ERRATA

p 3, section 1.3, line 10: "Fibulins" for "Fibulins"
p 6, 2nd para, line 6: "secretary" for "secretory"
p 7, section 1.3.4, line 5: "non polar" for "non-polar"
p 8, Fig 1.2: "hydrophobic" for "hydrophobic"
p 20, 2nd last line: "trans retinol" for "trans-retinol"
p 23, line 4: "down regulate" for "down-regulate"
p 41, line 4: "depariffinised" for "deparaffinised"
p 51, Fig 3.2, legend: "35 S" for "^{35}S"
p 70, Fig 4.2, legend, last line: "smooth muscle" for "smooth muscle layer"
p 112, 3rd line: "the difference the statistical" for "the difference in the statistical"

ADDENDUM

p 4, line 14: "true" for "precise"

p 11, Degradation and turnover of elastin in the lung, line 4: delete "(Shapiro et al., 1991)"

p 14, last line: "vast number" for "large number"

p 20, line 4-6: Insert reference "(Bassett & Thorburn, 1969)" after "The surge in fetal plasma cortisol concentrations that occur towards the end of gestation"

p 41, last line of para 1: "extrapolation" for "extrapolating"

p 42: Add at the end of para 1: "At least 2 tissue sections from different areas of the lung and at least 3 fields of view were examined per animal in each experimental group."

p 58, section 3.3, line 6: Insert reference "(Naguchi & Samaha 1991)" after "in rat lungs,"

p 60, 2nd para, line 4-14: Text altered to read:

"These changes in elastin content during development largely reflect changes in lung weight because elastin content is derived from elastin concentration (mg/g lung) multiplied by lung weight. Therefore the significant decrease in elastin content measured at 2 weeks of age may be a result of a small decrease in wet lung weight (due to decreased tissue water content) at this age (142d: 164.1 ± 12.9g, 2 wks: 121.7 ± 9.8g) rather than an actual decrease in elastin synthesis. The apparent decrease in wet lung weight at 2 weeks after birth is likely to be due to the lung's transition from a liquid-filled to an air-filled organ. Although fetal lungs were drained of lung liquid before being weighed, it is possible that some residual liquid remained within the

tissue and therefore contributed to lung weight, leading to an overestimation of fetal lung weight near term, compared with after birth; this accounts for the apparent decrease in lung weight at 2 weeks post-term. However, in this case, it would also be expected that the higher water content of fetal lungs would result in a lower elastin concentration at 142 days of gestation compared to 2 weeks postnatal age, but these values were not different. It is possible that the apparent discrepancy between the elastin concentration and elastin content could be a result of a greater loss of liquid from fetal lung tissue during the dissection procedure compared to that in postnatal lungs."

p 94, line 3: "gravimetrically" for "by gravity"

p 95, In situ hybridisation, line 7-8: "tropoelastin expression was increased both in the number expression sites expression and the amount of expression at each site as determined by silver grain density." for "tropoelastin expression was increased both in the number of expression sites and the amount of expression at each site as determined by qualitative visual inspection."

p 132, line 2: "absent" for "an absence of"

p 133, 2nd para, line 11: "at the tips of secondary septa." for "at the tips of secondary septa as in control animals"

Elastin synthesis in the fetal sheep lung in vivo: effects of physical, metabolic and endocrine factors

Belinda Jane Joyce

Bachelor of Science (Honours)

A thesis submitted for fulfillment of the requirements of the degree of Doctor of Philosophy

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January 2004

For my Nanna

Vera May Reynolds
November 1916 - December 2003

You were so brave

I wish you could've seen this

I love you forever

♥

Table of Contents

Summary

Lung growth and maturation in the later stages of fetal development are critical for postnatal survival and lung function. Adverse prenatal conditions resulting in altered lung growth, maturation and structure, or a shortened period of *in utero* development such as in pre-term birth, are major causes of neonatal morbidity and mortality, and can affect lung health into adulthood. One of the main features of lung maturation before birth in precocial species such as humans is the initiation of alveolar development and the structural maturation of the parenchymal region of the lung. Factors such as lung tissue stretch (mediated by the degree of fetal lung expansion), corticosteroids and adverse prenatal conditions resulting in low birth weight have all been shown to have an influence on lung structure and alveolar number. Because elastin synthesis is required for alveolar formation, the aim of experiments reported in this thesis was to determine the influence of the above factors on lung elastin synthesis and deposition *in vivo*.

In experiments described in this thesis, sheep were used as the ovine fetal environment can be easily manipulated, fetal physiological measurements can be made and sheep are a long gestation species in which alveolar formation begins before birth as in humans. There have been no previous studies of the temporal or spatial pattern of tropoelastin expression in the lung of fetal and postnatal sheep *in vivo*. In particular, there have been no previous studies of elastin expression from fetal to adult life in a long-gestation, long-lived species. Thus the first study reported in this thesis provides an overview of the ontogeny of elastin synthesis and content in the ovine lung *in vivo* between the canalicular period of lung development in late gestation and at maturity. An ontogenic assessment of elastin synthesis from tropoelastin expression to mature elastin deposition was performed to provide a foundation for subsequent studies in this thesis. Consistent

with the developmental pattern of elastin synthesis in rat lungs, the peak in elastin synthesis in the parenchyma of sheep lungs was found to be associated with alveolar development.

Sustained alterations in fetal lung expansion induced by changes in lung liquid volume profoundly affect lung growth and the structural development of the lung, including alveolar development. The synthesis of elastin in the developing lung is very closely associated with alveolar development and therefore it was hypothesised that alterations in fetal lung expansion would alter elastin synthesis in accordance with changes in alveolar development previously observed. The experiments described in this thesis have shown that sustained alterations in the basal degree of lung expansion in fetal sheep *in vivo*, induced by either tracheal obstruction or lung liquid drainage, cause alterations in both tropoelastin expression and elastin deposition in lung parenchymal tissue consistent with changes in alveolar development. Sustained increases in the level of fetal lung expansion resulted in a transient increase in tropoelastin mRNA levels whereas a prolonged decrease in the level of fetal lung expansion resulted in a profound reduction in tropoelastin mRNA levels. Elastin accumulation, however, as demonstrated by changes in elastin concentration and content in lung parenchymal tissue, did not appear to alter in proportion to the changes in fetal lung growth. However, an important finding of the study was the influence of fetal lung expansion on the manner in which elastin was deposited in the lung parenchyma. It was found that an adequate degree of fetal lung expansion is essential for the correct laying down of elastic fibres in lung parenchymal tissue, and hence normal alveolar development.

It is well established that both endogenous and exogenous corticosteroids have a major influence on fetal lung maturation, particularly on the structural maturation of the lung. However, information relating to the influence of corticosteroids on elastin synthesis during lung development is scant and difficult to interpret. Since alveolar development has been shown to increase in response to physiological levels of cortisol during fetal development, it was hypothesised that elastin synthesis would also be stimulated. In addition, the inability of physiological levels of cortisol to restore alveolar numbers in under-expanded fetal lungs led to a second hypothesis, that cortisol would be unable to counteract the detrimental effects of a decreased level of fetal lung expansion on elastin deposition and alveolar development. In agreement with these two hypotheses, it was found that physiological levels of fetal plasma cortisol increase tropoelastin mRNA

levels and elastin deposition consistent with changes in alveolar development in normally expanded lungs and that fetal lung under-expansion appears to override the influence of cortisol.

Intra-uterine growth restriction in sheep induced by placental insufficiency late in gestation (a critical period for elastin synthesis), results in persistent impairments in lung structure and function consistent with alterations in elastin synthesis. In addition, factors associated with IUGR such as hypoxia and undernutrition individually have been shown to affect elastin synthesis. Therefore, it was hypothesised that elastin synthesis would be altered as a result of IUGR. In this study however, factors associated with IUGR *in vivo* did not affect elastin synthesis or deposition in the fetal, early postnatal or adult sheep lung and therefore could not explain the changes in lung structure and function observed in related studies.

The studies reported in this thesis highlight the importance of the prenatal environment in the development of the lung before birth in relation to elastin deposition and alveolar development. In particular the physical environment in which the lungs develop *in vivo* has a major influence on lung development, elastin deposition and alveolar formation. These studies also highlight the implications of an altered prenatal environment on lung health from birth into adulthood and provide valuable information regarding alveolar development in normal and pathological conditions.

Declaration

I the undersigned, declare that to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text. No part of this thesis has been submitted for examination for any other degree or diploma at Monash University or any other university or institution.

[redacted]

Belinda J. Joyce

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This journey has been one of the most fulfilling and valuable experiences of my life which I could not have achieved without the support of all of these people.

Publications

Publications and abstracts arising from studies presented in this thesis

Publications

JOYCE, B. J., M. J. WALLACE, R. HARDING & S. B. HOOPER (2003). Sustained changes in lung expansion alter tropoelastin mRNA levels and elastic content in fetal sheep lungs. *Am J Physiol: Lung Cell Mol Physiol* 284(4): L643-L649.

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Publications

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Abbreviations

^{14}C	Radioactive carbon
^{32}P	Radioactive phosphate
^{35}S	Radioactive sulphur
A	Adenosine
Amp_r	Ampicillin resistance gene
ANOVA	Analysis of variance
APS	Ammonium persulphate
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
bp	Base pair
BPD	Bronchopulmonary dysplasia
C	Cytosine
CaO_2	Arterial oxygen content
cDNA	Complimentary deoxyribonucleic acid
Cl	Chloride
cmH_2O	Centimetres of water
cpm	Counts per minute
d	Day(s)
dCTP	Deoxycytidinetriphosphate
ddATP	Dideoxyadenosinetriphosphate
ddGTP	Dideoxyguanosinetriphosphate
ddTTP	Dideoxythymidinetriphosphate
ddUTP	Dideoxyuridinetriphosphate
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidetriphosphate
DTT	Dithiothreitol
EBP	Elastin binding protein
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra acetic acid
EGF	Epidermal growth factor
EMILIN	Elastin microfibril interface located protein
ETOH	Ethanol

fib-1	Fibrillin-1 gene
g	Grams / Force
G	Guanine
GA	Gestational age
H_2O	Water
hr	Hour
i.m.	Intramuscular
i.v.	Intravenous
IGF	Insulin-like growth factor
IPTG	Isopropylthio-β-D-galactoside
IUGR	Intrauterine growth restriction
K_2HPO_4	Dipotassium hydrogen phosphate
KCL	Potassium chloride
kDa	Kilo Daltons
kg	Kilogram(s)
KH_2PO_4	Potassium dihydrogen phosphate
LB	Luria-Bertani medium
LTBP	Latent transforming growth factor-beta binding protein
M	Molar
MAGP	Microfibril-associated glycoprotein
mg	Milligrams
$MgCl_2$	Magnesium chloride
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre
mM	Millimolar
MOPS	3-(N-Morpholino) propanesulfonic acid
MQ H_2O	MilliQ reverse osmosis purified water
mRNA	Messenger ribonucleic acid
n	Number / sample size
N_2O	Nitrogen
NaCl	Sodium chloride
$NaH_2PO_4.H_2O$	Sodium dihydrogen orthophosphate
NaOH	Sodium hydroxide
ng	Nanogram(s)
NIH	National Institute of Health
nm	Nanometres
O_2	Oxygen
OD	Optical density
p	p-value; level of significance
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PM	Postmortem
pmol	Picomoles
PNA	Postnatal age
RDS	Respiratory distress syndrome
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
s	Second(s)

s.c.	Subcutaneous
SaO_2	Arterial oxygen saturation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SSC	Saline-sodium citrate
SSPE	Saline-sodium phosphate EDTA
T	Thymine
TAE	Tris-acetate EDTA
TE	Tris EDTA
TGF-β	Transforming growth factor-beta
T_m	Melting temperature
TNF-α	Tumor necrosis factor-alpha
TO	Tracheal obstruction
TPE	Tropoelastin
U	Uracil
UPE	Umbilico-placental embolisation
UV	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume
wk	Week(s)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
yr	Year(s)
µg	Micrograms
µl	Microlitre
µm	Micrometres

Chapter 1

Literature review

1.1 Introduction

The successful transition from intra- to extra-uterine life is largely dependent upon the lung developing into an efficient gas exchange organ by the time of birth. For this to occur, the lung must have developed a sufficiently large surface area to sustain gas exchange (via the development of thin-walled alveoli) and must be both structurally and biochemically mature. One important feature of this process is the synthesis and deposition of pulmonary elastin. Elastin is a unique protein that is a major component of the lung extracellular matrix and is concentrated around the entrance of individual alveoli as well as in the walls of airways and blood vessels (Mecham, 1997). The physical properties of elastin allow the expansion and recoil of the lung that are essential to its mechanical performance. In the developing lung, elastin deposition occurs at the apex of secondary septal crests during the process of alveolarisation (Fukuda et al., 1983) and prior to alveogenesis, occurs in the mesenchyme surrounding the developing distal airways (Wendel et al., 2000). Evidence indicating that the synthesis and deposition of elastin plays an essential role in the structural development of the fetal lung is provided by the finding that a reduction or abolition of elastin expression impairs distal airway development (Wendel et al., 2000) and alveolar formation (Lindahl et al., 1997). The synthesis of elastin in the lung of mammalian species is confined to the late fetal and early neonatal periods of development (Anceschi et al., 1992; Mariani et al., 1997; Noguchi & Samaha, 1991; Shibahara et al., 1981) and turnover is minimal in the healthy adult lung, (Rucker & Dubick, 1984; Shapiro et al., 1991). Thus, the elastin laid down during this critical period of lung development must

support lung function for the normal lifespan of the organism. As a result, perturbations in lung development affecting elastin synthesis at this time could affect respiratory function for many years after birth.

The synthesis of elastin in the lung and other tissues can be regulated by a number of factors such as hypoxia (Berk et al., 1999) and undernutrition (Kalenga & Henquin, 1987; Sahebjami & MacGee, 1985) (both of which are commonly associated with intra-uterine growth restriction), as well as corticosteroids (Pierce et al., 1995) and mechanical stretch (Nakamura et al., 2000). However, little is known of the influence of these factors on elastin synthesis and deposition in the developing lung *in vivo*. The experiments performed in this thesis are therefore aimed at providing a better understanding of the regulation of elastin synthesis during normal lung development as well as in a sub-optimal intra-uterine environment such as that associated with intra-uterine growth restriction, using the ovine lung as a model.

1.2 The lung extracellular matrix

The extracellular matrix (ECM) of the lung is a network of collagens, elastin, glycoproteins and proteoglycans that form the structural framework of the lung, compartmentalize tissues, and regulate cell shape and behaviour via cell-matrix interactions (Chambers & Laurent, 1996). Collagen and elastin are the major constituents of the lung ECM and provide the structural support and physical properties essential to lung function. The collagens, of which there are at least 11 different types known to be present in the lung, confer tensile strength to lung tissue and provide a physical limit to lung tissue expansion while also allowing the flexibility and distensibility that are essential to lung mechanics (Chambers & Laurent, 1997). Elastin, the second of the major structural components of the lung ECM, provides the vasculature, conducting airways and alveoli with an elastic architecture that allows the lung to undergo repeated deformation and recoil and is also critical to lung structure and function (Mecham, 1997). While the role of collagen and elastin in determining lung structure and function is well established, the development of the lung ECM and the interrelationships between each of the components and lung cells also play an important role in lung development. Elastin in particular has been shown to be critical in airway branching (Wendel et al., 2000) and alveolar development (Bostrom et al., 1996; Lindahl et al., 1997).

Turnover of the majority of lung ECM components is rapid, allowing tissue remodelling during development following lung injury and throughout the aging process. Elastin however, is unique in that it is an extremely insoluble protein, with an extremely long physiological half-life and once laid down, turnover of mature cross-linked elastin is minimal (Rucker & Dubick, 1984; Shapiro et al., 1991). This coupled with its critical role in lung structure and function and its limited period of expression during fetal and neonatal development make it a particularly important protein in relation to the regulation of its synthesis and deposition during this critical period of lung development. In addition, little is known about the control of elastin development in the lung. Therefore, in this thesis, I have chosen to focus on elastin and factors that regulate its synthesis during lung development.

1.3 Elastic fibres

The elastic fibre is a complex structure comprised of two morphologically distinct components, the microfibrillar component and amorphous appearing elastin. "Elastin" is used to describe the amorphous component of elastic fibres and makes up more than 90% of the mature elastic fibre (Starcher, 1986). Also in association with elastic fibres is the enzyme lysyl oxidase, a copper-dependant enzyme required for cross-linking the soluble precursor protein, tropoelastin, to form mature insoluble elastin. Numerous other proteins have been found to be associated with elastic fibres, however, little is known of their role in elastic fibre synthesis and function. These are generally families of proteins which include the microfibril-associated glycoproteins (MAGPs) (Gibson et al., 1996; Gibson et al., 1991), Fibulins (Nakamura et al., 2002; Roark et al., 1995; Yanagisawa et al., 2002), latent transforming growth factor beta (TGF-β)-binding proteins (LTBPs) (Oklu & Hesketh, 2000), EMILINs (elastin microfibril interface located proteins) (Colombatti et al., 2000; Doliana et al., 2001; Doliana et al., 1999) and elastin binding protein (EBP)(Hinek & Rabinovitch, 1994).

1.3.1 Microfibrils

Elastin associated microfibrils form a component of elastic fibres that is both morphologically and biochemically distinct from amorphous appearing elastin. These microfibrils are 10- to 12- nm fibril structures and were first described as forming a sheath surrounding a core of amorphous elastin (Gross, 1949). During development,

microfibrils can be observed before the onset of tropoelastin production and can also be seen within mature elastic fibres. This observation has led to the popular hypothesis that microfibrils act as a scaffold for elastic fibre formation. However, microfibrils can also exist in the absence of an elastic component in non-elastic tissues such as the ciliary zonule of the eye (Streeten et al., 1981), which suggests a functional role of microfibrils independent of elastic fibre assembly. Furthermore, microfibrils also appear to have some endogenous elasticity (McConnell et al., 1996) and in evolution preceded appearance of the tropoelastin gene (Reber-Muller et al., 1995); therefore, it has been suggested that microfibrils may play a role in tissue elasticity as well as in elastogenesis. The main structural components of microfibrils are the glycoproteins fibrillin and microfibril-associated glycoprotein (MAGP). Recent advances in the characterisation of the major component of the microfibrils, the fibrillins, genetic mutations of these genes and gene targeting experiments are beginning to shed more light on the true function of elastin associated microfibrils.

Fibrillins

The fibrillins consist of two large 350-kDa glycoproteins fibrillin-1 and fibrillin-2 (Ramirez & Pereira, 1999). The fibrillins are mostly made up of repeating cysteine-rich domains homologous to those found in epidermal growth factor (EGF) and the latent TGF-β-binding proteins (LTBPs) (Ramirez & Pereira, 1999). Most EGF motifs contain the consensus sequences for calcium binding which appears to be important for stability of the microfibrillar structure (Kielty & Shuttleworth, 1995). The structures of the two fibrillin proteins are virtually identical with the only major difference between the two being the replacement of a proline-rich region in fibrillin-1 with a glycine-rich region in fibrillin-2 (Kielty & Shuttleworth, 1995). Both proteins also contain an identical RGD sequence that binds to αvβ3 integrin, and fibrillin-2 contains an additional RGD sequence that is not available for cell-matrix interaction (Ramirez & Pereira, 1999).

Fibrillin-1 and -2 are encoded by separate genes which are differentially expressed in relation to both developmental stage and tissue distribution (Zhang et al., 1995). Developmentally, fibrillin-1 appears to be expressed during late morphogenesis, whereas fibrillin-2 expression coincides with early morphogenesis and the beginning of elastogenesis (Zhang et al., 1995). Therefore it has been hypothesised that fibrillin-2 guides elastogenesis while fibrillin-1 provides force bearing structural support (Ramirez

& Pereira, 1999). This is supported by targeting of the gene encoding fibrillin-1 (Pereira et al., 1997), and manifestations of gene mutations of the fibrillin-1 and -2 genes that result in Marfan's syndrome (Dietz et al., 1991) and the related disease congenital contractural arachnadactyly (Putnam et al., 1995) respectively. Furthermore, studies in embryonic rat lung indicate that developmental expression of fibrillin-2 parallels that of tropoelastin, and fibrillin-2 also appears to be involved in branching morphogenesis of the lung (Yang et al., 1999).

Microfibril-Associated Glycoprotein (MAGP)

While there are numerous proteins that have been associated with microfibrils and elastic fibres in general, it is unclear for most of these proteins whether they are integral components of the elastic fibre structure, or loosely associated with the elastic fibres in the ECM. The MAGPs have however been localised to isolated microfibrils, and bind in a periodic manner down the length of the microfibril (Kielty & Shuttleworth, 1997). Therefore, these proteins may be directly involved in microfibrillar structure. The MAGPs are small glycoproteins of which there have been at least two identified MAGP-1 and MAGP-2. Protein interaction studies of MAGP-1 and MAGP-2 indicate that MAGPs interact with the fibrillins (Jensen et al., 2001; Penner et al., 2002) and tropoelastin (Jensen et al., 2001) suggesting a possible role for MAGPs in elastic fibre assembly.

1.3.2 Lysyl oxidase

Lysyl oxidase is a copper dependant enzyme that is immunolocalised to the interface between amorphous elastin and microfibrils (Parks et al., 1993). The function of lysyl oxidase is to facilitate cross-link formation between individual tropoelastin monomers to form mature insoluble elastin (Vrhovski & Weiss, 1998). Elastin cross-link formation is a critical process in elastogenesis, with copper deficiency and reduced lysyl oxidase activity resulting in connective tissue defects and emphysematous lung structure (Kida & Thurlbeck, 1980; O'Dell, 1981).

1.3.3 Other proteins associated with elastic fibres

The number of proteins identified as having an association with elastic fibres is increasing but the function of many of these proteins remains unclear. Recent gene

targeting studies in mice, however, have shed some light on the function of one such protein, Fibulin-5, also known as DANCE (Nakamura et al., 2002; Yanagisawa et al., 2002). These studies showed that disruption of the Fibuiln-5 gene results in disorganisation of elastic fibres resulting in loose skin, vascular abnormalities and emphysematous lung. Fibulin-5 was also shown to interact directly with elastic fibres and serve as a ligand for cell surface integrins, thus its function may be to act as a scaffold protein for anchoring elastic fibres to cells, in order to stabilise and organise elastic fibres in elastin rich tissues such as the lung (Nakamura et al., 2002; Yanagisawa et al., 2002).

Elastin binding protein (EBP) is another protein which is not directly involved in elastic fibre structure but appears to play an important role in elastic fibre assembly. EBP is colocalised with tropoelastin intracellularly and at the cell surface of elastin producing cells, as well as with mature elastic fibres in the ECM (Hinek et al., 1995). The proposed function of EBP is to bind tropoelastin and act as a chaperone, escorting it through the secretary pathways to the microfibrils in the ECM where it is cross-linked and incorporated into elastic fibres (Hinek & Rabinovitch, 1994). This process serves to protect tropoelastin from premature self aggregation (Vrhovski & Weiss, 1998) and proteolytic degradation (Hinek & Rabinovitch, 1994). Recycling of EBP back into elastin producing cells to bind to new tropoelastin molecules has also been shown to be an essential part of this process (Hinek et al., 1995). In addition to the role of EBP in tropoelastin secretion, it is likely that EBP in association with two other membrane bound proteins plays a direct role in elastic fibre assembly. EBP which is identical to an alternatively spliced form of β-galactosidase (Privitera et al., 1998) has binding sites for tropoelastin and lectin (Hinek et al., 1993) and forms a complex with two membrane bound proteins (Mecham et al., 1989). Binding of a galactosugar or a glycosaminoglycan to the lectin binding site, reduces the affinity of EBP for tropoelastin and the membrane bound complex and simultaneously causes release of tropoelastin from EBP and EBP from the membrane bound proteins (Mecham et al., 1991)(Figure 1.1). It has been proposed that the lectin binding site interacts with the highly glycosylated microfibrils hence resulting in the release of tropoelastin directly onto the surface of microfibrils for incorporation into elastic fibres (Hinek et al., 1988).

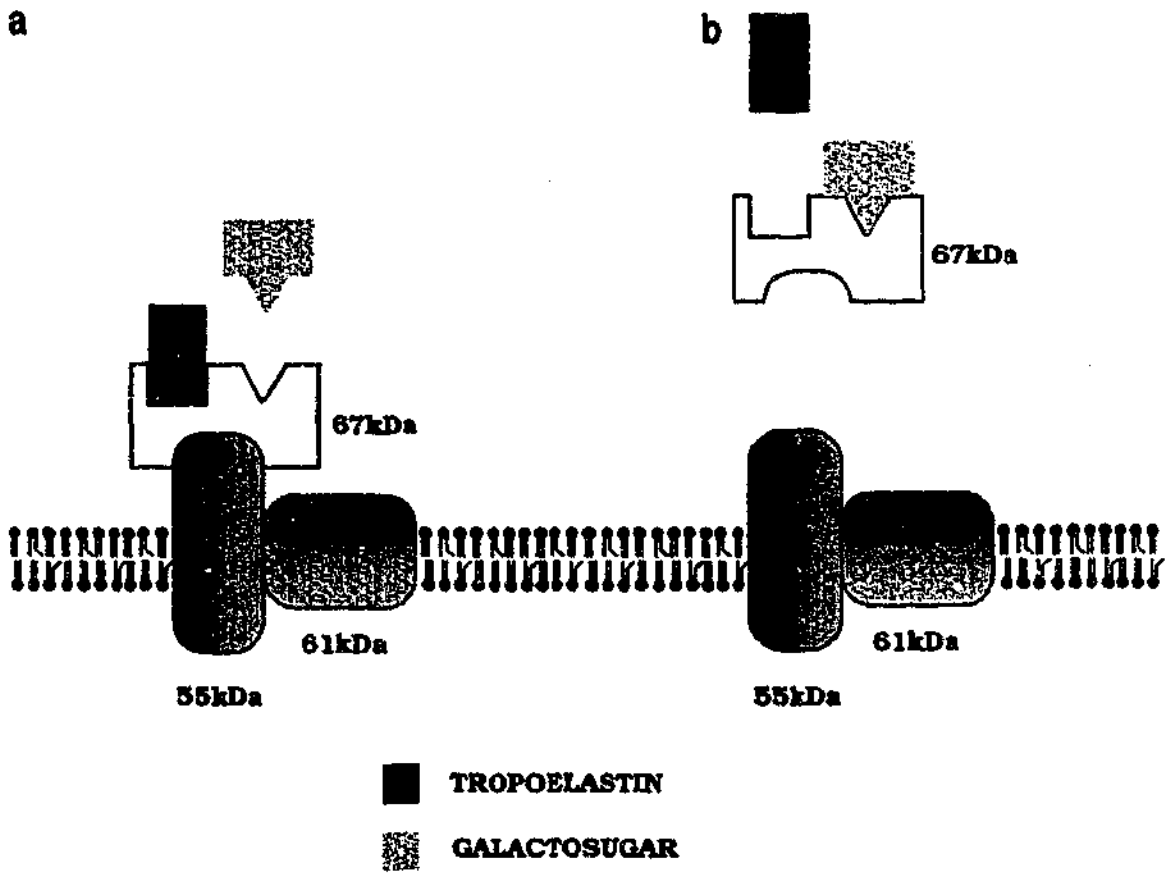


Figure 1.1 Model of the elastin binding protein (EBP) receptor complex
(a) The 67kDa protein EBP binds to a membrane bound complex consisting of two proteins (55kDa and 61kDa) and tropoelastin. (b) Upon binding of a galactosugar to the lectin binding site of EBP, its affinity for both tropoelastin and the membrane complex is lost resulting in simultaneous release of tropoelastin from EBP and EBP from the membrane bound proteins (Vrhovski & Weiss, 1998).

1.3.4 Tropoelastin

Tropoelastin is the soluble precursor protein of elastin and its sequence is highly conserved among mammalian species (Vrhovski & Weiss, 1998). Tropoelastin has a highly modular structure made up of alternating hydrophobic domains and hydrophilic cross-linking domains (Vrhovski & Weiss, 1998). The hydrophobic domains are rich in non polar amino acids such as glycine, valine, proline and alanine (Vrhovski & Weiss, 1998), and the cross-linking domains usually consist of stretches of alanine separated by two or three lysine residues (Mariani et al., 1997). The hydrophilic cross-linking domains are strictly conserved, particularly in relation to the position of lysine residues, which are always separated by precisely two or three alanine residues (Mariani &

Pierce, 1999). The hydrophobic domains on the other hand are less conserved in sequence, but the hydrophobicity of each domain is retained (Mariani & Pierce, 1999). Hydrophobic amino acids make up 90% of the tropoelastin sequence (Mariani & Pierce, 1999), indicating that the hydrophobicity of elastin is highly significant to its functional properties. Another important domain is a highly basic domain found at the C-terminus of tropoelastin that contains the only two cysteine residues in the protein and is highly conserved among species (Vrhovski & Weiss, 1998). *In vitro* studies indicate that this domain mediates interactions between tropoelastin and MAGP and therefore may be involved in elastic fibre assembly (Brown-Augsburger et al., 1994).

Tropoelastin is encoded by a single gene in all mammalian species studied, including sheep (Olliver et al., 1987). Structurally, the elastin gene consists of separate alternating exons that encode the hydrophobic and cross-linking domains of the protein (Mariani & Pierce, 1999). Alternative splicing of the elastin gene results in multiple closely related isoforms of tropoelastin of 60-70-kDa which appear to be developmentally regulated in some species such as rats, cows and chicks (Vrhovski & Weiss, 1998). Some tissue specificity of the different tropoelastin isoforms has been noted in rats however, tissue specificity in other species has not been clearly demonstrated (Vrhovski & Weiss, 1998). Thus the functional significance of the different tropoelastin isoforms in relation to elastic fibre structure and function remains unclear. Figure 1.2 below shows a diagrammatic representation of the typical structure of the tropoelastin cDNA coding sequence.

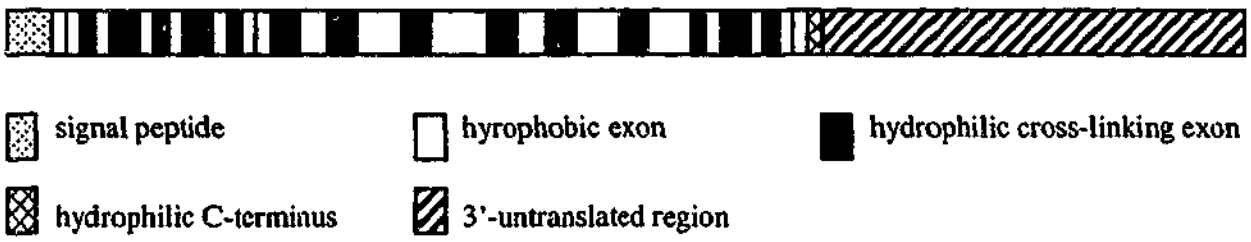


Figure 1.2 Typical structure of the tropoelastin cDNA coding sequence
Diagrammatic representation of the typical structure of the tropoelastin mRNA coding sequence showing the alternating exons that encode for hydrophobic and hydrophilic cross-linking regions of the tropoelastin protein (adapted from (Vrhovski & Weiss, 1998)).

Elastin cross-links

Cross-link formation between individual tropoelastin monomers by the enzyme lysyl oxidase results in mature insoluble elastin. Inter- and intra-chain covalent cross-links are formed by lysine residues in the cross-link domains of the tropoelastin protein (Parks et al., 1993). The two main cross-links formed are desmosine and isodesmosine which are unique to elastin and can therefore be used as markers for the presence and quantification of elastin. The first step in cross-link formation is the enzymatic deamination of lysine residues by lysyl oxidase to produce allysine, followed by the spontaneous condensation with lysine and allysine that results in formation of the cross-links desmosine and isodesmosine that are unique to elastin (Vrhovski & Weiss, 1998). Elastin cross-link formation is shown in Figure 1.3 below.

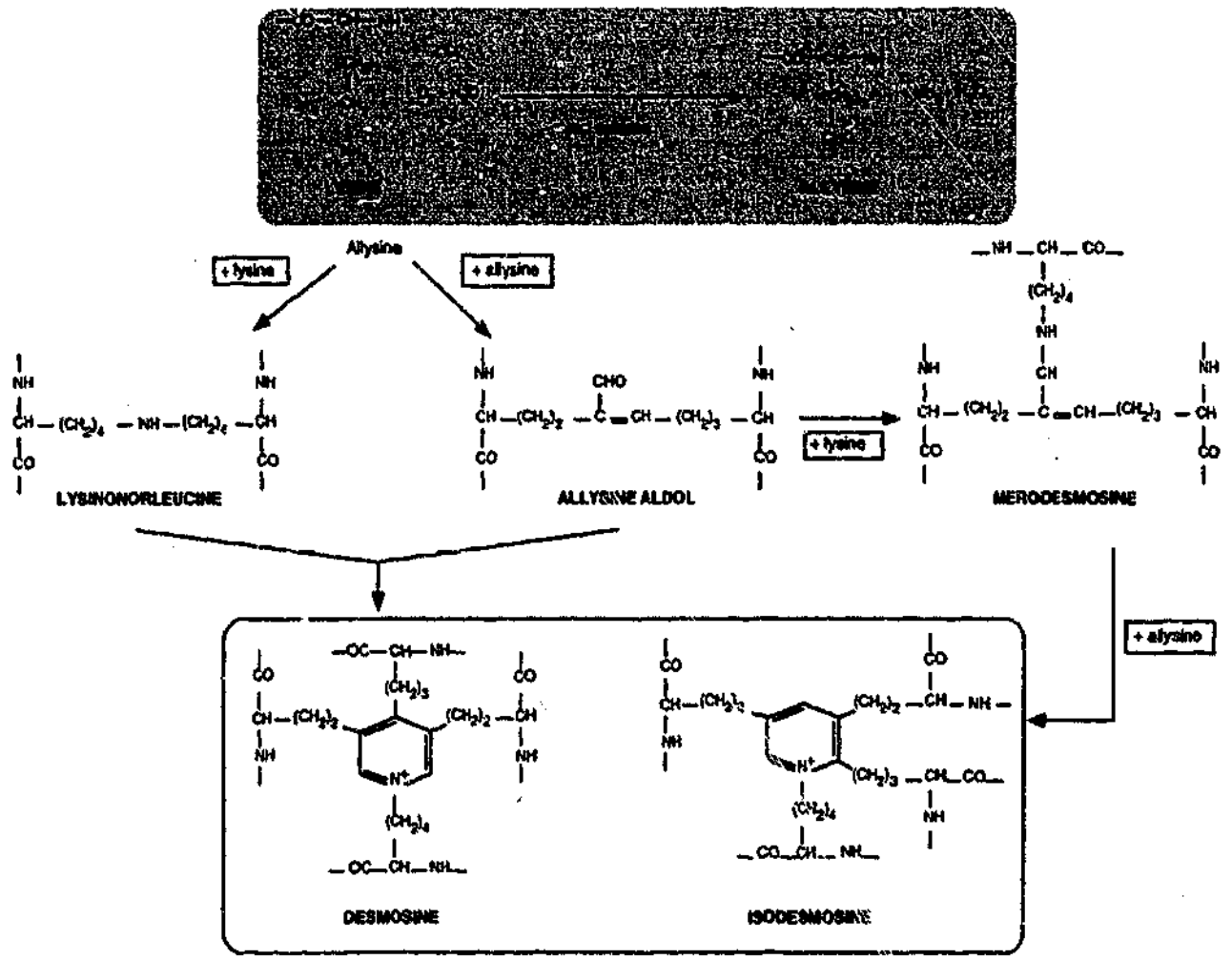


Figure 1.3 Elastin cross-link formation
Formation of elastin cross-links begins with the reaction catalysed by lysyl oxidase (shaded box) followed by subsequent spontaneous condensations between lysine and allysine to produce further cross-links including desmosine and isodesmosine (Vrhovski & Weiss, 1998).

1.3.5 Elastin

Elastin is the predominant component of mature elastic fibres and in cross-link formation is extremely hydrophobic and insoluble. Elastin is stable at extreme pH, temperature and pressure conditions and has been defined as the insoluble protein remaining after all other components have been solubilised. The unique properties of elastin, in particular the extensive covalent cross-linking, have made structural and chemical studies of the mature protein difficult. Thus the relationship between elastin structure and its functional elastic properties are still not well understood. A number of models to explain the mechanism of its elasticity have been proposed, with hydrophobic interactions a common theme in each model (Vrhovski & Weiss, 1998). The extreme hydrophobicity of elastin and the observation that it is only elastic when swollen in water suggest that hydrophobic interactions play a major role in the mechanism of elasticity of the protein (Gosline, 1978). It has been proposed that when elastin is stretched, there is an increase in the hydration of hydrophobic regions followed by exclusion of water molecules upon recoil to restore elastic fibres to an increased state of entropy (Gosline, 1978). Whatever the mechanism, the unique functional properties of elastin are crucial to lung structure and function.

Elastin and postnatal lung structure and function

The role of elastin in the maintenance of lung structure and function in the postnatal lung has been demonstrated by the experimental destruction of elastin by intra-tracheal instillation of proteolytic enzymes such as elastase and papain into mature rat lungs (Johanson & Pierce, 1972). Johanson and Pierce showed that the introduction of elastase and papain into rat lungs *in vitro* resulted in increased lung compliance, decreased lung elastic recoil and a marked distortion of pulmonary architecture (Johanson & Pierce, 1972). Focal regions of the lungs showed enlargement of airspaces with fewer alveoli and elastin staining in these areas was either absent or abnormal (Johanson & Pierce, 1972). While the proteolytic activity of the enzymes elastase and papain are not specific for elastin, regional alterations in lung structure corresponded with regional alterations in elastin staining and morphology and no change was observed in the staining or morphology of collagen fibres. Furthermore, collagenase did not alter lung structure or function in this study (Johanson & Pierce, 1972). Therefore, the experimental destruction of elastin using proteolytic enzymes such as elastase gives a good indication

of the importance of elastin for the structure and function of the lung. These effects however are confined only to the amorphous component of elastic fibres as elastases do not degrade the microfibrillar component.

Similar lung structural lesions and functional deficits can be observed clinically in human pulmonary emphysema. With the discovery of an association between deficiencies of the elastase inhibitor α_1-antitrypsin and emphysema (Eriksson, 1965), it became apparent that the destruction of elastic fibres may be involved in the pathogenesis of pulmonary emphysema. Abnormal elastic fibre morphology has also been observed in human emphysematous lungs (Fukuda et al., 1989). Interestingly, although emphysema appears to be associated with the destruction of elastic fibres in the lung, elastin content in emphysematous human lungs at autopsy has been shown to be normal (Pierce et al., 1961). This is likely to be due to the re-initiation of elastin synthesis that occurs following proteolytic destruction of elastic fibres (Kuhn et al., 1976). However, despite the restoration of normal elastin content, elastic fibre morphology and lung structure remain altered (Kuhn et al., 1976), indicating that elastogenesis does not occur in a normal manner following proteolytic lung injury in the mature lung. These data highlight the importance of a normal elastic fibre structural network in the maintenance of lung structure and function as well as the importance normal elastogenesis for the deposition of functional elastic fibres.

Degradation and turnover of elastin in the lung

Elastin is one of the most durable substances in the body and in the healthy adult lung, turnover of elastin is minimal. Shapiro *et al.* (1991) measured the elapsed time since synthesis of mature elastin in healthy human lung and found that the age of lung parenchymal elastin corresponded with the age of the subject (Shapiro et al., 1991). Animal studies have also shown by ^{14}C-labelling of lysine that the turnover of elastin in the lung is best estimated in years (Rucker & Dubick, 1984). These data indicate that in the healthy lung, the elastin synthesised during development supports the lung for the entire life of the organism. This point is of critical importance in conditions where elastin synthesis and deposition may be altered during development or in disease states such as emphysema where elastin is degraded and re-synthesis of elastin does not occur in a normal functional manner (Fukuda et al., 1989; Kuhn et al., 1976).

While elastin degradation and re-synthesis is negligible in the healthy adult lung, as mentioned in the previous section elastin re-synthesis can occur following proteolytic elastin degradation in animal models of emphysema (Kuhn et al., 1976) and pulmonary fibrosis (Mariani et al., 1995), but elastogenesis in these situations results in aberrant elastic fibre formation. One exception, however, is the elastogenesis that occurs following postpneumonectomy lung growth in adult rat lungs which apparently results in a normal pattern of elastogenesis and elastic fibre morphology (Koh et al., 1996). The reason for the ability of the lung to re-initiate normal elastogenesis in this unique situation is unknown but may involve the re-initiation of gene expression and synthesis of multiple elastic fibre proteins that may not occur as a result of lung injury.

1.3.6 Elastic fibre formation

As described earlier, there are numerous proteins that are either involved directly in elastic fibre structure or in its formation, however, relatively little is known of how each of these proteins interact in a coordinated manner to form mature functional elastic fibres. Studies relating to microfibrillar proteins and elastin binding protein (EBP) indicate that interactions between these proteins and tropoelastin are likely to be important for elastic fibre assembly. Disruption of the genes for both fibrillins and fibulin-5 has also been shown to result in aberrant elastic fibre formation. These studies highlight the importance of future studies related to the understanding of the complicated process of elastogenesis, and the role of elastic fibre proteins other than elastin in lung development and repair. Also, the identification of factors that regulate multiple elastic fibre proteins in a coordinated manner will be important for the understanding of normal elastogenesis in the lung and why this process does not occur normally following lung injury or alterations on fetal pulmonary development.

