Identification of Genes Differentially Expressed in Rat Alveolar Type I Cells

Katherine Dahlin, Edward M. Mager, Lennell Allen, Zachary Tigue, Lee Goodglick, Madhuri Wadehra, and Leland Dobbs

Departments of Medicine, Pediatrics, and Cardiovascular Research Institute, University of California San Francisco, San Francisco; and the David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California

Although ~ 98% of the internal surface area of the lung is lined by alveolar type I cells, little is known about the functions of this cell type. Using freshly isolated rat type I and type II cells, we created a subtraction library by suppression subtractive hybridization to identify genes differentially expressed by type I cells. We identified twelve genes of known function that are differentially expressed by type I cells. Differential expression of all 12 genes was confirmed by Northern blotting; we confirmed differential expression by immunocytochemistry for 3 genes for which suitable antibodies were available. Most of the genes code for proteins that are multifunctional. From the known functions of these genes, we infer that type I cells may play a role in the maintenance of normal alveolar homeostasis and protection from injury, lung development and remodeling, host defense, tumor/growth suppression, and surfactant metabolism, among other functions.

The alveolar region contains more than 99% of the large internal surface area of the lung; in rats, 98% of this surface area is lined by alveolar type I cells, the remainder by type II cells (1). Type II cells synthesize, secrete, and recycle pulmonary surfactant (2), transport ions (3, 4), participate in immune responses of the lung (5–7), and act as progenitor cells in lung injury (8, 9). In response to injury in rodent lungs, type II cells proliferate and transdifferentiate into type I cells (8); some evidence suggests that this process may occur during development (9).

We embarked on the current experiments with the goal of identifying genes differentially expressed by type I cells. The underlying rationale for the work is that little is known about the functions of type I cells. For many years, the accepted belief has been that, other than barrier functions, the type I cell has no important physiologic functions in the lung. In the last 5 years, type I cells have been shown to have a very high osmotic water permeability (10), to contain transport proteins (11, 12) and to have the capacity to transport ions (12), suggesting that type I cells may play an important role in lung liquid homeostasis. These results serve as a basis for the more general concept that type I cells, in addition to serving barrier functions, have other important functions. Previously known molecules that are expressed in type I cells (recently reviewed in Ref. 13) include RTI40 (T1α) (14–17), caveolin-1 (18, 19), aquaporin 5 (20), receptor for advanced glycation end products (RAGE) (21), carboxypeptidase M (22), and, more recently, plasminogen activator inhibitor (PAI-1), P2X purinoceptor (P2X4), and cyclin-dependent kinase 4 inhibitor 2B ($P15^{INK4B}$) (23). Some of these previously identified proteins are expressed by type II cells or other cell types, in addition to type I cells. We initially employed differential display PCR (DD-PCR) in an attempt both to identify cell-specific markers for each cellular phenotype and to provide insights about previously unanticipated functions of each cell type. We found, as others have, that DD-PCR yielded many candidate sequences, but also produced a high number of false positives (Mager, Vanderbilt, and Dobbs, unpublished observations). Although we were able to identify an additional marker for the type II cell phenotype (7), several novel candidate markers for the type I cell phenotype did not prove amenable to 5′ extension strategies to obtain sufficient sequence information for gene identification. For this reason, we turned to suppression subtractive hybridization (SSH) to generate a subtracted library to identify marker genes for the type I cell phenotype. Type II cells are believed to be progenitors for type I cells and, by virtue of this fact, the two cell types are believed to be closely related, although they have strikingly different morphologic characteristics. The rationale for making a subtraction library between type I and type II cells is based on the following: (*i*) very little is known about the type I cell, and any potential type I cell functions we can infer from gene discovery may be useful; (*ii*) determining genes differentially expressed in type I cells may be useful in generating testable hypotheses regarding different functions of these two cell types; (*iii*) differentially expressed genes can be used as additional molecular markers of the type I cell phenotype.

Some recent studies have used cultured type II cells, which express some markers of the type I cell phenotype, as a model of type I cells. However, it is unclear how similar the cultured type II cell model system is to native type I cells. Therefore, we used freshly isolated type I cells to generate a subtracted library to identify genes differentially expressed by type I cells, in comparison to type II cells. Of 576 screened clones, we identified 53 clones identifying candidate marker genes for the type I cell phenotype. Thirty-two of these 53 clones contained identical sequence to 14 genes of known function, and 21 clones did not show significant similarity to known genes in the public databases at the National Center for Biotechnology Information (NIH, Bethesda, MD). Twelve of the fourteen genes of known function were confirmed by Northern blotting to be expressed in type I cells, but not in type II cells or alveolar macrophages (AM). Two of the fourteen genes were not differentially expressed by Northern blotting. The presence of several genes of known functions in type I cells suggests potential functions for this cell type and may suggest new experimental approaches to the nascent field of type I cell biology. The results of these studies provide additional new markers for the type I cell phenotype and may lead to new directions for the study of type I cell functions.

(*Received in original form November 21, 2003 and in revised form June 10, 2004*)

Address correspondence to: Leland G. Dobbs, M.D., Suite 150, 3333 California St., San Francisco, CA 94118. Email: dobbs@itsa.ucsf.edu

Abbreviations: alveolar macrophage, AM; annexin VIII, ANXA8; α crystallin B, CRYAB; epithelial membrane protein 2, EMP2; epithelial sodium channel, ENaC; inducible nitric oxide synthase, iNOS; c-Jun N-terminal kinases, JNK; mitogen-activated protein kinase, MAPK; matrix metalloproteinase, MMP; group IIA secretory phospholipase A2, sPLA2-IIA; protein kinase C alpha, PKCα; phosphatidylserine, PS; receptor for advanced glycation end products, RAGE; semaphorin 3F, Sema3F; serum deprivation response protein, SDPR; suppression subtractive hybridization, SSH; tissue inhibitor of metalloproteinase 3, TIMP3; topoisomerase III alpha, TOP3A; tumor suppressor of lung cancer 1, TSLC1; vascular endothelial growth factor, VEGF.

Am. J. Respir. Cell Mol. Biol. Vol. 31, pp. 309–316, 2004
Originally Published in Press as DOI: 10.1165/rcmb.2003-0423OC on June 17, 2004
Internet address: www.atsjournals.org

Materials and Methods

Cell Preparations

Type II cells were isolated from the lungs of Sprague-Dawley specific pathogen–free rats (Simonsen Laboratories, Gilroy, CA) by previously described methods (24); type I cells were removed by negative selection with magnetic beads (25). The type II cell preparation used for these experiments contained 85% type II cells and $< 0.1\%$ type I cells by analysis of cytocentrifuged preparations stained with monoclonal antibodies specific for apical membranes of each cell type (14, 24) and then incubated with appropriate secondary antibodies (Alexa 594–anti-mouse IgG1; Molecular Probes, Eugene, OR; and FITC–anti-mouse IgG3; Cappel [ICN], Irvine, CA) (Figure 1). The remaining cells consisted of AM and lymphocytes, as identified by their typical appearance after modified Papanicolaou staining (24).

Type I cells were isolated as previously described (10). The preparations used in these experiments contained $\sim$ 80% type I cells and $< 2\%$ type II cells. The remaining cell constituents were macrophages and lymphocytes.

AM were obtained by bronchoalveolar lavage; the preparation contained 100% macrophages by modified Papanicolaou staining and no type I or type II cells by immunostaining.

