# DEVELOPMENTAL CHANGES IN THE HUMAN GROWTH HORMONE RECEPTOR AND ITS SIGNAL TRANSDUCTION PATHWAYS

by

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## ABSTRACT

Although tissue human growth hormone receptors (GHRs) have been identified from as early as the first trimester of fetal life, little is known about their functional role. We recently reported that fetal hGHRs appear to be relatively immature or have adapted to the *in utero* environment [194]. To further characterize these age-related differences, we have examined certain aspects of the hGHR structure as well as its signalling pathways. Initial cDNA sequencing and immunoblotting analyses showed no difference in hGHR molecular size. We then used RT-PCR to investigate levels of an alternatively spliced mRNA for a truncated (1-279) hGHR (T) which acts as a dominant negative to the full-length (FL) receptor; however, fetal hepatocytes showed T/FL ratios similar to postnatal liver (0.07±0.02 vs. 0.09±0.01, M±SEM). Western blotting was used to assess developmental differences in the relative abundance of downstream signalling factors. Stimulatory Jak and Stat proteins were detected in all fetal hepatocytes and postnatal livers tested (13 weeks fetal age to 62 years). However, the fetal hepatocytes showed much lower levels compared to postnatal liver (5-22%; Jak2 [$p<0.002$] and Stat5B [$p<0.02$]). Four Socs proteins, which negatively regulate hGHR action, were also detected in all samples: Socs1 (119% of postnatal liver), Socs2 (51%), Socs3 (239%, $p<0.03$) and Cis (200%, $p<0.03$).

Our results suggest that changes in the primary structure of the hGHR as well as the relative abundance of T/FL are not influencing hepatocyte responsiveness during fetal life. The relatively low levels of stimulatory Jak/Stat molecules, coincident with the high levels of at least two inhibitory Socs proteins, are more likely to be responsible for the fetal-specific hepatocyte responses to GH.

***To Russ, for your unconditional love and support…***
***thank you for always believing in me.***

"Never regard study as duty, but as the enviable opportunity to learn…for your own personal joy and to the profit of the community to which your later work belongs."
*-Albert Einstein (1879-1955)*

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# RÉSUMÉ

Bien que les récepteurs de l'hormone de croissance humaine (hGHR) aient été identifiés dans plusieurs tissus, leurs fonctions respectives durant le développement n'ont pas encore été clairement déterminées. Récemment, nous avons rapporté que les GHRs fœtaux présentent des caractéristiques d'une immaturité ou une adaptation *in utero* [194]. Pour mieux caractériser les différences d'expression de l'ARNm liées aux différents stades du développement, nous avons utilisé la RT-PCR. Celle-ci nous permettrait d'étudier l'expression d'une variante de l' ADNc dont l'exon 9 est alternativement épissé, il en résulte une forme tronquée (T) du hGHR (1-279). Cette forme tronquée pourrait agir comme un dominant négatif par rapport à la forme complète (FL) du récepteur. Nous avons effectivement montré que le ratio T/FL est deux fois plus élevé dans les tissus fœtaux que dans les tissus postnataux (0.07±0.02 vs. 0.09±0.01, M±SEM). Nous avons aussi utilisé la technique « Western blotting » pour caractériser les différences quant à la nature des facteurs de transduction impliqués durant le développement. Les facteurs Jak et Stat ont été trouvés aussi bien dans les hépatocytes fœtaux que dans les tissus de foie postnatal. Toutefois d'autres échantillons testés présentent des taux plus bas dans les hépatocytes fœtaux comparés au foie postnatal (5-22%; Jak2 [$p<0.002$] and Stat5B [$p<0.02$]). En plus, quatre Socs ont été aussi détectés: Socs 1 (119% de foie postnatal), Socs2 (51%), Socs3 (239%, $p<0.03$) et Cis (200%, $p< 0.03$).

En résumé, nos résultats suggèrent que l'abondance relative du hGHR (T/FL) dans le hepatocytes fœtaux ne contribue pas au niveau de la réponse des cellules hépatiques observé durant la vie fœtale. Les taux relativement bas des molécules activatrices Jak/Stat, coïncidant avec un niveau élevé des protéines de régulation négative

Socs, sont probablement responsables des réponses spécifiques du GH observées dans les hépatocytes fœtaux.

## ACKNOWLEDGEMENTS

It is amazing how fast time flies when you're having fun... Life as a graduate student has been a great experience, one that I will forever cherish.

First and foremost, I would like to thank my mentor, Dr. Cindy Goodyer, for giving me the opportunity to undertake this project and for all your guidance and sound advice. Your constant enthusiasm and dedication to science has inspired me to succeed the past two years. Thank you to all my lab mates: Joy, for your help in the lab, for fostering my chocolate addiction and especially for staying late all those Wednesday nights; Zak, for translating my abstract and for being such great company; Stasia, for all your good advice, for introducing me to new restaurants and always making me smile; David, for showing me what will power is all about; Hong, for always managing to find things I needed in the lab; and John, for making me laugh no matter what time of day.

I would also like to thank: the South-African "men" at Place Toulon, Dave and Greg, for all the great times we had just hanging out, eating lunch and 3:00 pm "T"; Luciano, for tiramisu, cheesecake and for always being yourself; Oriana, for being an inspiration at a whole new level of hard-work; and Alison, Jenn, Luc, Lynn, Mike, Olivier, Pablo and Suzanna, for making my time at the research institute an enjoyable one. Many thanks to my girls Carey and Patsy, for being such a great thesis-writing support group.....for yummy lunches, never-ending gossip sessions (about science, of course!) and for being so much more than just lab acquaintances. A special thanks to Russ, for inspiring me to get through the past three years and for all your help in putting together this thesis.

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Most of all, I would like to thank my loving parents: you are the reason I try my best every day and reach for the stars. Dad, you have taught me the true meaning of persistence and hard work. Mom, you are my angel and my lifelong friend. Thank you both for your constant love and encouragement…without you, I wouldn't be here.

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## PUBLICATIONS ARISING FROM THE WORK OF THIS THESIS

Manalo JA, Osafo J and Goodyer CG. **Developmental Changes in the Human Growth Hormone Receptor and its Signal Transduction Pathways.** (manuscript in preparation)

Manalo JA and Goodyer CG. **Developmental Changes in the Human Growth Hormone Receptor and its Signal Transduction Pathways.** June 2002, San Francisco, CA. Endocrine Society's 84th Annual Meeting, Abstract #P3-278.

Goodyer CG, Figueiredo RMO, Krackovich S, De Souza Li L, Manalo JA and Zogopoulos G. 2001. **Characterisation of the Growth Hormone Receptor in Human Dermal Fibroblasts and Liver during Development.** American Journal of Physiology: Endocrinology and Metabolism. 281: E1213-E1220.

The candidate was responsible for the truncated (1-279) and full-length hGHR mRNA studies, which were carried out in the laboratory of Dr. Goodyer.

Manalo JA and Goodyer CG. **Changes in the Human Growth Hormone (hGH) Receptor and hGH Signal Transduction Pathways during Development.** July 2001, Montreal, QC. Pediatric Endocrinology Montreal 2001 LWPES/ESPE 6th Joint Meeting, Abstract #P2-190.

## ABBREVIATIONS

| | |
|---|---|
| 3+: | exon 3-retained GHR |
| 3-: | exon 3-deleted GHR |
| ALS: | acid-labile subunit |
| ANOVA: | analysis of variance |
| ATCC: | American type culture collection |
| Asn: | asparagine |
| ATP: | adenosine triphosphate |
| $CaCl_2$: | calcium chloride |
| cDNA: | complementary deoxyribonucleic acid |
| CV1: | African Green monkey kidney fibroblast cell line |
| DMEM: | Dulbecco's Modified Eagle's Medium |
| dNTP: | deoxyribonucleotides |
| DTT: | dithiothreitol |
| EDTA: | ethylenediaminetetraacetic acid |
| EMEM: | Eagle's minimum essential medium |
| EPOR: | erythropoietin receptor |
| ERK: | extracellular signal regulated kinase |
| FBS: | fetal bovine serum |
| FL: | full-length GHR |
| GAS: | interferon-gamma activated sequence |
| GH: | growth hormone |
| GHBP: | growth hormone binding protein |
| GHR: | growth hormone receptor |
| GHRH: | growth hormone releasing hormone |
| GHS: | growth hormone secretagogue |
| GLE: | gas-like element |
| Grb: | growth factor receptor bound |
| HAL: | human adult liver |
| HBS: | HEPES buffer solution |
| HEK293: | human embryonic kidney cell line |
| HEPES: | hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| HepG2: | human hepatoma cell line |
| hGH: | human growth hormone |
| hGHR: | human growth hormone receptor |
| Huh7: | human hepatoma cell line |
| IFN: | interferon |
| IGF: | insulin-growth factor |
| IL: | interleukin |
| IMCD: | mouse inner medullary collecting duct cell line |
| IP: | immunoprecipitation |
| IRS: | insulin receptor substrate |
| LPS: | lipopolysaccharide |
| Jak: | janus kinase |
| KCl: | potassium chloride |

## ABBREVIATIONS (2)

| | |
|---|---|
| M: | mean |
| MAPK: | mitogen activated protein kinase |
| MEK: | mitogen activated protein-ERK |
| $MgCl_2$: | magnesium chloride |
| mRNA: | messenger ribonucleic acid |
| NaCl: | sodium chloride |
| NaF: | sodium fluoride |
| $Na_3VO_4$: | sodium vanadate |
| PBS: | phosphate buffer saline |
| PI3K: | phosphatidylinositol-3-kinase |
| PIAS: | protein inhibitor of activated stats |
| PKC: | protein kinase C |
| PMSF: | phenylmethylsulfonyl flouride |
| PRLR: | prolactin receptor |
| PTB: | phosphotyrosine binding |
| PVDF: | polyvinylidene difluoride |
| RLU: | relative light units |
| RT-PCR: | reverse transcription-polymerase chain reaction |
| SDS-PAGE: | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SEM: | standard error of the mean |
| SH2: | src-homology 2 |
| SHC: | src homology 2/α collagen-related |
| SHP: | src-homology 2-containing phosphatase |
| SIE: | sis inducible element |
| SIRP: | signal regulatory protein |
| Socs: | suppressor of cytokine signalling |
| Sos: | son of sevenless |
| SS: | somatostatin |
| Stat: | signal transducer and activator of transcription |
| T: | truncated GHR |
| TPOR: | thrombopoietin receptor |
| UbE: | ubiquitin endocytosis motif |
| UTR: | untranslated region |
| WB: | western blot |
| V: | variant 5'UTR hGHR exon |
| wks FA: | weeks fetal age |
| yr: | years old |

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# 1.GENERAL INTRODUCTION AND LITERATURE REVIEW

## 1.1 Growth Hormone (GH)

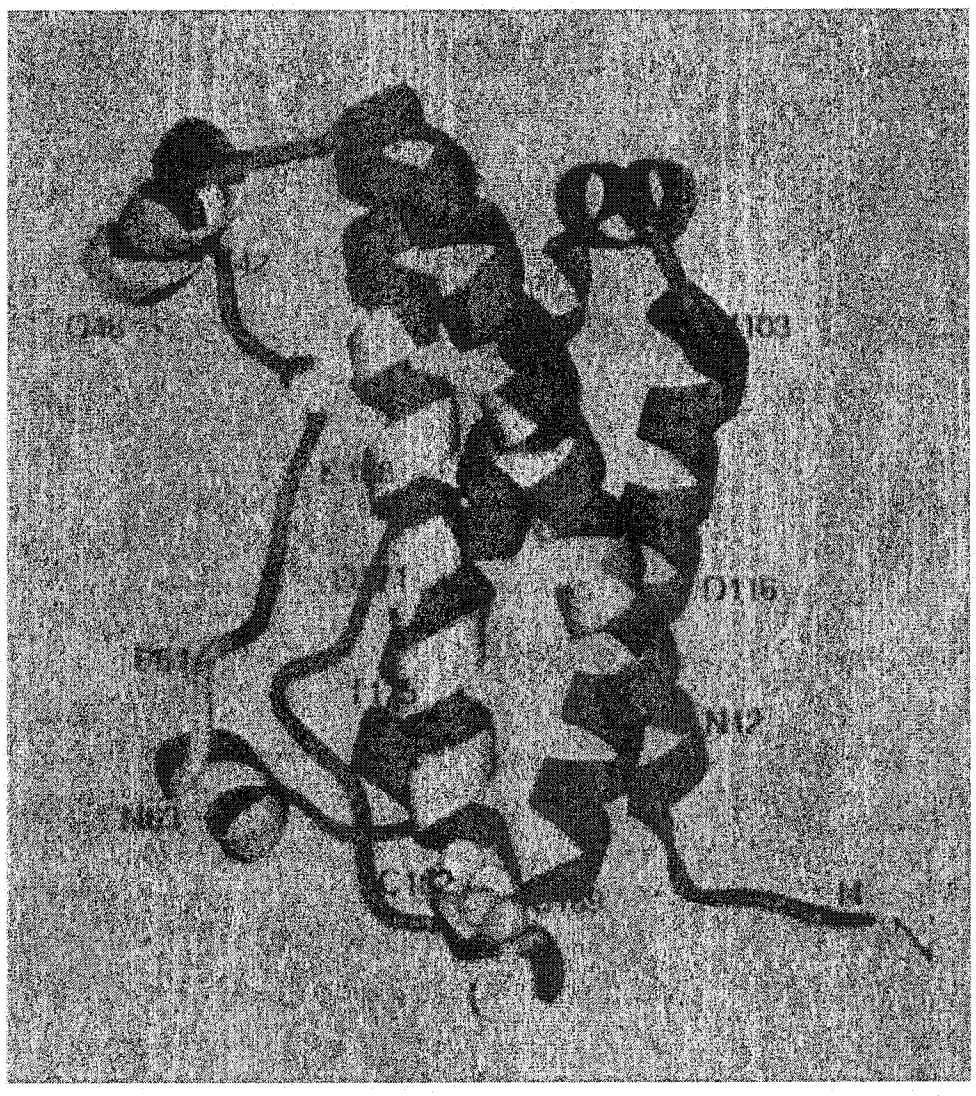
### 1.1.1 GH Structure

The major form of human GH (hGH) is a 191 amino acid (~22 kD) product of the hGH-N gene on chromosome 17 [1]. Alternative splicing of the hGH-N gene also occurs, resulting in an internal deletion of 15 amino acids and the formation of a 176 amino acid isoform of hGH [2]; this 20 kDa hGH represents ~15% of circulating hGH. Other variants of hGH (acetylated, phosphorylated, glycosylated, dimerized and oligomerized hGH) can be generated post-translationally but their functional significance is unknown. GH has a half-life of approximately 20 minutes and 50% circulates in the serum bound to the GH binding protein (GHBP) [3]. The three-dimensional structure of the hGH molecule is arranged as four antiparallel alpha-helical bundles, which run in an unusual pattern of "up-up-down-down" [4] (Figure 1).

### 1.1.2 Hypothalamic Regulation of GH secretion; GH-IGF axis

GH is an important regulator of somatic growth and metabolism in humans [5]. Release of GH from the pituitary gland is not constant over time, but rather is intermittent, or pulsatile. Maximal GH secretion occurs within 1 hour after the onset of deep sleep, reflecting a nocturnal surge of GH [6]. In humans, GH secretion begins at approximately the $10^{th}$ week of fetal life, increasing up to 120-150 ng/mL at midgestation: serum levels then gradually decrease to term but remain elevated for several weeks postnatally [7]. In the normal human adult, GH is secreted at an average

**Figure 1. Ribbon representation of the structure of hGH, viewed as perpendicular to the four helix bundle** [4]. The $NH_2$-terminus is marked N. the COOH-terminus, C. Residues in the interfaces between the hormone and the two GH receptors are coloured green (interface I) and blue (interface II), respectively.

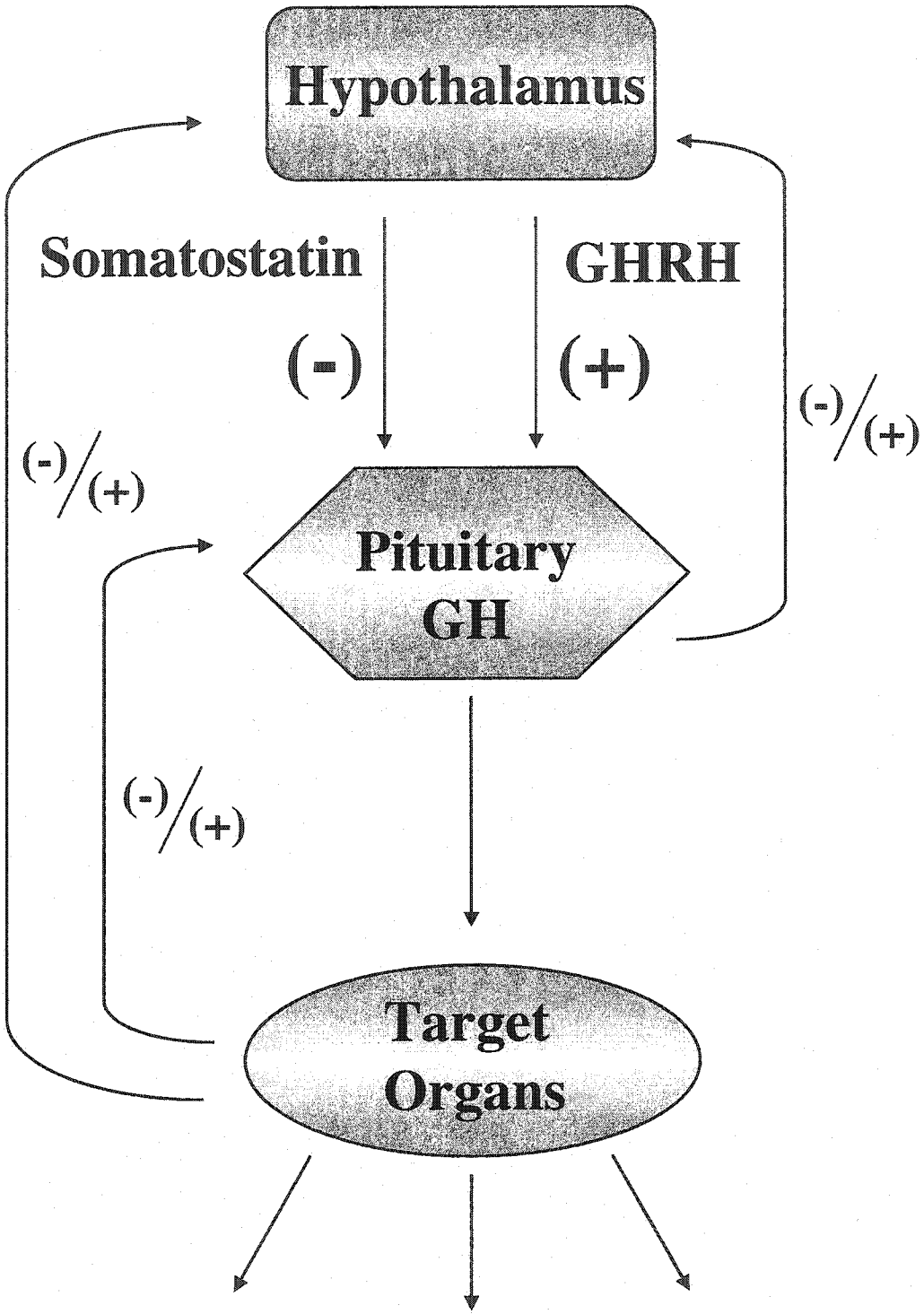

O4b
O3
O1b
N2
N3
H

rate of ~200 μg/day with an average of 7-10 episodic pulses per day. Serum levels of GH range from 0-10 ng/mL, where peak postnatal secretion occurs during puberty [8]. Notable sex differences have also been observed in humans [9]. Compared to men, premenopausal women have a two- to threefold higher daily GH secretion rate due to higher GH pulse amplitude. Despite this difference, both males and females exhibit the same GH pulse frequency. Both the pulsatility and nocturnal surge pattern of GH release is critical for normal human growth.

GH is primarily synthesized by somatotrophs in the anterior pituitary gland, under the regulation of two hypothalamic regulatory peptides, GH-releasing hormone (GHRH) and somatostatin (SS) [10] [11] (Figure 2). GHRH binds to its specific seven transmembrane G-protein coupled receptor at the level of the somatotrope to enhance GH synthesis and to stimulate GH release. It is also needed for normal pulsatile GH secretion [12]. SS, on the other hand, inhibits the release of GH [11]. SS affects the timing and amplitude of GH secretion and, unlike GHRH, is not believed to modulate GH synthesis [13].

Ghrelin, a 28 amino acid GH secretagogue (GHS), also plays an important role in the regulation of GH. Primarily produced in cells lining the stomach [14] [15], Ghrelin may act as a signal for the nutritional status of individuals. An increase in GH secretion has been shown to be stimulated by Ghrelin in two ways: 1) directly from the somatotrope and 2) indirectly by increasing GHRH release. Plasma GH levels are also affected by circulating insulin-like growth factor I (IGF-I), which regulates GH through feedback effects at the hypothalamus as well as pituitary [16]. IGF-I is produced and secreted by all tissues (but primarily the liver) in response to GH stimulation, and acts by

**Figure 2. Hypothalamic-GH-IGF axis** (modified from [17]). Pituitary hGH production and secretion is regulated by the hypothalamic hormones GHRH and SS. GH and IGF-1 both feedback at the level of SS and GHRH secretion by the hypothalamus, while IGF-I also has feedback effects at the pituitary somatotrope.


Hypothalamus
Somatostatin
GHRH
(-)
(+)
(-)/(+)
(-)/(+)
Pituitary
GH
(-)/(+)
Target
Organs
Growth and Metabolic
Effects
direct and indirect (via IGF-I)

binding to type I IGF receptors [18]. Other factors that influence the secretion of GH include sex hormones, stress and nutritional status.

### 1.1.3 Metabolic actions of GH

GH acts both directly on target tissues (eg. liver, bone, muscle and fat) and indirectly, by stimulating production of its "second messenger", IGF-I [19]. Major endocrine effects of GH include a reduction of glycolysis in the liver, increasing lipolysis in adipocytes, increasing amino acid transport in muscle, liver and adipocytes, and the stimulation of chondrocyte differentiation. It also stimulates IGF-I production in almost every tissue that has been examined to date, leading to proliferative and metabolic effects.

GH has both insulin-like and anti-insulin-like effects on cells and tissues. Acute exposure of GH to GH-starved cells of tissues stimulates insulin-like effects, while chronic exposure results in anti-insulin-like actions. Although a common signalling pathway for GH and insulin has not yet been discovered, it has been shown that GH is capable of stimulating the phosphorylation of insulin receptor substrates (IRS) [20] [21]. Phosphatidylinositol-3-kinase (PI3K) is an important downstream molecule of phosphorylated IRS-1 and IRS-2, which act as binding sites for the p85 subunit of PI3K in a variety of cell types in vitro and in vivo [22] [23]. PI3K activity is important for the insulin-like action of GH, as it has been shown that the inhibition of PI3K blocks GH-stimulated lipid synthesis and the anti-lipolytic action of GH. The anti-insulin-like effects that result from chronically elevated levels of GH promote insulin resistance and diabetes. Excess GH can lead to chronic activation of the IRS-PI3K pathway in liver,

reducing the degree of insulin-induced activation. GH has also been implicated in the inhibition of the expression of the gene encoding glucose transporter 1 [24].

Cell growth and differentiation, which require changes in cell shape and/or location, are positively regulated by GH. GH was recently shown to stimulate cell motility and spreading [25] [26], actin rearrangement [27], microtubule polymerization [28] and assembly of multiprotein complexes with several proteins involved in cell adhesion and/or movement [29]. SH2-Bβ, a src-homology 2 (SH2) domain-containing protein that binds Jak2, has also been implicated in GH regulation of the actin cytoskeleton [30].

### 1.1.4 Clinical Aspects of GH Action

The most interesting clinical disorders related to the GH-GHR-IGF axis are those that display significantly increased or decreased net GH action (Table 1). Acromegalic patients have chronically high levels of GH due to GH-secreting pituitary adenomas [31]. Certain acromegalics may also manifest type II diabetes mellitus, with detectable insulin resistance. Postpubertal acromegalics exhibit overgrowth of soft tissues while gigantism (excessive height) occurs in individuals whose tumours are active prior to pubertal closure of the epiphyseal growth plate in the long bones [19].

On the other hand, individuals with chronically low levels of GH are often characterized by short stature and abnormal skeletal and cartilage maturation. The apparent decreased net GH action can usually be explained by hereditary or early acquired GH deficiency, due to GH gene deletion, somatotroph abnormal development or destruction or dysregulation of GH secretion [32]. A mutation in the GH molecule has

**Table 1. Clinical syndromes related to GH pathology** [17]. The most striking clinical disorders related to the GH axis are those that manifest significantly increased or decreased net GH action.

| GH STATE | CLINICAL SYNDROME | CAUSES |
|---|---|---|
| ↑ **GH ACTION** | • ACROMEGALY (after puberty)<br>• GIGANTISM (before puberty) | • GH-Secreting Pituitary Adenoma<br>• Ectopic GHRH<br>• Ectopic GH |
| ↓ **GH ACTION** | • GH-deficiency | • Isolated Gene Defect<br>• Pituitary Failure<br>• Hypothalamic Dysfunction |
| | • Abnormal GH | • GH Mutations |
| | • GH Resistance (Laron Syndrome) | • GHR Mutations |
| | • "Normal Short Kids" | • ? |

also been described [33], which resulted in short stature due to the presence of abnormal GH.

Laron Syndrome (GH resistance) is a rare disease, similar in phenotype to GH deficiency [34]. Although circulating IGF-I levels are low in Laron patients, GH is present and often elevated. Thus, the observed hormone resistance in Laron syndrome is usually associated with a variety of defects in GH receptor (GHR) structure, function or cell surface expression. A mouse model of this clinical disorder (Laron Mouse) has been obtained by targeted disruption of the GHR gene [35]. The GHR-deficient mouse displays marked postnatal growth retardation, greatly decreased levels of serum IGF-I and elevated serum GH, mimicking the human Laron syndrome. While defects are typically caused by a gene deletion or mutation preventing proper GHR cell surface routing or GH binding, defects in GH-GHR downstream signal transduction pathways have also been observed [36] [37]. Certain Laron syndrome patients exhibit a specific GHR mutation D152H, which affects the net charge of the extracellular domain of receptor. This mutation, which occurs at the dimerization interface of receptor, prevents GH-induced GHR dimerization. The absence of formation of the GH/GHR complex at the cell surface prevents receptor stimulation and subsequent activation of downstream intracellular signalling molecules. Finally, the abnormal physiological mechanism responsible for "normal short kids" (ISS: GH insensitivity syndrome) is presently unknown. These children have normal GH and only rare heterozygous mutations of the GHR (<2%) [38].

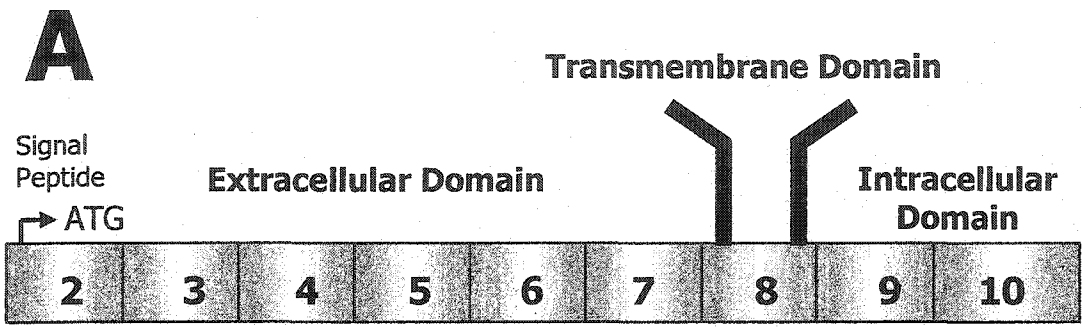
## 1.2 Growth Hormone Receptor

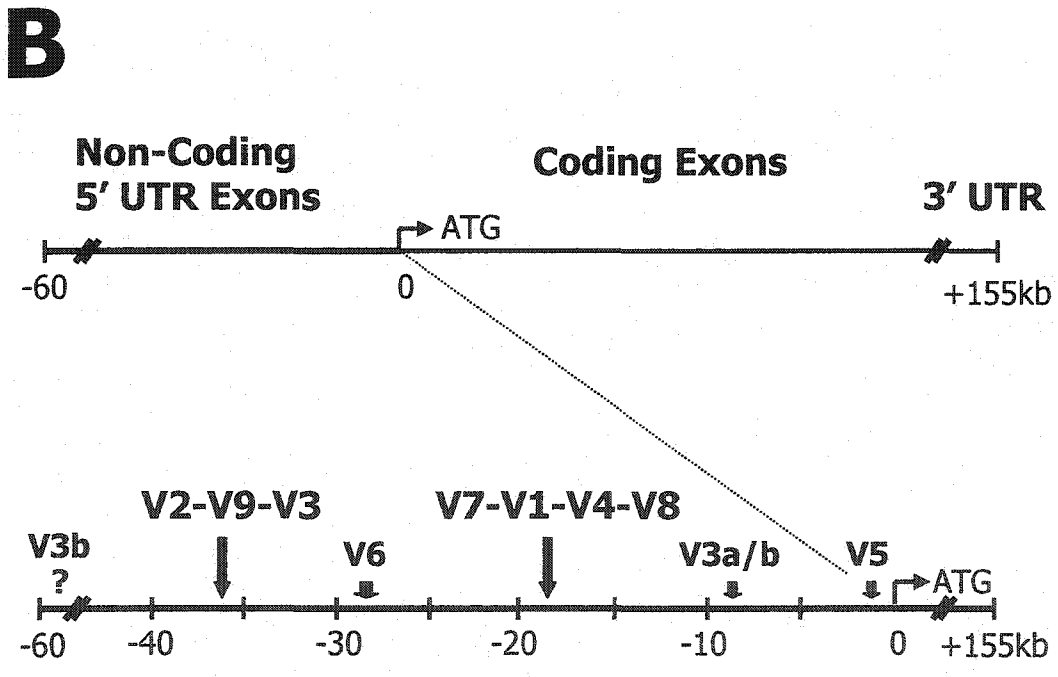
### 1.2.1 GHR gene expression

The GHR gene has been mapped to 5p12-13 on chromosome 5 [39]. The gene is approximately 150 kb long and codes for a mature protein of 620 amino acids [40]. The coding region is defined by nine exons (#2-10), with exon 2 containing 11 bp of the 5' UTRs, the translational start site and 23 bp of the signal sequence, exons 3-7 encoding the extracellular domain, exon 8 the transmembrane domain and exons 9-10 the intracellular domain (Figure 3A). The GHR gene is expressed in all tissues that have been examined, with mRNA levels being the highest in liver.

Transcriptional regulation of the human GHR gene is complex: eleven coding exons have been identified in the 5' UTR region of the hGHR gene that give rise to eleven mRNA transcripts [41] (Figure 3B). The sequences of the transcripts all splice into exon 2, 11 bp upstream of the ATG codon and thus, code for the same protein. However, the sequences upstream of the 11 bp are unique. These variants have been named V1-V9 (V3 has three mRNAs due to alternative splicing) according to the number of clones isolated in a human adult liver cDNA library, V1 being the most abundant [41]. Expression of these transcripts is tissue specific and developmentally regulated and differential promoter activity is hypothesized for each of the 5' UTR exons [42]. Ten of the known 5' UTR exons of the hGHR were mapped within approximately 40 kb of the 5' UTR flanking region [43]. There are two major clusters of non-coding exons within the 5' UTR region: V7-V1-V4-V8 and V2-V3-V9. V7-V1-V4-V8 occupies approximately 2 kb and is 13 kb [44] or 18 kb [43] upstream of the translational start site. mRNAs derived from the V7-V1-V4-V8 exons are found only in the postnatal liver. The

**Figure 3. Human GHR gene.** A) Coding exons [45] and B) the eleven known 5' UTR exons of the hGHR were mapped within approximately 40 kb of the 5' UTR flanking region [43]. There are two major clusters of non-coding exons within the 5' UTR region: the tissue and developmental-specific V7-V1-V4-V8 and the ubiquitously expressed V2-V3-V9.


A
Transmembrane Domain
Signal Peptide
ATG
Extracellular Domain
Intracellular Domain
2
3
4
5
6
7
8
9
10


B
Non-Coding 5′ UTR Exons
Coding Exons
3′ UTR
ATG
-60
0
+155kb
V2-V9-V3
V7-V1-V4-V8
V3b ?
V6
V3a/b
V5
ATG
-60
-40
-30
-20
-10
0
+155kb

second cluster, V2-V3-V9, occupies approximately 1.6 kb and is an estimated 33 kb away from the translational start site. This cluster exhibits ubiquitous expression in all human tissues examined to date.

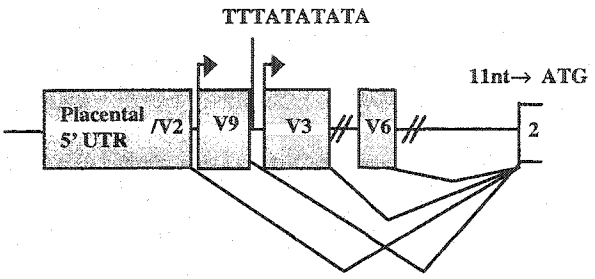
Multiple transcripts have also been identified in other species [46] [47] (Figure 4). Although the hGHR gene is the most complex, there are similar 5'UTR sequences as well as parallel expression patterns amongst all species. One GHR mRNA is always specific for postnatal liver: ovine and bovine 1A, mouse L1 and rat GHR1. Each sub-primate GHR gene also has at least one ubiquitously expressed mRNA: ovine 1B, bovine 1B and 1C, mouse L2-5, rat GHR2 and GHR4. Despite these structural similarities, the regulatory mechanisms are likely to be different. For example, expression of mouse liver L1 mRNA increases with pregnancy, rather than around the time of birth [48]. In addition, the developmental increase in ovine liver 1A occurs due to a surge in fetal cortisol levels during the last two weeks before birth [49], while human and baboon liver V1 levels are detectable only several weeks after birth [50] [51] (unpublished observations).

### 1.2.2 Cytokine Receptor Superfamily

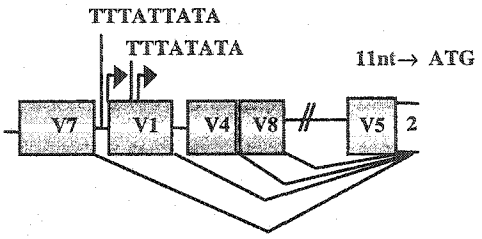
GHR is a member of the class I cytokine receptor superfamily (Figure 5), which is characterized by the absence of intrinsic catalytic activity within the carboxy terminus of the receptor [52]. Instead, the GHR is engaged in a noncovalent association with cytoplasmic tyrosine kinases to form multi-subunit receptor complexes. Other family members include the closely related prolactin receptor as well as receptors of various interleukins, colony-stimulating factors, erythropoietin, thrombopoietin and leptin. These

**Figure 4. Comparison of the GHR 5' flanking regions across five species [43].** Homologues to the ubiquitiously expressing human V2 exon have been characterized in the ovine (1B), bovine (1B) and rodent (L2 or GHR2). Human V9 homologues include 1C in the bovine and three exons in the mouse of GHR4 in the rat. Homologues of the liver-specific expressing human V1 exon include 1A in the ovine and bovine and L1 or GHR1 in the rodent. A V5 homologues has also been identified in mice (L5).

