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Growth Hormone in the Developing Lung

by

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ABSTRACT

Pituitary growth hormone (GH) is an endocrine regulator of growth and development. However, due to the late ontogenetic appearance of the pituitary gland, and the late detection of GH in fetal circulation, pituitary GH cannot be involved in fetal or embryonic growth. This paradox may reflect the recent discovery that the GH gene is widely expressed in many extrapituitary tissues during organogenesis, in which it is thought to have local autocrine or paracrine actions that stimulate tissue proliferation and differentiation. Since the lung has been shown to express GH receptors (GHRs) prior to the ontogeny of pituitary somatotrophs, the possibility that the lung may be a site of GH production and action during early lung development has been examined.

Expression of GH mRNA was first evaluated in the developing chick lung. GH mRNA and protein were found to be expressed in mesenchymal and epithelial cells throughout embryonic development, declining near hatch, with the onset of pituitary GH secretion. GHR mRNA and protein were also found in the chick lung during the same period and in the same tissues, suggesting autocrine/paracrine roles of GH during lung development. The rat lung was also found to similarly express GH during the saccular and alveolar stages of development, when GHRs are also expressed. The possibility that lung GH may have autocrine/paracrine actions in the lung during during perinatal development was therefore examined, using protein expression and a proteomics approach as a marker of GH action.

Altered GH signaling in the lung during the alveolarization period was achieved using intratracheal antisense oligodeoxynucleotides, to specifically downregulate lung GH expression, and by aerosolized GH transfected adenoviruses, to specifically overexpress the GH gene. Neonatal GHR knockout mice were also used as a model of GH deficiency. Changes in lung proteins, determined by 2D gel electophoresis and mass spectrometry, revealed roles of GH in the regulation of proliferative, metabolic, oxidative stress, and surfactant-related proteins in the lung during alveolarization.

These studies demonstrate the novel expression and action of GH in the developing lung and support the possibility that GH has important autocrine or endocrine roles in lung development.

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LIST OF ABBREVIATIONS

2-DE	two dimensional gel electrophoresis
ABC	avidin-biotin-peroxidase method
AdV	adenovirus
AE1 cell	type I epithelial cell
AE2 cell	type II epithelial cell
ALD	aldehyde reductase
ALS	acid labile subunit
AM	alveolar macrophages
AP	anteroposterior
APEN	apurinic endonuclease
Apoa	apolipoprotein A
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bFGF	basic fibroblast growth factor
bp	base pair(s)
BMP	bone morphogenetic protein
CacyBP	calcyclin binding protein
CAF	chromatin assembly factor
cDNA	complementary deoxyribonucleic acid
cGH	chicken growth hormone
CMV	cytomegalovirus
Cu-Zn SOD	copper-zinc superoxide dismutase

D	postnatal day
DAB	diaminobenzidine tetrahydrochloride
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOPE	dioleoylphosphatidyl-ethanol-amine
DOTAP	1,2-Dioleoyl-3-trimethylammonium-propane
DPPC	dipalmitoylphosphatidylcholine
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ED	embryonic day
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
FABP	fatty acid binding protein
FBM	fetal breathing movements
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GADD	growth arrest and DNA damage inducible protein
GD	gestational day
GFP	green fluorescent protein

GH	growth hormone
GHR	growth hormone receptor
GHRG	growth hormone response gene
GH-V	placental growth hormone (growth hormone variant)
gp	glycoprotein
Grtp	growth hormone regulated TBC protein
G S-T	glutathione s-transferase
HNF	hepatocyte nuclear factor
IEF	isoelectric focusing
IGF	insulin-like growth factor
IGF-R	insulin-like growth factor receptor
IGFBP	insulin-like growth factor binding protein
IHC	immunohistochemistry
IPG	immobilized pH gradient
IRF	interferon response factor
IRS	insulin-receptor substrate
ISH	*in situ* hybridization
IVF	*in vitro* fertilization
JAK	janus kinase
kDa	kilodalton
KO	knockout mouse
L2 cells	lung type II epithelial cell line
MAP	mitogen activated protein

MAPK	mitogen activated protein kinase
MCT	monocarboxylate transporter
mGH	mouse growth hormone
min	minute
Mr	relative molecular weight
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MT-MMP	membrane-type matrix metalloproteinase
MW	molecular weight
NBT/BCIP	nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate
NDPK	nucleoside diphosphate kinase B
NFκB	nuclear factor of kappa light chain gene enhancer in B cells
NGS	normal goat serum
NRS	normal rabbit serum
ODN	antisense oligodeoxynucleotide
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PD	postnatal day
Prdx	peroxiredoxin
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
RBP	retinoblastoma binding protein

rGH	rat growth hormone
RNase	ribonuclease
RT	reverse transcription
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation unit
SEM	standard error of the mean
SH	src-homology domain
Shh	sonic hedgehog
SOCS	suppressor of cytokine signaling
SP	surfactant protein
STAT	signal transducer and activator of transcription
TBS	tris buffered saline
T-TBS	tween-tris buffered saline
TE	tris-ethyldiamine tetraacetic acid
TGFβ	transforming growth factor beta
V	volt
Vh	volt hour
V-CAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WSXWS	tryptophan-serine-X-tryptophan-serine motif
YGEFS	tyrosine-glycine-glutamic acid-phenylalanine-serine motif

CHAPTER 1

LITERATURE REVIEW

1.1 Review of Selected Literature

1.1.1 Pituitary growth hormone (GH), insulin-like growth factor (IGF)-1, IGF-2 and growth

1.1.1.1 Pituitary GH and growth

Endocrine GH is produced and secreted into circulation by somatotroph cells in the anterior pituitary gland, and stimulates the growth and differentiation of distant target cells, tissues, and organs. GH has general roles in metabolism that promote growth of the individual. In particular, these include metabolism of protein (Moller and Norrelund, 2003; Hammarqvist et al., 2001; O'Leary et al., 2002; Bush et al., 2003), fat (Louveau and Gondret, 2004; Takahashi and Satozawa, 2002), glucose (Van der Lely, 2004), and bone (Ueland 2004; Guler et al.,1988). Given these general metabolic roles of GH, it follows that GH is critical for normal growth. For example, growth is impaired in hypopituitarism and hyposomatotropism (Gluckman et al., 1981; Cheng et al., 1983). These roles of GH in metabolism were once thought to be mediated by insulin-like growth factors (IGFs), which were originally named somatomedins.

The original version of the somatomedin hypothesis was that GH was an endocrine that stimulated the liver to produce IGF-1 and release it into the circulation, whereby IGF-1 in turn acted as an endocrine to promote somatic growth throughout the body (Le Roith et al., 2001b). In the current version of the somatomedin hypothesis (reviewed by Le Roith et al., 2001a), GH acts on tissues to stimulate local production of IGF-1, which leads to tissue growth. When GH is given to hypophysectomized animals, IGF-1 expression is detected in liver, pancreas, muscle,

intestine, kidney, brain, and adipose tissue (Roberts et al., 1987). This has led researchers to conclude that local tissue production and function of IGF-1 is strictly dependent on GH. However, recent evidence has shown that IGF-1 can also have functions on growth that are independent of GH.

1.1.1.2 GH-independent effects of IGF-1 on Growth

Genetic evidence has suggested that GH may not affect prenatal development, whereas IGF-1 plays a major role (Le Roith et al., 2001a). IGF-1 knockout (KO) mice are born small in size, few survive, and those that do survive grow poorly during postnatal development (Liu et al., 1998, Powell-Braxton et al., 1993, Liu et al., 1993). This implies that there may be other molecules controlling IGF-1 action other than GH.

IGF-1 expression is also regulated by other factors. These factors include: estrogen in the uterus, follicle-stimulating hormone in the ovary, parathyroid hormone and estrogen in bone, and thyroid hormone in the heart (Murphy et al., 1987, Hatey et al., 1992, Kupfer and Rubin, 1992). The plurality of IGF-1 (and the structurally similar IGF-2) regulators may be important in specific growth phases, particularly in the prenatal period, during which pituitary GH secretion is lacking until late in development.

1.1.1.3 IGF-1 and IGF-2 in Embryonic/Fetal Growth

To date, there is a considerable consensus that IGF-2 is an important systemic/paracrine factor in embryonic growth (Le Roith et al., 2001a, Haig and

Graham, 1991). IGF-2 exerts its actions through binding to the IGF-1 receptor (R), whereas the IGF-2R functions as a clearance receptor (Gluckman and Pinal, 2003). IGF-2 overexpression or IGF-2R KO leads to embryonic overgrowth, whereas IGF-2 KO leads to poor embryonic growth (Gluckman and Pinal, 2003).

The primary IGF in embryonic growth is thought to be IGF-2, and the primary IGF in fetal growth is IGF-1 (D'Ercole et al., 1980). Some researchers consider IGF-1 production in the fetus is not regulated by GH, since GHRs are only expressed at low levels in fetal tissues (Gluckman 1986, Gluckman et al., 1992, Klempt et al., 1993). In fact, fetal insulin has been proposed as the regulator of circulating fetal IGF-1 (Oliver et al., 1996). The importance placed on IGF-1 and 2 in development has led many to conclude the absence of a role for GH in prenatal growth. Although the potential prenatal roles of GH in growth are controversial (and will be discussed later), the role of GH in postnatal growth has been well documented.

1.1.1.4 Pituitary GH deficiency and growth

Postnatally, the major role of GH is to promote longitudinal bone growth (Boot 2003). GH deficiency results in a decreased height (Di Cesare et al., 2005), as defined by greater than 2 standard deviation (SD) units below the population mean. Upon administration of recombinant GH, children experience substantial catch-up growth (Carel et al., 2003). As discussed previously, GH has metabolic roles. GH deficiency is associated with increased fat mass and decreased lean body mass (Boot et al., 1997; Degerblad et al., 1992). Furthermore, lumbar spine bone mineral density is decreased in GH deficient children (Boot 2003). As studies have shown that pituitary GH

deficiency produces delayed normal growth, pituitary GH excess manifests instead as expedited growth.

1.1.1.5 Pituitary GH excess and growth

GH excess causes gigantism in juveniles and acromegaly in adults, which are disorders characterized by overgrowth of some tissues and abnormalities in metabolism (Battezzati and Bertoli, 2005, Drimmie et al., 2000) usually caused by a benign GH-producing tumour of the pituitary (Ferone et al., 2004). Acromegaly induces a decrease in body fat, and an increase in both bone density and water retention (Battezzati and Bertoli, 2005). When considered in light of the studies on GH deficiency, GH serum levels are directly related to bone mineral density and lean body mass, and inversely related to fat mass. These pathological conditions of GH deficiency and excess have been modeled in transgenic mice, with comparable results.

1.1.1.6 Ablated GH action in GHR knockout transgenic mice

Compared to controls, GHR knockout mice have decreased body weight, fasting serum glucose and insulin levels, and IGF-1 levels, and have an extended life span (Coschigano et al., 2003). In fact, GHR KO mice only reach a maximum body weight of approximately 40% of that of control mice (List et al., 2001). These mice have decreased bone growth, as demonstrated by shorter total body length and shorter femur length (Sjogren et al., 2000). Furthermore, the weights of several organs were decreased, including the lungs, liver, thymus, kidney, heart, and spleen (Zhou et al.,

1997). Many of the deficiencies found in the GHR KO mice are similar in GH antagonist mice, and manifest as excesses in the transgenic mice that overexpress GH.

1.1.1.7 GH antagonist transgenic mice

GH antagonist (GHA) mice are those that lack GH bioactivity, since they overexpress a GH antagonist which competes with GH for binding to GHR (Li et al., 2003). GHA mice are born with a smaller body weight and a decreased body length (Li et al., 2003), comparable to GHR KO mice. However, unlike GHR KO mice, GHA mice catch-up to their normal littermates in body weight (Li et al., 2003). The body lengths do not catch up, so adult GHA mice are not as long as their normal littermates. The differences between the GHA and the GHR KO mice are not completely understood, and require further clarification. GH deficient mice provide further support for roles of GH in growth.

1.1.1.8 GH deficient mice

GH "knockout" mice have been generated through expression of diphtheria toxin specifically in somatotrophs (Behringer et al., 1988). These mice have nearly complete ablation of somatotrophs in the pituitary, and thus do not have detectable circulating levels of GH. These transgenic mice are much smaller than their normal littermates, and cease growth at 10-15g body weight at 6 weeks of age (Behringer et al., 1988). This effect on body growth is thought to be caused by pituitary GH, since it is endocrine GH that is "knocked out" in this model. Lungs weights are also

decreased in these transgenic mice, proportionally to overall body weight (Behringer et al., 1990).

Ames mice are another model of GH-deficiency. These mice have a recessive loss of function mutation in Prop-1, a molecule essential for the pituitary-specific transcription factor that leads to GH expression, Pit-1 (Dutour 1997). Since Pit-1 is absent, GH is not expressed in the pituitary gland. These mice have reduced levels of IGF-1, thyroid hormones, insulin, and glucose, and have reduced body weight (Sornson et al., 1996; Bartke 2000). This supports the role of pituitary GH in growth and metabolism.

Snell dwarf mice are also GH-deficient. Whereas Ames mice have a mutation in Prop-1, Snell dwarf mice have a mutation in Pit-1 (Dutour 1997). Snell dwarfs also have decreased body weight (Flurkey et al., 2001), and decreased IGF-1 and insulin levels (Hsieh et al., 2002; Bartke et al., 2003). All of these models of GH-deficiency highlight the role of GH in body growth and metabolism. GH excess transgenic mice reveal opposite results to those obtained in the GHR KO mice and GH deficient mice.

1.1.1.9 GH excess in transgenic mice

In comparison to control mice, GH excess transgenic mice had increased total body weight, nose-rump length, and bone mineral content (in whole carcass, femur, tibia, humerus, and spine) (Eckstein et al., 2002). Prolonged exposure to GH excess causes overgrowth of many tissues. For example, chronic GH excess in transgenic mice causes adrenal cortical enlargement, through both hypertrophy and hyperplasia of zona fasciculata cells (Hoeflich et al., 2002). Furthermore, chronic GH elevation

causes increased serum cholesterol, IGF-1, and insulin, whereas it causes decreased serum glucose, free fatty acids, and triglycerides (Frick et al., 2001).

In summary, these studies reveal that GH is a potent regulator of postnatal growth. Currently, dogma in the literature states that IGF-1/-2 are much more important than GH in prenatal growth and development, and that they may not be regulated by GH. These theories have been prevalent in the literature for a long time, and have only been challenged recently with newer studies on prenatal roles of GH.

1.1.2 GH in development

1.1.2.1 Historical Perspective

The development literature has widely concluded that growth hormone (GH) has no role in development, since much of development occurs prior to the appearance of pituitary somatotrophs. Somatotrophs appear at embryonic day (ED) 19 of the 21 day gestation period in rats (Frawley et al., 1985; Hemming et al., 1986) and at ED16 of 21 day incubation period in chicks (Harvey et al., 1998). For this reason, fetal development has been referred to as a "growth without growth hormone syndrome"(Geffner 1996). This theory is supported by the normal development of decapitated chick embryos (decapitated at ED 1.5) until ED14.5, at which time the embryos were found to grow more slowly, an effect reversed through pituitary GH replacement (Thommes et al., 1992). Similarly, anencephalic human fetuses (Chard 1989) and hypophysectomised fetuses (Gluckman et al., 1981) grow at approximately normal rates without detectable circulating pituitary GH. Pituitary GH cannot therefore, participate in early embryonic or fetal growth. The pituitary is, however,

not the only tissue that expresses the GH gene (Table 1.1). Extrapituitary GH, therefore, may be acting as a local paracrine/autocrine growth and differentiation factor in early development, considering its cytokine-like functions (Harvey et al., 1998, Waters et al., 1999). Since GH is expressed locally in these tissues, it may be equally or even more important than pituitary GH in signaling through the GH receptors (GHR) that are found in these tissues.

GHR mRNA and protein are expressed in preimplantation mouse embryos and embryonic stem cells (Ohlsson et al., 1993, Pantaleon et al., 1997), with protein detected from the two cell stage by immunohistochemistry. The role of these GHR is proposed to be in glucose transport and protein synthesis (Pantaleon et al., 1997), as well as in decreased apoptosis (Kolle et al., 2003).

GHR has widespread distribution in the mid to late gestation fetal rat (Garcia-Aragon et al., 1992). Walker et al. (1992) have found that GHR transcripts declined from ED19 to postnatal day (PD) 7 in kidney, lung, and ileum, whereas GHR expression in the liver increased during this same period. A similar decline has also been reported in the rat brain (Lobie et al., 1993). These studies demonstrate that GHR has tissue-specific regulation. Unexpectedly, GHR and IGF-1 mRNA expression mostly do not overlap histologically, with GHR expression primarily expressed in epithelial layers and IGF-1 expressed in connective tissue (Edmonstone et al., 1995). Only in some connective tissues of the dermis, lung, and gut were GHR and IGF-1 coexpressed (Edmonstone et al., 1995). This reflects, once again, the theory that GH and IGF-1 can function independently in the fetus. Although local

GH made in the embryo/fetus may have roles in prenatal growth through GHR, maternal GH may also be important.

1.1.2.2 Maternal GH and growth

GH is an important determinant of litter size and birth length (Waters and Kaye, 2002) that acts during all stages of development to promote successful fetal development. In fact, maternal administration of GH in pigs and cattle increases embryo survival and growth (Gluckman and Pinal, 2002), and implies that one of the roles of GH in early embryogenesis may be to improve placental efficiency (Hill 1992, Rehfeldt et al., 2004). GH has also been shown to act directly on the early embryo (Waters and Kaye, 2002) through improvement of the reliability of cleavage and blastocyst formation in cattle (Izadyar et al., 1998) and mice (Fukaya et al., 1998). GH is known to have a stimulatory effect on oocyte maturation *in vitro* in cows (Izadyar et al., 1996), rats (Apa et al., 1994), pigs (Hagen and Graboski, 1990), rabbits (Yoshimura et al., 1993), and humans (Hassan et al., 2001). This could be a GH action mediated by local IGF-1, since GHR is expressed in germinal vesicle and metaphase II oocytes (Izadyar et al., 2000), and IGF-1 antisera blocks this effect (Apa et al., 1994). GH has practical roles in *in vitro* fertilization (IVF), as IVF rates in humans are more than doubled with prior GH administration to the mother (Hassan et al., 2001). These studies demonstrate that circulating GH from the mother is involved in embryonic/fetal growth. However, the placenta and the fetal pituitary may also be important sources of endocrine GH.

1.1.2.3 Placental GH roles in growth

In humans, placental GH (GH-V) is secreted from placental syncytiotrophoblasts in large quantities into maternal circulation, which regulates maternal metabolism (Waters and Kaye, 2002). Unlike in the human, the ovine placental GH enters fetal circulation, and may have roles in placental growth (Lacroix et al., 1999). Although placental GH appears to be important in fetal growth, many GH disorders result from disruptions in signaling of pituitary GH.

1.1.2.4 Fetal pituitary GH roles in growth

In several species (human, cow, sheep, pig), the pituitary begins to produce GH from the end of the first trimester, whereas in rodents pituitary GH does not appear until the final quarter of gestation (Waters and Kaye, 2002). For those species that demonstrate early pituitary GH, endocrine GH potentially has a role in fetal physiology.

Endocrine GH has several direct actions on fetal development. Laron dwarfs (which have inactivating GHR mutations) are born 2 standard deviation units (SD) shorter than normal (Laron et al., 1993), GH deficient babies are shorter but with normal birthweights (Gluckman et al., 1992), and GH deficient dwarf rats are proportionately reduced in size (Kim et al., 1993). The role of GH in these disease states is supported by *in vitro* and *in vivo* studies of the effects of GH on the fetus. *In vitro*, GH has been shown to have actions on fetal tissues. These include: increased release of insulin from human and rat pancreatic islets (Formby 1985), increased alkaline phosphatase and IGF-1 release from rat fetal tibia (Stracke et al.,

1984), increased proliferation of human hepatocytes (Strain et al., 1987), and increased myelin basic protein synthesis in fetal rat brain (Almazan et al., 1985). As well, several *in vivo* responses to GH have been established in the fetus. Some examples are inhibition of lipogenesis in the subcutaneous adipose deposits of hypophysectomized fetal sheep (Stevens and Alexander, 1986) and fetal pigs (Hausman et al., 1999), and stimulation of angiogenesis in the chick chorioallantoic membrane (Struman et al., 1999).

This summary has focused on general roles of GH in prenatal growth. However, a more detailed discussion of how GH acts at the subcellular (in the control of DNA synthesis) and the cellular levels is needed to understand how GH promotes prenatal growth and development.

1.1.2.5 GH and DNA synthesis

GH has general roles in regulating growth through stimulation of DNA synthesis. Hypophysectomised rats demonstrated decreased DNA synthesis in all tissues examined, and DNA synthesis was restored upon administration of exogenous GH (Goldspink and Goldberg, 1975). This is a function of endocrine pituitary GH, but GH also stimulates DNA synthesis in cell culture. This implies that GH may also act as an autocrine and/or paracrine in stimulating DNA synthesis. Supporting this, GH has been found to promote DNA synthesis in a wide range of cultured cell types, including hepatocytes (Strain et al., 1987), osteoblasts (Slootweg et al., 1988), pancreatic islet of Langerhans cells (Swenne and Hill, 1989), and chondrocytes (Ohlsson et al., 1992). As DNA synthesis is crucial to normal passage through the

cell cycle and replication of the genome, stimulation of DNA synthesis is a key role for GH in cell proliferation.

1.1.2.6 GH and cell proliferation/differentiation

GH is a stimulant of cell proliferation during development, acting as early as the oocyte (Kölle et al., 2003) and the blastocyst (Markham and Kaye, 2003) stages. In the blastocyst, GH regulates the number of cells in the trophectoderm independent of IGF-1. However, IGF-1 regulates the proliferation of the inner cell mass, possibly in concert with GH (Markham and Kaye, 2003). GH may also stimulate muscle cell proliferation in the chick later in development (Goddard et al., 1996, Halevy et al., 1996).

These effects of GH on proliferation may be through phosphatidylinositol 3-kinase intracellular signaling pathways (Jeay et al., 2001) and through p38 MAP kinase (Zhu and Lobie, 2000). The proliferative effects of GH and IGF-1 are mediated through distinct intracellular signaling pathways, at least in the Ba/F3 cell line (Baixeras et al., 2001). Intracellular signaling of GH will be discussed in more detail later in this chapter. The important point here is that these GH roles constitute the basis of GH control of proliferation and differentiation of tissues at the cellular level.

1.1.2.7 GH and tissue proliferation/differentiation

Given the ability of GH to stimulate such basic processes as DNA synthesis and cellular proliferation, it follows that GH would have key roles in regulating tissue

growth. The following are selected tissues chosen as examples to elucidate this assertion.

1.1.2.7.1 Neurogenesis and gliogenesis

GH is a promoter of neurogenesis and gliogenesis in developing rat cerebral cortical cells (Ajo et al., 2003). GH may also negatively regulate neuronal differentiation in some neuronal populations, through its regulation by suppressor of cytokine signaling 2 (SOCS2), suppresses intracellular cytokine signaling (Turnley et al., 2002). These seemingly conflicting findings await reconciliation, and may reflect different actions of GH in different cell populations.

1.1.2.7.2 Adipogenesis

GH levels may also affect adipogenesis (Gevers et al., 2002). GH directly upregulates the expression of several development regulatory molecules in preadipocytes and adipocytes, such as c-fos and c-jun (Sumantran et al., 1992), and members of the STAT family (Shang et al., 2002). GH is a stimulator of adipogenesis in 3T3-F442A fibroblasts (Guller et al., 1988), whereas GH appears to inhibit differentiation of bone stromal cells into adipocytes (Appiagyei-Dankah et al., 2003). Therefore, the role of GH in adipogenesis appears to be tissue specific and awaits further clarification.

1.1.2.7.3 Chondrogenesis

In cultured chick embryonic chondrocytes, GH stimulates chondrogenesis through increases in sulfation of chondroitin sulfate (Meier and Solursh, 1972). The role of GH is in promotion of cartilage differentiation and extracellular mineralization (Maor et al., 1989). In addition, epiphyseal plate chondrocytes express GHR that is capable of GH-binding (Monsonego et al., 1993), supporting a GH-GHR loop in embryonic chondrocytes. GH is therefore an important molecule in chondrogenesis.

In addition to direct tissue-specific roles of GH in proliferation, GH also supports tissue growth indirectly through establishment of tissue vasculature.

1.1.2.8 GH and Angiogenesis

Growth of tissues requires a supply of nutrients from the bloodstream. GH is important in the establishment of blood vessels within tissues.

GH is angiogenetic in the chorioalantoic membrane of the chick embryo (Gould et al., 1995), even though the GH molecule is composed of both angiogenic and antiangiogenic portions (Struman et al., 1999). As well, in transgenic mice overexpressing GH, angiogenesis is increased in wounds during wound healing (Thorey et al., 2004). Although the precise mechanism of GH-induced angiogenesis is not understood, this action of GH may be through an influence on endothelin-induced migration of endothelial cells (Ikeo et al., 2001). The function of GH in the promotion of angiogenesis is another mechanism by which GH leads to tissue proliferation. In addition to angiogenesis-mediated growth, GH directly controls the survival of cells within tissues.

1.1.2.9 GH and Cell Survival

If differentiating cells are not exposed to anti-apoptotic survival factors from neighbouring cells, apoptosis may ensue (Raff et al., 1994). GH has been shown extensively to be an anti-apoptotic survival factor *in vivo* and *in vitro* (Mylonas et al., 2000; Segard et al ., 2003; Gonzalez-Juanatey et al., 2004). Anti-apoptotic roles in development are seen from the oocyte stage (Kolle et al., 2003).

GH may promote cell survival through effects on the cytoskeleton and cell adhesion, as cell adhesion loss can lead to death of the cell (Frisch and Screaton, 2001), known as anoikis. GH can initiate both actin (Goh et al., 1997, Herrington et al., 2000) and microtubule (Goh et al., 1998) cytoskeletal reorganization, and can increase the expression of the cell adhesion molecule V-CAM (Hansen et al., 2004).

Most of the studies on anti-apoptotic roles of GH in development have focused on the neonate. GH has been shown to be neuroprotective in the neonatal rat brain (Shin et al., 2004), and has an *in vitro* protective effect on cardiac myocytes of the 1 day postnatal rat (Gu et al., 2001). These anti-apoptotic roles of GH may serve important functions in the development of these organs.

The potential of GH in anti-apoptotic actions presents the possibility that it may be an important survival factor in specific cell types of the developing lung, an organ that is heavily dependent on the balance of proliferation, differentiation, and apoptosis (Del Riccio et al., 2004).

The previous discussion has revealed many mechanisms through which GH regulates growth. However, for GH to have roles within a given tissue, the presence of functional GHR is required.

1.1.3 GH Receptor (GHR)

1.1.3.1 GHR in Fetal Development

The GH Receptor (GHR) has been localized during fetal development in the human, cow, rat, rabbit, and chicken (egs. Burnside and Cogburn, 1992, Hill et al., 1992, Scott et al., 1992, Ymer and Herrington, 1992, Batchelor et al., 1998). Furthermore, GHRs are present very early in development, from ED2 in the chick (Harvey et al., 2000), from early cleavage in the mouse (Terada et al., 1996, Panteleon et al., 1997), and from ED2 in the bovine embryo (Izadyar et al., 2000, Kolle et al., 2001). Since these studies have demonstrated GHRs prior to the differentiation of the pituitary gland, this raises the possibility that these GHRs may not be for endocrine pathways, but for the actions of GH produced locally that may function through an autocrine and/or a paracrine mechanism.

The importance of GHRs in fetal development has been shown by studies with GHR homozygous knockout (KO) mice. Among GHR KO homozygous matings, litter sizes are reduced to nearly 25% of wildtype matings. This may reflect decreased embryo survival and/or a decrease in the fertility of the dam (Danilovich et al., 1999). Furthermore, GHR KO mice display decreased plasma IGF-I, and a dwarf phenotype, as a result of reduced growth (Bartke et al., 1999). These findings reflect notable deficits in development due to the absence of functional GHRs.

Although GH is traditionally considered to be an endocrine molecule, it is in fact a member of the cytokine family, a group of molecules that are mainly considered to function as autocrines and/or paracrines. GHR is a cytokine receptor, of the receptor associated tyrosine kinase type.

1.1.3.2 GHR as a Member of the Family of Cytokine Receptors

The GHR was the first member of the class 1 cytokine receptors to be cloned (Leung et al., 1987). All of the receptors in this family possess the following five features: (i) they are single membrane pass receptors that function either as homodimers (GHR), or as heterodimer/oligomeric complexes with limited extracellular domain homology; (ii) the extracellular domains are made up of two or more fibronectin III modules (GHR - two fibronectin beta sandwich domains); (iii) there are two cysteine pairs and a conserved tryptophan adjacent to the cysteine in the N-terminal; (iv) there is a WSXWS or equivalent motif (GHR – equivalent YGEFS motif) found in the C-terminal; and (v) there are two conserved proximal membrane sequences in the cytoplasmic domain, which are referred to as boxes 1 (proline-rich and important for signaling) and 2 (Waters et al., 1999). GH, like the other ligands for class 1 cytokine receptors, forms a four alpha helical bundle (Abdel-Meguid et al., 1987). As GHR is structurally similar to other class 1 cytokine receptors, it also has functional similarities.

1.1.3.3 GHR as a Receptor-Associated Tyrosine Kinase

The GHR functions analogously to the tyrosine kinase receptors, except that the cytoplasmic domain of GHR does not have intrinsic tyrosine kinase activity. As such, the GHR dimer has two bound janus kinase (JAK) 2 molecules, one bound to each of the box 1 sequence (ie. one per each molecule of GHR) (Waters et al., 1999). Upon ligand binding, the two JAK2 molecules are juxtapositioned (Liu et al., 1998) and proceed to *trans*-phosphorylate each other, leading to JAK2 activation (Waters et al.,

1999), which gives kinase activity to the complex, commencing the intracellular signaling cascade.

1.1.3.4 GHR Intracellular Signaling

Upon JAK2 activation, JAK2 proceeds to phosphorylate the GHR, which then signal through signal transducers and activators of transcription (STAT) (particularly STAT5) that control transcription of GH-response genes, adaptor proteins such as insulin-receptor substrate (IRS) 1 and 2 that control glucose uptake, Shc that leads to ras and then mitogen activated protein (MAP) kinase pathway activation, and phospholipase C that leads to the control of calcium influx (Waters et al., 1999). The GHR also controls other intracellular signaling, reviewed elsewhere (eg. Postel-Vinay and Kelly, 1996, Waters et al., 1999). Through these pathways of intracellular signaling, GH regulates the expression of several genes.

1.1.4 GH Induction of Genes

GH is known to induce the expression of several genes. Traditionally recognized response genes to GH are: IGF-1 (Matthews et al., 1986), IGF binding protein (IGFBP)-3 (Lemmey et al., 1997), acid labile subunit (ALS) (Ooi et al., 1997), fatty acid binding protein (FABP) (Berry et al., 1993), epidermal growth factor (EGF) receptor (Ekberg et al., 1989), interferon response factor (IRF)-1 (Le Stunff and Rotwein, 1998), and hepatocyte nuclear factor (HNF)-6 (Lahuna et al., 1997).

Upon preadipocyte stimulation with GH, several transcription factors are upregulated, most notably c-*fos* and egr-1 (Chen et al., 1995). Analysis of the

promoter regions of these transcription factors reveals that GH uses STATs and multiple serum response factor, respectively, to activate their expression (Waters et al., 1999).

Of considerable interest is the ability of GH to induce expression of the suppressors of cytokine signaling (SOCS) (Adams et al., 1998). These proteins form a feedback loop that makes the tissue refractory to further stimulation by cytokines for hours.

Using microarray technology, GH was shown to induce expression of several genes in the liver. These were found to be fibrinogen beta, glycoprotein (gp) 130, STAT3, p38MAPK, growth arrest and DNA damage inducible protein 45 (GADD45), apurinic endonuclease (APEN), membrane-type 1 matrix metalloproteinase (MT1-MMP) and monocarboxylate transporter 1 (MCT1) (Thompson et al., 2000). Of these, gp130, STAT3, and p38MAPK are all transducers of cytokine and/or growth factor signaling; GADD45 and APEN control DNA damage in response to stress (Hollander et al., 1999, Ramana et al., 1998); MT1-MMP is a protein capable of degradation of extracellular matrix proteins (Nagase and Woessner, 1999); and MCT1 functions in lactate transport (Poole and Halestrap, 1993).

Other GH-responsive genes include proto-oncogenes (Yoon et al., 1990, Triest et al., 1995), protease inhibitors (Yoon et al., 1987, Warren et al., 1993), and steroid hydroxylases (Wells et al., 1994, Subramanian et al., 1995). As well, a new GH-response gene has been identified in chickens (Harvey et al., 2001).

Since GH can induce the expression of several genes (via GHR), many of which are transducers of cytokine signaling, GH may behave as a local cytokine. The local

expression of GH in many tissues other than the pituitary suggests that this is a possibility.

1.1.5 Extrapituitary Expression of GH

Since the pituitary gland does not secrete GH until late in rat gestation, pituitary GH is not thought to be involved in most of development. Therefore, given the widespread localization of GHR within embryonic and fetal tissues, perhaps GH is locally produced within extrapituitary tissues. In fact, the abundant and widespread production of GH in peripheral tissues of early embryonic chicks (Harvey et al., 1998) and mice (Pantaleon et al., 1997) suggests GH involvement in early development through autocrine or paracrine mechanisms (Harvey and Hull, 1997). Table 1 lists tissues expressing GH mRNA and/or GH-like proteins (modified from Harvey and Hull, 1997). The extrapituitary expression of GH suggests that GH may function as a cytokine, through autocrine and/or paracrine mechanisms.

1.1.6 GH as an autocrine/paracrine

1.1.6.1 Autocrine/paracrine actions of GH mediated through insulin-like growth factor 1 (IGF-1)

GH has been shown to act as an autocrine/paracrine through IGF-1. For example, GH stimulated human thymocytes display a marked increase in synthesis of IGF-1 (Sabharwal and Verma, 1996). This study also demonstrated that antisera to IGF-1 (polyclonal and monoclonal) inhibited the GH-stimulated proliferation of human primary thymic epithelial cell cultures. This is only one example of

autocrine/paracrine GH acting through IGF-1. However, it is important to note that IGF-1 is not always involved as a mediator of autocrine/paracrine GH.

1.1.6.2 Autocrine/paracrine actions of GH independent of IGF-1

As is true for endocrine GH, autocrine/paracrine GH is also subject to the dogma that IGF-1 is its major (and perhaps sole) mediator. Studies have found this not to be the case. For example, GH has been shown to be involved in the process of tooth development (Zhang et al., 1997). GH, GHR, IGF-1, and IGF-1 receptor (IGF-1R) immunoreactivity have been shown in the tooth throughout development (Zhang et al., 1992, Joseph et al., 1994, Zhang et al., 1997). Of interest in these studies is that GH has been shown *in vitro* and *in vivo* to increase the synthesis of bone morphogenetic proteins (BMP) 2 and 4 in pulpal fibroblasts (Li et al., 1998). As such, this demonstrates that autocrine/paracrine actions of GH may also be mediated by factors other than IGF-1, even though IGF-1 is present. In fact, many other local factors may also be induced by autocrine/paracrine GH. GH stimulates proliferation of avian growth plate chondrocytes by increasing the sensitivity of these cells to epidermal growth factor (Monsonego et al., 1995). As well, GH in human mammary carcinoma cells promotes cell proliferation through a down-regulation of transforming growth factor production (Graichen et al., 2001).

GH is also thought to have IGF-1 independent neuroprotective roles in neurogenesis (Harvey et al., 2003). Administration of exogenous GH reduces neuronal necrosis induced by local hypoxic-ischemic injury (Scheepens et al., 1999, 2000, 2001). GH immunoreactivity is increased in the cortical pyramidal neurons in

hemispheres that have suffered hypoxic-ischemic injury and is more pronounced in regions that normally express GHR (Scheepens et al., 1999, 2001). This appears to be an IGF-1 independent mechanism, since it occurs before IGF-1 expression is induced and in regions of the brain that have low IGF-1 tissue levels (Harvey et al., 2003).

Other IGF-1 independent effects of GH include expansion of the growth-plate germinal zone, since this still occurs in IGF-1 KO mice (Wang et al., 1999), and hepatomegaly in response to GH treatment in IGF-1 KO (Liu and Le Roith, 1999). Futhermore, treatment of normal humans and animals with GH increases lipolysis and serum free fatty acids, whereas this effect is not observed upon treatment with IGF-1 (Le Roith et al., 2001). Chronic elevations in GH lead to insulin resistance (Kopchick et al., 1999, Dominici et al., 1999), but similar increases in systemic IGF-1 increase insulin sensitivity (Froesch et al., 1996).

Of importance is that GH can act locally as an autocrine/paracrine, whether through IGF-1 or not. As such, both endocrine and autocrine/paracrine GH may have roles in our tissue of study, the lung.

1.1.7 GH and the lungs

1.1.7.1 Pituitary GH excess (acromegaly) and lung function

It has been observed that large lungs (Bartlett, 1971), upper airflow obstruction (Trotman-Dickenson et al., 1991), and narrowing of the small airways (Harrison et al., 1978) are present in GH excess (acromegalic) patients. Furthermore, respiratory disorders are common complications in acromegaly. Sleep apnea (a breathing

disturbance related sleep disorder), for example, affects 60-70% of acromegalics (Fatti et al., 2001). Overall mortality rate for acromegalics from lung disease is 2-3 times higher than it is in the general population (Orme et al., 1998). Persistent congestion of the nasal mucosa and nasal polyps are also common (Lund 1994, Petruson et al., 1988). It is believed that the hypertrophy of the nasal mucosa, due to elevated GH and IGF-1 levels, is responsible for these nasal characteristics in acromegalics (Hansson et al., 1991). As well, acromegaly results in pneumomegaly, which is an increase in proliferation in the lung and thus an increase in alveolar number (Brody et al., 1970). This produces an increase in lung capacity by 81% in males, and by 56% in females. These effects are due to excess *pituitary* GH, as opposed to locally produced lung GH, but they nonetheless demonstrate potential roles for GH in pulmonary function.

1.1.7.2 Pituitary GH deficiency and lung function

GH deficiency, on the other hand, displays a decrease in respiratory muscle strength, and a reduction in the maximum inspiratory and expiratory pressures (Merola et al. 1996, Merola et al. 1995). As well, total lung capacity and vital capacity are reduced in GH deficiency (Sofia et al., 1995). Again, this is pituitary GH, but implies more potential roles for GH in the lung. Furthermore, in Laron syndrome (GH-insensitivity) peak exercise O_2-uptake is reduced (Ben-Dov et al., 2003). This is another example of impaired lung function when GH signaling is reduced. Since GH excess and GH deficiency result in abnormal lung sequelae, the lung may be a GH target site.

1.1.7.3 The Lung as a Possible GH Target Site?

The possibility that the lung is a target site for pituitary GH action is indicated by the GH-induced production of superoxide by alveolar macrophages (Edwards et al., 1992), the GH-induced increase in circulating lung neutrophil activation during sepsis, and the accompanying increase in microvascular injury (Liu et al., 2002a). Exogenous GH also induces NFκB activation in the lung (Liu et al., 2002b), increases phosphorylase A activity (Jost et al., 1979) and stimulates the tyrosine phosphorylation of specific proteins in fetal lung epithelial cells (Batchelor et al., 1998). Considered in the context of the pathophysiological changes induced by pituitary GH excess and deficiency, and the active GHR in fetal lung epithelium (Batchelor et al., 1998), we believe the lung to be a GH target site, particularly during development.

1.1.8 Lung Development

1.1.8.1 Lung Development in Avian Species

Early in development (ED3), the primitive gut tube (consisting of endoderm and visceral mesoderm) is subdivided along the anteroposterior (AP) axis into distinct domains that each give rise to an organ primordium (Grapin-Botton and Melton, 2000, Roberts 2000). Organ-specific morphogenesis is then controlled by epithelial-mesenchymal interactions, and by interactions between adjacent primordia (Smith et al., 2000, Yasugi 1993), the latter establish borders between organs.

