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Centrifugal Migration of Mesenchymal Cells in Embryonic Lung

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Abstract

Murine lung development begins at embryonic day (E) 9.5. Normal lung structure and function depend on the patterns of localization of differentiated cells. Pulmonary mesenchymal cell lineages have been relatively unexplored. Importantly, there has been no prior evidence of clonality of any lung cells. Herein we use a definitive genetic approach to demonstrate a common origin for proximal and distal pulmonary mesenchymal cells. A retroviral library with 3,400 unique inserts was microinjected into the airway lumen of E11.5 lung buds. After 7–11 days of culture, buds were stained for placental alkaline phosphatase (PLAP). Most PLAP+ cells are peribronchial smooth muscle cells, initially localized laterally near the hilum, then migrating down airways to the subpleural region. Laser-capture microdissection and polymerase chain reaction confirm the clonal identities of PLAP+ cells proximally and distally. Our observation of this fundamental process during lung development opens new avenues for investigation of maladaptive mesenchymal responses in lung diseases.

Keywords

retrovirus; microinjection; alkaline phosphatase; smooth muscle; clonal analysis

INTRODUCTION

Mammalian epithelial cell differentiation during lung development proceeds in an orderly manner in both mice and humans: first, pulmonary neuroendocrine cells (PNECs) appear, followed by Clara cells, ciliated cells, and type II pneumocytes (Ten Have-Opbroek, 1991).

Epithelial cell lineages have been investigated primarily through the use of transgenic mouse models using lung epithelial cell-specific promoters (Hackett et al., 1996; Pan et al., 2002; Perl et al., 2002). In comparison, mesenchymal cell differentiation is less understood (Anjos-Afonso et al., 2004), despite the long-recognized importance of lung mesenchyme for branching morphogenesis and epithelial cell differentiation (Spooner and Wessells, 1970; Wessells, 1970; Shannon and Hyatt, 2004). The limited knowledge of mesenchymal cell differentiation is in part due to the lack of mesenchymal cell-specific promoters that are primarily or exclusively expressed in the lung. Using traditional histopathology, it has been learned that myofibroblasts are the proliferative, partially differentiated cell type that precedes terminally differentiated desmin-positive smooth muscle cells (Mitchell et al., 1990; Wright et al., 1999). In addition, capillaries originate by means of angiogenesis from the proximal pulmonary vasculature (Parera et al., 2005).

To investigate clonal lineages of airway-associated cells in developing lung, we chose a definitive genetic retroviral labeling approach. Most methods to study cell lineages are limited by uncertainties about clonal relationships between daughter cells (Le Douarin, 1984; Serbedzija et al., 1994). Cepko et al. have developed a new technology for analyzing cell lineages that allows the definitive identification of clonal progeny of a single precursor (Fields-Berry et al., 1992; Cepko et al., 1998; Cai et al., 2000), based on the labeling of dividing progenitor cells with a library of replication incompetent retroviruses, which is passed on only to daughter cells. The progeny are labeled with a histochemical marker such as placental alkaline phosphatase (PLAP) as a genetic tag. The most recent libraries (3.4×10^3 clones) optimize chances of identifying multiple clones in the same tissue preparation, leading to faster and more accurate results (Fields-Berry et al., 1992; Reid et al., 1995; Cepko et al., 1998; Cai et al., 2000). This approach has been instrumental in defining cell fate in the retina, where glial cells and neurons were identified as derived from a common precursor (Cepko et al., 1996), and in mapping widespread dispersion of neuronal clones across functional regions of developing cerebral cortex, diencephalon, and hypothalamus (Walsh and Cepko, 1992; Reid et al., 1995; Arnold-Aldea and Cepko, 1996; Golden and Cepko, 1996). We now apply this well-characterized genetic technology to the first clonal analysis of cell lineages and cell fates during embryonic lung development.

