Surfactant Protein-D Regulates Surfactant Phospholipid Homeostasis in Vivo*

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Surfactant protein D (SP-D) is a 43-kDa member of the collectin family of collagenous lectin domain-containing proteins that is expressed in epithelial cells of the lung. The SP-D gene was targeted by homologous recombination in embryonic stem cells that were used to produce SP-D (±) and SP-D (−/−) mice. Both SP-D (−/−) and SP-D (±) mice survived normally in the perinatal and postnatal periods. Whereas no abnormalities were observed in SP-D (±) mice, alveolar and tissue phosphatidylcholine pool sizes were markedly increased in SP-D (−/−) mice. Increased numbers of large foamy alveolar macrophages and enlarged alveoli were also observed in SP-D (−/−) mice. Phospholipid composition was unaltered in SP-D (−/−) mice, but surfactant morphology was abnormal, consisting of dense phospholipid membranous arrays with decreased tubular myelin. The pulmonary lipidosis in the SP-D (−/−) mice was not associated with accumulation of surfactant proteins B or C, or their mRNAs, distinguishing the disorder from alveolar proteinosis syndromes. Surfactant protein A mRNA was reduced and, SP-A protein appeared to be reduced in SP-D (−/−) compared with wild type mice. Targeting of the mouse SP-D gene caused accumulation of surfactant lipid and altered phospholipid structures, demonstrating a previously unsuspected role for SP-D in surfactant lipid homeostasis in vivo.

Pulmonary surfactant is essential for normal lung mechanics and gas exchange in the lung. Both quantitative and qualitative deficiencies in pulmonary surfactant are associated with neonatal respiratory distress (1), adult respiratory distress syndrome (2), and congenital deficiencies of surfactant protein B (3), demonstrating the important clinical consequences of abnormalities in surfactant. Alveolar surfactant pools are regulated at multiple levels including intracellular synthesis, secretion, re-uptake of lipids and proteins by type II epithelial cells, and the uptake and degradation of these components by alveolar macrophages (4). The synthesis and clearance of surfactant phospholipids and proteins is further influenced by developmental, mechanical, and humoral stimuli that serve to maintain steady-state surfactant concentrations after birth (1). Recent studies demonstrated the important role of surfactant catabolism in determining steady-state surfactant protein and lipid concentrations in the lung. Deficiency of granulocyte macrophage colony stimulating factor (GM-CSF)1 or GM-CSF receptors are both associated with extracellular accumulation of pulmonary surfactant lipids and proteins, causing pulmonary alveolar proteinosis both in transgenic mice and in humans (3, 5–7). Mechanisms selectively regulating lipid concentrations have not been identified, and a role for SP-D in regulating surfactant has not been previously reported. In vitro studies support the concept that surfactant proteins may be important in the regulation of surfactant homeostasis (8). Although the hydrophobic surfactant proteins SP-B and SP-C have roles in production of the surfactant monolayer, in vitro studies indicated that surfactant proteins A, B, and C may also facilitate surfactant uptake and/or secretion by type II epithelial cells (8). However, recent studies of SP-A null mice have not supported the primary role of surfactant protein A in surfactant secretion or re-uptake. The absence of SP-A does not lead to obvious physiologic or morphologic structural abnormalities of the lung. SP-A null mutant mice lack tubular myelin figures but produce highly functional surfactant that adsorbs rapidly and produces stable monolayers. Surfactant lipid synthesis, secretion, and re-uptake were essentially normal in SP-A null mice (9–11). In contrast, targeted deletion of SP-B caused abnormal processing of proSP-C, the absence of tubular myelin, and death from respiratory failure in neonatal SP-B (−/−) mice (12).

SP-D and SP-A are members of the collectin family of C-type lectins that include a number of molecules with known host defense functions. SP-A and SP-D enhance uptake of bacteria and viruses by alveolar macrophages and neutrophils and may influence macrophage function and signaling via collectin receptors present on target cells (13, 14). Various cellular binding sites for SP-A and SP-D have been identified on alveolar macrophages and, in the case of SP-A, on type II epithelial cells (13, 15, 16). The critical role of SP-A in host defense was supported by the observation that SP-A-deficient mice are susceptible to infections by both group B streptococcus and Pseudomonas aeruginosa in vivo (17, 18).

