

## Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice

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**ABSTRACT** Murine lung epithelial (MLE) cell lines representing the distal bronchiolar and alveolar epithelium were produced from lung tumors generated in transgenic mice harboring the viral oncogene simian virus 40 (SV40) large tumor antigen under transcriptional control of a promoter region from the human surfactant protein C (SP-C) gene. The cell lines exhibited rapid growth, lack of contact inhibition, and an epithelial cell morphology for 30–40 passages in culture. Microvilli, cytoplasmic multivesicular bodies, and multilamellar inclusion bodies (morphologic characteristics of alveolar type II cells) were detected in some of the MLE cell lines by electron microscopic analysis. The MLE cells also maintained functional characteristics of distal respiratory epithelial cells including the expression of surfactant proteins and mRNAs and the ability to secrete phospholipids. Expression of the exogenous SV40 large tumor antigen gene was detected in all of the generated cell lines. The SP-C/SV40 large tumor antigen transgenic mice and the MLE cell lines will be useful for the study of pulmonary surfactant production and regulation as well as lung development and tumorigenesis.

The respiratory epithelium in the mammalian lung is composed of a variety of cell types with distinct morphologic and biochemical characteristics. Nonciliated bronchiolar (Clara) cells and alveolar type I and type II cells are the predominant epithelial cell types in the bronchioalveolar region of the lung. Clara cells and type II cells produce pulmonary surfactant, a complex mixture of phospholipids (PLs) and proteins that is critical for reducing surface tension at the air–liquid interface of the distal lung to prevent alveolar collapse upon expiration. Deficiency of pulmonary surfactant in premature infants results in respiratory distress syndrome, and a reduction of surfactant components is associated with other pathological conditions including adult respiratory distress syndrome (1). Phosphatidylcholine is the predominant PL in pulmonary surfactant and is secreted into the alveolus by type II epithelial cells (for review, see ref. 2). Specific surfactant proteins (SPs), designated SP-A, SP-B, and SP-C, are closely associated with surfactant lipids and function in the organization, spreading, and stability of PL membranes (for review, see ref. 2). Study of the regulation of SP synthesis and PL secretion has been hampered by the loss of differentiated characteristics of distal respiratory epithelial cells after their isolation and primary culture. For example, type II epithelial cells lose apical microvilli (3), lamellar body inclusions (4), production of PLs (4), and synthesis of SPs (5) in culture.

Clara cells and type II epithelial cells also serve as progenitor cells of the distal respiratory epithelium in the adult lung (6, 7) and are believed to be the cell of origin for

pulmonary adenocarcinomas, a subtype of non-small-cell lung cancer (8). We have previously produced transgenic mice harboring the simian virus 40 (SV40) large tumor antigen (TAG) under the transcriptional control of regulatory sequences derived from the human SP-C promoter region to study the development of pulmonary adenocarcinomas *in vivo* (9). Transgenic mice bearing the exogenous SP-C/TAG chimeric gene developed pulmonary tumors within 4–6 months of age. The presence of microvilli and lamellar bodies by electron microscopy, as well as the presence of respiratory epithelial cell markers by *in situ* hybridization, were consistent with the identification of the tumor cells as both bronchiolar and alveolar subtypes *in vivo*. While cells of the proximal respiratory epithelium and lung epithelial cells of fetal origin (10) have been established in culture, distal respiratory epithelial cell lines that maintain a differentiated phenotype in culture have not been previously produced. In the current study, we describe the production and characterization of distal respiratory epithelial cell lines derived from the SP-C/TAG transgenic mice.

### METHODS

**SP-C/TAG Transgenic Mice.** The origins and characteristics of the SP-C/TAG transgenic mice have been described in detail (9). The chimeric transgene contains 3.7 kb of the 5' promoter region from the human SP-C gene fused to SV40 early region sequences encoding the TAG and small tumor antigen.