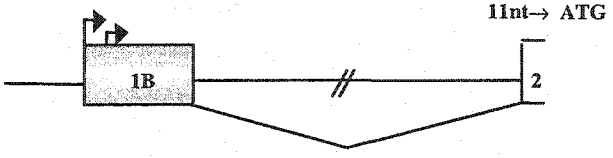
Human *GHR*



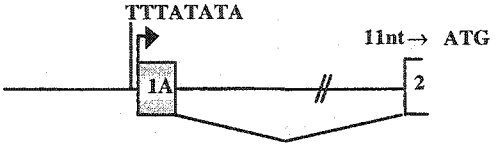

Human *GHR*



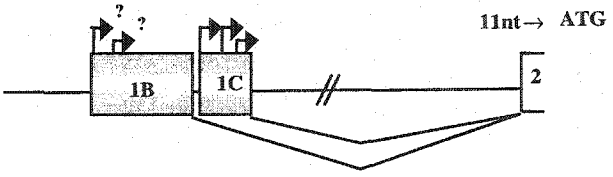

Ovine *GHR*



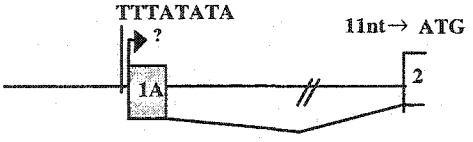

Ovine *GHR*



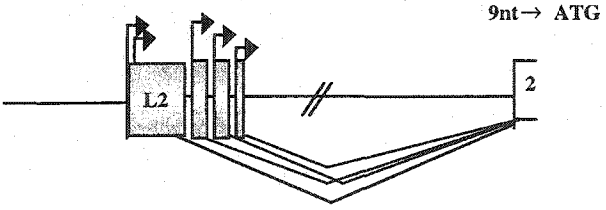

Bovine *GHR*



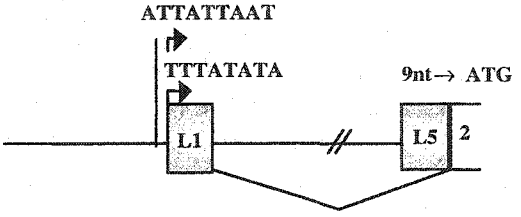

Bovine *GHR*



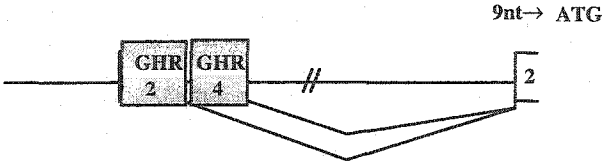

Mouse *GHR*




Mouse *GHR*




Rat *GHR*




Rat *GHR*

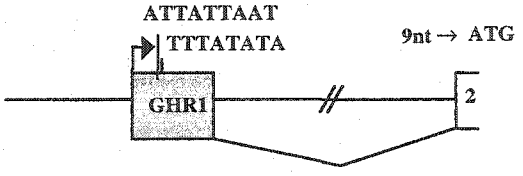
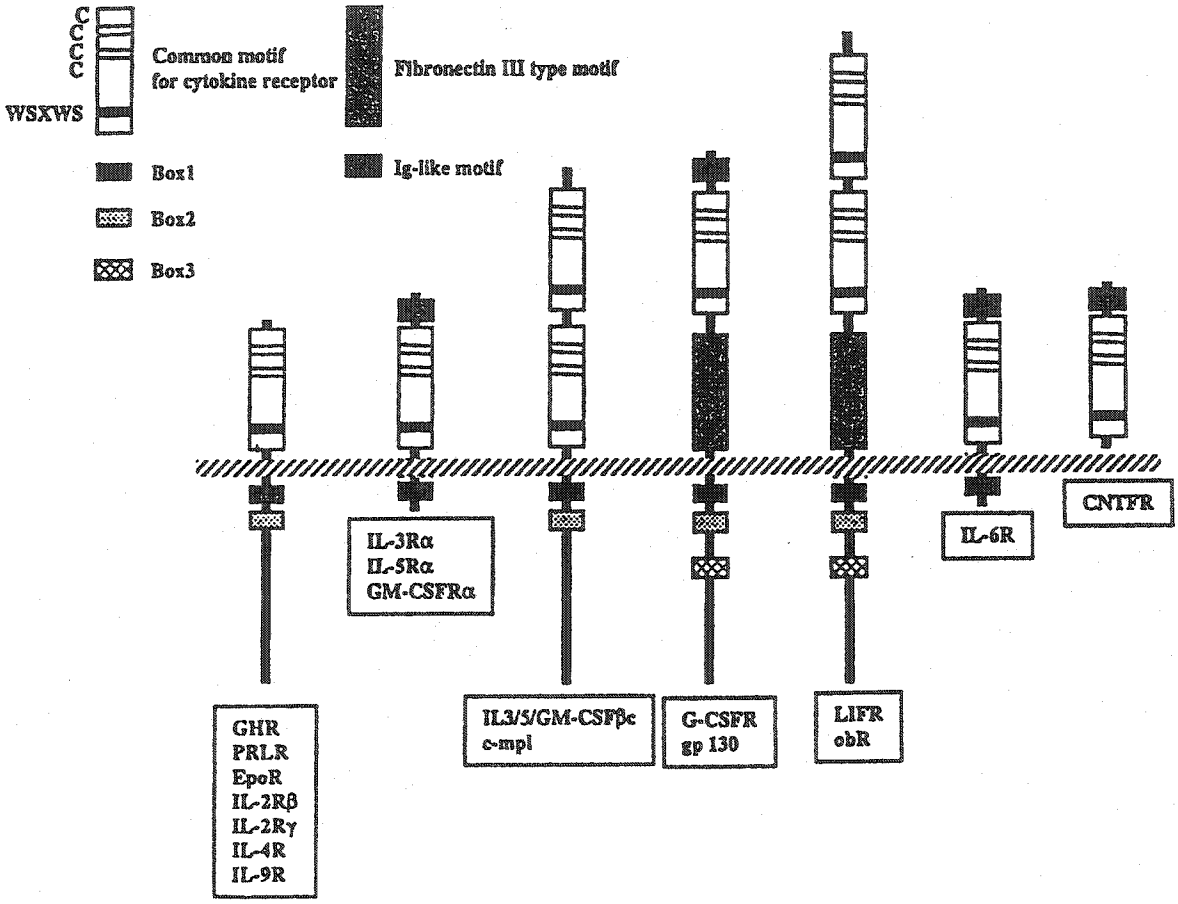


**Figure 5. Schematic representation of the general structure of some receptors in the GH/PRL hematopoietic cytokine receptor family [53].** The growth hormone receptor is a single transmembrane protein belonging to the cytokine receptor superfamily, which is characterized by the absence of catalytic activity within the carboxy terminus. Common features include the WSXWS motif in the extracellular domain (YGEFS in the GHR) and the Box 1 region in the cytoplasmic tail.


C
C
C
C
WSXWS
Common motif
for cytokine receptor
Fibronectin III type motif
Box1
Box2
Box3
Ig-like motif
IL-3Rα
IL-5Rα
GM-CSFRα
GHR
PRLR
EpoR
IL-2Rβ
IL-2Rγ
IL-4R
IL-9R
IL3/5/GM-CSFβc
c-mpl
G-CSFR
gp 130
LIFR
obR
IL-6R
CNTFR

class I cytokine receptors display a common WSXWS-motif (YGEFS in the GHR) in the extracellular domain, which is involved in ligand binding [54]. Class II receptors, which include receptors for interferons, IL-10 and IL-12, do not contain this structural motif. The GHR and other cytokine receptor family members also contain regions of homology in their cytoplasmic domains, such as the Box 1 and Box 2 elements, that confer similarities in their signalling mechanisms.

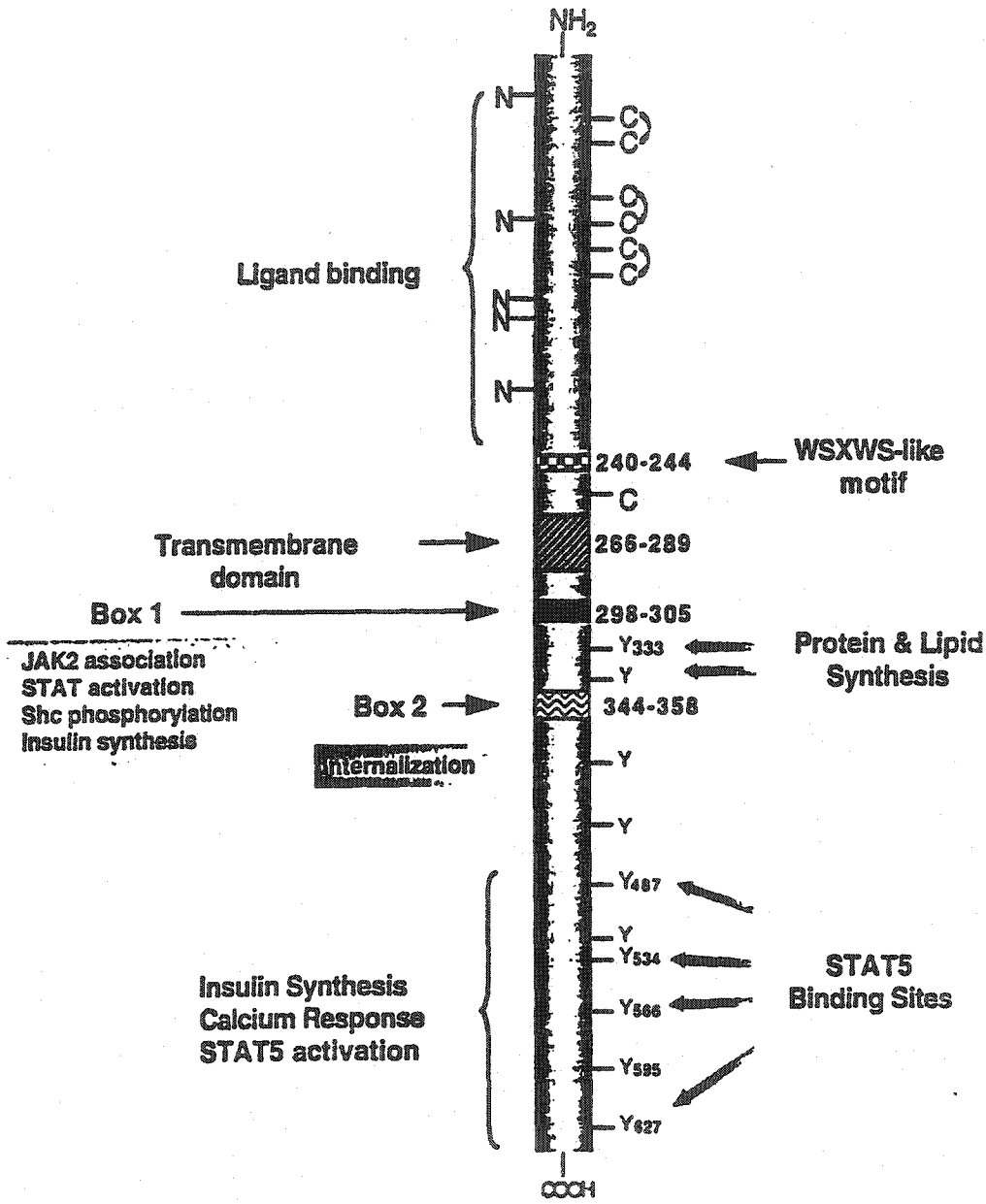
### 1.2.3 GHR protein structure

The major form of the mature GH-specific receptor is a 620 amino acid single polypeptide chain, containing an extracellular hormone binding domain of 246 residues, a 24 residue transmembrane domain and a large cytoplasmic domain of 350 residues (Figure 6). Human and rabbit GHR cDNAs were first isolated in 1987 from the liver, the most abundant source of the GHR [45]. The GHR contains several structural features that are of interest.

In mammalian GHRs, the WSXWS-like motif, represented by $Y^{222}GEFS^{226}$, plays an important role in GH binding and signal transduction. Alanine mutations (Y222A, S226A and G223A) resulted in diminished ligand binding and abolished signal transduction [54]. Because this motif does not make direct contact with GH [54], it is likely that the YGEFS motif plays a critical role in maintaining receptor conformation.

Three pairs of conserved cysteine residues, which form intrachain disulfide linkages, are also present in the extracellular domain of the GHR. These residues function with the WSXWS-like motif to help form and/or support the receptor's extracellular ligand binding pockets [54] [55]. The presence of five potential asparagine

**Figure 6. Growth Hormone receptor: key structural and functional features** [17]. Shown are the locations of five Asn-linked glycosylation sites (N) and seven cysteine residues (C) within the extracellular ligand binding domain of GHR. Ten tyrosine residues (Y) are present in the cytoplasmic region of rat GHR; the six tyrosines that are conserved across species are numbered. Also shown are the extracellular WSXWS-like motif and the intracellular Box 1 and Box 2 regions. Regions of GHR that are required for various functions, including Stat5B binding, are indicated using the rat GHR numbering system.

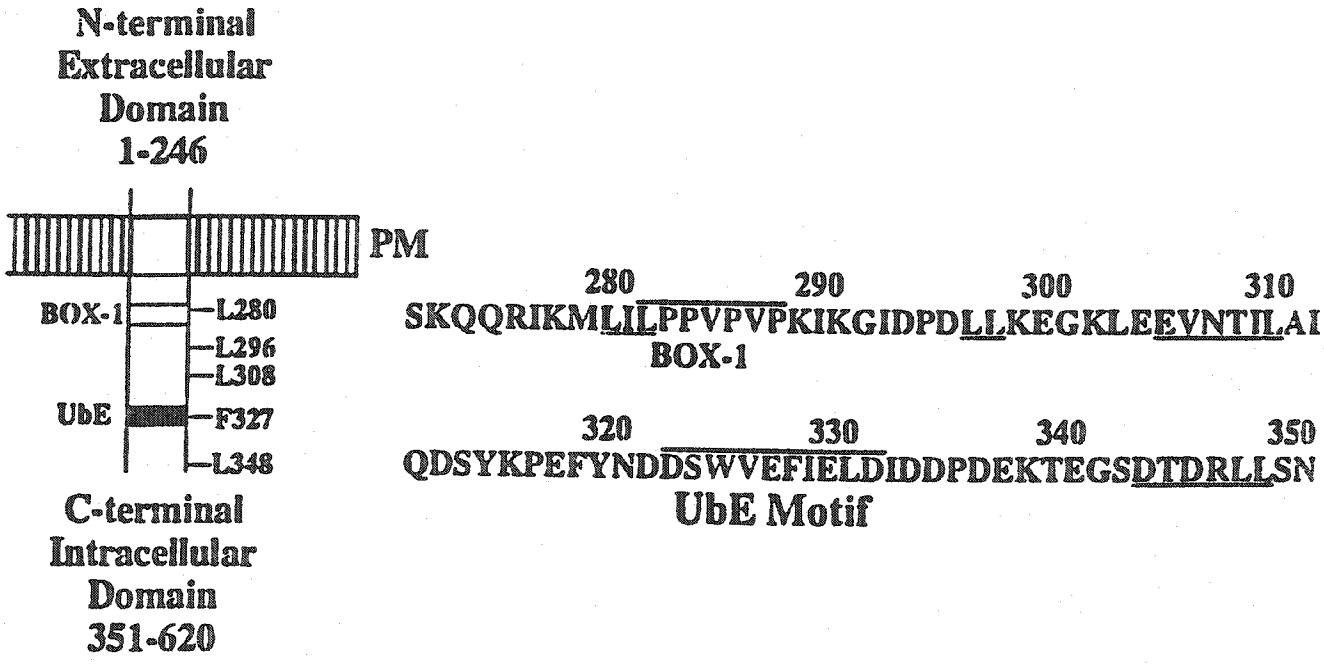

NH2
Ligand binding
240-244
WSXWS-like motif
C
Transmembrane domain
266-289
Box 1
298-305
JAK2 association
STAT activation
Shc phosphorylation
Insulin synthesis
Y333
Y
Protein & Lipid Synthesis
Box 2
344-358
Internalization
Y
Y
Y487
Y
Y534
STAT5 Binding Sites
Insulin Synthesis
Calcium Response
STAT5 activation
Y566
Y595
Y627
COOH

(Asn)-linked glycosylation sites, bearing the highly conserved consensus sequence Asn-X-Ser/Thr, are also necessary for the maintenance of a high affinity GH binding site. The role of the Asn-linked glycosylation in the GHR was defined by site-directed mutagenesis [56], where three out of five pGHR Asn residues were demonstrated to be essential for GH binding. It has also been shown that residues serine 145, histidine 150, aspartate 152, tyrosine 200, and serine 201 in the extracellular dimerization domain of the GHR are required for effective signal transduction [57] [36].

C-terminal to the 24 amino acid transmembrane domain is the 320 aa intracellular domain of the GHR (Figure 6). Similar to other members of the class I cytokine receptor superfamily, the cytoplasmic tail of the GHR contains no known intrinsic enzymatic activity that can initiate signalling. Instead, the required tyrosine kinase activity is provided by a receptor-associated cytoplasmic protein from the Janus kinase (Jak) family, Jak2 [58]. The presence of a proline-rich Box 1 element (ILPPVPVP) in the receptor's proximal cytoplasmic domain is crucial for the association of the GHR with Jak2 [59].

In addition, the 80 aa membrane-proximal region of the GHR, containing both Box 1 and the downstream Box 2 element, was shown to be important in Jak2 and Signal transducers and activators of transcription (Stat) activation [60]. Box 2 is located about 40 residues C-terminal to Box 1. It is a more loosely conserved region containing seven acidic residues, a ubiquitin endocytosis motif (UbE) motif (DSWVFIELD) and a terminal lysine (Figure 7). The cytoplasmic tail of the GHR has been examined in detail for its role in endocytosis, as binding of GH stimulates ubiquitination, internalization and degradation of the receptor [45] [61] [62] [63] [64]. *In vitro* studies have demonstrated that truncation of the cytoplasmic tail of the GHR at amino acid residue 349 results in

**Figure 7. Ubiquitin endocytosis motif (UbE) in GHR cytoplasmic tail.** [61]. Overlined, Box 1 and UbE motif; underlined, possible di-leucine internalization motifs involved in clathrin-mediated endocytosis.


N-terminal
Extracellular
Domain
1-246
PM
BOX-1
L280
L296
L308
UbE
F327
L348
C-terminal
Intracellular
Domain
351-620
280
290
300
310
SKQQRIKMLILPPVPVPKIKGIDPDLLKEGKLEEVNTILAI
BOX-1
320
330
340
350
QDSYKPEFYNDDSWVEFIELDIDDPDEKTEGSDTDRLLSN
UbE Motif

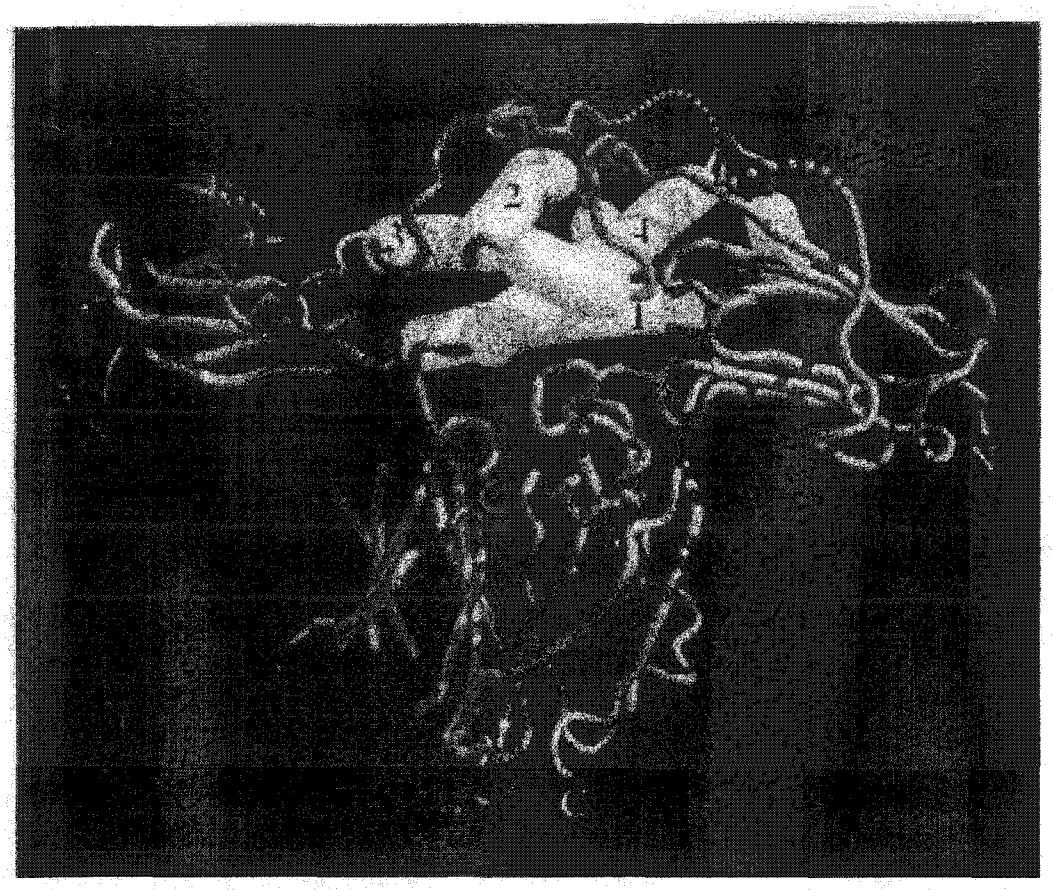
loss of ligand-induced uptake, despite the presence of three potential di-leucine internalization sequences upstream [65]. The UbE motif was determined to be required for proper ubiquitin conjugating activity and endocytosis of the GHR. Interestingly, this UbE motif does not resemble any other known ubiquitination domain [66]. Studies have also shown that a phenylalanine residue in the proximal one-fifth of the GHR cytoplasmic domain (Phe-346 in rat; Phe-327 in rabbit) is necessary for internalization [59] [67].

Additional truncations of the GHR intracellular domain have suggested functional specificity for different regions of the cytoplasmic tail. For example, six tyrosine residues present in the GHR are conserved across species: $Y^{rodent/human\&rabbit}$ ($Y^{333/314}$, $Y^{487/469}$, $Y^{534/516}$, $Y^{566/548}$, $Y^{595/577}$ and $Y^{627/609}$). Rodent Y333 and/or Y338 were shown to be phosphorylated upon GHR activation in response to GH, but not required for GHR:Jak2 association [68]. In addition, Lobie *et al.* have demonstrated a requirement for specific GHR tyrosine residues Y333 and Y338 for two GH-regulated cellular responses, lipogenesis and protein synthesis [69].

## 1.2.4 GH binding and receptor dimerization

The GHR binding protein (GHBP) corresponds to a soluble form of the GHR's extracellular domain. Examination of the three-dimensional crystal structure of human GHBP bound to human GH has demonstrated the existence of a complex consisting of two GH receptors and a single GH molecule [4] (Figure 8). The three dimensional structure of the GHR extracellular domain consists of two domains, each domain containing seven beta-strands that together form a sandwich of antiparallel beta-sheets. This N-terminal domain also contains three disulfide bridges, two of which link

**Figure 8. Backbone structure of the 1 hGH:2 hGHBP complex** [4]. The hormone is shown as yellow cylinders representing the helices connected by red tubes. The beta-strands of the binding proteins are shown in brown, the loops are green (hGHBP I) and blue (hGHBP II). The figure is a sideways view of the GH-GHR complex. In this orientation, the C-termini of the extracellular domains, and therefore the cell membrane, are at the bottom of the figure.


2
1

neighbouring strands and the third disulfide bridge cross-linking the two sheets of the sandwich. The conserved cysteines present in the extracellular domain of the GHR are therefore linked to form disulfide bonds that are buried in the interior of the beta-barrel. The major structural feature of the hGH molecule is a four-helical bundle with an unusual topology; the helices run up-up-down-down, in contrast to the more usual up-down-up-down scenario. The receptor binding sites of hGH are located on the faces of opposite sides of the four-helical bundle. Mutational analyses within each of the two sites of the GH molecule that are involved in the interaction with the GHRs suggest a multi-sequential event. The initial step is high-affinity binding of site 1 of GH to the first receptor. The first binding site on hGH has a concave character, formed by residues on exposed faces of mainly helix 4 but also of helix 1 (Figure 8). The second, relatively flat binding site of GH, made up of the exposed sides of helices 1 and 3, then contacts a second GHR, and the 1GH:2GHR complex is stabilized by interaction along the membrane-proximal halves of the C-terminal domains of the receptors [70]. Although the overall shapes of the two binding sites on the hormone are quite different, it is interesting to note that the residues on both receptors that interact with these sites are largely the same. Human GH mutants with site-specific alterations at site 2, namely G120R or G120K [19], are potent GH antagonists; they bind one GHR with high affinity, but are unable to bind the second receptor molecule or to stimulate cell proliferation. These experimental findings support the two-site model of GH-GHR interactions, and indicate that GH-induced receptor dimerization is required for a biological response to GH.

### 1.2.5 GHR Intracellular Signalling

Four major signalling cascades are initiated following the GH-GHR complex formation (Figure 9): 1) activation of signal transducers and activators of transcription (STAT) pathways that lead to a direct modulation of target gene transcription; 2) activation of the Ras-Raf-MEK-ERK pathway that results in the activation of mitogen-activated protein kinase (MAP kinase); 3) activation of phosphatidylinositol-3-kinase (PI3K), at least in part by tyrosine phosphorylation of insulin receptor substrates, IRS-1 and IRS-2; and 4) activation of PKC. In many cases, there is significant crosstalk between these four GH-activated pathways. One common feature of all four cascades is the initial activation of Jak2 and tyrosine phosphorylation of the GHR [71].

#### 1.2.5.1 Janus Kinase family and Activation of Jak2

Jak proteins are intracellular protein tyrosine kinases that range from 120-140 kDa in size. There are four members of the Jak family in mammals, namely Jak1, Jak2, Jak3 and Tyk2 [72]. While Jak1, Jak2 and Tyk2 are ubiquitously expressed, Jak3 is expressed predominantly in hematopoietic cells. The Jak family is characterized by seven Jak Homology (JH) regions of variable lengths, JH1-JH7 [72] (Figure 10A). JH1 is representative of the C-terminal tyrosine kinase domain. A highly conserved motif (Phe-Trp-Tyr) within this catalytic domain renders the JH1 domain identical in all Jak family members. JH1 also contains the crucial tyrosine residues that are phosphorylated upon Jak activation. JH2 represents the non-catalytic kinase-like (KL) domain, which is adjacent to the functional kinase domain. This unique "pseudokinase" domain appears to be required for the catalytic activity of JH1 [73]. Studies have also suggested that the KL

**Figure 9. Intracellular signalling pathways proposed to be activated by GH [74].** Growth hormone causes its receptor to dimerize, activating the Jak2 protein kinase. The activity of Jak2 mediates many of the downstream responses to growth hormone through phosphorylation of Stat transcription factors, MAP kinases, other kinase cascades and molecules involved in metabolism like IRS-1. Factors like Socs and SHP-1 appear to play a role in the down regulation of signaling by growth hormone and cytokines.

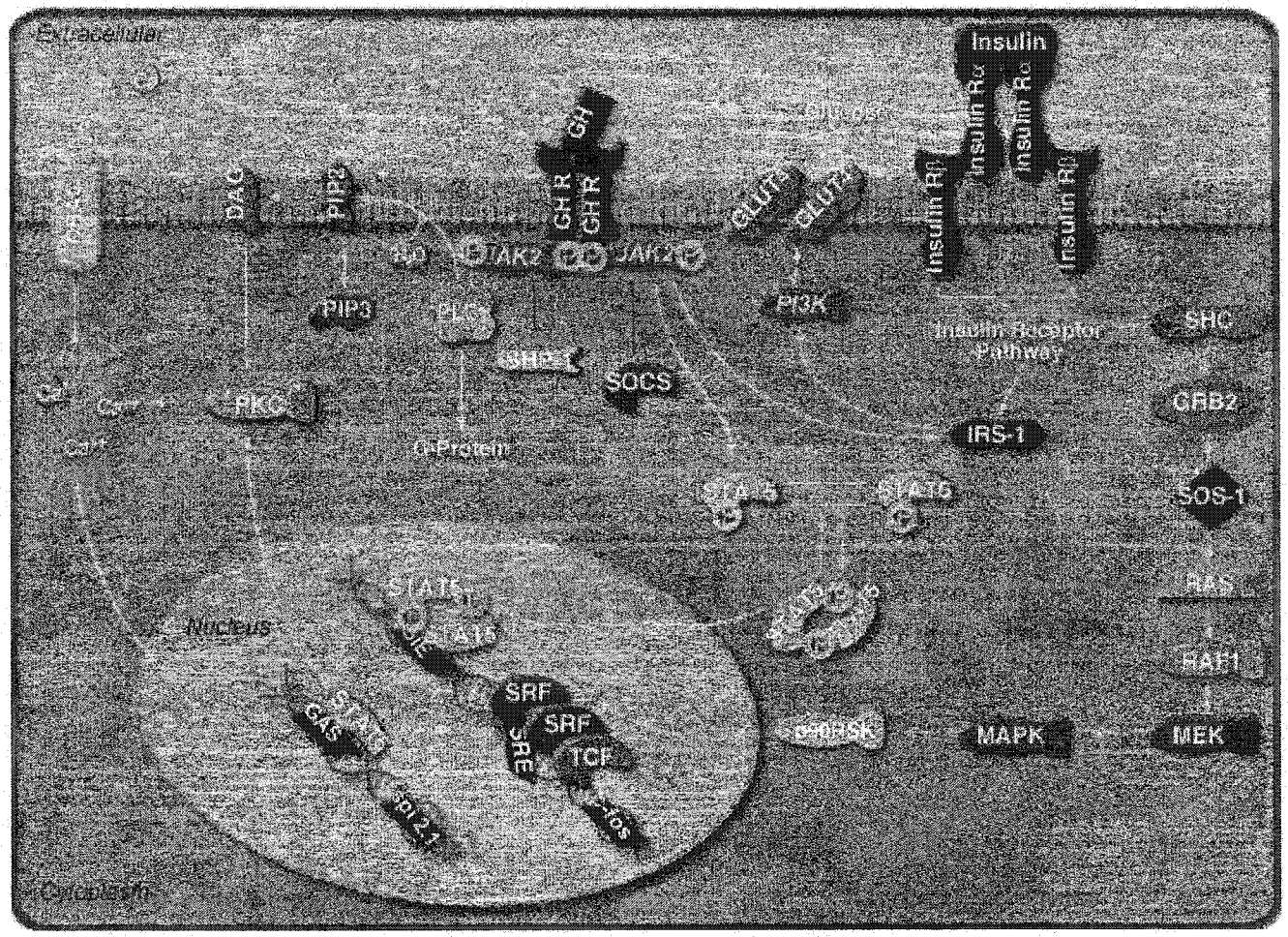
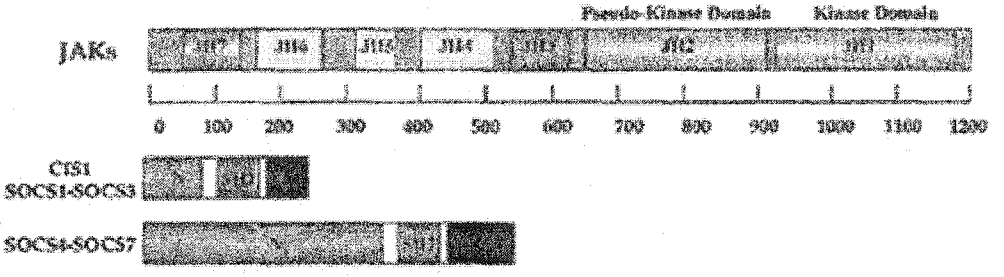
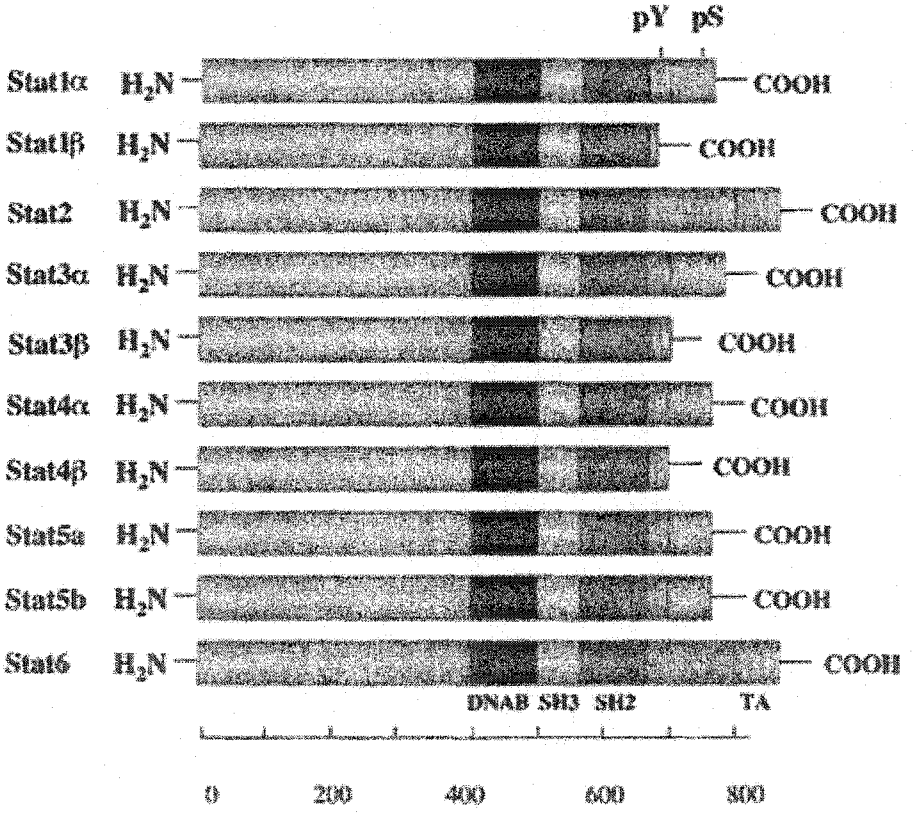

Extracellular
Insulin
Insulin R
Insulin R
Insulin R
Insulin R
GH
GH R
GH R
GLUT4
GLUT4
DAG
PIP2
JAK2
JAK2
PI3K
PIP3
PLC
SHP-2
SOCS
Insulin Receptor Pathway
SHC
GRB2
IRS-1
PKC
Ca2+
Ca2+
Ca2+
G-Protein
STAT 5
STAT5
SOS-1
RAS
RAF1
Nucleus
STAT5
STAT5
SRF
SRF
SRE
TCF
Fos
Jun
MAPK
MEK
Cytoplasm

**Figure 10. A) Structural organization of the Janus Kinases** [72]. The structural domains featured in the JAK kinase family are referred to as the JAK homology regions (JH1-JH7). JAK kinases, apart from featuring the functional kinase domain (JH1) at the carboxy terminus, also possess a pseudo-kinase domain (JH2). Sequences amino terminal to the kinase and kinase-like domains bear no resemblance to any characterized protein motif. Also shown are the structures of CIS/SOCS family of proteins that negatively regulate JAK kinase activity. **B) Stat protein structure** [72]. Cytokines signal via members of the STAT family of proteins. The various STAT proteins, many of which are derived as a result of alternative splicing events (e.g.: Stat 1, Stat 3, Stat 4 and Stat 5), are comprised of approximately 800 amino acid residues. TA, transactivation domain; DNAB, DNA binding region.

