

**Regulation of Human Pituitary Growth Hormone Gene (*hGH1*)  
Expression by Energy Homeostasis**

By

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***In the name of God, the Lord of Mercy, the Giver of Mercy***

*“Read in the name of your Lord who created.*

*Read, your Lord is the most bountiful one;*

*who taught by pen;*

*who taught man what he did not know.”*

*Quran - 96 Surat Al-`Alaq*

## ABSTRACT

**Background:** Activation of the human (h) pituitary growth hormone (GH) gene (*hGHI*) during embryonic development is facilitated by histone H3 and H4 hyperacetylation of a well-characterized remote locus control region (LCR). The LCR possesses enhancer activity and appears to exert its effect on the *hGHI* proximal promoter region via a long-range physical interaction involving “looping” of intervening sequences. The possibility that physiological cues, including energy homeostasis, regulate the postnatal expression of the *hGHI* by affecting physical intrachromosomal interactions essential for embryonic activation of the locus is explored in this thesis.

**Methodology/Principal Findings:** Excess caloric intake in the form of high fat diet and physical activity were used as physiological cues for two independent transgenic (hGH/CS) mouse lines containing the *hGHI* and expressing it preferentially in the pituitary. The chromosomal conformation capture (3C) assay was used to analyze the *hGHI* locus and long-range interactions between remote enhancer and proximal promoter regions through looping of the intervening 14 kilobases. This chromatin structure is associated with efficient *hGHI* promoter activity *in vivo* and hence is referred to here as an “expression” loop. Three days of a high fat diet created a state of excess insulin but not hyperglycemia or weight gain. A decrease in hGH1 production was observed. It was further demonstrated that *hGHI* expression is regulated directly by insulin at the level of the somatotroph; a significant and dose-dependent decrease in hGH transcript levels was detected in primary pituitary cell cultures from two independent hGH/CS transgenic

mouse lines with insulin treatment. This insulin regulation of the *hGHI* was mediated through an enhancer box DNA element located in the proximal promoter region. Furthermore, the effect on *hGHI* expression was associated with disruption of the hGH1 LCR/promoter “expression” loop and reduced histone H3/H4 hyperacetylation. Increased and decreased recruitment of nuclear receptor co-repressor (NCOR) and RNA polymerase II, respectively, were also observed. Collectively, these effects are consistent with reduced *hGHI* promoter function. Inclusion of physical activity, in the form of swimming, significantly muted the effect of excess caloric intake on the hyperinsulinemic state. There was also a corresponding blunting of the decrease in *hGHI* promoter hyperacetylation and *hGHI* expression seen in the absence of prescribed physical activity. Furthermore, these changes with physical activity were consistent with preservation of the “expression” loop.

**Conclusions/Significance:** The rapid down regulation of *hGHI* expression in response to acute excess caloric intake, offers a novel way (effect on synthesis) to explain the early and rapid suppression of serum hGH levels seen in healthy individuals who overate for three days. Observations presented in this thesis also provide the first evidence that energy homeostasis (energy intake and expenditure) targets hGH1 synthesis through dynamic changes in the three-dimensional chromatin structure of the *hGHI* locus. This is consistent with a fluid structure for cell type-specific chromosomal architecture and a capacity for reconfiguration as a component of postnatal gene regulation.

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Components of three manuscripts published in peer-reviewed scientific journals (as listed below) are presented in this dissertation. Hana Vakili-Tajareh performed all the experimental works except for contributions made by Ms. Yan Jin and these are identified in the respective figure legends as appropriate. In addition, Figure 1.6 presented in Chapter 1 was generated in collaboration with Dr. James I. Nagy (a microscopy specialist) and technical expertise of Mr. Brett McLean from the Department of Physiology & Pathophysiology, University of Manitoba.

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## ABBREVIATIONS

°	degree
3C	chromosome conformation capture
ACTH	adrenocorticotrophic hormone
ANOVA	analysis of variance
ARN	arcuate nucleus
ARNT	aryl hydrocarbon receptor nuclear translocator protein
B2M	β-2 microglobulin
bHLH	basic helix-loop-helix
Bmal1	brain and muscle ARNT-like 1
bp	base pairs
BSA	bovine serum albumin
C	centigrade
cAMP	cyclic adenosine monophosphate
CD79b	B-cell antigen receptor complex-associated protein β-chain
cDNA	complementary DNA
CE	cytosolic extract
cGMP	cyclic guanosine monophosphate
ChIP	chromatin immunoprecipitation
Clock	circadian locomotor output cycles kaput
cm	centimeters



CoCl <sub>2</sub>	cobalt (II) chloride hexahydrate
CRISPR	cas-based RNA-guided DNA endonucleases
CS	chorionic somatomammotropin
dcsFBS	double charcoal stripped FBS
dIdC	deoxyinosinic-deoxycytidylic
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E-box	enhancer box
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
EPO	erythropoietin
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GH	growth hormone
GH-R	GH receptor
GH-V	placental GH variant
GHBP	GH binding protein
GHD	growth hormone deficiency
GHRH	growth hormone releasing hormone

GHRH-R	GHRH receptor
GHS	growth hormone secretagogue
GHS-R	growth hormone secretagogue receptor
GM-CSF	granulocyte–macrophage-colony stimulating factor
h	human
HAc	hyperacetylated
HBS	hypoxia inducible factor binding site
HDAC	histone deacetylase
HEK293	human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	high fat diet
hGH1	human pituitary growth hormone protein
<i>hGH1</i>	human pituitary growth hormone gene (when in italics)
HIF-1 $\alpha$	hypoxia-inducible factor 1- alpha
HS	hypersensitive sites
IBlot	immune-blotting
IGF	insulin-like growth factor
IgG	immunoglobulin G
IGHD	isolated growth hormone deficiency
JAK2	janus kinase 2
kb	kilo bases
kcal	kilocalories
KCl	potassium chloride

kDa	kilo Dalton
LB	lysogeny broth
LCR	locus control region
LFD	low fat diet
LH	luteinizing hormone
Luc	luciferase
m	mouse
MgCl <sub>2</sub>	magnesium chloride
ml	milliliter
mut	mutated
NaCl	sodium chloride
NCOR	nuclear receptor co-repressor
NE	nuclear extract
nM	nanomolar
NO	nitric oxide
NP-40	nonyl phenoxyethoxyethanol
p	promoter
P value	probability value
PAS	Per-Arnt-Sim
PBS-CMF	calcium-free phosphate buffered saline
Per2	period circadian protein homolog 2
Pit-1	pituitary specific transcription factor
PKA	protein kinase A

pM	picomolar
PMSF	phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
POMC	proopiomelanocortin
POU	Pit-Oct-Unc
PRL	prolactin
PVN	paraventricular nucleus
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNA pol II	RNA polymerase II
rt-PCR	real-time reverse transcriptase-PCR
SCN4A	skeletal muscle sodium channel $\alpha$ -subunit
SDS	sodium dodecyl sulfate
Ser5-pRNA pol	phosphorylated ser 5 at the C-terminal domain of the large subunit
shRNA	short hairpin RNA
SS	somatostatin
sst	somatostatin receptor subtype
STAT5	signal transducer and activator of transcription 5
TALENs	transcription activator-like effector nucleases
TCAM-1	testicular adhesion molecule-1
TG	transgenic
TK	thymidine kinase
TSA	trichostatin A
TSH	thyroid-stimulating hormone

Untr6	untranscribed region of chromosome 6
USF-1	upstream stimulatory factor 1
VEGF	vascular endothelial growth factor
WT	wild type
$\alpha$ -MSH	$\alpha$ melanocyte-stimulating hormone
$\mu$ g	microgram
$\mu$ L	microliter
$\mu$ M	micromolar

# CHAPTER 1

## Introduction

### *1.1. The problem: The obesity epidemic and growth hormone (GH) insufficiency*

Obesity and its associated metabolic changes is a state brought on by excessive energy intake and less energy expenditure over a prolonged period (Frayn 2005). This can result in a higher weight/height ratio, in which the excess weight reflects increased fat accumulation, mainly in adipose tissue (Frayn 2005). The prevalence of obesity has increased considerably in developed and developing countries in all genders and age groups, and has reached epidemic proportions (Rokholm, Baker et al. 2010, Shcherbakova, Poriagina et al. 2010). Obesity is a major health concern for adults in North America, increasing the risk for diabetes and heart disease (Steinberger, Daniels et al. 2003). Unfortunately, obesity is also a growing problem in children, and it is noteworthy that the incidence of type 2 diabetes mellitus is increasing in teenagers in parallel with the prevalence of obesity (Arslanian 2002, Gungor and Arslanian 2002). Thus, the obesity epidemic requires complementary treatments and management strategies, which might be made possible by understanding the underlying factors and mechanisms by which they are regulated.

Pituitary growth hormone (GH) insufficiency is considered a typical feature of obesity, regardless of age and gender (Vanderschueren-Lodeweyckx 1993). This is in addition to a spectrum of other significant metabolic derangements including changes in levels and/or peripheral responsiveness to multiple endocrine factors, as defined by hyperinsulinemia, hyperleptinemia, hyperglycemia and high levels of free fatty acids (De Marinis, Bianchi et al. 2004, Pataky, Bobbioni-Harsch et al. 2010). Dysregulation of GH is expected to result in progression of more metabolic complications, given GH is a major metabolic homeostatic factor. GH is involved extensively in lipid metabolism and implicated in adipose tissue remodeling to help maintain a healthy balance between energy intake and expenditure via its strong lipolytic activity (De Marinis, Bianchi et al. 2004, Yang, Mulder et al. 2004, Pataky, Bobbioni-Harsch et al. 2010). Thus, there is a basis to understand the regulation of somatotrophs in this regard, which may open new avenues to potential therapeutic interventions for prevention and/or better treatments of obesity and metabolic syndrome (Johansen and Malmlof 2006). Therefore, *this thesis* is the result of an investigation of how human GH production is regulated in the context of excess caloric intake as the initial step towards progression of obesity.

## ***1.2. Somatotrophs of the anterior pituitary gland are the major source of GH***

Somatotrophs are the predominant cell population of the anterior pituitary gland representing approximately 40-50% of the total pituitary cell number (Perez-Castro, Renner et al. 2012). The pituitary gland is also known as *hypophysis cerebri* (Greek origin terminology), and was named by Andreas Vesalius based on its location as an

attachment beneath the brain (Costoff, Greeley et al. 1977). The pituitary gland is an anatomic organ that acts as a connector between the hypothalamus and peripheral target organs based on its anatomic location. Even though the adult human pituitary gland is normally less than 10 mm in dimension and its weight is less than 1 gram, it is complex and its functional significance in health and disease is enormous. Most of the physiological and pathophysiological roles of the pituitary gland were recognized at the beginning of 20<sup>th</sup> century, in large part as a result of extensive investigations by the neurosurgeon, Dr. Harvey Williams Cushing (Cushing 1909, Jane, Thapar et al. 2002, Pendleton, Adams et al. 2011, Pendleton, Redett et al. 2013).

The pituitary gland is composed of two functionally distinct entities, the neurohypophysis, also known as the posterior lobe and the adenohypophysis that includes the anterior and intermediate lobes (Amar and Weiss 2003). Each pituitary lobe is characterized by distinct cell-type populations, secretory products, and regulatory mechanisms (Amar and Weiss 2003).

The *posterior lobe* of the pituitary is formed as a collection of axonal terminals of the magnocellular neurons. Oxytocin and vasopressin are the two major products of these neurons, which are transported to the axonal terminals located in the posterior lobe and subsequently secreted into the general circulation (Zimmerman 1977).



The *intermediate lobe* contains a group of specialized cells known as melanotrophs, which secrete  $\alpha$  melanocyte-stimulating hormone ( $\alpha$ -MSH); required for regulation of melanin production and its distribution by melanocytes (Voitkevich 1967).

The *anterior pituitary gland* has a central role in growth, metabolism, lactation, stress and reproductive physiology. Five distinct cell populations are resident in the anterior pituitary gland (Zhu, Gleiberman et al. 2007), including:

- 1) Somatotrophs that secrete GH,
- 2) Lactotrophs that produce prolactin (PRL),
- 3) Corticotrophs that secrete adrenocorticotrophic hormone (ACTH), a proteolytic product of proopiomelanocortin (POMC),
- 4) Thyrotrophs that produce thyroid-stimulating hormone (TSH),
- 5) Gonadotrophs that produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

The vertebrate pituitary gland has a dual origin. The posterior lobe of the gland originates from neuroectoderm while the anterior and intermediate lobes derive from the hypophyseal placode, which is a thickened plate of ectoderm in the early embryo (Zhu, Gleiberman et al. 2007). Stratification of the temporally and spatially organized endocrine cell types appearance as well as position are coordinated by multiple factors that signal cell division, lineage commitment and fate within the anterior pituitary gland.

These factors and signals largely follow the same general principles in different species; reviewed in (Zhu, Gleiberman et al. 2007).

In the human fetus, components of the pituitary gland and more specifically somatotrophs are anatomically formed by mid-gestation. The anterior pituitary develops from Rathke's pouch, an ectodermal outpouching of *stomodeum* named after the German embryologist and anatomist, Martin Heinrich Rathke (1793-1860). Rathke's pouch appears at approximately three weeks of gestation. Somatotrophs can be detected by ultra structural analysis at 8 weeks of gestation (Suganuma, Seo et al. 1989). Pituitary GH transcript levels increase progressively throughout 16-27 weeks of gestation. The pituitary concentration of GH rises to peak level at 25-30 weeks of gestation and remains constant thereafter until birth (Kaplan, Grumbach et al. 1972).

Somatotrophs differentiate from precursor cells that are characterized by the ability to express Pit-1 (also known as POU1F1), a member of the POU (Pit-Oct-Unc) domain containing transcription factor family (Latchman 1999). Pit-1 contains an amino-terminal POU-specific domain and a COOH-terminal POU homeodomain. These two domains are essential for a high-affinity binding to AT-rich DNA elements (Yang, Jin et al. 2010); also reviewed in (Andersen and Rosenfeld 2001). Snell and Dwarf Jackson mice carry natural mutations in the Pit-1 gene, and are GH deficient and dwarf (Li, Crenshaw et al. 1990). Genetic studies with these mice revealed an absolute requirement of functional Pit-1 for efficient production of GH, PRL and TSH and hence differentiation of their respective specialized pituitary cell types (Camper, Saunders et al.

1990, Li, Crenshaw et al. 1990, Dasen, O'Connell et al. 1999). Pit-1 expression, itself, is dependent on Prophet of Pit-1 (Prop1); a paired-like homeodomain transcription factor (Gage, Brinkmeier et al. 1996, Sornson, Wu et al. 1996, Zhu, Gleiberman et al. 2007). Pit-1 production continues in somatotrophs throughout adult life (Zhu, Gleiberman et al. 2007), suggesting an essential role in maintaining GH expression, as one of its target genes.

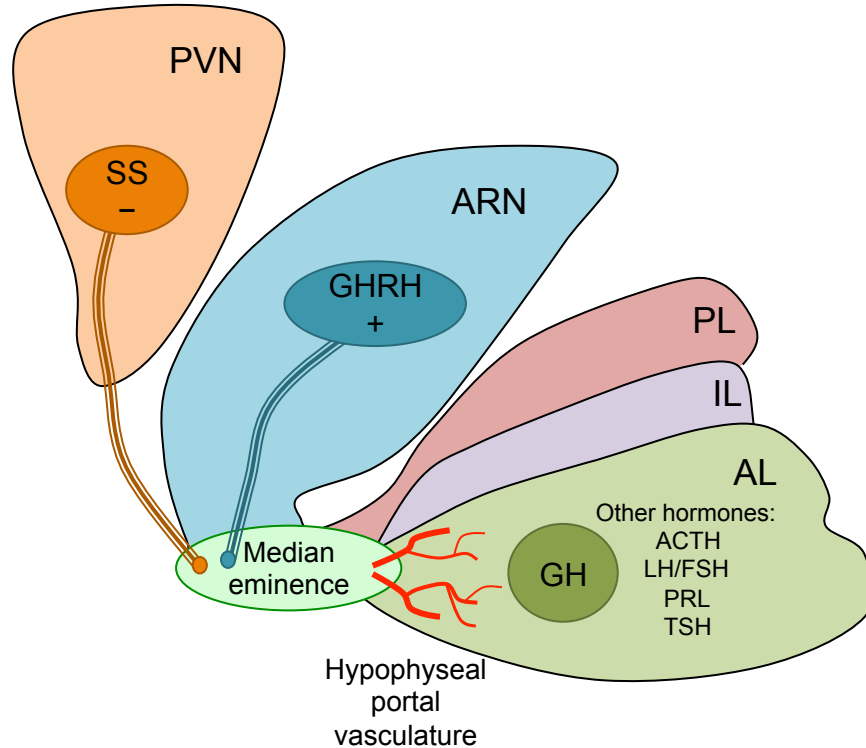
GH, also referred to as somatotropin, is the product of the somatotrophs of the anterior pituitary gland. It is the most abundant hormone produced by the anterior pituitary gland (Segrestaa, Gueris et al. 1975). GH is a 22-kilo Dalton (kDa) polypeptide hormone composed of 191 amino acid residues forming a single chain with four helical regions and two disulfide bridges (Frank 2001). Structurally, the helical bundles with loops of differing lengths between these helices are elements of a general topography that is characteristic of a large group of cytokines and colony stimulating factors. This group also includes erythropoietin (EPO) and granulocyte–macrophage-colony stimulating factor (GM-CSF) (Frank 2001). Pituitary GH is generally regarded to be an essential factor for regulating somatic growth in vertebrates from fishes to mammals (Kostyo and Reagan 1976, Reagan, Pardue et al. 1976).

### ***1.3. The hypothalamic/pituitary axis: Hypothalamic regulation of somatotrophs***

The hormones of the anterior pituitary are regulated by hypothalamic factors with stimulatory and inhibitory functions under normal physiological conditions (Figure 1.1). Growth hormone release from the pituitary is pulsatile and is regulated predominantly by two hypothalamic factors: growth hormone releasing hormone (GHRH), synthesized by neurons of the arcuate nucleus as well as somatostatin (SS), which is mainly synthesized by neurons in the periventricular nucleus. Hypothalamic neurons release GHRH and SS to the medial eminence where they are conveyed to the anterior pituitary by the hypophyseal portal vasculature. GHRH affects GH production through activating the GHRH receptor (GHRH-R), which is expressed by the pituitary somatotrophs. Activation of the GHRH-R which is a G-protein coupled receptor, elicits elevated intracellular cyclic adenosine monophosphate (cAMP) levels and subsequently activates the protein kinase A (PKA) pathway, which is subsequently leads to enhancement of Ca<sup>2+</sup> channels resulting in calcium influx (Lin, Lin et al. 1993, Takei, Takano et al. 1996, Xu, Lavinsky et al. 1998, Cohen, Hashimoto et al. 1999). The functional significance of the GHRH-R signaling pathway in somatotroph proliferation is revealed by both little (lit/lit) mice, which carry a single amino acid substitution in the GHRH-R leading to defective ligand binding, as well as in the GHRH gene null mice (Alba and Salvatori 2004). A hypoplastic pituitary gland and GH deficiency are characteristics of these mutant mice, which in turn result in a postnatal growth retarded phenotype (Godfrey, Rahal et al. 1993, Lin, Lin et al. 1993). In humans, mutations in the GHRH-R gene are also associated with isolated GH deficiency (IGHD); reviewed in (Mullis 2005). By contrast to GHRH, SS and its cognate

receptors exhibit a main inhibitory role in GH regulation (Burgus, Ling et al. 1973). SS signals through the G-protein coupled receptors, somatostatin receptor subtype 1 (sst1), sst2, sst3, sst4 and sst5. The sst2 is alternatively spliced to two isoforms, sst2a and sst2b (Panetta and Patel 1995). SS antagonizes the effect of GHRH through membrane hyperpolarization by opening potassium channels leading to depletion of intracellular calcium concentration, which in turn effectively inhibits GH exocytosis and secretion (Draznin, Dahl et al. 1988, Tsaneva-Atanasova, Sherman et al. 2007).

**Figure 1.1: The hypothalamic-pituitary axis**



*Schematic representation of a sagittal view of the pituitary gland and a part of the hypothalamus. The pituitary gland is composed of three anatomically distinct entities: (i) the posterior lobe (PL), (ii) the intermediate lobe (IL) and (iii) the anterior lobe (AL). The anterior lobe produces adrenocorticotrophic hormone (ACTH), growth hormone (GH), luteinizing hormone (LH), follicle-stimulating (FSH), prolactin (PRL), and thyroid stimulating hormone (TSH). GH production and secretion are regulated positively (+) and negatively (-) by GH releasing hormone (GHRH), synthesized by neurons of the arcuate nucleus (ARN), and somatostatin (SS) produced by the paraventricular nucleus (PVN), respectively. These two hypothalamic factors are released from the termini of their respective neurons to the median eminence and consequently transported to the anterior pituitary by the hypophyseal portal vascular system. Based on (Zhu, Gleiberman et al. 2007).*

#### ***1.4. Ghrelin: A GH secretagogue***

Ghrelin is a gastric peptide hormone that is produced by a subset of stomach cells, and is the only natural ligand of growth hormone secretagogue receptor (GHS-R) (Howard, Feighner et al. 1996). The stomach is the primary source, of ghrelin, however, it is also produced in many other tissues including intestine, colon, lung, heart, pancreas, kidney, testis, pituitary and hypothalamus (Date, Kojima et al. 2000, Hosoda, Kojima et al. 2000, Korbonits, Bustin et al. 2001, Gnanapavan, Kola et al. 2002, Sakata, Nakamura et al. 2002). There are many biological functions of ghrelin related to its metabolic property. These include stimulation of appetite and food intake, the modulation of gastric acid secretion and motility, and the modulation of the endocrine and exocrine pancreatic secretions (Tschop, Smiley et al. 2000, Kamegai, Tamura et al. 2001, Nakazato, Murakami et al. 2001, Shintani, Ogawa et al. 2001). A major and significant physiological property of ghrelin includes, however, the induction of GH secretion from the pituitary somatotrophs. GH secretion is induced by ghrelin through direct interaction with its receptor, GHS-R, and induction of both cyclic guanosine monophosphate (cGMP) and nitric oxide (NO) signaling pathways, resulting in increased intracellular calcium concentration and subsequently increased GH exocytosis (Takaya, Ariyasu et al. 2000).

## ***1.5. Physiological functions of GH: growth and metabolism***

### ***1.5.1. GH signaling pathway***

GH is complexed with GH binding protein (GHP) upon release from somatotrophs. GHP corresponds to the extracellular domain of the GH receptor (GH-R), and is found in the serum of many species (Baumann 1994). It is suggested that GHP enhances the biological effects of GH *in vivo* by increasing its half-life in the circulating compartment. The next step in GH action is binding to its specific cell surface receptor. GH-R is highly expressed in liver but receptors are also found in almost all tissues (Sorensen, Chaudhuri et al. 1992). The GH-R is a transmembrane protein and member of the cytokine receptor superfamily (Postel-Vinay and Finidori 1995). GH binding leads to dimerization of the receptor, which then activates several secondary messengers (Wells 1996). GH-R is associated with and activates the tyrosine kinase janus kinase 2 (JAK2) and signal transducer and activator of transcription 5 (STAT5) (Galsgaard, Gouilleux et al. 1996, Sotiropoulos, Moutoussamy et al. 1996, Zhu, Goh et al. 2001). Once activated, STAT5 acts as a transcriptional factor that can activate a number of intracellular pathways by affecting the expression levels of target genes (Postel-Vinay and Finidori 1995). GH signaling influences somatic growth, physical and metabolic adaptations (Roemmich and Rogol 1997), and central nervous system reactions that help to maintain a healthy homeostatic balance (Veldhuis, Anderson et al. 2001).



### **1.5.2. Growth**

GH is a dominant component of the growth process as well as of the physiology of puberty. The pubertal growth spurt is influenced by a number of factors including variety of hormones, nutritional status, and physical activity acting mostly in harmony to achieve optimal physical growth (Christoforidis, Maniadaki et al. 2005).

The GH signaling pathway drives the direction of specific sets of target genes expressed in their target organs. This is most profoundly in skeletal muscle, cartilage and bone, thereby promoting skeletal and muscular growth, and as such somatic growth (Werther, Haynes et al. 1990, Jorgensen, Jessen et al. 2006). The somatotrophic axis governs physical growth at puberty, consisting in transient surges in pituitary GH, somatomedins including insulin-like growth factor-1 (IGF-1) and IGF-2, their carrier proteins, and receptors (Renaville, Hammadi et al. 2002, LeRoith and Roberts 2003) (Figure 1.2). The plasma concentration of IGF-I rises to the highest levels following the peak in GH concentration in puberty and declines to low levels in old age. The role of IGF-2 has been best characterized in fetal growth before birth, however its role in pubertal growth in comparison to IGF-1 is less clear (Baker, Liu et al. 1993, Kaklamani, Linos et al. 1999). Most but not all growth promoting effects of GH are exerted indirectly through stimulation of hepatic IGF-1 or IGF-1 from other peripheral tissues (Adams and Haddad 1996, Lupu, Terwilliger et al. 2001, Yakar, Wu et al. 2002). Thus, the somatotrophic axis can be considered the GH/IGF-1 axis.

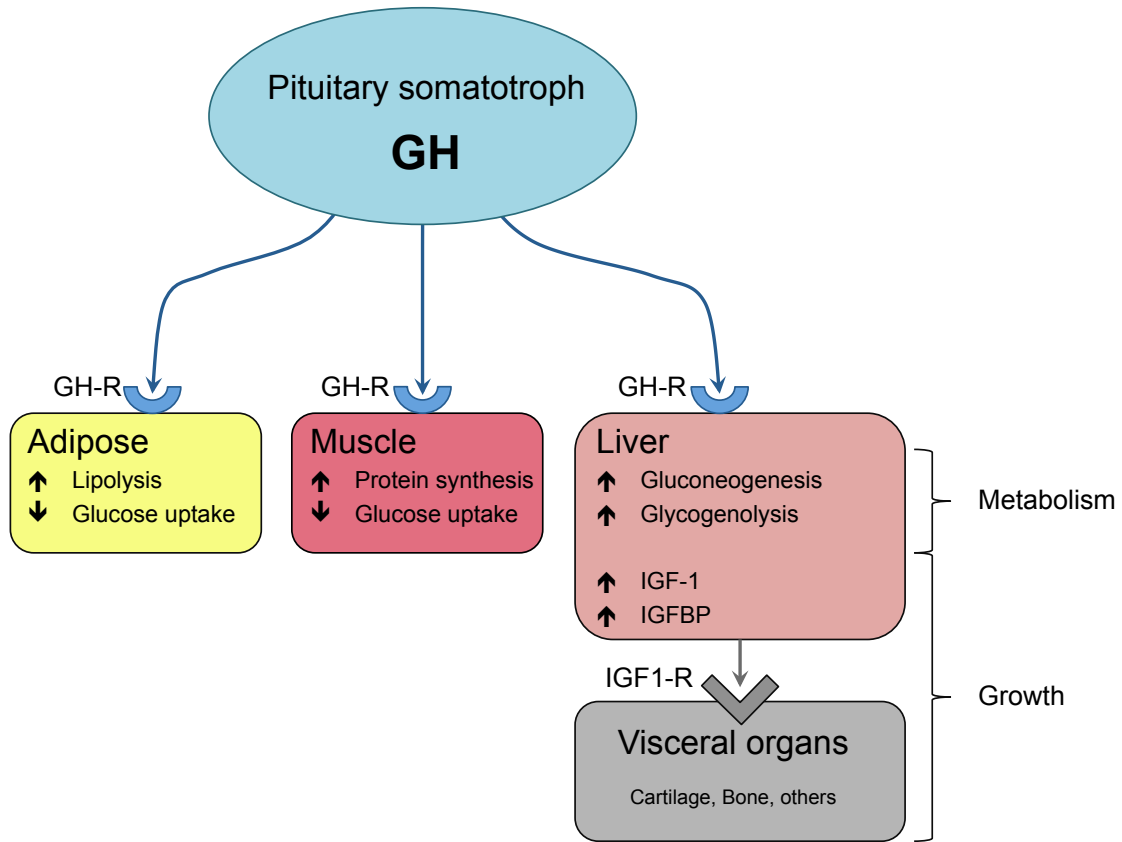
In humans, a gradual and progressive decline in spontaneous GH secretion occurs at a rate of 14% per decade in adulthood as somatic growth diminishes with GH deficiency occurring after 60 years of life; this phenomenon is known as *somatopause* (Iranmanesh, Lizarralde et al. 1991, Toogood, O'Neill et al. 1996, Toogood, Jones et al. 1998, Savine and Sonksen 1999).

### ***1.5.3. Metabolism***

The significance of the somatotropic axis, and more specifically GH, is not limited to pubertal growth. GH is also a metabolic factor involved in a complex series of events that are diverse, tissue-specific and can influence lean and fat mass. It exerts its effects by several means at the level of gene expression (transcriptional regulation) as well as acute changes in catalytic activity of several key enzymes involved in carbohydrate, lipid and protein metabolism (Moller, Jorgensen et al. 1995). It is well established that GH can stimulate hepatic glucose production by affecting gluconeogenesis and glycogenolysis (Brooks, Trent et al. 2007). GH also stimulates lipolysis in the adipose tissue, and predominantly the visceral and subcutaneous depots (Krag, Nielsen et al. 2008). Furthermore, changes in GH action are linked with alterations in the adipokine profile, which implicate the association of GH with adipose tissue remodeling in health and disease (Oliveira, Salvatori et al. 2010) (Figure 1.2). There is also evidence suggesting growth promotion by GH that is secondary to the substantial metabolic impact of the hormone (Moller, Jorgensen et al. 1995). GH leads to rapid increases not only in circulating levels of IGF-1 but also insulin, free fatty acids, ketone

bodies, and even glucose (Davidson 1987, Press 1988). These metabolic changes collectively have independent nitrogen-retaining effects, and therefore they can inherently facilitate the anabolic role of GH.

**Figure 1.2: “Growth hormone biology: growth and metabolism”**



*Growth hormone is a central component of growth and metabolism through its (i) direct action via activation of growth hormone receptor (GH-R) signaling, which is mostly attributed to its metabolic role or through (ii) indirect action via IGF-1 signaling mostly related to its growth promoting effect. In the human, the GH-R is highly expressed in visceral organs such as: liver, adipose tissue, heart, kidneys, intestine, lung, pancreas, cartilage and skeletal muscle where it promotes the synthesis of IGF-1 as a second messenger of the GH signaling. Based on Koeppen and Stanton: Berne and Levy Physiology, 6<sup>th</sup> Edition*

## ***1.6. GH that affects metabolism is also affected by metabolism***

### ***1.6.1. Excess caloric intake, obesity and GH***

GH exerts significant control over the total body metabolism by regulating lipids, protein and energy homeostasis as described above in section 1.5. This notion is supported by the fact that *growth hormone deficiency* (GHD) both in human and rodent models is highly associated with metabolic dysfunction as defined by dyslipidemia, obesity and reduced exercise capacity, suggesting a substantial involvement of GH in metabolism (Moller, Jorgensen et al. 1995, McMurray and Hackney 2005). Patients with GH deficiency are insulin resistant, compared with age and body mass index matched controls with normal levels of GH (Salomon, Cuneo et al. 1989, Johansson, Fowelin et al. 1995, Hew, Koschmann et al. 1996). Furthermore, the prevalence of impaired glucose tolerance is higher in GH deficient patients (Oliveira, Salvatori et al. 2012). The metabolic properties of GH are also supported by a number of clinical trials where correction of GHD with GH replacement therapy can rescue various aspects of malfunctioning metabolic processes affected by GHD (Powrie, Weissberger et al. 1995, McMurray and Hackney 2005, Trepp, Fluck et al. 2008). Furthermore, physiological as well as pathological factors such as body composition, sex, age, sleep, nutrients can significantly alter the production and pulsatile secretion of GH, which can then in turn influence the subsequent biological effects of GH (Djarova, Ilkov et al. 1986, Bray 2004). Collectively, these data suggest that limited GH availability may compromise the overall energy homeostasis, but also suggests that metabolic/endocrine alterations can cause

change in GH levels and/or function.

### ***1.6.2. GH deficiency is a characteristic of all stages of obesity progression***

Obesity is a complex chronic disease involving decades and cascades of pathophysiological changes and adaptation. Thus it is difficult to ascertain the exact mechanisms for this long-term process in humans. Development of obesity, however, can be divided into a continuum (the three O's) with three phases. The first phase (overfeeding), is normally initiated by excess caloric intake, with a low level of energy expenditure. This in turn promotes storage of excess energy in the form of adipose tissue, leading to weight gain and the second (overweight) phase. The third phase in this continuum is obesity itself (De Marinis, Bianchi et al. 2004, Pataky, Bobbioni-Harsch et al. 2010). It is noteworthy that GH levels are extremely sensitive to increased caloric intake within the continuum of obesity progression. A study in 2011 identified a 77% decrease in plasma GH levels in a group of healthy individuals of age 24 who overate for three days without any significant weight gain during this period (Cornford, Barkan et al. 2011). Furthermore, GH levels continued to be suppressed within two weeks of overeating by which point the subjects had gained significant weight (Cornford, Barkan et al. 2011). Thus, GH insufficiency appears to be a feature at all stages of obesity development.

### ***1.7. Negative correlation between GH and insulin levels in the context of overeating***

In humans, hyperinsulinemia was suggested to be the likely mediator of the rapid suppression of human GH production seen after three days of overeating (Cornford, Barkan et al. 2011). Blunted GH secretion in obese human population has been linked with metabolic derangements (De Marinis, Bianchi et al. 2004, Pataky, Bobbioni-Harsch et al. 2010), but in the three day overeating study there was no significant changes in body weight (Cornford, Barkan et al. 2011). Elevated levels of insulin as a component of metabolic syndrome might be a major contributor to the obesity-related reduction in GH secretion (Lanzi, Manzoni et al. 1997, Lanzi, Luzi et al. 1999). Abnormal GH production in obese patients is characterized by impaired spontaneous secretion, as well as a decline in pituitary somatotroph responsiveness to all known pharmacological provocative stimuli. Specifically, GH-producing somatotrophs become significantly hypo-responsive to exogenous secretagogues including GHRH, ghrelin/growth hormone secretagogue (GHS) (Maccario, Procopio et al. 1995, Procopio, Maccario et al. 1995, Pijl, Langendonk et al. 2001, Scacchi, Orsini et al. 2010). It was suggested that the effect of excess insulin as the result of short term overeating (three days) was to impair GH secretion (Cornford, Barkan et al. 2011). Importantly in the context of this thesis, their data do not exclude a negative effect of overeating on GH synthesis by pituitary somatotrophs. An effect at the level of synthesis might not only affect interpretation of existing data but also provide insight into a perceived lack of effectiveness of GH secretagogues as anti-obesity agents (Iranmanesh, Lizarralde et al. 1991, Veldhuis, Iranmanesh et al. 1991, Iranmanesh, South et al. 1998).

