Regulation of surfactant production by fetal type II pneumocytes and the characterization of fibroblast-pneumocyte factor.

by

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A straight line may be the shortest distance between two points,
but it is by no means the most interesting.

The Doctor, ‘Doctor Who’

No. Try not. Do or do not. There is no try.

Yoda, ‘The Empire Strikes Back’

Yes, and this is mine. My magnificent octopus.

Baldrick, ‘Blackadder the Third’

Declaration

I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution.

Garth Maker

Abstract

The fetal lung undergoes extensive physiological and biochemical maturation prior to birth in preparation for its postnatal function as an organ for gas exchange. Pulmonary surfactant, a substance that reduces surface tension and prevents alveolar collapse, is produced by type II pneumocytes within the lung. Reduced ability to produce surfactant leads to neonatal respiratory distress syndrome. Synthesis of the phospholipid component of surfactant, phosphatidylcholine (PC), is stimulated by fibroblast-pneumocyte factor (FPF), a protein expressed by fibroblast cells within the fetal lung. Although its function is well known, the identity of this important protein has remained a mystery. Recent research has suggested that FPF may be neuregulin-1, a growth factor found in many tissues during development.

Enhanced synthesis of PC (and therefore detection of FPF) is measured using a tissue culture-based method. Primary cultures of lung fibroblasts and type II pneumocytes are prepared, and fibroblast-conditioned medium (FCM) is exposed to the type II cells. Resultant PC synthesis is measured using radioisotope-labeled PC-precursor and a chloroform-based lipid extraction method. Initial results using this method were very inconsistent, so a study was undertaken to determine which parts of the method could be contributing to this inconsistency. Cell density of type II cultures (measured in μg DNA.plate^{-1}) was shown to have a significant effect on results. Treatment of fibroblasts with 100 nM dexamethasone and exposure of type II cultures to the resultant FCM caused a mean 9.17% increase in PC synthesis, but when only type II cultures with a cell density below 25 μg DNA.plate^{-1} were analyzed, this value increased to 17.56%. Type II cultures with cell density above this threshold value showed a mean increase in synthesis of only 3.39%. The consistent application of

[^{3}H]-choline chloride also had a significant effect on results. Experiments utilizing phorbol 12-myristate 13-acetate to stimulate fibroblasts were very inconsistent. The mean activity of the initial [^{3}H]-choline chloride solution prepared for these experiments was found to be 2.04 μCi.mL^{-1}, compared to a mean of 4.79 μCi.mL^{-1} for all other experiments. Observations from this section of the study led to considerable revision of the method used to measure PC synthesis.

Generation of FPF by fetal lung fibroblasts is stimulated by a number of compounds, and several were investigated in order to determine the best way to produce FPF for further analysis. One of these compounds was neuromedin C, which had been shown to cause a significant increase in surfactant secretion. Exposure of fibroblasts to 1 nM neuromedin C led to a mean 3.23% decrease in surfactant synthesis, but this was not significantly different from control. The lack of effect is most likely due to insufficient specific receptors that bind neuromedin C being present.

Quadrupolar ion trap mass spectrometry (MS) was used to analyze FCM and determine if neuregulin-1 (NRG1) could be FPF. A mass spectrum was obtained for recombinant NRG1, with predominant ions of 1068, 1142 and 1246 *m/z*. All three of these ions were also detected in both control and dexamethasone-treated FCM. Partial fragmentation of 1068 *m/z* of NRG1 was achieved using MS^{2}, and generated a base peak of 1047 *m/z*. This fragmentation was also observed in 1068 *m/z* from FCM. LC/MS was utilized to quantify NRG1 in FCM, using a standard curve generated using recombinant NRG1. Control FCM had a NRG1 concentration of 19.85 μg.mL^{-1}, while the concentration in dexamethasone-treated FCM was 41.59 μg.mL^{-1}. FCM which had given no positive response to dexamethasone when tested using the indirect cultured cell system had a control NRG1 concentration of 20.85 μg.mL^{-1}, and a dexamethasone-

treated concentration of 22.84 μg.mL^{-1}. These values were not significantly different from the control value for FCM in those fibroblast cultures that had generated a positive response to dexamethasone. Results of this section of the study have provided strong evidence that NRG1 is a major component of FPF, and a review of the NRG1 signaling pathway further supports this conclusion.

Insulin-like growth factors (IGFs) are functionally related to neuregulins and are known to be important in fetal development. The effect of IGF-II on synthesis of surfactant PC and its subsequent secretion from type II pneumocytes was studied. In terms of PC synthesis, IGF-II was tested at concentrations of 0.4, 0.6 and 0.8 μM. The mean increase in synthesis was found to be 6.00, 6.15 and 6.91%, respectively. These values were not significantly different from control values. Secretion of PC was tested over the concentration range of 0.1 to 1.6 μM, with no significant effect observed. Possible inhibition by IGF-II was also studied, using the known stimulants of secretion, neuromedin C and isoproterenol. No significant effect on the enhanced level of secretion was observed when IGF-II was added with either secretagogue. Lack of an appropriate receptor and/or the possibility that cultured cells may not exactly mimic the situation *in vivo* are probably the reasons IGF-II has no effect on either synthesis or secretion.

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Recommended soundtrack for reading this thesis: 'Between the Lines' by Mike Stern, 'The Central Park Concert' by Dave Matthews Band and 'Live Art' by Bèla Fleck and the Flecktones.

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Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSS	buffered salts solution
CID	collision-induced dissociation
DAD	diode array detector
DHT	dihydrotestosterone
DMSO	dimethylsulphoxide
DPPC	dipalmitoylphosphatidylcholine
EGF	epidermal growth factor
ESI	electrospray ionization
FCM	fibroblast-conditioned medium
FPF	fibroblast-pneumocyte factor
GRE	glucocorticoid response element
GRP	gastrin-releasing peptide
HPLC	high-performance liquid chromatography
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGFR	insulin-like growth factor receptor
IgG	immunoglobulin G
MEM	minimal essential medium
MS	mass spectrometry
MW	molecular weight
NBCS	newborn calf serum
NRDS	neonatal respiratory distress syndrome
NRG	neuregulin
PBS	phosphate buffered saline
PC	phosphatidylcholine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
SP	surfactant-associated protein

Units

°C	degrees Celsius
AU	absorbance units
Ci	curie
cm	centimetre
dpm	disintegrations per minute
g	gram
g	centrifugal force
IU	international unit
kDa	kilodalton
L	litre
M	moles.litre^{-1} (molar)
m	metre
m/z	mass to charge ratio
mAU	milli-absorbance units
mCi	millicurie
μCi	microcurie
mg	milligram
μg	microgram
mL	millilitre
μL	microlitre
mM	millimoles.litre^{-1} (millimolar)
μM	micromoles.litre^{-1} (micromolar)
mm	millimetre
μm	micrometre
ms	millisecond
ng	nanogram
nM	nanomoles.litre^{-1} (nanomolar)
nm	nanometre
psi	pound per square inch
V	volt

Chapter 1. General introduction

The air sac, capable of gas exchange with the atmosphere, first evolved during the Devonian period, and allowed primitive fish to move onto land (Possmayer, 1982). In terrestrial vertebrates, including mammals, this air sac is known as the lung. The mammalian lung consists of two lobes and is connected to the upper airway by the trachea and bronchi. Inside the lung, the branching of the bronchi forms the bronchial tree, with each branch terminating in an alveolus. The alveoli are the site of gas exchange with the atmosphere. Human lungs contain approximately 300 million alveoli, which gives the lungs a far greater internal than external surface area (Snell, 2007).

During development, human fetal lungs function within the fluid intrauterine environment, and must adapt to the extrauterine atmosphere at birth. There are many physiological events that must occur to allow this adaptation, and key among them is the induction of the pulmonary surfactant system. Pulmonary surfactant is a complex, surface-active material that is composed of phospholipids and four specific surfactant-associated proteins. The surfactant lines the alveolar surface of the lung (Griese, 1999) (Figure 1.1).

The air-liquid interface within the alveoli is subject to great surface tension which, if unmodified, would lead to progressive atelectasis and respiratory failure. The primary function of pulmonary surfactant is to reduce this surface tension to very low values, and dynamically alter the tension as surface area changes with inspiration and expiration (Johannson and Curstedt, 1997). Surfactant also functions to protect the lung from injury and infection that may be caused by the inhalation of particles and microorganisms (Griese, 1999).

The induction of the surfactant system does not occur until late in gestation, as the lungs are not involved in gas exchange until after birth. Infants that are born

prematurely have immature lungs containing little surfactant, which leads to neonatal respiratory distress syndrome (NRDS) (Griese, 1999). This condition causes extremely high surface tension in the alveoli during expiration, and they tend to collapse, greatly reducing the ability of the lung to obtain sufficient oxygen for bodily needs (Levine and Gordon, 1942).

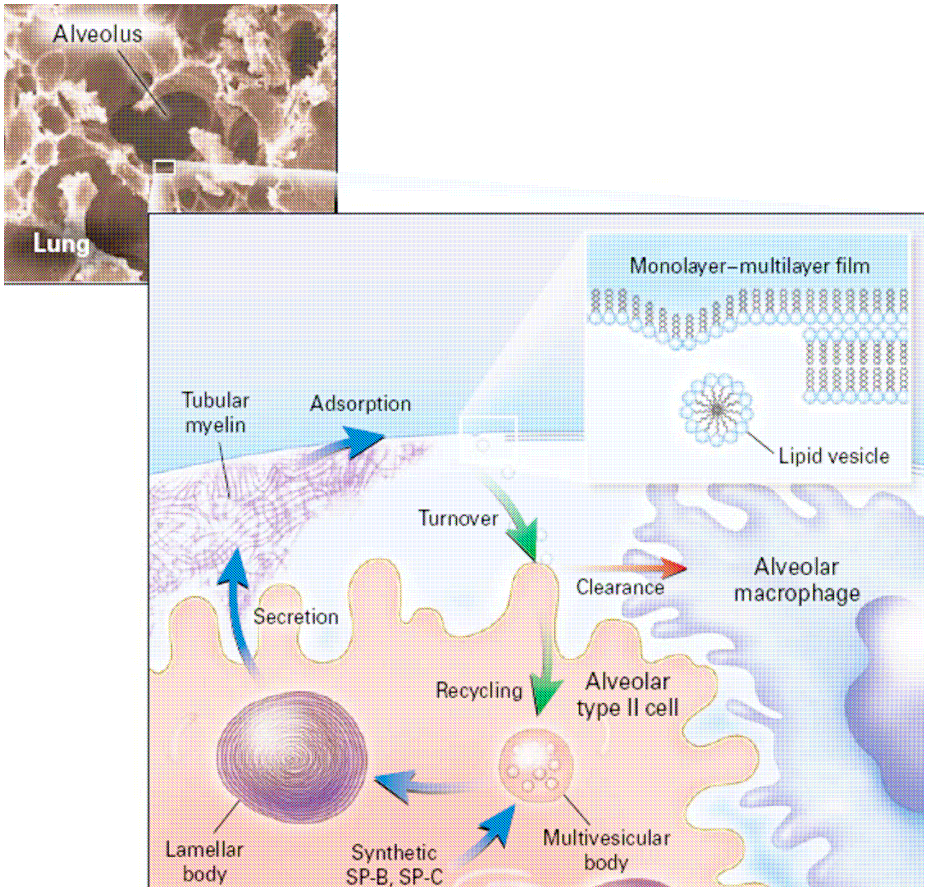

Figure 1.1: Micrograph of alveolar space with a magnified diagram of the air-liquid interface illustrating the surfactant system (Whitsett and Weaver, 2002).

1.1 History of the surfactant system

The discovery of the surfactant system began in 1805 with the publication of Young's paper 'An essay on the collision of fluids', which was followed a year later by Laplace's paper on the same topic. The relationship between force, surface tension and radii of curvature defined by these papers became known as the Young-Laplace law (Comroe, 1977). Over 100 years later, Langmuir developed a method to determine molecular dimensions using surface physics and chemistry (Langmuir, 1917), and this led to the development of equipment for measuring surface tension under dynamic conditions.

In 1929, von Neergaard theorized that a relationship might exist between the specialized material lining the lung and the Young-Laplace law. In spite of its potential importance, this work was not recognized until much later. In his 1954 paper, Radford found a ten-fold discrepancy in calculating lung surface area using physical measurement and histological methods. He argued that a surface active substance was unlikely to exist, so the lung must be in a semi-solid state (Radford, 1954). In the same year, Macklin published a paper describing the mucoid film that coats the alveoli. In his description he postulated that this film would maintain a constant surface tension (Macklin, 1954). The following year, Pattle proposed that, in order to prevent alveolar transudation and resulting pulmonary oedema, the surface tension in the lung must be maintained at a low level (Pattle, 1955).

The work of Radford and Pattle prompted Clements to derive a quantitative measurement of alveolar surface tension, under dynamic conditions. These measurements definitively showed the presence of a surface active material that maintained a constant surface tension (Clements, 1957). Clements' surface tension values were confirmed in 1959 by Avery and Mead, who studied 'hyaline membrane disease'. They found a three-fold increase in mean surface tension in infants who had

died from this condition (Avery and Mead, 1959). These results led to further studies of the development and induction of the surfactant system in mammals (Buckingham *et al.*, 1968; Comroe, 1977).

1.2 Lung cell types

The induction of the surfactant system is regulated by the interaction between two cell types within the lung: epithelial (type II pneumocyte) and mesenchymal (fibroblast) cells (Smith and Fletcher, 1979).

Type II cells are cuboid in shape and are located in the corners of the alveoli. They have a high number of lamellar bodies, as well as mitochondria, Golgi apparatus and rough endoplasmic reticulum (Ballard, 1986). The primary function of the type II cell is the synthesis and secretion of pulmonary surfactant. Surfactant is stored in the bell-shaped lamellar bodies until its secretion by exocytosis (Bangham and Horne, 1964). Each type II cell contains approximately 150 (± 30) lamellar bodies, with exocytosis occurring at a rate of 15 bodies per hour (Young *et al.*, 1981). The differentiation of the type II cell is a key step in lung maturation and surfactant production.

The ‘timer’ for this differentiation is within the tissue, and involves an interaction between the epithelial (pneumocyte) and mesenchymal (fibroblast) tissues (Smith and Post, 1989). This interaction appears to occur prior to day 13 in fetal rats, and commits the type II cells to their phenotype. A key indicator of this differentiative event is glycogen depletion, which provides an energy source for the formation of lamellar bodies (Smith and Post, 1989).

There are two types of cellular change – proliferation and development. Fibroblasts from the pseudo-glandular stage of lung development stimulate epithelial cell proliferation, while those from the saccular stage stimulate differentiation (Caniggia

et al., 1991). Also, fibroblasts in close proximity to the epithelial cells produce mainly differentiation factors while those further away produce mainly proliferation factors. Pneumocyte cells do not respond to proliferation factors once they have developed past the saccular stage (Caniggia *et al.*, 1991).

Differentiation of epithelial cells (type II pneumocytes) is triggered by the subjacent mesenchyme (fibroblasts). The fibroblasts respond to glucocorticoids and secrete the differentiation factor (Caniggia *et al.*, 1991). Glucocorticoids bind more readily to fibroblasts adjacent to pneumocytes than those located peripherally, because these adjacent fibroblasts are enriched with glucocorticoid receptors.

1.2.1 Tissue culture

The use of whole lungs in studies of cellular differentiation does not consider the cellular diversity found within those lungs (Post *et al.*, 1983), requiring individual cell types to be cultured separately. It has been found that isolated and cultured fetal alveolar type II cells retain characteristics of *in vivo* type II cells, in particular the ability to synthesize and secrete phosphatidylcholine (PC). These cultured cells also retain the ability to proliferate and respond to fibroblast-pneumocyte factor (FPF) (Post *et al.*, 1983).

Culture studies found several factors which will enhance the quality of cell culture. A higher purity of cells is obtained if collagenase is used to disperse cells, and fetal calf serum is essential for optimal growth (Post *et al.*, 1983). The optimal conditions for *in vitro* generation of FPF by fibroblasts were the use of primary cultures, maintenance of cultures in a medium that does not cause rapid cellular proliferation, and induction of pyridoxal deficiency to enhance responsiveness to glucocorticoids (Smith, 1981a).

1.3 The pulmonary surfactant system

1.3.1 Composition of surfactant

In the lungs surfactant exists in two pools – the intracellular and extracellular pools. Intracellular surfactant is stored in the type II cells in lamellar bodies (Wright and Clements, 1987). The extracellular pool of surfactant is the layer that lines the alveoli (Schurch *et al.*, 1995). Alveolar material, and that obtained through bronchoalveolar lavage, contains several different morphological and biochemical forms of surfactant. These forms include (Wright and Clements, 1987):

a) densely-packed multilamellar structures that resemble secreted lamellar contents;
b) unique lattice-like structures called tubular myelin;
c) the surface film;
d) multilamellar and unilamellar vesicles; and
e) distinct structures that resemble open-ended bilayers.

Of these forms, it is thought that the lamellar body contents expand and form the complex structure of tubular myelin upon contact with the alveolar space. This process is triggered by calcium, as well as the environmental conditions within the space (King *et al.*, 1983). The process by which the bilayer structures form a monolayer film is not understood.

While the properties of surfactant are well known, and can be readily identified, its exact *in vivo* chemical composition is not known. This is most likely because surfactant is heterogeneous in both chemical composition and morphology (Robertson, 1984). In addition, surfactant isolated for testing is likely to comprise active surfactant,

newly secreted surfactant and surfactant that is ready for recycling and removal from the alveolar lining.

Pulmonary surfactant contains several types of lipid, including phospholipids, triglycerides, cholesterol and fatty acids (Johannson and Curstedt, 1997). Of these, phospholipids are present in the greatest quantities. Phosphatidylcholine comprises 70-80% of surfactant lipids, and phosphatidylglycerol a further 5-10% (King, 1982). Approximately 60% of surfactant phosphatidylcholine is present in the disaturated form, with both fatty acids being palmitic acid. This lipid is known as dipalmitoylphosphatidylcholine (DPPC) (Hildebran *et al.*, 1979). The polar end of the DPPC molecule contains the choline residue, while the other, non-polar end contains the two palmitic acid residues. It is currently thought that DPPC is the component of surfactant responsible for reducing surface tension (Holm *et al.*, 1996).

The role of phosphatidylglycerol (PG) is less well understood. The level of PG is generally low during gestation and increases either prior to, or shortly after, birth (Hallman *et al.*, 1977; Egberts and Noort, 1986). Phosphatidylglycerol is the most variable surfactant component in different mammalian species. It is greatly reduced in species such as cats (Shelley *et al.*, 1984), and replaced with phosphatidylinositol (PI) in adult rhesus monkeys (Egberts *et al.*, 1987). Studies on PG-deficient adult rabbits have shown no obvious changes to surfactant properties or function, although these studies did use PI to substitute for PG (Beppu *et al.*, 1983; Hallman *et al.*, 1985). Human infants who are born prior to the pre-natal increase in PG levels are more likely to suffer from NRDS than those born after (Hallman *et al.*, 1977), suggesting a definite role for PG in the function of surfactant. It is possible that the presence of PG helps with alveolar stability (Hallman and Gluck, 1976), or in the association of proteins with phospholipids (King and Martin, 1980).

Surfactant generally contains 5-10% protein, and these surfactant-associated proteins (SP) interact extensively with the phospholipids. These interactions include changing the structure and properties of lipid layers and films (Yu and Possmayer, 1988; Dhand *et al.*, 1998). There are four surfactant proteins that have been identified: SP-A, SP-B, SP-C and SP-D. The general characteristics of each protein are summarized below (Table 1.1).

Table 1.1: Characteristics of the four surfactant-associated proteins, SP-A, SP-B, SP-C and SP-D (Yu and Possmayer, 1988).

Protein	Molecular weight range	Structure	MW of polypeptide chains (kDa)	Polarity
SP-A	high	octadecamer	26.0	hydrophilic
SP-B	low	dimer	8.7	hydrophobic
SP-C	low	monomer	4.0	hydrophobic
SP-D	high	dodecamer	39.0	hydrophilic

Due to differences in the methods used to isolate surfactant, a precise quantitation of the surfactant proteins has not been obtained. Recent research has shown that the formation and stability of surfactant are critically dependent on SP-B and SP-C (Weaver and Conkright, 2001), while SP-A is important for immune and inflammatory responses (Crouch and Wright, 2001). Other proteins have also been isolated with surfactant, including albumin, immunoglobulins A and G (King *et al.*, 1973; Paciga *et al.*, 1980) and uteroglobin (Guy *et al.*, 1992). The function of these additional proteins is not fully understood, although it is possible that they have roles in immunity and extending the functional life of surfactant (Guy *et al.*, 1992).

Several factors have been shown to affect the composition of surfactant, most notably diet. A deficiency of vitamin A can delay the maturation of lungs in newborn

rats (Chailley-Heu *et al.*, 1999) and reduce the surfactant phospholipid levels by more than 20%. This is probably due to the relationship between plasma retinol levels and expression of key enzymes in the lipid synthesis pathway. It has also been found that vitamin A deficiency can reduce the amounts of SP-A, SP-B and SP-C (Chailley-Heu *et al.*, 1999). Choline-deficient diets can lead to surfactant phosphatidylcholine levels that are nearly 50% lower than those in control rats (Yost *et al.*, 1985).

1.3.2 Biophysical function of surfactant

The surface tension that exists at the alveolar air-water interface opposes lung inflation. The action of pulmonary surfactant is to reduce this surface tension to effectively zero, meaning that the whole of the alveolar space remains open, even during expiration (Griese, 1999). If this did not occur, blood oxygenation would be reduced and the physical work required for adequate breathing would increase. One study has shown that the presence of surfactant may be required to maintain proper function of not only the alveoli, but also the bronchioles leading to them (Enhorning and Holm, 1993). Reduced surface tension is also important for ensuring that the alveoli do not become filled with fluid, as high surface tension can lead to a thick film developing and inundating the sites of gas exchange (Guyton *et al.*, 1984). Surfactant is involved in the removal of foreign material and discarded cells from the alveolar space, as well as possible immune cell functions (Jarstrand, 1984).

The monolayer film is the active form of surfactant. It is enriched with DPPC, indicating that DPPC is the component of surfactant responsible for surface tension-reducing properties (Hildebran *et al.*, 1979). Other lipids and proteins in surfactant are thought to facilitate lipid transport through the aqueous environment of the air-water interface at the alveolar surface. In particular, the hydrophobic proteins SP-B and SP-C play a role in this transport (Gil *et al.*, 1995). It has also been observed that calcium ions

are essential for transport processes in the alveoli (King *et al.*, 1983). The function of SP-A differs from the other proteins, and it is thought to alter the distribution and movement of surfactant within the alveoli, especially through acceleration of lipid movement. SP-A also has a protective function, by minimizing the inhibitory effect of blood proteins (Cockshutt *et al.*, 1990).

1.4 Synthesis of surfactant

1.4.1 Site and timing of synthesis

The two main surfactant phospholipids, dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol (PG), are found only in trace amounts in tissues outside of the lungs (Clements and King, 1976). There is a large body of evidence to suggest that surfactant synthesis takes place in type II pneumocyte cells within the alveoli. Type II cells comprise approximately 15% of all cells within the lung, and are characterized by the presence of a large number of lamellar bodies, which are the site of surfactant storage and the mechanism of secretion (via fusion with the cellular membrane) (Haagsman and van Golde, 1991). Analysis of the phospholipid content of lamellar bodies within type II cells shows that the lipids are almost identical to those found in pulmonary surfactant (Hallman *et al.*, 1981). Culturing of isolated type II cells, and treatment of these cultures with labeled phospholipid precursors produces phospholipids very similar in composition to surfactant (Dobbs *et al.*, 1982; Mason, 1987). It has been shown that immature type II cells have a greatly reduced ability to synthesize lipids, implying a relationship between the development of the lungs and the ability to produce surfactant.

Evidence does exist to suggest that lamellar bodies are not the sole organelle involved in surfactant synthesis. Only some of the enzymes required for surfactant phospholipid synthesis are found within these bodies, and certain key enzymes for

phosphatidylcholine production are absent (Baranska and van Golde, 1977). The autoradiographic study of Chevalier and Collet (1972) provided a wealth of information on the production and storage of pulmonary surfactant. The majority of surfactant lipids are synthesized in the endoplasmic reticulum and transferred to the Golgi apparatus. From here, small lamellar bodies transport the lipids to larger bodies, which are the site of storage and secretion (Chevalier and Collett, 1972). Surfactant proteins are also synthesized in the endoplasmic reticulum and transferred to the Golgi apparatus (Sorokin, 1967). The transfer from the Golgi apparatus to the lamellar bodies probably involves multivesicular bodies, which are found in high concentrations in type II cells. The lamellar bodies are the site where surfactant lipids and proteins are first associated and assembled into the final storage form of surfactant (Williams, 1977). The exact site of production of SP-D is not known (Voorhout *et al.*, 1992; Zhang *et al.*, 2006), implying that other organelles or even cell types may be involved.

In humans, type II cells are identifiable after 20-22 weeks of gestation, but secretion of surfactant is only detected after 30-32 weeks. These results are mirrored in all mammalian species studied to date, where surfactant synthesis seems to begin after the completion of 80-90% of gestational development. Fetal type II cells are actually capable of synthesis of surfactant from early in gestation, but only at low levels. This rises dramatically to that required late in gestation (Fraslon-Vanhulle *et al.*, 1994). Pulmonary fatty acid synthesis peaks during this period, and certain enzymes involved in lipid synthesis, such as fatty acid synthase and acetyl-CoA carboxylase, also show a marked rise in activity (Fraslon-Vanhulle *et al.*, 1994). The surfactant-associated proteins SP-A, SP-B and SP-C, as well as their respective mRNAs, appear during this period and increase in concentration until birth (Smith, 1984), stimulated by hormones such as leptin (Kirwin *et al.*, 2006). The level of mRNA for choline phosphate cytidylyltransferase also increases (Fraslon and Batenburg, 1993).

It has been observed that special pretranslational regulation of several key enzymes involved in increasing the rate of fatty acid synthesis occurs in the pulmonary system during development (Batenburg and Whitsett, 1989; Fraslon and Batenburg, 1993). However hormones, which are vitally important in controlling surfactant synthesis prior to birth, do not seem to initiate the commencement of this synthesis.

1.4.2 Synthesis of phospholipids

The production of surfactant phospholipids begins with a glycerol backbone that is produced from glucose obtained from the bloodstream. The most likely starting point for synthesis of the diacyl glycerolipids is the formation of glycerol-3-phosphate (Kennedy, 1986). Fetal type II cells contain large intracellular glycogen stores, which are the major source of this glycerol-3-phosphate. Fatty acids are obtained from the blood, as well as being synthesized *de novo* from lactate. In particular, lipogenesis is required for the production of palmitate. The enzymes involved in lipogenesis increase in activity towards birth.

Production of phospholipids can be accelerated by several factors. Glucocorticoids act on lung fibroblast cells to produce fibroblast-pneumocyte factor (FPF) (Smith, 1978). Epidermal growth factor (EGF) enhances the rate of production of PC by type II cells (Sen and Cake, 1991) apparently via enhanced release of FPF from lung fibroblasts. FPF is discussed in more detail in **1.5**.

1.4.2.1 Production of phosphatidylcholine

In mammals, phosphatidylcholine (PC) is produced by the CDP-choline pathway. Choline is endocytosed and phosphorylated to choline phosphate by choline kinase. A CDP residue is added, and the phosphate removed by the enzyme choline phosphate cytidylyltransferase. The phosphorylcholine moiety is then transferred to

diacylglycerol to produce phosphatidylcholine catalyzed by the enzyme cholinephosphotransferase. The key regulatory step in this pathway is believed to be choline phosphate cytidylyltransferase, which has a high binding affinity for lipids, implying a negative feedback mechanism (Dunne *et al.*, 1996). This pathway produces approximately 90% of the PC synthesized in a given cell (Epstein and Farrell, 1975). The alternative pathway for PC synthesis involves methylation of phosphatidylethanolamine (Batenburg, 1992). Synthesized PC is then converted to DPPC within type II cells. 1-saturated-2-unsaturated-PC is deacylated to lyso-PC and then reacylated with palmitoyl-CoA to produce DPPC (Haagsman and van Golde, 1991).

1.4.2.2 Regulation of PC synthesis

The study of regulation of PC synthesis began with the observation that administration of glucocorticoids to fetal lambs accelerated lung maturation (Liggins, 1969). Treatment of fetal rabbits with the same steroids enhanced the activity of surfactant and the appearance of type II cells (Whitsett *et al.*, 1985). In humans, the administration of glucocorticoids prior to normal maturation leads to increased use of glucose, acceleration of glycogenolysis, increased phospholipid synthesis and enhanced lung maturation (Smith, 1984). It has become clear that mesenchyme-derived factors have a major role in the differentiation and development of type II cells (Smith, 1979). The factor involved in lung development is called fibroblast-pneumocyte factor (FPF), and it acts to stimulate PC synthesis via increased intracellular cyclic AMP levels (Smith, 1979).

There are many different chemicals that can regulate synthesis of PC. Gross and Wilson (1982) found that dexamethasone, a synthetic glucocorticoid, causes a 2.5-fold increase in PC synthesis. This effect has been observed in rats (Gross and

Wilson, 1982), rabbits (Ballard *et al.*, 1984) and humans (Gonzales *et al.*, 1986). In addition, it has been found that the response to this hormone can be enhanced through an additive or synergistic effect with triiodothyronine (T_3) (Schellenburg *et al.*, 1988). The use of corticosteroids together with insulin and/or prolactin leads to a two-fold increase in lamellar body phospholipid concentration compared to controls (Mendelson *et al.*, 1981). Glucocorticoids also enhance expression of mRNA for SP-B (Ahmad *et al.*, 1996), as well as promoting stability of SP-A mRNA (Seidner *et al.*, 1996).

Dexamethasone has also been shown to block production of transforming growth factor β (TGFβ), which blocks the maturation of type II pneumocytes (Torday and Kourembanas, 1990; McDevitt *et al.*, 2007). It is therefore possible that dexamethasone simultaneously exerts both stimulatory and inhibitory effects on fetal lung fibroblasts.

1.4.3 Inhibition of the surfactant system

The ratio of male: female deaths is 4:1 in the first trimester, 2:1 in the second and nearly 1:1 in late pregnancy. However, after birth it rises again to 2:1, due to the incidence of neonatal respiratory distress syndrome (Torday and Nielsen, 1987). Male infants are at a greater risk of developing NRDS than female infants of the same age (Miller and Futrakul, 1968). This trend is found in most animal models, except avians, where the sex-determination system, and thus the trend in NRDS are reversed (Smith and Post, 1989).

The lecithin/sphingomyelin ratio, as well as the levels of PC and PG in the amniotic fluid of male fetuses is 1.5 to 2 weeks behind female fetuses (Torday *et al.*, 1981; Fleisher *et al.*, 1985). Male fetuses are less responsive to glucocorticoids (Ballard *et al.*, 1980) and fibroblasts derived from male fetuses produce less FPF than those from female fetuses (Torday, 1984). The lungs from female fetuses have greater stability

(Kotas and Avery, 1980), epithelial cells differentiate sooner to form type II cells (Adamson and King, 1984), and rates of surfactant synthesis are higher than in lungs from males (Nielsen *et al.*, 1982). The most likely cause of these differences are male androgens, which may inhibit lung development in some way. Administration of dihydrotestosterone (DHT) to fetal rabbits decreased the production of surfactant (Nielsen *et al.*, 1982). DHT exerts its effect at a pre-translational level and inhibits production of FPF by fibroblasts (Floros *et al.*, 1987), or reduces the response of type II cells to FPF (Torday and Nielsen, 1987). The fact that sex-based differences in response to steroids and surfactant production also occur in isolated cell cultures suggests that there is also a possible genetic basis for this difference.