The lung originates from the ventral wall of the esophagus-respiratory region of the foregut, which is positioned between the pharynx and the stomach (Sakiyama et

al., 2003). First, the primordium in the ventral wall swells, then bifurcates into a pair of primary lung buds by ED3.5 (Figure 3), and then each lung bud undergoes repeated budding and branching (Sakiyama et al., 2003), which forms the bronchial tree. The resultant lung consists of the bronchial tree, which functions in gas exchange, and the air sacs, which control air movement (Sakiyama et al., 2000).

1.1.8.2 Lung Development in Mammals

The process of lung development in mammals has been divided into five consecutive overlapping stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar (Fig. 4).

1.1.8.2.1 Embryonic Stage

The embryonic stage of development is the period during which the primitive lung forms as an outpocket on the ventral side of the foregut, which lasts from gestational days (GD) 0-13 (Burri 1997) in the rat. As this stage continues, the lung bud bifurcates into two branches that extend distally into the surrounding mesenchyme (Burri and Moschopulos, 1992). These two branches are the early bronchi, which will continue to grow and branch as they form the conducting airways of the lungs (Copland and Post, 2004).

1.1.8.2.2 Pseudoglandular Stage

The pseudoglandular stage takes place between GD 13-18 (Burri 1997), a stage characterized by rapid branching of the bronchi to form the bronchial tree, and finally,

the terminal bronchioles. The lung resembles an acinar gland throughout this period, which gives it its name (Burri 1984). Developing alongside the branching bronchial tree is a branching pulmonary vasculature tree (Stenmark and Abman, 2005). At this stage the primitive airway epithelium begins to differentiate, and it is the stage when neuroendocrine, ciliated, and goblet cells appear, and when mesenchymal cells begin to form cartilage and smooth muscle cells (Jeffery 1998). Throughout the development of the airways, there is a mass of undifferentiated cells that are found at the ends of the developing airways, and as the airways branch these cells accompany the new branches (Burri 1984). These cells are the precursor cells to the alveolar epithelium (Burri 1997). As well, fetal breathing movements can first be identified during the pseudoglandular stage (de Vries et al., 1982). This stage terminates with the formation of the acini and the onset of the canalicular stage.

1.1.8.2.3 Canalicular Stage

The canalicular stage occurs between GD 18-20, the stage in which the airways branching pattern is completed, but that is predominantly characterized by extensive vascular growth (Burri 1997) and the beginnings of the gas exchange region (Copland and Post, 2004, Burri 1997). This period is when the respiratory bronchioli appear, the interstitial tissue thins, peripheral mesenchyme vascularisation increases, and the differentiation of cuboidal epithelium into type 1 and type 2 cells leads to the beginning of surfactant production (DiFiore and Wilson, 1994). At the end of this stage (GD20), the acini have subdivided into saccules, which gives the lung a spongy appearance (Burri 1997).

1.1.8.2.4 Saccular Stage

The saccular stage takes place from GD21 to postnatal day (PD) 4. This is the stage during which extensive branching and widening of the airspaces in the lung periphery occurs (Burri 1984, 1997). This stage comprises the growth of the pulmonary parenchyma, connective tissue thinning between airspaces, and maturation of the surfactant system (Copland and Post, 2004). The lung is functional for air breathing at this stage, even though the alveoli are practically absent.

1.1.8.2.5 Alveolar Stage

The alveolar stage is primarily between PD4-14 (Burri 1997), during which time the saccules septate and the alveoli form. This stage is composed of two separate, but likely interrelated, processes. These are alveogenesis and angiogenesis. Alveogenesis is the growth of the lung and the development of alveoli, whereas angiogenesis is development of the pulmonary vasculature (Bhatt et al., 2001). The alveolar stage is characterized by the formation of buds (secondary septa) from along the walls of the terminal sac (primary septa) (Burri 1997). Following budding, the secondary septa elongate, eventually dividing the saccules (Bhatt et al., 2001). The division of the saccules occurs through coordinated actions with the surrounding capilliaries. Two capillaries (one along each side of the septal bud), which are separated by insterstitium, fuse upon elongation of the bud to form a single capilliary layer, thereby ending growth of the secondary septa (Figure 5; Burri et al., 1997). The overall result of this process is a substantial increase in the surface area of the lung, thereby increasing efficiency of gas-exchange. Upon completion of the alveolar

stage, the lung is composed of non-parenchymal (bronchi, vessels, pleura) and parenchymal (airspace and septa) tissue, with airspaces representing over 80% of the parenchymal tissue (Burri 1984).

1.1.8.3 Regulators of Mammalian Lung Development

1.1.8.3.1 Local Regulators

1.1.8.3.1.1 Fibroblast Growth Factors

The Fibroblast Growth Factors (FGF) have important roles in the development of many organs, including the development of the lungs. The FGF literature is quite extensive, so as such it will only be addressed here selectively.

FGF-10 and FGF Receptor (FGFR)-2 are involved in the regulation of airway branching (Cardoso 2001). The respiratory tract epithelium expresses FGFR-2 (Peters et al., 1992) from early lung development through branching morphogenesis. The mesenchyme expresses FGF-10 in close association with distal epithelial tubules in a dynamic fashion; FGF-10 expression precedes distal bud formation, and expression is down-regulated once the bud has formed (Bellusci et al., 1997). The importance of FGF-10 in lung development is demonstrated by the FGF-10 -/- KO mice. In FGF-10 KO mice, the lungs do not form, and the mice have a blunt-ended tracheal tube (Min et al., 1998, Sekine et al., 1999).

FGF-1 and FGF-7 also bind to FGFR-2 (Szebenyi and Fallon, 1999), so they could have similar effects to FGF-10. However, studies have shown that only FGF-1 can mimic the effect of FGF-10 (Bellusci et al., 1997a, Nogawa and Ito, 1995, Park et al., 1998), although it does not have the same chemotactic effects as FGF-10. FGF-7

appears to be more generally involved in epithelial cell proliferation (Cardoso et al., 1997). As well, FGF-7 and FGF-1 can induce type II cell differentiation, but only together (Shannon et al., 1999). Finally, FGF-2 has been shown to be an inducer of Surfactant Protein (SP)-A, -B, and -C (Matsui et al., 1999).

1.1.8.3.1.2 Sonic Hedgehog

Sonic Hedgehog (Shh) is expressed in the developing lung epithelium in a gradient fashion, with levels highest in cells at the tips (Cardoso 2001), and its receptor, Ptc1, is found in the mesenchyme (Bellusci et al., 1997a). Shh is a key protein in lung branching morphogenesis, as witnessed by the failure of the epithelial tubules of Shh -/- KO mice to branch properly, which is thought to involve increased mesenchymal cell death and decreased cell proliferation (Litingtung et al., 1998, Pepicelli et al., 1998). Of greater importance is the regulation of FGF-10 by Shh. The model proposed by Bellusci et al., (1997b) has the growing epithelial bud expressing high levels of Shh, which interact with the chemotactic source (FGF-10) in the distal mesenchyme to inhibit its local production of FGF-10 and thereby decrease its chemotactic effect.

1.1.8.3.1.3 Bone Morphogenetic Proteins (BMPs)

At least three members of the BMP family have been found in the developing lung (Bellusci et al., 1996), BMP-4, -5, and -7. BMP-4 is a key regulator of epithelial proliferation and proximal-distal cell fate during lung development (Cardoso 2001). BMP-4 is expressed dynamically in the distal epithelium of the branching airways,

with expression beginning after bud initiation, and levels increasing at the tips during bud extension (Weaver et al., 2000). This implies that BMP-4 does not have roles in bud induction. In mice expressing a dominant negative BMP receptor or the BMP-4 inhibitor Xnoggin, proximal cell types (such as ciliated cells) are seen in the distal lung (Weaver et al., 1999), highlighting the role of BMP-4 in regulating proximal-distal cell differentiation. FGF-10 in the mesenchyme up-regulates expression of BMP-4 in the distal epithelium (Lebeche et al., 1999), the BMP-4 then inhibits epithelial cell proliferation and prevents budding, and as such acts as an antagonist to FGF-10 (Weaver et al., 2000).

1.1.8.3.1.4 Transforming Growth Factor β-1

Transforming Growth Factor (TGF) β-1 is primarily involved in inhibition of epithelial branching in lung development (Zhao J et al., 1996, Serra and Moses, 1995). TGFβ-1 controls lung branching by three major mechanisms: limiting the proliferation of epithelial buds (Serra and Moses, 1995), inhibiting FGF-10 expression and thus inhibiting chemoattraction (Lebeche et al., 1999), and by synthesizing extracellular matrix components that promote cleft stability (Heine et al., 1990).

1.1.8.3.1.5 Retinoic Acid

Retinoic Acid (RA) is thought to be involved in lung development, although its role is not clearly understood. Vitamin A (from which RA is synthesized in the body) deprivation in pregnant dams results in blunt-ended tracheae and lung agenesis

(Dickman et al., 1997). Retinaldehyde dehydrogenase-2 (RALDH-2) is the main enzyme in RA generation (Niederreither et al., 1997, Niederreither et al., 1999, Ulven et al., 2000, Zhao D et al., 1996), and acts to synthesize RA in the mesenchyme when the primary lung buds are forming (Malpel et al., 2000), implicating RA in lung bud initiation. After lung bud initiation, RA does not appear to be important in branching morphogenesis, as it is down-regulated during this period of lung development (Cardoso 2001).

RA is also thought to be involved in alveolarization. Low plasma Vitamin A levels have been found in neonates who develop bronchopulmonary dysplasia (Shenai et al., 1985), which is a condition that involves impaired alveolarization. Furthermore, treatment of neonatal rats during alveolarization with RA increases the number of alveoli (Massaro and Massaro, 1996). Finally, RA can reverse the anatomical features of emphysema induced by elastase (Massaro and Massaro, 1997), a condition in which alveolar walls have been destroyed.

1.1.8.3.1.6 Insulin-like Growth Factor 1

IGF-1 mRNA is abundantly localized within fetal mesenchymal lung cells, especially those surrounding airway epithelium (Retsch-Bogart et al., 1996), although it is also present in airway epithelial cells (Wallen and Han, 1994). As the expression of IGF-1 in the developing lung was correlated with cellular proliferation and the maturation of connective tissue (Lallemand et al., 1995), it is possible that these actions reflect the upstream expression of GH in the same tissues and cells. The increased production of IGF-1 in the lungs of GH-treated rats (D'Ercole et al., 1984) supports this possibility.

As support for the role of IGF-1 in lung development, IGF-1 Receptor (IGF-1R) KO mice die at birth of respiratory failure (Liu et al., 1993).

Recently, a new role for IGF-1 in lung development has been proposed, that being as a mediator of vasculogenesis/angiogenesis. Upon inactivation of IGF-1R with a neutralizing antibody in human fetal lung explants, the developing lung displayed a loss of endothelial cells, changes in lung explant morphology, and apoptosis of numerous mesenchymal cells (Han et al., 2003).

1.1.8.3.1.7 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) has been generally found to be involved in the development of lung vasculature. The epithelial expression of VEGF-A during the pseudoglandular stage of development has been proposed to establish a morphological gradient that mediates interactions between the epithelial tubules and the vascular net (Ng et al., 2001; Park et al., 1993).

1.1.8.3.1.8 Calcyclin

Calcyclin, along with its binding protein Calcyclin Binding Protein (CacyBP) are expressed within the lung (Breen et al., 1999; Jastrzebska et al., 2000). The interaction between Calcyclin and CacyBP, however, is not yet understood. Calcyclin has been implicated in growth of the lungs due to mechanical stretch (Breen et al., 1999). CacyBP has been proposed in RA induced differentiation, but only in other tissues (Wu et al., 2003).

1.1.8.3.1.9 Epidermal Growth Factor

Epidermal Growth Factor (EGF) has been localized to both the mesenchyme and epithelium of the embryonic lung (Snead et al., 1989), although its expression seems to be localized to the mesenchyme (Ruocco et al., 1996). EGF is thought to have roles in lung maturation (Sundell et al., 1980), type II cell differentiation (Plopper et al., 1992), and in surfactant synthesis (Higuchi et al., 1989).

1.1.8.3.2 Endocrine Factors

1.1.8.3.2.1 Glucocorticoids

Glucocorticoids have been found to be important in lung development. They have roles in regulation of pulmonary surfactant (Ballard 1989), and in regulation of surfactant proteins (Weaver and Whitsett, 1991).

1.1.8.3.3 Mechanical Factors

During lung development, Fetal Breathing Movements (FBM) alternate with "apnoeic" periods in which FBM are absent. FBM oppose lung recoil, and thereby provide the amount of stretch necessary to promote normal lung growth and maturation (Harding 1997). During apnoeic periods, active laryngeal constriction prevents lung liquid from escaping, thereby preserving the stretch of the lungs (Harding 1997).

1.1.9 Relevant Distal Lung Cell Types

1.1.9.1 Type I Epithelial Cells

Alveolar type I epithelial cells (AE1 Cells) cover most of the alveolar surface area, and constitute the alveolar part of the extremely thin gaseous diffusion barrier (Young and Heath, 2000). These are the cell type in the alveoli that the respiratory gases pass through going into and out of the surrounding capillaries.

1.1.9.2 Type II Epithelial Cells

Alveolar type II epithelial cells (AE2 Cells) have two major functions: as a source of alveolar surfactant, and as the stem cell of the alveolar epithelium (Fehrenbach 2001). The surfactant produced by these cells functions to regulate surface tension and alveolar fluid balance, as well as to function in host defense. As a stem cell, AE2 cells function to produce new cells to replace damaged/aged AE2 and AE1 cells.

1.1.9.3. Mesenchymal Cells

Mesenchymal Cells surround the epithelial tubules early in development, and will eventually form the vasculature of the lung (Cardoso 2001). As well, the mesenchymal cells will also form cartilage and smooth muscle cells (Jeffery 1998).

1.1.9.4 Alveolar Macrophages

Alveolar macrophages (AM) are part of the lung's defense against inspired particles and pathogens. AM are large phagocytic cells found both in the alveolar wall and free in the alveolar space that engulf small particles and microorganisms (Young and

Heath, 2000). After the AM has engulfed foreign material, it migrates into the airways, is carried up the mucociliary escalator, and is then disposed of by coughing and swallowing.

1.1.9.5 Endothelial Cells

The endothelial cells of the lung form the capillaries and are the part of the pulmonary vasculature which gases diffuse through going into and out of the alveoli (Burri 1984).

1.2 Animal Models Used in these Studies

1.2.1 Chick

The chick is physically very large during the embryonic period (relative to rodents), and as such permits analysis of tissue at much earlier stages of development. Relevant to the aim of these studies, the lung bud can be visualized at ED4, which is just twelve hours after first appearance of the lung bud in the chick embryo at ED3.5 (Sakiyama et al., 2000). As such, the ED4 lung bud could be examined by both *in situ* hybridization (ISH) and immunohistochemistry (IHC). The avian lung does not use a diaphragm (Scheid and Piiper, 1989), has air sacs, has parabronchi instead of alveoli (Lopez et al., 1992), and it begins air breathing one day before hatch (Menna and Mortola, 2002). Furthermore, the chick is a well established model of embryogenesis, one that permits manipulations that are not possible in early mammalian development (reviewed in Mozdziak and Petitte, 2004). This model

therefore provides a unique opportunity to compare and contrast similarities and differences to mammalian lungs.

1.2.2 Rat

The studies in rats were, in contrast to those on the chick, focused on understanding late fetal and early postnatal development of the lung, especially the period of alveolarization from PD4-14 (Gomi et al., 1994). Since, unlike most other mammalian species, rats go through the stage of alveolarization postnatally and not *in utero*, this is a useful model to study alveolarization, as *in utero* manipulations are much more difficult and problematic.

1.3 Rational for these Studies

As noted, GH has been found to be expressed locally in many tissues. Studies on GH in the lung (Kyle et al., 1981, Costa et al., 1993, Allen et al., 2000) support the possibility that GH may be expressed locally in the lungs, particularly during development.

Since GH and GH mRNA are widespread in peripheral tissues of chick embryos (Harvey and Hull, 1997), and as GH is ubiquitous in preimplantation rodents (Pantaleon et al., 1997), we **hypothesized** that GH mRNA and protein are found within the chick throughout embryonic development and within the rat in fetal and early postnatal development. The **first specific aim** was to analyze embryonic chicks by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and ISH for presence and cellular localization of GH mRNA, and by Western blotting and IHC for

presence and cellular localization of GH protein. The **second specific aim** was to analyze rats from the pseudoglandular through to the end of the alveolar stage by RT-PCR and ISH for presence and cellular localization of GH mRNA, and by Western blotting and IHC for presence and cellular localization of GH protein.

The results of these two specific aims are documented in Chapters 2 and 3, respectively. Upon successful completion of these descriptive studies, the next step was to examine if GH had a role in lung development. We **hypothesized** that GH acts as a local autocrine/paracrine in the lung during the period of alveolarization. The **third specific aim** was to down-regulate expression of GH mRNA in the lung during the period of alveolarization using antisense oligodeoxynucleotides to rat GH. Following this treatment, lungs were to be examined by molecular biological techniques to determine changes in signaling pathways and potential roles for local GH. The results of this specific aim are found in Chapter 4. The **fourth specific aim** was to up-regulate expression of GH in the lung using a mouse GH adenovirus expressed during the period of alveolarization. Following this treatment, lungs were to be examined by molecular biological techniques to determine changes in signaling pathways. These results are in Chapter 5. The **fifth specific aim** was to compare GHR knockout (-/-) mice to normal control GHR (+/+) mice using 2-dimensional gel electrophoresis to examine changes in protein regulation in the lung in the absence of normal GH-signaling. These results are presented in Chapter 6.

The **significance** of these studies will be a documentation of the expression of GH in the lung during development and an understanding of some of the important

mediators of GH action in lung development, specifically in the process of alveolarization.

1.4 Table

Table 1.1 – Tissue Distribution of GH-Like Proteins and mRNA in Extrapituitary Sites in Human, Rat and Chicken. (modified from Harvey and Hull 1997).

Tissue	Species	GH	GH mRNA
Posterior pituitary	Rat	(Lechan *et al.*1983)	
Brain	Rat	(Hojvat *et al.*1982)	(Martinoli *et al.*1991)
	Chicken	(Render *et al.*1995)	(Render *et al.*1995)
Spinal Cord	Rat	(Lechan *et al.*1981)	
Neural Retina	Chicken	(Harvey *et al.*2004)	(Harvey *et al.*2004)
Placenta	Human	(Cooke and Liebhaber 1997)	(Cooke and Liebhaber 1997)
	Rat	(Ogilvie *et al.*1990)	
Amnion	Chicken	(Harvey *et al.*2000)	
Mammary Gland	Human	(Mol *et al.*1995)	
Wolffian Duct	Rat	(Nguyen *et al.*1996)	
	Chicken	(Harvey *et al.*2000)	
Mullerian Duct	Chicken	(Harvey *et al.*2000), (Wang 1989)	
Testis	Human		(Untergasser *et al.*1996)
Kidney	Rat	(Kyle *et al.*1981)	
Heart	Chicken	(Harvey *et al.*2000)	
Lung	Rat	(Kyle *et al.*1981)	(Allen *et al.*2000)
Liver	Chicken	(Harvey *et al.*2000)	
Gastrointestinal Tract	Rat	(Kyle *et al.*1981)	
Stomach	Chicken	(Harvey *et al.*2000)	
Spleen	Human		(Wu *et al.*1996)
	Chicken	(Render *et al.*1995)	(Render *et al.*1995)
Thymus	Human	(Sabarwal and Varma 1996)	(Wu *et al.*1996)
Lymphocytes	Human	(Varma *et al.*1993)	(Wu *et al.*1996)
	Rat	(Hattori *et al.*1994)	
Tonsils	Human		(Wu *et al.*1996)
Lymph Node	Human		(Wu *et al.*1996)
Limb bud	Chicken	(Harvey *et al.*2000)	
Skin	Human		(Slominski *et al.*2000)
Muscle	Human	(Kyle *et al.*1981)	(Wu *et al.*1996)

1.5 Figures

Figure 1.1 – Chicken GH mRNA and Protein Sequence. From Lamb *et al.*(1988).

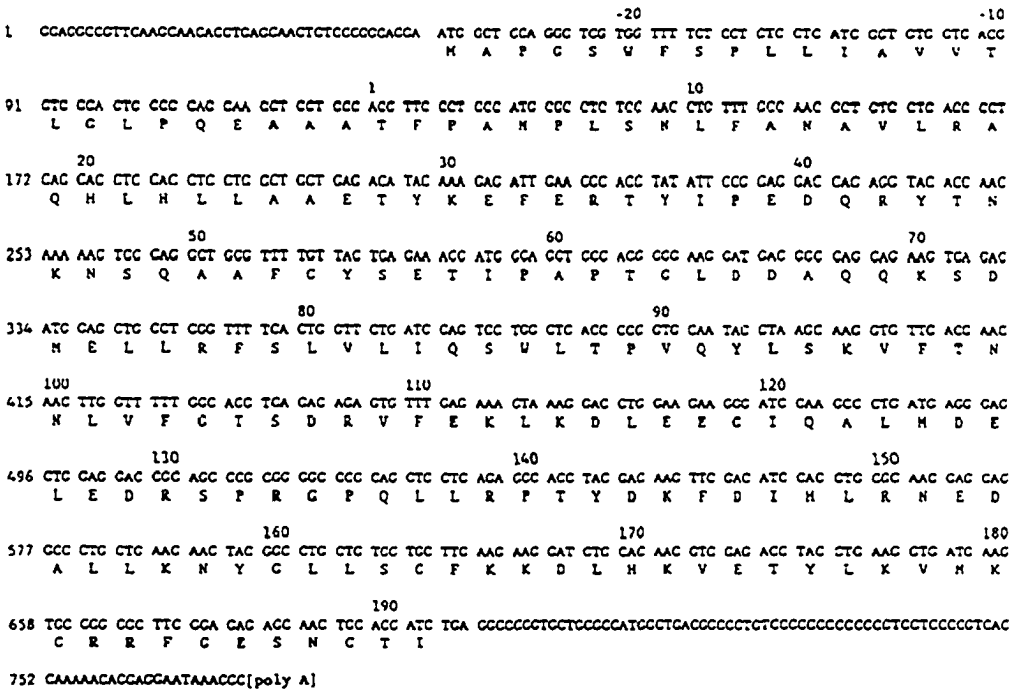
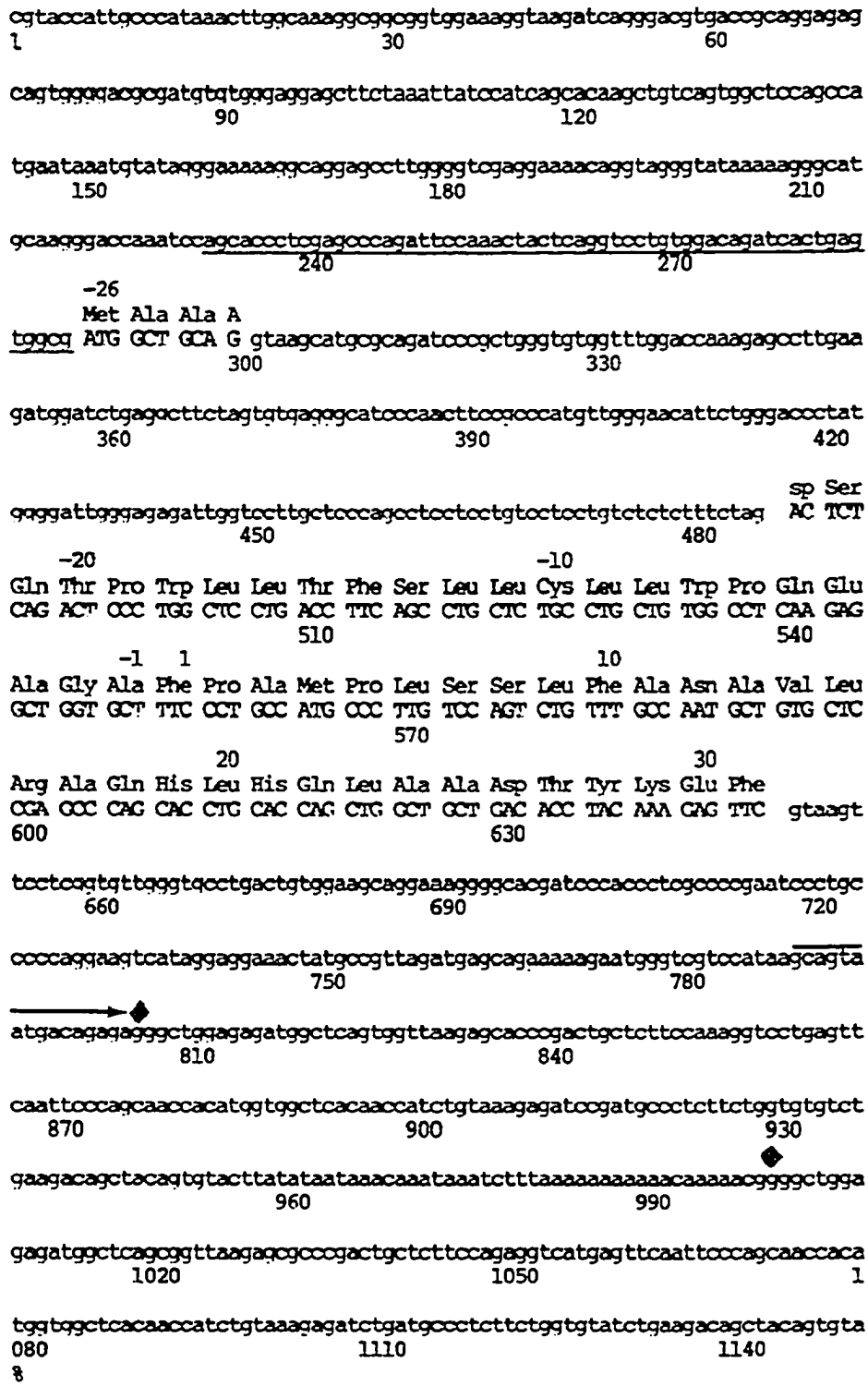


Figure 1.2 – Rat GH Gene, mRNA and Protein Sequence. From Page *et al.* (1981).



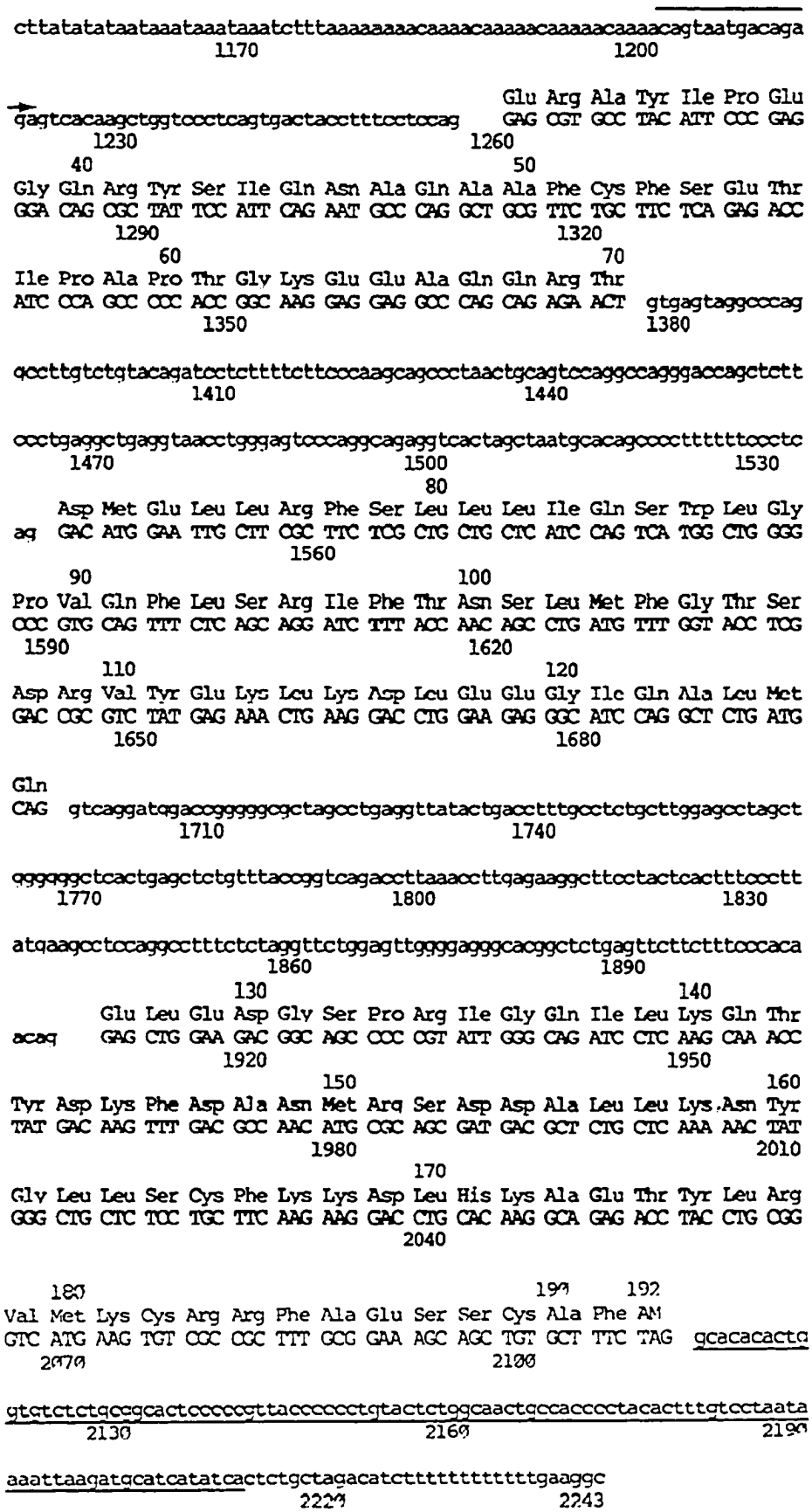


Figure 1.3 – Chick Lung Development. From Sakiyama *et al.*(2000).

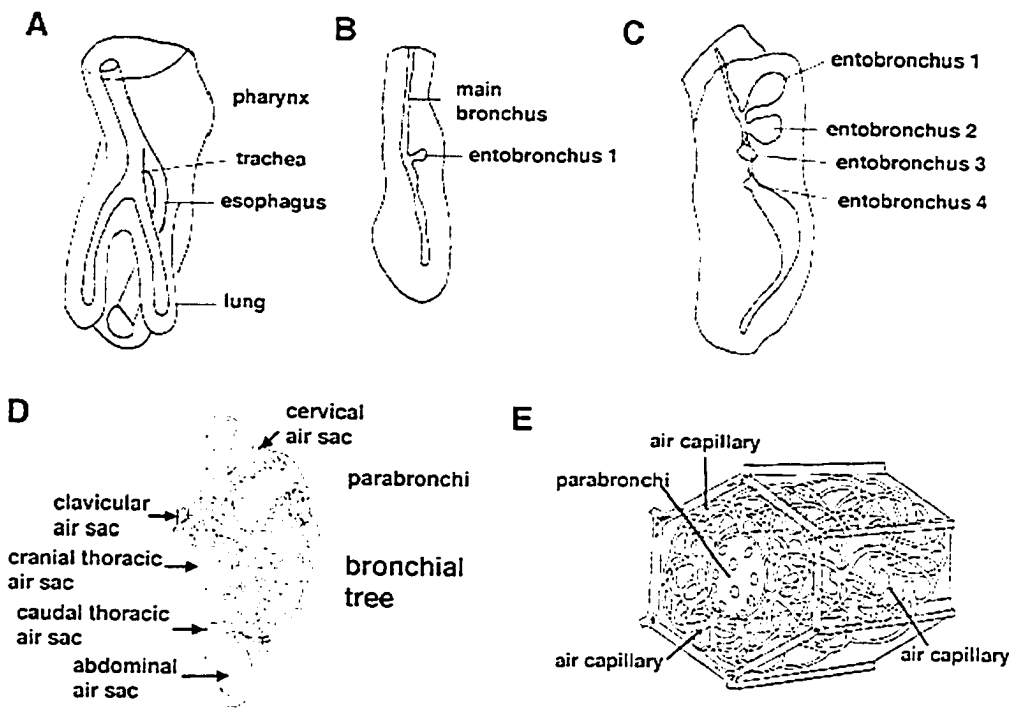
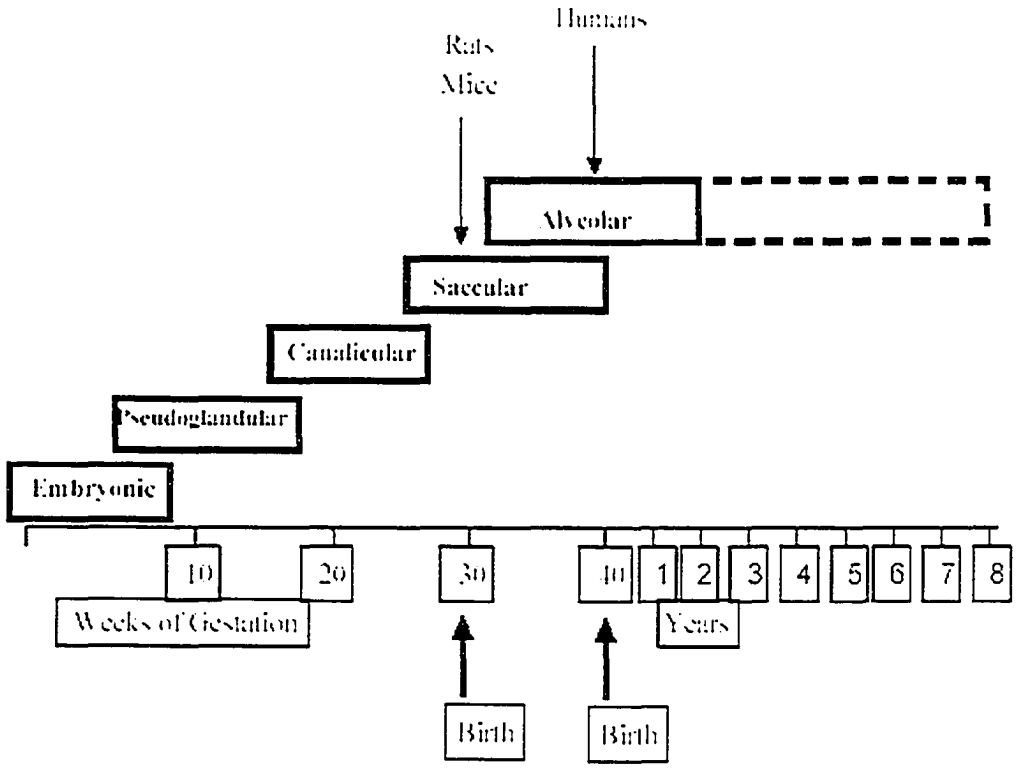


Figure 1.4 – Lung development in mammalian species based on timeframe in weeks for human development (Modified from Zeltner and Burri 1987).



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PART I: DESCRIPTIVE STUDIES

CHAPTER 2

GROWTH HORMONE (GH) AND GH RECEPTOR EXPRESSION IN THE LUNGS OF EMBRYONIC CHICKS

CHAPTER 2: GROWTH HORMONE (GH) AND GH RECEPTOR EXPRESSION IN THE LUNGS OF EMBRYONIC CHICKS[1]

2.1 INTRODUCTION

The lung is a postnatal site of growth hormone (GH) action, as reflected by its activation of lung NF_{kB} (Liu et al., 2002a), induction of phosphorylase A activity (Jost et al., 1979), phosphorylation of lung epithelial proteins (Batchelor et al., 1998), activation of alveolar macrophages (Edwards et al., 1992), and accumulation of lung neutrophils (Liu et al., 2000b). A pathological excess of GH secretion is, moreover, associated with large lungs (Barlett et al., 1971), upper airflow obstruction (Trotman-Dickenson et al., 1991) and a narrowing of the small airways (Harrison et al., 1978). A deficiency of GH is, conversely, associated with a decrease in respiratory muscle strength and a decrease in the maximum inspiratory and expiratory pressure (Merola et al., 1995; Merola et al., 1996). These actions likely reflect the endocrine actions of pituitary GH on pulmonary GH receptors (GHRs) (Tiong et al., 1989; Garcia-Aragon et al., 1992; Batchelor et al., 1998). These receptors are, however, present in fetal lungs before the ontogenic differentiation of pituitary somatotrophs. Early lung development therefore occurs in the absence of pituitary GH, although it may not be GH-independent.

Although somatotrophs are not present in embryonic chicks until embryonic day (ED) 16 of the 21-day incubation period (Porter 1997), and GH is not present in peripheral plasma until ED17 (Harvey et al., 1979), a specific GH-response gene

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(GHRG) is widely expressed in the lung buds of ED7 embryos (Harvey et al., 2002). As GHRG-1 is a marker of GH action in chickens, its expression in the embryonic lung supports the possibility that extrapituitary GH participates in early pulmonary function. Indeed, GH gene expression is now known to be widespread in many extrapituitary tissues of the chick embryo (Harvey and Hull, 1997; Harvey et al., 2000), although its occurrence in the lung has yet to be determined. GH mRNA has, however, previously been found in alveolar macrophages in adult rats (Allen et al., 2000) and trace amounts of GH-immunoreactivity are present in whole lung extracts of fetal and adult mammals (Kyle et al., 1981; Costa et al., 1993). The possible presence of GH and GH mRNA in the lungs of embryonic chicks has therefore been investigated in the present study.

2.2 MATERIALS AND METHODS

Animals/Tissues

Fertile White Leghorn eggs from the University of Alberta Poultry Unit were incubated at 37.5°C in a humid Hova-Bator™ Incubator (Miller Hatcheries, Edmonton, Alberta). Eggs were turned one-quarter of a revolution each day of incubation. The parabronchi of the chick lungs differentiate at ED8 (Sakiyama et al., 2000) and lung breathing commences at ED20 (Chiba et al., 2002). Chick lungs were therefore collected before (at ED7) and after (at ED11, ED13, ED15 and ED18) parabronchial development, and at the onset of lung breathing (at ED20). For comparison, the heart was collected from ED7 embryos, since it is known to possess GH immunoreactivity (Harvey et al., 2000; Murphy et al., 2002) and pituitary glands from the heads of adult slaughter-house fowl provided a

positive control. Pituitary glands were not used from embryonic chicks, as they do not express GH until late in fetal development.

For RT-PCR and Western Blotting, lungs were excised in PBS and immediately frozen in liquid nitrogen and RNA and protein extraction, respectively. For in situ hybridization (ISH) and immunohistochemistry (IHC), lungs were excised in PBS, then immediately transferred to freshly-prepared 4% paraformaldehyde in PBS. Lungs were fixed overnight in 4% paraformaldehyde at 4°C. The following day, lungs were dehydrated and cleared [PBS for 15 min x 2, 50% EtOH for 15 min, 70% EtOH for 15 min, 95% EtOH for 30 min, 100% EtOH for 30 min x 2, Citri-Solve (Fisher Scientific, Edmonton, Alberta, Canada) for 30 min x 2]. Lungs were then placed in paraffin wax at 60°C overnight, and embedded in paraffin wax the next morning. 8μm sections were made with a microtome, flattened on 42°C 0.1% DEPC (Sigma, Mississauga, Ontario, Canada) treated water, and mounted on Fisher Superfrost®/Plus slides (Fisher).

RT-PCR

Total RNA was extracted from frozen tissues using TRIzol® Reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada; 100mg/ml). Reverse transcription (RT) was performed with Thermoscript® RNase H^- reverse transcriptase (Invitrogen). The reaction was carried out in a 20μl volume containing 3μg total RNA, 2.5μM Oligo $(dT)_{20}$, 1mM each dNTP, 12 x Invitrogen cDNA Synthesis Buffer, 2U/:l RNase OUT® (Invitrogen),

5mM DTT, and 0.75U/μl Thermoscript® RNase H$^-$ reverse transcriptase. Oligo $(dT)_{20}$, RNA, and dNTPs were mixed and made up to 12μl volume with 0.1% (DEPC) (Sigma) treated water. To denature the RNA and olgio $(dT)_{20}$, the 12μl mix was incubated at 65°C for 5 min, then placed on ice. The remaining components of the reaction were then added to make a final volume of 20μl. Samples were transferred to a Techgene thermal cycler (Techne Ltd., Duxford, Cambridge, UK) preheated to 59°C. The reaction was incubated at 59°C for 60 min, then terminated by incubation at 85°C for 5 min, then transferred to ice.

