 15-day-old male mice SMGs paraffin embedded 5 μM sections. (A,B) TROMA-1 antibody (Boller and Kremler, 1983), which recognises cytokeratin 8, staining of adult (A) and 15-day-old (B) glands. All epithelial cells are stained (ductal, 1; acini, 2), except the GCT cells (3) of the adult gland. Staining is strongest at the apical domain of the ductal cells (1). (C,D) Anti-cytokeratin 19 antibody, staining of adult (C) and 15-day-old (D) glands. Acini are not stained (2) while ductal cells (1), except GCT cells (3), are labelled. The GCT cells are only beginning to differentiate in the 15-day-old gland (B,D), therefore, it is difficult to say with accuracy which ductal structures will give rise to this cell type, thus the (3) label is tentative. Bar, 100 μm.

cells were cultured for five days on filter supports. Confocal immunofluorescence microscopy, using an antibody specific to the α1 subunit of the protein (Pietrini et al., 1992), showed that the α subunit is restricted in its delivery to the basolateral domain of SIMS cells that have grown as a confluent monolayer (Fig. 5). The exclusive basolateral localisation was confirmed by an *xz* confocal image (lower panel of Fig. 5). These data suggest that SIMS cells sort the Na^+,K^+-ATPase protein in the Golgi complex: whether a mechanism of retention in the basolateral membrane also operates, as has been proposed for canine renal MDCK cells (Hammerton et al., 1991; Mays et al., 1995), remains to be tested. The localisation of the pump in these SMG-derived ductal epithelial cells is in agreement with the reported distribution of the pump in ductal cells of other exocrine and endocrine glands (Smith et al., 1987).

Expression of duct-specific epithelial markers, indicating the differentiated state of the cell lines SIMS and SIMP

The expression of cytokeratin intermediate-sized filaments is specific to epithelial cells and different epithelial cell types express different cytokeratin subunits. In addition, during development the subunit pair may change suggesting a link between subunit composition and the morphological and differentiated state of a particular cell type. In the pathology of human SMG tumours immunohistochemical cytokeratin detection is applied in diagnoses (Geiger et al., 1987; Born et al., 1987; Draeger et al., 1991) and we have used this information to determine the in vivo pattern of cytokeratin expression in the different cell types of the mouse SMG. Sections from 15-day-old and adult mouse SMG were examined using antibodies to cytokeratins 7, 8 and 19. The 15-day-old gland is largely immature both in size and structure and although acini, intercalated and striated ducts are present, neonatal terminal tubule structures still persist and the differentiation of GCT cells is just beginning. In contrast, the male adult gland contains a high (up to 50% of the total gland volume) proportion of GCT ductal structures (reviewed by Gresik, 1994). Both acini and duct cells in the adult glands stained positive for cytokeratin 8, using the antibody TROMA-1 (Boller and Kremler, 1983). Striated ducts stain strongly at the apical pole (Fig. 6A). The ducts

