EXPLORING THE SUITABILITY OF A SPECIFIC GLUCOCORTICOID RECEPTOR ANTAGONIST AS A TOOL IN THE STUDY OF THE REGULATION OF RAT LUNG ALVEOLARIZATION BY GLUCOCORTICOIDS

By
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ABSTRACT

Exploring the suitability of a specific glucocorticoid receptor antagonist as a tool in the study of the regulation of rat lung alveolarization by glucocorticoids

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Background: Intracellular glucocorticoid receptors (GRs) mediate the regulation of lung development, including alveolarization, by glucocorticoids (GCs). One potential approach to determining the role of GC-GR signalling in alveolar formation would be by pharmacologic blockade.

Hypothesis: CP472555, a novel GR antagonist with negligible anti-PR activity, is a suitable tool for the study of GC-GR regulation of rat alveolarization.

Design/Methods: CP472555 doses needed to block GR were estimated in vitro in fetal rat lung primary cultures. Postnatally, a variety of doses were administered intraperitoneally over a range of days.

Results: During postnatal days (PN)0-PN10, when GC levels are low, CP472555 induced changes consistent with GR agonist activity. While GC levels increase after PN11, animals exposed to CP472555 from PN11-PN21 exhibit changes consistent with anti-GR antagonist activity.

Conclusion: CP472555 causes a degree of GR blockade sufficient to permit further pharmacological investigation of the role of endogenous GC-GR signalling at the end of alveolarization.
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LIST OF ABBREVIATIONS

AEC: Alveolar epithelial cells
AFs: Adjacent fibroblasts
AP-1: Activator protein-1
AQP: Aquaporin
ATI: Alveolar type I
ATII: Alveolar type II
BPD: Bronchopulmonary dysplasia
BSA: Bovine serum albumin
BW: Body weight
Cav-1: Caveolin-1
CBG: Corticosteroid-binding globulin
CC10: Clara cell secretory protein 10
CCT: Choline-phosphate cytidylyltransferase
CDKI: Cyclin dependent kinase inhibitor
cDNA: Complementary DNA
CRH: Corticotrophin releasing hormone
DBD: DNA binding domain
Dex: Dexamethasone
DPPC: Dipalmitoyl phosphatidylcholine
DTT: Dithiothreitol
DNA: Deoxyribonucleic Acid
ECM: Extracellular matrix
ENaC: Epithelial sodium channel
FA: Fatty acid
FAS: Fatty acid synthase
FBMs: Fetal breathing movements
FGF: fibroblast growth factor
FOXA2: Forkhead homolog A2
GCs: Glucocorticoids
PFs: Peripheral fibroblasts
PDGF: platelet derived growth factor
PN: Postnatal day
RA: Retinoic acid
RDS: Respiratory distress syndrome
RNA: Ribonucleic acid
rRNA: Ribosomal RNA
RT-PCR: Reverse transcription PCR
SHRP: stress-hyporesponsive period
α-SMA: α-smooth muscle actin
SMC: Smooth muscle cell
SP: Surfactant protein
STAT: Signal transducer and activator of transcription
TFs: Transcription factors
TGF: Transforming growth factor
TTF-1: Thyroid transcription factor
THY-1: Thymocyte differentiation antigen 1
VEGF: vascular endothelial growth factor
Chapter One: Introduction
1.1 **Introduction: The Mammalian Lung**

The mature mammalian lung is made up of a system of airways that conduct air to the acinus and the alveoli, which are respectively the respiratory region of the lung and its gas-exchanging units. In humans there are 23 generations of airway branching, which begin at the primary bronchi and continue to the distal air sacs. Proceeding along the airways, airway diameter progressively diminishes as cross-sectional area increases. Surface area increases dramatically at the acinus, such that the alveolar surface area in the mature lung covers about 70m² (72). Moreover, the alveolo-capillary barrier, the tissue that separates the alveoli from the vasculature, is only 0.2 μm thick, allowing for sufficient gas exchange (170).

1.2 **Stages of Mammalian Lung Development**

The process of lung development is divided into stages based on anatomical and morphological appearance; it is nonetheless a continuous and centrifugal process, with gradual transitions and overlap from one stage to the next (44; 170). The experimentally accessible rat has served as a useful model for the study of mammalian lung development, with the basic mechanisms of the different stages well preserved relative to humans. While alveolarization begins during the prenatal period in humans, it occurs entirely postnatally in rats. The stages of lung development (Table 1), discussed below, are as follows: embryonic, pseudoglandular, canalicular, saccular, and alveolar. It is important to note that these developmental stages are influenced by glucocorticoids (GCs), which will be discussed in detail in section 1.7.
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Physiological Events
- Cell lineage determination
- Branching morphogenesis
- Epithelial cell differentiation
- Surfactant synthesis
- Blood-gas interface establishment
- Alveolarization

**Table 1. The five stages of lung development in rats and humans.** Units in days for rats and weeks for humans, unless otherwise noted.

### 1.2.1 Embryonic Period (4 – 7 weeks gestation in humans, gestational days 5 – 15.5 in rats)

The embryonic period is the developmental phase during which the lung and other organs are laid down. The lung arises from the laryngo-tracheal groove, a diverticulum of endoderm from the ventral primitive foregut. In humans, the paired endodermal lung buds originate at the end of week 4 from the laryngo-tracheal groove, at this point outpouching from the primitive anterior pharynx. At 5 to 6 weeks, the ventral laryngo-tracheal groove pinches away from the dorsal primitive esophagus to form the primitive trachea and simultaneously the two primary bronchial branches. Similarly, in the rat at gestational day 14.5, the laryngo-tracheal groove bifurcates into paired lung buds, which elongate as the anteriorly-directed ventral trachea pinches away from the pharyngeal region of the primitive foregut, separating from the dorsal esophagus. The two lung buds divide into lobar buds, two on the left and three on the right in humans (one on the left and four on the right in rodents), which define the prospective lobar bronchi and lung lobes. The epithelium-lined lung buds elongate into the surrounding primitive mesenchyme, which regulates the growth and branching of the epithelium.
1.2.2 Pseudoglandular (7 – 16 weeks gestation in humans, gestational days 15 – 17.5 in rats)

The pseudoglandular stage is so-called due to the lung’s glandular appearance upon histology. During this phase, the conductive tracheobronchial tree is formed as the lung bud undergoes reproducible branching via intricate mesenchymal-epithelial interactions (170). This repetitive branching results in completion of the trachea, bronchi and terminal bronchi out to 16 generations by the end of the 16th week; after this, branches can grow in size but new branches can no longer form (170; 328). The 7 remaining generations of branches develop during the latter part of gestation (328). The progressive branching is paralleled by active division of the epithelial cells in the developing lung buds (57). The epithelium takes on a tall columnar shape in the proximal airways and gradually transitions to single-layered cuboidal epithelium distally at the bronchioles (44). The first epithelial cells to differentiate are the neuroendocrine cells at 8 weeks’ gestation (76). By 10 weeks’ gestation, the epithelium appears as columnar cells superficial to irregular deeper basal layers on a basement membrane. By the 12th week, mucous glands form in the bottom of epithelial folds and then penetrate the well-developed and complete basement membrane. Mucosal tubules appear in the proximal glands at 14 weeks’ gestation. Goblet cells appear as vacuolated cells in the epithelial crypt folds at 13 weeks and are well established by 16 weeks’ gestation. The cilia appear centrally by the 13th week, and the majority of the aforementioned cells progress outward in their development and cytodifferentiation with further development of the peripheral bronchial segments (47; 57). The extreme periphery however is an exception, with the epithelium remaining undifferentiated until the alveolar period (44). In a similar centrifugal fashion, cartilage is first found centrally before appearing in segmental bronchi and then in the bronchioles near weeks 10, 12, and until week 25 respectively.
The cartilage differentiates from the mesenchyme adjacent to the endoderm-derived lung bud, as do the connective tissue, muscle, blood vessels, and lymphatics (170). The development of the diaphragm, the muscular structure regulating fetal breathing movements and responsible for driving postnatal lung ventilation, occurs during the 7th week. The major portion develops early on from the septum transversum, a ventral mesodermal fold about the growing abdominal organs. The lateral contribution of the diaphragm comes from the thoracic muscles and the pleuropitoneal membranes, which develop from the lateral thoracic wall and shut the canals connecting the pleural and peritoneal cavities. Additionally, the esophageal mesentery and the ventral pleural sac make minor contributions to the diaphragm. Embryologic development of the diaphragm is complete by the end of 7 weeks’ gestation (57), and by 10 weeks’ gestation, fetal breathing movements can be observed (170).

1.2.3 Canicular (16 – 24 weeks gestation in humans, gestational days 17 – 19.5 in rats)

The canicular stage encompasses the foundation and vascularization of the forthcoming gas exchange surface of the lung, the pulmonary acini. The end of the 17th week is marked by this newly defined acinus, which consists of a stem tubule, the prospective terminal bronchiole and respiratory bronchioles, and small clusters of short tubules and buds (43). With time, branching continues peripherally as tubular branches lengthen, resulting in about 3 additional generations of branching (43). This peripheral growth occurs in parallel with the rapid proliferation of capillaries, which are initially situated in a loose network within the mesenchyme (170). As the distal airspaces widen at the expense of the surrounding mesenchyme, the capillaries are consequently arranged in much closer proximity to the airway epithelium; the cuboidal epithelium at these points continues to flatten and a thin air-blood barrier is formed. At
22 to 24 weeks’ gestation, the acinar epithelium begins its differentiation into alveolar epithelial cells (AEC), more precisely into alveolar type II (ATII) cells. The fat, rounded ATII cells are responsible for the synthesis, storage and secretion of surfactant. Alveolar type I (ATI) cells, which are derived from ATII cells, in contrast take on a flat appearance, and mediate gas exchange across the alveoli. The end of the canalicular stage marks an important point in fetal development because of the potential for gas exchange to occur should the baby be born prematurely (44).

1.2.4 Saccular (24 – 36 weeks gestation in humans, gestational days 19.5 – postnatal day 8 in rats)

The saccular stage is characterized by a substantial increase in the size of the prospective gas-exchange parenchyma, whose development is necessary for successful adaptation to extrauterine life (258). This expansion is due to further lengthening and widening of the airways distal to the terminal bronchioles, as well as the formation of the final generations of the airway tree (44). The smooth proximal ends of the peripheral airways are interrupted by thick crests, which in turn protrude into clusters of terminal widened airspaces, or saccules. With the crests, or primary septa, introducing a capillary network into saccules, they are divided into subsaccules (170). As the airspaces widen there is a concurrent thinning in the intervening interstitial tissue; this, along with the continued flattening of the epithelium results in a marked thinning of the airspace walls (44; 170). Airspace wall thinning and an increase in vascular growth permits the capillary networks to become closer (44; 47). By the 36\textsuperscript{th} week, functional maturation of the lung is achieved as ATII cells have matured and secrete surfactant (or otherwise they have differentiated into ATI cells) (170).
1.2.5 Alveolar (36 weeks – at least 3 years after birth in humans, postnatal days 3 to 28 in rats)

The human lung is immature at birth, born during the phase of alveolarization. With 85% of over 300 million alveoli in the adult lung forming after birth, alveolar formation is considered mainly postnatal in humans; this is similar then to the entirely postnatal process in the rat (44; 46). The formation of alveoli first occurs dramatically during a period of bulk alveolarization, and continues afterwards at a much slower rate. In humans, bulk alveolarization is achieved by the 6th month, while the parallel event occurs in the rat from postnatal day (PN) 3 to PN14 (44; 45). Firm conclusions on the precise timing of the termination of alveolarization have been difficult to draw (44); however, what is believed is that while most of alveolarization is complete by 18 to 24 months in humans, it continues on to a lesser degree for 6 years thereafter (44; 170).

The walls of the channels and saccules (or, the primary septa) of the lung at birth are smoothly contoured and relatively thick, with a central sheath of connective tissue flanked on either side by a capillary layer (45). Alveolar formation begins with low ridges bulging from both sides of the saccular walls. This results in the incomplete partitioning of the saccular lung walls into smaller units: channels becoming alveolar ducts, and saccules becoming alveolar sacs, and thus alveoli arise. The low ridges which elongate into the primitive interalveolar walls, or secondary septa/crests, display a similar ultrastructure to that of the primary septa (containing a sheet of connective tissue flanked by a capillary layer at both sides) (44). Capillary loops over the edge of the septum sometimes interconnect capillary layers. Located at the tip of the secondary septum, or below the capillary loop, are elastin fibres, which have been deposited there by migrating alveolar myofibroblasts; these elastin fibres play a critical role in alveolarization (see section 1.4.2.1).
Figure 1.1. Alveolarization. A. Simplified schematic representation of secondary septation during alveolarization in the postnatal lung. New (secondary) septa are lifted off immature (primary) septa containing a double capillary network. B. Microvascular maturation. The interstitial tissue thins, and the capillaries fuse and transform the double capillary layer into a single capillary layer. Adapted from (82).

The primary and secondary septa are thick and contain double capillary layers early during alveolarization; this contrasts sharply with the single capillary layer in the thin interalveolar walls in the adult (45). The final stages of alveolarization then involve extensive septal thinning, followed by the maturation of the microvasculature and the development of the single capillary network (see section 1.4). The mature gas exchange units of the lung consist of thin ATI cells overlying the central single capillary layer, with cuboidal ATII cells distributed linearly along thick elastin fibers at the angles of the alveoli (127).

1.3 Development of the Pulmonary Vasculature

1.3.1 Fetal

Occurring in concert with the development of the airways, lung vascularization involves two processes, vasculogenesis and angiogenesis. The former involves the transdifferentiation of
mesodermal cells into endothelial cells, which proliferate into multicellular structures that form a single layer around a central lumen. This \textit{de novo} process occurs early on in the periphery of the embryonic lung. Angiogenesis, which leads to the development of the pulmonary artery and vein, is a process whereby new vascular structures form as extensions from previously formed vessels; this is achieved via further proliferation of differentiated endothelial cells (328). As early as 34 days gestation, each primitive lung bud is supplied by a pulmonary artery running parallel the trachea, and is connected to the prospective left atrium via a pulmonary vein (122).

During the pseudoglandular phase, vasculogenesis leads to the formation of the pre-acinar pulmonary arteries, which grow alongside the developing branches of the airway, and the pre-acinar pulmonary veins, which lie in the intersegmental plane. By 16 weeks gestation, much in the same way that all of the branches of the conductive airways are present at this point, new pre-acinar arteries branches can no longer form; existing branches will only grow via an increase in length and diameter. Development of the pre-acinar veins follows that of the arteries, but is nonetheless complete by 20 weeks gestation (43; 170).

Starting in the canalicular phase, the intra-acinar arteries and veins form via angiogenesis. In contrast to the pre-acinar vessels, they continue to increase in number after birth and follow the development of the alveoli. The intra-acinar vessels are located parallel to the respiratory bronchioles and prospective alveolar ducts or within prospective alveolar walls (143; 170). Vascularization, together with epithelial thinning (further promoted by the closeness of the underlying capillaries to the epithelium due to mesenchymal thinning), results in the formation the blood-gas barrier (122; 170).

A complete vascular circuit exists by the start of the saccular stage, formed via the fusion of the angiogenic and vasculogenic networks. As the capillary networks surrounding the future
airspaces come closer together, they form a double capillary network in the primary septa; the septa are characterised by a thin central sheet of connective tissue flanked on either side by a capillary layer (44; 122).

1.3.2 Postnatal

As described previously, the newborn lung is immature, characterized by primary septa. During the alveolar stage, secondary septa arise from primary septa via folding-up of one layer of the double-layered capillary network, which in turn gives rise to new, secondary septa containing their own double-layered capillary network (44; 258). It is evident that the vascular network plays an important role in alveolar development, and this extends to its final stages as well. During the process of microvascular maturation, there is an increase in the rate of apoptosis of the central layer of connective tissue in the septa such that the layer thins, resulting in increased proximity of the two capillary layers (44; 276). The two capillaries then fuse into a single capillary network, with the thinned connective tissue sheet on either its left or right side. After the process of capillary fusion, the capillaries undergo preferential growth of the fused areas, whereby the growth spreads to the immature areas that have not yet fused. This promotes a mature single capillary network and significantly extends the vasculature.
1.4 **Alveolarization**

### 1.4.1 Two phase model of alveolarization

Until recently, it was thought that once the double-layered capillaries in the alveolar septa were reduced to single-layered capillaries via the process of microvascular maturation, additional new alveolar septa could no longer arise from these single-capillary alveoli. Consequently, expansion of the gas-exchange area would have to occur via lung growth (48; 200). Supporting evidence for this concept arose from studies of postnatal lung development in the rat. First, Meyrick and Reid (212) found that there is an early spurt in alveolar multiplication, with alveolar density increasing between PN3 to PN8, and then remaining constant from PN8 onwards to the end of the study period at PN35. Subsequently, a study by Randell *et al.* (261) demonstrated that total alveolar number increased linearly until PN21. The two phase model of alveolarization arose from these findings. It proposes that in rats, the first phase of alveolar formation occurs via secondary septation in the first week of life. After this period and the maturation of the vasculature, the second phase of alveolar formation is not accomplished via septation, but rather through peripheral extension (48; 200).

Contrary to the two phase model of alveolarization, an *avant-garde* study offers evidence that alveolarization occurs via new septation following vascular maturation in the rat lung (277). Schitny *et al.* followed the growth of existing secondary septa and the appearance of new secondary septa throughout postnatal development by measuring their length. The formation of new secondary crests was delineated by short-length septa. Following the completion of microvascular maturation (PN21 in rats (269)), it was demonstrated that half of the secondary septa formed by lifting off of existing septa. This process continued throughout adolescence and
into young adulthood (PN60) (277). A similar observation in rhesus monkeys by Hyde et al. lends support to this concept. Although microvascular maturation is known to be essentially complete at birth in rhesus monkeys (277), the number of new alveoli counted continued to increase until young adulthood (130). Complementary to these findings are studies demonstrating new alveolar formation with refeeding after alveolar reduction induced by starvation in rodents (199; 204), and in compensatory lung growth following unilateral or partial pneumectomies in rodent and dog models (39; 343). With the growing evidence that new alveoli continue to form after pulmonary vascular maturation, a modified two phase model of alveolarization has been proposed.

The new model is still in accord with the current one in that a double-layered capillary network is required at the site of septation. The difference is that the double-layer need not be pre-existing, but rather may be formed by angiogenesis locally and immediately on demand (277). Two overlapping “phases of developmental alveolarization” are depicted, which distinguishes developmental alveolarization from that which may be regenerative. Developmental phase one involves rapid alveolar formation from immature septa with a double-layered capillary network (PN4 – PN21). This exceeds the growth of the lung parenchyma, in turn decreasing the size of the terminal air spaces. In comparison to the current classification, phase one of developmental alveolarization corresponds to the classical phase of alveolarization, from PN4 to PN14. Including new alveolar formation from mature pre-existing septa, phase two of the latest proposal begins at PN14 and continues on to young adulthood (277). In comparison to the current classification, phase two of developmental alveolarization corresponds to late
alveolarization, which is described as any kind of alveolar formation occurring after PN21, when microvascular maturation is complete (45; 277).

1.4.2 The alveolarization process

Although much remains to be understood about the process of alveolarization, it involves a plethora of interactions (e.g., cell-cell, cell-extracellular matrix) and mechanisms acting synchronously.

1.4.2.1 Extracellular matrix

The extracellular matrix (ECM) is an organized network of proteins, glycoproteins, proteoglycans, and glycosaminoglycans (e.g., collagens, laminins, fibronectin, tenascin, enactin) to which cells adhere (1; 87). It is an important component of the cellular environment, functioning not only as crucial structural support in the lung, but also in the organization, differentiation, and function of the lung’s cellular component. In particular, cell-ECM interactions are essential for pulmonary development and function.

In rat postnatal lung development, production and renewal of the ECM are augmented. One such component of the ECM is collagen. A delicate and complex collagen network allows for normal septation (197), while in models of lung injury, collagen content is altered with respect to its quantity, appearance or deposition (311). In the rodent, maximal interstitial collagen (e.g., collagen types I and III) and basement membrane collagen (i.e., type IV) gene expression coincide with alveolarization (195). At this time, 40% of the newly formed collagen degrades within a few hours (11). Rapid renewal of type IV collagen is of particular importance, since it is the major constituent of the basement membrane throughout the terminal airways and
airs. During rat lung development, there are considerable amounts of closures and
openings of basement membrane discontinuities; this discontinuity permits intercellular
communication between the AECs and the interstitial cells, critical for AEC differentiation (4).
In addition to a role in AEC differentiation, the basement membrane collagens play a role in the
vascularization of the alveoli (type IV collagen is also a major component of the basal lamina of
blood vessels), while the interstitial collagens are likely required for the elongation of the
alveolar septa (195).

