INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
NOTE TO USERS

This reproduction is the best copy available.

UMI
EPITHELIAL Na$^+$ CHANNEL EXPRESSION IN THE NORMAL AND ABNORMAL HUMAN FETAL LUNG

by

David Edward Smith, M.D.

A thesis submitted in conformity with the requirements for the Degree of Master in Science in the University of Toronto

Respiratory Research Division of the Hospital for Sick Children
and
The Institute of Medical Science, School of Graduate Studies,
University of Toronto, Toronto, Canada

© Copyright by David Edward Smith, 1998
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

0-612-40815-9
ABSTRACT

Epithelial Na⁺ Channel Expression in the Normal and Abnormal Human Fetal Lung

David E. Smith, M.D., M.Sc., 1998

Institute of Medical Science, School of Graduate Studies,
University of Toronto, Ontario, Canada

Active liquid secretion and absorption is critical to normal lung development and successful respiration at birth. The Epithelial Na⁺ Channel (ENaC) is the rate limiting step in Na⁺ absorption. Impaired ENaC activity in the perinatal period results in respiratory distress characterized by wet lung. We hypothesize that perinatal conditions associated with respiratory distress have deficient expression of ENaC. I have examined the normal ontogeny and cellular distribution of the αhENaC subunit mRNA in normal archival human fetal lung using a non-isotopic in situ hybridization technique. Archival cases of pulmonary immaturity were then compared to the normal lung data. My results are surprising. In normal lung, αhENaC expression is detected at the embryonic stage of lung development localized to the fetal lung bud epithelium. By late gestation, expression is found in the epithelium of all airways, serous cells of submucosal glands, and in the distal lung unit in an alveolar type II pattern. Expression of αhENaC mRNA is preserved in newborn lung diseases associated with respiratory distress. There are several possible explanations for these findings. The early expression of ENaC suggests that αhENaC mRNA may be constitutively
expressed. ENaC mRNA may be accumulated throughout gestation in preparation for when rapid translation and channel activation is required. The localization of ENaC mRNA is consistent with an important role for ENaC in the epithelium of all regions of the lung. The presence of ENaC mRNA in newborn lung diseases associated with deficient fluid absorption suggests ENaC function may be regulated by post transcriptional mechanisms. Alternatively, the developmental expression of one of the other two ENaC subunits may be impaired in certain newborn lung diseases. Finally, alternate Na⁺ Channels may be important to the absorption of fluid in humans during late gestation.
ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Hugh O’Brodovich, and the members of my supervisory committee: Drs Gail Otulakowski, Herman Yeger, Martin Post and Jim Hu who have skillfully guided me through this degree. I would like to acknowledge the members of the Thoracic Surgery department who provided me with samples and the Pathology departments, especially Dr Earnst Cutz, Lilly Marunaka and Wilson Chan who provided invaluable histology advice. I have been very fortunate to work with very generous colleagues whose advice and encouragement were much appreciated. In particular, I would like to express my gratitude to Brent Steer, Bijan Rafi, Harry Robert Bremner, and Jenny Kwong. A very special thanks to Marjorie Samuel, Julie Deimling, Peter Bray, and Nicholas Julian Cartel who went out of their way to help me at especially needy times of my project. I would be remiss not to mention the love and continuous support I have received from my wife Tanya. All of these people have taught me over the last two years what it takes to be an excellent researcher, professional and friend.

I am also grateful to Cystic Fibrosis Foundation, Glaxo, and the Medical Research Council of Canada for financial support.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................ II

ACKNOWLEDGEMENTS ................................................................................ IV

TABLE OF CONTENTS .................................................................................. V

LIST OF TABLES ............................................................................................. VII

LIST OF FIGURES ......................................................................................... VIII

LIST OF APPENDICES ................................................................................... VIII

LIST OF ABBREVIATIONS .............................................................................. IX

1 INTRODUCTION AND LITERATURE REVIEW ........................................ 1

1.1 LUNG DEVELOPMENT ............................................................................. 1
1.1.1 Stages Of Lung Development ............................................................... 1
1.1.2 Lung Epithelial Cell Differentiation ....................................................... 3
1.1.3 Lung Branching ................................................................................. 7
1.1.4 Influence Of Fetal Lung Fluid On Lung Growth ................................. 9
1.1.5 Perturbations Of Lung Growth In The Human .................................... 10

1.2 EPITHELIAL ION TRANSPORT AND FLUID MOVEMENT IN THE FETAL AND PERINATAL LUNG ................................................................. 14
1.2.1 Lung Liquid Secretion ...................................................................... 14
1.2.2 Lung Liquid Absorption .................................................................. 16
1.2.3 Site Of Fluid Absorption In The Lung ............................................... 18
1.2.4 Switch Of The Lung Epithelium From Predominantly Fluid Secreting To Predominantly Fluid Absorbing ...................................................... 19

1.3 AMILORIDE-SENSITIVE EPITHELIAL Na⁺ CHANNEL (ENaC) .................. 21
1.3.1 Cloning of ENaC and its Subunits ...................................................... 21
1.3.2 Structure Function Analysis Of ENaC ............................................... 23
1.3.3 ENaC in Lung Epithelia ..................................................................... 28
1.3.4 Ontogeny Of Lung ENaC Expression ................................................ 28
1.3.5 Relevance Of Epithelial Na⁺ Transport To Human Lung Diseases ...... 30

1.4 ASSESSMENTS OF mRNA LEVELS ......................................................... 33
1.4.1 Northern Blot Analysis ...................................................................... 33
1.4.2 RNase Protection Assay (RPA) ............................................................ 34
1.4.3 RT-PCR ............................................................................................ 34
1.4.4 In Situ Hybridization (ISH) ................................................................. 35

1.5 IN SITU HYBRIDIZATION ANALYSES OF LUNG ENaC EXPRESSION .... 37

1.6 EXPERIMENTAL HYPOTHESIS AND AIM ........................................ 40

2 MATERIALS AND METHODS ................................................................. 41

2.1 TISSUE SAMPLING ............................................................................... 41
2.1.1 Sample Collection for In Situ Hybridization ...................................... 41
2.1.2 Sample Collection for Northern Blot Analysis ................................... 43
2.2 **NORTHERN BLOT HYBRIDIZATION** ........................................... 44
2.3 **NON-ISOTOPIC IN SITU HYBRIDIZATION** ........................................... 46
  2.3.1 αhENaC Riboprobe Synthesis using Reverse Transcriptase .................. 46
  2.3.2 In Situ Hybridization Protocol ................................................. 47
2.4 **CYTOKERATIN IMMUNOHISTOCHEMISTRY** ........................................... 48

3 **RESULTS** ........................................................................................................... 50

3.1 **RIBOPROBE CHARACTERIZATION AND IN SITU HYBRIDIZATION CONTROLS** ........................................... 50
  3.1.1 Genebank Blast Homology Check ................................................. 50
  3.1.2 Northern Analysis Of Adult Human Lung, Kidney And Liver Total RNA Using αhENaC Riboprobe ........................................... 50
  3.1.3 In situ hybridization: Regional αhENaC subunit mRNA in positive control kidney tissue ........................................... 51
  3.1.4 In situ hybridization: Competition for target αhENaC mRNA between labeled and unlabeled antisense riboprobes ........................................... 51
  3.1.5 In situ hybridization: RNase A treatment of tissue sections before hybridization ........................................... 52

3.2 **EXPRESSION OF αhENaC mRNA DURING NORMAL HUMAN LUNG DEVELOPMENT AND IN NEWBORN LUNG DISEASE.** ........................................... 53
  3.2.1 Ontogeny and cellular distribution of the αhENaC subunit mRNA during normal human fetal lung development using in situ hybridization ........................................... 53
  3.2.2 Pattern of αhENaC expression in cases of oligohydramnios, CDH, and infant prematurity ........................................... 56

4 **DISCUSSION** .......................................................................................................... 68

6 **REFERENCES** ......................................................................................................... 76
LIST OF TABLES

TABLE 1: Summary of published studies of the localization of ENaC subunit mRNA in adult rat and human lung by ISH .................................................................39

TABLE 2: Sample sizes: Expression of αhENaC in normal human fetal lung ..................................................................................................................41

TABLE 3: Sample sizes: Expression of αhENaC in abnormal human fetal lung ..............................................................................................................43

TABLE 4: Ontogeny and localization of αhENaC mRNA expression in developing human lung by ISH ..........................................................56

TABLE 5: Localization of αhENaC mRNA in newborn lung disease by ISH .................................................................................................................57
LIST OF FIGURES

FIGURE 1: Lung fluid secretion: Major components of active Cl⁻ transport 15
FIGURE 2: Lung fluid absorption: Major components of active Na⁺ transport 17
FIGURE 3: Model of ENaC. 24
FIGURE 4: Northern blot of adult human kidney, lung and liver total RNA hybridized with αENaC antisense and sense riboprobes 58
FIGURE 5: In situ hybridization controls
5a) αENaC mRNA expression in human kidney tissue
5b) Cold competition ISH of αENaC mRNA in fetal lung 59
FIGURE 6: In situ hybridization of αENaC mRNA in embryonic human fetal lung 60
FIGURE 7: In situ hybridization of αENaC mRNA in pseudoglandular human fetal lung 61
FIGURE 8: In situ hybridization of αENaC mRNA in canalicular human fetal lung 62
FIGURE 9: In situ hybridization of αENaC mRNA in saccular human fetal lung 63
FIGURE 10: In situ hybridization of αENaC mRNA in alveolar human fetal lung 64
FIGURE 11: In situ hybridization of αENaC mRNA in pulmonary hypoplasia (Oligohydramnios) 65
FIGURE 12: In situ hybridization of αENaC mRNA in pulmonary hypoplasia (CDH) 66
FIGURE 13: In situ hybridization of αENaC mRNA in pulmonary immaturity (Infant Prematurity) 67

LIST OF APPENDICES

APPENDIX A: Patient Data: 75
LIST OF ABBREVIATIONS

ABC: avidin biotin complex
AS: amiloride sensitive
ATI: alveolar Type I
ATII: alveolar type II
ATP: adenosine triphosphate
AVP: arginine vasopressin
BNaC: brain Na⁺ channel
BSA: bovine serum albumin
cAMP: cyclic adenosine monophosphate
CDH: congenital diaphragmatic hernia
CFTR: cystic fibrosis transmembrane conductance regulator
CSPD: disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-5',
cloro)tricyclo[3.3.1.1³.⁷]decan]-4-yl)phenyl phosphate
DEG: degenerin
DEPC: diethyl pyrocarbonate
DIG: digoxigenin
DTT: dithiothreitol
ECM: extracellular matrix
EGF: epidermal growth factor
EIPA: N-ethyl-N-isopropyl-2'-4'-amiloride
ENaC: epithelial Na⁺ channel (rENaC = rat, mENaC = mouse, hENaC = human)
FDLE: fetal distal lung epithelium

H₂O₂: hydrogen peroxide

HCl: hydrochloric acid

HMD: hyaline membrane disease

HSC: Hospital for Sick Children

H-type: high amiloride affinity

ISH: in situ hybridization

kb: kilobases

LMW: low molecular weight

L-type: low amiloride affinity

MOPS: morpholino propanesulfonic acid

MVA: motor vehicle accident

Na⁺/K⁺ ATPase: sodium potassium adenosine triphosphatase

NHS: normal horse serum

PAS: periodic acid-schiff

PBS: phosphate buffered saline

PHA-1: pseudohypoaldosteronism type I

pS: picoSiemons

QRT-PCR: quantitative reverse transcriptase-polymerase chain reaction

R/T: room temperature

RAC: radial alveolar count

RDS: respiratory distress syndrome

RPA: RNase protection assay
SDS: sodium dodecyl sulfate

SP-A: surfactant protein A

SP-C: surfactant protein C

SSC: sodium chloride/sodium citrate buffer

T3: triiodothyronine

TBS: tris buffered saline

TRH: thyroid releasing hormone

TTN: transient tachypnea of the newborn

UTP: uridine triphosphate

UTR: untranslated region
1 INTRODUCTION AND LITERATURE REVIEW

1.1 LUNG DEVELOPMENT

Human fetal lung development is a complex process that involves both cell proliferation and differentiation. Lung development is divided into five stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar. The early stages of lung development deal primarily with airway development, it is not until later stages that the gas exchanging regions are formed. Both the airways and the gas exchanging structures are lined by an epithelium which plays an important role in lung function and development. The basic stages of lung development, described below, have been the subject of a recent review(1).

1.1.1 Stages Of Lung Development

During the embryonic stage, the fetal lung appears at 3-4 weeks gestation as a ventral diverticulum of the foregut. The diverticulum separates from the pharynx and gives rise to the trachea, which bifurcates distally to form the right and left lung buds. The lung bud continues to branch in a dichotomous fashion to form the future primary bronchi, lobar bronchi, and by 7 weeks gestation, the bronchopulmonary segments. The lung epithelium is of endodermal origin. It is uniform and composed of a single layer of tall columnar cells which are multipotent. These undifferentiated epithelial cells contain a large amount of glycogen and few organelles. The variable position of the nuclei gives the epithelium a pseudostratified appearance.

During the pseudoglandular stage from 6-16 weeks of gestation, most of the conducting airway and some of the respiratory airway development is completed by repeated dichotomous branching (16 - 25 generations). The small epithelial lined tubules surrounded by mesenchyme give the lung a glandular appearance. Mesoderm condenses around the larger bronchial tubes.
and gives rise to cartilage, smooth muscle cells and the vascular system. In this stage, dramatic changes in the airway epithelium begin to occur. Cells in the proximal airways become differentiated and the nuclei more localized near the lumenal surface. In the distal lung tubules, the transition from columnar cells to cuboidal cells demarcates the pulmonary acinus, the respiratory portion of the lung.

In the canalicular stage from 16-28 weeks of gestation, the primitive gas exchanging structure begins to develop. Interstitial tissue decreases and the cuboidal epithelium lining the potential airspace begins to flatten in preparation for formation of an air-blood interface. The epithelial cells lining the proximal airways contain less glycogen and resemble the postnatal lung from this point onwards. In the pulmonary acinus, a capillary network forms that will link up with the developing pulmonary arteries and veins. Capillary ingrowth is soon followed by epithelial differentiation of alveolar type I (ATI) and alveolar type II (ATII) pneumocytes. By the end of the canalicular period, structural development of the lung has developed to a point where gas exchange is possible.