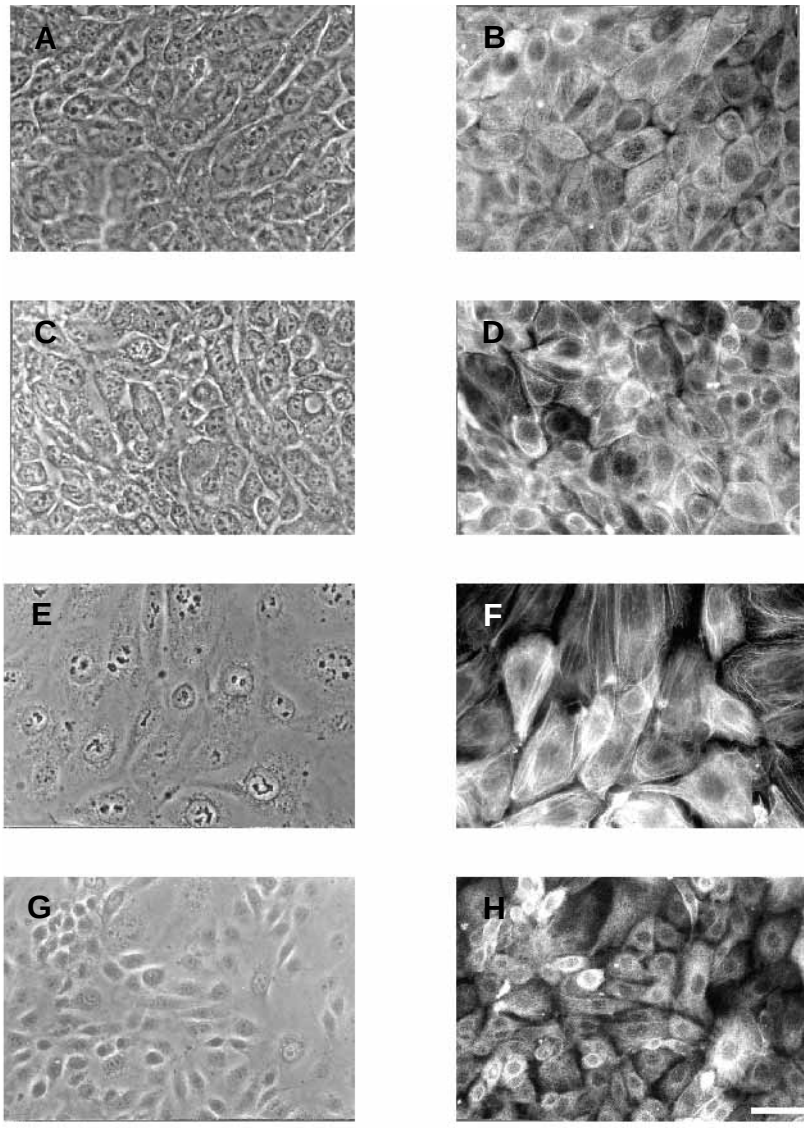

Fig. 7. SIMS and SIMP express duct-specific cytokeratins. Phase contrast microscopy (A,C,E,G) and fluorescence microscopy of the same field following immunohistochemical staining with anti-cytokeratin antibodies (B,D,F,H). (A-D) SIMS cells stained with TROMA-1 (B, cytokeratin 8-specific) and with anti-cytokeratin 7 (D). (E-H) SIMP cells stained with TROMA-1 (F, cytokeratin 8-specific) and with anti-cytokeratin 19 (H). (E,F) Same magnification (×40 objective) as SIMS cells (A-D) showing that SIMP cells are phenotypically distinct and significantly larger than SIMS cells in confluent monolayer cultures. Bars: A-F, 10 μM. (G-H) ×20 objective.

and acini of the 15-day-old gland are also positive (Fig. 6B). Examination of the staining pattern observed with anti-cytokeratin 7 and anti-cytokeratin 19 antibodies indicate that ductal cells are positively labelled in both the 15-day-old and the adult while acini are unstained (Fig. 6C-D, and data not shown). Thus, while cytokeratin 8 is a general marker of SMG epithelial cells, cytokeratins 7 and 19 are restricted to ductal cells and are, therefore, appropriate markers for this cell type. It is interesting to note that the GCT cells do not react with any of the antibodies. This is in agreement with previous reports that GCT cells stain poorly or negatively for cytokeratin proteins (Takai et al., 1985; Gresik, 1994). Using the same antibodies we then examined the cytokeratin pattern of SIMP and SIMS cells. The cells reacted strongly to all three antibodies (Fig. 7B,D,F), indicating the presence of the simple epithelial cytokeratin subunit 8, as well as the duct-specific cytokeratins 7 and 19. We can conclude, therefore, that both cell lines, although morphologically distinct from each other (Fig. 7A,C,E), represent differentiated epithelial cells of ductal orgin.

Epithelial plasticity of SIMS and SIMP cell lines

Following the establishment of our ductal cell lines, we investigated whether, in vitro, they can remodel within collagen gels to form three-dimensional structures. SIMS and SIMP were seeded in type I collagen and fed standard medium for two to three weeks. After three days in culture small clusters were seen within the gel. The continuous proliferation and migration of the cells eventually resulted in structures with a distinct defined periphery, easily visible by light microscopy (Fig. 8A).