To evaluate the importance of SP-D, transgenic mice lacking SP-D were generated. Whereas SP-D (−/−) and SP-D (±) mice

1 The abbreviations used are: GM-CSF, granulocyte macrophage colony stimulating factor; SP-A, -B, -C, -D, surfactant protein A, B, C, D, respectively; EM, electron microscopy; kb, kilobase(s); RT-PCR, reverse transcriptase polymerase chain reaction; Sat-PC, saturated phosphatidylcholine.
survive normally in the vivarium, surfactant phospholipid accumulation was observed in the SP-D (-/-) mice in the absence of increased surfactant proteins A, B, and C. These studies support the critical role of SP-D in surfactant lipid homeostasis in vivo.

EXPERIMENTAL PROCEDURES

Vector Construction—A mouse 129J genomic library was kindly provided by Dr. M. Shull at the University of Cincinnati. This library was screened with the rat cDNA clone for SP-D (19). Seven genomic clones were identified that contained homologous sequences. Two of these clones were contiguous, encoding approximately 28 kb of genomic DNA containing the entire SP-D gene. The SP-D gene was sequenced in its entirety. A mouse cDNA library purchased from Stratagene was also screened. 15 positive clones were identified and three clones were sequenced, and the sequenced data agreed with subsequently published information. Transcription initiation sites were identified by 5' RACE and also agree with published information (20). A targeting Vector was designed using pGKneo (21) by first subcloning a 5.1-kb blunt ended KpnI-tailed HindIII genomic fragment encoding intron 2 through exon 2 on an 6 into a KpnI site between the neomycin resistance cassette and the thymidine kinase cassette. Subsequently, a 1.5-kb genomic PacI fragment containing a portion of intron 1 was tailed with XhoI linkers and cloned into an XhoI site 5' from the neomycin resistance cassette (see Fig. 1).

Generation of SP-D Null Mutant Mice—ES cells (clone E14.1) generated from 129 Ola mice were grown on neomycin-resistant mouse embryonic fibroblast feeder layers. Using = 20 µg of the SP-D targeting construct, about 2 x 106 cells were electroporated. Targeted ES cells were selected by growth in G418 (150 µg/ml) and gancyclovir (2 µM) and identified as described in genotyping. Clone 93, a rapidly proliferating undifferentiated clone was microinjected into C57/B16 host blastocysts. Chimeric males were bred to NIH Swiss Black females. A single germ-line positive agouti female from over 1500 offspring of chimeric matings was identified and bred with an NIH Swiss Black male to generate a colony of SP-D (+/+) , SP-D (-/-) mice.

Genotyping—Double selected stem cell clones were screened by both 5' and 3' PCR and by DNA blot analysis. For DNA blot analysis, a HindIII digest was probed with a 5' genomic BamHI/PstI restriction fragment yielding a 9.3-kb band with targeted integration and a 2.3-kb band for wild type DNA. To identify heterozygotic and chimeric animals, an identical strategy was used for tail clip DNA. Once heterozygotes were identified, null animals were identified by using a cloned genomic DNA fragment encoding part of intron 2 and exon 2 as a probe and the G418 gene as a probe on BamHI digests of tail DNA. The G418 probe identifies a BamHI fragment of 7.5 kb, and the exon 2 probe identifies a fragment of 6.5 kb. Hybridization conditions were as described previously (9, 18).

Alveolar Lavage and Surfactant Characterization—Mice were injected intraperitoneally with pentobarbital to achieve deep anesthesia, and the distal aorta was cut to exsanguinate each animal. Alveolar lavage was performed four times for each lung as described previously (9, 18). Mice used for histological or EM studies were about 6 months of age. All mice had been maintained in barrier containment facilities, and at the time of study, all mice appeared healthy.

RESULTS

SP-D Null Mutant Mice—Integration of the targeting vector (Fig. 1) generates a deletion of the second exon of the SP-D gene, which includes removal of the initiating methionine and translation initiation sequences. Eight of 104 ES clones surviving the double selection process were correctly targeted as determined by both 5' and 3' PCR analyses. Clone 93, a highly undifferentiated and proliferative clone, was expanded and injected into C57/B16 blastocysts generating chimeric males. Chimeric males were bred to NIH Swiss Black females. A female bearing the targeted gene was obtained and bred to NIH Swiss Black males to generate normal SP-D (+/-) and SP-D (-/-) mice. The distribution of genotypes from heterozygotic matings followed a Mendelian pattern, with 30 (+/+) , 45 (-/-) , and 25% (-/-) ) of 115 offspring, indicating that there were no obvious abnormalities in survival related to SP-D alleles.

