Keratinocyte Growth Factor Modulates Alveolar Epithelial Cell Phenotype *In Vitro*: Expression of Aquaporin 5

Zea Borok, Richard L. Lubman, Spencer I. Danto, Xiao-Ling Zhang, Stephanie M. Zabski, Landon S. King, Douglas M. Lee, Peter Agre, and Edward D. Crandall

Will Rogers Institute Pulmonary Research Center, Division of Pulmonary and Critical Care Medicine, University of Southern California, Los Angeles, California; and Division of Pulmonary and Critical Care Medicine, and Department of Biological Chemistry, Johns Hopkins University, Baltimore, Maryland

We investigated the role of keratinocyte growth factor (KGF) in regulation of alveolar epithelial cell (AEC) phenotype *in vitro.* Effects of KGF on cell morphology, expression of surfactant apoproteins A, B, and C (SP-A, -B, and -C), and expression of aquaporin 5 (AQP5), a water channel present *in situ* on the apical surface of alveolar type I (AT1) cells but not expressed in alveolar type II (AT2) cells, were evaluated in AECs grown in primary culture. Observations were made on AEC monolayers grown in serum-free medium without KGF (control) or grown continuously in the presence of KGF (10 ng/ml) from either Day 0 (i.e., the time of plating) or Day 4 or 6 through Day 8 in culture. AECs monolayers express AQP5 only on their apical surfaces as determined by cell surface biotinylation studies. Control AECs grown in the absence of KGF through Day 8 express increasing levels of AQP5, consistent with transition toward the AT1 cell phenotype. Exposure of AECs to KGF from Day 0 results in decreased AQP5 expression, retention of a cuboidal morphology, and greater numbers of lamellar bodies relative to control on Day 8 in culture. AECs treated with KGF from Day 4 or 6 exhibit a decrease in AQP5 expression through subsequent days in culture, as well as an increase in expression of surfactant apoproteins. These data, showing that KGF both prevents and reverses the increase in AQP5 (and decrease in surfactant apoprotein) expression that accompanies progression of the AT2 toward the AT1 cell phenotype, support the concepts that transdifferentiation between AT2 and AT1 cell phenotypes is at least partially reversible and that KGF may play a major role in modulating AEC phenotype. **Borok, Z., R. L. Lubman, S. I. Danto, X.-L. Zhang, S. M. Zabski, L. S. King, D. M. Lee, P. Agre, and E. D. Crandall. 1998. Keratinocyte growth factor modulates alveolar epithelial cell phenotype** *in vitro***: expression of aquaporin 5. Am. J. Respir. Cell Mol. Biol. 18:554–561.**

The alveolar epithelium is composed of two morphologically distinct cell populations. Alveolar type I (AT1) cells are thin, flat cells that line most of the gas exchange surface of the lung. Alveolar type II (AT2) cells are cuboidal cells that are the primary sites of surfactant synthesis and secretion. Current evidence indicates that AT2 cells in the adult lung are capable of proliferation and differentiation into AT1 cells, whereas AT1 cells have been presumed to be terminally differentiated (1, 2).

The process of one highly differentiated cell type, such as the AT2 cell, undergoing a transition to another highly differentiated cell type, such as the AT1 cell, has been referred to as transdifferentiation. AT2 cells grown in primary culture can be observed to undergo such a transition *in vitro*, in that they acquire a type I cell-like appearance with time in culture (3). The cells also begin to express a number of AT1 cell-specific markers, concurrent with the loss of lamellar bodies and other hallmarks of the AT2 cell phenotype (4–8). These observations suggest that alveolar epithelial cells (AECs) transdifferentiate from the AT2 toward the AT1 cell phenotype *in vitro,* recapitulating the events that occur *in vivo* during normal cell turnover and following lung injury.

AT2 cells can be identified by their characteristic morphology, including the presence of lamellar bodies, and by

(*Received in original form November 20, 1996 and in revised form September 10, 1997*)

Address correspondence to: Zea Borok, M.B., Ch.B., Division of Pulmonary and Critical Care Medicine, University of Southern California, GNH 11-900, 2025 Zonal Avenue, Los Angeles, CA 90033. E-mail: zborok@hsc.usc.edu

Abbreviations: alveolar epithelial cells, AEC; alveolar type II, AT2; alveolar type I, AT1; aquaporin 5, AQP5; Dulbecco's modified Eagle's medium–Ham's F12 nutrient mixture, DME–F12; Engelbreth–Holm–Swarm tumor basement membrane, EHS; keratinocyte growth factor, KGF; minimal defined serum-free medium, MDSF; phosphate-buffered saline, PBS; sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE; surfactant apoprotein, SP; transmission electron microscopy, TEM; Tris-buffered saline, TBS.

a variety of biochemical, immunological, and molecular probes. The best characterized indicators of the fully differentiated AT2 cell phenotype are probes directed against surfactant proteins (9). In contrast, few specific markers of the AT1 cell phenotype have been available until recently. The monoclonal antibody (mAb) VIIIB2, which recognizes an undetermined type I cell surface epitope *in situ*, has been used as a marker of the AT1 phenotype *in vitro* (5, 10, 11). Probes recognizing T1α, a gene (and gene product) expressed in AT1 cells but not AT2 cells, have also been used for this purpose (7, 8). Other putative AT1 cell markers have generally been shown to exhibit only relative specificity for AT1 or AT2 cells, and have therefore proved less useful tools for studying AEC phenotype.

Keratinocyte growth factor (KGF) is an epithelial mitogen that acts as a paracrine mediator of mesenchymal–epithelial interactions in many different tissues (12–14). KGF promotes rat AT2 cell proliferation both *in vitro* and *in vivo* (15, 16), suggesting a role for KGF in repair of the alveolar epithelium following lung injury (17–19). Studies of AT2 cells cultured on Engelbreth–Holm–Swarm (EHS) tumor basement membrane suggest that KGF may influence aspects of the AT2 cell differentiated phenotype, as indicated by upregulation of SP-A and SP-B mRNA (20).

Aquaporin 5 (AQP5) is a mercury-sensitive water channel in salivary, lacrimal, and respiratory tissues (21). In adult alveolar epithelium, *in situ* studies indicate that AQP5 is expressed only on the apical surface of AT1 cells (22). Although its functional significance is thus far unconfirmed in AT1 cells, it is presumed to contribute to alveolar fluid homeostasis and/or cell volume regulation via its role as a water channel (23). To determine the effects of KGF on AEC phenotypic characteristics, we evaluated its influence on AEC morphology, expression of AQP5, and expression of the AT2 versus AT1 cell-specific surfactant apoproteins A, B, and C (SP-A, -B, and -C). Our results indicate that KGF promotes retention of the AT2 cell phenotype and can at least partially reverse transdifferentiation from the AT2 to AT1 cell phenotype by AECs in primary culture.