2μl of the reaction product, 2mM MgSO4, 1 x High Fidelity (Invitrogen) PCR Buffer, 0.2mM of each dNTP, IU of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen), and 0.2μM of sense and antisense oligonucleotide primers designed to amplify a 690 bp cDNA of the full-length chicken pituitary GH cDNA (Render et al. 1995). The forward primer (cCLR1;5'-CGTTCAAGCAACACCTGAGCAACTCTCCCG-3') and the reverse primer (cCLR2; 5'-GCCTCAGATGGTGCAGTTGCTCTC TCCGAA-3') were combined and the final reaction volume of 50μl was achieved using DEPC treated water. Touchdown PCR was performed as follows: 95°C for 1 min; then 5 cycles of 95°C for 30 min and 72°C for 2 min; then 35 cycles of 95°C for 30 sec and 65.6°C for 2 min; then 68°C for 10 min. Ten microlitres of each PCR product was visualized by ethidium bromide staining on a 1.5% agarose gel.

Sequencing

PCR products were purified by the High Pure® PCR Product Purification Kit (Roche Diagnostics Canada, Laval, Québec, Canada), then sequenced by the DNA Core Lab, Department of Biochemistry, University of Alberta. Sequences were analyzed by BLAST and compared with sequences in the NCBI database.

In Situ Hybridization

ISH was performed using full-length (690 bp) cGH antisense probes, with full-length (690 bp) cGH sense probes as a negative control. cCLR1 and cCLR2 primers were used to generate a 690bp cDNA, which was inserted into a pCR®II-TOPO Vector (Invitrogen) plasmid. The plasmid was transfected into One Shot TOP 10 chemically competent cells (Invitrogen) and cultured. Plasmids were restriction digested with either *SacI* or *NotI* (to make antisense or sense probes, respectively), then antisense or sense probes were transcribed with T7 or SP6 enzymes (Invitrogen), respectively in the presence of DIG RNA Labeling Mix (Roche). cGHR antisense and sense probes were made in an analogous fashion, using a 500bp cDNA generated from the extracellular portion of cGHR with khu9 (5' – CCTCGATTTGGATACCATATTGTGTTAAGC - 3') and khu10 (5' – CTG TTA CGG CCA GCC CAC ACA CTC CGA AG - 3') primers (Hull et al., 1995).

8μm 4% paraformaldehyde-fixed sections were deparaffinized and rehydrated (Citri-Solve [Fisher] for 5 min x 2, 100% EtOH for 2 min, 95% EtOH for 2 min, 70%

EtOH for 2 min, 0.1% DEPC water for 2 min, 0.1% DEPC-Phosphate Buffered Saline (PBS) for 5 min x 2). Slides were post-fixed in 4% formaldehyde in 0.1% DEPC-PBS for 10 min. RNase treatment was performed in 0.1% DEPC-PBS (DEPC added just before use) for 15 min x 2. Sections were treated in Proteinase K (Invitrogen) solution (1mg Proteinase K, 0.02M Tris, 0.01M Na_2EDTA, in 0.1% DEPC water) at 37°C for 1 min. Slides were equilibrated in 5 x SSC for 10 min x 2. Slides were prehybridized for 2 hours at 65°C with 120µl prehybridization solution (50% formamide, 5 x SSC, 0.5mg/ml salmon testes DNA (Sigma), in 0.1% DEPC water) under a cover slip, on an elevated platform in a sealed container with 75% formamide at the bottom of the container. Slides were hybridized for 16 hr at 65°C with 120µl of hybridization solution [prehybridization solution diluted 1:8 with either the antisense or sense digoxigenin (DIG)-labeled cGH riboprobes or with the antisense or sense DIG-labeled cGHR probes (for the mRNA sequences coding for the intracellular domain of the GHR). After hybridization, the slides were washed in 2 x SSC for 30 min at room temperature, in 2 x SSC for 60 min at 65°C, then finally in 0.1 x SSC for 60 min at 65°C. The slides were then washed for 5 min x 2 in Buffer One (0.01M Tris, 0.15M NaCl, pH 7.5), then incubated with anti-digoxigenin (Roche, at a final concentration of 1:2000) in 1% (w/v) blocking reagent (Roche) (1% w/v blocking reagent in 0.1M maleic acid, 0.15M NaCl, pH 7.5) for 2 hours. The slides were then washed for 15 min x 2 in Buffer One, then for 5 min in Buffer Two (0.1M Tris, 0.1M NaCl, 0.05M $MgCl_2$, pH 9.5) and developed in a commercial NBT/BCIP substrate [1:50 dilution of NBT/BCIP Stock Solution (Roche) in Buffer Two] for 30 min at 37°C. Color development was stopped by immersion in TE buffer for 5 min x 2. Sections were counterstained for 2 min with 0.5% (w/v) methyl

green in 3M NaOAc (pH 4.0), rinsed thrice in 0.1% DEPC water, rehydrated (35% EtOH for 2 min, 50% EtOH for 2 min, 75% EtOH for 2 min, 95% EtOH for 2 min, 100% EtOH for 2 min, Citri-Solve for 2 min x 2), then mounted with DPX (Fluka-Sigma). Digital images were collected using a SPOT Digital Microscope camera (Carsen Group, Markam, Ontario, Canada) mounted on an Olympus B x 40 microscope.

Western Blotting

Tissue samples were homogenized, protein content was estimated using a commercial assay (BioRad) and Western Immunoblotting was performed as described in Hosford et al., (2003), except that a 15% polyacrylamide gel was used. Immunoreactivity was detected using a specific rabbit antibody raised against chicken GH (αGH1, as described in Harvey et al., 2000).

Immunohistochemistry

Immunocytochemical staining was performed using the avidin-biotin-peroxidase (ABC) method, as described by Harvey et al., (2001), using αGH1 antibody, or with an antisera raised in rabbits against a synthetic fragment (CH17) of the extracellular domain of the chicken GHR (Hull et al., 1999). The staining of somatotrophs in the caudal lobe of adult chicken pituitary glands provided a positive control for the GH antisera, and the specificity of GH staining was demonstrated by its blockade following its preabsorption with recombinant cGH (Amgen, Thousand Oaks, California, USA) (1mg/ml for 2 hours)

and by the absence of staining in the cephalic lobe. The specificity of GHR staining was demonstrated using normal rabbit serum (NRS) as a negative control. Digital images were collected using a SPOT Digital Microscope camera (Carsen Group, Markam, Ontario, Canada) mounted on an Olympus B x 40 microscope.

2.3 RESULTS

RT-PCR

As expected (Render et al., 1995), a 690 bp cDNA was amplified from reverse-transcribed pituitary mRNA in the presence of oligonucleotide primers CLR1 and CLR2 (Fig. 2.1). With the same primers, cDNA fragments of identical size were also generated by the RT-PCR of mRNA from the lungs of ED11 and ED18 chick embryos (Fig. 2.1). In contrast, no cDNA fragments were generated in the negative controls in the absence of the reverse transcriptase (Fig. 2.1).

cDNA Sequencing

Nucleotide sequencing of the 690 bp cDNA amplified from ED11 lung mRNA demonstrated complete (100%) homology with the 690 bp cDNA amplified from adult chicken pituitary (data not shown). Both cDNA's were also >99.5% homologous to the sequence published by Tanaka et al., (1996) for chicken GH cDNA, differing only in a

base-pair substitution at position 588. This substitution would not, however, change the amino acid of the coded apoprotein.

Western Blotting

As expected (Aramburo et al., 2000) GH-immunoreactivity in the pituitary glands of adult chickens was primarily associated with a protein of 26 kDa (Fig. 2.2). GH-immunoreactive proteins of similar size were also present in the lungs of ED11-ED18 chickens (Fig. 2.2). A smaller protein of 15 kDa was additionally present in the lungs of ED13, ED15 and ED18 chicks, in which it was more abundant that the 26 kDa monomer. An immunoreactive protein of approximately 29 kDa was also present in the lungs of ED15 embryos, but only in trace amounts (Fig. 2.2).

GH In Situ Hybridization

Intense hybridization of the antisense probe to somatotrophs in the caudal lobe of the adult chicken pituitary gland was shown as a positive control (Fig. 2.3a,b). No hybridization was seen in the cephalic lobe and no hybridization to caudal lobe cells occurred in the negative controls in the presence of the sense probe (Fig. 2.3c).

Intense hybridization of the antisense probe was also seen in sections of the ED7 lung (Fig. 2.4a). Staining for GH mRNA was widespread and in most cells in the mesenchyme (Fig. 2.4b; Fig. 2.4d) and in the epithelia surrounding the developing

bronchi (Fig. 2.4c). In marked contrast, no specific staining was seen in ED7 controls in the presence of the sense probe (Fig. 2.4e-g). GH mRNA was similarly ubiquitous in the lungs of ED11 (Fig. 2.5a-c) and ED15 (Fig. 2.6a-c) embryos, but most intense in the epithelia lining the parabronchi (Fig. 2.5c; Fig. 2.6b) and in the endothelial cells of blood vessels surrounding the parabronchi (Fig. 2.5b; Fig. 2.6a). GH mRNA was, however, only barely detectable in the lungs of ED20 embryos, in which labeling with the sense (Fig. 2.7c) and antisense (Fig. 2.7a,b) probes was comparable. The specificity of the hybridization of the antisense probe to lung cells in the ED7, ED11 and ED15 embryos is also indicated by the lack of staining in blood cells that were present in the heart of ED7 embryos, within which strong hybridization was seen in cardiac muscle (Fig. 2.8a).

GHR In Situ Hybridization

Like GH mRNA, GHR mRNA was widespread in the ED7, 11, and 15 chick lung (Fig. 2.9, 2.10, 2.11). GHR mRNA staining was detected in the ED7 mesenchyme (Fig. 2.9b; Fig. 2.9d), and in the epithelia of the bronchi (Fig. 2.9c). GHR mRNA was also ubiquitous in the ED11 (Fig. 2.10a-c) and ED15 (Fig. 2.11a-c) lungs, particularly in the undifferentiated mesenchyme (Fig. 2.10c, 2.11b) and in the epithelium of the parabronchi (Fig. 2.10b, 2.11c). In marked contrast, no staining was observed using the GHR sense probe (Fig. 2.9e-g; Fig. 2.10d-e; Fig. 2.11d-e).

GH Immunocytochemistry

As expected, the cytoplasm of large cells clumped in the caudal lobe of the adult chicken pituitary gland were intensely labeled by the primary GH antiserum (Fig. 2.3d,e), and the specificity of this staining to somatotrophs was shown by its absence following the preabsorption of the antibody with excess recombinant GH (Fig. 2.3f) and by the absence of staining in the cephalic lobe (Fig. 2.3d).

GH-immunoreactivity was present in most cells in the ED7 lung (Fig. 2.4h), although GH staining was greatest in the mesenchymal cells (Fig. 2.4i, k) and epithelial cells (Fig. 2.4j) surrounding the bronchi and in the single layer of cells bordering the pleural cavity (Fig. 2.4h). GH staining was similarly widespread and abundant in the lungs of ED11 (Fig. 2.5f) and ED15 (Fig. 2.6f) embryos. In particular, staining was intense in the mesenchymal cells (Fig. 2.5h, Fig. 2.6g), the epithelial cells of the parabronchi (Fig. 2.5g, Fig. 2.6h), the endothelia of blood vessels (Fig. 2.5f), and in the smooth muscle cells surrounding the parabronchi (Fig. 2.6h). GH immunoreactivity was also present in some epithelial and mesenchymal lung cells of ED20 embryos (Fig. 2.7b), although the number of immunoreactive cells was less than that in earlier embryos. The specificity of the GH staining was shown by its blockade following the preabsorption of the GH antibody with recombinant GH (Fig. 2.4l-n; Fig. 2.5i-j; Fig. 2.6i-j; Fig. 2.7i-j) and by the absence of GH staining in the blood cells of ED7 embryos (Fig. 2.8b).

GHR Immunocytochemistry

Like GH, GHR immunoreactivity was widespread in the ED7, 11, and 15 chick lung (Fig. 2.9, 2.10, 2.11). GHR staining was detected in the ED7 mesenchyme (Fig. 2.9i; Fig. 2.9k), and in the epithelia of the bronchi (Fig. 2.9j). GHR was also ubiquitous in the ED11 (Fig. 2.10f-h) and ED15 (Fig. 2.11f-h) lungs, particularly in the mesenchyme (Fig. 2.10h, 2.10g) and in the epithelium of the parabronchi (Fig. 2.10g, 2.11h). Staining was absent upon replacement of the GHR antibody with normal rabbit serum (Fig. 2.9l-n; Fig. 2.10i-j; Fig. 2.11i-j).

2.4 DISCUSSION

These results clearly show, for the first time, the presence of GH and GH mRNA in the lungs of embryonic chicks, in which GHR and GHR mRNA are also present. These results therefore suggest the embryonic lung is a site of GH production and action.

The distribution of GH immunoreactivity and the DIG-labeled staining of GH mRNA in the embryonic lung were widespread and almost ubiquitous. The detection of GH and GH mRNA in the lung was not, however, due to methodological artifacts, since both cytochemical techniques similarly labeled somatotrophs in the caudal (but not cephalic) lobes of adult pituitary glands and because lung GH was not detected using preabsorbed GH-antisera and lung GH mRNA was not detected using a sense riboprobe. Moreover, GH and GH mRNA were not detected in all lung cells, nor in ED7 blood cells, further

indicating the specificity of the widespread GH and GH mRNA staining in chick embryos.

Within the embryonic chick lung, GH immunoreactivity was intense in the undifferentiated mesenchymal cells and in the bronchial and parabronchial epithelial cells, as well as in the smooth muscle cells surrounding the bronchi and the parabronchi, and in the endothelial cells lining the blood vessels that provide the vasculature of the lung. GH is similarly present and abundant in these cell-types in other embryonic tissues of early chicken embryos (Harvey et al., 2000; Murphy et al., 2003). The presence of GH immunoreactivity in the smooth muscle of rat blood vessels was similarly reported by Recher et al., (2001) and GH-immunoreactivity has also been found in the myocytes surrounding the seminiferous tubules of adult chickens (Luna et al., 2004), and is present in homogenates of the human colon (Kyle et al., 1981).

The GH immunoreactivity in the lung is likely to be derived from the local expression of GH mRNA, which was similarly widely distributed throughout the embryonic lung. Indeed, GH immunoreactivity in early embryos cannot be derived from the pituitary, since somatotroph differentiation does not occur until approximately ED12-ED14 (Porter 1997). Furthermore, as GH secretion does not occur until approximately ED16 (Porter 1997) and as GH is not present in peripheral plasma until ED17 (Harvey et al., 1979), its presence in the lung cannot reflect the sequestration of pituitary or plasma GH. The GH immunoreactivity in the embryonic lung is, moreover, mostly associated with a 15 kDa protein rather than the 26 kDa monomer that is most abundant in the pituitary gland. The 15 kDa GH variant is produced from monomer GH by proteolytic degradation (Aramburo et al., 2000). As the transcript for the full-length (26kDa) GH monomer is present in the

lungs of embryonic chicks, the abundance of the 15kDa GH variant in the lungs indicates tissue-specific differences exist in the processing or degradation of the full-length protein. The 15kDa GH variant is also present in the pituitary glands of perinatal embryos, in which its abundance (although far less than the monomer) is much greater than that in the pituitary glands of adult chickens (Aramburo et al., 2000). Its production may therefore be developmentally regulated. This, however, remains to be determined.

Whilst the factors regulating GH gene expression in extrapituitary tissues are largely unknown (Harvey and Hull, 1997), it was apparent that GH mRNA was barely detectable in the lungs of ED20 embryos. However, as pituitary GH secretion is autoregulated (Agoustsson and Bjorndddon, 2000; Asa et al., 2000), it is possible that this reflects downregulation by the ontogenetic appearance of GH in peripheral plasma, at ED 17 (Harvey et al., 1979). The continued presence of moderate GH immunoreactivity in the ED20 lung may reflect the greater stability of protein, in comparison with mRNA, or the sequestration of circulating GH by lung GHRs. Indeed, homogenates of adult human and rodent lungs contain trace amounts of GH immunoreactivity (Kyle et al., 1981: Costa et al., 1993) and the GH immunoreactivity in some adult tissues (eg the choroid plexus) is thought to be GHR bound (Nyberg 2000). This developmental loss of lung GH expression is also consistent with the loss of GH immunoreactivity in the liver of embryonic chicks between the first and second trimesters of incubation (Harvey et al., 2000), and suggests tissue-specific patterns in GH gene downregulation or extinction.

The finding of widespread GHR immunoreactivity and GHR mRNA in the embryonic lung suggests it is a target site for GH action. The widespread presence of GHRG-1, a GH-response gene that is a marker of GH action in birds (Radecki et al.,

1977) in the lungs of ED7 embryos (Harvey et al., 2002) supports this view. The presence of GHRs in the cytoplasm and nucleus of lung cells may also account for the accumulation of GH in these intracellular sites. Indeed, it is now well known that GHRs are predominately in nuclear locations of GH-target tissues (Fraser and Harvey, 1992: Lobie et al., 1994a, 1994b; Lincoln et al., 1998; Mertani et al., 2003). Moreover, GHR mRNA is also present in the nucleus of some GH target sites (Mertani et al., 1994; Kajimura et al., 2004), as in the lungs of embryonic chicks (eg. Fig. 11c), which may indicate a high level of GHR gene transcriptional activity (Morel et al., 1989).

The expression of the GHR gene occurs before the presence of GH in peripheral plasma, during late embryogenesis (Harvey et al., 1979). The local production of GH in the lung may thus have autocrine or paracrine roles in lung development or in pulmonary function. It is now well established that GH has local actions in many tissues (Harvey and Hull, 1997; Pantaleon et al., 1997; Waters and Kaye, 2002), which may or may not be mediated through an array of other growth factors (Waters et al., 1998; Sanders and Harvey, 2004). It may, therefore, be pertinent that insulin-like growth factor (IGF) is abundantly present in the lungs of embryonic chicks (Tanaka et al., 1996) in which transforming growth factor (TGF)-β-4 (Jakowlew et al., 1992), TGF-β-2 (Maina et al., 2003; Calvitti et al., 2004) and interleukin-1 (Calvitti et al., 2004) are also widely expressed and are thought to participate in lung development. Putative actions of pulmonary GH in lung development are, however, unlikely to be mediated via IGF-1, since IGF-1 expression in the embryonic lung is GH-independent and present in GHR deficient dwarf chicks (Tanaka et al., 1996).

It is also now well established that GH has functional actions in neonatal, juvenile and adult lungs of mammals, and is a target site for pituitary GH action (Bartlett et al.,1971; Harrison et al., 1978; Jost et al., 1979; Trotman-Dickenson et al., 1991; Edwards et al., 1992; Merola et al., 1995; Merola et al., 1996; Batchelor et al., 1998; Liu et al., 2002a; Liu et al., 2002b). It is therefore possible that pituitary GH also participates in lung development in perinatal chick embryos, following the ontogeny of pituitary GH secretion (Harvey et al., 1979; Porter 1997). This possibility is supported by the marked impairment in lung growth in hypophysectomized ED16-ED18 embryos (Marin et al., 1978; Hylka and Doneen, 1983), at a time when Type II pneomocytes normally differentiate (Sakiyama et al., 2000; Chiba et al., 2002) and when GH normally appears in peripheral plasma (Harvey et al., 1979). Furthermore, the ability of ectopic pituitary transplants to reverse the biochemical and morphological defects of the lung that accompany hypophysectomy provides evidence for pituitary hormone involvement in the maturation of the respiratory epithelium in perinatal chick embryos (Dameron and Marin, 1978; Marin et al., 1978; Hylka and Doneen, 1983).

In summary, these results show that prior to lung breathing, the embryonic chick lung is an extrapituitary site of GH production and GH action, which may involve hitherto unsuspected autocrine, paracrine and endocrine roles of lung GH in lung development or pulmonary function.

Figure 2.1. RT-PCR of reverse-transcribed mRNA from the lungs of embryonic day (ED) 11 and ED18 chicks (lanes 2 and 3, respectively) in comparison with reverse-transcribed mRNA from the pituitary glands (methodological control) of adult chickens (lane 1), using oligonucleotide primers designed to generate a 690bp chicken GH cDNA. Control reactions with mRNA (in the absence of reverse transcriptase) are shown in lanes 4 (for adult pituitary (pit) mRNA), 5 (for ED11 lung mRNA), and 6 (for ED18 lung mRNA). The data are representative of at least three RT-PCRs using different tissue extracts.

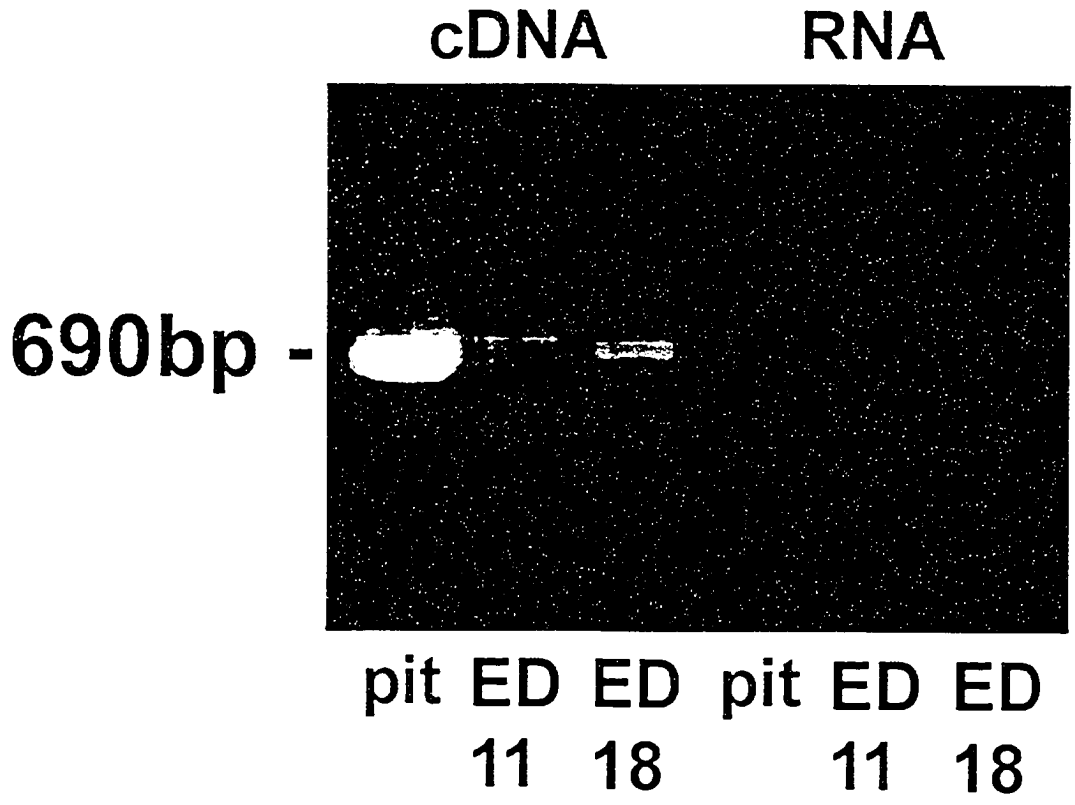


Figure 2.2. Western blotting of GH-immunoreactive proteins in the lungs of embryonic day (ED) 11, ED13, ED15, and ED18 chicks (lanes 2-5) in comparison with the pituitary glands of adult chickens (lane 1). Representative of at least two similar blots using different tissue extracts. 25μg of protein was loaded into lanes 2-5 (ED11, ED13, ED15, ED18), whereas 2μg of protein was loaded in lane 1.

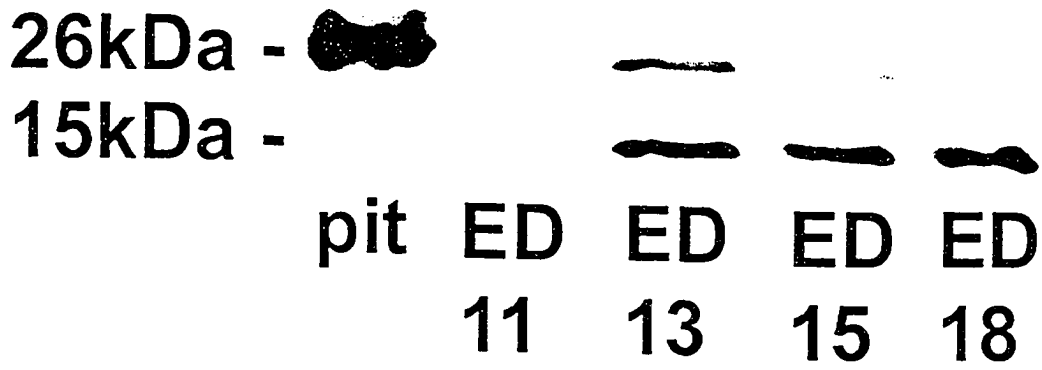


Figure 2.3. GH mRNA and GH-immunoreactivity in adult chicken pituitary glands. (a,b) In situ hybridization of GH mRNA using a 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA. Specific hybridization is shown in the cytoplasm of large rounded cells (somatotrophs) in the caudal lobe (Ca), but not in the cephalic lobe (Ce) of the pituitary gland, nor (c) when using the 690bp DIG-labeled *Not1* sense probe for chicken GH mRNA. (d,e) Immunocytochemical staining of GH cells in the caudal lobe (Ca, but not in the cephalic lobe, Ce) of adult chicken pituitary glands using a specific antibody raised in rabbits against native chicken pituitary GH. (f) Preabsorption of the primary antibody with excess recombinant chicken GH completely abolished pituitary staining. Magnifications are indicated by the bars (bar=10μm). Representative of at least three pituitary glands.

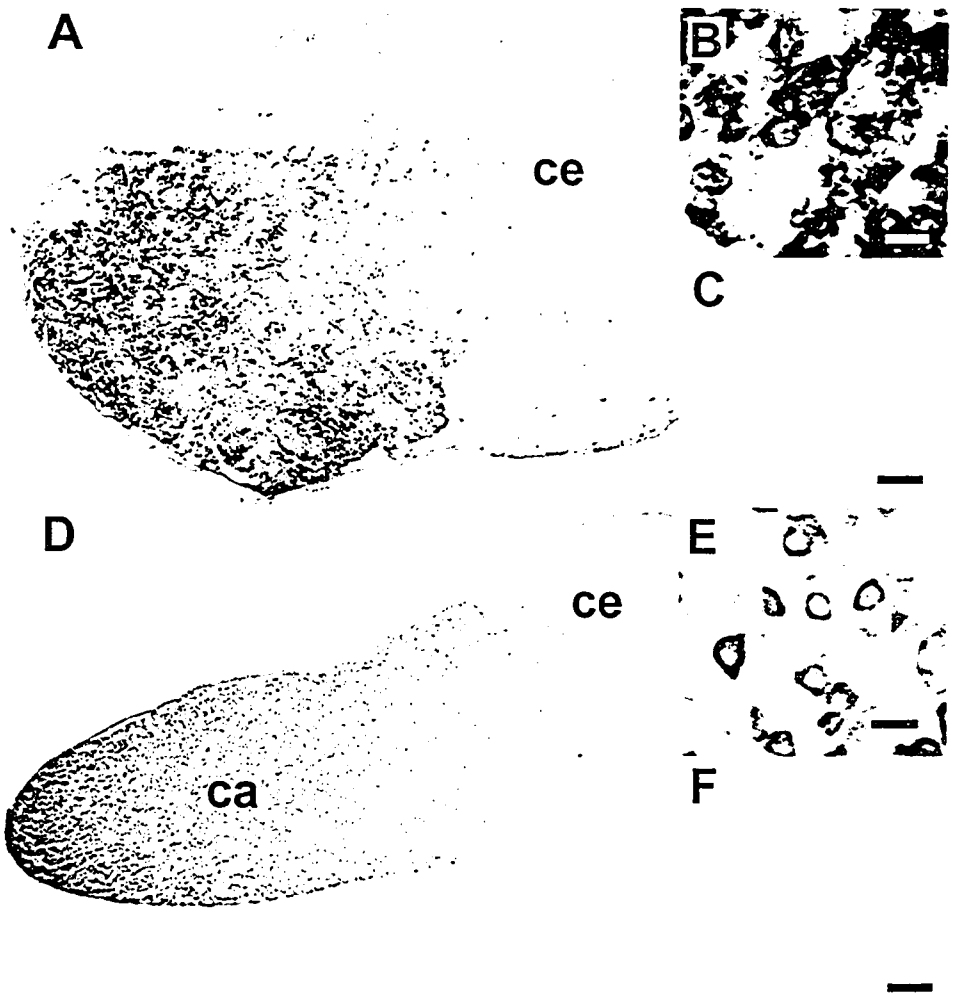


Figure 2.4. GH mRNA (a-g) and GH-immunoreactivity (h-n) in the lungs of embryonic day (ED) 7 chicks. (a) In situ hybridization of GH mRNA using a 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA. Specific hybridization is shown in the mesenchymal (mc) and epithelial (ep) cells upon use of 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA (a-d), but not (e-g) when using the 690bp DIG-labeled *Not1* sense probe for chicken GH mRNA. (h-k) Immunocytochemical staining of GH in mesenchymal and epithelial cells using a specific antibody raised in rabbits against native chicken pituitary GH. (l-n) Preabsorption of the primary antibody with excess recombinant chicken GH completely abolished all staining. Magnifications are indicated by the bars (a and h: bar=100μm, other bars=25μm). Representative of at least 4 embryos. Air sacs (as) and bronchioles (br) are indicated.

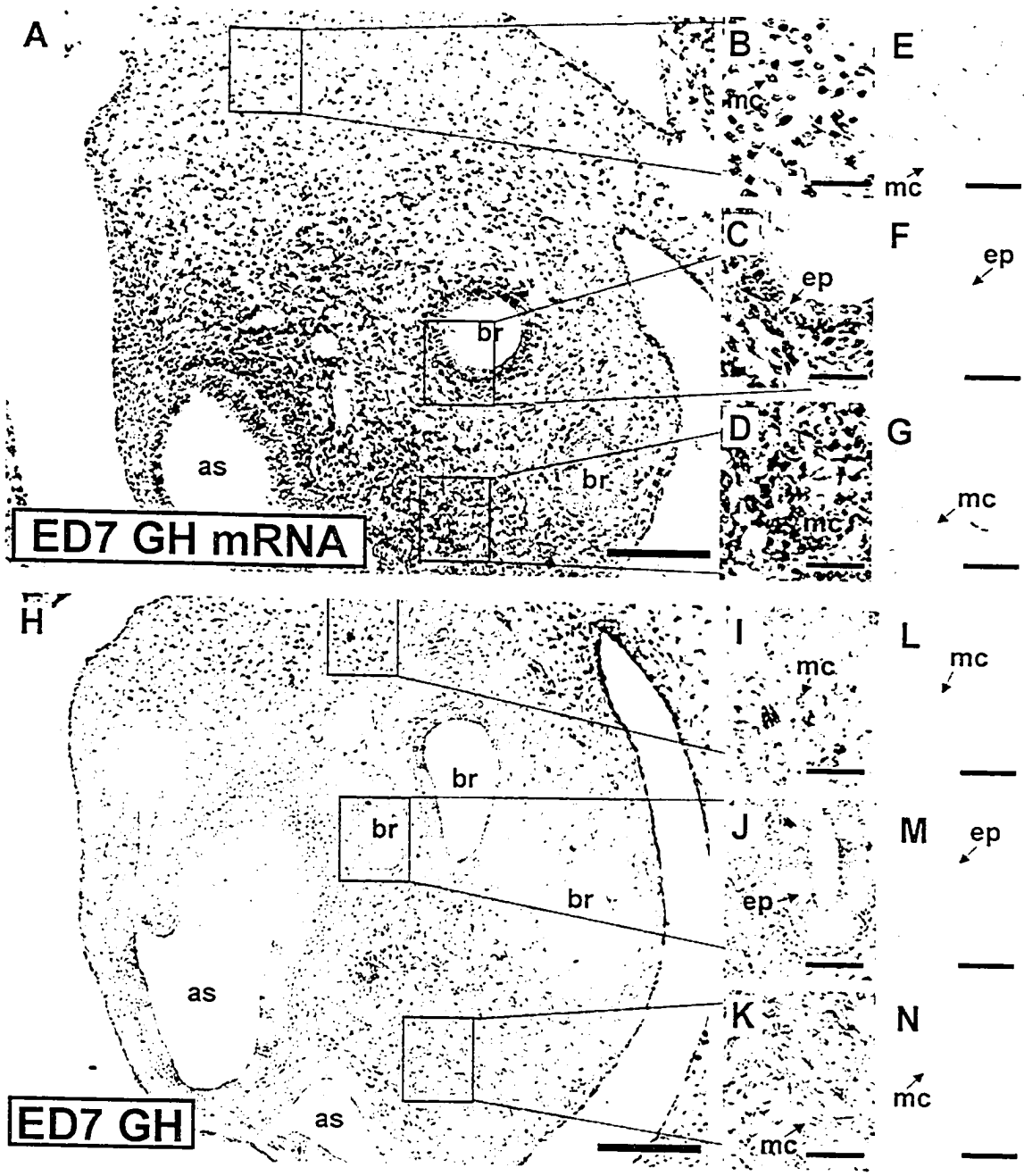


Figure 2.5. GH mRNA (a-e) and GH-immunoreactivity (f-j) in the lungs of embryonic day (ED) 11 chicks. (a) In situ hybridization of GH mRNA using a 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA. Specific hybridization is shown in the mesenchymal (mc) and epithelial (ep) cells, and in the blood vessels (bv) upon use of 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA (a-c), but not (d-e) when using the 690bp DIG-labeled *Not1* sense probe for chicken GH mRNA. (f-h) Immunocytochemical staining of GH in mesenchymal and epithelial cells using a specific antibody raised in rabbits against native chicken pituitary GH. (i-j) Preabsorption of the primary antibody with excess recombinant chicken GH completely abolished all staining. Magnifications are indicated by the bars (a and f: bar=25μm, all others: bar=20μm). Representative of at least 4 embryos.

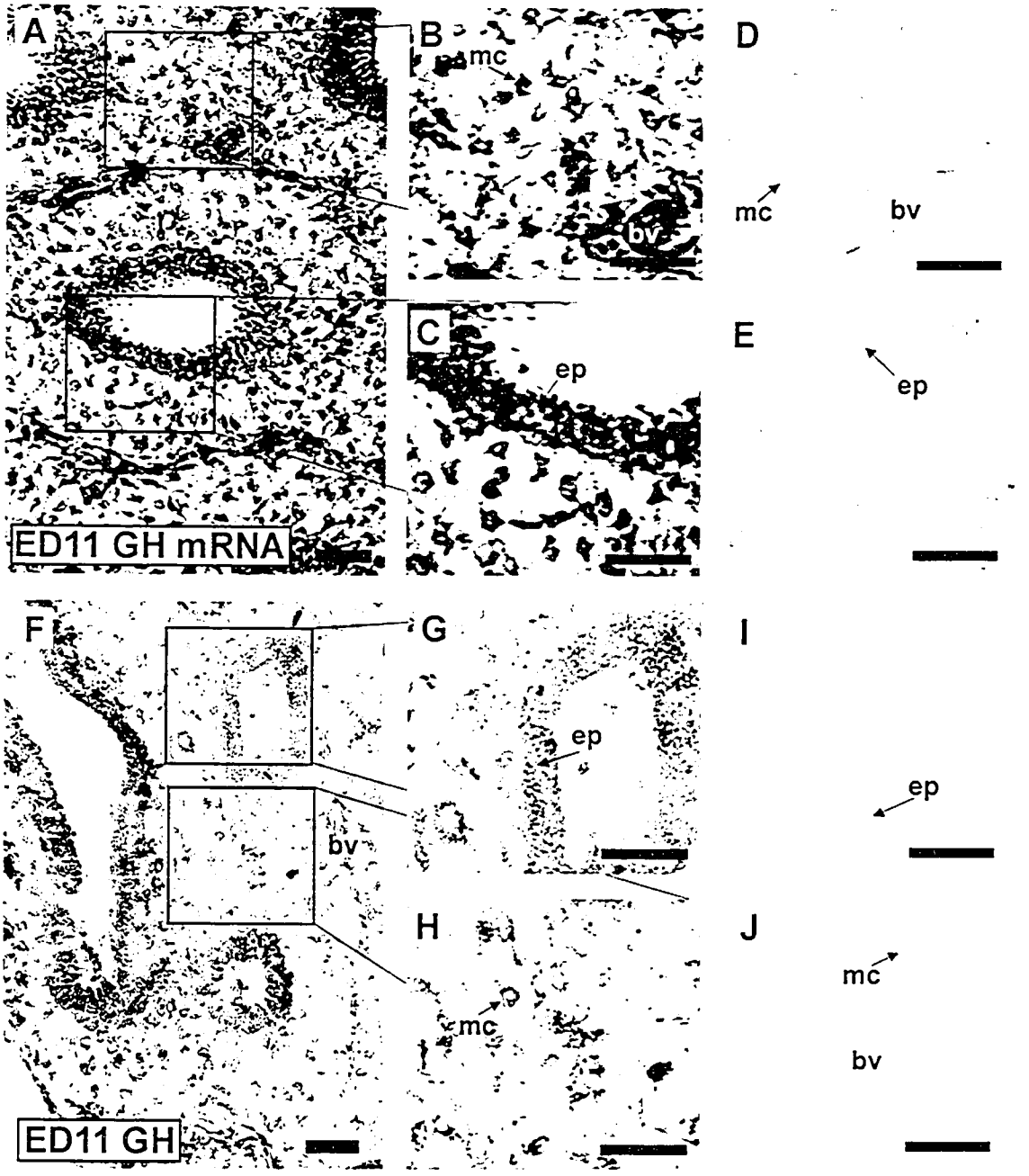


Figure 2.6. GH mRNA (a-e) and GH-immunoreactivity (f-j) in the lungs of embryonic day (ED) 15 chicks. (a) In situ hybridization of GH mRNA using a 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA. Specific hybridization is shown in the mesenchymal (mc) and epithelial (ep) cells upon use of 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA (a-c), but not (d-e) when using the 690bp DIG-labeled *Not1* sense probe for chicken GH mRNA. (f-h) Immunocytochemical staining of GH in mesenchymal, smooth muscle (sm), and epithelial cells using a specific antibody raised in rabbits against native chicken pituitary GH. (i-j) Preabsorption of the primary antibody with excess recombinant chicken GH completely abolished all staining. Magnifications are indicated by the bars (a and f: bar=25μm, all others: bar=20μm). Representative of at least 4 embryos.