1.3.7 Distribution of elastic fibres in the lung

Elastic fibres have a wide distribution throughout the lung and can be found in pulmonary arteries, airways, pleura and alveolar parenchyma. In pulmonary arteries, elastic fibres are arranged in concentric sheets (lamellae) perpendicular to blood flow. Fine elastic fibres can be found in the lamina propria of airways which is located between the epithelium and smooth muscle layer and the visceral pleura that lines the outer surface of the lung also contains a layer of fibrous supporting tissue containing

elastic fibres. However, the greatest percentage of elastic fibres in the lung is found in the parenchyma (Foster et al., 1989). In the parenchyma, elastic fibres are concentrated in a ring surrounding the entrance of individual alveoli which in cross section can be seen as concentrated deposits at the tips of alveolar septal walls (Figure 1.4).

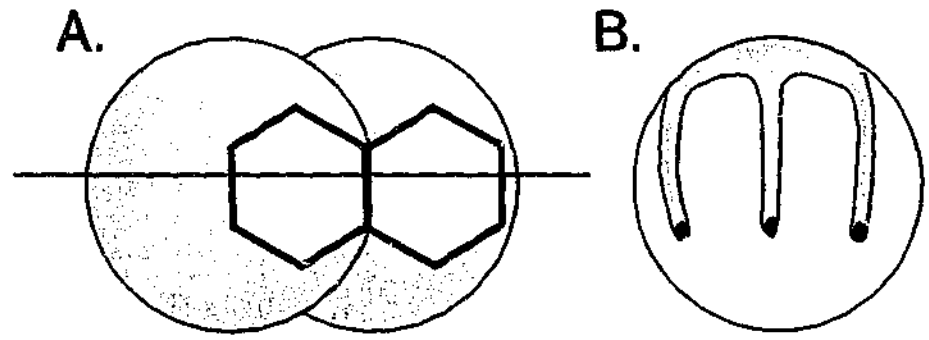


Figure 1.4 Elastin deposition in alveoli
(A) A schematic representation of alveoli showing elastin deposited in a ring surrounding the entrance of each alveolus and (B) how it can be seen in cross section as concentrated deposits at the tips of alveolar septal walls.

1.4 Elastin in lung development

1.4.1 Developmental expression of elastin in the lung

Elastin synthesis in the lung of mammalian species occurs primarily during late fetal and early neonatal periods of development. The timing of elastin synthesis in different species appears to be related to the stage of lung development rather than gestational or postnatal age. More specifically, peak tropoelastin expression or synthesis occurs during alveolarisation (Noguchi & Samaha, 1991; Shibahara et al., 1981).

Much of the data relating to the developmental expression of elastin in the lung has been derived from studies conducted in rats. In this species, *in situ* hybridisation studies have shown that tropoelastin mRNA is first detectable in the pseudoglandular period of lung development, peaks during alveolarisation in the neonate and is barely detectable in the adult lung (Mariani et al., 1997; Noguchi et al., 1990). As lung development progresses, the location and cell types responsible for tropoelastin expression and their relative contribution to total lung elastin production varies according to the lung structures developing at any one time. For example, during the pseudoglandular period of fetal lung development, tropoelastin mRNA is detectable in the smooth muscle of

pulmonary arteries but is minimal in mesenchymal tissue. Through the canalicular period tropoelastin mRNA can also be detected in the smooth muscle layer of developing airways. During the saccular period of lung development, tropoelastin expression is high in mesenchymal cells subjacent to differentiating epithelium of terminal airspaces, and during alveolarisation in neonatal rats, tropoelastin expression is concentrated at alveolar septal tips and at bends in alveolar walls (Mariani et al., 1997).

In experiments reported in this thesis, sheep have been used as a model of fetal lung development as the ovine fetal environment can be easily manipulated and fetal physiological measurements can be made in this species. More importantly, as the focus of this thesis is on the regulation of elastin synthesis in the developing lung *in utero*, and elastin expression and synthesis peaks during alveolarisation, sheep are the preferred model for extrapolation to human lung development over rats or mice in this regard. This is because of the timing of the alveolar period of lung development which begins prenatally in sheep (Alcorn et al., 1981) and humans (Langston et al., 1984), whereas alveolar development occurs entirely postnatally in rats and mice (Burri, 1974).

While there have been no previous studies that have evaluated the temporal and spatial expression patterns of tropoelastin in fetal and postnatal sheep lung, tropoelastin production in fetal and neonatal lung explant cultures has been shown to increase dramatically just prior to birth during the alveolar period of lung development and decline shortly after birth (Shibahara et al., 1981). In addition, a sharp increase in the mature elastin content of the lung just prior to birth has also been demonstrated in sheep (Schellenberg & Liggins, 1987; Shibahara et al., 1981). An evaluation of the ontogeny of tropoelastin expression and accumulation in fetal and postnatal sheep lung is presented in chapter 3 of this thesis.

1.4.2 Cell types responsible for elastin synthesis in the lung

The cell types responsible for elastin production and deposition in the lung vary depending on the location and tissue types. Elastin in pulmonary arteries and airways is produced predominantly by smooth muscle cells, however, elastin production in arteries also occurs in endothelial cells and adventitial fibroblasts (Parks et al., 1993). In the lung parenchyma, the identification of the cell types responsible for elastin production has been more difficult because of the vast number of different cell types found in lung

parenchymal tissue and the differentiation and phenotypic changes that occur in interstitial cells throughout development. For example, in rat lung, immature interstitial cells can differentiate into two phenotypically different fibroblasts, classified as lipid-filled and non-lipid filled fibroblasts (Vaccaro & Brody, 1978). Both cell types are capable of elastin synthesis in culture suggesting that both fibroblast types may have a role in elastin synthesis and deposition in the alveolar parenchyma. *In vivo* populations of the two types of fibroblasts differ in location, with lipid-filled fibroblasts located at the base of alveolar septa and non-lipid filled fibroblasts located at the tips of alveolar septa (Vaccaro & Brody, 1978). The phenotype of the non-lipid filled fibroblasts appears closely linked to the smooth muscle phenotype in that they contain myofilamentous structures. For this reason, the non-lipid filled fibroblasts have been termed alveolar septal myofibroblasts. These myofibroblasts which are phenotypically similar to smooth muscle cells also stain positive for smooth muscle isoactin. In fetal bovine lung *in vivo*, elastic fibre deposition during alveolar development was shown to be associated mainly with smooth muscle isoactin positive cells at the tips of developing secondary septae (Noguchi et al., 1989). Ultrastructural analysis in neonatal rat lung has also shown that elastin deposition is associated with alveolar myofibroblasts at the tips of developing septa (Vaccaro & Brody, 1978). Therefore it is likely that the alveolar septal myofibroblasts are the main cell type responsible for elastin deposition in the parenchyma during alveolar development. The role of the lipid-filled fibroblasts (lipofibroblasts) in elastin production is less clear, although studies of these cells in culture indicate that culture conditions that influence the lipid storage in pulmonary fibroblasts, also influence the expression of tropoelastin and smooth muscle isoactin such that increasing lipid storage decreases tropoelastin and smooth muscle isoactin expression and vice versa (McGowan et al., 1997b). These data suggest that it is possible that lipofibroblasts may be able to differentiate into myofibroblasts. Furthermore, lipofibroblasts have also been shown to store retinoids which are involved in the regulation of elastin synthesis and alveolar development and this suggests a role for this subclass of fibroblasts in elastin and alveolar development (McGowan & Torday, 1997).

1.4.3 Role of elastin synthesis during normal lung development

In addition to the role of elastin in the maintenance of lung structure and function in the adult, the close temporal and spatial association between elastogenesis and alveogenesis suggests a major role for elastin synthesis in alveolar formation during development. In support of this, studies that involve the interference of elastic fibre synthesis also result in disruptions in alveolar development. For example, inhibition of the cross-linking enzyme lysyl oxidase, interferes with elastin and therefore elastic fibre formation and results in an irreversible reduction in alveolar number and an increase in alveolar size, suggesting an inhibition of secondary septal formation (Kida & Thurlbeck, 1980). The complete loss of myofibroblasts in alveolar walls and the subsequent loss of alveolar septal elastic fibre deposition in platelet-derived growth factor (PDGF)-A-null mice also results in failed alveogenesis (Bostrom et al., 1996; Lindahl et al., 1997). Finally, deletions of retinoic acid receptors also result in both a reduction in elastin synthesis and alveolar formation (McGowan et al., 2000). While all of these studies provide strong evidence for a role of elastin and hence elastic fibre synthesis in alveolar development, the mechanisms remain unclear.

In addition to the role of elastin synthesis in alveogenesis, a complete disruption of elastin synthesis in mice results in failed development of the distal airways prior to the beginning of alveolar development (Wendel et al., 2000). This additional role of elastin synthesis in distal airway development prior to its role in secondary septal formation is consistent with the temporal and spatial expression patterns of elastin synthesis in the developing rat lung (refer to section 1.4.1).

1.5 The regulation of elastin synthesis in the developing lung

Cross-linked tropoelastin monomers that form mature amorphous elastin make up more than 90% of the mature elastic fibre (Starcher, 1986). Disruptions in the expression and synthesis of this protein during development result in impaired fetal lung development (Kida & Thurlbeck, 1980; Wendel et al., 2000) and due to its long half-life and minimal turnover once laid down, any impairment in elastin synthesis during its short intense period of synthesis in the fetus and neonate will have implications for future lung health. In addition, since elastogenesis is intimately associated with alveolar formation, and alveolar development begins before birth in humans as in sheep, it is important to

identify factors that regulate tropoelastin expression and elastin synthesis in the fetus both during normal development and as a result of adverse prenatal conditions.

Tropoelastin expression in the lung as mentioned earlier in Section 1.4.1 is confined to a short period of time during late fetal and early neonatal development. However, little is known of how the gene is regulated during development, or the influence of factors that are known to affect lung growth, maturation and structural development on tropoelastin expression and elastin synthesis *in vivo*. Studies in rat lung indicate that the induction and maintenance of tropoelastin expression is controlled at the level of transcription, whereas the cessation of expression in mature lungs is controlled by post-transcriptional mechanisms (Swee et al., 1995). This was indicated by the persistence of tropoelastin pre-mRNA levels in adult lung tissue in spite of the dramatic decrease in steady-state mRNA levels compared to peak levels in neonatal lung (Swee et al., 1995). A number of soluble and physical factors have been shown to have the ability to modulate tropoelastin expression, but the mechanisms or factors which control the age-specific induction and cessation of expression remain unknown. There is, however, evidence of a developmentally regulated cytosolic protein that is involved in the accelerated decay of the tropoelastin mRNA transcript in adult tissues (Zhang & Parks, 1999). The modulation of tropoelastin expression can occur by both transcriptional and post-transcriptional mechanisms such as for glucocorticoids (Pierce et al., 1995) and TGFβ (Kahari et al., 1992; McGowan, 1992) respectively.

While a number of factors influence tropoelastin gene expression, there are numerous other points at which elastin synthesis and deposition may be regulated, for example, the activity of lysyl oxidase may be altered which will not influence tropoelastin gene expression but instead will influence elastin cross-linking and hence the production of mature functional elastin. In addition, while the focus of the majority of studies to date, as well as the focus of experiments performed in this thesis, is on elastin, elastogenesis is a complex process involving many proteins in order to produce functional elastic fibres. Therefore, factors that regulate tropoelastin expression may also regulate the expression of other proteins involved in elastic fibre formation; at least during normal lung development. For example, retinoids reverse the defect in septation associated with the duplication of the central region of fib-1 in the tight skin mouse (Massaro & Massaro, 2000). Figure 1.5 is a diagrammatic representation showing the major steps

involved in the processing of elastin into mature elastic fibres and possible points of regulation.

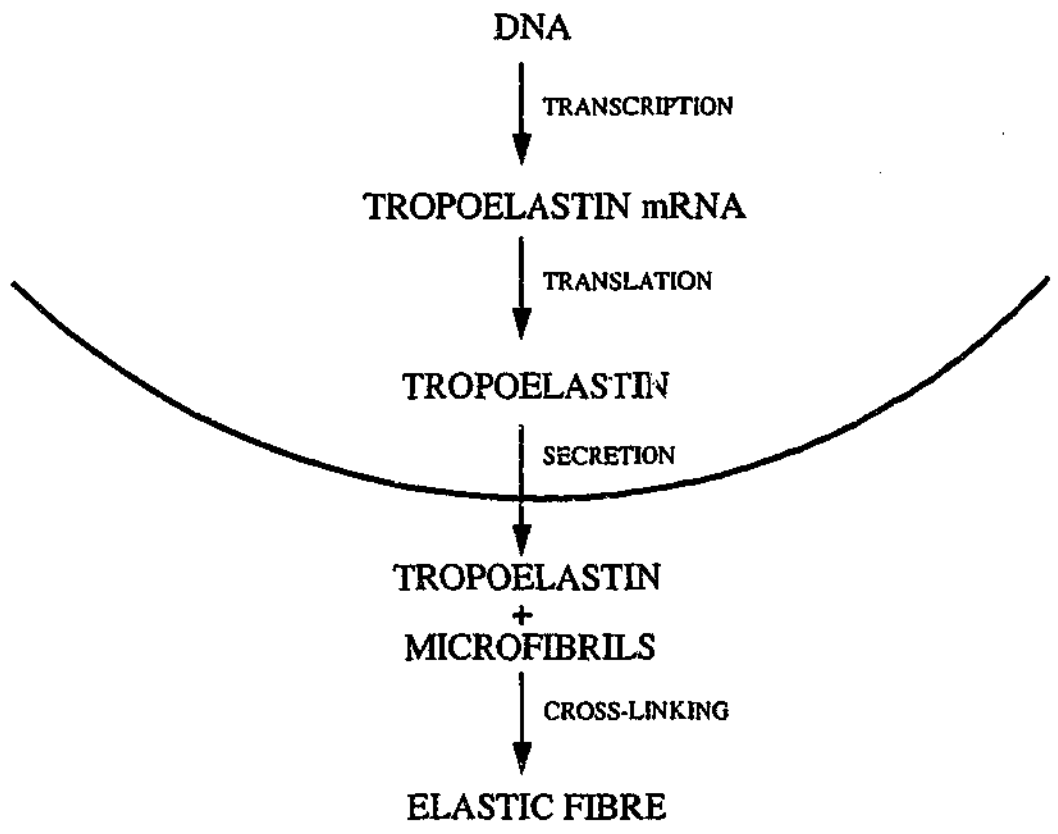


Figure 1.5 Elastin processing into elastic fibres
Diagrammatic representation of the major steps involved in the processing of elastin into mature elastic fibres.

1.5.1 Role of mechanical forces in lung elastin synthesis

Mechanical forces and lung tissue stretch during fetal development in sheep are known to play a major role in lung growth and maturation (Harding & Hooper, 1996). These forces are mediated by the degree of fetal lung expansion, which in turn is determined by the volume of lung liquid held within the lung during development (Hooper & Harding, 1995). Clinical conditions that lead to altered fetal lung expansion and fetal lung growth include tracheal atresia which leads to increased fetal lung expansion and congenital diaphragmatic hernia, oligohydramnios and impaired or absent fetal breathing movements which lead to decreased fetal lung expansion (Harding & Hooper, 1996).

Experimental studies have shown that sustained increases in fetal lung expansion are a potent stimulus of lung growth including alveolar development (Nardo et al., 1998; Nardo et al., 2000), whereas sustained decreases in fetal lung expansion result in a virtual cessation of lung growth and a substantial reduction in alveolar development. Based on the known association between elastin synthesis and alveolar development, it is likely that, as part of the global influence of mechanical forces on fetal lung growth and maturation, elastin synthesis will also be influenced by such physical factors.

Evidence for a role of mechanical forces in the regulation of elastin synthesis has been shown by increases in tropoelastin expression in pulmonary artery segments stretched *in vitro* (Tozzi et al., 1989) and in cultured fetal rat lung cells subjected to cyclical mechanical strain resulting in an increase in gene transcription (Nakamura et al., 2000). However, the relevance of this to the modulation of tropoelastin expression in the fetal lung *in vivo* is unclear. The stimulus used in the study by Nakamura *et al.* was of a cyclical nature which may not be as relevant to the nature of the physical forces that are acting on lung tissue in the fetal lung compared to the postnatal lung. The authors' rationale for the stimulus used is the coincident induction of fetal breathing movements and tropoelastin expression in fetal rat lung. The rhythmic contractions of respiratory muscles in the fetus that resemble postnatal breathing are referred to as fetal breathing movements. Abolition of fetal breathing movements is known to result in a decrease in lung growth and structural development (Wigglesworth & Desai, 1979), and studies *in vitro* led to the suggestion that the influence of fetal breathing movements on lung growth was mediated by phasic changes in lung tissue stretch (Liu et al., 1992). However, *in vivo* evidence suggests that fetal breathing movements result in very minor changes in lung volume compared to the changes in lung volume that occur after birth. Instead, the role of fetal breathing movements appears to be in the maintenance of lung liquid volume and therefore sustained rather than phasic lung tissue stretch (Hooper & Harding, 1995). Also, because the lung is a complex structure in which interactions between cells and matrix are important for development, and elastin *in vivo* appears to accumulate at points of high stress in alveolar walls (Mariani et al., 1997), mechanical forces may have different effects in the intact lung, in particular in relation to elastin deposition. There have been no previous studies that have evaluated the effects of mechanical forces on elastin synthesis in the fetal lung *in vivo*.

1.5.2 Cortisol

Cortisol is known to play a role in many aspects of fetal lung maturation and the preparation of the lung for air-breathing after birth. Part of this process is the development of thin walled alveoli and therefore, it is possible that cortisol may play a role in the regulation of lung elastin synthesis. The surge in fetal plasma cortisol concentrations that occur towards the end of gestation coincide with peak tropoelastin expression and thus it would seem likely that cortisol would have stimulatory influence on elastin synthesis at that stage of development. Previous studies have however produced contradictory results. Dexamethasone treatment of cultured fetal rat lung cells (Pierce et al., 1995) and neonatal rat pulmonary fibroblasts (Noguchi et al., 1990) indicate that corticosteroids up-regulate tropoelastin expression in an age and dose dependent manner which occurs at the level of gene transcription. Maternal Dexamethasone treatment in rats also led to an age-dependant increase in tropoelastin mRNA levels *in vivo* (Pierce et al., 1995). Other studies in rats have however shown that maternal Dexamethasone treatment of a similar dose resulted in postnatal decreases in lung desmosine concentration (an indicator of mature elastin concentration) (Schellenberg et al., 1987b). Also, some studies on rats have reported a decrease in alveolar formation following prenatal administration of corticosteroids (Blanco et al., 1989; Massaro & Massaro, 1992; Tschanz & Burri, 1997) which may be evidence for an inhibition of lung elastin synthesis by corticosteroids. In sheep, fetal betamethasone treatment in one study caused a decrease in parenchymal elastin density at 121 days of gestation but an increase at 135 days of gestation (Willet et al., 1999) and fetal cortisol treatment in another study resulted in an increase in alveolar number (Boland et al., 2004). The reason for the conflicting published data is most likely the wide variation in the animal species used, the stage of lung development at which corticosteroids were administered, and the type, dose and route of delivery of corticosteroids. This is because most studies are aimed at determining the effect of corticosteroids on lung development in relation to antenatal corticosteroid treatment. The role of cortisol in the regulation of lung elastin synthesis during normal development has not been addressed.

1.5.3 Retinoids

Retinoids are chemical derivatives of all-trans retinol which is commonly known as vitamin A (McGowan, 2002). Retinol is acquired from the diet and is converted to the

more biologically active form which is retinoic acid in most target tissues including the lung (McGowan et al., 1997a). Retinol that is not required immediately is stored primarily in the liver but is also stored in parenchymal lipofibroblasts in the lung (McGowan & Torday, 1997). Retinol stores in parenchymal lipofibroblasts decline during alveolar development in rats (McGowan & Torday, 1997) and therefore, utilisation of these stores could potentially be involved in alveolar formation. Studies in rats and mice have demonstrated that retinoids can enhance alveolarisation during neonatal alveolar development (Massaro & Massaro, 1996) as well as induce alveolar formation in adult rat lungs with elastase-induced emphysema (Massaro & Massaro, 1997) and mice with a pharmacologically induced or genetic failure of septation (Massaro & Massaro, 2000). One of the gene targets involved in this process is likely to be elastin as endogenous retinoids have been shown to increase elastin expression in cultured neonatal rat lung fibroblasts (McGowan et al., 1997a). Maternal vitamin A deficiency also results in altered lung morphology and reduced tropoelastin mRNA levels and elastin staining in fetal rat lungs (Antipatis et al., 1998) and deletions in retinoic acid receptors decrease tropoelastin mRNA and alveolar number (McGowan et al., 2000). Exogenous retinoids have also been shown to increase tropoelastin mRNA levels in neonatal rat lung fibroblasts at least in part due to an increase in gene transcription (Liu et al., 1993).

1.5.4 Growth factors

A number of cytokines and growth factors have been shown to have an influence on tropoelastin expression and/or elastin production but information regarding the regulation of elastin production in the lung by such factors is scant. Transforming growth factor-β_1 (TGF- β_1) is one of the most studied and has been shown to have a stimulatory effect on tropoelastin expression in cultured neonatal rat lung fibroblasts (McGowan et al., 1997c). The mechanism by which TGF-β_1 mediates this response is via the post-transcriptional regulation of mRNA turnover. It has been suggested that TGF-β_1 decreases the binding activity of a cytosolic protein that is involved in the age-dependant increase in tropoelastin mRNA degradation (Zhang et al., 1999), thus resulting in an increase in mRNA stability. Since the content of TGF-βs is greater in neonatal rat lung compared to adult rat lung, it is possible that TGF-βs play a role in the developmental expression of tropoelastin in the lung.

Platelet derived growth factor-A (PDGF-A) is another growth factor involved in the developmental regulation of lung elastin production and has been shown to have a major and specific effect on alveolar elastin production in the lung. Unlike TGF-β_1, PDGF-A does not modulate tropoelastin expression but rather the developmental proliferation and migration of the alveolar myofibroblasts that synthesise elastin in developing secondary septa. This has been demonstrated by the complete loss of alveolar myofibroblasts and septal elastin deposition following PDGF-A knockout (Bostrom et al., 1996; Lindahl et al., 1997).

Insulin-like growth factor-I (IGF-I) has been shown to have a stimulatory effect on tropoelastin expression via an increase in gene transcription in rat aortic smooth muscle, however, this response appears to be tissue specific as IGF-I does not appear to have an effect on tropoelastin expression in the rat lung *in vivo* (Foster & Curtiss, 1990).

While the above mentioned factors have a stimulatory or positive effect on tropoelastin expression and elastin production, there are also factors that have an inhibitory effect on elastin synthesis. Basic fibroblast growth factor (bFGF) decreases in rat lung coincident with increases in elastin synthesis, and decreases elastin production in cultured neonatal rat lung fibroblasts (Brettell & McGowan, 1994). Therefore it is possible that bFGF may also play a role in the developmental regulation of elastin synthesis in the lung.

Factors other than growth factors, such as the cytokine tumor necrosis factor-α (TNF-α), vitamin D_3 and phorbol esters, have also been shown to have inhibitory influences on elastin synthesis in cultured vascular smooth muscle cells, but their influence on elastin synthesis in the developing lung *in vivo* is unknown (Mariani et al., 1997).

1.5.5 Adverse prenatal conditions

While the above sections describe factors that regulate elastin synthesis mainly during normal lung development, there are also factors that may regulate and alter elastin synthesis and deposition in a sub optimal intra-uterine environment. Intrauterine growth restriction (IUGR) is a major cause of low birth weight, which increases the risk of respiratory illness after birth (Barker et al., 1991; Minior & Divon, 1998; Nikolajev et al., 1998; Rona et al., 1993; Stein et al., 1997; Tyson et al., 1995). IUGR in animal studies results in impairments in lung structure and function (Joyce et al., 2001; Maritz et al., 2001) that could possibly be attributed to alterations in elastin synthesis. IUGR is

associated with reduced oxygen and nutrient supply to the fetus, as well as elevated plasma cortisol concentrations (Nicolaides et al., 1989) and these factors have been shown individually either in culture or *in vivo* to affect elastin synthesis.

Hypoxia

Hypoxia in cultured neonatal rat lung fibroblasts has been shown to down regulate tropoelastin expression in a dose and time dependant manner by pretranslational mechanisms (Berk et al., 1999). In that study, very low levels of oxygen (<10%) were required to decrease tropoelastin mRNA below control levels. Lysyl oxidase activity on the other hand has been shown to increase with hypoxia (Brody et al., 1979). Whether these responses are relevant to the *in vivo* environment, for example as a result of fetal hypoxemia due to placental restriction, are unknown.

Undernutrition

Starvation in neonatal rats during the period of peak elastin synthesis results in altered lung mechanical properties and reduced elastin accumulation (Kalenga & Henquin, 1987; Sahebjami & MacGee, 1985). Nutritional deprivation in un-weaned rat pups via removal of the mother for two 24 hour periods also results in reduced intraseptal elastin accumulation and delayed alveolarisation (Das, 1984). The effect of prenatal nutrient restriction on elastin accumulation is unknown; however, maternal nutrient restriction during the last third of gestation in guinea pigs, in which alveolar development occurs before birth, results in decreased alveolarisation (Lechner, 1985). Lysyl oxidase activity in rat lung has been shown to be decreased as a result of starvation (Madia et al., 1979) which could contribute to a decrease in elastin synthesis observed in these models.

1.6 Specific aims of studies in this thesis

Study 1 – Ontogeny of elastin synthesis in the developing lung

The aim of study 1 (Chapter 3) was to characterise tropoelastin expression from approximately mid-gestation, in the early canalicular period of lung development, to maturity (approximately 2 years postnatal age) in sheep and relate levels of expression to changes in elastin content and staining over the same period.

Study 2 – Influence of lung expansion on elastin synthesis and deposition in the ovine fetal lung *in vivo*

The aim of study 2 (Chapter 4) was to determine the effect of sustained increases and decreases in lung expansion on elastin synthesis in the fetal sheep lung *in vivo*.

Study 3 – Effect of physiological levels of corticosteroids on elastin synthesis and deposition in the ovine fetal lung *in vivo*

The first aim of this study 3 (Chapter 5) was to examine the effect of corticosteroids at physiological levels on both elastin synthesis and deposition in the developing fetal lung.

The second aim of study 3 was to evaluate the effect of corticosteroids on elastin synthesis and deposition both in the presence and in the absence of an adequate degree of lung expansion.

Study 4 – Effect of intra-uterine growth restriction (IUGR) on lung elastin synthesis and deposition

The aim of study 4 (Chapter 6) was to evaluate the effects of IUGR induced by late gestational placental insufficiency on lung elastin synthesis and deposition in fetal and postnatal sheep.

Chapter 2

General Methods

2.1 Generation of an ovine specific tropoelastin cDNA probe

An ovine specific tropoelastin cDNA probe was generated for the detection and measurement of tropoelastin mRNA in ovine lung tissue. An ovine specific probe for tropoelastin was not previously available and therefore also provides a valuable resource for future studies.

2.1.1 Design of ovine cDNA primers specific for ovine tropoelastin

Two oligonucleotide primers were designed to amplify a 214 base pair fragment of the published coding sequence of tropoelastin in sheep (Figure 2.1) (Yoon et al., 1985). The first primer, designated TPE1F was 20 nucleotides in length, and was identical in sequence to nucleotide numbers 90 to 109 (5'-TGTGTCTCCAGCTGCAGCCTG-3') at the 5' end of the tropoelastin cDNA sequence. The second primer, designated TPE2R was 21 nucleotides in length, and was complementary in sequence to nucleotide numbers 305 to 285 (5'-TCACTTTCTCTTCCGGCCACA-3') at the 3' end of the tropoelastin cDNA sequence. The chosen sequence of each of the primers was compared to the Genbank Internet database to ensure that they were closely matched to sheep tropoelastin and had low homology with genes other than tropoelastin, to limit the amplification of non-specific polymerase chain reaction (PCR) products. The two primers were designed to have similar melting temperatures (T_m°C) which were calculated using the following formula:

$$\text{Estimated Tm (°C)} = 2 \times (A+T) + 4 \times (G+C)$$

where A,T,G and C are the number of each respective nucleotide in the primer. The estimated T_m of both TPE1F and TPE2R was 64°C. Complementary bases at the ends of primers were avoided to prevent dimerisation.

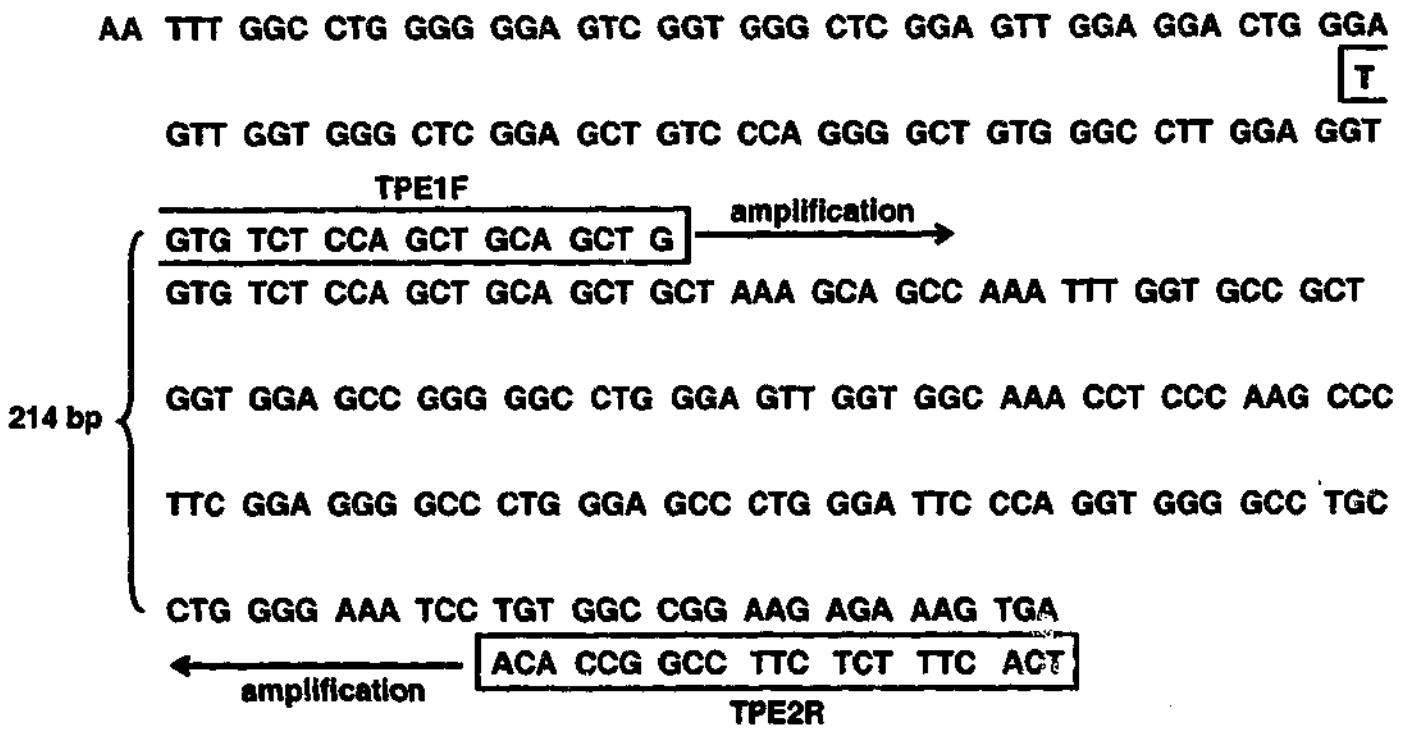


Figure 2.1 Sheep tropoelastin 3' region
Published coding sequence of sheep tropoelastin (Yoon et al., 1985) showing the two primers designed for the current study and the 214 bp fragment that should be amplified by these primers.

2.1.2 Extraction of total RNA

Total RNA was extracted from fetal and adult sheep lung using a modified phenol chloroform extraction method. Briefly, 0.5-1.0g of frozen lung was homogenised using a homogenising tool (Ultra Turrax T25, Janke and Kunkel, IKA-Labortechnik, 24,000 rpm) in 4M guanidine thiocyanate solution (Appendix 2.1) and kept on ice for 3-4 minutes. Sodium acetate (2M), water-saturated phenol and chloroform (at a ratio of 0.1, 1, and 0.4 of the homogenate respectively) were added to the homogenate and the solution was shaken vigorously for 30 seconds. The tubes were then cooled on ice for 15 minutes and then centrifuged for 20 minutes (4°C) at 14,500 x g (Sorvell RC-5B

centrifuge). Following centrifugation, the supernatant was carefully transferred into a clean tube and sodium acetate (2M, 0.1 volumes) and 2-propanol (1 volume) were added. The samples were then stored at -20°C overnight to precipitate the RNA. The following day, the samples were centrifuged at 14,500 x g for 30 minutes at 4°C to form a pellet of RNA. The supernatant was discarded and the pellet was re-suspended in 2.4ml of 4M guanidine thiocyanate solution and 2-propanol (2.4ml) was added. The samples were stored at -20°C for 30 minutes to again precipitate the RNA. The samples were centrifuged at 14,500g for 15 minutes at 4°C, the supernatant was discarded and the pellet washed with 100% and 80% ETOH respectively, each separated by a 15 minute centrifugation. The RNA pellet was re-suspended in 100-200µl of diethylpyrocarbonate (DEPC) treated H_2O and incubated at 65°C for 15 minutes. Prior to storage at -70°C, the samples were checked for degradation by electrophoresing them on a 1% agarose TAE gel (DNA grade agarose, Progen Industries Ltd., Australia; 1 x TAE, Appendix 2.1) containing ~60ng of ethidium bromide (Sigma, Australia) per ml of gel. Visualisation of distinct bands corresponding to 28S and 18S ribosomal RNA under ultraviolet (UV) light indicated that the RNA was not degraded. The concentration of the RNA samples was determined by measurement of the optical density (OD) at a wavelength of 260nm (Eppendorf Biophotometer, Germany), where an OD_{260} of 1.0 = 40µg/ml of RNA.

2.1.3 Reverse Transcription (RT)

DNase 1 treatment of RNA

Total RNA extracted from fetal lung tissue was DNase treated prior to reverse transcription to remove any DNA contamination that may have been present in the RNA sample. This procedure prevents the amplification of genomic DNA in the subsequent PCR step. The DNase enzyme, (10U; RQ1 RNase-free DNase1, Promega, Australia), an RNase inhibitor (10U; Rnasin, Promega, Australia), Tris-Cl pH 8-8.3 (10mM), KCL (50mM) and $MgCl_2$ (1.5mM) were added to 50µg of total RNA and the reaction was made up to a final volume of 100µl with sterile MilliQ reverse osmosis purified water (MQ H_2O) and incubated at 37°C for 30 minutes. Following incubation, the RNA was extracted by adding 100µl of a phenol / chloroform / isoamyl mixture (3 parts phenol : 1 part chloroform / isoamyl (24:1)) and centrifuging at 15,000 x g for 5 minutes. The

supernatant was transferred into a clean tube and 3M sodium acetate (10µl) and 100% ETOH (250µl) were added and the sample was stored at -20°C overnight to precipitate the RNA. The following day the sample was centrifuged, and the RNA pellet washed with 70% ETOH and then re-suspended in ~70µl of Tris EDTA (TE, Appendix 2.1) buffer. The sample was electrophoresed on a 1% agarose gel to check for degradation and its concentration determined by spectrophotometry (refer to section 2.1.2).

First Strand cDNA Synthesis

DNase-treated RNA (1µg) was reverse transcribed with one of 3 primers. The first was the specifically designed antisense primer TPE2R, the second was the non-specific primer T15 that binds to the poly-A tail of all mRNA species and the third was a mix of Random Hexamers consisting of random oligonucleotide primers 6 nucleotides in length that bind to homologous sites on mRNA species. Three primers were chosen to use in separate RT reactions to increase the chance of successfully amplifying the desired tropoelastin cDNA sequence by PCR. In addition to the three separate RT reactions, negative control (no RT) reactions (in which no reverse transcriptase was added) were produced for each of the primers to test for contaminating genomic DNA in the template RNA sample during the PCR process. Each RT reaction contained DNase-treated RNA (1µg), primer (50pmol), DTT (8mM), dNTPs (20mM), 1x AMV RT buffer, AMV reverse transcriptase (20U; Promega, Australia) and sterile MQ H_2O in a final reaction volume of 25µl. The reactions were incubated at 42°C for at least one hour (maximum 4 hours) to facilitate reverse transcription and then stored at -20°C.

2.1.4 Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a rapid method for enzymatic amplification of a specific portion of DNA. The technique relies on the continuous cycling of reactions through temperatures that facilitate the separation (denaturation) of DNA strands, followed by the annealing of specifically designed primers to each of the strands, and finally synthesis of a new strand of DNA by the addition of free nucleotides (dNTPs) by the enzyme DNA polymerase. Success of the procedure relies on the optimisation of the reaction conditions, such as magnesium chloride ($MgCl_2$) concentration and annealing temperature. Magnesium chloride stabilises primer annealing, and also forms a complex with dNTPs, which is essential for recognition by the enzyme Taq DNA Polymerase. In

this experiment, PCR reactions were performed with $MgCl_2$ concentrations ranging from 1.5mM to 5.0mM, and the optimal $MgCl_2$ concentration was found to be between 1.5 and 2.5mM. The annealing temperature used initially was 59°C (5°C less than the estimated Tm values for the sense (TPE1F) and antisense (TPE2R) primers). PCR reactions with annealing temperatures of 59°C and 61°C yielded only non-specific PCR products and therefore, further optimisation procedures were employed. Firstly, Taq DNA polymerase was added to the reaction after it had been denatured (Hot start PCR)(Chou et al., 1992) to prevent the amplification of non-specific PCR products by the DNA polymerase synthesising product from non-specifically annealed primers before the first denaturation step. Secondly 'touch-down' PCR (Hecker & Roux, 1996), was used to optimise the annealing temperature. This technique involves stepwise decreases in annealing temperature (0.5°C per PCR cycle for 10 cycles) followed by further amplification at the final annealing temperature. The aim of this procedure is to achieve the optimal annealing temperature during the first 10 cycles, resulting in sufficient specific PCR product for preferential amplification of that product during the remaining cycles.

Successful PCR reactions were obtained using template DNA from the reverse-transcription reaction that used the TPE2R primer (2μl) in combination with the following reagents: TPE1F sense primer (25pmol), TPE2R antisense primer (25pmol), dNTPs (1mM), 1x Taq polymerase buffer (containing 1.5mM $MgCl_2$), Taq DNA polymerase (2.5U, Invitrogen, Australia; added when reaction reached 94°C) and sterile MQ H_2O to a final reaction volume of 50µl. In addition to the "no RT" control, a second negative control was used in which no DNA template was added to the PCR reaction to test for DNA contamination of PCR reagents. PCR was carried out in a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer, Australia). Each cycle consisted of three steps; a) denaturation of cDNA (94°C for 30s), b) primer annealing to single stranded cDNA (at an initial temperature of 65°C for 30s and decreasing by 0.5°C per cycle for the first 10 cycles, followed by 25 cycles at 60°C), and c) synthesis of a new cDNA strand by Taq DNA polymerase (72°C for 1min). Loading buffer (Appendix 2.1) was added to 30µl of the PCR reaction and to a DNA ladder (DNA Mass Ladder, Invitrogen, Australia). Electrophoresis was used to separate PCR products according to size on a 1.5% agarose TAE gel containing ethidium bromide, and these were viewed under UV light and their size estimated in comparison to the DNA ladder. The remainder of the

PCR reaction was stored at -20°C. At least two bands were observed between 200 and 400bp in length (Figure 2.2); no PCR products were observed when RT reactions were performed with the T15 or Random Hexamer primers. As the correct fragment should have been 214bp, the brightest band closest to the 200bp marker of the DNA ladder was excised from the gel using a gel extraction kit (QIAEX II, QIAGEN, Germany). The purified PCR product was stored at -20°C.

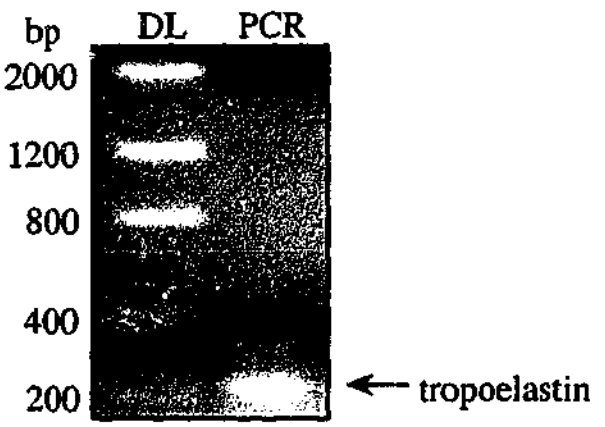


Figure 2.2 Electrophoresis of PCR products
An agarose gel showing a DNA ladder (DL) with bands of known base pair length (bp) in the left lane and PCR products (PCR) in the right lane. Of the PCR products, the brightest band closest to the 200bp band of the DNA ladder was considered likely to be the expected 214bp tropoelastin fragment and was sub-cloned into pGEM-T Easy, sequenced (refer to sections 2.1.5 to 2.1.8) and found to be 100% homologous with the published coding sequence of sheep tropoelastin.