**Isolation of Cells and Culture Techniques.** Lung tumors were resected from two F<sub>3</sub> generation SP-C/TAG transgenic mice at ≈5 months of age. The excised tumors and adjacent tissue were minced and washed with medium. Pooled washes and remaining tumor pieces were plated separately in T-25 tissue culture flasks in HITES medium (11) modified with transferrin (10 μg/ml), L-glutamine (original concentration plus 2 mM), and HEPES (10 mM), or ACL-4 medium (12). The medium was supplemented with 2% or 5% fetal bovine serum or used without addition of serum. Epithelial cells formed colonies with a three-dimensional configuration within 1–2 weeks. Cell aggregates from the apical surfaces of the colonies were released into the medium by tapping the flasks and were used to seed another flask. Cells were passaged two or three times in this manner to separate epithelial cells from fibroblasts. Cell populations were maintained separately as "mixed" epithelial cells and given an identification number. All cells were maintained in HITES medium supplemented with 2% fetal bovine serum and the antibiotics penicillin G (100 units/ml) and streptomycin (100 μg/ml) after the initial passages in culture. Clonal cell lines were derived by limiting

dilution cloning and designated as passage 1 at the time of cloning. Population doubling times were determined by plating the cells at two cell densities and obtaining cell counts at 24-hr intervals with a hemocytometer.

**Electron Microscopy.** Lung tissue from SP-C/TAg transgenic mice and murine lung epithelial (MLE) cells were processed and analyzed as described (13).

**RNA Extraction and Northern Blot Analysis.** Total RNA was isolated and Northern blot analysis was performed as described using Hybond-N membranes and hybridizing and washing conditions suggested by the manufacturer (Amersham) (9). Filters were sequentially hybridized with the following probes and stripped between probings if necessary: a murine SP-A cDNA 3' PCR fragment (14), a murine SP-B cDNA (clone 3.1a) (15), the murine SP-C cDNA (16), the SV40 TAg genomic region (9), and the human cytoplasmic  $\beta$ -actin cDNA (17).

**Immunoprecipitation and Isolation of SPs.** Cells were metabolically labeled for 30 min, immunoprecipitated with polyclonal antisera to SP-B or SP-C (R4595) (18), and subjected to SDS/PAGE in the presence of 2-mercaptoethanol (18). The SP-B antiserum (R28031) was generated in rabbits injected with purified SP-B active peptide isolated from bovine lung lavage (19). Mature SP-B and SP-C were isolated from MLE cells by extraction in methanol/chloroform/5 mM  $\text{CaCl}_2$  (1:2:0.4) (20), dialysis in acidified methanol/chloroform (1:1), separation on a 40- to 63- $\mu\text{m}$  Bio-Sil C<sub>8</sub> silica column (Bio-Rad), and elution with acidified methanol/chloroform (1:1) (procedure developed by John E. Baatz, Medical University of South Carolina). Proteins were immunoblotted with rabbit polyclonal antiserum R559 (21).

**PL Secretion and [<sup>3</sup>H]Choline Incorporation into PL Classes.** Secretion of PLs by cultured cells was assessed as described (22). The cells were incubated for 3 hr in forskolin (5  $\mu\text{M}$ ), ATP (10  $\mu\text{M}$ ), phorbol 12-myristate 13-acetate (PMA) (0.1  $\mu\text{M}$ ), Con A (10  $\mu\text{g}/\text{ml}$ ), and/or SP-A (1  $\mu\text{g}/\text{ml}$ ) isolated from rat lavage (23). Lactate dehydrogenase activity was determined in each sample to measure cytotoxicity (22).

To analyze incorporation of [<sup>3</sup>H]choline into PL classes, lipids were extracted from MLE-12 cells or primary isolates of rat type II cells (22) after addition of 50  $\mu\text{g}$  each of phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin as carriers. The lipid phase was dried, resuspended in 50  $\mu\text{l}$  of chloroform, spotted on silica gel plates (no. 06-600C; Fisher), and analyzed as described (24).

**Tumorigenicity Assays in Nude Mice.** Cells were injected subcutaneously into the flank of homozygous BALB/c *nu/nu* mice (Charles River Breeding Laboratories) at a concentration of  $3\text{--}5 \times 10^6$  cells per 0.2–0.4 ml of RPMI 1640 medium. Mice were monitored for visual and palpable tumors. Each cell population was tested in both flanks of two separate mice.

