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Surfactant Protein-C in the Maintenance of Lung Integrity and Function

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Abstract

Surfactant protein-C (SP-C) is a lung cell specific protein whose expression is identified from the earliest stages of mammalian lung development in a subset of developing epithelial cells and in the alveolar type II cell in the mature lung. Although SP-C gene expression is not critical and protein function is not necessary for the normal developing morphological patterning of the lung, studies of SP-C protein mutations and SP-C deficiency have revealed critical roles of SP-C in the maintenance and function of the preterm and mature lung during various forms of intrinsic or extrinsic lung injury. This review summarizes studies using *in vitro* experimental approaches, *in vivo* modeling in transgenic mice, and analysis of human disease pathogenesis. Collected data reveal an essential role for SP-C singly and in combination with other lung proteins, in maintenance of lung structure and pulmonary function of the immature and mature lung.

Introduction

Development of the lung begins during mid embryogenesis as an outgrowth of the foregut endoderm. Establishment of the respiratory tree involves the progressive growth and branching of endodermal tissue that invades pulmonary mesenchyme to produce an extensive conducting network that terminates in saccules. These saccular structures in turn must dilate and undergo septal extension, thinning, and division to form the acinar units that mature into efficient gas exchanging alveoli. Extension by the invading epithelium requires the simultaneous growth in parallel of the pulmonary vasculature to produce a fine lattice-like capillary bed that surrounds the developing alveolar structures. Lung development and alveologenesis does not terminate at birth. While initial alveolar maturation occurs prenatally in humans the expansion of the number of alveoli continues well into postnatal life [1]. Hypoxia and childhood illnesses influence the final number of alveoli produced [2]. Postnatal lung development is more extensive in mice than in humans. Alveolar units continue to be added after birth and the differentiation of the alveolar epithelium occurs perinatally in mice. Evaluation of term and postnatal septation in rodents indicates that two thirds of alveoli are formed after birth [3]. The mammalian lung is exposed to a variety of injurious substances. Both the developing lung in preterm infants and the mature lung can be injured by infection or inhalation of environmental toxicants which can result in aberrant lung remodeling and function leading to acute or chronic lung diseases. Studies of innate inmmune molecules in the lung show that many of these molecules including SP-C are produced in the prenatal developing lung.

In the mature lung, the proximal airway to distal alveolar surfaces of the lung are lined by a continuum of highly differentiated epithelial cell types. These cells synthesize and secrete material to preserve hydration, protect against inspired irritants or pathogens and maintain lung compliance. Pulmonary mucin imparts protective functions along the conducting surface of airways while pulmonary surfactant shields the alveolar surface and portions of the conducting airway. Pulmonary surfactant is the critical protective phospholipid - protein complex that reduces surface tension and prevents alveolar collapse at end expiration. Surfactant deficiency in the preterm infant is a major cause of neonatal respiratory distress syndrome (RDS) [4,5]. In full term infants impaired surfactant metabolism and production also cause acute respiratory failure. Important among the goals of pulmonary research has been the identification of critical genes and networks regulating lung development, function, and recovery from injury. These studies of the developing lung have led to identification of surfactant proteins which although produced in the developing lung also have roles in the maintenance of the mature lung. Such studies have been important in identifying necessary surfactant proteins in bovine and porcine surfactant extracts that are used in treating respiratory distress in infants.

Surfactant proteins (SP-A, SP-B, SP-C and SP-D) facilitate surface absorption and spreading of the surfactant phospholipid film or bind to microbial pathogens to enhance their clearance and prevent or reduce the severity of infection. However, all four of the surfactant proteins have distinctive structures, functional properties, and patterns of expression. SP-A and SP-D are aqueous soluble proteins that harbor collagen like domains and lectin-like binding domains and are thus commonly referred to as collectins. The collagen sequences form higher ordered oligomers while the lectin binding motifs mediate microbial binding and aggregation [6]. SP-B and SP-C are highly hydrophobic peptides that associate with the surfactant phospholipids. SP-B has multiple amphipathic helical domains and is essential for intra-cellular surfactant formation and extracellular surface activity [7]. SP-C is the most hydrophobic of the peptides and only 35 amino acids in length [4,8]. Cloned segments of the SP-C gene promoter have been used as probes of alveolar epithelial cell development and to identify transcription factors that determine cell specific expression. These SP-C promoter elements are widely used to alter lung development and model lung disease in transgenic mice [9]. Defects of SP-C expression underlie some forms of human lung disease and mutated or deficient SP-C cause interstitial lung disease (ILD) and idiopathic pulmonary

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fibrosis (IPF). The role of SP-C in human lung development, disease and application of the SP-C promoter to model injury is the subject of this review.