2.1.5 Sub-Cloning of PCR products

PCR products were sub-cloned into the plasmid pGEM-T Easy (Figure 2.3, Promega, Australia) so that they could be easily manipulated. The pGEM-T Easy plasmid is a pre-digested Ampicillin resistant 3015bp vector that contains a single "T-overhang" at the 3' ends of the insertion site. These ends are compatible with the "A-overhang" ends of PCR fragments generated using Taq DNA Polymerase, which has a tendency to add a single deoxyadenosine (A) nucleotide onto the 3' end. The manufacturers (Promega) protocol was followed for sub-cloning reactions. The amount of insert (purified PCR

product) required for the sub-cloning reaction that ligates the PCR cDNA fragment into the plasmid was calculated using the following formula:

$$\text{ng of PCR insert} = \frac{(3) \times (50\text{ng of plasmid}) \times (\text{basepairs of PCR insert})}{(\text{basepairs of plasmid})}$$

Ligation reactions contained 1x Rapid Ligation Buffer, pGEM-T Easy vector (50ng), PCR product (ng as calculated above), T4 DNA ligase (3U, Promega, Australia) and sterile MQ H_2O in a final volume of 10µl and were incubated overnight at 4°C to facilitate ligation.

2.1.6 Transformation of XL1 – Blue competent E. Coli cells

Transformation of plasmid DNA into competent bacterial cells takes advantage of the ability of bacteria to replicate plasmid DNA independently of the host chromosome, allowing further amplification of the target cDNA sequence contained within the recombinant plasmid. Transformation was achieved by incubating 1µl of the ligation reaction with 100µl of XL1-Blue competent cells on ice for 30 minutes and then at 42°C in a preheated water bath for exactly 90 seconds (to heat shock the bacterial cells) allowing them to take up the plasmid DNA. The cells were then placed immediately back on ice for 2 minutes. Following transformation, 900µl of sterile Luria-Bertani Medium (LB, Appendix 2.1) was added to the cells which were then incubated in a rotary shaker (200 cycles/min) at 37°C for 45 minutes. This step allows the cells that had taken up the plasmid sufficient time to express the Ampicillin (Sigma, Australia) resistance gene present in the pGEM-T Easy plasmid. 200µl of cells were then plated onto LB agar plates (LB media, 20g/L bacteriological agar) containing 100µg/ml of Ampicillin and coated with isopropylthio-β-D-galactoside (40µl 100mM IPTG, Progen, Australia) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (100µl 2% X-gal, Progen, Australia) and cultured upside down, overnight at 37°C. The Ampicillin in the agar plates allows for select growth of only bacterial cells that contain the pGEM-T Easy plasmid. The IPTG and X-gal allow for colour selection of colonies that contain the PCR insert within the plasmid, thus disrupting the LacZ gene which encodes for enzyme β-galactosidase. IPTG induces the synthesis of β-galactosidase in plasmids lacking a

PCR insert, which then cleaves the substrate X-gal to produce a blue colour. As the gene is disrupted in cells containing the PCR insert, the colonies remain white. The following day, white colonies were inoculated into 5ml of LB media containing Ampicillin (100μg/ml) in a rotary shaker (200 cycles/min) at 37°C overnight.

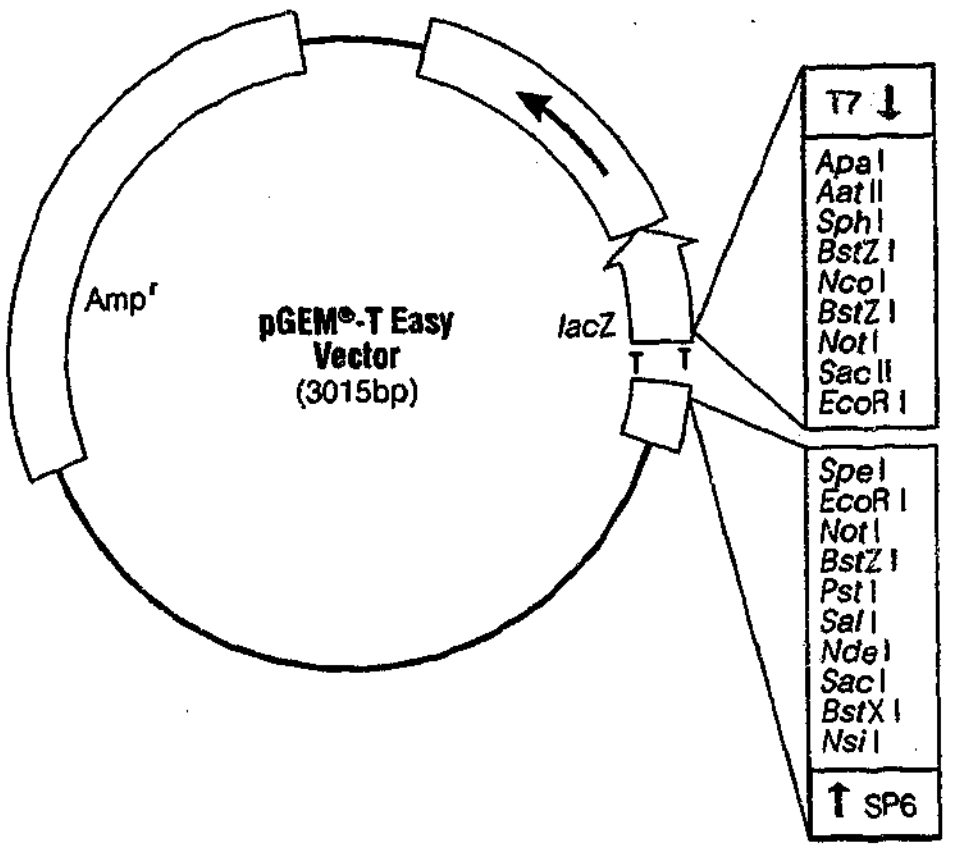


Figure 2.3 pGEM-T Easy plasmid vector
A schematic representation of the pGEM-T Easy vector used to sub-clone the PCR amplified region of sheep tropoelastin, showing the T-overhang within the LacZ gene (the site of PCR insert ligation), restriction enzyme cleavage sites and SP6 and T7 promoter sequences.

2.1.7 Extraction and digestion of plasmid (vector) DNA

Plasmid DNA was isolated from the bacterial culture using the Concert Rapid Plasmid Miniprep System (Life Technologies, Germany). The protocol and all solutions were provided by the manufacturer. Briefly, bacterial cells were pelleted by centrifugation (2000 x g, 5 mins), the LB media (supernatant) was removed, and the cells were lysed with the provided lysis buffer. The lysate was centrifuged to separate the bacterial cell walls and genomic DNA which form a pellet, from the plasmid DNA contained in the

supernatant. The supernatant was transferred to a spin cartridge in which the plasmid DNA is adsorbed to silica-based membranes and are contaminants washed away in subsequent washing steps. Finally, the DNA was eluted from the cartridge using warm (75°C) TE buffer and stored at - 20°C.

To confirm that the plasmid DNA extracted in the miniprep contained the insert (PCR product), a restriction digestion reaction was carried out on a sample of the miniprep plasmid DNA using the EcoR1 restriction enzyme. The reaction contained 4µl of miniprep DNA, 1x React 3 buffer (supplied with the enzyme), EcoR1 restriction enzyme (10U; Invitrogen, Australia) and sterile MQ H_2O in a final reaction volume of 20µl. The reaction was incubated at 37°C for 2 hours and then electrophoresed alongside a DNA mass ladder on a 1% agarose gel containing ethidium bromide. The gel was viewed under UV light and the fragment length of the excised band was confirmed as being the same as the original PCR product. An estimation of the yield of plasmid DNA obtained in the miniprep was also made by comparison of the intensity of the vector and insert bands relative to the intensity of the DNA ladder (DNA Mass Ladder, Invitrogen) bands which are of a known DNA mass.

2.1.8 DNA Sequencing

To verify that the subcloned RT-PCR product was 100% homologous with the 214bp region of the published coding sequence of sheep tropoelastin, sequence analysis was performed using a T7 Sequencing Kit (Amersham Biosciences, Australia). This kit is based on the dideoxy sequencing method. In Brief, the first step of the procedure was denaturation of the double stranded DNA template (miniprep DNA, refer section 2.1.7) followed by primer annealing. The primer used was complimentary in sequence to either the T7 or SP6 promoter sequences in the pGEM-T Easy plasmid near the insertion site (Figure 2.3). Denaturation of the Template DNA was achieved by adjusting the concentration such that 32µl contained 1.5-2µg of DNA, followed by addition of 2M NaOH (8µl) and incubation at room temperature for 10 minutes. After denaturation, the DNA template was ethanol precipitated and re-dissolved in 10µl of dH_2O before addition of primer (2µl, 5-10pmol) and Annealing Buffer (2µl). The mixture was incubated at 60°C for 10 minutes and then at room temperature for at least 10 minutes. The next step in the procedure was the labelling reaction which involved addition of Labelling Mix (3µl), labelled dNTP (1µl, [α-^{35}S]dATPαS), and diluted T7

(kit) or SP6 DNA Polymerase (Promega, Australia) (2µl) followed by incubation at room temperature for 5 minutes. Immediately following the 5 minute incubation period of the labelling reaction, termination reactions were initiated by addition of 4.5 µl of the labelling reaction to 4 pre-warmed (37°C) sequencing mixes each containing one of ddATP, ddCTP, ddGTP or ddTTP. The termination reactions were incubated for 5 minutes at 37°C after which they were terminated by addition of 5µl of Stop Solution. Flowing the sequencing reactions, a 3µl aliquot of each of the four reactions was resolved in separate lanes of a poly-acrylamide sequencing gel.

Sequencing Gel

A 6% poly-acrylamide sequencing gel (70ml 6% acrylamide, 2.5ml 10% ammonium persulphate (APS), 50µl Temed) was pre-electrophoresed using a BRL Sequencing System (Gibco, Model S2, Gaithersburg) at 1800 volts until the temperature of the gel reached 50°C. The radioactive sequencing reactions were denatured at 80°C for 2 minutes and loaded into separate wells and electrophoresed for approximately 1 hour until the xylene cyanol (purple dye front) had just reached the bottom of the gel. During electrophoresis, the voltage was adjusted to maintain a gel temperature of 50°C. The gel was carefully transferred onto 3MM Whatman chromatography paper (Whatman International, UK), covered with plastic wrap and dried under vacuum at 80°C for 2 hours (SpeedGel SG210D Savant Instruments, USA). Once dry, the gel was placed into a film cassette and exposed to autoradiographic film (BioMax MR, Kodak, Australia) overnight, before the film was developed, and the sequence read directly from the film placed on a light box. The sequence obtained was compared to the Genbank Internet database using the BLAST alignment algorithm provided by NIH (www.ncbi.nlm.nih.gov). Once the PCR product was confirmed to be 100% homologous with the published sheep tropoelastin cDNA sequence, larger quantities of the cDNA insert were amplified using the Perfect prep plasmid midi kit (Eppendorf, Australia) which is a larger-scale version of the miniprep kit described in section 2.1.7.

2.2 *Determination of tropoelastin mRNA levels by Northern blot analysis*

2.2.1 Electrophoresis of Total RNA

RNA was extracted from a portion of lung tissue from each animal using the method described in section 2.1.2. An aliquot containing 20µg of extracted RNA from the lung tissue of each animal was transferred to a sterile screw cap Eppendorf tube and the samples were vacuum dried (SpeedVac-Plus SC110A, Savant Pty Ltd, USA) and then re-suspended in 5.5µl of DEPC-treated water. To each sample, 20x Northern Buffer (Appendix 2.1, 1.0µl), 37% deionised formaldehyde (3.5µl) and deionised formamide (10µl) was added, and the samples were boiled for 5 minutes to denature the RNA and then placed on ice for 5 minutes to ensure that the RNA remained denatured. Northern loading buffer (Appendix 2.1) was then added to each sample before it was loaded into a separate well of a 1% agarose gel containing 2.2M deionised formaldehyde and 1 x Northern Buffer. The gel was electrophoresed at 22V for 16 hours in a Hoeffer electrophoresis chamber (Hoeffer Max Submarine Unit HE-99X, Hoeffer, USA) or an Amersham Biosciences electrophoresis chamber (GNA-200 Submarine unit, Amersham Biosciences, Australia) for larger gels, to allow separation of the RNA species according to molecular size.

2.2.2 Transfer of total RNA to nylon membrane

Following electrophoresis, the RNA was transferred from the gel to a nylon membrane (Duralon, Stratagene Biorad, Australia) by capillary transfer. To do this, the wells were trimmed from the gel and a notch cut into the top left-hand corner using a scalpel blade to help with orientation. Four pieces of 3MM Whatman paper and one piece of nylon membrane were cut to the exact dimensions of the gel and a notch cut into the top left-hand corner of the membrane to maintain alignment with the gel. The gel was washed in 2x standard saline citrate solution (SSC) (Appendix 2.1) for 10 minutes and 5x SSC for 10 minutes. The nylon membrane was washed in DEPC-treated H_2O for 10 minutes, and the Whatman paper soaked in 5x SSC. A flat rectangular piece of glass was placed across a baking dish filled with 5x SSC and a piece of Whatman paper saturated in 5x SSC was placed across the glass to act as a wick. The gel was placed face down on the

wick (with the notch at the top right-hand corner) and the nylon membrane and Whatman paper placed on the gel, ensuring at each step that there were no air bubbles to interfere with the RNA transfer. Plastic wrap was placed around the gel to ensure that capillary movement of buffer could only occur through the gel, and a stack of paper towels was placed on top weighed down by a flat piece of glass to facilitate capillary movement of buffer overnight.

Following overnight transfer of RNA to the membrane, the membrane was washed twice (10 minutes per wash) in 2x SSC before the membrane was air-dried. The RNA was UV cross-linked to the membrane by exposure to ultraviolet light (Hoeffer UVC 500, Hoeffer USA). The membrane was then washed in 5% acetic acid for 15 minutes and stained in methylene blue stain (0.04% methylene blue, 0.5M sodium acetate) for 10 minutes, to determine whether the transfer was successful and also to ensure that the RNA hadn't degraded during the transfer. The methylene blue stain binds reversibly to total RNA on the membrane and clear bands corresponding to 18S and 28S ribosomal RNA indicated intact RNA. Membranes were air-dried and stored in sealed plastic bags at -20°C.

2.2.3 Hybridisation procedure using a labeled cDNA probe

Prior to hybridisation with the tropoelastin cDNA probe, the membrane was pre-hybridised in hybridisation buffer (Appendix 2.1) for at least 4 hours at 42°C to reduce non-specific binding of the labeled probes. Pre-hybridisation and hybridisation was performed in glass hybridisation bottles with mesh to ensure adequate exposure of the entire membrane to the hybridisation solution.

The tropoelastin cDNA probe was labeled with [α^{32}P]-dCTP (Perkin-Elmer, Australia) using a random priming labelling kit (Oligolabelling Kit, Pharmacia, Australia or Rediprime™ II, Amersham Biosciences, Australia), according to kit instructions. The probe was then purified using Sephadex® G-50 DNA Grade columns (NICK™ columns, Pharmacia, Australia) according to manufacturer's instructions, to separate the ^{32}P-labeled probe from unincorporated nucleotides. This involved addition of the reaction mixture directly to the column, which had been pre-washed with TE buffer. TE buffer (400µl) was then added to the column and the resulting elution discarded. Another 400µl of TE buffer was added to the column and this eluant, containing the

labeled cDNA probe, was collected. A 1µl aliquot of the purified probe was counted using a beta liquid scintillation counter (Liquid scintillation systems, LS 5801, Beckman USA) to determine incorporation of ^{32}P into the cDNA probe. The labeled probe was stored at -20°C and used within 14 days.

Labeled cDNA probe was denatured prior to being added to the hybridisation bottle containing the membrane and fresh hybridisation buffer (2x 10^6 counts per minute (cpm)/ml of hybridisation buffer), and incubated for 24 hours at 42°C. The probe binds specifically to the RNA on the membrane by complimentary base pairing. After hybridisation, the membranes were washed twice (30 minutes each) in 1x SSC/0.1% sodium dodecyl sulphate (SDS) at 42°C, and once for 30 minutes in 0.1x SSC/0.1% SDS at 42°C to remove non-specifically bound probe.

2.2.4 Detection and quantification of radiolabelled bands

Following hybridisation, the membranes were air-dried and sealed in airtight plastic bags and placed in a cassette containing a storage phosphor screen (Kodak SO230, Molecular Dynamics, Sunnyvale, USA). The membranes were exposed to the screen for up to 48 hours, with the exposure time being dependant on the amount of radioactivity present on the membrane. During exposure, the ionising radiation on the membrane excites electrons in the phosphor screen to a higher, stable energy state. After the exposure period, the storage phosphor screen was scanned by a phosphoimaging machine (Storm 860, Molecular Dynamics, Sunnyvale, USA). The phosphoimager directs an HE-NE laser beam at the screen, which further excites the electrons in the screen to an unstable energy state from which they return to their ground energy state with the emission of a photon of light. These photons of light are detected by the phosphoimager and are converted into a digital image. The amount of light emitted is proportional to the amount of radioactivity and therefore the amount of probe bound to the RNA on the membrane. Phosphoimagery was chosen over autoradiography because the linear range for the storage phosphor screens is approximately 15 times that of film, making the period of exposure much less critical to ensure that band densities remain within the linear range.

After imaging, the cDNA probes were stripped from the membranes by washing in 0.01x SSC/0.5% SDS at 80°C for 1 hour (2 x 30 minute washes). The membranes were

then re-hybridised with a ^{32}P labeled probe for 18S ribosomal RNA. The 18S is a gene that is constitutively expressed and is used to correct for minor loading differences between the lanes in the gel.

The relative amounts of tropoelastin and 18S were quantified using ImageQuaNT software program (Molecular Dynamics, Sunnyvale, USA). The program measures the optical density of each band on the scanned image. The density of the tropoelastin bands were expressed as a proportion of the density of the corresponding 18S band for each lane.

2.3 Localisation of tropoelastin mRNA in lung tissue by in situ hybridisation

In situ hybridisation experiments were performed in the USA with the assistance of R.A. Pierce and C. Heintz using a bovine specific tropoelastin riboprobe that had previously been determined to bind specifically in ovine fetal lung tissue.

2.3.1 Preparation of ^{35}S-labeled riboprobe

A radiolabelled riboprobe was prepared using a bovine tropoelastin cDNA sequence as a template (Prosser et al., 1989). A 15µl aliquot of ^{35}S UTP was vacuum dried in a microfuge tube and the following were added: DNA template (1µg), 1x transcription buffer, DTT (10mM), Rnasin (1.6U), NTP's (0.5mM), T7 RNA Polymerase (5U, Promega) and Nuclease free H_2O (Ambion) to a final reaction volume of 10µl. The reaction was incubated for 1 hour at 37°C followed by the addition of another 5U of RNA polymerase and a further 1 hour incubation. The riboprobe was purified by addition of RQ1 DNase (2U, Promega) and incubated at 37°C for 15 minutes to degrade the DNA template. The RNA was then extracted by addition of 37.5µl of DEPC treated H_2O and 50µl of phenol/chloroform/isoamyl mixture (25µl phenol and 25µl chloroform/isoamyl (24:1)) and centrifugation at 15,000 x g for 5 minutes at 4°C. The supernatant was transferred to a clean tube and GlycoBlue (30µg, Ambion), 3M sodium acetate (pH 5.2, 5µl) and 95% ETOH (125µl) were added and the sample stored at -70°C overnight to precipitate the riboprobe. The following day, the sample was centrifuged at 4°C for 30 minutes and the RNA pellet was washed with 70% ETOH and

re-suspended in 50µl of DTT (20mM). Both anti-sense and sense riboprobes were prepared to test for probe specificity.

2.3.2 Pretreatment of tissue sections for hybridisation

Paraffin embedded lung tissue sections were cut at 5µm in thickness and mounted on Superfrost-plus (Menzel-Glaser, Germany) microscope slides. Tissue sections were then de-parafinised in Americlear (Allegiance Scientific Products, USA) for 30 minutes and re-hydrated by placing in 100% (x2), 95%, 70%, 50% and 30% solutions of ETOH (5 minutes per change). Following re-hydration, the slides were washed for 5 minutes in DEPC-treated PBS (PBS/d, Appendix 2.1) on an orbital shaker and then treated by incubation at 37°C for 30 minutes with nuclease-free Proteinase K (Sigma, 10µl of 10µg/ml stock to 200ml of pre-warmed PBS/d). The slides were transferred into 0.1M triethanolamine (Sigma) for 5 minutes with continuous stirring. After this time, 500µl of 0.25% acetic anhydride (Sigma) was added, and the slides bathed in the solution with stirring for a further 10 minutes. Finally, the slides were washed in 2 changes of 2x DEPC-treated SSC (SSC/d, Appendix 2.1) for 5 minutes each and dehydrated through graded ETOH (30%-100%; 5 minutes per change).

2.3.3 Hybridisation of tissue sections

Following pretreatment, the slides were baked for 30 minutes at 60°C and then air-dried to ensure firm adherence of the tissue sections to the slides. Anti-sense and sense probes were prepared such that the hybridisation solution (UltraHyb, Ambion) contained 40,000cpm/µl of probe and 20mM DTT. The hybridisation solution was then denatured by incubation at 69°C for at least 10 minutes. Denatured hybridisation solution (approximately 50µl) was added to each tissue section and the sections were covered with Parafilm (Pechiney plastic packaging, USA) fitting the tissue outline to prevent the solution from drying out or running off the tissue sections. The slides were placed in a sealed humidified container (containing paper towels soaked in 1:1 formamide/4x SSC/d) and hybridised overnight at 50°C.

The following day, the slides were washed and treated with RNase A (Promega, Australia) to remove any probe that had not bound specifically to tropoelastin mRNA in the tissue. The slides were washed in 4x SSC/d plus 10mM DTT for 15 minutes on an

orbital shaker followed by 5 minutes in 0.5x SSC/d plus 10mM DTT and then in 0.1x SSC/d plus 10mM DTT at 60°C for 15 minutes. The slides were then equilibrated in RNase A buffer (Appendix 2.1) at 37°C for 1 minute followed by RNase A treatment (20µl of 10µg/ml stock to 15ml of pre-warmed RNase A buffer) at 37°C for 30 minutes. After RNase A treatment, the slides were placed in RNase A buffer at 37°C for 30 minutes, 2xSSC/d at RT for 30 minutes, 0.1xSSC/d at 60°C for 15 minutes and finally 0.1xSSC/d at RT for 30 minutes before being dehydrated through graded ETOH (30%-100%) and then air-dried.

In order to estimate the appropriate exposure time to photographic emulsion, the slides were exposed to film overnight to visualise the intensity and pattern of radioactivity on the tissue sections. The slides were then dipped in photographic emulsion (NTB2 emulsion, Kodak, USA) and left to dry overnight before being placed in a plastic slide container wrapped in opaque plastic and left to expose at 4°C for the appropriate amount of time (1-6 weeks). At the end of the exposure period, the slides were developed by placing in developer (1:1 dilution, Kodak D19 developer) for 4 minutes, H_2O for 30 seconds, fixer (Kodak fixer) for 5 minutes and finally H_2O. Slides were counterstained with Haematoxylin and Eosin, viewed under a light microscope and images captured using a digital camera.

2.4 Determination of the elastin content of the lungs

2.4.1 Microdissection of tissue to remove larger airways and vessels

Lung tissue was micro-dissected to remove larger airways and blood vessels (>20µm in diameter) and pleural membrane before assaying for elastin in order to focus specifically on elastin concentration and content in the alveolar region of the lung.

2.4.2 Quantification of elastin concentration in parenchymal tissue

Elastin concentration in lung tissue was quantified using a modification of an established method (Naum & Morgan, 1973). Briefly, 0.8g to 1.0g of lung tissue was micro-dissected and re-weighed before being homogenised in 0.15M NaCl with a final volume of 10ml. The resulting homogenate was divided equally for duplicate analysis. The homogenate was centrifuged at 2600 x g and the supernatant removed. The

remaining pellet was re-suspended in 5M guanidine hydrochloride and incubated at 25°C for 24 hours and then centrifuged at 20,800 x g for 20 minutes. The pellet was then extracted for a further 24 hours in 5M guanidine hydrochloride as above to remove all soluble proteins from the tissue extract. Following guanidine hydrochloride treatment, the tissue extract was washed and re-suspended in distilled water and autoclaved at 121°C to solubilise and remove collagen. The pellet was again centrifuged and washed with distilled water. The remaining tissue extract, now considered to be elastin, was made soluble by elastase digestion (Sigma, porcine pancreas type III, 0.1mg/ml in 0.02M $NaHCO_3$ buffer, pH 8.8) and the resulting peptides were quantified using a standard protein assay. The intra and inter-assay coefficients of variation were 8.7% and 13.1% respectively. Amino acid analysis was performed (Auspep, Melbourne, Australia) on the guanidine and autoclave resistant tissue extract to confirm that it had an amino acid composition consistent with that of pure elastin. In these samples, both cysteine and methionine were undetectable which is consistent with the sample being pure elastin. In addition, the amino acid composition of the sample was compared to that of the predicted composition from the published cDNA sequence (Yoon et al., 1985) by regression analysis and the amino acid compositions were found to be significantly correlated ($p < 0.0001$, $r^2 = 0.79$). The elastin content of the lung was estimated by multiplying elastin concentration (mg/g lung) by lung weight. This value is an estimate of lung elastin content in alveolar parenchymal tissue only, as it is derived by extrapolation from a small amount of tissue in which airways and blood vessels had been removed.

2.5 Elastin staining

Elastin staining was performed in the USA with the assistance of R. A. Pierce and C. Heintz. Lung tissue sections, embedded in paraffin, were cut at 5µm in thickness, stained using the Hart's resorcin-fuchsin stain for elastin and counter stained with tartrazine 0.25% in saturated picric acid. Paraffin sections were de-pariffinised in Americlear for 30 minutes and re-hydrated to distilled water by placing in 100%, 95%, 70%, 50% and 30% solutions of ETOH followed by distilled water. The slides were then placed in a 0.25% Potassium permanganate solution (Sigma) for 5 minutes, rinsed in distilled water and placed in a 5% Oxalic acid solution (Sigma) until the sections were clear. Slides were rinsed in tap water for 5 minutes and then placed in distilled

water before leaving in resorcin-fuchsin working solution (10ml Resorcin-fuchsin solution (Polyscientific), 100ml 70% ETOH, 2ml hydrochloric acid) overnight. The following day, the slides were rinsed in tap water for 10 minutes, followed by distilled water, and counterstained with 0.25% tartrazine (Sigma) in saturated picric acid. Finally, slides were washed by immersing in 95% ETOH and 100% ETOH 20 times each, cleared twice in xylene and mounted. Stained sections were viewed under the light microscope and images were captured using a digital camera.

2.6 Data analysis

Data are presented as mean ± SEM. In all Northern blots, each lane contained total RNA from a single animal. The integrated density of the tropoelastin mRNA band was divided by that of the 18S rRNA band for each lane to adjust for minor loading differences between lanes. In most cases, comparisons were made between mRNA samples that were run on the same Northern blot, thereby exposing the mRNA to the same hybridisation conditions and exposure times. The only exception was for ontogeny (Chapter 3) where samples were run on three separate northern blots. In this case, the same five samples from 128 day fetuses were run on each blot and data was expressed as a percentage of mean 128 day levels to allow for comparison between different Northern blots. Statistical analyses of all data were performed using the Student's unpaired *t*-test, or one-way analysis of variance (ANOVA). Significant differences indicated by ANOVA were subjected to the Student Newman Keuls post-hoc test to detect significant differences between individual group means. Statistical significance was taken at $p<0.05$.

2.7 *Appendix*

2.7.1 Appendix 2.1 (Solutions)

4M guanidine thiocyanate solution
4M guanidine thiocyanate
0.02M N lauroyl sarcosine
0.02M Na Citrate
0.5% v/v Antifoam A
0.7% v/v β-mercaptoethanol (added immediately before use)

Tris EDTA (TE) buffer
10mM Tris
1mM EDTA
pH 7.5

Loading buffer / Northern loading buffer
0.25% bromphenol blue
0.25% xylene cyanol FF
30% glycerol

Luria-Bertani (LB) medium
10g/L NaCl
10g/L Tryptone
5g/L yeast extract
1% v/v 1N NaOH
pH 7.0

Northern buffer 20x stock solution
0.4M MOPS
4M Sodium acetate
0.01M EDTA

Saline-Sodium Citrate (SSC) 20x stock solution
3M NaCl
0.3M tri-sodium citrate

0.1% DEPC for DEPC treated working solutions (SSC/d)

Saline-Sodium Phosphate EDTA (SSPE) (20x stock solution)
3M NaCl
0.2M $NaH_2PO_4.H_2O$
0.02M EDTA

Hybridisation buffer
50% v/v deionised formamide
7% w/v sodium dodecyl sulphate (SDS)
5x SSPE
10mg/100ml denatured Salmon Sperm DNA (Roche diagnostics, Australia)

Phosphate Buffered Saline (PBS)
0.01M KH_2PO_4 (pH 7.4)
0.01M K_2HPO_4 (pH7.4)
0.15M NaCl

0.1% DEPC for DEPC treatment (PBS/d)

RNase A buffer
0.01M NaCl
10mM Tris
1mM EDTA
0.1% DEPC

Tris Acetic acid EDTA (TAE) buffer (50x stock)
0.5M Tris
57.1ml/L glacial acetic acid
0.05M EDTA

Chapter 3

Ontogeny of elastin synthesis in the developing sheep lung

3.1 Introduction

Elastin is a major structural component of the mammalian lung and is essential to its development and function. Its unique physical properties allow for the repeated deformation and recoil that is fundamental to the mechanical performance and function of the lung (Mariani et al., 1997). The distribution of elastic fibres within the lung is also essential to its structural integrity by providing supportive scaffolding for the delicate alveolar structures during respiration. In addition to its importance for mechanical performance, elastin also appears to play a critical role in the structural development of the lung, particularly in relation to the distal airways (Wendel et al., 2000) and alveoli (Lindahl et al., 1997). Elastin synthesis in the lung of mammalian species occurs primarily during the late fetal and early neonatal periods of development, and peaks during alveolarisation (Mariani et al., 1997). In rats, from which much of the data on elastin expression has been derived, tropoelastin mRNA is first detectable in the pseudoglandular period of lung development, peaks during alveolarisation in the neonate and is barely detectable in the adult (Noguchi & Samaha, 1991). This indicates that there is a critical period of lung development during which perturbations that affect elastin synthesis could potentially have long-term effects on lung structure and function. In contrast to lung development in rats, alveolarisation in long-lived, long-gestation species such as sheep (Alcorn et al., 1981) and humans (Langston et al., 1984) begins before birth and therefore the developmental timing of elastin synthesis is different. In

these species, factors that affect elastin synthesis during development may be expected to be different compared to short-gestation, altricial species such as rats and mice.

In experiments reported in this thesis, sheep have been used as the ovine fetal environment can be easily manipulated, fetal physiological measurements can be made and sheep are a long gestation species in which alveolar formation begins before birth as in humans (Alcorn et al., 1981; Langston et al., 1984). Tropoelastin production in lung explant cultures (Shibahara et al., 1981) and elastin accumulation in the sheep lung have been shown to rise dramatically before birth, during the alveolar period of lung development (Schellenberg & Liggins, 1987; Shibahara et al., 1981). Although the development of elastic fibres in the lung of fetal and postnatal sheep has also been characterised (Fukuda et al., 1984), there have been no previous studies of the temporal or spatial pattern of tropoelastin expression in the lung of fetal and postnatal sheep *in vivo*. In particular, there have been no previous studies of elastin expression from fetal to adult life in a long-gestation, long-lived species. Therefore, the aims of this study were to characterise tropoelastin expression from approximately mid-gestation, in the early canalicular period of lung development (Alcorn et al., 1981), to maturity (~2 years postnatal age) in sheep and to relate levels of expression to changes in elastin content and staining over the same period.

3.1.1 Experimental preparation

Lung tissue was obtained from fetuses of date-mated Border Leicester X Merino sheep at gestational ages of 90 days (n=5), 105 days (n=5), 111 days (n=4), 122 days (n=4), 125 days (n=5), 128 days (n=5), 138 days (n=4) and 142 days (n=5), from postnatal lambs at 6 hours (n=4), 2 weeks (n=5) and 8 weeks after birth (n=5) and from mature sheep at ~2 years of age. Term in this breed of sheep is approximately 147 days of gestation and sexual maturity is reached at 9-12 months. All lung tissue was obtained from either sham-operated or unoperated control animals which had not experienced any experimental manipulation. Ages of animals used in each experiment varied slightly depending on lung tissue available at the time each experiment was performed.

In all animals, the lungs were removed at autopsy and the left main bronchus ligated, after which portions of the left lobes were removed, frozen in liquid nitrogen and stored at -70°C. Pieces of the left lung were used for extraction of total RNA for Northern blot

analysis (section 2.2) and for the determination of elastin content (section 2.4). Lung tissue was micro-dissected prior to measurement of elastin content to remove larger airways and blood vessels so as to focus my findings on the alveolar region of the lung. The right lung was fixed via the trachea at 20 cmH_2O using 4% paraformaldehyde, sliced into 5mm sections and post-fixed in Zamboni's fixative overnight. All fixed tissue samples were embedded in paraffin and sectioned at 5μm for in situ hybridisation (section 2.3) and histochemical analysis (section 2.5) to localise elastin mRNA expression and elastin deposition in lung tissue.

3.2 Results

3.2.1 Tropoelastin mRNA levels

Northern blot analysis

As shown in figure 3.1, tropoelastin mRNA levels in the developing lung of sheep increased dramatically between 90 days of gestation and term. Maximum tropoelastin mRNA levels occurred just prior to term and levels remained elevated at 2 weeks after birth. By 8 weeks after birth, mRNA levels had decreased to levels similar to those of 111 day fetuses and were barely detectable in the adult sheep lung at ~2 years of age. Expressed as a percentage of mean values of 128 day fetuses, tropoelastin mRNA levels at 90 days gestational age (GA) were only 19.0 ± 2.8%, but increased significantly at 105 and 111 days GA to 53.6 ± 12.0% and 44.6 ± 6.3% respectively. Tropoelastin mRNA levels again increased significantly between 111 and 128 days GA and continued to rise to 125.0 ± 11.6% at 138 days GA, 169.8 ± 18.5% at 142 days GA and 177.8 ± 28.2% at 2 weeks after birth (Figure 3.1). By 8 weeks after birth, tropoelastin mRNA levels had significantly decreased to 56.0 ± 4.7%, and by ~2 years after birth were barely detectable at only 5.8 ± 0.7% of levels of 128 day fetuses (Figure 3.1). Overall, tropoelastin mRNA levels increased approximately 10 fold between 90 days GA and near term (142 days GA – 2 wks postnatal age (PNA)), when peak mRNA levels occurred, and these peak mRNA levels were approximately 30 fold higher than those of adult sheep lungs.

In situ hybridization

Tropoelastin expression was prominent in pulmonary arteries and minimal or not detectable in airways of fetal sheep lungs between 90 and 142 days of gestation and in neonatal sheep lungs at 6 hours after birth (Figure 3.2). Tropoelastin mRNA was not detectable in pulmonary arteries or airways in the adult sheep lung at ~2 years after birth (Figure 3.2). In the alveolar region of the lungs, tropoelastin mRNA could not be detected at 90 days of gestation, but by 111 days of gestation, tropoelastin mRNA was localised to the tips of developing secondary septa and at bends in alveolar walls where secondary septal formation would be expected to occur (Figure 3.3). Between 111 days of gestation and term, the number of sites of tropoelastin expression in the alveolar region of the lung increased at each age, and were highest in the near term fetus and at 6 hours after birth (Figure 3.3). At 2 years after birth in the adult sheep lung, tropoelastin expression was located only at a small number of sites in the alveolar region of the lung (Figure 3.3). With increasing gestational age, tropoelastin expression in pulmonary arteries appeared to decrease while parenchymal tropoelastin expression increased (relative to each other). However, in this study, it could not be established whether there were any differences in elastin expression between blood vessels of different sizes.

3.2.2 Elastin deposition

Elastin concentration and content

As shown in figure 3.4 the concentration of elastin in fetal lung parenchymal tissue increased significantly between 90 days (1.0 ± 0.1 mg/g lung) and 111 days GA (1.8 ± 0.02 mg/g lung), after which it continued to increase steadily until just prior to term at 142 days GA (3.0 ± 0.2 mg/g lung). Values between 111 days and 142 days of gestation were not significantly different when assessed by one-way ANOVA, but tended to increase over this time. Elastin concentration at 2 weeks after birth was similar to values in the near-term fetus (2.8 ± 0.6 mg/g lung) and then increased significantly by 8 weeks (6.5 ± 0.2 mg/g lung) and remained higher at 2 years postnatal age (5.2 ± 0.5 mg/g lung)(Figure 3.4).

The elastin content of the lungs increased significantly between 90 days (26.8 ± 3.4 mg) and 122 days GA (167.5 ± 44.0 mg) and again between 122 days and 142 days of GA (486.9 ± 40.0 mg). At 2 weeks after birth, lung elastin content was apparently lower

(326.7 ± 62.03 mg) than in the near term fetus, but was increased significantly at 8 weeks (1460.2 ± 149.0 mg) and 2 years postnatal age (2710.5 ± 299.2 mg) (Figure 3.4).

Elastin staining

Elastin staining in pulmonary arteries and airways was already well established in sheep fetuses at 90 days of gestation, and the nature of elastin staining in blood vessels and airways did not appear to change appreciably over gestation. Elastin staining in blood vessels and airways has not been presented in a figure because vessels and airways of comparable size could not be shown over the wide range of gestational ages with the tissue sections that were available. In addition, the focus of this study and subsequent studies in this thesis is on elastin synthesis in the later stages of fetal lung development in the alveolar parenchyma in relation to alveolar formation and development.

At 90 days of gestation in the sheep, during the canalicular period of lung development, elastin staining in the walls of the multiple airspaces (termed canals at this stage) (Alcorn et al., 1981) was barely detectable by Hart's stain under the light microscope at X20 magnification. At X100 magnification, however, minimal elastin staining could be detected at sites of subdivision and branching of the canals (Figure 3.5). By 111 days of gestation, elastin staining was clearly observed at the tips of newly developing secondary septa and in alveolar walls where septal formation would be expected to occur (Figure 3.5). Between 122 and 142 days of gestation alveolar development continued by formation and outgrowth of new secondary septa, such that by 142 days of gestation, a vast network of thin walled alveoli had been formed with elastin staining concentrated at the tips of secondary septa and at sites of newly forming secondary septa (Figure 3.5). Alveolar formation and elastin deposition after birth in the neonate occurred in a similar manner to that of the near term fetus. At 2 years after birth in the adult sheep lung, elastin staining was focused in discrete aggregates in the walls of mature alveoli (Figure 3.5).

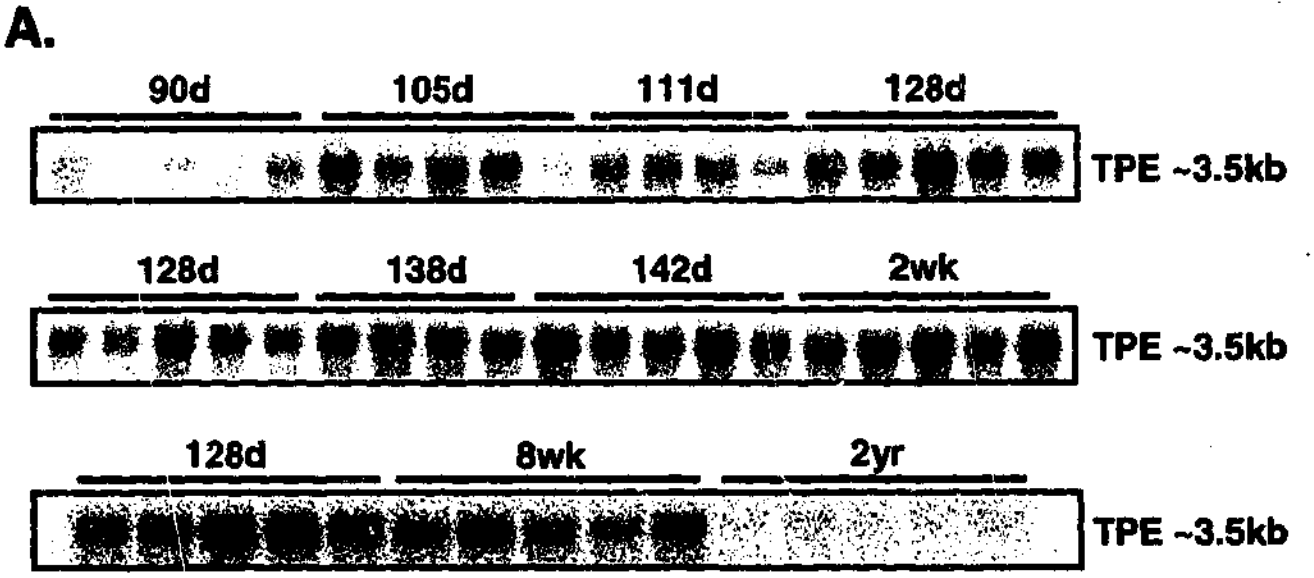


Figure 3.1 Ontogeny of tropoelastin (TPE) mRNA levels in the lung
A: Northern blot analysis of total RNA extracted from lung tissue collected from unmanipulated control fetal and postnatal sheep at increasing ages. *B*: Mean tropoelastin mRNA levels expressed as a ratio of the density of the corresponding 18S rRNA band (not shown for simplicity) and then expressed as a percentage of mean values at 128 days GA to allow comparison between different Northern blots. Values that do not share a common letter are significantly different from each other ($p<0.05$).

Figure 3.2 Ontogeny of tropoelastin mRNA of blood vessels and airways
In situ hybridisation for tropoelastin mRNA in blood vessels and airways in fetal and postnatal sheep lung at 90 days GA, 111 days GA, 142 days GA, 6 hours PNA (postnatal age) and 2 years PNA. Presented are paired bright- and dark-field photographs taken at X20 magnification of fetal and postnatal sheep lung hybridised with 35 S-labeled antisense RNA for tropoelastin mRNA. A strong positive signal for tropoelastin mRNA was detected in blood vessels (BV) of fetal sheep lung between 90 and 142 days of gestation. Relative to parenchymal tropoelastin mRNA, the signal in vessels appeared to decrease with gestation and was absent in lung from mature 2 year old sheep. Minimal or no signal for tropoelastin mRNA was detected in airways (A) of lungs at all ages.