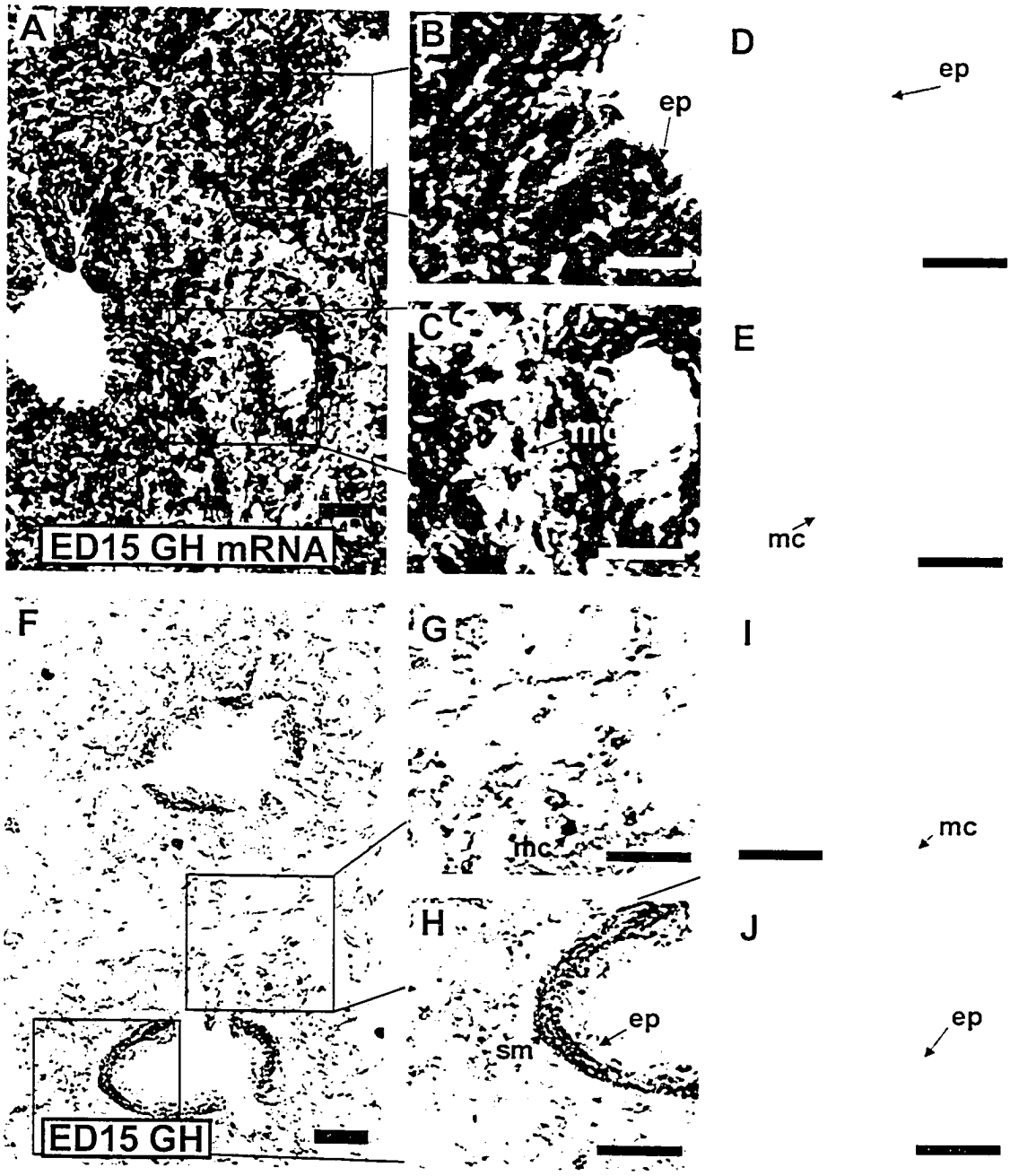


Figure 2.7. GH mRNA (a-e) and GH-immunoreactivity (f-j) in the lungs of embryonic day (ED) 20 chicks. (a) In situ hybridization of GH mRNA using a 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA. Specific hybridization is barely detectable in the mesenchymal (mc) and epithelial (ep) cells upon use of 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA (a-c). (d-e) Non-specific hybridization is shown when using the 690bp DIG-labeled *Not1* sense probe for chicken GH mRNA. (f-h) Immunocytochemical staining of GH in some mesenchymal, and epithelial cells using a specific antibody raised in rabbits against native chicken pituitary GH. Preabsorption of the primary antibody with excess recombinant chicken GH completely abolished all staining (i,j). Magnifications are indicated by the bars (a and f: bar=25μm, all others: bar=20μm). Representative of at least 4 embryos.

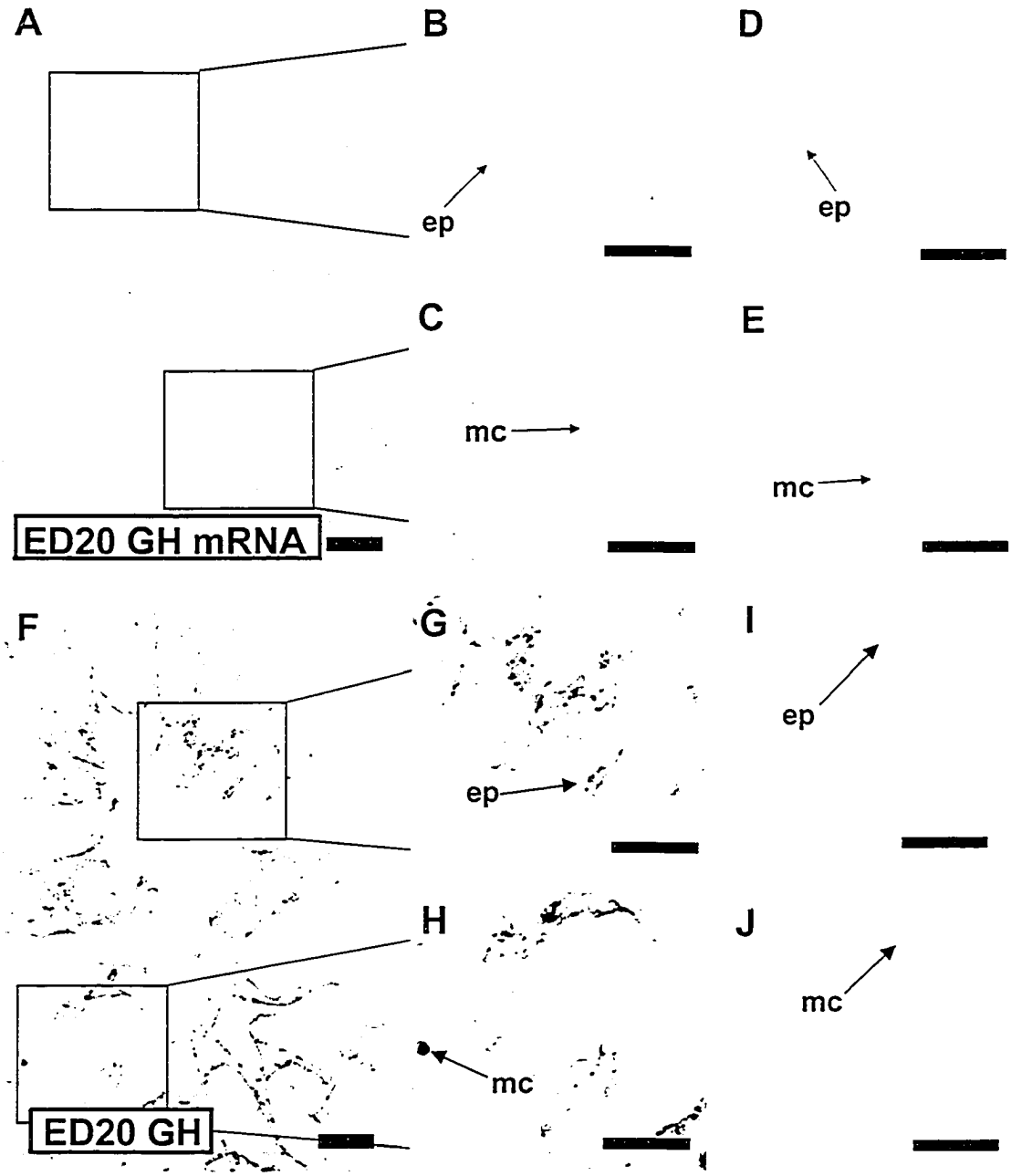


Figure 2.8. GH mRNA (a) and GH-immunoreactivity (b) in the heart of embryonic day (ED) 7 chicks. (a) In situ hybridization of GH mRNA using a 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA. Specific hybridization is shown in the cardiac muscle (cm) upon use of 690bp DIG-labeled *Sac1* antisense probe for chicken GH mRNA , but not in the red blood cells (rbc's). (b) Immunocytochemical staining of GH in the cardiac muscle but not red blood cells when using a specific antibody raised in rabbits against native chicken pituitary GH. Magnifications are indicated by the bars (a and f: bar=25μm, all others: bar=20μm). Representative of at least 4 embryos.

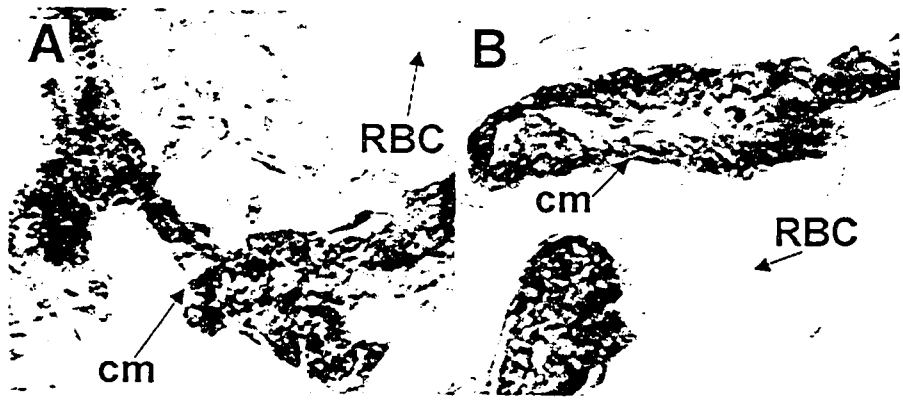


Figure 2.9. ED7 GHR mRNA (a-g) and GHR-immunoreactivity (h-n) in the lungs of embryonic day (ED) 7 chicks. (a) In situ hybridization of GHR mRNA using a 500bp DIG-labeled *Sac1* antisense probe for chicken GHR mRNA. Specific hybridization is shown in the mesenchymal (mc) and epithelial (ep) cells upon use of 500bp DIG-labeled *Sac1* antisense probe for chicken GHR mRNA (a-d), but not (e-g) when using the 500bp DIG-labeled *Not1* sense probe for chicken GHR mRNA. (h-k) Immunocytochemical staining of GHR in mesenchymal and epithelial cells using an antibody raised in rabbits against a synthetic fragment (CH17) of the extracellular domain of the chicken GHR. (l-n) Replacement of the primary antibody with normal rabbit serum completely abolished all staining. Magnifications are indicated by the bars (a and h: bar=100μm, other bars=25μm). Representative of at least 4 embryos. Air sacs (as) and bronchioles (br) are denoted.

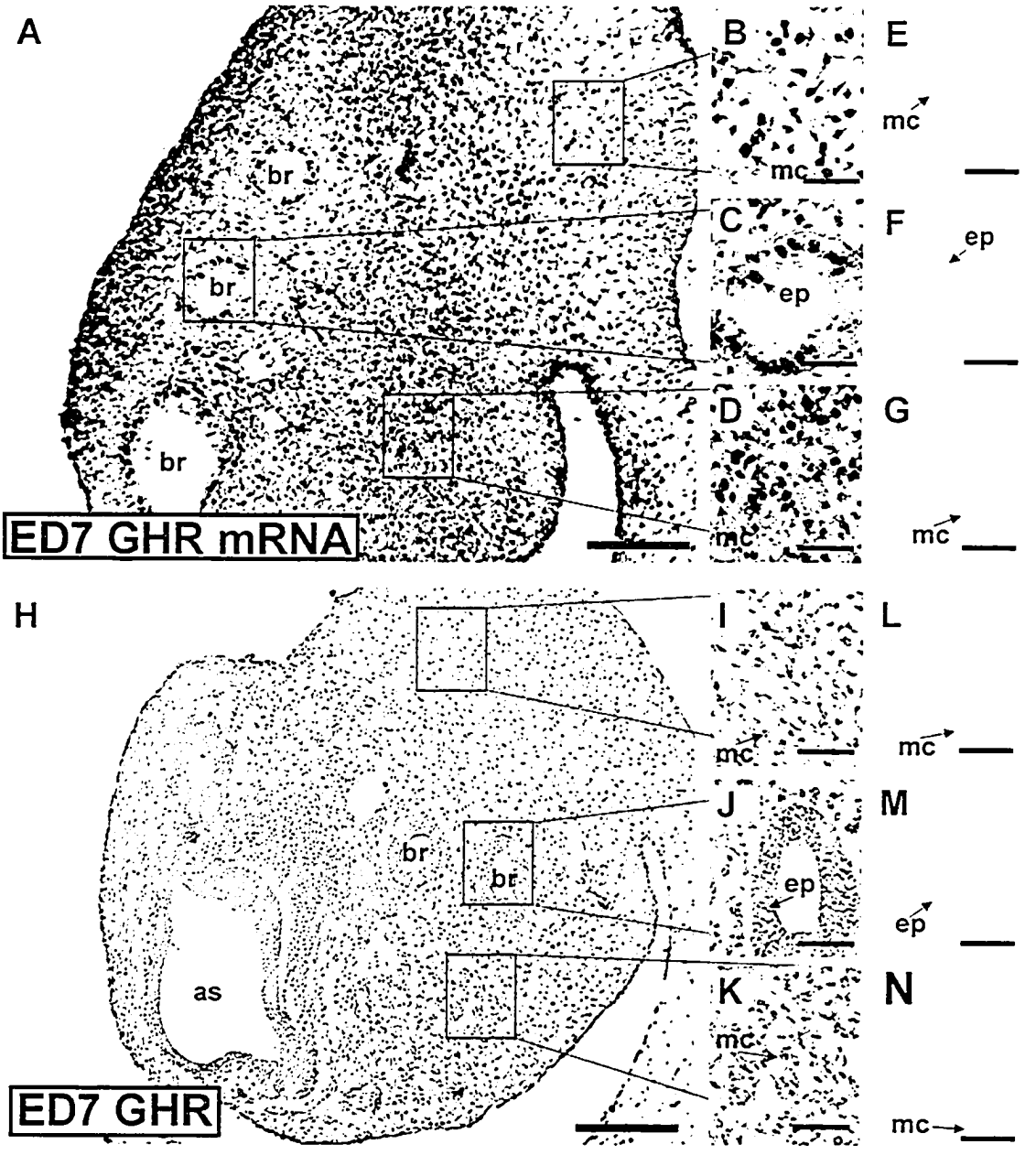


Figure 2.10. ED11 GHR mRNA (a-e) and GHR-immunoreactivity (f-j) in the lungs of embryonic day (ED) 11 chicks. (a) In situ hybridization of GHR mRNA using a 500bp DIG-labeled *Sac1* antisense probe for chicken GHR mRNA. Specific hybridization is shown in the mesenchymal (mc) and epithelial (ep) cells upon use of 500bp DIG-labeled *Sac1* antisense probe for chicken GHR mRNA (a-c), but not (d-e) when using the 500bp DIG-labeled *Not1* sense probe for chicken GHR mRNA. (f-h) Immunocytochemical staining of GHR in mesenchymal and epithelial cells using an antibody raised in rabbits against a synthetic fragment (CH17) of the extracellular domain of the chicken GHR. (i-j) Replacement of the primary antibody with normal rabbit serum completely abolished all staining. Magnifications are indicated by the bars (a and f: bar=25μm, all others: bar=20μm). Representative of at least 4 embryos.

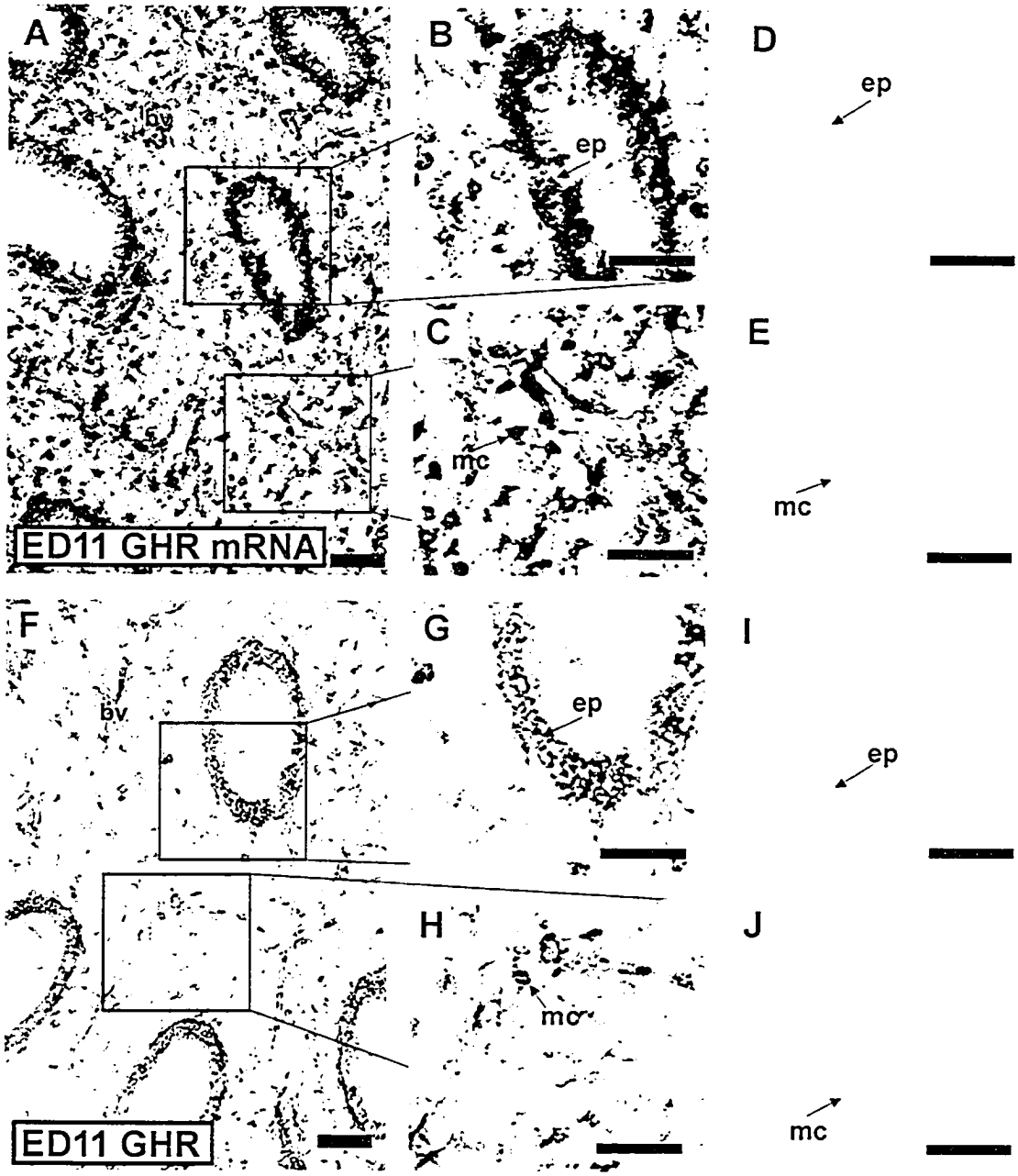
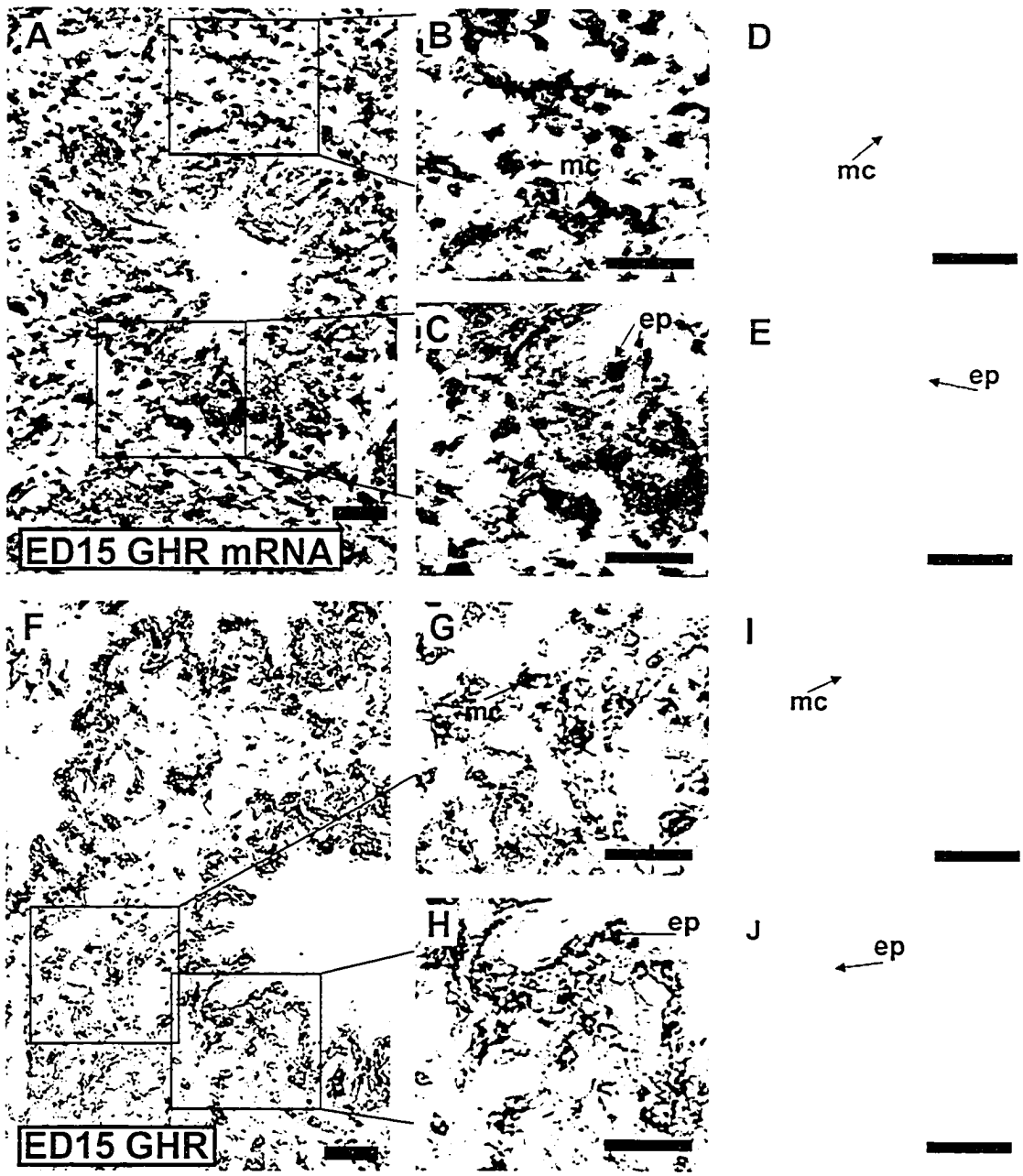


Figure 2.11. ED15 GHR mRNA (a-e) and GHR-immunoreactivity (f-j) in the lungs of embryonic day (ED) 15 chicks. (a) In situ hybridization of GHR mRNA using a 500bp DIG-labeled *Sac1* antisense probe for chicken GHR mRNA. Specific hybridization is shown in the mesenchymal (mc) and epithelial (ep) cells upon use of 500bp DIG-labeled *Sac1* antisense probe for chicken GHR mRNA (a-c), but not (d-e) when using the 500bp DIG-labeled *Not1* sense probe for chicken GHR mRNA. (f-h) Immunocytochemical staining of GHR in mesenchymal and epithelial cells using an antibody raised in rabbits against a synthetic fragment (CH17) of the extracellular domain of the chicken GHR. (i-j) Replacement of the primary antibody with normal rabbit serum completely abolished all staining. Μαγνιφιχατιονσ are indicated by the bars (a and f: bar=25μm, all others: bar=20μm). Representative of at least 4 embryos.



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CHAPTER 3

GROWTH HORMONE EXPRESSION IN THE PERINATAL AND POSTNATAL RAT LUNG

CHAPTER 3: GROWTH HORMONE EXPRESSION IN THE PERINATAL AND POSTNATAL RAT LUNG[1]

3.1 INTRODUCTION

A role for growth hormone (GH) in pulmonary function is indicated by the physiological and anatomical changes in the lung in pathophysiological states of GH excess and deficiency. For instance, large lungs (Bartlett, 1971), upper airflow obstruction (Trotman-Dickenson et al., 1991), and narrowing of the small airways (Harrison et al., 1978) accompany GH excess, whereas a decrease in muscle strength and reduction in the maximum inspiratory and expiratory pressure (Merola et al., 1996; Merola et al., 1995) is associated with GH deficiency. The possibility that the lung is a target site for GH action is also indicated by the GH-induced production of superoxide by alveolar macrophages (Edwards et al., 1992), the GH-induced increase in circulating lung neutrophil activation during sepsis and the accompanying increase in microvascular injury (Liu et al., 2002a). Exogenous GH also induces NFκB activation in the lung (Liu et al., 2002b), increases phosphorylase A activity (Jost et al., 1979) and stimulates the tyrosine phosphorylation of specific proteins in lung epithelial cells (Batchelor et al., 1998). The GH receptor (GHR) gene is also expressed in pulmonary tissues (eg. Batchelor et al., 1998; Garcia-Aragon et al., 1992; Tiong et al., 1989). Pituitary GH is thus likely to be an endocrine regulator of lung growth and function in juvenile development and in adulthood. It is, however, unlikely to be a regulator of pulmonary function in the early fetus.

[1] A version of this chapter has been published. Beyea, J.A., Olson, D.M., and Harvey, S. 2005. Dev Dyn 232:1037-46.

In many species, lung growth and differentiation occurs during ontogeny prior to the appearance of the pituitary gland and the differentiation of GH-secreting somatotrophs. For instance, lung buds appear at embryonic day (ED) 10 in fetal rats (with a 21d gestation period) (Young et al., 2002) and at ED3.5 (with a 21d incubation period) in developing chicks (Sakiyama et al., 2000), both of which occur before the presence of pituitary somatotrophs at ED19 in rats (Chatelain et al., 1979; Frawley et al., 1985; Hemming et al., 1986) and at ED16 in chicks (Harvey et al., 1998) . The expression of GH receptors in pulmonary tissues also occurs before the appearance of GH in peripheral plasma at ED19 (Strosser and Mialhe, 1975) in rats and at ED17 in chicks (Harvey et al., 1998). Early fetal development is therefore thought to be a *growth-without-GH-syndrome* (Geffner, 1996), independent of pituitary GH. The abundant and widespread production of GH in peripheral tissues of early embryonic chicks (Harvey et al., 1998) and mice (Pantaleon et al., 1997) nevertheless suggests GH involvement in early development through autocrine or paracrine mechanisms (Harvey and Hull, 1997). The presence of GH, GHR, and a GH-specific response gene (GH response gene-1) in the lungs of early chick embryos (Harvey et al., 2000, Harvey et al., 2001) supports this possibility, although the presence of GH in extrapituitary organs of the mammalian fetus is poorly documented. GH may, however, act as a local growth or differentiation factor in the mammalian lung, since GH mRNA, detected by RT-PCR, is present in the alveolar macrophages of adults (Allen et al., 2000) and trace amounts of GH immunoreactivity are present in whole lung extracts of fetal and adult lungs (Kyle et al., 1981; Costa et al., 1993). The possibility that GH production occurs in the rat lung during perinatal

and neonatal periods of lung development, therefore, has been determined in the present study.

3.2 MATERIALS AND METHODS

Animals/Tissues

Sprague-Dawley albino rat pups (Charles River Laboratories, St. Constant, Quebec, Canada) of both sexes were used. The pregnant dams were housed in the Health Sciences Laboratory Animal Service Department of the University of Alberta under veterinary supervision. Dams were maintained on regular rodent pellets and water ad libitum and were kept on a 12:12-h light-dark cycle. Dams were killed (when pups were at ED15, 17, 19, and 21) with an overdose of pentobarbital sodium injected intraperitoneally. Pups (n=4) were similarly sacrificed at ages day (D)2, D6, D9, and D14. These five ages were chosen to represent rat lung during the pseudoglandular (ED17), saccular (D2), and alveolarization (D6, D9, D14) periods of lung development (Gomi et al., 1994). The animal protocol was approved by the University of Alberta's Animal Policy and Welfare Committee.

For RT-PCR, lungs were excised in phosphate buffered saline (PBS) and immediately frozen in liquid nitrogen for RNA and protein extraction, respectively. For comparative purposes, samples of the pectoralis muscle were also collected.

For ISH and immunohistochemistry, pups were decapitated, torsos were rinsed in PBS, then immediately transferred to freshly-prepared 4% paraformaldehyde (Fisher Scientific, Edmonton, Alberta, Canada) in PBS. Torsos were fixed overnight in 4% paraformaldehyde at 4°C. The following day, torsos were

dehydrated and cleared [PBS for 15min x 2, 50% EtOH for 15min, 70% EtOH for 15min, 95% EtOH for 30min, 100% EtOH for 30min x 2, Citri-Solve (Fisher Scientific) for 30min x 2]. Torsos were then placed in paraffin wax at 60°C overnight, and embedded in paraffin wax the next morning. Postnatal lungs were perfused with 4% paraformaldehyde in PBS, at 20cm H_2O pressure for 5min. The tracheae were then tied closed and the lungs placed in 4% paraformaldehyde in PBS at 4°C overnight. The following day, the trachea were untied, the left and right lung lobes separated, and the lungs placed in tissue cassettes (Fisher Scientific). Lungs were washed for 3x 30min in cold PBS, and then left in 0.1M glycine (Fisher) in PBS at 4°C overnight. The next day, lungs were washed, for an hour each, in the following ethanol solutions: 70%, 80%, 90%, 95%, 100%, 100%, 100%, then cleared in xylene (Fisher Scientific) for 1hour. The next morning, lungs were embedded in paraffin wax. Tissue sections (8μm) were cut with a microtome, flattened on 42°C 0.1% DEPC (Sigma, Mississauga, Ontario, Canada) treated water, and mounted on Fisher Superfrost®/Plus slides (Fisher Scientific). For comparative purposes, ISH was similarly conducted with skeletal muscle (tongue) tissue sections.

RT-PCR

Total RNA extraction of liquid nitrogen-frozen whole lungs was performed using 100mg of tissue in 1ml TRIzol® Reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada), using the manufacturer's instructions. Reverse transcription was performed using 3μg RNA with Thermoscript™ RNase H⁻ Reverse Transcriptase (Invitrogen). The reaction was carried out according to the manufacturer's

instructions in a Techgene thermal cycler (Techne Ltd., Duxford, Cambridge, UK), using an extension temperature of 59°C. Following the reaction, cDNA products were transferred to ice.

After reverse transcription, the cDNA's were amplified by the polymerase chain reaction using 1U of Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) per reaction, according to the manufacturer's instructions, in the presence of 0.2μM of oligonucleotide primers designed to generate a 693bp rat (r) GH cDNA (Seeburg et al., 1977) (forward primer: JAB1: 5'– TGG ACA GAT CAC TGA GTG GCG –3'; and reverse primer: JAB2: 5'– CGC AGA GAC ACC AGT GTG TGC – 3'). Touchdown PCR was performed as follows: 95°C for 1min; then 5 cycles of 95°C for 30s and 65°C for 2min; then 35 cycles of 95°C for 30s and 56.2°C for 2min; followed by 68°C for 10min. Ten microliters of each PCR product was visualized by ethidium bromide staining in a 1.4% agarose gel. mRNA from adult rat pituitary glands was used as a positive control: negative controls utilized mRNA rather than cDNA.

Sequencing

PCR products were purified by the High Pure™ PCR Product Purification Kit (Roche Diagnostics Canada, Laval, Québec, Canada), then sequenced by the DNA Core lab, Department of Biochemistry, University of Alberta. Sequences were analyzed by BLAST and compared with sequences in the NCBI database.

In Situ Hybridization

Probes

In situ hybridization (ISH) was performed using a full-length rGH antisense probe, with a full-length rGH sense probe as a negative control. Both rGH probes were 693bp and spanned bases -21 to 672 of the GH mRNA (Seeburg et al., 1977), and were made from a PCR product generated from adult rat pituitary with JAB1 and JAB2. For RNA probe synthesis, PCR products were then cloned into pCR7 II-TOPO vectors (Invitrogen). The vectors were linearized with restriction endonucleases *BamHI* and *NotI* (for rGH, antisense and sense probes, respectively). Digoxigenin-labeled antisense and sense riboprobes were synthesized by *in vitro* transcription with T7 and SP6 RNA polymerase, respectively, and DIG RNA labeling mix (Roche), according to the manufacturer's instructions. Probe concentrations were determined by dilution and dot blot analysis.

Procedure

Briefly, 8μm 4% paraformaldehyde-fixed sections were deparaffinized in Citri-Solve (Fisher Scientific), and hydrated in a graded ethanol series. The sections were then washed in 0.1%DEPC water for 2min, and then washed in 0.1%DEPC-phosphate buffered saline (PBS) for 5min x 2. Slides were post-fixed in 4% formaldehyde in 0.1%DEPC-PBS for 10min. RNase treatment was performed by placing slides in fresh 0.1% DEPC-PBS (DEPC added just before use) for 15min x 2. Sections were treated in Proteinase K (Invitrogen) solution (1mg Proteinase K, 0.02M Tris, 0.01M Na_2EDTA, in 0.1%DEPC water) at 37°C for 1min. Slides were then equilibrated in

5X SSC for 10min x 2. The sections were then prehybridized for 2 hours at 65°C with 120μL prehybridization solution (50% formamide, 5X SSC, 0.5mg/ml salmon testes DNA [Sigma], in 0.1% DEPC water) under a cover slip, on an elevated platform in a sealed container with 75% formamide at the bottom of the container. Slides were then hybridized for 16h at 65°C with 120μL of hybridization solution [prehybridization solution with 1:8 diluted of either antisense digoxigenin (DIG)-labeled rGH probe, or with 1:8 diluted sense Digoxigenin-labeled rGH probe], under prehybridization conditions.

Slides were then washed in 2X SSC for 30min at room temperature, in 2X SSC for 60min at 65°C, then finally in 0.1X SSC for 60min at 65°C. Slides were washed for 5min x 2 in Buffer One (0.01M Tris, 0.15M NaCl, pH7.5), then transferred to antibody solution [1:2000 dilution of anti-digoxigenin, fab fragments (Roche) in 1% (w/v) blocking reagent (Roche) (1% w/v blocking reagent in 0.1M maleic acid, 0.15M NaCl, pH7.5)] for 2h. Slides were then washed for 15min x 2 in Buffer One, then for 5min in Buffer Two (0.1M Tris, 0.1M NaCl, 0.05M $MgCl_2$, pH9.5), and developed in NBT/BCIP solution [1:50 dilution of NBT/BCIP Stock Solution (Roche) in Buffer Two] for 30min at 37°C. Colour development was stopped by immersion in TE buffer for 5min x 2. Sections were counterstained for 2min with 0.5% (w/v) methyl green (Sigma) in 3M NaOAc (pH4.0), rinsed three times in 0.1% DEPC water, rehydrated in graded ethanol, then cleared in Citri-Solve for 2min x 2), then mounted with DPX Mounting Medium (Fluka - Sigma). Staining specificity was demonstrated using the adult rat anterior pituitary as a positive control (Figure 2A, B) in which intense cytoplasmic staining was clearly evident, in some but

not all, pituitary cells, whereas staining was not observed using the sense probe (Figure 2C). Digital images were collected using a SPOT Digital Microscope camera (Carsen Group, Markam, Ont., Canada) mounted on an Olympus B×40 microscope.

Immunohistochemistry

Immunocytochemical staining was performed using the avidin-biotin-peroxidase (ABC) method, as described in Harvey et al.(2000), and 1:500 diluted goat anti-human GH antibody. The specific goat antibody was raised against the amino terminus of human GH (lot#A091, Santa Cruz Biotechnology, Santa Cruz, California, USA), and has been shown by the manufacturer to cross react with both rat and mouse GH. The lack of sequence homology between the peptide to which the antibody was raised and the sequence of rat prolactin precludes cross-reactivity of the antibody with rat prolactin.

For immunocytochemistry, the slides were deparaffinized in Citri-Solve (Fisher) and hydrated in a graded ethanol series. Slides were then incubated for 30min in a 50%methanol/1% hydrogen peroxide solution. Slides were washed 3x5min in PBS, blocked in 10% normal goat serum (NGS) (Sigma) for one hour, then incubated overnight with the primary antibodies in 1% NGS. The following morning, slides were washed (as before) in PBS, and incubated for 1h in 1:500 diluted biotin-SP-conjugated donkey anti-goat (Lot#56255; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and then washed again in PBS. ABC reagent (Vector Laboratories, Burlingame, CA, USA) was prepared, and incubated on slides for 1h, followed by PBS washes. Staining was visualized using the chromogenic substrate,

diaminobenzidine tetrahydrochloride (DAB) (Sigma), which resulted in a brown precipitate. The adult rat anterior pituitary was used as a positive control for GH staining (Figure 2D, E) in which intense cytoplasmic staining was clearly evident, in some but not all, pituitary cells. The specificity of the staining was also established by preabsorption (2h, with shaking at room temperature) of the primary antibodies with GH blocking peptide (the sequence used for antisera generation; Santa Cruz Biotechnology), at final concentration of 80μg/mL, for 2h (pituitary control – Figure 2F). Digital images were collected using a SPOT Digital Microscope camera (Carsen Group, Markam, Ont., Canada) mounted on an Olympus B×40 microscope.

Western Blotting

Protein samples were homogenized, and protein content was estimated using Bio-Rad Protein Assay (Bio-Rad Life Sciences, Mississauga, Ontario, Canada). A total of 25μg of protein was loaded in each lane (except pituitary, for which 2μg of protein was loaded). Samples were run on a 15% polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked with 5% skim milk powder in 0.1% T-TBS. Blots were incubated overnight with the same commercial antibody used for immunohistochemistry and also at a dilution of 1:500. The blots were then incubated with peroxidase-conjugated anti-goat antibody (Jackson Immunoresearch Laboratories, West Grove, PA), at a dilution of 1:500. Immunoreactive bands were visualized by using ECL Western Blotting Detection Reagents (Amersham Biosciences, Baie d'Urfe, Québec, Canada). Pituitary was used as a positive control,

and heart, skeletal muscle, and liver from adult rats were used for comparative purposes.

3.3 RESULTS

GH mRNA

As expected, full-length GH (693bp) cDNA was detected in pituitary mRNA, following RT-PCR with the GH oligonucleotide primers (Fig. 3.1A, lane 1). cDNA moieties of identical size were similarly generated with mRNA from ED15 (Figure 1A, lane 2), ED17 (lane 3), ED19 (lane 4), and ED21 (lane 5) lungs. These moieties were not generated in the negative controls (lanes 7-11, respectively), nor in reverse transcribed skeletal muscle mRNA (Fig. 3.1B, lane 2).

Fetal Rat Lung cDNA Sequence

All of the fetal rat lung GH cDNA moieties were sequenced, and all were found to be 99.9% homologous with the published rat pituitary GH sequence (Accession #V01237 - 46). The only exception to the published sequence (for Norway Rat) was a substitution at base 81 (a C rather than A), which would result in Phe rather than Lys as the coded amino acid. This substitution was also present in the GH cDNA amplified from pituitary mRNA (data not shown) and hence was not specific to the lung.

In Situ Hybridization

Staining for rGH mRNA in the rat pituitary was demonstrated as a positive control (Fig. 3.2A, B). Intense cytoplasmic staining was clearly evident in some, but not all, pituitary cells, whereas staining was not observed using the rGH sense probe (Fig. 3.2C) nor in muscle sections (Fig. 3.2G,H). Staining for GH mRNA was also detected in the perinatal rat lung during the pseudoglandular stage (ED17; Fig. 3.3), in which intense staining is visible within mesenchymal (m), epithelial (ep), and smooth muscle (sm) cells (Fig. 3.3A-C). The specificity of the staining is indicated by the lack of staining in these cell types when using the sense probe (Fig. 3.3D, E). rGH mRNA staining was similarly intense and widespread within the postnatal day 2 (D2) lung during the saccular stage (Fig. 3.4A, B), and during early (D6; Fig. 3.5A, B), mid (D9; Fig. 3.6A, B), and late (D14; Fig. 3.7A, B) alveolarization. At each stage, rGH mRNA was localized to morphologically identified type I and II epithelial cells, pulmonary tissue macrophages, and alveolar macrophages.

