The role of the growth hormone/IGF-I system on islet cell growth and insulin action

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Dedication

This thesis is dedicated to my friends and family who supported me. I have to thank my parents and sisters for helping me whenever I needed help.

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Abstract

The study of diabetes mellitus is vital in this day and age because its incidence is increasing at an alarming rate. Diabetes results in the loss of function of β-cells within the pancreas. Insulin resistance contributes to diabetes but the human body can compensate in various ways such as increasing the islet cell mass, glucose disposal and insulin secretion, in order to prevent the onset of diabetes. Growth hormone (GH) and insulin-like growth factor-I (IGF-I) are two integral hormones important in both glucose homeostasis and islet cell growth. Early studies using cultured islet cells have demonstrated positive regulation of β-cell growth by both GH and IGF-I. To evaluate their relevance on normal β-cell growth, compensatory growth, as well as in insulin responsiveness, we have used two mouse models that represent opposite manipulations of the GH/IGF-I axis. Specifically, the growth hormone receptor gene deficient ($GHR^{-/-}$) and the IGF-I overexpression (MT-IGF) mice, to help understand the role of glucose homeostasis and islet cell growth in the GH/IGF-I axis. GH is essential for somatic growth and development as well as maintaining metabolic homeostasis. It is known that GH stimulates normal islet cell growth. Moreover, GH may also participate in islet cell overgrowth and compensate for insulin resistance induced by obesity. To determine whether the islet cell overgrowth is dependent on GH signaling, we studied the response of $GHR^{-/-}$ mice to high-fat diet (HFD)-induced obesity. We also studied the insulin responsiveness in $GHR^{-/-}$ mice. On the other hand, IGF-I promotes embryonic development, postnatal growth and the maturation of various organ systems. The notion that IGF-I stimulates islet cell

growth has been challenged in recent years by results from IGF-I and receptor gene targeted models. We have characterized MT-IGF mice which overexpress the IGF-I gene.

The results of our studies indicate that 1) GH is essential for normal islet cell growth, but not required for compensatory overgrowth of the islets in response to obesity, 2) GHR gene deficiency caused delayed insulin responsiveness in skeletal muscle; in contrast to elevated insulin sensitivity in the liver; 3) although overexpression does not stimulate islet cell growth, a chronic IGF-I elevation caused significant hypoglycemia, hypoinsulinemia, and improved glucose tolerance, 4) finally IGF-I overexpression mice are resistant to experimental diabetes.

Résumé

De nos jours, l'étude du diabète est essentielle puisque l'incidence de cette maladie augmente à un taux alarmant. Une des conséquences du diabète est la perte de la fonction des cellules bêta du pancréas. La résistance à l'insuline contribue au développement du diabète mais le corps humain peut compenser de diverses façons telles qu'en augmentant la masse des cellules des îlots du pancréas, en éliminant le glucose et en secrétant plus d'insuline afin de prévenir le développement du diabète. L'hormone de croissance (GH) et l'*insulin-like growth factor-I* (IGF-I) sont deux hormones importantes dans le maintient de l'homéostasie du glucose et la croissance des cellules des îlots pancréatiques. Les études premières utilisant les cellules d'îlot cultivé ont démontré le règlement positif de croissance par GH et IGF-I. Pour évaluer leur pertinence sur la croissance normale des cellules β, la croissance compensatoire ainsi que sur la réponse à l'insuline, nous avons utilisé deux modèles de souris qui représentent des manipulations opposées de l'axe GH/IGF-1. Spécifiquement, nous avons utilisé les souris déficientes pour le gène du récepteur de l'hormone de croissance ($GHR^{-/-}$) et les souris sur-exprimant le gène de l'IGF-I (MT-IGF) pour essayer de comprendre le rôle de l'homéostasie du glucose et de la croissance des cellules d'îlot dans l'axe GH/IGF-I. La GH est essentielle pour la croissance et le développement somatique ainsi que le maintient de l'homéostasie métabolique. De plus, il est connu que la GH stimule la croissance normale des cellules des îlots. Toutefois la GH peut aussi participer à la surcroissance des cellules des îlots afin de compenser la résistance à l'insuline induite par l'obésité. Pour déterminer

si la surcroissance des cellules des îlots est dépendante des voies de signalisation de la GH, nous avons étudié la réponse des souris $GHR^{-/-}$ soumises à un régime riche en gras (HFD). Les résultats ont démontrés que les souris $GHR^{-/-}$ avaient une masse des cellules bêta légèrement supérieure à celle des souris de type sauvage. D'autre part l'IGF-I favorise le développement embryonnaire, la croissance postnatale et la maturation de divers systèmes d'organe. La notion que l'IGF-I stimule la croissance des cellules d'îlot a été défiée récemment par les résultats provenant des modèles de gènes ciblant l'IGF-I et son (ses) récepteur (s). Nous avons caractérisé les souris MT-IGF qui sur-exprimant le gène de l'IGF-I. principalement dans les cellules pancréatiques des îlots.

Les résultats de nos études montrent que 1) la GH est essentielle pour la croissance normale des cellules d'îlot mais elle n'est pas requise pour la sur-croissance compensatoire de ces cellules en réponse à l'obésité, 2) la déficience du gène GHR cause un délai dans la réponse à l'insuline dans le muscle squelettique; contrairement à une augmentation de la sensibilité à l'insuline dans le foie; 3) bien que la sur-expression ne stimule pas la croissance des cellules d'îlot, une augmentation chronique de l'IGF-I cause une hypoglycémie et une hypoinsulinémie significatives et une tolérance de glucose améliorée, 4) finalement les souris sur-exprimant le gène de l'IGF-I sont résistantes au diabète expérimental.

Preface

The frequency of diabetes has been increasing at an alarming rate in the past 2 decades; the world health organization has predicted that 300 million people will become diabetic by 2025. The personal (heart disease, stroke, kidney failure) as well as the financial ($1,000-$15, 000/year) cost of this disease are staggering. It is no surprise that many researchers are interested in understanding more of this disease such as the mechanism and improved therapies. Diabetic patients have abnormal glucose homeostasis and a diminished β-cell mass and/or function. Growth hormone (GH) and insulin-like growth factor I (IGF-I) are two important hormones that play a role in both glucose homeostasis as well as islet cell growth. With the recent debate over whether IGF-I has a role in promoting islet growth, further study is needed to clarify the situation. The aim of this thesis is to understand the roles of GH and IGF-I in normal islet growth, compensatory islet growth as well as regenerative islet growth using 2 mouse models, the growth hormone receptor gene deficient ($GHR^{-/-}$) and IGF-I overexpression (MT-IGF) mice. Original findings are that GH does not play a role in the overgrowth of the islets when mice are subjected to a high-fat diet (HFD), as well as the fact that a general whole body IGF-I overexpression causes severe hypoglycemia, decreased gluconeogenesis, and resistance to streptozotocin induced diabetes. This thesis contains four chapters. The first chapter consists of an introduction pertaining to diabetes, pancreas development and the roles of GH and IGF-I on islet cell growth. The second chapter describes "Growth hormone receptor gene deficiency causes delayed insulin responsiveness in skeletal muscles without

affecting compensatory islet cell overgrowth in obese mice"; published in American Journal of Physiology: Endocrinology and Metabolism (**2006 291:E491-498**). The third chapter documents "A general overexpression of IGF-I results in normal islet cell growth, hypoglycemia and significant resistance to experimental diabetes", a manuscript in preparation. The final chapter is a discussion of the results as well as future directions.

Contribution of Authors

The experiments described in the first authored papers pertaining to myself (Chapter 2 and 3) were all performed by the first author except:

The Northern blot in figure 3.1 was performed by Yarong Lu.

The IGF concentration in table 3.1 was performed by Dr. A.F. Parlow.

Dr. John J. Kopchick provided the $GHR^{-/-}$ mice and commented on the manuscript.

Dr. P Kay Lund provided the MT-IGF mice and commented on the manuscript.

Dr. Su Qing provided assistance in the animal experiments.

Publication list

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1. **Robertson K**, Kopchick JJ, Liu JL: Growth hormone receptor gene deficiency causes delayed insulin responsiveness in skeletal muscles without affecting compensatory islet cell overgrowth in obese mice. *Am J Physiol Endocrinol Metab* 291:E491-498, 2006

2. **Robertson K**, Yarong Lu, Qing Su, P Kay Lund, and Jun-Li Liu: A general overexpression of IGF-I results in normal islet cell growth, hypoglycemia and significant resistance to experimental diabetes. (manuscript in preparation)

Coauthored (full text can be found in the appendix):

3. Liu JL, Coschigano KT, **Robertson K**, Lipsett M, Guo Y, Kopchick JJ, Kumar U, and Liu YL. Disruption of growth hormone receptor gene causes diminished pancreatic islet size and increased insulin sensitivity in mice. *Am J Physiol Endocrinol Metab* 287: E405–E413, 2004.

4. Guo Y, Lu Y, Houle D, **Robertson K**, Tang Z, Kopchick JJ, Liu YL, and Liu JL. Pancreatic islet-specific expression of an insulin-like growth factor-I transgene compensates islet cell growth in growth hormone receptor gene-deficient mice. *Endocrinology* 146: 2602–2609, 2005.

Meeting abstracts

1. **Robertson K**, Yarong Lu, Qing Su, P Kay Lund, and Jun-Li Liu: A general overexpression of IGF-I results in normal islet cell growth, hypoglycemia and significant resistance to experimental diabetes. American Diabetes Association's 67th Sessions Scientific , Chicago, IL, June 22-27, 2007.

2. **Robertson K**, Yarong Lu, P Kay Lund, A.F. Parlow, and Jun-Li Liu: Normal islet growth, hypoglycemia and anti-diabetic effect of IGF-I

overexpression in transgenic mice. Boston-Ithaca Islet Club, Boston, MA, November 4-5, 2006 (Oral presentation).

3. **Robertson K**, Yarong Lu, P Kay Lund, and Jun-Li Liu. A general, robust overexpression of IGF-I gene fails to affect pancreatic islet cell growth. The Endocrine Society's 88th Annual Meeting, Boston, MA, June 24-27, 2006 (Anthony R Means Basic Science Award-Oral presentation).

4. **Robertson K**, Yarong Lu, P Kay Lund, and Jun-Li Liu. An Overexpression of IGF-I Gene Fails to Affect Pancreatic Islet Cell Growth. The 2006 McGill Endocrine Retreat, May, 2006 Montreal,QC (Oral presentation)

4. **Robertson K**, Yarong Lu, P Kay Lund, and Jun-Li Liu. A General Robust Overexpression of IGF-I Gene Fails to Affect Pancreatic Islet Cell Growth 6th Annual McGill Biomedical Conference, February 2006 Montreal, QC

5. **Robertson K**, JJ Kopchick and JL Liu. Decreased insulin responsiveness in the skeletal muscle of growth hormone receptor gene deficient mice. The Endocrine Society's 87th annual meeting, June 4-7, 2005 San Diego, CA .

6. **Robertson K**, JJ Kopchick, and JL Liu. Decreased insulin responsiveness in the skeletal muscle of growth hormone receptor gene deficient mice. 5th Annual McGill Biomedical Conference, March 2005 Montral, QC.

7. Guo Y, Lu Y, Coschigano KT, Kopchick JJ, Tang Z, **Robertson K** and Liu JL. Pancreatic islet-specific expression of an IGF-I transgene increases islet cell mass in GHR-/- mice. American Diabetes Association, 64th Annual Scientific Sessions, June 4-8, 2004, Orange County Convention Center, Orlando, FL.

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Abbreviations

αPY	anti-phosphotyrosine antibody
AADC	L-amino acid decarboxylase
AKT/PKB	v-akt murine thymoma viral oncogene homolog 1/ protein kinase B
ALS	acid labile subunit
ATP	adenosine triphosphate
BAD	Bcl-2-antagonist of cell death protein
Bax	Bcl-2 associated X protein
Beta2/neuroD	neurogenic differentiation factor
Bcl-xL	B-cell leukemia/lymphoma xl protein
Bcl-2	B-cell leukemia/lymphoma 2
βIRKO	β-cell specific insulin receptor knockout mice
BMP	bone morphogenetic protein
Brn-4	brain-4 a POU domain, class 3, transcription factor 4
cAMP	cyclic adenosine monophosphate
CDK	cyclin-dependent kinases
CNS	central nervous system
CSF	cerebrospinal fluid
db/db	code for an obese mouse due to a mutation in leptin receptor
DNA	deoxyribonucleic acid
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
ER	endoplasmic reticulum
erbB-2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERK	extracellular signal-regulated kinases
ESC	embryonic stem cells
FFA	free fatty acids
FGF	fibroblast growth factor
Fn	fibronectin
Foxa-2	forkhead box A2
FKHR	forkhead receptor
GDP	guanosine diphosphate
GH	growth hormone
GHa	growth hormone antagonist
GHBP	growth hormone binding protein
GHR	growth hormone receptor
$GHR^{-/-}$	homozygous growth hormone receptor gene knockout
$GHR^{+/-}$	heterozygous growth hormone receptor
GHRH	growth hormone releasing hormone

GHRP	growth hormone releasing peptide
GLP-1	glucagon-like peptide-1
GLUT	glucose transporter
Grb2	growth factor receptor bound protein
GTP	guanosine triphosphate
GS	glycogen synthase
GSK-3	glycogen synthase kinase-3
G6P	glucose-6-phosphate
G6Pase	glucose-6-phosphatase
Hex	hematopoietically expressed homeobox
Hes1	hairy and enhancer of split 1
HFD	high-fat diet
Hlxb9	homeobox gene HB9
HNF	hepatocyte nuclear factor
Hox-8	homeobox 8
IFNγ	interferon gamma
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGF-IR	insulin-like growth factor-I receptor
IGFBP	insulin-like growth factor binding protein
IL-1	interleukin-1
INGAP	Islet neogenesis associated protein
INS-1	rat insulinoma-1 cell line
IR	insulin receptor
IRR	insulin-receptor-related receptor
IRS	insulin receptor substrate
Isl-1	islet-1
JAK	janus kinase
JH	JAK homology domain
LID	liver specific IGF-I gene deficiency
LIF	leukemia inhibitory factor
Lrh1	liver receptor homolog 1
MAPK	mitogen-activated protein kinases
Mek	mitogen-activated protein kinases / extracellular signal-regulated kinases kinase
Mist1	muscle, intestine, and stomach expression 1, helix-loop-helix
MKR	muscle specific IGF-IR dominant negative mouse
MODY	maturity onset diabetes of the young
MT-IGF	metallothionein promoter driven IGF-I overexpressed mice
Nck	non-catalytic region of tyrosine kinase adaptor protein
NF-κB	nuclear factor kappa B
NGF	nerve growth factor
Nkx-2.2	NK2 transcription factor related, locus 2

Nkx-6.1	NK2 transcription factor related, locus 1
NOD	non-obese diabetic
NPY	neuropeptide Y
NS	not significant
ob/ob	code for an obese mouse due to leptin gene deficiency
PAX	paired box gene
PDK1	phosphoinositide-dependent protein kinase 1
Pdx-1	pancreatic duodenal homeobox-1
PEPCK	Phosphoenolpryuvate carboxykinase
PH	pleckstrin homology
PI	phosphatidylinositol
PID	pancreatic-specific IGF-I gene deficiency
PI3K	phosphatidylinoside-3 kinase
PKA	protein kinase A
PKC	protein kinase C
Ptdins-3, 4, 5-P_3	Phosphatidylinositol-3, 4, 5-trisphosphate
PI-4-P	phosphatidylinositol-4-phosphate
PLC	protein lipase C
PP	pancreatic polypeptide
PPAR	peroxisome proliferator-activated receptors
PPG-1	glycogen associated protein phospatase-1
Pref-1	preadipocyte factor-1
Prox-1	prospero-related homeobox 1
PTF1	pancreas specific transcription factor-1
Ptf1a/p48	pancreas specific transcription factor-1 subunit 48
PTP	protein-tyrosine phosphatases
PYY	peptide tyrosine tyrosine
RA	retinoic acid
Rab-GTPases	ras in the brain-guanosine triphosphatases
Raf	v-raf-leukemia viral oncogene 1
Ras	Harvey rat sarcoma virus oncogene 1
Reg	the regeneration-associated gene
rh	recombinant human
RNA	ribonucleic acid
T1D	type 1 diabetes mellitus
T2D	type 2 diabetes mellitus
TNF-α	tumor necrosis factor alpha
TrkA	tyrosine kinase receptor type 1
SCAMPS	secretory carrier membrane proteins
Shc	Src homology 2 domain containing
SHH	sonic hedgehog
SH2	src homology 2 domain
SH3	src homology 3 domain
SHP	SH2 domain-containing protein-tyrosine-phosphatase

SIRP-α	signal-regulatory protein alpha
SE	standard error
SOCS	suppressor of cytokine signaling
SOS	son of sevenless homolog
Sox-17	SRY-box containing gene 17
STAT	signal transducer and activator of transcription
STZ	streptozotocin
TUNEL	terminal transferase dUTP nick end labeling
VAMPS	vesicle-associated membrane proteins

Chapter One

Introduction

1.1 Diabetes Mellitus

It is well-known that the incidence of both type 1 (T1D) and type 2 (T2D) diabetes is increasing at an alarming rate. There is an extensive ongoing research to discover better treatments, to understand the mechanisms involved and ways to possibly prevent this disease. Diabetes mellitus can be considered as a group of metabolic diseases characterized by hyperglycemia which can result from defects in insulin action, secretion or both [1]. Diabetes is associated with long-term damage due to the presence of chronic hyperglycemia, which can cause many complications such as deregulation and failure of various organs, notably the eyes, kidneys, blood vessels, heart, and nerves [1]. In both forms of diabetes a key feature is β-cell failure [2]. In T1D there is an autoimmune reaction against the β-cells, while in T2D β-cell dysfunction coupled with an inability to compensate for insulin resistance causes β-cell failure [2]. Two important hormones, growth hormone (GH) and insulin-like growth factor-I (IGF-I) interact with each other and their independent roles in pancreatic islet growth and insulin action will be studied and this thesis focuses primarily on these two hormones.

T1D, which used to be known as insulin-dependent diabetes is more often found in young adults or children. T1D is caused by an autoimmune reaction, which destroys the β-cells (i.e. lose their ability to secrete insulin) resulting in permanent insulin loss. In T1D there is a reduction of the β-cell mass of at least 80% [3]. The previous statement findings, correlate with patients data which shows a progressive decrease in the first phase of insulin secretion [4]. There are various mechanisms that can contribute to β-cell death. During insulitis,

mononuclear cells invade the islets causing an inflammatory reaction; β-cell death is most likely caused directly by the interaction with macrophages and T cells or by the interaction of the islet cells with the secretions of these cells such as cytokines, free radicals and nitric oxide [5]. β-cells exposed to interleukin (IL)-1β or tumor necrosis factor (TNF)-α and interferon (IFN)-γ in vitro exhibit increased insulin levels and a loss of the first-phase insulin secretion capacity in response to glucose; therefore, they are responding the same way as pre-diabetic patient β-cells [6]. This change is due to decreased intracellular insulin docking and fusing to the β-cell membrane caused by IL-1β, therefore decreased insulin release [7]. In T1D the main cause of cell death is apoptosis but necrosis also occurs. In human and rodents IL-1β can activate nuclear factor kappa B (NF-κB), a transcription factor involved in the death pathway [5] and downregulate pancreatic duodenal homeobox-1 (Pdx-1) and islet-1 (Isl-1), transcription factors involved in β-cell differentiation and function [2]. Purified human and rodent β-cells exposed to IL-1β will not undergo apoptosis unless also exposed to IFN-γ; dual exposure will cause a 50% apoptotic rate within 6-9 days [5]. The downstream pathway involves IFN-γ binding to its receptor which will lead to the activation of the tyrosine kinase Janus kinases (JAK)-1 and JAK-2. These kinases will phosphorylate signal transducer and activator of transcription (STAT)-1, a transcription factor which will dimerize, enter the nucleus and then cause cell death [5].

T2D, previously known as non-insulin dependent diabetes often occurs in older, overweight individuals, but this is starting to change due to the high level of

childhood obesity that is now occurring. In the initial stages of the disease the insulin target tissues do not effectively respond to insulin, resulting in decreased glucose disposal, increased hepatic gluconeogenesis and hyperglycemia [8]. Consequently, there is compensation by the β-cells to secrete more insulin but over time there is a decrease in β-cell function which causes glucose intolerance and ultimately T2D which will deteriorate if no further action is taken [9]. In T2D there is a loss of up to 50% of the β-cell mass along with a 3 fold increased rate of apoptosis of the β-cells and there are many complications [10, 11]. β-cell mass is negatively affected by dyslipidemia and hyperglycemia which increase the rate of apoptosis [12, 13]. Dyslipidemia is occurring more often today due to our sedentary lifestyle and high fat diets. A high level of free fatty acids (FFA) are a risk factor for T2D [14]. Hyperglycemia doesn't necessarily kill the β-cells but it does contribute to the progression from glucose intolerance to T2D. It has been shown that rodent β-cells cultured in high glucose exhibit various changes such as altered glucose-stimulated insulin secretion, cell growth, survival and even changes in gene expression [13, 15]. There are numerous mechanisms that can account for these changes including but not limited to; cytokine-, oxidative- and endoplasmic reticulum (ER)- stress as well as functional changes such as an increase in glycogen [12, 16-18].

1.2 Pancreatic Development

1.2.1 Pancreatic Structure

The word pancreas derives from its Greek root "pan" meaning "all" and "creas" meaning "flesh". The pancreas consists of three main types of cells excluding the stromal and vascular supporting cells; the exocrine cells, the endocrine cells and the ductal tree [19]. The developed pancreas in mammals and higher vertebrates can be divided into two main units; the exocrine and the endocrine unit with both units having physiologically separate roles. The exocrine pancreas produces digestive enzymes which are secreted into the gut and the endocrine pancreas synthesizes a number of hormones secreted into the bloodstream and is involved in metabolism and nutritional homeostasis [20].

The exocrine portion is the larger of the two, is branched, lobulated and consists of two cell types: the secretory acinar cells and the ductular cells. The acini are pyramidal in shape, composed of basal nuclei, a large golgi complex, rough endoplasmic reticulum arranged in rows, and many zymogen granules. These zymogen granules contain digestive enzymes: there are approximately 20 including but not limited to amylases, lipases, nucleases, and proteases. Most zymogens are secreted as inactive precursors which are activated in the duodenum [19]. Small cuboidal centroacinar cells are found at the junction of the ductal and acini cells, these cells are rich in mitochondria and are thought to secrete non-enzymatic pancreatic fluid such as bicarbonate. The ducts are lined with columnar epithelial cells, with the larger ducts containing brush and goblet cells. Hormonal

stimulation such as gastrin, secretin and cholecystokinin regulate secretion of the pancreatic fluid but neural stimuli also play a role [19].

The endocrine portion is quite small, only 2% of the total mass. The cells arrange themselves into the islets of Langerhans and contain five types of cells; α, β, PP, δ, and ε cells [20]. The islets are tight, compact, spheroidal clusters, embedded in the exocrine tissue. Each cell produces a different hormone. The α cells produce glucagon and are situated along the periphery of the islets. The β cells synthesize and secrete insulin and an insulin antagonist amylin and are found in the interior of the islet, they compose the majority of the cells within the islets. The PP cells produce pancreatic polypeptide and the δ cells produce somatostatin, the latter are found scattered throughout the periphery of the islets. PP-rich islets are found in the posterior head of the pancreas [19]. The ε cells have been discovered very recently, albeit in very low numbers but they increase upon loss of NK2 transcription factor related, locus 2 (Nkx-2.2) or paired box gene (PAX)-4 activities in the β-cells [21]. The ε cells produce ghrelin which regulates food uptake [22]. All types of islet cells also express gene products related to neuroendocrine cells such as but not restricted to tetanus toxin receptor, neuron-specific enolase, A2B5 antigen and the homeodomain LIM protein Isl-1 [23, 24].

1.2.2 Pancreatic Evolution

Comparative morphological and immunohistochemical studies during the 1980's have described the evolution of the pancreas. The pancreas in terms of a secretory organ having both endocrine and exocrine function is specific to

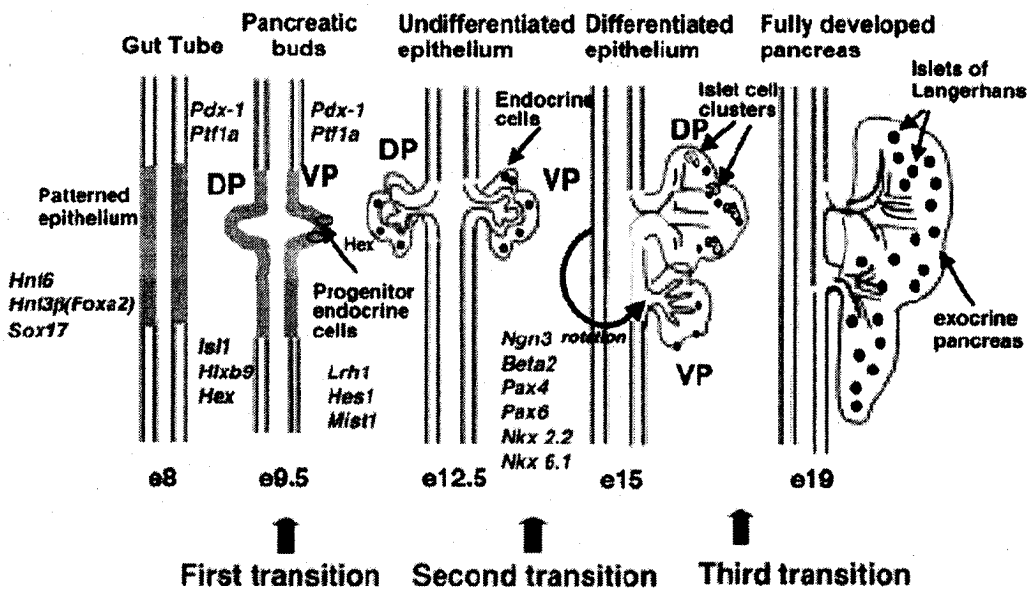
vertebrates since in insects the brain produces the gastrointestinal hormones [25]. Fish vary in the complexity and number of pancreatic structures; this is related to the evolution process [26]. The hagfish which represents primitive vertebrates, contains a primitive pancreatic organ in the gut mucosa; it does not contain any acinar cells, therefore it does not have an exocrine function, but it is composed primarily (99%) of insulin cells [27]. Holocephalan cartilaginous fish are an example of a primitive exocrine pancreas, in that it contains a pancreatic duct ending in the gut lumen and is connected to a structure made of exocrine cells and islets which produce insulin, glucagon and somatostatin [28]. The organization which we are familiar with is found in shark endocrine islets, that contain four classes of cells and produce insulin, glucagon, somatostatin and PP, along with luminal cells which comprise the exocrine portion [29].

1.2.3 Pancreatic Embryogenesis

A figure of rodent pancreatic development can be seen in **Figure 1.1.** The embryonic pancreas develops in the mouse relatively early in development, embryonic day 8.5 (E8.5) to E9.5, in the foregut epithelium from a dorsal bud and 2 ventral buds. Transcription factors are essential in the cellular differentiation process and are required at each developmental stage. The dorsal bud arises from the primitive gut endoderm in between the foregut and midgut and the ventral buds arise next to the hepatic diverticulum [19]. One ventral bud will atrophy and the other will grow [30].The two buds, which are only partially differentiated by E10.5 will develop by E12.5 into a ductal tree through branching morphogenesis.

This series of events is considered to be the first developmental transition and results in two pancreas organs made up of undifferentiated ductal epithelium [31]. Between E13 and E14 the stomach and duodenum rotate. The hepatopancreatic organ and ventral bud will come into contact with the dorsal bud, and fusion occurs. The ventral bud forms the posterior section of the head of the pancreas (duodenal section) and the dorsal bud forms the body of the pancreas (splenic section) [19]. The ductal pancreas differentiates into the exocrine pancreas between E14.5 and E15.5; at this stage acini can be seen within the ducts. The endocrine cells are present since E9.5; they are derived from stem/progenitors cells found in the gut endoderm, and at E14 they undergo a wave of proliferation. This is considered to be the second developmental transition. On E16 islet-like clusters begin to form. The islets are almost fully formed between E18-E19 just before birth, and remodel and develop for 2-3 weeks after birth during the third developmental transition [31]. The endocrine cells appear in a regulated fashion. In the foregut at E9.5, glucagon-expressing cells are present as well as neuropeptide Y (NPY), peptide tyrosine tyrosine (PYY) and pancreatic polypeptide (PP). At E10-E10.5 the cells will co-express insulin and glucagon, and then differentiate into β-cells and α-cells, which secrete insulin and glucagon respectively. The δ-cells which secrete somatostatin arise on E14 [31].

Figure 1.1 Illustration of rodent pancreatic development.
Prepatterned endodermal epithelium of the foregut first arises on E8, and forms dorsal (DP) and ventral buds (VP) by E9.5. The buds develop into branching ducts and undifferentiated epithelium by E12.5 which is considered the first developmental transition. The undifferentiated epithelium contains single endocrine cells. By E14 the buds begin to differentiate into endocrine and exocrine cellular lineages which proliferate and expand profusely, this is the second developmental transition. By E15 the 2 buds rotate, fuse and form an almost fully developed pancreas by E19, containing the endocrine cells arranged into clusters that will form the islets of Langerhans, this is the third and final developmental transition. This last transition continues for 2-3 weeks after birth and consists of the maturation of the endocrine cells. The important transcription factors are depicted in *blue* [31].
Habener, J.F., D.M. Kemp, and M.K. Thomas, *Minireview: transcriptional regulation in pancreatic development.* Endocrinology, 2005. **146**(3): p. 1025-34.



1.2.4 Endodermal Marker Genes

Regulation of pancreatic embryogenesis has been well studied and many extracellular signaling factors are known to be involved. In early stages retinoic acid (RA) is an important signaling molecule in vertebrates. Different animals depend on RA for different steps at different times. For example, the zebrafish depends on RA during the gastrulation stage whereas xenopus and mice require RA for the formation of the dorsal pancreatic bud [32-34]. An excess of RA in xenopus during gastrulation will increase the endocrine population and decrease the exocrine population. In the ventral endoderm augmented levels of RA will increase exocrine markers but the liver will not develop [33]. These results indicate that RA is important for the patterning of the endoderm which will later become the pancreas. The differentiation of the dorsal and ventral buds requires different signals which are believed to be provided by the mesoderm. Signals from the notochord such as fibroblast growth factor (FGF) repress sonic hedgehog (SHH) signaling in the dorsal pancreatic bud [35, 36]. Initially SHH inhibits pancreas development but is required for the formation of the stomach and duodenum. It is believed that notochord may play a role because it is in contact with the early dorsal gut endoderm although this is still a hypothesis since there is not a great deal of evidence to support this claim [37]. One must note that the mesenchyme surrounds the dorsal pancreatic bud during pancreatic development and it is known that mesenchyme induces pancreatic growth in vitro [38]. Positive signals from the notochord and signaling from the dorsal aorta {i.e. Isl-1, homeobox gene HB9 (Hlxb9), hematopoietically expressed homeobox (Hex) etc.}

are also required to form the dorsal bud. The ventral pancreatic bud receives its signals from the cardiac mesoderm. For example, (FGF), which inhibits the pancreatic development but promotes liver specification, and other transcription factors such as liver receptor homolog 1 (Lrh1), hairy and enhancer of split 1 (Hes1) and muscle, intestine, and stomach expression 1 (Mist1) are involved [39]. The notochord induces pancreatic genes by inhibiting SHH expression in the dorsal epithelium [35, 40]. Notch is also important for the differentiation of both exocrine and endocrine cells. Notch keeps pancreatic cells in an undifferentiated proliferative state and ectopic expression of Notch intracellular domain will lead to the inhibition of differentiation of both exocrine and endocrine cells [41, 42]. Notch inhibition results in increased numbers of endocrine cells and pancreatic hypoplasia. Hes1, a mediator of Notch signaling, when deficient leads to the same phenotype as notch inhibition [43, 44].

Transcription factors are required for cell differentiation initiation and maintenance. A schematic of the most important factors is presented in **Figure 1.1 and 1.2**. The differential expression of the endodermal marker gene, Pdx-1 plays an important role in pancreatic cell development. Pdx-1 is one of the earliest transcriptional factors detected and is expressed entirely throughout the primitive pancreatic organ and the surrounding gut tube [19]. Pdx-1 is a homolog of the Xenopus gene homeobox 8 (Hox-8), which is expressed in the same areas as Pdx-1 [45]. Pdx-1 is a very important homeodomain transcription factor since its loss causes pancreatic agenesis although insulin and glucagon cells remain in the early embryonic buds [37]. Pdx-1 expression patterns are maintained throughout

development and control spatial and temporal developments. The expression of Pdx-1 is detected at E8.5, before the appearance of insulin and glucagon, in the foregut endoderm. Pdx-1 can be seen in both dorsal and ventral buds on E9.5 [46]. Pdx-1 is expressed in the ductal branches between E11.5-E13.5. Between E14-E15 the exocrine pancreas develops and Pdx-1 can be seen in the forming islets. In the adult mouse Pdx-1 is only expressed in the β-cells and most likely is a transcription factor for insulin [19].

Ptf1a/p48 is another equally important transcription factor of the basic helix-loop-helix family, it is first detected at E15 and is present throughout life [31]. Ptf1a/p48 RNA is observed in the pancreas on E12 and is produced within the exocrine and endocrine pancreas but at later stages is restricted exclusively in exocrine cells [47]. Knockout of this gene will cause the pups to die shortly after birth and they lack the exocrine pancreas [48]. The inactivation of Ptf1a/p48 alters the pancreatic cells such that they develop into intestinal cells [49]. Pdx-1 and Ptf1a/p48 are both expressed in pancreatic precursor cells but Ptf1a/p48 is restricted to pancreatic cells while Pdx-1 can be found in the stomach and duodenum [46, 49]. These two transcription factors are extremely important for the appearance of the following transcription factors.

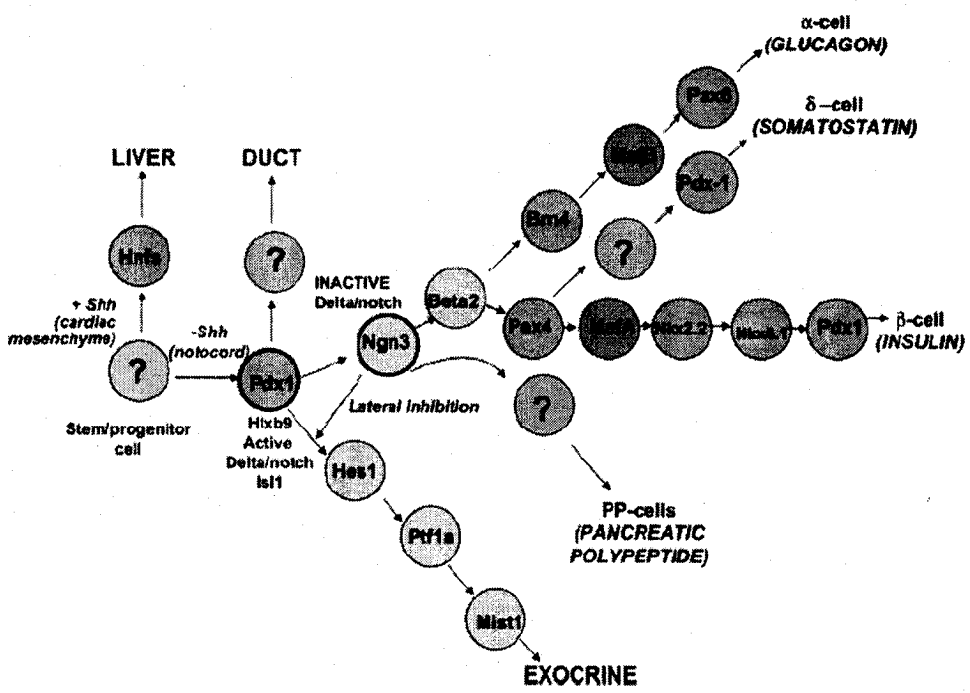
Isl-1, a LIM homeodomain protein, is present in the developing pancreas and in adults is expressed in all of the islet cells but in very low levels in the β-cells [24, 50]. Disruption of the Isl-1 gene is embryonic lethal due to arrest of embryogenesis on E9.5 [51]. Studies show that Isl-1 is important for development of the dorsal bud and differentiation of dorsal epithelium to endocrine cells [52].

NK2 transcription factor related, locus 1 (Nkx 6.1) a homeodomain protein, is initially expressed in a subset of cells in the pancreatic bud but is then restricted to the β-cells, indicating that it may play a role in β-cell differentiation and function [31, 53]. Many other transcription factors such as SRY-box containing gene 17 (Sox-17), Hes1, Neurogenin-3, Notch, neurogenic differentiation factor 1 (Beta2/NeuroD), Hex, prospero-related homeobox 1 (prox-1), Tlx-1, Mist1, brn-4, pax-4 and pax-6 are all expressed in the early pancreas [31, 54-56]. Each exocrine cell requires a specific pattern of transcription factors to form a fully functional cell. The whole primitive pancreatic organ also expresses the enzyme L-amino acid decarboxylase (AADC) which is later restricted to the islets after birth [57]. Disruptions of these transcription factors result in altered pancreatic develop such as impaired islet cell formation or maturity onset diabetes of the young (MODY).

Figure 1.2 Transcription factors expressed in the developing rodent pancreas.

A hierarchy of transcription factors determined based on their temporal arrival or studies from knockout experiments. The color scheme designates the type (class) of transcription factors involved in the proposed hierarchal cascade: *pink*, homeodomain proteins; *purple*, bHLH; *green*, Maf proteins; *orange*, Hnfs; and *gray*, unidentified factors [30].

Habener, J.F., D.M. Kemp, and M.K. Thomas, *Minireview: transcriptional regulation in pancreatic development.* Endocrinology, 2005. **146**(3): p. 1025-34. Copyright 2005, The Endocrine Society.



1.2.5 Pancreatic tissue regeneration

Under normal conditions the β-cells don't normally regenerate because the neogenesis factors are quiescent but once the pancreas has been damaged the acinar and β-cells can regenerate via the reactivation of precursor cells. Different experimental models can be used to study pancreatic regeneration.

Partial pancreatectomy is a common model used to study regeneration. New islet-like formations emerge after a partial pancreatectomy, they seem to differentiate from ductal epithelium very reminiscent of islet formation during embryogenesis which suggests precursor cells present in the adult can be activated [58]. The acinar cells and the islet cells show an increased mitotic index during the first two weeks after pancreatectomy [59]. Recently an in vivo genetic approach showed that pre-existing β-cells and not multipotent stem cells or pleuripotent precursor cells, make up the pool of new β-cells during adult life [60]. However, the theory that a small pool of insulin positive cells serves as precursor cells, which have the capacity to proliferate, and not being fully differentiated β-cells is still relevant. A recent study on this issue showed that a serum free culture led to the generation of various lineages with exocrine and endocrine pancreatic phenotypes. These precursor cells can be found in both pancreatic ducts as well as islets [61].

Another model used to study regeneration is partial duct ligation, which leads to exocrine juices flowing into the interstitial space and causing tissue damage. A week following the ligation, the ligated portion of the pancreas exhibits hyperplasia of the β-cells but with only a slight increase in β-cell

replication and the animals remain normoglycemic. Therefore neogenesis may be the main cause of the hyperplasia [62]. Pancreatic regeneration may occur through various processes i.e. proliferation of differentiated cells and activation of precursor cells.

Destruction of the islets by the use of streptozotocin, a deoxyribonucleic acid (DNA) alkylating agent, is a third model of β-cell regeneration. The specificity of the damage is due to the selective uptake of the drug by the β-cells, although at increased doses damage can occur to other organs [63]. The damage to the β-cells varies on the dose and injection site used. For example, a rat given 100 mg/kg intravenous dose on P1 will lose 90% of it's β-cell mass within 2 days, 3 weeks later the β-cell mass is still not restored and at 6 weeks the rats become glucose intolerant [64]. Streptozotocin given between P1-P5 greatly reduces the β-cells ability to regenerate due to a decrease in the rate of neogenesis [65]. In neonatal rats, if glucagon-like peptide-1 (GLP-1) is co-administered with the streptozotocin β-cell neogenesis is not inhibited and glucose homeostasis is maintained into adulthood [66]. In adult rodents if the β-cells are destroyed the animals usually remain diabetic due to lack of regeneration and eventually die without intervention [67].

Transdifferentiation

Transdifferentiation is classified as a switch from one differentiated cell type to another. In the pancreas severe damage leads to metaplasia, the acinar cells morph into duct-like cells [68, 69]. In vitro isolated adult acinar cells and an

embryonic acinar cell line can transdifferentiate into hepatocytes and the embryonic acinar cell line can even transdifferentiate into endocrine cells [70-72]. Transdifferentiation may also occur in vivo: in ductal ligation experiments the acinar cells turn into duct-like cells expressing endocrine markers and secrete insulin [73]. Recently, exocrine cells cultured with epidermal growth factor (EGF) and leukemia inhibitory factor (LIF) switched into functional β-cells (i.e. ability to secrete insulin) [74]. The ventral pancreas and the liver both derive from the same precursor cell population in the embryo. There have been instances where cells in the liver had a pancreatic phenotype: chemical or tumor formation in the liver can cause transdifferentiation of the liver into pancreas [75]. Pdx-1 gene transfer into the liver induces in the mouse both endocrine and exocrine gene expression [76, 77]. The loss of the transcription factor Hes-1 in embryos can also result in transdifferentiation of non-pancreatic endoderm into pancreatic tissue [78]. Still unknown is the origin of these cells: are they pleuripotent precursor cells or differentiated cells that need to be dedifferentiated.

1.2.6 Generation of pancreatic cells from precursor cells

Stem cells are multipotent, self-renewing cells. It is clear that in the liver stem cells, i.e. oval cells, do exist and they play an important role during liver regeneration [79]. The case is not so clear cut in the pancreas. Some studies claim that islets or exocrine ducts contain precursor cells but these are not true stem cells [80, 81]. Evidence for true stem cells residing in the pancreas has been shown recently. A report claimed that cells isolated from rodent pancreatic tissue

and subcultured for long periods of time can differentiate into insulin-producing, self-renewing cells which upon transplantation can reverse diabetes [82]. Another study reported that nestin-expressing, self-renewing cells can generate endocrine and exocrine cells [83]. Finally a claim was made that the pancreas is able to give rise to clonigenic cells which secrete and express insulin, although they have a finite renewal capacity [61]. It is not known if these cells actually play a role in β-cell differentiation, growth and expansion.

Cell therapy is an attractive treatment for diabetes. There are many protocols on how to generate insulin-producing cells from stem cells in vitro. The problem lies in the definition of a functional β-cell. Several groups have used the ectopic expression of Pax-4, a β-cell differentiation factor, or Pdx-1, a pancreatic precursor to generate β-cells from mouse embryonic stem cells [33, 84, 85]. Work still needs to be done to get functional β-cells in a large enough quantity in order to be used in transplantations.

1.3 Dynamics of the β-cell mass

The β-cell mass is indeed dynamic, determined by positive factors (β-cell hypertrophy, hyperplasia and neogenesis) and negative factors (β-cell hypotrophy and death) [86]. Hyperplasia and neogenesis can both contribute to the expansion of the β-cell mass [87]. Recent studies have shown that neogenesis is the main contributor to increase the β-cell mass [88, 89]. However an interesting report has shown that replication of pre-existing β-cells using radiolabeling (of β-cells) is the only contributor to the expansion of the β-cell mass [60]. In rodent models of

insulin resistance and T2D, proliferation is the main mechanism of the expansion of the β-cells whereas in humans there is evidence for increased neogenesis [11].

1.3.1 Fetal growth of the islets

The number of β-cells increases during the late fetal gestation period in rats, a doubling of the β-cell mass occurs everyday from E16 to birth [90]. Not all β-cells are replicating at once; in the rat approximately 10% of β-cells can participate in cell progression [91]. 10-20% of the new β-cells are formed from cell division; the other 80% are formed from neogenesis of precursor cells. The cells surrounding the islets exhibit a higher rate of DNA synthesis than the islets and these cells contain cytokeratin proteins that are usually found in mature exocrine ducts. At times these "duct cells" co-express insulin; these results are clear evidence for the hypothesis that duct cells can be the precursors of β-cells [92]. Similar results can be found in the human. In human fetal pancreata, the β-cells express cytokeratin-19, a protein normally found in duct cells [93]. The β-cell expansion, which occurs in early fetal life, is critical because it determines how many β-cells one will have later in life. A study in rats was performed using a model of intrauterine growth retardation by way of restricting blood flow to the fetus so that there were no differences between the β-cell mass at 1 and 7 weeks but at 15 weeks the intrauterine growth retarded rats had half of the β-cell mass and interestingly the growth retarded rats developed glucose intolerance perhaps due to islet-deficiency [93].

1.3.2 Postnatal growth of the β-cells

In rodents, neonatal growth of the β-cells does occur albeit at a lower frequency than during fetal growth [90, 94]. β-cell neogenesis and replication are both hypothesized to play a role. Certain duct-like structures have been reported to have a very high replication rate during the first weeks of life and then these ducts disappear leading some to believe that they are a potential source of neogenesis [64]. Transgenic models have been used to clarify which process occurs. Cyclin D2 knockout mice, which lack the positive regulator of cell cycle progression, exhibit a normal β-cell mass at birth but there is no expansion of β-cells between the first and second weeks after birth. However, they exhibit replicative acinar and ductal cells, therefore it was concluded that β-cell mass during the first week of life expands by replication and not neogenesis, although there is still controversy over this debate [95].

The growth of the β-cell mass slows down after weaning but still continues at a very low frequency, around 2-3% per day and the growth occurs by hypertrophy more than neogenesis [65, 96, 97]. The β-cell replication rate becomes quite low in 3 month old rats; only 0.25% of the β-cells are in the S-phase of the cell cycle [65]. The β-cell mass and body weight do exhibit a linear correlation but with increasing age there is a reduction in β-cell number per kilogram body weight [65]. In obese rats, the β-cell mass does increase correspondingly, for example, in the obese nondiabetic Zucker rats (mutation in fa/fa gene), their β-cell mass increases 4 fold versus wild-type rats [98]. The β-

cell mass increases quite significantly during pregnancy: in rats the β-cell mass increases 2.5 fold due to increased cell number and hypertrophy [99].

There are a number of hormones and growth factors which can regulate the β-cell mass. The following section will briefly describe some of the most important regulators of the β-cell mass; growth hormone (GH), insulin, insulin-like growth factor-I (IGF-I) and will be discussed in their respective sections (**1.4.6, 1.5.5,** and **1.7.5**).

1.3.2A Glucose

Glucose is known to have an effect on the β-cell mass. In vitro cultures of fetal islets subjected to high level glucose concentrations will increase the number of proliferating β-cells [100]. 24 h glucose infusion in rats will increase the β-cell mass by 50%; a week after the infusion the β-cell mass returns to normal due to increased rates of apoptosis [101]. In hyperglycemic-hyperinsulinemic rats and hyperglycemic-euinsulinemic rats there is a 50% increase in β-cell number within 48 h, due to increased neogenesis [102]. Increased β-cell replication and hypertrophy occur if the glucose infusion is continued and the β-cell mass will remain larger even when the infusion is arrested [103, 104]. High glucose levels have been shown to increase β-cell survival by inhibiting apoptotic mechanisms but it has also been shown that at the same level glucose can become toxic to β-cells due to the secretion of IL-1 [105, 106]. Chronic hyperglycemia is usually considered detrimental because it can exhaust the β-cells do to the continuous need to secrete insulin therefore causing cell death, leading to T2D [13].