Methods

Cell Isolation and Culture

AT2 cells were isolated from adult male Sprague-Dawley rats by disaggregation with elastase (2.0–2.5 U/ml) (Worthington Biochemical, Freehold, NJ) followed by panning on IgG-coated bacteriologic plates (24, 25). The enriched AT2 cells were resuspended in a defined serum-free medium (MDSF) consisting of Dulbecco's modified Eagle's medium and Ham's F12 nutrient mixture in a 1:1 ratio (DME–F12; Sigma Chemical, St. Louis, MO), supplemented with 1.25 mg/ml bovine serum albumin (BSA) (Collaborative Research, Bedford, MA), 10 mM Hepes, 0.1 mM nonessential amino acids, 2.0 mM glutamine, 100 U/ml sodium penicillin G, and 100 μg/ml streptomycin (24). Cells were seeded onto tissue culture-treated polycarbonate (Nuclepore) filter cups (Transwell; Corning-Costar, Cambridge, MA) at a density of 1.0×10^6 cells/cm^2. Media were changed on the third day after plating and every other day thereafter. Cultures were maintained in a humidified 5% CO_2 incubator at 37°C. AT2 cell purity ($\geq$ 90%) was assessed by staining freshly isolated cells for lamellar bodies with tannic acid (26). Cell viability ($>$ 90%) was measured by trypan blue dye exclusion. Cell culture media were purchased from Irvine Scientific (Santa Ana, CA). All other chemicals were purchased from Sigma Chemical and were of the highest commerical quality available.

Determination of Polarized Distribution of AQP5 by Cell Surface Biotinylation Studies

Cell surface biotinylation was performed essentially as described by Gottardi and Caplan (27). AEC monolayers grown on 24-mm Transwell Nuclepore filters (Day 5 in culture) were placed on ice and washed twice with ice-cold phosphate-buffered saline with calcium and magnesium (PBS-CM) (10 mM $NaPO_4$, 0.15 M NaCl, 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.2). Intact monolayers were then incubated with 1 mg/ml biotin (Sulfo-NHS-LC-Biotin; Pierce Chemical, Rockford, IL) in biotinylation buffer (10 mM triethanolamine, 2 mM $CaCl_2$, 150 mM NaCl, pH 9.0). For biotinylation of apical membrane proteins, 0.5 ml of biotinylation buffer with biotin was added to the apical surface of the monolayer, and 1.7 ml of the buffer without biotin was added to the basolateral side. The same volumes of buffer were added to each compartment for biotinylation of basolateral membrane proteins, or both apical and basolateral membrane proteins, except that biotin was added to the basolateral fluid only, or to apical and basolateral fluid, respectively. Biotinylation was carried out twice consecutively for 30 min at 4°C. The cells were subsequently washed four times with PBS-CM for 5 min each at 4°C and then solubilized in 0.5 ml of lysis buffer (LB), consisting of Tris-buffered saline (TBS) (50 mM Tris and 500 mM NaCl), 1% Nonidet P-40 (NP-40), and 1% BSA at pH 8.0, for 1 h on ice.

Lysates were centrifuged at 14,000 $\times$ *g* for 20 min at 4°C. The resulting supernatant was incubated with streptavidin beads (50 μl for each sample) for 1 h at 4°C. After incubation, the beads were briefly spun and collected, washed twice with LB, once with TBS (pH 8.0), and once with TBS (pH 6.8). Proteins were eluted from the beads in 2% sodium dodecyl sulfate (SDS) sample buffer (28) and heated at 100°C for 5 min before electrophoretic separation as described below.

Western Blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the buffer system of Laemmli (28), and immunoblotting was performed using procedures modified from Towbin and coworkers (29). Protein was extracted from freshly isolated AT2 cells and from control and KGF-treated monolayers in 2% SDS sample buffer. Equal amounts of cell protein in sample buffer (or constant volumes of each eluent for cell surface biotinylation studies) were resolved by SDS-PAGE under reducing conditions and electrophoretically blotted onto Immobilon-P (Millipore, Marlborough, MA). The blotted sheets were blocked for 2 h with 5% nonfat dry milk in TBS at pH 7.5, then incubated with the affinity-purified anti-AQP5 polyclonal antiserum (30). Blots were incubated with horseradish per-

oxidase-linked goat anti-rabbit IgG conjugates for 1 h, and antigen–Ab complexes were visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Membranes were purified by standard methods from rat lung and submandibular gland (31) to be included as positive controls for AQP5 (30). Protein concentrations were determined using the Bio-Rad DC protein assay (BIO-RAD, Hercules, CA), with BSA used as a standard.

RNA Isolation and Northern Analysis

Total RNA was isolated from freshly isolated AT2 cells and from control and KGF-treated monolayers by the acid phenol–guanidinium–chloroform method of Chomczynski and Sacchi (32). Equal amounts of RNA (5 or 10 μg) were denatured with formaldehyde, size fractionated by agarose gel electrophoresis under denaturing conditions, and transferred to nylon membranes. RNA was immobilized by ultraviolet (UV) cross-linking (Hybond N^+; Amersham Life Science, Cleveland, OH). Blots were prehybridized for 2 h at 65°C in 1 M $NaPO_4$ buffer (pH 7), 7% SDS, and 1% BSA. Hybridization was performed for 16 h at 65°C in the same buffer. Blots were probed with an AQP5-specific cDNA probe (21) labeled with [α-^{32}P]dCTP (Amersham) by the random-primer method using a commercially available kit (Boehringer Mannheim, Indianapolis, IN). Blots were washed at high stringency (0.5× SSC [75 mM NaCl, 7.5 mM sodium citrate, pH 7.0] with 0.1% SDS at 55°C) and visualized by autoradiography. In some experiments, blots were rehybridized with cDNA probes for SP-A, -B, and/or -C. Differences in RNA loading were normalized using a 24-mer oligonucleotide probe for 18S rRNA end labeled with [^{32}P]ATP.