There is evidence to suggest GH production is under insulin control. Excess insulin may act indirectly on the hypothalamus by affecting GHRH and somatostatin secretion tone, or directly at the pituitary level (Luque and Kineman 2006). Somatotrophs have also been implicated as the primary target of hyperinsulinemia in animal models. A reduction in GH synthesis and/or secretion was associated with increased circulating levels of insulin in both high fat diet-induced obesity and the leptin deficient ob/ob mice (Buettner, Newgard et al. 2000, Luque and Kineman 2006). The effect of insulin on GH, however, was not accompanied by any alteration in GHRH and somatostatin expression in the hypothalamus of the obese mice, supporting a more direct effect of insulin at the pituitary level (Luque and Kineman 2006). Certainly, the mouse pituitary gland is an insulin sensitive tissue, since insulin receptors are present at levels comparable to those detected in “classical” insulin sensitive tissues, such as adipose, liver and skeletal muscle (Luque and Kineman 2006). Furthermore, there is also evidence that pituitary cells continue to be sensitive to insulin while the cells from other peripheral tissues become more resistant from chronic hyperinsulinemia in the obese state (Luque and Kineman 2006, Brothers, Wu et al. 2010). Thus, the idea of somatotrophs as “sensors” with the ability to receive metabolic signals and respond to alterations in metabolic pathways has been suggested (Childs, Akhter et al. 2011). The mechanism, however, for this insulin-related suppression of GH synthesis is poorly understood. *Thus, a major goal of this thesis was to assess the effect of acute excess caloric intake, and by extension excess insulin, on human GH synthesis as well as secretion by the pituitary* (Chapters 3 and 4).



### ***1.8. Energy homeostasis and GH levels: a balance between energy intake and expenditure***

The rule of energy balance suggests that when energy intake is more than energy expended, weight gain and subsequently obesity and health related complications are the main results. Physical activity protects against the development of obesity through an increase in energy expenditure due to the cost of the activity itself as well as via changes in endocrine factors that lead to an increase in the resting metabolic rate (Poehlman 1989, Goran, Reynolds et al. 1999). More importantly, GH is a target for physical exercise; it is well known that a linear dose-response relationship exists between exercise intensity and the GH secretory pattern in healthy individuals (Weltman, Weltman et al. 1992, Veldhuis, Liem et al. 1995, Saito, Sone et al. 1997, Pritzlaff, Wideman et al. 2000). Targeting GH is suggested to be important due to its potent lipolytic effect, an anticipated further increase in energy expenditure and prevention of obesity (Pritzlaff, Wideman et al. 1999). ***Thus, an additional goal of this thesis was to assess the effect of excess caloric intake and physical activity on GH production by the pituitary.*** Specifically, the overall impact of incorporating prescribed physical activity (swimming) into a high fat diet treatment regimen to promote use of excess energy was assessed (Chapter 6).

### ***1.9. Primate versus rodent GHs: Differences in gene structure and biology***

Human (h) GH levels are known to fluctuate dynamically under different physiological conditions, including exercise, obesity, sleep, and aging, but the mechanism involved in this regulation has not been studied extensively beyond speculation regarding effects on secretion. This is because assessments of hGH production are restricted largely to measurements of hormone in the circulation. Investigation of hGH synthesis under physiological and pathophysiological conditions, and specifically at the level of gene expression versus secretion, is limited due to the inaccessibility of human pituitary samples.

Historically, murine (mouse and rat) systems have been used to model a variety of human diseases because of their anatomical, physiological and genomic similarities to humans. As such, rodents have been the primary source of models for investigations into the physiological regulation of GH, growth and metabolic impact of GH signaling. Although a limited number of non-human primate studies have been reported, this system is not readily accessible. As a result, our knowledge of GH gene control is largely derived from studies done using rodent GH-secreting pituitary cell lines (e.g., GC, GH1, GH3, GH4C1, MtT/S, and MtT/E) and the endogenous *rodent* GH gene (Samuels and Shapiro 1976, Ostlund, Leung et al. 1978, Leite, Cardoso et al. 1996, Nogami, Hiraoka et al. 2006). Efforts have been made to look at hormonal regulation of proximal *hGHI* promoter activity through transient gene transfer assays, by transfecting 0.5-5 kilobase (kb) fragments of the *hGHI* into rat pituitary tumor cell lines or assessing RNA levels in

human primary adenoma cells, but with variable results (Cattini, Anderson et al. 1986, Isaacs, Findell et al. 1987, Morin, Louette et al. 1990, Zhang, Brooks et al. 1992). In addition, any extrapolation of data on murine (non-primate) GH to hGH needs further consideration, as there are a number of fundamental differences between primate and non-primates GHs.

There are distinct differences in structure between the human (primate) and mouse (non-primate) GH genes, including in both the flanking and coding DNA. As a result, hGH and rodent GH have the potential for different regulatory control and function (Nickel, Kardami et al. 1990, Strasburger 1990, Lira, Kalla et al. 1993, Wells, Cunningham et al. 1993, Wells and de Vos 1993, Jones, Monks et al. 1995, Yi, Bernat et al. 2002).

In terms of evidence to support differences in primate and non-primate GH gene structure, only 0.3 kilobases of rat GH upstream DNA is needed to direct efficient pituitary expression of a transgene in mice, whereas the equivalent result requires 14.5 kb of *hGHI* promoter and upstream DNA (Lira, Kalla et al. 1993, Jones, Monks et al. 1995).

In terms of protein sequence and based on phylogenic analyses, primate GH diverged, evolutionarily, from the other eutherian (placental mammals) orders approximately 75 million years ago (Ohta 1993, Wallis 1994, Liu, Makova et al. 2001). This divergence resulted in a 59–63 amino acids difference in the peptide sequence of 190 amino acids, which accounts for a difference of ~33% between primate and rodent

GHs (Ohta 1993, Wallis 1994, Liu, Makova et al. 2001). This is in comparison to most of the other non-primate GHs, which differ from each other by a maximum of four amino acids in their mature peptide (Ohta 1993, Wallis 1994). Thus, it is reasonable to expect differences between the biological properties of GHs in primates and rodent due to their distinct amino acid sequences and structure. In fact, this is the case and it is clear that specificities in binding of GHs to their respective receptors vary markedly (Wallis 1980, Hughes and Friesen 1985). Thus, in terms of coding sequences and function, hGH can bind both the GH and prolactin receptors while mouse and rat GH bind only the GH receptor (and weakly to the hGH receptor) (Chadwick, Folley et al. 1961, Wells, Cunningham et al. 1993, Wells and de Vos 1993). As a result hGH but not murine GHs possess lactogenic in addition to their somatogenic function.

Furthermore there is evidence for differential effects on downstream signaling and thus distinct physiological control. Human GH plays a crucial role in preserving energy balance by suppressing lipogenesis (Oscarsson, Ottosson et al. 1999, Richelsen 1999) and adipogenesis (Carrel and Allen 2000, Carrel and Allen 2000) and stimulating lipolysis (Raben and Hollenberg 1959). Thus, under the conditions of energy deprivation and fasting, levels of hGH secretion are elevated and hence can induce the release of free fatty acids to be consumed as a source of energy fuel to maintain a healthy metabolic homeostasis (Ho, Veldhuis et al. 1988, Hartman, Veldhuis et al. 1992, Vance, Hartman et al. 1992). In contrast, fasting inhibits the pulsatile secretion of mouse GH, and the induced release of free fatty acids with fasting does not follow an elevation in GH release in mice (Steyn, Leong et al. 2012).

Thus, a caveat with extrapolating from rodent GH to hGH is that non-primate and primate GHs are structurally and functionally distinct, with differences extending beyond the coding to regulatory sequences (Nickel, Kardami et al. 1990, Strasburger 1990, Lira, Kalla et al. 1993, Wells, Cunningham et al. 1993, Wells and de Vos 1993, Jones, Monks et al. 1995, Yi, Bernat et al. 2002). Therefore, there might be variation in the signals/mechanisms under which production and/or action of rodent and primate GH are subjected, as well as how they are regulated.

Transgenesis was pursued in response to the lack of a readily available system to study the hGH1 gene (*hGHI*) in pituitary cells *in situ*. Transgenic (TG) mice were generated by Ms. Yan Jin and Ms. Agnes Fresnoza in the laboratory of Dr. Peter A. Cattini (University of Manitoba) containing the intact *hGHI* and locus control region (LCR) in “large” (greater than 100 kb) fragments of human chromosome 17; the two lines maintained are referred to as 141hGH/CS and 171hGH/CS TG mice (Jin, Lu et al. 2009). Further characterization of these TG mice will be discussed below in Section 1.12.

### ***1.10. Human GH gene family***

Duplications of the pituitary GH gene in higher primates have given rise to a family of GH-related genes, only one of which is expressed preferentially in the pituitary. The GH-related gene family consists of five members including pituitary growth hormone (*GHI*) (the major focus of this thesis) as well as placental GH (*GH-V*), and the chorionic somatomammotropin (CS) genes (*CS-A*, *CS-B* and *CS-L*), contained within a

single 47 kb locus on chromosome 17 (Chen, Liao et al. 1989) (Figure 1.3). The family is flanked upstream by the lymphocyte-specific CD79b and skeletal muscle sodium channel  $\alpha$ -subunit SCN4A genes, and downstream by the testicular adhesion molecule-1 (TCAM-1) gene (Bennani-Baiti, Jones et al. 1995, Bennani-Baiti, Cooke et al. 1998). Polypeptide products of all members of the GH gene family are highly related based on their structure and biochemical properties (Seeburg, Shine et al. 1977, Shine, Seeburg et al. 1977, Martial, Hallewell et al. 1979, Martial, Seeburg et al. 1979). Despite their structural relatedness, these hormones are produced in a tissue-specific manner. Human *GHI* is expressed predominantly in the somatotrophs of the anterior pituitary gland, whereas the placental members, chorionic somatomammotropin (CS; also known as placental lactogen) and placental growth hormone (GH-V), are produced by syncytiotrophoblasts in the placenta during pregnancy (Chen, Liao et al. 1989).

Efficient and/or tissue-specific expression of the human (h) GH/CS genes is associated with the presence of five DNase I hypersensitive sites (HS) I-V, which also constitute the LCR (Figure 1.3). DNase I HS in chromatin were first identified 26 years ago as regions where putative regulatory DNA elements might have heightened accessibility (Gross and Garrard 1988). This would allow binding of sequence-specific transcriptional regulatory factors in place of a canonical nucleosome, leading to chromatin remodeling and cell-selective activation of target genes (Thurman, Rynes et al. 2012). Hypersensitive sites I-V of the hGH/CS locus were discovered as relatively “open” domains of chromatin 14 to 32 kb upstream of the *hGHI*, defining the LCR (Jones, Monks et al. 1995). The LCR can act as a tissue or cell-specific enhancer, but

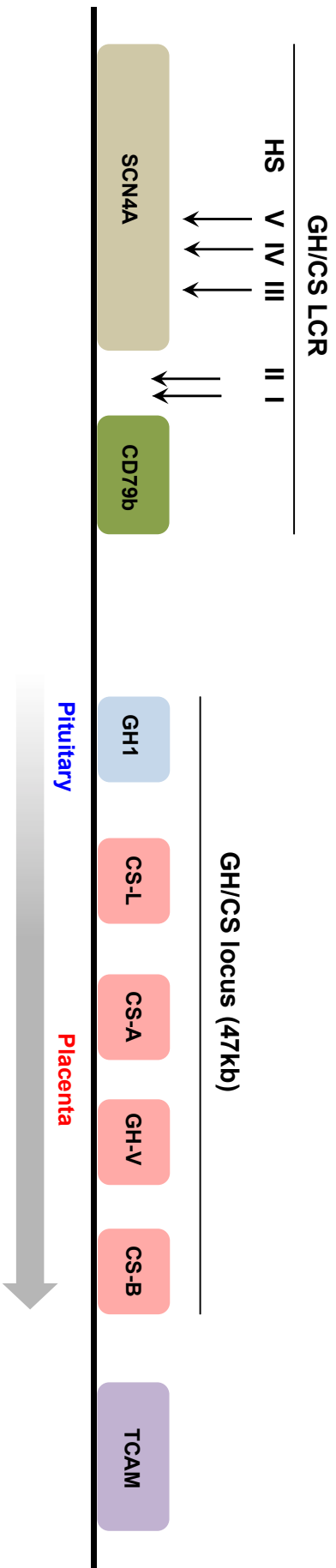
importantly is also a novel eukaryotic genetic regulatory element that is sufficient to confer appropriate site of integration-independent expression of a gene, including a transgene (Grosveld, van Assendelft et al. 1987, Elefant, Cooke et al. 2000, Jin, Lu et al. 2009, Vakili, Jin et al. 2011). Hypersensitive sites I and II are restricted to the pituitary, and HS IV is placenta-specific (Jones, Monks et al. 1995). HS III-V are found in a region 28-32 kb upstream of the *hGHI* within the *SCN4A* gene (Figure 1.3). These sites were described initially as being limited to pituitary and placenta chromatin, however, HS III has now been identified outside pituitary and placenta chromatin as well (Kimura, Sizova et al. 2007).

Hypersensitive sites I and II are located about 15 kb upstream of the *hGHI* in the 5'-flanking sequences of the adjacent B-cell antigen receptor complex-associated protein  $\beta$ -chain (CD79b) gene (Figure 1.3). Extensive characterization of HS I/II has revealed that this region of the hGH LCR possess pituitary enhancer activity *in vitro* and *in vivo*, and characterized the pituitary-specific nature of HS I/II by demonstrating they contain binding sites for the pituitary-specific protein Pit-1 (Jin, Surabhi et al. 1999, Jin, Norquay et al. 2004). It is suggested that Pit-1 participates in the generation of the pituitary-specific hypersensitive sites, and hence in the remodeling of chromatin (Jin, Surabhi et al. 1999). This enhancer activity of HS I/II was subsequently confirmed by others who added that both HS I and II were reestablished in the pituitaries of transgenic mice (Shewchuk, Asa et al. 1999).

**Figure 1.3: Schematic representation of the human GH/CS locus and nearby genes located on chromosome 17**

*Genomic organization of the human GH-related gene family members (GH-1, CS-L, CS-A, GH-V and CS-B) contained within a single 47 kb locus on chromosome 17. These genes are flanked upstream by the lymphocyte-specific CD79b and skeletal muscle sodium channel  $\alpha$ -subunit SCN4A genes, and downstream by the testicular adhesion molecule-1 (TCAM-1) gene and the relative position of hypersensitivities sites (HS) and GH/CS genes are shown; HS I and II are pituitary-specific, HS IV is placenta-specific, and HS III and HS V appear to be present constitutively.*





### ***1.11. Transcriptional activation of hGH1 involves chromatin remodeling***

Histones are basic proteins that facilitate folding and condensation of negatively charged eukaryotic genomic DNA in the form of chromatin, consisting of nucleosomal units. Each nucleosomal unit consists of 147 base pairs of DNA wrapped around an octamer of histone proteins consisting of dimers of histones H2A, H2B, H3 and H4 (Fernandez-Morera, Rodriguez-Rodero et al. 2010). These nucleosomes with their inter-nucleosomal linker DNA and linker histone (H1) can support transcription and more importantly facilitate further condensation of chromatin into higher order structures. Histone H1 as well as the amino (N) -terminal (tail) regions of the core histones are important in stabilizing chromatin higher-order structures and 30-nanometer fibers, which at its extreme includes the transcriptionally inactive metaphase chromosome (Manuelidis and Chen 1990).

Regulation of transcription in response to intrinsic/environmental cues is dependent on separation of the two strands of genomic DNA momentarily, thus allowing access of RNA polymerases to the DNA molecule. The folded-chromatin in the form of 30-nanometer fibers however acts as a barrier and makes the DNA molecules inaccessible to the transcription machinery and initiation (Fischle, Tseng et al. 2005). Fine-tuning of the transcription process is mostly mediated by reversible modifications to the N-terminal tails of core histones such as acetylation, methylation, sumoylation, or ubiquitination of basic lysine and arginine residues, but most often of lysine residues. These modifications can “loosen” or “compact” the DNA and making it more or less accessible to the transcription machinery (Fernandez-Morera, Rodriguez-Rodero et al.

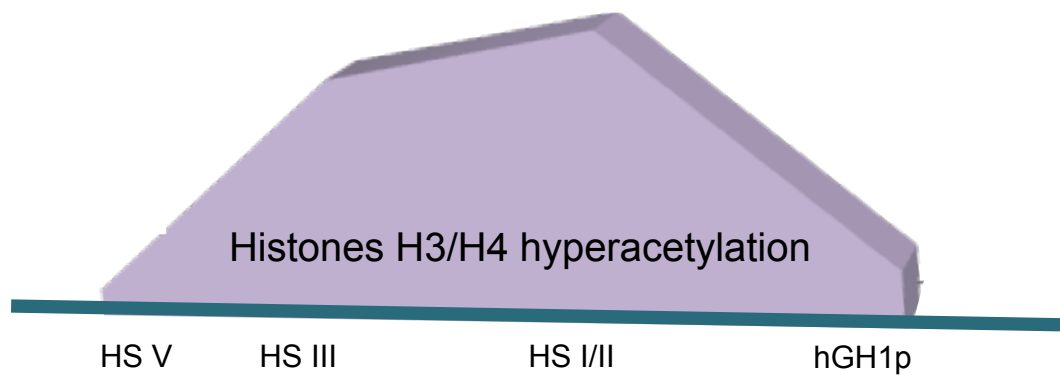
2010). In general H3/H4 hyperacetylation is associated with a more open chromatin structure and thus transcriptional activation, whereas specific patterns of H3 trimethylation can be linked to an active promoter (as in tri-methylation of lysine 4) or gene repression/silencing (as in tri-methylation of lysines 9 or 27) (Lachner, O'Sullivan et al. 2003, Shahbazian and Grunstein 2007, Nottke, Colaiacovo et al. 2009, Christophersen and Helin 2010, Fernandez-Morera, Rodriguez-Rodero et al. 2010). Thus, multiple histone modifications act in combination to regulate transcription (Wang Z 2008, Fernandez-Morera, Rodriguez-Rodero et al. 2010)

In spite of the fact that every single cell of multicellular organisms contains the same genomic material, different cells display distinct phenotypes and as a result diverse functions. This phenomenon that is characterized by distinct gene expression patterns, is established during development through chromosomal configuration in the interphase stage of terminally differentiated cells (Cremer and Cremer 2010). This process allows activation of cell-specific genes through making appropriate chromatin-chromatin physical contacts and thus spatial arrangements of chromosomes within the interphase nucleus of differentiated cells (Goetze, Mateos-Langerak et al. 2007, Goetze, Mateos-Langerak et al. 2007). This phenomenon ensures specialized cells to accomplish their respective unique three-dimensional chromosomal architecture (Goetze, Mateos-Langerak et al. 2007, Goetze, Mateos-Langerak et al. 2007). Based on elegant transgenic mouse studies, activation of the hGH locus in the somatotrophs of the anterior pituitary gland requires “global” acetylation of a 32 kb chromatin domain centered and greatest at HS I/II, and “tracking” outwards in an ‘umbrella-like’ manner to include HS V upstream

and the *hGHI* promoter downstream during embryonic development (Ho, Elefant et al. 2002, Ho, Tadevosyan et al. 2008). (Figure 1.4, based on Vakili, Jin et al. 2012). Hyperacetylation of histones H3 and H4 catalyzed by histone acetyltransferase regulate the transcriptional activity of genes by determining the level of acetylation of the amino-terminal domains of nucleosomal histones of chromatin due to changes in electrostatic properties of histones (Vignali, Hassan et al. 2000). Increased histone hyperacetylation is associated with “open” and transcriptionally active chromatin (Iizuka and Smith 2003). This hyperacetylation pattern is dependent on the pituitary specific transcription factor Pit-1 binding at the HS I/II (Ho, Elefant et al. 2002). Thus, chromatin remodeling at HS I/II is permissive for the establishment of pituitary-specific expression of *hGHI*.

**Figure 1.4: Schematic representation of the histone hyperacetylation pattern for the human GH/CS locus in the pituitary**

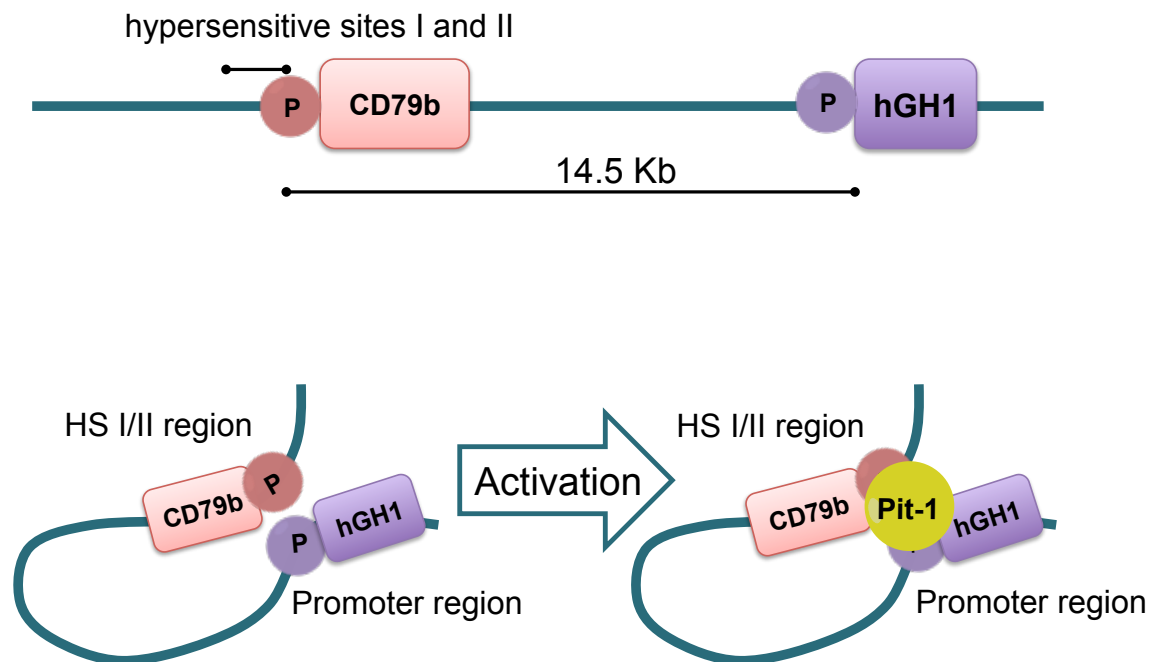
*The “global” histone hyperacetylation pattern for the 32 kb of chromatin domain centered and extending outwards from HS I/II to HS V upstream and the hGH1 promoter (p) downstream in pituitary somatotroph, with the highest level at the HS I/II is shown.*



In the case of the hGH/CS locus, the distant upstream LCR is critical for tissue/cell-specific activation of the pituitary *hGHI* promoter during embryonic development (Jones, Monks et al. 1995, Fleetwood, Ho et al. 2012). HS I/II possesses pituitary enhancer activity *in vitro* and *in vivo*, and is the major determinant of efficient expression (Jin, Surabhi et al. 1999). This HS I/II enhancer activity is located in the upstream-flanking DNA of the adjacent CD79b gene, but exerts its effect on the *hGHI* promoter about 15 kb downstream via a long-range interaction involving “looping” of intervening chromatin between these two regions (Ho, Elefant et al. 2002, Ho, Tadevosyan et al. 2008). Formation of this “expression” loop, and resulting physical interaction between pituitary transcription factor Pit-1 binding sites at HS I/II and the proximal promoter region is essential for *hGHI* activation during development (Jones, Monks et al. 1995, Ho, Tadevosyan et al. 2008) (Figure 1.5).

**Figure 1.5: Schematic representation of chromosomal looping implicated in pituitary hGH1 gene activation and expression**

*Formation of the “expression loop” occurs as a result of physical interaction between the HS I/II and the hGH1 promoter (p) regions and looping out of the intervening chromatin (14.5 kb). This looping is associated with activation of the locus and efficient expression. Previous studies demonstrated that this physical interaction is mediated by pituitary specific transcription factor Pit-1 at cognate sites both at HS I/II located within the promoter (p) of both the CD79b and hGH1 genes (Hunsaker, Jefferson et al. 2012).*



There are only few gene loci where specific interactions required for expression *in vivo* between a remote LCR and proximal promoter sequences separated by long distances (often thousands of base pairs) have been described. Examples of such gene loci are: **(i)** the  $\beta$ -globin locus (Grosveld, van Assendelft et al. 1987), **(ii)** the erythroid-specific alpha globin locus (Hanscombe, Vidal et al. 1989, Higgs, Wood et al. 1990), **(iii)** the T cell specific CD2 locus (Greaves, Wilson et al. 1989), **(iv)** the macrophage-specific lysozyme gene (Bonifer, Vidal et al. 1990), **(v)** human red and green visual pigment genes (Wang, Macke et al. 1992), **(vi)** T cell receptor alpha/delta locus (Diaz, Cado et al. 1994), and of course the human GH/CS gene locus (Su, Liebhaber et al. 2000). Among these, only the globin and hGH/CS loci chromosomal conformation have been studied extensively, and critical regulatory components within each locus have been identified. The hGH/CS locus is distinct from the globin locus in that it presents a distinctive model to study the molecular mechanisms by which a group of closely related and recently evolved genes has become specialized to express related products in two separate tissues. This contrasts with the  $\beta$ -globin locus, where expression is restricted to a single (erythroid) tissue type with diverse expression during development (Bulger, van Doorninck et al. 1999). It should be noted, however, the commonality of underlying mechanisms as how these loci are activated has played a critical role towards the expansion of our knowledge in regards to one another.

Despite the essential role chromatin structure and the “expression” loop plays in the specific activation of the hGH/CS locus in the developing pituitary, the continued importance of this chromosomal configuration or its components in terminally



differentiated pituitary cells (somatotrophs) has not been established. This chromosomal arrangement of the hGH locus may lend itself to further regulatory control and thus may serve as a target point for post-developmental transcriptional regulation of *hGHI*. **As such, the question asked in this thesis is whether the physical contact between a remote LCR and more local regulatory promoter element is “fixed” or is subject to modification and perhaps reconfiguration as part of the post-developmental transcriptional regulatory process by physiological but also environmental cues (overeating and prescribed physical activity) (Chapters 5 and 6).** Any insight obtained by studying the hGH/CS locus may contribute to our uncovering the mystery in biology of how our genome integrates intrinsic and environmental signals, and responds at the transcriptional level accordingly to maintain a homeostatic balance.

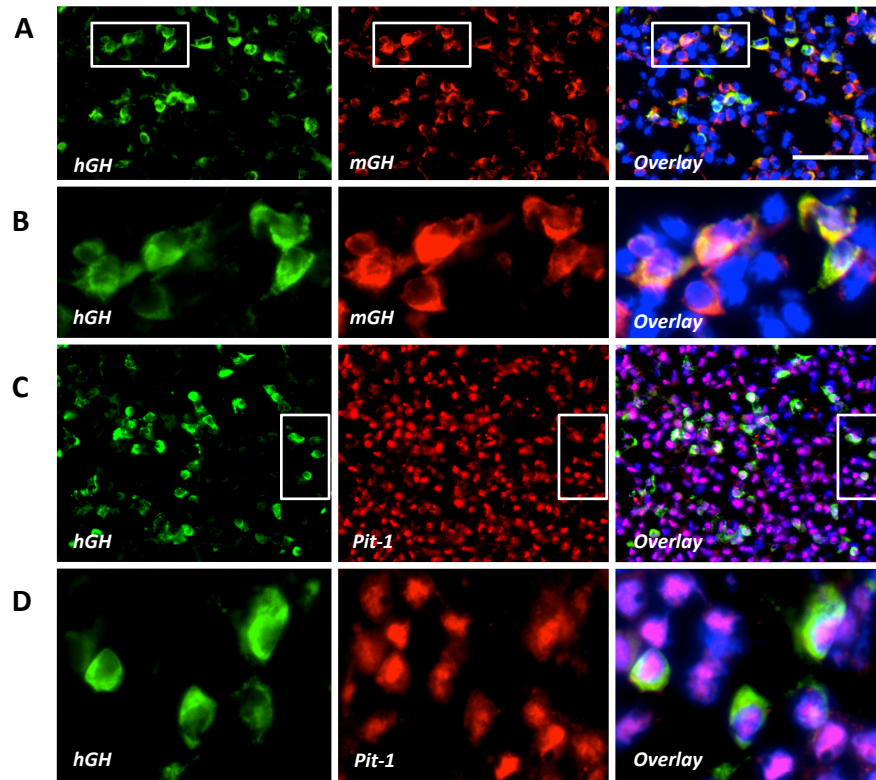
### ***1.12. Human GH/CS transgenic mice***

In the absence of access to human pituitary samples, transient transfection of (pituitary) cell lines has offered a relatively rapid and useful means to assess possible DNA elements for effects on promoter activity and, by extension, implicate protein interactions in hGH gene regulation. This system does not lend itself readily, however, to studies linked to those described above (Section 1.8) and specifically studies: **(i)** of *hGHI* related chromatin remodeling: **(ii)** that satisfy additional and complementary requirements for inclusion of the intact *hGHI* LCR, promoter and gene, as well as **(iii)** with pituitary cells of a non-tumor origin.

In response to the lack of a model system or “pituitary context”, transgenic mice were generated containing the intact *hGHI* and LCR (Jones, Monks et al. 1995, Jin, Lu et al. 2009). As discussed, the LCR is sufficient to confer appropriate site of integration-independent expression of a gene, including a transgene (Grosveld, van Assendelft et al. 1987, Jones, Monks et al. 1995, Elefant, Cooke et al. 2000, Jin, Lu et al. 2009, Vakili, Jin et al. 2011). As a result, others and we observed site of integration-independent and/or pituitary expression of *hGHI* in multiple independent transgenic mouse lines with inclusion of the hGH LCR (Jones, Monks et al. 1995, Jin, Lu et al. 2009, Vakili, Jin et al. 2011). Cells producing hGH in pituitaries from hGH/CS TG mice were assessed by immunocytochemistry in tissue sections and in primary pituitary cell culture. Our data indicate that greater than 95% of hGH-positive cells are also positive for mGH *in situ*, and represent greater than 10% of anterior pituitary cells (Vakili, Jin et al. 2011). The

hGH-positive cells are also somatotrophs based on being positive for Pit-1 as well as GH releasing hormone (GHRH) receptor (GHRH-R) (Vakili, Jin et al. 2011) (Figure 1.6), which are both somatotroph markers in the context of the anterior pituitary (Mayo, Godfrey et al. 1995, Miller, Godfrey et al. 1999, Urbano, Suarez-Penaranda et al. 2000).

**Figure 1.6 <sup>1</sup>: Double-labeling and immunohistochemical detection of hGH, mGH and Pit-1 in sections of 171 hGH/CS TG mouse pituitary *in situ***



(A) Staining for both hGH using Alexa488 secondary antibody (green) and mGH using Cy3 (red). (B) High power images of the boxed regions in (A) showing GH-positive cells with variable intensity of hGH versus mGH staining. (C) Human GH-positive cells co-stained consistently for Pit-1, which was co-localized with DAPI (blue) staining of the nucleus. (D) High power images of the boxed regions in (C), which are rotated 90° in clockwise direction. Bar = 50  $\mu$ m (A and C) and 15.5  $\mu$ m (B and D).

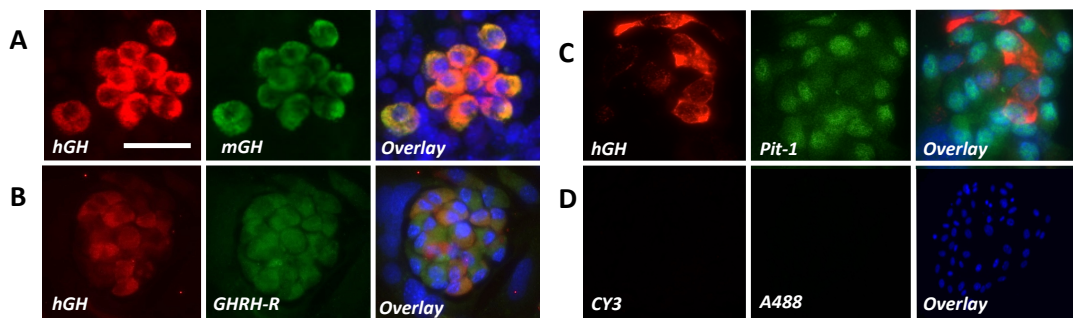
<sup>1</sup> Reprinted figure: Vakili H, Jin Y, Nagy JI, Cattini PA, **Transgenic mice expressing the human growth hormone gene provide a model system to study human growth hormone synthesis and secretion in nontumor-derived pituitary cells: Differential effects of dexamethasone and thyroid hormone**, *Molecular and Cellular Endocrinology*, 345:48-57, 2011. © Elsevier

Primary pituitary cell cultures were developed as a component of a characterization of hGH/CS TG mice. The hGH-producing cells were positive for mouse GH, GHRH receptor and pituitary transcription factor Pit-1 in culture (Figure 1.7) and the latter was also detected in TG mouse pituitary gland sections *in situ* (Figure 1.6). An interesting observation was the suggestion that a variable contribution of mGH and hGH to the total cellular GH levels was present in sections and monolayer culture by immunodetection. Examples of GH-producing cells spanning the spectrum from hGH-positive/mGH-negative through hGH-positive/mGH-positive to hGH-negative/mGH-positive were observed. The co-expression of mGH and hGH was expected based on the inclusion of the intact hGH LCR (Jin, Lu et al. 2009). The latter implies that the hGH1 transgene and its product are also subject to regulatory or feedback control together with the endogenous mGH. This would be consistent with the varied contribution of hGH to the total GH (hGH and mGH) observed in sections through the anterior pituitary *in situ* and confirmed by immunocytochemistry in primary cultures. While this would explain the detection of hGH-positive/mGH-negative somatotrophs in primary cultures, their presence also raises the alternative possibility that non-somatotroph cells are capable of expressing hGH. The positive co-staining for Pit-1, however, indicates these alternative cells would include only lactotrophs and/or thyrotrophs (Li, Crenshaw et al. 1990, Radovick, Nations et al. 1992). Furthermore, these cells were also positive for GHRH receptor, which is a somatotroph marker in the context of the anterior pituitary (Mayo, Godfrey et al. 1995, Miller, Godfrey et al. 1999, Urbano, Suarez-Penaranda et al. 2000, Japon, Urbano et al. 2002). On this basis, the hGH-positive/mGH-negative cells are somatotrophs. Thus, the variable contribution of hGH and mGH to the total GH staining

of somatotrophs in culture, either relates: **(i)** to a pre-culture/tissue effect or, more likely, **(ii)** reflects production or accumulation of GH in response to local feedback involving the GH/IGF-1 axis, and that hGH or mGH can be produced to meet feedback requirements. Certainly, IGF-1, IGF-1 receptor and GH receptor have been detected in normal human and mouse pituitary tissues (Schwartz 2000, Beuschlein, Hancke et al. 2005), and more importantly GH receptor has been localized by *in situ* hybridization and immunohistochemistry within the anterior pituitary to somatotrophs (Mertani, Delehay-Zervas et al. 1995, Mertani and Morel 1995, Mertani, Pechoux et al. 1995). Therefore, it is reasonable to suggest the existence of an ultra-short loop feedback mechanism that could contribute to such paracrine/autocrine regulation of both human and mouse GH expression. The significance of such an intra-pituitary ultra-short feedback loop in the pituitary remains to be determined. Primary pituitary cells isolated from TG mice were also used to investigate stimulation and repression of hGH production by dexamethasone and thyroid treatments, respectively, which suggests the capacity of the system for assessment of hormonal regulation (Vakili, Jin et al. 2011).