Elastin is another component of the ECM that is important for maintaining the lung’s
structural and functional integrity. In the mature lung, elastin fibres are concentrated in a ring
around the mouth of each alveolus, conferring resilience as they stretch and contract during
respiration (333). As one of the body’s most stable proteins, elastin fibres will undergo little
remodelling in adult life under normal conditions (75).

In the developing lung, elastin fibres also play an essential role in alveolarization, as is
suggested by their appearance in the lung immediately prior to alveolar formation and by their
localization to the tips of the secondary crests. In fact, maximal elastin precursor synthesis and
secretion by myofibroblasts corresponds to the maximal period of secondary septa formation
(230). Tropoelastin, the non-polymerized elastin precursor, is a soluble protein of approximately
70kDA in molecular weight with many cross-linking domains. Cross links in collagen and
elastin are formed via a reaction catalyzed by the enzyme lysyl oxidase (LOX) (75). Treatment
of rat pups postnatally with β-aminopropionitrile, a LOX inhibitor, to reduce cross-linking of
collagen and elastin results in the inhibition of septation and reduces the total number of alveoli
(78). In the platelet derived growth factor (PDGF)-A null mouse model (see sections 1.5.2.2 and
where myofibroblasts fail to form thereby leading to the absence of elastin expression in the pulmonary parenchyma postnatally, the mice appear normal until PN4. After PN4, the failure of alveolarization becomes evident as the distal air spaces appear dilated and thin-walled (34). In another mouse model of complete disruption of elastin expression, the result was failed development of the terminal airway branches during the perinatal period, and consequently in fewer, dilated distal air sacs at birth and nonsurvival past PN4. This demonstrates an important role for elastin during fetal pulmonary development, in addition to the well-established postnatal role of elastin in the structure and function of mature alveoli (333).

Elastin gene expression has been shown to be modified by various factors, including TGF-β, basic fibroblast growth factor, retinoic acid and GCs (75). As rat lung development progresses from fetal to early and late neonatal stages, tropoelastin mRNA levels increase. Furthermore, administration of GCs to pregnant dams stimulates elastin production in addition to accelerating lung development (254; 304). The increase in mRNA levels is accompanied by a similar increase in pre-mRNA levels, suggesting that GCs influence tropoelastin transcription in the developing lung (304). The 5’ flanking segment of tropoelastin has GREs (268). Furthermore, GCs modulate LOX activity. In some developing tissues, GCs stimulate LOX expression (290). In the lung however, GCs are known to alter its structural integrity by inhibiting LOX activity, leading to a reduction in cross-linking between the collagen and elastin fibre networks (24).

1.4.2.2 Proliferation and apoptosis during lung development

Lung morphogenesis entails structural changes involving the tightly regulated processes of cell proliferation, apoptosis, and differentiation. The rates of these three processes are crucial
for ultimately establishing an effective gas-exchange area and for maintaining lung tissue homeostasis (277; 319).

1.4.2.2.1 Proliferation

In the developing lung, proliferation of cells occurs first in the central tubular regions undergoing morphogenetic changes, and progresses to the periphery. Proliferation of epithelial cells increases initially during fetal development, but declines at late gestation, corresponding to an increase in epithelial cell differentiation. The amount of mesenchymal cells undergoing cell division decreases initially during fetal development but, as capillary formation emerges in the canalicular stage, there is a sharp rise in proliferation. Although fibroblast growth slowly declines during late gestation, the rate of vascular growth continues rapidly throughout late gestation such that mesenchymal cells constitute the main dividing cell type near term (307). Overall, epithelial and mesenchymal cell proliferation rates both decrease as the lung develops, but they do so at variance. Such differences in proliferation rates can be illustrated by comparing the ratio of epithelial to mesenchymal cell numbers: early on at the pseudoglandular stage the ratio is 1:4; it subsequently increases to 1:1 by the end of the canalicular stage, and decreases again at term (3). During alveolarization, both epithelial and mesenchymal cells undergo a rapid phase of proliferation.

Fibroblast number increases 4-fold in rats, with 14% of fibroblast cells proliferating at this point (compared to less than 1% after alveolar multiplication). Similarly, the percentage of AECs proliferating is 5-6%, compared to a percentage that is otherwise quite low (82). Occurring primarily in septal crests, interstitial fibroblast cell proliferation is predominant early on, while endothelial proliferation is rapid throughout (307). Occurring in septal buds and walls,
both ATII and ATI cell populations increase; however only the ATII cells are proliferating, while ATI arise via differentiation from ATII cells.

1.4.2.2 Apoptosis

Apoptosis, a physiologic programmed cell death, is a regulated process whereby most cells follow a typical pattern of morphologic changes including cell shrinkage, reorganization and segmentation of the nucleus, membrane blebbing, and fragmentation of the cell into membrane-enclosed apoptotic bodies. Neighbouring cells remain free from injury and all cells will die without an inflammatory reaction, thus distinguishing the process from necrosis (276). Apoptosis is necessary to achieve progressing and final morphologies in fetal and postnatal pulmonary development, as well as in the restoration of epithelium following lung injury. Apoptosis in the lung, and in other organs such as the thymus (132; 340), is under GC control.

Apoptosis is detected in the mesenchymal tissue as early as the embryonic stage (167). Beginning in the canalicular stage and continuing onwards, there is not only a shift towards apoptosis of both epithelial and mesenchymal tissues, but also a rapid increase in the apoptotic rate which remains elevated until shortly after term. Changes in proliferation and apoptosis at this point in morphogenesis are thought to be stimulated by changes in hormonal levels, parturition, and fetal breathing movements which result in mechanical strain and a change in cell shape (167).

After birth, the rate of apoptosis in the postnatal rat lung remains elevated due to involvement in vascular maturation and the requisite for thinning of the gas-exchange tissue. By the end of the third postnatal week in rats, there is an excess of ATII cells. This may serve to increase surfactant production and/or to provide sufficient progenitor cells for differentiation to
ATI cells to contribute to the increasing area of the gas-exchange surface of the alveoli (276). During the third postnatal week in rats, the lung undergoes remodelling of the secondary septa and microvascular maturation, whereby the cells in the connective tissue separating the double-capillary layer are reduced in number via typical apoptosis. There is also evidence that ATII cells undergo apoptosis, however not all of the typical morphologic signs appear. This is thought to be due to advanced removal of cells by macrophages at an early stage of cell death (276). Thus, total ATII cell number is reduced not only by differentiation to ATI cells (319), but also via apoptosis (276).

1.5 **Lung Cell Types: Epithelial and Mesenchymal Cells**

The mammalian lung consists of various cell types that function collectively in order to provide the machinery required for respiration. These include alveolar epithelial cells and mesenchymal cells.

1.5.1 **Alveolar epithelial cells (AECs)**

The functions of the alveolar epithelium include fluid homeostasis, surfactant synthesis, immune protection and gas exchange. It is comprised of two distinct cell types, alveolar type I (ATI) cells and alveolar type II (ATII) cells, and they subserve different functions. While only about one third of the mature alveolar epithelial cells are ATI cells, they occupy around 95% of the alveolar surface area. Mature ATII cells make up the remaining two-thirds of the alveolar epithelium, covering only 5% of the alveolar surface area (198). While the distributions of various cell types in the alveolar region are relatively constant across species (73), the percentage
of the alveolar surface area occupied by ATI and ATII cells varies slightly; in humans the proportion of surface area covered is 93% and 7% for ATI and ATII cells, respectively (72), while in the rat respective proportions of surface area covered are 97.5% and 2.5% (109).

Markers of AECs appear as early as the embryonic period, suggesting that both cell types may be derived from an unknown common progenitor cell (209). By mid-gestation, markers specific to each cell type are already expressed in the undifferentiated epithelium of the lung. While this may signify that cell fate is determined relatively early for the prospective alveoli, the morphological characteristics of ATI and ATII cells are not discernable until much later on in fetal development (192).

1.5.1.1 ATI cells

ATI are large, highly flattened epithelial cells with long cellular extensions (279); this phenotype is a morphogenetic hallmark of ATI cells that does not occur until the last 2 to 3 days of gestation in rodents (337). Contiguous with one another, ATI cells are also juxtaposed to the avesicular zone of the endothelium of the alveolar capillaries, which is thin and has few or no plasmalemmal vesicles (294). With their basal laminae often fused and due to their thin nature, diffusion distance is minimized for the function of gas exchange across the air-blood barrier of the alveolar air space and the pulmonary capillary blood.

Although ATI cells lack organelles, they still contain many proteins and structures important in lung function. They express the aquaporin (AQP) water channels (e.g., AQP5, an ATI cell-specific marker) and transepithelial sodium (Na⁺) transport machinery (epithelial sodium channels (ENaCs) and Na⁺-K⁺-ATPase channels) that are important in alveolar fluid
resorption during the transition at birth from an intrauterine to air environment, as well as in the resolution of pulmonary edema in adults (142). It has been suggested that active Na\(^+\) transport in ATI cells in vivo seems unlikely because they lack the mitochondrial density necessary to provide the energy for vigorous Na\(^+\) transport (198). The T1 alpha (T1α) protein is also an important cell-specific marker for ATI cells. The importance of T1α is exemplified by T1α null mice, which demonstrate impeded ATI differentiation, deficient alveolar sac development, and an inability to inflate their lungs, which results in death due to respiratory insufficiency (213; 260).

ATI cells are also marked by the presence of caveolae, plasma membrane invaginations opening into the alveolar lumen, of which the primary structural component is the caveolae-1 (cav-1) protein (262). Caveolae are important in normal lung function, as cav-1 null mice demonstrated a marked thickening of alveolar septa and hyper-cellularity (as seen in T1α null mice) and poor lung function (86). Caveolae are thought to be important in the regulation of signalling transduction via the compartmentalization of receptors [e.g., PDGF receptor (PDGFR)], and expression of signalling molecules and enzymes (e.g., nitric oxide synthase), as well as transporters for small molecular weight substances (e.g., hormones) (86; 262; 337). ATI cells also express carboxypeptidase M, a protein localized to the apical membrane, which through cleavage, may activate or inactivate peptides that enter the alveolar lumen [e.g., endothelial growth factor (EGF)] (337). Finally, ATI cells express intercellular adhesion molecule-1 (ICAM), which is situated at the cell’s periphery near the intercellular junctions with ATII cells. ICAM-1 is thought to contribute to functioning of alveolar macrophages by supporting their activity and accelerating their motility (337).
1.5.1.2 ATII cells

ATII are cuboidal cells with apical microvilli, situated at the corners of the alveoli and bulging into the alveolar lumen (319). Contrary to ATI cells, ATIIIs have cytoplasmic organelles, including lamellar bodies which serve for surfactant storage (198). ATII cells are the primary sites of surfactant synthesis, storage and secretion, and also function in fluid homeostasis and in immune protection.

Pulmonary surfactant is a complex proteolipid mixture, essential for stabilizing the surface tension in the alveoli and thereby preventing alveolar collapse at end expiration and allowing for ease of distension of alveoli during respiration. A surge in surfactant towards the end of gestation is particularly crucial for the start of respiration after birth (at low lung volumes) (235; 319). Premature birth or lung injury resulting in a lack of pulmonary surfactant can lead to lethal respiratory distress syndrome (RDS). At higher lung volumes pulmonary surfactant is less crucial, since lung inflation can be restricted by the collagen and elastin fibre network (235).

In the transition to the air environment, fetal lung fluid must be cleared from potential airspaces of the lung. ATII cells have Na\(^+\)-K\(^+\)-ATPase channels and ENaCs (demonstrated in vivo and in vitro) as well as the mitochondrial density required for their vigorous function, which are responsible for fluid absorption from the lung via Na\(^+\) transport from the apical to the basolateral surface (198), potentially alongside those of ATI cells (demonstrated in vitro) (142).

The lung is exposed to harmful substances inhaled from the atmosphere (e.g., microorganisms, toxic gases, and particulates), and still remains free of chronic inflammation. ATII cells appear to be the primary contributors to this innate immunity, providing a defense by secreting anti-inflammatory proteins (e.g., the collectins surfactant protein (SP)-A and SP-D) and antimicrobial substances (e.g., lysozyme) into the alveolar fluid. When these defenses are
overcome or cells become damaged, ATII cells can boost the inflammatory response by upregulating antimicrobial protein production, and by releasing chemokines and cytokines. With viral infections, AECs also secrete interferons, which increase chemokine synthesis and surface expression of ICAM-1 (on ATIs), which facilitate movement of alveolar macrophages (337), reviewed in (198).

1.5.1.2.1 ATII to ATI differentiation

The ATII cell is considered the stem cell of the alveolar epithelium: it can divide and proliferate to provide undifferentiated daughter cells (90), which in turn may differentiate to ATI cells or remain as ATII cells (319). This is important not only in postnatal growth and normal cell turnover, but also as a repair mechanism after lung injury when AECs are destroyed in order to restore normal tissue architecture and lung function. The ability of this process to restore ATI cells is particularly important, since they are more vulnerable (198) and thought to be terminally-differentiated (319).

Recently recognized is the ability of ATI cells to transdifferentiate to ATII cells via an intermediate cell type under certain conditions (33). However, no evidence exists demonstrating that ATI cell division can occurs, so cell division and proliferation would still have to occur via transdifferentiation to the ATII cell type (319).

1.5.2 Mesenchymal cells

The mesenchymal cells of the lung include smooth muscle, pericytes, endothelial cells, and fibroblast cells which, while sharing some characteristics, can nonetheless be distinguished by their morphology and location. This is also true of the pulmonary fibroblasts, of which there
are diverse subpopulations (210). A defining distinguishing characteristic between fibroblast subpopulations is the expression of Thy-1, a glycosylphosphatidylinositol-linked outer membrane glycoprotein. Thy-1 positive fibroblasts (Thy-1+) are spindly, lipid-laden, have many cell-cell contacts, and synthesize considerable quantities of collagen and moderate amounts of fibronectin. In contrast, fibroblasts that do not express Thy-1 (Thy-1⁻) are rounder and well-spread, do not contain intracellular lipid or filopodia, exhibit greater microfilament and microtubule networks, secrete less collagen and more fibronectin, and express significantly more \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) protein in comparison to Thy-1⁺ cells (250; 253; 272). Thy-1⁺ cells are also known as lipofibroblasts, while Thy-1⁻ cells have been identified as myofibroblasts (210; 272).

1.5.2.1 Lipofibroblasts

The lipid-laden lipofibroblasts are situated in the thick portion of the pulmonary interstitium at the base of the alveolar septum (155), and are sometimes found adjacent to ATII cells. Constituting half of the cells in the alveolar walls, LFs are particularly important in the synthesis of surfactant as an accessory to ATII cells. LFs recruit neutral lipids from the circulation, such that these cells are glycogen-rich and have high triglyceride content; triglycerides are then transported to ATII cells for the synthesis of surfactant phospholipids (210). LFs also play critical roles in the synthesis of extracellular matrix (ECM) structural proteins during alveolar formation (97; 210), in offering cytoprotection against oxidant injury via their high triglyceride content (314), and in the storage of retinoids, which affect alveolarization (see section 1.6.3) (208).
1.5.2.2 Myofibroblasts

Myofibroblasts are contractile cells in the alveolar interstitium that contribute significant amounts of α-SMA and collagen to the ECM. They play critical roles in both fetal and postnatal lung development. During the process of branching morphogenesis, prospective alveolar myofibroblast cells (that are PDGFR-α positive) are responsible for stabilizing and restricting expansion of the developing bronchioles (214). During alveolarization, myofibroblasts are embedded in elastin fibers in the alveolar interstitium, and are important for elastin deposition in secondary septation (160). Critical to this role is PDGFR-α and its activation by its ligand PDGF-A, which is required for the prospective alveolar myofibroblasts to multiply and spread from the future bronchiolar walls to the interstitium of the terminal sac walls (179). In the PDGF-A null mouse model, postnatal survivors display a lack of myofibroblasts and elastin deposition, and in turn failed alveolar septation (34). This is thought to be due to the failure of the prospective myofibroblasts to migrate to the terminal airspace walls (179).

1.5.2.3 Adjacent and peripheral fibroblasts

Adjacent and peripheral fibroblasts (AFs and PFs, respectively) are two morphologically distinct subpopulations of fibroblasts classified with respect to their proximity to the epithelium. When fetal rat lung cells are isolated in primary culture, AFs are situated beneath the distal airway epithelial cells and in direct contact with them, whereas PFs are more distant. The cell types produce different factors, with those of the AFs primarily promoting cell differentiation, while the PF factors promote epithelial cell proliferation (49).
1.6 **Important Morphogenetic Factors in Lung Development**

The regulation of pulmonary morphogenesis is complex and depends on both physical and biochemical factors. Some factors affecting fetal lung development involve epithelial-mesenchymal interactions and include physical forces, transcription factors (TF), growth factors (GF) and morphogens, and hormones.

**1.6.1 Physical factors influencing lung development**

Physical factors that play important roles in pulmonary development include: sufficient thoracic area for lung distension and growth, fetal breathing movements (FBMs), and lung and amniotic fluid volumes.

A generous intrathoracic space is required for distension of the lungs during FBMs. Restriction of this space can decrease the distending force in the lungs, reducing lung extension and growth, resulting in lung hypoplasia. Thoracic cavity restriction can be caused by a mass, effusion or external compression (152); infants born with congenital diaphragmatic hernias, for instance, present with pulmonary hypoplasia (104; 123; 152).

FBMs involve an ‘inspiratory’ and ‘expiratory’ phase (196). The former is defined as the contraction of the diaphragm in the downward/caudal direction, accompanied by an inward movement of the chest wall and outward movement of the fetal abdomen, while the latter involves the return of these structures to their initial positions (196). Early on, FBMs are spontaneous and sporadic; they become more regular and sustained from 28 weeks gestation onwards. As the fetus progresses towards term, there is an increase in tracheal fluid volume (146) and spontaneous breathing movements increase up to 30% of the time (170), due to
maturation of the lung as well as the functional development of the fetal respiratory and sleep centres in the brain (120). GCs not only influence lung development, but also are crucial in the development of the brain (addressed in section 1.7.4). Restricted thoracic space can suppress FBMs, and complete abolition of FBMs can also lead to diminished lung growth and alterations in fetal lung fluid volume, as demonstrated in sheep whose FBMs were obliterated via high spinal cord transections in order to paralyze the diaphragm (113).

Fetal and neonatal lungs undergo tonic and phasic / cyclic stretch; if stretch is reduced, lung growth is also suppressed (271). FBMs are important in inducing stretch in the lung. AECs are vulnerable to cyclic stretch, as it promotes surfactant (88) and cytokine release (322), Na+ pump trafficking to the basolateral membrane (93), ATII apoptosis (88) and differentiation to ATI cells (271) and modifications of the cytoskeleton (308). Tonic stretch, on the other hand, has the opposite effect. When induced by tracheal occlusion (increasing the intraluminal pressure), tonic stretch leads to enhanced pulmonary tissue growth and AEC maturation (94).

Fetal intrapulmonary fluid, which contributes up to a third of amniotic fluid volume, is concentrated with chloride ions and produced by the lung epithelium via active transepithelial transport into the trachea-bronchial lumen(170). Fluid volume and ion content are well regulated, as it establishes positive pressure within the developing lung, and chloride content reduction results in lung hypoplasia (170). Tracheal occlusion also causes an increase in lung fluid volume, which results in tissue growth and larger lungs (8), AEC maturation (94), increased ATII apoptosis (80) and thinning of prospective alveolar walls (8). Lung fluid drainage results in smaller lungs and immature AECs (95). Fluid clearance of the lung near birth requires the epithelium to transform from a secretory to an absorptive condition, which is known to be influenced by GCs (discussed in section 1.7.4). The transformation is achieved by increasing
Na\(^+\) absorption across the alveolar epithelium, predominantly via ENaC (136). The importance of fetal lung fluid clearance is demonstrated by transgenic animals lacking ENaC, which fail to clear the fluid and die shortly after birth (128; 207), and by those lacking the ATI cell AQP-5 channels, which demonstrate reduced water absorption but survive (187). The importance of amniotic fluid in lung development is suggested by experimental induction of oligohydramnios (lack of amniotic fluid) (227; 338), which interferes with lung development and is associated with pulmonary hypoplasia (170; 312).

Physical forces acting on the postnatal lung, such as ventilation and oxygen exposure, influence its development as well. Immature ferrets tracheotomized and subjected to a continuous positive airway pressure (6 cm H\(_{2}\)O) for two weeks demonstrated accelerated lung growth (increased total lung capacity, lung weight and total lung protein and DNA content, with negligible changes in lung recoil) (347). Nevertheless, mechanical ventilation of newborns can also insult the lung with barotrauma and volutrauma due to over distension (55; 193). Minimizing over distention by using shorter inspiratory times reduces the incidence of extra-alveolar air leakage and death (256). As in the fetal lung, postnatal expansion and mechanical strain induced ATII cell proliferation and differentiation of ATII to ATI cells (85; 88). However, mechanical stretch also causes ATII cell apoptosis, which is thought to be an important mechanism of ventilation-induced lung injury (88; 112). Increased oxygen tension also influences postnatal lung development, as newborns are exposed to an environment which, compared to that in utero, has a higher oxygen tension. Massaro et al. (203) demonstrated that maintenance of neonatal rats in low oxygen chambers resulted in irreversible reductions in secondary septation. However in addition to reduced gas-exchange surface area, impaired septation was also seen in neonatal rats exposed to hyperoxic environments (41), thus
demonstrating the sensitivity of postnatal pulmonary development to anything other than normoxic environments.