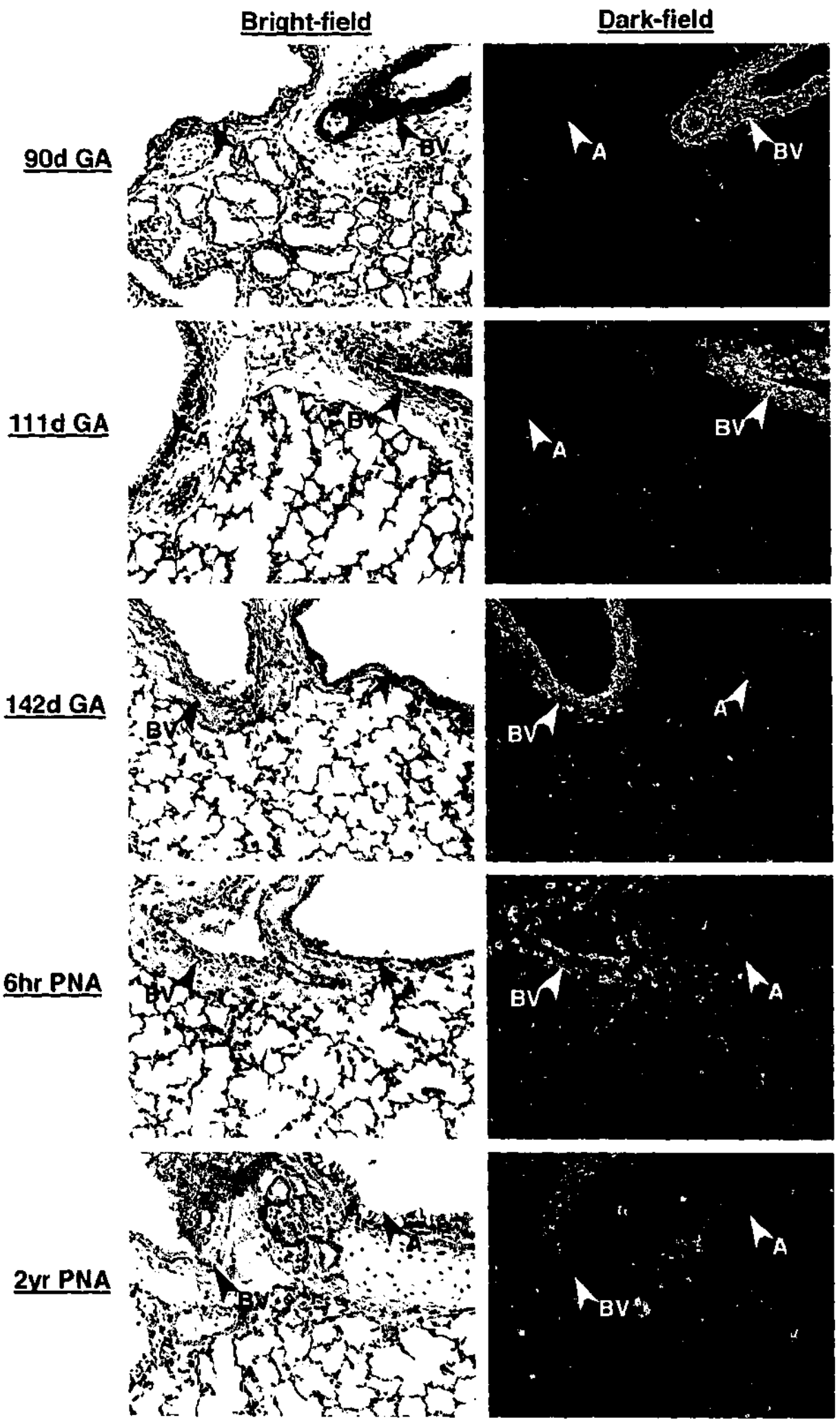
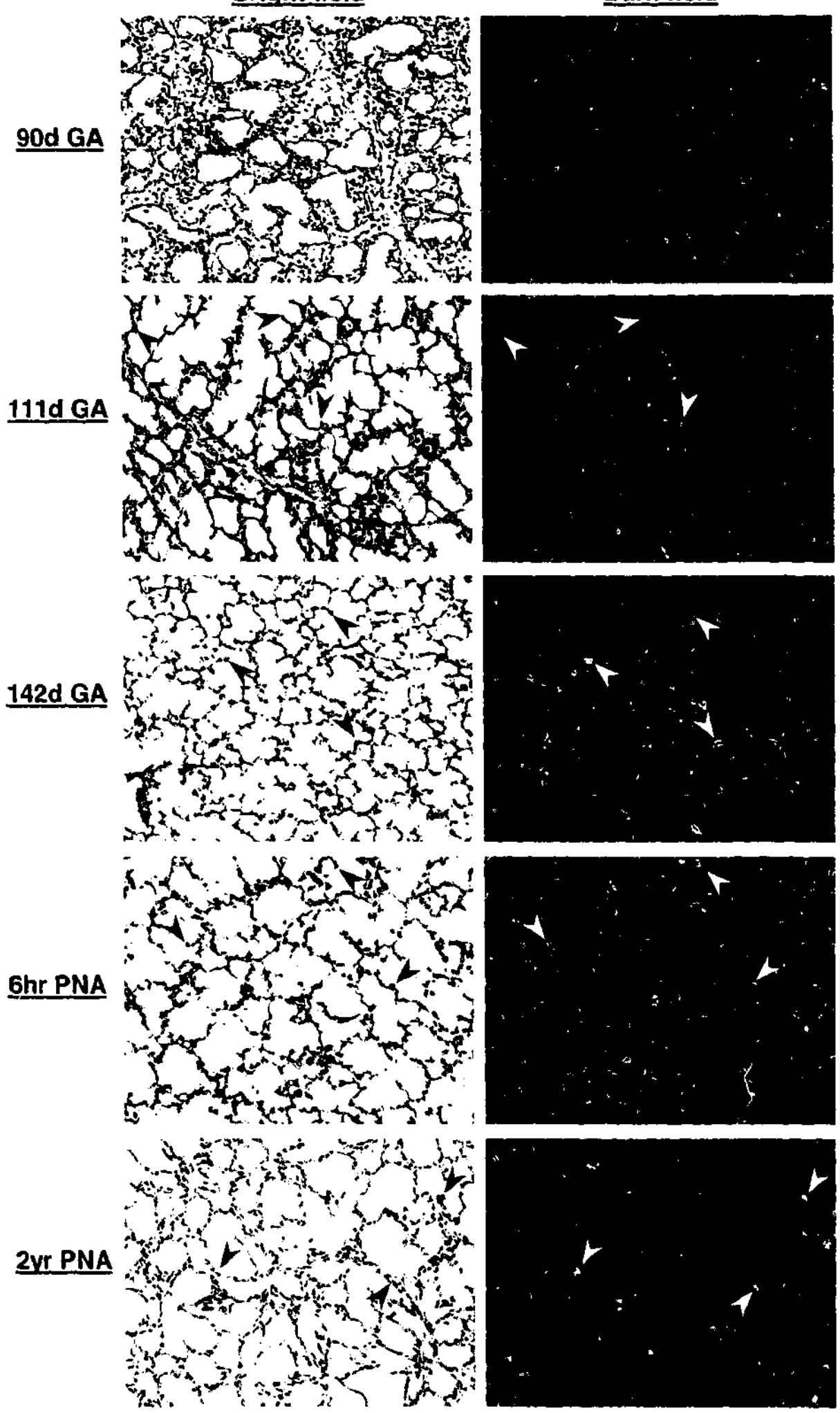

Bright-field
Dark-field
90d GA
111d GA
142d GA
6hr PNA
2yr PNA
A
BV

Figure 3.3 Ontogeny of tropoelastin mRNA in the alveolar region of the lungs
In situ hybridisation for tropoelastin mRNA in lung parenchymal tissue Presented are paired bright- and dark-field photographs taken at X20 magnification of fetal and postnatal sheep lung hybridised with ^{35}S-labeled antisense RNA for tropoelastin mRNA. No signal was detected in parenchymal tissue from 90 day fetuses. At 111 days GA, tropoelastin mRNA signal was detected at the tips of newly developing secondary septa (arrow heads). The number of sites of tropoelastin mRNA signal increased with gestational age and was highest at 142 days GA and 6 hours after birth (6hr PNA). A small number of sites with a positive signal for tropoelastin were detected in alveolar walls of lungs from 2 year old sheep (2yr PNA).


Bright-field
Dark-field
90d GA
111d GA
142d GA
6hr PNA
2yr PNA

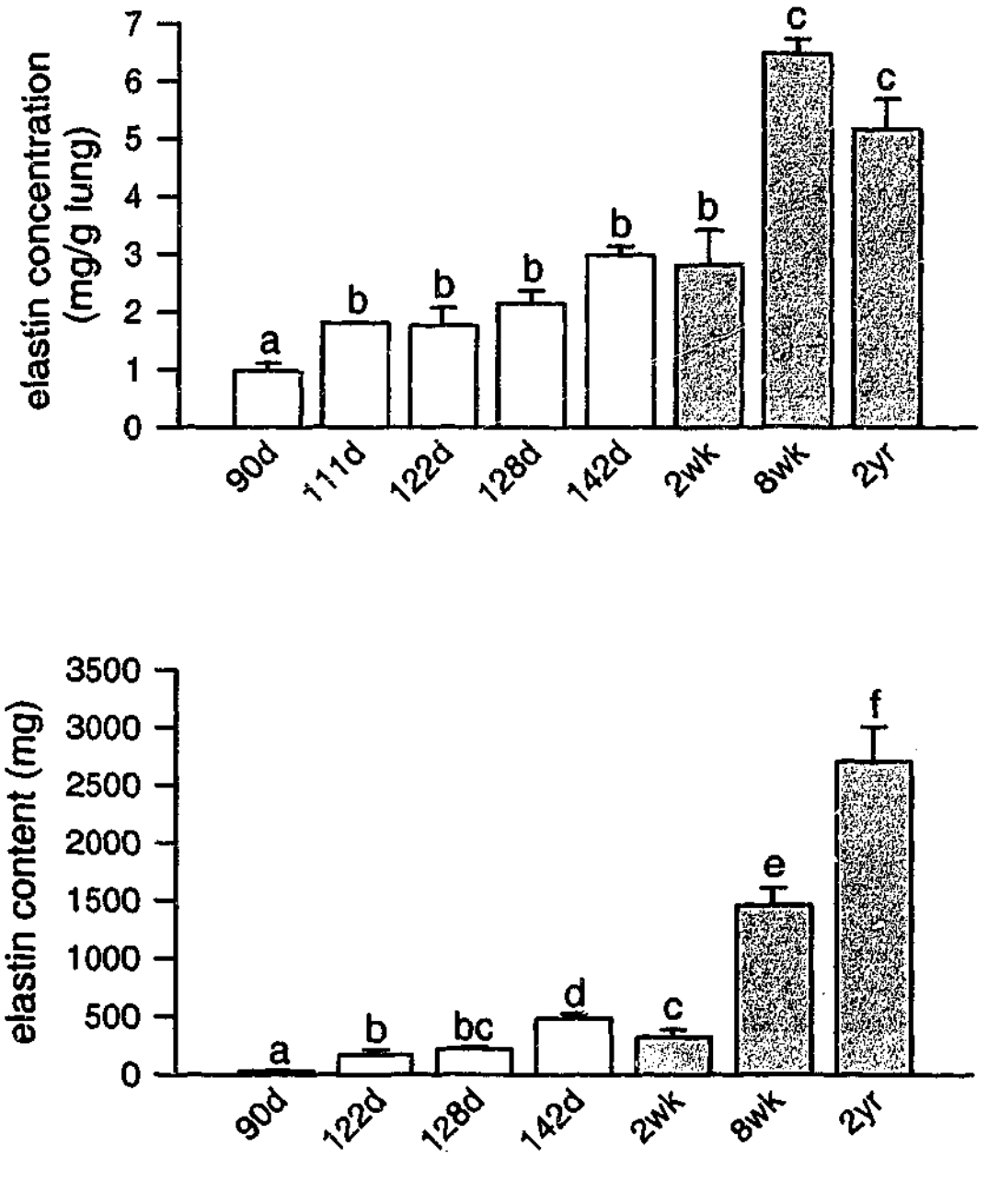
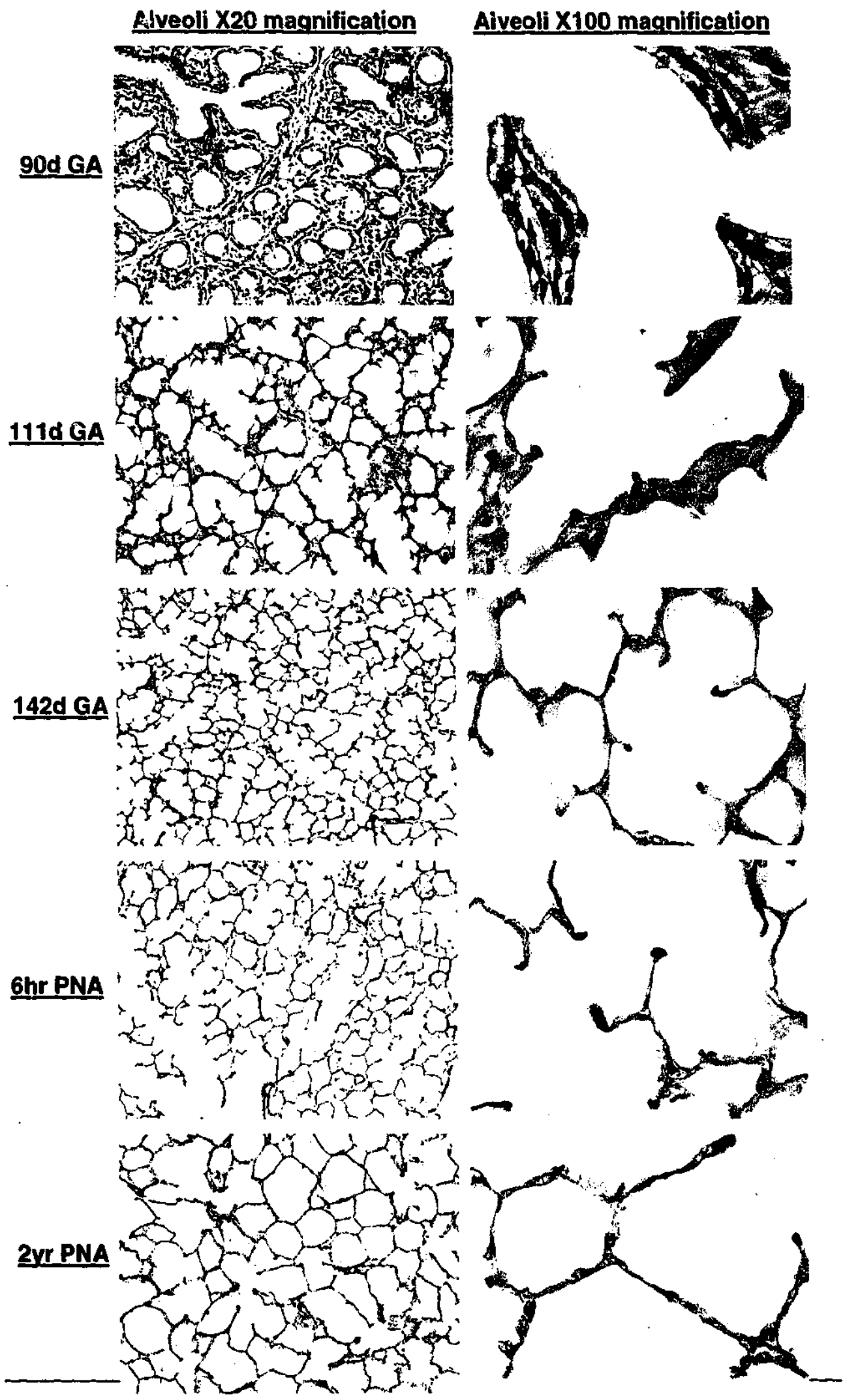


Figure 3.4 Ontogeny of elastin concentration and elastin content of the lungs in sheep

Elastin concentration and elastin content of the lungs of unmanipulated control fetuses and postnatal sheep at various ages. Values that do not share a common letter are significantly different from each other ($p<0.05$).

Figure 3.5 Ontogeny of elastin staining in the alveolar region of the lungs in fetal and postnatal sheep

Hart's resorcin-fuchsin stain for elastin in lung tissue sections from fetal and postnatal sheep at 90 days GA, 111 days GA, 142 days GA, 6 hours after birth and 2 years after birth. Photographs taken at X20 and X100 magnification of the alveolar region of the lung are presented for each age group. Elastin is stained dark purple-black and is focused at the tips of secondary septal crests from 111 days GA. At 90 days of gestation elastin staining in the walls of the multiple airspaces termed canals at this stage was barely detectable by Hart's stain under the light microscope at X20 magnification, but at X100 magnification elastin staining could just be detected at sites of subdivision and branching of the canals.


Alveoli X20 magnification
Alveoli X100 magnification
90d GA
111d GA
142d GA
6hr PNA
2yr PNA

3.3 *Discussion*

This study provides an overview of the ontogeny of elastin synthesis and content in the ovine lung *in vivo* between the canalicular period of lung development in late gestation and at maturity. An ontogenic assessment of elastin synthesis from tropoelastin expression to mature elastin deposition was performed to provide a foundation for subsequent studies in this thesis. Such a study had not previously been performed in sheep. Consistent with the developmental pattern of elastin synthesis in rat lungs, the peak in elastin synthesis in the parenchyma of sheep lungs was found to be associated with alveolar development.

3.3.1 Tropoelastin expression

Tropoelastin mRNA levels assessed by Northern blot analysis in the fetal and postnatal sheep lung *in vivo* increased dramatically during the later stages of fetal lung development and were highest near term (at 142 days of gestation) and in the neonate at 2 weeks after birth. Between 2 and 8 weeks after birth, tropoelastin mRNA levels rapidly decreased to levels similar to those of 111 day old fetuses and were barely detectable in the mature sheep lung at ~2 years after birth. When assessed by *in situ* hybridisation, it became apparent that, while the majority of the tropoelastin mRNA detected by Northern blot at 90 days of gestation in the fetus was contributed by vascular tropoelastin mRNA, from 111 days of gestation onwards, tropoelastin mRNA in the alveolar region of the lung increased relative to the vasculature. These data indicate that the steep rise in tropoelastin mRNA levels towards the end of gestation in sheep, as in rats, is primarily associated with the rapid increase in septal wall formation and alveolar development that occurs at this time. This pattern of tropoelastin expression in the developing sheep lung is consistent with the increase in the rate of tropoelastin production observed from 112 days of gestation until term in cultured fetal sheep lung explants that had been dissected free of major airways and blood vessels (Shibahara et al., 1981). Interestingly, although elastin synthesis was barely detectable in the mature sheep lung by Northern blot analysis at 2 years after birth, *in situ* hybridisation results indicate that a small number of sites of tropoelastin expression are still present in the alveoli of mature sheep. The temporal and spatial pattern of tropoelastin expression observed in sheep lung in this study is similar to that observed in rats, however, the developmental timing of tropoelastin expression is different. In a

similar ontogenic study in rats, *in situ* hybridisation studies showed that tropoelastin expression began at gestational day 17, during the pseudoglandular period of lung development, peaked between postnatal days 7 and 11, during the period of alveolarisation, declined substantially by postnatal day 21 and was virtually absent in the adult rat lung (Noguchi & Samaha, 1991). As in the present study, in which tropoelastin expression in the pulmonary vasculature appeared to peak before the peak in tropoelastin expression in the alveolar region of the lung, peak tropoelastin expression in pulmonary vessels in rats occurred between day 19 of gestation and postnatal day 1 (Noguchi & Samaha, 1991).

3.3.2 Elastin deposition

Elastin concentration and content

The elastin concentration (mg/g of lung) of lung tissue following the removal of larger vessels and airways, increased significantly between 90 days of gestation and 142 days of gestation (near term), similar to the rise in tropoelastin mRNA levels. The increase in elastin concentration before term, however, did not appear to be as steep as the increase in tropoelastin mRNA levels and, although elastin concentration apparently increased between 111 and 142 days of gestation, this increase was not statistically significant when assessed by one-way ANOVA. This was an unexpected result since *in situ* hybridisation experiments showed that the major increase in tropoelastin expression late in gestation occurred in the alveolar region of the lung and the rate of increase in elastin concentration in fetal sheep lung in another study was found to be greater in dissected tissue than in non-dissected tissue (Schellenberg & Liggins, 1987). The failure to detect a significant difference in elastin concentration in fetal lung tissue between 111 days of gestation and term in this study may be attributable to the sensitivity of the assay used to measure elastin. In the study by Shellenberg and Liggins (1987), elastin concentration was measured by a radioimmunoassay for desmosine (a cross-linking amino acid specific to elastin) and is a more sensitive method for assessing elastin concentration than the method used in this study. The radioimmunoassay for desmosine was unfortunately unavailable to me for use in this study.

At 2 weeks after birth, pulmonary elastin concentration, as for tropoelastin expression, was similar to that measured at 142 days of gestation in the near term fetus.

Surprisingly, in contrast to the changes observed in tropoelastin expression, elastin concentration between 2 and 8 weeks after birth increased significantly and elastin concentration was similar at 8 weeks and 2 years after birth. The increase in elastin concentration between 2 and 8 weeks after birth suggests that elastin synthesis and alveolar development in sheep continues up to, and beyond, 8 weeks after birth. This is supported by a study that has shown that alveolar number in neonatal sheep doubles between birth and 8 weeks (Davey et al., 2001). The magnitude of the increase in elastin concentration at 8 weeks, despite a significant drop in tropoelastin mRNA levels, is surprising; however it is likely that elastin concentration at 8 weeks and 2 years after birth may have been overestimated due to the difficulty in dissection of airways and vessels from lung tissue at these ages. Also, although there is a large difference in tropoelastin mRNA levels in whole lung between 2 and 8 weeks after birth, the proportion of mRNA contributed by the alveolar region of the lung versus vascular tissue is likely to be higher at 8 weeks. This is supported by data obtained from my *in situ* hybridisation experiments showing that tropoelastin expression appears to decrease in the vasculature and increase in the alveolar region of the lung with advancing gestational and postnatal age.

The elastin content of the lungs before birth increased significantly between 90 and 122 days of gestation and between 122 and 142 days of gestation. After birth, elastin content apparently decreased at 2 weeks after birth and then increased markedly at 8 weeks after birth and again at ~2 years after birth. These changes in elastin content over gestation largely reflect changes in lung weight because elastin content is a derivation of elastin concentration (mg/g lung) multiplied by lung weight. Therefore the significant decrease in elastin content measured at 2 weeks of age is likely to be a result of a small decrease (not statistically significant) in wet lung weight at this age rather than an actual decrease in elastin synthesis. The apparent decrease in wet lung weight at 2 weeks after birth is likely to be due to the lungs transition from a liquid filled to an air filled organ. Although fetal lungs were drained of lung liquid before weighing, it is possible that some liquid remained trapped in alveoli and therefore contributed to lung weight leading to an overestimation of fetal lung weight near term, and therefore an apparent decrease in lung weight at 2 weeks post-term. In combination with elastin concentration, these data indicate that elastin synthesis in lung parenchymal tissue continues between 8 weeks and 2 years after birth in sheep, albeit at a substantially slower rate than in the

fetus and neonate. Estimates of elastin accumulation rates (elastin content g/day) indicate that elastin accumulation appears to increase approximately 4 fold between 90 days of gestation and 142 days (near term). Elastin accumulation then appears to continue at a similar rate throughout the neonatal period to 8 weeks and decreases approximately 10 fold between 8 weeks and 2 years postnatal age.

Elastin staining

Elastin staining in pulmonary arteries was already well established at 90 days of gestation during the canalicular period of lung development in the sheep fetus. This is consistent with the high level of tropoelastin mRNA in blood vessels compared to parenchymal tissue in the 90 day old fetus and further highlights the difference in the developmental timing of tropoelastin expression in blood vessels compared to alveoli. Elastin staining in the parenchyma of 90 day old fetal lungs by the Hart's method was only just discernable at X100 magnification. Previous studies in sheep, however, have demonstrated elastic fibres by transmission electron microscopy as early as 47 days of gestation during the pseudoglandular stage of lung development (Fukuda et al., 1983). In that study, elastin staining in the primitive alveolar region of the lung prior to alveolar development was primarily associated with bifurcations of the primitive airspaces (Fukuda et al., 1983). This observation is similar to the staining observed in the present study at 90 days under the light microscope at X100 magnification, where staining was apparent at bifurcations of canals in lung parenchyma. Elastin staining at 111 days of ovine gestation showed the beginning of alveolar development in the late canalicular stage of lung development. Elastin staining at this stage of development was concentrated at sites of secondary septal crest formation and at the tips of developing secondary septa. From 111 days of gestation, in parallel with increases in both alveolar tropoelastin expression and elastin concentration and content, elastin staining continued to be associated with the formation of secondary septa. Changes in elastin staining in the airways and vasculature after 90 days in fetal and postnatal sheep if present were unable to be detected in this study most likely because elastin deposition in these structures is established at an earlier stage of lung development.

3.3.3 Conclusions

This study has shown that elastin synthesis from 90 days of gestation in the ovine fetal lung *in vivo* rises sharply towards term and is maximal during the period of alveolarisation which occurs before birth and shortly after birth in this species. This rise in elastin expression and deposition also appeared to be mainly associated with alveolar development rather than vascular development at this stage. Therefore, perturbations in the fetal environment that affect elastin synthesis during this stage of development are likely to have a major effect on alveolar development. In addition, the tight window during which elastin synthesis occurs in the lung, and the minimal synthesis and turnover of elastin in the healthy adult lung, indicate that altered elastin synthesis during this critical period of development could have long lasting effects on lung structure and function.

Chapter 4

Influence of lung expansion on elastin synthesis and deposition in the ovine fetal lung in vivo

4.1 Introduction

It is well established that mechanical forces within the lungs during fetal life play an integral role in lung growth and maturation (Harding & Hooper, 1996). In the fetus, the lungs are filled with a liquid that is secreted across the pulmonary epithelium into the future airspaces and leaves the lungs via the trachea. This liquid maintains the lungs in an expanded state and provides the tissue stretch that is essential for normal lung development (Alcorn et al., 1977). Sustained increases in lung liquid volume by obstruction of the fetal trachea in sheep result in an accumulation of lung liquid within the lung's "airspaces", imposing an additional stretch stimulus on lung tissue, which is a potent stimulus of lung growth (Nardo et al., 1998). DNA synthesis rates in the fetal lung have been shown to increase by 777% following 2 days of tracheal obstruction (Nardo et al., 1998). Conversely, prolonged reductions in lung liquid volume cause a decrease in fetal lung expansion and a virtual cessation of lung growth (Moessinger et al., 1990; Nardo et al., 1995). Alterations in fetal lung expansion not only alter the rate of lung growth but also influence structural maturation, including alveolar development (Alcorn et al., 1977; Nardo et al., 2000). Alveolar number is increased following sustained increases in fetal lung expansion (Nardo et al., 2000) and decreased following prolonged periods of decreased lung expansion (Boland et al., 2004).

The location and timing of elastin synthesis and deposition in the lung during development indicates a major role for elastin in alveolar development. Focal

accumulations of elastin in the walls of saccules appear to be the first step in the development of secondary septal crests that form new alveoli (Bostrom et al., 1996). In addition, reductions in elastin expression have been shown to impair alveolar formation (Lindahl et al., 1997). Given the close association between elastin synthesis and alveolar development, as well as structural changes in the lung parenchyma induced by alterations in fetal lung expansion, it was hypothesised that sustained alterations in the degree of fetal lung expansion would alter elastin synthesis and hence elastin content in the fetal lung.

An *in vitro* study has shown that cyclical mechanical strain increases tropoelastin mRNA expression in cultured fetal rat lung cells (Nakamura et al., 2000). However, the relevance of this to the *in vivo* environment is unclear as this stimulus was also shown to stimulate surfactant protein-C expression, which is inhibited by a sustained increase in expansion of the fetal lung *in vivo* (Lines et al., 1999). There have been no studies that have demonstrated an influence of mechanical forces on tropoelastin expression in the fetal lung *in vivo* where the major mechanical forces affecting lung tissue are not likely to be of a cyclical nature.

In order to gain a better understanding of the regulation of elastin synthesis in the developing lung, particularly in relation to alveolar development, more information is required about the control of elastin synthesis by physical factors, such as the degree to which the lung is expanded. Therefore, the aim of this study was to determine the effect of sustained increases and decreases in lung expansion on elastin synthesis in the fetal sheep lung *in vivo*.

4.2 Materials and methods

4.2.1 Experimental preparation

Fetal lung tissue was obtained from experiments performed previously on chronically catheterised fetal sheep in our laboratory (Boland et al., 2004; Keramidaris et al., 1996; Nardo et al., 1998). In those studies, aseptic surgery was performed on pregnant Border Leicester X Merino ewes between 107 and 115 days of gestation (term ~ 147 days). Under general anesthesia (1.5% halothane in O_2 - N_2O, 50:50 v/v), two large-diameter saline-filled catheters were inserted into the midcervical trachea of the fetus; one was

directed towards the lungs, and the other was directed towards, but did not enter the larynx. These two catheters were exteriorised and joined together to form a continuous exteriorized tracheal loop that allowed the normal flow of tracheal fluid (Hooper et al., 1988). Fetal arterial and venous catheters were also inserted for blood sampling and antibiotic administration respectively. The ewe and fetus were allowed to recover from surgery for at least 5 days before the beginning of experiments.

To increase fetal lung expansion (Nardo et al., 1998), the re-entrant tracheal catheter was obstructed for either 2 days (n=5 fetuses), 4 days (n=5 fetuses), 7 days (n=5 fetuses), or 10 days (n=5 fetuses), with all experimental periods finishing on day 128 of gestation. In a separate group of age-matched control fetuses (n=5), the trachea was cannulated but remained unobstructed, allowing the normal flow of lung liquid. To reduce fetal lung expansion (Boland et al., 2004), lung liquid was continuously drained by gravity from the descending tracheal catheter into an external bag for 20 days (111-131 days of gestation)(n=5). In age-matched control fetuses, the trachea was cannulated but lung liquid was not drained (n=5). At the end of the experimental periods, all ewes and fetuses were painlessly killed using an overdose of pentobarbital sodium (i.v.) administered to the ewe.

At postmortem (128 days GA), the lungs were removed from the fetus and the left main bronchus ligated before portions of the left lobes were removed and frozen in liquid nitrogen and stored at -70°C. Pieces of the left lung were used for extraction of total RNA for Northern blot analysis (section 2.2) and for the determination of elastin content (section 2.4). Lung tissue was micro-dissected prior to measurement of elastin content to remove larger airways and blood vessels so as to focus my findings on the alveolar region of the lung. The right lung was fixed via the trachea at 20 cmH_2O using 4% paraformaldehyde, sliced into 5mm sections and post-fixed in Zamboni's fixative overnight; some samples were fixed in 4% paraformaldehyde and 0.2% gluteraldehyde without post-fixing, as this was the standard practice for tissue processing in the lab at the time when those animals were produced. All fixed tissue samples were embedded in paraffin and sectioned at 5µm for in situ hybridisation (section 2.3) and histochemical analysis (section 2.5) to localise elastin mRNA expression and elastin deposition in lung tissue.

4.3 Results

4.3.1 Responses to sustained increases in lung expansion

Tropoelastin mRNA levels

Following 2 days of increased fetal lung expansion induced by tracheal obstruction (TO), pulmonary tropoelastin mRNA levels (33.9 ± 8.7 arbitrary units) were significantly greater than in control fetuses (13.6 ± 1.7). After 4 days of TO, tropoelastin mRNA levels were similar to those in control fetuses (control, 14.9 ± 2.4 vs 4d TO, 16.6 ± 2.7) and this was also the case at 7 days of TO (control, 8.3 ± 1.0 vs 7d TO, 9.6 ± 2.5) and 10 days of TO (control, 14.2 ± 2.6 vs 10d TO, 16.8 ± 1.7). Expressed as a percentage of control values, pulmonary tropoelastin mRNA levels were significantly elevated at 2 days of TO (249.8 ± 8.7%), but then declined to 111.7 ± 18.1% at 4 days, 115.1 ± 30.3% at 7 days and 118.1 ± 11.7% at 10 days of TO (Figure 4.1).

In situ hybridisation

Tropoelastin expression was prominent in pulmonary arteries and minimal in airways in the lungs of control animals at 128 days of gestation (Figure 4.2). In the alveolar region of the lungs of control animals, tropoelastin mRNA was localised to the tips of developing secondary septa, and at bends in alveolar walls where secondary septal formation would be expected to occur (Figure 4.3). Following 2 days of increased lung expansion, tropoelastin mRNA expression in the walls of pulmonary arteries and in airway walls was increased compared to lungs of control fetuses (Figure 4.2). In the alveolar region of the lung, 2 days of increased lung expansion increased both the number of sites of tropoelastin expression, and the amount of expression at each site compared to control lungs (Figure 4.3).

Elastin concentration and content

The concentration of elastin in lung parenchymal tissue was not different following 2 days of TO but was significantly lower following 4 days of TO (1.6 ± 0.0 mg/g lung) and 10 days of TO (1.4 ± 0.1 mg/g lung) compared to control lung tissue (1.9 ± 0.1 mg/g lung). Expressed as a percentage of mean control values, elastin concentration was 96.0 ± 5.3%, 82.4 ± 1.9% and 74.8 ± 2.6% of control at 2, 4 and 10 days of TO

respectively (Figure 4.4). The elastin content of the lungs however, was not significantly different following 2 days (298.3 ± 35.3 mg), 4 days (300.3 ± 49.2 mg) or 10 days of TO (265.8 ± 13.3 mg) compared to that of controls (224.8 ± 28.3 mg). Expressed as a percentage of control values, elastin content was 132.7 ± 15.7%, 133.6 ± 21.9% and 118.2 ± 5.9% of control at 2, 4 and 10 days of TO respectively (Figure 4.4).

Elastin staining

In fetuses exposed to TO, lung tissue stained for elastin appeared to show an increase in the thickness of the elastic lamina surrounding blood vessels (Figure 4.5). However in this study, it was unable to be determined whether this reflects a change in elastin deposition resulting from an increase in tropoelastin expression, or variation between vessels of different sizes or from different areas of the lung. There were no apparent differences in the elastin staining of airways (Figure 4.5) or alveolar compartments (Figure 4.6) compared to controls. Elastin staining was focused at the tips of secondary septal crests and, therefore, it appeared that secondary septal formation was occurring in a similar manner to that observed in control fetuses (Figure 4.6).

4.3.2 Responses to a sustained decrease in lung expansion

Tropoelastin mRNA levels

Following 20 days of decreased lung expansion, tropoelastin mRNA levels in lung tissue were significantly lower than in control fetuses (reduced lung expansion, 13.8 ± 1.9 vs control, 31.5 ± 5.8). Tropoelastin mRNA levels in treated fetuses were 43.7 ± 5.9% of values in control fetuses (Figure 4.7).

In situ hybridisation

Tropoelastin expression was prominent in pulmonary arteries and minimal in airways in the lungs of both control animals, and animals subjected to 20 days of decreased lung expansion (Figure 4.8). Relative to parenchymal tropoelastin expression, tropoelastin expression in the walls of pulmonary arteries did not appear to be affected by a decreased level of fetal lung expansion. In control lungs, tropoelastin expression in the alveolar region of the lungs was focused at the tips of developing secondary septa and at bends in alveolar walls where secondary septal formation would be expected to occur

(Figure 4.9). In contrast, following 20 days of decreased fetal lung expansion, tropoelastin expression in the alveolar region of the lung was substantially reduced in both the number of sites of tropoelastin expression and the amount of expression at each site compared to normally expanded lungs. Furthermore, focal points of tropoelastin expression were not apparent following decreased lung expansion as they were in control lungs (Figure 4.9).

Elastin concentration and content

After 20 days of decreased lung expansion, the elastin concentration of the lungs was not different from that in control fetuses (reduced lung expansion, 1.9 ± 0.3 vs control, 1.6 ± 0.2), whereas the elastin content of the lungs (97.6 ± 22.9 mg) was almost significantly lower (p=0.06) than in age-matched control lungs (194.4 ± 37.7 mg). Expressed as a percentage of control values, elastin concentration was 121.1 ± 18.4% and elastin content was 46.7 ± 11.7% of control values following 20 days of decreased lung expansion (Figure 4.10).

Elastin staining

Elastin deposition did not appear to be altered in blood vessels and airways following prolonged lung liquid drainage (Figure 4.11). However, elastin deposition was altered in the alveolar walls of lungs subjected to 20 days of lung liquid drainage, compared to lungs from age-matched control fetuses. In particular, focal aggregates of elastin occurred at closer intervals within the saccular walls, and elastic fibres appeared to be located along the saccule wall rather than being focused at the tips of secondary septa. It appeared that alveolarisation had failed, as many focal sites of elastin aggregation were not associated with the formation of secondary septal crests (Figure 4.12).

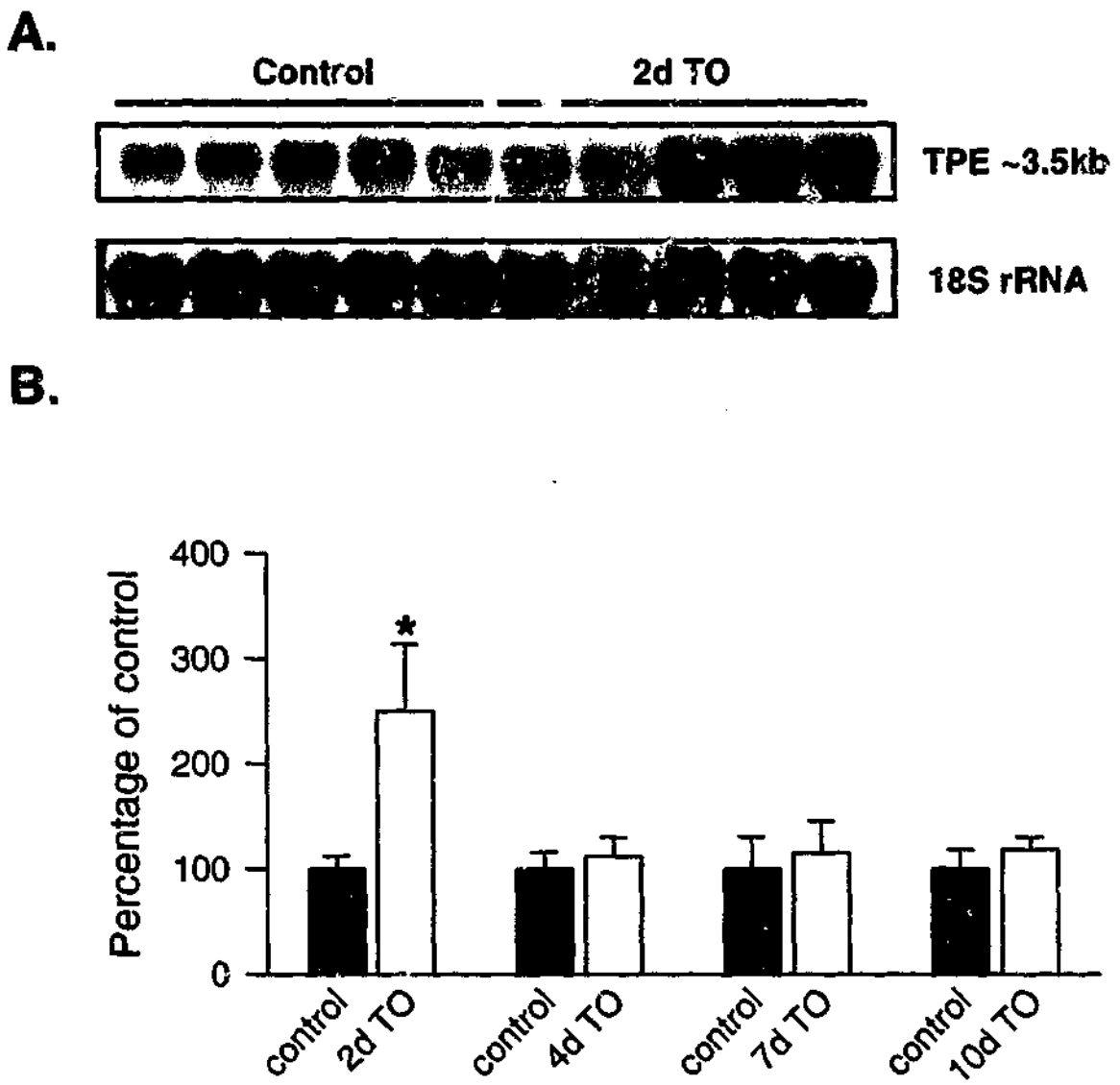


Figure 4.1 Tropoelastin (TPE) mRNA levels following increased fetal lung expansion

A: Northern blot analysis of total RNA extracted from lung tissue collected from control fetuses and fetuses exposed to 2 days of TO. *B*: Mean tropoelastin mRNA levels in control fetuses and fetuses exposed to 2, 4, 7 or 10 days of TO where each pair of bars represents data from a single Northern blot. The density of each tropoelastin mRNA band was expressed as a ratio of the density of the corresponding 18S rRNA band and then expressed as a percentage of mean control values. The asterisk (*) indicates a difference between the groups ($p<0.05$) when analysed by the unpaired *t*-test.