Creation of SSH Libraries

Total RNA extracted from each of the cell preparations was used to construct an SSH library employing the PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA); 1 μg of type I cell RNA was used as the "tester" and 1 μg of RNA obtained from a pool of cells containing 75% type II cells and 25% AM was used as the "driver." The macrophages were added to the type II cells to ensure adequate subtraction of this cell type, which is present in preparations of both type I and type II cells. The oligo-dT primer (CDS) was used in synthesizing first-strand cDNA, which was then processed according to the protocol. The resulting double-stranded cDNAs were ligated into the pT-Adv vector (Clontech) and transformed into Electromax DH5α-E cells (Invitrogen, Carlsbad, CA).

Screening of Subtracted Libraries

For screening, equal amounts of the PCR-amplified inserts from 576 clones were spotted onto four identical filters. These were screened with ^{32}P-labeled probes made from cDNAs of type I cell, type II cell, "forward" (type I-type II), or "reverse" (type II-type I) subtracted RNAs. Candidate genes were selected by comparing results of screening with these probes. Two criteria were used to select promising candidate genes: (*i*) detection by forward subtracted probe [type I-type II], and (*ii*) detection by type I and type II unsubtracted probes. No clones were selected that hybridized with either type II unsubtracted or the type II-type I subtracted probes. A total of 53 clones were selected as candidate genes differentially expressed by type I cells.

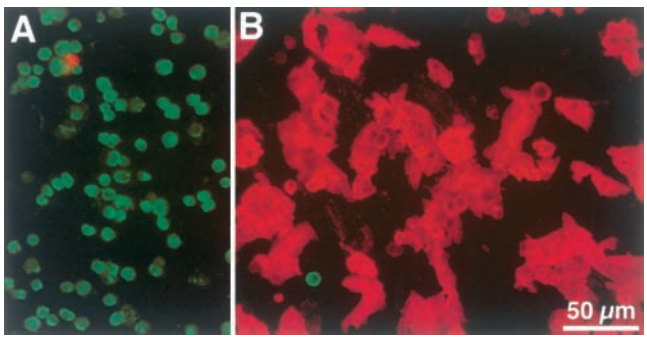

Figure 1. Double-label immunofluorescence of cytocentrifuged preparations of TI and TII cells. Cytocentrifuged preparations of TII cells (*A*) and TI cells (*B*) were doubly stained with antibodies against RTI40 and RTII70 and appropriate secondary antibodies. RTI40 (TI cell) is *red*, RTII70 (TII) is *green*. (For details, *see* text.)

Northern Blot Analysis

Total RNA was extracted from preparations of type I cells, type II cells, and AM using QIAGEN's RNeasy Total RNA Isolation Kit (QIAGEN Inc, Valencia, CA). We obtained ~ 0.5 μg RNA/10^6 TI cells and ~ 0.2 μg RNA/10^6 TII cells. One microgram of RNA from each cell preparation was used for Northern analysis, and a total of three replicate Northern blots were performed.

Immunocytochemistry

Tissue was fixed, frozen, sectioned, and processed for immunocytochemistry as previously described (26). Thin 2-μm cryostat sections were incubated overnight with the following primary antibodies: anti-HTI56 (mouse monoclonal antibody directed against an integral membrane protein specific in human lung to type I cells [26]) at a 1:1 dilution, anti–epithelial membrane 2 (EM2) rabbit polyclonal, (27, 28) (1:400), anti–semaphorin 3F (Sema3F) rabbit polyclonal (1:100; Chemicon International Inc., Temecula, CA), and anti–tissue inhibitor of metalloproteinase 3 (TIMP3) rabbit polyclonal (1:100; Chemicon International). Proteins were visualized by incubating sections with goat anti-rabbit or goat anti-mouse IgG conjugated to Alexa 594 at 1:3,000 (Molecular Probes Inc.). Fluorescence and phase contrast images were captured at 2,600 × 2,060 dpi with a Leica DC500 camera on a Leica Orthoplan microscope.

Results

Representative double-label immunofluorescence images of cytocentrifuged preparations of TI and TII cells are shown in Figure 1. Cells were stained with antibodies specific for TI cells (RTI40) and TII cells (RTI70); TI cells can be identified by *red staining*; type II cells by *green staining*. The TI cells are larger than TII cells, as documented for TI cells *in situ* (1). Although the preparations of TII cells we used contained $< 0.1\%$ TI cells, we have shown a field containing one TI cell ($\sim 1\%$) so that the double-staining will be readily apparent and the magnification suitable for recognizing individual cells. The field shown of the TI cell preparation contains $\sim 1.5\%$ TII cells.

Fifty-three clones were selected from promising candidate clones by screening (Figure 2) and were sequenced; insert sizes ranged from 189–874 bp. Of these, 32 clones were identical in sequence to 14 genes with previously ascribed functions. Eleven of these clones were in coding regions, the rest in 3′-UTRs or in overlapping coding and 3′-UTR regions. Some of these genes were identified by multiple clones (up to seven separate clones for caveolin-1); seven genes were identified by a single clone each; differential expression was not confirmed for two of these genes (*see* Table 1).

Differential expression in type I cells (compared with type

TABLE 1. Number of clones identifying type I cell-specific genes

Gene	No. of Clones
ANXA8	2
CRYAB	4
Caveolin-1	7
EMP2	3
sPLA2-IIA	3
RAGE	1
RT140	4
SEMA3F	1
SDPR	1
TIMP3	1
TOP3A	1
TSLC1	2
Not confirmed	2

Definition of abbreviations: ANXA8, annexin VIII; CRYAB, α crystallin B; EMP2, epithelial membrane protein 2; RAGE, receptor for advanced glycation end products; SDPR, serum deprivation response protein; SEMA3F, semaphorin 3F; TIMP3, tissue inhibitor of metalloproteinase 3; TOP3A, topoisomerase III α; TSLC1, tumor suppressor of lung cancer 1.

II cells and alveolar macrophages) was confirmed for 12 (out of the 14 candidate genes) by Northern blotting (Figure 3); two candidate genes were not differentially expressed by Northern blotting. Three genes, caveolin-1, RTI-40, and RAGE, have previously been shown to be expressed in type I cells; the remaining nine identified genes were not previously known to be differentially expressed in type I cells. There is a report of RAGE expression by type II cells by *in situ* hybridization (29), but our results corroborate at the mRNA level the finding by Fehrenbach and coworkers (21), who used immunoelectron microscopy and colocalization with cell-specific markers to localize RAGE expression in the lung to type I cells. In addition to the 14 candidate genes of known function, we found 21 clones, all in noncoding regions, that had no significant homology with known genes when all nonredundant databases of the basic local alignment and search tools (BLAST) at the National Center for Biotechnology Information (NIH) were searched. Northern blots of these genes have not been performed and therefore differential expression is not confirmed and these results are of uncertain significance.

To confirm whether candidate gene products were differentially expressed not only in isolated cells, but also in the lung *in situ*, we performed immunocytochemistry with antibodies against those gene products for which appropriate antibodies were available. We used human lung tissue because the antibodies worked best with human tissue. We confirmed differential expression patterns in type I cells for EMP2, TIMP3, and Sema3F. In Figures 4A and 4B, phase contrast views are paired with staining by HT156, an antibody against an apical membrane protein of human type I cells (26); the type II cells, recognized by their lucent lamellar bodies, do not show staining. Similar patterns are seen in Figures 4C and 4D (antibody against EMP2); Figures 4E and 4F (SEMA3F); and Figures 4G and 4H (TIMP3). Type II cells are negative in each of these panels.