### 1.6.2 Epithelial-mesenchymal interactions

Pulmonary development is dependent on tightly regulated paracrine interactions between lung epithelium and mesenchyme. The importance of mesenchymal signalling in epithelial development, with the epithelium responding to an instructive soluble factor secreted by the mesenchyme, is well noted (287). Spooner and Wessells (302) demonstrated that while various mesoderm types cultured with isolated endoderm (committed to lung development) will induce lung bud formation, such buds require bronchial mesoderm specifically in order to proceed with branching. A complimentary classical study demonstrated not only the ability of fetal tracheal epithelium to branch when denuded of tracheal mesenchyme and replaced with bronchial mesenchyme, but also the specificity of branching even within the lung: bronchial epithelium experimentally exposed to tracheal mesoderm will not branch, despite branching of nearby bronchi covered with bronchial mesoderm (335). Additionally, grafted distal pulmonary mesenchyme onto fetal tracheal epithelium induced ATII cell differentiation (287). Moreover, recent advances in transgenic technologies have made transgenic and knockout (KO) mice models available to investigate factors involved in epithelial-mesenchymal interactions during fetal pulmonary development (37).

Conversely, there is also growing evidence demonstrating the reciprocal importance of lung epithelium in the development and maintenance of the surrounding mesenchyme. In order to investigate this effect of the epithelium on fibroblasts, primary cultures of both cell types have been used. Taderera (305) was among the first to observe in vitro that lung mesenchyme failed
to develop specialized cell types (vascular and stromal cells) in the absence of the epithelium. More recently, Gebb and Shannon (103) observed that in the absence of epithelium, early fetal rat distal lung mesenchyme cultures degenerated and few that stained positively for vascular endothelium were maintained. However, when recombined with the epithelium, an abundance of positively-stained vascular endothelial cells were arranged in a tissue specific manner near the epithelium, similar to *in vivo* organization (103). These studies, among others (313; 330), demonstrate the importance of epithelial interactions with mesenchymal tissues in the growth and maintenance of the pulmonary vasculature. The epithelium’s role in mesenchymal proliferation is elucidated in experiments involving tracheas denuded of epithelial cells, which had their lumina completely obliterated with connective tissue upon examination 1 to 6 weeks later. However, inoculation of denuded tracheas with isolated epithelial cells resulted in re-epithelialization and the reestablishment of a nearly normal epithelial lining of the trachea (310). Furthermore, when the process of re-epithelialization following lung damage is disturbed, fibroblast proliferation takes place and leads to pulmonary fibrosis (114). When cultured with a filter between them, epithelial cells secrete a factor that suppresses fibroblast growth (2), and coculture of both cell types in conditioned medium of epithelial cells inhibits fetal lung fibroblast proliferation (50). This suppression was possibly achieved via a lipid soluble, low molecular weight peptide, called fibroblast growth inhibitory factor, acting in a paracrine fashion (5; 50). Another coculture system demonstrated that a soluble factor secreted by ATII cells stimulated fibroblasts to produce prostaglandin E₂, which in turn acted as an autocrine signal to directly impede fibroblast proliferation (249).

It is known that the mesodermal signalling factor fibroblast growth factor (FGF)-10 is crucial for lung development; however in transgenic embryos lacking its receptor, which is
expressed in the endoderm, primary bronchi will develop normally but secondary or tertiary buds are absent (125). Although many aspects of epithelial-mesenchymal interactions remain incompletely explored, insight into this complex activity is offered by a network of signalling cues which include ECM molecules, TFs and GFs/morphogens.

1.6.3 Transcription factors

Transcription factors (TFs) are nuclear proteins that influence the transcription of target genes, either by binding to regulatory regions of target genes via DNA-binding domains, or indirectly by binding to other nuclear TFs. TFs implicated in the process of alveolarization include forkhead homolog A2 (FOXA2), GATA binding protein 6 (GATA-6), thyroid TF-1 (TTF-1) and SMAD family member 3 (SMAD3) (189).

Foa2, part of the forkhead/winged helix domain family of TFs, is expressed in select cells of the respiratory epithelium pre- and postnatally. Conditional deletion of Foxa2 in mice resulted in inhibited ATII cell differentiation and neonatal respiratory failure; the mice presented characteristics of RDS, including atelectasis and surfactant deficiency (325). Foxa2 is also involved in the regulation of alveolarization. Deletion of Foxa2 in late gestation decreased secondary septation and resulted in airspace enlargement (325).

GATA-6 is a zinc-finger TF expressed in the epithelial cells of the developing lung airways epithelium. The need for GATA-6 activity in the maturation of the terminal airways and alveoli before birth was demonstrated with inhibition of GATA-6 from mid-gestation onwards, which decreased expression of AQP-5 mRNA and surfactant proteins, delayed peripheral airway wall thinning, and interfered with differentiation of ATII and ATI cells (181). Interestingly,
expressing elevated levels of GATA-6 postnatally disrupted alveolarization, as transgenic mice demonstrated impaired alveolar septation and persistent air space enlargement (180). The timing and spatial regulation of GATA-6 is critical for normal postnatal pulmonary morphogenesis.

Changes in TTF1 and Smad3 expression can also alter alveolar formation. TTF1 is a member of the Nkx2 family of homeodomain-containing TFs. It is expressed differentially in lung epithelial cells and is critical throughout lung morphogenesis. The precise regulation of TTF-1 is crucial for pulmonary homeostasis in the postnatal period, as evidenced by overexpression of TTF-1 at various levels; at one level it interfered with alveolar septation and at the highest level it caused pulmonary inflammation (334).

Smad3 is one of many downstream signal transducers of the extracellular Transforming Growth Factor (TGF)-β (see section 1.6.4.1). Smad3 null mutant mice demonstrate a decrease in cell proliferation in the peripheral airways as well as retarded lung alveolarization from PN7 to PN28. Thereafter and into adulthood, the mice developed centrilobular emphysema and demonstrated decreased tropoelastin mRNA expression and increased matrix metalloproteinase-9 protein expression and activity (58). Smad3 models the interrelationship between TFs and other factors upstream or downstream of them, including target genes such as GFs and ECM molecules.

1.6.4 Growth factors and morphogens

GFs/morphogens are diffusible polypeptides that mediate signalling; acting within the vicinity of their source, their local concentrations lend information to neighbouring cells regarding GF position. Together, stimulatory and inhibitory GF signalling pathways regulate
cellular growth, proliferation and differentiation of the developing lung via epithelial-mesenchymal interactions [reviewed in (52; 251; 328)].

1.6.4.1 TGF-β

TGF-β1, 2, and 3, of the TGF-β superfamily, play critical roles in lung development. TGF-β1 inhibits pulmonary morphogenesis, as shown by TGF-β1 overloading, either *in vitro* through exogenous administration to fetal lung tissues (284) and cells (49) or *in vivo* through overexpression in transgenic mice which display neonatal lethality (349). TGF-β1 inhibits proliferation of epithelial cells (244; 284) and selectively promotes deposition of ECM components (244). It also reduces synthesis of proteases involved in connective tissue degradation (e.g., collagenase and elastase) and increases production of factors that inhibit ECM degradation (140; 244). In contrast to TGF-β1 transgenic mice, the prenatal lungs of TGF-β2 null mice have normal morphology and unaffected epithelial differentiation. However, postnatally the mice experience respiratory failure due to collapsed terminal airways and alveoli (273). TGF-β3-deficient mice also show postnatal defects in the conducting airways (e.g., lack of alveolar septal formation and alveolar hypoplasia) and die within 20 hours of birth; however, in contrast to the TGF-β2 deficient mice, this may be due to defects in fetal pulmonary development. These mice demonstrate delayed pulmonary development, including defects in branching morphogenesis and epithelial cell differentiation, and decreased expression of SP-C (145; 273).

Levels of endogenous TGF-β1 and 2 mRNA are downregulated by exogenous GCs in both fibroblast (332) and epithelial cells (206), accompanied by increased expression of TTF-1 and surfactant proteins (206). By contrast, TGF-β3 gene expression in fetal lung fibroblasts is induced by GCs without an effect on epithelial cell differentiation (139; 326). TGF-β3 treatment
does influence epithelial cells indirectly however, by inhibiting the increased gene expressions of surfactant proteins induced by dexamethasone in fetal fibroblasts (327). However, GC administration to TGF-β3 deficient mice still promotes pulmonary maturation (291), suggesting a GC-mediated effect on pulmonary development independent of TGF-β3. Taken together, these results demonstrate GC-mediated effects on the lung, potentially independently and dependently on TGFβs and epithelial differentiation (as demonstrated by TGF-β1 and 2 responses).

1.6.4.2 Retinoic Acid

RA is a lipophilic morphogen of low molecular mass (300 Da). In the lung, it is stored among other retinoids in LF cells and binds to nuclear receptors [retinoic acid receptors (RARs) –α, -β, -γ and retinoid X receptors]. RA plays a role in lung development in utero and postnatally, modulating branching morphogenesis, epithelial cell differentiation, and alveolarization (61; 188). In the perinatal and neonatal period, RA-producing enzymes, RARs and cytoplasmic binding proteins are present and there is a change in pulmonary retinoid content, involving a decline in storage forms and an increase in the active forms, which includes RA (188). RA increases expression of the elastin gene in primary cultures of neonatal rat lung fibroblasts (185). Mice deficient in the RAR-γ gene demonstrated defects in alveolar formation; they had a reduction in tropoelastin mRNA levels in LFs at PN12, and a decrease in whole lung elastin content and alveolar number at PN28. Interestingly however, septation is premature in RAR-β KO mice; during bulk alveolarization alveolar formation occurs at double the rate compared to controls, but after bulk alveolarization the rate is similar to controls. Conversely, treatment of newborn rats with an RAR-β agonist impairs septation (202). RAR-β null mice also displayed decreased levels of PDGF-A protein postnatally, suggesting that deletion of the RAR-β
gene may impair alveolar formation via a pathway involving PDGF-A (298). Finally, as demonstrated in RAR-α null mice, although RAR-α was not needed for alveolarization for the 2 weeks after birth, it was required for the effective production of alveoli with normal volume, surface area, and number thereafter until PN50 (201). These transgenic RAR models suggest that, acting via different RARs, RA can serve as a positive or negative regulator of alveolarization and can modulate alveolar formation during and after septation.

Of interest is the RA antagonism of GC-stimulated effects on pulmonary morphology and gene expression. For instance, RA counteracts GC-induced increases in SP-A, SP-B and Clara cell secretory protein 10 (CC10) mRNA levels, reduced lung growth and mesenchymal tissue, and formation of large airspaces. Postnatally, RA antagonizes the inhibition of alveolar formation caused by GC treatment and increases the number of alveoli (242). There is a critical role of balanced actions of endogenous RA and GC in normal pulmonary development, particularly during alveolarization, and recent evidence suggests that midkine (MK) may act as an important regulator.

1.6.4.3 Midkine

MK is a 13kDa heparin-binding growth factor and a GC- and RA-responsive gene thought to be involved in epithelial-mesenchymal interactions, terminal lung differentiation, and alveolar formation/vascularization (345). While absent in adult lungs, MK elicits a bimodal temporal-spatial expression pattern in the developing mouse lung: it is high early in development and primarily in the epithelium and AFs, decreases towards term, and then increases again at PN2 and peaks at PN4, and remains maximal from PN5 to PN15, localized postnatally to the stroma and blood vessels (215; 345). GR KO mice demonstrate increased levels of MK.
expression (98). Postnatally, in rat pups treated from PN1 to PN15 with dexamethasone or RA, MK expression was decreased or increased, respectively, and RA inhibited the GC-induced decrease in MK when both treatments were given in concert. In addition, similar effects of exogenous GC and RA treatment on MK expression were seen in human fetal ATII-like cells in culture (345). Reduced MK levels are consistent with increased GC and decreased RA signals, such as in late gestation, and are consistent with hormonal effects on alveolarization during postnatal lung development as well (345).

1.6.4.4 LGL1

Late gestation lung 1 (LGL1) is an essential, GC-inducible gene expressed in the mesenchyme in the fetal lung; it is a secreted glycoprotein synthesized in AFs in the pseudoglandular stage and imported by the distal lung epithelium in late gestation. LGL1 has been implicated in branching morphogenesis, GC-induced mesenchymal-epithelial interactions, surfactant synthesis, and postnatal lung development (168; 224; 245; 246). In rodents, LGL1 was found to be concentrated at the tips of secondary septa at PN7 and PN14. (168; 223). With LGL1 null mice demonstrating embryonic lethality, a complex phenotype of heterozygote mice was observed postnatally. Neonatal LGL1 heterozygote mice displayed features of inflammation, increased interstitial tissue and disorganized elastin architecture. At maturity (PN28), lung mechanics were altered with a reduction in lung elastance and an associated increase in tropoelastin mRNA levels (168). In hyperoxia-exposed rat models of BPD, LGL1 levels were considerably diminished but subsequently restored during air recovery (223). That LGL1 is subject to coordinate transcriptional regulation by GC, RA, and vitamin D suggests that LGL1 might modulate alveolarization (224).
1.6.4.5 IGF

Named after their sequence homology to insulin, insulin-like growth factors (IGFs), their binding proteins (IGFBP) and their receptors function in promoting cellular proliferation and differentiation (303). Their expression in the lung indirectly suggests a role in lung development and remodelling (228; 267). Gene expression of IGF-I is localized to mesenchymal cells, IGF-II predominantly to epithelial cells, the type 1 IGF receptor to all lung cells and the type 2 IGF receptor to mesenchyme and part of the intrapulmonary vessels. The IGFBPs elicit a complex pattern of expression at various locations, such that they may be modulators of IGF action in a site-specific manner (267). IGFBPs are GC-responsive, with IGFBP-2 to -5 mRNA levels decreasing and IGFBP-6 increasing in response to exogenous GC administration in fetal lung explants (164). In mice deficient of either type 1 or type 2 IGF receptor expression, branching morphogenesis is unaffected. Type 1 IGF receptor KO mice have hypoplastic lungs and die from respiratory failure (183), while type 2 KO mice have abnormal alveoli and die from cardiac defects (225). During and even in recovery from hyperoxia-mediated lung injury, expression of IGFs, type 1 IGF receptor and certain IGFBPs is increased (59; 228). Hyperoxia-induced mesenchymal proliferation is stimulated by increasing IGF-I in the neonatal rat lung, and this was inhibited by RA administration (59). A role for the IGF signalling pathway has also been implicated in alveolar epithelial cell proliferation and differentiation during tissue remodelling, as evidenced by increased expression of IGF-I and IGF-II during ATII to ATI differentiation, and partial inhibition of both processes in cells exposed to anti-type 1 IGF receptor and anti-IGF-I antibodies (228).
**1.6.4.6 HGF**

Hepatocyte GF (HGF) is expressed in the mesenchyme of the perinatal lung, while its receptor (c-Met) is expressed in the pulmonary epithelium. Disruption of HGF gene expression in mice results in developmental abnormalities causing embryonic lethality, however lung morphology is normal (318). HGF plays a role in the morphogenesis of alveolar and bronchial epithelia. This was evidenced by inhibition of epithelial morphogenesis in embryonic rat lung explants with the interference of endogenous expression of HGF, and by stimulation of morphogenesis with exogenous HGF exposure (135). Fetal rat lung epithelial explants with the surrounding mesenchyme removed did not have branching restored when exposed to exogenous HGF, suggesting that HGF is not required for pulmonary branching. However, HGF may play an essential role in postnatal alveolar formation, as PN6 rat pups administered with neutralizing antibody to HGF displayed simpler alveoli, with inhibited DNA synthesis in secondary crests, and reduced alveolar number (248).

**1.6.4.7 FGF**

Fibroblast growth factors (FGFs) make up a large family with 4 FGF receptors. Their role in fetal lung development is well established, particularly in lung bud formation, branching and cellular differentiation via mesenchymal-epithelial interactions (283; 286; 288). Postnatal involvement includes a cooperative role for FGFR-3 and -4 in regulation of alveolarization (331). The lungs of mice doubly homozygous for mutations of FGFR-3 and -4 display a complete lack of secondary septation and fail to downregulate postnatal elastin deposition; this complete blockage of alveolarization consequently leads to postnatal death due to respiratory failure (331). In neonatal rats exposed to a hyperoxic environment from PN0 to PN7, there were decreased levels of basic FGF (bFGF), but not its receptor FGFR-1. Levels were subsequently
increased during recovery, suggesting a role for bFGF in secondary septation (138). Finally, a role for FGFs in alveolarization is further supported by the inhibition of FGF-7 in rats from PN3 to PN5. This lead to alveolar defects involving cessation of secondary septa formation and a reduction in small blood vessels, suggesting FGF-7 inhibition likely influences vascular development (247).

1.6.4.8 VEGF

The vascular endothelial growth factor (VEGF) is encoded by a gene family that includes placental GF, VEGF-A, -B, -C, and -D, the first three of which are important in the formation of blood vessels, while the latter two are important in the formation of lymphatic vessels (144). These GFs bind in different combinations to VEGF receptors (VEGFR) -1, -2, and/or -3; VEGF-A, for example, binds to both VEGFR -1 and -2, albeit with a 10 times greater affinity to VEGF-1, while VEGF-B potentially binds only to VEGFR-1 (238). While VEGFR-1 is the principal receptor in signalling for angiogenesis (211), VEGFR-2 also plays a role in embryonic formation of vascular beds (285). In fact, VEGF is critical for vascular bed formation in organogenesis, including that of the lung. Newborn VEGF-deficient mice have immature lungs, with less complex alveoli compared to controls (105). Conversely, VEGF excess by transgenic expression (at higher levels and earlier development stages) in the respiratory epithelium resulted in concomitant VEGFR-2 overexpression, increased pulmonary vasculogenesis, disrupted branching morphogenesis and inhibited differentiation of ATI cells, resulting in neonatal death (344). In the lung, VEGF can be found in smooth muscle cells, bronchial epithelium, and alveolar epithelium and macrophages (105; 222), while VEGFR-1 and -2 are found in endothelial cells and the pulmonary mesenchyme (105; 222). VEGF overexpression changed blood vessel growth and may have altered intricate interactions (cell-cell or cell-ECM) involved
in development of the acini. Smooth muscle and myofibroblast differentiation and organization may have been altered as well, as evidenced by a reduction in bronchiolar α-smooth muscle actin (105). VEGF is also critical in postnatal lung development. Infants dying of BPD demonstrate disrupted pulmonary vasculature and reduced levels of VEGF and VEGFR-1 (27). Of interest is that exogenous administration of GCs to neonatal rats increases gene expression of VEGF and VEGFR-2 (26). Postnatal inhibition of VEGF by blockade of VEGFR-2 impairs alveolarization by attenuating lung vascular growth and reducing alveolar formation in the neonatal rat (108; 137). This effect, however, is reversible with time (108) or can be rescued by VEGF administration (69).

1.6.4.9 PDGF

The platelet derived GF (PDGF) family consists of A-, B-, C-, and D- polypeptide chains, disulfide-bonded. The GF exists as 30 kDa dimers, the five established ones being PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD, and as such they bind two receptors at the same time, which then dimerize (30; 117; 293). The α-receptor (PDGFR-α) binds with high affinity to the A-, B- and C- chains, while the β-receptor (PDGFRβ) binds with high affinity to the B- and D- chains only (154; 293). Target cell response depends on the receptors it expresses. Fibroblast and smooth muscle cells express both PDGFR-α and PDGFR-β, but a greater amount of the latter, while capillary endothelial cell expression is limited to PDGFR-β (296; 320). Essential for development is the normal expression of PDGFs and PDGFRs, which are localized to epithelial-mesenchymal interaction sites, including those of the lung. Of particular interest, PDGF-A is produced by epithelial cells and PDGFR-α is expressed by the mesenchyme (179; 241), while in the developing vasculature, endothelial cells produce PDGF-B and smooth muscle cells express PDGFR-β (118). As alluded to earlier, PDGF-A null mice that died postnatally
demonstrated immature alveolar development (half died prior to embryonic day 10) (34). Thought to be due to the failure of prospective myofibroblasts to migrate to terminal airspace walls (179), this animal model demonstrates that PDGF-A signalling is essential in pulmonary myofibroblast and alveolar development (see sections 1.4.2.1 and 1.5.2.2). PDGFRα (but not PDGF-A) was shown to be GC-responsive, as evidenced by increases in PDGFRα mRNA and protein in rodent fibroblast cell lines exposed to Dex (329). PDGF-B deficient mice demonstrated a non-overlapping phenotype (lung abnormalities were not specified) with heart defects that were worse than those of PDGFR-β null mice, implicating that PDGF-BB may also act via the PDGFR-α during development (174). In an animal model of BPD, where secondary septation was inhibited by continuous exposure to 60% oxygen for 2 weeks, rat lungs had delayed expression of PDGF-BB, PDGF-AA and PDGFR-β, and intraperitoneal injection of truncated soluble receptors for PDGFR-β and neutralizing antibody to PDGF-BB impaired lung cell DNA synthesis (40).