Confocal microscopy, following staining of the nuclei with propidium iodide and of the cytoplasmic actin with FITC labelled phalloidin, revealed that the cells organise in a polarised manner to form symmetrical cysts. A wide lumen is present and the cysts vary in thickness from 30 μM to 80 μM. The structure of one half of a cyst can be clearly seen in Fig. 9: the cyst appears externally to contain relatively compacted cells with large amounts of actin visible, in particular at the outer edges of the cyst. As the images focus into the centre, at 2 μM intervals, the lumen becomes evident with a single layer of polarised cells surrounding it, having actin filaments at both the basal and apical surfaces.

The cell line SIMP also formed similar cysts of about 30 μM thickness but unorganised structures were also evident (Fig. 8B). The cell line SIMS, on the otherhand, showed a more homogenous population of cysts. It is important to note here that SMG cell populations from PyLT male mice and from E1A-T male mice of different ages form branching structures in collagen gels (Fig. 8C). However, electron microscopy and confocal microscopy demonstrated that these branching structures, although appearing superficially to mimic tubule formation in vivo, consist of unpolarised cells which do not organise to form a lumen (unpublished observations). In contrast, the basal surfaces of polarised SIMS and SIMP cells are in contact with the collagen while the apical surfaces face the lumen, as is the case in vivo. The plasticity of the cells is due, at least partly, to their ability to form contacts with their neighbouring cells and with the collagen substratum; subsequently, the cells migrate and divide to form characteristic epithelial cysts.

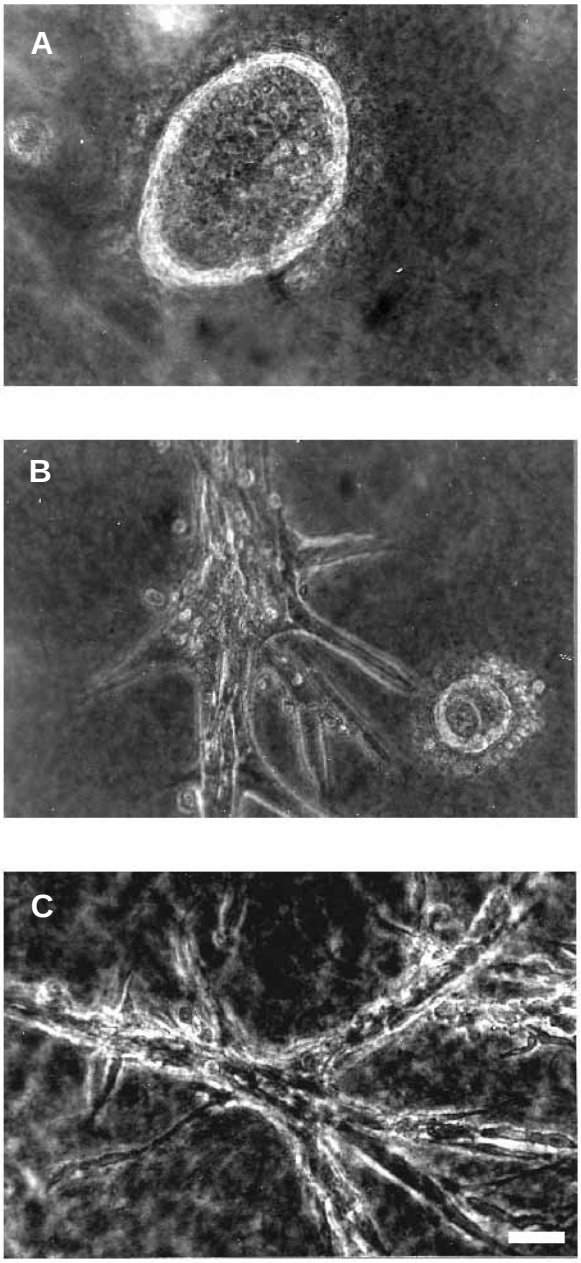

Fig. 8. Growth of SMG cultures in three-dimensional collagen type I gels. The cell lines SIMS and SIMP forms spherical structures (A, SIMS), and branching filaments (B, SIMP). SMG cell populations form branching structures without a lumen (C). Bar, 10 μm.