Infants of diabetic women born at full-term have a higher incidence of NRDS than infants of non-diabetic women (Robert *et al.*, 1976). It has been found that in these infants, PG is greatly reduced or even entirely absent from the surfactant (Cunningham *et al.*, 1978). It has been proposed that fetal hyperinsulinemia associated with maternal diabetes can antagonize lung development (Obenshain *et al.*, 1970). Insulin has been shown to reduce the stimulatory effect of corticosteroids on PC synthesis (Smith, 1978), although this has not been supported by other work (Mendelson *et al.*, 1981). Mendelson and colleagues (1981) also could not show inhibition of PG synthesis by insulin. Insulin appears to affect the levels of surfactant-associated proteins, as SP-A concentration is significantly lower in the amniotic fluid of diabetic mothers (Katyal *et al.*, 1984). Snyder and Mendelson (1987) showed that inhibition of SP-A synthesis in human lungs by insulin is dose-dependent. In some species fetal hyperglycemia in the absence of hyperinsulinemia has also been seen to block lung maturation (Carlson *et al.*, 1984).

1.5 Fibroblast-pneumocyte factor

1.5.1 Characteristics of fibroblast-pneumocyte factor

Fibroblast-pneumo(no)cyte factor (FPF) is a polypeptide differentiation factor that is present in the culture medium of glucocorticoid-treated lung fibroblasts (Smith and Post, 1989). It is a heat-stable, dialyzable oligopeptide (Smith, 1979) that is inactivated by trypsin (Smith and Post, 1989). It has only one known activity, although there may be more. FPF has an apparent molecular mass in the range of 5 to 15 kDa (Smith and Post, 1989), although it is unknown whether this represents a form of the protein that differs from the active form (Scott and Das, 1993). Studies suggest that both the FPF peptide and its mRNA precursor occur in very low abundance *in vivo* (Floros *et al.*, 1985).

Epithelial-mesenchyme interactions, such as that facilitated by FPF, are highly organ-specific, but not species-specific, and occur widely (Smith and Fletcher, 1979). FPF has been isolated from human lung fibroblast cultures (Scott and Das, 1993) and similar activity has been found in cats, rabbits, rats, two species of monkey and chickens (Smith and Fletcher, 1979). This activity was not found in newborn rabbits or rats, where the lungs are already developed and the function of FPF is no longer required (Smith and Fletcher, 1979).

FPF activity has been detected in human amniotic fluid from 27 to 40 weeks gestation, although the activity declined with advancing gestation and was inversely related to cell number (Seybold and Smith, 1980). The actual presence of FPF in amniotic fluid is not related to lung maturation in the fetus, and its decrease follows the decrease in total protein content in late gestation (Seybold and Smith, 1980). There is also an inverse relationship between cortisol and FPF levels in human amniotic fluid (Seybold and Smith, 1980), indicating that as the level of cortisol decreases (implying usage within the fetus), the amount of FPF produced increases.

A similar differentiation factor has been found to be responsible for epithelial-mesenchyme interactions in the liver and has been termed fibroblast-hepatocyte factor. It is a heat-stable, low molecular weight protein that is antigenically different from FPF (Dow *et al.*, 1983). Stretch-stimulated surfactant synthesis is known to occur through another epithelial-mesenchyme interaction, mediated by parathyroid hormone-related protein (Torday and Rehan, 2002).

Recent research (Dammann *et al.*, 2003) has suggested that neuregulin-1β (NRG-1), a growth factor involved in many different stages of human development, may be FPF. They found that both fibroblast-conditioned medium (FCM) and NRG-1 had the same stimulatory effect on surfactant synthesis in type II cells, and that the activity of both was blocked by an antibody to NRG-1. Histological observation revealed that lung fibroblast cells secrete NRG-1, and that this secretion increased as surfactant synthesis increased prior to parturition (Dammann *et al.*, 2003). The receptors for neuregulins, ErbB receptors, were up-regulated in type II pneumocyte cells by exposure to dexamethasone (Dammann *et al.*, 2006). This research has provided the strongest evidence yet for the identity of FPF.

1.5.2 Induction of FPF

Surfactant synthesis is under multi-hormonal control and local cell and tissue interactions modulate the endocrine signals (Smith and Post, 1989). The central role is played by endogenous fetal glucocorticoids. In the fetal lung, inactive cortisone is metabolised to the active form cortisol (Smith and Post, 1989). This glucocorticoid activity acts on the fibroblast to produce FPF, which acts on the type II pneumocyte cell (Smith and Post, 1989). The glucocorticoid hormone binds to cytosolic receptors (GR) which are then translocated to the nucleus of the fibroblast (Smith and Post, 1993). There, they bind to glucocorticoid response elements, leading to the induction or

repression of gene transcription ((Smith and Post, 1993). In the rat, glucocorticoid receptor mRNA levels in the whole lung rapidly increase between days 18 and 19 of gestation and decrease again by days 20-22, and following day 19 there is a gradual rise in GR-binding activity (Smith and Post, 1993).

The effect of glucocorticoids on fibroblast cells is inhibited by the presence of actinomycin D during the first, but not the second, 24 hours of incubation, implying that the response involves transcriptional events (Floros *et al.*, 1985). Fractionation of mRNA and subsequent translation produces a peptide that possesses FPF activity (Floros *et al.*, 1985). The glucocorticoid effect is also blocked by the presence of cycloheximide during the first and second 24 hours of incubation, indicating that protein synthesis is required (Floros *et al.*, 1985). The mRNA and protein products are both stable, existing in the cell for some time (Smith and Post, 1989). These results suggest that glucocorticoids act at a pre-translational level to induce production of FPF and that the primary translation products are biologically active (Floros *et al.*, 1985). However, monoclonal antibody to FPF will not recognize the protein unless it has undergone post-translational modification, implying significant changes to the *in vivo* peptide (Smith and Post, 1989). The low detected activity of translation products indicates that both the mRNA and FPF protein are present in very low abundance (Floros *et al.*, 1985).

Dexamethasone has no overall impact on the activity of fetal lung choline kinase or choline phosphotransferase. Direct exposure of mixed fetal lung cell cultures to cortisol results in a 1.6-fold increase in the incorporation of labelled choline into PC (Post *et al.*, 1986). The effect of glucocorticoids can still be seen in pure type II cell cultures, but a 10-fold increase in hormone concentration is required. Thus, FPF acts not only as a signal transducer, but also as an amplifier (Smith and Post, 1991). The action of glucocorticoids on the fibroblasts is slow, which limits their use as therapy for NRDS through the stimulation of surfactant production and secretion (Smith and Post, 1989).

Epidermal growth factor (EGF) also enhances choline incorporation into PC in type II cells by an indirect mechanism (Sen and Cake, 1991). When glucocorticoids and EGF are added together the effect is not additive and maximal stimulation is the same as with the steroid alone (Sen and Cake, 1991). Both act via a similar mechanism – they produce a stimulatory factor that has a similar chromatographic elution profile. However, EGF does not appear to increase choline phosphate cytidylyltransferase activity so it is possible that it does not act via FPF (Gross *et al.*, 1986).

Thyroid hormones (e.g. triiodothyronine (T_3)) appear to enhance the responsiveness of type II cells to FPF (Smith and Post, 1989). Glucocorticoids acting on the mesenchyme and T_3 acting on epithelia have a synergistic effect (Smith and Sabry, 1983). T_3 has no effect on FPF production, but potentiates the effects of both glucocorticoids and FPF (Smith and Sabry, 1983).

In an analogous situation liver cells from chick embryos grown in mixed cultures, tyrosine aminotransferase (TAT) activity is induced by cortisol stimulation (Dow *et al.*, 1983). However, pure hepatocyte cultures do not respond to cortisol. If cortisol is incubated with mixed cultures and the conditioned medium transferred to pure hepatocytes, TAT activity is detected (Dow *et al.*, 1983). A substance produced by the fibroblasts when exposed to cortisol is capable of inducing TAT activity in the hepatocytes (Dow *et al.*, 1983) and this is known as the fibroblast-hepatocyte factor.

1.5.3 Action of FPF

FPF-enhanced maturation does not lead to changes in lung weight (Smith, 1979). Rather, it is a physiological ‘on switch’ that prepares the type II cells for their postnatal function (Smith and Post, 1989). As little as 1 μg of partially-purified FPF can accelerate lung maturation in fetal rats (Smith, 1979). Exposure of fetal type II cells to FPF enhances the incorporation of radioactively-labelled choline, glycerol and palmitate

into phosphatidylcholine (Post and Smith, 1984a). It also stimulates the formation of phosphatidylglycerol from labelled glycerol and palmitate. However, it does not affect the synthesis of other phospholipids, specifically stimulating only the production of surfactant phospholipids (Post and Smith, 1984a). The activities of enzymes in this pathway are not significantly altered except for a two-fold increase in the activity of choline phosphate cytidylyltransferase (Post *et al.*, 1986), the rate-limiting enzyme in surfactant phospholipid synthesis (Post and Smith, 1984b). Exposure of fetal type II cultures to cortisol-conditioned fibroblast medium results in a 1.5-fold increase in choline incorporation into PC (Post *et al.*, 1986). This correlates well with the doubling of the choline phosphate cytidylyltransferase activity. Thus, the action of FPF can be measured by increased conversion of choline phosphate to CDP-choline, and decreased cellular levels of choline phosphate (Post *et al.*, 1986).

The effect of FPF is rapid (60 minutes) suggesting that choline phosphate cytidylyltransferase is controlled by enzyme-modulator interactions rather than by changes in the number of enzyme molecules (Post *et al.*, 1986). It is most likely a post-translational effect such as the translocation of inactive enzyme from the cytosol to the endoplasmic reticulum, where it is activated. This is probably controlled by reversible phosphorylation or the effects of long-chain fatty acids or their CoA derivatives on the enzyme (Post *et al.*, 1986). Thus, the general timeline is: glucocorticoid induction of FPF in fibroblasts → FPF induction of cyclic AMP in epithelial cells → enhanced production of saturated phosphatidylcholine (Smith and Sabry, 1983).

1.5.4 Inhibition of FPF

As discussed above, male infants are at a far greater risk of developing NRDS than female infants. Significantly higher 11-oxidoreductase activity and FPF activity are found in cultures derived from female fetuses (Torday, 1984). Mixed cultures from

female lungs synthesized twice as much PC as male cultures, while there were no sex-specific differences in PC synthesis in pure type II cultures (Torday, 1984). Thus, the effect of testosterone is on the fibroblast cells, and their indirect interaction with the type II cells. FPF production is delayed in fibroblasts derived from male fetuses, because of the presence of dihydrotestosterone. This effect cannot be reversed, even if cells are subsequently stimulated with cortisol (Floros *et al.*, 1987). DHT completely blocks cortisol stimulation of PC synthesis (Torday, 1985). Dihydrotestosterone appears to affect events in FPF production that occur at a pre-translational level (Floros *et al.*, 1987).

Insulin also inhibits production of FPF, leading to an increased incidence of NRDS in children of diabetic mothers (Smith and Post, 1989). The stimulatory effect of cortisol on FPF production is abolished in the presence of insulin (Carlson *et al.*, 1984). It was initially thought that insulin blocked activity of choline phosphate cytidylyltransferase, but this is the enzyme that FPF acts on, and it is the production of FPF that is blocked by insulin (Carlson *et al.*, 1984). Fetal hyperglycemia results in pancreatic islet cell hyperplasia and fetal hyperinsulinemia. The hyperinsulinemic fetal state and the associated antagonism of glucocorticoid action leads to a greater incidence of NRDS (Carlson *et al.*, 1984).

There are several other compounds that can inhibit the production or action of FPF. Cortisol stimulation of PC synthesis was reduced by the presence of monoclonal antibodies to FPF. The presence of these antibodies also delays lung maturation *in vivo* (Post *et al.*, 1984). In late gestation, cortisol inhibits the production of FPF instead of stimulating it (Floros *et al.*, 1987). It has also been suggested that FPF is produced by adult lung fibroblasts, however in adults it appears to inhibit PC synthesis by type II cells (Floros *et al.*, 1987). The anti-glucocorticoid RU486 delays fetal lung maturation,

confirming the vital role of these hormones in natural development (Smith and Post, 1989).

1.6 Secretion of surfactant

Secretion from cells can occur via both constitutive and regulated pathways. Regulated secretion is where a stimulus causes material that has been previously synthesized and stored to be released from a cell. This process is known as classical exocytosis (Michael *et al.*, 2006). The general sequence of events that occurs for secretion of surfactant from the lamellar bodies of type II cells is as follows (Dietl and Haller, 2005):

1. Extracellular stimuli modulate intracellular chemical events;
2. These events cause movement of lamellar bodies towards the apical surface of the cell;
3. The lamellar bodies position themselves adjacent to the apical plasma membrane; and
4. Upon fusion with the apical membrane, the contents of the lamellar bodies are extruded into the alveoli.

The steps linking the initial stimulus to final secretion are not well understood.

Many different models have been used to study secretion. Chemical methods are the most common, and they generally focus on only one component of surfactant, even though surfactant is a heterogeneous mixture. Most studies have used the lipid components to monitor secretion, using whole animals, isolated lung slices and cultured type II cells (e.g. Dobbs *et al.*, 1982; Whitsett *et al.*, 1985). The main drawback to using whole animals is that direct effects on the type II cells cannot be differentiated from

indirect effects via other cells. However, when using cultured type II cells, artifacts can be introduced and effects observed *in vitro* may be more or less important than *in vivo*.

1.6.1 Factors affecting secretion of surfactant

The mechanism of surfactant secretion is less well understood than that of surfactant synthesis. Several different types of stimuli are known to enhance secretion, including:

1. hyperventilation (Klass, 1979)
2. cholinergic agents (Corbet *et al.*, 1976)
3. prostaglandins (Oyarzun and Clements, 1978)
4. thyroxine (Redding *et al.*, 1972)
5. leukotrienes (Gilfillan and Rooney, 1986)
6. agents known to elevate cyclic-AMP levels in type II cells, such as β-adrenergic agonists (Brown and Longmore, 1981; Mescher *et al.*, 1983)
7. agents that elevate protein kinase C activity (Sano *et al.*, 1985)
8. agents that increase the intracellular Ca^{2+} concentration (Dobbs *et al.*, 1986)
9. agents that activate purinoceptors (Gilfillan and Rooney, 1987).

Images of lamellar bodies engaged in exocytosis have been shown in secretion studies involving microscopy (Plate 1.1). The membrane that surrounds the lamellar body fuses with the plasma membrane of the cell (Kliewer *et al.*, 1985). Evidence exists to suggest that most components of surfactant are secreted simultaneously. Specific activity-time curves for PC, DPPC, PG and cholesterol are all very similar (Wright and Clements, 1987), and proteins SP-B and SP-C are secreted with phospholipids (Henry *et al.*, 1996). SP-A and SP-D are secreted independently of the lamellar bodies (Rooney,

2001). Synthesis and secretion are coupled, with high levels of surfactant components leading to inhibition of secretion (Dobbs *et al.*, 1987).

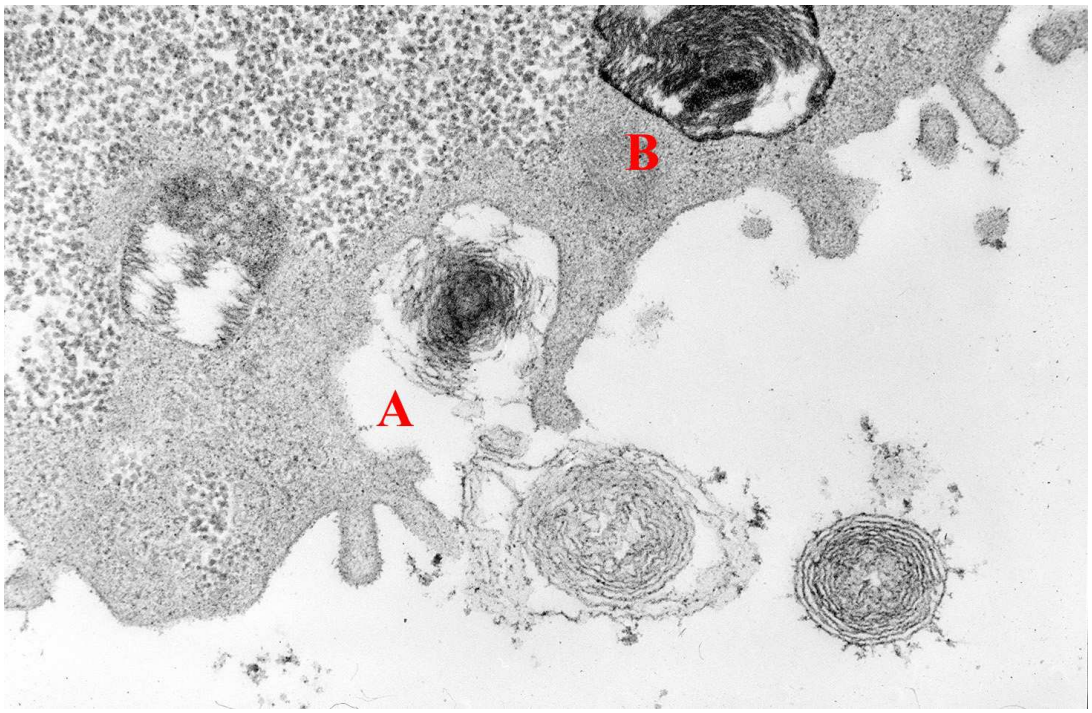

Plate 1.1: Transmission electron micrograph of a cultured fetal type II pneumocyte engaged in secretion of surfactant. A represents a lamellar body that has fused with the cell membrane and is secreting its contents into the alveolar space. B represents an intact lamellar body within the cytosol. Total magnification including photographic enlargement = 46,500x.

Although the mechanism of secretion is not completely known, interaction with several different chemicals gives some information that may be used to build a partial picture of the process. The β-adrenergic agonist terbutaline increases secretion, and it is known to activate cyclic AMP-dependent protein kinase, meaning that a

phosphorylation reaction is likely to be involved (Griese *et al.*, 1992). Cell actin, which has been shown to be closely associated with lamellar bodies, can be phosphorylated via a cyclic AMP-dependent protein kinase. Treatment of type II cells with cyclic AMP alters the intracellular distribution of cytoskeletal β-actin. This evidence suggests that the pathway of secretion involves cyclic AMP-dependent protein kinase-mediated phosphorylation of actin as a key step (Wright and Clements, 1987). Use of A23187 on cells increases cyclic AMP as well as Ca^{2+}, indicating that the intracellular Ca^{2+} concentration may also affect surfactant secretion (Dobbs *et al.*, 1986). Treatment of cells with ATP causes generation of inositol-1,4,5-trisphosphate and diacylglycerol and these compounds lead to the activation of Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C (PKC) (Griese *et al.*, 1991; Chander *et al.*, 1995), although down-regulation of PKC has also been observed (Chander *et al.*, 1998). Several regulatory peptides, known to activate these two protein kinases, have also been shown to have an effect on surfactant secretion (Asokananthan and Cake, 1996). Certain compounds, including compound 40/80 and substance P, have been shown to inhibit secretion from type II cells (Rice and Whitsett, 1984; Rice and Singleton, 1986a).

Administration of β-adrenergic agonists prior to delivery has been shown to lower the risk of NRDS (Bergman *et al.*, 1982), which is consistent with the observation that they stimulate secretion of PC. These include isoproterenol, terbutaline, salbutamol and isoxsuprine (Abdel-Latif and Hollingsworth, 1980; Brown and Longmore, 1981; Chander, 1989). Activation of cell-surface receptors by these agonists is likely to activate the Gs protein, which stimulates adenylate cyclase and therefore elevates the level of cyclic AMP (Brown and Longmore, 1981). A further indication that cyclic AMP can elevate secretion is that a permeable analogue, 8-bromoadenosine-3’5’-cyclic monophosphate, can also increase secretion (Brown and Longmore, 1981).

Purinoceptor agonists are classified as either P1 or P2, depending on the potency of response to ATP, ADP, AMP and adenosine (Brown *et al.*, 1977). Secretion increases in response to P1 agonists, namely AMP, adenosine and non-metabolizable adenosine analogues, as well as P2 agonists, namely ATP and ADP (Chander, 1989). P1 agonists act to increase cyclic AMP levels within type II cells, whereas the action of P2 agonists is less clear. One proposed mechanism is that P2 agonists increase hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), which produces diacylglycerol leading to activation of protein kinase C (Fisher *et al.*, 1984). P2 agonists are more potent than P1, causing a five-fold increase in secretion (Rice and Singleton, 1986b).

Phorbol 12-myristate 13-acetate (PMA) is a tetracyclic diterpene isolated from croton oil (Hecker, 1968), and it is a potent tumor promoter. PMA induces secretion of PC, in both a time-dependent and dose-dependent fashion (Dobbs *et al.*, 1982). The molecular structure of PMA is similar to diacylglycerol, which is known to activate protein kinase C, leading to cell-specific phosphorylation (Nishizuka, 1984). These compounds increase the affinity of the enzyme for Ca^{2+}, which is required for its activation.

The calcium ion (Ca^{2+}) may be a secondary messenger in the regulation of secretion. Calcium ionophores increase the cytosolic Ca^{2+} in type II cells (Page-Robberts, 1972), and this increases the rate of secretion (Marino and Rooney, 1980; Dobbs *et al.*, 1986). Stimulation of type II cells with ATP also increases intracellular Ca^{2+} and PC secretion (Rice and Singleton, 1987).

Treatment of fetal rabbits *in vivo* with epidermal growth factor (EGF) on day 24 of gestation led to greater lung distensibility and increased deflation stability by day 27. Morphological maturation of the alveoli was enhanced, and the number of type II cells and the number of lamellar bodies per cell were also increased (Catterton *et al.*, 1979). EGF has been shown to act directly on the lung to stimulate the synthesis of PC from

choline (Gross and Dynia, 1984), as well as increasing secretion from fetal rat type II cells (Sen and Cake, 1991). The mechanism by which EGF enhances secretion is not well understood, although it has been observed that treatment with EGF increases intracellular Ca^{2+} concentration and protein kinase C activity (Berridge, 1987).

1.7 Clearance and recycling of surfactant

Secreted surfactant is removed from the alveoli via three different mechanisms (Bourbon, 1991):

a) Degradation – components are broken down and used to synthesize new surfactant lipids and proteins;
b) Recycling (or Resorption) – components are not degraded, but are taken up by type II cells, incorporated into new lamellar bodies and re-secreted; and
c) Removal – surfactant is removed from the alveolar region either as intact molecules or partially-degraded compounds.

It is possible that if material is not removed from the alveolar region then it could build up to dangerous levels. Removal of surfactant does not occur via the lymphatic system (Tarpey *et al.*, 1983), although macrophages can digest surfactant compounds (King and Martin, 1980). Resorption of surfactant by type II cells is responsible for the majority of the clearance from the alveolar region (Hallman *et al.*, 1981), and isolated type II cells in culture can ingest PC (Chander *et al.*, 1983). It is possible that uptake involves it specifically binding to receptors, followed by endocytosis (Williams *et al.*, 1984). A portion of surfactant is taken up into type II cells, degraded by lysosomes and the resulting degradation products transported to the endoplasmic reticulum for re-synthesis into surfactant (Chander *et al.*, 1983).

The majority of surfactant is reused without being degraded. In rabbits, it has been estimated that as much as 85% of surfactant is reutilized (Magoon *et al.*, 1983). Phosphatidylcholine turnover in the alveoli occurs every 10.1 hours, but the biological half-life of the compound is 41 hours (Jacobs *et al.*, 1983). This difference clearly indicates that PC is reused within the type II cells. PC is thought to be deposited as intact molecules back into lamellar bodies once it has been resorbed (Hallman *et al.*, 1981). Multivesicular bodies are formed as precursor compartments in this recycling process (Jacobs *et al.*, 1985; Fisher *et al.*, 1987).

It is likely to be more energy efficient to recycle surfactant material that has already been produced, rather than to degrade and re-synthesize it. It has been estimated that approximately 10% of the alveolar pool of surfactant is recycled per hour (Jacobs *et al.*, 1985), although the efficiency of reuse varies greatly with age (Jobe *et al.*, 1989). Radiolabelled lipids remain in the alveolar space for several days. The specific activity of lavage and lamellar body lipids gradually becomes equal, and does not fall to zero until over 100 hours post-injection (Hallman *et al.*, 1981). It is possible that recycling is stimulated by β-adrenergic agonists and cyclic AMP (Fisher *et al.*, 1987).

1.8 Respiratory Distress Syndrome

Premature birth is the primary cause of morbidity and mortality in infants less than one month old (Griese and Westerburg, 1998). The major factor contributing to these deaths is neonatal respiratory distress syndrome (NRDS), which is caused by the immaturity of the lungs and a deficiency of pulmonary surfactant (Griese and Westerburg, 1998). Other health effects of NRDS include pulmonary oedema and leakage of plasma contents into the airway (Cott *et al.*, 1987; Liu *et al.*, 1997; Wang and Notter, 1998). There is also a risk of bronchiolar over-distension during inspiration, which can cause epithelial disruption in the airways (Robertson, 1984). Levels of

oedema and unresorbed pulmonary fluid are greater during the early stages of the disease (DeSa, 1969). Most cases of NRDS are caused by a functionally immature respiratory system, which produces far less surfactant than is required (Griese, 1999).

Infants with NRDS exhibit a classic histopathological pattern, with liquid- and fibrin-filled and/or collapsed alveoli and over-distended, aerated terminal airways (Robertson, 1984). This pattern is known as atelectasis and is caused as the air-liquid interface rises to the bronchiolar level at end-expiration, as well as increased resistance to aeration due to capillarity in the conducting airways. NRDS is also known as hyaline membrane disease, as hyaline membranes are found lining the terminal airways (Griese, 1999).

1.8.1 Biochemistry of NRDS

Avery and Mead (1959) were the first to observe a deficiency of surfactant in the watery lung extract of infants with what was, at the time, known as hyaline membrane disease. Infants with NRDS have a complete lack, or greatly reduced amount, of both PC and PG as well as decreased levels of surfactant phospholipids and SP-A. These result in an increased alveolar surface tension (Griese, 1999). Infants with NRDS who die within the first 48 hours of life have been shown to have a specific lack of SP-A (Markgraf *et al.*, 1990). Other surfactant-associated proteins are not as reduced in concentration as the lipids, which can modify the activity of surfactant and cause further problems (Ikegami *et al.*, 1984; Seeger *et al.*, 1985). These problems possibly arise due to competition or chemical interactions between the proteins and other components of surfactant.

A hereditary deficiency of SP-B can also cause NRDS, due to changes in the surfactant protein composition (Nogee *et al.*, 1994). A single amino acid substitution in

the gene coding for SP-B is sufficient to cause NRDS symptoms. Deficiency of SP-C can also reduce the stability of surfactant (Glasser *et al.*, 2001).

1.9 Proposed study

The purpose of the present study was to attempt to identify and better understand the nature of fibroblast-pneumocyte factor. The direct use of FPF to treat NRDS would present a viable alternative to current treatments, and avoid problems associated with inhibition of FPF production by compounds such as insulin and dihydrotestosterone. The study required the optimization of existing protocols for production and detection of the protein via tissue culture. From this, a refined method was established which will improve reproducibility of future results. Several possible stimulants of FPF production were tested to determine which would have the greatest indirect effect on surfactant synthesis. Conditioned medium produced by exposure of cultured fetal lung fibroblasts to these stimulants was then analyzed in an attempt to identify FPF.

Recent literature had indicated that neuregulin-1β is the most likely candidate for FPF (Dammann *et al.*, 2003). Other studies had identified a probable role for NRG1 in lung development (Patel *et al.*, 2000; Liu *et al.*, 2004), and further study of the neuregulin system showed that it could fulfill the functions of FPF. This protein was analyzed using HPLC and quadrupolar ion trap mass spectrometry, and the resultant spectra used to search for FPF in the fibroblast-conditioned media (FCM) produced using the tissue culture system. This method was also used to quantify the amount of neuregulin-1β present in FCM, and to detect any change in its concentration when fibroblasts were exposed to dexamethasone.

The observation that NRG1 could indeed be FPF prompted the examination of similar proteins for possible roles in the surfactant system. The effect of insulin on the production of surfactant has been well-researched (Gross *et al.*, 1980; Snyder and

Mendelson, 1987), and due to similarities to both insulin and the neuregulins (Zorzano *et al.*, 2003) and a demonstrated role in lung development (Maitre *et al.*, 1995), it is possible that insulin-like growth factors (IGFs) may also exert an influence on both synthesis and secretion of surfactant. Tissue culture-based experiments involving the addition of IGF-II, as well as other known agonists and antagonists, were conducted.

Chapter 2. Materials and methods

2.1 Animals

Nineteen-day pregnant rats of the Wistar strain of *Rattus norvegicus* were used for all experiments. The full gestation period of these animals is 22 days, so fetuses were delivered approximately three days premature. Animals were supplied by the Animal Resource Centre (Murdoch, Western Australia). Their mating protocol involves caging male and female rats overnight, followed by vaginal smearing the next morning. If sperm is detected, then conception is considered to have occurred. This is accurate to within eight hours of actual conception and is considered to be day zero. All experiments complied with National Health and Medical Research Council guidelines and were approved by the Murdoch University Animal Experimentation Ethics Committee.

2.2 Preparation of materials for cell culture

2.2.1 Materials

Eagle's minimal essential medium (MEM) and newborn bovine serum were obtained from Thermo Fisher Scientific, Waltham, MA, USA. Penicillin G, streptomycin sulfate, solubilised β-amphotericin (Fungizone) and 3,5-diaminobenzoic acid were supplied by Sigma-Aldrich, St. Louis, MO, USA. L-glutamine was purchased from Invitrogen Corp., Carlsbad, CA, USA. Chloramphenicol was obtained from NBL Gene Sciences Ltd., Northumberland, UK. Collagenase A (*Clostridium histolyticum*) was a product of F. Hoffman-La Roche Ltd., Basel, Switzerland. FSA Laboratories, Leicester, UK supplied 'Hi-Safe II' scintillant. Filters were purchased from Millipore Pty. Ltd., Billerica, MA, USA. All radioisotope-labelled

compounds were supplied by GE Healthcare, Little Chalfont, UK. All other reagents were of analytical grade.

2.2.2 Charcoal treatment of newborn calf serum

Newborn calf serum (NBCS) was incubated with sterile, acid-washed charcoal (50 $mg.mL^{-1}$ serum) for 30 minutes in a 37°C shaking water bath, to remove endogenous steroids. Charcoal was then removed by two successive centrifugations at 27000 *g* for 45 minutes at 4°C in a Beckman J2-21M/E centrifuge using a JA-20 rotor (Beckman Instruments, Palo Alto, CA, USA). Serum was filtered through a 0.44 μm Millipore filter to remove remaining charcoal, and then through a 0.22 μm Millipore filter to sterilize. Charcoal-treated newborn calf serum was stored in 11.5 mL aliquots at -20°C.

2.2.3 Preparation of rat immunoglobulin G

Rat immunoglobulin G (IgG) was purified using the method of Hudson and Hay (1976). Serum was obtained from whole blood via centrifugation, and stored at -20°C until purification. The serum was diluted 1:2 with 0.145 M NaCl, followed by the addition of 100% (w/v) $(NH_4)_2SO_4$ (buffered to pH 7.2) to a final concentration of 45%. This mixture was stirred for 30 minutes at room temperature and the precipitate collected via centrifugation (at 1000 *g* for 15 minutes). The precipitate was washed with 45% $(NH_4)_2SO_4$ and re-dissolved to the original volume in phosphate-buffered saline (PBS) (pH 7.2). PBS contains 136.9 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl and 1.47 mM K_2HPO_4. This new mixture was centrifuged at 1000 *g* for 15 minutes to remove any remaining insoluble material. The supernatant was adjusted to 40% $(NH_4)_2SO_4$ and the precipitate washed with a 40% $(NH_4)_2SO_4$ solution. This precipitate was re-dissolved in a minimum volume of PBS and dialyzed at 4°C against

two changes of PBS (at least 12 hours per change). Any precipitate was removed via centrifugation and the purified material was lyophilized and stored at 4°C.

2.2.4 Preparation of balanced salts solution

Balanced salts solution (BSS) was prepared by mixing 900 mL MilliQ water, 50 mL 20x saline solution and 50 mL of 20x buffer solution. The solution was adjusted to pH 7.4 and filtered through a 0.22 μm Millipore filter to sterilize. 20x saline solution contains 2.74 M NaCl, 54 mM KCl, 20 mM $CaCl_2$ and 20 mM $MgCl_2$. 20x buffer solution contains 119 mM $NaHCO_3$, 111 mM D-glucose, 27 mM Na_2HPO_4 and 3 mM NaH_2PO_4.