A myriad of growth factors are involved in postnatal lung development. As alluded to throughout, growth factors and their effects upon lung development are influenced by GCs, among other hormones.

1.6.5 Hormones

It is well established that hormones such as sex hormones, thyroid hormones, insulin, and GCs influence lung development. Thyroid hormones may promote pulmonary maturation without promoting lung growth. The role of sex hormones in the regulation of lung development is evidenced by animal studies. Androgens exert inhibitory effects, while estrogens exert stimulatory effects. For instance, by comparing surfactant production, lung maturation is
delayed in male fetuses compared to females in many species, and this difference is thought to be
due to higher levels of androgens in males. During fetal development, compared to males,
female rat lungs not only have a higher rate of lung growth, but also have more cuboidal
epithelial cells prior to epithelial differentiation. [Reviewed in (51; 53; 54)].

1.7 Role of Glucocorticoids during Lung Development

1.7.1 The glucocorticoid receptor and gene transcription

In the cell, GC effects are exerted by the intracellular GR (64), of which the highest
levels found in the body are within the lung (151). GR is a 94 kDa polypeptide (13) which
functions as a ligand-dependent transcription factor (64). It has three defined domains, which
include (1) a transcriptional activation domain at the amino-terminus, (2) the central DNA-
binding domain (DBD), which targets GR to GC response elements (GREs) within very specific
DNA sequences, and (3) the ligand binding domain (LBD) at the multifunctional carboxy-
terminus (13; 234; 237). GR-α and -β isoforms, 94 and 90 kDa respectively, are achieved via
alternative splicing (126; 234); the former is located in the cytoplasm and functions as the
mediator of GC effects on target tissues, while the latter is situated in the nucleus and has been
shown to antagonize the function of the –α isoform (233; 234). When the GR ligand is not
present, GR is maintained in the cytosol in an inactive complex, with transport to the nucleus
prevented through interactions with chaperone proteins such as heat shock protein (HSP) 90 and
HSP70 (32; 139) at GR carboxy-terminus sequences (234). HSPs also allow GR to be available
for GCs by maintaining them in a high affinity state, and they are released upon ligand binding,
which induces a conformational change (13). The lipophilic nature of GCs allows them to
diffuse across the cell membrane and interact at the GR LBD. The activated GC-GR complex homodimerizes, undergoes nuclear translocation, and changes target gene transcription therein. Modulations of gene expression by GR are achieved by classical and non-classical transcriptional mechanisms. In the classical mechanism, the GC-GR complex binds directly to target gene promoter regions at GREs. Binding specificity is achieved by interaction of the P-box in the first zinc-finger of the DBD with the palindromic GRE, which then leads to transactivation. Binding to the less conserved negative GREs (nGREs) leads to transrepression, however this is less common (266). The non-classical mechanism occurs indirectly via protein-protein tethering interactions by cross-talk of GR with transcription factors, such as AP-1, NF-κB, and GATA-1 (266), which generally inhibit transactivation (265; 266). This transrepression is likely exerted by GR monomers, and both the LBD and DBD are known to be involved in this interaction as shown via mutational analysis (264).

In GC-GR mediated changes in transcriptional activity in the lung, the classical mode was thought to play a more prominent role. However the importance of the non-classical mode was elucidated by the production of transgenic mice with dimerization-defective GR, in which GRE-mediated transactivation was not inducible but protein-protein interactions were unaffected. These mice displayed abnormalities of many physiological functions of GR; however, their lungs did not have any irregularities and importantly, the mice were viable. This study demonstrated that GR activities via DNA-binding are not essential for survival and that the non-classical mode of GR modulation of transcriptional activity was likely responsible for the viability of these mice (264).
Figure 1.2. **Modes of GC-GR signalling modifying transcription.** In the cytoplasm, GCs bind to GR and HSPs are released. The classical pathway involves the GC-GR dimer binding to a GRE leading to transcriptional activation or repression. The protein-protein interactions of the non-classical pathway involve GR cross-talk with other TFs to modify transcription. Together with GR, AP-1 and NF-κβ repress transcription, while STAT5 and STAT3 activate transcription. Adapted from (266).

1.7.2 **GCs in prenatal lung development**

In late gestation, there is a surge in circulating GCs. This is due to enhanced GC synthesis by the fetal adrenal cortex and a change in the conversion of GCs from inactive to active forms (101). In humans, near term there is a reduced rate of deactivation of cortisol, the active form of GC, via oxidation into its cortisone, its inactive metabolite, by type 2 11β-hydroxysteroid dehydrogenase (HSD) (101; 336). In rodents, the HSD activity differs such that near term there is an increase in the conversion of the inactive GC form, 11-dehydrocorticosterone, to the active corticosterone. Nonetheless, in both human and rodent fetal
lungs it seems that HSD activity may function to increase local productions of active GCs (101). This pre-term surge is necessary for the closing stages of the lung parenchyma’s structural and functional differentiation of in utero, preparing the animal for the transition to the extra-uterine environment (175). Thus, it is relevant to note that in the antenatal period, GCs significantly influence various aspects of lung development. Moreover, while not yet well understood, it is becoming progressively more evident that the high preterm levels of Corticotrophin Releasing Hormone (CRH) and cortisol are likely to be important regulators involved in the process of human parturition [reviewed in (153)]. GCs have been shown to stimulate surfactant synthesis, influence ion and water transport in fluid clearance, and influence morphological maturation.

GCs increase the production of fatty acids (FA), which are an integral part of the lipid components of surfactant [e.g., phosphotidylcholine (PC) and dipalmitoyl phosphatidylcholine (DPPC)]. This is achieved by accelerating rate-limiting step enzymatic activity of de novo FA (235) and PC synthesis (20), likely by a direct influence on fatty acid synthase (FAS) and subsequently choline-phosphate cytidylyltransferase (CCT) indirectly, involved the former and latter process respectively (20; 341). Exogenous GCs (i.e., Dex) have been shown to influence prenatal lung development by decreasing epithelial cell proliferation, increasing ATII differentiation (marked by an increase in LBs and glycogen (158; 242; 243; 342)), enhancing surfactant proteins and CC10 transcription and/or mRNA stability (235; 242; 243; 306), and enhancing ATII to ATI differentiation (marked by an increase in appearance of squamous cells and ATI cell markers (e.g., cav-1)(16; 74)).

Just prior to parturition there is a switch from fluid secretion to absorption and a decrease in fetal lung fluid. Together with thyroid hormones, GCs prime the fetal lung for epinephrine-
induced reabsorption of lung liquid (324). Circulating GCs increase ENaC expression during maturation, although this effect is species specific. In CRH KO mice, GC deficiency was related to a reduction in the expression of all ENaC subunits. In particular, ENaC-α mRNA expression was more sensitive to exogenous GCs than the β and γ subunits (226), while in the rat, exogenous GCs only induced ENaC-α mRNA expression during the canalicular stage (309). Taken together, this suggests that in rodents, ENaC-α is particularly more sensitive to GCs (226).

In cultured human fetal lung however, Dex induced all three subunit mRNA equally (321). In the alveolar epithelium A549 cell line, which originated from an explant culture of an adult lung carcinomatous tissue, ENaC-β and -γ mRNA and protein expression were increased with GC stimulation. Any change in ENaC-α was limited to a non-significant increase in mRNA expression (171). In addition to changes in ENaCs, exogenous GCs have also been shown to upregulate the Na-K-ATPase in rat AECs (18) and the water channel AQP1 in rat fetal lungs (161), as well as the water channel AQP5 in fetal sheep (182) and A549 cells (22).

Exogenous GC administration accelerates lung maturation by inducing morphological changes related to the mesenchyme, a main target for GCs in late gestation (158; 257). As evidenced by in vitro studies (242; 243) and in vivo (158; 255), these changes include enlarged air spaces, primitive septa, reduced tissue to airspace ratios due to mesenchymal condensation between epithelial tubules, and a decrease in alveolar wall thickness, which are effects subsequently consistent with facilitated gas exchange. In the lung, the reduction of mesenchymal tissue is thought to occur by way of GC-regulated inhibition of cell division,
evidenced by GC-mediated reduction of $^{3}$H-thymidine incorporation into DNA (158) and stimulation of cyclin dependent kinase inhibitors (CDKIs), inhibitors of cell division (56; 71).

1.7.3 Transgenic mouse models

Transgenic GC or GR deficient mice models have offered further insight into the role of GC-GR signalling in fetal and postnatal lung development. These include CRH KO mice, in which the CRH gene and mRNA products are completely knocked out (219); GR$^{null}$ mice, in which exons 1C and 2 have been excised (36); GR$^{hypo}$ mice, in which the GR protein is severely truncated at the amino-terminal as a result of a PGK neomycin cassette insertion into exon 2 of the GR gene (65; 68; 315); GR$^{hypo-epi+}$ mice, in which a fully functional GR is expressed exclusively in the distal lung epithelium a on a GR$^{hypo}$ background (98); and GR$^{epi-}$ in which GR is fully functional except in the distal lung epithelium (194).

CRH-KO murine pups derived from CRH-KO parents, such that they are deficient of maternal and fetal GCs, develop cyanosis and die of respiratory insufficiency within 24 hours following birth. This results from abnormal lung development, where lungs begin to deviate from normal morphology after day 16.5 (coinciding with the GC surge in mice, which peaks at around day 16(17)), and by day 17.5, septal thinning and formation of the airspaces failed to occur such that the lungs were hypercellular and maintained a pseudoglandular appearance. These differences in morphology are due to the distal epithelial and proximal epithelial and mesenchymal compartments undergoing continuous proliferation (and not failed apoptosis), contrary to wild-type mice in which cell division ceases by day 18.5. In addition, while expression of SP-A, SP-B, FAS, and CC10 mRNAs were delayed (lower at day 16.5 and normal
by day 18.5), surfactant phospholipid synthesis was not, as demonstrated by unaffected DPPC and LB levels. Neonatal fatality in CRH KO mice may reflect the essential role of endogenous GCs and their critical timing in pulmonary maturation rather than the effect on surfactant proteins and lipids, which was minimal (219; 220).

All GRnull mice (278) and most (>80%) GRhypo (63; 66; 98) display neonatal mortality and abnormalities in lung architecture (hypercellularity and atelectasis) similar to the GC-deficient mice. GRhypo lungs, which were indeed hypercellular, also demonstrated increased cell proliferation rates and no change in apoptosis at late gestation (day 18.5 gestation). Expression of the GC-responsive negative cell cycle regulator CDKI p21CIP1 gene was reduced, while the growth factor MK was increased (29). A similar increase on MK expression was seen in GRnull mice at the same time point and PN0.5 as well (156). In GRhypo mice, surfactant production and secretion were unremarkable. In contrast, the proportion of ATI cells markedly decreased in consort with a drastic decrease in the gas exchange surface area, suggestive of a role in neonatal respiratory failure. However, that the proportion ATII cells increased suggests that while GC-GR signalling facilitates transdifferentiation of ATII into ATI cells, it is not required for ATII differentiation. Moreover, the impairment of fluid absorption in these mice may have also contributed to respiratory dysfunction, as evidenced by a reduction in the expression levels of the GC-responsive genes ENaC-γ and AQPI (67).

Interestingly, while GRhypo-epi+ mice displayed neonatal mortality similar to that of GRhypo, both tissue cellularity and expression of MK were comparable to wild-type mice (98). Conversely, when GR was expressed with the exception of the distal lung epithelium, about half of GR-epi- mice demonstrate decreased viability (194). Late gestation lungs were immature with
greater cellularity, and demonstrated increased epithelial cell glycogen pools and decreased expression of surfactant proteins, AQP5, ENaC–β and –γ. These two models demonstrate that GR in the lung epithelium is essential in normal pulmonary development, the former elucidating that GC-GR responsive genes of the distal lung epithelium are important in normal mesenchymal remodelling in the fetal lung (98) and the latter that impaired epithelial differentiation might contribute to reduced neonatal viability of newborn mice with suppressed pulmonary epithelial GR (194).

1.7.4 GCs during postnatal lung development

1.7.4.1 GC-GR effects on early postnatal lung development in transgenic GR animal models

As previously mentioned, all GR\textsuperscript{null} mice (278) and most (>80%) GR\textsuperscript{hypo} (63; 66; 98) and GR\textsuperscript{hypo-epi+} (98) and about half of GR\textsuperscript{epi-} (194) mice demonstrate respiratory insufficiency and die within hours of birth. While all homozygous GR hypomorphic mice (GR\textsuperscript{hypo}) on an isogenic 129 sv genetic background also died at birth (68), correlating with delayed mesenchymal thinning near term, Nemati \textit{et al.} (229) demonstrated 40% of newborn GR\textsuperscript{hypo} mice on a mixed C57Bl6/129 sv background were alive and healthy at birth. As such, this study has been a useful transgenic mouse model in studying the effects of GR deficiency in the neonatal period by assessing survivors and comparing surviving and non-surviving GR\textsuperscript{hypo} mice. Newborn non-survivors demonstrated 50% thicker airspace walls and a 46% reduction in secondary crest counts compared to either survivor or wild-type littermates. Both GR\textsuperscript{hypo} mice groups (not expressing wild-type GR) had increased tissue to airspace ratio compared to mice expressing wild-type GR. The morphology of survivor GR\textsuperscript{hypo} lungs was more mature relative to those of
non-survivors (thinner mesenchyme and a lower tissue-to-airspace ratio), indicating that a partial decrease in mesenchymal thickness is still compatible with viable lung function and postnatal lung development, including capillary remodelling and alveolar septation. Since mesenchymal thinning is required prior to alveolarization, delayed thinning in the non-survivors may relate to a subsequent reduction in secondary crests. That α-actin expression in SMCs surrounding airways and blood vessels and vWF staining in endothelial cells of large proximal vessels were not different across groups suggests that the classical mechanism of GC-GR signalling may not contribute significantly to SMC development or distribution in the distal lung near term (229). Thus this model may also be useful in differentiating between GC-GR signalling dependent and independent genes.

In addition, reduced neonatal survival in GR\textsuperscript{epi} mice, where GR was eliminated exclusively in the alveolar epithelium \textit{in utero}, demonstrates a novel appreciation for the role of the GC responsive genes in the epithelium in perinatal lung development (194).

\subsection{1.7.4.2 Effect of exogenous GCs on postnatal lung development}

Since postnatal studies on transgenic models have so far been limited, experimental studies involving exogenous GCs have offered a plethora of information regarding their effects on pulmonary development during the postnatal period.

It is well established that postnatal treatment of lungs with exogenous GCs, particularly the potent and often-used synthetic GC Dexamethasone (Dex), will inhibit alveolar formation via premature maturation of the alveolar septa, transpiring as accelerated lung development. This impairment in alveolarization was shown to be a permanent effect, lasting into adulthood (PN60 (200; 316) or PN99(270)), when Dex was administered to neonatal rats in a minute dose (e.g.,
0.1 µg/animal) daily over 10 to 14 days during the first two postnatal weeks. The adult distal
lung demonstrated impaired alveolar septation, with larger, less complex, and fewer airspaces.
When Dex was given earlier on in a high dose (e.g. 0.01 - 0.01 µg/g of BW) daily from PN1 to
PN4 the impairment in alveolarization was found to be temporary (70; 186; 269; 282; 317). At
PN4, the distal lung was characterized by larger and fewer airspaces, significantly thinner inter-
 airspace walls, and premature maturation of septa (thinner and reduced number of emerging
 secondary crests). Tschanz et al. (317) also looked at the septal ultrastructure of these Dex-
treated lungs at PN4, and observed that single capillary networks were predominant with less
 interstitial tissue; the effect was reversed by PN10. This morphological observation suggesting
 that Dex treatment from PN1 to PN4 induced premature microvascular maturation on PN4, was
 further supported by Roth-Kleiner et al. using the same model with morphometric data (i.e.,
 intersection counting on transmission electron microscopy and vascular casts) demonstrating
 accelerated wall thinning and accelerated microvascular maturation (increased number of areas
 with single-capillary layer) at PN4 in Dex treated animals. Interestingly, the Dex-treated group
 exhibited a temporary arrest of microvascular maturation thereafter, such that both Dex and
 control groups displayed the same microvascular maturity at PN10. This evidence lends further
 support to the hypothesis that Dex accelerates maturation of the capillary bed, thereby
 contributing to the reduction in inter-airspace wall thickness (269; 316). That 30% of the
 microvasculature had matured at PN10 in either group suggests that there is an overlap between
 alveolarization and microvascular maturation, and that the remaining 70% still to occur was
 sufficient for normal alveolar formation thereafter. Dexamethasone (high dose PN1-PN4) was
 also shown to alter the balance between cell proliferation and apoptosis, decreasing apoptosis
 from PN3 to PN4 and drastically reducing cell proliferation from PN2 to PN4 (186). The peak
of apoptosis normally observed at PN19 and PN21 was also suppressed, but since Dex (half-life of 1 day) would have been eliminated by then, it was able to exert a secondary effect. The authors suggested that if a surplus of cells is established during the first two postnatal weeks to serve as a backup, which would be removed via apoptosis later on upon maturation of the alveolar septa, then Dex may have prevented this surplus from forming early on such that removal of cells at the time of maturation might no longer be required (186). Complementing this observed inhibition of proliferation at PN4 was a study analyzing dex-induced changes on cell cycle control mechanisms during postnatal lung development, particularly the key regulator of cell cycle machinery, the cyclin dependent kinase (CDK) system, and its regulation of cell proliferation. Interestingly, along with the expected thinning of septa at PN4, there was an increase in cyclin dependent kinase inhibitors (CDKIs), and P27KIP1 (at PN4 only) and p21CIP1 (from PN1 through PN10). So, impairment of G1 cyclin/CDK complex activation may be a means by which Dex/GCs influence lung maturation (70). Dex also alters the expression of various genes involved in alveolarization. A similar exposure of mice to Dex early on at a high dose demonstrated that at PN4, mRNA and protein levels of VEGFR-2 (a major mediator of angiogenic and mitogenic effects of VEGF (refer to section 1.6.4.8), were downregulated. This implicated a role for VEGFR-2 and a blockage of angiogenesis in a Dex-induced inhibition of septation (62). The consequences of Dex treatment from PN1 to PN4 in rats are well studied due to observations suggesting that a short course of exogenous GCs is more beneficial than a long one prenatally in women at high risk of preterm delivery (see section 1.8). PN1 to PN4 in rats is about the same as the 34th to 36th weeks of pregnancy in humans (186).
1.7.4.3 Regulation of GC action

The action of GCs at target tissues is regulated by a few mechanisms, including the local metabolism of GCs, the expression of GR, the binding of cortisol to corticosteroid-binding globulin (CBG), and the physiological feedback mechanisms regulating GC biosynthesis and secretion via activity of the hypothalamic-pituitary-adrenal (HPA) axis. As touched upon previously, the local metabolism of GCs plays a role in their availability at that particular tissue. In the human fetal lung, there is a continuous conversion of cortisol to cortisone throughout gestation, a reaction thought to be achieved by type 2 HSD. Indeed, type 2 HSD protein has been found within cells of the developing lung, excluding the AECs (101). On the other hand, the conversion of cortisone to cortisol (type 1 HSD activity) does not occur in human fetal lung and while type 1 HSD mRNA has been reported in the fetal lung, reports of its protein being expressed are inconclusive (101). While the role of type 2 HSD in the fetal lung remains to be explored, its role in the human adult lung has been studied. In adults, type 2 HSD is highly expressed in bronchial epithelial cells, and inhibition of its activity in primary culture was shown to increase the potency of cortisol (92).

1.7.4.3.1 GR expression in the lung

Although GCs can autoregulate GR expression and thereby change the cellular sensitivity to the hormone, the cellular concentration of GR in particular is considered a limiting factor in the GC-GR signal transduction pathway (232; 236). In the adult lung, GCs downregulate expression of GR mRNA and protein via a classical feedback mechanism (9; 150; 151). On the other hand, GCs upregulate GR expression in the late gestation fetal lung in vivo, and in d20 primary cultures of fibroblast cells alone. However, while primary cultures of distal lung
epithelial cells of the same age demonstrate decreased GR expression, the removal of the mesenchymal-epithelial interaction is thought to account for this direct downregulation of GR by GCs (134; 239).

