Characterization of Mesangial Cell Lines Established from Nontransgenic (NT) and Growth Hormone Receptor Knockout (GKO) Mice

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This thesis titled
Characterization of Mesangial Cell Lines Established from Nontransgenic (NT) and
Growth Hormone Receptor Knockout (GKO) Mice

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ABSTRACT

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Characterization of Mesangial Cell Lines Established from Nontransgenic (NT) and Growth Hormone Receptor Knockout (GKO) Mice (87 pp.)

Director of Thesis: Karen T. Coschigano

Growth hormone (GH) is a peptide hormone that is secreted from the anterior pituitary under the regulation of different proteins, hormones, amino acids and nutrients. GH and its signaling pathways regulate cellular growth, development and metabolism. Involvement of GH in damaging the kidney, especially in diabetes, is a matter of concern as diabetic nephropathy has been regarded as an increasing health threat. Establishment of mesangial cell lines could provide an effective in vitro model to investigate a connection between individual/multiple downstream signaling pathway/s of GH signaling and damage of kidney mesangium and thus was the topic of this thesis. The primary step of establishing this in vitro model was to characterize the presence or absence of functional GH signaling in mesangial cell lines established from the nontransgenic (NT) and growth hormone receptor gene-disrupted or knockout (GKO) mice. Glomeruli from the kidneys of NT and GKO mice were isolated and used to establish two mesangial cell lines. Specific morphology, selective media and immunohistochemistry confirmed the identity of the cells as mesangial cells. Next, expressed GHR transcripts were characterized in the mesangial cell lines by performing RT/PCR and gel electrophoresis. Expression of an intact GHR transcript in NT mesangial cells and a stable but mutated GHR transcript in GKO cells was demonstrated. Finally, the presence or absence of GH
signaling in NT and GKO mesangial cell lines was tested by assaying the induction of RNA transcripts or phosphorylated proteins of downstream signaling pathways in response to exogenous GH stimulation using real-time RT/PCR, western blotting analysis or ELISA. The only response observed was GH stimulation of iNOS and STAT5b mRNA expression in both the NT and GKO mesangial cell cultures, suggesting that mesangial cells from the NT mice might be useful for studying the GH-stimulated expression of specific GH responsive genes but the cells from the GKO mice may not provide the expected contrast of lack of GH signaling. It will be important to further evaluate GH signaling and responses in both the NT and GKO mesangial cells to firmly establish the role of GH in mesangial cells.

Approved:_______________________________

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CHAPTER 1 INTRODUCTION

1.1 Growth Hormone and Its Signaling

Growth hormone (GH) is a single chain polypeptide hormone composed of 191 amino acids, which is secreted from the somatotrophs of the anterior pituitary in a pulsatile manner. Secretion occurs more frequently in the female (approximately every hour in a 24 h cycle) than in the male (approximately every 3 h in a 24 h cycle), but the peak values are considerably higher in the male than in the female (Ross and Buchanan, 1990; Terry et al., 1977). The secretion of GH is regulated by growth hormone releasing hormone, somatostatin, ghrelin, neurotransmitters, metabolic factors, and thyroid and steroid hormones. GH regulates cell development, metabolism and growth by binding with its receptor (GHR), which triggers several downstream signaling pathways (Lanning and Carter-Su, 2006; Lodish et al., 2007).

GHR structurally belongs to the cytokine haematopoietin superfamily, which includes receptors for prolactin, erythropoietin, leptin, thrombopoietin, interleukin 3/5/6, and interferon (Rosenfeld and Hwa, 2009). GHR mRNA has been cloned in several species, including human and mouse, and deduced amino acid sequences give a clear idea of the structure of GHR, varying little among species (Rosenfeld and Hwa, 2009). In general, GHR is a trans-membrane glycoprotein of 620 amino acids. Initially, a pre-peptide of 640 amino acids is formed that is targeted to the cell membrane due to the presence of an N-
terminal signal peptide. After removal of the signal peptide and partial passage through
the membrane, the mature GHR possesses three domains: an extracellular domain of
approximately 245 amino acids that varies based on species and interacts with GH, a
hydrophobic transmembrane domain of 24 amino acids, and an intracellular domain of
approximately 350 amino acids (Edens and Talamantes, 1998). In humans, the ATG and
most of the signal sequence is located in exon 2, the membrane-spanning region is in
exon 8 and the stop codon is in exon 10. The mouse gene is nearly identical to the human
GHR gene with two exceptions. The addition of exon 4B between exons 4 and 5 adds a
unique eight amino acid region to the mouse GHR extracellular region. The addition of
exon 8A between exons 7 and 8 introduces a hydrophilic tail and stop codon that
provides the means for making the GH binding protein (GHBP) via alternative splicing
rather than via proteolytic cleavage of the GHR protein as seen in humans. This binding
protein circulates throughout the body with a binding affinity for GH similar to that of the
receptor. The actual role for GHBP is still unknown, but it may increase the activity of
GH \textit{in vivo} by complexing with GH and enhancing its half life or it may attenuate cellular
responses to GH as GHBP competes with circulating GH in binding GHR (Edens and
Talamantes, 1998).

Binding of GH with its receptor is an essential, critical step in triggering downstream
signaling cascades. Predimerized GHR binds to the asymmetrically located binding sites
on GH and is followed by a rotation of helices of the GHR (Lichanska and Waters, 2008).
This repositioning of the receptor dimers creates a conformational change that transmits
through the transmembrane domain to the intracellular domains, leading to the alignment of two pre-bound Janus Kinase (JAK) 2 molecules. The adjacent JAK2 molecules transphosphorylate each other and then phosphorylate tyrosine residues in the intracellular domains of GHR (Brown et al., 2005; Rosenfeld and Hwa, 2009). Phosphorylated tyrosines of the GHR act as docking sites for downstream signaling molecules like signal transducer and activator of transcription (STAT) 5 and Sarcoma homology 2a collagen-related (Shc), and binding of these downstream molecules triggers signaling cascades that activate additional downstream genes and/or effector molecules like mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K). Activation of STAT5 and MAPK by GH was used to assay the presence of functional GH signaling in the current study.

STAT5 was first isolated as a single gene but later two separate genes encoding STAT5a and STAT5b were isolated. STAT5a and STAT5b share 95% identity in the amino acid sequences (Lanning and Carter-Su, 2006; Teglund et al., 1998). Activation of the STAT proteins takes place by binding of STAT5 to the phosphorylated GHR tyrosines followed by phosphorylation of STAT5 tyrosine residues by JAK2 (Figure 1) (Lanning and Carter-Su, 2006; Rosenfeld and Hwa, 2009). Numerous studies have demonstrated activation of STAT5 phosphorylation upon GH stimulation. One such study showed STAT5 phosphorylation upon treatment of mouse fibroblast cells stably expressing porcine GHR with porcine or bovine GH (Wang et al., 1994). Phosphorylation of STAT5 was also seen in vivo after injection of mice with human GH (Gevers et al., 2009; List,
The phosphorylated STAT5 molecules dimerize and translocate to the nucleus where they initiate transcription of genes necessary for cell growth and development (Lanning and Carter-Su, 2006). For example, STAT5a is an imperative factor in developing mammary gland and lactogenesis (formation of milk) during pregnancy and STAT5b regulates the sexual dimorphic expression of several cytochrome P450 molecules (Lichanska and Waters, 2008; Liu et al., 1997). STAT5b has also been implicated in the GH-dependent transcriptional activation of insulin-like growth factor -1 (IGF-1) (Chia et al., 2006; Woelfle et al., 2003). Induction of IGF-1 RNA expression by GH was also used to assay the presence of functional GH signaling in the current study.

Figure 1: GH and two of its downstream signaling pathways. GH binds to the transmembrane GHR and triggers downstream signaling cascades such as GHR-JAK2 mediated STAT5 and MAPK phosphorylation to activate gene expression within the nucleus. TF, transcription factor. This figure was adapted from Kopchick et. al (1999).
MAPK is another downstream molecule activated by GH. The pathway to activate MAPK starts with the binding of the adaptor protein Shc with the phosphorylated GHR-JAK2 molecule (Figure 1). JAK2 phosphorylates Shc, which then binds growth factor receptor-bound protein 2 (Grb2), a cytosolic protein constitutively bound with Son of sevenless (SOS), a guanidino nucleotide exchange factor (Lanning and Carter-Su, 2006; VanderKuur et al., 1995). The Shc-Grb2-SOS complex then binds the membrane-bound protein rat sarcoma viral oncogene homolog (Ras) (Lodish et al., 2007). Ras, a monomeric protein, is inactive in its GDP-bound state. The Shc-Grb2-SOS complex induces a conformational change in Ras that releases the GDP and allows Ras to bind GTP. GTP bound active Ras moves into the cytosol to activate downstream serine/threonine kinase Raf (Lodish et al., 2007). Raf thereby phosphorylates MEK (MAPK kinase or MAP/ERK kinase), which in turn phosphorylates one tyrosine (Y204) and one threonine (T202) residue on MAPK (Argetsinger and Carter-Su, 1996; Lodish et al., 2007). Active MAPK is a serine/threonine kinase that is also known as extracellular-signal-regulated kinase or ERK1/2 (Lodish et al., 2007). MAPK phosphorylates various proteins such as phospholipase A, s6 kinase p70^{rsk}, p90^{rsk} and transcription factors including c-jun and p62^{TCF}-ELK1 that are involved in cell growth and development, adipocyte differentiation, and expression of cytochrome enzymes (Lanning and Carter-Su, 2006). MAPK phosphorylation upon hGH stimulation was observed in a mouse myeloid cell line and pGH stimulation was seen in a mouse macrophage cell line (Rowlinson et al., 2008; Tripathi and Sodhi, 2009).
Many of the studies defining the various signaling pathways of GH were performed *in vitro*. In order to study GH signaling *in vivo*, mice with a disrupted growth hormone signaling receptor gene (growth hormone receptor knockout or GKO) were created (Zhou et al., 1997). These mice also served as a mammalian model of a special kind of dwarfism called Laron syndrome, which is characterized by genetic growth hormone resistance along with decreased serum IGF-1 and elevated GH levels and decreased GH signaling in patients (Zhou et al., 1997). Laron syndrome results from mutations such as deletion, frameshift, missense, nonsense and splice site mutations in the GHR gene exons, especially within exon 4, which encodes a portion of the extracellular GH binding domain of the GHR protein (Zhou et al., 1997). Creation of the mouse model of Laron syndrome involved deleting the 3’ end of exon 4 and part of the following intron, targeting exon 4 as was often seen in Laron syndrome patients (Zhou et al., 1997). Mice homozygous for the GHR exon 4 deletion lacked detectable GHR mRNA and protein and were dwarf with low serum IGF-1 and high serum GH levels, as seen in the human patients (Zhou et al., 1997). These mice, when compared with their normal, wildtype littermates, provide a valuable means of evaluating the role of GH signaling in the whole body, in an organ, or in a cell. For example, amounts of fat deposition differed by location between NT and GKO mice, suggesting that GH signaling plays different roles in different parts of the body (Berryman et al., 2004). Additionally, GKO mice lived longer than NT littermates, indicating that GH signaling affects longevity (Coschigano et al., 2000). Thus, NT and GKO mice, and perhaps even specific tissue cultures from them,
should serve as good models to compare the effect of presence and absence of GH signaling in characterizing the multiple roles of GH.