2.2.5 Preparation of culture media

Eagle's minimal essential medium was reconstituted as specified by the manufacturer, and supplemented with 0.2% $NaHCO_3$. This mixture was then adjusted to pH 7.4. L-glutamine, penicillin G and streptomycin sulphate were added to final concentrations of 2.6 mM, 100 $IU.mL^{-1}$ and 100 $\mu g.mL^{-1}$, respectively. Medium was sterilized by filtration through a 0.22 μm Millipore filter and 3.23 $\mu g.mL^{-1}$ of Fungizone was then added. This solution is termed MEM^{+}. To produce MEM-complete (MEM^{c}), newborn calf serum was added to a concentration of 10%. Due to a persistent contamination by a chloramphenicol-sensitive bacterium in late 2005, 50 $\mu g.mL^{-1}$ of this antibiotic was added to the medium from November 2005.

2.3 Isolation of fibroblasts and type II pneumocytes

Sterile techniques were used during all cell isolation procedures, all of which were performed at room temperature unless otherwise stated. Nineteen-day pregnant rats were killed by anaesthesia with 80:20 O_2:CO_2 followed by asphyxiation with

100% CO_2. Fetuses were delivered by Caesarean section. Fetal lungs were removed and placed in a Petri dish of warm BSS. Any other tissues, such as the heart and thymus, were removed and discarded. Lungs were subsequently minced using a McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Surrey, UK) set at 0.1 mm with two traverses, one perpendicular to the other. Tissue was then placed in a sterile flask containing 0.75 mL warm BSS per lung and collagenase (approximately 0.26 $U.mg^{-1}$, slight variation between batches) was added to a final concentration of 0.2 $mg.mL^{-1}$. The flask was gassed with carbogen (95:5 O_2:CO_2) for 90 seconds to ensure cell survival, and incubated for 20 minutes in a 37°C shaking water bath.

Following incubation the tissue was filtered through two layers of sterile French *voile* gauze into centrifuge tubes. Additional warm BSS was added to ensure that all tissue was transferred from the culture flask to the tube. The mixture was centrifuged at 20 *g* for two minutes, supernatant was removed and replaced with warm BSS, and the washing procedure repeated. Cells were resuspended in warm serum-free MEM^+ (10 mL per 20 lungs) and plated onto 6 cm (diameter) Corning tissue culture plates (3 mL per plate). These plates were incubated for 30 minutes at 37°C in a humidified CO_2 incubator (5% CO_2, 95% air) to allow adhesion of fibroblasts. Following incubation, non-adhering cells were suspended by gentle swirling and removed from the plates. To each plate, 3 mL of MEM-complete was added.

The non-adhering cells were used to isolate type II pneumocytes, according to the method of Dobbs *et al.* (1986). Type II cells lack F_c receptors, whereas most other lung cells possess these receptors and so adhere to plates coated with IgG. Bacteriological plates (Falcon 1001, Falcon Labware, Becton Dickinson, Lincoln Park, NJ, USA) were coated with 3 mL of rat IgG solution (0.5 $mg.mL^{-1}$) at least 24 hours prior to use, and dried at room temperature under ultraviolet light to maintain sterility. Plates were washed twice with 10 mL of BSS and non-adhering cells from the fibroblast

cultures were transferred onto these plates. IgG plates were incubated in the humidified CO_2 incubator for 60 minutes. Following incubation, plates were removed from the incubator and type II cells were resuspended by gentle panning (so as not to detach other cell types). Type II cells were transferred to a sterile flask and diluted with MEM^+ to a concentration of 0.185 lungs.mL^{-1}. NBCS was then added to a final concentration of 10%. 3 mL of this cellular suspension (equivalent to 0.5 lungs) was plated onto 6 cm (diameter) Primaria tissue culture plates. After 2-3 days these plates are considered to be confluent cultures consisting of at least 90% type II cells (Plates 2.1, 2.2). This method was validated previously using Papanicolaou staining (Sen, 1991).

2.4 Conditions of cell culture

Cultures were maintained in a water-saturated atmosphere of 5% CO_2 and 95% air and at a temperature of 37°C. The incubator was a Forma Scientific 3157 water-jacketed CO_2 incubator (now supplied by Thermo Fisher Scientific, Waltham, MA, USA). The carbon dioxide level was monitored using a Bacharach Fyrite test kit (Bacarach Instruments Co., Pittsburg, PA, USA). To help maintain a sterile environment, 500 mL of Microshield 5 antiseptic solution (Johnson & Johnson, East London, UK) was added to the water pan, which was changed every month.

Type II cultures were given a media change at 24 hours post-plating. This involved removing media from the plates, washing with 2 mL of BSS and adding 3 mL of MEM^c to each plate. Fibroblast cultures were given a media change after 48 hours of culture, and every 48 hours (72 hours over weekends) after that, for 14 days. BSS and MEM^c were pipetted into the corner of the plate, so as not to detach cells.

Since the conclusion of tissue culture experiments for this project, it has come to light that there was an error in the laboratory techniques that had been provided at the beginning of the project. Both type II and fibroblast cultures should receive a media

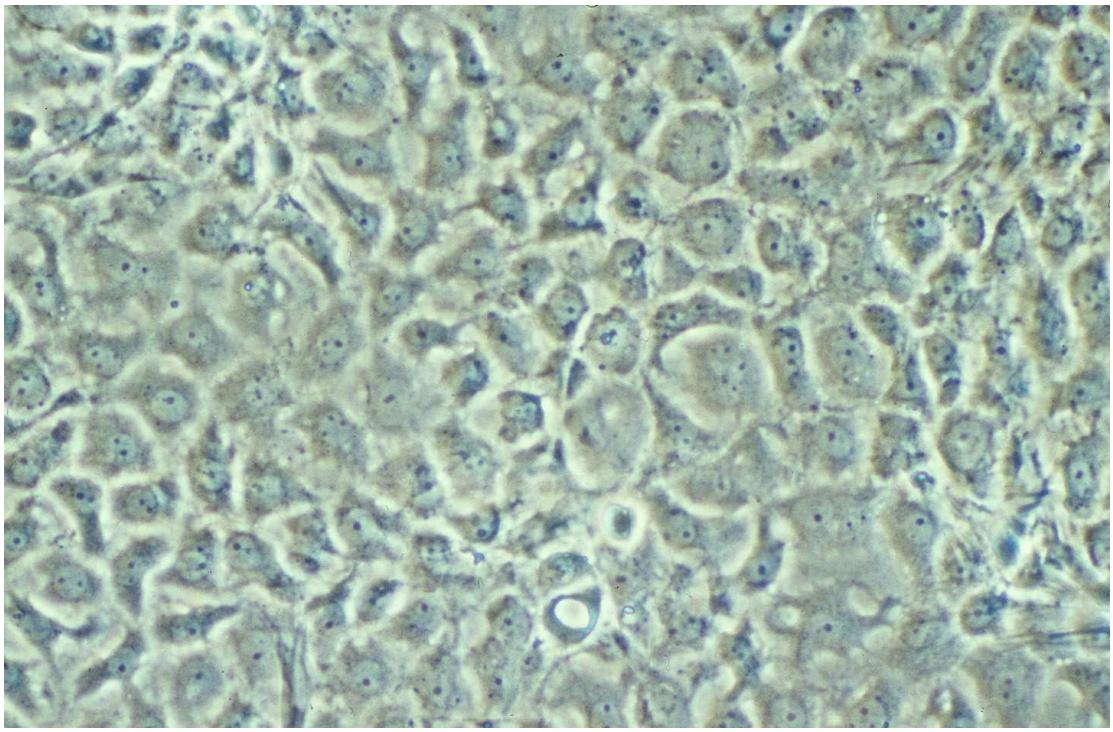

Plate 2.1: Phase contrast photomicrograph of a typical 19-day fetal type II pneumocyte culture.

Type II pneumocytes were isolated and cultured from 19-day fetal rats. A phase contrast photomicrograph of cells was taken after four days in culture using a Kodak Ektachrome 160 tungsten film on an Olympus IMT-2 inverted phase microscope. The overall magnification (including photographic enlargement) was 880x.

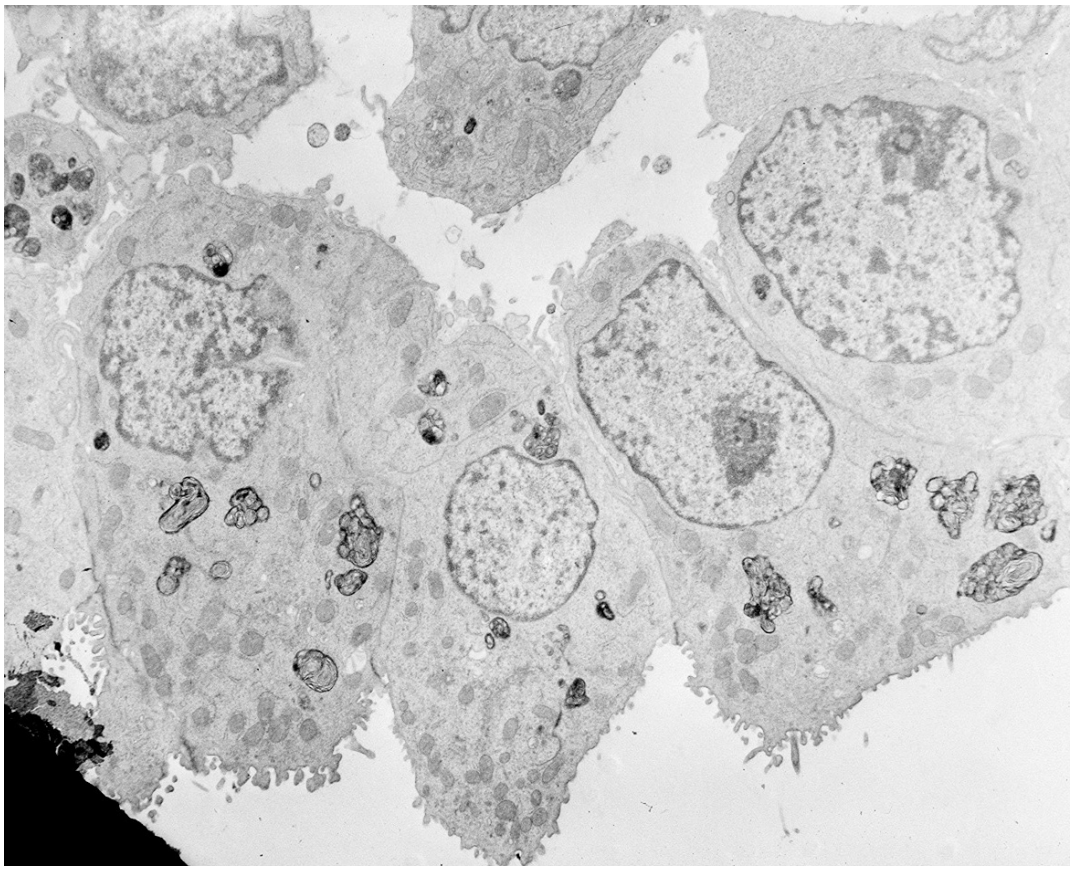

Plate 2.2: Transmission electron micrograph of a typical 19-day fetal type II pneumocyte culture.

Type II pneumocytes were isolated and cultured from 19-day fetal rats. A transmission electron micrograph of cells was taken after four days in culture using a Philips CM100 electron microscope. The overall magnification (including photographic enlargement) was 4680x.

change 24 hours after plating, and then every 48 hours, regardless of weekends. Failure to follow this regimen can greatly affect the yield of cells on fibroblast plates.

2.5 Preparation of fibroblast-conditioned medium

After 15 days in culture, fibroblast plates were considered confluent, and free of any type II cell contamination (Plate 2.3), as judged by microscopy. At this time, media was removed from the plates and they were washed twice with 2 mL of BSS. 1.7 mL of MEM^+ was added to each plate as well as 17 μL of additive + vehicle (test) or vehicle only (control). Plates were returned to the incubator for a further 24 hours, after which fibroblast-conditioned medium was collected, transferred to sterile bottles and stored at -80°C. Cells were then harvested from the plates for DNA assay using the method described for synthesis experiments (**2.6**).

2.6 Initial synthesis experimental method

Fibroblast-conditioned medium (FCM) produced by the above method should contain fibroblast-pneumocyte factor (FPF) (Smith and Post, 1989). To confirm the presence and activity of the protein, it was necessary to test its ability to stimulate synthesis of phosphatidylcholine (PC) by type II cells. The following method has been used in this laboratory for over a decade, and is based on the lipid extraction method of Bligh and Dyer (1959).

FCM was removed from the -80°C freezer and thawed at room temperature. This was demonstrated by Smith (1981a) to have no effect on activity of FPF. FCM was then heat-treated in a 65°C water bath for 60 minutes to remove known inhibitory factors. After cooling to room temperature, it was filtered through a 0.22 μm Millipore syringe filter and diluted 1:4 with MEM^+ (0.8 mL FCM: 2.4 mL MEM^+). To this diluted

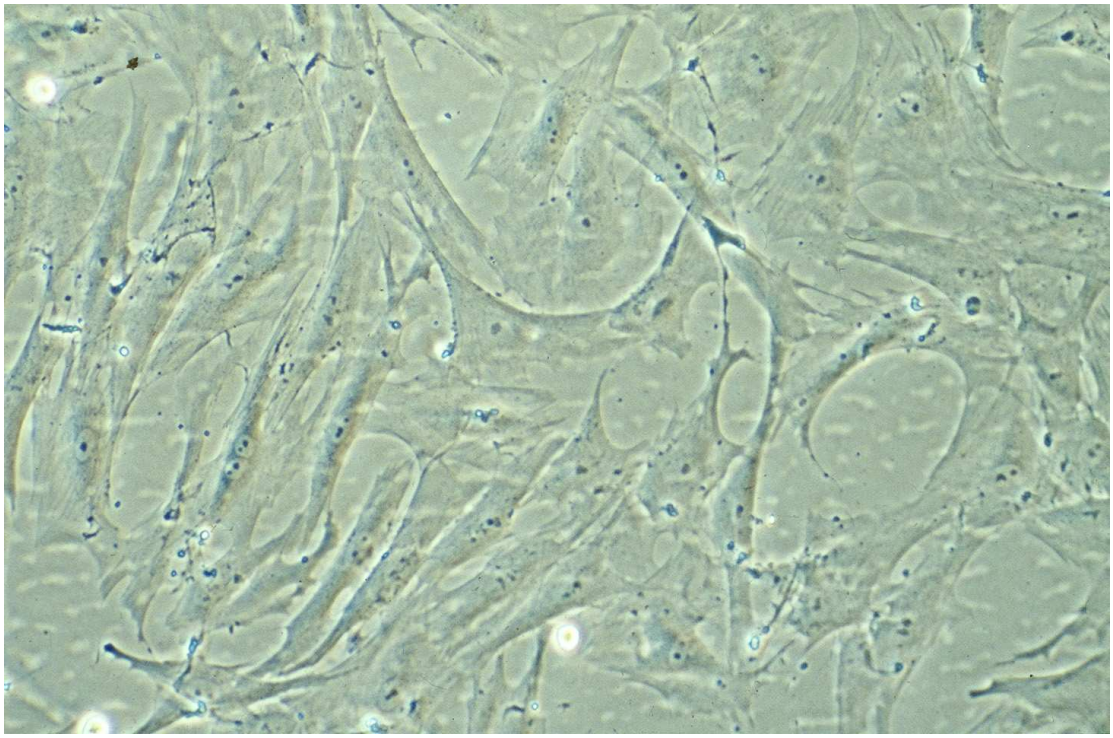

Plate 2.3: Phase contrast photomicrograph of a typical 19-day fetal fibroblast culture.

Pulmonary fibroblasts were isolated and cultured from 19-day fetal rats. A phase contrast photomicrograph of the cells was taken after 15 days culture using a Kodak Ektachrome 160 tungsten film on an Olympus IMT-2 inverted phase microscope. The overall magnification (including photographic enlargement) was 1240x.

media, 24 μL of either additive + vehicle or vehicle only was added, depending on the FCM required. Bottles were thoroughly vortexed to ensure adequate mixing.

Type II plates were removed from the incubator after approximately 72 hours of culture and the media removed. Plates were washed twice with 2 mL of BSS and 3 mL of diluted, supplemented fibroblast-conditioned media was added to each plate. In addition, control plates were produced by adding 3 mL of MEM^+ plus 30 μL of either additive + vehicle or vehicle only. Plates were returned to the incubator for a further 21 hours incubation.

A solution of 7 $\mu Ci.mL^{-1}$ [methyl-^{3}H]-choline chloride in MEM^+ was prepared. The labeled choline, supplied as an ethanol solution, was dried under nitrogen prior to the addition of MEM^+. Type II plates were removed from the incubator and 500 μL of this [^{3}H]-choline chloride solution was added to each. Plates were swirled to mix and returned to the incubator for a further 6 hours. This incubation time was used as it represents the middle of the linear phase of choline incorporation.

At the conclusion of incubation, plates were removed from the incubator and placed on ice to prevent further lipid synthesis. Media was removed from the plates and discarded. Plates were washed twice with 1 mL of BSS, with cells being scraped from the plate with a spatula at each step. The wash buffer was collected, along with the cells, into a pre-weighed conical centrifuge tube, which was kept on ice until all plates had been harvested. Weighing of tubes provides a preliminary indication of cultures with a poor yield. Tubes were centrifuged at 1000 *g* and 4°C for two minutes and the supernatant was removed by aspiration and discarded. Centrifuge tubes were again weighed, to allow calculation of the weight of cells harvested from each plate. 400 μL of BSS was added to each tube and the tubes were sonicated using a Braunsonic 1510 sonicator with a microprobe (B. Braun, Melsungen, Germany) at 10 watts for 10 seconds to disrupt the cells.

From this sonicate, two 50 μL aliquots were taken and dried in a 50°C oven to determine DNA content. Lipids were extracted from cells using the method of Bligh and Dyer (1959) as follows: A further 200 μL aliquot of the sonicate was taken and added to a tube containing 2 mL methanol, 1 mL chloroform, 600 μL of MilliQ-H_2O, 50 μL of L-3-phosphatidylcholine-1,2-di[1-^{14}C]palmitoyl (approximately 10000 dpm) as a recovery standard and 20 μL of 2.5 mg.mL^{-1} phosphatidylcholine as a carrier. A further 1 mL of chloroform and 1 mL of MilliQ-H_2O were added, the tubes vortexed for 10 seconds and placed in a 4°C refrigerator for at least 48 hours.

The amount of DNA was determined using a method adapted from Hinegardner (1971). DNA assay tubes were removed from the oven after at least 24 hours and 100 μL of a 0.3 g.mL^{-1} 3,5-diaminobenzoic acid solution was added to each. Tubes were placed back in the 50°C oven for a further 45 minutes to allow product development. After this, 1.5 mL of 1.0 M HCl was added to each tube. Tubes were read in a Perkin-Elmer LS-50 luminescence spectrofluorometer (Perkin-Elmer, Waltham, MA, USA) with excitation wavelength at 415 nm and emission wavelength at 505 nm.

During the 48 hours in the refrigerator, the chloroform:methanol extract separated into two layers, with the lower layer containing lipid. The upper water/methanol layer was removed via aspiration and a 400 μL aliquot of the lower layer was taken and placed into a scintillation vial. The contents of the vial were dried by evaporation for two hours and 3 mL of scintillant was added to each vial. Vials were vortexed and left to stand at room temperature for 24 hours prior to counting in a Beckman LS3801 scintillation counter (Beckman Instruments, Palo Alto, CA, USA). Three 50 μL samples from the [^{14}C]-labelled phosphatidylcholine standard were also counted. Tubes were counted for both ^{3}H and ^{14}C. To determine synthesis of phospholipids, several calculations were performed using data from the experiment.

The first calculation was:

$$^{3}\text{H incorporated (dpm)} \quad \text{x} \quad \frac{^{14}\text{C input (dpm)}}{^{14}\text{C recovered (dpm)}}$$

This determined the recovery of ^{14}C, which was used to obtain the full count for ^{3}H at 100% recovery. DNA, which was calculated from 50 μL of sonicate, was multiplied by four to give the total from 200 μL of sonicate, the same volume used for lipid extraction. The ^{3}H value, which had been corrected for recovery, was then divided by the DNA in 200 μL to give dpm incorporated.ng DNA^{-1}. Duplicates were performed for all experiments, and these were averaged to give a mean value. By comparing the control and test values for both direct and indirect treatments, the percentage increase due to FPF was determined. The choline-labelled material produced using this method was previously characterised by TLC (Sen, 1991) and shown to consist almost exclusively (>85%) of phosphatidylcholine.

2.7 Early synthesis results

2.7.1 Dexamethasone

Dexamethasone has been demonstrated to cause a 2.5-fold increase in PC synthesis in rat lung cultures (Gross and Wilson, 1982), but direct exposure of type II cells to dexamethasone has no effect. However, when fibroblasts cells are exposed to dexamethasone and the resultant conditioned medium exposed to type II cultures, PC synthesis is augmented (Smith and Post, 1989). Dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) was stored at 4°C as a 10 μM solution in propylene glycol (Ajax Chemicals, Auburn, NSW, Australia). Following the method described in **2.5**, dexamethasone was added to confluent fibroblast plates to a final concentration of 100 nM, as this concentration had previously been shown to give maximal stimulation

(Sen, 1991). Control plates substituted propylene glycol. The resultant FCM was tested for its ability to increase PC synthesis according to the method described in **2.6**.

Thirteen pairs of control and test FCM were tested and the results obtained were very inconsistent (Table 2.1). Rather than the expected increase in synthesis, both the direct and indirect treatments showed a mean decrease (14.91 and 26.07% respectively).

Table 2.1: The direct and indirect effect of dexamethasone on the augmentation of PC synthesis by type II pneumocytes.

Treatment	PC Synthesis (dpm/ng DNA)	Difference	P-value	Mean type II pneumocyte DNA (μg/plate)	Mean fibroblast DNA level (μg/plate)
Direct (13)					
Control	40.05 ± 7.61				
Dexamethasone	34.08 ± 6.56	↓ 14.91%		22.64	
Indirect (13)			0.18		
Control	36.29 ± 7.16				
Dexamethasone	26.83 ± 5.84	↓ 26.07%		24.21	15.37

Primary cultures of lung fibroblasts were grown to near confluence, followed by 24 hours incubation in the presence or absence of 100 nM dexamethasone in MEM^+. The fibroblast-conditioned media (FCM) were collected and stored at -80°C. Prior to use, the media were thawed and incubated at 65°C for one hour, cooled to room temperature and filter-sterilized to remove detached cells and cellular debris. The media were then diluted 1:4 with MEM^+. The effect on PC synthesis by type II pneumocytes was investigated by exposing the cells either to the FCM (indirect) or to MEM^+, with and without dexamethasone (direct). The data represent the mean ± SE. The number of replicates examined for each condition is indicated in parentheses, and each replicate comprised four cultures. Data were compared using single-factor ANOVA.

PC synthesis values (expressed in dpm.ng DNA^{-1}) were very high for these experiments, being almost double the magnitude previously observed (Sen and Cake, 1991). Standard error values were also very high, as much as 21.8% in the case of indirect dexamethasone treatments. Single-factor ANOVA ($p = 0.18$) indicated no significant difference between the direct and indirect treatments.

The manufacturer of the dexamethasone was contacted and confirmed that, while the product has no specific 'expiry date', its activity could not be guaranteed more than six months after purchase. As the dexamethasone used was far older than this, it is likely that poor quality reagents were the cause of the inconsistent results obtained in this set of experiments.

2.7.2 PMA

The inconsistent results obtained with dexamethasone led to consideration of possible alternative stimulants of FPF production. 12-O-Tetradecanoylphorbol-13-acetate, more commonly known as phorbol 12-myristate 13-acetate (PMA), is a tetracyclic diterpene isolated from croton oil (Hecker, 1968) that has previously been shown to have a significant effect on PC synthesis via fibroblasts (Sen, 1991). PMA acts via protein kinase C, meaning that it is likely to stimulate FPF production via a different mechanism than glucocorticoids. These factors made it an ideal choice for a second series of experiments. PMA was stored at 4°C as a 0.5 $mg.mL^{-1}$ solution in dimethylsulphoxide (DMSO) (both from Sigma-Aldrich, St Louis, MO, USA). This solution was diluted with 1% DMSO in BSS to give a working concentration of 2 μg $PMA.mL^{-1}$. The vehicle added to the plates was 1% DMSO in BSS. FCM was generated as described in **2.5** and tested as in **2.6**.

Fourteen pairs of control and test FCM were tested, and again the results were very inconsistent (Table 2.2). Both the direct and indirect tests showed a mean decrease

Table 2.2: The direct and indirect effect of phorbol 12-myristate 13-acetate on the augmentation of PC synthesis by type II pneumocytes.

Treatment	PC Synthesis (dpm/ng DNA)	Difference	P-value	Mean type II pneumocyte DNA (μg/plate)	Mean fibroblast DNA level (μg/plate)
Direct (14)					
Control	21.07 ± 6.57				
PMA	15.94 ± 3.31	↓ 24.35%		35.59	
Indirect (14)			0.93		
Control	21.97 ± 5.54				
PMA	18.75 ± 4.07	↓ 14.66%		37.25	16.08

Primary cultures of lung fibroblasts were grown to near confluence, followed by 24 hours incubation in the presence or absence of 2 $\mu g.mL^{-1}$ phorbol 12-myristate 13-acetate (PMA) in MEM^{+}. The fibroblast-conditioned media (FCM) were collected and stored at -80°C. Prior to use, the media were thawed and incubated at 65°C for one hour, cooled to room temperature and filter-sterilized to remove detached cells and cellular debris. The media were then diluted 1:4 with MEM^{+}. The effect on PC synthesis by type II pneumocytes was investigated by exposing the cells either to the FCM (indirect) or to MEM^{+}, with and without PMA (direct). The data represent the mean ± SE. The number of replicates examined for each condition is indicated in parentheses, and each replicate comprised four cultures. Data were compared using single-factor ANOVA.

in PC synthesis (24.35 and 14.66% respectively). Standard error values were again very high, with direct control treatments having an error of 31.8%. Single-factor ANOVA ($p = 0.93$) showed no significant difference between direct and indirect response to PMA.

2.8 Final synthesis experimental method

The erratic results obtained from the first 29 experiments indicated a possible problem with the experimental methodology. To improve accuracy, several steps were added to the method, mostly to monitor the addition of [^{3}H]-choline chloride to the plates. The entire method for synthesis experiments is presented again, with alterations and additions noted in bold.

FCM was removed from the -80°C freezer and thawed at room temperature. This was demonstrated by Smith (1981a) to have no effect on activity of FPF. FCM was then heat-treated in a 65°C water bath for 60 minutes to remove known inhibitory factors.After cooling to room temperature, it was filtered through a 0.22 μm Millipore syringe filter and diluted 1:4 with MEM$^+$ (0.8 mL FCM: 2.4 mL MEM$^+$). To this diluted media, 24 μL of either additive + vehicle or vehicle only was added, depending on the FCM. Bottles were thoroughly vortexed to ensure adequate mixing.

Type II plates were removed from the incubator after approximately 72 hours of culture and the media removed. Plates were washed twice with 2 mL of BSS and 3 mL of diluted, supplemented fibroblast-conditioned media was added to each plate. In addition, control plates were produced by adding 3 mL of MEM$^+$ plus 30 μL of either additive + vehicle or vehicle only. Plates were returned to the incubator for a further 21 hours incubation.

A solution of 7 μCi.mL^{-1} [methyl-^{3}H] choline chloride in MEM$^+$ was prepared. The labeled choline, supplied as an ethanol solution, was dried under nitrogen prior to

the addition of MEM^+. **The flask was incubated in a 37°C shaking water bath for 10 minutes to ensure all of the choline chloride had gone into solution. Media was removed from type II plates to sterile tubes, and 500 μL of [^{3}H]-choline chloride solution added to each. Tubes were vortexed and a 50 μL sample of each taken for determination of initial ^{3}H content. In addition, three 50 μL samples of the [^{3}H]-choline chloride solution were taken to be counted as well. Media was returned to the plates,** which were placed in the incubator for a further 6 hours. **Samples for counting were placed in scintillation vials, 3 mL of scintillant was added to each, vials were vortexed and they were left in the dark for at least 24 hours prior to counting.**

At the conclusion of incubation, plates were removed from the incubator and placed on ice to prevent further lipid synthesis. Media was removed from the plates and discarded. Plates were washed twice with 1 mL of BSS, with cells being scraped from the plate with a spatula at each step. The wash buffer was collected, along with the cells, into a pre-weighed conical centrifuge tube, which was kept on ice until all plates had been harvested. Weighing of tubes provides a preliminary indication of cultures with a poor yield. Tubes were centrifuged at 1000 *g* and 4°C for two minutes and the supernatant was removed by aspiration and discarded. Centrifuge tubes were again weighed, to allow calculation of the weight of cells harvested from each plate. 400 μL of BSS was added to each tube and the tubes were sonicated using a Braunsonic 1510 with a microprobe at 10 watts for 10 seconds to disrupt the cells.

From this sonicate, two 50 μL aliquots were taken and dried in a 50°C oven to determine DNA content. Lipids were extracted from cells using the method of Bligh and Dyer (1959) as follows: A further 200 μL aliquot of the sonicate was taken and added to a tube containing 2 mL methanol, 1 mL chloroform, 600 μL of MilliQ-H_2O, 50 μL of L-3-phosphatidylcholine-1,2-di[1-^{14}C]palmitoyl (approximately 10000 dpm) as a

recovery standard and 20 μL of 2.5 $mg.mL^{-1}$ phosphatidylcholine as a carrier. **This mixture was vortexed for 10 seconds and left to stand at room temperature for 10 minutes.** A further 1 mL of chloroform and 1 mL of MilliQ-H_2O were added, the tubes vortexed for 10 seconds and placed in a 4°C refrigerator for at least 48 hours. The total volume of the organic layer was 2.05 mL.

The amount of DNA was determined using a method adapted from Hinegardner (1971). DNA assay tubes were removed from the oven after at least 24 hours and 100 μL of a 0.3 $g.mL^{-1}$ 3,5-diaminobenzoic acid solution was added to each. Tubes were placed back in the 50°C oven for a further 45 minutes to allow product development. After this, 1.5 mL of 1.0 M HCl was added to each tube. Tubes were read in a Perkin-Elmer LS-50 luminescence spectrofluorometer (Perkin-Elmer, Waltham, MA, USA) with excitation wavelength at 415 nm and emission wavelength at 505 nm.

During the 48 hours in the refrigerator, the chloroform:methanol extract separated into two layers, with the lower layer containing lipid. The upper water/methanol layer was removed via aspiration and a 400 μL aliquot of the lower layer was taken and placed into a scintillation vial. The contents of the vial were dried by evaporation for two hours and 3 mL of scintillant was added to each vial. Vials were vortexed and left **in the dark** at room temperature for 24 hours prior to counting in a Beckman LS3801 scintillation counter (Beckman Instruments, Palo Alto, CA, USA). Three 50 μL samples from the ^{14}C-labelled phosphatidylcholine standard were also counted. Tubes were counted for both ^{3}H and ^{14}C, **and in most cases were counted either two or three times to confirm values**.

To determine synthesis of phospholipids, several calculations were performed using data from the experiment. The first calculation was:

$$^{3}H \text{ incorporated (dpm)} \quad x \quad \frac{^{14}C \text{ input (dpm)}}{^{14}C \text{ recovered (dpm)}}$$

This determined the recovery of ^{14}C, which was used to obtain the full count for ^{3}H at 100% recovery. DNA, which was calculated from 50 μL of sonicate, was multiplied by four to give the total from 200 μL of sonicate, the same volume used for lipid extraction. The ^{3}H value, which had been corrected for recovery, was then divided by the DNA in 200 μL to give dpm incorporated.ng DNA^{-1}. Duplicates were performed for all experiments and these were averaged to give a mean value. By comparing the control and test values for both direct and indirect treatments, the percentage increase due to FPF was determined. The choline-labelled material produced using this method was previously characterised by TLC (Sen, 1991) and shown to consist almost exclusively (>85%) of phosphatidylcholine.