Coincident with the pre-term GC surge, GR mRNA increases as fetal development progresses (21), thereby allowing the lung to be very responsive to the effects of GCs. The expression of GR in the rodent lung has been shown to be the highest relative to all other tissues (150), as well as the most steady throughout late fetal and postnatal development (151). Tissue-specific differences exist after adrenalectomy and Dex treatment. In the adult rat lung, while adrenalectomy did not exert an effect on GR mRNA levels, the latter resulted in an approximately 50% decrease of GR gene expression (150). The developmental pattern of GR gene expression is also tissue-specific; drawing focus on the rodent lung, no significant changes in GR mRNA levels occur throughout development (i.e., in fetal lungs, in neonatal lungs (PN1, 2, 3, 5, 7, 10, 14, 21) and onwards at 1, 3, 5, 9, and 20 months) (151). In investigations of the fetal and adult human lung (neonatal human lung specimens were not included in the study), although no significant changes were observed, expression of GRα mRNA demonstrated a pattern of low expression at the pseudoglandular stage, increasing during gestation, and lower in adults (259).

1.7.4.3.2 GC levels during alveolarization

The total plasma corticosterone (the active GC in rodents) levels during the first 2 to 3 weeks of life in the neonatal rat are known to be relatively low. However, the reported developmental profiles and precise total plasma corticosterone levels over this early neonatal nadir have been quite variable, dispersed across a 40-fold range. Moreover, the developmental
timing of the postnatal rise in GC levels has also been inconsistent. In 1966, Haltmayer et al. (111) reported high, but decreasing plasma corticosterone levels from PN1 through PN5 (about 10-22µg/100ml), and lower, steady levels onwards till PN21 (about 7 µg/100ml). Two years later, Bartova (19) described a different trend in changing plasma corticosterone concentration, wherein at PN2, levels were lower than in adults (5.2µg/100 ml), but decline significantly towards PN11 (3.6µg/100ml), and rise again at 16 days (6.2µg /100 ml) increasing up to day 24 (7.7µg/100 ml), nearing adult values. A decade after Haltmayer, Redman and Sreebny (263) described low plasma corticosterone at PN6 and PN10 (just under 20µg/100 ml), followed by a steady, sizeable increase until PN21(reaching just over 40µg/100 ml), and minimal fluctuations thereafter. The use of fluorimetric assays in the aforementioned rat studies was concerning however, since this method was found to lead to substantial overestimation of mouse plasma corticosterone due to nonspecificity (84). Using a more specific method of competitive protein binding , Daniels et al. (77), reported low concentrations from PN5 until PN15 (between 1-2µg/100mL). This then showed a tendency to increase after PN16, followed by a 2-fold increase between PN18 and PN19, where the mean value remained between 5-7µg/100mL until PN22; after PN22, there was a considerable increase in plasma corticosterone concentration up to 15µg/100mL on PN28, which then steadied near adult values (~6.4µg/100mL). However, it is possible that gastric intubation 4 hours prior to sacrifice may have elevated the corticosterone levels above resting values. Thus, while this study overcame the potential issue of corticosterone non-specificity, it did not address the effect of stress as others previously had (111; 263).

Moreover, Henning (119) demonstrated that sequential removal of rat pups from a single cage after PN12 was sufficient to activate the adrenal stress response (see below). In order to acquire truer basal values of plasma corticosterone for the first 28 postnatal days, a competitive protein
binding technique was used in combination with experiments having been completed using many litters in parallel, such that on any given day only one pup was removed from the cage (119). It was found that total corticosterone levels remained steady below 1µg/dL until PN12, at which point levels continued to rise, reaching a 3-fold increase at PN14, 7µg/dL by PN21 and a peak at PN24 (11µg/dL), after which levels declined and reached adult levels by PN28 (Figure 1.3).

![Figure 1.3. Developmental patterns for total (●—●) and free (△—△) corticosterone in plasma from rat pups. Values are given as means ± SE (n = 5 at every age). Reproduced from Henning, Am J Physiol, 1978. Am Physiol Soc, with permission.](image)

The rise in plasma corticosterone during the 3rd week of life is paralleled by a rise in plasma concentrations of CBG which, when bound, serves as an inactive reservoir of
corticosterone in the circulation (119; 173). The developmental rise is thought to be due to reduced removal of corticosterone from the circulation, rather than an increased production (173). Leeper et al. (173) examined the physiologic determinants underlying the developmental rise in corticosterone levels, including the corticosterone half-life of disappearance ($t_{1/2}$), the apparent volume of distribution ($V_d$), and the metabolic clearance rate (MCR, where $MCR=\ln 2 V_d/ t_{1/2}$). A decreased $t_{1/2}$ was associated with increased binding to CBG. Between PN12 and PN22, there was an overall decrease in MCR, due primarily to a steep decline in the $V_d$, which outweighed the decline in $t_{1/2}$. In a follow-up study (281), the decrease in plasma clearance was found to be sufficient to account entirely for the developmental rise in corticosterone, and the decline in $V_d$ was again attributed to the concurrent increase in plasma CBG and increased association of corticosterone to CBG (173; 281).

The total serum corticosterone level includes GCs both bound to CBG and unbound. The common consensus is that only the free (unbound) fraction (<10% of the total plasma concentration) is considered to be available for entry into target tissues, while the rest remains reversibly bound to CBGs. However, evidence suggests that at least in some cases, CBG may facilitate corticosteroid entry into target tissues, regulating its availability at target tissues (35). On the other hand, in one study where exogenous GCs were administered to fetal and neonatal rats, pulmonary corticosteroid 11-reductase activity was elevated, in turn inactivating the active GC at the tissue level (295).

1.7.4.3.3 Modulators of basal GC-levels

The HPA axis involves the release of CRH via the hypophyseal stalk to the anterior pituitary, where it stimulates the secretion of stored ACTH to the circulation. At the cortex of
the adrenal gland, ACTH stimulates the biosynthesis of corticosteroids. Release of CRH from the hypothalamus involves complex interactions, and is influenced by stress, GC blood levels, and a circadian rhythm. In adult mammals, the HPA axis is an essential part of the integrated response to stress (221), whereby adrenal steroids increase cardiovascular, metabolic and respiratory activity as a means of coping with a range of potentially life-threatening stressors (10). In the typical adult circadian rhythm of the nocturnal rat (housed in a 12:12 hour light:dark cycle), the levels of corticosterone are highest at lights off and lowest at lights on. On the other hand, the neonatal rat pup at 2 weeks of age does not exhibit this plasma corticosterone rhythm, but does so by 3 weeks of age (6). Interestingly, there is a significant basal level of non-rhythmic corticosterone secretion from neonatal adrenals into the plasma, thought to be important in differentiation of brain and other tissues (10).

The adrenal response to ACTH is suppressed in order to minimize the chance of large fluctuations in circulating GCs, which could otherwise be detrimental to the development of the central nervous system if too high. Neonates display a “stress-hyporesponsive period” (SHRP), wherein response to mild stress is minimal during the first two weeks of life (280; 289). Henning (119) demonstrated that in litters where one pup was removed over several days across the postnatal period from PN0 to PN28, the stress response in pups remaining in the cage, as indicated by an increase in circulating GCs, was not activated until PN12 (119). The adrenal in particular was identified as the unresponsive fraction of the neonatal rat’s HPA axis during the SHRP, while the rest of the HPA axis was shown to be capable of responding to a mild stress (e.g., intra-peritoneal injection of isotonic saline) during this time (83). The overall effect of the SHRP is that the relatively low GC levels are not increased by stress.
1.8 **GCs and Lung Diseases of Prematurity**

### 1.8.1 Respiratory Distress Syndrome

Births prior to 37 completed weeks of gestation are considered premature. Furthermore, births before 34 weeks gestation are considered very premature, those prior to 27 weeks are extremely premature, and delivery before 22 completed weeks is usually fatal (218). Premature birth is generally associated with structural and biochemical maturation of the fetal lung that is insufficient for ventilation in the extrauterine environment. This structural immaturity contributes to neonatal respiratory distress syndrome (RDS) in premature infants, which is characterized by surfactant deficiency and results in uneven and unstable lung inflation causing atelectasis, as well as insufficient clearance of liquid from the airspaces, all leading to insufficient respiratory function (25).

### 1.8.2 The “old” Bronchopulmonary Dysplasia

In the past, strenuous mechanical ventilation and high levels of oxygen treatment of preterm infants with RDS resulted in injury of their immature lungs. Due to an insufficient capacity for lung repair, they consequently developed a condition of chronic lung injury known as bronchopulmonary dysplasia (BPD) (231). First characterized in 1967, this “classic” or “old” BPD involved airway injury, interstitial and alveolar edema, inhomogeneous inflation, persistent inflammation and extensive fibrosis, accompanied by impaired alveolar and vascular development (25). It was seen in infants born at 30 to 33 weeks gestation, with their lungs still rendering a saccular appearance. However, these relatively large preterm infants seldom develop BPD nowadays, as perinatal therapies have evolved (e.g., more tolerable ventilation, exogenous...
surfactant administration and steroid treatments) and in turn have improved survival of more premature infants.

1.8.3 The “new” Bronchopulmonary Dysplasia

The increasing number of premature infants surviving includes extremely preterm infants with more immature lungs (14). As such, it is now the case that in these smaller premature infants, born at 24 to 26 weeks of gestation with canalicular lungs, emerges the development of a different condition known as the “new BPD”. The “new BPD” is characterized by impaired secondary septation of the alveoli leading to a permanent overall reduction in alveolar number and gas-exchange surface area, as well as interstitial thickening, decreased vascular development and large simplified alveoli with diminished fibrosis, more uniform inflation, and minimal lung injury (25; 141). As oppose to the “old BDP”, this pathology may develop without prior exposure to mechanical ventilation or oxygen therapy (141). Even with mechanical ventilation, while low birth weight infants had immature lungs with fewer and larger alveoli, they demonstrated less fibrosis and inflammation compared to those in the “old BPD” (124; 129). Thus, while the “old BPD” was the consequence of lung injury, the “new BPD” reflects impaired lung growth (162).

1.8.4 BPD and GC treatment

In the late 1960s, two complementary studies (176; 177) first demonstrated that administration of antenatal steroids to fetuses not only led to preterm labour in sheep, but also importantly accelerated lung maturation via increased surfactant production, which in turn improved respiration of the preterm lambs. Benefits of this therapy on preterm neonates include
reduced requirements of supplemental oxygen, exogenous surfactant administration, and time spent on mechanical ventilation (297). However, the positive effects are restricted within the limits of timing and duration. Effective administration of antenatal corticosteroid therapy is limited to within 2 to 7 days prior to labour (178), and a single course is accepted (297). However, the benefit of repetitive GC treatments over longer durations is controversial; while many have associated it to adverse effects such as reduced bodyweight and growth, impaired brain development, and adrenal suppression (15; 141; 205; 292), others have reported no adverse effects and no benefit of repetitive GC treatment (297). Furthermore, administration of GCs just prior to alveolarization in rats has been shown to induce premature arrest of alveolarization (269). That being said, the postnatal use of multiple courses of high-concentration steroids in premature newborns is not favourable and thus is not recommended (292).

1.9 Glucocorticoid Receptor Antagonists

1.9.1 RU486 (Mifepristone)

RU486 is a synthetic steroid, also known as mifepristone, that binds with high affinity to both the progesterone and glucocorticoids receptors (PR and GR), and thus functions as an effective antagonist to progesterone and GC actions (172; 275). RU486 also has been demonstrated to exhibit partial agonist activity at both the PR and GR (89; 301; 348).

RU486 is a derivative of the progestational agonist norethindrone, a synthetic 19-nor-steroid (190; 275). The PR is primarily located in the cytosol, where it is maintained as an inactive monomeric protein bound to HSPs (e.g., HSP90). Binding of progesterone to PR leads
to dimerization of receptor monomers, followed by nuclear translocation, protein
phosphorylation, interaction of the hormone-receptor complex to DNA at progesterone response
elements (PREs) and transactivation of target genes (131). RU486 binds PR with greater affinity
(5Xs) than progesterone (131; 190). Interestingly, RU486 mimics progesterone in its mechanism
of action in as much as the following: dissociation of PR from HSPs, dimerization, nuclear
translocation and DNA footprint at the PRE where the complex binds. Distinct from
progesterone-PR interactions are the amino acids within the ligand-binding pocket of PR to
which RU486 forms contacts (23), and the induction of an alternate three-dimensional
conformation of the PR at its carboxy-terminal LBD (89). The unique conformation of the
RU486-PR complex promotes repression by engaging co-repressor proteins (323) and does not
allow for binding to transcriptional coactivator proteins (240), thus eliminating the
transactivation of progesterone-dependent genes.

The mechanism of RU486’s anti-GC activity is also incompletely understood. Similarly
to PR antagonism, RU486 binding to the GR LBD induces a conformational change, wherein the
coactivator pocket becomes covered by helix 12 such that coactivation is inhibited while co-
repressor proteins are recruited, ultimately leading to antagonism of GC action (159). Moreover,
Lee et al. (172) recently demonstrated that the carboxy-terminus region of the AF1 domain and
an effect on GR motility in the nucleus are associated with RU486-induced GR antagonism.
Other effects of RU486 on GR are increased stability of GR-HSP interactions, reduced affinity
of RU486-GR for DNA versus Dex-GR, and different recycling of GR to the cytoplasm when
complexed with RU486 versus Dex (131).
The molecular weight of RU486 is 429.6, and while soluble in methanol, chloroform and acetone, it is poorly soluble in water (131). It is absorbed orally with a 69% absorption rate from the gut (275) after a 20mg dose. Plasma concentrations of RU486 increased by raising the single dose in increments from 50 up to 100 mg, after which no increases are seen between 100 to 800 mg. While this is true for the RU486 parent compound, the mono-demethylated, di-demethylated (which are generated by CYP3A4 enzymatic activity) and hydroxylated metabolites, which are pharmacologically active, do increase with all dose escalations. In the plasma, RU486 is 98% bound to albumin and α1-acid glycoproteins (131). The half-life of RU486 in humans is 20 to 40 hours. Since this is relatively long, it effectively competes with PR- or GR- agonists and binds to the receptor itself, while the agonists are eliminated from the target cell or metabolized in situ (190). Furthermore, the RU486 metabolites may significantly contribute to the anti-PR, and more so to anti-GR action of the antagonist, at 23-30% and 47-61% respectively (115). The main mechanisms of elimination are metabolism in the liver, enterohepatic circulation, followed by fecal excretion (131).

The main clinical use of RU486’s anti-PR activity is to inhibit progesterone activity in the first few weeks after conception leading to abortion of the fetus, however it has also been implicated in the induction of labour without apparent toxicity, as a highly effective postcoital contraceptive agent, as treatment of endometriosis, and as a potential therapy for certain tumours (190; 300).

As evidenced in vitro and in vivo, RU486 is an effective antagonist of GC action at high concentrations (172). An in vitro study demonstrated the RU486 inhibition of Dex-induced up-regulation of the p11 protein (a gene in the prefrontal cortex with known GREs) expression in
SH-SY5Y cells (346). In investigation of the regulation of synaptic plasticity in the rat, RU486 administration to block GR just after exposure to acute stress prevented stress-induced impairment of both hippocampal (12) and prefrontal long-term potentiation (191). Moreover, in a rat model of depression wherein the expression of hippocampal synapsin I and depression-associated behaviour were induced by chronic unpredictable mild stress, RU486 treatment for one week restored both parameters (339). In men, RU486 had maximal anti-GC activity (disinhibition of the human pituitary-adrenal system) during the morning hours of the circadian rhythm (99). Another clinical study in patients with bipolar disorder and schizophrenia utilized the anti-GC drug effect of RU486 to demonstrate persistent reductions in the HPA axis activity (measured by peripheral cortisol levels) lasting two weeks after treatment (100). Clinically, the anti-GR activity of RU486 has possible uses such as in the management of Cushing’s syndrome, an endocrine disorder caused by hypercorticism (60), and in the treatment of neuropsychiatric disorders and depression (81).

1.9.2 CP472555

RU486 was one of two GR antagonists used in tandem as a foundation for a three-dimensional pharmacophore model upon which the design of two potent, nonsteroidal and selective GR ligands, CP394531 and CP409069, was based (217). However, they demonstrated little anti-GC activity in vivo, as evidenced by their limited bioavailability and high clearance rate in animal studies. As such, the GR antagonist CP472555 was developed, which is a compound with potent and highly selective anti-GR activity in animal models (216). Moreover, the use of CP472555 as a GR antagonist is advantageous over that of RU486, since it is specific for GR and demonstrates negligible anti-PR activity (216).
Arriving at CP472555 required various experiments. Phenols, including CP394531, undergo rapid clearance in vivo via phase II metabolism, which often renders inactive metabolites (31). This conjugation at the phenol of CP394531 was shown to be a major route of the high plasma clearance (84mL/min/Kg) in \textit{in vivo} rat pharmacokinetic studies. Furthermore, examination of rat liver slices and rat bile, urine and plasma following intravenous administration of 15mg/kg of CP394531, demonstrated that minimal traces of unchanged drug remained; the major metabolite was its primary glucuronide conjugate, while additional minor metabolites (five glucuronide conjugates of the hydroxylated parent compound) present suggested oxidation followed by glucoronidation as an additional mechanism of clearance. In deriving a more useful GR antagonist with \textit{in vivo} function, the phenol conjugation that was present in the parent compounds was blocked in their derivatives. Moreover, a moiety at the A-ring phenol that contained a hydrogen-bond acceptor or donor/acceptor, such as the amide in CP472555, permitted potent GR antagonism.
Figure 1.4. Molecular structures of RU486 and CP472555. The structure of RU486 was modified in the synthesis of CP472555. A hydrogen-bond acceptor or an acceptor-donor pair at the A-ring phenol were desirable for potent GR binding and antagonism. The amide (R1) in CP472555 not only demonstrated this feature, but this additional phenol surrogate also blocked the phenol conjugation that limited the *in vivo* function of the CP472555 parent compounds.

The particular potencies were determined by examining binding affinities and cellular functional antagonism, which appeared to be species-specific. The former were assessed by measuring IC$_{50}$s, the concentration required to inhibit 50% of the binding of [$^3$H]-Dex, while the latter used IC$_{50}$s that were determined from concentration compound curves using whole cell functional assays (7; 216). Binding affinity and cellular functional antagonism for CP472555 at the human GR were 2nM and 30nM, respectively, while at the mouse GR they were 50nM and 40 to 600nM, respectively. As was the case with its parent compounds derived and optimized from RU486, CP472555 demonstrates potencies comparable to RU486 and a >200-fold
selectivity for GR versus other steroid receptors (human PR, mineralocorticoid receptor, androgen receptor, and estrogen receptor) (7).

The molecular weight of CP472555 is 501.07, and it is soluble in 100% ethanol and propylene glycol (148). While details of its parent compound’s metabolism in the rat were readily available as described above, the bioavailability and metabolism of CP472555 were not provided. Moreover, although the literature to date does not offer a description of its precise mechanism of action in GR antagonism, it is conceivable that it acts in a similar way to the compound from which it was derived, RU486.

As alluded to above, CP472555 functions as a full antagonist at the human GR, and is more potent at the hGR than the murine GR. Moreover, in vitro experiments with L929 cells containing endogenous murine GR demonstrated partial agonism at the receptor in the absence of GCs in whole-cell functional assays (216). Nevertheless, when utilized in vivo in rodent models, GR antagonism is still evident. Kale et al. (148) induced hypoglycemia with insulin injections, in turn inducing hypercorticosteronemia, which they suggested modulates CNS efferent autonomic and neuroendocrine motor responses. The group utilized intracerebroventricular (i.c.v.) administration of CP472555 into rats prior to inducing hypoglycemia to inhibit GR, demonstrating that during recurring insulin-induced hypoglycemia (RIIH), antecedent activation of central GR is required to aggravate hypoglycemia and that central GRs mediate the effects of hypoglycemic hypercorticosteronemia on autonomic efferent responses. Doses of CP472555 were administered daily for 3 days, and the effect was achieved in a dose dependent fashion, with escalating doses from 25ng to 100ng having no effect, and 500ng being sufficient to effectively inhibit GR. Kale et al. (147) then used the same
experimental model with CP472555 administration to prevent RIIH-associated decreases in colabeling of lateral hypothalamic area orexin-A neurons (a component of the central circuitry that regulates energy homeostasis), demonstrating that central GR-dependent mechanisms are involved. Subsequently, Kale et al. (149) used CP472555 antagonism of GR to reverse decreased responsiveness of certain areas of the brain during RIIH, lending support to the view that GC-sensitive neurons in specific brain loci might be directly modified by GCs in the central neural response to recurring hypoglycemia. Recently, the group showed that adaptive changes in paraventricular glucokinase and oxytocin gene activity (but not CRH or vasopressin) during chronic insulin exposure are reversed by pre-treatment with CP472555, implicating GR in the former but not the latter transcriptional adaptations to recurring glucoprivation (38).

