EPITHELIAL-MESENCHYMAL INTERACTIONS AND THE NOTCH PATHWAY IN MAMMALIAN LUNG DEVELOPMENT

by

Julie F. Deimling

A thesis submitted with the requirements for the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

© Copyright by J. F. Deimling 1999
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.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
ABSTRACT

Epithelial-Mesenchymal Interactions and the Notch Pathway in Mammalian Lung Development

M.Sc., 1999

Julie F. Deimling

Department of Laboratory Medicine and Pathobiology

University of Toronto

Interactions between the pulmonary epithelium and its surrounding mesenchyme are critical for regulating developmental processes during early lung development. However, less is known about the importance of these tissue interactions during late lung development. In this study we employed a novel cell recombination model to examine the role of epithelial-mesenchymal interactions in both lung epithelial morphogenesis and cellular differentiation during late fetal gestation. Using tissue specific marker antibodies, immunohistochemical analysis revealed that, even during late lung development, lung epithelial cells could be reorganized into different morphological patterns depending on the type of mesenchymal tissue present. Moreover, *in situ* hybridization analysis of Surfactant Protein-C, Surfactant Protein-B and Clara Cell 10 kDa Secretory Protein gene expression revealed that interactions between lung epithelium and specifically lung mesenchyme are essential for cell differentiation and the maintenance of the alveolar type II cell phenotype during late lung development. These results suggest that at late fetal gestation, lung epithelial cells are not fully committed to a specific phenotype and still have the plasticity to respond to various signals originating from the surrounding mesenchyme.
We have also begun to investigate a potential role for the Notch signaling pathway in mammalian lung development. The Notch pathway is an evolutionarily conserved mechanism for regulating cell differentiation in populations of equipotent cells. Since there must be mechanisms which control the specification of lung epithelial cell fate during lung development, we analyzed the spatial and temporal mRNA expression patterns of some of the Notch receptors, ligands and various pathway mediators, by non-radioactive in situ hybridization. These results show strong mRNA expression of a number of the Notch pathway components and suggest a potential role for the Notch pathway throughout lung development. In addition, a comparison between the mRNA expression of the Notch signaling molecules and lung epithelial cell specific markers, demonstrated that the expression of SP-C, SP-B and CC10 increase at approximately the same time that Notch pathway transcripts decrease in the developing lung. These results further suggest a role for the Notch pathway in mammalian lung development and possibly a specific role in regulating lung epithelial cell differentiation.
ACKNOWLEDGEMENTS

Throughout the last few years I have had the privilege of working with a number of highly skilled and knowledgeable scientists, colleagues and friends. Each and every one of these people has contributed to making my time in graduate school a truly valuable and memorable experience.

First, I would like to extend many thanks to my supervisor, Dr. Martin Post. His scientific enthusiasm, guidance and skills as a principle investigator have been truly exemplary. I am also very grateful to my thesis committee members, Dr. Keith Tanswell and Dr. Sean Egan, whose comments and suggestions have been very helpful in the completion of these studies.

In addition, throughout the last two years I have been very fortunate to work with a number of wonderful laboratory colleagues whose friendship, intellectual stimulation and technical assistance has made my time in the laboratory so much more enjoyable. In particular, I would like to thank Jinxia Wang, Jason Liu, Irene Tseu and Maciek Kulizewski for their patience, advice and help in teaching me new laboratory techniques. I also thank Dr. David Koehler, Dr. Sharon Unger, Dr. Richard Keijzer, Ross Ridsdale and Nicholas Cartel for always keeping me amused and making sure that I laugh at least a dozen times a day!

Finally, I would like to acknowledge the financial support received from the Hospital for Sick Children and the University of Toronto.
TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS v
LIST OF TABLES vii
LIST OF FIGURES viii
LIST OF ABBREVIATIONS x

CHAPTER 1. INTRODUCTION

1.1 Lung Organogenesis 1

1.2 Epithelial-Mesenchymal Interactions in Lung Development 4

1.3 Transcription Factors in Lung Development 8

1.4 Morphogens in Lung Development 11

1.5 Growth Factors in Lung Development 12

1.6 The Notch Signaling Pathway 14

1.7 Elements of Notch Signaling 14

1.8 Rationale for Studying Notch Signaling in Lung Development 21

1.9 Aims of Study 23

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials 25

2.2 Primary Cell Culture: Isolation and separation of fetal day-13 cells 26
CHAPTER 2. METHODS

2.3 Primary Cell Culture: Isolation and separation of fetal day-19 and 21-cells

2.4 Primary Epithelial Cell Transfection

2.5 Generation of Homotypic and Heterotypic Cell Recombinants

2.6 Reporter Gene Assays

2.7 Synthesis of Non-radioactive Riboprobes

2.8 Non-radioactive In Situ Hybridization

2.9 Immunohistochemistry

CHAPTER 3. RESULTS

3.1 Tissue Morphology in Early Gestation Recombinants

3.2 Tissue Morphology in Late Gestation Recombinants

3.3 Lung Epithelial Cell Differentiation in Early and Late Gestation Recombinants

3.4 Temporal and Spatial Expression Patterns of the Notch Pathway Components

CHAPTER 4. DISCUSSION

4.1 Epithelial-Mesenchymal Interactions in Lung Epithelial Morphogenesis and Differentiation

4.2 The Notch Pathway in Mammalian Lung Development

REFERENCES
LIST OF TABLES

Table 1. The Stages of Mammalian Lung Development ........................................ 1

Table 2. Nomenclature for the Proteins in the Notch Signaling Pathway .............. 17

Table 3. Descriptions of Riboprobes used for In Situ Hybridization .................. 32

Table 4. The In Vitro Transcription Reaction .................................................. 33
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>Diagrammatic model of the Notch signaling pathway</td>
<td>20</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Immunostaining to determine tissue morphology of homotypic and heterotypic 13-day cell recombinants</td>
<td>38</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Immunostaining to determine tissue morphology of homotypic and heterotypic 19-day recombinants</td>
<td>42</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>mRNA expression patterns of SP-C, SP-B and CC10 in the developing rat lung</td>
<td>45</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>mRNA expression of the lung epithelial cell markers, SP-C and CC10 in 13-day homotypic and heterotypic recombinants</td>
<td>47</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>mRNA expression of the bronchiolar epithelial cell marker, CC10, in 19-day homotypic and heterotypic recombinants</td>
<td>50</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>mRNA expression of the lung epithelial cell markers, SP-C and SP-B, in 19-day homotypic and heterotypic recombinants</td>
<td>52</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>mRNA expression of the bronchiolar epithelial cell marker, CC10, in 21-day homotypic and heterotypic recombinants</td>
<td>54</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>mRNA expression of the lung epithelial cell markers, SP-C and SP-B, in 21-day homotypic and heterotypic recombinants</td>
<td>56</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>SP-C promoter activity in epithelial cells of homotypic and heterotypic 19-day recombinants</td>
<td>59</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>Spatial and temporal mRNA expression patterns of the Notch receptors in the developing rat lung</td>
<td>62</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>Spatial and temporal mRNA expression patterns of the Notch ligands in the developing rat lung</td>
<td>64</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>Jagged-1 and Jagged-2 protein localization</td>
<td>66</td>
</tr>
</tbody>
</table>
Figure 14. Spatial and temporal expression patterns of the Mammalian Fringe homologues in the developing rat lung.

Figure 15. A comparison of the mRNA expression patterns of the downstream Notch pathway mediator, Hes5, and lung epithelial cell markers, SP-C and CC10.
LIST OF ABBREVIATIONS

BSA: bovine serum albumin
CC10: clara cell (10 kDa) secretory protein
DEPC: diethyl pyrocarbonate
DIG: digoxigenin
DMEM: dulbecco’s modified eagle medium
FBS: fetal bovine serum
FGF: fibroblast growth factor
HBSS(-): hank’s balanced salt solution
ICD: intracellular domain
MEM: eagle’s minimal essential medium
MOPS: morpholino propanesulfonic acid
NGS: normal goat serum
PBS: phosphate buffered saline
PCR: polymerase chain reaction
SP-B: pulmonary surfactant protein-B
SP-C: pulmonary surfactant protein-C
SSC: sodium chloride / sodium citrate buffer
TBE: tris borate EDTA buffer
TBS: tris buffered saline
CHAPTER 1. INTRODUCTION

1.1 Lung Organogenesis

Development of the fetal lung is divided into five distinct stages: embryonic, pseudoglandular, canalicular, saccular and alveolar. Although there is considerable overlap between these stages, each is marked by specific anatomical and morphological characteristics. The early stages of lung development primarily involve branching morphogenesis and airway development, whereas the later stages are active phases of pulmonary cell differentiation and maturation of the gas exchange structures of the lung. The different stages of mouse (term = 19 days), rat (term = 22 days) and human (term = 40 weeks) lung development are described in the following table (reviewed by Burri, 1997).

Table 1. The Stages of Mammalian Lung Development

<table>
<thead>
<tr>
<th>Stage</th>
<th>Gestational Age</th>
<th>Major Events</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
<td>Rat</td>
</tr>
<tr>
<td>Embryonic</td>
<td>26d-6w</td>
<td>12-14d</td>
</tr>
<tr>
<td>Pseudoglandular</td>
<td>6-16w</td>
<td>15-18d</td>
</tr>
<tr>
<td>Canalicular</td>
<td>16-28w</td>
<td>19-20d</td>
</tr>
<tr>
<td>Saccular</td>
<td>26-36w</td>
<td>21d-post-natal</td>
</tr>
<tr>
<td>Alveolar</td>
<td>36w-post-natal</td>
<td>post-natal</td>
</tr>
</tbody>
</table>

During the embryonic stage of rat lung development, the respiratory system first develops as paired endodermal buds in the ventral half of the primitive foregut, just proximal to the
developing stomach at approximately 10 days of gestation. As the two buds elongate into the surrounding splanchnic mesenchyme forming the left and right primary bronchi, the tubular foregut simultaneously begins to pinch into two separate tubes; the dorsal esophagus and the ventral trachea. Each primary bronchus then begins to divide monopodially giving rise to stem or lobar buds that correspond to the mature lung lobes. In the rodent, the right lung characteristically has four major lobes and the left lung consists of only one smaller lobe. These epithelial lobar buds are destined to undergo successive branching and, in conjunction with the surrounding mesenchyme, will give rise to the definitive pulmonary lobes that characterize adult lung organization. During this stage of development the primitive lung buds are lined by an endodermally derived pluripotent epithelium that will later differentiate into both the airway epithelium and the specialized alveolar epithelium. The lung buds grow into the surrounding mesodermal tissue which will later provide the vascular system, cartilage, smooth muscle and other connective tissue of the mature lung.

During the pseudoglandular period of rat lung development the conducting airways are formed by repeated dichotomous branching of the epithelial tubules. During this stage, the lung has a characteristic glandular appearance created by its system of branched tubules surrounded by abundant mesenchyme. These tubules are lined with columnar epithelial cells in the proximal airway generations and, as branching proceeds, the height of these lining cells decreases finally to more cuboidal shaped cells in the terminal branches. In addition, these epithelial cells contain large amounts of glycogen, which provides the fuel for later cytodifferentiation. At the end of the pseudoglandular stage, formation of the tracheobronchial tree is nearing completion.

The canalicular phase of rat lung development involves the formation and vascularization of the primitive gas exchange structures. The future air spaces begin to expand and the cuboidal epithelial cells lining these structures begin to flatten in preparation for their function as the gas
exchange surface of the mature lung. As the canaliculor stage progresses the amount of interstitial tissue decreases and capillary growth beneath the epithelial cells which line the primitive gas-exchange channels begins. Capillary growth is soon followed by initiation of epithelial cell differentiation towards alveolar type I and alveolar type II pneumocytes. By the end of this stage of development the fetal lung has developed to a point where gas exchange is possible.

The saccular stage of rat lung development involves a marked decrease of interstitial tissue and a substantial thinning of the airspace walls. These events serve to facilitate a close link between pulmonary capillaries and the alveolar epithelium. This period is also marked by subdivision of the distal airspace regions into saccules. Although alveolar epithelial cells morphologically resemble mature cells of the adult lung, alveolar type II cells remain biochemically immature.