Results were tabulated and graphed using Microsoft Excel 2003 (v11.8117) and statistics calculated using SPSS for Windows 14.0.1.

2.9 Results from modified method

2.9.1 Dexamethasone

A new batch of dexamethasone (also from Sigma-Aldrich) was purchased and used as described in **2.7.1**. Sixteen pairs of control and test FCM were tested using the modified method (**2.8**) and the results obtained were much more consistent (Table 2.3). Direct treatment with dexamethasone showed a mean increase of 1.77%, while indirect treatment yielded a 9.17% mean increase. Standard error values were much lower than in the earlier experiments, with the highest being 9.9% over 16 experiments. PC synthesis values (expressed in dpm.ng DNA^{-1}) for these experiments were of similar magnitude as those observed previously (Sen and Cake, 1991). Single-factor ANOVA ($p = 0.02$) showed a significant difference between the stimulatory effect of dexamethasone exposure in direct and indirect treatments, indicating that the synthesis

experiments were now a reliable method for detecting the presence of FPF. FCM from these experiments was stored at -80°C for future use in isolating FPF (**3.2.1**).

Table 2.3: The direct and indirect effect of dexamethasone on the augmentation of PC synthesis by type II pneumocytes, utilizing the modified experimental method.

Treatment	PC Synthesis (dpm/ng DNA)	Difference	P-value	Mean type II pneumocyte DNA (μg/plate)	Mean fibroblast DNA level (μg/plate)
Direct (16)					
Control	28.34 ± 2.77				
Dexamethasone	28.84 ± 2.84	↑ 1.77%		31.04	
Indirect (16)			0.02		
Control	26.82 ± 2.54				
Dexamethasone	29.28 ± 2.65	↑ 9.17%		26.88	12.84

Primary cultures of lung fibroblasts were grown to near confluence, followed by 24 hours incubation in the presence or absence of 100 nM dexamethasone in MEM^+. The fibroblast-conditioned media (FCM) were collected and stored at -80°C. Prior to use, the media were thawed and incubated at 65°C for one hour, cooled to room temperature and filter-sterilized to remove detached cells and cellular debris. The media were then diluted 1:4 with MEM^+. The effect on PC synthesis by type II pneumocytes was investigated by exposing the cells either to the FCM (indirect) or to MEM^+, with and without dexamethasone (direct). The data represent the mean ± SE. The number of replicates examined for each condition is indicated in parentheses, and each replicate comprised four cultures. Data were compared using single-factor ANOVA.

2.10 Experimental reliability

Several values were calculated to monitor the reliability of the experiments. Type II DNA values were converted to the total DNA per plate, to monitor cell yield from week to week. The mean amount of DNA per plate was also calculated for the fibroblast plates used in a given experiment. By taking a mean of ^{14}C recovered and dividing it by the mean ^{14}C input, the percentage recovery was calculated. This indicated what percentage of total lipids was being extracted and counted for each experiment. The three samples of the [^{3}H]-choline chloride solution were also averaged to monitor the actual amount of ^{3}H being added to the plates for each experiment. The samples taken from each plate immediately after the addition of ^{3}H showed how much ^{3}H was present on each plate at the beginning of incubation. Both of these ^{3}H values were multiplied by 20 and divided by 2.22×10^{6} to convert to $\mu Ci.mL^{-1}$. The ratio of total and plate ^{3}H radioactivity was also monitored to ensure an accurate 1:7 dilution onto the plate.

It should be noted that although these changes were not implemented until the final five experiments using PMA, data for plate DNA and percentage recovery of lipids were being collected from the first dexamethasone experiment. This allowed these three values to be calculated for all experiments. Values relating to ^{3}H radioactivity could only be calculated for the final five PMA experiments and the second batch of dexamethasone experiments.

To determine if any of the monitored parameters were influencing the results of the synthesis experiments, values for those experiments which gave a positive indirect effect (of FCM on PC secretion by type II cells) were compared with those which showed no indirect effect. Values were also compared between data sets (e.g. dexamethasone, PMA) to determine if the erratic results were due to these

parameters. All statistical comparisons were achieved using single-factor ANOVA, with a confidence level of 95%.

2.10.1 Type II pneumocyte cell density

Comparison of experiments that gave a positive indirect effect in response to the added agent with those that were unresponsive showed that overall there was no significant difference in type II cell density (p= 0.42) (Table 2.4). Standard error values were small, with a mean of 15.3%, indicating that density was consistent within experimental sets. However, there was a clear difference for the second batch of dexamethasone experiments, where the cultures which did not respond to the FCM had a significantly higher cell density of 36.20 $\mu g.plate^{-1}$, compared to 24.06 $\mu g.plate^{-1}$ for those plates which had a positive response (p = 0.04).

Table 2.4: Mean DNA level of type II cultures ($\mu g.plate^{-1}$) for experiments which gave a positive indirect response and those which did not. P-values were obtained using a single-factor ANOVA.

Additive	DNA level ($\mu g.plate^{-1}$)		P-value
	Positive response	No response	
Dexamethasone	17.93 ± 2.31	26.16 ± 6.19	0.50
PMA	32.53 ± 5.48	39.14 ± 5.39	0.42
Dexamethasone (new)	24.06 ± 3.16	36.20 ± 4.36	**0.04**

Type II pneumocytes do not culture well from single cells, requiring large groups of cells to be plated. These clumps then spread out across the plate as culture time progresses (Tajbakhsh and Cake, unpublished observations). If the large clumps have not spread out by the time the plates are considered confluent and used for

experiments (i.e. after four days), results will be affected. Cells located on the exterior of the clumps will behave as expected, but those on the interior will not have the same exposure to stimulants (e.g. dexamethasone, FPF) and radiolabelled choline precursor. This means that both synthesis of PC and incorporation of ^{3}H into that PC would be reduced. These interior cells would however contribute to the DNA extracted from the plate, and would therefore decrease the apparent synthesis of PC, which is calculated as dpm.ng DNA^{-1}. Visual observation of cultures has shown that for plates with very high cell density (often in excess of 40 $\mu g.plate^{-1}$) the cells are not distributed evenly across the plate, with many of these cellular clumps still present.

Purification of type II cells prior to plating is achieved through binding of other cells types to IgG, leaving only type II cells in suspension. Large clumps of cells are likely to contain other cells types, such as fibroblasts, and these will be transferred to the type II plate, as they are not exposed to IgG. This means that the type II plates will also contain contaminating fibroblasts which will be exposed as culture time progresses and the clumps spread out. This will have an effect on apparent PC synthesis for two reasons. Firstly, as with clumped type II cells, fibroblasts will not contribute to synthesis but will add to cell density, thereby reducing the calculated level of synthesis. Secondly, when exposed to dexamethasone any fibroblasts cells will produce FPF and thereby increase synthesis of PC in neighbouring type II cells. This latter effect should be cancelled out because of the addition of dexamethasone to all cultures (supplementation), but it is possible that response to dexamethasone may be reduced when fibroblast contamination is high. Confluent cell cultures have also been observed to down-regulate expression of receptors for growth factors (e.g. Csordas *et al.*, 2000).

Following this investigation, the results from the second batch of dexamethasone experiments were analyzed again, this time separating them into those cultures where the cell density was less than 25 $\mu g.plate^{-1}$ and those where it exceeded this value

(Table 2.5). This threshold value was determined by analysis based on response to dexamethasone and cell density. The indirect cultures with high cell density (mean = 35.05 $\mu g.plate^{-1}$) showed a 3.39% increase in synthesis from exposure to FCM, compared to those with cell density below the threshold value (mean = 17.80 $\mu g.plate^{-1}$) which showed a 17.56% increase. This result is highly significant ($p = 0.003$) and indicates that cell density had a considerable impact on the sensitivity of the cultures. Further investigation using the FCM generated and tested via this method focused only on those samples that had been tested on type II cultures with a cell density below 25 $\mu g.plate^{-1}$.

The density of cells on the type II plate may also have influenced the response of experiments using PMA, as the mean cell density for these experiments was 35.84 $\mu g.plate^{-1}$. Comparison of cell density between experimental sets indicated that the mean concentration for PMA was significantly higher than for either of the dexamethasone sets ($p = 0.01$). In the case of the PMA experiments, where results were very inconsistent, it is possible that cell density was a major factor that was not being considered prior to the introduction of the revised method. Analysis of cell density indicates that this value should definitely be monitored in all future experiments to ensure consistency of results. Unfortunately, attempts to produce primary type II cultures with a consistent cell density have not been successful. Primary fibroblast cultures are required, as line cells do not show the same response (Smith, 1981a). It is possible that a cell line could be used to generate type II cultures, and this may improve reliability. Given that this would require a major alteration to the tissue culture procedures in use, this alternative was not explored in this study.

Table 2.5: The effect of dexamethasone on PC synthesis in type II pneumocytes taking the density of the type II pneumocyte cultures into consideration.

Treatment	PC Synthesis (dpm/ng DNA)	Difference	P-value	Mean type II pneumocyte DNA (μg/plate)	Mean fibroblast DNA level (μg/plate)
< 25 μg DNA.plate^{-1}					
Direct (8)					
Control	27.99 ± 5.82				
Dexamethasone	29.21 ± 6.08	↑ 4.36%		16.29	
Indirect (8)			0.003		
Control	22.95 ± 3.55				
Dexamethasone	26.98 ± 4.14	↑ 17.56%		17.80	15.43
> 25 μg DNA.plate^{-1}					
Direct (8)					
Control	29.20 ± 3.31				
Dexamethasone	28.79 ± 3.37	↓ 1.4%		42.51	
Indirect (8)			0.24		
Control	30.31 ± 4.07				
Dexamethasone	31.34 ± 4.15	↑ 3.39%		35.05	17.46

Primary cultures of lung fibroblasts were grown to near confluence, followed by 24 hours incubation in the presence or absence of 100 nM dexamethasone in MEM^{+}. The fibroblast-conditioned media (FCM) were collected and stored at -80°C. Prior to use, the media were thawed and incubated at 65°C for one hour, cooled to room temperature and filter-sterilized to remove detached cells and cellular debris. The media were then diluted 1:4 with MEM^{+}. The effect on PC synthesis by type II pneumocytes was investigated by exposing the cells either to the FCM (indirect) or to MEM^{+}, with and without dexamethasone (direct). The data represent the mean ± SE. The number of replicates examined for each condition is indicated in parentheses, and each replicate comprised four cultures. Data were compared using single-factor ANOVA.

2.10.2 Fibroblast plate cell density

Comparison of those cultures that gave a positive indirect effect with those that were unresponsive showed that overall there was no significant difference in cell density of the fibroblast cultures ($p = 0.90$) (Table 2.6). Standard error values were small, with a mean value of 15.1%, indicating that density was consistent between experiments using a given additive. A difference was found for the initial dexamethasone experiments, where positive experiments had a fibroblast density of 10.33 $\mu g.plate^{-1}$ compared to 16.88 $\mu g.plate^{-1}$ for those that did not respond ($p = 0.03$). These experiments were very inconsistent, and it is possible that the density of the fibroblasts was one of many factors adversely influencing the results. It is not clear how fibroblast cell density might affect the results, but it is likely to be due to similar issues as those discussed in **2.10.1**. The basal level of FPF may be much higher in dense fibroblast cultures, potentially diminishing the observed response to dexamethasone. Conversely, insufficient numbers of cells on a given plate will mean that not enough FPF is produced to stimulate PC synthesis, while too many may limit exposure to stimulants.

A study currently being conducted by an Honours student under my co-supervision has indicated that the level of DNA per plate of fibroblast cultures may not be a reliable indicator of the quality of fibroblasts (in terms of their ability to proliferate). It has been observed that if media changes are not performed every 48 hours, the health of the fibroblast cells can be greatly affected (Ellison, unpublished data). These poor quality cells would still contribute to cell density as calculated using the amount of DNA, but are unlikely to contribute to FPF production. Given that the initial method used for these experiments did not stipulate media changes exactly 48 hours apart, it is possible that some of the fibroblast cultures were in poor health by the time FCM was being generated.

Table 2.6: Mean DNA level of fibroblast cultures (μg.plate^{-1}) for experiments which gave a positive indirect response and those which did not. P-values were obtained using a single-factor ANOVA.

Additive	DNA level (μg.plate^{-1})		P-value
	Positive response	No response	
Dexamethasone	10.33 ± 1.80	16.88 ± 1.34	**0.03**
PMA	17.48 ± 1.91	15.02 ± 1.73	0.36
Dexamethasone (new)	14.02 ± 3.34	10.81 ± 2.66	0.52

2.10.3 Percentage recovery using [^{14}C]-DPPC

Comparison of tests that gave a positive indirect effect with those that did not respond showed that overall there was no significant difference in the percentage recovery of the [^{14}C]-DPPC internal standard ($p = 0.90$) (Table 2.7). Standard error values were very small with a mean of 4.4%, indicating that percentage recovery was extremely consistent throughout the entire study. Assuming complete recovery of [^{14}C]-labelled standard and taking into account that only 400 μL of the chloroform layer was counted, the expected recovery is 100%. The mean value for recovery was 91.78%, showing that the method used is a very reliable way to assess surfactant synthesis. None of the individual experimental sets showed any significant variation in recovery, and comparison between data sets also showed no significant difference ($p = 0.55$). Percentage recovery did not influence the results obtained in any of the experiments conducted. This further indicates the reliability of the lipid extraction method, as the amount extracted from one experiment to another was consistent.

Table 2.7: Mean percentage recovery of the [^{14}C]-DPPC internal standard for experiments which gave a positive indirect response and those which did not. P-values were obtained using a single-factor ANOVA.

Additive	[^{14}C]-DPPC recovery (%)		P-value
	Positive mean	No response mean	
Dexamethasone	95.55 ± 5.95	89.05 ± 7.80	0.63
PMA	87.05 ± 1.60	89.75 ± 1.25	0.21
Dexamethasone (new)	94.30 ± 2.40	95.00 ± 5.00	0.89

2.10.4 Initial [^{3}H]-choline chloride radioactivity

Comparison of tests that gave a positive indirect effect with those that did not respond showed that overall there was no significant difference in the radioactivity of the [^{3}H]-choline chloride solution that was prepared for synthesis experiments ($p = 0.15$) (Table 2.8). Standard error values were low, with a mean of 6.4%, indicating consistency between experiments using a given additive. A contributor to the inconsistency in the results for these experiments may be due to variability in the amount of ^{3}H added to plates during each experiment.

The radioactivity of this stock ^{3}H solution was significantly lower for the PMA experiments than for dexamethasone ($p = 0.000002$). The mean activity for the PMA experiments was 2.04 $\mu Ci.mL^{-1}$ compared with 5.09 $\mu Ci.mL^{-1}$ for dexamethasone. If insufficient [^{3}H]-choline chloride was supplied at the beginning of an experiment, it would mean that synthesis of labelled PC would be greatly limited, indicating that ^{3}H activity may have been a major factor in the inconsistent results obtained during these experiments. The random nature of scintillation counting also means that lower values are more difficult to count accurately.

Table 2.8: Mean radioactivity of stock [^{3}H]-choline chloride solution (μCi.mL^{-1}) for experiments which gave a positive indirect response and those which did not. P-values were obtained using a single-factor ANOVA.

Additive	[^{3}H]-choline chloride radioactivity (μCi.mL^{-1})		P-value
	Positive mean	No response mean	
PMA	1.90 ± 0.09	2.17 ± 0.34	0.58
Dexamethasone (new)	4.80 ± 0.32	5.38 ± 0.16	0.20

The process of drying under nitrogen means that the [^{3}H]-choline will be dried onto the interior surface of the bottle and early results indicated that it does not go back into solution as quickly as previously thought. A study was undertaken to determine the optimum solubilization time for the [^{3}H]-choline solution. Triplicate [^{3}H]-choline solutions were prepared exactly as described in **2.8**, and these solutions were placed in a shaking water bath at 37°C. Samples were taken for counting at 0, 5, 10, 15, 20, 30, 45 and 60 minutes. Scintillant was added and the samples were kept in the dark for 24 hours prior to counting. The study showed that the solution reached its highest activity (13.5% increase) after 10 minutes incubation (Figure 2.1), so it was decided in all future experiments to incubate the solution for 10 minutes prior to use. The addition of this step to the method meant that the activity could be monitored and kept consistent.

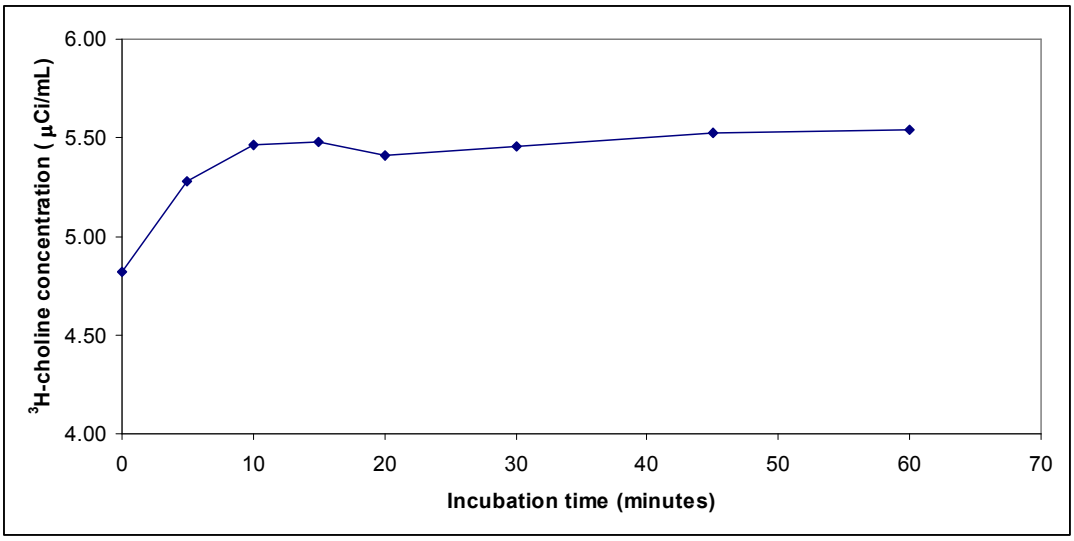

Figure 2.1 Radioactivity of [^{3}H]-choline chloride in MEM$^+$ solution at different times of incubation in a 37°C shaking water bath.

It should be noted that the intended activity of [^{3}H]-choline chloride in this stock solution was 7.0 µCi.mL^{-1}, which is much greater than the mean value of 4.55 µCi.mL^{-1} obtained for these experiments. While the values obtained were very consistent, a value closer to the intended activity was not reliably obtained. This is possibly due to insufficient time being allowed for the [^{3}H]-choline to go back into solution following drying, or possibly due to a pipetting error. Pipettes were calibrated regularly, but [^{3}H]-choline chloride is provided in ethanol, and volatile solvents are often difficult to pipette accurately. It is also possible that the activity of the [^{3}H]-choline chloride solution changed over time. However, given that it is supplied as an ethanol solution, activity would be expected to increase over time as ethanol evaporated. For these experiments, it was decided that a consistent value was more important than the desired radioactivity, so the issue was not explored further.

2.10.5 Plate [^{3}H]-choline chloride radioactivity

Comparison of tests that gave a positive indirect effect with those that did not respond showed that overall there was no significant difference in the radioactivity of the [^{3}H]-choline chloride on each plate at the beginning of an experiment ($p = 0.35$) (Table 2.9). Standard error values were very low, with a mean of 4.7%, indicating consistency between experiments using a given additive. This indicates that the amount of [^{3}H]-choline added to each plate was not likely to have been affecting the results obtained in any given experimental set. As with the activity of the stock ^{3}H solution, values for this parameter were less than the expected value of 1.0 μCi.mL^{-1}.

The activity of ^{3}H solution in the cultures was significantly lower for the PMA experiments than for dexamethasone ($p = 0.001$). The mean activity for the PMA experiments was 0.47 μCi.mL^{-1} compared with 0.69 μCi.mL^{-1} for dexamethasone. This is clearly related to the lower activity of the initial [^{3}H]-choline chloride solution, and will be discussed further in **2.10.6**.

Table 2.9: Mean radioactivity of [^{3}H]-choline chloride solution on individual plates (μCi.mL^{-1}) for experiments which gave a positive indirect response and those which did not. P-values were obtained using a single-factor ANOVA.

Additive	[^{3}H]-choline chloride radioactivity (μCi.mL^{-1})		P-value
	Positive mean	No response mean	
PMA	0.51 ± 0.01	0.44 ± 0.04	0.31
Dexamethasone (new)	0.65 ± 0.04	0.73 ± 0.02	0.18

2.10.6 ^{3}H Ratio

From analysis of the plate ^{3}H radioactivity, it is clear that none of the values were close to the desired value of 1.0 $\mu Ci.mL^{-1}$. However, because the initial activity was much lower than expected, this value cannot be taken alone as an indication of consistency. Instead, the ratio of the two values must be considered to determine if the appropriate dilution is being made onto the plate. This value is expressed as:

$$\frac{\text{plate radioactivity}}{\text{stock radioactivity}}$$

The desired value is a ratio of 0.143, with values higher than this representing a higher plate concentration than expected and lower values indicating a low radioactivity.

Comparison of tests that gave a positive indirect effect with those that did not respond showed that overall there was no significant difference in the ratio of the activity on the individual plates and activity of the initial ^{3}H solution ($p = 0.16$) (Table 2.10). Standard error values were low, with a mean of 8.9%, indicating consistency between experimentals using a given additive. The mean value is close to 0.143, being 0.183 ± 0.016, indicating that even though the initial activity is lower than expected, the dilution onto the plate is consistent and close to 1:7. This confirms that the radioactivity of the initial ^{3}H solution is likely to have been more important to the consistency of results than transfer to plates.

The ratio of plate to initial ^{3}H activity was significantly higher for the PMA experiments than for dexamethasone. This shows that, in spite of the fact that the activity of the initial ^{3}H solution was lower, the amount being transferred to plates was on average greater than the 1:7 dilution expected. This is most likely due to pipetting errors. Analysis of the results in **2.10.5** indicates that while the ratio was higher, the actual radioactivity added to each plate was still significantly lower than for the other

experimental set monitored, and so was still likely to have been a major factor in the inconsistency of these results.

Table 2.10: Mean ratio of plate ^{3}H and stock [^{3}H]-choline chloride solution radioactivity for experiments which gave a positive indirect response and those which did not. Expected ratio is 0.143. P-values were obtained using a single-factor ANOVA.

Additive	Plate:stock [^{3}H]-choline chloride radioactivity ratio		P-value
	Positive mean	No response mean	
PMA	0.266 ± 0.010	0.190 ± 0.051	0.48
Dexamethasone	0.136 ± 0.002	0.137 ± 0.002	0.97

2.11 Discussion

Refinement of the methodology used in tissue culture experiments meant that a consistent indirect response to dexamethasone could be obtained. The response observed was also consistent with previous results (Smith, 1978; Gross and Wilson, 1982). Dexamethasone is considered to interact with cytoplasmic glucocorticoid receptors within the fibroblast cell, inducing the steroid-receptor complexes to translocate to the nucleus where they bind to glucocorticoid response elements and enhance transcription of the gene for FPF and other glucocorticoid-regulated proteins (Smith and Post, 1993). Consistency of results for these experiments was very important, as FCM produced by this method has been shown to contain FPF (Smith and Post, 1989), and samples that reliably contained FPF were essential for further attempts at identification.

The indirect response observed with PMA was very inconsistent, and did not reflect previous results (Sen, 1991). The most likely reasons for this have already been

discussed, and it is likely that the lack of response in this study was due to variations in cell density and inconsistencies in the addition of radioisotope-labelled compounds, rather than in the actual biochemical interaction of PMA with fibroblast cells.

The early experiments conducted during this study indicated that the tissue culture system used to test for the presence and activity of FPF was affected by a number of factors. The amount of DNA per plate, used as an indication of the number of cells on a given culture plate, can have a significant effect, and it has become apparent that there is an upper limit to this value for type II cultures. Too many cells on a plate can greatly reduce synthesis of PC, and non-contributing cells will reduce apparent synthesis due to the way this value is calculated. Feedback mechanisms that exist to regulate the surfactant system are also likely to have an impact at high cell density. High density type II pneumocytes would mean increased secretion of surfactant during incubation, and a high level of secreted surfactant has been shown to inhibit further synthesis (Dobbs *et al.*, 1987). The effect of fibroblast cell density is less clear, but is likely to be similar to that for type II cells. The health of fibroblast cells may also be an important factor.

Type II cell density contributed to the lack of response from the cultures with a density above 25 μg DNA.plate^{-1} in the second batch of dexamethasone experiments, which used the refined method. Indirect experiments with type II cultures with a cell density below the threshold (mean density = 17.8 μg DNA.plate^{-1}) showed a dexamethasone-induced increase of 17.56% in PC synthesis. Those with cell density above 25 μg DNA.plate^{-1} (mean density = 35.05 μg DNA.plate^{-1}) showed a 3.39% increase. The threshold value was used to analyze the results of similar experiments conducted by an Honours student under my co-supervision (Peh, 2006), and a similar trend was revealed (Table 2.11). Indirect experiments with type II culture cell density below 25 μg DNA.plate^{-1} (mean density = 14.8 μg DNA.plate^{-1}) showed an increase of

18.1% in PC synthesis, while those with cell density above the threshold (mean density = 40.5 μg DNA.plate^{-1}) showed a 13.3% decrease. These results are consistent with those of this study, further highlighting the effect of type II pneumocyte cell density on PC synthesis as discussed in **2.10.1**.

The consistent application of radioisotope-labelled compounds is also very important. Percentage recovery, as measured by the use of a [^{14}C]-labelled internal standard, was very consistent, and so is unlikely to have influenced the results. Accurate delivery of [^{3}H]-choline chloride is also important, and is likely to have caused the erratic results observed when using PMA as a stimulant. The amount of ^{3}H applied to each plate in these experiments very inconsistent, meaning that even if synthesis was stimulated, it would not have been detected at accurate levels.

The modification of the synthesis method has greatly improved reproducibility of results, and has allowed the accurate monitoring of several parameters that had not previously been considered as having a significant effect on the results. It has also meant that the indirect response on type II pneumocytes by FCM produced by exposing fibroblasts to dexamethasone is consistent. The revised method has now been adopted within the laboratory, reducing the likelihood of miscommunication of methodology in future. Any further experiments should utilize the method as described, and should also attempt to further improve the reliability of the monitored values.

Table 2.11: The effect of dexamethasone on PC synthesis in type II pneumocytes taking the density of the type II pneumocyte cultures into consideration (Peh, 2006).

Treatment	PC Synthesis (dpm/ng DNA)	Difference	Mean type II pneumocyte DNA (μg/plate)	Mean fibroblast DNA level (μg/plate)
< 25 μg DNA.plate^{-1}				
Direct (9)				
Control	29.0 ± 5.8			
Dexamethasone	26.6 ± 4.7	↓ 8.2%	13.3	
Indirect (9)				
Control	26.8 ± 5.2			
Dexamethasone	31.6 ± 5.5	↑ 18.1%	14.8	3.6
> 25 μg DNA.plate^{-1}				
Direct (5)				
Control	22.2 ± 4.5			
Dexamethasone	22.1 ± 4.3	↓ 0.4%	41.2	
Indirect (5)				
Control	18.7 ± 3.6			
Dexamethasone	16.2 ± 4.8	↓ 13.3%	40.5	7.5

Primary cultures of lung fibroblasts were grown to near confluence, followed by 24 hours incubation in the presence or absence of 100 nM dexamethasone in MEM^{+}. The fibroblast-conditioned media (FCM) were collected and stored at -80°C. Prior to use, the media were thawed and incubated at 65°C for one hour, cooled to room temperature and filter-sterilized to remove detached cells and cellular debris. The media were then diluted 1:4 with MEM^{+}. The effect on PC synthesis by type II pneumocytes was investigated by exposing the cells either to the diluted FCM or to MEM^{+}, with and without dexamethasone. The data represent the mean ± SE. The number of replicates examined for each condition is indicated in parentheses, and each replicate comprised four cultures.

Chapter 3. Identification of the fibroblast-pneumocyte factor

3.1 Introduction

The function of fibroblast-pneumocyte factor (FPF) is well known, but its identity has remained a mystery. Smith (1979) described a heat-stable oligopeptide with only one known activity. It has an apparent molecular mass in the range of 5 to 15 kDa (Smith and Post, 1989) and antibodies have been raised against it (Post *et al.*, 1984). In spite of this information, there was little evidence (or even speculation) as to the actual identity of FPF. It was not known whether it represented a novel protein or one that had been previously described in another system. In order to attempt to identify it, information would need to be gathered to provide a starting point for the investigation.

3.1.1 Glucocorticoid response elements

The effect of glucocorticoid hormones such as dexamethasone on surfactant synthesis is well known, and it is thought that this process is mediated by FPF. Steroid hormones, including glucocorticoids as well as estrogens, progestins and androgens, all mediate their effects by regulating the initiation of transcription of target genes. These hormones cross the cell membrane via diffusion and encounter specific receptors (sequence-specific DNA-binding transcription factors) within the cell (Bentley, 1998). The receptors for steroid hormones are members of a superfamily of large proteins that contain specific functional domains: the ligand- or hormone-binding domain, the DNA-binding domain and the transcription activation domain. The DNA-binding domain mediates the interaction of the receptor with its specific binding site upstream of the promoter region of the target gene. These specific binding sites are known as hormone response elements (HREs) (Bentley, 1998).

The original work in characterizing the response element of glucocorticoids was done by Scheidereit and Beato (1984) on mouse mammary tumour virus (MMTV). The hormone receptor binds to four sites within the glucocorticoid response element (GRE) -72 to -192 bases upstream of the primary transcription initiation site. The GRE is defined as an inverted (or palindromic) repeat of a hexamer sequence separated by three nucleotides and under the standard system this is classified as IR3. These GRE sequences have been found in the promoters of many glucocorticoid-responsive genes, including MMTV and tyrosine aminotransferase (Cooney and Tsai, 1994). A consensus sequence has been generated from the combination of a large number of glucocorticoid-responsive genes (Beato, 1989) and has been found to be:

5'-G-G-T-A-C-A-N-N-N-T-G-T-T-C-T-3'

3'-C-C-A-T-G-T-N-N-N-A-C-A-A-G-A-5'

Expression of the gene encoding FPF is enhanced by dexamethasone, most likely through the GRE. In order to identify potential candidate proteins for FPF, both human and rat genomes were Blast searched for this consensus sequence. Unfortunately, this yielded far too many results to sufficiently narrow the search, so this line of investigation was not pursued any further.

3.1.2 The smoking gun

Work by Dammann *et al.* (2003) found that neuregulin-1β had the same effect on surfactant synthesis by type II pneumocytes as FCM containing FPF. They also found that the addition of a neuregulin-1β antibody to FCM inhibited its effect. This research presented the most compelling evidence yet as to the identity of FPF. The focus of the current study was shifted to focus on determining if neuregulin-1β was in fact FPF.

3.1.3 Neuregulins

The neuregulins are a family of growth factors that continue to be discovered in an increasing number of systems and organisms. The first neuregulin was purified from brain tissue and named the glial growth factor (Brockes *et al.*, 1980). It was also discovered in other mammalian tissues and named acetylcholine receptor inducing activity (Falls *et al.*, 1990), neu differentiation factor (Peles *et al.*, 1992) and heregulin (Holmes *et al.*, 1992). It was not until the early 1990s (Marchionni *et al.*, 1993) that all of these different compounds were determined to be the same protein, which was given the consensus name neuregulin (and is now known as neuregulin-1). The multiple isoforms of neuregulin, many of which differ significantly in molecular weight and have great diversity of activity (Lemke, 1996), elicit cellular responses ranging from proliferation and differentiation to migration and apoptosis (Falls, 2003).