Morphological Evaluation

To compare the morphological changes occurring with time in culture in the presence or absence of KGF, representative monolayers were examined by transmission electron microscopy (TEM). Filters were washed with cold PBS at pH 7.2, fixed in 2.5% glutaraldehyde in PBS for 1 h, washed once in PBS, and fixed in 1.5% osmium in PBS overnight. Cells were then stained with 2% tannic acid in PBS, rinsed, dehydrated in graded ethanols, and infiltrated with resin (SPURR). The filters were removed from the cups, embedded in a flat embedding mold, and polymerized overnight at 70°C. Ultrathin (silver–gray) sections were cut with a diamond knife on a Sorvall (Norwalk, CT) MT-2B ultramicrotome and viewed with a JEOL (Tokyo, Japan) 1200ex transmission electron microscope.

Experimental Design

RNA and protein were harvested from freshly isolated AT2 cells (Day 0) and from AECs grown in MDSF on polycarbonate filters for 1 or 8 d to evaluate changes in AQP5 and surfactant apoprotein expression between early and late days in culture. To evaluate the inhibitory effects of KGF on progression of AT2 cells toward the AT1 cell phenotype with time in culture, cells were plated in either MDSF (control) or MDSF supplemented with KGF (10 ng/ml) (R&D Systems, Minneapolis, MN) from the time of plating (Day 0). Monolayers in MDSF ± KGF were harvested on Day 8 for analysis of mRNA and protein or processing for electron microscopy. To determine whether the transition toward the AT1 cell phenotype could be reversed by KGF, cells were maintained in MDSF until Day 4 or Day 6 and media supplemented with KGF (10 ng/ml) thereafter. RNA and protein were isolated from monolayers maintained in MDSF before addition of KGF on Day 4, and from control and KGF-treated monolayers on subsequent days until Day 8. Monolayers were processed for electron microscopy on Days 4 and 8.

Results

Detection of AQP5 and Determination of Polarized Distribution in AEC Monolayers

AEC monolayers cultivated under conditions that have previously been shown to promote expression of AT1 cell markers over time (10, 11) were subjected to cell surface biotinylation on either apical, basolateral, or both cell surfaces on Day 5 in culture. As illustrated in Figure 1, biotinylated AQP5 (∼ 28 kD) was detected on the apical, but not basolateral, surface of the AEC monolayers. Recovery of biotinylated AQP5 protein was equivalent when biotinylation was performed on the apical surface alone, or on both apical and basolateral surfaces, further consistent with the absence of AQP5 protein on the basolateral surface of the monolayers. These results demonstrate the presence of AQP5 in cultured AECs and confirm its predominant, if not exclusive, location on the apical surface of the cells.

Time Course of AQP5 mRNA and Protein Expression in Cultured AEC Monolayers

Expression of AQP5 mRNA and protein was analyzed by Northern and Western blotting, respectively, in freshly isolated AT2 cells, and in AECs cultivated under conditions that have previously been shown to promote expres-

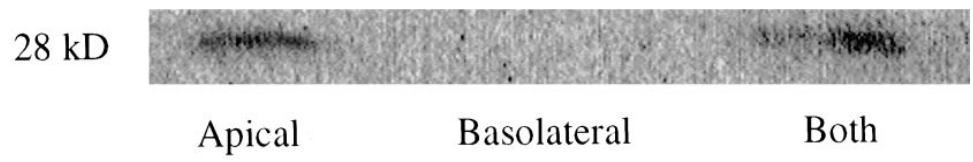

Figure 1. Immunodetection and polarized distribution of AQP5 in AEC monolayers. AEC monolayers were subjected to cell surface biotinylation on either apical, basolateral, or both cell surfaces on Day 5 in culture. As shown in this representative Western blot, labeled AQP5 was detected on the apical (but not basolateral) surface of the AEC monolayers. Recovery of AQP5 protein was equivalent when biotinylation was performed on the apical surface alone, or on both apical and basolateral surfaces, consistent with its exclusively apical location in AECs (n = 3).

sion of AT1 cell markers over time. As illustrated in Figure 2a, low levels of AQP5 mRNA were detectable in populations of freshly isolated AT2 cells. AECs grown in MDSF on tissue culture-treated polycarbonate filters for 1 d also expressed low levels of AQP5 mRNA. A marked increase in AQP5 mRNA was noted between Days 1 and 8 in culture. As shown in Figure 2b, AQP5 protein was only faintly detectable in freshly isolated AT2 cells. An increase in AQP5 protein was noted from Day 1 to Day 8 in culture, indicating that the increase in mRNA was associated with increased AQP5 protein levels. Similar results (data not shown) were also obtained using a different affinity-purified anti-AQP5 antiserum (Alpha Diagnostic International, San Antonio, TX). These results confirm the presence of AQP5 mRNA in AECs and demonstrate the utility of AQP5 as a marker of AEC differentiation *in vitro.* These findings provide further support for previous observations suggesting that AECs in primary culture on inflexible substrata undergo at least partial transition toward the AT1 cell phenotype.

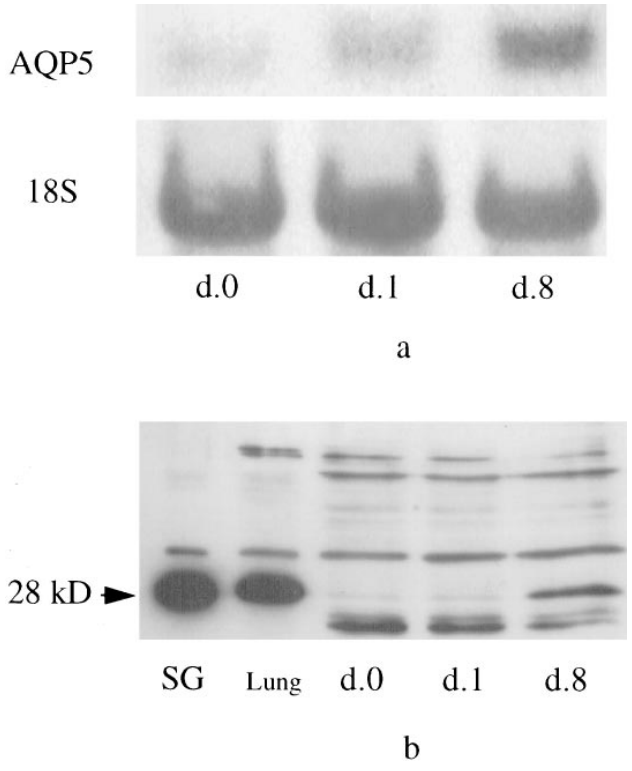

Figure 2. Expression of AQP5 by alveolar epithelial cells. (*a*) Expression of AQP5 mRNA by AECs. AQP5 mRNA is present at low levels in freshly isolated cells and after 1 d in culture, as shown in this representative Northern blot (n = 8). Levels of AQP5 mRNA increase dramatically between Days 1 and 8. Expression of 18S rRNA is similar under all conditions. (*b*) Expression of AQP5 protein by AECs. In this representative Western blot (n = 3), AQP5 protein is faintly detectable in freshly isolated cells and on Day 1, with a substantial increase noted on Day 8. Protein loading for AECs is similar under all conditions. Rat submandibular gland and lung membranes (5 μg/lane) are shown as positive controls.