**Figure 1.7 <sup>2</sup>: Detection of hGH and mGH in somatotrophs in 171 hGH/CS TG mouse pituitary cultures**

(A) Staining for both hGH (Cy3, red) and mGH (Alexa488, green) in pituitary cell culture. Co-staining of hGH (red) with somatotroph-related proteins including: (B) G-coupled protein GH releasing hormone (GHRH) receptor (green); and (C) POU-homeodomain transcription factor Pit-1 (green). (D) Primary antibodies were omitted as a negative control. Cell nuclei were also stained with DAPI (blue) in all cases. Bar = 15.5  $\mu\text{m}$  (A-C) and 50  $\mu\text{m}$  (D).



<sup>2</sup> Reprinted figure: Vakili H, Jin Y, Nagy JI, Cattini PA, **Transgenic mice expressing the human growth hormone gene provide a model system to study human growth hormone synthesis and secretion in nontumor-derived pituitary cells: Differential effects of dexamethasone and thyroid hormone**, *Molecular and Cellular Endocrinology*, 345:48-57, 2011. © Elsevier

These hGH/CS TG mice express appropriate levels of GH, grow normally and show no evidence of gigantism due to overproduction of “total” GH (Jones, Monks et al. 1995, Su, Liebhaber et al. 2000, Ho, Elefant et al. 2006, Jin, Lu et al. 2009, Vakili, Jin et al. 2011, Vakili, Jin et al. 2012). This is reflected in an unchanged number of somatotrophs in hGH/CS TG versus wild type mice (Vakili, Jin et al. 2011), which is consistent with normal pituitary development. This suggests the presence of functional somatotrophs, and appropriate regulatory or feedback control of hGH1 together with endogenous mGH. These transgenic mice have been maintained for more than twenty eight generations and continue to express the hGH1 preferentially in pituitary somatotrophs under the control of the hGH LCR, with no apparent change in the pattern of expression (Jones, Monks et al. 1995, Su, Liebhaber et al. 2000, Ho, Elefant et al. 2006, Jin, Lu et al. 2009, Vakili, Jin et al. 2011).

The hGH/CS TG mouse model system offers a number of research opportunities:

- (i)** It provides a combined heterologous and homologous model system to study both transgenic *hGHI* and endogenous mGH gene expression.
- (ii)** This animal model is more accessible than non-human primate cells, which share more similarities with the *hGHI*.
- (iii)** Isolated primary pituitary cells from this animal model provide an alternative to pituitary tumor cell cultures, and can be used to investigate the effect of a variety of endocrine and cytokine factors on the *hGHI*. More importantly, this culture system can



be manipulated experimentally for dissecting molecular signaling pathways. This includes knock down or overexpression of genes, and/or inhibition or stimulation of numerous signaling pathways that are suspected to mediate an effect of a studied factor on the *hGHI*.

*(iv)* The pituitary tissue and derived cell cultures lend themselves to studies of *hGHI* locus/LCR-related chromatin remodeling, which is not possible in transient transfection model systems before (as presented in this thesis).

### ***1.13. Overall hypothesis and research objectives***

As reviewed in previous sections, a negative correlation exists between GH and insulin levels as obesity progresses. It is hypothesized that high levels of circulatory insulin regulate hGH production during the initial stages of high fat diet-induced obesity development in hGH/CS TG mice. It is further hypothesized that this regulation occurs at the transcriptional level and involves changes in the local three-dimensional chromatin structure of the *hGHI* locus control (with known enhancer activity) and promoter regions. This structure is manifest as a chromatin loop, reflecting a physical interaction between the remote enhancer and promoter regions, which is critical for transcriptional activity. Thus, this three dimensional structure is referred to as “expression loop”. Moreover, these changes are associated with a decrease in *hGHI* expression. Finally, the change in *hGHI*-related chromatin induced by excess caloric intake can be influenced by to all intents and purposes an intervention in *lifestyle* or more specifically incorporation of increased physical activity in a treatment regimen.

Specific ***research objectives*** are:

1. To assess the impact of acute excess caloric intake on hGH synthesis and secretion in hGH/CS TG mice fed a high fat diet for three days (Chapter 3)
2. To investigate direct regulation of *hGHI* by insulin in an isolated primary pituitary cell cultures derived from hGH/CS TG mice, and the underlying mechanism (Chapter 4)

3. To investigate the impact of high fat diet induced hyperinsulinemic state *in vivo*, and a direct effect of insulin *in vitro* on the hGH locus chromatin remodeling and chromosomal architecture (Chapter 5)

4. To determine whether introduction of acute swim activity will prevent (or modify) the negative effect of excess caloric intake on hGH production as well as any changes observed in the organization of the chromatin associated with the hGH locus (Chapter 6)

## CHAPTER 2

### Materials and methods

#### *2.1. Animal ethics approval*

All procedures involving animals, their tissues and cells conform to the Guide for the Care and Use of Laboratory Animals published by the Canadian Council on Animal Care, and was approved by the Animal Protocol Management and Review Committee at the University of Manitoba. Animals were individually housed with *ad libitum* access to food and water in an environmentally controlled room maintained on a 12-hour light/dark cycle (6:00 pm lights off / 6:00 am lights on).

#### *2.2. Transgenic mouse model*

Transgenic (TG) mice were generated containing the intact *hGH1* and locus control region (LCR) corresponding to sequences 14.5-32 kb upstream in 171 kb or 141 kb fragments of human chromosome 17 in a CD1 genetic background. These are termed 171hGH/CS TG and 141hGH/CS TG mice, and were described previously as F-74 and F-81 mice, respectively (Jin, Lu et al. 2009). Both 171hGH/CS TG and 141hGH/CS TG mice express hGH1 specifically in the pituitary and hCS in the placental labyrinth (Jin, Lu et al. 2009). Use of these mice, referred to as 171 and 141 hGH/CS TG mice, are

specified in each figure legend. Wild type CD1 mice were also used to assess mGH and as a non-transgenic/non hGH-producing control.

### ***2.3. Animals and diet***

Transgenic as well as wild type CD1 mice were housed under a 12 h light-dark cycle with ad libitum access to water and regular rodent chow. For experiments, control (LFD; fat, 10 kcal%; carbohydrate, 70 kcal%; protein, 20 kcal% from Research Diets, New Brunswick, NJ) or high-fat (HFD; fat, 60 kcal%; carbohydrate, 20 kcal%; protein, 20 kcal% from Research Diets) diets were supplied as palatable pellets for three days, three weeks and 10 weeks to four-week-old male mice. Body weights were recorded prior and daily during the diet intervention. Daily food and energy intake was estimated by weighing remaining food at the end of each day/week and was used to calculate average daily caloric intake. Mice were maintained on their respective diets until time of euthanization and assay.

### ***2.4. Swimming protocol***

Swimming as a prescribed form of physical activity was conducted as reported previously with minor modifications (Jimenez, Jassal et al. 2011). Four-week-old male hGH/CS TG mice on a standard diet were acclimatized initially in a water bath (50 x 30 x 20 cm - 6 mice per water bath) filled with fresh water (34–37° C) for increasing 10-minute intervals over 5 days with continuous monitoring. Mice were placed under a heat

lamp on clean dry towels after swimming, to help shorten time for drying fur and to help keep their body temperature from dropping. Once animals were dry, they were returned to their designated cages. Post five days of acclimation, mice were allowed to rest for two days prior to the onset of the study. Mice then swam twice daily for three days for 50 minutes (2 x 25 minutes per day) while they were on a LFD or HFD. Mice were weighed daily and their daily calorie intake was also measured throughout the study.

### ***2.5. Blood chemistry***

Trunk blood was collected from fed mice and allowed to clot at 4° C for 15 minutes and serum was separated by centrifugation (9300 x g for 5 minutes). Serum samples were stored at -80 ° C until assessed by ELISA (human GH, 22 ALPCO, 22-HGHHUU-E01; mouse GH, Millipore, EZRMGH-45K; mouse insulin, mouse/rat insulin ELISA, Millipore, EZRMI-13K; mouse C-peptide, mouse/rat C-peptide ELISA, Millipore, EZRMCP2; mouse total IGF-1, R&D Systems, MG200). Blood glucose level was measured by a handheld glucometer (OneTouch Ultramini, Lifescan, Inc). The specificity of the hGH ELISA kit was confirmed using wild type CD1 serum as a negative control and data obtained were comparable to the “blank” control, and thus levels of hGH in wild type CD1 mice is considered “not detectable”. Cross reactivity of hGH and mGH using the mGH ELISA kit from Millipore (EZRMGH-45K) is reported by the company as not detected.

## ***2.6. Primary pituitary cell culture***

For primary pituitary cell cultures, ten adult male and female (8-10 week) mice were euthanized by cervical dislocation. The anterior pituitary glands were dissected and placed in (2 ml) calcium-free phosphate buffered saline (PBS-CMF) with 0.1% bovine serum albumin (0.1% BSA/PBS). The tissue fragments were rinsed with 0.1% BSA/PBS and incubated in 0.1% BSA/PBS solution containing hyaluronidase (1mg/ml), trypsin inhibitor (0.5 mg/ml), pancreatin (0.5 mg/ml) and collagenase type I (1 mg/ml) for 10 min at 37 °C in a shaking water bath. The digestate was collected in 5 ml and added to 20 ml of fetal bovine serum (FBS). The digestion of the tissue fragments was done for total three times. Digestion was completed by adding 150 units of DNase I per 50 ml of digested supernatant, with gentle mixing for 2 min at room temperature. After filtering, cells were pelleted (1200 x g for 5 min) before resuspension in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS (10% FBS-DMEM). Trypan blue dye exclusion was used to determine the viability of cells. Survival was routinely greater than 90%. Cells were plated at  $1 \times 10^5$  cells/well in 24-well plates, with or without a poly L-lysine-coated coverslip, in 1 ml of 10% FBS-DMEM with antibiotics (10 UI/ml penicillin, 10 mg/ml streptomycin), and maintained in this medium for 72 hours.

## ***2.7. Hormonal/chemical treatment of primary pituitary cell cultures***

For insulin treatment, cells were re-fed DMEM supplemented with 1% double charcoal stripped FBS (1% dcsFBS) for 24 hours, and then treated with 1-200 nM insulin (Sigma, Oakville, ON, I9278) for 24 hours.

To induce HIF-1 $\alpha$  protein levels, cells were incubated with 150-500  $\mu$ M cobalt (II) chloride hexahydrate (CoCl<sub>2</sub>; Sigma, C2644) for 5 and/or 24 hours. To interfere with HIF-1 $\alpha$  DNA binding, cultures were pre-incubated for 60 minutes with 10 or 20 nM echinomycin (Enzo Life Sciences, PA, USA, ALX-380-201) or vehicle (dimethyl sulfoxide) prior to insulin treatment. Similarly, inhibition of histone deacetylases (HDACs) class I, II or III was done using pretreatment with trichostatin A (300 nM, Sigma, T1952) or nicotinamide (15 mM, Sigma, N0636), respectively, for 60 minutes.

## ***2.8. Lentivirus-mediated short hairpin RNA (shRNA) treatment***

HIF-1 $\alpha$  shRNA (Santa Cruz, sc-35562-V) and control/scrambled shRNA lentiviral particles (Santa Cruz, sc-108080) were used for HIF-1 $\alpha$  “knock down” as previously reported (Nam, Ko et al. 2011). The viral infection was performed according to manufacturer’s instructions. Briefly, cultures were incubated with DMEM containing lentiviral particles in the presence of Polybrene (5  $\mu$ g/ml, Santa Cruz) for 24 hours.



Lentiviral particles were washed away and cells were incubated for a further 48 hours. Medium was then changed to 1% dcsFBS-DMEM for 24 hours before treatment with insulin for 24 hours, harvesting and analysis.

## ***2.9. RNA preparation and quantitative real-time reverse transcriptase-PCR***

Total RNA was isolated using QIA shredder and RNeasy Plus Mini Kit (Qiagen, ON, Canada, 79656, 74136). Quality of the extracted RNA was assessed by agarose gel electrophoresis. Total RNA (1 µg) was transformed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, 205314) according to the manufacturer's instructions. Quantitative real-time reverse transcriptase (RT) -PCR (qPCR) analyses were done in a 7500 system (AB Applied Biosystems, Warrington, UK) with specific primers (Table 2.2). Reactions (20 µl) included 10 µl of Power SYBR green Master mix (Applied Biosystems), 1µl of forward and reverse primers (0.5 pM) and 0.1 µg of cDNA. Thermal cycling was started with 5 min denaturation at 95 °C, followed by 40 cycles of 95 °C for 30 seconds, annealing at 60 °C for 15 seconds and 72 °C for 30 seconds. Standard curves were generated using plasmids containing the amplicon sequences for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Minus RT controls were done using the same PCR primers and PCR conditions as a control for genomic DNA contamination. Specific amplifications were identified by a single peak in the melting curve and a single band in the final PCR product visualized on 1% agarose gel. The gene expression level in each sample was calculated from a standard curve (for each primer set) and normalized to

mouse GAPDH and/or  $\beta$ -2 microglobulin (B2M) expression as appropriate. Tests were run at least in duplicate and on three independent samples for each experiment.

### ***2.10. Plasmid constructs***

Digested plasmids with restriction enzymes (obtained from New England BioLabs (NEB) or Promega) were purified from 1% agarose gels using a gel purification kit (Qiagen). Ligations were done with T4 ligase (NEB) at room temperature after overnight (~16 hours) digestions.

#### ***2.10.1. Hybrid reporter constructs***

The plasmid Renilla Luciferase (pRL-TKp-Luc) was purchased from Promega. The pXP1 luciferase plasmids were a kind gift from Dr. Steve Nordeen from Department of Pathology, University of Colorado.

#### **1) Human GH1 promoter luciferase construct (hGH1p-WT and hGH1p-Mut):**

Mutation of E-box element (wild type (WT) 5'-CCACGTGACC- 3') into mutant (5'-CCAgcTGACC- 3') in the 0.5 kb *hGH1* promoter region was generated using a two-step PCR reaction approach and use of specific primers, as described previously (Jin,

Oomah et al. 2011). In brief, the two corresponding double-stranded components of the fragment to be modified were generated containing a 21-nucleotide overlap of the region to be mutated. After mixing equimolar amounts of each fragment, melting, annealing and a second round of PCR was done using primers to introduce *Bam*H1 (5'-GGATCC- 3') and *Hind*III (5'-AAGCTT- 3') sites for subcloning into the *Bam*H1 and *Hind*III sites of the firefly luciferase vector, pXP1-Luc, under the control of a minimal thymidine kinase (TK) promoter (TKp).

## **2) Mouse GH promoter luciferase construct (mGHp-Luc):**

The 0.5 kb of the *mGH* promoter region was generated by PCR using a set of primers (Forward: 5'-GATCGGGATCCTCCCCAAAAGTTATCTTCT- 3', Reverse: 5'-CGATCAAGCTTGGAACTCTGGACTCTAGGATG- 3') that introduce *Bam*H1 (5'-GGATCC- 3') and *Hind*III (5'-AAGCTT- 3') sites for subcloning into the *Bam*H1 and *Hind*III sites of the firefly luciferase vector, pXP1-Luc, under the control of a minimal thymidine kinase (TK) promoter (TKp).

The PCR product was purified using a QIAquick PCR product purification kit (Qiagen), digested with *Bam*H1 and *Hind*III, isolated and inserted into the same restriction enzyme site of pXP1-Luc.

### **3) Human period circadian protein homolog 2 (Per2) luciferase construct (pGL3-hPer2-Luc):**

The construct was kindly provided by Dr. Louis Ptacek (University of California, San Francisco) (Xu, Toh et al. 2007).

#### ***2.10.2. Expression vectors***

The cDNA expression vectors for mouse circadian locomotor output cycles kaput (Clock), previously reported as Gal4-Clock (UA256), and brain and muscle ARNT-like 1 (Bmal1), previously reported as (Gal4-Bmal1/UA255), were kindly provided by Dr. Urs Albrecht (University of Fribourg, Switzerland) (Langmesser, Tallone et al. 2008).

#### ***2.11. Plasmid transformation***

The DH5 $\alpha$  strain of *Escherichia coli* was used for the production of all plasmids. The MAX Efficiency® DH5 $\alpha$ ™ Competent Cells were obtained from Invitrogen (18258-012) and introduction of plasmid DNA was done according to the manufacturer's instructions. The transformed DH5 $\alpha$  cells were then plated onto LB/Agar plates containing the appropriate selected antibiotics.

### ***2.12. Plasmid isolation***

Plasmid DNA was extracted using the QIAprep® Spin Miniprep Kit (Qiagen) based on the manufacturer's instructions. For small-scale plasmid preparation, single bacterial colonies were incubated in 2 mL of L-Broth containing appropriate antibiotics and were grown at 37 °C with vigorous shaking for ~16 hours. For larger scale plasmid preparation, single bacterial colonies were selected and were incubated in 5 mL of L-Broth with appropriate antibiotics and were grown ~16 hours at 37 °C. Five mL of the starter culture was then transferred to 250 mL L-Broth with appropriate antibiotics for a larger culture, grown at 37 °C for 12-16 hours with vigorous shaking. Plasmid DNA was extracted using the Plasmid Maxi Kit (Qiagen). Final DNA pellets were re-suspended in TE buffer (0.1M Tris-HCl pH 8.0, 1mM EDTA).

### ***2.13. Transient transfection of human embryonic kidney 293 cells***

Human embryonic kidney 293 (HEK293) cells were maintained in a monolayer culture in Dulbecco's modified Eagle's Medium (DMEM) (Invitrogen Corp., 12100-061) supplemented with 500 µM glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, supplemented with 10% (v/v) FBS in a humidified air/CO<sub>2</sub> (19:1) atmosphere, at 37°C (Graham, Smiley et al. 1977). Cells were washed with phosphate buffered saline (PBS). Cells were harvested using trypsin- EDTA after a single wash in PBS.

For transient transfection of HEK293 cells, a Mirus Trans-IT293 kit (Mirus Biol Corp., MIR 2700) was used. In brief cells were plated at a density of  $1 \times 10^5$  cells in a 12-well plate 24 hours prior to transfection. The “Trans-IT293 reagent” was diluted with serum-free DMEM. Expression vectors, or alternatively, hybrid reporter gene and pRL-TKp-Luc (as a control for DNA uptake) vectors were mixed into the diluted reagent. For expression of cDNA vectors,  $1 \times 10^6$  cells were plated per 100 mm plate. Up to 4  $\mu$ g of plasmid DNA (expressing vector) were used in each 100 mm plate. For reporter gene vectors,  $2.5 \times 10^5$  cells were plated into each well of a 6-well plate. Up to 0.5  $\mu$ g of reporter gene plasmid and 10 ng pRL-TKp-Luc plasmid were used in each well. Complex of DNA/reagent was then added to each plate. DNA was washed away from cells 24 hours post transfection and cells were collected 72 hours after gene transfer. Protein extraction and luciferase assay were done immediately after harvesting.

#### ***2.14. Luciferase assay***

Luciferase activity was measured using the Dual-luciferase assay system (Promega Corp., PRE1960), according to manufacturers instructions, using a photon counting luminometer (LUMAT LB9507, EG&G Berthold). In brief, cell pellets were resuspended in 50  $\mu$ L of lysis buffer (100 mM Tris- HCl pH 7.8, 0.1% Triton X-100) for 10 minutes on ice, and centrifuged at  $15800 \times g$  for 15 minutes at 4° C. Values were normalized through co-transfection with pRL-TKp-Luc and assessment of Renilla luciferase in a dual assay as well as with protein concentration, which was assessed using

the Bradford Protein assay.

### ***2.15. Nuclear protein extraction and detection***

Nuclear extraction from cells in cultures and mouse pituitary tissue was performed using a nuclear extraction kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA, 54001) (Aza-Carmona, Shears et al. 2011). Briefly, following treatment, cells were suspended in hypotonic buffer (20 mM HEPES, pH 7.5, 5 mM sodium fluoride, 10  $\mu$ M sodium molybdate, and 0.1 mM ethylenediaminetetraacetic acid) containing phosphatase inhibitors and incubated on ice for 15 minutes. Detergent (Nonidet P-40) was added to a final concentration of 0.5%, and incubation was continued for 15 minutes. Nuclei were separated by centrifugation (14,000  $\times$  g for 30 seconds at 4°C), and the nuclear pellet was next extracted in a hypertonic lysis buffer for 30 minutes on ice. Following centrifugation (14,000  $\times$  g for 10 minutes at 4°C), the supernatant containing the pure nuclear fraction was collected. Protein concentration was determined by a Bradford protein assay (Bio-Rad, Richmond, CA, USA).

For detection of HIF-1 $\alpha$ , 25  $\mu$ g (after CoCl<sub>2</sub> treatment) or 100  $\mu$ g (after insulin treatment) of whole cell protein or nuclear extracted proteins was analyzed by protein immunoblotting as previously described (Norquay, Yang et al. 2006). For detection of Clock, Bmal1 and USF-1, 20  $\mu$ g of nuclear and cytoplasmic proteins isolated from HEK293 cells expressing the expression cDNA vectors were immunoblotted. Proteins

were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane and immunoblotted with anti-HIF-1 $\alpha$  Clock, Bmal1, and USF-1 antibodies as appropriate. Beta ( $\beta$ )-tubulin was used as control for protein loading for whole cell lysates (Table 2.1). Lamin B, USF-1, and Histone H1 were also assessed as controls for protein loading for nuclear proteins (Table 2.1). Each protein was visualized using horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) secondary antibody and ECL plus immunoblotting detection reagents (Thermo Fisher Scientific Inc, ON, Canada).

#### ***2.16. Electrophoretic mobility shift assay (EMSA)***

EMSA and competition with oligonucleotides was performed essentially as previously described (Norquay, Yang et al. 2006). Briefly, 500 ng of recombinant HIF-1 $\alpha$  and 500 ng of HIF-1 $\beta$  or 4  $\mu$ g of nuclear extract isolated from HEK293 cells were incubated with EMSA buffer containing 2- $\mu$ g poly-dIdC for 5 minutes. One ng of radiolabeled oligonucleotide probes was then added and the reactions were incubated for a further 10 minutes at room temperature. In competition experiments, 2.5, 5, and 50-fold molar excess of unlabeled oligonucleotide duplexes were added during a pre-incubation period. The DNA-protein complexes were resolved in non-denaturing 5% (w/v) polyacrylamide gels, which was then visualized by autoradiography.



### ***2.17. Chromatin immunoprecipitation (ChIP) assay***

ChIP assay with primary pituitary cell cultures treated with or without insulin or CoCl<sub>2</sub> were done as previously described (Yang, Jin et al. 2010). In brief, 2 x 10<sup>6</sup> cells were harvested 24 hours after insulin or CoCl<sub>2</sub> treatments in cold phosphate buffered saline buffer and cross-linked with 1% of formaldehyde for 10 minutes before lysis. ChIP assay was also done with mouse anterior pituitaries (8-10 pituitary glands from male hGH TG mice/ChIP assay) using EZ-Magna ChIP kit (Millipore, 17-10086) according to the manufacturer's instructions. In brief, pituitaries were cross-linked with 1% of formaldehyde at room temperature for 30 minutes before lysis. ChIP assay was also done using primary pituitary cell culture with a pool of 3 preparations (total of 30 TG male and female mice). Primary pituitary cells were harvested 24 hours after insulin or CoCl<sub>2</sub> treatments in cold phosphate buffered saline buffer and cross-linked with 1% of formaldehyde before lysis for 15 minutes. Chromatin was fragmented by sonication (100 seconds in 10-second pulses and 40% amplitude). Insoluble material was removed by centrifugation. DNA content was then measured by spectrophotometry. Soluble chromatin was pre-cleared for 1 hour and then immunoprecipitated with 5 µg of specific antibodies as indicated in figure legends and Table 2.3 overnight with rotation at 4°C. Protein A/G plus agarose (Santa Cruz) was added to immunoprecipitation reaction with rotation for 1 hour at 4°C to collect antibody/chromatin complexes. The agarose-antibody complexes were subjected to a series of washes and elution (Yang, Jin et al. 2010). The eluted antibody complexes were reverse cross-linked at 65 °C overnight, and DNA was isolated using QIAquick PCR purification kit (Qiagen). Quantitative PCR was performed

in a 7500 Fast Real Time PCR system (Applied Biosystems), under conditions standardized for each primer set (Table 2.4). Each qPCR reaction was carried out in duplicate in a 20  $\mu$ L reaction volume by using 5  $\mu$ L of the 1% of input DNA and 5  $\mu$ L of pre-amplified (15 cycles) eluted immunoprecipitated DNA and 10  $\mu$ L of Power SYBR green Master mix (Applied Biosystems). Dissociation curves were analyzed as a means to ensure the quality of amplicons and to monitor primer dimers. Final PCR products were visualized as a single band on 1% agarose gel. ChIP Enrichment was determined based on a percent input method (Yochum, McWeeney et al. 2007). Briefly, the signals obtained from the immunoprecipitated DNA amplification were divided by the signals obtained from an input sample. As the starting input fraction was 1%, a dilution factor of 100 or 6.644 cycles (i.e.,  $\log_2$  of 100) was subtracted from the CT value of diluted input.

Enrichment was calculated based on the formula,  $100 \times 2^{(CT \text{ adjusted Input} - CT \text{ Enriched})}$  and data are presented as percent input and relative fold change compared to the control which is arbitrarily set to 1. For ChIP assay of histone acetylation in the hGH locus, hyperacetylation levels are presented as relative fold-changes compared to levels detected at HS V, which are arbitrarily set to 1.

### ***2.18. Chromosome conformation capture (3C) assay***

The 3C assay was performed essentially as described (Dekker, Rippe et al. 2002, Miele and Dekker 2009). Briefly, mouse pituitaries (4 pituitary glands/3C test) were homogenized in a tissue nuclei isolation buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM PMSF, 0.1% NP-40) supplemented with complete protease inhibitors (Thermo Scientific) to obtain cell suspension. The cells were cross-linked for 10 minutes at room temperature using 1% formaldehyde-HEPES buffer and the reaction was quenched by addition of 0.125 M glycine. The cells were washed with PBS, and lysed in 1 ml of ice-cold lysis buffer (10 mM Tris-Cl; pH 8.0, 10 mM NaCl, 0.2% NP-40) supplemented with complete protease inhibitors (Thermo Scientific). Isolated nuclei were washed with NEB buffer 3 (50 mM Tris-HCl pH 7.9, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM DTT; New England BioLabs) and were resuspended in 120 µL of NEB buffer 3 containing 0.1% sodium dodecyl sulfate (SDS), and incubated for 30 minutes at 37° C with shaking. Triton X-100 was added (1% final), and then the nuclei were further incubated for 30 minutes at 37° C. Cross-linked DNA was then digested overnight with the restriction enzyme *Bgl*II (500 units) in a total volume of 200 µl. Selection of *Bgl*II for 3C analysis was based on appropriately located restriction sites in the vicinity of chosen DNA targets. An aliquot of DNA was collected before and after the digestion to determine the digestion efficiency. The restriction enzyme was inactivated by adding SDS (1% final) and incubating at 65° C for 20 minutes. Two µg of digested chromatin was diluted in a final volume of 0.8 mL containing 1% Triton-X. Ligation reaction was carried out at 16° C for 4 hours and followed by incubation at room temperature for a

further 30 minutes in the presence of 4000 units of T4 DNA ligase (New England BioLabs). A “no ligation control” without addition of T4 DNA ligase was set up under identical condition. Reverse cross-linking of ligated DNA was carried out overnight at 65 °C in the presence of proteinase K with the final concentration of 10 ng/mL. The following day, samples were incubated for 30 minutes at 37 °C with RNase A (10 ng/ml) followed by purification of DNA using phenol/chloroform extraction and ethanol precipitation method. DNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific). Nested primers were used with two rounds of PCR to increase sensitivity, which was followed by qPCR using a primer set that encompassed the *Bg*III restriction cut site (Table 2.5) under the following conditions using the Power SYBR PCR Kit (Applied Biosystems): the initial denaturation for 5 minutes at 95 °C, 30 cycles of 95 °C for 15 sec, 60 °C for 15 sec, and 72 °C for 30 sec. The results were normalized with amplified GAPDH of digested and undigested samples. Digestion efficiency for each sample was >84%. The ligated 3C qPCR product was sequenced to confirm the identity of the product (Robarts Institute, London, ON, Canada). Interaction between HS I/II and the *hGHI* promoter (GHp) was determined by calculating the ligation frequency according to the formula:

$$\underline{[Ligation\ frequency = (level\ of\ ligation\ product/loading\ control) - (level\ of\ non-ligation\ product/loading\ control)]}$$

### ***2.19. Statistical analysis***

Statistical analysis was performed using GraphPad InStat® and Prism® software. For single comparisons, paired t-tests were applied, and for multiple comparisons, one-way ANOVA was used with the Tukey-Kramer or Bonferroni post-test as appropriate. For multiple comparisons with more than one variable, data were analyzed using two-way ANOVA with the Bonferroni post-test. A value of  $p < 0.05$  is considered statistically significant and represented in figures as: \* or #,  $p < 0.05$ ; \*\* or ##,  $p < 0.01$ ; \*\*\* or ###,  $p < 0.001$ .

**Table 2.1: Antibodies used for immunoblotting (IBlot)**

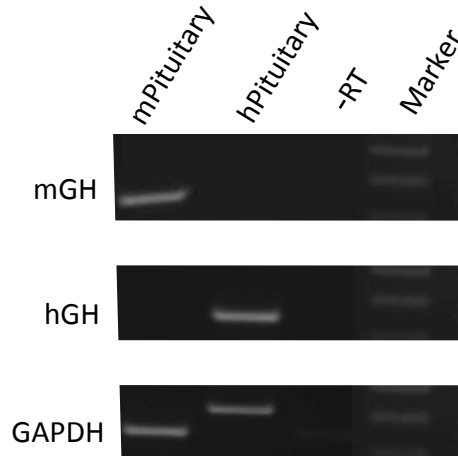
<b>Antibody</b>	<b>Species</b>	<b>Source</b>	<b>Dilution</b>
HIF-1 $\alpha$	Rabbit	Novus Biologicals (NB100-134)	IBlot (1:2000)
USF-1	Rabbit	Santa Cruz (sc-229)	IBlot (1:1000)
Histone H1	Goat	Santa Cruz (sc-8616)	IBlot (1:1000)
Clock	Rabbit	Abcam (ab3517)	IBlot (1:2000)
Bmal1	Rabbit	Abcam (ab3350)	IBlot (1:1000)
$\beta$ -tubulin	Rabbit	Santa Cruz (sc-9104)	IBlot (1:1000)
Lamin B	Goat	Santa Cruz (sc-6217)	IBlot (1:500)

**Table 2.2: Primers used for quantitative polymerase chain reaction (qPCR)**

h = human, m = mouse

<b>Transcript</b>	<b>Primer sequence</b>
m B2M	For: GCTATCCAGAAAACCCCTCAA Rev: GCGGGTGGAAGTCTGTTCG
m/h GAPDH	For: TCACCACCATGGAGAAGGC Rev: CCTAAGCAGTTGGTGGTGCA
h GH1*	For: CCTAGAGGAAGGCATCCAAA Rev: GCAGCCCGTAGTTCTTGAGTAG
m GH*	For: ACGCGCTGCTCAAAAATAT Rev: CACAGGAGAGTGCAGCAGAG
m GHRH	For: TGTGATCCTCATCCTCACCA Rev: ATCCTCTCCCCTTGCTTGTT
m GHRH-R	For: TCCTGTGCAAGGTCTCTGTG Rev: CCAGCACTCAGTGCCTCAA
m GHS-R	For: CTGGACAAAGTCGAGCATCA Rev: CTGCCATCTGGCTCTACTC
m Somatostatin	For: TCTGCATCGTCCTGGCTTT Rev: CTTGGCCAGTTCCTGTTTCC
m SST1	For: TGCCCTTTCTGGTCACTTCC Rev: AGCGGTCCACACTAAGCACA
m SST2A	For: CCCATCCTGTACGCCTTCTT Rev: GTCTCATTAGCCGGGATTT
m SST2B	For: TGATCCTCACCTATGCCAACA Rev: CTGCCTTGACCAAGCAAAGA
m SST3	For: GCCTTCTTCGGCCTCTACTT Rev: GAATGCGACGTGATGGTCTT
m SST4	For: AGGCTCGTGCTAATGGTGGT Rev: GGATGAGGGACACATGGTTG
mSST5	For: ACCCCCTGCTCTATGGCTTT Rev: GCTCTATGGCATCTGCATCCT
m TSH $\beta$	For: TCTGTGCTGGGTATTGTATGAC Rev: GCGGCTTGGTGCAGTAGTTG
m VEGF	For: AGCACAGCAGATGTGAATGC Rev: AATGCTTTCTCCGCTCTGAA

\* **Figure 2.1: Specificity of primers to human and mouse GH transcripts** <sup>3</sup>



*To test the primers specific to human (h) and mouse (m) GH transcripts, total RNA was extracted using QIA shredder and RNeasy Plus Mini Kit (Qiagen) from wild type CDI mouse and human pituitary tissues. Post-mortem and anonymised human pituitary tissue was obtained from the Human Pituitary Repository, Protein and Polypeptide Laboratory, University of Manitoba as described previously (Norquay, Yang et al. 2006, Yang, Jin et al. 2010) Total RNA (1 µg) was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Reactions (20 µL) included 10 µL of Power SYBR green Master mix, 1 µL of forward and reverse primers (0.5 pM) and 0.1 µg of cDNA as discussed in methods section. PCR products from mouse pituitary and human pituitary and GAPDH amplicons were visualized on a 2% agarose gel. The gel electrophoresis revealed presence of GAPDH amplicons in all the cell samples suggesting equal loading. No evidence of cross-reactivity was observed between the primers used for detection of hGH1 and mGH transcripts.*

<sup>3</sup> Reprinted figure: Vakili H, Jin Y, Nagy JI, Cattini PA, **Transgenic mice expressing the human growth hormone gene provide a model system to study human growth hormone synthesis and secretion in nontumor-derived pituitary cells: Differential effects of dexamethasone and thyroid hormone**, *Molecular and Cellular Endocrinology*, 345:48-57, 2011. © Elsevier



**Table 2.3: Antibodies used for chromatin immunoprecipitation (ChIP)**

<b>Antibody</b>	<b>Species</b>	<b>Source</b>
HIF-1 $\alpha$	Rabbit	Novus Biologicals (NB100-134)
NCOR	Rabbit	Thermo Scientific (PA1-844A)
Total RNA pol II	Rabbit	Santa Cruz (sc-899)
Ser5-pRNA pol II	Mouse	Covance (MMS-134R)
Hyperacetylated Histone H3	Rabbit	Upstate Biotechnology (Cat# 06-599)
Hyperacetylated Histone H4	Rabbit	Upstate Biotechnology (Cat# 06-598)

**Table 2.4: Primers used for ChIP-qPCR**

h = human, m = mouse

<b>Genomic regions</b>	<b>Primer sequence</b>
hGH1p-HBS	For: CACAGAGTGTCAGCCAGAGATA Rev: GGATGTGGTTCGGTAGGGGGT
-0.5 kb hGH1 promoter	For: CCCCTTCTCTCCCCTGTTG Rev: AACCTCACAACACTGGTGAC
-2 kb hGH1 promoter	For: CTGTGTCCACCTGCAGAGTT Rev: AGCTTCTTCCATGTTCTCC
HS I/II	For: CATGGGCCTCAAGCTGACCT Rev: CGTTCCGGGCAGCCCCAGAT
HS III	For: CACTGATGAGCTTGGCGTCAC Rev: CCTGCCACTTCCGCTCTCCA
HS V	For: TCCCTCGGACCAGAACAC Rev: CCCAGGTAAAAGCAGCATGT
mGH promoter	For: ATGGTCTCAGAGGACACACC Rev: TCATGTCCCTGACCTTATCGT
Untr6	For: TCAGGCATGAACCACCATAC Rev: AACATCCACACGTCCAGTGA

**Table 2.5: Primers used for the chromosome conformation capture (3C) assay**

<b>Genomic regions</b>	<b>Primer sequence</b>
Nested PCR HS I/II-GH1p	HS I/II: ACAGCACCCATGAGGCAACA GHp: CAGGATAGCCAGTCCTTGAGAC
qPCR HS I/II-GH1p	HS I/II: CCATTCTTAACCTCTCAGTGACCA GHp: GAAAAAGAAAAAGAAAGATGCCCTGT

## CHAPTER 3

### **Human growth hormone synthesis is rapidly suppressed by acute excess caloric intake: A possible effect of hyperinsulinemia**

#### ***3.1. Rationale and specific aim***

Growth hormone insufficiency is considered a typical feature of obesity, regardless of age and gender (Vanderschueren-Lodeweyckx 1993). This is in addition to a spectrum of other significant metabolic derangements including changes in levels and/or peripheral responsiveness to multiple endocrine factors, as defined by hyperinsulinemia, hyperleptinemia, hyperglycemia and high levels of free fatty acids (De Marinis, Bianchi et al. 2004, Pataky, Bobbioni-Harsch et al. 2010).