RESULTS

Whole-Mount PLAP Staining

In a few lung buds harvested after 1–5 days, no PLAP+ staining was visible (data not shown). After 7 days of culture, PLAP+ cells are visible along either lateral side of the trachea, spatially restricted to the trachea, main-stem bronchi, and first one to two airway generations of all eight lung buds examined at this time point (arrow in Fig. 1a indicates the distal-most PLAP+ cells), apparently tracking along the airways below the epithelium, suggesting localization to the peribronchial mesenchyme. After 7 days, in eight lung buds examined, PLAP+ cells rarely cross the midline (Fig. 1b, arrows), and the distal-most PLAP+ cells extend to $41 \pm 4\%$ of the distance from the carina to the pleural surface (Fig. 2). After 9 days (Fig. 2c,d), PLAP+ cells cross the midline in all nine lung buds examined at this time point (indicated by arrowhead in Fig. 1c), and the distal-most PLAP+ cells extend to $58 \pm 6\%$ of the distance from the carina to the pleural surface (Figs. 1d, 2). After 11 days in culture (Fig. 1e,f), the PLAP+ cells extend outward to the pleural surface in all nine lung buds examined (indicated by white arrows in Fig. 1e), with PLAP+ cells at $97 \pm 1\%$ of the distance from the carina to the pleural surface (Fig. 2). By day 11, the PLAP+ cells are visible as dark linear markings throughout the interstitium (white arrows in Fig. 1f) in the sub-pleural region (edge of pleura indicated by asterisk in Fig. 1f). In a few cultures maintained for 13 days, the lung buds were smaller and had evidence of apoptosis on hematoxylin and eosin staining, consistent with involution. The highest lactate

dehydrogenase levels, measured daily in the media, occurred during the first 1–2 days of culture; then these levels dropped and did not rise again through day 11 (data not shown).

Histochemistry on Lung Sections

After whole-mount PLAP staining, lung buds were embedded in paraffin and sections were counterstained with eosin. Representative photomicrographs showing PLAP staining retained in the sections are given in Figure 3. After 7 days in culture (Fig. 3a,b), PLAP staining is visible only in mesenchymal cells immediately adjacent to the airway epithelium with the distal-most PLAP+ cells present in the first- or second-generation bronchi (Fig. 3a, arrow). At day 7, the PLAP+ cells are present only on either side of the midline, consistent with developing airway smooth muscle (Fig. 3a,b), and are consistently absent inferior to the tracheal bifurcation (asterisks in Fig. 3a,b). At day 9, PLAP+ cells extend approximately halfway to the pleura (Fig. 3c,d, arrows indicating distal-most PLAP+ cells). PLAP+ cells remain consistently absent inferior to the tracheal bifurcation (asterisks in Fig. 3c,d) but do cross the mid-line (arrowhead on left in Fig. 3d), with the trachea pointing to the left. At day 11, PLAP+ cells are distributed throughout the bronchial tree (some indicated by arrows in Fig. 3e,f). In the same lung bud, there are sub-pleural foci of PLAP+ cells (arrowheads on right of Fig. 3f).

Cytokeratin immunostaining is continuous, consistent with PLAP+ cells being nonepithelial (Fig. 4a). The dark PLAP staining quenches fluorescence and immunohistochemical chromogens. From Figure 4b, it is clear that the PLAP+ cell distribution is essentially identical to that of airway smooth muscle: α -smooth muscle actin-positive (SMA+; red arrows on left in Fig. 4b), and desmin-positive (Fig. 4c,e). A few cells are double-positive for SMA and PLAP (Fig. 4b, arrows on right and inset panel). The PLAP+ signal is typically so intense that colocalization with any other cellular marker is usually impossible (Cepko et al., 1998). We were fortunate to identify regions where the PLAP+ staining was less intense so that we could demonstrate some cells with colocalization for SMA and PLAP (Fig. 4b). Subsequently, at day 11 many of the subpleural PLAP+ cells stained for SMA, but not desmin (Fig. 4f, long arrows) and/or were closely adjacent to SMA+ cells (Fig. 4f, arrowheads). It should be noted that only a small number of PLAP+ cells occur near the pleura on day 11 of culture. Similarly, very few SMA+ cells occur in this location (Fig. 4f), and these are all desmin-negative.