1.3.2B GLP-1

GLP-1 is an incretin, which affects β-cell function, neogenesis, replication, and apoptosis. In the ob/ob (leptin deficient, hyperglycemic, obese) mouse model an analog of GLP-1 can increase β-cell replication, islet cell mass and improve glycemia [107]. db/db (mutation in leptin receptor) diabetic mice given GLP-1 have delayed onset of diabetes and, if mice are diabetic, GLP-1 causes neogenesis of the β-cells from ductal cells expressing Pdx-1 and decreases glycemia [108, 109]. GLP-1 is able to differentiate rat and human ductal precursor cells into β-cells which express Pdx-1 and secrete insulin [110, 111]. GLP-1 can decrease blood glucose levels by inhibiting appetite and reducing mobility of the gastrointestinal tract as well as decreasing glucagon release and increasing glucose-stimulated insulin release [112]. GLP-1 also has the ability to stimulate β-cell neogenesis in human progenitor cells [113].

1.3.2C INGAP

Islet neogenesis associated protein (INGAP) is a member of the Reg family proteins involved in β-cell expansion. It has been shown that the in vivo addition of a portion of INGAP, namely residues 104-118, can cause an increase in β-cell mass in a dose-dependent manner. In a mouse model of T1D the addition of INGAP 104-118 can actually reverse the diabetes [89]. It has recently been shown that INGAP 104-118 can cause human derived duct-like structures to differentiate into glucose responsive islets [114]. This is intriguing because human derived duct-like structures are progenitor cells, which are highly

proliferative therefore they represent a population of cells, which can potentially be used in islet transplantation.

1.3.2D Growth Factors

The addition of both gastrin and EGF will increase the β-cell mass in rodents; likely due to increased neogenesis [88]. In human islets this dual addition of gastrin and EGF will also increase the expansion of the β-cells, most likely through the same mechanism as in rodents [115]. This study also shows that gastrin and EGF increase β-cell mass in vivo as well as in vitro. Non-obese diabetic (NOD) mice receiving transplanted human pancreatic islets displayed increased glucagon and insulin contents upon treatment with gastrin and EGF [115].

Insulin-like growth factor-II (IGF-II) plays an important role in the embryogenesis and neonatal life of the β-cell since it has an anti-apoptotic effect, although in the adult, addition of IGF-II to cultured β-cells does not lead to an increase in β-cells [116, 117]. Hepatocyte growth factor may increase β-cell number because addition of it to cultured islet cells stimulates human islet cell growth; but there are some reports that claim it is the islet ductal precursor cells which undergo expansion and not the β-cells themselves [118, 119].

1.3.2E Cell cycle regulators

The cell cycle is regulated by positive influences such as cyclins and cyclin-dependent kinases (CDK) and negative regulators like CDK inhibitors and tumor

suppressors. The majority of adult β-cells are resistant to mitotic stimulation. Cyclin D2 is necessary for neonatal replication of β-cells, but not necessary for embryonic development of β-cells revealed by the cyclin D2 knockout mouse [95]. Growth hormone and prolactin through the JAK-2/STAT-5 pathway increase cyclin D2 expression [120]. In vitro and transgenic CDK-4 overexpression results in increased β-cell proliferation while maintaining β-cell function [121, 122]. β-cell specific overexpression of B-cell leukemia/lymphoma xl protein (Bcl-xL) , an anti-apoptotic factor, prevents β-cell death but results in impaired insulin secretion and hyperglycemia [123]. Cell cycle regulators can be used to alter the β-cell mass, potentially offering a therapeutic use.

1.4 Growth Hormone

1.4.1 Structure of growth hormone

Growth hormone (GH), also known as somatotropin, was first identified in the 1930's by Lee and Shaeffer and Li and Evans [124]. It is a member of the GH/cytokine gene family which includes prolactin and placental lactogen. GH displays structural and sequence homology between most species. GH is encoded by a single gene approximately 2 kb in length consisting of 5 exons and 4 introns [125]. In mammals duplications of the GH gene has given rise to gene clusters. In humans a cluster exists of 5 GH-like genes on the long arm of chromosome 17. At the 5' end, the hGH-N gene is found which secretes the classical GH and is expressed in the pituitary. The other four genes are expressed in the placenta [126, 127]. GH is a polypeptide composed of 191 amino acids; it is synthesized

primarily by the anterior pituitary gland as well as in many extrapituitary tissues. The GH molecule consists of two disulfide bonds, four alpha helices that run up-up-down-down, opposite of what is normally seen, up-down-up-down and three shorter helices. GH is post-translationally modified in the pituitary and circulation; it undergoes glycosylation, acetylation, phosphorylation, and deamidation. Many of GH's proteolytically modified forms retain some functionality [128]. The secreted GH forms are 22 kDa and 20 kDa, which account for 90% and 10% of the circulating GH levels, respectively. The 22 kDa form is the typical GH; it consists of amino acids 1-191 and promotes growth, anabolic lipolysis and other energy/metabolic processes [124]. The 20 kDa form lacks amino acids 32-46 due to alternative splicing in exon 3 and is more somatogenic than the 22 kDa form [124]. Both forms of GH are secreted in bursts from the pituitary in a constant molar ratio, although circulating levels vary among species and gender. The crystallographic structure shows that GH binds 2 molecules of the growth hormone receptor (GHR) [128]. The receptor binding sites for the GHR are located on opposite sides of the GH molecule.

There is also the presence of the growth hormone binding protein (GHBP), it is the truncated version of GHR consisting solely of the extracellular domain [129]. In humans the GHBP is formed due to proteolytic cleavage of the GHR. In rodents alternative splicing of the GHR mRNA forms the GHBP and this results in the receptor transmembrane and cytoplasmic domain being replaced by a short hydrophilic peptide not present on the full length GHR. [130]. The role of GHBP is not exactly clear but it may sequester GH from the cell surface, stabilize GH

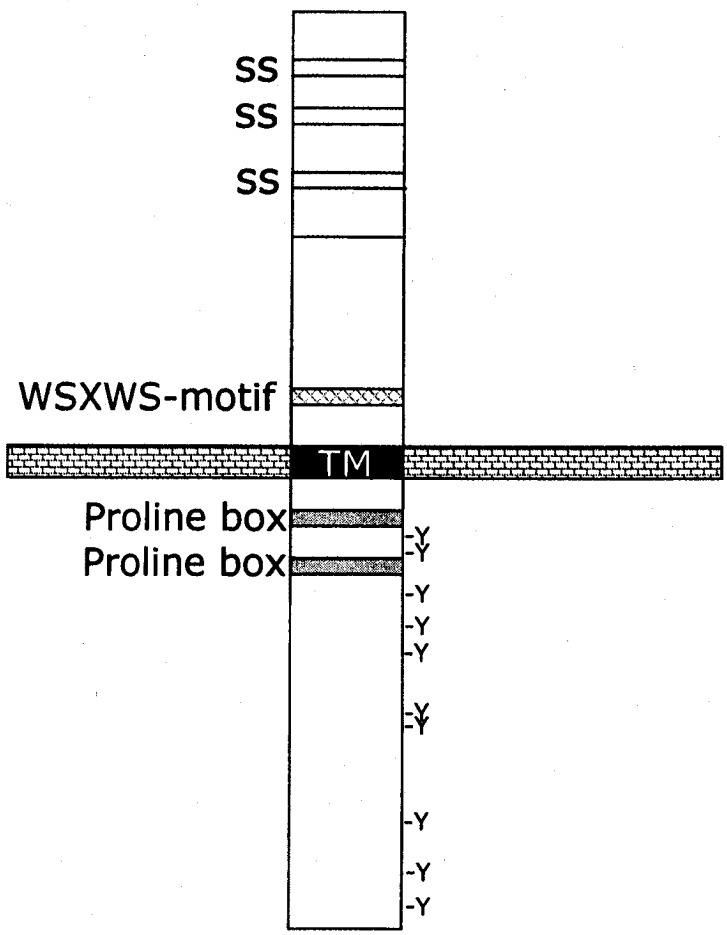
bioavailability, or form complexes with GH and GHR to prevent GH from signaling [131, 132].

1.4.2 Structure of the GHR

The GHR is a class 1 cytokine receptor, 620 amino acids long, present on the surface of almost all cells. The receptor is synthesized as a 640 amino acid preprotein with an N-terminal signal peptide [133]. The structure of the GHR is presented in **Figure 1.3.** Cleavage of the signal peptide produces the mature GHR which has a single transmembrane portion of 24 amino acids, with an extracellular ligand binding domain consisting of 246 amino acids and a 350 residue intracellular cytoplasmic domain responsible for signaling, internalization and receptor downregulation [130]. Like other members of the cytokine receptor family the GHR has 3 pairs of disulphide-linked cysteines in the N-terminal extracellular domain residues which play important functional and structural roles, a WSXWS extracellular motif for ligand specificity, multiple tyrosine phosphorylation sites, and two cytoplasmic proline rich boxes, necessary for signal transduction [134, 135]. In the humans there are 14 variants of the GHR mRNA with differences in their 5' untranslated region [136, 137].

Two truncated membrane bound forms of the GHR (GHR 1-277 and GHR 1-279) exist. They are due to alternative splicing and their levels are variable in extracellular tissues. The roles of the short forms of the GHR are not very clear but they are easily proteolysed and may account for the majority of the

Figure 1.3 Schematic representation of the GHR.
The receptor consists of an extracellular domain with three disulfide linked cysteines (SS) important for functional and structural actions, a WSXWS motif essential for ligand specificity, a single spanning transmembrane domain, and an intracellular region with 2 proline rich boxes critical for signal transduction and multiple tyrosine phosphorylation sites necessary to bind downstream signaling molecules [136].



GHBP. They can also act as natural dominant negative proteins therefore inhibiting GH actions [138, 139].

The GHR is expressed primarily in the liver but can be found in the muscle, kidney, bone, adipose, mammary gland, and embryonic stem cells [140]. GHR synthesis can be influenced at different levels, including; transcription, translation, and posttranslational modifications [141].

1.4.3 GH signaling pathway

A single GH molecule binds to the extracellular domain of 2 dimerized GHRs via 2 contact sites, at sites I and II [128]. The GHR intracellular portion does not contain any intrinsic kinase activity. A cartoon of the GH signaling pathway is depicted in **Figure 1.4**. The tyrosine kinase JAK, in the case of GHR, primarily JAK-2, although JAK-1 and JAK-3 have been reported to be used, binds to a proline rich sequence on the cytoplasmic domain of each receptor. This brings the two kinases close enough to each other to allow for transphosphorylation and thus JAK activation [142, 143]. JAK kinases are unique because they lack either a src homology 2 domain (SH2) or src homology 3 domain (SH3) domains and instead, have 7 conserved JAK homology domains (JH) regions. JH1 consists of the catalytic domain and JH2 of a pseudokinase domain, which acts as a negative regulator of JAK-2. Almost all pathways, triggered by GH activation require the activation of JAK-2. JAKs are associated with the receptors at all times and the binding of GH stabilizes the complex. In some cases JAK can activate a nearby receptor such as v-erb-b2 erythroblastic

leukemia viral oncogene homolog 2 (erbB-2) [144]. The proline rich box on GHR is required to activate JAK-2 [145].

Once JAK-2 is activated, it will phosphorylate the intracellular domain of the GHR. The phosphorylated JAK-2 as well as the phosphorylated GHR act as docking sites for SH2- and PTB-containing molecules. SH2-containing proteins such as STATs are transcription factors which will homo- or hetero-dimerize after phosphorylation on conserved tyrosines by JAK-2. Dimerized STATs can enter the nucleus to target gene activation [146]. STATs bind to γ sequence elements consisting of 9 base pair palidromic sequences [147, 148]. There are 7 STATs (-1, -2, -3, -4, -5a, -5b and -6) four of which (-1, -3, -5a, and -5b) can become activated in response to GH [149]. The functions of STATs are to produce the majority of GH effects. STAT-5b plays an important role in the regulation of gene expression needed for the masculinization of the liver in the male rat and growth [150].

GHR is also able to activate the mitogen-activated protein kinases (MAPK) signaling pathway that plays an important role in cell growth and proliferation. Src homology 2 domain containing (Shc), an adaptor molecule, binds to the phosphorylated JAK and then becomes phosphorylated itself. Through a series of recruitments and phosphorylations, i.e. of growth factor receptor bound protein (Grb2), son of sevenless homolog (SOS), Harvey rat sarcoma virus oncogene 1 (Ras), v-raf-leukemia viral oncogene 1 (Raf), and MAPK/ERK kinase (MEK) cell cycle and growth promoting genes are activated [151].

Insulin receptor substrate (IRS)-1 and IRS-2 can also interact with GHR complexes [152, 153]. GHR can phosphorylate IRS-1 and IRS-2 via JAK-2. Once phosphorylated, IRS-1 and IRS-2 are docking sites for SH2 containing proteins such as the p85 subunit of phosphatidylinositol 3 kinase (PI3K). PI3K phosphorylates inositol lipids to generate polyphosphoinositides such as Phosphatidylinositol-3,4,5-trisphosphate (Ptdins-3, 4, 5-P_3) which recruits phosphoinositide-dependent protein kinase 1 (PDK1), v-akt murine thymoma viral oncogene homolog 1/ protein kinase B (AKT/PKB) and protein kinase C (PKC) to the cell membrane [154]. PI3K is involved in many processes such as DNA synthesis, glucose uptake, cell cycle regulation and inhibition of apoptosis. Therefore most of the metabolic effects of GH seem to occur via the IRS molecules [155, 156].

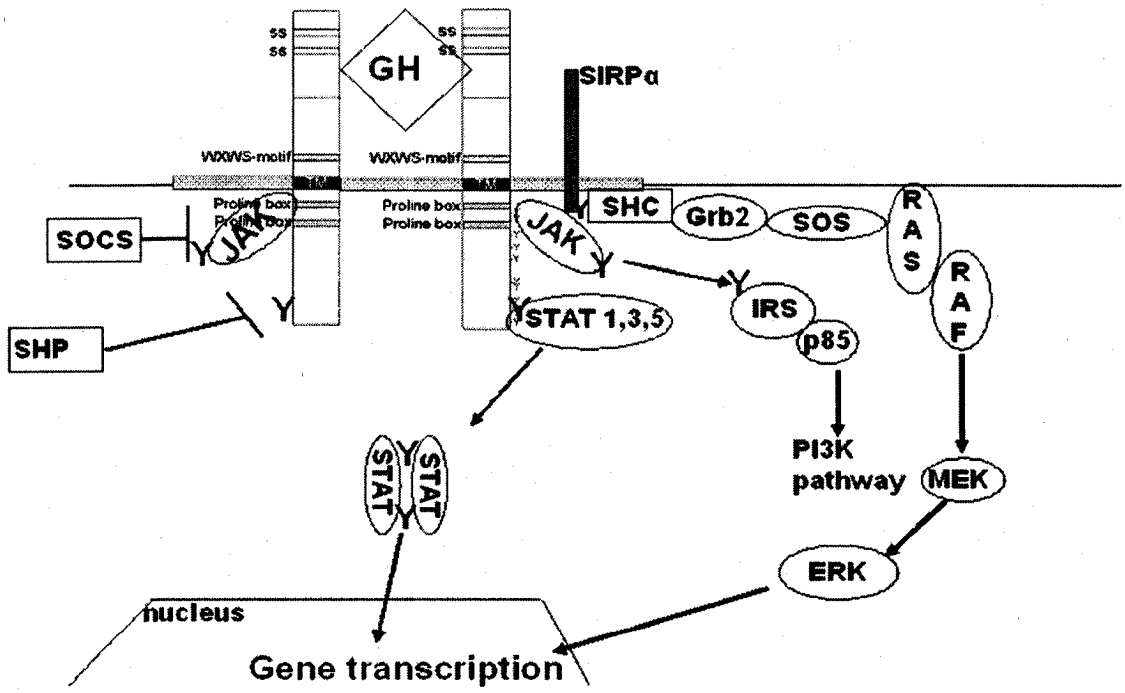
PKC also plays a role in GH signal transduction. A transient increase in diacylglycerol, a mediator of PKC, occurs after GH stimulation. In the kidneys GH also increases inositol trisphosphate [157, 158]. GH stimulated lipogenesis, c-fos induction, and activation of CCAAT binding proteins can be inhibited with the use of PKC inhibitors [159-161].

GH is known to regulate the expression of IGF-I and insulin-like growth factor binding proteins (IGFBP) genes. As part of the IGF/BP complex, the acid labile subunit (ALS) which complexes with IGFBP-3 and STAT-5a/b have known GH responsive elements [162, 163].

Figure 1.4 GH signaling pathway

GH binds to the dimerized cell surface receptors with subsequent association and phosphorylation of JAK-2 with the GH receptor. JAK-2 is then responsible for subsequent phosphorylation of numerous signaling molecules (including GHR). These include: (1) STAT family members including STAT-1, STAT-3, STAT-5a and STAT-5b. The STATs dimerize and are translocated to the nucleus to activate gene transcription (2) adaptor proteins such as Shc, and Grb2 lead to the activation of the MAPK family (3) and members of the IRS group including IRS-1 and IRS-2, which may act as docking proteins for further activation of signaling molecules including phosphatidylinositol-3 kinase. Negative regulation is present in the forms of SOCS,SHP and SIRPα.

JAK; Janus kinase; STAT, signal transducers and activators of transcription; SOCS, suppressors of cytokine signaling; SHP2, protein tyrosine phosphatase [142].



1.4.4 Downregulation of GH signaling

The primary determinant of GH responsiveness is the level of GHR at the cell membrane. Downregulation of GH signaling can occur at several levels and by different stimuli. GHR can be eliminated from the cell surface by endocytosis, the first step in receptor downregulation [164]. GHR is internalized constantly but binding to GH increases the rate of internalization and ubiquitination, through caveolae and clathrin-coated pits [165, 166]. Internalization requires a functional ubiquitin system and ubquitination commences at the cell membrane [167, 168]. The receptor ligand complex is degraded in the proteasome or lysosome and there is no recycling of the receptor to the cell surface [169]. The 26S proteasome complex is an important site to degrade the ubiquitinalyted GHR, since blocking the proteasome will enhance the GHR phosphorylation and JAK-2/STAT-5 activity and block GHR internalization and degradation [170-172].

Homologous desensitization occurs when excessive GH inactivates and desensitizes further activation of the GHR, this mechanism is attributed to various proteins. The suppressor of cytokine signaling (SOCS) family of regulatory proteins and several protein tyrosine phosphatases are believed to play a role in decreasing the levels of the GHR or decreasing the level of phosphorylation of the GHR or JAK-2 [173, 174]. SOCS are usually found in low concentrations in the cell but increase significantly after stimulation with GH [175]. SOCS binds to the receptor/JAK complex, and competes with downstream substrates, and can also inhibit STAT dimerization and nuclear entry, all of which lead to an inhibition of GH signaling (**Figure 1.4**) [176, 177]. SOCS2 knockout mice are giants, due to

increased organ size and bone growth, providing further evidence that SOCS plays a negative role in GH signal transduction [178, 179].

SH2B-β, a JAK-2-associating protein, increases JAK-2 activation and manipulating the level of SH2B-β can indirectly effect GH signaling [180, 181]. Protein phosphatases such as src homology phosphatase (SHP)-1, protein tyrosine phosphatase (PTP)-1B and PTP-H1 are important molecules in turning off GH signaling [141]. Phosphotyrosine phosphatase inhibitors increase the length of time that JAK-2 and STAT-5 are phosphorylated [182]. GH stimulates SHP-1 expression, and causes it to enter the nucleus, bind to STAT-5b and inhibit its activity [183]. PTP1B dephosphorylates JAK-2, which shuts off GH signaling [184]. PTP-H1 can dephosphorylate the GHR itself, impairing it's ability to signal [185]. Signal-regulatory protein (SIRP)-α is a transmembrane glycoprotein associated with SHP-2, Grb-2 and SHP-1 [186]. JAK-2 phosphorylates SIRP-α allowing binding to SHP-2, (**Figure 1.4**) [187]. Overexpression of SIRP-α negatively regulates GH stimulated MAPK signaling by inhibiting phosphorylation of JAK-2, STAT-3, STAT-5b, extracellular signal-regulated kinases (ERK)-1 and ERK-2 [186].

Heterologous desensitization occurs when other stimuli dampen GH's signals. For example insulin has an effect on GH signaling. A study has shown that in rat osteoblasts insulin reduces GH binding to the GHR without affecting GHR levels. The authors suggest that insulin causes a decrease in the translocation of the GHR [188]. There is also crosstalk at the level of MAPK/IRS-

1 between the insulin and GH signaling pathways which can influence each others activities e.g. GH can induce phosphorylation of IRS-1 to -3 [144].

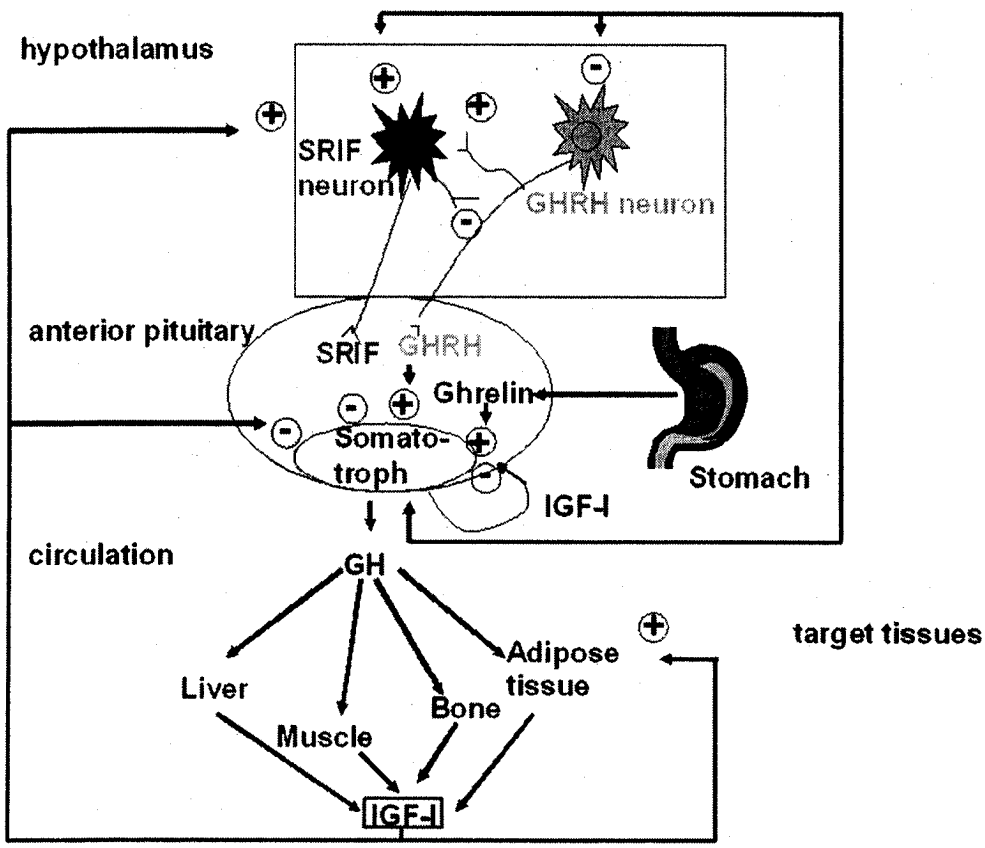
1.4.5 The actions of GH

GH, an anabolic hormone, plays a role in many organs and systems; it affects longitudinal growth, protein, lipid and carbohydrate metabolism [189]. In adult rodents GH is secreted in a sexually dimorphic pattern, males secrete GH every 3-4 hours with a distinct trough during the nadir while females secrete GH more randomly with low peak and high trough levels. The secretion differences are due to sexual differences in the secretion of growth hormone releasing hormone (GHRH) and somatostatin [190]. It has been demonstrated that the pulsatile pattern of GH secretion is more effective for stimulating growth and IGF-I levels than a continuous secretion [191, 192]. Hyper- or hypo-secretion can lead to medical disorders [193]. **Figure 1.5** depicts the regulation of GH secretion. The Somatomedin hypothesis states that the observed effects of GH are mediated via a growth factor- somatomedin now termed IGF-I [143]. It is now believed that not all actions of growth hormone are mediated by IGF-I. GH secretion is regulated by the hypothalamic positive stimulating factor, GHRH and the negative regulating factor, somatostatin. There are other regulators such as ghrelin and IGF-I. Ghrelin, a 28 amino acid peptide is expressed in the endocrine cells of the stomach and at low levels in the arcuate nucleus, stimulates GH secretion. IGF-I present in the circulation acts in a feedback loop and inhibits GH secretion and influences GH actions on peripheral tissues. [189, 193, 194].

There are many other factors, which influence GH secretion. FFA directly inhibit GH secretion via the pituitary, constituting a feedback loop which occurs because GH induces lipid mobilization [195, 196]. Leptin acts via the hypothalamus to stimulate GH secretion; it may also act through NPY since leptin inhibits NPY expression and NPY inhibits GH secretion [197-199]. There is evidence that GH is synthesized in areas other than the pituitary such as the placenta, neutrophils, lateral hypothalamus, lymphocytes and mammary tissues [200-204]. These extrapituitary sites may be important for IGF-independent or perhaps autocrine and paracrine effects.

Most somatic growth, except intrauterine growth, is dependent on the pulsatile pattern of GH secretion, especially during pubertal growth [205]. GH has a variety of physiological effects; a few will be briefly described. Patients with GH-deficiency exhibit muscle dystrophy, a condition that can be rescued by giving recombinant human (rh)GH, restore muscle size [206, 207]. GH was thought to have it's most important role in the skeletal muscle because it stimulates amino acid uptake but there is evidence that in other tissues GH plays an equally important role in nitrogen balance [208, 209]. Long term treatment of GH increases lean body weight, kinetic studies indicate that this is due to stimulation of protein synthesis and not inhibition of protein degradation [210]. The GH effects on muscles are largely dependent on IGF-I [211]. GH also inhibits fat mass accumulation and increases lipid mobilization, often seen in GH treated geriatric and GH-deficient patients [212]. GH is necessary for bone growth and

Figure 1.5 Schematic representation of the regulation of GH
Somatostatin (SRIF) and GH releasing hormone (GHRH) hypothalamic neurons control each other directly. SRIF and GHRH are released from the median eminence of the hypothalamus and reach somatotrophic cells of the pituitary via the portal vessels and inhibit or stimulate GH secretion, respectively. GH regulates its own secretion either by stimulating or inhibiting the secretion of SRIF and GHRH. GH inhibits GHRH mRNA synthesis and GHRH release in the hypothalamus, and stimulates mRNA synthesis of SRIF and SRIF release. GH also stimulates IGF-I secretion from somatotrophic cells (ultra short feedback loop). GH is released into the circulation as secretory spikes. The circulating GH then binds to GHRs on target tissues including liver, muscle, bone and adipose. GH promotes paracrine or endocrine secretion of IGF-I that, in turn with GH, stimulates tissue proliferation. IGF-I produced in the target tissues, in the long feedback loop scheme, will inhibit GH secretion either directly at the somatotrophs or indirectly by stimulation of SRIF release. Ghrelin, is secreted from the stomach and is believed to act on somatotrophic cells to stimulate GH secretion [191].



development. GH, through an increase in both local and circulating concentrations of IGF-I, stimulates long bone growth via chrondrocyte proliferation, differentiation and calcification [213]. GH depends on IGF-I to stimulate osteoblast maturation and type I collagen formation [214].

GH given for a short term stimulates glucose uptake but chronic administration leads to hyperinsulinemia and insulin resistance due to impairments in insulin signaling [215]. The short-term, insulin-like effect is not dependent on IGF-I but on GH induced phosphorylation of IRS-1 and IRS-2 may play a role [216, 217]. Consequently, chronic administration of GH results in increased hepatic gluconeogenesis and glycogenolysis due to GH stimulated lipolysis and inhibition of insulin activity through increased free fatty acids [218].

GH has IGF-I independent effects. The most convincing evidence is the observation that IGF-I knockout mice have enlarged growth plates and GH administration to these mice results in hepatomegaly [219, 220]. Hepatic growth is largely dependent on GH alone. GH and IGF-I both induce bone morphogenetic protein (BMP) expression but GH along with anti-IGF antibodies will still increase the expression of BMPs, demonstrating an IGF-I independent effect [221]. There have been other experiments showing that β-cell proliferation and growth of epiphyseal chrondocytes can be achieved by GH alone [222, 223].

1.4.6 GH effects on β-cells

The expression of the GHR gene can be detected in normal islets as well as in islet-derived cell lines [224]. Early experiments suggested that GH plays a role

in stimulating β-cell proliferation, since rats receiving transplanted GH producing tumors had increased β-cell proliferation [225]. When rat pancreatic islets were cultured with GH, it was shown that GH could stimulate β-cell proliferation, glucose-stimulated insulin release and insulin gene expression and synthesis [226, 227]. GH activates STAT-5a/b, STAT-1, and STAT-3 in pancreatic islets or islet-derived tumor cells [228, 229]. It was shown using a dominant negative mutant, that STAT-5 activation is essential for the mitogenic effect of GH in β-cells [230] and is sufficient to drive transcriptional induction of cyclin D2, which promotes the G1 to S phase transition in the cell cycle [231]. In extra-islet cells, GH increases the activity and/or protein level of forkhead box A2 (Foxa-2) and hepatocyte nuclear factor (HNF)-1α, both of them being important factors in β-cell growth and proliferation [232, 233]. Activation of STAT-5 will also inhibit apoptosis by inducing Bcl-xL and B-cell leukemia/lymphoma 2 (Bcl-2), and by decreasing Bcl-2 associated X protein (Bax) gene expression [234-236]. GH can increase the levels of nerve growth factor (NGF) and its receptor, tyrosine kinase receptor type 1 (Trk A), which regulates islet β-cells in an autocrine fashion [237, 238]. GH also induces expression of the preadipocyte factor-1 (Pref-1) in neonatal islets [239]. Pref-1 is a cell surface protein present in adipose and pancreatic tissues [232]. GH induces Foxa-2 and Pref-1 expression during adipocyte differentiation [232]. Pref-1 is present in a subpopulation of islet β-cells and its expression in the pancreas is high in early embryos and during early postnatal days. It can also be induced in the β-cell during pregnancy [239].

GH effect is not limited to β-cell growth; it can also stimulate insulin secretion from islet β-cells. Although the original studies showed that hypophysectomy blunted insulin release [240], it has now been shown that GH induces a strong stimulatory effect on insulin secretion from pancreatic islets of hypophysectomized rats [241, 242]. Using cultured human islet cells, GH increased β-cell growth and insulin release reaching 10-20 fold over a 3-month period [243]. The increase in insulin level was still very significant even after considering increased cell numbers. GH seems to act directly on β-cells since both JAK-2 and STAT-5 are detectable in rat insulinoma cell line (INS-1) cells and GH treatment results in a time-dependent nuclear translocation of STAT-5 [244].

1.5 Insulin

1.5.1 Structure of insulin

Banting and Best at the University of Toronto discovered insulin in 1921. The study of insulin played an important role in the early years of protein chemistry, in 1955 its amino acid sequence was completed and it was the first protein synthesized chemically [245, 246]. The human insulin gene is located on chromosome 11p15.5 in between the IGF-II and tyrosine hydroxylase genes [247], and is composed of 3 exons and 2 introns. Insulin is a peptide of approximately 6 kDa, composed of 2 chains (alpha 21 amino acids and beta 30 amino acids) linked together by two amino acid disulfide bonds at position of A7-B7 and A20-B19 [248]. The A chain consists of an N terminal helix linked to a C terminal helix which forms an internal disulfide bond and the B chain has a

central helix with N and C terminal helices protruding out from it [249]. Insulin dimerizes at micromolar concentrations and these dimer form further hexamers [248]. As a rare example, C elegans present a divergent sequence where insulin contains a hydrophobic core with two surrounding nonpolar areas; one is flat and aromatic forming an antiparallel beta sheet, the other forms hexamers but the basic insulin fold is still present [249].

The transcript of preproinsulin mRNA is 446 bp in length and is translated into a single chain peptide precursor and the removal of its signal peptide sequence during insertion into the endoplasmic reticulum generates proinsulin. Proinsulin is further processed by the removal of the C-chain and the generation of the mature insulin [19]. Most animals contain 1 copy of the gene except the rat and mouse which contain 2 copies each [250]. The mature insulin is stored in secretory granules; the level of its secretion is largely dependent on the level of blood glucose [19].

The promoter sequence of insulin genes is evolutionarily conserved and is important for insulin expression by mediating the rate of transcription initiation. Up to 4 kb upstream of the transcriptional start site is needed for the initiation and regulation of the transcription [251, 252]. Insulin gene expression is exclusive to the β-cells and cannot be transcribed in any other cell type due to the fact that the insulin promoter contains cis elements and the combinations of transcription factors that can bind to these elements are unique to the β-cells [253].

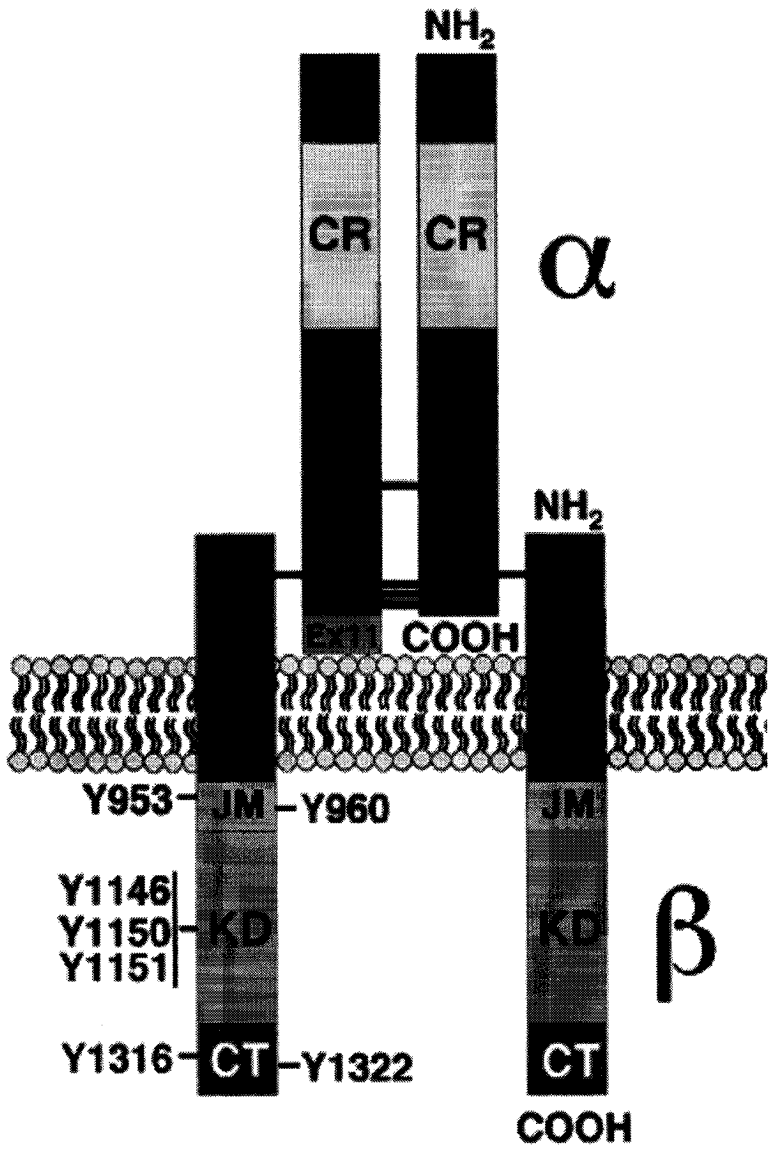
1.5.2 Structure of the insulin receptor

The insulin receptor (IR) is composed of 2 α- and 2 β-subunits covalently linked by disulfide bonds; a schematic is represented in **Figure 1.6**. Each of the subunits performs a different function. The α-subunit contains the ligand-binding domain and the β-subunit contains the tyrosine kinase catalytic domain. The IR is a tyrosine kinase that can transfer a γ-phosphate of adenosine triphosphate (ATP) onto itself. The IR falls into class II of the tyrosine kinase receptor superfamily because of the two cysteine-rich motifs in the extracellular domain; however it, as well as the IGF-I receptor, are different from other class II members in that they are preformed dimers even in the absence of their ligands [254, 255].

The IR is encoded by a gene of 22 exons and 21 introns. Exon 11 can be alternatively spliced resulting in two isoforms, A and B (which lacks or contains exon 11 respectively), which have slightly different affinities for insulin. IR-A is expressed in the development of embryos and in tumor cells and has a higher affinity for IGF-I and IGF-II than the B isoform. IR-B is expressed in adult insulin target cells and has a 100 fold lower affinity for IGFs than insulin [256]. The receptors are synthesized as precursors in single chains that then undergo multiple posttranslational modifications such as glycosylation, folding and dimerization to yield the mature form of the receptor (tetramer of 2 alpha and 2 beta subunits).

The crystal structure of IR has been determined and it shows that the N terminal half of the receptor consists of 2 large homologous domains, L1 and L2, separated by a cysteine rich region consisting of disulfide linked molecules [257].

Figure 1.6 A representation of the structure of the insulin receptor
The *left side* illustrates IR-B, which includes the 12-amino acid alternatively spliced exon11 (Ex11) at the carboxyl terminus of the α-subunit. The *right side* depicts IR-A. The extracellular α- and intracellular β-subunits are depicted. The *horizontal black bars* represent disulfide bonds.
CR, Cysteine-rich domain; JM, juxtamembrane domain; KD, kinase domain; CT, carboxylterminal domain. The positions of the tyrosine autophosphorylation sites are indicated [253].
Watson, R.T., M. Kanzaki, and J.E. Pessin, *Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes.* Endocr Rev, 2004. **25**(2): p. 177-204. Copyright 2004, The Endocrine Society.



The C terminal portion contains 3 fibronectin (Fn) type III domains. An insert domain is found within the second Fn type III domain, its function is unknown but it contains the site of cleavage between the two subunits. The intracellular portion of the β-subunit contains the juxtamembrane region, which has a role in docking IRS-1 to -4 and Shc as well as a role in internalization, 2 regulatory domains, the kinase domain and a C terminal tail. Each portion of the intracellular subunits contain multiple tyrosine phosphorylation sites [248].

In 1989 the orphan insulin-receptor-related receptor (IRR) was identified. It is expressed in pancreatic islets and differentiated β-cell lines. [258]. A chimeric receptor consisting of the extracellular domain of IR and the intracellular domain of IRR was created to help identify its signaling pathway because the cognate ligand of the IRR is unknown [259]. The addition of insulin caused the IRR to become phosphorylated as well as IRS-1 and IRS-2, suggesting that the IRSs serve as substrates for IRR in intact cells [260, 261]. IRR forms heterodimers with both IR and insulin-like growth factor-I receptor (IGF-IR), therefore it might modulate IR and/or IGF-IR signaling [259]. Although a mouse model of the IRR knockout exhibits no phenotype, the lack of phenotype may be explained by gene redundancy. A triple knockout of the IR/IGF-IR/IRR gives some clues to what the orphan receptor may do because the mice examined at E18.5 exhibit gonadal dysgenesis and somatic sex reversal [258].

1.5.3 Insulin signaling pathway

Insulin actions can be either metabolic (e.g. promoting glucose uptake) or mitogenic (e.g. stimulating growth), but in either case to have any action the insulin signal must be transduced. A cartoon of the transduction process can be viewed in **Figure 1.7**. Insulin binding to the receptor leads to the activation of the tyrosine kinase domain, resulting in the autophosphorylation of its tyrosine residues in the β-subunit [262]. The autophosphorylation of the IR is necessary for recruitment and phosphorylation of other cytoplasmic substrates that leads to downstream signaling and will be discussed below [263, 264]. IRs are tightly controlled and once activated by insulin binding undergo rapid endocytosis. Internalization of the insulin-IR complex is regarded as the major mechanism of insulin degradation and downregulation of cell surface receptors.

IR activity is negatively regulated by dephosphorylation of their tyrosine residues by protein-tyrosine phosphatases (PTP) [265]. PTP1B was shown to interact with and be activated by the autophosphorylated IR, in the process it dephosphorylates the receptor thereby reducing it's activity [266, 267]. PTP1B knockouts improve insulin sensitivity via increased signaling [268]. Other molecules, such as SOCS1, SOCS3, and GRB10 inhibit insulin signaling by physically blocking the IR interaction with IRS-1 or interacting with the IR kinase domain [269, 270]. The SOCS are important because, in the state of insulin resistance, they are upregulated [271].

Once the IR is activated, i.e. phosphorylated, the message must be propagated. There are two models, which are not mutually exclusive. The first

model involves the tyrosine phosphorylation of downstream intracellular substrates IRS-1, IRS-2, IRS-3 and IRS-4 [272-275] and the second model consists of tyrosine phosphorylated receptor and noncovalent interactions with other signaling molecules [276]. The IRSs interact with SH2-containing proteins to cause the propagation of the signal, different than most ligand-activated receptors [277].

There are 6 members in the IRS family. IRS-1 and -2 are found in many tissues and cells, IRS-3 is specific to the brain and adipocytes, IRS-4 is limited to embryonic tissues and IRS-5 and -6 are limited to a few tissues [278]. IRS-1 is the primary substrate of the IR tyrosine kinase and contains more than 20-22 tyrosine and 40 serine/threonine residues, which may represent potential protein binding sites to initiate various downstream signaling pathways such the PKC, protein kinase A (PKA), and MAPK pathways [272, 276]. These signaling cascades play a role in coordinating the regulation of kinases and phosphatases important in glycogen metabolism. The N terminus of IRS proteins contains pleckstrin homology (PH) and PTB domains enabling association with the IR, the middle and C termini contain tyrosine phosphorylation sites. On the other hand, serine phosphorylation of IRS in the C-terminus, by insulin abrogates IRS signaling [279].

Tyrosine phosphorylated IRS-1 leads to the activation of PI3K, a heterodimeric enzyme composed of a regulatory subunit (p85) and a catalytic subunit (p110). The p85α subunit contains two SH2 domains and a SH3 domain; this subunit has effects on cell growth and metabolism. PI3K catalyzes the

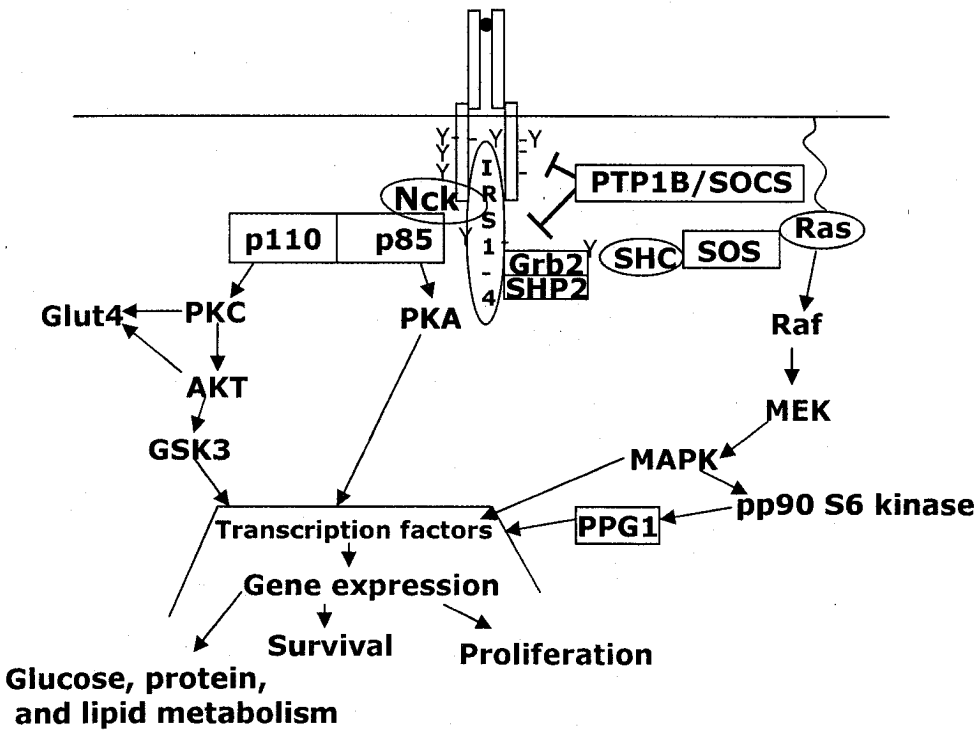
phosphorylation of phosphatidylinositol (PI), phosphatidyinositol-4-phosphate (PI-4-P), PI-4-5-P_2 to PI-3-P, PI-3,4-P_2 and PI-3,4,5-P_3 [280]. The products of the PI3K reaction have an unknown role; they are not substrates for phospholipases so they may generate their signals as intact phospholipids. PI-3, 4,5-P_3 was shown to activate protein lipase C (PLC)ζ in vitro [281]. PKC and AKT/PKB are serine/threonine kinases downstream of PI3K. PKC is involved in insulin stimulated glucose uptake. Most metabolic events of insulin are mediated through AKT. AKT phosphorylates glycogen synthase kinase-3 (GSK-3) which inhibits its activity thereby increasing glycogen synthesis [282]. AKT phosphorylates and therefore inhibits ras in the brain-guanosine triphosphatases (Rab-GTPase) activating protein which in turn regulates glucose transport by activating Rab and allowing glucose transporter (GLUT)-4 access to the plasma membrane [283].

p21ras is a membrane-associated guanosine triphosphate (GTP)-binding protein which has GTPase activity and plays a role in the regulation of cell growth, tumor formation, protein trafficking and vesicular transport [284]. The cycling of guanosine diphosphate (GDP)-bound inactive form to GTP-bound active form regulates the activity of these proteins. Growth factors, including insulin, stimulate tyrosine phosphorylation of Shc, which provides a binding site for Grb2 therefore forming a Shc/Grb2/SOS complex; this results in a rapid increase in the amount of GTP-bound p21ras and leads to the phosphorylation and activation of RAF, MEK and the MAPK. This pathway regulates cell growth and metabolism [285, 286].

Much research has also been focused on the signaling events starting from the IR to activation of the pp90 S6 kinase pathway and the resultant effects on glycogen synthesis. The initial steps of glycogen synthesis are: 1) insulin activates IRS-1; 2) Grb2 links IRS-1 to the guanine exchange factor SOS, which activates p21ras, 3) Raf-1 kinase is activated by the active p21ras (GTP bound form) [287]; 4) Raf-1 in turn phosphorylates and activates MEK [288], which phosphorylates MAPK/ERK-1 [289] that phosphorylates and activates pp90 S6 kinase [290]. When pp90 S6 kinase is stimulated by insulin, the glycogen associated protein phosphatase-1 (PPG-1) is activated. PPG-1 dephosphorylates and activates glycogen synthase and dephosphorylates and inactivates both phosphorylase kinase and glycogen phosphorylase [291].

There are at least three other SH2-containing proteins that associate with IRS-1. The PI3K pathway mentioned above involves the p85α isoform. There is also a β form, which associates with the same p110 catalytic subunit, but the effects of this association are unknown [280]. SH2 domain-containing protein-tyrosine-phosphatase (SHP)-2/Syp is a protein tyrosine phosphatase that has two SH2 domains and also binds to IRS-1 [292]. Since SHP2 is a phosphatase, it may enable a mechanism of downregulating the insulin signal. Non-catalytic region of tyrosine kinase adaptor protein (Nck) is an adaptor protein composed of three SH3 domains and one SH2 domain. Nck binds to the IRS-1 molecule and its overexpression results in cellular transformation; therefore it may play a role in deregulation of cell proliferation [281, 293].