Discussion

Suppression subtractive hybridization is a powerful method for identifying differentially expressed genes that may be in low abundance; the prolonged time used for secondary hybridization is important in the identification of candidate low abundance mRNAs. We used freshly isolated rat alveolar type I and type II cells to create subtraction libraries by SSH. The preparations of type II cells contained $< 0.1\%$ type I cells and the type I cell preparations $< 2\%$ type II cells by immunocytochemical evaluation with cell-specific antibodies. By screening dot blots with pooled RNA from type I cells, type II cells, "forward" (type I-type II), or "reverse" (type II-type I) subtracted probes, a total of 53 clones, out of 576 screened, were selected as candidate genes differentially expressed by type I cells. Thirty-two of these clones contained sequences identical to 14 known genes. By Northern blotting, 12 of these 14 genes were confirmed to be expressed preferentially in type I cells, in comparison with type II cells or alveolar macrophages. The genes identified in this study do not represent an exhaustive list of differentially expressed type I cell genes. Three of these genes, RTI40, caveolin-1, and RAGE, had previously been reported to be expressed in type I cells; RT-140 and caveolin were found in four and seven different clones, respectively. Aquaporin 5, which is known to be expressed in type I cells and not type II cells (20) was not detected in our subtraction library. The remaining nine genes are of potential use as new markers of the type I cell phenotype and as potential indicators of previously unknown functions of type I cells. Although most of these genes are multifunctional, taken together, they can be useful in broadening our understand-

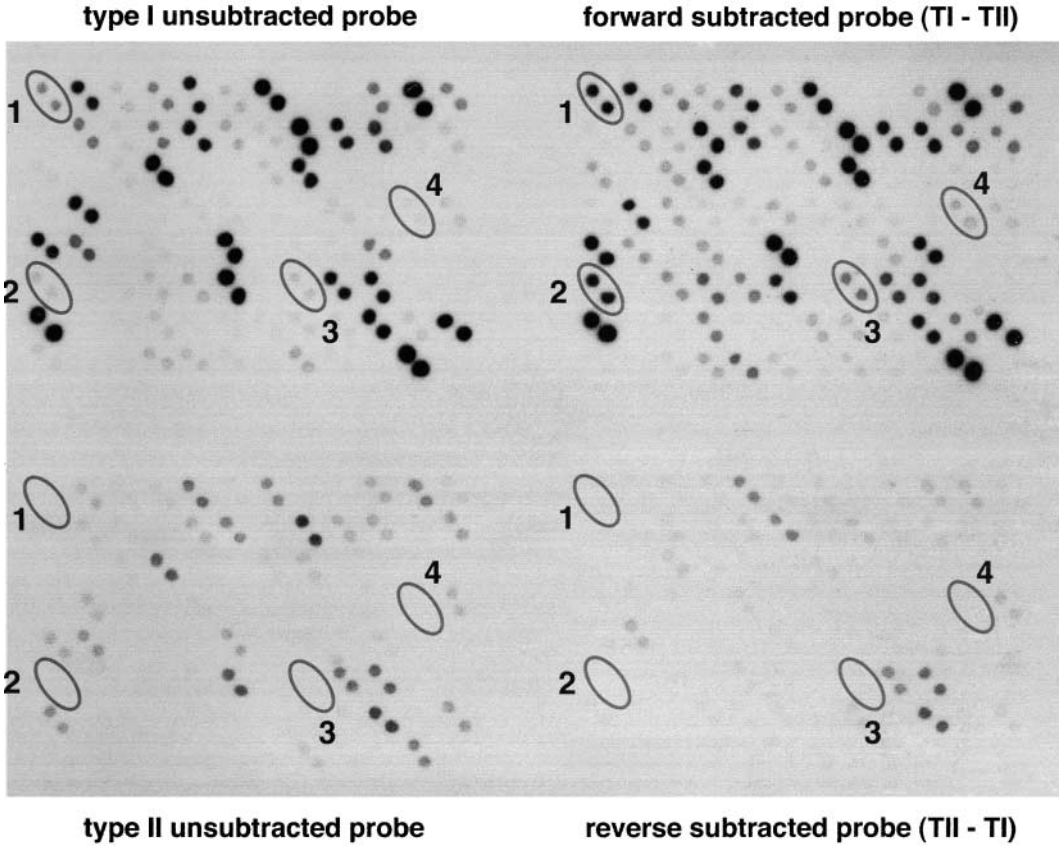

Figure 2. Differential screening of amplimers generated by SSH. Clones were spotted in duplicate onto four membranes which were then hybridized with different radiolabeled cDNA probes, as described in Materials and Methods. *Circles* identify examples of positive clones chosen for further screening; the numbers indicate genes subsequently identified through Blast analysis. (*1*) α Crystallin B, (*2*) Phospholipase A2 group IIA, (*3*) RT140, (*4*) ANXA8.

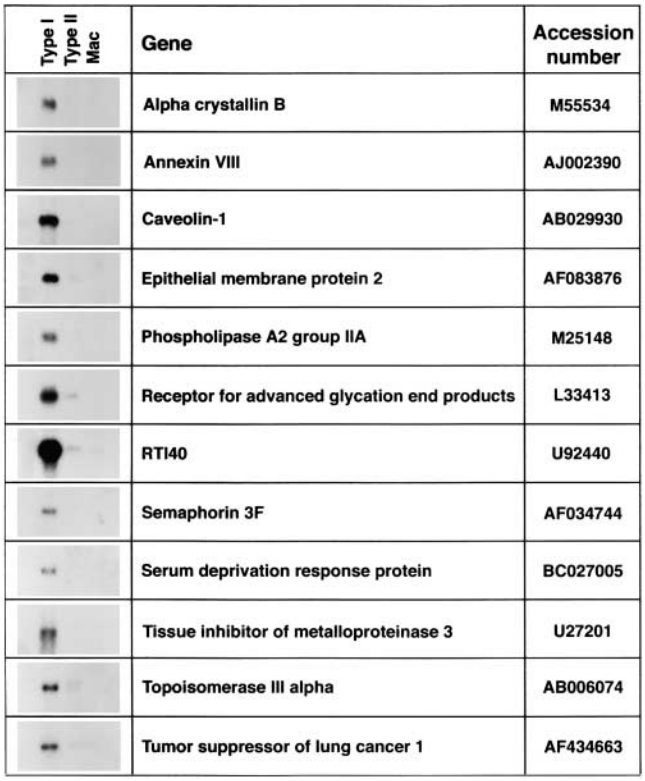

Figure 3. Northern blots of type I differentially expressed genes. Northern blotting was performed as described in MATERIALS AND METHODS. The preparations of TI cells contained < 2% type II cells, the preparations of TII cells contained < 0.1% type I cells. The preparations of alveolar macrophages contained no detectable TI or TII cells.

ing of potential functions of type I cells (Table 2). We have grouped the genes rather arbitrarily into six functional categories; however, the following categories do not contain an exhaustive description nor all of the potential functions of each gene. Figure 5 contains a cartoon illustrating known and hypothetical functions of type I cells.