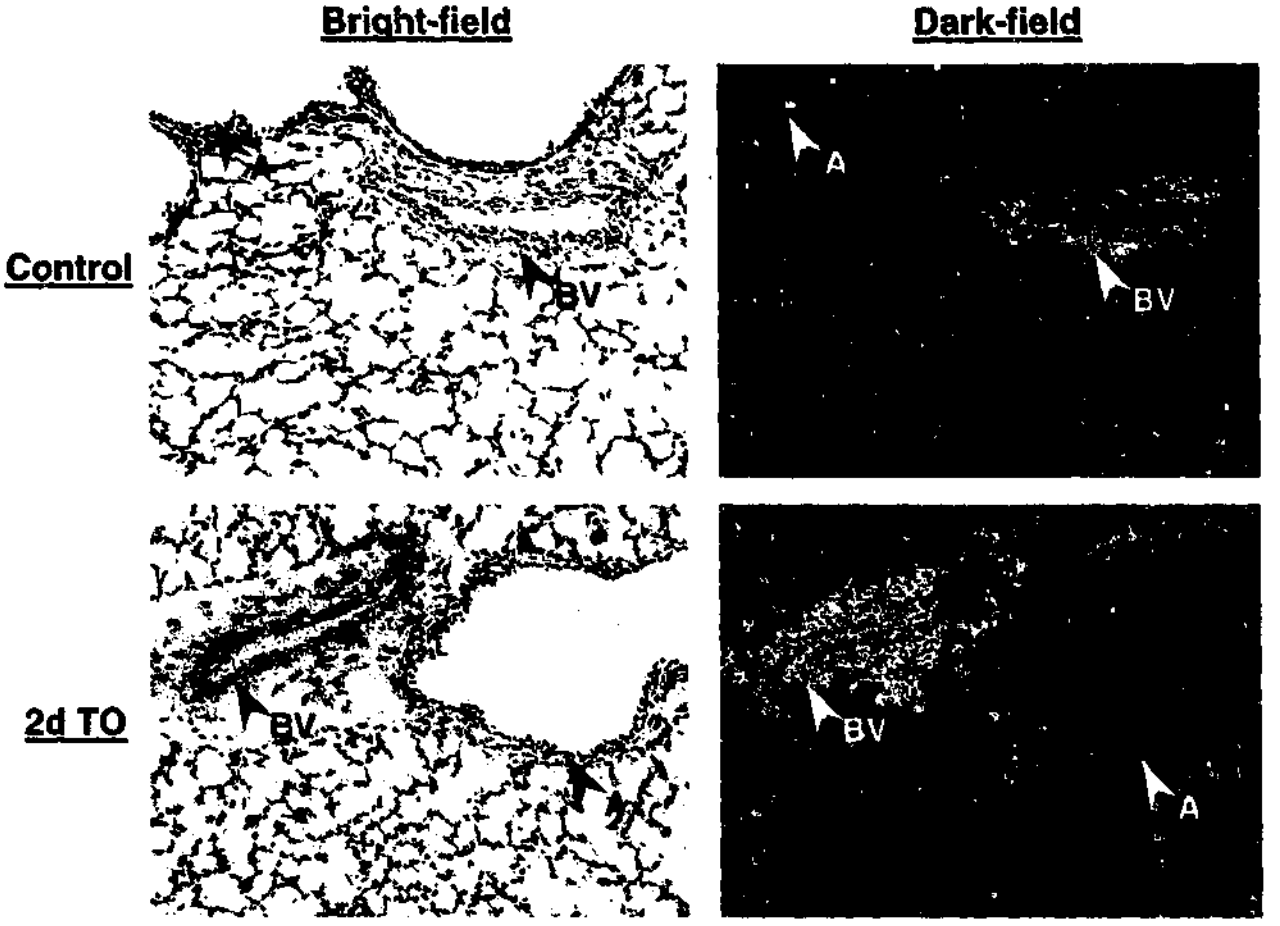


Figure 4.2 Tropoelastin mRNA in blood vessels and airways following 2 days of increased lung expansion

In situ hybridisation for tropoelastin mRNA in blood vessels (BV) and airways (A) in lungs of control fetuses and fetuses exposed to 2 days of increased lung expansion. Presented are paired bright- and dark-field photographs taken at X20 magnification of fetal lung hybridised with ^{35}S-labeled antisense RNA for tropoelastin mRNA. A strong positive signal was detected in blood vessels and minimal or no signal in airways of control fetuses at 128 days of gestation. Following 2 days of increased lung expansion, signal for tropoelastin mRNA was increased in the walls of pulmonary arteries and in smooth muscle in the walls of airways.

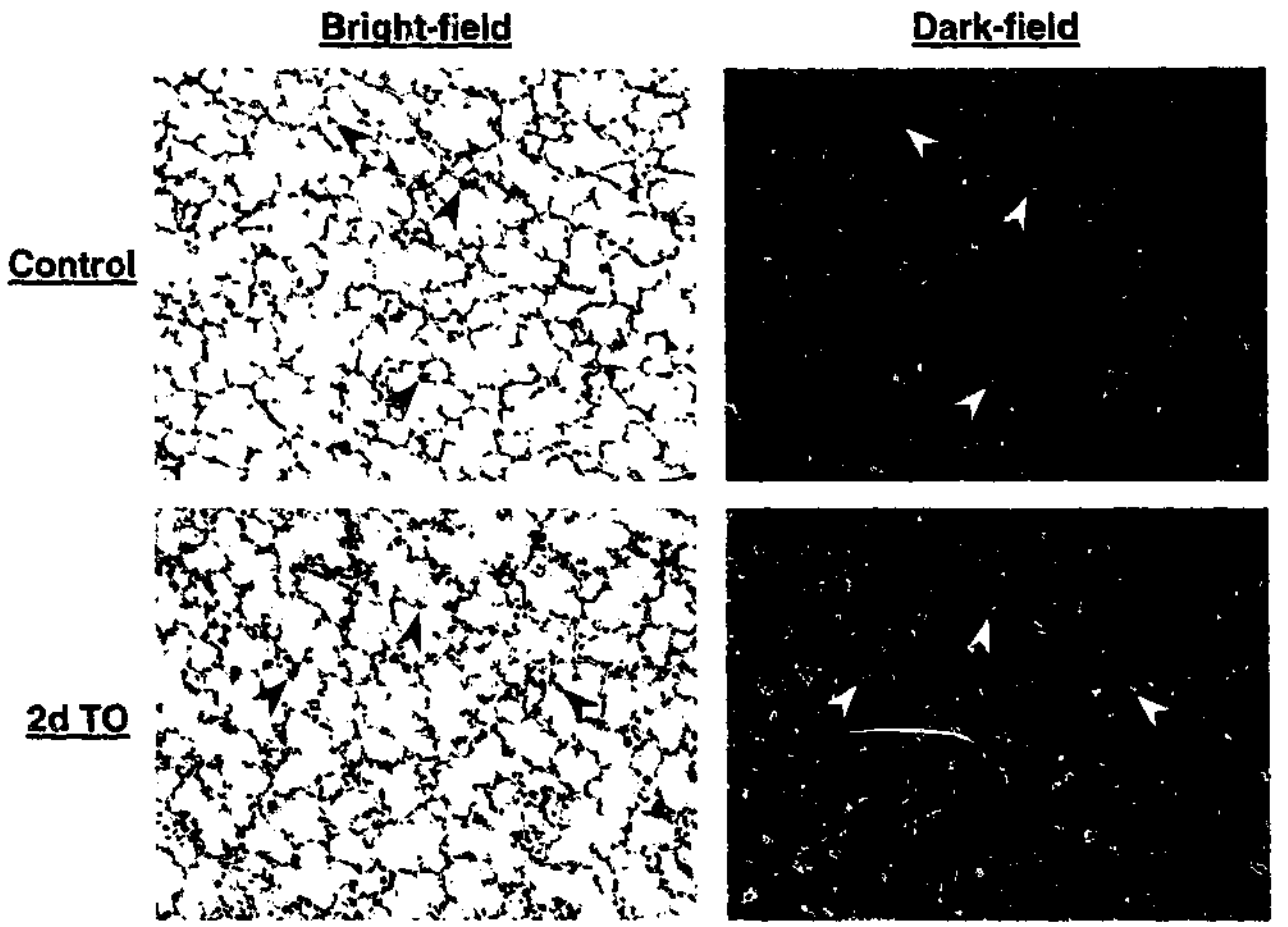
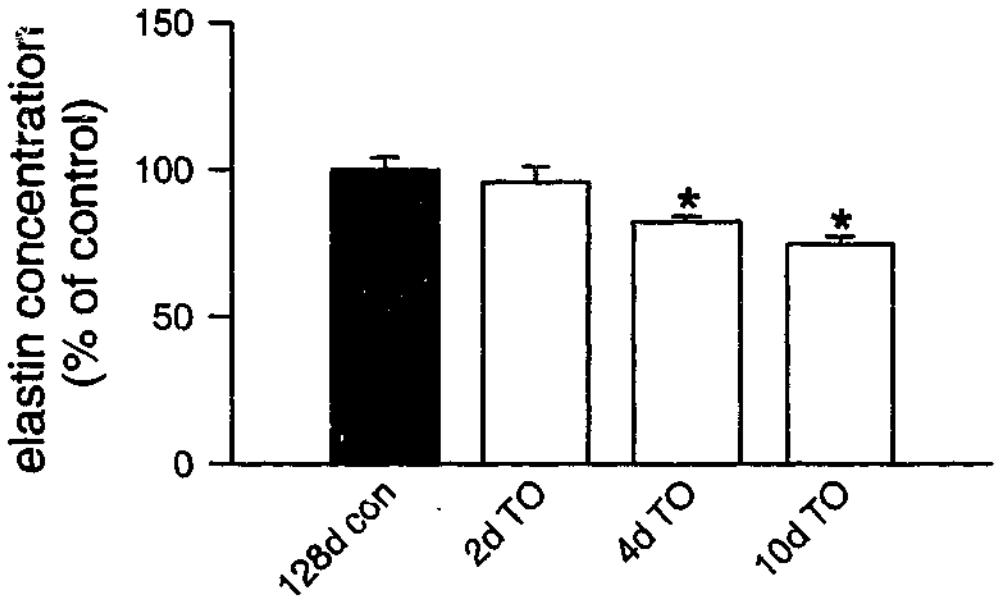


Figure 4.3 Tropoelastin mRNA in the alveolar region of the lungs following 2 days of increased lung expansion

In situ hybridisation for tropoelastin mRNA in lung parenchymal tissue of control fetuses and fetuses exposed to 2 days of increased lung expansion. Presented are paired bright- and dark-field photographs taken at X20 magnification of fetal lung hybridised with ^{35}S-labeled antisense RNA for tropoelastin mRNA. Positive signal for tropoelastin mRNA was detected at the tips of developing secondary septa in control lungs (arrow heads). Following 2 days of increased lung expansion, both the number of sites positive for tropoelastin mRNA and the density of silver grains at each site was increased.



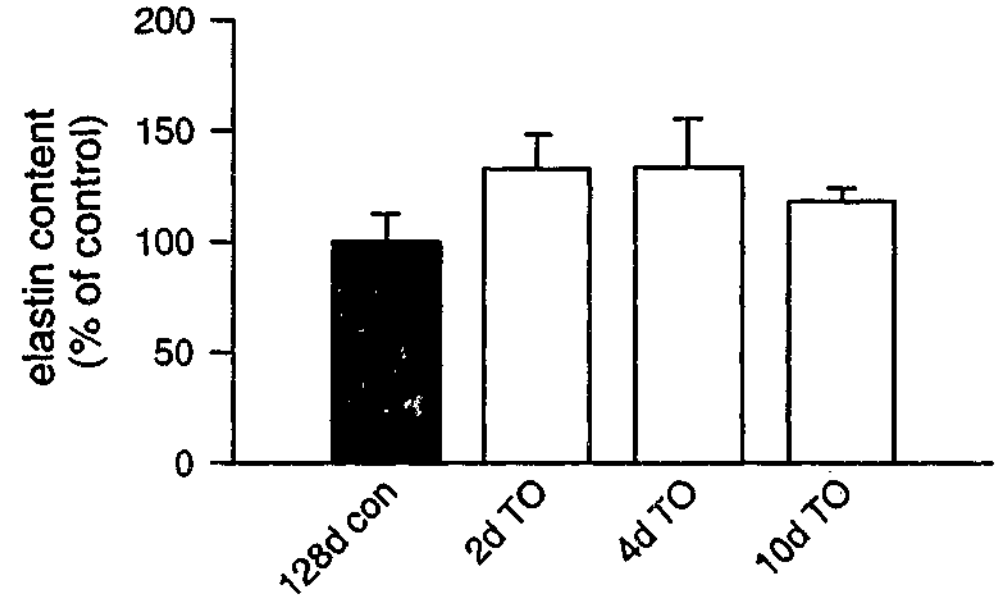


Figure 4.4 Elastin concentration and elastin content of the lungs following increased lung expansion

Elastin concentration and elastin content of the lungs of control fetuses and fetuses exposed to 2, 4 or 10 days of TO, expressed as a percentage of control values. Asterisks (*) indicate values different from control values ($p<0.05$) when analysed by one-way ANOVA.

Figure 4.5 Elastin staining of blood vessels and airways in fetal lungs following increased lung expansion
Hart's resorcin-fuchsin stain for elastin in lung tissue sections taken from control lung (128 days GA) and lung from fetuses exposed to 2, 4 or 10 days TO. Photographs taken at X20 magnification of a pulmonary artery and a cartilaginous airway are presented for each treatment group. Elastin is stained dark purple-black and can be seen as thick layers in the walls of pulmonary arteries and finer layers in the walls of large airways.

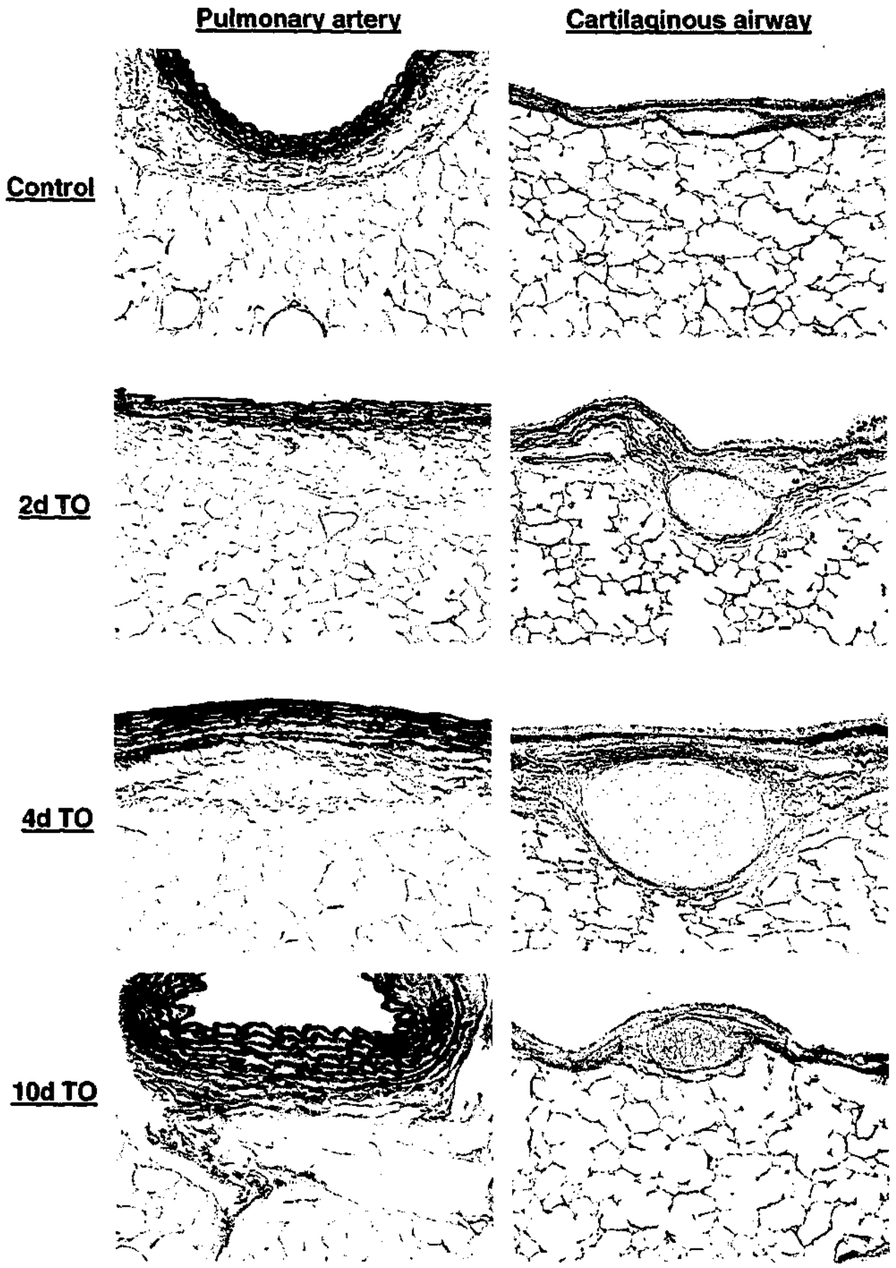
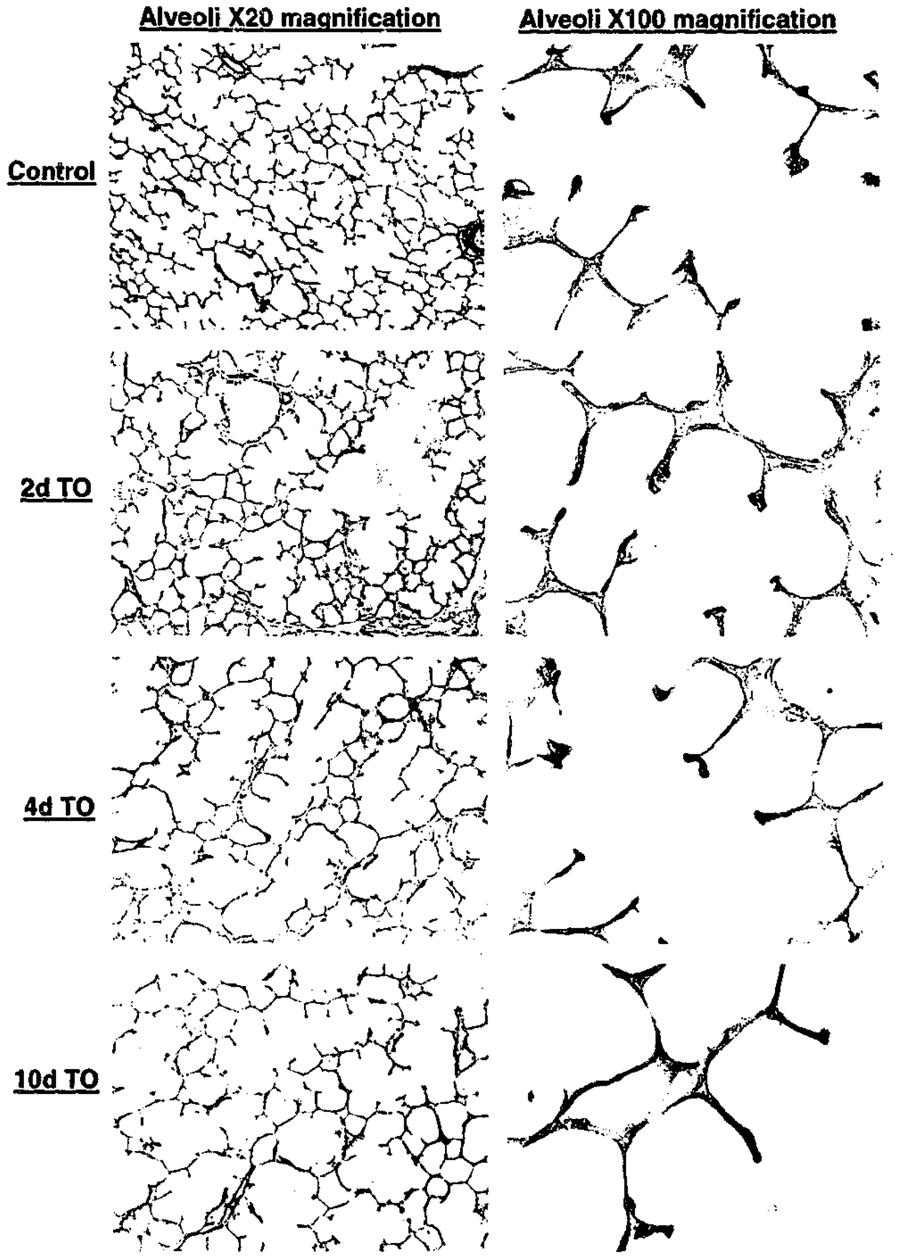


Figure 4.6 Elastin staining in the alveolar region of fetal lungs following increased lung expansion
Hart's resorcin-fuchsin stain for elastin in lung tissue sections taken from control lung (128 days GA) and lung from a fetuses exposed to 2, 4 or 10 days of TO. Photographs taken at X20 and X100 magnification of the alveolar region of the lung are presented for each treatment group. Elastin is stained dark purple-black and is focused at the tips of secondary septal crests in both control lungs and lungs from fetuses exposed to TO.



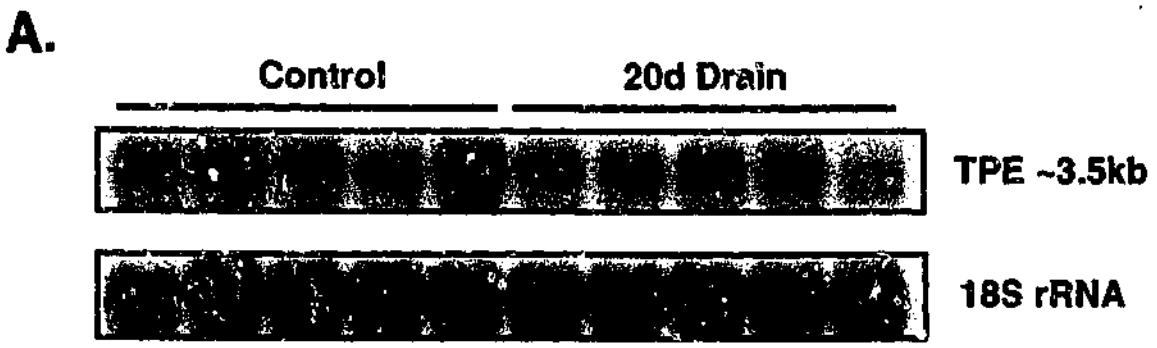


Figure 4.7 Tropoelastin (TPE) mRNA levels following decreased fetal lung expansion

A: Northern blot analysis of total RNA extracted from lung tissue collected from control fetuses and fetuses exposed to 20 days of lung liquid drainage B: Mean tropoelastin mRNA levels in control fetuses and fetuses exposed to 20 days of lung liquid drainage. The density of each tropoelastin mRNA band was expressed as a ratio of the density of the corresponding 18S rRNA band and then expressed as a percentage of mean control values. The asterisk (*) indicates a difference between the groups ($p<0.05$) when analysed by the unpaired t-test.

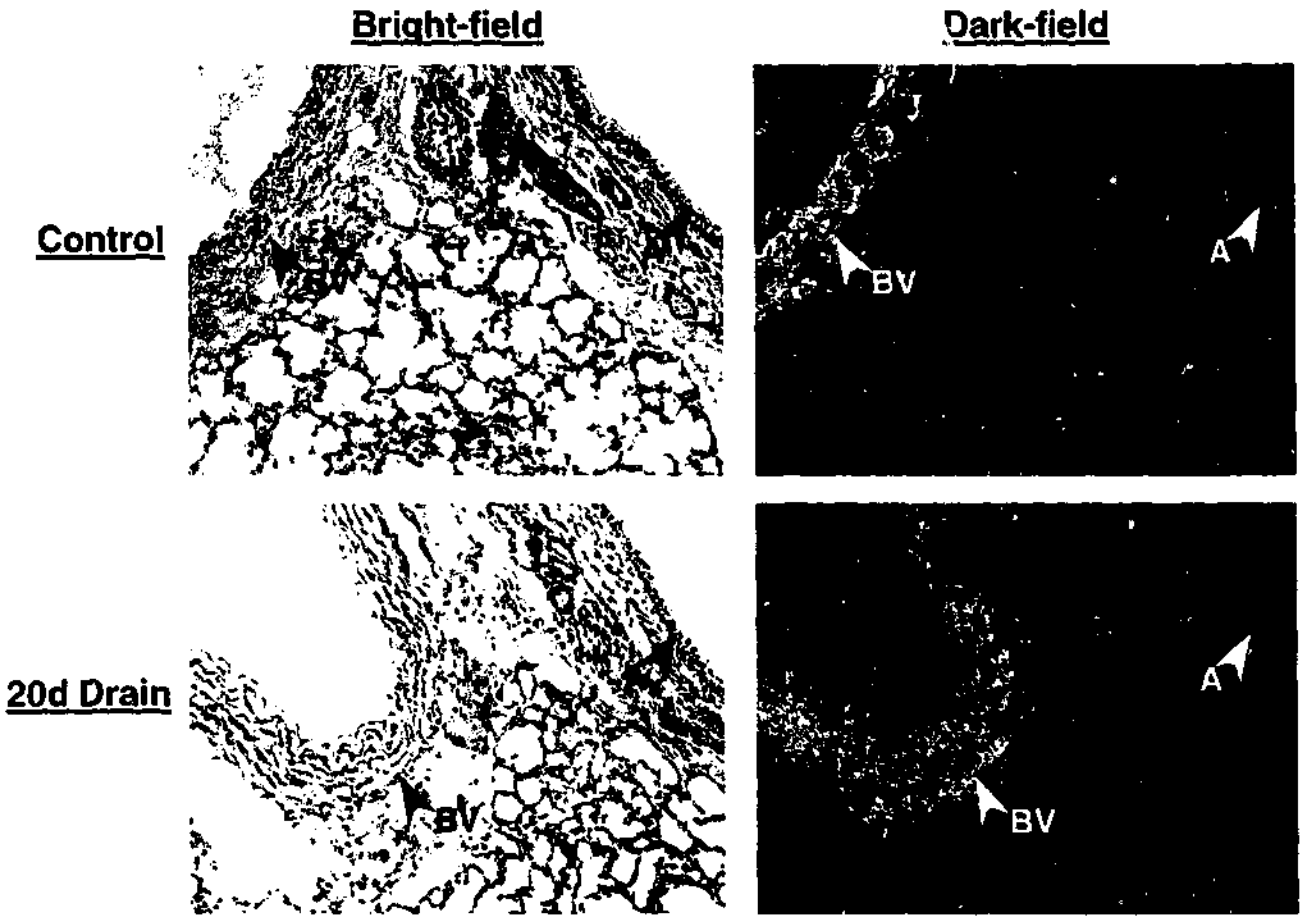


Figure 4.8 Tropoelastin mRNA in blood vessels and airways following decreased lung expansion

In situ hybridisation for tropoelastin mRNA in vessels (BV) and airways (A) in lungs of control fetuses (131 days GA) and fetuses exposed to 20 days of lung liquid drainage. Presented are paired bright- and dark-field photographs taken at X20 magnification of fetal lung hybridised with ^{35}S-labeled antisense RNA for tropoelastin mRNA. A strong positive signal was detected surrounding blood vessels and minimal or no signal was detected in airways of control fetuses at 131 days of gestation. Following 20 days of decreased lung expansion the signal for tropoelastin mRNA was similar in blood vessels and airways to that of control fetuses.

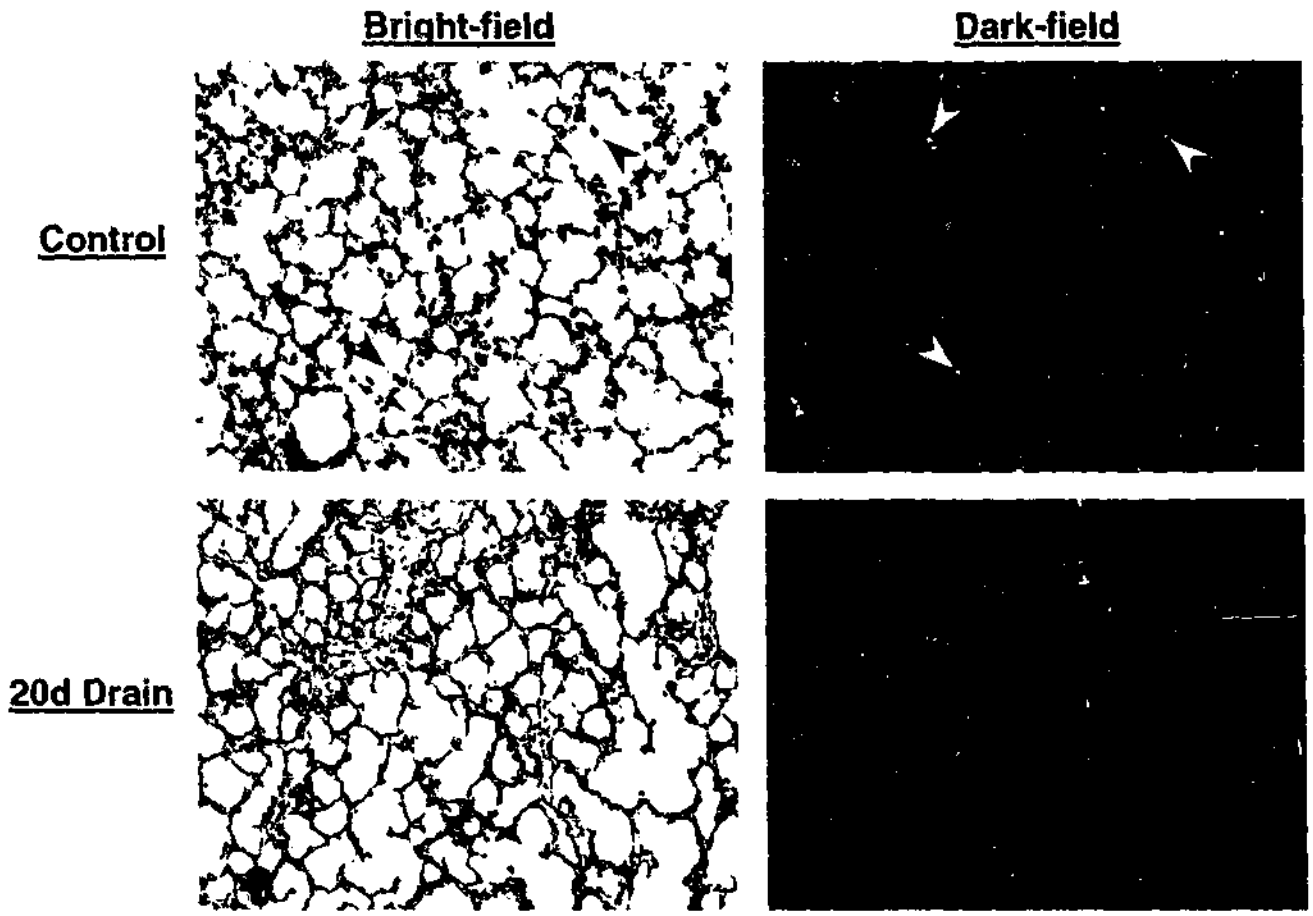
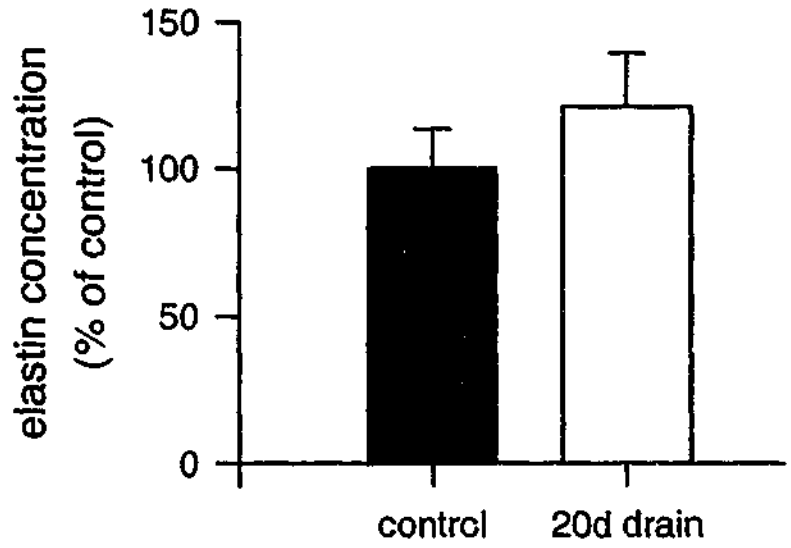


Figure 4.9 Tropoelastin mRNA in the alveolar region of fetal lungs following decreased lung expansion

In situ hybridisation for tropoelastin mRNA in lung parenchymal tissue of control fetuses (131 days GA) and fetuses exposed to 20 days of lung liquid drainage. Presented are paired bright- and dark-field photographs taken at X20 magnification of fetal lung hybridised with ^{35}S-labeled antisense RNA for tropoelastin mRNA. Positive signal for tropoelastin mRNA was detected at the tips of developing secondary septa in control lungs (arrow heads). Following 20 days of decreased lung expansion, positive signal for tropoelastin mRNA was substantially reduced, and focal points of tropoelastin expression were not apparent as in control lungs.



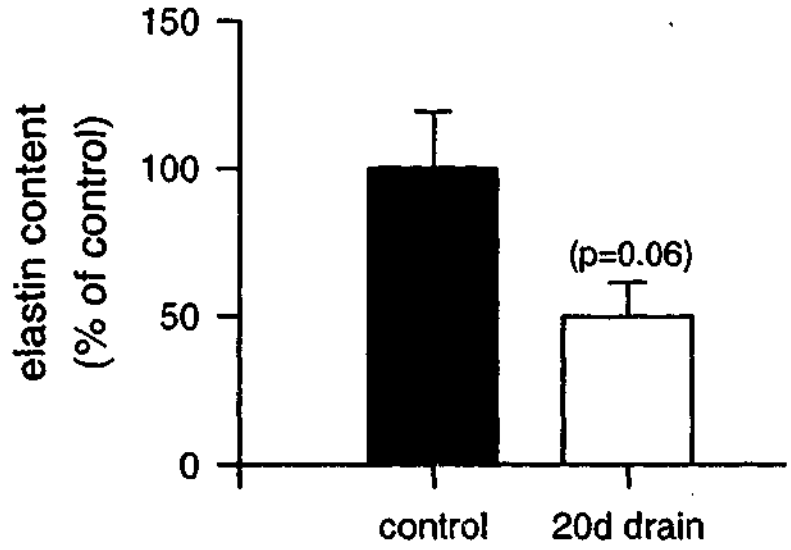


Figure 4.10 Elastin concentration and elastin content of the lungs following decreased fetal lung expansion
Elastin concentration and elastin content of the lungs of control fetuses and fetuses exposed to 20 days of lung liquid drainage expressed as a percentage of control values.

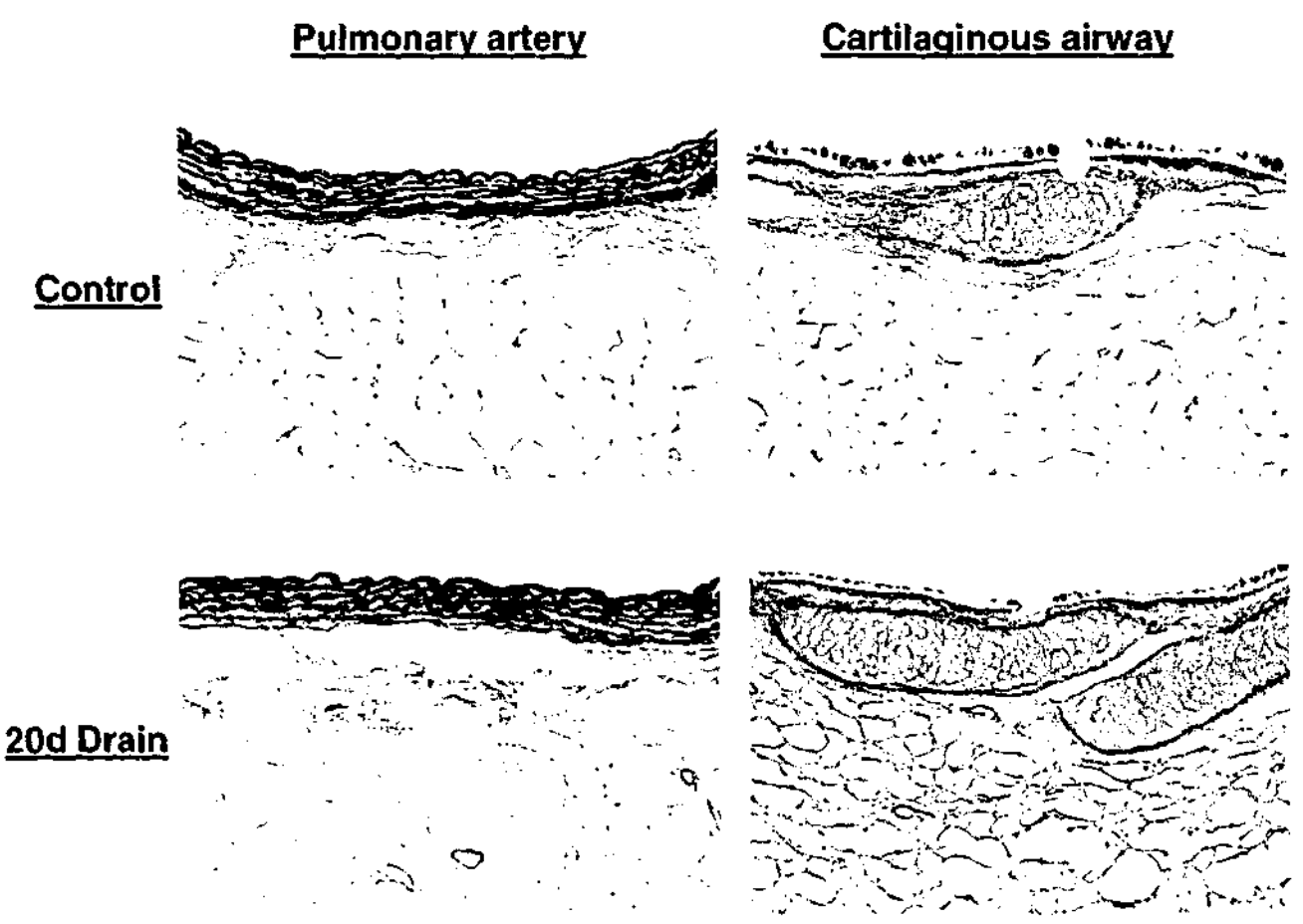


Figure 4.11 Elastin staining of blood vessels and airways in fetal lungs following decreased lung expansion

Hart's resorcin-fuchsin stain for elastin in lung tissue sections taken from a control fetus (131 days GA) and from a fetus exposed to 20 days of lung liquid drainage (20d Drain). Photographs taken at X20 magnification of a pulmonary artery and a cartilaginous airway are presented for each treatment group. Elastin is stained dark purple-black and can be seen as thick layers in the walls of pulmonary arteries and finer layers in the walls of large airways.

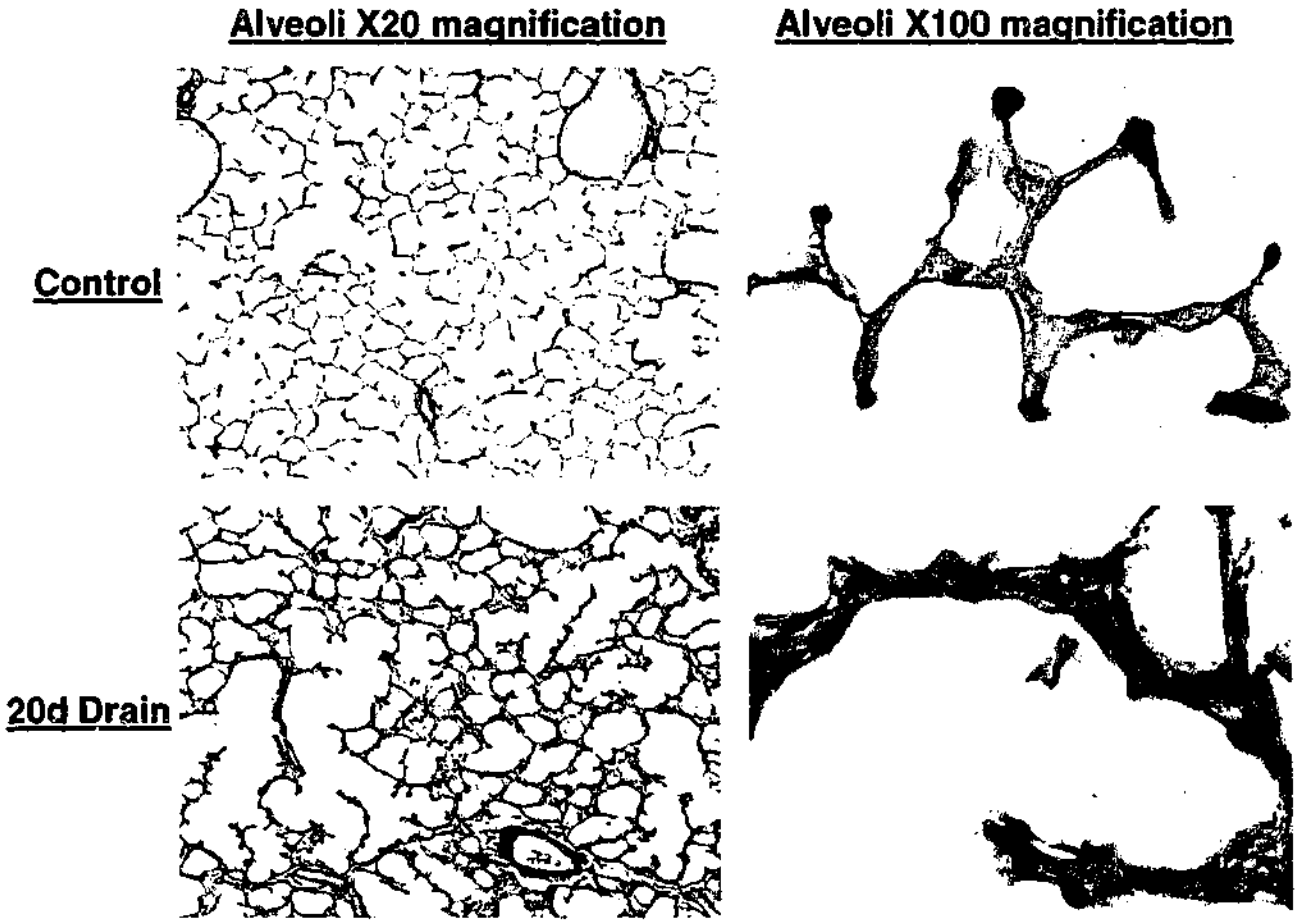


Figure 4.12 Elastin staining in the alveolar region of fetal lungs following decreased lung expansion

Hart's resorcin-fuchsin stain for elastin in lung tissue sections taken from a control fetus (131 days GA) and from a fetus exposed to 20 days of lung liquid drainage (20d Drain). Photographs taken at X20 and X100 magnification of the alveolar region of the lung are presented for each treatment group. Elastin is stained dark purple-black and is focused at the tips of secondary septal crests in control lungs. In lungs exposed to 20 days of under-expansion, focal aggregates of elastin occur at much shorter distances from each other and secondary septation appears to have failed. At X100 magnification, elastin can be seen along the alveolar walls rather than primarily being focused at the tips of secondary septae.