SIMS and SIMP express a set of differentiation markers, characteristic of GCT cells in vivo

In the 1960s the initial discovery and purification of nerve growth factor (NGF; Cohen, 1960) and of epidermal growth factor (EGF; Cohen, 1962) was from the SMG material of male mice. It was correctly assumed that the GCT cells are high producers of these peptides (Gresik and Barka, 1977). It was also noted that certain strains of mice synthesise an SMG-specific renin enzyme which is found in the same secretory granules of the GCT cells as EGF and NGF. The cDNA for this renin, *Ren*-2, was subsequently cloned (Panthier et al.,

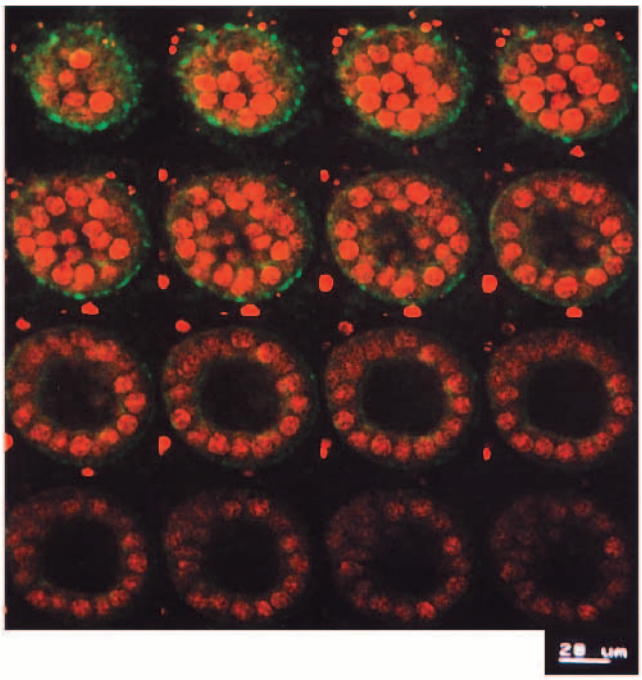

Fig. 9. SIMP and SIMS cells form cysts in collagen gels. Confocal images of one half of a cyst (40 μM) formed by SIMS in collagen gels after three weeks in culture. Cell nuclei were stained with propidium iodide (in red) and FITC-labelled phalloidin was used for actin staining (in green). Sixteen sequential images (from top to bottom and left to right), separated by 2 μM or 4 μM, show the bottom of a cyst (closest to the glass slide, top left corner of figure) which is composed of compacted cells through to the centre of the cyst which shows the cells polarised with the apical surface facing the lumen.

1982) and it was established that it is distinct from *Ren*-1 which is found in all strains of mice. To determine whether our cell lines express these GCT-specific markers we firstly carried out RT-PCR analysis using primers specific for EGF, NGF and renin (see Materials and Methods). Interestingly, all of the SMG cell populations tested express NGF and EGF (Fig. 10A,B). However, renin expression is more restricted and may require a more differentiated cell phenotype. SIMP and SIMS express all three markers and, using an internal *Alu*I restriction site present only in the *Ren*-2 gene to determine whether the renin transcripts are *Ren*-1 or *Ren*-2 specific, we found that *Ren*-2 specific PCR products are present in both cases (lower band in Fig. 10C). Immunohistochemical staining of cell lines SIMP and SIMS demonstrate that NGF and renin immunoreactive material is present in the cells (Fig. 10D-E). Both cell lines also stained positive for EGF (data not shown). Immunoreactivity was observed throughout the cytoplasm and was occasionally perinuclear but, as expected, was not found in the nucleus. Not all cells stained with the same intensity, however, and some appeared to be free of reaction product. This pattern is similar to the in vivo pattern of expression of these markers, as determined by in situ hybridization, which shows cell to cell variation (reviewed by Gresik, 1994).