During the saccular stage from 26 - 36 weeks of gestation, a marked decrease in the prominence of the interstitial tissue and flattening of the acinar epithelium facilitates a close link between capillaries and epithelial cells. There is also subdivision of the terminal gas exchanging region to form “saccules”. The alveolar pneumocytes are now morphologically indistinguishable from the corresponding cells described in adult human lung tissue. Biochemically however, the ATII cells are still immature and produce surfactant rich in phosphatidylinositol. Late in gestation, ATII cells produce surfactant rich in phosphatidylglycerol.

The alveolar stage overlaps with the saccular phase from 32 weeks of gestation and continues postnatally for several years. The alveolar stage is characterized by further division of
the saccules into "alveoli" plus microvascular maturation. The alveoli are thin walled, multifaceted structures which greatly increase the gas exchanging surface area. The ATII pneumocytes are now fully mature and have characteristic lamellar bodies.

1.1.2 Lung Epithelial Cell Differentiation

Early in gestation, the respiratory epithelium consists of a single columnar cell type that is pluripotential(2). During development, the epithelial cells differentiate and acquire specialized shapes and functions. Morphologically, the epithelium eventually acquires a columnar shape in proximal airways, a cuboidal shape starting at the distal bronchioli, and a mix of squamous and cuboidal cells in the alveolus(3). Functionally, the embryonic lung epithelium differentiates into cells specialized for mucus production, mucus movement, surfactant production, gas-exchange and ion transport. The maturation process follows a centrifugal pattern, starting in proximal airways and progressing distally.

Ciliated and goblet cells make up the majority of the tracheobronchial epithelium in approximately a 5:1 ratio. Ciliated cells are present appearing as early as 11 weeks gestation and increase until 24 weeks gestation when the surface epithelium resembles that of adults(4). They are roughly columnar in shape tapering at the base where they meet the basement membrane. The cell cytoplasm is electron lucent because it does not contain secretory granules or many ribosomes. On the luminal surface there are hundreds of cilia attached to each cell. Interspersed between these cilia are microvilli and fine cytoplasmic processes giving the luminal surface a very characteristic appearance(3). The length and number of cilia vary at different stages of lung development. The main function of ciliated cells is to propel respiratory secretions toward the pharynx.
Mucus producing goblet cells begin to appear at around 16 weeks of gestation (4). They represent approximately 15% of the epithelial cell population in the proximal airways (approx. 7,000/mm²) but decrease in number peripherally until very few are detected in normal bronchioles. The prevalence of goblet cells increases between 15-19 weeks gestation and with airway disease. The cell name is based on the characteristic shape of the mature goblet cell in which only a narrow part of the tapered basal cytoplasm touches the basement membrane. Goblet cells contain many well-developed organelles for protein synthesis and are filled with electron lucent mucus granules. Mature goblet cells stain purple with alcian blue-periodic acid-schiff (AB-PAS) consistent with an acidic, mucin type glycoprotein content (3).

Submucosal glands also contribute to the secretion of mucus. The glands are embedded in the airway wall and open into the lumen via a duct. They first appear around 12-14 weeks gestation and increase in number until 23 weeks. In humans, they are found from the trachea down to the small bronchi (3). Three regions, the ciliated duct, the collecting duct, and the secretory tubules, have been identified in the human mucous gland. The ciliated duct is lined by airway surface epithelium. The larger collecting duct is fusiform in shape and the site where the secretory tubules empty. The collecting duct epithelium changes from airway epithelium to tall columnar (eosinophilic) cells with centrally placed nuclei. They exhibit evidence of high metabolic activity but do not produce mucus. It has been hypothesized and the presence of both the epithelial Na⁺ channel and cystic fibrosis transmembrane regulator (CFTR) suggest that these cells may adjust the water and ion concentration of the secreted mucus. The secretory tubule system is composed of mucous and serous tubules (3). Mucous cells containing large electron lucent granules are located in the proximal (mucous) tubules and are morphologically similar to goblet cells (PAS positive). Serous cells containing small electron dense granules are pyramidal
in shape and found in the more distal (serous) tubules. Serous cells have also been described in the superficial epithelium of the upper respiratory tract in human fetal lung.

The amine and peptide (ie serotonin) producing neuroendocrine cells are the first cell type to differentiate and appear at about 8 weeks gestation as solitary pyramidal shaped cells distributed along the entire length of the tracheobronchial tree. Innervated clusters, neuroepithelial bodies are found mainly within the intrapulmonary airways up to the bronchiolar-alveolar junction(2).

Basal cells differentiate around 12 weeks gestation and are found in all except the most peripheral airways(2). The basal cells are arranged in one or two rows of round polygonal cells, immediately adjacent to the basement membrane and not extending to the lumen surface. They are the progenitor cells in the upper airways forming mucous, ciliated and other cells.

The mature epithelium of terminal bronchioles is composed mainly of low ciliated and taller nonciliated cells. Almost all of the nonciliated cells are clara cells which appear around 22-24 weeks gestation(4). These secretory cells have a deeply invaginated central nucleus and protrude beyond the ciliated cells. The cell apex is often crowned by a round process and rests on the basement membrane. Clara cells secrete a variety of low molecular weight proteins (including surfactant related proteins) and are therefore distinct from mucous secretory cells(5). Clara cell secreted proteins may be involved in regulation of inflammation in the lung(5). Several studies suggest that clara cells are the major progenitor cell of the small airway during development and following injury(5,6).

As the lung begins to develop its distal airspaces, the fetal distal lung epithelium (FDLE) which lines this area will undergo maturation as the alveolus develops. The alveoli are largely lined by squamous ATI pneumocytes interspersed with cuboidal ATII pneumocytes which begin
to appear at around 24 weeks gestation(4). The production of surfactant products, cuboidal cell shape and corner localization within the alveolus characterize the ATII cells. ATII cells are more numerous than ATI cells but cover only 7% of the alveolar surface area in the adult(7). ATII cells are the only cells in the lung responsible for the production, storage (lamellar bodies) and secretion of pulmonary surfactant. Surfactant reduces alveolar surface tension, preventing airspaces from collapsing at low lung volumes. During lung maturation, many changes in the surfactant system take place. These characteristic changes can be used as markers of lung maturity. ATI cells are extremely thin which minimizes the thickness of the blood air barrier. Although less numerous than the ATII cells, ATI cells cover approximately 93% of the alveolar surface in the adult. ATI cells possess minimal metabolic activity and require the division of a progenitor cell, in this case the ATII cell, for renewal of the cell population(6).

The acquisition of specialized features (shape and function) by the undifferentiated epithelium is controlled by genetic factors within the cell, extracellular signals including soluble growth factors or hormones, and the extracellular matrix (ECM) which is a complex of both soluble and structural proteins. All cells undergoing morphogenesis produce and deposit an insoluble mesh of structural proteins, the ECM. This matrix can affect cell shape by providing the necessary physical interaction with adjacent epithelial surfaces. The importance of this insoluble mesh to cell shape is demonstrated by the effects of different cell culture conditions. Cuboidal ATII cells cultured on plastic tissue culture dishes quickly flatten. If grown on a basement membrane-like matrix, they can retain their cell shape and many of the ATII cell features(8). The presence of matrix secreted by cultures of immature lung cells modulate the ion transporting phenotype of FDLE(9). Interactions between the epithelium and mesenchyme can
also be mediated via the ECM. Alternatively, the ECM may act as a reservoir for certain growth hormone peptides that could subsequently affect epithelial cell differentiation.

Corticosteroids and thyroxine accelerate the formation of alveoli and differentiation of respiratory cell types in organ culture and in-utero(10). The critical importance of glucocorticoids in lung development has been recently demonstrated in genetic experiments where the mouse glucocorticoid receptor was knocked out. Glucocorticoid effects on the maturation of the surfactant synthetic pathway are mediated through a fibroblast-derived polypeptide named fibroblast-pneumocyte factor(11,12). It can accelerate anatomic changes in the alveoli and surfactant synthesis(13). Retinoic acids exert their influence through the steroid hormone receptor superfamily and induce and maintain a more proximal epithelial cell phenotype in the rabbit(14).

There are several families of growth factors that can affect epithelial cell differentiation. One example is the epidermal growth factor (EGF) family(15). The temporal and spatial expression of these genes suggest their role in epithelial differentiation. In cell culture, EGF increases the production of surfactant protein A (SP-A) by ATII cells(16). Recent studies using gene knock-out techniques demonstrate that the growth hormone EGF plays a significant role in epithelial cell proliferation and differentiation. Knock-out of the EGF receptor resulted in severe epithelial immaturity in the respiratory bronchioles and alveoli, thickening of the alveolar septa and multiorgan failure including the lung(15,17).

1.1.3 Lung Branching

During the embryonic stage, the branching pattern establishes the number of lobes in the right and left lungs. The primitive trachea bifurcates distally to form the right and left lung buds.
*In vitro* rat lung morphogenesis studies suggest that these second generation airways are formed by a process of monopodial (in which a lateral branch arises from a continuous axis) budding from the main airway and that these airways grow in length simultaneously with branching(18). The exact role of monopodial budding in human lung branching is uncertain. The remaining airways are believed to undergo branching in a dichotomous (when the axis divides into 2) fashion. In the pseudoglandular stage, the entire conducting airway system and some of the respiratory airway system develops. In the canalicular stage, further subdivision of the distal airways occurs. Branching morphogenesis is a complex process that involves cell proliferation, migration, and differentiation. Although the molecular signals are incompletely understood, epithelial-mesenchymal interactions are known to influence the branching properties of the primitive lung epithelium(19). The pattern of branching is always determined by the associate mesenchyme. Tracheal mesenchyme inhibits branching in the trachea or bronchus while bronchial mesenchyme induces bronchial branching in both regions(20). The amount of branching is directly proportional to the quantity of bronchial mesenchyme surrounding the buds(21). When the mesenchyme of one species is attached to the endoderm of another, the branching pattern is that of the species providing the mesoderm(20-22). The mechanism by which the mesenchyme exerts this control over branching is uncertain. Components of the ECM, such as collagens, proteoglycans, and glycoproteins, are also suspected to determine the way the mesenchyme influences lung branching(19). Most of the matrix components have binding sites for growth factors and may sequester branching factors in critical sites(23). Collagen may act as a physical barrier in the lamina propria preventing branching except in places where there is a gap(24). Alternatively, the collagen meshwork could act as a filter, permitting selected
molecules to penetrate the epithelial surface by regulation of the spaces within the mesh, charge density, or hydrophobicity(25).

1.1.4 Influence Of Fetal Lung Fluid On Lung Growth

Throughout gestation, liquid is actively secreted by the pulmonary epithelium. This lung liquid is critical to normal lung growth. In the normal fetus, the lung liquid volume is carefully regulated by the transpulmonary pressure which is determined by the pleural pressure and the secretion pressure of lung liquid acting against the resistance to outflow in the upper airway and the amniotic fluid pressure(26). The importance of lung liquid to normal lung growth was demonstrated by the work of Alcorn(27). This group restricted fluid outflow from fetal sheep lungs by tracheal ligation during the canalicual period and noted a significant increase in lung growth and alveolar structural maturity. Specifically, ligated lungs were associated with increased tissue growth (increased size and weight) and thinning of the alveolar walls and an increase in airspace fraction (ASF). Interestingly, they report a delay in ATII cell differentiation. In humans, obstruction of lung liquid drainage at the upper airway has been shown to similarly enhance lung growth(28).

Compromise of the transpulmonary pressure gradient, leading to decreased lung liquid volumes, is associated with incomplete lung growth and delayed alveolar structural maturity. Fetal tracheostomy and lung liquid drainage during the canalicual period resulted in lung hypoplasia(27). Specifically, drained lungs exhibited tissue growth inhibition (decreased size and weight) and alveolar wall thickening. Interestingly, they report enhanced differentiation of ATII cells. Other models of lung hypoplasia are not associated with an increased number of ATII cells(29).
Moessinger devised a model where manipulations of lung liquid volume could be studied in a single fetal sheep(26). Again, chronic fetal lung distention by lung liquid retention resulted in lung hyperplasia as determined by measurements of lung weight, and DNA content (a measure of cell number) while lung liquid drainage caused lung hypoplasia. Biochemical measurements of lung maturation including surfactant profiles were not affected by the manipulations of lung liquid volume. It is possible that pulmonary biochemical lung maturation is regulated independently of pulmonary growth, perhaps by systemic factors.

The specific mechanisms by which lung liquid controls lung growth is uncertain. One theory is that pressure or volume regulation of lung growth occurs with the lung liquid acting as an internal splint. In sheep at approximately 112-115 days gestation there is a significant increase in the rate of lung liquid secretion, and intrapulmonary pressure (IPP), which coincides with the most rapid period of lung growth(30,31). The mechanical stretch generated by the increased lung liquid volumes in tracheal ligation and compromised by lung liquid drainage would explain the abnormalities of lung growth seen in these conditions. Intermittent stretch increases fetal rat lung cell division and DNA synthesis in organotypic culture(32,33). The events that are regulated by stretch and directly effect lung growth are currently being studied.

1.1.5 Perturbations Of Lung Growth In The Human

1.1.5.1 Lung Hypoplasia

Pulmonary hypoplasia is defined as incomplete lung development (growth or maturation). The etiology and timing of the insult to lung development determine the features that characterize the hypoplasia for each case(34). In normal lung development, most of the conducting airways are developed by 16 weeks gestation followed by progressive increases in the acinar structural
complexity and maturity until term. Epithelial differentiation is a complex process that occurs throughout gestation. ‘Early’ onset pulmonary hypoplasia is typically characterized by abnormalities of early and late gestational development (airway branching, epithelial differentiation, and acinar complexity) while ‘late’ onset pulmonary hypoplasia tends to be limited more to developmental abnormalities of the acinus and epithelium. Despite such efforts to broadly categorize the features of hypoplasia, variability in severity and timing of any insult (even of the same etiology) makes this difficult and helps to explain inconsistencies in the literature. Thurlbeck(35) has suggested that it is important to analyze any given case by the degree of hypoplasia or hyperplasia using assessments of lung growth and maturation. Lung growth is routinely evaluated by determining lung:body weight ratio, lung volume, DNA and protein measurement, total alveolar number, and number of bronchiolar branchings. Lung maturation has both structural and biochemical components and is assessed by looking at acinar complexity (radial alveolar count-RAC), tissue maturity: (subjective assessments of duration of gestation by appearance ie. connective tissue thickness, percent airspace fraction) and cell maturity (glycogen content, lamellar bodies, ATII:ATI cell ratio, surfactant profiles)(35). Most studies of lung growth look at a subgroup of these parameters for practical reasons. Two clinical intrauterine events known to inhibit lung growth are congenital diaphragmatic hernia (CDH) and oligohydramnious(26,28).