CP472555 has also been used in ob/ob mice, a rodent model of obesity, in order to explore the anti-obesity and anti-diabetic activity of attenuating GC action. RU486 administration to ob/ob mice has been shown to ameliorate diabetes symptoms, reducing blood glucose levels to those seen in lean mice, and it also reduced serum insulin by about 50% (106). CP472555 treatment resulted in a cumulative (2 day), dose dependent decrease in food intake, with 25mg/kg resulting in an 18% reduction, and 50mg/kg resulting in a 55% reduction. At the end of the experiment (18 hours after the last 50mg/kg dose of CP472555), there was also a 33% reduction in blood glucose. These ob/ob mice were dosed orally for 2 days, twice daily. Furthermore, GR antagonism was confirmed in normal rats by inhibition of Dex-induced increase in liver tyrosine amino transferase activity, whose gene is known to have GREs. Oral dosing resulted in a dose-responsive inhibition, with 10mg/kg, 20mg/kg and 40mg/kg resulting in 9%, 80%, and 92% suppression, respectively (216).
1.7 **Rationale and Hypothesis**

Studies of GR and GC deficient mice have confirmed the principle that GC-GR signalling is necessary for maturation of the lung. A role for endogenous GC signalling in alveolar development in the postnatal rat lung has been suggested, but empirical data to support this contention are lacking. One potential approach to determining the role of GC-GR signalling in alveolar formation would be by pharmacologic blockade. RU486, a readily available GR antagonist is, inconveniently, also a potent progesterone receptor antagonist. Use of a specific GR antagonist, CP472555, would in theory permit specific GR blockade in order to further investigate the role of GC-GR signalling in postnatal rat lung development.

Eliminating gaps in knowledge about the timing and downstream effector molecules of GC-GR signalling in alveolarization may eventually permit the design of safer treatment or prophylaxis for BPD. Ultimately, it is hoped that this information may facilitate the design of better therapeutic strategies that avoid adverse effects of GCs on the lungs and other developing vital organs, including the brain.

**Hypothesis:** CP472555, a novel GR antagonist with negligible anti-PR activity, is a suitable tool for the study of GC-GR regulation of alveolarization in the rat.

**Specific Aims:** (1) To demonstrate blockade of GC-GR signalling by CP472555 in fetal rat lung fibroblast and epithelial cells; (2) To demonstrate blockade of GC-GR signalling by CP472555 in live newborn rat pups; and (3) To identify the effects of GC-GR blockade on rat alveolarization.
Chapter Two: **Materials and Methods**
2.1 Materials

2.1.1 In vivo interventions

- Timed-pregnant Wistar rats from Charles River (Saint-Constant, QC, Canada)
- Diethyl ether (Caledon Laboratories, Georgetown, ON, Canada)
- Sodium Pentobarbitol (CEVA Santé Animale, Libourne, France)
- 30\(^{1/2}\) gauge needle (Becton Dickinson, NJ, USA)
- Dexamethasone (Sigma, St. Louis, MO, USA)
- Mifepristone (RU486)(Sigma, St. Louis, MO, USA)
- CP-472555-01 (Pfizer Inc., Groton, CT, USA)
- 100% Ethanol (Commercial Alcohols, Brampton, ON, Canada)
- Propylene Glycol (1,2-Propanediol, 99.5+%, A.C.S. Reagent) (Sigma-Aldrich, St. Louis, MO, USA)

2.1.1.1 Isolation of fetal rat fibroblast and distal lung epithelial cells

- Collagenase (Worthington Biochemical Corp., Freehold, NJ, USA)
- DNAsé (Worthington Biochemical Corp., Freehold, NJ, USA)
- Trypsin (Gibco, Grand Island, NY, USA), (Multicell, Wisent Inc., Saint-Bruno, QC, Canada)
- Nitex Mesh (Tetko Inc., Elsmford, NY, USA)
- 1X Hank’s Balanced Salt Solution- ***without Mg\(^{2+}\) and Ca\(^{2+}\) [HBSS(-)](Gibco, Grand Island, NY, USA), (Multicell, Wisent Inc., Saint-Bruno, QC, Canada)
- Dulbecco’s Modified Minimum Essential Media (DMEM) (Gibco, Grand Island, NY, USA)
- Fetal Bovine Serum (FBS) (Gibco, Grand Island, NY, USA)
- 100X Pen-Strep (P/S) (Gibco, Grand Island, NY, USA)

2.1.1.2 Bacterial plasmid preparation and SEAP assay

- QIAfilter Plasmid Midi Kit (25) (QIAGEN Sciences, Mississauga, ON, Canada)
- SEAP Assay Kit (Tropix, Bedford, MA, USA)
  - 5X Dilution Buffer, Assay Buffer, 20X CSPD Substrate (chemiluminescent alkalinephosphatase substrate), Reaction Buffer
- Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada)
- EG&G Berthold Luminometer (Montreal Biotech, Inc, Kirkland, QC, Canada)

2.1.2 Tissue collection

- SofSilk™ suture thread (United States Surgical, Norwalk, Connecticut, USA)
- 10% buffered formalin (BDH Inc., Toronto, ON, Canada)
- Gauze (Johnson & Johnson Medical Limited, Skipton, UK)
- 27\(^{1/2}\) gauge needle (Becton Dickinson, NJ, USA)
2.1.3 Reagents and enzymes used for RNA analyses

- Isopropanol, TRIzol, oligo(dT) primers, MMLV Reverse Transcriptase, 5X First Strand Buffer (Invitrogen Life Technologies, Burlington, ON, Canada)
- MicroAmp 96 well plates, MicroAmp Optical 8-tube Strip, Optical caps, Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA)
- 2X SYBR® Green reaction buffer [dNTPs HotGoldStar DNA polymerase, MgCl₂ (5mM final concentration), Uracil-N-Glycosylase, SYBR® Green I, stabilizers and passive reference] (Eurogentec, San Diego, CA, USA)
- ABI 7700 and ABI 7900 sequence detection system including SDS software (Applied Biosystems, Foster City, CA, USA)

2.1.4 Paraffin embedding

- Paraffin (McCormick Scientific, St. Louis, MO, USA)
- Xylene (Sigma, St. Louis, MO, USA)
- 100% Ethanol (Commercial Alcohols, Brampton, ON, Canada)
- OmniSette® tissue cassettes (Fisher Scientific, Markham, ON, Canada)
- Permount and Superfrost® Plus microscope slides (Fisher Scientific, Markham, ON, Canada)
- Glass coverslips (Fisher Scientific, Markham, ON, Canada)

2.1.5 Reagents used for histological and morphometric analyses

- Bovine serum albumin (BSA) (Vector Laboratories, Burlingame, CA, USA)
- Phosphate buffered saline (PBS): 10X PBS prepared with 80g NaCl, 2g KCl, 6.1g Na₂PO₄ and KH₂PO₄ dissolved in ultra pure water (pH 7.4), diluted to 1X PBS for use (Reagents from BioShop, Burlington, ON, Canada)
- Tris buffered saline (TBS): 10X TBS prepared with 0.5M Tris HCl and 1.5M NaCl in UP water (pH 7.4) diluted to 1X TBS for use (Reagents from BioShop, Burlington, ON, Canada)
- Triton-X-100 (BioShop, Burlington, ON, Canada)
- Eosin Stain (Sigma, St. Louis, MO, USA)
- Hematoxylin Gill II formula (Vector Laboratories, Burlingame, CA, USA)
- Resorcin-fuchsin solution (Rowley Biochemical Institute, Danvers, MA, USA)
- Accustain® Tartrazine Solution (Sigma-Aldrich, St. Louis, MO, USA)
2.2 Experimental Procedures

2.2.1 In vivo interventions

All animal experiments were conducted according to Canadian Council for Animal Care guidelines. Protocols used were approved by the Animal Care Review Committee of the Research Institute at the Hospital for Sick Children. Animals were housed on a 12h:12h light:dark cycle in pathogen-free conditions with *ad libitum* access to rodent food pellets and water. For cell culture experiments, timed-gestation (day 20, term = 21.5 days) Wistar dams were killed by diethyl ether excess and the fetuses removed. For *in vivo* studies, newborn Wistar rats (10-12 pups/litter) were used. Pups received *intraperitoneal* (i.p.) injections of either vehicle (propylene glycol) or CP-472555.

The *in vivo* pharmacokinetic properties of CP472555 were not available, and thus the optimal dose, frequency and developmental period over which CP472555 would inhibit GR in the postnatal rat lung had to be determined empirically. Dose administration (dose and frequency) and the developmental period over which CP472555 treatment was applied were plenty. Once daily injections were performed each morning. Twice daily (BID) injections were done every 10 – 12 hours. CP472555 doses used were at each step from 5mg/kg decreasing in 10-fold dilutions until 0.5μg/kg, inclusive. Developmental periods examined were from PN0 until PN10, and from PN11 until PN16, 18 and PN21. The various combinations of treatment schedule and administration are listed in Table 2.1. From PN11 onwards, where pups were injected with CP472555 in a two-log excess of total plasma GCs, CP472555 was maintained at such a concentration according to the increase in total plasma GCs (which is not proportional to the increase in body weight). Total plasma volume for a given weight was estimated based on the formula from Bijsterbosch (28), *Plasma Volume (mL) = 0.0291 X BW(g) + 2.54.* Total
plasma GC levels (free and CBG-bound GCs) were used in preference to free GC levels to ensure that sufficient CP472555 was available to block all GCs present locally in the lung.

Pups were sacrificed by i.p. injection of 0.1 mL/10g BW of sodium pentobarbital (54.7 mg/mL). Propylene glycol and CP-472555 (powder and in vehicle) were stored at room temperature (RT) protected from light.

<table>
<thead>
<tr>
<th>Developmental Period</th>
<th>PN0 – PN10 (low blood corticosterone levels)</th>
<th>PN11 – PN21 (rising corticosterone levels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>PN0 - 4 5 or 0.5 mg/kg</td>
<td>PN0 - 10 50, 5, or 0.5µg/kg</td>
</tr>
<tr>
<td></td>
<td>PN0 - 4 50, 5, or 0.5µg/kg</td>
<td>PN0 - 7 0.5mg/kg</td>
</tr>
<tr>
<td>Frequency</td>
<td>Once Daily</td>
<td>BID</td>
</tr>
</tbody>
</table>

Table 2.1. Administration of CP472555 in vivo. CP472555 treatments covered two periods of development. In the first period, postnatal days (PN) 0 – 10, circulating levels of corticosterone (the main GC in the rat) remain low. In the second, PN 11 – 21, corticosterone levels are rising.

2.2.1.1 Isolation of fetal rat fibroblast and distal lung epithelial cells

Pregnant Wistar dams were killed by diethyl ether excess and day 20 fetuses were aseptically removed. Lungs were dissected from the fetuses, separated from the heart, trachea and other major airways and vessels, and placed in cold HBSS(-). The lungs were washed 3 times in HBSS(-), minced diligently and suspended in HBSS(-). Minced tissue was vortexed to remove red blood cells and centrifuged 3 times at 135 X g for 3 min until the supernatant was clear.

The pellet was suspended into 40 mL HBSS(-) with 0.125% trypsin and 0.02% DNAse. The mixture was transferred to a 75mL trypsinizing flask and stirred for 20 min in a 37°C water
bath. After the 20 minutes (min), 0.2 mL DNase was added and the mixture was pipetted 5
times (10 mL pipette) to break up DNA. Any remaining undispersed tissue was trypsinized for
an additional 10 min. The mixture was filtered through a Nitex mesh into a 50 mL centrifuge
tube, divided equally into two 50 mL tubes, and equal volumes of DMEM + 10% (vol/vol) FBS
were added to both tubes to neutralize trypsin action. Cells were centrifuged at 135 X g for 3
min, and repeated until the supernatant was clear. The pellets were pooled, resuspended into 35
mL of DMEM without serum, and centrifuged at 135 X g for 3 min. The pellet was resuspended
into 30 mL of pre-warmed DMEM with 0.1% collagenase and 0.02% DNAse, and incubated for
15 min in a 37°C water bath. The mixture was divided equally into two 50 mL tubes, and equal
amounts of DMEM + 10% FBS were added to neutralized the collagenase. After centrifuging,
the pellet was resuspended into 2 mL DMEM + 10% FBS per 4 fetuses, and 2 mL of this cell
suspension was then added to 13 mL DMEM + 10% FBS per T75 flask.

The flasks were incubated for 30 min in a 37°C tissue culture chamber with 5% CO₂ to
allow for adherence of adjacent fibroblasts. The contents of the flask (loose epithelial cells) were
transferred into 50 mL tubes (2 flasks per tube) and centrifuged. Cells were seeded again into
half as many flasks and incubated for 1 hour (hr). The flasks with the adjacent fibroblasts were
shaken vigorously to remove adherent epithelial cells, washed 3 times with HBSS(−), and left in
10 mL fresh DMEM + 10% FBS undisturbed in the tissue culture incubator. Media was changed
every 2-3 days.

After the hour incubation, flasks with epithelial cells were emptied into 50 mL tubes (3
flasks into 2 tubes) with DMEM. After centrifuging, the pellet was rinsed until white in colour.
Pooled pellet was resuspended in 5 – 8 mL DMEM + 10% FBS depending on expected yield.
Cells were counted with a haemocytometer and cell concentration was adjusted to 10 million
cells/mL with DMEM + 10% FBS. In T25 flasks, 20 million cells were seeded with the final volume adjusted to 3.5 mL, and incubated at 37°C tissue culture chamber with 5% CO₂. After 24 hours, epithelial cells were rinsed with HBSS(-) and new media added (DMEM + 10% FBS).

2.2.2 Cell transfection and SEAP assay

Once cells were confluent, flasks were rinsed 2X with HBSS(-). Cells were incubated in 2 mL trypsin for several minutes until cells detached from flask. Two mL of DMEM + 10% FBS (without P/S) were added to inactivate trypsin, the flask contents transferred to a 50 mL tube, and the volume was brought to 10 mL total with HBSS(-). Cells were centrifuged and the supernatant discarded. After adding 10 mL DMEM, cells were counted with a haemocytometer. Primary culture adjacent fibroblast (AF) cells were seeded in 6-well plates (Costar, Corning, NY, USA) at 373,000 cells/well (optimized seeding density for next-day 80-90% confluency). Primary culture epithelial cell optimized seeding density was 680,000 cells/well. To each well in the 6-well plate, 2 mL DMEM and the determined volume of cell mixture for optimized seeding density was added. Cells were incubated at 37°C, 5% CO₂ overnight.

Cells were transfected with a plkNEO-SEAP construct, while lipofectamine 2000 was used as a means of altering the cell membrane to allow the plkNEO-SEAP plasmid to cross into the cytoplasm. The plkNEO-SEAP-to-lipofectamine ratios were optimized to 0.5µg : 1.5µL for AFs and 2.4µg : 5µL for epithelial cells. In a 15 mL tube, plkNEO-SEAP construct stock preparation was diluted in DMEM to a total volume of 250 µL per well. In a 15 mL tube, lipofectamine was added to DMEM to a total volume of 250 µl per well. Lipofectamine mixture was left at RT for 4 min (no longer than 5 min). After 4 min, the plkNEO-SEAP mixture was added to the Lipofectamine mixture, mixed gently, and left at RT for at least 20 min. Using 6-
well plates, 2 mL of DMEM and 500 µL of the plkNEO-SEAP-lipofectamine were added to each well. Cells were incubated at 37°C for 4 hours to allow the transfection to occur. Cells were rinsed twice with HBSS(-) and incubated in 2 mL of fresh DMEM + 10% CS-FBS + P/S per well over night or until confluent.

Cells were stimulated with various concentrations of dexamethasone (Dex) alone (MW = 392.5g/mol), or in combination with either RU486 (MW = 429.6g/mol) or CP-472555 (MW = 501.07 g/mol). Dexamethasone was used as a GR agonist; the antagonist RU486 was used as a positive control for comparison with the novel GR antagonist CP472555. Ethanol was used as the solvent and ethanol-treatment was carefully used as control value. A 10 mM stock solution of Dex was prepared with 3.1 mg Dex in 790 µL of 100% ethanol. A 20 mM stock solution of RU486 was prepared with 6.9 mg RU486 in 803 µL of 100% ethanol. A 10mM stock solution of CP was prepared with 1.5 mg CP in 299 µL of 100% ethanol. Serial dilutions in 100% ethanol were made to desired concentrations. For example, for cell treatment with a final concentration of 0.1 nM of Dex (or Dex 10⁻⁷ M), 2 µL of 0.1µM stock of Dex was added to 2 mL DMEM + 10% CS-FBS + P/S per well.

Cells were rinsed and treatments were added in duplicate. Following 24-hour stimulation, cell culture medium was collected from each well, from which the amount of SEAP was quantified. The media were centrifuged at 14,000 rpm for 5 min, and 200 µL were collected. The media were mixed 1:3 with supplied dilution buffer and incubated at 65°C for 30 min. Assay buffer was added to the luminometer plate (50 µL per well) first, then 50 µL of medium with treatment was mixed in each well in duplicate, and left incubating at RT for 5 min. Meanwhile, CSPD was mixed into cold reaction buffer diluents, 1:20 (vol/vol). At the 5 min mark, 50 µL of cold reaction buffer was added to each well in 10 sec intervals, using a new
pipette tip for each well. After 20 min at RT from the initial addition of reaction buffer to the first well, the plate was read at the microplate luminometer.

2.2.2.1 Bacterial plasmid preparation

The HindIII – XbaI fragment encoding SEAP from pSEAP Basic vector was previously sub-cloned into the HindIII site of the plk-NEO vector as per manufacturer’s protocol (CLONTech Laboratories, Palo Alto, CA). This is a highly inducible expression vector that is glucocorticoid-responsive (121). Plasmid-containing bacterial colonies were amplified in liquid culture overnight at 37°C. Plasmid DNA was purified by the QIAGEN Plasmid Midi Purification kit, according to the manufacturer’s instructions. dsDNA concentrations were determined by spectrometry [absorbance at 260nm (A_{260}), where 1 \ A_{260} = 50 \mu g/mL dsDNA)] using a dilution of 1/250.

The SEAP dephosphorylates the CSPD substrate, which results in unstable anion that decomposes and emits light. The light signal in turn is quantified in the luminometer. It is proportional to the concentration of SEAP, and thus to the activity of glucocorticoid receptor.

2.2.3 Tissue collection

Pups were weighed prior to sacrifice. The abdomen was opened and a cut was made along the midline up to the thorax. The muscle layer over the trachea was carefully removed and suture thread was passed under the trachea and left in a loose knot for trachea ligation later. After pup gender was noted by identification of gonads, an incision was made in the diaphragm to collapse the lungs. The diaphragm was removed and the rib cage cut up the midline, carefully opened and cut off. The right lobes were ligated with suture thread. A small cut was made in the
left atrial appendage of the heart to allow for fluid flushed out of the left pulmonary circulation to drain into the open cavity instead of returning to the left ventricle. A 10 mL syringe was filled with 1X PBS and a 25\(\frac{5}{8}\) gauge needle was used to penetrate the right ventricle (RV). To clear the left lobe of blood, it was then flushed via the RV with 1X PBS until white in colour. Once flushed, the trachea was cannulated with a blunted 27\(\frac{1}{2}\) gauge needle and fixed using 10% (wt/vol) neutral-buffered formalin (formalin), under constant airway pressure of 20 cm H\(_2\)O. The left lobe was simultaneously fixed and inflated with the formalin and stopped when it was visually apparent that the lobe apices were pointed and the lung looked translucent. The trachea was then ligated. The right lobes were quickly excised and briefly washed in 1X PBS. The four right lobes were split into two pairs, flash frozen in liquid nitrogen and stored at -80°C until needed for mRNA analysis. The trachea, left lobe and heart were carefully removed from the chest cavity en bloc, immersed in formalin and processed for histology.

2.2.4 RNA isolation

Total RNA was isolated from fetal rat lung cells and frozen lung samples using TRIzol reagent according to the manufacturer’s instructions. Briefly, 1.2 mL TRIzol was added to each T75 flask (1 mL for T25 flasks) of fetal rat lung cells; cells were detached with a cell scraper and lysed by repetitive pipetting and transferred to a 1.5 mL Eppendorf tube. Whole lung samples were homogenized in 1mL TRIzol. For each mL of TRIzol, 200μL of chloroform was added, vigorously mixed by inversion for 15 sec and then left at RT for 10 min. Samples were then centrifuged at 14,000 X g for 15 min at 4°C. Approximately 600 μL of the upper of the three resulting phases was then carefully transferred to fresh Eppendorf tubes and 500 μL of isopropanol added. The tubes were gently mixed by inversion and left at room temperature for 5 min. The samples were then centrifuged at 14,000 X g for 10min at 4°C and the isopropanol
aspirated. The RNA pellets were washed 3 times in 1000μL of ice cold 75% ethanol followed by centrifugation at 14,000 X g for 5 min at 4°C. After the 3rd wash, the 75% ethanol was carefully aspirated and the pellets were left to air dry for 10-15 min, and then re-dissolved in 35 μL RNase-free DEPC-treated water at 60°C for 15 min. RNA concentrations were determined by spectrometry [absorbance at 260nm (A_{260}), where 1 A_{260} = 40μg/mL RNA)] using a dilution of 1/100 in RNase-free DEPC-treated water for each sample. Samples were stored at -80°C until needed.