Finally, the alveolar stage of rat lung development occurs postnatally and involves further division and development of the epithelial lined saccules into mature alveoli. These structures are thin walled and lined with extremely thin alveolar type I cells as well as more cuboidal alveolar type II cells. The type I cell is responsible for gas exchange and the now morphologically and biochemically mature type II cell is responsible for the production and secretion of pulmonary surfactant into the alveolar airspace. There is still considerable debate over the duration of this phase of development however it is thought that the process of alveolarization extends significantly into postnatal life.
1.2 Epithelial-Mesenchymal Interactions in Lung Development

Interactions between the epithelial and mesenchymal tissue layers are required for the development of many organs including those of the gastrointestinal, urogenital, integumental and respiratory systems (Yasugi and Mizuno, 1990; Lehtonen, 1975; Grobstein and Cohen, 1965; Kratochwil, 1969 and Masters, 1976). It has been established for some time that interactions between the pulmonary epithelium and its surrounding mesenchyme are critical for both branching morphogenesis and cellular differentiation during mammalian lung development. However, it is only recently that we have begun to define the specific nature of these interactions and to identify some of the specific signaling molecules involved. It is like that the regulatory molecules involved in epithelial-mesenchymal signaling include components of the extracellular matrix (ECM), cell-associated signaling peptides and various diffusible factors such as hormones, growth factors and cytokines (reviewed in Keijzer and Post, 1999; Shannon and Deterding, 1997).

The importance of these interactions in lung organogenesis has been appreciated since the early studies by Rudnick were conducted in the 1930's. These studies demonstrated that when embryonic chicken lung rudiments were grafted onto chorioallantoic membranes in ovo, lung development proceeds in grafts in which the lung bud had just emerged as well as in those in which primary and secondary branches had been established. However, more interesting was the observation that all development ceased when the mesenchyme surrounding the lung rudiment was removed prior to grafting (Rudnick, 1933). Further tissue recombination studies have confirmed and extended these early results in the development of both avian and mammalian respiratory systems (Tadera, 1967; Spooner and Wessels, 1970; Masters, 1976).
Recent studies into the role of epithelial-mesenchymal interactions during mammalian lung development have been facilitated by the advent of a number of techniques for studying lung development in vitro. The first and perhaps most important of these arose from the observation that, when cultured under specific conditions, embryonic lungs are able to undergo both branching morphogenesis and lung cell differentiation in an in vitro tissue culture system. The culture conditions that have been successful in supporting these developmental processes vary greatly in both their contents and effectiveness. The second useful technique has arisen from experiments using tissue recombination models. In these experiments, embryonic organs are isolated at a specific point in gestation, usually early in organogenesis. The epithelial and mesenchymal tissue layers are then separated by a combination of enzymatic treatments and mechanical separation. The separated epithelium can then be recombined with mesenchyme from the same (homotypic recombinant) or an alternate organ source (heterotypic recombinant) and vice versa.

Tissue recombination experiments to date have led to the determination of several facts regarding the role of interactions between the epithelial and mesenchymal tissue layers in lung development. The first of these is that lung epithelial cells require the presence of mesenchyme for their very survival. If cultured alone for extended periods of time they will undergo necrosis. In addition, Masters (1976) determined that epithelial growth, branching morphogenesis and cytodifferentiation is dependent on the amount of lung mesenchyme present in culture. Fetal lung epithelium also survives when recombined with mesenchyme from an alternate source (i.e. trachea, salivary gland et.). However, cellular proliferation and branching of the epithelial tubules is arrested. When either rat or mouse epithelial cells are isolated in the early pseudoglandular stage of lung development and subsequently recombined with salivary gland mesenchyme, epithelial growth is completely ablated (Lawson, 1972; Lawson, 1974; Ball,
Similarly, when recombined with mouse tracheal mesenchyme, embryonic mouse epithelium does not branch (Hilfer, 1985). Finally, experiments involving the recombination of mouse or rat salivary gland epithelium with lung mesenchyme demonstrated that lung mesenchyme can support copious branching of a heterotypic epithelium. These results suggest that embryonic lung epithelium has a strict requirement for lung mesenchyme and the specific factors provided by this tissue for the specification of lung developmental processes (Lawson, 1972; Lawson, 1974; Ball, 1974).

The tracheal graft model has also been a useful system to study the supportive role of lung mesenchyme in epithelial branching during early lung development. In this system a portion of embryonic (day 11-12 in the mouse) tracheal mesenchyme is surgically removed and replaced by mesenchyme from an alternate source. When distal lung mesenchyme is grafted onto tracheal epithelium branching occurs in a pattern which is remarkably similar to that seen in the developing lung (Alescio and Cassini, 1962). However, studies in which tracheal epithelium was cultured with grafted mesenchyme from intestine, skin, salivary gland and mammary gland demonstrated simple bud formation but no significant branching was observed (Wessells, 1970). Further, the grafting of tracheal mesenchyme to distal epithelium denuded of its own mesenchyme inhibits all distal epithelial branching (Wessels, 1970). These studies clearly demonstrated a key difference in the instructive capabilities of embryonic lung and tracheal mesenchyme and suggest a specific requirement of lung epithelial cells for lung mesenchyme in order for proper branching morphogenesis to occur.

More recent studies indicate that epithelial-mesenchymal interactions are also important for lung epithelial cell differentiation. Tracheal graft studies have demonstrated that distal lung mesenchyme is a potent inducer of the distal epithelial phenotype when recombined with tracheal epithelium. The induced tracheal epithelium adopted an alveolar type II phenotype as
determined by the presence of lamellar bodies, large glycogen deposits and the expression of surfactant protein C (SP-C), a specific marker for alveolar type II cell differentiation (Shannon, J.M., 1994). Similarly, tracheal mesenchyme induces a tracheal epithelial phenotype, determined by the loss of lamellar bodies and SP-C expression and positive mucin immunostaining, when grafted to distal lung epithelium (Shannon, J.M. et al., 1998). This data suggests that during early lung development, epithelium of the respiratory lineage has the plasticity to respond to various mesenchyme originating from the respiratory tract.

The effects of lung mesenchyme in stimulating lung epithelial proliferation, branching and differentiation have also been studied using trans-filter tissue recombination experiments modeled after those done in the kidney and salivary gland. (Grobstein, 1956; Grobstein and Cohen, 1965) These studies have demonstrated that the effects of lung mesenchyme appear to be short range and mediated by diffusible factors. In this experimental system, lung epithelial and mesenchymal rudiments are cultured on opposite sides of a Millipore filter. A filter thickness of 25 μm and pore size of 0.45 μm is used to allow passage of macromolecules between the two side of the filter, but to restrict direct cell-cell contact. When mouse fetal day-12 lung epithelium is cultured opposite from day-12 lung mesenchyme the epithelial rudiment branches robustly. However, epithelial rudiments cultured alone simply spread over the surface of the filter but do not branch at all (Taderera, 1967). Interestingly, when day-12 mouse epithelial cells are cultured opposite day-5 chick lung mesenchyme, the epithelial tissue branches in a similar pattern to that cultured with mouse lung mesenchyme, indicating that the inductive molecules involved in branching morphogenesis are evolutionarily conserved between birds and mammals. In addition, when mouse lung epithelium is directly combined and cultured with chick mesenchyme the epithelial cells contain copious amounts of lamellar bodies, indicating
conservation of inductive mesenchymal effects for alveolar type II cell differentiation (Hilfer, 1985).

In summary, evidence to date suggests that during early lung development (i.e. embryonic and early pseudoglandular stages) interactions between the pulmonary epithelium and mesenchyme are an absolute requirement for proper branching morphogenesis and cellular differentiation to occur. Although the importance of these cell-cell communications is appreciated at these early gestations, much less is know about the importance of epithelial-mesenchymal signaling in lung maturation at later gestation (i.e. canalicular, saccular and alveolar periods). Future studies such as those presented here will be important in elucidating the role of these interactions during late lung development.

1.3 Transcription Factors in Lung Development

There is an abundance of evidence to date which suggests that differentiation of the embryonic foregut endoderm is associated with the temporally and spatially restricted expression of a number of distinct organ- and cell-specific transcription factors. In broad terms, transcription factors can be defined as DNA-binding proteins that control the expression of various downstream target genes. There are several families of transcription factors which are based on common motifs (i.e. helix-turn-helix, basic-helix-loop-helix, winged-helix, zinc-finger and leucine-zipper) within their DNA-binding domains (reviewed in Pabo and Sauer, 1992). Some transcription factors are ubiquitously expressed and are needed for the transcriptional control of a number of genes, whereas others may have specific functions and are required for tissue- and cell-specific gene expression.
Homeodomain-containing (Homeobox or Hox) proteins make up a large family of transcription factors which share a common “homeodomain”, or more precisely, a 60-amino acid motif related to the helix-turn-helix motif of prokaryotic DNA-binding proteins (reviewed in Cardoso, 1995). Hox genes are arranged in four clusters (a,b,c and d) on separate chromosomes and their 3’ to 5’ position within a cluster corresponds to their expression along the anterior-posterior axis of the developing embryo (Cardoso, 1995; Kappen, 1996; Krumlauf, 1994). There is evidence that Hox genes play a role in mammalian lung morphogenesis as many of these genes are expressed in the lung during early development and their expression seems to be developmentally regulated. Specifically, Hoxb genes have been found to be expressed in specific portions of the developing lung (Bogue et al., 1994) The expression of Hoxb-2 and b-5 is restricted to the distal mesenchyme, whereas, Hoxb-3 and b-4 are expressed in both proximal and distal mesenchyme. These specific regional expression patterns suggest that Hoxb genes may be involved in specifying regional differences in mesenchyme along the anterior-posterior axis of the developing lung.

Thyroid transcription factor-1 (TTF-1) is another transcriptional regulator belonging to the homeodomain class of transcription factors which is thought to play a critical role in differential gene activation during lung morphogenesis (Minoo et al., 1997). Several lines of evidence suggest that the actions of TTF-1 are absolutely crucial for both proper branching morphogenesis and the establishment of the distal epithelial cell phenotype. Disruption of TTF-1 translation using antisense oligonucleotides in a lung explant system results in the complete inhibition of branching in vitro (Minoo et al., 1995). In addition, TTF-1 null mice die at birth and lack proper lung parenchyma (Kimura et al., 1996). Finally, TTF-1 functions upstream of the pulmonary surfactant proteins SP-A, SP-B, SP-C (Bohinsky et al., 1994; Bruno et al., 1995)
and Clara Cell Secretory protein (i.e. CC10) as a transcriptional regulator (Bohinsky et al., 1994; Toonen, et al., 1996).

Finally, studies in Drosophila have identified a number of transcription factors that play important roles in the development and branching of the fly tracheal (respiratory) system. The Drosophila tracheal system is an enormous network of branched ectodermal tubules that conduct oxygen from the spiracular openings to the internal tissues of the fly (Samakovlis et al., 1996). The tracheal system arises from clusters of ectodermal cells which invaginate into the mesoderm at mid-gestation and form 20 epithelial sacs of about 80 cells each. Each sac then branches into successively finer tubules, eventually generating a tree-like structure. Remarkably, the development of the fly tracheal system occurs in the absence of cell proliferation and results exclusively from cell migration and changes in cell shape (reviewed in Metzger and Krasnow, 1999). Recently a member of the basic-helix-loop-helix/PAS (bHLH-PAS) protein family of transcription factors, tracheless (trh), was cloned and identified as a master controller for tracheal cell fate and tubulogenesis (Wilk et al., 1996). Trh is expressed in all tracheal cells during development and in the absence of trh the tracheal system fails to develop (Isaac et al., 1996; Wilk et al., 1996). In addition, the homeotic gene Spalt encodes a zinc finger transcription factor and functions during fly development as a suppressor of tracheal cell fate (Kuhnlein and Schuh, 1996). Finally, the Drosophila transcription factors pointed (pnt) and driffer (drf) are involved in branching of the tracheal system and in the absence of pnt and drf activity no secondary branches develop (Klambt, 1993; Anderson et al., 1995). Whether there are mammalian homologues of these transcription factors which play similar roles in the development of the mammalian respiratory system remains to be established.
1.4 Morphogens in Lung Development

Morphogens are defined as molecules that diffuse away from their source and give positional information to surrounding cells based on their local concentration. Small differences in morphogen concentration may lead to differences in the pattern of gene expression in neighboring cells, thereby resulting in cells closer to the morphogen source adopting a different phenotype from those further away (reviewed in Lawrence and Struhl, 1996). It is postulated that morphogen activity plays an important role in regulating lung epithelial cell differentiation and morphogenesis. This is likely since, epithelial cells in the early lung bud must receive some informative signals with regards to their position relative to their neighbors, in order to ensure proper distribution and pattern of cell and tissue differentiation along the proximo-distal axis of the developing airways. Several members of the Hedgehog (Ingham, 1995), Wnt (Parr, 1994), Fibroblast growth factor (Kengaku, 1995) and Transforming Growth Factor-β (Wall, 1994) protein families have been identified as possible morphogens based on their concentration dependent activity in the developing mouse embryo. It is likely that some or all of these morphogenic factors in conjunction with others play important roles in directing developmental processes in the mammalian lung.
1.5 Growth Factors in Lung Development

Recently a number of families of growth factors have been shown to function as inductive signaling molecules in the developing lung. A number of these factors play an important role in mediating epithelial-mesenchymal interactions and aid in directing branching morphogenesis and cellular differentiation in the embryonic lung (reviewed in Shannon and Deterding, 1997; Minoo and King, 1994 and Keijzer and Post, 1999).