1.1.5.1.1 Congenital Diaphragmatic Hernia

In CDH, abdominal contents are shifted into the thorax and compete for space with the developing lung(36). The most popular theory is that the pleuriperitoneal canal fails to close at 8-10 weeks gestation creating a primary diaphragmatic defect. There is a wide spectrum of
severity of hypoplasia in CDH ranging from minimal lung growth to no symptoms. Severe cases of CDH usually cause bilateral lung hypoplasia(37). Features of lung hypoplasia in CDH have been reported to include: 1) a reduction in the number of conducting airways(38) 2) acinar hypoplasia with decreased numbers of alveoli(39) and preacinar pulmonary vessels(40) 3) subjective lung immaturity(39,41); and 4) biochemical cell immaturity(34,42). The reported effect of CDH on lung biochemical maturity, however, is variable.

Hypoplasia of the lung in CDH is presumably due to compression from abdominal contents within the thoracic cavity. As CDH occurs during the early pseudoglandular stage of lung development, it is not surprising that both early (branching) and late (acinar complexity) lung developmental events are affected. Maintenance of lung liquid volumes have been shown to reverse both the structural and physiological sequelae of hypoplasia in CDH(31,41).

1.1.5.1.2 Oligohydramnios

Reduced amniotic fluid volumes can be caused by impaired amniotic fluid production (renal agenesis or dysplasia) or increased fluid loss from the amniotic cavity (prolonged rupture of membranes). The absence of kidneys in Potter’s syndrome eliminates the fetal urine contribution to amniotic fluid volume(43). Lung hypoplasia in these cases has been primarily characterized by acinar hypoplasia (both fewer and smaller alveoli)(34). A diminished number of airway generations has also been described(34). Several studies have reported delayed alveolar maturation in models of oligohydramnios induced lung hypoplasia with extensive persistence of cuboidal epithelium, delay in the of thinning air-blood barriers, and deficiency of surfactant(34,44-46).
The mechanism of the pulmonary hypoplasia seen in oligohydramnious is controversial. Inhibition of fetal breathing movements or increased loss of fetal lung fluid into the amniotic space have been suggested as two possible mechanisms. Even when fetal breathing is eliminated by cord transection, oligohydramnios causes further lung hypoplasia, suggesting that restricted fetal breathing is not the predominant mechanism of lung hypoplasia in oligohydramnios(47). There is evidence that oligohydramnious leads to a decrease in lung liquid secretion(48) and that the hypoplastic effect of oligohydramnios can be reversed by tracheal ligation(28,47). It is also possible that the decrease in the amniotic fluid hydrostatic pressure contributes to increased egress of lung liquid and impaired lung growth. Collectively, these studies suggest that lung hypoplasia associated with oligohydramnios is related to the loss of the internal fluid splint.

1.1.5.2 Lung Hyperplasia

Upper airway obstruction is known to have a positive effect on lung growth(27). When evaluating changes in lung growth; the ratio of total DNA (an estimate of total nuclear material) to total protein (an estimate of total cytoplasmic and extranuclear material) can be used to determine if changes in lung growth are due to changes in proliferation (cell division) rates vs trophic (cell shape) changes. A normal DNA/protein ratio indicates that the cells have a normal ratio of nucleus to cytoplasm, and are therefore of normal size. Increases in lung size can therefore be attributed to cell division and not by hypertrophy. Clinical intrauterine events known to accelerate lung growth include laryngeal atresia and tracheal agenesis(28). Morphometric studies and lung tissue weight/body weight ratios show that these lungs consist of increased amount of tissue and not simply increased lung liquid(28). A report of human laryngeal atresia demonstrated precocious lung development. The fetal lungs (30 weeks
gestation) were three times the expected weight and showed accelerated alveolarization appropriate for three weeks postnatal age(49).

1.2 EPITHELIAL ION TRANSPORT AND FLUID MOVEMENT IN THE FETAL AND PERINATAL LUNG

1.2.1 Lung Liquid Secretion

Liquid secretion by the lung epithelium is an important determinant of lung growth in the fetus and is required for humidification and mucociliary clearance in the postnatal lung. Early researchers thought that fetal lung liquid was aspirated from the amniotic cavity. Jost and Picard disproved this theory and showed that liquid is produced within the lung(50). Later, it was shown that the lung fluid was the product of active transport by the developing lung epithelium(51). It is now appreciated that the fetal lung is a liquid secretory organ, producing about 5 ml/kg/hr liquid at full term, a flow nearly as great as that produced by the fetal kidney(52).
The major driving force for the fluid secretion is active transepithelial Cl⁻ transport from the interstitium to the lumen(30). In the most commonly accepted model, Cl⁻ enters the basolateral epithelial cell with Na⁺ and K⁺ via the bumetanide-sensitive Na⁺/K⁺/2Cl⁻ cotransportor. Na⁺ and K⁺ are actively exchanged by the basolateral sodium potassium adenosine triphosphatase (Na⁺/K⁺ ATPase) (3 Na⁺ exchanged for 2 K⁺) which combined with basolateral K⁺ channels generates an electrochemical gradient favoring Cl⁻ secretion into the lumen(53). The osmotic force generated by this Cl⁻ transport causes fluid flux into the lung lumen(53). The specific molecular identities of fetal Cl⁻ channels have not been fully elucidated. Both mRNA and protein for the volume-activated chloride channel, CIC-2, have been detected in
mid to late gestation rat lung along the airway epithelium suggesting it may be involved in fluid secretion during normal lung morphogenesis(54). Another possible pathway for Cl\(^{-}\) secretion across the pulmonary epithelium is the cystic fibrosis transmembrane conductance regulator (CFTR)(55-57). CFTR is an apical membrane Cl\(^{-}\) channel regulated by cyclic adenosine monophosphate (cAMP)-dependent phosphorylation and by intracellular adenosine triphosphate (ATP)(58). Mid gestation human alveolar epithelium explant cultures exhibit cAMP-stimulated fluid secretion(57). First trimester fetal conducting airway epithelium in submersion organ culture(59) also secretes lung fluid. The secretion of Cl\(^{-}\) and fluid from cultured mid gestation human fetal lung can be stimulated by β agonists and by cAMP(59). In late gestation, β agonists and cAMP decrease the rate of fluid secretion and induces fluid absorption(53). The volume of lung lumen fluid diminishes before the onset of labor in lambs(53). The exact contribution of decreased liquid secretion versus increased fluid absorption to this decreased lung liquid volume is not known. There is evidence of decreased gene expression and functional activity of the ClC-2 (54) and CFTR Cl\(^{-}\) channels in late gestation(55).

### 1.2.2 Lung Liquid Absorption

Towards late gestation, Na\(^{+}\)/fluid absorption in the fetal lung epithelium can be induced with cAMP and β agonists(30,53). Active liquid absorption is critical for the transition from a liquid-filled fetal lung to an air-filled postnatal lung at birth. Active liquid absorption is also a major mechanism in the prevention of and recovery from pulmonary edema(60,61). The driving force for movement of fluid from the lumenal to the interstitial side of the epithelium is generated by Na\(^{+}\) transport(60,62). Critical components in the active transport of Na\(^{+}\) include the
apically located Na$^+$ channel, the basolateral Na$^+$-K$^+$-ATPase, and the interepithelial tight junctions. The apical Na$^+$ channel is believed to be the rate limiting step(63).

**FIGURE 2:** Lung fluid absorption: Major components of active Na$^+$ transport

![Diagram of lung fluid absorption](image)

Na$^+$ ions are pumped out of the epithelial cell at the basolateral membrane by the enzyme Na$^+$-K$^+$ ATPase creating a low Na$^+$ concentration inside the cell (intracellular Na$^+$ concentration is approximately 10mM). The Na$^+$-K$^+$ ATPase combined with Na$^+$, K$^+$, and other ion channels also cause the intracellular membrane potential to be negative relative to the outside of the cell. This electrochemical gradient favors the flow of Na$^+$ through specialized apical pathways into the cell(64). The major pathway for Na$^+$ absorption across the apical membrane is the amiloride-sensitive epithelial Na$^+$ channel (ENaC). The movement of Na$^+$ across the membrane then results in Cl$^-$ moving passively via the intracellular tight junctions, with water following via paracellular pathways or transcellularly through water (aquaporin) channels(65,66). In the mature fetal and postnatal lung, fluid absorption can be increased by beta adrenergic agonists
which upregulate ENaC activity via the intracellular second messenger cAMP(53). Most of the increase in Na$^+$ transport induced by beta agonists is inhibited by the ENaC blocker amiloride(67). The remaining Na$^+$ transport is speculated to be mediated via amiloride insensitive pathways although whether this occurs via channels or transporters is unknown.

1.2.3 Site Of Fluid Absorption In The Lung

Many attempts have been made to identify the quantitatively important site of active Na$^+$ transport in the mature fetal and postnatal lung. Difficulties that are encountered relate to the presence of many different cell types in the adult lung epithelium(3) as well as the dramatic maturational changes that occur in the epithelial lining of the developing lung. Epithelial cell culture techniques represent one system to examine the function of individual cell types. One requires caution when interpreting such results since these are only ‘models’ of the biology of cells in vivo and may not reproduce every characteristic of differentiated cells in vivo. It is well known that ATII cells in standard cell culture conditions undergo a series of changes in which they lose many of the specific features of the ATII cell phenotype(68). Whole lung studies can be used to estimate the contribution of various regions of the lung to ion transport but not for the study of individual cells.

All regions of the postnatal lung appear to be capable of amiloride-sensitive Na$^+$ absorption but the relative contribution of different regions to liquid homeostasis is not known. Primary cultures of adult human proximal airway epithelial cells exhibit Na$^+$ absorptive capabilities(69,70). Animal but not human fetal airway epithelia has been studied(71). In cell cultures, late gestation fetal(72,73) and adult rat(74) ATII cells produce fluid-filled domelike structures which represent a net fluid absorptive flow, and generate a significant basal $I_{SC}$ when
placed in Ussing chambers. This current is increased by β2 agonists and inhibited by the ENaC blocker amiloride. Patch clamp studies have also verified the existence of Na⁺ permeant ion channels in the apical membrane of FDLE (75). Primary cultures of adult lung Clara cells (76) have also been shown to actively transport Na⁺. The contribution of ATI cells and other cell types to Na⁺ absorption is unknown and at present further studies are limited by researchers inability to isolate many of the relevant cell types in primary culture. The enormous surface area of the distal lung unit makes it a likely candidate as the quantitatively important site of active Na⁺ transport in the mature fetal and post-natal lung.

1.2.4 Switch Of The Lung Epithelium From Predominantly Fluid Secreting To Predominantly Fluid Absorbing

The factors that regulate, on a short and longer term basis, the transition of the lung epithelium from a secretory to an absorptive state are being elucidated. The developmental maturity of the lung epithelium determines its responsiveness to these 'absorptive triggers'. Thyroid and glucocorticosteroid hormones are capable of priming the fetal lung epithelium to make it respond to absorptive stimuli (77,78). Glucocorticoids are also known to increase and advance the expression of αENaC, but not β or γENaC, in primary cultures of rat FDLE (78,79) whereas they upregulate all three subunits in human fetal lung epithelium (80). These hormones are known to have a maturational effect on fetal lungs which includes both morphologic and biochemical changes (81). In the human fetus, both thyroid and cortisol levels increase progressively from about 30 weeks of gestation (52). Clinical studies have demonstrated that prenatal combined corticosteroid and thyroid-releasing hormone therapy can diminish respiratory distress syndrome in prematurely born human infants (82).
To date, the perinatal surge of endogenous fetal catecholamines and changes in oxygen tension have emerged as strong candidates to control perinatal lung liquid flow. Fetal plasma epinephrine levels are elevated during labour and β- or β2-receptor agonists can, within minutes, convert the mature fetal lung from a fluid-secreting to a fluid-absorbing organ(83,84). This does not occur in immature lung and which aspect of the Na⁺ absorptive pathway is not functioning in a "postnatal" pattern in immature fetuses is unknown. It could be any of the steps from the β-receptor, adenyl cyclase, cAMP-dependent kinase, or the Na⁺ channel and its regulatory proteins. The absorptive response is blocked by amiloride, suggesting that the ENaC plays a critical role in this effect. However, β-blockers do not prevent the absorption of lung liquid during late gestation(53) suggesting that there are additional factors that regulate this switch in the mature fetal lung.

Changes in oxygen tension at birth could contribute to the permanent switch of the lung epithelium from secretion to absorption(85,86). Alveolar PO₂ in the fetal lung is around 25 mmHg and in the postnatal lung it is about 100 mmHg. Pitkanen maintained primary cultures of FDLE isolated from late gestational fetal rats under a ‘fetal’ 3% oxygen environment(85). These monolayers had little amiloride sensitive Na⁺ transport and very low levels of mRNA coding for α, β, and γ rENaC. Exposure of comparable monolayers to "postnatal" 21% oxygen resulted in marked increases in both mRNA levels and amiloride sensitive Na⁺ transport. Barker also demonstrated that liquid secretion by fetal distal rat lung explants in late gestation is regulated by gas composition(87). Exposure of 22 day fetal distal rat lung epithelial to fetal gases (3% O₂-8% CO₂) supported liquid secretion as evidenced by cyst formation whereas ‘postnatal’ gases prevented cyst formation. When ATII cells are isolated from adult rats, decreased alveolar oxygen tension diminishes vectorial Na⁺ transport and ENaC mRNA in a time dependent
manner(88). These changes were reversed by re-oxygenation (21% O₂). Collectively, these studies suggest that oxygen tension regulates Na+ channel expression and that increased oxygen may serve as an indicator of postnatal life and as a signal for increased Na+ transport.