Immunohistochemistry

As a positive control, GH immunoreactivity was demonstrated in the rat pituitary gland (Fig. 3.2D, E). The staining was confined to the cytoplasm (Fig. 3.2E) and was specific, being completely abolished upon preabsorption with the blocking peptide (Fig. 3.2F). In contrast, no staining was seen in muscle, as a negative control (Fig. 3.2I, J). Staining for GH was also seen in the pseudoglandular ED17 lung (Fig. 3.3F-H), and was most intense in the mesenchymal (m), epithelial (ep), and smooth muscle (sm) cells (Fig. 3.3G, H). The specificity of staining is indicated by the lack of

staining in these cells types when the antibody is preabsorbed with blocking peptide (Fig. 3.3I, J). Widespread staining was also present in the newborn in the first 2 weeks of postnatal development, during the saccular period (D2; Fig. 3.4D, E), and during early (D6; Fig. 3.5D, E), mid (D9; Fig. 3.6D, E), and late (D14; Fig. 3.7D, E) alveolarization. rGH was again localized to type I and II epithelial cells, and to pulmonary tissue macrophages and alveolar macrophages, similar to the rGH mRNA localization. The staining for GH in these tissues was completely lost after the preabsorption of the GH antibody with the blocking peptide (Fig. 3.4-3.7F).

Western Blotting

As expected, the GH immunoreactivity in the pituitary gland was associated with a 22kDa protein (Fig. 3.8, lane 1). This same protein was present in the postnatal day 14 lung (Fig. 3.8, lanes 2-4), and in the heart (lane 5), in which an additional protein of 15kDa was also present. In contrast, no GH immunoreactivity was present in extracts of skeletal muscle (lane 6) and liver (lane 7).

3.4 DISCUSSION

These results clearly demonstrate, for the first time, GH gene expression in the lungs of perinatal and neonatal rats. The GH mRNA in the fetal and neonatal rat lung was identical to that expressed in the adult pituitary gland of these rats. The GH transcript in immune (Rohn and Weigent, 1995) and neural (Baudet et al., 2003) tissues of the rat is also homologous to pituitary GH mRNA. In contrast, tissue-specific GH transcripts are present in central (neural retina) and peripheral (heart) tissues of

perinatal chickens that differ from pituitary GH mRNA (Takeuchi et al., 2001). The GH mRNA sequence in the pituitary glands and lungs of our (Sprague-Dawley) rats differed from the published sequence for the Norway rat (Seeburg et al., 1977), but this may reflect the presence of polymorphisms in the GH gene (eg. Malveiro et al., 2001; Sorensen et al., 2002).

GH gene expression has previously been demonstrated in adult human lungs (Allen et al., 2000), although this was only in isolated activated alveolar macrophages and was only characterized by RT-PCR. The present study is therefore the first to localize GH mRNA within lung epithelia, smooth muscle, mesenchyme and Type I and II cells.

The presence of GH mRNA in the lung provides strong evidence for its synthesis within this tissue during development. The presence of GH-releasing hormone (GHRH) (Shibasaki et al., 1984; Allen et al., 2000) and ghrelin (Gnanapavan et al., 2002; Volante et al., 2002) in the lung may indicate the involvement of these GH secretagogues in the expression of the lung GH gene, as in the pituitary gland.

Within the lung, GH mRNA was present from ED15, prior to its ontogenetic appearance in the pituitary gland at ED19 (Chatelain et al., 1979; Frawley et al., 1985; Hemming et al., 1986). The widespread presence of GH in the ED17 lung is thus unlikely to be of pituitary origin, especially as the circulating GH concentration is not detectable at this age (Strosser and Mialhe, 1975). The abundance and localization of GH-immunoreactivity in the mesenchymal, epithelial, and smooth

muscle cells of the fetal lung is also unlikely to reflect its sequestration from GH-producing macrophages or monocytes in the alveoli or airways of the developing lung (Allen et al., 2000). Although GH can pass from bronchial airways into the lung interstitium and retain its biological activity (Patton et al., 1989), the GH immunoreactivity in the interstitial cells of the perinatal and neonatal lung is far more abundant than in the cells of the bronchial associated lymphoid tissue inside the airway lumen. Indeed, the GH immunoreactivity in pulmonary macrophages reflect its uptake and degradation (Patton et al., 1989), suggesting GH release from lung airway epithelial cells might contribute to the GH found in airway macrophages. Alveolar macrophages are therefore unlikely to be the source of the GH immunoreactivity in the perinatal and neonatal lung.

Our finding of GH immunoreactivity in the lung is in agreement with earlier studies that measured radioimmunoassayable GH in whole-lung extracts in the adult lungs (Kyle et al., 1981) and in the fetal lungs (Costa et al., 1993) of humans. In the latter study, the concentration of immunoreactive GH in the fetal lung was >5 fold higher than in adults, suggesting its presence was ontogenetically regulated and possibly linked to lung growth and development.

It is now well established that the lung is a target site for GH action. Indeed, radioligand binding sites for GH have been demonstrated in adult and fetal rabbit lungs (Amit et al., 1987; Labbe et al., 1992). Northern blotting has also demonstrated the presence of GHR mRNA in the lungs of fetal rats (Walker et al., 1992) and rabbits (Tiong et al., 1989). GHR mRNA is also expressed in the human lung from the first trimester of gestation (Zogopoulos et al., 1996). Immunocytochemistry and *in situ*

hybridization have also localized GHR and GHR mRNA within fetal rat lung epithelial cells (Garcia-Aragon et al., 1992; Edmondson et al., 1995) and GHRs have also been identified by Western blotting in fetal (Walker et al., 1992) and adult (Frick et al., 1998) rat lungs. Batchelor et al., (1998) also found that the GHR mRNA was present in the rat lung from ED16-21, corresponding to part of the pseudoglandular, the canalicular, and part of the saccular stages of rat lung development, and found that it was 50% more abundant than in the liver, which is a recognized GH target site (Baumbach et al., 1989). Moreover, these authors showed that GH stimulation of the receptor induced tyrosine kinase activity, indicating that fetal rat lung GHR is functional. Shoba et al., (1999) similarly correlated GHR abundance in the rat lung with the activity of proteins involved in GHR signaling (STAT-1, -3, -5 and JAK 2) and Lu et al., (2001) found a GH-regulated gene (Grtp1) was also present in the rat lung. The presence of GH and GHR in the developing lungs of perinatal and neonatal rats therefore suggests GH actions in lung development or in pulmonary function.

Actions of exogenous GH in promoting lung development have been shown in neonatal rats (Dubreuil and Morisset, 1986) and hypophysectomized mice (Sondergaard et al., 2003). Pathological pituitary GH excess in acromegaly is similarly correlated with lung hypertrophy (Trotman-Dickenson et al., 1991), particularly in alveolar size and alveolar surface area (Donnelly et al., 1995), whereas a decrease in lung size is a characteristic of pituitary GH deficiency (De Troyer et al., 1980). Exogenous GH also increases respiratory muscle strength (Felbinger et al., 1999) in GH Deficiency patients. Other actions of GH within the rat lung include an increase in the activity of pulmonary guanylate cyclase (Vesely, 1981), increased

activities of the antioxidant glutathione (Youn et al., 1998) and a decrease in lung lipid peroxidation (Weigent et al., 1992). Whereas roles for lung GH in lung development are currently unknown, it may participate in its vascularization, as GH stimulates angiogenesis in other tissues (Struman et al., 1999; Corbacho et al., 2002). It may also participate in cellular differentiation (Sanders and Harvey, 2004) or regulate immune function within the lung (Batchelor et al., 1998; Waters et al., 1999; Allen et al., 2000).

The abundance and widespread distribution of GH and GH mRNA in the perinatal and neonatal lung suggests GH action during development is of physiological significance. Moreover, as GH expression occurs in the lung before its appearance in the pituitary gland at ED19, (Chatelain et al., 1979; Frawley et al., 1985; Hemming et al., 1986) and the presence of GH in systemic circulation at ED19 (Strosser and Mialhe, 1975), actions of GH in the developing lung are likely to result from local autocrine or paracrine actions, at least in the late pseudoglandular stage. Local actions of GH within immune (van Garderen et al., 1997), mammary (Zhang et al., 1997), and orthodontic tissues (Mertani et al., 2001) and GH-expressing cell lines (eg. Kaulsay et al., 1999) are now well established and a similar local mechanism may be operative in the lung during development. Such actions, however, may be indirect and mediated by an array of growth mediators (Sanders and Harvey, 2004), including insulin-like growth factors (IGFs).

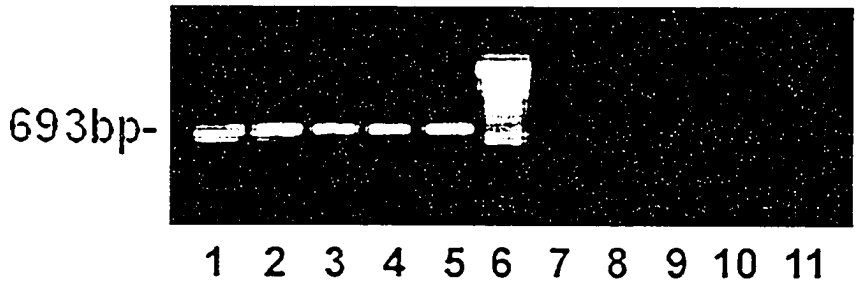
The possibility that actions of GH within the developing lung might be mediated by an IGF-I dependent mechanism is supported by the distribution of IGF-I mRNA in the fetal rat lung, since it is comparable to our findings of GH and GH

mRNA localization. IGF-I mRNA is abundantly localized within fetal mesenchymal lung cells, especially those surrounding airway epithelium (Retsch-Bogart et al., 1996), although it is also present in airway epithelial cells (Wallen and Han, 1994). As the expression of IGF-I in the developing lung was correlated with cellular proliferation and the maturation of connective tissue (Lallemand et al., 1995), it is possible that these actions reflect the upstream expression of GH in the same tissues and cells. The increased production of IGF-I in the lungs of GH-treated rats (D'Ercole et al., 1984) supports this possibility.

In summary, these results demonstrate the expression of GH mRNA, and GH-immunoreactivity, within the lungs of perinatal and neonatal rats and suggest autocrine/paracrine actions of pulmonary GH are involved in the pseudoglandular through the alveolarization stages of development of this tissue.

Figure 3.1. A. RT-PCR of full-length rat growth hormone (rGH) mRNA in prenatal rat lungs. Positives (lanes 1-5), 100bp ladder (lanes 6), and negatives (no reverse transcriptase) (lanes 7-11) are shown. Samples are pituitary control (lanes 1 and 7), ED15 lung (lanes 2 and 8), ED17 lung (lanes 3 and 9), ED19 lung (lanes 4 and 10), ED21 lung (lanes 5 and 11). B. RT-PCR of full-length rat growth hormone mRNA in pectoralis muscle. Positives (lanes 1, 2), 100bp ladder (lane 3), and negative (no reverse transcriptase (lanes 4, 5) are shown. Samples are pituitary positive control (lanes 1 and 4), and pectoralis muscle negative control (lanes 2 and 5). Each gel is representative of at least 5 similar gels.

A



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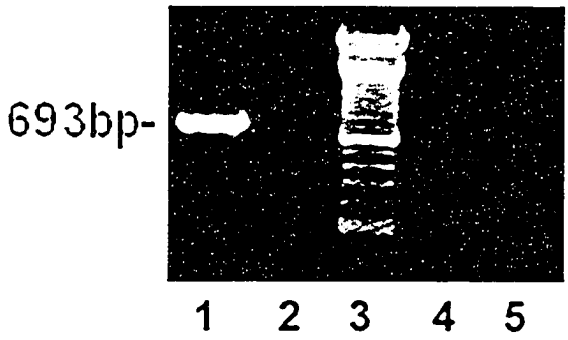


Figure 3.2. *In situ* hybridization of rGH mRNA in the pituitary glands (A-C) and skeletal muscle (G, H) of adult rats using full-length rGH RNA probes. Staining for rGH mRNA with the full-length antisense rGH probe is present in the pituitary somatotrophs (A, and arrow B), and absent upon use of the full-length rGH sense probe (arrow C). Staining is also absent in skeletal muscle using both the antisense (G) and sense (H) rGH probes. Immunohistochemistry of rGH in the pituitary glands (D-F) and skeletal muscle (I-J) of adult rats using a specific antibody against rGH. Cytoplasmic staining for rGH is present in the pituitary somatotrophs (D, and arrow E), and absent upon preabsorption of the antibody with the specific blocking peptide (arrow F). Staining is also absent in skeletal muscle with anti-GH antibody (I) and preabsorbed anti-GH antibody (J). All sections are representative of at least 4 adult rats.

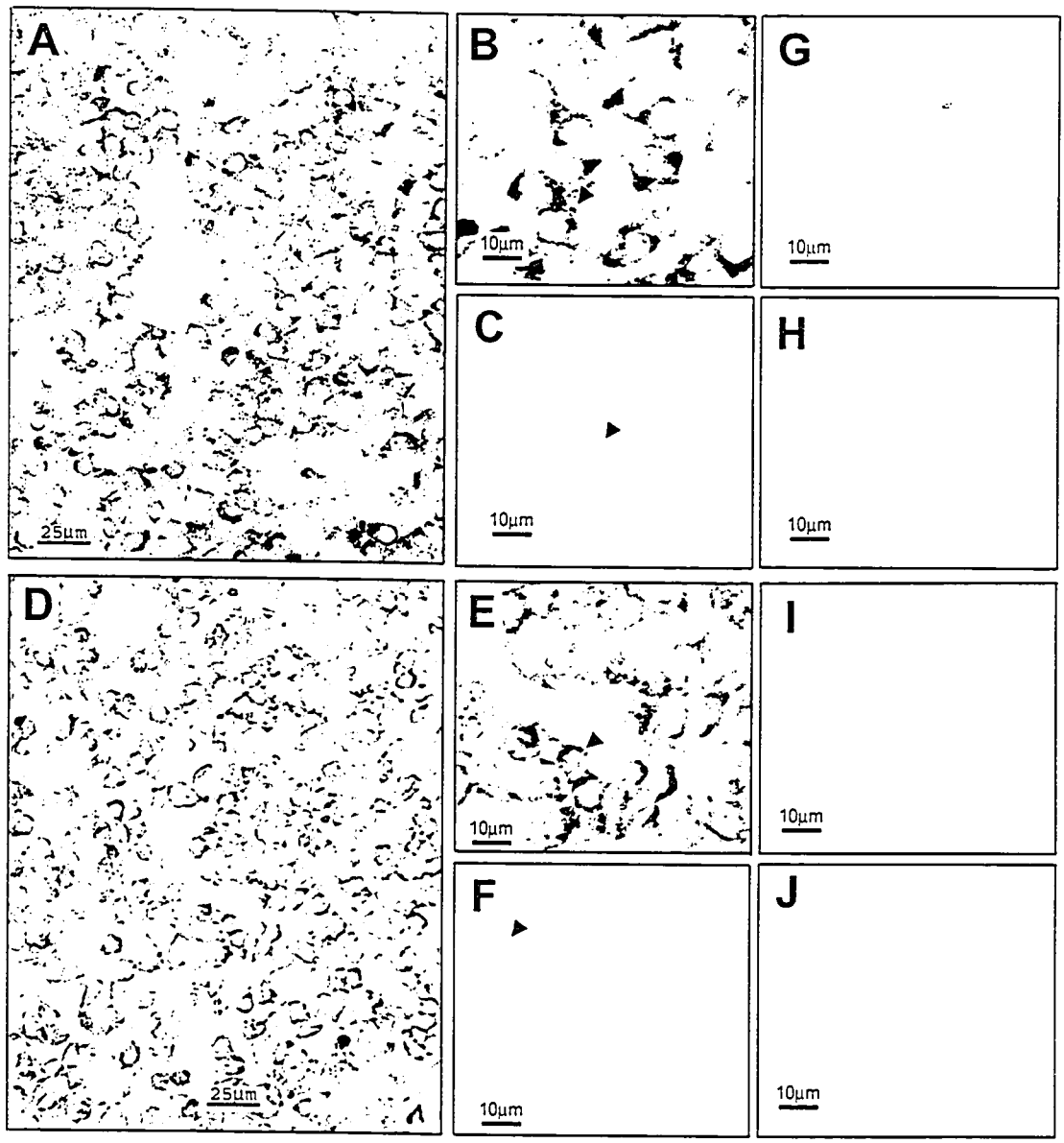


Figure 3.3. *In situ* hybridization (A-E) of rGH mRNA in pseudoglandular (ED17) rat lung using full-length rGH RNA probes. Using the full-length rGH antisense probe, staining is present in the ED17 fetal lung (A), specifically in the epithelial (ep), mesenchymal (m) (B), and smooth muscle (sm) cells (C). Staining in these cell types is absent (D, E) upon use of the full-length rGH sense probe. Immunohistochemistry (F-J) of rGH in pseudoglandular (ED17) rat lung using a specific antibody against rGH. Staining is present in the ED17 fetal lung (F), specifically in the smooth muscle (sm), mesenchymal (m) (G), and epithelial cells (ep) (H). Staining in these cell types is absent (I, J) upon preabsorption of the antibody with the specific blocking peptide. Representative sections are shown from at least 4 pups.

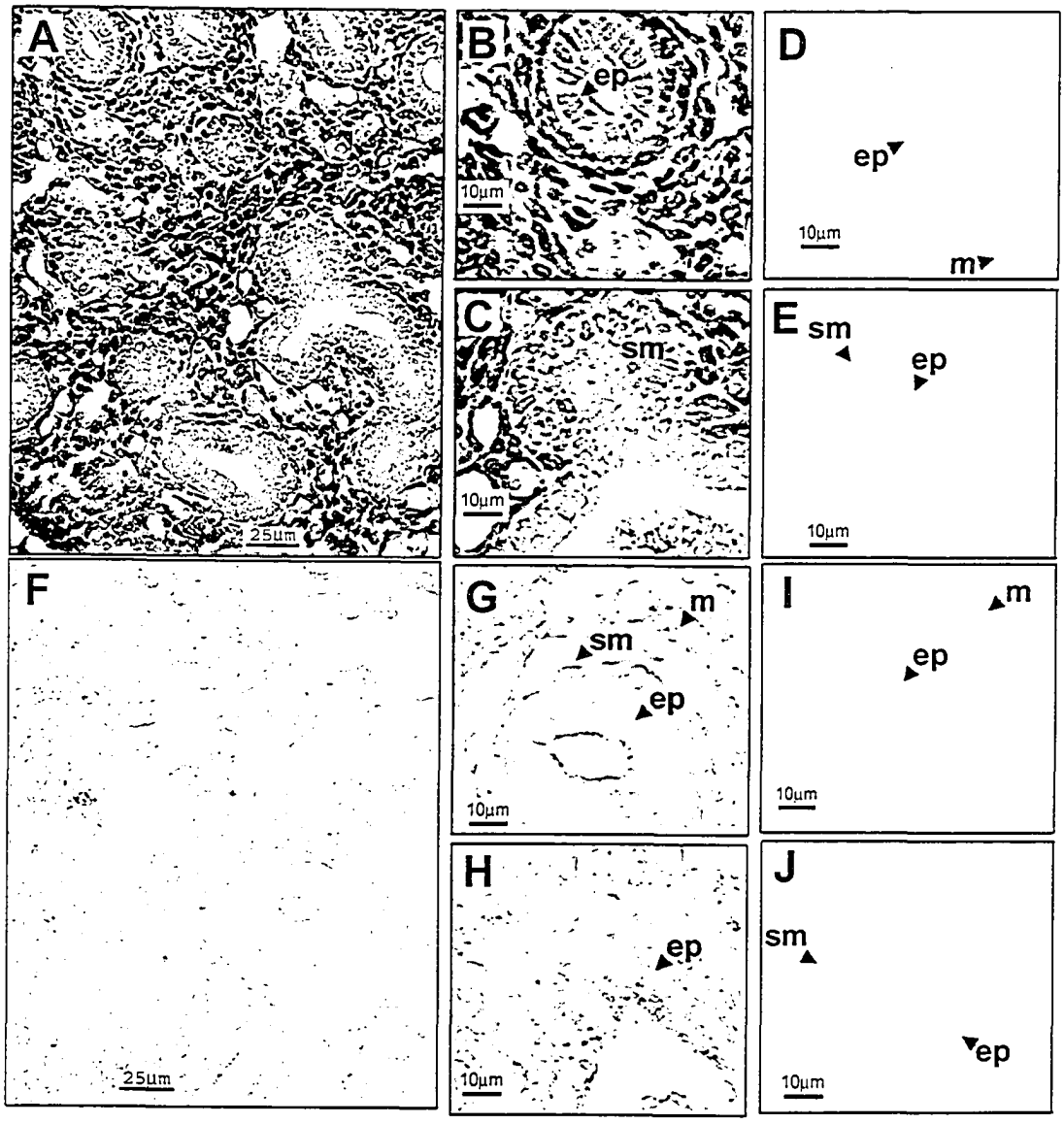


Figure 3.4. *In situ* hybridization (A-C) of rGH mRNA in saccular (D2) rat lung using full-length rGH RNA probes. Using the full-length rGH antisense probe, staining is present in the D2 postnatal lung (A), specifically in the type I (I), type II (II), and pulmonary tissue macrophage (p) cells (B). Staining in these cell types is absent (C) upon use of the full-length rGH sense probe. Immunohistochemistry (D-F) of rGH in saccular (D2) rat lung using a specific antibody against rGH. Staining is present in the D2 postnatal lung (D), specifically in the type I (I), type II (II), and pulmonary tissue macrophage (p) cells (E). Staining is lost (F) upon preabsorption of the antibody with the specific blocking peptide. Representative sections are shown of at least 4 pups.

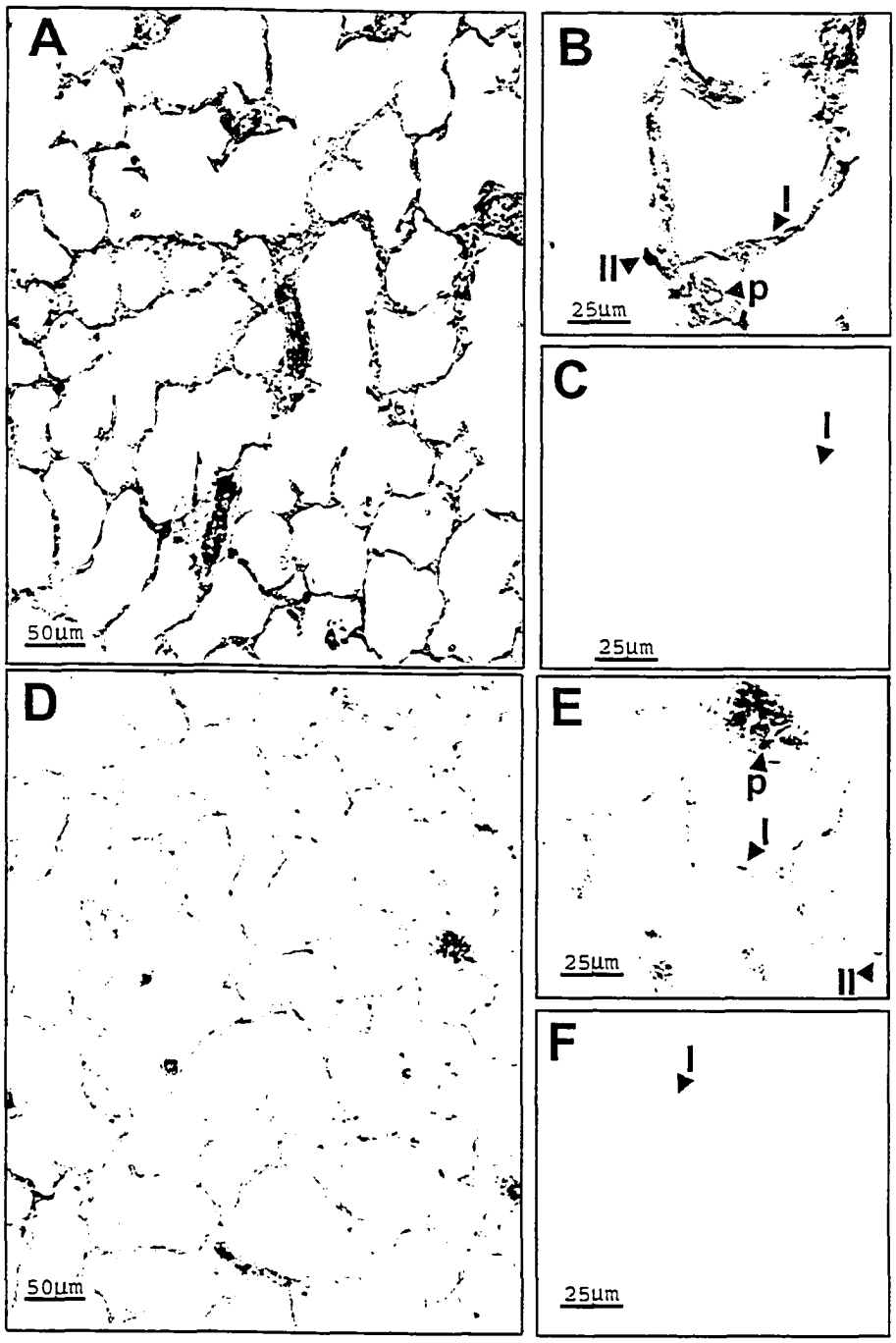


Figure 3.5. *In situ* hybridization (A-C) of rGH mRNA in early-alveolariztion (D6) rat lung using full-length rGH RNA probes. Using the full-length rGH antisense probe, staining is present in the D6 postnatal lung (A), specifically in the type I (I), and type II (II) cells (B). Staining in these cell types is absent (C) upon use of the full-length rGH sense probe. Immunohistochemistry (D-F) of rGH in early-alveolarization (D6) rat lung using a specific antibody against rGH. Staining is present in the D6 postnatal lung (D), specifically in the type I (I), type II (II), and alveolar macrophage (a) cells (E). Staining is lost (F) upon preabsorption of the antibody with the specific blocking peptide. Representative sections are shown from at least 4 pups.

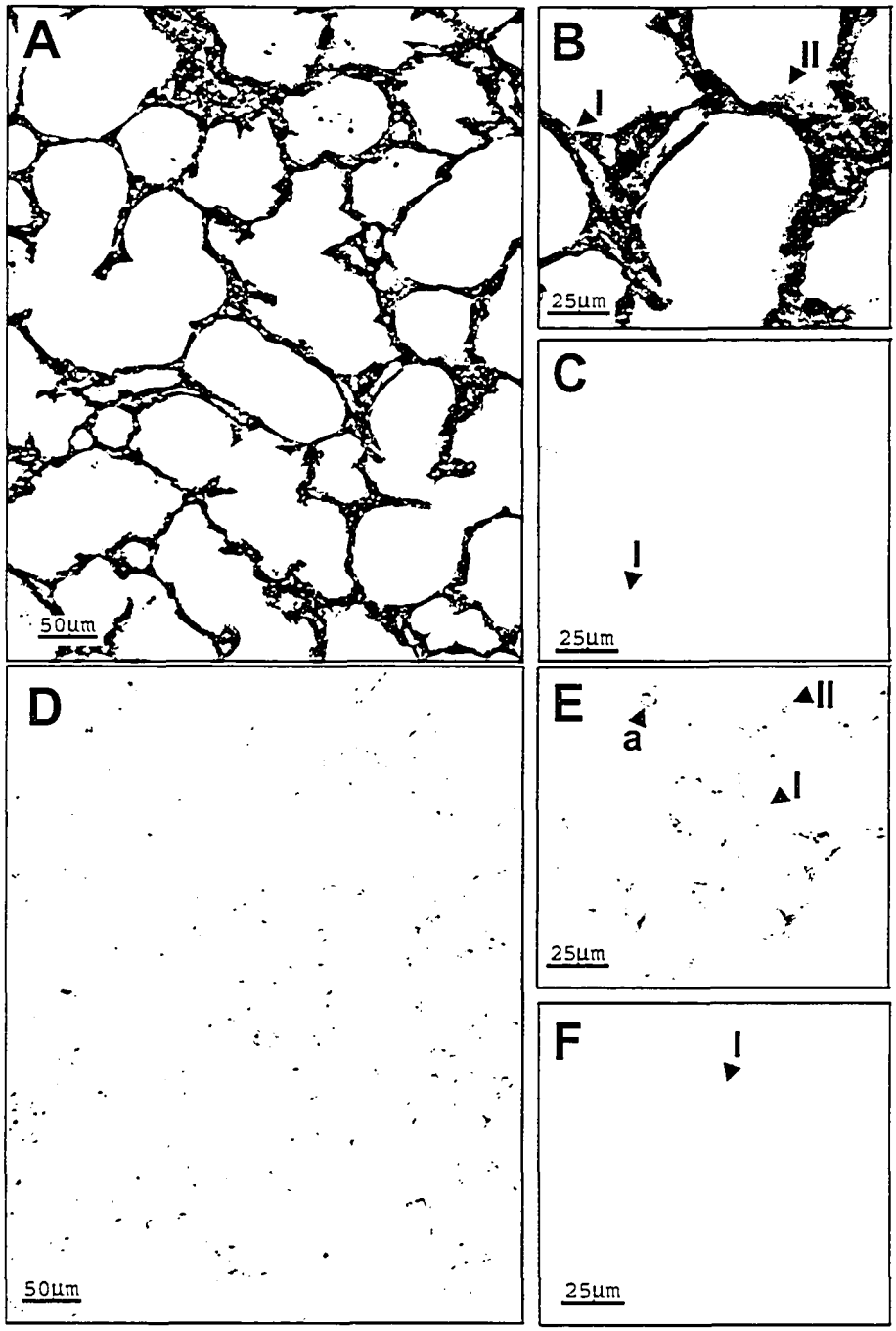


Figure 3.6. *In situ* hybridization (A-C) of rGH mRNA in mid-alveolarization (D9) rat lung using full-length rGH RNA probes. Using the full-length rGH antisense probe, staining is present in the D9 postnatal lung (A), specifically in the type I (I) and type II (II) cells (B). Staining in these cell types is absent (C) upon use of the full-length rGH sense probe. Immunohistochemistry (D-F) of rGH in mid-alveolarization (D9) rat lung using a specific antibody against rGH. Staining is present in the D9 postnatal lung (D), specifically in the type I (I), type II (II), and alveolar macrophage (a) cells (E). Staining is lost (F) upon preabsorption of the antibody with the specific blocking peptide. Representative sections are shown from at least 4 pups.

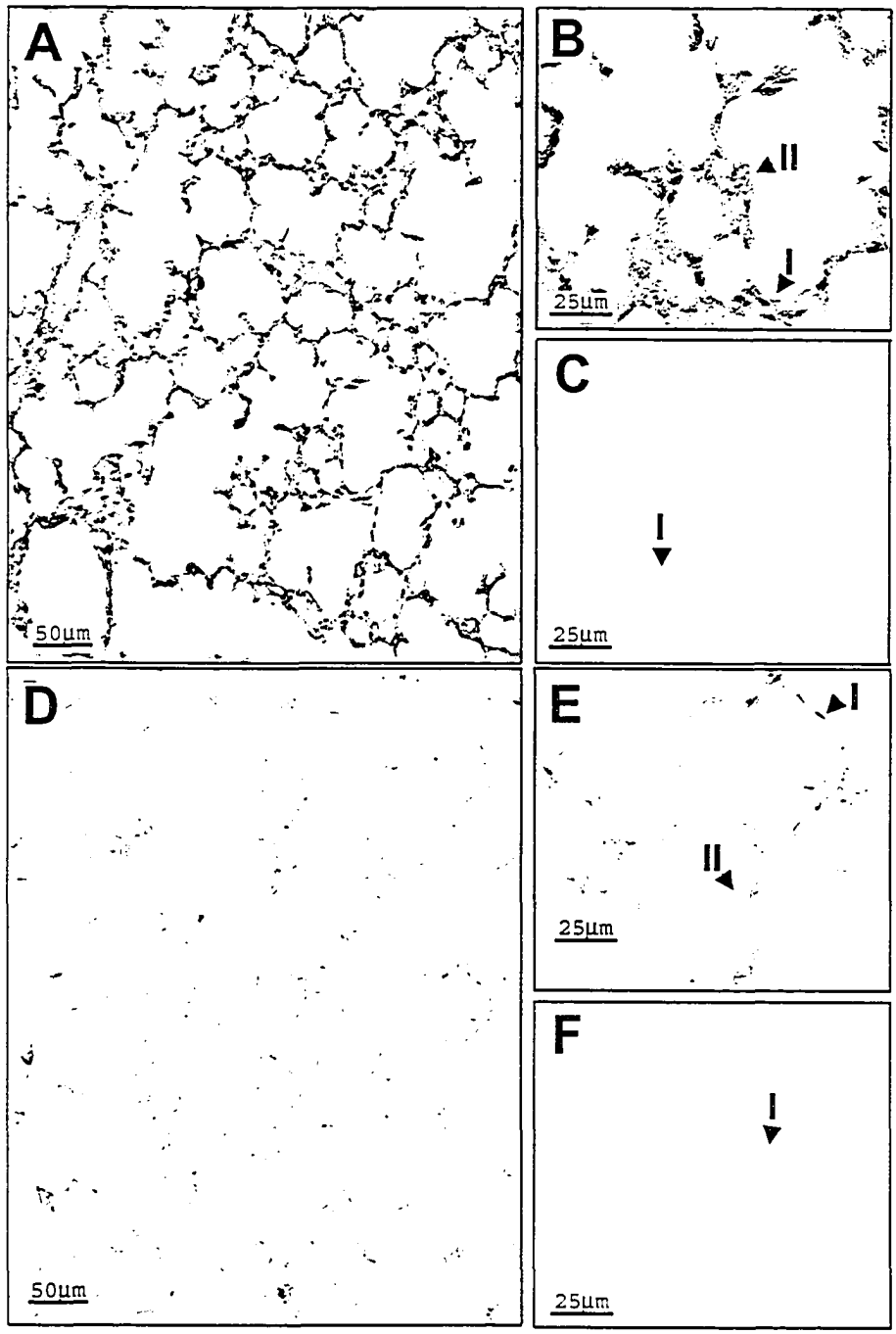


Figure 3.7. *In situ* hybridization (A-C) of rGH mRNA in late-alveolarization (D14) rat lung using full-length rGH RNA probes. Using the full-length rGH antisense probe, staining is present in the D14 postnatal lung (A), specifically in the type I (I), type II (II), and alveolar macrophage (a) cells (B). Staining in these cell types is absent (C) upon use of the full-length rGH sense probe. Immunohistochemistry (D-F) of rGH in late-alveolarization (D14) rat lung using a specific antibody against rGH. Staining is present in the D14 postnatal lung (D), specifically in the type I (I), type II (II), and alveolar macrophage (a) cells (E). Staining is lost (F) upon preabsorption of the antibody with the specific blocking peptide. Representative sections are shown from at least 4 pups.

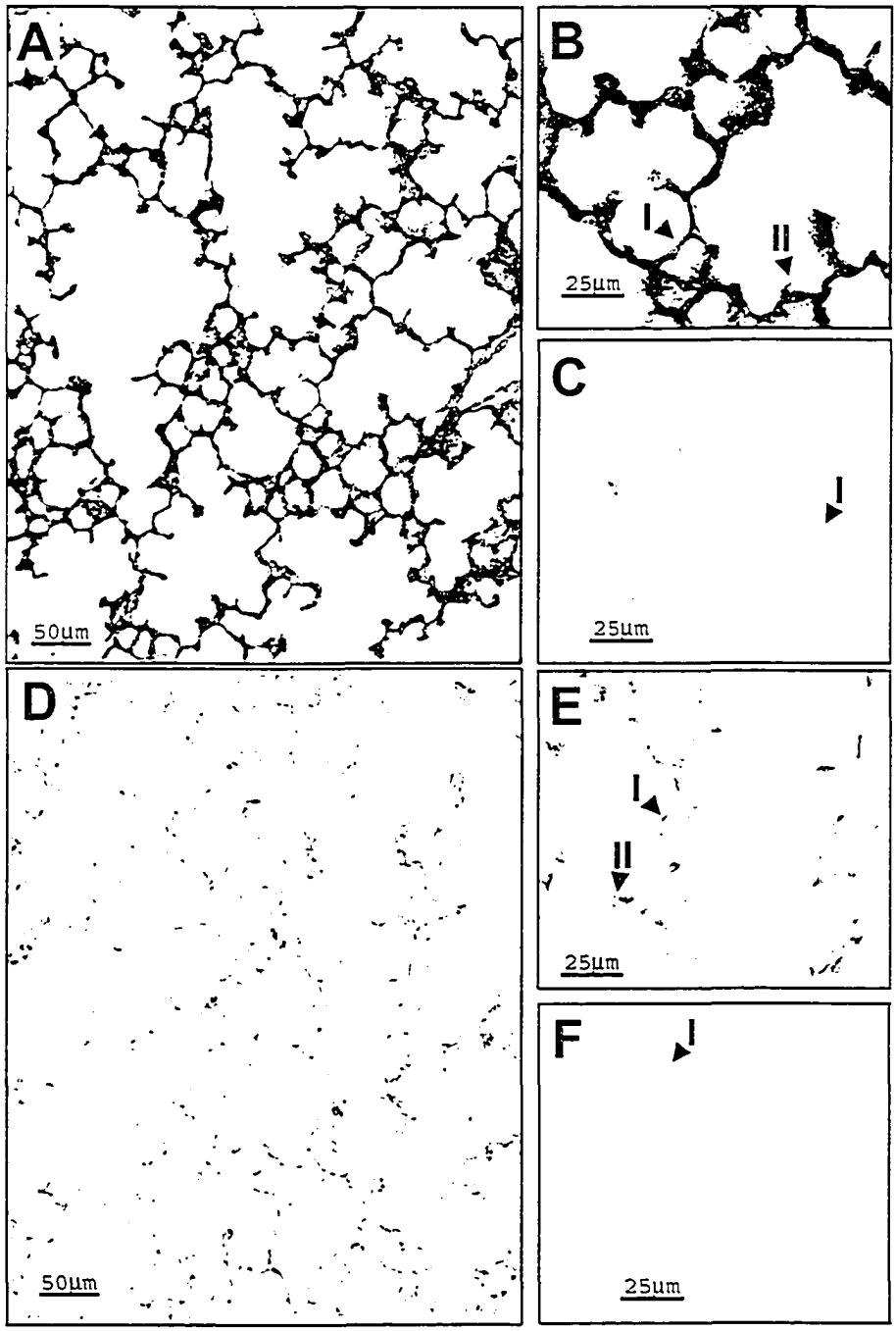
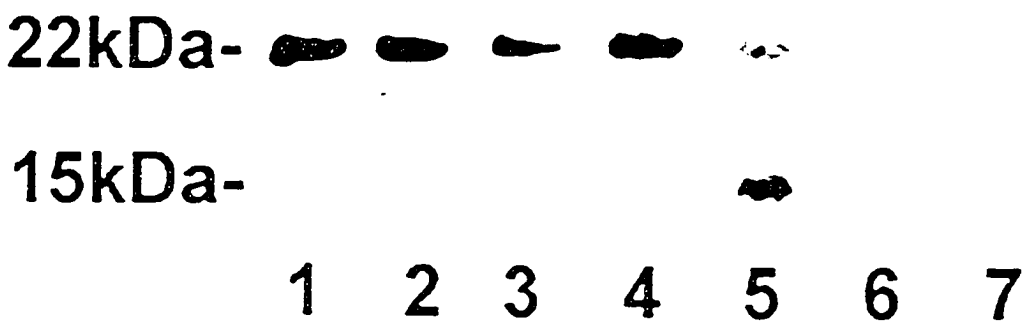


Figure 3.8. Western blotting for rat GH-immunoreactivity. An immunoreactive band of 22kDa is seen in the adult pituitary (lane 1), postnatal day 14 lung (lanes 2-4), and in adult heart (lane 5), in which an additional immunoreactive 15 kDa protein is also present. In contrast, no immunoreactivity is seen in adult skeletal muscle (lane 6), or adult liver (lane7). Representative of at least 5 gels.