Control of Surfactant Synthesis and Catabolism is Essential for Respiratory Function and Alveolar Stasis

The production of pulmonary surfactant is a highly structured biosynthetic process. Pulmonary surfactant is synthesized and secreted by a distinct subset of cuboidal epithelial cells (type II cells) in the alveolus while gas exchange occurs across thin highly attenuated type I epithelial cells. Phospholipids and surfactant proteins are assembled into multivesicular bodies that coalesce into concentric membranous lamellae containing organelles. These lamellar bodies serve as the intracellular storage form of surfactant in the type II cells (Figure 1). Exocytosis of the lamellar body into the extracellular space results in a rapid unraveling and release of phospholipid rich material that ultimately reorganizes into a monolayer across the surface of the alveolus [10,11]. The compression and expansion of the phospholipids in the surfactant film lower surface tension and impart resistance to collapse during respiration. During repetitive breathing extracellular forms of surfactant are altered and depleted of SP-B/SP-C. Surfactant is internalized by type II cells in a recycling pathway for re-incorporation into surface-active material. Rare forms of newborn or childhood interstitial lung disease are caused by mutations that impede surfactant production and metabolism. Altered type II cell specific phospholipid routing disrupts lamellar body formation and results in a fatal surfactant dysfunction. Alveolar surfactant levels are also regulated by macrophages that phagocytose and degrade surfactant. Impaired surfactant catabolism results in a clearance disorder termed alveolar proteinosis where the alveolar lumens fill with surfactant material and alters respiratory function [12].

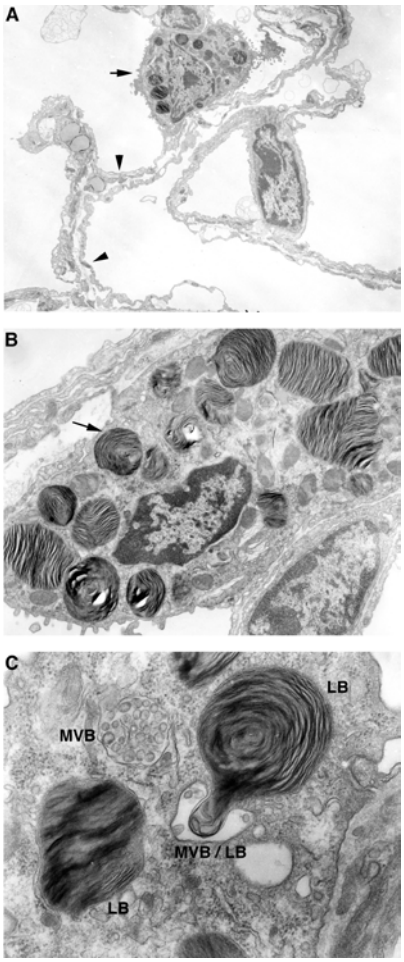

Figure 1: Ultrastructural detail of alveolus, type II cell and surfactant biosynthetic elements

A: The alveolar surface is line by an epithelium of two distinct differentiated cell populations. A small set of cuboidal type II epithelial cells (arrow) produce and secrete surfactant onto the alveolar lumen, note surface microvilli and perinuclear granules. The surfactant film confers alveolar stability during respiratory cycles and prevents injury to the thin extended type I epithelial cells (arrowheads).

B: Higher magnification image of a type II cell. The type II cell is the singular site of SP-C synthesis. The mature type II cell contains numerous cytoplasmic lamellar bodies (arrow) formed by concentric lipid sheets. Lamellar bodies are the mature stored surfactant secretory granules.

C: High magnification depicts maturation of the lamellar body. Pre-lamellar multivesicular body (MVB) contains small membrane bound vesicles that fuse to form the lamellar body. MVB/LB indicates a transition structure of MVB partially incorporated into the growing lamellar body (LB) via continuous outer limiting membrane and internal vesicle-to-lamellar membranes. SP-C has been localized to the MVB and LB [11].
Magnifications: A: 2,700X B: 8,000X C: 27,000X Micrographs courtesy of Dr. Cheng-Lun Na.