## RESULTS

**Production of Lung Epithelial Cell Lines.** Lung tumor tissue from two SP-C/TAg transgenic mice was dispersed and placed in culture. Mixed epithelial cell populations and 29 clonal cell lines were obtained from the explanted tissue. The lung epithelial cell lines have been designated MLE followed by an identification number (e.g., MLE-7). The cell lines described in the current study represent a mixed cell population (MLE-7) and two clonal cell lines (MLE-12 and MLE-15), which have been maintained for 30–40 passages in culture. Population doubling times of 7–9 hr were determined for MLE-7 (passage 16) and MLE-12 (passage 12) cell lines.

**Morphological Analysis of Tumors and MLE Cells.** Light microscopic analysis of lung tumors from a SP-C/TAg transgenic mouse at  $\approx 4$  months of age demonstrated lepidic, papillary, and solid adenocarcinomas, consistent with previ-

ous studies of this mouse line (9). At the ultrastructural level, the tumors consisted of a heterogeneous population of cells. Lamellar bodies, characteristic of type II alveolar cells, were apparent in some of the tumor cells (Fig. 1). Other regions of tumor contained cells with prominent, pleomorphic nuclei and were devoid of ultrastructural characteristics associated with differentiated distal respiratory epithelial cells. Heterogeneity of tumor cells was seen both among tumors and within an individual tumor.

The MLE cell lines maintained a typical polygonal epithelial cell morphology by light microscopy at all passages in culture. At the ultrastructural level, microvilli and cytoplasmic multivesicular bodies were detected in MLE-7 (passages 17 and 18), MLE-12 (passage 15), and MLE-15 (passage 17) cells (Fig. 1; data not shown). In addition, multilamellar inclusion bodies were detected in a subset of cells from the MLE-15 cell line (Fig. 1).

**Synthesis of Lung Epithelial Cell-Specific mRNAs and Proteins.** SP-A, SP-B, and SP-C mRNAs were detected in the MLE cells by Northern blot analysis (Fig. 2) and varied among the cell populations examined. MLE-7 cells maintained expression of SP-A, SP-B, and SP-C mRNAs at all passages examined. SP-B and SP-C mRNA were detected in the MLE-15 and MLE-12 cell lines although at different levels. SP-A mRNA was not detected in the MLE-12 cell line but was detected at low levels in the MLE-15 cell line. The SP mRNAs detected in the cell lines comigrated with the corresponding mRNAs in the mouse lung, verifying the

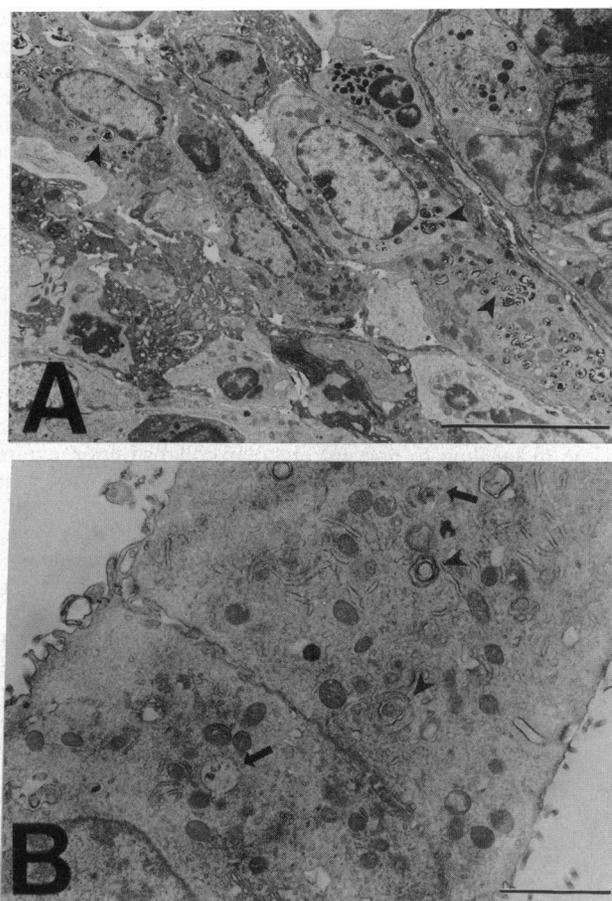


FIG. 1. Ultrastructural analysis of tumors from SP-C/TAg transgenic mice and MLE cell lines. (A) Region of a lung tumor *in vivo*. Cells containing lamellar bodies (arrowheads) were apparent. (Bar = 10  $\mu\text{m}$ .) (B) Cellular detail of the MLE-15 cell line at passage 17 in culture. Microvilli, multivesicular bodies (arrows), and multilamellar inclusion bodies (arrowheads) were detected. (Bar = 2  $\mu\text{m}$ .)