1.2 Physiology of the Kidney

The kidney is a large, bean-shaped organ, two of which are located near the back of the abdominal cavity (Eroschenko, 2007). Blood from the body enters into the kidney via the renal artery and leaves via the renal vein. The kidney is covered by a thick, connective tissue and has two regions inside, the cortex and medulla, which are the outer and inner sections, respectively (Figure 2). The main role of the kidney is to maintain homeostasis, including regulation of blood pressure, blood composition, fluid volume and acid-base balance (Eroschenko, 2007). One outcome of this is urine production, which involves three main processes: a.) filtration of blood in the glomeruli; b.) resorption of nutrients and other needed substances from the filtrate; and c.) secretion of metabolic waste products and unwanted molecules into the filtrate for excretion as urine (Eroschenko, 2007). Glomerular filtration rate is a measure of flow rate of the filtrate through the kidney and is a useful indication of kidney function.
The nephron is the functional unit of the kidney, made up of a corpuscle, or glomerulus, and surrounding Bowman’s capsule, and a renal tubule (Figure 2). The glomerulus contains a tuft of capillaries where the initial filtration occurs (Figure 3) (Eroschenko, 2007). The resident cell types of the glomerulus are endothelial cells, parietal epithelial cells, visceral epithelial cells or podocytes, and mesangial cells. Endothelial cells form the lining of the capillaries and, through fenestrations, or transcytoplasmic holes, allow the filtration of low-molecular-weight waste products from the blood and provide a barrier to larger molecules (Satchell and Braet, 2009). The function of parietal epithelial cells, which line the Bowman’s capsule, is not clear but they may serve to confine macromolecules such as proteins in the glomerular filtrate to the Bowman’s space and prevent leakage into the extraglomerular space (Ohse et al., 2009). The function of podocytes includes turnover of glomerular basement membrane, regulating the
glomerular filtration and also supporting the glomerular capillaries (Mundel and Kriz, 1995).

The fourth type of cells in the glomerulus, and the cell type of interest in this thesis, is mesangial cells. They have characteristics of smooth muscle cells and thus are enriched in actin and myosin filaments that enable mesangial cells to constrict and dilate under the influence of vasoconstrictors and vasodilators as observed in vitro. Thus, mesangial cells can regulate the movement of associated glomerular capillaries and can influence the blood flow, blood pressure and glomerular filtration rate (Mene, 1996; Schlondorff, 1987). Apart from the smooth muscle activity that is contraction and expansion, mesangial cells produce extracellular matrix, which also takes part in blood filtration because of its three dimensional meshwork structure (Mene, 1996). Additionally,
mesangial cells produce prostaglandin and cytokines and are involved in the formation and breakdown of glomerular membrane and also take up macromolecules including immune complexes (Schlondorff, 1987). Mesangial cells can serve as a good \textit{in vitro} model if cultured to prevent the growth of other glomerular cell types. One main advantage of culturing mesangial cells, but not other glomerular cells, is a multi-layered growth pattern ("hills and valleys") that is not subject to contact inhibition. Thus, mesangial cells are able to easily proliferate in growth media (MacKay et al., 1988; Wilson and Stewart, 2005).

The filtration function of the kidney can be disrupted due to damage or disease in the kidney, the source of which could be either congenital or acquired (Emanuel Rubin, Howard M. Reisner, 2008). Congenital or inherited kidney diseases include horseshoe (fused) kidney and autosomal dominant or recessive polycystic kidney diseases. The acquired kidney diseases include diabetic nephropathy, lupus nephropathy, and kidney damage caused by inflammation and immune complexes. Decreased glomerular filtration rate, proteinuria (release of proteins in urine), and hematuria (release of blood in urine) indicate the disruption of kidney function. Although the pathophysiology of damaged kidneys varies depending on the type of disease, it often includes cysts (in the case of congenital kidney diseases), necrosis, lesions and damage of kidney capillaries, and hyperplasia with gross enlargement of glomeruli (Emanuel Rubin, Howard M. Reisner, 2008). Among the kidney diseases, diabetic nephropathy, which is caused by diabetes mellitus, has been regarded as one of the main reasons of end stage renal disease or
kidney failure (Tervaert et al., 2010). The stages of the advancement of diabetic nephropathy are physiologically classified as thickening of glomerular basement membrane, mild mesangium expansion, severe mesangium expansion, nodular sclerosis and finally advanced diabetic glomerulosclerosis (Tervaert et al., 2010). Mesangium expansion is characterized by increased deposition of mesangial extracellular matrix and an increase in the volume of mesangial cells, or cellular hypertrophy (Tervaert et al., 2010). The mild or severe expansion of the mesangium matrix could be in response to insulin, glucose, angiotensin, or different growth factors such as platelet derived growth factor A or transforming growth factor beta, as well as to nitric oxide production by nitric oxide synthase from arginine (Abrass, 1995; Narita et al., 1995). Moreover, reactive oxygen species generated by the hyperglycemic state in diabetes also contribute to the extracellular matrix deposition in diabetic kidney glomeruli (Lee et al., 2003). The dysfunction of the kidney in the early stages of diabetic nephropathy is suggested to be characterized by hyperfiltration and microalbuminuria, which has been associated with increased production of nitric oxide (Prabhakar, 2004).

1.3 Involvement of GH and Its Signaling in Kidney Damage

Involvement of GH in kidney damage has been suggested through different studies. Expression of GHR has been seen in renal proximal tubules, podocytes and mesangial cells, suggesting that these cells are capable of responding to GH (Doi et al., 2000; Rabkin and Schaefer, 2004; Reddy et al., 2007). It has been shown that GH can
specifically stimulate the synthesis of nitric oxide as well as the enzyme that produces it, nitric oxide synthase, in mesangial cells (Doi et al., 2000). As mesangial matrix production is stimulated by nitric oxide activity, the authors suggested that GH may be playing a role in this stimulation (Doi et al., 2000). GH signaling was observed in podocytes where it is involved in the development and polymerization of the cytoskeleton and activation of downstream JAK2/MAPK and STAT5 pathways (Reddy et al., 2007). This same report also demonstrated that GH treatment can lead to increased levels of reactive oxygen species and thus suggested a role for GH in the development of diabetic kidney damage (Reddy et al., 2007). Previous reports have shown that transgenic mice over-expressing bovine GH developed glomerulosclerosis, including lesions in the mesangium and expansion of mesangium and also increased accumulation of the extracellular matrix protein collagen IV in comparison to the normal littermates (Doi et al., 1988; Yang et al., 1993a). In contrast, mesangium expansion was attenuated in diabetic mice with decreased or disrupted growth hormone signaling in comparison to the diabetic littermates having intact growth hormone signaling (Bellush et al., 2000; Yang et al., 1993b). Thus, GH and its signaling pathways may play a role in damaging the kidney whereas disruption of GH signaling possibly protects the kidney from damage.
CHAPTER 2 HYPOTHESIS AND SPECIFIC AIMS

It could be postulated that GH might have involvement in kidney damage, perhaps through a single or multiple downstream pathways. However, direct involvement of GH signaling in kidney damage has not yet been investigated. As diabetic nephropathy is an increasing health threat, and expansion of the mesangial matrix is a hallmark event for this disease, investigation of involvement of specific downstream signaling pathways of GH in the expansion of the mesangium is critical. Establishment of mesangial cell lines, one with intact GH signaling and another with disrupted GH signaling, could be an effective *in vitro* model. The primary step of utilizing this model was to establish the presence or absence of functional GH signaling in mesangial cell lines established from the normal and GHR gene-disrupted mice, respectively, as this had never before been done. Thus it was hypothesized that a *mesangial cell line established from nontransgenic (NT) mice will exhibit functional growth hormone (GH) signaling whereas a mesangial cell line established from growth hormone receptor knock out (GKO) mice will not exhibit functional GH signaling.*

**Specific aim 1:** To establish primary cell cultures and demonstrate the identity of the cultured cells as mesangial cells by morphological appearance, growth on D-valine containing selective growth media and presence/absence of specific characteristic proteins by immunohistochemistry.
Specific aim 2: To characterize the GHR mRNA transcripts produced in the NT and GKO mesangial cell cultures using RT/PCR and specific primer pairs.

Specific aim 3: To demonstrate the presence or absence of functional GH signaling in NT and GKO mesangial cells, respectively, by assaying the presence of RNA transcripts or phosphorylated proteins of downstream signaling pathways in response to exogenous GH stimulation using real-time RT/PCR, western blotting analysis or ELISA.
CHAPTER 3 METHODOLOGIES

3.1 Animals

The growth hormone receptor/binding protein gene-disrupted mouse line used in this study was descended from chimera #8 of the original line created by Zhou et al. (1997). For this study, a colony was maintained in the Ohio University Life Science Building animal facility. Mice were housed in microisolator cages on a 14 h light/10 h dark cycle and had free access to water and standard rodent chow (Prolab RMH 3000, PMI Nutrition International, Inc., Brentwood, NJ). All of the mice were of the same mixed genetic background, a combination of 129 Ola and Balb/C, but the nontransgenic (NT or +/-) mice in the study came from crosses of +/- males and females while the homozygous knockout mice (-/-; henceforth referred to as growth hormone receptor knockout or GKO) mice came from crosses of -/- males and females. All procedures were approved by Ohio University’s Institutional Animal Care and Use Committee and met local, state and federal guidelines.

3.2 Glomerular Isolation

Glomeruli were isolated from a single 55 days old NT male and a single 51 days old GKO male essentially as described by Takemoto et al. (2002) (Figure 4). In short, each mouse was administered 17 µl avertin (1.4% solution of avertin stock [25 g
tribromoethanol + 19.5 ml tert-amylalcohol] in water) per g body weight by intraperitoneal injection. The unconscious mouse was perfused via the heart by gravity flow (~3 ml per minute) with 8×10^7 inactivated 4.5 µm Dynabeads (Dynabeads® M-450 Tosylactivated, Invitrogen, Carlsbad, CA) in 30 ml phosphate-buffered saline (PBS). After perfusion, the kidneys were removed, minced well and then digested in 1 mg/ml collagenase A (Roche Scientific, Indianapolis, IN), 100 U/ml deoxyribonuclease I (Invitrogen) in Hanks’ Balanced Salt Solution (HBSS; BioWhittaker, Walkersville, MD) at 37°C for 30 minutes with gentle agitation. The digested mixture was passed sequentially through two 100 µm cell strainers (BD Falcon, Franklin Lakes, NJ) with gentle pressing. The filtered material was centrifuged at 1200 rpm (IEC CENTRA 7R, Kent City, MI) at 4°C for 10 minutes. The cellular pellets were resuspended in ice-cold
HBSS and the glomeruli were collected using a Magnetic Particle Concentrator (Invitrogen, Carlsbad, CA), washed twice with ice-cold HBSS and then resuspended in 500 µl HBSS. The glomeruli were counted manually in five 10 µl drops using bright field microscopy at 40X magnification, averaged, and then the number adjusted for total volume. Approximately 8100 and 6900 glomeruli were retrieved from the NT and GKO kidneys, respectively.