SP-C Structure, Processing, and Function

SP-C is expressed as a 197 amino acid proprotein of approximately 21 kD that consists of three distinct domains: 1) a short 23 amino acid N-terminal peptide required for late endosome routing, 2) a hydrophobic helical transmembrane domain which corresponds to the mature, secreted form of SP-C, and 3) a 14kD C-terminal peptide containing a BRICHOS 'chaperone' domain. The BRICHOS related region of proSP-C is a structurally similar domain found in previously unrelated rare degenerative diseases and forms of cancer [8,13]. After transport from the ER to the Golgi, the adjacent cysteines of the transmembrane domain are palmitoylated. This in turn initiates endocytic translocation to multivesicular bodies where sequential proteolysis steps eliminate the long C-terminal domain. Upon condensation of the multivesicular bodies into densely packed lamellar bodies, the short N-terminal domain is proteolytically removed yielding a 35 amino acid peptide of 3.7kD that is the mature form of SP-C secreted into the airspace.

The central hydrophobic domain of mature SP-C is comprised of a valine rich region (10 of 16 residues) flanked by additional hydrophobic amino acids. This domain is configured as a trans-membrane spanning alpha helix with covalent attachment of palmitoyl groups at two adjacent amino terminal cysteine residues. Thus SP-C is a true lipopeptide able to associate with the surfactant phospholipid film, cellular membrane structures, or membrane associated proteins via the hydrophobic helical domain and/or the palmitoyl side chains. Historically, the function of SP-C has been associated with surface active properties imparted by both the helical domain and the palmitoyl chains. Insertion of SP-C alters phospholipid packing and facilitates the rapid spreading of surfactant lipids and imparts stability of surfactant monolayer films [4,8,10]. While SP-C does exhibit surface tension lowering properties, these do not approach the efficiency exhibited by the amphipathic SP-B to lower surface tension to near-zero (mN/m) values. However, fully palmitoylated SP-C is required for optimal surface active properties and has been further shown to exhibit synergistic surface activity with

SP-B [14]. It should also be emphasized that the palmitoyl groups and adjacent amino acids have also been shown to promote binding to phosphatidylglycerol and lipopolysaccharide, the latter of which is unrelated to its surface active properties. Palmitoylation of several proteins, including G proteins, facilitates interaction with membrane associated receptor proteins, offering an additional potential role for the palmitoyl groups of SP-C.

Owing to the surface active properties of SP-B and SP-C, extracts of porcine and bovine surfactant are now used clinically to rescue neonatal respiratory distress. These surfactant extracts contain variable amounts of SP-B and are enriched in SP-C [15]. Instillation of SP-C-phospholipid preparations into the lungs of surfactant deficient animals improved the outcome of experimental RDS. Recombinant SP-C based preparations decreased injury in both a preterm animal model of pulmonary surfactant deficiency and animals with acute lung injury induced by lavage depletion of surfactant [16-18]. A synthetic surfactant comprised of recombinant SP-C and synthetic phospholipids has been tested in trials for adult RDS with limited efficacy [19]. The poor outcome of the clinical trials may reflect the complex origins, advanced injury, and spectrum of disorders grouped into adult RDS relative to the developmental deficiency that is newborn RDS.

As is discussed in latter sections, several lung diseases are associated with alterations in the stability of either the helical domain or acylated cysteines of mature SP-C. For example, SP-C isolated from broncho-alveolar lavage of patients with pulmonary alveolar proteinosis exists as misfolded amyloid-like fibrils, where the helical region of SP-C is transformed into beta-sheet aggregates. The beta transformation results in SP-C fibril structures that are associated with plaque induced injury [20,21]. Such structures have been recapitulated *in vitro* by deacylation and acidification [20]. Comparative analysis of native and deacylated SP-C indicates that acylation is required to stabilize the helical conformation of mature SP-C and increases surface phospholipid interactions [22,23]. In addition, the BRICHOS related region of proSP-C appears to function as an intramolecular chaperone that induces proper SP-C (helical) conformation of the transmembrane region (corresponding to mature SP-C) during the early stages of