For example, several members of the fibroblast growth factor family and their receptors have recently been implicated in mouse lung development. Three of the four known FGF receptor genes (*Fgfr2, Fgfr3* and *Fgfr4*) are expressed in the early lung epithelium and transgenic mice expressing a dominant negative *Fgfr2 IIIb* splice variant under the control of the SP-C promoter exhibit severe defects in lung branching (Peters *et al.*, 1994). Consistent with the idea that FGFs are involved in interactions between the epithelium and its surrounding mesenchyme, the ligands, FGF1, FGF7 (KGF) and FGF10 are all expressed in the mesenchyme, particularly distal mesenchyme, in the developing lung (Bellusci *et al.*, 1997). Transgenic misexpression of *Fgf7* in mouse distal lung epithelium by way of the SP-C promoter results in the formation of abnormal lungs composed largely of undifferentiated columnar epithelial cells (Simonet *et al.*, 1995). Consistent with these results and a role for FGFs in epithelial cell differentiation are the observations that expression of the surfactant proteins is altered in response to treatment of lung cultures with FGF1 or FGF7. In addition, inhibition of FGF7 function using antisense oligonucleotides inhibits embryonic lung branching *in vitro* (Post *et al.*, 1996). However, in spite of the evidence for a role for FGF7 in lung development, mice bearing a null-mutation in the *fgf7* gene show no obvious lung abnormality, suggesting that other FGF family members can compensate for the loss of FGF7 *in vivo* (Guo *et al.*, 1996). Finally,
exposure of lung explants to FGF10 results in increased epithelial branching (Bellusci et al., 1997) while the generation of FGF10 deficient transgenic mice results in pups which die at birth due to a complete disruption in early lung development (Sekine et al., 1999). Together, these data suggest that at least three members (FGF1, FGF7 and FGF10) of the expanding FGF family (i.e. FGF1-18) and one FGF receptor (FgfR2) are likely involved in regulating developmental processes in the mammalian lung.

In addition to the Fibroblast Growth Factors, it has been suggested that a number of other growth factor families are actively involved in lung development. Studies examining the role of Epidermal Growth Factor, Transforming Growth Factor-α and -β, Platelet-Derived Growth Factors, Insulin-like Growth Factors, Hepatocyte Growth Factor and Vascular Endothelial Growth Factor suggest that development of the mammalian lung is a result of a complex interplay of a number of different growth factor signaling pathways (reviewed in Shannon and Deterding, 1997; Minoo and King, 1994; Keijzer and Post, 1999).
1.6 The Notch Signaling Pathway

The Notch signaling pathway is an evolutionarily conserved pathway which is believed to play a central role in the specification of cell fate through cell-cell communications in both vertebrate and invertebrate organisms. It is now widely accepted that during many mammalian developmental processes, the generation of differentiated cells from a population of undifferentiated progenitors is regulated by Notch signaling (reviewed in Weinmaster, 1998; Artavanis-Tsakonas et al., 1999). Notch is a transmembrane receptor that is activated through direct contact with a cell-surface ligand on a neighboring cell. It is thought that the activation of the Notch receptor, through these cell-cell interactions, functions in developmental processes such as neurogenesis (De la Pompa et al., 1997), myogenesis (Nye et al., 1994) and hematopoiesis (Milner et al., 1996 and Bigas, 1998) to suppress undifferentiated precursor cells from adopting a more differentiated state.

1.7 Elements of Notch Signaling

In the developing imaginal wing disc of Drosophila, activation of Notch, by its ligands Delta and Serrate, is required at the dorsoventral boundary for specification of the wing margin (DeCelis et al., 1996; Flemming et al., 1997; Panin et al., 1997). Delta and Serrate are both membrane bound ligands and are required on opposite sides of the presumptive wing margin. Delta, expressed in ventral cells will bind and activate Notch receptors on the surface of dorsal cells, whereas Serrate in dorsal cells will activate Notch in ventral cells. This activation of Notch at the Dorsal/Ventral boundary is required for expression of wing margin-specific genes such as wingless, cut, and vestigial and subsequent wing development. Proper specification of the
margin requires Notch activation by both of the known ligands, and interestingly, both Serrate and Delta elicit compartment specific responses through Notch.

The *Drosophila* gene, *fringe*, has recently been suggested to play a key role in regulation of the Notch signaling pathway (Flemming *et al.*, 1997; Cohen *et al.*, 1997; Panin *et al.*, 1997). *Fringe* encodes a putative secreted protein and is expressed exclusively in dorsal cells of the wing disc during the second and third larval instars of *Drosophila* development. The Fringe protein has been proposed to function as a boundary determining molecule in the wing margin and it is believed to act upstream of Serrate to inhibit Notch-Serrate interactions while potentiating Notch-Delta interactions (Flemming *et al.*, 1997; Panin *et al.*, 1997). Fringe shares a high sequence homology and similar predicted secondary structure to bacterial glycosyltransferases. Therefore, *fringe* may control wing development by altering the glycosylation patterns of other signaling molecules (Yuan *et al.*, 1997).

Genetic and molecular analysis have identified some of the downstream components of this pathway in Drosophila. Deltex (Dx) is a cytoplasmic protein, whereas the transcription factor Suppressor of Hairless (Su(H)) and the basic helix-loop-helix (bHLH) genes encoded by the *Enhancer of split* (E(sp1)) locus are downstream nuclear components of notch signaling (reviewed in Artavanis-Tsakonas, 1999). Recent evidence suggests that the intracellular transmission of notch signaling involves a ligand dependent cleavage event, which releases the intracellular domain (ICD) of the notch receptor and allows it to translocate to the nucleus (Schroeter *et al.*, 1998; Struhl and Adachi, 1998). Although the protease responsible for cleavage of the Notch receptor has not been identified, recent data indicate that the Presenilin proteins may play an important role in regulating this event (Ray *et al.*, 1999; DeStrooper *et al.*, 1999; Ye *et al.*, 1999; Struhl and Greenwald, 1999; Song *et al.*, 1999). Mutations in the presenilin genes are a major cause of early-onset familial Alzheimer’s disease and there is
mounting evidence which suggests that the same mutations in presenilin-1, which are associated with Alzheimer's disease, also lead to impaired proteolytic release of the Notch ICD and its subsequent nuclear translocation (Song et al., 1999). After its release, Notch ICD can then interact with Su(H). This new complex (notch ICD/Su(H)) then binds to its specific binding site in the 5'-flanking regions of the E(spl) complex. Here, Su(H) functions as a transcriptional activator, upregulating the expression of the basic helix-loop-helix transcription factors encoded by the E(spl) complex (Jerriault et al., 1995). The E(spl) gene products often function as transcriptional repressors themselves, suppressing the expression of various genes involved in cellular differentiation. For example, *Drosophila* neuroblastic differentiation is associated with a requirement for the gene products of the *achaete-scute* gene complex, which encodes tissue specific transcription factors (Goodburn, 1995). Ligand activation of Notch suppresses the expression of these genes through activation of the transcriptional repressors within the *Enhancer of split* gene complex. By this mechanism, those cells in which Notch is activated are prevented from undergoing terminal differentiation into neuroblasts.

Recent studies have demonstrated the pleiotropic nature of Notch activity and the functional requirement of the Notch pathway throughout development in several vertebrate and invertebrate species. In Drosophila, the Notch pathway has been implicated in a multitude of developmental processes including neurogenesis, mesoderm formation, larval malpighian tubule formation, eye formation and wing development (Artavanis-Tsakonas et al., 1995). Mammalian homologues of Notch, Delta, Serrate and Fringe have been suggested to play similar developmental functions. To date, four Notch homologues (Notch1 to Notch4), two Delta homologues (Dll1, Dll3), two Serrate homologues (Jagged1, Jagged2) and three Fringe homologues (Lunatic, Manic and Radical fringe) have been cloned and identified (Nye, 1995;
Shawber et al., 1996; Cohen et al., 1997; Johnson et al., 1997; Panin et al., 1997). Table 2 contains a list of the nomenclature for proteins in the Notch signaling pathway.

Table 2. Nomenclature for Proteins in the Notch Signaling Pathway

<table>
<thead>
<tr>
<th></th>
<th>Drosophila</th>
<th>Xenopus</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptors</td>
<td>Notch</td>
<td>xNotch1/Xotch</td>
<td>Notch-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Notch-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Notch-3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Notch-4</td>
</tr>
<tr>
<td>Ligands</td>
<td>Delta</td>
<td>xDelta-1</td>
<td>DII1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>xDelta-2</td>
<td>DII3</td>
</tr>
<tr>
<td></td>
<td>Serrate</td>
<td></td>
<td>Jagged-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jagged-2</td>
</tr>
<tr>
<td>Intracellular</td>
<td>Suppressor of Hairless Su(H)</td>
<td>xSu(H)</td>
<td>RBP-Jk/KBF2/CFB1</td>
</tr>
<tr>
<td>mediators</td>
<td>Enhancer of Split E(spl)</td>
<td></td>
<td>Hes1, Hes5</td>
</tr>
</tbody>
</table>

Genetic analysis in mice revealed that Notch1 and Dll1 are both required for neurogenesis and somitogenesis (Conlon et al., 1995; de la Pompa et al., 1997; Harabe de Angelis et al., 1997). Both null mutants die during early to mid-gestation with severe neural and somite defects. In contrast, Jagged2 mutant mice die shortly after birth with limb, craniofacial and thymic defects (Jiang et al., 1998) and Jagged-1 null mutants have embryonic lethality by 10.5 days of gestation due to severe defects in the embryonic vasculature (Xue et al., 1999). Finally, recent studies have demonstrated that Lunatic fringe is also required for somitogenesis (Zhang and Gridley, 1998; Evard et al., 1998). These observations suggest that Lunatic fringe is likely an essential component of the Notch signaling pathway. Unexpectedly, mutant mice lacking Lunatic fringe function are viable, indicating that some of its developmental functions might be compensated by other fringe homologues (Zhang and Gridley, 1998; Evard et al., 1998).
Signaling events downstream of Notch activation in the mammalian system are thought to be similar to those observed in Drosophila. During mammalian neurogenesis and myogenesis, Notch signaling is thought to regulate cellular differentiation by activating the transcription of downstream bHLH transcription factors (Jerriault et al., 1995). Genetic analysis in mice has indicated that a mammalian Su(H) homologue, RBP-Jk/KBF2/CBF1, is essential for neurogenesis and somitogenesis as mice with a null mutation in RBP-Jk present with phenotypes similar to those observed in Notch1 and Dll1 mutants (de la Pompa et al., 1997). Interestingly, when compared with Notch1 mutant embryos, RBP-Jk mutant embryos display a similar but more pronounced neural and somite phenotype, which is consistent with the notion that there are some functional redundancies between the Notch receptors during early embryogenesis and that RBP-Jk acts as a common downstream mediator of Notch receptors (de la Pompa et al., 1997).

Of particular interest is the recent identification of a lung specific homologue of RBP-Jk, RBP-L. Although the role of RBP-L in Notch signaling is unknown, it can bind specific DNA sequences similar to RBP-Jk (Minoguchi et al., 1997).

The Hes family of transcriptional repressors are mammalian homologues of Drosophila Hairy and Enhancer of Split [E(spl)] bHLH transcription factors. All Hes-related proteins are complexed with corepressors and function as transcriptional repressors in order to block cellular differentiation processes in cells actively involved in Notch signaling. Activation of Notch leads to cleavage of its intracellular domain (ICD) and subsequent translocation to the nucleus. This peptide then complexes with RBP-Jk and the complex activates downstream Hes expression. Levels of Hes expression may then serve as markers for Notch activation within given cell populations. To date, four mammalian Hes genes have been identified (i.e. Hes1, 2, 3 and 5) (Nishimura et al., 1998). However, recent evidence suggests that only two of these (i.e. Hes1 and Hes5) are regulated by Notch activation (Nishimura et al., 1998; Ohtsuka et al., 1999).
These results suggest that Hes genes may be functionally classified into two groups, those regulated by Notch activation and those that aren't (Nishimura et al., 1998). Targeted disruption of Hes1 results in an embryonic lethal phenotype consisting of severe nerulation defects and premature neurogenesis (Ishibashi et al., 1995). Figure 1 shows a schematic which represents the current model of the Notch signaling pathway.
Upon ligand stimulation, an intracellular proteolysis releases the intracellular domain (ICD) of the Notch receptor. Notch ICD is then translocated to the nucleus where it interacts with Su(H)/RBP-Jk. This new complex (i.e. Notch ICD/RBP-Jk) functions as a transcriptional activator to upregulate the expression of the basic helix-loop-helix encoded by the E(spl)/Hes genes. These upregulated gene products then often serve as transcriptional repressors, suppressing the expression of various genes involved in cellular differentiation.