3.3 Mesangial Cell Culture

To establish mesangial cell lines, the glomeruli isolated from the NT and GKO mice were seeded in 20% FBS DMEM-HAM’s media [DMEM-HAM’s F12 (3:1) (Biowhittaker, Walkersville, MD); 20% FBS (16000-069, Invitrogen, Carlsbad, CA); 100 U/ml penicillin, 100 µg/ml streptomycin (Cellgro, Manassas, VA); 1 mM glutamine, 0.75% sodium carbonate (Invitrogen); and 11 mM glucose (EMD, Gibbstown, NJ)] in T25 flasks (Nunc, Rochester, NY) pre-coated with fibronectin (1.5 ml of 0.05 mg/ml fibronectin (Thermo Fisher Scientific, Pittsburgh, PA) per T25 flask incubated for 1 h at room temperature, rinsed with sterile water and then air-dried) and grown at 37°C, 5% CO₂. Three days after seeding, the growing media was supplemented with fresh 20% FBS DMEM HAM’s media. Four days later, the cells were detached from the flasks using 0.25% trypsin-EDTA (Invitrogen), rinsed and transferred to T25 flasks in 20% FBS DMEM-HAM’s media (passage 1). Four days later they were again transferred to new T25 flasks (passage 2). At ~80% confluence, the cells were transferred to T75 flasks.
At ~80% confluence, the cells in each T75 were split into four T75 flasks. Five days later, a portion of the cells was transferred to 20% FBS DMEM-HAM’s media containing 10% DMSO (ATCC, Manassas, VA) and stored in 2 million cells/ml aliquots in liquid nitrogen. ~2 months later, an aliquot for each cell line was revived from the frozen state by quickly thawing, diluting 10-fold in 20% FBS DMEM-HAM’s media, centrifuging at 1200 rpm (IEC CENTRA 7R, Kent City, MI) at 4°C for 10 minutes, washing once more with media, and then seeding in fibronectin-coated T75 flasks (passage 5). Cells were again frozen and stored in liquid nitrogen at passage 7. Six months later, aliquots of each cell line were revived from the frozen state and grown in fibronectin-coated T75 flasks (passage 8). At ~80% confluence, they were split 1:3 into the selective MEM Eagle D-Valine Modification w/L-glutamine, D-Valine media (US Biological, Swampscott, MA) supplemented with 20% FBS (Invitrogen, Carlsbad, CA); 100 U/ml penicillin, 100 µg/ml streptomycin (Cellgro, Manassas, VA); 2.53 mM glutamine, 0.75% sodium carbonate (Invitrogen); and 10.246 mM glucose (EMD, Gibbstown, NJ) in T75 flasks (passage 9). At ~80% confluence, they were transferred back to the 20% FBS DMEM-HAM’s media and subsequently used for the RNA and protein assays as well as the GHR transcript characterization. Separate aliquots of passage 7 of each cell line were revived and grown in 20% FBS DMEM-HAM’s media in fibronectin-coated T75 flasks. After growth in selective D-valine media for one passage followed by growth in 20% FBS DMEM-HAM’s media for multiple passages, cells from passage 16 were used for immunohistochemistry assays.
3.4 E6 Cells

E6 cells generated by Dr. Edward List (List, 2001) were provided by Elahu Gosney and Dr. John Kopchick (Ohio University). These cells had been created by transfecting mouse fibroblast L cells with a GHR “mini-gene” that contained a mouse GHR cDNA along with exon 8a and its flanking introns for expression of both GHR and GHBP (List, 2001). Stable cell lines were established by Dr. List and bGH and hGH binding assays and functional assays measuring STAT5 activation by bGH were used to verify both the presence and activity of the GHR in the transfected cells (List, 2001). The cell line generated from the 6th clone exhibited a high level of GHRs per cell (170,000) and was hence named E6 (List, 2001). E6 cells also showed STAT5 phosphorylation upon hGH stimulation that progressively decreased with increasing growth hormone antagonist (GHA) concentration (E. Gosney, personal communication). For the current study, E6 cells were grown in DMEM media (Invitrogen) containing 10% Nu-serum (BD Biosciences, San Jose, CA) and 50 µg/ml gentamicin (Sigma Aldrich, St. Louis, MO) at 37°C, 5% CO₂.

3.5 Immunohistochemistry

The following method was performed by Cecilia Courreges but is included due to relevance to the thesis. ~20,000 NT and GKO mesangial cells were seeded in each well of an 8 well Lab-Tek II Chamber Slide System (Nalge Nunc International, Rochester,
NY) in 20% FBS DMEM-HAM’s media. After overnight incubation at 37°C, 5% CO₂, cells were fixed by 10 minute incubation with pre-chilled 4% formaldehyde followed by permeabilization with 0.1% Triton X-100 for 10 minutes (Sigma Aldrich, St. Louis, MO) at room temperature. After washing with PBS, endogenous peroxidases were quenched by a 10 minute incubation with 3% H₂O₂ (Sigma Aldrich) at room temperature. Blocking was performed by incubation in 1% BSA in PBS (Sigma Aldrich) for 30 minutes followed by two successive 15 minute incubations, first with avidin (70 µg/ml) followed by biotin (20 µg/ml) (Avidin/Biotin blocking kit-SP2001,Vector Laboratories, Burlingame, CA).

Primary antibody incubation was performed for 1h at room temperature. Mouse anti-human actin (alpha smooth muscle isoform) monoclonal antibody (0.05 mg/ml stock; used at 1:2000 dilution) (Millipore, Billerica, MA) and rabbit anti-chicken desmin polyclonal antibody (2-3 mg/ml; used at 1:80 dilution) (Millipore) were used as primary antibodies to identify mesangial cells. Rabbit anti-human von Willebrand factor polyclonal antibody (1 mg/ml; used at 1:100 dilution) (Millipore) and mouse anti-cytokeratin pan monoclonal antibody (1 mg/ml; used at 1:50 dilution) (Millipore) were used as primary antibodies to detect contamination by endothelial and epithelial cells, respectively. Isotype controls were run in parallel for each primary antibody with final concentrations similar to that used for each specific antibody (mouse IgG2a, kappa monoclonal [MG2a-53] (0.5 mg/ml) (Abcam, Cambridge, MA) - isotype control for actin; Normal Rabbit Serum (60 mg/ml, Jackson Immunoresearch Laboratories, West
After the primary antibody incubation and a PBS wash, slides were incubated for 15 minutes at room temperature with LSAB + Kit /HRP, Rabbit/Mouse/Goat (Dako, Glostrup, Denmark) as the source of secondary antibody and streptavidin-HRP. The reaction was developed using DAB (3,3’-diaminobenzidine tetrachloride enhanced liquid substrate system, Sigma Aldrich) until the color appeared in the slides. Slides were then counterstained with hematoxylin and mounted. Slides were observed at 200X and 400X magnification with a compound light microscope (Eclipse 80i, Nikon Instruments Inc., Melville, NY). Endothelial and epithelial cell lines from Dr. Fabian Benencia’s lab (Ohio University) were used as positive controls to test the effectiveness of the rabbit anti-human von Willebrand factor polyclonal antibody and mouse anti-cytokeratin pan monoclonal antibody.

3.6 Porcine and Human Growth Hormone Stocks

Porcine growth hormone (pGH) was obtained from Prospec (CYT-519, Rehovot, Israel). 500 µg of pGH powder was resuspended in 1000 µl sterile deionized water and single use aliquots were stored at -20°C, as recommended by the manufacturer. The presence of lipopolysaccharide was not tested.
Human growth hormone (hGH) was prepared by Elahu Gosney in Dr. John Kopchick’s lab (Ohio University) (Gosney et al., 2008). In short, a hGH cDNA clone (Open Biosystems, Huntsville, AL) was sub-cloned in a pET/D-TOPO expression vector (Invitrogen, Carlsbad, CA) and expressed in BL21 Star (DE3) *E. coli* (Invitrogen), inclusion bodies were isolated from the bacterial cultures by sonication and deoxycholate washes, the protein pellet was solubilized using a low concentration of urea at high pH, refolding was performed by rapid dilution of the denatured protein, and then renatured protein was applied to an ion exchange column, eluted in 200 mM NaCl, diafiltered with regenerated cellulose membranes and stored at -80°C (Gosney et al., 2008) (E. Gosney, personal communication). Activity was demonstrated using a STAT5 phosphorylation assay, an IM-9 cell proliferation assay and an adipocyte differentiation assay (Gosney et al., 2008) (E. Gosney, personal communication). For the current studies, purified hGH was stored as 800 ng/µl in 200 mM NaCl, 50 mM Tris, pH 8.0 as single use aliquots at -20°C. The presence of lipopolysaccharide was not tested.

3.7 Cell Growth Conditions for RNA Assays

For characterization of the GHR transcripts, ~300,000 NT and GKO mesangial cells were seeded in quadruplicate in 20% FBS DMEM-HAM’s media in T25 flasks (Nunc). The cells were incubated at 37°C, 5% CO₂ for 24 h before harvesting for RNA extraction. This experiment was performed once.
For testing the **single GH dose** dependent RNA expression of iNOS and IGF-1, ~300,000 NT and GKO mesangial cells were seeded in quadruplicate in 5% FBS DMEM media (no HAM’s F-12, all other constituents except FBS were at same concentration as used in 20% FBS DMEM-HAM’s media) in T25 flasks. 24 h after seeding, a frozen aliquot of pGH (500 ng/μl) was thawed at room temperature and then diluted to 5 ng/μl using sterile distilled water. The diluted pGH was further diluted into 5% FBS DMEM media to a final concentration of 50 ng/ml. The growth media in each T25 flask was replaced with the freshly prepared media containing pGH (50 ng/ml) to treat NT and GKO mesangial cells for 24 h as previously described (Doi et al., 2000). “No GH” treatment controls received fresh 5% FBS DMEM media only. This experiment was performed once.