GH is a major metabolic homeostatic factor, which is involved extensively in lipid metabolism and implicated in adipose tissue remodeling to help maintain a healthy balance between energy intake and expenditure via its strong lipolytic activity (De Marinis, Bianchi et al. 2004, Yang, Mulder et al. 2004, Pataky, Bobbioni-Harsch et al. 2010). Obesity and its associated metabolic changes are brought on by excessive energy intake with insufficient energy expenditure over a prolonged period. This leads to a greater excess weight/height ratio, reflecting additional adipose tissue expansion (Frayn 2005). It is noteworthy that GH levels are extremely sensitive to increased caloric intake within the continuum of obesity progression. A recent study identified a 77% decrease in plasma GH levels, without any significant weight gain, in a group of healthy individuals

of age 24 who overate for three days (Cornford, Barkan et al. 2011). It was suggested that hyperinsulinemia is the likely mediator of the rapid suppression of human GH production seen at three days, which occurs independent of significant changes in body weight (Cornford, Barkan et al. 2011). Furthermore, GH levels continued to be suppressed within two weeks of overeating by which the subjects had gained significant weight (Cornford, Barkan et al. 2011). Thus, GH insufficiency appears to be a feature at all stages of obesity development. The events characterizing this suppression are incompletely understood. It was suggested that the effect of excess insulin was to impair GH secretion, however, the data do not exclude a negative effect of overeating on GH synthesis by pituitary somatotrophs. This is an important point since an effect on synthesis might affect interpretation of data and specifically the perceived effectiveness of GH secretagogues as anti-obesity agents.

Thus, in this study, the effect of acute excess caloric intake and possible associated hyperinsulinemic state through use of high fat diet on the regulation of human GH synthesis as well as secretion by the pituitary was determined in two independent lines of TG mice (171 and 141 hGH/CS TG).

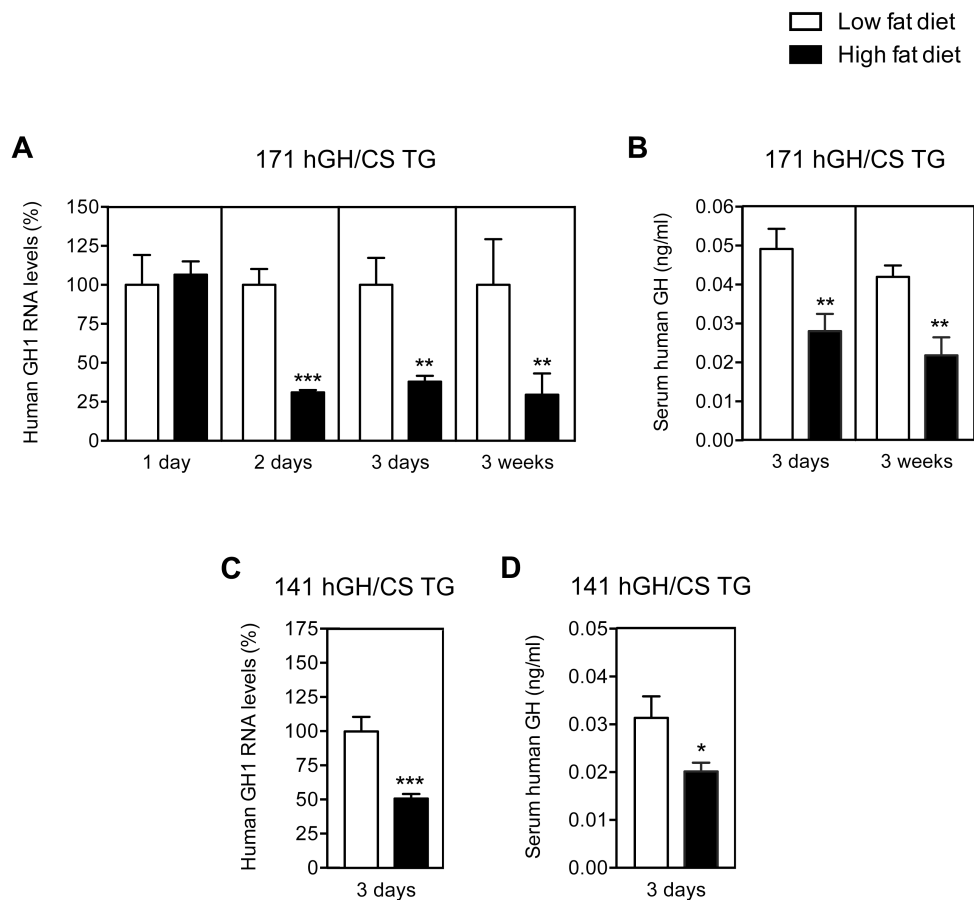
## RESULTS

### ***3.2. Human GH synthesis and secretion are decreased within three days on a high fat diet***

To determine the impact of acute high caloric intake on hGH production, hGH/CS TG mice were fed either a high fat diet (HFD) or a control low fat diet (LFD) at four weeks of age. Use of 60% HFD is accepted as a means of inducing a rodent model of HFD-induced obesity with associated metabolic dysfunctions (West, Waguespack et al. 1995). Mice were maintained on their respective diets until time of euthanization and assay. One day after introduction of the HFD, there were no detectable differences seen in *hGHI* RNA levels of mice on the LFD and HFD as assessed by reverse transcription and quantitative polymerase chain reaction (qPCR). Starting from the second day, however, there was a significant 75% reduction in *hGHI* transcript levels ( $p < 0.001$ ,  $n=4-12$ ). This reduction remained significantly low on the third day and was still evident after three weeks on the HFD ( $p < 0.01$ ,  $n=6$ ) (Figure 3.1A). Serum hGH levels in mice on the HFD were also reduced to 50% of those detected in mice on the control LFD throughout the study period (three days and three weeks) as assessed by enzyme-linked immunosorbent assay (ELISA) ( $p < 0.01$ ,  $n=6-12$ ) (Figure 3.1B). A similar decrease in hGH RNA (50%,  $p < 0.001$ ,  $n=4-6$ ) and serum hGH (32%,  $p < 0.05$ ,  $n=4-5$ ) levels was also observed in a second hGH/CS TG line (141hGH/CS) in response to three days on a HFD (Figure 3.1C and D).

**Figure 3.1<sup>4</sup>: Decreased hGH synthesis and secretion within three days on a HFD**

(A) Total pituitary RNA from 171hGH/CS TG mice fed a HFD (black-filled) versus a LFD (white-filled) was assessed for hGH RNA levels by qPCR. The results are expressed as the mean (percentage)  $\pm$  standard error of mean relative to the mean value determined for LFD, which is arbitrarily set to 100%. The significance of any effect was assessed for each time point by t-test (n=4-12). (B) Secreted hGH levels were also assessed by ELISA at three days and three weeks. The results at each time point are expressed as mean concentration (ng/ml)  $\pm$  standard error of mean, and significance was assessed as above. The effect of a HFD versus LFD for three days on hGH (C) RNA and (D) serum levels was also assessed in a second (141 hGH/CS) TG mouse line as described in (A) and (B) above (n=4-5).



<sup>4</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

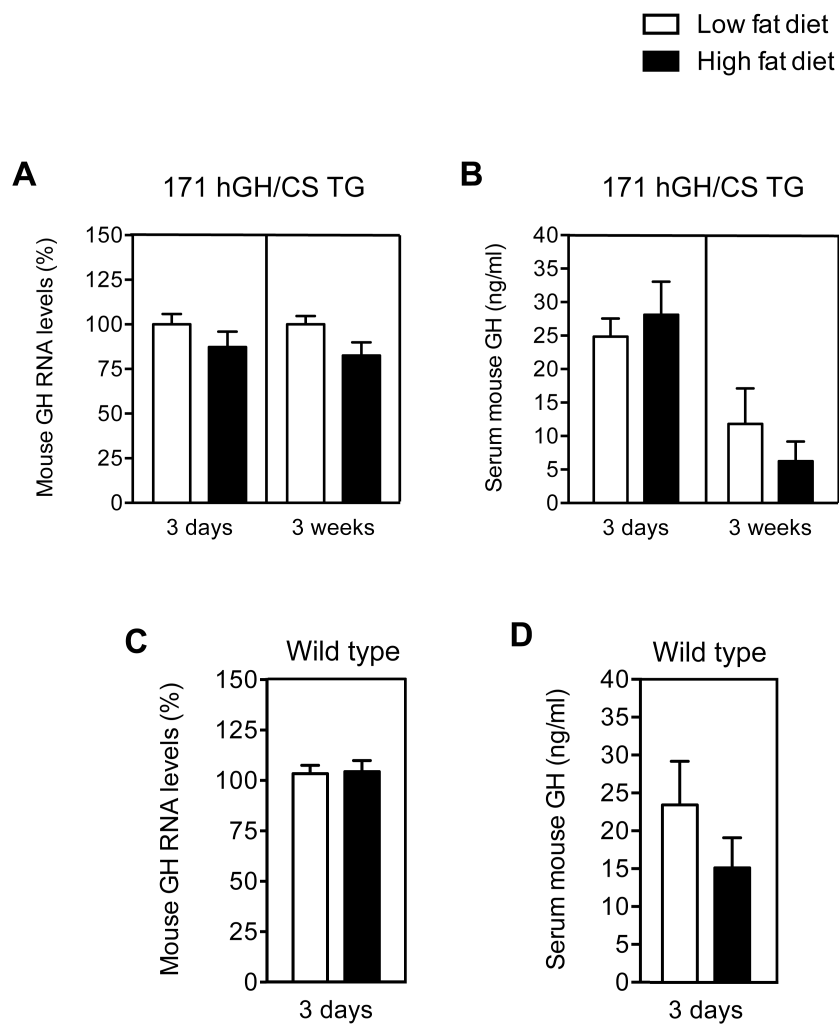
### **3.3. Mouse (m) GH production was not affected by three days of a HFD challenge**

The availability of hGH/CS TG mice provides a combined heterologous and homologous model system to study both transgenic human *GHI* and endogenous mouse *GH* gene expression. Thus, the impact of the three days and three weeks of high fat diet regimen on mouse GH production (transcript levels in the pituitary and protein levels in serum) was investigated. Unlike human *GHI*, there were no detectable differences seen in mGH RNA levels in mice on the LFD versus HFD for three days as assessed by qPCR (Figure 3.2A). This lack of response was sustained in the hGH/CS TG mice even after three weeks on the HFD; a decreasing trend was observed, but this did not become statistically significant. There was also no significant effect of the HFD versus LFD on secreted mGH levels as assessed by ELISA. There was, however, an apparent reduction in secreted mGH levels after three weeks on the HFD, but as with RNA levels, this did not reach statistical significance (Figure 3.2B). Like the hGH/CS TG mice, there was also no detectable differences seen in mGH RNA and serum levels in wild type CD1 mice fed the HFD versus LFD for three days as assessed by qPCR and ELISA (Figure 3.2C and D).



**Figure 3.2<sup>5</sup>: Mouse GH production is not affected by three days of a HFD challenge**

Mouse pituitary GH RNA and serum protein levels were assessed in 171hGH/CS TG mice fed a HFD (black-filled) versus LFD (white-filled) for three days or three weeks by qPCR and ELISA, respectively. (A) The results for RNA are expressed as the mean (percentage)  $\pm$  standard error of mean relative to the LFD mean value, which is arbitrarily set to 100% (n=4-12). (B) For serum mGH, the results are expressed as mean concentration (ng/ml)  $\pm$  standard error of mean. The levels of mGH RNA (C) and secreted protein (D) were also assessed in wild type CD1 mice at three days as above (n=6). The significance of any effect of HFD versus LFD was assessed at each independent time point by t-test.

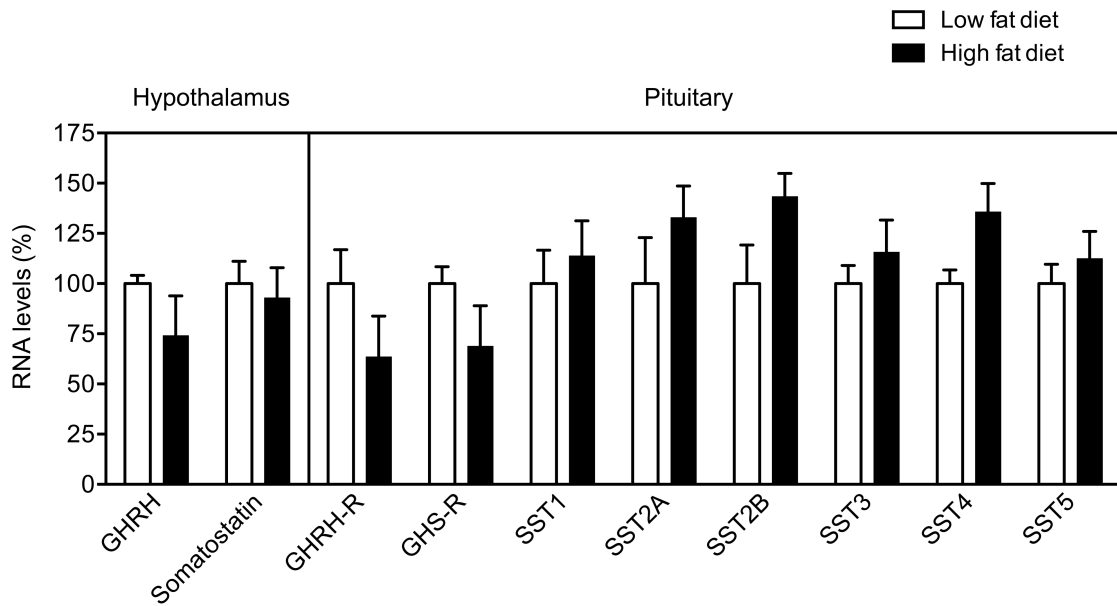


<sup>5</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

***3.4. The HFD-induced reduction in hGH production is not associated with significant changes in hypothalamic factor/receptor gene expression at the pituitary level***

Growth hormone release from the pituitary is pulsatile and is predominantly regulated by two hypothalamic factors: growth hormone releasing hormone (GHRH) and somatostatin. GHRH is synthesized by neurons of the arcuate nucleus and somatostatin, is mainly synthesized by neurons in the periventricular nucleus of hypothalamus (Lin, Lin et al. 1993, Xu, Lavinsky et al. 1998, Cohen, Hashimoto et al. 1999). RNA levels for GHRH and the GH inhibitor somatostatin in the hypothalamus, and their corresponding receptors GHRH-R and SST 1-5 in the pituitary, were assessed in hGH/CS TG mice fed a LFD versus HFD for three days. Overeating had no significant effect on GHRH, somatostatin or their respective receptor RNA levels (Figure 3.3). A decreasing trend in the levels of GHRH-R and GH secretagogues receptor (GHS-R) RNAs is suggested, as well as an increase in SST transcripts, however, these did not reach statistical significance with 9-12 mice per diet group (Figure 3.3).

**Figure 3.3<sup>6</sup>: HFD had no significant effect on GHRH, somatostatin or their respective receptor RNA levels**



*Total pituitary and hypothalamic RNA from 171hGH/CS TG mice fed a high fat (black-filled) versus low fat (white-filled) diet was assessed by qPCR using specific and independent primer sets for mouse GHRH and somatostatin transcripts, as well as GHRH receptor (GHRHR), GH secretagogues receptor (GHS-R) and somatostatin (SST) receptor RNAs. The results are expressed as mean (percentage)  $\pm$  standard error of mean relative to the value determined for LFD, which is arbitrarily set to 100%. Significance was assessed by t-test ( $n=9-12$ ).*

<sup>6</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

### ***3.5. HFD for three days created a state of excess insulin but not hyperglycemia***

A significant increase in caloric intake (1.5-fold,  $p < 0.001$ ) was observed in hGH/CS TG mice fed a HFD for three days (Table 3.1). ELISA was used to determine non-fasting serum insulin and C-peptide levels, as a surrogate marker for insulin secretion (O'Rahilly, Burnett et al. 1987, Clark and Hales 1994). Blood glucose levels were measured with a glucometer, and changes in body and gonadal fat pad weight were assessed as a measure of adipose tissue expansion. The HFD for three days was associated with a state of excess insulin and C-peptide but not blood glucose, IGF-1 or significant changes in body and gonadal fat pad weight (Table 3.1).

**Table 3.1<sup>7</sup>: Systemic effects of three days of HFD consumption**

*Measurement of daily caloric intake, weight gain, fat mass as well as insulin, C-peptide, random blood glucose and IGF-1 levels in serums collected from fed 171hGH/CS TG mice on a HFD versus LFD for three days. Values are presented as mean  $\pm$  standard error of mean, and significance (*P* value less than 0.05) is assessed by *t*-test (*n*=5-12).*

	<b>LFD</b>	<b>HFD</b>	<b>P value LFD vs. HFD</b>
<b>Daily caloric intake (kcal)</b>	13.68 $\pm$ 0.51	19.03 $\pm$ 0.56	<0.001
<b>Weight gain (g)</b>	0.64 $\pm$ 0.28	0.82 $\pm$ 0.16	0.58
<b>Epididymal fat mass (g)</b>	0.51 $\pm$ 0.04	0.56 $\pm$ 0.04	0.30
<b>Insulin (ng/ml)</b>	0.95 $\pm$ 0.21	3.01 $\pm$ 0.67	<0.05
<b>C-peptide (pM)</b>	357.95 $\pm$ 24.92	561.13 $\pm$ 35.82	<0.01
<b>Blood glucose (mM)</b>	12.88 $\pm$ 0.71	13.96 $\pm$ 0.47	0.18
<b>IGF-1 (ng/ml)</b>	812.44 $\pm$ 59.92	776.38 $\pm$ 153.60	0.81

<sup>7</sup> Reprinted table: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

### ***3.6. Chapter discussion***

In this chapter, transgenic mice (hGH/CS mice) containing the human GH gene (*hGHI*) locus were used to assess the effect of high caloric intake on the *hGHI* expression. Our data emphasize that human (h) GH synthesis as well as secretion is extremely sensitive to increased caloric intake as early as after two days on a HFD. Interestingly, the GH level remains suppressed within the continuum of obesity progression (three weeks). In support of the latter, in the human study, persistent overeating for two weeks resulted in significant increased body weight and fat mass, and GH secretion remained suppressed (Cornford, Barkan et al. 2011).

Impaired spontaneous secretion of hGH and a decline in somatotroph responsiveness to all known pharmacological provocative stimuli is reported in obese humans (Vanderschueren-Lodeweyckx 1993, Maccario, Procopio et al. 1995, Procopio, Maccario et al. 1995, Pijl, Langendonk et al. 2001, Scacchi, Orsini et al. 2010). This suppression of hGH can occur because of impaired GH secretagogue signaling at the somatotroph level, however, this reduction can also be described in terms of a decrease in GH synthesis and thus insufficient reserves to be secreted in response to stimuli (Vakili and Cattini 2012). The data are consistent with the latter, and a negative impact of a HFD for three days on hGH production. The lack of a significant effect on hypothalamic GHRH and somatostatin transcripts and those of their respective pituitary receptors further supports a direct effect of overeating on hGH synthesis. Thus, it is possible that a HFD for three days does not impair GH secretagogue signaling significantly in the mouse

pituitary.

Short term overeating can cause a complex network of endocrine responses that can modulate endocrine and metabolic systems in favor of repressed GH levels (Cornford, Barkan et al. 2011). Nonetheless, a major finding of the *in vivo* studies here demonstrates a significant role for insulin regulation of hGH. Higher levels of serum insulin and C-peptide (surrogate marker for insulin synthesis) were observed in conjunction with the reduction in hGH levels in hGH/CS TG mice in response to short-term excess caloric intake. Not surprisingly and consistent with the hGH/CS TG mouse study, the hyperinsulinemia seen after three days in the human study was the only significant event identified to explain a potent suppressive impact of overeating on GH levels; there were no major changes measured in cortisol, total IGF-1, free fatty acids or glucose levels (Cornford, Barkan et al. 2011). Direct insulin regulation of human GH gene expression is explored in Chapter 4.

### ***3.7. Summary of the results***

- *Acute high caloric intake for three days results in hyperinsulinemia without hyperglycemia, and is associated with a decrease in hGH synthesis and secretion but not significant changes in mouse GH gene expression and/or secretion.*



## CHAPTER 4

### **Human growth hormone gene expression is negatively regulated by insulin**

#### *4.1. Rationale and specific aim*

A reduction in human (h) GH synthesis is associated with increased circulating levels of insulin in response to a short-term excess caloric intake (data presented in Chapter 3). Review of the literature provides evidence supporting a direct role for insulin acting at the pituitary level and regulating GH gene expression in rodent models of obesity induced by long term excess caloric intake (Luque and Kineman 2006). This regulation was not accompanied by any alteration in GHRH and somatostatin expression in the hypothalamus of the obese mice (Luque and Kineman 2006). These data are also consistent with observations made on the effect of short-term excess caloric intake on hGH production with no significant effects on GH-related hypothalamic factors and their respective receptors (as presented in Chapter 3).

Certainly, the mouse pituitary gland appears to be an insulin sensitive tissue since insulin receptors are present at levels comparable to “classical” insulin sensitive tissues, such as adipose, liver and skeletal muscle (Luque and Kineman 2006). Furthermore, there is evidence that pituitary cells continue to be sensitive to insulin while the cells from other peripheral tissues become more resistant from chronic hyperinsulinemia in the

obese state (Luque and Kineman 2006, Brothers, Wu et al. 2010).

Thus, in this study, the impact of insulin treatment in primary monolayer cultures of transgenic mouse pituitary cells containing the intact *hGH1* and GH LCR was investigated. This *in vitro* culture serves as a complementary system to the *in vivo* transgenic mouse model for investigating hGH1 gene/protein regulation. Furthermore, it allows the potential for dissection of the molecular mechanism mediating insulin regulation of both hGH and mGH genes, as well as, perhaps, for identifying the basis for the differential impact of acute excess caloric intake on hGH versus mGH gene expression (as described in Chapter 3).

## RESULTS

### ***4.2. Endogenous hGH and mGH RNA levels are negatively regulated by insulin***

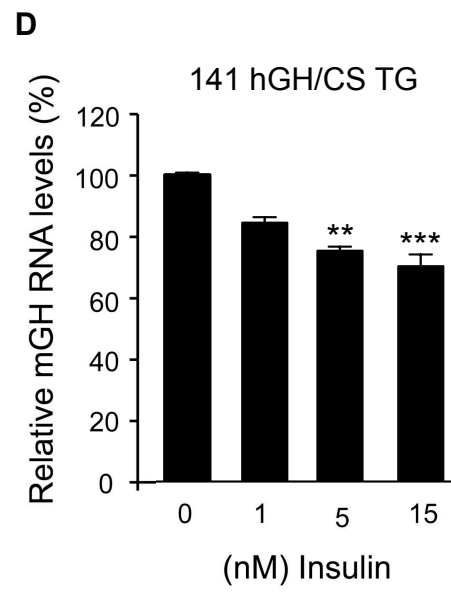
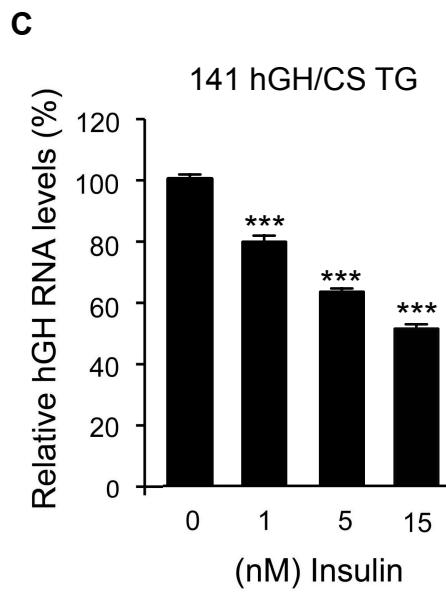
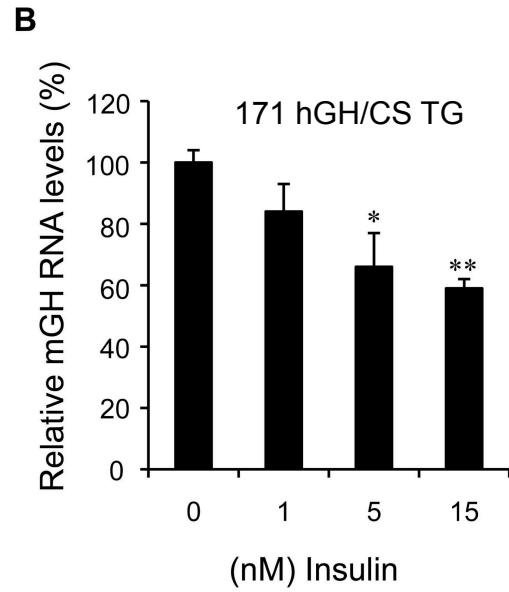
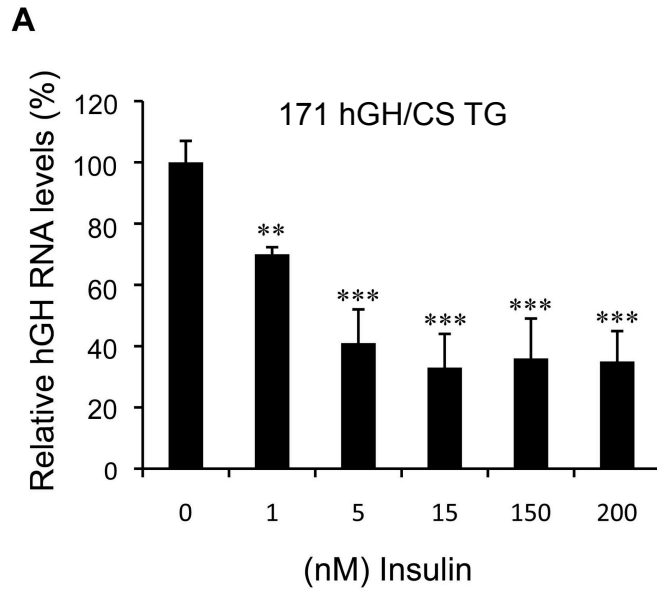
Cultures were generated from hGH/CS TG mouse pituitaries as has been described previously (Vakili, Jin et al. 2011), and treated with insulin to determine whether the “endogenous” and intact *hGHI* is responsive. Primary pituitary cells were de-induced in 1% dcsFBS-DMEM for 24 hours and treated with 1, 5, 15, 150 or 200 nM insulin for 24 hours. Levels of hGH or mGH RNA were assessed in each sample by qPCR using absolute quantification and normalization to mouse B2M RNA. Human GH RNA levels decreased significantly in response to both physiological (1nM) and pharmacological (200 nM) doses of insulin (Figure 4.1A). This decrease reached a plateau (~60% decrease) at 5 nM ( $p<0.001$ ,  $n=12$ ). A similar decrease in hGH RNA levels was observed in cultures from a second independent (141hGH/CS TG) mouse line (Jin, Lu et al. 2009) with insulin (Figure 4.1C). A decrease in mGH transcripts was also seen with 1 nM insulin treatment but unlike hGH RNA levels was not significant (Figure 4.1B and D), however, a significant and increasing ~40% reduction in mGH RNA levels was also observed with 5 and 15 nM insulin ( $p<0.01$ ,  $n=6$ ).

**Figure 4.1 <sup>8</sup>: Endogenous hGH and mGH RNA levels are negatively regulated by insulin in two independent TG lines**

*(A) Human GH RNA levels were decreased at physiological (1 nM) and pharmacological (200 nM) concentrations of insulin. (B) Mouse GH transcripts were also reduced in response to insulin treatment, but was only significant at insulin concentrations above 1 nM. RNA levels in each sample (absolute quantification) were calculated from the standard curve and normalized to mouse B2M RNA. The effect of insulin treatment on (C) hGH RNA and (D) mGH RNA levels was also assessed in a second (141 hGH/CS) TG mouse line as described in (A) and (B) above (n=4-5). The results are expressed as relative mean change, plus or minus standard error of the mean, compared to the control (0) value, which is arbitrarily set to 100%. Data were assessed by one-way ANOVA with the Tukey-Kramer post-test. A value of  $p < 0.05$  is considered statistically significant:  $p < 0.05$ , \*;  $p < 0.01$ , \*\*;  $p < 0.001$ , \*\*\*.*

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<sup>8</sup> Reprinted modified figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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### ***4.3. Detection of an enhancer box (E-Box) element in the proximal hGHI promoter region***

There are no available data on the effect of insulin on endogenous *hGHI* expression, in spite of evidence for negative control of human and non-human primate GH by excess insulin as well as obesity (Prager and Melmed 1988, De Marinis, Bianchi et al. 2004, Luque, Gahete et al. 2006). The proximal region (nucleotides -496/+1) of the *hGHI* promoter was shown, however, to respond negatively to insulin treatment after transfection into rat pituitary tumor cells (Lefevre, Imagawa et al. 1987, Peritz, Fodor et al. 1988, Prager and Melmed 1988, Prager, Gebremedhin et al. 1990). This correlates with a study in which insulin treatment was associated with induction of a DNA-protein interaction at nucleotides -308/-235 in the proximal *hGHI* promoter region (Lefevre, Imagawa et al. 1987, Peritz, Fodor et al. 1988, Prager and Melmed 1988, Prager, Gebremedhin et al. 1990). Additionally, protein binding to this region has been shown by nuclease protection assay using pituitary rat GC cell and human non-pituitary HeLa cell (cervical epithelium carcinoma) nuclear extracts, however no tissue specificity was observed (Lefevre, Imagawa et al. 1987). The last data points to involvement of a more ubiquitous transcription factor as opposed to a tissue and/or cell specific transcription factor. Inspection of these sequences reveals an Enhancer Box (E-box), which includes a palindromic hexanucleotide sequence (CACGTG) at position -264 to -259 that is not present in the equivalent mGH sequences (Figure 4.2). As such, it was hypothesized that these sequences might play a role in the differential regulation of hGH and mGH genes as reflected in Chapter 3 in response to excess caloric intake.

**Figure 4.2<sup>9</sup>: An enhancer box (E-Box) DNA element is located within nucleotides -278/-250 of the proximal *hGH* promoter region**

*Alignment and comparison of human (nucleotides -327/-1) and mouse (nucleotides -355/-1) GH gene proximal promoter regions (Krawczak, Chuzhanova et al. 1999). Conserved TATA box and Pit-1 binding sites are indicated. The human (*hGH*) but not the mouse *GH* (*mGH*) promoter region contains a palindromic hexanucleotide region (Enhancer box).*

```
GCTATCTGACATCCTTCGCCGCGTGCAG::::GTTGG::CCACCATGG::::::::::CCTGCCG:CCAGA -273 hGH
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
GATATCC:::TACCCTT::::::::::GGATTCAAAATGGTCTCAGAGGACACACCCGGGTGGGCTCCAGA -299 mGH

Enhancer box
GGGCACCCACCTGACCCCTT:::::AAA:::::GAGAGGACAAGTTGGGTGGTATCTCTGGCTGACACTCTGTGCACA -206 hGH
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
:GT:ATCC::::TACCCTTGGATTCAAAATGGTCTCAGAGGACACACCCGGGTGGGCTCTGTGCTGGGA:TCT:TGCATA -227 mGH

ACC::::CTCACAACTGTTGACGGTGG:GAAGGAAAGATGACAA:GCCAGGGGCATGATCCCA:::::::::: -145 hGH
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
ACCAATGCCATAAGCCTGGCAAAGGTGGCGATG:::AGACGATAAGGTCA:GGGACATGACCGCAGAAGAGGAGTGGG -151 mGH

Pit-1 binding site Pit-1 binding site
:::GC:ATGTGTGGGAGGAGCTTCTAAATTATCCATTAGCACAAAGCCCGTCAGTGGCCCC:::ATGCATAAATGTACAC -72 hGH
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
GACCGCATGAGTGGGAGGAGCTTCTAAATTATCCATCAGCACAAAGC:TGTCAGTGGCCCGAGCCATGAATAAATGTATAG -73 mGH

TATA Box
A:GAAA::CAGG::::TGGGGTCAACAGTGGGAGAGAAGGGCCAGGGTATAAAAGGGCCACAAAGAGACCAGCT:CA -1 hGH
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
GGGAAAGGCAGGAGCCTTGGGGTC:::::GAGGAAAACAGGT:AGGGTATAAAAGGGCACGCAAGGGACCAAGTCCA -1 mGH
```

<sup>9</sup> Reprinted modified figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, *Journal of Biological Chemistry*, 287:33282-33292, 2012.  
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#### ***4.4. Interference with E-box related factor binding reduces the effect of insulin***

The effect of inhibiting E-box related transcription factor binding on hGH RNA levels in primary pituitary cell cultures was assessed. Echinomycin is a cyclic peptide, antibiotic and specific DNA binding factor with a strong affinity for the 5'-ACGTG-3' sequence. This sequence is at the core of E-box (Formica and Waring 1983, Van Dyke and Dervan 1984). Cultures were incubated with 10 nM echinomycin an hour prior to insulin treatment. Human GH, mGH RNAs were assessed by qPCR after 24 hours. The mouse GHS-R RNA levels were also assessed as a positive control since GHS-R is a known insulin-responsive gene (Luque, Gahete et al. 2006, Luque and Kineman 2006). Pretreatment with echinomycin interfered significantly with the negative effect of 1, 5 and 15 nM insulin on hGH transcript levels (Figure 4.3A); this effect was greater than 50% at all doses of insulin. In contrast, the significant decreases in mGH and GHS-R RNA levels were not affected by echinomycin pretreatment ( $p < 0.01$ ,  $n = 6$ ) (Figure 4.3B and C).