# A


Pseudo-Kinase Domain
Kinase Domain
JAKs
JH4
JH3
JH2
JH1
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
CIS1
SOCS1-SOCS3
SOCS4-SOCS7

# B


pY
pS
Stat1α
Stat1β
Stat2
Stat3α
Stat3β
Stat4α
Stat4β
Stat5a
Stat5b
Stat6
H2N
COOH
DNAB
SH3
SH2
TA
0
200
400
600
800

domain may play a role in Jak-Stat interactions [75]. While the N-terminal JH3-JH7 domains are highly divergent among Jak family members, JH6 and JH7 may serve as minimal receptor interaction elements for Jak2 and Jak3 [76] [77].

Jak kinases act as mediators of multiple signalling pathways and are essential for the normal function of mammals. Studies have shown the importance of Jak1 in signaling by members of the IL-2, IL-4, gp130 and class II cytokine receptor families. Jak1 deficient cells are defective in signaling, and knockout mice exhibit perinatal lethality [78], suggesting a critical role for Jak1 in the mediation of early developmental biological responses to several major cytokine receptor families. As Jak3 expression is largely restricted to hematopoietic cells, Jak3 -/- mice demonstrate severe defects in lymphopoiesis, and Jak3 deficient humans manifest severe combined immunodeficiency (SCID) [79]. Tyk2 appears to be most important in mediating the biological response to IL-12 and LPS, as shown by the relatively subtle defects found in IFN-α/β signaling in Tyk2 -/- mice [80]. Jak2 has been implicated in signaling by members of a number of receptors, including growth hormone (GHR), prolactin (PRLR), erythropoietin (EPOR) and thrombopoietin (TPOR). Targeted disruption of Jak2 results in an embryonic lethal phenotype caused by a severe disruption in hematopoiesis, which is similar to what has been observed in the EPO -/- knockout mouse [81] [82].

Although GHR has been shown to associate with [83], and result in, tyrosine phosphorylation of Jak1 [84] [85], Jak3 [86] and Tyk2 [87] in response to GH, Jak2 is the preferred kinase that is activated by the GHR. The GHR and Jak2 interact through the membrane-proximal proline-rich Box 1 region of the GHR cytoplasmic domain and the N-terminal region of Jak2. Studies have shown that JH6-JH7 serve as minimal receptor

interaction elements for Jak2, as deletion of these two JH domains abrogates the association of Jak2 with the GHR [88]. Deletion of the Jak2 JH1 kinase domain eliminated functional coupling but not physical association of Jak2 with the GHR [88] [89], suggesting that JH1 also plays an important role. Upon ligand binding, GHR dimerization at the cell surface results in rapid and transient activation of the tyrosine kinase activity of Jak2 [71] by transphosphorylation of GHR-associated Jak2 monomers brought into closer proximity. Jak2 activation results in subsequent phosphorylation of the GHR intracellular domain at multiple tyrosine residues, which activates the receptor and leads to a cascade of phosphorylation of other intracellular proteins.

#### 1.2.5.2 Signal Transducers and Activators of Transcription (Stat) pathway

To date, seven signal transducers and activators of transcription (Stat) have been identified in mammals. These latent cytoplasmic transcription factors, which range in size from 750 to 850 amino acids, share several common domains (Figure 10B). The N-terminal domain is conserved among all family members and it is responsible for N-terminal dimerization of Stat proteins, promoting cooperative binding to tandem response elements on target genes [90] [91]. Other suggested functions of the N-terminal domain include interaction with transcriptional coactivators [92] , the PIAS (Protein Inhibitors of Activated Stats) family of proteins [93], contact with receptor domains [94], and regulation of nuclear translocation [95]. Adjacent to the N-terminal domain is the coiled-coil domain, which has been implicated in binding to other helical proteins, receptor binding, tyrosine phosphorylation and nuclear export [96]. Further towards the carboxy terminus lies the DNA-binding domain. The linker domain connects the DNA-binding

domain with the src-homology 2 (SH2)/dimerization domain. This SH2 region, the most highly conserved Stat domain, plays a crucial role in signal transduction through its capacity to bind to specific phosphotyrosine motifs. These SH2-phosphotyrosine interactions are a critical step in determining the specificity of receptor-mediated Stat activation. For each receptor, there are particular cytokine receptor tyrosine motifs that are implicated in mediating the recruitment of specific Stats (Table 2). Residues most involved in defining the specificity of the interaction between the SH2 domain and the tyrosine motif are located at positions -1, -3 and +5, +6, +7 to the phosphotyrosine [97]. The recognition of precise phosphotyrosine motifs by the SH2 domain is essential in binding of Stat monomers to the activating Jak [98], as well as in Stat homo- or heterodimerization [99]. The C-terminal domain, which encodes a transcriptional activation domain (TAD), is the least conserved among Stat family members. A detailed understanding of how the Stat carboxy-terminus regulates transcription is not yet defined. Recent studies have suggested modulation through serine phosphorylation [100], which appears to enhance the transcription of some, but not all, target genes.

Four members of the Stat family have been implicated in GHR signalling: Stat1, Stat3, Stat5a and Stat5b [84]. The generation of Stat1 knockout mice, which were defective in IFN-dependent immune responses to both viral and microbial agents, confirmed the involvement of Stat1 in IFN signalling [101] [102]. Disruption of the Stat3 gene results in an early embryonic lethal phenotype [103], highlighting its importance in cell growth. Mice deficient in Stat5A and/or Stat5B have provided the greatest insight into the role of Stat proteins in GH action. Stat5A and Stat5B are encoded by two separate genes, which share 96% identity and differ at their carboxy

**Table 2. Cytokine receptor tyrosine motifs implicated in mediating the recruitment of specific Stats** [104].

| STAT | Receptor | STAT-binding tyrosine motif[a] | Reference[b] |
|---|---|---|---|
| Stat1 | IFN-γ | YDKPH | 1 |
| Stat2 | IFN-α | YVFFP | 2 |
| Stat3 | IL-6, LIF, IL-10 | YXXQ | 3,4,5,6 |
| Stat1, Stat3 | IL-6 | YXPQ | 3,5 |
| Stat4 | IL-12 | YLPSNID | 7 |
| Stat5 | IL-2 | YLSLQ | 8,9,10 |
| | | YCTFP | 8,9,10 |
| | | YFFFH | 8,9,10 |
| | IL-7 | YVTMS | 8 |
| | IL-9 | YLPQE | 11,12 |
| | EPO | YLVLD | 9,13,14,15 |
| | | YTILD | 13 |
| | PRL | YLDPT | 16 |
| | | YVEIH | 9 |
| | GH | YVSTD | 17,18 |
| | | YFCEA | 17,18 |
| | | YITTE | 17,10,18 |
| | | YTSIH | 10 |
| | GM-CSF | YLSLP | 9 |
| | | YLCLP | 19 |
| | | YVSSA | 19 |
| | | YVELP | 19 |
| | | YCFLP | 19 |
| Stat 6 | IL-4 | YKAFS | 20,21 |
| | | YKPFQ | 20,21 |

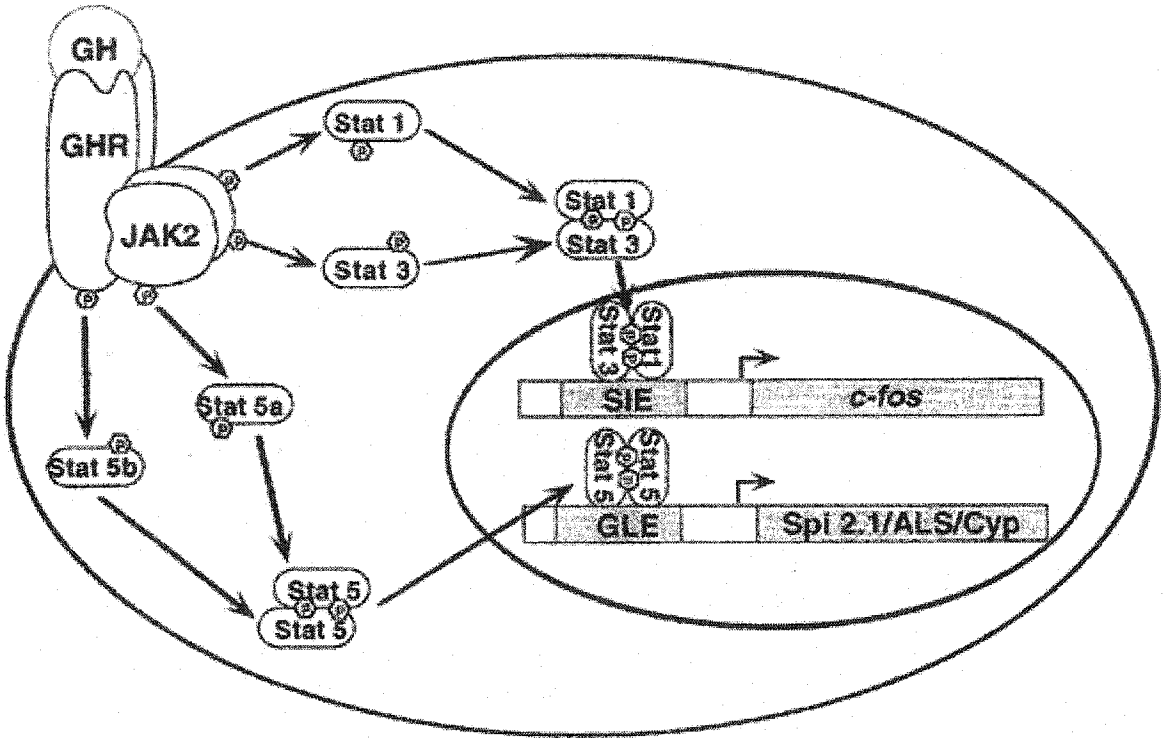
[a] The tyrosine is not conserved in the murine system.

[b] 1, Greenlund et al. (1994); 2, Yan et al. (1996); 3, Stahl et al. (1995); 4, Hemmann et al. (1996); 5, Gerhartz et al. (1996); 6, Weber-Nordt et al. (1996); 7, Naeger et al. (1999) #2699; 8, Lin et al. (1995); 9, May et al. (1996); 10, Gaffen et al. (1996); 11, Demoulin et al. (1996); 12, Bauer et al. (1998); 13, Gobert et al. (1996); 14, Quelle et al. (1996); 15, Damen et al. (1995); 16, Lebrun et al. (1995); 17, Hansen et al. (1996); 18, Hansen et al. (1997); 19, Itoh et al. (1998); 20, Hou et al. (1994); 21, Ryan et al. (1998).

termini [105]. Despite their structural and functional similarities, single Stat5 knockout mice have distinct phenotypes. Stat5a knockout mice are predominantly defective in PRL-dependent mammary gland development [106], whereas mice deficient in Stat5B have pronounced impairment of body growth, especially in males [107]. Stat5a/Stat5b double knockout mice, on the other hand, support the view for redundancy in function of these proteins in GH action. The most severe defects in liver gene expression and body growth, affecting both sexes, are found when mice lack both Stat5A and Stat5B [108]. Although Stat1, Stat3 and both isoforms of Stat5 have been shown to become tyrosine phosphorylated in response to GH, it has been demonstrated that, at least in the rodent male, the Stat5b isoform plays a critical role in mediating the activation of hepatic GH-specific genes and allowing normal linear growth as well as adipose tissue formation [109] [110].

Upon GH binding, specific tyrosines on associated Jak2 molecules as well as the cytoplasmic tail of the GHR are phosphorylated. Stats are then recruited to the GH-GHR-Jak2 complex and associate with the phosphotyrosine residues via their SH2 domains. GH activation of Stats 1 and 3 involves binding interactions with Jak2 but does not require interactions with any GHR cytoplasmic domain tyrosine residues [111]. Full activation of Stat5A and Stat5B by GH requires both activation by Jak2 and direct interaction with the GHR [112]. Once phosphorylated by Jak2, Stat proteins form homo- or heterodimers and translocate to the nucleus. Here, they interact with particular consensus sequences in the promoter regions of responsive genes, thereby regulating their transcription (Figure 11).

**Figure 11. GHR signalling via Stat proteins** [113]. GH has been demonstrated to stimulate the formation of Stat1/Stat3 heterodimers, which bind to the Sis inducible element (SIE) of the *c-fos* gene. Stat5A and Stat5B have been implicated in GH-dependent activation of *spi.2.1, ALS and p450 CYP3A 6 beta-hydroxylase* genes, by binding to IFN-γ activated sequence (GAS)-like elements (GLE) on gene promoters.

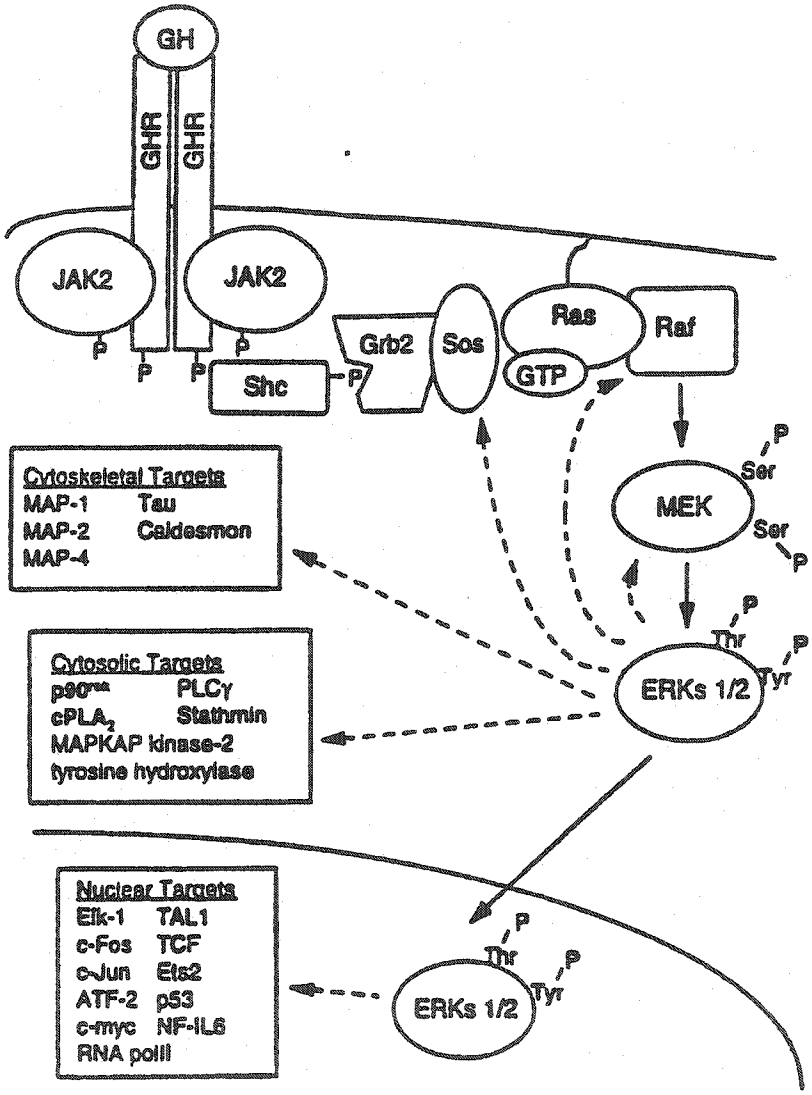

GH
GHR
JAK2
Stat 1
Stat 3
Stat 1
Stat 3
Stat 5a
Stat 5b
Stat 5
Stat 5
Stat 3
Stat 1
SIE
c-fos
Stat 5
Stat 5
GLE
Spi 2.1/ALS/Cyp

There exist specific Stat DNA binding elements found within promoter regions of several GH-inducible target genes. GH has been demonstrated to stimulate the formation of Stat1 homodimers, Stat3 homodimers and Stat1/Stat3 heterodimers all binding to the Sis inducible element (SIE) of the *c-fos* gene [114] [115]. GH induces the binding of Stat5 proteins to IFN-γ activated sequence (GAS)-like elements (GLE) in several different genes, including *spi.2.1, Insulin 1 and p450 CYP3A 6 beta-hydroxylase* genes. In addition, Stat5A and Stat5B were recently shown to bind to a GH-responsive DNA sequence (ALS-GAS1) in the Acid Labile Subunit (ALS) gene promoter and mediate transcriptional activation [116]; ALS is a serum protein that recruits IGF-I into a complex with IGF binding protein-3 [117]. Stat5 has also been implicated in GH-dependent activation of the β-casein gene via a GLE [118] [119], as well as stimulation of the salmon IGF-I promoter in Hep3B cells expressing the GHR [120].

### 1.2.5.3 Mitogen Activated Protein Kinase (MAPK) pathway

MAP kinases belong to a family of serine/threonine tyrosine kinases that play an important role as mediators of cellular responses to a variety of extracellular stimuli and growth factors, including GH [121] [122]. GH-induced MAPK activation involves activation of the GHR-bound tyrosine kinase Jak2 [123] (Figure 12). Although pathways leading to MAPK activation in response to GH may vary, GH has been reported to activate p44/p42 MAPK (also named extracellular signal-regulated kinases or ERKs) [124] [125], *c-jun* N-terminal kinases (also names SAPKs) [29] and p38 MAPK [123]. Activation of ERKs by GH seems to be cell type specific: western blotting and *in vitro* kinase assays have demonstrated that although GH (200 ng/ml) activates MAPK in 3T3-

**Figure 12. GHR signalling via the MAP kinases ERKs 1 and 2** [126]. A signalling cascade leading from GHR to the MAPK and activation of subsequent targets is shown. Solid arrows and signalling molecules and bolded targets indicate pathways and proteins regulated by GH. The dotted arrows and targets in plain text indicate pathways, feedback mechanisms and target utilized in cells treated with other cytokine and growth factors that activate MAPK or detected *in vitro*. These have not yet been shown to be involved in GH signal transduction.


GH
GHR
GHR
JAK2
JAK2
P
P
P
P
P
Shc
P
Grb2
Sos
Ras
GTP
Raf
Cytoskeletal Targets
MAP-1 Tau
MAP-2 Caldesmon
MAP-4
P
Ser
MEK
Ser
P
P
Thr
P
Tyr
ERKs 1/2
Cytosolic Targets
p90rsk PLCγ
cPLA2 Stathmin
MAPKAP kinase-2
tyrosine hydroxylase
Nuclear Targets
Elk-1 TAL1
c-Fos TCF
c-Jun Ets2
ATF-2 p53
c-myc NF-IL6
RNA polII
P
Thr
P
Tyr
ERKs 1/2

F442A cells, it does not activate MAPK in IM-9 lymphocytes [127]. In general, the MAPK pathway involves the adapter molecule SHC (src homology 2/α collagen-related), the docking protein Grb2 (growth factor receptor-bound 2), a guanine nucleotide exchange factor Sos (son of Sevenless), the small guanosine triphosphate-binding protein Ras, its downstream effector Raf and a serine/threonine kinase that activates MAP-ERK (MEK) [128] [129]. MEK1 then activates, by phosphorylation, MAP serine-threonine kinases ERK1 and ERK2 (extracellular signal-regulated kinases 1 and 2). ERK1 and ERK2 activate several enzymes, such as the S6 serine/threonine kinases p70rsk and p90rsk, as well as transcription factors. In 3T3-F442A cells, MAPK activation appears to be critical in allowing GH-induced *c-fos* and *egr-1* gene transcription as well as activation of the Elk-1 transcription factor [130]. MAPK has also been demonstrated to regulate Stat-mediated transcription by association with and phosphorylation of Stat molecules on serine residues [131]. Other signal transduction pathways regulate the ability of GH to activate MAPK: protein kinase C [132] and PI3K [133] activities are both required for full GH activation of the MAPK in 3T3-F442A preadipocytes.

#### 1.2.5.4 Phosphatidylinositol-3-Kinase (PI3K) pathway

GH and insulin share many cellular effects in common including the stimulation of amino acid transport, protein synthesis, glucose transport, lipogenesis, gene expression, mitogenesis and reorganization of cytoskeletal structure [134]. Thus, GH and insulin can be expected to utilize common components of intracellular signal transduction pathways. Insulin receptor substrates-1 and -2 (IRS-1 and –2) are nontransmembrane proteins that are tyrosine-phosphorylated at multiple sites by insulin, interleukin-4,

several other cytokines and IGF-I. Phosphorylated tyrosine residues on IRS-1 and IRS-2 act as docking sites for associations with SH2-containing signalling molecules such as Grb-2, SHP-2 and the p85 regulatory subunit of PI3K. In fact, in 3T3-F442A fibroblasts GH promotes the tyrosine phosphorylation of both IRS-1 and IRS-2 [20] [21] and their subsequent association with p85, activating PI3K. The IRS-mediated, insulin-induced activation of PI3K is of potential importance for insulin-induced glucose transport. This suggests a possible mechanism to account for some of the insulin-like and anti-insulin-like metabolic effects of GH. Interestingly, primary rat adipocyte GHR does not contain the NPXY consensus sequence required for association of IRS proteins with receptors for insulin, IGF-I and IL-4, and no specific tyrosine residues are required for phosphorylation of IRS-1 or IRS-2 [135]. Since Jak2 is also devoid of the NPXY consensus sequence, it is more likely that the IRS proteins associate with Jak2 via an adaptor molecule such as Grb2 [136]. PI3K activity also seems to be required for GH-stimulated actin cytoskeletal reorganization [27], GH-mediated GLUT4 translocation in Chinese hamster ovary (CHO) cells [137] and primary rat adipocyte GH-stimulated lipogenesis [138].

#### 1.2.5.5 Phospholipase C (PLC)/ Protein Kinase C (PKC)/ $Ca^{2+}$ pathway

A ubiquitous signalling mechanism for members of the cytokine receptor superfamily is the PLC-catalyzed hydrolysis of phosphatidylinositol 4,5 bisphosphate to produce the second messenger molecules 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). While IP3 stimulates an increase in intracellular free calcium ($Ca^{2+}$), DAG serves as a major activator of PKC. Although the role of PKC in GH signalling has not yet been clearly defined, GH has been shown to increase levels of DAG in rat

hepatocytes, thus activating PKC [139]. In CHO cells stably expressing rabbit GH receptor, GH has also been reported to increase intracellular calcium levels [140]; this effect requires the C-terminal domain of the GHR, but not the Box 1 region [141]. This GH-activated increase in cellular calcium seems to be PKC-dependent, as inhibitors of PKC block calcium uptake seen in fat cells [142]. Therefore, it is possible that GH promotes direct PKC-catalyzed phosphorylation of the calcium channels. PKC has also been implicated in several other GH responses, including GH-induced MAP kinase activation [132], stimulation of lipogenesis [143], and downregulation of cell surface GHR levels [144].

## 1.2.6 Regulation of GHR-Jak2 Signalling

### 1.2.6.1 SH2B-β

Yeast two hybrid screens initially identified the SH2B-β protein as capable of interacting with tyrosine phosphorylated Jak2 via its SH2 domain [145]. While the structure of SH2B-β suggests a role as an adaptor protein, studies have demonstrated it to be a potent enhancer of GH signal transduction pathways by increasing the GH-stimulated kinase activity of Jak2 as well as the activation of a number of downstream signalling proteins, including tyrosine phosphorylation of Stat3 and Stat5B [146]. In addition, mutation of a critical lysine in the FLVR domain of SH2B-β inhibits the migration of Stat5B to the nucleus in overexpressed 3T3-F442A cells [30]. Thus, SH2B-β may play an important role in the activation of GHR intracellular Jak/Stat signalling pathways.

### 1.2.6.2 Suppressors of Cytokine Signalling (Socs)

Suppressors of cytokine signalling (Socs), which belong to a family of cytokine-inducible genes, are also important in the regulation of GHR-Jak2 signalling [147]. Structurally, the N-terminal regions of Socs proteins are variable in length, while the C-terminal domain contains a conserved motif referred to as the Socs box [148] (Figure 13). Socs expression is tightly regulated at the transcriptional level. It is now apparent that the Stat family of transcription factors contributes significantly to the transcriptional upregulation of the *cis*, *socs1* and *socs3* genes. The promoter of the *cis* gene contains four Stat5-binding sites [149], the *socs1* promoter contains putative binding sites for Stat1, Stat3 and Stat6 [147], and the *socs3* gene promoter contains a Stat1/Stat3 binding element, which is also capable of binding Stat5b [150]. It is likely that *socs2* expression is also regulated by the Stats; however, this remains to be confirmed.

Of the eight known family members, Socs1, Socs2, Socs3 and Cis expression are induced by GH in the rat liver with different kinetics [151] [152] [153]. Biochemical studies demonstrate that the SH2 domains of Socs1 compared to Socs3 and Cis recognize different targets (Figure 14): Socs1 inhibits Jak2 directly by binding to the kinase activation loop of Jak2 and inhibiting its kinase activity [154] [155]. Socs3 interacts with tyrosine residues on the cytoplasmic domain of the receptor, indirectly inhibiting Jak2 kinase activity. Cis also binds to GHR phosphorylated tyrosines, preventing STAT binding [156]. A motif within the N-terminal domain of Socs1, Socs3 and Cis resembles the activation loop of Jaks, potentially acting as a Jak pseudosubstrate, and inhibiting kinase activity.

**Figure 13. Structural organization of Socs/Cis proteins** [147]. The domain structure and alternative names for each Socs family member are shown. In addition, the cytokines that each Socs protein negatively regulates in vitro are listed.

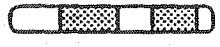
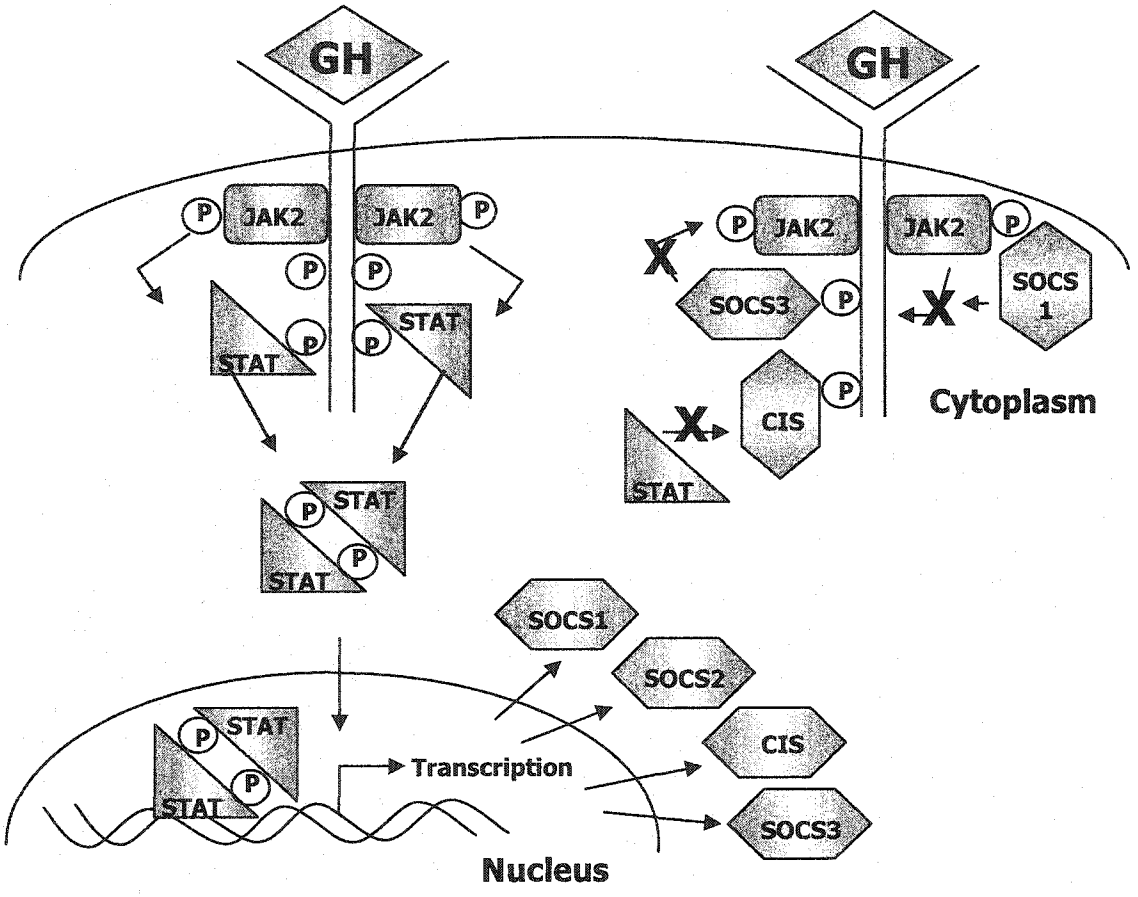
| NAME | STRUCTURE | INHIBITS SIGNALING BY |
|---|---|---|
| CIS / CIS1 |   | IL-2 (1), IL-3 (2), GH (1), prolactin (1), Epo (2) |
| SOCS1 / JAB / SSI1 |  | IL-2 (3), IL-3 (3), IL-4 (4), IL-6 (5), GH (6), prolactin (7), Epo (3), LIF (5), IFNγ (5), IFNα (8), OSM (5), TSLP (9), Tpo (5) |
| SOCS2 / CIS2 / SSI2 |  | GH (10), IL-6 (12), LIF (11) |
| SOCS3 / CIS3 / SSI3 |  | IL-2 (13), IL-3 (13), IL-4 (4), IL-6 (12), IL-11 (14), GH (6), prolactin (7), Epo (15), LIF (12), IFNγ (8), IFNα (8), CNTF (16), leptin (17), OSM (8) |
| SOCS4 |  | not tested |
| SOCS5 / CIS6 |  | IL-6 (12) |
| SOCS6 / CIS4 |  | does not inhibit in systems tested |
| SOCS7 / CIS5 |  | not tested |

**Figure 14. The molecular mechanism by which Socs proteins negatively regulate cytokine signalling** (modified from [147]).

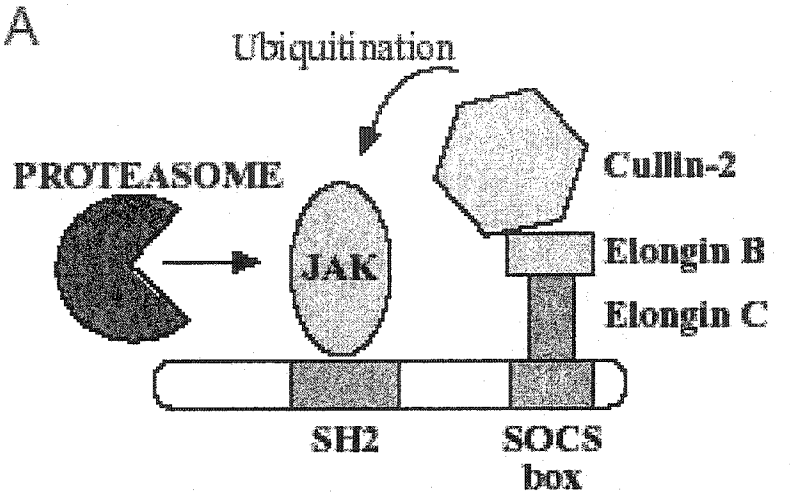

GH
GH
JAK2
JAK2
P
STAT
SOCS3
SOCS 1
CIS
Cytoplasm
SOCS1
SOCS2
CIS
SOCS3
Transcription
Nucleus

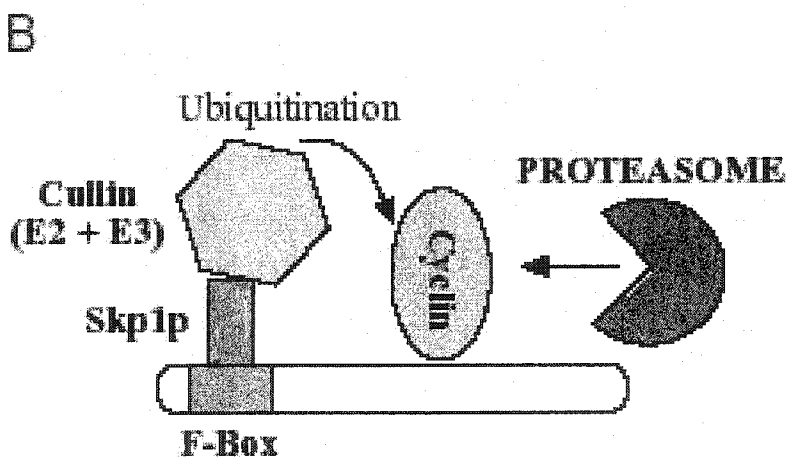
The physiological importance of Socs1, Socs2 and Socs3 has been investigated in knockout mice. *Socs1* -/- mice, though normal at birth, exhibit stunted growth and die at 3 weeks of age [157] [158]. The neonatal defects exhibited by *socs1* -/- mice appear to occur primarily as a result of uncontrolled IFN-γ signalling, suggesting that Socs1 plays a key role in the negative regulation of signalling by IFN-γ. Mice deficient in Socs2 display gigantism [159], suggesting that Socs2 plays an important role in the termination of GHR signalling. Interestingly, Socs3 transgenic mice have no fetal erythropoiesis, whereas *socs3* -/- embryos exhibit marked erythrocytosis [160]. In both cases, mice die during the embryonic stage of development, thus, effects on growth are unknown. To date, no phenotype has been reported in *Cis* -/- mice, which are viable and fertile [92]. Studies have also shown that SOCS proteins target activated signalling proteins for degradation by the proteosome [161]. In this model, Socs proteins associate with a complex containing elongins B and C through their Socs box [162] (Figure 15). The elongin BC complex then binds to a putative E3 ubiquitin ligase, which is necessary for ubiquitination of the proximal signalling proteins and degradation by the proteasome.