For testing the **multiple GH dose** dependent expression of iNOS, IGF-1, STAT5b and GHR, ~300,000 NT and GKO mesangial cells were seeded in triplicate in 0.1% FBS DMEM media in T25 flasks. 24 h after seeding, an aliquot of hGH (800 ng/μl) was thawed at room temperature and then diluted in 0.1% FBS DMEM media (no HAM’s F-12, all other constituents except FBS were at same concentration as used in 20% FBS DMEM-HAM’s media) to final concentrations of 250 ng/ml, 50 ng/ml, 10 ng/ml and 1 ng/ml. The growth media in each flask was replaced with the freshly prepared media containing the diluted hGH. “No GH” treatment controls received fresh 0.1% FBS DMEM media only. The NT and GKO mesangial cells were further incubated at 37°C, 5% CO₂ for 48 h before harvesting for RNA extraction. This experiment was performed once.
3.8 RNA Extraction and Purification

Total RNA was extracted from NT and GKO mesangial cells using 1 ml of RNA STAT60 Total RNA/mRNA Isolation Reagent per T25 flask according to the manufacturer’s instructions (Tel-Test, Inc., Friendswood, TX). In short, cells were lysed for ~5 minutes at room temperature in the reagent, which contained phenol and guanidinium thiocyanate, and then transferred to a 1.5 ml microcentrifuge tube. 200 µl of chloroform was added followed by centrifugation at 12000 rpm (Hermle Z233 M2, Labnet, Edison, NJ) at 4°C for 15 minutes. The aqueous layer was transferred to a new tube and 5 µl of 20 mg/ml glycogen (Fermentas Inc., Glen Burnie, MD) and 500 µl isopropanol were added. After incubation at room temperature for 5-7 minutes, the RNA was precipitated by centrifugation at 12000 rpm at 4°C for 15 minutes. The pellet was washed with 70% ethanol, air-dried briefly and resuspended in 16 µl of nuclease-free water. The resuspended RNA was quantified using the Nanodrop (ND-1000 spectrophotometer, Thermo Scientific, Pittsburgh, PA) in the Ohio University Genomic Facility. A 10 µl aliquot of each RNA sample was prepared, adjusting the concentration to equal the concentration of the most dilute sample. Genomic DNA was then removed by DNase I digestion using the DNA-free™ DNase Treatment and Removal Kit as recommended by the manufacturer (Ambion, Austin, TX). After inactivation of the DNase, 10µl of supernatant was transferred to a new tube and used for cDNA synthesis.
3.9 cDNA Synthesis

cDNA was created from the purified RNA using the Bio-Rad iScript™ cDNA Synthesis Kit (Hercules, CA) essentially as described by the manufacturer, using a concentration of 1 µg RNA per 20 µl reaction. The reaction mixes were incubated in the Bio-Rad MyiQ thermocycler (BioRad, Hercules, CA) for 5 minutes at 25°C, 30 minutes at 42°C, and then 5 minutes at 85°C. The prepared cDNA was stored at -20°C.

3.10 Real-time RT/PCR and Gel Electrophoresis

Real-time RT/PCR was performed using iQ™SYBR® Green Supermix (BioRad, Hercules, CA), 0.5 - 1 µl of cDNA and 2 µM gene-specific primers (Tables 1 and 2; Integrated DNA Technologies, Coralville, IA) in duplicate in a MyiQ™ Single Color Real-Time PCR Detection System (BioRad, Hercules, CA). After averaging the duplicates, the Ct values were transformed into relative quantity values using the delta Ct (also known as comparative Ct) method, with the highest expression level set to 1, using the following equation:

\[ Q = E^{(\text{minCt} - \text{sampleCt})} \]

\( Q = \) sample quantity relative to sample with highest expression

\( E = \) amplification efficiency (2 = 100%)

\( \text{minCt} = \) lowest Ct value = Ct value of sample with highest expression
Table 1

**Primers Used for RT/PCR to Detect and Characterize the GHR Transcript**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>GHR Exon Bound</th>
<th>Sequence</th>
<th>Melting Temperature (°C)</th>
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<tr>
<td>2+1</td>
<td>2</td>
<td>TGTCAGGTCTTCTTAACCTTG</td>
<td>54.3</td>
</tr>
<tr>
<td>4-2</td>
<td>4</td>
<td>CAGGATTATCTCCTTCTGTCC</td>
<td>54.4</td>
</tr>
<tr>
<td>5-2</td>
<td>5</td>
<td>GGGTATCCAAATGGAGGTAT</td>
<td>54.0</td>
</tr>
<tr>
<td>6-2</td>
<td>6</td>
<td>CCACGAATCCCGGTCAAACT</td>
<td>59.1</td>
</tr>
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<td>γACTIN-F</td>
<td>control</td>
<td>ACCAACAGCAGACTTCCAGG</td>
<td>58.5</td>
</tr>
<tr>
<td>γACTIN-R</td>
<td>control</td>
<td>AGACTGGCAAGAAGGAGTG</td>
<td>57.8</td>
</tr>
</tbody>
</table>

Table 2

**Primers Used for Real-Time RT/PCR to Assay GH Dose Dependent RNA Expression of Genes**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Melting Temperature (°C)</th>
<th>Primer efficiency (%)</th>
</tr>
</thead>
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<tr>
<td>2+1</td>
<td>TGTCAGGTCTTCTTAACCTTG</td>
<td>54.3</td>
<td>97.9</td>
</tr>
<tr>
<td>4-2</td>
<td>CAGGATTATCTCCTTCTGTCC</td>
<td>58.0</td>
<td></td>
</tr>
<tr>
<td>iNOS F</td>
<td>TGCATGGACCAATAGCCAAAGGC</td>
<td>59.4</td>
<td>97.5</td>
</tr>
<tr>
<td>iNOS R</td>
<td>GCTTCTGGTGTCAAGACATGGG</td>
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<tr>
<td>STAT5b F</td>
<td>CGATGCCCTTCACCAGATG</td>
<td>55.9</td>
<td>99.2</td>
</tr>
<tr>
<td>STAT5b R</td>
<td>AGCTGGATGCTTTAACATGTTC</td>
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<td></td>
</tr>
<tr>
<td>IGF-1C F</td>
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<tr>
<td>IGF-1C R</td>
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<td>γACTIN R</td>
<td>AGACTGGCAAGAAGGAGTGTA</td>
<td>57.8</td>
<td></td>
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</table>

Quantities were then normalized using the geometric mean of expression of GAPDH and γ-actin, both of which had previously had been identified using the NormFinder program as being the best normalization pair for these RNA samples (Andersen et al., 2004). This experiment was performed once.
To assess the molecular weights of the products generated using GHR primers (Table 1), 11 µl of the amplified gene products were electrophoresed along with a molecular weight ladder (exACTGene 100 bp PCR DNA ladder, Fisher Scientific, Pittsburgh, PA) through a 2% agarose, 0.2 µg/ml ethidium bromide, 1×TAE horizontal gel at 100V for 30 minutes. The products were visualized by UV transillumination using a Geldoc digital imager (Biorad, Hercules, CA). This experiment was performed once.

3.11 Enzyme Linked Immunosorbent Assay (ELISA) and Growth Hormone Treatment for STAT5 Phosphorylation

Human/Mouse Phospho STAT5 (699) Immunoassay utilizing phospho-STAT5 (Y699) and total STAT5 primary antibodies (KCB4190, R & D Systems, Minneapolis, MN) was used to test STAT5 phosphorylation upon pGH and hGH stimulation. For the low GH dose dependent STAT5 phosphorylation assay, ~15,000 NT and GKO mesangial cells and positive control E6 cells were seeded in duplicate in a 96 well ELISA plate in 100 µl of serum-free DMEM media (Figure 5). 24 h after seeding, previously frozen pGH (500 ng/µl) and hGH (800 ng/µl) were thawed at room temperature and diluted into serum-free DMEM media to final concentrations of 11 ng/µl, 5.5 ng/µl, 2.75 ng/µl, 0.55 ng/µl, 0.11 ng/µl and 0.0275 ng/µl and kept on ice. 10 µl of the appropriately diluted GH was added to each well to achieve final concentrations of 1000 ng/ml, 500 ng/ml, 250 ng/ml, 50 ng/ml, 10 ng/ml, and 2.5 ng/ml pGH and hGH according to the depicted set-up (Figure 5). Addition of serum-free media without GH served as the 0 ng/ml control. Following
Serum-free DMEM media

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<td>GKO</td>
<td>E6</td>
<td>E6</td>
<td>1000 No 1º antibody control</td>
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Figure 5: ELISA set-up for testing STAT5 phosphorylation using lower doses of pGH and hGH.

incubation for 10 minutes at room temperature, cells were fixed, permeabilized and incubated with primary antibody, enzyme conjugated secondary antibody and fluorogenic substrates as described in the manufacturer’s protocol. Total STAT5 was measured at 360 nm excitation/450 nm emission and phosphorylated STAT5 was measured at 540 nm excitation/585 nm emission using a Micromax 384 Microwell Plate reader (SelectScience Ltd, Corston, United Kingdom). The duplicate samples were averaged and then the fluorescence of the phosphorylated STAT5 was normalized to the fluorescence of total
STAT5 by dividing the fluorescence of phosphorylated STAT5 by the fluorescence of total STAT5 for each sample. This experiment was performed once.

For the high GH dose dependent phosphorylation of STAT5 assay, ~60,000 NT and GKO mesangial cells were seeded in duplicate in the 96 well ELISA plate in 100 µl of 0.1% FBS DMEM media (Figure 6). An equal number of E6 cells were seeded in duplicate in either 0.1% FBS DMEM media or serum-free DMEM media as indicated (Figure 6). 24 h after seeding, previously frozen pGH (500 ng/µl) and hGH (800 ng/µl) were thawed at room temperature and diluted into serum-free DMEM media to final concentrations of 44 ng/µl, 22 ng/µl, 11 ng/µl, 5.5 ng/µl and 2.75 ng/µl and kept on ice. 10 µl of the appropriately diluted GH was added to each well to achieve final concentrations of 4000 ng/ml, 2000 ng/ml, 1000 ng/ml, 500 ng/ml, 250 ng/ml, 50 ng/ml according to the depicted set-up (Figure 6). Addition of serum-free media without GH served as the 0 ng/ml control. Following incubation for 12 minutes at room temperature, the assay was continued as described for the low GH dose experiment. This experiment was performed once.
Figure 6: ELISA set-up for testing STAT5 phosphorylation using higher doses of pGH and hGH.