We also observe abundant subpleural SMA-positive/desmin-negative cells in developing murine lungs in vivo at embryonic day (E) 12.5. However, the frequency of these cells declines markedly by E14.5 and there are only rare subpleural SMA+ cells present at E18.5. SMA+ subpleural cells are not detected in lung after birth. In culture, the E11.5 lung bud explants are maintained with high structural integrity and no LDH release into the supernatant between 3 and 11 days of culture (data not shown). The distal-most end of the airways is separated from the pleural surface by primitive alveoli lined by SPC-immunopositive cells. It is not until day 13 of culture that there is some histological evidence of involution of the lung buds, with mesenchymal cell death and shrinkage of the lung buds (data not shown).

Laser-Capture Microdissection and Clonal Analysis

To assess the clonality of different PLAP+ cells, we used laser capture microdissection to remove small clusters of prominent PLAP+ cells, followed by two rounds of polymerase chain reaction (PCR) amplification (40 cycles each) (Signoretti et al., 1999). PCR products were subcloned into the TA plasmid (Zhou and Gomez-Sanchez, 2000). Four colonies were sequenced for each microdissected focus, with eight or nine foci captured per lung bud. In all three lung buds, identical sequences were obtained for multiple cells located both

proximally and distally, as shown in Figure 5 and Table 1. A representative lung bud is shown in Figure 5a, with three distinct proximal foci (#19, 20, and 21), and three separate subpleural foci (#22, 23, and 26). In Figure 5a, identical sequences are shown in the same color. Thus, focus #20 contains one clone (blue) identical to a clone in focus #21 (blue center), indicating that the same cell did in fact cross the mid-line. A different clone in focus #21 (red rim) is identical to a clone (red) at focus #22 (subpleural). Similarly, a clone at proximal focus #19 is identical to a distal clone at focus #26. These two matches (#19 and 26, and #21 and 22) indicate sequence identity between subpleural clones and proximal clones. There were, in addition, 37 unique sequences such as at focus #23 that were distributed randomly and evenly throughout the bronchial tree (Fig. 5a). All together, subclones of 27 laser capture samples from three lung buds yielded 47 sequences, of which 10 matched a sequence in one or more other distinct foci (Table 1). In two lung buds, multiple very proximal foci had sequences that matched multiple subpleural foci. These sequence identities indicate that proximal and distal mesenchymal cells can be derived from the same precursor, providing definitive evidence for both clonality and centrifugal cell migration during lung development.

DISCUSSION

The present study demonstrates that daughter cells from a single pulmonary mesenchymal clone can be distributed both paratracheally and distally, migrating centrifugally along developing airways, establishing a direct link between subepithelial smooth muscle and subpleural mesenchymal cell lineages. At least some of the subpleural mesenchymal cells are SMA+ but desmin-negative, consistent with myofibroblasts. In most lineage investigations in other laboratories using the same library, the identity of PLAP+ cells was determined exclusively by cell morphology and by the localization of cells within a given tissue (Cepko et al., 1996). We initially carried out the experiments in the hopes of analyzing lineages of the airway epithelial cells. We have no obvious explanation for the localization of the retroviral library in the subepithelial mesenchyme. Nonetheless, this was the site of PLAP+ staining, and the precise localization of PLAP+ cells was highly reproducible, despite the filling of the entire tracheobronchial tree with the retroviral library. It is possible that the intraluminal DAP retroviral stock might be extruded along either side of the trachea, which could in part explain the proximal localization of the earliest PLAP staining on day 7. Despite this being a loosely consolidated tissue, we carried out clonal analyses using fine tools: laser-capture microdissection followed by PCR amplification of DNA inserts and subcloning to distinguish individual cells. There is only one retroviral integration event per cell 99% of the time (Price et al., 1987). The mesenchymal localization of the integrated retroviral library could reflect a higher rate of proliferation of mesenchymal cells in embryonic lung buds (as measured by bromodeoxyuridine incorporation). Retroviral integration requires ongoing cell division. Preliminary observations indicate that some of the dye injected into the tracheal lumen of E12.5 lung buds is detected in the surrounding mesenchyme, suggesting that the embryonic epithelium is leaky to small particles (Warburton and Tefft, data not shown). Furthermore, uptake of retrovirus by epithelial cells in the lung is orders of magnitude less efficient than epithelial uptake of adenovirus under the same conditions, either in vivo or in vitro, whereas uptake of retrovirus into fibroblasts in culture is relatively efficient, possibly because the fibroblasts proliferate much faster than epithelial cells in culture (Warburton and Tefft, data not shown). In embryonic lung in vivo, cell proliferation is higher in the mesenchyme than in the epithelium (Haley et al., 1997). Differential expression of retroviral receptors is unlikely, because these receptors are ubiquitous (Rapp and Marshall, 1980). We cannot rule out the possibility that epithelial-to-mesenchymal transformation might contribute to the mesenchymal localization of integrated retrovirus in lung bud cultures (Kim et al., 2006). Finally, we cannot exclude the possibility that progenitor cells might occur in other regions of the developing lung buds. We can only