### 1.2.6.3 Protein Tyrosine Phosphatases

Termination of GH signal transduction pathways presumably involves recruitment and/or activation of protein tyrosine phosphatases to GHR/Jak2 complexes. These phosphatases likely dephosphorylate GHR, Jak2 and/or Stats to terminate recruitment of signalling molecules to the complex. Indeed, there are two candidate phosphatases that are thought to regulate GH-activated signalling: the SH2 domain-containing

**Figure 15. Socs family members might target signalling proteins for degradation by the proteasome: similarity between Socs proteins and F-box proteins** [147]. A) All Socs proteins tested bind the elongin BC complex through their Socs box. In turn, the elongin BC complex associates with the putative ubiquitin ligase cullin-2. Signalling proteins associated with the N-terminal or SH2 domains of Socs proteins could be ubiquitinated by cullin-2, targeting them for degradation by the proteasome. B) Socs proteins may be analogous to F-box proteins that operate in the yeast phosphoprotein ubiquitin ligase complex. F-box proteins contain an N-terminal F-box motif that associates with the elongin C homolog Skp1p and the cullin-2 ubiquitin ligase homologs E2 and E3. Yeast F-box family members bind cyclins in response to serine phosphorylation, thus targeting them for ubiquitination and degradation by the proteasome. This permits cell cycle progression from G1 to S.


A
Ubiquitination
PROTEASOME
Cullin-2
JAK
Elongin B
Elongin C
SH2
SOCS
box


B
Ubiquitination
Cullin
(E2 + E3)
PROTEASOME
Cyclin
Skp1p
F-Box

phosphatases, SHP-1 and SHP-2 [163]. SHP-1 has been demonstrated in mouse hematopoietic cells and Cos-7 cells to negatively regulate Jak/Stat signalling by direct association with Jak2 [164]. In response to GH in the liver, SHP-1 dephosphorylates the activating tyrosine residue within the kinase domain of Jak2, deactivating it [165]. In contrast to the negative regulatory role of SHP-1 in GH signalling, comparison of wild-type SHP-2 and a catalytically inactive SHP-2 mutant demonstrated a positive effect of SHP-2 on GH-induced gene activation [166]. SHP-2 has also been proposed to have a positive effect on other signaling pathways, including receptors for the platelet-derived growth factor receptor [167], insulin [168] and prolactin [169]. While studies in 3T3-F442A fibroblasts have shown that SHP-2 associates with GHR through its SH2 domain in response to GH [170], mutation of GHR tyrosine residues that serve as binding sites for SHP-2 seems to prolongs tyrosine phosphorylation of GHR, Jak2 and STAT5B [171]. This suggests that SHP-2 binding to specific tyrosines in GHR may also regulate GH signaling in a negative manner. Dephosphorylation of GHR may also signal its internalization and degradation [172, 173].

Other proteins may be involved in the dephosphorylation of GHR, Jak2 and STATs. Signal regulatory proteins (SIRPs) are receptor-like transmembrane glycoproteins, the majority of which contain a cytoplasmic domain with four tyrosine residues and a proline-rich region. In 3T3-F442A fibroblasts, SIRP-α becomes tyrosine phosphorylated by Jak2, to which it is constitutively bound [171]. Its subsequent association with SHP-2 results in dephosphorylation of Jak2 [170]. Overexpression studies demonstrated that SIRP-α also reduces GH-induced phosphorylation of ERK1,

ERK2, STAT3 and STAT5B [171], suggesting that SIRP-α is a negative regulator of GH signalling.

#### 1.2.6.4 Protein Inhibitors of activated STATs (PIAS)

PIAS are constitutively expressed inhibitors of STAT proteins with differential preference for the individual STATs [174, 175]. Recent studies have shown that PIAS proteins bind to phosphorylated STAT dimers, preventing DNA recognition [176]. Although PIAS3 has been reported to be involved in the negative regulation of prolactin signal transduction [175], it remains to be determined if GH utilizes PIAS for the regulation of STAT-mediated transcription.

#### 1.2.6.5 Grb-10

Overexpression studies have also implicated the adaptor protein Grb-10 as another regulator of GH signalling. Member of a family of SH2 domain-containing proteins, Grb10 has been demonstrated to associate with the cytoplasmic tail region (454-620 aa) of tyrosine phosphorylated GHR, and to inhibit transcriptional activity of c-fos and spi2.1 reporter genes [177]. Interestingly, Grb-10 had no effect on a reporter gene containing only STAT5 elements [178], suggesting that Grb-10 down-regulates some GH signalling pathways downstream of Jak2 and independently of STAT5.

## 1.3 GHR Isoforms

### 1.3.1 Growth Hormone Binding Protein (GHBP)

Nearly 50% of the circulating GH in humans is complexed to a short isoform of the GHR, which corresponds to the extracellular domain of the full-length (FL) receptor. The mechanism underlying the generation of this high-affinity growth hormone binding protein (GHBP) differs among species. In rats and mice, an alternative GHR mRNA is generated due to splicing in of an alternative exon (8A) that introduces a premature stop codon; this mRNA encodes a secreted GHBP [179]. In contrast, the generation of GHBP in humans and rabbits involves proteolytic cleavage of the GHR at the cell surface [180] (Figure 16). The GHBP binds GH with the same affinity as the intact receptor [181] and may protect GH from degradation and renal clearance, allowing a longer half-life than that of free GH. GHBP can also block the actions of GH by competing with the membrane-bound receptor for GH, preventing access of GH to the membrane-bound receptors and subsequent signal transduction [182]. Such inhibitory effects of GHBP have been demonstrated *in vitro*. The physiological importance of GHBP *in vivo* is not completely clear, although the amount of GHBP is thought to be a measure of the relative tissue levels of GHR [183].

### 1.3.2 Truncated (1-277) and (1-279) GHR isoforms

Two other truncated forms, namely GHR (1-277) and GHR (1-279), have been identified at approximately 1% and 10%, respectively, of total GHR levels in GH target tissues [184] [185]. Due to the use of alternative acceptor splice sites during post-

**Figure 16. Mechanisms of GH binding protein generation** [186]. A) Rabbit and human GHBPs arise by proteolysis of the transmembrane GHR with shedding of the extracellular domain. B) In rodents, GHBP is principally the product of an alternatively spliced GHR mRNA in which a hydrophilic peptide replaces the transmembrane and cytoplasmic domains.

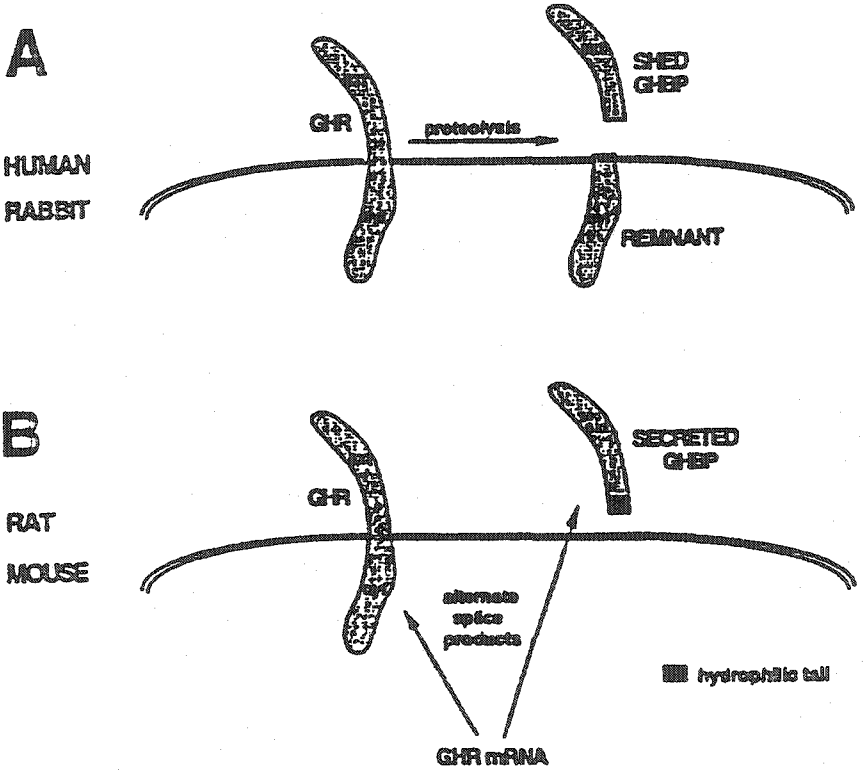
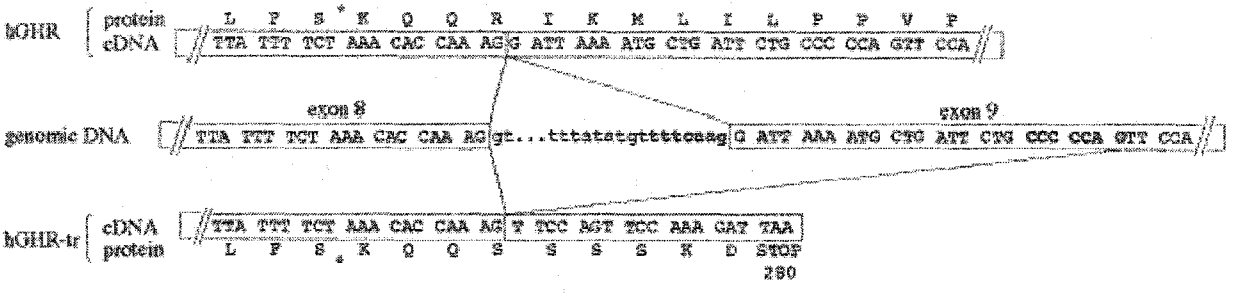
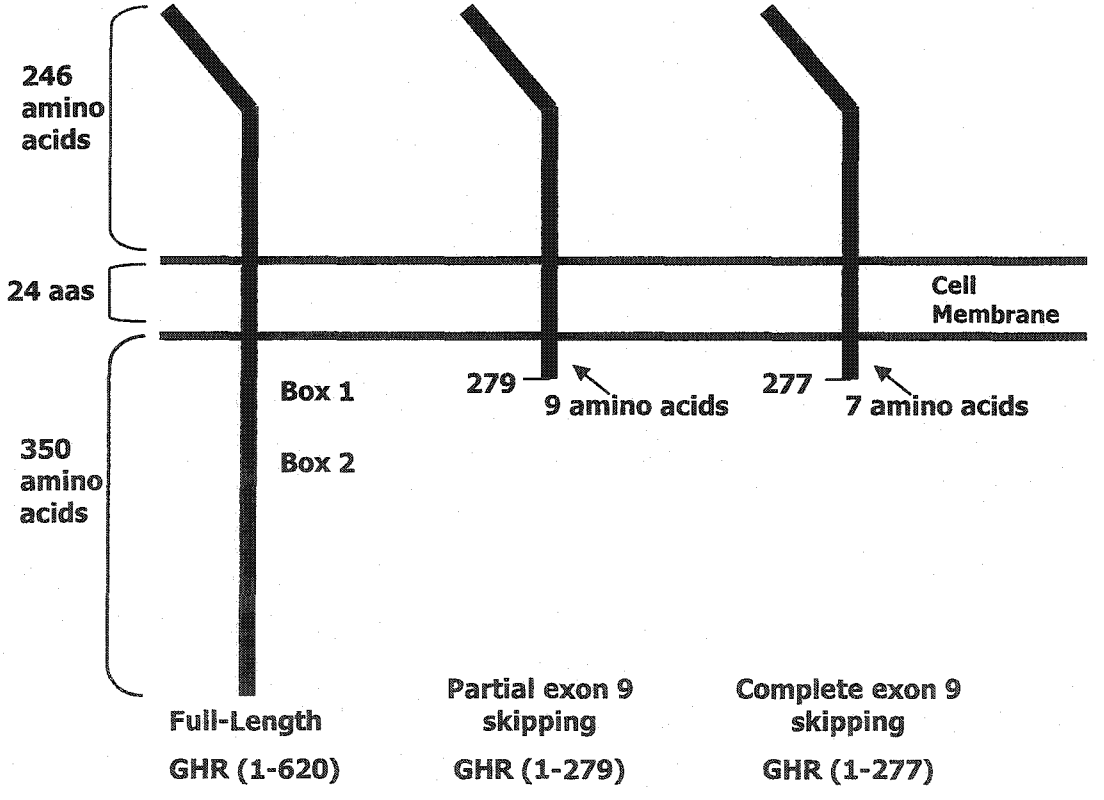

A
HUMAN
RABBIT
GHR
proteolysis
SHED
GHBP
REMNANT
B
RAT
MOUSE
GHR
SECRETED
GHBP
alternate
splice
products
hydrophilic tail
GHR mRNA

**Figure 17. Full-length and truncated isoforms of hGHR.** A) Alternative splicing mechanisms generating FL and T (1-277) and T (1-279) hGHR transcripts [187]. Schematic representation of the genomic DNA fragment involved in the generation of hGHR and hGHR-tr transcripts. Intron and exon sequences are denoted by lowercase type and boxed uppercase type, respectively. Resulting amino acid sequences are shown above and below. The transmembrane-cytoplasmic junction is marked by an asterisk in the amino acid sequence. B) FL and T GHR proteins resulting from alternative splicing.

# A


hGHR
protein
cDNA
L F S K Q Q R I K M L I L P P V P
TTA TTT TCT AAA CAC CAA AGG ATT AAA ATG CTG ATT CTG CCC CCA GTT CCA
exon 8
exon 9
genomic DNA
TTA TTT TCT AAA CAC CAA AG gt...tttatatgttttcaag G ATT AAA ATG CTG ATT CTG CCC CCA GTT CCA
hGHR-tr
cDNA
protein
TTA TTT TCT AAA CAC CAA AG T TCC AGT TCC AAA GAT TAA
L F S K Q Q S S S S K D STOP
280

# B


246 amino acids
24 aas
350 amino acids
Cell Membrane
Box 1
Box 2
279
9 amino acids
277
7 amino acids
Full-Length
GHR (1-620)
Partial exon 9 skipping
GHR (1-279)
Complete exon 9 skipping
GHR (1-277)

transcriptional processing, GHR (1-279) lacks the first 26 bp of exon 9 of the full-length GHR mRNA whereas exon 9 is completely deleted in GHR (1-277) (Figure 17A). Both alternative splicing events result in a frame shift and a premature stop codon. The resultant mRNAs encode GHR isoforms with intact extracellular and transmembrane domains but lacking >90% of the intracellular domain (Figure 17B). Although both variants are capable of binding GH with high affinity, they are devoid of intracellular signalling capacity. It has also been shown that GHR (1-279) can inhibit GH action mediated by the FL GHR in a dominant-negative manner [188] (Figure 18). Ross *et al.* previously demonstrated a dose-related inhibition of Stat5-dependent transcriptional activity mediated by GHR in the presence of increasing amounts of the T (1-279) isoform [188]: while a T/FL ratio of 0.1 resulted in a 10-30% inhibition of Stat5-dependent transcriptional activity, activity was diminished by 40-80% with a 1:1 T/FL ratio and abolished with a 10:1 ratio. In addition, when cells are transfected with the truncated GHR isoform cDNAs, large amounts of GHBP are produced. The increased capacity to generate a greater amount of soluble GHBP compared to full-length GHR is suggested to be a consequence of their markedly reduced internalization and, hence, longer residence on the plasma membrane [188]. The physiological role of these truncated GHR isoforms during development has not been investigated to date.

### 1.3.3 Exon 3-retained and –deleted GHR

The exon 3-deleted form of the GHR, where exon 3 encodes for 22 amino acids in the N-terminal extracellular domain of the receptor, is present in about 30% of postnatal individuals due to recombination [189] (Figure 19). Although the absence of exon 3 from

**Figure 18. Dominant negative action of truncated hGHR isoform.** Truncated hGHR (1-279) is capable of dimerizing to full-length (FL) hGHR but inhibits hGH action mediated by the FL hGHR in a dominant-negative manner.

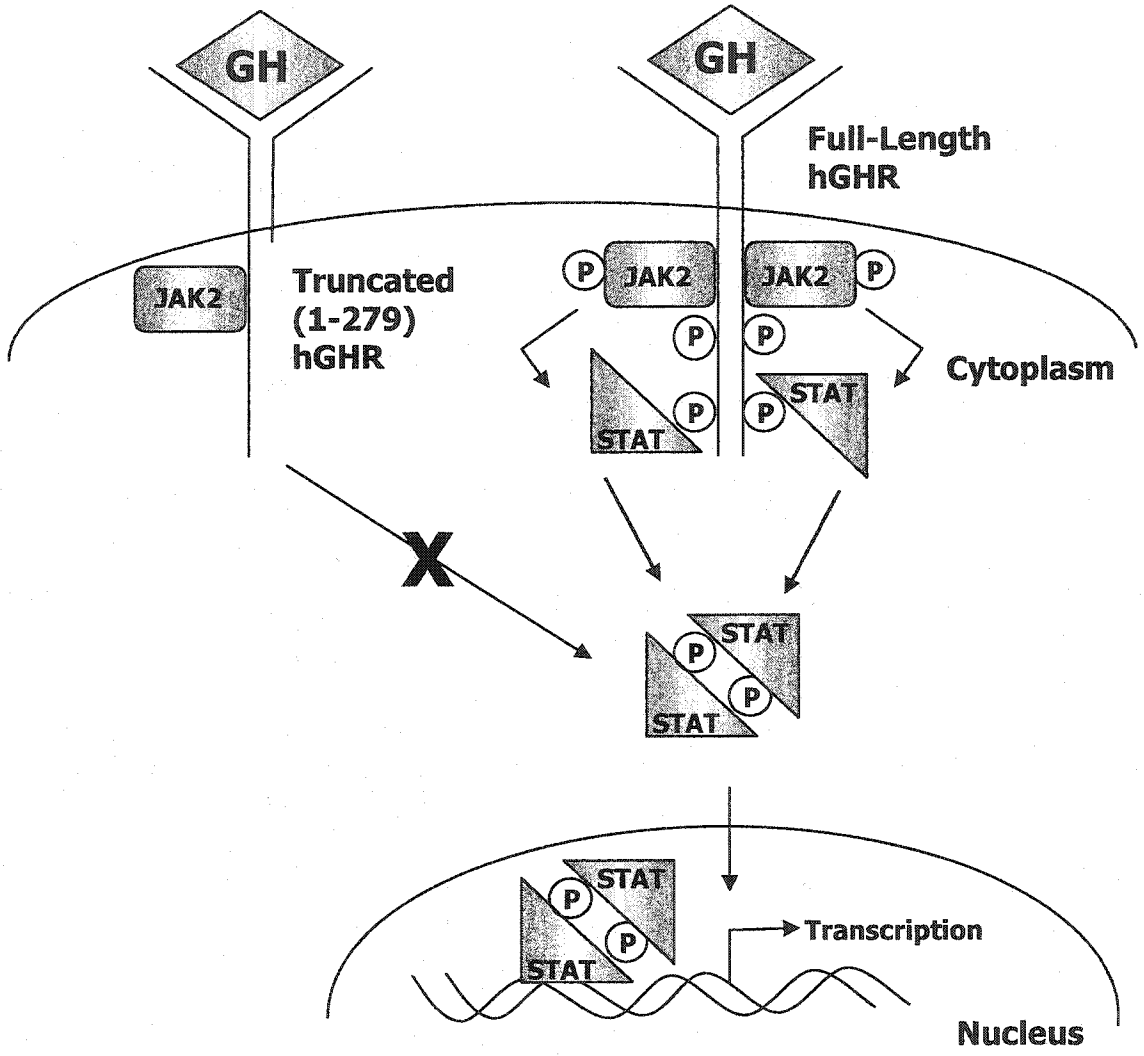
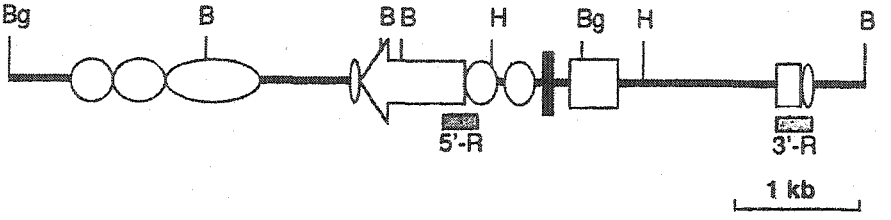
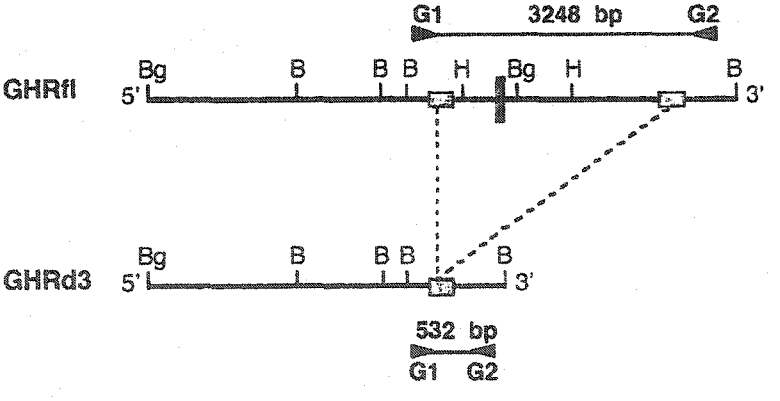

GH
GH
Full-Length
hGHR
JAK2
Truncated
(1-279)
hGHR
P
JAK2
JAK2
P
P
P
STAT
P
P
STAT
Cytoplasm
X
STAT
P
P
STAT
STAT
P
P
STAT
Transcription
Nucleus

**Figure 19. Genomic organization of the human GHR locus in the vicinity of exon 3** [189]. A) GHR exon 3 locus in an individual expressing full-length GHR transcripts. Exon 3 is shown as a *black box*. The long terminal repeat (LTR) sequences originating from the human endogenous retrovirus HERV-P are indicated by an *arrow* (complete LTR sequence) or *rectangle* (partial LTR sequence). Medium reiteration frequency (MER) elements and the mammalian LTR transposon element are indicated by *ellipses* and a *square*, respectively. A partial restriction enzyme map of the region is given. Bg, BglII; B, BanII; H, HindIII. B) Schematic representation of human GHRfl and GHRd3alleles. A partial restriction enzyme map of the region is given. The repeated elements are shown as gray boxes. Two repeated elements flank exon 3 on GHRfl alleles, whereas a single copy of the repeat is present on GHRd3 alleles. The meeting point of the two dashed lines shows the intragenic recombination breakpoint.

A


Bg
B
B B
H
Bg
H
B
5'-R
3'-R
1 kb

B


G1
3248 bp
G2
GHRfl
5'
Bg
B
B B
H
Bg
H
B
3'
GHRd3
5'
Bg
B
B B
B
3'
532 bp
G1
G2

GHR mRNA causes an in-frame deletion of 22 amino acids, the resulting receptor does not appear to be functionally different from the full-length form, since it is able to bind the appropriate ligands with similar affinities [190]. Population studies have shown that exon 3-deficient mRNA is expressed in an individual-, rather than tissue-, specific manner [191] [192].

## 1.4 GH and GHR during development

Although pivotal roles for GH and its receptor have been well established in postnatal tissues, their function during human gestation remains unclear. Plasma levels of immunoreactive GH are significantly elevated in the fetus (up to 150 ng/mL at midgestation [7]) relative to the normal adult (0-10 ng/mL). Interestingly, high levels of GH in the fetal circulation are correlated with low levels of IGF-I and IGF-II. This physiological pattern seems to mimic that observed in Laron syndrome patients, who manifest a resistance to GH due to a mutation at the level of the GHR or in downstream signalling pathways [34] [36] [37]. Postnatally, lower levels of GH are detected, as well as increased levels of IGF-I (GH-dependent) and IGF-II (nutrition-dependent).

GHR mRNA and protein have been identified in a wide variety of human tissues from as early as the first trimester of fetal life [50] [191] [193]. By mid-gestation, the tissue pattern of expression of the GHR resembles that of postnatal tissues. We recently demonstrated that the human GHR (hGHR) present in fetal hepatocytes specifically bind GH and have biological responses after chronic exposure to GH. However, although the cells possess functional GHR, these receptors (and/or their signalling pathways) are immature or have adapted to the *in utero* environment: 1) affinity cross-linking

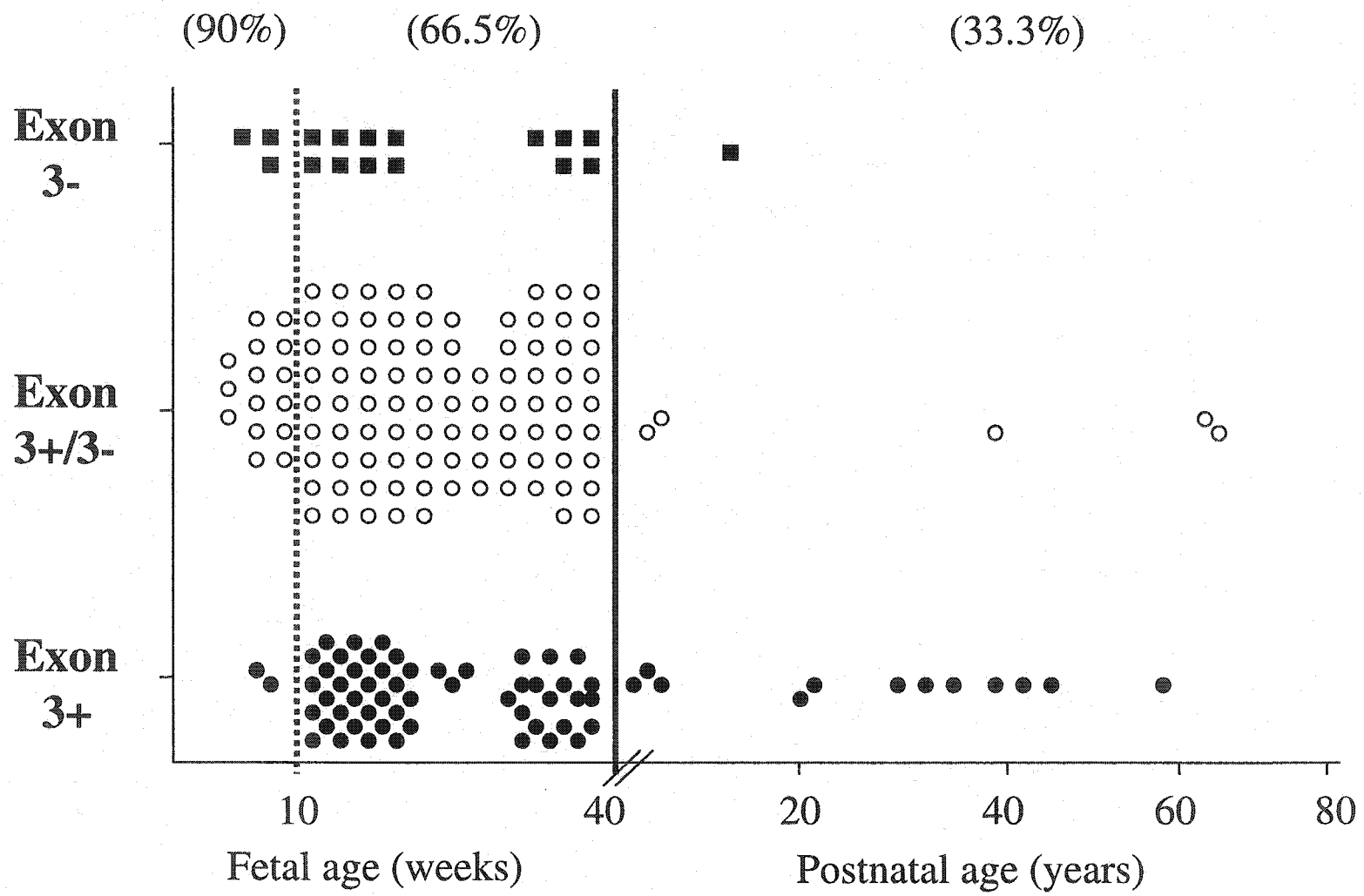
experiments showed that $^{125}$I-hGH bound to different molecular mass species of hGHR in human fetal (~112 kD) vs. postnatal (~80-100 kD) liver; 2) fetal hepatocytes produced 10 times more IGF-II than IGF-I, and responded to hGH with a small but significant increase in IGF-II rather than IGF-I; and 3) fetal dermal fibroblasts demonstrated a lack of responsiveness of hGH binding following pretreatments with hGH or dexamethasone [194]. A detailed description of our findings is summarized in Table 3.

The relative abundance of GHR isoforms during development also differ. In the fetus and newborn, serum concentrations of GHBP are the lowest. GHBP levels increase rapidly during the first two to three years of infancy, and gradually plateau during childhood, reaching a maximum during puberty. These observations suggest a major increase in tissue levels of GHR during early infancy [195]. Exon 3-deficient mRNA can also be detected in fetal tissues from as early as 4 weeks fetal age (wks FA) and there seems to be a predominant expression of the exon 3-deficient transcript during fetal life [191]: expression of the exon 3-deleted transcript (either alone or together with the exon 3-retaining transcript) decreases with developmental age from 90% (4-9 wks FA) to 69% (10-40 wks FA) to 33% (1 week to 43 years postnatal age) (Figure 20). As GH-induced receptor dimerization has been demonstrated to be important for activation of signal transduction mechanisms, a change in the conformation of the receptor complex (3+/3- heterodimer vs. 3+ or 3- homodimers) may modulate the activation process. No studies have examined the relative abundance of truncated (1-277) and (1-279) hGHR isoforms during development.

**Table 3. Summary of immature and/or adaptive responses observed in fetal vs. postnatal tissues** [194] [196].

| BIOLOGICAL ENDPOINT | FETAL | | POSTNATAL |
|---|---|---|---|
| **Total hGHR mRNA (Liver)** | x | < | 6x |
| **hGH binding (Liver)** | y | < | 4y |
| **Total FL hGHR mRNA (Liver)** | z | < | 1.5z |
| **T/FL hGHR mRNA (Liver)** | = | = | = |
| **hGH-hGHR complex (Liver)** | 112 kDa | > | 80-100 kD |
| **IGFs (Hepatocytes)** | IGF-II 10x > IGF-I<br>(+) GH = ↑ IGF-II only | | (+) GH = ↑ IGF-I |
| **IGFBPs (Hepatocytes)** | IGFBP 1, 2 and 4<br>(+) GH = no change [BPs] | | IFGBP 1, 2, 3 and 4<br>(+) GH = ↑ [BP3] |
| **Glucose Uptake (Hepatocytes)** | (+) GH = small but significant increase | < | (+) GH = major increase |
| **hGH binding (Dermal Fibroblasts)** | =<br><br>(+) GH = no change<br><br>(+) dexamethasone = no change | = | =<br><br>(+) GH = ↓ binding<br><br>(+) dexamethasone = ↑ binding |

**Figure 20. Expression of exon 3-deleted isoform during human development** [191]. Expression of the exon 3-deleted transcript (either alone or together with the exon 3-retaining transcript) decreases during gestation and postnatally.


(90%)
(66.5%)
(33.3%)
Exon 3-
Exon 3+/3-
Exon 3+
10
40
20
40
60
80
Fetal age (weeks)
Postnatal age (years)

# 2. RESEARCH PROPOSAL

The hGH plays an essential role in the growing child, through multiple growth-promoting as well as metabolic effects. Its actions are intimately linked to the availability of a functional hGHR. In the fetus, however, the role of hGH in the regulation of human fetal growth is poorly understood. When fetal hGH production is low or absent (GH deficiency) or the hGHR is dysfunctional (Laron syndrome), growth failure is often observed in newborns. This suggests an important role for hGH and its specific receptor in regulating growth at all stages of development.

This project addresses a complex stage in human development, the fetus. hGH and its receptor undergo dramatic shifts in expression, including changes in the molecular forms of hGH produced by the pituitary [197], the expression of multiple variants of hGHR mRNA in tissues [50] [191] [42] and the biological responses to hGH in these tissues [194].

Our recent data demonstrates that fetal hGHRs present in hepatocytes specifically bind hGH and have biological responses after chronic exposure to hGH [194]. However, despite the presence of functional hGHRs, these fetal receptors (and/or their signalling pathways) are immature or have adapted in the *in utero* environment. Based on these results, we hypothesize that there are **fetal-specific hepatocyte differences in hGHR and/or its downstream signal transduction pathways that can explain the relatively immature or adaptational biological responses documented.**

To examine this hypothesis, the immediate objectives of this project have been:

1) To determine if the fetal-specific biological responses could be explained by differences in the **protein structure** of the fetal GHR, due to:
    a) post-translational modifications, which may account for the differences in hGHR molecular weight previously shown by affinity crosslinking [194].
    b) the relative abundance of a dominant-negative truncated hGHR isoform in fetal hepatocytes.
    c) the functional significance of the predominant expression of the exon 3-deleted hGHR isoform.
2) To determine if there are differences in the relative abundance of **GHR downstream signal transduction molecules** that can account for the immature biological responses detected during fetal life, due to:
    a) the relative abundance of stimulatory molecules of the Jak/Stat signal transduction pathway.
    b) the relative abundance of inhibitory molecules of the Socs pathway.

This project was originally intended for a Ph.D. thesis. I have decided to complete graduate school with an M.Sc. because of my desire to pursue a career outside of academia. Subsequently, some aspects of this project (research proposals 1a and 1c) have not been fully investigated: 1a) the only available GHR antibody (Mab 263), which was previously used for immunohistochemistry assays [193], was demonstrated to be inappropriate for our immunoprecipitation and immunoblotting studies. We were not able to obtain appropriate antibodies against the GHR until March 2002 and

subsequently, this part of the project was only recently undertaken; 1c) technical difficulties in creating stable cell lines (please see section 4.6 for details) delayed the advancement of this work. Despite this, I have completed a series of critical preliminary experiments for these studies. Several recommendations for future experiments for this project have also been made throughout the thesis.

# 3. MATERIALS AND METHODS

## 3.1 Materials

Antibodies directed against the GHR: AL-47 (which recognizes the intracellular domain of the GHR) and 74.3 (extracellular domain) were kindly provided by Dr. Stuart Frank (Department of Medicine, University of Alabama, Birmingham, USA) and GHR AS-15E (extracellular domain) by Dr. Jurgen Kratzsch (Institute of Laboratory Medicine, Clinical Chemistry, and Molecular Diagnostics, 04103 Leipzig, Germany). Antibodies directed against GH-signalling proteins were obtained from the following commercial sources: Jak1 (sc-7228), Jak2 (sc-294), Jak3 (sc-513), Tyk2 (sc-169) Socs1 (sc-7005), Socs2 (sc-9022), Socs3 (sc-9023) and Cis (sc-1529) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); Jak2 (06-255), Stat1 (06-501), Stat3 (06-596), Stat5A (06-968) and Stat5B (06-969) from Upstate Biotechnology, Inc. (Lake Placid, NY); calnexin (C45520) from Transduction Laboratories (Lexington, KY); ubiquitin (U5379) from Sigma-Aldrich, Inc., (Saint Louis, MO).

## 3.2 Tissues

Human fetal tissues were obtained at the time of therapeutic abortion (10.5-19.5 wk of fetal age(FA)); FA was determined by foot length [198]. Postnatal liver specimens, which were perfused to remove all blood cells, were collected within 4-10 hours after removal of organs for transplantation (11-62 yr). Ethical approval for the study was obtained from local institutional research ethics boards and informed consent was obtained in each case.