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<td>E6</td>
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0.1% FBS DMEM media

Serum-free DMEM media

pGH

hGH

GH (ng/ml)

0 Control

50

250

500

1000

2000

4000

4000 No 1° antibody control
3.12 Enzyme Linked Immunosorbent Assay (ELISA) and Growth Hormone Treatment for MAPK Phosphorylation

Human/Mouse/Rat Phospho ERK1/ERK2 (T202/Y204) Immunoassay utilizing phospho-ERK1/ERK2 and total ERK1/ERK2 primary antibodies (KCB1018, R & D Systems, Minneapolis, MN) was used to test MAPK phosphorylation upon pGH and hGH stimulation. ~60,000 NT and GKO mesangial cells were seeded in duplicate in the 96 well ELISA plate in 100 µl of 0.1% FBS DMEM media (Figure 7). An equal number of E6 cells were seeded in duplicate in either 0.1% FBS DMEM media or serum-free DMEM media as indicated (Figure 7). 24 h after seeding, previously frozen pGH (500 ng/µl) and hGH (800 ng/µl) were thawed at room temperature and diluted into serum-free DMEM media to final concentrations of 11 ng/µl, 5.5 ng/µl, 2.75 ng/µl, 0.55 ng/µl, 0.11 ng/µl and 0.0275 ng/µl and kept on ice. 10 µl of the appropriately diluted GH was added to each well to achieve final concentrations of 1000 ng/ml, 500 ng/ml, 250 ng/ml, 50 ng/ml, 10 ng/ml, and 2.5 ng/ml pGH and hGH according to the depicted set-up (Figure 7). Addition of serum-free media without GH served as the 0 ng/ml control. Following incubation for 12 minutes at room temperature, the assay was continued as described for the STAT5 assays. This experiment was performed once.
**Figure 7: ELISA set-up for testing MAPK phosphorylation using pGH and hGH.**

### 3.13 Western Blot Assay

Western blot analysis was performed using Phospho-STAT5 (Tyr 694) antibody (9351, Cell Signaling Technology, Danvers, MA) to identify phosphorylation of STAT5 upon GH stimulation. ~100,000 NT and GKO mesangial cells were seeded in 0.1% FBS DMEM media in twelve wells plates and kept at 37°C, 5% CO₂ for 24h. At the same time, similar numbers of E6 cells were seeded in serum-free DMEM media in twelve
wells plate and kept at 37°C, 5% CO₂ for 24 h. The next day, a previously frozen aliquot of hGH (800 ng/µl) was diluted into serum-free DMEM media to final concentrations of 100 nM (2200 ng/ml), 20 nM (440 ng/ml), 10 nM (220 ng/ml) and 2 nM (44 ng/ml). The cells were washed three times with the respective media without GH and then the GH-containing media was added: 2 nM (44 ng/ml), 10 nM (220 ng/ml), 20 nM (440 ng/ml) and 100 nM (2200 ng/ml) hGH for the NT cells; 10 nM (220 ng/ml), 20 nM (440 ng/ml) and 100 nM (2200 ng/ml) hGH for the GKO cells; 10 nM (220 ng/ml) and 20 nM (440 ng/ml) hGH for the E6 cells. Addition of serum-free media without GH served as the 0 ng/ml control for each cell line. After a 10 minute incubation at 37°C, 5% CO₂, the cells were washed with 1× PBS and then lysed in 200 µl 1× RIPA buffer (Cell Signaling Technology) on ice for 5 minutes. The cells were then sonicated (Branson® 2510 sonicator, Danbury, CT) for 2 minutes and the lysates centrifuged at 12,800 rpm (Marathon 60 K, Fisher Scientific, Pittsburgh, PA) for 10 minutes at 4°C. The supernatants were transferred to new tubes and the proteins were quantified using the MicroBCA Protein Assay kit (Thermo Fisher Scientific, Pittsburgh, PA). 4.3 µg protein for each sample was brought to a total volume of 15 µl with H₂O, mixed with 15 µl 2× Laemmli buffer (containing 5% beta-mercaptoethanol) (Bio-Rad, Hercules, CA), heated for 5 minutes at 100°C, and then loaded in a stacking gel (3.9 % acrylamide) on top of a separating/ denaturing gel (12.5 % acrylamide, 0.1% SDS). The E6 and mesangial cell samples were loaded on separate gels. Both gels were electrophoresed along with Fermentas Spectra multicolor broad range protein ladder (Fermentas Inc., Glen Burnie, MD) for 1.30 h at 35 amps/150 volts. After electrophoresis, the gels were soaked in
transfer buffer containing 10% methanol for 20 minutes. The proteins were transferred to West Clear Nitrocellulose membranes (GenScript Corporation, Piscataway, NJ) at 20V overnight at 4°C. The next day, the membranes were treated, in separate containers, with Tris-glycine transfer buffer for 5 minutes followed by blocking buffer (5% dry milk powder, 0.1% Tween-20 in Tris Buffered Saline or TBS) for 1 h. The membranes were then washed three times with 0.1% Tween-20 in TBS for 5 minutes at room temperature. Primary phospho-STAT5 (Tyr 694) antibody (9351, Cell Signaling Technology, Danvers, MA) was added to the membranes (1000-fold dilution in primary antibody dilution buffer [5% BSA, 0.1% Tween-20 in TBS]) and incubated on a Rotator (Lab Line, Melrose Park, IL) overnight at 4°C. The next day the membranes were washed three times with 0.1% Tween-20 in TBS with gentle shaking and then incubated with anti-rabbit secondary antibody (RPN 2124, ECL plus™ western Blotting Reagent pack, GE healthcare, Piscataway, NJ) in blocking buffer for 1 h with gentle shaking followed by three washes with gentle shaking using 0.1% Tween-20 in TBS. Substrate, Lumingen PS-3 detection reagent (RPN 2132, ECL plus™ western Blotting detection system, GE healthcare, Piscataway, NJ) was added to the membranes at room temperature and incubated for 5 minutes. The membranes were then exposed to X-ray film (Hy-band, Denville Scientific, Metuchen, NJ) in the dark for 2 minutes and developed in the Hope Micro-max film developer (Warminster, PA). This experiment was performed once.
3.14 Statistical Analyses

Data are presented as means ± SE. SPSS general linear model was used to construct custom hypotheses using the l matrix procedure for analyzing the RNA expression of iNOS and IGF-1 by single GH dose (section 4.3) as recommended by Dr Victor Heh (OUCOM biostatistician). Statistical significance of RNA expression of iNOS, IGF-1, STAT5b and GHR in response to different doses of GH (section 4.7) was determined by analysis of variance using SPSS followed by Tukey’s post-hoc analysis when appropriate, as recommended by Dr. Kumika Toma. P<0.05 was considered to be significant.
4.1 Establishment of Mesangial Cell Lines from NT and GKO Mice

Mass isolation of glomeruli from the kidneys of a single NT and GKO mouse was accomplished utilizing cardiac perfusion of dynabeads (Figure 8 and data not shown). Approximately 8100 and 6900 glomeruli were retrieved from the NT and GKO kidneys, respectively. In order to establish mesangial cell lines from each mouse, the isolated glomeruli were cultured in media containing 20% FBS. Stellate-shaped cells phenotypically similar to mesangial cells (Wilson and Stewart, 2005) were the predominant cell type present in the cultures (Figure 9). After the cultures were well established but before additional experiments were performed, the cells were grown for a generation in selective media containing D-valine, which prevented the growth of phenotypically similar fibroblast cells (Wilson and Stewart, 2005).
Figure 8: Glomerular preps shown at three different magnifications. The isolated glomeruli are shown at 40X (A), 100X (B) and 400X (C). The Dynabead spheres are indicated in each figure. Photos were taken by Brett Buller.

Figure 9: Cell cultures established from isolated glomeruli. The predominant cells exhibit the stellate shape characteristic of mesangial cells. 100X magnification.
Immunohistochemistry was used to confirm the identity of the cells in culture as mesangial cells (Mene, 2000; Wilson and Stewart, 2005). The cells stained positively for alpha smooth muscle actin and desmin (Figure 10A and C). No staining was seen for the isotype controls (Figure 10B and D). The presence of epithelial cells, another cell type within the glomerulus and potentially within the culture, was eliminated because of the negative staining result using a specific antibody against cytokeratin, which is a characteristic protein in epithelial cells (data not shown). Likewise, the presence of another glomerular cell, the endothelial cell, was eliminated due to the negative result obtained using an antibody specific to the endothelial specific protein, von Willebrand factor (data not shown). Overall, using distinct morphology, selective media and immunohistochemistry, the grown cells were identified as mesangial cells and thus mesangial cell lines were established from NT and GKO mice.
Figure 10: Immunohistochemistry staining of NT and GKO mesangial cells at 200X magnification.

A. NT and GKO mesangial cells staining positively for alpha smooth muscle actin. B. Isotype control for the actin antibody. C. NT and GKO mesangial cells staining positively for desmin. D. Isotype control for the desmin antibody. Photos were taken by Cecilia Courreges.
4.2 Detection of GHR Transcript in Mesangial Cells

Gel electrophoresis and visualization of RT/PCR products was performed to detect GHR transcripts present in the mesangial cells using four sets of primers with binding sites in exons 2, 4, 5 and 6 of the GHR gene (Table 1) (Figure 11A). Observation of the 214 bp product in duplicate reactions of NT RNA using primer 2+1 and primer 4-2 corroborated the presence of RNA transcribed from the intact GHR sequence (Figure 11B, lanes 2, 3). Likewise, observation of the ~387 bp product using primer pair 2+1/ 5-2 (Figure 11B, lanes 6, 7), as well as the ~529 bp product using primer pair 2+1/ 6-2 (Figure 11B, lanes 10, 11), further supported the presence of RNA transcribed from the intact GHR sequence in NT mesangial cells. In contrast, the absence of product in duplicate reactions of GKO RNA using primer 2+1 and primer 4-2 supported the absence of stable RNA transcribed from the deleted region of the GHR gene in GKO mesangial cells (Figure 11B, lanes 4, 5). Surprisingly, an ~285 bp product using primer pair 2+1/ 5-2 and an ~427 bp product using primer pair 2+1/ 6-2, corresponding to the predicted sizes of transcripts spanning the exon 4 deletion, were observed in GKO mesangial cells (Figure 11B, lanes 8, 9 and lanes 12, 13), indicating that a stable RNA transcript could be synthesized from the remaining portion of the mutated GHR gene. The 76 bp product of the γ-actin control reaction was observed in both NT and GKO mesangial cells (Figure 11B, lanes 14, 15, 16, 17). Overall, these results suggest that intact GHR transcripts are expressed in NT mesangial cells and mutated but stable GHR transcripts are expressed in GKO mesangial cells.
Figure 11: Detection of GHR transcripts from NT and GKO mesangial cells.
A. Schematic representation of protein coding exons within the GHR gene (not to scale). Arrows indicate the approximate binding site and orientation of each specific primer. “×” indicates the deletion of most of exon 4 within the GHR gene in GKO mice. B. Agarose gel electrophoresis and ethidium bromide visualization of RT/PCR products obtained from NT and GKO mesangial cells (performed in duplicate) using the indicated primer pairs. L: 100 bp ladder. γ-actin: gamma actin control.
4.3 Measurement of iNOS and IGF-1 RNA Expression in NT and GKO Mesangial Cells after Treatment with pGH

Doi et al. had previously demonstrated the ability of pGH to induce expression of iNOS (inducible nitric oxide synthase) in mesangial cells isolated from wild type mice (Doi et al., 2000). They used a range of pGH doses (0 - 50 ng/ml) and observed consistently increasing expression of iNOS mRNA with the increasing pGH doses. The highest expression of iNOS (10 fold higher in comparison to the untreated control) occurred at the highest pGH dose (50 ng/ml). In the current study, in an effort to assess the response of the NT and GKO cell cultures to GH treatment, real time RT/PCR was performed to measure RNA expression of iNOS in quadruplicate cultures of NT and GKO mesangial cells after treatment with the highest dose of pGH used in the previous study (50 ng/ml) and using the same primer pair targeting the iNOS gene (Doi et al., 2000). Although iNOS RNA expression appeared to be modestly upregulated in the GH treated NT and GKO cells in comparison to the untreated controls, the increases did not reach statistical significance (Figure 12A). This was in stark contrast to the previous results obtained by Doi et al. (2000).

