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Progenitor cells of the adult human airway involved in submucosal gland development

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SUMMARY

A bronchial xenograft model of the human airway was used to identify submucosal gland progenitor cells within the surface airway epithelium. Lineage analysis using recombinant retroviruses has demonstrated considerable diversity in the cellular composition of expanded clones within reconstituted xenograft airway epithelium. These findings provide evidence for the existence of multiple progenitors in the airway with either limited or pluripotent capacity for differentiation. Furthermore, the development of transgene-expressing submucosal glands was associated with a single subset of surface airway epithelial clones. This gland progenitor cell demonstrated two discernible characteristics consistent with the identification of an airway stem cell including: (1) pluripotent capacity for airway differentiation and (2) a two-fold higher proliferative rate than other observed clone types. The number of progenitor cells involved in gland development was also assessed by clonal analysis using alkaline phosphatase and β-galactosidase transgenes. These studies demonstrated that more than one airway progenitor cell is involved in the initial stages of gland development. A second explanation for the high prevalence of non-clonality in developing glands was suggested from three-dimensional reconstruction of transgene marked glands. These reconstruction experiments demonstrated that 27% of glands contained more than one duct to the surface airway epithelium. This observation suggests a novel mechanism of gland morphogenesis by which independently formed glands interact to join glandular lumens. Such a mechanism of glandular development and morphogenesis may play an important role in normal submucosal gland development and/or the progression of hypersecretory diseases of the adult human airway as seen in cystic fibrosis, chronic bronchitis and asthma. The identification of progenitor cells with the capacity to form submucosal glands has implications on the targets for gene therapy in cystic fibrosis.

Key words: airway, glands, morphogenesis, retrovirus, lineage, xenografts

INTRODUCTION

Submucosal glands in the adult human airway are composed of numerous interconnecting ducts and tubules which regulate the flow of fluid and mucus secretions into the airway lumen. Secretory products of the submucosal glands move vectorally from the most distal regions of the serous tubules through mucus tubules, collecting ducts, and lastly ciliated ducts which exit into the surface airway lumen (Meyrick and Reid, 1970). The morphologic progression of submucosal gland formation in the fetal human airway has been extensively studied. Initial stages of gland development have been correlated with the progression of gland forming 'buds' from the surface airway epithelium and subsequent lateral morphogenesis within the interstitial layers of the airway (Thurlbeck et al., 1961; Tos, 1960, 1968). In vitro collagen matrix models of the initial stages of gland formation (Infeld et al., 1993) have suggested that these processes may act via mechanisms of epithelial-mesenchymal interactions similar to those seen in embryologic tubulogenesis in the kidney, mammary gland, and lung airways (Spooner et al., 1980; Hilfer et al., 1985; Howlett et al., 1993; Bluemink et al., 1976). Such mechanisms of epithelial invasion have been shown to be regulated by a dynamic interaction of microenvironmental cues and signals which control epithelial cell growth, differentiation and morphogenesis. Mediators such as mesenchymally expressed cytokines (EGF and TGFα) affect the secretion of metalloproteinases (extracellular matrix degrading enzymes) by epithelial cells and have been implicated in these processes (Taub et al., 1990; Ganser et al., 1991). Alternatively, cytokines such as TGFβ1 have been shown to inhibit processes of epithelial duct growth by mediating mesenchymal deposition of extracellular matrix (Roberts et al., 1986; Ignotz et al., 1986) and inhibiting its degradation (Lund et al., 1987; Edwards et al., 1987). In summary, the microenvironmental cues which involve the dynamic and reciprocal interaction of soluble cytokines, cytokine receptors, extracel-

lular matrix (ECM), and adhesion molecules have been proposed to be mediated by three potential mechanisms: (1) epithelial-mesenchymal cellular contact interactions, (2) cellular interactions with ECM components, and (3) diffusion of epithelial or mesenchymally secreted soluble factors such as cytokines.

Little is presently known about the progenitor cells of submucosal glands or the process by which gland development and morphogenesis occur in the airway. It is generally accepted that both secretory and basal cells have the capacity to divide within the surface airway epithelium. In contrast ciliated cells are thought to be terminally differentiated and non-dividing (Breuer et al., 1990; Donnelly et al., 1982; Randell et al., 1991; Inayama et al., 1988). Several approaches have been used to identify the pluripotent progenitor cells in the tracheas of rats, including radiolabeling with [^{3}H]thymidine (Breuer et al., 1990; Donnelly et al., 1982) and cell purification strategies followed by reconstitution in denuded tracheal grafts within immune incompetent hosts (Randell et al., 1991; Inayama et al., 1988). However, the pluripotent capacity of basal and secretory cells remains subject to general debate.

Current hypotheses suggest that the majority of submucosal gland development occurs in utero during development of the human airway. By definition this infers that a progenitor (or stem cell) exists during in utero development of the airway which retains the ability to differentiate into both the cellular components of the surface airway epithelium and submucosal glands. One potential model proposes that these gland progenitor cells have a transient existence during proximal airway development. Alternatively, these gland progenitor cells may also exist in the adult airway and play an important role in diseases such as cystic fibrosis, chronic bronchitis and asthma where submucosal gland hyperplasia and hypertrophy occur. In support of this alternative hypothesis is evidence for the extension of submucosal glands more distally in the airways of cystic fibrosis patients (Oppenheimer et al., 1975). This finding may result from the growth of new submucosal glands from surface airway epithelial progenitor cells (hyperplasia) or the expansion of existing glands within the interstitium (hypertrophy). In vivo models of induced de novo submucosal gland formation in the adult rat trachea (Lim et al., 1993) and adult hamster cheek pouch (Covent and Hardy, 1990) have been described and lend credibility to the concept of de novo gland development in the adult human airway. The lack of information regarding this process in the adult human airway is predominantly due to the lack of in vivo models and the limited extent of differentiation in vitro. For example, matrix embedded polarized epithelial cell cultures have demonstrated the ability of surface airway epithelial cells to invaginate in the early stages of gland development (Infeld et al., 1993). However, these studies are limited in their ability to address questions pertaining to gland progenitor cells by the lack of serous tubule, mucous tubule, and duct differentiation.

Several approaches have been extensively used to analyze progenitor-progeny relationships in model systems such as the brain, retina, and intestine. One such approach has included the use of recombinant retroviral marker genes to track the migration, proliferation, and differentiation of infected progenitors (for reviews see Price, 1987; Cepko, 1988; and Sanes, 1989). Such techniques have been applied in the retina (Fields-Berry et al., 1992; Turner and Cepko, 1987) and brain (Walsh and Cepko, 1990).

To better define the cell types in the surface airway epithelium that are involved in gland development, we have developed a human bronchial xenograft model system of the proximal airway which also demonstrates glandular reconstitution. Replication defective recombinant retroviruses were used to follow the development and morphogenesis of submucosal glands. This approach has led to the identification of a pluripotent progenitor cell within the airway epithelium which also retains the capacity for gland development. Furthermore, by studying the clonality of transgene expression within submucosal glands by three-dimensional reconstructions, we determined that more than one progenitor cell is involved in gland formation.

MATERIALS AND METHODS

Retroviral vectors

Three replication defective retroviral vectors (Fig. 1A) were used to produce amphotropic virus including: (1) LTR-*lacZ*, in which the retroviral LTR is responsible for *LacZ* transcription (also called BAG; Turner and Cepko, 1987), CMVβA-*lacZ,* which contains the cytomegalovirus (CMV) enhancer and chicken β-actin (BA) promoter driving the cytoplasmic *lacZ* reporter gene (Engelhardt et al., 1991), and LTR-Alkphos which contains the LTR promoter driving the cytoplasmic alkaline phosphatase reporter gene (Schreiber et al., 1993). Amphotropic virus was produced in the ψ crip packaging line as described previously (Danos and Mulligan, 1988) and used at a titer of 1×10^6 cfu/ml. The viral stocks used in this study were free of replication competent virus based on the previously described *lacZ* mobilization assay (Danos and Mulligan, 1988).

Retroviral infection of human bronchial epithelial cell primary cultures and generation of bronchial xenografts

Primary bronchial epithelial cells and human bronchial xenografts were generated as previously described (Engelhardt et al., 1992b) from human bronchial tissue acquired at the time of lung transplantation. Primary epithelial cells were cultured in hormonally defined F12 medium and infected 2, 3, and 4 days post-seeding with 5 mls of 16- to 18-hour conditioned retroviral producer medium in the presence of 2 μg/ml polybrene for 2 hours. Virus was passed through a 0.45 μm filter to remove cellular debris prior to infection of primaries. Following each retroviral infection the cells were washed twice with Hams F12 medium without serum or hormonal supplements prior to the addition of hormonally supplemented medium. On the fifth day the cells were trypsinized, neutralized with 10% FCS, and $0.5\text{-}1\times10^6$ cells placed in 25 μl of hormonally defined medium prior to seeding into denuded rat tracheas. Rat tracheas were obtained from 150 to 200 g Fisher rats and denuded by repeated freeze/thawing followed by rinsing with medium three times prior to seeding transduced epithelial cells. Grafts were allowed to regenerate for a period of 5-6 weeks prior to their excision for morphological and cytochemical analysis.