RNA was reversed transcribed using oligo (dT) primers to prepare cDNA used in quantitative RT-PCR assays. RNA samples were diluted to 200 μg per mL; then 2 μL of the RNA dilution, 6.4 μL of water, and 2μL of random primers were incubated at 70°C for 5 min to denature the RNA and then placed directly on ice to prevent RNA re-coiling. The reverse transcription reaction mixture was prepared with 1.6μL of dNTPs, 1μL Ribolock, 4μL of 5X standard buffer, 2μL of 0.1M dithiothreitol (DTT), and 1μL MMLV per sample; 9.6 μL of this mixture was added to each sample tube to bring the total reaction volume to 20μL. The mixture was incubated at 37°C for 1 hr. The reaction was then stopped by incubating the samples at 70°C for 15 min. Samples were stored at -20°C until needed.

2.2.5 Real-time PCR assays

All real-time quantitative reverse transcription polymerase chain reactions (RT-qPCR) were executed in the ABI 7700 and ABI 7900 sequence detection system. Reactions were carried out in 96-well plates with total reaction volumes of 25 μL per well. First reaction mixtures (21 μL, excluding diluted cDNA) were pipette into wells and then 4 μL of diluted cDNA were added
in duplicates per sample. Primer sequences, reaction mixture contents and reaction parameters for various genes are listed in Tables 2.2, 2.3, 2.4 respectively.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFbp6</td>
<td>TTCTACCGAAAGCAGCAGTGTC</td>
<td>TGACCATCTGGAGACACTGGCA</td>
<td>104</td>
</tr>
<tr>
<td>LGL1</td>
<td>CATCTACGCTGACACTTC</td>
<td>ATCACATCTGCATAGCCACCA</td>
<td>87</td>
</tr>
<tr>
<td>Midkine</td>
<td>AGCCCTGCACCTCCAAGAC</td>
<td>AACTCTCTGGCTCTGACTCA</td>
<td>129</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>TGCAGTTGCCTACGACTCGAAT</td>
<td>AGCCACCTTCATTACAGGGTGGA</td>
<td>143</td>
</tr>
</tbody>
</table>

**Table 2.2.** Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFbp6, LGL1, Midkine, PDGFRα</td>
<td>7.5 μL of RNase-free water, 12.5 μL of 2X SYBR® Green reaction buffer, 0.5 μL each of forward and reverse primers, 4μL of diluted (1/8 dilution) cDNA</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>8.125μL of RNase-free water, 12.5μL of 2X Taqman® Universal PCR Master Mix, 0.125 μL each of ribosomal probe, forward primer, reverse primer, 4 μL of diluted (1/8000 dilution) cDNA</td>
</tr>
</tbody>
</table>

**Table 2.3.** Reaction mixtures.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Annealing</th>
<th>Step 5</th>
<th>Cycles</th>
<th>Dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFbp6, Midkine, PDGFRα</td>
<td>50°C-2min</td>
<td>95°C-10min</td>
<td>95°C-15sec</td>
<td>60°C-30sec</td>
<td>72°C-30sec</td>
<td>40</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>LGL1</td>
<td>50°C-2min</td>
<td>95°C-15min</td>
<td>95°C-15sec</td>
<td>56°C-30sec</td>
<td>72°C-30sec</td>
<td>40</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>50°C-2min</td>
<td>95°C-10min</td>
<td>95°C-15sec</td>
<td>60°C-30sec</td>
<td>72°C-30sec</td>
<td>40</td>
<td>VIC</td>
</tr>
</tbody>
</table>

**Table 2.4.** Reaction parameters.
To quantitate differences in mRNA levels, a comparison was made of the number of PCR cycles required to achieve a threshold of fluorescent activity above background during the reaction’s exponential phase. Since all reactions were run in duplicates and given that the Ct values did not vary by more than 0.5, the average Ct value was used. Relative gene copy number was determined using the $2^{-\Delta\Delta Ct}$ method, with all values normalized to 18S rRNA (184).

2.2.6 Preparation of paraffin embedded lung sections

The left lobes of the rat pup lungs were fixed in formalin for 24 hours. Lungs were then dehydrated by immersion in a series of increasing ethanol concentrations (70%, 80%, 90%, 95% for 45 min each, and 3 X 100% for 1 hr each, with the heart and trachea removed before the 2nd 100% ethanol step), the lungs then cleared in 100% xylene followed by 1:1 xylene:paraffin, both for 1 hr, and then embedded in paraffin. Using a microtome, coronal sections of either 5 or 10 μm in thickness were cut and mounted on coated glass slides. Slides were air dried before baking at 43°C overnight, and then stored at RT until needed.

2.2.7 Hematoxylin and eosin staining

Hematoxylin and eosin stained sections were used to illustrate gross lung morphology. Left lobe sections of 5μm in thickness were de-paraffinized in xylene (3 x 5 min), rehydrated with 100% ethanol (3 x 5 min), and then immersed for 5 minutes each in 95%, 90%, 80%, 70%, 50% ethanol and distilled H$_2$O. These were then stained with hematoxylin for 5 sec and rinsed in lukewarm running water for 20 minutes. Sections were then dehydrated in ethanol solutions with increasing concentrations (70%, 80%, 90%, 95%) for 5 min each, counterstained in eosin
for 10 sec, washed in 95% and quickly in 100% ethanol, and then submerged in 100% ethanol (3 x 5 min) and cleared in xylene (2 X 5 min). Sections were mounted using 70:30 xylene:permount mounting medium and glass coverslips.

2.2.8 Hart’s elastin staining

Left lobe sections (5 μm) were stained as follows to localize secondary crests. They were de-paraffinized in xylene (3 x 10 min) and rehydrated in 100% ethanol (2 x 5 min), followed by immersions of 5 min each in 95%, 90%, 80%, 70%, 50%, 35% ethanol and distilled H₂O. The sections were left in Weigert’s resorcin-fuchsin solution diluted in acidic 70% ethanol for 22 hours protected from light, then washed in distilled H₂O for 10 min, and counterstained with tartrazine for 10 min. They were then rinsed in distilled water for 1 sec and briefly in 70% ethanol, followed by dehydration by immersion in increasing ethanol concentrations (70%, 80%, 90%, 95%, 3 x 100%) for 5 min each and cleared in xylene (3 x 15 min). Sections were mounted using 70:30 xylene:permount mounting medium and glass coverslips.

2.2.9 Morphometric analysis: Secondary crest measurements

Evenly inflated lungs were used for morphometric analysis. Images of stained sections were digitally captured using a Leica DC200 camera and Leica DC Viewer software. Magnification used was 250X. For each animal, 10 random non-overlapping images were obtained from each of 4 tissue sections. The number of elastin-stained secondary crest tips, irrespective of their length, were counted per image. This value was divided by the area of the screen (91,120 μm²) to determine the number of secondary crests per unit area. The average of
the 40 counts was used to calculate the individual animal’s average. Mean ± standard error of the mean (SEM) calculated from the 4 animal values was then used for group results.

2.2.10 Morphometric analysis: Mean linear intercept

Mean linear intercept (Lm) is a measure of the average diameter of alveoli. To determine Lm, a cross of two perpendicular lines each 200 μm in length was superimposed over an image. Then, the number of times the cross intersects with tissue was counted (1 count for distal lung tissue intercept and 0.5 for airway or vessel wall intercept). To determine Lm (in micrometers), the length of the two lines (400 μm) was then divided by the number of intersections.

2.2.11 Statistical analysis

Data from SEAP assays are presented as mean of treatment duplicates ± SEM. Data from real-time RT-PCR are presented as mean $2^{-\Delta\Delta Ct}$ ± SEM. Statistical significance was defined as $p<0.05$, and was determined using Sigma Stat 3.0 software (SPSS, Jandel Scientific, Chicago, IL, U.S.A.). Comparisons between 3 or more groups were made using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test. Comparisons between two groups were made with either Student’s t-test or a Mann-Whitney test for data that failed a normality test.
Chapter Three: **Results**
3.1 In Vitro GC-GR Signalling Blockade

Overall, CP472555 in a 2-log excess of GC provided effective pharmacological blockade of GC-GR signalling in fetal rat lung cells in vitro.

To test for stimulation of GR activity by Dex and for effective blockade of this stimulation by GR antagonists RU486 or CP472555, primary cultures of either rat lung adjacent fibroblast or epithelial cells on fetal day 20 (term=21.5 days) were transfected with the GR-reporter gene SEAP. In fibroblasts, a 10-fold molar excess of RU486 [10^{-7}M] over Dex [10^{-8}M] reduced the SEAP mRNA levels from those of Dex-treated cells to those of cells treated with either RU486 or (control) medium alone (Figure 3.1, n = 3; p<0.001, by one-way ANOVA, followed by Tukey test). In fibroblast cells, CP472555 antagonism occurred in a dose-dependent fashion: a 100-fold molar excess of CP472555 [10^{-6}M] over Dex [10^{-8}M] reduced the SEAP mRNA levels to those of cells treated with either CP472555 antagonist or control medium alone (Figure 3.2, n = 8; p<0.05, by one-way ANOVA, followed by Tukey test). A 10-fold molar excess of CP472555 [10^{-7}M] to Dex [10^{-8}M] partially reduced the effect of Dex, and equal concentrations of CP472555 to Dex did not significantly reduce Dex stimulation. In epithelial cells, Dex at a concentration of 10^{-7}M was necessary to stimulate GR activity, while a 10-fold molar excess of CP472555 [10^{-6}M] over Dex [10^{-7}M] completely reduced the effect of Dex to the levels of CP472555 and control alone (Figure 3.3, n = 3; p<0.001, by one-way ANOVA, followed by Tukey test). Thus, for both fibroblast and epithelial cells, CP472555 effectively blocked GR at a dose of 10^{-6}M.

GR blockade was also confirmed by assessing mRNA levels of the GC-inducible mesenchymal marker LGL1 in fibroblast cells. Dex [10^{-8}M] increased LGL1 mRNA levels
approximately 4-fold, an effect inhibited by CP472555 [10^{-6}M] (Figure 3.4, \( n = 3; \ p<0.05 \), by one-way ANOVA, followed by Tukey test).
Figure 3.1. **RU486 inhibits GR-dependent SEAP activity in rat fetal day 20 lung fibroblasts.** A 10-fold molar excess of RU486 [10^{-7} M] to Dex [10^{-8} M] completely abrogated the stimulatory effect of Dex [10^{-8} M] on SEAP activity. Equimolar RU486 reduced but did not completely block the Dex effect. All values normalized to Dex [10^{-8} M] treatment value. Bars represent the Mean ± SEM; n=3; *p<0.001 versus all other groups by one-way ANOVA, followed by Tukey test.
Figure 3.2. CP472555 inhibits GR-dependent SEAP activity in fetal rat day 20 lung fibroblasts in a dose dependent fashion. A 100-fold molar excess of CP [10^{-6}M] to Dex [10^{-8}M] completely abrogated the stimulatory effect of Dex. Bars represent the Mean ± SEM; n=8; **p<0.001 versus other Dex [10^{-8}M] groups; *p<0.05 versus Dex [10^{-8}M] alone and with CP [10^{-8}M] by one-way ANOVA, followed by Tukey test.
Figure 3.3. CP472555 inhibits GR-dependent SEAP activity in rat fetal day 20 lung epithelial cells. A 10-fold molar excess of CP [10^{-6}M] to Dex [10^{-7}M] blocked the stimulatory effect of Dex. Bars represent the Mean ± SEM; n=3; **p<0.001 versus all groups by one-way ANOVA, followed by Tukey test.
Figure 3.4. Rat fetal day 20 lung fibroblast cells: LGL1 mRNA levels are upregulated by Dex and Dex effect is blocked by CP472555. A 100-fold molar excess of CP [10^{-6}M] to Dex [10^{-8}M] blocked the stimulatory effect of Dex. Cortisol is shown as a positive control since it also stimulates LGL mRNA expression (157). Fold change is relative to ETOH (control), and normalized to 18S rRNA. Bars represent the Mean ± SEM; n=3; *p<0.05 versus all groups by one-way ANOVA, followed by Tukey test.
3.2 *In Vivo* GC-GR Signalling Blockade

To evaluate the blockade of GC-GR signalling *in vivo*, two main analyses were used. The mRNA levels of a number of GC responsive markers important in lung development were assessed by quantitative real time PCR (conditions and primers specified in the Methods section) of 2 right lobes in antagonist treated and littermate controls rat pups treated with solvent vehicle alone. Expression of LGL1, PDGFRα, IGFbp6, and midkine mRNA were assessed, with 18S ribosomal RNA levels used to normalize all values. GCs have been found to increase the mRNA expression of the LGL1 (245), PDGFRα (329), and IGFbp6 (164), and downregulate the expression of midkine (345).

Histological observation as well as quantification of secondary crest numbers in elastin-stained whole left lobe sections were used to compare CP472555-treated and control littermates.

### 3.2.1 CP472555 administration

The *in vivo* pharmacokinetic properties of CP472555 were not available. Therefore, the initial injection frequency was based upon the once daily frequency described for the commonly used GR antagonist RU486, from which CP472555 was derived, consistent with initial reports of the use of CP472555 to block GR in organs other than the lung. However, once daily administration had variable effects, so I then tried injecting twice daily (BID), based on reports of BID administration of another newly reported GR antagonist, ORG34850 (not available to us) (299), as well as a single report of BID oral administration of CP472555 (216). Duration of treatment and doses used are summarized in Table 3.1.
<table>
<thead>
<tr>
<th>Developmental Period</th>
<th>Frequency</th>
<th>Dose</th>
<th>Differences between CP472555 and control groups</th>
<th>CP472555 effect on GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN0 to PN6 - PN10</td>
<td>Twice daily</td>
<td>50µg/kg, 5µg/kg or 0.5µg/kg</td>
<td>LGL1, PDGFRα, and IGFbp6 mRNA levels upregulated, midkine downregulated (Fig. 3.5)</td>
<td>Partial Agonism</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Simple airspaces (Fig. 3.6B) (Similarity to Dex treatment)</td>
<td></td>
</tr>
<tr>
<td>PN0 to PN4</td>
<td>Once daily</td>
<td>5mg/kg or 0.5mg/kg</td>
<td>Reduced secondary crest count (Fig. 3.7)</td>
<td>Partial Agonism</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Simple airspaces (Fig. 3.8B)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Similarity to Dex treatment (Fig. 3.8C)</td>
<td></td>
</tr>
<tr>
<td>PN11 to PN16</td>
<td>Twice daily</td>
<td>100-molar excess over endogenous corticosterone</td>
<td>Trend towards LGL1, PDGFRα, and IGFbp6 mRNA levels upregulated (Fig. 3.9A)</td>
<td>Partial Agonism</td>
</tr>
<tr>
<td>PN11 to PN18</td>
<td>Twice daily</td>
<td>100-molar excess over endogenous corticosterone</td>
<td>None (Fig. 3.9B)</td>
<td>No net effect</td>
</tr>
<tr>
<td>PN11 to PN21</td>
<td>Twice daily</td>
<td>100-molar excess over endogenous corticosterone</td>
<td>LGL1 and PDGFRα mRNA levels downregulated (Fig. 3.9C)</td>
<td>Antagonism</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Abnormal elastin deposition at secondary crest tips (Fig 3.11)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1. CP472555 *in vivo* from PN0 - PN10, at relatively low circulating corticosterone levels, and while levels are increasing from PN11 – PN21. Once daily administrations of either 50µg/kg, 5µg/kg or 0.5µg/kg prior to PN10 (from PN0 - PN4, and PN0 - PN10), did not exert any effects on CP472555 treated rat pups. Twice daily
administrations prior to PN10 of 5mg/kg (from PN0 – PN4) or 0.5mg/kg (from PN0 – PN7) resulted in non-specific toxicity.

3.2.2 CP472555 injections during the period PN0 to PN10

3.2.2.1 CP472555 administered from birth until the mid-alveolarization period exerted GC agonist-like effects on mRNA levels and secondary septation

Pups treated with a variety of CP472555 doses (50μg/kg, 5μg/kg, or 0.5μg/kg) from PN0 until PN10 had significantly decreased viability compared to control pups (Table 3.2; p<0.001 by Chi Square). Body weights (BWs) of some pups treated with CP472555 before PN10 were significantly decreased compared to control pups on day of sacrifice (Table 3.3; p<0.05 by Student’s t-test). Animals were observed twice daily from PN0 until day of sacrifice. At no time were there any signs of respiratory distress (i.e., not cyanotic, not breathing fast).

Irrespective of the dose used or day of sacrifice, mRNA analyses in most cases demonstrated upregulation in Lgl1, PDGFRα, and IGFbp6, and a downregulation in midkine mRNA expression (Figure 3.5; p< 0.05 by Student’s t-test). In addition, elastin stained tissue sections revealed simpler airspaces in CP472555 treated pups versus vehicle controls at PN7 and at PN10 (Figure 3.6). These results are consistent with the agonist effect of a similar course of Dexamethasone treatment in rat pups.
Table 1. Total neonatal viability prior to PN10. CP472555 treated pups (BID) had significantly decreased viability compared to control pups. p<0.001, by Chi Square.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CP472555 Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead</td>
<td>16</td>
<td>48</td>
</tr>
<tr>
<td>Alive</td>
<td>83</td>
<td>67</td>
</tr>
</tbody>
</table>

Table 2. Body weights. Rat pups treated with 50 µg/kg, 5 µg/kg, or 0.5 µg/kg, BID, and sacrificed between PN6 and PN10. *Values are means ± SEM for n animals in parenthesis. BW = body weight. PN = postnatal day. † p<0.001, ‡ p<0.01, § p<0.05 by Student’s t-test.
Figure 3.5. Partial GC-agonist effect of CP472555 when administered before PN10 on GC-responsive genes. Real-time PCR analysis of lung mRNA from right lobes for LGL1, PDGFRα, IGFbp6 and midkine mRNA. For 5µg/kg administered from PN0-PN10, IGFbp6 and midkine mRNA levels were not investigated because differences in LGL1 and PDGFRα mRNA levels already confirmed partial agonist activity. Values normalized to 18S rRNA (arbitrary units). Representative experiments per dose. Bars represent the Mean ± SEM; *p<0.05 by Student’s t-test.
Figure 3.6. Representative images of Hart’s elastin-stained lung sections from rat pups treated twice daily until PN7 and PN10. CP472555 treated animals (either 50µg/kg, 5µg/kg or 0.5µg/kg) demonstrated simple airspaces compared to control groups. Pups treated with CP472555 are similar in appearance to published results of Dex treated pups (200; 282; 316).
3.2.2.2 CP472555 administered from birth until PN4 altered secondary septation consistent with a GC agonist effect

Once daily injections with CP472555 (5mg/kg or 0.5mg/kg) from PN0 to PN4 were associated with changes in histology. Secondary crest numbers were quantified in elastin stained whole left lobe sections from CP472555 treated and control littermates. The number of secondary crests per unit area (normalized to control), determined via counting those crests stained with elastin per image and dividing this number by the area of the screen (91,120 μm²), was significantly reduced in pups injected with 5mg/kg (0.77 ± 0.05) or 0.5mg/kg (0.81 ± 0.04) of CP472555, compared to control pups (1.00 ± 0.06) (Figure 3.7, n = 4 in each group; p<0.05 by one-way ANOVA followed by Tukey test). Mean linear intercepts in either CP472555 treated group were not statistically different compared to the control group. Representative sections from CP472555 treated and control pups are shown in Figures 3.8A and 3.8B, respectively. CP472555 treated rat pups showed thinner interalveolar septa compared to the control group. These results are consistent with the agonist effect of a similar course of Dex treatment in rat pups (Figure 3.8C).
Figure 3.7. Elastin-stained tips of secondary crests quantified on lung tissue sections from rat pups treated once daily, from PN0 to PN4. A reduction in secondary crest per unit area (normalized to the mean of the no-treatment control group) was seen in rat pups treated with either 5mg/kg or 0.5mg/kg of CP472555. Bars represent the Mean ± SEM; n=4; *p<0.05 vs controls by one-way ANOVA followed by Tukey test.
Figure 3.8. Hart’s elastin-stain, rat pup lung. CP472555 on PN0 – PN4 [5mg/kg or 0.5mg/kg] (B) caused thinner interalveolar septa and fewer secondary crests compared to control (A). Secondary crest tips are elastin-positive in control image (A) (arrows). Dexamethasone (0.04mg/kg) (C) are similar in appearance to CP472555 treated pups.
3.2.3 CP472555 injections during the period PN11 to PN21

Endogenous plasma GC levels spontaneously rise from PN12 onwards (119). Injections of a 100-molar excess of CP472555 over the published total plasma GC concentration at each day after PN11 were well tolerated and all animals survived, in contrast to the injections prior to PN10. BWs were not significantly different compared to control rat pups at PN16, PN18 or PN21.

