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**LUNG DEVELOPMENT AND NEONATAL LUNG DISEASE:
SURFACTANT PROTEINS AS STUDY MODEL**

A Thesis in
Integrative Biosciences

by
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ABSTRACT

There are approximately 400,000 infants born prematurely each year in the United States alone. Recent technological advances in perinatal and neonatal medicine have decreased morbidity and increased survival rates of prematurely born infants. Modern therapies, such as surfactant, steroids and gentler ventilation have lead to a drastically decreased age and weight of surviving newborns. As a result, there has been a change in incidence of neonatal pulmonary disease and its pathophysiology. Although current neonatal therapies have many advantages, they can cause long-term changes in lung physiology. Even though ventilatory support of premature neonates is essential, ventilation of the anatomically and biochemically immature lung structure results in alveolar simplification and damage. Neonatal steroid therapy, although beneficial for surfactant production, leads to the arrest of alveolarization that results in decreased number of alveoli. These changes have been proposed to have long-term consequences that may extend into adulthood. Furthermore, the underlying genetic background appears to affect the outcome of premature birth and the differential response to therapy, such as steroids.

There is a compelling need to study normal lung development, lung pathology due to premature birth, and the effects of current and novel therapeutic treatments on both states. In order to shed light on these processes, a multifaceted but integrated approach is needed. Such an approach should incorporate knowledge gained from models of lung development and lung disease, as well as knowledge derived from clinical and translational studies of neonatal pulmonary disease.

The work presented in this thesis describes; 1) development of an *in vivo* model of human fetal lung development; 2) development of a pyrosequencing genotyping method; and 3) an association of candidate genes with neonatal pulmonary lung disease. This *in vivo* model allows for the study of human lung developmental processes and could be used to study the effects of current and novel therapeutic modalities on lung development and pathogenesis. In addition, the specific effects of these modalities could be studied by choosing fetal lungs with the genetic background of interest. It is anticipated that the work presented in this thesis will provide a starting point for study of a number of processes related to lung development and therapies during premature birth.

The focus here has been on the role of surfactant proteins in lung development and neonatal lung disease. These proteins are good candidate genes for these studies due to their essential role in lung development, lung maturation, and host defense. Development of a functional alveolar epithelium capable of gas exchange and surfactant secretion is essential for successful adaptation of the fetus to extra-uterine life. Premature birth is commonly accompanied by a deficiency and perturbation of the surfactant system. Pulmonary surfactant is a lipoprotein complex produced by type II pneumocytes that acts to reduce at low lung volumes surface tension at the air-liquid interface in the alveolus and, thereby, prevents atelectasis. Surfactant proteins (SP)-A, SP-B, SP-C, and SP-D have multiple important roles within the alveolus and are subject to developmentally and hormonally regulated expression.

Specific aim I of this thesis describes an *in vivo* xenograft model of human fetal lung development. In this model, human fetal lung tissues were grafted either beneath

the renal capsule or the skin of athymic mice (NCR-nu). Tissues were analyzed from 3 to 42 days post-grafting for morphological alterations by light and electron microscopy (EM), and for mRNA and protein content of surfactant proteins by reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry (ICC), respectively. The changes observed mimic those of human lung development *in utero* in many respects, including the differentiation of epithelium to the saccular stage. However, each stage of development occurred over approximately one week in the graft in contrast to the eight weeks of *in utero* development. At all time points examined, all four surfactant proteins (SP-A, SP-B, SP-C, and SP-D) were detected in the epithelium by ICC. Lamellar bodies were first identified by EM in 14 day xenografts. By day 21, a significant increase was observed in both the number of lamellar bodies per cell and lamellar body positive cells. Cellular proliferation, as marked by proliferating cell nuclear antigen (PCNA) ICC and elastic fiber deposition resembled those of canalicular and saccular *in utero* development. Tissues that were grafted longer than 28 days, started to undergo distention of alveoli, presumably due to the accumulation of fluid. These findings indicate that the fetal lung xenograft model can serve as a valuable tool in the study of human fetal lung development. This model can provide the means to study the impact of various pharmacological agents on the development of human fetal lungs in general, and on the surfactant proteins in particular.

In order to better study the role of surfactant protein genetic variants in neonatal respiratory disease, rapid and accurate methods of genotyping are necessary. In this respect, **Specific aim IIa** of this thesis describes the development of a novel

pyrosequencing based method for genotyping of single nucleotide polymorphisms of SP-A, SP-B, and SP-D. This primer extension sequencing method has been used to develop the following assays: **1)** simplex and multiplex pyrosequencing assays for SP-A1 and SP-A2; **2)** molecular haplotype assay for AA19(C/T) and AA50(C/G) SNPs of SP-A1; **3)** simplex pyrosequencing assays for SP-B SNP B1580(C/T); **4)** simplex and multiplex pyrosequencing assays for SP-D SNPs DA11(C/T) and DA160(A/G); and **5)** assays for allele frequency determination in genomic DNA pools for DA11(C/T) and DA160(A/G) SNPs of SP-D. These assays greatly accelerate individual genotype analysis of surfactant proteins thus enabling efficient and reliable genotyping of SPs in samples from individuals with various pulmonary diseases.

This high-throughput method of genotyping was applied to the study of surfactant protein genetic variants in Bronchopulmonary dysplasia (BPD) in **Specific aim IIb**. BPD is a chronic lung disease of light weight prematurely born infants that are mechanically ventilated from birth. It has been suggested that genetic factors contribute to BPD pathogenesis. We hypothesized that genetic variants of surfactant proteins are differentially responsive to disruption of surfactant homeostasis in premature birth and are either protection or susceptibility factors for BPD.

In order to study genetic associations of surfactant protein (SP) genetic variants and microsatellite markers linked to SP-B, a family based association study was conducted using the Transmission Disequilibrium Test (TDT) and Family Based Association Test (FBAT). The study group consisted of 61 families with 71 BPD affected infants. SNP B-18_C of the SP-B gene was identified as susceptibility factor in

BPD at 36 weeks. Allele 6 of SP-B linked microsatellite marker AAGG was found to be a susceptibility factor in BPD. Haplotype analysis revealed two SP-A-SP-D susceptibility haplotypes, and ten susceptibility and one protective haplotypes for SP-B and SP-B-linked microsatellite markers.

Functional analysis of these variants should be investigated to better understand their role in BPD and point to mechanisms involved in BPD pathogenesis.

Taken together, these studies provide a model that may be used to study lung development and response of the developing lung to therapeutic interventions, and associations of surfactant protein genetic variants with BPD.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
AEC	Alveolar epithelial cells
ALS	Antilymphocytic serum
AS	Antisense
BAL	Bronchoalveolar lavage
BPD	Bronchopulmonary dysplasia
CRD	Carbohydrate recognition domain
CCD	Charge-coupled device
CLD	Chronic lung disease
COPD	Chronic obstructive pulmonary disease
CAP	Congenital alveolar proteinosis
dNTP	Deoxyribonucleotide triphosphate
DPPC	Dipalmitoyl phosphatidylcholine
EM	Electron microscopy
ETDT	Extended transmission disequilibrium test
ELBW	Extremely low birthweight
FBAT	Family based association test
FP	Fluorescence polarization
FRET	Fluorescence resonance energy transfer
GS	Gene specific

ICC	Immunocytochemistry
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
LB	Lamellar body
LPS	Lipopolysaccharide
MBP	Mannose binding protein
NICHHD	National Institute of Child Health and Human

Development

NF- κ B	Nuclear factor kappa B
PAMP	Pathogen associated molecular pattern
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PG	Phosphatidylglycerol
PS	Phosphatidylserine
PCR	Polymerase chain reaction
PCR-cRFLP	Polymerase chain reaction converted restriction

fragment length polymorphism

PIC	Polymorphism information content
PCNA	Proliferating cell nuclear antigen
PPi	Pyrophosphate
RDS	Respiratory distress syndrome

RSV	Respiratory syncytial virus
RFLP	Restriction fragment length polymorphisms
RT-PCR	Reverse transcription-polymerase chain reaction
S	Sense
SCID	Severe-combined immunodeficient
SC	Subcutaneous
SIRP α	Signal inhibitory regulatory protein α
SNP	Single nucleotide polymorphism
SP	Surfactant protein
SR	Subrenal
SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
SP-D	Surfactant protein D
TDT	Transmission disequilibrium test (TDT)
TM	Tubular myelin
TNF- α	Tumor necrosis factor alpha
UTR	Untranslated region
VLBW	Very low birthweight

Chapter 1

Literature review

1.1 Literature review – Section I

1.1.1 Lung and pulmonary surfactant

The primary function of the lung is gas exchange. Due to its function, the lung is in constant and direct contact with the environment, demanding an elaborate system to maintain fluid balance of the alveolar lining and functions of innate host defense. The surface tension produced over the extensive gas-exchange surface area in the lung could lead to alveolar collapse and prevent ventilation. This problem is avoided by the presence of pulmonary surfactant, a lipid-rich lipoprotein complex found in several morphological forms, which lowers surface tension. The functional form of surfactant that reduces surface tension is found at the interface between alveolar gas and the liquid on the surface of epithelial cells (Floros, 1997). Surfactant reduces the surface tension forces at the air-liquid interface about eight-fold, from about 80 milliNewtons/meter (mN/m) to about 10 mN/m (Clements, 1977). This process occurs in an alveolar-size dependent manner, where the surface tension is preferentially reduced in small alveoli, preventing the emptying of small alveoli into larger ones. Surfactant therefore stabilizes the alveoli by maintaining lung volumes at end-expiration, thus reducing the work of breathing.

Studies performed during the past decade have identified surfactant components as active players in the innate immunity of the lung (Borron et al., 2000; Crouch and Wright, 2001; Kremlev et al., 1994; LeVine et al., 1997; LeVine et al., 2002; LeVine et

al., 1998; Malherbe et al., 2005; Mikerov et al., 2005; Phelps, 2001; Restrepo et al., 1999; Senft et al., 2005; Tino and Wright, 1999; Wang et al., 2000; Wang et al., 2002; Wright and Youmans, 1993). Due to their essential multiple roles in lung physiology, surfactant and its components have been implicated as major players in respiratory disorders. Perturbations of pulmonary surfactant homeostasis, whether caused by premature birth, lung injury or mutations in genes critical to surfactant production or function, can lead to respiratory pathology of both neonates and adults.

1.1.2 Surfactant composition

Pulmonary surfactant, a multifunctional complex, is composed of 90% lipids and 10% proteins (King et al., 1973). The lipid fraction of surfactant consists mostly of phospholipids, 80% of which are represented by phosphatidylcholine (PC), 10% by phosphatidylglycerol (PG), while the remaining 10% consist of phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatidylcholine and sphingomyelin (Goerke, 1974). Dipalmitoyl phosphatidylcholine (DPPC), which makes up 40-70% of PC (Hildebran et al., 1979), plays a key role in the reduction of surface tension. The non-phosphorylated lipid fraction consists of cholesterol, triacylglycerol, and free fatty acids.

The protein fraction of surfactant consists mostly of surfactant-specific proteins and some serum proteins. Surfactant proteins are necessary for optimal functioning of the lung, and disruption of their homeostasis can lead to pulmonary pathology. Some of these proteins are essential for the formation of the surfactant surface film, which is

essential for the reduction of surface tension, or the process of surface film formation, whereas others may not participate at all in surfactant-related functions (Cochrane and Revak, 1991; King et al., 1973; Oosterlaken-Dijksterhuis et al., 1991a; Rodriguez-Capote et al., 2001; Wright et al., 1989). The surfactant associated proteins are generally divided into two groups based on their function. Surfactant protein B (SP-B) and surfactant protein C (SP-C) are hydrophobic proteins that are mostly involved in the surface tension reduction (Cochrane and Revak, 1991; Fisher et al., 1988a; Glasser et al., 2001; Glasser et al., 1990; Lin et al., 2000a; Noguee et al., 2001; Noguee et al., 1994; Pilot-Matias et al., 1989). Surfactant protein A (SP-A) and surfactant protein D (SP-D) are hydrophilic proteins that are involved in the first line of defense against pathogens (Crouch et al., 1994; Crouch and Wright, 2001; Kremlev et al., 1994; Phelps, 2001; Wright and Youmans, 1993; Wu et al., 2003), although SP-A also plays a role in surfactant related functions. For example, SP-A inhibits surfactant secretion by type II cells (Dobbs et al., 1987; Wang et al., 2004) and in co-operation with SP-B promotes formation of the phospholipid surface film (Rodriguez-Capote et al., 2001). Although SP-D is not present in the surface film, it is referred to as surfactant protein because it co-isolates with surfactant (Persson et al., 1990; Persson et al., 1992; Voorhout et al., 1992).

1.1. 3 Surfactant storage, secretion, and extracellular metabolism

Pulmonary surfactant is synthesized and secreted by type II cells in the lung by a highly regulated process as illustrated in Figure 1.1. Surfactant reservoirs in the lung are commonly divided into two compartments, the intracellular and the extracellular

(Sanderson and Vatter, 1977; Williams et al., 1971; Williams, 1977). Surfactant pools in both compartments are stored within highly organized structures. The primary storage site of intracellular surfactant is within the lamellar bodies of type II cells. Lamellar bodies are specialized acidic intracellular organelles rich in surfactant lipids and proteins (SP-A, SP-B, and SP-C), that are secreted into the fluid layer lining the alveoli (Chander et al., 1986). These structures are secreted from type II cells in a highly regulated process of exocytosis (Chander and Fisher, 1990). SP-A may also be secreted via a lamellar independent pathway (Rooney et al., 1993).

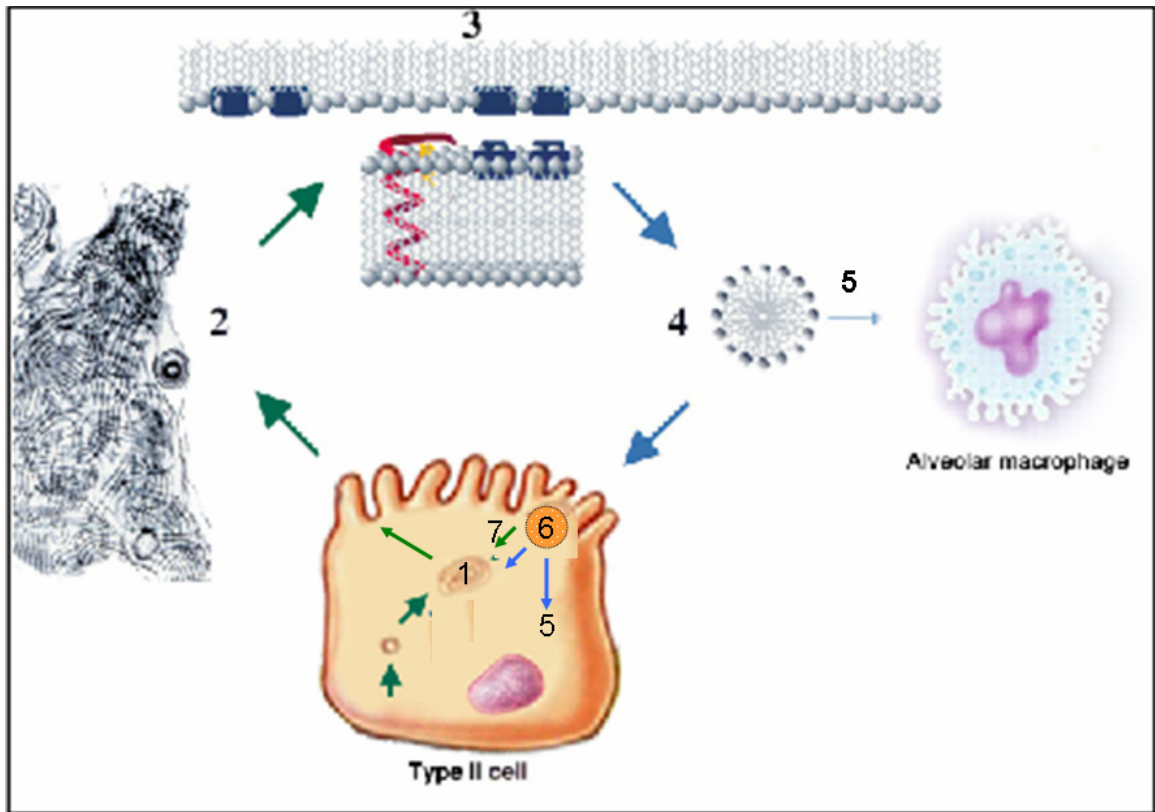


Figure 1.1: **Surfactant homeostasis**

1) Lamellar body within type II pneumocyte. 2) Green arrow represents the exocytosis of lamellar bodies and formation of tubular myelin structures and large aggregates of surfactant in alveolar hypophase. 3) Formation of functionally active surfactant monolayer at the air-liquid interface. Spreading of surfactant lipids is facilitated by SP-B and SP-C (SP-B presented in blue and SP-C in red). 4) Inactivated surfactant is converted into surface-inactive small aggregates. 5) Small aggregates are ingested and broken down by alveolar macrophages, and/or are taken up by the type II cells for degradation. 6) Alternatively, multivesicular bodies are formed within type II cells and 7) recycling of surfactant occurs. Anabolic pathways are outlined with green arrows while catabolic pathways are outlined with blue arrows. Adapted from Weaver and Conkright 2001 (Weaver and Conkright, 2001).

Secretion is stimulated by a number of factors, such as, hyperventilation (Jain et al., 2003; Nicholas and Barr, 1983), variety of β -adrenergic agonists (Brown and Longmore, 1981), TPA, diacylglycerol, ATP, calcium ionophores (Mason and Voelker, 1998). Once exocytosis is triggered by a stimulus it leads to the release of the lamellar body into the aqueous lining layer of the alveolus. Following secretion lamellar bodies unravel to form highly organized lattice-like structures called tubular myelin (Williams, 1977). These structures lead to the formation of mono- and multi-layered, phospholipid-rich sheets and vesicles (Poulain et al., 1992). At this point large and small aggregates are formed. The functionally active surfactant monolayer must occupy the air-liquid interface during inflation of the lung.

For adequate decrease of the surface tension at end expiration, the surfactant monolayer must become enriched with DPPC (Hildebran et al., 1979; Veldhuizen et al., 2000). This is most likely achieved by selective exclusion of other surfactant components from the surface film, thus resulting in a surface monolayer of almost pure DPPC (Pastrana-Rios et al., 1994; Schurch et al., 1992). At the beginning of inhalation rapid re-spreading of lipids occurs, facilitated by SP-B and SP-C (Oosterlaken-Dijksterhuis et al., 1991a). The large aggregates of lamellar bodies and tubular myelin have surface-active properties, while the inactivated surfactant is converted into small aggregates of lamellar bodies that are surface-inactive (Veldhuizen et al., 1999). Small aggregates are then taken up by the type II cells for degradation and/or recycling (Oosterlaken-Dijksterhuis et al., 1991b). In addition, alveolar macrophages also regulate the catabolism of both lipids and proteins by ingestion and presumably breakdown of the

surfactant components (Stern et al., 1986). In addition to being secreted via the lamellar body pathway, SP-A is also secreted via non-lamellar body secretory vesicles (Osanai et al., 2006).

1.1.4 Surfactant proteins: structure and function

1.1.4.1 SP-A

SP-A is the predominant protein by weight of pulmonary surfactant (Ballard et al., 2003). Together with SP-D, it belongs to the collectin family of proteins that are characterized by an N-terminal collagen-like domain and a C-terminal carbohydrate recognition domain. It was the first surfactant-associated protein to be characterized (Hawgood et al., 1985; King et al., 1973), have its precursor molecule identified (Floros et al., 1986a; Floros et al., 1985), and its cDNA and gene cloned (Floros et al., 1986b; White et al., 1985). SP-A has been subsequently identified in all vertebrate species studied, as well as in the lungs and swim bladders of some species of lungfish and goldfish (Rubio et al., 1996; Sullivan et al., 1998). More recently SP-A has been found in several extra-pulmonary tissues (Lee et al., 2006; Leth-Larsen et al., 2004; Lin et al., 2001; MacNeill et al., 2004; Madsen et al., 2003; Paananen et al., 2001; Rubio et al., 1995). Although the role of SP-A in these tissues has not yet been elucidated, presence of the molecule on these numerous mucosal surfaces supports the role of SP-A in innate defense not only of the lung, but of other tissues as well. Comparisons of the primary structure of SP-A isolated from a variety of species reveals extensive sequence similarity

(Benson et al., 1985; Benson and Hawgood, 1985; Boggaram et al., 1988; Fisher et al., 1988b; Glasser et al., 1988b; Korfhagen et al., 1992; White et al., 1985).

In its monomeric form, human SP-A is a 28-36 kDa molecule consisting of 248 amino acids. The primary structure of SP-A consists of four domains: an N-terminal domain, a collagen-like domain, a hydrophobic “neck” domain, and a carbohydrate recognition domain (CRD) as presented in Figure **1.2** (Floros and Wang, 2001; Hawgood and Shiffer, 1991; Wang et al., 2004).

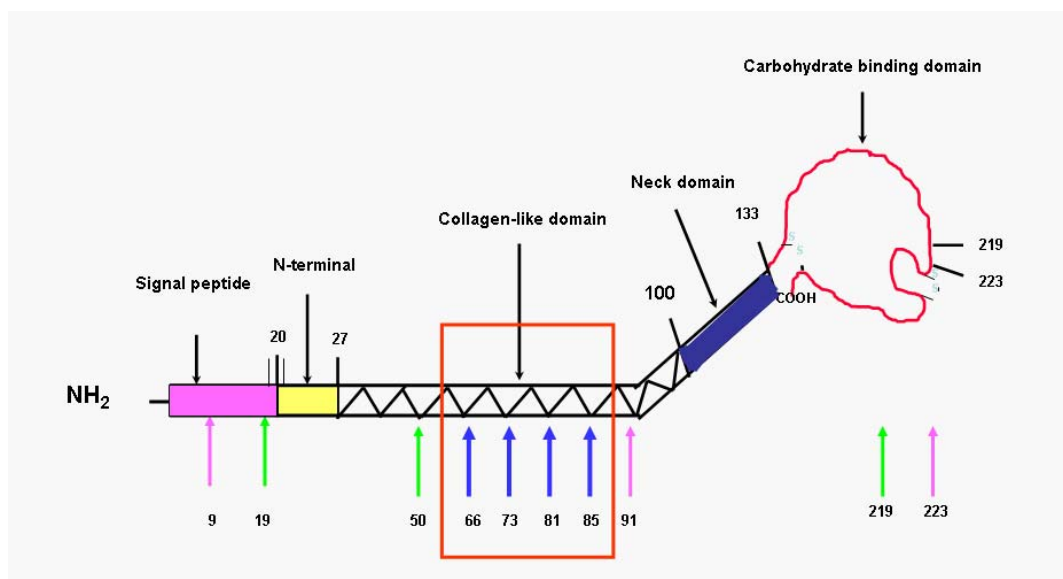


Figure 1.2: Primary structure of human SP-A

Specific domains of SP-A are presented in the Figure 1.2. Signal peptide (in pink) and N-terminal domain (in yellow) make up the first 27 amino acids. N-terminal sequence isoforms have been identified where cleavage can occur anywhere between amino acids 18 and 21, resulting in the variation of the N-terminal sequence length (Wang et al., 2004). Collagen-like domain (hatched pattern) spans from amino acids 27 to amino acid 100, and consists of 23 gly-x-y repeats. Neck domain (in blue) spans 33 amino acids, from aa 100 to aa 133. Carbohydrate recognition domain (CRD) spans 115 amino acids, from aa 133 to aa 248. Blue arrows highlight the amino acids (66, 73, 81, and 85) that vary between the two SP-A genes, SP-A1 and SP-A2. Green arrows point to amino acids (19, 50, and 219) that vary among different SP-A1 alleles. Pink arrows point to amino acids (9, 91, and 223) that vary among different SP-A2 alleles.

Adapted from McCormack et al. 1998 (McCormack, 1998).

The N-terminal domain of SP-A is involved in protein oligomerization and binding of phospholipids (Palaniyar et al., 2002). This domain is generated by cleavage of the signal peptide prior to secretion from type II cells. The resulting N-terminal domain in humans is a seven to ten amino acid long segment containing two cysteine residues that have been shown to participate in intrachain disulfide bond formation and to be required for SP-A multimer formation.

With respect to human SP-A, several isoforms have been identified in *in vitro* expressed single and co-expressed SP-A1 and SP-A2 products (Wang et al., 2004). In these isoforms, cleavage can occur anywhere between amino acids 18 and 21, resulting in the variation of the N-terminal sequence length. Cys20 was identified as the dominant isoform. While more than 95% of molecules of SP-A2 variant 1A⁰ have Cys20 (being the first amino acid), this isoform was found in only about 50% of SP-A1 molecules, specifically variant 6A². Cys20 is not essential for SP-A interactions with phospholipids and type II cell, or for formation of higher order oligomers in rat (Zhang et al., 1998). However, it is essential for complete disulfide dependent SP-A oligomer formation (Elhalwagi et al., 1997). Formation of these disulfide bonds has been proposed to promote 'parallel' orientation of trimeric subunits that facilitates the formation of the entire octadecamer. Evidence for this was also shown in the Wang et al. study where *in vitro* expressed variants of human SP-A1 and SP-A2 devoid of Cys20 were not found in higher size oligomers (Wang et al., 2004). Heterogeneity of isoforms and presence of Cys20 have also been identified in the Bronchoalveolar lavage (BAL) from both normal volunteers and alveolar proteinosis patients (Hickling et al., 1998).

Site directed mutagenesis of cysteine at amino acid 26 in mammalian cell-expressed human SP-A1, showed that this mutant was able to form octadecamers, dodecamers, and nonamers. However, the mutant was more susceptible to proteolytic degradation than the wild type SP-A, did not self-aggregate in Ca^{+2} dependent fashion, was unable to induce aggregation of rough LPS in the presence of Ca^{+2} , and failed to promote adsorption of SP-B/SP-C-surfactant membranes to an air-liquid interface (Sanchez-Barbero et al., 2005).

The collagen-like domain of human SP-A is a 73 amino acid long sequence that consists of a series of 23 repeating glycine-Xaa-Yaa tripeptides (Haagsman et al., 1989). This region is critical for the formation of the higher oligomeric structures of SP-A, its deletion leading to the loss of SP-A aggregation (Haagsman et al., 1990). It was proposed that the collagen domains consist of two molecules of SP-A1 and one molecule of SP-A2 that form a triple helix and six of these helices together form an octadecamer as presented in Figure 1.3 (Voss et al., 1991). Electron microscopy studies have shown that the octadecamer has a highly ordered structure resembling a bouquet of tulips (Voss et al., 1988).

Recent studies have expanded on this model. Experiments with human SP-A variants expressed in insect cells have shown that individual gene products (SP-A1 and SP-A2) and co-expressed products have different patterns of oligomerization when analyzed by native gel electrophoresis (Garcia-Verdugo et al., 2002). SP-A2 was found to form higher size forms than SP-A1, and the co-expressed products favored oligomers with 18 polypeptide chains. Differences in oligomerization between two gene products

were in part contributed to by the additional cysteine at amino acid 85 in SP-A1. It was proposed that this cysteine could form interchain disulfide bonds that would in-turn lead to increased complexity of oligomerization of SP-A1 as compared to SP-A2. Wang et al. have showed that variants of human SP-A1 and SP-A2 expressed *in vitro* in mammalian cell line form homo- and hetero-oligomers as presented in Figure 1.3 (Wang et al., 2004). Higher order multimers were observed for SP-A1 than SP-A2, with the majority of SP-A1 oligomers found in the form of trimers and hexamers, while SP-A2 was primarily found in the form of dimers and trimers.

Thus, a new model of SP-A oligomerization based on disulfide bond formation was proposed, where the extra cysteine at amino acid 85 contributes to formation of an additional inter-trimeric disulfide bond between trimers of SP-A1, thus leading to formation of hexamers and higher order multimers (Wang et al., 2004). Although the functional differences between oligomers of SP-A1 and SP-A2 are yet undetermined, the oligomerization state is likely to have numerous functional consequences. It has been shown that various oligomers of SP-A are found in different disease states, with a trend toward lower oligomeric forms observed in alveolar proteinosis and pollen allergy patients as compared to normal controls (Hickling et al., 1998). Similar observations were made by Wang et al. where low molecular size forms were found in BAL from alveolar proteinosis patients, but not from normal controls (Wang et al., 2004). These investigators proposed that the oligomerization status of SP-A may play a crucial role in the extrapulmonary sites of expression, where the opsonic function of SP-A may provide protection from a diversity of pathogens/stressors.

The neck domain, a hydrophobic region consisting of 33 amino acids, is primarily involved in phospholipid binding of SP-A (Ross et al., 1986). A synthetic peptide analog of this domain has been shown to enhance surface activity and pulmonary compliance in pre-term newborn rabbits when combined with synthetic phospholipids (Walther et al., 1996). Based on the crystallographic analysis of the SP-A, CRD-neck angle interdomain, it has been suggested that the trimer of SP-A has a larger hydrophobic surface than other collectin molecules and thus binds lipophilic membrane components with greater affinity (Head et al., 2003). This domain may facilitate ligand binding to SP-A trimers by spatial orientation of its CRD domains.

The CRD, consisting of 115 amino acids, is the longest domain of SP-A and together with the collagen domain mediates the majority of its biological functions. The primary function of this domain is Ca^{+2} dependent binding of carbohydrates which mediates immune defense related functions of SP-A (Benne et al., 1995; Van Iwaarden et al., 1994), discussed below. This domain also mediates the binding of SP-A to its receptors on type II cells (Kuroki et al., 1988).

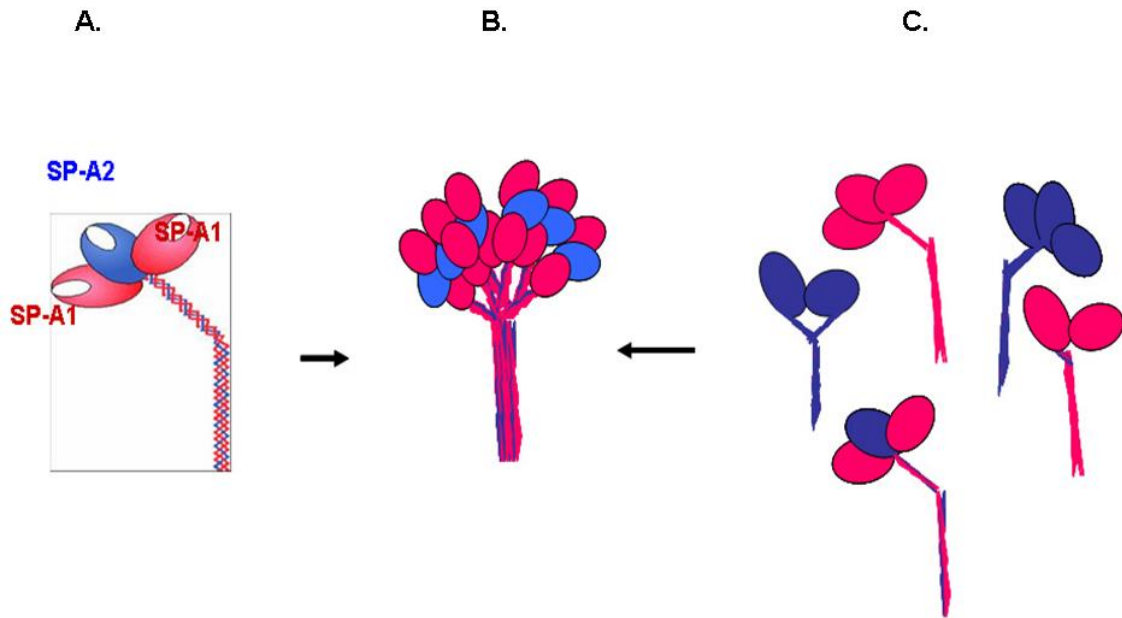


Figure 1.3: **SP-A structure**

SP-A is found in monomeric, trimeric, and multimeric forms. Panel A: Three SP-A monomers (two of SP-A1 (in red) and one of SP-A2 (in blue)) come together through their collagen like domains to form a triple helix. Panel B: Six trimers together form an octadecamer that resembles a bouquet of tulips (Voss et al., 1991). Recent studies have shown that SP-A can be found in both homo and hetero dimers and trimers, where the oligomer is composed only of SP-A1 or SP-A2 monomers, or of combination of SP-A1 and SP-A2 monomers, as presented in Panel C above. Adapted from Wang et al. 2004 (Wang et al., 2004).

A role of SP-A in local host defense of the lung was initially proposed by sequence comparison studies that revealed extensive structural similarity with other collectins involved in host defense (Drickamer et al., 1986; Hansen and Holmskov, 1998). This family of proteins includes SP-A, SP-D, serum mannose-binding protein (MBP) (Drickamer et al., 1986), conglutinin (Lee et al., 1991), collectin-43 (Lim et al., 1994), and collectin CL-L1 (Ohtani et al., 1999). These molecules have the ability to bind to complex carbohydrates and lipids on the surface of various pathogens, and thus facilitate the clearance of these pathogens. This notion was further supported by the phenotypic characterization of the SP-A knockout mouse. The SP-A knockout mouse is viable and does not suffer from respiratory problems under normal conditions, but shows increased susceptibility when challenged with microorganisms, such as group B Streptococci, *P. aeruginosa*, and Respiratory Syncytial Virus (RSV) (LeVine et al., 1997; LeVine et al., 1999a; LeVine et al., 2002; LeVine et al., 1998; LeVine et al., 1999b). In addition, SP-A knockouts clear pathogens more slowly (Korfhagen et al., 1998; LeVine et al., 1997; Wert et al., 2000) and shows higher levels of pro-inflammatory cytokines in the lavage fluid upon pathogen colonization compared to the wild type mouse. These mice are also more susceptible to the acute lung injury caused by lipopolysaccharide (LPS) (Borron et al., 2000; Quintero et al., 2002; Wu et al., 2003).

A number of studies have indicated that SP-A modulates the inflammatory response in the lung by affecting a variety of immune cells. Differential regulation of alveolar macrophages and macrophage-like cell lines by SP-A has been reported, where both inhibitory and stimulatory effects have been observed. SP-A has been shown to

stimulate chemotaxis (Tino and Wright, 1999; Wright and Youmans, 1993), production of cytokines (Kremlev and Phelps, 1994; Kremlev et al., 1997), expression of cell surface proteins (Kremlev and Phelps, 1997; Kremlev et al., 1994) and phagocytosis by alveolar macrophages and neutrophils (Giannoni et al., 2006; Kudo et al., 2004; LeVine et al., 1999b; Mikerov et al., 2005; Mikerov et al., 2007; Schagat et al., 2003).

SP-A also binds, via its CRD domain, to carbohydrates on the surface of Gram-positive and Gram-negative bacteria (Hartshorn et al., 1998; Van Iwaarden et al., 1994). While it binds rough lipopolysaccharide (LPS) and not smooth LPS (Sano et al., 1999; Van Iwaarden et al., 1994), it does appear to play a modulatory role to smooth LPS response, by inhibiting cytokine production by smooth LPS stimulated macrophages *in vitro* (Arias-Diaz et al., 2000; Gardai et al., 2003) and stimulating greater increase in cytokine production in SP-A^{-/-} mice than in wild type controls (Borron et al., 2000). In addition, SP-A has anti-viral and anti-fungal properties (Atochina et al., 2004; Barr et al., 2000; Hartshorn et al., 1997; LeVine et al., 2002; Sano et al., 2003; White et al., 2005). Depending on the nature of the pathogen it is bound to, it can either cause its agglutination or facilitate killing of the pathogen by permeabilizing its cellular walls and stimulating opsonization and phagocytosis by macrophages (Kabha et al., 1997; LeVine et al., 2000; Madan et al., 1997; Pikaar et al., 1995).

Opsonization is primarily mediated by the CRD of SP-A. Binding of the CRD to pathogens leads to phagocytosis of the SP-A-pathogen complex by alveolar macrophages (van Iwaarden et al., 1990). In addition, SP-A leads to increased ingestion of non-opsonized bacteria by alveolar macrophages, thus facilitating phagocytosis and clearance (Gaynor et al., 1995; Kabha et al., 1997).