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PART II: FUNCTIONAL STUDIES

CHAPTER 4

PROTEIN CHANGES IN THE RAT LUNG FOLLOWING ANTISENSE SUPPRESSION OF LUNG GROWTH HORMONE

CHAPTER 4: PROTEIN CHANGES IN THE RAT LUNG FOLLOWING ANTISENSE SUPPRESSION OF LUNG GROWTH HORMONE

4.1 INTRODUCTION

Growth hormone (GH) is principally produced in the pituitary gland, although GH mRNA has been demonstrated in numerous extrapituitary sites, in which it may exert autocrine or paracrine actions (Harvey and Hull, 1997; Waters et al., 1999). For instance, in recent years the lung has been identified as a site of GH production, since GH and GH mRNA have been detected in alveolar macrophages (Allen et al., 2000; Beyea et al., 2005), in Type I and Type II epithelial cells and in mesenchymal cells (Beyea et al., 2005) during the period of lung alveolarization. As GH receptor (GHR) and GHR mRNA are also expressed in these cells (Garcia-Aragon et al., 1992), GH may act as an autocrine or paracrine during lung development, especially as exogenous GH has been shown to induce lung function (Batchelor et al., 1998; Liu et al., 2002a; Liu et al., 2002b). This possibility was therefore examined in the present study in which antisense GH oligodeoxynucleotides (ODNs) were used to knock down GH gene expression in the lung and a proteomic approach was used to determine changes in lung function.

4.2 MATERIALS AND METHODS

Animals

Sprague-Dawley albino rat pups (Charles river Laboratories, St. Constant, Quebec, Canada) of both sexes were used. The pregnant dams were housed in the Health

Sciences Laboratory Animal Service Department of the University of the Alberta under veterinary supervision. Dams were maintained on regular rodent pellets and water ad libitum and were kept on a 12:12-h light-dark cycle. Pups were sacrificed by pentobarbital overdose through intraperitoneal injection at postnatal day (D) 14, at the end of the alveolarization period, after dynamic changes in lung structure and function have occurred. We chose the period of lung alveolarization to administer the antisense GH because it is the period of lung maturation (Copland and Post, 2004), that involves rapid structural, functional, and biochemical development. During such a critical time the likelihood of altering the expression of lung proteins should be optimal. The animal protocol was approved by the University of Alberta's Animal Policy and Welfare Committee in accordance with guidelines of the Canadian Council of Animal Care.

Reagents

Anti-rat GH and control scrambled ODN's were prepared by Sigma Genosys (Sigma-Genosys, The Woodlands, Texas, USA). 1,2-Dioleoyl-3 trimethylammonium-propane (DOTAP) and dioleoylphosphatidyl-ethanol-amine (DOPE) phospholipids were purchased from Avanti Polar Lipids (Avanti Polar Lipids, Alabaster, Alabama, USA). Culture media (F12K Nutrient Mixture (Kaighn's Modification, 1X), fetal bovine serum (FBS) and antibiotic/antimycotic (100X) reagents were purchased from Gibco (Invitrogen Canada Inc., Burlington, Ontario, Canada). Rat lung epithelial L2 cells (Type II Epithelial Cells) (ATCC Number CCL-149) were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Oligonucleotide primers were synthesized by the DNA COR Laboratory, Department of Biochemistry,

University of Alberta. Platinum Taq DNA polymerase was purchased from Invitrogen. SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, California, USA). OCTEIA Rat/Mouse IGF-1 ELISA Kit was purchased from GroPep (GroPep Limited, Thebarton, South Australia, Australia). All reagents for 2-dimensional gel electrophoresis were purchased from Bio-Rad (Life Science Research Division, Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada). Vector Hematoxylin QS stain was purchased from Vector (Vector Laboratories, Burlingame, California, USA).

Antisense Rat GH and Scrambled ODN's

20mer phosphorothioate ODN's were designed according to specifications of Agrawal (1999). The antisense directed against the rat GH mRNA (5'-GAGAGTCTGCAGCCATCGCC-3', and the scrambled ODN control (5'-AAGGATACCTAGGACCCGCC-3') were synthesized from phosphorothioate bases to prolong their half life in vivo.

Preparation of Liposomes

Liposomes used in this study were prepared using a protocol modified from Legendre and Szoka (1992). One milligram DOTAP and one milligram DOPE (both in chloroform) were mixed, and the chloroform evaporated. Two milliliters of sterile saline was added to the DOTAP/DOPE phospholipids, to achieve a solution that was 1mg/ml (0.5mg DOTAP/ml and 0.5mg DOPE/ml). The liposomes were dispersed in an ultrasonic bath (for approximately 10 min) until they were approximately 200nm in

diameter. Liposome diameters were determined by dynamic light scattering using a Brookhaven (B190) particle sizer (Brookhaven Instruments, Holtsville, New York, U.S.A).

ODN/Liposome Co-Incubation

The DOTAP/DOPE liposomes are cationic, since DOTAP phospholipids have cationic heads. This positive charge permits a complex between the cationic liposomes and the anionic ODN (Stenton et al., 2000). These complexes were formed through incubation of a 2.5:1 ratio of liposome (2.5mg) to ODN (1mg) (Anti-rat GH or scrambled) for 45 min at room temperature in sterile saline (Stenton et al., 2000). For cell culture, these complexes were diluted in F12K Nutrient Mixture to a final ODN concentration of 1μM, consistent with ODN concentrations used in other studies (Baker et al., 2001). For in vivo aerosolization, the complexes were diluted in sterile saline to a final concentration of 0.05mg/ml in a final volume of 18ml, comparable with the dosage of an aerosolized anti-Syk kinase ODN used by Stenton et al., (2000).

Antisense GH-ODN in vitro

The effectiveness of the antisense GH-ODN in inhibiting GH gene expression was first determined in the rat lung epithelial L2 cell line that constitutively expresses the GH gene (Fig. 1). L2 cells were grown in 90% F12K Nutrient Mixture and 10% FBS; supplemented with antibiotic-antimycotic (100U penicillin/ml, 100:g streptomycin/ml and 25μg amphotericin B/ml), at 37°C in 5% CO_2. Cells were grown in 75cm^2 flasks, then 1 x 10^6 cells were seeded in each well of a 6-well plate. Twenty-four hours later,

the media were removed and the cells rinsed with PBS. The cells were then starved for 24h in media lacking FBS. Control (scrambled) and GH-antisense ODN/liposome complexes were then added so that the concentration of ODN was 1μM in the 2ml of media in each well. RNA was extracted from cells using Trizol (Invitrogen) 0, 3, 6, 12, 18 and 24h after the ODN/liposome complexes were added.

Antisense GH-ODN in vivo

Fourteen rat pups were evenly spaced in a sealed plastic container for the duration of the aerosolization. The liposome/ODN mixtures (18ml in both cases) were nebulized for 45 min (using a Nebulizer model 8901, Salter Labs, Vital Aire, Mississauga, Ontario, Canada) into sealed plastic containers that the rat pups. This aerosolization treatment was performed once daily, starting on postnatal day 4 and ending on postnatal day 13.

Tissue Preparation

On postnatal day 14 the pups were euthanized with an intraperitoneal injection (0.15ml) of pentobarbital sodium (Euthanyl, Bimeda-Mtc Animal Health Inc., Cambridge, ON). The lungs were removed and frozen in liquid nitrogen. RNA was extracted from frozen lungs with Trizol (Invitrogen). For protein determination the frozen lungs were homogenized in Rehydration Buffer (Bio-Rad), and quantified by Bio-Rad assay.

Validation of Liposome/ODN Delivery

To confirm delivery of the liposome/ODN complexes to the lower airways, a DOTAP/DOPE combination that contained fluorescent transfection reagent (Avanti

Polar Lipids, Alabaster, Alabama, U.S.A.) was aerosolized under identical conditions to visualize the localization of the aerosolized liposome/ODN complexes. The animals were sacrificed 6h after aerosolization, the lungs collected in liquid nitrogen, and 8μm frozen sections were subsequently obtained using a cryostat.

RT-PCR for Rat GH in L2 Cells

Total RNA was extracted from duplicate wells (in six-well plates) of L2 cells using 1ml TRIzol® Reagent (Invitrogen) using the manufacturer's instructions. Reverse transcription was performed using 3μg RNA with Superscript II® RNase H-Reverse Transcriptase (Invitrogen). The reaction was carried out according to the manufacturer's instructions. Following reverse transcription, the cDNA products were transferred to ice and the cDNAs were amplified by the polymerase chain reaction, using 1U of Platinum® Taq DNA Polymerase (Invitrogen) in the presence of 0.2:m of oligonucleotide primers designed to generate a 693 bp rat GH cDNA (Seeburg et al., 1977) (forward primer: JAB1: 5'-TGG ACA GAT CAC TGA GTG GCG-3' and reverse primer: JAB2: 5'-CGC AGA GAC ACC AGT GTG TGC-3'). PCR was performed as follows: 94°C for 1 min; then 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; followed by 72°C for 10 min. Ten microlitres of each PCR product was visualized by ethidium bromide staining in a 1.0% agarose gel.

2-Dimensional Gel Electrophoresis

Lung proteins were separated by 2-dimensional gel electrophoresis, as detailed by Sawicki et al., (2003). Protein (200μg) was applied to each 11cm IPG (linear pH

gradient range 3-10) strip (5 samples on 5 separate strips for each treatment group) (Bio-Rad), with rehydration, for 16-18h at 20°C. For isoelectric focusing, the Bio-Rad Protean IEF Cell was used with the following conditions at 20°C, using fast-voltage ramping: step 1 – 15 min with an ending voltage of 250V, step 2 – 150 min with an ending voltage of 8,000V, and step 3 – 35,000Vh (approximately 240 min). Strips were equilibrated according to the Bio-Rad protocol. The second dimension was performed with Criterion precast gels (8-16%)(Bio-Rad) in a Criterion Dodeca Cell (Bio-Rad). Upon completion of the second dimension, the proteins were stained with Coomassie blue. In order to minimize variation in staining between gels, all 10 gels were stained simultaneously in the same bath. The reproducibility of the protein resolution using this protocol is very high (intra-assay and inter-assay coefficients of variation of approximately 5 and 10%, respectively), as detailed previously (Sawicki et al., 2003; Sawicki and Jugdutt, 2004). In our laboratory, the intensity of staining for a single protein when run on separate gels differs by <5% and the correlation coefficient (r^2) for the staining intensities of multiple protein samples measured on different gels is >0.97 (Sawicki et al., 2003). This procedure therefore obviates the need for replicate determinations, as previously established (Sawicki et al., 2003; Sawicki and Jugdutt, 2004; Jugdutt and Sawicki, 2004; Sawicki et al., 2004). Developed gels were scanned with a GS-800 calibrated densitometer (Bio-Rad). Image analysis and mass spectrometry were performed according to Sawicki and Jugdutt, (2004). Mass spectrometry and identification of protein spots was performed by the Institute for Biomolecular Design, University of Alberta.

IGF-1 ELISA

IGF-1 levels were evaluated, since IGF-1 is a measure of GH action (Le Roith et al., 2001) and its turnover is less dynamic than that of GH itself (Veldhuis et al., 1993; Gillespi et al., 1996). A rat/mouse IGF-1 ELISA kit (GroPep Limited, Adelaide, South Australia, Australia) was used to determine immunoreactive IGF-1 concentrations in the protein extracts. Samples (n=4) were run in duplicate using 300μg protein/sample, according to manufacturer's instructions. Briefly, samples were diluted in dilution buffer. 25μl of diluted sample was added to the plate, then 100μl of anti-rat IGF-1 Biotin was added to each well of the plate. The plate was then incubated at room temperature for 2h while shaking, washed and 200μl of Enzyme Conjugate was then added to each well. The plate was then incubated at room temperature for 30 min, washed and 200μl of TMB substrate was then added to each well. The plates were then incubated for 20 min at room temperature, after which 100μl of stop solution was added to each well. The plates were then read at 450nm (with reference at 650nm).

4.3 RESULTS

Antisense GH ODN in Rat Lung Epithelial L2 Cells

Upon incubation of rat lung epithelial L2 Cells with the antisense GH ODN, expression of the rGH gene was completely eliminated within 12h, and remained suppressed at 24h (Fig. 4.1), whereas expression of rGH in the control (scrambled ODN) group did not change over the course of the experiment.

Validation of Liposome/ODN Delivery

6h after the aerosolization of the liposome/ODN complex, the fluorescent transfection reagent was widespread within lung alveolar cells. Representative sections from upper, middle and lower sections of the caudal lobe are shown in Fig. 4.2. This confirms results obtained using aerosolized methylene blue in adult rats by Stenton et al., (2000).

2-Dimensional Gel Electrophoresis

Representative gels are shown in Fig. 4.3A, B. Analysis of the 2D gels showed that 45 proteins in the lungs of the antisense GH-ODN treated rats changed in concentration ($P<0.05$) relative to the corresponding proteins in the control group (35 proteins were increased, 10 proteins were decreased in concentration). Eleven of these proteins had highly significant ($P<0.01$) increases in relative content and, of these, eight were present at intensities on the gels that permitted their identification by mass spectrophotometry. These proteins were the alpha and beta subunits of ATP synthase and electron transfer flavoprotein, albumin, calcyclin binding, protein, superoxide dismutase 2, and RNA binding protein motif 3. Relative quantities of these proteins are given in Table 4.1.

IGF-1 ELISA

24h after the final antisense GH ODN administration, immunoreactive IGF-1 levels in the lung were decreased ($P<0.05$) by >50% (Fig. 4.4).

4.4 DISCUSSION

The results of this study demonstrate actions of endogenous GH in the rat lung that suggest it has functional autocrine and paracrine roles during the alveolarization period. The local production of GH in the nervous system (Harvey and Hull, 2003), immune system (Weigent et al., 1991; Sabharwal and Varma, 1996; Arnold and Weigent 2004), reproductive system (Hull and Harvey, 2000; Luna et al., 2004), mammary glands (van Garderen et al., 1997) and teeth (Zhang et al., 1997) is similarly thought to induce paracrine or autocrine actions involved in tissue development or differentiation.

It is now well established that the lung is a target site for the GH action, since GHR expression occurs in fetal and adult lungs (Garcia-Aragon et al., 1992; Batchelor et al., 1998; Tiong et al., 1989). Moreover, a role for GH in pulmonary function is indicated by physiological and anatomical changes in the lung in pathophysiological states of pituitary GH excess and deficiency. For instance, large lungs (Bartlett 1971), upper airflow obstruction (Trotman-Dickenson et al., 1991) and narrowing of the small airways (Harrison et al., 1978) accompany GH excess, whereas a decrease in muscle strength and a reduction in the maximum inspiratory and expiratory pressure (Merola et al., 1995; Merola et al., 1996) is associated with pituitary GH deficiency. Exogenous GH has also been shown to induce superoxide production by alveolar macrophages (Edwards et al., 1992), to activate lung neutrophils during sepsis (Liu et al., 2002a), to induce NF_KB activation (Liu et al., 2002b) and to increase phosphorylase A phosphorylation of specific (but unidentified) proteins in lung epithelial cells (Batchelor et al., 1998). Exogenous GH also increases the production of IGF-binding protein (IGF-BP)-2 mRNA and the binding of IGF-1 to IGF-BP-2 lung epithelial cells (Batchelor et

al., 1998). It is therefore possible that these actions also reflect local actions of GH produced in the lung.

The presence of GH, in trace amounts, in the mammalian lung was first demonstrated by Kyle et al. (1981) and subsequently confirmed by Costa et al. (1993). It has also been detected in the chicken lung (Harvey et al., 2000). More recent studies have localized GH and GH mRNA within most epithelial and mesenchymal cells of neonatal rat lungs during the period of alveolarization (Beyea et al., 2005). The expression of the GH gene in the rat lung was confirmed in the present study, in which GH expression was also demonstrated in a rat lung epithelial cell line (L2 cells). As GHRs are also present in rat lung epithelial and mesenchymal cells (Garcia-Aragon et al., 1992), GH may act as an autocrine or paracrine factor during lung alveolarization. This possibility is supported in the present study by the biochemical consequences following the inhalation of an aerosolized GH ODN.

Antisense GH transgenic expression is an effective approach to block pituitary GH production and to induce dwarfism (Matsumoto et al., 1995; Shimokawa et al., 2003). Antisense deoxynucleotides to GH have also been shown to inhibit the production of GH by rat lymphocytes in vitro and to block autocrine or paracrine actions of lymphocyte GH on cell proliferation (Weigent et al., 1991) and cell survival (Arnold and Weigent, 2004). The results of the present study show that GH expression by rat lung epithelial cells in vitro is completely blocked by an antisense GH ODN. The same antisense GH ODN was aerosolized and inhaled by neonatal rats. This provides a unique experimental model to assess putative autocrine/paracrine actions of GH in the

lung. Stenton et al., (2000) used a similar experimental approach to down-regulate expression of Syk kinase in the alveolar macrophages of the lung.

At least 45 proteins (out of more than 200 detected by 2D gel electrophoresis) in the lungs of the rats treated with the aerosolized antisense GH ODN had altered concentrations at the end of the treatment period. This suggests the transcription or translation of the genes coding for these proteins are normally under GH-inhibition or GH-stimulation.

GH responsive genes have been identified (Flores-Morales et al., 2001), including genes involved in the regulation of metabolism, signal transduction, transcription, protein turnover, transport, detoxification, cell structure or replication. Numerous other GH-responsive genes have also been identified in the rat liver (Thompson et al., 2000), including proto-oncogenes (Triest et al., 1995; Yoon et al., 1990), protease inhibitors (Yoon et al., 1997; Warren et al., 1993) and steroid hydroxylases (Wells et al., 1994; Subramanian et al., 1995). Of these, it is of interest that the genes for ATP synthase (Flores-Morales et al., 2001; Tollet-Egnell et al., 2000) and albumin (Flores-Morales et al., 2001) were found to be GH- regulated, since ATP synthase and albumin levels were similarly increased in the lungs of our antisense GH-ODN treated rats.

In addition to these proteins, electron transfer flavoprotein (a metabolic enzyme), calcyclin binding protein (possibly involved in cell cycle regulation), manganese superoxide dismutase 2 (involved in free radical destruction) and RNA binding protein motif 3 (involved in gene transcription) were also upregulated in the lung by antisense GH ODN administration. The significance of these findings is

uncertain, since they have not previously been identified as GH-responsive proteins. Calcyclin binding protein mRNA has, however, been detected in the lung (Jastrzebska et al., 2000) and calcyclin, a protein associated with cell differentiation, is a regulator of the cell cycle in lung fibroblasts (Breen et al., 1999). It is therefore possible that GH participates in cell cycle regulation in the lung.

In contrast to the proteins upregulated by antisense GH-ODN administration, the lung concentration of IGF-1 was reduced. This result was expected, since IGF-1 is a well established GH-response gene (Le Roith et al., 2001). As IGF-1 is thought to have roles in angiogenesis and vasculogenesis in the lung (Han et al., 2003), lung GH may be therefore be an upstream regulator of these processes during development.

Autocrine or paracrine actions of GH upregulating or downregulating numerous genes have also been demonstrated in mammary carcinoma cells stably transfected with the GH gene, in which GHR blockade abrogates GH-induced transcriptional activation or suppression and autocrine/paracrine actions of GH inducing cell proliferation, differentiation and cell spreading (Kaulsay et al., 1999; Kaulsay et al., 2000; Liu et al., 1997; Graichen et al., 2002; Mertani et al., 2001). The immunoneutralization of endogenous GH has similarly established autocrine/paracrine actions of GH in mouse blastocysts (Markham and Kaye, 2003), in thymic epithelial cells (Sabharwal and Varma, 1996), and in the Wolffian duct during fetal development (Nguyen et al., 1996). Autocrine or paracrine actions of GH in many tissues are therefore well established.

In summary, these results demonstrate biochemical changes in the rat lung during alveolarization following antisense-ODN administration. Furthermore, they support the use of the Stenton et al., (2000) model for downregulation of lung gene expression, and

propose 2D gel electrophoresis as a means for detection of changes in multiple lung proteins in response to a given treatment. These results suggest that autocrine or paracrine actions of GH are involved in early lung development.

Table 4.1 – Lung Proteins Responsive to Treatment with Anti-rGH Antisense Oligodeoxynucleotides (ODN's) .

Proteins[a]	Scrambled ODN	Anti-rGH ODN
1. ATP Synthase (β-subunit)	†100.00±9.09	*145.45±9.09
2. Albumin	100.00±22.88	*211.54±24.51
3. Calcyclin Binding Protein	100.00±10.46	*202.70±22.52
4. Electron Transfer Flavoprotein (β-subunit)	100.00±17.50	*157.14±14.29
5. ATP Synthase (α-subunit)	100.00±14.71	*201.73±35.30
6. Manganese Superoxide Dismutase 2	100.00±4.00	*132.5±6.05
7. RNA Binding Protein Motif 3	100.00±9.42	*184.62±22.43
8. Electron Transfer Flavoprotein (α-subunit)	100.00±9.54	*160.70±27.64

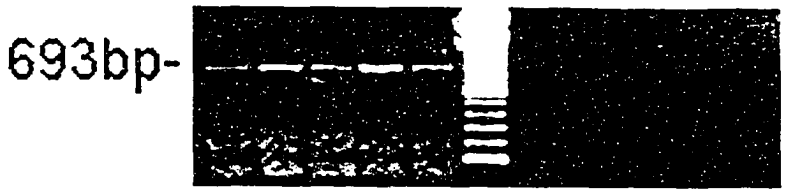
[a]Identified by 2D gel electrophoresis and mass spectrophotometry.

†All values are given as percent of control (Scrambled ODN). Mean±standard error; n=5 for each of the two treatment groups.

*Significantly different from control at $p<0.01$ (Student's t-test).

Figure 4.1. Detection of cDNA from rGH mRNA by RT-PCR in L2 epithelial type II cell culture at 12 (A), 18 (B), and 24 (C) hours following treatment with scrambled or anti-rat GH ODN. rGH mRNA (band=693bp) expression is unaltered by treatment with scrambled ODN (lanes 1-5 of A, B, and C). rGH mRNA expression is absent in anti-rGH ODN treated cells (lanes 7-11 of A, B, and C). 100bp ladder is shown on all gels (lane 6 of A, B, and C).

A



1 2 3 4 5 6 7 8 9 10 11

Scrambled Anti-rGH

B

1 2 3 4 5 6 7 8 9 10 11

Scrambled Anti-rGH

C

1 2 3 4 5 6 7 8 9 10 11

Scrambled Anti-rGH

Figure 4.2. Detection of fluorescent labeled liposomes 6hr after aerosolization. Liposomes only (A) are shown on a slide. These liposomes are detected in the upper (B), mid (C) and lower (D) caudal lobe. Selected alveoli (alv) are denoted.

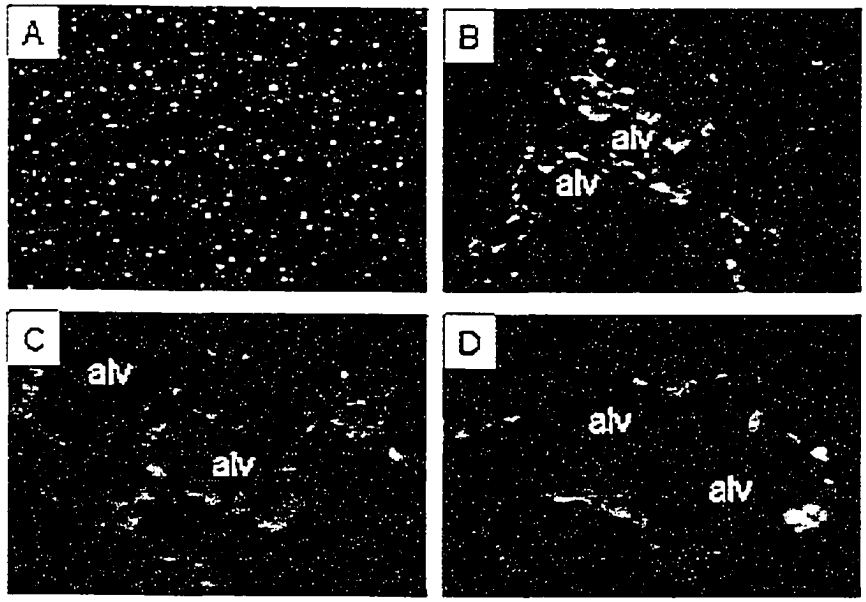


Figure 4.3. Representative 2D gel electrophoresis of whole lung homogenate for scrambled control treated (A) and anti-rGH treated (B) rats at postnatal day fourteen. Numbers (B) indicate proteins whose levels have changed in the anti-rGH group relative to the control. These numbers correspond to the numbers of the eight proteins in Table 4.1.

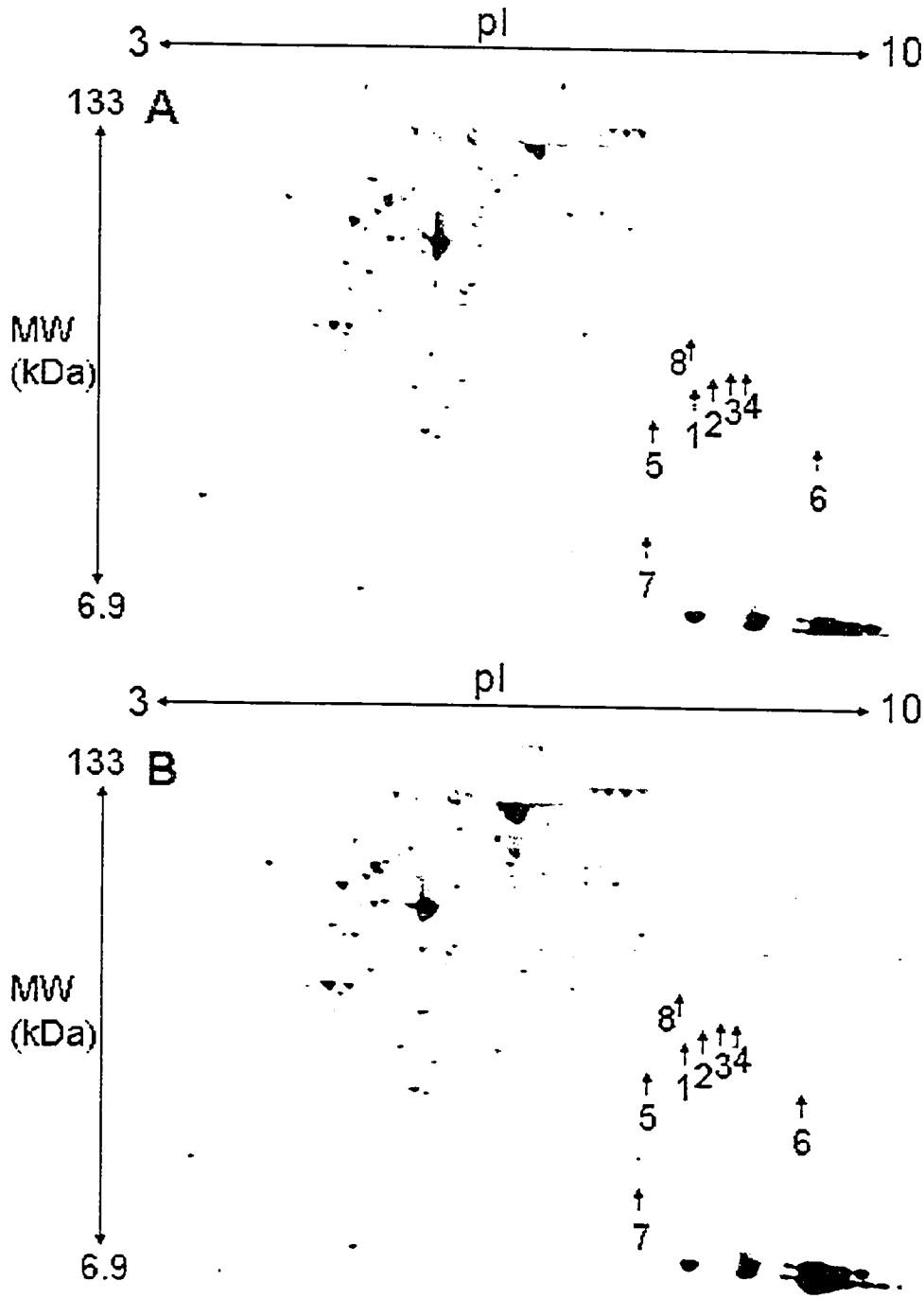
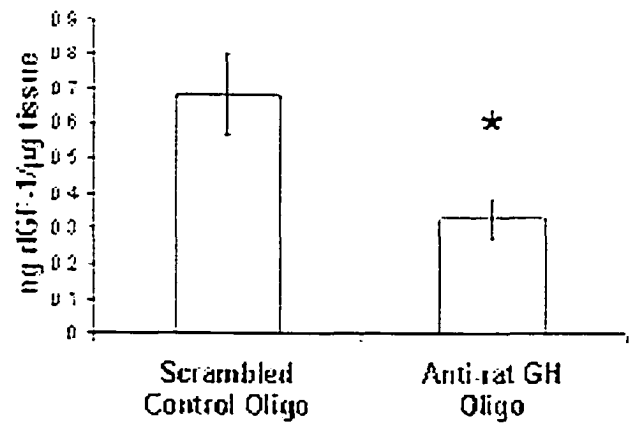


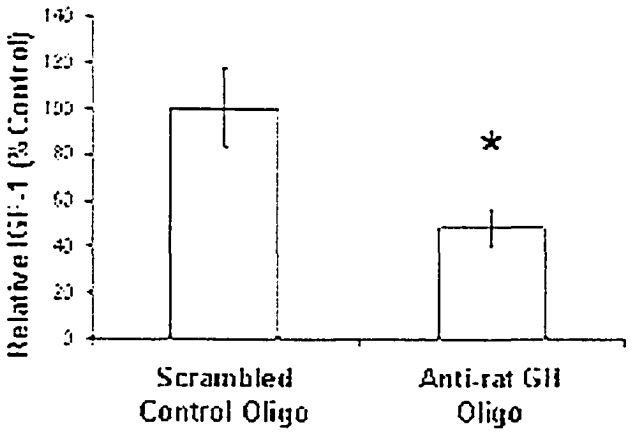
Figure 4.4. IGF-1 absolute levels (A) and percent control (B) in scrambled and anti-rGH treated postnatal day 14 lungs. Asterisks indicate statistical differences ($p<0.05$, student's t-test) between groups. Means ± SEM's (n=4).

A



*p<0.05

B



*p<0.05

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CHAPTER 5

GROWTH HORMONE (GH) ACTION IN THE DEVELOPING LUNG: CHANGES IN LUNG PROTEINS FOLLOWING ADENOVIRAL GH OVEREXPRESSION

CHAPTER 5: GROWTH HORMONE (GH) ACTION IN THE DEVELOPING LUNG: CHANGES IN LUNG PROTEINS FOLLOWING ADENOVIRAL GH OVEREXPRESSION[1]

5.1 INTRODUCTION

The lung is a target site of growth hormone (GH) action. GH receptors (GHRs) are present in the lung (Tiong et al., 1989; Garcia-Aragon et al., 1992; Batchelor et al., 1998) and exogenous GH induces the production of superoxide (Edwards et al., 1982), increases the activity of NFκB (Liu et al., 2002b) and phosphorylase A (Jost et al., 1979) and stimulates the tyrosine phosphorylation of specific, but unidentified, proteins in lung epithelial cells (Batchelor et al., 1998). Pathological changes in pituitary GH secretion also result in changes in lung function, since large lungs (Bartlett 1971), upper airflow obstruction (Trotman-Dickenson et al., 1991) and small airway narrowing (Harrison et al., 1978) accompany acromegaly, whilst a decrease in muscle strength and a reduction in maximum inspiratory and expiratory pressure accompany GH deficiency (Merola et al., 1995, 1996). The pituitary gland is not, however, the only site of GH gene expression and the lung may not only be responsive to GH in peripheral cir[1]culation, but to GH produced locally as well.

It is now well established that GH is produced in many tissues in which it may have local autocrine/paracrine actions rather than endocrine roles (Harvey and Hull, 1997; Fukaya et al., 1998; Waters et al., 1999). Indeed, GH and GH mRNA are present in most mesenchymal and epithelial cells of the neonatal rat lung during the period of

[1]A version of this chapter has been accepted for publication: Beyea, J.A., Olson, D.M., and Harvey, S. Dev Dyn

alveolarization (days 4-14; Beyea et al., 2005). The possible functional importance of lung GH in lung function has therefore been further assessed in the present study, in which overexpression of GH in the neonatal lung during the alveolarization period has been induced by the administration of an aerosolized GH-expressing adenovirus. If GH has local roles in lung function, we hypothesized that overexpression of GH in the lung would alter the production of GH-responsive proteins in this tissue.

5.2 MATERIALS AND METHODS

Animals

Sprague-Dawley albino rat pups (Charles River Laboratories, St. Constant, Quebec, Canada) of both sexes were used. The pregnant dams were housed in the Health Sciences Laboratory Animal Service Department of the University of Alberta under veterinary supervision. Dams were maintained on regular rodent pellets and water *ad libitum* and were kept on a 12:12-h light-dark cycle. Pups were sacrificed by pentobarbital overdose through intraperitoneal injection at postnatal day (D) 14. The animal protocol was approved by the University of Alberta's Animal Policy and Welfare Committee in accordance with guidelines of the Canadian Council of Animal Care.

Mouse GH Adenovirus

The mouse GH adenovirus (AdV) which was used in these studies was generously provided by Dr. Bruce J. Baum (National Institute of Dental and Craniofacial Research, NIH, Bethesda, Maryland). This vector (AdCMVmGH) contains the cytomegalovirus (CMV) promoter-enhancer and is replication deficient. It is well established that this vector is expressed translated and biologically active in rat epithelial cells *in vitro* and *in*

vivo in rats and mice (Marmary et al., 1999). An adenovirus expressing Green Fluorescent Protein (AdGFP) (Sambandam et al., 2004) was used as a control.

Expression of Mouse GH AdV in Rat Lung Epithelial L2 Cells in Vitro

In preliminary studies, the expression of the mouse GH AdV was determined *in vitro* in the rat lung epithelial Type II L2 cell line (American Type Culture Collection, Manassas, Virginia, USA). L2 cells were grown in 90% F12K Nutrient Mixture (Gibco, Mississauga, Ontario, Canada) with 10% FBS (fetal bovine serum, Gibco) and antibiotic-antimycotic (Gibco; 100U penicillin/ml, 100µg streptomycin/ml, and 0.25µg amphotericin B/ml), at 37°C in 5% CO_2. Cells were grown in 75cm^2 flasks, then 1.67 x 10^6 cells were seeded in each well of a 6-well plate. Twenty-four hours later, the media was removed and the cells rinsed with PBS. The cells were then starved of FBS for 24h prior to infection with the mGH Adv or its control, expressing green fluorescent protein. Infection was achieved with 30 copies of the viruses (2.5 x 10^7 pfu/ml in 2ml media) per cell. RNA was then extracted from the cells using Trizol (Invitrogen Canada Inc., Burlington, Ontario, Canada) before, 24, 48 and 72h after the mouse GH AdV was added, to ensure its stable expression.

Expression of Mouse GH AdV In Vivo

Under halothane anaesthesia, 4d-old rat pups (at the beginning of the alveolarization period) were injected intratrachealy (using a 22 gauge needle) with 25ul of 1.8 x 10^{10} pfu/ml of the mGH adenovirus or its GFP control. This procedure has been shown to effectively restrict adenoviral gene expression to the lungs (Kanaan et al., 2002; Pozeg et al., 2003; Chicoine et al., 2004). The rats were sutured with 3-0 Dexon II suture, and

then were allowed to recover under a lamp. No adverse effects were experienced and once the rats had regained consciousness, they were returned to their mothers. Adenoviral delivery of genes directly to the lungs by this technique has been shown not to affect mortality, lung toxicity or inflammation of the lungs (Kanaan et al., 2002; Pozeg et al., 2003). The pups were sacrificed when they were 14 days of age, at the end of the alveolarization period, at a time when the lung expresses GH in Type I and Type II epithelial cells (Beyea et al., 2005). The lungs were removed from the rats and frozen in liquid nitrogen. For protein determination, some of the lung tissue was homogenized in Rehydrated Buffer (Bio-Rad Life Science Research Division, Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada), and quantified by Bio-Rad protein assay. For GFP immunofluorescence studies, the 14 day-old pups were euthanized with an intraperitoneal injection of 150ul of pentobarbital sodium (Euthanyl, Bimeda-Mtc Inc, Cambridge, ON). A midline laparotomy was performed in the upper abdomen and the internal organs were moved to reveal the diaphragm, which was punctured to create a bilateral pneumothorax. An incision was made to expose the trachea; a slit was made on its ventral side, and a small catheter was inserted and tied to allow 4% paraformaldehyde to perfuse the tissue for 5 min at 20 cm H_2O. Perfusions were made to a closed chest so the lungs would inflate to a natural state. After 5 min the catheter was removed, the trachea was tied off, the rib cage was carefully opened, and the lungs removed, fixed with paraformaldehyde, and processed as in Beyea et al., (2005). Slides were examined for GFP fluorescence. Green fluorescence was viewed using a blue exciter filter BP 460 - 490 and a bandpass barrier filter BA 515 – 550. Images were captured using an Olympus Microscope System (Model BX40) (Olympus America Inc., Melville, NY, USA). Lung mass (wet weight) was determined in separate tissues by similarly removing them *en bloc*

from the control and experimental animals and blotting them dry. The lungs were then put into a slow-oven and dried until constant weight. The ratio of dry weight to wet weight was then determined.

RT-PCR for Mouse GH mRNA

Total RNA was extracted from frozen whole lungs using Trizol Reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada; 100mg/ml), according to the manufacturer's protocol. Reverse transcription was performed using 3μg RNA with Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen), and the cDNA products were transferred to ice. The cDNAs were then amplified by the polymerase chain reaction using 1U of Platinum7 *Taq* DNA Polymerase (Invitrogen) per reaction, in the presence of 0.2μM of oligonucleotide primers designed to generate a 651 bp mouse GH cDNA (Linzer and Talamantes, 1985) (forward primer: JAB3: 5'-ATG GCT ACA GAC TCT CGG ACC-3'; and reverse primer: JAB4: 5'-CTA GAA GGC ACA GCT GCT TTC-3'). These oligonucleotides are completely specific for mouse GH cDNA and do not amplify cDNA moieties in the presence of rat mRNA. PCR was performed as follows: 94°C for 1 min; then 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; followed by 72°C for 10 min. Ten microlitres of each PCR product was visualized by ethidium bromide staining in a 1.0% agarose gel.

Real-Time PCR for Mouse GH in L2 Cells

Total RNA in L2 cells in duplicate wells of the 6-well plates was extracted by the TRIzol7 Reagent (Invitrogen). Reverse transcription was performed using 3μg RNA

with Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen) and the cDNA products were transferred to ice. Real-Time PCR (n=6) was performed using 2X SYBR7 Green PCR Master Mix (Applied Biosystems, Foster City, California, U.S.A.). Primers used were JAB3 and JAB4. Cycling was as follows: 10 min at 95°C; 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; 95°C for 1 min; 55°C for 1 min; 40 cycles of temperature ramp at +0.5°C/12 sec. Threshold cycle values were compared against those obtained from a standard cDNA curve of a GFP control sample (made form 3,000ng, 1,000ng, 500ng, 200ng, 20ng of RNA). PCR products were purified by the High Pure7 PCR Product Purification Kit (Roche Diagnostics Canada, Laval, Quebec, Canada), then sequenced by the DNA Core Lab, Department of Biochemistry, University of Alberta. Sequences were analyzed by BLAST and compared with sequences in the NCBI database.

2-Dimensional Gel Electrophoresis

Lung proteins were separated by 2-dimensional gel electrophoresis, as detailed by Sawicki et al., (2003). Protein (200μg) was applied to 11cm IPG (linear pH gradient range 3-10) strips (6 samples on 6 separate strips for each treatment group) (Bio-Rad), with rehydration, for 16-18h at 20EC. For isoelectric focusing, the Bio-Rad protean IEF Cell was used with the following conditions at 20°C, using fast-voltage ramping: step 1 - 15 min with an ending voltage of 250V, step 2 -150 min with an ending voltage of 8,000V, and step 3 -35,000Vh (approximately 250 min). The strips were equilibrated according to the Bio-Rad protocol. The second dimension was performed with Criterion precise gel (8-16%) (Bio-Rad) in a Criterion Dodeca Cell (Bio-Rad). Upon completion of the second dimension, the proteins were stained with Coomassie Blue. In order to

minimize variation in staining between gels, all 12 gels were stained simultaneously in the same bath. Reproducibility of the protein resolution using this protocol is very high (intra-assay and inter-assay coefficients of variation of approximately 5 and 10%, respectively). Developed gels were scanned with a GH-800 calibrated densitometer (Bio-Rad). Image analysis and mass spectrometry were performed according to Sawicki and Jugdutt, (2004).