1. Maintenance of Normal Alveolar Architectural Homeostasis and Protection from Injury: CRYAB, TIMP3, ANXA8

Several of the genes differentially expressed by type I cells have "cell-protective" functions. α Crystallin B (CRYAB), a small heat shock protein upregulated by various stress stimuli, protects enzymes from inactivation (30); overexpression is protective against ischemia-induced damage and stress-induced apoptosis (31, 32). Tissue inhibitor of metalloproteinase 3 (TIMP3), by inhibiting ADAM17 (TNF-α convertase) (33), may prevent activation of the proinflammatory cascade. In addition, TIMP3 inhibits matrix metalloproteinase (MMP) activity, maintaining extracellular matrix (ECM) integrity. The TIMP3 null mutant mouse, in which MMP activity is increased, exhibits progressive airspace enlargement without apparent inflammation and results in early lethality (34), demonstrating the importance of TIMP3 in the maintenance of normal alveolar architecture. TIMP3 also blocks binding of VEGF to VEGFR2 in a chick chorioallantoic membrane model system (35), thereby inhibiting downstream signaling and angiogenesis. Annexin VIII (ANXA8) inhibits coagulation and PLA2 activity (36). We infer from the functions of this group of genes that they may act either to prevent damage to type I cells or to promote alveolar structural homeostasis.

2. Host Defense and Proinflammatory Properties: sPLA2-IIA, RAGE

The protective functions described in the last paragraph may be in balance with potential proinflammatory functions. Expression of secretory phospholipase A2 group IIA protein (sPLA2-IIA), which is bactericidal (37), is increased after acute lung injury (38). The type I cell, as well as activated or cultured macrophages (39) (but not quiescent macrophages; *see* Northern blot in Figure 3) is a potential source for phospholipase A2. sPLA2-IIA causes an increase in production of chemokines and adhesion molecules in endothelial cells (40), induces iNOS (inducible NO synthase) (41), and can act as a mitogen (42). These functions of sPLA2-IIA suggest that type I cells may play a role in innate host defense. RAGE, a multiligand receptor of the immunoglobulin superfamily, can mediate proinflammatory functions by a variety of different mechanisms. These include mediating the effects of AGEs that activate metalloproteinases (43).

3. Development and Remodeling: SEMA3F, RTI40, EMP2, Caveolin-1, RAGE, TIMP3

Some of these genes have the capacity to modulate tissue development and remodeling. Sema3F mRNA is weakly expressed in distal epithelium and mesenchyme in early lung development. Later in gestation, expression is increased (44). In lung bud cultures, Sema3F stimulates branching morphogenesis and cell proliferation, the latter by promoting progression into S-phase (45). Although the function of RTI40 remains unknown, experiments with null-mutant mice have demonstrated that the gene is important for normal lung development (46). Epithelial membrane protein 2 (EMP2) interacts with β1 integrins and selectively increases surface expression of α6β1, altering adhesion of cells to matrix proteins (47). Caveolin-1, which is found in both endothelial cells and type I cells (19), is important for normal development. Null mutant mice for caveolin-1 develop cardiac hypertrophy and abnormally thickened septae (48). Because it can bind ligands such as amphoterin (chromatin proteins which can be secreted) (49), and S100 proteins (50), RAGE can modulate important biological processes in development such as cell motility (50), cell survival (50, 51), and differentiation (51). TIMP3, as discussed earlier, prevents matrix degradation by MMPs and may also alter the functions of growth factors bound to matrix (reviewed in Ref. 52).

4. Tumor/Growth Suppression: TSLC1, TIMP3, EMP2, SEMA3F, Caveolin-1, sPLA2-IIA

Suppression of cellular proliferation is a function shared by many of these genes. Tumor suppressor of lung cancer 1 (TSLC1), which is identical to three independently characterized genes, IGSF4, SglGSF, and SynCAM, has reduced or absent expression in lung cancer cell lines; reduced expression of TSLC1 is correlated with promoter hypermethylation and the formation of epithelial tumors (53). Promoter hypermethylation of TIMP3 has also been observed in numerous human cancers (54). EMP2, a member of the growth arrest–specific 3 (GAS3/PMP22) family of small hydrophobic membrane proteins, suppresses B-cell lymphoma tumorigenicity, and induces apoptosis under conditions of growth factor withdrawal (28). Semaphorin 3F (SEMA3F), in contrast to its stimulatory effects on cell proliferation in normal developing lung, can inhibit cell attachment and spreading in tumor cell lines (55). Expression of caveolin-1 negatively regulates cell cycle progression by inducing G0/G1 arrest via a p53/p21-dependent mechanism (56). In several types of cancers, sPLA2-IIA expression is upregulated in an apparent host defense and exerts a tumor-suppressing effect (57). Interestingly, half of the genes of known function that we identified have