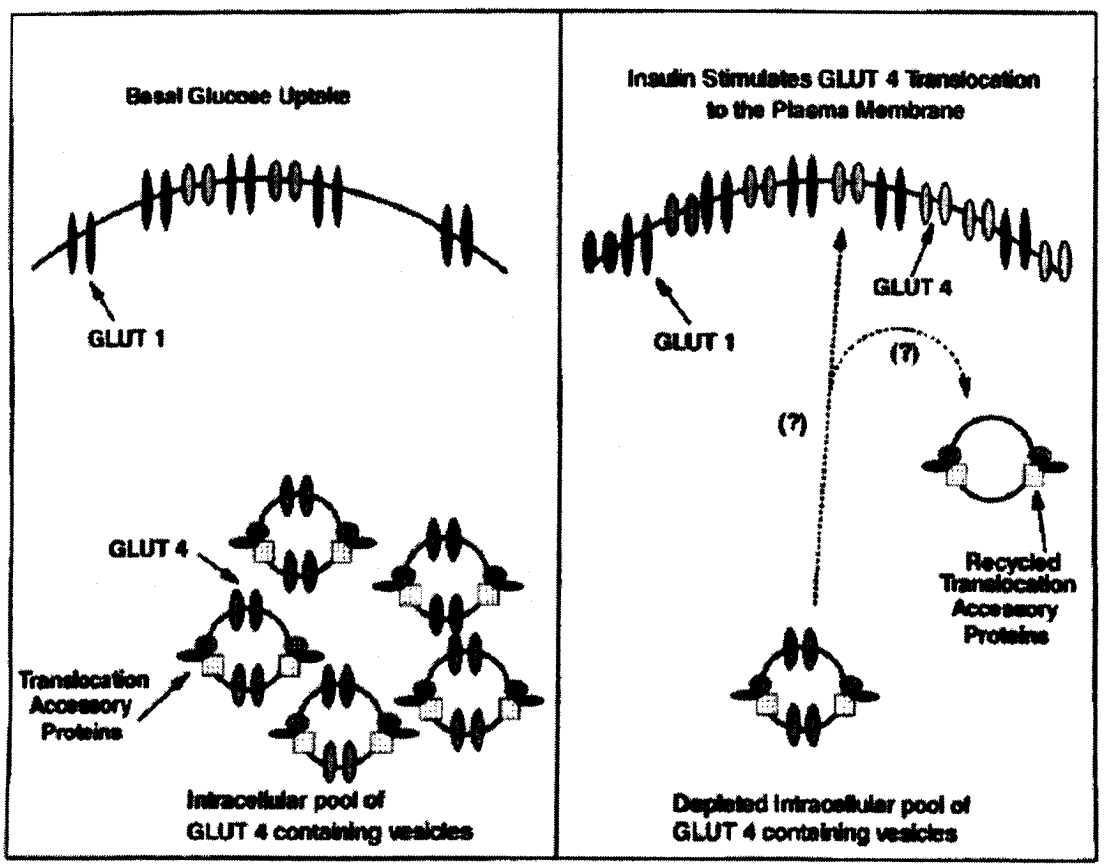
Figure 1.7 A simplified representation of the insulin signaling pathway. Activation of the insulin receptor by insulin induces autophosphorylation of the receptor, creating docking sites for downstream interacting proteins, such as IRS-1-4, which are phosphorylated themselves and provide further docking sites for SH2 domain-containing proteins. Two important signaling pathways are the PI3K and MAPK pathways. The PI3K pathway involves the p85 subunit of PI3K which binds to IRS, which initiates the downstream activation of PKC, AKT, and inhibition of GSK3 leading to glucose, protein and lipid metabolism as well as cell survival. The MAPK pathway involves the phosphorylation of Shc, which associates with Grb2 and SOS which leads to the activation of Ras which then activates the MAP kinases leading to cell proliferation. There are many inhibitory (PTB1B, SOCS, and SHP) and stimulatory (Nck) proteins involved which regulate insulin signaling. These events allow the transmission of the signals that ultimately result in the diverse biological effects of insulin [261,262].



1.5.4 Insulin regulated glucose transport

The uptake of glucose is one of the most important roles of insulin since it has a major effect on glucose homeostasis. Glucose uptake is performed by two types of glucose transporters: Na^+-dependent (in intestinal tract, not regulated by insulin) and facilitative glucose transporters [294]. There are five facilitative glucose transporters (GLUT1-5). GLUT-4 is the only transporter that is sensitive to insulin. It is found in the muscle and adipose cells. A summary of glucose transport is pictured in **Figure 1.8**. In the basal states the majority of glucose transport action is performed by GLUT-1 because GLUT-4 resides in vesicles in an intracellular pool, although they do cycle back and forth from the intracellular vesicles to the plasma membrane [276, 295]. After treatment with insulin, these vesicles move to the plasma membrane, via an increased rate of GLUT-4 vesicle exocytosis and a smaller decrease in the rate of internalization via endocytosis, and this causes a 10-20 fold increase in glucose uptake due to the fact that more GLUT-4 is present on the cell surface [296, 297]. The exact molecular mechanisms of GLUT-4 translocation are not known, but secretory carrier membrane proteins (SCAMPS), vesicle-associated membrane proteins (VAMPS), a newly identified 160 kDa protein, Rab4, potentially other small GTP-binding proteins (since all of these proteins are found within the GLUT-4 containing vesicles), and the actin cytoskeleton and microtubule network are believed to be involved [276, 298]. After initiating glucose uptake, insulin levels decrease and GLUT-4 is internalized by clathrin-coated pits and recycled to intracellular pools.

Figure 1.8 A cartoon of insulin stimulated glucose uptake
In the absence of insulin GLUT-1 performs the majority of the glucose uptake, in the presence of insulin GLUT-4 is translocated to the plasma membrane and increases glucose uptake 10-20 fold. GLUT 4-containing vesicles also contain other associated accessory proteins which appear to be involved in the translocation process; these include secretory carrier membrane proteins (SCAMPS), vesicle-associated membrane proteins (VAMPS), a novel, newly identified 160 kDa protein, and Rab4 and potentially other small GTP-binding proteins [274].
Cheatham, B. and C.R. Kahn, *Insulin action and the insulin signaling network.* Endocr Rev, 1995. **16**(2): p. 117-142. Copyright 1995, the Endocrine Society.



A major area of research is to identify the insulin regulated signaling pathways that are involved in mediating the translocation of GLUT-4 vesicles. Insulin regulates various signaling cascades that may be involved in the translocation/activation of GLUT-4. Current thinking is that PI3K is involved downstream of IRS-1 and upstream of pp70 S6 kinase.

Another pathway that may account for the translocation of GLUT-4 involves the already mentioned p21ras pathway, which causes activation of pp90 S6 kinase, although this view is controversial. Overexpression of constititutively active p21ras in 3T3-L1 adipocytes results in translocation of GLUT-4 to the plasma membrane and stimulation of glucose uptake therefore mimicking insulin's actions [299]. However, EGF/thrombin activation of the p21ras/MAPK pathway doesn't alter glucose uptake or GLUT-4 translocation unlike insulin [300]. If activation of p21ras pathway is involved in insulin-stimulated glucose uptake, it is probably independent of the activations of Raf-1 and MAPK.

1.5.5 Insulin and β-cells

Insulin is also an important growth factor. Insulin is detected quite early in the pancreas, being first detected on E8.5 in the mouse, and it's expression continues throughout adulthood where it is mainly limited to the pancreatic β-cells [301]. The IR is expressed in the β-cells and is detected throughout the pancreas as well as in muscle, liver and fat [302].

It has been hypothesized that insulin secreted from β-cells can stimulate its own synthesis and secretion [303-305]. Amperometry on single β-cells shows

that insulin can cause an autocrine stimulation of insulin secretion, which only requires physiological levels of insulin (~4 nM) and this result can be blocked by IR antibodies [306]. This effect seems to be mediated by IRS-1, PI3K and the release of intracellular calcium stores [307]. β-cell specific IR knockout mice (βIRKO) showed no difference in the islet cell mass within the first 2 months, after which the islet mass started to decrease in association with impairments in glucose-stimulated insulin release and glucose tolerance. For example, βIRKO mice show an 18% reduction in β-cell mass, which becomes more severe (31% reduction) in the diabetic βIRKO mice vs. age-matched control littermates. Loss of the IR in the β-cells leads to altered glucokinase expression and impaired insulin secretion, resembling T2D [308].

The effects of insulin signaling on β-cell proliferation are still controversial but a recent study showed that when the IR was abrogated by 80% in MIN6 insulinoma cells, the G0/G1 to S phase transition was delayed and there was decreased proliferation indicating that insulin may have a positive effect on β-cell growth [309]. Insulin promotes islet regeneration (neogenesis and proliferation) in neonatal diabetic rats treated with streptozotocin [80, 310]. The defects in β-cell secretion and growth are likely mediated via IRS-1 and IRS-2 respectively, because complete knockout of IRS-1 leads to defective insulin secretion in response to glucose and amino acids [311], while inactivation of IRS-2 leads to impaired β-cell neogenesis and proliferation [312]. It is thought that insulin stimulates β-cell proliferation and neogenesis via IRS-2 by relieving Foxo1 inhibition of Pdx-1 [313]. However studies have also shown that mice

lacking both insulin genes die 48 h after birth due to ketoacidosis; examination of their pancreas in late gestation reveals an increase in islet cell mass due to a decrease in apoptosis and an increase in alpha and beta cell proliferation [314]. The relative hyperplasia of the islets in embryos examined in late gestation may be related to increased vascularization of the pancreas [314]. These conflicting results make it difficult to understand if insulin has a positive or negative effect on islet growth, since compensatory mechanisms may also play a role.

Whole body IR knockout mice are born with a slight growth retardation (~10%) but without apparent metabolic abnormalities [315, 316]. After birth, metabolic problems arise: glucose levels increase upon feeding and insulin levels rise up to a thousand fold above normal and β-cell failure occurs within days, characterized by the disappearance of insulin secretory granules within the cytoplasm and followed by death of the animals due to diabetic ketoacidosis. A study of the pancreas in the late gestational stages of the IR/IGF-IR double knockout shows that the alpha and beta cells are in normal proportions compared to the wild-type mice [317]. This indicates that signaling through the IR or IGF-IR is not necessary for the formation of the β-cells.

1.5.6 Insulin Resistance

Among its many roles, insulin is the most important hormone to prevent hyperglycemia. Insulin resistance is a condition where the target tissues become insensitive to insulin actions. Insulin sensitivity is defined by how well insulin can lower blood glucose levels through the inhibition of hepatic gluconeogenesis and

by increasing glucose uptake in muscle and fat [318]. Insulin resistance is linked to many health problems such as obesity, infection, hypertension, and cardiovascular disease [319]. Compensation of insulin resistance may be achieved in some individuals by increasing circulating levels of insulin, by increasing insulin secretion or decreasing insulin clearance. Insulin resistance is present in all T2D patients whether lean or obese [318]. T2D obese patients have increased insulin concentrations to maintain glucose tolerance, and insulin secretion rates are 3 fold higher than in lean controls [320].

The mechanisms of insulin resistance are varied and numerous. There can be decreased receptor level, receptor kinase activity, phosphorylation of substrates, glucose transporters, translocation and intracellular enzymes. There are 3 main sites of insulin resistance, skeletal muscle, liver and fat, each will be further explained below.

Skeletal muscle

Defects in early steps of the insulin signal transduction

T2D patients exhibit a reduction in insulin-stimulated glucose transport in muscle and it has been questioned whether the defects lie in the insulin signaling pathway [321-325] or from impaired GLUT-4 translocation [326, 327]. In obese rodents, there are decreases in IR and IRS-1 protein levels, IR and IRS-1 phosphorylation, and P13K activity. These effects may very well contribute to the reduced insulin-mediated glucose transport in the skeletal muscles [328-331]. Zucker Fatty rats have a mutation in fa/fa gene and they are hyperphagic and

obese and it has been shown in these mice that in the liver and muscle the protein expression of the p85 subunit of PI3K is altered [332]. On the other hand, streptozotocin-induced diabetic rats exhibit decreased insulin-mediated glucose transport but increased skeletal muscle IR and IRS-1 phosphorylation, along with increased PI3K activity [330, 331, 333]. These defects may not be the cause of the insulin resistance but may be secondary changes due to the altered metabolic state.

T2D patients who are not obese exhibit either unchanged or decreased IR phosphorylation in the skeletal muscles compared to healthy subjects [325, 334]. Those who are moderately obese show reduced IRS-1 phosphorylation and decreased PI3K activity in skeletal muscles, with the decreased IRS-1 phosphorylation not relating to the decreased IRS-1 levels [335]. These results link decreased IRS-1 phosphorylation and decreased PI3K activity in moderately obese T2D patients with reduced insulin mediated glucose transport in skeletal muscles, but it is still unclear whether these results are the cause of insulin resistance or secondary changes due to the altered metabolic state.

Defects in the intermediate steps of the insulin signal transduction

AKT is a serine/threonine kinase, downstream of and activated by PI3K. In skeletal muscles of non-obese T2D patients, insulin stimulated AKT phosphorylation is decreased versus normal individuals, although in the basal state there is no difference in AKT phosphorylation between the 2 groups [322]. While in obese T2D patients insulin stimulated activity of AKT was unchanged in

the skeletal muscle versus controls, even though IRS-1 and IRS-2 associated PI3K activity was reduced [336]. In T2D patients the insulin resistance seen at the level of AKT does not account for the reduction in insulin-stimulated glucose transport.

There is evidence in many animal models that suggests that various PKC isoforms in the skeletal muscle are altered with diabetes [337]. Long-term changes can occur due to increased glucose, lipid, and insulin concentrations. Rats fed a high fat diet exhibit increases in PKCθ and PKCε in their skeletal muscles and these changes are due to alterations in fatty acid concentrations [338]. The levels and activity of the various isoforms of PKC are increased in the membrane fraction and diminished in the cytosolic fraction of muscle in the non-obese Goto-Kakizaki diabetic rat [339]. The effects of the altered levels of PKC on intracellular signaling are not known but it has been reported that extracellular high glucose levels inhibit insulin action due to PKC mediated serine phosphorylation [340]. It is also interesting to note that increased concentrations of non-esterified fatty acids can activate PKCθ which can decrease IRS-1 associated PI3K activity [341]. Therefore, increased concentrations of non-esterified fatty acids or glucose can activate PKC that can then impair insulin signal transduction in T2D patients.

Defects in the translocation of GLUT-4

Impairments in GLUT-4 translocation may have an impact on whole body insulin-mediated glucose uptake [326, 327, 342]. Reduced GLUT-4 expression in skeletal muscles is found in morbidly obese patients, this may account for the

decreased glucose uptake [343]. In T2D patients who are not obese, GLUT-4 expression in the skeletal muscles is normal, therefore impaired action of GLUT-4 is probably not the cause of the altered glucose uptake [342, 344, 345]. However in all insulin resistant patients, GLUT-4 exhibits abnormal localization. Therefore impairments in GLUT-4 distribution, translocation and trafficking are indeed a cause of insulin resistance in the skeletal muscles [326]. It has been shown that high fat diet impairs GLUT-4 fusion with the plasma membrane due to defects in the early insulin signal transduction pathway [346].

Liver

Hepatic Glucose Metabolism

In many organs and cells, especially the CNS, glucose is the main source of energy. A number of different functions rely on a set amount of glucose entering from the bloodstream; therefore regulation of the blood glucose level is critical. The liver plays an important role in maintaining the blood glucose levels by controlling the rate of glucose uptake or output depending on the state i.e. fed or fasted, respectively [347]. After a meal, insulin stimulates glucose uptake. This glucose can then be stored as glycogen (glycogenesis) or can be oxidized to be used as fuel (glycolysis). In a fasted state glycogen is degraded to produce glucose (glycogenolysis) or glucose can be produced from glycerol, pyruvate, and lactate (gluconeogenesis) [347] . Glycogenolysis produces glucose within a few hours of fasting and is suppressed by insulin within a relatively short time (1 h) after a meal [348]. Gluconeogenesis produces glucose during fasts of prolonged time

periods (12-14 h) once the liver glycogen stores are depleted [349]. Gluconeogenesis may also occur in states where there is a low concentration of insulin, such as in T1D, or where liver insulin resistance has developed, for example in T2D and obesity.

Metabolic Pathways

Metabolic conditions will have an effect on the fate of the intracellular glucose. In times of feeding, glucose enters the cells and is either oxidized or stored as glycogen. Glucose metabolism occurs in the liver and consists of many steps, the first being the conversion of glucose into the most important regulator, glucose-6-phosphate (G6P), by the enzyme glucokinase [350]. G6P can then enter numerous metabolic pathways. G6P can be oxidized to form pyruvate, and then acetylCoA that can enter the Krebs cycle to generate energy. G6P may also be stored in the form of glycogen. Glycogen synthase kinase-3 (GSK-3) and glycogen synthase (GS) are two important enzymes for the conversion of G6P into glycogen and they can be phosphorylated (inhibits GSK3 activity) or dephosphorylated (activates GSK3 activity), respectively by the PI3K pathway [351].

During the fasted state, glycogen phosphorylase converts glycogen into G6P and then glucose-6-phosphatase (G6Pase) converts G6P into glucose. Glucose is then released into the bloodstream and can be supplied to extrahepatic tissues. Glycogen will eventually be depleted. Therefore, while glycogenolysis is occurring, gluconeogenesis quickly follows and is accelerated. The

gluconeogenic precursors, excluding glycerol, enter the pathway through pyruvate and eventually are converted into G6P. Glycerol in the form of dihydroxyacetone phosphate will be converted into G6P. Phosphoenolpryuvate carboxykinase (PEPCK) is the major enzyme catalyzing this process [351].

In insulin resistant states insulin does not suppress G6Pase and PEPCK effectively; therefore glucose uptake is decreased and glucose production is increased which leads to hyperglycemia [352-354].

Pathogenesis of liver insulin resistance

In healthy individuals after an overnight fast, glucose is produced at a rate that is sufficient to meet the body's metabolic demands. The hepatic glucose output depends on the amount of lean body mass and level of peripheral insulin utilization [355]. After a meal insulin is released and will suppress the glucose output. Insulin resistance occurs when the liver is unable to "read" the signal. The suppressive effect of insulin can occur through the hepatic IR directly or indirectly via a decrease in gluconeogenic precursors [356].

Intracellular signaling defects can suppress glucose uptake. Excessive insulin, glucose, and FFA can impair insulin signaling in obese and T2D patients [321, 357]. Hepatic insulin resistance may also be caused by liver steatosis [358, 359]. Trauma, chronic disease and sepsis can all cause insulin resistance as well as other physiological states: pregnancy, aging, and puberty. Visceral obesity is the main environmental cause of insulin resistance. Insulin resistance along with hyperinsulinemia will lead to increased fat mass, FFA and lipolysis. These effects

in turn will lead to a further impairment of insulin signaling, lipotoxicity, increased hepatic glucose production, β-cell exhaustion and diabetes [360].

Adipose

The adipose tissue is very important in maintaining metabolic equilibrium; this is demonstrated in cases of lipoatrophy. Mice without any fat are susceptible to glucose intolerance, hyperphagia, insulin insensitivity, and high triglyceride levels and fat transplantation rescues most of these defects [361]. The white adipocytes are the preferential depots for storing triglycerides [362]. Adipose tissue controls glucose levels by acting as a buffer to regulate fatty acid flux by controlling the balance between non-esterified fatty acids and release and the clearance of triglycerides [363]. Therefore, in the fasting state adipocytes release fatty acids to be used as substrates and in the fed state adipocytes absorb fatty acids from triglycerides. In cases of obesity adipose tissue function is altered. Obesity is the most important contributor to insulin resistance. In insulin resistant states the adipose tissue is resistant to insulin's antilipolytic effect and therefore excessive FFAs are produced [364]. The adipose tissue also plays an important role in glucose uptake. Glucose uptake is the rate-limiting step in insulin-stimulated glucose disposal. Disturbances in fatty acid homeostasis and uptake play a role in causing insulin resistance and T2D [365].

Adipose tissue is no longer considered an inert storage tissue. Today adipose tissue is known to synthesize and secrete numerous hormones, termed adipokines, with numerous roles in energy expenditure and metabolism. Adipokines are

secreted from adipose tissue usually act on the muscle or liver to induce a state of insulin resistance or to promote insulin sensitivity. Elevated FFA are hypothesized to play a role in insulin resistance because they inhibit glucose uptake, oxidation and glycogen synthesis. Increased FFA are known to decrease IRS-1 phosphorylation and association with PI3K. The connection between insulin resistance and FFA may involve the increased aggregation of triglycerides, ceramides, and diacylglycerol in the liver and muscle [318]. TNF-α is present at high levels in obese rodents and inhibits IR activity by phosphorylating IRS-1 on a serine residue thus inducing insulin resistance [366]. TNF expression in adipose tissue is greater in obese individuals and decreases after weight loss [367]. Further evidence for the role of TNF in insulin resistance is described in the ob/ob mice, which are leptin deficient insulin resistant mice. It was shown that some mice lacked the TNF receptor and had improved insulin sensitivity versus ob/ob mice with the TNF receptor, when challenged with a high fat diet [368].

Adiponectin is mainly synthesized in the adipose tissues but the skeletal muscles, endothelial cells, and cardiac myocytes do express adiponectin albeit at lower levels [369, 370]. Adiponectin is decreased in states of obesity in humans and mice, and administration leads to improvement in insulin sensitivity and a decrease in FFA and triglyceride levels in the muscle and liver [371]. Conversely, adiponectin knockout mice develop insulin resistance when fed a high fat/high sugar diet due to reduced hepatic insulin sensitivity and decreased responsiveness to peroxisome proliferator-activated receptors (PPAR)γ agonists [372]. Activation

of PPARγ leads to an increase in adiponectin levels in vivo [373]. These examples provide evidence that adiponectin plays a prominent role in insulin sensitivity.

Resistin mRNA is present in many tissues such as adipose, skeletal muscle, gastrointestinal tract, pancreas and hypothalamus. Resistin levels are increased in obese mice and administration of thiazolidinedione, an anti-diabetic drug, decreases resistin levels. Improvement in blood glucose and insulin action in obese mice is achieved with the administration of anti-resistin antibodies [374, 375]. However, the role of resistin in humans is still in debate.

Leptin is mainly produced by the adipose tissue but the placenta, stomach, bone marrow, and brain may also synthesize leptin [376]. Leptin is considered to be a pro-inflammatory cytokine yet its main role seems to be the control of appetite. Mice deficient in leptin (ob/ob) or the leptin receptor (db/db) are obese [377]. Leptin levels are proportional to the amount of adipose tissue in humans and mice. Leptin levels also are increased during inflammatory conditions [378]. Leptin administration increases the progression of diabetes in non-obese female mice, and will cause insulin resistance in obese mice [377]. The interaction of different adipokines is not well understood and their interactions may have a large impact on the types of disorders arising from obesity.

1.6 Glucose Metabolism

1.6.1 Insulin and hypoglycemia

In a normal individual, after a meal or any other occurrence which increases blood glucose, insulin is released automatically and the amount secreted

is adjusted based on the need [135, 379]. Insulin released by the pancreatic β-cells first enters the liver by the portal vein in order to stimulate glycogen synthesis and inhibit gluconeogenesis. The liver eliminates half of the insulin therefore there is only 50% of the original insulin concentration to enter the periphery circulation and to stimulate glucose disposal or inhibit lipolysis in the skeletal muscles or adipose tissues, respectively [380, 381]. In diabetic patients, insulin must be administered and the levels are not auto-regulated therefore they only deplete when the subcutaneous depot is depleted but during this time the blood glucose levels may already be too low [379]. The route of administered insulin is reversed compared to endogenous insulin and enters the circulation at a far slower rate, therefore elevated insulin levels persist for a longer period of time.

1.6.2 Normal glucose counter-regulation

In a normal person a decrease in blood glucose levels causes a set of responses. The first reaction is that insulin secretion is suppressed, then the decrease in peripheral glucose uptake and increase in hepatic glucose production is halted; these steps prevent the glucose levels from plummeting too low hence the avoidance of true hypoglycemia [382, 383]. The decrease in blood glucose levels will also stimulate the α-cells to secrete glucagon which can promote hepatic glucose production via glycogenolysis and gluconeogenesis [384, 385]. In cases of severe hypoglycemia the central nervous system (CNS) is activated and triggers sweating, palpitations, and hunger, all warning signs designed to stimulate food seeking and ingestion behaviour before severe defects such as

cognitive failure ensues [379].

1.6.3 Diabetic glucose counter-regulation

T1D patients have impaired glucose counter-regulatory mechanisms. T1D patients are unable to secrete insulin therefore they can not counteract the falling blood glucose levels, in addition they also lack the paracrine mechanisms which un-inhibit the α-cells to secrete glucagon [379]. Without the activation of glucagon to prevent hypoglycemia, it is essential that the CNS warning symptoms be paid attention to. Iatrogenic hypoglycemia decreases the CNS symptoms and shifts the level of glucose even lower before these warning symptoms appear [386, 387], this causes a cycle which may lead the patient to be unaware that they are hypoglycemic. This "hypoglycemic unawareness" results in the onset of neuroglycopenia before the CNS warning symptoms appear which can lead patients into seizures or comas [388].

In T2D patients with remaining β-cell function they can promote a defense against hypoglycemia, because their glucagon response and most counter-regulatory mechanisms are functional [379]. There is the problem of some T2D patients having insufficient insulin secretion, which can cause glucagon not to respond properly and result in hypoglycemia.

1.7 Insulin-like growth factor (IGF)

1.7.1 The origin of IGF-I

50 years ago, Salmon and Daughaday first studied the role of a pituitary regulated substance that had growth promoting effects using the incorporation of radiolabeled sulfate into chondroitin sulfate in costal cartilage [389]. Hypophysectomy was later shown to decrease the incorporation, but injections of pituitary extracts and bovine growth hormone restored the incorporation of sulfate although bovine growth hormone added directly to cartilage in vitro caused very little effect [390, 391]. This opened the doors to the suggestion that there was an intermediary factor between GH and its effect on cartilage and led to the term "sulfation factor". Additional experiments showed that rat serum was able to cause the observed effect but hypophysectomized rat serum could not, unless the hypophysectomized rats were injected with bovine GH. Five years and many experiments later it was shown that the growth effects of bovine GH could be replicated by a purified fraction of the "sulfation factor" from acromegalic patients, giving birth to the term somatomedin [392].

1.7.2 Structure and similarity of IGF to Insulin

The somatomedins were purified and divided up into subgroups according to molecular weights and new terminology was suggested: insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II) since these somatomedins were able to stimulate glucose uptake as well [393]. IGF-I is regulated by circulating GH [394, 395]. IGF-I is a member of the IGF family of

growth factors which is composed of 3 hormones (IGF-I, IGF-II, and insulin), 3 trans-membrane receptors (IGF-IR, IR and IRR) and specific binding proteins for IGFs (IGFBPs 1-6) [396]. The sequence and tertiary structure of the IGFs are closely related to proinsulin (e.g. the IGF's share 50% sequence homology with insulin); they may be derived from gene duplications of a common ancestor. IGF-I is highly conserved among mammals and is composed of a linear polypeptide consisting of 70 amino acids linked by three disulfide bridges. IGF-I can be subdivided into four continuous domains: the amino terminal B (29 amino acids), central C (12 amino acids), A (21 amino acids), and carboxy terminal D (8 amino acids) domains. Insulin is formed by the cleavage of proinsulin and it consists of 2 domains, A and B, linked by two disulfide bonds. The B and A domains of IGF-I are homologous to the insulin B and A domains, respectively. Insulin contains 19 conserved residues only two of which; glutamine A5 and asparagine A21 are replaced in IGF-I with glutamic acid and alanine, respectively [395]. Due to alternative splicing, IGF-I mRNAs consist of E domains attached to the C domains, a conserved feature amongst mammals. The human/rodent single copy IGF-I gene is composed of six exons and 5 introns and maps to chromosome 12 (human) [397]. Variant IGF-I mRNAs are produced by alternative splicing at both the 5' and 3' ends.

IGF-II is primarily a fetal hormone in rodents with roles in promoting growth, differentiation and survival. IGF-II is composed of a single polypeptide chain of 67 amino acids, with a high degree of homology to IGF-I and proinsulin. The IGF-II gene consists of 10 exons and can be mapped to chromosome

11p15.5, adjacent to insulin, in humans [398]. In mice IGF-II is found on chromosome 7 [399]. IGF-II contains an N-terminal B domain (28 amino acids), C domain (12 amino acids), A domain (21 amino acids), and a D domain (6 amino acids) present at the carboxyl terminal. There are multiple variant IGF-II mRNAs present which arise through alternative splicing and the protein products account for 25% of circulating IGF-II levels; the significance of these variants is not clear [400]. IGF-II, similar to IGF-I, is synthesized as a precursor, with an E domain jutting off from the C terminal; however, the E region is less conserved and its function is unknown, similar to IGF-I. Four promoters are located within the IGF-II gene in rodents [401]. The expression patterns of IGF-II differ between rodents and humans. In rodents IGF-II expression diminishes after the first two weeks of life but in humans IGF-II is present throughout life at relatively high concentrations due to continuous activity of a specific promoter within the liver [402, 403]. The coding region is located over three exons, with the first two containing the mature IGF-II molecule, signal peptide and partial residues of the E domain; the final exon contains the remaining residues of the E domain and the 3' untranslated portion of IGF-II mRNA.

1.7.3 Structure of the IGF receptors

The IGF-IR and the IR are similar in structure, showing a 60% amino acid homology [404]. The IGF-IR belongs to the class II receptor tyrosine kinase family due to the conserved cysteine residues in the extracellular domain and since it has the ability to transfer a γ phosphate of ATP onto itself. As is the case

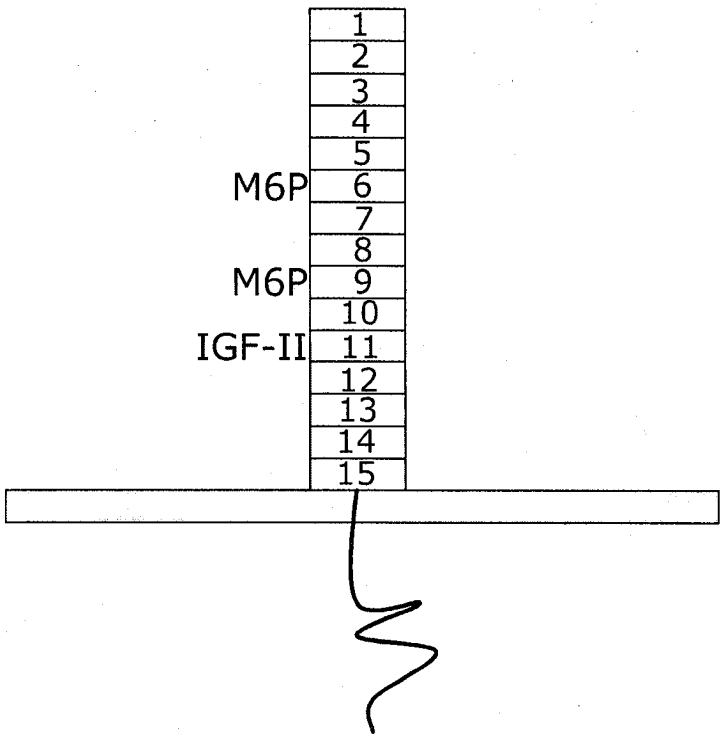
with the IR, it is composed of 2 α- and 2 β-subunits covalently linked by disulfide bridges; the α-subunit contains the ligand-binding domain and the β-subunit contains the tyrosine kinase catalytic activity. The receptor is different from other class II members because it is a preformed dimer even without a ligand. [254]. The human IGF-IR gene is a single copy present on chromosome 15 [404]. The cDNA for the human IGF-IR was cloned in 1986; it consists of 4989 nucleotides and codes for a 1367 amino acid precursor. It contains a signal peptide residue (30 residues) and a furin cleavage site which results in the α- and β-chains. The extracellular portion of the IGF-IR is composed of the α-chain and 156 residues of the β-chain, which contain 16 potential glycosylation sites. There is a single transmembrane domain and a 408-residue cytoplasmic domain which houses the tyrosine kinase [404]. The human IGF-IR gene is composed of 21 exons, 10 coding the α-chain and 11 coding the β-chain, and is greater than 100 kb in length [405]. Unlike the IR, there is no evidence of an alternatively spliced exon 11. The N terminal half of the IGF-IR ectodomain contains two homologous regions, L1 and L2, separated by cysteine rich regions [257]. The C terminal half consists of three fibronectin type III domains [406]. Intracellularly there is a tyrosine kinase domain flanked by a juxtamembrane and a C-tail domain both of which are regulatory domains. The C-tail serves as a binding site for phosphotyrosine containing signaling molecules [254]. In humans, IGF-IR mRNA consists of two bands, a major (11 kb) and minor (7 kb) form; in rodents only the major form is detected [407]. The IGF-IR is expressed by all tissues and cells, in agreement with the role of IGFs as mitogens. High levels of IGF-IR are

present during embryonic development, due to the high level of activity and growth of cells, while postnatally the levels decrease [408]. Surprisingly the liver has virtually no IGF-IR although IGF-I expression is greatest in this organ. This phenomenon can be explained by downregulation of the receptor by locally produced IGF-I [409, 410].

The IGF-II/mannose-6-phosphate receptor, illustrated in **Figure 1.9**, is composed of a 300 kDa single chain polypeptide; in the mouse it is found on chromosome 17 and in humans on chromosome 6. It consists of a large extracellular domain composed of 15 repeating domains, with 1 binding site for IGF-II and 2 binding sites for mannose-6-phosphate containing ligands and a very short cytoplasmic domain necessary for lysosomal enzyme sorting but with no intrinsic enzymatic activity [411-413]. There is receptor homology amongst most species. The receptors are present in all cells; most of the IGF-II receptors are found within the cell, while 10% are found on the cell surface. The major function of this receptor is to bind and transport lysosomal enzymes but it can also bind, internalize and degrade IGF-II [414, 415].

The IR and IGF-IR are expressed in the majority of tissues and frequently within the same cells. In cells where both receptors are expressed, commonly the skeletal muscle, it is possible that an insulin/IGF-I receptor hybrid can form consisting of an IGF-I α-β hemireceptor linked via a disulfide bond to an IR α-β hemireceptor. IGF-I binds to the hybrid receptors more readily than insulin and can stimulate the phosphorylation of both β chains [416]. It is unknown whether the downstream signaling pathway differs from the single receptors. The hybrid

Figure 1.9 A schematic of the IGF-II mannose-6-phosphate receptor
The IGF-II mannose-6-phosphate receptor is composed of a 300 kDa single chain polypeptide. It consists of a large extracellular domain composed of 15 repeating domains, with 1 binding site for IGF-II and 2 binding sites for mannose-6-phosphate containing ligands and a very short cytoplasmic domain necessary for lysosomal enzyme sorting but with no enzymatic activity [392].



receptors behave like the IGF-IR in terms of internalization and degradation, undergoing recycling and not degradation [417].

The signal transduction pathway of IGF-IR is very similar to the insulin signaling pathway since the receptors are part of the same family and have similar effects, please refer to section **1.5.3/Figure 1.7** or a very interesting review by Butler et al [418].

1.7.4 IGF-binding proteins

Unlike insulin, IGFs are bound to binding proteins in the circulation and these IGF-binding proteins (IGFBP) were first discovered over 25 years ago. IGFBPs can be subdivided into three domains based on their primary structure. The amino terminus, which contains the IGF binding site, and the carboxyl terminus are highly conserved and contain cysteine residues that form intramolecular disulfide bonds. The linker domain is the least conserved of the three domains showing less than 30% primary sequence homology [419]. There are six members of IGFBPs and they have 4 main roles: 1) to transport the IGFs in the plasma, 2) to prolong the half-lives of IGFs, 3) to provide tissue and cell type localization of the IGFs, and 4) to control interactions between IGFs and their receptors. The IGFBPs are able to fulfill these roles since IGF-I and IGF-II have a greater affinity for the IGFBP than for the IGF-IR [420].

In the serum the majority (85%) of IGF-I and IGF-II exist in a 150 kDa complex: through their C domains they bind to IGFBP-3, the most prevalent binding protein, and the acid labile subunit (ALS) [421]. The serum IGFBP-3

levels peak at puberty and then slowly decrease. Many conditions such as GH deficiency, diabetes, and malnutrition can decrease IGFBP-3 levels whereas GH and IGF-I can increase its levels. IGFBP-3 is a 46-53 kDa protein containing 3 glycosylation sites. ALS is an 88 kDa glycoprotein, rich in leucine, and known to be important for regulating protein-protein interactions within the serum [422]. This ternary complex doesn't allow the IGFs to leave the vascular complex and it also increases the half-life of free IGF binding proteins and free IGFs from 30-90 min and 10 min to 12-15 h, respectively [423].

There are 5 other binding proteins that are 25.3 kDa to 34 kDa in size and form lower molecular weight complexes without the involvement of ALS. IGFBP-1, 25.3 kDa, is predominantly expressed in the liver and amniotic fluid and binds to IGF-I and IGF-II with equal affinity [424]. IGFBP-1 usually inhibits IGF-I actions. IGFBP-2, 34 kDa, is found at very high levels postnatally but decreases significantly in adults [425]. IGFBP-2 inhibits IGF-II actions and in some cells may mimic IGF-I actions. IGFBP-4, 26 kDa, is produced by bone cells, and inhibits IGF-I actions; it may actually serve a role to protect the cells from IGF-I overstimulation and encourage the use of alternate signaling pathways, which are inhibited by IGF-I. IGFBP-5, 29-31 kDa, is expressed in almost all peripheral tissues and is the major IGFBP found in bone; it binds to fibroblast extracellular matrix (ECM) and increases DNA synthesis. IGFBP-6, 28-34 kDa, is present in serum and cerebrospinal fluid (CSF) and may have an anti-gonadotropin effect in the ovary [422].

Recently it has been shown that IGFBPs may also have IGF-independent effects. IGFBP-1 can stimulate cell migration through the fibronectin receptor; it can also inhibit cell motility in metastatic breast cancer cells, indicating that it's actions may be cell/tissue specific [426, 427]. Via proteoglycan interactions IGFBP-2 can interact with the extracellular matrix and with the membranes of neuroblastoma cells [428, 429]. IGFBP-3 can stimulate apoptosis in prostate cancer cells [430]. IGFBP-5 through its heparin binding region can stimulate mesangial cell migration [431].

1.7.5 IGF-I and β-cells

There is a great deal of evidence which supports a role for IGFs in islet cell proliferation [432]. IGF-I and its receptor IGF-IR are expressed in various cell types of the endocrine pancreas. IGF-I induces DNA synthesis in a glucose-dependent manner in islet cells [433-436]. The β-cell mass of neonatal rodents undergoes a phase of remodeling, including a wave of apoptosis, which peaks at approximately 2 weeks of age [437]. IGF-I expression is low in islets during the remodeling phase and increases after 2 weeks of age. Thus, the neonatal β-cell apoptosis is most likely caused by a lack of IGF signaling [438].

IGF-I is known to promote β-cell replication as well as survival through IGF-IR activation, recruitment and phosphorylation of IRS-2 [439]. IRS-2 has an important role in the maintenance of a normal β-cell population [440], via activation of PI3K and AKT [441]. Increased IRS-2 in vitro promotes β-cell replication, neogenesis and survival and decreased IRS-2 induces β-cell apoptosis

[442]. In vivo overexpression of IRS-2 promotes β-cell survival [443] while IRS-2 knockout mice exhibit a decrease in β-cell mass [312]. Islet-specific IGF-I overexpression promotes islet cell regeneration in diabetic mice [444].

However in recent years, results from several gene-targeted mice have shown that endogenous IGF-I does not promote islet cell growth. Inactivation of both the IR and IGF-IR or IGF-I and IGF-II genes in early embryos results in normal islet development [317]; islet β-cell-specific inactivation of the IGF-IR gene causes no change in β-cell mass [445, 446]; studies of liver- and pancreatic-specific IGF-I gene deficiency (LID and PID) mice even suggest that IGF-I exerts an inhibitory effect on islet cell growth [447-449]. Many factors need to be considered to understand these conflicting results such as gene redundancy, the responses of IGFBPs and even the properties of the transgenic promoters.

IGF-I is known to be anti-apoptotic. Administration of IGF-I prevents Fas-mediated autoimmune β-cell destruction and delays the onset of diabetes in non-obese diabetic (NOD) mice by minimizing insulitis [434, 435, 450, 451]. The IGF-I-treated animals show a greater percentage of intact islets (49% vs. 2%), a decrease of lymphocytic infiltrated islets and an increased β-cell mass. The anti-apoptotic effect contributes to a stable β-cell mass, prevents autoimmune destruction of the islets and delays the onset of diabetes in non-obese diabetic mice [434, 435, 450, 451]. The anti-apoptotic effect involves PI3K activation and phosphorylation of AKT [452]. In INS-1 and MIN6 β-cell lines, IGF-I prevents cell death by increasing the phosphorylation of AKT and various other PI3K

substrates such as GSK-3β, Bcl-2-antagonist of cell death protein (BAD), forkhead receptor (FKHR), and p70 S6kinase.

In vivo and pancreatic perfusion studies suggest that IGF-I inhibits insulin secretion. IGF-I inhibits insulin secretion from isolated perfused rat pancreas, in response to either glucose or arginine [453]; as well as in conscious rats under high glucose conditions [454]. The mechanism involves activation of phosphodiesterase 3B and AKT and a reduction in intracellular cyclic adenosine monophosphate (cAMP) levels [455, 456].

IGF-I signaling is important for the glucose-response in β-cells. Inactivation of the IGF-IR gene in islet β-cells decreases the secretory response to glucose stimulation, due to decreased GLUT-2 and glucokinase gene expression [445, 446]. In MIN6 insulinoma cells loss of IGF-IR causes alterations in mitochondrial ATP synthesis and blocks glucose-stimulated insulin secretion [457]. This defect is most likely caused by decreased expression of glucokinase, GLUT-2 and/or the voltage-sensitive calcium channel subunit [457]. In all, IGF-I might stimulate islet cell proliferation, promotes β-cell survival, and seems to inhibit insulin secretion.

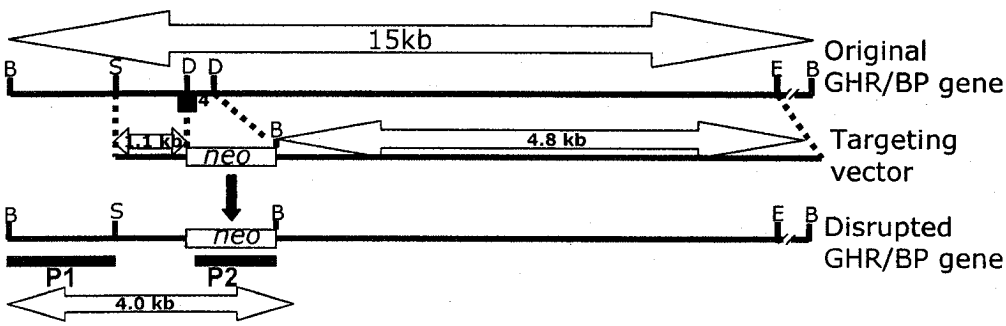
1.8 Gene targeting in mouse models

1.8.1 Gene knockouts

Gene knockouts are useful to study the function of specific genes as well as to understand the involvement of genes in different pathological states. Knockout mice can be produced by homologous recombination. The process

described below is for the construction of a GHR knockout ($GHR^{-/-}$) mouse. A cartoon of the gene targeting mechanism is depicted in **Figure 1.10**. Embryonic stem cells (ESC) are removed from the inner cell mass of blastocysts. The ESC colonies are cultured and grown on plates until an ESC line is chosen. The selected clone then undergoes gene targeting. The GHR consists of 10 exons, with exon 4 encoding a section of the GHR/binding protein. A Dra III fragment, which contains the 3' end of exon 4 and a selectable marker, a neomycin cassette, replaces the 5' section of intron 4/5. Exon 4 is selected because some Laron patients (similar to GHR knockout mice) have a heterogeneous mutation in exon 4. Exon 4 is also chosen because if alternative splicing occurred between exon 3-5, a frameshift would occur with a stop codon in exon 5. An EcoRI fragment consisting of exon 4 is isolated from a C57/Bl6 genomic library. The GHR/binding protein-targeting vector is fabricated with a SacI/EcorI fragment containing a 1.1 kb neomycin resistant gene cassette instead of the 500 bp Dra III fragment via blunt end ligation. This targeting vector lacks the majority of exon 4 as well as a 500 bp section of intron 4/5 of the GHR/binding protein gene. Adjacent to the neomcycin cassette on both sides are the mouse GHR/binding protein gene homologous sequences. A negative marker in the form of a herpes simplex virus thymidine kinase gene is placed downstream at the 3' end of the GHR/binding gene homologous sequence. ESC of 129/Ola mouse strain are transfected with the GHR/binding protein-targeting vector via electroporation and then positively selected. Homologous recombination occurs in some ESC and they are selected for. Heterozygous GHR/binding protein disrupted ESC are

Figure 1.10 Strategy for gene disruption of the mGHR/BP gene.
The targeting vector was constructed using a *Dra*III–*Dra*III fragment containing a major portion of GHR exon 4 and 500 bp of intron 4/5 was replaced by the neomycin resistance (*neo*) gene. A herpes simplex virus thymidine kinase gene cassette (not shown) was placed at the 3' end of the targeting vector for negative selection. The length (kilobases) of the left and right arms of the targeting vector and the important restriction fragments are indicated in the figure.
B, *Bam*HI; D, *Dra* III; E, *Eco*RI; and S, *Sac*I. P1 and P2 denote hybridization probes [449].



injected into blastocyts and reimplanted into BALB-c5 foster mothers. Male chimeras, detected by coat colors and other factors are selected and crossed with BALB-c5 females to produce GHR heterozygous mice [458]. The end result is a mouse lacking the GHR gene.

1.8.2 Growth hormone receptor knockout ($GHR^{-/-}$) mice

$GHR^{-/-}$ mice exhibit delayed puberty, reduced fertility and increased postnatal mortality. Although being born normal size, starting from the time of weaning, the $GHR^{-/-}$ mice weigh considerably less and are shorter than wild-type littermates. The $GHR^{-/-}$ mice have significantly smaller organs except for the brain when compared to body weight and no gross malformations are apparent. GH and IGFBP-2 are significantly elevated and IGF-I and IGFBP-1, -3, and -4 significantly reduced. The fasting serum levels of insulin and glucose are significantly reduced in the $GHR^{-/-}$ mice. Interestingly, the $GHR^{-/-}$ mice live significantly longer compared to controls [458, 459].

1.8.3 Transgenic overexpression

Transgenic mice are produced in a similar manner to gene knockouts except a gene is stably introduced into the animal; this technique allows the understanding of the function of the gene or to produce a model of disease. The most common method for generation of transgenics is the introduction of the gene by manipulation of ESC or microinjection of cloned DNA into eggs, as in the case of metallothionein promoter IGF-I overexpressed mice (MT-IGF mice). A

detailed description of the generation of MT-IGF mice will follow. A fusion gene pMIG65 containing a mouse metallothionein I promoter, rat somatostatin sequence (to ensure secretion), hIGF-I-A cDNA and the 3' end of hGH ribonucleic acid (RNA) processing elements is constructed. This construct is first stably expressed in baby hamster kidney cells. Northern blot is performed to verify if correct transcriptional processing occurs. Western blot identifies a peptide identical to human IGF-I being secreted in the medium, therefore ensuring correct cleavage occurs. A restriction fragment, KpnI-EcoRI containing the desired construct is obtained from the vector and injected into the pronuclei of fertilized eggs which are then implanted into foster mothers and selection of the pups containing the foreign DNA is determined by dot hybridization [460]. This produces a whole body IGF-I overexpression.

1.8.4 MT-IGF mice

MT-IGF mice have not been characterized in depth even though they were first produced in 1988. MT-IGF mice weigh 1.3 fold more than wild-type mice, starting from the age of 2 months, although bone length is not significantly different. Serum IGF-I levels are increased 1.5 fold. Most of the major organ weights corrected by body weight, such as spleen, pancreas, brain, skeletal muscle, are increased in MT-IGF mice due to hyperplasia since DNA content is increased. There are no apparent differences in the weight of the major fat pads. GH levels are significantly decreased due to the feedback inhibition of IGF-I [460]. IGFBP-3 is increased in MT-IGF mice [461]. The organs do not exhibit

any gross anatomical lesions but the kidneys of MT-IGF mice have larger glomeruli, the skin exhibits microscopic abnormalities such as a thickened adipose layer and disrupted collagen bundles, and the spleen exhibits mild red cell hematopoiesis. The serum was analyzed and protein, albumin, and cholesterol levels are normal but triglyceride levels are elevated and insulin levels are reduced [462].