Mass spectrometry

Mass spectrometry was performed according to Sawicki and Jugdutt, (2004) in the Institute for Biomolecular Design at the University of Alberta. Electrophoretically-separated proteins from the spots that demonstrated statistically significant changes in intensity and were consistent in all experiments were excised from the gel and pooled. In-Gel digestion was performed on a MassPrep Station (MicroMass, UK), using the method supplied by the manufacturer. Excised gel pieces were destained, reduced with DTT, cysteine residues reacted with iodoacetamide, digested with trypsin (Promega sequencing grade) and extracted. The extract was analyzed via LC/MS/MS. LC was performed on a Waters CapLC (Milford, MA), using a water/acetonitrile (0.2% formic acid) gradient, on a PicoFrit capillary column (New Objectives, Woburn, MA) (BioBasis C18, 5 micron particle size, 10 cm x 75 micron ID, 15 micron tip). The eluted peptides were then electropsprayed and analyzed on a MicroMass Q-ToF 2 using automated data dependent MS to MS/MS switching. The resultant MS/MS data were searched against NCBInr and Swiss Prot databases for identification of the protein. A mass deviation of 0.2 was tolerated and 0 missed cleavage sites were allowed in the searches. The Mascot (www.matrixscience.com) search engine was then utilized to analyze the NBCInr protein

database for protein identification. The Mousse scoring algorithm (Perkins et al., 1999) was used for justification of accuracy of protein identification and is incorporated in the Mascot search engine.

5.3 RESULTS

Expression of Mouse GH AdV in Rat Lung Epithelial L2 Cells

Mouse GH mRNA was not detectable in the L2 cells before infection with the mouse GH adenovirus (Fig. 5.1). However, in lung epithelial cells infected with the mouse GH Adv, mouse GH mRNA was, as expected (Marmary et al., 1999), readily detected after 24h and for at least another 48h thereafter. In marked contrast, mouse GH mRNA was not seen in the Green Fluorescent Protein (GFP) expressing controls (Fig. 5.1).

Expression of Mouse GH Transgene in the Lungs

Expression of the mouse GH adenovirus in the lungs of six 14 day-old rats was clearly demonstrated 10 days after intratracheal administration (on day 4), indicating a relatively stable transfection of lung cells (Fig. 5.2; lanes 1-6). In each case, a single 651 bp cDNA was readily detectable after RT-PCR with the JAB3/JAB4 primer set, whereas this transcript was not present in mRNA extracted from the GFP expressing controls (Fig. 5.2; lanes 7-12). Sequencing of these cDNA moieties confirmed that they had 100% identity with mouse GH cDNA.

The widespread localization of GFP in the air-exchange parenchyma of the lung on day 14 confirmed the uptake and expression of the adenovirus in the lung (Fig. 5.3). The absence of GFP fluorescence in surrounding extra pulmonary tissues (data not shown)

indicates, as expected (Pozeg et al., 2003; Chicoine et al., 2004), that adenoviral expression after intratracheal administration was restricted to the lung. There were also no overt indications of pulmonary inflammation, such as increased levels of alveolar macrophages in bronchioalveolar lavage, nor in the dry weight/wet weight ratios of the lungs, which did not differ between the mouse GH Adv and control groups (data not shown).

2-Dimensional Gel Electrophoresis

Numerous proteins (>200) were resolved following 2D-gel electrophoresis of the proteins extracted from the lungs of the control and mouse GH Adv treated rats (representative gels are shown in Fig. 5.4). Of these proteins, the concentration of 13 in the lungs of rats overexpressing mGH were significantly different ($P < 0.05$, by approximately -50 to +175%) from the GFP controls and 11 of these proteins had intensities sufficiently high enough for identification by mass spectrophometry (Table 1). Raw spot intensity data is shown (Fig. 5.5) and relative protein quantities are listed as percent of GFP control for each protein (Table 5.2).

Infection of the lung with the mouse GH AdV increased ($P<0.05$) the relative concentration of at least nine proteins (Table 5.2; Fig. 5.5). These proteins included enzymes (nucleotide diphosphate kinase B, Cu/Zn superoxide dismutase, glutathiones s-transferase and aldehyde reductase 1) and structural proteins (beta-5 tubulin), as well as proteins involved in signal transduction (G-protein beta subunit and nucleoside diphosphate kinase B) and cell proliferation (retinoblastoma binding protein 4, major acute phase protein and calponin 2). In contrast, infection of the lung with the mouse GH AdV reduced ($P<0.05$) the relative concentration of haptoglobin and major acute phase alpha-1 protein (Table 5.2; Fig. 5.5).

5.4 DISCUSSION

The results of this study therefore clearly show the transgenic expression of the mouse GH gene in the rat lung. The amount of mouse GH expressed was not quantified, but mouse GH cDNA was readily amplified by RT-PCR and Marmary et al., (1999) reported a 10 fold increase in circulating GH concentrations in rats 4 days after the intravenous administration of the same mouse GH- expressing adenovirus, although these authors could not distinguish between mouse and rat GH immunoreactivity in peripheral plasma.

The localized expression of the mouse GH gene in the rat lung demonstrates that this is an effective method for inducing targeted GH overexpression (Jayasankar et al., 2004). Indeed, Pozeg et al., (2003) and Chicoine et al., (2004) used a similar experimental approach to specifically induce the expression of other genes in the lung, between 1 and 13 days after the intratracheal administration of the adenoviruses. Kanaan et al., (2002) also found that intratracheal administration was the best experimental approach for the lung-specific overexpression of adenoviral encoded genes, with negligible effects (if any) on gene expression in other organs or on the serum concentration of the coded proteins. This experimental approach has also been shown to induce gene expression in all the cell types forming lung airway epithelia (Mastrangeli et al., 1993), with little (if any) inflammation (Waszak et al., 2002; Kanaan et al., 2002; Pozeg et al., 2003). Our model of adenoviral gene delivery is therefore a useful tool to study the role of GH gene expression in lung development, particularly in the postnatal alveolarization stage.

GH is similarly known to induce or repress the activity of numerous genes (particularly in the liver), including genes involved in regulating metabolism,

transcription, signal transduction, protein turnover, transport, detoxification, cell structure or replication (Flores-Morales et al., 2001). Other GH-responsive genes in the rat liver include proto-oncogene (Triest et al., 1995; Yoon et al., 1990), protease inhibitors (Yoon et al., 1987; Warren et al ., 1993) and steroid hydroxylases (Wells et al., 1994; Subramanian et al., 1995). Changes in protein content in the neonatal lung therefore provide a marker for GH action in this tissue.

Of the GH responsive proteins in lung, aldehyde reductase 1 (involved in glucose metabolism) was increased over two-fold in response to mouse GH adenoviral expression This is consistent with its induction in the livers of transgenic mice overexpressing the bovine GH gene (Olsson et al., 2003). It may, therefore, be pertinent that this enzyme (also known as aldose reductase) is also induced by basic fibroblast growth factor (bFGF) (Laeng et al., 1995), as bFGF is induced by GH (Izumi et al., 1995) and GH and bFGF act together to promote growth (Edmondson et al., 1999; Waters et al., 1999). In addition to aldose reductase, glutathione-S-transferase was also increased almost two fold by mouse GH adenoviral expression, consistent with the responsiveness of the enzyme in rat livers to exogenous GH treatment (Tollet-Egnell et al., 2000). The increased concentrations of G-protein subunit in the lungs of the rats expressing the mouse GH adenovirus are also in agreement with increased G-protein subunit levels in human somatotrophic pituitary tumors (Hamacher et al., 1998). However, none of the other 8 proteins that were GH-responsive in the rats expressing the mouse GH adenovirus have previously been identified as markers of GH action.

Retinoblastoma binding protein 4 (also known as chromatin assembly factor 1 subunit C or CAF-1 subunit C) was found to be doubled in response to mouse GH overexpression in the rat lung. CAF-1 is a marker of proliferating cells (Polo et al., 2004) and subunit C

is a subunit of the histone deacetylase complex (Taunton et al., 1996). GH may thus be involved cell proliferation during alveolarization. This possibility is supported by the decreased levels of major acute phase protein (also known as T-kininogen) following mouse GH adenoviral expression, as this protein inhibits cell proliferation, especially in fibroblasts (Torres et al., 2001). Similarly the increase in calponin 2 concentrations might be expected to promote cellular proliferation, since it regulates the actin cytoskeleton in proliferating cells (Hossain et al., 2003). Although we did not assess cellular proliferation in the lungs of the control and experimental rats, it is of interest that GH is thought to promote lung growth in acromegalics and in GH-treated rats through an increase in cell hypertrophy rather than increased cell hyperplasia (Barlett 1971; Brody and Buhain, 1972; Garcia-Rio et al., 2001).

Adenoviral expression of mouse GH in the developing rat lung was also associated with increased concentrations of two proteins involved in intracellular signaling: Nucleoside diphosphate kinase B (NDPK B) and G-protein beta subunit, with which it complexes (Cuello et al., 2003). The upregulation of these two proteins could, therefore, provide a novel pathway through which GH could increase signaling through G-protein coupled pathways. Indeed, GH has similarly been found to increase the activity of guanylate cyclase in the lung (Vesely 1981).

Fetuin A concentrations in the neonatal lung were also found to be increased by mouse GH adenoviral expression. Fetuin A is an inhibitor of the insulin-receptor tyrosine kinase (Rauth et al., 1992) and is involved in some types of insulin resistance (Kalabay et al., 2002), which is classically induced by GH overexpression (eg. Dominici et al., 1998, Takano et al., 2001). While fetuin A is often considered a serum protein, its expression has previously been demonstrated in the developing mouse lung (Yang et al., 1992).

Cu-Zn superoxide dismutase (Cu-Zn SOD) is also known to be expressed in the human lung during development (Strange et al ., 1988), in which it is an important antioxidant (Peskin 1997). Indeed, Cu-Zn SOD expression and enzymatic activity rise late in gestation and are important for degradation of superoxide upon transition to air breathing and for oxygen scavenging in rats postnatally (Clerch et al., 1992; Nozik-Grayck et al., 2000). The increased Cu-Zn SOD in the lungs of rats expressing the mouse GH adenovirus therefore suggests that GH may normally promote this perinatal oxygen-scavenging mechanism during the transition of the lung to air breathing. GH is a well established regulator of superoxide production (Edwards et al., 1992) and its effect on Cu-Zn SOD in the developing lung is consistent with its role in tissue protection and immune regulation (Arnold and Weigent, 2003; Arnold and Weigent, 2004).

In contrast with these GH-responsive proteins, haptoglobin concentrations were decreased in the lungs of the mouse GH-expressing rats. This protein is known to be locally expressed within the fetal lung and is believed to be produced in alveolar Type II epithelial cells in response to infection (Yang et al., 1995). It is therefore pertinent that GH and has cytokine actions (Waters et al., 1999) and is also produced in Type II epithelial cells (Beyea et al., 2005). An inhibitory effect of GH on haptoglobin production is also indicated by the decrease in serum haptoglobin levels in response to recombinant human GH treatment in burn-victim rats (Jeschke et al., 1999).

The expression of the mouse GH adenovirus in the rat lung would thus appear to have biological activity, since changes in gene expression and tissue protein concentrations are well established markers of GH action (eg. Thompson et al., 2000; Flores-Morales et al., 2001). As the expression of the adenovirus was confined to the lung, its overexpression is likely to reflect the actions of endogenous GH produced in the lung. These results

therefore support the possibility (Beyea et al., 2005) that GH expression during alveolarization promotes autocrine/paracrine actions involved in lung function.

5.5 TABLES

Table 5.1 – Growth hormone responsive lung proteins

Spot Number	Probability Based Mouse Score** Threshold (P<0.05)	Observed Score	*Protein Identity
#1	75	402	Nucleoside Diphosphate Kinase B
#2	77	230	Cu/Zn Superoxide Dismutase
#3	77	118	Haptoglobin
#4	77	423	Glutathione S-Transferase
#5	77	246	G-Protein (Beta subunit)
#6	77	105	Calponin 2
#7	77	186	Aldehyde Reductase 1
#8	77	124	Beta-5 Tubulin
#9	77	113	Retinoblastoma Binding Protein 4
#10	77	183	Fetuin A
#11	77	358	Major Acute Phase alpha-1 Protein

*Identified by mass spectrophotometry and Mascot search engine.

**-10 log(P) where P is the probability that the observed match is a random event (P<0.05). Individual scores >75 indicate identity or extensive homology.

Table 5.2 – Relative quantities of Proteins Responsive to GH Overexpression in the Lung.

Protein[a]	GFP AdV	Mouse GH AdV
1. Nucleoside Diphosphate Kinase B	†100.0±13.8	*168.2±23.0
2. Cu/Zn Superoxide Dismutase	100.0±3.3	*126.9±11.1
3. Haptoglobin	100.0±12.4	*51.19±11.2
4. Glutathione S-Transferase	100.0±17.0	*178.1±21.2
5. G-Protein (Beta subunit)	100.0±14.6	*172.3±26.2
6. Calponin 2	100.0±7.1	*147.4±14.6
7. Aldehyde Reductase 1	100.0±28.8	*265.4±67.0
8. Beta-5 Tubulin	100.0±36.6	*276.6±30.9
9. Retinoblastoma Binding Protein 4	100.0±21.5	*212.3±40.7
10. Fetuin A	100.0±13.1	*157.9±25.8
11. Major Acute Phase alpha-1 Protein	100.0±12.7	*61.4±8.5

[a] Identified by 2D gel electrophoresis and mass spectrophotometry.

†All values are given as percent of control (GFP Control). Mean±standard error; n=6 for each of the two treatment groups.

*Significantly different from control, $p<0.05$ (Student's t-test.)

Figure 5.1. Real-time PCR for mouse GH in rat lung epithelial L2 cells. Quantities are shown at 0hours, 24hours, 48hours, and 72hours for both GFP infected (control) and mouse GH infected (GH) cells. Mouse GH is not detected in the GFP control group throughout the time course. Expression of mouse GH mRNA is detected at 24hours post-infection, and continues to increase through 48 and 72hours. Different letters mean that the group is significantly different ($P<0.05$) from all other groups by ANOVA.

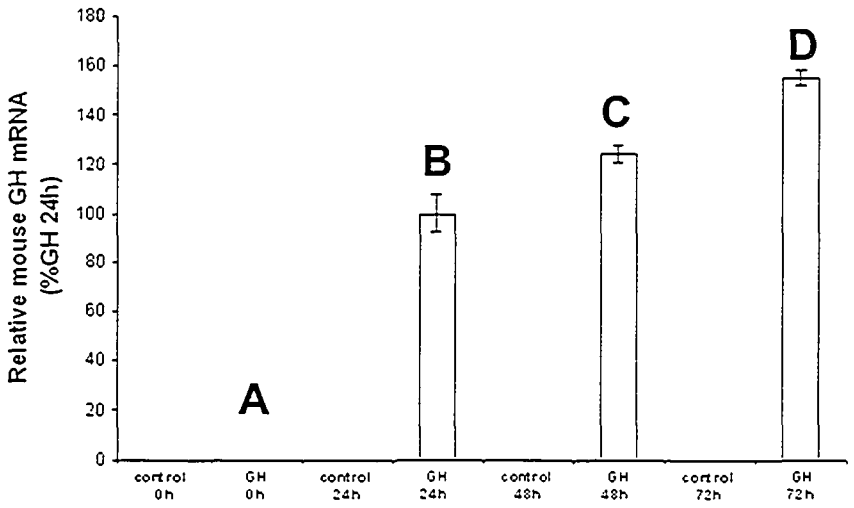


Figure 5.2. Postnatal day 14 expression of mouse GH adenovirus in lungs. A 651bp transcript is amplified in all six mouse GH adenovirus treated lungs (lanes 1-6), and is absent from all six GFP adenovirus treated lungs (lanes 8-13). A 100bp ladder is shown for comparison (lane 7).

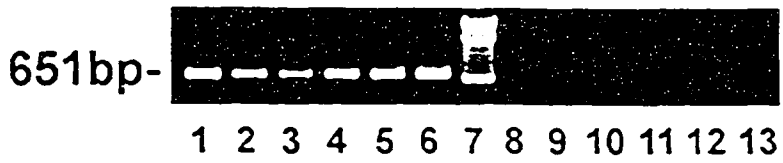


Figure 5.3. Representative sections from upper (A), middle (B) and lower (C) portions of the caudal lobe of postnatal day 14 GFP control, demonstrating widespread localization of the GFP protein.

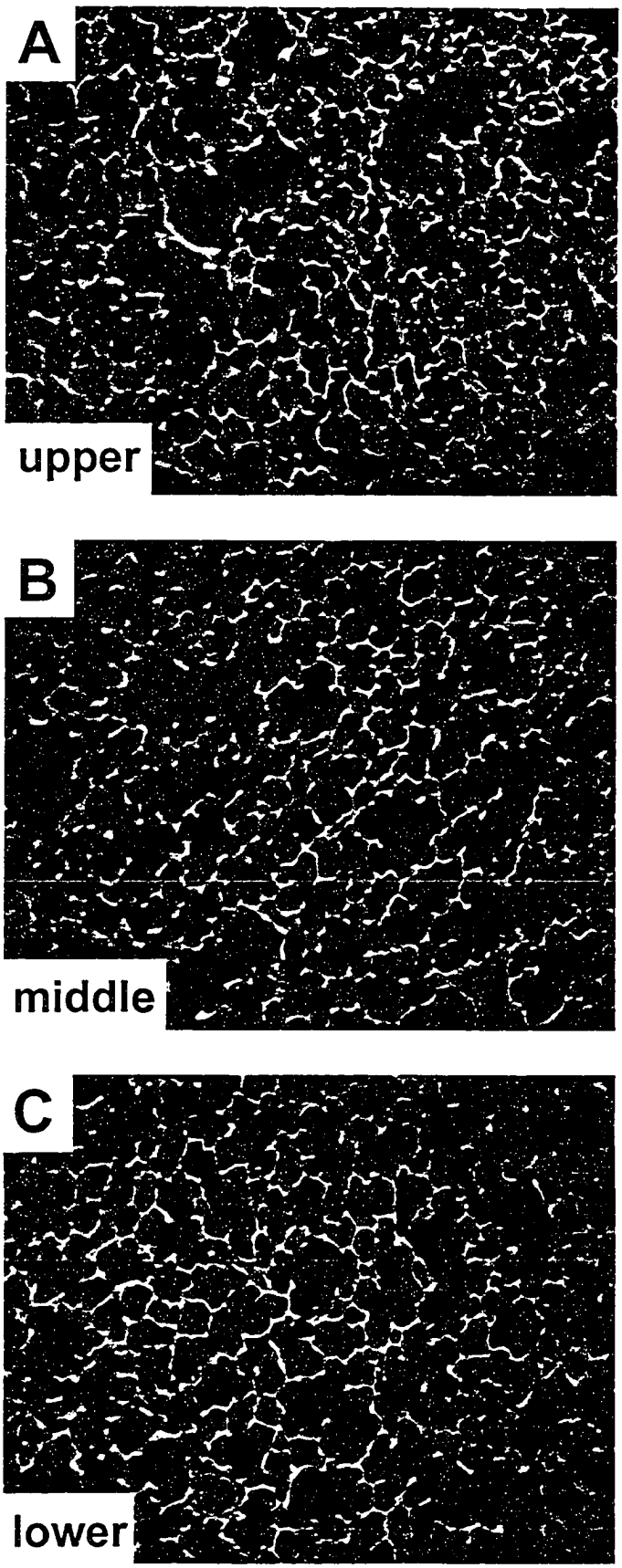


Figure 5.4. Representative 2D gel electrophoresis of whole lung homogenate for GFP adenovirus control treated (A) and mouse GH adenovirus treated (B) rats at postnatal day 14. Numbers indicate proteins whose levels have changed in the mouse GH adenovirus group relative to the GFP control. These numbers correspond to the numbers of the eleven proteins in Table 1.

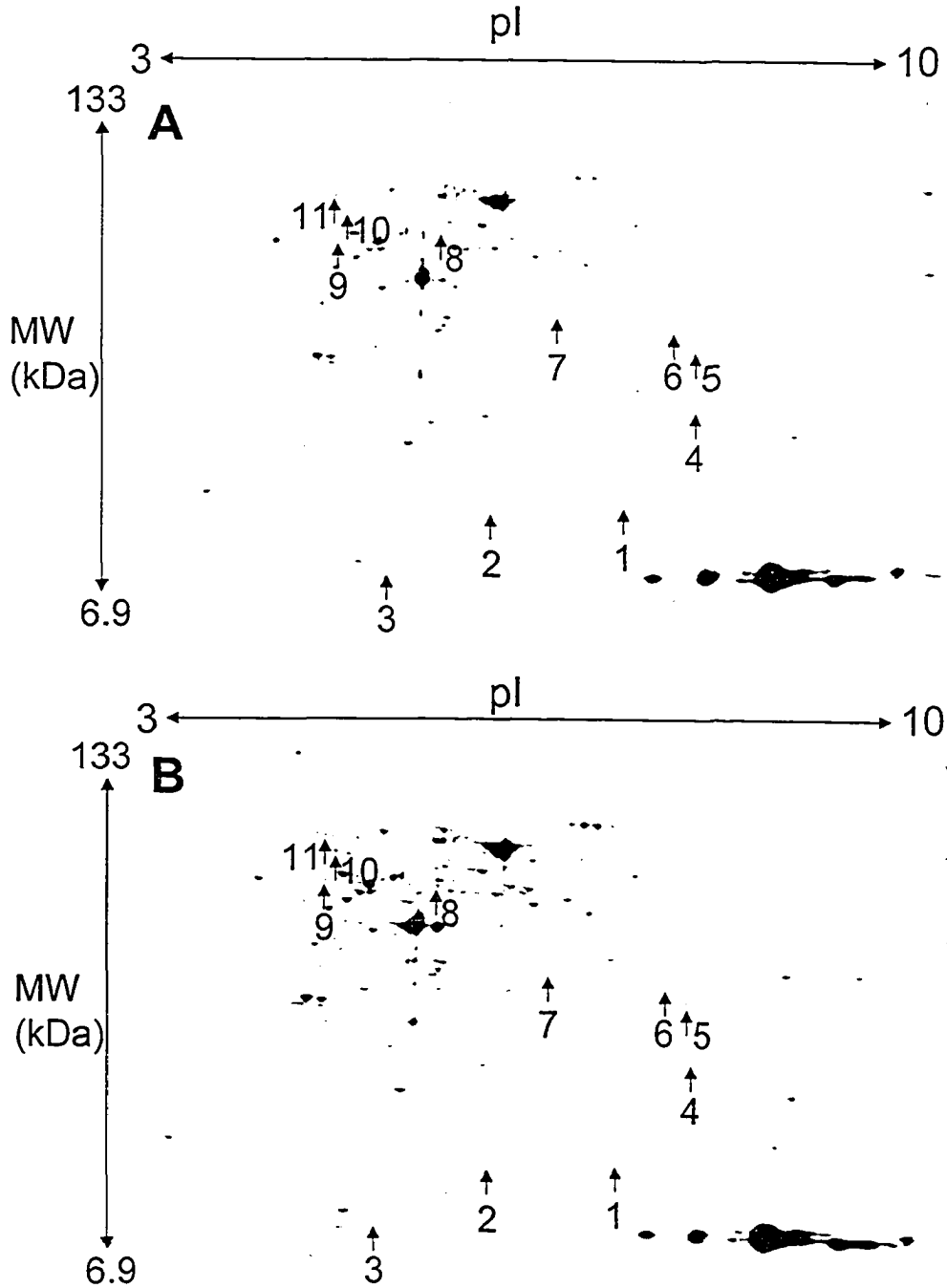
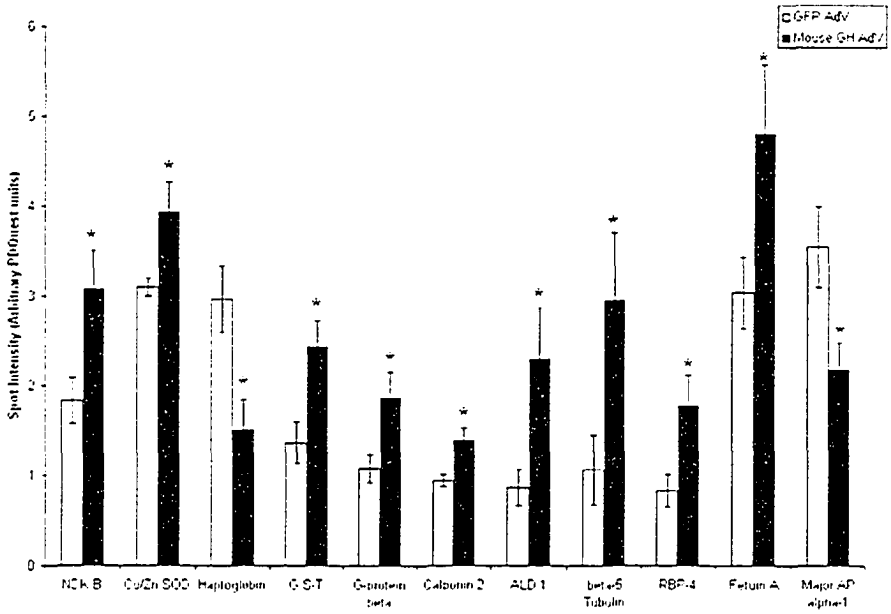


Figure 5.5. Spot intensity of proteins identified in Table 1 for GFP adenovirus and mouse GH adenovirus treatment groups. Intensities are given as arbitrary units provided by the PDQuest7 software. Abbreviations for proteins are NDK B (Nucleoside diphosphate kinase B), Cu/Zn SOD (Cu/Zn superoxide dismutase), G S-T (glutathione s-transferase), ALD 1 (aldehyde reductase 1), RBP-4 (retinoblastoma binding protein 4), and Major AP alpha-1 (major acute phase alpha-1 protein). Mean spot intensities for all mouse GH adenovirus treated lungs are significantly different than mean spot intensities of GFP control adenovirus treated lungs (*$P<0.05$).



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CHAPTER 6

GROWTH HORMONE (GH) RECEPTOR KNOCKOUT MICE REVEAL ACTIONS OF GH IN LUNG DEVELOPMENT

CHAPTER 6: GROWTH HORMONE (GH) RECEPTOR KNOCKOUT MICE REVEAL ACTIONS OF GH IN LUNG DEVELOPMENT[1]

6.1 INTRODUCTION

The lung is a target site for growth hormone (GH) action, since the GH receptor (GHR) gene is expressed in pulmonary tissues (Tiong et al., 1989; Garcia-Aragon et al., 1992; Batchelor et al., 1998). Indeed, a role for GH in pulmonary function is indicated by the anatomical and biophysical changes in the lung in pathological states of pituitary GH excess and deficiency. For instance, in acromegaly (see Colao et al., 2004 for review) the lungs are large (Bartlett 1971), upper airflow is obstructed (Trotman-Dickenson et al., 1991) and the small airways are narrowed (Harrison et al., 1978). In contrast a decrease in respiratory muscle strength (Merola et al., 1996) and a reduction in the maximum inspiratory and expiratory pressure (Merola et al., 1995) accompanies GH deficiency. Moreover, exogenous GH induces the production of superoxide by alveolar macrophages (Edwards et al., 1992), activates lung neutrophils (Liu et al., 2002a), induces NF_KB production (Liu et al., 2002b), increases lung phosphorylase A activity (Jost et al., 1979) and stimulates the tyrosine phosphorylation of specific but unidentified proteins in lung epithelial cells (Batchelor et al., 1998). The physiological importance of endogenous GH in lung development is, however, unknown.

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Lung development involves cell proliferation, differentiation and survival and the process of alveolarization, in particular, involves airway branching and widening and angiogenesis of the pulmonary vasculature (Copland and Post, 2004). Numerous local growth factors, including fibroblast growth factors, sonic hedgehog, and retinoic acid (Cardoso 2001) have established roles in lung development and in alveolarization in particular (Shenai et al., 1985; Massaro and Massaro, 1996, 1997). Since the GH gene is widely expressed in Type I and Type II epithelial cells of the rat lung during the period of alveolarization (Beyea et al., 2005), it may also participate in lung development, especially as local GH expression has autocrine/paracrine roles rather than endocrine actions in other tissues (Waters et al., 1999; Sanders and Harvey, 2004). The possibility that pituitary and/or pulmonary GH have physiological roles in lung development has therefore been investigated in GHR knockout mice (List et al., 2001), using a proteomic approach to determine if an absence of GH-signaling affected the proteome of the developing lung. Actions of GH in skeletal muscle (Chrysis et al., 2002) have similarly been determined using a proteomic approach, as have the actions of GH in the livers of mice with deficient GH-signaling (Dozmorov et al., 2001; Dozmorov et al., 2002; Miller et al., 2002; Murakami et al., 2003; Boylston et al., 2004).

6.2 MATERIALS AND METHODS

Animals

The mice used in these studies were females, housed in the transgenic facility at the Edison Biotechnology Institute and have been described previously (Zhou et al.,

1997). GHR-knockout (-/-, KO) mice and their controls (+/+) were obtained from the inbreeding of GHR (+/-) mice. Genotypes were confirmed by RT-PCR of total RNA from the tails of mice collected at autopsy (Chandrachekar et al., 1999). Animals were housed in a room with a controlled photoperiod of 12 hr light: 12 hr darkness (lights on from 0600 to 1800 hr) and a temperature 22-23°C. Mice were given free access to a nutritionally balanced diet (Lab Diet: PM1 Feeds, St. Louis, MO, USA) and tap water. All experiments were approved by the University Animal Care and Utilization Committee, and were conducted in accordance with NIH Animal Care guidelines.

Tissues

GHR-KO (-/-) (n=5) and GHR (+/+) (n=6) mice were sacrificed on postnatal day 14, late in the alveolarization period (Goncalves et al., 2001). Lungs were excised and flash frozen in liquid nitrogen. Protein samples for 2-dimensional electrophoresis (2-DE) were prepared by mixing the powdered lungs with rehydration buffer (8M urea, 4% CHAPS, 10 mM dithiothreitol (DTT), 0.2% Bio-Lytes 3/10, Bio Rad, Mississauga, Ontario, Canada), which was sonicated twice for 5 seconds and centrifuged for 10 minutes at 15,000 rpm at room temperature, to remove any insoluble particles.

2-Dimensional Polyacrylamide Gel Electrophoresis

The protein content of lung samples in rehydration buffer was measured using the BioRad protein assay after suitable dilution of the samples. 0.2 mg protein was

applied to 11 cm immobilized pH gradient (IPG) strips, which had a linear pH gradient from 3-10 (BioRad). For isoelectric focusing (IEF) the BioRad protean IEF cell was used at 20°C with fast voltage ramping: step 1: 15 min with an end voltage of 250 V; step 2: 150 min with an end voltage of 8,000 V; step 3: 35,000 V hr (approximately 260 min). After IEF, the strips were equilibrated according to the BioRad protocol. The second dimension of 2DE was carried out using Criterion precast gets (8-16%) (BioRad) in a Criterion Dodeca Cell (BioRad). After separation, the proteins were detected using Commassie Brilliant Blue R-250 (BioRad). To minimize variation in staining, all gels were stained in the same bath. The reproducibility of protein resolution using this 2-DE technique and staining procedure is very high, as detailed previously (Sawicki et al., 2003; Sawicki and Jugdutt, 2004). In our laboratory, the intensity of staining for a single protein when run on separate gels differs by <5% and the correlation coefficient (r^2) for the staining intensities of multiple protein samples measured on different gels is >0.97 (Sawicki et al., 2003). This procedure therefore obviates the need for replicate determinations, as previously established (Sawicki et al., 2003; Sawicki and Jugdutt, 2004; Jugdutt and Sawicki, 2004; Sawicki et al., 2004).

Image Analysis

Developed gels were scanned using a GS-800 calibrated densitometer (BioRad). Quantitative analysis of spot intensity was performed using PDQuest 7.1 software (BioRad). The protein spot sensitivity threshold was used to determine significant changes in protein spot size and density. This was based on 4 parameters: minimum

peak volume sensitivity, smallest spot area, largest spot area and the noise filter level. Only spots with relative intensities between 2-200 arbitrary units were considered for mass spectrometry analysis.

Mass Spectrometry

Electrophoretically-separated proteins from the spots that demonstrated statistically significant changes in intensity (at the $P<0.02$ level) were excised from the gels and separately pooled. In-Gel digestion was performed on a MassPrep Station (Micromass, Manchester, UK), using the method supplied by the manufacturer. Excised gel pieces were destained, reduced with DTT and the cysteine residues reacted with iodoacetamide, before digestion with trypsin (Promega sequencing grade) and extraction. The extract was then analyzed via LC/MS/MS. Liquid chromatography was performed on a Waters CapLC (Milford, MA), using a water/acetonitrile (0.2% formic acid) gradient, on a PicoFrit Capillary column (New Objectives, Woburn, MA) (BioBasic C18, 5 micron particle size, 10 cm x 75 micron ID, 15 micron tip). The eluted peptides were then electrosprayed and analyzed on a MicroMass Q-ToF 2, using automated data- dependent MS to MS/MS switching (Sawicki and Jugdutt, 2004). The resultant MS/MS data were searched against NCBInr and Swiss Prot databases for identification of the protein. A mass deviation of 0.2 was tolerated and 0 missed cleavage sites were allowed in the searches. The Mascot (www.matrixscience.com) search engine was used to search the NCBInr protein database for protein identification. The Mowse scoring algorithm (Perkins et

al., 1999) was used for justification of accuracy of protein identification and is incorporated in the Mascot search engine.

6.3 RESULTS

Representative electrophoregrams for proteins in the lungs of GHR (+/+) and GHR (-/-) mice are shown in Fig. 6.1. 2-DE gel analysis detected >600 proteins, of which 39 spots (approximately 7%) differed significantly in protein content at the $P<0.05$ level (6 were of higher abundance in the GHR (-/-) group, 33 were of lower abundance). Of these proteins, the abundance of 17 were significantly different at the $P<0.02$ level (5 of higher abundance in the GHR (-/-) group, 12 of lower abundance). These spots were excised from the gels for mass spectrometry analysis. Of these 17 spots, 14 were sufficiently resolved on the gels to permit accurate excision and 7 could be definitively identified by MS. The protein identity, the accuracy of protein identification (Mowse score), and the number of matched peptides for these proteins are summarized in Table 6.1. Mean spot intensities of proteins from GHR (+/+) and GHR-KO (-/-) lungs are displayed for vimentin (Fig. 6.2, lung content reduced by 75.0% in the GHR (-/-) group), SH3 domain-binding glutamic acid-rich-like protein (Fig. 6.2, lung content reduced by 88.3%), proteasome 26S ATPase subunit 4 (Fig. 6.2, lung content reduced by 69.2%), apolipoprotein A IV(Apoa4) (Fig. 6.2, lung content reduced by 72.7%), peroxiredoxin 6 (Prdx6) (Fig. 6.3, lung content reduced by 81.0%), isocitrate dehydrogenase 1 (Fig. 6.3, lung content reduced by 69.6%), and electron transfer flavoprotein alpha subunit (Fig. 6.3, lung content reduced by 48.6%), along with representative spots from the GHR (+/+) and GHR-KO (-/-) 2D gels.

6.4 DISCUSSION

This study demonstrates, for the first time, differences between normal and GHR-KO mice in tissue proteome content, in particular differences in the abundance of lung proteins. Since significant differences were seen in the abundance of approximately 7% of the detected proteins, these results suggest that GH signaling is of physiological importance in lung development.

The 7 proteins that were definitively identified in this study include proteins involved in growth (vimentin), proteins involved in oxidative stress (SH3 domain-binding glutamic acid-rich-like protein, Prdx6 and isocitrate dehydrogenase 1), proteins involved in lipid metabolism (Apoa4 and Prdx6), and proteins involved in general metabolism/cell function (electron transfer flavoprotein alpha subunit and proteasome 26S ATPase subunit 4). The decreased content of these proteins in the lungs of GHR-KO (-/-) mice suggests their synthesis is normally dependent upon GH stimulation through the lung GHR.

These proteins were definitively identified by mass spectrometry, obviating the need to identify them by immunoblotting techniques, which are less sensitive and precise. Indeed, immunoblotting is dependent on the detection of specific epitopes of the proteins, which may undergo different post-translational modifications in different genetic or treatment groups. Actin and myosin light chain 1 (Sawicki et al., 2003) measurements in mice are, for instance, are discrepant when determined by mass spectrometry and Western blotting.

Of the proteins identified, vimentin is an intermediate filament expressed in fibroblasts, mesenchymal cells and endothelial cells (Wang and Stamenovic, 2002),

including those that are responsive to GH action (Tseng et al., 1997). It is thought to have roles in development, since it promotes cell migration and proliferation (Wang and Stamenovic, 2002; Pelosi et al., 2003). Although null mutation of the vimentin gene results in mice without any obvious phenotypic defect (Colucci-Guyon et al., 1994), this may reflect the redundancy of growth factors involved in cytoplasmic filament function and the induction of compensatory mechanisms. In the lung, vimentin expression occurs in Type I and Type II epithelial cells (Kasper et al., 1993; Koslowski et al., 2004) and in the endothelia of arteries and veins (Kaarteenaaho-Wiik et al., 2004). The amount of vimentin in the lung proteome is reduced by disease (Walburg et al., 2004), whereas vimentin expression is increased in lung repair following injury (Kasper et al., 1993). Vimentin is thus thought to be involved in lung growth. The reduced vimentin levels in the lungs of GHR-KO mice would thus suggest that they have poorly developed lungs, although this has yet to be determined. This possibility is, however, supported by the reduced amounts of antioxidant proteins in the lungs of GHR-KO mice, since deficient antioxidative proteins occur in the proteome of diseased, poorly functioning lung cells (Waldburg et al., 2004). This is the first report to indicate a role for GH in vimentin regulation in the lung, although GH is thought to be involved in lung growth and repair (Fitzgerald et al., 1998) and the autocrine production of GH in human mammary carcinoma cells is accompanied by an induction of vimentin expression (Mukhina et al., 2004).

Oxidative stress is particularly important in the lung and the postnatal period is when the lung acquires its ability to deal with this stress (Land and Wilson, 2005). One of the antioxidant proteins in the lung is the SH3 domain-binding, glutamic acid-

rich-like protein, a member of the SH3BGR family that belongs to the thioredoxin super-family (Mazzocco et al., 2002). Thioredoxin is a vital component of the lung antioxidant machinery and *in vivo* delivery of recombinant thioredoxin has been proposed as a therapeutic approach for treating oxidative stress-associated lung disorders (Nakamura et al., 2005). The reduced amount of SH3 domain-binding glutamic acid-rich-like protein in the lungs of the GHR-KO mice therefore suggests they would have reduced antioxidant activity. This possibility is also supported by the reduced content of Prdx6 (also known as acidic calcium-independent phospholipase A2), an enzyme involved in oxidative defense against reactive oxygen species (Wang et al., 2004). Prdx6 is found predominantly in the lung, specifically within alveolar Type II cells, alveolar macrophages and in bronchiolar epithelium (Kim et al., 1998). Its antioxidant role is indicated by the protection against hyperoxic injury that occurs in mouse lungs following the adenoviral overexpression of Prdx6 (Wang et al., 2004). Furthermore, antisense oligonucleotide suppression of Prdx6 expression in lung L2 epithelia cells results in increased oxidant sensitivity and apoptosis (Pak et al., 2002) and Prdx6 (-/-) KO mice show increased lung injury and mortality in hyperoxia (Wang et al., 2004).

Isocitrate dehydrogenase 1 is another antioxidant that was reduced in content in the lungs of GHR-KO mice. This enzyme produces reduced NADP, which is then used to regenerate reduced glutathione (Murakami and Yoshino, 2004), which protects the lungs from oxidative stress (Rahman 1999). GH has previously been shown to interact with the glutathione-antioxidant system, by inducing glutathione synthesis and by modulating glutathione degradation (Brown-Borg et al., 2004;

Brown-Borg et al., 2005). In addition, cells genetically engineered to produce human GH were found to overproduce glutathione (Cherbonnier et al., 2003). The activity of this enzyme has also been shown to be increased by exogenous GH in the liver of chickens (Roseborough et al., 1991). The reduced content of isocitrate dehydrogenase 1 in the lungs of GHR-KO mice is consistent with the reduced levels of SH3BGR and Prdx6 proteins and a hitherto unknown role for GH in oxidative protection of the developing lung.