Modulation of AQP5 Expression by KGF

To determine whether expression of AQP5 over time in culture could be modulated by KGF, AQP5 mRNA and protein expression were evaluated in AEC monolayers cultivated in MDSF ± KGF from the time of plating (Day 0). AQP5 mRNA levels were markedly reduced in KGF-treated monolayers on Day 8 (Figure 3a, *lane 6*) compared with untreated controls maintained in MDSF (Figure 3a, *lane 4*), and were similar to levels on Day 1 (not shown). These effects of KGF on AQP5 expression in AECs were also reflected at the protein level. KGF-treated cells showed a signal for AQP5 that was only slightly above background on Day 8 (Figure 3b, *lane 7*).

The ability of KGF to modulate expression of AQP5 once transition toward the AT1 cell phenotype had already commenced was evaluated. Addition of KGF to AEC monolayers from Day 4 (at which time AQP5 mRNA was readily detectable) resulted in a progressive decline in AQP5 mRNA on subsequent days in culture. AQP5 mRNA levels in AECs treated with KGF on Day 4 were decreased by Day 6 (Figure 3a, *lane 3*) relative to Day 4 (Figure 3a, *lane 1*) and Day 6 (Figure 3a, *lane 2*) controls, with a further decrease noted on Day 8 (Figure 3a, *lane 5*). Similar effects of KGF on AQP5 protein are seen following addition on Day 4 (Figure 3b, *lane 6*). Even when KGF is added as late as Day 6, a small decrease in AQP5 is noted on Day 8 (Figure 3b, *lane 5*). Together, these results indicate that KGF was able to reverse, at least in part, the transition between AT2 and AT1 cell phenotype with time *in vitro.*

Effects of KGF on Surfactant Apoprotein Expression

To assess the effects of KGF on reciprocal expression of the AT2 cell phenotype, blots from some experiments were also probed for SP-A, -B, and/or -C. Figure 4 shows a typical experiment in which KGF was added to the culture medium on Day 4, by which time SP-A and SP-B were no longer detectable. Treatment with KGF resulted in increases in SP-A, -B, and -C expression by Day 6 (Figure 4, *lane 3*) relative to controls grown in MDSF on Day 4 (Figure 4, *lane 1*) or Day 6 (Figure 4, *lane 2*). Further large increases in SP-A and -B expression occurred by Day 8 (Figure 4, *lane 5*) versus AEC controls grown in MDSF alone (Figure 4, *lane 4*). Surfactant apoprotein expression when AECs were treated with KGF from Day 0 to Day 8 was either the same or slightly reduced compared with AECs treated from Day 4 to Day 8 (data not shown). Although some differences were noted in the degree by which each surfactant apoprotein was upregulated, KGF clearly upregulates all three of these AT2 cell markers concurrent with downregulation of the AT1 cell marker AQP5 in AEC monolayers.

Effects of KGF on AEC Morphology

Morphologic evaluations of AECs cultured in MDSF ± KGF from either Day 0 or Day 4 are shown in representative electron micrographs. As shown in Figure 5, AECs grown in MDSF on polycarbonate filters lose the morphologic hallmarks of AT2 cell differentiation. By Day 4 (Figure 5A), the cells have lost their lamellar bodies and have

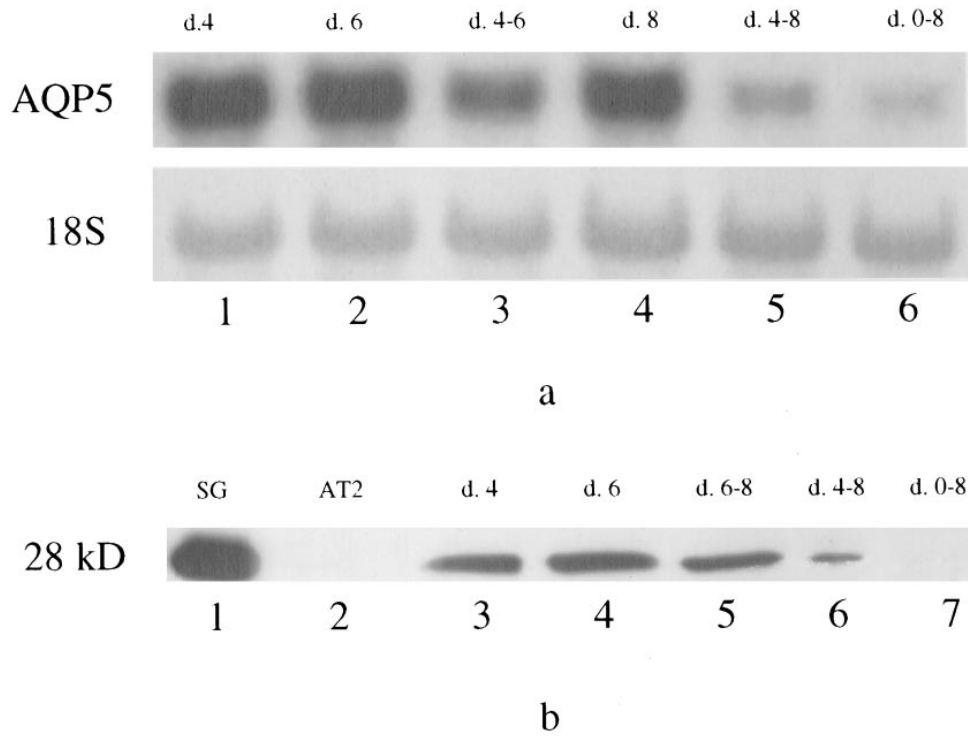

Figure 3. Inhibition and reversal of transition toward the AT1 cell phenotype by KGF. (*a*) Northern blotting. AECs grown in MDSF express approximately constant levels of AQP5 mRNA on Day 4, 6, or 8 (*lanes 1, 2,* and *4,* respectively). AECs grown in MDSF plus KGF from Day 4 express decreased levels of AQP5 mRNA by Day 6 (*lane 3*) compared with cells grown in MDSF alone (*lane 2*), with expression further diminished by Day 8 (*lane 5*). AECs grown in MDSF plus KGF from Day 0 show minimal AQP5 expression on Day 8 (*lane 6*) compared with cells exposed to KGF from Day 4 (*lane 5*) or cells grown in MDSF alone (*lane 4*). Expression of 18S rRNA is similar under all conditions. (*b*) Western blotting. In the experiment illustrated here (n = 3), AQP5 appears to be absent in freshly isolated AT2 cells (*lane 2*), but is readily detectable in AECs grown in MDSF on Days 4 and 6 (*lanes 3* and *4,* respectively). AECs treated with KGF from Day 6 through Day 8 show a diminished level of AQP5 (*lane 5*), with even greater loss of AQP5 seen in AECs treated with KGF from Day 4 through Day 8 (*lane 6*). AQP5 is only faintly detectable in AECs treated with KGF from Day 0 through Day 8 (*lane 7*). Protein loading for AECs is similar under all conditions. Rat submandibular gland is shown as a positive control (*lane 1*).