1.8.5 Other mouse models

Using transgenic approaches there are numerous mouse models available to study glucose homeostasis, insulin resistance and diabetes. Deletion of GLUT-4 specifically in the muscles leads to hyperglycemia and insulin resistance. Overexpression of GLUT-4 in transgenic mice results in hypoglycemia, hypoinsulinemia and increased glucose clearance and isolated skeletal muscles and adipocytes revealed an increase in both basal and insulin-stimulated glucose transport [463]. Overexpression of GLUT-4 specifically in the muscles results in increased glucose uptake, glycolysis and glycogen synthesis [464]. Inactivation of IRS-1 results in growth retardation and insulin resistance that does not progress to diabetes, therefore the mitogenic function is more important for this substrate [465]. IRS-2 knockouts develop diabetes due to impaired β-cell growth [312]. IRS-3 knockout exhibit no phenotype this may be due to gene redundancies and IRS-4 knockout mice resemble IRS-1 knockout mice albeit with a milder phenotype [466, 467]. Muscle specific IR knockout did not result in any metabolic changes even though they presented with decreased IR signaling [468].

A muscle specific IGF-IR dominant negative mouse (MKR) has been produced. These mice develop insulin resistance specifically in the muscle due to lack of IGF-I and insulin-stimulated glucose uptake, although insulin resistance does occur in the liver and adipose tissue. Hyperinsulinemia occurs and by 6 weeks the mice resemble human type 2 diabetics. It is interesting to note that the phenotype is much more severe than muscle specific IR knockout mice perhaps due to the fact that the IGF-IR is still functional in these latter mice [469, 470].

1.8.6 Differences between humans and mice

Transgenic mouse models of disease are not always perfect replicas of human disease. For example $GHR^{-/-}$ mice originally were the animal models for human Laron syndrome but they differ in 3 significant aspects. Laron patients exhibit insulin resistance, abdominal obesity, and increased insulin levels while $GHR^{-/-}$ mice exhibit insulin hypersensitivity, no obesity and decreased insulin levels [471]. Various explanations have been provided to explain the discrepancies: different species, gestation periods, interference from selection markers, compensatory genes, lifespan differences between mice and humans, and functional redundancies, etc. [472]. In the case of $GHR^{-/-}$ mice it has been reported that the differences may be due to the level of the mutation in the GHR, $GHR^{-/-}$ mice lack the entire GHR while Laron patients have a mutation in the receptor; or perhaps the differences may be due to mice and humans exhibiting different gestational periods and downstream mediators such as IGF-II which have different expression patterns [471].

1.9 Hypothesis and objectives

The GH/IGF-I axis has been studied for many years but recently the focus has been on islet cell growth. Various transgenic models have challenged the view that IGF-I is a necessary growth factor. We hypothesized that both GH and IGF-I are necessary for islet cell growth. Our aims have been to study the insulin responsiveness in the liver and skeletal muscle of $GHR^{-/-}$ mice and challenge them with a high-fat diet (HFD) to determine if GH signaling plays a role in compensatory islet growth. We also wanted to study the effect of IGF-I expression on islet cell growth, glucose metabolism and test the effect of IGF-I overexpression on streptozotocin-induced T1D.

Chapter Two

Growth hormone receptor deficiency causes delayed insulin responsiveness in skeletal muscles without affecting compensatory islet cell overgrowth in obese mice

2.1 Abstract

Growth hormone (GH), acting through its receptor (GHR), is essential for somatic growth and development and maintaining metabolic homeostasis. GHR gene deficient ($GHR^{-/-}$) mice exhibit drastically diminished IGF-I levels, proportional growth retardation, elevated insulin sensitivity and reduced islet β-cell mass. Unlike the liver which is mostly unaffected by changes in the IGF-I level, skeletal muscles express high levels of IGF-I receptor (IGF-IR). The net result of a concurrent deficiency in the actions of both GH and IGF-I, which exert opposite influences on insulin responsiveness, has not been evaluated. Here we have studied insulin-stimulated early responses in the insulin receptor (IR), IRS-1 and p85 subunit of PI3K. Upon in vivo insulin stimulation, the skeletal muscles of $GHR^{-/-}$ mice exhibit transient delayed responses in IR and IRS-1 phosphorylation, but normal level of p85 association with IRS-1. This is in contrast to normal/elevated insulin responses in hepatocytes and indicates tissue-specific effects of GHR gene deficiency. In addition to stimulating normal islet cell growth, GH might participate in islet cell overgrowth, which compensates for insulin resistance induced by obesity. To study whether the islet cell overgrowth is dependent on GH signaling, we have studied the response of male $GHR^{-/-}$ mice to high-fat diet (HFD)-induced obesity. After 17 weeks on a HFD, $GHR^{-/-}$ mice became more significantly obese than the wild-type mice and exhibited increased β-cell mass to a slightly higher extent. This data demonstrates that GH signaling is not required for compensatory islet growth. Thus, in both muscle insulin

responsiveness and islet growth compensation, normal levels of GH signals do not seem to play a dominant role.

2.2 Introduction

Growth hormone (GH), acting through its receptor (GHR), is essential for somatic growth and development and maintaining metabolic homeostasis [473, 474]. Concerning insulin production and action, GH is known to stimulate islet cell growth and insulin secretion [225, 475-477]. On the other hand, GH counteracts insulin actions and causes insulin resistance, when in excess, on insulin target tissues. Consequently, GHR gene deficient ($GHR^{-/-}$) mice exhibit proportional growth retardation; elevated insulin sensitivity partly due to increased insulin receptor (IR) level and receptor activation in hepatocytes; reduced islet β-cell mass due to decreased islet cell proliferation; and selective increases in fat pad weights [458, 471, 478, 479]. GHR gene deficiency also causes a concurrent decrease in the production of insulin-like growth factor I (IGF-I) which plays a role in islet cell growth, insulin secretion and maintaining insulin sensitivity [448, 455, 470, 480, 481]. Unlike the liver which is mostly unaffected by IGF-I, skeletal muscles express high levels of IGF-I receptor (IGF-IR) [470]. Both tissues are prime targets of GH actions. The net result of a concurrent deficiency in the actions of both GH and IGF-I, which normally exert opposite influences on insulin responsiveness, has not been evaluated in the $GHR^{-/-}$ mouse. Maintaining islet β-cell mass and adequate insulin secretion to meet metabolic demands is crucial to avoid the development of type 2 diabetes [8, 482-

484]. In this aspect, in contrast to human Laron syndrome where GH insensitivity is characterized by hyperinsulinemia, insulin resistance and trunkal obesity, $GHR^{-/-}$ mice exhibit decreased serum insulin levels, increased insulin sensitivity and are not grossly obese [478, 485, 486]. This may be caused by species-specific characteristics of GH physiology but more likely by the fact that mice are not exposed to the "buffet-type" human diet, which favors obesity and insulin resistance. In addition to stimulating normal islet cell growth, GH might participate in islet cell overgrowth, which compensates for insulin resistance induced by obesity. To our knowledge, a response of $GHR^{-/-}$ mice to diet induced obesity, its possible effects on insulin responsiveness and islet compensation has not been reported. In order to extend our early studies on $GHR^{-/-}$ mice in the two key aspects, i.e. increased insulin sensitivity and decreased islet cell growth, the current study was designed to explore the interplay of GH and IGF-I in regulating insulin responsiveness of the skeletal muscles; and the response of $GHR^{-/-}$ mice to high-fat diet-induced obesity, insulin resistance and islet overgrowth.

2.3 Material and Methods

Materials.

The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). The monoclonal anti-phosphotyrosine antibody (αPY, PY99) and the polyclonal anti-IR β-subunit antibody (αIR, C-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-rat carboxyl-terminal insulin receptor substrate-1 (IRS-1; αIRS-1 antibody), and

the antibody to the p85 subunit of phosphatidylinositol 3-kinase (PI3K; αp85) were purchased from Upstate Biotechnology (Lake Placid, NY). The The insulin antibody (H-86) used in immunohistochemistry was

obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animal Procedures.

$GHR^{-/-}$ mice carry a targeted disruption of exon 4 of the mouse GHR/binding protein (GHR/BP) gene, as previously reported [458]. Offspring (wild types as controls and $GHR^{-/-}$) derived from heterozygous ($GHR^{+/-}$) mating pairs on a hybrid 129/Ola-BALB/c-C57BL/6 background were used in experiments. To determine genotype, we isolated genomic DNA from tail clips with standard methods. Primers In4-1 (5'-CCC TGA GAC CTC CTC AGT TC), In3-1 (5'-CCT CCC AGA GAG ACT GGC TT), and Neo-3 (5'-GCT CGA CAT TGG GTG GAA ACA T) were used in PCR reactions, which yield a 390-base band for the wild-type allele and 290/200 double bands for the knockout allele, as previously reported [479]. The animals were maintained in 12:12-h dark-light cycles at room temperature with free access to food and water or, when indicated, were food deprived for 24 h with free access to water. For the study of insulin response, $GHR^{-/-}$ mice and their wild-type littermates (6 mo of age) were fasted for 24 h and anesthetized by intraperitoneal administration of a cocktail of ketamine-xylazine-acepromazine. The mice were injected with insulin (10 IU/kg, ip) or saline; after 5 or 15 minutes they were killed and their soleus and gastrocnemius muscles were removed along with the liver to prepare cell lysate. All animal-handling procedures were approved by the McGill University Animal

Care Committee. Serum concentrations of insulin (Linco Research) were determined using a radioimmunoassay kit.

Immunoprecipitation and Western blots.

The tissues were homogenized in 5 ml protein extraction Buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 0.5% Nonidet P-40, 100mM NaF, 10mM sodium pyrophosphate, 10mM sodium orthovanadate and protease inhibitors (Complete Mini EDTA-free; Roche, Indianapolis, IN) at 4° C [481]. The samples were incubated on ice for 30 min and centrifuged at 600 rpm for 20 min at 4° C, and then the supernatant was removed and centrifuged at 13,000 rpm for 45 min at 4°C in a Beckman JA20-1 rotor. The Bradford Assay (Bio-Rad) was used to measured protein concentration and the sample was aliquoted and stored at –80° C.

Tissue lysates containing 2 mg of total protein were immunoprecipitated with the antibodies αIR or αIRS-1 (2 μg/ml final concentration) overnight at 4° C. The next day, 40 μl protein G-Sepharose (Roche) were added, and samples were incubated for another 1.5 h on a rocking platform at 4° C and centrifuged at 14,000 rpm for 2 min at 4° C. The precipitate was washed three times with the protein extraction buffer. The final precipitate was boiled for 5 min in 40 μl of Laemmli Sample Buffer (Bio-Rad).

Samples were loaded onto a 7% SDS-polyacrylamide gel with the use of a Mini Protean apparatus (Bio-Rad). Transfer of proteins from the gel to 0.2 μm nitrocellulose (Trans-Blot transfer medium; Bio-Rad) was performed for 1.5 h at 100 V using the Bio-Rad mini transfer apparatus in a transfer buffer consisting of

25 mM Tris, 192 mM glycine, and 20% methanol. The membranes were blocked for 1 h at room temperature to reduce non-specific binding in a TBS-T buffer (composed of 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.6) containing 3% BSA for phosphotyrosine detection or 2% ECL Advance blocking agent (Amersham Biosciences, Anhersham, UK) for protein detection. The membranes were incubated overnight at 4°C with αPY, αIR, or αIRS-1 (all at 1:1000) each and diluted in corresponding blocking buffer. The membranes were washed, incubated with secondary antibodies, and detected by chemiluminescence using the ECL Advance Western blotting detection kit (Amersham). To detect the amount of p85 associated with IRS-1, the blots that were used to detect αIRS-1 were stripped with Re-Blot Plus (Chemicon International), washed in TBS-T for 5 min, blocked for 30 min, and then probed with αp85 antibody (1:1,000) and detected using chemiluminescence. Images were captured using a Fluorchem 8900 imager (Alpha Innotech, San Leandro, CA) and densitometry was carried out using AlphaEase software (Alpha Innotech).

High-fat diet-induced obesity.

Male $GHR^{-/-}$ mice and their male wild-type littermates (3.5 mo of age) were fed for 17 weeks with a high-fat diet (HFD; Research Diets, New Brunswick, NJ). Their body weight was measured once a week and blood glucose levels every 3 wk, using a OneTouch blood glucose meter (Lifescan, Burnaby, BC, Canada). At 15 wk, an insulin tolerance test was performed. Animals were injected with recombinant human insulin (0.75 IU/kg ip; Roche), and blood glucose levels were measured at 0, 20, 40, and 60 min afterward. At 17 weeks the

mice were anesthetized by intraperitoneal administration of a cocktail of ketamine-xylazine-acepromazine, and then body length and weight were measured and fat pads were removed and weighed. The mice were killed by cervical dislocation and then blood was collected for serum preparation and pancreata were rapidly removed for histochemical analysis.

Immunohistochemistry and islet cell mass measurement.

Pancreatic sections were stained with an insulin antibodies (H-86; Santa Cruz Biotechnology) using diaminobenzidine substrate, which resulted in a brown immunoreactive signal with a hematoxylin counterstain (blue) of cell nuclei. The β-cell mass and average cell size were determined as previously reported [86, 471].

Statistical analysis.

Data are expressed as mean ± SE. Student's *t*-test (unpaired and paired) was performed using InStat software version 3 (GraphPad Software, San Diego, CA).

2.4 Results

A general characterization of $GHR^{-/-}$ mice was reported by our group recently [471, 487]. Because of a lack of GH signaling, the body weight of adult $GHR^{-/-}$ mice is only one-half that of their wild-type littermates, confirming severe growth retardation; blood glucose and serum insulin levels are significantly reduced to 80% and 40% of the normal values, respectively; serum level of the total IGF-I is reduced to one-half because of a lack of GH stimulation; and $GHR^{-/-}$

mice exhibit significantly increased sensitivity to insulin tolerance tests [471]. Most of these characteristics have also been reported by other groups [458, 478, 488, 489] and were unchanged in the animals used in this study (data not shown).

Delayed and diminished insulin receptor phosphorylation in skeletal muscle.

$GHR^{-/-}$ mice exhibit elevated insulin responsiveness including increased IR levels and activation in the hepatocytes [458, 488]. To study whether this phenomena is tissue-specific, we have tested insulin-stimulated early responses in the skeletal muscle and liver. $GHR^{-/-}$ mice and control littermates were injected with insulin (10 IU/kg, ip) or saline, and muscle or liver homogenates were subjected to immunoprecipitation with the use of an antibody against the IR β-subunit (αIR), followed by immunoblotting with the same antibody and that against the tyrosine phosphorylation (αPY). After densitometry analysis was completed, the amount of tyrosine phosphorylation of the IR β-subunit was normalized using the total receptor levels. As shown in **Figure 2.1**, in skeletal muscles of wild-type mice, 5 min of insulin treatment caused a significant 3.5-fold increase in IR phosphorylation (**A & B, left**) over that of untreated animals. However, upon insulin stimulation of $GHR^{-/-}$ mice, IR phosphorylation was only marginally elevated 1.3 fold [not significant (NS)]; significantly lower than in wild-type mice. In contrast, in the liver (**Figure 2.1, A and B, right**), insulin treatment caused significantly increased phosphorylation in both groups of mice, e.g., 7.5- and 6.8-fold in wild-type and $GHR^{-/-}$ mice, respectively. Later, at 15 min, wild-type mice maintained a significant elevation in IR phosphorylation (4.3-fold; **Figure 2.1, C and D, left**), which was only marginally elevated 1.9-

fold in $GHR^{-/-}$ mice ($P<0.05$); lower than in wild-type mice mostly because of an elevated basal activity. AS a control, in the liver (**Figure 2.1, C and D, right**), insulin treatment caused fourfold significantly increased phosphorylation in both the wild-type and $GHR^{-/-}$ mice compared with untreated controls. The IR responses in the liver of $GHR^{-/-}$ mice, almost identical to that of wild-type mice, were slightly lower than reported previously [488].

Delayed IRS-1 Phosphorylation in skeletal muscle.

The same sets of muscle/liver proteins were used to determine the level of IRS-1 phosphorylation. The muscle or liver homogenates were subjected to immunoprecipitation with an antibody against IRS-1 (αIRS-1), followed by immunoblotting with the same antibody and another against the tyrosine phosphorylation (αPY). The data were normalized with the amount of total IRS-1 levels (**Figure 2.2**). In skeletal muscles of wild-type mice, 5 min of insulin treatment caused a significant increase in IRS-1 phosphorylation (2.1-fold), that was virtually abolished in the $GHR^{-/-}$ mice (**Figure 2.2, A and B, left**). As a positive control for insulin action in **Figure 2.2, A and B, right**, IRS-1 phosphorylation in liver samples displayed a 1.5 fold increase in both wild-type and $GHR^{-/-}$ mice compared with untreated controls. Later at 15 min, the muscles of both wild-type and $GHR^{-/-}$ mice showed comparable and significant elevations of IRS-1 phosphorylation of 2.0- and 1.6-fold respectively (**Figure 2.2, C and D, left**). The liver samples (**Figure 2.2, C and D, right**) exhibited an increase in IRS-1 phosphorylation of 1.7- or 3.5-fold in wild-type or $GHR^{-/-}$ mice, respectively. The IRS-1 responses in the liver of $GHR^{-/-}$ mice were slightly higher

than reported previously [488]. The results of IR and IRS-1 phosphorylation in the skeletal muscles are in contrast to that reported previously and to what we have demonstrated in the hepatocytes, which exhibited increased IR protein level, normal or slightly increased (rather than delayed or decreased) receptor activation, and IRS-1 phosphorylation in $GHR^{-/-}$ mice [488].

Normal p85 stimulation in skeletal muscle.

PI3K is activated after insulin stimulation and IRS-1 phosphorylation. In this study, the PI3K activation was measured as the amount of p85 protein associated with IRS-1 in an immunoprecipitate with the use of anti-IRS-1. With the same muscle or liver homogenates that were immunoprecipitated with αIRS-1 (**Figure 2.2, C and D**), the proteins were immunoblotted with an antibody against p85 (αp85). The values were normalized by the total IRS-1 levels (**Figure 2.3B**). In the muscle, p85 levels were increased similarly (2.6- and 2.2- fold) in both wild-type and $GHR^{-/-}$ mice, when stimulated by insulin; i.e., the signaling pathway leading to the activation of the PI3K by insulin was unaltered in the skeletal muscles of $GHR^{-/-}$ mice. As positive controls, increased p85 association with IRS-1 was demonstrated in the livers of both wild-type and $GHR^{-/-}$ mice, as reported previously [488].

HFD-induced obesity in the $GHR^{-/-}$ mice.

To study whether GH signaling is involved in the compensatory growth of pancreatic islets in response to obesity-induced insulin resistance, we challenged male $GHR^{-/-}$ mice and their wild-type littermates with a HFD for 17 wk. Their body weights were measured once a week (**Figure 2.4A**) and their blood glucose

levels every 3 wk (data not shown). Both wild-type and $GHR^{-/-}$ mice gained significant weight with the HFD, 21% and 31% of their initial body weights, respectively (**Figure 2.4B**). To further demonstrate increased fat mass and reveal potential depot-specific effect, various fat pads were excised, weighed and corrected for total body weight. As shown in **Figure 2.4C**, both the $GHR^{-/-}$ mice and their wild-type littermates significantly increased the weight of each fat pad (except the renal in the $GHR^{-/-}$), indicating that $GHR^{-/-}$ mice are not resistant to HFD-induced obesity. Normally, $GHR^{-/-}$ mice are hypersensitive to insulin, in contrast to human Laron patients. To determine whether the mice have decreased insulin responsiveness as a result of obesity, we performed an insulin tolerance test on all animals, on either HFD or normal diet, 2 wk before the end of the study. In both wild-type and $GHR^{-/-}$ mice, a 15-wk HFD failed to cause a significant change in insulin sensitivity due to obesity. Serum insulin levels, another indirect indicator of insulin resistance, were unaffected as well (data not shown). Thus, HFD for 17 weeks created obesity, but not insulin resistance, in both wild-type and $GHR^{-/-}$ mice.

Evaluation of islet cell overgrowth due to obesity.

To study whether the HFD-induced obesity can cause a compensatory islet overgrowth in the $GHR^{-/-}$ mice, we measured β-cell mass at 17 wk from pancreatic sections stained with insulin. As shown in **Figure 2.4D**, consistent with our previous report [471], $GHR^{-/-}$ mice on the normal diet had only 45% of the β-cell mass compared with wild-type littermates; after the HFD, however, both types of mice displayed similar extents of islet compensation, e.g. 2.8-fold in wild-type

and 3.3-fold in $GHR^{-/-}$ mice, respectively. Representative islets are illustrated in **Figure 2.4E**. In both wild-type and $GHR^{-/-}$ mice, HFD caused a significant enlargement of the islet size. The islet compensation was likely caused by cell hyperplasia, because the average cell size (representing hypertrophy) and islet density per tissue area (representing islet neogenesis) were unaffected (data not shown). Thus, obese $GHR^{-/-}$ mice exhibited a normal, compensatory overgrowth of islet cells.

2.5 Discussion

Using $GHR^{-/-}$ mice, our group recently demonstrated that GH signaling is essential for maintaining pancreatic islet growth, stimulating islet hormone production and maintaining normal insulin sensitivity and glucose homeostasis [471]. Through islet-specific overexpression of IGF-I, we were able to rescue some of the islet defects, suggesting that IGF-I mediates some GH actions on islet growth [487]. In the current study, we further characterized $GHR^{-/-}$ mice in insulin responsiveness and in islet cell growth. Specifically, we have shown that GH signaling does not play a dominant role in either insulin responsiveness in the skeletal muscles and in islet growth compensation due to obesity. Antagonizing insulin's actions, GH decreases glucose uptake, maintains hepatic glucose production, decreases responsiveness of target tissues to insulin and diminishes the conversion of glucose to fat. Conversely, $GHR^{-/-}$ mice are clearly hypersensitive to insulin's actions [471, 488]. As for specific target tissues affected, it has been reported [488] that in the hepatocytes of $GHR^{-/-}$ mice, both

the basal levels of IR protein and the response in IR phosphorylation are elevated. As another important target tissue of insulin action, the role of skeletal muscles has not been evaluated in $GHR^{-/-}$ mice. In a similar system, GH-deficient Ames dwarfs, also hypersensitive to insulin, exhibit reduced insulin-stimulated phosphorylation of IR and IRS-1 in the skeletal muscles [490]. Our results are thus consistent with those of Ames mice, perhaps because of a common deficiency in endogenous IGF-I production.

As a primary insulin target, skeletal muscles express high level of receptors for insulin, GH and IGF-I [491]. Insulin causes IR autophosphorylation, which recruits IRS-1 and other SH2-containing docking molecules. Among other signaling pathways, insulin stimulation causes dissociation of the p85 subunit from PI3K and, thereby, activation of the p110 subunit, which is a major mediator of insulin's actions [492, 493]. These early responses cause activation of downstream molecules such as Akt/PKB, recruitment of the glucose transporter GLUT4 into plasma membrane, and increased glucose uptake into the muscle cells. The current study was designed to reveal possible changes in early insulin responses in the skeletal muscle and to compare them with those of hepatocytes in $GHR^{-/-}$ mice. In contrast to the elevated or normal insulin responsiveness exhibited in the liver of $GHR^{-/-}$ mice [488], our results indicate that upon to insulin stimulation, there is no elevated insulin response; i.e., skeletal muscles do not contribute to increased insulin sensitivity in $GHR^{-/-}$ mice. If anything, there were transiently delayed and/or diminished responses in the early phase of IR or IRS-1 phosphorylation. One of the underlying difference is that hepatocytes do

not express IGF-I receptor [494] and are unaffected by secondary deficiency in IGF-I production in these $GHR^{-/-}$ mice. It seems that IGF-I plays a greater role in skeletal muscles in maintaining insulin responsiveness: its deficiency causes diminished insulin response. IGF-I does not bind to hepatocytes or adipocytes, and therefore its primary insulin-like action is believed to be mediated through the skeletal muscles [495]. Indeed, in newborn IR deficient mice, IGF-I directly activates PI3K and, presumably glucose uptake in the muscles, because it corrects the hyperglycemia [496]. It has been established that in the skeletal muscles, GLUT-4 translocation to the cell membrane is stimulated by both insulin and IGF-I through their cognate receptors, a crucial process in postprandial glucose disposal. The importance of this mechanism is clearly demonstrated by muscle-specific ablation of the GLUT-4 gene, which causes severe insulin resistance and glucose intolerance [497]. Moreover, muscle-specific inactivation of both IR and IGF-IR in MKR mice, by overexpressing a dominant negative protein, creates an even more severe phenotype by causing an early onset of diabetes [470]. To understand the relative contributions of either IR or IGF-IR to this defect, note that muscle-specific inactivation of IR gene alone (using Cre/loxP system) is insufficient to cause significant insulin resistance or glucose intolerance [498], indicating that IGF-IR, or IGF-IR in conjunction with IR, plays a potent role in stimulating glucose uptake in the skeletal muscles. Of course, for more conclusive proof one would have to create a specific ablation of IGF-IR gene alone in the muscle cells. In the meantime, our results indicate that GH signaling is not dominant in counteracting insulin's actions in the muscles and that the major site

at which GH antagonizes insulin's action is the liver [495]. This supports tissue-specific influences of GH on in vivo insulin responsiveness.

There is no doubt that GH has an insulin-counterregulatory role in skeletal muscle and our results are, in general, consistent with other related models of GH deficiency or excess. Transgenic mice overexpressing GH antagonist (GHa) exhibit elevated insulin sensitivity and decreased blood glucose and serum insulin levels, similar to $GHR^{-/-}$ and Ames dwarfs, although their growth retardation is much milder [499]. They maintain normal rate of glucose uptake in skeletal muscles and brown adipose tissues, and exhibit normal (rather than elevated) insulin responses in IR, IRS-1 and Akt in the skeletal muscles, similar to our $GHR^{-/-}$ mice [499]. The enhanced insulin sensitivity in GHa mice seems to be caused by significantly improved glucose uptake in white adipose tissues, resembling that occurring in the liver of $GHR^{-/-}$ mice. In this study, slightly differently from that in GHa mice, the delayed insulin responsiveness in the skeletal muscles was transient and limited to IR and IRS-1 only. By 15 min after insulin stimulation, all parameters including PI3K were normalized. Whether this phenomenon has any physiological implications needs to be addressed in future studies. For instance, as a primary organ for glucose disposal, insulin-stimulated glucose uptake could be affected, albeit transiently. Even then, an accumulated effect may consequently contribute to the phenotypes such as longevity of these mice [490]. This study and the reports of insulin responsiveness in the liver of $GHR^{-/-}$ mice and in the muscles of Ames dwarfs suggest that normal levels of GH signals do not antagonize insulin responsiveness in the skeletal

muscles [488, 490]. This is not necessarily contradictory to the finding that GH antagonist corrects the insulin resistance in LID mice, because the latter finding only implies that excessive GH secretion causes insulin resistance in skeletal muscles [499].

In addition to effects on insulin action, GH is an important growth factor for islet cells [446, 455]. Downstream of the GHR/Jak2 interaction, signal transducer and activator of transcription 5 activation and consequent induction of cyclin D2 are essential for the mitogenic effect of GH on β-cells [225, 231]. In non-islet cells, GH increases the activity or protein level of Foxa-2 and hepatocyte nuclear factor 1α, key molecules in β-cell growth [232, 233]. These factors likely mediate GH-stimulated islet cell growth. However, it is unclear whether GH signals are involved in compensatory overgrowth of islet cells such as in pregnancy or obesity. In this aspect, GH secretion is markedly diminished due to obesity [500], which may contribute to the eventual failure of β-cells.

Thus, we were interested in studying whether $GHR^{-/-}$ mice would be able to increase β-cell growth in response to obesity. On the other hand, $GHR^{-/-}$ mice are not grossly obese, unlike human Laron syndrome [471]. Another question was whether $GHR^{-/-}$ mice would be resistant to obesity itself. For these purposes, we have successfully induced obesity in two sets of mice at 3-4 and 9-10 mo of age. Under the experimental conditions, the obesity was not severe enough to cause significant changes in insulin tolerance, let alone diabetes. Nevertheless, our results clearly demonstrate that $GHR^{-/-}$ mice respond more efficiently to the HFD in becoming obese and exhibit a significant increase in islet cell growth, slightly

higher than wild-type mice. Thus, GH signals are not essential for the compensatory growth of islet cells in response to obesity. The causes of enlarged β-cell mass in nondiabetic obese humans and rodents include increased islet cell replication, neogenesis and cellular hypertrophy [442, 501, 502]. Currently, there are many other factors for islet cell growth that are potentially involved in islet compensation. They include cyclins D1 and D2 [503], nutrients such as glucose, hormones such as a combination of epidermal growth factor and gastrin [115], glucagon-like peptide-1, and several growth factors including fibroblast growth factor and hepatocyte growth factor/scatter factor [504, 505]. As we wrote this report, we were unaware of any positive involvement of these factors in obesity-induced islet compensation.

In summary, in response to in vivo insulin stimulation, the skeletal muscles of $GHR^{-/-}$ mice exhibit transient delayed and/or diminished response in IR and IRS-1 phosphorylation. This finding is in contrast to elevated or normal insulin responses in hepatocytes, perhaps because of a concurrent decrease in IGF-I effect. When challenged with a HFD, $GHR^{-/-}$ mice became more significantly obese, in contrast to the human Laron syndrome of GH insensitivity, which displays default obesity over the normal population. As a consequence of obesity, $GHR^{-/-}$ mice displayed an enhanced β-cell compensation, slightly greater than that of wild-type mice, demonstrating that GH signals are not required for compensatory islet growth. Thus, in both muscle insulin responsiveness and islet compensation, GH does not seem to play a dominant role.

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Figure 2.1. Skeletal muscles exhibit delayed and/or diminished responses in insulin-stimulated insulin receptor (IR) phosphorylation in growth hormone receptor-deficient ($GHR^{-/-}$) mice.

Mice fasted for 24 h were injected with insulin (10 IU/kg, ip) for 5 (A and B) or 15 min (C and D) before being killed, and their muscles or livers were removed to prepare cell lysates. A and C: lysates were precipitated with IR antibody and probed with the same antibody as well as with anti-phospho-tyrosine (IR-P-tyr) in Western blots. A representative blot is illustrated from experiments of n=8 for skeletal muscles and n=3 for liver. B and D: densitometric quantification of the IR phosphorylation levels, corrected by total IR protein level. *P < 0.05; **P < 0.01; ***P < 0.001vs untreated controls.

Figure 2.1

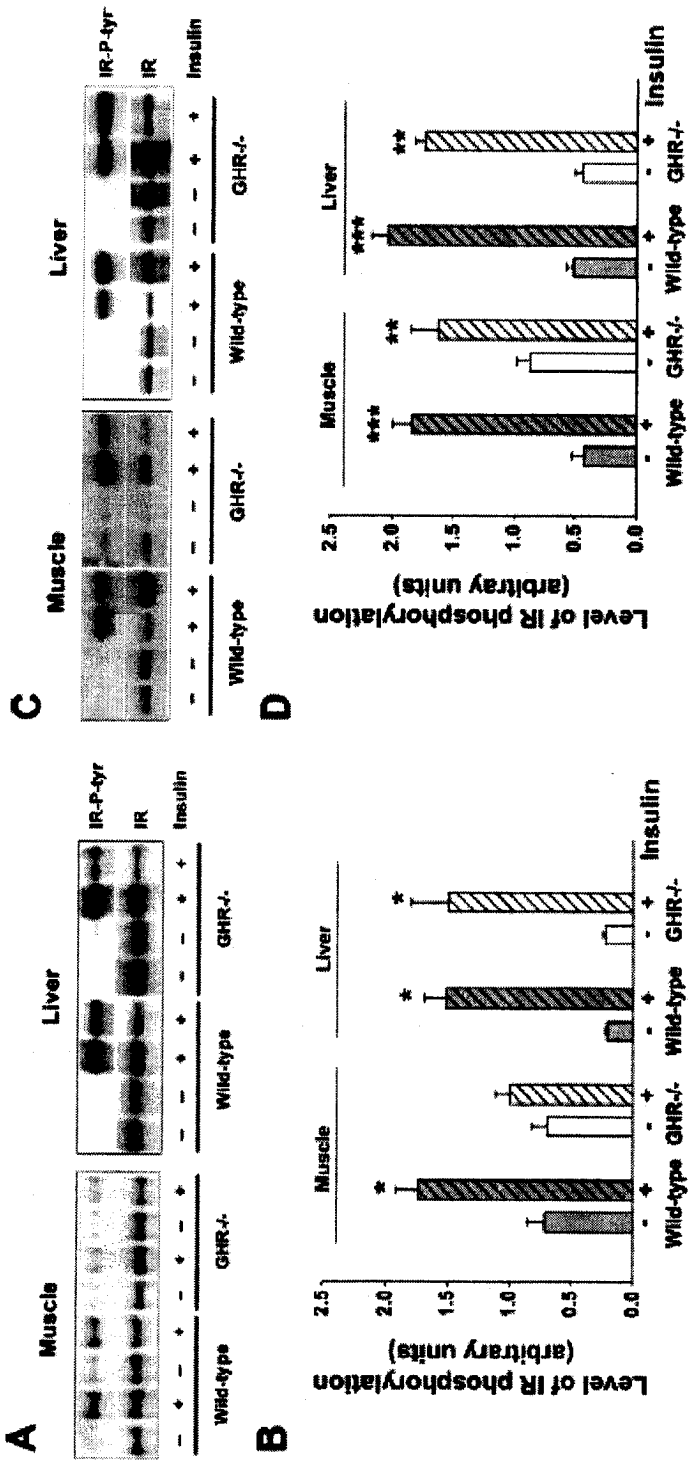

A
Muscle
Liver
IR-P-tyr
IR
Insulin
Wild-type
GHR-/-
Wild-type
GHR-/-
B
Level of IR phosphorylation (arbitrary units)
Muscle
Liver
Insulin
C
Muscle
Liver
IR-P-tyr
IR
Insulin
D
Level of IR phosphorylation (arbitrary units)
Muscle
Liver
Insulin

Figure 2.2. Skeletal muscles exhibit a delayed response in insulin-stimulated insulin receptor substrate-1 (IRS- 1) phosphorylation in $GHR^{-/-}$ mice.

Mice fasted for 24 h were injected with insulin (10 IU/kg, ip) for 5 (A and B) or 15 min (C and D) before being killed, and their muscles or livers were removed to prepare cell lysate. A and C: lysates were precipitated with IRS-1 antibody and probed with the same antibody as well as with anti-phospho-tyrosine (IRS-1-P-tyr) in Western blots. A representation blot is illustrated from experiments of n=8 at 5 min and n=5 at 15 min for skeletal muscles and n=3 for liver. B and D: densitometric quantification of the IRS-1 phosphorylation levels corrected by total IRS-1 protein level. *P<0.05; **P < 0.01 vs untreated controls.

Figure 2.2

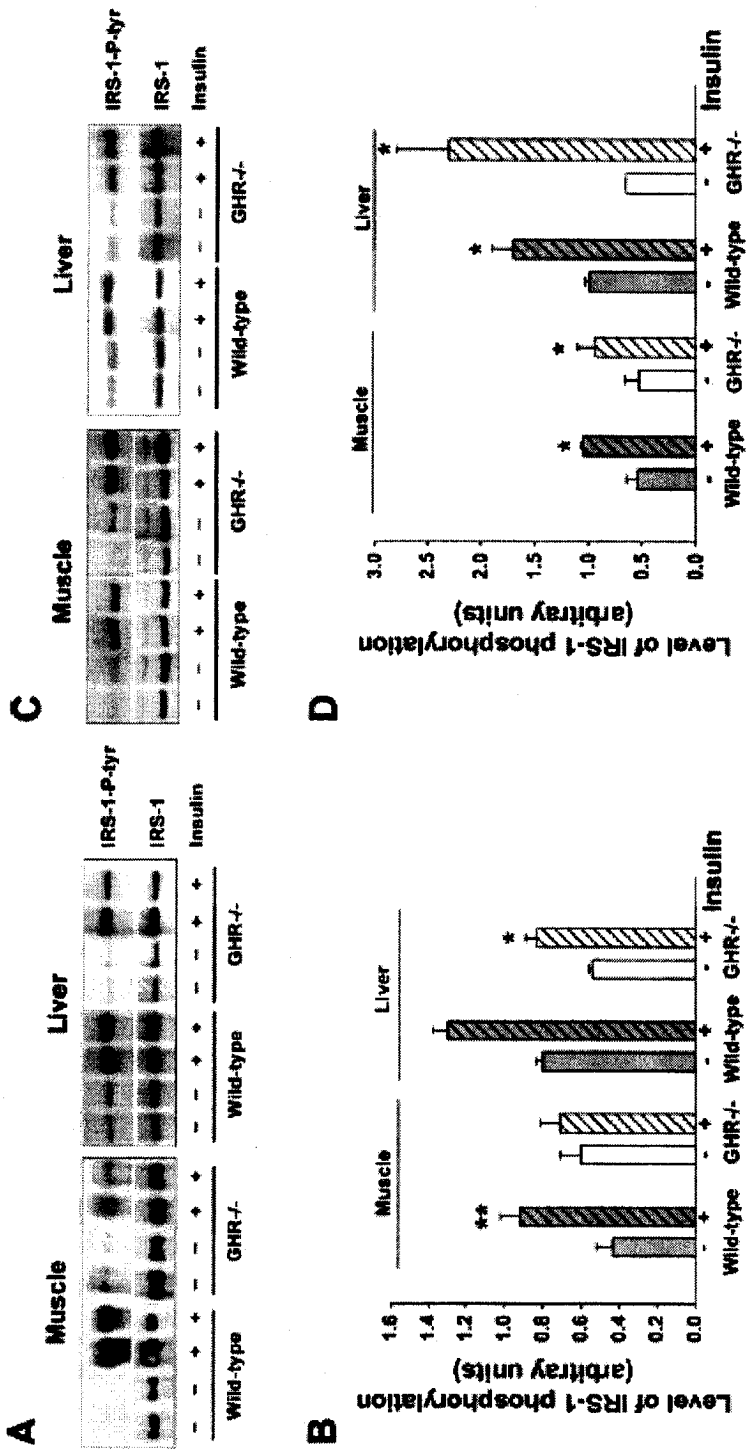

A
Muscle
Liver
IRS-1-P-tyr
IRS-1
Insulin
Wild-type
GHR-/-
Wild-type
GHR-/-
C
Muscle
Liver
IRS-1-P-tyr
IRS-1
Insulin
Wild-type
GHR-/-
Wild-type
GHR-/-
B
Level of IRS-1 phosphorylation (arbitrary units)
Muscle
Liver
Insulin
Wild-type
GHR-/-
Wild-type
GHR-/-
D
Level of IRS-1 phosphorylation (arbitrary units)
Muscle
Liver
Insulin
Wild-type
GHR-/-
Wild-type
GHR-/-

Figure 2.3. Insulin-stimulated p85 association with IRS-1 in skeletal muscles of $GHR^{-/-}$ mice and their wild-type littermates.

Mice fasted for 24 h were injected with insulin (10 IU/kg, ip) for 15 min before being killed, and their muscles or livers were removed to prepare cell lysate. A: lysates (same as in Figure 2.2C) were precipitated with IRS-1 antibody and probed with the same antibody, stripped and then probed with p85 antibody in Western blots. A representative blot is illustrated from experiments of n=5 for skeletal muscles and n=3 for liver. B: densitometric quantification of the p85 levels corrected by total IRS-1 protein level. *P < 0.05 vs untreated controls.

Figure 2.3

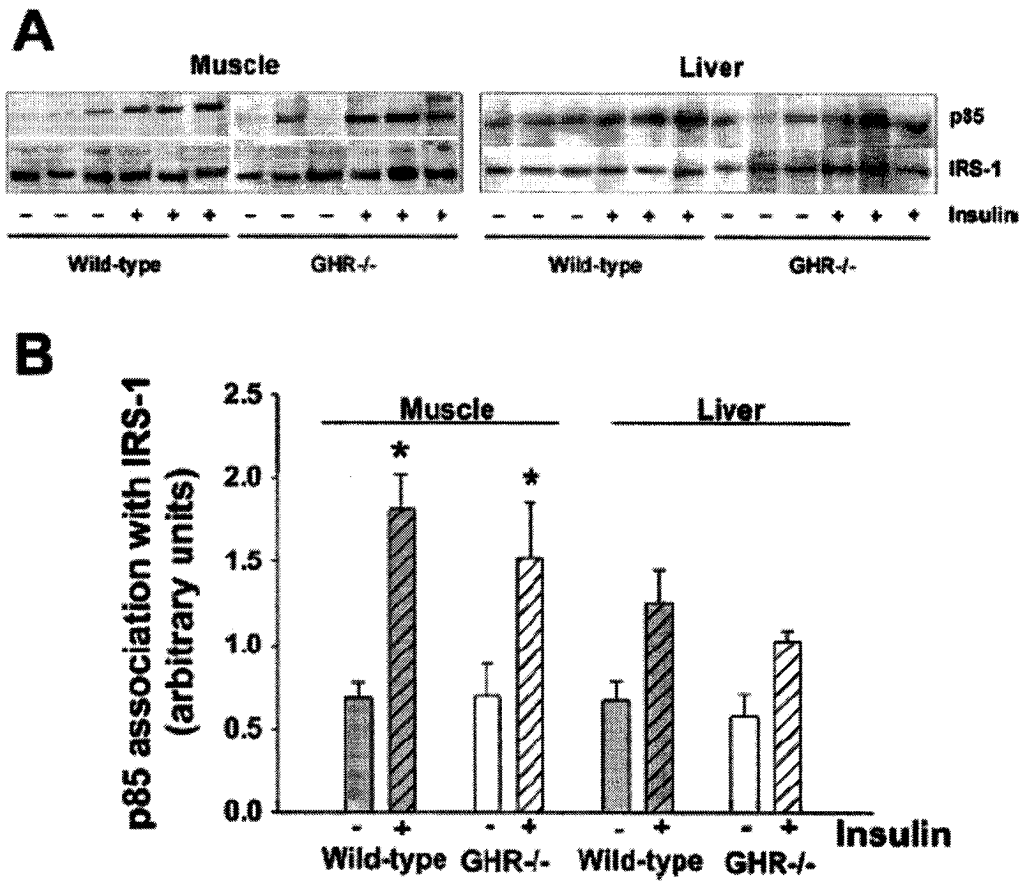

A
Muscle
Liver
p85
IRS-1
Insulin
Wild-type
GHR-/-
Wild-type
GHR-/-
B
p85 association with IRS-1 (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
Muscle
Liver
Insulin
Wild-type
GHR-/-
Wild-type
GHR-/-

Figure 2.4. High-fat diet (HFD)-induced obesity and pancreatic islet overgrowth in GHR$^{-/-}$ mice.

A: HFD induced steady increases in body weight in both wild-type and GHR$^{-/-}$ mice. Male GHR$^{-/-}$ and their wild-type littermates (3.5 mo) were fed normal or HFD for 17 wk. Their body weight was measured once a week and plotted against the number of weeks on the diet. n=4~5. KO, knockout mice. *P<0.05 vs normal diet. B: percent body weight gain (per initial weight) after 17 wk on HFD or normal diet. *P < 0.05; **P<0.01 vs normal diet; @ P < 0.05 vs wild-type on HFD. C: HFD caused increases in fat pad weights (per total body weight) in wild-type and GHR$^{-/-}$ mice. After 17 wk on HFD, 4 fat pads were excised and measured. BW, body weight. *P < 0.05 vs normal diet. D: changes in β-cell mass after HFD, per total body weight. There was a 2.8-fold increase in wild-type mice after HFD. GHR$^{-/-}$ mice normally exhibit diminished β-cell mass (45% of wild-type level) but displayed a 3.3-fold increase after HFD. *P < 0.05 vs normal diet; @P < 0.05 vs wild-type on normal diet. E: changes in pancreatic islet morphology after HFD. Pancreatic sections were stained for insulin (brown) and counter-stained for cell nuclei (blue). In both wild-type and GHR$^{-/-}$ mice, HFD caused a significant enlargement in the islet size. Representative results for 2 experiments are shown. Scale bar, 100 μm

Figure 2.4

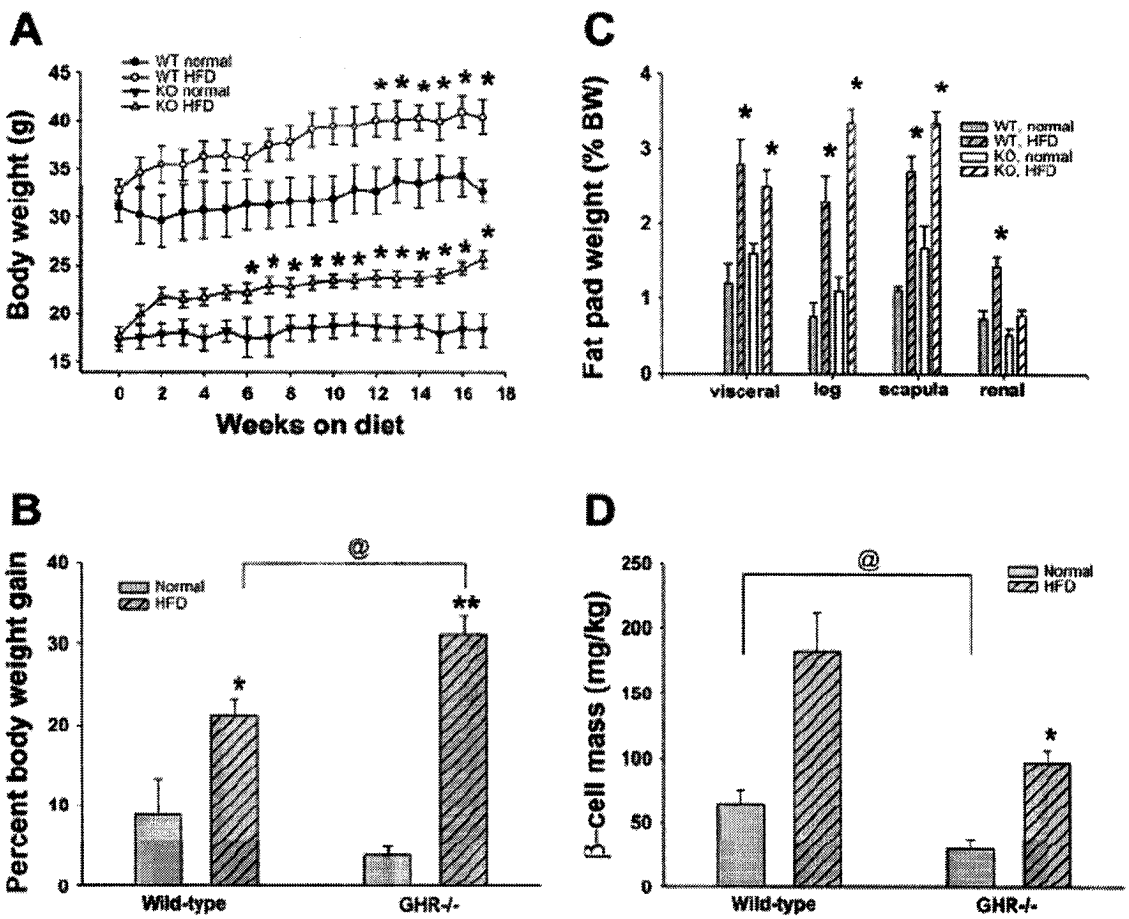
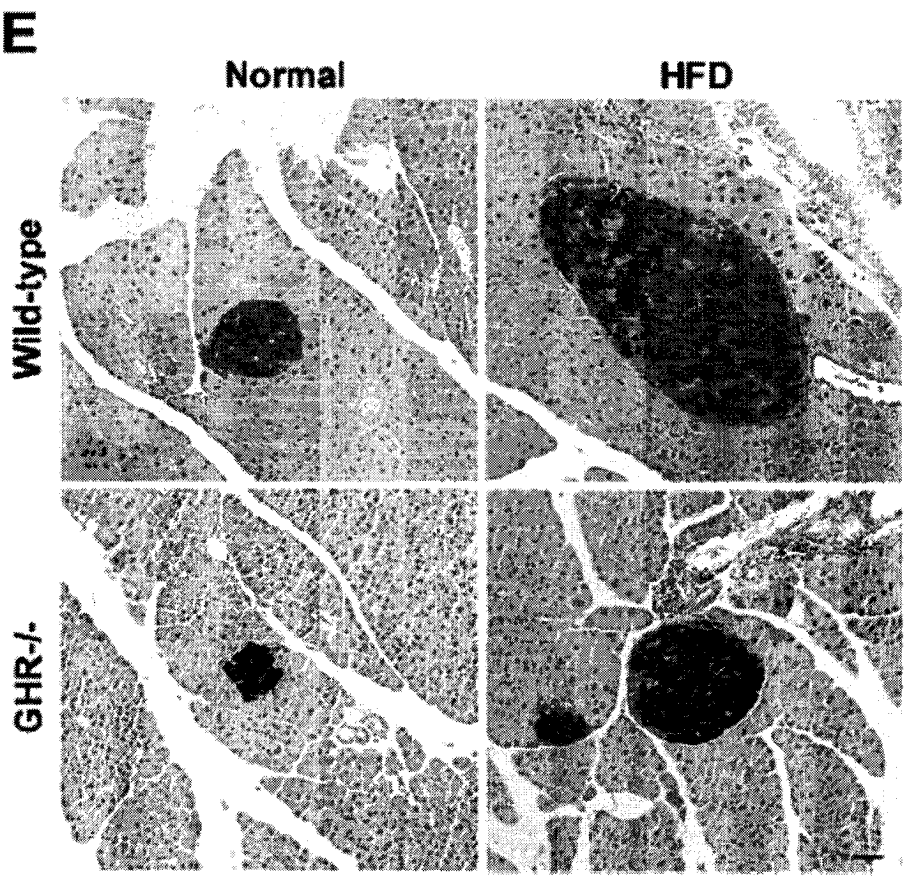

A
WT normal
WT HFD
KO normal
KO HFD
Body weight (g)
Weeks on diet
C
Fat pad weight (% BW)
WT, normal
WT, HFD
KO, normal
KO, HFD
visceral
leg
scapula
renal
B
Percent body weight gain
Normal
HFD
Wild-type
GHR-/-
D
β-cell mass (mg/kg)
Normal
HFD
Wild-type
GHR-/-

Figure 2.4



Connecting Text

Our initial hypothesis was that both GH and IGF-I are involved in islet cell growth and IGF-I mediates the effects of GH. To validate this, we have demonstrated that GH signals are indeed crucial for normal islet growth, using $GHR^{-/-}$ mice [471]. As IGF-I production from the liver and every other tissues was affected, we thought to rescue the phenotype using IGF-I overexpression. Our next plan was to use whole body IGF-I overexpression (MT-IGF) mice to rescue the islet size in $GHR^{-/-}$ mice by creating double transgenic mice. First we needed to characterize the MT-IGF mice in regards to islet cell growth and insulin responsiveness. This second part of study becomes the next chapter. After that, our original goal was to create double transgenic mice that are deficient in GHR but overexpress IGF-I, although we were unsuccessful because none of the double transgenic pups survived. This obstacle was unexpected as the double crossing had been achieved although the models utilized were different (GH deficient mice versus $GHR^{-/-}$ mice) [506]. We thus finished the project after characterizing the MT-IGF mice.