To determine genotype, DNA from tail clips was digested with BamHI and probed with a PCR product derived from genomic mouse DNA, and containing exon 2 and part of intron 2, and with the G418 resistance cDNA clone. Fig. 2 demonstrates simultaneous loss of exon 2 with appearance of sequences encoding G418 resistance in SP-D (-/-) and SP-D (-/-) mice. Null, normal, and heterozygous mice displayed no obvious abnormalities in the paraffin for sectioning and then immunostained for SP-B using rabbit polyclonal SP-B antisera as described previously (27).

Western Blot Analysis—For Western blot analysis, cellular debris was pelleted from alveolar washes at 250 x g for 10 min, and surfactant pellets were recovered by centrifugation at 12,000 x g for 20 min. Immunoblots of surfactant samples were prepared as described previously (9). Blots were incubated with anti-protein antisera as follows. SP-B and SP-A antibodies were raised against SP-D produced from a rat SP-D cDNA clone (19) using a baculovirus expression system, kindly provided by Dr. Frank McCormack, University of Cincinnati. Antibodies used for SP-A, SP-B, and SP-C detection were as described previously (9). Blots were rinsed and an appropriate horseradish peroxidase-conjugated secondary antibody (Calbiochem, Inc.) was added for 4 h. Blots were rinsed and developed using ECL detection reagents (Amersham Pharmacia Biotech). Immunoreactive bands were identified by exposing blots to XAR film (Kodak). Relative band intensity was determined using an IS 1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA).

Mouse Husbandry—Mice used in experimental procedures were handled in accordance with approved protocols through the Institutional Animal Care and Use Committee at Children's Hospital, Cincinnati. Mice used for biochemical studies were between 8 and 12 weeks of age. Mice used for histological or EM studies were about 6 months of age. All mice had been maintained in barrier containment facilities, and at the time of study, all mice appeared healthy.

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To demonstrate that SP-D was not expressed in null animals, RNA blot analysis was conducted with total lung RNA from null, normal, and heterozygotic animals. Fig. 3 demonstrates an approximately 50% reduction in the intensity of the SP-D hybridization band in heterozygous animals with a total absence of normally sized SP-D mRNA in null animals. After prolonged exposure, a diffuse mRNA band approximately 150 nucleotides smaller than the normal SP-D mRNA was detected (data not shown). By scanning densitometry, this band repre
Identification of a methionine in-frame at nucleotide 216, 18 base pairs 5' to the beginning of the collagenous domain. This methionine was not in a nucleotide context, predicting efficient initiation of translation (28).

Lung homogenates were subjected to Western blot analysis using rabbit anti-rat SP-D antiserum (Fig. 4). SP-D was reduced approximately 50% in heterozygous SP-D (+/–) mice and was absent in SP-D (–/–) mice. No unusually sized immunoreactive proteins were detected in homogenates from SP-D (–/–) null mice.

Lung Structure—To determine whether absence of SP-D expression led to structural abnormalities, lungs from null, normal, and heterozygous mice were inflation fixed, and sections were evaluated by light microscopy. There was no evidence of infection and no obvious alterations in airway epithelial cells at the level of light microscopy. However, heterogeneous abnormalities in lung parenchyma, with enlarged alveoli, were consistently observed in the SP-D (–/–) but not SP-D (+/+) or SP-D (+/–) controls. Increased bronchial-associated lymphocytic tissue (BALT) was noted in the SP-D (–/–) mice. Intensity of SP-B immunostaining in type II cells was similar among the three genotypes. However, there were focal areas of increased numbers of large, foamy intra-alveolar cells, which appeared to be alveolar macrophages containing abundant cytoplasmic vesicles. These cells increased in size as a result of increasing number and volume of cytoplasmic vesicles. The vesicles stained with Nile Red and fluoresced when excited with 520–550 nm green light after staining with Nile Blue and thus contained lipid or phospholipid. These macrophages were also stained by SP-B antiserum (Fig. 5). In alveolar lavage, approximately 4-fold more macrophages (1.2 × 10^6 per mouse) were observed in SP-D (–/–) compared with normal mice (0.36 × 10^6/mouse), but there were no changes in relative neutrophil or lymphocyte cell counts. Macrophage size was estimated from the diameter of fixed and stained macrophages from cytospin preparations sedimented onto glass slides at 1500 × g for 2 min. Mean diameter of macrophages from (+/+) was 11.75 ± 1.75 μm compared with (–/–) mice 18.75 ± 7.25 μm. Abnormally large macrophages, defined as those with a diameter of twice normal, comprised 22.4 ± 0.6% of the macrophages from (–/–) mice compared with 1.8 ± 1.0% from (+/+) mice. Numbers and morphology of alveolar macrophages were not different in SP-D (±) mice. Ultrastructural characteristics of type II