## 3.3 Cell Lines

Five different cell lines were analyzed: CV1 (African green monkey kidney ibroblasts), HEK293 (human embryonic kidney epithelial cells), mIMCD (mouse inner medullary collecting duct cells) and two human postnatal hepatoma cell lines, HepG2 (ATCC) and Huh7 (kindly provided by Dr. Ken K. Ho, Garvan Institute of Medical Research, Sydney, New South Wales, Australia). CV1, HEK293 and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Corporation) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin G and 1.6 mg/mL gentamycin sulfate (complete DMEM). mIMCDs were cultured in DMEM:Nutrient Mixture F-12 (D-MEM/F-12) (Invitrogen Corporation) supplemented with 5% fetal bovine serum, 100 IU/mL penicillin G and 1.6 mg/mL gentamycin sulfate, while Huh7 cells were cultured in Earle's MEM (EMEM) (Invitrogen Corporation) supplemented with 10% fetal bovine serum, 7.5 mM HEPES and 500 IU/mL penicillin. All cells were cultured at 37°C with 5% $CO_2$ in a humid environment. Cells were washed twice with ice-cold 1x phosphate buffer solution (PBS) and collected by scraping. Proteins were extracted with lysis buffer and after incubation on ice for 15 min, the cell lysate was centrifuged at 13 000 x *g* for 10 min to remove insoluble materials. Supernatants were transferred to fresh tubes, and stored at -20°C. Alternatively, cells were centrifuged at 1000 x *g* for 10 min and pellets extracted for RNA using the TriZol method (Invitrogen Corporation).

## 3.4 Hepatocyte Preparations

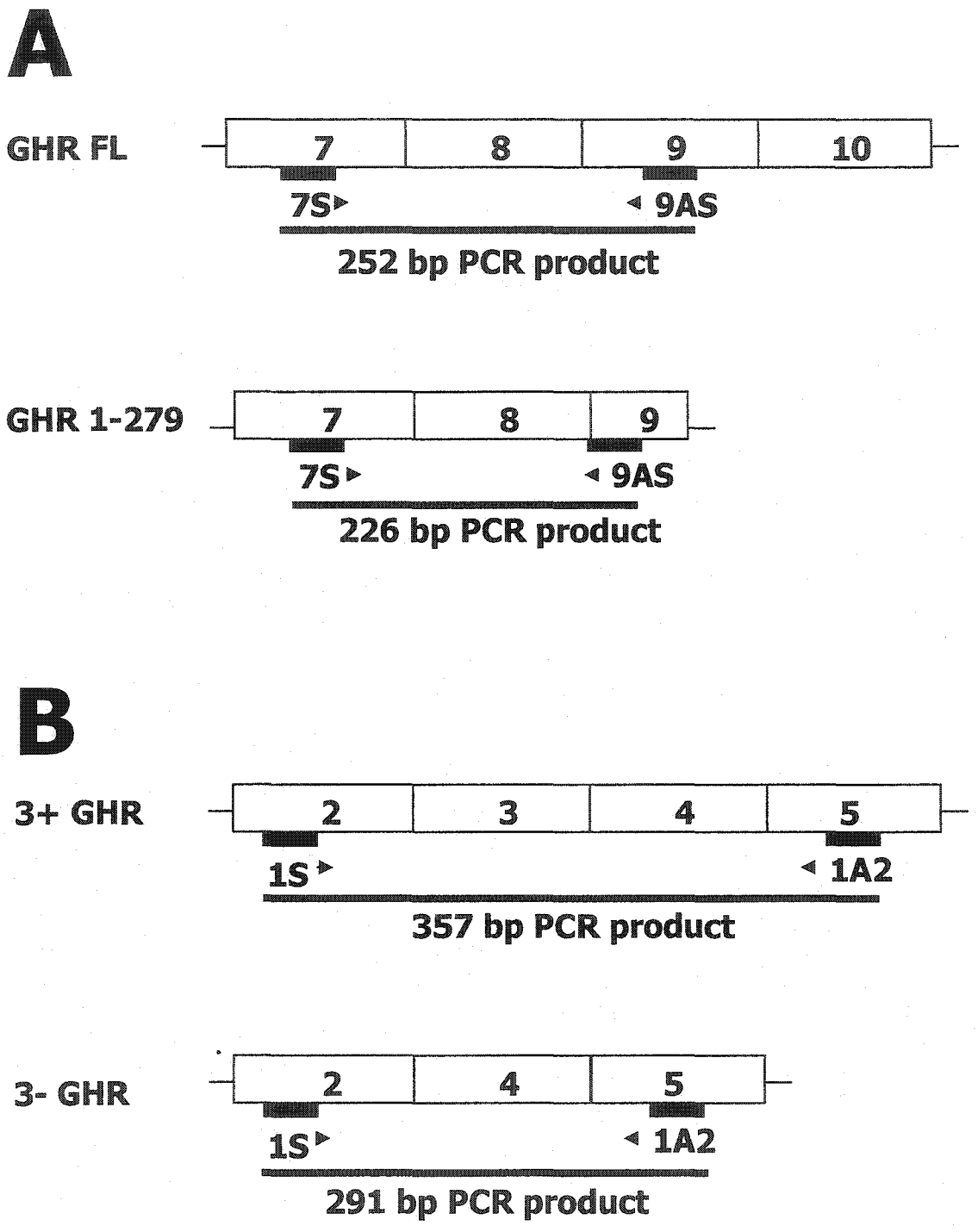
The postnatal liver tissues used for these studies had been perfused (for transplantation) and therefore, did not contain blood cells. Since the fetal liver is the primary hematopoietic organ during this stage of human development, it was therefore necessary to isolate fetal hepatocytes from intact fetal liver to remove blood cells, to permit a more accurate comparison of fetal to postnatal liver. For each experiment, approximately five grams of human fetal liver tissue were minced in 10 mL of warm (37°C) solution I (10 mM HEPES (pH 7.4), 0.142 M NaCl, 6.7 mM KCl and 1 mM EDTA•4Na, adjusted to pH 7.4 and filter sterilized). Solution I was removed, the minced tissue transferred to a flat-bottom glass vial with a mini-stir bar, and 10 mL of warm (37°C) solution II (100 mM HEPES (pH 7.4), 67 mM NaCl, 6.7 mM KCl, 4.8 mM $CaCl_2 \cdot 2H_2O$, adjusted to pH 7.4, filter sterilized with 2 mg/mL collagenase type I (Invitrogen Corporation, Carlsbad, CA) and 0.2 mg/mL DNase I (Roche Diagnostics Corporation, Indianapolis, IN)) were added to the minced tissue and allowed to stir at 37°C for 20 min. Following triturition with a pasteur pipette, the resultant suspension was allowed to settle at unit gravity for 20 min at room temperature. The supernatant was discarded and this step was repeated once more. The settled cells were resuspended in 5 mL of warm (37°C) solution III (Minimum Essential Medium (Invitrogen Corporation) and 0.96 mM EDTA, adjusted to pH 7.4, filter sterilized, with 0.16 mg/mL DNase I) and centrifuged at 500 x g for 10 min at room temperature. The supernatant was discarded and the resultant cell pellet was washed by unit gravity two times for 20 min each with 5 mL of warm (37°C) solution III. The final layer of sedimented cells, containing primarily (>95%) hepatocytes, was resuspended in William's E medium (Invitrogen Corporation)

supplemented with 10% fetal bovine serum, 100 IU/mL penicillin G, 16 μg/mL gentamycin sulfate and 39.2 μg/mL dexamethasone. The isolated hepatocytes were plated on collagen-coated petri dishes. 2 h after plating, the hepatocytes were rinsed thoroughly (3-5 times) with 1x phosphate buffer saline (PBS) to remove all remaining traces of hematopoietic cells and then collected by scraping for isolation of protein or RNA. For ubiquitin and GH-treatment studies, hepatocytes were rinsed thoroughly (3-5 times) with 1x PBS to remove all remaining traces of hematopoietic cells, replaced with fresh, complete William's E medium with 39.2 μg/mL dexamethasone and incubated overnight. 24 h later, hepatocytes were treated with 150 ng/mL for 15 min (GH-treatment studies) or simply washed with 1x PBS (ubiquitin studies) and collected by scraping. Proteins were extracted with lysis buffer and after incubation on ice for 15 min, the cell lysate was centrifuged at 13 000 x *g* for 10 min. Supernatants were transferred to fresh tubes, and stored at -20°C. Alternatively, cells were centrifuged at 1000 x *g* for 10 min and pellets extracted for RNA using the TriZol method (Invitrogen Corporation).

## 3.5 RT-PCR

*Full length vs. Truncated hGHR [194].* For each reaction, five micrograms of hepatic total RNA were reverse transcribed for 1 h at 42°C in the presence of 200U of Superscript II (Invitrogen Corporation), 200 μM deoxyribonucleotides (dNTPs) (Invitrogen Corporation), 10 mM dithiothreitol (DTT) (Sigma Aldrich) and a specific exon 9 reverse primer (9AS) designed to recognize both the full-length and truncated (1-279) hGHR mRNAs (Figure 21A). Prior to the reverse transcription reaction, the RNA

**Figure 21. Schematic diagram of primers used for RT and PCR assays.** A) A specific primer in Exon 9 (9AS) was designed to recognize both the full-length and truncated (1-279) forms of hGHR mRNA. B) 3+ and 3- hGHR mRNAs were detected using primers 1S (Exon 2) and 1A2 (Exon 5).


A
GHR FL
7
8
9
10
7S
9AS
252 bp PCR product
GHR 1-279
7
8
9
7S
9AS
226 bp PCR product
B
3+ GHR
2
3
4
5
1S
1A2
357 bp PCR product
3- GHR
2
4
5
1S
1A2
291 bp PCR product

was heated at 65°C for 5 min to disrupt any secondary structure. Three microliters of RT product were amplified for 35 cycles with 1.25U *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA), 200 μM dNTPs, 1.5 mM $MgCl_2$ and 0.5 μM hGHR exon 7 sense (7S) and antisense hGHR exon 9AS primers. The cycles consisted of one cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1 min, ending with a final elongation at 72°C for 10 min. In each assay, an aliquot of $H_2O$ was amplified in parallel as a negative PCR control. PCR products were separated on 10% polyacrylamide gels and stained with Sybr Gold (Molecular Probes, Eugene, Oregon). Densitometric analyses were carried out using the Bio-Rad GelDoc system (Mississauga, ON, Canada).

*Exon 3-retained vs. –deleted hGHR [191].* Following the above-described method, a specific exon antisense primer (1A2) was used for reverse transcription (Figure 21B). Three microliters of RT product were amplified for 35 cycles with 1.25U *Taq* DNA polymerase (Invitrogen Corporation), 0.5 mM dNTPs, 3 mM $MgCl_2$ and 0.5 μM hGHR sense (1S) and antisense 1A2 primers. The reaction consisted of one cycle at 92°C for 3 min, 61°C for 1 min and 72°C for 3 min, followed by 35 cycles of 92°C for 30 s, 61°C for 1 min and 72°C for 1 min 30 s, ending with a final elongation at 72°C for 5 min. In each assay, an aliquot of $H_2O$ was amplified in parallel as a negative PCR control.

| *Primers* | |
|---|---|
| 7S | 5'-ATAAGGAATATGAAGTGCGTGTGAG-3' |
| 9AS | 5'-TAATCTTTGGAACTGGAACT-3' |
| 1S | 5'-CTGCTGTTGACCTTGGCACTGGC-3' |
| 1A2 | 5'-AGGTATCCAGATGGAGGTAAACG-3' |

## 3.6 Tissue Cytosolic and Membrane Fractionation

Approximately 5 gms of human fetal liver were homogenized in ice-cold lysis buffer (50 mM Tris (pH 7.5), 0.1% Triton X-100, 150 mM NaCl and 2 mM EDTA) containing protease and phosphatase inhibitors (1 mM $Na_3VO_4$, 1 mM PMSF, 10 μg/mL each aprotinin, leupeptin, pepstatin and 5 mM NaF) for 15 min on ice. Homogenates were centrifuged at 1 000 x g for 10 min to remove insoluble materials. Supernatants were then centrifuged at 100 000 x g for 1 hour. Cytoplasmic proteins present in the supernatant from this high-speed centrifugation were aliquoted and frozen at -20°C. The cell pellets, containing membrane proteins, were solubilized in ice-cold lysis buffer, aliquoted and frozen at -20°C.

## 3.7 Immunoprecipitation and Immunoblotting

Tissue fragments or cell pellets were solubilized in ice-cold lysis buffer (50 mM Tris (pH 7.5), 0.1% Triton X-100, 150 mM NaCl and 2 mM EDTA) containing protease and phosphatase inhibitors (1 mM $Na_3VO_4$, 1 mM PMSF, 10 μg/mL each aprotinin,

leupeptin, pepstatin and 5 mM NaF) for 15 min on ice. To remove insoluble material, lysates were centrifuged at 13 000 x g for 10 min at 4°C. The supernatants were collected and protein concentrations were measured with a Bradford dye-binding assay kit (Bio-Rad Laboratories Inc., Hercules, CA); BSA was used as a standard. For immunoprecipitation, 0.5-1 mg of liver lysate protein were incubated by rotation in the lysis buffer described above with anti-GHR antibodies (αAL-47, 1:250) overnight at 4°C, followed by the addition of 50 μL protein G-agarose beads (Amersham Biosciences, Baie D'Urfé, QC, Canada) for 1 h at 4°C. Antibody complexes were washed three times in lysis buffer followed by a single wash in 50 mM Tris (pH 8.0). Immunoprecipitated extracts or 50-100 μg of whole cell extracts were boiled for 5 min in SDS polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (50 mM Tris (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol and 100 mM DTT) and proteins were resolved by SDS-PAGE (8%-15%) and transferred (Mini Trans-blot Cell, Bio-Rad Laboratories, Inc.) onto polyvinylidene difluoride (PVDF) Immobilon-P transfer membranes (Millipore Corporation, Mississauga, ON, Canada).

Proteins were immunodetected with antibodies to AL-47 (1:2000), GHR 74.3 (1:2000), GHR AS-15E (1:5000), Jak1 (1:1000), Jak2 (1:1000), Jak3 (1:500), Tyk2 (1:500), Stat1 (1:2000), Stat3 (1:2000), Stat5A (1:1200), Stat5B (1:1200), Socs1 (1:100), Socs2 (1:200), Socs3 (1:200), Cis (1:100), calnexin (1:1000) or ubiquitin (1:50) for 1 h at room temperature. The membranes were incubated with an anti-mouse (1:1000) or anti-rabbit (1:1000) IgG conjugated to horseradish peroxidase (Perkin Elmer Life Sciences Inc., Boston, MA) for 1 h at room temperature and visualized by an enhanced chemiluminescence (ECL) detection system (Perkin Elmer Life Sciences Inc.). For

reprobing with another antibody, membranes were rehydrated in methanol, rinsed in distilled water and incubated for 30 min at 50°C with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris (pH 6.8)). Densitometric analyses were carried out using the Bio-Rad GelDoc system.

To account for errors in sample loading, membranes were initially stripped and re-probed with antibodies against actin or GAPDH. But since immunoblotting experiments demonstrated that these proteins were not expressed at consistent levels during development (data not shown), we used an antibody against calnexin, an integral membrane protein of the endoplasmic reticulum (ER) that functions as a molecular chaperone [199]. Immunoblotting experiments with increasing concentrations of protein at all stages of development demonstrated that calnexin was the more appropriate antibody to use as a loading control for our studies (data not shown). Data are therefore expressed in arbitrary densitometric units as a ratio of GH-signalling molecule to calnexin, which was usually detected as a doublet at ~90 kDa [200].

## 3.8 Plasmids

Exon 3-retained (full-length, FL) and –deleted (Δ3) hGHR expression plasmids were kindly provided by Dr. Marie-Laure Sobrier (INSERM, Créteil, France) [190]. The FL and Δ3 hGHR cDNA coding sequences were cloned into the expression vector pECE at a *SalI* restriction site at the 5'end (5'to the ATG translation initiation codon) and an *SstI* restriction site at the 3'end.

For analysis of the relative biological responsiveness of the 3+ vs. 3- hGHRs, the Spi2.1 promoter-luciferase (Spi2.1-luc) plasmid was obtained from Dr. Nils Billestrup

(Novo Nordisk, Denmark) [201]. Specifically, two copies of the *XhoI* site of the Spi2.1-Gas-like element (GLE: 5'-tcgaACGCTTCTACTAATCCATGTTCTGAGAAATCATC CAGTCTGCCCA-3') were ligated into the *XhoI* site of the pGL2 vector (Promega), upstream of the luciferase gene.

## 3.9 Transient Transfection

Approximately 1.0 x $10^5$ HepG2 cells (P110-P115) were plated per well in 12-well plates in complete DMEM for 24 h and transfected the following day. 60-70% confluent cells were transfected with 1.5 μg Spi2.1-luc pGL2 basic vector, 3.0 μg RSV-β-galactosidase-encoding plasmid and various combinations of FL hGHR pECE and Δ3 hGHR pECE (to a total of 3.0 μg DNA) using the calcium phosphate method. All experiments were performed in triplicate. A DNA solution consisting of plasmids and autoclaved water was prepared and 75 μL (per triplicate) of 4x $CaCl_2$ (500 mM $CaCl_2$, 2 mM Tris-HCl, 0.2 mM EDTA) was added dropwise to each tube. 150 μL (per triplicate) of 2x HBS (50 mM HEPES, 280 mM NaCl, 1.5 mM $Na_2HPO_4$) was introduced to the bottom of the tube, pipetting 5-6 times to produce bubbles. The solution was incubated at room temperature for 30-45 min to allow the DNA precipitate to form. Cells were washed with 1x PBS and medium was replaced with fresh, warm (37°C) complete DMEM. 100 μL of DNA precipitate were distributed evenly to each well and the cells were incubated at 37°C for 24h. The medium was then removed, cells were washed with 1x PBS and replaced with fresh, warm (37°C) complete DMEM. Untreated cells recovered in complete DMEM for an additional 24 h. For GH-treatment, cells were allowed to recover in complete DMEM for up to 8 hours. Cells were then serum-starved overnight

by replacing medium with serum-free DMEM. The following morning, cells were stimulated with 10 or 150 ng/mL hGH (Protropin, Genentech Canada Inc.) for up to 6 h, after which luciferase and β-galactosidase assays were performed. Cells were also collected to isolate 3+ and 3- hGHR mRNA and protein, for RT-PCR and WB analysis, respectively.

## 3.10 β-Galactosidase and Luciferase Assays

Media were aspirated from wells and the cells were washed with ice-cold 1x PBS. Cells were harvested in 200 μL of lysis buffer (0.01 M DTT, 0.1 M Tris (pH 8.0), 0.5% NP-40) per well and rotated at room temperature for 15 minutes to allow complete lysis of cells to occur. For the β-galactosidase assay, 10 μL of cell lysate were added to wells of a microtiter plate with 100 μL β-galactosidase solution (0.1 mM β-galactosidase substrate in Reaction Buffer Diluent: 100 mM sodium phosphate (pH 7.5), 1 mM $MgCl_2$, 5% Sapphire-II enhancer (Tropix Galacton-Star, Bedford, MA)), covered in plastic wrap and incubated at room temperature for 1 h. For the luciferase assay, 100 μL of cell lysate were added to wells of a microtiter plate and a 1x luciferin solution was prepared from a 10x luciferin solution (0.1 mM Coenzyme A, 2.5 mM ATP, 1 mM luciferin, 5 mM $MgCl_2$, and 500 mM Tris-HCl (pH 7.9)). Both β-galactosidase and luciferase activity was measured using WinGlow system (EG&G Berthold, Bad Wildbad, Germany) and data were expressed as a ratio of luciferase activity (relative light units (RLU)) to β-galactosidase activity.

## 3.11 Statistical Analyses

Differences between groups (by age or treatment) were analyzed by Student's *t*-test if there were two groups or by ANOVA followed by Bonferroni's multiple range test if multiple groups. Data are presented as the mean ± SEM. $P<0.05$ was considered to be significant.

# 4. ANALYSIS OF GHR PROTEIN STRUCTURE

## 4.1 Preface

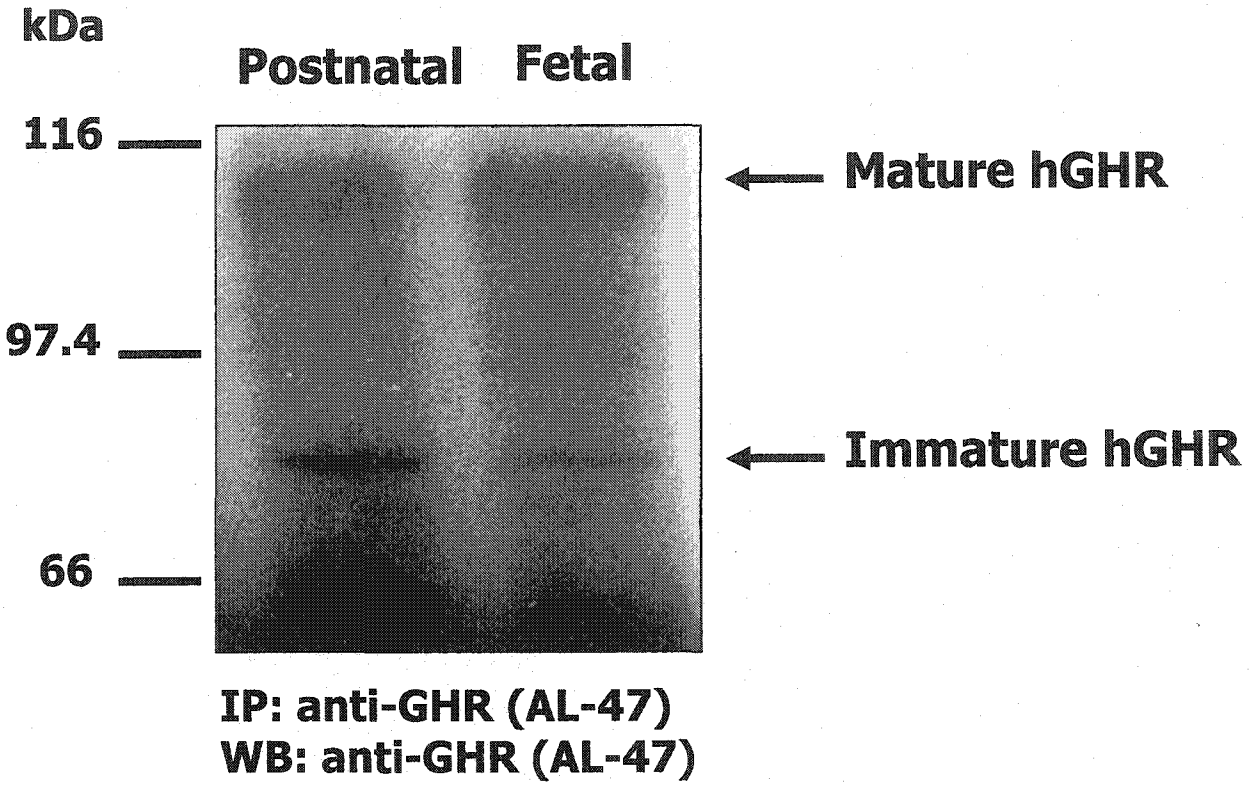
Previous affinity crosslinking experiments had shown that $^{125}$I-hGH bound to different molecular mass species of hGHR in fetal (~110 kD) compared to postnatal (~95 kD) liver [194]. This difference in molecular mass of the fetal hepatic hGHR complex needed to be explored to determine what role this difference may play in restricting hGH responsiveness during fetal development. hGHR mRNA from both fetal and postnatal samples have been sequenced and found to be identical, suggesting that the basic protein must be similar [202]. We hypothesized that the size difference might be due to either a) post-translational modifications (i.e. glycosylation or ubiquitination of the hGHR extracellular domain) or b) differential association of the hGHR with additional molecules (e.g. Socs). One other logical question to ask was whether the relative abundance of the dominant-negative truncated (1-279) hGHR isoform in fetal cells may play an important role in the immature responses. Finally, the predominant expression of the hGHR exon 3-deficient transcript during fetal life was of interest: expression of the exon 3-deleted transcript (either alone or together with the exon 3-retaining transcript) is 90% from 4-9 wks FA, 69% from 10 wks FA to term and 33% postnatally [191]. Therefore, we decided to analyze the downstream effects of different hGHR complexes (i.e. 3+/3- heterodimer vs. 3+ or 3- homodimers) in a human hepatoma cell line that lacked endogenous hGHR, using a known GH response element in a luciferase reporter vector (spi2.1/luc) [201].

## 4.2 Analysis of hGHR molecular mass in human fetal hepatocytes vs. postnatal liver

To determine whether the hGHR size differences were due to post-translational modifications, fetal hepatocytes (n=2; 12.7 and 13.75 wks FA) and intact postnatal liver (n=2; 37 and 62 yr) samples were analyzed by immunoprecipitation and immunoblotting with an antibody directed against the intracellular domain of hGHR. Both immature (~70 kD) and mature (~110 kD) forms of the hGHR were detected in fetal hepatocytes and postnatal liver (Figure 22). While there was an approximate two-fold increase in the immature form of hGHR in postnatal liver, both the immature and mature forms were identical in molecular size to those observed in fetal hepatocytes, suggesting that differential post-translational modifications (e.g. glycosylation, ubiquitination) were not the reason for the difference in affinity cross-linking molecular weights of the hGHR.

To further confirm IP/WB: anti-GHR data (Figure 22), that the size differences previously seen in affinity cross-linked hGHR are not due to post-translational modifications, two future sets of experiments should be undertaken: 1) deglycosylation of GHRs at both stages of development; and 2) IP: anti-GHR/WB: anti-ubiquitin to assess for the ubiquitinated status of the two receptors. These studies will also allow us to address whether there is a differential ratio of glycosylation or ubiquitination in the fetal vs. postnatal hepatic hGHRs. Because fetal hepatocytes were previously prepared from freshly dispersed cells, where there may be minimal amounts of cell surface GHR, ongoing experiments to examine the ubiquitinated state of the hGHR should focus on cells allowed to incubate overnight prior to harvesting.

**Figure 22. Representative Immunoprecipitation (IP) and Western Blot (WB) analysis of hGHR in fetal hepatocytes (n=2; 12.7 and 13.75 wks FA) vs. postnatal liver (n=2; 37 and 62 yr).**


kDa
Postnatal
Fetal
116
97.4
66
Mature hGHR
Immature hGHR
IP: anti-GHR (AL-47)
WB: anti-GHR (AL-47)

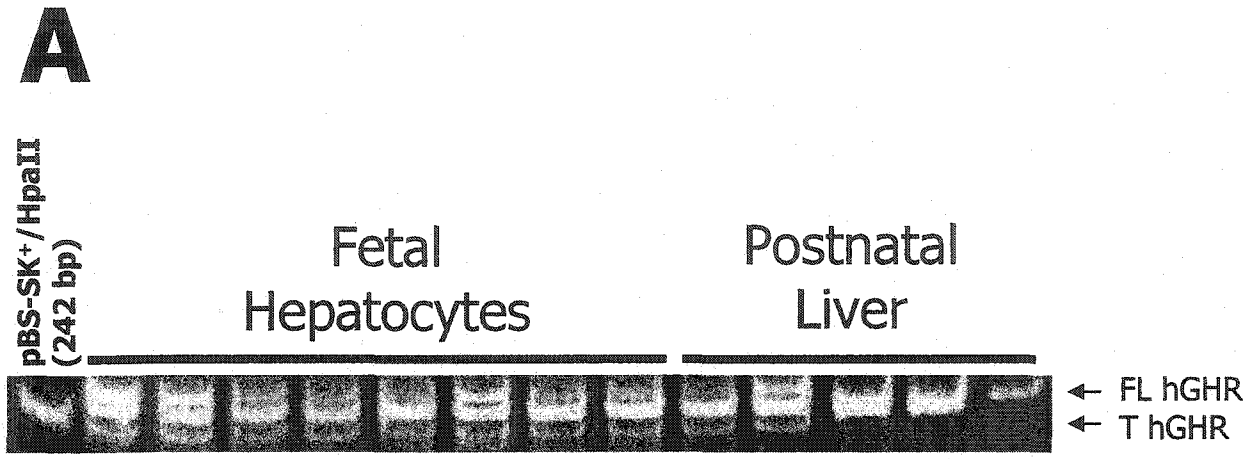
## 4.3 Truncated (1-279) hGHR mRNA in human fetal hepatocytes vs. postnatal liver

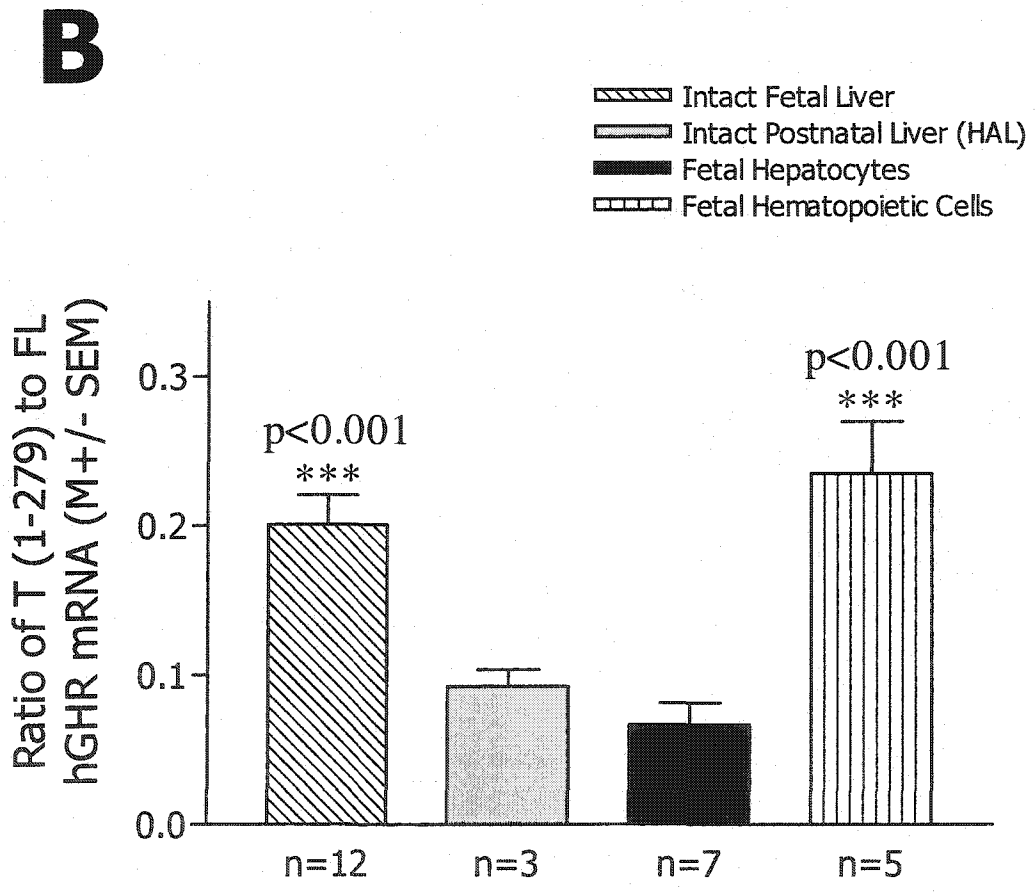
To analyze whether the major dominant-negative truncated (1-279) (T) form of the hGHR plays a role in fetal-specific biological responses, we determined mRNAs for both the full-length (FL) and T form of the hGHR using RT-PCR. We designed an antisense primer that is capable of recognizing both the FL and the T hGHR mRNA, resulting in PCR product sizes of 252 bp and 226 bp, respectively. This allowed us to examine two distinct bands that could easily be separated on the same polyacrylamide gel (Figure 23A).

Initially, ratios of T to FL (T/FL) hGHR mRNA were analyzed in intact fetal liver (0.20±0.02; n=12; 12.7-19.5 wks FA) vs. intact postnatal liver (0.09±0.01; n=3; 37-62 yr). While the FL transcript always predominated, there was a trend for fetal liver to have a two-fold higher ratio of T/FL hGHR mRNAs (Figure 23B): because of a two-to three-fold variability within each age group, the difference did not reach statistical significance.

However, since the fetal liver is the primary hematopoietic organ during this stage of development, it was decided to dissect the intact fetal liver into its two major cell components, hepatocytes and hematopoietic cells, since the latter contain a number of cytokine receptors, including the GHR. This isolation permitted us to make a more accurate comparison of fetal to postnatal liver: the postnatal liver tissues used for these studies had been perfused for transplantation and, therefore, did not contain blood cells. When intact fetal liver, fetal hematopoietic cells, isolated fetal hepatocytes and intact postnatal liver were compared, the highest T/FL hGHR mRNA ratio was detected in fetal hematopoietic cells (0.24±0.04; n=5; 12.7-19.5 wks FA), while fetal hepatocytes

**Figure 23. Comparison of full-length (FL) and truncated (T) (1-279) hGHR mRNAs in fetal (10.5-19.5 wks FA) vs. postnatal liver by RT-PCR.** A) Representative polyacrylamide gel of PCR products. Full-length (FL) hGHR mRNA is detected at 252 bp and truncated (1-279) (T) hGHR mRNA at 226 bp. pBS-SK$^+$/Hpa II marker is indicated on the left. B) Comparative data for intact fetal liver, intact postnatal liver, fetal hepatocytes and fetal hematopoietic cells: each sample was tested using three different RNAs and the final data are expressed as a ratio of truncated to full-length (T/FL) transcripts. Statistical differences are expressed relative to fetal hepatocytes.


A
pBS-SK+/HpaII
(242 bp)
Fetal
Hepatocytes
Postnatal
Liver
FL hGHR
T hGHR


B
Intact Fetal Liver
Intact Postnatal Liver (HAL)
Fetal Hepatocytes
Fetal Hematopoietic Cells
Ratio of T (1-279) to FL
hGHR mRNA (M+/- SEM)
0.3
0.2
0.1
0.0
p<0.001
***
p<0.001
***
n=12
n=3
n=7
n=5

(0.07±0.02; n=7; 11.5-17.5 wks FA) showed ratios similar to postnatal liver (Figure 23B). The comparable T/FL ratios detected in hepatocytes at the two stages of development suggest that the dominant-negative T hGHRs present in fetal hepatocytes are not likely to contribute to the fetal-specific biological responses.