Figure 1. Diagramatic model of the Notch signaling pathway. Upon ligand stimulation, intracellular proteolysis releases the intracellular domain (ICD) of the Notch receptor. Notch ICD is then translocated to the nucleus where it interacts with Su(H)/RBP-Jk. This new complex (i.e. Notch ICD/RBP-Jk) functions as a transcriptional activator to upregulate the expression of the basic helix-loop-helix encoded by the E(spl)/Hes genes. These upregulated gene products then often serve as transcriptional repressors, suppressing the expression of various genes involved in cellular differentiation.
1.8 Rationale for Studying Notch Signaling in Lung Development

As mentioned earlier, the mammalian respiratory system originates as two endodermal buds arising from the primitive embryonic foregut. These buds grow and dichotomously branch into the surrounding splanchnic mesenchyme, eventually giving rise to the mature pulmonary tree. During early lung development, the primitive lung is lined by an endodermally derived pluripotent epithelium that will differentiate throughout development into the many different cell types which constitute the mature airway epithelium and the specialized alveolar epithelial cells. Although the occurrence of this cellular differentiation process is appreciated and the mature cell types have been identified and well characterized, the mechanisms and factors which are involved in regulating this process are less clear. What mechanisms exist in the developing lung to determine which cells, from a pluripotent epithelial cell population, are destined to become, for example, alveolar epithelial cells as opposed to bronchiolar epithelial cells? What signals determine the specific number and location of cells that differentiate into alveolar type II cells versus type I cells in the alveolar airspace? It is specifically because the evolutionarily conserved function of the Notch pathway is to regulate the generation of cellular differentiation within an equipotent population of cells, that we hypothesize a potential role for Notch signaling in mammalian lung development. Perhaps the activation of one or more of the mammalian Notch receptors results in a signaling cascade which functions in the control and designation of cell fate during lung epithelial cell differentiation.

Recent evidence suggests that indeed the Notch pathway does play an important role in regulating both branching morphogenesis and cell fate specification in the Drosophila tracheal system (Llimargas, 1999). The fly tracheal system is a branched system of tubular epithelium that functions to transport oxygen to the insect’s tissues. Development of the Drosophila
tracheal system and the mammalian respiratory system share several important organizational and molecular features, therefore, studying the various developmental processes involved in tracheal development has proven useful in identifying the various factors that are involved in mammalian lung development (reviewed in Metzger and Krasnow, 1999). In the developing trachea, Notch signaling serves to regulate the specification of fusion cell fate and the generation of Notch null mutants results in the allocation of extra fusion cells and the consequent formation of inappropriate tracheal branch fusions (Llimargas, 1999). In addition, Notch function is also required to repress the tracheal terminal cell fate, as the ablation of Notch activity produces ectopic terminal branches in the embryonic trachea (Llimargas, 1999). Since the Drosophila tracheal system is an excellent model for use in the analysis of many of the developmental processes in the mammalian lung, it stands to reason that the Notch pathway may also play similar roles in the development of the mammalian respiratory system.

Finally, recent studies have demonstrated that expression of Notch-1, -2 and -3 as well as Jagged-1 are regulated by epithelial-mesenchymal interactions in the developing tooth (Mitsiadis et al., 1995; 1997). These data show that Notch expression is downregulated in epithelial cells which are juxtaposed to mesenchyme in the developing tooth. Homo- and heterotypic explant experiments indicated that indeed the epithelium requires a mesenchyme derived signal in order to maintain the downregulation of Notch and this signal may be critical for the determination of the ameloblast fate (Mitsiadis et al., 1995). Similarly, the same group of investigators has recently determined that specific mesenchymal signals downregulate Jagged-1 expression in adjacent epithelial or preameloblasts (Mitsiadis et al., 1997) however, the expression of the ligand Delta1 is unaffected by interactions between the dental epithelium and its surrounding mesenchyme (Mitsiadis et al., 1998). Since it is now well known that epithelial-mesenchymal interactions play a crucial role in regulating mammalian lung development, we can also postulate
that Notch signaling is involved in these tissue interactions and perhaps epithelial-mesenchymal signaling regulates the expression of some of the Notch pathway components.

1.9 Aims of the Present Study

Interactions between the pulmonary epithelium and its surrounding mesenchyme are required for proper development of the mammalian lung. The importance of these cell-cell interactions in branching morphogenesis and cellular differentiation during early lung development is now appreciated. However, the role of epithelial-mesenchymal interactions in developmental processes during late lung development is less well understood. We hypothesized that interactions between the pulmonary epithelium and mesenchyme are important for lung epithelial cell morphogenesis and differentiation, not only during early fetal gestation (i.e. 12 to 15 days in the rat), but also at relative late fetal gestation (i.e. 19 and 21 days of fetal rat gestation) as well.

The specific aims of this portion of the study were:

1) To determine the effect of both homotypic (i.e. from the same tissue) and heterotypic (i.e. from a different tissue type) mesenchyme on lung epithelial cell morphology at relative late fetal gestation.

2) To determine the effect of homotypic and heterotypic mesenchyme on lung epithelial cell differentiation at relative late fetal gestation.

A novel in vitro cell recombinant model was used to examine the effects of mesenchymal type on lung epithelial cell morphology and differentiation. The morphology of the cell recombinants was examined by immunocytochemistry using both anti-cytokeratin and antivimentin antibodies. The effect of mesenchyme on lung epithelial cell differentiation was
determined by assessing the expression patterns of the lung epithelial cell markers, SP-C, SP-B and CC10. Expression levels were examined by non-radioactive in situ hybridization using specific digoxigenin-labeled riboprobes.

One candidate for involvement in lung epithelial cell differentiation, at both early and late fetal gestation, is the evolutionarily conserved Notch signaling pathway. Since there is now an abundance of evidence which suggest that the Notch pathway plays an important role in regulating cellular differentiation during many mammalian developmental processes, I hypothesized that activation of the Notch pathway is involved in the regulation of lung epithelial cell differentiation. In order to begin characterizing the role of the Notch pathway in embryonic lung development, we first examined the expression patterns of some of the signaling molecules within the pathway.

The specific aim of this portion of the study was:

1) To characterize the spatial and temporal expression patterns of the components of the Notch pathway throughout fetal rat lung development.

The expression patterns of Notch-1, 2 and 3, Jagged-1 and 2, Delta-1, Lunatic, Manic and Radical Fringe and Hes5 were determined by non-radioactive in situ hybridization using specific digoxigenin-labeled RNA probes.
CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Female (200-250 g) and male (250-300 g) Wistar rats were obtained from Charles River (St. Constant, Quebec) and were bred in our animal facilities. Rats were sacrificed at days 19 and 21 of gestation (term = 22 days). Culture media, antibiotics, dispase, fetal bovine serum and ascorbic acid were from Gibco (Grand Islands, NY). Twenty-four well cell culture plates were from Nunc (Intermed, Denmark) and culture inserts were from Millipore (Bedford, MA). Lipofectamine used for epithelial cell transfections was from Gibco (Grand Islands, NY). The Phospha-Light kit for detection of the SEAP2 reporter gene was from Tropix (Bedford, MA) and the β-galactosidase enzyme assay system was obtained from Promega (Madison, WI). The 3.7 Kb SP-C promoter was a gift from Dr. J. Whitsett (Cincinnati, OH).

Mouse monoclonal antibodies to vimentin were from Serotec (Missisauga, ON) and the anti-cytokeratin monoclonal antibody (AE1/AE3 clone) was from Boehringer Manheim (Laval, QC). Rabbit polyclonal antibodies against human proSP-C and rat CC10 were kind gifts from Dr. J. Whitsett (Cincinnati, OH) and Dr. Kaytal (Pittsburg, PA), respectively. The goat anti-rabbit and sheep anti-mouse biotinylated secondary antibodies were obtained from Calbiochem (LaJolla, CA) and Amersham (Burlington, ON). All Notch receptor, ligand and fringe probes were kind gifts from Dr. Sean Egan (Toronto, ON). Polyclonal antibodies against Jagged-1 and Jagged-2 were obtained from Santa Cruz (Santa Cruz, CA). The DIG-RNA labeling kit and the DIG nucleic acid detection kit were from Boehringer Manheim (Laval, QC) and the Qiagen gel extraction kit was from Qiagen (Valencia, CA). Finally, a kit for avidin-biotin peroxidase complex immunostaining was purchased from Vector Laboratories (Burlingame, CA).
2.2 Primary Cell Culture: Isolation and Separation of Fetal Day-13 Cells

Timed-gestation Wistar rats were killed by diethyl ether excess, and the fetuses were aseptically removed from the mothers. The day-13 embryonic lungs were surgically dissected from the fetus and transferred in 200 μl of Hanks Balanced Salt Solution (-) (HBSS(-)) to a small glass petri dish containing 800 μl of HBSS(-). Both 200 μl of 100% (v/v) Dispase and 12 μl of 0.01 mg/ml DNase were added to the solution and the resulting suspension was incubated at 37 °C for 45 minutes. Enzymatic activity was then neutralized by adding 1 ml of DMEM/F12 + 5% (v/v) FBS medium containing 0.1% penicillin/streptomycin and 0.25 mg/ml ascorbic acid. Finally, lung rudiments were transferred to a large glass petri dish and the epithelial and mesenchymal components were separated using 0.27-gauge needles under a dissecting microscope.

2.3 Primary Cell Culture: Isolation and Separation of Fetal Day-19 and -21 Cells

A sterile technique was used throughout this protocol. Timed-gestation Wistar rats (average was 4 rats per experiment) were killed by diethyl ether excess, and the fetuses were aseptically removed from the mothers. The fetal lungs, skin and intestine were removed and placed in separate petri dishes containing cold HBSS(-) without calcium and magnesium [HBSS(-)]. At all times, lung, skin and intestine tissues were kept separately. The tissues were then cleaned of any excess adherent tissue (i.e. heart, trachea, connective tissue) and washed twice in HBSS(-). Using sharp scissors the tissues were minced into very fine pieces, resuspended in 35 ml of HBSS(-) and transferred to a 50 ml plastic tube. The minced tissues were then vortexed gently in order to remove erythrocytes and centrifuged at 100 xg for one
minute. The supernatant was discarded and this step was repeated several times until the medium supernatant was clear. After the final wash in HBSS(-) the minced lung, skin and intestine tissues were centrifuged at 420 xg for 5 minutes and the supernatants were discarded. The pellets were resuspended in 40 ml of trypsinization solution [0.125% (w/v) Trypsin and 0.01mg/ml DNase in HBSS(-)] and transferred to sterile trypsinizing flasks containing magnetic stir bars. The minced tissues in solution were then incubated for 20 minutes at 37 ºC under constant stirring. The tissue trypsinization was stopped by adding Eagle’s Minimal Essential Medium (MEM) containing 50 µg/ml gentamycin, 0.625 µg/ml amphotericin B and 5% (v/v) fetal bovine serum (FBS). The neutralized cell suspensions were then filtered through sterile mesh filters (pore size of 100 µm) into clean 50ml tubes. The filtered cell suspensions were then pelleted at 420 xg for 5 minutes, and the resulting supernatants discarded. The cell pellets were then washed with 35 ml of MEM and centrifuged again. The resulting cell pellets were then resuspended in 30 ml of 0.1% (w/v) collagenase and 0.01 mg/ml DNase in MEM and placed in a 37 ºC water-bath for 15 minutes. An equal amount of MEM + 5% (v/v) FBS was added to neutralize the collagenase activity and the suspension was centrifuged at 420 xg for 5 minutes. The cell pellets were then resuspended in the appropriate amount of MEM + 5% (v/v) FBS and the cell suspensions were divided (approximately 15 ml per flask) into T75 tissue culture flasks. The flasks were then left to incubate for 1 hour at 37 ºC, in an atmosphere of 5% CO₂ in air, in order to allow fibroblasts to adhere. After 1 hour the non-adherent cell suspensions were collected and the flasks were rinsed with fresh MEM twice. Both the non-adherent cell suspensions and MEM rinses were collected in 50 ml tubes and subsequently centrifuged at 420 xg for 5 minutes. The pellets were again resuspended in MEM + 5% (v/v) FBS and seeded into another set of T75 tissue culture flasks. The flasks were incubated for a second time at 37 ºC in
order to remove residual fibroblasts from the epithelial cell suspensions. After a 1 hour incubation, the non-adherent cell suspensions and the subsequent MEM washes were collected and centrifuged at 200 xg for 3 minutes. The supernatants were discarded and the cell pellets were washed by gentle vortexing in 35 ml of MEM. Again, the suspensions were spun at 200 xg for 3 minutes and this step was repeated 3 times. The final epithelial cell pellets were resuspended in MEM + 5% (v/v) FBS and seeded into T75 tissue culture flasks. The adherence of epithelial cells to the tissue culture flasks required an overnight incubation at 37 °C, 16 to 18 hours, following which pure lung epithelial and fibroblast cell populations were available for further studies.