Analysis of clonality using differentially localized reporter transgenes

Clonal boundaries were defined using two independent histochemical markers, β-galactosidase and alkaline phosphatase. Human bronchial primary epithelial cells were infected with a 1:1 mixture containing 0.5×10^6 cfu/ml of LTR-*lacZ* and LTR-Alkphos retrovirus. Xenografts repopulated with *lacZ* and Alkphos-infected primary cells were histochemically stained *en bloc* in X-gal for 4 hours followed by staining for alkaline phosphatase for 30 minutes (Engelhardt et al., 1991;

Schreiber et al., 1993). Briefly, tissue was fixed in 0.5% glutaraldehyde in PBS for 10-15 minutes followed by rinsing in 1 mM $MgCl_2$/PBS two times for 15 minutes followed by staining for 4 hours in X-gal solution (Engelhardt et al., 1991). When alkaline phosphatase was colocalized, tissue was heat inactivated in 1 mM $MgCl_2$/PBS for 30 minutes at 65°C immediately following X-gal staining (Fields-Berry et al., 1992; Schreiber et al., 1993). Following heat inactivation, tissue was stained in 0.1 M Tris-HCl (pH 9.5) 0.1 M NaCl, 50 mM $MgCl_2$, 0.33 mg/ml nitroblue tetrazolium (NBT), 0.22 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 30 minutes at 37°C. Clonal expansion of retrovirally infected progenitor cells was determined by quantitating the number of *lacZ*, Alkphos-, and *lacZ*/Alkphos-expressing arrays from four independent xenograft experiments. The % error in assigning clonality was predicted based on a previously derived formula {% error in assignment = [(No. of bicolored arrays)/(2p(B)p(P)]/Total No. of arrays scored]} where p(B) and p(P) refer to the proportion of the primary cell inoculum which was infected with *lacZ* and Alkphos retrovirus, respectively (for derivation of this formula see Fields-Berry et al., 1992). Since primary cells were co-infected with equivalent titers of LTR-*lacZ* and LTR-Alkphos virus, p(B) and p(P) are both equal to 0.5. The percentage of primary cells infected with both retroviruses was confirmed by histochemical staining of primary cells prior to seeding in xenografts.

Analysis of clone phenotype

The phenotypic classification of surface airway epithelial clones was evaluated by morphological identification of cell types in GMA sections. Four independent tissue samples were used to generate eight xenografts for this analysis. In these experiments the β-galactosidase reporter gene was expressed from both the CMVβA-*lacZ* and LTR-*lacZ* retroviral constructs. Morphologic criteria used for the classification of transgene-expressing cell types in the surface airway epithelium included the identification of: (1) basal cells (defined as cuboidal cells residing on the basement membrane with no contact with the lumenal surface); (2) ciliated cells (defined as columnar cells with cilia); (3) goblet cells (defined as columnar cells with visible secretory granules); and (4) intermediate cells (defined as columnar cells residing in the lower half of the surface epithelium without lumenal contact or visible secretory granules). X-gal-expressing clones within the surface airway epithelium were evaluated for their constituent cell types by the above criteria in 4 μm GMA sections taken at 60 μm intervals. This analysis led to the quantification of approximately 50 cross sections per xenograft and a total of 1115 clones.

Surface airway epithelial clone size was analyzed to compare the relative proliferative capacity of various clone types. Because of the difficulty in judging overlapping cells in adjacent sections, we chose to quantitate the number of nuclei per cross-section of non-overlapping clones. For this analysis nuclei were quantitated for all clones contained in 4 μm GMA sections separated by 60 μm intervals. A total of 260 clones were analyzed from both LTR-*lacZ* and CMVβA-*lacZ* infected xenografts. Three independent xenografts generated from three different bronchial tissue samples were analyzed.

Submucosal gland reconstructions using serial 4 μm GMA sections were generated to assess the number of ducts which lead to the surface airway epithelium and the percentage of transgene reconstitution. Reconstructions of 51 submucosal glands by serial sections were evaluated from 12 independent xenografts generated from 6 bronchial tissue specimens. In these studies, which evaluated xenografts infected with either LTR-*lacZ* or CMVβA-*lacZ* retroviruses, 30 out of 51 glands partially expressed transgene. The clonal origin of glands was determined in the subset of glands, which contained only a single duct to the surface airway epithelium. To determine conclusively the number of progenitor cells that contribute to gland formation, transgene expression was evaluated in glands from xenografts co-infected with LTR-Alkphos and LTR-*lacZ* retroviruses. To achieve glands with both *lacZ* and Alkphos transgene expression it was necessary to achieve high levels (>75%) of infection in primary cells. This was accomplished by serial infection of cells that had been passaged once prior to infection. This culturing modification led to a more homogeneous and highly proliferative cell type.

Immunocytochemical localization of cytokeratins and serous cell specific proteins in human bronchial xenografts and primary cell preparations

Human bronchial xenografts were harvested at 5-6 weeks post-transplantation and divided into 3 mm rings. One half of the tissue was frozen in OCT embedding medium and evaluated for expression of epithelial specific markers including CK-14, lactoferrin, lysozyme and mucin. The remaining tissue was analyzed for histochemical expression of *lacZ* as described above. Immunocytochemical studies were performed on cytospun cells or frozen bronchial sections (6 μm) fixed in −20°C methanol for 10 minutes, blocked in 20% donkey serum/PBS, and incubated with primary antibody (monoclonal mouse anti-CK-14 hybridoma supernatant (gift from Dr C. S. Ramaekers), polyclonal rabbit anti-human lysozyme (BioGenex), polyclonal rabbit anti-human lactoferrin (BioGenex), and monoclonal mouse anti-human mucin (gift from Dr R. Wu) for 90 minutes. Sections were washed 3 times in 1.5% DS/PBS followed by incubation in 25 μg/ml of purified donkey FITC-labeled secondary antibody for 30 minutes in 1.5% DS/PBS. Sections were washed and mounted in Citifluor followed by fluorescent microscopic analysis.

RESULTS

Clonal expansion of transgene marked surface airway epithelial progenitor cells

We have previously demonstrated that primary human bronchial epithelial cells give rise to a fully differentiated bronchial epithelium when seeded onto denuded rat tracheas and transplanted subcutaneously in athymic mice (Engelhardt et al., 1992b,a). The cellular distribution of basal, intermediate, goblet, and ciliated cells within these xenografts is indistinguishable from that found in native human bronchus (Engelhardt et al., 1992a). In the present studies we have attempted to identify airway epithelial progenitor cells involved in submucosal gland development using traditional retroviral lineage analysis. Clonal expansion of retrovirally marked progenitor cells was confirmed using *lacZ* and Alkphos histochemical markers. These studies are based on the premise that clusters of transgene-expressing epithelial cells, which express a single histochemical marker, reflect the expansion of a single progenitor cell which was genetically tagged in vitro. When populations of primary cells were infected at levels of 5-10% with a mixture of CMVβA-*lacZ* and βA-Alkphos retrovirus, the resultant epithelium was composed predominantly of epithelial clusters of cells which expressed either Alkphos or *lacZ* transgenes (Fig. 1B,C). The predicted error in clonal assignment from four independent experiments averaged less than 2.0% (Table 1). These results demonstrate that following reconstitution of xenografts, transgene-expressing progenitor cells expand clonally within the xenograft epithelium without substantial cellular migration. Although care is taken during trypsinization of primary cells to achieve a single cell suspension, we cannot rule out that seeded clones are derived from smaller clumps of in vitro clonally expanded progenitor cells.

High levels of transgene reconstitution in xenografts (>75%) led to visibly adjoining clones (Fig. 1D-F). Although this level of transduction was not useful for clonal analysis in the surface

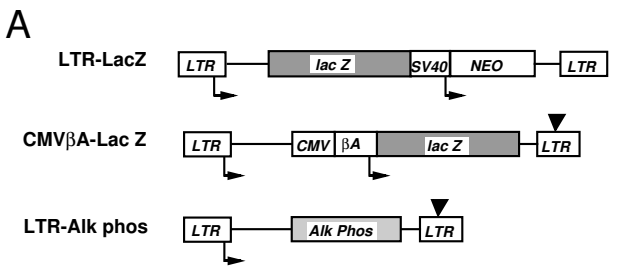

Fig. 1. Clonal expansion of transgene-expressing progenitor cells within surface airway epithelium and submucosal glands of xenografts. A schematic representation of the three retroviral vectors used in lineage analysis is shown in A. In clonal analysis experiments, primary bronchial epithelial cells were co-infected with either CMVβA-*lacZ*/LTR-Alkphos (B and C) or LTR-*lacZ*/LTR-Alkphos (D-G) retroviruses and seeded at a density of 1×10^6 cells per denuded rat trachea. Xenografts were harvested 5 weeks post transplantation and histochemically stained for β-galactosidase and alkaline phosphatase in frozen (6 μm) sections. Retroviral transduction at levels of approximately 5% were used to demonstrate the clonal expansion of CMVβA-*lacZ* (closed arrow) and LTR-Alkphos (open arrow) infected progenitor cells within the surface airway epithelium (B,C). C shows a high power photomicrograph of *lacZ* and Alkphos clones within the surface airway epithelium of the section in B. In contrast, the clonality of submucosal glands was assessed at higher levels of transduction greater than 90% (D-G). (D,F) High levels of transgene-expressing epithelium with adjacent *lacZ* (closed arrow) and Alkphos (open arrow) clones. The presence of both *lacZ*- (closed arrow) and Alkphos- (open arrow) expressing glandular epithelium in the sections in E, H and G demonstrates that multiple progenitor cells are involved in gland development. A higher power photomicrograph of the gland in H (adjacent section of the gland in E) better represents the differential staining patterns of Alkphos and *lacZ*. This gland shows a unique pattern of Alkphos expression within myoepithelium (open arrow) and *lacZ* expression within all cell types contained within the adjacent ductal region (closed arrow).