Pro-inflammatory functions of SP-A are counteracted by inhibitory effects of SP-A. SP-A has been shown to inhibit LPS induced cytokine production by macrophages (McIntosh et al., 1996; Sano et al., 1999), and inhibit inflammation following viral and fungal infection (Harrod et al., 1999; Rosseau et al., 1999). In addition to playing a role in the innate immune defense, SP-A also affects cells of the adaptive immunity. It has been shown to have a dual role in proliferation of lymphocytes induced by mitogens, to inhibit allergens (Kremlev et al., 1994; Wang et al., 1998; Wright and Youmans, 1993) and prevent dendritic cell maturation (Brinker et al., 2003).

As Gardai et al. recently proposed, a model that addresses the apparent dual role of SP-A as both pro-inflammatory and anti-inflammatory molecule (Gardai et al., 2003). In this model, in the absence of pathogens SP-A binds to signal inhibitory regulatory protein α (SIRP α) on the surface of alveolar macrophages through its C-lectin domain in order to suppress the immunological response caused by insults of everyday living (Gardai et al., 2003). In this way SP-A acts as an anti-inflammatory molecule that works to maintain tissue homeostasis. In the presence of pathogens, C-lectin domain of SP-A binds pathogen associated molecular patterns (PAMPs) on foreign pathogens, apoptotic cells, and cellular debris. The free collagen-like domains then aggregate and can interact with calreticulin/CD91 complex on the surface of alveolar macrophages. This interaction leads to activation of pro-inflammatory response (Gardai et al., 2003). These studies indicate that SP-A has an immunomodulatory effect in the lung and perturbation of this SP-A activity may have an impact on the pathogenesis and/or severity of various lung diseases.

Although SP-A does not appear to be directly involved in the surface tension reducing properties of surfactant, it does play a role in the regulation of this function by modulating lipid uptake and secretion, insertion of phospholipids into the surfactant monolayer, aggregation of lipids (Garcia-Verdugo et al., 2002; Meyboom et al., 1999; Oosterlaken-Dijksterhuis et al., 1991a; Tsunazawa et al., 1998), and tubular myelin formation (Ohtani et al., 1999; Poulain et al., 1992; Taeusch et al., 1986). SP-A knockout mice do not form tubular myelin structures (Ikegami et al., 1998) and tubular myelin is rarely found in SP-A deficient babies affected with RDS (deMello et al., 1993; deMello et al., 1989).

1.1.4.2 SP-B

Presence of hydrophobic surfactant proteins was first described in 1972 (King et al., 1973) and was followed a few years later by characterization of these proteins as major components of lamellar bodies (Phizackerley et al., 1979). This protein was subsequently identified and named SP-B. SP-B is a small hydrophobic protein of 6 kDa and with a nonreduced molecular mass of 18 kDa. The primary translation product (42 kDa) (Jacobs et al., 1987) undergoes an intracellular cleavage that does not appear to be cell-specific cleavage to 25 kDa (Guttentag et al., 1998), followed by a series of cleavages that are either type II cell- (Hawgood et al., 1993; Korimilli et al., 2000) or Clara cell-specific (Wikenheiser et al., 1992), where anionic amino- and carboxy-terminal domains are cleaved in order to form a cationic mature SP-B (Wang et al., 2003a; Weaver and Whitsett, 1989). The mature SP-B consists of 79 amino acids. SP-B

homodimer is rich in cysteines that form a pattern of three intramolecular disulfide bonds and one intermolecular disulfide bond, which are necessary to stabilize the protein (Johansson et al., 1991; Johansson et al., 1992).

SP-B is primarily involved in the biophysical properties of surfactant lipids and thus contributes to the decrease in surface tension at the alveolar air-liquid interface. It is essential for the formation of lamellar bodies, and together with SP-A, the formation of tubular myelin. Deficiency of this protein is not compatible with life as illustrated by respiratory failure at birth of SP-B knockout mice and fatal respiratory failure of SP-B deficient human neonates (Thompson, 2001; Tokieda et al., 1999b). SP-B deficiency in neonates leads to congenital alveolar proteinosis (CAP) and fatal respiratory failure (Ballard et al., 1995; deMello et al., 1994a; deMello et al., 1994b; Hamvas et al., 1994; Lin et al., 1998; Nogee et al., 1994). These infants have an abundance of concentric multilamellar bodies and a deficiency of tubular myelin. SP-B knockout mice lack lamellar bodies and display an abundance of small vesicular inclusions and electron-dense masses (Stahlman et al., 2000; Tokieda et al., 1997).

SP-B facilitates rapid insertion of phospholipids into the surfactant monolayer, thus maintaining the alveolar integrity at the air-liquid interface (Hawgood et al., 1987; Kobayashi et al., 1991; Oosterlaken-Dijksterhuis et al., 1991a). The insertion of SP-B into the phospholipid layer increases the inter- and intra-molecular ordering of the lipid membranes (Cochrane and Revak, 1991; Vincent et al., 1991). It has been suggested that SP-B reduces the surface tension by increasing the lateral stability of the phospholipid monolayer (Cochrane and Revak, 1991).

Recently, SP-B has been shown to play a role in lung host defense. When transgenic mice that overexpress SP-B were treated with endotoxin there was a decreased recruitment of inflammatory cells and pro-inflammatory cytokines as compared to control mice (Epaud et al., 2003). Induction of SP-B deficiency in adult mice leads to pulmonary inflammation and severe respiratory failure within four days. Once SP-B expression is restored lung inflammation subsides and lung function returns (Ikegami et al., 2005).

1.1.4.3 SP-C

SP-C is a hydrophobic protein that is mostly involved in surface tension-reducing properties of surfactant. The primary translation product of 22 kDa undergoes post-translational cleavage of the amino- and carboxy-termini to form the 4.2 kDa mature protein (Glasser et al., 1988a). The mature protein is 36 amino acids long and is characterized by a high valine content. The secondary structure of SP-C is mostly a regular α -helix (Hawgood and Shiffer, 1991; Johansson et al., 1994; Pastrana-Rios et al., 1994), which can span the DPPC bilayer (Morrow et al., 1993). Both monomers and dimers of SP-C have been observed. Dimeric SP-C is mostly found in the form of a β -sheets that enhance surface tension lowering properties of surfactant (Tokieda et al., 1999a; Wustneck et al., 2003). Although the specific consequences of the *in vitro* conversion of α -helix to β -sheet are unknown, it has been postulated that the resulting SP-C fibril formation and aggregation may be associated with lung diseases (Johansson, 2001; Wustneck et al., 2003) similar to other diseases where there is extracellular deposition of amyloid-like fibrils (Guijarro et al., 1998; Ohnishi and Takano, 2004).

These insoluble aggregates, as seen in patients with pulmonary alveolar proteinosis, are thought to contribute to a potential cellular toxic effect of SP-C (Gustafsson et al., 1999).

Like SP-B, SP-C is involved in promotion of rapid phospholipid insertion into the air-liquid interface and regulation of phospholipid ordering (Oosterlaken-Dijksterhuis et al., 1991b; Perez-Gil et al., 1992; Takahashi and Fujiwara, 1986; Williams et al., 1991). In contrast to SP-B, SP-C can disrupt the lipid structure of the monolayer in its immediate vicinity and thereby increase the miscibility of the phospholipid mixture (Oosterlaken-Dijksterhuis et al., 1991a). Unlike SP-B, SP-C deficiency is compatible with life. The SP-C knockout mice are viable at birth and have been observed to grow to adulthood without obvious pulmonary abnormalities (Glasser et al., 2001). More recently, a condition resembling interstitial lung disease has been described in these mice with emphysema, monocytic infiltrates, epithelial cell dysplasia, and atypical accumulations of intracellular lipids in type II epithelial cells and alveolar macrophages observed (Glasser et al., 2003). In addition, when exposed to bleomycin, these mice have increased and prolonged pulmonary fibrosis as compared to wild type mice (Lawson et al., 2005).

Studies of lung mechanics have shown abnormalities in lung hysteresis at low, positive-end, expiratory respiratory pressures. Studies of SP-C deficient surfactant from the knockout mice indicate that SP-C plays a key role in stabilizing the surfactant complex at low lung volumes (Glasser et al., 2001; Ikegami et al., 2002). While the importance of this function might be negligible in normal physiological states, it is likely to be vital in low lung volume states such as those associated with respiratory distress.

While other SPs have been found in variety of tissues, SP-C is the only surfactant protein that is exclusively expressed in type II pneumocytes (Phelps and Floros, 1991).

1.1.4.4 SP-D

The latest surfactant-associated protein to be identified, SP-D (Crouch et al., 1991; Phelps and Taeusch, 1985) is, together with SP-A, a member of the collagenous family of calcium-dependent lectins, commonly called collectins. In its reduced state, it is a 43 kDa protein of 355 amino acids. Like SP-A, SP-D forms four structural domains and is found in both monomeric and oligomeric forms. Each molecule of SP-D is made up of three monomers that form a trimer. Trimeric subunits associate through their N-termini to form oligomers. Most preparations of SP-D are predominantly made up of dodecamers (four trimers) (Crouch et al., 1994), although higher order structures of up to 32 (or more) trimeric subunits have been found. The collagen domain of SP-D is longer than that of SP-A (59 Gly-X-Y repeats) (Lu et al., 1992) and lacks the interruption in the repeating sequence, resulting in the stretched out extended structure of the molecule. The collagen domain contributes to oligomeric assembly of SP-D and mediates interactions with cellular receptors. The CRD mediates the high-affinity binding to carbohydrate ligands (Hakansson et al., 1999; Kishore et al., 1996).

Compared to the other surfactant proteins, SP-D is not involved in the reduction of surface tension in the lung, but is, together with SP-A, an important modulator of pulmonary host defense. Purified SP-D binds phosphatidylinositol of the surfactant lipid fraction with high affinity (in a calcium-dependent manner) through the co-operative

activity of the N-terminal domain and collagenous domain (Ogasawara et al., 1992; Ogasawara et al., 1994; Ogasawara and Voelker, 1995; Persson et al., 1992). It also binds saturated, unsaturated and hydroxylated fatty acids through its neck and CRD domain (DeSilva et al., 2003). Both of these properties are postulated to be important for the immune-related functions of the protein. Like SP-A, SP-D regulates cytokine and free radical production and stimulates phagocytosis and chemotaxis by a number of immune cells.

As compared to SP-A, SP-D only moderately affects clearance of *P. aeruginosa* by alveolar macrophages and reduces uptake of *M. tuberculosis* (Ferguson et al., 1999; Restrepo et al., 1999). It binds rough LPS, mycobacterium lipoarabinomannan, and *M. pneumoniae* (Chiba et al., 2002; Ferguson et al., 1999; Kuan et al., 1992). SP-D can bind to a variety of both bacterial and viral glycoconjugates (LeVine et al., 2001; Wert et al., 2000) and, due to its tertiary structure, is a potent agent of bacterial agglutination (Kuan et al., 1992). Together with SP-A, SP-D has recently been shown to have a direct anti-microbial and anti-fungal activity (McCormack et al., 2003; Wu et al., 2003). SP-D seems to be a more potent stimulator of the inflammatory response than SP-A, not only by enhancing phagocytosis, chemotaxis, cytokine and free radical production of macrophages and neutrophils, but also by augmenting the antigen uptake and presentation by dendritic cells (Brinker et al., 2003).

The SP-D knockout mice (SP-D^{-/-}) are viable and do not show changes in surfactant surface activity or post-natal respiratory function (Botas et al., 1998). However, in contrast to SP-A^{-/-} these mice have altered surfactant homeostasis with

increased surfactant lipid pools. They are susceptible to viral pathogens and although they are able to clear bacterial pathogens, they have increased inflammation and inflammatory cell recruitment (LeVine et al., 2004; LeVine et al., 2000; LeVine et al., 2001; Wert et al., 2000). While SP-A^{-/-} mice show impaired clearance of numerous pathogens, SP-D^{-/-} mice show no change in pathogen load after *Group B streptococcus* and *H. influenzae* challenge (LeVine et al., 2000). In contrast to SP-A^{-/-} mice, these mice produce increased levels of reactive oxygen species, thus leading to more effective pathogen clearance (LeVine et al., 2000). The increased oxygen stress in these animals has been shown to lead to induction of NF-κB and matrix metalloproteinases (Yoshida et al., 2001).

Adult SP-D^{-/-} mice develop progressive emphysema marked by an increase in reactive oxidant species and metalloproteinases (Wert et al., 2000). When SP-D is replaced in adult SP-D^{-/-} mice, abnormalities in surfactant phospholipid homeostasis and alveolar macrophages are readily corrected, but emphysema can not be reversed (Filali et al., 2002; Zhang et al., 2002a; Zhang et al., 2002b). This suggests a possible use of recombinant SP-D as therapy in people at risk for or in beginning stages of chronic obstructive pulmonary disease (COPD).

1.1.5 Surfactant proteins: genomics

1.1.5.1 SP-A

Human SP-A is encoded by two closely linked, highly polymorphic genes, SP-A1 (White et al., 1985) and SP-A2 (Katyal et al., 1992). Baboons are the only other species that have been found to have two functional SP-A genes (Gao et al., 1996). Other species, such as dog (Benson et al., 1985), rabbit (Boggaram et al., 1988), rat (Fisher et al., 1988b), and mouse (Korfhagen et al., 1992) have only a single gene, that is distinct from either of the human SP-A genes, but appears to have characteristics of both. For example, rat SP-A is more similar to SP-A1 by sequence comparison, but like SP-A2 lacks a cysteine at amino acid 85 (Fisher et al., 1988b; Floros et al., 1986b; Karinch and Floros, 1995a).

The human SP-A locus is located on chromosome 10, q22–q23 (Bruns et al., 1987). It encodes, in addition to the two functional genes, a nonfunctional pseudogene (Korfhagen et al., 1991). Physical and radiation hybrid mapping have revealed that the pseudogene is located between the two functional genes, and is in same orientation as SP-A2, while SP-A1 is in the opposite orientation (Hoover and Floros, 1998). In addition, SP-D and mannose binding protein (MBP) have also been mapped to the long arm of chromosome 10 (Crouch et al., 1993; Kolble et al., 1993; Sastry et al., 1989), and the order of these genes from the centromere to the telomere is MBP, SP-D, SP-A2, pseudogene, and SP-A1 (Floros and Hoover, 1998).

The two primary translation products of SP-A were identified in 1985 (Floros et al., 1985) followed by the cloning of cDNA sequences (1A, 6A) of each in 1986 (Floros et al., 1986b). The 6A cDNA sequence is represented by SP-A1 genomic sequence described by White et al. (White et al., 1985) and the 1A cDNA sequence is represented by SP-A2 genomic sequence described by Katyal et al. (Katyal et al., 1992). Due to historic reasons, SP-A variants have been denoted as 6Aⁿ for the SP-A1 gene and 1Aⁿ for the SP-A2 gene. These variants are classified with respect to differences within coding region sequences (DiAngelo et al., 1999).

Haplotypes of SP-A1 are defined by various combinations of five SNPs at amino acids 19, 50, 62, 133, and 219, where nucleotide changes in the codon sequence result in amino acid change for amino acid 19, 50, and 219, while the nucleotide change is silent at amino acid 62 and 133. Four SNPs are involved in SP-A2 haplotypes, where amino acid change occurs due to nucleotide change at amino acid 9, 91, and 223, while the codon nucleotide change at amino acid 140 is silent. Based on these differences more than 30 variants of SP-A1 and SP-A2 have been fully or partially characterized (DiAngelo et al., 1999; Floros et al., 1996; Hoover and Floros, 1998; Karinch and Floros, 1995a). Four SP-A1 (6A, 6A², 6A³, 6A⁴) and six SP-A2 (1A, 1A⁰, 1A¹, 1A², 1A³, 1A⁵) variants are frequently observed (>1%) in the general population (DiAngelo et al., 1999; Floros, 2001; Floros et al., 1996). Figure 1.4 presents the two SP-A genes and their most commonly found variants.

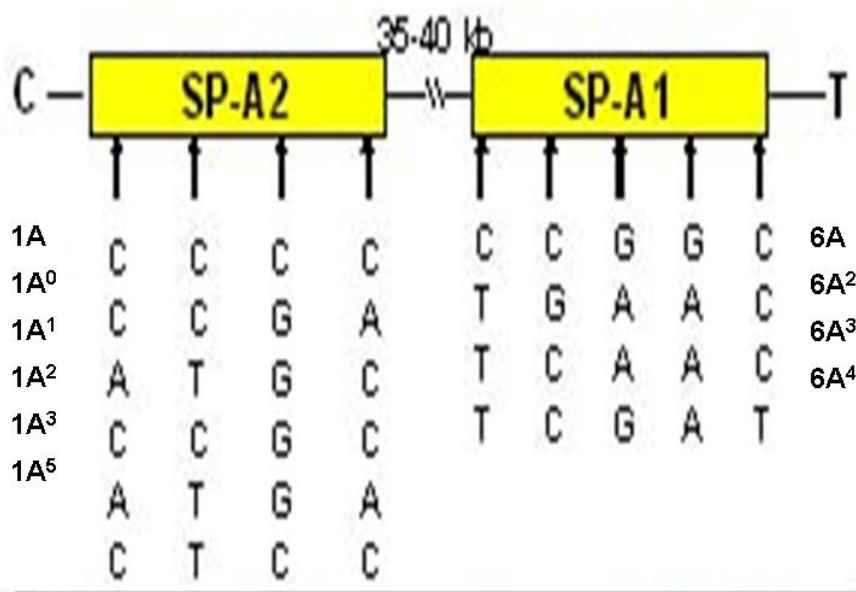


Figure 1.4: SP-A1 and SP-A2 variants

The two SP-A genes are in opposite orientation to one another on human chromosome 10. Variants of each gene are represented in blue and nucleotide sequences noted. The corresponding amino acid number is highlighted in purple.

Adapted from Floros 2001 (Floros, 2001).

The coding sequence of the two SP-A genes and the corresponding variants are distinguished by the “core” of invariant nucleotides or amino acids in exon 2 (Karinch and Floros, 1995a). These core differences are found at amino acid residues 66, 73, 81 and 85 of the collagen domain of SP-A. SP-A sequence variability is also found in both the 5' untranslated region (5' UTR) and the 3' untranslated region (3'UTR). The 5'

untranslated regions (5'UTRs) splice in different configurations giving rise to a number of different SP-A1 and SP-A2 transcripts (Karinch and Floros, 1995a; Karinch and Floros, 1995b; McCormick and Mendelson, 1994). The 3' UTR contains sequence differences, such as an 11 bp sequence that is found in the most common variants of SP-A1 (i.e. 6A²) and in all of the SP-A2 variants (Hoover and Floros, 1999).

Genetic complexity of SP-A1 and SP-A2 points to the multitude of potential qualitative and quantitative difference among the variants of these genes that are further discussed in section 1.3.3.

1.1.5.2 SP-B

SP-B is located on the short arm of chromosome 2p12-p11.2 (Vamvakopoulos et al., 1995). It is a relatively small gene that consists of 11 exons where the 11th exon is untranslated (Pilot-Matias et al., 1989). The mature protein is encoded by exons 6 and 7 of the gene. The cDNA sequences of SP-B from a number of species are highly conserved with a greater than 80% similarity. Several human SP-B variants have been identified, and the distribution of these variants differs among racial groups (Floros et al., 1995; Veletza et al., 1996).

A number of coding and non-coding sequence variants of SP-B have been identified in patients with SP-B deficiency (deMello and Lin, 2001). The first mutation of SP-B was identified in exon 4 of the gene in patients with congenital pulmonary alveolar proteinosis. This frameshift mutation consists of a substitution of GAA for C in codon 121 of SP-B and is commonly referred to as 121ins2 (Nogee et al., 1994). Another

frameshift mutation due to the deletion of a T nucleotide at position 1533 within exon IV (amino acid 122delT) causes a premature stop codon that results in complete absence of mature SP-B (Hickling et al., 1998). A missense polymorphism at position 4380(C/T), changes amino acid 236 from arginine to cysteine resulting in partial SP-B deficiency (Ballard et al., 1995). The exon 4 SNP B1580(C/T) causes a change in amino acid 131 (Ile131Thr) from isoleucine (encoded by T) to threonine (encoded by C) and might have important functional consequences since the substitution of isoleucine for threonine abolishes a consensus signal for N-linked glycosylation at this site (Wang et al., 2003a)

Particularly diverse are the size variants of intron 4. These are length polymorphisms of (CA)_n microsatellite sequences, characterized by deletions or insertions of specific sequence motifs (Floros et al., 1995) and may play a role in regulation of SP-B expression (Lin et al., 2005). Other single nucleotide polymorphisms (SNPs) of SP-B, such as B-18(A/C) in the 5' flanking region, B1013(A/C) at the intron 2/exon 3 splicing junction, and B9306(A/G) at the 3' end, most likely, if important, play a role in the regulation of gene expression.

1.1.5.3 SP-C

SP-C is located on a short arm of chromosome 8 (Fisher et al., 1988b; Glasser et al., 1988a). The gene consists of six exons, where the last exon is untranslated (Glasser et al., 1988a). The mature SP-C is encoded only by exon 2. Although exon 2 does not exhibit polymorphisms, SNPs found in exons that encode proSP-C may play a role in protein function by affecting the processing and trafficking of the immature protein. A

mutation of SP-C that results in hereditary respiratory failure was identified by Noguee et al. (Noguee et al., 2001). This single base substitution in intron 4 of SP-C results in shortened proprotein due to skipping of exon 4. A missense mutation in exon 5 was found to associate with autosomal dominant familial pulmonary fibrosis with a variable clinical course (Thomas et al., 2002). Two coding sequence variations have been characterized in SP-C, CA138(A/C) that results in either asparagine (A) or threonine (C), and CA186(A/G) that results in asparagine (A) or serine (G). Although functional consequences of these variants are currently unknown, since they are found in the C-terminus of pro-SP-C, they are likely to play a role in processing and trafficking of the immature protein.

1.1.5.4 SP-D

As has been already mentioned, SP-D is located on chromosome 10q22-q23 about 80-100 kb from SP-A (Crouch et al., 1993; Hoover and Floros, 1998). Organization of human SP-D suggests a close evolutionary relationship to SP-A (Drickamer et al., 1986). The gene consists of eight exons, where two commonly found SNPs have been identified in the coding sequence: 1) SNP DA11(C/T) of amino acid 11 (following signal peptide cleavage) in the amino-terminal part of the protein results in a substitution of threonine (C) for methionine (T) and 2) SNP DA160(A/G) in the collagenous domain of the SP-D-gene results in threonine (A) to alanine (G) substitution at amino acid 160 (Crouch et al., 1993; DiAngelo et al., 1999). In addition, a polymorphism at amino acid 270 (Ser270Thr) in the carbohydrate recognition domain of SP-D has recently been identified

(Lahti et al., 2002). The potential functional significance of these variants is presently unknown. As for SP-A, no mutations of SP-D have been identified to associate with disease.

1.2 Literature review – Section II

1.2.1 Lung development

Prenatal development of the human fetal lung is divided into following stages: 1) embryonic stage; 2) pseudoglandular stage; 3) canalicular stage; 4) saccular stage; and 5) alveolar stage. These same stages are seen in other species, but the alveolar stage is an exclusively post-natal process in a number of species (mouse, rat) (Burri, 1984).

Each stage of development is characterized by the appearance of specific structures of the future alveolar spaces and progression of vascularization as presented in Figure 1.5. During the embryonic stage (up to 7 weeks of gestation) formation of proximal airways first occurs with the appearance of the lung bud as a ventral diverticulum of the foregut. The lung bud is lined by endoderm which gives rise to the specialized epithelial cells of the lung. The endoderm forms two lung buds as it divides into the surrounding mesenchyme that will give rise to supporting structures of the airway. By 6 weeks of gestation the two lungs can be distinguished as separate structures in the thorax. The airways branch out and develop to the level of the bronchopulmonary segments at about the 16th generation of branching.

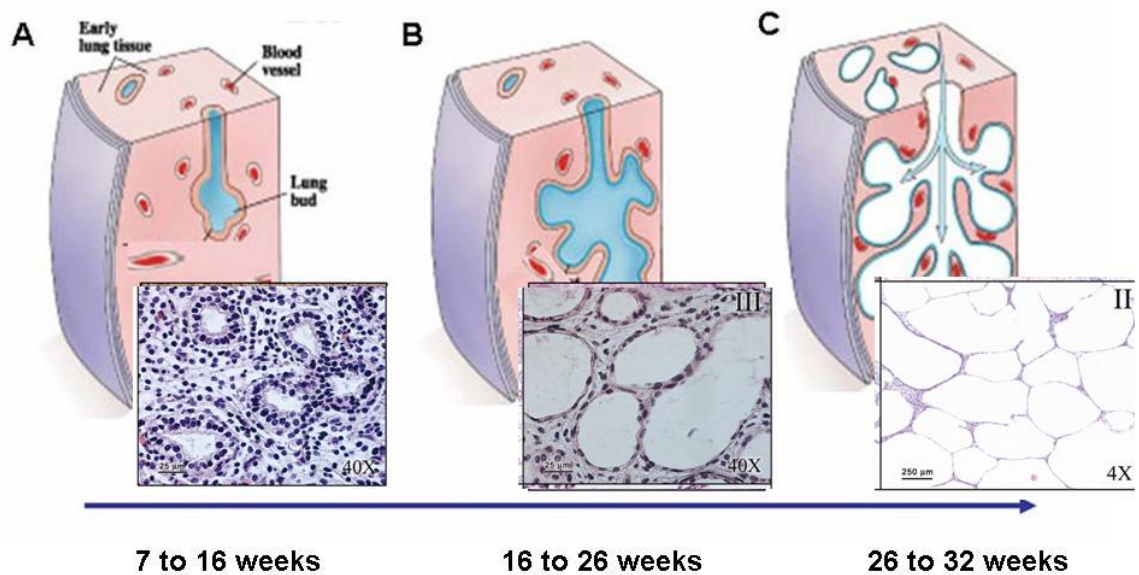


Figure 1.5: Sequential progression of human fetal lung development

Panel A is representative of pseudoglandular stage of tissue development. Panels B and C present canalicular and saccular stages of development, respectively. (adapted from

<http://www.seifmedgraphics.com/images/discovery/D070101images/exI.jpg>. See below

for details.

By the end of the pseudoglandular stage (from 7–16 weeks of gestation, Panel A, Figure 1.5) all preacinar airways to the level of the terminal bronchioli are formed and the angiogenic development of preacinar vasculature is completed. The cells of airway walls differentiate to form smooth muscle, cartilage, submucosal glands, and connective tissue.

The acinar structures (future alveoli) are lined with a uniform population of high columnar epithelium. The peripheral airways are lined with ciliated, goblet, basal cells, and Clara cells. Clara cells produce a 10 kDa cell specific protein CC10, and have also been shown to produce SP-A in some species (Auten et al., 1990) and SP-D (Voorhout et al., 1992).

The canalicular stage of development (from 16 to 26-28 weeks of gestation, Panel B, Figure 1.5) is marked by formation of acini and extensive development of distal pulmonary circulation. As the capillaries appear in the mesoderm, they connect with the pulmonary arteries. The thinning and differentiation of the tissue bring into apposition the capillary endothelial cells and epithelial cells of the future alveoli, forming the structures of the blood-gas barrier by 19 to 20 weeks of gestation (DiMaio et al., 1989). The extent of vascularization of the lung at this point has been suggested to be sufficient to sustain the lives of even extremely premature infants (DiMaio et al., 1989). In the early canalicular phase, the future alveoli are lined with cuboidal epithelium which is represented by non-differentiated stem cells of alveolar epithelial cells (AECs). The alveolar epithelial stem cells first differentiate into type II cells which then divide to produce more type II cells and further differentiate into type I cells (Uhal, 1997). Towards the end of the canalicular phase both type II and type I AECs can be seen (Flecknoe et al., 2003).

The saccular stage of development extends from 26-28 to 32-36 weeks of gestation (Panel C, Figure 1.5). Time ranges for each stage are due to the gradients of development within the lung, development proceeds from proximal to distal and from

apical to basal, thus making it possible to find different stages of development in the lung at the same time. Saccular stage is characterized by the appearance of secondary crests, which start to divide the primary saccules into subsaccules or primitive alveoli. There is a marked decrease in the interstitial space of the saccular walls and formation of the double capillary network within the crests. The epithelium undergoes dramatic flattening with the increase of lung volume resulting in increased expression of type I AECs (Flecknoe et al., 2000).

In contrast to mice and rats, where alveolar development is a post-natal process, the human alveolar stage of development starts between 32 and 36 weeks of gestation and continues up to at least 3 years of age (Burri, 1984; Zeltner and Burri, 1987). Some authors have suggested that the alveolar stage is not fully completed until about 8 years of age (Reid, 1984; Thurlbeck, 1975). During this stage, a further thinning of the interstitium takes place. The alveoli are formed through a septation process that greatly increases the surface area available for gas exchange (Burri, 1984; Zeltner and Burri, 1987). In order to facilitate gas exchange a single capillary network is established, where one capillary bulges into both alveoli with which it associates.

The process of alveolarization starts distally and proceeds proximally in the lung. At term, the terminal air spaces are made up of about 150 million alveoli (Hislop et al., 1986). Although the alveolar growth slows down during the first few months of life, there is a rapid increase in the number of alveoli by the first year of life and reaches the approximate adult number of 300 million alveoli by three years of age (Crapo et al., 1982; Reid, 1984; Thurlbeck, 1982).

1.2.2 Pulmonary cells during development

The type II pneumocytes are round, cuboidal cells that produce, store, and secrete pulmonary surfactant. In the adult lung they account for 60% of epithelial cells, but they cover only about 3% of the alveolar surface (Crapo et al., 1982). They have prominent microvilli and granular appearance on EM due to numerous lamellar bodies.

Type I cells make up the bulk of the gas exchange surface. These cells cover 95% of alveolar surface area of the adult lung and account for about 40% of the overall epithelial cells in the lung (Crapo et al., 1982). Type I cells are flat with broad cytoplasmic flaps and a perinuclear zone where the nucleus and organelles are clustered. Aside from gas diffusion, type I cells also control fluid movement between the interstitium and the airspace. Injury to these cells (a common consequence of mechanical ventilation) results in infusion of fluid exudates into the airspaces.

The long standing scientific dogma has been that type I cells are terminally differentiated and that as lung development progresses type II cells irreversibly differentiate into type I cell. However, recent *in vitro* (Danto et al., 1995) and *in vivo* (Flecknoe et al., 2000) studies have shown that type I cells can trans-differentiate into type II cells. This has prompted a speculation that the proportion of type II and type I cells can change during pre- and post-natal development depending on the factors that influence cellular differentiation/transdifferentiation. This process seems to be primarily dependent on the degree of mechanical strain experienced by AECs. Flecknoe et al. (Flecknoe et al., 2003) were able to show that in late saccular stage of development of fetal sheep significant portion of AECs (63%) had differentiated into type I cell

phenotype, and that very few type II cells were present (about 4%). In post-natal sheep, two weeks following birth, as the expansion of fetal lungs decreased, type I cells decreased to about 44% and type II cells increased to about 53%. This study points to the flexibility of pulmonary cell differentiation where cellular phenotype is rapidly influenced by structural changes in the lung.

1.2.3 Pulmonary surfactant during development

Synthesis of surfactant has been reported to begin toward the end of the second trimester as the type II cells differentiate from AEC stem cells. Lamellar bodies can be seen at about 24 weeks of gestation (Ivie et al., 1987; Snyder et al., 1983) and saturated phosphatidylcholine (PC) is present even earlier in whole lung extracts (Ballard, 1989). Active secretion of surfactant is detectable after 30-32 weeks, as shown by amniotic fluid measurements (Clements, 1977; Oulton et al., 1980). As the development of fetal lung advances, the phosphatidylinositol (PI), which is abundant in the early form of surfactant, is gradually replaced by phosphatidylglycerol, which is representative of the mature surfactant form (Hallman et al., 1976; Kulovich et al., 1979). At 30-32 weeks of gestation, dipalmitoyl phosphatidylcholine (DPPC) and phosphatidylglycerol (PG) content increase substantially (Hallman and Gluck, 1977). Although the functional consequences of this change are not yet clear (Beppu et al., 1983), the shift from a PI to a PG enriched surfactant is used as a marker of fetal lung maturation. Lamellar body count in amniotic fluid has also gained acceptance as a test of fetal lung maturity, where maturity is suggested by a count of 50,000 lamellar bodies/ μ L or greater, while counts of

15,000 lamellar bodies/ μL or less are indicative of immaturity. Although lamellar body count greater than 50,000 has repeatedly been associated with lung maturity (Bahasadri and Changizi, 2005; Khazardoost et al., 2005; Piazze et al., 2005), recent studies that have addressed the cut-off values for fetal lung maturity have reported a range from 10,000 (Bahasadri and Changizi, 2005) to 22,000 lamellar bodies/ μL (Piazze et al., 2005).

With respect to the developmental regulation of surfactant proteins, studies in a number of species have shown that expression of SP-B and SP-C precedes that of SP-A and SP-D (Ballard, 1984). SP-B and SP-C transcripts have been found in human lung tissue as early as 13 weeks of development, and SP-B protein has been found in amniotic fluid after 13 weeks of gestation (Liley et al., 1989; Whitsett et al., 1987). Studies from a number of animal systems have shown that SP-B and SP-C mRNAs are present in the pseudoglandular stage of development and that they increase rapidly to term, reaching near adult levels at birth (Khour et al., 1994; Schellhase et al., 1989; Wert et al., 1993; Whitsett et al., 1987; Wohlford-Lenane et al., 1992; Wohlford-Lenane and Snyder, 1992).

The long standing dogma that SP-A is not expressed in human fetal lungs until the appearance of lamellar bodies in type II cells, has recently been refuted by a number of studies that have shown expression of SP-A as early as 14 weeks of gestation (Khour et al., 1996; Otto-Verberne et al., 1990). SP-A is thought to be predominantly secreted by a lamellar body independent pathway (Froh et al., 1993; Rooney et al., 1993).

Concentration of SP-A increases in amniotic fluid with progression of gestation (Hallman et al., 1988; Pryhuber et al., 1991; Shimizu et al., 1989).

1.2.4 Models available for study of human lung development

Human fetal lung explants have been the primary model of human lung development used in the literature (Beers et al., 1995; Dulkerian et al., 1996; Goss et al., 1998; Karinch et al., 1998; Mendelson et al., 1991; Mendelson and Boggaram, 1991; Solarin et al., 1997). Potential uses of this model are limited by the highly accelerated morphological development and limited viability of explants in cell culture. In particular, studies of changes specific for particular stages of lung development and studies over longer periods of time, are not feasible in this model.

Recently models of human fetal tissue grafts have been proposed as models that could provide for more adequate studies of human organ development. Studies of grafts of human cancer cells and tissues in immunocompromised mice (xenografts) have been extensively used in cancer research. Both mechanisms of carcinogenesis and the effects of different pharmacological agents on human tumor growth have been studied in these models (Giovanella and Fogh, 1985; Malkinson, 2001). In addition, these models have been used to study transmission of infection in various tissues by pathogens (Howett et al., 1997; Howett et al., 1990; Howett et al., 1999; Madan et al., 1997; Miller et al., 1994).

The first study to use immunocompromised mice for human lung development was performed by Phillips and Gazet, where human fetal lungs were grafted into a mouse treated with anti-lymphocytic serum (Phillips and Gazet, 1969). These lungs developed and grew in the host for 90 days. Allograft models of mouse lung development, where whole embryonic mouse lungs were grafted into immunocompromised mice, showed a sequential progression of structural development with subcutaneous grafts showing accelerated timing of development (Schwarz et al., 2000) and timing of renal subcapsular graft development closely following that of *in utero* development (Vu et al., 2003).

Studies of human fetal lung xenografts have focused primarily on the endpoints of upper airway tissue development, rather than on the characterization of individual stages. (Delplanque et al., 2000; Puchelle and Peault, 2000) (Engelhardt et al., 1993; Filali et al., 2002). Study by Peault et al. showed that subcutaneous grafts of human fetal lung rudiments from 7.5 to 16 weeks of gestation showed mature bronchioles and alveoli after 6-8 weeks of grafting, regardless of the starting gestational age of the tissue (Peault et al., 1994). A different study reported that subcutaneous grafts of fetal lungs from 10 -14 weeks of gestation reached alveolar stage of development at 8 weeks post-grafting (Groscurth and Tondury, 1982).