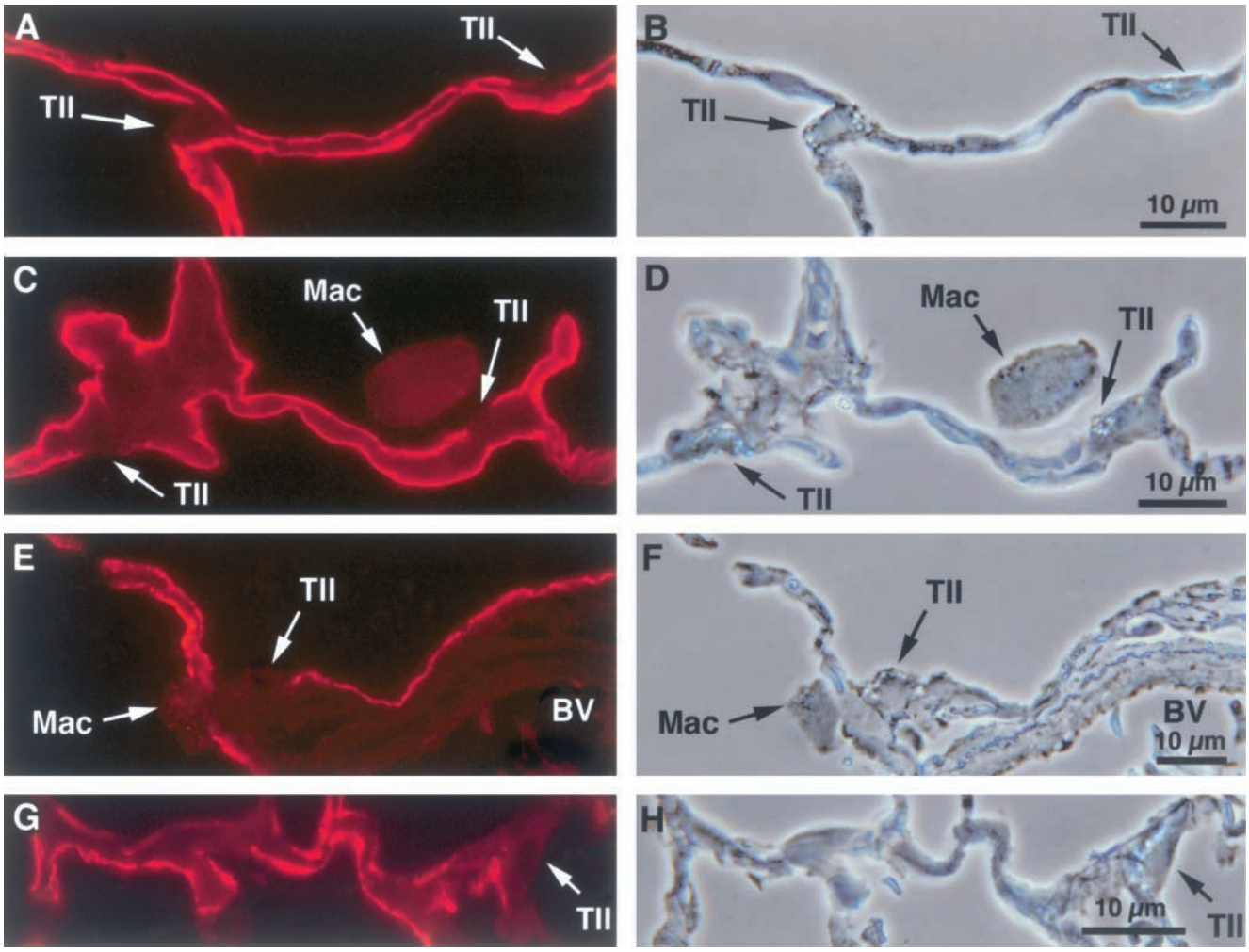

Figure 4. Immunocytochemistry of differentially expressed genes in human lung tissue. Immunocytochemistry was performed as described in Materials and Methods; *panels* shown are matching fluorescence and phase contrast views for each antibody. Type II cells are recognized in these 2-μ-thick cryostat sections by their typical intracellular lucent lamellar bodies. Alveolar macrophages can be recognized by their intraluminal location in normal lung. The linear staining pattern (*red*) is typical of localization to TI cells. (*A*, *B*) Matching fluorescence and phase contrast images of human lung tissue stained with HTI56, an antibody specific for type I cells in the human lung, demonstrating staining of type I cells but not type II cells. (*C*, *D*) Fluorescence pattern of epithelial membrane protein 2 (EMP2); (*E*, *F*) Semaphorin 3F localization. (*G*, *H*) Localization of tissue inhibitor of metalloproteinase 3 (TIMP3); staining is more patchy than with the previous three antibodies, but still clearly localized to TI cells.

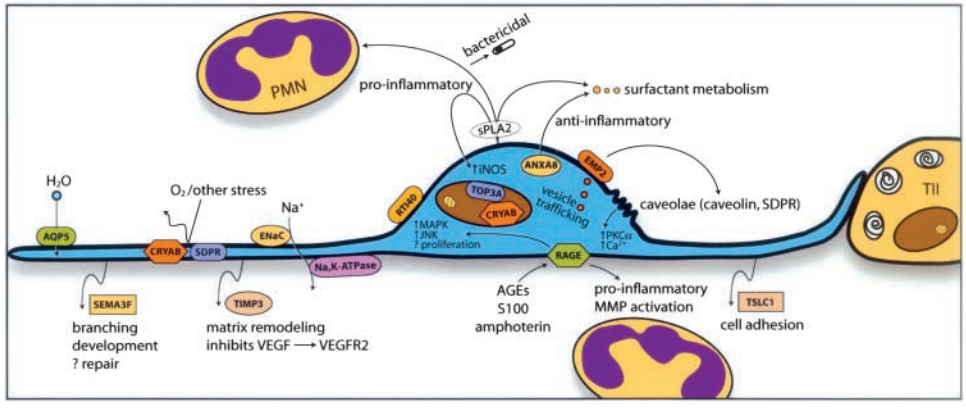

Figure 5. Cartoon representing known and hypothetical functions of type I cells. Water transport across the apical surface occurs via AQP5 (aquaporin 5); Na^+ and K+ transport via ENaC (epithelial sodium channel) and Na,K-ATPase. TIMP3 is critical for maintenance of normal extracellular matrix and in preventing emphysema. CRYAB and SDRP may be important in the defense against oxidative and other stress. TSLC1, a member of the immunoglobulin superfamily, mediates cell adhesion and cell–cell interactions. The function of RTI40 is unknown, but the gene has been shown to be important for normal lung development. RAGE is localized mainly to the basal membrane; the receptor binds various proteins such as AGEs, S100, and amphoterin, resulting in diverse downstream biologic events, such as stimulating MAPK and JNK pathways, proliferation, activation of MMPs, and proinflammatory responses. EMP2 has complex properties, including facilitating protein trafficking, altering adhesion of cells to matrix proteins, and inhibiting caveolin-1 expression. sPLA2 also has many potential functions, including bactericidal and proinflammatory functions, stimulation of intracellular iNOS, and surfactant metabolism. Of the genes shown in this diagram, all with the exception of ENaC and Na,K-ATPase are expressed in type I cells but not type II cells; ENaC and Na, K-ATPase are expressed in both cell types. Details of gene functions and references are described in the text and Table 2. The diagram is not to scale.

TABLE 2. Characteristics of differentially expressed genes

Gene	Tissue Expression	Cellular Location	Functions
ANXA8	Highest in lung; also in skin, liver, and kidney (71)	Cytoplasm	Anticoagulant (36) Inhibitor of PLA2 (36) Binds lamellar bodies in vitro (68) Unknown
Caveolin-1	Highest in white adipose tissue, also in lung, with lesser expression in muscle (58)	Integral membrane protein caveola, lipid raft	Signal transduction (58) Scaffolding protein (58) Trafficking (58) Cholesterol regulation (58) Tumor suppression (58)
CRYAB	Wide distribution; particularly lens epithelium, lung, heart, kidney, brain, and skeletal muscle (72)	Cytoplasm, nucleus	Chaperone (72) Heat shock (72) Structural protein (72) Stress resiliency (72) Preserving genomic stability (73)
EMP2	Highest in lung, moderate expression in eye, heart, thyroid, and uterus (28)	Integral membrane protein	Cell proliferation (28) Cell–cell interactions (47) Cell–matrix interactions (47) Tumor suppression (28)
sPLA2-IIA	Highest in small intestine. Moderate expression in large intestine and spleen and in specific cell types (74)	Secreted	Host defense (42) Signal transduction (42)
RT140	Highest in lung, some expression in eye and brain (75)	Integral membrane protein	Unknown, important for normal lung development (46)
RAGE	Wide distribution; highest in lung (76)	Integral membrane protein and secreted	Signal transduction (50) Host defense (43) Tissue remodeling (50)
SDPR	Wide distribution, highest in heart and lung (61)	Plasma membrane, caveola	Signal transduction (61)
SEMA3F	High expression in lung, kidney, fetal brain, and mammary. Also in heart and liver (77)	Secreted	Cell motility (44) Cell proliferation (44) Signal transduction (77) Tumor suppression (77)
TIMP3	Highest in kidney, lung, and brain (78)	Secreted	Tissue remodeling (78) Tumor suppression (54)
TOP3A	Highest in testis; also in thymus, brain, heart, and lung (80)	Nucleus, mitochondria	DNA metabolism (79) Preserving genomic stability (79)
TSLC1	Wide tissue distribution (53)	Integral membrane protein, synapse	Cell adhesion (59) Signal transduction (59) Tumor suppression (53)

been reported to exhibit tumor suppressing activity. It is unclear whether, as a whole, this group of genes may also act to prevent proliferation of normal growth which might explain, in part, the observation that type I cells may not proliferate in normal lungs.