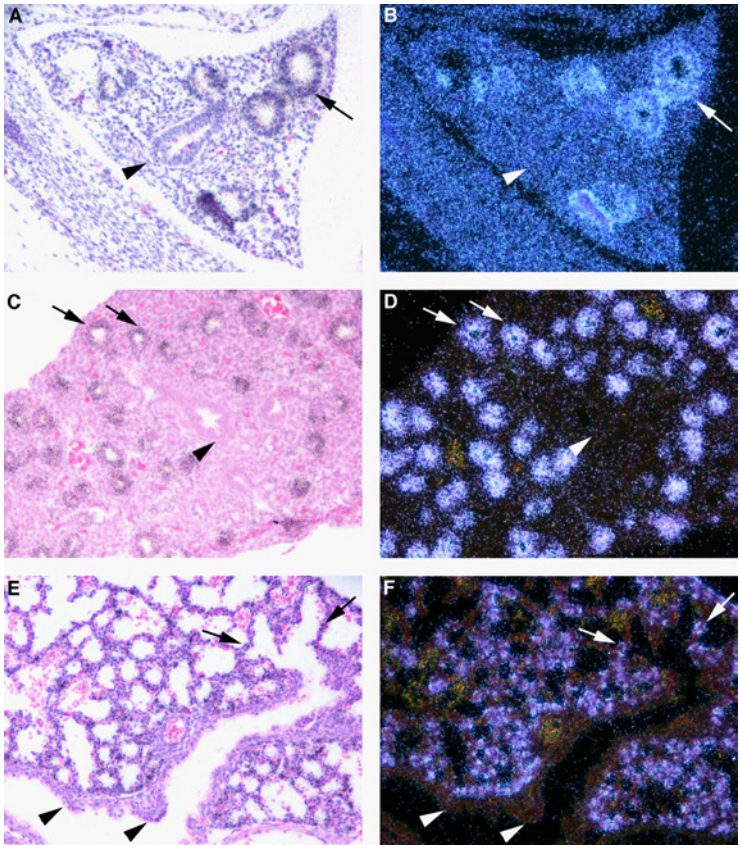

Figure 2: Pattern of SP-C expression during lung development

In situ hybridization was used to identify sites of SP-C expression in the developing mouse lung: embryonic day (ED) ED13 (panels of A, B), ED15 (panels of C, D), ED18 (panels of E, F).
A, C, E are bright field images to visualize tissue morphology.
B, D, F are corresponding dark field image where hybridization of the SP-C riboprobe to SP-C mRNA appears as light granules. Arrowheads indicate central airway epithelia, arrows indicate peripheral extension of the growing airway(s) epithelia where SP-C is expression is sustained.
Weak SP-C expression is detected in cells of the primitive airways of the ED13 lung rudiment where branch formation has begun. SP-C expression is already decreased in the central most tubule (arrowhead panels A, B).
The relative expression of SP-C has increased and localizes to the extending epithelium in ED15 lung. As branching morphogenesis proceeds SP-C expression is silenced in cells lining the larger central airways (arrowhead) and restricted to the most peripheral aspects of the invading epithelium.
By ED18 lung mesenchyme has thinned and septation has begun. SP-C expression is extinguished in the conducting airway epithelia and is only detected as strong focal sites of expression in the alveolarizing compartment. All images 20X original magnification.

processing [24,25]. The BRICHOS region of proSP-C has been shown to bind to the unfolded poly-valine region and facilitate alpha helical formation of mature SP-C [19,20]. Genetic mutations leading to specific amino acid substitutions in the BRICHOS region of proSP-C have been associated with RDS and familial SP-C deficiencies.

The pattern of SP-C expression is developmentally regulated

Each surfactant protein has distinct patterns of expression in a subset of respiratory epithelial cells that include the alveolar type II cell. Type II cells have regenerative properties, proliferating to renew the alveolar epithelial surface following injury. SP-C is the most restricted of the surfactant proteins being detected only in the surfactant producing type II cell in the adult lung while SP-A, B, and D are expressed in tracheal gland cells (SP-A) and the bronchiolar -Clara cell epithelial population as well as alveolar type II cells. SP-C is the earliest expressed surfactant protein. It is produced at high abundance early in organogenesis [26]. In the mouse embryo the lung rudiment emerges from the endodermal foregut as distinct buds at embryonic day (ED) 9.5 -10.0. SP-C mRNA is detected as early as ED11. SP-C mRNA is readily detected by *in situ* hybridization in the most distal subset of cells lining the primitive airway rudiments (Figure 2A, B). SP-B expression is detected approximately 2 days later and SP-A, SP-D still later. As lung morphogenesis progresses SP-C expression is sustained in cells at the tips of peripheral elongating branches and extinguished in the cells lining the central and proximal tubules (Figure 2, C-F) [26]. The relative expression of SP-C increases throughout development most likely reflecting the increase in the number of terminal buds generated by branching morphogenesis harboring SP-C positive cells. Ultimately SP-C gene expression is detected in a focal pattern throughout the alveolar-parenchymal compartment of the adult mouse lung that is consistent with the location of differentiating type II cells.

SP-C expression is intimately linked to differentiation and maintenance of the type II cell *in vivo*. In a model of forced ectopic differentiation SP-C expression is induced when heterologous non-alveolar tissue is reprogramed *in vitro* to assume a distal parenchymal appearance. When embryonic tracheal epithelial rudiments are excised, stripped of mesenchyme, and cultured in a defined matrix-media that supports type II cell differentiation the tracheal epithelium buds and branches to form alveolar-like structures. At the ultrastructure level the cells lining the tracheal buds trans-differentiate into surfactant producing type II cells. SP-C expression is detected by *in situ* hybridization at the tips of distal extending airways in a pattern that matches the intrinsic SP-C expression observed during embryonic lung maturation [27]. Thus SP-C is the earliest surfactant marker of the pre-type II cells during organogenesis, is the only surfactant protein gene expressed just in the alveolar type II cell and serves as the most specific marker of alveolar maturation, injury, or successful remodeling.