The neuregulins are part of the epidermal growth factor (EGF) family (Prigent and Lemoine, 1992), which consists of a large number of ligands. The isoforms of neuregulin-1 are all generated from a single gene (*nrg1*) (Lemke, 1996), however three other genes encoding neuregulins have also been discovered: *nrg2*, *nrg3* and *nrg4* (Busfield *et al.*, 1997; Zhang *et al.*, 1997; Harari *et al.*, 1999). Little is known about the function of the proteins encoded by these three genes (termed neuregulin-2, 3 and 4, respectively), although they are known to bind to the same receptors as neuregulin-1 (Falls, 2003). NRG2 has been shown to elicit different responses to NRG1 when binding to the same receptor (Crovello *et al.*, 1998). Unless otherwise stated, this review will focus solely on neuregulin-1 (NRG1).

3.1.3.1 Functions of neuregulin-1

Neuregulin-1 was first identified in the neuromuscular system, where it functions as a potent mitogen (Brockes *et al.*, 1980). It is produced by embryonic

neurons shortly after birth (Marchionni *et al.*, 1993; Chen *et al.*, 1994) and is thought to be involved in nerve cell proliferation, differentiation and survival (Lemke, 1996). In skeletal muscle cells, it can increase expression of specific genes (Harris *et al.*, 1988) and also contributes to neuronal control of muscle cell gene expression (Fischbach *et al.*, 1994). Much of the work on NRG1 has used mammary epithelial cells, where they perform mitogenic, differentiation and transformation functions (Lupu *et al.*, 1995). NRG1 is also important in the development of the cardiac (Meyer and Birchmeier, 1995) and pulmonary systems (Patel *et al.*, 2000). The role of NRG1 in lung development is discussed further in **3.1.3.5**. Over-expression of NRG1 is important in a number of cancers, including breast cancer (Lemke, 1996). Problems with NRG1 signaling are also thought to be involved in a number of disease states, including schizophrenia (Stefansson *et al.*, 2002).

3.1.3.2 Structure of neuregulin-1

The gene for neuregulin-1 is located on the short arm of human chromosome 8 (Orr-Urtreger *et al.*, 1993). It is 1.4 megabases long and less than 0.3% of this length actually encodes the functional protein (Stefansson *et al.*, 2002). At least sixteen different isoforms of NRG1 are formed from this single gene through a combination of alternative splicing and multiple promoter usage (Steinthorsdottir *et al.*, 2004). Four splicing hotspots have been identified which seem to produce most of the known variants (Lemke, 1996), although six exons have recently been identified as likely to generate further isoforms (Steinthorsdottir *et al.*, 2004). The biological reason for such a large number of variants is not currently known, although some display different binding affinity for certain receptors (Kita *et al.*, 1995) and some cell types express only specific isoforms (Lemke, 1996), implying cell- or tissue-specific functions.

The three main distinguishing characteristics of the NRG1 isoforms are the type of EGF-like domain (α or β), the N-terminal sequence (I, II or III) and whether the protein is transmembrane or soluble (Falls, 2003). The EGF-like domain is the only part of NRG1 that is well characterized and it is very similar to the bioactive domain of epidermal-growth factor (EGF) (Carraway and Burden, 1995). All isoforms of NRG1 contain this domain, meaning that they are all able to interact with the receptors of the EGF family, known as ErbB receptors (Lemke, 1996). This domain has been demonstrated as being essential for activity in all neuregulins (Carraway *et al.*, 1994; 1995). NRG1 isoforms are divided into three types based on differences in the N-terminus. Type I NRG1s have an extracellular heparin-binding, immunoglobulin-like domain. Type II NRG1s share this domain and also possess a kringle domain, while type III NRG1s have a cysteine-rich domain (Esper *et al.*, 2006). All NRG1 isoforms are generated as transmembrane forms, and proteolysis of these generates the soluble forms (Burgess *et al.*, 1995).

3.1.3.3 Neuregulin receptors

Neuregulins interact with receptors in the ErbB family, which contains four distinct receptors (ErbB1-4) that are glycosylated transmembrane proteins and are regarded as the stereotype for receptor tyrosine kinase activity (Citri and Yarden, 2006). The ErbB receptors have been described as a signaling network, with interactions within and between individual receptors (Falls, 2003). The generalized structure of an ErbB receptor is an extracellular domain with two cysteine-rich regions, a transmembrane domain, an intracellular juxtamembrane domain and a cytoplasmic domain with tyrosine kinase activity (Lemke, 1996). This activity catalyzes phosphorylation of tyrosine residues in polypeptide sequences. Proteins then bind to the newly created phosphotyrosine residues and lead to the downstream reactions (van der Greer *et al.*,

1994). Ligands for ErbB receptors contain an EGF-like sequence with six cysteine residues which is recognized by the receptor (Citri and Yarden, 2006). Binding of a ligand leads to a conformational change in receptor subunits and formation of dimers (Moriki *et al.*, 2001). For example, in the case of ErbB1, this dimer consists of two ErbB1 receptors to which two ligand molecules bind (Lemmon *et al.*, 1997). No NRG1 isoforms have been found that interact with ErbB1, so the focus here will be on the remaining ErbB receptors.

ErbB2 is the best-characterized receptor in the ErbB family, and is also known as c-neu or HER2 (Lemke, 1996). The extracellular structure of ErbB2 is very similar to the ligand-bound form of ErbB1, preventing it from binding any ligands (Garrett *et al.*, 2002). This unusual structure means that it is primed for interaction with other ligand-bound receptors, making it the preferred partner for heterodimerization (Tzahar *et al.*, 1996). It is also incapable of forming homodimers (Plowman *et al.*, 1990). ErbB2 appears to function as an amplifier for the NRG1 signal (Citri and Yarden, 2006), as it is able to interact with a greater number of phosphotyrosine-binding proteins than any of the other receptors (Jones *et al.*, 2006).

Two other ErbB receptors have been identified: ErbB3 (Kraus *et al.*, 1989) and ErbB4 (Plowman *et al.*, 1990). ErbB3 and 4 are very similar to ErbB2 and participate with this receptor in NRG1 signaling. ErbB3 has a defective tyrosine kinase activity due to substitutions in its cytoplasmic domain (Guy *et al.*, 1994), meaning that it cannot transduce a signal on its own. ErbB4 binds a large number of ligands (Citri and Yarden, 2006) and also exists in a number of different isoforms (Rio *et al.*, 2000), making it a very versatile receptor. Heterodimers of ErbB2/ErbB3 and ErbB2/ErbB4 are capable of binding NRG1, as are homodimers of ErbB4 (Sliwkowksi *et al.*, 1994; Tzahar *et al.*, 1994; Chan *et al.*, 1995; Horan *et al.*, 1995; Wallasch *et al.*, 1995). The complexity of interactions within the ErbB family is further increased as receptors interact with each

other, leading to transphosphorylation (Carraway and Cantley, 1994). Studies with knockout mice have indicated that genes for these three receptors and NRG1 itself are essential for embryonic development to proceed past day 10 (Lemke, 1996).

Regulation of these receptors is also complex, with both positive and negative feedback systems. Positive feedback loops exist and prolong the activity of signaling pathways, as well as enhancing the amplitude of the signal (Freeman, 2000). Positive feedback also occurs at a transcriptional level, where genes for other ErbB ligands are up-regulated following receptor-ligand binding (Schulze *et al.*, 2001). Multiple negative feedback systems also exist, and are up-regulated after ligand binding to receptors. Most of these act more slowly than the positive feedback systems (Citri and Yarden, 2006).

3.1.3.4 ErbB2/ErbB3 heterodimers

In terms of cell growth and transformation, ErbB2/ErbB3 heterodimers are the most potent in the ErbB family (Pinkas-Kramarski *et al.*, 1996). ErbB2 cannot bind any known ligands, while ErbB3 cannot transduce a signal, but together they form a functional and powerful receptor (Figure 3.1). Both ErbB2 and 3 are expressed in most epithelial cell layers, while mesenchymal tissues are a source of ligands such as neuregulin (Citri *et al.*, 2003). The potency of these heterodimers is explained by four main factors: the slow rate of ligand dissociation (Karunagaran *et al.*, 1996); relaxed specificity of ligands (Pinkas-Kramarski *et al.*, 1998); ability to limit endocytosis (Worthylake *et al.*, 1999); and coupling to a number of signaling pathways.

Heterodimers of ErbB2/ErbB3 activate several signaling pathways: mitogen-activated protein kinase (MAPK) (Ben-Levy *et al.*, 1994), phosphatidylinositol-3-kinase (PI3K) (Prigent and Gullick, 1994), phospholipase-C_γ (PLC_γ) (Peles *et al.*, 1991), protein kinase C and the Janus kinase (Jak-STAT) (Liu and Kern, 2002). The most significant of these are MAPK, which is involved in cell proliferation, and PI3K, which

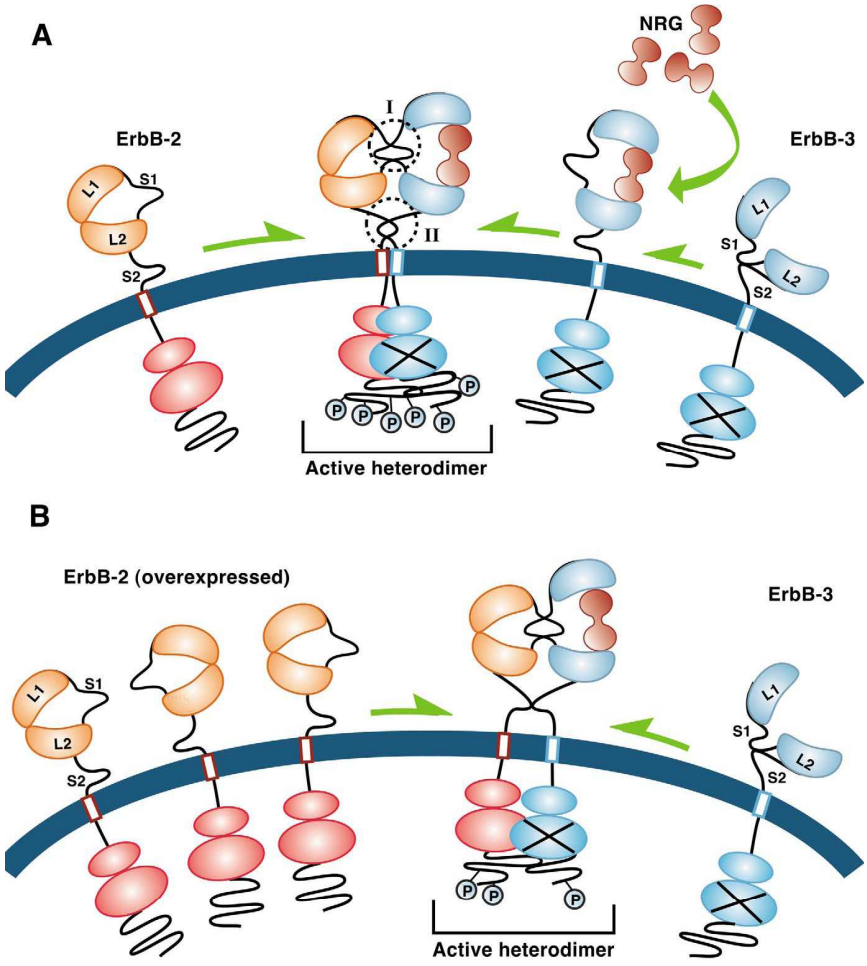

Figure 3.1: The proposed mechanism of neuregulin induced ErbB2/ErbB3 heterodimer formation (Citri *et al.*, 2003).

is a cell survival pathway (Citri *et al.*, 2003). Unlike ErbB1 homodimers, these heterodimers do not interact with pathways that down-regulate mitogenesis, such as Ras-specific GTPase-activating protein (Ras-GAP) (Bar-Sagi and Hall, 2000).

3.1.3.5 Role of neuregulin in lung development

Neuregulin-1 has been discovered in many systems, where it plays a crucial role in development. Its role in the early stages of development in the fetal lung has been partially characterized, such as the triggering of branching morphogenesis (Liu *et al.*, 2004). Functions for NRG1 in the later stages of development, such as in the stimulation of surfactant production, are less well understood. It is only recently that the importance of this signaling has begun to be uncovered.

Research has shown that factors that regulate other facets of lung development, including retinoic acid (RA) and dihydrotestosterone (DHT), have been shown to affect expression of ErbB receptors (Dammann and Nielsen, 1998). Dexamethasone treatment leads to an increase in expression of these receptors and modification of dimerization (Dammann *et al.*, 2006). In the second trimester of gestation, both ErbB2 and ErbB3 can be detected in fetal lung, but not ErbB4 (Patel *et al.*, 2000). Recent observations (Liu *et al.*, 2004) have confirmed that only ErbB2 and ErbB3 are present in the developing lung. Both of these receptors are localized to the epithelial cells, such as type II pneumocytes (Patel *et al.*, 2000). Exposure to recombinant NRG1 caused a four-fold increase in receptor phosphotyrosine content, indicating receptor activation. Increases were also observed in epithelial cell density and proliferation, as well as a decrease in SP-A levels (Patel *et al.*, 2000). These observations are consistent with NRG1 activation of the ErbB2/ErbB3 heterodimer. Activation of both PI3K (Liu *et al.*, 2004) and Jak-STAT (Liu and Kern, 2002) through ErbB2/ErbB3 has been demonstrated in cultures of fetal lung cells.

As indicated in **3.1.2**, it was the work of Dammann *et al.* (2003) that redirected this study towards neuregulin-1. In their work, Dammann *et al.* used immunostaining to detect NRG1β, and confirmed that it increased in concentration in fetal lung fibroblast cells at the same time as surfactant synthesis commenced in type II cells. These fibroblasts secreted the NRG1β and this stimulated synthesis of surfactant in these type II cells. Fibroblast-conditioned medium and recombinant NRG1β were both observed to increase synthesis and ErbB2 receptor phosphorylation, and this activity was blocked in both cases by an antibody to NRG1β. Immunoprecipitation was used to confirm the presence of NRG1β in FCM (Dammann *et al.*, 2003). This work, combined with the presence of the necessary receptors, presented a compelling case for NRG1β to be FPF, and led to this study's attempts to confirm this using HPLC and LC/MS.

3.1.4 HPLC and LC/MS

A general introduction to the two techniques used in this chapter is required to indicate why these particular instruments were chosen for this investigation. High-performance liquid chromatography (HPLC) is a widely-used separation technique, while liquid chromatography/mass spectrometry (LC/MS) is a similarly powerful technique for separation and mass analysis of compounds. Both of these techniques are now used in many types of protein and proteomic research.

3.1.4.1 High-performance liquid chromatography

The principles and applications of high-performance liquid chromatography (HPLC) have been widely reviewed, and so only a general overview will be presented here. For further reading, useful books on the topic include Snyder *et al.* (1997) and Meyer (2004).

The modern form of HPLC is reversed-phase HPLC (RP-HPLC), which involves the use of two phases: a mobile phase (polar) and a stationary phase (non-polar) (Meyer, 2004). The mobile phase is comprised of solvents which are moved through the stationary phase, contained in a packed column, to effect the separation. The mobile phase then moves through a detector, usually utilizing ultraviolet/visible (UV/Vis) spectroscopy, to enable the separated compounds to be visualized (Meyer, 2004). Most mobile phases use a combination of water and a non-polar organic solvent such as acetonitrile or methanol, although many variations have been used for different analyses. There is a similarly large range of stationary phases available, varying in parameters such as composition, hydrophobicity and pore size. The most commonly used stationary phase is silica, which is treated to generate R-groups of a defined length (often 8 (C_8) or 18 (C_{18}) carbons) (Snyder *et al.*, 1997).

The basis of the RP-HPLC separation is hydrophobic interaction (Snyder *et al.*, 1997). The relatively non-polar analytes interact with the non-polar stationary phase, with more hydrophobic compounds interacting more strongly. As the concentration of organic solvent in the mobile phase is increased, compounds will elute in order of increasing hydrophobicity. Variation of mobile phase parameters such as composition, pH buffering, and gradient, as well as column chemistry, will affect the retention time of analytes. The properties of the analytes, as well as sample preparation techniques, also have a large impact on separation (Snyder *et al.*, 1997).

HPLC has been widely used in protein and proteomic research, both for analysis of samples and as a cleanup method for other analytical techniques. An excellent review of current progress and advances in the use of HPLC in proteomics was written by Mitulovic and Mechtler (2006).

3.1.4.2 Mass spectrometry

Mass spectrometry is a general term used for a number of instruments that analyze samples based on their mass to charge ratio. In the context of this study, it refers to electrospray ionization-quadrupole ion trap mass spectrometry (ESI-MS). This system is often coupled with an HPLC instrument, and is then termed LC/MS. As with HPLC, only a general overview of the technique will be given. For further reading, Cole (1997) offers a thorough introduction to both electrospray ionization and associated mass spectrometry. Pramanik *et al.*. (2002) also covers a number of advances and applications of this technology.

Prior to mass analysis in a mass analyzer, the sample must be ionized. A range of methods exist for the ionization of compounds, including electron, chemical and photo-ionization. One of the most widely used, and generally considered to be the best for use with proteins, is electrospray ionization (ESI) (Kebarle and Ho, 1997). ESI was developed for ionization of large biological molecules (Fenn *et al.*, 1989), and John Fenn was awarded the 2002 Nobel Prize in Chemistry for its development. The sample, consisting of analytes in solvent (often from an HPLC system), is forced through a small capillary (Figure 3.2). This capillary has a high electric field applied, and this causes the sample to nebulize. Nitrogen gas is also added to assist in the nebulization process. Solvent is evaporated through the use of a heated drying gas (usually nitrogen) and eventually analyte ions free of solvent are obtained. The exact mechanism of ionization is not completely understood, but involves the transfer of charge from the solvent droplets to analyte molecules as droplets become smaller and smaller (Kebarle, 2000). The analyte ions are quasimolecular ions, usually due to the addition ($[M+H]^+$) or loss ($[M-H]^-$) of proton. Other adducts can form, such as addition of sodium ($[M+Na]^+$), due to the chemical composition of the sample (Kebarle and Ho, 1997).

Due to this ionization, the mass spectrometer analyses mass-to-charge ratios (*m/z*), rather than molecular masses.

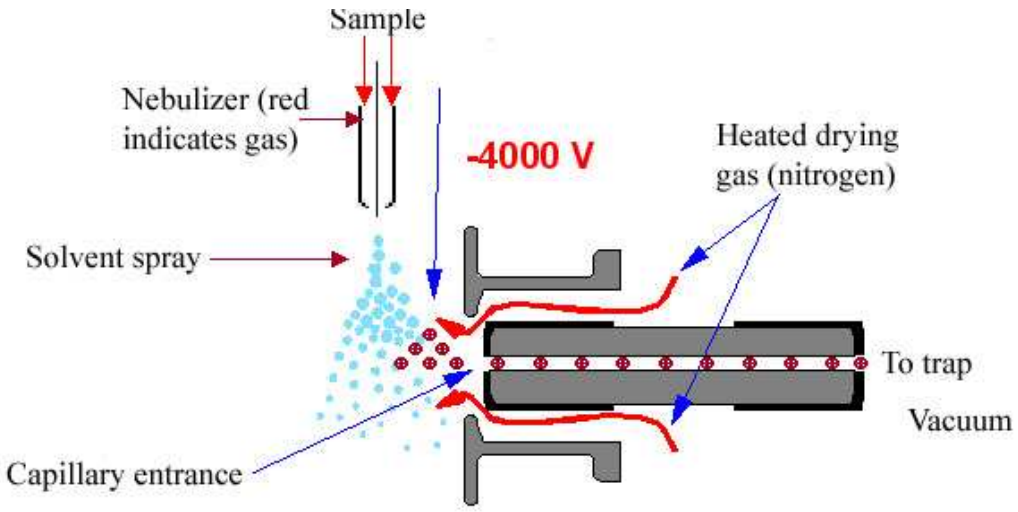

Figure 3.2: Schematic of an electrospray ion source, indicating flow of solvent and drying gas (adapted from Agilent, 2003).

The ESI source is interfaced with the trap itself via a capillary (Figure 3.3). Following this are two skimmer lenses, an octopole and a series of lenses, which serve to focus the ions and remove any contaminants and neutral species.

Quadrupole ion trap mass spectrometry (herein referred to as ion trap MS) is a modification of the quadrupole mass spectrometer (QMS). The QMS sets up a radio frequency (RF) quadrupolar field and uses an electrical field to stabilize ions passing through this field (McEwen and Larsen, 1997). In an ion trap MS, the linear quadrupole has been made circular, allowing ions to be selectively trapped and/or ejected from the field (Bier and Schwartz, 1997). The trap itself consists of two end-cap electrodes with a

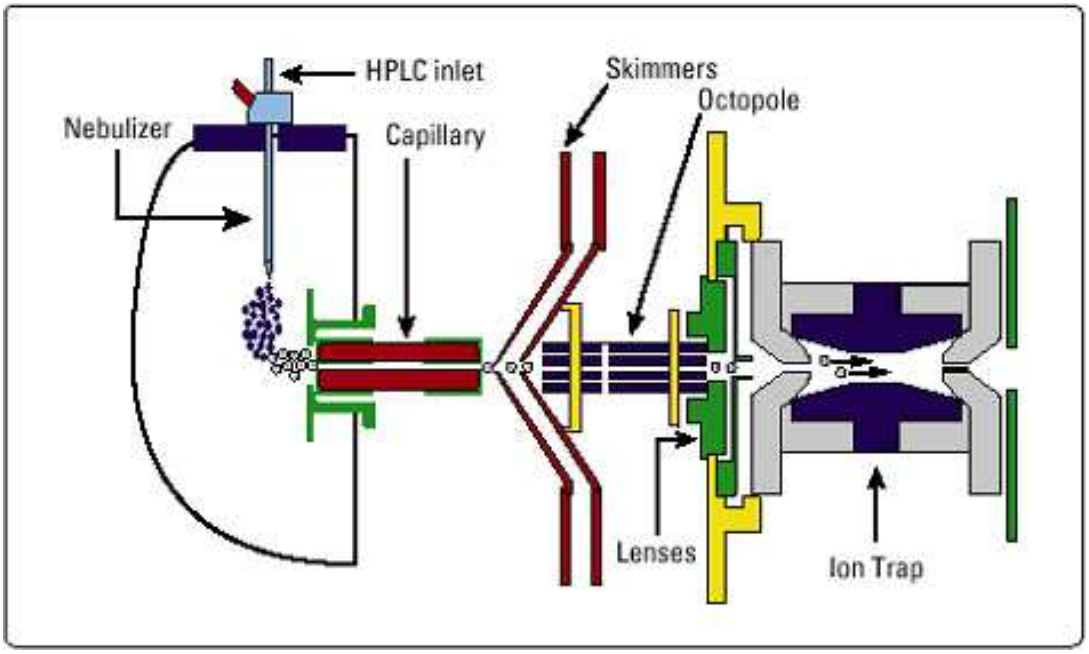

Figure 3.3: Schematic indicating the general structure of a quadrupole ion trap mass spectrometer (Agilent, 2003).

ring electrode halfway between them, forming a spherical space (Figure 3.4). Ions are trapped within this space using electrical fields, and resonance excitation (using the end-cap electrodes) is used to selectively eject ions from the trap (March, 2000). This functionality allows the instrument to be used for MS^n. In classical MS^n, a single mass is isolated and collision-induced dissociation is performed (usually using helium). The resulting fragments are analyzed and this process can be repeated a number of times (with the number defining the n value) to provide information on the structure and identity of the isolated compound (Robert, 1994).

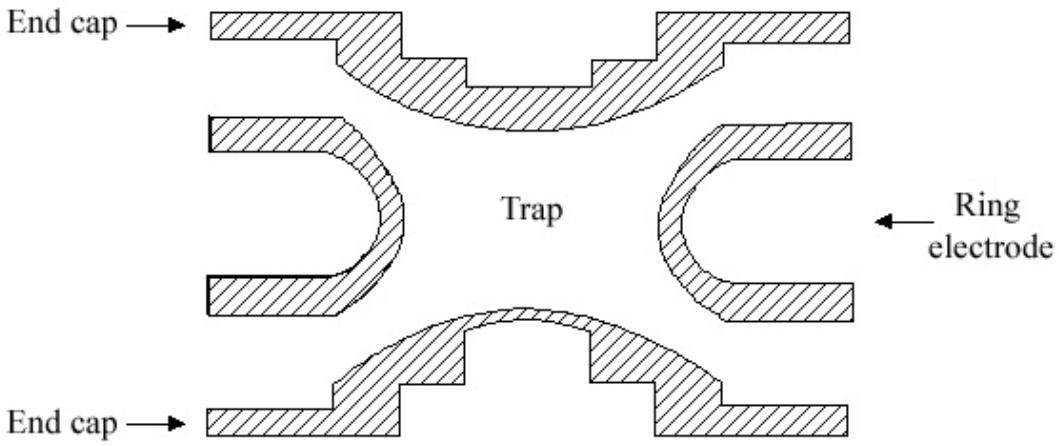

Figure 3.4: Schematic cross-section through an ion trap, showing the two end caps and the central ring electrode (adapted from Agilent, 2003).

Ion trap MS is a popular technique in proteomic research, for several reasons. Electrospray ionization allows the generation of ions directly from solution and is able to impart sufficient energy to ionize intact proteins (Kebarle and Ho, 1997). The ion trap can be used to determine the molecular weight of unknown proteins, and fragmentation can be used in peptide sequencing (Aebersold and Goodlett, 2001). Comprehensive reviews of the many uses of mass spectrometry in proteomics include Aebersold and Goodlett (2001), Griffiths *et al.* (2001) and Aebersold and Mann (2003).

3.2 Methods

3.2.1 Pretreatment of FCM

Fibroblast-conditioned media from dexamethasone exposed cell cultures, with a mean cell density below 25 μg.DNA plate^{-1}, were used for all analyses using LC/MS. It is likely that FCM from other experiments also contained FPF, but as this was not

demonstrable by the indirect method (where fibroblasts were exposed to an agonist and the resulting FCM harvested and exposed to type II pneumocytes to observe any effect on surfactant phospholipid synthesis), they were excluded. Prior to use in this analysis, FCM had been stored at -80°C immediately after collection from cultures. Treatment of FCM for LC/MS was almost identical to the treatment used prior to tissue culture experiments. FCM was thawed at room temperature, before being heat-treated in a 65°C water bath for 60 minutes. After cooling to room temperature, it was filtered through a 0.22 μm Millipore syringe filter and diluted 1:4 with MilliQ-H_2O.

3.2.2 HPLC

Initial attempts to identify FPF were undertaken using HPLC only, in an attempt to separate the multitude of compounds present in FCM prior to LC/MS. Snyder *et al.* (1997) have indicated the parameters that should be investigated in initial HPLC method development. These are illustrated in Table 3.1.

Table 3.1: Variables that should be investigated in initial HPLC method development (Snyder *et al.*, 1997).

Component	Variable
column	dimensions, particle size, stationary phase
mobile phase	solvents, percentage of organic solvent, buffers
flow rate	-
temperature	controlled, not controlled
sample size	injection volume
detector	wavelengths monitored

Most of these parameters were tested using FCM which had been pretreated as described in **3.2.1**. Work was conducted on an Agilent 1100 Series HPLC (Agilent Technologies Ltd., Alpharetta, GA, USA) with a quaternary pump, column thermostat

and diode array detector. The software employed was Agilent Chemstation for LC 3D, Rev. B.09.03. The range of variables which were tested is shown in Table 3.2. All solvents and buffers were HPLC grade.

Table 3.2: Variables tested during HPLC method development, indicating the range tested for each.

Variable	Range Tested
column length	150, 250 mm
column internal diameter	2.1, 4.6 mm
column particle size	5 μm only
column stationary phase	C_{18} only
mobile phase solvents	water with acetonitrile or methanol
mobile phase % organic	initial concentration of 1, 5, 10%
	gradient to 100% organic from 10 to 60 min
mobile phase buffers	trifluoroacetic acid, formic acid, acetic acid
	buffer concentrations of 0.1 to 2%
flow rate	0.8 to 5.0 $mL.min^{-1}$
temperature (column)	not controlled, 25 and 35°C
injection volume	1.0 to 25.0 μL
detector	205 to 300 nm, plus full UV spectrum

Particle size and stationary phase composition of the columns were the only parameters for which a range was not tested. Due to the high cost of HPLC columns, all of the above tests were run using two Vydac 'Protein and Peptide' C_{18} columns: a 218TP5415 (5 μm particle size, 4.6 mm i.d., 150 mm length) and a 218TP52 (5 μm particle size, 2.1 mm i.d., 250 mm length). These columns contain polymerically bonded, endcapped n-octadecyl bond on silica. The pore size on both of these columns is 30 nm, which is required for work with large molecules such as proteins. Additional columns would have been purchased to expand the study, but the emphasis shifted to separation using MS, as detailed in **3.2.4**.

Results obtained using HPLC showed a poor separation of the compounds in FCM. An example chromatogram is included to illustrate the problems encountered (Figure 3.5). The majority of the compounds were un-retained and eluted at the beginning of the run, with very little material being effectively separated.

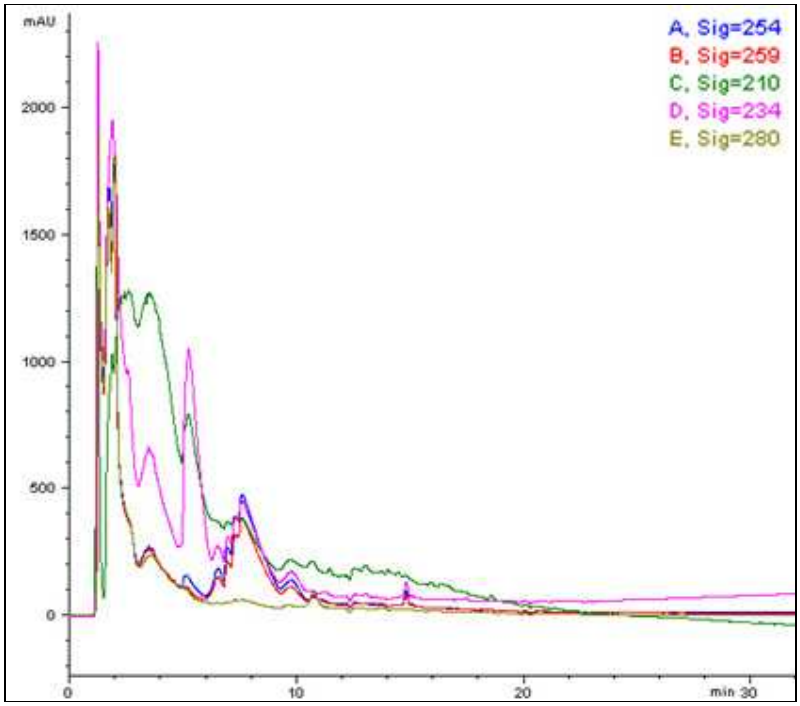

Figure 3.5: Chromatogram of fibroblast-conditioned medium obtained using HPLC. Response is shown in milli-absorbance units (mAU) as a function of time. Detection wavelengths (nm) are indicated by different colours.

Attempts to separate the components of FCM using HPLC proved to be problematic and time-consuming, primarily due to the fact that there was a large number of individual components and because most of the compounds of interest are likely to be present in very low concentrations. A second option for separation involves using a mass spectrometer, such as an ion trap MS, to perform the separation based on mass

rather than properties such as hydrophobicity. This is becoming a more popular option as more complex systems are investigated (e.g. Lu *et al.*, 2007).

3.2.3 Neuregulin standard

Publication of data indicating that neuregulin-1β (NRG1) was possibly FPF (Dammann *et al.*, 2003) provided a new approach for LC/MS analysis of FCM. A mass spectrum for NRG1 could be obtained and used to search FCM. Recombinant human neuregulin-1β (sold as heregulin-β1) (PeproTech, Rocky Hill, NJ, USA) is a 7.5 kDa form of NRG1 which comprises only the EGF-like domain (the bioactive domain). NRG1 was supplied as a lyophilized powder, was reconstituted in MilliQ-H_2O to a concentration of 100 $\mu g.mL^{-1}$ and stored at -20°C until use. Further dilutions were also undertaken with MilliQ-H_2O.

3.2.4 Mass spectrometry

Ion trap mass spectrometry was undertaken utilizing an Agilent 1100 Series LC/MSD Trap (Agilent Technologies Ltd., Alpharetta, GA, USA), operating with Bruker-Daltonik MSD Trap Control software (v 5.1) (Bruker-Daltonik, Bremen, Germany). The MS was calibrated with a 1:5 dilution of Agilent ES Tuning Mix.