## 4.4 Truncated (1-279) hGHR mRNA in Fetal Tissues

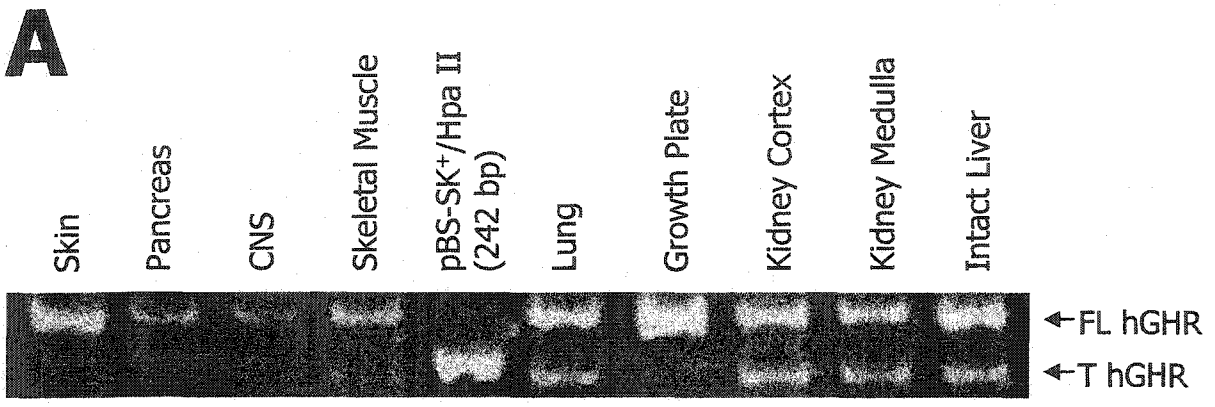
The relative abundance of truncated (1-279) to full-length hGHR mRNA was assessed in other developing tissues in order to place the hepatic T/FL hGHR mRNA data into context. Like the fetal liver, eight other tissues predominantly expressed the full-length form of the hGHR (Figure 24A). The dominant-negative T hGHR isoform was also detected in all tissues, but T/FL hGHR mRNA ratios varied considerably: 0.002 in skin, 0.03±0.01 in skeletal muscle, 0.09±0.04 in pancreas, 0.12±0.02 in growth plate, 0.18±0.07 in thymus, 0.20±0.02 in intact liver, 0.30±0.13 in CNS, 0.51±0.07 in lung, 0.53±0.06 in kidney cortex and 0.65±0.09 in kidney medulla (Figure 24B). Thus, although the dominant-negative hGHR does not likely play a role in the immature responses found in the fetal hepatocytes, the relatively high levels of T (1-279) hGHR in other fetal tissues, such as the kidney, may have more functional significance.

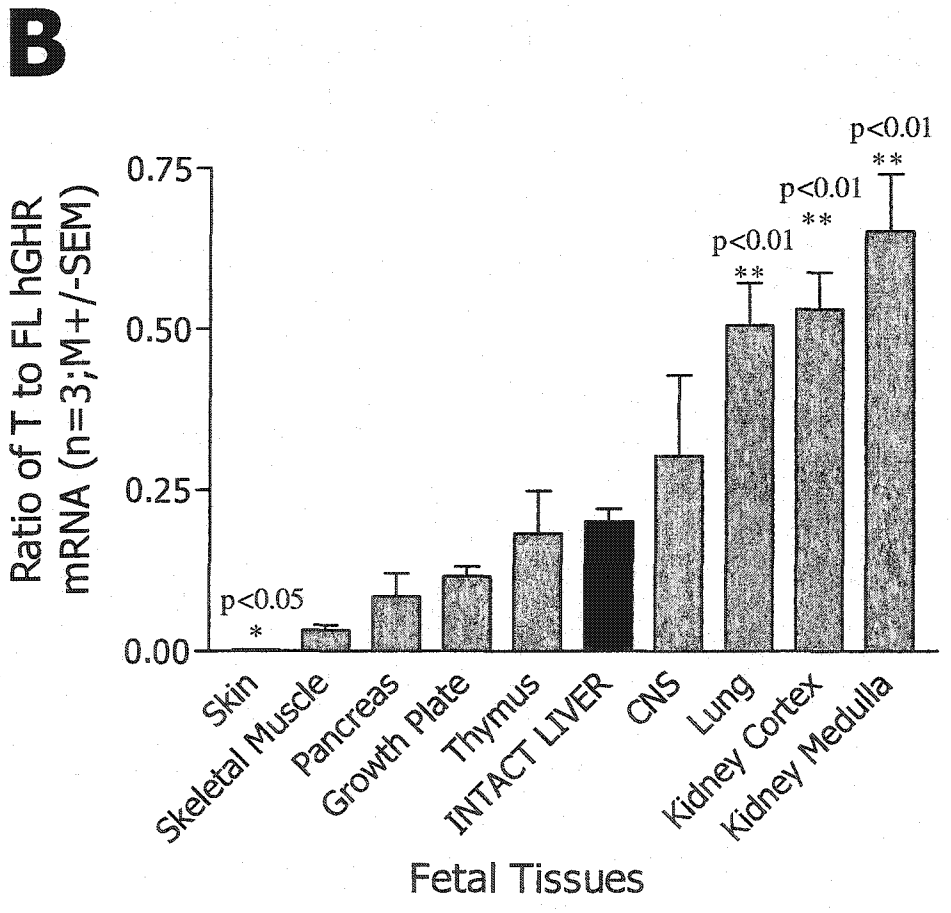
## 4.5 Characterization of GHR isoforms in various cell lines

### 4.5.1 T/ FL hGHR mRNA

Four different primate cell lines were analyzed: CV1 (African green monkey kidney fibroblasts), HEK293 (human embryonic kidney epithelial cells), HepG2 and Huh7 (both

**Figure 24. Comparison of full-length (FL) and truncated (T) (1-279) hGHR mRNAs in various fetal tissues.** A) Representative polyacrylamide gel of PCR products. pBS-$SK^+$/Hpa II marker is indicated at 242 bp. CNS, central nervous system. B) T (1-279) hGHR mRNA is detected in a wide variety of fetal tissues. The T/FL ratios vary considerably. Statistical differences are expressed relative to intact liver.


A
Skin
Pancreas
CNS
Skeletal Muscle
pBS-SK+/Hpa II (242 bp)
Lung
Growth Plate
Kidney Cortex
Kidney Medulla
Intact Liver
FL hGHR
T hGHR

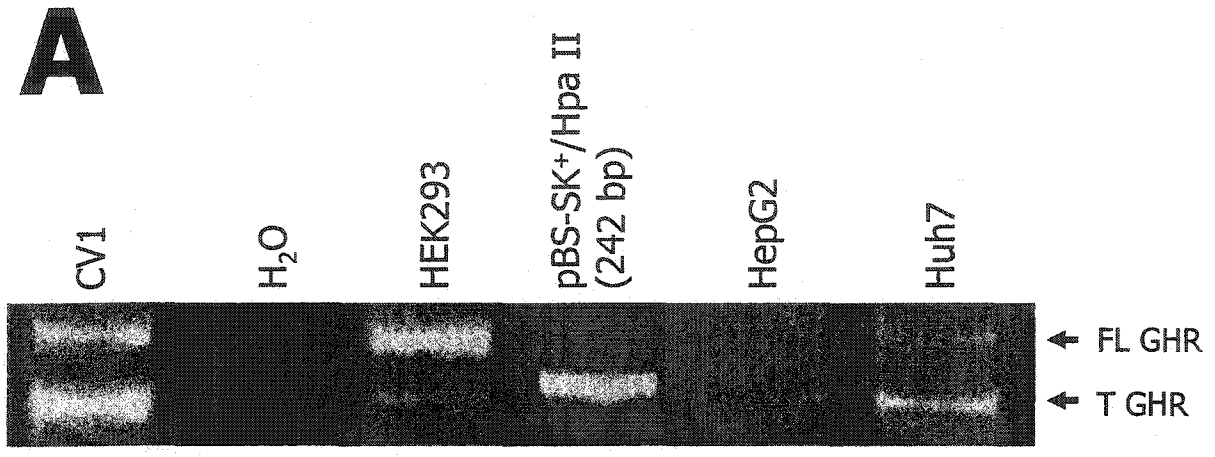

B
Ratio of T to FL hGHR mRNA (n=3;M+/-SEM)
0.75
0.50
0.25
0.00
p<0.05
*
p<0.01
**
p<0.01
**
p<0.01
**
Skin
Skeletal Muscle
Pancreas
Growth Plate
Thymus
INTACT LIVER
CNS
Lung
Kidney Cortex
Kidney Medulla
Fetal Tissues

human postnatal hepatoma cell lines). Interestingly, the hepatoma cell lines demonstrated very high T/FL ratios (HepG2: 3.00±0.94, Huh7: 1.71±0.30). T/FL ratios of HEK293 cells were quite low (0.21±0.05), while CV1 showed intermediate values (1.27±0.19) (Figure 25). These data suggest that the T form of the hGHR may have a physiological role as a dominant-negative receptor in hepatoma cells as well as a lesser role in HEK293 cells.

### 4.5.2 Exon 3+/3- hGHR mRNA

The same cell lines were characterized for the presence of exon 3-retained (3+) and/or exon 3-deleted (3-) GHR mRNA in order to determine in which cell line to carry out the exon 3+/3- functional studies. A human postnatal sample known to contain both 3+ and 3- hGHR mRNA was used as a positive control (HAL37) (Figure 26). Using RT-PCR, HEK293 and Huh7 cells consistently demonstrated the presence of only the 3+ GHR mRNA. hGHR mRNA was barely, if at all, detectable in HepG2 cells; only one out of four attempts detected a faint band of 3+ hGHR. Two bands were detected in IMCDs (see legend for details). Surprisingly, neither form of the GHR was seen in CV1 fibroblasts. As discussed in section 4.5.1, FL hGHR mRNA was detected in CV1 cells using primers in exon 7 and exon 9 of the hGHR gene. Through BLAST analysis of human, baboon and macaque sequences, we determined that the antisense 1A2 primer used for these studies had two mismatched nucleotides for monkey GHR mRNA. Thus, the absence of 3+ or 3- hGHR bands in CV1 fibroblasts was likely a technical artifact and not biologically significant. Based on these results, we decided to use the HepG2 hepatoma cell line due to the undetectable levels of endogenous GHR mRNA, to analyze

**Figure 25. Comparison of full-length (FL) and truncated (T) (1-279) hGHR mRNAs in various primate cell lines.** A) Representative polyacrylamide gel of PCR products. pBS-SK$^+$/Hpa II marker is indicated at 242 bp. B) Graph analysis of T/FL hGHR mRNA in four cell lines expressed relative to human adult liver (HAL). Each sample was tested using three different RNAs and the final data are expressed as a ratio of truncated to full-length (T/FL) transcripts.


A
CV1
$H_2O$
HEK293
pBS-SK+/Hpa II (242 bp)
HepG2
Huh7
FL GHR
T GHR

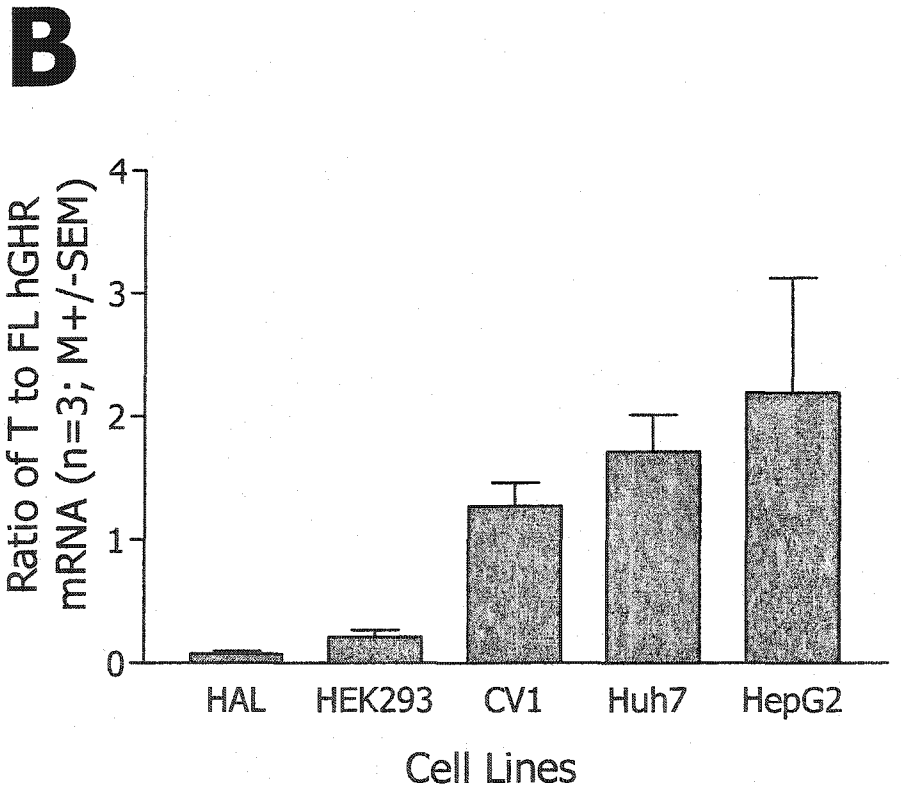

B
Ratio of T to FL hGHR mRNA (n=3; M+/-SEM)
0
1
2
3
4
HAL
HEK293
CV1
Huh7
HepG2
Cell Lines

**Figure 26. Comparison of Exon 3 -retained (3+) and -deleted (3-) hGHR mRNAs in various cell lines.** Representative polyacrylamide gel of PCR products. Human adult liver (HAL37) was used as a positive control for both 3+ and 3- hGHR mRNA isoforms. Due to the absence of exon 3 alternative splicing in the mouse gene [203], only the 3+ hGHR band was expected in IMCD cells. Several additional RT-PCR experiments were performed with murine-specific primers to confirm the presence of only 3+ hGHR mRNA (data not shown). The lower band detected with the human sequence primers is suspected to be nonspecific due to a lack of primer specificity. The upper IMCD band is higher in migration due to the presence of a novel exon (4A) in mouse GHR mRNA.

3+ GHR
3- GHR
H2O
HepG2
HAL37
IMCD
HEK293
CV1
Huh7
100 bp
400
300

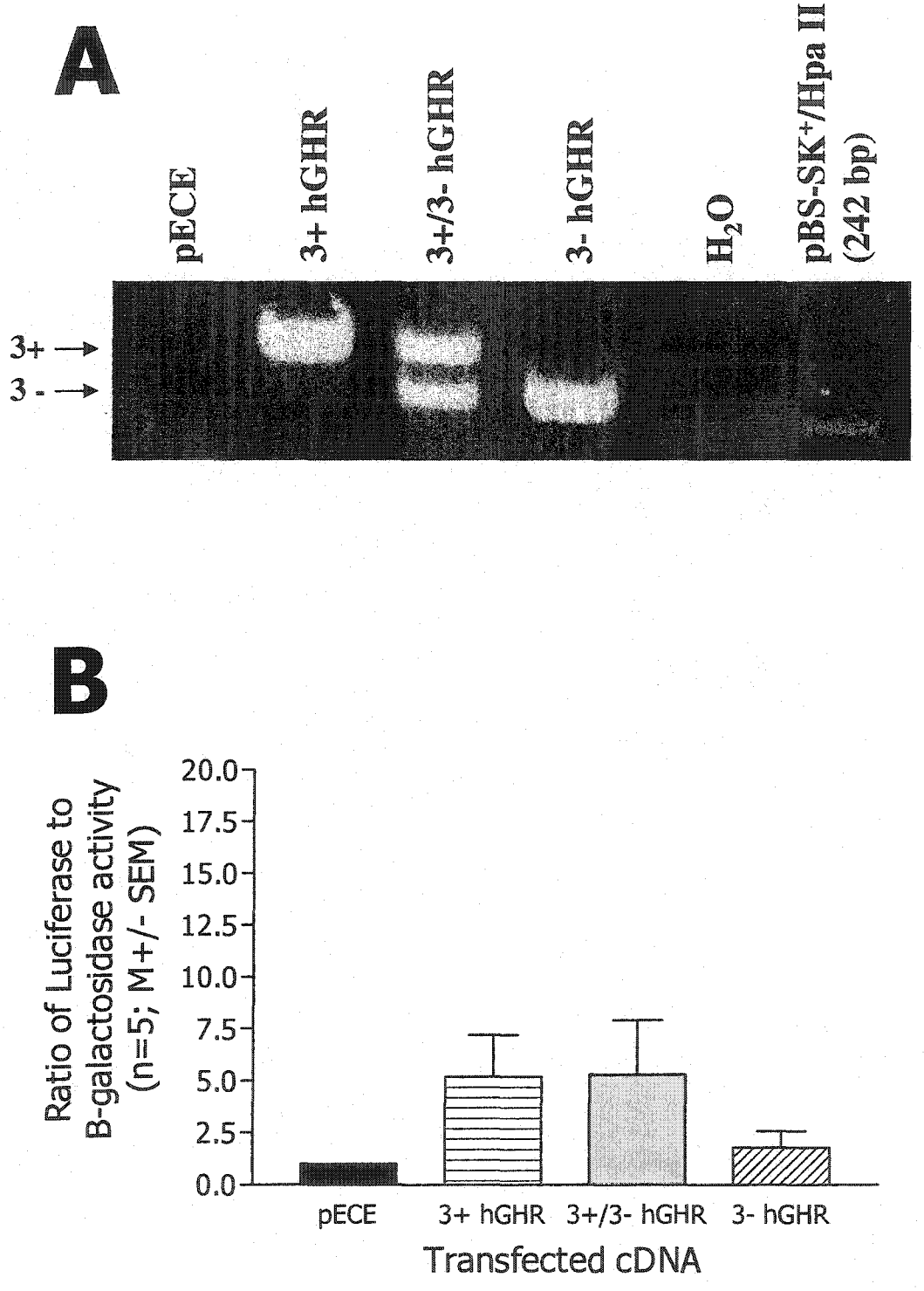
the functional significance, if any, of the exon 3-deleted isoform of the GHR.

## 4.6 Functional Analysis of Exon 3-deleted hGHR

Due to the predominant expression of the exon 3-deficient (3-) hGHR transcript during fetal life, we wanted to determine whether there was a functional significance of this hGHR isoform. Our initial goal was to create three stable HepG2 cell lines for these studies, expressing 3+ only, 3- only or 3+/3-. Exon 3-retained and –deleted pECE plasmids were digested by restriction enzymes *SalI* and *SstI* to remove 3+ and 3- hGHR cDNA coding sequences for subcloning into the pcDNA3.1 expression vector. However, after numerous attempts, we were unable to transform the constructs into competent *E.coli*. Subsequently, Dr. Stuart J. Frank (Department of Medicine, University of Alabama, Birmingham, USA) informed us of a "poison sequence" located in the transmembrane domain of the hGHR that was preventing transformation of the pcDNA3.1 vector constructs by the competent cells. For this reason, we decided to perform transient transfections with the 3+ and 3- pECE plasmids instead.

When cells were transiently transfected with 3+, 3- or 3+/3- hGHR cDNAs and the spi2.1/luciferase reporter vector containing two copies of a GH (Stat) response element, RT-PCR experiments showed equivalent expression of transfected constructs at the mRNA level (Figure 27A). Relative to the empty vector pECE, HepG2s transfected with 3+ hGHR (5.20±2.01), 3+/3- (5.32±2.61) or 3- hGHR (1.79±0.76) showed small, but insignificant increases in spi2.1/luciferase activity. Future experiments should include an immunoblotting analysis of transfected HepG2s to confirm equivalent expression of the 3+ and 3- hGHRs and a study of the functional effects on spi2.1/luciferase transactivation

**Figure 27. Exon 3+/3- hGHR functional studies.** A) Exon 3+ and 3- mRNA levels in transfected HepG2 cells analyzed by RT-PCR. 3+ hGHR mRNA is detected at 357 bp and 3- hGHR mRNA at 291 bp. pBS-SK$^+$/Hpa II marker is indicated on the right. B) Relative spi2.1/luciferase activity in transiently transfected HepG2 cells. Data are expressed as a ratio of luciferase activity (relative light units) to β-galactosidase activity.


A
pECE
3+ hGHR
3+/3- hGHR
3- hGHR
H2O
pBS-SK+/Hpa II
(242 bp)
3+
3 -
B
Ratio of Luciferase to
B-galactosidase activity
(n=5; M+/- SEM)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
pECE
3+ hGHR
3+/3- hGHR
3- hGHR
Transfected cDNA

following treatment with various concentrations (0, 10, 50, 150 ng/mL) of recombinant 22KDa hGH.

# 5. ANALYSIS OF GHR SIGNALLING PATHWAYS

## 5.1 Preface

We recently demonstrated differential biological responses in fetal vs. postnatal liver and dermal fibroblasts following chronic exposure to hGH [194]. Certain responses appear to be an adaptation to the fetal environment: in response to pretreatment with hGH, fetal hepatocytes surprisingly demonstrated no change in IGF-I but an increased production of IGF-II. Other responses may simply be due to an immature cell system: fetal dermal fibroblasts demonstrated a lack of responsiveness in hGH binding following treatment with either hGH or dexamethasone. We hypothesize that these adaptive and/or immature biological responses during fetal life may be due to differences in the relative abundance of GHR downstream signal transduction molecules, such as the stimulatory molecules of the Jak/Stat signal transduction pathway or the inhibitory molecules (e.g. Socs proteins).

## 5.2 Expression of Jak/Stat proteins in intact fetal vs. postnatal liver

At the beginning of this project, protein extracts of intact fetal and postnatal liver tissues were analyzed to compare the relative abundance of Jak/Stat family members. At this time, multiple western blot experiments were being performed to test for the optimal dilution of Jak/Stat antibodies as well as an appropriate loading control. Thus, we were only able to analyze these samples for the presence or absence of a small number of Jak/Stat proteins since we still lacked an appropriate control protein to account for errors

in sample loading and there were technical problems with several of the antibodies. Jak2, Tyk2 and Stat1 were found to be present at both stages of development (data not shown).

## 5.3 Expression of Jak/Stat proteins in cytosolic vs. membrane fractions of human intact fetal and postnatal liver

Early in the project, we also asked the question of whether there are developmental differences in the relative abundance of stimulatory Jak/Stat proteins at the level of the cytosol vs. membrane in human liver. Although only a few samples were tested, all of the Jak/Stat proteins were detected at relatively similar levels in fetal (Jak1: 148% of postnatal liver, Jak2: 134%, Jak3: 106%, Tyk2: 131%, Stat1: 184%, Stat3: 104% and Stat5A: 116%) compared to postnatal liver (Figure 28 and 29A; WB data normalized to calnexin). While Stat1 (84% of postnatal liver) and Stat3 (105%) were also detected in membrane fractions, there was a striking difference in Jak 1 and Jak2 levels (1006% and 450%, respectively) in fetal liver membrane fractions compared to postnatal samples (Figure 28 and 29B), suggesting increased levels of Jak proteins in fetal membranes. However, given our results from the T/FL hGHR studies (Figure 23), we decided that it was critical to dissect the intact fetal liver into its two major cell components, hepatocytes and hematopoietic cells prior to continuing our Jak/Stat analyses. Subsequent studies were limited to whole cell extracts of isolated hepatocytes, due to the large amounts of protein required for cytosolic and membrane fractionation and the limited availability of hepatocyte samples.

**Figure 28. Expression of Jak2 protein in cytosolic vs. membrane fractions of human fetal (n=2; 18 and 19.5 wks FA) and postnatal (n=2; 37 and 62 yr) liver.**

A) Representative Jak2 immunoblots of fetal and postnatal liver cytosolic and membrane protein extracts. SDS-PAGE Molecular Weight Standard Broad Band markers (Bio-Rad Laboratories Inc.) were used. Molecular weights of Jak2 and calnexin are indicated on the left.

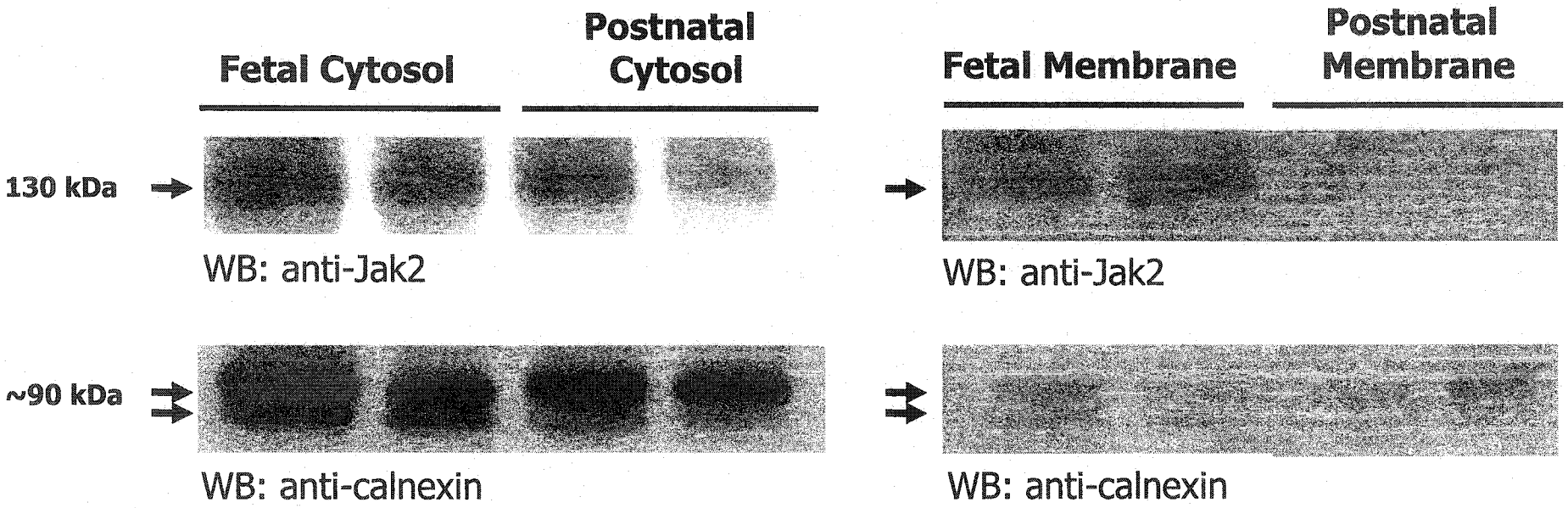
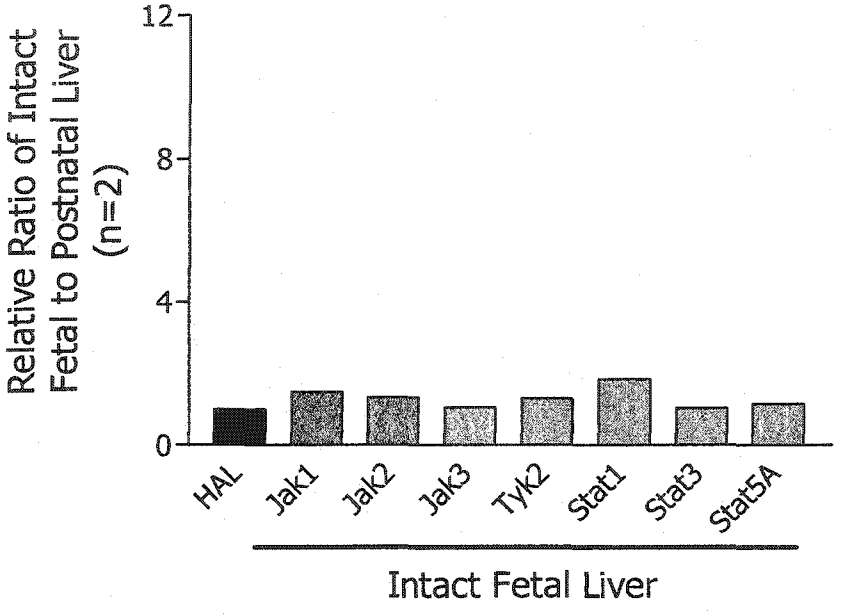

Fetal Cytosol
Postnatal Cytosol
Fetal Membrane
Postnatal Membrane
130 kDa
WB: anti-Jak2
WB: anti-Jak2
~90 kDa
WB: anti-calnexin
WB: anti-calnexin

**Figure 29. Comparative data of Jak/Stat proteins in cytosolic and membrane fractions in fetal (n=2; 18 and 19.5 wks FA) vs. postnatal (n=2; 37 and 62 yr) liver.** A) Analysis of cytosolic fractions of human liver. Abundance of Jak/Stat proteins is expressed as a ratio of Jak/Stat to calnexin. No data are available for Stat5B due to technical difficulties with the Stat5B antibody at the time. B) Analysis of membrane fractions of human liver. The absence of data for Jak3, Tyk2, Stat5A and Stat5B was due to technical difficulties with the antibodies at the time. Statistical differences are expressed relative to human adult liver (HAL).

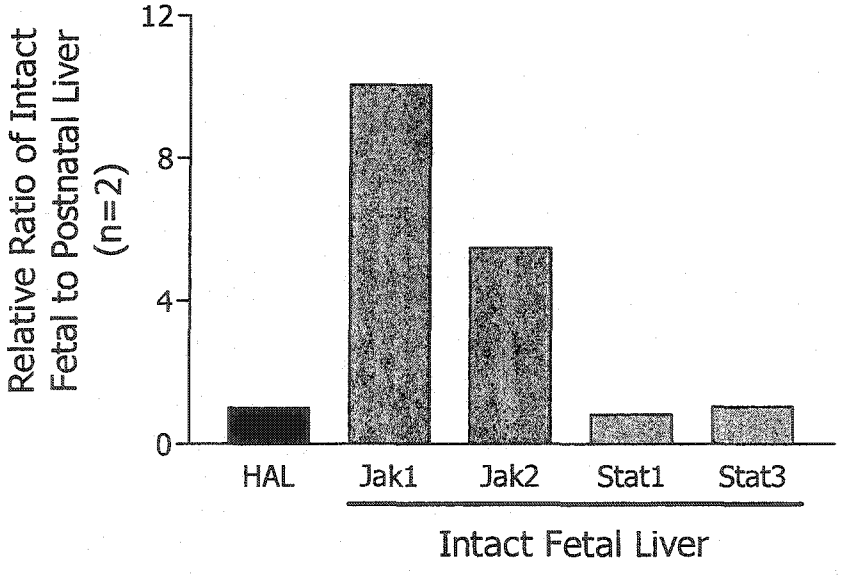
**A**

## Abundance of Jak/Stat proteins in Cytosolic Fractions of Intact Human Liver




**B**

## Abundance of Jak/Stat proteins in Membrane Fractions of Intact Human Liver



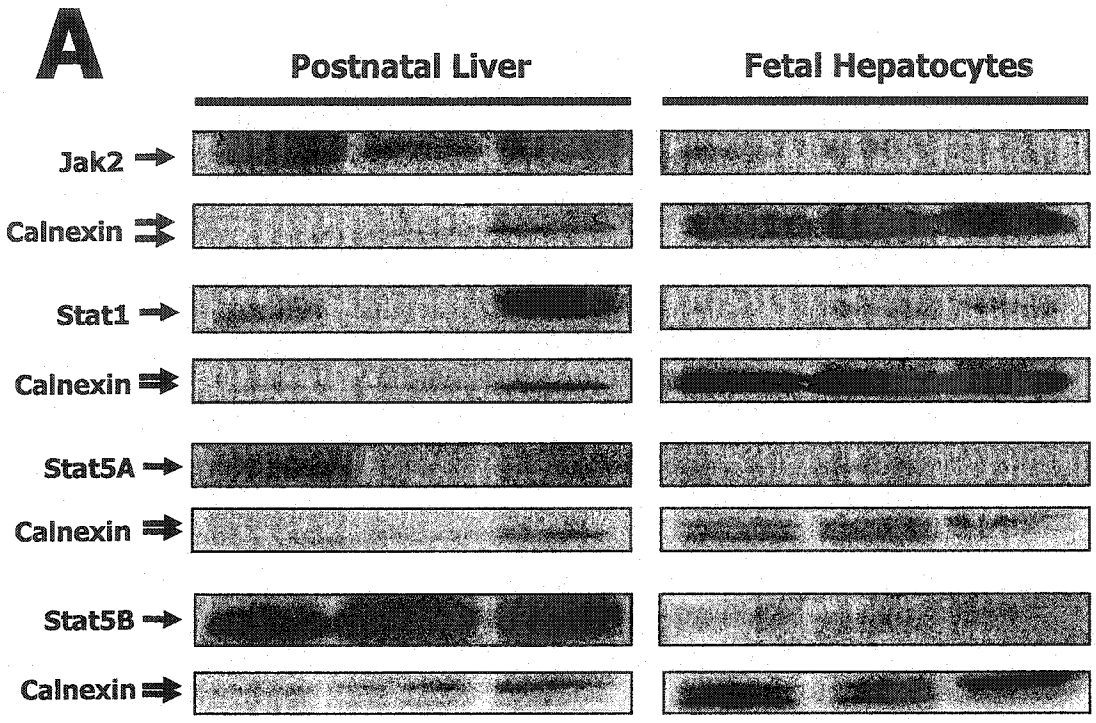
## 5.4 Expression of Jak/Stat proteins in human fetal hepatocytes vs. postnatal liver

Stimulatory Jak (Jak2) and Stat (Stat1, Stat3, Stat5A and Stat5B) proteins were detected in whole cell extracts of all human fetal hepatocytes (n=3; 12.7-17.5 wks FA) and postnatal livers (n=3; 37-62 yr) tested (Figure 30A). Interestingly, normalized Jak/Stat samples showed a general decrease in fetal hepatocytes compared to postnatal liver, ranging from 5-22% of postnatal liver. Those proteins best known to be involved in hGHR signalling demonstrated significantly lower levels in fetal hepatocytes: Jak2 (22% of postnatal liver, $^{**}p<0.002$), Stat5A (11%, $^{*}p<0.03$) and Stat5B (5%, $^{*}p<0.02$) (Figure 30B). Thus, both human fetal and postnatal hepatocytes contain the Jak/Stat proteins known to be involved in hGHR signal transduction from as early as 12.7 wks FA. The significantly lower levels of Jak2, Stat5A and Stat5B, in particular, may be responsible for the fetal-specific hepatocyte responses detected in fetal vs. postnatal liver cells.

## 5.5 Expression of Socs proteins in human fetal hepatocytes vs. postnatal liver

To assess whether the relative abundance of negative regulatory molecules in the GHR signalling pathway might be responsible for the differential fetal biological responses, we determined the levels of inhibitory Socs proteins in human fetal hepatocytes (n=3; 18-19 wks FA) and postnatal liver (n=3; 37-62 yr). Using SDS-PAGE and WB of whole cell extracts, we detected Socs1, Socs2, Socs3 and Cis at all stages of development (Figure 31A). Relatively similar levels of Socs1 were detected in fetal hepatocytes (119% of postnatal liver), whereas Socs2 demonstrated a marked decrease (51%). Interestingly, a significant two-fold increase in the levels of both SOCS3 (239%

**Figure 30. Relative abundance of Jak/Stat proteins in fetal hepatocytes (n=3; 12.6-17.5 wks FA) and postnatal liver (n=3; 37-62 yr).** A) Immunoblots of Jak2, Stat1, Stat5A and Stat5B. Membranes were stripped and reprobed with calnexin, which was used as a loading control. B) Abundance of Jak/Stat proteins is expressed as a ratio of Jak/Stat to calnexin. Statistical differences are expressed relative to human adult liver (HAL).