3.2.3.1 CP472555 injected BID from PN11 until PN16 exerted GC agonist-like effects on mRNA levels

There was a trend towards upregulation of Lgl1, PDGFRα, and IGFbp6, and no difference in midkine mRNA expression (Figure 3.9A). Since n=2 for the PN16 control group, statistical comparison was not possible. As such, these data are presented as mean ± range.

3.2.3.2 CP472555 injected BID from PN11 until PN18 did not exert GC agonist-like effects on mRNA levels

There were no differences between treated and control pups in the mRNA levels of the specified GC-inducible genes (Figure 3.9B, n = at least 3 per group). This suggests that there is no net effect with drug administration, and perhaps an equilibrium between partial agonist and antagonist effects.

3.2.3.3 CP472555 injected BID from PN11 until PN21 (end of secondary septation and microvascular maturation) exerted anti-GR antagonist activity on mRNA levels

When pups were injected with CP472555 BID from PN11 to PN21, LGL1 and PDGFRα mRNA levels were reduced 2-fold and 1.6-fold, respectively (Figure 3.9C, n = 4; p<0.05 by
Student’s t-test). Midkine mRNA levels were not different between CP472555-treated and control pups. Since the former two genes are upregulated by GC-GR signalling, CP472555 appears to be displaying anti-GR antagonist activity during the period of increased circulating endogenous GC levels. These results are consistent with upregulation of these genes during late alveolarization by endogenous GCs.

Sections from the CP472555-treated PN21 pups were stained with Hart’s elastin solution. While secondary crest counts per unit area and mean linear intercept results were not affected by the CP472555 treatment, elastin fibres at some of the secondary crest tips appear abnormal and fragmented compared to those found in the control group (Figure 3.10).
Figure 3.9. Effect of CP472555 (100-molar excess over endogenous corticosterone) BID from PN11 to PN16 (A), PN18 (B), or PN21 (C) on mRNA levels of GC-responsive genes in lungs of neonatal rat pups. Real-time PCR analysis of lung mRNA from right lobes for LGL1, PDGFRα, IGFbp6 and midkine mRNA. Values normalized to 18S rRNA (arbitrary units). Bars represent the Mean ± SEM; for n=2, presented as Mean ± Range. *p<0.05 by Student’s t-test.
Figure 3.10. **Elastin deposits at the tips of secondary crests.** Elastin fragments at the tips of secondary crests in CP472555-treated rat pups (PN11 – PN21, BID) are frayed and spread out (arrows), compared to the dense and round elastin at the tips of secondary crests in control rat pups (arrows).
3.2.3.4 RU486 injected BID from PN11 until PN21 exerted anti-GR antagonist activity on mRNA levels

To further test the effects of GR antagonism from PN11 to PN21, rat pups were treated with RU486 in a 100-fold molar excess over total plasma GCs. Gene expression of LGL1 was significantly decreased approximately 2-fold (Figure 3.11, n = at least 4; p<0.05 by Student’s t-test). PDGFRα mRNA trended towards decreased levels. Midkine mRNA levels were not different between RU486-treated and control pups. These results are consistent with those observed with CP472555, above.
Figure 3.11. Effect of RU486 (100-molar excess over endogenous corticosterone) BID from PN11 to PN21 on mRNA levels of GC-responsive genes in lungs of neonatal rat pups. Real-time PCR analysis of lung mRNA from right lobes for LGL1, PDGFRα, and midkine mRNA. Values normalized to 18S rRNA (arbitrary units). Bars represent the Mean ± SEM; *p<0.05 by Student’s t-test.
Chapter Four: *Discussion, Conclusion, and Future Directions*
4.1 **Discussion**

The purpose of this research was to determine the suitability of the compound CP472555 for the blockade of GC-GR signalling in neonatal rat lung *in vivo*. The principal finding of this study is that CP472555 exhibits anti-GR antagonist activity in the developing rat lung at PN21, as demonstrated by decreased mRNA levels of LGL1 and PDGFRα, two GC-inducible genes known to be upregulated by GCs. This finding is important because it provides evidence that endogenous GCs normally upregulate LGL1 and PDGFRα during this period of alveolarization. Although Zhang and colleagues (345) have suggested that endogenous GCs influence lung development strictly in the post-natal period, there are no empirical data to support this contention. Further investigation of the effect of GR blockade in alveolarization from PN18 onwards is now feasible with the administration of CP472555 as presented herein.

The present study is the first to use CP472555 for pharmacological blockade of GR in postnatal lung of any species. Importantly, this GR antagonist is potent and selective. GR can also be inhibited in the lung at PN21 by blockade with RU486, but the specificity of CP472555 simplifies the interpretation of results compared to the use of RU486, which exerts both anti-GR and confounding anti-PR antagonist activity.

While GR antagonism occurred at PN21, use of CP472555 prior to PN16 exerted GC agonist activity. This was evidenced by differences in gene expression and changes in histology. Up to and including PN16, CP472555 increased LGL1, PDGFRα, and IGFbp6 mRNA levels and decreased midkine mRNA levels, the first three known to be upregulated and the latter known to
be downregulated by GCs. Moreover, changes in histological appearance included simpler airspaces with thinner inter-airspace walls and a reduced number of secondary septa.

CP472555 may act in the rat as a weak partial agonist that competes directly with endogenous GCs for rat (as opposed to human) pulmonary GRs. An analogy might be the suggested mechanism by which RU486 acts as a PR agonist. When circulating progesterone levels are very low, as in postmenopausal women and ovariectomized monkeys, RU486 acts as a progestin agonist. When progesterone levels are higher, RU486 blocks the progesterone receptor and hence progesterone effects (301). Assuming CP472555 to be a weak, partial GR agonist in neonatal rat lung would explain my present results. Early on, when the total and free circulating GC levels are relatively low, CP472555 has GC agonist activity. The circulating GC levels remain low until PN11 and gradually rise thereafter (119). By contrast to the agonist activity, when GC levels are higher later on in the postnatal period, CP472555 blocks the GR and hence GC effects. In the presence of pathologically raised GC levels, CP472555 has been used to inhibit GR, such as in an obese mouse model (216), in hypercorticosteronemia resulting from insulin-induced hypoglycemia (147-149), just as RU486 has been used in obese mice (106), in hypercorticism in Cushing’s syndrome (60), in stress-induced increases in GCs (339), and in changes in the hypothalamic-pituitary-adrenal (HPA) axis in neuropsychiatric disorders and depression (81; 100).

The mechanism(s) by which CP472555 modulates GC-GR signalling are as yet not completely understood; however, one may speculate about conceivable mechanisms. These could include competitive antagonism with underlying partial agonist activity, displacement of
GCs from CBG and mechanism(s) similar to RU486. When circulating endogenous GC levels are low, if CP472555 is a weak partial GR agonist, it may stimulate GR activity more than the endogenous GCs. However, when endogenous GC levels are higher, CP472555 would be expected to serve primarily as a competitive antagonist to the endogenous GCs in binding to GR. If CP472555 were considered a partial agonist exclusively, when both full agonist (GCs) and partial agonist (CP472555) are present, the partial agonist might compete with the full agonist for receptor occupancy, thus producing a net reduction in receptor activation when compared to the full agonist alone (107). Another potential effect could involve the displacement of GCs from CBG by CP472555, thus releasing GCs and increasing the free amount of GCs available in the circulation. By analogy to RU486 (used in the synthesis of CP472555), one could also speculate that they may share similar mechanisms of anti-GC activity. These include, but are not limited to (i.) the induction of a conformational change upon binding to the GR LBD, such that the coactivator pocket on GR is blocked and co-repressors are recruited; (ii.) increased stability of GR-HSP interactions; (iii.) an inhibitory effect on GR’s motility to the nucleus; (iv.) reduced affinity of CP472555-GR for DNA; and (v.) a change in GR recycling back to the nucleus (see section 1.9.1). Since GCs also bind the mineralocorticoid receptor (MR), one could also speculate that CP472555 may have non-GR effects. However, any effect that GCs have on the MR would not be directly touched on by CP472555, since its affinity for MR is very low (>300-fold affinity for GR versus MR) (7).

Previous studies of GC deficiency in the early postnatal period due to adrenalectomy (132; 133) have investigated the role of GCs in the maturation of the thymus and gastrointestinal enzymatic activity, but did not assess lung development. When Kraml et al. (165) administered
onapristone (a compound structurally related to RU486 with high affinity for both GR and PR) from PN9 to PN11, endogenous GCs were inhibited as was evidenced by an increase in thymus weight and early inhibition of brush border enzymatic activity at PN12. By contrast, when onapristone or RU486 were used alone in another study, agonist effects on thymus weight and thymocyte counts, but not other measures, were dose-dependent and limited to the postnatal period from PN12 to PN17, whereas the opposite (antagonist) effect was observed at PN19 (166). These investigators did not study the period of development before PN9, nor did they assess lung development.

In the absence of specific experience with CP472555 treatment of newborn rat pups, the dosing used in studies of adult rodents was considered. While Kleiber’s law states that drug clearance rate increases as the body weight of the animal decreases (163), this law excludes neonates. Neonatal animals differ from adults in a number of important respects, including slower hepatic metabolism and inefficient renal excretion. In neonates, the hepatic metabolism of lipid-soluble drugs occurs at a slower rate, since the microsomal oxidative reactions and glucuronide conjugation are still developing (91; 169). As such, the drug may have a longer half-life in neonates than in the adult (169). Additionally, neonates synthesize acidic urine, which prolongs the duration of action of certain lipid-soluble drugs by promoting their tubular reabsorption (91). Moreover, with a greater percentage of body water and less fat compared to adults, as well as hypoproteinemia which allows for greater peripheral drug distribution, neonates usually have a greater volume of distribution than adults (169). Keeping all of this in mind, generally neonates may require a larger initial dose and reduced frequency of administration to account for the reduced drug elimination rate (91; 169). Due to uncertainty
concerning absorption and elimination in the neonatal animal, dose amounts and frequency often
must be determined empirically (91), and the present study was no exception.

Administration of CP472555 prior to PN10 had an adverse effect in some individual rat
pups. During the neonatal period, the adrenal constitutively secretes corticosterone to promote
differentiation of brain and other tissues (10; 274). Circulating levels are maintained within a
narrow range, because GC levels outside this range are detrimental to the developing CNS (274).
Early corticosteroid therapy in preterm infants (prior to 8 days) causes acute adverse effects
including gastrointestinal bleeding, intestinal perforation, hyperglycaemia, hypertension,
hypertrophic cardiomyopathy and growth failure, as well as long term effects on the CNS (110).
Thus, CP472555 administration prior to PN10 may have had deleterious effects on the CNS and
other systems. While administration of Dex, a full GR agonist, in neonatal rat lung studies
within the first two postnatal weeks is associated with growth failure (200), it was not reported to
cause mortality (200; 269). In addition to the adverse effects known to be caused by exogenous
GR agonists, it is possible that CP472555 may have other, as yet unknown, toxic effects upon the
rat during the period PN0 – PN10.

A potential factor contributing to the apparent systemic GR agonist activity of CP472555
during the period PN0 - PN16 in the rat may relate to GR in the pituitary. GR blockade in the
pituitary might be expected to cause a disinhibition of adrenal GC secretion. In adult women
(99; 116) and obese adult rats (252), RU486 administration has been shown to disinhibit the
HPA axis, such that adrenal GC secretion is augmented. This disinhibition in humans only
occurs at a specific time of day however, demonstrating the importance of the circadian rhythm
(99). It is important to consider the differences between the adult and neonatal HPA axis and circadian rhythm at this point. The adrenocortical circadian rhythm, on the basis of blood corticosterone, is not observed in rats until PN21-PN25, and as early as PN16 when handled or stressed (6). Neonates also exhibit the stress hyporesponsive period during the first two postnatal weeks, where the HPA axis response to mild stress is maintained at a minimum. In one report, an increase in circulating corticosterone as a response to significant stress was not elicited until after PN12 (119). Taken together, these data suggest that increased adrenal secretion of GCs due to disinhibition of the HPA axis would be unlikely to be the cause of the agonist effect seen prior to PN16.

Another factor to consider is that some of the rat pups treated with CP472555 prior to PN10 may have been malnourished, given that their body weights (BW) were significantly lower than control pups. Malnutrition can retard lung growth and delay alveolarization (reviewed in (102)), depending on the severity of the malnutrition and the postnatal day of study.

In one study where rat pups were starved (food deprivation for 24 hours) twice on PN1 and PN5, the rat pups displayed both diminished lung growth and BWs, but their lungs were structurally similar to control lungs when sacrificed at PN7 (79). Pups sacrificed at PN14 on the other hand displayed striking morphologic differences (e.g., large alveoli reduced in number, thick alveolar walls, and reduced elastin fibre counts), which may have been due to severe starvation that permanently impeded alveolarization. Starvation affected elastic tissue quantity in the lung at PN14, but not at PN7. In a different model of experimental malnutrition, the lungs of rat pups that were underfed (achieved by increasing litter size) until PN7 were not significantly different in histological or morphometric analyses (96). These undernourished pups
displayed no lethality and similar reductions in lung and BW (21 and 23%, respectively).

Massaro et al. (200) underfed rat pups until PN14, and found that alveolar septation and alveolar size were unaltered. However, alveolar surface was reduced compared to control pups, and they suggested this was due to an undetermined mechanism not depending on septation.

Should CP472555 administration indeed have had an effect on nutrition of the rat pups, it may have been due to an adverse secondary effect similar to those of Dex. For instance, Dex treatment is associated with gastrointestinal bleeding and spontaneous gastrointestinal perforation in newborns (110); this could conceivably contribute to malnutrition and reduced BWs while still exerting its agonist effects on the lung. While the potential for malnutrition having exerted this effect on these lungs cannot be entirely ruled out, that the partial agonist effect was still apparent at PN16 but the BWs were not different in CP472555 treated pups compared to controls suggests that the agonist affect may have still occurred prior to PN10 even if the BWs had remained unchanged.

In the in vitro investigation of GC-GR signalling blockade, CP472555 in a 100-fold excess of GCs achieved pharmacological blockade. As reflected by the GR reporter SEAP, the levels of GR activity in either lung fibroblasts or epithelial cells were similar in both the control and CP472555-alone treated groups. This suggests that GR agonist activity of CP472555 in the doses used was absent in primary cultures of rat lung cells. Although CP472555 exerts GR agonist activity in mouse cells in vitro, this effect was not present in human cells in vitro (216). This observation underlines the species-specific differences in GR activity by CP472555 in vitro.
Had CP472555 blocked GR in the PN0–PN10 period, we might have expected to observe abnormal lung development similar to that of GR\textsuperscript{hypo} mice, in which GR protein is aberrant throughout gestational development as well as in the newborn period. GR\textsuperscript{hypo} newborn non-survivors demonstrated thicker interalveolar septa and a reduction in secondary crest counts compared to either GR\textsuperscript{hypo} survivor mice or wild-type littermates (229). However, it was not possible to achieve GR blockade using CP472555 during this period, where treated pups displayed differences in histology and changes in mRNA levels that were similar to pups treated with exogenous GC agonists. The lungs of rats treated with Dex in the first 4 postnatal days and within the first two postnatal weeks demonstrate thinner interalveolar septa and premature maturation of the alveolar septa compared to controls (200; 269). Since alveolar septation involves replication of various cell types, this inhibitory effect of GCs on septation may be due to their ability to inhibit cell proliferation (70; 200). Dex administration also accelerates maturation of the capillary bed, thereby contributing to the reduction in inter-airspace wall thickness (269; 316). Just as exogenous GCs inhibit septation, it has been suggested that endogenous GCs might be inhibitors of septation (345). This is in light of the fact that GC levels remain low when the process of secondary septation is maximal, and rise as septation ends and microvascular maturation occurs (119).

If indeed the physiological rise in endogenous GC levels beginning on PN12 in the rat inhibits secondary alveolar septation as is currently thought, I expected pharmacological blockade of GC-GR signalling at this time to cause a prolongation of the period of secondary septation. CP472555 at least partially blocked GR at PN21, as evidenced by reductions in LGL1 and PDGFR\textalpha mRNA expression levels. Interestingly, both LGL1 and PDGFR\textalpha deficiencies
have been associated with irregularities in elastin deposition (168; 179), which is required for septation to occur. Some forming secondary septa of CP472555 treated pups at PN21 had fragmented, aberrant elastin deposition at the tips compared to control pups. Moreover, quantitative changes in histology might have been expected. Had an inhibitory effect of endogenous GCs on septation been prevented by CP472555, then CP472555 treated pups should have demonstrated ongoing septation, whereas inhibition would already have begun in controls pups. Perhaps this is what I would have found if CP472555 had been administered exclusively after the point at which it last displayed agonist activity (PN16), rather than beginning at PN11.

Postnatal GC-GR signalling could be disrupted in a variety of ways, including transgenic mouse models with inducible GR suppression or GR small inhibitory (si)RNA. However, these alternative methods are associated with their own drawbacks; those for inducible GR suppression include background gene expression (gene of interest not maintained in fully repressed “off” state), incomplete recombination, and toxicity of some inducing reagents, whereas siRNA might not exert a complete blockade in all cell types.

4.2 Conclusion

In conclusion, this research investigated the suitability of the compound CP472555 in the blockade of GC-GR signalling in the neonatal rat lung in vivo. At PN21, CP472555 exhibits anti-GR antagonist activity in the developing lung. By contrast, use of CP472555 prior to PN16 exerted GC agonist activity. Moreover, CP472555 injections prior to PN10 resulted in adverse effects and mortality in some individual neonatal rat pups younger than PN10. As such, use of this compound in neonatal rat pups younger than PN10 may not be advised. In vitro studies of GR antagonism demonstrated that CP472555 in a 100-fold excess of GCs exerted a complete
pharmacological blockade. Interestingly, agonist activity of CP472555 in primary culture of rat lung cells was absent. The effects of anti-GR antagonist activity in the developing lung at PN21, near the termination of septation and at the time of microvascular maturation, support physiological roles for endogenous GC-GR signalling at this time, and further investigation of the effect of GR inhibition is now feasible pharmacologically with the administration of CP472555 as presented herein.

4.3 Future Directions

Inhibition by CP472555 of GC-GR signalling during the postnatal period in the rat when GC levels are rising should facilitate investigation of GC-responsive factors that regulate late alveolarization and microvascular maturation. Furthermore, it might be interesting to see if CP472555 could be used to block of GC-GR signalling in other systems, such as a means of preventing pre-term labour.

Given my finding that GR-blockade during this time may alter elastin deposition, it would be of interest to evaluate the effects of such blockade on the expression of tropoelastin and PDGFs (PDGF-A, PDGF-B and its beta-receptor) as well as on the migration/localization of alveolar myofibroblasts. It would also be of interest to assess the expression and activity of enzymes and proteins involved in elastin synthesis and assembly (e.g., LOX, LOX like-1, LOX like-2, fibulin-5, fibrillin-1, fibrillin-2). Looking at other ECM proteins might shed light on how they might be influenced by endogenous GCs during the period of alveolar formation in vivo in the rat. Particularly, other factors implicated in alveolar development of interest would be tenascin-C, HGF, FGF7, VEGFR-2, VEGFR-1 and VEGF-A. These can be investigated by
screening for changes in mRNA levels via qRT-PCR and protein levels with western blot and immunohistochemistry.

Transmission electron micrographs of the interalveolar septa at and near PN21 would be useful in visualizing septal characteristics (e.g., thick versus thin, smooth versus rugged and irregular), as well as those of the capillary system (e.g., degree of maturation into the single capillary network, and whether it is located at one side of the septum). Moreover, changes in the timing and rate of apoptosis of the central layer of connective tissue (which normally peaks at PN19 - PN21) might be studied by applying the TdT-mediated dUTP nick end labelling (TUNEL) apoptosis assay or assessing apoptosis markers (e.g., caspase 3 and 9). Capillary endothelial cell proliferation expected after the process of capillary fusion may also be assessed by looking at markers of cellular proliferation (such as thymidine incorporation, immunostaining for proliferation cell nuclear antigen, bromodeoxyuridine or phospho-Histone H3) and cell cycle genes (e.g., CDKIs such as P27KIP1 and p21CIP1).

Since exogenous GCs are known to arrest alveolarization and accelerate capillary maturation, the above studies would be of interest to provide insight into how endogenous GCs influence maturation of the postnatal rat lung.
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