Fig. 1. A, representation of the targeting construct; B, intron/exon structure of murine SP-D; C, targeted integration of the vector is indicated as a bold line. Restriction sites used for genotyping are shown.

Fig. 2. Southern blot of genomic DNA. Genomic DNA from tail clips was digested to completion with BamHI and probed with a cDNA for G418^R or exon 2. SP-D (–/–) mice have a single band of 7.5 kb corresponding to the targeted allele, and SP-D (+/+ ) mice have a single band of 6.5 kb corresponding to the normal. Both bands are detected in SP-D (±) mice.

Fig. 3. SP-A and SP-D mRNA in SP-D (+/+ ), (±), and (–/–) mice. Total lung RNA was probed with a cDNA for SP-A (A) or SP-D (B), and then the membranes were stripped and reprobed for 18 S RNA. The ratio of SP-A RNA to 18 S RNA was determined by measuring the level of 32P corresponding to each band using a PhosphorImager. SP-A:18 S ratio was 1.31 ± 0.39 in SP-D (–/–), 1.95 ± 0.24 in SP-D (±), and 2.20 ± 0.26 in SP-D (+/+) mice (p < .05). Presented data are representative of mRNA levels in 14 mice for SP-D and in 6 mice for SP-A of each genotype.

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cells were similar in SP-D (−/−) compared with SP-D (+/+)
mice (data not shown).

Saturated Phosphatidylcholine Pool Sizes and Composition—

There were no differences in body weight of SP-D (+/+), (±), or 
(−/−) mice. Alveolar, tissue and total Sat-PC (p < 0.001) was 
increased about 3-fold in SP-D (−/−) mice (Fig. 6). Levels of 
Sat-PC were not altered in SP-D (±) mice. For alveolar lavage 
phospholipid composition analysis, two–four samples consist-
ing the pooled lavage from two–three mice were evaluated 
for the relative abundance of phosphatidylcholine, phosphati-
dyethanolamine, phosphatidylglycerol, phosphatidylinositol, 
spingomyelin, and lyso-bis-phosphatidic acid. Phospholipid 
composition did not differ among genotypes (data not shown).

Incorporation of [3H]choline into total lung Sat-PC was slightly 
increased 8 h following injection, incorporation being approxi-
mately 20% greater in SP-D (−/−) mice (p < 0.05).

Structure of Isolated Surfactant—Large aggregate surfac-
tant was isolated from pooled alveolar lavage of SP-D (−/−) 
and SP-D (+/+ ) mice and examined by EM. Lipid aggregates in 
SP-D (−/−) mice were enlarged and organized into electron 
dense phospholipid arrays and contained less tubular myelin 
compared with SP-D (+/+ ) mice (Fig. 7).

Surfactant Proteins—No differences in SP-B and SP-C 
mRNAs or proteins were observed in SP-D (−/−) mice. In 
contrast, SP-A mRNA was reduced in SP-D (−/−) mice (Fig. 3). 
Consistent with the reduction in SP-A mRNA, BAL SP-A pro-
tein was apparently reduced by about 25% in SP-D (−/−) mice 
as assessed by Western blot analysis of alveolar lavage from 
three mice but did not reach statistical significance(Fig. 8). 
Total protein content in BAL fluid was similar in all genotypes 
(data not shown).

DISCUSSION

A null mutation in the murine SP-D gene caused the accumu-
lation of surfactant phospholipids, but not proteins, in the 
lung of SP-D (−/−) mice, demonstrating a previously unex-
pected role for SP-D in lung phospholipid homeostasis. The 
pulmonary lipoidosis in the SP-D (−/−) mice is distinct from 
previously described alveolar proteinosis syndromes in experi-
mental animal models and in humans who have marked accu-
mulations of both surfactant proteins and lipids. Thus, the 
SP-D null mouse demonstrates for the first time that surfac-
tant lipids and protein homeostasis can be dissociated in vivo.