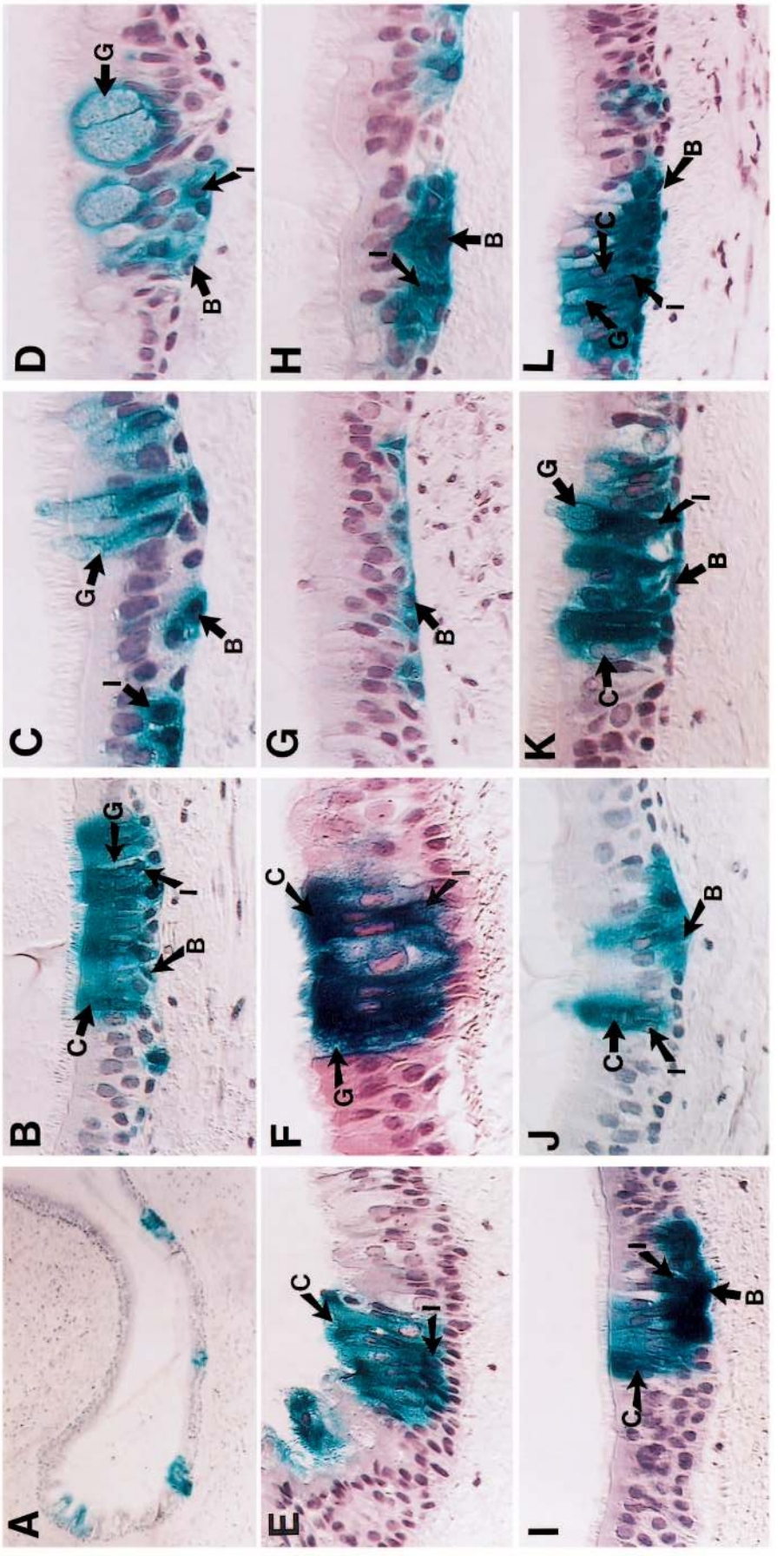

Fig. 2. Surface airway epithelial clone types in retrovirally infected human bronchial xenograft. Primary bronchial epithelial cells were infected with either LTR-*lacZ* or CMV/βA-*lacZ* recombinant retrovirus to give a final level of transduction between 5-10%. Grafts were harvested 6 weeks post-transplantation in athymic mice and histochemically stained *en bloc* in X-gal followed by embedding in GMA. Clonal analysis was performed on 4 μm GMA sections counterstained in hematoxylin. A shows a low power photomicrograph with 4 *lacZ*-expressing clones within the reconstituted xenograft epithelium. Examples of various clone types based on morphologic criteria of cellular composition included: (C,D) basal, intermediate, and goblet; (E) intermediate and ciliated; (F) intermediate, goblet, and ciliated; (G) basal alone; (H) basal and intermediate; (I,J) basal, intermediate, and ciliated; (B,K,L) basal, intermediate, goblet, and ciliated. The morphological identification of cell types was performed as described in the Methods and are marked: B, basal cell; I, intermediate cell; G, goblet cell; C, ciliated cell.

Table 1. Percent Error in Clonal Assignment

Xenograft Exp*	*lacZ* arrays	Alkphos arrays	*lacZ*&Alkphos arrays	% error in clonal assignment†
1	83	48	4	3.9%
2	28	22	0	0%
3	33	16	1	3.8%
4	6	13	0	0%

*Primary cells used for reconstituting xenografts were co-infected with a mixture of amphotropic producer supernatants containing 0.5×10^6 pfu/ml LTR-*lacZ* and 0.5×10^6 pfu/ml LTR-Alkphos retroviruses. The total percentage transgene reconstitution (*lacZ* + Alkphos) within xenograft epithelium varied from approximately 20% (Exp 1) to 5-10% (Exp 2-4). Approximately 1/2 to 1/4 of the xenograft was analyzed in each experiment.

†The percentage error in clonal assignment was determined as {[No. of *lacZ*&Alkphos Arrays]/[2*P*(B)*P*(P)]}/{Total No. of arrays scored} where *P*(B)=0.5 and *P*(P)=0.5. The average percentage error in clonal assignment from these four independent experiments was 2.0%.

airway epithelium, it did prove useful in determining the number of progenitor cells involved in gland development (discussed below). For this reason, in experiments concerning surface airway epithelial clone phenotypes, we chose a level of transduction in primaries (approx. 5-10 % of the epithelium) which led to visibly non-adjacent clones (Figs 1B, 2A).

Classification of airway epithelial progenitor cells

To gain insight into the potential heterogeneity of progenitor cells within the surface airway epithelium, *lacZ*-expressing clones were classified according to their cellular compositions. This analysis demonstrated that multiple progenitors exist within primary cultures of surface airway epithelial cells that have either limited or pluripotent capacity to differentiate into basal, intermediate, goblet, and ciliated cells (Table 2). Studies utilized two independent retroviral constructs with LTR and CMVβA promoters driving the *lacZ* gene. We found that CMVβA-*lacZ* gave the most intense and evenly distributed staining within all cell types of the epithelium. For this reason, the majority of analyses were performed using the CMVβA-*lacZ* construct. Eight xenografts generated from 4 independent primary bronchial tissue specimens were analyzed. Cellular identification of non-overlapping clones was based on the morphological criteria described in the Methods and assessed in serial 4 μm thick GMA sections separated by 60 μm. Table 2 depicts the percentage (mean ± s.e.m.) of each clone type from individual experiments analyzing a total of 1115 clones. The observed clone frequencies were as follows: (1) basal, intermediate, goblet and ciliated cells (43±2.4%); (2) basal, intermediate, and ciliated (26±3.0%); (3) intermediate, goblet, and ciliated (10±2.0%); (4) basal, intermediate and goblet (6.6±2.4%); (5) basal and intermediate (6.8±1.3%); (6) intermediate and ciliated (5.7±1.8%); and (7) basal cell alone (2.5±0.4%). Examples of these clone types are shown in Fig. 2. These findings suggest that multiple progenitor cells exist within the surface airway epithelium, each of which has defined capacities for differentiation. Furthermore, the two vectors (LTR-*lacZ* and CMVβA-*lacZ*) used in this analysis showed no consistent differences in the distribution of clone types observed (Table 2). However, the absence of less frequent clone types from some of the xenografts suggests that some level of variability may be present between different tissue specimens.

The proliferative capacity of various surface airway epithelial clones was evaluated by quantitating clone size. Our first approach attempted to quantitate the total number of cells in a given clone. However, in more densely packed clones it was difficult to assess what cells overlapped in serial section. Therefore, we chose to quantitate the average density of nuclei within cross-sections of non-overlapping clones. Fig. 3 demonstrates the distribution of clone sizes for the various clone phenotypes. The largest clone types contained basal, intermediate, goblet, and ciliated cell with an average of 21±10 nuclei/cross-section. These clones were statistically larger than all other clone types ($P<0.001$). In contrast, basal cell clones contained the fewest number (3±0.7) of nuclei per cross-section.

Submucosal gland development in human bronchial xenografts

In addition to their capacity to regenerate a fully differentiated surface airway epithelium, the isolated populations of surface airway epithelial progenitor cells also demonstrated the ability to develop submucosal glands. Morphological evaluation of xenograft submucosal glands demonstrated several identifiable characteristics of native bronchial submucosal glands

Table 2. Distribution of Clone Phenotypes

Vector*	Bronchial sample	B/I/G/C†	B/I/C	I/G/C	B/I/G	B/I	I/C	B	Total clones
CMVβA-*lacZ*	1	119 (43%)	61 (22%)	0 (0%)	52 (19%)	34 (13%)	0 (0%)	11 (4.0%)	277
CMVβA-*lacZ*	1	63 (43%)	35 (24%)	6 (4.1%)	20 (14%)	15 (10%)	2 (1.4%)	5 (3.4%)	76
CMVβA-*lacZ*	2	32 (42%)	28 (37%)	12 (16%)	0 (0%)	3 (4.0%)	0 (0%)	1 (1.3%)	106
CMVβA-*lacZ*	3	40 (38%)	38 (36%)	11 (10%)	2 (1.9%)	7 (7.0%)	6 (6.0%)	2 (1.9%)	118
CMVβA-*lacZ*	3	37 (31%)	36 (31%)	15 (13%)	3 (2.5%)	11 (9.3%)	11 (9.3%)	5 (4.2%)	141
CMVβA-*lacZ*	3	72 (51%)	23 (16%)	19 (14%)	4 (2.8%)	9 (6.4%)	12 (8.5%)	2 (1.4%)	164
LTR-*lacZ*	4	87 (53%)	37 (23%)	17 (10%)	7 (4.3%)	4 (2.4%)	10 (6.1%)	2 (1.2%)	87
LTR-*lacZ*	4	38 (44%)	13 (15%)	13 (15%)	7 (8.0%)	2 (2.3%)	12 (14%)	2 (2.3%)	146
Mean % ± s.e.m.‡	1,2,3,4	43% ±2.4	26% ±3.0	10% ±2.0	6.6% ±2.4	6.8% ±1.3	5.7% ±1.8	2.5% ±0.4%	1115

*The cellular composition of *lacZ*-expressing clones were analyzed from xenografts infected with either CMVβA-*lacZ* or LTR-*lacZ* retroviral vectors. Four independent bronchial tissue samples were used to generate a total of eight xenografts.