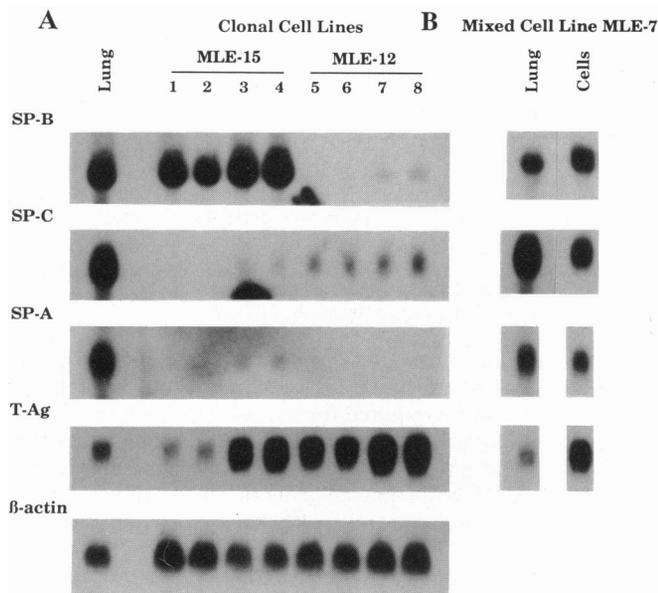


FIG. 2. Expression of cell-specific mRNAs in MLE cells. Total cellular RNA (15 or 20  $\mu$ g) was isolated and Northern blot analysis was performed. (A) Total lung RNA from a SP-C/TA9 transgenic mouse (lung) and total cellular RNA from MLE-15 cells at passage 8 (lanes 1 and 2) or passage 25 (lanes 3 and 4) in culture and MLE-12 cells at passage 10 (lanes 5 and 6) or passage 25 (lanes 7 and 8) in culture. The signal for SP-B mRNA in lanes 5 and 6 was more apparent after longer exposure periods.  $\beta$ -Actin mRNA was used as a control for equal loading of lanes. (B) Total lung RNA from a SP-C/TA9 transgenic mouse (lung) and total cellular RNA from MLE-7 cells. Results are representative of analysis of cells at passages 13, 16, and 23 in culture.

identity of the mRNAs and supporting the conclusion that the genes were correctly transcribed and the mRNAs were correctly processed by the MLE cells. CC10 mRNA, a nonciliated bronchiolar (Clara) cell marker, was detected in a mixed MLE cell population at an early passage in culture in the presence of 50 nM dexamethasone (data not shown). Despite this, CC10 mRNA was not detected in any of the established mixed or clonal cell populations grown in the presence or absence of dexamethasone at later passages in culture (data not shown). SV40 TAG mRNA was detected in every mixed and clonal MLE cell line (Fig. 2; data not shown). Moreover, SV40 TAG mRNA appeared to be more abundant in the clonal cell lines at the higher passages in

culture than the corresponding cell populations at lower passages (Fig. 2).

ProSP-B, migrating at  $\approx$ 40 kDa, was readily detected in the MLE-7 and MLE-15 cells and at a lower level in the MLE-12 cell line (Fig. 3). Two isoforms of proSP-C, migrating at 21 and 24 kDa, were detected in the MLE-7 and MLE-12 cells, whereas a single species of proSP-C migrating at 21 kDa was detected in the MLE-15 cell line (Fig. 3).

SP-B and SP-C undergo extensive proteolytic processing to generate the mature, hydrophobic peptides detected in bronchioalveolar lavage (for review, see ref. 2). To determine whether the MLE cells processed SP-B and SP-C to the mature peptides, organic solvent extracts of MLE-7 and MLE-12 cells were analyzed for SP-B and SP-C. SP-B was detected in organic solvent extracts of MLE-7 cells migrating at 18 kDa under nonreducing conditions (Fig. 3) and at 8 kDa under reducing conditions (data not shown). In contrast, SP-B was not detected in organic solvent extracts of MLE-12 cells (data not shown) despite the synthesis of proSP-B by these cells (Fig. 3). SP-C, migrating at 6 kDa under reducing and nonreducing conditions, was detected in organic solvent extracts of MLE-7 (data not shown) and MLE-12 cells (Fig. 3).