Settings for the mass spectrometer were adapted from Mandel and Lopez (2000) and Zumwalt (2005) and then optimized for NRG1. The MS was run in standard mode and at normal scan resolution, scanning for positive ions. The ESI nebulizer was set at 15 psi, with drying gas (N_2) flow at 5 $L.min^{-1}$ and 325°C. Target mass was initially set to 1000 *m/z* and changed to 1068 *m/z* once NRG1 had been detected. Compound stability was 35% and the trap drive level 100%. Scan range was initially 50 to 2200 *m/z*, although this was reduced to 500 to 1500 *m/z* when NRG1 was detected. Scan averaging was set at 5, with rolling averages off. Ion Charge Control (ICC) was on

and set to collect $3x10^4$ ions, with a maximum accumulation time of 300 ms. In expert mode, Skim 1 voltage was changed to 15 V, Skim 2 to 6 V and Cap Exit Offset to 60 V. Collision-induced dissociation utilized helium as the collision gas.

Both NRG1 and pretreated FCM were infused directly into the MS at 5 $\mu L.min^{-1}$using a KD Scientific 100 syringe pump (KD Scientific Inc., Holliston, MA, USA) and a 500 μL GasTight syringe (Hamilton Company, Reno, NV, USA). The ESI source and spray shield were cleaned after every sample with a 50:50 solution of isopropanol and H_2O. Every 24 hours, the source and trap were cleaned by infusing 500 μL of a cleaning solution composed of 50% acetonitrile, 25% isopropanol, 15% cyclohexane and 10% dichloromethane, followed by 500 μL of 50:50 isopropanol and water.

3.2.5 Quantitation of neuregulin

An Agilent 1100 Series capillary HPLC (Agilent Technologies Ltd., Alpharetta, GA, USA), with two binary pumps configured to operate at a maximum flow rate of 20 $\mu L.min^{-1}$, was connected to the ion trap MS. This LC was equipped with both column and autosampler thermostats and a diode array detector (DAD). This instrument was running Agilent Chemstation for LC 3D, Rev. A.09.01 software. The column used for all work on this instrument was an Agilent Zorbax 300SB-C_{18} capillary column (3.5 μm particle size, 0.3 mm i.d., 100 mm length).

Mobile phase solvents were water and acetonitrile, both buffered with 0.1% formic acid. Flow rate was set at a 5 $\mu L.min^{-1}$ with a maximum pressure of 400 bar. A 35-minute gradient was used as indicated in Table 3.3, with a 10-minute post-time to ensure the column was completely flushed prior to the subsequent run.

Table 3.3: Gradient used in capillary HPLC runs, indicating percentage of acetonitrile (ACN) at different times in the run. The transition from 10 to 100% is linear over 15 minutes.

Time (min)	% ACN
0 - 10	10
10 - 25	10 - 100
25 - 35	100

A volume of 1 μL of sample was injected at the start of each run, and the autosampler was kept at 4°C to prevent sample degradation. Column temperature was also kept at 25°C to ensure it remained constant. The DAD was used to collect full spectra (190 to 600 nm) as well as specifically monitoring 254 and 280 nm.

This HPLC method was used in conjunction with the MS method outlined in **3.2.4** to quantify the amount of NRG1 present in FCM. Multiple reaction monitoring (MRM) was used, meaning that only ions at 1068, 1142 and 1246 *m/z* were monitored. This greatly improved the signal to noise ratio compared to the full scans of FCM.

3.3 Results

3.3.1 Direct infusion mass spectrometry

NRG1 solution (10 $ng.mL^{-1}$) was infused directly into the MS and showed a mass spectrum characteristic of a protein (Figure 3.6). The protein exists in multiple charge states, due to the many potential binding sites for protons on the molecule. The predominant ions were 1068, 1142 and 1246 *m/z*. Deconvolution of this spectrum suggested a molecular weight of 7.480 kDa, very close to the actual value of 7.478 kDa. MS^2 using CID was attempted for 1068 *m/z* (Figure 3.7), but was only partially successful due to the large amount of energy required to fragment an intact protein.

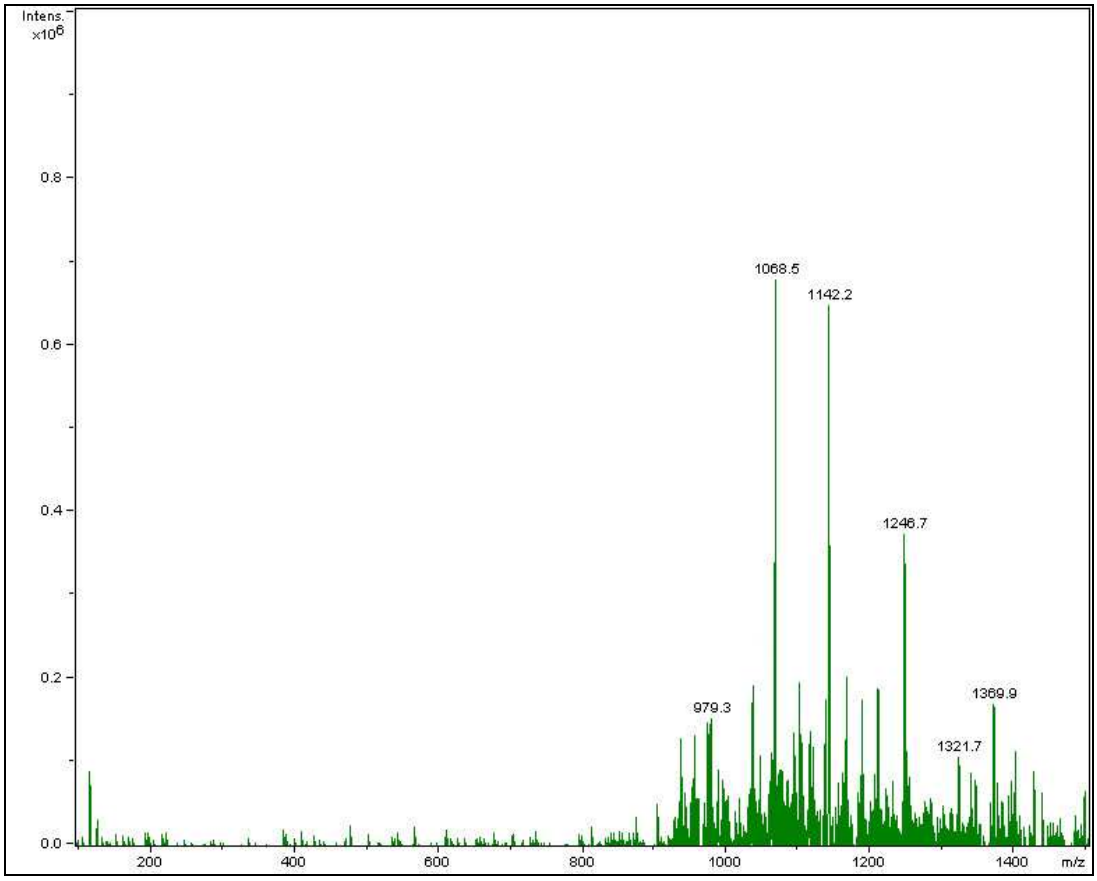

Figure 3.6: Mass spectrum for neuregulin-1β (10 ng.mL^{-1}) obtained using direct infusion into a quadrupole ion trap mass spectrometer. Intensity is an arbitrary scale and indicates relative abundance of ions in the trap for a given scan.

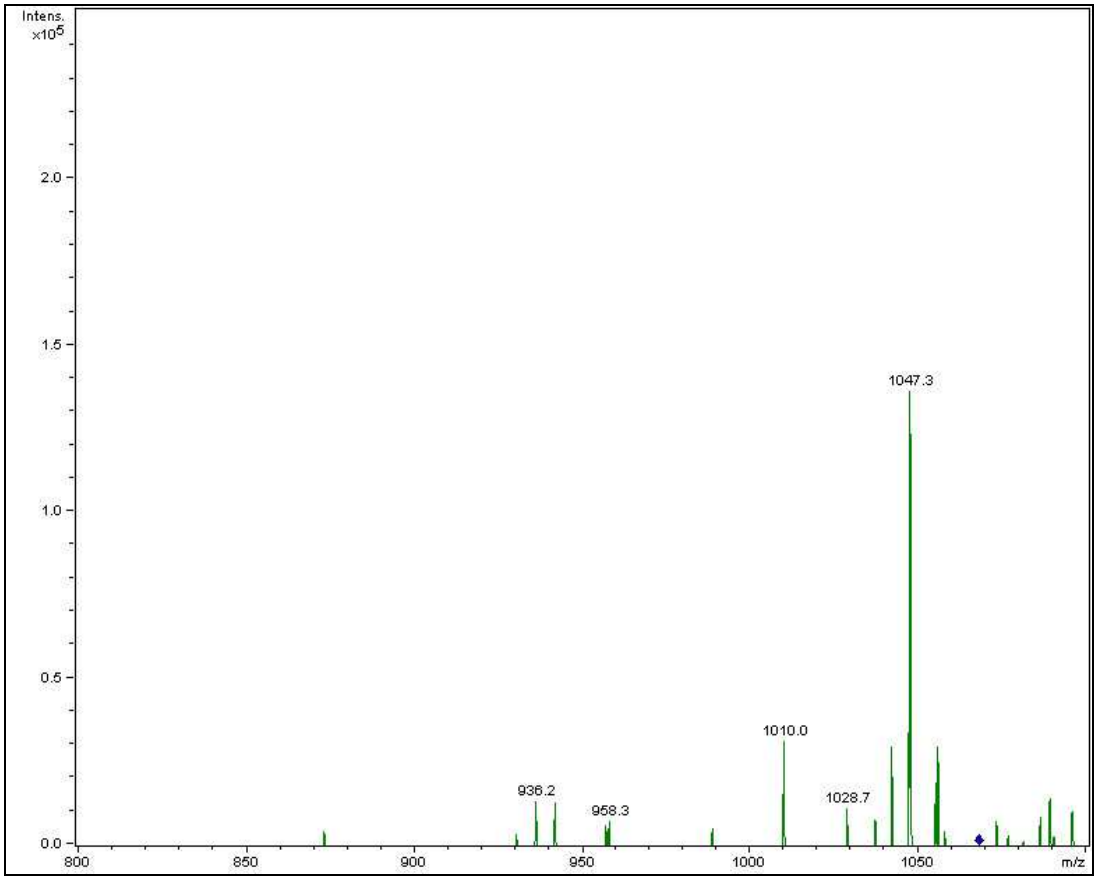

Figure 3.7: Mass spectrum for collision-induced dissociation of the 1068 *m/z* ion of neuregulin-1β obtained using direct infusion into a quadrupole ion trap mass spectrometer. This spectrum represents MS^2. Intensity is an arbitrary scale and indicates relative abundance of ions in the trap for a given scan.

The primary fragment ion was 1047.3 *m/z*, indicating a neutral loss of 20.7 *m/z* from the original molecule. Other fragment ions included 958.3, 1010.0 and 1028.7 *m/z*.

FCM of both control and dexamethasone (test) treatments was also infused directly into the MS. Mass spectra for both types of FCM were complex, with many compounds present, and many of those in multiple charge states (Figures 3.8, 3.9). A key difference is that 1068 *m/z* is clearly visible in the test FCM, but not in the control FCM. MS^2 indicated that ions of 1068, 1142 and 1246 *m/z* were all present in both types of FCM, but these results indicated that the concentration of these ions was likely to be greater in the test FCM. MS^2 using CID was attempted for 1068 *m/z*, and the response was identical for both FCM types, indicating that this ion corresponds to the same compound. The resultant fragmentation spectrum (Figure 3.10) displayed ions of 958.3, 1016.5, 1028.7 and 1047.2 *m/z*. Several of these ions are in common with those generated from NRG1 standard (Figure 3.7), providing strong evidence that the ion at 1068 *m/z* in FCM is NRG1.

3.3.2 Capillary HPLC

Quantitation of NRG1 in FCM required the use of an HPLC system to deliver the entire sample into the MS simultaneously. A method was developed for NRG1 and subsequently used for the FCM samples (see **3.2.5**). The 280 nm chromatogram for 10 $ng.mL^{-1}$ NRG1 (Figure 3.11) showed that NRG1 was largely un-retained by the column and eluted with a retention time of approximately 2.5 minutes. Control FCM was also run with this method to ensure that it was suitable, and the 280 nm chromatogram for this sample was overlaid with that for NRG1 (Figure 3.12). This comparison showed that if NRG1 was present in FCM, then it was likely to be co-eluting with a large number of other compounds that absorbed strongly at 280 nm.

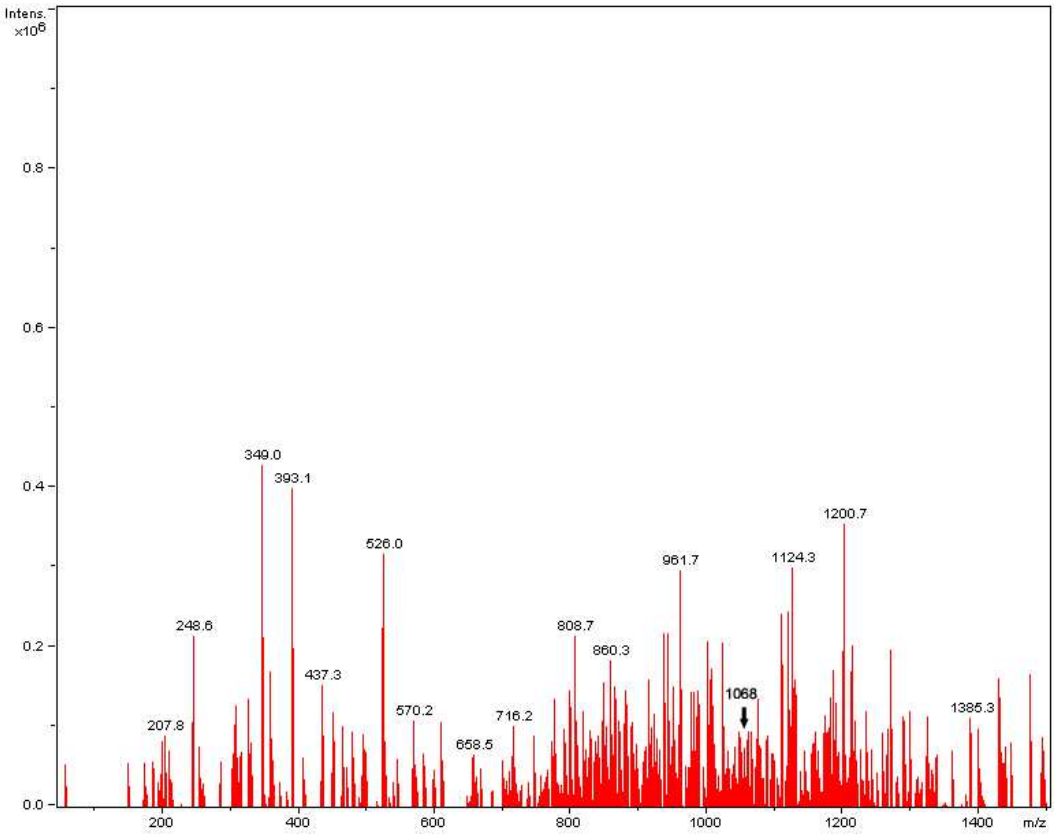

Figure 3.8: **Mass spectrum for fibroblast-conditioned medium from control cultures obtained using direct infusion into a quadrupole ion trap mass spectrometer. Intensity is an arbitrary scale and indicates relative abundance of ions in the trap for a given scan.**

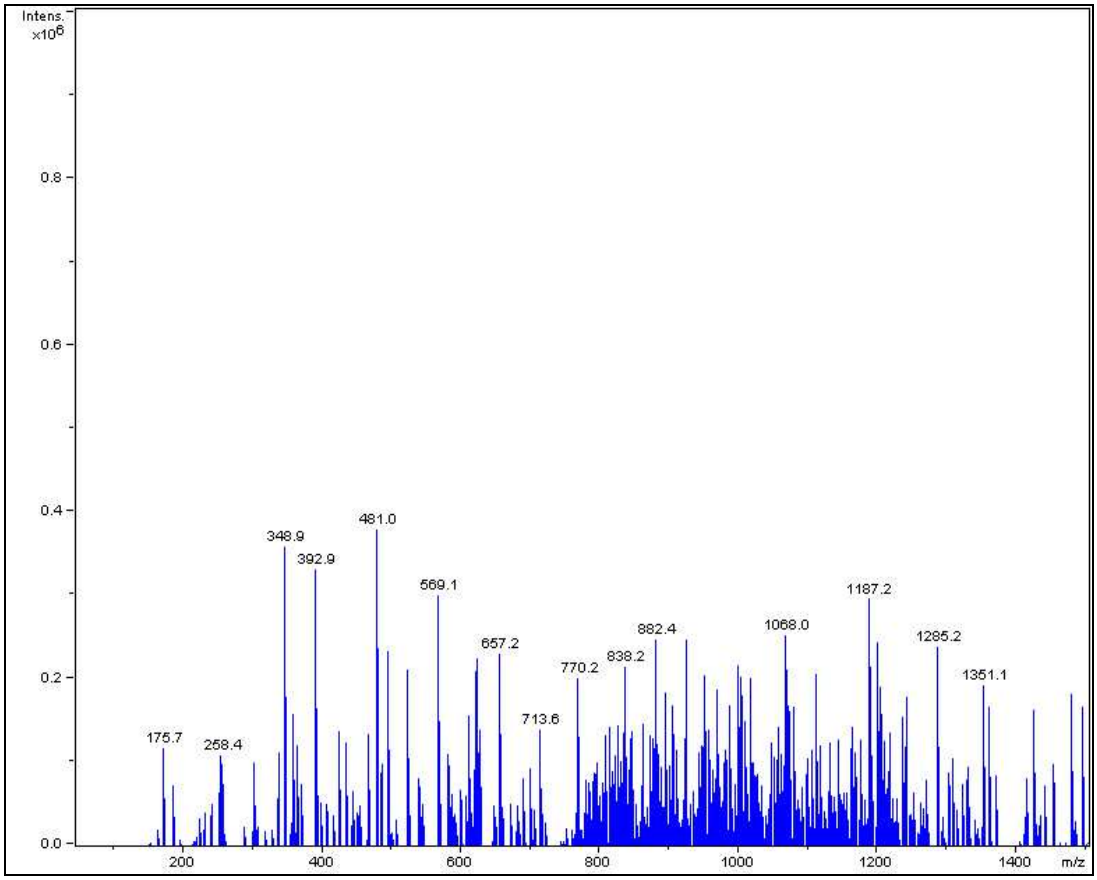

Figure 3.9: Mass spectrum for fibroblast-conditioned medium from dexamethasone-treated cultures obtained using direct infusion into a quadrupole ion trap mass spectrometer. Intensity is an arbitrary scale and indicates relative abundance of ions in the trap for a given scan.

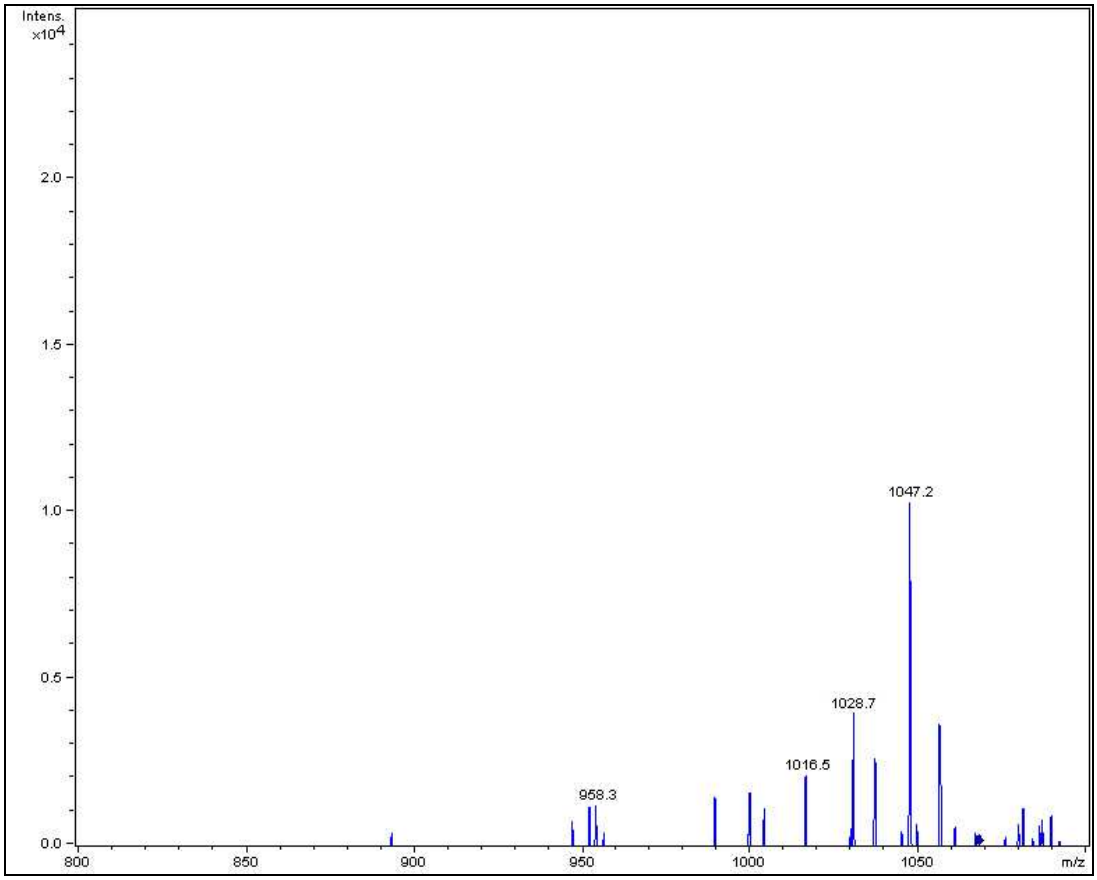

Figure 3.10: Mass spectrum for collision-induced dissociation of the 1068 *m/z* ion of fibroblast-conditioned medium obtained using direct infusion into a quadrupole ion trap mass spectrometer. This spectrum represents MS^2. Intensity is an arbitrary scale and indicates relative abundance of ions in the trap for a given scan.

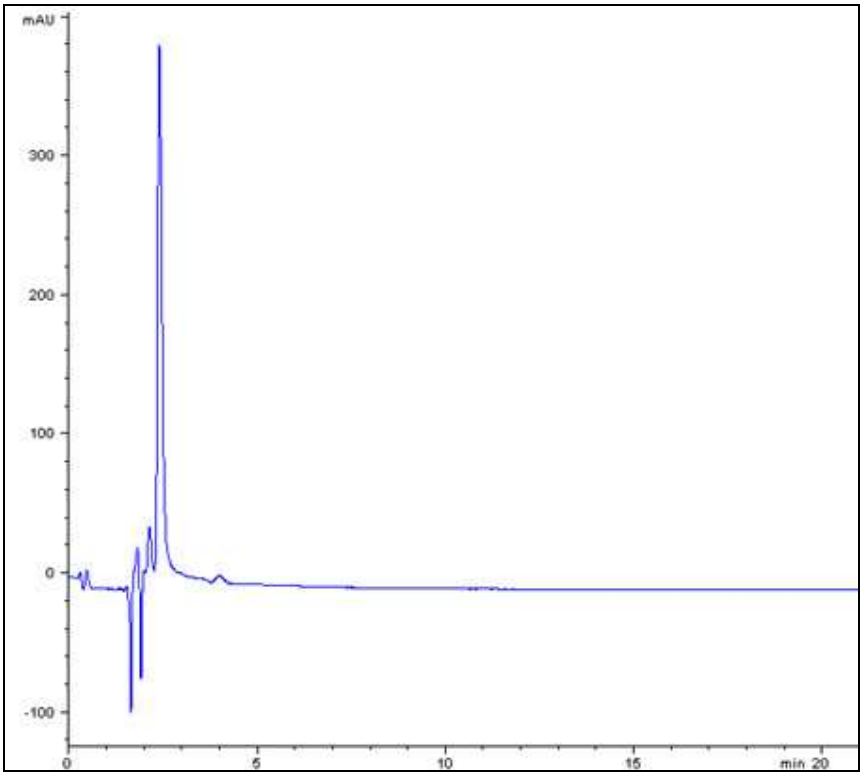

Figure 3.11: Chromatogram of neuregulin-1β (10 ng.mL^{-1}) obtained using capillary HPLC. Response is shown in milli-Absorbance units (mAU) as a function of time. Detection wavelength was 280 nm.

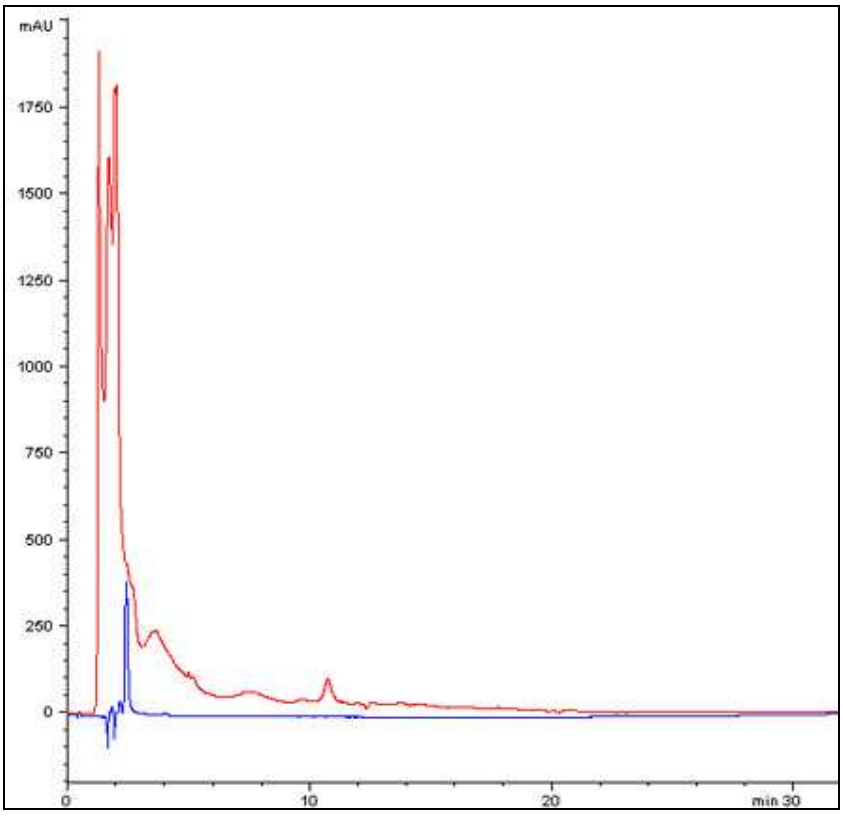

Figure 3.12: Chromatogram of neuregulin-1β (blue) overlaid on chromatogram of fibroblast-conditioned medium (red) obtained using capillary HPLC. Response is shown in milli-Absorbance units (mAU) as a function of time. Detection wavelength was 280 nm for both analyses.

3.3.3 Quantitation of NRG1

Solutions of NRG1 at concentrations of 0.1, 0.5, 1, 5, 10, 50 and 100 ng.mL^{-1} were run using the capillary HPLC method described in **3.2.5** and the MS conditions outlined in **3.2.4**. The resultant mass spectrum chromatograms were then used to generate a standard curve for NRG1 in the ion trap MS (Figure 3.13). The area under the peak was calculated using the mass spectrometry software, and this area plotted against NRG1 concentration. Samples were run in triplicate and this generated a standard curve with a very strong correlation (R = 0.9978).

Six pairs of control and dexamethasone (test) treatment FCM were analyzed using the same method, and chromatograms generated. To provide comparison, three pairs of FCM from experiments which had shown no indirect response to dexamethasone, in spite of being below the cell density threshold, were also chosen and analyzed. As no suitable internal standard was available, all samples were run in triplicate to reduce standard error. The area under the peak for each sample was calculated and the standard curve used to calculate NRG1 concentration. Results of this analysis showed that mean NRG1 concentration in the control treatment FCM was 19.85 ng.mL^{-1} (± 1.73 ng.mL^{-1}), while in the test treatment FCM it was 41.59 ng.mL^{-1} (± 1.44 ng.mL^{-1}) (Figure 3.14). For FCM which had shown no indirect response, control FCM contained 20.85 ng.mL^{-1} (± 3.03 ng.mL^{-1}) NRG1, while test FCM had a concentration of 22.84 ng.mL^{-1} (± 1.67 ng.mL^{-1}). Single-factor ANOVA of these results indicated a significant difference between the test treatment FCM (positive indirect response) and the remaining three FCM samples ($p = 7.51 \times 10^{-7}$).

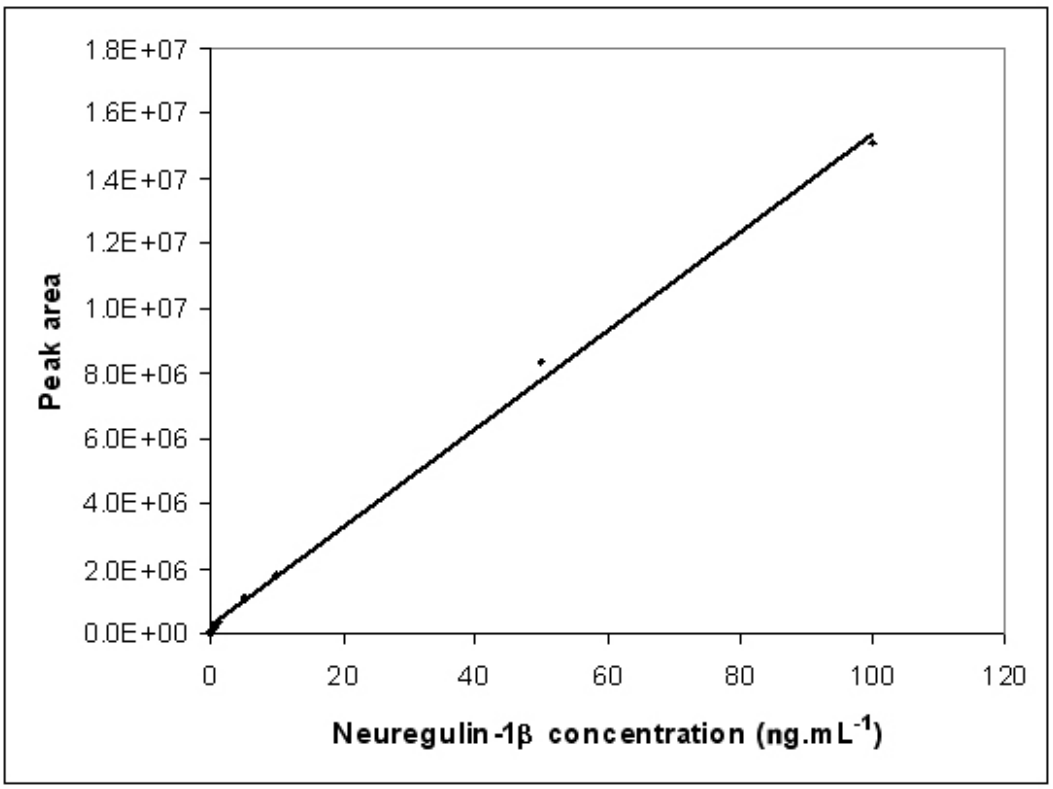

Figure 3.13: Standard curve for response of neuregulin-1β in quadrupole ion trap mass spectrometer. Peak area is calculated by the mass spectrometer software and has no units. The line is a trend line with a correlation of 0.9978.