†Clones are grouped by the cell types they contain: B-basal; I-intermediate; G-goblet; and C-ciliated cells. Results show the absolute number of clones observed for each xenograft with the per cent shown in brackets.

‡The mean per cent distribution of each clone type ± s.e.m. summarize the findings from the 1115 clones analyzed.

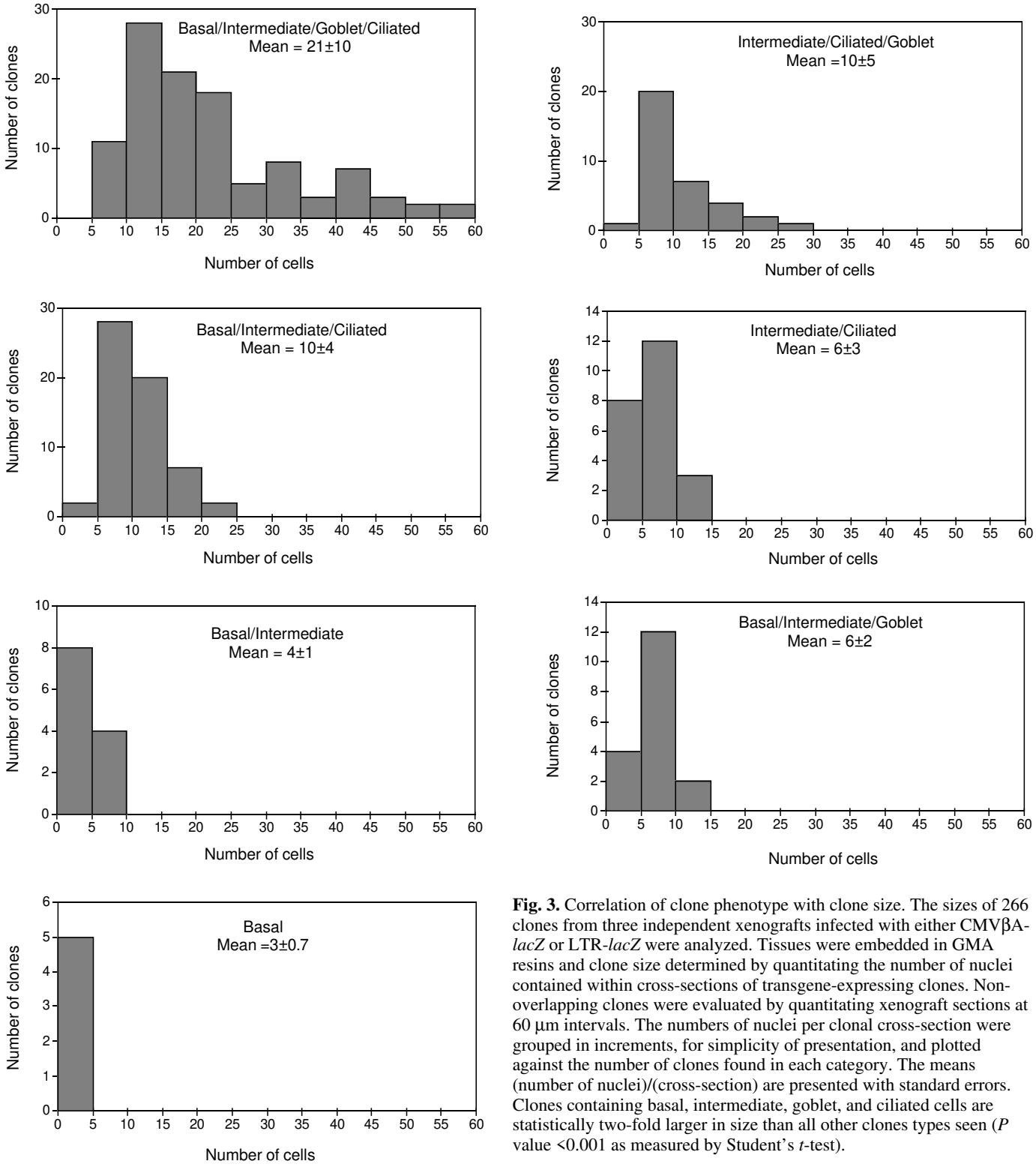

Fig. 3. Correlation of clone phenotype with clone size. The sizes of 266 clones from three independent xenografts infected with either CMVβA-*lacZ* or LTR-*lacZ* were analyzed. Tissues were embedded in GMA resins and clone size determined by quantitating the number of nuclei contained within cross-sections of transgene-expressing clones. Non-overlapping clones were evaluated by quantitating xenograft sections at 60 μm intervals. The numbers of nuclei per clonal cross-section were grouped in increments, for simplicity of presentation, and plotted against the number of clones found in each category. The means (number of nuclei)/(cross-section) are presented with standard errors. Clones containing basal, intermediate, goblet, and ciliated cells are statistically two-fold larger in size than all other clones types seen (P value <0.001 as measured by Student's t-test).

including ducts, mucous tubules, and serous tubules (Fig. 4A-D). Interestingly, previous studies using isolated cultures of rat tracheal epithelial cells seeded into xenografts showed no submucosal gland development (Engelhardt et al., 1991) and probably reflects the extremely low abundance of these structures in native rat tracheal airway. This finding suggests a species-specific predisposition of human surface airway epithelial cells to develop submucosal glands rather than an environmentally stimulated process related to host-graft interaction. To evaluate more closely the differentiated state of submucosal glands formed in human bronchial xenografts, we sought to localize several proteins known to be expressed

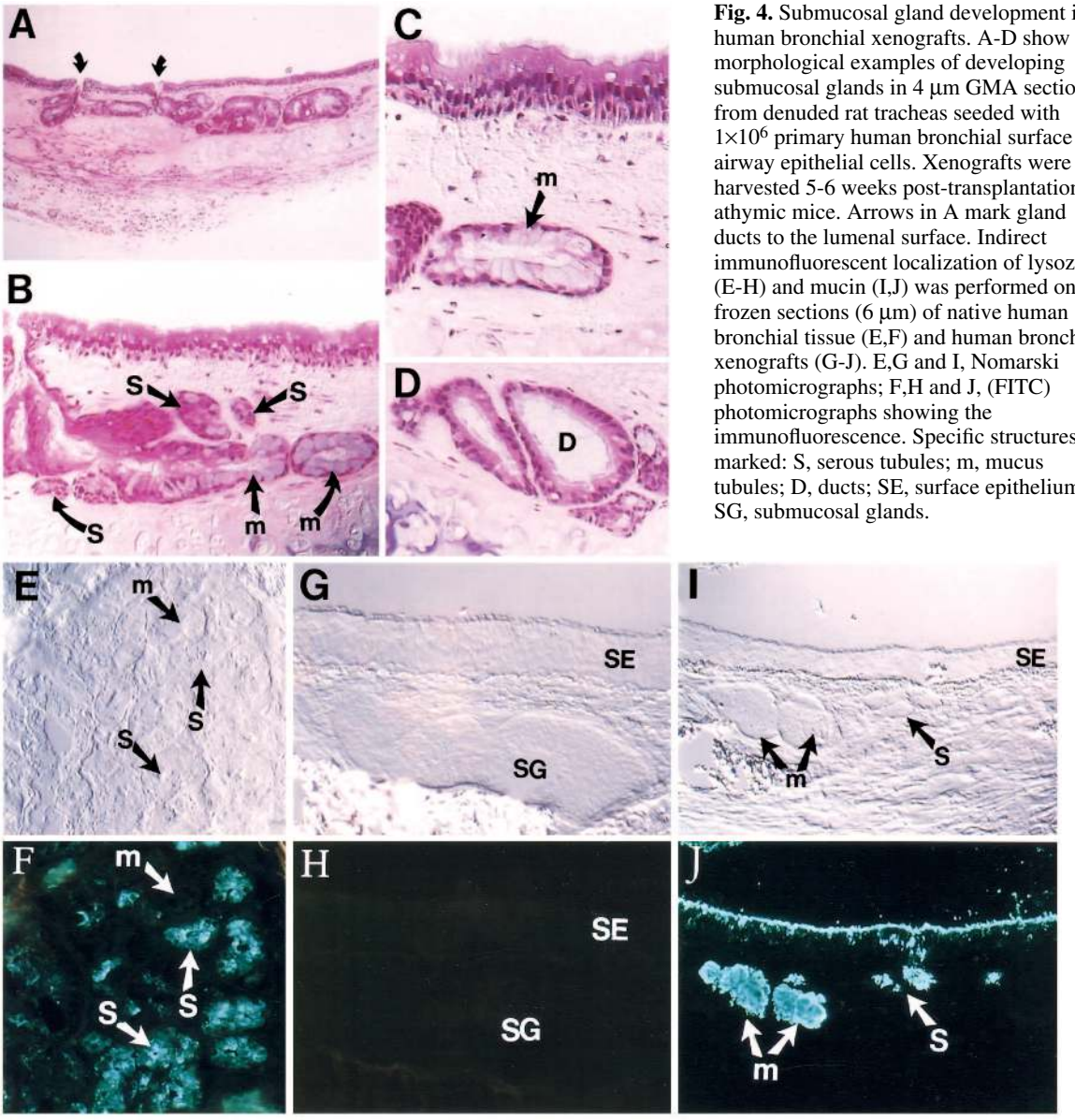

Fig. 4. Submucosal gland development in human bronchial xenografts. A-D show morphological examples of developing submucosal glands in 4 μm GMA sections from denuded rat tracheas seeded with 1×10^6 primary human bronchial surface airway epithelial cells. Xenografts were harvested 5-6 weeks post-transplantation in athymic mice. Arrows in A mark gland ducts to the lumenal surface. Indirect immunofluorescent localization of lysozyme (E-H) and mucin (I,J) was performed on frozen sections (6 μm) of native human bronchial tissue (E,F) and human bronchial xenografts (G-J). E,G and I, Nomarski photomicrographs; F,H and J, (FITC) photomicrographs showing the immunofluorescence. Specific structures are marked: S, serous tubules; m, mucus tubules; D, ducts; SE, surface epithelium; SG, submucosal glands.

within native bronchial submucosal glands (lysozyme, lactoferrin, mucin and cytokeratin-14) and compared the native pattern of localization to that found in xenografts.