2.4 Primary Epithelial Cell Transfection

Freshly isolated day-19 lung epithelial cells were co-transfected with 0.9 μg of SPC-SEAP2 fusion plasmid and 0.1 μg of internal control plasmid (pCMV-βgal) per 10 cm² dish, using cationic liposomes (Lipofectamine) and DNA at a ratio of 12:1. Transfected cells were incubated for 24 hours prior to recombination with mesenchymal cells. Expression levels of SEAP2 would later be used to determine the effect of mesenchyme on activation of the SP-C promoter.
2.5 Generation of Homotypic and Heterotypic Cell Recombinants

After separation of epithelial and mesenchymal cells, transfected and untransfected lung epithelial cells were recombined in ratios of 1:1 and 1:3 with either lung, skin or intestine mesenchymal cells of the same or different gestation. We recombined day-19 epithelial cells with day-19 mesenchymal cells. However, day-21 epithelial cells were recombined with either day-19 or day-21 mesenchymal cells. Recombinations of day-19 mesenchymal cells with day-13 epithelial cells were also used. In order to recombine the tissue, individual cell populations of late gestation (i.e. day-19 and -21) were first trypsinized from tissue culture flasks and counted by Coulter counter. Approximately, $3 \times 10^6$ epithelial cells were mixed with the appropriate number of mesenchymal cells (i.e. $3 \times 10^6$ or $9 \times 10^6$ cells) and the cell mixtures were then centrifuged at 420 xg for 5 minutes. Excess medium was removed and the cell aggregates were then incubated at $37^\circ$C, in air with 5% CO$_2$ for 1 hour. Aggregates were then gently loosened by stirring with the tip of a micro-pipette and transferred to porous membrane inserts (4 μm pore size) in 24-well tissue culture plates. Membranes were presoaked in MEM for 1 hour before addition of cells. A small amount of Dulbecco’s Modified Eagle Medium (DMEM) + 5% (v/v) FBS (approximately 200 μl) was then added to each well so that it just moistened the surface of the cells but the aggregates were not submerged (i.e. a semi-dry culture). Cell recombinants were then cultured at 37 °C in an atmosphere of 5% CO$_2$ in air for 5 days. Our cell recombination and culture methods are modifications of those previously described by Fukuda et al.(1994).
2.6 Reporter Gene Assays

After 5 days in culture, the media from the day-19 recombinants, containing SPC-SEAP2 transfected epithelium were collected and frozen at -20 °C until used in the SEAP assay. The β-galactosidase assay was performed on recombinant tissue first. The cell aggregates were washed with 1X phosphate buffered saline (PBS) and cells were lysed by adding 200 μl of reporter lysis buffer (provided in assay kit) and incubating at room temperature for 15 minutes. The lysates were collected and briefly sonicated to ensure complete separation and lysis of all cells. A small amount of each sample (100 μl) was then mixed with 50 μl of reporter lysis buffer and 150 μl of 2x assay buffer (both are provided in assay kit). The mixture was gently vortexed and placed in 37 °C heating block to allow for colour development. Colour detection required overnight development (approximately 20 hours) at which time absorbance at 420 nm was read and compared with a generated standard curve to determine enzyme concentration.

The protocol used in the SEAP assay follows that provided by the Phospha-Light Kit. Briefly, the collected media from the recombinants were diluted and heated for 1 hour at 65° C in order to inactivate endogenous alkaline phosphatases. Equal volumes (25 μl) of sample, assay buffer and reaction buffer (provided in kit) were added to the wells of a luminometer plate. Luminescence was then measured by luminometer. SEAP2 levels were subsequently normalized to β-galactosidase levels to account for transfection efficiency.
2.7 Synthesis of Non-Radioactive Riboprobes

Short PCR fragments, complementary to rat SP-C and CC10 (330 and 315 bases respectively) were generated and inserted into the EcoRI site of the PCR 2.1 expression vector. The identity of the PCR sequences was confirmed by DNA sequencing using a Li-Cor automated sequencer. As mentioned earlier, all probes for the Notch pathway components were obtained from Dr. S. Egan (Hospital for Sick Children, Toronto, ON).

All plasmids containing cDNA probe inserts were linearized using the appropriate restriction endonuclease. The linear plasmid was then separated from uncut plasmid on a 1.5% (w/v) Tris-borate-EDTA (TBE) agarose gel and subsequently isolated from the gel using a Qiagen Gel Extraction Kit. Single stranded sense and antisense digoxigenin (DIG)-labeled riboprobes were then synthesized, by in vitro transcription, using approximately 1 μg of linearized template and 40 units of the appropriate RNA polymerase (i.e. depending on the orientation of the cDNA insert within the cloning site of the vector). Table 3 lists all of the probes used in this study and describes the restriction endonucleases used for linearization and RNA polymerases used for DIG-labeling.
Table 3. Descriptions of all Riboprobes used for *In Situ* Hybridization

<table>
<thead>
<tr>
<th>CDNA Probe</th>
<th>Vector</th>
<th>Insert Size (bp)</th>
<th>Restriction Enuclease</th>
<th>RNA Polymerase</th>
<th>Probe Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-C</td>
<td>PCR 2.1</td>
<td>330 bp</td>
<td>Hind III</td>
<td>T7</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hind III</td>
<td>T7</td>
<td>Antisense</td>
</tr>
<tr>
<td>CC10</td>
<td>PCR 2.1</td>
<td>315 bp</td>
<td>Hind III Xba I</td>
<td>T7</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SP6</td>
<td>Antisense</td>
</tr>
<tr>
<td>Notch-1</td>
<td>PGEM-T</td>
<td>709 bp</td>
<td>Not I Nco I</td>
<td>T7</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SP6</td>
<td>Antisense</td>
</tr>
<tr>
<td>Notch-2</td>
<td>PGEM-T</td>
<td>407 bp</td>
<td>Nco I Not I</td>
<td>SP6</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T7</td>
<td>Antisense</td>
</tr>
<tr>
<td>Notch-3</td>
<td>PGEM-T</td>
<td>403 bp</td>
<td>Not I Nco I</td>
<td>T7</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SP6</td>
<td>Antisense</td>
</tr>
<tr>
<td>Delta-1</td>
<td>PBK-CMV</td>
<td>777 bp</td>
<td>Pst I Sca I</td>
<td>T3</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T7</td>
<td>antisense</td>
</tr>
<tr>
<td>Jagged-1</td>
<td>PBluescript(K)</td>
<td>310 bp</td>
<td>Bam HI Hind III</td>
<td>T3</td>
<td>sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T7</td>
<td>antisense</td>
</tr>
<tr>
<td>Jagged-2</td>
<td>PBK-CMV</td>
<td>1400 bp</td>
<td>Bam HI EcoRI</td>
<td>T7</td>
<td>sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T3</td>
<td>antisense</td>
</tr>
<tr>
<td>Lunatic Fringe</td>
<td>PGEM-T</td>
<td>756 bp</td>
<td>Bgl II Eco RI</td>
<td>SP6</td>
<td>sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T7</td>
<td>antisense</td>
</tr>
<tr>
<td>Radical Fringe</td>
<td>PBluescript</td>
<td>709 bp</td>
<td>Kpn I Eco RI</td>
<td>T3</td>
<td>sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T7</td>
<td>antisense</td>
</tr>
<tr>
<td>Manic Fringe</td>
<td>PBluescript</td>
<td>462 bp</td>
<td>Hind III Xba I</td>
<td>T3</td>
<td>sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T7</td>
<td>antisense</td>
</tr>
<tr>
<td>HES-5</td>
<td>PBluescript</td>
<td>540 bp</td>
<td>EcoRI</td>
<td>T7</td>
<td>antisense</td>
</tr>
</tbody>
</table>

The labeling reaction required a 2 hour incubation at 37 °C and the presence of DIG-labeled uridine triphosphate (UTP). The reagents used in this protocol were provided in the DIG RNA Labeling Kit from Boehringer Manheim. Table 4 lists the necessary reagents and the final concentrations necessary for the *in vitro* transcription.
Table 4. \textit{In Vitro} Transcription Reaction

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
<th>Volume (Control Reaction)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified DNA template or Control DNA</td>
<td>Variable (1µg)</td>
<td>4 µl</td>
<td>0.05 µg/µl</td>
</tr>
<tr>
<td>NTP Labeling Mix (10x)</td>
<td>2 µl</td>
<td>2 µl</td>
<td>1x</td>
</tr>
<tr>
<td>10x Transcription Buffer</td>
<td>2 µl</td>
<td>2 µl</td>
<td>1x</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>To 18 µl</td>
<td>2 µl</td>
<td></td>
</tr>
<tr>
<td>RNA Polymerase (SP6, T7 or T3)</td>
<td>2 µl</td>
<td>2 µl</td>
<td>2 units/µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
<td>20 µl</td>
<td></td>
</tr>
</tbody>
</table>

The labeled riboprobes were then ethanol precipitated and resuspended in diethylpyrocarbonate (DEPC)-treated water. Small aliquots (i.e. approximately 5 µl) of the probe were run on a 2% (w/v) denaturing formaldehyde MOPS agarose gel in order to analyze the integrity of the RNA probe and to gather a rough estimate of its final concentration. In addition, the concentration of labeled riboprobe was approximated by dot-blotting a small amount and comparing it for direct colorimetric detection against a dilution series of labeled control RNA (provided in the DIG RNA Labeling Kit by Boehringer Manheim).

2.8 Non-Radioactive \textit{In Situ} Hybridization

Paraffin sections were prepared as described for immunohistochemical analysis. Tissue sections were de-waxed in xylene for 20 minutes and then rehydrated in a decreasing ethanol series (i.e. 100, 95, 90, 80, 70 and 50% for one minute each). Slides were then washed in PBS twice for 5 minutes each. Tissue was then fixed in 4% (w/v) paraformaldehyde for 20 minutes and washed twice more in PBS. Tissue permeabilization was then achieved by treating the tissue sections with 20 µg/ml of proteinase K in 50 mM tris pH 8.0, 5 mM EDTA for approximately 17 minutes at room temperature. Tissue sections were then washed twice in PBS and fixed in 4%
(w/v) paraformaldehyde for 5 minutes. The slides were then rinsed quickly in DEPC-treated water and subsequently washed in 0.1M triethanolamine, 0.25% (v/v) acetic anhydride for 10 minutes at room temperature. Tissues were then washed in PBS, dehydrated through an increasing ethanol series (i.e. 50-100% ethanol) and then air-dried before the addition of the hybridization solution. Digoxigenin (DIG) labeled riboprobes were then added to the freshly prepared hybridization solution (50% (v/v) deionized formamide, 10% (w/v) dextran sulfate, 1.5x Denhardt's reagent, 0.5 mg/ml yeast tRNA, 0.3M NaCl, 0.005 M EDTA, 0.025 Tris pH 7.6) at a concentration of approximately 1 ng/μl. The hybridization solution, containing the appropriate probe was then denatured at 95 °C for 3 minutes and cooled briefly on ice. Finally, the hybridization solution was applied to the tissue sections and slides were incubated in a humid chamber at 55 °C overnight (i.e. 16 to 18 hours). The following morning, the tissue sections were washed in glass Coplin jars containing 5x SSC, 50% (v/v) formamide for 1 hour at 55 °C. The slides were then transferred to coplin jars which were heated only to room temperature and washed in 0.5 M NaCl, 10 mM Tris pH 7.5, 5 mM EDTA for 15 minutes at room temperature. The same wash was then repeated twice, for 10 minutes each, at 37 °C using pre-heated buffer. Tissue sections were then treated with RNase A (10 μg/ml) in the same buffer, at 37 °C, for 30 minutes. This wash served to digest non-specifically bound riboprobe, thereby effectively lowering background staining. Slides were then washed in the same buffer (i.e. 0.5 M NaCl, 10 mM Tris pH 7.5, 5 mM EDTA) for 15 minutes at 37 °C. Finally, the tissue was washed stringently twice in both 2x SSC followed by 0.1x SSC at 50 °C. All subsequent washes and incubations were carried out at room temperature. Slides were incubated in 1 % (w/v) blocking solution (provided in DIG-nucleic acid detection kit from Boehringer Manheim) diluted in 100 mM maleic acid, 150 mM NaCl, pH 7.5 for 1 hour. This step was performed to block non-
specific binding of the primary antibody used to detect the DIG label. A 1:500 dilution of the alkaline phosphatase conjugated anti-DIG antibody, diluted in the same blocking buffer was applied to the tissue sections and left for 1.5 to 2 hours at room temperature. Alternatively, this step was left at 4 °C for 12 to 16 hours (i.e. overnight) in a humid chamber. Slides were then washed in 100 mM maleic acid, 150 mM NaCl, pH 7.5 twice for 15 minutes in order to remove unbound antibody. The pH of the tissue sections was then equilibrated to 9.5 by washing briefly in detection buffer (i.e. 0.1 M Tris-HCL pH 9.5, 0.1 M NaCl, 50 mM MgCl). Colour detection using 175 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 337.5 µg/ml nitroblue tetrazolium salt (NBT) in detection buffer then required approximately 2 hours in the dark. Colour development was then stopped by immersing the slides in distilled water. Finally, the tissue was counter-stained using either methyl green or nuclear fast red, dehydrated (i.e. from 50% ethanol up to xylene) and mounted with Permount and a glass coverslip.