Information available on lung xenograft models (Cobb, 1975; Delplanque et al., 2000; Deutsch G., 1997; Engelhardt et al., 1993; Filali et al., 2002; Phillips and Gazet, 1969; Pilewski et al., 1994; Puchelle and Peault, 2000; Schwarz et al., 2000) led to the hypothesis that human fetal lung tissue of lower airways grafted into immunocompromised mice would undergo changes that mimic, in many respects, the

stages of *in utero* lung development. This assumption was studied within the Specific Aim I of this thesis and is presented in Chapter 2.

1.2.5 Adaptation of the lung to the extrauterine life

In order for the fetus to complete the transition from intrauterine to extrauterine life, the lung fluid must be cleared soon after birth. During development lung liquid is actively produced, as has been demonstrated by the increase in lung volume in models of tracheal ligation (Blewett et al., 1996; Carmel et al., 1965; Papadakis et al., 1998; Papadakis et al., 1997; Piedboeuf et al., 1997). Lung fluid is distinct from the amniotic fluid and plasma. It is almost devoid of proteins, has high potassium and chloride concentrations and an acidic pH (6.27) (Bland, 1990; Pachi et al., 2001). The fetal lung movements, which are commonly referred to as fetal breathing, do not move much lung fluid. The fetal breath rate varies between 30 to 70 breaths per minute (Dawes, 1984) (6 month baby respiratory rate 25-50). Fetal breathing is suppressed as labor approaches, and remains suppressed during active labor (Boylan and Lewis, 1980). During the later part of the third semester, lung liquid clearance gradually increases, but the phenotypic switch of the lung epithelium from net secretion to net absorption does not occur until the commencement of labor (Barker and Olver, 2002). At birth, the pulmonary epithelium changes from a chloride-secreting membrane to a sodium-absorbing membrane with the reversal of the direction of flow of lung fluid.

1.2.6 Preterm labor and premature birth

Preterm deliveries are the leading cause of perinatal morbidity and mortality in the United States (Jobe, 2002). Preterm birth, is birth that occurs before 37 weeks of gestation. However, since the degree of prematurity varies based on the gestational age and weight of the infant, this definition has been expanded into several sub-categories, based on the gestational age and weight of the infant. While preterm birth occurs before 37 weeks of gestation, very preterm birth is most commonly defined as birth that occurs at lower than 32 weeks of gestation (previous definitions have included 33 and 34 weeks of gestation). Premature infants who weigh lower than 2500 g are defined as low birthweight, while those that weigh lower than 1500 g and 1000 g are defined as very low (VLBW) and extremely low birthweight (ELBW), respectively (Jobe and Ikegami, 2000; Lemons et al., 2001; Luke et al., 1993).

Current yearly expenditures for preterm deliveries and preterm birth are over \$3 billion. The majority of preterm deliveries occur spontaneously and are due to preterm labor associated with infection, decidual hemorrhage or pathological uterine distention commonly seen in multiple gestations. There is a strong link to sociodemographic factors, including maternal age, race, ethnicity, and socioeconomic status. Still, in most cases, the precise causes of preterm labor are not known.

There are approximately 400,000 infants born prematurely each year in the United States alone (Ventura et al., 2000). Current rates of premature birth vary between 8 to 10 percent, with greatest number of premature births occurring between 32 and 37 weeks (11.6%) and only 1.96% occurring at a gestational age of 32 weeks or lower (Martin et

al., 2003). The high percentage of preterm births between 32 and 36 weeks of development is primarily due to the higher rate of multiple gestation pregnancies, since these pregnancies are prone to preterm delivery (MacDorman et al., 2005).

Recent technological advances in perinatal and neonatal medicine have decreased the morbidity and increased survival rates of prematurely born infants. The survival rates are dramatically increased with an increase in gestational age and birthweight (Hack and Fanaroff, 1999). Infants born at 23 weeks of gestation have a 28% survival, while those born at 29 weeks of gestation have a 92% survival. Infants born with birth weights of 501 to 750 g have 54% chance of survival, while infants born at 1250 g to 1500 g have a 97% survival (Lemons et al., 2001). Hence, the need for improved perinatal treatment is imperative. This underscores a need for better indicators to identify women who will develop preterm labor and those who will deliver before term.

1.2.7 Lung-related consequences of preterm birth

Premature infants commonly develop complications due to anatomic and/or functional immaturity (Lemons et al., 2001; Markestad et al., 2005; Mikkola et al., 2005; Tommiska et al., 2001). The successful adaptation of the infant to extrauterine life is dependent on a number of factors, of which the development of normal pulmonary alveolar epithelium is essential. Fetal lung fluid secreted by the alveolar epithelium must be cleared for adequate gas exchange to commence. This process occurs with impending labor by decreased production of lung fluid and increased clearance of fluid from the lung (Bland, 1990; Helve et al., 2005). In order to prepare the lungs for air breathing,

synthesis of surfactant and antioxidant enzymes also increase. Failure of these perinatal adaptations to occur results in respiratory distress (Clark et al., 2001; Helve et al., 2004; O'Brodovich, 1996).

Since the lungs are one of the last organs to develop, respiratory abnormalities are common: 60% of very low birthweight (VLBW) infants who were studied by the centers of National Institute of Child Health and Human Development (NICHD) Neonatal Research Network between 1995 and 1996 required endotracheal intubation (Lemons et al., 2001). Respiratory distress is therefore a common sequela of premature birth that commonly abates several days after birth, but can also progress to more complicated illnesses such as pulmonary air leak and BPD.

1.2.8 Neonatal respiratory disease

1.2.8.1 Respiratory Distress Syndrome (RDS)

Respiratory distress syndrome (RDS) is the most common cause of respiratory distress in premature infants (Ainsworth, 2005; Farrell and Wood, 1976). RDS is primarily due to a deficiency of surfactant in the immature lung (Avery and Mead, 1959). Gender, ethnicity, and maternal disease (such as diabetes) are also known risk factors of RDS (Farrell and Kotas, 1976; Verma, 1995). In RDS, lack of surfactant leads to increased surface tension (especially at low volumes), alveolar collapse, and diffuse progressive atelectasis. As blood bypasses atelectatic air spaces, mismatch of ventilation

and perfusion results in hypoxemia (Jobe, 1991). Moreover, atelectasis triggers an inflammatory cascade that results in cytokine increase, followed by neutrophil recruitment into the lung and endothelial cell damage (Murch et al., 1996; Ogden et al., 1984). The inflammatory process and vascular leakage lead to accumulation of protein-rich fluid in the lung, resulting in pulmonary edema. Influx of the protein-rich fluid inactivates the already deficient tissue surfactant pools, leading to the impaired compliance and exacerbation of RDS (Kobayashi et al., 1991).

1.2.8.2 Clinical course of RDS

Clinically, respiratory distress and cyanosis occur immediately or within a few hours after premature birth. Infants develop tachypnea and labored breathing, followed by nasal flaring (reduces nasal resistance and reflects increased use of accessory muscles of respiration), grunting (compensatory effort to prevent end-expiratory alveolar collapse), and intercostal and subcostal muscle retractions due to a highly compliant chest wall that is drawn in during inspiration in an effort to expand the poorly compliant lung (Jobe, 1991). Due to the resulting hypoxemia, patients often require supplemental oxygen (Gregory et al., 1971). Failure to expand lungs leads to atelectasis, this in turn is recognizable by the classic findings of a diffuse, reticulogranular, ground glass appearance with air bronchograms on a chest radiograph (Dimitriou et al., 1995; Giffin et al., 1995; Isdale and Thomson, 1979; Yuksel et al., 1991). Natural progression of uncomplicated, untreated RDS typically continues for 48-72 hours (Jobe, 1991). By the

end of the first week of life improvement in respiratory function occurs due to the significant increase in endogenous surfactant production.

Although use of ante-natal steroids has decreased the incidence of RDS by about 50% (Crowley et al., 1990; Jobe and Soll, 2004) the overall rate of RDS is still around 10% of premature births. Administration of exogenous surfactant to infants reduces mortality from RDS by 40% (Schwartz et al., 1994). Surfactant therapy improves oxygenation, decreases air leaks, and improves overall pulmonary function (Bose et al., 1990; Katyal et al., 1992).

1.2.9 Neonatal respiratory disease: Bronchopulmonary Dysplasia (BPD)

Bronchopulmonary dysplasia (BPD), first described in 1967 by William Northway, is a chronic lung disease (CLD) of premature babies who require intensive oxygen therapy in order to survive (Northway et al., 1967). Traditionally, the pathology of BPD/CLD has been described as severe lung fibrosis with significant cellular proliferation, affecting both parenchyma and airways with alterations of normal growth and development of the lung (Jobe and Bancalari, 2001; O'Brodovich and Mellins, 1985). Recent introduction of modern therapies, such as surfactant, corticosteroids, and improved ventilation strategies, have led to an increase in survival of very premature, VLBW infants with BPD/CLD as well as a change in pathophysiology of the disease (Bancalari et al., 2003; Rojas et al., 1995). The “new” BPD is characterized by less fibrosis, with large and small airways free of epithelial metaplasia and smooth muscle hypertrophy. Nonetheless, the disease is still characterized by a decrease in

alveolarization, and with septation and microvascular development characteristic of arrested lung maturation (Ehrenkranz et al., 2005).

However, the overall incidence of BPD has not decreased, but rather increased. The increase in BPD incidence is primarily due to the increased survival (due to steroid and surfactant therapy) of very premature, very low birthweight (VLBW) infants who are at highest risk for BPD (Ehrenkranz et al., 2005). It has been suggested that there is not only a difference in quantity, but also in quality of BPD; where the incidence of “old” BPD (mostly caused by harsh ventilation strategies) is declining, and the incidence of “new” BPD (milder form affecting VLBW infants) is increasing (Bancalari et al., 2003). Currently, the overall incidence of BPD is 2-3 infants per 1000 live births (Ehrenkranz et al., 2005). The incidence rate sharply increases as the birthweight decreases. Infants weighing lower than 1200 g have a 22% BPD incidence rate, while those born weighing 1000g or lower have a 78% incidence. BPD infrequently affects infants with a birth weight of more than 1250 g or gestational age greater than 30 weeks (Berger et al., 2004; Clark et al., 2001). The primary risk factors include lung immaturity, oxygen toxicity, baro- and volutrauma, mechanical ventilation, prenatal and nosocomial infections, and increased pulmonary blood flow secondary to a patent ductus arteriosus (Abele-Horn et al., 1998; Chambers and van Velzen, 1989; Choi et al., 2006; Clark et al., 2001; van de Bor et al., 1988). Since the immature lungs have a poorly developed antioxidant system, they are at a heightened risk of oxygen free radical damage (O'Donovan and Fernandes, 2000).

1.2.9.1 BPD etiology

The common understanding has been that BPD is preceded by RDS in VLBW infants (Van Lierde et al., 1991). The traditional opinion that BPD occurs due to the stress of mechanical ventilation superimposed on the immature surfactant-deficient lung, has been challenged by the findings that infants who have no initial lung disease can develop BPD or develop BPD once RDS has been resolved (Charafeddine et al., 1999). These infants are most likely exposed to chorioamnionitis or early postnatal pulmonary or systemic infection (Speer, 2003). This has led to a view of BPD as a disorder resulting from intra-uterine inflammation superimposed on the immature lung structure that results in alveolar simplification (decreased number of septations). In a recent review, Jobe proposed that intrauterine infection leads to early lung maturation. This protects the infants from RDS but may lead to abnormal structural development, ultimately resulting in BPD (Jobe, 2003).

1.2.10 Inflammation and surfactant proteins in neonatal lung disease

Preterm birth is strongly associated with intrauterine infection (chorioamnionitis) (Goldenberg et al., 2000). Timing of gestation and parturition is very specific in humans, however mechanisms involved in preterm labor and parturition are mostly unknown. DNA microarrays of human uterine tissues showed that many of the genes whose expression was upregulated in both pre-term and term uterine samples, encoded for inflammatory proteins (Bethin et al., 2003). NF- κ B has been implicated by a number of studies as the key player in parturition and particularly in activation of pre-term labor

(Allport et al., 2001; Lee et al., 2003). It has been found to be activated at higher levels in pre-term than term labor. Induction of NF- κ B by IL-1 leads to activation of inflammatory pathways that lead to progression of labor. Activation of NF- κ B can be due to a number of factors, such as bacterial toxins, allergens, and stretching of the uterine muscle (Bennett et al., 2000; Bytautiene et al., 2004; Silver et al., 1995).

Furthermore, a recent study suggests that secretion of SP-A by a maturing fetal lung into the amniotic fluid leads to onset of labor (Condon et al., 2004). Condon et al. were able to show that increased secretion of SP-A by the developing mouse fetal lung into the amniotic fluid activates macrophages of fetal origin to migrate to the uterus to induce production of cytokines, IL-1 β in particular, leading to activation of NF- κ B thus stimulating the onset of labor. Injection of SP-A into the amniotic fluid of pregnant mice induced preterm delivery of mouse fetuses within 6-24 hours, while injection of SP-A antibody or NF- κ B inhibitor into the amniotic fluid delayed labor by more than 24 hours (Condon et al., 2004). Based on these findings authors concluded that SP-A in addition to being a marker of fetal lung maturation plays a role in initiation of parturition in mice by activating macrophages of fetal origin to migrate to the uterus. When uterine myometrium of women who had undergone spontaneous labor at term was examined, fetal macrophages were not found in maternal tissue, raising the question of whether the activation of parturition seen in mice occurs in humans (Kim et al., 2006). However, a study by Han et al. showed that both message and protein of SP-A are expressed in amniotic epithelial cells, macrophages, and trophoblasts in chorioamniotic membranes of women with pre-term and term labor, with or without histologic chorioamnionitis (Han et

al., 2007). Specifically, expression of SP-A1 mRNA in resident macrophages of chorioamniotic membranes was increased with progression of gestation as well as in the presence of chorioamnionitis, prompting the authors to conclude that SP-A and alveolar macrophages interacted in an autocrine/paracrine loop in activation of parturition. This was strengthened by the findings of a recent study where presence of two uterine binding sites for SP-A was shown in rat myometrium and endometrium as well as in myometrial cells in culture (Garcia-Verdugo et al., 2007). Presence of these binding sites was not influenced by pregnancy-related changes, and addition of LPS to SP-A blocked the binding of SP-A to these sites. These interactions point to the dual role (pro- and anti-inflammatory) that SP-A is likely to play in parturition related events.

The traditional thought has been that the fetus is immunologically naïve and that the introduction of infection and/or inflammation into the fetal compartment leads to an inappropriate fetal response and possibly pre-term birth. Although chronic inflammation is still seen as generally detrimental to the fetus, recent studies have shown that the fetal lungs can benefit from antenatal inflammatory stimulation (Cornette, 2005; Kramer and Jobe, 2005). While an excessive pro-inflammatory response is damaging to the adult lung, fetal lung development is dependent on the dominance of pro-inflammatory cytokines, which are substituted by anti-inflammatory cytokines after birth (Dammann and Leviton, 2001).

Pro-inflammatory cytokines are spontaneously produced by fetal and umbilical cord mononuclear cells during the second and third trimesters of pregnancy, reaching peak levels around 30 weeks of gestation and declining steadily thereafter (Taeusch et al.,

1986). The effect of cytokines on lung development has been studied in a variety of animal models. In fetal sheep, intra-amniotic injection of endotoxin caused an increase in expression of IL-1b, IL-6, IL-8, and proliferation of inflammatory cells (Kallapur et al., 2001). Chronic exposure (for 28 days) of fetal sheep to endotoxin did not cause progressive structural abnormalities of the lung, thus suggesting that the fetal lung is able to down-regulate prolonged inflammatory stimulus (Kallapur et al., 2005). However, seven days of treatment with endotoxin in this model, led to decreased alveolar septation and microvascular development that was mild BPD-like (Kallapur et al., 2004). When the sheep were exposed to a single dose of endotoxin, these changes were not observed. Moreover, increased surfactant production and improved lung function were detected (Jobe et al., 2000; Sosenko et al., 2006).

Administration of IL-1 has been shown to induce the expression of surfactant protein mRNA (SP-A and SP-B primarily) in preterm rabbits and preterm lambs (Bry et al., 1997; Emerson et al., 1997) and in rabbit explant cultures (Dhar et al., 1997; Glumoff et al., 2000; Seppanen et al., 2005) as well as expression of surfactant lipids in preterm rabbits. A single injection of 20 mg of endotoxin (which induces IL-1, IL-6, IL-8 and TNF- α production) into the amniotic fluid of fetal sheep leads to a several fold increase in surfactant protein (SP-A) mRNA by day 1 which persists for 15 days. These mechanisms are indicative of lung maturation processes that support lung development and cellular differentiation, and thus may protect prematurely born infants from neonatal lung disease. Moreover, the studies of rabbit fetal lung explants indicate that the cytokine effect is gestation stage-specific, because *in vitro* IL-1 induces expression of SP-A, SP-B, and SP-

C mRNA in rabbit lung explants from 19 and 22 day fetuses, while it suppresses SPs message in explants from 27 and 30 day fetuses (Glumoff, Vayrynen 2000). These observations may shed light on the unexpected finding that maternal chorioamnionitis has been shown to be negatively associated with RDS prevalence and positively associated with BPD prevalence (Watterberg et al., 1996). The report by Watterberg et al. showed that the incidence of RDS decreased and the incidence of BPD increased in infants born to women with histologic chorioamnionitis, compared to infants born to women without chorioamnionitis. Also, preterm infants colonized with *Ureaplasma urealyticum* (an organism commonly associated with chorioamnionitis and BPD) had a significantly lower incidence of RDS in the early neonatal period than the uncolonized controls. These infants later progressed to have significantly greater rates of BPD than uncolonized infants (Hannaford et al., 1999).

Various studies have reported an increase in levels of IL-6, IL-1, IL-8, TNF- α and multiple other promoters and products of inflammation in airways of infants who developed BPD (Inwald et al., 1998; Jones et al., 1996; Munshi et al., 1997; Tauscher et al., 2003). In addition, infants who develop RDS and/or BPD/CLD have low SP-A levels (deMello et al., 1993; deMello et al., 1989; Hallman et al., 1991). While exogenous surfactant therapy reduces the consequences of BPD it does not prevent the development of the disease.

1.3 Literature review – Section III

1.3.1 Genetic diversity as it relates to human disease

Natural variations in DNA sequence have been used as essential tools in genetic studies of human disease. Since the original identification in 1900 of the polymorphic phenomenon describing the blood group ABO system (Landsteiner, 1900), a very large number of genetic variations have been characterized (Kantha, 1995). Moreover, identification of DNA- based polymorphisms has transformed human genetic mapping, as it provides a way of identifying species-specific differences in the non-coding parts of DNA (evolutionarily less conserved than coding regions). Although detection of human sequence variation was not an objective of the preliminary Human Genome Project (1993-1998), the latest version of the project has mapped more than 100,000 SNPs. The expansion of this field has been facilitated by the development of new molecular biology tools, in particular by introduction of automated polymerase chain reaction (PCR). Prior to the wide introduction of PCR, identification of DNA polymorphisms was essentially limited to restriction fragment length polymorphisms (RFLPs). The first comprehensive human genetic map was developed based on almost 400 such markers (Donis-Keller et al., 1987).

There are several types of DNA polymorphisms in the human genome, including tandem repeat polymorphisms, single nucleotide polymorphisms (SNPs), and insertion/deletion polymorphisms (Langley and Aquadro, 1987; Miyashita and Langley, 1988; Schumm et al., 1988). Usefulness of these markers is dependent on: 1) degree of

polymorphic informativeness; 2) frequency of distribution through the genome/putative distance of the marker from the disease locus; and 3) ease of analysis. Marker informativeness is of special interest, since a highly informative marker can dramatically reduce the number of individuals that must be typed to map disease genes.

Tandem repeat polymorphisms are generally divided into two groups: minisatellites and microsatellites. Minisatellite markers are highly polymorphic, but their utility in linkage mapping is limited due to their predominant location on the telomeric ends of chromosomes. Microsatellites are characterized by blocks of short tandem repeats, such as $(dA)_n \cdot (dT)_n$, $(dC-dA)_n \cdot (dG-dT)_n$, $(dT-dC)_n \cdot (dA-dG)_n$, $(dTtA)_n \cdot (dAAT)_n$ (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989; Zuliani and Hobbs, 1990), with the majority of the blocks having 25 or fewer repeats (Hamada et al., 1984). Due to the uniform distribution of microsatellites in the genome, marker abundance parallels chromosome size (Weber and May, 1989). The polymorphism information content (PIC) is extremely high, with average PIC values of 0.61 (Weber, 1990). Overall, these microsatellite markers are abundant, highly informative, and dispersed throughout genome. As such, they have been extensively used as markers in linkage analysis in order to study the tendency of genes to be inherited together in families. However, alleles of microsatellite markers differ by only a single repeat, which makes the analysis and scoring of these markers particularly challenging. Moreover, the density of microsatellite markers is limited to about one per 30 kb, precluding their use in detailed genetic mapping.

Although single nucleotide polymorphisms (SNPs) have a decreased polymorphism information content (average PIC about 0.3) (Weber and May, 1989) compared to microsatellites, they are extremely useful markers in genetic analysis due to their abundance in the genome. These variations in single nucleotide substitutions feature a biallelic situation in which the alternative bases occur at a frequency higher than 1% (Schafer and Hawkins, 1998). SNPs are the most common variants of human genetic diversity. Their frequency ranges from 1/300 to 1/1000 nucleotides, resulting in between 3 and 6×10^6 SNPs in a normal human genome (Cargill et al., 1999; Halushka et al., 1999). They represent a virtually unlimited resource of molecular markers that can be used to distinguish all individuals except for monozygotic twins.

The dense distribution of SNPs and the availability of the human SNP map facilitate high resolution mapping of genetic variations that underlie complex diseases. In some cases a single SNP is directly responsible for a particular genetic disease. However, the majority of diseases have complex genetic backgrounds where SNPs can be used as indirect markers of disease traits and/or to identify genetic elements directly involved in specific diseases. Moreover, there are examples where a single SNP can not be associated with a particular disease risk or drug response. Since SNPs do not exist in isolation, the combination of several SNPs on single chromosomes, the variant, is more informative. Therefore, disease susceptibility and outcomes may depend upon the interactions of multiple SNPs closely linked on the same chromosome (molecular variant). In this respect, the focus of genetic association studies has turned from single

SNPs to variants, which are expected to offer greater power to identify causative loci for multifactorial complex diseases.

1.3.2 Molecular techniques used for the study of genetic diversity

Multiple SNPs that need to be typed and the large number of DNA samples needed in association studies of complex diseases, entail a time-consuming and costly effort (Chanock, 2001; Halushka et al., 1999; Shastry, 2002). Therefore, there is a great need to develop methods that enable rapid, accurate, and economical identification of genetic polymorphisms. Moreover, such high-throughput methods are essential for application of pharmacogenetics to drug development. Genotyping of SNPs in large scale pharmacogenetic studies is expected to enable selection of the right drugs for the right patients at the right doses.

Current and emerging technologies for assessing DNA sequence variations are based on diverse detection methods. These methods can be separated into two broad groups: those that are detected in a sequence-specific way and those detected in sequence-nonspecific way. Sequence-nonspecific detection takes advantage of physical differences between mismatched heteroduplexes formed between allelic DNA molecules or single-stranded DNA molecules. It is based on the capture, cleavage, or mobility change during electrophoresis (as in RFLP analysis) or liquid chromatography of these variants (Kwok, 2000). Although these methods have been widely used in polymorphism/mutation discovery, they provide inferred genotypes rather than direct readings of true genotypes.

The alternative to these approaches has been provided by a number of methods that employ sequence-specific detection of SNPs. These methods are based on four general mechanisms for allelic discrimination: 1) allele-specific hybridization; 2) allele-specific oligonucleotide ligation; 3) allele-specific invasive cleavage (Kwok, 2000; Kwok, 2001; Kwok, 2002; Kwok and Chen, 2003); and 4) allele-specific nucleotide incorporation (primer extension). All four mechanisms are reliable, but the first three demand the design of highly specific probes/assays that can be particularly challenging for GC rich sequences.

Primer-extension based assays have been the evolving methods of choice in SNP analysis. These assays are based on the ability of DNA polymerase to incorporate specific nucleotide bases complementary to the sequence of the template DNA. Primer-extension based assays are highly flexible and in general require the least number of primers and/or probes.

Genotyping methods are based on a series of biochemical steps and a final product detection step. While biochemical steps employ a wide array of technologies, the detection step is most commonly based on light emission. Luminescence, fluorescence, fluorescence resonance energy transfer (FRET), and fluorescence polarization (FP) have all been used to monitor the light emitted by the specific allelic products.

Pyrosequencing, the method of SNP genotyping used in this thesis, is a primer based DNA sequencing method, where DNA synthesis is monitored by four different enzymes and detected through luminescence in the form of a light pulse. In this method, light is observed as nucleotides are added to the growing chain of DNA. Since the

sequencing reaction allows for detection of multiple bases in the vicinity of the SNP of interest, this allows for flexibility in primer positioning, thereby providing for ability to multiplex and accommodate difficult sequences.

1.3.3. Genetics of surfactant proteins in neonatal pulmonary disease

The multifactorial role of surfactant proteins in lung development, surfactant homeostasis and lung innate immunity, makes them good candidate genes for the study of neonatal respiratory disease. Polymorphisms of these proteins have been implicated in a number of adult and neonatal pulmonary disorders (Floros et al., 2001a; Floros et al., 2001b; Floros and Hoover, 1998; Floros and Kala, 1998; Floros et al., 2006; Guo et al., 2001; Lin et al., 2000b; Liu et al., 2003; Pavlovic et al., 2006; Seifart et al., 2005; Seifart et al., 2002; Selman et al., 2003).

Genetic studies of neonatal pulmonary disease and RDS in particular, have focused on genetic variants of SP-A and SP-B. A case-control study conducted by Floros et al. was the first to identify an association of the SP-B intron 4 polymorphism with RDS (Floros et al., 1995). Another case-control study by the same group confirmed the previous results and found increased frequency of the 1A⁰ variant of SP-A2 in a subgroup of RDS, as compared to the control group (Kala et al., 1998). In addition, the frequency of combined polymorphisms of SP-A2 (1A⁰) and SP-B (intron 4) was also increased in the RDS group, indicating a synergistic effect of two polymorphisms (Kala et al., 1998). However, neither deletion nor insertion variants of intron 4 were found to associate with RDS in the Finnish group (Haataja et al., 2000; Rova et al., 2004).

A number of case-control and family-based association studies have supported associations between SP-A and SP-B alleles and RDS (Floros et al., 2001a; Floros et al., 2001b; Floros et al., 2006; Floros et al., 1995; Haataja et al., 2001; Haataja et al., 2000; Kala et al., 1998; Marttila et al., 2003a; Ramet et al., 2000). These studies have been conducted in both heterogeneous African-American and various Caucasian populations and in a relatively homogeneous population of Finnish ancestry.

A family-based association study by Floros et al. indicated linkage between SP-A and RDS, by showing through transmission disequilibrium test (TDT) that haplotypes $1A^0$, $6A^2$, and $1A^0/6A^2$ had increased transmission from parents to the affected offspring, thus indicating that these are RDS susceptibility factors (Floros et al., 2001b). In the same study transmission of haplotypes $1A^5$, $6A^4$, and $1A^5/6A^4$, was decreased from parents to offspring, indicating that these are RDS protection factors. Furthermore, this study revealed possible race differences among RDS subpopulations. In black infants between 31 and 35 weeks (controls) of gestation the $6A^3$ variant was found more frequently than in the younger RDS affected babies, while white infants with RDS who received antenatal steroids had a higher frequency of the $6A^3/6A^3$ genotype. A family-based study of a Finnish population confirmed the association of the $1A^0$ and $6A^2$ variants of SP-A with RDS (Haataja et al., 2001). In a different study by the same Finnish group $1A^0$ and $6A^2$ variants were associated with increased risk of RDS, while variant $6A^3$ was associated with decreased risk of RDS. These associations were restricted to a subgroup of RDS babies born earlier than 32 weeks of gestation that were homozygous for SP-B SNP 1580(C/C) (Haataja et al., 2000). Overall, variants $1A^0$ of SP-A2 and $6A^2$ of SP-A1

have consistently been found to associate with susceptibility to disease (Floros et al., 2001a; Floros et al., 2001b; Floros et al., 2006; Floros et al., 1995; Haataja et al., 2001; Haataja et al., 2000; Kala et al., 1998; Marttila et al., 2003a; Ramet et al., 2000). These findings are interesting in light of the fact that the $1A^0$ variant has been found to associate with low to moderate SP-A mRNA levels in fetal and adult tissues (Karinch et al., 1997) and that RDS has been consistently associated with low levels of SP-A (deMello et al., 1993; deMello et al., 1989; Moya et al., 1994).

Recent studies of twins with RDS by the Finnish group indicate an association with $1A^0$ and $6A^2$ variants of SP-A opposite of that seen in singletons, where $1A^0$ and $6A^2$ variants are found more frequently in healthy premature twins compared to twins with RDS (Marttila et al., 2003a; Marttila et al., 2003b). The apparent discrepancy of twin studies compared to that in singletons has been attributed to the difference in the size of the uterus and the length of gestation at birth.

In addition to being associated with RDS (Floros et al., 2006), SP-B has recently been associated with BPD (Pavlovic et al., 2006; Rova et al., 2004). A case control study by Rova et al. has shown that SP-B intron 4 deletion variants are associated with BPD even when essential external confounding factors were included in the analysis (Rova et al., 2004). The study presented in chapter 4 of this thesis shows that SNP B-18_C of SP-B associated with susceptibility in the more severe subgroup of BPD (BPD at 36 weeks) (Pavlovic et al., 2006). Furthermore, variant analysis revealed ten susceptibility and one protective variant for SP-B and SP-B-linked microsatellite markers that associated with

BPD and two SP-A-SP-D haplotypes that associated with protection in the less severe group of BPD (BPD at 28 days) (Pavlovic et al., 2006).

In addition to association of SP-B polymorphisms with neonatal respiratory disease, rare mutations of SP-B have been identified as causes of fatal respiratory disease. As mentioned previously, Noguee et al. first identified deficiency of SP-B to be due to a frameshift mutation caused by a 2 base-pair insertion (121ins2) in exon 4 that results in a premature stop codon (Noguee et al., 1994). Overall, more than 22 distinct SP-B mutations that result in respiratory failure have been identified. These mutations are due to several basic molecular defects; insertions or deletions that result in premature stop codon (Ballard et al., 1995; Griese et al., 2005; Klein et al., 1998; Noguee et al., 1994; Noguee et al., 2000) or in alternatively spliced or aberrant mRNA (Lin et al., 2000b). While mutations of SP-B and mutations of SP-C (Noguee et al., 2001) are causes of hereditary fatal respiratory failure, no such mutations of SP-A and SP-D have been found so far.

1.3.4 Functional consequences of heterogeneity of surfactant protein genetic variants

Extensive genetic complexity of these genes holds the potential for both qualitative (functional) and quantitative (regulatory) differences among SP-A variants. Coding region differences that result in amino acid differences are likely to affect functional characteristics of the proteins, while differences in 5' UTR splice variants and 3'UTR sequence differences affect the regulation of these variants.

Functional and regulatory differences have been observed for SP-A variants, with respect to biochemical properties (Garcia-Verdugo et al., 2002; Wang et al., 2004), function of SP-A (Huang et al., 2004; Mikerov et al., 2005; Mikerov et al., 2007; Wang et al., 2000; Wang et al., 2002) and regulation of SP-A (Hoover and Floros, 1999; Hoover et al., 1999; Wang et al., 2003b; Wang et al., 2005). Furthermore, regulatory differences between genetic variants of SP-A may account for considerable variability in normal SP-A mRNA levels that have been seen among different adult individuals (Floros et al., 1991; Karinch et al., 1997; McCormick and Mendelson, 1994). For example, the 6A²6A²:1A⁰1A⁰ genotype of SP-A1:SP-A2 was first associated with low to moderate levels of SP-A mRNA in adult lungs of unrelated individuals (Karinch et al., 1997). Subsequent studies confirmed these findings in fetal lungs of unrelated individuals (Karinch et al., 1998). Moreover, given the functional differences observed between SP-A1 and SP-A2 in *in vitro* expressed variants (Huang et al., 2004; Mikerov et al., 2005; Mikerov et al., 2007; Wang et al., 2004; Wang et al., 2000; Wang et al., 2002), a varying SP-A1/SP-A2 ratio may contribute to the overall SP-A function. If the protein ratio of

SP-A1/SP-A2 follows the pattern observed at the mRNA level then it is possible that the same amount of total SP-A may differ in its activity if the ratio of SP-A1/SP-A2 differs. A recent study showed that the ratio of SP-A1 to total SP-A protein differed for example in individuals with cystic fibrosis.

In addition, homodimers, homotrimers, and/or homooligomers resulting from a single gene product may play a differential role in SP-A function in various tissues. Furthermore, the different sites of expression of the two proteins in the lung, where SP-A2 is expressed in both type II cells and tracheal and bronchial submucosal glands, but SP-A1 is expressed only in type I cells (Goss et al., 1998; Saitoh et al., 1998) might be another indication of differential function of these proteins. Recently, SP-A1 mRNA was detected in amniotic fetal membranes of pregnant women, while no SP-A2 message was detected in these tissues (Han et al., 2007). How the expression level of a particular gene and/or gene variants of surfactant proteins may influence neonatal disease, parturition, and pre-term labor is yet unknown.

1.4 References

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Chapter 2

Development and Characterization of a Human Fetal Lung Xenograft Model

2.1 Introduction

Development of a functional alveolar epithelium capable of gas exchange and surfactant secretion is essential for successful adaptation of the fetus to extra-uterine life. Because lung maturation is a late gestational event, respiratory distress is frequent in babies born prematurely. Premature infants are most commonly born during the sacular stage of lung development (26–34 wk of gestation) when lung pneumocytes are not yet completely differentiated. These infants often exhibit a deficiency of surfactant production that leads to insufficient gas exchange. Due primarily to a higher rate of multiple gestations, the overall number of premature live births (Mercer et al., 1999) has increased by 26% during the last three decades. This has led to a significant increase in neonatal respiratory disease, including Respiratory Distress Syndrome (RDS) and Bronchopulmonary Dysplasia (BPD) (Clark et al., 2001; Jobe and Ikegami, 2000).

Premature birth is commonly marked by immaturity of the gas exchange regions, poorly developed blood supply, and a deficiency and perturbation of the surfactant system. Pulmonary surfactant is a lipoprotein complex produced by type II pneumocytes that acts to reduce surface tension at the air-liquid interface in the alveolus and thereby, prevent atelectasis (Floros, 1997; Phelps, 2001). Surfactant proteins (SPs) may contribute to surfactant function (SP-A, SP-B, SP-C) or associate with the surfactant complex but not contribute to its function (SP-D). Each SP has multiple important roles within the alveolus (Floros and Hoover, 1998; Phelps, 2001; Weaver and Whitsett, 1989) and is subject to developmentally- and hormonally-regulated expression (Ballard, 1984;

Ballard et al., 1995; Dulkerian et al., 1996; Hoover et al., 1999; Karinch et al., 1998; Kumar and Snyder, 1998; Mendelson et al., 1991; Mendelson and Boggaram, 1991; Solarin et al., 1997; Wang et al., 2003). SP production starts early in development (pseudoglandular stage), but active secretion of functional surfactant (predominantly in the form of lamellar bodies) is initiated during the sacular stage of development, after almost 75% of gestation is completed (Ballard, 1984).

While lung development has been extensively studied in multiple models involving different animal species, studies of human lung development have primarily been limited to fetal lung explants (Dulkerian et al., 1996; Karinch et al., 1998; Kumar and Snyder, 1998; Mendelson et al., 1991; Mendelson and Boggaram, 1991; Solarin et al., 1997). Although the importance of the fetal lung explant model is undisputed, the highly accelerated morphological development and limited viability of explants in cell culture (on the order of days) limits their use in studies of cellular and molecular changes specific for particular stages of lung development and studies over longer periods of time.