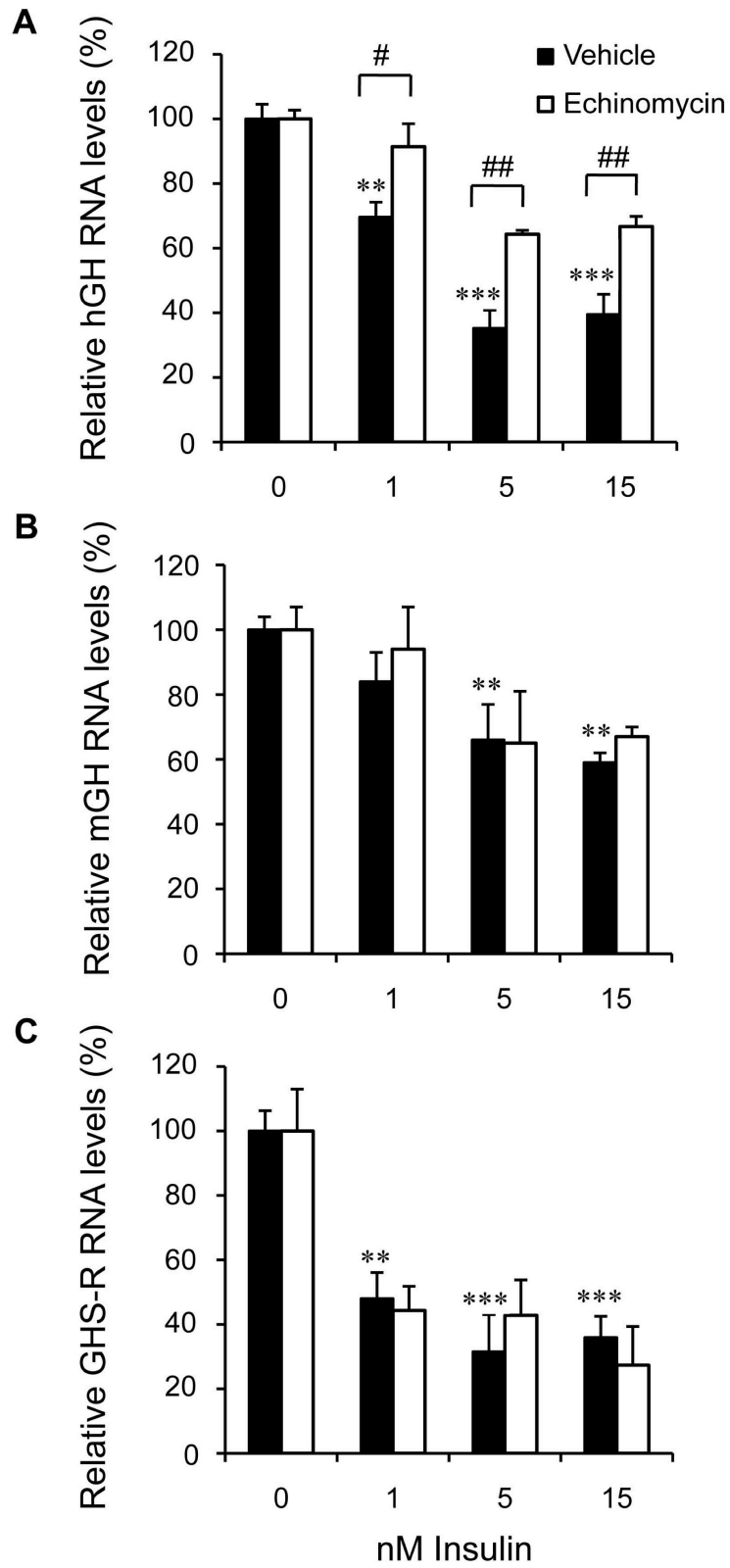


**Figure 4.3 <sup>10</sup>: Interference with E-box binding reduces the effect of insulin on hGH RNA levels**

*(A) Human GH, (B) mGH and (C) GHS-R RNA levels were assessed by qPCR 24 hours post insulin treatment. Dose dependent decreases in hGH, mGH and GHS-R RNA levels were observed, however, the decrease in hGH RNA was the only one affected significantly by echinomycin treatment. Results are expressed as relative mean change, plus or minus standard error of the mean, compared to control values, which are arbitrarily set to 100. Data were analyzed by two-way ANOVA with the Bonferroni post-test. Significant differences between groups at each concentration of insulin are indicated with brackets;  $p < 0.05$ , #;  $p < 0.01$  ##. Significant differences between insulin treatments compared with no insulin group (0) as control, are indicated by  $p < 0.01$ , \*\*;  $p < 0.001$ , \*\*\*.*

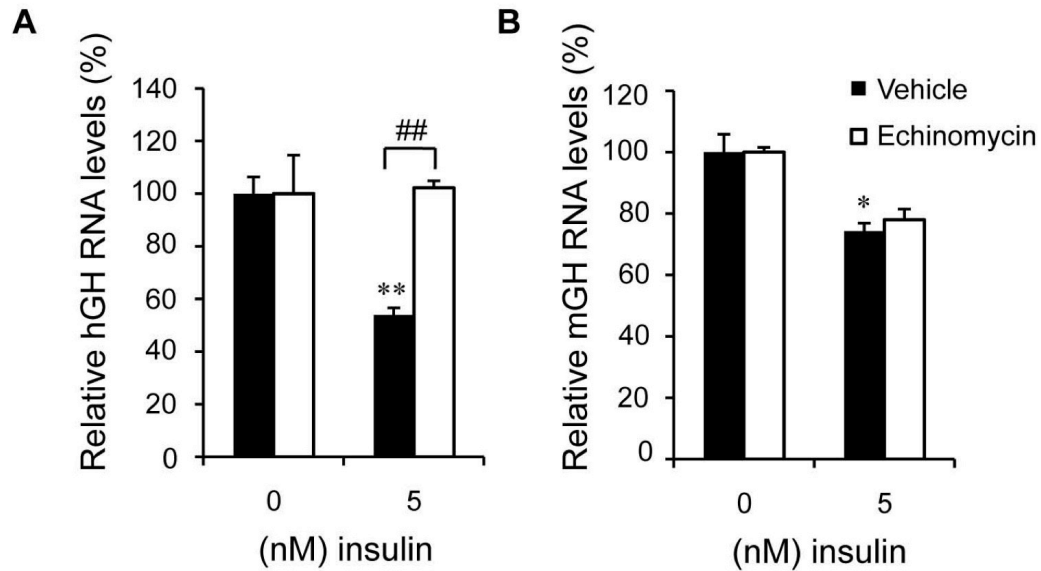
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<sup>10</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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It is important to note that just greater than 50% of the negative effect of insulin on hGH1 RNA levels was inhibited at 10 nM echinomycin. To rule out whether interference with E-box binding by echinomycin is a dose-dependent phenomenon, primary pituitary cells were then exposed to higher 20 nM concentration of echinomycin for 16 instead of 24 hours. The shorter exposure time was an attempt to off-set possible negative effects of echinomycin treatment on cultures. Pretreatment with a higher concentration of echinomycin (20 nM) led to 100% inhibition of the negative effect of 5 nM insulin on hGH1 RNA levels. Again, pretreatment with 20 nM echinomycin had no effect on insulin regulation of mGH transcripts (Figure 4.4A and B).

**Figure 4.4 <sup>11</sup>: Pretreatment with 20 nM echinomycin had no effect on insulin regulation of mGH transcripts**



Primary pituitary cells were incubated without (solid columns) or with (open columns) 20 nM echinomycin an hour prior to insulin (5 nM) treatment. (A) Human GH and (B) mGH RNA levels were assessed by qPCR 16 hours post insulin treatment. Results are expressed as relative mean change, plus or minus standard error of the mean, compared to control values, which are arbitrarily set to 100. Data were analyzed by two-way ANOVA with the Bonferroni post-test. Significant differences between groups at each concentration of insulin are indicated with brackets;  $p < 0.01$  ##. Significant differences between insulin treatment compared with no insulin group (0) as control, are indicated by  $p < 0.05$ , \*;  $p < 0.01$ , \*\*.

<sup>11</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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#### **4.5. Human GH promoter E-box and insulin regulation**

Inhibition of E-box DNA binding using echinomycin blunted the negative effect of insulin on *hGHI* expression. As such, E-box transcription factors are candidates for facilitating the insulin response. The CACGTG palindromic E-boxes regularly serve as binding sites for several basic helix-loop-helix (bHLH) transcription factors (Chan, Leung et al. 2012). Owing to the large number of bHLH proteins, approximately 242 members, which have been described as ubiquitous transcription factors thus far, the identification of a specific E-box transcription factor(s) that may involve in *hGHI* regulation is extremely challenging (Kvietikova, Wenger et al. 1995, Swanson, Chan et al. 1995, Grandori, Mac et al. 1996, Viollet, Lefrancois-Martinez et al. 1996, Atchley and Fitch 1997, Zelzer, Levy et al. 1998, Swanson and Yang 1999, Grandori, Cowley et al. 2000, Massari and Murre 2000, Kietzmann, Samoylenko et al. 2003, Chakrabarti, Turley et al. 2004, Turley, Wykoff et al. 2004, Wang, Zhou et al. 2010, Schodel, Oikonomopoulos et al. 2011). Another level of complexity in understanding the biology of E-box associated transcription factors is their ability to heterodimerize in variety of combinations under different conditions with a distinct tissue/cell specificity (Massari and Murre 2000).

#### ***4.6. Insulin induces the HIF-1 $\alpha$ , an E-box associated transcription factor complex***

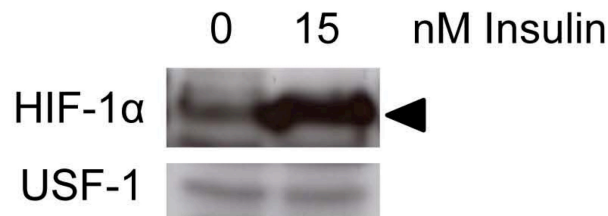
A review of the literature revealed that insulin shares the ability with hypoxia to induce the hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) transcription factor complex, which is considered to be a downstream molecule of insulin signaling (Zelzer, Levy et al. 1998, Kietzmann, Samoylenko et al. 2003, Wang, Zhou et al. 2010). HIF-1 $\alpha$  has also been detected in the pituitary (Yoshida, Kim et al. 2006, Zhang, Jin et al. 2009). The ability of insulin to induce accumulation of HIF-1  $\alpha$  also correlates with the study in which insulin treatment was associated with induction of a DNA-protein interaction at nucleotides -308/-235 in the proximal *hGHI* promoter region, which contains an E-box element (Lefevre, Imagawa et al. 1987, Peritz, Fodor et al. 1988, Prager and Melmed 1988, Prager, Gebremedhin et al. 1990). Thus, these properties make HIF-1 $\alpha$  a candidate to mediate insulin regulation of *hGHI* expression. HIF-1 $\alpha$  and  $\beta$  are members of the bHLH transcription factor family, which contain two Per-Arnt-Sim (PAS) domains, A and B, both of which are important for heterodimerization (Chapman-Smith and Whitelaw 2006, Park, Kong et al. 2006). Under normoxic conditions, HIF-1 $\alpha$  protein is ubiquitinated and subject to continuous proteosomal degradation, because it contains an oxygen-dependent degradation domain targeted by a specific HIF-prolyl hydroxylase (HPH1-3 also referred to as PHD1-3). Hypoxia, however, attenuates hydroxylation and HIF-1 $\alpha$  protein is stabilized. HIF-1 $\alpha$  is then able to heterodimerize with its constitutively present HIF-1 $\beta$  partner, and form a functional complex that regulates genes via a specific hypoxia responsive element, which is an E-box (CACGTG) (Huang, Gu et al. 1998, Zelzer, Levy et al. 1998, Kietzmann, Samoylenko et al. 2003, Lofstedt, Jogi et al. 2004, Wang, Zhou et

al. 2010, Schodel, Oikonomopoulos et al. 2011).

To assess whether insulin affects HIF-1 $\alpha$  levels in primary pituitary cells, nuclear extracts were isolated from cultures treated with or without 15 nM insulin for 24 hours, and analyzed by protein immunoblotting using antibodies to HIF-1 $\alpha$ . Levels of upstream stimulatory factor 1 (USF-1), a ubiquitously expressed transcription factor, were also assessed (Sirito, Lin et al. 1994). This was done because USF-1 is an E-box-related factor but also because others suggested it previously as a potential regulator of *hGHI* (Lemaigre, Courtois et al. 1989). A band of the expected size for HIF-1 $\alpha$  (120 kDa) was detected, and levels were stimulated reproducibly by insulin; a representative result is shown (Figure 4.5)

**Figure 4.5 <sup>12</sup>: Insulin induces the HIF-1 $\alpha$  transcription factor protein in primary pituitary cell cultures**

*Protein immunoblotting was done to assess HIF-1 $\alpha$  protein in nuclear extracts (100  $\mu$ g) of primary pituitary cells treated with 15 nM insulin for 24 hours. Nuclear proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoreactive proteins were detected by chemiluminescence. The HIF-1 $\alpha$  protein band (~120 kDa) is indicated by black arrowhead. USF-1 (43 kDa) from the same samples is also shown for comparison (n=3).*



<sup>12</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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#### **4.7. Human GH promoter E-box is a HIF-1 $\alpha$ binding sequence (HBS)**

EMSA was done to confirm that CACGTG DNA sequence in the *hGHI* proximal promoter region is a potential HIF binding sequence (HBS). Specifically, radiolabeled *hGHI* nucleotides -278/-250 were used in combination with recombinant HIF-1 $\alpha$  and HIF-1 $\beta$  protein (Figure 4.6). A known HBS from the EPO gene was used as a positive control (Lofstedt, Jogi et al. 2004), and RF-1 sequences (Lytras and Cattini 1994) were also radiolabeled as a negative control. A single specific complex was observed with both hGH and EPO gene but not RF-1 sequences in the presence of HIF-1 $\alpha$  protein (Figure 4.6). The complex was more readily detected with hGH than EPO gene sequences. In addition, the complex formed on nucleotides -278/-250 of *hGHI* was competed efficiently by non-labeled *hGHI* sequences compared to the EPO HBS, suggesting a higher affinity/specificity for the HBS in -278/-250 *hGHI* sequences.

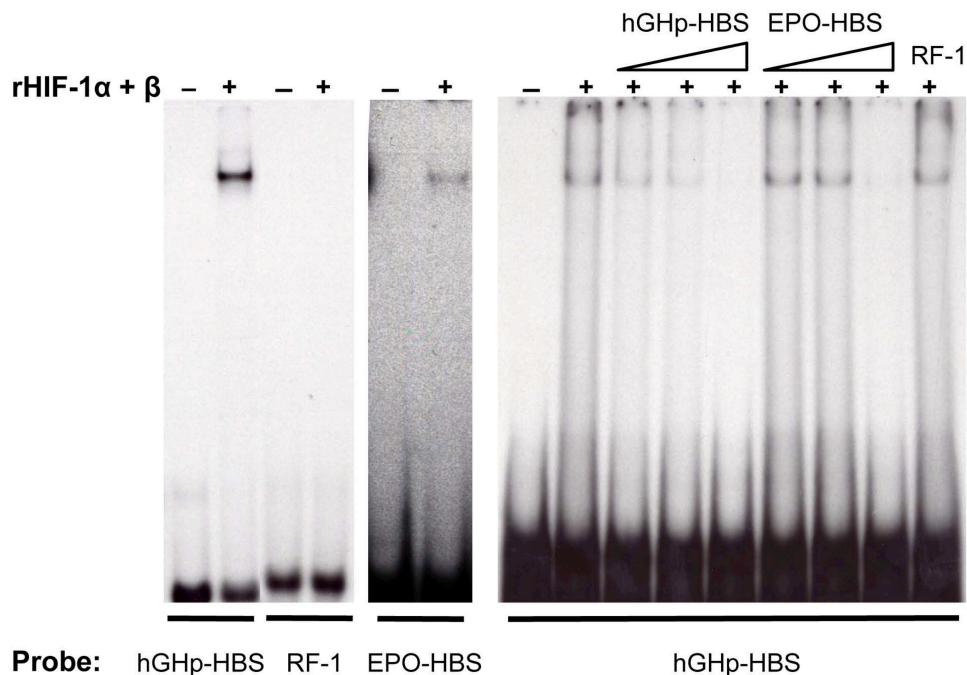
**Figure 4.6<sup>13</sup>: Human GH promoter E-box is a hypoxia inducible factor-1 alpha (HIF-1 $\alpha$ ) binding sequence (HBS)**

*EMSA was done with a combination of recombinant (r) HIF-1 $\alpha$  and HIF-1 $\beta$  protein together with radiolabeled probes: hGHp-HBS, EPO-HBS (positive control) and RF1 (negative control), with sequences as indicated. A single complex was seen in the presence of recombinant protein with both hGHp-HBS and EPO-HBS probes by autoradiography, but required prolonged exposure to detect the EPO-HBS/protein complex. Competition of the hGHp-HBS/protein complex with a 2.5, 5 and 50-fold molar excess of unlabeled hGHp-HBS and EPO-HBS oligonucleotide, as well as a 50-fold molar excess of RF-1 oligonucleotides, was also performed to assess affinity/specificity. Competition was detected with hGHp-HBS and EPO-HBS, but was more evident with a 5-fold molar excess of unlabeled hGHp-HBS than EPO-HBS; no competition with RF1 was observed.*

**hGHp-HBS:** GGCCAGAGGGCACCCACGTGACCCTTAAA

**EPO-HBS:** GCCCTACGTGCTGTCTCA

**RF-1:** CTCATCAACTTGGTGTGGACGGC



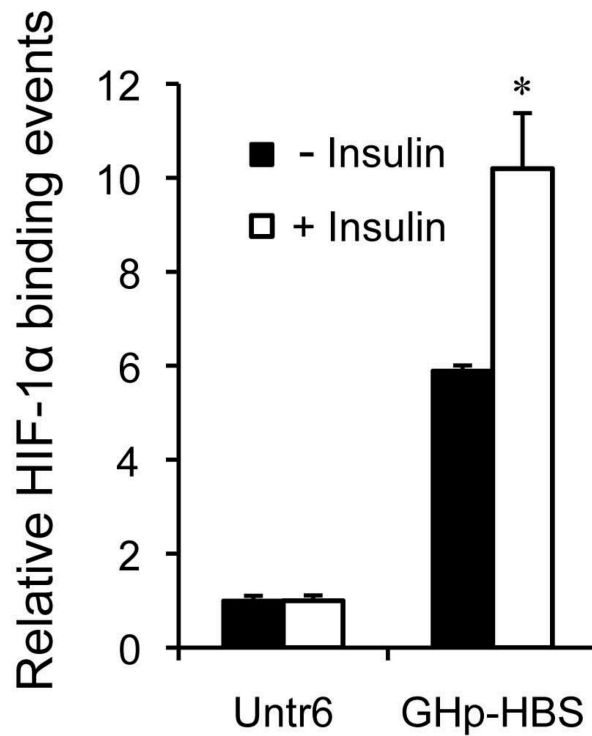
<sup>13</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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#### ***4.8. Insulin increases HIF-1 $\alpha$ transcription factor association with the hGHI promoter chromatin in situ***

ChIP assay was used to assess HIF-1 $\alpha$  association with the region containing the E-box element of the *hGHI* proximal promoter region in the context of pituitary cell chromatin *in situ*. Anti-HIF-1 $\alpha$  antibody was used for independent immunoprecipitations, and normal rabbit IgG was employed as a negative control. PCR primers (Table 2.3) were designed to specifically amplify the -308/-235 region of *hGHI* promoter and an untranscribed region on chromosome 6 (Untr6). The latter served as a measure of non-specific association of DNA/protein complex detected by ChIP assay. HIF-1 $\alpha$  associates preferentially with the *hGHI* proximal promoter region by ChIP assay, relative to the signal seen with Untr6 control sequences (Figure 4.7). The level of association increased significantly 1.7 fold following insulin (15 nM) treatment of pituitary cell cultures for 24 hours ( $p < 0.05$ ,  $n = 4$ ).

**Figure 4.7 <sup>14</sup>: Insulin increases the HIF-1 $\alpha$  transcription factor association with the *hGH1* promoter chromatin *in situ***

*ChIP assay was performed with an anti-HIF1 $\alpha$  antibody, on chromatin isolated from primary pituitary cells treated with 15 nM insulin for 24 hours. “Binding events” were calculated based on the signals obtained from the immunoprecipitated/input DNA amplification, using specific primers to *hGHp-HBS* and the control untranscribed region (*Untr6*) by qPCR. The results are expressed as relative mean change, plus or minus standard error of the mean, compared to the control (0 nM insulin) *Untr6* value, which is arbitrarily set to 1. Significant difference was assessed by t-test and is indicated by  $p < 0.05$ , \*.*



<sup>14</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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#### ***4.9. Induction of HIF-1 $\alpha$ by CoCl<sub>2</sub> mimics the effect of insulin on hGH1 RNA***

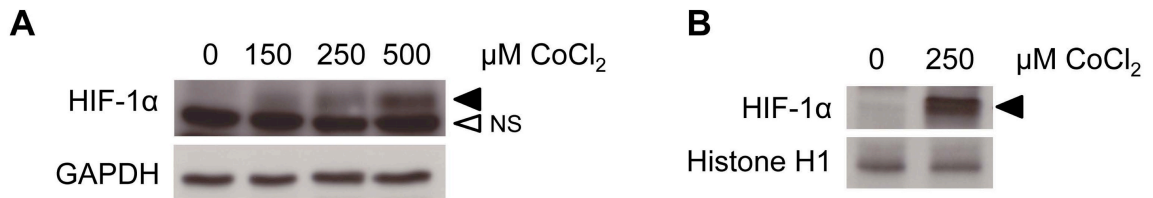
The effect of increased HIF-1 $\alpha$  availability on hGH1 RNA levels was examined by treating primary pituitary cells with CoCl<sub>2</sub>, which can mimic the effects of hypoxia by inhibiting prolyl hydroxylase activity (Takayanagi and Eguchi 2011). This is the major enzyme responsible for hydroxylation of HIF-1 $\alpha$  under normoxic condition, which is a mark for subsequent proteasomal degradation of HIF-1 $\alpha$  protein (Bruick and McKnight 2001). As a result, inhibition of prolyl hydroxylase stabilizes HIF-1 $\alpha$  (Xi, Taher et al. 2004). Cultures were treated with or without 150-500  $\mu$ M CoCl<sub>2</sub> for 5 hours in serum substitute, and whole cell or nuclear extract (250  $\mu$ M only) was assessed for HIF-1 $\alpha$  by protein immunoblotting (Figure 4.8A and B). A dose-dependent stimulation of a band of the expected size (120 kDa) was observed in the whole cell extract and was also detectable in nuclear protein.

The effect of 250 and 500  $\mu$ M CoCl<sub>2</sub> on hGH1 and mGH RNA was also assessed by qPCR after 24 hours treatment. Vascular endothelial growth factor (VEGF) expression was also assessed as positive control. VEGF expression is up-regulated by hypoxia through direct involvement of HIF-1 $\alpha$  (Fukuda, Kelly et al. 2003), and is also produced by hormone-producing as well as capillary endothelial cells of the anterior pituitary gland (Vidal, Lloyd et al. 2002, Lombardero, Vidal et al. 2006). CoCl<sub>2</sub> decreased hGH1 RNA levels significantly ( $p < 0.001$ ,  $n = 6$ ) and by a similar extent (~60%) to that observed with insulin treatment (Figure 4.8C). In contrast, there was no significant effect of CoCl<sub>2</sub>

treatment on endogenous mGH RNA levels (Figure 4.8D).

Primary pituitary cells were treated with  $\text{CoCl}_2$  for 24 hours and assessed by ChIP assay for HIF-1 $\alpha$  association with the HBS-containing *hGHI* proximal promoter region (Figure 4.8E). The level of association increased significantly 4-5 fold compared with the control group ( $p < 0.001$ ,  $n = 4$ ).

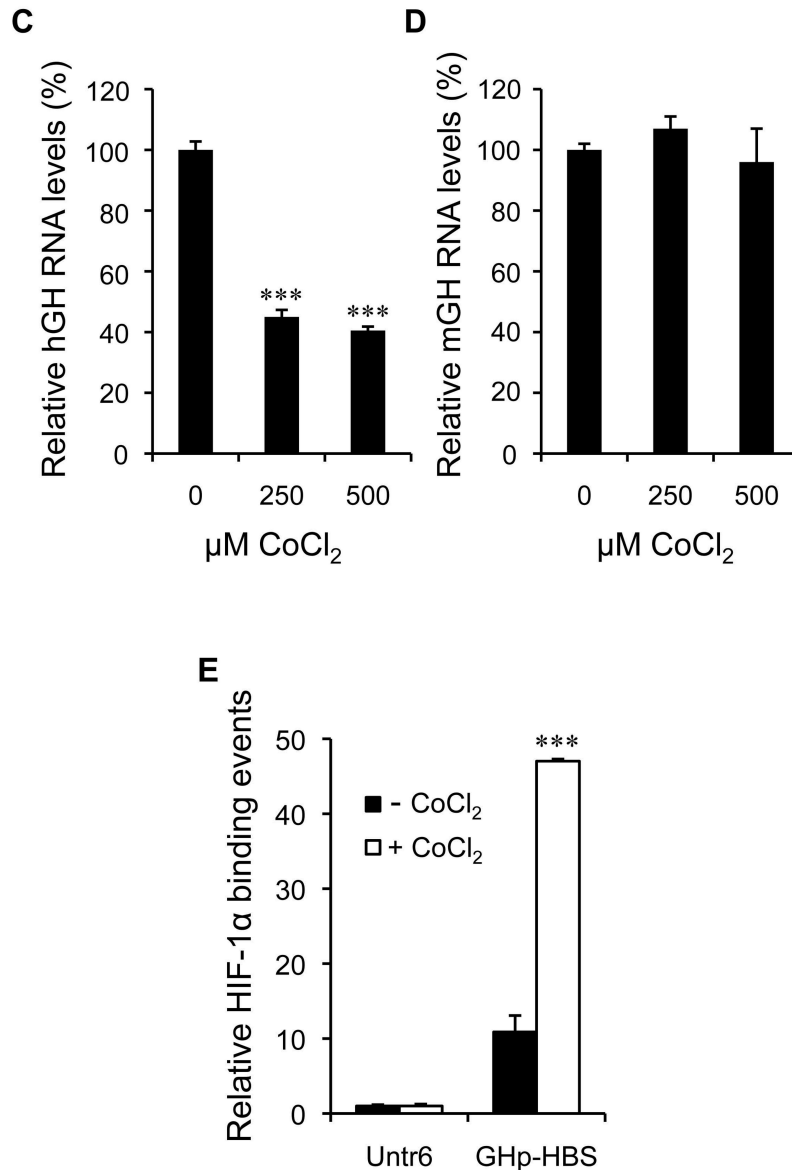
**Figure 4.8<sup>15</sup>: Induction of HIF-1 $\alpha$  protein by CoCl<sub>2</sub> treatment mimics the effect of insulin on hGH1 RNA levels**



*Immunodetection of induced HIF-1 $\alpha$  (120 kDa) in 25  $\mu\text{g}$  of whole cell lysate (A) and 15  $\mu\text{g}$  of nuclear extracts (B) of primary pituitary cells treated with 150-500  $\mu\text{M}$  CoCl<sub>2</sub> for 5 hours. GAPDH (37 kDa) and histone H1 (32 kDa) were used as loading controls for whole cell and nuclear proteins, respectively. The HIF-1 $\alpha$  protein band (black arrowhead) and the nonspecific protein band (NS), which was only detected in whole cell lysate, are indicated (n=3).*

<sup>15</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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**Figure 4.8**<sup>16</sup>: The effects of 250 and 500  $\mu\text{M}$   $\text{CoCl}_2$  treatment on (C) hGH and (D) mGH RNA levels were assessed by qPCR after 24 hours. Significant decreases in hGH but not mGH RNAs were observed. (E) ChIP assay was performed with an anti-HIF1 $\alpha$  antibody, on chromatin isolated from primary pituitary cells treated with 250  $\mu\text{M}$   $\text{CoCl}_2$  for 24 hours. “Binding events” were calculated and are expressed as relative mean change, based on the signals obtained from the immunoprecipitated/input DNA amplification, using specific primers to hGhp-HBS and the control untranscribed region (Untr6) by qPCR. Significant differences are indicated by  $p < 0.001$ , \*\*\*.

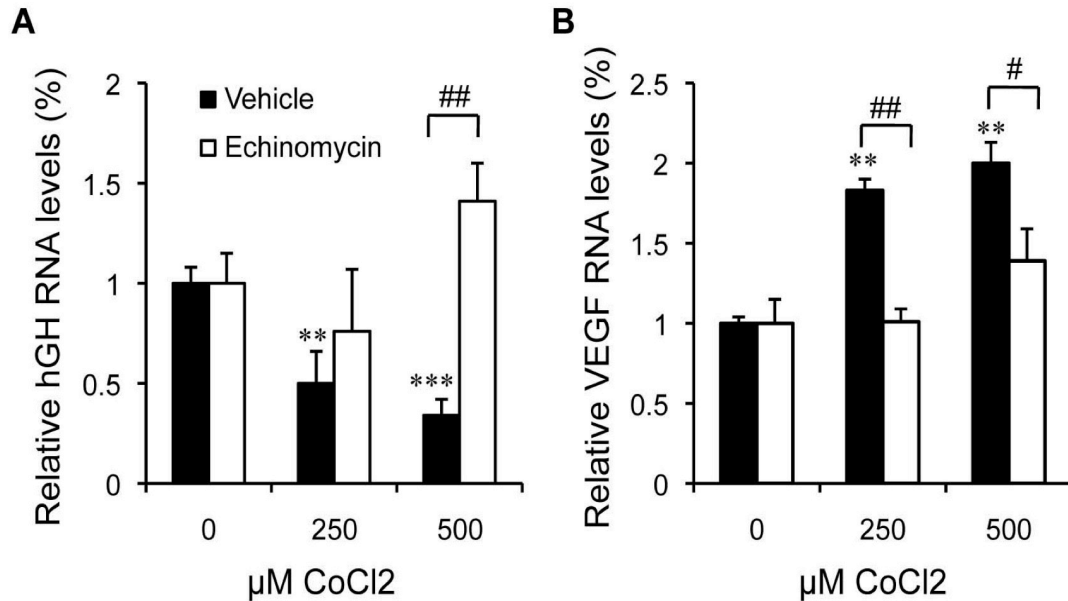


<sup>16</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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It was also noted that the decrease in hGH RNA (Figure 4.8C) and increase in VEGF RNA levels (served as a positive control) seen in response to  $\text{CoCl}_2$  were blocked by echinomycin pretreatment ( $p < 0.01$ ,  $n = 3$ ) (Figure 4.9).

**Figure 4.9 <sup>17</sup>: Decrease in hGH RNA and increase in VEGF RNA levels seen in response to CoCl<sub>2</sub> were blocked by echinomycin pretreatment**



Primary pituitary cells were incubated without (solid columns) or with (open columns) 20 nM echinomycin an hour prior to CoCl<sub>2</sub> (250 and 500 μM) treatment. (A) Human GH and (B) VEGF RNA levels were assessed by qPCR 24 hours post CoCl<sub>2</sub> treatment. Results are expressed as relative mean change, plus or minus standard error of the mean, compared to control values, which are arbitrarily set to 1. Data were analyzed by two-way ANOVA with the Bonferroni post-test. Significant differences between groups at each concentration of insulin are indicated with brackets;  $p < 0.05$  #,  $p < 0.01$  ##. Significant differences between CoCl<sub>2</sub> treatment compared with no CoCl<sub>2</sub> group (0) as control, are indicated by  $p < 0.01$ , \*\*;  $p < 0.001$ , \*\*\*.

<sup>17</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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#### ***4.10. Treatment with HIF-1 $\alpha$ shRNA reduces the effect of insulin on hGH1 RNA levels***

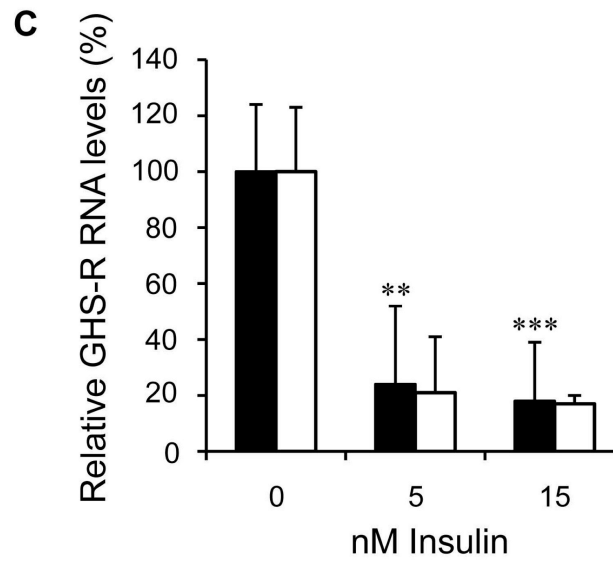
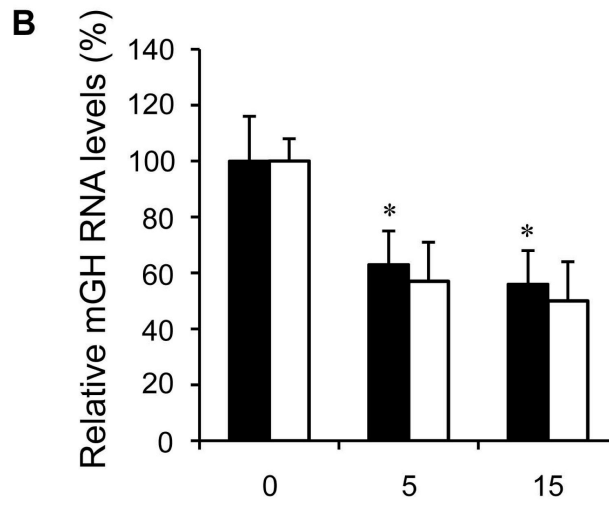
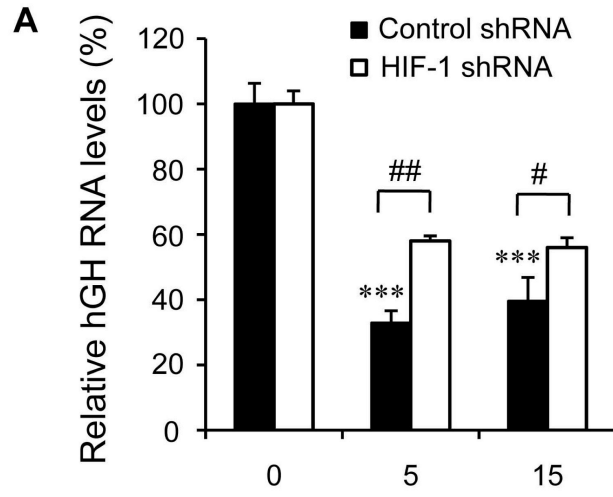
Lentiviral transduction particles of shRNA against HIF-1 $\alpha$  were used to limit HIF-1 $\alpha$  induction in response to insulin, and examine the effect on hGH1 RNA levels. Pituitary cell cultures were treated with HIF-1 $\alpha$  and control/scrambled shRNA for 24 hours, maintained for 72 hours and then treated for 24 hours with insulin. Human GH1, mGH and mGHS-R RNAs were assessed by qPCR. Partial but significant reduction in negative effect of 5 and 15 nM insulin on hGH RNA levels was observed with HIF-1 $\alpha$  shRNA versus control treatment ( $p < 0.01$ ,  $n = 5$ ) (Figure 4.10A). As with echinomycin (Figure 4.3B and C), however, there was no significant effect on the insulin responsiveness of mGH and mGHS-R transcript levels (Figure 4.10B, C).