The ECM is known to play an important role in lung organogenesis including the regulation of branching patterns and epithelial cell differentiation. To explore if ECM had a regulatory effect on ion transport in the developing lung, Pitkanen et al (9) examined the effect of different gestational aged fetal mixed lung cell derived matrix preparations on FDLE ion transport. The matrix produced by mixed lung cell isolated at the canalicular gestational stage decreased the FDLE’s sensitivity to amiloride. These results suggest that the ECM is an additional source of ion transport regulatory signals in the developing fetal alveolar epithelium.

1.3 AMILORIDE-SENSITIVE EPITHELIUM Na⁺ CHANNEL (ENaC)

1.3.1 Cloning of ENaC and its Subunits

In 1993, Canessa(89) and Lingueglia(90) successfully used expression cloning to isolate a cDNA (αrENaC) capable of inducing amiloride-sensitive (AS) Na+ currents in a Xenopus oocyte model from a rat colon cDNA library. The following year, Canessa identified the β and γENaC subunits using a functional complementation strategy(91). These three ENaC subunits were subsequently cloned from several other species including humans(92-94). Equivalent rat and human ENaC (rENaC and hENaC respectively) subunits show 83-85% similarity in their amino acid sequences(92,94). The α, β, and γENaC subunits share 33-37% amino acid identity with each other which is consistent with evolution from a common ancestor gene(91). The αhENaC gene has been mapped to chromosome 12 while both β, and γhENaC were mapped to chromosome 16 (92,94).
The α, β, and γENaC mRNAs have been detected by Northern analysis as unique bands of 3.7, 2.2, and 3.2 kilobases (kb) respectively(91). In the human, two different transcript sizes approximately 3.8 & 3.4 kb, have been reported for αhENaC(92,93). The different transcript sizes may represent variable transcription start sites, different polyadenylation sites or alternatively spliced mRNA. The α, β and γhENaC cDNAs have open reading frames of 2,007, 1,920, and 1,947 nucleotides respectively which code for proteins predicted to contain 669, 640, and 649 amino acids respectively(92,94).

In rat, there are high concentrations of ENaC mRNAs in epithelial tissues such as renal cortex and medulla, distal colon, lung, urinary bladder, placenta, and salivary glands (91,93). Transcript levels are lower in proximal colon, uterus, thyroid, and intestine. No signal is detected in liver, stomach, duodenum, smooth and striated muscle, brain or blood-brain microvessels. Using antisera of questionable specificity for endogenous ENaC protein, similar patterns of expression were observed for α, β, and γENaC proteins in immunolabelling experiments (95-97). In the human, α, β and γhENaC mRNAs were found in the kidney and lung(94).

Identification of the ion channel subunit cDNAs for ENaC has also revealed similarity with a family of proteins previously identified in the nematode Caenorhabditis elegans. There was 12 % amino acid identity observed between ENaC subunits and the degenerin (DEG) proteins MEC-4, MEC-10, and DEG-1, subunits of a putative ion channel that might be involved in mechanosensation(89,98). More recently additional branches of this new DEG/ENaC superfamily have been identified. A channel from the human brain (BNaCl & BNaC2) has recently been cloned(99) but the function of this channel is unknown. Other members of the DEG/ENaC/BNaC superfamily continue to be revealed. All the proteins in this evolving gene family are characterized by the presence of two large hydrophobic domains, a large extracellular
loop with a conserved 19 amino acid region, and the presence of one or two cysteine-rich regions (98). A human δENaC subunit has been cloned which structurally resembles the αENaC subunit and also requires co-expression of the β and γ subunits for significant expression. The δENaC subunit is not epithelial, being most highly expressed in testis, ovary, pancreas and brain (100).

1.3.2 Structure Function Analysis Of ENaC

The epithelial Na\(^+\) channel is a heteromultimeric protein made up of three homologous subunits, α, β, γ (91). Each individual ENaC subunit has an amino-terminus which is 50-100 residues long and is oriented on the cytoplasmic side of the membrane (101). Two hydrophobic domains are separated by a large hydrophilic domain, the extracellular loop. The extracellular loop has a highly conserved cysteine-rich region and at least four N-glycosylation sites. The glycosylation of αrENaC does not play a significant role in the functional expression of the channel (101). The hydrophobic domains are suspected transmembrane spanning regions, predicted to adopt an α-helical structure (101). Preceding the second transmembrane domain, a short segment shows a high degree of homology among all the members of the ENaC gene family. It has been postulated that this segment may be involved in pore formation (102). Finally there is a carboxy-terminal segment which is 20-100 residues long and also located in the cytoplasm (101). The carboxy terminus of the three subunits each contains two proline-rich sequences, P1 and P2. The P2 sequence is conserved between rat and human sequences (91, 93) and is an important site involved in the interaction of ENaC with various regulatory proteins.
The regions of ENaC involved in the ion permeation pathway have not been clearly defined. The ENaC channel pore has been predicted to be a progressively narrowing funnel that allows only the smallest monovalent cations such as H\textsuperscript{+}, Li\textsuperscript{+}, or Na\textsuperscript{+} to permeate the channel.

The subunit stoichiometry of the epithelial Na\textsuperscript{+} channel remains controversial. Although an amiloride sensitive Na\textsuperscript{+} current has been detected with the $\alpha$ENaC subunit alone, the $\beta$ and $\gamma$ subunits are required for maximal functional expression\cite{91}. The $\beta$ and $\gamma$ subunits cannot by themselves induce a functional channel\cite{91}. Two very different models have been proposed. Firsov et al predict a two $\alpha$, one $\beta$, and one $\gamma$ subunit model with the two $\alpha$ subunits separated by
β and γ subunits, and arranged in a ring-like structure around the channel pore(103). In contrast, Spyder et al predict that ENaC is composed of a nine subunit stoichiometry (3α, 3β, 3γ)(104).

The biophysical and pharmacological properties of the ENaC in the kidney and *Xenopus oocytes* include: a slow gating channel with openings and closings on the time scale of 1 second; a unitary channel conductance of 5 picoSiemons (pS) for Na⁺ ions; and a typical ion selectivity sequence of Li⁺>Na⁺>>>K⁺(105). In contrast to the voltage-gated Na⁺ channels present in excitable tissues, the epithelial Na⁺ channel is little affected by membrane potential although the open probability tends to increase at high negative potentials(105). In the lung, several different types of ENaC have been described (section 1.3.3). ENaCs from cultured rat FDLE have different biophysical profiles characterized by unitary conductances of 25 pS and 12 pS. The 25 pS channels are equally permeant to Na⁺ and K⁺ while the 12 pS channel is more permeant to Na⁺ over K⁺. Both are highly selective for these cations over anions(106,107). Amiloride and its analogues block transport through ENaC by binding to high affinity ligand binding sites(108).

Various proteins are known to regulate ENaC activity. The cytoskeletal protein α-spectrin is suspected to mediate the apical localization of ENaC(109). Nedd4, (neural precursor cells expressed developmentally downregulated), is a protein that has been implicated in the ubiquitination of ENaC via an interaction with the P2 region of the β and γ ENaC subunits(110). Mutations in this P2 region in β and γ subunits in humans leads to a hypertensive (Liddle’s) syndrome sensitive to a low sodium diet and amiloride(111,112) and characterized by upregulated ENaC channel activity(113). CFTR exhibits an inhibitory effect on ENaC activity(114,115). The mechanisms of CFTR downregulation of ENaC currents are not fully understood.
Vasopressin and aldosterone are the two main hormones that control Na+ reabsorption and homeostasis in the kidney. In the lung glucocorticoids and β adrenergic agonists predominately regulate Na transport.

Vasopressin is a potent modulator of Na+ absorption in the renal cortical collecting duct. The exact mechanism by which vasopressin increases Na+ absorption is not clear. There are data indicating that vasopressin, by way of V2-receptor stimulation increases adenylate cyclase activity, cAMP and protein kinase activity. The principle effect of vasopressin is to increase the channel number via translocation of ENaC from intracellular pools to the apical membrane, thereby promoting an increase in Na+ entry(116).

The mineralocorticoid aldosterone is the primary hormone responsible for maintaining total body salt and water balance. Aldosterone immediately increases Na+ channel activity primarily by increasing the open time (Po) of the channels in the apical membrane(117). Aldosterone can modulate channel activity over several hours by altering gene expression and inducing de novo synthesis of particular proteins (ie other ENaC subunits) that stabilize the channel(118). The aldosterone effect is mediated by two cytoplasmic receptors with high affinity (type I, mineralocorticoid) and low affinity (type II, glucocorticoid)(79) to aldosterone. The induction of ENaC is tissue specific and variable with the individual subunits. In the rat colon β- and γENaC but not αENaC are upregulated by aldosterone(119).

In the lung, steroid regulation of ENaC subunits has been well described. O’Brodovich used whole lung Northern blot analysis to show that combined thyroid-releasing hormone (TRH) and dexamethasone induces the expression of lung αENaC in fetal rats(78). Champigny examined the mechanism of corticosteroid regulation in primary culture of FDLE. Both aldosterone and dexamethasone induces αENaC mRNA expression and also causes a large
increase in the activity of the amiloride sensitive current(79). Using specific agonists and antagonists for mineralocorticoid and glucocorticoid receptors, they show that these “aldosterone effects” are mediated via the glucocorticoid type II receptor. Triiodothyronine (T3), known to modulate steroid action in several tissues, individually has no effect on either the level of αENaC mRNA or the amiloride sensitive current, but potentiates the effect of the dexamethasone. Tchepichev used an in vivo model to examine the effect of corticosteroids on all three ENaC subunits(120). He shows that, in the fetal rat, there is a differential response by individual ENaC subunits to treatment with corticosteroids which is dependent upon fetal maturity. In-vivo administration of these agents increases αENaC expression only if given during the canalicular period. TRH and/or dexamethasone increases fetal lung αENaC levels, however, none of these treatments increases expression of β or γENaC. Venkatesh showed that in cultured human fetal lung, all three subunit genes are coordinately upregulated by dexamethasone(80). Glucocorticoids increase in vivo around birth(120) and it is speculated that they upregulate ENaC expression to facilitate lung fluid absorption in preparation for respiration at birth. This upregulation of ENaC by glucocorticoids may contribute to the beneficial effects of antenatal glucocorticoids in premature babies(82). Sweezey demonstrates that the female gender hormones progesterone and estradiol combined augment levels of α and γENaC mRNA in sexually immature female rats. Sex hormones increase ENaC activity as assessed by amiloride sensitive current in epithelial monolayers(121). These findings suggest a gender-dependent influence on the lungs ability to recover from pulmonary edema and on the degree of airway fluid hydration in cystic fibrosis.
1.3.3 ENaC in Lung Epithelia

There are data suggesting the presence of a more than one type of Na\(^+\) channel in the lung epithelium(106,122). A pharmacologic classification utilizes binding affinity for amiloride and its analogues; N-ethyl-N-isopropyl-2'-4'-amiloride (EIPA), which has greater potency for Na\(^+\)/H\(^+\) exchangers, or benzamil and phenamil, which have a greater affinity for Na\(^+\) channels. The high affinity, H-type channels, have much greater affinity for amiloride and benzamil relative to EIPA(123). The low affinity, L-type channels, have a lower affinity for amiloride and benzamil and EIPA can inhibit Na\(^+\) transport in these channels as well or more effectively than amiloride. Rat FDLE epithelial cells contain both L-type and H-type channels(122).

Na permeant ion channels can also be classified according to their electrophysiologic properties. In the rat FLDE patch clamp studies have identified both a 25 pS nonselective cation(107) and a 12-pS highly Na\(^+\) selective Na\(^+\) channel(106). Both of these Na\(^+\)-permeant ion channels are amiloride sensitive. At least two different Na\(^+\) channels have been identified in human airway epithelium(124). In addition, there has been one report of a 4 pS highly selective Na channel in lung epithelium(93) although other groups failed to find such a channel.

Whether different channel types can be found with in the same cell types, whether they arise from different genes or represent artifacts of culture is unknown. The contribution of various types of channels to overall Na\(^+\) transport is unknown.

1.3.4 Ontogeny Of Lung ENaC Expression

Aspects of the ontogeny of ENaC subunit mRNA expression have been reported in the rat(78,120), mouse(125), and human(92). Rat \(\alpha\)ENaC mRNA has been detected by Northern blot analysis of whole lung isolated during the canalicular period(120) and noted to surge in the
saccular stage of lung development(78,120,125). αrENaC mRNA levels fall slightly after birth but return to peak levels in the adult rat(78). β and γrENaC are not detected until the saccular stage of lung development(120) and show only a modest prenatal increase followed by a continued increase in the postnatal period to adult levels(120). At all times the α subunit abundance was several fold greater than that of the β or γ subunits(120). The pre- and post-natal patterns of αrENaC mRNA expression parallel the endogenous glucocorticoid rise in the fetus and rat pups’ early postnatal corticosteroid resistance. Corticosteroids have been shown to increase expression of α, but not β and γrENaC subunits(120) in the lung via glucocorticoid receptors.

In the human fetal lung, a large increase in expression of αhENaC mRNA was detected from fetal canalicular (20-25 weeks) to adult stage by Northern blot analysis of 1.2 μg of polyA mRNA(93). In other studies, αhENaC mRNA were detected in first and second trimester human fetal lung while performing Northern blot analysis on 30 μg of total RNA(92). A recent study using cultured human fetal canalicular (20-24 weeks) lung tissue detected expression of all three Na$^+$ channel subunit genes at this stage using reverse transcriptase-polymerase chain reaction (RT-PCR). α, β, and γENaC subunit mRNA levels in the second trimester were determined by RNase protection assay (RPA) to be 13, 26 and 32 % the adult expression levels respectively(80). In the human, the canalicular stage corresponds to 17 - 24 weeks gestation which is comparable to 19-20 days gestation in the rat.

In the adult human, Otulakowski examined the relative amounts of α, β and γ subunits in the nasal epithelium of healthy men. Using a competitive quantitative RT-PCR assay, they were able to show that the αhENaC subunit mRNA was expressed at approximately a 5 fold greater
level than the βhENaC subunit mRNA which was expressed at approximately a 4 fold greater level than the γhENaC subunit mRNA(126).