**Secretion of PLs.** MLE-12 cells secreted [ $^3$ H]choline-labeled PLs in response to treatment with ATP and PMA at passages 10–15 in culture (Table 1). Moreover, the PMA-stimulated secretion was inhibited by the lectin Con A, similar to the response of primary cultures of rat type II cells (25). The PMA-stimulated PL secretion was also detected in the cells at passages 25–40; however, secretion was not inhibited by Con A or purified rat SP-A. In contrast to the ATP-stimulated PL secretion by the MLE-12 cells at the earlier passages in culture, ATP did not stimulate PL secretion at later passages in culture.

Phosphatidylcholine was the predominant [ $^3$ H]choline-labeled PL detected in the MLE-12 (passages 25–40) cells and in primary cultures of rat type II cells containing  $79.7\% \pm 1.1\%$  (mean  $\pm$  SEM;  $n = 12$ ) and  $89.3\% \pm 2.2\%$  (mean  $\pm$  SEM;  $n = 6$ ) respectively. Sphingomyelin ( $3.1\% \pm 0.2\%$  vs.  $5.6\% \pm 1.2\%$ ) and lysophosphatidylcholine ( $14.1\% \pm 1.1\%$  vs.  $4.7\% \pm 1.7\%$ ) were also labeled in both MLE-12 and type II cells. Percentage incorporation of [ $^3$ H]choline into the PL classes analyzed demonstrated statistically significant differences between the MLE-12 and rat type II cells (ANOVA;  $P < 0.05$ ).

**Tumorigenicity of MLE Cells.** The MLE cells were tested for tumorigenicity by subcutaneous injection into nude mice. Tumors were generated in all four injection sites of the mice inoculated with MLE-15 (passage 28) and MLE-7 (passage

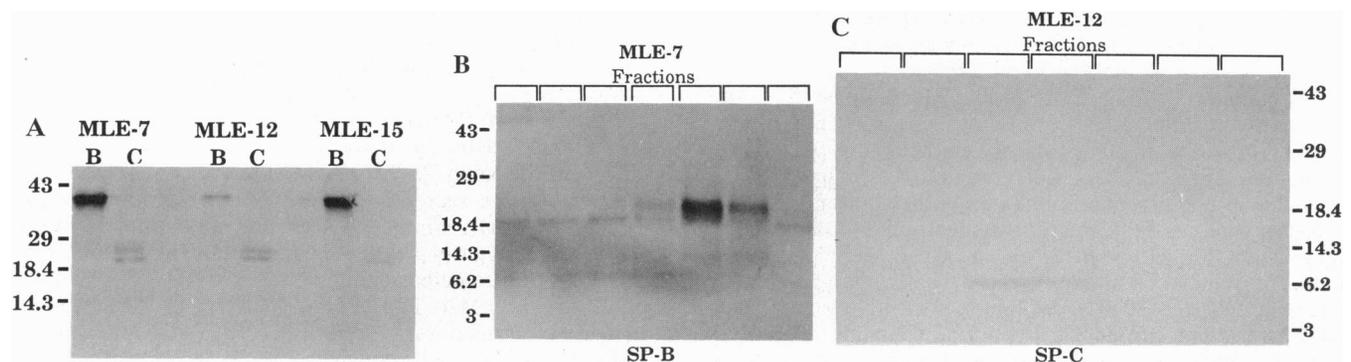


FIG. 3. Expression and processing of SPs in MLE cells. (A) MLE-7 (passage 22), MLE-12 (passage 20), and MLE-15 (passage 21) cells were metabolically labeled and cell extracts were immunoprecipitated with rabbit antisera generated against SP-B (lanes B) or SP-C (lanes C). Immunoprecipitated proteins were separated by SDS/PAGE in the presence of 2-mercaptoethanol, transferred to a membrane, and subjected to autoradiography. Molecular mass markers are indicated in kDa. (B and C) Column fractions of organic extracts from MLE-7 (B) (passage 25 or 35) or MLE-12 (C) (passage 28) cells were subjected to SDS/PAGE in the absence of reducing agents. Western blot analysis was performed with the polyclonal antiserum R559, which detects mature SP-B and SP-C (21). Molecular mass markers are indicated in kDa.