5. Cell Signaling: Caveolin-1, TSLC1, SDPR, ANXA8, sPLA2-IIA, SEMA3F, RAGE

Several of the genes may participate in cell signaling. There is considerable support for a role for caveolin-1 in cell signaling. A 20–amino acid scaffolding domain of the protein has been found to bind to numerous signaling molecules and it is thought that caveolin-1 functions to regulate their activity (reviewed in Ref. 58). TSLC1 is a cell adhesion molecule of the immunoglobulin superfamily. In the brain, it is localized to both sides of the synapse and mediates transsynaptic signaling (59), suggesting the potential for its involvement in signal transduction events in other tissues. Serum deprivation response protein (SDPR) binds protein kinase C α in caveolae and also binds phosphatidylserine in a Ca^{2+}-independent manner (60, 61). Although the physiologic role of ANXA8 is currently unknown, cellular localization by immunofluorescence staining and subcellular fractionation indicate that it is primarily associated with the plasma membrane (62). The annexin family has been implicated in vesicle trafficking and intracellular Ca^{2+} signaling, the latter by acting both as atypical ion channels and as modulators of ion channel activity (63). Annexin I has been implicated in intracellular signaling regulated by chloride concentration (64). sPLA2-IIA binds to a receptor that is a member of the C-type multilectin mannose receptor family (reviewed in Ref. 42) and can induce iNOS via downstream effects of receptor binding on phosphatidylinositol 3-kinase and Akt (41). SEMA3F acts as a signaling molecule in developing neurons (65). Activated glycation end products (AGEs), by binding to RAGE, can activate SMAD signaling by both TGF-β–dependent and –independent mechanisms involving MAPKs (66). The downstream effects of these signaling pathways are a subject of speculation.

6. Potential Role in Lipid/Surfactant Metabolism: sPLA2-IIA, ANXA8

sPLA2-IIA is presumably secreted by the type I cell into the airspace. Although studies of surfactant recycling have been

performed with type II cells, the expression of sPLA2-IIA by type I cells points to the possibility that type I cells also participate in the recycling of surfactant components. Type I cells contain numerous small vesicles, which might result from either endocytic or synthetic pathways. Several of the annexins have been implicated in surfactant metabolism (67–69). Although to our knowledge it has not been localized to type II cells, ANXA8 has been reported to bind to isolated lamellar bodies (70).

Summary

To date there have been eight genes of known function described in type I cells (13, 21, 23), although it has not been clear that all of these genes are differentially expressed in type I cells. The identification of an additional nine genes of known function will serve to extend the parameters of what is known about the type I cell and provide additional markers to differentiate the two cellular phenotypes. Although most of the differentially expressed genes reported in this study are multifunctional, the functions of these genes suggest potential roles of type I cells in the dynamic maintenance of normal alveolar homeostasis and protection from injury, lung development and remodeling, host defense, tumor/growth suppression, and surfactant metabolism, among other functions. These observations may serve as a basis for future experiments regarding the elucidation of type I cell functions in the lung.

Acknowledgments: This work is supported in part by NIH R01 57426 and HL-24075.

References

1. Stone, K. C., R. R. Mercer, B. A. Freeman, L. Y. Chang, and J. D. Crapo. 1992. Distribution of lung cell numbers and volumes between alveolar and nonalveolar tissue. *Am. Rev. Respir. Dis.* 146:454–456.
2. Wright, J. R., and S. Hawgood. 1989. Pulmonary surfactant metabolism. *Clin. Chest Med.* 10:83–93.
3. Mason, R. J., M. C. Williams, J. H. Widdicombe, M. J. Sanders, D. S. Misfeldt, and L. C. Berry, Jr. 1982. Transepithelial transport by pulmonary alveolar type II cells in primary culture. *Proc. Natl. Acad. Sci. USA* 79:6033–6037.
4. Goodman, B. E., and E. D. Crandall. 1982. Dome formation in primary cultured monolayers of alveolar epithelial cells. *Am. J. Physiol.* 243:C96–100.
5. Harbeck, R. J., N. W. Gegen, D. Struhar, and R. Mason. 1988. Class II molecules on rat alveolar type II epithelial cells. *Cell. Immunol.* 111:139–147.
6. Paine, R., III, M. W. Rolfe, T. J. Standiford, M. D. Burdick, B. J. Rollins, and R. M. Strieter. 1993. MCP-1 expression by rat type II alveolar epithelial cells in primary culture. *J. Immunol.* 150:4561–4570.
7. Vanderbilt, J. N., E. M. Mager, L. Allen, T. Sawa, J. Wiener-Kronish, R. F. Gonzalez, and L. G. Dobbs. 2003. CXC chemokines and their receptor are expressed in Type II Cells and upregulated by injury. *Am. J. Respir. Cell Mol. Biol.* 29:661–668.
8. Evans, M. J., L. J. Cabral, R. J. Stephens, and G. Freeman. 1975. Transformation of alveolar type 2 cells to type 1 cells following exposure to NO2. 22:142–150.
9. Adamson, I. Y., and D. H. Bowden. 1975. Derivation of type 1 epithelium from type 2 cells in the developing rat lung. *Lab. Invest.* 32:736–745.
10. Dobbs, L. G., R. Gonzalez, M. A. Matthay, E. P. Carter, L. Allen, and A. S. Verkman. 1998. Highly water-permeable type I alveolar epithelial cells confer high water permeability between the airspace and vasculature in rat lung. *Proc. Natl. Acad. Sci. USA* 95:2991–2996.
11. Borok, Z., J. M. Liebler, R. L. Lubman, M. J. Foster, B. Zhou, X. Li, S. M. Zabski, K. J. Kim, and E. D. Crandall. 2002. Na transport proteins are expressed by rat alveolar epithelial type I cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 282:L599–L608.