1.3.5 Relevance Of Epithelial Na⁺ Transport To Human Lung Diseases

1.3.5.1 Respiratory Distress Syndrome (RDS)

Respiratory Distress Syndrome (RDS), a lung disorder which occurs with increasing frequency in premature infants, is characterized by varying degrees of pulmonary edema, surfactant deficiency and structural lung immaturity. Respiratory disease of the premature newborn is commonly divided into 2 broad categories, RDS type I and RDS type II based on the severity of the disease. The most severe form, RDS I, is noted for combined surfactant deficiency(127), delayed lung fluid clearance from immaturity of the Na⁺ transport system(128-130) and abnormal lung morphology. The airspaces may be fluid filled or atelectatic(129) and infants dying from RDS have markedly increased lung water contents(128). These cases have a characteristic pulmonary histology, known as hyaline membrane disease (HMD). The hyaline membranes appear as smooth homogenous pink membranes lining the terminal and respiratory bronchioles and alveolar ducts. There is increased fibrous tissue around vessels. The hyaline membrane formation is an indicator of acute lung injury which results from damage to the epithelial-endothelial integrity(131) and the resulting protein deposition. Epithelial regenerative activity may be seen as cuboidal cells flatten out and spread beneath the hyaline membranes. Other epithelial cells become abnormally thick and squamous and can present a significant barrier for gas exchange.

RDS II, also known as transient tachypnea of the newborn (TTN) or wet lung, is a milder form of disease characterized by delayed clearance of fetal lung fluids but normal surfactant
levels and lung morphology. It is most frequently seen in term infants. Failure to clear lung fluid results in defective gas exchange and decreased lung compliance. This syndrome is more frequently diagnosed in infants delivered by cesarian section. Insufficient Na\(^+\) transport mechanisms may explain the delayed liquid clearance in these cases(132).

The critical role of ENaC in perinatal liquid clearance is demonstrated by several studies. The Na\(^+\) channel blocker amiloride, when instilled into the fluid filled airspaces of full term guinea pigs, caused delayed clearance of lung liquid and respiratory distress(133). Knock-out mice, deficient of \(\alpha\)ENaC, were unable to clear liquid from their airspaces and died from respiratory distress within 40 hours of birth(134). Human observations have shown that the AS drop in potential difference between the apical membrane of the nasal epithelium and the subcutaneous space (an indicator of ENaC activity) is decreased in newborn infants with RDS, and returns to normal as the infants respiratory condition improved(130). Collectively, these studies show that inadequate/immature epithelial Na\(^+\) transport, in addition to surfactant deficiency, contributes to the pathogenesis of RDS.

1.3.5.2 Pulmonary Edema

Post-natally, the airspace can again become fluid filled during the course of high pressure (cardiogenic) or high permeability (ARDS) pulmonary edema. The importance of AS epithelial Na\(^+\) transport in the clearance of alveolar pulmonary edema has been demonstrated in several animal studies(84,135). Berthiaume demonstrated that liquid clearance from anesthetized ventilated dogs and sheep was increased by \(\beta\) adrenergic agonists. The increased liquid clearance was blocked by the Na\(^+\) channel blocker amiloride. Similar results were obtained in studies of alveolar fluid clearance in the resected human lung(67). Finally, recovery from either high
permeability or high pressure pulmonary edema has been correlated with the lungs ability to concentrate its airspace fluid, presumably by actively absorbing Na\(^+\) and water(136).

1.3.5.3 Cystic Fibrosis

Cystic Fibrosis (CF) is a common, life threatening, autosomal recessive disease. The primary defect in CF is abnormal function of the cystic fibrosis transmembrane conductance regulator (CFTR), which is a cAMP-regulated chloride channel(137,138). However, a classic finding in these patients is enhanced AS-Na\(^+\) absorption and osmotically linked water absorption across the airway epithelia(139,140). The most favored hypothesis as to the mechanism leading to CF lung disease is that abnormal electrolyte and fluid transport causes an increased viscosity of CF respiratory mucous which in turn decreases airway clearance of bacteria and leads to increased infection and exacerbated CF lung disease. In normal respiratory epithelium, CFTR exerts an inhibitory effect on ENaC(114). Mutations of CFTR which occur in CF abolish CFTR-dependent down regulation of ENaC resulting in an increase in Na\(^+\) reabsorption(115,141). These electrolyte transport abnormalities have been shown by Jiang to lead to altered fluid transport by CF respiratory epithelium(139).

1.3.5.4 ENaC Dysfunction In Other Tissues

Proper function of ENaC, located at the apical membrane of the Na\(^+\)-transporting epithelia, is essential in the control of salt and fluid homeostasis in the kidney and colon. Several inherited human disorders were recently linked by genetic analysis to mutations in the ENaC subunits. In the salt-wasting disease pseudohypoaldosteronism type I (PHA-I)(142,143), there is an inactivating mutation to ENaC. With Liddle's syndrome, an inherited autosomal dominant form of human hypertension(144,145), the mutation causes increased activity of ENaC.
1.4 ASSESSMENTS OF mRNA LEVELS

Methods of RNA analysis include Northern blot hybridization, RNase protection assay (RPA), Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and In Situ Hybridization (ISH).

1.4.1 Northern Blot Analysis

Northern analysis is a traditional method of assessing mRNA expression. The technique involves three steps. First, the RNA is size separated by electrophoresis under denaturing conditions. The RNA is then transferred from the gel to a nitrocellulose or nylon membrane by upward capillary transfer. Finally, hybridization analysis of the RNA sequences is performed using a labeled cDNA or RNA probe which has a nucleic acid sequence complementary to the mRNA of interest. This method is valuable for determining tissue specificity and size of a specific mRNA transcript. There are four limitations: 1) relatively large amounts of RNA is required (10-40μg of total RNA or 1-4μg polyA+ mRNA), 2) only relative, not absolute, measurements can be made, 3) mRNA levels are averaged from all the cells contained in the original sample and 4) cell relationships are lost with this technique. Slot blots are a variation of the Northern blot technique that allows immobilization of unfractionated RNA on a membrane after which hybridization analysis is carried out as above to determine the relative abundance of the targeted mRNA sequences in the blotted samples. Although slot blots are a little more sensitive, they do not yield information on mRNA size like Northern analysis does. Densitometry is more accurate in slot blot analysis because sharp bands are generated.
1.4.2 RNase Protection Assay (RPA)

In RPA, a labeled RNA probe, complementary to part of the RNA to be analyzed, is hybridized with sample RNA. After hybridization, the mixture is treated with ribonuclease to degrade the single stranded, unhybridized probe. Labeled probe that hybridized to complementary RNA in the sample mixture will be protected from ribonuclease digestion and can be separated on a gel, visualized by autoradiography, and quantitated. The amount is directly proportional to the amount of complementary RNA in the sample mixture. The sensitivity of RPA allows one to quantitate a specific mRNA from as little as 1μg of total RNA. Difficulties estimating the relative abundance of different mRNAs using RPA or Northern hybridization relate to the different hybridization efficiencies and specific activities of individual probes.

1.4.3 RT-PCR

RT-PCR is a rapid procedure for the detection of a specific segment of RNA. This PCR technology is the most sensitive assay for rare sequences. The technique involves the synthesis of a cDNA copy of the RNA sequence of interest using reverse transcriptase. Enzymatic amplification of this cDNA is then performed using appropriate primers, a heat stable DNA polymerase, deoxyribonucleoside triphosphates (dNTPs), a buffer and salts. Theoretically, a single mRNA is sufficient for detection by this technique. Several potential downsides of this technique include 1) the risk of contamination by infinitesimal amounts of unwanted exogenous sequences, 2) the exponential nature of the reaction creates the risk of detecting mRNA's which are not present at physiologically significant levels, 3) cell relationships are lost and 4) mRNA levels are averaged from all the cells contained within the original sample.
With the careful development of controls, competitive quantitative RT-PCR can be developed and used to calculate the actual number of mRNA per cell. In this assay, cellular ENaC mRNA is compared with a known amount of added internal standard RNA. In order to control for differences in the RT step, the internal standard used should also be RNA. To control for amplification differences based on the primers, the internal standard RNA should be the same sequence as the cellular mRNA, except for a significant internal deletion, so that amplification of both RNAs may be performed with the same primers. The deletion in the standard RNA allows the DNA products to be separated on an ethidium gel and the band intensities determined using computer analysis. A set of reactions with serial dilutions of the cellular RNA are performed and the equivalence point (where the amount of cellular mRNA equals the amount of internal standard control RNA) determined. Using the linear relationship that exists between the log of the ratio (cellular mRNA / standard RNA) and the log of the input total cellular RNA, absolute determination of cellular ENaC is determined from the equivalence point. The primary advantage of this methodology is that quantitation may be performed from very small samples. Otulakowski has developed such a technique for the accurate measurement of ENaC subunit mRNA expression in very small (50ng total RNA) human epithelial samples(126). In a study of adult human nasal epithelium, Otulakowski was able to show that the amount of α, β, and γhENaC subunit mRNAs (each normalized to cytokeratin 18) respectively was; 39±4, 7.5±0.92, and 1.8±0.25 attomol/fmol cytokeratin mRNA(126).

1.4.4 In Situ Hybridization (ISH)

ISH is a valuable cytological technique that provides descriptive information about the temporal and spatial expression patterns of specific mRNA sequences in tissue sections at the
cell level. The major advantage of ISH techniques is the ability to determine which cells of mixed populations or tissues are expressing the mRNA of interest. The technique applies a similar principle to Northern blot analysis and depends on the hybridization of a specifically labeled nucleic acid probe to the target cellular RNA in individual cells on tissue sections. ISH is sensitive and can localize mRNA to a single cell(146). One limitation of this technique is the difficulty obtaining quantitative results. Both isotopic and non-isotopic probes can be used for this technique. The advantage of radiolabeled probes is their ability to detect very low levels of transcript, whereas their limitations include long exposure times, poor resolution, short half-life times, and the associated health hazards and costs related to their handling and disposal. Nonradioactive labeled probes (ie digoxigenin) are safe, significantly improve histologic resolution and shorten development times(147). The color precipitate generated as signal is stable providing a permanent ISH signal for specimens that are to be archived. Because the signal is a color precipitate, background stains can be applied to allow analysis of histologic detail concurrently with analysis of ISH signals.
1.5 IN SITU HYBRIDIZATION ANALYSES OF LUNG ENaC EXPRESSION

In the last few years, several groups have employed in situ hybridization to examine the cellular expression of α, β, and γENaC subunit mRNA in the lung (Table 1). Most of these studies have been in the rat or mouse. All reported studies, except Yue(86), have used isotopic probe labeling ³⁵S techniques. All studies, including my own, indicate that ENaC expression is localized to the epithelium of expressing tissues.

Matsushita(148) examined the distribution of the three subunit mRNAs in adult rat. αENaC was detected in the tracheal, bronchial, and bronchiolar epithelium. Based upon the αENaC distribution pattern compared to that of surfactant protein-C (SP-C) in the acinus, they concluded that αENaC was primarily expressed in the alveolus in a pattern consistent with the known distribution of ATII epithelium. β and γENaC expression was mainly expressed in the bronchi and bronchioles with minimal expression in the trachea and alveoli.

Farman(149) examined the distribution of the three subunit mRNAs in adult rat. Interestingly all three subunits were found in the trachea with considerably more α, and γ expression than β. This same α, γ and >> β expression pattern was reported in nasal and tracheal gland acini plus a subgroup of alveolar cells presumed to be ATII cells. Equivalent levels of all three subunits were found in the bronchiolar epithelium and the rat nasal gland duct epithelium. No data were reported for the expression in the bronchus. In a study of adult rats exposed to hyperoxia using a ³²P-labeled αENaC riboprobe, α subunit mRNA was localized to the bronchial epithelium and in the alveolus in an ATII cell pattern(86).
Burch (150) localized the three ENaC subunit mRNA's in adult human normal and cystic fibrosis nasal and bronchial airways. They reported that all three subunit mRNAs were expressed diffusely in the surface epithelium of nasal and bronchial epithelia and interestingly, that α, and βhENaC were also expressed in the epithelia of submucosal gland ducts and acini. In the submucosal gland acini, α and β hENaC expression appeared to be in both the serous and mucinous cells. ENaC expression in human trachea and alveoli was not studied.

In our study, the ontogeny and cellular localization of αhENaC during normal and abnormal lung development was examined using an in situ hybridization technique. Although the previous studies have examined the localization of ENaC in the lung by in situ hybridization(86,148-150), our study provides several important pieces of new information. This is the first in situ study of human lung which examines the distribution of αhENaC in the upper and lower airways, as well as the distal lung unit. This study looks at normal lung development as well as αhENaC expression in specific newborn lung disorders characterized by pulmonary hypoplasia and immaturity. Finally, we have used a non radioactive in situ hybridization technique which provides more exact cellular localization.
**TABLE 1:** Summary of published studies of the localization of ENaC subunit mRNA in adult rat and human lung by ISH

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference(s)</th>
<th>Probe</th>
<th>Nasal Epith</th>
<th>Nasal gland duct</th>
<th>Nasal gland acini</th>
<th>Trach. Epith</th>
<th>Trach gland duct</th>
<th>Trach gland acini</th>
<th>Bronch Epith</th>
<th>Bronch ile Epith</th>
<th>Alveolus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Rat</td>
<td>(149)</td>
<td>α-ENaC</td>
<td>++</td>
<td>+++</td>
<td>+/−</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>NS</td>
<td>++</td>
<td>++∗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-ENaC</td>
<td>−</td>
<td>+++</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>NS</td>
<td>++</td>
<td>++/−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>γ-ENaC</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>NS</td>
<td>++</td>
<td>++∗</td>
</tr>
<tr>
<td>Adult Rat</td>
<td>(148)</td>
<td>α-ENaC</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>+/−</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>++</td>
<td>++∗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-ENaC</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>+/−</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>++</td>
<td>++/−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>γ-ENaC</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>+/−</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>++</td>
<td>++/−</td>
</tr>
<tr>
<td>Adult Rat</td>
<td>(86)</td>
<td>α-ENaC</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>+</td>
<td>NS</td>
<td>+</td>
<td>NS</td>
<td>NS</td>
<td>+∗</td>
</tr>
<tr>
<td>Adult human</td>
<td>(150)</td>
<td>α-ENaC</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>NS</td>
<td>++</td>
<td>++</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-ENaC</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>NS</td>
<td>++</td>
<td>+</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>γ-ENaC</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>NS</td>
<td>−</td>
<td>−</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Legend:**

NS: Not Studied

†: Original authors verbally describe the relative intensity of ENaC.

(+): system approximated by D. Smith based on description.