conclude that progenitor cells are present on either side of the trachea under the given experimental conditions and that some of these cells migrate into the subpleural region by day 11 of culture.

Our observations are novel and distinct from results from other laboratories studying genetically altered mice. Mailleux et al. showed that a pool of Fgf10+ cells in the subpleural mesenchyme of the lung contains progenitor cells which contribute to peribronchial smooth muscle in the bronchioles by moving proximally relative to the epithelium as the epithelium extends peripherally during branching morphogenesis (Mailleux et al., 2005). Our observations are not in conflict with the findings of Mailleux et al. (2005) because we are analyzing a distinct subset of mesenchymal cells initially located in the perihilar region and migrating distally along the developing airways. Thus, our findings do not exclude a role for derivatives of the splanchnic mesoderm in formation of intrapulmonary mesenchymal lineages. In the present study, the airway-associated mesenchymal cells are concentrated in the first few generations of the tracheobronchial tree, with relatively small numbers of PLAP+ cells migrating outward. Furthermore, del Moral et al. demonstrated that Fgf9, expressed in the pleural mesothelium, in turn regulates Fgf10 expression within the pool of subpleural smooth muscle progenitors, most likely through Tbx4 and 5 (del Moral et al., 2006). Fgf9 also controls the expression of Hedgehog targets Ptc and Gli in a hedgehog-independent manner and suppresses smooth muscle differentiation in the subepithelial as opposed to the sub-pleural domains of the peripheral lung mesenchyme (del Moral et al., 2006; White et al., 2006). It remains to be seen whether the novel pool of mesenchymal progenitors reported herein that migrate from proximal to distal locations along the airways are also controlled by these or other developmental positional and differentiation signals (Mailleux et al., 2005).

Our unprecedented observations could have implications for lung pathobiology as well as lung development. Mesenchymal stem cells are present in airways of adults as well as developing lung (Sabatini et al., 2005). Mesenchymal cell migration could contribute to the progression of a variety of lung diseases, such as chronic lung disease in newborns (Jobe, 2003; Chess et al., 2006), remodeling in asthmatics (Holgate et al., 2004), and progression of idiopathic pulmonary fibrosis (White et al., 2003). Future investigations will be required to test these hypotheses in animal models.

EXPERIMENTAL PROCEDURES

Library

The DAP library we used was obtained directly from the laboratories of Dr. Christopher Walsh and Dr. Connie Cepko. The complexity of this library is 3,400 (Reid et al., 1995, 1997). There appears to be even representation of tags within this library, with <0.5% of the clones being duplicated (Fields-Berry et al., 1992; Cepko et al., 1998). Only one retroviral vector integrates in a given cell; thus, the unique insert becomes a specific cell marker. Using competent *Escherichia coli* DH5 α and kanamycin, a large pool of colonies was selected to ensure that all vectors were expressed. The CR7 amphotropic packaging line was used to obtain ~60,000 transient viral particles. This supernatant was then used to make stable retroviral producer cell lines in the ecotropic packaging cell line ψ 2 as described (Fields-Berry, et al., 1992; Reid et al., 1995). We carried out rigorous analyses for helper virus. No helper virus was present in the library that we used for microinjection in the current study (data not shown), following a published protocol (Cepko et al., 1998).