Chapter Three

A general overexpression of IGF-I results in normal islet cell growth, hypoglycemia and significant resistance to experimental diabetes

3.1 Abstract

Insulin-like growth factor I (IGF-I) is normally produced from hepatocytes and various other cells and tissues, including the pancreas. However, the notion that IGF-I stimulates islet cell growth has been challenged recently by studies using IGF-I and IGF-I receptor gene targeting. In order to test the effects of a general, more profound increase in circulating IGF-I on islet cell growth and glucose homeostasis, we have characterized MT-IGF mice which overexpress the IGF-I gene under the metallothionein I promoter. In previous reports, a 1.5-fold elevated serum IGF-I level caused accelerated somatic growth and pancreatic enlargement. In the present study we demonstrated that the transgene expression, although widespread, was highly concentrated in the pancreatic islets. Yet, islet cell percentage and morphology were unaffected. IGF-I overexpression also resulted in significant hypoglycemia, hypoinsulinemia, increased glucose tolerance and normal insulin sensitivity. Pyruvate tolerance tests indicated suppressed hepatic gluconeogenesis, which might have caused the severe fasting hypoglycemia. MT-IGF mice were significantly resistant to streptozotocin-induced diabetes, with diminished hyperglycemia, weight loss and death this may be due to a partial prevention of β-cell death and/or the insulin-like effects of IGF-I overexpression. Thus, although IGF-I does not promote islet cell growth, its overexpression results in anti-diabetic effects by improving islet cell survival and insulin responsiveness.

3.2 Introduction

Insulin-like growth factor I (IGF-I) is normally produced from hepatocytes and various other cells and tissues, including the pancreas. Acting through its receptor, IGF-IR, IGF-I promotes embryonic development, postnatal growth and maturation of major organ systems. IGF-I is known to stimulate islet cell replication in vitro, prevent Fas-mediated autoimmune β-cell destruction and delay the onset of diabetes in non-obese diabetic (NOD) mice [434, 435, 507, 508]. Although pancreatic islet-specific IGF-I overexpression in RIP-IGF mice did not increase islet cell growth, it promoted islet cell regeneration and a faster recovery from diabetes [444]. Moreover, in MODY3 diabetes of reduced β-cell mass, diminished IGF-I expression seems to play a key role [509]. Together these results indicate that IGF-I is a growth factor for pancreatic islet cells.

However, we recently discovered that liver- and pancreatic-specific IGF-I gene deficiency in LID and PID mice caused increased islet β-cell mass, suggesting that IGF-I exerts an *inhibitory* effect on islet cell growth, albeit indirectly, involving growth hormone and Reg family proteins [447-449, 510]. In addition mice with β-cell-specific IGF-IR gene deficiency exhibited normal islet cell mass, indicating that IGFs are not required for normal islet cell growth [445, 446]. Although β-cell-specific dual deficiency of insulin receptor and IGF-IR genes resulted in diminished islet cell growth and early diabetes, insulin signaling seems to have a greater effect than IGF-IR [511].

These discrepancies suggest the need for a reevaluation of the effect of IGF-I on islet cell growth in a more effective system. Previously, in pancreatic

islet-specific RIP-IGF mice, IGF-I expression was limited to the islet cells and/or only at a modest scale [444, 512]. In order to test whether a general and more profound increase in local production and in circulating IGF-I might affect islet cell growth, we have characterized MT-IGF mice which overexpress the IGF-I gene driven by the metallothionein I promoter [460-462, 506, 513].

In addition, IGF-I has insulin-like effects in adipose tissues and skeletal muscles resulting in increased glucose transport, lipogenesis and glycogenesis and decreased lipolysis [514]. In the past decade, it has been explored as a diabetic intervention for improving insulin responsiveness and activating insulin receptor substrates directly [515-519]. IGF-I and the IGFBP-3 complex reduced basal glucose production and peripheral glucose uptake during a hyperglycemic clamp in subjects of type 1 diabetes (T1D) [519]. However, a study of long-term, systemic elevations in circulating IGF-I levels on glucose homeostasis has not been done in transgenic mice. Unexpectedly in this study, we demonstrated a general yet *islet*-enriched IGF-I overexpression in MT-IGF mice, which had no effect on islet cell growth but caused severe hypoglycemia due to suppressed gluconeogenesis and enabled mice to be resistant to streptozotocin-induced β-cell damage and diabetes.

3.3 Research design and methods

The MT-IGF mice

Mice with germline integration of a human IGF-I cDNA driven by the mouse Metallothionein 1 promoter (MT-IGF) were studied along with their wild-type

littermates, on a mixed C57BL/6 background [461, 513]. To determine genotype, genomic DNA was isolated from tail clips with standard methods. Primers MT-1 (5'-GCA TGT CAC TCT TCA CTC CTC AGG) and MT-2 (5'-TCT GCA TCG TCC TGG CTT TG) were used in PCR reactions, which yield a 500-bp band for the transgenic allele. The animals were maintained in 12 h dark/light cycles at room temperature with free access to food and water. At the desired age, the mice were anesthetized with a cocktail of ketamine/xylazine/acepromazine, and sacrificed by cervical dislocation. Serum was collected, and the pancreata removed to perform pancreatic RNA analysis and/or histology. Glycogen content in liver and muscles under fasted conditions was quantified as previously reported [520]. The McGill University Animal Care Committee approved all animal-handling procedures.

RNA isolation and Northern blots

Total RNA was isolated by acid guanidinium isothiocyanate-phenol-chloroform extraction. In order to minimize RNA degradation, the mice were anesthetized; their pancreas was dissected and homogenized immediately before being sacrificed. Northern blot analysis was performed as reported using rat IGF-I, mouse insulin and β-actin probes [448, 449, 512].

Blood chemistry and in vivo procedures

Serum or plasma concentrations of insulin and glucagon were determined using RIA kits (Linco Research, St. Charles, MO). Blood glucose levels were measured using the OneTouch blood glucose meter (LifeScan Canada, Burnaby, BC). For insulin tolerance testing, animals were injected with recombinant human insulin

(0.75 IU/kg i.p.; Roche), and blood glucose levels were measured at 0, 20, 40, and 60 min afterward. For glucose tolerance testing, mice were fasted 24 h and injected with glucose (1 g/kg i.p.) and blood glucose levels measured at 0, 15, 30, 60, and 120 min afterward; for pyruvate and glutamine tolerance testing, mice were fasted for 24 h and injected with pyruvate (2 g/kg i.p.) or L-glutamine (1.5 g/kg i.p.) respectively. For insulin secretion, mice were fasted for 19 h, injected with glucose (3 g/kg, i.p.) and sacrificed at 5 and 15 min to collect blood.

Immunohistochemistry and islet cell mass measurement

Pancreatic sections were stained with insulin, glucagon (Monosan, Uden, The Netherlands) or IGF-I antibodies (clone Sm1.2, Upstate) using diaminobenzidine substrate. Images of all pancreatic sections were captured with a Retiga 1300 digital camera (Q Imaging, Burnaby, BC) using Northern Eclipse software version 6.0 (Empix Imaging, Mississauga, ON). The islet density, average islet cell size, and β-cell percentage were determined as previously reported [448, 471].

Streptozotocin-induced islet cell damage and diabetes

MT-IGF and wild-type littermates, 3-month-old males and females, were injected daily for 5 days with streptozotocin (Sigma; 80 mg/kg for females and 75 mg/kg for males; i.p.) prepared fresh in 0.1 M sodium citrate, pH 4.5 [447, 448]. Blood glucose levels from tail vein and body weight were measured every 3 d after the initial injection. As mice became diabetic by 22 days, they were sacrificed to determine serum insulin level and perform pancreatic immunohistochemistry. To detect islet cell apoptosis prior to the onset of hyperglycemia, a separate set of

mice were injected with streptozotocin twice and sacrificed at 48 h; dewaxed paraffin sections of the pancreas were labeled with an in situ cell death detection kit (Roche) and insulin antibody by immunofluorescence, according to the manufacturer's instructions and a previous report [448].

Statistical Analysis

Data are expressed as mean ± SE. The graphs were prepared using SigmaPlot software, version 10 (Systat, San Jose, CA). The Student's *t*-test (unpaired and paired) was performed using InStat software version 3 (GraphPad Software, San Diego, CA).

3.4 Results

A general, robust yet pancreatic islet-enriched IGF-I overexpression

Normally, metallothioneins are synthesized primarily in the liver and kidney. However, in an early characterization, another independent transgenic line of MT-IGF mice displayed IGF-I overexpression not only in the liver and kidney but also in the pancreas [460]. In fact, the resulting level of IGF-I mRNA was 31 fold higher in the pancreas than in the liver; IGF-I content was increased 5,200 fold in the pancreas, with the absolute content 344 fold higher than in the liver [460]. Because of this robust overexpression, serum IGF-I level was increased 1.5 fold, with a consequent reduction in the level of growth hormone. Moreover, overexpressed IGF-I resulted in selective organomegaly in the spleen and pancreas (2.0 and 1.8 fold weight increases, respectively) and a 1.4 fold increase in the total body mass [460, 461, 521]. Consistent with previous reports, including

the one using the same transgenic line [461] as in this study, serum concentration of IGF-I increased 1.5 fold in adult MT-IGF mice vs. wild-type littermates (Table 1). By Northern blot analysis, the level of IGF-I mRNA exhibited a 422 fold increase in the pancreas, because the basal level was virtually non detectable, confirming a pancreatic-enriched expression (**Figure 3.1**).

In order to reveal what types of cells (exocrine/endocrine) in the pancreas express the transgene, immunohistochemistry was used to characterize IGF-I expression in the pancreas (**Figure 3.2**). Surprisingly, the IGF-I staining in MT-IGF mice was highly concentrated in the islets, resembling that of insulin, while in the wild-type pancreas, staining was essentially undetectable in the islets, with some positive signals in blood cells. Although the metallothionein promoter is active in islet cells, such a high level and specific activity was not expected. Consistent with previous reports, the general and robust IGF-I overexpression accelerated somatic growth in MT-IGF mice [460, 462]. Adult body weights were significantly increased, although the increase in length did not reach statistical significance (**Table 3.1** and data not shown). Our tests not only confirmed IGF-I overexpression in these mice but also for the first time revealed a very strong islet-specific expression pattern.

Normal pancreatic islets, hypoinsulinemia and hypoglycemia

As a prominent growth factor, IGF-I overexpression within the pancreatic islets was expected to accelerate the growth of islet cells and increase the production of insulin. However, serum insulin levels were markedly decreased by 49% (**Table 3.1**) and pancreatic insulin mRNA by 44% (**Figure 3.1**) in MT-IGF

mice. Evaluation of hemotoxylin-eosin stained pancreatic sections and immunohistochemistry for insulin revealed no obvious abnormality in islet morphology. There was no change in the percent distribution of small, medium or large islets **(Figure 3.3, panels A & B)**, in the average islet cell size (surface area), or islet density (data not shown). As an indication of β-cell mass, we measured β-cell percentage and found no change in MT-IGF mice **(Figure 3.3C)**. Thus, MT-IGF mice did not display any increase in islet cell growth but exhibited decreased (rather than increased) insulin gene expression and plasma insulin levels. The absolute β-cell mass was not used as it would be differentially affected by the increases in pancreatic and total body masses in MT-IGF mice. As a measure of insulin secretion, the plasma insulin levels in MT-IGF mice were 60% lower than wild-type littermates 5 min after glucose injection (3 g/kg, i.p.; N=5; data not shown).

In association with the changes in islet function and/or increased IGF-I, adult MT-IGF mice exhibited lower blood glucose levels vs. wild-type littermates at random fed status (Table 1). After being fasted for 24 h, the difference was greatly exaggerated, such that MT-IGF mice had a 47% decrease in blood glucose level which even induced hypoglycemic coma in some animals **(Table 3.1).** This effect has not been reported in MT-IGF mice and occurred despite decreased serum insulin levels suggesting that the hypoglycemia could be a direct consequence of IGF-I production and its insulin-like effects.

Normal insulin sensitivity but significantly improved glucose tolerance

A possible cause of hypoglycemia is elevated insulin sensitivity, as we

have reported in mice deficient in the growth hormone receptor gene [471]. However, MT-IGF mice showed no change in insulin tolerance (**Figure 3.4A**). To further explore possible changes in glucose homeostasis, a glucose tolerance test was performed. As shown in **Figure 3.4B**, following a glucose injection wild-type mice exhibited a sharp increase in blood glucose level, peaking between 15 and 30 min, which was not normalized before 120 min. However, MT-IGF mice started with a significantly lower glucose level, displayed a much reduced glucose escalation after the injection and a faster decline to baseline by 60 min. This significant contrast indicates the presence of a much more efficient mechanism of glucose disposal in MT-IGF mice, which is not associated with improved insulin secretion or insulin sensitivity.

Suppressed hepatic gluconeogenesis by pyruvate tolerance test

The increased glucose disposal as a consequence of "insulin-mimicking" IGF-I stimulation would explain the slightly decreased glucose level under random fed status. The more severe hypoglycemia after 24 h fasting in MT-IGF mice suggests additional consequences such as decreased gluconeogenesis and/or glycogenolysis. The conversions of pyruvate to glucose in the liver and glutamine to glucose in the kidney and the small intestine are key steps in gluconeogenesis and can be measured by tolerance tests [522]. As shown in **Figure 3.4C**, after pyruvate injection, wild-type mice showed a rapid increase in blood glucose level, peaking at 30 min, which remained elevated beyond 120 min. On the other hand, MT-IGF mice started with a significantly lower glucose level and displayed a much diminished elevation following pyruvate injection, which rapidly returned

to baseline by 120 min. Considering the different basal glucose levels, MT-IGF mice displayed a significantly smaller peak-increase of 2.2- vs. 2.7-fold in wild-type littermates ($P<0.01$). The result of the glutamine tolerance test is illustrated in **Figure3. 4D**. In wild-type mice, blood glucose levels were elevated step-wise after glutamine injection, reached a peak level of 200 mg/dL and did not recede significantly within the 2 h. In comparison, MT-IGF mice displayed a smaller and gradual increase and peaked only at 100 mg/dL. Nevertheless, when corrected by their respective basal glucose levels, the relative fold increases were actually very close, i.e. 2.8 fold in MT-IGF vs. 3.1 fold in wild-type mice. The obvious difference in glucose production induced by pyruvate indicates a suppressed hepatic gluconeogenesis; although an increased glucose disposal (as reflected in **Figure 3.4B**) may also contribute to a smaller extent since the animals were fasted. As insulin levels were decreased and insulin sensitivity remained normal, this effect is likely a direct consequence of IGF-I overexpression. Indirectly, decreased gluconeogenesis can be the result of decreased growth hormone level in MT-IGF mice [460], while the glucagon level was not decreased and the ratio of islet α-cells actually showed a slight increase compared to wild-type littermates (**Table 3.1 and Figure 3.3D**). On the other hand, the rate of glycogenolysis seemed normal as no change was detected in the glycogen contents of either the liver or skeletal muscles in MT-IGF mice after 24 h fasting (**Table 3.1**).

MT-IGF mice were resistant to streptozotocin-induced β-cell death and diabetes

Since IGF-I is known to inhibit cell apoptosis, the robust islet-specific overexpression in MT-IGF mice should provide a significant protection against

islet damage and prevent the onset of diabetes; additionally, the enhanced "insulin actions" as a result of IGF-I overexpression should relieve the diabetic symptoms once the diabetes has been induced. To test these possibilities, we have challenged MT-IGF mice with a multiple-low-dose injection of streptozotocin. In wild-type mice, both male and female, streptozotocin induced a rapid onset of hyperglycemia from day 3, with continued increases up to day 18 or 21 (**Figure 3.5A and B**). The mean peak glucose levels reached 550-600 mg/dL. Three weeks after streptozotocin, the wild-type mice had lost ~20% of body weight and 30-38% of them actually died towards the latter half of the study (**Figure 3.5 C, D, E and F**). In contrast, MT-IGF mice exhibited significantly smaller increases in the glucose level at most time points, from day 3 till the end of the 21 days; levels peaked at ~450 mg/dL, indicating a delayed onset of diabetes and/or improved symptoms of the disease (**Figure 3.5A and B**). The weight loss was only ~8% in both male and female MT-IGF mice and there was no death caused by streptozotocin administration (**Figure 3.5C, D, E and F**), further indicating a significant protection and/or relief of the diabetic symptoms. To confirm islet damage at the end of 21 days, pancreatic immunohistochemistry was performed using an insulin antibody (**Figure 3.6G**). Without streptozotocin treatment, there was no significant difference in islet histology and insulin staining in MT-IGF and wild-type littermates. Streptozotocin caused a drastic decrease in islet size and in the level of insulin staining in wild-type mice while the islets in MT-IGF mice were better preserved, e.g. larger in size, although with similarly reduced levels of insulin staining (data not shown).

DNA damage and cellular apoptosis is a key aspect of streptozotocin-induced islet β-cell damage [448, 523-526]. To confirm that the delayed onset of diabetes was due to IGF-I-induced islet cell survival, we studied islet cell apoptosis after streptozotocin but before the onset of hyperglycemia. Pancreatic sections were prepared from wild-type and MT-IGF mice, 48 h after streptozotocin administration (or no administration), and double-stained for immunofluorescence against insulin (red Cy-3) and terminal transferase dUTP nick end labeling (TUNEL) (green fluorescein). As shown in **Figure 3.6**, no apoptotic nuclei can be detected in untreated wild-type mice (upper middle panel). After streptozotocin, many islet cells underwent apoptosis in wild-type mice (white arrow in center panel), at 2.0 ± 0.3 (N=22) cells per 1000 μm^2 islet surface area. In contrast, fewer cells were apoptotic in MT-IGF mice, with the ratio decreased to 1.2 ± 0.2 (N=21; P=0.04) (bottom panels). This significant protection of islet cells against apoptosis by IGF-I overexpression seems to explain why MT-IGF mice had a delayed onset of hyperglycemia after streptozotocin. Thus, IGF-I overexpression in MT-IGF mice delayed the onset of diabetes and/or improved the diabetic symptoms, at least partly due to improved survival and function of the islet cells.

3.5 Discussion

The role of IGF-I in diabetes has been addressed extensively. For example, T1D is associated with reduced serum IGF-I levels [527]. MODY3 diabetes, caused by a mutation of hepatocyte nuclear factor-1α gene, exhibits

reduced IGF-I expression and β-cell growth [509]. IGF-I treatment is effective in both T1D and type 2 diabetes (T2D) [515, 519]. Islet β-cell mass is a key element in the development of autoimmunity-induced T1D and in compensating for insulin resistance in T2D. It has been demonstrated that IGF-I stimulates islet cell growth and promotes the survival of transplanted islet cells in rodents [444, 507, 508, 528-530]. Recently, however, this notion has been challenged by several reports of tissue-specific IGF-I and IGF-IR gene targeting, including one from us [317, 445, 446, 448]. They indicate that IGF-I is not involved in normal islet cell growth and undermine its potential applications in islet expansion, protection and transplantation. Current studies using a robust and islet-enriched IGF-I overexpression mouse model further support the new consensus that IGF-I does not stimulate islet cell growth. To reconcile the differences, earlier in vitro reports indicated that high levels of glucose are required for IGF-I effects, an unlikely condition in normal animals [433, 507, 508]. Furthermore, unlike in cultured islet cells, the effect of increased free IGF-I in transgenic mice would be limited by corresponding increases in binding proteins. On the other hand, because of the high level of expression, the MT-IGF mice provide a unique opportunity to reevaluate the in vivo insulin-like effects and the effects of IGF-I on insulin secretion and protecting islet cells against damage.

Several reports indicate that IGF-I inhibits insulin secretion, by a mechanism associated with the activation of phosphodiesterase 3B and protein kinase B [453, 455, 456]. Consistently, MT-IGF mice exhibited a significantly lower level of serum insulin, decreased in vivo insulin secretion to glucose

stimulation and a reduction in insulin gene expression. In addition to a direct inhibition of insulin secretion by IGF-I, severe hypoglycemia and/or the insulin receptor upregulation in the liver and muscles (data not shown) in MT-IGF mice might act indirectly to cause decreased insulin secretion and/or gene expression. On the other hand, decreased insulin levels in MT-IGF mice were not associated with elevated insulin sensitivity. Finally, the unexpected high level of IGF-I production in the *islet* cells of MT-IGF mice, driven by the metallothionein promoter, may also create a non-specific competition for the same transcription/translation/secretion machinery against insulin secretion in islet β-cells. Although metallothionein may be involved in pancreatic hormone synthesis and secretion, in addition to zinc homeostasis and detoxification [531], it has never been reported that the metallothionein promoter in a transgenic system would have such a high level expression in islet β-cells (**Figure 3.2**). The relatively enriched zinc ions in β-cells might have increased the promoter activities.

Although excess IGF-II, such as in hormone-producing tumors, is known to cause hypoglycemia [532], reports of hypoglycemia caused by a chronic *IGF-I* excess, either in rodents or human, are very rare [533]. Previously, a 6- or 14-d IGF-I infusion into normal rats caused no change in normal glycemia [534]; 6-d IGF-I administration to hypophysectomized rats only caused transient hypoglycemia [535] while 6- or 7-d IGF-I treatment of streptozotocin-induced diabetic rats caused no significant amelioration of hyperglycemia [536, 537]. The severe hypoglycemia in this study is likely the result of IGF-I cross-activating

insulin receptors, as well as its cognate effect mediated by IGF-IR itself. By sequence alignment, IGF-I and -II are highly homologous to proinsulin and may derive from gene duplications of a common ancestor. Together, they form a complex family of 3 hormones, 3 trans-membrane tyrosine kinase receptors (IR, IGF-IR and IRR), and specific binding proteins for IGFs (IGFBPs) [396]. IGF-I has much lower affinity for insulin receptor than insulin itself. The highly homologous IGF-IR is also a heterotetrameric tyrosine kinase, ubiquitously expressed in cell types derived from all three embryonic lineages and can be activated by both IGF-I and IGF-II, as well as insulin albeit with much lower affinity. Upon ligand binding, both the insulin receptor and the IGF-IR undergo autophosphorylation on intracellular tyrosine residues and activation of their intrinsic tyrosine kinases.

It is well known that IGF-I mimics insulin actions especially at high concentrations, and vice versa [396]. In this study, the total serum IGF-I level in wild-type mice, in molar concentrations, was 475 fold of that of insulin (Table 1); considering only ~4% of total IGF-I being free [538], the free hormone would be 19 fold higher than insulin and insufficient to significantly activate insulin receptor (Kd for IGF-I 10-100 nM vs. for insulin 0.1-0.2 nM). In MT-IGF mice, with a concurrent increase in IGF-I and decrease in insulin levels, the free IGF-I would become 55 fold higher than the insulin level, reaching a threshold required for activating the insulin receptor with a lower affinity. Normally most of the circulating IGF-I exists in complexes with binding proteins, protecting against its hypoglycemic effect. In MT-IGF mice, 2.1-2.9 fold increases have been reported

in serum IGFBP-3 and IGFBP-2 levels, which help to limit the surge of free IGF-I [539]. Acting through the insulin receptors on skeletal muscles, hepatocytes and other insulin targets, overexpressed IGF-I would stimulate glucose uptake and inhibit glucose production. In contrast to insulin though, the levels of overexpressed IGF-I in MT-IGF mice are not regulated by fasting and refeeding such that the (insulin) receptor activation would be persistent regardless of the nutritional status. Even with fasting, when elevated glucose production is desired, the overexpressed IGF-I would still inhibit the process, resulting in severe hypoglycemia. Supporting this possibility, the levels of insulin receptor protein in the liver and skeletal muscles were significantly elevated in MT-IGF mice (data not shown). This unusual upregulation of the receptor levels would be consistent with an over stimulation and has not been previously reported for IGF-I, while hyperinsulinemia actually *down* regulates the level of insulin receptor [540]. Because serum insulin levels were diminished and insulin sensitivity was unchanged, the contribution of endogenous insulin in causing the hypoglycemia can be excluded. Although there was no change in serum glucagon levels, the decrease in growth hormone levels that occurs in MT-IGF mice could contribute to a certain extent [460]. Decreased glucose production in MT-IGF mice seems to be caused by suppressed hepatic gluconeogenesis but not by a change in glycogenolysis. Moreover, the reduction in gluconeogenesis could also be caused indirectly by decreased proteolysis due to IGF-I overexpression and thus a reduced supply of amino acids [541]. Fasting hyperglycemia as a result of enhanced overnight gluconeogenesis is a common problem in T2D that is often

uncontrolled by bedtime insulin. Our results support a therapeutic potential of IGF-I in this regard. Finally, since IGF-IR is also capable of mediating insulin-like effects including direct inhibition on gluconeogenesis [496, 519, 522, 542], it would be worthwhile to determine in future studies to what extent the hypoglycemia in this model was caused by IGF-IR-mediated events.

In this report, MT-IGF mice were significantly resistant to streptozotocin-induced diabetes. It is expected that once the diabetes has been induced, persistent elevated IGF-I in the circulation would manifest an "insulin-like treatment" and thus improve the symptoms, including hyperglycemia, weight loss or animal death. In the longer term, overexpressed IGF-I promoted a faster recovery from diabetes through accelerated islet cell regeneration [444]. Our current study supports another possibility, that IGF-I may help to prevent β-cell destruction through an apparent anti-apoptotic effect. This was indicated by the findings that in the first 6 (female) or 3 days (male) after streptozotocin treatment, MT-IGF mice displayed a significantly delayed onset of hyperglycemia (**Figure 3.5A, B**). More directly, streptozotocin-induced islet cell apoptosis, i.e. DNA fragmentation was significantly diminished in IGF-I overexpression (**Figure 3.6**). Streptozotocin is known to cause β-cell apoptosis in vivo and in vitro [523-526]. It has been known that IGF-I prevents islet cell apoptosis, autoimmune destruction of islets and delays onset of diabetes in NOD mice [434, 435, 450, 451]. Thus, although IGF-I may not be effective in promoting islet cell growth in vivo, it can still be potentially useful in combating T1D. Either at diabetic prevention, treatment or islet recovery, IGF-I-based therapy is still potentially valuable. As for the

concerns of side effects and tumor incidence, we should consider targeting specific cells and/or intracellular substrates.

In summary, although MT-IGF mice have been created almost two decades ago, this study revealed a novel, islet-enriched IGF-I overexpression. The resulting normal islet cell growth supports the recent findings from conditional targeting of the IGF-I and IGF-I receptor genes and argues against the notion that IGF-I is an islet growth factor. We further discovered significant alterations in glucose homeostasis, including hypoglycemia, hypoinsulinemia and improved glucose tolerance. Pyruvate tolerance test indicated suppressed hepatic gluconeogenesis. Chronic IGF-I overexpression thus confirmed strong, persistent insulin-like effects on glucose disposal and production. Finally, MT-IGF mice were significantly resistant to streptozotocin-induced diabetes likely due to prevention of β-cell damage and/or the insulin-like "treatment". Our results support a new consensus that IGF-I does not promote islet cell growth and inhibits β-cell apoptosis, insulin secretion and hepatic glucose production. Thus, although IGF-I may not promote islet cell growth in vivo, its overexpression is clearly anti-diabetic by mimicking insulin actions and/or by eliciting directs IGF-I effects.

3.6 Acknowledgments

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Table 3.1. Changes in body weight, serum chemistry and glycogen contents in MT-IGF mice.

Values were given in Mean ± standard error (SE); Numbers were in parenthesis; P values were derived from unpaired t-tests; NS: not significant; M/F: male and female.

	Wild-type	MT-IGF	P value
Body weight (g), 3-month-old			
Male	29.7 ± 0.4 (9)	34.8 ± 1.1 (9)	0.0007
Female	18.9 ± 0.4 (7)	23.9 ± 1.0 (9)	< 0.001
Blood glucose concentration (mg/dL), 2.5-month-old			
M/F 24-h fasted	72 ± 3 (8)	38 ± 5 (12)	0.0007
Male fed	149 ± 4 (9)	128 ± 3 (9)	0.0009
Female fed	139 ± 7 (7)	124 ± 3 (8)	NS
Serum hormone level (ng/ml), 3-month-old, M/F, random fed			
Total IGF-I	506 ± 37 (7)	740 ± 40 (9)	0.017
Insulin	0.81 ± 0.06 (10)	0.41 ± 0.02 (11)	< 0.0001
Plasma glucagon level (pg/ml), 4 month-old, 20 h fasted			
Male	58 ± 8 (5)	62 ± 8 (5)	NS
Female	92 ± 11 (9)	84 ± 13 (9)	NS
Glycogen content (mg/g tissue), 5-month-old, M/F, 24-h fasted			
Liver	28.6 ± 3.8 (5)	26.0 ± 5.0 (5)	NS
Skeletal muscles	26.8 ± 3.5 (5)	24.8 ± 2.3 (5)	NS

Figure 3.1. Increased IGF-I but decreased insulin mRNA levels in the pancreas of MT-IGF mice.

10 μg pancreatic RNA from wild-type or MT-IGF mice, 3-month-old littermates, was hybridized with riboprobes against insulin and IGF-I mRNAs. A representative blot was depicted from six mice/samples from each group. The level of β-actin mRNA was used as a loading control. Relative mRNA abundances in MT-IGF mice after β-actin corrected densitometry: Insulin 55.6%, IGF-I 422-fold, vs. wild-type littermates.

Figure 3.1

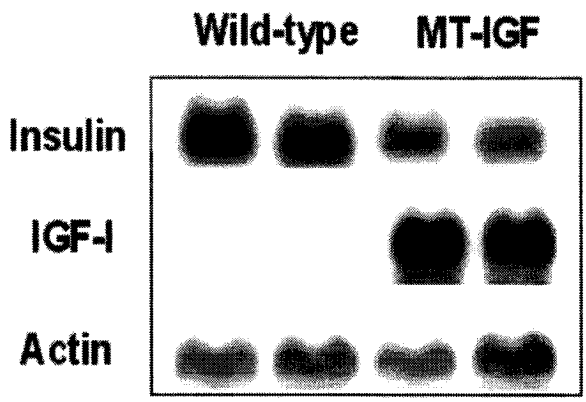


Figure 3.2 Pancreatic islet-specific IGF-I expression revealed by immunohistochemistry.

Pancreatic sections prepared from 3-month-old mice of wild-type and MT-IGF genotypes were stained for IGF-I using the diaminobenzidine complex. IGF-I staining was shown as *brown* pigmentation within the islets. Cell nuclei were counterstained with hematoxylin. Images were representatives of at least 10-15 mature islets from each group and have been recorded at 400X. Scale bar = 50 microns.

Figure 3.2

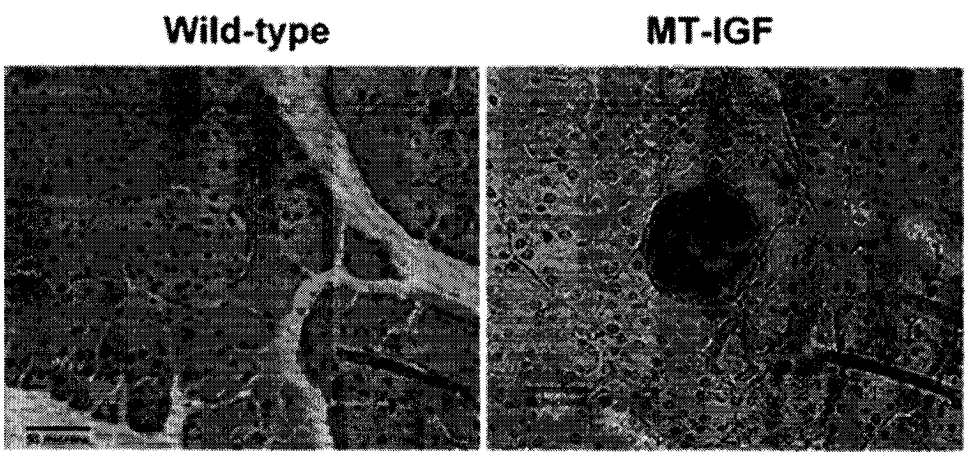


Figure 3.3 MT-IGF mice displayed normal pancreatic islets.

Mice of 3-month-old, mixed sex, were used for histology. A, Immunohistochemistry using insulin antibody. Images were recorded in 100X magnification and analyzed using Northern Eclipse software. Representative small, medium and large islets were illustrated from wild-type and MT-IGF littermates. Scale bar =100 microns. B, Summary of islet size distribution. In both wild-type and MT-IGF mice, islets were divided into 3 groups according to their size (in 1000 μm^2). *Y*-axis represents % of total islets distributed in each of three size groups. ($N \geq 35$) C, Summary of β-cell percentage, as means ± SE. N = 5. D, Immunohistochemistry using glucagon antibody. Images were recorded in 400X, representative mature islets were illustrated. Scale bar = 50 microns.

Figure 3.3

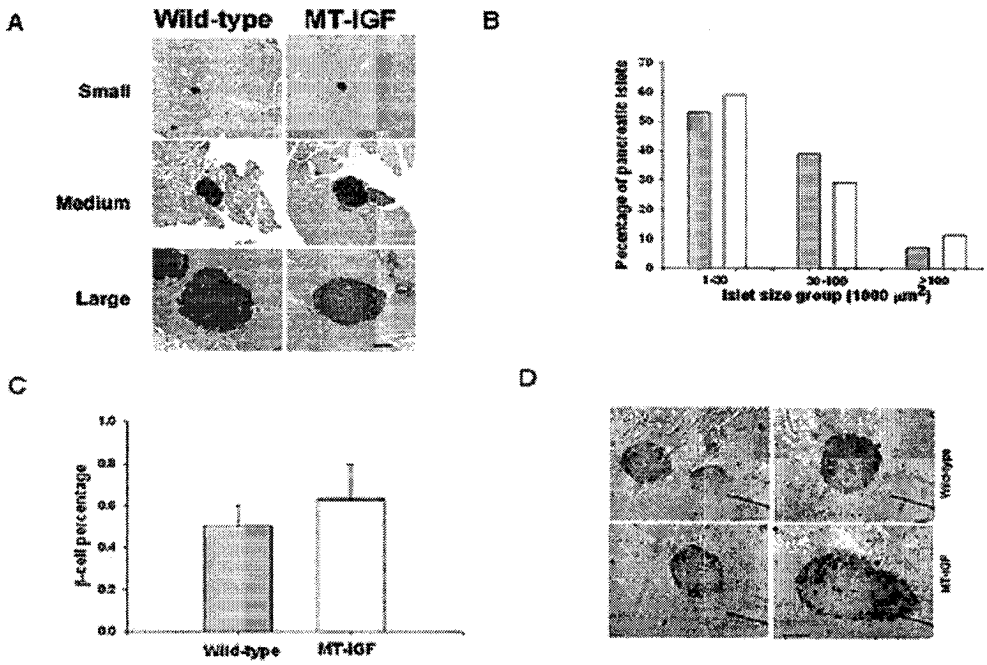

A
Wild-type
MT-IGF
Small
Medium
Large
B
Pecentage of pancreatic islets
Islet size group (1000 μm²)
C
β-cell percentage
Wild-type
MT-IGF
D
Wild-type
MT-IGF

Figure 3.4 Normal insulin sensitivity, increased glucose clearance and decreased gluconeogenesis in MT-IGF-I mice:

Results of insulin-, glucose-, pyruvate-, and glutamine-tolerance tests. Mice of 2.5-month-old, mixed male and female, were used. A, Insulin tolerance test. Wild-type and MT-IGF mice in random fed state were injected with insulin (0.75 IU/kg ip). Blood glucose levels were measured at 0, 20, 40 and 60 min. Percentage values relative to *time 0* were expressed as means ± SE. (N = 4) B, Glucose tolerance tests. Mice were fasted for 24 h, injected with glucose (1.0 g/kg ip). Blood glucose levels were measured at 0, 15, 30, 60 and 120 min after injection and expressed as means ± SE. ***P<0.001 vs. wild-type littermates. (N = 4~6) C, Pyruvate tolerance tests. Mice were fasted for 24 h, injected with pyruvate (2.0 g/kg ip). Blood glucose levels were measured. *P<0.05, **P<0.01 vs. wild-type littermates. (N = 4~5) D, Glutamine tolerance tests. Mice were fasted for 24 h, injected with L-glutamine (1.5 g/kg ip). *P<0.05, **P<0.01 vs. wild-type littermates. N = 7~12.

Figure 3.4

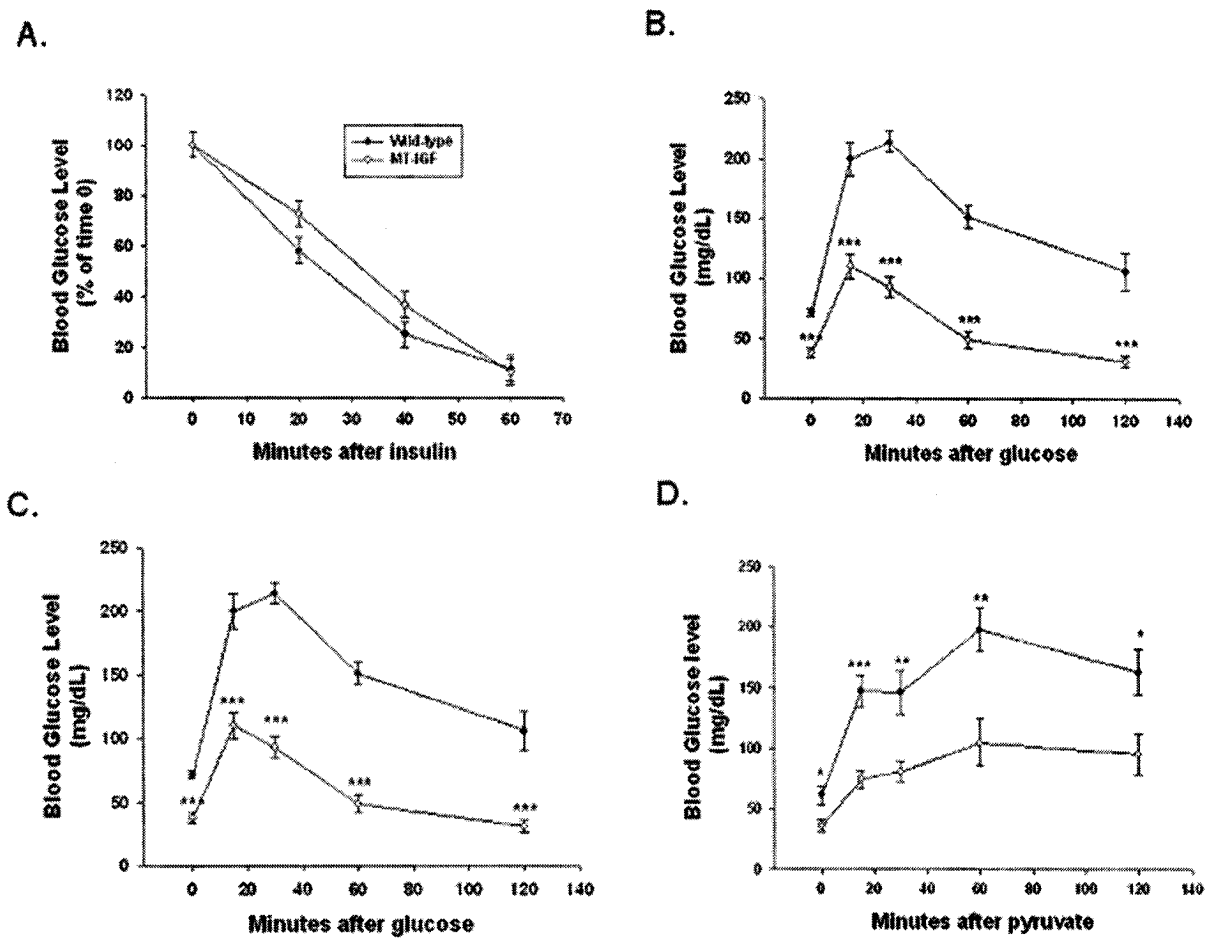

A.
Blood Glucose Level (% of time 0)
Wild-type
MT-IGF
Minutes after insulin
B.
Blood Glucose Level (mg/dL)
Minutes after glucose
C.
Blood Glucose Level (mg/dL)
Minutes after glucose
D.
Blood Glucose level (mg/dL)
Minutes after pyruvate

Figure 3.5 MT-IGF mice were resistant to streptozotocin-induced diabetes.

Both male and female mice, 3 month old, were injected with streptozotocin (female 80 mg/kg q.d., i.p.; male 75 mg/kg) for 5 days. Blood glucose levels from tail vein were measured at 0, 3, 6, 9, 12, 15, 18 and 21 d after the initial injection. A & B, Changes in blood glucose levels, illustrated in means ± SE, in female and male mice respectively. MT-IGF mice displayed continuous and significantly lower glucose levels than wild-type littermates. C & D, Changes in body weight, expressed as percent of initial weights. MT-IGF mice exhibited more stabilized body weight than wild-type littermates after streptozotocin. E & F, Differences in accumulated survival rates. Compared to greater than 30% death rates in wild-type mice after streptozotocin, MT-IGF mice had all survived the experiment. *$P<0.05$ vs. wild-type littermates. Data in panels A, C & E were female, N=7; B, D & F were male, N=7~9. G, Pancreatic islet damage in MT-IGF and wild-type mice. Twenty-two days after streptozotocin administration, the mice were sacrificed and their pancreatic sections were studied by immunohistochemistry using insulin antibody. Representative islets from each group (bottom panels) and untreated control mice (upper panels) were recorded as 100X images. On average, MT-IGF mice exhibited better preserved islets (larger) and the same ratio of insulin staining versus their wild-type littermates after streptozotocin. Similar results were obtained from both male and female animals. Scale bar =100 microns.