Surfactant lipids and protein concentrations are maintained 
at precise physiological concentrations by the regulation of 
synthesis, secretion, recycling, and catabolism by type II epi-
thelial cells and catabolism by alveolar macrophages. Clara 
cells synthesize SP-A and SP-B but not lipids, and their role in 
recycling and catabolism of lipids and proteins is not known (8).

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protein and surfactant proteins B and C were unchanged, and there was a modest apparent decrease in SP-A levels. The ultrastructure of the phospholipid-rich material isolated from BAL in the SP-D (−/−) mice was markedly abnormal, containing reduced quantities of tubular myelin and forming unique, densely packed lipid structures. SP-D, therefore, plays a role in the structural organization of alveolar lipids. Whether the changes in structure are mediated indirectly by the changes in the stoichiometry of the lipid-protein complex or by direct interactions of SP-D with surfactant constituents remains to be clarified.

The present findings clearly distinguish the SP-D (−/−) mice from alveolar proteinosis in which both lipids and proteins accumulate in material rich in tubular myelin. Recent studies in transgenic mice and in humans support the critical role of clearance mechanisms in surfactant homeostasis. GM-CSF and its receptor regulate both surfactant lipid and protein clearance in the lung (5–7). In the absence of GM-CSF or the common β-chain of the GM-CSF receptor, SP-A, -B, -C, and -D, as well as surfactant lipids, accumulate to high concentrations (5, 7). Although the levels of lipid accumulation in the GM (−/−) mice are similar to those seen in the SP-D (−/−) mice, lack of changes in surfactant proteins distinguish the SP-D (−/−) mice from the alveolar proteinosis models. Surfactant accumulation in the GM-CSF-deficient mouse is caused by decreased surfactant lipid and protein clearances that are associated primarily with abnormalities in alveolar macrophage maturation and function, distinct from the lipoidosis seen in the SP-D (−/−) mouse.

The numbers and mean size of alveolar macrophages were increased in the SP-D (−/−) mice. It remains unclear whether this observation represents a primary abnormality or reflects changes secondary to increased surfactant lipids. The changes in macrophage morphology observed in the SP-D (−/−) mice were heterogeneous within regions of the lung parenchyma. Both SP-D and SP-A are known to interact with alveolar macrophages and monocytes via a variety of surface receptors (4, 14, 15). The accumulation of surfactant lipids and the abnormalities in alveolar macrophage morphology seen in SP-D (−/−) mice were not observed in SP-A (−/−) mice (9). Abundant surfactant protein B staining and lipid accumulations were noted in the SP-D (−/−) alveolar macrophages, demonstrating the ability of these macrophages to take up surfactant. It remains unclear whether the abnormalities in alveolar macrophage numbers and size reflect primary changes in surfactant catabolism or are caused by changes in lipid clearance or production by type II cells.

The Sat-PC pool size was increased in lung lavage from the SP-D (−/−) mice, and phospholipid composition was not changed. Whereas surfactant proteins SP-B and SP-C mRNAs and proteins were unaltered in the SP-D (−/−) mice, SP-A protein and mRNAs were reduced approximately 25–40% in SP-D (−/−) mice (9). Abundant surfactant protein A deficiency is not associated with alterations in surfactant lipid concentrations, the pulmonary lipoidosis seen in the SP-D (−/−) mice is not likely to be directly related to changes in SP-A concentrations, although interactions between two proteins remain possible. A role for SP-A in surfactant homeostasis was postulated from in vitro experiments, but surfactant lipid levels, synthesis, and clearance were unperturbed in the SP-A (−/−) mice in vivo, although tubular myelin figures were virtually absent in these mice (9–11). There were no differences in lipid content, choline incorporation, or recombinant protein or mRNAs in the heterozygous SP-D (±) mice. Choline incorporation into Sat-PC was slightly increased in the SP-D (−/−) mice, a small effect that is unlikely to directly account for the observed 3-fold