**Figure 4.10<sup>18</sup>: Treatment with HIF-1 $\alpha$  shRNA reduces the effect of insulin on hGH1 RNA levels**

*Primary pituitary cells were treated with lentiviral transduction particles of scrambled/control (solid columns) or HIF-1 $\alpha$  shRNA (open columns) prior to insulin (5 and 15 nM) treatment. (A) Human GH, (B) mGH and (C) GHS-R RNA levels were assessed by qPCR 24 hours post insulin treatment. Significant decreases in hGH, mGH and GHS-R RNA levels were seen. Partial but significant blockage of the inhibitory effect of insulin on hGH RNA levels was observed with HIF-1 $\alpha$  but not control shRNA. There was no significant effect of shRNA on mGH GHS-R RNA levels in response to insulin. Results are expressed as relative mean change, plus or minus standard error of the mean, compared to control values, which are arbitrarily set to 100. Data were analyzed by two-way ANOVA with the Bonferroni post-test. Significant differences between groups at each concentration of insulin are indicated with brackets;  $p < 0.05$ , #;  $p < 0.01$  ##. Significant differences between insulin treatments compared with no insulin group (0) as control, are indicated by  $p < 0.05$  \*,  $p < 0.01$ , \*\* and  $p < 0.001$ , \*\*\*.*

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<sup>18</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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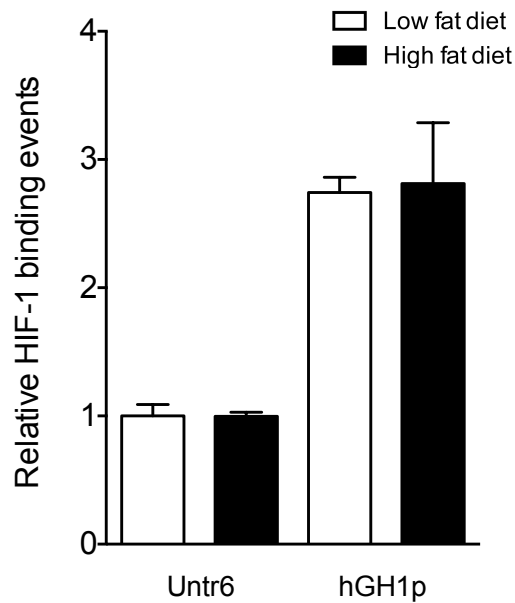


***4.11. Acute excess caloric intake regulation of hGH1 expression was not accompanied by increased association of HIF-1 $\alpha$  with hGH1 promoter chromatin in situ***

The possible effect of a hyperinsulinemic state via acute excess caloric intake on HIF-1 $\alpha$  recruitment to the *hGH1* promoter (hGH1p) region was investigated in mice. Specifically, would there be a similar induction to that seen with insulin treatment *in vitro*. CHIP assay was performed with an anti-HIF1 $\alpha$  antibody on chromatin isolated from pituitary tissues of mice fed with a low or high fat diet for three days. Surprisingly and unlike the primary pituitary culture treated with insulin, there was no significant effect of excess caloric intake for three days on association of HIF-1 $\alpha$  with the hGH1p chromatin *in situ* (Figure 4.11).

**Figure 4.11: No increase in association of HIF-1 $\alpha$  with hGH1p chromatin *in situ* was detected by excess caloric intake**

*ChIP assay was performed with an anti-HIF1 $\alpha$  antibody, on chromatin isolated from pituitary tissues of mice fed with a high fat diet for three days. “Binding events” were calculated based on the signals obtained from the immunoprecipitated/input DNA amplification, using specific primers to hGHp1 and the control untranscribed region (Untr6) by qPCR. The results are expressed as relative mean, plus or minus standard error of the mean, compared to the control (low fat diet) Untr6 value, which is arbitrarily set to 1. Significant differences were assessed by one-way ANOVA, n=3.*



***4.12. Reduction seen in hGHI RNA levels with overeating corresponds to an effect on “brain and muscle ARNT-like 1” recruitment to the hGHI promoter in situ***

Lack of HIF-1 response to excess caloric intake in mice fed a high fat diet for three days, led to an investigation of a potential role for other members of the bHLH superfamily. Specifically members which contain a PAS domain (Chapman-Smith and Whitelaw 2006) and may participate in the regulation of *hGHI* in response to overeating through E-box element. The presence of a PAS domain results in selective dimerization and a strong affinity for E-box DNA element interaction that distinguish this bHLH-PAS proteins subfamily from the broader bHLH superfamily (Taylor and Zhulin 1999, Massari and Murre 2000). A closely related transcription factor to HIF-1 $\alpha$  is brain and muscle ARNT-like 1 (Bmal1), which is a PAS domain-containing transcription factor (Dunlap 1999, Panda, Hogenesch et al. 2002, Reppert and Weaver 2002, Zhang and Kay 2010).

HIF-1 $\alpha$  and Bmal1 share conserved sequences in their DNA binding and dimerization domains, and have very similar DNA recognition specificities, leading to redundant roles as suggested by a crosstalk between hypoxia and circadian signaling (Labrecque, Prefontaine et al. 2013). To what extent inter-pathway regulation plays a role in transcriptional regulation is still poorly understood; however, there is some evidence that HIF-1 $\alpha$  can compete with ARNT-like 1 protein in DNA binding (Gu, Hogenesch et al. 2000).

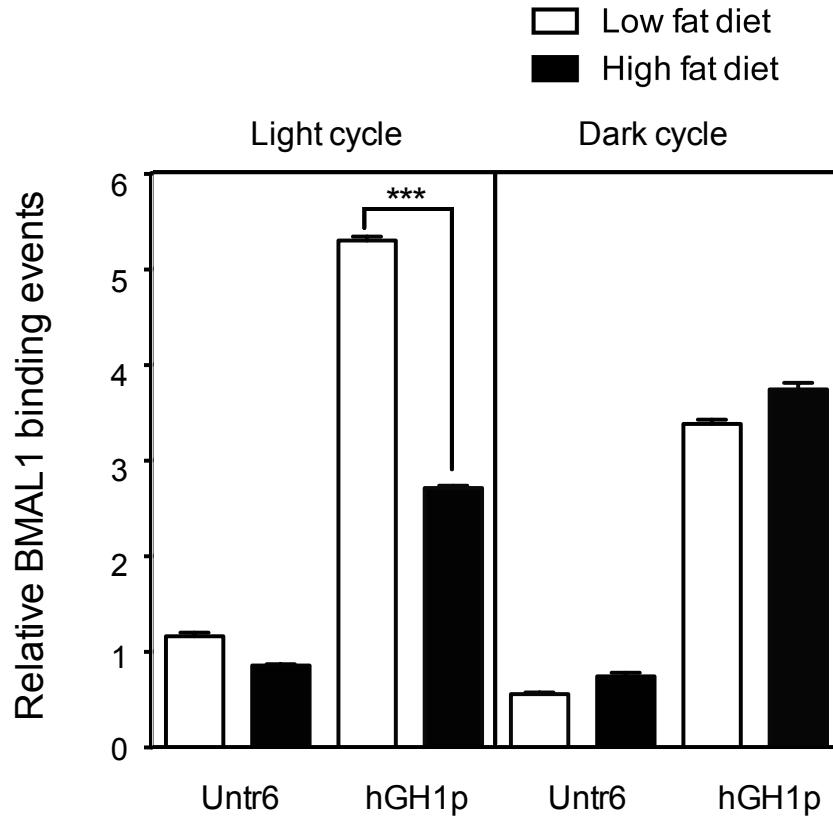
Heterodimerization of Bmal1 with its partner Clock (the circadian locomotor



output cycles kaput) is an essential component of circadian physiology through binding an E-Box DNA element (Gekakis, Staknis et al. 1998, Hogenesch, Gu et al. 1998). Therefore, the presence of the E-Box DNA element within the proximal promoter region of the *hGHI* is consistent with the possibility that it is a target of Clock and Bmal1 proteins. Thus, it is reasonable to speculate that hGH transcription and by extension synthesis is under the control of a circadian rhythm. In fact secreted hGH levels detected in serum are reported to show a circadian cycle in the presence of sleep (Linkowski, Mendlewicz et al. 1987, Linkowski, Kerkhofs et al. 1994). If the sleep pattern is disrupted, however, compensatory and largely unpredictable pulses of GH release will still occur during the awake period as demonstrated in shift-workers (Weibel, Follenius et al. 1997, Brandenberger and Weibel 2004). This suggests that GH production is not solely and simply under the control of the sleep/awake cycle, but is regulated at a higher level of complexity by circadian machinery. Furthermore, in a recent study, it was reported that consumption of a HFD for just three days in a mouse model, resulted in disruption of Bmal1 chromatin binding on its target hepatic genes *in situ* (Eckel-Mahan, Patel et al. 2013). As such it was concluded that acute excess caloric intake for three days is sufficient to reprogram the circadian rhythm (Eckel-Mahan, Patel et al. 2013). Thus, the impact of the three-day HFD-feeding paradigm on Bmal1 occupancy on the *hGHI* promoter region was assessed by ChIP assay. This revealed that occupancy by Bmal1 was reduced at the *hGHI* promoter region during the light cycle (pituitary tissues were collected at 10 a.m.) (Figure 4.12). Intriguingly, the reduction in Bmal1 binding observed during the light cycle with the HFD regimen, was not evident during the dark cycle (pituitary tissues were collected at 10 p.m.) (Figure 4.12).

**Figure 4.12: Reduction in Bmal1 association with the *hGH1* promoter chromatin *in situ* with excess caloric intake**

ChIP assay was performed with an anti-Bmal1 antibody, on chromatin isolated from pituitary tissues of mice fed with a high fat diet for three days and isolated during light cycle (10 a.m.) or dark cycle (10 p.m.). “Binding events” were calculated based on the signals obtained from the immunoprecipitated/input DNA amplification, using specific primers to *hGH1p* and the control untranscribed region (*Untr6*) by qPCR. The results are expressed as relative mean, plus or minus standard error of the mean, compared to the control (low fat diet) *Untr6* value. Significant differences were assessed by student’s *t*-test as indicated by \*\*\*;  $p < 0.001$ ,  $n = 3$ .

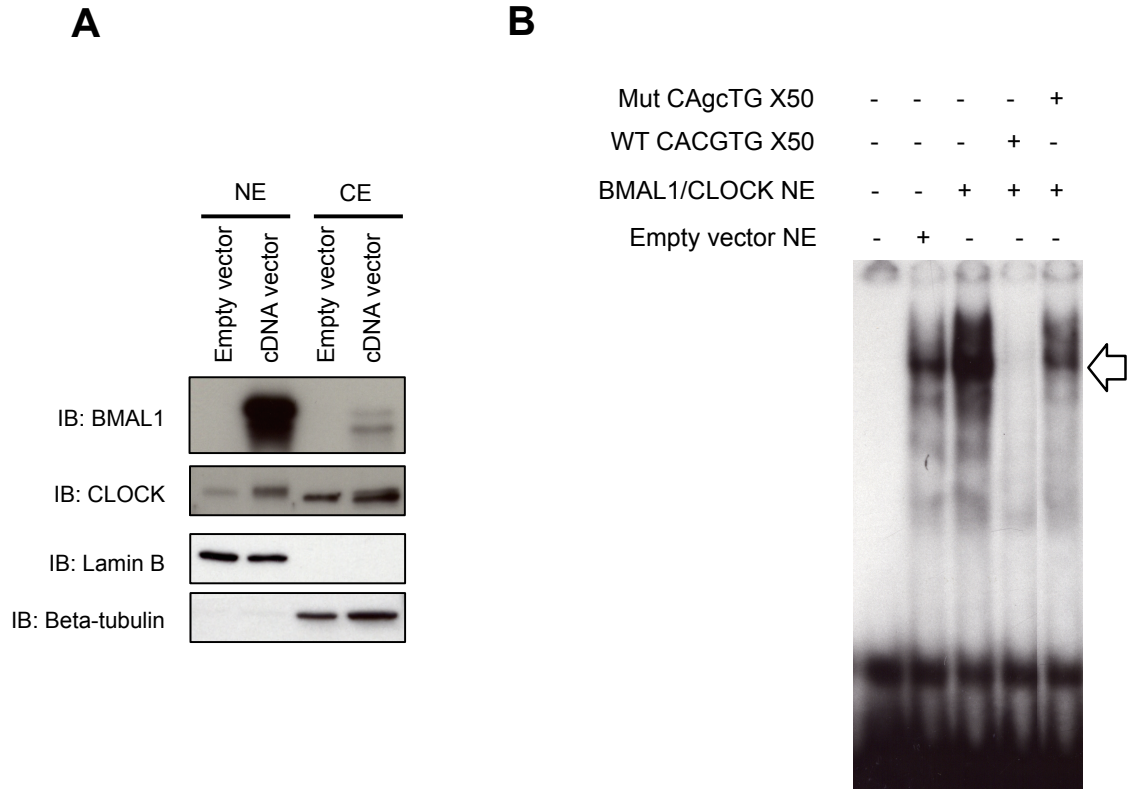


#### **4.13. Human *GH* promoter E-box supports Bmal1/Clock heterodimer binding**

EMSA was done to confirm that CACGTG DNA sequences in the *hGH1* proximal promoter region can support Bmal1 binding. Nuclear protein extracts from HEK293 cells overexpressing Bmal1 and Clock proteins were combined with radiolabeled *hGH1* promoter (hGH1p) E-Box element (nucleotides -278/-250) as a probe. To confirm efficient overexpression of Bmal1 and Clock by HEK293 cells, 5 µg of the nuclear and cytosolic extract (NE and CE) proteins were initially resolved by SDS-PAGE and immunoblotted using both Bmal1 and Clock antibodies (Figure 4.13A). Dramatic increases in both Bmal1 and Clock proteins in transfected cells in both NE and CE fractions were observed. EMSA was done using radiolabeled hGH1p E-Box element as a probe combined with NE from HEK293 cells transfected with or without Bmal1/Clock expression vectors. A major complex was seen using the HEK293 cell NE without expression vectors. This may represent the endogenous proteins, however, the intensity of this major complex was increased when using Bmal1/Clock NE and hGH1p probe by autoradiography (Figure 4.13B). Competition of the hGH1p/protein complex with a 50-fold molar excess of unlabeled wild type (WT) hGH1p CACGTG versus mutated (Mut) hGH1p CAgcTG oligonucleotides was also performed to assess affinity/specificity. This mutation has been reported to disrupt binding and *trans*-activation of the E-Box DNA element (Arpiainen, Lamsa et al. 2007). Efficient competition was detected with WT hGH1p (in -278/-250 *hGH1* sequences), but was less efficient with mutant (Mut CAgcTG) oligonucleotide (Figure 4.13B).

**Figure 4.13: Human GH promoter E-box supports Bmal1/Clock binding**

(A) The expression vectors *Bmal1* and *Clock* were transfected into HEK293 cells. Five  $\mu$ g of the nuclear and cytoplasmic extract (NE and CE) proteins were resolved by SDS-PAGE and immunoblotted using both *Bmal1* and *Clock* antibodies as well as lamin B and  $\beta$ -tubulin as loading controls for nuclear and cytoplasmic proteins, respectively. (B) EMSA was done using the radiolabeled hGH1 promoter (p) E-Box element as a probe and 1  $\mu$ g of nuclear extracts (NE) from transfected HEK293 cell without and with *Bmal1*/*Clock* expression vectors. A major complex was seen using the NE without expression vectors, shown by an arrow, however, this major complex was increased when using *Bmal1*/*Clock* NE and hGH1p probe by autoradiography. Competition of the hGH1p/protein complex with a 50-fold molar excess of unlabeled intact or wild type (WT) hGH1p (CACGTG element) and disrupted or mutated (Mut) hGH1p (CAgcTG) oligonucleotide was also performed to assess affinity/specificity.



#### ***4.14. Bmal1/Clock overexpression in HEK293 cells trans-activates hGH1p-luciferase reporter via association with an E-box DNA element***

The ability of Bmal1/Clock to *trans*-activate the *hGH1* promoter (hGH1p) with an intact or mutated E-box DNA element was assessed using transfected hybrid luciferase genes. This was done having established efficient overexpression of Bmal1 and its partner Clock in transfected HEK293 cells, and their ability to heterodimerize and bind. A hybrid reporter gene driven by 0.5 kb of the mGH promoter, which contains no E-box DNA element, was also assessed as a negative control. A human Per2 promoter luciferase hybrid reporter, which contains three E-box DNA elements, was also used as a positive control (Xu, Toh et al. 2007). In all cases, cells were co-transfected with Renilla-TKp-Luc to allow normalization for DNA uptake.

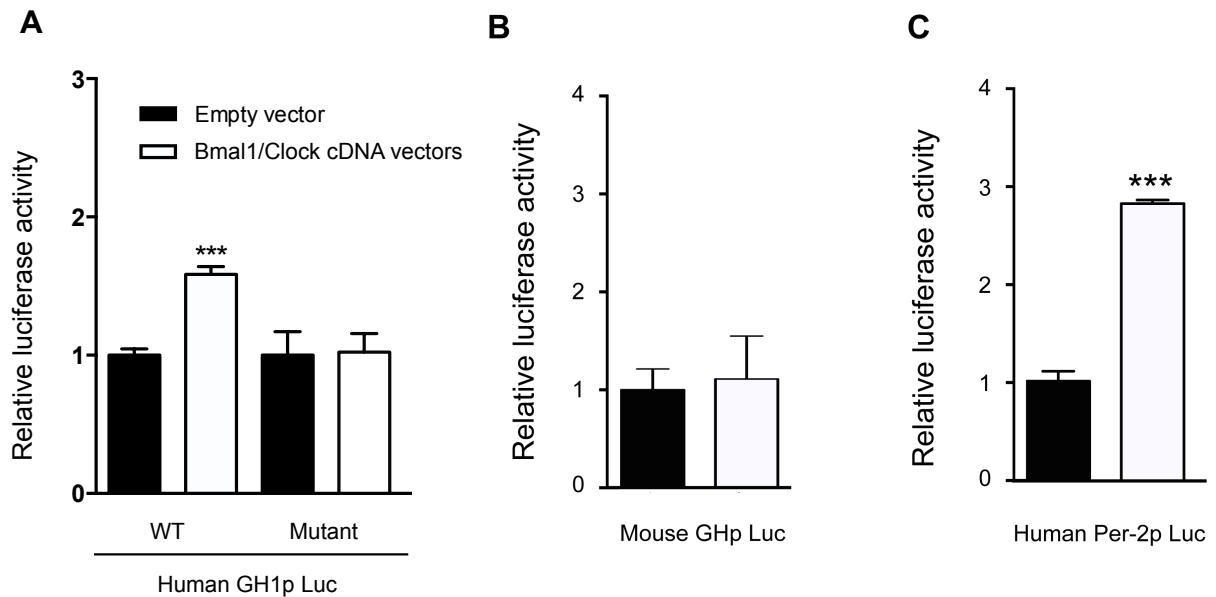
HEK293 cells were transiently transfected with hybrid hGH1p-Luc genes containing the wild type or mutated E-Box DNA element (introduced by PCR-based site-directed mutagenesis). Both hybrid Luc genes were co-transfected with either control empty vector or Bmal1/Clock expression vector. The values with control vector were arbitrarily set to 1, and relative values for Bmal/Clock expression are expressed as fold effect (Figure 4.14A). A significant increase in hGH1p-Luc expression in the presence of the wild type E-Box DNA element was observed ( $p < 0.001$ ,  $n=9$  by *t-test*). By contrast, no significant effect on hGH1p-Luc gene activity containing the mutant E-box DNA element was seen in response to Bmal1/Clock overexpression (Figure 4.14A).

Furthermore, a hybrid mouse GH promoter/luciferase gene was not responsive to overexpression of Bmal1/Clock proteins in transiently transfected HEK293 cells (Figure

4.14B). In contrast, and as expected, the activity of the Per2 promoter luciferase reporter gene was increased significantly by Bmal1/Clock protein overexpression ( $p < 0.001$ ,  $n = 6$ ), as reported previously (Xu, Toh et al. 2007).

**Figure 4.14: Co-expression of Bmal1/Clock in HEK293 cells *trans*-activates the *hGH1* promoter (p), but mutation of the E-box element limits this response**

Hybrid luciferase (*Luc*) reporter genes including (A) human *GH1p Luc* (WT and Mutant), (B) mouse *GHp Luc* and (C) human *Per2-p Luc* were used to assess the effect of co-expressed *Bmal1/Clock* in transfected HEK293 cells. Firefly luciferase counts were corrected by protein concentration as per  $\mu\text{g}$  protein lysate. Values are expressed as fold change over cells transfected with empty vector, which is arbitrarily set to 1. Statistical analysis was performed by student's *t*-test. \*\*\*,  $p < 0.001$  ( $n = 6-9$ ). Bars represent standard error of the mean (SEM).



#### **4.15. Summary of the results**

In this chapter, the impact of insulin treatment on transcriptional regulation of the intact *hGHI* and GH LCR in primary monolayer cultures of transgenic mouse pituitary cells *in situ* was investigated. Observations made from this study are:

- *Human GH1 transcription is regulated directly by physiological as well as supra-physiological concentrations of insulin at the somatotroph level. Insulin treatment results in a significant and dose-dependent decrease in hGHI transcript levels in primary pituitary cell cultures from two independent lines of transgenic mice capable of expressing hGHI.*
- *Mouse (m) GH RNA levels, like those of hGHI, were regulated by insulin in vitro. This is in contrast with no response of mGH to the hyperinsulinemic condition induced by acute excess caloric intake in vivo as described in Chapter 3.*
- *The presence of an enhancer box DNA element in the proximal hGHI promoter region and its absence from equivalent region of the mGH promoter suggests a potential role for E-Box associated transcription factors in mediating the differential response of human and mouse GH genes to hyperinsulinemic conditions induced by acute excess caloric in vivo. Chemical interference with Enhancer-box related factor binding using echinomycin reduced the effect of insulin on hGHI but not mGH RNA levels.*



- *Insulin induced the HIF-1 $\alpha$  protein, an E-box associated transcription factor complex, and increased its association with the hGH1 proximal promoter region in situ.*
- *Increased HIF-1 $\alpha$  availability with a hypoxia mimetic, significantly decreased hGH RNA levels, and was accompanied by recruitment of HIF-1 $\alpha$  to the hGH1 promoter in situ as seen with insulin. However, chemical induction of increased HIF-1 $\alpha$  had no significant effect on mGH production.*
- *Limiting availability of HIF-1 $\alpha$  by RNA interference blunted the negative effect of insulin on hGH1 but not mGH.*
- *There was no increased association of HIF-1 $\alpha$  with the hGH1 promoter chromatin in situ after three days on a HFD, equivalent to that observed with insulin treatment in vitro. The data do not exclude the possibility, however, of a transient induction and increase in association of HIF-1 $\alpha$  with the hGH1 locus at an earlier time point. This apparent discrepancy between in vivo and in vitro observations will be discussed in the Chapter 7 (Section 7.7).*
- *The data support a role for disruption of Bmal1 (as an E-box associated transcription factor with high structural and functional similarity to HIF-1) association with the hGH1 promoter region that is affected by acute excess caloric intake.*

## CHAPTER 5

### **Chromosomal reconfiguration of the human growth hormone gene locus by excess caloric intake correlates with a chromatin-remodeling event**

#### ***5.1 Rationale and specific aim***

Control of gene expression involves the orchestrated action of multiple regulatory regions of the genome, which may be separated by tens of thousands of base pairs. Promoter activity and efficient gene expression may require physical interaction among these DNA elements, which can lead, presumably, to looping of the chromatin fiber (Lanctot, Cheutin et al. 2007). The hGH gene locus offers an excellent example. The HS I/II region located in the upstream-flanking DNA of the adjacent CD79b gene exerts its enhancer activity on the *hGHI* promoter about 15 kb downstream via a long-range interaction involving “looping” of intervening chromatin between these two regions (Ho, Elefant et al. 2002, Ho, Tadevosyan et al. 2008). Formation of this “expression” loop, and resulting interaction between pituitary transcription factor Pit-1 binding sites at HS I/II and the promoter is essential for *hGHI* activation during development (Jones, Monks et al. 1995, Ho, Tadevosyan et al. 2008). Recent studies suggest that this interaction also continues to be important for efficient postnatal *hGHI* expression into adult life (Ho, Shewchuk et al. 2013).

This tissue/cell-specific chromosomal configuration and chromatin looping and as a result activation of the pituitary *hGHI* promoter during embryonic development is

facilitated by histone H3 and H4 hyperacetylation (Jones, Monks et al. 1995, Fleetwood, Ho et al. 2012). This hyperacetylation is associated with a more “open” chromatin configuration (Iizuka and Smith 2003). For the hGH gene locus, histone hyperacetylation is centered at HS I/II but tracks out to include HS V upstream and the *hGHI* promoter downstream (Elefant, Cooke et al. 2000, Yang, Jin et al. 2010). HS I/II possesses pituitary enhancer activity both *in vitro* and *in vivo*, and is the major determinant of efficient expression (Jin, Surabhi et al. 1999). Thus, the three-dimensional chromosomal architecture, and specifically the presence of the “expression” loop together with relative H3/H4 hyperacetylation can be seen as markers of efficient *hGHI* expression (Jones, Monks et al. 1995, Ho, Tadevosyan et al. 2008). As such, these “markers” were assessed and used as a functional endpoints in this Chapter.

In this chapter, the impact of excess caloric intake *in vivo* and insulin treatment *in vitro* on transcriptional regulation of *hGHI* is explored. Specifically, effects on chromatin remodeling and changes in chromosomal conformation of the intact *hGHI* and the GH LCR are examined.

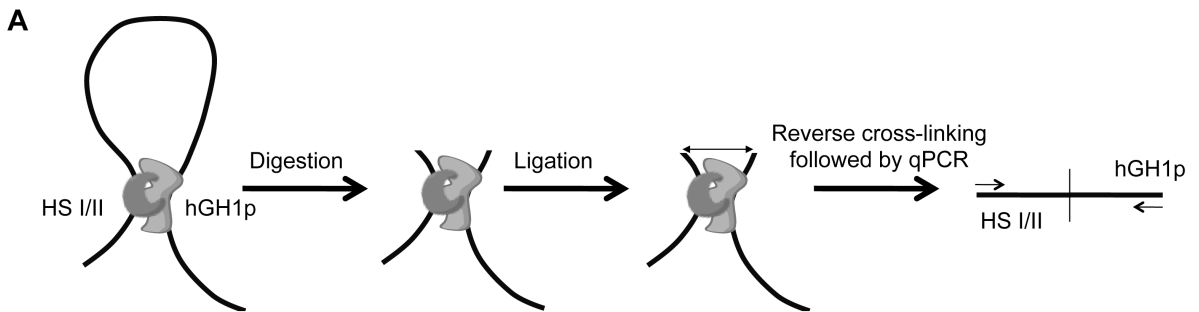
## RESULTS

### ***5.2. Excess caloric intake is associated with disruption of the chromosomal architecture involving the hGH LCR and proximal hGHI promoter region***

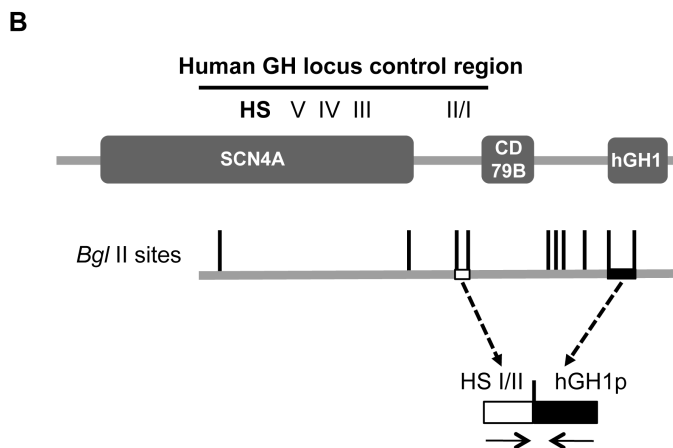
The effect of a HFD versus LFD for three days on the integrity of the “expression” loop in the pituitaries of hGH/CS TG mice was assessed by quantitative 3C assay (Figure 5.1A). Cross-linked hGH locus chromatin was digested with *Bgl*II restriction enzyme, ligated and frequency of ligation between the HS I/II and *hGHI* promoter region fragments was assessed using specific primers to amplify the combined ligated product by qPCR (Figure 5.1B). There was no detectable ligated product representing combined fragments containing HS I/II and *hGHI* promoter (hGHp) regions in the “no ligase treatment” control of digested chromatin (Figure 5.1C). The 3C assay was also performed on heart tissue taken from the same mice to assess for (pituitary) tissue-specificity of ‘expression’ loop formation (Figure 5.1D). A significant 75% reduction ( $p < 0.001$ ) in the “expression” loop (or ligation frequency of the HS I/II and hGHp fragment) was seen in pituitaries of mice with excess caloric intake from a HFD (Figure 5.1E).

**Figure 5.1<sup>19</sup>: Disruption of the interaction between HS I/II and the hGH1 promoter regions by excess caloric intake**

(A) Schematic outline of the 3C assay, which involves: (1) crosslinking the interacting chromatin with formaldehyde; (2) digestion of DNA by an appropriate restriction enzyme; and (3) ligation of digested cross-linked chromatin fragments that can be quantified by qPCR.

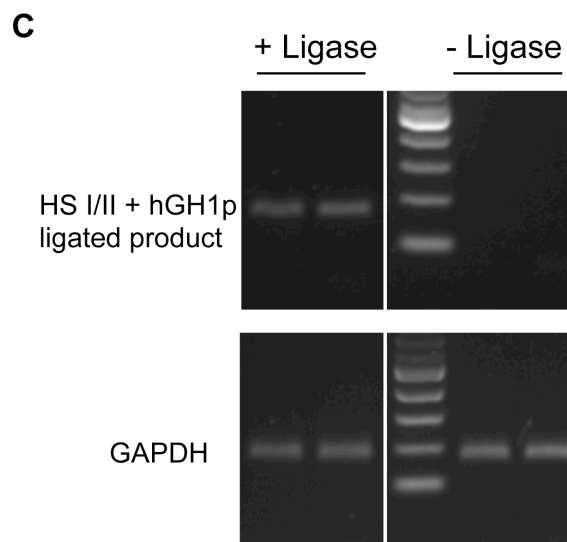


(B) Long-range chromatin-chromatin interactions that allow efficient expression of pituitary hGH1 in vivo and in response to a HFD were assessed. The map of BglII restriction fragments and positions of primers (arrows) used to determine and quantify the ligated products representing HS I/II and hGH1 promoter (hGH1p) physical interaction are shown below the locus. The formula for calculating the ligation frequency between HS I/II and hGH1p is: “(Ligation frequency = ligation product/loading control - level of non-ligation product/loading control)”



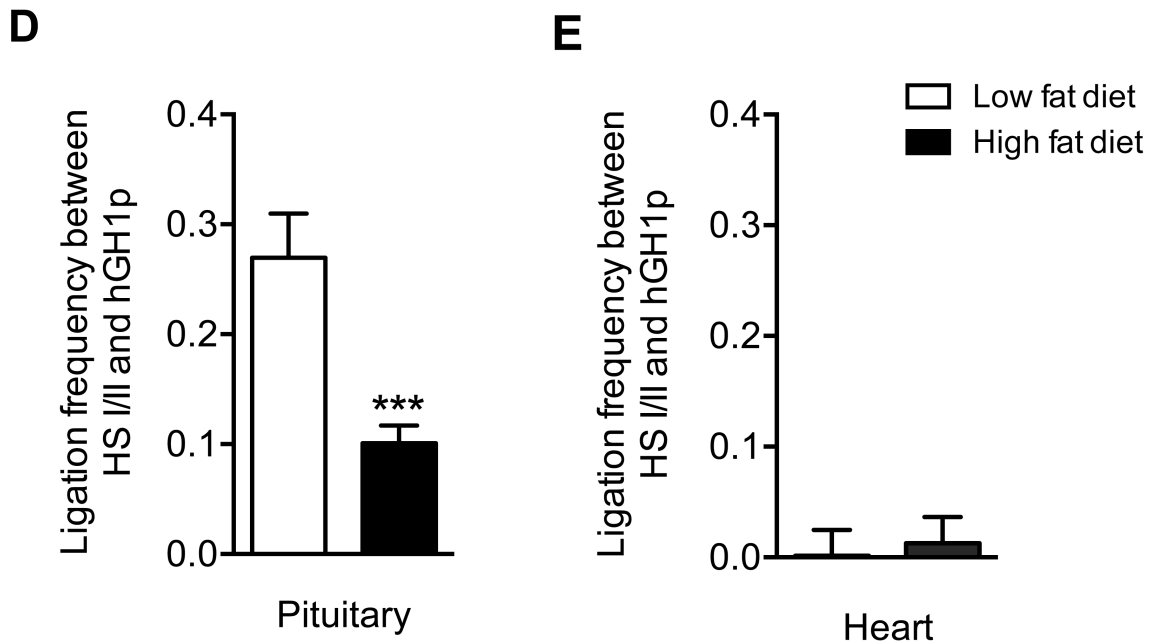
<sup>19</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

**Figure 5.1**<sup>20</sup>: (C) Ligated *Bgl*II fragments resulting from physical interaction between HS I/II and *hGH1* promoter (*hGH1p*) visualized in an agarose gel as a single band in the final PCR product. There was no detectable ligated product representing combined fragments containing HS I/II and *hGH1p* regions in our “no ligase treatment” control of digested chromatin.



<sup>20</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, *Journal of Clinical Investigation*, 2014. © American Society for Clinical Investigation

**Figure 5.1**<sup>21</sup>: **(D)** Pituitary and **(E)** heart tissue from 171hGH/CS TG mice fed a high fat versus low fat diet for three days; Ligation frequency = ligation product/loading control - level of non-ligation product/loading control. The results are obtained from three independent samples and two ligation/sample and are expressed as relative mean change  $\pm$  standard error of the mean. Significance was assessed by t-test,  $P < 0.001$ , \*\*\*,  $n = 6$ . Data provided by Ms. Yan Jin.