Models of organ development have been developed using immunocompromised mice as hosts for grafted fetal tissue (Cobb, 1975; Phillips and Gazet, 1969). Various xenograft models have been used extensively in cancer research to study mechanisms of carcinogenesis and the effects of different pharmacological agents on human tumor growth (Giovannella and Fogh, 1985; Malkinson, 2001), as well as in studies of transmission of infection in various tissues by pathogens (Howett et al., 1997; Howett et al., 1990; Howett et al., 1999; Kish et al., 2001; Kreider et al., 1985). With respect to lung embryogenesis, immunocompromised mice have been used to study whole organ lung development in mice (Schwarz et al., 2000; Vu et al., 2003), as well as lower

(Groscurth and Tondury, 1982; Peault et al., 1994) and upper airway development in humans (Delplanque et al., 2000; Deutsch G., 1997; Engelhardt et al., 1993; Filali et al., 2002; Pilewski et al., 1994; Puchelle and Peault, 2000). Allograft models have been used where whole embryonic mouse lungs were grafted into immunodeficient mice (Schwarz et al., 2000). Progression of structural development as well as appearance of mature alveoli was observed 14 days after grafting. It has also been observed that (Phillips and Gazet, 1969) human fetal lung tissues grafted into a mouse treated with antilymphocytic serum (ALS, lymphocyte suppressing antibodies) could develop and grow in the host for up to 90 days. Others observed differentiation of lung-specific cell types in two, five, and eight week old transplant grafts (Groscurth and Tondury, 1982). More recently, a number of studies involving xenograft models of human upper airway development have been reported (Delplanque et al., 2000; Puchelle and Peault, 2000). The focus in these studies was on the development of bronchial xenografts to study the pathogenesis of cystic fibrosis (Engelhardt et al., 1993; Filali et al., 2002). Although these studies have clearly shown that human embryonic lung tissue can be successfully differentiated in immunocompromised mice, the focus was on the endpoint of tissue development, rather than on the characterization of developmental processes.

Our goal was to characterize a model for the study of successive stages of development of the human lower airways, with particular emphasis on the expression of surfactant components. Based on the knowledge available from a number of lung xenograft models (Cobb, 1975; Delplanque et al., 2000; Deutsch G., 1997; Engelhardt et al., 1993; Filali et al., 2002; Groscurth and Tondury, 1982; Peault et al., 1994; Phillips and Gazet, 1969; Pilewski et al., 1994; Puchelle and Peault, 2000; Schwarz et al., 2000;

Vu et al., 2003), we hypothesized that human fetal lung parenchyma grafted into immunocompromised mice will undergo changes that mimic the stages of *in utero* lung development in many respects. The assumption was that although grafted tissue may undergo accelerated maturation, this acceleration would not be as rapid as that seen in fetal lung explants, enabling one to study stage-specific processes of lung development. In the present study, we characterized these stage-specific processes with respect to the progressive cytodifferentiation of a model of human fetal lower airway development.

2.2 Material and Methods

2.2.1 Fetal lung xenografts

Six to fourteen week-old female NCr-nu (nude) (Taconic Farms, Germantown, NY) were used for both renal capsular and subcutaneous grafts. Animals were maintained under pathogen-free conditions and were fed sterilized rodent Purina 5K52 diet. Research involving all animals followed the "Guiding Principles in the Care and Use of Animals" by the Council of the American Physiological Society and was approved by The Penn State College of Medicine Institutional Animal Care and Use Committee. Human fetal lung tissues (n=5), ranging from 13 to 17 weeks of gestation, were obtained according to the protocol approved by the Penn State College of Medicine Institutional Review Board. Fetal age was obtained from clinical information and confirmed by fetal foot-length measurements. To ensure that graft tissue was derived from fetal lung, only

whole lung lobes were used for preparation of xenograft tissue. Fresh lower airways were cut under sterile conditions into 1-3 mm³ pieces. Surgery was performed on nude mice anesthetized by intraperitoneal injection of 100 mg/kg Ketaset and 5 mg/kg Xylazine. Human fetal lung tissue was placed either under the dorsal fold of skin or beneath the renal capsule of mice. Each mouse received only one type of graft. Human fetal lung tissue was placed either under the dorsal fold of skin (four pieces) or beneath the renal capsule of each mouse (one piece). The subcutaneous grafts were planted both in the anterior and posterior portions of a given mouse's back. Xenografts were harvested 3-42 days after grafting. A schematic drawing of the experimental approach is presented in Fig. 2.1. Mice were anesthetized with halothane and sacrificed by cervical dislocation.

2.2.2 Histology/Light microscopy

Tissue harvested at 3, 6, 10, 14, 21, 28, 35 and 42 days after grafting was rapidly removed from under the mouse skin or kidney capsule, fixed in neutral buffered formalin fixed and paraffin embedded. Tissues were cut into 4 µm thick sections and stained with hematoxylin and eosin. Additionally, selected tissues were stained with Verhoff's-van Gieson's stain for collagen and elastic fibers (Tassler et al., 1994) for morphological examination. Using light microscopy, tissue sections were examined for morphological characteristics of lung development. All tissues were examined independently and together by at least two researchers, who assigned the developmental stage to each section. The sections were viewed using a Nikon Eclipse E600 microscope and recorded using either a Spot digital camera (Diagnostic Instruments, Inc.) and Image Pro Plus

version 3.0 software or a Nikon Digital Camera DXM 1200 and Nikon, ACT1 version 2 software. Microscope magnification is 50x, to this is added magnification of individual lens, as stated in the figures.

Elastic fiber staining: Verhoff's-van Gieson's staining was performed as previously described (Tassler et al., 1994).

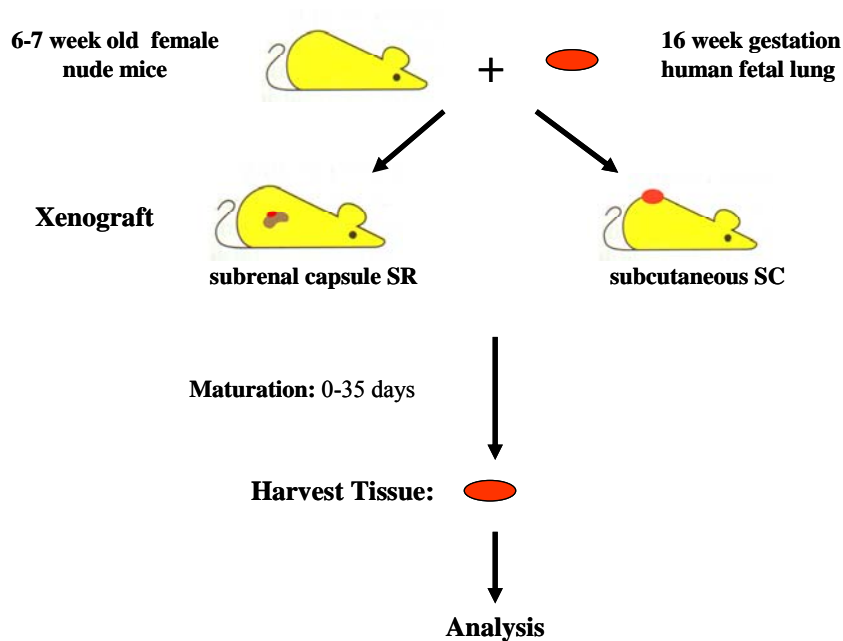


Figure 2.1: Schematic drawing of renal subcapsular and subcutaneous grafting

2.2.3 Electron microscopy

Xenograft tissues were fixed for 3 hr in 2% glutaraldehyde in 85 mM cacodylate buffer at room temperature, and post-fixed overnight in buffered osmium tetroxide (1%) at 4°C. Following dehydration in graded ethanol, the tissue was infiltrated with propylene oxide and EMBED 812. After an overnight rotation in propylene oxide: EMBED 812 (1:1), tissues were rotated and embedded in 100% EMBED 812. Semi-thin sections were stained with toluidine blue and examined under a light microscope. If the sections appeared satisfactory by light microscopy, blocks were mounted and sectioned on a Sorvall MT2-B ultramicrotome at 0.08 μm (800Å) in preparation for electron microscopy. These ultra thin sections were mounted on square 200 mesh copper grids, stained with uranyl acetate and lead citrate and examined with a Philips 400 transmission electron microscope.

2.2.4 Immunocytochemical Analyses (ICC)

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and stained for the presence of human SPs using the following rabbit polyclonal antibodies: anti-SP-A (1:250) (Phelps and Floros, 1991), anti-SP-B (1:250, Chemicon, Temecula, CA), anti-pro-SP-C (1:100, Chemicon), and anti-SP-D (1: 500, Chemicon). A low-temperature antigen retrieval procedure (Brown, 1998) was applied to deparaffinized and rehydrated tissue sections using 0.1 mol/L citrate buffer, pH 6.0, for 1 hr at 80°C followed by dehydration and rehydration by incubation in xylene and graded ethanol series. Endogenous alkaline phosphatase was inhibited by immersing the slides in 0.2 N HCl for

5 min. Samples were blocked with 15% normal goat serum (Vector Laboratories, Burlingame, CA) in 1X PBS and 0.3% TX-100. Following overnight incubation at 4°C with each selected primary antibody, tissue sections were incubated with biotinylated goat anti-rabbit secondary antibody for 1 hour at room temperature. All subsequent steps were performed at room temperature. The signal from the secondary antibody was visualized using Vectastain ABC with alkaline phosphatase as the reporter, and Vector Red Alkaline Phosphatase Substrate Kit I reagent (Vector Laboratories, Burlingame, CA) as the chromogen.

For ICC with anti-human antibodies to proliferating cell nuclear antigen (PCNA) and endothelial cell markers PECAM-1 (same as CD31), Mouse on Mouse DAKO Ark kit (DAKOCytomation, Carpinteria, CA) was used as per manufacturer's instructions. Both primary antibodies (anti-human PCNA and anti-human CD31) were mouse monoclonal antibodies that are specific for the human antigen. The primary antibodies (PCNA 1:50, and CD31 1:20, DAKOCytomation) were pre-incubated with the secondary antibody in equimolar amounts in order to avoid the staining of mouse immunoglobulin within the human grafts with the secondary anti-mouse IgG antibody. Control mouse tissues were negative for PCNA and CD31 staining using this protocol (not shown). Images of tissue sections were captured as described above.

2.2.5 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from snap-frozen, non-fixed fetal lung tissue, graft tissue, and adult human lung tissue (obtained from pneumonectomies performed at Hershey Medical Center), using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Briefly, tissue was first disrupted using a hand held mortar and pestle, homogenized in Qiashtredder, and processed as described by the manufacturer. Extracted RNA (1 μ g of each sample) was DNase I treated and reverse transcribed using a gene-specific oligonucleotide (oligo) for each surfactant protein (see below). The RT procedure was performed as described previously (Karinch and Floros, 1995). RNA from human spleen tissue (Ambion, Austin, TX) was used as the negative control. The orientation, gene specificity, and nucleotide position of each oligo used in the present study are shown in Table 1. For SP-A, RT was performed using oligo 68A, followed by the first amplification with oligos 780/781, and 1 μ l of this template was used for a nested reaction with primers 1321/781 resulting in a 100 bp long template. For SP-B, oligo 110 was used for RT, followed by a first cycle of PCR with oligos 70A/603, followed by nested PCR with oligos 172/189 to create a 51bp template. RT for SP-C was performed using oligo 86A, oligos 1108/1106A were used in the first round of PCR, while oligos 1108/1116 were used in the nested reaction producing a 142 bp long amplicon. Finally, oligo 961 was used for SP-D RT, followed by first round of PCR with oligos 825/925, and nested PCR with oligos 915/925 resulting in a 291 bp long template. All PCR reactions were performed using 4 μ g cDNA from the gene-specific RT reaction in final concentrations of 0.5 X buffer 1 (Roche, Indianapolis, IN) and 0.5 X buffer 2 (Roche), 2 ng of each primer, 100 μ M of each dNTP and 0.15 μ l

of Taq Polymerase (Roche). The PCR profile was as follows: one cycle of 95°C for 2 min, followed by 35 cycles of: 95°C for 30 seconds, 58°C for 1 min, and 72°C for 1 min, this was followed by one cycle of 72°C for 5 min. DNA fragments from the first PCR reaction were used for nested PCR, with the same PCR profile. Amplified SP gene-specific fragments were then resolved on 8% PAGE gels, and specific bands visualized.

Table 2.1: Primers used in the study

Oligo number	orientation	Gene specificity	Sequence from 5' to 3'	Nucleotide position
780	S	SP-A	GACGTTTGTGTTGGAAGCCCTGG	White et al.: 3603-3625 (White et al., 1985)
68A	AS (RT)	SP-A	TGCCACAGAGACCTCAGAGT	White et al.: 6333-6352 (White et al., 1985)
781	AS	SP-A	GGTACCAGTTGGTGTAGTTCACAG	White et al.: 5688-5711 (White et al., 1985)
1321	S	SP-A	AAGTACAACACATATGCCTAT	White et al.: 5612-5632 (White et al., 1985)
110	AS (RT)	SP-B	GAATTCACCAGTGGAAAGTGACTCCCGAGG	Pilot-Matias et al.: 9685-9715 (Pilot-Matias et al., 1989)
603	AS	SP-B	TGGCTGCCGGGATTTGCACA	Pilot-Matias et al.: 3478-3497 (Pilot-Matias et al., 1989)
70A	S	SP-B	CAAAGCCTGGAGCAAGCATT	Pilot-Matias et al.: 1484-1503 (Pilot-Matias et al., 1989)
172	S	SP-B	CTGGTCATCGACTACTTCCA	Pilot-Matias et al.: 2591-2610 (Pilot-Matias et al., 1989)
189	AS	SP-B	CATACAGATGCCGTTTGAGTC	Pilot-Matias et al.: 3447-3467 (Pilot-Matias et al., 1989)
86A	AS (RT)	SP-C	CGCGGATCCACAGAGGGCGAATGGAAA	Glasser et al.: 3327-3353 (Glasser et al., 1988)
1116	AS	SP-C	GCACCTCGCCACACAGGGAG	Glasser et al.: 2773-2792 (Glasser et al., 1988)
1108	S	SP-C	GATGGAATGCTCTCTGCAGG	Glasser et al.: 2650-2669 (Glasser et al., 1988)
1106A	AS	SP-C	AAATCAGGCTGCTTTATTCTG	Glasser et al.: 3210-3230 (Glasser et al., 1988)
915	S	SP-D	AGGAGCTGCAGGGCAAGCAG	Rust et al.: 843-862 (Rust et al., 1991)
825	S	SP-D	CTGGAAGCAGAAATGAAGAC	Rust et al.: 262-281 (Rust et al., 1991)
925	AS	SP-D	TCCCTTAGGGCCTGCGAGGC	Rust et al.: 1690-1709 (Rust et al., 1991)
961	AS (RT)	SP-D	GAGCTATACCACACTGGCTAAG	Rust et al.: 4473-4494 (Rust et al., 1991)

Legend: sense (S), antisense (AS); reverse transcription (RT)

2.3 Results

2.3.1 Both renal subcapsular and subcutaneous sites of graftment support graft growth and development

Human fetal lung tissue from fetuses of 13 to 17 weeks of gestation was grafted underneath the renal capsule (subcapsular) or subcutaneously in nude mice and harvested after 3, 6, 10, 14, 21, 28, 35 and 42 days. Each mouse received only one type of graft.

Xenografts established at both transplant sites underwent lung development to the saccular stage (Fig. 2.2. D). Subcutaneous grafts underwent a substantial macroscopic growth with increased time of transplantation, at times reaching a volume of 4-5 mm³. The increase in macroscopic size of subcapsular grafts was less pronounced, presumably due to the lack of space to grow (Fig. 2.2. II and Fig. 2.2. I, respectively). Subcutaneous xenografts from the anterior part of the mouse back appeared to develop faster and initiate morphological changes by day three post-grafting, whereas such changes in the posterior grafts were observed at day six post-grafting. By six days post-grafting, the developmental progression of both anterior and posterior dorsal subcutaneous xenografts was equivalent to that of subcapsular grafts (Fig. 2.2. CI and CII). Limiting the initial size of subcutaneous grafts to about 1 mm³ minimized the lack of uniformity in tissue differentiation observed in larger grafts (not shown).

To ensure that a sufficient quantity of xenograft tissue was available for post-grafting analysis, three to four 1 mm³ pieces were placed into each subcutaneous pocket on the back of the nude mouse. These were generally found to be fused together 2-4 weeks after grafting, contributing to the larger size of subcutaneous grafts. Our overall

observations indicate that both subcutaneous and subcapsular grafts represent adequate models of human fetal lung tissue development (Fig. 2.2. I and Fig. 2.2. II). However, subcutaneous grafts (Fig. 2.2. II) have the advantage of greater ease of engraftment, better access to graft tissue, and larger size of grafts.

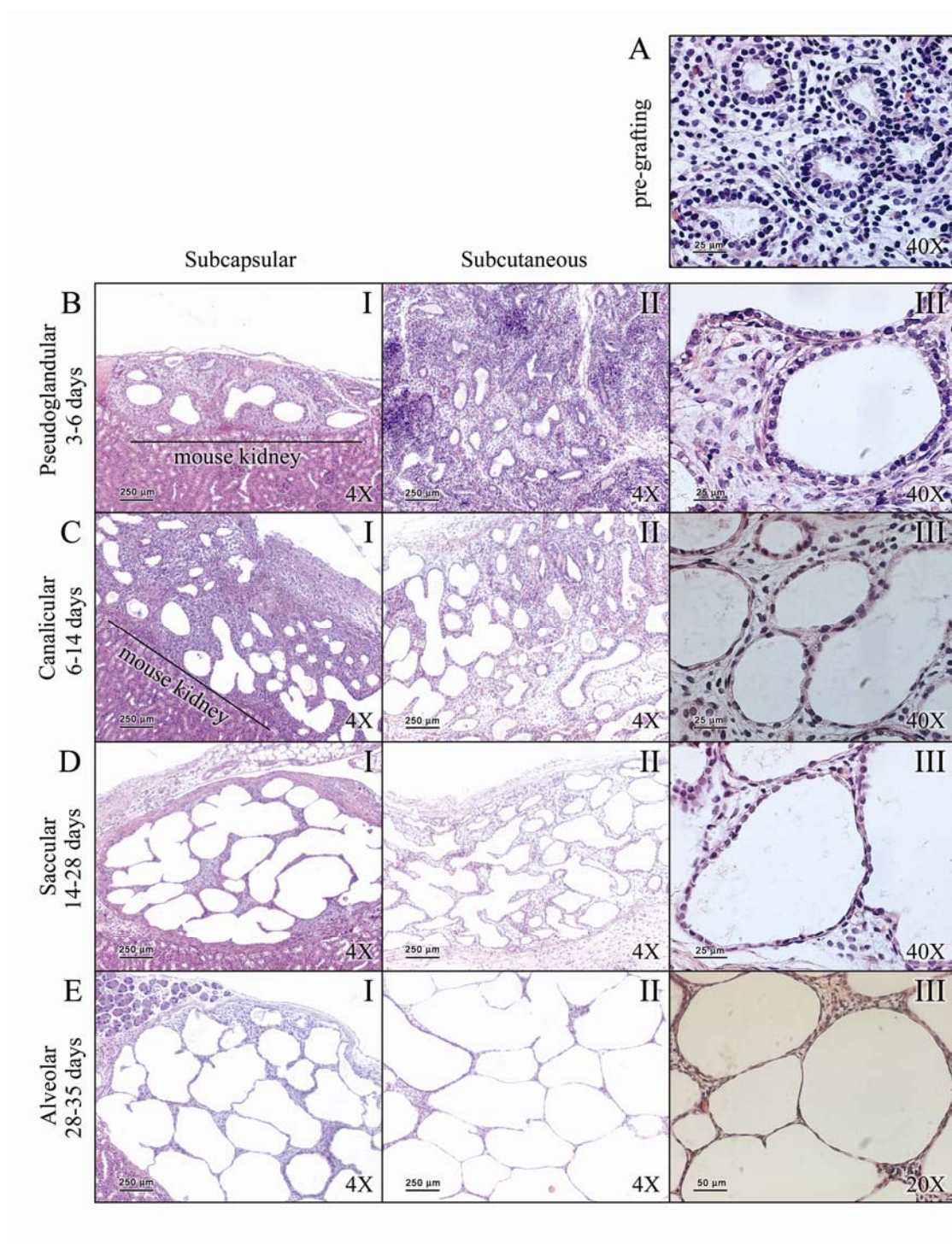


Figure 2.2: Both subcapsular and subcutaneous grafts recapitulate the sequential

stages of *in utero* lung development

A: Starting fetal lung tissue (16 weeks gestation). Panel B: Epithelial thinning and future air space enlargement seen after 3 days of grafting. Panel C: Grafts between 6 and 14 days show progression of differentiation. Panel D: Post 14 day grafts with flattening of epithelium. Panel E: Distended saccules with flattened epithelium are observed after 28 days of grafting.

Panel A is characteristic of pseudoglandular stage of development. Changes in Panel B are consistent with the late pseudoglandular stage. In Panel C grafts resemble the canalicular stage of development, forming acini and increased number of capillaries. In Panel D grafts resemble saccular stage of *in utero* development display flattening of the epithelium, and differentiation of pre-type II cells into type II cells, and further differentiation onto type I cells. Alveolar spaces are considerably enlarged, and prominent alveolar septation is evident. In Panel E, differentiation of the epithelium to type I cells, thinning of alveolar walls, and neovascularization are observed. Legend: I: low magnification (4x) of subcapsular grafts; II: low magnification (4x) of subcutaneous grafts; III: high magnification (A, B, C, and D at 40X; E at 20x) of subcutaneous grafts.

2.3.2 Human fetal lung grafts mimic sequential stages of lung development *in utero*

The starting tissue for both subcapsular and subcutaneous grafts was at the pseudoglandular stage of development (Fig. 2.2. A). The progression of tissue grafts through subsequent stages of lung development was observed at different time points post-grafting. The pseudoglandular stage was characterized by the formation of conducting airways that were lined by cuboidal or low columnar epithelium (Fig. 2.2. B). There is a limited number of proliferating cells at this stage of development, as indicated by proliferating cell nuclear antigen (PCNA) immunoreactivity (Fig. 2.3. B). Staining for collagen and elastic fibers with Verhoff's-van Gieson's stain, shows a collagen-rich tissue lacking elastic fibers (Fig. 2.4. A).

After 3 days of grafting small increases in future alveolar spaces were observed as well as thinning of the epithelium, a change that represents the late pseudoglandular phase (Fig. 2.2. B.III). Grafts harvested after 6 and 10 days of engraftment resemble the canalicular stage of lung development; six day old xenografts resemble the early canalicular stage, and 10 day old grafts had characteristics of late canalicular phase (Fig. 2.2. C). During the late canalicular phase of lung development, lung acini were formed (Fig. 2.2. C.III), and the numbers of capillaries visible in the mesoderm increased. The latter process was clearly visible by light microscopy, but not readily apparent in the corresponding photographic images.

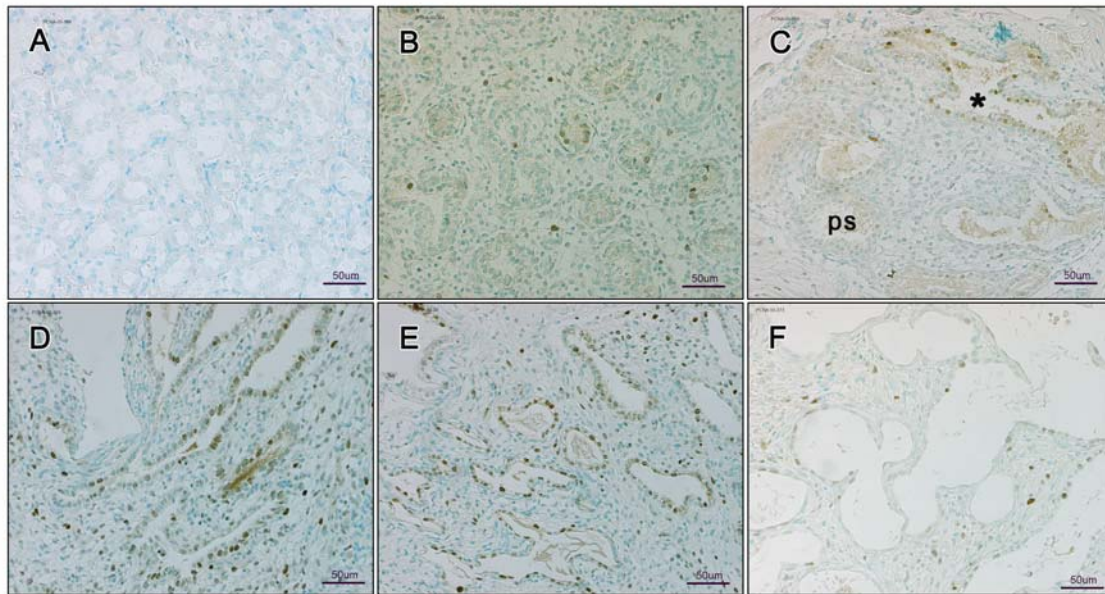


Figure 2.3: Cellular proliferation is present throughout graft development as shown by immunocytochemistry (ICC) of the proliferating cell nuclear antigen (PCNA)

Panel A: negative control - mouse kidney of subcapsular graft. Panel B: limited PCNA immunopositive cells at 16 weeks development. Panel C: 6 day graft with majority of PCNA immunopositive cells in cannalicular stage of development (marked with *). Panel D and Panel E: 10 and 14 day grafts, respectively, abundant with PCNA immunopositive cells. Panel F: 21 day graft. In Panel A there is no staining within mouse tissue section. No brown cells are detectable indicating complete inhibition of endogenous peroxidase and lack of specific PCNA staining. Therefore, the PCNA positive cells in the other sections are of human and not mouse origin. In Panel C, PCNA immunopositive cells were observed within the tissue sections entering the cannalicular stage of development (marked with *), rather than those still in pseudoglandular stage (marked ps). In Panel D and E changes in the morphology of tissue are represented by the increasing number of PCNA immunopositive cells. Panel F shows a 21 day graft entering the saccular stage of development with sparsely distributed PCNA positive cells.

Pseudoglandular and cannalicular stages are defined in Figure legend 1. Figures are representative of three experiments. All pictures are at 20x magnification.

Appearance of early primary saccules was observed in 10 day grafts. This process was marked by a dramatic increase in PCNA positive cells within the saccular spaces (Fig. 2.3. D). Alveolar sacs in these grafts were lined by flattened cuboidal epithelium and closely resembled the morphological appearance of a 20+ week old human fetus (Copland and Post, 2004).

Fetal lung grafts harvested 14 and 21 days post-grafting resemble the early and late saccular stage, respectively. In these grafts, the epithelium was flattened, representing the differentiation of pre-type II cuboidal cells from the canalicular phase into maturing type II cells, and further differentiation of the type II cells into putative type I pneumocytes (Fig. 2.2. D.III). Cellular proliferation was particularly evident in 14 day grafts based on PCNA immunostaining (Fig. 2.3. E), while the 21 day grafts had very few PCNA immunopositive cells (Fig. 2.3. F) indicating terminal differentiation of the tissue. As a result of cellular differentiation and the reduction of interstitial tissue, alveolar crests were formed and divided primary saccules into subsaccules or primitive alveoli (Fig. 2.2. D.I and 2.2. D.II). Elastic fiber deposition was seen in 14 day grafts as punctate dots at tips of developing alveolar crests (Fig. 2.4. B). Elastic fibers were not apparent in all graft tissues (not shown) and were scarce in representative sections of 21 day grafts (not shown). Notably, there were no elastic fibers seen in 28 or 35 day old grafts. Ciliated and non-ciliated cells were observed in the conducting airways in these grafts. Moreover, a significant increase in the macroscopic growth of graft tissue was readily observed after 14 days of engraftment.

Human fetal lungs transplanted for longer than 28 days (up to 42 days) (Fig. 2.2. E.I and E.II) had thin walls, with limited amounts of interstitium (Fig. 2.2. E.III), suggesting their progression past the saccular stage of lung development. The airspaces were significantly enlarged compared to earlier graft time points, and were lined with flattened epithelium, presumably consisting of putative type I cells (Fig. 2.2. E.III). Although the grafts developed in a limited space particularly in the case of renal subcapsular grafts, alveolar spaces were never collapsed. The alveolar spaces were always opened and filled with liquid, presumably fluid secreted by the lung tissue.

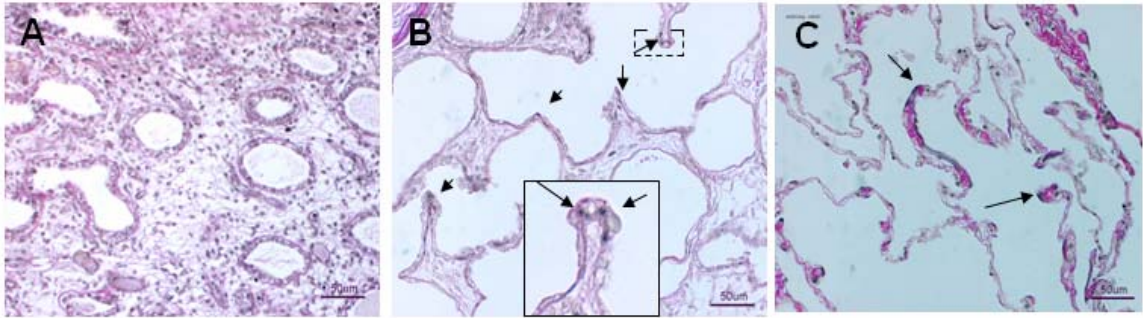


Figure 2.4: Elastic fiber proliferation is detectable in the saccular stage of development

Panel A: 6 day graft lacking elastic fiber staining as would be expected in pseudoglandular/cannalicular stage of development. Panel B: 14 day graft with elastic fibers visible as black punctate dots (arrows) at the tips of developing alveolar crests. Panel C: adult lung tissue (positive control) has an abundance of layered elastic fibers within the thin alveolar walls (arrows). The inset in panel B (dotted line) shows a high magnification (40x) of an alveolar crest with elastic staining (arrows). Hardly any staining was observed in 21 day graft (not shown). There is an abundance of collagen fibers in all tissues (red). Figures are representative of three experiments. Panels A, B, and C are at 20x magnification.

2.3.3 Lamellar bodies are present in graft tissue, consistent with *in utero* lung development

Based on light microscopy (Fig. 2.2.), we conclude that the grafts follow time dependent structural and morphological development. To determine whether these structural changes were also complemented by specific cellular and biochemical changes, grafts were examined by EM for the presence of lamellar bodies and surfactant proteins. The starting graft tissue in the pseudoglandular stage of development did not contain lamellar bodies, but had abundant glycogen stores, as indicated by the dense gray areas (arrow, Fig. 2.5. A2). As the glycogen stores started to diminish, lamellar bodies were detected in 14 day grafts. At the same time, morphological differentiation of the epithelium into mature type II pneumocytes was observed. Fig. 2.5. B2 shows lamellar bodies (long arrow) and the apparent beginnings of microvilli (thick arrow) at the apical cell surface in the 14 day grafts, while Fig. 2.5. C2 shows a significant increase in the number of lamellar bodies per cell (long arrow, Fig. 2.5. C2), and an abundance of microvilli on the apical membrane in the 21 day graft. Note that even though Fig. 2.5. C1 is at lower magnification than Fig. 2.5. B1, the abundant microvilli are readily detectable (area marked by a dotted line). Numerous secreted lamellar bodies and some unraveling of lamellar bodies were observed in the saccular spaces, but no tubular myelin structures (extracellular structural forms of surfactant) were observed in graft tissue. Overall, these images closely resemble the progression of pneumocyte differentiation during *in utero* lung development.

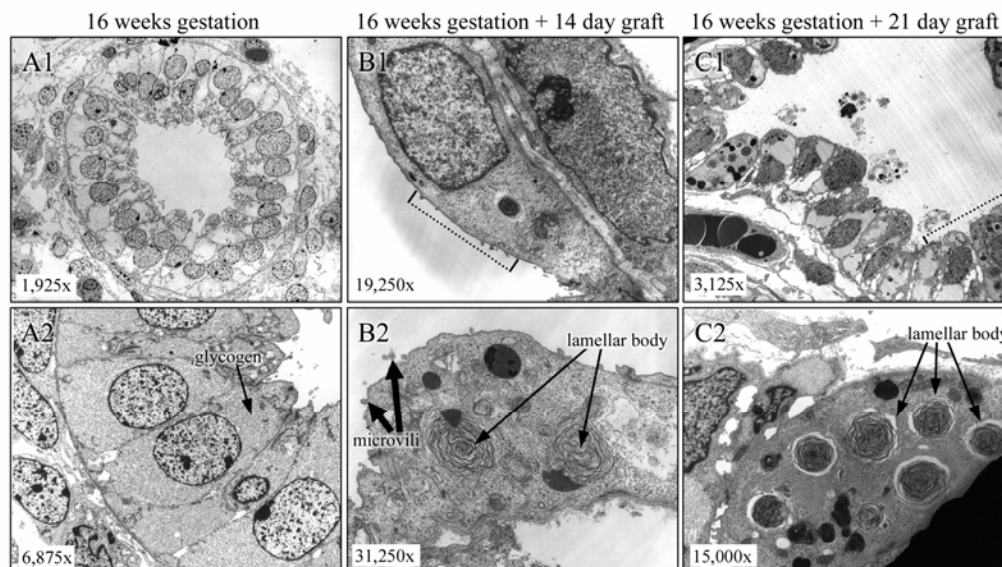


Figure 2.5: Lamellar bodies are present in graft tissues, consistent with *in utero* development

Panel A1 and A2: Lamellar bodies are absent in starting fetal lung tissue. Panel B1 and B2: Lamellar bodies are first seen in 14 day grafts. Panel C1 and C2: 21 days post-grafting, the number of lamellar body positive cells and lamellar bodies per cell is sharply increased. In Panel A, pre-type II columnar cells are rich with glycogen. As shown in Panel B, 14 day grafts are in early saccular stage of development, as evidenced by flattening of epithelial cells and scarce microvilli. In Panel C, numerous secreted lamellar bodies are seen in the alveolar spaces, but no tubular myelin-like structures are observed.

2.3.4 Surfactant proteins are present in graft tissue, consistent with *in utero* lung development

mRNA for all four proteins was present in the starting fetal lung at 16 weeks of gestation and message persisted during the graft development (not shown). However, adequate quantification of mRNA across the developmental stages could not be done due to the heterogeneity and sampling of the xenograft tissue and the fact that RT-PCR does not provide a quantitative measure. In order to study the pattern of SP expression throughout graft development, all time points of graft transplantation were examined for the presence of SPs by ICC. Starting fetal tissue was positive for all four surfactant proteins as assessed by ICC using a specific antibody for each surfactant protein as shown in Figure 2.6. Pre-type II cells of early graft time points (3, 6, 10, and 14 day grafts) showed a presence of all four surfactant proteins. Fig. 2.7 shows 21 day grafts for all of the proteins.

Localization of SP-D was limited to the apical surface of cells, whereas SP-A, SP-B, and SP-C displayed more uniform patterns of distribution within the cell (Fig. 2.6 and Fig. 2.7 high mag. insert). It is of interest that on one occasion, ciliated cells of conducting airways in addition to type II pneumocytes were also found to express SP-D by ICC. Although these cells were immunonegative for other SPs, a number of other cells of unknown type were seen to be positive for all four SPs as determined by ICC. Moreover, post-translational processing and modification of the primary translation products to more mature and multimeric forms of SP-A or SP-D could not be studied due to the limited amount of tissue available.

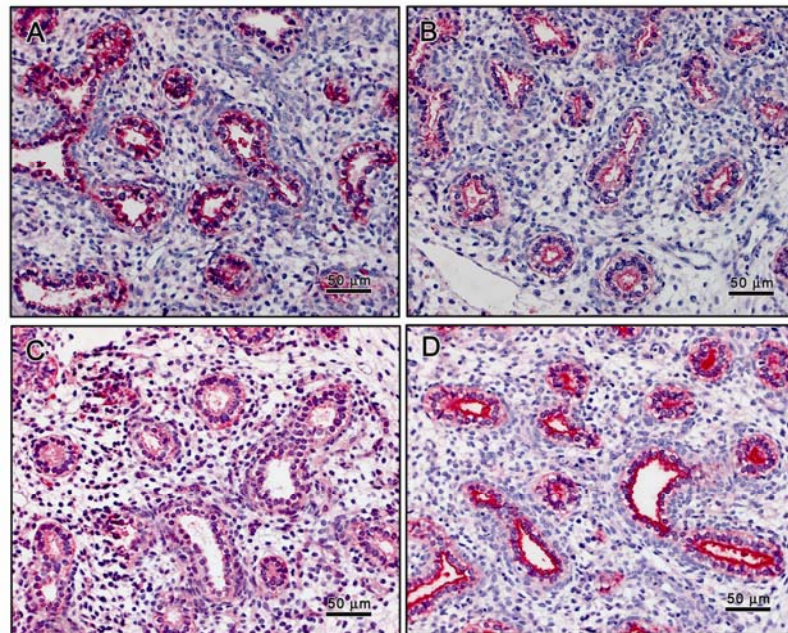


Figure 2.6: Surfactant proteins are present in starting graft tissue as shown by immunocytochemistry (ICC)

SPs are abundant in starting graft tissues at pseudoglandular stage of development. The primordia of alveoli are lined by cuboidal pre-type II cells that are immunopositive for SPs. Localization of SP-A (Panel A), SP-B (Panel B), and SP-C (Panel C) is uniform within the cells. Localization of SP-D (Panel D) is limited to the apical surface of the cells. Panels are shown at medium magnification (20x).