*: distribution in a pattern suggestive of ATII cell localization
1.6 EXPERIMENTAL HYPOTHESIS AND AIM

- ENaC is known to play a critical role in the absorption of lung liquid at birth. The mature, but not immature, fetal lung is able to begin Na\(^+\) absorption in response to β agonists. The expression of the three ENaC subunits is known to be differentially regulated during development. Studies by Northern blot analysis show there is a surge in the expression of αENaC in the rat and mouse towards late gestation. Several types of newborn lung diseases are characterized by incomplete lung growth and are associated with respiratory distress syndromes. We hypothesized that the expression of αENaC varies during normal human lung development and in newborn lung disease.

The specific aims of this study were:

1) To determine the ontogeny and cellular distribution of the αENaC subunit mRNA during normal human fetal lung development using *in situ* hybridization.

2) To examine the pattern of αENaC expression in cases of oligohydramnios, CDH, and infant prematurity.

Studies were performed using archival tissues. A non-isotopic *in situ* hybridization technique was employed because it provides information about the spatial distribution of ENaC mRNA at the individual cell level in complex tissues. Cytokeratin immunohistochemistry was used to identify the lung epithelium and to differentiate cell types in the distal lung unit. Hematoxylin and eosin staining provided histologic information about structural changes in the lung parenchyma during development and in disease. Serial lung tissue sections were used for all studies.
2 MATERIALS AND METHODS

2.1 TISSUE SAMPLING

2.1.1 Sample Collection for In Situ Hybridization

Human fetal lung and kidney tissues were obtained from archival samples in The Hospital For Sick Children Pathology Department (Table 2). Tissues were routinely fixed in 10% formalin fixative and then embedded in paraffin blocks. Clinical patient data is presented in appendix A.

2.1.1.1 Normal fetal and newborn lungs

All early gestation tissues, embryonic (n=2) pseudoglandular (n=2) canalicular stage tissues (n=3), were obtained from terminated pregnancies. Saccular stage tissues (n=2) were obtained from the autopsy of stillbirths where the mother was involved in a motor vehicle accident. Alveolar stage tissues were obtained from cases of infant death following birth trauma (n=2, umbilical cord asphyxia), or late gestation placental abruption (n=2). All lung samples were evaluated by pathologists as normal for their respective gestational age. As indicated in table 2, aHENAxC expression was assessed in 13 cases of normal lung development.

TABLE 2: Samples sizes: Expression of aHENAxC in normal human fetal lung

<table>
<thead>
<tr>
<th>Number of Samples</th>
<th>Embryonic</th>
<th>Pseudoglandular</th>
<th>Canalicular</th>
<th>Saccular</th>
<th>Alveolar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Gestational Age(wks)</td>
<td>5</td>
<td>10</td>
<td>19</td>
<td>30</td>
<td>40</td>
</tr>
</tbody>
</table>
2.1.1.2 Abnormal fetal and newborn lungs.

Three preterm infants had experienced RDS after birth. Two infants succumbed to their respiratory restrictions within hours while the third died 4 days after birth secondary to intraventricular hemorrhage. Only one infant had received exogenous surfactant therapy. All lung samples were assessed by the staff pathologist who reported hyaline membrane disease with coexistant immature pulmonary parenchyma consistent with their gestational age.

The lungs of 2 infants with oligohydramnios due to renal malformations were studied. One infant had cystic renal dysplasia and the other renal agenesis (Potters Syndrome). Both infants died within hours due to respiratory insufficiency associated with pulmonary hypoplasia. Despite their full term gestation, the lungs were only approximately 45% the normal lung weight for their body size. The pathologist describes a decreased number of alveolar units and extension of airways close to the pleural surface.

Three infants with congenital diaphragmatic hernias all had extensive abdominal contents within the pleural space and severe bilateral pulmonary hypoplasia incompatible with life. Despite their full term gestation, the lungs were only 10-15% the normal lung weight for the given body size. The pathologist reports significant alveolar hypoplasia and collapse with large sized airways close to the pleura.

As indicated in Table 3 below, αhENaC expression was assessed in 8 cases of abnormal lung development.
TABLE 3: Samples sizes: Expression of αhENaC in abnormal human fetal lung

<table>
<thead>
<tr>
<th>Newborn Lung Disease</th>
<th>Prematurity</th>
<th>Oligo-hydramnios</th>
<th>Congenital Diaphragmatic Hernia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Samples</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Average Gestational Age(wks)</td>
<td>29</td>
<td>40</td>
<td>41</td>
</tr>
</tbody>
</table>

2.1.2 Sample Collection for Northern Blot Analysis

To verify the specificity of the riboprobes for the αhENaC mRNA transcript, adult human lung, kidney and liver tissues were collected from adult human surgical specimens resected for malignancy. The research protocol was approved by The Toronto Hospital Committee for Research on Human Subjects. Care was taken to sample the normal tissue from a site removed from any gross pathology. Tissues were immediately frozen in liquid nitrogen and stored until total RNA isolation was performed using Trizol RNA isolation reagent as per manufacturers instruction (GIBCO BRL, Burlington Ont).
2.2 NORTHERN BLOT HYBRIDIZATION

Total RNA (20μg) was denatured (5 minutes at 65°C) in 3X RNA sample buffer (formamide 7.2 ml, 10X morpholino propanesulfonic acid (MOPS) 1.5 ml, formaldehyde 2.6 ml, diethylpyrocarbonate (DEPC) treated water 1.8 ml, 80% sterile glycerol 1 ml) size fractionated on a 1% agarose formaldehyde-gel using a 1% MOPS buffer, transferred to a Hybond-N+ Nylon membrane (Amersham, Buckinghamshire, UK) and fixed on the membrane by UV cross-linking and baking (80°C for 10 minutes). The membrane was pre-hybridized in digoxigenin (DIG) EASY HYB solution (Boehringer Mannheim - Cat. No. 1603558) for 30 minutes at 68°C. The DIG labeled αhENaC riboprobe was denatured at 100°C for 10 minutes and filtered through a sterile 0.45μm Millex®-HA filter (Millipore corp. Bedford, MA) immediately prior to hybridization. Hybridization was performed overnight at 68°C in DIG EASY HYB with a probe concentration of 100ng/ml. The membrane was washed sequentially; twice for 5 minutes in 2x sodium chloride/sodium citrate buffer (SSC)/0.1% sodium deoxy chloride (SDS) at R/T, twice for 15 minutes in 0.1xSSC/0.1% SDS at 68°C. Chemiluminescent detection was then performed using standard Boehringer Mannheim DIG detection solutions and protocol. Briefly, the membrane was equilibrated for 5 minutes in washing buffer, and then blocked in 1xblocking buffer for 30 minutes. Following incubation with alkaline phosphatase conjugated anti-DIG-labeled antibody (Boehringer Mannheim) at 1:10,000 dilution in blocking buffer for 30 minutes, unbound antibodies were washed off twice with washing buffer for 15 minutes each. The pH of the membrane was equilibrated to 9.5 by soaking twice in detection (alkaline phosphatase) buffer for 2 minutes each. The membranes were covered with transparency and incubated with disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^3,7]decan]-4-yl)phenyl phosphate (CSPD) substrate 1:100 dilution in detection buffer for 5 minutes at R/T and then 10
minutes at 37°C. The membrane was then placed in an X-ray cassette with an autoradiographic film (Kodak X-OMAT AR) and exposed for 20 minutes. The length of the transcript detected by the αhENaC riboprobe was calculated by comparison to an RNA ladder that was run concurrently on the gel.
2.3. NON-ISOTOPIC IN SITU HYBRIDIZATION

2.3.1 αhENaC Riboprobe Synthesis using Reverse Transcriptase

Using total RNA from human lung, reverse transcription of the RNA was primed with an oligonucleotide specific for the 3' untranslated region (UTR) of the αhENaC sequence. The cDNA from this reaction was used to amplify, via PCR, a 319 bp fragment (nt 2169-2488) of the αhENaC subunit. The PCR primers were designed to incorporate an EcoRI site. The amplified fragment was cut with EcoRI, electrophoresed, and purified from a 0.7% low melting point agarose gel. Following DNA purification, the cut fragment was subcloned into pGEM α 3Zf(+/−) (Promega, Madison, WI.) in both the sense and antisense orientation. The fragment was sequenced and confirmed to correspond to the GENbank entry for αhENaC. The 3' UTR was selected to reduce nonspecific hybridization with homologous β and γ subunit mRNAs.

Plasmids containing the αhENaC probe sequence were linearized with SacI and the 3’ overhang end blunted with T4 DNA polymerase. The linearized plasmids were then separated from uncut plasmid in a 1% TAE low melting point agarose gel and isolated using a Wizard plasmid purification kit (Promega). Single stranded sense and antisense riboprobes were synthesized by in vitro transcription of 1 μg linearized vector with 20 units of T7 RNA polymerase (Boehringer, Mannheim), for 2 hours at 37° Celcius (C) using DIG-labeled uridine triphosphate (UTP) according to manufacturer’s protocol (Boehringer, Mannheim). The labeled probes were ethanol precipitated and resuspended in DEPC treated water. Riboprobes were characterized by electrophoresis on a 2% denaturing formaldehyde MOPS agarose gel, transferred to Hybond-N+ Nylon membrane (Amersham) and visualized by direct detection of DIG-UTP using the standard immunodetection procedures to ensure that the labeling reaction
resulted in the appropriate sized transcript (data not shown). The amount of labeled riboprobe was approximated by comparison with a dilution series of labeled control RNA (Boehringer Mannheim) by direct colorimetric detection.

2.3.2 *In Situ* Hybridization Protocol

Five μm tissue sections were mounted on Fisher Superfrost slides under RNase free conditions. Tissues were then deparaffinized in xylene and rehydrated through a graded series of decreasing ethanol concentrations in DEPC-treated water. Enhancement of signal was performed by transferring sections into a pressure cooker containing 1 liter of 0.1 M Tris-HCl, pH 8.0 and heating at maximum power for 18 minutes in a microwave. Following the heating step, the slides were left in the pressure cooker with the lid on for 15 minutes, and then an additional 30 minutes with the lid off. The sections were prehybridized in 50% formamide(v/v)/2xSSC (NaCl 0.30M, Na-Citrate 0.03M) in a 37°C incubator for 1 hour. Hybridization mix with a probe concentration of 500ng/ml was used for the hybridization step. The hybridization solution contained 50% deionized formamide/1xSSC/0.01M Tris-HCl pH 8.0/1xDenhardt’s/250ng/ml salmon sperm DNA/5% dextran sulfate/0.01M dithiothreitol (DTT). The probe was denatured immediately before use by heating at 80°C for 2 minutes followed by a quick chill on ice. Probe was applied to the sections and covered with an acetate coverslip. Sections were then hybridized overnight in a moist environment (2xSSC). Positive controls, consisting of kidney sections, were performed concurrently for every *in situ* hybridization experiment on lung sections. Coverslips were removed by soaking in 2xSSC at 37°C and the slides were then stringently washed sequentially in 2xSSC at 37°C, 1xSSC at 37°C, 0.5xSSC at 47°C, and 0.1xSSC at 47°C for 15 minutes each wash. All subsequent incubations were carried out at R/T. Slides were blocked in
3% blocker solution (deglycosylated casin)(Boehringer Mannheim Canada, Laval, Quebec) in 100mM maleic acid / 150mM NaCl, pH 7.5 for 30 minutes and then washed twice in TBS buffer (50 mM Tris HCl, pH 7.5/150 mM NaCl) for 2 minutes each. Following incubation with alkaline phosphatase conjugated anti-DIG-labeled antibody (Boehringer Mannheim) at 1:500 dilution in tris buffered saline (TBS) buffer for 1 hour, unbound antibodies were washed off twice with TBS buffer for 5 minutes each. The pH of the sections was equilibrated to 9.5 by soaking twice in Buffer B (0.1M Tris-HCl pH 9.5/50 mM MgCl₂/0.1M NaCl) for 10 minutes each. Sections were incubated in 375μg/ml nitroblue tetrazolium salt (NBT) and 180μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) plus 50μM levamisole (to block endogenous alkaline phosphatase) in 1 ml of Buffer B for 2 hours in the dark. Colour development was stopped by washing the slides in water for 15 minutes. Slides where then allowed to air dry and mounted with Crystal/mount™ (Biomed Corp. Foster City, CA) at 65°C and then coverslipped under Permount (Fisher Scientific, Fairlawn, New Jersey) at R/T.

Serial sections were used for sense and antisense in situ hybridizations, hematoxylin and eosin staining and cytokeratin immunohistochemistry. Photomicroscopy was performed using Kodak Ektachrome 160 ET135 film and a binocular microscope (Leitz Laborolux D) using a 35mm camera attachment (Vario-orthomat 2) photomicroscope.

2.4 CYTOKERATIN IMMUNOHISTOCHEMISTRY

Cytokeratins 8, 18, and 19 were immunolocalized on tissue sections using anti-low molecular weight (LMW) cytokeratin antibodies. Paraffin sections (5μm) were deparaffinized in xylene and rehydrated through a graded series of decreasing ethanol concentrations in distilled water. Antigen retrieval was performed in 0.5% pepsin (Sigma P7012) in 0.01N hydrochloric
acid (HCl) at 37°C for 30 minutes. Endogenous peroxidase was blocked with 1.5% (v/v)
hydrogen peroxide (H₂O₂) in absolute methanol for 30 minutes at R/T followed by washes in 2x
phosphate buffered saline (PBS)-0.07% bovine serum albumin (BSA) pH 7.2-7.4. Next,
blocking in normal horse serum (NHS) (16μl/ml) for mouse antibody in PBS-BSA was
performed for 10 minutes. Subsequently, anti-LMW cytokeratin clone CAM 5.2 (Becton
Dickinson Immunocytometry Systems, CA) was applied in PBS-BSA at 1:30 dilution for one
hour at R/T. This was followed by 3x 10 minute washes in PBS-BSA, incubation with
biotinylated horse anti-mouse IgG (5μl/ml) + 15μl NHS/ml in PBS-BSA for 30 minutes at R/T,
and a further wash in 2x PBS-BSA. Elite ABC (Avidin Biotin Complex) solution (Vector
Laboratories, Burlingame CA), containing the avidin and biotinylated horseradish peroxidase
macromolecular complex, was mixed (10μl solution A + 10 μl solution B per ml of PBS-BSA)
and allowed to sit for 10 minutes. Slides were incubated in elite ABC solution in PBS-BSA for
30 minutes at RT followed by a wash in 2x PBS-BSA and then a quick rinse in 2x Tris buffer
(0.05M, pH 7.6). The peroxidase enzyme reaction was developed in a 0.05% diaminobenzidine
tetrahydrochloride/0.01% hydrogen peroxide (DAB-H₂O₂) pH 7.2 solution (Sigma. St Louis) for
10 minutes at R/T followed by counterstaining with hematoxylin for 45 seconds, water washing,
and dehydration in a graded series of increasing ethanol concentrations and mounting with
Entellan (BDH) medium.
3 RESULTS

3.1 RIBOPROBE CHARACTERIZATION AND IN SITU HYBRIDIZATION CONTROLS

3.1.1 Genebank Blast Homology Check

No detectable homology between the selected alpha sequence and the structurally similar beta and gamma ENaC sequences was detected by GENEBANK BLAST homology check.