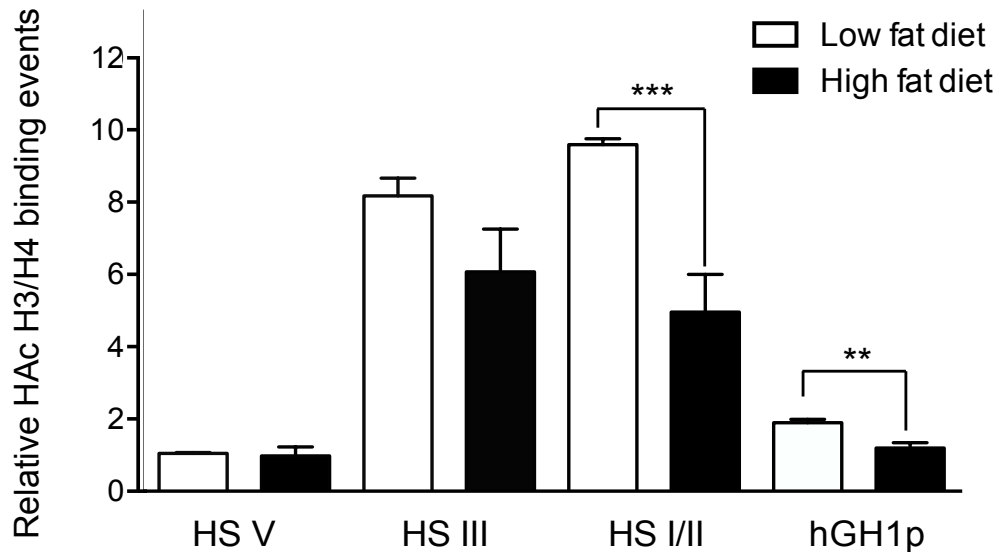
<sup>21</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

### ***5.3. Histone H3/H4 hyperacetylation is reduced by excess caloric intake at HS I/II and the hGH1 promoter***

The relative level of histone H3/H4 hyperacetylation across the hGH LCR and promoter in pituitary tissue from hGH/CS TG mice fed with LFD and HFD was assessed by ChIP assay. As expected, hyperacetylation showed the characteristic pituitary cell pattern with the highest levels centered and extending out from HS I/II (Figure 5.2A). There was a decrease in H3/H4 hyperacetylation associated with HS III, HS I/II and the *hGH1* promoter, which was modest at HS III (Figure 5.2A) and statistically significant at HS I/II and the proximal promoter regions in mice on the HFD (Figure 5.2B and C) ( $p < 0.01$ ,  $n=6$ ).



**Figure 5.2<sup>22</sup>: Histone H3/H4 hyperacetylation is reduced by excess caloric intake at HS I/II and the *hGH1* promoter**



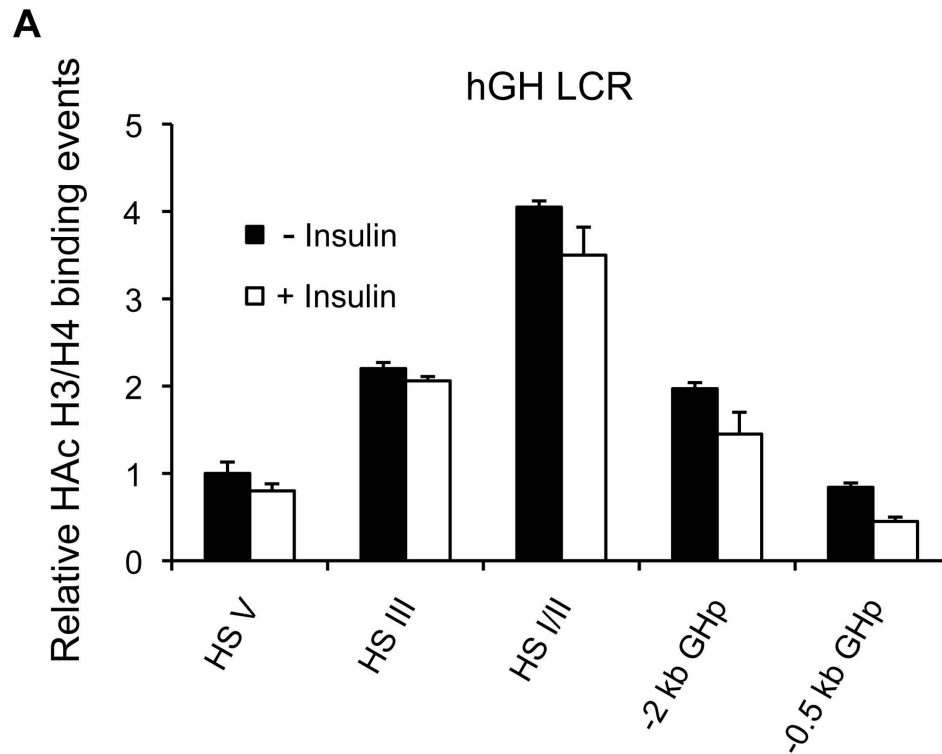
*Relative levels of hyperacetylated (HAc) histone H3 and H4 were assessed in pituitary chromatin from 171hGH/CS TG mice fed a HFD (black-filled) versus and LFD (white-filled) by ChIP assay and qPCR. Input and immunoprecipitated (bound) chromatin fractions were assessed using specific PCR primer sets for upstream HS V, HS III, HS I/II, and proximal (-0.5 kb) hGH1 promoter (p) regions. The results are expressed as HAc H3/H4 “binding events” relative to the LFD value for HS V, which is arbitrarily set to 1. Significance was assessed by one-way ANOVA with the Bonferroni post-test (n=4).*

<sup>22</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

***5.4. Treatment of primary pituitary cells in culture with insulin is also associated with a decrease in histone hyperacetylation at the hGHI proximal promoter region***

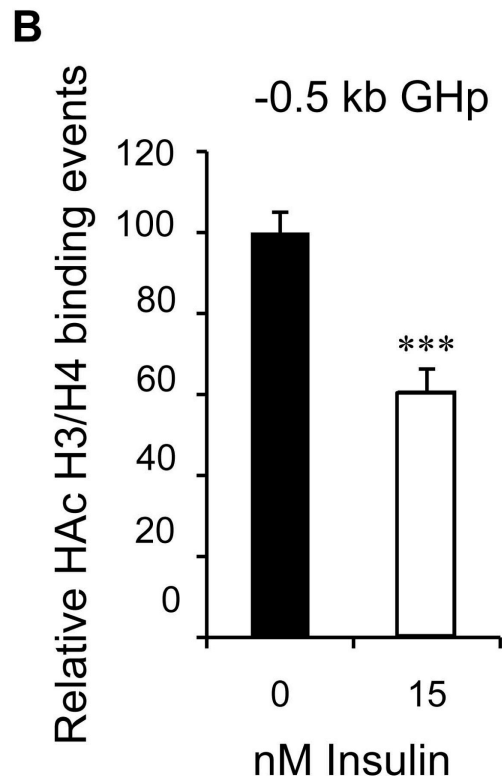
The possibility that a **hyperinsulinemic state** associated with excess caloric intake is a major event that can facilitate chromosomal reconfiguration of the hGH locus through **chromatin remodeling**, and more specifically histone acetylation, was pursued. Isolated primary pituitary cells from 171hGH/CS TG mice were treated with insulin for 24 hours, chromatin isolated and assessed for histone H3/H4 hyperacetylation levels across the locus. As expected and consistent with results obtained *in situ* (Figure 5.2A), hyperacetylation showed the characteristic pituitary cell pattern with the highest levels centered and extending out from HS I/II (Figure 5.3A). This pattern was also seen after insulin treatment; however, there was a significant ~40% decrease in H3/H4 hyperacetylation at the proximal (not more distal) *hGHI* promoter region after insulin treatment ( $p < 0.001$ ,  $n = 4$ ) (Figure 5.3B). There was also the suggestion of a modest decrease in hyperacetylation in the region spanning HS I/II and the *hGHI* promoter.

**Figure 5.3<sup>23</sup>: Histone hyperacetylation decreases in the *hGH* proximal promoter region with insulin treatment**



(A) ChIP assay was performed with antibodies specific to the hyperacetylated (HAc) histone H3 and H4 (three or more acetylated lysine residues), on chromatin isolated from primary pituitary cells treated without (solid columns) or with 15 nM insulin (open columns) for 24 hours. Quantitative PCR was performed on both input and immunoprecipitated (bound) chromatin fractions with primer sets designed to detect upstream HS V, HS III, HS I/II, as well as distal (-2 kb) and proximal (-0.5 kb) *hGH* promoter regions. Results are presented as relative HAc H3/H4 “binding events” compared to the control value for HS V, which is arbitrarily set to 1. A peak for HAc H3/H4 is seen at HS I/II in the presence and absence of insulin.

<sup>23</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, *Journal of Biological Chemistry*, 287:33282-33292, 2012.  
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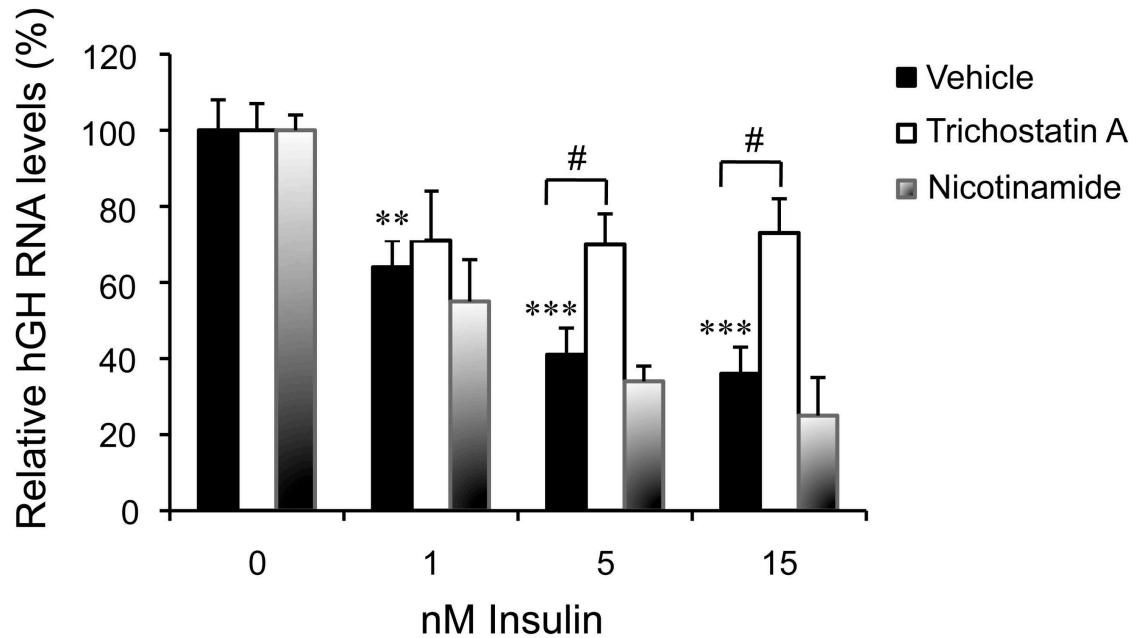
(B) When re-graphed (control arbitrarily set to 100), a significant ~40% decrease in HAC H3/H4 is detected only in the proximal hGH1 promoter (GHp) region. Significance was assessed by *t*-test,  $P < 0.001$ , \*\*\*.

### ***5.5. The negative effect of insulin on hGH RNA levels is trichostatin A sensitive***

Deacetylation of lysine residues of histone proteins is facilitated by histone deacetylases (HDACs), which remove the acetyl groups and as such restore the positive charge (Kouzarides 2007). This change in electrical property of the histone proteins can cause a condensation of chromatin, known as heterochromatin, and thus lead to repression of transcription.

A potential role for involvement of histone deacetylases (HDACs) on histone acetylation status in *hGHI* insulin responsiveness was examined. Pituitary cell cultures were treated with increasing concentrations of insulin in the presence of the class I and II HDAC inhibitor, trichostatin A, and class III HDAC (sirtuins) inhibitor, nicotinamide (Yoshida, Horinouchi et al. 1995, Bitterman, Anderson et al. 2002). A dose-dependent decrease in hGH RNA was observed with increasing insulin concentration as assessed by qPCR, however, the decreases with 5 and 15 nM insulin were blunted significantly in response to trichostatin A but not nicotinamide treatments ( $p < 0.05$ ,  $n = 6$ ) (Figure 5.4).

**Figure 5.4 <sup>24</sup>: The negative effect of insulin on hGH RNA levels is trichostatin A sensitive**



*Primary pituitary cells were treated with HDACs inhibitors, trichostatin A (300 nM), nicotinamide (15 mM) or vehicle one hour prior to insulin (1, 5 and 15 nM) treatment. Human GH RNA was assessed by qPCR 24 hours post insulin treatment. Trichostatin A but not nicotinamide muted the insulin effect on hGH RNA levels, and was significant with 5 and 15 nM insulin. RNA levels in each sample (absolute quantification) were calculated from the standard curve and normalized to mouse B2M RNA. Data were analyzed by two-way analysis of variance with the Bonferroni post-test. Significant differences between groups at each concentration of insulin are indicated with brackets; #,  $p < 0.05$ . Significant differences between insulin treatments compared with no insulin group (0) as control are indicated by \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  ( $n = 6$ ).*

<sup>24</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Negative Regulation of Human Growth Hormone Gene Expression by Insulin is Dependent on Hypoxia-Inducible Factor Binding in Primary Non-Tumor Pituitary Cells**, Journal of Biological Chemistry, 287:33282-33292, 2012.  
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***5.6. HFD-related reduction in hGH RNA levels is consistent with increased association of the NCOR factor with the proximal hGH1 promoter region***

Transcription factors are the key mediators in physiological/homeostatic routes, as they receive and process overall environmental signals into changes in gene promoter activity and expression. This is consistent with a role for the insulin-induced E-Box associated transcription factor HIF-1 $\alpha$  in negative regulation of *hGH1* as described in the previous chapter. Transcriptional co-regulators (co-activators and co-repressors) have also emerged as equally crucial modulators of these adaptive transcriptional responses as a part of larger complexes that integrate complex signaling pathways (Desvergne, Michalik et al. 2006, Feige, Gelman et al. 2006, Tudor, Feige et al. 2007). These transcriptional co-regulators act as scaffolding proteins that provide a platform for the assembly and/or separation of the transcriptional machinery as well as chromatin modifier enzymes (Frietze and Farnham 2011). The specificity of transcriptional regulatory process by these co-regulators is supported by their ability to interact with a variety of cell/tissue specific transcription factors (Frietze and Farnham 2011).

Generally, transcriptional repression is mediated by recruitment of co-repressor complexes, which can lead to chromatin remodeling by directing histone modification enzymes to specific site of regulatory elements of the target gene. Among the most well characterized transcriptional co-repressors of the pituitary gland is the nuclear receptor co-repressor (NCOR). It is known to interact with the pituitary transcription factor Pit-1 in addition to nuclear receptors (Scully, Jacobson et al. 2000, Jepsen and Rosenfeld 2002,

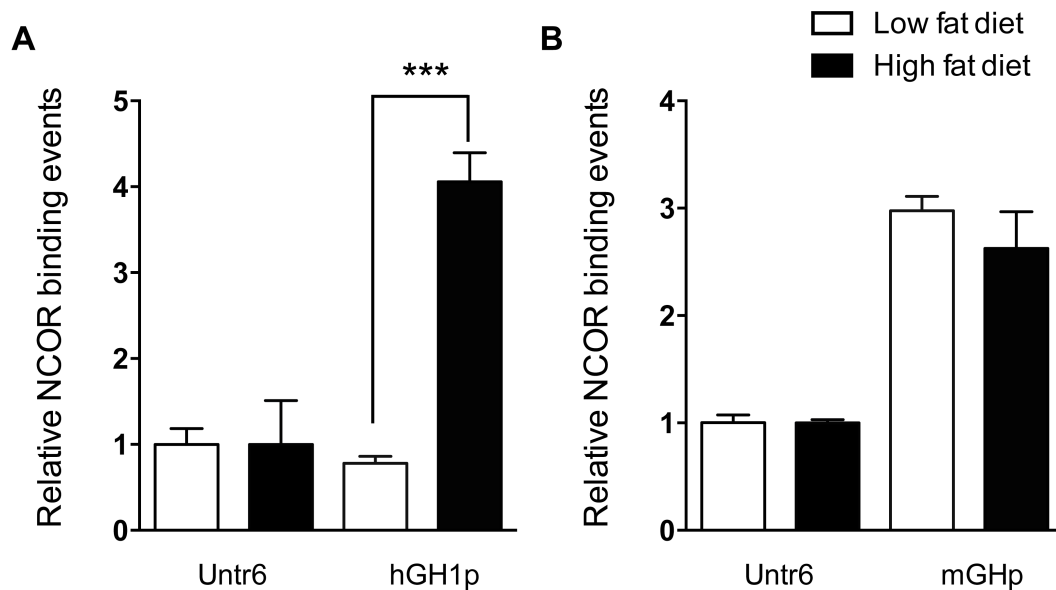
Voss, Demarco et al. 2005). An interaction between NCOR and Pit-1 was suggested as a mechanism to silence *hGHI* in Pit-1-producing lactotrophs during embryonic differentiation of the anterior pituitary (Scully, Jacobson et al. 2000, Voss, Demarco et al. 2005). This property of NCOR identifies it as a potential mediator of the transcriptional repression of *hGHI* in somatotrophs/pituitary from 171hGH/CS TG mice overfed for three days. Involvement of NCOR would also implicate co-repressor pathways that incorporate several histone deacetylases including class I (HDAC 3), class II (HDAC 4, 5, 7, and 9) (Perissi, Jepsen et al. 2010). HIF-1 $\alpha$ , like NCOR, is also reported to interact with members of class I, II, III HDACs and to recruit them to regulatory elements in target genes (Kato, Tamamizu-Kato et al. 2004, Qian, Kachhap et al. 2006, Lee, Lee et al. 2010, Perissi, Jepsen et al. 2010, Patsopoulos, Bayer Pharma et al. 2011, Rajendran, Garva et al. 2011, Du, Chen et al. 2012). Thus, a role for NCOR as a co-repressor involved in the postnatal transcriptional suppression of the hGH gene was explored.

Excess caloric intake resulted in a 4-fold increase in association of NCOR with the *hGHI* promoter region in the pituitary as assessed by CHIP assay ( $p < 0.01$ ,  $n=3$ ) (Figure 5.5A). In contrast, there was no significant effect of the HFD on association of NCOR with the *mGH* promoter region (Figure 5.5B).



**Figure 5.5<sup>25</sup>: Increased association of the NCOR factor with the human but not mouse GH promoter region in response to excess caloric intake for three days**

*Pituitary chromatin from 171 hGH/CS TG mice fed a HFD versus LFD for three days was assessed by ChIP assay using an anti-NCOR antibody and qPCR. “Binding events” were calculated based on the signals obtained from the immunoprecipitated/input DNA amplification, using specific PCR primers for the (A) hGH1 and (B) mGH promoter (p) regions as well as a control untranscribed region of mouse chromosome 6 (Untr6). The results are expressed as mean binding events  $\pm$  standard error of the mean, relative to the value determined for Untr6 in the LFD group, which is arbitrarily set to 1. Significance was assessed by one-way ANOVA with the Bonferroni post-test ( $n=3$ ). Data provided by Ms. Yan Jin.*



<sup>25</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

### 5.7. Summary of the results

In this chapter, the impact of excess caloric intake *in vivo* and insulin treatment in primary monolayer cultures *in vitro* on transcriptional regulation of the intact *hGH1* and GH LCR was investigated. The following observations were made:

- *Histone modifications are implicated in the transcriptional regulation of the hGH1 locus. Reduced histone hyperacetylation is detected in two major regulatory regions, specifically HS I/II and the promoter regions in response to excess caloric intake through use of a high fat diet in vivo as well as in response to insulin treatment in vitro.*
- *The evidence also supports a role for the class I HDAC family in the reduced hyperacetylation seen in the hGH locus and, thus, by extension in the negative regulation of hGH1 expression in response to insulin.*
- *Furthermore, the data support a role for recruitment of a transcriptional co-repressor in the regulation of postnatal hGH synthesis. This correlates with changes in the three dimensional-architecture of the hGH locus in terminally differentiated pituitary cells. Specifically, the evidence supports a loss in integrity of a chromatin loop, a critical physical interaction between the hGH locus enhancer (HS I/II) and promoter, which is associated with a decrease in gene expression.*

## CHAPTER 6

### **A negative impact of overeating on human growth hormone gene expression is limited by physical activity *in vivo***

#### ***6.1. Rationale and specific aim***

Exercise is a physical activity that has a great impact on practically every organ and tissue of an organism. Physical activity confers many health benefits and lack of exercise is implicated in many chronic diseases and pathophysiological conditions (Baldwin 2000, Booth, Gordon et al. 2000).

GH is among the endocrine factors targeted by physical exercise; it is well known that a linear dose-response relationship exists between exercise intensity and the GH secretory pattern in healthy individuals (Weltman, Weltman et al. 1992, Veldhuis, Liem et al. 1995, Saito, Sone et al. 1997, Pritzlaff, Wideman et al. 2000). GH is suggested to be an important factor due to its potent lipolytic effect, and will result in further increased energy expenditure and prevention of obesity (Pritzlaff, Wideman et al. 1999). More importantly, physical activity protects against the development of obesity through an increase in energy expenditure due to the cost of the activity itself as well as via changes in endocrine factors to increase the resting metabolic rate (Poehlman 1989, Goran, Reynolds et al. 1999).

Treadmill running, voluntary wheel running and swimming are three exercise modalities widely used in biomedical research (Kaplan, Cheslow et al. 1994). Swimming rather than running was selected as a model in this thesis because of its efficiency in promoting energy expenditure in comparison to treadmill and/or wheel running. Swimming exercise in rodents such as mice requires the activity of a large volume of muscle mass, which translates into an efficient promotion of energy use (Kaplan, Cheslow et al. 1994). In addition, the duration of the physical activity can be managed to an extent in comparison to voluntary wheel running (Kaplan, Cheslow et al. 1994). More importantly, in general, swimming exercise of mice has the same acute physiological effects as treadmill or wheel running. These physiological impacts of swimming include increased heart rate and oxygen consumption, changes in insulin and glucagon secretion rates consistent with the increased demand of skeletal muscle, energy expenditure as well as changes in sympathetic nervous system activity (Karlsson and Ahren 1990, Yoshimura, Shimomura et al. 1996, Kirchhof, Fabritz et al. 2003).

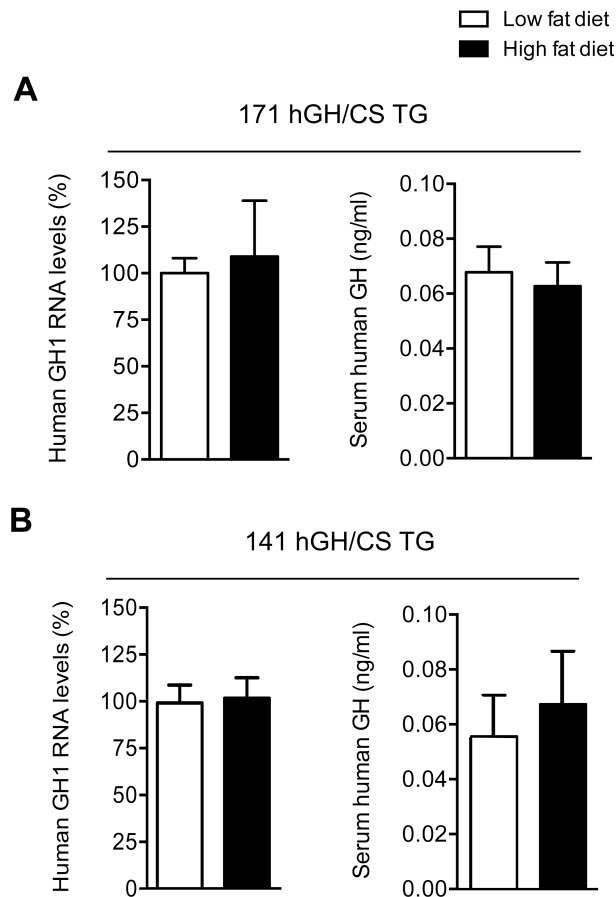
Thus, the overall goal in this chapter is to investigate the impact of prescribed physical activity (swimming), to promote use of excess energy from the HFD treatment regimen, on transcriptional regulation of *hGHI* and total hGH production.

***6.2. Prescribed physical activity blocks the repressive effect of excess caloric intake on hGH synthesis and secretion***

The effect of incorporating prescribed physical activity (50 minutes of swimming) daily into the acute (three-day) HFD and LFD feeding regimen was assessed in hGH/CS TG mice. Addition of swimming completely muted the decrease in hGH RNA and serum protein levels observed with acute excess caloric intake, as assessed by qPCR and ELISA (Figure 6.1A and B).

**Figure 6.1<sup>26</sup>: Prescribed physical activity blocks the repressive effect of excess caloric intake on hGH synthesis and secretion**

*Assessment of hGH RNA and serum protein levels, in (A) 171hGH/CS and (B) 141hGH/CS TG mice fed a HFD or LFD for three days with the incorporation of physical activity. Total pituitary RNA isolated from TG mice fed a HFD (black-filled) versus a LFD (white-filled) was assessed for hGH RNA levels by qPCR. The results are expressed as the mean (percentage)  $\pm$  standard error of mean relative to the mean value determined for LFD, which is arbitrarily set to 100%. The significance of any effect was assessed by student's t-test (n=4-9). Secreted hGH levels were also assessed by ELISA. The results are expressed as mean concentration (ng/ml)  $\pm$  standard error of mean. Significance was assessed by t-test, (n=4-10).*

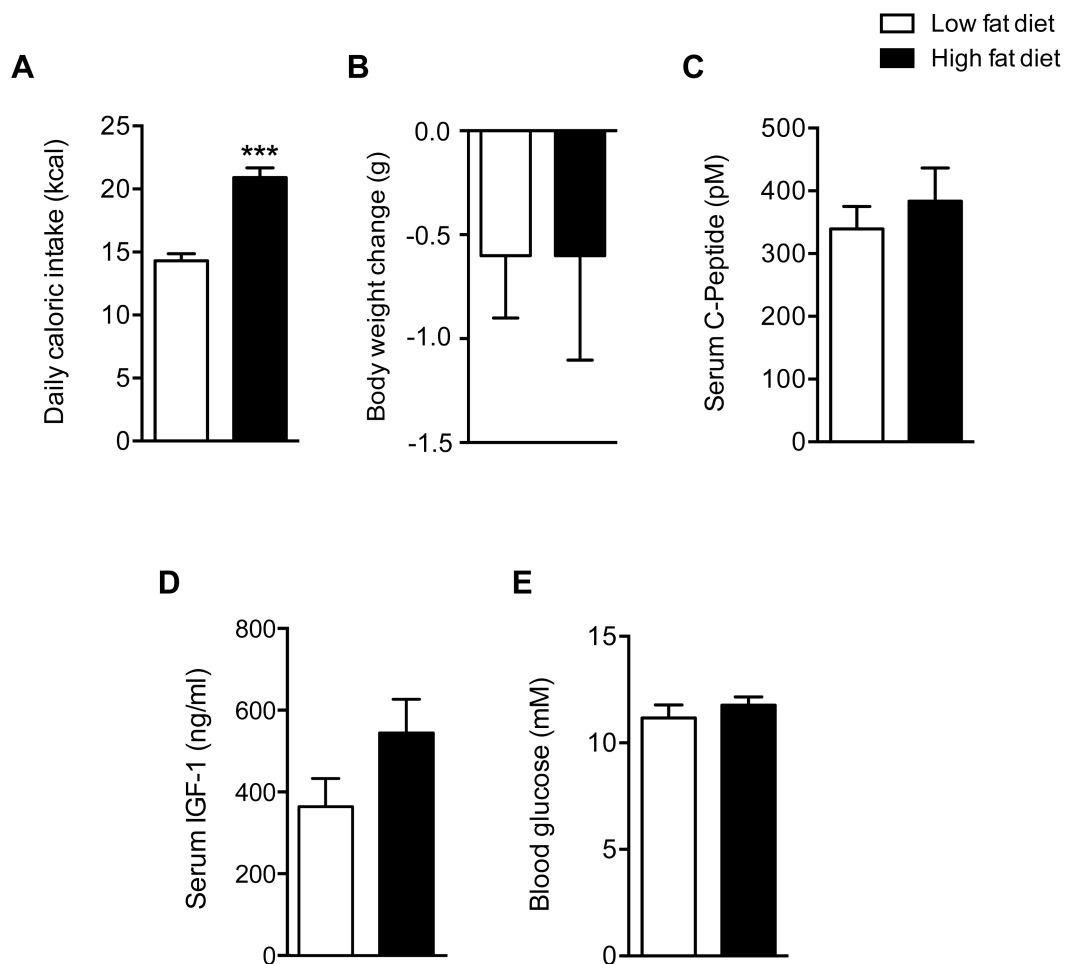


<sup>26</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

There was, however, no significant difference between caloric intake of mice on the HFD with and without physical activity (Figure 6.2A). Introduction of physical activity resulted in reduced body weight in both LFD and HFD groups (Figure 6.2B), however there was no significant difference between the two groups of mice. In spite of the high caloric intake by the HFD group, swimming muted the effect of the three-day HFD on C-peptide levels significantly (Figure 6.2C). In contrast, there was no significant difference between total IGF-1 levels in mice fed the HFD and LFD with addition of swimming, as assessed by ELISA (Figure 6.2D). These changes were not associated with glycemic levels as there was no difference between the random blood glucose levels in the mice on a LFD and HFD, which received the same physical activity regimen (Figure 6.2E).

**Figure 6.2 <sup>27</sup>: Prescribed physical activity prevents the hyperinsulinemic state associated with excess caloric intake**

Assessment of (A) daily caloric intake (kcal), (B) body weight (g), (C) serum C-peptide (pM), (D) serum IGF-1 (ng/ml) and (E) blood glucose (mM) in 171 hGH/CS TG mice fed a HFD or LFD for three days with the incorporation of physical activity. Values represent the mean  $\pm$  standard error of the mean. Significance was assessed by t-test, (n=4-10).



<sup>27</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation

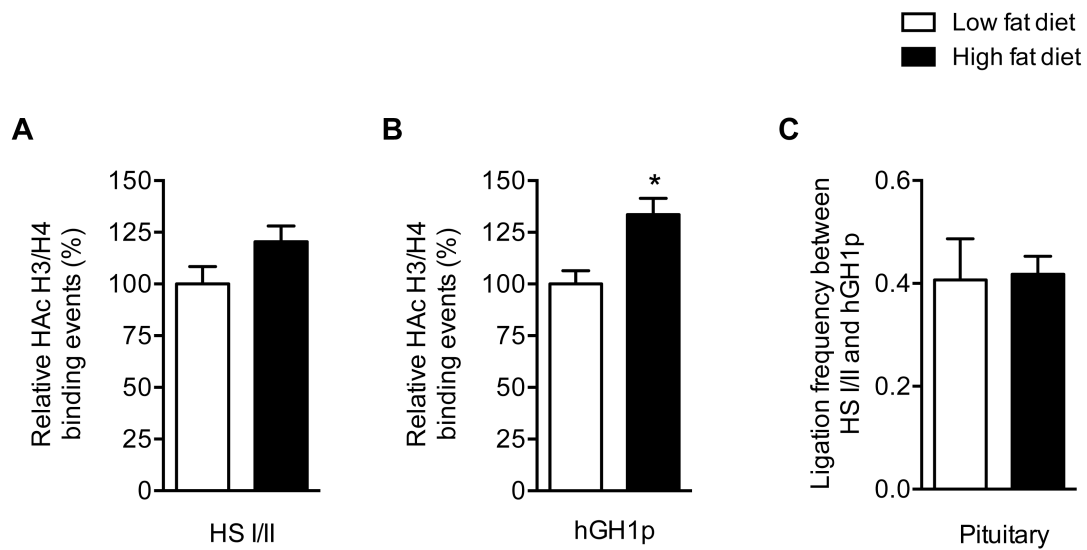


### ***6.3. Physical activity effectively preserves the histone H3/H4 hyperacetylation pattern and integrity of the “expression” loop in the hGH locus***

The effect of incorporating daily physical activity in the acute (three-day) HFD feeding regimen on histone H3/H4 hyperacetylation of the hGH LCR and proximal promoter region was examined by ChIP-qPCR assay. The reduction in H3/H4 hyperacetylation at both HS I/II and *hGHI* promoter sequences associated with the consumption of HFD was not observed (Figure 6.3A and B). Furthermore, inclusion of swimming in the treatment regimen significantly muted the effect of short term overeating on the “expression” loop as detected by 3C assay. This is consistent with maintenance of an intact chromatin-chromatin interaction between HS I/II and *hGHI* promoter regulatory regions (Figure 6.3C).