In marked contrast, it is generally thought that the longevity of dwarf mice with deficient GH signaling (Ames and Snell mice) reflects increased antioxidant defense and reduced oxidative stress (Hauck and Bartke, 2001). However, while catalase activity in these dwarf mice is greatly increased in numerous tissues (Brown-Borg et al., 1999; Brown-Borg and Rakoczy, 2000; Hauck and Bartke, 2000), glutathione levels are actively decreased in the livers of dwarf mice (Brown-Borg et al., 1999). Indeed, in these mutant mice, the glutathione level is increased in the liver, brain and muscle following exogenous GH treatment (Brown-Borg and Rakoczy, 2003). Tissue levels of antioxidant proteins in dwarf mice and their response to exogenous GH are therefore protein specific and possibly also tissue-specific and modified by age (Brown-Borg and Rakoczy, 2004; Brown-Borg and Rakoczy, 2005; Hauck and Bartke, 2001). Moreover, in contrast with Ames and Snell mice, catalase activity in the liver and kidneys of GHR-KO mice is decreased rather than increased (Hauck et al., 2002). The longevity in these mice (Coschigano et al., 2000) is therefore not a result of increased free radical scavenging. Similarly, GH-resistance in the Hnf-1 alpha knockout "Laron" mouse (Lee et al., 1998) is also associated with

reduced catalase and glutathione peroxidase activity in the liver and other tissues (Muppala et al., 2000). These findings are therefore consistent with our demonstration of reduced antioxidant protein levels in the lungs of GHR (-/-) mice. There is, however, no evidence of lung dysfunction in GHR-KO mice, which live 40% longer than their wild-type siblings (Zhou et al., 1997; Bartke and Brown-Borg, 2004). The physiological consequences of having decreased lung proteins involved in antioxidant defense are thus not life-threatening. Indeed, without information on the activity of these antioxidative enzymes, the level of antioxidative defense in the lungs of the GHR-KO mice is uncertain. In the absence of lung dysfunction, it is thus likely that there is a redundancy of antioxidative defense systems and compensatory mechanisms.

In addition to its role as an antioxidant, Prdx6 is also involved in lung surfactant production. Dipalmitoylphosphatidylcholine (DPPC) is the major phospholipid constituent of lung surfactant, with 50% of DPPC synthesized through a phosphatidylcholine (PC) remodeling pathway in Type II pneumocytes that is largely regulated by Prdx6 (Fisher and Dodia, 1997). Lipid metabolism and the production of surfactant is of major importance in the lung (Griese et al., 1999) and GH may therefore be involved in this process, especially as the lung content of Apoa4 was also reduced in the GHR-KO mice. Apoa4 is primarily involved in the metabolism of triglycerides and high-density lipoproteins (Bai et al., 1996) and Apoa4 is expressed in the rabbit (Lenich et al., 1988) and mouse (Srivastava et al., 1991) lung. The decreased Apoa4 content in GHR-KO mice suggests its production is normally dependent upon GH-signaling, a possibility supported by the decreased Apoa4

expression in the liver of GH-deficient Snell dwarf mice (Boylston et al., 2004). Indeed, of 50 hepatic genes in Snell mice that differ from normal mice in transcriptional amount, Apoa4 is the most affected by the lack of GH signaling (Boylston et al., 2004). The expression of apolipoprotein E is similarly increased by exogenous GH in rat skeletal muscle (Tollet-Egnell et al., 2004).

In the absence of GHR-mediated GH signaling, the lung contents of 26SATPase subunit 4 and electron transfer flavoprotein alpha subunit were also reduced, indicating that their production is normally GH-dependent. The 26S proteasome complex plays a major role in the ubiquitin-proteasome pathway that mediates the non-lysosmal degradation of intracellular proteins (Mason et al., 1998; Naujokat and Hoffmann, 2002). ATPase subunit 4 is part of the 19S regulatory subunit of the 26S proteasome and this promotes substrate unfolding and translocation (Braun et al., 1999). Proteasome 26S ATPase 4 is widely employed as a 'housekeeper' gene (Szabo et al., 2004), although exogenous GH has previously been found to increase proteasome subunit mRNAs (C-2, C-3, C-5, C-6, C-8 and C-9) in rat skeletal muscle (Chrysis et al., 2002) and in rat liver (for C-2, C-3 and C-8) (Chrysis et al., 2002). Proteosomal ATPase has also been identified as a GH-response gene in the rat liver (Tollet-Egnell et al., 2000). Electron transfer flavoprotein alpha subunit transfers electrons between several mitochondrial dehydrogenases and the main respiratory chain (Purevjav et al., 2002). It has not previously been thought to be GH-regulated.

In summary, the results of this study identify proteins in the lungs of neonatal GHR-KO mice that are less abundant than in their normal controls. These results

therefore suggest that these proteins are normally dependent upon GH-signaling and that GH is normally involved in promoting vimentin-induced lung growth, increasing SH3BGH, Prdx6 and isocitrate dehydrogenase-induced antioxidant protection, increasing Prdx6 and Apoa4 lipid metabolism and increasing lung proteasomal activity. These actions may reflect endocrine actions of pituitary GH and/or local autocrine/paracrine actions of GH produced within the lung (Beyea et al., 2005).

6.5 TABLE

Table 6.1 – Identification of lung proteins.

Spot Number	Probability Based Mowse Score*		Peptides Matched	Protein Identity (n)
	Threshold	Observed Score		
1	29	517	16	Vimentin
2	29	88	2	SH3 domain-binding glutamic acid-rich-like protein
3	30	160	7	Proteasome 26S ATPase subunit 4
4	31	338	9	Apolipoprotein A-IV
5	30	75	2	Peroxiredoxin 6
6	29	139	4	Isocitrate dehydrogenase 1
7	30	86	2	Electron transfer flavoprotein alpha subunit

*-10 log(P) where P is the probability that the observed match is a random event. Individual scores >29, >30, or >31 indicate identity or extensive homology ($P<0.02$).

Figure 6.1. Representative 2D gel electrophoresis of proteins extracted from the lungs of GHR normal (+/+) (A) and GHR-KO (-/-) (B) mice at postnatal day 14. Numbers indicate the seven identified proteins (listed in Table 1) that differed in abundance.

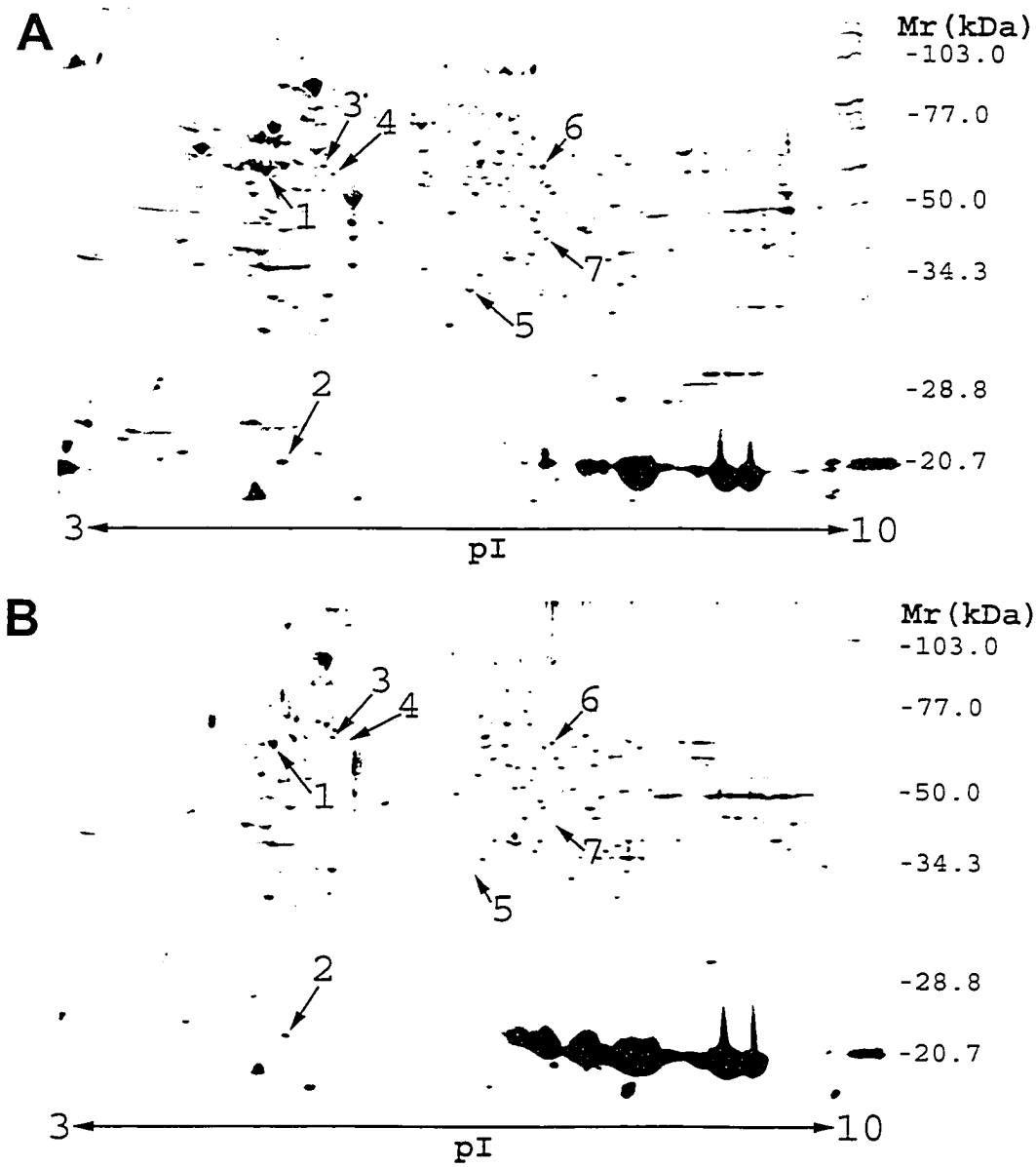


Figure 6.2. (A, C, E, G) Spot intensity (arbitrary units) for identified proteins in the lungs of GHR normal (+/+) (n=6) and GHR-KO (-/-) mice (n=5) (Means +/- SEM, asterisks indicate significant differences between the groups, $P<0.02$) and (B, D, F,H) representative 2D gel electrophoresis highlighting individual protein spots (1-4). (Protein 1 = vimentin (A,B), Protein 2 = SH3 domain-binding glutamic acid-rich-like protein (C,D), Protein 3 = proteosome 26S ATPase subunit 4 (E,F), Protein 4 = apolipoprotein A-IV (G,H)).

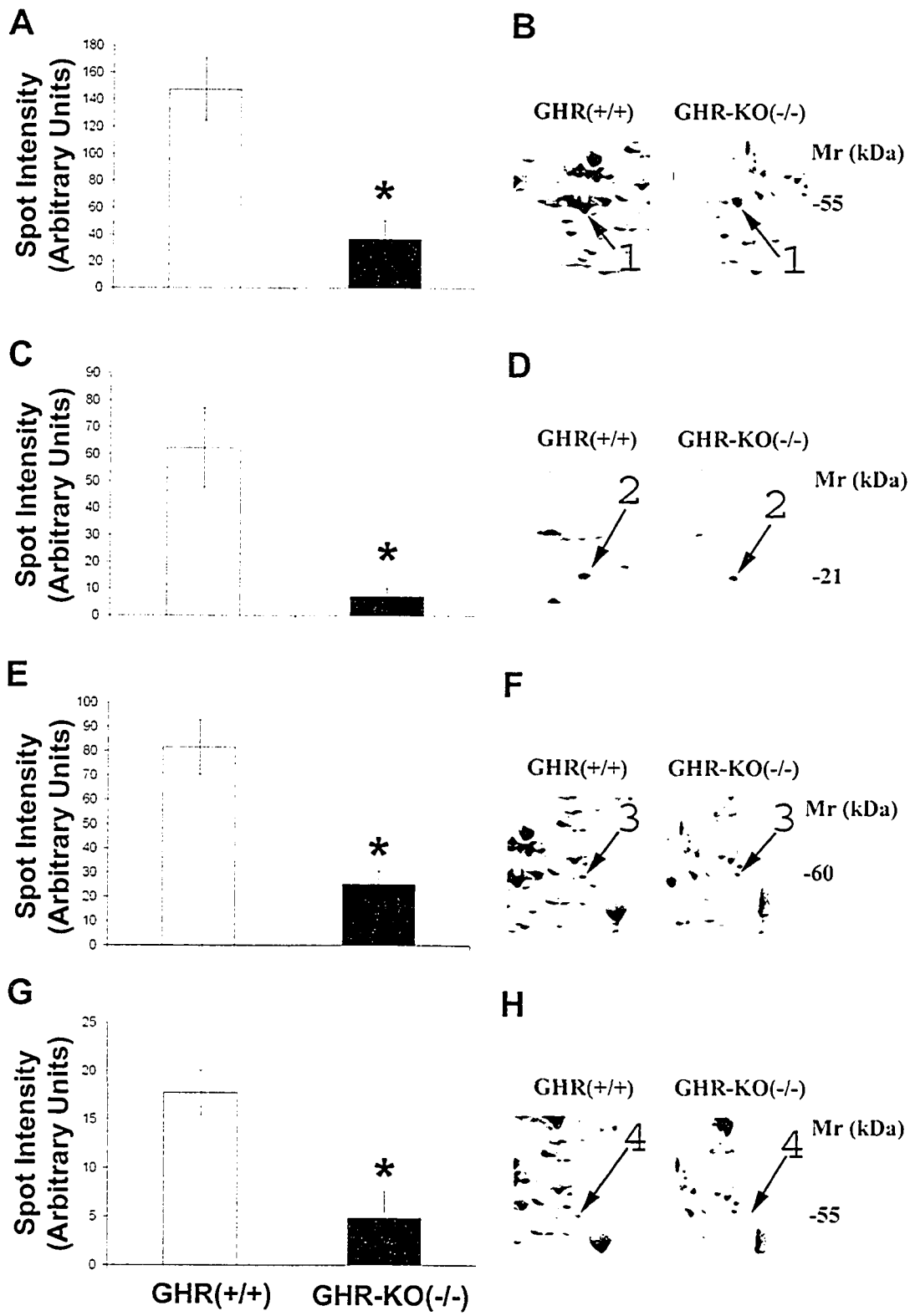
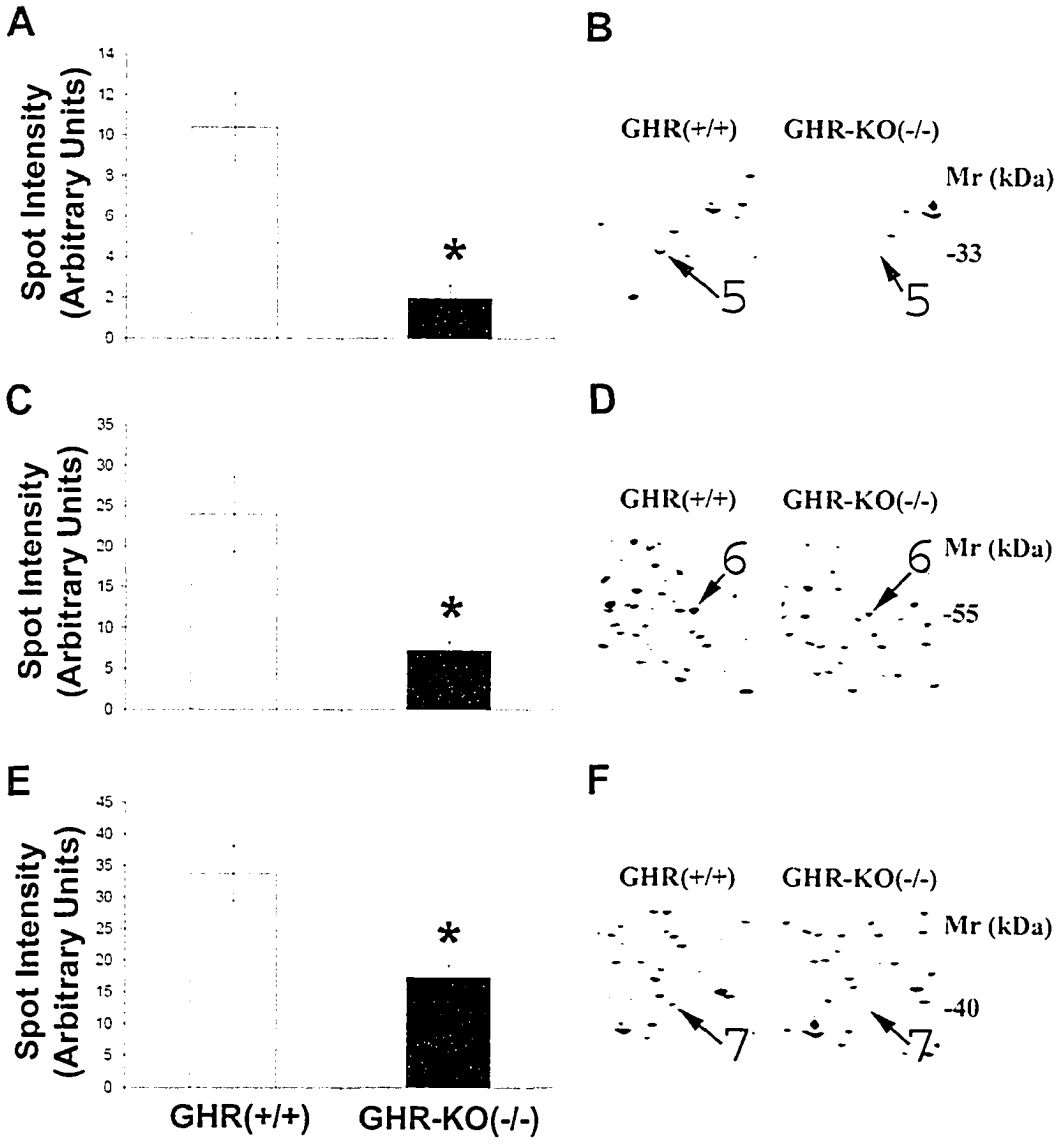


Figure 6.3. (A, C, E) Spot intensity (arbitrary units) for identified proteins in the lungs of GHR normal (+/+) (n=6) and GHR-KO (-/-) mice (n=5) (Means +/- SEM, asterisks indicate significant differences between the groups, $P<0.02$) and (B, D, F) representative 2D gel electrophoresis highlighting individual protein spots (5-7). (Protein 5 = peroxiredoxin 6 (A, B), Protein 6 = isocitrate dehydrogenase (C, D), Protein 7 = electron transfer flavoprotein alpha subunit (E, F)).



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CHAPTER 7

CONCLUSION

CHAPTER 7: CONCLUSION

7.1 Overall Summary

These studies addressed the hypotheses that (7.1.1) the developing lung is an extrapituitary site of GH expression, and that (7.1.2) altered local GH signaling through GHR changes the expression of important lung proteins during the development of the lung.

7.1.1 GH in the lung: descriptive studies

GH has been demonstrated in many extrapituitary tissues (reviewed in Harvey and Hull, 1997) of the chick. In chapter 2, our studies expanded these tissues to include the developing chick lung. Our data demonstrate that GH mRNA and protein are expressed in the chick lung from ED7 to ED15, and decline before hatch, at ED20. GH mRNA and protein were localized to mesenchymal, epithelial, and smooth muscle cells, and were also found in blood vessels. The full-length 690bp mRNA transcript was detected, with a sequence identical to the pituitary GH transcript. GH-immunoreactivity was associated primarily with a smaller 15kDa GH-moiety; however, the larger 22kDa isoform was also present. This study clearly demonstrated that the embryonic chick lung is an extrapituitary site of GH expression, and implies potential roles for local GH in chick lung development.

As extrapituitary GH expression in the lung may be species-specific given the notable structural and physiological differences between the avian and the mammalian lung, we chose next to examine the developing rat lung for extrapituitary

GH. Chapter 3 documents GH mRNA and protein within the developing rat lung from ED17 to postnatal day 14. Prenatally, GH mRNA and protein was localized to airway epithelial, smooth muscle, and mesenchymal cells. Postnatally, GH mRNA and protein was detected in Type I and II epithelial cells, and in pulmonary tissue macrophages and alveolar macrophages. The lung GH mRNA sequence was the full length 693bp transcript, with an identical nucleotide sequence to that of pituitary GH mRNA. GH-immunoreactivity was solely with a 22kDa moiety, the same isoform as is found in adult rat pituitary. This study was the first demonstration in the literature of GH expression locally within the developing lung. To date, only GH immunoreactivity from adult and fetal lung extracts has been shown (Kyle et al., 1981; Costa et al., 1993). GH mRNA has been demonstrated in the alveolar macrophages of adult rats. These past studies demonstrated only trace quantities of GH immunoreactive protein and GH mRNA, and only the Allen et al. (2000) study elucidated the source of the GH.

These two descriptive studies on extrapituitary lung GH (chapters 2 and 3) have documented the lung as a GH-producing tissue, and lay the framework for the functional studies of chapters 4-6.

7.1.2 GH in the lung: functional studies

To reveal potential roles for local GH in the lung, we used two models of altered GH expression (antisense oligodeoxynucleotide downregulation of GH expression, and adenovirus-mediated overexpression of GH), and one model of altered GH signaling (GHR knockout mice). We chose the period of alveolarization, the final stage of lung

development, as the time to examine these roles, as it is most conducive to experimental manipulations. These models were examined using a proteomics approach followed by mass spectrometry to determine changes in lung proteins.

Antisense oligodeoxynucleotides to rat GH were used to examine changes resultant from decreased local expression of GH in the lung during the period of alveolarization. This experimental procedure revealed that 45 proteins were changed in relative abundance upon GH-underexpression. Of these, eight proteins were sequenced and found to be metabolic proteins (ATP synthase α and β subunits, electron transfer flavoprotein α and β subunits), a regulator of the cell cycle (calcyclin binding protein), an enzyme for free radical distruction (superoxide dismutase 2), a gene transcription protein (RNA binding protein 3), and albumin. As well, IGF-1 levels were found to be decreased by ELISA. IGF-1 is thought to have roles in angiogenesis and vasculogenesis. These proteins demonstrate potential GH actions in the lung

A mouse GH adenovirus, generously provided by Dr. Bruce J. Baum (Marmary et al., 1999), was used to overexpress full-length mouse GH in the lungs of day 4 postnatal rat lungs. This resulted in altered expression of thirteen lung proteins. These proteins included enzymes (nucleotide diphosphate kinase B, Cu/Zn superoxide dismutase, glutathione s-transferase and aldehyde reductase 1) and structural proteins (beta-5 tubulin), as well as proteins involved in signal transduction (G-protein beta subunit and nucleoside diphosphate kinase B), cell proliferation (retinoblastoma binding protein 4, major acute phase protein and calponin 2), immune regulation (major acute phase alpha-1 protein), and heme scavenging (haptoglobin).

These results support the possibility that GH expression during alveolarization promotes autocrine/paracrine actions involved in lung function.

Finally, we analyzed a GHR knockout mouse, obtained through a collaboration with Dr. John J. Kopchick (Zhou et al., 1997). This mouse has a homozygous disrupted GHR gene. As GHR is the only GH receptor, absence of GHR ablates GH signaling (Zhou et al., 1997). Thirty-nine lung proteins were altered in abundance in this experiment. Of these, seven proteins were sufficiently resolved on the gels and were conducive to mass spectrometry. These were proteins involved in growth (vimentin), proteins involved in oxidative stress (SH3 domain-binding glutamic acid-rich-like protein, Prdx6 and isocitrate dehydrogenase 1), proteins involved in lipid metabolism (Apoa4 and Prdx6), and proteins involved in general metabolism/cell function (electron transfer flavoprotein alpha subunit and proteasome 26S ATPase subunit 4). This study suggests that these proteins are involved in normal signaling of GH through GHR in the lung.

These three functional studies reveal potential metabolic, oxidative stress, and surfactant roles of GH in the developing lung. As locally expressed GH has not been previously suggested in the literature, the descriptive and functional studies reported here form the basis for further investigation into local GH in the lung.

7.1.3 Limitations of these studies

The descriptive studies present a foundation for further understanding of the pattern of GH expression in the developing lung. However, these studies are not comprehensive. They do not examine the earliest stages of lung development (from

the onset of lung budding from the primitive foregut). This means that the original cells to express GH and the stage of development at which GH expression begins is unknown. Analogously, it is not known when (or if) GH expression is turned off, as the latest stage to be examined is late embryonic development in the chick and two weeks postnatally in the rat. An understanding of GH in adult chicks and rats was not sought in these studies, and as such it is unclear whether or not GH has roles in the lung other than during development.

GHR is still poorly understood during lung development. Although GHR mRNA and protein have been shown in the chick lung (chapter 2) and previously in the rat lung (Garcia-Aragon et al., 1992; Batchelor et al., 1998), it is unclear if GHR is inserted into the membrane, and if this GHR binds GH. Induction of proteins following GH administration to fetal rat lungs has been shown (Batchelor et al., 1998). This suggests a functional GHR in the late fetal rat lung, but further GH-GHR binding studies in the lung are required.

As liposomes only have the potential to merge with the membrane of one cell and the mouse GH adenovirus is replication deficient, changes in proteins in chapters 4 and 5 likely reflect effects only on the type I and II epithelial cells of the lung. Given that GH is widely expressed in the rat lung (chapter 3), new models need to be designed that can alter GH expression in all parts of the lung. This is especially true for the mesenchymal cells, as much of the lung (including the pulmonary vasculature) develops from these GH expressing cells.

The GHR knockout mouse model has non-functional GHR in all tissues, not specifically the lung. However, GH expression is not altered. The results obtained in

Chapter 6 may be a summation of effects of both pituitary GH and local lung GH on lung GHR. To confirm that the changes in protein levels in the lung are due solely to lung GH, a lung-specific ablation of GHR must be designed.

7.2 Overall Discussion

7.2.1 Extrapituitary GH

Expression of GH in sites other than the pituitary gland has been demonstrated in many tissues in the chick (Harvey and Hull, 1997; Harvey et al., 2000). Expression of GH mRNA is detected in the chick brain (Render et al., 1995), neural retina (Harvey et al., 2004), and spleen (Render et al., 1995). Extrapituitary GH in these tissues is thought to act through autocrine/paracrine mechanisms (Harvey et al., 2000). Data presented in Chapter 2 supports a similar mechanism for chick lung GH. As GH is expressed in mesenchymal, epithelial, and smooth muscle cells throughout embryonic development, it follows that locally expressed GH is involved in chick lung development. The theory of autocrine/paracrine actions for extrapituitary GH is less well developed in the rat.

GH mRNA in the rat has been detected in the brain (Martinoli et al., 1991), mononuclear leukocytes (Weigent et al., 1992), thymus, liver, spleen, ileum, smooth muscle and endothelial cells of blood vessels (Recher et al., 2001), and in resident alveolar macrophages (Allen et al., 2000). As such, our data (Chapter 3) demonstrate the first instance of GH mRNA expression within the lungs. However, presence of GH mRNA and protein in the developing rat lung do not imply action. To examine if local GH in the rat lung has actions, we designed three functional studies (Chapters 4-

6). We proposed that extrapituitary GH in the developing rat lung acts as an autocrine/paracrine.

7.2.2 GH as an autocrine/paracrine in the lung

GH has been suggested as an autocrine/paracrine in many tissues. The local production of GH in the nervous system (Harvey and Hull, 2003), immune system (Weigent et al., 1991; Sabharwal and Varma, 1996; Arnold and Weigent 2004), reproductive system (Hull and Harvey, 2000; Luna et al., 2004), mammary glands (van Garderen et al., 1997) and teeth (Zhang et al., 1997) is thought to induce autocrine or paracrine actions involved in tissue development or differentiation.

Some studies have directly demonstrated autocrine/paracrine actions of local GH. Human thymocytes produce and secrete GH (Sabharwal and Varma, 1996). Upon use of a GH polyclonal antibody in human thymocyte cell culture, thymocyte proliferation was markedly inhibited (Sabharwal and Varma, 1996). This is a demonstration of the GH-GHR autocrine/paracrine loop, since the antibody-GH interaction prevents GH binding to the GHR in the same and neighbouring cells. Similar results have been found through the use of anti-GH antibodies in cultured myoblasts (Segard et al., 2003). This study, however, involved overexpression of GHR. Myoblasts treated with GH did not proliferate, whereas those that overexpressed GHR proliferated in response to GH. This effect was ablated on use of anti-GH antibodies. Since the importance of GHR has been demonstrated in this model, this study supports autocrine/paracrine actions of GH.

Local GH expression in the rat lung (chapter 3) has led us to propose a similar role for lung GH. Since GH mRNA expression and GH-immunoreactivity are widespread in the late fetal and early postnatal rat lung, it appears that GH is important in many cell types, and not specific to either the mesoderm or the endoderm. This is in contrast to many molecules in lung development, such as FGF-10 and Shh, which are specifically expressed in the mesoderm (Bellusci et al., 1997a) and endoderm (Cardoso 2001), respectively. The role of GH may in fact be more akin to that of EGF, which like GH, is expressed throughout the developing lung, in the mesoderm and the endoderm (Snead et al., 1989). EGF is also believed to have roles in lung maturation (Sundell et al., 1980) and surfactant synthesis (Higuchi et al., 1989). Given the distribution of GH in the developing lung, it is likely that GH behaves as a growth and differentiation factor like EGF, rather than a signaling/patterning molecule like FGF-10 and Shh. Regardless of the resultant roles of GH, the mechanism of action is likely autocrine and/or paracrine.

Functional studies (Chapters 4-6) designed to test the hypothesis that GH acts as an autocrine/paracrine in the developing lung resulted in the elucidation of many proteins which are GH regulated. The alteration of lung proteins involved in such essential lung functions as anti-oxidant defense and surfactant metabolism establish local GH as an intricate part of the development of the functional rat lung.

7.2.3 Local GH in the development of the rat lung

Lung development is currently thought to be mainly regulated by fibroblast growth factors (Bellusci et al., 1997b; Cardoso 2001), sonic hedgehog (Litingtung et al.,

1998; Pepicelli et al., 1998), bone morphogenetic proteins (Bellusci et al., 1996), transforming growth factor beta (Serra and Moses, 1995; Zhao J et al., 1996), retinoic acid (Dickman et al., 1997), insulin-like growth factors (Lallemand et al., 1995), vascular endothelial growth factor (Park et al., 1993; Ng et al., 2001), and epidermal growth factor (Sundell et al., 1980). Since prenatal growth is considered by many to be a "growth without growth hormone" syndrome (Geffner 1996), potential roles for GH early in development of organs have been widely ignored. With the documentation of local GH expression in the developing lungs, combined with the recognition of the ability of GH to act as a local cytokine (Waters et al., 1999), GH should now be considered a molecule with roles in lung development. Of the several roles of GH in lung development which we propose through our functional studies, the discussion here will be limited to those which I believe to be the most important: response to oxidative stress, lipid/surfactant metabolism, and general metabolism/growth.

Oxidative stress is particularly important in the normal functioning of the lung. During the postnatal period, the lung acquires its ability to deal with this stress (Land and Wilson, 2005). Our studies have shown that Cu/Zn superoxide dismutase (SOD), SH3 domain-binding glutamic acid-rich-like protein, peroxiredoxin 6 and isocitrate dehydrogenase to all be controlled by lung GH. These proteins are essential components of the lung's protection against oxidative stress. Cu-Zn SOD expression and enzymatic activity have been shown to rise late in gestation, and are important for degradation of superoxide upon transition to air breathing and for oxygen scavenging in rats postnatally (Clerch et al., 1992; Nozik-Grayck et al., 2000). Studies on the

prenatal and postnatal rat (Chapter 3) have demonstrated GH expression during this time frame, supporting a role for GH in control of Cu/Zn SOD expression in the lung. SH3 domain-binding glutamic acid-rich-like protein is a member of the thioredoxin superfamily, which is a major component of the lung anti-oxidant machinery Nakamura et al., 2005). Peroxiredoxin 6 is an enzyme that is involved in oxidative defense against reactive oxygen species (Wang et al., 2004). It is found predominantly in the lung, specifically within alveolar Type II cells, alveolar macrophages and in bronchiolar epithelium (Kim et al., 1998), cell types that we have shown to express the GH gene (Chapter 3). Finally, isocitrate dehydrogenase was also GH controlled. Isocitrate dehydrogenase generates reduced glutathione (Murakami and Yoshino, 2004), which protects the lungs from oxidative stress (Rahman 1999). Through the actions of all of these GH-regulated proteins, it is evident that GH has important roles in the development of the lung's ability to deal with oxidative stress. Although the above proteins have been identified by our studies, conclusions drawn here are likely simplistic. GH is also known to activate macrophages to produce reactive oxygen species (Edwards et al., 1992). As alveolar macrophages are resident in the alveoli, and since they have been shown to contain GH (Chapter 3) they may also be involved. Perhaps GH can both activate alveolar macrophages to produce reactive oxygen species that destroy invading microorganisms, and control the production of enzymes that degrade reactive oxygen species and thereby protect the epithelium from damage. The lung is very prone to infection. GH may constitute part of the lung's defense against foreign microbes.

Lipid metabolism and surfactant production are essential to the maintenance of alveolar stability and of efficient gas exchange (Berhard et al., 2001). The GHR knockout mouse revealed that peroxiredoxin 6 (Prdx6) and apolipoprotein A IV(Apoa4) are controlled by GH signaling in the developing lung. In addition to the roles of Prdx6 in the control of lung defenses against reactive oxygen species, it is also a potent regulator of phosphatidylcholine synthesis, a precursor of lung surfactant (Fisher and Dodia, 1997). Through its action on Prdx6, GH may be an upstream regulator of phospholipid synthesis in the developing rat lungs. Another stage at which GH may control surfactant is indirectly through lipid metabolism. Apoa4 was found to be GH-responsive, and is primarily involved in the metabolism of triglycerides and high-density lipoproteins (Bai et al., 1996). Through its control of lipid metabolism and synthesis of surfactant precursors, GH may have important roles in development of the lung surfactant system.

Finally, the models of altered GH signaling that have been described in our studies have revealed proteins with general metabolic and growth roles that are controlled by local GH in lung development. These include aldehyde reductase 1, chromatin assembly factor 1 (CAF1), major acute phase protein 1, and vimentin. Aldehyde reductase 1 is an enzyme that is induced by basic fibroblast growth factor (bFGF) (Laeng et al., 1995). bFGF is induced by GH (Izumi et al., 1995) and GH and bFGF act together to promote growth (Edmondson et al., 1999; Waters et al., 1999). Through this GH-bFGF-aldehyde reductase 1 pathway, we believe that GH promotes lung growth. This needs to be further elucidated with studies on bFGF. CAF-1 is a marker of proliferating cells (Polo et al., 2004). GH may thus be involved cell

proliferation during alveolarization. Major acute phase protein 1 was found to decrease in response to GH overexpression. This protein normally inhibits cell proliferation, especially in fibroblasts (Torres et al., 2001). The downregulation of this protein in response to elevated GH may be an indirect mechanism through which GH promotes cell proliferation. Vimentin is an intermediate filament expressed in fibroblasts, mesenchymal cells and endothelial cells (Wang and Stamenovic, 2002), including those that are responsive to GH action (Tseng et al., 1997). Vimentin promotes cell migration and proliferation (Wang and Stamenovic, 2002; Pelosi et al., 2003). Expression of vimentin has been localized to Type I and Type II epithelial cells (Kasper et al., 1993; Koslowski et al., 2004), cells that we have shown to express GH (chapter 3). Through GH-control of the expression of these four proteins, GH is important in the control of lung growth through effects on metabolism and cell proliferation. GH has been shown numerous times, and in many tissues to be a potent regulator of metabolism and growth. The lung is likely not an exception. The widespread distribution of GH in the lung implies that it has more general growth and/or metabolic roles that are important to all cells in the developing lung. In addition to the specific roles for GH previously discussed, these general roles may be just as important.

Our studies form the foundation for understanding potential roles for local GH in the developing lung and will guide future research in this field. I will close with a brief discussion of potential future clinical applications and studies that I believe are logical next steps in an understanding of the role of local GH in lung development.

7.2.4 Future clinical applications

The identification of a novel molecule involved in the growth of a particular tissue invokes interesting possibilities for clinical applications. Many disorders of the lung have been recognized, from those that are known to involve altered GH levels (acromegalics, growth hormone deficiency), to many other disorders that may indirectly involve GH but have not yet been recognized as such. Our functional studies have documented two methods to alter gene expression in the lung (antisense oligodeoxynucleotide mediated gene downregulation, and adenoviral mediated transgene delivery). These are potential ways to alter GH expression clinically in the lung. However, these methods are limited in scope in that they are designed for short term experimental manipulations, not for the long term treatment of chronic disease. Thus, other methodology may be more amenable to clinical practice.

Selective overexpression of GH has been used previously to alter physiological parameters. Selective cerebral overexpression of GH alters cardiac function, morphology, and energy metabolism (Bohlooly-Y et al., 2005). Although it is unclear the particular mechanism by which overexpression of GH in the brain controls heart function, this study demonstrated that experimentally altered GH expression has definitive physiological consequences. Perhaps in an analogous manner, overexpression of GH in the medulla (which controls breathing rate) may have distinct consequences in pulmonary function. More relevant to autocrine/paracrine lung GH, altered GH expression in the lung may be useful in treating disorders in lung function. The lung is a major gene therapy target for the treatment of chronic disorders that are both genetically inherited and those that have

been acquired through lifestyle (Gill et al., 2001). The problem with most gene therapy in the lung is that viral and non-viral expression vectors are short-lived, which makes them ideal for experimental manipulations but insufficient for the treatment time frame of years for chronic diseases. Due to this limitation, researchers have designed plasmid vectors that link transgenes to promoters of genes that are constitutively expressed in the lung, such as ubiquitin C (Gill et al., 2001). Strategies such as this have resulted in more stable transgene expression over time. This could be employed with GH. For disorders that result as from insufficient GH, GH could be linked to this promoter and transfected into the lung. For disorders that are caused by excess GH (eg. Acromegaly), a GH-antagonist could be linked to this promoter. These models could be used to control GH levels in the lung, and perhaps provide therapy for clinical conditions that are resultant from local GH imbalance. For instance, decreased/normalized GH expression in acromegalics may be used to prevent upper airflow obstruction and narrowing of the small airways. As GH-levels are probably closely regulated in the lung, mastery of gene therapy techniques to achieve predictable and constant levels of GH requires extensive experimentation. This type of approach may not be feasible for some years to come, and would rely on a much deeper understanding of the role of GH in the lung during development, and throughout life. Further experimentation on the basic physiology of GH in the lung may provide these answers.

7.2.5 Suggested future studies

Studies presented here (Chapters 2-6) have revealed both the expression of GH in the developing lungs, and potential roles for local GH in lung development. As this topic is novel, there are many aspects of this system that are currently unanswered. Further experimentation with clarify the part that local GH plays in lung development.

In addition to the experimental manipulations that we have performed, there are many other possibilities. Other models include Ames and Snell dwarf mice, GH antagonist mice, and targeted gene therapy (described above). These models can be assessed through a proteomics approach, as we have used, as well as the analysis of many other endpoints.

Our studies did not provide information on tissue growth of the lungs. The lung weights and lung to body weight ratios of these models need to be assessed to suggest general roles of GH in lung growth. Furthermore, lung architecture and maturity can be assessed through histological and morphological studies. These would reveal if GH expression levels were related to lung maturation. In addition to these studies, data is required regarding pulmonary function.

Pulmonary function endpoints will provide distinct physiological clues as to the potential results that therapeutic treatment of chronic disorders could provide. Endpoints such as vital capacity, tidal volume, and forced expiratory volume are clinically relevant measures of lung function. As well, surfactant production should be measured in altered GH states to confirm our theory regarding GH and surfactant synthesis. These studies represent only first steps in an understanding of lung GH.

The results provided by these experiments will guide further research and understanding in this field.

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