Evaluation of the mucus secretory component of xenograft submucosal glands was confirmed by three criteria including: (1) identifiable lucent secretory granules under Nomarski optics; (2) immunoreactivity to mucus antibodies; and (3) alcian blue/PAS histochemical staining. The result of immunofluorescent localization with a polyclonal antibody generated against human mucin demonstrates compartmentalization of mucus secretory tubules (Fig. 4I,J). These studies confirm the morphological identification of mucus tubules in hematoxylin and eosin stained sections (Fig. 4B,C). Evaluation of xenograft submucosal glands for cytokeratin-14 expression demonstrated staining within myoepithelium and basal cells which was identical to that seen in the surface airway epithelium and submucosal glands of native human bronchial tissue (data not shown). Immunocytochemical evaluation of the serous cell markers lysozyme and lactoferrin in frozen sections demonstrated diminished or absent levels of staining in xenograft submucosal glands (lysozyme, Fig. 4G,H; lactoferrin, data not shown) as compared to native human bronchial serous cells (lysozyme, Fig. 4E,F; lactoferrin, data not shown). These findings suggest that this cellular component of submucosal glands may be derived from other progenitor cells than those harvested in our primary cultures of surface airway epithelial cells. Alternatively, the observation that in utero human submucosal gland development takes >10 weeks to achieve fully differentiated tubule functions (Tos, 1960) supports the hypothesis that xenograft glands may represent a less mature phenotype (i.e. less than 6 weeks).

Identification of submucosal gland progenitor cells

The finding that primary airway epithelial cells reconstitute both a differentiated airway epithelium and submucosal glands suggests that a common airway progenitor cell(s) may exist for both of these structures. Alternatively, the possibility remained that both airway epithelial and submucosal gland cells were

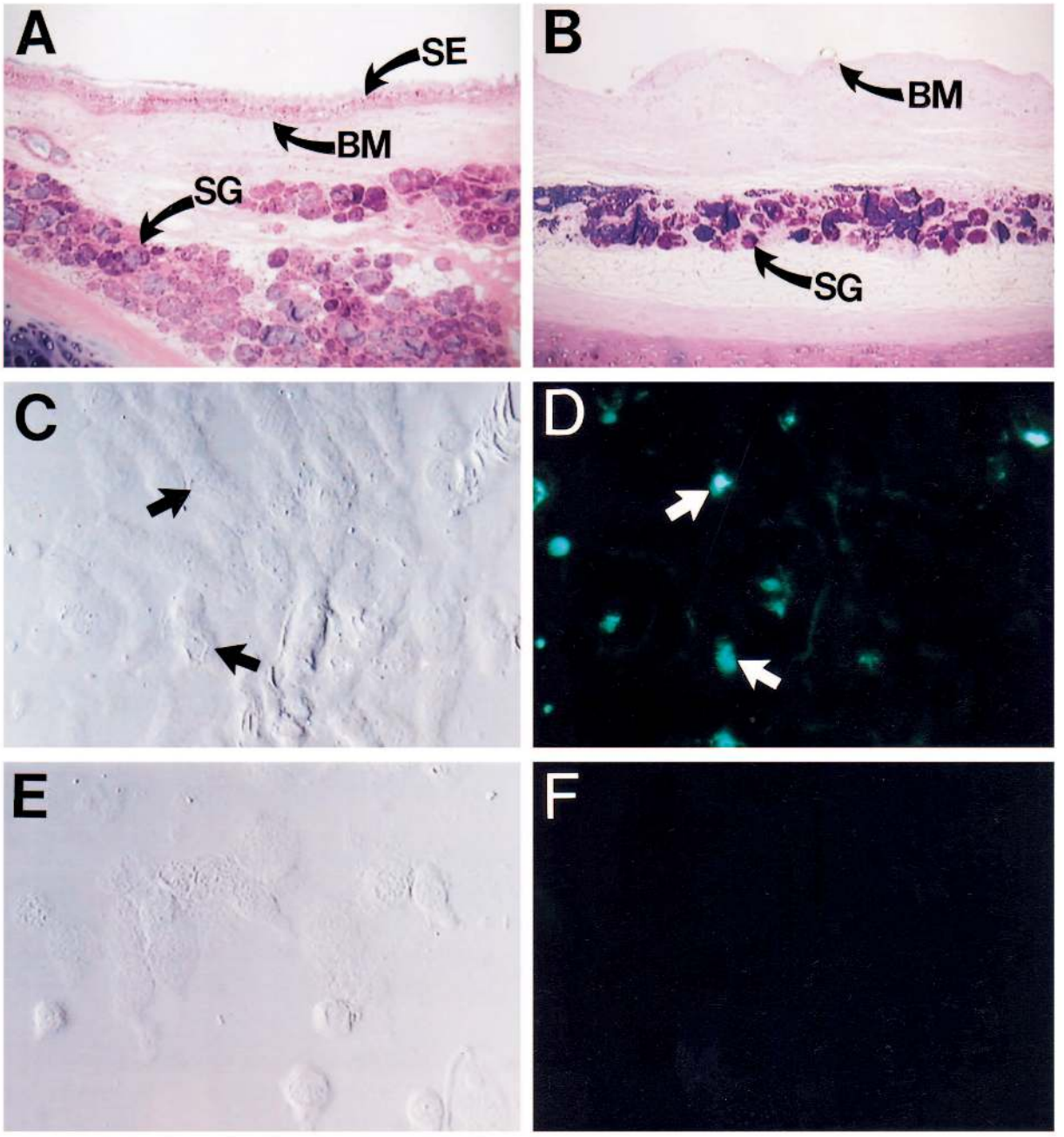

Fig. 5. Assessment of submucosal gland cell contamination within surface airway epithelial cell preparations. Human bronchial tissues were treated with protease XIV for 36 hours followed by harvesting of surface airway epithelial cells by agitation. Tissues were fixed prior to (A) and following surface airway epithelial cell harvesting by enzymatic digestion (B). Fixed tissues were processed for GMA sectioning (at 4 μm) and staining in alcian blue/PAS. Freshly isolated submucosal gland cells were harvested by collagenase/elastase treatment of surface epithelial-stripped bronchial tissue and cytospun on glass slides for indirect immunofluorescent localization of lysozyme (C,D). Freshly isolated surface airway epithelial cells were harvested by protease XIV treatment of bronchial tissue (E,F) and similarly stained for lysozyme protein. C and E, Nomarski photomicrographs; D and F, photomicrographs of fluorescent (FITC) anti-lysozyme staining. Arrows in C and D mark lysozyme-expressing primary gland serous cells. Specific structures are marked: SE, surface epithelium; BM, basement membrane; SG, submucosal glands.

isolated in our surface airway epithelial preparations. To address this concern we used three approaches to define the origin of progenitor cells in our primary airway epithelial preparations including: (1) the evaluation of submucosal gland integrity following harvesting of surface airway epithelial cells to assess contamination of submucosal gland cells in surface airway epithelial cell preparation; (2) the purity of surface airway epithelial cell preparation by analysis with specific markers for submucosal glands including lysozyme and lactoferrin, and (3) genetic marking of progenitor cells with recombinant retroviral vectors followed by analysis of clonal cellular compositions.

Studies evaluating the extent of submucosal gland cell contamination in surface bronchial epithelial cell preparations analyzed the extent of alcian blue/PAS-positive submucosal glands in sections of bronchial tissue before and after protease XIV digestion. These results demonstrate the integrity of submucosal gland structures following removal of surface airway epithelial cells (compare Fig. 5A with B). To evaluate further potentially low levels of submucosal gland contamination, we used a more sensitive assay which localized the extent of submucosal gland-specific markers (lysozyme and lactoferrin) within preparations of surface airway epithelial cells. In these studies, freshly isolated preparations of surface airway epithelial cells were cytospun onto glass slides and evaluated by immunocytochemistry for the extent of staining with antibodies specific for lysozyme and lactoferrin. As a control for the extent of lysozyme staining in freshly isolated primary cultures of epithelial cells, we also performed gland cell isolation by collagenase/elastase treatment of bronchial samples following removal of the surface airway epithelium by sharp dissection. Findings from these studies clearly demonstrate the absence of detectable lysozyme (Fig. 5E,F) and lactoferrin (data not shown) staining in isolated surface epithelial cell populations as compared to staining in submucosal gland serous cells of native human bronchus (Fig. 4F). In contrast, freshly isolated submucosal gland cells demonstrated considerable staining for lysozyme (Fig. 5C,D) and lactoferrin (data not shown), confirming that the absence of immunoreactivity in surface epithelial preparations was not a result of the cell isolation procedure. Together these findings suggest that the extent of submucosal gland cell contamination with preparations of surface airway epithelial cells likely represents a minor or absent component in our preparations.