4.4 *Discussion*

Sustained alterations in fetal lung expansion induced by changes in lung liquid volume profoundly affect lung growth and the structural development of the lung, including alveolar development. The synthesis of elastin in the developing lung is very closely associated with alveolar development and therefore it was hypothesised that alterations in fetal lung expansion would alter elastin synthesis in accordance with changes in alveolar development previously observed. This study has shown that sustained alterations in the basal degree of lung expansion in fetal sheep *in vivo*, induced by TO and lung liquid drainage, cause alterations in both tropoelastin expression and elastin deposition in lung parenchymal tissue consistent with changes in alveolar development. Sustained increases in the level of fetal lung expansion resulted in a transient increase in tropoelastin mRNA levels whereas a prolonged decrease in the level of fetal lung expansion resulted in a profound reduction in tropoelastin mRNA levels. Elastin accumulation, however, as demonstrated by changes in elastin concentration and content in lung parenchymal tissue, did not appear to alter proportionately with the changes observed in fetal lung growth. However, an important finding of this study was the influence of fetal lung expansion on the deposition of elastin in the parenchymal region of the lung. It was found that an adequate degree of fetal lung expansion is essential for the correct laying down of elastic fibres in lung parenchymal tissue, and hence normal alveolar development.

4.4.1 Effect of alterations in fetal lung expansion on tropoelastin expression

A sustained increase in fetal lung expansion, induced by TO, caused a large but transient increase in tropoelastin mRNA levels at 2 days. However, by 4 days, mRNA levels for tropoelastin had returned to control levels and did not alter for up to 10 days of increased lung expansion. The transient nature of the increase in tropoelastin expression observed in response to a sustained increase in fetal lung expansion was a surprising finding. It is not fully understood why the increase in tropoelastin expression was not sustained throughout the period of increased lung expansion. However, it is interesting that the time-course for the changes in tropoelastin expression is very similar to the time course for the changes in pulmonary DNA synthesis rates in response to

sustained TO, where there is a large transient increase in DNA synthesis rates at 2 days of TO (Nardo et al., 1998)(Figure 4.13).

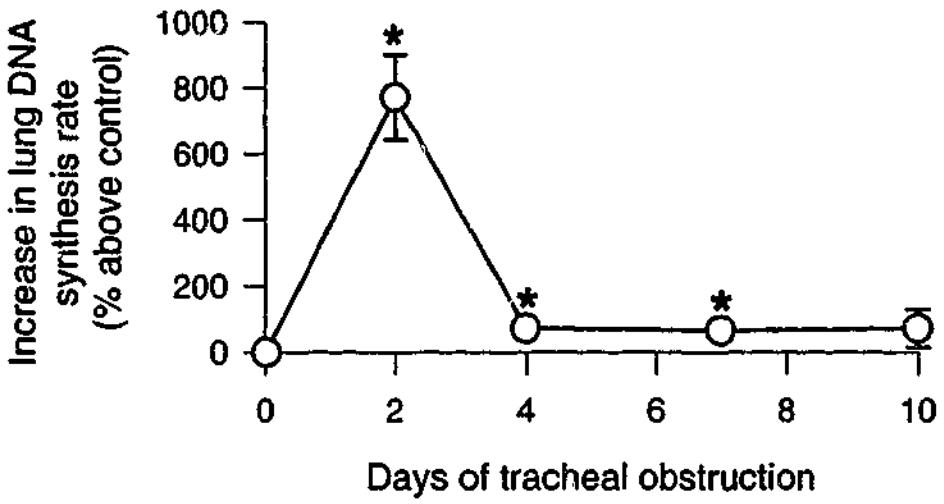


Figure 4.13 DNA synthesis rates following TO
Mean DNA synthesis rates following 2, 4, 7 and 10 days of TO. Asterisks (*) indicate a significant increase above control values ($p<0.05$); the values at 4 and 7 days are significantly increased, although this is obscured by the large range of the y axis. (Data from Nardo et al., 1995)

The large transient peak in DNA synthesis and tropoelastin expression at 2 days of TO suggests that the stretch stimulus applied to responding cells, induced by increased lung volume, is maximal at this time; similar results have also been observed for the expression of the calmodulin-2 gene (Gillett et al., 2002). One possible explanation for the transient nature of this response is the differential process by which the lung expands during extended periods of TO, thereby altering the stretch stimulus provided to lung cells. Nardo *et al.* (1995) have previously shown that, over the first day of TO, fetal lung luminal volume increases approximately 2-fold, from ~30ml/kg to ~55ml/kg. However, between days 1 and 2, the increase in lung expansion temporarily ceases and lung volume does not increase further until after day 2; the lungs then continue to expand again until day 7, when they reach a maximum volume of ~95ml/kg (Nardo et al., 1998)(Figure 4.14). Further expansion does not occur, presumably owing to the structural limits imposed by the chest wall.

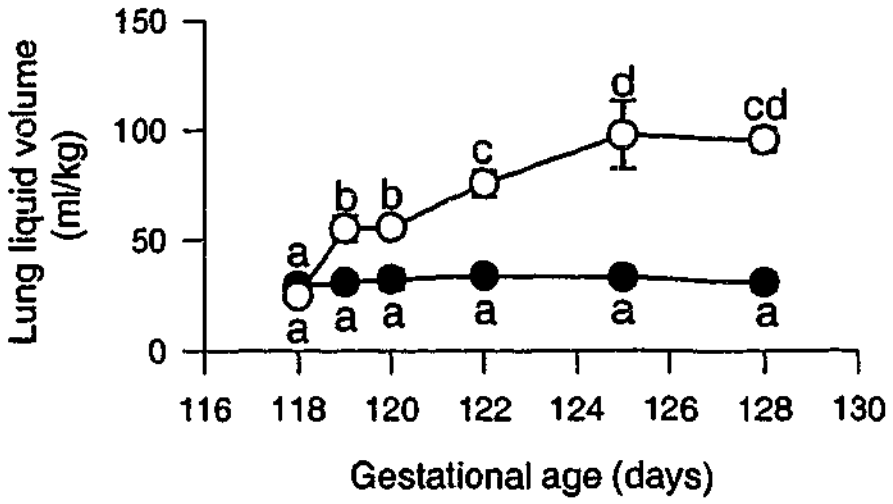


Figure 4.14 Lung liquid volume following TO
Mean lung liquid volume adjusted for fetal body weight of control fetuses (solid circles), and fetuses exposed to 2, 4, 7 or 10 days of TO (open circles). Values that do not share a common letter are significantly different ($p<0.05$) from each other. (Data from Nardo *et al.*, 1995)

The interpretation of these changes in lung volume is that over the first day of TO, the lung expands to a limit imposed by the structural framework of the lung tissue and further expansion (i.e. between days 2 and 7) is dependent upon remodeling of this framework. As the majority of lung cells are attached to, and closely interact with this structural framework, it is likely that the stretch-stimulus is maximal when the structural framework is initially stretched to its limit. However, after this initial 2 day period, extension or remodelling of the framework, perhaps due to increased collagen (Nardo et al., 1998) and elastin synthesis, is unlikely to impose the same level of mechanical load to the same number of lung cells as the initial period of lung expansion (days 0-2).

In contrast to an increase in fetal lung expansion induced by TO, continuous lung deflation suppressed parenchymal tropoelastin expression for up to 20 days. This most likely results from the large decrease in the basal degree of lung expansion and it is likely that the suppression of tropoelastin expression will be sustained for as long as the lung continues to be deflated. While the decrease in fetal lung expansion resulted in a substantial reduction (almost 50%) in tropoelastin mRNA levels, a complete ablation of tropoelastin expression did not occur despite a virtual cessation in lung growth. This indicates that although the level of fetal lung expansion has a major influence on tropoelastin expression, some other factor or factors other than mechanical forces must

determine the basal level of tropoelastin expression at least during the alveolar period of lung development. The finding that a sustained reduction in lung expansion decreased tropoelastin expression, whereas an increase in lung expansion stimulated tropoelastin expression, demonstrates the sensitivity of elastin synthesis to sustained alterations in fetal lung expansion.

Further assessment of the influence of alterations in fetal lung expansion on tropoelastin expression by *in situ* hybridisation revealed that tropoelastin expression was increased in pulmonary arteries, airways and alveoli following an increase in fetal lung expansion. However, following a decrease in fetal lung expansion, tropoelastin was reduced in alveoli but did not appear to be altered in pulmonary arteries or airways. These data indicate that while the basal level of fetal lung expansion does not appear to have a major influence on tropoelastin expression in the walls of pulmonary arteries and airways, an increase in fetal lung expansion can increase tropoelastin expression in both structures.

4.4.2 Effect of sustained alterations in fetal lung expansion on elastin concentration and elastin content in lung parenchymal tissue

Despite the large increase in tropoelastin expression following 2 days of TO, elastin concentration (mg/g of lung) was unchanged at 2 days and was significantly reduced by 4 and 10 days of TO. The elastin content (mg) of the lungs was not significantly different between the groups. Taken together, these data suggest that following 2 days of TO, when the stretch stimulus applied to the lungs is apparently maximal; elastin expression is increased causing an increase in elastin synthesis maintaining the concentration of elastin in lung parenchymal tissue at a level appropriate for the size of the lung. However, by 4 days of TO, elastin expression returns to control levels and elastin synthesis slows such that the concentration of elastin in the lung parenchymal tissue does not continue to increase at a rate appropriate for the rate of lung growth that is occurring. This is possibly due to the lung having a limited capacity to increase elastin synthesis in lung parenchyma above that which occurs during normal lung development, when the level of lung expansion is presumably optimal. It must be noted also that a proportion of the increase observed in tropoelastin mRNA levels was due to

an increase in vessels and airways as determined by *in situ* hybridisation experiments, whereas, elastin concentration and content was measured in parenchymal tissue from which vessels and airways had been removed.

In contrast, the sustained reduction in tropoelastin expression induced by reduced lung expansion resulted in a reduction in elastin content ($p=0.06$) and no change in the elastin concentration of lung parenchymal tissue compared with control animals. The failure of the reduction in total elastin content to reach statistical significance at $p<0.05$, despite a 50-60% reduction in lung weight, may either be a result of the variation in the data, or the fact that, while elastin expression is reduced by approximately 50%, lung growth virtually ceases. Therefore, as with changes in elastin synthesis following increases in fetal lung expansion, the rate of elastin synthesis may not appropriately match the altered rate of lung growth. It is considered that the reduction in elastin content resulted from the associated reduction in elastin synthesis although it is possible that an increase in elastin metabolism contributed to the decrease in content. Consistent with the suggestion that alterations in lung expansion can alter elastin synthesis, observations in human fetal lungs have demonstrated an apparent advance in elastin maturation in fetuses with laryngeal atresias which cause an accumulation of lung liquid and increased levels of lung expansion (Wigglesworth et al., 1987). Also, reduced elastin staining in alveolar septal crests by electron microscopy has been observed in hypoplastic human fetal lungs associated with oligohydramnios (Haidar et al., 1991); the oligohydramnios most probably results in a prolonged reduction in fetal lung expansion (Dickson & Harding, 1989; Harding et al., 1990).

4.4.3 Elastin synthesis and alveolar development

In support of the hypothesis that changes in elastin synthesis would occur in parallel with changes in alveolar development, the transient increase in tropoelastin expression at 2 days of TO is consistent with the increase in alveolar number observed between 2 and 4 days of TO (Nardo et al., 2000). Furthermore, the decrease in tropoelastin expression to control levels at 4 days of TO coincides with the absence of a further increase in alveolar number between 4 and 10 days of increased lung expansion (Nardo et al., 2000)(Figure 4.15). Although alveolar numbers do not increase between 4 and 10 days of TO, the luminal surface area does, suggesting that elongation of the inter-alveolar septa has occurred (Nardo et al., 2000). Also, the finding that elastin synthesis

and content, as well as alveolar number (Boland et al., 2004)(Figure 4.15), are reduced in response to sustained reductions in lung expansion is consistent with the concept that alveolarisation and elastin synthesis are closely related.

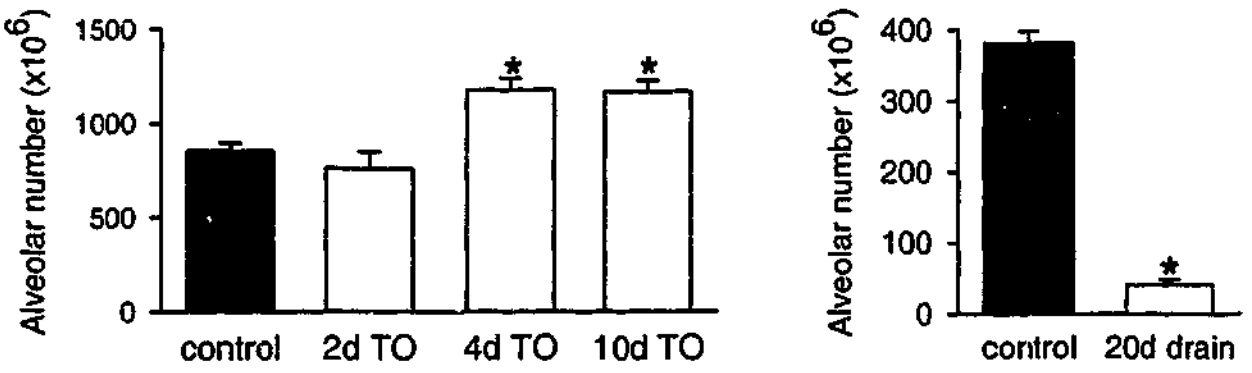


Figure 4.15 Changes in total alveolar number following TO and lung liquid drainage
Mean alveolar number in fetuses exposed to 2, 4 or 10 days of TO, or 20 days of lung liquid drainage and in age-matched control fetuses. Asterisks (*) indicate a difference from control values ($p<0.05$). (Data from Nardo et al. 1998 and Boland et al. 2004)

In addition to alterations in elastin expression and content resulting from changes in fetal lung expansion, changes in elastin deposition and secondary septal crest formation were observed in lungs from fetuses with a sustained reduction in lung expansion. In those fetuses, elastin was laid down in a disorderly manner in the alveolar walls and at focused points where secondary septal crests would be expected to form; this observation is consistent with the suggestion that septation is inhibited in these animals. In contrast, in fetuses exposed to increased lung expansion, elastin deposition occurred predominantly at the tips of septal crests, as in control fetuses. Thus, it appears that the increase in alveolar number induced by an increase in fetal lung expansion (Nardo et al., 2000) is associated with normal elastin deposition within the secondary septal crests, although the associated increase in alveolar number may not be appropriate for the increase in lung growth observed in this model. These findings indicate that sustained stretch of the lung prenatally is important for the correct laying down of elastic fibres and for alveolar formation. In addition, one study has shown disordered pulmonary elastin deposition and alveolar septation in 125 day old lambs that have been born pre-term and mechanically ventilated (Pierce et al., 1997). This further supports the notion that mechanical forces play a critical role in elastin deposition and formation,

particularly during the critical period of lung development when elastin synthesis is occurring.

4.4.4 Lung tissue stretch and elastin synthesis

In accordance with the findings of the present study, previous studies have shown that phasic mechanical strain increases tropoelastin mRNA expression in cultured fetal rat lung cells (Nakamura et al., 2000). Furthermore, mechanical stress-dependant changes in elastin synthesis have been shown in pulmonary artery segments tonically stretched *in vitro* (Tozzi et al., 1989), suggesting a major role for mechanical forces in the regulation of elastin synthesis in different cell types. Studies in fetal cows (Noguchi et al., 1989) and developing mice (Bostrom et al., 1996) indicate that elastin in the alveolar region of the lung is produced primarily by cells staining positive for α-smooth muscle actin (alveolar myofibroblasts) located at sites of alveolar septal formation. The mechanisms by which these cells respond to mechanical stimuli to alter elastin expression are unknown, but are likely to involve various complex cell-matrix interactions. It is possible that mechanical forces could directly alter the transcription of a gene by deformation of the nucleus via cytoskeletal elements (Maniotis et al., 1997). Elastin expression may also be altered by various soluble mediators such as growth factors released from surrounding cells. It has been suggested that alveolar epithelial cells have the ability to regulate lung fibroblast tropoelastin expression via an unknown soluble factor (Mariani et al., 1998). The influence of alterations in lung expansion on a number of growth factors thought to play a role in the regulation of tropoelastin synthesis is unknown. However, TGF-β_1 is unlikely to be involved, as pulmonary TGFβ_1 mRNA levels are increased only at 10 days of TO (M.J. Wallace unpublished observation) and do not coincide with the transient increase in tropoelastin expression observed at 2 days of TO. During normal lung development, PDGF-A appears to play a critical role in the migration and differentiation of alveolar myofibroblasts and in alveogenesis (Bostrom et al., 1996), and therefore PDGF-A released from surrounding cells or alveolar myofibroblasts themselves could alter the number of elastin producing cells. Evaluation of tropoelastin expression by *in situ* hybridisation in the present study showed that in alveolar structures, both the amount of tropoelastin expression and the number of sites of expression increased, indicating a potential alteration in the number, differentiation or migration of alveolar myofibroblasts. However, the influence of lung

expansion on PDGF-A in the lung is unknown. Other than the changes in elastin expression that occurred in the alveoli, this study showed that an increase in the level of fetal lung expansion also resulted in an increase in tropoelastin expression in the walls of pulmonary arteries and airways. Thus it is likely that there is more than one mechanism by which the lung increases elastin synthesis in response to lung tissue stretch and these mechanisms may be different for the different lung structures. Whatever the specific mechanisms, the finding that sustained alterations in lung expansion in fetal sheep can affect tropoelastin expression is important for the understanding of normal lung development. In particular, the apparent relationship between alterations in elastin synthesis and alveolar development may have implications for infants born following oligohydramnios as well as preterm infants who may be born without experiencing the high levels of lung expansion that occur *in utero* during alveolar development.

4.4.5 Conclusions

This study shows that, in developing fetal lung, the prevailing physical environment affects tropoelastin expression and elastin synthesis and deposition. A sustained increase in lung expansion transiently stimulates tropoelastin expression in vessels, airways and alveoli. In contrast, a sustained reduction in fetal lung expansion reduces tropoelastin expression and content in alveoli, and although it still appears to be deposited in aggregates, these aggregates are not associated with secondary septal crests. These findings provide further evidence that the basal degree of lung expansion *in utero* is an important determinant of the growth and structural development of the lung, in particular, alveolar architecture.

Chapter 5

Effect of physiological levels of corticosteroids on elastin synthesis and deposition in the ovine fetal lung in vivo

5.1 Introduction

During normal development, fetal plasma cortisol concentrations increase significantly towards the end of gestation (Bassett & Thorburn, 1969), which plays an important role in the maturation of many fetal organ systems including the lungs. Liggins first observed the maturational effects of corticosteroids on the lungs in 1969 when conducting experiments on glucocorticoid induction of premature labour. During this study, he observed that glucocorticoid treatment resulted in better lung stability and compliance in premature lambs than was expected at that gestational age (Liggins, 1969). This study led to many others documenting the effects of corticosteroids on lung maturation and the wide use of antenatal corticosteroids to induce *in utero* lung maturation in fetuses expected to be born prematurely, to reduce the risk of respiratory distress syndrome (RDS). The maturational effects of corticosteroids on the fetal lung were primarily attributed to the maturation of the surfactant system (Liggins, 1969), however, both endogenous and exogenous corticosteroids have also been shown to stimulate the structural maturation of the lung (Crone et al., 1983; Willet et al., 1999). This enhanced structural maturation has often been considered to be at the expense of lung growth and alveolar septation, and indeed a number of studies have reported that lung growth and/or alveolar formation are inhibited by prenatal administration of corticosteroids (Blanco et al., 1989; Massaro & Massaro, 1986; Massaro & Massaro,

1992; Tschanz & Burri, 1997). However, the majority of these observations were derived from studies in which pharmacological doses of synthetic corticosteroids (~30 fold greater bioactivity than cortisol) were administered either maternally, to the fetus just prior to delivery, or postnatally. In addition, corticosteroid administration in previous studies has generally not been designed to result in fetal plasma cortisol levels analogous to those that would occur during normal development. In contrast, a recent study using fetal sheep, in which doses of cortisol (designed to mimic the pre-partum rise in circulating cortisol concentration that occurs before labour in sheep) given between 122 and 131 days of gestation, resulted in no inhibition of fetal lung growth, and an increase in alveolar number (Boland et al., 2004). This suggests that physiological levels of cortisol may play a role in alveolar formation during normal lung development.

As discussed in the previous chapter, there is a strong link between alveolar septal wall formation and elastin deposition. Previous studies that have examined the effects of corticosteroids on elastin synthesis in the lung have produced varying results. Some studies report a decrease in lung elastin concentration (Schellenberg et al., 1987b; Willet et al., 1999), while others report an increase in elastin gene expression or protein concentration (Anceschi et al., 1992; Noguchi et al., 1990; Pierce et al., 1995; Schellenberg et al., 1987a) following corticosteroid treatment. In these studies, the species, the dose of corticosteroids, and the timing and method of administration of corticosteroids all varied. In addition, no study has examined the effect of corticosteroids at physiological levels on both elastin synthesis and deposition in the developing fetal lung. This, therefore, was the first aim of this study. Since elastin synthesis in the lung of fetal sheep increases towards the end of gestation, coincident with the increase in fetal plasma cortisol concentration that occurs during normal development, and alveolar number has been shown to increase in the model used in this study (Boland et al., 2004), it was hypothesised that elastin synthesis would also be increased.

In addition, experiments performed in studies reported in Chapter 4 indicate that, not only is mechanical stretch of lung tissue an important regulator of elastin synthesis during fetal development, an adequate degree of lung expansion is essential for the correct laying down of elastic fibres and hence alveolar septal wall formation. Therefore, in order to further elucidate the potential role of corticosteroids in elastin

synthesis and deposition, and in alveolar development, the second aim of this study was to evaluate the effect of corticosteroids on elastin synthesis and deposition both in the presence and absence of an adequate degree of fetal lung expansion. Previous studies using the same model have shown that, although cortisol increases alveolar number in under-expanded fetal lungs, alveolar number remains substantially lower than in normally expanded lungs (Boland et al., 2004). Therefore it was hypothesised that cortisol would increase elastin synthesis, but would be unable to counteract the detrimental effects of a decreased level of fetal lung expansion on elastin deposition and alveolar development.

5.2 Materials and methods

5.2.1 Experimental preparation

Fetal lung tissue was obtained from experiments performed previously on chronically catheterised fetal sheep in our laboratory (Boland et al., 2004). Aseptic surgery was performed on pregnant Border Leicester X Merino ewes at approximately 110 days of gestation (term ~ 147 days) to insert fetal tracheal catheters for the manipulation of lung fluid volume as described in section 4.2.1. Fetal vascular catheters were also inserted for arterial blood sampling and for the intravenous infusion of saline and cortisol.

To determine the influence of cortisol treatment on elastin synthesis in normal fetal lungs and fetal lungs that have experienced a sustained reduction in the level of lung expansion, the following four treatment groups were used:

Group 1) A *saline-only* group in which saline was infused at 1.2 ml/hr i.v. into the fetus for a period of 9 days (122-131 days GA, n=5).

Group 2) A *cortisol-only* group in which cortisol (hydrocortisone sodium succinate, Solu Cortef, Upjohn Pty Ltd, Australia) was infused i.v. into the fetus at increasing concentrations for a period of 9 days (122-131 days GA, n=5). Cortisol doses were chosen to induce a rise in fetal plasma cortisol concentration to levels that are similar to those observed in the last 10 days of gestation before the onset of labour in the sheep, but without inducing labour (Magyar et al., 1980). Cortisol was dissolved in heparinised saline and delivered at a rate of 1.2 ml/hr using an infusion pump as follows: 1.5mg/day

on 122-123 days GA, 2.5mg/day on 124-125 days GA, 3.0mg/day on 126-127 days GA, 3.5 mg/day on 128-129 days GA and 4.0 mg/day on 130-131 days GA.

Group 3) A *drain & saline* group in which lung liquid was drained gravimetrically into a sterile bag for 20 days (111-131 days GA) and saline was infused as in Group 1 (n=5).

Group 4) A *drain & cortisol* group in which fetal lung liquid was drained as in Group 3 (111-131 days GA) and cortisol was infused for 9 days as in Group 2 (122-131 days GA)(n=5).

At postmortem (131 days GA), the lungs were removed from the fetus and the left main bronchus ligated before portions of the left lobes were removed and frozen in liquid nitrogen and stored at -70°C. Pieces of the left lung were used for extraction of total RNA for Northern blot analysis (section 2.2) and for the determination of elastin content (section 2.4). Lung tissue was micro-dissected prior to the measurement of elastin content to remove larger airways and blood vessels so as to focus my findings on the alveolar region of the lung. The right lung was fixed via the trachea at 20 cmH_2O using 4% paraformaldehyde, sliced into 5mm sections and post-fixed in Zamboni's fixative overnight. All fixed tissue samples were embedded in paraffin and sectioned at 5µm for in situ hybridisation (section 2.3) and histochemical analysis (section 2.5) to localise elastin mRNA expression and elastin deposition in lung tissue respectively.

5.3 Results

5.3.1 Tropoelastin mRNA levels

Northern blot analysis

Following a 9 day cortisol infusion (between days 122 and 131 of gestation) designed to mimic the pre-partum rise in fetal plasma cortisol concentrations, tropoelastin mRNA levels in lung tissue were significantly greater than in *saline-only* (vehicle) infused fetuses (*cortisol-only*: 75.4 ± 10.4 vs *saline-only* 38.9 ± 5.7 arbitrary units).

After 20 days of reduced lung expansion combined with a cortisol infusion, tropoelastin mRNA levels (*drain & cortisol*: 32.7 ± 3.5) were not significantly different from those of fetuses that underwent lung liquid drainage alone (*drain & saline*: 26.4 ± 1.4) or

saline-only infusion (32.9 ± 5.7). Tropoelastin mRNA levels were, however, significantly lower than in fetuses infused with cortisol in the presence of a normal degree of fetal lung expansion (*cortisol-only*: 75.4 ± 10.4). Expressed as a percentage of mean values of the *saline-only* group, pulmonary tropoelastin mRNA levels following *cortisol-only* infusion were 194.1 ± 26.6%. Following cortisol infusion plus 20 days of lung liquid drainage (*drain & cortisol*), tropoelastin mRNA levels were 84.1 ± 8.9% of mean values of the *saline-only* group (Figure. 5.1) (Table 5.1).

Table 5.1 Tropoelastin mRNA levels

Mean tropoelastin mRNA levels ± SEM (arbitrary units), measured at 130.8 ± 0.1 days of gestation in the *saline-only*, *drain & saline*, *cortisol-only* and *drain & cortisol* groups of fetuses. Values that do not share a common letter are significantly different from each other (P<0.05).

	saline-only (n=5)	*drain & saline* (n=5)	*cortisol-only* (n=5)	*drain & cortisol* (n=5)
tropoelastin mRNA levels (arbitrary units)	38.9 ± 5.7^{a}	26.4 ± 1.4^{a}	75.4 ± 10.4^{b}	32.7 ± 3.5^{a}
% of mean *saline-only* values	100 ± 14.78^{a}	67.84 ± 3.6^{a}	194.1 ± 26.6^{b}	84.1 ± 8.9^{a}

In situ hybridisation

Tropoelastin expression was prominent in pulmonary arteries and minimal in airways in the lungs of all treatment groups. Physiological levels of corticosteroids did not appear to affect tropoelastin expression in blood vessels or airways in normally expanded or under-expanded lungs (Figure 5.2). Tropoelastin expression in the alveolar region of the lungs was focused at the tips of developing secondary septa and at bends in alveolar walls where secondary septal formation would be expected to occur. Following *cortisol-only* treatment, tropoelastin expression was increased both in the number expression sites expression and the amount of expression at each site as determined by silver grain density. Tropoelastin expression in both groups of fetuses that underwent a period of lung liquid drainage (*drain & saline* and *drain & cortisol*) was reduced both in the number of tropoelastin expression sites and the amount of expression at each site. Focal points of tropoelastin expression were also not as apparent following decreased lung expansion compared to normally expanded lungs. Tropoelastin expression in the

alveolar region of the lung appeared to be similar in under-expanded fetal lungs regardless of cortisol treatment (Figure 5.3).

5.3.2 Elastin deposition

Elastin concentration and content

The concentration (mg/g lung tissue) of elastin in lung parenchymal tissue was not significantly different between any of the four treatment groups. However, the mean elastin content of the lungs was significantly lower in both the *drain & saline* (97.6 ± 23.0 mg) and *drain & cortisol* (98.9 ± 14.6 mg) groups of fetuses than in *saline-only* (196.4 ± 37.7 mg) or *cortisol-only* (255.9 ± 15.9 mg) treatments. Cortisol infusion in lung liquid drained fetuses did not alter elastin content compared to lung liquid drainage alone. Expressed as a percentage of mean values in *saline-only* treated fetuses, elastin content was 130.3 ± 8.1% in *cortisol-only* fetuses, 49.7 ± 11.7% in *drain & saline* fetuses and 50.3 ± 7.4% in *drain & cortisol* fetuses (Figure. 5.4).

Table 5.2 Elastin concentration and content

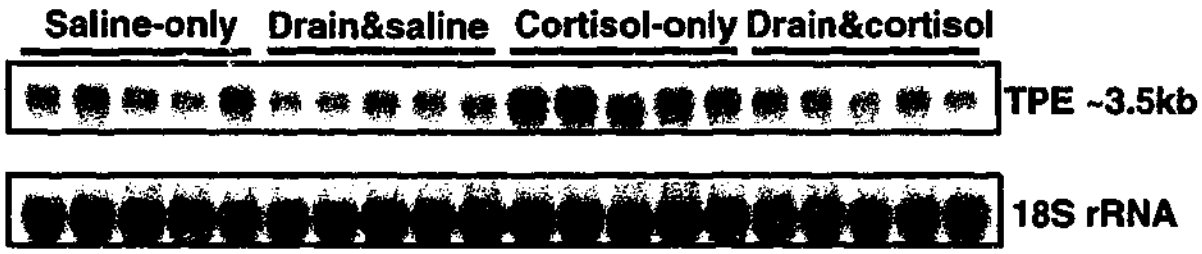
Mean elastin concentration ± SEM (mg/g lung tissue) and elastin content ± SEM (mg), at 130.8 ± 0.1 days of gestation in the *saline-only*, *drain & saline*, *cortisol-only* and *drain & cortisol* groups of fetuses. Values that do not share a common letter are significantly different from each other ($p<0.05$).

	saline-only (n=5)	*drain & saline* (n=5)	*cortisol-only* (n=5)	*drain & cortisol* (n=5)
elastin concentration (mg/g lung)	1.6 ± 0.2	1.9 ± 0.3	2.0 ± 0.1	1.9 ± 0.2
% of mean *saline-only* values	100.0 ± 13.4	121.0 ± 18.3	128.1 ± 6.6	124.2 ± 15.6
elastin content (mg)	196.4 ± 37.7[a]	97.6 ± 23.0[b]	255.9 ± 15.9[a]	98.9 ± 14.6[b]
% of mean *saline-only* values	100.0 ± 19.2[a]	49.7 ± 11.7[b]	130.3 ± 8.1[a]	50.3 ± 7.4[b]

Elastin staining

In cortisol treated fetuses with normally expanded lungs (*cortisol-only*), lung tissue stained for elastin showed no apparent differences in elastin deposition in blood vessels and airways (Figure 5.5) or alveolar compartments (Figure 5.6) compared to *saline-only* treated fetuses. Elastin staining was focused at the tips of secondary septal crests and, therefore it appeared that secondary septal formation was occurring in a manner similar to that observed in *saline-only* treated fetuses (Figure 5.6). When cortisol treatment was combined with under-expansion of the fetal lungs (*drain & cortisol*), elastin deposition was altered in the alveolar walls of lungs similar to that seen in under-expanded lungs without cortisol treatment (*drain & saline*). As in *drain & saline* fetuses, focal aggregates of elastin were not regularly spaced, and many were not associated with the formation of secondary septal crests (Figure 5.6). Elastin deposition in blood vessels and airways however, did not appear to be altered by cortisol treatment in under-expanded lungs (Figure 5.5).

A.



B.

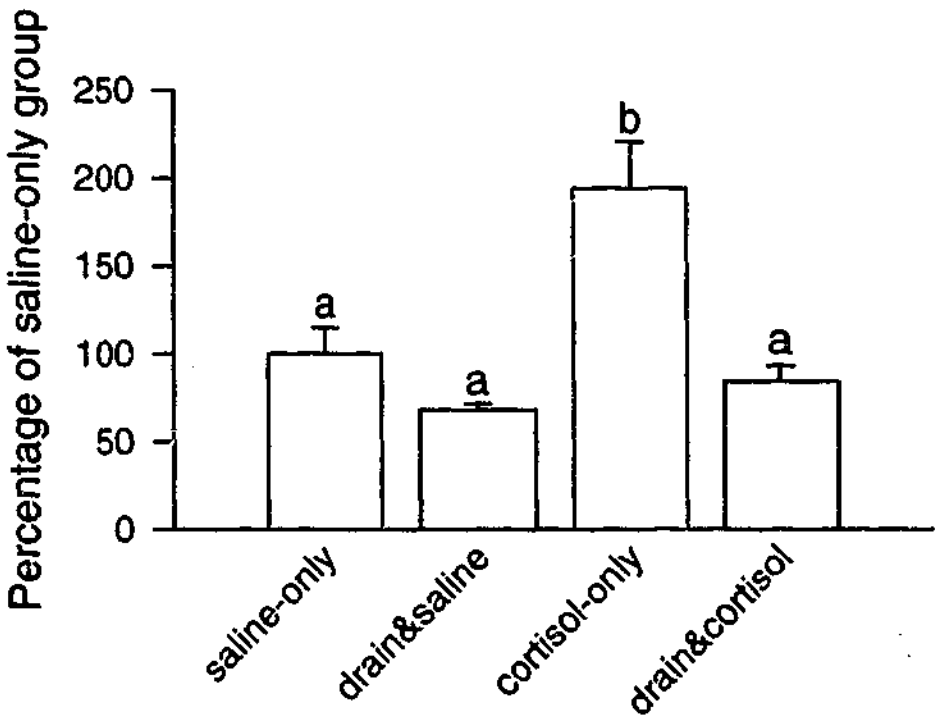


Figure 5.1 Tropoelastin (TPE) mRNA levels following cortisol treatment of normally expanded and under-expanded fetal lungs

A: Northern blot analysis of total RNA extracted from lung tissue collected from saline treated (*saline-only*), saline treated fetuses exposed to 20 days of lung liquid drainage (*drain & saline*), cortisol treated fetuses (*cortisol-only*) and cortisol treated fetuses also exposed to lung liquid drainage (*drain & cortisol*). *B*: Mean tropoelastin mRNA levels where the density of each tropoelastin mRNA band was expressed as a ratio of the density of the corresponding 18S rRNA band and then expressed as a percentage of mean values of the saline-only group. Values that do not share a common letter are significantly different to each other ($p<0.05$) when analysed by one-way ANOVA.

Figure 5.2 Tropoelastin mRNA in blood vessels and airways following cortisol treatment in normal and under-expanded fetal lungs
In situ hybridisation for tropoelastin mRNA in blood vessels (BV) and airways (A) in lungs of *saline-only*, *drain & saline*, *cortisol-only*, and *drain & cortisol* treated fetuses. Presented are paired bright- and dark-field photographs taken at X20 magnification of fetal lung hybridised with ^{35}S-labeled antisense RNA for tropoelastin mRNA. A strong positive signal was detected in blood vessels and minimal or no signal was detected in airways of fetuses in all treatment groups.