Microinjection

E11.5 lung buds from timed-pregnant Swiss-Webster mice (Charles River Laboratories) were immobilized in wells on agarose plates, with the trachea pointing upward at ~45

degrees. The DAP retroviral stock was prepared at $\sim 10^5$ pfu/ml with polybrene 80 $\mu\text{g/ml}$ and 1% trypan blue dye. A micropipette was positioned just inside the trachea and $\sim 1\text{-}\mu\text{l}$ of the retroviral stock was infused into the lumen over ~ 15 min using a peristaltic pump (Fig. 6). Lung buds were then transferred and cultured on 0.3- μm pore-size Transwell filters in six-well plates as described, overlying BGJb medium containing ascorbic acid, 2.5% bovine fetal calf serum, HITES, 2% L-glutamine, and 10 ml of penicillin–streptomycin at 37°C in a humidified incubator in 5% CO_2 , as described (20). The virus is known to have a half-life of 4 hr at 37°C (Walsh and Cepko, 1992), and a high concentration of polybrene is necessary for viral adhesion to cells, so the treatment is essentially a single pulse with no significant carryover of virus into later stages (>24 hr) of the 7–11 day culture. Buds were harvested 7 to 11 days later by fixation followed by whole-mount PLAP staining (Arnold-Aldea and Cepko, 1996).

Immunoperoxidase

After whole-mount PLAP staining, lung buds were processed manually into paraffin blocks as described (Kong et al., 2004). Each lung bud was sectioned through completely in 5- μm slices and every fourth slide was counterstained with eosin. Immunoperoxidase staining for cytokeratin and desmin was carried out with the ABC method (Vector Laboratories, Burlingame, CA), as previously described (Kong et al., 2004) using: 1:100 dilution of rabbit anti-bovine cytokeratin (broad-spectrum; Dako Laboratories, Inc., Carpinteria, CA); or 1:100 rabbit antidesmin (Sigma Laboratories, St. Louis, MO). Immunoperoxidase staining for α -smooth muscle actin (SMA) was carried out using 1:60 dilution of alkaline-phosphatase–conjugated mouse monoclonal anti-SMA purified IgG (clone 1A4, Sigma Laboratories). In brief, slides were pre-treated with 0.3% Triton X-100 in phosphate buffered saline for 10 min followed by 20 min with 1:200 normal serum (goat for rabbit IgG, horse for mouse IgG) before the primary antibody, which is incubated at 4°C overnight. After washing, the biotinylated anti-IgG is incubated at 4°C for 2 hr, then endogenous peroxidase is blocked with 0.3% H_2O_2 in methanol for 30 min. The ABC reagent is applied for 45 min, then developed using diaminobenzidine (Sigma Laboratories, St. Louis, MO) or NovaRed (Vector Laboratories, Burlingame, CA). NovaRed provides better contrast against the dark PLAP staining. For SMA immunostaining, the secondary antibody and blocking steps (normal horse serum and methanol-peroxide) were omitted and the alkaline phosphatase Vector-Red substrate was applied for 5 min at room temperature according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA).

Laser-Capture Microdissection and PCR

Five-micrometer sections of lung buds were placed on uncoated glass slides for laser-capture microdissection as previously described (Signoretti et al., 1999). To assess the clonality of different PLAP+ cells, we used laser capture microdissection to remove small clusters of cells (foci $\sim 10\text{ }\mu\text{m}$ in diameter) from these lung bud sections. DNA was prepared from microdissected cells, followed by 2×40 cycles of PCR amplification (Signoretti et al., 1999) using nested primers for the DAP library (Walsh and Cepko, 1992): First round, PBR4 (5'-GCGGAGCCTATGGAAAAACGCCAGC-3') and BND3 (5'-TGAGTGGCCATTAGAGCAGTAGTCCCTGTTC-3'); Second round, PBR5 (5'-CGGGTTTCGCCACCTCTGACTTGAGCGTCG-3') and BND4 (5'-TCTACTGCGGCTTGGAGCTGCTGGAATTGC-3'). PCR products were subcloned into the Promega TA plasmid as described (Zhou and Gomez-Sanchez, 2000). DNA from isolated white colonies was sequenced at the Duke University DNA Analysis Facility using the Applied Biosystems Dye Terminator Cycle Sequencing system with Big Dye terminator v1.1 sequencing chemistry combined with ABI 3730 PRISM DNA Sequencing instruments. Sequence comparisons were carried out using MacVector 7.0 software.