2.9 Immunohistochemistry

Cell recombinants, with untransfected epithelial cells, and fetal rat lungs were fixed in 4% paraformaldehyde in PBS for 24 hours and subsequently paraffin wax embedded. Tissues were cut in series into 5 µm sections and placed on aminopropyltriethoxysilane-coated glass microscope slides. Tissue sections were de-waxed in xylene and dehydrated through a decreasing ethanol series (i.e. 100 to 50% ethanol). Tissue permeabilization was achieved by boiling the sections in 10 mM sodium citrate for five minutes in the microwave. The slides were allowed to cool for 20 minutes in the buffer and were then boiled for another 3 minutes. After cooling for another 15 minutes the slides were washed in PBS twice and endogenous peroxidase activity was blocked by treatment with 3% (v/v) hydrogen peroxide in methanol for 10 minutes.
The slides were then incubated in PBS containing 5% (w/v) normal goat serum (NGS) and 1% (w/v) bovine serum albumin (BSA) for 1 hour in order to block non-specific binding of antibodies. Tissue sections were then washed in PBS and incubated at room temperature for 1 hour with anti-cytokeratin (diluted 1:5 in PBS containing 5% (w/v) NGS and 1% (w/v) BSA), anti-vimentin (neat), anti-proSP-C (1:500), anti-CC10 (1:2000), anti-jagged-1 (1:25) or anti-jagged-2 (1:25). Unbound primary antibody was removed with PBS washes and tissue sections were then incubated for another hour with the appropriate biotinylated secondary antibody. Goat anti-rabbit IgG-biotin was diluted 1:300 and sheep anti-mouse IgG-biotin was diluted 1:200 in PBS containing 5% NGS and 1% BSA. Slides were again washed in PBS and incubated for a further 2 hours with a strepavidin-biotinylated peroxidase complex (ABC). Finally a 10 to 15 minute incubation with 3,3'-diaminobenzidine (DAB) in Tris-buffered saline (TBS, pH 7.6) with 0.001% (v/v) H₂O₂ yielded the colour necessary to detect the protein of interest. Tissue sections were then counter-stained with Carrazzi haemotoxylin, dehydrated and prepared for viewing.
CHAPTER 3. RESULTS

3.1 Tissue Morphology in Early Gestation Recombinants

The morphology of day-13 homotypic and heterotypic recombinants was assessed by immunostaining using monoclonal antibodies against cytokeratin, an epithelial cell-specific marker, and vimentin, a mesenchymal cell-specific marker. Antibody staining revealed that in all cases, lung epithelial cell morphogenesis was determined by the type of inducing mesenchyme present (Figure 2). After 5 days in culture, cell recombinants in which 13-day lung epithelial cells were combined with 19-day lung mesenchyme adopted a lung-like morphology, consisting of open, saccular structures that were lined with positive cytokeratin stained cells (Fig. 2A). In these lung homotypic recombinants, vimentin immunostaining showed the presence of mesenchymal cells in the interstitial matrix of the tissue recombinants (Fig 2D). When 13-day lung epithelium was combined with 19-day skin mesenchyme, organization of the epithelial cells into a skin-like morphology was apparent after 5 days in culture. Cytokeratin staining in these recombinants reveals epithelial cell organization in patterns resembling epidermal cell layers (Fig 2B). In contrast, when 13-day epithelium was recombined with 19-day intestinal mesenchyme, epithelial cells were organized into cytokeratin-positive structures that resembled intestinal villi (Fig. 2C). Finally, the mesenchymal cells surrounding the epithelial structures in both skin and intestine heterotypic recombinants stained positively for vimentin (Fig 2E and F).
Figure 2. Immunostaining to determine cell morphology of homotypic and heterotypic day 13 cell recombinants. Immunocytochemistry, using α–cytokeratin (A-C) and α–vimentin (D–E) monoclonal antibodies, was performed on homotypic and heterotypic recombinants to analyze tissue morphology. The dark brown colour indicates positive staining. The 13-day lung epithelium/19-day lung mesenchyme (A and E) homotypic recombinants display a saccular, lung-like morphology. In contrast, when 13-day lung epithelial cells were recombined with 19-day skin (B and D) or intestinal (C and E) mesenchyme, the recombinant tissue adopted a morphological pattern that is specific to the mesenchymal cell origin (i.e. skin-like and intestine-like morphologies).
3.2 Tissue Morphology in Late Gestation Recombinants

The morphology of late-gestation (i.e. day-19) heterotypic and homotypic recombinants was assessed by immunostaining using monoclonal antibodies against cytokeratin and vimentin. These immunostainings revealed that lung epithelial morphogenesis was also determined by the type of inducing mesenchyme present at late fetal gestation (Fig. 3). Immediately after recombination no specific epithelial cell organization was apparent. However, after 2 to 3 days in culture, organization of epithelial cells into patterns specific to the inducing mesenchyme present was evident by light microscopy observation. After 5 days in culture, epithelial cells of day-19 homologous recombinants (i.e. lung epithelium + lung mesenchyme) were organized into structures having a saccular appearance. These alveolar-like structures stained positively for cytokeratin (Figure 3A) Lung epithelial cells recombined with skin mesenchyme, however, formed structures resembling a more skin-like morphology. An epidermal-like layer of cytokeratin-positive epithelial cells, which rested upon multiple layers of mesenchymal cells, was the characteristic morphology of these recombinants (Figure 3B). Lung epithelial cells, which were combined with intestine mesenchymal cells, formed open cytokeratin-positive structures which remarkably resembled intestinal villi. (Figure 3C) Occasional saccular epithelial structures were also noted in some heterotypic recombinants (i.e. lung epithelium + skin or intestine mesenchyme). Vimentin staining patterns in both homo- and heterotypic recombinants (Figure 3D-F) complemented cytokeratin immunostaining. That is, those cells that surrounded cytokeratin-positive cells stained strongly for vimentin. Similar results were found when the morphology of both homotypic and heterotypic day-21 recombinants was examined. It is also worth mentioning that immunostaining using the anti-vimentin antibody was performed on lung epithelial cells which were cultured alone as controls, in order to assess the purity of our
epithelial cell cultures. Results indicated that the epithelial cultures were free of fibroblast contamination.
Figure 3. Immunostaining to determine cell morphology of homotypic and heterotypic day 19 cell recombinants. Immunocytochemistry, using α–Cytokeratin (A-C) and α–Vimentin (D-F) monoclonal antibodies, was performed on homotypic and heterotypic day-19 cell recombinants to analyze tissue morphology. The dark brown colour indicates positive immunolocalization. Epithelial cells in homotypic lung epithelium/ lung mesenchyme recombinants (A and D) were organized into structures that had a saccular, alveolar-like appearance. Whereas, lung epithelial cells in lung epithelium/ skin mesenchyme (B and E) and lung epithelium/ intestine mesenchyme (C and F) heterotypic recombinants adopted was morphology that specific to the mesenchymal cell origin (i.e. skin and intestine-like morphologies).
3.3 Lung Epithelial Cell Differentiation in Early and Late Gestation Recombinants

To determine the effect of epithelial-mesenchymal interactions on lung epithelial cell differentiation we examined the expression patterns of Surfactant Protein-C (SP-C), Surfactant Protein-B (SP-B) and Clara Cell Protein-10 (CC10) in homotypic and heterotypic recombinants. SP-C is a distal epithelial marker in the developing lung and an alveolar type II cellular differentiation marker in the adult lung. SP-B transcript is normally found in both distal alveolar epithelial cells and some more proximal bronchiolar epithelial cells. CC10 is a bronchiolar epithelial cell marker. Figure 3 shows the expression patterns of these cell specific markers as analyzed by non-radioactive in situ hybridization. The expression patterns of SP-C, SP-B and CC10, in the developing rodent lung, have previously been established (Singh, G. et al., 1986; Kalina, M. et al., 1992; Wollford-Lenane, C.L et al., 1992) and are further confirmed by our study (Fig. 4). The expression of all of these genes increases with advancing gestational age and all are highly expressed at the time of isolation for late-gestation recombinants (i.e. 19 and 21 days of gestation). In addition, at late fetal gestation these cellular differentiation markers are clearly expressed in distinct cell populations. SP-C and SP-B mRNA are primarily localized to the distal epithelial cells (Fig. 4A to E, F to J) whereas CC10 is detected in more proximal epithelial cells (Fig. 4K to O) which line the major airways of the lung.

After confirming the expression patterns of SP-C, SP-B and CC10 in the developing lung, the same riboprobes were used to assess the influence of homotypic and heterotypic mesenchyme on lung epithelial cell differentiation in cells of early (i.e. 13 days) and late (i.e. 19 and 21 days) of fetal gestation. Non-radioactive in situ hybridization results revealed that CC10 transcript was undetectable in the epithelial cells of all 13-day homotypic and heterotypic recombinants (Fig. 5D-F). In contrast, SP-C was weakly expressed in the lung epithelial cells of 13-day lung epithelium/19-day lung mesenchyme (Fig. 5A) homotypic recombinants, but
Figure 4. mRNA expression patterns of SP-C, SP-B and CC10 in the developing rat lung.

A non-radioactive in situ hybridization technique used digoxigenin-labeled RNA probes to analyze SP-C (A-E), SP-B (F-J) and CC10 (K-O) transcript levels in fetal rat lung sections. Positive staining is indicated by the dark blue colour. Sections from rat embryonic day 13 (A,F,K), 16 (B,G,L), 18 (C,H,M), 20 (D,I,N) and 22 (E,J,O) lungs were analyzed for expression levels of all three lung epithelial cell markers. The expression of all lung epithelial cell specific markers increased with increasing gestation age.
Figure 5. Expression of the lung epithelial cell markers SP-C and CC10 in 13 day homotypic and heterotypic recombinants. Non-radioactive in situ hybridization using digoxigenin-labeled SP-C (A-C) and CC10 (D-F) riboprobes was performed on 13-day lung epithelium/19-day lung mesenchyme homotypic recombinants (A and D), 13-day lung epithelium/19-day skin mesenchyme heterotypic recombinants (B and E) and 13-day lung epithelium/19-day intestinal mesenchyme heterotypic recombinants (C and F). Dark blue colour indicates positive staining.
SP-C mRNA was undetectable in both 13-day skin (Fig. 4E) and intestinal (Fig. 4F) heterotypic recombinants.

When CC10 transcript levels were examined in late-stage recombinants (i.e. 19 and 21 days) we found that CC10 mRNA was highly expressed in the epithelial cells of both homotypic and heterotypic day-19 recombinants (Fig. 6A-C). Immunostaining, using a specific anti-CC10 polyclonal antibody confirms the in situ hybridization results (Fig. 6D-F). Expression of CC10 mRNA and protein in the epithelial cells of homotypic and heterotypic day-21 recombinants is comparable in location and intensity, to expression levels in day-19 recombinants (Fig. 8). It is worth mentioning that figure 8 shows only results of day-21 epithelial cells recombined with day 19 mesenchyme, however identical CC10 expression patterns were obtained when day-21 epithelial cells were combined with 21-day mesenchymal cells.