3.1.2 Northern Analysis Of Adult Human Lung, Kidney And Liver Total RNA Using αhENaC Riboprobe

Northern analysis of total RNA isolated from adult human lung, kidney and liver preparations (Fig. 4) demonstrated that the highest levels of αhENaC subunit mRNA were found in kidney, more weakly in lung, and was not detectable in liver. The αhENaC mRNA length, determined by comparison to an RNA ladder, was 3700bp. Similar sized αhENaC mRNA transcripts have been previously described in kidney and lung tissues in both rat(91,148) and human studies(93,150). There was no signal detected with the sense αhENaC riboprobe. The above findings suggest that the probe generated for this study is specific for the αhENaC subunit mRNA.
3.1.3 In situ hybridization: Regional αhENaC subunit mRNA in positive control kidney tissue

We performed in situ hybridization in human kidney tissue to confirm the specificity of the riboprobes (Fig 5A). It has previously been reported that the mRNA for all three ENaC subunits are co-expressed in the distal convoluted tubule and the cortical collecting duct with diminishing intensity in the outer medullary collecting ducts. In contrast, no significant ENaC expression was detected in the glomerulus, the proximal tubule and the medullary thick ascending limb of Henle’s loop(95). Using our αhENaC riboprobe and in situ hybridization protocol, αhENaC mRNA expression was detected in the distal nephron but absent in the glomerulus (Fig 5A). There was minimal or no background using the sense αhENaC riboprobe.

3.1.4 In situ hybridization: Competition for target αhENaC mRNA between labeled and unlabeled antisense riboprobes

We performed a series of in situ hybridization experiments on human fetal lung tissue using a fixed amount of DIG-labeled αhENaC antisense riboprobe and increasing amounts of unlabeled αhENaC antisense riboprobe. Since both probes compete for the same target αhENaC mRNA in the tissue sections, the DIG-labeled probe signal should be diluted out by increasing amounts of unlabeled probe. As expected, the DIG-labeled probe signal intensity decreased with increasing amounts of unlabeled probe (Fig. 5B). These results provided further evidence of the probe and in situ hybridization protocol specificity.
3.1.5 *In situ* hybridization: RNase A treatment of tissue sections before hybridization.

We pretreated lung and kidney tissue sections with RNase A (20ng/ml) for 30 minutes at 37°C followed by washes in pre-hybridization buffer solution (2 x 30 minutes). Hybridization with αhENaC antisense probe (including RNA guard protection) was then performed. Since the target mRNA is eliminated by this treatment no probe hybridization should occur. As expected no signal over background was detected (data not shown).
3.2 EXPRESSION OF αhENaC mRNA DURING NORMAL HUMAN LUNG DEVELOPMENT AND IN NEWBORN LUNG DISEASE.

3.2.1 Ontogeny and cellular distribution of the αhENaC subunit mRNA during normal human fetal lung development using in situ hybridization.

In this study, which is the first such study in the human, fetal lungs were analyzed for αhENaC mRNA expression using a non-isotopic high resolution in situ hybridization technique. Minimal signal was present in all sense in situ hybridization experiments performed. Lung developmental structural and epithelial changes were assessed using hematoxylin and eosin (H&E) staining and cytokeratin (CK) immunohistochemistry. Serial sections were employed for all studies. Results for both normal and abnormal human fetal lung studies are summarized in tables 4 and 5 respectively and described in more detail below. Reference to ‘large airways’ in this study includes all bronchi (1°, 2°, 3°), characterized by their large diameter, (>1mm in fully developed lungs), tall columnar epithelium, and/or the presence of cartilage, submucosal glands and goblet cells. The large airway designation includes the lung bud in the embryonic stage. ‘Small airways’ in this study includes all generations of bronchioles including the terminal and respiratory bronchioles. These bronchioles are characterized by a small diameter, (≤1mm in fully developed lungs), low columnar to cuboidal epithelium, and the absence of cartilage, submucosal glands, and goblet cells. The ‘distal lung unit’ designation refers to all structures distal to the small airways such as the alveolar ducts, alveolar sacs and alveoli. The characteristic distal lung unit with airspaces is not present until the late canalicular stage however the small airway endpoints in the early canalicular stage have been shown in previous studies to be precursors of
the future saccules and alveoli. The peripheral structures in the canalicular sections are thus also included in this ‘distal lung unit’ designation.

### 3.2.1.1 Embryonic stage

In the embryonic human lung studies (Fig 6), the epithelial cells at this stage are known to be filled with glycogen. The αhENaC mRNA was detected by ISH and was uniformly distributed throughout all the epithelial cells with a trend for localization at in the basal or apical aspect of the cell. The appearance of the αhENaC mRNA may reflect the minimal amount of cytoplasm that exists at this stage and the space occupying effect of intracellular glycogen. The H&E stains demonstrate that the lung consists of solitary bud lined by undifferentiated pseudostatified epithelium. CK immunohistochemistry identified the epithelium lining the lung bud (Fig 6).

### 3.2.1.2 Pseudoglandular stage

In the pseudoglandular stage human lung studies (Fig 7), airway development is evident, however, the epithelium remains undifferentiated. The αhENaC antisense signal persisted in a similar uniform pattern in the large and small airway epithelium.

### 3.2.1.3 Canalicular stage

In the canalicular stage human lung studies (Fig 8), the large airway epithelium morphologically resembles the postnatal lung with the appearance of ciliated and secretory cells. In the small airways, the transition from columnar to cuboidal cells demarcates the future respiratory portion of the lung. The αhENaC antisense signal was present throughout the airway epithelium and notably absent from the vascular tissues. The (proximal) large, more differentiated airway epithelium exhibited a more patchy αhENaC antisense signal compared with the signal in the distal (small) airway epithelium.
3.2.1.4 Saccular stage

In the saccular stage human lung studies (Fig 9), the interstitial tissue has greatly decreased and saccules are clearly present. The (proximal) large airway epithelium is well differentiated. The cuboidal epithelium lining the distal lung unit has begun to flatten forming an early air-blood interface. Cuboidal, ATII pneumocytes are detected. Flattening of cuboidal epithelial cells may also represent the differentiation of ATII into ATI cells. Submucosal glands are present. The αhENaC antisense signal was present in most of the superficial airway epithelia and in the serous cells of the submucosal gland acini. The αhENaC antisense signal in the distal lung unit was localized to the corner cells in an ATII cell pattern.

3.2.1.5 Alveolar stage

In the alveolar stage human lung studies (Fig 10), the alveolus is thin walled and the alveolar epithelial differentiation is advanced. Submucosal glands are very prevalent in the large airways. The αhENaC antisense signal in all airway epithelium and the serous cells of the submucosal gland acini continued to be strong. Alveolar expression of αhENaC antisense signal was consistent with an ATII cell pattern.
TABLE 4: Ontogeny and localization of αhENaC mRNA expression in developing human lung by ISH

<table>
<thead>
<tr>
<th>α-hENaC</th>
<th>Gestational Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Embryonic</td>
</tr>
<tr>
<td>• large airway epithelium</td>
<td>+</td>
</tr>
<tr>
<td>• submucosal gland acini</td>
<td>NP</td>
</tr>
<tr>
<td>• small airway epithelium</td>
<td>NP</td>
</tr>
<tr>
<td>• distal lung unit epithelium</td>
<td>NP</td>
</tr>
</tbody>
</table>

Legend
*: distribution in a pattern suggestive of ATII cell localization
**: signal in serous cells but not mucous cells
***: patchy signal
NP: not present

3.2.2 Pattern of αhENaC expression in cases of oligohydramnios, CDH, and infant prematurity

3.2.2.1 Oligohydramnios

In the studies of pulmonary hypoplasia associated with oligohydramnios there was histologic evidence of a retarded alveolar development (Fig 11). The interstitium is much thicker and the mesenchymal cells more abundant than what is seen in normal lung at term gestation. Despite the morphologic immaturity, the αhENaC antisense signal was observed in the corner cells of the distal lung unit epithelium and the superficial airway epithelium.

3.2.2.2 Congenital Diaphragmatic Hernia

In the studies of pulmonary hypoplasia associated with CHD, the alveoli are hypoplastic and collapsed (Fig 12). Mesenchymal cells were more abundant in these lungs compared to normal lungs at the same term gestation. The αhENaC antisense signal is distributed in a patchy
fashion throughout the large airway epithelium. Small discrete areas of labeling are present within the collapsed alveolar parenchyma; this is speculated to correspond to the signal seen in the corner cells of other expanded distal lung units.

3.2.2.3 Infant Prematurity

In studies of pulmonary immaturity secondary to infant prematurity, the interstitial thickness and alveolar complexity are compatible with that of the normal fetal lung at the saccular stage of development (Fig 13). αhENaC antisense signal is patchy in the large airway epithelium. The αhENaC antisense signal in the distal lung unit is localized to the corner cells in an ATII cell pattern.

TABLE 5: Localization of αhENaC mRNA in newborn lung disease by ISH

<table>
<thead>
<tr>
<th>α-hENaC</th>
<th>Newborn Lung Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prematurity</td>
</tr>
<tr>
<td>large airway epithelium</td>
<td>+***</td>
</tr>
<tr>
<td>Submucosal gland acini</td>
<td>+**</td>
</tr>
<tr>
<td>small airway epithelium</td>
<td>+</td>
</tr>
<tr>
<td>distal lung unit epithelium</td>
<td>+*</td>
</tr>
</tbody>
</table>

Legend
*: distribution in a pattern suggestive of ATII cell localization
**: signal in serous cells but not mucous cells
***: patchy signal
Figure 4. Northern Blot of adult human kidney, lung and liver total RNA (20μg) hybridized with αhENaC antisense or sense riboprobes. A 3,700bp transcript, consistent with αhENaC, is detected with the antisense probe in the kidney and lung while, as expected, no signal is detected with the sense probe.
Figure 5A. In situ hybridization of αhENaC mRNA in kidney. Antisense (AS) and sense (S) images are shown for representative distal nephron (←) and glomerular (<> regions. αhENaC antisense signal is shown over the distal nephron epithelium while the signal in the glomerulus is minimal.

Figure 5B. Cold competition in situ hybridization of αhENaC mRNA in fetal lung. Hybridization was performed with a fixed amount of DIG-labeled αhENaC riboprobe and increasing amounts of unlabeled αhENaC riboprobe. The ratio of DIG:unlabeled probe is shown (1:0), (1:1), (1:5). αhENaC antisense signal decreases with increasing amounts of unlabeled probe.
Figure 6. In situ hybridization of αhENaC mRNA in embryonic human fetal lung at 5 weeks gestation. Antisense (AS) and sense (S) images are shown on the top. Cytokeratin (CK) immunohistochemistry and hematoxylin and eosin (H&E) stain on the bottom. A strong and uniform αhENaC hybridization signal is shown over the lung bud epithelium.
Figure 7. *In situ* hybridization of αhENaC mRNA in pseudoglandular human fetal lung section at 11 weeks gestation. Antisense (AS) and sense (S) images are shown. A strong and uniform αhENaC hybridization signal is shown over the airway epithelium.
Figure 8. *In situ* hybridization of αhENaC mRNA in canalicular lung at 18 weeks gestation. Antisense (AS) and sense (S) images plus cytokeratin (CK) immunohistochemistry are shown for representative (I) small, (II) medium and (III) large airways.

A uniform antisense αhENaC signal is shown in the small airway epithelium and a more patchy signal is seen in the larger, better differentiated airway epithelium. No signal is seen in the arterial structures (a).
Figure 9. *In situ* hybridization of αhENaC mRNA in saccular human lung at 32 weeks gestation. Antisense (AS), sense (S) images plus cytokeratin (CK) immunohistochemistry are shown for representative saccular and large airway regions. αhENaC signal is shown in the corners of the saccular regions in an alveolar type II pattern, in the superficial epithelium of the large airways and in the serous cells of the submucosal glands.
Figure 10. *In situ* hybridization of αENaC mRNA in alveolar human fetal lung at 41 weeks gestation. Antisense (AS) and sense (S) images plus cytokeratin (CK) immunohistochemistry are shown for representative (I) alveolar and (II) large airway regions. αENaC antisense signal is shown in the alveolar region in an alveolar type II pattern (††), in the superficial epithelium of the large airway and in the serous cells of the submucosal glands (¬¬¬).
Figure 11. *In situ* hybridization of αhENaC mRNA in a lung section from a term infant with Potter's Syndrome. Antisense (AS) and sense (S) images plus cyto-keratin (CK) immunohistochemistry are shown for representative (I) distal lung unit and (II) large airway regions. The retarded alveolar development is shown. αhENaC antisense signal is shown in the corner cells of the distal lung unit and the superficial epithelium of the large airway.
Figure 12. *In situ* hybridization of αhENaC mRNA in a lung section from a term infant with congenital diaphragmatic hernia. Antisense (AS) and sense (S) images plus cytokeratin (CK) immunohistochemistry are shown for representative (I) distal lung unit and (II) large airway regions. The collapsed distal lung unit is shown. αhENaC antisense signal is present within the collapsed parenchyma (->) and in the superficial airway epithelium.
Figure 13. *In situ* hybridization of αhENaC mRNA in a lung section from a premature infant born at 28 weeks gestational age. Antisense (AS) and sense (S) images plus cytokeratin (CK) immunohistochemistry are shown for representative (I) distal lung unit and (II) large airway regions. The histologic immaturity of the distal lung unit is shown. αhENaC antisense signal is shown in the distal lung unit and the superficial epithelium of the large airway.
4 DISCUSSION

In our study, the ontogeny and cellular localization of αhENaC mRNA during normal and abnormal lung development was examined using a non-radioactive in situ hybridization technique. Our results show that αhENaC subunit mRNA is expressed in the epithelium from a very early stage of lung development. Expression is found in the epithelium of conductive and respiratory airways, serous cells of submucosal glands, and in the distal lung unit in a pattern consistent with the known distribution of ATII epithelium. Expression of αhENaC mRNA is preserved in newborn lung diseases associated with respiratory distress syndrome. These were unexpected results.