DISCUSSION

Here we report the immortalisation of mouse SMG epithelial cell lines, derived from male mice transgenic for the large T antigen. The mouse SMG develops as a result of epithelial-mesenchymal interactions whereby the epithelial anlage grows into the surrounding mesenchymal tissue and develops into a

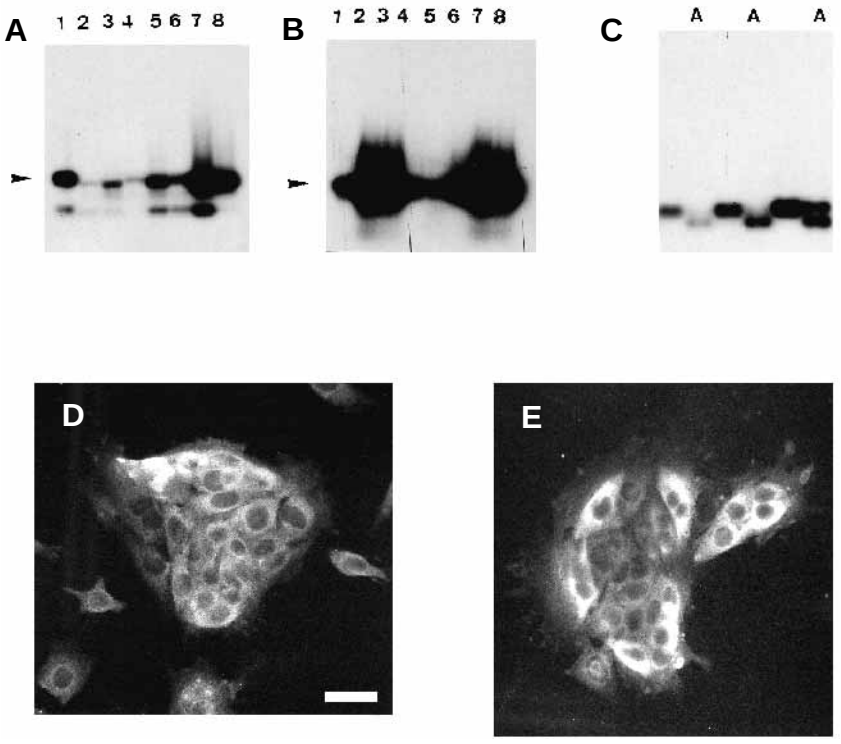

Fig. 10. SIMP and SIMS express a set of differentiation markers. RT-PCR analysis using SMG specific markers as primers. DNA was isolated from various cell cultures, cDNAs were prepared and PCR reactions were carried out using standard conditions (see Materials and Methods). (A) Lanes 1-8: RT-PCR reactions using EGF specific primers. Lanes 1-5 contain the reaction products using different SMG cultures which were not used for further studies. All SMG cell populations synthesise EGF mRNA. Lane 6, SIMS product; lane 7, SIMP product. Lane 8 is a positive control using RNA isolated from a male mouse SMG. EGF-specific bands are indicated by an arrowhead. (B) Lanes 1-8: RT-PCR reactions using NGF specific primers. Lanes 1-5 contain the reaction products using different SMG cultures which were not used for further studies. All SMG cell populations also synthesise NGF mRNA (arrowhead). Lane 6, SIMS PCR product; lane 7, SIMP PCR product. Lane 8 is a positive control using RNA isolated from a male mouse SMG. (C) RT-PCR reactions using renin specific primers. The first and third lanes contain the reaction products of SIMP and SIMS cells, respectively. 'A' represents the same cultures except that the corresponding PCR products were digested with *Alu*I before loading onto the gel. The last two lanes show SMG samples. (D-E) Immunocytochemistry using antibodies to renin and NGF: indirect immunofluorescence with anti-renin antibodies (D) and anti-NGF antibodies (E) on the cell line SIMS. Staining is heterogenous. Bar, 10 μm.