Analysis of SP-C and SP-B mRNA expression levels in 19-day recombinants yielded more surprising results (Fig. 7). Epithelial cells of homotypic lung recombinants expressed high levels of SP-C and SP-B mRNA (Fig. 7A,G) However, in epithelial cells recombined with either skin or intestine mesenchyme both SP-C (Fig. 7B,C) and SP-B (Fig. 7H,I) mRNA expression fell below detectable levels. Similar results were obtained with all day-21 recombinant (Fig. 9). Again, figure 9 shows only staining patterns in 21-day lung epithelial cells recombined with 19-day mesenchyme but identical SP-C and SP-B expression patterns were seen in 21-day cells when cultured in the presence of both 21-day homotypic and heterotypic mesenchymal cells. Finally, when epithelial cells were cultured alone (i.e. no mesenchyme present ) as controls and stained for SP-C and CC10 mRNA expression by in situ hybridization, expression levels of both epithelial markers were similar to those observed in heterotypic recombinations. That is, CC10 mRNA expression remained quite high in the absence of mesenchyme whereas SP-C mRNA expression was no longer detectable in lung epithelial cells cultured alone. Analysis of proSP-C
Figure 6. Expression of the bronchiolar epithelial cell marker, CC10, in day-19 homotypic and heterotypic recombinants. Non-radioactive in situ hybridization using a digoxigenin-labeled CC10 riboprobe was performed on 19-day lung epithelial/lung mesenchyme homotypic recombinants (A) and 19-day, lung epithelial/skin mesenchyme (B) and lung epithelial/intestine mesenchyme (C) heterotypic recombinants. Epithelial cells of all three tissues contained high levels of CC10 transcript. Similarly, immunostaining using an αCC10 polyclonal antibody detected high protein levels in corresponding cell types (D-F). Dark blue and brown colours indicate positive staining.
Figure 7. Expression of the lung epithelial cell markers, SP-C and SP-B in 19-day homotypic and heterotypic recombinants. Non-radioactive in situ hybridization using SP-C (A-C) and SP-B (G-I) digoxigenin-labeled riboprobes was performed on day-19 lung homotypic (A and G) and skin (B and H) and intestine (C and I) heterotypic recombinants. SP-C and SP-B transcripts were only detectable in lung homotypic recombinants (A and G). Immunostaining using an αSP-C polyclonal antibody detected SP-C protein in epithelial cells of both homotypic and heterotypic tissue (D-F). The dark blue and brown colours indicate positive staining.
Figure 8. Expression of the bronchiolar epithelial cell marker, CC10, in 21-day homotypic and heterotypic cell recombinants. Non-radioactive in situ hybridization using a digoxigenin-labeled CC10 riboprobe was performed on day-21 homotypic (A) recombinants and 21-day skin (B) and intestine (C) heterotypic recombinants. Epithelial cells of all three recombinant types contained high levels of CC10 transcript. Similarly, immunostaining using an αCC10 polyclonal antibody detected CC10 protein in the same cells (D-F). Dark blue and brown colour indicates positive staining.
Figure 9. Expression of the lung epithelial cell markers, SP-C and SP-B in 21-day homotypic and heterotypic recombinants. Non-radioactive in situ hybridization using SP-C (A-C) and SP-B (G-I) digoxigenin-labeled riboprobes was performed on day-19 lung homotypic (A and G) and skin (B and H) and intestine (C and I) heterotypic recombinants. SP-C and SP-B transcripts are only detectable in lung homotypic recombinants (A and G). Immunostaining using an α-SP-C polyclonal antibody (D-F) detects high levels of SP-C protein in epithelial cells of lung homotypic recombinants (D) and much lower levels in skin (E) and intestine (F) heterotypic tissue. The dark blue and brown colours indicate positive staining.
protein levels in both 19 (Fig. 7D) and 21 (Fig. 9D) day recombinants yielded positive staining in patterns identical to mRNA localizations in lung homotypic recombinants. However, in contrast to in situ hybridization results, proSP-C protein was detected in the lung epithelial cells of day-19 (Fig. 7E and F) and -21 (Fig. 9E and F) skin and intestine heterotypic recombinants.

The effect of varying mesenchyme on the activation of the SP-C promoter was determined by measuring the activity of SEAP after transient transfection of day-19 epithelial cells with a SEAP2 reporter gene construct under the control of the 3.7 kb human SP-C promoter. SP-C promoter activity as assessed by SEAP luminescence was at consistently low levels in epithelial cells cultured alone, as well as in both skin and intestinal heterotypic recombinants. In contrast, promoter activity was 10- to 100-fold greater in 19-day lung epithelial cells which were recombined with 19-day lung mesenchymal cells (Fig. 10. In all experiments SP-C-SEAP2 constructs were co-transfected with a β-galactosidase construct and all SEAP levels were later normalized to β-galactosidase levels in order to account for transfection efficiencies. In addition, all promoter analysis studies were done in duplicate over 4 separate experiments.
Figure 10. SP-C promoter activity in epithelial cells of homotypic and heterotypic day-19 recombinants. Day-19 lung epithelial cells were transiently transfected with an SP-C-SEAP2 construct and then recombined with either lung homotypic mesenchyme or skin or intestinal heterotypic mesenchyme. Promoter activity was then assessed by measuring SEAP levels in the culture medium. SP-C promoter activation results were significantly different (P<0.05) as analyzed by ANOVA.
3.4 Temporal and Spatial Expression Patterns of the Notch Pathway Components

The expression patterns of all Notch pathway components were determined by non-radioactive in situ hybridization using DIG-labeled riboprobes. Figure 11 shows the expression pattern of the receptors, Notch-1 (A-D), Notch-2 (E-H) and Notch-3 (I-L) throughout rat lung development. From these results it appears that Notch-3 is the most abundant Notch receptor in the developing lung. At relatively early fetal gestation (i.e. 13 and 16 days) Notch-3 transcript was detected in both pulmonary epithelium and mesenchyme (Fig. 11A,B) and by late gestation (20 and 22 days) it was expressed at lower levels, in a more epithelial-specific fashion (Fig. 11K,L). Notch-1 and Notch-2 transcripts were detected at lower levels in the developing lung and had an ubiquitous expression at early gestation (i.e. 13 and 16 days), and later, an epithelial-specific expression which disappeared before birth (i.e. by 22 days of gestation).

The Notch ligands, Jagged-1, Jagged-2 and Delta-1 are all highly expressed in the developing rat lung (Fig. 12). The three ligands showed similar expression patterns which closely resembled that of the Notch receptors. All three ligands were detected in both epithelium and mesenchyme at early gestation (i.e. 13 and 16 days) and by late gestation (i.e. 20 and 22 days) were expressed in a more epithelial-specific pattern. Jagged-1, however, is the only ligand which could be detected in lung epithelial cells at 22 days of gestation, just before birth (Fig. 12D). Jagged-1 and Jagged-2 protein was also detected by immunocytochemistry, using specific polyclonal antibodies, in the same spatial and temporal expression pattern as its corresponding mRNA (Fig. 13).

Examination of the temporal and spatial expression of mammalian Fringe homologues indicates that Lunatic Fringe and Radical Fringe were dynamically expressed in similar patterns throughout rat lung development (Fig. 14A-D, E-H). At 13 days of gestation, both transcripts
Figure 11. Spatial and temporal mRNA expression patterns of the Notch receptors in the developing rat lung. Non-radioactive in situ hybridization was used to determine the temporal and spatial expression patterns of the Notch receptors, Notch-1 (A-D), Notch-2 (E-H) and Notch-3 (I-L) throughout lung development. Tissue sections from fetal rat gestational day 13 (A,E,I), 16 (B,F,J), 20 (C,G,K) and 22 (D,H,L) were used to examine transcript distribution. Dark purple/blue colour indicates positive mRNA staining.
Figure 12. Spatial and temporal mRNA expression patterns of the Notch ligands in the developing rat lung. Non-radioactive in situ hybridization was used to determine the temporal and spatial expression patterns of the ligands, Jagged-1 (A-D), Jagged-2 (E-H) and Delta-1 (I-L) throughout rat lung development. Tissue sections from fetal gestational day 13 (A,E,I), 16 (B,F,J), 20 (C,G,K) and 22 (D,H,L) were used to examine transcript distribution. Dark blue colour indicates positive mRNA staining.
Figure 13. **Jagged-1 and Jagged-2 protein localization.** Immunostaining using specific Jagged-1 (A-D) and Jagged-2 (E-H) polyclonal antibodies indicated that ligand protein could be detected in the same spatial and temporal pattern as its corresponding mRNA (see Fig. 12). Lung tissue sections from fetal day 13 (A,E), 16 (B, F), 20 (C,G) and 22 (D,H) were used to examine protein distribution. Brown colour indicates positive staining.
were predominantly detected in early lung mesenchyme. However, by 16 days, Lunatic and Radical Fringe were expressed strongly in both lung epithelium and mesenchyme. Finally, at 20 days of gestation, both transcripts were expressed in a more epithelial-specific fashion and only very weakly expressed in lung epithelium just before birth (i.e. at 22 days). Manic fringe was weakly expressed in the early rat lung but no appreciable expression could be detected by 20 and 22 days of gestation (Fig. 14I-L).

Finally, the mRNA expression pattern of HES-5, a downstream component of the Notch pathway, was also determined by in situ hybridization (Fig. 15A-D). At 13 days of gestation HES-5 was expressed predominantly in the early lung mesenchyme (Fig. 15A). However, by 16 days, HES-5 transcript was strongly expressed in lung epithelium. Finally, by 20 days of gestation, HES-5 transcript was only weakly detectable in pulmonary epithelium. Figure 15 also shows a comparison of HES-5 mRNA expression patterns to that of lung epithelial markers SP-C (Fig. 15E-H) and CC10 (Fig. 15I-L). It is of interest to note that mRNA expression of both SP-C and CC10 increased at approximately the same time that HES-5 transcript levels decreased.
Figure 14. Spatial and temporal expression patterns of the mammalian Fringe homologues in the developing rat lung. Non-radioactive in situ hybridization was used to determine the temporal and spatial expression patterns of Lunatic Fringe (A-D), Radical Fringe (E-H) and Manic Fringe (I-L) throughout rat lung development. Tissue sections from fetal gestational day 13 (A,E,I), 16 (B,F,J), 20 (C,G,K) and 22 (D,H,L) were analyzed for transcript distribution. Dark blue colour indicates positive mRNA staining.
Figure 15. A comparison of the mRNA expression patterns of the downstream Notch pathway mediator Hes5 and lung epithelial cell markers SP-C and CC10. Non-radioactive
*in situ* hybridization was used to determine the temporal and spatial mRNA expression of Hes5
(A-D) throughout rat lung development. Distribution of Hes5 transcript was then compared to
SP-C (E-H) and CC10 (I-L) mRNA expression patterns. Lung tissue sections from fetal days 13
(A,E,I), 16 (B,F,J), 20 (C,G,K) and 22 (D,H,L) were used to determine mRNA distribution.
Dark blue colour indicates positive staining.
DISCUSSION

4.1 Epithelial-Mesenchymal Interactions in Lung Epithelial Morphogenesis and Differentiation

In the present study we have introduced a novel cell recombination model which may prove useful in future analysis of essential cell-cell interactions during lung organogenesis. Using this system we were able to determine the importance of epithelial-mesenchymal interactions for both epithelial cell morphogenesis and somewhat surprisingly for alveolar type II epithelial cell differentiation at late fetal gestation. There is mounting evidence which suggests that these interactions are critical for proper branching morphogenesis and cell differentiation in the early fetal lung (reviewed in Minoo and King, 1994; Shannon and Deterding, 1997). However, very little is known about the role of interactions between the pulmonary epithelium and mesenchyme during late lung development.

When we examined the morphology of the lung epithelial cells in both the homotypic (lung epithelium/ lung mesenchyme) and heterotypic (lung epithelium/skin mesenchyme and lung epithelium/intestine mesenchyme) 13, 19 and 21 day recombinants, it is clear that epithelial morphogenesis, even at late gestation, is determined by the type of inducing mesenchyme present. These results are consistent with many earlier studies which established that recombining distal lung epithelium with mesenchyme from another source resulted in a cessation of normal lung epithelial branching morphogenesis. Lung epithelial cells in the lung homotypic recombinants displayed a lung-like morphology, whereas lung epithelial cells from skin and intestine heterotypic recombinants adopted a more skin and intestine-like morphology, respectively. To our knowledge this is the first observation that suggests that late gestation lung
epithelium can be reprogrammed to organize into different structures not resembling a normal lung morphology.

To examine the effect of heterotypic mesenchyme on lung epithelial differentiation we examined the mRNA expression patterns of the lung epithelial cell markers, Pulmonary Surfactant Protein-C (SP-C), Pulmonary Surfactant-B (SP-B) and Clara Cell Secretory Protein-10 (CC10). When SP-C transcript levels were examined in day-13 recombinants, after 5 days in culture, we could detect low levels of SP-C mRNA in the epithelial cells of day-13 lung epithelial/day-19 lung mesenchymal homotypic recombinants. These results indicate that although SP-C transcript was not detectable by in situ hybridization in day-13 lung epithelial cells, when these cells were recombined with lung mesenchymal cells of a later stage in development (i.e. 19 days of gestation) the expression of Surfactant Protein-C can be "turned-on" or induced in day-13 epithelial cells. In contrast, when 13-day lung epithelial cells were recombined with either 19-day skin or intestine mesenchyme and cultured for 5 days, SP-C mRNA expression cannot be detected in the lung epithelium. Therefore, these results suggest that the specific developmental signals needed for the induction of SP-C expression, and perhaps the alveolar type II cell phenotype, in early gestation lung epithelial cells (i.e. 13-days) are provided only by late-gestation (i.e. 19-days) lung mesenchyme. However, when CC10 mRNA expression was assessed in day-13 epithelial/day-19 mesenchyme homo- and heterotypic recombinants, CC10 transcript was undetectable by in situ hybridization in all three recombinant types. That CC10 mRNA was absent in epithelial cells of even the 13-day lung epithelium/19-day lung mesenchyme homotypic recombinants was surprising. However, it is well known that developmental processes, including cellular differentiation, occur more slowly in vitro that they do in vivo. Therefore, since CC10 transcript was not detectable in the developing rat lung until 17 days of gestation, and our recombinant model used 13-day epithelial cells which were
cultured for 5 days (the equivalent to 18 days of fetal gestation), it is plausible to suggest that CC10 expression may have been detected in the epithelial cells of homotypic and even heterotypic day-13 recombinants had they been cultured for longer periods of time (i.e. perhaps 7 days in culture).