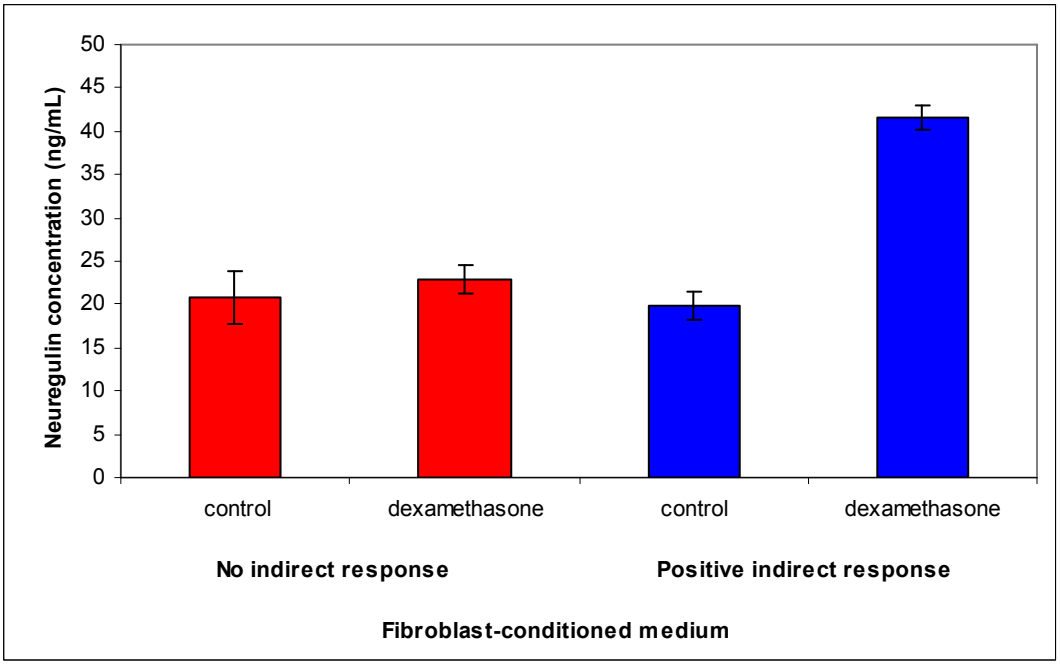

Figure 3.14: Concentration of neuregulin-1β (ng.mL^{-1}), calculated using ion trap mass spectrometry, in fibroblast-conditioned media (FCM) generated in the absence (control) and presence of 100nM dexamethasone (dexamethasone). Red indicates FCM for which no indirect response to dexamethasone was detected, blue represent FCM for which a positive response was observed.

3.4 Discussion

This study has provided further evidence of a significant role for neuregulin-1 (NRG1) in the fibroblast-pneumocyte factor response observed in fetal lung cells. The presence of this growth factor in such a large number of systems during fetal development shows the breadth of its activity, so its involvement in the surfactant system is unsurprising. It is also known to be involved in earlier stages of lung development, including the stimulation of branching morphogenesis (Liu *et al.*, 2004). In fact, given the number of systems in which NRG1 has been identified, it is surprising that a role in this system was not postulated prior to 2003.

Neuregulins are related to epidermal growth factor, which has previously been shown to have a significant impact on surfactant synthesis (Sen and Cake, 1991), and the two factors share the same receptors, the ErbB family. Receptors for NRG1 have been shown to be present in the fetal lung at the same time that surfactant synthesis is initiated (Liu *et al.*, 2004). Furthermore, these receptors have only been detected on epithelial cells (i.e. type II pneumocytes) (Patel *et al.*, 2000), while NRG1 expression has been detected in mesenchymal cells (i.e. fibroblasts) (Citri *et al.*, 2003). This localization implies that NRG1 is secreted from fibroblast cells and acts in a paracrine fashion on neighboring type II pneumocytes, exactly as FPF has been proposed to act (Smith and Post, 1989).

Further study of ErbB receptors has found that only ErbB2 and ErbB3 are expressed in the fetal lung (Liu *et al.*, 2004). This implies that the action of NRG1 on type II pneumocytes would be via the ErbB2/ErbB3 heterodimer. Activation of PI3K (Liu *et al.*, 2004) and Jak-STAT (Liu and Kern, 2002) via ErbB2/ErbB3 has been demonstrated in fetal lung cells, but stimulation of surfactant synthesis by NRG1 is likely to occur via protein kinase C (PKC), as known activators of PKC such as EGF (Sawyer and Cohen, 1981) and PMA (Sen, 1991) have been shown to stimulate

surfactant synthesis. Activation of PKC by ErbB2/ErbB3 signaling has been noted (Citri *et al.*, 2003), although it is not one of the primary pathways for this receptor system. It is likely that ErbB2/ErbB3 increases the activity of phospholipase C_{γ}, which would increase hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol-1,4,5-trisphosphate. The latter compound elevates the intracellular level of calcium and this, together with diacylglycerol, regulates the activity of PKC (Spitaler and Cantrell, 2004). It has been proposed that activity of choline phosphate cytidylyltransferase (CPCT), the rate-limiting enzyme of surfactant synthesis, is regulated by enzyme modulator interactions (e.g. phosphorylation) rather than transcriptional regulation (Post *et al.*, 1986). If NRG1 is acting via PLC and PKC, then it is possible that PKC then acts on CPCT and causes an increase in its activity.

One of the main pathways by which ErbB2/ErbB3 transduces signals involves mitogen-activated protein kinase. The MAPK signaling cascade leads to increased mitogenesis and cellular proliferation (Seger and Krebs, 1995). If NRG1 action on type II cells leads to an increase in MAPK activity as well as PKC, it is likely that it causes type II cell numbers to increase. This mitogenic effect may contribute to the stimulation of surfactant synthesis prior to parturition *in vivo*, but is unlikely to have an effect in the culture system, as results are expressed as dpm.ng DNA^{-1}.

Elevation of protein kinase C activity (via diacylglycerol) has also been shown to increase secretion of surfactant (Sano *et al.*, 1985; Asokananthan and Cake, 1996). Direct activation of protein kinase C via the ErbB2/ErbB3 heterodimer may lead to an increase in secretion from type II cells. FPF has always been postulated as a direct control on synthesis of surfactant, but not secretion. If FPF is NRG1, then the action of this protein could in fact regulate both synthesis and secretion, and could be considered a regulator for the entire pulmonary surfactant system.

A discrepancy between this work and that of Dammann *et al.* (2003) is the molecular weight observed for NRG1. Dammann *et al.* isolated NRG1 with a molecular weight of 44 kDa, while this study focused on the much smaller mass of 7.5 kDa. The 44 kDa form is thought to be a proteolysed soluble form of the 75 kDa transmembrane isoform of NRG1. The 7.5 kDa form is known to comprise only the EGF-like bioactive domain, the only domain possessed by all known isoforms of NRG1. Only this domain is required for the activity of the protein, and so this molecular weight likely represents a fully-processed form of NRG1. It is therefore possible that the quantitation of NRG1 obtained using ions of the 7.5 kDa isoform does not represent the total concentration of NRG1 present in FCM. Other, larger isoforms may be present and are likely to ionize very differently. Some isoforms may not ionize at all if electrospray ionization cannot impart sufficient energy.

Expression of the protein NRG1 by fetal lung fibroblasts is enhanced by dexamethasone. The gene for NRG1, *nrg1*, therefore probably possesses at least one glucocorticoid response element. These are located upstream of the promoter for a given gene, providing a binding site for glucocorticoid receptors (Bentley, 1998). The gene *nrg1* is known to possess multiple promoters that can generate different isoforms of NRG1 (Steinthorsdottir *et al.*, 2004). Depending on the degree of separation of these promoters, it is possible that only particular promoters are preceded by a GRE, meaning that only certain isoforms are expressed when cells are exposed to glucocorticoids. This may mean that compounds other than glucocorticoids that stimulate surfactant synthesis indirectly may act via different promoters, leading to the production of different NRG1 isoforms with different activity. Such convoluted regulation is probably unlikely, but due to the complexity of this system, all aspects of the system must be considered.

Another possibility that must be considered is that in addition to enhancing expression of growth factors, dexamethasone may also suppress expression of inhibitory factors. Torday and Kourembanas (1990) observed that fetal lung fibroblasts produced a growth factor homologous to transforming growth factor β (TGFβ) that blocked the maturation of type II pneumocytes. This has been supported by recent work (McDevitt *et al.*, 2007) which used RT-PCR and microarrays to confirm the expression of TGFβ by fibroblasts. Any increase in synthesis with dexamethasone treatment may therefore be due to a combination of enhanced expression of FPF and inhibition of expression of TGFβ. The compelling evidence that exists for both regulatory pathways indicates that signaling in the developing lung is more complex than initially thought.

The main evidence to indicate that the ions observed in FCM were ions of NRG1 is the partial fragmentation pattern obtained. The pattern matched that of the NRG1 standard for several ions. A more detailed fragmentation pattern, preferably over several MS^n steps would be desired for a definitive identification, but insufficient energy was available through CID to allow for further fragmentation of an intact protein molecule such as NRG1. Thus, it is possible (although unlikely) that the ions observed in FCM were ions of an unrelated compound with an identical *m/z*. The best approach would be to use a more powerful mass spectrometry technique such as Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). The use of high strength magnetic fields means that this instrument can provide molecular weights accurate to five decimal places, sufficient for definitive identification of a compound (Marshall *et al.*, 1998). This type of high-resolution analysis, combined with proteomic techniques such as Multidimensional Protein Identification Technology (Washburn *et al.*, 2001), would remove any doubt as to the identity of the ions in FCM.

The concentration of NRG1 in FCM from experiments which exhibited no indirect response to glucocorticoids showed no significant difference from control FCM. This clearly established a strong correlation between NRG1 concentration in FCM and increased surfactant synthesis. The reason for the lack of response in some cultures is not clear, and is possibly related to the sensitivity of the tissue culture system that was highlighted in **Chapter 2**. This particular observation warrants further investigation to determine if a particular aspect of the system can prevent the cells from responding to dexamethasone and secreting NRG1.

Attempts to separate the components of FCM using HPLC were largely unsuccessful. This can be attributed to the complexity of FCM, both in terms of the array of compounds secreted from the cultured lung fibroblasts into the medium, and the composition of MEM^{+} itself. Compounds of interest, mostly secreted proteins, are also likely to be present in much lower concentrations than other components. For this reason, further research into NRG1 in this system would be greatly advantaged by improved separation. This may require protein purification techniques to extract the proteins from solution, although important non-protein compounds may be overlooked. A technique such as solid phase extraction may be of use, as it allows compounds to be separated initially on the basis of solubility in different solvents. Other possibilities include HPLC using two columns of differing chemistry, or possibly a column of larger pore size (e.g. 100 nm); use of two-dimensional gel electrophoresis to separate proteins prior to mass spectral analysis; or the use of other forms of mass spectrometry, as detailed earlier. FT-ICR-MS would remove the need for adequate separation on HPLC and allow all of the components of FCM to be identified using mass spectrometry. Identification of all of the compounds present in FCM would be highly advantageous, as

it would provide a much clearer picture of exactly what is secreted from fetal lung fibroblasts.

This study has provided further evidence that FPF, whose molecular identity has been unknown, is most likely NRG1, or at least includes NRG1 as a significant component. The concentration of a particular NRG1 isoform in fibroblast-conditioned medium was shown to increase significantly when fibroblast cultures derived from fetal rat lung were exposed to dexamethasone. Previous findings about the localization of NRG1 and its receptors, the ErbB family, show that the appropriate receptor-ligand system exists for NRG1 to have the effects previously observed for FPF in the surfactant system. Transduction of the signal by ErbB2/ErbB3 can occur via a number of pathways, and it is likely that phospholipase C_{γ}/protein kinase C provides the signaling pathway in type II pneumocytes. The complexity of regulation of the NRG1 system, from transcription of the *nrg1* gene through to receptor interactions, has made it difficult to generate a complete picture of this system from a single study. More research is required into NRG1 isoforms and receptors to fully understand the role of NRG1 in the surfactant system. In spite of the remaining ambiguity, it does seem likely that neuregulin-1 is a major component of the activity of the compound identified as 'fibroblast-pneumocyte factor'.

Chapter 4. Effect of insulin-like growth factor II on surfactant synthesis and secretion

4.1 Introduction

4.1.1 Insulin-like growth factors

Insulin-like growth factors (IGFs) are a group of growth factors that share similarities in function with the neuregulins (Zorzano *et al.*, 2003). IGFs are involved in many biochemical processes, including cell differentiation, proliferation and apoptosis (Jones and Clemmons, 1995). Two IGFs have been identified to date: insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II). Specific IGF receptors (IGFRs) and binding proteins (IGFBPs) have also been identified (Yu and Rohan, 2000) (Figure 4.1). Other components of the system include an acid labile subunit (ALS) and several low-affinity IGFBP-related proteins (IGFBP-rPs) (Frystyk, 2004).

Insulin-like growth factor I is a 70-amino acid polypeptide that has been found to be important in growth and injury repair in a variety of organs, including the lung. It shares 49% homology with insulin (Ullrich *et al.*, 1986). Insulin-like growth factor II is a 67-amino acid polypeptide with 47% homology with insulin and 61% with IGF-I (Rinderknecht and Humbel, 1978). The concentration of IGF-II in adult human serum is 660 ± 124 ng.mL^{-1}, while in adult rat serum it is 11.4 ± 0.7 ng.mL^{-1} (Bowsher *et al.*, 1991). Expression and regulation of IGFs is not well understood. IGF concentration is regulated by a number of hormones, including estrogen, follicle-stimulating hormone and luteinising hormone (Yu and Rohan, 2000), but there are likely to be many other mechanisms of regulation that have not yet been identified.

Considerable evidence exists to indicate that IGF-II plays a key role in mammalian development, and is more important in early life than IGF-I (Brissenden *et al.*, 1984). It has been suggested that the main functions of IGF-II occur during fetal

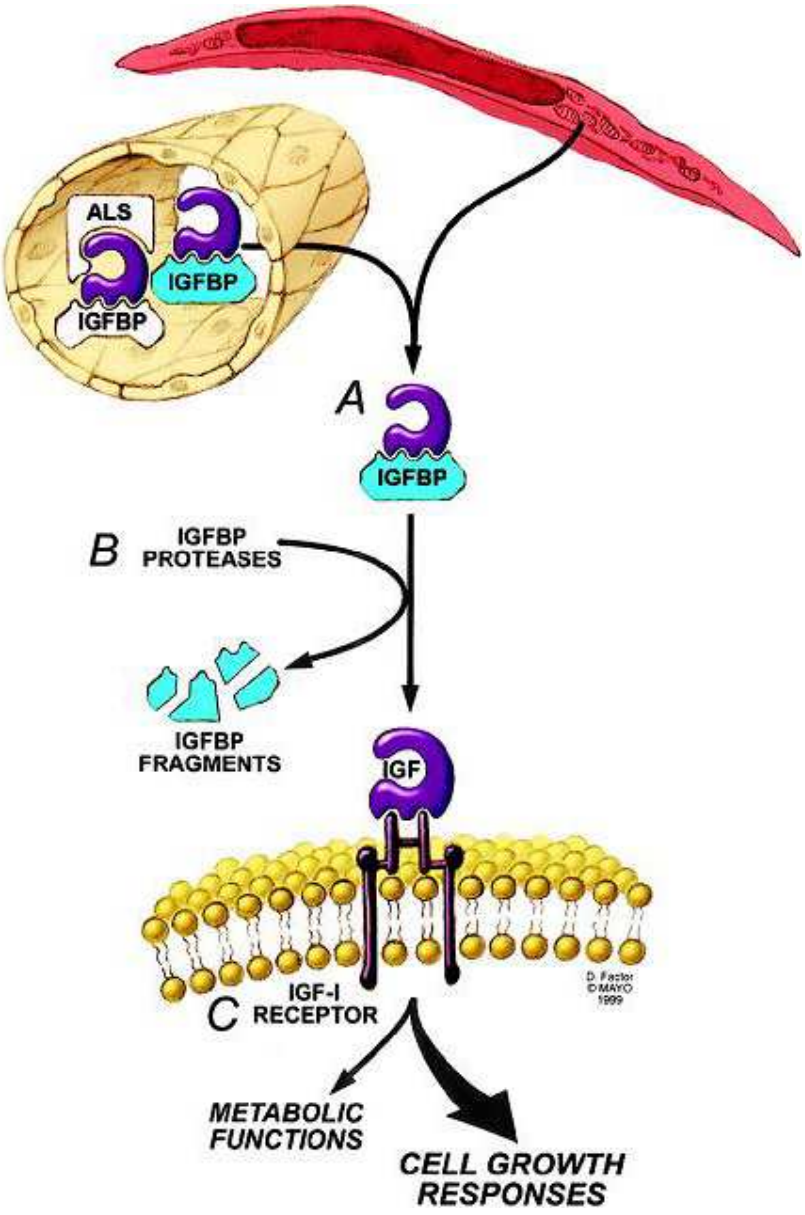

Figure 4.1: Schematic representation of the IGF axis (Bayes-Genis *et al.*, 2000).

The IGFs are present in circulation as binary complexes with IGFBPs. IGFBPs modulate cell response to IGFs (A); and specific proteases act on IGFBPs to release IGFs (B). IGF can then interact with transmembrane receptors (C).

development and that these roles are taken over by IGF-I post-parturition (Stewart and Rotwein, 1996). The IGF-II gene is expressed during fetal life (Brown *et al.*, 1986; Graham *et al.*, 1986), and IGF-II mRNA has been detected in a number of fetal tissues (Beck *et al.*, 1987). Expression of the IGF-II gene in rat liver is highest during fetal development, begins to decrease post-parturition and is undetectable after weaning (Carr *et al.*, 1995; Langford *et al.*, 1998).

4.1.2 Insulin-like growth factor receptors

Two distinct transmembrane insulin-like growth factor receptors (IGFRs) have been identified: type 1 and type 2.

The type 1 IGF receptor (IGFR-1) is a glycoprotein with a molecular weight of approximately 325 kDa, composed of four subunits (Sara and Hall, 1990). IGFR-1 has 60% structural and functional homology with the insulin receptor (Ullrich *et al.*, 1986), and transduces its effect intracellularly via tyrosine kinase (Humbel, 1990). Both IGFs can bind to IGFR-1, but IGF-I shows a greater affinity than IGF-II (Rechler and Nissley, 1985). IGFR-1 has been detected in a number of systems during fetal development (Humbel, 1990; Baker *et al.*, 1993) and its role is likely to be an essential one, as null mutations in IGFR-1 are lethal (Liu *et al.*, 1993). Levels of IGFR-1 decline post-parturition, but it is still detectable (Baker *et al.*, 1993).

The type 2 IGF receptor (IGFR-2) is a single-chain polypeptide with a molecular weight of approximately 250 kDa, composed of three domains (Sara and Hall, 1990). It is structurally unrelated to either the insulin receptor or IGFR-1, but appears to be identical to the mannose-6-phosphate cation-independent receptor (Kornfeld, 1992). As a result, it is able to bind mannose-6-phosphate-containing proteins such as renin, proliferin and thyroglobulin (Jones and Clemmons, 1995). IGFR-2 has a high affinity for IGF-II and is unable to bind insulin (Massague and Czech, 1982). IGFR-2 acts via a

G-protein secondary messenger system (Nishimoto *et al.*, 1989), but there is much evidence to suggest that IGFR-2 is not involved in any signal transduction pathways (Sakano *et al.*, 1991; Kornfeld, 1992). Instead, IGFR-2 is likely to be involved primarily in regulation of IGF-II levels (Filson *et al.*, 1993).

Insulin and the IGFs are able to bind to each other's receptors, although with a greatly reduced affinity (Frattali and Pessin, 1993). A hybrid of the insulin receptor and IGFR-1 has also been identified, but its expression is inconsistent (Jones and Clemmons, 1995). This hybrid receptor has a higher affinity for IGF-I than for insulin, but its biological function is not known.

4.1.3 Insulin-like growth factor binding proteins

Circulating IGFs are almost exclusively bound to one of the insulin-like growth factor binding proteins (IGFBPs). Six IGFBPs (IGFBP-1 to IGFBP-6) have been purified and they have a high affinity for both IGFs (Wallen *et al.*, 1997). The amino acid sequences of the IGFBPs show 47% to 60% homology (Murphy, 1998). Most circulating IGF-I is bound to IGFBP-3, forming a 150 kDa complex that regulates transport of IGF-I (Bayes-Genis *et al.*, 2000). The four main functions of IGFBPs are (Jones and Clemmons, 1995):

a) transport and storage
b) clearance and recycling
c) tissue-specific and cell-specific localization
d) modulation of the interaction of IGFs with IGFRs

The affinity of IGFBPs for IGFs is modulated by IGFBP proteases (Blat *et al.*, 1994). Consequently, these proteases are very important in determining the bioavailability of IGFs at a cellular level (Rajah *et al.*, 1995). Several different

proteases have been identified as being able to cleave IGFBPs, and there are likely to be many others that have not been identified. An example is cathepsin D, a lysosomal protease, which can act on all six known IGFBPs (Claussen *et al.*, 1997).

4.1.4 The role of insulin-like growth factors in lung development

Considerable evidence exists to indicate that IGFs play an important regulatory role in the development of the fetal lung. mRNA and peptides for both IGF-I and IGF-II have been detected in lung tissue (Retsch-Bogart *et al.*, 1996), and abundance of these varies with developmental stages (Klempt *et al.*, 1992). Fetal lung cells in culture have been shown to produce both IGFs and IGFBPs (Maitre *et al.*, 1995), and IGFs have been demonstrated to be involved in mitogenesis in similar cultures (Mouhieddine *et al.*, 1994).

IGF-II mRNA is most abundant during early fetal lung development in rats (up to days 16-18), and declines until four weeks post-partum (Klempt *et al.*, 1992; Wallen and Han, 1994). Peptide fragments of IGF-II are also found in the lung, although their abundance varies greatly in different parts of the lung and different developmental stages. The highest levels of these peptides are observed in the time immediately after birth (Kauffman *et al.*, 1974). Elevated cortisol levels decrease the IGF-II concentration in the lung (Li *et al.*, 1993), so it is possible that the rise in cortisol observed in fetal plasma prior to birth may be the cause of the gradual decline in IGF-II levels.

Receptors for IGFs are present in the fetal lung, but the pattern of expression is difficult to interpret. IGFR-1 is detectable in day 14 rat epithelial cells, but not day 18 cells (Maitre *et al.*, 1995), and its expression is almost negligible in human fetal lung cells (Chetty *et al.*, 2004). IGFR-1 is largely expressed post-parturition, when IGF-II is not detected, indicating that IGF-I is likely to be more important to postnatal growth than IGF-II (Maitre *et al.*, 1995). IGFR-2 is expressed in epithelial cells of fetal

rat lung during the early stages of gestation, but it cannot be detected at day 18 or later. It can be found in mesenchymal tissues at this time, but IGF-II is present only in epithelial tissues at this stage of development (Maitre *et al.*, 1995). This evidence indicates that if IGF-II has a role in the surfactant system at birth, it is likely to be through a receptor other than the IGFRs.

Human insulin receptor (IR) is expressed in two distinct forms, IR-A and IR-B. IR-B is the dominant form, and is thought to be essential for the metabolic effects of insulin (Mosthaf *et al.*, 1990). IR-A binds to both insulin and IGF-II with high affinity and is expressed primarily in tumours and fetal tissues (Frasca *et al.*, 1999). It is therefore possible that IR-A is the main receptor for the IGF system in fetal rat lung. In hepatoma cells, dexamethasone causes expression to switch from IR-A to IR-B (Kosaki and Webster, 1993), so the increase in glucocorticoid expression in late gestation may cause the tissues to change to the adult expression of IR-B.

IGFBPs have also been identified in the developing lung, particularly in the later stages of development (Delhanty and Han, 1993). Genes for IGFBP-2 to IGFBP-6 are expressed in fetal rat lung (Wallen *et al.*, 1997) and mRNA for IGFBP-2 to IGFBP-5 has been detected in lung cultures, although the expression appears to be at a different rate to that observed *in vivo* (van de Wetering *et al.*, 1997). This indicates that regulation of IGFBPs in the developing lung may be controlled by factors extrinsic to the tissue. The most abundant IGFBP in the fetal rat lung is IGFBP-2, which is expressed most during late gestation (Klempt *et al.*, 1992). It is possible that increased production of IGFBPs occurs to bind IGFs and prevent them from interacting with their receptors (Mouhieddine *et al.*, 1994). A potentially important observation made by Mouhieddine *et al.* (1994) is that expression of IGF-II, IGFR-2 and IGFBP-2 is

increased in growth arrested type II pneumocytes. This may be important for experiments using cultured type II pneumocytes, such as in this study.

The presence of IGFs, IGFRs and IGFBPs in the developing lung, and the changes in distribution and abundance observed indicate that the IGFs are likely to be involved in many different processes during lung growth and maturation. It is therefore possible that they are also involved in regulation of the surfactant system. This potential role for IGFs has not been previously investigated, and given that IGFs are functionally similar to neuregulins, a study was undertaken to determine if IGFs have an effect on synthesis or secretion of pulmonary surfactant.

4.2 Methods

4.2.1 IGF-II preparation

Insulin-like growth factor-II (IGF-II) (Novozymes GroPep, Thebarton, SA, Australia) was supplied as a lyophilized powder and dissolved in 10 mM hydrochloric acid (HCl) to a concentration of 100 μM IGF-II. This stock solution was stored at -80°C until use. This solution was further diluted with 10 mM HCl to provide the required working concentration for each experiment. Control plates in these experiments included 10 mM HCl.

4.2.2 Surfactant synthesis experiments

Tissue culture and surfactant synthesis experiments were carried out as described in the revised method of **2.8**. The circulating concentration of IGF-II in fetal rats prior to parturition has been determined to be in the range of 0.4 to 0.7 μM (Daughaday *et al.*, 1982), so experiments focused on the following concentrations: 0.4, 0.6 and 0.8 μM.

4.2.3 Surfactant secretion experiments

In addition to studying the effect of IGF-II on surfactant synthesis, the effect on secretion of surfactant from type II pneumocytes was also examined. Insulin is known to stimulate the rate of surfactant secretion (Snyder and Mendelson, 1987), so it is possible that related compounds like IGF-II may exert a similar effect. As with synthesis, IGF-II was studied at 0.4, 0.6 and 0.8 μM, as well as 0.1, 0.2 and 1.6 μM as part of a concentration curve.

After 72 hours in culture, type II cells were taken from the incubator, media was removed and plates washed twice with 2.0 mL of BSS. A solution of MEM^+ containing 2 $\mu Ci.mL^{-1}$ [methyl-^{3}H]-choline chloride was prepared and placed in a 37°C shaking water bath for 10 minutes. 2.0 mL of this solution was added to each type II plate and plates were returned to the incubator for 24 hours.

Following incubation, plates were washed with BSS to remove unincorporated choline. 1.7 mL of MEM^+ was added to all plates and plates were equilibrated in the incubator for a further 60 minutes. Two plates were then removed from the incubator to determine the amount of phospholipid in media and cells at 'time zero'. To the remaining plates 17 μL of IGF-II + vehicle (test) or vehicle only (control) were added. Plates were returned to the incubator for a further three hours.

Plates were removed from the incubator after three hours and briefly stored on ice. Media was transferred to a centrifuge tube and plates washed three times with 0.83 mL of BSS. Wash buffer was also transferred to the same centrifuge tube and plates were placed back on ice. Tubes were centrifuged at 1000 *g* for two minutes and a 3.5 mL aliquot of the supernatant was added to a screw-capped tube. To this tube, 50 μL of L-3-phosphatidylcholine-1,2-di[1-^{14}C] palmitoyl was added as a recovery standard and 20 μL of 2.5 $mg.mL^{-1}$ phosphatidylcholine was added as a carrier. Following this, 13.125 mL of 1:2 chlorform:methanol was added, tubes vortexed and

the mixture left to stand at room temperature. After 10 minutes, 4.375 mL of chloroform was added, tubes vortexed and the contents left to stand for a further 10 minutes. Finally, 4.375 mL of MilliQ-H_2O was added, tubes vortexed and placed in a 4°C refrigerator for at least 48 hours. Cells were also harvested for both lipid and DNA assays using the revised method described above for synthesis experiments in **2.8**.

Lipid assays for media and cells were completed as described above, with 400 μL samples being taken into scintillation vials, dried, vials filled with scintillant, vortexed and left in the dark for 24 hours before being counted for both ^{3}H and ^{14}C. DNA assays were completed as in **2.8**. Calculations to determine mean dpm.ng DNA^{-1}, cell density and percentage recovery were also undertaken as described previously. The calculation for percentage secretion was:

$$\% \text{ secretion} = \frac{\text{dpm in media phospholipids}}{(\text{dpm in media phospholipids}) + (\text{dpm in cellular phospholipids})} \times 100$$

Results were tabulated and graphed using Microsoft Excel 2003 (v11.8117) and statistics calculated using SPSS for Windows 14.0.1.

4.3 Results

4.3.1 Surfactant synthesis

A total of 31 pairs of control and test FCM were tested, ten at 0.4 μM IGF-II, seven at 0.6 μM and fourteen at 0.8 μM (Table 4.1). Response to IGF-II was small, with direct treatment showing a mean increase in synthesis of 3.34%, and indirect treatment a mean increase of 3.60%. Single-factor ANOVA showed no significant difference between direct and indirect treatments at any of the concentrations tested

Table 4.1: The direct and indirect effect of IGF-II on the augmentation of PC synthesis by type II pneumocytes.

Treatment	PC Synthesis (dpm/ng DNA)	Difference	P-value	Mean type II pneumocyte DNA (μg/plate)	Mean fibroblast DNA level (μg/plate)
IGF-II 0.4 μM					
Direct (10)					
Control	21.24 ± 3.26				
IGF-II	22.01 ± 3.65	↑ 3.60%		25.54	
Indirect (10)			0.86		
Control	19.65 ± 3.54				
IGF-II	20.17 ± 4.06	↑ 2.65%		26.75	8.30
IGF-II 0.6 μM					
Direct (7)					
Control	25.51 ± 7.41				
IGF-II	26.39 ± 6.66	↑ 3.44%		20.59	
Indirect (7)			0.99		
Control	18.44 ± 3.69				
IGF-II	19.35 ± 4.47	↑ 4.94%		27.26	4.19
IGF-II 0.8 μM					
Direct (14)					
Control	26.92 ± 4.81				
IGF-II	27.73 ± 4.25	↑ 2.99%		23.18	
Indirect (14)			0.98		
Control	23.43 ± 3.63				
IGF-II	24.19 ± 4.04	↑ 3.22%		24.82	5.08

Primary cultures of lung fibroblasts were grown to near confluence, followed by 24 hours incubation in the presence or absence of either 0.4, 0.6 or 0.8 μM in MEM^+. The fibroblast-conditioned media (FCM) were collected and stored at -80°C. Prior to use, the media were thawed and incubated at 65°C for one hour, cooled to room temperature and filter-sterilized to remove detached cells and cellular debris. The media were then diluted 1:4 with MEM^+. The effect on PC synthesis by type II pneumocytes was investigated by exposing the cells either to the FCM (indirect) or to MEM^+, with and without IGF-II (direct). The data represent the mean ± SE. The number of replicates examined for each condition is indicated in parentheses, and each replicate comprised four cultures. Data were compared using single-factor ANOVA.

(p = 0.86, 0.99 and 0.98 for 0.4, 0.6 and 0.8 μM IGF-II, respectively). As with the results for dexamethasone (**2.9**), experiments with a type II cell density above the threshold value of 25 $\mu g.plate^{-1}$ were removed from the data set and the results recalculated.

In the revised data set, a total of 22 pairs of control and test FCM were tested, seven at 0.4 μM IGF-II, six at 0.6 μM and nine at 0.8 μM (Table 4.2). Response at the three different concentrations was very similar, with direct treatment showing a mean increase in synthesis of 4.65%, and indirect treatment a mean increase of 6.35%. The lowest indirect response was at 0.4 μM (6.00%) and the highest at 0.8 μM (6.91%). Standard error values were high, with an error of 31.5% for direct controls at 0.6 μM. PC synthesis values (expressed in $dpm.ng\ DNA^{-1}$) for these experiments were generally consistent with those observed previously (Sen and Cake, 1991). Single-factor ANOVA showed no significant difference between the direct and indirect treatments at any of the concentrations tested (p = 0.90, 0.93 and 0.89 for 0.4, 0.6 and 0.8 μM IGF-II, respectively). While still not significant, the level of response to the various concentrations of IGF-II for the revised data set was much more consistent, indicating that type II cell density was affecting the calculated results of synthesis experiments.