A
Postnatal Liver
Fetal Hepatocytes
Jak2
Calnexin
Stat1
Calnexin
Stat5A
Calnexin
Stat5B
Calnexin

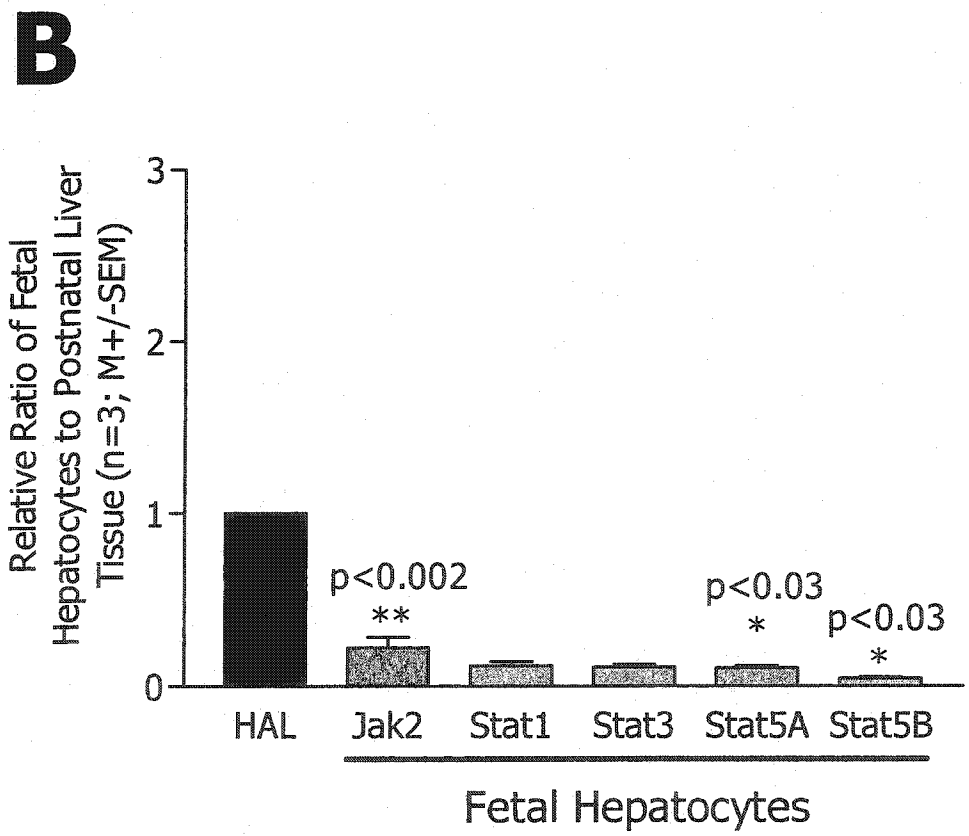
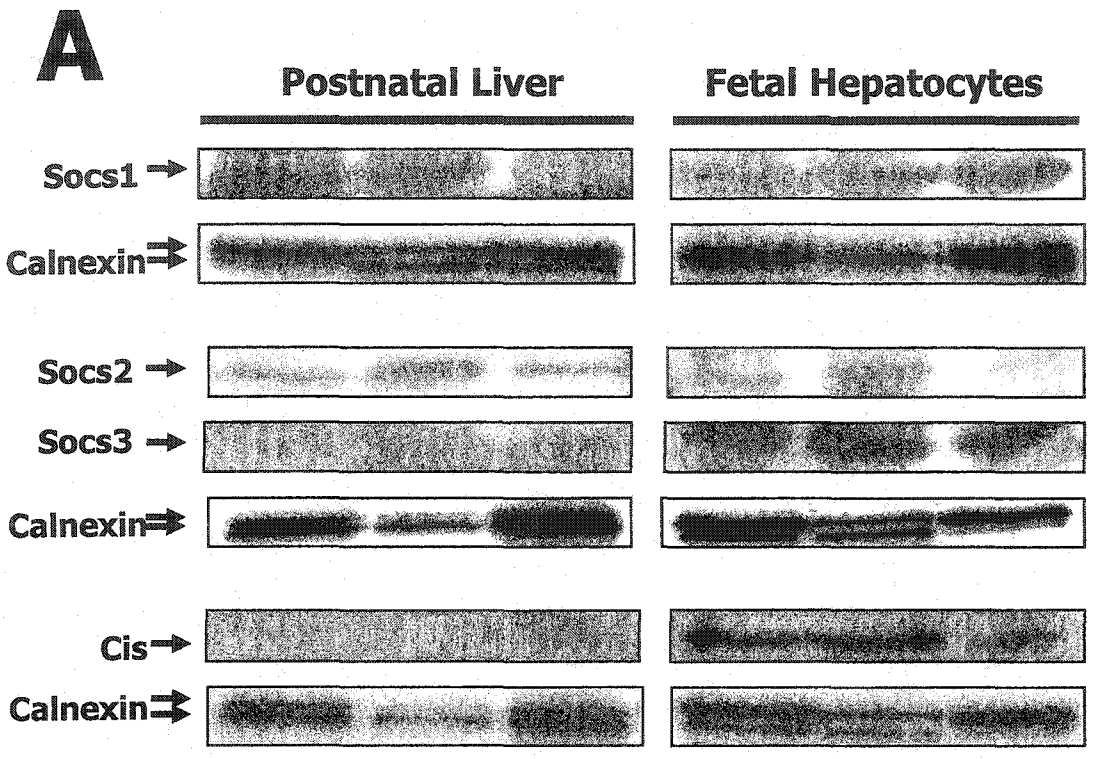
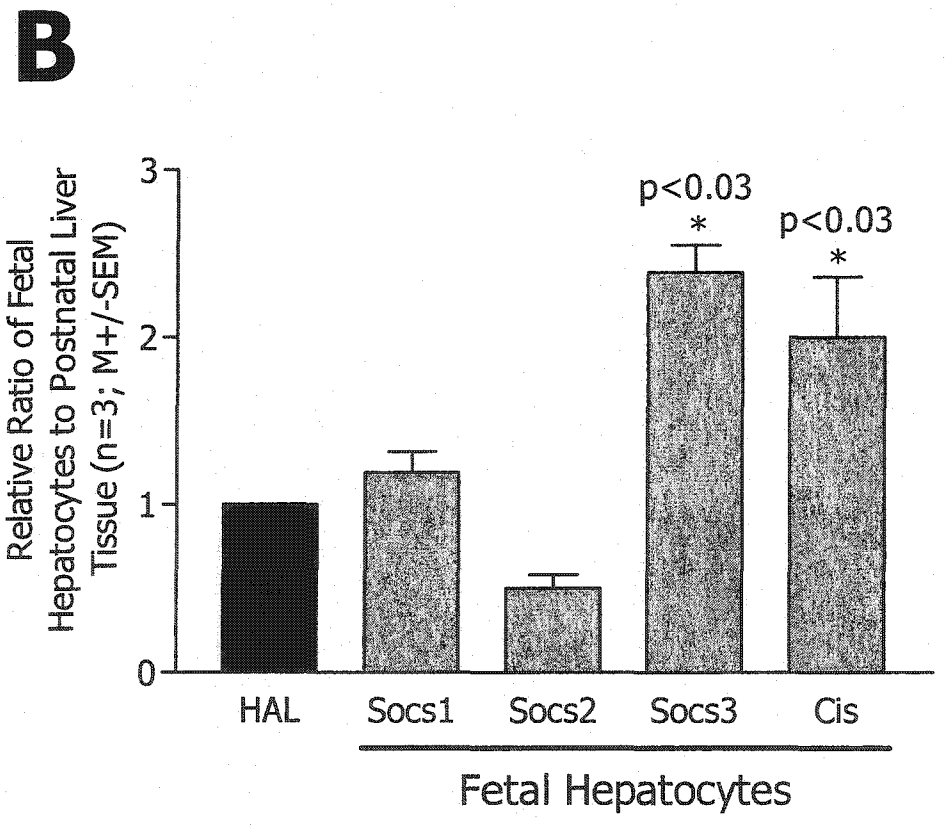

B
Relative Ratio of Fetal Hepatocytes to Postnatal Liver Tissue (n=3; M+/-SEM)
3
2
1
0
p<0.002
**
p<0.03
*
p<0.03
*
HAL
Jak2
Stat1
Stat3
Stat5A
Stat5B
Fetal Hepatocytes

**Figure 31. Relative abundance of Socs proteins in fetal hepatocytes (n=3; 18-19 wks FA) and postnatal liver (n=3; 37-62 yr).** A) Immunoblots of Socs1, Socs2, Socs3 and Cis. Membranes were stripped and reprobed with calnexin, which was used as a loading control. B) Abundance of Socs proteins is expressed as a ratio of Socs to calnexin. Statistical differences are expressed relative to human adult liver (HAL).


A
Postnatal Liver
Fetal Hepatocytes
Socs1
Calnexin
Socs2
Socs3
Calnexin
Cis
Calnexin


B
Relative Ratio of Fetal Hepatocytes to Postnatal Liver Tissue (n=3; M+/-SEM)
3
2
1
0
p<0.03
*
p<0.03
*
HAL
Socs1
Socs2
Socs3
Cis
Fetal Hepatocytes

of postnatal liver, **p<0.03) and CIS (200%, **p<0.03) were seen in fetal hepatocytes (Figure 31B). Human fetal hepatocytes, therefore, show significantly higher levels of at least two negative regulatory Socs proteins, Socs-3 and Cis, suggesting that they may play an important role in the fetal-specific responses to hGH.

# 6. DISCUSSION

hGH acts by binding to its high-affinity receptor on target cells. The hGHR is a 620 amino acid single-chain polypeptide which dimerizes with a second hGHR in the presence of one molecule of hGH [186] [113]. Three hGHR isoforms have been reported to date. Two truncated hGHR mRNAs, 1-277 and 1-279, are produced at low levels in all human tissues examined to date [194] [202] [present data]. These truncated receptors are capable of binding hGH with high affinity but do not dimerize or activate intracellular signal transduction pathways and, in fact, act as dominant negative receptors [188]. Exon 3-deleted hGHR mRNA has also been detected in fetal tissues from as early as the $8^{th}$ week of gestation [191] [204]. This transcript is expressed in an individual- rather than tissue-specific manner, and translates into a high affinity hGHR that binds specific hormones with similar affinities as the full-length receptor [190] [191] [192]; further functional studies of this truncated receptor have not been carried out.

Formation of the 1:2 hGH/hGHR complex at the cell surface results in receptor stimulation followed by a cascade of phosphorylation of intracellular proteins. Tyrosine phosphorylation of the non-receptor tyrosine kinase Jak2 is the first step leading to activation of multiple pathways, including the phosphorylation, dimerization and nuclear translocation of activated Stats which bind to specific DNA-response elements to activate gene transcription [205]. hGHR signalling is negatively regulated by members of a family known as suppressors of cytokine signalling (Socs) as well as phosphatases (SHP-1) and ubiquitination followed by internalization [147].

Although pivotal roles for GH, GHR and its downstream signal transduction pathways have been well established in postnatal tissues, their function during human

gestation remains unclear [206]. Fetal serum levels are high in both primates and subprimates [207]. However, while numerous studies have shown the onset of significant GHR mRNA expression only around the time of birth in subprimates [208] [209] [210], ubiquitous transcription of the hGHR gene has been documented from as early as the first trimester in the human fetus [43] [50] [191]. In addition, immunohistochemical [193] and immunoblotting experiments [present study] have identified hGHR protein in human tissues from as early as 13 wks FA. These fetal hGHRs appear to have immature or adaptive biological responses to chronic exposure to hGH [194].

In the present study, we have addressed the question of what may be responsible for these fetal-specific biological responses, by beginning a detailed comparison of the hGHR structure and its downstream signalling pathways in fetal hepatocytes vs. postnatal liver. While initial studies were performed with intact fetal liver, it was decided to dissect the intact fetal liver into its two major cell components, hepatocytes and hematopoietic cells, since the fetal liver is the primary hematopoietic organ during this stage of development. Hematopoietic cells contain a number of cytokine receptors, including the GHR, making interpretation of intact liver analyses very difficult. This isolation permitted us to make a more accurate comparison of fetal to postnatal liver: the postnatal liver tissues used for these studies had been perfused for transplantation and, therefore, also did not contain blood cells.

## 6.1 Analysis of GHR Protein Structure

Previous affinity cross-linking studies detected the fetal hGHR at ~112 kDa compared to the postnatal hGHR at ~95 kDa [194], suggesting either a larger receptor or the presence of additional molecules bound to the GH/GHR complex in fetal liver. Subsequent mRNA sequencing [202] and now immunoprecipitation/ immunoblotting experiments indicate no major size differences between the fetal and postnatal hepatic hGHR: both the immature and mature forms of the fetal hGHR in hepatocytes have the same SDS-PAGE migration pattern as the postnatal hGHR (Figure 22).

Based on its amino acid sequence, the GHR is predicted to be approximately 70 kDa in size but, when resolved on SDS-PAGE gels under reducing conditions, the mature GHR migrates at 100-140 kDa. This difference is due primarily to the presence of five potential asparagine-linked glycosylation sites within the extracellular domain, several which are glycosylated [56]. In addition, GHR can be modified by ubiquitination, a step that may target the receptor for internalization and degradation [61] [62]. Our IP/WB detection of the hGHR at similar molecular masses in fetal hepatocytes and postnatal liver suggests that the differences previously found were not due to these post-translational mechanisms. Instead, additional molecules non-covalently bound to the fetal GH-GHR complex (e.g. Socs, Grb10, SHP-1) may account for the shift in molecular weight. Exactly which molecule(s) is present remains to be determined, as well as the relative glycosylated and ubiquitinated state of the fetal vs. postnatal hepatic GHR.

It should also be noted that the previous cross-linking experiments were performed with intact fetal liver, which contains a large number of hematopoietic cells. The band detected at ~112 kDa may therefore be explained by the presence of a large

population of hematopoietic hGHR, which may be more heavily glycosylated than the postnatal form. Future experiments need to examine the molecular size of the hepatic hematopoietic hGHR by IP/WB.

It will be necessary, in parallel, to confirm the molecular size of the fetal hepatocyte GH/GHR complex, using a combination of affinity cross-linking and IP/WB techniques. If a larger molecular weight species is still observed with isolated fetal hepatocytes, it will then be important to assess which non-covalently linked molecules are present in this complex, by immunoblotting with antibodies against various intracellular proteins known to bind the GHR and inhibit its activity (e.g. Socs, SHP-1, Grb-10). In addition, while it has been shown that Jak2 constitutively interacts with the GHR in postnatal liver [83], this has yet to be studied in fetal hepatocytes. It is possible that the low levels of Jak2 in fetal hepatocytes result in a decreased association of Jak2 with the GHR. If so, activation of these fetal GHRs may be a much less efficient process, providing one further explanation for the immature responses observed during fetal life.

Similar low T/FL hGHR mRNA ratios were detected in fetal hepatocytes and postnatal liver (Figure 23). It is therefore unlikely that the presence of the dominant-negative hGHR isoform plays a role in the fetal hepatocyte immature responses. However, it is interesting that we observed relatively high T/FL hGHR mRNA ratios in hepatic hematopoietic cells, certain fetal tissues and primate cell lines (Figures 23, 24 and 25, respectively). Ross *et al.* previously demonstrated that a T/FL ratio of 1:10 resulted in a 10-30% inhibition of FL GHR activity, a 1:1 T/FL ratio gave a 40-80% inhibition and a 10:1 ratio completely blocked activity of the FL GHR [188]. Certain fetal tissues that we studied (e.g. kidney) as well as CV1 cells had a ~1:1 ratio while the hepatoma

cells had ratios of ~2:1 to 3:1. Thus, the high levels of T (1-279) hGHR mRNA that we found in some fetal tissues and cell lines may play a functionally significant role.

## 6.2 Analysis of GHR Signalling Pathways

Our analysis of the GHR downstream signalling factors suggests that developmental difference in these pathways may explain, at least in part, the fetal-specific immature/adaptive responses to GH: fetal hepatocytes contain significantly lower levels of the stimulatory Jak/Stat proteins and increased levels of the inhibitory Socs3 and Cis compared to postnatal liver.

The decreased levels of Jak2, Stat5A and Stat5B could be due to levels of gene expression and/or insensitivity to GHR signalling. Although fetal hepatocytes contain significant levels of inhibitory Socs proteins, the increased amounts do not account for the relatively low levels of Jak/Stat molecules: Socs3 inhibits the kinase activity of Jak2 and Cis prevents the recruitment of Stats to the receptor, but overall cytoplasmic levels Jak/Stat proteins are unaffected by Socs [147].

*In vitro* studies have demonstrated a strong correlation between GH binding and GH biological effects [211], suggesting that any reduction in GH binding in liver may result in decreased GH intracellular action [212]. Thus, the lower levels of GH binding that we detected in fetal liver [194], coincident with the relatively low levels of Jak/Stat proteins in fetal hepatocytes, may simply limit the ability of GH to activate the Jak/Stat pathway. However, since our results represent only the total amount of cellular Jak or Stat protein, it will be critical in the future to compare the relative phosphorylation of these Jak/Stat molecules following GH treatment in fetal hepatocytes and postnatal liver.

This has been shown to be important in studies of GH resistance in fasting [212] and endotoxin-induced sepsis [213]. In both cases, there was a marked decrease in the GH-stimulated tyrosine phosphorylation of GHR, Jak2 and Stat5 and an up-regulation of Socs3 mRNA with no change in GHR/Jak2/Stat5 total protein content.

Our immunoblotting experiments of fetal hepatocytes and postnatal liver whole cell extracts showed significantly higher levels of Socs3 and Cis, but not Socs1 and Socs2, in fetal cells. Socs3 binds to the hGHR to indirectly inhibit Jak2 kinase activity, whereas Cis binds the receptor to prevent the recruitment of Stat molecules to the activated complex. The different mechanistic actions of these two Socs proteins may function to ensure efficient inhibition at two different levels of receptor signalling.

Socs1 was present at relatively similar levels at both stages of development. Although Socs1 has been implicated in the regulation of GHR signalling [147], studies have suggested a more prominent role for Socs1 in the negative regulation of signalling by IFN-γ, as neonatal defects exhibited by *socs1* -/- mice appear to occur primarily as a result of uncontrolled IFN-γ signalling [157] [158]. Interestingly, the *socs1* gene promoter has been reported to contain binding sites for only Stat1, Stat3 and Stat6 [147]. In contrast, the promoter of *socs3* gene contains a Stat1/Stat3 binding element that is capable of binding Stat5 and the Cis gene promoter also contains Stat5 binding sites. Because all of our studies were carried out in the liver, where Stat5 is the predominant Stat activated in the GHR signalling pathway, it is possible that Socs1 may play a more important role in the negative regulation of GHR signalling in other tissues.

Regulation of the *socs2* gene promoter is less defined. However, the relatively low levels of Socs2 we detected in fetal hepatocytes vs postnatal liver may also be

modulating hGHR signalling in a negative manner. Tomic *et al.* previously reported that, unlike Socs1 and Socs3, Socs2 had no inhibitory effects on tyrosine phosphorylation of the PRLR and was unable to abolish the ability of the PRLR to induce β-casein gene promoter activity [214]. In GHR signalling, however, Socs2 seems to have dose-dependent opposite effects: at low concentrations it inhibits GH-induced Stat5-dependent gene transcription, but restoration of GH signalling was observed at higher concentrations [215]. At high concentrations, Socs2 is able block the inhibitory effect of Socs1 but not that of Socs3 on GH signalling [215], suggesting that Socs2 functions by restoring the sensitivity to GH by overcoming the initial inhibitory effects of other endogenous Socs molecules. It may well be that the lower levels of Socs2 in fetal cells are also be acting as an inhibitor of GHR signalling.

*Socs3* is a major transcriptional target of GH action. Studies have shown that GH rapidly induces strong expression of *Socs3* and to a lesser extent *Socs1*, *Socs2* and *Cis* in murine liver [151] [152]. This preferential induction of *Socs3* mRNA by GH is mediated by Stat5b [150]. Paradoxically, several models of GH "resistance" have demonstrated a different scenario: fasting [212] and endotoxin-induced sepsis [213] show low levels of tyrosine phosphorylated GHR, Jak2 and Stat5 and increased Socs3 mRNA levels. Our results also demonstrate low levels of Jak2 and Stat5 protein coincident with high levels of Socs3 and Cis. These data suggest that, under certain conditions (i.e. fasting, sepsis, *in utero* environment), an alternate activator of *Socs3* gene expression is involved. While the mechanisms by which *Socs3* is induced under these conditions remains to be determined, studies have demonstrated that hormones such as leptin and insulin may play a role [216] [217].

While increased levels of Socs3 and Cis in fetal hepatocytes most likely act to inhibit signalling, it is also possible that the relatively high levels of Socs3 play a role in regulating fetal erythropoiesis. Transgenic mice lacking Socs3 demonstrate marked erythrocytosis [160], suggesting a critical role for Socs3 in the regulation of fetal liver erythropoiesis. Indeed, as the fetal liver is the major hematopoietic organ during fetal life, Socs3 may be present in fetal hepatocytes to regulate erythrocytosis in surrounding progenitor blood cells.

Studies have also shown that degradation of activated signalling proteins by the proteosome may be a consequence of SOCS proteins, which are capable of targeting proteins for ubiquitination [161] [162]. Therefore, the significant levels of Socs proteins detected in fetal hepatocytes vs postnatal liver may reflect a greater incidence of GHR ubiquitination and turnover. This, in turn, may provide at least one explanation for the immature/adaptive responses to hGH found during fetal life. Studies to assess the ubiquitinated state of GHR in fetal vs. postnatal liver should begin to help resolve this question.

Gonzalez *et al.* [218] recently investigated the effects of high and continuous levels of homologous GH on GH signalling in GHRH transgenic mice. The involvement of certain Socs proteins in the desensitization of GH signalling caused by such pattern of GH secretion was also determined. Interestingly, Cis was shown to be associated with the inhibition of GH-stimulated tyrosine phosphorylation of GHR, Jak2, Stat5a and Stat5b, leading to a desensitization of the GH signalling pathway. Although this may serve as an appropriate model for our studies, since plasma levels of immunoreactive GH are also chronically elevated in the fetus, the relative abundance of active, phosphorylated

hGHR/Jaks/Stats in fetal vs. postnatal hepatocytes following 22 KDa hGH treatment has yet to be examined. In addition, high levels of hGH in the fetal circulation are correlated with low levels of IGF-I and IGF-II whereas, postnatally, increased amounts of IGF-I are detected. Thus, gene transcription of GH-regulated genes, including IGF-I, upon GH treatment needs to be assessed. It will also be important to compare the relative activity of alternative ligands for the hGHR (20 KDa hGH, hPL) that are present during fetal life.

In conclusion, our studies to date demonstrate that there may be significant fetal-specific hepatocyte differences in the hGH/hGHR complex and its downstream signal transduction pathways. The relative abundance of T/FL hGHR in fetal hepatocytes is not likely to be responsible for the immature or adaptive fetal biological responses to 22 kDa GH. However, the significantly lower levels of stimulatory Jak2, Stat5A and Stat5B in fetal hepatocytes, coincident with the higher levels of inhibitory Socs3 and Cis, may well play an important role in the fetal-specific biological responses to hGH.

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# 8. APPENDIX

Goodyer CG, Figueiredo RMO, Krackovich S, De Souza Li L, **Manalo JA** and Zogopoulos G. 2001. **Characterisation of the Growth Hormone Receptor in Human Dermal Fibroblasts and Liver during Development.** American Journal of Physiology: Endocrinology and Metabolism. 281: E1213-E1220.

**Manalo JA** and Goodyer CG. **Developmental Changes in the Human Growth Hormone Receptor and its Signal Transduction Pathways.** June 2002, San Francisco, CA. Endocrine Society's 84th Annual Meeting, Abstract #P3-278.

**Manalo JA** and Goodyer CG. **Changes in the Human Growth Hormone (hGH) Receptor and hGH Signal Transduction Pathways during Development.** July 2001, Montreal, QC. Pediatric Endocrinology Montreal 2001 LWPES/ESPE 6th Joint Meeting, Abstract #P2-190.

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# Characterization of the growth hormone receptor in human dermal fibroblasts and liver during development

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**Goodyer, Cynthia G., Rilene M. O. Figueiredo, Stephanie Krackovitch, Lilia De Souza Li, Jennifer A. Manalo, and George Zogopoulos.** Characterization of the growth hormone receptor in human dermal fibroblasts and liver during development. *Am J Physiol Endocrinol Metab* 281: E1213–E1220, 2001.—Human tissues express growth hormone receptors (hGHR) by the 3rd mo of gestation. We assessed developmental changes in hGHR function in fibroblasts and liver, testing binding and hormonal response. Fetal cells showed low but reproducible hGH binding. No age-related changes occurred in fibroblasts (9 wk–34 yr). In contrast, there was a fourfold increase in hGH binding in postnatal liver, with a sixfold increase in hGHR mRNA. Both full-length and truncated hGHR mRNAs were detected in all livers. Cross-linking revealed a larger hGH/receptor complex in fetal liver. Fetal hepatocytes produced 10 times more insulin-like growth factor (IGF)-II than IGF-I, and responded to hGH (150 ng/ml) with a significant increase in IGF-II. Fetal hepatocytes secreted three IGF-binding proteins (IGFBPs), including IGFBP1, but not IGFBP3. hGH did not alter fetal hepatocyte IGFBPs but stimulated glucose uptake. Exposure of fibroblasts to hGH decreased hGH binding only in >1-yr postnatal fibroblasts, whereas treatment with dexamethasone (100–400 nM) increased binding only in postnatal cells. Thus, although fetal hepatocytes and fibroblasts possess functional hGHR, these receptors (and/or their signaling pathways) are immature or have adapted to the in utero environment.

fetal; postnatal; insulin-like growth factors; insulin-like growth factor-binding proteins

GROWTH HORMONE (GH) is required for normal postnatal growth, having a critical role in bone growth as well as important regulatory effects on protein, carbohydrate, and lipid metabolism (49). The actions of human (h)GH are primarily through the stimulation of insulin-like growth factor (IGF-I and IGF-II) production in target tissues. Although synthesis of IGF-I has been demonstrated to be hGH- and nutritionally regulated, IGF-II is thought to be more of a constitutive (and fetal) growth factor (6, 10, 45). The IGFs circulate bound to specific IGF binding proteins (IGFBPs) (32) and work in an autocrine, paracrine, or endocrine fashion by binding to specific receptors (39).

In contrast, the role of hGH during fetal development is not clearly defined. Plasma levels of immunoreactive hGH are markedly elevated in the fetus relative to the normal adult, up to 150 ng/ml at midgestation (34). We (19, 54, 66, 67) and others (26, 62) have identified hGH receptors (hGHR) at the mRNA and peptide level in fetal tissues as early as the first trimester. hGH stimulated proliferation and IGF production in cultured fetal hepatocytes but had a variable effect on insulin and IGF production by islets and no effect on fetal myoblasts, fibroblasts, or cartilage explants (13, 24, 46, 56, 57). Despite inconsistencies in the functional studies, the data indicate that hGH may have important functions during differentiation of specific fetal tissues.

Clinical studies also suggest that hGH begins to have an influence before birth. The median birth length of a large cohort of idiopathic hGH-deficient children was found to be 0.83 below that of controls, and 21% were >2 standard deviation scores (SDS) shorter than normal newborns (16). In addition, certain infants with hGHR defects are shorter than normal controls at term (−1.59 SDS), and their clinical phenotypes indicate that the hGHR is critical for normal maturation of specific fetal tissues such as skin, liver, kidney, gonads, and muscle (3, 50, 52).

To examine this question further, we have characterized several functional aspects of the hGHR in human dermal fibroblasts and liver during development, including hGH binding and biological responsiveness to regulatory hormones (hGH, dexamethasone). These tissues were chosen because of their relative availability at both fetal and postnatal stages in development. Our investigations confirm the presence of functional hGHR in fetal fibroblasts and hepatocytes but suggest that the fetal receptors (and/or their activated pathways) are still in an immature form or that they have adapted to the fetal milieu.

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## MATERIALS AND METHODS

*Tissues.* Human fetal tissues were obtained at the time of therapeutic abortion [9–20 wk of fetal age (FA)]; FA was determined by foot length (43). Specimens from premature newborns (23–33 wk FA) and postnatal donors (2 mo–64 yr) were obtained within 6–10 h after death. The three premature newborns died within 1–3 days after birth due to a variety of pathologies (oligohydramnios, diaphragmatic hernia, respiratory distress syndrome). Postnatal liver samples were obtained from adult transplant donors who had died of acute head injuries ($n$ = 5). Postnatal skin samples were obtained from pediatric patients at the time of surgery (inguinal or abdominal hernia) or from our local Genetics Cell Bank collection. Skin samples were placed in explant culture, and liver tissues were flash frozen in a dry ice-acetone bath and stored at −70°C. Rat livers were from adult male Sprague-Dawley rats (Charles River, Montreal, QC, Canada). Ethical approval for the study was obtained from local institutional research ethics and animal care committees.

*Cultured cells.* Dermal fibroblasts from fetal and postnatal donors were grown as monolayer cultures in Ham's F-10-DMEM (1:1; Life Technologies, Gaithersburg, MD) with 10% fetal/newborn calf serum (1:1; Hyclone, Logan, UT), 100 U/ml of penicillin G (Marsam Canada Pharmaceutical, Montreal, QC, Canada), 16 μg/ml of gentamycin sulfate (Schering Canada, Pointe Claire, QC, Canada), and 5 μg/ml of amphotericin B (Squibb Canada, Montreal, QC, Canada). Fibroblasts from the 10th–20th passages were used for experiments. The monolayers were refed every 3 days (until 80% confluence was achieved) as well as 24 h before being tested. IM-9 cells were grown in suspension in RPMI-1640 medium (Life Technologies) with 10% fetal/newborn calf serum (1:1) and antibiotics and tested while in the exponential phase of growth, 24–48 h after refeeding. Cells used for hormonal experiments were preincubated overnight in Ham's-DMEM with 0.1% BSA (Sigma, no. 4378; Missassauga, ON, Canada). Triplicate cultures were treated with recombinant 22-kDa hGH (5–150 ng/ml) (Eli Lilly, Indianapolis, IN) or dexamethasone (100–400 nM; Sabex International, Montreal, QC, Canada) in BSA-medium for 48 h.

Hepatic tissues were minced and dispersed by collagenase, followed by gravity separation, yielding >95% pure hepatocytes (assessed by cytokeratin, albumin, and α-fetoprotein staining), as described previously (67). Cells were plated at ~80% confluence on collagen-coated P100 (IGF/IGFBP studies) or 6-well (glucose uptake studies) dishes in William's E medium (Life Technologies) with 10% FBS, antibiotics, and 2 μM hydrocortisone (Upjohn Canada, Mississauga, ON, Canada). After 24 h, triplicate dishes or wells of hepatocytes were treated with hGH (50–150 ng/ml) for 72 h. The primary cultures were changed to serum-free medium for the final 24 h in vitro for analyses of α-fetoprotein, IGFs, and IGFBPs.

*Microsomal membrane preparations.* Human and rat livers were homogenized (1:5, wt/vol) on ice in 0.3 M sucrose containing 0.7 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF; ICN, Mississauga, ON, Canada), filtered through mesh cloth, and centrifuged at 1,500 $g$ for 10 min at 4°C. The resultant supernatants were spun first at 15,000 $g$ for 20 min at 4°C, followed by 30 min at 400,000 $g$ at 4°C. The 400,000-$g$ pellets were resuspended in 3 mM $MgCl_2$, incubated for 15 min on ice to remove endogenously bound hGH, and then recentrifuged at 400,000 $g$ for 30 min, resuspended in 25 mM Tris·HCl and 10 mM $MgCl_2$, and respun at 400,000 $g$ for 30 min at 4°C. Final pellets were resuspended in Tris binding buffer and frozen at −70°C. IM-9 cells were sonicated on ice in 25 mM Tris·HCl, 10 mM $MgCl_2$, and 0.7 mM AEBSF and spun at 15,000 $g$ for 25 min, followed by centrifugation at 400,000 $g$ for 30 min. Pellets were treated with $MgCl_2$, rinsed twice, and frozen at −70°C.

*Radioreceptor assays.* hGH (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) and IGF-II (Eli Lilly) were labeled with $^{125}I$ by the chloramine-T method (specific activity: 42–145 μCi/μg), as previously described (18) and used only if >90% trichloroacetic acid precipitable.

Fibroblast monolayers were washed four times (15 min, 37°C, 5% $CO_2$) with 5 ml of binding buffer (DMEM, 25 mM HEPES, 25 mM Tris·HCl, pH 7.4, and 0.1% BSA). A total of 15,000 cpm/ml (0.1 ng/ml) of iodinated peptide with or without excess unlabeled hGH (22 kDa, Eli Lilly) was added to each dish and incubated for 2 h at 30°C. The reaction was terminated by washing the monolayers three times with 4 ml of ice-cold PBS-0.1% BSA. The fibroblasts were solubilized with 0.6 N NaOH for 6–12 h, and aliquots were analyzed for radioactivity and protein. All assays were performed in quadruplicate. Binding assays with IM-9 cells were run in parallel with the use of triplicate tubes of 750 μg (protein) of cells in suspension per 0.5 ml of final volume. The reaction was terminated by adding ice-cold PBS-0.1% crude BSA and centrifuging at 3,000 rpm for 30 min. Viability of the fibroblasts and IM-9 cells was monitored using light microscopy as well as the trypan blue exclusion test.

Radioreceptor assays with liver or IM-9 microsomal membranes were carried out in triplicate for 18–22 h at 4°C. A total of 100 μg/ml of IM-9 membranes or 200 μg/ml of liver membranes was incubated in 150 μl of binding buffer [25 mM Tris·HCl, pH 7.6, 10 mM $MgCl_2$, 0.1% BSA, 1 μM aprotonin, 0.7 mM AEBSF, 21 μM leupeptin, 1 μM pepstatin, and 100,000 cpm of $^{125}I$-labeled hGH (0.7 ng)]. Nonspecific binding was determined by addition of excess unlabeled hGH (10 μg/ml). The assay was terminated by adding ice-cold Tris·HCl and $MgCl_2$ buffer with 0.1% BSA and centrifuging at 15,000 $g$ for 30 min at 4°C. Radioreceptor assays with primary hepatocytes were not carried out due to the limited amounts of fetal and adult liver samples available.

*Affinity cross-linking.* Microsomal membranes (200–1,000 μg) were incubated with $0.2–1.0 \times 10^6$ cpm of $^{125}I$-hGH in 0.5 ml of binding buffer for 18–22 h at 4°C with or without excess (10 μg/ml) unlabeled hGH. Binding was terminated by addition of ice-cold Tris·HCl buffer and centrifugation at 400,000 $g$ for 30 min. The pellets were resuspended in PBS containing 0.5 mM disuccinimidyl suberate (Pierce, Rockford, IL) and incubated at room temperature for 15 min. Cross-linking was stopped by addition of 1 mM Tris-0.2 M EDTA and recentrifugation at 400,000 $g$ for 30 min. Pellets were dissolved in sample buffer (0.05 M Tris·HCl, pH 6.8, 10% glycerol, 0.05% bromphenol blue, 10% SDS, and 5% β-mercaptoethanol) and run on 7.5% polyacrylamide gels. The gels were dried, and the receptor complexes were revealed by autoradiography. Liver, but not fibroblast, microsomal membranes gave sufficiently high specific binding for cross-linking results.

*α-Fetoprotein and IGF RIAs.* α-Fetoprotein levels were measured using the Amerlex-M RIA kit (Johnson and Johnson Clinical Diagnostics, Rochester, NY). IGF-I and IGF-II concentrations were determined by DSL RIA kits (Webster, TX) after formic acid-acetone extraction (7).