To demonstrate conclusively the presence of progenitor cells within the surface airway epithelium which retain the capacity to differentiate into both airway epithelial and submucosal gland cell lineages, we used recombinant retroviral vectors to genetically mark progenitor cells. The number of progenitor cells involved in reconstituting the cellular components of xenograft submucosal glands was evaluated in xenografts infected with both alkaline phosphatase and β-galactosidase retroviral vectors. In these studies it was necessary to use significantly higher levels of transduction than that used for clonal analysis of the surface airway epithelium. Fig. 1D-F shows representative photomicrographs in which the surface epithelium is almost completely reconstituted with adjacent *lacZ*- and Alkphos-positive clones. At this level of transduction, glands could be found which express both types of transgenes. These results conclusively demonstrate that more than one progenitor cell is involved in gland development. As expected, at levels of transgene expression used for clone composition analysis in the airway epithelium (5-10%), glands showed only partial transgene reconstitution (Fig. 6B-F). Furthermore, two independent promoter elements (LTR and CMVβA) from three different retroviral vectors gave similar patterns of partial glandular transgene expression. These results suggest that the phenomenon of partial glandular transgene expression is due to the contribution of multiple gland progenitor cells rather than transcriptional inactivation.

Analysis of surface airway epithelial clones for which transgene expression extended into the submucosal glands (for example see Fig. 6D) gave insight into the progenitor cells involved in gland development. Of the 30 transgene-expressing glands analyzed by serial section, the majority of *lacZ*-expressing glands were associated with *lacZ*-expressing surface airway epithelium at the neck of the gland. All the surface airway epithelial clones associated with transgene-expressing gland ducts demonstrated a defined cellular composition composed of basal, intermediate, goblet, and ciliated cells (Figs 1G and 6D). These findings suggest that a specific subset of progenitor cells retains the capacity for both pluripotent airway differentiation and submucosal gland development.

Novel mechanisms of gland morphogenesis

To address further the finding that a population of transgene-expressing glands could be found without transgene-expressing surface airway epithelium at the gland duct (Fig. 6E), we followed the compartmentalization of transgene expression throughout glands in serial sections. We hypothesized that if multiple progenitor cells were involved in the initial stages of gland development de novo from the surface airway epithelium, then transgene- and non-transgene-expressing regions would expand clonally during gland morphogenesis. In these studies, xenografts were seeded with *lacZ* retrovirally tagged surface airway epithelial cells and the glands analyzed in 4 μm serial sections. Unexpectedly, these experiments demonstrated the presence of multiple ducts which led to the surface airway epithelium within a single gland (Figs 6E,G-I, 4A). Of 51 glands analyzed (both transgene and non-transgene containing glands) from 12 independent xenografts, 27% contained 2 or more ducts leading to the surface airway epithelium. The average gland size was 156±13 μm (n=51) on the longest axis. Furthermore, in those glands that expressed β-galactosidase (59%, n=51), transgene reconstitution was predominantly compartmentally localized and continuous with the extension of *lacZ*-expressing surface airway epithelial clones (Figs 6G-I, 7). Fig. 7 depicts representative serial photomicrographs of a gland partially reconstituted with *lacZ* transgene-labeled cells. In this example it can be seen that three ducts lead to the surface airway epithelium, one of which originates at a transgene-expressing clone at the lumenal surface (Fig. 7M, N). Fig. 8A depicts a schematic glandular reconstruction based on the serial sections from the gland presented in Fig. 7. Transgene expression within this gland was predominantly confined to the region of the gland continuous with transgene-expressing surface airway epithelium. This profile of clonal expansion within a gland which contains multiple ducts suggests that the origin of this gland may be derived from two or more independently formed glands. Such a hypothesis would predict that the epithelia of independently formed submucosal glands may communicate within the interstitium to join glandular lumens and may in part explain findings of partial transgene reconstitution within glandular components. An alternative mechanism by which glands communicate with the surface airway epithelium may also partially explain these results.

DISCUSSION

A subset of pluripotent surface airway progenitor cells retains the capacity to develop submucosal glands

The results presented in this report are an attempt to define the progenitors within the adult human bronchial surface airway

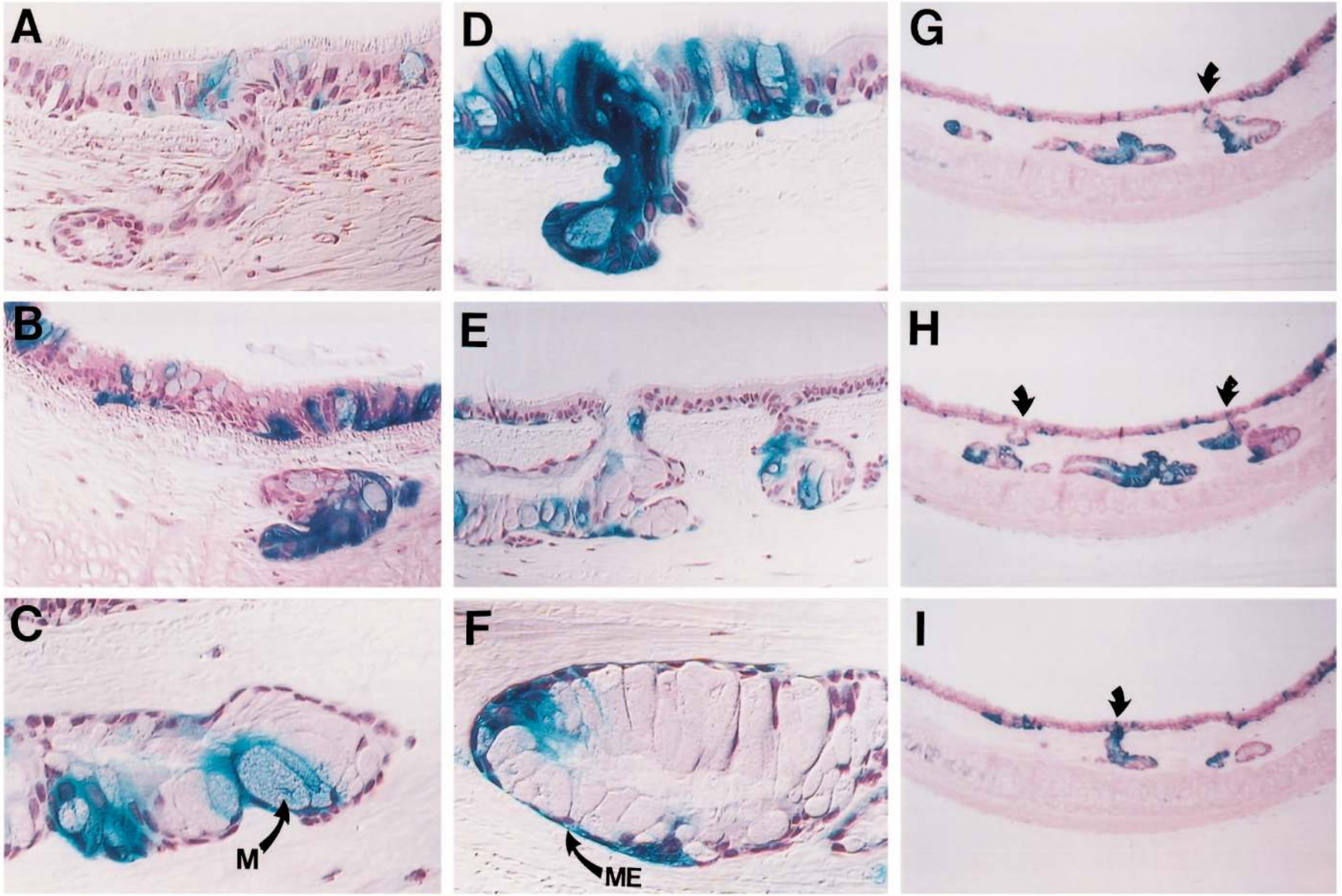

Fig. 6. Expansion of *lacZ* retrovirally marked gland progenitor cells within human bronchial xenografts. Xenografts were seeded with CMVβA-*lacZ* infected primary surface airway epithelial cells, harvested at 5 weeks post-transplantation in athymic mice, and histochemically stained *en bloc* with X-gal. Tissues were processed in GMA, sectioned at 4 μm, and counterstained with hematoxylin and/or eosin. A and D show examples of non-transgene and transgene-expressing gland ducts at the surface epithelium, respectively. Partial transgene reconstitution within gland cell types is shown in B-I. Note the cell type-specific differences in transgene expression seen within mucous cells (C) and myoepithelial cells (F) of gland mucous tubules which partially express transgene. Serial sections separated by approximately 30-40 μm from a single transgene-expressing gland with multiple ducts (marked by arrows) to the surface airway epithelium are shown in G-I (Note the clonality of transgene expression centered around the middle transgene-expressing duct). M, mucous cell; ME, myoepithelium.