Table 1. Secretion of [<sup>3</sup>H]PL by MLE-12 cells

Cell passage in culture	% [ <sup>3</sup> H]PL released					
	Control	Forskolin	ATP	PMA	PMA + Con A	PMA + SP-A
10–15	3.2 ± 0.3	ND	6.2 ± 0.8*	5.1 ± 0.3*	3.5 ± 0.1 <sup>†</sup>	ND
25–40	2.4 ± 0.3	2.3 ± 0.2	2.5 ± 0.3	3.7 ± 0.3*	3.9 ± 0.2*	3.7 ± 0.3*

MLE-12 cells at passages 10–15 or 25–40 were labeled with [<sup>3</sup>H]choline, and [<sup>3</sup>H]PL secretion was determined. Data represent means ± SEM for two (passages 10–15) or eight (passages 25–40) separate preparations of cells, each in triplicate. ND, not determined.

\*Statistical significance (ANOVA with Newman–Keuls test;  $P < 0.05$ ) as compared to untreated control.

<sup>†</sup>Statistical significance (ANOVA with Newman–Keuls test;  $P < 0.05$ ) as compared to PMA treatment alone.

30) cells 39–41 days after injection. In contrast to the MLE-15 and MLE-7 cells, no tumors were generated in the mice inoculated with the MLE-12 cells at passage 11 or 29 in culture in the 85-day observation period to date.

## DISCUSSION

MLE cell lines were generated from transgenic mice harboring the SV40 TAG under the control of the human SP-C promoter region. The MLE cell lines maintained morphologic and functional characteristics of distal respiratory epithelial cells normally lost after isolation and primary culture. These results contrast with previous unsuccessful attempts at establishing differentiated distal respiratory epithelial cells by stable introduction of an oncogene into primary cells in culture. The poor proliferation rate of distal respiratory epithelial cells *in vitro* (26) may account for the lack of stable integration of exogenous oncogenes into the cellular DNA of the cells in culture. We utilized the strategy of directing expression of a viral oncogene *in vivo* and subsequent propagation of lung cells in culture to develop distal respiratory epithelial cell lines. The bronchioalveolar specificity of the SP-C promoter (27) and the maintenance of distal pulmonary epithelial cell characteristics of the cell lines support the conclusion that the cells originated from the distal respiratory epithelium.

**Expression of SV40 TAG mRNA.** SV40 TAG mRNA was detected in all cell populations generated from the SP-C/TAG transgenic mice. This observation, in addition to the increased expression of SV40 TAG mRNA at later cell passages in culture, suggests that there was a selective pressure for the continued expression of the exogenous oncogene in culture. These findings are similar to previous observations reported for cell lines generated by transformation with SV40 TAG (28, 29). Direct demonstration of the requirement for SV40 TAG expression in intestinal cell lines generated from transgenic mice was recently reported by Whitehead *et al.* (29). Thus, expression of SV40 TAG appears to be required for the initial transformation of epithelial cells as well as maintenance of the transformed phenotype in culture.

**Expression of Lung Cell-Specific Genes.** The SPs and mRNAs were detected at various levels in the MLE cell lines, consistent with a distal respiratory epithelial cell phenotype. SP-A and SP-B mRNAs were previously localized to the bronchiolar and alveolar epithelium in the adult murine lung (14, 15), whereas SP-C mRNA was restricted to the alveolar epithelium (9). Although SP-A and SP-B were previously detected in lung cell lines generated from human pulmonary adenocarcinomas (30, 31), cell lines constitutively expressing SP-C were not previously reported. Clement *et al.* (10) recently described the production of a neonatal rat distal respiratory epithelial cell line (SV40-T2) by transfection with the SV40 TAG in culture. SP-A, SP-B, and SP-C mRNAs were not detected in the SV40-T2 cell line despite the presence of microvilli and lamellar bodies and the ability of the cells to secrete PLs. Recent studies demonstrated that SP mRNA expression by primary cultures of rat type II epithelial

cells was dependent on growth on floating collagen gels (5), suggesting that extracellular matrix components and cellular architecture may be required for the continued expression of SP mRNAs. Although extracellular matrix components and loss of adherence to plastic substrates may alter SP expression by type II epithelial cells in primary culture, these factors are not required for SP mRNA expression in the MLE cell lines.