spread considerably. They progressively acquire the morphologic features typical of AT1 cells *in vivo,* developing markedly attenuated cytoplasmic processes by Day 8 (Figure 5B). In contrast, these changes are largely prevented in the presence of KGF. AECs maintained in KGF from Day 0 remain more cuboidal in shape on Day 4 (Figure 5C) through Day 8 (Figure 5D), contain numerous lamellar bodies (Figure 5F), and spread to a lesser degree than do control AECs. Morphologic features on Day 8 are similar when KGF is added to AEC culture medium from Day 4 (Figure 5E), with some increase in lamellar bodies and relative thickening of the cytoplasmic processes evi-

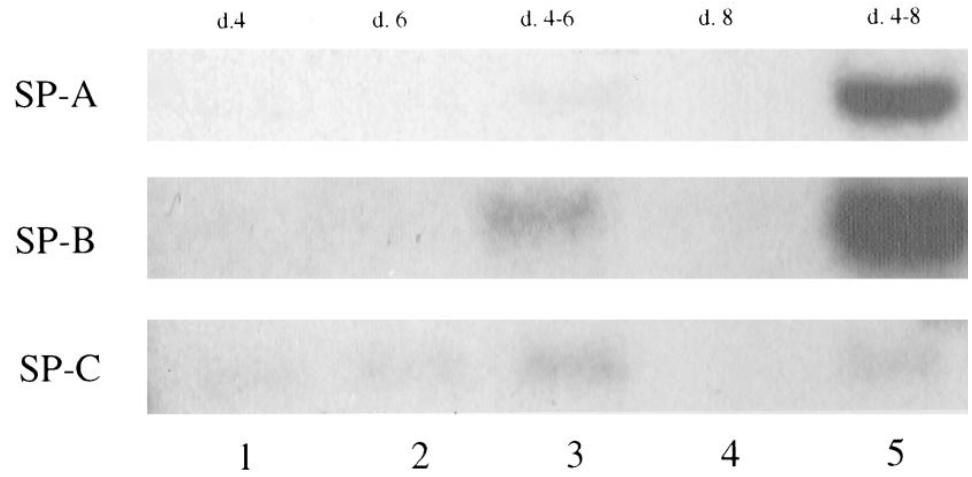

Figure 4. Effects of KGF on expression of mRNA for surfactant apoproteins -A, -B, and -C. In this representative Northern blot (n = 3), SP-A and -B are undetectable in AECs grown in MDSF on Day 4, 6, or 8 (*lanes 1, 2,* and *4,* respectively). SP-C is detectable at a very low level on Days 4 and 6 and undetectable on Day 8. AECs grown in MDSF plus KGF from Day 4 express increased levels of SP-A, -B, and -C mRNA by Day 6 (*lane 3*) compared with cells grown in MDSF alone (*lane 2*), with expression further increased for SP-A and -B by Day 8 (*lane 6*). Results shown are from the same blot as in Figure 3a, which has been reprobed for SP-A, -B, and -C. Expression of 18S rRNA is similar under all conditions.

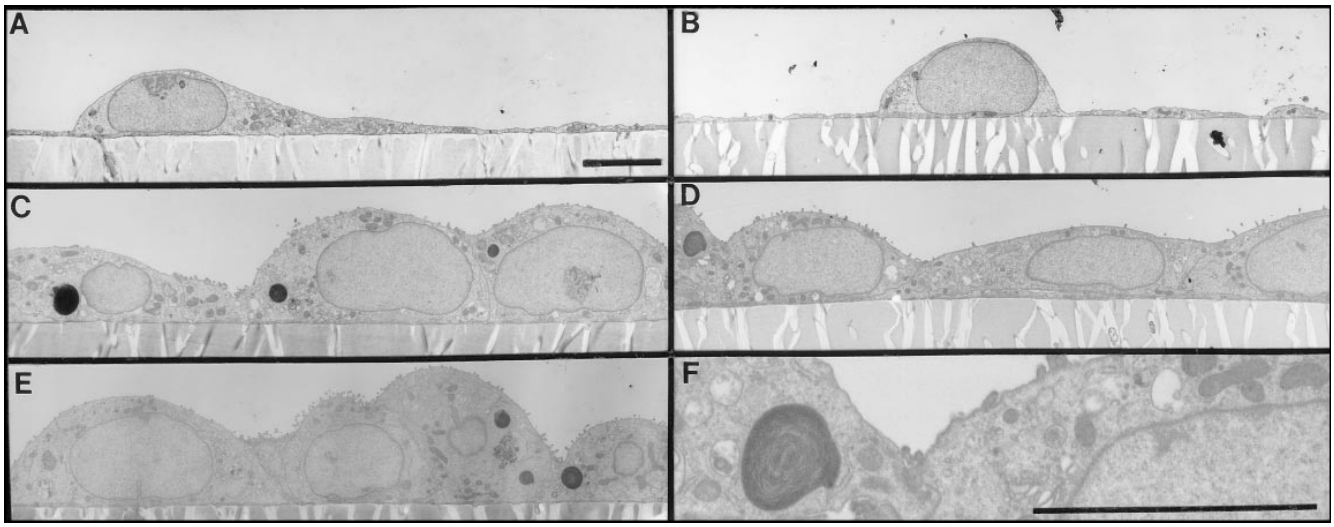

Figure 5. Effects of KGF on AEC morphology in primary culture. AECs maintained in MDSF for 4 d lose their lamellar bodies and begin to spread (*A*). The cells resemble AT1 cells on Day 8, developing prominent nuclei and attenuated cytoplasmic processes (*B*). In the presence of KGF from the time of plating, these changes are largely prevented. KGF-treated cells remain more cuboidal, with significant retention of lamellar bodies and absence of spreading on Day 4 (*C*) through Day 8 (*D*). *F* is a magnified view of *D*, showing a typical lamellar body in greater detail. Addition of KGF from Day 4 results in partial reversal of the Day 4 phenotypic changes seen in MDSF, with reappearance of some lamellar bodies and thickening of the cytoplasmic processes on Day 8 (*E*). Scale bars: (*A–E*) 5 μm; (*F*) 5 μm.

dent. The number of lamellar bodies in AECs grown in the presence of KGF was clearly greater than in AECs grown in the absence of KGF, although the KGF-treated cells did not appear to contain as many lamellar bodies as freshly isolated AT2 cells. Differences between KGF-treated and -untreated AECs were especially striking in Day 8 cells, in which no lamellar bodies were typically seen in cells grown in the absence of KGF. These morphologic results provide further evidence for the modulation of AEC phenotype by KGF *in vitro*.