Experiments in numerous models support the concept that the synthesis of surfactant lipids and proteins by type II cells is regulated in a highly independent manner in vivo. Despite complex humoral and physiological regulation of surfactant lipids and proteins, pathways by which surfactant proteins and lipid pools are regulated independently have not been previously identified. In the present studies, surfactant lipids were markedly increased in lung tissue and within the airway of the SP-D (−/−) mice. Although [3H]choline incorporation into Sat-PC was slightly increased in SP-D (−/−) mice, the small increase in synthesis is not likely to be sufficient to account for the 3-fold increase in Sat-PC pool sizes. However, total BAL

Fig. 7. Ultrastructure of isolated large aggregate surfactant. Tubular myelin figures were abundant and readily detected in large aggregate pools of SP-D (+/+ ) mice (A). Although tubular myelin figures were present in large aggregate pools of SP-D (−/−) mice, most lipid structures were composed of electron dense phospholipid arrays (B). Pools were prepared from three mice of each genotype. Bar = 1 μm.

Fig. 8. Immunoblot of SP-A BAL fluid. Lung alveolar lavage was prepared from SP-D (+/+ ), (±), and (+/+ ) mice. BAL proteins were normalized, subjected to SDS-PAGE, and blotted with SP-A antiserum, as described. 20–34 kDa SP-A protein was reduced approximately 25% in SP-D (−/−) mice as determined by densitometric assessment. Data represent analyses of BAL from three mice of each genotype.
increase in phospholipid content seen in the SP-D (−/−) mice. The mechanism and physiologic relevance of the decreased SP-A in the SP-D (−/−) mice is unclear, but provides the first evidence that SP-D may play a regulatory role in SP-A gene expression or homeostasis. A reciprocal relationship was not apparent in the SP-A (−/−) mice because SP-D levels were not changed (9).

The gene targeting construct used in the present study was designed to replace the second exon of the SP-D gene with the gene encoding G418 resistance. Because SP-D translation is initiated in exon 2, we expected either no SP-D mRNA or a fusion transcript, including neomycin resistance coding sequence, to be possible. Whereas no normal SP-D mRNA was detected in the SP-D (−/−) mice, low levels of a transcript arising from splicing of exon 1 to exon 3 (lacking the G418 resistance gene) was identified by RT-PCR, consistent with an open reading frame beginning in exon 3. The nearest in-frame methionine occurs at nucleotide 216; however, this methionine is not in a context favoring translation (28). We were unable to detect an alternative SP-D protein fragment by Western blot, and the abundance of the alternative truncated mRNA was low, making it unlikely that the alternative transcript generates a physiologically functional protein.

No differences in weight gain or mortality have been observed in the SP-D (−/−) mice. The colony has been maintained in filtered cages for more than six months. Histologic examination of the lungs has not revealed an increased abundance of neutrophils or signs of infection. Alveolar size was consistently increased in the SP-D (−/−) mice. Whether the differences in alveolar size in the SP-D (−/−) mice reflects changes in inflammation related to altered surfactant or indicate a possible role of SP-D in the regulation of alveolarization remains to be assessed. The present findings demonstrate that SP-D is not required for either perinatal respiratory adaptation or postnatal survival under vivarium conditions. Because SP-D is known to bind to and enhance the opsonization of a number of infectious organisms, including bacteria, pneumocystis, and influenza virus, it will be important to evaluate the role of SP-D in host defense in vivo (13).

In summary, the present study demonstrates a remarkable and unexpected role of SP-D in the selective regulation of surfactant phospholipid homeostasis and the structure of alveolar surfactant in the lung. Overt abnormalities detected in the SP-D (−/−) mice have been limited to the pulmonary system, wherein increased surfactant phospholipids were observed in association with increased alveolar size and increased numbers of alveolar macrophages. It remains unclear whether the SP-D modulates surfactant phospholipid homeostasis via changes in intracellular or extracellular pathways, and specifically whether SP-D regulates surfactant homeostasis at the level of synthesis and/or catalysis by type II epithelial cells, or catalytic pathways by alveolar macrophage. Gene targeting of SP-D has revealed a novel pathway mediating surfactant lipid homeostasis.