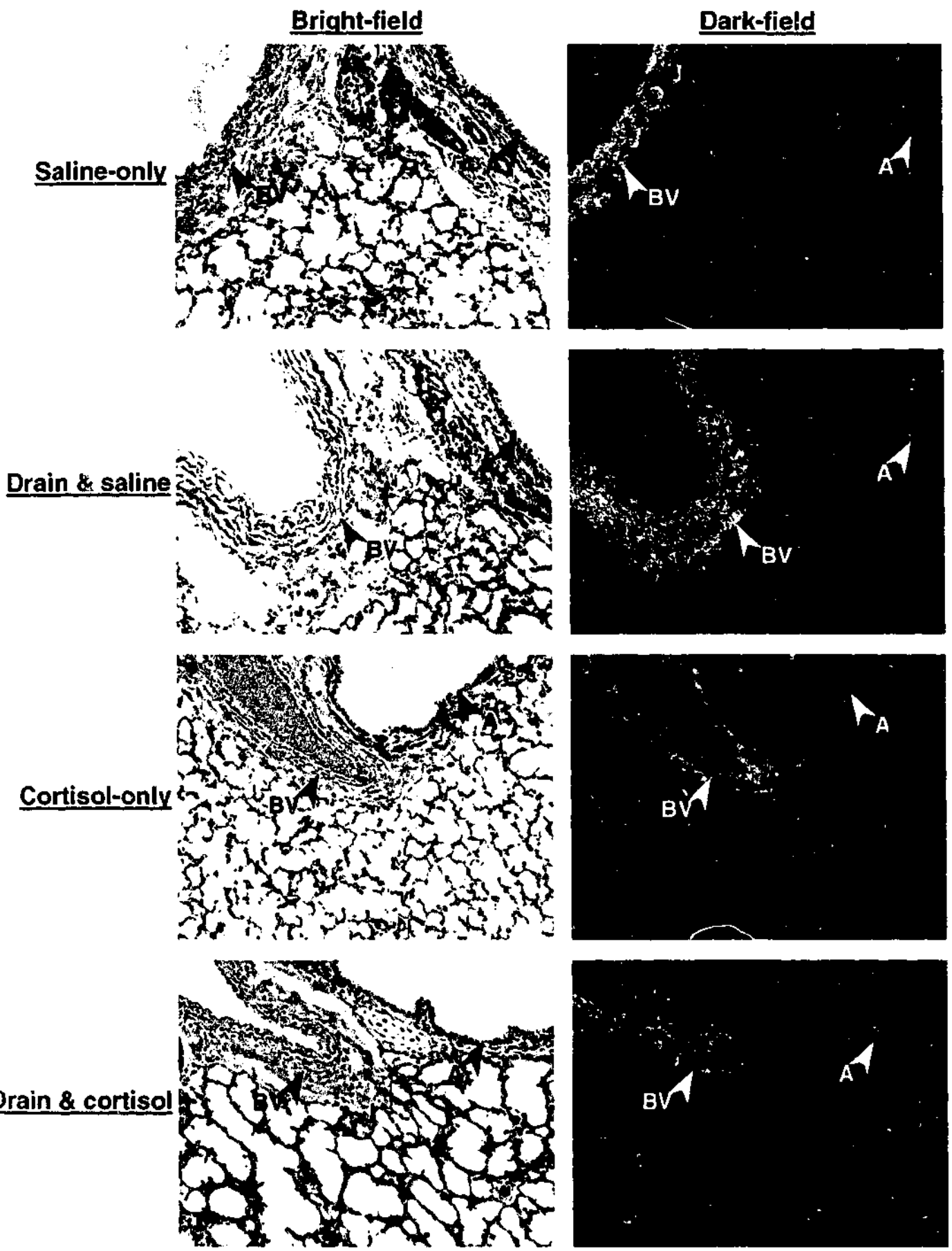
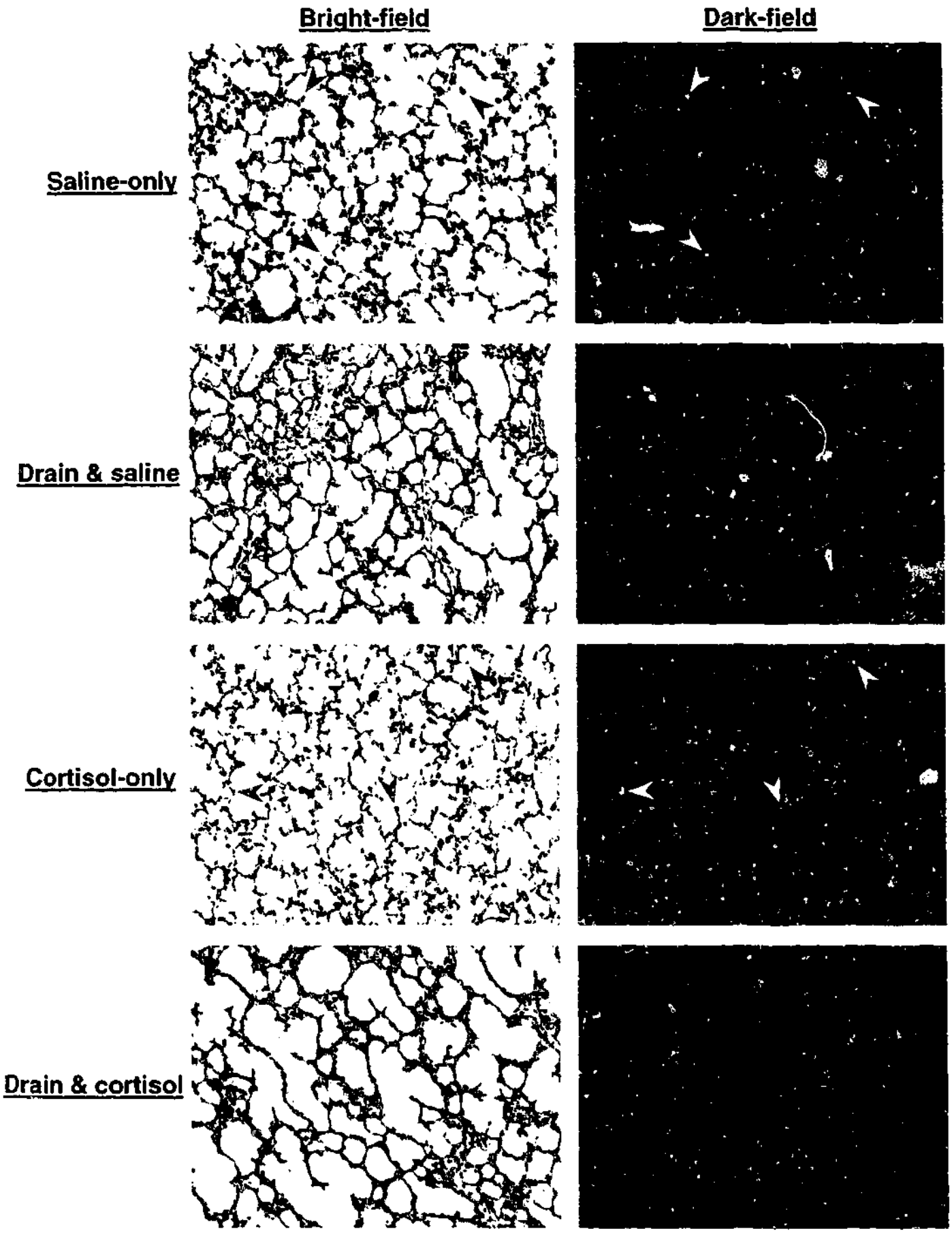

Bright-field
Dark-field
Saline-only
Drain & saline
Cortisol-only
Drain & cortisol
BV
A

Figure 5.3 Tropoelastin mRNA in the alveolar region of the lungs following cortisol treatment in normal and under-expanded fetal lungs

In situ hybridisation for tropoelastin mRNA in lung parenchymal tissue of *saline-only*, *drain & saline*, *cortisol-only*, and *drain & cortisol* treated fetuses. Presented are paired bright- and dark-field photographs taken at X20 magnification of fetal lung hybridised with ^{35}S-labeled antisense RNA for tropoelastin mRNA. Positive signal for tropoelastin mRNA was detected at the tips of developing secondary septa in normally expanded lungs (arrow heads). Following *cortisol-only* treatment, both the number of sites positive for tropoelastin mRNA and the density of silver grains at each site was increased. Following fetal lung under-expansion, positive signal for tropoelastin mRNA was substantially reduced, and focal points of tropoelastin expression were not apparent as in normally expanded lungs.


Bright-field
Dark-field
Saline-only
Drain & saline
Cortisol-only
Drain & cortisol

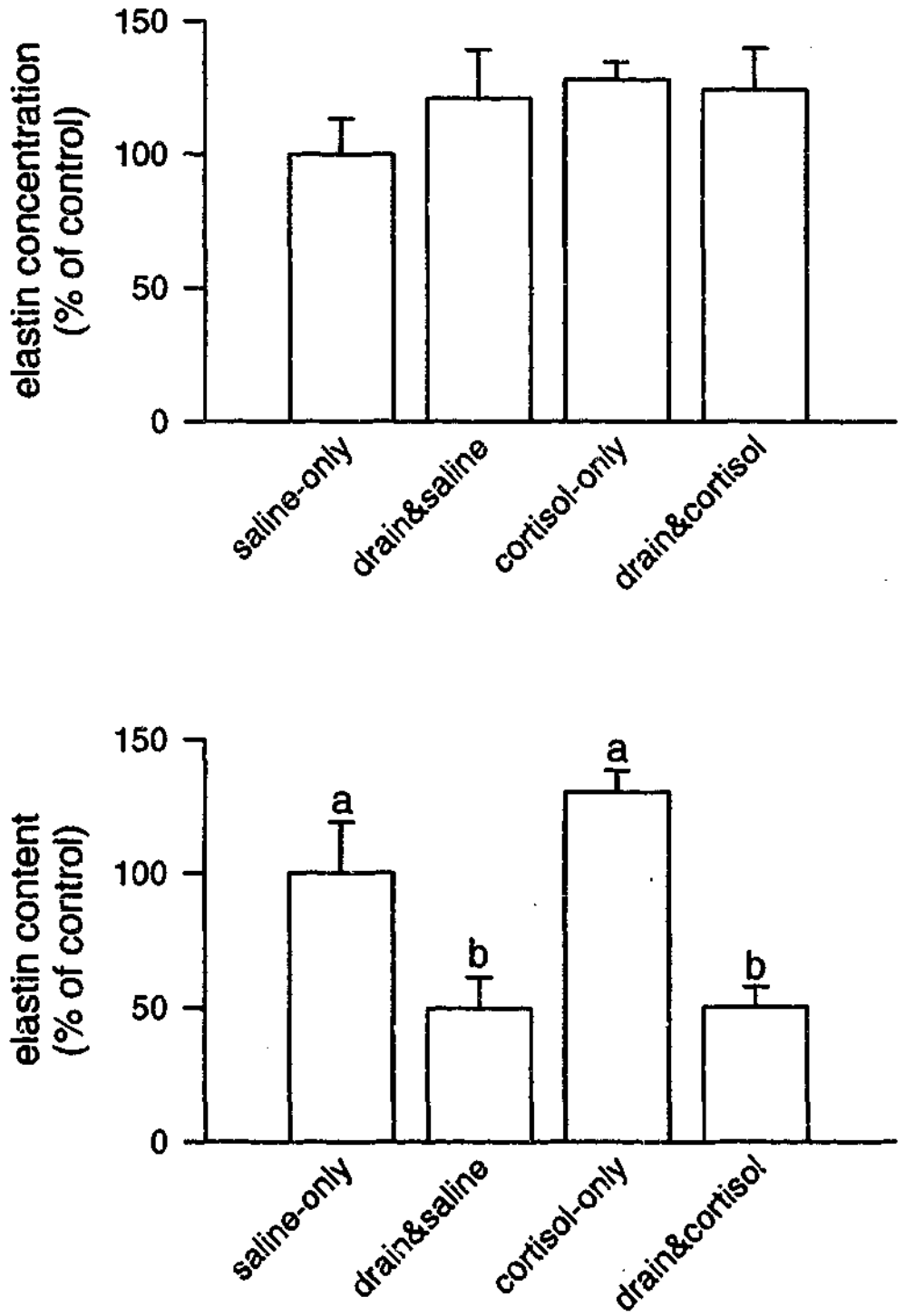


Figure 5.4 Elastin concentration and elastin content of the lungs following cortisol treatment of normally expanded and under-expanded fetal lungs
Elastin concentration and elastin content of the lungs of saline treated (saline-only), fetuses exposed to 20 days of lung liquid drainage (drain & saline), cortisol treated fetuses (cortisol-only) and cortisol treated fetuses also exposed to lung liquid drainage (drain & cortisol). Values were expressed as a percentage of the mean values of the saline-only group. Values that do not share a common letter are significantly different to each other ($p<0.05$) when analysed by one-way ANOVA.

Figure 5.5 Elastin staining of blood vessels and airways following cortisol treatment in normal and under-expanded fetal lungs

Hart's resorcin-fuchsin stain for elastin in lung tissue sections. Photographs taken at X20 magnification of a pulmonary artery and a cartilaginous airway are presented for each treatment group. Elastin is stained dark purple-black and can be seen as thick layers in the walls of pulmonary arteries and finer layers in the walls of large airways.

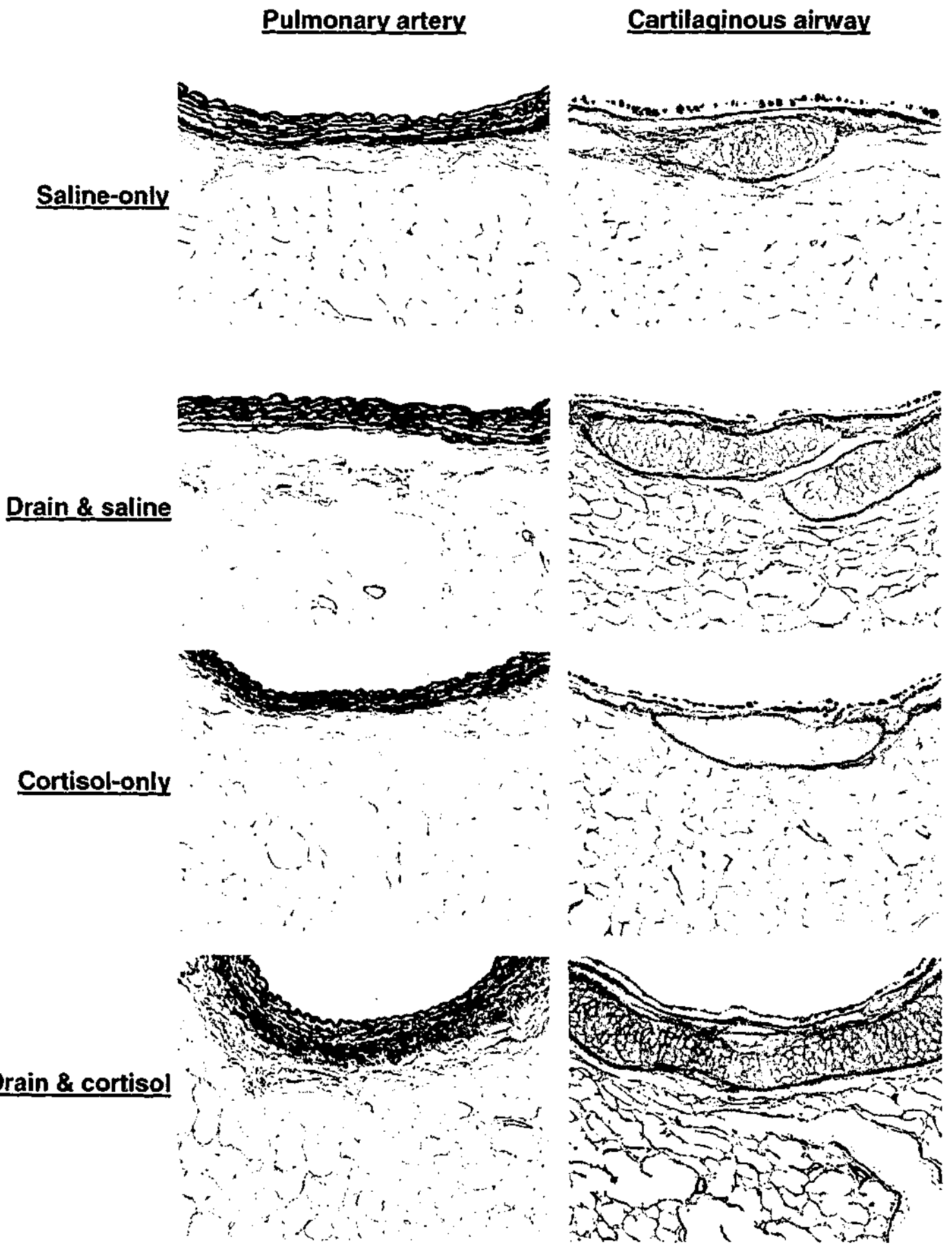
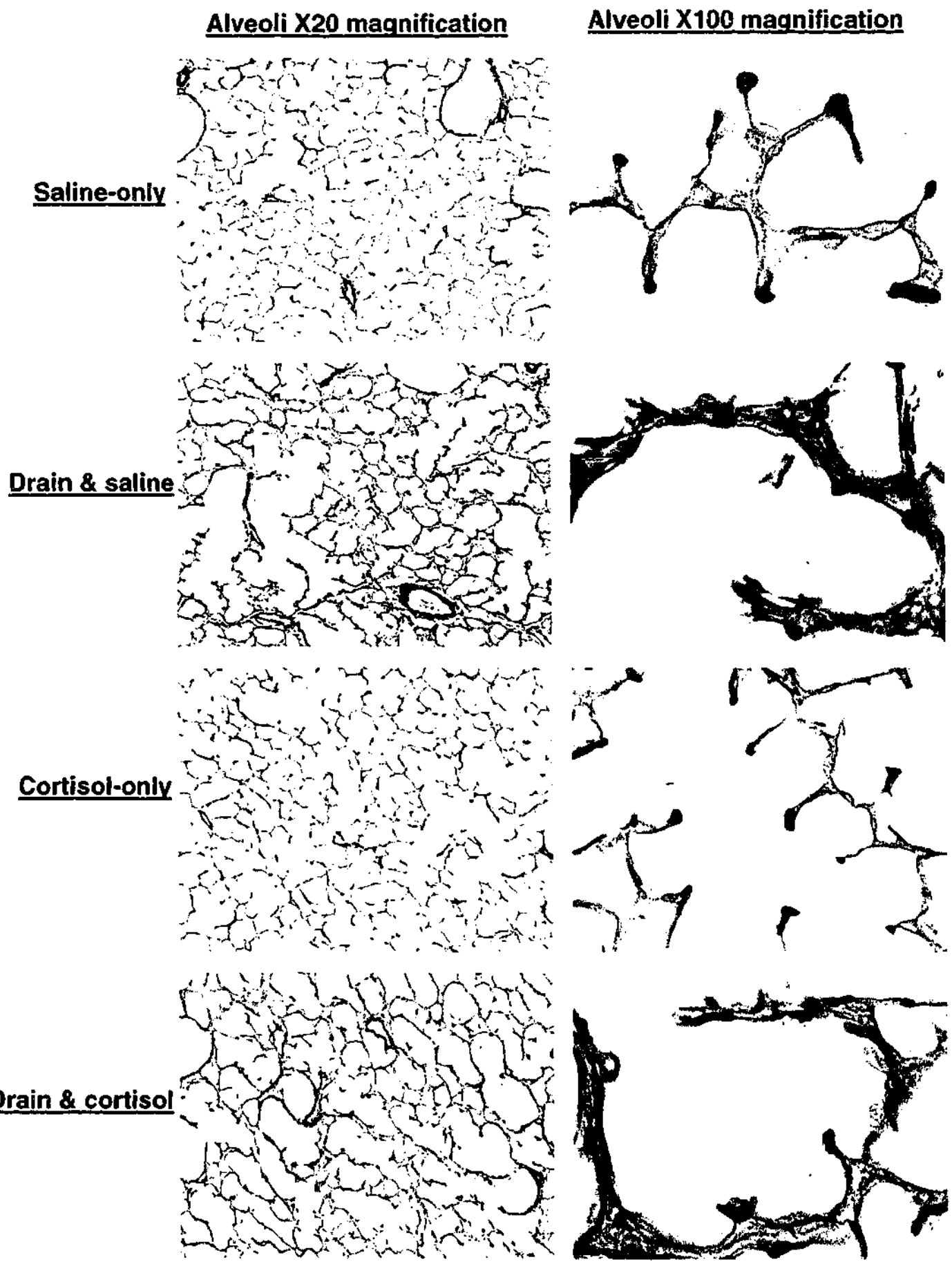

Pulmonary artery
Cartilaginous airway
Saline-only
Drain & saline
Cortisol-only
Drain & cortisol

Figure 5.6 Elastin staining in the alveolar region of the lungs following cortisol treatment in normal and under-expanded fetal lungs

Hart's resorcin-fuchsin stain for elastin in lung tissue sections. Photographs taken at X20 and X100 magnification of the alveolar region of the lung are presented for each treatment group. Elastin is stained dark purple-black and is focused at the tips of secondary septal crests in control lungs. In *drain & saline* lungs, focal aggregates of elastin occur at much shorter distances from each other and secondary septation appears to have failed. At X100 magnification, elastin can be seen along the alveolar walls rather than primarily being focused at the tips of secondary septae. Elastin deposition in *cortisol-only* treated lungs appears similar to that of *saline-only* treated lungs, and elastin deposition in *drain & cortisol* lungs appears similar to that of *drain & saline* lungs.


Alveoli X20 magnification
Alveoli X100 magnification
Saline-only
Drain & saline
Cortisol-only
Drain & cortisol

5.4 Discussion

It is well established that both endogenous and exogenous corticosteroids have a major influence on fetal lung maturation, particularly on the structural maturation of the lung. However the information relating to the influence of corticosteroids on elastin synthesis during lung development is scant and difficult to interpret. Since alveolar development has been shown to increase in response to physiological levels of cortisol during fetal development, it was hypothesised that elastin synthesis would also be stimulated. In addition, the inability of physiological levels of cortisol to restore alveolar numbers in under-expanded fetal lungs led to a second hypothesis, that cortisol would be unable to counteract the detrimental effects of a decreased level of fetal lung expansion on elastin deposition and alveolar development. In agreement with these two hypotheses, this study has shown that physiological levels of fetal plasma cortisol increase tropoelastin mRNA levels and elastin deposition consistent with changes in alveolar development in normally expanded lungs and that fetal lung under-expansion appears to override the influence of cortisol.

5.4.1 Fetal plasma cortisol concentrations

Previous studies that have examined the influence of corticosteroids on pulmonary elastin synthesis have been conducted in various animal species, at different stages of lung development and using various doses and methods of administration of corticosteroids (summarised in Table 5.3). As a result, the observations derived from these studies have been varied and often contradictory, and are therefore difficult to interpret in terms of the role that corticosteroids play in elastin synthesis during normal lung development. In addition, no study investigating corticosteroids and elastin synthesis has attempted to mimic *in vivo*, fetal plasma cortisol concentrations that occur during normal lung development, and none have reported fetal plasma cortisol concentrations resulting from the administration of corticosteroids. In this study, however, corticosteroids administered via a fetal intravenous infusion in increasing doses over a 9-day period (122-131 days of gestation) produced fetal plasma cortisol concentrations similar to those that occur endogenously in the 10 days prior to labour in sheep (Magyar et al., 1980). This model is therefore more appropriate than those used in previous studies for gaining a better understanding of the normal role of corticosteroids

in elastin and alveolar development. There have been no studies to date that have evaluated the influence of endogenous corticosteroids on pulmonary elastin synthesis. Figure 5.7 below shows fetal plasma cortisol concentrations that were achieved in the fetuses used in this study.

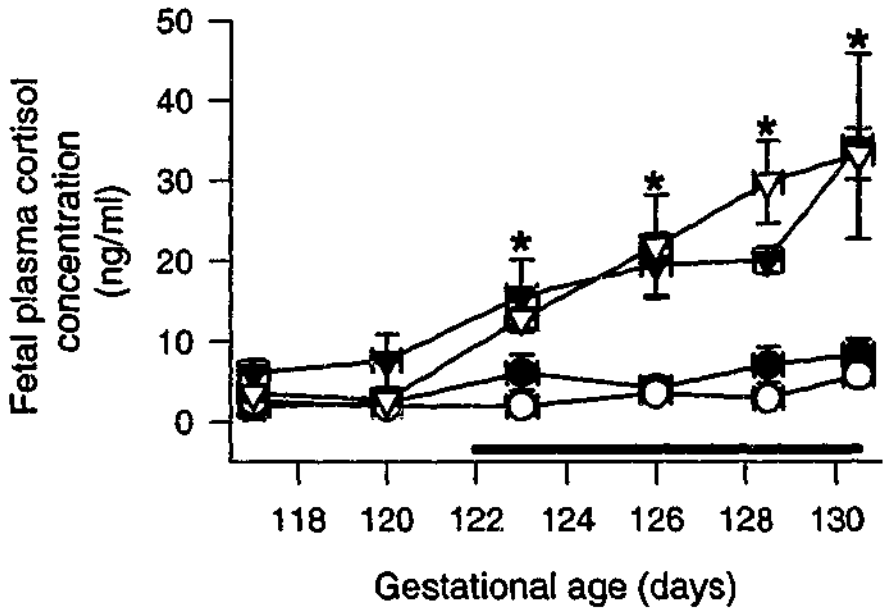


Figure 5.7 Fetal plasma cortisol concentrations
Cortisol concentrations (mean±SEM) in fetal plasma of saline-infused (circles) and cortisol-infused (triangles) fetuses. Open symbols represent fetuses that have undergone a 20-day period of lung liquid drainage whereas the closed symbols represent values from fetuses not exposed to lung liquid drainage. The closed bar represents the period during which the cortisol and saline infusions were administered. The asterisks indicate mean plasma cortisol concentrations in the cortisol-infused fetuses that are significantly different ($p<0.05$) from both of the saline-infused groups at the corresponding gestational age. (Data from Boland *et al.* 2004)

5.4.2 Effect of physiological levels of corticosteroids on tropoelastin expression

A 9-day fetal cortisol infusion between days 122 and 131 of gestation in intact fetuses resulted in a dramatic increase in tropoelastin mRNA levels. These results are consistent with increases in tropoelastin mRNA levels observed in fetal rat lung cells following maternal Dexamethasone administration (Pierce et al., 1995) and, following Dexamethasone treatment of neonatal rat pulmonary fibroblasts (Noguchi et al., 1990) and fetal rat lung cells (Pierce et al., 1995) in culture. Both of these studies indicate that the stimulation of tropoelastin expression by corticosteroids is age dependant,

suggesting that at least in rats there are specific windows of time in which corticosteroids are capable of stimulating tropoelastin expression. It is unclear from the present study however, whether such specific windows (when tropoelastin expression is inducible by corticosteroids) exist during fetal lung development in the sheep. This is because tropoelastin expression was only measured at one time point, and expression could be induced during a period where endogenous corticosteroid concentrations are normally very low. Willet *et al.* (1999) observed age dependant changes in the density of parenchymal elastin staining following corticosteroid treatment, indicating that this may be the case; however, tropoelastin expression was not measured in that study, and the differences in corticosteroid administration make it difficult to make comparisons with the present study.

In fetal sheep, tropoelastin mRNA levels increase throughout the canalicular and alveolar periods of lung development (~ day 80 until term) and peak in the late fetal and early neonatal period (Chapter 3, Figure 3.1). Fetal plasma cortisol concentrations however do not begin to rise until approximately 135 days of gestation and peak just prior to labour (Magyar et al., 1980), indicating that corticosteroids are not responsible for the induction of elastin expression during fetal lung development but are capable of modifying elastin production by cells already producing elastin. The ability of physiological levels of cortisol to induce tropoelastin expression as shown in the present study suggests a role for endogenous corticosteroids in the modulation of elastin synthesis during normal fetal lung development in sheep. The extent to which the endogenous rise in fetal plasma cortisol concentration influences elastin expression during normal development is, however, unknown. Since the naturally occurring rise in endogenous fetal plasma cortisol concentrations occurs at a later stage than those induced in this study, and there is evidence of an age dependant response to corticosteroids in the rat, it cannot be assumed that the response to cortisol observed in this study reflects that which occurs endogenously. However, the offset in the plasma cortisol rise is only approximately 13 days, and the stage of lung development is the same as that which occurs during the endogenous cortisol surge; therefore, it is likely that the influence of endogenous corticosteroids on pulmonary elastin synthesis would be similar. The measurement of tropoelastin expression following fetal adrenalectomy would further clarify the influence of endogenous corticosteroids during normal fetal lung development.

Studies in rat lung cells *in vitro* indicate that the mechanism by which corticosteroids upregulate tropoelastin expression during development is at the level of transcription (Noguchi et al., 1990; Pierce et al., 1995). This may be via a glucocorticoid response element (GRE) in the promoter region of the tropoelastin gene. Pierce *et al.* (1995) have also suggested from *in situ* hybridisation experiments in rat lung, that the stimulation of tropoelastin expression by glucocorticoids involves both an increase in the number of elastin producing cells as well as an increase in tropoelastin expression per cell (Pierce et al., 1995). In the present study, *in situ* hybridisation experiments also showed an apparent increase in both the number of sites of tropoelastin expression and the amount of expression at each site. Alterations in tropoelastin expression in the study by Pierce *et al.* (1995) were observed in cells surrounding developing airways and were associated with airway branching during the pseudoglandular and canalicular periods of lung development in the rat. In the present study however, changes in tropoelastin occurred predominantly in the alveolar region of the lung and were associated with alveolar formation. There were no differences observed in tropoelastin expression in the airways, thus indicating that the influence of corticosteroids on elastin expression during lung development in airways and alveoli may be age-dependent.

When cortisol treatment was combined with a decreased level of fetal lung expansion, pulmonary tropoelastin mRNA levels were not different from those in *drain & saline* treated fetuses or *saline-only* treated fetuses, but were substantially lower than in *cortisol-only* treated fetuses. This suggests that the decrease in pulmonary elastin expression caused by under-expansion of the fetal lung overrides the cortisol-induced increase in elastin expression; therefore, lung tissue stretch appears to be a more dominant regulator of tropoelastin expression during late fetal lung development than fetal plasma cortisol levels. It is also possible that some of the effects of cortisol on tropoelastin mRNA levels are mediated by an increase in lung expansion as cortisol in this model has also been shown to increase lung liquid volume and thus lung expansion (Wallace et al., 1995). Furthermore, adrenalectomy has been shown to prevent age related increases in lung volume (Wallace et al., 1996).

It is important to note that although the data presented in this chapter does not show a significant decrease in tropoelastin expression in the *drain & saline* treatment group compared to the *saline-only* group, this reduction just failed to reach statistical significance when analysed by the unpaired *t*-test, with a p-value of 0.06. However, the

data are derived from the same animals as those presented in Chapter 4 in which there was a statistically significant difference between the groups. While the difference between the groups is most probably physiologically significant, the difference the statistical significance of the data is most likely a result of inter-experimental variation.

5.4.3 Effect of physiological levels of corticosteroids on elastin concentration and elastin content in lung parenchymal tissue

Despite the large increase in pulmonary tropoelastin expression stimulated by cortisol treatment, elastin concentration and content were not significantly different in *cortisol-only* treated fetuses compared to *saline-only* treated fetuses. However, there was a tendency for an increase, with p-values for the increases in elastin concentration and content being 0.1 and 0.2 respectively. This was an unexpected result, since alveolar numbers were increased in these animals as a result of cortisol treatment (Boland et al., 2004), and elastin synthesis is thought to be associated with alveolar development. An explanation for this discrepancy may be that the change in elastin synthesis associated with a small increase in alveolar number may be too subtle to be detected by the assay used in this study. What is clear, however, is that physiological levels of plasma cortisol in the sheep fetus do not decrease elastin synthesis. In contrast, a study conducted in rats reported that desmosine concentration (a cross-linked amino acid specific to elastin) was reduced in neonatal but not fetal rat lungs following maternal Dexamethasone administration, equivalent to 1mg/kg over 24 hours, during the pseudoglandular period of lung development (Schellenberg et al., 1987b). This decrease in desmosine concentration was, however, also associated with a decrease in lung and body weights in the neonatal rats and, therefore, could be a result of a decrease in the number of elastin producing cells in the lungs of these animals. Recent studies in sheep indicate that decreases in lung and body growth associated with corticosteroid treatment are associated with maternal administration betamethasone and not cortisol (Jobe et al., 2003; Newnham et al., 1999). The stage of lung development at which treatment was given in the present study was also much earlier than the stage at which endogenous fetal plasma cortisol levels rise in both the sheep and human. Willet *et al.* (1999) observed a decrease in parenchymal elastin density following a single fetal intra-muscular injection of betamethasone, but this was only at 121 days of gestation, whereas elastin density was actually increased at 135 days following betamethasone

treatment, consistent with that which might occur during normal fetal lung development.

When cortisol treatment was combined with a decreased level of fetal lung expansion, pulmonary elastin concentration and content were similar to those of the *drain & saline* group of fetuses, and were consistent with the changes seen in tropoelastin expression. As with the changes observed in tropoelastin expression, these data indicate that the dominant influence on elastin synthesis is the degree of fetal lung expansion rather than cortisol.

5.4.4 Corticosteroids, elastin and alveolar development

As with alterations in fetal lung expansion (Chapter 4), increases in tropoelastin expression induced by physiological levels of corticosteroids were consistent with the increase in alveolar number previously observed in these animals (Boland et al., 2004) (Figure 5.8). These data support a role for corticosteroids in elastin synthesis and alveolar development during normal fetal lung development as well as adding further support to the importance of elastin synthesis in alveolar development.

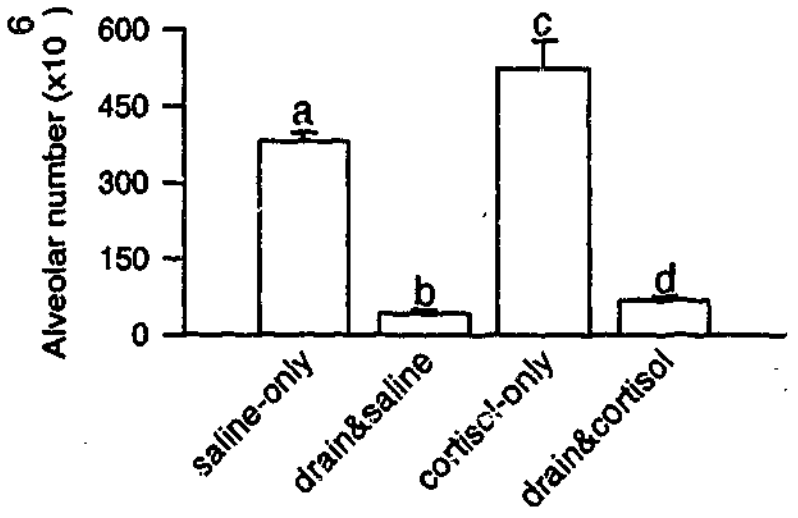


Figure 5.8 Alveolar number following cortisol treatment of normally expanded and under-expanded fetal lungs

Alveolar number (mean ± SEM) in saline treated fetuses (*saline-only*), fetuses exposed to 20 days of lung liquid drainage (*drain & saline*), cortisol treated fetuses (*cortisol-only*) and cortisol treated fetuses also exposed to lung liquid drainage (*drain & cortisol*). Values that do not share a common letter are significantly different from each other ($P<0.05$) (Data from Boland *et al.* 2004).

Previous studies in the sheep indicate that there is an age-dependant effect of corticosteroids on elastin synthesis, with a decrease in parenchymal elastin density following corticosteroid treatment at 121 days of gestation, but an increase following treatment at 135 days of gestation (Willet et al., 1999). Therefore, the influence of corticosteroids on alveolar development may also be age-dependant. In the study by Willet *et al.* (1999), secondary septal number was not significantly different in lungs of fetuses exposed to antenatal corticosteroid treatment compared to controls of either age group. These measurements however, were made just 48 hours after corticosteroid treatment and, therefore, there may not have been sufficient time for changes in septal formation to be detectable. A study in the fetal rhesus monkey has reported an inhibition of alveolar septal formation by corticosteroids during the pseudoglandular period of lung development. It was also noted that there was a stimulation of alveolar septal formation at a later gestational age during the canalicular stage of lung development (Bunton & Plopper, 1984). However, it is unknown whether the changes in alveolar development observed by Bunton *et al.* (1984) were related to changes in elastin synthesis or deposition because the influence of corticosteroids on elastin was not assessed in that study.

When cortisol treatment was combined with a decreased level of fetal lung expansion (*drain & cortisol*), elastin deposition did not appear different to that observed in the lungs of *drain & saline* treated fetuses. This suggests that while corticosteroids appear to play a role in elastin synthesis and alveolar development, it is apparent that an adequate degree of lung expansion is more important than cortisol for elastin deposition and alveolar formation. This is in agreement with the hypothesis that cortisol is unable to reverse the detrimental effects of a decreased level of fetal lung expansion on pulmonary elastin deposition and alveolar development.

5.4.5 Conclusions

This study shows that physiological levels of corticosteroids are likely to play a role in elastin synthesis and alveolar development during normal lung development since tropoelastin expression was stimulated following cortisol treatment in normally expanded lungs. However, the influence of fetal lung expansion on elastin synthesis and deposition appears to be more dominant than that of corticosteroids during lung development.

Table 5.3 Summary of effects of corticosteroids on elastin synthesis

Summary of the effects of corticosteroids on elastin synthesis obtained from published literature to date

Source	Species	Stage of lung Development	Administration	Dose	Summary of results
Schellenberg *et al.* 1987	Sheep	Saccular / alveolar	Fetal i.v. 4d infusion	Cortisol 1mg/hr (in conjunction with other hormones)	Desmosine concentration ↑ at 128d GA in more distensible lungs
Schellenberg *et al.* 1987	Rats	Pseudoglandular / canalicular	Maternal s.c. (3 doses over 24hrs)	Dexamethasone 1mg/kg/day	Desmosine concentration ↓ postnatally but was not altered in fetal lungs
Noguchi *et al.* 1990	Rats	Saccular / alveolar	*in vitro* pulmonary fibroblast culture	Dexamethasone 10^{-11} to 10^{-5}M (40 hrs)	Age and dose dependant ↑ in tropoelastin mRNA and protein
Anceshi *et al.* 1992	Rabbits	Canalicular	Maternal i.m. 48hr before delivery	Betamethasone 0.2 mg/kg	Age dependant ↑ in desmosine concentration
Pierce et al. 1995	Rats	Pseudoglandular / canalicular	Maternal i.m. 3d before delivery or *in vitro* lung culture	Dexamethasone 1mg/kg or Cortisol (*in vitro*) 10^{-11} to 10^{-7}M	*in vivo* age dependant ↑ in tropoelastin mRNA and *in vitro* ↑ in tropoelastin mRNA
Willet *et al.* 1999	Sheep	Saccular / alveolar	Fetal i.m. 48hr before delivery	Betamethasone 0.5mg/kg	Parenchymal elastin density - ↓ at 121d GA, ↑slightly at 135d GA

Chapter 6

Effect of intra-uterine growth restriction (IUGR) on lung elastin synthesis and deposition

6.1 Introduction

Epidemiological evidence indicates that exposure to a sub-optimal intra-uterine environment resulting in low birth weight increases the risk of respiratory illness in affected infants (Minior & Divon, 1998; Tyson et al., 1995), children (Nikolajev et al., 1998; Rona et al., 1993) and adults (Barker et al., 1991; Stein et al., 1997). These studies suggest that lung development may be impaired by IUGR, a major cause of low birth weight, and that these impairments may persist into adulthood. In support of this, recent studies in the sheep have shown that IUGR induced by late gestational placental insufficiency results in alterations in lung function that persist until at least 8 weeks after birth (Joyce et al., 2001), and adverse changes in lung structure that persist until at least 2 years after birth (Maritz et al., 2001; Maritz et al., 2004). The underlying processes behind these changes in lung structure and function are unclear; however, changes such as a decrease in lung compliance and alveolar number are consistent with alterations in connective tissue elements such as elastin. Therefore the aim of this study was to evaluate the effects of IUGR induced by late gestational placental insufficiency on lung elastin synthesis and deposition in fetal and postnatal sheep.

Placental insufficiency results in reduced oxygen and nutrient delivery to the fetus and is a common cause of IUGR (Hoogland, 1982). Causes of placental insufficiency in human pregnancy include maternal tobacco smoking, pregnancy-induced hypertension and placental pathology (Kramer, 1998; Owens et al., 1995). Blood samples taken from

the umbilical cord in human cases of IUGR indicate that growth restriction is associated with fetal hypoxemia, hypoglycemia and elevated plasma cortisol concentrations (Nicolaides et al., 1989). All of these factors are known to affect elastin synthesis and alveolar development in the rat lung; hypoxia (Berk et al., 1999) and undernutrition (Kalenga & Henquin, 1987; Sahebjami & MacGee, 1985) have been shown to result in decreases in elastin mRNA levels and mature elastin accumulation respectively, while cortisol has been shown to increase elastin gene expression (Pierce et al., 1995).

Placental insufficiency in this study, and related studies (Joyce et al., 2001; Maritz et al., 2001; Maritz et al., 2004), was induced late in gestation by umbilico-placental embolisation (UPE). UPE reduces the exchange of oxygen and nutrients across the placenta and results in fetal hypoxemia, hypoglycemia and growth restriction (Cock et al., 2001; Cock & Harding, 1997; Joyce et al., 2001), and thus replicates metabolic changes that occur in human IUGR (Nicolaides et al., 1989). The UPE period coincided with the saccular and alveolar stages of lung development in the sheep, a time critical to elastin synthesis and deposition in the lung. Given that fetal hypoxemia and hypoglycemia are the dominant effects of UPE, and that alveolar number has been shown to decrease in this model, it was hypothesised that elastin synthesis and deposition would be decreased as a result of IUGR.

6.2 Materials and methods

6.2.1 Experimental preparation

Lung tissue was obtained from experiments performed previously on chronically catheterised fetal and postnatal sheep (Cock et al., 2001; Gagnon et al., 2002; Joyce et al., 2001; Louey et al., 2000). Aseptic surgery was performed on date-mated pregnant ewes at approximately 116 days of gestation (term ~147 days). Under general anesthesia (1.5% halothane in O_2 - N_2O, 50:50 v/v), saline-filled polyvinyl catheters were inserted into the fetal femoral artery and vein. The arterial catheter was inserted approximately 6-7cm such that its tip lay in the abdominal aorta below the renal arteries; this catheter was used for blood sampling and injection of microspheres for placental embolisation (Cock & Harding, 1997; Gagnon et al., 2002). The venous catheter was used for administration of drugs before and after birth.

Intrauterine growth restriction was induced by umbilico-placental embolisation (UPE) in four groups of animals:

Group 1) A *128d fetal* IUGR group (n=6) that underwent 5 days of UPE (~123 - 128 days GA) by injection of 15μm microspheres (diluted with sterile saline solution) in boluses of 1 to 2 million into the fetal abdominal aorta. Microsphere injections began 5 - 6 days postoperatively, and were made every 15 to 20 minutes over a 2 hour period until the fetal arterial oxygen content (CaO_2) was reduced to 50% of the pre-embolisation value. These experiments were conducted in the Department of Reproductive Medicine, University of California, San Diego, USA (Gagnon et al., 2002).

Group 2) A *140d fetal* IUGR group (n=6) that underwent 20 days of UPE (120-140 days GA) by injection of 40-70μm insoluble mucopolysaccharide microspheres (Sephadex G25, Pharmacia, Uppsala, Sweden, suspended at 1% w/v in heparinised saline with 0.02% Tween 80) into the fetal abdominal aorta. Daily injections of microspheres were made in boluses of 0.1 to 1 million to reduce the fetal arterial oxygen saturation (SaO_2) to 25-35% (i.e. a reduction of approximately 50%). These experiments were conducted by myself, S. Louey and M. L. Cock in the Department of Physiology, Monash University, Australia between 1997 and 1999 (Cock et al., 2001; Cock & Harding, 1997).

Group 3) An *8wk postnatal* IUGR group (n=6) that underwent 25.2 ± 1.0 days of UPE (120 days GA - birth; term ~147 days) as in Group 2. In this group, UPE was continued until birth and lambs were born spontaneously at term. Lambs were raised by their mothers, but if necessary, they were supplemented with formula milk suitable for lambs (Veanavite Pty Ltd, Australia). Preparation of animals in this group was conducted by myself and S. Louey in the Department of Physiology, Monash University, Australia between 1998 and 1999 (Joyce et al., 2001).

Group 4) A *2yr postnatal* IUGR group (n=6) that underwent 22.3 ± 0.21 days of UPE (120 days GA – birth) as in Group 3. Lambs in this group were raised by their mothers as in group 3 until 8 weeks of age, when they were weaned. At 12 weeks of age the lambs were sent out to pasture until the end of experiment at ~ 2 years of age. Preparation of animals in this group was conducted by M. L. Cock and S. Louey in the Department of Physiology, Monash University, Australia between 1998 and 1999.

Age-matched controls (n=6) received saline injections during the embolisation period.