Analysis of the SP-C promoter has identified enhancers and transcription factors that dictate cell selective gene expression

Functionally testing regions of the human SP-C gene promoter in transgenic mice identified the segments of DNA that directed lung specific gene expression. 3.7 kB of the human SP-C promoter proximal genomic DNA conferred lung specific expression of a linked reporter gene [28,29]. Transgene expression was localized to distal respiratory and alveolar epithelial cells. 4.8kB of the mouse SP-C gene promoter also produced strong lung specific expression in transgenic mice. *In vivo* deletion analysis localized an essential lung specific element to 318 bp of DNA adjacent to the basal promoter. Lung expression from the 318bp SP-C transgenic lines was extremely weak and inferred that a strong enhancer resides in the more distal region of the 4.8kB of genomic DNA [30]. Cell transfection experiments confirmed the existence and nature of the distal enhancer region. In transient cell transfection assays the 4.8kB SP-C promoter constructs produced low-level expression while in stable cell transfection experiments the promoter activity increased by almost two orders of magnitude. An increase in expression after stable integration did not occur with the 318 bp SP-C construct. Increased SP-C driven expression following stable transfection indicates that the upstream region of the 4.8kB SP-C promoter construct contains an enhancer that requires chromatin integration to impart the high level SP-C expression.

The 318 bp of SP-C genomic DNA that directed *in vivo* lung specific expression has been used to identify transcription factors that activate SP-C expression. Cell transfection experiments with a 318bp SP-C promoter-luciferase reporter plasmid in combination with candidate transcription factor constructs revealed that the homeodomain factor Nkx2.1 stimulates the SP-C promoter by interaction at two adjacent binding sites [31]. Nkx2.1 is expressed in a limited set of organs including the brain, thyroid, and lung. Nkx2.1 is expressed in the earliest developing lung rudiment and loss of the Nkx2.1 expression *in vivo* disrupts lung morphogenesis indicating that Nkx2.1 is essential for lung organogenesis [32-34]. Nkx2.1 activates the SP-A and SP-B promoters as well, indicating that it has a central role in the regulation of surfactant protein gene expression. The transcription factors Gata-6, NF1, TAZ, and Erm-1 stimulate SP-C expression and bind in close approximation to the Nkx2.1 sites. Similar to Nkx2.1, Gata-6 is a driver of distal lung morphogenesis and is expressed in alveolar type II cells. Over-expression of Gata-6 or expression of dominant negative Gata-6 impaired alveologenesis in the developing lung [35,36]. Gata-6, TAZ, NF1 and Erm-1 were shown to interact directly with Nkx2.1 and synergistically stimulate SP-C expression [37-40]. Collectively these findings identify a set of factors that are critical for directing lung formation and also directing SP-C gene transcription.

SP-C deficiency and mutations are associated with rare familial ILD and increase susceptibility to injury

Although uncommon some full term infants have respiratory distress at birth while others will develop respiratory diseases in later infancy and childhood. While phenotypically heterogeneous, these disease are linked to mutations that inhibit surfactant function and are collectively termed surfactant dysfunction diseases [41,42]. Mutations that alter SP-B structure or expression result in an immediate fatal RDS at birth [43]. Ultrastructure studies from infants with SP-B mutations or mice with an inactivated SP-B gene reveal common features of incomplete intracellular lamellar body formation and reduced airspace surfactant that has poor surface activity. Infants with SP-B mutations also have misprocessed proSP-C indicating that both surfactant proteins are depleted and that SP-B and SP-C processing are interdependent [44]. Individuals with familial forms of interstitial lung disease (ILD) were linked to impaired SP-C expression. The pulmonary phenotype associated with abnormalities of SP-C expression is more heterogeneous than neonatal SP-B deficiency. SP-C related respiratory decline may occur as an acute post partum crisis, emerge slowly in childhood or go undetected until later in life. The clinical profile of adult onset SP-C related lung disease varies from mild ILD to severe pulmonary fibrosis [45]. SP-C deficiency has also been attributed to the onset of bovine and canine pulmonary fibrosis [46,47].