Discussion

In this study, we have identified the water channel aquaporin 5 (AQP5) as an apical membrane protein in cultured alveolar epithelial cells and demonstrated that its expression can be regulated by the mitogenic peptide keratinocyte growth factor (KGF). Further, we have demonstrated reciprocal changes in the expression of AQP5 and the surfactant apoproteins effected by KGF, consistent with the notion of AQP5 as an AT1 cell marker *in vitro* as well as *in situ*, and with the role of KGF in modulating AEC phenotype. Finally, we have correlated KGF-induced changes in AQP5 and surfactant apoprotein expression with changes in AEC morphology, demonstrating that KGF causes decreases in AQP5 expression in parallel with a more AT2 cell-like morphology and increased SP-A, -B, and -C expression. These effects occur even in AEC monolayers that have already lost AT2 cell phenotypic properties, supporting the concept that AT1 cells can undergo reversible transdifferentiation toward the AT2 cell phenotype *in vitro*.

Evidence that AQP5 is useful as a marker of the AT1 cell phenotype in cultured AECs comes from several different types of data. First, Nielsen and coworkers (22) have obtained immunoelectron micrographic data demonstrating that rat AT1 cells express AQP5 on their apical membranes *in situ*. No expression of AQP5 was observed in AT2 cells, as indicated by absence of immunogold labelling using the same anti-AQP5 antibody reagent as used in the present study. Second, our current data indicate that freshly isolated AT2 cells express little or no AQP5, whereas cultured AEC monolayers express AQP5 predominantly, if not solely, on their apical surfaces. AQP5 expression in AEC monolayers thus parallels AQP5 expression in AT1 cells *in situ*. AQP5 has also been demonstrated to be present in the apical (but not basolateral) membranes of submandibular gland acinar cells, although it is entirely absent from intralobular ducts of the glands (33). Thus, AQP5 displays a polarized and cell-specific distribution in the lung that parallels its distribution in submandibular gland. Finally, our current data indicate that AQP5 expression increases in AECs with time in culture, in a fashion similar to other well-established markers of AT1 cell phenotype (i.e., VIIIB2 and T1α), in parallel with AT1 cell-like morphologic changes and inversely to changes in expression of AT2 cell markers. These findings confirm the notion that AQP5 is a specific marker of the AT1 cell phenotype both *in vitro* and *in situ*.

We detected low levels of AQP5 mRNA in freshly isolated AT2 cells in the experiments described herein, whereas AQP5 protein was either absent or faintly detectable in some cell preparations on Day 0. Because AQP5 is expressed only by AT1 cells in the adult rat alveolar epithelium *in situ*, the minor amount of AQP5 found could arise from the small degree of AT1 cell membrane contamination probably present in the freshly isolated AT2

cell suspensions. Alternatively, rapid induction of AQP5 during the steps involved in cell isolation could explain these findings, as similarly observed with T1α (8). The near total suppression of AQP5 on Day 8 for cells treated with KGF from Day 0, however, is most consistent with the concept that AQP5 expression is induced by factors that promote the AT1 cell phenotype and suppressed by those that promote the AT2 cell phenotype.

Evidence for the role of KGF as a modulator of cell phenotype comes from both *in vivo* and *in vitro* data. KGF has been shown to play a role in the development of epithelial tissues and in maintenance of epithelial integrity, and also directly influences the morphologic appearance and phenotypic characteristics of a number of epithelial cell types during development and adult life (12–14, 34–37). For example, the skin of transgenic mice in which a dominant-negative KGF receptor transgene is expressed in basal keratinocytes shows epidermal atrophy, abnormalities in the hair follicles, and dermal hyperthickening (36). Overexpression of KGF in livers of transgenic mice causes abnormalities in epithelial growth and differentiation within multiple organ systems and results in perinatal lethality (37). The resulting high systemic levels of KGF during the latter stages of embryogenesis are responsible for several phenotypic abnormalities, including epithelial hyperplasia, in kidney, lung, liver, pancreas, and urinary bladder. KGF is expressed in many tissues at increased levels following injury, consistent with a role for KGF in normal wound healing and repair (36). Abnormal expression of KGF may also be associated with the pathologic cycles of injury and repair in conditions such as inflammatory bowel disease, where KGF has been reported to be overexpressed in surgical specimens from affected patients (38).

Current data suggest that KGF plays a critical role in normal lung growth and development. KGF and KGF receptor (KGFR) are abundantly expressed in both fetal and adult rat lung (39). Inhibition of ligand binding via targeted expression of a dominant negative fibroblast growth factor (FGF) receptor in developing lung epithelium results in disruption of normal branching morphogenesis and alveolar epithelial cell cytodifferentiation (40). Animals described in this study died preterm, as did transgenic mice expressing KGF driven by the SP-C promoter in the embryonic lung (41). Embryonic rat lungs treated with KGF also show elevated expression of SP-C and distention of the distal epithelium into cystlike structures containing AT2 cells and/or their progenitors. Most recently, KGF was shown to stimulate fetal rat pulmonary epithelial cell growth and to promote the differentiation of fetal distal lung epithelium (FDLE) toward a distal alveolar epithelial cell phenotype (42). Thus, KGF may exert major effects on both airway and alveolar epithelial cell differentiation during development.

Data suggest that KGF can also modulate adult alveolar epithelial cell phenotype. Sugahara and colleagues (20) found that KGF induced a two- to threefold increase in steady state levels of mRNAs for SP-A and SP-B in cultured AECs. These effects were time dependent, as in our current study, and could be mimicked by treatment with acidic FGF (aFGF). In contrast, these investigators found no increase in SP-C mRNA in AECs after exposure to KGF. This discrepancy may have resulted from differences in culture conditions in the earlier study (e.g., use of EHS tumor basement membrane, which promotes retention of the AT2 cell phenotype) versus those in the present study, where increases in SP-C were noted to occur in cells that had already acquired AT1 cell properties (i.e., increased expression of AQP5 and AT1 cell-like morphology).