Figure 3.5

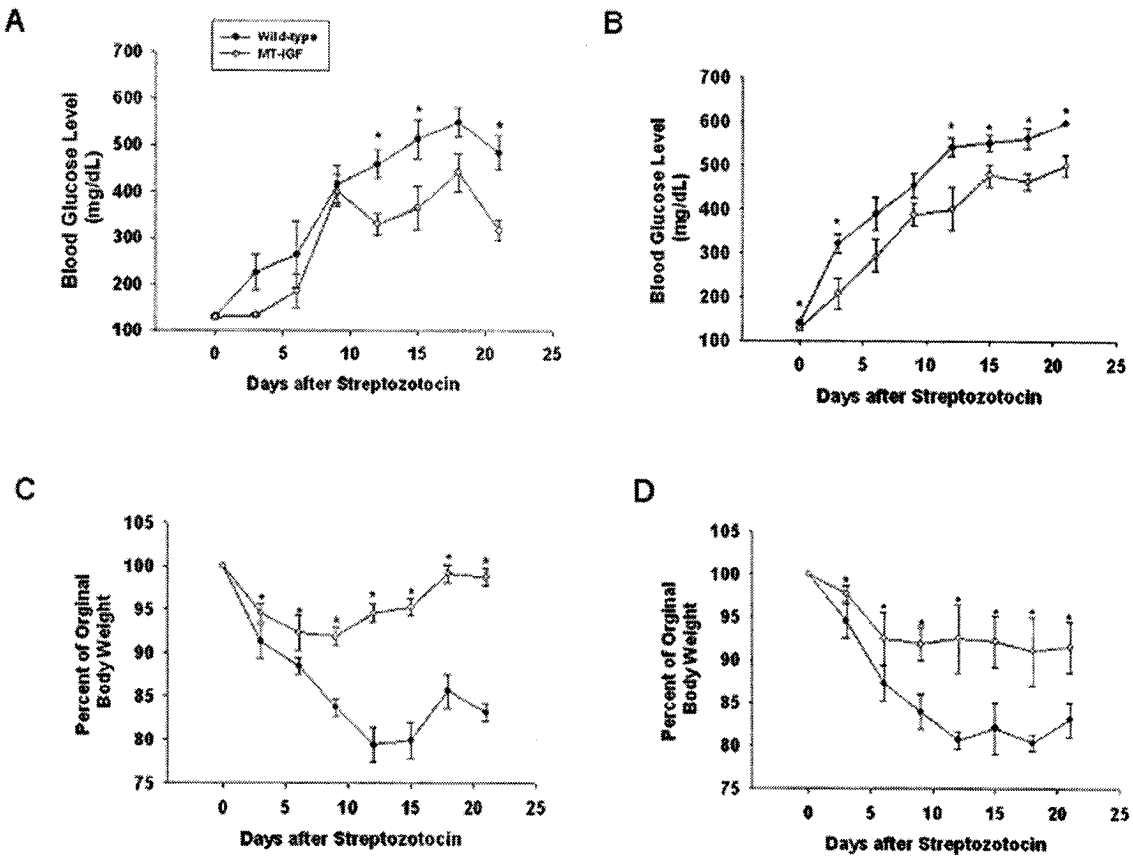

A
Wild-type
IMT-IGF
Blood Glucose Level (mg/dL)
Days after Streptozotocin
B
Blood Glucose Level (mg/dL)
Days after Streptozotocin
C
Percent of Orginal Body Weight
Days after Streptozotocin
D
Percent of Orginal Body Weight
Days after Streptozotocin

Figure 3.5

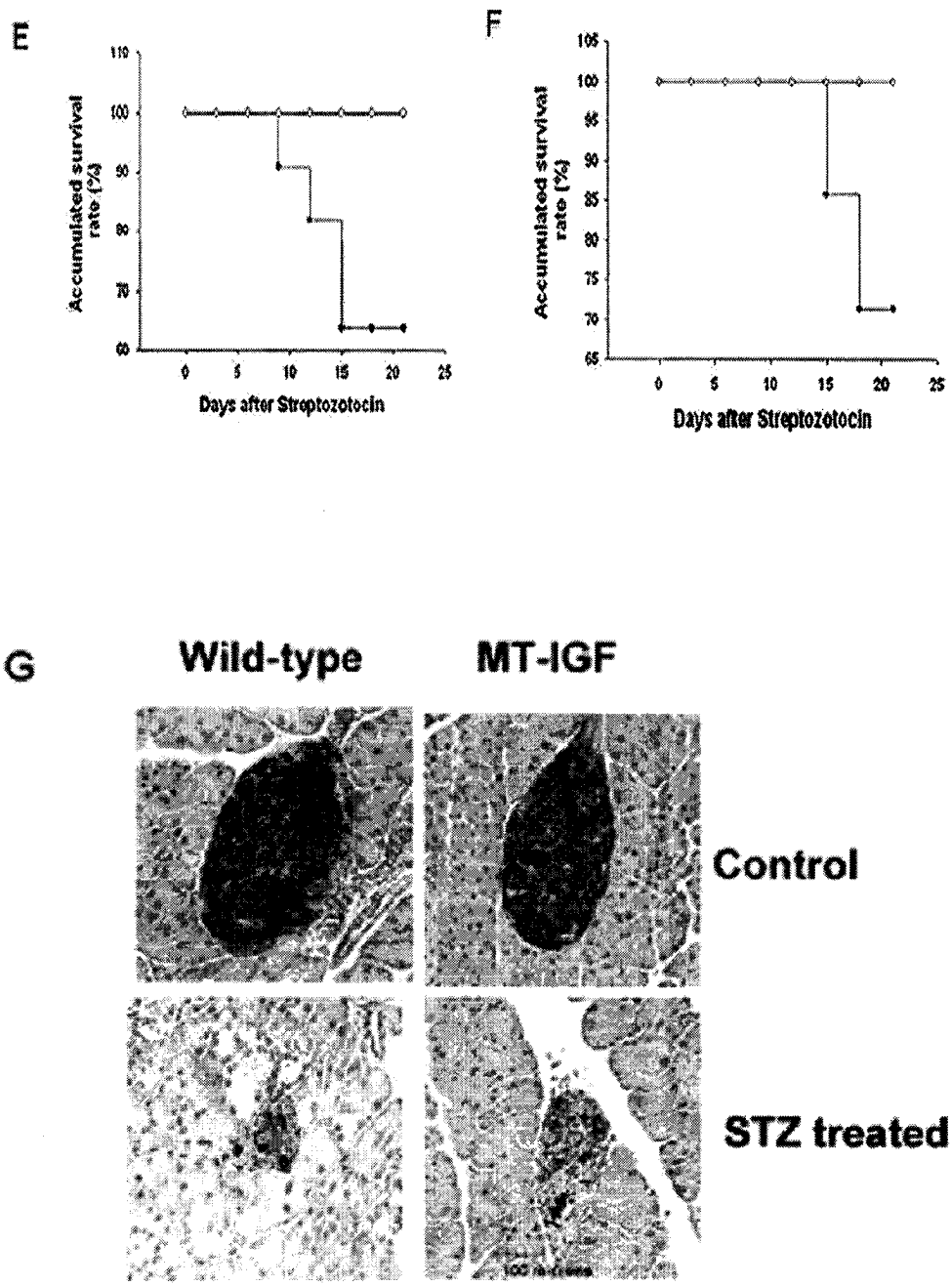
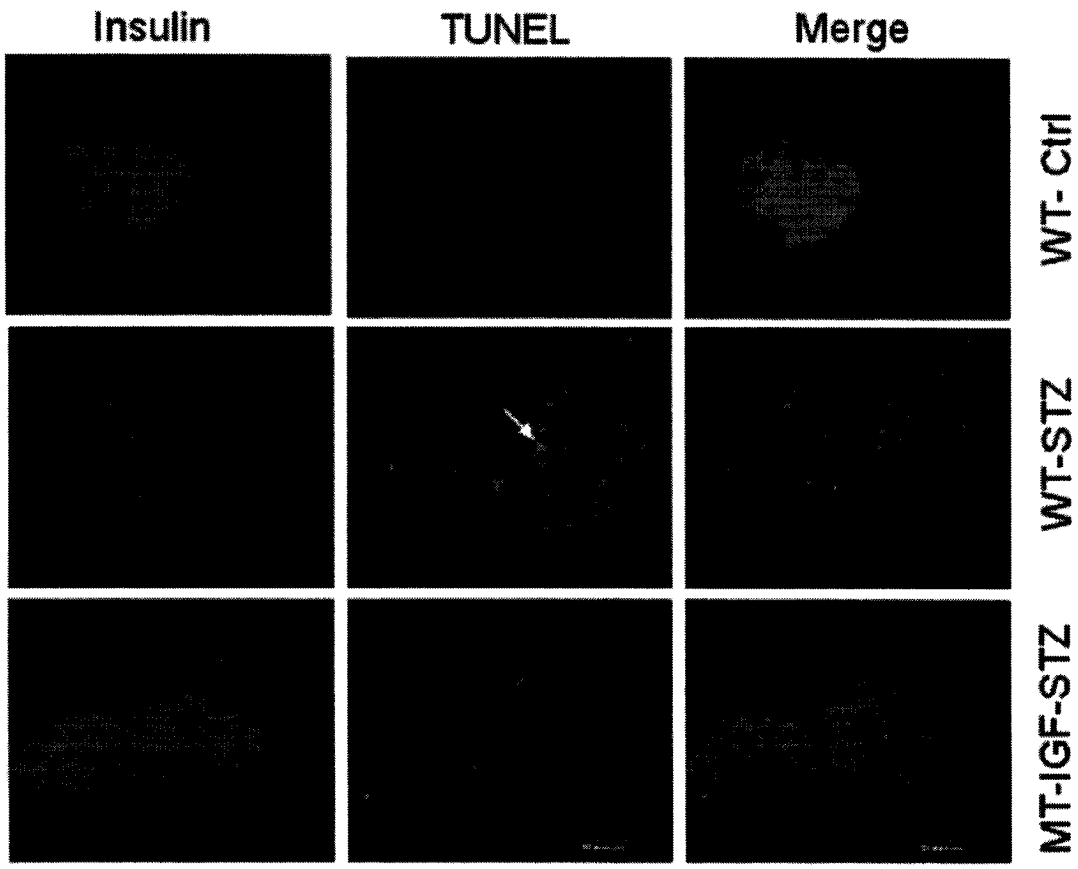

E
Accumulated survival rate (%)
Days after Streptozotocin
F
Accumulated survival rate (%)
Days after Streptozotocin
G
Wild-type
MT-IGF
Control
STZ treated

Figure 3.6 MT-IGF mice were resistant to streptozotocin-induced islet β-cell death. Pancreatic sections were prepared from 3-month-old wild-type (WT) or MT-IGF (MT) mice 48 h after streptozotocin (STZ) administration, or without treatment (Ctrl). Double-labeled immunoflurescence against insulin (red Cy-3; left column) and TUNEL (green fluorescein; middle column) was performed. Representative islets from each group are illustrated as 400X images, with the bar indicating 50 microns. The white arrow in the middle panel indicates TUNEL signal. Compared to untreated wild-type (WT-Ctrl, top row) islets, streptozotocin treatment (WT-STZ, middle row) caused decreased insulin staining and appearance of apoptotic islet cells; in MT-IGF mice (MT-STZ, bottom row), the insulin level was preserved and apoptotic rate diminished significantly.

Figure 3.6



Chapter Four

General Discussion

4.1 Significance and relevance

Diabetes mellitus is a metabolic disorder, characterized by hyperglycemia and β-cell dysfunction. Changes in pancreatic islet cell mass, insulin secretion and the capacities of glucose disposal and production compensate for insulin resistance and prevent the onset of diabetes. It has long been recognized that the GH/IGF-I axis can influence islet cell biology and insulin action. Numerous in vivo and in vitro models of diabetes have been studied and results of often conflicting nature reported. Such differences may have arisen due to the differences in in vitro and in vivo effects, the complexity in the IGF/insulin family, gene redundancies, and interference from growth hormone or IGFBPs. Understanding how growth hormone and IGF-I affect these processes may provide useful information in developing new effective treatments. The ever-increasing chronicity and hitherto incurable disease of diabetes necessitates the development of novel and effective treatments.

The destruction of pancreatic islet β-cells is a key factor in the development of autoimmunity-induced T1D and the inability to compensate for insulin resistance is a critical factor in T2D. β-cell mass can be stimulated by various growth factors. Two important hormones that have been known to stimulate islet cell growth, inhibit cell apoptosis and regulate insulin biosynthesis and secretion are GH and IGF-I. GH and IGF-I are both potent regulators of cell growth, differentiation, and metabolism, and are crucial for postnatal growth in mammals. GH can stimulate β-cell proliferation, glucose- stimulated insulin release and insulin gene expression and synthesis. The interaction between GH

and IGF-I in regulating growth and development has been well defined: GH stimulates postnatal growth through activating IGF-I production in target tissues (GH-local IGF-I axis) as well as through IGF-I-independent, direct actions. Their inter-dependent and/or independent roles in regulating pancreatic islet cell growth, insulin secretion and actions on target tissues have not been fully defined.

There are numerous examples of GH-IGF-I interactions, most notably the interaction at the growth plate [143]. In $GHR^{-/-}$ mice, cortical and longitudinal bone growth and bone turnover are all reduced. Short term administration of IGF-I via micropumps can substantially reverse many of these alterations, suggesting that the main defect may relate to reduced IGF-I levels due to the absence of GHR [543, 544]. GH and IGF-I exert opposite effects on target tissue sensitivities to insulin. On the other hand, GH actions are usually the opposite of insulin actions. The long-term effect of excess GH is hyperinsulinemia, which leads to a secondary reduction of IR levels and impaired receptor tyrosine phosphorylation, because GH does not affect the levels of IR directly in cultured cells. It has been previously reported that, in the liver of $GHR^{-/-}$ mice, increased IR levels and increased IR and IRS-I phosphorylation contribute to the increased insulin sensitivity. Unlike the liver, which is mostly unaffected by IGF-I, skeletal muscles express high levels of IGF-IR [470]. Both tissues are prime targets of GH actions. The net result of a concurrent deficiency in the actions of both GH and IGF-I, which normally exert opposite influences on insulin responsiveness, has not been evaluated in the $GHR^{-/-}$ mouse. In order to avoid the development of T2D, islet β-cell mass and adequate insulin secretion must be maintained [8, 482-484]. In

contrast to the human Laron syndrome where GH insensitivity is characterized by hyperinsulinemia, insulin resistance and abdominal obesity, $GHR^{-/-}$ mice exhibit decreased serum insulin levels, increased insulin sensitivity and are not grossly obese [478, 485, 486]. There are several explanations why the two models differ in this aspect. While species-specific characteristics of GH physiology may account in part for such differences, it is likely that the mice are spared from the energy rich nature of human diet which favors obesity and insulin resistance.

In studies described in Chapter II, I demonstrated that changes in insulin sensitivity is tissue specific, because in the skeletal muscles of $GHR^{-/-}$ mice there was a delayed/diminished phosphorylation of both IR and IRS-1 but normal association of p85 to IRS-1. We believe the differences between the liver and skeletal muscle are due to the presence of IGF-IR in the skeletal muscles and the lack of the receptors in the liver which in turn causes the hepatocytes to be unaffected by a secondary deficiency in IGF-I production in these $GHR^{-/-}$ mice. IGF-I does not bind to hepatocytes [409, 410] and binds to a much lesser extent than insulin to adipocytes [545], therefore its primary insulin-like action is believed to be mediated through the skeletal muscles. IGF-I plays a greater role in skeletal muscles in maintaining insulin responsiveness: its deficiency causes diminished insulin response. These results enable us to show that GH signaling does not play a dominant role in insulin responsiveness in the skeletal muscles. T2D is a major research focus nowadays. To understand what role GH plays in diet-induced islet overgrowth, we challenged the $GHR^{-/-}$ mice with a HFD. Not only did the $GHR^{-/-}$ mice become obese, they gained more weight on the HFD

than wild-type littermates and exhibited a significant increase in islet cell growth, slightly greater than wild-type mice. Thus, GH signaling is not essential for the compensatory growth of islet cells in response to obesity. There are numerous factors involved in islet cell growth but the factors involved in HFD-induced compensatory growth are not clear at this time but it can be speculated that insulin, glucose and prolactin may be involved.

IGF-I is known to stimulate embryonic development, postnatal growth and maturation of major organ systems. IGF-I can stimulate glucose disposal in skeletal muscles and was thought to stimulate islet cell growth. The role of IGF-I on islet cell growth has been debated for many years. IGF-I and IGF-IR are both expressed in various cell types of the endocrine pancreas. IGF-I has been considered a growth factor because in in vitro systems it induces DNA synthesis of islet cells in a glucose-dependent manner and inhibits apoptosis. However in recent years novel transgenic mice have shown that 1) combined inactivation of the IR and IGF-IR or IGF-I and IGF-II genes in early embryos results in normal islet development; 2) islet β-cell-specific inactivation of IGF-IR gene causes no change in β-cell mass; and 3) liver- and pancreatic-specific IGF-I gene deficiency (LID and PID) mice suggest that IGF-I exerts an inhibitory effect on islet cell growth through indirect mechanisms, i.e. inhibiting either growth hormone release or expression of growth promoting genes, such as the Reg family proteins. These results indicate that IGF-I does not promote normal islet cell growth.

To further evaluate these controversial findings, we investigated the effects of chronic elevation of circulating IGF-I in MT-IGF mice, which

overexpress the IGF-I gene under the metallothionein promoter, on islet cell growth. Our results indicated that the transgene expression, although widespread, was highly concentrated in the islets but surprisingly the islet cell percentage and morphology were unaffected. In the meantime, IGF-I overexpression resulted in significant hypoglycemia, hypoinsulinemia, improved glucose tolerance and normal insulin sensitivity, indicating a strong insulin-like effect. MT-IGF mice were significantly resistant to streptozotocin-induced diabetes, with diminished hyperglycemia, weight loss and death, likely due to a partial prevention of β-cell death and/or the insulin-like effects of IGF-I overexpression. The onset of islet damage, reflected by hyperglycemia after streptozotocin, seems to be delayed because IGF-I overexpressing MT-IGF mice are significantly resistant to islet cell apoptosis. Once the animals have become diabetic, the IGF-I mimicked insulin actions and further relieved the symptoms. Thus, IGF-I overexpression was anti-diabetic by promoting islet cell survival and by mimicking insulin actions. The strong insulin-like effects are not due to increased insulin release or sensitivity, but may be caused at least partially by suppressing the release of insulin-antagonizing hormones such as growth hormone and glucagon. In our assays growth hormone levels are reduced although glucagon levels are unchanged. It is interesting to note that our results are in sharp contrast, in several aspects, to that of islet-specific IGF-II overexpression that caused 3 fold enlarged but disorganized islets and increased insulin secretion [546], while a whole body IGF-II overexpression caused a 25% reduced body mass, normal growth hormone levels and a 34% increase in hepatic glucose production [547, 548]. These

different outcomes cannot be explained by the general belief that IGF-II and IGF-I share the same receptor and signaling pathways, and suggest that IGF-I does not promote islet cell growth but that its overexpression is clearly anti-diabetic by improving islet cell survival and insulin responsiveness.

Our results further support that IGF-I may have a therapeutic potential in T1D treatment. It is known that in T1D patients serum IGF-I concentrations are reduced due to decreased insulin levels in the portal vein, as insulin is required to maintain IGF-I production by stimulating hepatic GHR and IGF-I gene expression [549]. Replacement of IGF-I (chronic and acute) in T1D patients results in an improved insulin response and a decreased rate of glucose production [527, 550, 551]. Co-administration with IGFBP-3 has similar beneficial effects without some of the adverse side effects associated with IGF-I alone [517, 518]; the formation of a complex with the binding protein prolongs the half-life and avoids IGF-I toxicity [552]. IGF-I/IGFBP-3 treatment is associated with the inhibition of endogenous glucose production and lowers the overnight insulin requirements [519]. Likewise, in T1D patients, IGF-I administration decreases hepatic glucose production, independent of changes in GH, glucagon or insulin [542]. Our results thus give credence to the importance of IGF-I in glycemic control on account of its ability to preserve islet integrity and/or suppress overnight hyperglycemia.

My studies have provided insight into the role of the GH/IGF-I axis in diabetes. We provide evidence that the insulin hypersensitivity seen in the $GHR^{-/-}$ mice is liver-specific since skeletal muscles do not contribute to the increased insulin sensitivity present. It is also interesting to note that GH is not needed for

islet compensatory islet overgrowth. Concerning MT-IGF mice, clearly IGF-I does not play a role in islet growth but is important in protecting islets against T1D due to its anti-diabetic effects. The role of IGF-I should be reevaluated for in T1D.

4.2 Conclusion

The GH-IGF-I axis has been studied for fifty years, yet there are still questions today regarding the interdependent effects of these hormones. GHR-mutant Laron patients are known to differ from $GHR^{-/-}$ mice in aspects of insulin sensitivity, insulin level, and obesity. In order to study the differences, the first step was to consider the differences in diet. When we placed $GHR^{-/-}$ mice on a HFD, they gained more weight compared to their wild-type littermates. Surprisingly their islets grew normally, because previously we reported that $GHR^{-/-}$ mice have significantly smaller islets than wild-type littermates. Our finding that GH is not necessary for islet cell compensation has enhanced our understanding of GH actions on the pancreatic islets. Recent reports indicated that the liver of $GHR^{-/-}$ mice expressed elevated levels of IR and increased phosphorylation of IR and IRS-I. Given the inherent differences of the level of IGF-IR receptor between the liver and skeletal muscle, we compared the changes in these tissues induced by GHR deficiency. Examining the skeletal muscles led to some interesting results, e.g. the opposite of what was observed in the liver. We demonstrated decreased IR and IRS-I phosphorylation albeit transiently; the decreased sensitivity is due to the presence of the IGF-IRs in the skeletal muscle. We thus demonstrated that there is tissue specific insulin sensitivity in the $GHR^{-/-}$

mice.

To our great surprise, in the second part of the study, the IGF-I transgene was expressed in multiple tissues in the MT-IGF mice but was highly concentrated within the islets yet there was no change in islet cell size, density or morphology. This study reconfirms other gene-targeted studies where IGF-I did not seem to play a positive role in islet cell growth [447-449, 510]. As a prominent growth factor, IGF-I was expected to increase the production of insulin but the MT-IGF mice exhibited reduced serum insulin concentrations. Despite decreased insulin levels, our studies revealed a significantly decreased blood glucose levels in the fasted state in MT-IGF mice. Decreased blood glucose level may occur due to increased insulin sensitivity as seen in our $GHR^{-/-}$ mice, but insulin sensitivity was unchanged in these mice. The MT-IGF mice showed an increase in glucose disposal, most probably due to IGF-I acting like insulin. Hepatic glucose production was significantly decreased in the MT-IGF mice. This is probably causing the decreased blood glucose levels and this effect is likely related to indirect effect of IGF-I suppressing GH. IGF-I has strong insulin-like effects as well as islet survival effects. MT-IGF mice should provide a significant protection against islet damage and prevent the onset of diabetes. When we challenged MT-IGF mice with multiple-low-dose injections of streptozotocin, MT-IGF mice were indeed protected against hyperglycemia, body weight loses, and islet cell apoptosis indicating a delayed onset of diabetes and/or improved symptoms of the disease. These results indicate that IGF-I should be considered once again for the treatment of diabetes especially in case where patients are

resistant to insulin.

4.3 Future Studies

We have provided evidence that GH is important for islet cell growth in $GHR^{-/-}$ mice. To further reveal target genes affected by GH signals, it would be interesting to examine the alterations in the pancreatic profile in the $GHR^{-/-}$ versus wild-type controls to identify which factors are necessary for islet cell growth. A DNA microarray experiment comparing islets from $GHR^{-/-}$ and wild-type mice will likely identify islet cell genes that are affected by GHR gene deficiency. One could then focus on the genes with the most significantly altered expression that are known to be involved in islet cell growth, and novel genes with potential therapeutic applications. For the target genes that are specifically affected and known to participate in islet cell growth/differentiation, we would explore mechanisms of how GH signals could affect their expression. This is relatively straightforward as both intracellular (JAKS/STATS/MAPK/PI3K) and in vivo mechanisms (through IGF-I, NGF or Pref-1) of GH actions are largely understood, and these target genes are expected to interact with GH or IGF-I signaling factors.

The MT-IGF mice exhibit decreased gluconeogenesis but the mechanism remains unknown. There are two key enzymes involved in gluconeogenesis, glucose-6-phosphatase (G6Pase) and phospho-enolpyruvate carboxykinase (PEPCK). The levels of G6Pase and PEPCK mRNAs could be determined using real-time PCR to elucidate their changes. It is possible to examine the glucose

counter-regulatory hormones and examine what happens to gluconeogenesis if we restore glucagon, GH, and/or catecholamines since they would all be expected to have an influence on islet growth and glucose homeostasis.

Our result of lack of islet overgrowth in MT-IGF mice seems to support the notion that the IGF-I effect is dependent on high-levels of glucose or the possibility that the in vivo overexpressed IGF-I was neutralized by corresponding increases in IGFBPs. I propose to further examine the IGF-I effect directly using isolated primary islets and to study the effect of IGF-I overexpression and high glucose on islet cell replication.

Regardless of the debate on whether and how IGF-I stimulates islet cell replication, its anti-apoptotic potential will no doubt contribute to better survival of β-cells and a stable cell mass, either during normal development or against a cytotoxic attack (such as the autoimmune destruction in T1D). The effect of IGF-I has not been tested in a transgenic in vivo environment; it will be interesting to examine the mechanism of anti-apoptosis. A mixture of IL-1, TNF-α, and IFN-γ is known to cause β-cell apoptosis, this can be injected into MT-IGF and wild-type mice, after a few days their pancreata will be removed and apoptotic cells determined by a TUNEL assay, a comparison of the MT-IGF and wild-type mice will be determined to see if there is any protective effect of the enhanced IGF-I.

4.4 Claim of Original Research

I have further characterized the $GHR^{-/-}$ mice in response to insulin sensitivity and specifically revealed; 1) delayed/diminished IR phosphorylation in skeletal muscle of $GHR^{-/-}$ mice; 2) delayed/diminished IRS-1 phosphorylation in skeletal muscle of $GHR^{-/-}$ mice; and 3) normal p85 association with IRS-1 in skeletal muscle of $GHR^{-/-}$ mice. I have also characterized the $GHR^{-/-}$ mice on a HFD and showed that $GHR^{-/-}$ mice are not resistant to HFD-induced obesity and GH signals are not required for compensatory islet growth response to obesity.

I have examined MT-IGF mice in regards to pancreatic islet cell growth, glucose homeostasis and resistance to diabetes and revealed that MT-IGF mice exhibit 1) islet-enriched expression of the transgene; 2) normal pancreatic islet size; 3) severe hypoglycemia, especially after fasting; 4) normal insulin sensitivity but significantly improved glucose tolerance; 5) decreased rate of hepatic gluconeogenesis (pyruvate tolerance); and 6) resistance to streptozotocin-induced diabetes.

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Appendix

by decapitation, blood was collected for serum preparation, and pancreata were rapidly removed for biochemical or histological analysis. All animal handling procedures were approved by the McGill University Animal Care Committee.

Blood chemistry and in vivo procedures. Serum concentrations of insulin and glucagon were determined using RIA kits obtained from Linco Research (St. Charles, MO). Blood glucose levels were measured using a SureStep blood glucose meter and strips (LifeScan Canada, Burnaby, BC, Canada). The Clinical Chemistry Department of McGill University Health Centre performed blood biochemistry profiles, which included determination of albumin, total protein, sodium, potassium, chloride, creatinine, urea, HDL, triglyceride, cholesterol, alkaline phosphatase, alanine aminotransferase, total bilirubin, calcium, uric acid, creatine kinase, and γ-glutamyltransferase levels in serum. Insulin tolerance tests were performed on animals in the random-fed state. Animals were injected with human insulin (1 IU/kg ip, Sigma-Aldrich, St. Louis, MO), and blood glucose levels were measured at 0, 20, 40, and 60 min after the injection.

RNA preparation and analysis. Total RNA was isolated from fresh tissues by acid guanidinium isothiocyanate-phenol-chloroform extraction (5). RNA concentration was determined by spectrophotometry at 260/280 nm. For Northern blot analysis, 5–30 μg of total RNA were subjected to electrophoresis on 1.5% agarose formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH), and the RNA blots were hybridized for 18 h at 60°C in a solution of 50% formamide (vol/vol). ^{32}P-labeled antisense RNA probes were transcribed from a mouse insulin I cDNA (*RsaI/Eco*RI fragment, extending from 48 to 725 bp) (42) and from the pTRI-β-actin-mouse plasmid (Ambion, Austin, TX). The blots were exposed to X-ray films for 1 to 2 days. For the RNase protection assay, 50 μg of total RNA were hybridized to ^{32}P-labeled antisense RNA probes using the 182-bp mouse IGF-I exon 4 and pTRI-β-actin-mouse. The reaction mixture was treated with RNase A, RNase T1, proteinase K, and phenol-chloroform and then precipitated. Protected probes were denatured, electrophoresed on an 8% polyacrylamide gel, and exposed to X-ray film (43). The intensity of the hybridization signals on the autoradiogram was analyzed using an Astra 2200 scanner (UMAX Technologies, Fremont, CA) with Scion Image 4 software (Scion, Frederick, MD).

Immunohistochemistry. Pancreata were removed from 2-mo-old GHR$^{-/-}$ mice and their wild-type littermates (n = 4 in each group). The tissue was fixed, embedded in paraffin, and cut into 5-μm sections (34). The sections were then subjected to immunohistochemical staining for insulin and glucagon with rabbit polyclonal antibodies (Monosan, Uden, Netherlands) by use of the ABC (avidin-biotin-peroxidase complex) technique, which results in a red immunoreactive signal with a nuclear counterstain from the use of methyl green (21, 34). Images of all pancreatic islets were captured with a Retiga 1300 digital camera (Q imaging, Burnaby, BC, Canada) at magnifications of 25×, 100×, or 400×. The whole pancreatic sections were digitally recorded by multiple 25× microscopic fields. The area of the pancreatic tissue was measured using Northern Eclipse computer software, version 6.0 (Empix imaging, Mississauga, ON, Canada). The number of insulin-stained pancreatic islets in each image was manually counted using Adobe Photoshop 7.0 computer software. Average islet density (number of islets per unit area of pancreatic tissue) was derived. The size of individual islets was measured in 100× images with Northern Eclipse computer software.

To study the rate of islet cell proliferation, pancreatic sections taken from 3-day-old GHR$^{-/-}$ pups and from their wild-type littermates (n = 4 for each genotype) were double stained for Ki67 (rat anti-murine Ki67 monoclonal antibody TEC-3, 1/45; Dako) and insulin with a standard immunofluorescence technique. Ki67 is a large nuclear protein that is expressed in proliferating cells and may be required for maintaining cell proliferation (39). It has been used as a marker for cell proliferation of solid tumors, some hematological malignancies, and pancreatic islets (12, 14, 32). The rate of islet cell replication for each islet (n = 8–9 per animal) was calculated using the number of Ki67-positive cells divided by the islet area. To reflect individual islet cell growth in adult (2-mo-old) mice, average cell size in hematoxylin and eosin-stained ×400 images was calculated using total islet area divided by the number of cell nuclei. For this purpose, a minimum of 10 mature islets were chosen from both GHR$^{-/-}$ and their wild-type littermates.

β- and α-Cell mass determination. Mouse pancreatic slides were stained with anti-insulin antibody and analyzed using a light microscope (Zeiss Axioskop 40, final magnification ×140) and Northern Eclipse image analysis software. β-Cell mass (defined as all cells staining positive for the hormone insulin) was determined by initially weighing the excised pancreatic tissue and then determining the percentage of the excised organ that was insulin positive (2). All insulin-positive β-cell clusters (islets) were loosely traced, and the insulin immunoreactive area was determined by use of the thresholding option. Total tissue area was also quantified with the threshold option to select the stained areas but not selecting unstained areas (white space). β-Cell percentage was determined by dividing the total insulin area by the total tissue area for each animal. The β-cell mass for each animal was then derived by multiplying the β-cell percentage by the excised pancreas tissue weight. Two slides, 120 μm apart, were examined, resulting in 265 ± 27 fields of view and 211 ± 25 mm^2 of total tissue analyzed per mouse. α-Cell mass was determined in the same fashion by the glucagon antibody.

Statistics. Data are expressed as means ± SE. Student's t-test (unpaired and paired) was performed using InStat software version 3 (GraphPad Software, San Diego, CA).

RESULTS

Decreased blood levels of insulin, glucagon, and glucose. As previously reported (9, 18, 46), adult 2-mo-old GHR$^{-/-}$ mice exhibited severe growth retardation and decreased blood glucose (80% of control) and serum insulin concentrations (40% of control) compared with their wild-type littermates under random-fed status (Fig. 1, *B* and *C*, 3rd time points). Additionally, serum glucagon levels (pg/ml) were reduced significantly, although to a lesser degree than those of insulin [wild-type (WT): 148 ± 10, n = 7 vs. GHR$^{-/-}$: 116 ± 9, n = 6, P = 0.04]. When the mice were fasted for 24 h, the decrease in blood glucose levels in GHR$^{-/-}$ mice was still significant, compared with their wild-type littermates (Fig. 1*C*). GHR$^{+/-}$ littermates exhibited marginal growth retardation (10% reduction in mean body wt vs. WT; not significant) and no change in blood glucose levels when random-fed or 24-h-fasted mice were measured (data not shown).

Decreased insulin and IGF-I gene expression. Consistent with decreased serum insulin concentration, insulin mRNA levels in the pancreas (determined by Northern blots) were markedly reduced to 40% in GHR$^{-/-}$ mice vs. their wild-type littermates (Fig. 2). Because IGF-I mediates many of the growth hormone actions in target tissues, and secretion of IGF-I is virtually abolished in GHR$^{-/-}$ mice (6, 46), the expression of IGF-I mRNA in liver and pancreas was also studied. Pancreatic IGF-I mRNA levels (determined by RNase protection assay) were reduced to 38% of that of wild-type littermates, to a similar extent as the insulin message. Liver IGF-I mRNA levels, determined by Northern blots, which exhibit a 0.7-kb major form and a 7-kb minor form as reported (31), were reduced to 37 and 24% of those of wild-type littermates, respectively (Fig. 2).

Increased insulin sensitivity. Insulin tolerance tests were performed in 7-mo-old mice under random-fed status. GHR$^{-/-}$

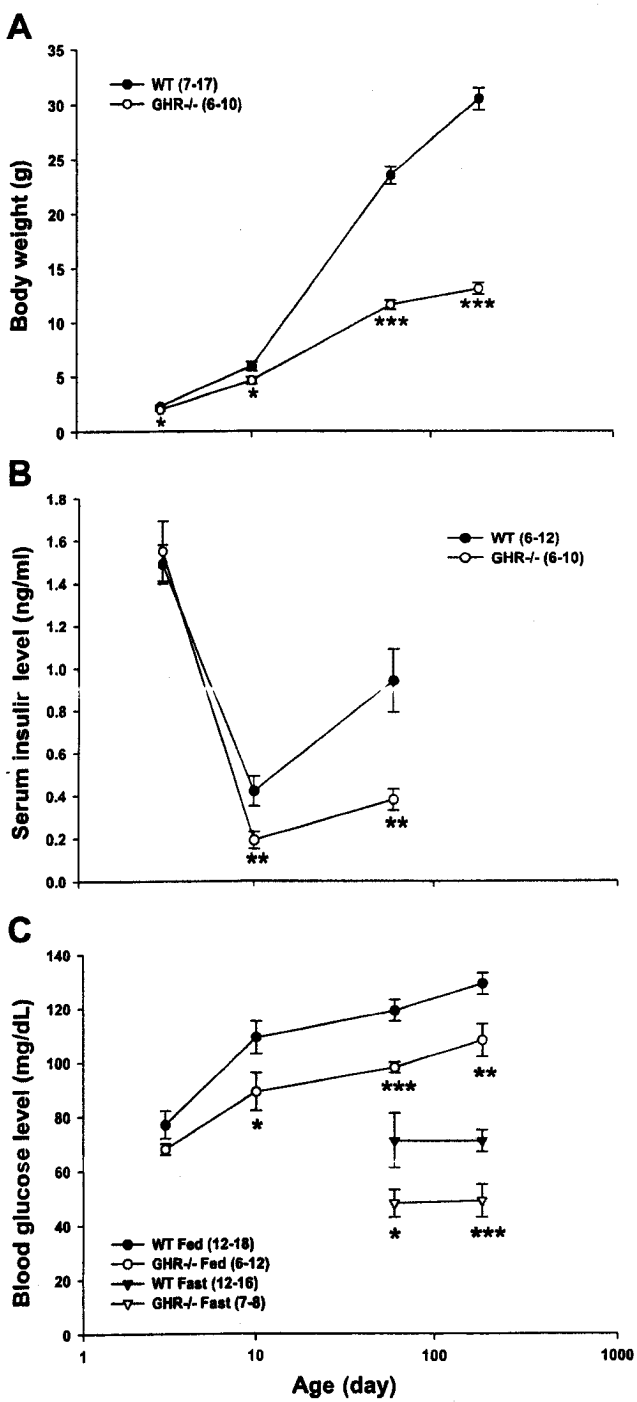

Fig. 1. Age-dependent changes in body wt and insulin and glucose levels in growth hormone receptor-deficient (GHR$^{-/-}$) mice. Mice were studied at the ages of 3, 10, 60, and 180 days. Serum levels of insulin and blood glucose concentrations were determined in random-fed GHR$^{-/-}$ mice and their wild-type (WT) littermates. As indicated, some glucose assays were also performed on 24-h-fasted mice. Data are expressed as means ± SE. Nos. of mice in each group are indicated in parentheses. *P* values are derived from comparison with WT littermates by unpaired *t*-tests. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. WT littermates.

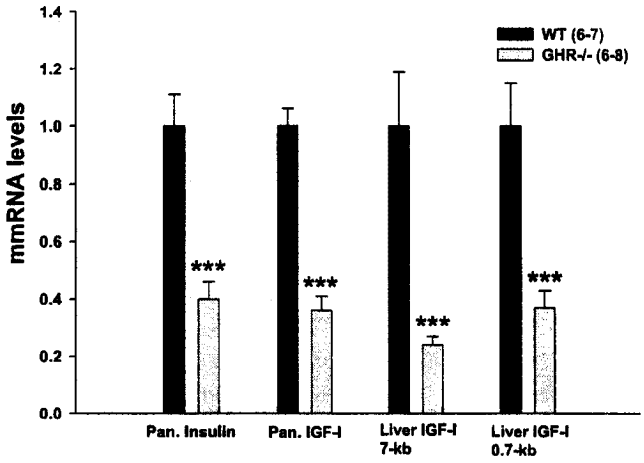

Fig. 2. Changes in the level of insulin and IGF-I gene expression in GHR$^{-/-}$ mice. WT littermates and GHR$^{-/-}$ mice were killed at 2–3 mo of age, and pancreas (Pan.) and liver were removed to prepare total RNA. Pancreatic insulin and liver IGF-I mRNA levels were analyzed using Northern blot hybridization. Pancreatic IGF-I mRNA levels were measured using an RNase protection assay. All relative intensity values were expressed as means ± SE after correction for abundance of β-actin mRNA. No. of measurements is indicated in parentheses. $***P < 0.001$ vs. WT littermates by unpaired *t*-tests.

mice exhibited hypoglycemia compared with their wild-type littermates. Insulin injection caused a significant 26–40% reduction in blood glucose levels from 20 to 60 min in wild-type littermates (Fig. 3). GHR$^{-/-}$ mice exhibited further decreased blood glucose levels of 60 and 67% at 40 and 60 min, significantly lower than those of control mice, thus exhibiting elevated sensitivity to insulin.

Diminished pancreatic islet size. To investigate the phenotype of pancreatic islets of GHR$^{-/-}$ mice, we performed immunohistochemistry by use of insulin antibody in 2-mo-old mice. The size of insulin-staining islets was measured in both wild-type and GHR$^{-/-}$ mice. In wild-type mice, there was an

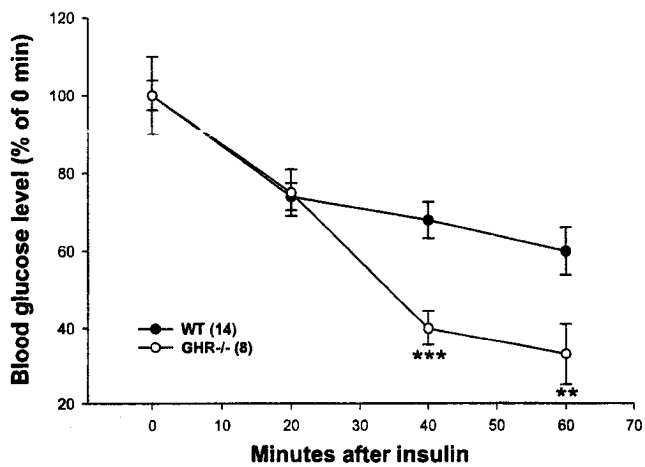

Fig. 3. Increased insulin sensitivity in adult GHR$^{-/-}$ mice. Insulin tolerance tests were performed in 7-mo-old mice. WT and GHR$^{-/-}$ mice of both sexes in random fed state were injected with insulin (1 IU/kg ip). Blood glucose levels were measured from tail blood at 0, 20, 40, and 60 min after injection. Percentage values relative to *time 0* are expressed as means ± SE; $**P < 0.01$, $***P < 0.001$ vs. WT mice in unpaired *t*-test. Similar results were obtained in a separate group of 3-mo-old mice.

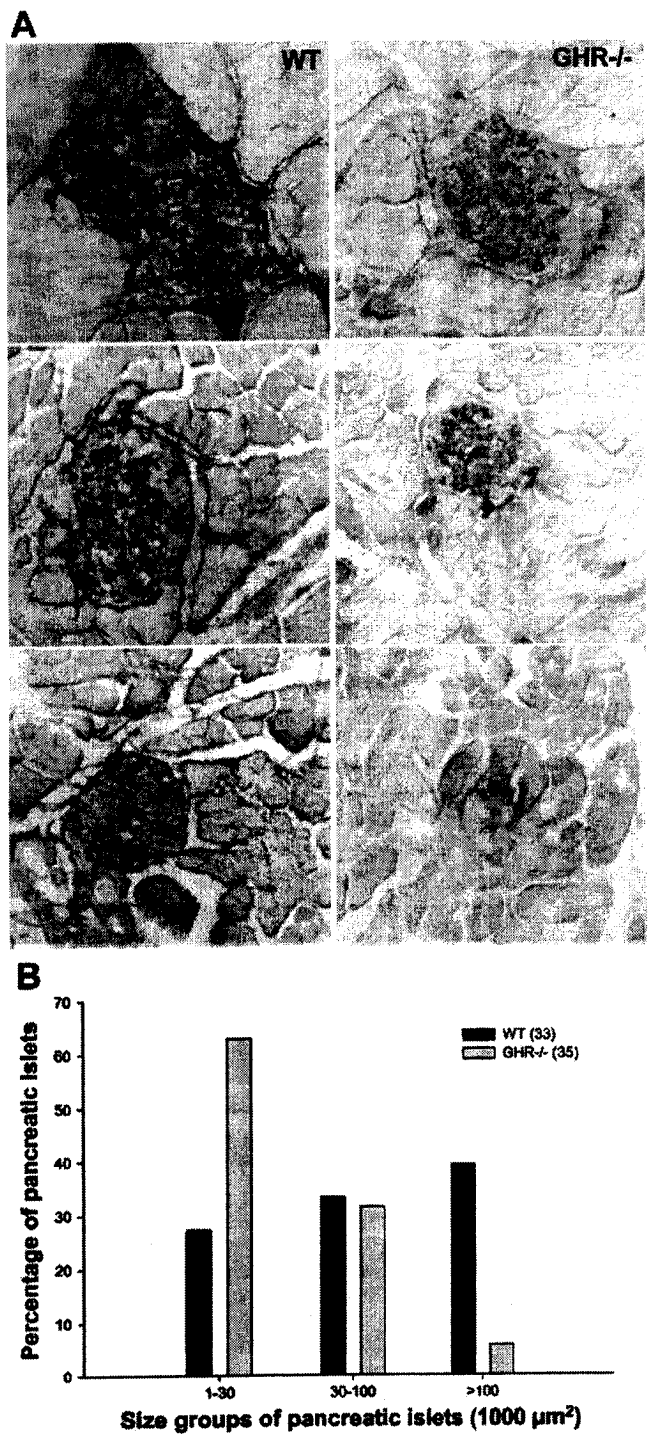

Fig. 4. Diminished pancreatic islet size in GHR$^{-/-}$ mice. *A*: pancreatic islet size distribution in 2-mo-old mice. Images of pancreatic islet immunohistochemistry by use of insulin antibody were recorded in ×100 magnification and analyzed using Northern Eclipse software. They are divided into 3 groups according to their sizes in both WT (*left*) and GHR$^{-/-}$ mice (*right*). One representative islet from each of 3 size groups was illustrated. *B*: summary of pancreatic islet distribution. In both WT and GHR$^{-/-}$ mice, islets were divided into 3 groups according to their size (in μm²). *Y*-axis represents % of total islets distributed in each of the 3 size groups.

even distribution of pancreatic islet sizes, from small to "giant" islets that differ 5- to 10-fold in diameter (Fig. 4, *A* and *B*). In GHR$^{-/-}$ mice, this distribution pattern was shifted toward the smaller islets, without the presence of "giant" ones (Fig. 4, *A* and *B*). As a result, the average islet size for GHR$^{-/-}$ mice decreased by 68% ($P < 0.0001$) compared with that in wild-type littermates (pancreatic islet size: WT 84.5 ± 11.5, $n = 33$ vs. GHR$^{-/-}$ 29.0 ± 4.9, $n = 35$; units expressed as $\times 10^3 \cdot \mu m^2$). The distribution of islets per unit pancreas area, measured in nonoverlapping ×25 images of the pancreatic sections, was unchanged (data not shown).

Total β-cell mass, determined by insulin antibody staining, was 4.5-fold lower in GHR$^{-/-}$ than in wild-type animals (Table 1). β-Cell mass-to-body weight ratios were also 50% lower in knockout than in wild-type mice, indicating that the reduced β-cell mass in knockout mice is due not only to their decreased body size. Pancreas mass-to-body weight ratios exhibited no significant decrease. In contrast, total α-cell mass (in mg) was unchanged in GHR$^{-/-}$ dwarf mice, as shown in Table 1 by a 2-fold increase in ratio of α-cell mass to body weight. There was no sexual dimorphism in these parameters.

Early onset of abnormalities in glucose homeostasis. To exclude the possibility that pancreatic islet growth in GHR$^{-/-}$ mice was proportionally reduced as a result of general growth retardation and to explore how early one can detect defects in pancreatic islet function and glucose homeostasis, we studied young pups 3 and 10 days after birth (before onset of the peripubertal growth spurt in the 3rd wk). At 3 days of age, GHR$^{-/-}$ pups were already retarded by 15% in mean body weight (WT 2.31 ± 0.09 g vs. GHR$^{-/-}$ 1.97 ± 0.08 g; $n = 6$–17, $P < 0.05$), although serum insulin and glucose concentrations remained normal (Fig. 1, 1st time points). At 10 days of age, GHR$^{-/-}$ pups showed further growth retardation (by 22% in mean body weight), and blood glucose and serum insulin levels were markedly reduced, to a similar extent as found in adult GHR$^{-/-}$ dwarfs (Fig. 1, 2nd time points). Measurement of pancreatic islet size in 10-day-old pups by use of immunohistochemistry with insulin antibody revealed a significant 28% reduction in GHR$^{-/-}$ islet size (pancreatic islet size: WT 12.8 ± 1.3, $n = 18$ vs. GHR$^{-/-}$ 9.2 ± 1.0, $n = 23$, $P = 0.03$; units expressed as $\times 10^3 \cdot \mu m^2$). Eventually the growth retardation of GHR$^{-/-}$ mice, as reflected by body weight, reached 51% in 2-mo-old adults (Fig. 1, 3rd time points).

Table 1. *Changes in pancreatic tissue and islet β- and α-cell mass in GHR$^{-/-}$ mice*

	WT (9)	GHR$^{-/-}$ (7)	*P* Value
Body weight, g	32.3±0.6	14.4±0.7	<0.00001
Pancreas mass, g	0.277±0.025	0.107±0.008	0.00005
β-Cell mass, mg	1.70±0.26	0.37±0.07	0.0006
β-Cell mass/body wt, mg/kg	53±8	25±3	0.007
Pancreas mass/body wt, %	0.86±0.07	0.75±0.04	NS
α-Cell mass, mg	0.032±0.002	0.029±0.013	NS
α-Cell mass/body wt, mg/kg	0.9±0.1	1.8±0.3	0.022

Wild-type (WT; 4 male, 5 female) and growth hormone receptor-deficient (GHR$^{-/-}$; 3 male, 4 female) 10- to 11-mo-old mice were analyzed. Values are means ± SE; nos. of mice are indicated in parentheses. *P* values are derived from comparison with WT littermates by unpaired *t*-tests. NS, nonsignificant.

Decreased cell proliferation and growth in pancreatic islets. To investigate how lack of growth hormone signaling may affect pancreatic islet growth, we studied cell proliferation with immunofluorescence and a Ki67 replication marker in pancreatic sections prepared from 3-day-old mice. As shown in Fig. 5, in wild-type mice (*left*) the proliferating cell nuclei (Ki67 in green) were easily detectable in endocrine islets double-labeled with insulin antibody (in red). On the other hand, in islets of GHR$^{-/-}$ mice (*right*), the distribution of Ki67-positive cells was more scattered. Islet cells that are undergoing replication, measured by Ki67-positive nuclei per given area of the pancreatic islets, exhibited a 68% reduction in GHR$^{-/-}$ vs. wild-type littermates (WT 4.8 ± 0.6 cells per 1,000 μm^2 of islet area, $n = 9$ vs. GHR$^{-/-}$ 1.5 ± 0.2, $n = 8$, $P < 0.001$). This indicates that a lower percentage of islet cells were undergoing replication in the GHR$^{-/-}$ mice at this age, although insulin and glucose levels were perfectly normal (see above). Attempts in older mice (14-day and 4-mo) were unsuccessful, because the replication rate in wild-type mice was too low for a meaningful comparison (data not shown).