Mutations in the SP-C gene (*SFTPC*) are the underlying molecular lesions of SP-C dysfunction disease [5,45]. Mutations alter proSP-C

structure and reduce levels of mature SP-C. The inability to correctly process aberrant forms of proSP-C can generate a cellular injury derived from misfolded protein stress responses, the accumulation of aggregated forms of pro-SP-C or from the absence of mature SP-C. The majority of *SFTPC* mutations map to the carboxy terminal region of proSP-C that harbors the BRICHOS domain. The BRICHOS region can bind the polyvaline hydrophobic domain of SP-C and induce proper helical formation inferring the BRICHOS segment of proSP-C functions as a molecular chaperon. The predominance of the BRICHOS mutants indicates an essential role for this domain in SP-C maturation and assimilation into lamellar bodies. Several *SFTPC* mutations occur more frequently in affected individuals. The most common variants include single nucleotide changes that eliminate an mRNA splice site to delete exon 4 (DelEx4), substitutions of a single amino acid leucine to glutamine at position 188 (L188Q in exon 5) or isoleucine to threonine position 73 (I73T in exon 3) [48,45,49]. The "molecular consequences" of these structural alterations vary. Del Ex4 and L188Q mutants were found to localize as perinuclear endoplasmic reticulum aggregates along with increased components of a misfolded protein response. Expression of the non-BRICHOS I73T proSP-C generates abnormal vesicular structures that are misrouted to plasma membrane - endosomes and have reduced recycling of phospholipids [50]. Normally type II cells actively clear and reprocess pulmonary surfactant from the alveolar lumen. Individuals with I73T mutations have an accumulation of surfactant in the airspaces that is consistent with the observed misrouting and decreased reutilization of surfactant phospholipids.

SFTPC associated mutations are more frequently identified in familial forms of pulmonary fibrosis, 5 of 20 patients sequenced compared to 1 mutation from 135 patients sequenced and 0 of 35 patients with nonfamilial, sporadic IPF [51-53]. Gene sequencing of a cohort of 121 children with diffuse lung disease identified 18 *SFTPC* mutations with hereditary transmission for 9 individuals [54]. The fibrosis resulting from SP-C mutation/dysfunction provides compelling evidence that mutations that compromise the respiratory epithelium underlie idiopathic forms of pulmonary fibrosis. Additional mutations that support this premise are summarized in the final subsections.

Additional defects underlie SP-C related ILD. SP-C deficient disease has been identified in a smaller subset of individuals without any structural *SFTPC* mutation. One report identifies familial childhood ILD with intermittent oxygen requirements and frequent exacerbations and in another report the clinical manifestation was pulmonary fibrosis [55,56]. Three non-coding sequence polymorphisms (presumptive mutations) were found upstream of the *SFTPC* gene in infants with SP-C related respiratory distress. The introduction of these changes in *SFTPC* promoter constructs reduced the transcriptional activity in transient cell expression assays thus demonstrating that these variants were mutations capable of decreasing SP-C expression [57]. These studies support the concept that abnormal transcriptional regulatory elements are important causes of human lung diseases. The mutations are found at positions -2385 to -1167 bp from the basal *SFTPC* promoter and fall within a conserved region of the human and mouse *SFTPC* promoters that direct strong expression in transgenic mice [30]. These relatively uncommon forms of the SP-C dysfunction disease have decreased SP-C expression without altering proSP-C/SP-C structure that could generate a misfolded protein cellular injury. Such SP-C null mutations show that the observed clinical disease can be multifactorial arising from an SP-C deficient state or from cellular injury due to SP-C variants that impose a misfolding, aggregation, or misrouting injury.