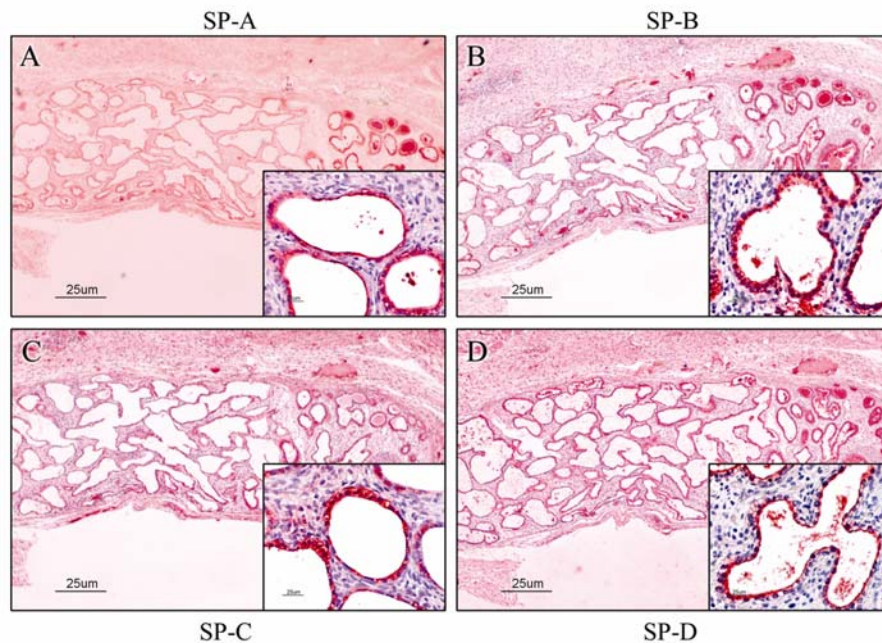


Figure 2.7: Surfactant proteins are present during graft development as shown by immunocytochemistry (ICC)

Surfactant proteins are abundant in 21 day grafts shown. Panel A: immunostaining for SP-A; Panel B: immunostaining for SP-B; Panel C: immunostaining for SP-C; and Panel D: immunostaining for SP-D. Localization of SP-A, SP-B, and SP-C (insert) is uniform within the cell. Localization of SP-D is limited to the apical surface of the cells (insert). After longer periods of grafting, cytodifferentiation is observed and fewer cells found to stain positive for surfactant proteins. This would be expected in a developing epithelium, where immunopositive pre-type II cells are differentiating into type II cells (immunopositive) which can then become type I cells (immunonegative) (not shown). Panels are shown in low magnification (4x) and inserts are in high magnification (40x).

We speculate that these cells are in intermediary stages of development of known and readily identifiable lung cell types. As cytodifferentiation progressed, SP-positive pre-type II cells differentiated into type II cells that further differentiated into putative type I cells and the number of SP-A, SP-B, SP-C, and SP-D positive cells per terminal airway decreased. Figure 2.8 depicts SP-A ICC at various graft time points (6, 10, 14, and 28 day grafts).

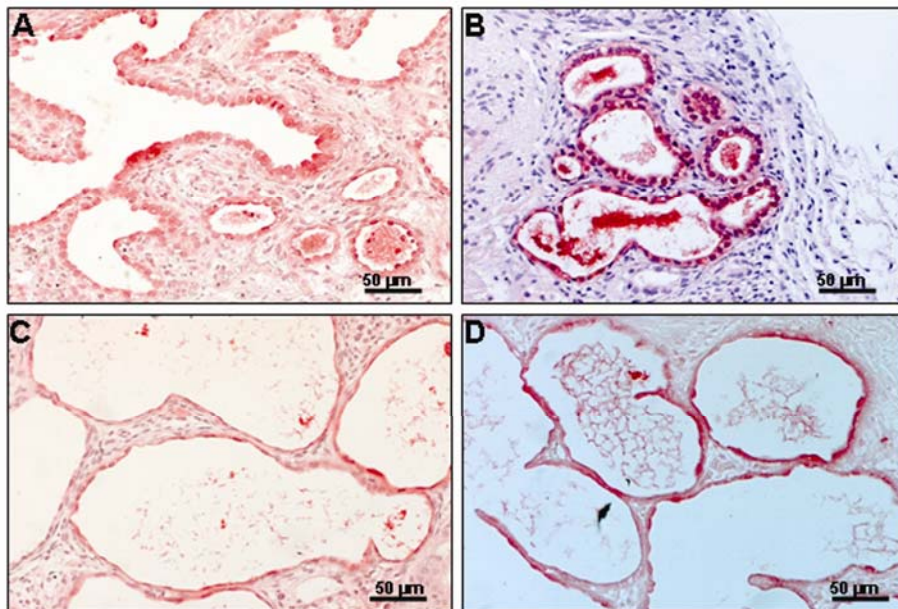


Figure 2.8: SP-A is present throughout graft development by ICC

Panel A: 6 day graft with pre-type II cuboidal cells immunopositive for SP-A. Panel B: 10 day graft with thinning epithelial cells predominantly SP-A immunopositive. Panel C: 14 day graft with saccular-like structures and differentiation of pre-type II into type II cells (both SP-A immunopositive). Panel D: 28 day graft with thinning saccules and septae. Panels shown at medium magnification (20x).

2.3.5 Blood vessels are present in the grafts as shown by ICC for human PECAM-1

In order to assess the vascularization of the subcutaneous grafts and origin of blood supply within the grafts, sections were immunostained with endothelial cell marker PECAM-1 specific for human antigen. In 3 day grafts, a network of blood vessels immunopositive for PECAM-1 was observed within the mesenchyme as shown in Fig. **2.9.A** (arrows). With the progression of tissue differentiation, thinning and branching of the PECAM-1 immunopositive vessels (arrowhead) was seen in the 6 day (Fig. **2.9.B**) graft. PECAM-1 staining was considerably decreased in all other graft time points. By 14 days staining was predominantly visible in the subpleural areas, as shown in Fig. **2.9.C**. There was undetectable or very little human PECAM-1 positive staining visible in the interstitium of later graft time points.

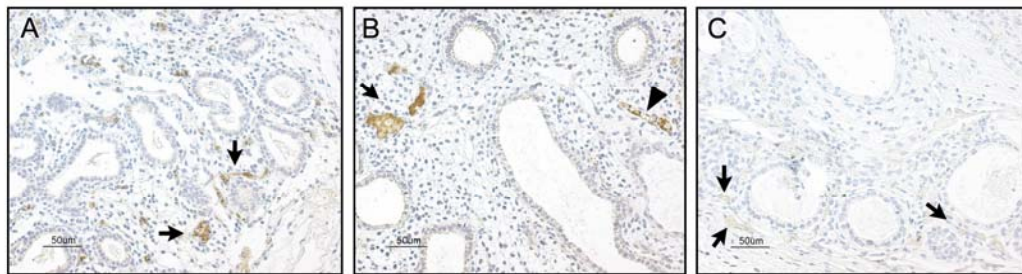


Figure 2.9: Human PECAM-1 is detectable by immunocytochemistry in early stage grafts

Panel A: Presence of blood vessels of human origin within the mesenchyme of a 3 day graft is indicated by PECAM-1 immunopositive cells (brown, arrows). Panel B: Branching of the PECAM-1 immunopositive clusters of cells (arrow) and formation of blood vessels (arrowhead) is evident in 6 day graft. Panel C: As differentiation progresses PECAM-1 staining becomes significantly fainter and mostly visible in the subpleural areas of the 14 day graft (as indicated by arrows). All panels are shown at medium magnification (20x).

Together, these observations indicate that human fetal lower airways grafted under renal capsule and subcutaneous tissues of nude mice can provide a model for study of human fetal lung development.

2.4 Discussion

Although a number of experimental models for the study of human fetal lung development are available, we undertook the current study to establish and characterize a model that could parallel normal intrauterine lung development in a time course more amenable to the study of stage-specific lung development. The goal of our study was to extend the characterization of currently available models and characterize a human fetal lung xenograft model, where successive stages of lung development could be studied with respect to morphological changes characteristic of cellular differentiation and the expression of the surfactant system. We observed that fetal lungs grafted under the renal capsule or subcutaneously in nude mice undergo maturation and differentiation where SPs and lamellar bodies are produced in a cell- and developmental stage-specific manner.

Overall macroscopic growth was observed in both types of grafts with increasing engraftment time, and this was particularly pronounced in subcutaneous grafts. Given the size restrictions of graft tissue, measurements of weight for comparisons of dry and wet tissue weights were impractical, although larger grafts were noted to have more lung fluid by gross observation. A considerable increase in the size of grafts after longer grafting times has been observed in both renal subcapsular (Vu et al., 2003) and subcutaneous (Schwarz et al., 2000) mouse fetal lung allograft models, although a comparison of these two engraftment sites has not been done previously. In the present study, initial transplantation of smaller pieces at both graft sites (subcapsular and subcutaneous) resulted in a more rapid vascularization of the entire area of the graft tissue and thus uniform growth and differentiation. The greater abundance and uniformity of blood

supply to all cellular structures in smaller grafts are presumed to eliminate initial necrosis at early graft time points and prevent disruption of development in later time points.

The xenograft model characterized in the present study offers advantages over the fetal lung explant model, because the development of the xenografted tissues is conducive to stage-specific studies. Although xenograft development is accelerated compared to *in utero* development, in this model each developmental stage occurs over one week. This one week interval makes it amenable to the study of stage-specific cellular and biochemical processes. A comparative study of fetal lung explant and mouse renal subcapsular fetal lung allograft models showed the advantages of the latter. While mouse fetal lung grafts undergo extensive proximal and distal differentiation of the epithelium that follows closely the temporal differentiation *in utero*, the lung explant cultures rapidly undergo branching morphogenesis at first (after 3 days), but after longer times in culture (5 days) cease development and start to deteriorate (Vu et al., 2003).

Factors responsible for the accelerated lung development observed *in vitro* and/or *in vivo* are not known. Removal of tissue from putative inhibitory factor(s) present *in utero* is a possible explanation (Gross, 1983). Moreover, cellular and biochemical changes of lung structure may be under tight microenvironmental and hormonal control, and any changes in these conditions may have downstream effects on lung development (Ballard, 1984; Mendelson and Boggaram, 1991). Estrogen is known to be one of the regulators of fetal lung development and surfactant synthesis. However, in studies where production of estrogen was suppressed in pregnant baboons (Pepe et al., 2003) the fetal lungs continued to grow and exhibited normal morphology. Moreover, female nude mice had relatively low levels of primary estrogens compared to human females (Witorsch,

2002), indicating that the accelerated lung development may not be due to the estrogen status of nude mice, but perhaps to other factors not yet well understood.

However, although tissue differentiation was accelerated compared to the *in utero* time scale, the changes in tissue morphology resembled the stages of human *in utero* lung development (pseudoglandular, canalicular and saccular). Each stage of lung development (about 8 weeks of *in utero* development) appeared to require about one week of graft development, with grafts reaching the early saccular stage after 14 days of engraftment. This is in contrast to the previous studies that have examined human graft development at multiple time points. In one study where fetal lungs from 10-14 weeks of gestation were subcutaneously grafted into nude mice, grafts resembling canalicular stage of development were observed at 8 weeks post-grafting (Cobb, 1975; Groscurth and Tondury, 1982; Peault et al., 1994; Phillips and Gazet, 1969). The time course of tissue differentiation also appears to be accelerated in the present model, as compared to another study where fetal lungs ranging from 8-12 weeks of gestation were grafted for 3, 6, 11, and 19 weeks (Cobb, 1975; Groscurth and Tondury, 1982; Peault et al., 1994; Phillips and Gazet, 1969). Saccular stage-like changes were observed in 11 weeks post-engraftment as compared to 3 weeks in our study. The accelerated timing observed in the present study is comparable to the subcutaneous mouse allograft model, where E14.5 mouse fetal lungs required two weeks to differentiate to the saccular stage (Schwarz et al., 2000). On the other hand, both of these are in contrast to the renal subcapsular mouse allograft model (Vu et al., 2003), where timing of developmental stages of E12.5 mouse embryonic lungs closely follows that of *in utero* development. The observation that renal subcapsular lung allografts develop faster than the subcutaneous grafts suggests that the

extra space provided for subcutaneous graft development and extra fluid accumulation within these grafts are not key factors in tissue development.

Differences in timing are probably due to a number of factors. Whether differences in developmental timing are dependent on the starting gestational age in human lung development is still unclear. Studies of human fetal grafts focusing on the end-point of fetal lung development (i.e. structural morphogenesis and appearance of alveolar-like structures) resemble the longer time points of our model (>28 days of grafting) with expanded alveoli that are presumably fluid-filled. Human fetal lung rudiments from 7.5 to 16 weeks grafted subcutaneously into SCID mice all showed mature bronchioles and alveoli after 6-8 weeks, regardless of their starting gestational age (Delplanque et al., 2000). This argues against a significant role of the starting gestational age in timing of lung development in these models.

Lung epithelial differentiation and branching are intimately tied to vascularization of the lung. Vascular development in the embryonic lung takes place through two simultaneous mechanisms. Angiogenesis is characterized by budding and branching of new vessels and their invasion of the pulmonary parenchyma, while vasculogenesis occurs by differentiation and organization of endothelial cells in lung mesenchyme that is necessary for the coordinated development of airways and blood vessels (deMello et al., 1997). Since blood vessels are formed in coordination with the epithelium and the epithelial branching is determined by epithelial-mesenchymal interactions, differentiation of epithelial cells in the model should be accompanied by changes in vasculature. In the present study, endothelial cell changes as assessed by human antigen specific endothelial

cell marker PECAM-1, suggest significant remodeling over the course of differentiation of the graft. As tissue differentiation progressed and endothelial cells became more attenuated, the PECAM-1 staining became less intense. In later day grafts (≥ 14 days), PECAM-1 immunopositive structures persisted in subpleural areas, however the staining was virtually absent from the extensively remodeled interstitium. In the areas where interstitium had been extensively remodeled and reduced in mass, with evidence of continued differentiation of the epithelium and active cell division and no evidence of necrosis, no PECAM-1 staining was observed.

Since graft tissue can not continue to be viable, differentiate, and grow without a blood supply, it is reasonable to deduce that at later time points host angiogenesis may be sufficiently developed to infiltrate the graft, and in turn take over the support of its growth. Studies in mouse allograft models have provided evidence for both sources of blood supply to the graft. While Schwartz et al. showed that the grafts were vascularized by vessels originating from endogenous endothelial cells, Vu et al. observed that host blood vessels provide circulation to the graft in addition to that of endogenous endothelial structures. The present PECAM-1 ICC data support the notion that graft endogenous endothelial cells are initially present and at later stages of development these are replaced by host vascular structures. Mechanisms of embryonic vasculogenesis are poorly understood. It is possible that in the later stages of graft development, where extensive remodeling has already taken place, the reservoir of native graft (human) endothelial stem cells becomes depleted and the host vasculature has had sufficient time to

completely penetrate the tissue and thus provide the blood supply needed to support further growth and differentiation of the grafted tissue.

Both mRNA and protein of all four SPs were expressed in the starting fetal lung tissues and their expression was preserved during graft development. The presence of SPs in tissues of early graft time points and therefore in pre-type II cells was observed by immunostaining in open airways throughout the lung. However, with progression of gestation the relative number of SP-positive cells per terminal air space decreased as the SP-positive pre-type II cells differentiated into SP-negative type I cells. This is in agreement with reported findings (Vu et al., 2003) in the mouse renal subcapsular model where a decrease in a number of pro-SP-C-positive cells with developmental progression of tissues was observed.

There has been an apparent discrepancy in the literature regarding the expression patterns of SPs during development. The assumption has been that SP-B and SP-C are not found in human fetal tissues until about 18-20 weeks of gestation, and that SP-A and SP-D are not expressed until about 24-26 weeks of gestation (Ballard, 1984; Beers et al., 1995; Mendelson et al., 1991; Mendelson and Boggaram, 1991). These observations differ from results in the present study and a number of other recent studies where expression of these proteins was observed either in early or late pseudoglandular phase of development. Expression of SP-A and SP-D has been observed as early as 10-12 weeks of human gestation (Otto-Verberne et al., 1990; Stahlman et al., 2002), while after 15 weeks of gestation expression of all four proteins has been readily detected (Khour et al., 1993; Khour et al., 1994). Because the focus in these studies was on the identification of SPs in

tissues of various gestational ages and not on the functions of these proteins, it is currently unknown whether the presence of these proteins at such early time points in gestation has any functional significance. The time interval between tissue procurement and experimentation, as well as the method of pregnancy termination may be some of the factors contributing to the above-mentioned discrepancies in SP expression. The time interval between tissue procurement and experimentation in the present study was rather short (three to eight hours).

Normal fetal lung development is dependent on sustained lung expansion due to the presence of lung fluid. Tracheal obstruction in animal models has been shown to accelerate fetal lung growth (Alcorn and Mendelson, 1993; Piedboeuf et al., 1997). Conditions that are associated with lung liquid drainage are characterized by pulmonary hypoplasia and an excess of type II cells (Flecknoe et al., 2003; Laudy and Wladimiroff, 2000). Hypoplasia is reversible with tracheal ligation, where presumed accumulation of lung fluid induces rapid lung growth and changes in alveolar structure (Flecknoe et al., 2000). In order to differentiate between the actual cellular growth of the graft vs. the increase in size due to fluid accumulation and/or fusion of grafts, ICC for proliferation marker (PCNA) was performed. Although the xenograft model described in the present study resembles models of tracheal obstruction with respect to microscopic changes and dilatation of alveoli observed at longer times of engraftment. However, the cellular proliferation patterns of graft tissues are more similar to those of control animals than to those of tracheal occlusion animals (Maltais et al., 2003). Compared to animals with tracheal obstruction showing a rapid increase in proliferation after 36 hours, lung grafts

initially have slightly increased proliferation rates (as shown by PCNA immunopositivity) followed by higher rates of proliferation after 10 and 14 days of engraftment. Therefore, according to the presence of PCNA immunopositive cells, proliferation of graft tissues is most prominent in the canalicular and saccular stage, indicative of actual and stages appropriate tissue differentiation and growth. Tracheal obstruction is also marked by increased elastin synthesis and tissue deposition (Joyce et al., 2003). In contrast to this, in our model elastic fibers were found only at the tips of the developing alveolar secondary crests, as seen during *in utero* development (Sobin et al., 1988). Moreover, the appearance and secretion of energy-demanding, highly-regulated cellular organelles such as lamellar bodies, specific for the type II pneumocyte, further underscores the similarity of graft development to *in utero* development.

In summary, through morphological and biochemical analysis of fetal lung tissue development, we were able to show that cytodifferentiation of human fetal lung xenografts resembles stages of human *in utero* development. This model is likely to serve as a valuable tool for the study of specific stages of human lung development and the temporal and spatial changes in expression and regulation of surfactant proteins through different stages.

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Chapter 3

Pyrosequencing method for genotyping of surfactant proteins

3.1 Introduction

Recent developments in molecular genetics have seen a growing interest in the correlation of genetic variation and individual differences in biological function and susceptibility to disease (Ahmadian et al., 2000). Single-nucleotide polymorphisms (SNPs) represent the most common variant of human genetic diversity, and have therefore become important indirect markers of disease as well as essential tools in the quest to identify genetic elements that are associated with complex diseases and/or individual responses to drugs (Elahi et al., 2004).

A variety of methods have been developed for SNP genotyping, each with specific advantages and drawbacks. Pyrosequencing, a primer extension based method, was recently introduced as an approach to SNP analysis (Ronaghi, 2003). It is a real-time, non-electrophoretic technique that depends on multiple enzymatic reactions in order to generate quantitative signals. This versatile technique has already been used for a variety of biological applications, including SNP genotyping, SNP discovery, haplotyping, insertion/deletion studies, methylation studies, and allele frequency studies (Neve et al., 2002; Pettersson et al., 2003; Pourmand et al., 2002; Wasson et al., 2002). We decided to apply pyrosequencing to investigate SNPs of surfactant protein (SP) genes (SP-A, SP-B, and SP-D).

The goal was to use pyrosequencing to design new genotyping assays for surfactant proteins that would improve upon our previously published PCR-cRFLP

method (DiAngelo et al., 1999). The Pyrosequencing SNP assay provides a more flexible, versatile, and high-throughput assay that reduces the time necessary to obtain data, and substantially decreases the hands-on time and technical support needed for such analysis.

3.2 Methods

3.2.1 Background to the methodology

Pyrosequencing is a sequencing-by-synthesis method based on a number of coupled enzymatic reactions. This method allows for monitoring of assays in real-time, and dispenses with the need for time consuming electrophoresis.

As presented in Figure 3.1, pyrosequencing is performed on single stranded DNA templates where sequencing primer hybridization to the complementary bases on the PCR template strand initiates DNA polymerase catalyzed nucleotide incorporation. As each new nucleotide is incorporated, a pyrophosphate (PPi) group is released in an equimolar proportion to the amount of the incorporated nucleotide. This pyrophosphate is converted to ATP by the ATP sulfurylase, which in turn drives the conversion of luciferin to oxyluciferin by luciferase. The light produced as a result of this reaction is detected by a charge-coupled device (CCD) camera. Each light signal is proportional to the number of nucleotides incorporated onto the DNA strand. As each nucleotide is dispensed, the complementary strand is synthesized and its signals generate a

PyrogramTM. Each light signal corresponds to a single peak in the pyrogram. Pyrograms are scored by pattern-recognition software that compares the predicted SNP pattern (histogram) to the observed pattern (pyrogram) (Pyrosequencing AB, Uppsala, Sweden). The ability to quantitate each peak of the pyrogram provides for numerous advantages. For example, efficiency of the PCR amplification can be determined from the peak height. Moreover, specificity of the template or any non-specific allelic amplification can be monitored by ensuring appropriate heights of specific peaks, and lack of incorporation of negative control nucleotides.

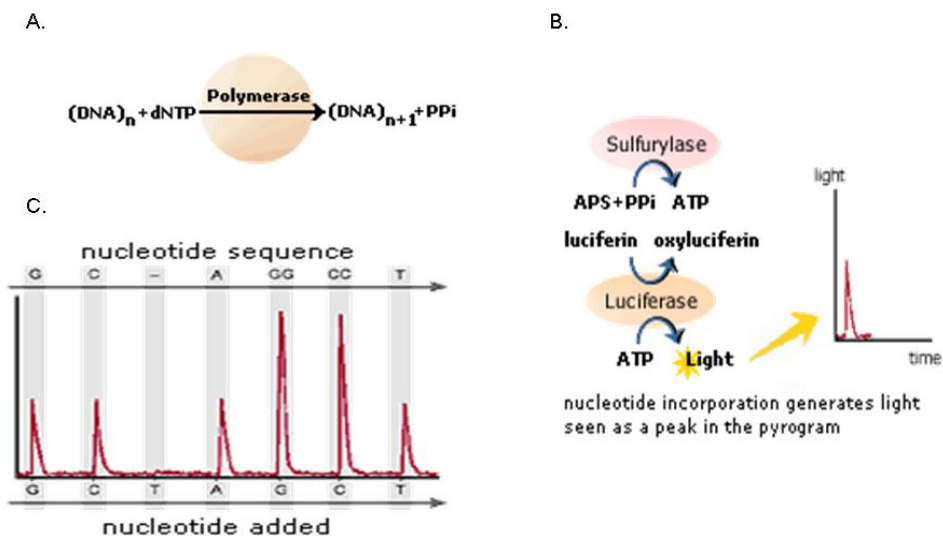


Figure 3.1: Pyrosequencing methodology: enzyme cascade and light detection

Pyrosequencing is a primer based DNA sequencing method. Panel A: Nucleotides are incorporated onto a single stranded DNA template produced by PCR and separation of the

biotinylated single strands. The pyrophosphate group released during the nucleotide incorporation is converted to a light signal through a series of enzyme reactions, as presented in Panel B. These light signals are detected by a highly sensitive camera and used to generate graphic representation of the sequencing reaction referred to as Pyrogram. Panel C shows a Pyrogram of the following sequence: GCAGGCCT. Each light signal represents a single peak in the program that corresponds to the representative nucleotide. In this case a single light peak of nucleotide G indicates that the first nucleotide of the sequence segment of interest is G, followed by a single light peak of C, extending the sequence to GC. When T nucleotide (a negative control) is released, no light signal is generated, indicating that sequencing reaction is specific. Release of A nucleotide generates another single peak, extending the sequence to GCA. Release of a G nucleotide followed by a C nucleotide, result in single G and C peaks of double height, respectively. This indicates that there are two nucleotides of the same type next to one another, resulting in sequence GCAGGCC. Finally, a release of a T nucleotide generates a single peak, rounding up the sequence of this DNA strand of interest to GCAGGCCT.

3.2.2 General methodology

3.2.2.1 Source and isolation of DNA

DNA was extracted from 200 μ l of blood using the QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. DNA was eluted in the final step using 200 μ l of DNase free dH₂O (Ambion). Upon extraction DNA was stored at -20°C until further handling.

3.2.2.2 SNPs of interest

Table 3.1 represents the SNPs for the four genes of surfactant proteins studied.

Table 3.1: SNPs of surfactant protein genes studied

SP-A1	SP-A2	SP-B	SP-D
AA19(C/T)	AA9(A/C)		DA11(C/T)
AA50(C/G)	AA91(C/G)		DA160(A/G)
AA62(A/G)	AA140(C/T)	B1580(C/T)	
AA133(A/G)	AA223(A/C)		
AA219(C/T)			

3.2.2.3 PCR based converted Restriction Fragment Length Polymorphisms (cRFLP)

PCR-cRFLP was performed as previously described (DiAngelo et al., 1999) using a 3.3 kb gene-specific (GS)-PCR template on 100 individual samples in order to compare accuracy and reproducibility of these two methods.

3.2.2.4 PCR and sequencing primer design

PCR primers were designed using commercially available software DNASTar (www.dnastar.com) or Pro-oligo (www.changbioscience.com). Recently, a Pyrosequencing assay design software has been developed (<http://www.biotagebio.com/DynPage.aspx?id=7261>) that provides for integrated assay design and eliminates the need of using multiple software and additional troubleshooting. Since this software was not available at the time of completion of this work, multiplex PCR and/or multiplex pyrosequencing primers were designed to ensure that no homo and/or heterodimers occurred, and that there were no false priming sites for either of the primers on either template. Biotin label was added to the 5' end of one of the primers in each primer pair. The biotin labeling of the primers is currently carried out by Biomers (www.biomers.com), although other companies have been used in the past. Sequencing primers for the pyrosequencing reaction were designed using the SNP Primer Design Software (technical support web site of Pyrosequencing AB, Uppsala, Sweden). These primers anneal directly in front of the SNP position (or several nucleotides before it) on the reverse strand of the PCR template to be analyzed as presented in Figure 3.2. Depending on the position of the sequence primer docking, the dispensation order of nucleotides is determined by the Pyrosequencing SNP analysis software. Nucleotides that are not present in the sequence, can serve as negative controls, and are dispensed, if possible, at the beginning of the sequencing reaction as well as in positions flanking the SNP position. The negative control ensures the specificity of the priming and sequencing reaction. The DNA strand to be analyzed is elongated through the SNP site. PCR primers and sequencing primers used in analysis

of each SNP are represented in Table 3.2.

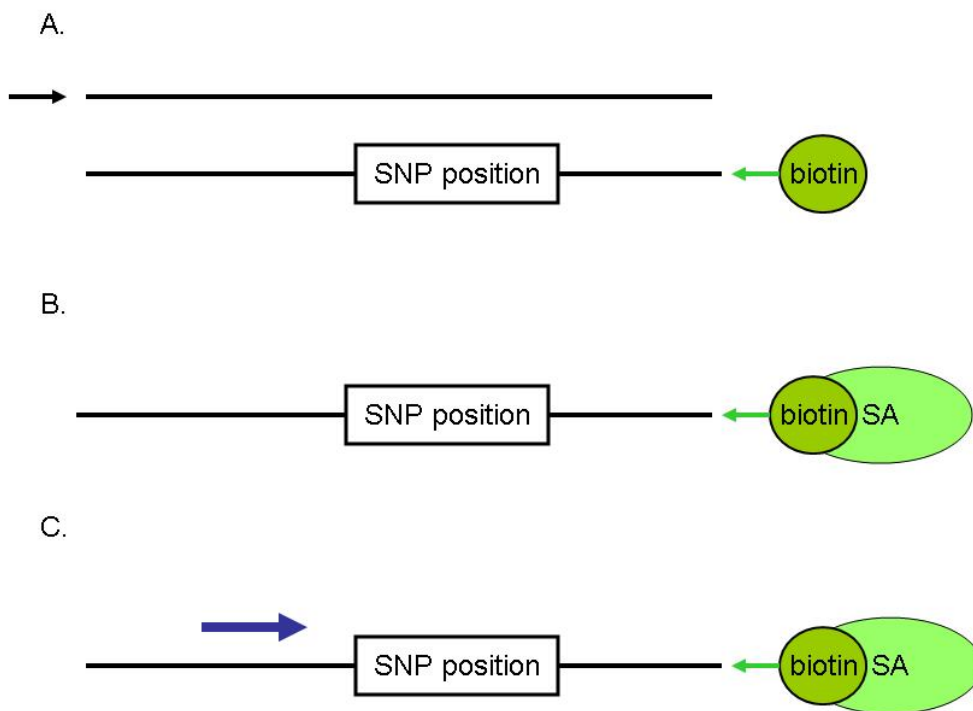


Figure 3.2: Pyrosequencing methodology: PCR and sequencing primer design

Panel A: PCR is performed using two primers of which one is biotin labeled (mustard green). Panel B: Denaturation of the double DNA strand and biotin labeled single stranded DNA bound to streptavidin-sepharose beads (light green). Panel C: Annealing of the sequencing primer (blue) immediately or several nucleotides in front of the SNP site initiates pyrosequencing reaction.

Table 3.2: PCR primers and sequencing primers used in analysis of each SNP

SNP	Gene	F #	F primer	R #	R primer	Seq #	S primer	Direction *	Simplex/multiplex
AA19(C/T)	SP-A1	1327A	cgactgacccagagccatgic	1328	B-ctggaggccagggtctcctt	1331	cagcctctggtgctg	Forward	S and M with AA50
AA50(C/G)	SP-A1	1327A	cgactgacccagagccatgic	1328	B-ctggaggccagggtctcctt	1332	caggagcggagag	Forward	S and M with AA19
AA62(A/G)	SP-A1	1344	B-tagctccaaccagttgtggigac	1345	tgatttccaggagagacatggca	1346	catggcatttctccag	Reverse	S only
AA133(A/G)	SP-A1	1233	B-agtctgcagggtccataatga	28A	accctcagtcaggctaccat	1338	ggagaagaccctctctcc	Reverse	S and M with AA219
AA219(C/T)	SP-A1	1321	aagtcacaacataatgacctat	494	B-tcagaactcacagatggtca	1339	agggagcccgcga	Forward	S and M with AA133
AA9(A/C)	SP-A2	1327A	cgactgacccagagccatgic	1328	B-ctggaggccagggtctcctt	1333	gccctctggcccctca	Forward	S only
AA91(C/G)	SP-A2	1397	tgggaataatggctgcctg	1398	B-ctgacttcagggtcgtctgig	1399	gagagaagggggag	Forward	S only
AA140(C/T)	SP-A2	1233	B-agtctgcagggtccataatga	28A	accctcagtcaggctaccat	1336	tggactgcccattg	Reverse	S and M with AA223
AA223(A/C)	SP-A2	1321	B-aagttacaacacalalgcctat	494	tcagaactcacagatggtca	1337	tgtgtacatctccacact	Reverse	S and M with AA140
B1580(T/C)	SP-B	1313	tcccaggacacgatgaggaaag	1390	B-gcaggcaggaggtgagc	1341	tacttcagaacca	Forward	S only
CA138(A/C)	SP-C	1393	B-tccagagatcctccagtc	1394	glagtagagcggcactcgc	1395	tgaaagtgtggactttt	Reverse	S only
CA186(A/G)	SP-C	1318	gggctgcacatgggatagaaactc	1319	B-ccctgggatcacactgctcacc	1320	ggcatggcccgtga	Forward	S only
DA11(C/T)	SP-D	1305	gaagcagaataagaagacctactcc	1301	B-gccactctccactgagctacaca	1302	aagacctctcccacagaa	Forward	S and M with AA160
DA160(A/G)	SP-D	1306	gaggttccctgggagcgt	1307	B-gtgggtggaggggggia	1308	gagtcctctggaaac	Forward	S and M with AA11

All primer sequences are 5' to 3'. Forward (F) primers are in sense and reverse (R) primers in anti-sense orientation. For forward and reverse sequencing the sequencing (Seq) primers are in sense and antisense orientation, respectively. * The forward and reverse direction of the sequencing assay are noted. In a multiplex reaction two SNPs can be sequenced from a single template (as for AA19/AA50) or from two different templates, as is the case in all other multiplex reactions showed. Primer numbers are arbitrary laboratory numbers. B stands for the biotin label at the 5' end of the primer. S denotes simplex reaction, M denotes multiplex reaction.

3.2.2.5 Template preparation and plate set-up for pyrosequencing

Twenty μl of biotinylated PCR product was immobilized to streptavidin-Sepharose HP beads (Amersham Biosciences) and incubated with binding buffer, 10 mmol/L Tris-HCl, 2 mol/L NaCl, 1 mmol/L EDTA, 1 mL/L Tween 207 in a 96-well microtiter plate for 10 min at 25°C with mixing at 1400 rpm. The 96-well plate was then processed through vacuum filtration using the Vacuum Prep Tool (Pyrosequencing AB), where the bead-bound PCR products were transferred to the filter at the end of each 96-Filter probe, and the remaining liquid was removed by vacuum filtration. The Vacuum Prep Tool was passed through a series of solutions including the denaturation solution (0.2 mol/L NaOH) that removed non-biotinylated strands. Sepharose bead-bound biotinylated strands were retained on the filter and rinsed in washing buffer (10 mmol/L Tris-acetate, pH 7.6). The vacuum was removed to release the beads from the filter. Bead-bound single-stranded DNA templates for each SNP were resuspended in annealing buffer (20 mM Tris-acetate, pH 7.6; 2 mM magnesium acetate) containing 20 pmol of each respective sequencing primer, in wells of a PSQ 96 plate (Pyrosequencing AB). This mixture was then heated at 80°C for 2 min on a compact heat block and cooled to room temperature for 10 min to facilitate annealing of the Pyrosequencing primers to templates.

The plate was placed into the PSQ HS 96MA System (Pyrosequencing AB) for analysis. Simplex pyrosequencing was performed for genotyping SNPs of all four genes. Where multiplexing could be achieved, duplexing of two SNPs of the same gene was designed, with the sequencing reaction taking place from either one or two PCR templates.

3.2.2.6 PCR based converted Restriction Fragment Length Polymorphisms (cRFLP)

PCR-cRFLP was performed as previously described (DiAngelo et al., 1999) using a 3.3 kb gene-specific (GS)-PCR template on 100 individual genomic DNA samples in order to compare accuracy and reproducibility of these two methods.