multilobed structure surrounded by neuronal and vascular elements (Nakanishi et al., 1986; reviewed by Fitch and Linsenmayer, 1994). Other glands, including the mammary gland and the lung, also undergo branching morphogenesis and the importance of the mesenchymal capsule in directly controlling the development of the branching pattern is now well established (reviewed by Fitch and Linsenmayer, 1994). After morphogenesis of the gland has taken place, the development of specialised epithelial cell types occurs (Pinkstaff, 1980). Cytodifferentiation, at least in vivo, also requires factors produced by the mesenchyme as well as extracellular matrix components (Pinkstaff, 1980; Fitch and Linsenmayer, 1994). The secretory and absorptive epithelia have a crucial role in fluid and ion transport (Turner, 1993). The secretory response of the SMG is mediated via the autonomous sympathetic and parasympathetic pathways; receptors on the epithelial cell membrane are activated following the release of neurotransmitters including norepinephrine and acetylcholine. In addition, Ca^{2+} is required for the stimulation of secretion and related ion fluxes (Turner, 1993).

We chose the mouse SMG because of the interesting properties of the gland, per se, and because of the usefulness of such an epithelial model to study a variety of functions universal to epithelial cells. We determined the functional and structural characteristics of two cell lines which were derived from mice transgenic either for the polyoma or SV40 T antigen, the latter under the control of the adenovirus 5 E1A promoter-enhancer. The PyLT gene has been reported to be an immortalising rather than a transforming gene and has been successfully utilised for the establishment of Sertoli cell lines (Rassoulzadegan et al., 1983, 1993; Paquis-Flucklinger et al., 1993). The transgenic mice reported in this paper, carrying the SV40 T antigen, E1A-T, exhibit little tumour formation and usually with advanced age. The construct has been shown to promote immortalisation of embryonic stem cells while still allowing differentiation along multiple pathways to occur (Kellermann and Kelly, 1986; Kellermann et al., 1987, 1990; Buc-Caron et al., 1990; Poliard et al., 1993). Immortalised SMG epithelial cell lines were obtained from both types of mice: SIMP, derived from a 12-day-old PyLT mouse and SIMS derived from a 22-day-old E1A-T mouse. It is important to stress, firstly, that the presence of the T antigen does not prevent the establishment of differentiated cell lines, at least in the cases described here. Secondly, the 12-day-old mouse is prepubertal and the gland is still immature, with remnants of the embryonic terminal tubule structures still persisting as well as developing acini and ductal structures. On the other hand, the 22-day-old mouse has fully developed acini and due to the presence of circulating androgens the differentiation of GCT ductal cells has begun to accelerate. Thus, we have chosen a gland which exhibits sexual dimorphism and postnatal development, combined with the advantages of an immortalising but non-transformed phenotype, to select and establish SMG epithelial cell lines.

Both cell lines display characteristics of differentiated ductal epithelial cells: they polarise, forming tight junctions, microvilli and intermediate junctions; they grow as tight monolayers on filter supports and as three-dimensional cysts when embedded in collagen gels; they express duct-specific cytokeratins and a set of SMG specific differentiation markers. SIMS cells are capable of ion transport and shows restricted basolateral distribution of the sodium pump (Na^+,K^+-ATPase). This is the first report, to our knowledge, of non-transformed immortalised SMG cells showing differentiated epithelial characteristics in long term culture. The cells have been in culture for more than two years and retain both structural and functional properties. SIMS cells, in particular, are highly functional in terms of structure, transport and SMG specific gene expression. We are now interested in studying the hormonal control of cytodifferentiation of GCT cells by androgens, thyroxine and adrenergic agents and are presently defining growth conditions in a defined serum-free medium in an attempt to recruit the terminal differentiation of the cell lines under in vitro conditions. We are also studying the interaction of these cell lines with cells of mesenchymal origin to determine the effect of such interactions on their growth and migration as well as on the regulation of gene expression. In the future we will use SIMS and SIMP cell lines to study hormonal and neurotransmitter control of secretion, for additional studies of fluid and ion transport and for transcytosis experiments. These cell lines can also be used for transfection studies to determine the androgen regulation of SMG specific genes.

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