To reflect changes in individual cell growth caused by lack of growth hormone signaling, we further measured islet cell size in pancreatic sections. In normal adults (2 mo old), the average islet cell size in GHR$^{-/-}$ mice (843 ± 32 μm^2/cell, $n = 10$) was significantly reduced by 20% compared with wild-type littermates (1,061 ± 40 μm^2 per cell; $n = 10$, $P = 0.0004$), suggesting decreased cell growth in GHR$^{-/-}$ islets.

Blood biochemical profile. Alterations in insulin responsiveness and pancreatic islet function in GHR$^{-/-}$ mice might be part of a more profound change in general metabolism. To detect other possible abnormalities, we analyzed 17 biochemical parameters in the blood of 3- to 4-mo-old, normally fed mice. Although GHR$^{-/-}$ mice had normal lipid profiles, they exhibited significant elevations in serum levels of chloride, albumin, urea, alanine aminotransferase, and creatine kinase. Other parameters were not significantly altered (Table 2).

Are GHR$^{-/-}$ mice obese? Unlike humans with Laron syndrome, which is accompanied by marked trunkal obesity (1, 28), GHR$^{-/-}$ mice were not obviously obese up to 4 mo of age. The mean body mass index (derived by dividing the body weight in kilograms by the square of the body length in meters, from nose to anus) of GHR$^{-/-}$ mice was not increased (Table 3). Blood biochemistry assays revealed normal lipid profiles (serum cholesterol, triglyceride, and HDL cholesterol levels) (Table 2). Nevertheless, careful measurement of the relative weight (to total body wt) of 3 fat pads revealed a selective enlargement in subcutaneous fat mass. The relative weight of the subcutaneous, lateral abdominal fat increased 2.5-fold compared with wild-type littermates. Other visceral fat pads found in the abdomen and surrounding the kidney were virtually unchanged (Table 3). As expected, GHR$^{-/-}$ mice exhibited a significant 22% decrease in relative weight of their livers (to total body wt) (Table 3) (41, 46). These changes exhibited no

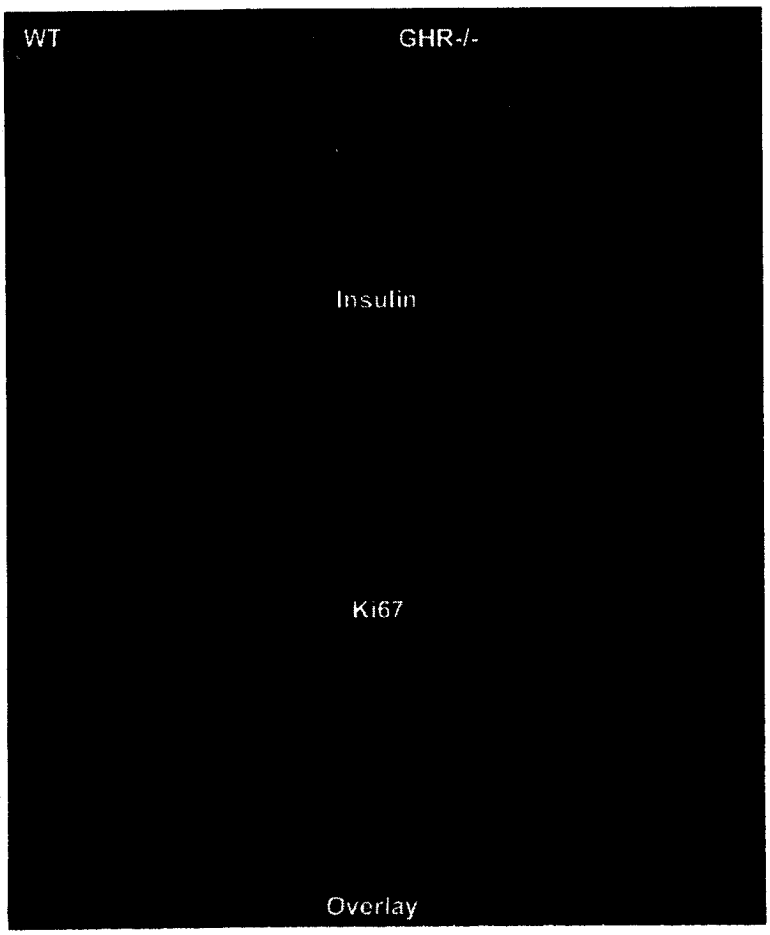

Fig. 5. GHR$^{-/-}$ mice exhibit decreased proliferation in pancreatic islet cells. Pancreatic sections taken from 3-day-old GHR$^{-/-}$ mice (*right*) and their WT littermates (*left*) were double-labeled with insulin (red) and Ki67 antibodies (green fluorescence). From a total of 8–9 islets examined in each genotype group, representative islet images (in ×400) are presented. *Top*: anti-insulin only; *middle*: anti-Ki67 only; *bottom*: computer-merged images.

Table 2. *Changes in blood chemistry in GHR$^{-/-}$ mice*

	WT	*n*	GHR$^{-/-}$	*n*	*P* Value
Sodium, mM	148±1	19	149±1	6	0.47
Potassium, mM	6.8±0.3	19	7.2±0.4	6	0.53
Chloride, mM*	115.1±0.5	19	117.8±0.8	6	0.0097
Albumin, g/l*	14.7±0.4	19	17.2±0.4	6	0.004
Total protein, g/l	46.8±0.8	19	44±1.2	6	0.074
Albumin/total protein	0.315±0.008	19	0.391±0.007	6	< 0.0001
Creatinine, μM	21.9±0.9	19	23.5±1.3	6	0.37
Urea, mM*	7.2±0.6	19	12.2±1.6	6	0.0011
HDL cholesterol, mM	2.47±0.13	19	1.99±0.15	6	0.06
Triglyceride, mM	1.24±0.1	19	1.00±0.11	6	0.21
Cholesterol, mM	2.56±0.13	19	2.17±0.14	6	0.13
Alkaline phosphatase, IU/l	90±4	19	104±6	6	0.11
Alanine aminotransferase, IU/l*	30±4	19	60±12	6	0.0062
Total bilirubin, μM	6.8±0.4	19	5.8±0.6	6	0.18
Calcium, mM	2.38±0.02	19	2.34±0.04	5	0.23
Uric acid, μM	132±9	19	154±11	6	0.22
Creatine kinase, IU/l*	848±122	16	1556±290	5	0.0165
γ-Glutamyl transferase, IU/l	2.4±0.3	18	1.6±0.4	5	0.2

Sera were prepared from GHR$^{-/-}$ mice and their WT littermates at 3–4 mo of age, both males and females, at random-fed status. Values are are means ± SE; *n*, nos. of mice. *P* values are derived from comparison with WT littermates using unpaired *t*-tests. *Independent parameters exhibiting significant changes in GHR$^{-/-}$ mice.

sexual dimorphism when the comparisons were made according to sex groups.

DISCUSSION

The classical form of Laron syndrome is caused by deletions or mutations in the GHR gene, resulting in dysfunction of the receptor and thus growth retardation, trunkal obesity, insulin resistance, and hyperinsulinemia (28, 29). When first created, GHR$^{-/-}$ mice were named Laron mice, because they seemed to share common cause and defects that include deficiency in growth hormone signaling and congenital IGF-I deficiency (46). Recent reports of decreased fasting glucose and insulin levels, increased sensitivity to insulin, as well as decreased glucose tolerance in GHR$^{-/-}$ mice (9, and Coschigano et al. at the 1999 Endocrine Society Annual Meeting) have made it necessary to further study their glucose homeostasis and pancreatic islet function. In this study, we demonstrated that lack of growth hormone signals caused diminished pancreatic islet size and β-cell mass, accompanied by elevated sensitivity to insulin. Islet hypotrophy is likely a primary cause of decreased pancreatic insulin mRNA and serum insulin levels. Glucagon production was also affected, although to a lesser extent and with no decrease in α-cell mass. The islet change was greater in proportion than the body growth retardation of the GHR$^{-/-}$ mice and was detectable as early as 10 days of age (islet replication decrease seen in 3-day-old mice), before the onset of the peripubertal growth spurt, suggesting specific effects independent of growth retardation. Double-stained immunofluorescence experiments suggest that decreased cell replication might contribute to the islet hypotrophy. Notably, GHR$^{-/-}$ mice exhibited the opposite characteristics of insulin secretion and sensitivity, as well as of fat mass, compared with human Laron syndrome patients.

Table 3. *Changes in fat pad weight in GHR$^{-/-}$ mice in relation to liver and body weights*

	WT		GHR$^{-/-}$	
	Male (6)	Female (8)	Male (3)	Female (7)
Body wt, g	30.2±1.2	23.6±0.7†	15.0±1.0§	14.1±0.8§
Body length, cm	9.5±0.2	9.2±0.1	7.3±0.2§	7.0±0.1§
Liver wt, % body wt	4.21±0.14	5.03±0.23*	2.70±0.55‡	4.06±0.16‡
Body mass index	3.3±0.2	2.8±0.1*	2.8±0.1	2.8±0.1
Fat wt, % body wt				
Visceral	1.14±0.30	1.33±0.30	1.32±0.10	1.40±0.22
Subcutaneous	0.37±0.02	0.45±0.05	1.29±0.22§	0.98±0.15‡
Renal	0.40±0.24	0.22±0.11	0.35±0.11	0.14±0.02

Three fat pads and the liver were dissected from 4-mo-old GHR$^{-/-}$ mice and their WT littermates. Total body wt, wet tissue wt, and body length (nose to anus) were measured. Data are expressed as means ± SE; nos. of mice/group are indicated in parentheses. *P* values are derived from comparison with WT littermates by unpaired *t*- tests (*$P < 0.05$, †$P < 0.001$, female vs. male WT; ‡$P < 0.01$, §$P < 0.001$ vs. same sex WT littermates).

Results of this study, demonstrating diminished pancreatic islet size and insulin mRNA accumulation in GHR$^{-/-}$ mice, indicate that the normal growth of the pancreatic islets and the level of insulin production were both affected by the lack of growth hormone signals. Consequently, basal levels of serum insulin under random-fed status or after 24 h of fasting were proportionally diminished. The decrease in pancreatic islet size seems to be disproportionately greater than the general growth retardation in GHR$^{-/-}$ mice, because, as body weight (mass) represents a 3-dimensional measurement (assuming mass represents volume when the object is homogeneous), its reduction to 49% of that of wild-type littermates (at 2 mo of age) would have caused reductions to 79% in 1-dimensional (such as body length) and 62% in 2-dimensional measurements (such as pancreatic islet area). [If $x^3 = 0.49$, then $x = (0.49)^{1/3} = 0.79$, and $x^2 = 0.62$, where x represents 1-dimensional reduction.] In fact, GHR$^{-/-}$ mice exhibited an islet size of 32% of wild-type littermates, significantly smaller than the calculated proportion of 62%, indicating a greater than proportional reduction in size. Our age-dependent studies, e.g., the relationships of body

weight and islet size in 10-day-old pups, and body weight/length in 4-mo-old GHR$^{-/-}$ mice vs. their wild-type littermates, further support this notion. More directly, our measurement of β-cell mass (Table 1) demonstrates a net deficit of the endocrine pancreas, significantly greater than proportionate growth retardation. Reductions in pancreatic islet size and serum insulin levels were observed as early as 10 days in GHR$^{-/-}$ mice, before onset of the profound peripubertal growth retardation. Growth hormone is known to promote islet cell growth and to prevent apoptosis in monocytes and tumor cells (15, 16). Diminished pancreatic islet size in adult GHR$^{-/-}$ mice might be attributed to either decreased proliferation or increased apoptosis of pancreatic islet cells. One of the primary changes was decreased islet cell proliferation, possibly due to lack of growth hormone signaling and/or concomitant lack of IGF-I production. The size of individual islet cells in adult GHR$^{-/-}$ mice was also significantly reduced by 20%, which further supports a reduced cell growth and might also contribute to a decreased islet cell mass.

Growth hormone maintains glucose utilization and hepatic glucose production, decreases responsiveness of target tissues to insulin, and diminishes the conversion of glucose to fat, all contradictory to insulin effects. Children and adults with growth hormone deficiency exhibit fasting hypoglycemia (decreased hepatic production of glucose), increased insulin sensitivity, and diminished insulin secretion (22). Rodent models of chronic growth hormone excess are useful tools to investigate the mechanism by which growth hormone induces insulin resistance. Decreased insulin receptor (IR), IRS-1, and IRS-2 tyrosyl phosphorylation in response to insulin was found in skeletal muscles, whereas a chronic activation of the IRS-phosphatidylinositol 3-kinase pathway was found in the liver of growth hormone transgenic mice (11). In contrast, both growth hormone-deficient Ames dwarf and GHR$^{-/-}$ mice exhibit a state of hypersensitivity to insulin, associated with increased insulin receptor abundance and receptor phosphorylation activity in hepatocytes (9–11). Prolactin and its receptor (PRLR) are highly homologous to growth hormone and GHR, and PRLR$^{-/-}$ mice exhibited very similar results, i.e., decreased islet cell mass, insulin mRNA level, islet insulin content, and glucose tolerance (13). Under basal conditions in GHR$^{-/-}$ mice, the reductions in glucose concentration likely reflect a reduction in hepatic glucose production (due in part to lack of growth hormone signals). Under random-fed status with reduced insulin levels, GHR$^{-/-}$ mice are still hypoglycemic, probably due to increased insulin sensitivity, which overcompensates for the reduced insulin secretion.

IGF-I mediates many growth-promoting effects of growth hormone (30). GHR$^{-/-}$ mice exhibit lack of both growth hormone action and IGF-I production (46). Growth hormone has been shown to stimulate β-cell proliferation, glucose-stimulated insulin release, and insulin biosynthesis in cultured rat islets (35). These actions on islet cells are not necessarily all mediated through IGF-I expression (7), because IGF-I stimulates β-cell proliferation but inhibits glucose-stimulated insulin secretion and insulin biosynthesis (19, 45). Recent reports of pancreatic islet β-cell-specific gene targeting demonstrated that lack of IGF-I receptor on β-cells caused no change in normal islet growth and β-cell mass, reduced expression of GLUT2 and glucokinase genes, and impaired insulin secretion upon stimulation (26, 44). It remains to be determined whether the islet hypotrophy observed in GHR$^{-/-}$ mice is due directly to a lack of growth hormone signal or indirectly to a lack of IGF-I production. We have attempted to rescue islet defects by islet-specific IGF-I overexpression by use of a rat insulin promoter IGF-I transgene (using rat insulin promoter 1). Although the transgene itself caused no change in general growth and pancreatic islet development, it increased islet cell mass 3.8-fold and essentially restored it to wild-type level, supporting the notion that IGF-I mediates the growth hormone effects on islet cells (Guo Y, Lu Y, Coschigano KT, Kopchick JJ, Tang Z, Robertson K, and Liu JL presented at the American Diabetes Association 64th Scientific Sessions in June 2004). On the other hand, diminished insulin biosynthesis seems to have been caused by the lack of growth hormone signaling and cannot be explained by IGF-I deficiency. Furthermore, because growth hormone and IGF-I affect insulin sensitivity in opposite ways, increased insulin sensitivity in GHR$^{-/-}$ mice suggests a direct effect of growth hormone deficiency.

GHR$^{-/-}$ mice resemble human Laron syndrome patients in growth retardation and other key elements (25, 46). As part of this study, we clearly demonstrate that these mice are oversensitive to insulin, hypoinsulinemic, and not obviously obese. In contrast, Laron syndrome patients are known to exhibit hyperinsulinemia, insulin resistance (27), and trunkal obesity (1, 28). These important distinctions might reflect a difference in species as well as in the etiology of defects. Rodents are born at a developmental stage corresponding to ~26 wk of human gestation (23). A human embryo deficient in GHR gene expression would be influenced significantly during the "prolonged" intrauterine growth in the third trimester. As another well-documented example, humans lacking insulin receptors show severe intrauterine growth retardation and hypoglycemia, in contrast to insulin receptor gene-deficient mice (23). Moreover, the Laron syndrome is caused by heterogeneous GHR mutations (usually partial defects) vs. complete gene inactivation in GHR$^{-/-}$ mice. Finally, downstream mediators of growth hormone action, such as the interplay of IGF-I and IGF-II, might also contribute differently in human and mouse. For instance, IGF-II production is maintained throughout life in humans but virtually ceases after birth in rodents (8, 17, 33).

Our results, extending those of previous reports, demonstrate that in addition to causing general growth retardation, GHR gene deficiency induces diminished pancreatic islet size (and β-cell mass), insulin gene expression, and serum levels. Hepatic as well as pancreatic expression of the IGF-I gene is also drastically reduced. Blood glucose and serum glucagon levels are significantly reduced. GHR$^{-/-}$ mice exhibit increased insulin sensitivity. The abnormalities in glucose homeostasis occur as early as 10 days after birth, when growth retardation in GHR$^{-/-}$ mice was relatively mild. Diminished pancreatic islet mass appears to be related to decreases in proliferation and cell growth. Finally, GHR$^{-/-}$ mice are different from the patients with Laron syndrome in serum insulin level, insulin responsiveness, and obesity. We conclude that growth hormone signaling is essential for maintaining pancreatic islet size, stimulating islet hormone production, and maintaining normal insulin sensitivity and glucose homeostasis.

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Pancreatic Islet-Specific Expression of an Insulin-Like Growth Factor-I Transgene Compensates Islet Cell Growth in Growth Hormone Receptor Gene-Deficient Mice

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Both GH and IGF-I stimulate islet cell growth, inhibit cell apoptosis, and regulate insulin biosynthesis and secretion. GH receptor gene deficiency ($GHR^{-/-}$) caused diminished pancreatic islet cell mass and serum insulin level and elevated insulin sensitivity. Because IGF-I gene expression was nearly abolished in these mice, we sought to determine whether that had caused the islet defects. To restore IGF-I level, we have generated transgenic mice that express rat IGF-I cDNA under the direction of rat insulin promoter 1 (RIP-IGF). Using RNase protection assay and immunohistochemistry, the IGF-I transgene expression was revealed specifically in pancreatic islets of the RIP-IGF mice, which exhibited normal growth and development and possess no abnormalities in glucose homeostasis, insulin production, and islet cell mass. $GHR^{-/-}$ mice exhibited 50% reduction in the ratio of islet cell mass to body weight and increased insulin sensitivity but impaired glucose tolerance. Compared with $GHR^{-/-}$ alone, IGF-I overexpression on a $GHR^{-/-}$ background caused no change in the diminished blood glucose and serum insulin levels, pancreatic insulin contents, and insulin tolerance but improved glucose tolerance and insulin secretion. Remarkably, islet-specific overexpression of IGF-I gene in $GHR^{-/-}$ mice restored islet cell mass, at least partially through cell hypertrophy. Interestingly, double-transgenic male mice demonstrated a transient rescue in growth rates *vs.* $GHR^{-/-}$ alone, at 2–3 months of age. Our results suggest that IGF-I deficiency is part of the underlying mechanism of diminished islet growth in $GHR^{-/-}$ mice and are consistent with the notion that IGF-I mediates GH-induced islet cell growth. (*Endocrinology* 146: 2602–2609, 2005)

GH AND IGF-I ARE POTENT regulators of cell growth, differentiation, and metabolism and are essential for postnatal growth in mammals (1). Receptors for both GH and IGF-I are expressed in the pancreatic islet cells (2, 3). Acting on their own, both GH and IGF-I promote islet cell growth, inhibit apoptosis, and are potentially involved in normal islet growth and maintenance (4–6), but it is unclear whether GH and IGF-I interact with each other in regulating pancreatic islet function and how IGF-I is involved in GH-stimulated islet growth and insulin biosynthesis and secretion. IGF-I produced either from the liver or locally within the pancreatic islets might mediate GH actions. We and others have recently demonstrated that GH receptor gene deficiency ($GHR^{-/-}$) caused diminished pancreatic islet cell mass and serum insulin level and elevated insulin sensitivity (7, 8). Because IGF-I gene expression was nearly abolished in $GHR^{-/-}$ mice, we sought to determine whether that had caused the islet defects (9). For this purpose, we have generated transgenic mice RIP-IGF that express an IGF-I transgene under the direction of rat insulin promoter 1 (RIP) and studied whether the islet defects in $GHR^{-/-}$ mice can be rescued. As a result, local expression of the IGF-I transgene restored pancreatic islet cell mass and improved glucose tolerance in $GHR^{-/-}$ mice, which is consistent with the notion that IGF-I mediates the GH-induced growth-promoting effect on pancreatic islet cells.

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Abbreviations: $GHR^{-/-}$, GH receptor gene deficiency; IGF-IR, IGF-I receptor; RIP, rat insulin promoter 1.

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Materials and Methods

Creation of RIP-IGF transgenic mice

A transgenic line has been developed to overexpress IGF-I cDNA driven by an insulin promoter (RIP-IGF). The promoter was chosen based on its high level and β-cell-restricted expression in driving IGF-II and Glut-2 antisense (10, 11). Briefly, a 0.5-kb *Pvu*II/*Ava*I fragment of a rat prepro-IGF-I cDNA from a pGEM4Z vector (12) was subcloned into a *Bam*HI/*Eco*RI site downstream of RIP in a pKS-RIP/globin vector (Fig. 1) (11). The integrity of the transgenic construct, pKS-RIP-IGF6, was confirmed by sequencing and restriction analysis before it was injected into the pronucleus of fertilized mouse ova. The manipulated ova were transferred into the oviducts of recipient female mice, which gave birth to founder mice.

To identify founder mice (on a mixed C3H and C57BL/6 background) and offspring that carry the transgene, a PCR, using the primers Tg1 (5′-GGT GAT ATT GGC AGG TGT TCC-3′) and Tg2 (5′-CAA ATC GGC AAA GTC CAG G-3′), generated a product of 600 bp corresponding to the entire cDNA. To probe the transgene expression by Northern blots and RNase protection assays, a 0.7-kb *Pst*I/*Eco*RI fragment that contains the cDNA including the two primer sites was subcloned into pSP72

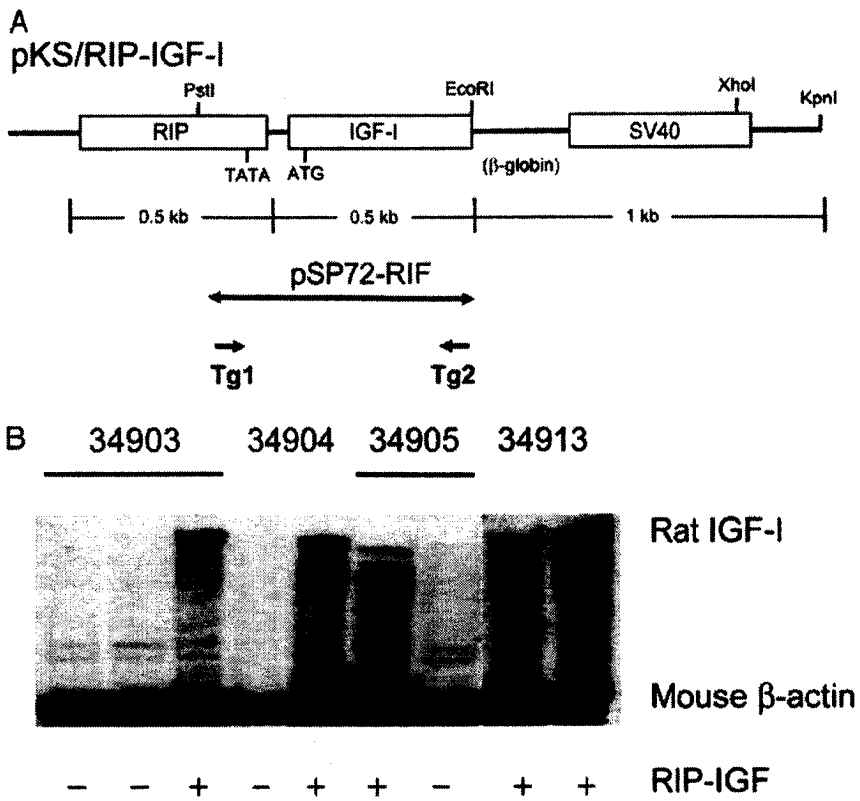

FIG. 1. Pancreatic islet-specific IGF-I overexpression: characterization of transgenic lines. A, Diagram of transgenic DNA vector: pKS/RIP-IGF-I. Based on pKS-RIP/globin vector, rat insulin promoter 1 (RIP) was used to drive expression of an intact rat prepro-IGF-I cDNA. The *arrows* mark the region covered by the pSP72-RIF probe and primers Tg1 and Tg2. B, Detection of transgene expression by RNase protection assay. Total pancreatic RNA prepared from transgenic mice (+) or wild-type littermates (−), determined by PCR, was hybridized to rat IGF-I and mouse β-actin probes. A protected band with rat IGF-I probe indicates transgene expression. Endogenous mouse IGF-I mRNA was unprotected by the probe thus destroyed by RNase treatment. Results from four representative families (codes 34903 *etc.*) are illustrated.

vector (Promega, Madison, WI). The resulting vector, named pSP72-RIF, was linearized by *Hin*dIII to direct synthesis of an antisense RNA probe using T7 polymerase.

Intercross with GHR$^{-/-}$ mice and genotyping

GHR$^{-/-}$ mice carry a targeted disruption of exon 4 of the mouse GHR/BP gene, as previously reported (8, 9). To intercross with RIP-IGF mice, heterozygous (GHR$^{+/-}$) mice, on a hybrid 129/Ola-BALB/c-C57BL/6 background, were used. To genotype the offspring, genomic DNA was isolated from tail clips using standard methods. Primers In4-1 (5′-CCC TGA GAC CTC CTC AGT TC-3′), In3+1 (5′-CCT CCC AGA GAG ACT GGC TT-3′), and Neo-3 (5′-GCT CGA CAT TGG GTG GAA ACA T-3′) were used in PCR, which yield a 390-base band for the wild-type allele, and 290/200 double bands for the GHR knockout allele, as previously reported (13). Offspring of four genotypes were selected: wild type, RIP-IGF (GHR$^{+/+}$), GHR$^{-/-}$ (no RIP-IGF), and GHR$^{-/-}$ plus RIP-IGF (GHR+RIP). All heterozygous (GHR$^{+/-}$) animals were excluded.

Animal procedures

The animals were maintained in 12-h dark, 12-h light cycles at room temperature with free access to food and water or when indicated, food deprived for 24 h with free access to water. At the desired age, the mice were anesthetized with a cocktail of ketamine/xylazine/acepromazine, bled via periorbital puncture, and killed by cervical dislocation. Blood was collected for serum preparation, and pancreata were rapidly removed for biochemical or histological analysis. All animal-handling procedures were approved by the McGill University Animal Care Committee.

Serum concentrations of insulin and glucagon were determined using RIA kits obtained from Linco Research Inc. (St. Charles, MO). IGF-I was determined using an RIA kit obtained from Diagnostic Systems Laboratories (Webster, TX). Blood glucose levels were measured using the OneTouch blood glucose meter and strips (LifeScan Canada, Burnaby, British Columbia, Canada). For the insulin tolerance test, animals were injected with human insulin (0.75 IU/kg, ip; Roche Applied Science, Penzberg, Germany), and blood glucose levels were measured at 0, 20, 40, and 60 min after the injection. For the glucose tolerance test, mice were fasted 24 h and injected with glucose (1 g/kg, ip), and blood glucose levels were measured at 0, 15, 30, 60, and 120 min after the injection.

Immunohistochemistry and islet cell mass determination

Pancreata were removed from 2-month-old mice (n = 4 in each group) and fixed, embedded in paraffin, and cut into 5-μm sections (14). The sections were then subjected to immunohistochemical staining for insulin and glucagon with rabbit polyclonal antibodies (Monosan, Uden, The Netherlands) using the avidin-biotin-peroxidase complex technique, which results in a red immunoreactive signal with a nuclear counterstain using methyl green, or diaminobenzidine substrate, which resulted in a brown immunoreactive signal with a hematoxylin counterstain (blue) of cell nuclei (14, 15). Mouse monoclonal IgG against human IGF-I (clone Sm1.2; Upstate USA Inc., Charlottesville, VA) was used to reveal IGF-I overexpression. Images of all pancreatic islets were captured with a Retiga 1300 digital camera (Q Imaging, Burnaby, British Columbia, Canada) at magnifications of ×25, ×100, or ×400. The area of the pancreatic tissue was measured using Northern Eclipse computer software, version 6.0 (Empix imaging, Mississauga, Ontario, Canada). The number of insulin-stained pancreatic islets in each image was manually counted using Adobe Photoshop 7.0 computer software.

The islet cell mass (defined as all cells staining positive for the hormone insulin) was determined by initially weighing the excised pancreatic tissue and then determining the percentage of the excised organ that was insulin positive (16). All insulin-positive β-cell clusters (islets) were loosely traced, and the insulin-immunoreactive area was determined using the thresholding option. Total tissue area was also quantified using the threshold option to select the stained areas while not selecting unstained areas (white space). Islet cell percent was determined by dividing the total insulin area by the total tissue area for each animal. The islet cell mass for each animal was then derived by multiplying the islet cell percent by the excised pancreas tissue weight. Each mouse pancreas was examined in one slide of approximately 40 fields of view and approximately 12 mm^2 of total tissue.

To reflect individual islet cell growth in adult (2 month old) mice, average cell size was calculated in hematoxylin-eosin-stained ×400 images using total islet area divided by the number of cell nuclei. For this purpose, a minimum of 10 mature islets were chosen from each genotype group.

Northern blot and RNase protection assay

RNA isolation and Northern blot analysis were as reported except with digoxigenin-labeled probes (Roche) (17, 18). RNase protection assay was as reported using ^{32}P-labeled probes (19, 20). The intensity of the hybridization signals on the autoradiogram was analyzed using a FluorChem 8900 imaging system (Alpha Innotech, San Leandro, CA).

Insulin and glucagon secretion

Mice at age 2–4 months, both male and female, were fasted 24 h and injected with glucose (3 g/kg, ip) (21). At 0 (without stimulation), 5, 15, or 30 min, they were anesthetized, bled via periorbital puncture, and killed. Blood was collected for serum preparation. Insulin and glucagon concentrations were determined by RIA.

Statistics and data plotting

Data are expressed as the mean ± SE. The Student's *t* test (unpaired and paired) and one-way ANOVA were performed using InStat software version 3 (GraphPad Software Inc., San Diego, CA). Data were plotted into curves, and the area under curve was calculated using SigmaPlot software version 9 (Systat Software, Inc., Point Richmond, CA).

Results

Pancreatic islet β-cell-specific IGF-I overexpression in RIP-IGF mice

To overexpress IGF-I in most cells of the pancreatic islets, we have used an insulin promoter to drive the transgenic expression of rat IGF-I cDNA (Fig. 1A). Multiple founder lines were created and screened for genomic integration of the transgene by specific PCR. Using immunohistochemistry and RNase protection, two mouse families (3 and 13) exhibited high levels IGF-I expression, which was specific in pancreatic islets. As shown in Fig. 1B, rat IGF-I mRNA could be detected in the pancreatic RNA prepared from RIP-IGF transgenic mice but not in nontransgenic littermates. As shown by immunohistochemistry in Fig. 2A (*top*), pancreatic islets in wild-type mice only exhibited scattered IGF-I staining in very few islet cells; the levels of IGF-I staining (brown pigmentation) was drastically elevated in RIP-IGF mice. Judging from the ratio of IGF-I-positive cells, it seems that not all β-cells express the transgene. Except nonspecific staining of the blood cells, the transgenic expression was relatively specific and not seen in exocrine acinar cells.

Normal growth and islet formation in RIP-IGF mice

RIP-IGF mice exhibited normal growth and development and possessed no abnormalities in blood glucose (fasted or fed), serum insulin, and glucagon levels, as shown in Table 1. Northern blot analysis revealed normal levels of insulin mRNA in transgenic *vs.* wild-type mice (data not shown). Pancreatic insulin content was unaltered in RIP-IGF mice (Table 2). Immunohistochemistry showed no obvious abnormality in islet morphology and α- and β-cell distribution patterns within the islets (data not shown). As shown in Fig. 2C (*columns* 1 and 2), RIP-IGF mice had normal islet cell mass. When challenged with a bolus injection of glucose, RIP-IGF mice exhibited an unaltered glucose clearance curve *vs.* wild-type littermates. Likewise, RIP-IGF mice showed no significant difference in their glucose-lowering response to insulin injection, compared with wild-type littermates (data not shown).

Effects of IGF-I overexpression on animal growth and glucose and insulin levels in GHR$^{-/-}$ mice

GHR$^{-/-}$ mice exhibited severe growth retardation, decreased blood glucose and serum insulin levels, increased insulin sensitivity, and diminished islet cell mass (7, 8). To investigate whether restored IGF-I expression in the pancreatic islets can rescue the islet defects, we intercrossed GHR$^{+/-}$ with RIP-IGF mice and studied second-generation offspring of four genotypes, *i.e.* wild type, RIP-IGF, GHR$^{-/-}$, and GHR+RIP. Compared with GHR$^{-/-}$ alone, islet IGF-I expression was indeed significantly increased in GHR+RIP mice as shown by immunohistochemistry (Fig. 2A, *bottom*). Computer-assisted image analysis indicated that, on average, the IGF-I-stained area increased from 4 ± 1% (n = 10) of total islet area in GHR$^{-/-}$ mice to 21 ± 3% (n = 11; $P < 0.05$) in GHR+RIP mice. Because the transgenic expression was limited to the islet cells at a moderate level, compared with similar reports (10, 22), and caused no change in serum IGF-I level (Table 2), it was not expected to have an impact on the growth retardation of GHR$^{-/-}$ mice. Nevertheless, we had measured their body weight from 1–4 months of age. As shown in Fig. 2B, RIP-IGF expression alone caused no change in growth *vs.* wild-type littermates. GHR$^{-/-}$ mice exhibited severe growth retardation with only approximately one half of the wild-type body weight at adult age. Interestingly, the double-transgenic male mice demonstrated a significant partial rescue in growth rates *vs.* GHR$^{-/-}$ mice at 2–3 months of age (*i.e.* ~19% increased body weight). This effect was transient, was not seen in females, and did not last beyond 4 months of age.

As expected, GHR$^{-/-}$ mice exhibited drastic reductions in serum insulin level (−33%) and pancreatic insulin content (−52%), suggesting reduced insulin production. RIP-IGF expression on this GHR$^{-/-}$ background failed to normalize serum insulin, pancreatic insulin content, and insulin mRNA to wild-type levels (Table 2 and data not shown).

Transgenic IGF-I overexpression restored islet cell mass in GHR$^{-/-}$ mice

Total islet cell mass, determined by insulin staining, was decreased 6.2-fold in GHR$^{-/-}$ mice *vs.* wild-type littermates. When corrected for body weight, the decrease was 2.9-fold (Fig. 2C, *column* 3). In double-transgenic GHR$^{-/-}$ mice that express the IGF-I transgene (GHR+RIP, *column* 4), total islet cell mass was increased 3.8-fold *vs.* GHR$^{-/-}$ mice alone. When corrected for body weight, the increase had effectively restored the islet cell mass to the level of wild-type mice (GHR+RIP 61 ± 22 *vs.* WT 46 ± 13 mg/kg; n = 5) (Fig. 2C, *column* 4).

Average islet cell size is a measure of islet cell hypotrophy (such as in GHR$^{-/-}$ mice) or hypertrophy (such as in β-cell compensation to type 2 diabetes) and a reflection of cell

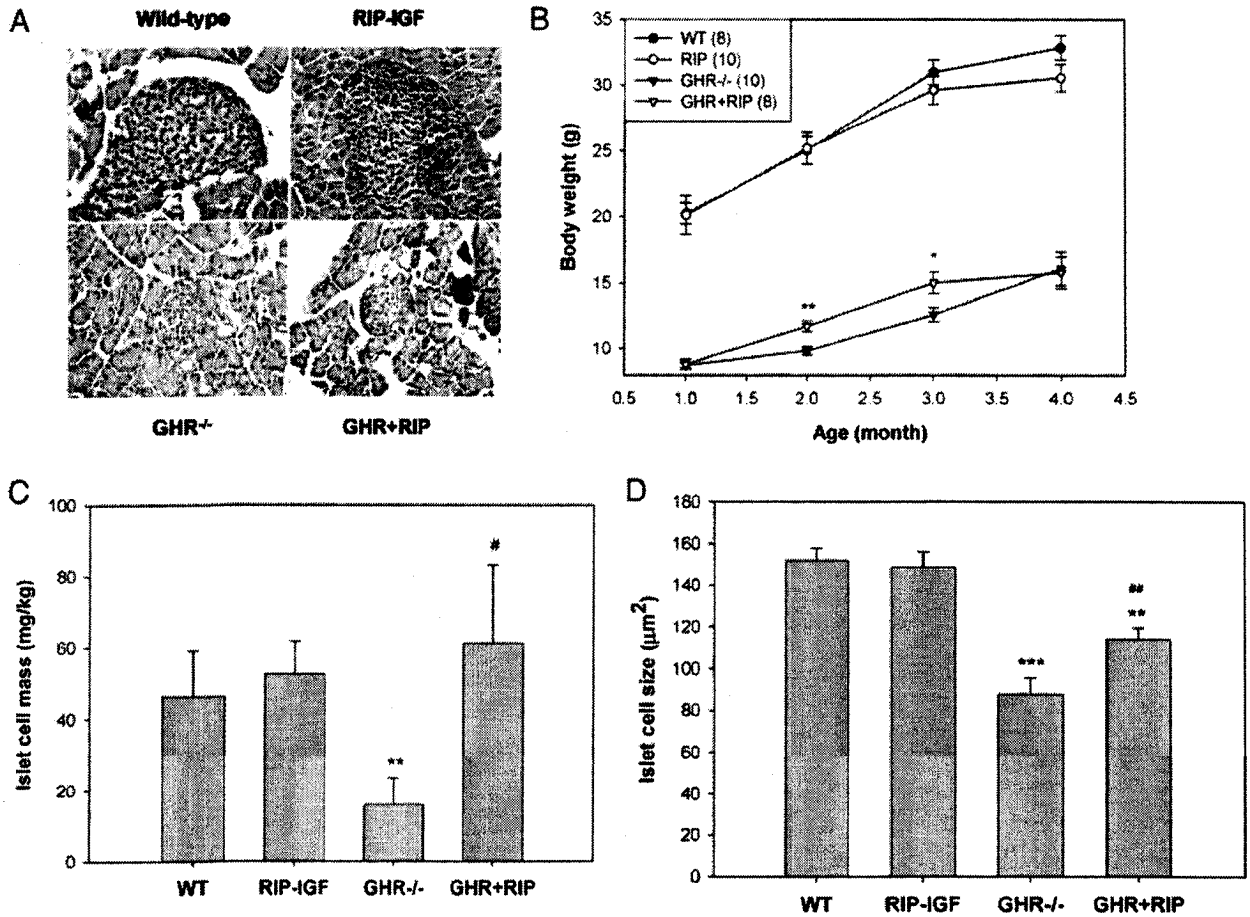

FIG. 2. Increased pancreatic islet cell mass and improved body growth caused by islet-specific IGF-I overexpression in male GHR$^{-/-}$ mice. A, Islet-specific transgenic expression revealed by immunohistochemistry. Pancreatic sections prepared from 2-month-old mice of four genotype groups were stained for IGF-I using the diaminobenzidine complex. IGF-I staining was shown as *brown* pigmentation within the islets. Cell nuclei were counterstained with hematoxylin. Images are representatives of at least 15–20 mature islets from each mouse and have been recorded at ×400. B, Postnatal growth rates in mice of various genotypes. Body weight was measured at 1, 2, 3, and 4 months of age and illustrated as mean ± SE. Numbers of animals are shown in *parentheses*. *, $P < 0.05$; **, $P < 0.01$ *vs.* GHR$^{-/-}$ alone. C, Changes in pancreatic islet cell mass, corrected for total body weight (n = 5). **, $P < 0.01$ *vs.* wild-type (WT); #, $P < 0.05$ *vs.* GHR$^{-/-}$ alone. ANOVA: $P = 0.006$; WT *vs.* GHR$^{-/-}$, $P < 0.05$; GHR$^{-/-}$ *vs.* GHR+RIP, $P < 0.05$. D, Pancreatic islet-specific overexpression of IGF-I increased the average islet cell size in GHR$^{-/-}$ mice (islet cell hypertrophy). From each genotype group of mice at age 2–3 months, 11–12 mature islets stained with hematoxylin-eosin were analyzed. The islet size and the number of cell nuclei were determined using Northern Eclipse software. ANOVA of four groups: $P < 0.001$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* WT littermates; ##, $P < 0.01$ *vs.* GHR$^{-/-}$ alone. Except that of B, similar results were obtained using female mice (not shown).

health and activity (8, 23). As shown in Fig. 2D, compared with wild-type littermates, IGF-I overexpression alone in RIP-IGF mice did not change islet cell size; as previously reported, GHR$^{-/-}$ mice exhibited a 42% reduction in islet cell size (8); in GHR$^{-/-}$ mice that overexpress IGF-I in islet cells, the average islet cell size was increased 29%, exhibiting a partial rescue in pancreatic islet cell growth. Similar results were obtained from both male and female mice.

Transgenic IGF-I overexpression improved glucose tolerance in GHR$^{-/-}$ mice

GHR$^{-/-}$ mice exhibit glucose intolerance and elevated insulin sensitivity, because of specific changes within the pancreatic islets and insulin target tissues (Coschigano, K.T., *et al.*, 1999, 81st Annual Meeting of The Endocrine Society, San Diego, CA) (8). In double-transgenic GHR+RIP mice, with restored IGF-I production, their glucose tolerance was largely restored (Fig. 3A). At 40 and 60 min after glucose injection, GHR+RIP mice exhibited significantly reduced blood glucose level *vs.* GHR$^{-/-}$ alone. Their rate of glucose disposal was almost as efficient as wild-type littermates (Fig. 3A).

Animals of the four genotypes were tested for insulin tolerance. As shown in Fig. 3B, compared with wild-type littermates, GHR$^{-/-}$ mice exhibited significantly decreased glucose levels at 20, 40, and 60 min after insulin injection. GHR+RIP mice exhibited less deviation from wild-type mice, but the differences were insignificant from either group. Thus, pancreatic islet-specific IGF-I overexpression did not affect the phenotype of insulin hypersensitivity.

Changes in glucose-stimulated insulin and glucagon secretion

Reduced glucose tolerance in GHR$^{-/-}$ mice, in the face of increased insulin sensitivity, suggests possible defects in in-

TABLE 1. Changes in body weight and glucose homeostasis in RIP-IGF mice *vs.* wild-type controls

	Wild type		RIP-IGF	
	Male (16)	Female (14)	Male (14)	Female (12)
Body weight (g)	23.5 ± 0.6	17.6 ± 0.3	23.7 ± 0.6	16.9 ± 0.6
Blood glucose level (mg/dl)	167 ± 7	157 ± 5	175 ± 8	147 ± 5
Blood glucose level, fasted (mg/dl)	113 ± 7	99 ± 6	105 ± 9	89 ± 6
Serum insulin level (ng/ml)	0.60 ± 0.08	0.39 ± 0.06	0.64 ± 0.21	0.47 ± 0.12
Serum glucagon level (pg/ml)	68 ± 7	127 ± 23	60 ± 6	146 ± 52

Mice were 2–3 months old and under random feeding (except in one group marked fasted, for 24 h). The number of animals in each group is illustrated in *parentheses*.

sulin secretion. Likewise, improved glucose tolerance in GHR+RIP mice would indicate elevated insulin secretion *vs.* GHR$^{-/-}$ alone. To verify these speculations, we have measured serum insulin levels after a glucose load (Fig. 4A). Insulin secretion exhibited 2.7- to 4.9-fold increases in wild-type mice at 5 and 15 min after glucose stimulation, which was virtually restored to normal by 30 min. RIP mice exhibited a similar response, except with a delayed return to basal level at 30 min. In contrast, the secretion in GHR$^{-/-}$ mice was drastically diminished and reached to only 2.2- and 2.7-fold at 5 and 15 min *vs.* 0 min, even after being corrected for their low basal level. Interestingly, at 5 min after glucose stimulation, GHR+RIP mice were able to demonstrate a transient enhancement in serum insulin level *vs.* GHR$^{-/-}$ mice alone (which did not last till 15 and 30 min), suggesting a certain degree of improvement in insulin secretion associated with islet-specific IGF-I overexpression. In the same experiment, serum glucagon levels exhibited no significant reductions in wild-type and RIP mice within 15 min of glucose injection (Fig. 4B). In GHR$^{-/-}$ and GHR+RIP mice, however, glucagon levels were reduced approximately 40% at 5 min after glucose stimulation, significantly lower than wild-type mice.

Discussion

Both GH and IGF-I stimulate islet cell growth and inhibit apoptosis and thus are potentially involved in normal islet development. GH stimulated insulin and glucagon secretion and pancreatic islet cell proliferation (24–27). The stimulation of cell replication in neonatal rat pancreatic monolayer cultures by GH was independent of glucose concentration (28). More recently, in primary cultures of pancreatic islet cells, GH stimulated β-cell proliferation, insulin gene transcription and insulin secretion (4). Among the various post-receptor substrates, Stat5a/b, Stat1, and Stat3 were found to be activated in pancreatic islet or islet-derived tumor cells (29, 30). GH overexpression *in vivo* increased pancreatic islet number and volume in transgenic mice (31). In a previous report, we have demonstrated reduced islet cell mass and enhanced insulin sensitivity in GHR$^{-/-}$ mice (8). Because IGF-I gene expression in liver and pancreas was severely affected and IGF-I is known to stimulate islet cell growth, we believe that lack of IGF-I production in GHR$^{-/-}$ mice had contributed to islet growth defect. In this study, we have created transgenic mice (RIP-IGF) that overexpressed the IGF-I gene in pancreatic islet cells and exhibited no obvious effect on islet growth by themselves. Crossing them with GHR$^{-/-}$ mice restored local production of IGF-I in the islet cells and rescued to various degrees islet cell mass, average cell size, glucose tolerance, and even transiently somatic growth. It is consistent with the notion that locally produced IGF-I mediates GH-stimulated islet cell growth. Glucose intolerance in GHR$^{-/-}$ mice is likely caused by insufficient (amount and speed) release of insulin in response to glucose (first), especially so in the face of increased insulin sensitivity. A significant improvement in GHR+RIP mice *vs.* GHR$^{-/-}$ alone (Fig. 3A) would indicate improvement in insulin secretion (second). Our *in vivo* insulin secretion study clearly confirmed the *first* possibility. The improvement in GHR+RIP mice, although marginal because it occurred only at 5 min (Fig. 4A), was supportive of the *second* possibility as well. On the other hand, it is unlikely that overexpressed IGF-I, coreleased with insulin, would increase hypoglycemic activity because total IGF-I level was unaffected (Table 2) and any increase in free (and active) IGF-I would be first neutralized by IGF binding proteins.

The interaction between GH and IGF-I in regulating growth and development has been well defined by the somatomedin hypothesis (1). More recently, GH has been known to have IGF-I-independent, direct actions, in addition to its interactions with locally produced IGF-I. According to the dual-effectors hypothesis, GH acts directly at the epiphyseal plate to stimulate linear growth, and GH, IGF-I, and IGF-II each has a unique and complementary role in augmenting long-bone growth (1). As recently reported, cortical

TABLE 2. Effects of IGF-I overexpression on serum chemistry and pancreatic insulin content in GHR$^{-/-}$ mice

	WT	RIP	GHR$^{-/-}$	GHR+RIP
Blood glucose level (mg/dl)	145 ± 8 (7)	137 ± 2 (9)	126 ± 7 (9)[a]	129 ± 8 (9)[a]
Serum insulin level (ng/ml)	0.49 ± 0.09 (8)	0.43 ± 0.13 (12)	0.33 ± 0.09 (15)[a]	0.30 ± 0.06 (9)[a]
Serum IGF-I level (ng/ml)	415 ± 34 (5)	419 ± 69 (5)	231 ± 6 (5)[b]	224 ± 12 (10)[b]
Pancreatic insulin content (ng/μg protein)	3.1 ± 0.4 (5)	2.9 ± 0.8 (6)	1.5 ± 0.3 (5)[a]	1.8 ± 0.4 (6)[a]

Male mice at age 2–3 months were used at random fed. The number of animals in each group is illustrated in *parentheses*. Serum IGF-I was determined in separate RIA experiments; thus, the absolute values of the first two and last two groups are incomparable. Similar results were obtained using female mice (not shown).