In the current study, IGF-1 expression was also measured as IGF-1 expression has been shown to be inducible by GH (Chia et al., 2006). Once again, the levels of IGF-1 RNA in the pGH treated samples did not differ significantly from the untreated controls (Figure 12B). The current study failed to demonstrate a response of either the NT or GKO
mesangial cell cultures to the pGH treatment in the induction of iNOS and IGF-1 RNA expression.

Figure 12: RNA expression of iNOS (A) and IGF-1 (B) assayed by real-time RT/PCR after treatment of NT and GKO mesangial cells with pGH.

NT and GKO mesangial cell cultures were grown in quadruplicate either in the absence or presence of 50 ng/ml pGH for 24 h before RNA isolation and cDNA preparation. Each sample was assayed for both iNOS and IGF-1 expression. Means±SE for each quadruplicate set are presented. * Significantly different (P<0.05) from corresponding NT control. None of the results for the GH-treated samples differed significantly from the corresponding untreated controls.
4.4 STAT5 Phosphorylation was not Detected in NT and GKO Mesangial Cells but was Detected in E6 Cells upon GH Stimulation in Western Blot Assay

STAT5, a ~95 kD protein, is known to be a downstream effector molecule in the GH signaling pathway. Stimulation of STAT5 phosphorylation by GH has been demonstrated in cultures of mouse fibroblast cells, but stimulation is dependent on expression of GHR (Lanning and Carter-Su, 2006; Wang et al., 1994; Xu et al., 1996). In the current study, stimulation of STAT5 phosphorylation by treatment of NT and GKO mesangial cell cultures with GH was used to assess the ability of the mesangial cells to respond to GH. The E6 cell line, which is a mouse fibroblast cell line stably transformed to express GHR and as such demonstrates hGH-stimulated phosphorylation of STAT5 (Gosney et al., 2008) (List, 2001) (personal communication with E. Gosney), served as a positive control for the current study. Single cultures of NT and GKO mesangial cells, as well as E6 cells, were treated with different doses of stock hGH (800 ng/µl) under similar conditions. Western blot analysis was then performed using an antibody against phosphorylated STAT5. STAT5 phosphorylation was observed in E6 cells after treatment with 10 nM (220 ng/ml) and 20 nM (440 ng/ml) hGH (Figure 13A). No phosphorylated STAT5 was observed in NT or GKO mesangial cells, even after treatment with a higher dose of hGH (100 nM or 2200 ng/ml) (Figure 13B). The current study failed to demonstrate a response of either the NT or GKO mesangial cell cultures to the hGH treatments in the stimulation of STAT5 phosphorylation.
Figure 13: Detection of STAT5 phosphorylation upon hGH stimulation by western blot assay in E6 cells (A) and in NT and GKO mesangial cells (B). E6 cell cultures and NT and GKO mesangial cell cultures were grown singly either in the absence or presence of the indicated amount of hGH for 10 minutes before preparation of protein lysates, SDS-PAGE and western blot assay of phosphorylated STAT5 protein (~95 kDa band). Signals corresponding to GH-stimulated STAT5 phosphorylation were observed in E6 cells but not in NT or GKO mesangial cells.

4.5 STAT5 Phosphorylation was not Detected in NT or GKO Mesangial Cells or E6 Cells upon GH Stimulation in ELISA Assay

ELISA was performed as another technique to assay STAT5 phosphorylation upon GH stimulation in cultures of NT and GKO mesangial cells as well as E6 cells. Initially, duplicate cultures of NT and GKO mesangial cells and E6 cells were treated with a low dose range (0 – 1000 ng/ml) of pGH or hGH in serum-free DMEM media for 10 minutes. In a follow-up experiment, duplicate cultures of NT and GKO mesangial cells were treated with a high dose range (0 – 4000 ng/ml) of pGH or hGH in 0.1% FBS DMEM media for 12 minutes while duplicated E6 cell cultures were treated with the high dose range (0 – 4000 ng/ml) of only hGH in either serum-free or 0.1% FBS DMEM media, also for 12 minutes. 0.1% FBS was included in the second experiment to aid the survival of the NT and GKO mesangial cells. A comparison of the influence of serum-free versus
0.1% FBS media on STAT5 phosphorylation was performed with the E6 cells as they appeared to grow equally well in either medium. The slight change in treatment time was made to accommodate differing numbers of samples in the treatment groups so that all samples within an assay were incubated for the same amount of time. The difference in incubation time was not expected to result in a significant difference in stimulation as 10-30 minutes previously was found to be an effective incubation period for observing GH-stimulated STAT5 phosphorylation (Ji et al., 2002; Wang et al., 1994). Both pGH and hGH previously were demonstrated to stimulate STAT5 phosphorylation in a dose dependent manner in vitro in mouse fibroblast cells expressing porcine GHR (Wang et al., 1994; Xu et al., 1996). Additionally, hGH has been demonstrated to stimulate STAT5 phosphorylation in vitro in E6 cells and also in vivo in mice (personal communication with E. Gosney) (Gevers et al., 2009; List, 2001).

In the current experiments, variations in the levels of phosphorylated STAT5 (expressed as a percentage of total STAT5) were observed at different GH doses in the NT and GKO mesangial cells for the low dose (Figure 14) and high dose (Figure 15) experiments, suggesting possible responses to GH. However, when the low dose and high dose experiments were plotted using the same scales, no consistent trends in response to GH treatment were seen (data not shown). Similarly, in E6 cells, although a trend toward increased STAT5 phosphorylation was observed in the low dose experiment (Figure 16A and B), the same response was not observed when the high dose range was tried (Figure 16C). The current study failed to demonstrate a consistent response of either the NT or
GKO mesangial cell cultures to GH treatments (pGH or hGH) in the stimulation of STAT5 phosphorylation as assayed by this ELISA. The dose response did not generate the expected bell-shaped curve that is typically seen with increasing GH or growth factor doses (Rosenfeld and Hwa, 2009). Surprisingly, E6 cell cultures also failed to demonstrate a consistent response to GH treatments in these assays, in contrast to the results obtained by western blot assay. It is not immediately clear what could account for this difference in response.
Figure 14: Detection of STAT5 phosphorylation by ELISA assay in NT and GKO mesangial cells using a low pGH (A) or hGH (B) dose range.
Duplicate cultures of NT and GKO mesangial cells were treated for 10 minutes with a low dose range (0, 2.5, 10, 50, 250, 500, 1000 ng/ml) of either pGH (panel A) or hGH (panel B). The amount of STAT5 phosphorylation was assessed by comparing the amount of phosphorylated STAT5 protein to total STAT5 protein in each culture using a combination of two different antibodies. The means for the duplicate cultures are presented.
Figure 15: Detection of STAT5 phosphorylation by ELISA assay in NT and GKO mesangial cells using a high pGH (A) or hGH (B) dose range. Duplicate cultures of NT and GKO mesangial cells were treated for 12 minutes with a high dose range (0, 50, 250, 500, 1000, 2000, 4000 ng/ml) of either pGH (panel A) or hGH (panel B). The amount of STAT5 phosphorylation was assessed by comparing the amount of phosphorylated STAT5 protein to total STAT5 protein in each culture using a combination of two different antibodies. The means for the duplicate cultures are presented.
Figure 16: Detection of STAT5 phosphorylation by ELISA assay in E6 cells using a low pGH dose range (A), a low hGH dose range (B), or a high hGH dose range (C). Duplicate cultures of E6 cells were treated for 10 minutes in serum-free media with a low dose range (0, 2.5, 10, 50, 250, 500, 1000 ng/ml) of either pGH (panel A) or hGH (panel B) or 12 minutes in serum-free or 0.1% FBS media with a high dose range (0, 50, 250, 500, 1000, 2000, 4000 ng/ml) of hGH (panel C). The amount of STAT5 phosphorylation was assessed by comparing the amount of phosphorylated STAT5 protein to total STAT5 protein in each culture using a combination of two different antibodies. The means for the duplicate cultures are presented.
4.6 MAPK Phosphorylation was not Detected in NT or GKO Mesangial Cells or E6 Cells upon GH Stimulation in ELISA Assay

ELISA was performed to assay MAPK phosphorylation upon GH stimulation in NT and GKO mesangial and E6 cells. Previously, MAPK phosphorylation in response to hGH stimulation was observed *in vitro* in mouse myeloid cell line (Rowlinson et al., 2008; Tripathi and Sodhi, 2009). MAPK phosphorylation in response to pGH was also seen in a mouse macrophage cell line (Rowlinson et al., 2008; Tripathi and Sodhi, 2009). In the current study, the E6 cell line could not be considered as a positive control for the NT and GKO mesangial cell cultures as MAPK phosphorylation had not been previously assayed in E6 cells (personal communication, E. Gosney and E. List). Duplicate NT and GKO mesangial cell cultures were treated with a dose range of 0-1000 ng/ml pGH (*Figure 17A*) or hGH (*Figure 17B*) in 0.1% FBS DMEM media for 12 minutes. E6 cells were treated with the same doses of hGH for 12 minutes, but in 0.1% FBS and serum-free DMEM media (*Figure 17C*). MAPK phosphorylation varied between 20% and 50% for the pGH treatments and between 60% and 90% for the hGH treatments in the NT and GKO mesangial cells (*Figure 17A and B*). Phosphorylation of MAPK was observed to be between 20% and 30% for the hGH treatments of E6 cells in either the 0.1% FBS or serum-free DMEM media. Once again, the current study failed to demonstrate a consistent response of either the NT or GKO mesangial cell cultures or the E6 cells to GH treatments (pGH or hGH) in the stimulation of MAPK phosphorylation as assayed by
ELISA. Again, the dose response did not generate the expected bell-shaped curve that is typically seen with increasing GH or growth factor doses (Rosenfeld and Hwa, 2009).
Figure 17: Detection of MAPK phosphorylation by ELISA assay in NT and GKO mesangial cells using a pGH (A) or hGH (B) dose range or in E6 cells using a hGH dose range (C).