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Fig. 1.

Whole-mount alkaline phosphatase staining of cultured lung buds. Microinjected buds were cultured on Transwell filters overlying BGJb medium with ascorbic acid, 2.5% bovine fetal calf serum, HITES, 2% L-glutamine, and 10 ml of penicillin–streptomycin at 37°C in a humidified incubator in 5% CO₂, as described (Golden and Cepko, 1996). Buds were harvested 7 to 11 days later by fixation followed by whole-mount placental alkaline phosphatase (PLAP) staining as described (Arnold-Aldea and Cepko, 1996). **a,b:** Embryonic day (E) 11.5 lung bud cultured for 7 days before whole-mount PLAP staining. **a:** Arrow indicates the distal-most detectable PLAP+ cells. **b:** Arrows indicate location of PLAP+

cells along either side of trachea, but not crossing the midline. **c,d:** E11.5 lung bud cultured for 9 days. Long black arrows indicate distal-most extension of PLAP+ cells. Arrowhead in c indicates PLAP+ cells crossing the midline. e,f: E11.5 lung bud cultured for 11 days. **e:** White arrows indicate site of PLAP+ cells in the subpleural region. **f:** White arrows indicate linear arrays of PLAP+ cells on the pleural surface, tracking along the interstitium.

Fig. 2.

Quantitative analysis of proximal-to-distal extension of placental alkaline phosphatase–positive (PLAP+) cells in whole-mount-stained lung buds. Embryonic day (E) 11.5 lung buds were microinjected with the BOLAP library and cultured as described in the legend to Figure 1. The numbers of lung buds examined were as follows: $n = 8$ at 7 days, $n = 9$ at 9 days, and $n = 9$ at 11 days. The distal-most extension of PLAP+ cells was determined as the greatest distance traveled by PLAP+ cells relative to the carina as a percentage of the total distance from the carina to the pleural surface in the same trajectory. * $P < 0.0001$ compared with day 7 or day 9; ** $P < 0.01$ compared with day 7.

Fig. 3.

Histopathological analyses of placental alkaline phosphatase-positive (PLAP+) cells. Five-micron paraffin sections of PLAP-stained lung buds were counterstained with eosin. Asterisks are at the carina. **a,b:** Sections of embryonic day (E) 11.5 lung bud cultured for 7-days. **a:** The arrow indicates the distal-most extension of PLAP+ cells immediately adjacent to a second-generation cartilaginous airway. **b:** Arrows indicate a few PLAP+ cells scattered along the subepithelial region of a first-generation cartilaginous airway. **c,d:** Sections of E11.5 lung bud cultured for 9 days. **c:** Arrow indicates the distal-most extension of PLAP+ cells along a third-generation noncartilaginous airway. The asterisk indicates the pleural surface. **d:** The arrowhead indicates PLAP+ cells in the mesenchyme crossing the midline of the distal-most trachea. Arrow indicates the distal-most extension of PLAP+ cells along a second-generation noncartilaginous airway up to the junction with a third-generation airway. **e,f:** Sections of E11.5 lung bud cultured for 11 days. **e:** Arrows indicate PLAP+ foci scattered along the bronchial tree. **f:** Arrowheads indicate PLAP+ cells tracking along subpleural region between arrows (the asterisk indicates the pleural surface) of same lung bud as shown in e (same arrow indicated by #1, same arrowheads indicated by #2 and #3). Scale bar = 50 μ m in a,d,f, 25 μ m in b, 100 μ m in c,e.

Fig. 4.