[a] $P < 0.05$.

[b] $P < 0.001$ *vs.* wild-type (WT) mice.

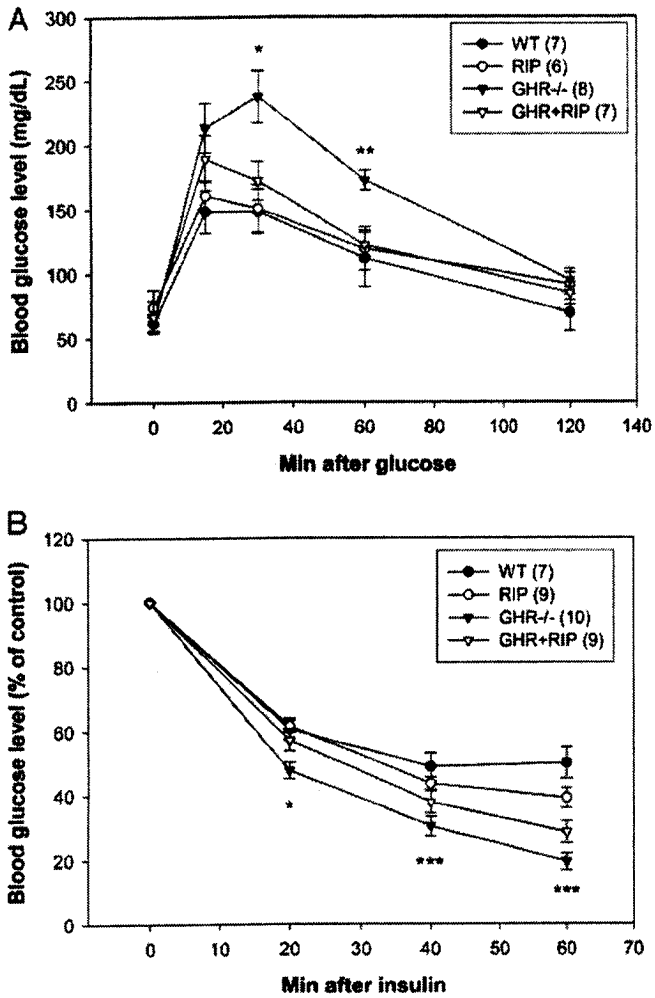

FIG. 3. Improved glucose tolerance but unaffected insulin sensitivity caused by islet-specific IGF-I overexpression in male GHR$^{-/-}$ mice. A, Glucose tolerance test. Mice (9–10 wk old) were fasted for 24 h, and glucose (1 g/kg) was injected ip. Blood glucose was measured at 0, 15, 30, 60, and 120 min after the injection. *, $P < 0.05$; **, $P < 0.01$ *vs.* wild-type (WT) littermates in unpaired *t* test. The number of animals in each group is indicated in *parentheses*. B, Insulin tolerance test. Mice (11–12 wk old) were injected with insulin (0.75 U/kg, ip), and blood glucose was measure at 0, 20, 40, and 60 min after. The percentage values relative to 0 time were expressed as mean ± SE. *, $P < 0.05$; ***, $P < 0.001$ *vs.* WT control mice. Similar results were obtained using female mice (not shown).

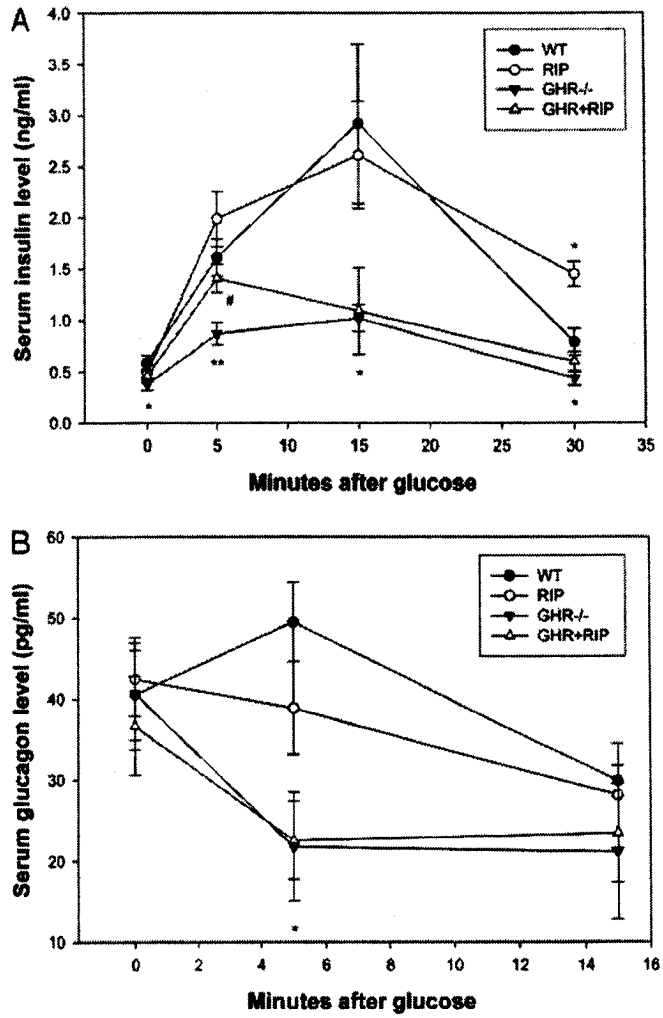

FIG. 4. Changes in glucose-stimulated insulin and glucagon secretion caused by islet-specific IGF-I overexpression in GHR$^{-/-}$ mice. A, Serum insulin level. Mice at age 3–4 months, both male and female, were fasted for 24 h before being stimulated with glucose (3 g/kg, ip). Serum insulin concentrations were measured at 0, 5, 15, and 30 min (n = 5–14). *, $P < 0.05$; **, $P < 0.01$ *vs.* wild-type (WT) mice; #, $P < 0.05$ *vs.* GHR$^{-/-}$ alone by unpaired *t* tests. The area under curve values were 56.0 for WT, 69.7 for RIP, 23.5 for GHR$^{-/-}$, and 29.9 for GHR+RIP. B, Serum glucagon levels. The values at 30 min are not presented because of insufficient samples.

and longitudinal bone growth and bone turnover were all reduced in GHR$^{-/-}$ mice. Short-term administration of IGF-I substantially reversed many of these defects, suggesting a main mechanism of reduced IGF-I levels in the absence of GHR (32). On the other hand, GH is clearly not essential for the differentiation of adipocytes, which were abundant in GHR gene-deficient mice and humans (1). Also from GHR$^{-/-}$ mice, the actions of GH on follicular growth seem independent of circulating IGF-I (33). Although in isolated pancreatic islets, GH stimulated cell growth partially through IGF-I release, it is unclear under normal conditions how IGF-I is involved in GH-stimulated islet growth and insulin biosynthesis and secretion (34, 35).

Precise colocalization by immunohistochemistry had indicated that IGF-I is normally produced in the α- and δ-cells of pancreatic islets, which perhaps act on the β-cells in a paracrine manner, whereas IGF-II is coproduced in β-cells with insulin (36, 37). Northern blot analysis showed that IGF-II is the major IGF expressed in the fetal and neonatal rat pancreas, the expression of which is replaced by IGF-I by the second postnatal week. Isolated rat islet α- and β-cells as well as islet-derived cell lines expressed high-affinity IGF-I receptors (IGF-IRs) (3). Although under intense study, the role of IGF-I in normal islet cell growth is still unclear. Transgenic IGF-II promoted islet growth, whereas IGF-I acted solely during regeneration after islet cell damage (10, 22, 38). At the cellular level, IGF-I induced proliferation of rat insulinoma-1 (INS-1) cells in a glucose-dependent manner via insulin receptor substrate-induced phosphatidylinositol 3-kinase ac-

tivity and downstream activation of p70^{S6K} (5). On the other hand, total deficiency in IGF-I or IGF-IR genes as well as islet β-cell-specific inactivation of IGF-IR gene caused no change in β-cell mass, suggesting that IGF signaling is not essential for normal growth and development of pancreatic islets (39, 40). In our recent studies, liver-specific IGF-I gene-deficient mice exhibited islet hyperplasia and hyperinsulinemia caused by compensatory GH hypersecretion (41–43). Furthermore, pancreatic-specific IGF-I gene-deficient mice exhibited increased islet cell mass probably because of indirect compensations (44). Of course, there might be possible defects in these studies such as gene redundancy, promoter limitation, indirect effects, and limited sample numbers that need to be addressed in future studies.

Nevertheless, this study does not exclude a direct action of GH on pancreatic islets because, first, the rescue was incomplete and limited to increased islet cell growth that restored islet cell mass and glucose tolerance. Restoring local production of IGF-I in pancreatic islets of GHR$^{-/-}$ mice failed to rescue other defects including serum insulin and glucagon levels, hypoglycemia, and insulin sensitivity. Although endocrine IGF-I is known to increase insulin sensitivity, locally produced IGF-I within the islet cells might be insufficient to cause improved insulin responsiveness in target tissues such as the skeletal muscles (thus unaltered insulin tolerance). Second, the level of IGF-I transgenic overexpression was only moderate compared with similar reports often showing 50-fold increase over endogenous levels (22). The choice of insulin promoter (which is presumably severely inhibited in GHR$^{-/-}$ mice) might be a restriction. Third, islet β-cells seem to have an intrinsic network capable of responding to GH directly. Activation of Stat5 was sufficient to drive transcriptional induction of cyclin D2 gene and proliferation of rat pancreatic β-cells. Cell-cycle regulatory factor cyclin D2 acts as a growth factor sensor for cell transition from G1 to S phase (45). Fourth, it has been reported that the stimulatory effect of GH on β-cell proliferation cannot be prevented by IGF-I antiserum and was additive to the IGF-I effect (46, 47). On the other hand, GH antagonizes insulin actions and GHR gene deficiency causes significantly elevated insulin sensitivity and hypoglycemia, which might contribute to an adaptive hypotrophy of islet cells, independent of direct actions of either GH or IGF-I.

The level of IGF-I in GHR$^{-/-}$ mice can also be compensated via short-term administration, which has almost completely rescued all defects on both bone growth and remodeling (premature reduction in chondrocyte proliferation and cortical bone growth as well as reduced trabecular bone turnover), supporting a direct effect of IGF-I on both osteoblasts and chondrocytes (32). Its effect on islet cell growth has not been studied. There has been a previous report on islet-specific IGF-I gene overexpression. As in our study, those transgenic mice exhibited similar islet cell mass, normal insulinemia and glycemia, and similar levels of insulin mRNA to wild-type control mice (22). The IGF-I overexpression was without effect until the mice were challenged with type 1 diabetes.

In summary, we have created a transgenic line in which the IGF-I gene was overexpressed in pancreatic islet β-cells and crossed the mice on to a GHR$^{-/-}$ background. As previously reported, islet-specific IGF-I overexpression alone caused no obvious change in islet cell growth and insulin production. Compared with GHR$^{-/-}$ mice, IGF-I overexpression on a GHR$^{-/-}$ background increased IGF-I production in the islet cells and caused no change in the diminished blood glucose and serum insulin levels and pancreatic insulin contents but improved glucose tolerance and insulin secretion. More remarkably, islet-specific overexpression of IGF-I gene in GHR$^{-/-}$ mice restored islet cell mass through cell hyperplasia and/or hypertrophy. Our results seem to suggest that IGF-I deficiency is part of the underlying mechanism of diminished islet cell growth in GHR$^{-/-}$ mice, consistent with the notion that IGF-I mediates the islet cell growth effect caused by GH release.

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Growth hormone receptor gene deficiency causes delayed insulin responsiveness in skeletal muscles without affecting compensatory islet cell overgrowth in obese mice

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body to the p85 subunit of phosphatidylinositol 3-kinase (PI3K; αp85 antibody) were purchased from Upstate Biotechnology (Lake Placid, NY). The insulin antibody (H-86) used in immunohistochemistry was obtained from Santa Cruz Biotechnology.

Animal procedures. GHR$^{-/-}$ mice carry a targeted disruption of exon 4 of the mouse GHR/binding protein (GHR/BP) gene, as previously reported (46). Offspring (wild types as controls and GHR$^{-/-}$) derived from heterozygous (GHR$^{+/-}$) mating pairs on a hybrid 129/Ola-BALB/c-C57BL/6 background were used in experiments. To determine genotype, we isolated genomic DNA from tail clips with standard methods. Primers In4-1 (5′-CCC TGA GAC CTC CTC AGT TC), In3-1 (5′-CCT CCC AGA GAG ACT GGC TT), and Neo-3 (5′-GCT CGA CAT TGG GTG GAA ACA T) were used in PCR reactions, which yield a 390-base band for the wild-type allele and 290/200-base double bands for the knockout allele, as previously reported (5). The animals were maintained in 12:12-h dark-light cycles at room temperature with free access to food and water or, when indicated, were food deprived for 24 h with free access to water. For the study of insulin response, GHR$^{-/-}$ mice and their wild-type littermates (6 mo of age) were fasted for 24 h and anesthetized by intraperitoneal administration of a cocktail of ketamine-xylazine-acepromazine. The mice were injected with insulin (10 IU/kg ip) or saline; after 5 or 15 min, they were killed and their soleus and gastrocnemius muscles were removed along with the liver to prepare cell lysate. All animal-handling procedures were approved by the McGill University Animal Care Committee. Serum concentrations of insulin (Linco Research) were determined using a radioimmunoassay kit.

Immunoprecipitation and Western blots. The tissues were homogenized in 5 ml of protein extraction buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Nonidet P-40, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, and protease inhibitors (Complete Mini EDTA-free; Roche, Indianapolis, IN) at 4°C (43). The samples were incubated on ice for 30 min and centrifuged at 600 rpm for 20 min at 4°C, and then the supernatant was removed and centrifuged at 13,000 rpm for 45 min at 4°C in a Beckman JA20–1 rotor. The Bradford assay (Bio-Rad) was used to measure protein concentration, and the sample was aliquoted and stored at −80°C.

Tissue lysates containing 2 mg of total protein were immunoprecipitated with the antibodies αIR or αIRS-1 (2 μg/ml final concentration) overnight at 4°C. The next day, 40 μl of protein G-Sepharose (Roche) were added, and samples were incubated for another 1.5 h on a rocking platform at 4°C and centrifuged at 14,000 rpm for 2 min at 4°C. The precipitate was washed three times with the protein extraction buffer. The final precipitate was boiled for 5 min in 40 μl of Laemmli sample buffer (Bio-Rad).

Samples were loaded onto a 7% SDS-polyacrylamide gel with the use of a Mini Protean apparatus (Bio-Rad). Transfer of proteins from the gel to 0.2-μm nitrocellulose (Trans-Blot transfer medium; Bio-Rad) was performed for 1.5 h at 100 V by using the Bio-Rad mini transfer apparatus in a transfer buffer consisting of 25 mM Tris, 192 mM glycine, and 20% methanol. The membranes were blocked for 1 h at room temperature to reduce nonspecific binding in a TBS-T buffer (composed of 10 mM Tris·HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.6) containing 3% BSA for phosphotyrosine detection or 2% ECL Advance blocking agent (Amersham Biosciences, Amersham, UK) for protein detection. The membranes were incubated overnight at 4°C with αPY, αIR, or αIRS-1 (all at 1:1,000) each and diluted in corresponding blocking buffer. The membranes were washed, incubated with secondary antibodies, and detected by chemiluminescence using the ECL Advance Western blotting detection kit (Amersham). To detect the amount of p85 associated with IRS-1, the blots that were used to detect αIRS-1 were stripped with Re-Blot Plus (Chemicon International), washed in TBS-T for 5 min, blocked for 30 min, and then probed with αp85 antibody (1:1,000) and detected using chemiluminescence. Images were captured using a Fluorchem 8900 imager (Alpha Innotech, San Leandro, CA), and densitometry was carried out using AlphaEase software (Alpha Innotech).

High-fat diet-induced obesity. Male GHR$^{-/-}$ mice and their male wild-type littermates (3.5 mo of age) were fed for 17 wk with a high-fat diet (HFD; Research Diets, New Brunswick, NJ). Their body weight was measured once a week, and blood glucose levels every 3 wk, using a OneTouch blood glucose meter (Lifescan, Burnaby, BC, Canada). At 15 wk, an insulin tolerance test was performed. Animals were injected with recombinant human insulin (0.75 IU/kg ip; Roche), and blood glucose levels were measured at 0, 20, 40, and 60 min afterward. At 17 wk, the mice were anesthetized by intraperitoneal administration of a cocktail of ketamine-xylazine-acepromazine, and then body length and weight were measured and fat pads were removed and weighed. The mice were killed by cervical dislocation, and then blood was collected for serum preparation and pancreata were rapidly removed for histochemical analysis.

Immunohistochemistry and islet cell mass measurement. Pancreatic sections were stained with an insulin antibody (H-86; Santa Cruz Biotechnology) using diaminobenzidine substrate, which resulted in a brown immunoreactive signal with a hematoxylin counterstain (blue) of cell nuclei. The β-cell mass and average cell size were determined as previously reported (2, 26).

Statistical analysis. Data are expressed as means ± SE. Student's *t*-test (unpaired and paired) was performed using InStat software version 3 (GraphPad Software, San Diego, CA).

RESULTS

A general characterization of GHR$^{-/-}$ mice was reported by our group recently (16, 26). Because of a lack of GH signaling, the body weight of adult GHR$^{-/-}$ mice is only one-half that of their wild-type littermates, confirming severe growth retardation; blood glucose and serum insulin levels are significantly reduced to 80 and 40% of the normal values, respectively; serum level of the total IGF-I is reduced to one-half because of a lack of GH stimulation; and GHR$^{-/-}$ mice exhibit significantly increased sensitivity to insulin tolerance tests (16, 26). Most of these characteristics have also been reported by other groups (7, 11, 12, 46) and were unchanged in the animals used in this study (data not shown).

Delayed and diminished insulin receptor phosphorylation in skeletal muscle. GHR$^{-/-}$ mice exhibit elevated insulin responsiveness, including increased IR levels and activation in the hepatocytes (11, 46). To study whether this phenomenon is tissue specific, we have tested insulin-stimulated early responses in the skeletal muscles and liver. GHR$^{-/-}$ mice and control littermates were injected with insulin (10 IU/kg ip) or saline, and muscle or liver homogenates were subjected to immunoprecipitation with the use of an antibody against the IR β-subunit (αIR), followed by immunoblotting with the same antibody and that against the tyrosine phosphorylation (αPY). After densitometry analysis was completed, the amount of tyrosine phosphorylation of the IR β-subunit was normalized using the total receptor levels. As shown in Fig. 1, in skeletal muscles of wild-type mice, 5 min of insulin treatment caused a significant 3.5-fold increase in IR phosphorylation (*A* and *B*, *left*) over that of untreated animals. However, upon insulin stimulation of GHR$^{-/-}$ mice, IR phosphorylation was only marginally elevated 1.3-fold [not significant (NS)], significantly lower than in wild-type mice. In contrast, in the liver (Fig. 1, *A* and *B*, *right*), insulin treatment caused significantly increased phosphorylation in both groups of mice, e.g., 7.5- and 6.8-fold in wild-type and GHR$^{-/-}$ mice, respectively, compared with untreated controls. Later, at 15 min, wild-type

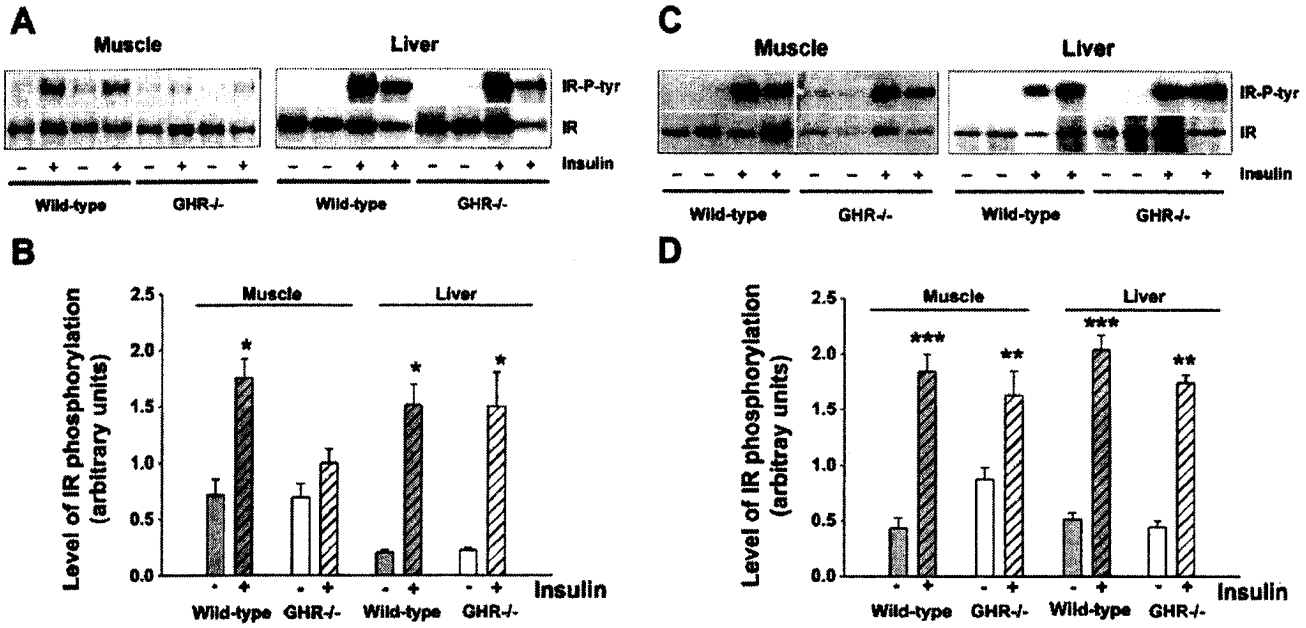

Fig. 1. Skeletal muscles exhibit delayed and/or diminished responses in insulin-stimulated insulin receptor (IR) phosphorylation in growth hormone receptor-deficient (GHR$^{-/-}$) mice. Mice fasted for 24 h were injected with insulin (10 IU/kg ip) for 5 (*A* and *B*) or 15 min (*C* and *D*) before being killed, and their muscles or livers were removed to prepare cell lysates. *A* and *C*: lysates were precipitated with IR antibody and probed with the same antibody as well as with anti-phosphotyrosine (IR-P-tyr) in Western blots. A representative blot is illustrated from experiments of $n = 8$ for skeletal muscles and $n = 3$ for liver. *B* and *D*: densitometric quantification of the IR phosphorylation levels corrected by total IR protein level. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. untreated controls.

mice maintained a significant elevation in IR phosphorylation (4.3-fold; Fig. 1, *C* and *D*, *left*), which was only marginally elevated 1.9-fold in GHR$^{-/-}$ mice ($P < 0.05$), lower than in wild-type mice mostly because of an elevated basal activity. As a control, in the liver (Fig. 1, *C* and *D*, *right*), insulin treatment caused fourfold significantly increased phosphorylation in both the wild-type and GHR$^{-/-}$ mice compared with untreated controls. The IR responses in the liver of GHR$^{-/-}$ mice, almost identical to that of wild-type mice, were slightly lower than reported previously (11).

Delayed IRS-1 phosphorylation in skeletal muscle. The same sets of muscle/liver proteins were used to determine the level of IRS-1 phosphorylation. The muscle or liver homogenates were subjected to immunoprecipitation with an antibody against IRS-1 (αIRS-1), followed by immunoblotting with the same antibody and another against the tyrosine phosphorylation (αPY). The data were normalized with the amount of total IRS-1 levels (Fig. 2). In skeletal muscles of wild-type mice, 5 min of insulin treatment caused a significant increase in IRS-1 phosphorylation (2.1-fold) that was virtually abolished in the GHR$^{-/-}$ mice (Fig. 2, *A* and *B*, *left*). As a positive control for insulin action in Fig. 2, *A* and *B*, *right*, IRS-1 phosphorylation in liver samples displayed a 1.5-fold increase in both wild-type and GHR$^{-/-}$ mice compared with untreated controls. Later, at 15 min, the muscles of both wild-type and GHR$^{-/-}$ mice showed comparable and significant elevations of IRS-1 phosphorylation of 2.0- and 1.6-fold, respectively (Fig. 2, *C* and *D*, *left*). The liver samples (Fig. 2, *C* and *D*, *right*) exhibited an increase in IRS-1 phosphorylation of 1.7- or 3.5-fold in wild-type or GHR$^{-/-}$ mice, respectively. The IRS-1 responses in the liver of GHR$^{-/-}$ mice were slightly higher than reported previously (11). The results of IR and IRS-1 phosphorylation in the skeletal muscles are in contrast to that reported previously and to what we have demonstrated in the hepatocytes, which exhibited increased IR protein level, normal or slightly increased (rather than delayed or decreased) receptor activation, and IRS-1 phosphorylation in GHR$^{-/-}$ mice (11).

Normal p85 stimulation in skeletal muscle. PI3K is activated after insulin stimulation and IRS-1 phosphorylation. In this study, the PI3K activation was measured as the amount of p85 protein associated with IRS-1 in an immunoprecipitate with the use of anti-IRS-1. With the same muscle or liver homogenates that were immunoprecipitated with αIRS-1 (Fig. 2, *C* and *D*), the proteins were immunoblotted with an antibody against p85 (αp85). The values were normalized by the total IRS-1 levels (Fig. 3*B*). In the muscle, p85 levels were increased similarly (2.6- and 2.2-fold) in both wild-type and GHR$^{-/-}$ mice when stimulated by insulin; i.e., the signaling pathway leading to the activation of the PI3K by insulin was unaltered in the skeletal muscle of GHR$^{-/-}$ mice. As positive controls, increased p85 association with IRS-1 was demonstrated in the livers of both wild-type and GHR$^{-/-}$ mice, as reported previously (11).

HFD-induced obesity in GHR$^{-/-}$ mice. To study whether GH signaling is involved in the compensatory growth of pancreatic islets in response to obesity-induced insulin resistance, we challenged male GHR$^{-/-}$ mice and their wild-type littermates with a HFD for 17 wk. Their body weights were measured once a week (Fig. 4*A*) and their blood glucose levels every 3 wk (data not shown). Both wild-type and GHR$^{-/-}$ mice gained significant weight with the HFD, 21 and 31% of their initial body weights, respectively (Fig. 4*B*). To further demonstrate increased fat mass and reveal potential depot-specific effect, various fat pads were excised, weighed, and corrected for total body weight. As shown in Fig. 4*C*, both the

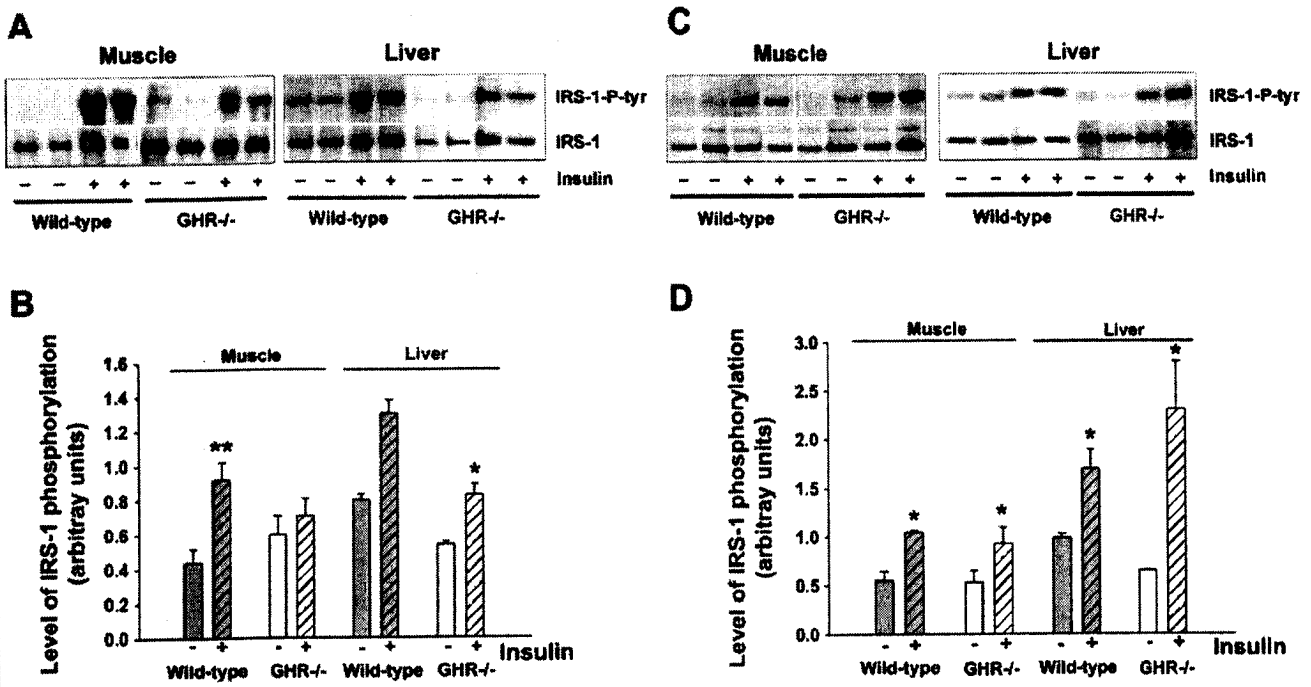

Fig. 2. Skeletal muscles exhibit a delayed response in insulin-stimulated insulin receptor substrate-1 (IRS-1) phosphorylation in GHR$^{-/-}$ mice. Mice fasted for 24 h were injected with insulin (10 IU/kg ip) for 5 (*A* and *B*) or 15 min (*C* and *D*) before being killed, and their muscles or livers were removed to prepare cell lysate. *A* and *C*: lysates were precipitated with IRS-1 antibody and probed with the same antibody as well as with anti-phosphotyrosine (IRS-1-P-tyr) in Western blots. A representative blot is illustrated from experiments of $n = 8$ at 5 min and $n = 5$ at 15 min for skeletal muscles and $n = 3$ for liver. *B* and *D*: densitometric quantification of the IRS-1 phosphorylation levels corrected by total IRS-1 protein level. $*P < 0.05$; $**P < 0.01$ vs. untreated controls.

GHR$^{-/-}$ mice and their wild-type littermates significantly increased the weight of each fat pad (except the renal in the GHR$^{-/-}$), indicating that GHR$^{-/-}$ mice are not resistant to HFD-induced obesity. Normally, GHR$^{-/-}$ mice are hypersensitive to insulin, in contrast to human Laron patients. To determine whether the mice have decreased insulin responsiveness as a result of obesity, we performed an insulin tolerance test on all animals, on either HFD or normal diet, 2 wk before the end of the study. In both wild-type and GHR$^{-/-}$ mice, a 15-wk HFD failed to cause a significant change in insulin sensitivity due to obesity. Serum insulin levels, another indirect indicator of insulin resistance, were unaffected as well (data not shown). Thus HFD for 17 wk created obesity, but not insulin resistance, in both wild-type and GHR$^{-/-}$ mice.

Evaluation of islet cell overgrowth due to obesity. To study whether the HFD-induced obesity can cause a compensatory islet overgrowth in the GHR$^{-/-}$ mice, we measured β-cell mass at 17 wk from pancreatic sections stained with insulin. As shown in Fig. 4*D*, consistent with our previous report (26), GHR$^{-/-}$ mice on the normal diet had only 45% of the β-cell mass compared with wild-type littermates; after the HFD, however, both types of mice displayed similar extents of islet compensation, e.g., 2.8-fold in wild-type and 3.3-fold in GHR$^{-/-}$ mice, respectively. Representative islets are illustrated in Fig. 4*E*. In both wild-type and GHR$^{-/-}$ mice, HFD caused a significant enlargement of the islet size. The islet compensation was likely caused by cell hyperplasia, because the average cell size (representing hypertrophy) and islet density per tissue area (representing islet neogenesis) were unaffected (data not shown). Thus obese GHR$^{-/-}$ mice exhibited a normal, compensatory overgrowth of islet cells.

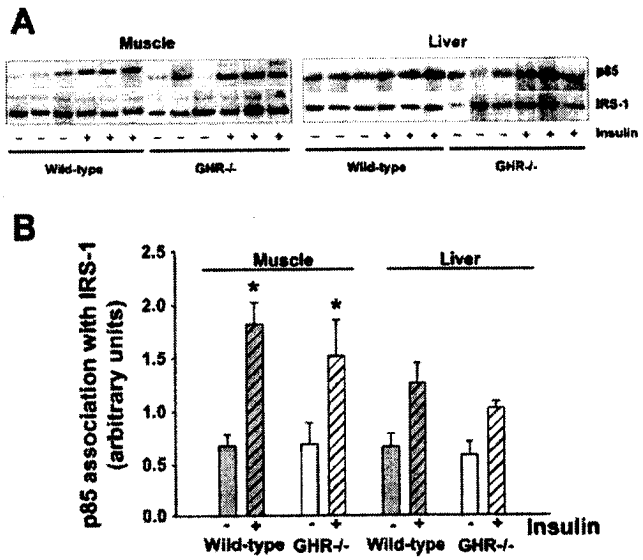

Fig. 3. Insulin-stimulated p85 association with IRS-1 in skeletal muscles of GHR$^{-/-}$ mice and their wild-type littermates. Mice fasted for 24 h were injected with insulin (10 IU/kg ip) for 15 min before being killed, and their muscles or livers were removed to prepare cell lysate. *A*: lysates (same as in Fig. 2*C*) were precipitated with IRS-1 antibody and probed with the same antibody, stripped, and then probed with p85 antibody in Western blots. A representative blot is illustrated from experiments of $n = 5$ for skeletal muscles and $n = 3$ for liver. *B*: densitometric quantification of the p85 levels corrected by total IRS-1 protein level. $*P < 0.05$ vs. untreated controls.

DISCUSSION

Using GHR$^{-/-}$ mice, our group recently demonstrated that GH signaling is essential for maintaining pancreatic islet growth, stimulating islet hormone production, and maintaining normal insulin sensitivity and glucose homeostasis (26). Through islet-specific overexpression of IGF-I, we were able to rescue some of the islet defects, suggesting that IGF-I mediates some GH actions on islet growth (16). In the current study, we have further characterized GHR$^{-/-}$ mice in insulin responsiveness and in islet cell growth. Specifically, we have shown that GH signaling does not play a dominant role in either insulin responsiveness in the skeletal muscles or in islet growth compensation due to obesity. Antagonizing insulin's actions, GH decreases glucose uptake, maintains hepatic glucose production, decreases responsiveness of target tissues to insulin, and diminishes the conversion of glucose to fat. Conversely, GHR$^{-/-}$ mice are clearly hypersensitive to insulin's actions (11, 26). As for specific target tissues affected, it has been reported (11) that in the hepatocytes of GHR$^{-/-}$ mice, both the basal levels of IR protein and the response in IR phosphorylation are elevated. As another important target tissue of insulin action, the role of skeletal muscles has not been evaluated in GHR$^{-/-}$ mice. In a similar system, GH-deficient Ames dwarfs, also hypersensitive to insulin, exhibit reduced

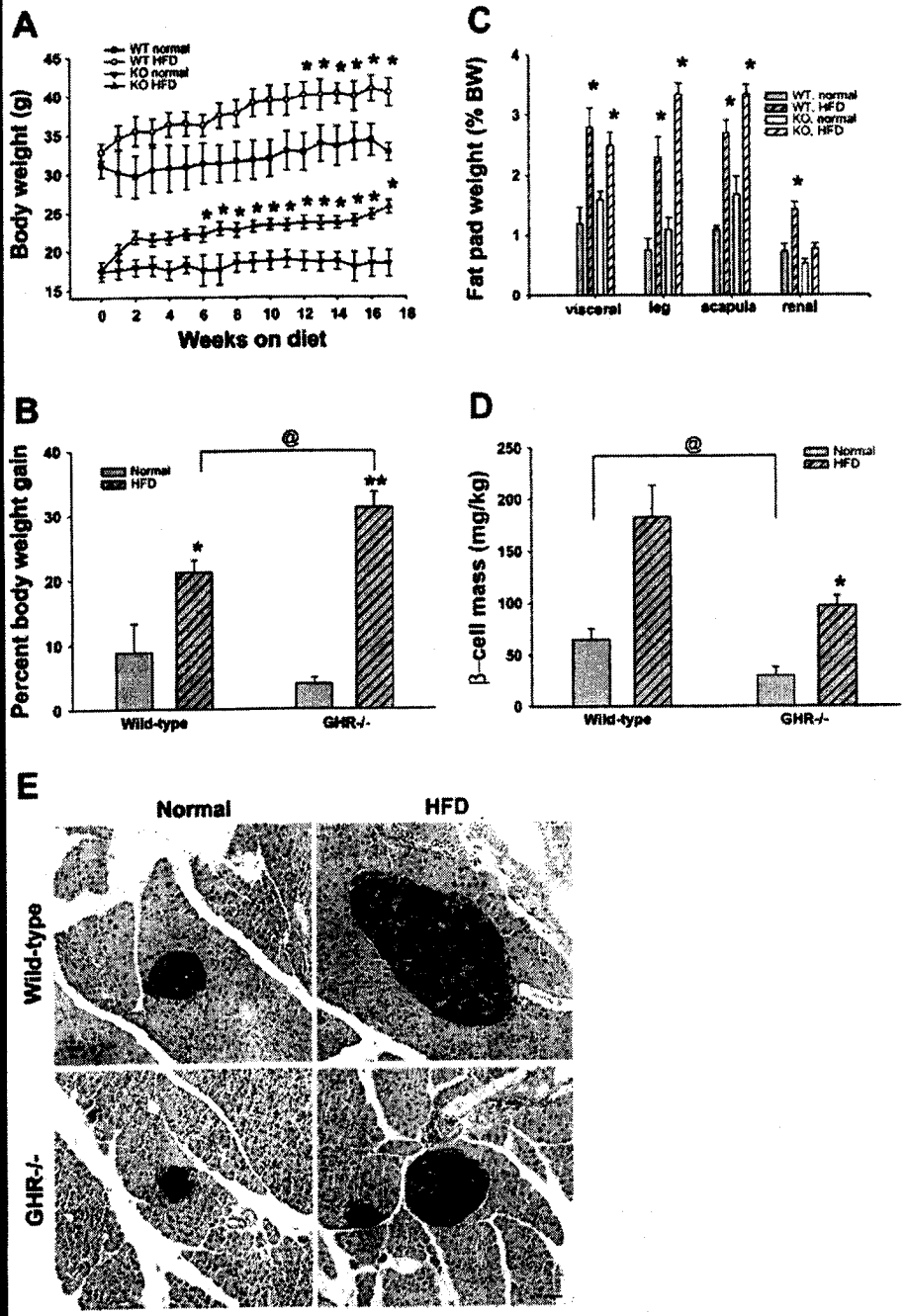

Fig. 4. High-fat diet (HFD)-induced obesity and pancreatic islet overgrowth in GHR$^{-/-}$ mice. *A*: HFD induced steady increases in body weight in both wild-type and GHR$^{-/-}$ mice. Male GHR$^{-/-}$ and their wild-type littermates (3.5 mo) were fed normal or HFD for 17 wk. Their body weight was measured once a week and plotted against the number of weeks on the diet; $n = 4$–5. KO, knockout mice. $^{*}P < 0.05$ vs. normal diet. *B*: percent body weight gain (per initial weight) after 17 wk on HFD or normal diet. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. normal diet; $^{@}P < 0.05$ vs. wild-type on HFD. *C*: HFD caused increases in fat pad weights (per total body weight) in wild-type and GHR$^{-/-}$ mice. After 17 wk on HFD, 4 fat pads were excised and measured. BW, body weight. $^{*}P < 0.05$ vs. normal diet. *D*: changes in β-cell mass after HFD per total body weight. There was a 2.8-fold increase in wild-type mice after HFD. GHR$^{-/-}$ mice normally exhibit diminished β-cell mass (45% of wild-type level) but displayed a 3.3-fold increase after HFD. $^{*}P < 0.05$ vs. normal diet; $^{@}P < 0.05$ vs. wild-type on normal diet. *E*: changes in pancreatic islet morphology after HFD. Pancreatic sections were stained for insulin (brown) and counterstained for cell nuclei (blue). In both wild-type and GHR$^{-/-}$ mice, HFD caused a significant enlargement in the islet size. Representative results for 2 experiments are shown. Scale bar, 100 μm.

insulin-stimulated phosphorylation of IR and IRS-1 in the skeletal muscles (10). Our results are thus consistent with those of Ames mice, perhaps because of a common deficiency in endogenous IGF-I production.

As a primary insulin target, skeletal muscles express high levels of receptors for insulin, GH, and IGF-I (14). Insulin causes IR autophosphorylation, which recruits IRS-1 and other SH2-containing docking molecules. Among other signaling pathways, insulin stimulation causes dissociation of the p85 subunit from PI3K and, thereby, activation of the p110 subunit, which is a major mediator of insulin's actions (39, 40). These early responses cause activation of downstream molecules such as Akt/PKB, recruitment of the glucose transporter GLUT4 into the plasma membrane, and increased glucose uptake into the muscle cells. The current study was designed to reveal possible changes in early insulin responses in the skeletal muscle and to compare them with those of hepatocytes in GHR$^{-/-}$ mice. In contrast to the elevated or normal insulin responsiveness exhibited in the liver of GHR$^{-/-}$ mice (11), our results indicate that upon insulin stimulation, there is no elevated insulin response: i.e., skeletal muscles do not contribute to increased insulin sensitivity in GHR$^{-/-}$ mice. If anything, there were transiently delayed and/or diminished responses in the early phase of IR or IRS-1 phosphorylation. One of the underlying differences is that hepatocytes do not express IGF-I receptor (29) and are unaffected by secondary deficiency in IGF-I production in GHR$^{-/-}$ mice. It seems that IGF-I plays a greater role in skeletal muscles in maintaining insulin responsiveness: its deficiency causes diminished insulin response. IGF-I does not bind to hepatocytes or adipocytes, and therefore its primary insulin-like action is believed to be mediated through the skeletal muscles (6). Indeed, in newborn IR-deficient mice, IGF-I directly activates PI3K and, presumably, glucose uptake in the muscles, because it corrects the hyperglycemia (8). It has been established that in the skeletal muscles, GLUT4 translocation to the cell membrane is stimulated by both insulin and IGF-I through their cognate receptors, a crucial process in postprandial glucose disposal. The importance of this mechanism is clearly demonstrated by muscle-specific ablation of the GLUT4 gene, which causes severe insulin resistance and glucose intolerance (47). Moreover, muscle-specific inactivation of both IR and IGF-IR in MKR mice, by overexpressing a dominant negative protein, creates an even more severe phenotype by causing an early onset of diabetes (13). To understand the relative contributions of either IR or IGF-IR to this defect, note that muscle-specific inactivation of IR gene alone (using Cre/loxP system) is insufficient to cause significant insulin resistance or glucose intolerance (4), indicating that IGF-IR, or IGF-IR in conjunction with IR, plays a potent role in stimulating glucose uptake in the skeletal muscles. Of course, for more conclusive proof one would have to create a specific ablation of IGF-IR gene alone in the muscle cells. In the meantime, our results indicate that GH signaling is not dominant in counteracting insulin's actions in the muscles and that the major site at which GH antagonizes insulin's actions is the liver (6). This supports tissue-specific influences of GH on in vivo insulin responsiveness.

There is no doubt that GH has an insulin-counterregulatory role in skeletal muscle, and our results are, in general, consistent with other related models of GH deficiency or excess. Transgenic mice overexpressing GH antagonist (GHa) exhibit elevated insulin sensitivity and decreased blood glucose and serum insulin levels, similar to GHR$^{-/-}$ and Ames dwarfs, although their growth retardation is much milder (44). They maintain a normal rate of glucose uptake in skeletal muscles and brown adipose tissues and exhibit normal (rather than elevated) insulin responses in IR, IRS-1, and Akt in the skeletal muscles, similar to our GHR$^{-/-}$ mice (44). The enhanced insulin sensitivity in GHa mice seems to be caused by significantly improved glucose uptake in white adipose tissues, resembling that occurring in the liver of GHR$^{-/-}$ mice. In this study, slightly differently from that in GHa mice, the delayed insulin responsiveness in the skeletal muscles was transient and limited to IR and IRS-1 only. By 15 min after insulin stimulation, all parameters including PI3K were normalized. Whether this phenomenon has any physiological implications needs to be addressed in future studies. For instance, as a primary organ for glucose disposal, insulin-stimulated glucose uptake could be affected, albeit transiently. Even then, an accumulated effect may consequently contribute to the phenotypes such as longevity of these mice (10). This study and the reports of insulin responsiveness in the liver of GHR$^{-/-}$ mice and in the muscles of Ames dwarfs suggest that normal levels of GH signals do not antagonize insulin responsiveness in the skeletal muscles (10, 11). This is not necessarily contradictory to the finding that GH antagonist corrects the insulin resistance in LID mice, because the latter finding only implies that excessive GH secretion causes insulin resistance in skeletal muscles (44).

In addition to effects on insulin action, GH is an important growth factor for islet cells (42, 45). Downstream of the GHR/Jak2 interaction, signal transducer and activator of transcription 5 activation and consequent induction of cyclin D2 are essential for the mitogenic effect of GH on β-cells (15, 31). In nonislet cells, GH increases the activity or protein level of Foxa-2 and hepatocyte nuclear factor-1α, key molecules in β-cell growth (30, 41). These factors likely mediate GH-stimulated islet cell growth. However, it is unclear whether GH signals are involved in compensatory overgrowth of islet cells such as in pregnancy or obesity. In this aspect, GH secretion is markedly diminished due to obesity (35), which may contribute to the eventual failure of β-cells. Thus we were interested in studying whether GHR$^{-/-}$ mice would be able to increase β-cell growth in response to obesity. On the other hand, GHR$^{-/-}$ mice are not grossly obese, unlike human Laron syndrome (26). Another question was whether GHR$^{-/-}$ mice would be resistant to obesity itself. For these purposes, we have successfully induced obesity in two sets of mice at 3–4 and 9–10 mo of age. Under the experimental conditions, the obesity was not severe enough to cause significant changes in insulin tolerance, let alone diabetes. Nevertheless, our results clearly demonstrate that GHR$^{-/-}$ mice respond more efficiently to the HFD in becoming obese and exhibit a significant increase in islet cell growth, slightly higher than wild-type mice. Thus GH signals are not essential for the compensatory growth of islet cells in response to obesity. The causes of enlarged β-cell mass in nondiabetic obese humans and rodents include increased islet cell replication, neogenesis, and cellular hypertrophy (9, 18, 27). Currently, there are many other factors for islet cell growth that are potentially involved in islet compensation. They include cyclins D1 and D2 (23), nutrients such as glucose, hormones such as a combination of epidermal

growth factor and gastrin (37), glucagon-like peptide-1, and several growth factors including fibroblast growth factor and hepatocyte growth factor (3, 22). As we wrote this report, we were unaware of any positive involvement of these factors in obesity-induced islet compensation.

In summary, in response to in vivo insulin stimulation, the skeletal muscles of GHR$^{-/-}$ mice exhibit transient delayed and/or diminished responses in IR and IRS-1 phosphorylation. This finding is in contrast to elevated or normal insulin responses in hepatocytes, perhaps because of a concurrent decrease in IGF-I effect. When challenged with a HFD, GHR$^{-/-}$ mice became more significantly obese, in contrast to the human Laron syndrome of GH insensitivity, which displays default obesity over the normal population. As a consequence of obesity, GHR$^{-/-}$ mice displayed an enhanced β-cell compensation, slightly greater than that of wild-type mice, demonstrating that GH signals are not required for compensatory islet growth. Thus, in both muscle insulin responsiveness and islet compensation, GH does not seem to play a dominant role.

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