Duplicate cultures of NT and GKO mesangial cells were treated for 12 minutes with a range (0, 2.5, 10, 50, 250, 500, 1000 ng/ml) of either pGH (panel A) or hGH (panel B) in 0.1% FBS media. Duplicate cultures of E6 cells were treated for 12 minutes with the same range of hGH but in either 0.1% FBS or serum-free media (panel C). The amount of MAPK phosphorylation was assessed by comparing the amount of phosphorylated MAPK protein to total MAPK protein in each culture using a combination of two different antibodies. The means for the duplicate cultures are presented.
4.7 Measurement of iNOS, IGF-1, STAT5b and GHR RNA Expression in NT and GKO Mesangial Cells after Treatment with hGH

In a final effort to assess the response of the NT and GKO mesangial cell cultures to GH treatment, real-time RT/PCR was performed to measure RNA expression of several genes (iNOS, IGF-1, STAT5b and GHR) in triplicate cultures of NT and GKO mesangial cells after a 48 h treatment with different doses (0, 1, 10, 50, 250 ng/ml) of hGH. GHR transcript expression as detected using the 2+1/4-2 primer pair was essentially the same for all hGH doses in the NT cells and was undetectable in the GKO cells, verifying the presence or absence of the exon 4 region in the NT and GKO cells, respectively (Figure 18A). Significant upregulation of iNOS RNA expression was observed at the highest (250 ng/ml) dose of hGH added in both NT and GKO cells, although the RNA expression was higher in the NT cells (Figure 18B). Significant upregulation of STAT5b RNA expression was observed with addition of the 10 ng/ml dose of hGH in both NT and GKO cells (Figure 18C), reminiscent of the expected bell-shaped curve that is typically seen with increasing GH or growth factor doses (Rosenfeld and Hwa, 2009). IGF-1 did not show any significant changes in RNA expression in NT and GKO cells at any of the hGH doses (Figure 18D). Overall, this final study appeared to demonstrate a response of both the NT and GKO mesangial cell cultures to hGH treatment in the induction of iNOS and STAT5b RNA expression.
Figure 18: RNA expression of GHR (A), iNOS (B), STAT5b (C), and IGF-1 (D) assayed by real time RT/PCR after treatment of NT and GKO mesangial cells with increasing doses of hGH.

NT and GKO mesangial cell cultures were grown in triplicate in different doses (0, 1, 10, 50, 250 ng/ml) of hGH for 48h before RNA isolation and cDNA preparation. Each sample was assayed for GHR, iNOS, STAT5b, and IGF-1 expression. Means±SE for each triplicate set are presented. Significant upregulation of iNOS and STAT5b RNA was observed. *: significantly different (P<0.05) from 0 ng/ml hGH control. +: significantly different (P<0.05) from corresponding NT sample.
Establishment of two mesangial cell lines was an important primary aim to achieve in order to test the hypothesis that a mesangial cell line established from an NT mouse will exhibit functional GH signaling while a mesangial cell line established from a GKO mouse will not exhibit functional GH signaling. Verification of the cell type in the cultures established from isolated glomeruli of the NT and GKO mice was critical because of the possible growth of other glomerular cell types such as endothelial and epithelial cells, along with fibroblasts, in addition to the desired mesangial cells. A factor that helped in the establishment of the mesangial cell cultures was the fact that, in vitro, epithelial and endothelial cells are subject to contact inhibition because of their monolayer growth pattern (MacKay et al., 1988). Therefore, mesangial cells generally become the prominent cell type after seeding of the isolated glomeruli (Wilson and Stewart, 2005). In the current study, grown cells were identified as mesangial cells because of their characteristic stellate shape, which is distinct from the cobblestone structure of glomerular endothelial or epithelial cells grown in culture (MacKay et al., 1988; Mentzel et al., 1997; Yaoita et al., 1999).

Morphology alone did not validate the presence of mesangial cells because fibroblasts could also have been present in the glomerular preps. They exhibit a similar stellate structure and can flourish in growth media as they are not subject to contact inhibition (Wilson and Stewart, 2005). Therefore, using selective media was a useful method to
prevent any fibroblast contamination in the mesangial cell cultures. The selective media contained D-Valine as one of the nutritional essential amino acids. This differed from the normal growth media that contained the L isoform of all of the essential amino acids. Presence of the D isoform of valine instead of the L isoform in the selective media prevented the growth of fibroblasts as they lack D amino oxidase, the enzyme that converts the D form of amino acids into the L form (Gilbert and Migeon, 1975; Lazzaro et al., 1992). Thus, the flourishing cell type from the seeded glomeruli was identified as the mesangial cell by observing the characteristic morphology and using a selective media.

Additionally, immunohistochemistry results supported the above results. Overall, the characterization of mesangial cell is generally based on “exclusion criteria” in excluding the possibilities of presence of any fibroblast, epithelial or endothelial contamination (Mene, 2000). The grown cells were identified as mesangial cells because of the positive staining results for alpha smooth muscle actin and desmin. Positive staining for alpha smooth muscle actin, a cytoskeletal protein, indicates the presence of mesangial cells, which have characteristics of smooth muscle in regulating the glomerular hemodynamics through the contraction and dilation of associated blood capillaries (Mene, 2000). Positive staining for desmin, another cytoskeletal protein, in cell culture also indicates the presence of mesangial cells (Floge et al., 1994). Negative staining results for von Willebrand factor and cytokeratin eliminated the presence of endothelial and epithelial cells, respectively.
After establishing the mesangial cell cultures, it was important to determine whether the mesangial cells expressed GHR transcripts as the GHR is a necessary component of GH signaling. Although determination of GHR protein expression would have been more directly relevant, the lab did not have the tools needed for GHR protein assessment but did have the tools needed to assess GHR mRNA expression. In a previous study, Doi et al. had detected the presence of GHR mRNA in a mesangial cell culture (Doi et al., 2000). Thus, in the current study, detection of GHR transcripts was expected in the NT mesangial cell line. In contrast, the original paper describing the generation of the GKO mice reported that GHR mRNA was undetectable by RT/PCR in liver tissue (Zhou et al., 1997). Thus, in the current study, detection of GHR transcripts was not expected in the GKO mesangial cell line. In support of the published observations, GHR mRNA was detected by RT/PCR in NT mesangial cells but was not detected in GKO mesangial cells when a primer binding in the gene-disrupted region, exon 4, was used. Surprisingly, a smaller than the wild type product was observed in the GKO cells when primers downstream of the deletion were used. The results indicated that a stable GHR transcript lacking the deleted exon 4 region but intact beyond that region (at least containing exons 5 and 6) was being expressed in the GKO cells. The observation of the mutated yet stable GHR transcript in the GKO contradicted the published article, which did not detect any GHR mRNA in the GKO mice (Zhou et al., 1997). However, the authors did not mention the sequences of primers used for the RT/PCR. It is possible that a primer specific to the deleted region of exon 4 was used, detecting no GHR mRNA in the GKO mice, just as observed in the current study. Perhaps additional primers beyond the deleted region were
not used, which would have indicated the presence of a mutated GHR transcript, as seen in the current study. It is important to determine if the observed stable, mutated GHR mRNA is able to generate a functional GHR protein. The authors stated that even if RNA was produced, alternative splicing from exon 3 to exon 5 would result in a frameshift and the presence of a stop codon in exon 5, preventing production of the GHR protein (Zhou et al., 1997). The original paper did not detect GHR protein by western blot assay when using an antibody to the intracellular region of the GHR (exons 9 and 10), but they did report detection of a low level of GH binding activity in the GKO mice (Zhou et al., 1997). These observations, i.e. the low level of GH binding activity detected in GKO mice and the presence of stable, mutated GHR mRNA detected in GKO mesangial cells, call for further experiments to check the possibility of the generation of a functional GHR protein in GKO mesangial cells and GKO mice in order to determine whether they are truly null for functional GH signaling. The dwarf size of the GKO mice suggests that the GHR protein is not functioning normally, but it is not clear if it is completely nonfunctional (and completely absent) or partially functional (and mutated or reduced in amount).

As cryptic splicing or alternative splicing between remaining exons on each side of the deletion could be possible (and not restricted to splicing between exons 3 and 5), determination of the mutated GHR transcript’s sequence could be informative. Primer pair 2+1/5-2 or primer pair 2+1/6-2 could be used to amplify the mutated region by RT/PCR and then the amplified product could be subcloned and sequenced. The
sequence of the mutated region could be compared with the known mouse GHR sequence to determine what sequence is remaining and to determine the extent of an open reading frame. The predicted protein sequence could be compared to the known protein sequence and domains in an effort to predict the functionality of any translated product. Western blot analysis also could be performed to check the presence of any GHR protein in the GKO mesangial cells using a different antibody, e.g. an antibody specific to the extracellular domain of the GHR protein or an antibody specific to a different intracellular region of the GHR protein than used previously to test the presence of the GHR protein in the GKO mice (Zhou et al., 1997). Knowledge of the general portions of expressed GHR protein could also help predict the functionality of the expressed protein.

Although sequencing the mutated GHR transcript or investigating the presence of GHR protein by western blot analysis could help predict the functionality of any expressed GHR protein, these methods do not definitively demonstrate the functionality of the expressed GHR protein. Thus, the rest of the experiments of the current study designed to assess the presence or absence of functional GH signaling in the established NT and GKO mesangial cell lines, respectively, took on an added importance. The hypothesis had been based on the assumption that the GKO mice were lacking functional GHR protein and thus would be incapable of exhibiting functional GH signaling. It was expected that the GKO mesangial cells would serve as a negative control in the tests for functional GH signaling. With observation of expression of a mutated GHR transcript in GKO mesangial cells, inclusion of the GKO cells in the assessments of functional GH
signaling provided a means of determining whether the GKO cells expressed a fully functional, partially functional, or nonfunctional GHR protein, at least in the context of the specific assays. Unfortunately, the results of the functionality assays remain inconclusive, but if the assays are improved and/or validated using the changes or controls described in the following paragraphs, these assays could be used to determine the functionality of GH signaling in the GKO mesangial cells.

In cell culture experiments, especially those studying the effects of cytokines, and growth hormone is a cytokine, results could be skewed due to stimulation or inhibition by contaminating bacterial lipopolysaccharide (LPS). The source of LPS in cell culture experiments is commonly found to be water (Hasiwa et al., 2007). Bacteria can grow in storage containers of water as well as in water purification systems, especially units utilizing deionizing cartridges or carbon filters. Routine autoclaving (121°C/ 20-30 minute) has been shown to be ineffective in removing LPS contamination. In the current study, possible LPS contamination could have occurred through the preparation of pGH and hGH dilutions using water.

LPS contamination can result in the induction of different cytokines, such as IL-1, IL-6, or TNFα, which have been shown to suppress GHR mRNA expression and the effect of GH stimulation in murine macrophage and human kidney embryonic cells (Ahmed et al., 2007; Dejkhamron et al., 2007; Sawa et al., 2008). In other studies, IL-6 was shown to inhibit GH mediated IGF-1 expression in hepatocytes (Ahmed et al., 2007; Dejkhamron
et al., 2007; Sawa et al., 2008) and LPS was found to decrease hGH stimulated STAT5 phosphorylation in rat liver (Hong-Brown et al., 2003). As a possible mechanism, reduced levels of GHR mRNA could lead to reduced levels of GHR protein that in turn could lead to the reduced response to GH. This could also account for the observed unresponsiveness to GH stimulation in most of the assays of the current study. In contrast, LPS has been found to effectively stimulate iNOS mRNA and protein expression in rat glomeruli \textit{in vivo} as well as stimulate iNOS mRNA expression in mouse macrophage cells \textit{in vitro} (Lee et al., 2002; Rafi et al., 2007). A similar effect could account for the increased iNOS mRNA expression seen at the highest level of hGH stimulation in the current study. LPS was also found to stimulate MAPK phosphorylation in THP-1, a human monocytic leukemia cell line (Wu et al., 2008). This effect was not observed in the current study. In light of the possible effects of LPS, it would be valuable to repeat the current study using sources of water, reagents and plastic-ware that are guaranteed to be low in LPS contamination or measuring the level of LPS contamination in the various sources of water or reagents used in culturing and treating the cells. In addition, polymyxin B, an inhibitor of LPS action, could be included in the various assays to eliminate or investigate the effect of LPS contamination (Dejkhamron et al., 2007).