The detection of αhENaC subunit mRNA during the embryonic stage of lung development contrasts with previous studies of ENaC ontogeny in the rat and mouse(78,120,125) using other molecular techniques. αENaC subunit mRNA is first detected by Northern analysis in the canalicular stage of lung development and levels surge with advancing gestation. β and γ ENaC subunit mRNAs are expressed at low levels in late gestation and only increase postnatally. Human ontogeny studies are less complete; however, studies using Northern analysis(93) and RPA(80), that suggest ENaC expression is detectable at least as early as the second trimester. In our study, it is interesting that we detect αhENaC expression during the embryonic stage of lung development. One possible explanation for the earlier detection of αhENaC in our study is the increased sensitivity of in situ hybridization compared to Northern analysis. During in situ hybridization, epithelial and mesenchymal cell compartments remain distinct and the technique is sensitive enough to detect the amount of RNA present in a single cell. In contrast, the RNA collected for Northern analysis is averaged from both mesenchymal and epithelial cell origins.
Undetectable levels of αhENaC mRNA (expressed in the epithelial cells) in early gestational stages likely relates to the relative abundance of mesenchymal cells which do not express αhENaC mRNA. As gestation proceeds, the epithelial cell contribution to the overall lung tissue rapidly increases, which may explain why ENaC is more readily detected by Northern analysis at these stages. This mesenchyme ‘dampening’ effect has been previously described in a study of Na⁺-K⁺ATPase expression in the normal and abnormal developing rat lung(152). Another possible explanation for our findings is that species differences exist and that ENaC expression is earlier in humans compared to rodents. Between species there are important differences regarding the time limits of various developmental stages. In general, lung development in the human is more advanced by end gestation compared to the rat where almost all alveolar development occurs post natally(2,4).

Initial ontogeny studies of ENaC by Northern analysis(78,120) had lead us to believe that ENaC mRNA expression is ‘linked’ to the maturation of the respiratory epithelium. The finding that ENaC mRNA is expressed from early gestation suggests that these two processes are separate events. Precedent for both linked and unlinked expression of alveolar epithelial cell proteins exists within the surfactant system. SP-C is expression begins early in human fetal lung development(8) and increases gradually throughout development. In contrast, SP-A expression is low before 34 weeks gestation(153) and increases rapidly during late gestation at a time of dramatic pulmonary epithelial cell (ATII) differentiation and maturation (surfactant phospholipids)(13,154). Interestingly, Coleman et. al. show that levels of the constitutively expressed SP-C are not affected in mouse models of pulmonary hypoplasia and epithelial immaturity secondary to CDH(42). In contrast, the developmentally regulated SP-A mRNA
expression is significantly reduced in the same model of pulmonary hypoplasia and in humans with CDH(42,153). Our findings of αENaC mRNA expression from very early in lung development are similar to the ontogeny of surfactant protein C (SP-C). Similar to Coleman’s findings for SP-C expression, we do not detect a change in the expression of αENaC mRNA in our cases of lung hypoplasia and pulmonary immaturity. We speculate that αENaC mRNA is constitutively expressed throughout lung development and is separate (unlinked) to the process of pulmonary epithelial maturation and differentiation.

The cellular localization of ENaC has previously been studied in the adult rat and mouse(86,148,149) and human(150) using radioactive probes. In the rodent, αENaC mRNA expression is described as diffuse in the epithelia of the trachea, bronchi, and bronchioles and in nasal and tracheal submucosal glands. In the alveoli, the use of radioactive probes prevents exact cellular localization, however αENaC antisense signal has been reported to be in a pattern suggestive of the known distribution of ATII epithelium. In the single previous human study by Burch(150), in situ hybridization analysis was limited to nasal cavity and larger bronchi. αhENaC expression was detected in the superficial epithelium of the upper airways and in submucosal gland ductular and acinar epithelium. It was suggested (in contrast with the serous cell-specific CFTR expression in gland acini), that αhENaC is expressed in both serous and mucous cells in the gland acini. In our study, αhENaC expression in the full term fetal lung was consistent with that reported for adult rat and human with a couple of minor exceptions. Similar to these studies, αhENaC expression was detected within the submucosal gland acini, however unlike Burch’s adult human study(150), expression in our study of fetal human lung was limited to the serous cells while mucous cells were negative. In the distal lung unit, the antisense signal
was in a pattern consistent with the known distribution of ATII epithelium. The resolution in these regions using the non-radioactive probes was excellent allowing us to be quite confident about localizing the signal. It is possible that the difference between these two studies is not a resolution issue but that the expression of αhENaC in the submucosal gland acini differs in fetal vs adult human. Collectively, these results are consistent with an important role for ENaC in the absorption of Na⁺ and fluid across the pulmonary epithelium in all regions of the lung.

The alveolus has long been assumed to be the quantitatively important site of fluid absorption based on the huge surface area represented by both ATI and ATII cells in this region and the relatively surface area represented by the other lung regions. In the adult human lung, the alveolar surface area (80-140 m²) is massive compared to that of the bronchioles (2.2 m²)(60). My results suggest that only the ATII cells express ENaC. Crapo has shown that the ATII cell covers only 7% of the alveolar epithelial surface area while the ATI cells cover the remaining 93%(7). The absence of ATI localized ENaC mRNA diminishes the ‘effective’ surface area in the alveolus expressing ENaC (5-10 m²). The ENaC expression in the smaller airways appears to be relatively diffuse implicating a role for all the epithelial cells in this region (primarily Clara and ciliated cells). The small airways may therefore represent a quantitatively more important site of Na⁺ and fluid absorption than previously predicted. Surface area determinations for the different regions of the fetal human lung do not exist and are subject to the ongoing changes inherent to lung development. The small airways however, represent a relatively greater percent of the total surface area in the incompletely developed lung as airway development precedes that of the distal lung unit.
Our study shows αENaC subunit mRNA is present in newborn lung diseases associated with respiratory distress syndrome. These results, at first glance, contrary to previous observations that Na⁺ channel transport and function is deficient or ineffective in newborns with hypoplastic and/or immature lungs characterized by wet lung. There are several possible explanations for these findings. First, it is well known that the functional sodium channel is composed of three subunits α, β, and γ(91). It is possible that in cases of incomplete lung growth, expression of one of the other two subunits is deficient and causes the impaired Na⁺/fluid absorption seen in these cases. The β and γ subunits are known to be required for maximal functional expression(91) in a *Xenopus oocyte* model. Transcriptional regulation of β and γ, but not αENaC subunits in kidney cell culture by AVP has been shown to be a key regulatory mechanism(155).

It is also recognized that a direct relationship between mRNA and functional protein cannot be predicted. It is possible that ENaC function is regulated by post-transcriptional mechanisms. Alternate potential sites of regulation include; α, β, γ subunit translation, ENaC subunit assembly and stability, ENaC transport to the membrane and/or control of ENaC function at the membrane surface. Translational regulation of mRNAs is a well known phenomenon in development(156). It may involve the interaction of regulatory proteins with the 5’ or 3’ untranslated regions (UTR) of the mRNAs or sequestration of mRNAs in messenger ribonucleoprotein particles inaccessible to translation. The only evidence to date of possible similar translational regulation of ENaC comes from preliminary studies in our lab where the presence of a long 5’UTR in αENaC mRNA has been noted to be more prevalent in fetal lung compared with adult lung or kidney(157). mRNAs with long G-C rich 5’UTR are invariably
translated less efficiently than those with short 5' UTRs lacking secondary structure or upstream AUG codons. In adult tissues where the 5'UTR is short, this translational regulation may be removed. The activation (at term) of proteins (initiation factors) capable of releasing this translational suppression would be one way to rapidly upregulate ENaC function at birth. This type of translational regulation would permit the lung to respond more rapidly than by de novo transcription of the gene.

In vitro studies suggest the γ subunit plays an important role in ENaC subunit assembly(158). Both β and γ subunits have been implicated in the regulation of the transport of ENaC to the membrane surface(118,159,160) in *Xenopus oocyte* systems.

Finally, it is possible that Na+ channels other than ENaC play an important role in fluid transport in the human fetal lung and that we failed to detect a significant abnormality in newborn lung disease mRNA states because we were not looking at the most important channel. There is data suggesting the presence of more than one Na+ channel in the lung epithelium(161). The overall contribution of the different channels to Na+/fluid transport remains to be determined.

There are several limitations to the present study that may serve as the basis of some future studies. In the present study the individual group sample sizes are small (n = 2-4) due to limited tissue availability. The finding that the *in situ* signal is constant throughout gestation provides support to our conclusions. Archival samples were used in this study as this greatly increased tissue sampling opportunities. The one disadvantage of archival tissues is the increased risk of tissue degradation. In this study we used gross histologic observations (ie the lack of epithelial sloughing) and cytokeratin immunohistochemistry (to verify protein signals
were still intact) to assess tissue preservation. Additional ways to more directly evaluate mRNA preservation include in situ analysis of ribosomal RNA or a housekeeping gene such as actin.

The present study has been limited to the analysis of the alpha hENaC subunit even though the functional protein consists of alpha, beta and gamma subunits. Future studies that will advance our knowledge of transcriptional regulation of ENaC include similar mRNA analyses for β and γ ENaC subunits. As both secretory and absorptive processes contribute to the maintenance of lung fluid volume, *in situ* hybridization studies of CFTR would also be an interesting extension of this study.

To fully understand the developmental expression of ENaC, it would be of particular value to examine protein expression in the same model. Unfortunately presently available antibodies for ENaC detect multiple nonspecific bands on Western analysis of native tissues and therefore are not considered reliable. Future studies should include the generation of sensitive and specific ENaC antibodies.

Information is still lacking regarding what is the relative contribution of ‘individual’ cell types to Na\(^+\) transport and which is the most important region of the lung for overall fluid absorption. The extension of our present ISH technique to immunohistochemistry work should include double labeling techniques with specific cell markers. In this study, alpha hENaC signal was found primarily in the epithelial structures. In some sections however, nuclear signal is present in the mesenchymal cells. This likely represents nonspecific background signal or hybridization of the riboprobe to DNA in the nucleus. To date there is no evidence that ENaC has a functional role in these mesenchymal tissues however this cannot be proven or disregarded by these studies.
APPENDIX A: Patient Data:

<table>
<thead>
<tr>
<th>Lung Samples:</th>
<th>Gestational age: (weeks)</th>
<th>Cause of Death:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>5</td>
<td>Terminated pregnancy: social reasons</td>
</tr>
<tr>
<td>#2</td>
<td>5</td>
<td>Terminated pregnancy: social reasons</td>
</tr>
<tr>
<td>Pseudoglandular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>10</td>
<td>Terminated pregnancy: social reasons</td>
</tr>
<tr>
<td>#2</td>
<td>10</td>
<td>Terminated pregnancy: social reasons</td>
</tr>
<tr>
<td>Canalicular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>20</td>
<td>Terminated pregnancy: Nuchal Hygroma</td>
</tr>
<tr>
<td>#2</td>
<td>19</td>
<td>Terminated pregnancy: Hydrocephalus</td>
</tr>
<tr>
<td>#3</td>
<td>19</td>
<td>Terminated pregnancy: social reasons</td>
</tr>
<tr>
<td>Saccular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>28</td>
<td>Stillbirth: maternal involvement in a motor vehicle accident (MVA)</td>
</tr>
<tr>
<td>#2</td>
<td>32</td>
<td>Stillbirth: maternal involvement in a MVA</td>
</tr>
<tr>
<td>Alveolar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>41</td>
<td>Birth trauma, death at 5 hours of age</td>
</tr>
<tr>
<td>#2</td>
<td>38</td>
<td>Stillbirth: maternal involvement in MVA</td>
</tr>
<tr>
<td>#3</td>
<td>40</td>
<td>Intrauterine asphyxia by umbilical cord</td>
</tr>
<tr>
<td>#4</td>
<td>40</td>
<td>Infanticide (drowning at birth)</td>
</tr>
<tr>
<td>Oligohydramnios</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>40</td>
<td>Bilateral cystic renal dysplasia death at 2 hours of age (respiratory failure)</td>
</tr>
<tr>
<td>#2</td>
<td>40</td>
<td>Bilateral cystic renal dysplasia (respiratory failure)</td>
</tr>
<tr>
<td>CDH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>42</td>
<td>Bilateral diaphragmatic hernia death at 1.5 hours of age (respiratory failure)</td>
</tr>
<tr>
<td>#2</td>
<td>41</td>
<td>Rt diaphragmatic hernia death at 2 hours of age (respiratory failure)</td>
</tr>
<tr>
<td>#3</td>
<td>41</td>
<td>Lt diaphragmatic hernia death at 1 hour of age (respiratory failure)</td>
</tr>
<tr>
<td>Infant prematurity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>26</td>
<td>prematurity death at 4 days of age (ventricular hemorrhage)</td>
</tr>
<tr>
<td>#2</td>
<td>28</td>
<td>prematurity death at 6 hours of age (respiratory failure)</td>
</tr>
<tr>
<td>#3</td>
<td>32</td>
<td>prematurity death at 1 hour at age (respiratory failure)</td>
</tr>
</tbody>
</table>
6 REFERENCES


cystic fibrosis transmembrane conductance regulator functional expression in isolated rat


Expression of CFTR and a cAMP-stimulated chloride secretory current in cultured human

1991. Nucleoside triphosphates are required to open the CFTR chloride channel. Cell
67:775-784.


261:C555-C564.


nor Na-glucose symport inhibitors slow neonatal lung water clearance. Am. J. Respir. Cell

transvascular water absorption: a study on isolated rat lung. J. Physiol. (Lond.) 384:311-
324.

64. O'Brodovich, H. M. 1995. The role of active Na+ transport by lung epithelium in the clearance


tissues. II. Subcellular distribution in respiratory and glandular tissues of rat. Am. J. Physiol.
273:C1549-C1561.