12. Johnson, M. D., J. H. Widdicombe, L. Allen, P. Barbry, and L. G. Dobbs. 2002. Alveolar epithelial type I cells contain transport proteins and transport sodium, supporting an active role for type I cells in regulation of lung liquid homeostasis. PG - 1966–71. *Proc. Natl. Acad. Sci. USA* 99:1966–1971.
13. Williams, M. C. 2003. Alveolar type I cells: molecular phenotype and development. *Annu. Rev. Physiol.* 65:669–695.
14. Dobbs, L. G., M. C. Williams, and R. Gonzalez. 1988. Monoclonal antibodies 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.
15. 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 development expression of a rat lung alveolar type I cell gene in embryonic endodermal and neural derivatives. *Dev. Biol.* 167:294–306.
16. Gonzalez, R. F., and L. G. Dobbs. 1998. Purification and analysis of RTI40, a type I alveolar epithelial cell apical membrane protein. *Biochim. Biophys. Acta* 1429:208–216.
17. Vanderbilt, J. N., and L. G. Dobbs. 1998. Characterization of the gene and promoter for RTI40, a differentiation marker of type I alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 19:662–671.
18. Kasper, M., T. Reimann, U. Hempel, K. W. Wenzel, A. Bierhaus, D. Schuh, V. Dimmer, G. Haroske, and M. Muller. 1998. Loss of caveolin expression in type I pneumocytes as an indicator of subcellular alterations during lung fibrogenesis. *Histochem. Cell Biol.* 109:41–48.
19. Newman, G. R., L. Campbell, C. von Ruhland, B. Jasani, and M. Gumbleton. 1999. Caveolin and its cellular and subcellular immunolocalisation in lung alveolar epithelium: implications for alveolar epithelial type I cell function. *Cell Tissue Res.* 295:111–120.
20. 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.* 273:C1549–C1561.
21. Fehrenbach, H., M. Kasper, T. Tschernig, M. S. Shearman, D. Schuh, and M. Muller. 1998. Receptor for advanced glycation endproducts (RAGE) exhibits highly differential cellular and subcellular localisation in rat and human lung. *Cell Mol. Biol. (Noisy-le-grand)* 44:1147–1157.
22. Nagae, A., M. Abe, R. P. Becker, P. A. Deddish, R. A. Skidgel, and E. G. Erdos. 1993. High concentration of carboxypeptidase M in lungs: presence of the enzyme in alveolar type I cells. *Am. J. Respir. Cell Mol. Biol.* 9:221–229.
23. Qiao, R., B. Zhou, J. M. Liebler, X. Li, E. D. Crandall, and Z. Borok. 2003. Identification of three genes of known function expressed by alveolar epithelial type I cells. *Am. J. Respir. Cell Mol. Biol.* 29:98–105.
24. Dobbs, L. G., and R. F. Gonzalez. 2002. Isolation and culture of pulmonary alveolar epithelial type II cells. *In* Culture of Epithelial Cells, 2nd ed. R. I. and M. G. F. Freshney, editors. Wiley-Liss, New York.
25. Gutierrez, J. A., R. F. Gonzalez, and L. G. Dobbs. 1998. Mechanical distension modulates pulmonary alveolar epithelial phenotypic expression in vitro. *Am. J. Physiol.* 274:L196–L202.
26. Dobbs, L. G., R. F. Gonzalez, L. Allen, and D. K. Froh. 1999. HTI56, an integral membrane protein specific to human alveolar type I cells. *J. Histochem. Cytochem.* 47:129–137.
27. Wadehra, M., G. G. Sulur, J. Braun, L. K. Gordon, and L. Goodglick. 2003. Epithelial membrane protein-2 is expressed in discrete anatomical regions of the eye. *Exp. Mol. Pathol.* 74:106–112.
28. Wang, C. X., M. Wadehra, B. C. Fisk, L. Goodglick, and J. Braun. 2001. Epithelial membrane protein 2, a 4-transmembrane protein that suppresses B-cell lymphoma tumorigenicity. *Blood* 97:3890–3895.
29. Katsuoka, F., Y. Kawakami, T. Arai, H. Imuta, M. Fujiwara, H. Kanma, and K. Yamashita. 1997. Type II alveolar epithelial cells in lung express receptor for advanced glycation end products (RAGE) gene. *Biochem. Biophys. Res. Commun.* 238:512–516.
30. Hess, J. F., and P. G. FitzGerald. 1998. Protection of a restriction enzyme from heat inactivation by [alpha]-crystallin. *Mol. Vis.* 4:29–32.
31. Bluhm, W. F., J. L. Martin, R. Mestril, and W. H. Dillmann. 1998. Specific heat shock proteins protect microtubules during simulated ischemia in cardiac myocytes. *Am. J. Physiol.* 275:H2243–H2249.
32. Morrison, L. E., H. E. Hoover, D. J. Thuerauf, and C. C. Glembotski. 2003. Mimicking phosphorylation of alphaB-crystallin on serine-59 is necessary and sufficient to provide maximal protection of cardiac myocytes from apoptosis. *Circ. Res.* 92:203–211.
33. Amour, A., P. M. Slocombe, A. Webster, M. Butler, C. G. Knight, B. J. Smith, P. E. Stephens, C. Shelley, M. Hutton, V. Knauper, A. J. Docherty, and G. Murphy. 1998. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett.* 435:39–44.
34. Leco, K. J., P. Waterhouse, O. H. Sanchez, K. L. Gowing, A. R. Poole, A. Wakeham, T. W. Mak, and R. Khokha. 2001. Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). *J. Clin. Invest.* 108:817–829.
35. Qi, J. H., Q. Ebrahem, N. Moore, G. Murphy, L. Claesson-Welsh, M. Bond, A. Baker, and B. Anand-Apte. 2003. A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. *Nat. Med.* 9:407–415.
36. Hauptmann, R., I. Maurer-Fogy, E. Krystek, G. Bodo, H. Andree, and C. P. Reutelingsperger. 1989. Vascular anticoagulant beta: a novel human Ca2+/phospholipid binding protein that inhibits coagulation and phospholipase A2 activity: its molecular cloning, expression and comparison with VAC-alpha. *Eur. J. Biochem.* 185:63–71.
37. Laine, V. J., D. S. Grass, and T. J. Nevalainen. 1999. Protection by group II phospholipase A2 against Staphylococcus aureus. *J. Immunol.* 162:7402–7408.
38. Touqui, L., and L. Arbibe. 1999. A role for phospholipase A2 in ARDS pathogenesis. *Mol. Med. Today* 5:244–249.
39. Arbibe, L., D. Vial, I. Rosinski-Chupin, N. Havet, M. Huerre, B. B. Vargaftig, and L. Touqui. 1997. Endotoxin induces expression of type II phospholipase A2 in macrophages during acute lung injury in guinea pigs: involvement of TNF-alpha in lipopolysaccharide-induced type II phospholipase A2 synthesis. *J. Immunol.* 159:391–400.
40. Beck, G., B. A. Yard, J. Schulte, M. Haak, K. van Ackern, F. J. van der Woude, and M. Kaszkin. 2003. Secreted phospholipases A2 induce the