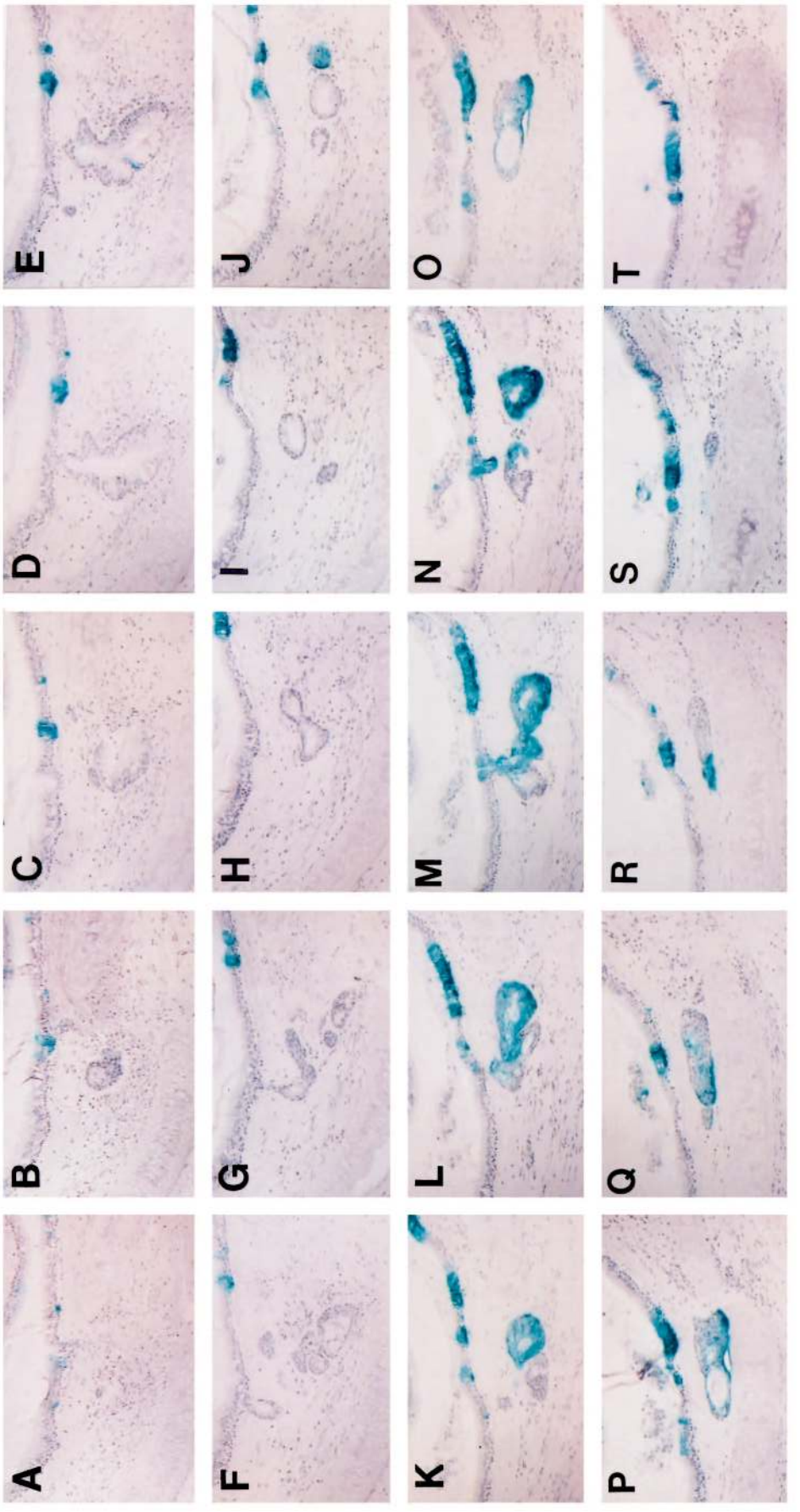

Fig. 7. Analysis of a transgene-expressing gland with multiple ducts to the surface airway epithelium by serial sections. This xenograft was reconstituted with CMVβA-*lacZ* infected primary bronchial epithelial cells and histochemically stained with X-gal at 5 weeks post-transplantation. 67 (4 μm) serial sections from this xenograft were used to reconstruct the morphological distribution of transgene expression in a gland with multiple ducts to the lumenal surface. Photomicrographs show serial sections (A through T) separated by approximately 12-16 μm, except for regions of gland ducts which were spaced every 4-8 μm to allow for more accurate assessment of these regions. Gland ducts leading to the surface airway epithelium are visible in D, F and M.

epithelium that retain a capacity for submucosal gland development. Lineage analysis using ex vivo retrovirally tagged progenitor cells was used to assess the cellular composition of clones within the surface airway epithelium which have the capacity to form submucosal glands. To confirm that clones within the surface airway epithelium represent the expansion of a single retroviral integration event, we analyzed the histochemical staining profile of expanded clones following co-infection with alkaline phosphatase and *lacZ* recombinant retroviruses. At levels of transduction between 5-10%, this analysis confirmed the clonality of expanded epithelial progenitors with a percentage error in clonal assignment of 2.0% (Fig. 1B,C, Table 1). This type of analysis was modeled on previous studies in the retina (Fields-Berry et al., 1992) and substantiates findings by others who performed morphometric quantification of clonality in the bronchial xenograft model system (Zepeda et al., 1995).

Phenotypic analysis of the cell types found within transgene-expressing clones has identified several populations of progenitor cells with either limited or pluripotent capacities to differentiate into the various cell types found in the proximal airway (Table 2). The most commonly seen clone type in all xenografts analyzed included those composed of basal, intermediate, goblet, and ciliated cells (43±2.4%). The second most common clone type was composed of basal, intermediate and ciliated cells (26±3.0%). Five other clone types based on the cellular composition of basal, intermediate, goblet, and ciliated cells were present at frequencies less than 10% (Table 2). Since the error in clonal assignment was <2.0%, small differences in clone percentages should be evaluated cautiously. This error in clonal assignment also suggests that less frequent cell restricted clones (i.e., those that contain only basal cells) may be more abundant than determined in this study due to overlap with more abundant unrestricted clones (i.e., those that contain basal, intermediate, goblet and ciliated cells). Evaluation of clone size demonstrated a considerable heterogeneity within the various clone phenotypes. The most common clone type (basal, intermediate, goblet, ciliated cell containing) was two-fold larger (ave size 21±10 nuclei/cross-section) than any of the other clones found within the epithelium. In contrast, clones which only contained basal cells were smallest in size (average size 3±0.7 nuclei/cross section). These findings demonstrate that certain clone phenotypes are associated with a high capacity to regenerate and substantiate similar studies evaluating pluripotent progenitor cells capable of reconstituting a significant proportion of xenograft surface epithelium (Zepeda et al., 1995).

To identify the airway progenitor cells that were involved in submucosal gland formation, we evaluated the surface epithelial clone phenotypes which demonstrated association with transgene-expressing glands at the lumenal ducts. These

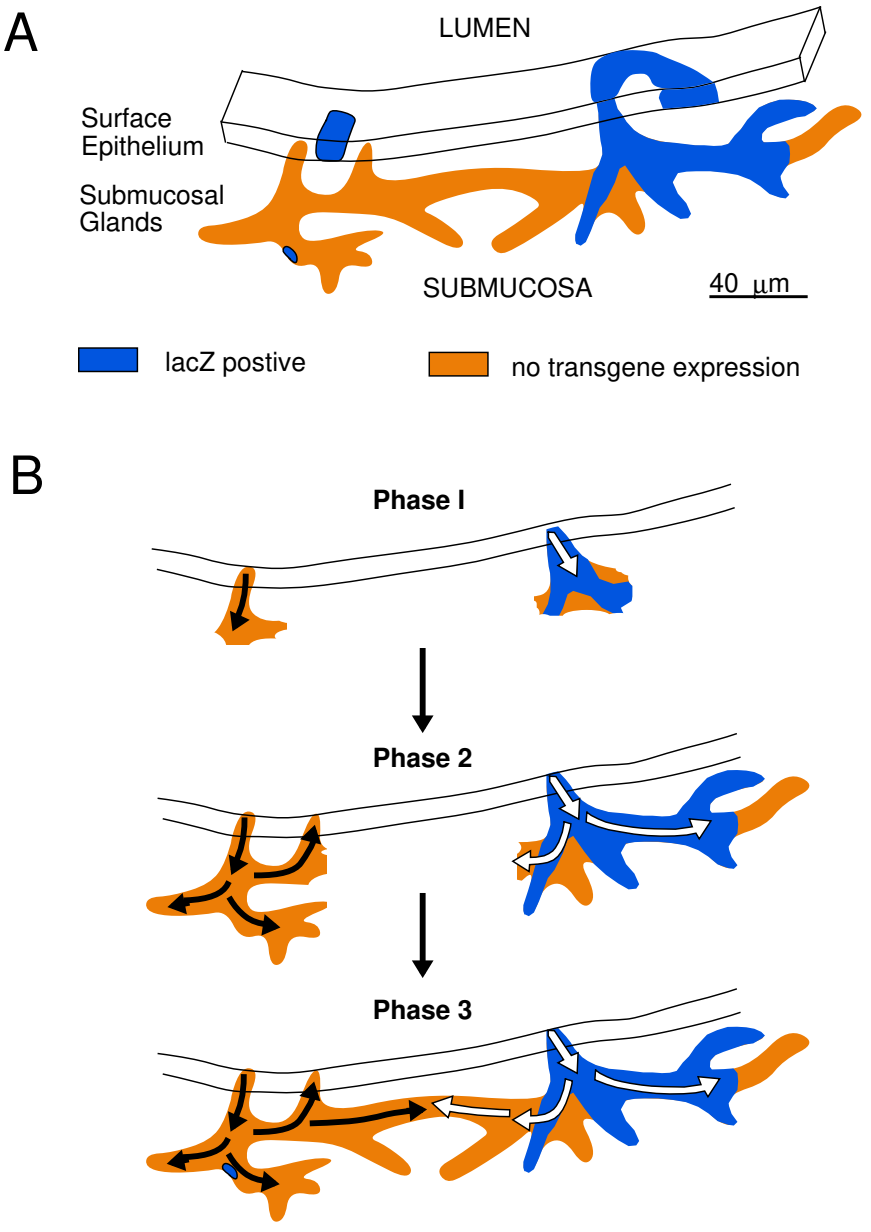

Fig. 8. Schematic reconstruction of submucosal glands by serial sections. The 67 serial sections analyzed in Fig. 7 were used to reconstruct a schematic representation of gland tubules and clonal transgene expression (A). Note the presence of several *lacZ*-expressing cells not contiguous with the bulk of the transgene-expressing gland clone which is likely due to cellular migration. Three potential phases of gland development and morphogenesis are proposed in panel B and are based on the pattern of transgene expression. These include: phase 1 – the formation of two initial glands, only one of which expresses transgene; phase 2 – lateral expansion of glands within the submucosa and communication of non-transgene-expressing glandular epithelium with the surface airway epithelium to form a new duct; phase 3 – lateral expansion of glands within the submucosa followed by lumenal joining of the two independently formed glands to generate one gland with multiple ducts to the surface airway epithelium.