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REFERENCES
1. Crewe, L. A., van Golde, L. M., and Haagsman, H. P. (1997) Lung 173, 1–39
2. Verbrugge, S. J., Sorm, V., and Lachmann, B. (1997) J. Physiol. Pharmacol. 48, 537–447
3. Nogee, L. M. (1997) Chest 111, (suppl.) 1298S–1358
4. Whitsett, J. A., and Dobbs, L. G. (1997) J. Clin. Invest. 100, 2211–2217
5. Ikegami, M., Ueda, T., Hull, W., Whitsett, J. A., Mulligan, R. C., Dranoff, G., and Jobe, A. H. (1996) Am. J. Physiol. 270, L650–L658
6. Ishizaka, K., Nakayama, N., Hiraishi, Y., Inoue, T., Aud, D., Korhagen, T. R., Arum, A., Miyajima, A., and Murray, R. (1995) Immunity 2, 211–222
7. Dirksen, U., Ishizaka, K., Gronert, P., Hattenhorst, U., Nogee, L., Murray, R., and Burdach, S. (1997) J. Clin. Invest. 100, 2211–2217
8. Hawgood, S., and Poulin, F. R. (1995) Pediatr. Pulmonol. 19, 99–104
9. Korhagen, T. R., Bruno, M. D., Ross, G. F., Huelmans, R. M., Ikegami, M., Jobe, A. H., Wert, S. E., Stripp, B. R., Morris, R. E., Glasser, S. W., Bachurski, C. J., Iwamoto, H. S., and Whitsett, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9594–9599
10. Ikegami, M., Korhagen, T. R., Bruno, M. D., Whitsett, J. A., and Jobe, A. H. (1997) Am. J. Physiol. 272, L479–L485
11. Ikegami, J., Korhagen, J. R., Whitsett, J. A., Bruno, M. D., Wert, S. E., Wada, K., and Jobe, A. H. (1998) Am. J. Physiol. 274, L247–L254
12. Clarke, J. C., Wert, S. E., Bachurski, C. J., Stahlman, M. T., Stripp, B. R., Weaver, T. E., and Whitsett, J. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7794–7798
13. Wright, J. R. (1997) Physiol. Rev. 77, 931–962
14. Harshborth, K. L., Crouch, E., White, M. R., Colamussi, M. L., Kakkashnutt, A., Tauber, B., Shepherd, V., and Sastry, K. W. (1998) Am. J. Physiol. 274, L368–L369
15. Holmskov, U., Lawson, P., Teisner, B., Tornoe, I., Willis, A. C., Morgan, C., Koch, C., and Reid, K. B. (1997) J. Biol. Chem. 272, 13743–13749
16. Chronopoulou, Z. C., Abdolrasulnia, R., Whitsett, J. A., Rice, W. R., and Shepherd, S. M. (1996) J. Biol. Chem. 271, 28137–28142
17. LeVine, A. M., Bruno, M. D., Huelmans, K. M., Ross, G. F., Whitsett, J. A., and Korhagen, T. R. (1997) J. Immunol. 158, 4336–4340
18. Reinecke, U., David, J. C., Huelmans, K. M., Korhagen, T. R. (1998) Am. J. Respir. Cell Mol. Biol., in press
19. Shimizu, H., Fisher, J. D., Papst, P., Benson, B., Lau, K., Mason, R. J., and Voelker, D. R. (1992) J. Biol. Chem. 267, 1853–1857
20. Rust, M., Bingle, L., Maranovich, W., Person, A., and Crouch, E. C. (1996) Am. J. Respir. Cell Mol. Biol. 14, 121–130
21. Terry, R. W., Kwiti, L., Baldwin, H. S., and Laibow, M. A. (1997) Transgenic Res. 6, 349–356
22. Mason, R. J., Nellenbogen, J., and Clements, J. A. (1976) J. Lipid Res. 17, 281–284
23. Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468
24. Kolesnikov, M. V., Hallman, M., and Gluck, L. (1979) Am. J. Obstet. Gynecol. 135, 57–63
25. Zhou, L., Dey, C. R., Wert, S. E., Yan, C., Costa, R. H., and Whitsett, J. A. (1998) Dev. Dyn. 210, 305–314
26. Horovitz, A. D., Korak, R., Mousavarnia, B., Whitsett, J. A., Wert, S. E., Hull, W. M., McNanie, J., and Ikegami, M. (1997) Am. J. Physiol. 273, L468–L477
27. Verbrugge, S. J., Profitt, S. A., Nogee, L. M., and Whitsett, J. A. (1995) Am. J. Physiol. 268, L647–L656
28. Pain, V. M. (1996) Eur. J. Biochem. 236, 747–771