In the adult lung, AT2 cells are believed to be the progenitors of AT1 cells (1, 2). Although AT1 cells have been thought to be incapable of proliferation and to be terminally differentiated, this concept has come under question. Previous studies from our group (10) and from Shannon and colleagues (43) have shown that AECs grown on collagen gels demonstrate reciprocal changes in expression of AT2 and AT1 cell markers. These changes are to some extent reversed when cell shape is altered, implying a role of the cytoskeleton in transducing signals that promote or suppress cell differentiation in AECs. The potential reversibility of the AEC phenotype resulting from the effects of other modulators (11, 44) is currently unknown. Our current data, which show that treatment of AECs by KGF on later days in culture results in loss of AT1 cell characteristics, provide further evidence for reversibility between the AT2 and AT1 cell phenotypes.

In summary, expression of AQP5, a specific marker for AT1 cells *in situ*, increases in AECs as a function of time in culture. These results are consistent with transition of AT2 cells toward an AT1 cell phenotype. KGF is able both to prevent and reverse the transition toward the AT1 cell phenotype, as determined by expression of markers of AT1 (AQP5) and AT2 (surfactant apoproteins) cell phenotypes and by cell morphology. These findings lend support to the concepts that transdifferentiation between AT2 and AT1 cell phenotypes is at least partially reversible and that KGF can play a major role in modulating the AEC phenotype.

Acknowledgments: The authors note with appreciation the expert technical support of Martha Jean Foster and Monica Flores, and thank Dr. John Shannon for cDNA probes for SP-A, -B, and -C. This work was supported in part by the American Lung Association, the American Heart Association, the American Heart Association–Greater Los Angeles Affiliate, National Heart, Lung and Blood Institute NRSA HL09119 (L.S.K.), CIDA HL02836 (Z.B.), F32 HL09420 and the Allen & Hanburys Pulmonary Fellowship Award (D.M.L.), National Institutes of Health Research Grants HL03609 and HL51928 (R.L.L.), HL38578 and HL38621 (E.D.C.), and HL33991, HL48268, and EY11239 (P.A.), and the Hastings Foundation. Dr. Crandall is Hastings Professor of Medicine and Kenneth T. Norris, Jr., Chair of Medicine.

References

1. Evans, M. J., R. J. Stephens, and G. Freeman. 1975. Transformation of alveolar type II cells to type I cells following exposure to NO_2. *Exp. Mol. Pathol.* 22:142–150.
2. Adamson, Y. R., and D. H. Bowden. 1975. Derivation of type I cell epithelium from type II cells in the developing rat lung. *Lab. Invest.* 32:738–745.
3. Cheek, J. M., M. J. Evans, and E. D. Crandall. 1989. Type I cell-like morphology in tight alveolar epithelial monolayers. *Exp. Cell Res.* 84:375–387.
4. Christensen, P. J., S. Kim, R. H. Simon, G. B. Toews, and R. Paine, III. 1993. Differentiation-related expression of ICAM-1 by rat alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 8:9–15.
5. Danto, S. I., S. M. Zabski, and E. D. Crandall. 1992. Reactivity of alveolar epithelial cells in primary culture with type I cell monoclonal antibodies. *Am. J. Respir. Cell Mol. Biol.* 6:296–306.
6. Dobbs, L. G., M. C. Williams, and A. E. Brandt. 1985. Changes in biochemical characteristics and pattern of lectin binding of alveolar type II cells with time in culture. *Biochim. Biophys. Acta* 713:118–127.
7. Dobbs, L. G., M. C. Williams, and R. Gonzalez. 1988. Monoclonal antibod-

ies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. *Biochim. Biophys. Acta* 970:146–156.