Postmortem examinations and tissue collection were performed at 128 days GA (Group 1), 140 days GA (Group 2), 8 weeks postnatal age (Group 3) or ~2 years postnatal age (Group 4). In all groups, the lungs were removed from the fetus or lamb, and portions of the left lobes were removed and frozen in liquid nitrogen and stored at -70°C. Frozen lung tissue was used for extraction of total RNA for Northern blot analysis (section 2.2), and for the determination of elastin content (section 2.4). Lung tissue was micro-dissected prior to measurement of elastin content to remove larger airways and blood vessels so as to focus my findings on the alveolar region of the lung. The right lung was fixed via the trachea at 20 cmH_2O using 4% paraformaldehyde, sliced into 5mm sections and post-fixed in Zamboni's fixative overnight (Group 4), or 4% paraformaldehyde and 0.2% gluteraldehyde only (Groups 2 and 3). All fixed tissue samples were embedded in paraffin and sectioned at 5μm for histochemical analysis (section 2.5) to localise elastin deposition in lung tissue.

6.3 Results

6.3.1 Fetal oxygenation, glucose, and lung and body weights

During 5 days of UPE (~123 - 128 days GA, Group 1), fetal arterial oxygen content (CaO_2) was significantly lower in treated fetuses compared to controls. Mean CaO_2 values on day 5 were 3.4 ± 0.2 mmol/l in controls and 2.5 ± 0.3 mmol/l in UPE treated fetuses. Blood glucose concentrations were not different between the groups during 5 days of UPE (Gagnon et al., 2002).

During 20 days of UPE (120 – 140 days GA, Groups 2-4), fetal arterial oxygen saturation (SaO_2) and blood glucose concentration was significantly lower in treated fetuses compared to controls. Mean fetal SaO_2 was 59.6 ± 3.3% in control animals and 33.4 ± 1.0% in fetuses subjected to 20+ days of UPE. Mean blood glucose concentrations were 0.7 ± 0.05 mmol/L and 0.4 ± 0.03 mmol/L in control and treated fetuses respectively.

Both 5 (Group 1) and 20+ days of UPE (Groups 2-4) resulted in IUGR, with body weights of UPE treated fetuses, and lambs at birth significantly lower than in control

fetuses and lambs (Table 6.1). At 8 weeks postnatally (Group 3), the mean body weights of IUGR lambs remained lower than those of controls, but by 2.3 – 2.4 years of age (Group 4), body weights were not different between the groups (Table 6.1).

Following 5 days of UPE (Group 1), lung weights at 128 days GA tended to be lower in IUGR fetuses compared to controls (p=0.07), and lung weight adjusted for body weight was significantly lower in IUGR fetuses compared to controls. Following 20+ days of UPE, lung weights were not different at 140 days GA (Group 2), or 8 weeks after birth (Group 3) but were significantly higher in mature IUGR sheep compared to controls at 2 years of age (Group 4). Lung weight adjusted for body weight was not different at 140 days or 8 weeks following 20+ days of UPE, but was higher at 2 years after birth (Table 6.1).

Table 6.1 Body and lung weights

Mean body weight ± SEM (kg) at birth in postnatal animals and at postmortem (PM) in all animals and mean lung weight ± SEM (g) and lung weight adjusted for body weight (g/kg of body weight). Asterisk (*) indicates a difference between the groups ($p<0.05$) when analysed by the unpaired t-test.

		128d fetal	*140d fetal*	*8wk postnatal*	*2yr postnatal*
Birth weight (kg)	Control	N/A	N/A	4.3 ± 0.2	4.4 ± 0.3
	IUGR			2.7 ± 0.3*	2.5 ± 0.1*
Body weight at PM (kg)	Control	2.9 ± 0.2	4.6 ± 0.3	16.7 ± 1.0	58.3 ± 1.4
	IUGR	2.4 ± 0.1*	3.1 ± 0.3*	12.6 ± 1.1*	58.5 ± 1.5
Lung weight (g)	Control	109.3 ± 13.9	127.7 ± 6.2	224.2 ± 20.3	526.6 ± 26.2
	IUGR	82.1 ± 4.1	106.3 ± 14.4	177.8 ± 24.5	755.5 ± 59.4*
Lung / body weight (g/kg)	Control	2.77 ± 0.1	31.3 ± 1.6	13.3 ± 0.6	9.1 ± 0.4
	IUGR	2.35 ± 0.1*	34.0 ± 3.4	14.1 ± 1.1	12.9 ± 1.1*

6.3.2 Tropoelastin mRNA levels

At 128 days of gestation, following 5 days of UPE, pulmonary tropoelastin mRNA levels were not different between control and treated fetuses (*Control*: 30.0 ± 9.1 vs *128d IUGR*: 38.6 ± 7.1 arbitrary units; Figure 6.1). Following 20 or more days of UPE, tropoelastin mRNA levels were also not different between control and treated fetuses both near term at 140 days GA (*Control*: 7.1 ± 0.7 vs *140d IUGR*: 5.5 ± 0.8 arbitrary units; Figure 6.2) and at 8 weeks postnatal age (*Control*: 15.6 ± 1.4 vs *8wk IUGR*: 15.9

± 3.2 arbitrary units; Figure 6.3). Expressed as a percentage of control values, tropoelastin mRNA levels were 128.6 ± 23.7%, 76.8 ± 10.6% and 102.1 ± 20.2% in *128d IUGR*, *140d IUGR* and *8wk IUGR* animals respectively. At 2 years postnatal age, following ~25 days of UPE, tropoelastin mRNA levels were not measurable in control or IUGR animals. Figure 3.1 (Chapter 3) shows the negligible tropoelastin mRNA levels in control sheep lungs at 2 years.

6.3.3 Elastin deposition

Elastin concentration and content

The concentration (mg/g lung tissue) of elastin in lung parenchymal tissue was not significantly different between control and IUGR fetuses and lambs at 128 days GA (*Control*: 2.3 ± 0.1 vs *128d IUGR*: 2.2 ± 0.1 mg/g lung), 140 days GA (*Control*: 2.3 ± 0.2 vs *140d IUGR*: 2.0 ± 0.1 mg/g lung), 8 weeks postnatal age (*Control*: 6.5 ± 0.2 vs *8wk IUGR*: 6.1 ± 0.4 mg/g lung) and 2 years postnatal age (*Control*: 5.6 ± 0.2 vs *2yr IUGR*: 5.2 ± 0.4 mg/g lung). Expressed as a percentage of control values, the elastin concentration in lung parenchymal tissue was 96.6 ± 3.0%, 87.5 ± 5.6%, 94.0 ± 16.2% and 92.2 ± 6.9% in *128d IUGR*, *140d IUGR*, *8wk IUGR* and *2yr IUGR* animals respectively (Figure 6.4).

The elastin content of the lungs tended to be lower in *128d IUGR* fetuses (*Control*: 248.4 ± 38.5 vs *128d IUGR*: 181.2 ± 11.9 mg; p=0.1) and *8wk IUGR* lambs (*Control*: 1460.1 ± 149.0 vs *8wk IUGR*: 1073.2 ± 135.1 mg; p=0.09) and was significantly lower in *140d IUGR* fetuses (*Control*: 292.8 ± 26.5 vs *140d IUGR*: 207.1 ± 25.3 mg; p<0.05) compared to age-matched control lungs (Figure 6.4). In *2yr IUGR* sheep, the elastin content of the lungs tended to be higher than in age-matched control lungs (*Control*: 2957.3 ± 170.5 vs *2yr IUGR*: 3877.8 ± 388.5 mg; p=0.06)(Figure 6.4). Expressed as a percentage of control values, the elastin content of the lungs was 73.0 ± 4.8%, 70.7 ± 8.7%, 73.5 ± 9.3% and 131.1 ± 13.1% in *128d IUGR*, *140d IUGR*, *8wk IUGR* and *2yr IUGR* animals respectively (Figure 6.4).

Table 6.2 Elastin concentration and content

Mean elastin concentration ± SEM (mg/g lung tissue) and elastin content ± SEM (mg), measured in *128d fetal*, *140d fetal*, *8wk postnatal* and *2yr postnatal* control and IUGR sheep. Asterisk (*) indicates a difference between the groups ($p<0.05$) when analysed by the unpaired *t*-test.

		128d fetal	*140d fetal*	*8wk postnatal*	*2yr postnatal*
elastin concentration (mg/g lung)	Control	2.3 ± 0.1	2.3 ± 0.2	6.5 ± 0.2	5.6 ± 0.2
	IUGR	2.2 ± 0.1	2.0 ± 0.1	6.1 ± 0.4	5.2 ± 0.4
% of control values	Control	100 ± 3.7	100 ± 7.4	100 ± 3.7	100 ± 4.1
	IUGR	96.6 ± 3.0	87.5 ± 5.6	94.0 ± 16.2	92.2 ± 6.9
elastin content (mg)	Control	248.4 ± 38.5	292.8 ± 26.5	1460.1 ± 149.0	2957.3 ± 170.5
	IUGR	181.2 ± 11.9	207.1 ± 25.3*	1073.2 ± 135.1	3877.8 ± 388.5
% of control values	Control	100 ± 15.5	100 ± 9.0	100 ± 10.2	100 ± 5.8
	IUGR	73.0 ± 4.8	70.7 ± 8.7	73.5 ± 9.3	131.1 ± 13.1

Elastin staining

In IUGR fetuses and lambs, lung tissue stained for elastin showed no apparent differences in elastin deposition in blood vessels and airways (not shown) or alveolar compartments (Figure 6.5) compared to age-matched control fetuses. Elastin appeared to be deposited at the tips of secondary septal crests and in the walls of alveoli, in a similar manner in both control and IUGR fetuses, lambs and 2 year old sheep.

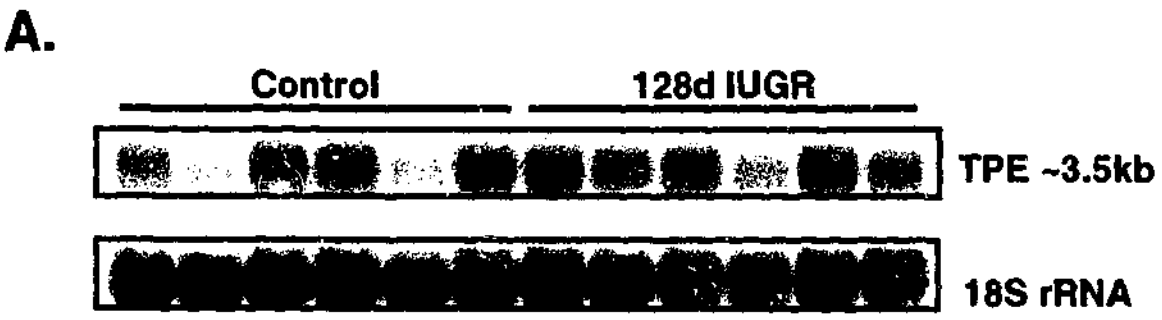


Figure 6.1 Tropoelastin (TPE) mRNA levels at 128 days GA following 5 days of UPE

A: Northern blot analysis of total RNA extracted from lung tissue collected from control fetuses and IUGR fetuses at 128 days GA. *B*: Mean tropoelastin mRNA levels in control fetuses and *128d* IUGR fetuses, where the density of each tropoelastin mRNA band was expressed as a ratio of the density of the corresponding 18S rRNA band and then expressed as a percentage of mean control values.

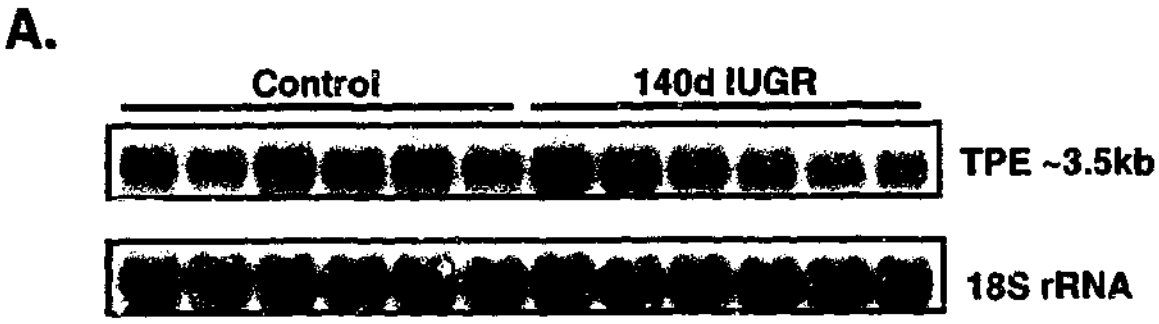


Figure 6.2 Tropoelastin (TPE) mRNA levels at 140 days GA following 20 days of UPE

A: Northern blot analysis of total RNA extracted from lung tissue collected from control fetuses and IUGR fetuses at 140 days GA. *B*: Mean tropoelastin mRNA levels in control fetuses and *140d* IUGR fetuses, where the density of each tropoelastin mRNA band was expressed as a ratio of the density of the corresponding 18S rRNA band and then expressed as a percentage of mean control values.

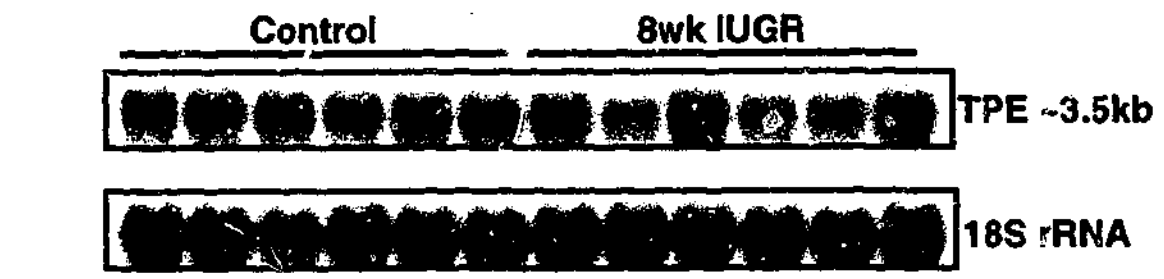


Figure 6.3 Tropoelastin (TPE) mRNA levels at 8 weeks postnatal age following ~ 25 days of UPE

A: Northern blot analysis of total RNA extracted from lung tissue collected from control lambs and IUGR lambs at 8 weeks postnatal age. *B*: Mean tropoelastin mRNA levels in control fetuses and *8wk* IUGR lambs, where the density of each tropoelastin mRNA band was expressed as a ratio of the density of the corresponding 18S rRNA band and then expressed as a percentage of mean control values.

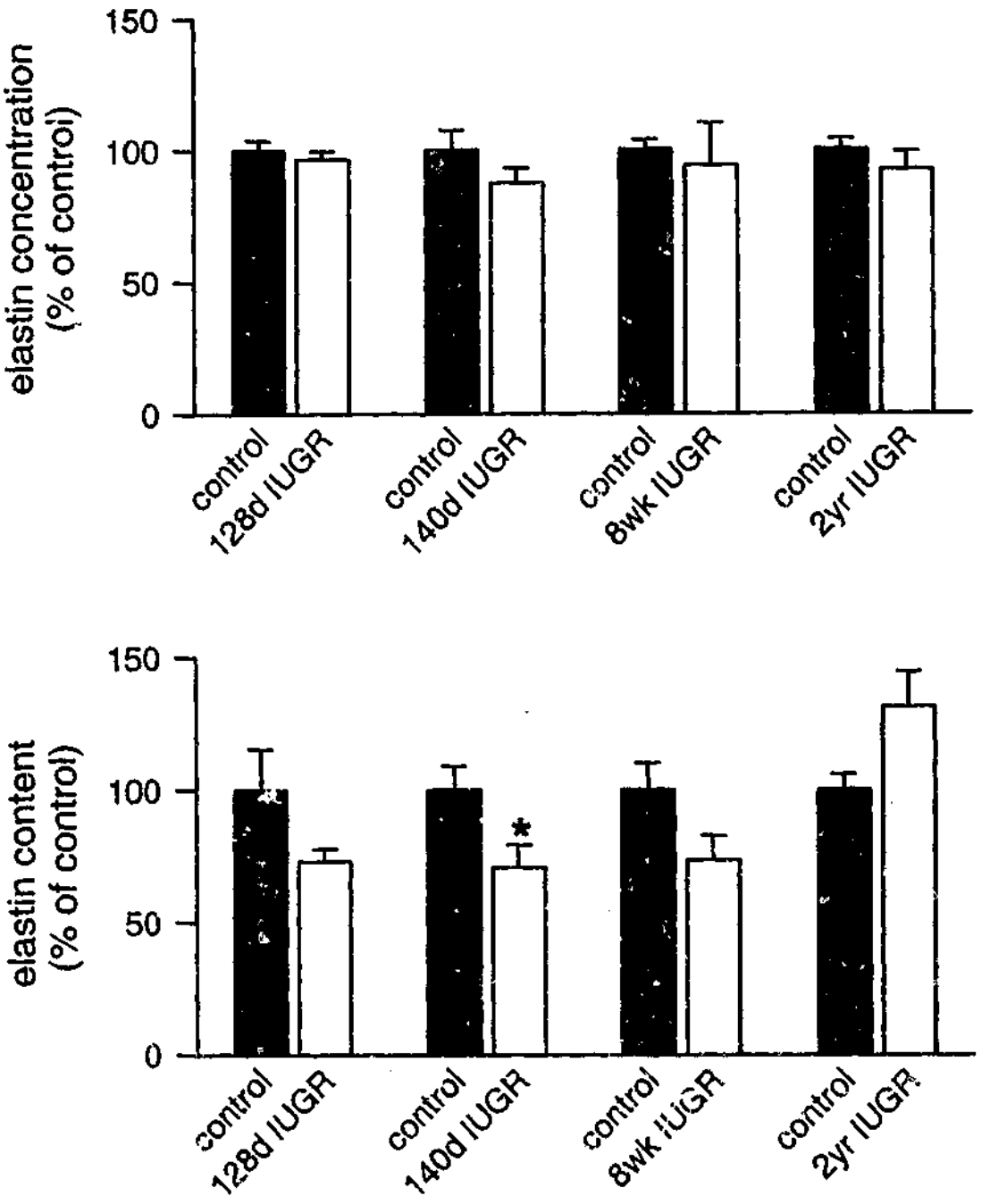


Figure 6.4 Elastin concentration and elastin content of the lungs following 5 days or 20+ days of UPE

Elastin concentration (mg/g lung) and elastin content (mg) of the lungs following 5 days of UPE at 128 days of GA, and 20+ days of UPE at 140 days GA, 8 weeks postnatal age, and 2 years postnatal age. Values are expressed as a percentage of mean control values. The asterisk (*) indicates a difference between the groups ($p<0.05$) when analysed by the unpaired t-test.

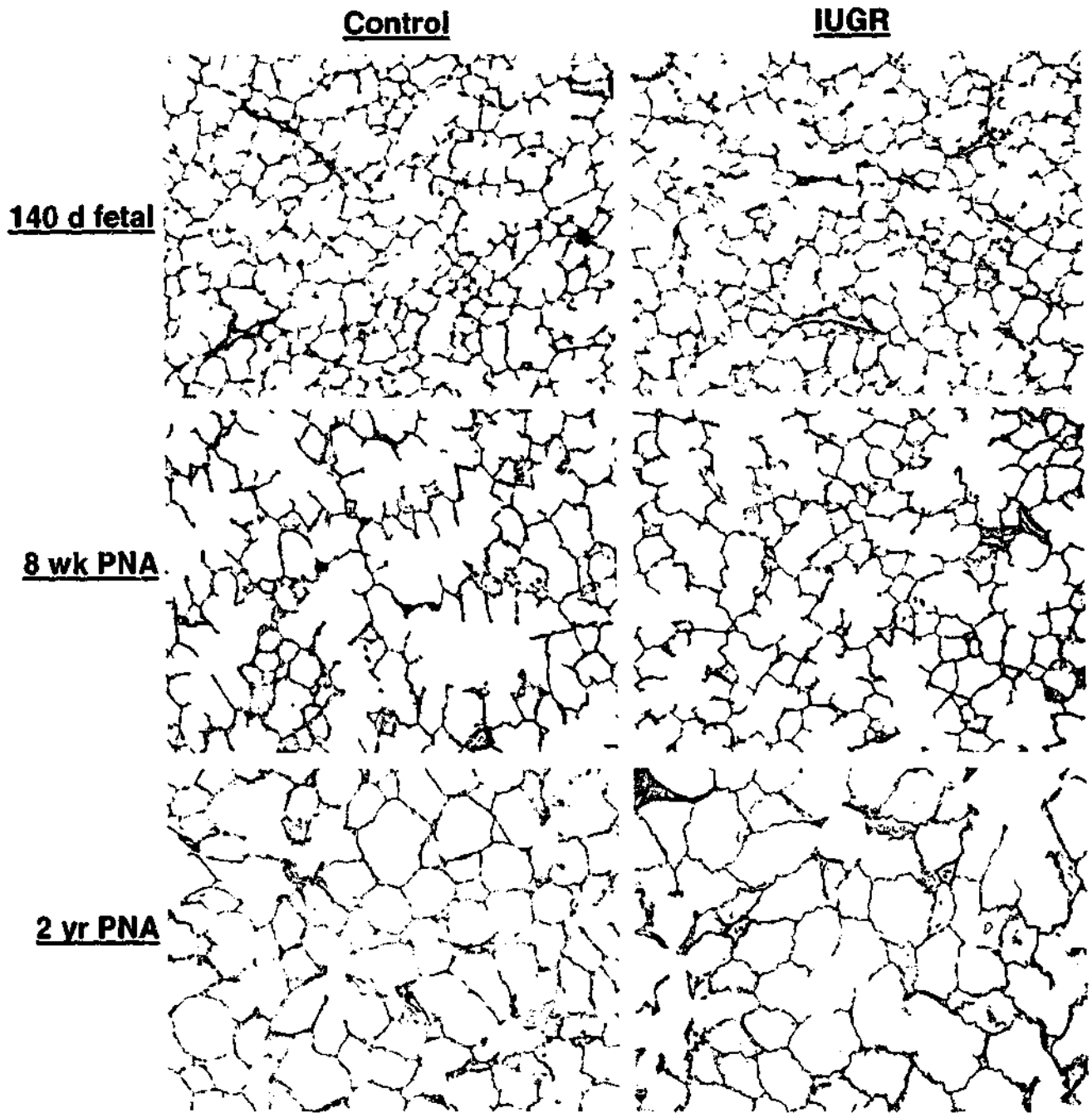


Figure 6.5 Elastin staining in the alveolar region of the lungs following 20+ days of UPE

Hart's resorcin-fuchsin stain for elastin in lung tissue sections taken from control lung and lung from fetal and postnatal (PNA) sheep exposed to 20+ days of UPE *in utero*. Presented are photographs taken at X20 magnification of the alveolar region of the lung at 140 days GA, 8 weeks, and 2 years after birth. Elastin is stained dark purple-black and is focused at the tips of secondary septal crests in control lungs and IUGR lungs. No apparent differences were observed in the deposition or distribution of elastic fibres between control lungs and lungs from IUGR sheep.

6.4 *Discussion*

Intra-uterine growth restriction in sheep, induced by placental insufficiency late in gestation (a critical period for elastin synthesis), results in impairments in lung structure (Maritz et al., 2001· Maritz et al., 2004) and function (Joyce et al., 2001) consistent with alterations in elastin synthesis. In addition, factors associated with IUGR such as hypoxia and undernutrition individually have been shown to affect elastin synthesis. Therefore, it was hypothesised that elastin synthesis would be altered as a result of IUGR. In this study however, factors associated with IUGR *in vivo* did not affect elastin synthesis or deposition in the fetal, early postnatal or adult sheep lung and therefore could not explain the changes in lung structure and function observed in related studies.

6.4.1 Effect of IUGR on lung tropoelastin expression

Despite the severe, chronic hypoxemia experienced by UPE treated fetuses *in utero* (approximately 50% reduction in CaO_2 (5 days of UPE) or SaO_2 (20+ days of UPE)) tropoelastin mRNA levels were not significantly different between control and IUGR fetuses at any age. This was an unexpected result since hypoxia in neonatal rat lung fibroblasts *in vitro* has been shown to decrease tropoelastin mRNA levels (Berk et al., 1999). One explanation for this is that, *in vivo*, the level of hypoxia experienced by elastin producing cells may not have been sufficient to alter tropoelastin mRNA levels. Consistent with this finding is that Berk *et al.* (1999) observed a dose and time dependant effect of decreased O_2 on cultured rat lung fibroblasts. In that study, it was found that incubation of cells in 10% O_2 did not significantly reduce tropoelastin mRNA levels; O_2 levels as low as 3% were required before a reduction in tropoelastin mRNA was observed (Berk et al., 1999).

Another potential explanation for my negative findings is that at 140 days GA, following 20 days of UPE, increased fetal plasma cortisol concentrations in IUGR fetuses used in this study (Cock et al., 2001), may have counteracted the inhibitory effects of hypoxia on tropoelastin mRNA levels, as cortisol increases tropoelastin expression (as discussed in Chapter 5). A similar level of hypoxia induced between 123 and 128 days of gestation (before the endogenous rise in fetal plasma cortisol levels (Magyar et al., 1980)) also failed to significantly affect tropoelastin mRNA levels; in fact tropoelastin mRNA levels tended to be higher in IUGR fetuses at this age.

Although hypoxia was induced before the endogenous rise in fetal plasma cortisol, experiments reported in Chapter 5 indicate that tropoelastin expression is inducible by physiological levels of fetal plasma cortisol at a similar age (131 days GA) and therefore, elevated fetal cortisol levels may also counteract any negative effect of hypoxia on tropoelastin mRNA levels in 128 day fetuses. Unfortunately, fetal plasma cortisol concentrations in the 128 day old fetuses were not measured. The duration of the hypoxia induced at this time was also much less (i.e. 5 days vs 20 or more days), however, Berk *et al.* (1999) showed that tropoelastin mRNA levels in rat lung fibroblasts *in vitro* were decreased substantially by 36 hours in response to 0% O_2, and thus, 5 days of hypoxemia should have been sufficient time to reduce tropoelastin mRNA levels. Finally, the Northern data from both control and IUGR animals at 128 days of gestation was quite variable; possible explanations include a large amount of variation in the density of blood vessels in tissue pieces taken from different animals for RNA extraction, or a large variation in fetal plasma cortisol levels. Neither of these is likely to lead to a false negative result but care should be taken when interpreting these data.

The results of this study suggest that the decrease in tropoelastin mRNA levels in neonatal rat lung fibroblasts *in vitro*, in response to extremely low O_2 levels (0-3%), may not be physiologically significant, since the severe level of hypoxemia experienced by our sheep fetuses *in vivo* did not result in any change in pulmonary tropoelastin mRNA levels. Furthermore, IUGR can be associated with both hypoxia and increased fetal plasma cortisol concentrations which have opposing effects on tropoelastin expression *in vitro* and therefore may counteract each other resulting in no net change in steady state mRNA levels in the lungs of IUGR fetuses.

6.4.2 Effect of IUGR on elastin concentration, elastin content and elastin deposition in lung parenchymal tissue

IUGR induced by 5 or 20+ days of UPE did not alter the concentration of elastin in lung parenchymal tissue at any age, which is consistent with the lack of change in tropoelastin mRNA levels. However, the elastin content in lung parenchymal tissue tended to be lower in *128d IUGR* and *8wk IUGR* animals, and was significantly lower in *140d IUGR* animals compared to age-matched controls. At 2 years after birth, elastin content tended to be higher in IUGR animals compared to controls. These differences

in lung elastin content appeared to reflect the trends in lung weight rather than a change in elastin synthesis. However, while there was a significant difference in elastin content in *140d IUGR* fetuses compared to controls, there was no significant difference in lung weight in these animals, but there was a tendency for lung weights to be lower in *IUGR* fetuses compared to controls. The lack of difference in parenchymal elastin concentration suggests that the significant difference in elastin content at 140 days GA is the result of the IUGR fetuses having smaller lungs. In addition, the mean percentage volume density of elastin, assessed by morphometric techniques, in lung parenchyma in IUGR fetuses at 140 days GA was also not different from that of age-matched controls (G.S. Maritz unpublished observations), and there was no difference in alveolar number at this age (Maritz et al., 2001).

Elastin staining in the lung parenchyma of IUGR and control animals also appeared to be similar both before birth (140 days GA) and postnatally at 8 weeks and ~2 years. There was no evidence of any impairment in elastin deposition in relation to alveolar development that could explain the decrease in alveolar number previously observed in 8 week (Maritz et al., 2001) and 2 year old IUGR sheep (Maritz et al., 2004).

These results indicate that the impairments observed in lung structure and function such as increased septal wall thickness, decreased alveolar number at 8 weeks and 2 years of age (Maritz et al., 2001; Maritz et al., 2004), and decreased lung compliance (Joyce et al., 2001), were not a result of a change in elastin synthesis or deposition. This finding is consistent with a study that reported that elastin accumulation in developing human lungs was not affected by fetal growth restriction (Desai et al., 1988).

6.4.3 Conclusions

This study shows that while factors such as hypoxia, undernutrition, and corticosteroids have been shown to individually affect elastin synthesis, when associated with IUGR *in vivo*, elastin synthesis and deposition are unaffected, and therefore cannot explain impairments in lung structure and function caused by IUGR.

Chapter 7

General Discussion

Lung growth and maturation in the later stages of fetal development are critical for postnatal survival and future lung health. Adverse prenatal conditions resulting in altered lung growth, maturation and structure, or a shortened period of *in utero* development such as in pre-term birth, are major causes of neonatal morbidity and mortality, and can potentially affect lung health into adulthood. One of the main features of the lung maturational process in precocial species such as humans is the initiation of alveolar development and the structural maturation of the parenchymal region of the lung before birth. Factors such as lung tissue stretch (mediated by the degree of fetal lung expansion), corticosteroids and adverse prenatal conditions resulting in low birth weight have all been shown to influence lung structure and alveolar number. Because elastin synthesis is required for alveolar formation, the broad aim of experiments reported in this thesis was to determine the influence of the above factors on lung elastin synthesis and deposition *in vivo*. The following is a discussion of the significance of major findings arising from this thesis in relation to current knowledge and human health issues, and possible directions for future research will also be considered.

7.1 Mechanical forces and elastin deposition

The degree to which the fetal lung is expanded *in utero* is a major determinant of lung growth including alveolar development. There are a number of clinical conditions that influence the level of fetal lung expansion during development hence resulting in altered fetal lung growth and development. Conditions such as laryngeal atresia result in

increased fetal lung expansion, whereas congenital diaphragmatic hernia, oligohydramnios and impaired or absent fetal breathing movements result in a decrease in the level of fetal lung expansion (Harding & Hooper, 1996). Experimental studies in sheep have shown a clear relationship between fetal lung expansion and lung growth and development including alveolar development (Boland et al., 2004; Nardo et al., 1998; Nardo et al., 2000). Given the link between the degree of fetal lung expansion and alveolar development and between elastin synthesis and alveolar development, as well as the increase in elastin synthesis observed in cultured fetal rat lung cells exposed to mechanical strain (Nakamura et al., 2000), it was hypothesised that alterations in fetal lung expansion would influence elastin synthesis *in vivo*. In agreement with this hypothesis, experiments reported in this thesis showed that sustained increases in fetal lung expansion stimulated elastin synthesis whereas a sustained reduction in fetal lung expansion reduced elastin synthesis.

Probably the most significant finding, however, was the effect of a prolonged very low degree of fetal lung expansion and hence decreased lung tissue stretch, on alveolar elastin deposition. These studies indicate that while elastin synthesis is required for alveolar formation, appropriate mechanical forces also appear to be imperative for the correct laying down of functional elastic fibres and the formation of alveolar septal walls. This conclusion also appears to be true postnatally in ventilated preterm lambs (Pierce et al., 1997) and following postpneumonectomy lung growth in the adult rat lung (Hsia et al., 2001). In a study by Pierce *et al.* mechanical ventilation in preterm lambs resulted in regional increases in elastin accumulation, with excessive production of elastic fibres of abnormal morphology and impaired development of secondary septal crests (Pierce et al., 1997). It is interesting to note that compensatory lung growth following partial pneumonectomy in adult rats results in an increase in lung growth with apparently normal elastin deposition and alveolar morphology (Koh et al., 1996), whereas prevention of expansion of the remaining lung by implantation of a silicone prosthesis following pneumonectomy in dogs, results in areas of septal crowding and impaired septal wall formation (Hsia et al., 2001). Furthermore, elastin accumulation during normal lung development appears to occur at points of maximal stress in alveolar walls (Mariani et al., 1997). These studies raise the question of the importance of not only the presence of mechanical expanding forces but also the nature of the mechanical forces on normal elastic fibre deposition. Further they also raise the possibility that

inappropriate mechanical forces, potentially due to uneven ventilation in preterm lungs, may contribute to preterm lung pathophysiology and bronchopulmonary dysplasia (BPD). The regional alterations in elastin deposition in ventilated preterm lambs also indicate that it is the local tissue forces that are likely to be important for correct elastic fibre deposition. Inappropriate or lack of mechanical expanding stimuli could also play a role in the inability of the lung to regenerate normal elastic fibres and lung structure in the adult lung following lung injury, such as in emphysema. These findings also have implications in the case of fetal lung hypoplasia resulting from prenatal conditions such as oligohydramnios, in which alveolar development may be permanently altered as a result of a prolonged decrease in fetal lung expansion. A better understanding of the role and mechanisms by which local mechanical forces regulate elastic fibre synthesis and deposition is required to address these possibilities and may be beneficial in the consideration of future therapies for lung hypoplasia, prematurity and emphysema.

The mechanisms by which mechanical forces alter elastin expression in the alveolar region of the lung appears to involve both an alteration in the amount of tropoelastin mRNA at each site of expression (e.g. the tips of developing secondary septa) and also the number of sites at which tropoelastin is being expressed. This raises the possibility that mechanical forces may have the ability to directly influence transcription of the tropoelastin gene as well as migration and/or differentiation of alveolar myofibroblasts that produce elastin. In the lungs of fetal sheep that had experienced prolonged periods of reduced fetal lung expansion, the distribution of elastin deposition was altered in that focal aggregates of elastin occurred at closer intervals within the saccular walls and elastic fibres were also located along saccule walls rather than being focused primarily at the tips of developing secondary septa. Thus, it is possible that the migration of elastin producing cells to appropriate locations could be altered by local mechanical forces. Studies which assess the effect of mechanical forces on alveolar myofibroblasts, or factors such as PDGF-A that influence these cells (Lindahl et al., 1997) would be useful to further clarify the mechanisms by which mechanical forces influence elastin deposition. The effect of mechanical forces on other proteins involved in elastic fibre assembly such as Fibulin-5, which has been shown to influence the organisation of elastic fibres (Nakamura et al., 2002; Yanagisawa et al., 2002), may also be of interest since elastic fibre organisation appears to be altered by a sustained decrease in fetal lung expansion.

7.2 *Corticosteroids and lung development*

Corticosteroids are commonly used to stimulate the maturation of the fetal lungs before premature birth; however, a number of animal studies suggest detrimental effects of antenatal corticosteroid treatment on lung growth and alveolar development. Furthermore, a new hypothesis regarding the pathogenesis of BPD involves an arrest of alveolar development induced by postnatal ventilation (Jobe, 1999). Therefore, any detrimental effects of corticosteroid treatment on alveolar development before birth may preclude or further exacerbate this problem. Experiments described in this thesis and in a related study (Boland et al., 2004) indicate that physiological levels of cortisol before birth in sheep do not inhibit lung elastin synthesis or alveolar development and that cortisol is likely to have a positive influence on elastin synthesis and alveolar development during normal fetal lung development. Recent studies in sheep indicate that detrimental effects of corticosteroids on fetal body and lung growth may be specific for the type and route of delivery of corticosteroid administered (Jobe et al., 2003; Newnham et al., 1999). That is, detrimental effects of antenatal corticosteroids were specific to maternal administration of relatively large doses of the synthetic corticosteroid betamethasone rather than cortisol. On the other hand, maternal cortisol administration did not induce fetal lung maturation perhaps because fetal plasma cortisol concentrations achieved were too low (Jobe et al., 2003). Unfortunately, alveolar development was not assessed in these studies. Some studies also indicate that the timing and dose of corticosteroid administration may result in differential effects on fetal lung development (Vyas & Kotecha, 1997). Therefore, while results from my study indicate that endogenous cortisol at physiological levels is likely to have a stimulatory rather than inhibitory influence on elastin synthesis and alveolar development, more studies are required to further dissect the differences between endogenous cortisol and synthetic corticosteroids used to accelerate lung development clinically. Further studies are also needed to determine whether these observations can be applied to improve antenatal corticosteroid treatment in relation to prematurity, lung maturation and alveolar development. A greater emphasis on the effect of dose, agent used and route of administration on alveolar development, in particular, would be beneficial in regard to the hypothesis of alveolar arrest in the pathogenesis of BPD.

One unique feature of the experiments reported in Chapter 5 of this thesis, and in the related study by Boland *et al.* (2004), was the emphasis on the effects of physiological

levels of cortisol on elastin and alveolar development during normal lung development which had not been addressed previously. However a limitation of this study is that, although the results indicate that it is likely that endogenous fetal cortisol has a stimulatory effect on elastin synthesis and alveolar development, the extent to which endogenous fetal plasma cortisol actually influences elastin synthesis and alveolar formation during normal lung development remains unknown. This is due to the necessary offset in gestational age of the induced fetal plasma cortisol surge compared to that which occurs during normal development. Further experiments which assess elastin synthesis and alveolar development following fetal adrenalectomy are required to fully answer this question.

7.3 IUGR and lung development

Intra-uterine growth restriction is associated with impaired lung function and respiratory illness in humans (Barker et al., 1991; Minior & Divon, 1998; Rona et al., 1993; Stein et al., 1997; Tyson et al., 1995) and persistent impairments in lung structure and function in sheep (Joyce et al., 2001; Maritz et al., 2001; Maritz et al., 2004) consistent with an alteration in pulmonary elastin. This combined with the negative effects of factors such as hypoxia and undernutrition that are associated with IUGR on pulmonary elastin expression and accumulation led to the hypothesis that lung elastin synthesis and deposition would be decreased by IUGR. However, IUGR had no apparent effect on lung elastin synthesis or deposition in fetal or postnatal sheep. This most likely relates to the differences in the level of hypoxia that elastin producing cells are exposed to *in vivo* as a result of IUGR compared to those which inhibit elastin expression in cell culture. Also, IUGR involves a number of metabolic and endocrine changes which can have differential effects elastin synthesis. The study highlights the importance of whole animal experiments in addition to *in vitro* cell culture experiments.

While it seems apparent that changes in elastin synthesis and deposition cannot explain impairments observed in lung structure as a result of IUGR, the mechanisms underlying these lung structural changes remain unknown. Of particular interest is the persistent reduction in alveolar number despite no apparent change in pulmonary elastin. It is possible that elastin deposition may be altered resulting in altered alveolar formation despite no change in elastin synthesis. However, elastin staining at the light microscope level did not suggest any alteration in elastin deposition. It is interesting to note that

alterations in lung structure appear to worsen with age and therefore postnatal rather than prenatal factors are likely to be more important. To elucidate the mechanisms behind altered lung structural development following IUGR, further studies are required to elucidate the effect of IUGR on other extracellular matrix proteins in the lungs such as the collagens and their influence on alveolar septal structure. Furthermore, also of interest are postnatal factors, such as undernutrition or hypoxia that could result in the worsening of lung structural changes present in the near term IUGR fetus over time. This point is of particular clinical relevance as it has important implications for pulmonary health in growth restricted individuals if this trend continues into adulthood.

7.4 Elastin synthesis and pulmonary vascular and airway development

Experiments described in this thesis were designed to evaluate the regulation of elastin synthesis and deposition in the alveolar region of the lung by factors that have previously been shown to alter alveolar development. Tropoelastin expression and elastin deposition in pulmonary vessels and airways were used as a means of evaluating the relative changes in tropoelastin expression and deposition that occur in alveoli. However, experiments reported in chapter 4 of this thesis indicate that increases in fetal lung expansion result in an increase in tropoelastin expression in the vasculature and airways as well as in alveoli. These data are difficult to interpret because a limitation of these studies is that tissue samples used did not include a large enough number of vessels and airways of comparable sizes and location to rule out an influence of any difference in vessel and airway size and location on tropoelastin expression pattern or deposition. Furthermore, studies in developing rat lung indicate that there is differential expression of tropoelastin, as evaluated by *in situ* hybridisation, in blood vessels and airways of varying sizes (Crouch et al., 1997). It has also been shown that the expression pattern of tropoelastin in the vasculature changes as development progresses (Crouch et al., 1997). While no obvious changes were detected in the vasculature or airways in response to cortisol treatment in studies reported in this thesis, it is still possible that endogenous corticosteroids do have an influence on the maturation of elastin synthesis in the developing vasculature. Therefore, it would be interesting to conduct further studies to evaluate the effects of stretch and cortisol on elastin

development in the vasculature and airways and any resulting influences on lung vasculature and airway development and function.

7.5 Summary

In summary, I have shown that prenatal factors such as the degree to which the fetal lung is expanded and fetal plasma cortisol levels can influence elastin expression and synthesis and these changes are likely to be related to previously observed alterations in alveolar development. In addition to the changes observed in elastin synthesis, however, it was found that expanding forces not only influence expression and synthesis of elastin but also have a profound influence on the manner in which elastin is deposited in the lung which in turn has a major influence on alveolar development and architecture. While physiological levels of fetal plasma cortisol were found to increase elastin synthesis in the presence of a normal level of fetal lung expansion, they could not reverse the detrimental effects of an inadequate degree of fetal lung expansion on elastin synthesis, deposition and alveolar formation. Elastin synthesis and deposition did not appear to be altered by IUGR and therefore can not explain the alterations in the structure of the lung following IUGR. My studies have provided basic information on the regulation of elastin synthesis and deposition in the fetal lung, and form a basis for future studies regarding alveolar development in normal and pathological conditions.

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