expression of chemokines in microvascular endothelium. *Biochem. Biophys. Res. Commun.* 300:731–737.
41. Park, D. W., J. R. Kim, S. Y. Kim, J. K. Sonn, O. S. Bang, S. S. Kang, J. H. Kim, and S. H. Baek. 2003. Akt as a mediator of secretory phospholipase A2 receptor-involved inducible nitric oxide synthase expression. *J. Immunol.* 170:2093–2099.
42. Fuentes, L., M. Hernandez, M. L. Nieto, and M. Sanchez Crespo. 2002. Biological effects of group IIA secreted phosholipase A(2). *FEBS Lett.* 531:7–11.
43. Schmidt, A. M., S. D. Yan, S. F. Yan, and D. M. Stern. 2001. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J. Clin. Invest.* 108:949–955.
44. Kagoshima, M., and T. Ito. 2001. Diverse gene expression and function of semaphorins in developing lung: positive and negative regulatory roles of semaphorins in lung branching morphogenesis. *Genes Cells* 6:559–571.
45. Ito, T., M. Kagoshima, Y. Sasaki, C. Li, N. Udaka, T. Kitsukawa, H. Fujisawa, M. Taniguchi, T. Yagi, H. Kitamura, and Y. Goshima. 2000. Repulsive axon guidance molecule Sema3A inhibits branching morphogenesis of fetal mouse lung. *Mech. Dev.* 97:35–45.
46. Ramirez, M. I., G. Millien, A. Hinds, Y. Cao, D. C. Seldin, and M. C. Williams. 2003. T1alpha, a lung type I cell differentiation gene, is required for normal lung cell proliferation and alveolus formation at birth. *Dev. Biol.* 256:61–72.
47. Wadehra, M., R. Iyer, L. Goodglick, and J. Braun. 2002. The tetraspan protein epithelial membrane protein-2 interacts with beta1 integrins and regulates adhesion. *J. Biol. Chem.* 277:41094–41100.
48. Drab, M., P. Verkade, M. Elger, M. Kasper, M. Lohn, B. Lauterbach, J. Menne, C. Lindschau, F. Mende, F. C. Luft, A. Schedl, H. Haller, and T. V. Kurzchalia. 2001. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* 293:2449–2452.
49. Palumbo, R., M. Sampaolesi, F. De Marchis, R. Tonlorenzi, S. Colombetti, A. Mondino, G. Cossu, and M. E. Bianchi. 2004. Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. *J. Cell Biol.* 164:441–449.
50. Huttunen, H. J., J. Kuja-Panula, G. Sorci, A. L. Agneletti, R. Donato, and H. Rauvala. 2000. Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J. Biol. Chem.* 275:40096–40105.
51. Muller, S., P. Scaffidi, B. Degryse, T. Bonaldi, L. Ronfani, A. Agresti, M. Beltrame, and M. E. Bianchi. 2001. New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. *EMBO J.* 20:4337–4340.
52. Stamenkovic, I. 2003. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J. Pathol.* 200:448–464.
53. Kuramochi, M., H. Fukuhara, T. Nobukuni, T. Kanbe, T. Maruyama, H. P. Ghosh, M. Pletcher, M. Isomura, M. Onizuka, T. Kitamura, T. Sekiya, R. H. Reeves, and Y. Murakami. 2001. TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. *Nat. Genet.* 27:427–430.
54. Bachman, K. E., J. G. Herman, P. G. Corn, A. Merlo, J. F. Costello, W. K. Cavenee, S. B. Baylin, and J. R. Graff. 1999. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer Res.* 59:798–802.
55. Nasarre, P., B. Constantin, L. Rouhaud, T. Harnois, G. Raymond, H. A. Drabkin, N. Bourmeyster, and J. Roche. 2003. Semaphorin SEMA3F and VEGF have opposing effects on cell attachment and spreading. *Neoplasia* 5:83–92.
56. Galbiati, F., D. Volonte, J. Liu, F. Capozza, P. G. Frank, L. Zhu, R. G. Pestell, and M. P. Lisanti. 2001. Caveolin-1 expression negatively regulates cell cycle progression by inducing G(0)/G(1) arrest via a p53/p21(WAF1/Cip1)-dependent mechanism. *Mol. Biol. Cell* 12:2229–2244.
57. Leung, S. Y., X. Chen, K. M. Chu, S. T. Yuen, J. Mathy, J. Ji, A. S. Chan, R. Li, S. Law, O. G. Troyanskaya, I. P. Tu, J. Wong, S. So, D. Botstein, and P. O. Brown. 2002. Phospholipase A2 group IIA expression in gastric adenocarcinoma is associated with prolonged survival and less frequent metastasis. *Proc. Natl. Acad. Sci. USA* 99:16203–16208.
58. Razani, B., S. E. Woodman, and M. P. Lisanti. 2002. Caveolae: from cell biology to animal physiology. *Pharmacol. Rev.* 54:431–467.
59. Biederer, T., Y. Sara, M. Mozhayeva, D. Atasoy, X. Liu, E. T. Kavalali, and T. C. Sudhof. 2002. SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 297:1525–1531.
60. Mineo, C., Y. S. Ying, C. Chapline, S. Jaken, and R. G. Anderson. 1998. Targeting of protein kinase Calpha to caveolae. *J. Cell Biol.* 141:601–610.
61. Gustincich, S., P. Vatta, S. Goruppi, M. Wolf, S. Saccone, G. Della Valle, M. Baggiolini, and C. Schneider. 1999. The human serum deprivation response gene (SDPR) maps to 2q32-q33 and codes for a phosphatidylserine-binding protein. *Genomics* 57:120–129.
62. Sarkar, A., P. Yang, Y. H. Fan, Z. M. Mu, R. Hauptmann, G. R. Adolf, S. A. Stass, and K. S. Chang. 1994. Regulation of the expression of annexin VIII in acute promyelocytic leukemia. *Blood* 84:279–286.
63. Hawkins, T. E., C. J. Merrifield, and S. E. Moss. 2000. Calcium signaling and annexins. *Cell Biochem. Biophys.* 33:275–296.
64. Muimo, R., Z. Hornickova, C. E. Riemen, V. Gerke, H. Matthews, and A. Mehta. 2000. Histidine phosphorylation of annexin I in airway epithelia. *J. Biol. Chem.* 275:36632–36636.
65. Dontchev, V. D., and P. C. Letourneau. 2003. Growth cones integrate signaling from multiple guidance cues. *J. Histochem. Cytochem.* 51:435–444.
66. Li, J. H., X. R. Huang, H. J. Zhu, M. Oldfield, M. Cooper, L. D. Truong, R. J. Johnson, and H. Y. Lan. 2004. Advanced glycation end products activate Smad signaling via TGF-beta-dependent and independent mechanisms: implications for diabetic renal and vascular disease. *FASEB J.* 18:176–178.
67. Liu, L., M. Wang, A. B. Fisher, and U. J. Zimmerman. 1996. Involvement of annexin II in exocytosis of lamellar bodies from alveolar epithelial type II cells. *Am. J. Physiol.* 270:L668–L676.
68. Sohma, H., N. Matsushima, T. Watanabe, A. Hattori, Y. Kuroki, and T. Akino. 1995. Ca(2+)-dependent binding of annexin IV to surfactant protein A and lamellar bodies in alveolar type II cells. *Biochem. J.* 312:175–181.
69. Chander, A., and R. D. Wu. 1991. In vitro fusion of lung lamellar bodies and plasma membrane is augmented by lung synexin. *Biochim. Biophys. Acta* 1086:157–166.
70. Sohma, H., H. Ohkawa, T. Akino, and Y. Kuroki. 2001. Binding of annexins to lung lamellar bodies and the PMA-stimulated secretion of annexin V from alveolar type II cells. *J. Biochem. (Tokyo)* 130:449–455.
71. Reutelingsperger, C. P., W. van Heerde, R. Hauptmann, C. Maassen, R. G. van Gool, P. de Leeuw, and A. Tiebosch. 1994. Differential tissue expression of Annexin VIII in human. *FEBS Lett.* 349:120–124.
72. Derham, B. K., and J. J. Harding. 1999. Alpha-crystallin as a molecular chaperone. *Prog. Retin. Eye Res.* 18:463–509.
73. van den Ijssel, P., R. Wheelock, A. Prescott, P. Russell, and R. A. Quinlan. 2003. Nuclear speckle localisation of the small heat shock protein alpha B-crystallin and its inhibition by the R120G cardiomyopathy-linked mutation. *Exp. Cell Res.* 287:249–261.
74. Nyman, K. M., P. Ojala, V. J. Laine, and T. J. Nevalainen. 2000. Distribution of group II phospholipase A2 protein and mRNA in rat tissues. *J. Histochem. Cytochem.* 48:1469–1478.
75. Williams, M. C., Y. Cao, A. Hinds, A. K. Rishi, and A. Wetterwald. 1996. T1a protein is developmentally regulated and expressed by alveolar type I cells, choroid plexus, and ciliary epithelia of adult rats. *Am. J. Respir. Cell Mol. Biol.* 14:577–585.
76. Brett, J., A. M. Schmidt, S. D. Yan, Y. S. Zou, E. Weidman, D. Pinsky, R. Nowygrod, M. Neeper, C. Przysiecki, and A. Shaw. 1993. Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. *Am. J. Pathol.* 143:1699–1712.
77. Xiang, R. H., C. H. Hensel, D. K. Garcia, H. C. Carlson, K. Kok, M. C. Daly, K. Kerbacher, A. van den Berg, P. Veldhuis, C. H. Buys, and S. L. Naylor. 1996. Isolation of the human semaphorin III/F gene (SEMA3F) at chromosome 3p21, a region deleted in lung cancer. *Genomics* 32:39–48.
78. Leco, K. J., R. Khokha, N. Pavloff, S. P. Hawkes, and D. R. Edwards. 1994. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J. Biol. Chem.* 269:9352–9360.
79. Wang, Y., Y. L. Lyu, and J. C. Wang. 2002. Dual localization of human DNA topoisomerase IIIalpha to mitochondria and nucleus. *Proc. Natl. Acad. Sci. USA* 99:12114–12119.
80. Seki, T., M. Seki, T. Katada, and T. Enomoto. 1998. Isolation of a cDNA encoding mouse DNA topoisomerase III which is highly expressed at the mRNA level in the testis. *Biochim. Biophys. Acta* 1396:127–131.