8. Rishi, A. K., M. Joyce-Brady, J. Fisher, L. G. Dobbs, J. Floros, J. VanderSpek, J. S. Brody, and M. C. Williams. 1995. Cloning, characterization, and developmental expression of a rat lung alveolar type I cell gene in embryonic endodermal and neural derivatives. *Dev. Biol.* 167:294–306.
9. Weaver, T. A., and J. A. Whitsett. 1991. Function and regulation of expression of pulmonary surfactant-associated proteins. *Biochem. J.* 273:249–264.
10. Danto, S. I., J. M. Shannon, Z. Borok, S. M. Zabski, and E. D. Crandall. 1995. Reversible transdifferentiation of alveolar epithelial cells. *Am. Rev. Respir. Cell Mol. Biol.* 12:497–502.
11. Borok, Z., A. Hami, S. I. Danto, S. M. Zabski, and E. D. Crandall. 1995. Rat serum inhibits progression of alveolar epithelial cells toward the type I cell phenotype *in vitro*. *Am. Rev. Respir. Cell Mol. Biol.* 12:50–55.
12. Finch, P. W., J. S. Rubin, T. Miki, D. Ron, and S. A. Aaronson. 1989. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science* 245:752–755.
13. Housley, R. M., C. F. Morris, W. Boyle, B. Ring, R. Blitz, J. E. Tarpley, S. L. Ackerman, P. L. Devine, R. H. Whitehead, and G. F. Pierce. 1994. Keratinocyte growth factor induces proliferation of hepatocytes and epithelial cells throughout the rat gastrointestinal tract. *J. Clin. Invest.* 94:1764–1777.
14. Pierce, G. F., D. Yanagihara, K. Klopchin, D. M. Danilenko, E. Hsu, W. C. Kenney, and C. F. Morris. 1994. Stimulation of all epithelial elements during skin regeneration by keratinocyte growth factor. *J. Exp. Med.* 179:831–840.
15. Ulrich, T. R., E. S. Yi, K. Longmuir, S. Yin, R. Blitz, C. F. Morris, R. M. Housley, and G. F. Pierce. 1994. Keratinocyte growth factor is a growth factor for type II pneumocytes *in vivo*. *J. Clin. Invest.* 93:1298–1306.
16. Panos, R. J., J. S. Rubin, K. G. Csaky, S. A. Aaronson, and R. J. Mason. 1993. Keratinocyte growth factor and hepatocyte growth factor/scatter factor are heparin-binding growth factors for alveolar type II cells in fibroblast-conditioned medium. *J. Clin. Invest.* 92:969–977.
17. Panos, R. J., P. M. Bak, W. S. Simonet, J. S. Rubin, and L. J. Smith. 1995. Intratracheal instillation of keratinocyte growth factor decreases hyperoxia-induced mortality of rats. *J. Clin. Invest.* 96:2026–2033.
18. Mason, C. M., B. P. Guery, W. R. Summer, and S. Nelson. 1996. Keratinocyte growth factor attenuates lung leak induced by alpha-naphthylthiourea in rats. *Crit. Care Med.* 24:925–931.
19. Toshiyuki, Y., R. R. Deterding, W. S. Simonet, J. M. Shannon, and R. J. Mason. 1996. Keratinocyte growth factor reduces lung damage due to acid instillation in rats. *Am. J. Respir. Cell Mol. Biol.* 15:433–443.
20. Sugahara, K., J. S. Rubin, R. J. Mason, E. L. Aronsen, and J. M. Shannon. 1995. Keratinocyte growth factor increases mRNAs for SP-A and SP-B in adult rat alveolar type II cells in culture. *Am. J. Physiol.* 269:L344–L350.
21. Raina, S., G. M. Preston, W. B. Guggino, and P. Agre. 1995. Molecular cloning and characterization of an aquaporin cDNA from salivary, lacrimal, and respiratory tissues. *J. Biol. Chem.* 270:1908–1912.
22. Nielsen, S., L. S. King, B. M. Christensen, and P. Agre. 1997. Aquaporins in complex tissues: II. Subcellular distribution in respiratory and glandular tissues of rat. *Am. J. Physiol.* 42:C1549–C1561.
23. Carter, E. P., M. A. Matthay, J. Farinas, and A. S. Verkman. 1996. Transalveolar osmotic and diffusional water permeability in intact mouse lung measured by a novel surface fluorescence method. *J. Gen. Physiol.* 108:133–142.
24. Dobbs, L. G., R. Gonzalez, and M. C. Williams. 1986. An improved method for isolating type II cells in high yield and purity. *Am. Rev. Respir. Dis.* 134:141–145.
25. Borok, Z., S. I. Danto, S. M. Zabski, and E. D. Crandall. 1994. Defined medium for primary culture de novo of adult rat alveolar epithelial cells. *In Vitro Cell Dev. Biol.* 30A:99–104.
26. Mason, R. J., S. R. Walker, B. A. Shields, and J. E. Henson. 1985. Identification of rat alveolar type II epithelial cells with a tannic acid and polychrome stain. *Am. Rev. Respir. Dis.* 131:786–788.
27. Gottardi, C. J., and M. J. Caplan. 1992. Cell surface biotinylation in the determination of epithelial membrane polarity. *J. Tissue Cult. Methods* 14: 173–180.
28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature (London)* 227:680–685.
29. Towbin, H. H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA* 76:43–50.
30. King, L. S., S. Nielsen, and P. Agre. 1997. Expression of aquaporin water channel proteins in complex tissues: I. Developmental patterns in respiratory tract and glandular tissue of rat. *Am. J. Physiol.* 42:C1541–C1548.
31. Zhang, X.-L., S. I. Danto, Z. Borok, J. T. Eber, P. Martín-Vasallo, and R. L. Lubman. 1996. Identification of Na^+,K^+-ATPase β subunit in alveolar epithelial cells. *Am. J. Physiol.* 272:L85–L94.
32. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* 162:156–159.
33. He, X., C.-M. Tse, M. Donowitz, S. L. Alper, S. E. Gabriel, and B. J. Baum. 1997. Polarized distribution of key membrane transport proteins in the rat submandibular gland. *Pflügers Arch.* 433:260–268.
34. Rubin, J. S., D. P. Bottaro, M. Chedid, T. Miki, D. Ron, H.-G. Cheon, W. G. Taylor, E. Fortney, H. Sakata, P. W. Finch, and W. J. LaRochelle. 1995. Keratinocyte growth factor. *Cell Biol. Int.* 19:399–411.
35. Peehl, D. M., S. T. Wong, and J. S. Rubin. 1996. KGF and EGF differentially regulate the phenotype of prostatic epithelial cells. *Growth Regul.* 6:22–31.
36. Werner, S., H. Smola, X. Liao, M. T. Longaker, T. Krieg, P. H. Hofschneider, and L. T. Williams. 1994. The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. *Science* 266:819–822.
37. Nguyen, H. Q., D. M. Danilenko, N. Bucay, M. L. DeRose, G. Y. Van, A. Thomason, and W. S. Simonet. 1996. Expression of keratinocyte growth factor in embryonic liver of transgenic mice causes changes in epithelial growth and differentiation resulting in polycystic kidneys and other organ malformations. *Oncogene* 12:2109–2119.
38. Brauchle, M., M. Madlener, A. D. Wagner, K. Angermeyer, U. Lauer, P. H. Hofschneider, M. Gregor, and S. Werner. 1996. Keratinocyte growth factor is highly overexpressed in inflammatory bowel disease. *Am. J. Pathol.* 149:521–529.
39. Shiratori, M., E. Oshika, L. P. Ung, G. Singh, H. Shinozuka, D. Warburton, G. Michalopoulos, and S. L. Katyal. 1996. Keratinocyte growth factor and embryonic rat lung morphogenesis. *Am. J. Respir. Cell Mol. Biol.* 15:328–338.
40. Peters, K., S. Werner, X. Liao, S. Wert, J. Whitsett, and L. Williams. 1994. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. *EMBO J.* 13:3296–3301.
41. Simonet, W. S., M. L. DeRose, N. Bucay, H. Q. Nguyen, S. E. Wert, L. Zhou, T. R. Ulich, A. Thomason, D. A. Danilenko, and J. A. Whitsett. 1995. Pulmonary malformation in transgenic mice expressing human keratinocyte growth factor in the lung. *Proc. Natl. Acad. Sci. USA* 92:12461–12465.
42. Deterding, R. R., C. R. Jacoby, and J. M. Shannon. 1996. Acidic fibroblast growth factor and keratinocyte growth factor stimulate fetal rat pulmonary epithelial growth. *Am. J. Physiol.* 271:L495–L505.
43. Shannon, J. M., S. D. Jennings, and L. D. Nielsen. 1992. Modulation of alveolar type II cell differentiated function in vitro. *Am. J. Physiol.* 262:L427–L436.
44. Hastings, R. H., D. Summers-Torres, T. C. Cheung, L. S. Ditmer, E. M. Petrin, D. W. Burton, R. G. Spragg, J. Li, and L. J. Deftos. 1996. Parathyroid hormone-related peptide, an autocrine regulatory factor in alveolar epithelial cells. *Am. J. Physiol.* 270:L353–L362.