Mouse models reproduce features of SP-C related lung disease

SP-C deficient (*Sftpc* -/-) mice have been generated by gene targeting techniques. The gene disruption completely ablates SP-C mRNA production and subsequent protein expression. The *Sftpc* -/- mice serve as a model of SP-C loss-of-function without the potential injury induced by the malformed proSP-C. The surfactant from *Sftpc*-/- mice was found to have subtle alterations in surface stability *in vitro* [58]. *Sftpc* -/- mice on a specific inbred background developed irregular lung inflammation and remodeling with features of ILD consistent with genetic influences modifying the human disease phenotype [59]. Genetic variability appears to complicate human *SFTPC* related disease wherein affected individuals within a family exhibit dramatic differences in the severity and time of onset even though they carry the same mutation [45]. Both the lack of SP-C and the overproduction of a proSP-C mutant predispose the animals to lung injury. *Sftpc* -/- mice had an increase fibrotic response and impaired recovery to challenge with the toxicant bleomycin [60]. Transgenic over-expression of a representative human *SFTPC* mutation deleting exon4 (DelEx4) profoundly disrupted normal lung morphogenesis and caused death. The injury was proportional to the level of DelEx4 expression and included hypoplasia, reduced branching morphogenesis, cystic terminal airspaces and reduced epithelial cell maturation [61]. The severe phenotype likely reflects the unrestricted early embryonic expression of DelEx4 conferred by the conventional 3.7kb SP-C promoter construct. To control the onset and duration of mis-expressed proSP-C, transgenic mice were generated where the target gene (proSPC) is silent until specifically activated in the respiratory epithelium by administration of doxycycline. The doxycycline-induced activation of the human L188Q mutant proSP-C in the lungs of adult transgenic mice produced no observable lung pathology, inferring that this variant was not cytotoxic. However the L188Q mice were more sensitive to bleomycin induced lung fibrosis. Stimulation of endoplasmic reticulum stress by administration of tunicamycin further exacerbated the bleomycin induced L188Q fibrosis [62]. In summary the absence of mature SP-C (*Sftpc* -/- mice) or presence of aberrant precursors of SP-C (L188Q) can predispose the lung to fibrotic injury. This inability to resolve inflammation or injury may be a component of disease pathogenesis.

SP-C confers an immunoprotective role against pulmonary pathogens

Singular and recurrent lung infections have been reported to complicate the health of individuals with *SFTPC* mutation or non-mutation related disease [45,53,54,63]. The reports primarily identify viral related exacerbation that includes RSV, parainfluenza, and influenza, as well as less common bacterial pathogens. SP-C has been shown to bind to bacterial lipopolysaccharide (LPS) and to enhance the recognition of influenza-derived immunogenic peptides by the nasal epithelium and reduced influenza infectivity upon challenge [64,65]. These observations support the hypothesis that SP-C has anti microbial and immuno-protective activity in the alveolar and upper airways. The non-alveolar functions likely reflect that in a healthy lung alveolar surfactant migrates up the airway and can act at distant epithelia.

Infection of strain-matched *Sftpc* +/+ and -/- mice was used to test if the loss of SP-C influenced susceptibility to respiratory pathogens. *Sftpc* -/- mice had reduced clearance of the bacterial pathogen *Pseudomonas aeruginosa*. The influx of inflammatory cells was increased and tissue injury included interstitial thickening, septal fragmentation, and goblet cell hyperplasia. Alveolar macrophages had altered morphology and

decreased phagocytic activity. The recovered *Sftpc* -/- macrophages expressed markers of alternatively activated macrophages and had increased production and secretion of matrix metaloproteinases that modify the extra cellular matrix [66]. This decreased antimicrobial response and expression of remodeling-related markers suggests that SP-C regulates the sentinel status of alveolar macrophages.

Sftpc -/-mice had an impaired response to challenge with respiratory syncytial virus (RSV). Viral clearance was decreased. Pulmonary pathology was increased in *Sftpc* -/- mice that included expanded alveolar interstitium, increased inflammatory cell infiltrates, and a goblet cell hyperplasia similar to the airway injury following P. aeruginosa infection [67]. The increased viral inflammation in *Sftpc* -/- mice was mediated by the innate immune receptor TLR3. *In vitro*, SP-C preparations decreased synthetic double stranded RNA activation of TLR3. The infection studies in *Sftpc* -/- mice compliment the clinical findings that individuals with reduced levels of SP-C or specific *SFTPC* mutations have compromised innate defense. SP-C blocks activation of viral innate receptors during infection and regulates the responsive status of alveolar macrophages. These studies show a direct role of SP-C in protecting the alveolus from microbial injury.

Other pulmonary epithelial specific gene dysfunctions induce fibrotic lung disease

SP-C associated ILD /IPF supports the concept that fibrotic lung disease can be initiated by epithelial cell crisis that eventually stimulates aberrant repair activity by fibroblasts. Recent population studies link defects of the pulmonary mucin MUC5B, surfactant protein A2 (*SFTPA2*), *ELMOD2* and telomerase genes (*TERC* and *TERT*) as a cause of ILD and pulmonary fibrosis [68-71]. MUC5B is expressed in airway epithelial cells. A *MUC5B* promoter polymorphism was identified in affected individuals from an extensive population study of 82 enrolled families. MUC5B immunostaining was increased in the airway epithelium inferring that the polymorphism increases mucin expression and accounts for the epithelial directed onset of IPF. SP-A is expressed in alveolar and airway epithelial cells and submucosal glands. Distinct mutations within the SP-A2 carbohydrate recognition/lectin-binding domain were detected in two index families with a variable phenotype of pulmonary fibrosis or cancer. The telomerase complex maintains chromosomal integrity during cell replication to control proliferation. Type II cells in morphologically normal regions of lung adjacent to fibrotic areas were found to have reduced telomerase expression and increased cellular apoptosis suggesting that reparative type II cell proliferation is reduced in emerging fibrosis [72]. ELMOD2 is a GTPase that is selectively expressed in alveolar macrophages and type II cells and regulates TLR3 mediated viral responses indicating an overlap of mechanisms with the reduced TLR3 response in *Sftpc* -/- mice [73]. The mutations linked to these various human disease pedigrees adversely affect epithelial function and support the general concept that the epithelium drives ILD/fibrosis.