findings demonstrate that all glands form from expansion of surface airway epithelial clones containing basal, intermediate, goblet, and ciliated cells. Three characteristics of this progenitor cell are consistent with an identified stem cell including: (1) pluripotent capacity for surface airway epithelium differentiation; (2) ability to form submucosal glands, and (3) highest regenerative capacity of clone types evaluated. Of interest was the unusually high susceptibility of this gland progenitor to retroviral infection (59% of glands analyzed expressed some level of transgene), as compared to the surface airway epithelium (5-10% of epithelial cells expressed transgene). This may reflect the high regenerative capacity of this progenitor cell in culture and supports the notion of this cell type as a pluripotent progenitor and potential stem cell in the airway.

Multiple progenitor cells contribute to de novo formation of submucosal glands from the surface airway epithelium

Using xenografts infected with both *lacZ* and Alkphos retroviruses we sought to assess the number of progenitor cells involved in submucosal gland development. These findings, which demonstrate simultaneous *lacZ* and Alkphos expression in glands with a single duct to the surface airway epithelium, confirm that multiple progenitor cells are involved in gland development (Fig. 1E,H,G). In support of this finding, experiments utilizing a single *lacZ* retrovirus demonstrated a consistent finding of partial transgene expression in the glands. Together, these findings demonstrate that at least two progenitor cells interact to form submucosal glands in vivo. Such a mechanism may be of general significance during in utero gland development or may represent a specific functional capacity of adult airway progenitor cells in these processes. Of interest is the phenotype diversity of transgene-expressing clones within glandular epithelium (Figs 1H, 6C,F). In these examples it is apparent that myoepithelial restricted and mucous containing clones can exist within the same gland. Since all glands cells are derived from two or more presumably identical surface airway epithelial cells, these data suggest that each progenitor may take on a defined capacity for differentiation once gland morphogenesis has been initiated. Alternatively, local environmental cues may influence the fate of gland cell phenotype.

Lineage model of surface airway and submucosal gland epithelial differentiation

Previous models of airway progenitors in the rat have proposed that both basal and goblet cells have the capacity for pluripotent differentiation (Randell et al., 1992). This model suggests that basal and goblet cells dedifferentiate to a common progenitor capable of differentiation into basal (B), goblet (G), intermediate (I) and ciliated (C) cells. Results from the present study would suggest an added level of complexity in airway progenitor-progeny relationships by defining multiple progenitor cells with either limited or pluripotent capacity for differentiation. One potential model to explain our findings is presented in Fig. 9 and suggests that all cells in the airway are derived from a common stem cell B_1. This model takes into account the fact that certain clone types were never found within reconstituted xenograft epithelium (Table 2). Specifically the following categories of clone types were never observed: (1) I-, G-, C-only clones; (2) two cell clones containing goblet cells, and (3) three cell clones composed of B/G/C cells. This observation helps to restrict the possible progenitor types in the cell inoculum. The model proposes that B_1 stem cells have the capacity to differentiate into both B_2 cells and submucosal glands. B_2 cells in turn can divide to form two types of intermediate cells: I_1 cells (which can divide to reproduce itself and ciliated cells) and I_2 cells (which can only divide to produce two goblet cells). This model also proposes that only basal cells (B_1 and B_2) and intermediate (I_1) cells can survive the transplantation into xenograft epithelium or alternatively are the only cells that can divide in culture to produce effective retroviral integration. Although these studies do not discount the possibility that goblet cells divide within the xenograft epithelium, it does support our findings that in this model system these cells do not undergo extensive self renewal. Furthermore, it is likely that environmental effects also influence clone phenotypes by restricting the extent of differentiation of B_2 in the formation of B/I/C, I/G/C and B/I/G clones (Table 2). The pathway for submucosal gland development in this model proposes that two different pluripotent progenitors (B_1 and B_2) must interact in the formation of glands. This hypothesis is based on two findings including: (1) only fully differentiated surface airway epithelial clones were associated with transgene-expressing gland ducts and (2) more than one pluripotent progenitor cell must give rise to glands (as evident by clonal experiments in lacZ/Alkphos co-infected xenografts). Based on the pluripotent phenotype of gland associated surface airway epithelial clones, this model hypothesizes that B_2 cells must be activated to divide into I_1 and I_2 to initiate the process of gland formation. Together with the fact that B/I/G/C containing clones have the largest capacity for regeneration, this model is consistent with the identification of a stem cell (B_1) in the airway with pluripotent capacity for surface airway epithelial and submucosal gland development.

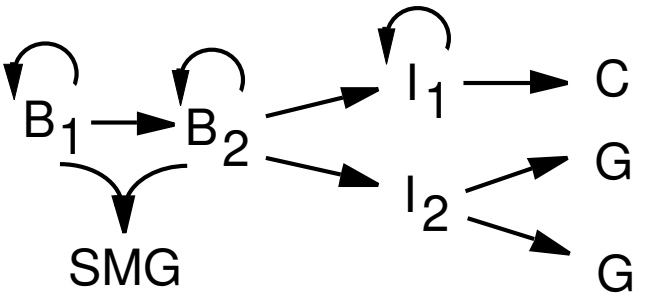

Fig. 9. Lineage model of airway epithelial differentiation. This model attempts to incorporate two findings from this study: (1) only certain cell types (B_1, B_2, and I_1 marked by circular arrows) have the capacity to divide in culture and hence integrate retroviral vectors, and (2) the absence of I-, G-, C-only, I/G, and B/G/C clones from reconstituted xenografts. Results from clonality experiments with *lacZ*/Alkphos co-infected xenografts have demonstrated that submucosal glands originate from more than one pluripotent progenitor cell. We hypothesize that gland development occurs as a result of an interaction between B_1 and B_2. This interaction may involve an activation of B_2 to divide and differentiate into I_1 and I_2, as suggested by the association of transgene-expressing gland ducts with only fully differentiated clones containing B/I/G/C cells. B, basal cells; I, intermediate cells; G, goblet cells; C, ciliated cells; and SMG, submucosal glands.

Future studies addressing the interaction of B_1 and B_2 cells may aid in the elucidation of signals necessary for gland formation in the airway.

Bronchial submucosal glands communicate within the interstitium in a novel mechanism of gland morphogenesis

In an attempt to define further the mechanisms that lead to partial transgene reconstitution within submucosal glands, we performed three dimensional reconstructions of transgene-expressing glands by serial sections. Results from these studies demonstrated two prominent findings: (1) transgene expression was predominantly compartmentally localized within partially transduced glands, and (2) approximately 20% of glands that partially expressed *lacZ* transgene contained multiple ducts leading to the surface airway epithelium. This latter observation suggests a novel mechanism for partial clonality of submucosal glands in which independently developed submucosal glands communicate within the interstitium to join lumens. Additionally, these results could be explained by submucosal gland reassociation with the surface airway epithelium. A schematic representation of the potential mechanisms involved in morphogenesis can be derived from the clonal patterns of gland transgene expression (Fig. 8B). Traditional models of in utero submucosal gland morphogenesis are proposed as unilateral processes in which newly formed glands retain only a single ductal access to the lumenal surface (Thurlbeck et al., 1961; Tos, 1960, 1968). Our findings suggest an alternative mechanism(s) by which surface airway epithelium and submucosal glands communicate through an interactive process of gland morphogenesis. Such mechanisms may be specific to gland development within the adult airway epithelium (i.e. gland hyperplasia) or may also exist in utero.

Possible implications for the pathological development of hypersecretory diseases

Submucosal gland hypertrophy has been shown to play an important role in the progression of hypersecretory diseases such as chronic bronchitis and cystic fibrosis. These processes involve the expansion of existing glands within the submucosa, which are associated with dynamic changes in the cellular architecture of the glands and increased numbers of mucus secretory cells (Boat et al., 1989; Oppenheimer and Esterly, 1975). These effects caused by environmental stimuli are thought to be the primary cause of increased mucus production from the glands in the hypersecretory state (Boat et al., 1989). Additionally, submucosal gland hyperplasia (the formation of new glands de novo from the surface airway epithelium) has been proposed to play a role in the pathophysiological progression of these diseases. In support of this notion, the presence of glands more distally located, in the non-cartilaginous regions of the airways, occurs in cystic fibrosis (Oppenheimer and Esterly, 1975). The mechanisms by which submucosal gland hyperplasia occur are largely unknown but may represent an induction of airway progenitor cell migration into the interstitium following signals from the surrounding pathological microenvironment. These studies have begun to evaluate the programmed capacity for submucosal gland development within a subset of adult surface airway epithelial progenitor cells. Our findings, which demonstrate the interactive joining of glands within the submucosa, suggest new mechanisms in gland morphogenesis. Insights into these mechanisms will enhance our understanding of the pathophysiologic progression of hypersecretory disease states and the development of strategies for preventing hypersecretory responses in chronic bronchitis, asthma and cystic fibrosis.

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