3.2.3 Allele quantification

3.2.3.1 Concentration measurements of DNA

Genomic DNA was quantitated using PicoGreen reagent (Molecular Probes, Eugene, OR) as per manufacturer's instructions. Briefly, genomic DNA was diluted in TE, and aliquots of 100 μ l were transferred in duplicates to Microfluor B black base 96 well plate. PicoGreen reagent was diluted in TE, and 100 μ l of dilution were added to each DNA sample. Following brief shaking, the plate was incubated in dark at room temperature for about 10 min. The plate was then read in the Tecan (Tecan Systems, San Jose, CA) plate reader at 485 nm excitation and 535 nm emission. Standard curves were constructed by using serial dilutions λ DNA as the standard. Standard was provided with the PicoGreen reagent (Molecular Probes, Eugene, OR). Final DNA concentrations were determined by averaging results of duplicate readings derived from the standard curve.

3.2.3.2 DNA pools and allele quantification

Equal amounts (50 ng) of DNAs were pooled into small and large size (10, 50, 70, and 100 samples) DNA pools and amplified in triplicate. These were then analyzed for allele frequencies by simplex pyrosequencing for amino acid 11 and amino acid 160 of SP-D. To insure accuracy of allele quantification, standard curves for peak height linearity were set up for both SNP sites (DA11(C/T) and DA160(A/G)) using DNA in various percentages (from 100%/0% to 0%/100%) from two samples representing both homozygous genotypes for each SNP. Triplicates of each DNA proportion of each respective SNP nucleotide (i.e. for DA11(C/T): 100%CC/0%TT, 80%CC/20%TT, 60%CC/40%TT, 50%CC/50%TT, 40%CC/60%TT, 20%CC/80%TT, and 0%CC/100%TT) were averaged and plotted for the linear regression best-fit line. Linear regression was used to adjust the observed allele frequency to the actual theoretical allele frequency.

3.3 Application of the pyrosequencing methodology to surfactant proteins

3.3.1 SP-A genotyping

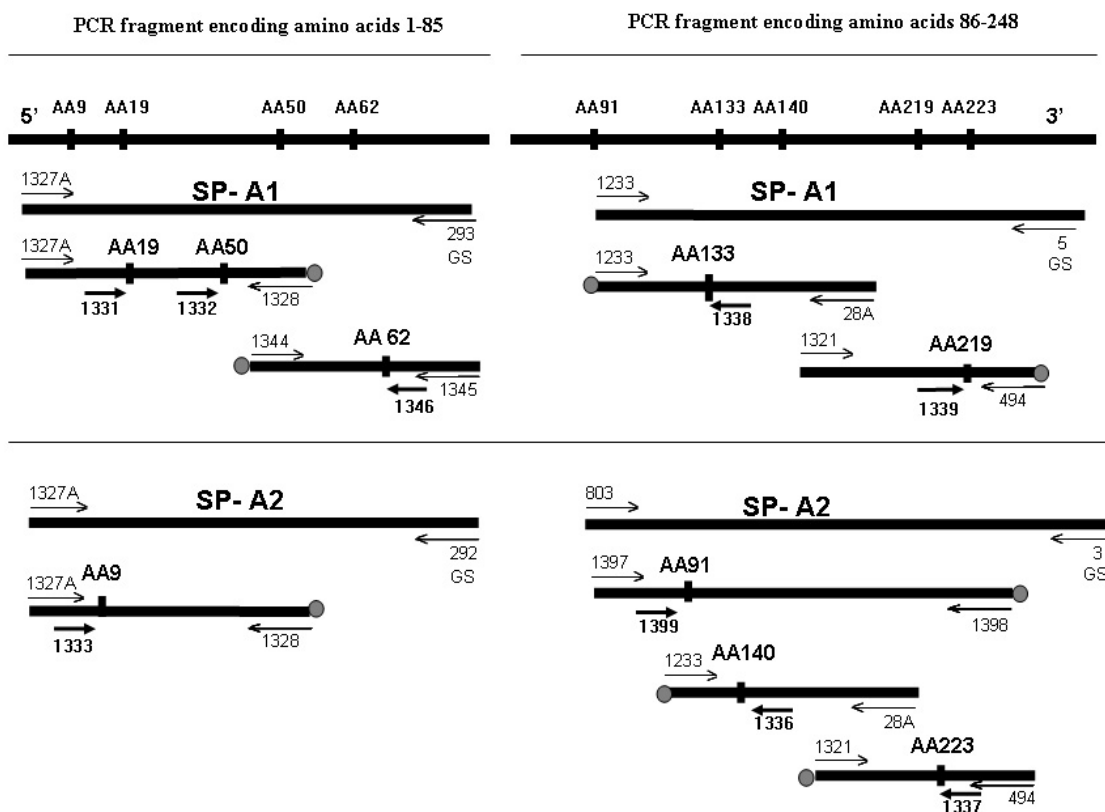
3.3.1.1 General, gene-specific, and nested PCR

All DNA samples were individually amplified from non-quantified genomic DNA by PCR using Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN). Two

μl of individual DNA samples were combined with 1 μl of 1.25 mM deoxynucleotide triphosphate (dNTP) mix, various amounts of each PCR primer (25 ng/ μl to 100 ng/ μl), 2.5 μl 10x Buffer 1 and 2.5 μl of 10x Buffer 2 (Roche) in 50 μl overall volume (this was done for all the SNPs of all four SP genes). Cycling conditions for SP-A (gene-specific reaction) were as follows: 95°C for 2 min followed by 35 cycles of 95°C for 30', 58°C for 1 min, and 72°C for 1 min. This was followed by one cycle of 72°C for 5 min. PCR profiles for SP-A nested reaction were as follows: 95°C for 2 min followed by 35 cycles of 95°C for 30', 60°C for 30', and 72°C for 30'. This was followed by one cycle of 72°C for 5 min. An aliquot of the PCR reaction was run on 8% PAGE gel to verify size and quantity of PCR product, and most importantly to ensure the presence of a single PCR band in simplex PCR or two bands in multiplex PCR. The outline of the SP-A SNP genotyping approach is presented in Figure 3.3. Advantage was taken of the area of highest gene-specificity (difference) found between the two genes, amino acids 66 to 85, in order to generate the gene-specific templates. As outlined in Figure 3.3, two gene-specific fragments were made for each SP-A gene and used as templates in nested PCR reactions to amplify short fragments around each SNP for pyrosequencing.

Figure 3.3. Schematic representation of SP-A PCR and pyrosequencing for PCR fragments encoding amino acids 1-248

Forward and reverse primers are presented with thin arrows, and GS (gene specific) stands for gene specific primers (SP-A1 or SP-A2 specific). Grey circles represent biotin label at the primer end. Sequencing primers are presented with thick arrows.



i) **SP-A1:** primer pair 1327A/293 was used to generate a 549 bp long PCR product, which was used as template for nested reactions for AA19, AA50, and AA62. Due to the spatial proximity of AA19 and AA50, these SNPs were amplified on the single DNA strand of a 187 bases product generated with 1327A/1328 primer pair. The 549 bp PCR product was also used as a template in a nested reaction with primers 1344/1345 to generate a fragment for analysis of AA62. The second gene specific fragment of SP-A1 was amplified using primers 1233/5 to generate a 422 bp fragment. Two nested reactions were performed on this template; a 200bp fragment for AA133 with primer pair 1233/28A and a 209 bp fragment for AA219 with primer pair 1321/494.

ii) **SP-A2:** A 541 bp long PCR product was amplified with primer pair 1327A/292 and used as template in nested PCR with primer pair 1327A/1328 to generate a 186 bp fragment for AA9 analysis. The second gene specific fragment of SP-A2 was amplified using primers 803/3 to generate a 1700 bp fragment. This fragment was used as a template in three nested PCR reactions: 1) with primers 1397/1398 to generate a 129 bp fragment for AA91 pyrosequencing, 2) with primers 1233/28A to generate a 200 bp fragment for AA140 pyrosequencing, 3) with primers 1321/494 to generate a 223 bp fragment for AA223 pyrosequencing. Due to the cost of biotinylated primers, the objective was to have as many as possible overlapping primers in the nested reactions for the two genes. Therefore, the same primer pairs were used for PCR of AA9, AA19, and AA50. Second set of primers was used for AA133 and AA140. Third set of primers was used for AA223 and AA219.

3.3.1.2 SP-A pyrosequencing

All pyrosequencing reactions were first performed in simplex, where a single SNP was analyzed at a time, using 20 μ l of the PCR reaction. Where possible, cost effective multiplex pyrosequencing assays were designed. For SP-A1, AA19 and AA50 were sequenced in duplex using 20 μ l of a singular template and both SNPs were analyzed in forward assays as presented. A reverse AA133 assay was performed in multiplex with a forward AA219 assay, using 15 μ l of AA133 PCR template and 25 μ l of AA219 template, to achieve optimal intensity in the ratio of the signal from each template. Multiplex assays for these SNPs are schematically presented in Figure 3.4. For SP-A2, 20 μ l of AA140 PCR product was pyrosequenced in multiplex with 20 μ l of AA223 PCR product. Both SNPs were pyrosequenced either in simplex or multiplex assays as schematically presented in the Fig. 3.5. AA9, AA62, and AA91 were pyrosequenced, by simplex assays, using 20 μ l of each template. The representative histograms and pyrograms are presented in Figure 3.6. Overall, complete genotyping of both SP-A genes requires 12 PCR reactions and 6 pyrosequencing reactions.

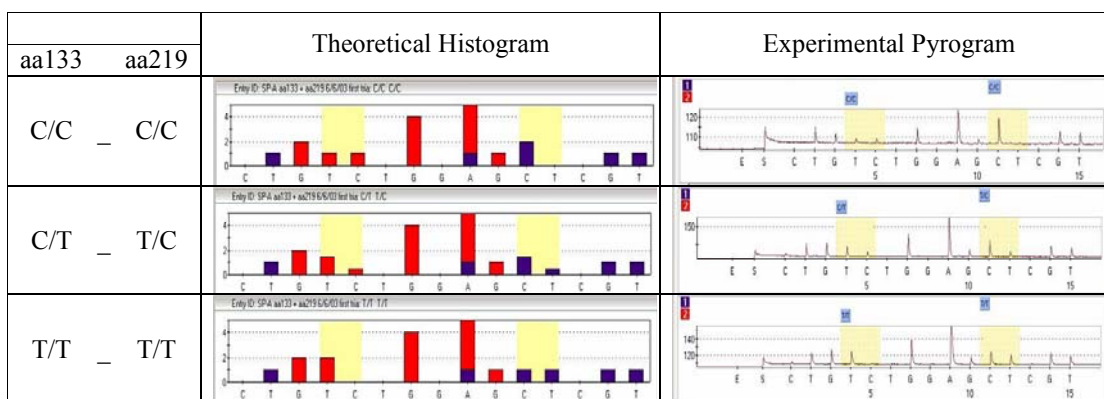


Figure 3.4: Multiplex assays for AA133 and AA219

Expected patterns for multiplex pyrosequencing analysis of two SNPs (AA133 and AA219) are presented in the left panel as theoretical histograms. The results of genotype analyses of the SNPs are presented in the right panel as experimental pyrograms. There are three possible patterns of nucleotide distribution in simplex pyrosequencing for each SNP (ie. for AA133: C/C, C/T, and T/T and for AA219: C/C, C/T, and T/T), while nine combinations are possible for multiplex pyrosequencing (only three are presented here). The positions of the SNPs of interest are highlighted in yellow. As presented in the figure, both SNPs (AA133 and AA219) are sequenced at once in a multiplex reaction. A C nucleotide is dispensed first as a negative control for both SNP strands, followed by a T nucleotide that corresponds to two nucleotides in front of the SNP site for AA219. AA133 SNP is sequenced first (red bars), followed by a negative control T. Dispensation of the following A nucleotide, sequences through both AA133 and AA219 sequence at once (red and blue bars overlap). Dispensation of additional C and T nucleotides, sequences through the AA219 SNP site (blue bars). This SNP site is followed by a negative control (C nucleotide). The remaining stretch of nucleotides is the surrounding sequence for AA219 (GT, blue bars).

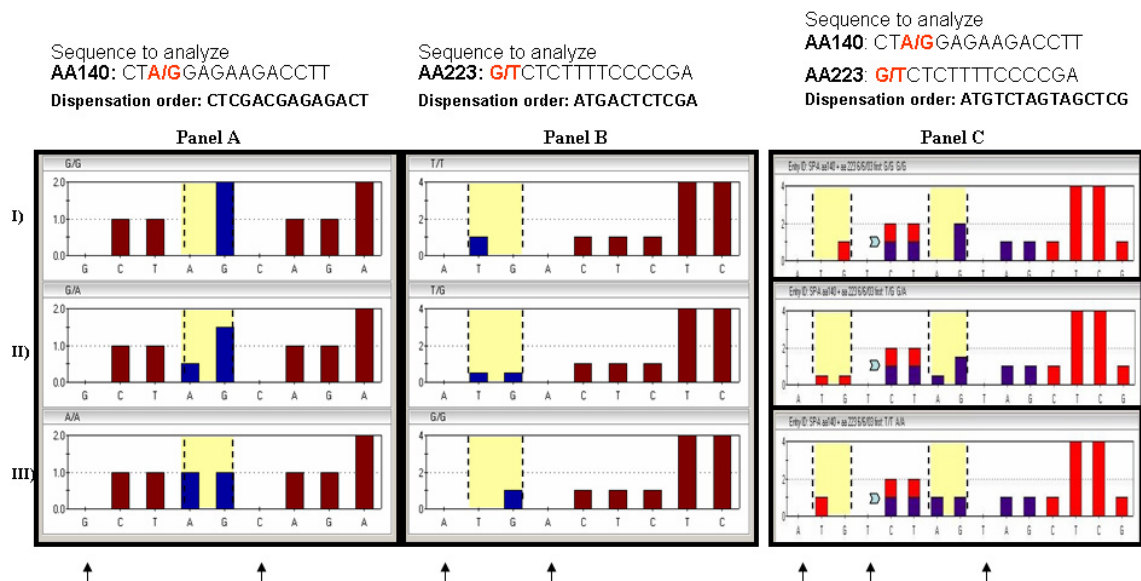


Figure 3.5: Multiplex assays for AA140 and AA223

Expected patterns from simplex pyrosequencing analysis (panels A and B) and multiplex analysis (panel C) of two SNPs (AA140 and AA223) are presented as histograms. There are three possible patterns of nucleotide distribution in simplex pyrosequencing for each SNP (panel A for AA140 SNP; panel B for AA223 SNP), while nine combinations are possible for multiplex pyrosequencing (only three are represented in panel C). The positions of the SNPs of interest are highlighted in yellow and outlined with a dashed line. **Panel B:** The sequencing primer for the AA223 SNP (G/T) is right in front of the SNP, and this makes it the most straight forward type of sequencing reaction (as compared to that in panel A). Since the AA223 sequence (**G/T**CTCTTTTCCCC, Panel B), does not contain an A nucleotide, this can serve as negative control. The nucleotides serving as negative controls are identified by an arrow at the bottom of each panel. Whenever possible, the negative control nucleotide is dispensed first. For

example, in panel B, the nucleotide dispensation order is as follows: the negative control (A) is followed by a T and then a G nucleotide (constituting the SNP). These are followed by another negative control A nucleotide (arrow). The dispensation order then continues to sequence through a short segment of the surrounding DNA sequence, in this case CTCTTTTCCCC (panel B), in order to ensure that the SNP analyzed is within the DNA segment of interest. Note the difference in peak height when a single T or C is dispensed versus four Ts or four Cs are dispensed in sequence. Panel portions I and III depict homozygous SNP patterns (G and T, respectively in panel B), while the II portion of each panel depicts a heterozygous SNP pattern (G/T in panel B). **Panel A:** For AA140 SNP, the sequencing primer is not right in front of the SNP site, as it is for the AA223 SNP (panel B). In this case two nucleotides (C and T) precede the SNP (A/G). Therefore, in the sequencing reaction, a G nucleotide (negative control, arrow) is dispensed. This is followed by dispensation of a C and a T that sequence the CT preceding the A/G SNP. Due to the fact that another G nucleotide is immediately adjacent to the A/G SNP, the specific SNP signal for G overlaps with the flanking G, as represented by the double peak height in panel A.I. A negative control C nucleotide (arrow) is dispensed following the A/GG sequence, and sequencing of surrounding bases continues as in panel B. **Panel C:** Both SNPs (AA140 and AA223) are sequenced at once. Due to the dispensation order set-up AA223 SNP is sequenced first (red bars), as in panel B, followed by a negative control T. The dispensation of the following C and T nucleotides, sequences through both AA223 and AA140 sequence at once (block arrow, red and blue bars overlap). Dispensation of the A and G nucleotides, sequences through the AA140 SNP site (blue bars). This SNP site is followed by a negative control (T

nucleotide, arrow). The remaining stretch of nucleotides is the surrounding sequence for both AA140 (AG, blue bar) and AA223 (CTTTTCCCCG, red bar).

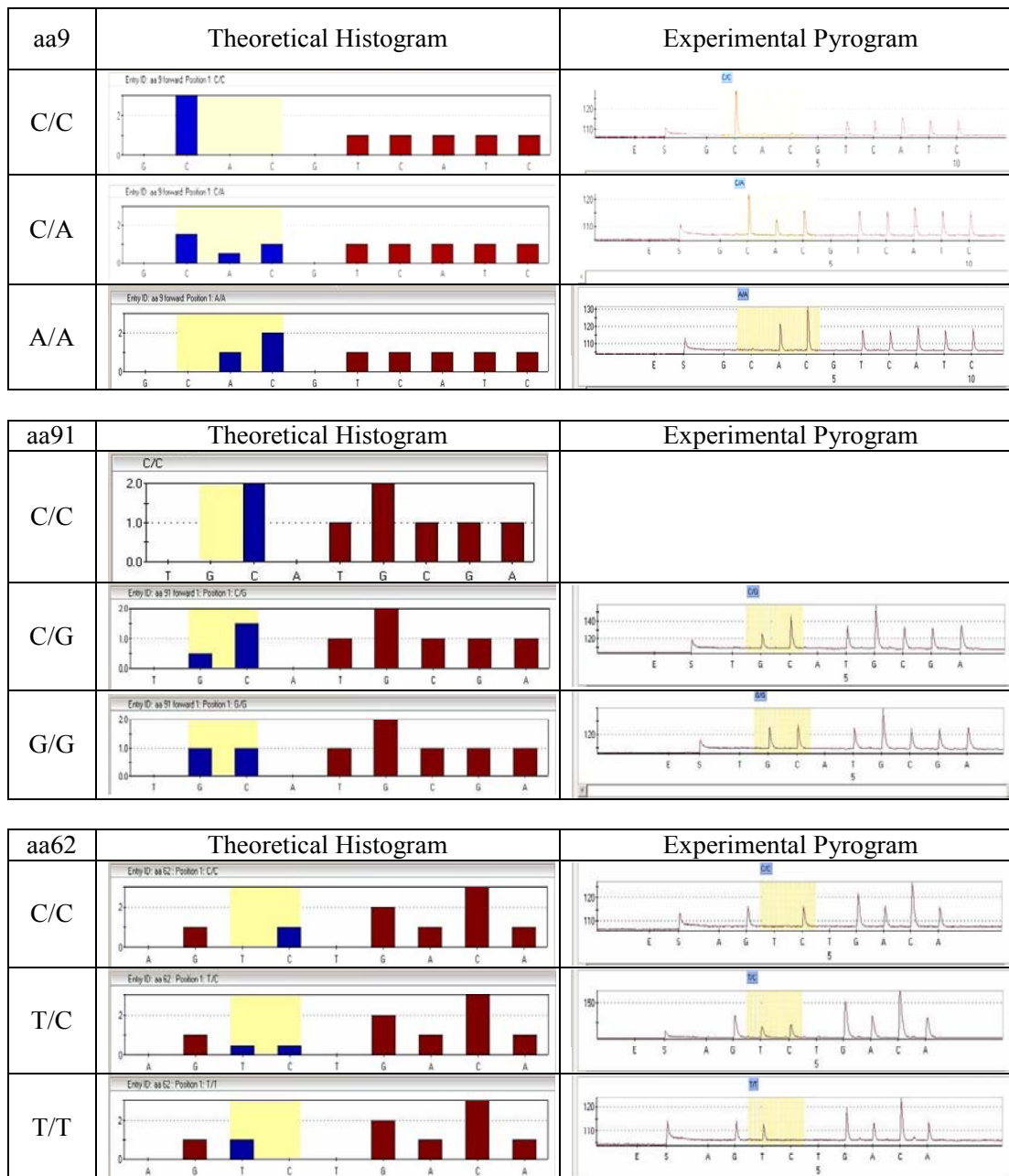


Figure 3.6: Simplex assays for AA9, AA62 and AA91

Figure 3.6: Expected patterns for simplex pyrosequencing analysis of each SNP (AA9, AA62, and AA91) are presented in the left panel as theoretical histograms. The results of genotype analyses of the SNPs are presented in the right panel as experimental pyrograms. The position of the SNP of interest is highlighted in yellow. There are three possible patterns of nucleotide distribution in simplex pyrosequencing for each SNP (ie. for AA9: C/C, C/A, and A/A; for AA62: C/C, C/T, and T/T, and for AA91: C/C, C/G, and G/G). For example, for AA9 sequencing primer for the SNP (C/A) is right in front of the SNP. Since the AA9 sequence (C/ACTCATC, first panel), does not contain a G nucleotide, this can serve as negative control. G nucleotide is dispensed first, followed by a C and then an A nucleotide (constituting the SNP). These are followed by another C, which is the nucleotide immediately following the SNP site and another negative control G nucleotide. The dispensation order then continues to sequence through the surrounding DNA sequence TCATC. SNP sequencing for AA91 and AA62 is performed in a similar fashion as illustrated above.

3.3.1.3 SP-A molecular haplotypes

The advantage of being able to pyrosequence AA19 and AA50 on a single DNA strand, allows for direct molecular haplotyping of these two SNPs. In SP-A1, the AA19(C)AA50(G) haplotype was not observed in 152 individual DNA samples genotyped by pyrosequencing and in 100 samples genotyped by PCR-cRFLP. Family based association test (FBAT) was used to estimate frequencies of SP-A1, AA19(C/T)AA50(C/G), haplotypes in 1534 individuals. The frequency of AA19(T)AA50(G) haplotype was 0.532, the frequency of AA19(T)AA50(C) was 0.383, the frequency of AA19(C)AA50(C) was 0.083, while the frequency of AA19(C)AA50(G) was 0.001. Moreover, this observation was confirmed by inferred haplotype analysis of 100 samples analyzed by PCR-cRFLP method, previously used in the laboratory. From these analyses, it can be concluded that the allele AA19(C), which encodes alanine, is in linkage with AA50(C) allele which encodes leucine. These observations together indicate that the SP-A1 haplotype AA19(C)AA50(G) is extremely rare (approximately 1 in 1000).

The representative histograms and pyrograms of all other haplotypes are presented in Figure 3.7.

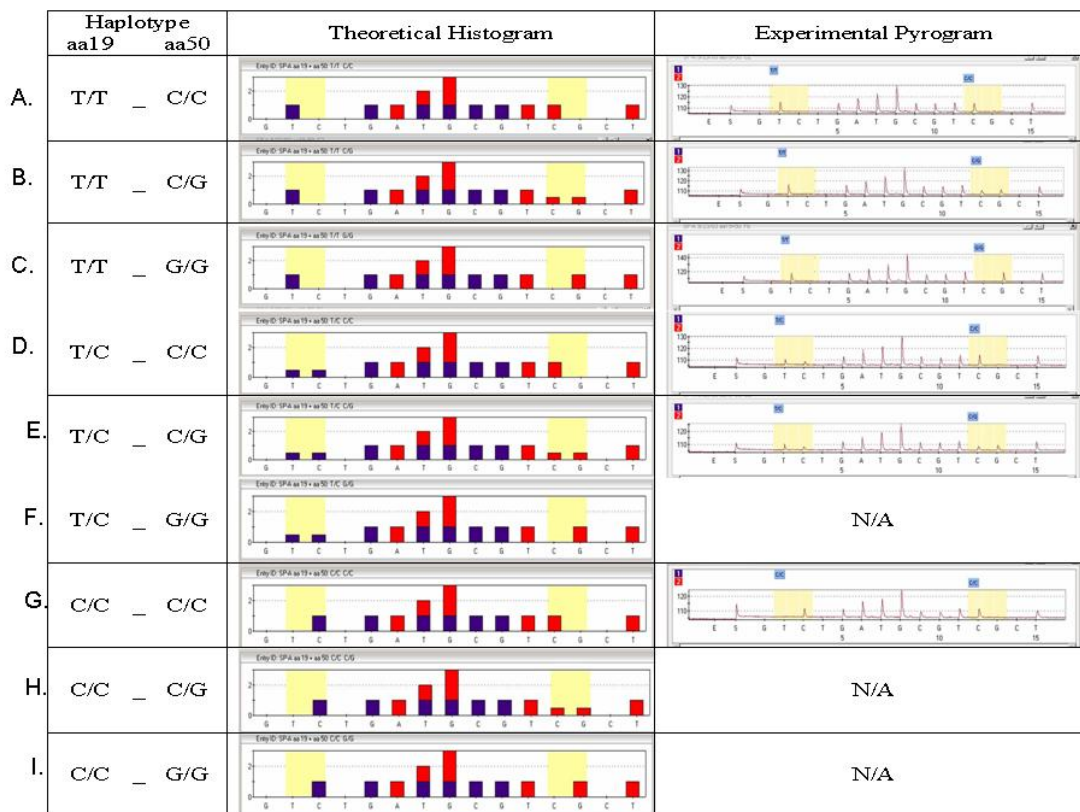


Figure 3.7: Multiplex assays for AA19 and AA50: molecular haplotype

Expected patterns for multiplex pyrosequencing for the two SNPs (AA19 and AA50) are presented in the left panel as theoretical histograms. The results of genotype analyses of the SNPs are presented in the right panel as experimental pyrograms. The position of the SNP of interest is highlighted in yellow. There are nine possible haplotypes. Both SNPs (AA19 and AA50) are sequenced at once from the same DNA strand. The SNP for AA19 is sequenced first (blue bars), followed by simultaneous sequencing of DNA stretches surrounding both SNP positions (overlap of blue and red bars) and followed by sequencing of AA50 SNP (red bars). The sequencing primer for the AA19 SNP (C/T) is right in front of the SNP, while the sequencing primer for AA50

SNP (C/G) is five nucleotides (ATGGT) in front of the SNP site. Panels A, C, G, and I are representative of the four haplotypes where both SNPs are homozygous. Panels B, D, F, and H represent haplotypes where one SNP is homozygous and one is heterozygous. Panel E is representative of the haplotype where both SNPs are heterozygous on the same DNA strand. As indicated by corresponding pyrograms on the right hand of the figure, no samples were observed that fit the haplotype represented by pyrograms in Panel F, H, and I, indicating that SP-A1 haplotype AA19(C)AA50(G) was not observed.

3.3.1.4 Pyrosequencing vs. PCR-cRFLP genotyping for SP-A

When the same 100 individual DNA samples were analyzed by both cRFLP and pyrosequencing, close to 100% concordance was found for AA19, AA50, AA62, AA91, AA133, AA219, and AA223. A 10% discrepancy in AA9 genotypes was observed that could not be explained. A marked discrepancy in AA140 genotype was identified to be due to the inconsistency of the restriction enzyme activity used in PCR-cRFLP. The T (enzyme cut) allele was undetectable in certain batches of samples genotyped by cRFLP, while readily detectable by pyrosequencing.

3.3.2 SP-B genotyping and pyrosequencing

PCR for marker B1580(T/C) was performed using oligos 1313/1390 and cycling conditions were as follows: 95°C for 2 min followed by 35 cycles of 95°C for 30', 58°C for 1 min, and 72°C for 1 min. This was followed by one cycle of 72°C for 5 min. An aliquot of the PCR reaction was run on 8% PAGE gel to verify size and quantity of PCR product, and most importantly to ensure the presence of a single PCR band. Pyrosequencing of the PCR product was done using sequencing with primer 1341.

3.3.3 SP-D genotyping and pyrosequencing

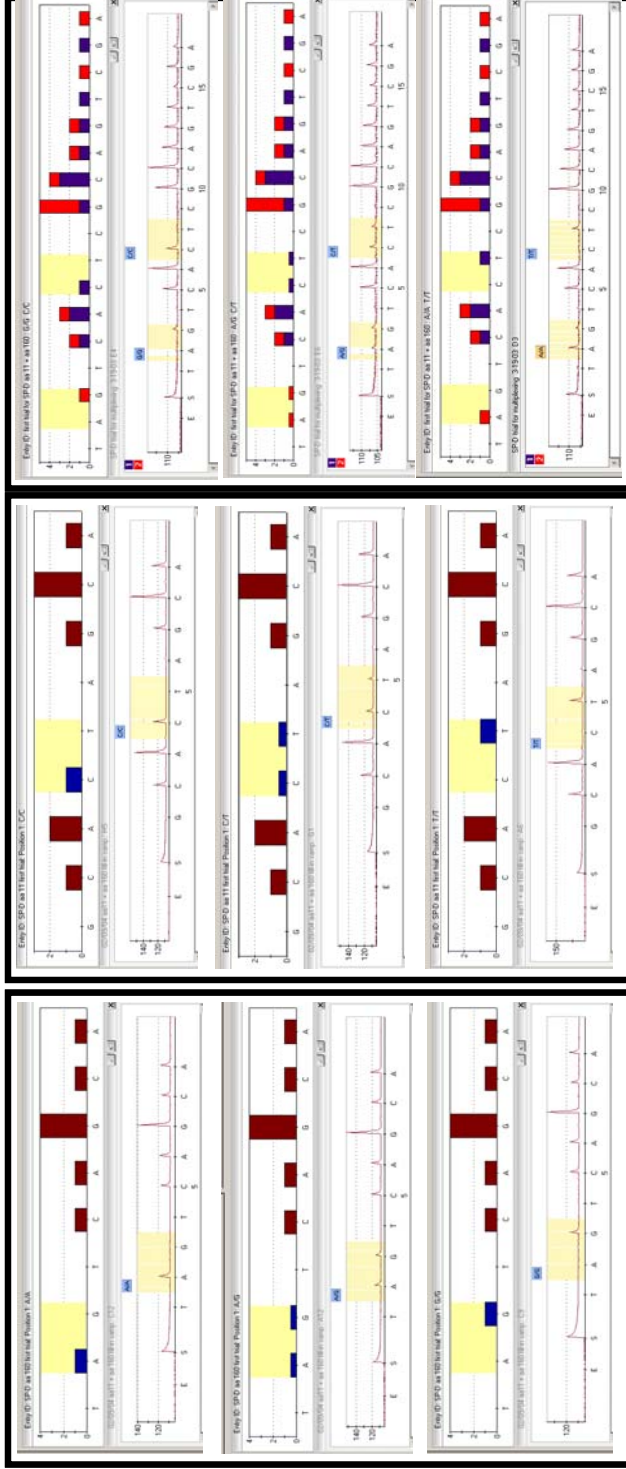
Simplex or multiplex PCR for DA11 and DA160 of SP-D was performed using primer pair 1301/1305 for DA11 and primer pair 1306/1307 for DA160. PCR profiles for SP-D both simplex and multiplex reaction were as follows: 95°C for 2 min followed by 35 cycles of 95°C for 30', 59°C for 30', and 72°C for 30'. This was followed by one cycle of 72°C for 5 min. An aliquot of the PCR reaction was run on 8% PAGE gel to verify size and quantity of PCR product, and most importantly to ensure the presence of a single PCR band in simplex PCR or two bands in multiplex PCR. These SNPs were pyrosequenced either in simplex or multiplex forward assays using oligos 1301 and 1308, respectively. Both SNPs were pyrosequenced either in simplex or multiplex forward assays as presented by histograms and programs in Figure 3.8.

Figure 3.8. Simplex and multiplex assays for SP-D

Sequence to analyze:
DA11: CAAC**T**GCCCCAGTGCT
DA160: A/GCAGGGGCAGCA
 Dispensation order: GCACTAGCA

Sequence to analyze:
DA160: A/GCAGGGGCAGCA
 Dispensation order: TAGTCAGCA

Sequence to analyze:
DA11: CAAC**T**GCCCCAGTGCT
DA160: A/GCAGGGGCAGCA
 Dispensation order: TAGTCACTCGCAGTCCA



Patterns expected from simplex pyrosequencing analysis (panel A and B) and multiplex analysis (panel C) of the two SNPs are represented in the histogram panels, and corresponding genotypes are in lower panel pyrograms. In simplex pyrosequencing, there are three possible patterns of nucleotide distribution (for each amino acid, panel A and B), while there are 9 possible combinations for multiplex pyrosequencing (only three are represented in panel C). The positions of the SNPs of interest are highlighted in yellow.

Expected patterns from simplex pyrosequencing analysis (panels A and B) and multiplex analysis (panel C) of two SNPs (DA11 and DA160) are presented as histograms. There are three possible patterns of nucleotide distribution in simplex pyrosequencing for each SNP (panel A for DA11 SNP; panel B for DA160 SNP), while nine combinations are possible for multiplex pyrosequencing (only three are represented in panel C). The positions of the SNPs of interest are highlighted in yellow. **Panel B:** The sequencing primer for the DA160 SNP (A/G) is right in front of the SNP, and this makes it the most straight forward type of sequencing reaction (as compared to that in panel A). Since the DA160 sequence (A/GCAGGGGCAGCA, Panel B), does not contain a T nucleotide, this can serve as negative control. Whenever possible, the negative control nucleotide is dispensed first. For example, in panel B, the nucleotide dispensation order is as follows: the negative control (T) is followed by a A and then a G nucleotide (constituting the SNP). These are followed by another negative control T nucleotide. The dispensation order then continues to sequence through a short segment of the surrounding DNA sequence, in this case CAGGGGCA (panel B), in order to ensure that the SNP analyzed is within the DNA segment of interest. Note the difference in peak height when a single nucleotide is dispensed versus four nucleotides dispensed in sequence. Panel portions I and III depict homozygous SNP patterns (A and G, respectively in panel B), while the II portion of each panel depicts a heterozygous SNP pattern (A/G in panel B). **Panel A:** For DA11 SNP, the sequencing primer is not right in front of the SNP site, as it is for the DA160 SNP (panel B). In this case three nucleotides (C, A, and A) precede the SNP (C/T). Therefore, in the sequencing reaction, a G nucleotide (negative control) is dispensed first. This is followed by dispensation of a C

and two As that sequence the CAA preceding the C/T SNP. A negative control A nucleotide is dispensed following the C/T sequence, and sequencing of surrounding bases continues as in panel B. **Panel C:** Both SNPs (DA11 and DA160) are sequenced at once. Due to the dispensation order set-up DA160 SNP is sequenced first (red bars), as in panel B, followed by a negative control T. The dispensation of the following C and A nucleotides, sequences through both DA11 and DA160 sequence at once (red and blue bars overlap). Dispensation of the C and T nucleotides, sequences through the DA11 SNP site (blue bars). This SNP site is followed by a negative control (C nucleotide). The remaining stretch of nucleotides is the surrounding sequence for both DA11 (blue bar) and DA160 (red bar).

3.3.3.1 Allele quantification for SP-D

In order to be able to accurately quantitate AA11 and AA160 gene frequencies, standard curves for peak height linearity were set up for both SNP sites. The actual allele frequencies for DNA pools were then adjusted using linear regression analysis and averaged values. It can be concluded that: **a)** allele frequencies for cRFLP-PCR genotyping of individual DNA samples (n=100) were in concordance with their respective allele frequencies from DNA sample pools (n=100) by pyrosequencing for both alleles of each SNP (DA11(C/T) or DA160(A/G); **b)** allele frequencies for genotyping of individual DNA samples with pyrosequencing (n=152) were in concordance with allele frequencies determined in DNA sample pools (n=152) for

DA160(A/G) alleles; **c**) an 8.9% discrepancy was observed in allele frequency of individual DNA samples genotyped with pyrosequencing (n=152) and allele frequency determined in DNA sample pools for DA11(C/T) alleles. This discrepancy may be due to small differences in DNA concentration and/or copy numbers between the two homozygous samples used to set up the standard curves. We speculate that this apparent discrepancy will be minimized by using several different homozygous samples for establishing the standard curve.