Secondary mutations that influence SFTPC disease

The natural course of parenchymal injury with SP-C related disease may be altered by additional mutations that impact surfactant synthesis and secretion. One candidate modifier is ABCA3. ABCA3 is a phospholipid transporter that is expressed specifically in alveolar epithelial cells and localizes to the lamellar body. Surfactant from the lungs of infants with ABCA3 gene mutations have altered phospholipid composition and reduced surface activity. Ultrastructure of the lungs from affected infants show malformed lamellar bodies in the type II cells consistent with ABCA3 as critical for phospholipid transport and incorporation [74]. ABCA3 gene targeted mice lack ABCA3 and have similar pathology with lamellar body defects and decreased surfactant biophysical activity. Unrelated infants with a history of lung disease were found to be heterozygous for both *SFTPC* and *ABCA3*. The compound *SFTPC/ABCA3* children had more severe lung disease than infants with just the specific *SFTPC* mutation [75]. A separate study of familial ILD detected subclinical interstitial changes in an *SFTPC / ABCA3* individual by high resolution computed tomography imaging. The early parenchymal alterations had not developed in the *SFTPC* only sibling supporting the interpretation that ABCA3 increases the severity of tissue destruction from the *SFTPC* defect [76].

A second potential modifier is the essential developmental control gene Nkx2.1. As detailed the Nkx2.1 gene is essential for both lung development and surfactant protein gene activation. Mutations that disable the Nkx2.1 gene produce a variable triad of thyroid, lung, and brain developmental deficits. Newborn respiratory failure is associated with approximately one half of the affected individuals. One recent report of fatal Nkx2.1 disease demonstrated a selective decrease in surfactant phospholipid and SP-C levels while the level of SP-B (also Nkx2.1 regulated) was unchanged [77]. This finding indicates that Nkx2.1 controls unknown steps in the surfactant phospholipid synthetic pathways and may impose a greater influence on *SFTPC* transcription than other Nkx2.1 responsive genes. The mechanism for this unequal loss of expression of an Nkx2.1 target gene (SP-C but not SP-B)is unknown. Perhaps other transcription factors compensate for the decreased Nkx2.1 activity to sustain SP-B expression. Although there was a clear selective loss of SP-C the pathogenic changes may be from yet-to-be defined co-regulators of Nkx2.1.

Summary

SP-C is expressed in a highly selective tissue and developmental pattern marking early presumptive distal lung epithelium and differentiated alveolar type II cells in the mature lung. Analysis of the SP-C promoter has identified genetic motifs that guide lung specific expression. Cloned SP-C promoter sequences have been used to target gene expression in the lung in order to model a host of lung disease processes. Disorders of SP-C expression may injure alveolar cells, modifying their functions leading to heterogeneous diffuse lung disease as well as pulmonary fibrosis. The molecular pathogenesis of SP-C dysfunction disease may be a composite of cellular injury generated by toxic proSP-C and alveolar injury from diminished airspace SP-C. Animal models validate the link of SP-C dysfunction with the human ILD. Infections in the SP-C null mice indicate that SP-C is immuno-protective suppressing bacterial and viral infection.

Abnormalities in SP-C dysfunction can be exacerbated by mutations in other genes. Homozygous recessive mutations for phospholipid transporter ABCA3 induce severe disease and even a single defective *ABCA3* allele increases the severity of *SFTPC* mutations. Mutations that ablate SP-B expression generate aberrant forms of proSP-C indicating that proper SP-B and SP-C processing are linked. ProSP-C binds and induces helical conformation of mature SP-C. Collectively these results provide evidence for dynamic protein interactions to achieve proper structure and assembly of surfactant in the type II cells. These protein interactions show that some disease processes are due to multiple steps in surfactant biosynthesis, composition or metabolism. Although the causes of many infant and childhood interstitial lung diseases remain unknown studies of surfactant proteins, in particular SP-C and other lung expressed proteins including ABCA3 and NKX 2.1 show that

single or multiple gene mutations and their altered protein function in the pathogenesis of significant human lung diseases.

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