*IGFBP Western ligand blots.* IGFBP profiles were analyzed according to the method of Hossenlopp et al. (29), with modifications. Conditioned media were concentrated 10-fold and electrophoresed through a 12.5% SDS-polyacrylamide gel, and the proteins were transferred to a nitrocellulose membrane. Blots were probed overnight with $^{125}I$-IGF-II, and the IGFBPs were visualized by autoradiography.

*[$^{14}$C]glucose uptake studies.* Assays were carried out in triplicate on *day 5*, following 72 h with or without 22-kDa hGH treatment. Cultures were incubated in Krebs-Ringer phosphate with 0.1% BSA with or without test factors (150 ng/ml hGH, 300 nM insulin) for 30 min, and then 0.375 μCi of D-[U-$^{14}$C]glucose (25 mCi/mmol; New England Nuclear) was added to each well for 30, 60, or 90 min. Glucose uptake was stopped by rinsing 3 times with ice-cold PBS. One milliliter of 0.6 N NaOH was added to each well, the cells were solubilized overnight at 37°C, and aliquots were analyzed for radioactivity and protein (Bio-Rad, Hercules, CA).

*RNA extractions.* The guanidium-thiocyanate-phenol method (9) was used to extract total RNA from human fetal and postnatal tissues.

*Semiquantitative RT-PCR.* Five micrograms of total RNA were reverse transcribed for 1 h at 48°C in the presence of 2.5 U of AMV-RT (Life Technologies), 80 U of RNAsin (Promega, Madison, WI), 71.4 ng/μl random primers (Life Technologies), 0.48 mM deoxyribonucleotides (dNTPs) (Pharmacia Biotech, Baie D'Urfe, QC, Canada), 10 mM $MgCl_2$, 10 mM dithiothreitol, 100 mM Tris·HCl, pH 8.3, and 50 mM KCl. Two microliters of RT product were amplified for 23 cycles with varying amounts of internal standard ($0.9–48.0 \times 10^{-4}$ fmol), 2.5 U of *Taq* DNA polymerase (Life Technologies), 0.5 mM dNTPs, 0.5 μM hGH receptor sense (exon 7: 5′-CCA GTG TAC TCA TTG AAA GTG GAT-3′) and antisense (exon 10: 5′-GTC TGA TTC CTC AGT CTT TTC ATC-3′) primers, 3 mM $MgCl_2$, 20 mM Tris·HCl, pH 8.4, and 50 mM KCl. The first cycle consisted of 3 min at 92°C, 1 min at 61°C, and 3 min at 72°C; subsequent cycles were 30 s at 92°C, 1 min at 61°C, and 1.5 min at 72°C, terminating with a final elongation of 5 min at 72°C. As a control, RNA samples were incubated in the absence of AMV-RT and then amplified in the presence of $1.4 \times 10^{-3}$ fmol of internal standard. Initial experiments (data not shown) showed that the PCR reaction was in an exponential phase at 23 cycles. The internal PCR standard was constructed according Jin et al. (33), as previously described (66). PCR fragments were electrophoresed through 1.5–2% agarose gels and transferred to 0.45-μm positively charged nylon membranes (Schleicher & Schuell, Keene, NH), as previously described (66, 67). Blots were hybridized overnight using the nested oligonucleotide (exon 10: 5′-GCT AAG ATT GTG TTC ACC TCC TC-3′) end labeled with [γ-$^{32}$P]ATP (NEN), and the bands were quantified using a Fuji phosphorimager (Stamford, CT) to obtain the molecules of hGHR mRNA per microgram of total RNA, as described (66).

*Full-length vs. truncated hGHR mRNA.* Five micrograms of hepatic total RNA were reverse transcribed using Superscript II (Life Technologies) and a specific exon 9 reverse primer (9AS) designed to recognize both the full-length and 1–279 hGHR mRNAs (5′-TAATCTTTGGAACTGGAACT-3′). RT products were amplified using an exon 7 sense (7S) primer (5′-ATAAGGAATATGAAGTGCGTGTGAG-3′) and the exon 9AS primer (see Fig. 2*A*), under the following conditions for 35 cycles: 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, ending with 72°C for 10 min. PCR products were separated on 10% polyacrylamide gels (Fig. 2*B*) and stained with Sybr gold, and densitometric analyses were carried out using the Bio-Rad GelDoc system (Mississauga, ON, Canada).

*Statistical analyses.* Differences between groups (by age or treatment) were analyzed by Student's *t*-test if there were two groups or by ANOVA followed by Duncan's multiple range test if multiple groups.

## RESULTS

*Developmental changes in tissue hGHR mRNA and microsomal membrane binding.* Using semiquantitative RT-PCR assays, we determined total hGHR mRNA levels in four pairs of human fetal and postnatal tissues. Although there was a general decrease in postnatal lung ($P < 0.05$), kidney, and small intestine compared with their fetal counterparts, the hGHR mRNA in postnatal liver increased sixfold ($P < 0.01$; Fig. 1*A*). We also detected mRNAs for both the full-length and the major truncated (GHR279) GHR in fetal ($n = 8$; 14–18 wk) and postnatal ($n = 3$; 21–54 yr) liver tissues. The full-length transcript was always predominant (Fig. 2*B*). Although there was a trend for fetal liver to have a twofold higher ratio of truncated to full-length mRNAs, there was two- to threefold variability within each age group, and the difference did not reach statistical significance (Fig. 2*C*).

The developmental increase in hepatic hGHR mRNA was accompanied by a fourfold ($P < 0.02$) increase in

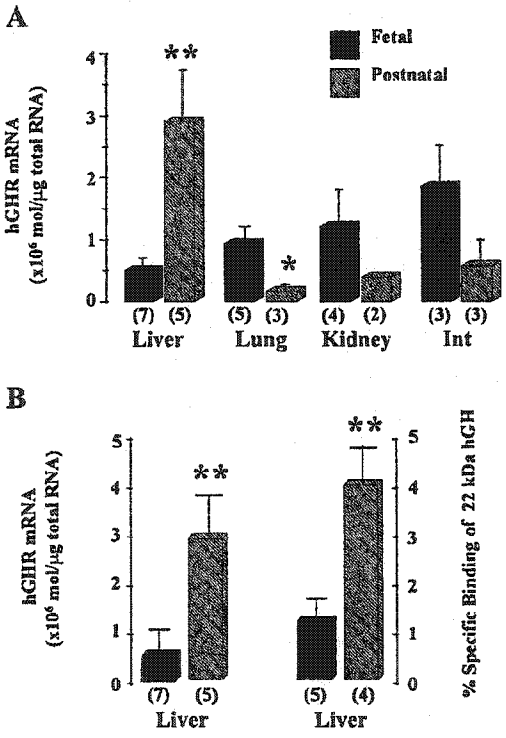


Fig. 1. Tissue- and developmental-specific expression of the human growth hormone receptor (hGHR). Nos. in parentheses indicate nos. of samples. *A*: fetal (11–17 wk) and postnatal (11–84 yr) tissues were analyzed by semiquantitative RT-PCR for total hGHR mRNA. There was a significant (6-fold: $^{**}P < 0.01$) increase in postnatal liver and a general decrease in postnatal lung, kidney, and small intestine (Int) (6-fold in lung: $P < 0.05$). *B*: fetal (14–20 wk) and postnatal (20–42 yr) liver samples were analyzed for specific $^{125}$I-labeled hGH binding. Data were obtained from 1–3 assays per microsomal preparation and represent percent total radioactivity in the binding buffer/200 μg protein (means ± SE). Controls included membranes prepared from adult male rat liver ($n = 10$; 31.0 ± 3.8%) and IM-9 cells ($n = 13$; 11.8 ± 1.6%). There was a significant ($^{*}P < 0.02$) 4-fold increase in postnatal hepatic hGH binding. Thus there are parallel increases in hGHR gene and receptor protein expression in postnatal liver.

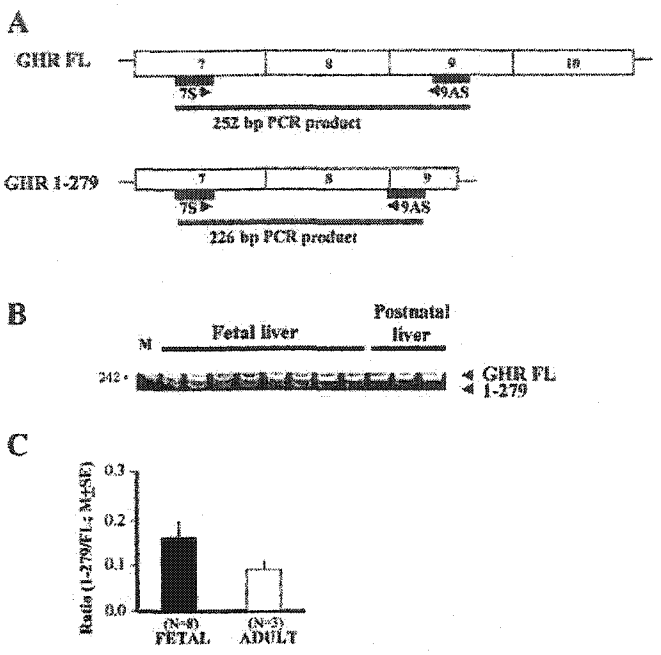


Fig. 2. Comparison of full-length (FL) and truncated (1–279) hGHR mRNAs in fetal vs. postnatal liver. *A*: schema showing the positions of the PCR primers used and sizes of the expected PCR products. 7S, exon 7 sense primer; 9AS, exon 9 antisense primer. *B*: representative gel of the PCR products from 3 adult and 8 fetal liver samples. Bp size of the relevant BS-*Hpa*II marker is indicated on the *left*. Positions of the two hGHR bands are indicated by arrows on the *right*. *C*: cumulative data for fetal vs. postnatal liver: each sample was tested using 3 different RT products, and the final data are expressed as a ratio of truncated to full-length hGHR transcripts [means (M) ± SE].

hGH binding to postnatal hepatic membranes (Fig. 1*B*), suggesting parallel changes in hGHR gene and protein expression. Scatchard analyses were carried out on one fetal and two adult samples (Table 1). The samples were limited but showed that fetal GHR binding capacity was ~50% that of the adult (90 vs. 165 fmol/mg protein), whereas binding affinity was ~10-fold less. Binding to control membranes prepared from rat liver and IM-9 cells was severalfold higher (see details in legend of Fig. 1), as reported previously (20, 31, 40, 64).

*Affinity cross-linking.* Cross-linking experiments showed that $^{125}$I-hGH bound to different molecular mass species of hGHR in human fetal, human adult, or rat adult liver (Fig. 3). In human fetal liver, the $^{125}$I-hGH/hGHR band was ~112 kDa; excess unlabeled 22-kDa hGH decreased, but did not eliminate, $^{125}$I-hGH binding (Fig. 3*A*). In human adult liver, the $^{125}$I-hGH/receptor complex was a broad band at ~80–100 kDa, which disappeared with excess unlabeled hGH (Fig. 3*B*). In rat liver, the hGH tracer was part of an ~65-kDa receptor complex; excess unlabeled hGH completely eliminated $^{125}$I-hGH binding to this band (Fig. 3*C*).

Table 1. *Scatchard analyses of $^{125}$I-labeled hGH binding to microsomal membranes (22-kDa hGH displacement)*

| | |
|---|---|
| Human fetal liver | $K_A$: $0.3\times10^{-9}$ M |
| 18 wk ($n$ = 1) | $B_{max}$: $1.2\times10^{-10}$ M |
| Human adult liver | $K_A$: $2.6\times10^{-9}$ M |
| 40 and 43 yr ($n$ = 2) | $B_{max}$: $2.2\times10^{-10}$ M |
| Controls | |
| Adult rat liver ($n$ = 2) | $K_A$: $2.3\times10^{-9}$ M |
| | $B_{max}$: $6.0\times10^{-10}$ M |
| IM-9 cells ($n$ = 3) | $K_A$: $7.6\times10^{-9}$ M |
| | $B_{max}$: $3.6\times10^{-10}$ M |

$K_A$, association constant; $B_{max}$, maximum binding capacity.

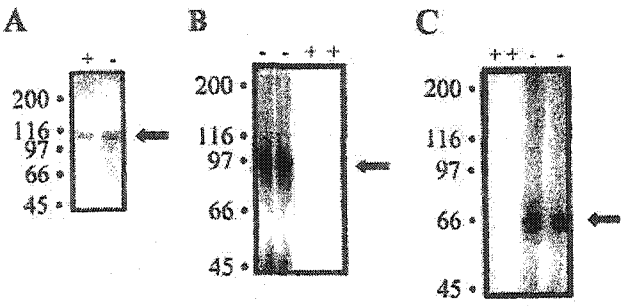


Fig. 3. Representative affinity cross-linking experiments with $^{125}$I-hGH with or without unlabeled 22-kDa hGH and human fetal liver (*A*; pool of $n$ = 3; 15.5–16.5 wk fetal age), human adult liver (*B*; $n$ = 2; 34 and 40 yr), and adult rat liver (*C*; $n$ = 2) microsomal membranes under reducing conditions. Nos. on *left*, molecular mass markers; arrows, radioactive hGH/receptor complexes; + and −, presence or absence of excess hGH (10 μg/ml), respectively.

*Fetal hepatocyte α-fetoprotein, IGF, and IGFBP production and [$^{14}$C]glucose uptake.* Fetal hepatocyte cultures were monitored for α-fetoprotein production on *day 5* in vitro. Levels in the conditioned serum-free media were quite consistent [$n$ = 9; 149.4 ± 23 ng/ml (means ± SE); blank medium = <6 ng/ml]. IGF-I and IGF-II concentrations were also assessed on *day 5*. There was a striking 10-fold greater production of IGF-II than IGF-I ($n$ = 9; 15.3–20 wk; 4.2 ± 0.3 vs. 0.43 ± 0.05 ng/ml). Pretreatment with 150 ng/ml of hGH for 72 h resulted in a small, significant increase in IGF-II (5.2 ± 0.4; $P < 0.04$) but not in IGF-I (0.42 ± 0.05). Western ligand blot analyses of the same *day 5*-conditioned media revealed the presence of three IGFBPs (32, 28, and 24 kDa; data not shown). The middle band was identified as IGFBP1 by Dr. J. W. van Neck (personal communication) (53), whereas the two others correspond to the molecular masses of IGFBP2 and IGFBP4, respectively. IGFBP3 was readily detectable in the control normal serum pool but was not present in the fetal hepatocyte-conditioned media (data not shown). hGH (50–150 ng/ml) treatment for 72 h had no effect on IGFBP profiles. In contrast, hepatocytes pretreated for 72 h with 150 ng/ml hGH showed a small, significant, time-dependent ($P < 0.01$) increase in [$^{14}$C]glucose uptake (Fig. 4*A*). The level of uptake was similar to that observed after acute (30-min) treatment with 300 nM insulin ($P < 0.05$; Fig. 4*B*).

*Fibroblast radioreceptor assays.* Previous studies demonstrated hGHR mRNA in human fetal (10–38 wk) and postnatal (4 mo-35 yr) skin as well as cultured fibroblasts through 12 generations in vitro (67). In the

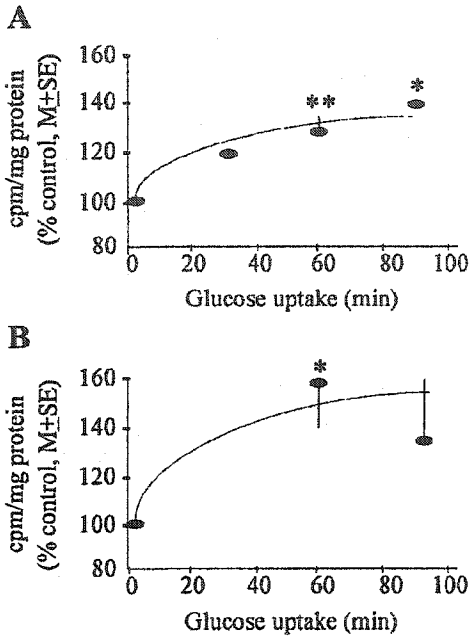


Fig. 4. [$^{14}$C]glucose uptake by human fetal hepatocytes ($n$ = 5; 13.75–20 wk) after chronic 72-h treatment with 150 ng/ml hGH ($A$) or acute 30-min treatment with 300 nM insulin ($B$), analyzed after 30, 60, or 90min. *$P$ < 0.05; **$P$ < 0.01.

present study, we determined that human dermal fibroblasts specifically bound $^{125}$I-hGH from as early as the 9th wk of fetal life. When a large fetal ($n$ = 23; 9–33 wk FA) and postnatal ($n$ = 11; 2 mo–34 yr) series was compared, we observed a small increase in both total (2.2 ± 0.1 vs. 3.2 ± 0.3%) and nonspecific (0.9 ± 0.09 vs. 1.6 ± 0.2%) binding of $^{125}$I-hGH ($P \leq 0.01$) but no significant change in specific binding (1.3 ±0.1 vs. 1.6 ±0.3%) as a function of donor age. Control IM-9 cells exhibited much higher total (8.8 ±0.3%; $n$ = 34) and specific (4.9 ± 0.3%) binding of $^{125}$I-hGH, although the percentage of nonspecific (3.7 ± 0.2%) binding was similar. The low level of fibroblast binding precluded Scatchard analyses.

*Fibroblast responses to hormonal treatments.* When fibroblast cultures were exposed to hGH (5–150 ng/ml) for 48 h, there were significant age-related differences in their ability to subsequently bind $^{125}$I-hGH (Fig. 5$A$). The fetal and early (<1 yr) postnatal cells showed no change in percentage of specific $^{125}$I-hGH binding, even when the highest hGH concentration, 150 ng/ml, was tested. In contrast, the older (>1 yr) postnatal fibroblast series showed a significant ($P$ < 0.05) dose-related decrease in binding. Control IM-9 cells showed the expected highly significant ($P$ < 0.01) decrease in $^{125}$I-hGH binding (40).

Experiments using dexamethasone pretreatment for 48 h also showed age-related differences in the fibroblasts' ability to subsequently bind $^{125}$I-hGH (Fig. 5$B$). Whereas the fetal series of cultures showed no change, even with the highest dose tested (400 nM), the postnatal cells showed a significant ($P$ < 0.02) increase in $^{125}$I-hGH binding after exposure to 100 nM dexamethasone.

## DISCUSSION

hGH acts by binding to its high-affinity receptor on target cells (47). The hGHR, a single-chain (620 amino acid) polypeptide member of the hGH/human prolactin/cytokine receptor superfamily, dimerizes in the presence of one molecule of hGH, activating specific intracellular signal transduction pathways and leading to gene regulation (14, 21, 35). The hGHR is encoded by a single gene spanning >150 kb on chromosome 5p13.1–12 (5, 17, 19, 41). The coding region is defined by exons 2–10, with exon 2 contributing the translation start site and signal sequence. Three hGHR isoforms have been reported. Exon 3-deficient mRNA can be detected in fetal tissues from as early as the 8th wk of fetal life (11, 59, 66, 67). This transcript, expressed in an individual- rather than a tissue-specific manner, translates into a high-affinity hGHR that binds hGH and related hormones with similar affini-

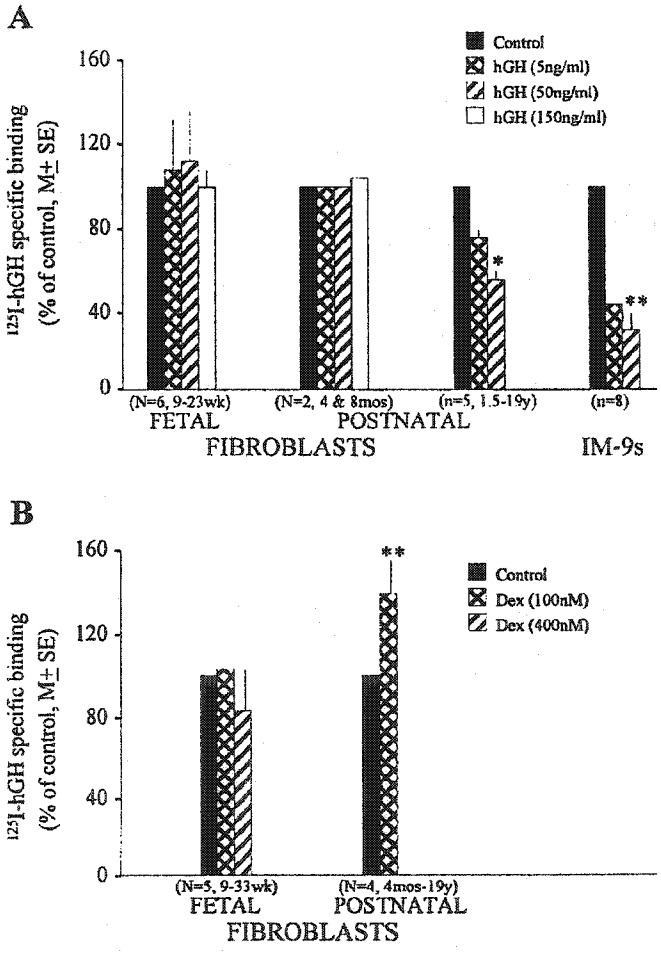


Fig. 5. Effects of 48-h pretreatment with either hGH ($A$) or dexamethasone (Dex; $B$) on the subsequent ability of fetal and postnatal fibroblast cultures or control IM-9 cells to bind $^{125}$I-hGH. Experiments were carried out in quadruplicate within each fibroblast or IM-9 series. *$P$ < 0.05; **$P$ < 0.02.

ties as the full-length receptor (55, 60, 63, 67). Two truncated forms of hGHR, 1–277 and 1–279, have also been demonstrated, with 1–279 being the major (10- to 20-fold more prevalent) form (2, 51). mRNAs for these truncated forms are produced at low levels in all human tissues examined to date due to variant splicing within exon 9 (4). The truncated receptors bind hGH with high affinity but do not dimerize or activate intracellular signal transduction pathways and thus may act as dominant negative receptors (51).

Although pivotal roles for hGH and its receptor have been well established in postnatal tissues, their function during human gestation remains unclear (22). Fetal serum GH levels are high in both primates and subprimates (15). However, although numerous studies have shown that onset of significant GHR mRNA expression occurs only around the time of birth in subprimates (1, 8, 36, 42, 58, 61, 65), ubiquitous transcription of the hGHR gene has been documented from the first trimester in the human fetus (19, 66, 67). In addition, immunohistochemical studies have identified hGHR protein in human tissues from 8 wk FA and shown that, by midgestation, the pattern of immunostaining is often identical to that found in the adult (26, 54). Thus both hGH and its receptor are present at very early stages in human development.

Whether the fetal hGHR are functional has been a controversial issue due to the variability of results from different research groups and the lack of correlation between hGHR mRNA/immunopeptide data and hGH responsiveness in certain tissues. There are reports of low hGH binding in fetal liver, chondrocytes, lung, and fibroblast cell lines, but no binding in muscle (25, 37, 44, 62). In one study, hGH treatment of fetal hepatocytes increased cell proliferation and IGF production (56). However, there have been inconsistent effects reported on pancreatic islet cells and negative effects observed with fetal myoblasts, cartilage explants, and dermal fibroblasts (13, 23, 46, 57). In the present study, we have addressed this controversy by focusing on two cell types, hepatocytes and dermal fibroblasts, and wherever possible, comparing hGHR functional characteristics in fetal vs. postnatal cells.

Our initial RT-PCR analysis demonstrated changes in total hGHR mRNA in four different sets of tissues: liver, kidney, lung, and small intestine. All but liver showed a decrease in hGHR mRNA in postnatal compared with fetal samples. In lung, this change was statistically significant and paralleled the previous observation of low hGH binding in fetal lung membranes and no binding in postnatal samples (37). In contrast, postnatal liver showed a striking increase in hGHR mRNA and a parallel increase in hGH specific binding. Thus there are marked tissue-specific differences in regulation of hGHR expression during development.

Cross-linking experiments indicate structural differences between the fetal and postnatal hepatic hGHR: the fetal receptor runs as a higher molecular mass band than either the human or rat adult liver GHR (27, 28, 30). Because fetal hepatic hGHR mRNAs are homologous to those found in postnatal liver (4, 67, present study), it is unlikely that this size difference is due to increased length of the basic receptor protein. Two other explanations seem more appropriate: increased glycosylation of the extracellular domain of the fetal hGHR and/or complexing with additional molecules.

The fetal hepatocytes produced IGFs and IGFBPs in an appropriate manner, with significantly higher IGF-II than IGF-I and no IGFBP3 (32, 39, 48). When pretreated with hGH, fetal hepatocytes showed an unusual response: no change in IGF-I but an increased production of IGF-II. Strain et al. (56) previously reported increased IGF-I secretion by human fetal hepatocytes after exposure to similar concentrations of hGH. However, the antibody they used to assay IGF-I has since been shown to also recognize IGF-II (6). Because IGF-II is the predominant IGF found in fetal tissues and circulation throughout gestation (6, 38, 45), it is likely that it was, in fact, IGF-II that was measured. There was no effect of hGH on IGFBP profiles. IGFBP3, the one IGFBP known to be responsive to hGH, is not produced until the final third of gestation (39, 48). Our data suggest that either the early hepatocytes cannot be induced to synthesise IGFBP3 after only 72 h of exposure to hGH or that hGH is not the appropriate inducing factor. The hepatocytes did respond to hGH with enhanced [$^{14}$C]glucose uptake. This increase was similar to the effect observed with acute insulin exposure, but much lower than has been observed in postnatal liver (12).

Dermal fibroblasts showed no age-related changes in hGH binding. However, there were marked changes in their responsiveness to both hGH and dexamethasone. Chronic exposure to hGH is well known to cause desensitization of hGH target cells due to loss of hGHR from the cell surface (40, 64). This response was exhibited by postnatal fibroblasts, but only those from infants >1 yr of age, suggesting that maturation of the downregulatory effect of hGH is not linked to birth and independence from the in utero environment. In contrast, the ability of dexamethasone to increase hGH binding was observed in postnatal fibroblasts as early as 4 mo after birth.

In conclusion, our studies demonstrate that fetal hepatocytes and fibroblasts do have functional hGHR, in that these cells specifically bind hGH and have biological responses after chronic exposure to hGH. Certain of the responses appear to be an adaptation to the fetal environment (increased IGF-II rather than IGF-I), whereas others (low glucose uptake, no change in hGHR in response to hGH or dexamethasone) may be simply due to immature cell systems (internalization/receptor cycling, signal transduction pathways, gene regulatory mechanisms), none of which has been investigated to date. The fact that the fetal hepatic hGHR is a different molecular size is also intriguing and needs to be explored to determine what role this difference may play in restricting hGH responsiveness during fetal development.

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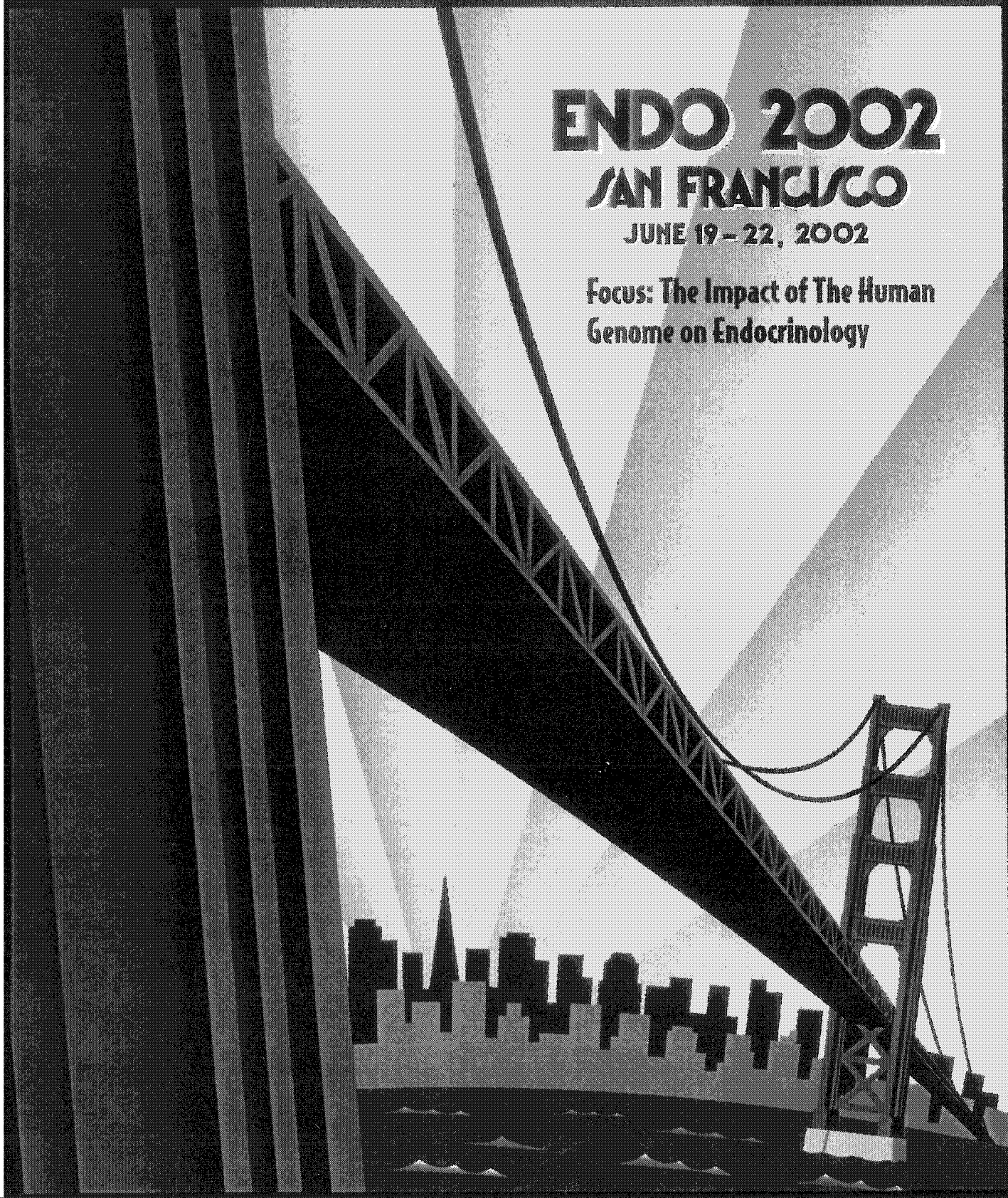
# MEETING & EXHIBIT GUIDE



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THE ENDOCRINE SOCIETY

**P3-278. Developmental Changes in the Human Growth Hormone Receptor (hGHR) and Its Signal Transduction Pathways.**

Jennifer A Manalo*[1], Cynthia G Goodyer[1]. [1]*Medicine and Pediatrics, McGill University, Montreal, Quebec, Canada*

Although tissue hGHRs have been identified from as early as the first trimester of fetal life, little is known about their functional role during early development. We recently reported that fetal hGHRs show characteristics of relative immaturity or adaptation to the *in utero* environment (*Am J Physiol Endocrinol Metab.* 281:E1213-E1220, 2001). To further characterise these age-related differences, we used RT-PCR to investigate the expression of an alternatively spliced exon 9 mRNA variant, producing a truncated (1-279) hGHR (T), which can act as a dominant negative to the full-length (FL) receptor. A two-fold greater ratio of T/FL hGHR transcripts was initially found in intact fetal (0.20±0.07) vs. postnatal liver (0.09±0.01) ($p>0.05$). However, the liver is the major hematopoietic organ during fetal life. When fetal liver tissue was enzymatically dissociated, a higher ratio of T/FL mRNA was detected in fetal hematopoietic cells (0.24±0.08), while fetal hepatocytes (0.07±0.04) showed ratios similar to postnatal liver ($p>0.05$). We also analyzed the T/FL ratio in ten other fetal tissues: these ranged from 0.002 in skin to 0.53±0.10 in kidney cortex and 0.65±0.15 in kidney medulla.

In addition, Western blotting was used to assess for developmental differences in the relative abundance of downstream signal transduction factors. Stimulatory Jak (Jak1, Jak2) and Stat (Stat1, Stat3, Stat5A, Stat5B) proteins were detected in all fetal hepatocytes and postnatal livers tested. However, the samples, normalized to calnexin, showed much lower levels in fetal hepatocytes compared to postnatal liver (5-22%), including molecules best known to be involved in hGHR signalling: Jak2 (22% of postnatal liver, $p<0.002$) and Stat5B (5%, $p<0.02$). Four suppressors of cytokine signalling (Socs), which negatively regulate hGHR action, were also detected in all samples: Socs1 (119% of postnatal liver), Socs2 (51%), Socs3 (239%, $p<0.03$) and CIS (200%, $p<0.03$).

In summary, our results suggest that the relative abundance of T/FL hGHR in fetal hepatocytes is not contributing to the levels of hepatocyte responsiveness observed during fetal life, although this may be an important factor in other fetal tisues (eg. Kidney). The relatively low levels of stimulatory Jak/Stat molecules, coincident with the high level of at least two negative regulatory Socs proteins, are more likely to be responsible for the fetal-specific hepatocytes responses to GH.

SUPPLEMENT TO:

# PEDIATRIC RESEARCH

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## ABSTRACTS ISSUE

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P2-190

**Changes in the Human Growth Hormone (hGH) Receptor and hGH Signal Transduction Pathways during Development**

Jennifer A Manalo, Cynthia G Goodyer. Pediatrics, McGill University, Montreal, Canada.

hGH receptors (hGHR) have been identified in human tissues as early as the first trimester of human life. Several subtypes have been found but little is known about their functional role or their intracellular signalling pathways, especially during fetal development.

Using RT-PCR, we determined that exon 3+ and 3- isoforms of hGHR mRNA are expressed at all stages in life, but there is greater expression of the 3- form prenatally (69% vs 33% postnatal, $p<0.01$). Furthermore, we used RTMultiplex PCR assays to investigate the expression of an alternatively spliced exon 9 mRNA variant, producing a truncated hGHR (1-279), in fetal and postnatal liver. Full-length and truncated hGHR mRNAs were detected in all samples, although fetal samples showed an approximately two-fold greater proportion of truncated to full-length transcripts (fetal: 46% vs. postnatal: 26%).

We also used Western blotting to analyse the abundance of downstream signalling molecules known to be involved following hGHR activation, Janus Kinase (Jak) (Jak1, Jak2) and Signal Transducers and Activators of Transcription (Stat) (Stat1, Stat3, Stat5A and Stat5B) family members were present in cytosol and membrane fractions of both fetal and postnatal liver tissues. Samples, normalized to calnexin, showed 26-71% greater levels of Jak1, Jak2, Stat1 and Stat5A in fetal than postnatal cytosol fractions. A more significant difference (310 and 450%, respectively) in Jak1 and Jak2 levels was observed in fetal compared to postnatal membrane fractions.

hGH-induced receptor dimerization is essential for turning on signal transduction pathways. Our results suggest that the presence of specific hGHR isoforms in fetal vs postnatal tissues may be important in determining which receptor dimmer complexes form in target cells at different developmental stages. In turn, this may result in the activation of alternate transduction pathways and difference biological responses.