3.4 Conclusion

In summary, the goal of this study was to develop a novel, high-throughput method for surfactant protein genotyping that would facilitate the execution of ongoing studies in the laboratory. To this end, we have developed: **1**) simplex and multiplex pyrosequencing assays for SP-A1 and SP-A2, using gene-specific PCR (GS-PCR) templates for nested PCR reactions with biotinylated primers; **2**) molecular haplotype assay for AA19 and AA50 SNPs of SP-A1; **3**) simplex pyrosequencing assays for SP-B SNP B1580(C/T); **4**) simplex and multiplex pyrosequencing assays for SP-D SNPs DA11(C/T) and DA160(A/G), on templates generated in either simplex or multiplex PCR, respectively; and **5**) assays for allele frequency determination in genomic DNA pools for DA11(C/T) and DA160(A/G) SNPs of SP-D.

These assays should accelerate individual genotype analysis of surfactant proteins and greatly facilitate execution of pilot studies of SP-D SNPs using large DNA pools. Also, haplotype analysis derived using standard statistical models can be correlated with

experimental observations from SP-A1 pyrosequencing. Allele quantification in DNA pools will be particularly useful for rapid comparison of allele frequencies between disease and control groups in pilot studies and allow for fast determination of SNPs of interest that would be worth pursuing by individual genotyping. These methodologies will enhance our ability to more efficiently and reliably genotype samples from individuals with various pulmonary diseases.

3.5 References

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Chapter 4

Genetic Variants of Surfactant Proteins in Bronchopulmonary Dysplasia

4.1 Introduction

Bronchopulmonary dysplasia (BPD) is the most common cause of morbidity in prematurely born infants who require prolonged mechanical ventilation (Jobe, 1999). BPD is almost exclusively a disease of severely premature (24-28 weeks of gestation), extremely low birth weight (ELBW, less than 1000 gr) infants who have been exposed to high airway pressures and/or high inspired oxygen concentrations (Jobe, 2003; Makhoul, 2002). Despite the dramatic improvements in neonatal care the incidence of BPD is on the rise due to the increased survival of the smallest infants (Berger et al., 2004). BPD infants have structurally and biochemically immature lungs, the development of which is disrupted due to a number of harmful stimuli (Jobe and Ikegami, 1998; Jobe, 1999). These processes lead to an impaired alveolar and capillary growth and overall an abnormal lung structural development that can have long term consequences (Blayney et al., 1991; Filippone et al., 2003).

It has been noted that genetic factors may contribute to BPD (Jobe and Bancalari, 2001). Gene expression profile studies of an animal model of BPD implicated several genes in the pathogenesis of this disease (Wagenaar et al., 2004). Candidate gene and linkage analysis are approaches used to identify genes associated with multifactorial diseases such as BPD. Although there have been very few studies on the genetic background of BPD in humans, preliminary reports have shown an association between BPD and surfactant proteins (Makri et al., 2002; Pavlovic et al., 2006; Weber et al.,

2000). A recent case control study has demonstrated that the frequency of SP-B intron 4 deletion variant alleles is increased in BPD versus control infants even when essential external confounding factors, such as birth order, are included in the analysis (Rova et al., 2004).

Surfactant proteins (SPs) are good candidate genes of neonatal disease due to their role in lung development and maturation (Boggaram, 2003; Floros, 1990; Floros and Kala, 1998; Wilder, 2004). These proteins are components of pulmonary surfactant, a lipoprotein complex necessary for lung function (Floros, 1990). Surfactant protein (SP-) B and SP-C play important roles in surfactant structure and surface tension lowering properties (Weaver and Conkright, 2001), while SP-A and SP-D play a role in local host defense and regulation of inflammatory processes in the lung (Crouch, 2000; Phelps, 2001; Wright et al., 2001). Deficiency of surfactant can result in Respiratory Distress Syndrome (RDS) in premature infants. The lungs of these infants have been identified to have low SP levels (deMello et al., 1993; deMello et al., 1989). Association of genetic variants of SP-A and SP-B with RDS has been demonstrated by both case control and family-based linkage studies (Floros and Fan, 2001; Floros et al., 2001a; Floros et al., 2001b; Floros and Kala, 1998; Haataja et al., 2001; Haataja et al., 2000; Marttila et al., 2003a; Marttila et al., 2003b). Since BPD is commonly preceded by RDS, overlapping underlying mechanisms regulated by the same genetic factors may play a role in the etiology of both diseases.

In this report, we sought to perform a family based association study in order to identify whether alleles and/or haplotypes of surfactant protein genes (SP-A, SP-B, SP-C, and SP-D) and SP-B-linked microsatellite markers are susceptibility or protective factors

in BPD. Associations between single nucleotide polymorphisms (SNPs) of surfactant proteins and BPD were tested using Transmission Disequilibrium Test (TDT) (Spielman and Ewens, 1996) and Family Based Association Test (FBAT) (Horvath et al., 2001; Laird et al., 2000; Rabinowitz and Laird, 2000). The extended TDT (ETDT) (Curtis, 1997; Sham and Curtis, 1995) and multi-allelic FBAT (Horvath et al., 2004) were used to test for markers with multiple alleles.

To accelerate genotype analysis necessary for this study, we adapted our previously described PCR-cRFLP method (DiAngelo et al., 1999) to the recently introduced pyrosequencing method. Pyrosequencing is a primer extension based method that has been used for a variety of biological applications, including SNP genotyping, SNP discovery, haplotyping, insertion/deletion studies, methylation studies and allele frequency studies (Elahi and Ronaghi, 2004; Ronaghi, 2003). This flexible, high-throughput method of genotyping improves on our previously published PCR-cRFLP method (DiAngelo et al., 1999) by reducing the time and effort necessary for data acquisition.

4.2 Materials and Methods

4.2.1 Study population

The study population consisted of 60 families from Greece with 71 affected infants, as outlined in Table **4.1**. All infants required supplemental oxygen at 28 days of

life. All infants were intubated and required $FiO_2 > 0.30$ on day 1 of life. Sixty four of them had RDS and were treated with surfactant. Out of 71 affected infants, 52 fulfilled the criteria for BPD at 28 days as described by Bancalari et al (Bancalari et al., 1979). All infants with BPD at the 28 days category met the Bancalari et al. criteria of supplemental oxygen requirement 28 days after birth, persistent abnormalities in the chest radiograph, and tachypnea in the presence of rales or retractions. This subgroup of infants is referred to here as BPD_28D. Nineteen infants went on to be oxygen dependent at 36 weeks of post-menstrual age (Shennan et al., 1988). This subgroup of infants is referred to here as BPD_36W. When the two subgroups are combined for analysis, they are referred to as BPD_28D/36W. The study protocol was approved by institutional committees of the participating hospitals and written parental consent obtained from each family.

Table 4.1: Characteristics of population used in the study

<i>Number of children per family</i>	<i>BPD at 28 days</i>	<i>BPD at 36 weeks</i>
1	35[‡] + 6*	11[€] + 4*
2	4	2
3	1	0
Total number of families	46	17

* These families have a second child that was diagnosed with either RDS or the other form of BPD

[‡] Two families have only mothers and no fathers.

[€] One family has only mother and no father.

4.2.2 Isolation of DNA

DNA was extracted from 200 μ l of blood using the QIAamp DNA Blood Midi kit (Qiagen, Valencia, CA) according to manufacturer's instructions. DNA was eluted in the final step using 200 μ l of DNase free dH₂O (Ambion). Upon extraction DNA was stored at -20°C until further handling.

4.2.3 SNPs of interest

Table **4.2** shows the SNPs studied for all four SP genes and the SP-B linked microsatellite markers.

Table 4.2: SNPs of surfactant protein genes studied.

SP-A1	SP-A2	microsatellite markers	SP-B	SP-C	SP-D
AA19(C/T)	AA9(A/C)	D2S388	B-18(A/C)	CA138(A/C)	DA11(C/T)
AA50(C/G)	AA91(C/G)	D2S2232	B1013(A/C)	CA186(A/G)	DA160(A/G)
AA62(A/G)	AA140(C/T)	AAGG	B1580(C/T)		
AA133(A/G)	AA223(A/C)		B9306(A/G)		
AA219(C/T)					

SP-A1, SP-A2, SP-C, and SP-D SNP positions are based on the amino acid number of the amino acid sequence deduced from the cDNA (for SP-D the amino acid numbering starts after cleavage of signal peptide, while for SP-A and SP-C starts prior to the signal peptide cleavage). The first letter denotes the gene (A for SP-A, B for SP-B, C for SP-C, and D for SP-D), and the second letter (A) stands for amino acid. For SP-B SNPs, the numbers refer to the nucleotide position of the polymorphism (Kala, Koptides et al. 1997). The polymorphic variant of each SNP is given in parenthesis. For SP-A, haplotypes consisting of SNPs within the coding sequence that may or may not change the encoded amino acid are denoted as 6A or 1A for SP-A1 and SP-A2, respectively. For example, the SP-A2 1A2 haplotype referred to in Figure 4.1. consists of SNPs encoding amino acids 9, 91, 140, and 223 (AA9_C/AA91_G/AA140_C/AA223_C) (DiAngelo, Lin et al. 1999). The SP-D locus is in physical proximity with the SP-A locus (Hoover and Floros 1998) and in Figure 4.1 the co-transmission of the SP-D SNPs with the 1A² haplotype are shown.

4.2.4 PCR based converted Restriction Fragment Length Polymorphisms (cRFLP)

PCR-cRFLP was performed as previously described (DiAngelo et al., 1999) for all SPs markers for the first 100 samples of the 252 samples used in the study. SP-C genotyping and SP-B genotyping of B-18(A/C), B1013(A/C), and B9306(A/G) were performed for all samples using the PCR-cRFLP method.

4.2.5 Microsatellite genotyping

Microsatellite genotyping was performed for SP-B-linked microsatellites AAGG, D2S2232 and D2S388 as described previously (Kala et al., 1997).

4.2.6 Genotyping of surfactant protein SNPs by pyrosequencing

Pyrosequencing of SP-A, SP-B, and SP-D SNPs was performed as outlined in Chapter 3.

4.2.7 Statistical analysis

PedCheck program was used to check the compatibility of genotypes at each marker locus within families, prior to analysis (O'Connell and Weeks, 1998). Marker loci with incompatible parental and offspring genotypes, were treated as missing in those families. Association between alleles of surfactant protein genes and BPD was tested using the Transmission Disequilibrium Test (TDT) (Spielman et al., 1993) and Family

Based Association Test FBAT (Horvath et al., 2001; Laird et al., 2000; Rabinowitz and Laird, 2000).

TDT analysis was performed using GENEHUNTER (Kruglyak et al., 1996) (Whitehead Institute for Biomedical Research, MIT) to determine, transmission of individual surfactant protein (SP-A, SP-B, SP-C, and SP-D) marker alleles and SP-B-linked microsatellite marker alleles from heterozygous parents to affected offspring, and also to test for transmission of SP-B haplotypes of two, three, and four marker loci. In this analysis it is sufficient for only one parent to be heterozygous for the marker in order for a family to be considered informative and thus used in the study. Extended TDT (ETDT) analysis was performed to assess linkage of a multi-allele locus to the disease locus (Curtis, 1997; Sham and Curtis, 1995).

FBAT analysis was performed using the online program, <http://www.biostat.harvard.edu/~fbat/fbat.htm>. Bi-allelic FBAT analysis was performed for SP SNP markers and selected microsatellites (Horvath et al., 2001; Laird et al., 2000; Rabinowitz and Laird, 2000). Multi-allelic analysis, where association of the entire locus with disease is examined, was also performed for marker loci with multiple alleles (Horvath et al., 2001). All of the FBAT analyses were performed assuming an additive model. The minimum size [minsize] of FBAT analyses was set to 4 indicating that the test statistic was not computed when the number of informative families available was fewer than 4. Informative families refer to families with non-zero contribution to the FBAT statistic. For each FBAT marker, the Z-statistic and the corresponding p value are listed. A significant p value and a positive Z-statistic are indicative of a susceptibility marker allele for disease, while a significant p value and a negative Z-statistic is

indicative of a protective marker allele for disease. TDT and FBAT analyses were performed for the following groups: BPD_28D, BPD_36W, and BPD_28D/36W.

Stratified FBAT analyses were also performed within each group based on baby's steroid treatment, mother's steroid treatment, and baby's surfactant therapy. Haplotype analyses were performed using the FBAT program, which implements an EM based algorithm (Horvath et al., 2004). EM algorithm is a general method of finding the maximum-likelihood estimate of the parameters of an underlying distribution from a given data set when the data set is incomplete or has missing values. For single marker analysis, FBAT and TDT gave the same results, although some differences were observed in the haplotype analysis, perhaps due to subtle differences in the assumptions each method makes (see discussion). For both TDT and FBAT analysis, significant results were noted when p was smaller or approaching 0.01. Results were not corrected for multiple comparisons.

Inferred haplotype reconstruction was performed using FBAT, where the most likely haplotypes were assigned for each individual infant. FBAT compares the distribution of test statistics using the conditional offspring genotype distribution under the null hypothesis (Horvath et al., 2004). The offspring genotype probabilities were computed given the parental mating type.

4.3 Results

We examined transmission of individual SNPs for all five surfactant protein genes and SP-B-linked microsatellites markers (as listed in Table 4.2).

4.3.1 Family-based allele association analysis:

a) **SP SNPs marker alleles and SP-B-linked microsatellites:** Tables 4.3A and 4.3B, summarize the results of TDT and bi-allelic FBAT analysis, of SP-B and SP-B-linked markers, respectively. In both TDT and FBAT analysis, allele 6 of the AAGG marker was found to be transmitted more frequently to the affected offspring in BPD_28D/36W ($p=0.011$, Tables 4.3). The SP-B marker locus B-18(A/C) was found to be associated with BPD_36W group by both TDT and FBAT ($p=0.018$), where the A allele was associated with protection and the C allele with susceptibility to disease. No significant associations were found for individual allelic variants of SP-A, SP-C, and SP-D by either TDT or FBAT.

Table 4.3A: TDT analysis of SP-B marker alleles

Disease group	Markers	allele	# of transmitted alleles	# of non-transmitted alleles	χ^2	p-value	effect
BPD_36W [¥]	B-18(A/C)	C	14	4	5.56	0.0180	susceptibility
BPD_28D/36W [€]	AAGG	6	9	1	6.4	0.0114	susceptibility

Table 4.3B: Bi-allelic FBAT analysis of SP-B marker alleles

Disease group	marker	allele	Family #	Z	p value	minsize*	effect
BPD_36W [¥]	B-18(A/C)	C	11	2.357	0.0180	10	susceptibility
BPD_28D/36W [€]	AAGG	6	7	2.53	0.0114	10	susceptibility

Disease groups: 1) ¥ denotes BPD at 36 weeks post-conceptual age;

2) € denotes combined group of BPD at 28 days and BPD at 36 weeks post-conceptual age;

* Minsize denotes the minimum number of informative families necessary to compute the

b) SP marker alleles stratified for pre-natal/neo-natal treatments: Stratified bi-allelic FBAT analysis was performed to investigate the possible effect of pre-natal/neo-natal treatments on the outcome of BPD babies. Analyses were stratified according to the three co-variables: 1) lack or presence of pre-natal steroids, 2) lack or presence of post-natal steroids, and 3) lack or presence of surfactant therapy. Of significance is that in the combined BPD group and BPD_28D, allele 4 of the D2S388 marker locus was associated with protection from disease in babies that did not receive pre-natal steroid treatment ($p=0.01$). When the effect of post-natal steroids was investigated, allele 7 of the AAGG marker was associated with a protective effect (in BPD_28D, $p=0.014$) while allele AAGG_6 (in the combined BPD group, $p=0.019$) and B-18_C (in the BPD_36W, $p=0.018$) was associated with susceptibility to disease in babies treated with post-natal steroids. Allele AAGG_6 was also found to be related to susceptibility in the combined BPD group that received surfactant therapy ($p=0.008$).

These results are in concordance with those of non-stratified analysis, as would be expected, since the majority of infants have received post-natal steroid treatment. On the other hand, in the smaller subgroup of BPD_28D (babies that did not receive post-natal surfactant therapy) allele 4 of the D2S388 marker was associated with a protective effect from disease ($p=0.008$). The functional significance of these associations remains to be determined, particularly since the sample number of families under study is small for all, except for BPD at 28 days post-natal steroid treatment group.

4.3.2 Family-based haplotype association analysis SP-A and SP-D SNP haplotypes

a) **SP-A and SP-D SNP haplotypes:** Fig. 4.1 depicts the findings of association between haplotypes of SP-A2 and SP-D genes and disease groups by TDT. In the BPD_28D group the DA160(G)SP-A2(1A²) (p=0.005) and the DA11(C)DA160(G)SP-A2(1A²) (p=0.014) haplotypes of SP-D/SP-A2 were not transmitted to the affected child. Therefore these haplotypes may act as protective factors in the BPD_28D subgroup. No significant associations were found for SP-A and SP-D haplotypes in the BPD_36W subgroup. Neither ETDT, nor bi and multi-allelic FBAT analysis showed any significant association between individual SP-A alleles and BPD disease subgroups.

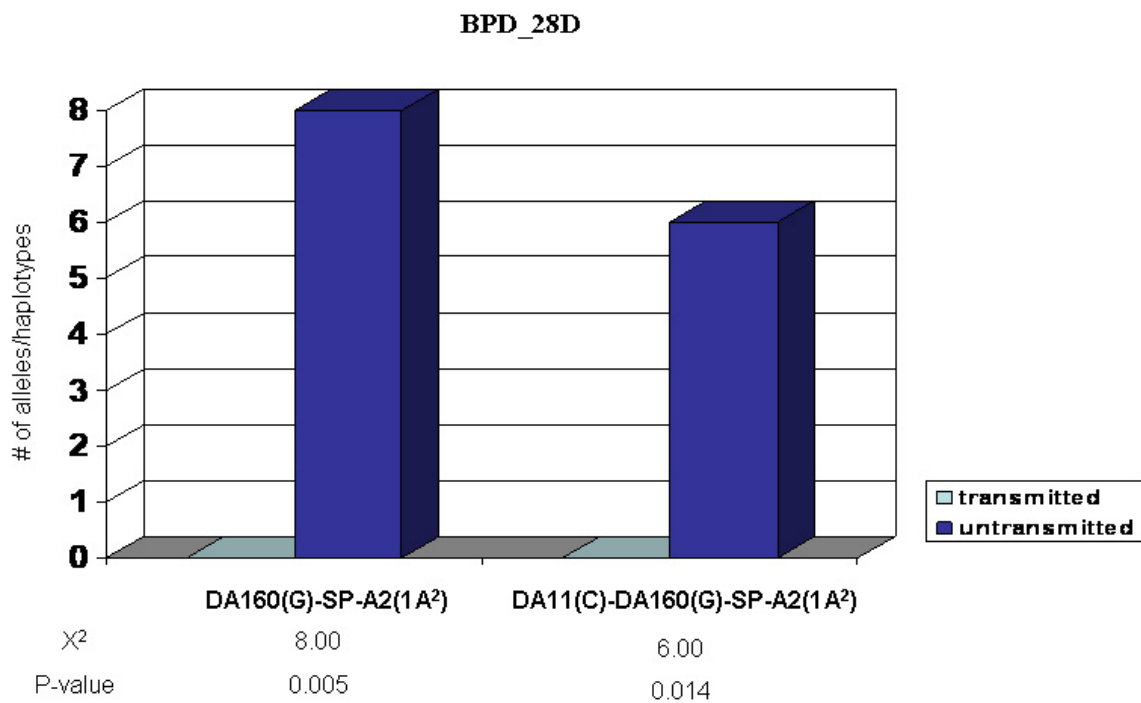


Figure 4.1: TDT analysis of SP-A haplotypes:

Significant haplotypes for BPD_28D/36W and BPD_36W are presented where p values approach 0.01. All 60 families were used in the analyses (n= 52 for BPD_28D; n=19 for BPD_36W).

b) Haplotype analysis of SP-B SNPs markers alleles and SP-B linked

microsatellite markers: Haplotype analyses were performed by both TDT (generated by Genehunter) and FBAT for two, three, and four markers at the time, and by FBAT for five, six, and seven markers at the time. The results of TDT and FBAT analyses are listed in Table **4.4A** and Table **4.4B**, respectively. Of note is that in most cases individual alleles found to be associated with either protection or susceptibility from disease in Table **3**, were also found to form haplotypes that were associated with the same overall effect.

SP-B-linked microsatellite markers were found in several disease associated haplotypes. The frequency of transmission of haplotype AAGG_6/B-18_C to babies in the BPD_28D/36W group was increased by TDT ($p=0.008$), as well as the AAGG_6/B-18_C/B1013_A haplotype ($p=0.014$). Moreover, the frequency of transmission of the AAGG_7-containing haplotypes to babies in all three subgroups was decreased by TDT ($p=0.02$), and the D2S2232_1/AAGG_7 haplotype was found less frequently in BPD_28D/36W group ($p=0.014$).

Table 4.4A: TDT analysis of SP-B marker haplotypes

Disease group	Markers	haplotype	# transmitted	# non-transmitted	χ^2	p-value	effect
BPD_28D/36W [€]	AAGG-B-18(A/C)	6 2	7	0	7	0.008	susceptibility
BPD_28D/36W	D2S2232-AAGG	1 7	0	6	6	0.014	protection
BPD_28D [*]	D2S2232-AAGG	2 3	13	3	6.25	0.013	susceptibility
BPD_36W [¥]	B-18(A/C)-B1013(A/C)	2 1	10	0	10	0.002	susceptibility
BPD_28D [*]	AAGG-B-18(A/C)-B1013(A/C)	7 2 1	8	21	5.83	0.016	protection
BPD_28D/36W	AAGG-B-18(A/C)-B1013(A/C)	6 2 1	6	0	6	0.014	susceptibility
BPD_28D/36W	AAGG-B-18(A/C)-B1013(A/C)	7 1 2	1	8	5.44	0.019	protection
BPD_36W	B-18(A/C)-B1013(A/C)-B1580(C/T)	2 1 4	6	0	6	0.014	susceptibility
BPD_36W	B-18(A/C)-B1013(A/C)-B1580(C/T)-B9306(A/G)	2 1 4 1	6	0	6	0.014	susceptibility

Disease groups: 1) * denotes BPD at 28 days; 2) ¥ denotes BPD at 36 weeks post-conceptual age; 3) € denotes combined group of BPD at 28 days and BPD at 36 weeks post-conceptual age. For B-18(A/C), B1013(A/C), and B1580(C/T): 1 denotes A variant, 2 (C variant), and 4 (T variant). # transmitted and # non-transmitted denotes the # of haplotypes.

Table 4.4B: FBAT analysis of SP-B marker haplotypes

Disease group	marker	allele	Family #	Z	p value	minsize*	effect
BPD_36W [¥]	B-18(A/C)-B-1013(A/C)	2 1	7	2.554	0.014	4	susceptibility
BPD_28D [*]	D2S388-D2S2232-AAGG	5 2 3	5	2.558	0.010	4	susceptibility
BPD_28D/36W	D2S2232-AAGG	1 7	7	-2.376	0.017	4	protection
BPD_28D/36W [€]	AAGG-B-18(A/C)	6 2	6	2.333	0.019	4	susceptibility
BPD_28D/36W	AAGG-B-18(A/C)-B1013(A/C)	6 2 1	6	2.333	0.019	4	susceptibility
BPD_28D/36W [€]	D2S388-D2S2232-AAGG	5 2 3	5	2.558	0.010	4	susceptibility
BPD_28D/36W [€]	D2S388-D2S2232-AAGG-B-18(A/C)	5 2 3 1	5	2.558	0.010	4	susceptibility

Disease groups: 1) * denotes BPD at 28 days; 2) ¥ denotes BPD at 36 weeks post-conceptual age; 3) € denotes combined group of BPD at 28 days and BPD at 36 weeks post-conceptual age. For B-18(A/C), B1013(A/C), and B1580(C/T): 1 denotes A variant, 2 (C variant). * Minsize denotes the minimum number of informative families necessary to compute the test statistics.

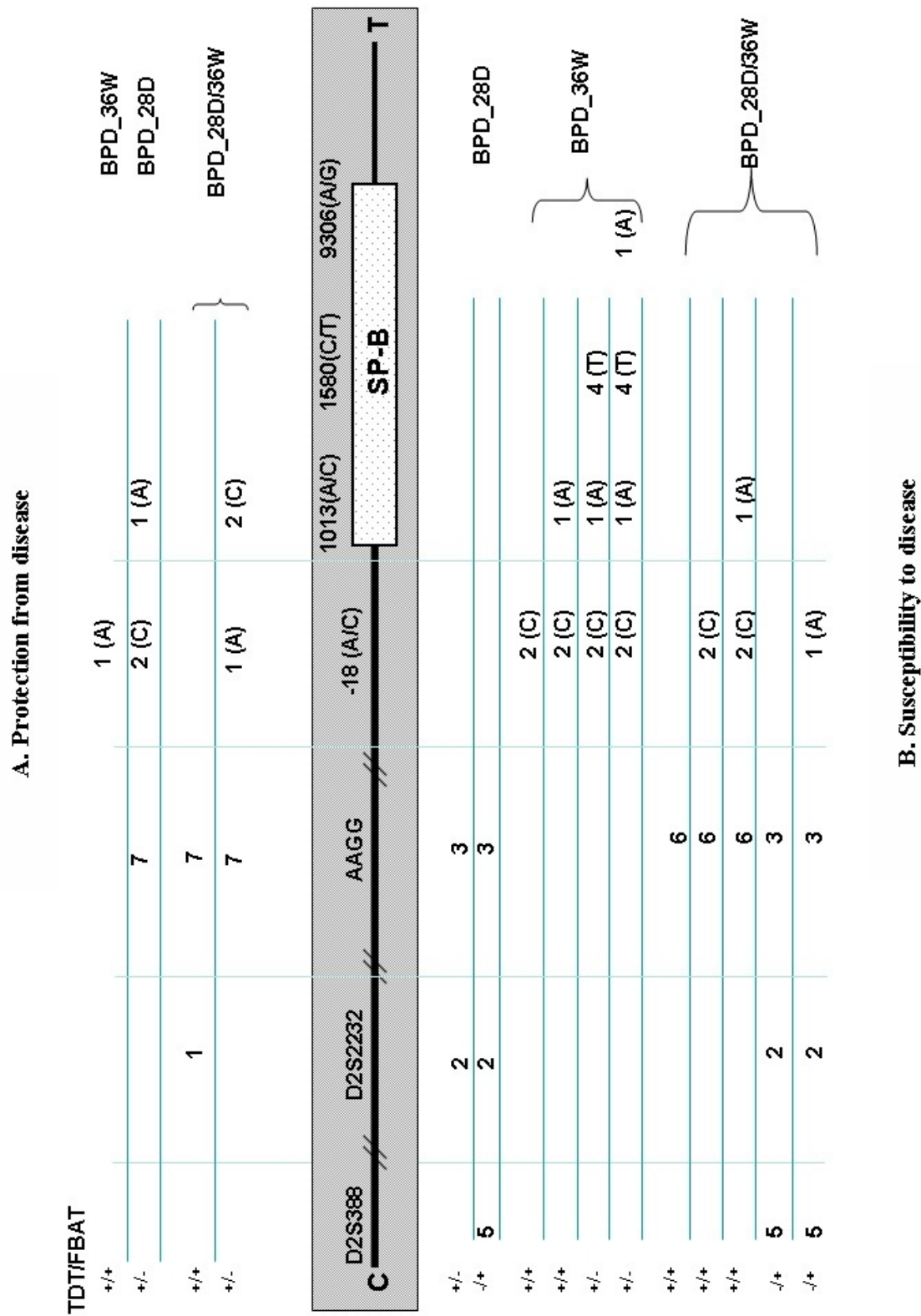
Figure 4.2 provides a schematic presentation of haplotypes among different disease subgroups by the two different tests, TDT and FBAT. Presence of AAGG₆ allele in haplotypes was consistently associated with susceptibility to disease, while the presence of AAGG₇ allele was associated with a protective effect from disease. Although, AAGG₃ allele was found in three susceptibility haplotype combinations, this allele may not be informative as the AAGG₆ and AAGG₇ alleles may be, because it

was also found in protection associated haplotypes (not shown: $p \leq 0.05$). The AAGG_3 containing haplotypes are, D2S2232_2/AAGG_3 found to associate with BPD_28D group by TDT, D2S388_5/D2S2232_2/AAGG_3 found to associate with both the BPD_28D and BPD_28D/36W by FBAT (in both $p=0.01$), and D2S388_5/D2S2232_2/AAGG_3/B-18_A found to associate with the BPD_28D/36W group by FBAT ($p=0.01$).

SP-B SNP marker haplotype, B-18_C/B1013_A was associated with susceptibility by both TDT and FBAT analysis in BPD_36W ($p=0.002$ and $p=0.014$, respectively) whereas B-18_C/B1013_A/B1580_T and B-18_C/B1013_A/B1580_T/B9306_A haplotypes were associated with susceptibility in the same group only by TDT.

Overall, both individual allele and haplotype analysis of SP-B markers indicate an association of the microsatellite marker AAGG_6 allele with susceptibility to disease in infants affected with BPD_28D/36W. SNP marker B-18(A/C) alleles were associated with the more severely affected babies, where the C allele associated with susceptibility and the A allele with protection from BPD_36W.

Figure 4.2: Schematic presentation of susceptibility and protection haplotypes.



A schematic representation of four SP-B SNPs B_18(A/C), B1013(A/C), B1580(C/T), B9306(A/G), and SP-B-linked microsatellite markers, is shown in the middle of the Figure. The centromeric (C) and telomeric (T) orientation of this DNA segment is also noted. The microsatellite marker AAGG is 27 kb from SP-B (Kala et al., 1997). Markers D2S2232 and D2S388 are 120 kb and 195 kb from SP-B (according to the current version CH 2 map http://www.ensembl.org:80/Homo_sapiens/geneview?gene=ENSG00000168878). Above the schematic presentation, protection haplotypes are shown (Table 4.4A) and below the schematic presentation susceptibility haplotypes are noted (Table 4.4B). The nucleotide for each SNP is also noted by a number: A=1, C=2, G=3, and T=4. For example in BPD_36W susceptibility haplotype B_18_C/B1013_A/B1580_T/B9306_A is also noted as haplotype 2/1/4/1. The alleles for each microsatellite marker locus have been defined previously (Kala et al., 1997) and are noted by numbers 1, 2, 3, etc. The haplotypes noted here are described in detail in Tables 4.4A and 4.4B. Only significant findings where p value approaches 0.01 are presented. The plus (+) sign in the left hand column denotes a haplotype that was found by either TDT or FBAT, while the minus (-) sign denotes that a particular haplotype was not found to have a significant p value by either TDT or FBAT.

4.4 Discussion

BPD is a multifactorial disease of preterm infants where multiple risk factors contribute to permanent pathophysiologic changes. Numerous genes required for neonatal lung adaptation are likely to be involved in the etiology of this disease. Since surfactant proteins are essential for normal lung function and play a role in both gas exchange and innate immune mechanisms of the lung, they are likely to play a role in the pathogenesis of BPD. Susceptibility to RDS, the common precursor of BPD has been shown to be linked to surfactant proteins, and SP-A in particular (Floros and Fan, 2001; Floros et al., 2001a; Floros et al., 2001b; Haataja and Hallman, 2002; Haataja et al., 2001; Ramet et al., 2000). Recently, in a case-control study, an SP-B intron 4 deletion variant was identified as a risk factor in BPD (Rova et al., 2004). In order to avoid spurious associations secondary to population stratification, characteristic of case-control studies, we performed family based association analyses. Such analyses are not confounded by population characteristics and provide a more powerful analysis even in small sample sizes (Shih and Whittemore, 2002). The data from TDT and FBAT (using two powerful family based association tests) revealed, a) association of SP-B with susceptibility to BPD_36W; and b) association of the microsatellite marker AAGG_6 with susceptibility in the BPD_28D/36W group; c) Ten susceptibility and one protective haplotypes for SP-B and SP-linked microsatellite markers were detected for all three subgroups (BPD_28D, BPD_36W, and BPD_28D/36W); d) TDT analysis revealed two SP-A/SP-D susceptibility haplotypes for BPD_28D.

Of the 71 infants in the study, 52 had the less severe BPD_{28D}, while 19 infants were affected with the more severe BPD_{36W}. Although the sample size in BPD_{36W} may be small, the use of the family based association approach may mitigate the margin of error due to small sample size (Shih and Whittemore, 2002). However, it should be noted that the majority of observations in the BPD_{28D/36W} group overlap with those for the BPD_{28D} sub-group, perhaps due to the predominance of the sample size of this group.

The SP-A2/SP-D haplotypes found to associate with protection from BPD_{28D} have not been previously studied. The lack of association with SP-A in the Finnish BPD study is most likely due to the fact that their study group consisted of more severely affected babies (86 infants had BPD at 36 weeks, and 21 had BPD at 28 days). The inflammatory components that appear to be at work in the early stages of the disease could be modulated by SP-A and SP-D (Jobe and Ikegami, 2000), which may be consistent with their immune related functions (Jobe and Ikegami, 2000; Phelps, 2001). Although it is unknown how the SP-A2(1A²)/SP-D haplotypes may protect from BPD, we speculate that this protection is likely due to adequate levels of SP-A and/or SP-D at a critical developmental stage of the lung. *In-vitro* studies have shown genotype-dependent levels of SP-A mRNA (Wang et al., 2003b) as well as differences among SP-A2 variants in 3'UTR mediated expression (Karinch et al., 1997). It is unlikely that the association reflects functional differences, because the SP-A2, 1A² variant is identical in the amino acid sequence of the mature protein with several other SP-A2 variants (DiAngelo et al., 1999). With regards to SP-D, the DA11(C/T) SNP was recently identified to affect oligomerization, function, and serum concentrations of the protein (Leth-Larsen et al.,

2005). Individuals homozygous for the DA11(C/C) variant were found to have significantly lower SP-D serum levels than the DA11(T/T) homozygous individuals. Polymorphisms of SP-A were not investigated in this study (Leth-Larsen et al., 2005).

SP-B SNP variant alleles and/or haplotypes on the other hand were only associated with the BPD_{36W}. Specifically, the C variant of the B-18(A/C) polymorphism within the 5'UTR of SP-B was found, either alone or in various haplotypes, to associate with susceptibility to BPD_{36W} by both TDT and bi- and multi-allelic FBAT. The finding of the SP-B intron 4 polymorphism association with BPD in a Finish study group (Rova et al., 2004), along with the present findings support a role of SP-B in BPD pathogenesis. Recently, a dual role for SP-B, as a protein essential for surfactant function and as an anti-inflammatory mediator, has been proposed that could explain involvement of this protein in BPD (Epaud et al., 2003) at multiple levels. Regulatory roles in SP-B gene expression of SP-B that B-18(A/C) SNP and intron 4 variants (Rova et al., 2004) may have can only be speculated. Inadequate levels of SP-B may contribute to the progression of BPD especially in the later stages of the disease progression. Infants that require continued ventilatory support, as in BPD, experience transient episodes of surfactant dysfunction that are associated with deficiency in SP-B (Merrill et al., 2004).

Multiallelic SP-B-linked microsatellite markers were also used to increase resolution in the definition of the genetic background of BPD subgroups. Of the three markers studied, AAGG and specifically allele AAGG₆ was found to associate with BPD_{28D/36W}. This microsatellite marker is located 26 kb upstream from SP-B, and allele 6 consists of 24 AAGG repeats (Kala et al., 1997). No difference in transcription

factor binding among the alleles of AAGG was found by current bioinformatics techniques (JASPAR transcription factor data base). It is currently unknown whether this marker imparts a direct functional effect on SP-B in relation to BPD, or whether it is linked to another gene and/or to a functional genetic variant(s) that is located in, or close to the SP-B gene.

To identify genes between the microsatellite marker AAGG locus and the SP-B SNPs an ensemble gene search (www.ensembl.org) was conducted. The only known gene located within this region is granulysin, a T cell activation gene. Although granulysin has not been implicated in the pathogenesis of BPD, this gene plays a role in immunity against intracellular pathogens, and may therefore be involved in the modulation of inflammatory responses in BPD (Pena et al., 1997). However, the disease locus does not have to be limited to this region, and may in fact be much further upstream or downstream. Although association data may identify and implicate genes in the clinical course of a disease, functional analysis of variants of interest should be conducted to better understand their role in BPD pathogenesis.