4.3.2 Surfactant secretion

Initial secretion experiments tested a range of IGF-II concentrations from 0.1 to 1.6 μM to determine if there was any significant response (Figure 4.2). The control cultures had a mean secretion rate of 1.48% (± 0.07%), while the greatest response from IGF-II was observed at 0.4 μM with a secretion rate of 1.69% (± 0.08%). A mean value of only 1.18% (± 0.15%) was seen at 1.6 μM IGF-II. The secretion rate for the other three concentrations tested (0.1, 0.2 and 0.8 μM) fell between these two values. Single-factor ANOVA revealed no significant differences in secretory response between any of

Table 4.2: The effect of IGF-II on PC synthesis in type II pneumocytes for type II cultures with cell density below 25 μg.plate^{-1}.

Treatment	PC Synthesis (dpm/ng DNA)	Difference	P-value	Mean type II pneumocyte DNA (μg/plate)	Mean fibroblast DNA level (μg/plate)
IGF-II 0.4 μM					
Direct (7)					
Control	23.61 ± 3.99				
IGF-II	24.76 ± 4.52	↑ 4.90%		13.40	
Indirect (7)			0.12		
Control	22.85 ± 4.35				
IGF-II	24.22 ± 5.01	↑ 6.00%		14.68	4.86
IGF-II 0.6 μM					
Direct (6)					
Control	29.30 ± 9.23				
IGF-II	30.61 ± 8.31	↑ 4.47%		14.28	
Indirect (6)			0.12		
Control	28.11 ± 6.37				
IGF-II	29.84 ± 7.24	↑ 6.15%		15.01	4.43
IGF-II 0.8 μM					
Direct (9)					
Control	29.48 ± 6.37				
IGF-II	30.83 ± 5.40	↑ 4.57%		13.77	
Indirect (9)			0.12		
Control	25.47 ± 5.20				
IGF-II	27.23 ± 5.82	↑ 6.91%		13.67	3.23

Primary cultures of lung fibroblasts were grown to near confluence, followed by 24 hours incubation in the presence or absence of either 0.4, 0.6 or 0.8 μM in MEM^{+}. The fibroblast-conditioned media (FCM) were collected and stored at -80°C. Prior to use, the media were thawed and incubated at 65°C for one hour, cooled to room temperature and filter-sterilized to remove detached cells and cellular debris. The media were then diluted 1:4 with MEM^{+}. The effect on PC synthesis by type II pneumocytes was investigated by exposing the cells either to the FCM (indirect) or to MEM^{+}, with and without IGF-II (direct). The data represent the mean ± SE. The number of replicates examined for each condition is indicated in parentheses, and each replicate comprised four cultures. Data were compared using single-factor ANOVA.

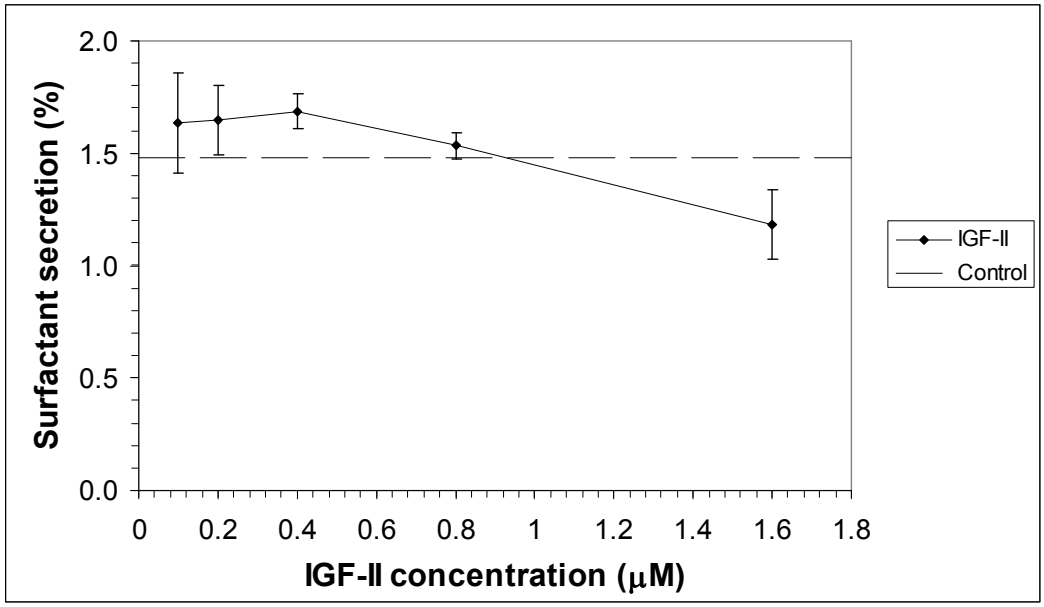

Figure 4.2: Effect of IGF-II on secretion of surfactant-associated phospholipids from cultured type II pneumocytes.

Type II cells were isolated from lungs of 19-day fetal rats and, after reaching confluence, were incubated for a further 24 hours with [^{3}H]-choline chloride in order to label cellular phosphatidylcholine. The cells were then washed thoroughly and equilibrated for one hour at 37°C in serum-free medium prior to the addition of the indicated concentrations of IGF-II. The percentage of total cellular [^{3}H]-phosphatidylcholine secreted into the media in the subsequent three hours was determined using the method outlined in **4.2.3**. The data represent the mean ± SE. For comparison, the mean control response is also shown.

the concentrations tested ($p = 0.12$). Standard error values were small, indicating consistency between experiments.

The initial experiments showed that IGF-II had no significant effect on surfactant secretion. However, some compounds have been shown to act synergistically or antagonistically with other secretagogues (Sullivan *et al.*, 2003). To investigate this possibility, the effect of IGF-II was examined with two known stimulants of secretion, neuromedin C and isoproterenol. Both compounds have been shown to increase surfactant secretion from type II pneumocytes at 1.0 nM (neuromedin C) (Asokananthan, 1996) and 1.0 μM (isoproterenol) (Asokananthan and Cake, 1996). Neuromedin C is the biologically active C-terminal end of gastrin releasing peptide, a peptide with a wide range of signaling roles in mammalian systems ((Sunday *et al.*, 1988). Isoproterenol (also known as isoprenaline) is a β-adrenergic agonist that is synthetically derived from noradrenalin (Gogarten *et al.*, 2001). Both of these compounds have been previously shown in this laboratory to have no effect on cell viability, using media LDH as a reporter.

Neuromedin C at 1.0 nM gave rise to a mean secretion rate of 4.59% (Figure 4.3), which was significantly higher than the mean control value of 1.55% ($p = 4.76 \times 10^{-7}$). IGF-II at 0.4 μM showed a mean secretion rate identical to the control value. The exposure of type II cells to the combined effects of 1.0 nM neuromedin C and 0.4 μM IGF-II yielded a secretion rate (4.24%) which was not significantly different ($p = 0.26$) from the elevated level seen with 1.0 nM neuromedin C alone. Standard error values were small, indicating consistency between experiments.

Exposure to isoproterenol at 1.0 μM resulted in a mean PC secretion rate of 4.44% (Figure 4.4), which was significantly higher than the mean control value of 1.56% ($p = 7.63 \times 10^{-8}$). The effects of IGF-II at 0.4 μM and 0.8 μM were almost

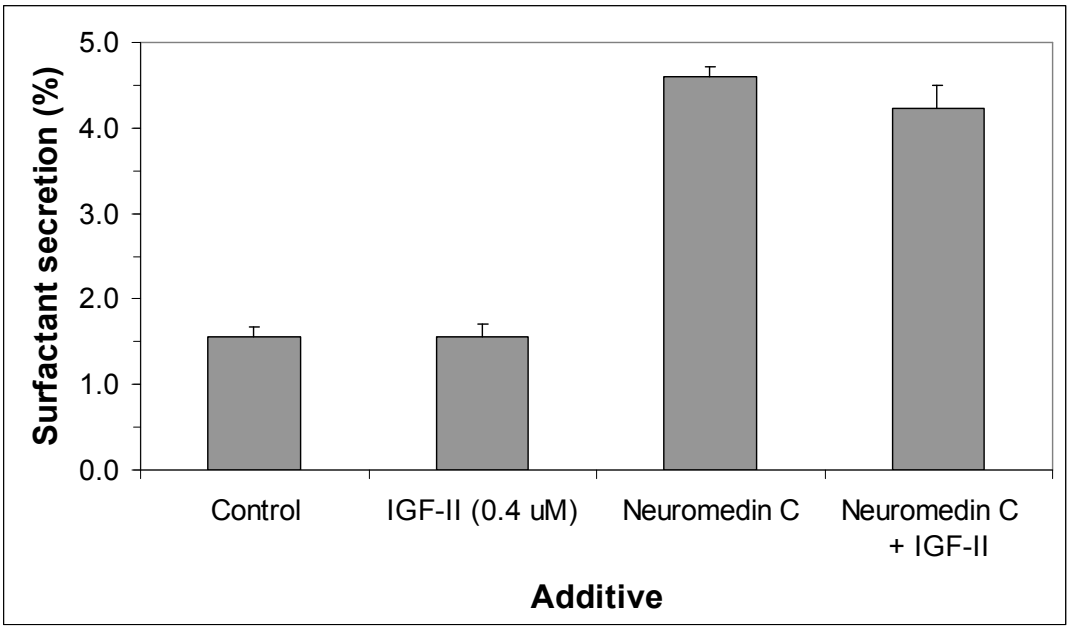

Figure 4.3: Effect of IGF-II on secretion of surfactant-associated phospholipids from cultured type II pneumocytes in response to neuromedin C.

Type II cells were isolated from lungs of 19-day fetal rats and, after reaching confluence, were incubated for a further 24 hours with [^{3}H]-choline chloride in order to label cellular phosphatidylcholine. The cells were then washed thoroughly and equilibrated for one hour at 37°C in serum-free medium prior to the addition of IGF-II (0.4 μM) and/or neuromedin C (1.0 nM). The percentage of total cellular [^{3}H]-phosphatidylcholine secreted into the media in the subsequent three hours was determined using the method outlined in **4.2.3**. The data represent the mean ± SE.

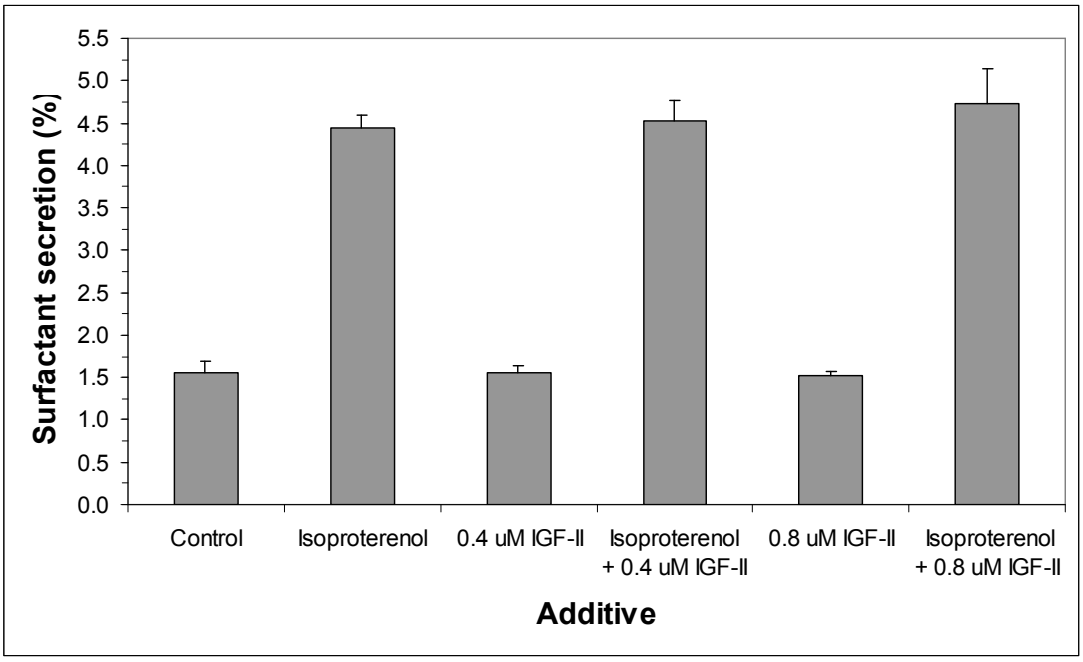

Figure 4.4: Effect of IGF-II on secretion of surfactant-associated phospholipids from cultured type II pneumocytes in response to isoproterenol.

Type II cells were isolated from lungs of 19-day fetal rats and, after reaching confluence, were incubated for a further 24 hours with [^{3}H]-choline chloride in order to label cellular phosphatidylcholine. The cells were then washed thoroughly and equilibrated for one hour at 37°C in serum-free medium prior to the addition of isoproterenol (1.0 μM) and/or the indicated concentration of IGF-II . The percentage of total cellular [^{3}H]-phosphatidylcholine secreted into the media in the subsequent three hours was determined using the method outlined in **4.2.3**. The data represent the mean ± SE.

identical to the mean control value, with secretion rates of 1.55% and 1.52%, respectively. The exposure of type II cells to the combined effects of 1.0 μM isoproterenol and 0.4 μM IGF-II yielded a secretion rate (4.53%) which was not significantly different ($p = 0.75$) from the elevated level seen with 1.0 μM isoproterenol alone. The combination of 1.0 μM isoproterenol and 0.8 μM IGF-II yielded a secretion rate of 4.74%, which was also not significantly different ($p = 0.42$) from the rate observed with 1.0 μM isoproterenol.

4.4 Discussion

The previously-described effects of insulin on surfactant synthesis and secretion, and the relationship between insulin and IGFs, suggested that IGF-II might have an impact on the surfactant system. Insulin delays maturation of fetal rat lungs and decreases the number of lamellar bodies present in type II cells (Gross *et al.*, 1980). It also causes an increase in SP-B levels, at the expense of SP-A, which leads to an increase in secretion (Snyder and Mendelson, 1987). If insulin and IGF-II act through the same or similar mechanisms in the fetal lung, IGF-II would be expected to decrease synthesis and increase secretion of surfactant.

The results of this study indicate that IGF-II had no significant effect on synthesis in either a direct or indirect manner, nor did it have any significant effect on secretion. The main receptor for the biological action of IGF-II in most systems is thought to be IGFR-1, and it is likely that this is the primary receptor in type II pneumocytes immediately post-parturition. However, IGFR-1 is not present in detectable concentrations in type II pneumocytes derived from fetal rats at day 18 of gestation (Maitre *et al.*, 1995), suggesting that it may not be present in the type II cultures used in this study. The absence of this receptor may account for the lack of a significant effect on synthesis or secretion of surfactant. It has been shown that, at this

stage of development, IGF-II may act through IR-A (Frasca *et al.*, 1999). This receptor does have a high affinity for IGF-II, but its abundance in fetal lung tissue has not been characterized. It is therefore possible that IR-A may not be present in fetal lung fibroblasts and type II pneumocytes, or may exist only at a very low level, meaning that IGF-II is unable to exert a significant effect on the surfactant system through this receptor.

Recent research (Siddle and Soos, 1999) has suggested that there are many atypical insulin and IGF receptors that are yet to be fully characterized. These receptors may arise from processes such as glycosylation, splicing or hybridization (Le Roith *et al.*, 1995). Some atypical receptors are able to bind IGF-II with reasonable affinity, and so may be important in IGF signaling. An example is the insulin-receptor-related receptor (IRR), which shares 55% homology with IR-B and IGFR-1 (Shier and Watt, 1989). The possible absence of the primary receptors for IGF-II in lung cells may indicate that receptors such as IRR are involved in the IGF system in fetal lung cells. The variable affinity and effect of these receptors (e.g. Zhang and Roth, 1992) may have contributed to the lack of response to IGF-II observed in this study.

The important role of IGFBPs in modulating the action of IGF-II must also be considered. The expression of IGFBPs is different in culture than it is *in vivo*, implying that control of this system is exogenous to the tissue (van de Wetering *et al.*, 1997). The IGFBPs may not have been present in the tissue cultures used in these experiments or may have been expressed at much higher levels than normal, possibly affecting the action of IGF-II. The potential absence/presence in excess of the other proteins of the IGF system must also be considered. The role of the IGFBP-related proteins is still not known and they may be involved in regulation of the effects of IGF-II. Further study is

needed to determine if any IGF-related proteins are present in either fibroblast or type II pneumocyte cultures and if so, which, and at what levels.

Previous research (Mouhieddine *et al.*, 1994) has shown that growth-arrested type II cells show an increase in expression of IGFR-2 and IGFBP-2. Confluent type II pneumocyte cultures may be considered to be non-proliferating, as they occupy the entire culture plate. The main role of IGFR-2 and IGFBP-2 appears to be to bind IGF-II and remove it from circulation, rather than being involved in any signaling pathways (Filson *et al.*, 1993; Mouhieddine *et al.*, 1994). As the regulation of some components of the IGF axis have been shown to be exogenously controlled, binding of IGF-II by receptors and binding proteins may not be solely concentration-dependent. If the type II cultures used in this study up-regulated IGFR-2 and/or IGFBP-2, then the IGF-II used in these experiments may have bound to either the IGFR-2 receptor or the binding protein and not been able to exert any significant effect on the synthesis or secretion of surfactant. The IGFBP proteases, required to free IGF-II from the IGFBPs, may not have been present in the system meaning that, once bound, the IGF-II could not be freed to interact with appropriate receptors. The lack of any significant effect of IGF-II observed in this culture system may therefore not be representative of that which occurs *in vivo*.

The primary role of IGF-II in some tissues is the enhancement of cellular activities such as proliferation and differentiation (Jones and Clemmons, 1995), so it was thought that IGF-II might cause an increase in surfactant synthesis and secretion through enhanced proliferation of cultured cells. However, research by Fraslon and Bourbon (1992) has shown that IGF-II does not lead to increased proliferation of type II cells. Increased cell density on type II plates would, in fact, probably have had a negative effect on synthesis, as detailed in **2.10.1**. Fraslon and Bourbon (1992) did find

that IGF-II could increase lung fibroblast cell numbers, but mean cell density of fibroblast cultures for IGF-II treatments was lower than for experiments with other compounds (as discussed in **2.10.2**). The results obtained indicate that the mitogenic effects of IGF-II were unlikely to have been a significant factor in these experiments.

Analysis of the results for surfactant synthesis indicated that cell density of type II cultures was again likely to be affecting the results observed. While removal of cultures with a density above 25 μg.DNA plate^{-1} did not reveal significant differences between direct and indirect treatments, the calculated results were far more consistent. This consistency at three different concentrations provides further evidence that IGF-II had no effect on the surfactant system. The density of type II cultures clearly had an effect on the reliability of the results obtained, further reinforcing the need to develop a method of producing primary type II cultures with a consistent density.

Insulin-like growth factor II did not behave as expected in the surfactant system, with no significant effect on either synthesis or secretion. As has been discussed, the complexity of the IGF system means that there are many possible reasons for this lack of effect. The most likely reason is the absence of the preferred receptor, IGFR-1. However, the receptors that IGF-II acts through in the fetal lung have not been definitively identified, although there are several possible candidates. These receptors, such as IR-A, have differing abundance and affinity for IGF-II and these factors may have greatly reduced the response of this system to IGF-II. The potential up-regulation of IGFR-2 and IGFBPs may have removed IGF-II from the system and prevented it from interacting with other receptors. IGF-II is clearly involved in many important developmental processes in the fetus, including lung development (Maitre *et al.*, 1995), but the results of this study indicate that it is unlikely to be a significant factor in modulating surfactant production.

Chapter 5. General discussion

As it is studied further, the complexity of the pulmonary surfactant system is revealed, both in terms of cellular interaction and regulation. One of the most perplexing mysteries of this system has been the identity of the fibroblast-pneumocyte factor (FPF), the fibroblast-expressed protein that triggers surfactant synthesis in type II pneumocytes. This study has provided further evidence that neuregulin-1 (NRG1), a growth factor found in many stages of development, is at least one of the components, if not the major component, of FPF. It also provided more insight into the surfactant system, and the method that is commonly used to study synthesis and secretion of surfactant.

This study used capillary HPLC coupled with quadrupole ion trap mass spectrometry to analyze fibroblast-conditioned medium (FCM) for FPF. Previous research (Dammann *et al.*, 2003) had indicated that NRG1 was possibly FPF, and that it possessed the same activity when exposed to type II pneumocyte cells, so analysis was focused on this protein. The concentration of NRG1 was significantly higher in FCM from fibroblast cultures treated with dexamethasone than control cultures. Importantly, FCM generated in the presence of dexamethasone, but which failed to stimulate PC synthesis in type II cells, was shown to contain a level of NRG1 comparable to controls. These findings strongly indicated that NRG1 is at the very least a major component of the response to FPF that has been observed in the past, if not FPF itself.

The finding that NRG1 is involved in this stage of the development of the surfactant system is not surprising, and indeed logical when neuregulin signaling is examined. Expression of NRG1 has been demonstrated in mesenchymal cells (Citri *et*

al., 2003), such as the fibroblast cells that express FPF. Receptors for NRG1, the ErbB receptors, have been observed in epithelial cells (Patel *et al.*, 2000), such as the type II pneumocytes in which FPF is known to stimulate surfactant synthesis. The timing of this stimulation also fits well with previous observations, as ErbB receptors have been shown to be present in the fetal lung at this time (Liu *et al.*, 2004). The findings of this study have shown that dexamethasone enhances expression of NRG1 by fibroblasts. Combination of all of this information allows a hypothetical picture of this system to be proposed.

Dexamethasone (and likely other glucocorticoids) acts on fibroblast cells through its receptor, leading to increased expression of a number of glucocorticoid-responsive genes. One of these genes is *nrg1*, which is expressed as NRG1, in a transmembrane isoform. This isoform is proteolysed to an unknown number of soluble forms, including at least a 44 kDa form (Dammann *et al.*, 2003) and the 7.5 kDa form isolated in this study. These soluble isoforms are secreted by the cell, via some form of exocytosis, where they interact with adjacent type II pneumocytes. These type II cells contain both ErbB2 and ErbB3 receptors (Liu *et al.*, 2004). NRG1 binds to ErbB3, which in turn binds to ErbB2 to form the ErbB2/ErbB3 heterodimer. This heterodimeric receptor then transduces its effects on the cell via a number of possible pathways. The most likely pathway for effect on surfactant synthesis (and possibly secretion) is activation of phospholipase C_γ (PLC). PLC increases hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol-1,4,5-trisphosphates which in turn regulates cellular levels of calcium (Ca^{2+}). These compounds regulate activity of protein kinase C (PKC) (Spitaler and Cantrell, 2004), which phosphorylates choline phosphate cytidylyltransferase (CPCT), the rate-limiting enzyme of surfactant synthesis. If this pathway, or something very similar,

exists in the fetal lung, then it is essentially what would be expected of FPF. This makes the case even stronger for NRG1 being FPF.

It is also possible that PKC acts on other enzymes, including those involved in surfactant secretion. This indicates a possible role for NRG1 in regulation of both synthesis of surfactant and its subsequent secretion into the alveolar space. FPF was originally proposed as a direct regulator of synthesis, but any role in secretion was not studied. If NRG1 is acting via a pathway similar to that described above, it is distinctly possible that it actually represents a regulator for the entire surfactant system. This would make it a very powerful compound and highlight its importance in the final stages of lung development around parturition. The fact that NRG1 is essential for lung maturation is certainly feasible given the vital roles for NRG1 in other developmental events, such as cardiac morphogenesis.

The proposed role for NRG1 as a 'super-regulator' of the surfactant system means that it could potentially be a very effective treatment for infants suffering from neonatal respiratory distress syndrome (NRDS). If NRG1 was used as a direct treatment, it would avoid the problems encountered with dihydrotestosterone, and possibly insulin, and the side-effects from current treatment procedures involving glucocorticoids. It would also provide a more rapid response, as it could act directly on the type II pneumocytes, as opposed to glucocorticoids which act on the fibroblasts to enhance expression of FPF. Treatment with NRG1 may also enhance secretion and possibly even have mitogenic effects on the lung tissues. Of course, care must be taken to establish if NRG1 is involved in other pathways at this specific stage of development, and if there exists the possibility for over-dose of NRG1 in these systems. The findings of this study provides a potential new option for treatment of NRDS, which warrant further exploration. There is currently no evidence as to whether NRG1 can cross the placenta, but it is known to be involved in signaling during implantation (Brown *et al.*,

2004). If it is able to cross the placenta, it may be able to administer NRG1 at the onset of premature labor, rather than post-partum, when NRDS may already have occurred.

Insulin-like growth factors (IGFs) are related to neuregulins in terms of function, so the effect of IGF-II on surfactant synthesis and secretion was also investigated. It was expected to have a similar effect to insulin, in that it would decrease synthesis (Gross *et al.*, 1980) and increase secretion (Snyder and Mendelson, 1987). These expectations were not met, with no significant effect being observed on either synthesis or secretion. The complexity of the IGF system means that there are a number of possible reasons for this lack of an effect.

The primary receptor for IGFs is IGFR-1, but this receptor is not found in fetal lung cells at the time of stimulation of surfactant synthesis (Maitre *et al.*, 1995). The secondary IGFR, IGFR-2, is thought to function primarily in binding IGF-II and removing it from circulation (Filson *et al.*, 1993). The most likely receptor for action of IGF-II in the fetal lung in late gestation is an isoform of the insulin receptor, known as IR-A. This receptor has a lower affinity for IGF-II than for insulin, which may explain why no effect was observed. It has also been found that growth-arrested type II pneumocyte cells increase expression of IGFR-2 and IGFBP-2, one of the IGF binding proteins (Mouhieddine *et al.*, 1994). The function of both of these molecules is to bind IGFs and remove them from circulation, implying that increased expression of these may have prevented IGF-II from having any significant effect. It is therefore likely that the combination of a lack of an appropriate receptor and a regulatory system that is different in culture to *in vivo* meant that IGF-II showed no significant effect on either synthesis or secretion of surfactant.

Analysis of FCM by mass spectrometry could not have been undertaken without a reliable method to generate FCM known to contain FPF. Early results from experiments to confirm the presence of FPF were very inconsistent. This led to a thorough examination of all of the steps of tissue culture and related experimental methods to determine which could be contributing to the unreliability of the experiments. Several parameters were identified, mostly related to the density of cultures or the addition of radioisotopes used in the experiments. This study led to the creation of a revised method, which was used for all subsequent experiments, including all those for which FCM was collected for analysis. This revised method can now be used for all future studies in this laboratory, and will reduce the variation that has been observed in the past.

The most significant effect on reliability was due to variations in the cell density of the type II pneumocyte cultures. This work, done in conjunction with an Honours student (Peh, 2006), found that cultures with a cell density above a threshold value of 25 $\mu g.mL^{-1}$ showed a mean decrease in phosphatidylcholine (PC) synthesis in response to dexamethasone-treated FCM, while those with density below the threshold showed a mean increase. When the results of experiments involving FCM generated in the presence of dexamethasone were separated on this basis, the mean indirect response to dexamethasone was elevated from a 9.17% to a 17.56% increase. Results for the PC synthesis experiments using IGF-II were also subdivided according to cell density, and response to IGF-II was higher for cultures below the threshold. The reasons for these density-related effects are not clear, although they are likely to be due to the nature of type II cell cultures. Type II pneumocyte cultures are generated from clumps of cells (Tajbakhsh and Cake, unpublished observations), and if these do not spread evenly across the plate, it is possible that not all cells will be exposed to the agonist. These cells will not contribute to synthesis, but will contribute to the level of DNA, causing an

apparent decrease in response. Fibroblast cell density affected results of some experiments, but much less so and not as consistently.

This section of the study also found that the application of the radioisotope-labeled precursor was not as consistent as previously thought, and that it could have been having a major effect on the variability of results. The erratic results of experiments using phorbol 12-myristate 13-acetate were due in part to inconsistent application of [^{3}H]-choline chloride. If insufficient precursor was available to cells, the response detected would have been reduced. In general, this part of the study served to highlight the sensitivity of the method used to even slight variations in some parameters, providing an understanding of why consistent results were initially difficult to obtain.

The findings of this study have provided more evidence that NRG1 is a major component of FPF. A study is currently in progress to examine the direct and indirect effect of recombinant NRG1 on synthesis and secretion of surfactant phospholipids. Fibroblast cells do not contain receptors for NRG1 at this stage of development (Patel *et al.*, 2000), so it is unlikely that treatment of fibroblast cells with NRG1 will yield a significant indirect response. It is more likely that the direct effect of NRG1 on type II cells will be significant, as this should be equivalent to exposing the cells to FPF. It is also possible that NRG1 will enhance surfactant secretion from type II cells, acting through Ca^{2+} and/or protein kinase C.

Further avenues for research also exist in studying NRG1 isoforms and the ErbB receptors. This study focused on only one of at least 16 different isoforms, and one other has already been observed in FCM (Dammann *et al.*, 2003). Different isoforms are known to interact differently with receptors (Kita *et al.*, 1995), so the presence of more than one form in FCM indicates the possibility of multiple roles for NRG1 in this

intercellular signaling pathway. The identification of all NRG1 isoforms that are involved in this system would be a major step, as would determining whether exposure of fibroblasts to different compounds (e.g. dexamethasone, EGF) leads to expression of different isoforms of NRG1. Localization of specific ErbB receptors may also be an important aspect of this signaling system. While ErbB2 and ErbB3 have been identified as present in these tissues at this stage of development, the cultures used should be analyzed to ensure that this expression pattern is consistent. It is possible that the conditions of cell culture led to modified receptor expression (as observed for IGF-II), and if ErbB4 is involved in this system, it could mean that subsequent signaling pathways are quite different. A full study of expression of both NRG1 and ErbB receptors by these cultures is important to provide a clear picture of the total involvement of NRG1 in regulation of the surfactant system.

As detailed in **3.4**, the use of a more powerful mass spectrometry technique, such as Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), would allow some of the limitations of ion trap MS to be overcome. Use of FT-ICR-MS would allow the majority of compounds present in FCM to be separated with sufficient resolution for positive identification (Marshall *et al.*, 1998). However, it must be considered that while a high-resolution technique such as FT-ICR-MS would certainly provide many answers, it is expensive. For this reason, further work on separation of the components of FCM, using a combination of HPLC and other separation/purification/fractionation techniques should be undertaken. If a reliable method can be developed, future work on these important secretions would be greatly advantaged.

As outlined in **2.11**, more work is also required on the tissue culture methodology used in this study. Primarily, a procedure for obtaining primary type II pneumocyte cultures with cell density consistently below 25 $\mu g.plate^{-1}$ is needed to

ensure that results of experiments are not affected by density. Considerable time has been spent on this problem, but a dedicated study has not been undertaken. All of the conditions of culture, from setup to maintenance, should be examined to determine if such consistency is attainable. Primary cultures are definitely required for fibroblasts, but it may be possible to use cell line cultures for type II pneumocytes. Results obtained from indirect experiments using primary and cell line type II cultures should be compared to assess whether cell line cultures could be used in future.

The role of FPF has been known for three decades, but its existence has remained a mystery. Confirmation of the identity of FPF represents a significant step in research into the surfactant system, and this study has contributed to that identification, making a strong case for NRG1. Research into the important role of FPF has dwindled in the last two decades, possibly because its unknown identity has meant that the work lacked direction. The findings of this study have provided a new direction for this research, allowing the focus to be shifted to NRG1 and its ErbB receptors. The complexity of the neuregulin system means that there is a considerable scope for future work, and many questions to answer before a more complete picture can be uncovered.

This study has provided a considerable amount of new information related to the surfactant system. It has examined the effects of insulin-like growth factor II and neuromedin C on surfactant synthesis and, in the case of IGF-II, surfactant secretion. A revised method has been developed for tissue culture and related experiments, which will provide future research with more reliable results. Most significantly, it has achieved its primary goal of confirming the identity of the fibroblast-pneumocyte factor as neuregulin-1, and has also provided more information on the role of neuregulin-1 in the pulmonary surfactant system.

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