Expression patterns of the lung epithelial cell markers, SP-C, SP-B and CC10 in both homo- and heterotypic 19- and 21-day recombinants revealed that CC10 mRNA and protein were expressed in the epithelial cells of all three recombinant types (i.e. lung homotypic recombinants and skin and intestine heterotypic recombinants). However, SP-C and SP-B transcripts were only detected in those epithelial cells which were cultured in the presence of lung mesenchyme, even though mRNA expression of both epithelial markers was high in distal lung epithelial cells at the time of isolation (i.e. 19 and 21 days of fetal rat gestation). Although no SP-C transcript could be detected in epithelial cells of heterotypic recombinants by in situ hybridization, immunostaining using an SP-C specific polyclonal antibody detects the presence of SP-C protein in both 19-and 21-day homotypic and heterotypic recombinants. These results identified a discrepancy between mRNA and protein expression. One possible explanation for this discrepancy may be that there exists a difference in mRNA and protein stability, and our antibody staining detected the presence of residual protein remaining after transcription has ceased. Alternatively, our antibody may be exhibiting some sort of specificity problems. In order to sort out this discrepancy, the specificity of our antibody must be examined and our immunostaining results must be repeated and confirmed perhaps using a different SP-C specific antibody. In addition, it would be helpful to confirm our mRNA expression analysis by additional techniques, such as RT-PCR and/or northern blotting.

Similar to in situ hybridization results, promoter activation studies revealed that the level of SP-C promoter activation, in lung epithelial cells, was significantly lower in 19-day skin and
intestine heterotypic recombinants then compared to their homotypic counterparts. These results suggest that at a relatively late fetal gestation, lung epithelial-mesenchymal interactions do not affect CC10 mRNA expression or, perhaps, the bronchiolar epithelial cell phenotype. However, interactions between the epithelium and specifically lung mesenchyme are critical for the maintenance of SP-C and SP-B mRNA expression or, more importantly, the maintenance of the alveolar type II epithelial cell phenotype at relative late fetal gestation.

These data were surprising. We expected that at late gestation, lung epithelial cells would be committed to a specific phenotype and this “differentiated state” would not be subject to changes resulting from various developmental and environmental signals. Hence, it may be appropriate to hypothesize that lung alveolar epithelial cells, particularly alveolar type II cells, require specific factors provided only by lung mesenchyme in order to maintain the alveolar type II cell phenotype during lung development and perhaps even later in the mature lung. Further, these cells may never reach what may be referred to as a “terminally differentiated” state. That is, these cells may maintain the plasticity to respond to specific signals from surrounding mesenchymal and epithelial cells indefinitely. There is strong evidence which suggests that indeed the alveolar type II cell functions as a sort of stem cell in lung injury and repair even in the adult lung (Adamson and Bowden, 1974; 1975). Upon lung injury, the damaged alveolar type I cells undergo cell death and are shed from the epithelial basement membrane. The remaining type II cells in the area then begin to proliferate, thereby reconstituting the region of injury with alveolar type II cells. It is then believed that a portion of these type II cells undergo differentiation into new alveolar type I cells.

It has long been believed that the term “cellular differentiation” represents a progressive process by which options for gene expression are limited within a given cell population as a result of specific and controlled developmental processes. However, Blau and Baltimore (1991)
have argued that in many cases, the maintenance of the differentiated state requires continuous regulation. Their view suggests that cellular differentiation requires active rather than passive inactivation of genes, and maintenance of the differentiated state requires continuous suppression of various genes that are not normally expressed in that particular cell type. As a result, those cells that are being actively maintained in a differentiated state maintain the ability to express other differentiation-related genes and, therefore, can adopt an alternate phenotype under the appropriate conditions. This view provides a genetic mechanism for the phenomenon of cellular plasticity, which according to results presented here, may be an important characteristic of lung epithelial cells, and more specifically, alveolar type II cells.

If we hypothesize that lung alveolar type II cells are maintained in a state of plasticity, even in the mature lung, the important question we must ask then is; what are the factors involved in the maintenance of this state? Are they of mesenchymal origin, epithelial origin, or both? Clearly, some essential factor(s) are provided by specifically lung mesenchyme for both the induction and the maintenance of the alveolar type II cell phenotype in lung epithelial cells. There are a number of studies that support the concept that the differentiated state of lung epithelial cells requires the presence of specific components of the ECM. These studies have demonstrated that alveolar type II cells in culture will lose their cell specific phenotype unless cultured on collagen-gels (Dobbs et al., 1988; Danto et al., 1992) or an ECM extracted from the Engelbreth-Holm-Swarm (EHS) tumor (Shannon, 1992). In addition, others report the importance of various soluble growth factors, cytokines and hormones in both the induction and the maintenance of alveolar type II cell differentiation. Members of the EGF (epidermal growth factor) and FGF (fibroblast growth factor) soluble growth factor families are now thought to be some of the most important factors involved in regulating type II cell differentiation. Taking the results presented here and those from the literature, one might suggest that the mesenchymal
direction and regulation of type II cell differentiation is an extremely complex process which involves the interplay of numerous signaling molecules both during lung development and possibly continuing into adult life. Further studies to determine the specific mechanisms and factors involved in lung epithelial-mesenchymal interactions will lead to a greater understanding of the role of pulmonary mesenchyme in lung epithelial morphogenesis and differentiation.
4.2 The Notch Pathway in Embryonic Lung Development

In light of the Notch pathway’s role in the regulation of cellular differentiation and recent evidence which suggests it plays an important role in *Drosophila* tracheal development (Llimargas, 1999), it is reasonable to hypothesize that perhaps it plays an important role in lung development, and specifically in lung epithelial cell differentiation. In order to begin investigating a potential role for the Notch pathway in fetal lung development we used a non-radioactive *in situ* hybridization technique to characterize the spatial and temporal mRNA expression patterns of the Notch pathway components during fetal rat lung development. Digoxigenin-labeled riboprobes, complimentary to specific regions of the Notch-1, -2 and -3 receptors, Jagged-1,-2 and Delta-1 ligands, Lunatic, Radical and Manic Fringe and the downstream mediator, Hes5, were generated and used to determine which of these signaling molecules are present in the developing lung. Unfortunately, specific antibodies against all the Notch pathway components are not yet available. So, with the exception of Jagged-1 and -2, our analysis has been limited to only mRNA expression patterns. However, as these antibodies become available we will be able to correlate our mRNA expression patterns to protein expression patterns by immunocytochemistry. In addition, specific Notch-4 and Delta-3 probes were not available at the time of completion of this study. Therefore in order to complete our mRNA expression pattern analysis we will obtain or generate these probes in the future.

It is of interest to note that while *in situ* hybridization is not a particularly quantitative measurement of transcript levels, we have taken certain steps to ensure that our experiments allowed us to make semi-quantitative statements about the mRNA expression levels of each of these genes. In other words, when looking at the mRNA expression pattern of a particular gene we performed *in situ* hybridization on tissue sections from all gestational ages in the same
experiment using the same probe concentration on all tissue. In addition, all NBT/BCIP colour
detection steps of the experiments were stopped at the same time in all tissues. That is, colour
detection in tissue from 22 days of gestation was stopped at the same time as, for example,
detection in gestational day 13 tissue when using a single probe. These steps allowed us to make
comparisons regarding the relative mRNA expression level of a single gene at early gestation
versus its levels at late gestation (i.e. Lunatic Fringe expression levels at 13 days of gestation
versus 22 days of gestation). However, comparison of mRNA expression levels between
different genes is more difficult (i.e. Lunatic fringe expression levels versus Radical Fringe
expression levels at 13 days of gestation). Every effort was made to use the same concentration
between different probes, but there are still small inaccuracies which may occur as a result of
differences in in vitro transcription efficiency (i.e. probe labeling efficiency) and in probe
hybridization efficiency, which make comparisons of mRNA expression levels between different
genes difficult. Finally, it is of importance to mention that the mRNA expression levels shown in
our studies only represent those that are detectable within a 2- to 3-hour colour detection
incubation. Therefore, some genes seem not to be expressed at all by the end of gestation (i.e. 22
days). However, if these colour detections were carried out overnight (i.e. for 12 to 16 hours) it
is likely, or at least possible, that some mRNA expression would be detected. For example, our
preliminary studies indicated that after a 16 hour colour detection, Lunatic Fringe mRNA
expression could be detected in both fetal day 22 and post-natal lung tissue, however, after a 2
hour incubation no expression was detected. Therefore it is important to keep in mind that the
expression levels we show here are only those which are detectable through a relatively short
colour detection.

The results of our Notch pathway mRNA expression pattern analysis suggested that a
number of the pathway components are highly expressed in the developing rat lung. All three of
the Notch receptors studied (i.e. Notch-1, Notch-2 and Notch-3) are expressed in the fetal lung, however, the Notch-3 expression pattern suggests that it may be the most important of these receptors for a proposed function during mammalian lung development. In addition, the three ligands studied (i.e. Jagged-1, Jagged-2 and Delta-1) all seem to be highly expressed in the fetal lung in patterns which largely overlap with Notch-3 mRNA expression patterns. The three ligands and the Notch-3 receptor are ubiquitously expressed at early gestation (<16 days of gestation) and later in development all three genes show an epithelial-specific expression pattern (i.e. by 20 days of gestation). Thus, it may be appropriate to hypothesize that Notch signaling may be involved in either or both epithelial-mesenchymal interactions or epithelial-epithelial cell signaling during early lung development, and epithelial-epithelial interactions in order to regulate cell differentiation during late lung development. In addition, Notch signaling and the latter proposed epithelial-epithelial cell interactions may be important for regulating alveolar type II cell to alveolar type I cell conversion.

The expression patterns of the mammalian Fringe homologues in the developing lung indicate that both Lunatic Fringe and Radical Fringe may play important role in regulating Notch-ligand interactions throughout lung development. Both genes are expressed in similar dynamic patterns which consist of mainly mesenchymal mRNA expression at 13 days of gestation, moving to both epithelial and mesenchymal expression by mid-gestation (i.e. 16 days), and later switching to an epithelial-specific pattern by late lung development (i.e. 20 days). Since both Fringe homologues display almost identical expression, it is possible that Lunatic and Radical fringe serve redundant functions in Notch regulation of fetal lung development.

Finally, the expression pattern of the downstream Notch pathway mediator, Hes5, suggests that its transcription may be regulated by Notch activation during early lung development. Hes5 is ubiquitously expressed in the lung primordium (i.e. 13 days of gestation)
and is predominantly expressed in undifferentiated lung epithelium at 16 days of gestation. However, its expression levels fall below detectable levels in differentiating lung epithelium during later lung development. These results then suggest the possibility that Hes5, through the activation of the Notch receptor, functions as an antagonist of epithelial cell differentiation during early lung development. When we compare the expression patterns of the lung epithelial cell specific markers, SP-C and CC10, to the development pattern of Hes5 expression, a striking difference is immediately apparent. A sharp reduction in Hes5 mRNA expression, as compared to its earlier expression levels, is seen just as lung epithelial cells begin to express cell specific markers, indicating epithelial cell differentiation has occurred. Therefore, the comparison of these cell marker expression patterns to Hes5 expression patterns further supports a potential role for Hes5 and the Notch pathway in the control of lung epithelial cell differentiation.

In summary, our analysis of the temporal and spatial expression patterns of the Notch pathway components indicates a potential role for the Notch pathway in regulating lung epithelial cell differentiation during mammalian lung development. Further studies, using both transgenic mouse over-expression and genetic knock-out models, will help us to better understand the specific function of the Notch pathway in embryonic lung development and at what times during development it plays important roles in regulating lung cell differentiation. Further, specific in vitro models, such as the cell recombinant model discussed earlier, may prove useful in determining whether Notch signaling and the expression of the pathway components, are regulated by epithelial-mesenchymal interactions in the developing mammalian lung.
REFERENCES