Multilocus haplotype association analyses are advantageous because they provide a stronger power of association than the single nucleotide methods (Zhao et al., 2003). The results from these analyses confirmed and extended on the trends seen by individual SNP analysis. Combinations of B-18_C, B1013_A and B1580_T were found in susceptibility haplotypes by both TDT and FBAT. Moreover, significant three marker linkage disequilibrium for B-18(A/C), B1013(A/C), and B1580(C/T) has been reported in several populations (Liu et al., 2003). In the Greek population the frequency of the B-18_C/B1013_A/B1580_T and B-18_A/B1013_C/B1580_C haplotypes of these three

marker loci is the same (0.274) (Liu et al., 2003). Therefore, association of the CAT haplotype of these marker loci with susceptibility to BPD in this study is not an artifact of the overall population characteristic, but it may be rather disease related.

Although FBAT and TDT analyses identified similar alleles and/or haplotypes associated with BPD, different findings were also observed between these two analyses. For example, FBAT revealed haplotypes with additional upstream loci (D2S388_5/D2S2232_2/AAGG_3 and D2S388_5/D2S2232_2/AAGG_3/B-18_A) for BPD_28D and BPD28D/36W. The basis for the observed differences is currently unknown, but it may be due to differences in approach and/or the underlying assumptions of the two tests (TDT and FBAT). Haplotype TDT uses the maximum likelihood approach based on the Lander-Green hidden-Markov-model (HMM) (Kruglyak et al., 1996), while the FBAT uses a weighted conditional approach (Horvath et al., 2004). Therefore, the differences in results are more likely to occur for low frequency haplotypes. Moreover, haplotype FBAT assumes no recombination among the marker loci (Horvath et al., 2004) and haplotype TDT assumes no linkage disequilibrium (Kruglyak et al., 1996). Recent studies have used these two tests in complement for either single nucleotide polymorphism analysis (Bougacha-Elleuch et al., 2004; Manor et al., 2004; Wong et al., 2004) or haplotype analysis (Deffenbacher et al., 2004). However, the partial discrepancies observed in the results by these two different family based association tests in these studies as well as the results presented in this study, point to the need for future studies to compare different analytical methods in parallel. Such efforts could identify differences in approach and assumption, understand the implications of such differences and clearly define advantages and disadvantages of each approach.

Due to the presumed overlap between BPD and RDS, it was hypothesized that similar genetic association findings would be seen in these two diseases (Floros et al., 2001a; Haataja et al., 2000). It was, therefore, surprising that the B1580_T variant was found in the susceptibility haplotypes in the present study. This SNP codes for the non-glycosylated Ile (ATT) variant (Wang et al., 2003a). This variant has been associated with decreased risk of RDS in blacks in the presence of the SP-A1 allele (6A³) (Floros et al., 2001a), whereas the glycosylated Thr (ACT) variant has been shown to associate with susceptibility to RDS especially in the first born and male infant in a study of prematurely born twins (Floros et al., 2001a; Marttila et al., 2003a). However, this is consistent with previous observations where factors that may protect from RDS, increase risk for BPD. For example, prenatal inflammation (chorioamnionitis) and increased post-natal lung inflammation associate with a decreased incidence of RDS among preterm babies, but these are associated with an increased risk for chronic lung injury following preterm delivery (Watterberg et al., 1996).

In summary, factors involved in predisposing infants to develop BPD following RDS are likely complex and presumably involve several loci with small effects, effectively making the traditional genetic studies difficult. In the present study, using two different family based association tests, we show that the SP-A/SP-D locus associates with BPD_28D, and the SP-B and SP-B-linked microsatellite marker loci with BPD_36W and BPD_28D/36W. However, larger sample size studies with various BPD subgroups are needed to extend these results and enable localization and characterization of genetic factors.

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Chapter 5

Conclusion

5.1 Summary of Major Results

The following is the summary of major findings, observations and conclusions reported in **CH 2 with respect to Specific Aim I: Development and Characterization of a Human Fetal Lung Xenograft Model:**

1. Morphological analysis revealed graft growth and development at both renal subcapsular and subcutaneous graft sites.
2. Grafts underwent macroscopic growth with increased time of transplantation.
3. Grafts underwent morphological changes in a developmental stage-specific manner, where each stage of lung development occurred over approximately a one week long period in the graft.
4. Tissue differentiation was characterized by cytodifferentiation accompanied by the thinning of epithelium and neo-vascularization.
5. Morphological analysis showed that 3-6 day grafts were most consistent with the pseudoglandular stage of lung development, 6-14 day grafts were representative of the canalicular stage, and 14-28 day grafts were consistent with the sacular stage of development. Longer graft times resulted in dilation of alveoli characteristic of lung distention due to fluid accumulation.
6. Verhoef's stain revealed abundance of collagen fibers in all graft tissues. Elastic fibers were absent in starting fetal lung tissue, 6 day, and 10 day old grafts, as would be

expected in pseudoglandular and canalicular stages of *in utero* development. Elastic fibers were observed as punctuate dots at the tips of developing alveolar crests in 14 day grafts, as is consistent with saccular stage of development.

7. Cellular proliferation was present throughout graft development as shown by immunocytochemistry (ICC) for the proliferating cell nuclear antigen (PCNA). PCNA immunostaining was most intense in 10 and 14 day grafts, indicative of active cellular growth and differentiation of tissue. The intensity of PCNA immunostaining was decreased in later stages of graft development, indicative of terminal cellular proliferation and differentiation.

8. Starting fetal lung tissue and graft tissues harvested at earlier time points after engraftment, were glycogen-rich and devoid of lamellar bodies as observed by electron microscopy. Lamellar bodies were first observed in 14 day old grafts. Starting fetal lung tissue and earlier graft tissues were glycogen-rich and devoid of lamellar bodies. Number of lamellar body positive cells and lamellar bodies per cell was dramatically increased in 21 day grafts. Numerous secreted lamellar bodies were present in saccular spaces, but no tubular myelin structures were seen.

9. mRNA and protein expression of surfactant proteins were detected at all graft time points examined by reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry (ICC), respectively. Localization of SP-D was limited to apical surface of cells, whereas SP-A, SP-B, and SP-C displayed more uniform patterns of distribution within the cells.

10. Presence of blood vessels of human origin was detected in 3 day grafts by ICC for PECAM-1. Formation of blood vessels was observed in 6 day grafts. PECAM-1 staining was significantly fainter in 14 day grafts and hardly detectable in later day grafts.

These studies conclude that the fetal lung xenograft model mimics lung development and can be used as a viable model of human fetal lung development. Moreover, this model could serve as an unique *in vivo* study system for human neonatal lung diseases. The relevance of a human model is emphasized by the fact that the time course of developmental stages and regulation of surfactant components are in part species-specific.

The following is the summary of findings reported in **CH 3 with respect to Specific Aim II: Pyrosequencing technology as a method of genotyping of surfactant proteins:**

The following reactions/assays were developed:

1. Gene-specific PCR for SP-A1 and SP-A2 templates that can accommodate genotyping from inadequate DNA preparations.
2. Nested PCR reactions for pyrosequencing of SP-A1 and SP-A2 SNPs.
3. Simplex pyrosequencing assays for AA19(A/C), AA50(C/G), AA62(A/G), AA133(A/G), and AA219(C/T) single nucleotide polymorphisms (SNPs) of SP-A1 and AA9(A/C), AA91(C/G), AA140(C/T), and AA223(A/C) SNPs of SP-A2.
4. Multiplex pyrosequencing assay for AA19(A/C) and AA50(C/G) SNPs of SP-A1.
5. Multiplex pyrosequencing assay for AA133(A/G) and AA219(C/T) SNPs of SP-A1.

6. Multiplex pyrosequencing assay for AA140(C/T) and AA223(A/C) SNPs of SP-A2.
7. Molecular haplotyping analysis for AA19(A/C) and AA50(C/G) SNPs of SP-A1.
8. Simplex PCR and simplex pyrosequencing assays for B1580(C/T) SNP of SP-B
9. Simplex and multiplex PCR and simplex and multiplex pyrosequencing assays for DA11(C/T) and DA160A/G) SNPs of SP-D
10. Allele frequency assays for DA11(C/T) and DA160A/G) SNPs of SP-D in DNA pools

These studies demonstrate the ability to use pyrosequencing methodology for surfactant protein SNP genotyping, haplotyping, and allelic frequency studies. Therefore, the pyrosequencing assays developed as a part of this thesis, dramatically decrease the time necessary for individual genotyping of surfactant proteins and greatly facilitate execution of pilot studies of SP-D SNPs using large DNA pools. Also, molecular haplotyping of AA19(A/C) and AA50(C/G) SP-A2 SNPs provides another important tool for analysis of surfactant protein genetics. Overall, pyrosequencing is accurate and reliable and avoids the common pitfalls of the converted PCR genotyping method. Moreover, the pyrosequencing assays were effectively used for the execution of Specific Aim II b.

The following is the summary of major findings, observations and conclusions reported in **CH 4 with respect to Specific Aim II b: Genetic variants of surfactant proteins in Bronchopulmonary dysplasia (BPD):**

1. Variants of surfactant proteins are involved in pathogenesis of BPD.
2. B-18_A allele of SP-B is a protection factor in BPD at 36 weeks, while B-18_C allele is a susceptibility factor in BPD_36W by both TDT and Family Based Association Test (FBAT).
3. Allele 6 of AAGG is a susceptibility factor in combined group BPD at 28 days and BPD_36W (BPD_28D/36W) by both TDT and Family based association test (FBAT).
4. Linkage of B-18(A/C) with BPD_28D/36W was confirmed and extended by multi-allelic Family based association test (FBAT).
5. Alleles B-18_A and AAGG_7 were consistently found in protection haplotypes, while alleles B-18_C and AAGG_6 were consistently found in susceptibility haplotypes by Transmission Disequilibrium test (TDT).
6. Findings of stratified analysis for 1) lack or presence of pre-natal steroids, 2) lack or presence of post-natal steroids, and 3) lack or presence of surfactant therapy; were in concordance with those of non-stratified analysis.
7. Stratified analysis for the lack or presence of surfactant therapy, revealed association with allele 4 of D2S388 in the subgroup of babies with BPD_28D.
8. No significant associations were found between SNPs of SP-C and SP-D and subgroups of BPD.

These studies demonstrate linkage between individual variants and haplotypes of surfactant proteins and subgroups of BPD in a family-based association study. Susceptibility and protection alleles and haplotypes of these genes were identified with respect to their association to BPD. Moreover, association of SP-B with only the severe

form of disease supports the original hypothesis that inflammation-related insults are determining factors in the less severe form of disease (BPD_28D), while structurally related proteins, such as SP-B, play a role in the more severe form (BPD_36W).

5.2 Discussion

Since lung organogenesis is a late gestational event, respiratory abnormalities are among the most common complications of premature birth. The rise in the rate of premature births and infants affected with neonatal pulmonary disease puts the need to better understand mechanisms of lung development at the forefront of neonatal research. Lung maturity, coupled with the capacity to produce surfactant at birth, is essential for successful adaptation of the newborn fetus to extrauterine life (Avery and Mead, 1959). Premature babies are born with lungs in the saccular stage of lung development, making it the critical stage to study with respect to developmental processes and regulation by pharmacologic agents. Thus, the damage incurred in neonatal respiratory distress is superimposed upon the saccular stage of the underdeveloped lung. Factors involved in the regulation of this critical stage of lung development have not been elucidated, mostly due to the lack of stage-appropriate models.

Human lung development studies have thus far been limited to fetal lung explants (Beers et al., 1995; Karinch et al., 1998; Mendelson and Boggaram, 1991; Solarin et al., 1997). Although the knowledge gained from this *in vitro* model has been enormously valuable, the dramatically accelerated tissue development in this system prevents identification of saccular stage-appropriate developmental mechanisms. In addition, lung

development and regulation of surfactant components have been extensively studied in various animal species. However, the time course and staging of development vary among different species, emphasizing the importance of a human model system. Moreover, differences in genetics of surfactant proteins between humans and that of commonly used animal species, further underlie a critical need for a model that mimics human *in utero* development.

Immaturity of the lung is the main determinant of neonatal lung disease incidence and outcomes. Recent improvement in antenatal and neonatal intensive care have significantly increased the survival rates of very preterm babies (Jobe, 2003). However, the rate of preterm birth has steadily increased over the last decade (Blickstein and Keith, 2002), leading to an increased number of infants with neonatal lung disease and children with potential long-term complications of neonatal respiratory distress. Production of functional surfactant is an essential function of the maturing lung, since appropriate production and regulation of the surfactant complex is essential for proper lung development and function (Ballard et al., 2003). Although production of surfactant components begins early in fetal lung development, secretion of functional surfactant does not occur until the saccular stage (Clements, 1977; Oulton et al., 1980). Therefore, prematurely born babies are deficient in functional surfactant components and consequently experience respiratory distress at birth (Jobe, 1991). Furthermore, there is significant variability in the amount of surfactant expression (Floros et al., 1991; Karinch et al., 1997) and rate of surfactant secretion (Hallman et al., 1992; Hallman et al., 1989) in individual fetal lungs.

In order to further study human lung development, an appropriate model that would provide for human stage specific studies in an *in vivo* environment is needed. Such a model would provide the hormone and growth factor conditions necessary for appropriate lung organogenesis that would approximate *in utero* development as close as possible. The fetal lung xenograft model presented in Chapter 2 of this thesis approximates this ideal, although not without drawbacks. While previous studies involving human fetal lung grafts have focused on the endpoint of lung tissue development, the model presented here allowed us to investigate the progression and timing of each of the four stages of lung development.

The sequential differentiation through each stage has been previously observed in mouse allograft models, but not in human graft models (Schwarz et al., 2000; Vu et al., 2003). When a mouse renal subcapsular allograft model was compared directly to mouse fetal lung explants, extensive proximal and distal differentiation of the epithelium was observed throughout graft development which closely followed *in utero* development, while explant cultures ceased to develop and started to deteriorate after five days. Direct comparison of graft development to fetal lung explant development was not performed in this study. Based on previous studies of fetal lung explants it can be deduced that changes that occur in the xenograft model over a period of three weeks occur in a fetal explant in about three days, and while the graft systems continues to be viable after prolonged grafting periods, explant cultures deteriorate after about five days (Ballard et al., 2003).

The viability of graft tissues is supported by ICC for proliferation marker PCNA. These tissues were shown to have highest proliferation rates in the canalicular and

saccular stages indicative of appropriate tissue differentiation and growth. The blood supply that is necessary for maintenance of advanced growth of tissues is most likely provided by the host in this model, since the staining for human specific endothelial cell marker was virtually absent in saccular stage grafts. Embryonic vasculogenesis of human lungs is poorly understood. As shown in studies of mouse allograft models, both endogenous endothelial cells and host vessels can provide circulation to the graft (Schwarz et al., 2000; Vu et al., 2003). The vasculogenesis of human lungs in the model presented in Chapter 2 remains to be further explored. It would be of interest to determine the timing and pattern of host (mouse) vascularization of the lung. From the findings presented here it would be expected that as the signal for human PECAM-1 endothelial cell marker is lost, the signal for mouse PECAM-1 should be observed.

With respect to timing of tissue development, other human graft studies have not focused on timing of each gestational stage. However, most studies have shown that grafts reach the sacculo-alveolar stage of development after 8 weeks of gestation, regardless of the gestational age of the starting tissue (Filali et al., 2002; Groscurth and Tondury, 1982; Peault et al., 1994; Phillips and Gazet, 1969; Pilewski et al., 1994). Therefore, the gestational age of the starting lung tissue is not likely to play a significant role, as long as the tissue is in pseudoglandular stage of development. This is an important factor considering that human fetal tissues are extremely scarce and that a demand to match exact gestational ages would further complicate the practical utility of the model.

In addition, direct comparison of subrenal and subcutaneous grafts was not examined in any of the previous mouse or human graft studies (Filali et al., 2002; Peault

et al., 1994; Phillips and Gazet, 1969; Pilewski et al., 1994; Schwarz et al., 2000; Vu et al., 2003). Observations from the model described in this work, indicate that subcutaneous grafts are comparable to the renal subcapsular grafts, and due to the easier methodology and fewer growth space restrictions are the preferred model of study.

With respect to the expression of surfactant proteins, all four proteins were detected in the model throughout graft development. Presence of surfactant proteins has not been investigated in other human lung graft models. Pattern of pro-SP-C expression in the mouse renal subcapsular allograft model is in concordance with the patterns of SPs expression observed in the presented model, where grafts in early stages of lung development show positive immunostaining throughout tissue (Vu et al., 2003). With advancement of graft development, SP-positive pre-type II cells differentiate into SP-negative type I cells. This decrease in number of SP immuno-positive cells is in concordance with *in utero* expression patterns. However, the exact amount of surfactant message/protein expressed in graft tissues is not known due to limited amount of graft tissue available for study. This would be of interest in order to be able to study regulation of surfactant system in this model.

However, the small size of grafts limits their use in full scale experiments. Particularly important would be to analyze the same graft tissue in multiple assays. However, this is not possible with the current size of grafts harvested from nude mice. Although subcutaneous grafts are in this respect advantageous compared to renal subcapsular grafts, the amount of graft tissue available is limited. Currently available technologies do not provide for obvious ways to accommodate larger graft size/growth. Grafting larger pieces of tissue is not a viable option, presumably because the host blood

supply is not able to uniformly penetrate the grafts, thus leading to different gradients of differentiation throughout tissue, as has been observed in this study. Differential sampling of starting lung tissue further adds diversity to tissue development. In order to resolve this issue, the use of the dissecting microscope has been tried with limited success.

Another drawback of the model is the inappropriate alveolar enlargement in the alveolarization stage of development. Presumably, the accumulation of alveolar fluid causes excessive dilatation of alveoli after 28 days of graft development. However, up to saccular stage of development, differentiation of grafts corresponds to that of *in utero* development.

Since study of the regulation of the surfactant system has thus far been limited to fetal lung explants, it would be of interest to examine it in the graft model described here. However, the scarce amount of tissue available and the variability of sampling makes mRNA quantification of specific SPs at specific developmental stages challenging in fetal lung grafts. Particularly in the case of SP-A, where significant variability of overall expression and differences in the relative expression of the two SP-A genes have been observed, using the graft model developed here, it could be possible and of great interest to follow relative amounts of each SP-A gene throughout gestational development in each individual tissue. This would provide further insight into the specific functional consequences of different SP-A genetic variants.

In order to understand preterm birth and neonatal lung disease, comprehensive studies are needed to investigate the role of inflammation in their development as a

function of lung immaturity. Lung xenografts provide a unique model that could enable such study in human tissues. Exact factors that interfere with lung development in preterm birth are not known, however knowledge derived from transgenic mouse models supports the notion that inflammatory mediators interfere with postnatal alveolarization.

The phenotypic diversity observed in humans with respect to respiratory distress associated with premature birth is likely due to a number of environmental and genetic factors. Surfactant protein genes have been shown to be one of the genetic factors involved in mediating this phenotypic diversity. For example, case-control and association studies have repeatedly shown linkage of SP-A and SP-B with neonatal RDS, where certain variants appear to impart protection or susceptibility to neonatal respiratory outcomes (Floros et al., 2001a; Floros et al., 2001b; Floros and Kala, 1998; Floros et al., 2006; Hallman et al., 2002; Marttila et al., 2003a; Marttila et al., 2003b; Rova et al., 2004). Therefore, surfactant proteins are likely candidate genes for other neonatal pulmonary diseases.

To best study the effect of genetic variation on disease, rapid and accurate detection of SNPs is necessary. Previous methods of surfactant protein genotyping involved labor intensive, non-direct SNP detection by introducing an artificial restriction enzyme digestion site through converted PCR (DiAngelo et al., 1999). Chapter 3 of this thesis describes the development of genotyping assays for variants of surfactant proteins using the primer based pyrosequencing method. This high throughput detection method involves direct sequencing of SNPs from gene specific PCR templates. Moreover, it accommodates both simplex and/or multiplex reactions and allows for rapid screening to obtain allele frequencies of candidate genes in DNA pools.

The pyrosequencing method of SNP detection was used for genotyping of surfactant proteins in an attempt to investigate their potential linkage to BPD. Chapter 4 of this thesis, describes a family-based association study to determine linkage of surfactant proteins to BPD. BPD is a neonatal respiratory disease, that involves inflammatory processes and volu- and baro-trauma imposed on an underdeveloped saccular stage lung (Bancalari et al., 2003). Since RDS has been implicated as a precursor of BPD, and severity of RDS increases risk of BPD, we hypothesized that surfactant proteins are candidate genes in BPD development.

Nine SNPs of SP-A, four SNPs of SP-B, two SNPs of SP-C, and two SNPs of SP-D were examined in the population of infants with BPD presented in Chapter 4. Of these, only SNP B_18(A/C) SNP was found to associate with BPD, specifically in the more severe subgroup of BPD infants at 36 weeks. Microsatellite marker AAGG_6 associated with susceptibility in both subgroups of BPD. Microsatellite markers have not previously been studied in BPD. This marker is located 26 kb upstream from SP-B and although no direct role of this marker in function/regulation of SP-B is known, it might be linked to another gene that might play a role in the pathogenesis of BPD or that might regulate SP-B (Kala et al., 1997). It has been proposed that microsatellites promote interaction with trans-acting factors by facilitating the formation of Z-DNA conformation (McLean and Wells, 1988).

Furthermore, (although not at the same level of significance) AAGG_7 allele was found to associate with protection from BPD and was also consistently found in haplotypes that associated with protection from BPD. This marker varies in one copy of the AAGG repeats from AAGG_6. A scan of transcription factor data base did not

reveal any differences in transcription factor binding between these two variants. In a recent study of RDS by the Floros group, neither allele AAGG_6 or AAGG-7 were found to associate with RDS, while allele AAGG_8 was found to associate with protection from RDS (Floros et al., 2006). It is possible that these differences might be due to spurious associations due to the relatively small size of the study groups. However, repeated association of the particular AAGG marker with the multiple protection or susceptibility haplotypes makes this less likely. The differences found in association of these markers with RDS and BPD are in accordance with the previously suggested inflammatory vs. structural pathogenesis of subgroups of disease.

SNP B_18(A/C) was found to associate with BPD. It is located in the 5'UTR of the SP-B, between the TATA box and the transcriptional initiation site of the promoter (Pilot-Matias et al., 1989), and is therefore most likely to play a role in the transcriptional regulation of SP-B. This SNP has not been previously identified to associate with neonatal disease in either BPD or RDS. A recent study explored the effect of the individual variants of this SNP on SP-B promoter function in both *in vitro* and *in vivo* assay (Steagall et al., 2007). Assays using human adult adenocarcinoma cell line NCI-H441, suggested that the B-18_C allele increased transcription almost two times more than allele B-18_A. This was thought to be due to preferential binding of transcription activation factor Sp-1 to allele C as compared to allele A. This study also found that the CC genotype associated with approximately threefold higher amount of SP-B than the AA genotype at SNP B-18(A/C) in both healthy volunteers and patients with lymphangio-leiomyomatosis. This study illustrates how single nucleotide sequence difference can result in significant quantitative differences in protein expression. The

study presented in this thesis (Pavlovic et al., 2006) found that allele C of B₁₈(A/C) associates with susceptibility to BPD at 36 weeks, while allele A is a protection factor from disease. Although these findings are in contradiction to the conclusions of the Steagall et al. study, it is possible that transcriptional regulation and therefore protein expression might be differentially regulated in the fetus than adult. In addition multiple factors may be at play and regulatory differences may exist between studies utilizing a cell culture system and human lung *in vivo*. Furthermore, the insult of ventilation on the premature lung, as is the case in BPD, may result in activation of alternate pathways of regulation.

The other polymorphism of BPD that has been identified by the Finnish group, intron 4, also plays a regulatory role in SP-B expression (Rova et al., 2004). Intron 4 is a polymorphism of (CA)_n microsatellite sequences (Pilot-Matias et al., 1989), which includes variants with insertions and/or deletions (Floros et al., 1995). This polymorphism was not examined in this study. It would be of interest to determine whether intron 4 is associated with BPD in the group of BPD infants studied here. It is of note that the B1580(C/T) polymorphism by itself was not associated with BPD in either the Finnish study group or our study group (Pavlovic et al., 2006; Rova et al., 2004). However, we found the B1580_T variant in several haplotypes that associated with BPD. This is in contrast to the previous observations in RDS, where this variant has been associated with protection from RDS in specific subgroups (Floros et al., 2001a; Marttila et al., 2003a).

The B₁₅₈₀(C/T) variant is found at the end of exon 4 and it results in Ile131Thr amino acid change which affects N-linked glycosylation of SP-B proprotein. The

B1580_T variant codes for isoleucine (Ile(AT \underline{T})) (Wang et al., 2003a), which does not support N-linked glycosylation. Although functional consequences of this variant are not yet known these changes are likely to affect protein folding, processing, sorting, and secretion. The association of haplotypes that contain the B1580_T variant of SP-B with BPD, while the B1580_C variant that enables N-linked glycosylation associated with RDS may be indicative of different underlying roles these variants may play in specific processes that are involved in pathogenesis of these neonatal diseases.

The association of the B_18(A/C) SNP and its haplotypes with the more severe subgroup of BPD is in concordance with the hypothesis that the early stages of BPD, (BPD at 28 days), are primarily due to inflammation superimposed on an immature lung, while the more severe variant of disease is due to further damage due to mechanical ventilation. Improved ventilatory techniques have minimized the severe structural damage to the lung that has lead to the pathology commonly found in “old” BPD. However, “new” BPD is still characterized by decreased alveolarization, arrested maturation, as well as transient surfactant dysfunction and deficiency of SP-B during continued mechanical ventilation of these infants (Bancalari et al., 2003; Merrill et al., 2004). The population used in the Finnish study of BPD consisted solely of infants with BPD at 36 weeks. The B_18(A/C) SNP was not examined in this study.

Infants examined in this study best fit the model of “new” BPD. Damage of the lungs in these infants is likely to be primarily due to inflammatory insult on the immature lung. In addition, immature lung have a poorly developed antioxidant system, therefore they are at heightened risk for oxygen free radical damage (O'Donovan and Fernandes, 2000). Decreased levels of SP-B predispose to oxygen toxicity (Tokieda et al., 1999).

Heterozygous SP-B deficient mice (SP-B +/-) have 50% lower SP-B levels than controls and are thus susceptible to oxygen induced damage (Tokieda et al., 1999). In addition, SP-B production is (decreased or increased) by LPS and pro-inflammatory cytokines in fetal and newborn lungs (Vayrynen et al., 2002). In a recent study by animals that were overexpressing SP-B were protected from various inflammatory events after endotoxin exposure, compared to SP-B-deficient mice (Epaud et al., 2003). In addition, inflammatory cells and proinflammatory cytokines were reduced in the lungs of SP-B-overexpressing mice compared with heterozygous SP-B-deficient mice.

SP-B may contribute to BPD in multiple ways. Specific genotype of SP-B may modulate the function of certain variants of molecules primarily involved in immunological roles, such as SP-A.

While no individual SP-A SNPs or haplotypes were found to associate with BPD, two SP-A-SP-D haplotypes were found to be protection factors in BPD at 28 days. These haplotypes have not been previously studied. It is likely that the inflammatory components that appear to be at work in early stages of neonatal pulmonary disease are modulated by SP-A and SP-D (Jobe and Ikegami, 2000). Although it is unknown how the SP-A2(1A²)/SP-D haplotypes may protect from BPD, we speculate that this protection is likely due to adequate levels of SP-A and/or SP-D at a critical developmental stage of the lung. *In vitro* studies have shown genotype-dependent levels of SP-A mRNA (Wang et al., 2003b) as well as differences among SP-A2 variants in 3'UTR mediated expression (Karinch et al., 1997). It is unlikely that the association reflects functional differences, because the SP-A2, 1A² variant is identical in the amino acid sequence of the mature protein with several other SP-A2 variants (DiAngelo et al.,

1999). With regards to SP-D, the DA11(C/T) SNP was recently identified to affect oligomerization, function, and serum concentrations of the protein (Leth-Larsen et al., 2005). Individuals homozygous for the DA11(C/C) variant were found to have significantly lower SP-D serum levels than the DA11(T/T) homozygous individuals. Polymorphisms of SP-A were not investigated in this study (Leth-Larsen et al., 2005).

It has been proposed that the elevation in pro-inflammatory cytokines in these infants might be due to an inadequate expression of anti-inflammatory cytokines, and thus an inability of the fetus to modulate inflammation (Brus et al., 1996; Jones et al., 1996; Munshi et al., 1997). However, since the fetus is commonly exposed to infection/inflammation, complex interactions of the fetal-maternal-external environment are likely at play in order to modulate fetal inflammatory response.

Increase in levels of products of inflammation in airways of infants with BPD has been reported in a number of studies (Tauscher et al., 2003). The associations between BPD, premature birth, and surfactant protein genotype and haplotypes may be due to inflammatory events. It is unknown how specific polymorphisms or haplotypes of surfactant proteins affect the interaction between inflammatory factors and lung maturation.

Recent studies have implicated SP-A in initiation of parturition. The study by Condon et al. showed that SP-A can activate macrophages of fetal origin to migrate to uterus and induce production of IL-1b and other cytokines, thus leading to activation of NF-kB and onset of labor (Condon et al., 2004). However, fetal macrophages were not found in uteri of woman who delivered at term (Kim et al., 2006). Women with chorioamnionitis and women with advanced pregnancies were found to have elevated

expression of SP-A1 mRNA (Han et al., 2007). Although role of SP-A in these tissues is not clear, two receptors of SP-A have been found in rat myometrium and endometrium and their expression was not related to pregnancy induced changes in the tissue (Garcia-Verdugo et al., 2007). Together, these observations can lead to a hypothesis that a **threshold quantity of SP-A is needed to signal partuition.** Since major stimulators of SP-A production are progression of gestational age and inflammation, this threshold quantity can be presumably attained either with progression of pregnancy resulting in term or near-term delivery, or with inflammation (most likely chorioamnionitis) resulting in pre-term delivery.

It is of interest that SP-A1 was only found to be expressed in myometrium and that it was elevated in the setting of chorioamnionitis, since SP-A1 is the predominant gene expressed in human fetal lungs while SP-A2 predominates in adult lungs (Goss et al., 1998; Han et al., 2007; McCormick et al., 1994; McCormick and Mendelson, 1994; Scavo et al., 1998). Since injection of SP-A into the amniotic fluid induced partuition in mice (Condon et al., 2004), it would be of interest to determine whether single gene products of SP-A1 or SP-A2 would induce partuition, and whether different quantities of each would be necessary. In addition, preterm dysfunction of the surfactant system might be due to the fact that the single gene product may not provide optimal function to neonates.

Qualitative differences between individual surfactant protein variants are unlikely to be of significance in intact physiological states. However, when quantity of SPs is not sufficient, qualitative differences may become important. This would be of particular importance in those infants where one gene is primarily expressed and thus any

qualitative deficiency of one gene can not be counterbalanced by the products of the other SP-A gene.

Together these studies point to the complex role of inflammation in lung maturation and onset of labor. Although the exact role of individual surfactant proteins and their specific genotypes in these processes is yet unknown, it is reasonable to speculate that SP-A and SP-D might have both pro- and anti-inflammatory properties depending on the stage of development and extent of inflammation, while SP-B and SP-C are likely to be anti-inflammatory mediators in accordance with their hydrophobic nature and their close interactions with surfactant lipids.

5.3 Conclusion

Chapter 2 successfully demonstrated development and characterization of a model to study human lung development that improves upon previously available models. This *in vivo* model of grafted human lung tissue, allows for the study of specific mechanisms involved in each stage of development. In addition to the study of normal developmental processes, this model can potentially be adapted to study pathophysiology of neonatal disease, and the treatment modalities used for them. Moreover, this model can help elucidate the role of genetic variation in disease outcome as well as individual response to therapeutics. For example, studies on the effects of pharmacological treatments on the xenografted lung of specific surfactant protein genetic variants, could

help gain insight into approaches that would help lead to predictive and personalized medicine.

To date, this model has been characterized with respect to stage-specific morphological changes, markers of developmental processes, as well as expression of surfactant proteins. **Overall, the advantages of the xenograft model can be summarized as following:** **i)** longer time of tissue viability than observed in the explant system currently in use; **ii)** ability to study different stages of fetal lung development; **iii)** ability to simulate diseases of the developing fetal lung; **iv)** ability to test the therapeutic and/or preventive potential of already established and/or experimental agents; **v)** ability to reconstitute the grafts with selected immune cell populations; **vi)** ability to deliver a systemic signal to the mice in order to manipulate the induction of surfactant components and/or inflammatory cytokines.

In order for this model to be used in studies of regulation of developmental mechanisms in the future, the differences in the apparent quantity of surfactant protein message present at different time points post-graftment, must be addressed. These are thought to be primarily due to inconsistent tissue sampling (type of tissue grafted) and various success rates of tissue differentiation. Since these drawbacks can not be avoided with currently available technologies, it is imperative that morphological characterization of each graft used for future mRNA analysis be performed. Moreover, morphometric analysis would be needed in order to be able to extrapolate findings between different tissues.

The potential drawbacks to wider use of this model are 1) lack of human fetal lung availability; 2) variability between tissues in terms of developmental age and extent of

differentiation; 3) heterogeneity of available human lung tissue; 4) inability to determine starting graft tissue morphology; 5) variations in individual graft progression through developmental stages.

Chapter 3 describes a development of a method for high throughput genotyping of SPs. These assays accelerate individual genotype analysis of surfactant proteins and should greatly facilitate execution of pilot studies of SP-D SNPs using large DNA pools. Allele quantification in DNA pools would be most useful for rapid comparison of allele frequencies between disease and control groups in pilot studies. This could allow for high-throughput screening of hundreds of samples at once. These methodologies will enhance our ability to more efficiently and reliably genotype samples from individuals with various pulmonary diseases. Moreover, the ability to perform molecular haplotyping for amino acids 19 and 50 of SP-A1, provides a valuable genetic tool that can be used as an anchor in various inferred haplotype analysis. There are several advantages to this methodology compared with the previously used method: **(i)** Pyrosequencing obtains DNA sequence data directly, thereby providing the same accuracy as conventional sequencing methods; **(ii)** the results are observed in real-time and multiple SNPs can be analyzed simultaneously; **(iii)** there is no need for labor intensive gel-electrophoresis; **(iv)** large number of samples can be analyzed in a short time; **(v)** multiplexing of samples increases the efficiency and cost of genotyping; **(vi)** molecular haplotyping provides for reliable haplotype determination in individual samples; **(vii)** allele frequency assays facilitate genotyping of hundreds of samples and execution of pilot genotyping studies; **(viii)** scoring of samples is machine-based and not

human-dependent; **ix)** electronic data are generated that are easily accessible in downstream analysis.

Although SNP genotyping by pyrosequencing is very versatile, the strict requirements of the technology in terms of primer and template specifications place great demand on assay design and can limit possibilities for multiplexing. For example, the necessity to have a relatively short sequencing template requires use of multiple nested reactions in SP-A genotyping. In addition, the 3' looping of the single stranded DNA provides numerous challenges to assay design, and can significantly limit the options of primer and assay design.

Chapter 4 effectively applied methods developed in CH 3 to investigate the role of genetic variation of surfactant proteins on Bronchopulmonary dysplasia (BPD), a neonatal lung disease. Although the sample size was relatively small, this was mitigated by the family based association approach. The contrast between the alleles of SP-A that are risk factors in pathogenesis of BPD and those that have been implicated as risk factors for RDS is of particular interest. It has long been postulated that due to the presumed overlap between RDS and BPD similar genetic association findings should be seen in these two diseases. However, the changing characteristics of the BPD population point to a heterogeneous group of infants, that seem to share similar responses to the inflammatory stimuli. These responses appear to be in contrast to those of the infants that develop RDS. Our findings support the notion that RDS and BPD have unique underlying mechanisms, and that the surfactant proteins might be differentially involved in these neonatal respiratory disease.

5.4 Ethical issues involving human fetal tissue related research

Although often controversial, fetal tissue research has served an instrumental role in the development of a number of lifesaving treatments available today. All such research must be conducted according to the guidelines of the NIH Revitalization Act of 1993. This act requires that the women sign the elective abortion consent form before the option of fetal tissue donation is discussed and among other things prohibits the researcher from taking part in any decision making that may affect the way in which pregnancy is terminated. Ultimately, the decision to donate fetal tissue belongs to each individual woman, as does the right to elect the abortion procedure.

Fetal tissue research can provide a venue for a potential social good in the midst of a great personal loss. The ethical responsibility and the ultimate goal of the scientific community are to create the safest and most effective treatments available whose full utility and possible detrimental side effects can not be assessed without the use of the appropriate *in vivo* human developmental model(s).

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