SP expression correlated with SP mRNA expression in the MLE cell lines. The apparent molecular masses of the SP-B and SP-C proproteins were consistent with the molecular masses predicted by the murine cDNAs (15, 16). The 21- and 24-kDa proSP-C isoforms were also consistent with the two proSP-C species previously identified in murine fetal lung explants (18). SP-B and SP-C proproteins and processed intermediates, but not the active peptides, were previously demonstrated in human pulmonary adenocarcinoma cell lines and Chinese hamster ovary cells expressing recombinant SP-B or SP-C (refs. 18, 30, and 31; unpublished observations). The MLE cell lines processed the SP-B and SP-C proproteins to the mature peptides present in pulmonary surfactant. The MLE cells represent a description of continuous cell lines capable of producing the mature SP-B and SP-C peptides in culture. Therefore, the MLE cells will be useful in elucidating the pathway of SP-B and SP-C protein processing and may be useful for preparation of SPs for treatment of surfactant-deficient states such as respiratory distress syndrome and congenital alveolar proteinosis.

CC10 mRNA was previously localized to the tracheal and bronchiolar, but not alveolar, epithelium in the adult murine lung (9), consistent with the localization of Clara cells in the mouse (32). CC10 mRNA was not detected in the established cell lines despite high levels of expression in the tumor cells *in vivo* (9) and the detection of CC10 mRNA in the cells at early passages in culture. Selective pressures imposed by the culture protocol used to generate the cell lines may result in the loss of CC10-expressing cells. Alternatively, expression of CC10 mRNA may require humoral factors, cell to cell interactions, or contact with components of the extracellular matrix.

**PL Secretion.** Primary cultures of type II cells secrete PLs in response to the secretagogues ATP and PMA and the diterpene forskolin (22, 25, 33). MLE-12 cells secreted PLs in response to the secretagogues PMA and ATP, but not forskolin. The PMA-stimulated secretion was inhibited by the lectin Con A at early passages in culture, but inhibition by the lectin was not detected at later passages in culture. Con A and SP-A inhibit PMA- and ATP-stimulated PL secretion by primary cultures of rat type II cells (25, 33). The mechanism of inhibition is unclear; however, the data are consistent with inhibition mediated through cell surface receptors. Thus, the lack of effect of Con A and rat SP-A on PL secretion by MLE-12 cells at later passages in culture may relate to a loss of surface receptors. Likewise, the loss of ATP-stimulated PL secretion may result from the loss of P<sub>1</sub> and/or P<sub>2</sub> purinoceptors.

**Phenotypic Heterogeneity in MLE Cell Lines.** The MLE cell lines maintained morphologic characteristics and gene expression patterns consistent with that seen in noncultured bronchiolar and alveolar type II epithelial cells. However, the morphologic and functional characteristics associated with an individual cell type did not always coexist in a clonal cell line. For example, MLE-12 cells expressed SP-C mRNA indicative of alveolar type II cells in the adult mouse (9); however, MLE-12 cells did not share other characteristics with alveolar type II cells, such as expression of SP-A mRNA (14) or the presence of lamellar bodies. The heterogeneity of cellular markers thought to be characteristic of type II cells may be related to the immortalization of cells at distinct stages of development since the SP-C promoter element was previously shown to direct high levels of expression of the reporter gene chloramphenicol acetyltransferase to the distal epithelium throughout lung development (34). Alternatively, the MLE cell lines may represent distinct subtypes of distal respiratory epithelial cells. Subpopulations of Clara cells were previously described based on morphological characteristics (32) and susceptibility to naphthalene-induced cytotoxicity (35).

In summary, lung epithelial cells representing the distal respiratory epithelium were generated by targeted expression of SV40 TAg in the lungs of transgenic mice and propagation of tumor cells in culture. The MLE cell lines offer a useful model system for the study of several aspects of lung biology, including the regulation of surfactant synthesis and secretion as well as pulmonary tumorigenesis.

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