Another possible explanation for the suppression of GH stimulation in most of the experiments of the current study could be the inclusion of FBS in many of the assays. FBS is rich in different growth factors and cytokines, and sometimes even LPS, and thus
the inclusion of FBS could potentially suppress the stimulatory effect of added GH in the mesangial cell assays (Li et al., 1996; Strle et al., 2004). Growth of the mesangial cells in serum-free media was tried for several of the assays, but a decrease in cell viability was seen with a later passage of cells (passage 14 in comparison to passage 9). Therefore, to create a balance between cell viability and the possible inhibitory effect of cytokines present in the FBS, cells in later experiments were seeded and exposed to GH using 0.1% FBS in the media. In future experiments, cell viability can be tested simultaneously by assaying the release of lactate dehydrogenase in the culture media as an increase in lactate dehydrogenase correlates with the loss of culture viability (Racher et al., 1990).

Two types of GH, pGH and hGH, were also utilized in the experiments of the current study. The choice of pGH and hGH was based in part on previous experiments by other groups. For example, a number of published studies used pGH to induce responses in cell culture, including in mesangial cells. The assessment of pGH-induced iNOS mRNA expression was directly based on a similar study by Doi et al. (Doi et al., 2000). pGH was also used in mouse fibroblasts to induce STAT5 phosphorylation (Wang et al., 1994; Xu et al., 1996). The pGH used in the current studies was obtained from a commercial source though it was not certified to be free of LPS. hGH was included in some of the studies because it had been shown to be effective in stimulating the phosphorylation of STAT5 in E6 cells (personal communication with E. Gosney). The E6 cells and hGH were included in some of the experiments of the current study to serve as a positive control. A complication of using hGH is that hGH can also bind the prolactin receptor (Dogusan et
al., 2001; Galsgaard et al., 2001). Since the only GH-stimulated response observed in the
current study was an increase in iNOS and STAT5b mRNA after hGH treatment, a
control needs to be included to demonstrate that the GH signaling is occurring through
the GHR and not the prolactin receptor. For example, an antibody that prevents the
functioning of the GHR (a GHR neutralizing antibody) could be included. Anti-GHR_{ext-
mAb} is an antibody that was created to bind to the extracellular domain of GHR to inhibit
GH binding in order to block downstream signaling pathways (Jiang et al., 2004).
Likewise, even though expression of the prolactin receptor in mesangial cells has not
been reported, inclusion of an antagonist or neutralizing antibody of the prolactin
receptor could also be used in the experiments with hGH to separate responses dependent
on the GHR versus responses dependent on the prolactin receptor. A third method would
be to include the GH antagonist and demonstrate the loss of GH-induced effects.
Mutations in the GHR binding sites of GH generates GH antagonists that compete with
GH in binding to GHR with the same affinity and result in little or no effective
downstream cascades that are usually induced by GH (Yang et al., 2008). One such
antagonist is G120R, which is a mutant version of GH that showed antagonistic effects \textit{in
vitro} by inhibiting GH-mediated phosphorylation of a ~93kD molecule (Chen et al.,
1994). Transgenic mice with this mutation developed dwarf phenotype with elevated
level of this antagonist, G120R in the serum indicating suppression of GH induced
growth promoting signaling pathways (Chen et al., 1994; Yang et al., 2008).
Unfortunately, this antagonist possibly would not distinguish signaling through GHR
versus prolactin receptor as it is derived from the human form of GH and has been
demonstrated to also antagonize the human prolactin receptor (Sundstrom et al., 1996). An antagonist derived from a non-primate form of GH would specifically recognize GHR and not the prolactin receptor, but this antagonist is not commercially available.

An inconsistency that occurred in the current study was the use of varying GH treatment times. This resulted in part due to the response being measured (mRNA production versus protein phosphorylation), in part due to procedural modifications implemented to elicit a response to the GH treatment when the expected response was not observed, and in part due to slight changes in the experimental setup that necessitated a change in incubation time. In the first situation, reports in the literature employed GH treatments of hours to days when assaying RNA production but only tens of minutes when assaying protein phosphorylation (McElhinney et al., 2004; Mrak et al., 2007; Sang et al., 2010; Wang et al., 1994; Xu et al., 1996) and thus similar parameters were followed for the current studies. In the second situation, treatment times were varied (e.g. 24 h versus 48 h) under the supposition that the peak signal had been missed. In the third situation, two GH incubation periods, 10 minutes versus 12 minutes, were utilized in the ELISAs due to a change in sample setup and in an effort to treat samples within a group all together and in a similar manner. The expectation was that the change of two minutes would not result in a significant change in measured outcome (phosphorylation) as a previous report had demonstrated that GH stimulated STAT5 phosphorylation reached a maximum intensity at 10 minutes, remained phosphorylated with the same intensity for the next 20 minutes, and then phosphorylation decreased after that (Ji et al., 2002; Wang et al., 1994). Most of
these changes in GH treatment times could have been avoided if responses to the GH treatments had been readily observed with the initial conditions used.

In the western blot assay, a response to hGH stimulation was seen in the positive control E6 cells, but not in the NT or GKO mesangial cells. Although all of the cells were treated similarly and equal amounts of total protein were analyzed for each sample, analysis of the E6 cell lysates was performed on a separate gel and membrane from the NT and GKO mesangial cell lysates. The E6 membrane had positive signals while the NT/GKO membrane was completely blank. This assay should be repeated with the positive control E6 lysates included on the same gel and membrane as the NT and GKO cell lysates to rule out a problem with the gel or membrane. In addition, variations in the GH dose or treatment time or amount of lysate utilized could all be tried.

Overall, the ELISA results were inconclusive for both STAT5 and MAPK phosphorylation in NT and GKO mesangial cells and also in E6 cells. The basal STAT5 phosphorylation level (4% - 5%) seen in the current study was quite similar to the basal level of STAT5 phosphorylation (5% - 7%) reported in wild type mouse liver prior to ovine GH treatment (Miquet et al., 2005). In E6 cells specifically, the basal level of 4.5% seen in this study fell in the range (4% - 6%) previously observed for these cells (personal communication with E. Gosney). Thus, it appears that only a basal level of STAT5, and probably also MAPK, phosphorylation was seen in the NT and GKO mesangial cell cultures. The reasons for not observing any GH-stimulated STAT5 or MAPK phosphorylation, not
even for the positive control E6 cell line that showed hGH stimulation of STAT5 phosphorylation by western blot assay, is not clear, even though a similar range of GH doses was used for each type of assay. The ELISA kit used in the current study was not a conventional sandwich ELISA where the wells are first coated with primary antibody and then protein extracts are added for detection. Rather, the cells were grown, treated with GH and then permeabilized before addition of primary and secondary antibodies and visualization of signal. In the future, a conventional sandwich ELISA should be tried using lysates as prepared for the western blot assays. Little can be concluded without a working positive control, which was lacking in the current study.

Finally, a variety of GH-induced effects were assayed in this study. The main reason for this was due to a lack of obtaining expected results as suggested by results in other cell types or situations. For example, phosphorylation of STAT5 and MAPK is often used to assess GH signaling in a variety of cell types (Rowlinson et al., 2008; Tripathi and Sodhi, 2009; Wang et al., 1994; Xu et al., 1996), though neither had been demonstrated previously in mesangial cells. STAT5 mRNA was reported to be induced by hGH in the liver and thymus of male and female rats \textit{in vivo} using a probe specific to STAT5b (Ortiz et al., 2000). IGF-1 mRNA was induced by hGH in human fibroblast cell \textit{in vitro} (Sang et al., 2010). Induction of iNOS and GHR mRNA by pGH was reported in primary mouse mesangial cells (Doi et al., 2000). Each of these responses had the possibility to serve as an indicator of GH signaling in the current study, especially the ones demonstrated previously in mouse mesangial cells. In addition to inclusion of the controls
and changes described in the previous sections, it may be necessary to include additional GH-induced effects to analyze. For example, GH-stimulated phosphorylation of the MAPK and STAT5 molecules relies on stimulation of GHR and JAK2 phosphorylation (Lanning and Carter-Su, 2006; Lodish et al., 2007). Therefore, phosphorylation of GHR and JAK2 along with MAPK and STAT5 phosphorylation upon GH stimulation could also be tested by ELISA or western blot assay to indicate active GH signaling in mesangial cells. GH signaling can also be tested by performing real-time RT/PCR to assay the RNA expression of Suppressor of cytokine signaling (SOCS) 1, 2, and 3 and Cytokine induced Src homology 2 (CIS) molecules as gene expression of these molecules is dependent on GH induced JAK2/STAT5 phosphorylation pathway (Lanning and Carter-Su, 2006). Observing the GH regulation of these additional molecules could also provide clues as to why the assays of downstream molecules in the current study did not work.

In summary, the goal of this study was to test the hypothesis that a mesangial cell line established from NT mice will exhibit functional GH signaling whereas a mesangial cell line established from GKO mice will not exhibit functional GH signaling. The first specific aim, to establish primary cell cultures and demonstrate the identity of the cultured cells as mesangial cells by morphological appearance, growth on a selective media, and presence/absence of specific characteristic proteins were achieved. Two mesangial cell lines were established, one from an NT mouse and one from a GKO mouse. The second specific aim, to characterize the GHR mRNA transcripts produced in
the NT and GKO mesangial cell cultures using RT/PCR and specific primer pairs, was also achieved. Expression of an intact GHR transcript in NT mesangial cells was demonstrated. Expression of a stable but mutated GHR transcript in GKO cells was also demonstrated. The final specific aim, to demonstrate the presence or absence of functional GH signaling in NT and GKO mesangial cells, respectively, by assaying the presence of RNA transcripts or phosphorylated proteins of downstream signaling pathways in response to exogenous GH stimulation using real-time RT/PCR, western blotting analysis or ELISA, was not achieved. A response to hGH treatment was observed in terms of stimulation of iNOS and STAT5b mRNA expression in both the NT and GKO mesangial cell cultures by real-time RT/PCR, but the response cannot be definitively attributed to GH signaling through the GHR and downstream pathways without additional controls to exclude the possibilities of contaminant LPS-mediated induction or involvement of the prolactin receptor. A response in terms of phosphorylation of STAT5 or MAPK was never consistently seen. Future research should focus on establishing and verifying assays of functional GH signaling in the mesangial cell cultures and then using these assays to determine the functionality of GH signaling in the GKO mesangial cells.
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