Immunohistochemical characterization of placental alkaline phosphatase-positive (PLAP+) cells. Immunohistochemical staining was carried out as detailed in the Experimental Procedures section. L, airway lumen. **a:** Cytokeratin immunostaining of embryonic day (E) 11.5 lung bud cultured for 9 days, with diaminobenzidine as substrate. Large cleft arrowheads demarcate PLAP+ cells extending along one stretch of cytokeratin-positive epithelium. The small triangular arrowhead in the left lower corner indicates a fold in the tissue section. **b:** Smooth muscle actin (SMA) immunostaining of E11.5 lung bud cultured for 9 days, with NovoRed as substrate. Representative SMA+ cells are indicated by short red arrows; several PLAP+ cells are indicated by black arrowheads. Long black arrows indicate a few cells with visible colocalization of both red SMA immunostaining and purple PLAP

staining. One cluster, indicated by dotted lines, is shown at higher power in the inset in the lower right hand corner. **c:** Desmin immunostaining of E11.5 lung bud cultured for 9 days, with NovoRed as substrate. Representative desmin+ cells are indicated by short red arrows, and several PLAP+ cells are indicated by black arrowheads. **d:** Another desmin-immunostained E11.5 lung bud cultured for 9 days is shown at lower power, with developing smooth muscle visible below the carina (*) and with PLAP+ staining laterally, between the epithelium and the cartilage. **e:** A representative negative control section adjacent to d was stained in parallel with d using normal rabbit IgG instead of rabbit antidesmin IgG. Note the absence of red immunostaining. **f:** A section of an E11.5 lung bud cultured for 11 days is shown, with the pleural surface at the top (indicated by three asterisks). Long arrows indicate PLAP+ cells and arrowheads indicate SMA+ immunostaining. Note the partial overlap of SMA and PLAP staining in some cells and the close association between SMA+ and PLAP+ cells. Less than half of the subpleural PLAP+ cells were SMA+ (data not shown). Scale bars = 25 μ m.

Fig. 5.

Laser-capture microdissection of placental alkaline phosphatase-positive (PLAP+) cells. **a:** A representative 5- μ m section of a PLAP-stained lung bud on an uncoated glass slide was used for laser-capture microdissection as previously described (Walsh and Cepko, 1992). The asterisk indicate the pleural surface. **b,c:** A pair of proximal images representing the same field containing focus #19 before and after microdissection. **d,e:** The same field with focus #23 before and after microdissection. Foci #19 and #26 contain identical clone, which differs from the identical sequence present in cells from foci #21 and #22. P, proximal; D, distal.

Fig. 6. Embryonic day (E) 11.5 lung bud after retroviral microinjection. Photograph of a representative whole-mount E11.5 lung bud immediately after microinjection of the retrovirus-dye mixture into the embryonic tracheal lumen. Note the dye is filling the future airway lumen throughout most of the bronchial tree.

TABLE 1

Clonal Identities in E11.5 Lung Buds Cultured for 11 Days

Lung bud	Proximal focus #	Proximal sequence #	Proximal focus #	Proximal sequence #	Midway focus #	Midway sequence #	Distal focus #	Distal sequence #
E11.5+11 #1	1	1	0	0	0	0	6	22
	1	3	2	6	0	0	0	0
	2	7	0	0	0	0	3	9, 12
	2	7	0	0	0	0	4	13
	2	7	0	0	0	0	8	30
E11.5+11 #3	3	10	0	0	0	0	7	26
	9	33	10	37	0	0	0	0
	10	39	13	50	0	0	0	0
	11	44	0	0	12	46	0	0
E11.5+11 #9	18	72	19	73	0	0	0	0
	19	76	0	0	0	0	26	101
	20	79	0	0	21	81	0	0
	0	0	0	0	21	84	22	85
	0	0	0	0	0	0	0	0

E11.5 lung buds were injected intraluminally with a DAP retroviral library containing 3,400 unique inserts. After 11 days of culture, buds were fixed and stained for PLAP. Buds# 1, 3, and 9 are shown here. The focus number represents the fragment of tissue that was laser-captured. After subcloning, four clones from each focus were sequenced. The horizontal bars (in different colours) indicate foci containing identical sequences. The locations of the foci are divided into: Proximal (trachea, mainstem bronchi, and cartilaginous airways); Midway (all noncartilaginous airways and primitive airspaces, excluding those within 50 μ of the pleural surface; and Distal, which includes cells within 50 μ of the pleural surface (see Fig. 5a). E, embryonic day; PLAP, placental alkaline phosphatase.