**Figure 6.3<sup>28</sup>: Physical activity effectively preserves the histone H3/H4 hyperacetylation pattern of the hGH locus and integrity of the “expression” loop**

*Detection of histone H3/H4 hyperacetylation (HAc) at (A) HS I/II and (B) the hGH1 promoter (p) regions in pituitary chromatin from 171hGH/CS TG mice fed a HFD (black-filled) or LFD (white-filled) for three days with the addition of physical activity by ChIP assay. “Binding events” were assessed as described in Figure 5. (C) The interaction (ligation frequency) between HS I/II and the hGH1p was also assessed as described in Figure 5.1. Significance was assessed by t-test (n=6). Data provided by Ms. Yan Jin.*



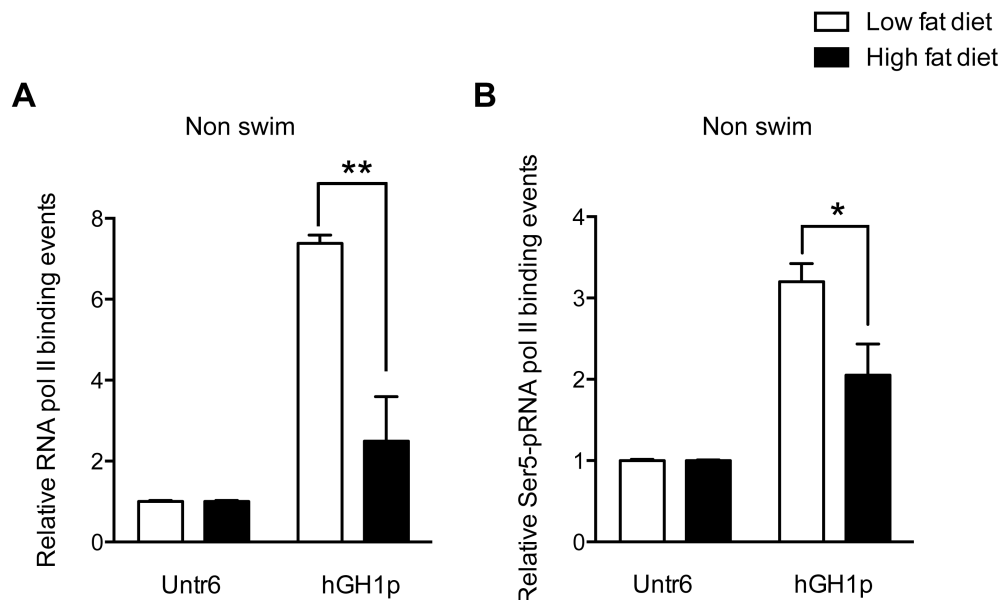
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***6.4. A reduction in association of RNA polymerase II at the hGH1 promoter with excess caloric intake is blunted with addition of prescribed physical activity***

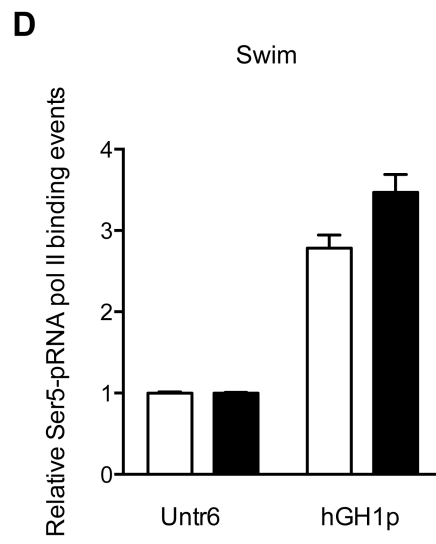
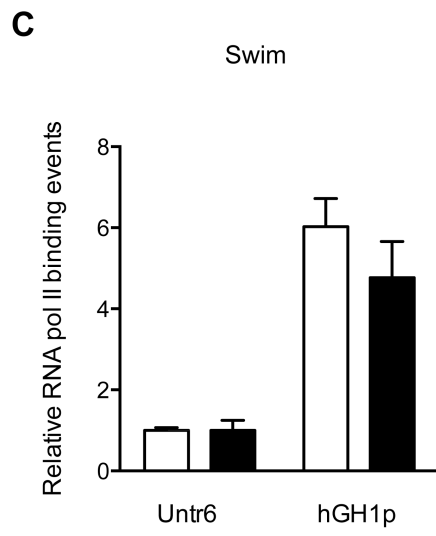
RNA polymerase (pol) II interaction with the *hGH1* promoter region was examined as an indication of corresponding changes in promoter accessibility and potential activity (Ronsch, Jager et al. 2011). Chromatin isolated from the pituitaries of hGH/CS TG mice fed a HFD versus control LFD for three days with and without prescribed physical activity was assessed by ChIP-qPCR. Specific antibodies were used to RNA pol II as well as RNA pol II with phosphorylated serine 5 at the C-terminal domain of the large subunit (Ser5-pRNA pol II). The latter is associated with polymerase that is more readily detected at the transcription start site and primed for transcriptional initiation (Phatnani and Greenleaf 2006, Hsin and Manley 2012). Short-term excess caloric intake resulted in a significant (70%) reduction of RNA pol II and Ser5-pRNA pol II associated with the *hGH1* promoter ( $p < 0.05$ ,  $n=3-6$ ) compared to mice on the control diet (Figure 6.4A and B). Furthermore, these reductions in RNA pol II association were not observed when swimming was added to the treatment regimen (Figures 6.4C and D).

**Figure 6.4 <sup>29</sup>: Reduced association of RNA polymerase (pol) II at the *hGH1* promoter with excess caloric intake is blunted with addition of physical activity**

*Assessment of (A, C) RNA polymerase (pol) II and (B, D) the large subunit of RNA pol II phosphorylated at serine 5 of the C-terminus domain (Ser5-pRNA pol II) by ChIP, as indicators of the transcription complex at the *hGH1* promoter and transcriptional initiation/elongation in 171 *hGH/CS* TG mice fed a HFD or LFD for three days (A, B) without (Non swim) or (C, D) with the incorporation of physical activity (Swim). “Binding events” were calculated based on the signals obtained from the immunoprecipitated/input DNA amplification, using specific primers for the *hGH1* promoter (p) region and the control *Untr6* by qPCR. The results are expressed as the mean  $\pm$  standard error of the mean, relative to the *Untr6* value from the LFD group, which is arbitrarily set to 1. Significance was assessed by one-way ANOVA using the Bonferroni post-test ( $n=3-6$ ). Data provided by Ms. Yan Jin.*



<sup>29</sup> Reprinted figure: Vakili H, Jin Y, Cattini PA, **Energy homeostasis targets chromosomal reconfiguration of the human GH1 locus**, Journal of Clinical Investigation, 2014. © American Society for Clinical Investigation



### ***6.5. Summary of the results***

The impact of incorporating prescribed physical activity in the treatment regimen was examined in this chapter. Thus, the effect of swim and excess caloric intake on transcriptional regulation of the intact *hGHI* and GH LCR was investigated *in vivo*. The following observations were made:

- *Prescribed physical activity mitigated the increase in circulatory insulin levels associated with the acute excess caloric intake. Thus, incorporation of swimming into the diet regimen muted the negative impact of a HFD on the chromosomal structure containing the hGH gene locus, association of the RNA pol II with the hGHI promoter region and, as a result, hGH RNA levels.*

## CHAPTER 7

### Discussion

The work presented here is focused on the postnatal regulation of the endogenous pituitary human growth hormone gene (*hGHI*). This was done in the context of an intact gene locus, in response to physiological stimuli that can affect energy balance (excess caloric intake and physical activity), by using a transgenic mouse model (hGH/CS TG) *in vivo*. These studies provide novel insights into how *hGHI* is regulated in the context of chromatin and influenced by hormonal changes in an intact endocrine system *in vivo* and *in vitro*. This includes dynamic changes in the association of enhancer and promoter regions, suggesting remodeling of a chromosomal structure that is linked to initial activation of the *hGHI* locus during embryonic development.

#### ***7.1. Summary of the results***

Transgenic mice containing the intact hGH locus from human chromosome 17 were used to study the effects of excess caloric intake and physical activity on local chromosomal organization, and its specific relationship to efficient *hGHI* expression via reorganization in response to environmental influence. High caloric intake for three days resulted in hyperinsulinemia without hyperglycemia and a decrease in both hGH

synthesis and secretion. By contrast, there was no significant effect on mGH levels in the same mice. Incorporation of physical activity, however, muted the effects of excess caloric intake on insulin levels as well as hGH production. Thus, a role for insulin in regulation of hGH was explored. Using cultures derived from hGH/CS TG mouse, hGH RNA synthesis was shown, for the first time, to be negatively regulated by insulin in primary non-tumor pituitary cells *in vitro*. In addition, postnatal hGH synthesis is regulated in response to a high fat diet known to induce a hyperinsulinemic state. The decrease in expression observed correlates with a change in three dimensional chromatin structure of the hGH locus in terminally differentiated pituitary cells *in vivo*. Furthermore, this change with diet is muted in response to prescribed physical activity. This reorganization in chromatin structure is associated with histone modifications and a decrease in apparent hGH enhancer and promoter region interaction. This interaction is essential for initial activation of the *hGHI* locus during somatotroph development (Ho, Elefant et al. 2006, Ho, Tadevosyan et al. 2008, Ho, Shewchuk et al. 2013). These results are discussed in relation to hGH and energy homeostasis as well as the molecular basis governing this rapid regulation of hGH production.

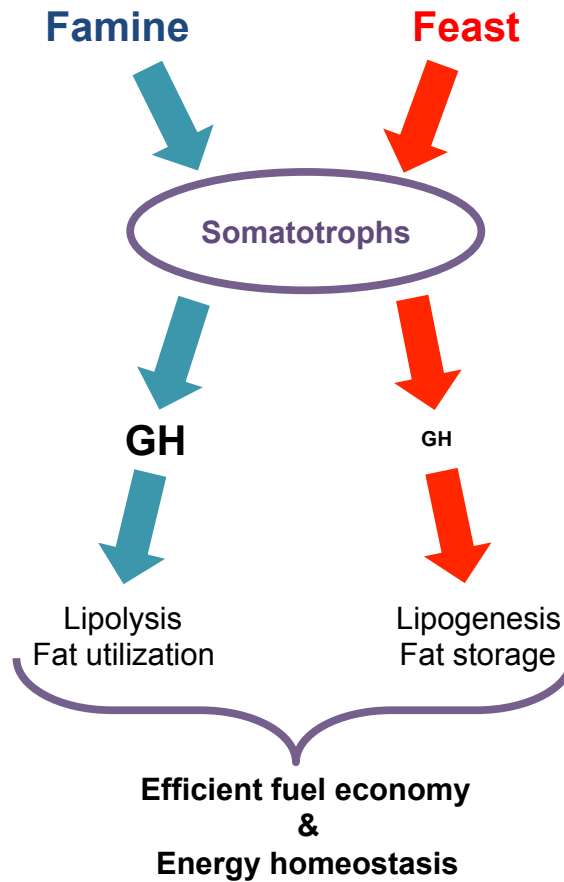
## ***7.2. Somatotrophs and GH - A cornerstone of catabolic and anabolic adaptations in metabolic homeostasis***

Survival during feast and famine cycles involves regulation on both sides of the energy balance equation, or anabolism and catabolism. In order to maintain a healthy metabolic status, signals originating from peripheral tissues such as endocrine factors,



and also extrinsic factors such as nutrition and their derivative metabolites, become integrated and analyzed to determine the ultimate reaction of an organism to the physiological condition experienced. In the “feast and famine cycle”, GH has been described as the major anabolic hormone during famine and stress (Rabinowitz and Zierler 1963). GH helps to ensure adequate sparing of glucose and protein at the expense of lipids as the main source of fuel by suppressing lipogenesis (Oscarsson, Ottosson et al. 1999, Richelsen 1999) and adipogenesis (Carrel and Allen 2000, Carrel and Allen 2000) as well as stimulating lipolysis (Raben and Hollenberg 1959) (Figure 7.1). Therefore under the conditions of energy deprivation and fasting, levels of hGH secretion are elevated. Hence, they can induce the release of free fatty acids to be consumed as a source of energy fuel to maintain a healthy metabolic homeostasis (Ho, Veldhuis et al. 1988, Hartman, Veldhuis et al. 1992, Vance, Hartman et al. 1992).

**Figure 7.1: Physiological involvement of somatotrophs and GH in fine-tuning during feast and famine cycles to ensure an efficient metabolic status**



*The level of GH rises during periods of negative energy balance (famine). As a result, the balance of activity between anabolic and catabolic pathways is altered to favor increased anabolic activity. Ultimately, increased anabolic activity results in elevation of food intake (feast) and GH levels fall in response. This results in further deposition of excess calories in the form of adipose tissue due to loss of enhancer impact of GH on lipolytic enzymes gene expression/activity. Limited function of lipolytic enzymes translates into enhancement of lipogenesis, which is associated with expansion of adipose tissue and thus adiposity. Collectively, an orchestrated balance between these two pathways contributes to overall metabolic health.*

This physiological regulation of GH levels has been investigated extensively in humans. Interestingly, recent studies suggest that GH levels might be regulated differently in primates versus murine species. Fasting in mice inhibits the pulsatile GH secretion pattern, and furthermore, a fasting-induced release of free fatty acids in mice does not follow an elevation in GH secretion (Steyn, Leong et al. 2012). Thus, there appears to be some variation in signals and mechanisms under which production and action of primate and rodent growth hormones are subjected and regulated (Vakili, Jin et al. 2011). This divergence may also result from sequence differences between primate and non-primate GH genes, which raises the possibility of distinct regulatory control of expression.

The other side of the energy balance equation is excess caloric intake during feasting as a regulator of the GH production, which is a major focus of this thesis. During feasting, storage of extra calories is favored and this condition is expected to have an impact on GH levels/action to ensure an appropriate fine-tuning of the energy balance by promoting fat storage and adipogenesis. In fact blunted GH secretion is one of the major characteristics associated with obesity (as an example of extreme and long term excess caloric intake) in humans (De Marinis, Bianchi et al. 2004, Pataky, Bobbioni-Harsch et al. 2010).

A negative effect of obesity on GH secretion in mice has also been observed, and thus appears to model the situation in the human population (Luque and Kineman 2006). This convergence of response in rodents and primates to obesity and associated

complications contrasts with what has been reported in regard to the impact of fasting/famine on GH production, as discussed above. There are, however, differences between humans and rodents, including the magnitude of response and how fast the response is detected. While chronic excess caloric intake (16 weeks) is associated with a decrease in mouse GH RNA levels, the effect of short-term excess caloric intake is not reported (Luque and Kineman 2006).

Strikingly, GH levels are reduced in humans within a short period of time by caloric overload (Cornford, Barkan et al. 2011). Normal healthy individuals that indulge in short term overeating display a dramatic suppression of serum GH levels. Interestingly, this phenomenon happens when obesity is not an issue (Cornford, Barkan et al. 2011). It was suggested that the effect of excess caloric intake was to impair GH secretion, however, the data did not exclude a negative effect of overeating on GH synthesis by pituitary somatotrophs. An effect on synthesis might affect interpretation of data and specifically the perceived effectiveness of GH secretagogues as anti-obesity agents. Data presented in Chapter 3 emphasize that hGH production is indeed extremely sensitive to increased caloric intake within the continuum of obesity progression.

The prevalence of obesity is high in both genders and in all age groups. The study reported in Chapter 3 was provoked, however, by the clinical report focused on healthy non-obese young men who displayed a dramatic decline in growth hormone levels as a result of overeating for a few days (Cornford, Barkan et al. 2011). The aim was to try to model this response in the hGH/CS transgenic mice, and thus, the initial focus had to be

on the use of young male mice. The intent was to extend this model and look at overeating as a stage in developing obesity. Extension of this study will ultimately include examination of the role of obesity and related factors on growth hormone production in female mice.

Impaired spontaneous secretion of hGH and a decline in somatotroph responsiveness to all known pharmacological provocative stimuli are reported in obese humans (Vanderschueren-Lodeweyckx 1993, Maccario, Procopio et al. 1995, Procopio, Maccario et al. 1995, Pijl, Langendonk et al. 2001, Scacchi, Orsini et al. 2010). Specifically, GH producing somatotrophs become significantly hypo-responsive to exogenous secretagogues including GHRH, ghrelin and arginine (Maccario, Procopio et al. 1995, Procopio, Maccario et al. 1995, Pijl, Langendonk et al. 2001, Scacchi, Orsini et al. 2010). Thus, most of our understanding of hGH levels under pathophysiological conditions of obesity and associated complications is heavily based on serum levels, without an appreciation that a negative effect on GH synthesis could ultimately contribute to the limited secretory capacity of somatotrophs. Thus, the suppression of hGH can occur as a result of impaired GH secretagogue signaling at the level of the somatotroph, however, it can also be explained in terms of a decrease in hGH synthesis and insufficient reserves to be secreted in response to a treatment with the GH secretagogue. Evidence presented in this thesis is consistent with the latter. Specifically, there was a significant decrease in hGH synthesis within three days on a HFD. In contrast there was no significant effect on hypothalamic GHRH and somatostatin transcripts and those of their respective pituitary receptors within the same time frame. Even though the secreted levels

of GHRH and/or somatostatin and the activity of their respective receptors were not measured, the above mentioned data regarding no significant change of these factors at the transcript levels further suggests a direct effect of overeating on hGH synthesis. Thus, it is possible that a HFD for three days does not impair GH secretagogue signaling significantly in the mouse pituitary, but rather that the effect on serum hGH levels is exerted at the level of synthesis.

### ***7.3. Somatotrophs can sense excess insulin as an obesity signal in vivo***

The significance of somatotrophs as a sensor with the ability to receive metabolic signals and respond to alterations in metabolic pathways has been suggested previously (Childs, Akhter et al. 2011). Data presented in Chapters 3 and 4 are consistent with a role for pituitary somatotrophs as the primary sensors of circulating insulin, which could determine the GH output under conditions where serum insulin levels are elevated, as with overeating and the HFD.

Short term overeating can create a complex network of endocrine responses that can modulate metabolism and ultimately repress GH levels as a consequence (Forbes, Brown et al. 1986, Forbes, Brown et al. 1989, Diaz, Prentice et al. 1992, Groop, Bonadonna et al. 1992). A major finding of the *in vivo* studies reported here (Chapter 3), however, is a role for insulin regulation of hGH. Higher levels of serum insulin and C-peptide (surrogate marker for insulin secretion) were observed in conjunction with the reduction in hGH levels in hGH/CS TG mice in response to short-term excess caloric intake. Total IGF-1 levels were also assessed and no significant change in response to

short-term excess caloric intake was observed. This is consistent with a previous report in which healthy individuals who overate for three days displayed a profound hyperinsulinemia and no significant changes in the total IGF-1 levels (as one of the main regulators of GH production via negative feedback) (Cornford, Barkan et al. 2011). Thus, it was suggested that the hyperinsulinemic condition associated with overeating is the potential mediator of the decrease in plasma hGH levels (Cornford, Barkan et al. 2011). A negative correlation between insulin and GH levels is supported by other human and rodent studies (Coleman and Bell 1976, DeFronzo, Ferrannini et al. 1981, Luque, Gahete et al. 2006, Luque and Kineman 2006). Circulating hormones including GH, insulin, corticosterone, ghrelin, IGF-I and metabolites such as glucose and free fatty acids were assessed in mice fed a HFD for 10 weeks (Luque and Kineman 2006). As expected mouse GH levels were reduced in mice fed the HFD compared to control mice, and this decrease was only associated with insulin levels (Luque and Kineman 2006).

Since high fat diet-induced obesity is accompanied by hyperleptinemia as well as GH deficiency, a role for leptin signaling and leptin involvement in reduction of GH levels associated with obesity can be suggested (Hoffler, Hobbie et al. 2009). However, based on the fact that obese leptin deficient ob/ob mice are also GH deficient in the absence of any leptin signaling, this possibility can be excluded (Buettner, Newgard et al. 2000, Luque and Kineman 2006). In fact the state of GH deficiency seen in ob/ob mice correlates with increased circulating levels of insulin (Buettner, Newgard et al. 2000, Luque and Kineman 2006).

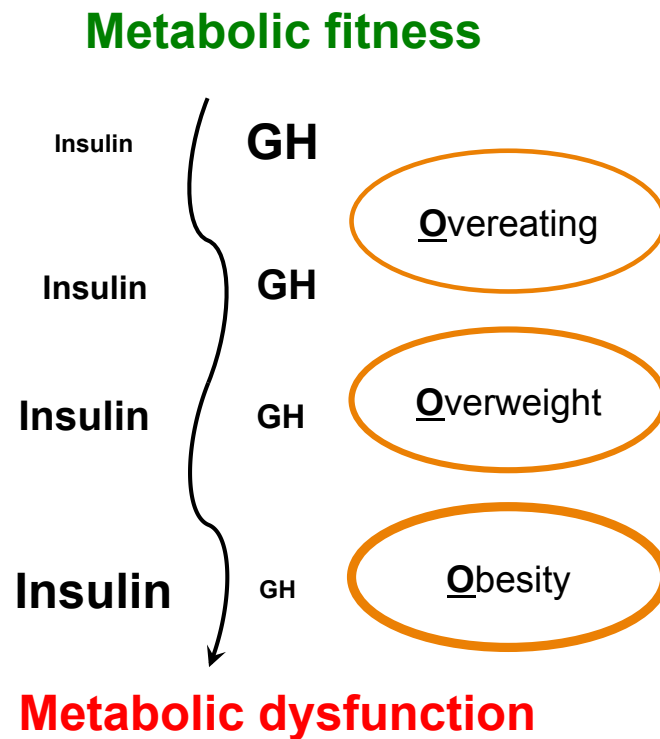
Further support for direct regulation of somatotrophs and GH expression by insulin comes from a transgenic mouse study by Gahete and colleagues. They observed elevated GH levels following a somatotroph-specific loss of insulin receptors in mice *in vivo* (Gahete, Cordoba-Chacon et al. 2011). This would be consistent with a loss of any inhibitory signal by insulin and its receptor on somatotroph function and GH production. The effect of short term overeating in humans is also consistent with a rapid suppression of GH levels that correlates with significant hyperinsulinemia. By contrast, there were no major changes in other measured factors such as cortisol, IGF-1, free fatty acids and glucose levels that appeared linked to the decrease in GH (Cornford, Barkan et al. 2011).

Thus, in the continuum of obesity, there is a negative correlation between GH and insulin levels. As such, a rise in insulin levels would lead to a reduction in GH levels, and persistent low GH levels may then contribute to metabolic syndrome through a disturbance in metabolic homeostasis. This speculation is summarized in Figure 7.2.

Thus far, overeating in humans has been modeled successfully in hGH/CS TG mice using a high fat feeding paradigm. Furthermore, there is direct evidence using this model that hGH synthesis at the RNA level as well as secretion is rapidly suppressed by overeating.



**Figure 7.2: Schematic representation of the relationship between GH and insulin in the continuum of obesity development**



*In the continuum of obesity, a negative correlation between GH and insulin levels exists, it is as such that rise in insulin levels consequent to excess caloric intake, with and without limited energy expenditure, leads to reduction in GH levels and these metabolic changes if persists may contribute to metabolic syndrome through disturbance in metabolic homeostasis.*

#### ***7.4. A role for somatotrophs as primary sensors of insulin –Evidence from insulin regulation of GH production in vitro***

A role for pituitary somatotrophs as the primary sensors of circulating insulin is suggested here. This property could determine the GH output under conditions where serum insulin levels are elevated, as with overeating and the HFD. Melmed and colleagues were the first to report direct regulation of GH levels by insulin in rat primary pituitary cell cultures and rodent GH producing pituitary cell lines (Melmed 1984, Melmed, Neilson et al. 1985, Melmed and Slanina 1985). Subsequently, they assessed effects on the hGH gene in a transient transfection system using pituitary cell lines (Melmed, Neilson et al. 1985, Melmed and Slanina 1985, Yamashita and Melmed 1986, Yamashita and Melmed 1986, Prager, Gebremedhin et al. 1990). They further provided evidence for direct regulation of GH transcription by inhibiting the insulin-signaling pathway through immune-neutralization of the insulin receptor (Melmed 1984, Yamashita and Melmed 1986). These studies were extended by others who demonstrated the direct inhibitory actions of insulin on somatotroph function in non-human primates (Luque, Gahete et al. 2006, Luque and Kineman 2006). In agreement, and as discussed in section 5.9.1 above, results presented in Chapter 3 using hGH/CS TG mice suggest that insulin downregulates hGH synthesis at the transcriptional level. Thus, the mechanism underlying the direct negative effect of insulin on human and endogenous mGH levels (gene expression in the context of native chromatin) was explored in isolated primary pituitary cultures from hGH/CS TG mice (in Chapter 4).

The data indicate that physiological concentrations (1-10 nM) of insulin negatively regulate hGH transcript levels in isolated primary pituitary cell cultures. This supports a direct role for insulin in the regulation of *hGHI* expression *in vivo* (Chapter 3). Furthermore, this is consistent with the existing negative correlation between GH production and the hyperinsulinemic state associated with HFD consumption over three days in transgenic mice.

An interesting observation, however, is the differential response of hGH versus mGH to short-term excess caloric intake and its associated hyperinsulinemic state. Unlike *hGHI*, the endogenous mGH gene displayed no negative response to the hyperinsulinemic conditions induced by acute excess caloric intake in hGH/CS TG and wild type CD1 mice *in vivo*. Negative regulation of mGH was observed, however, after treatment of primary pituitary cell cultures with insulin. At first glance there appears to be a divergence in terms of primate versus rodent GH genes in response to hyperinsulinemia *in vivo*, and a convergence *in vitro*. There are at least three considerations that might help explain this apparent inconsistency:

**(i)** Even though human and mouse GHs are both negatively affected by insulin treatment, a higher dose of insulin is required to exert a significant decrease in mouse versus human GH gene expression in isolated primary pituitary cell cultures from hGH/CS TG mice (Vakili, Jin et al. 2012).

**(ii)** A longer exposure to excess insulin *in vivo* might be required to observe a reduction in mGH RNA levels. This would be consistent with previous data showing a decreasing trend in GH levels after three weeks on the HFD, but which did not reach

statistical significance. Thus, the contradiction between presented results (*in vivo*) and the data found in the literature may arise from the duration of the HFD consumption as well as the sensitivity of mGH versus hGH.

(iii) There is also the potential for a differential impact of downstream molecules (transcription factors) in the insulin signaling pathway on *hGHI* versus *mGH* gene expression.

### ***7.5. Insulin induction of a DNA-protein interaction at the proximal promoter region of the hGH gene***

A possible explanation for divergence between the response of human and mouse GH genes to a high fat dietary challenge is the presence of a distinct regulatory DNA element(s). In regard to the *hGHI*, the proximal region (nucleotides -496/+1) of the *hGHI* promoter was downregulated by insulin after transfection into a rat pituitary (GC) cell line (Lefevre, Imagawa et al. 1987, Peritz, Fodor et al. 1988, Prager and Melmed 1988, Prager, Gebremedhin et al. 1990). This decrease correlated with induction of a DNA-protein interaction at nucleotides -308/-235 in the *hGHI* flanking DNA as assessed by nuclease protection assay (Lefevre, Imagawa et al. 1987, Peritz, Fodor et al. 1988, Prager and Melmed 1988, Prager, Gebremedhin et al. 1990). This site of DNA/protein interaction (nucleotides -308/-235) was visually comparable using nuclear extracts from pituitary and non-pituitary cell types. As such it was concluded that this DNA/protein interaction is not a cell or tissue-specific event, but rather implies participation of a more ubiquitous protein/DNA interaction (Lefevre, Imagawa et al. 1987, Peritz, Fodor et al.

1988, Prager, Gebremedhin et al. 1990). Furthermore, the possibility for redundant involvement of different members of a transcription factor family, which might have cell/tissue expression pattern, cannot be excluded here. Inspection of these sequences using database search (MatInspector, Genomatix) software identified a putative binding site for an Enhancer Box (E-box) transcription factor in the *hGHI* promoter region. This palindromic hexanucleotide sequence 5'-CCACGTGAC-3' (underlined) is located at position -264 to -259 and corresponds to the site of an insulin-induced DNA/protein interaction (Prager, Gebremedhin et al. 1990). There are multiple E-box factors and many of these are ubiquitous (Massari and Murre 2000, Ripperger and Schibler 2006, Biggs, Wan et al. 2007, Hu, Stiehl et al. 2011, Tang, Luo et al. 2011, Goriki, Hatanaka et al. 2014). As such, there is the potential for significant redundancy in terms of the responsiveness of an E-Box DNA element. A candidate E-Box factor to mediate the response of *hGHI* to insulin is hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), given its known responsiveness to insulin (Zelzer, Levy et al. 1998, Richard, Berra et al. 2000, Jiang, Jiang et al. 2001, Stiehl, Jelkmann et al. 2002, Treins, Giorgetti-Peraldi et al. 2002). Furthermore, analysis of the E-box element present in the sequences -264 to -259 of *hGHI* promoter region suggests similarity to those in known insulin sensitive genes. This includes the amylase and phosphoenolpyruvate carboxykinase genes, which are regulated by insulin via the E-Box elements and HIF-1 $\alpha$  (Prager, Gebremedhin et al. 1990, Treins, Giorgetti-Peraldi et al. 2002, Choi, Park et al. 2005, Tajima, Goda et al. 2009).

Similar to hypoxia, insulin provokes the stabilization and accumulation of HIF-1 $\alpha$  protein via phosphatidylinositol 3 kinase/Akt-dependent and/or mitogen-activated protein kinase signaling pathways (Treins, Giorgetti-Peraldi et al. 2002). Thus, HIF-1 $\alpha$ 's role was

investigated as a potential mediator of insulin regulation of *hGHI*. Insulin treatment of isolated primary pituitary cell cultures for 24 hours increased HIF-1 $\alpha$  protein levels as expected. In addition, insulin regulation of *hGHI* was shown to be dependent on the HIF-1 $\alpha$  transcription factor, since interference with HIF-1 $\alpha$  levels with HIF-1 $\alpha$  shRNA were consistent with blunting the negative response of *hGHI* to insulin significantly. Furthermore, chemical interference with E-box related factor binding by using echinomycin, reduces the effect of insulin as well as chemically-induced HIF-1 $\alpha$  on *hGHI* RNA levels. As such, E-box transcription factors, including HIF-1 $\alpha$ , are candidates for facilitating the observed response of *hGHI* to insulin treatment.

By contrast to *hGH*, the negative effect of insulin on *mGH* RNA levels was not responsive to changes in HIF-1 $\alpha$  levels or interference with HIF DNA binding (E-box). This is similar to the effect of insulin on *mGHS-R* RNA levels, which was decreased in agreement with previous reports using non-human primate pituitary cultures (Luque, Gahete et al. 2006). Insulin regulation of both *mGH* and *mGHS-R* appears to be independent of HIF-1 $\alpha$ . An alignment of *mGH* with *hGHI* promoter regions, including nucleotides -279/-250, revealed no putative E-box and as such no HIF-1 $\alpha$  binding site in equivalent *mGH* sequences; no binding site was detected when this was extended to include 2 kb of upstream *mGH* and *mGHS-R* flanking DNA. Thus, while there appears to be convergence in terms of the overall response of the *hGH* and *mGH* genes to insulin, *hGHI* and by extension human somatotrophs, may be more sensitive to insulin levels. This may reflect distinct regulatory mechanisms, and the involvement of E-Box

associated transcription factors(s) in hGH but not mGH synthesis in response to a hyperinsulinemic state induced by excess caloric intake.

#### ***7.6. A metabolic role for insulin-induced HIF-1 $\alpha$ and reduced hGH production***

HIF-1 $\alpha$  plays a significant role in developmental and physiological events (Ratan, Siddiq et al. 2004, Weidemann and Johnson 2008, Adams, Difazio et al. 2009), including energy metabolism (Jiang, Qu et al. 2011, Patsopoulos, Bayer Pharma et al. 2011, Zhang, Zhang et al. 2011, Krishnan, Danzer et al. 2012). Deregulation of HIF-1 $\alpha$  predominantly in relation to effects on pancreatic gene expression and  $\beta$ -cell function has been linked to the development of type 2 diabetes (Cheng, Ho et al. 2010). Furthermore, elevated levels of HIF-1 $\alpha$  in adipose tissue are postulated to contribute to obesity-related insulin resistance and metabolic dysfunction. As such, HIF-1 $\alpha$  is considered a participant of an obesity related transcription factor complex (Weng, Zhang et al. 2010, Zhang, Lam et al. 2010, Erman, Wabitsch et al. 2011). High concentrations of insulin can provoke the accumulation of HIF-1 $\alpha$  via phosphatidylinositol 3 kinase/Akt-dependent and/or mitogen-activated protein kinase signaling pathways (Treins, Giorgetti-Peraldi et al. 2002).

If “overeating” is seen as a stage of developing “obesity”, then the initial decrease in GH might be interpreted as a positive response by increasing the whole body insulin sensitivity for a limited period of time, due to its anti-insulin activity (Yakar, Setser et al. 2004, Luque, Lin et al. 2011). This was suggested based on the observation that adult onset isolated GH deficiency in mice, was associated with higher insulin sensitivity

(Luque, Lin et al. 2011). If conditions of excess caloric intake were to persist, however, then the decrease in GH is expected to be a primary or secondary cause of obesity by contributing to further weight gain, due to loss of lipolytic and anabolic activities of GH as well as impaired insulin output (Scacchi, Pincelli et al. 1999, Takahashi and Satozawa 2002, Moller, Gjedsted et al. 2003, Christoforidis, Maniadaki et al. 2005, Gahete, Cordoba-Chacon et al. 2011). If, however, the initial response to excess insulin is a decrease in GH and a compensatory increase in insulin sensitivity, then this would be consistent with HIF-1 $\alpha$  involvement. Thus, HIF-1 $\alpha$  as a signaling molecule induced by elevated levels of insulin (hyperinsulinemia) offers an explanation for the blunted GH levels seen in obese individuals (Jiang, Qu et al. 2011, Patsopoulos, Bayer Pharma et al. 2011, Zhang, Zhang et al. 2011, Krishnan, Danzer et al. 2012).

***7.7. A discrepancy between in vitro and in vivo insulin regulation of hGHI in regards to the identity of the E-box transcription factor(s)***

There is an apparent discrepancy between the *in vitro* and *in vivo* effects of insulin on regulation of *hGHI*. In contrast to a strong role suggested for HIF-1 $\alpha$  in the suppression of *hGHI* in response to insulin treatment in primary pituitary cell culture, no increase in association of HIF-1 $\alpha$  with the hGH locus was observed after three days of high fat diet regimen in hGH/CS TG mice *in vivo*. A possible explanation includes the differences seen in chromatin remodeling (histone hyperacetylation levels) of the hGH locus in response to excess insulin. Insulin treatment of isolated primary pituitary cells *in vitro* decreased the histone hyperacetylation only in the *hGHI* proximal promoter region



with insulin treatment. There was also a dramatic decrease in H3/H4 hyperacetylation associated with *hGHI* promoter in the *in vivo* study, but unlike cells in culture there was also a reduction at the HS I/II region in mice on the HFD. Given the evidence for a physical interaction between the HS I/II and the proximal promoter regions, decreased histone hyperacetylation may influence the accessibility of the promoter region to transcription factors significantly under the hyperinsulinemic state associated with acute excess caloric intake.

Another possible explanation for the difference and lack of HIF-1 detection *in vivo* relates to severity of hyperinsulinemic state as well as the timing of assessment. Serum insulin level detected after 3 days on HFD (approximately 0.5 nM) as indicated in Table 3.1) was lower than the lowest insulin concentration (1 nM) used in the *in vitro* assessment of insulin regulation of hGH levels experiments. Furthermore, the potential exists for early but transient induction and by extension association of HIF-1 $\alpha$  with the hGH1 locus. Thus, an effect might be detected at an earlier time point but binding may not persist for three days, the time point that pituitary tissues were assessed for HIF-1 $\alpha$  association by ChIP assay (Chapter 4). This pattern of transient HIF-1 $\alpha$  elevation that is sufficient to trigger its biological function has been described to explain transient induction of HIF-1 $\alpha$  in hypoxic vascular system (Kai, Kuwahara et al. 2002). Although HIF-1 $\alpha$  returns to insignificant levels, the physiological/pathophysiological outcome related to the transient presence of HIF-1 $\alpha$  persists as long as the influential condition is stable. Thus, it is possible that HIF-1 $\alpha$  may act as an initial and transient trigger of transcriptional repression of *hGHI*, perhaps including recruitment of additional repressor factor(s), in response to elevated levels of insulin as a result of excess caloric intake *in*

*vivo*. In order to test this possibility, a time course study could be done. Specifically, HIF-1 $\alpha$  induction and/or association with the hGH locus *in situ* could be assessed at 24, 48 and 72 hours post introduction of a HFD to hGH/CS TG mice.

### ***7.8. A role for disrupted circadian machinery at the E-box located in the proximal promoter region of hGH1 by excess caloric intake***

Evidence for the direct involvement of HIF-1 $\alpha$  in the response of *hGH1* to a HFD for three days *in vivo* was not obtained (Chapter 4). While this does not rule out an early and transient role for HIF-1 $\alpha$ , a role for disruption of Bmal1 association with the *hGH1* promoter region was supported. As indicated previously, consumption by mice of a HFD for just three days resulted in disruption of Bmal1 binding to target hepatic genes in chromatin *in situ* (Eckel-Mahan, Patel et al. 2013). Furthermore, this occurred without any significant changes in the level of Bmal1 RNA and/or protein levels (Eckel-Mahan, Patel et al. 2013). As such it was concluded that acute excess caloric intake for three days is sufficient to reprogram the circadian rhythm (Eckel-Mahan, Patel et al. 2013).

Results from EMSA also support the ability of Bmal1/Clock and *hGH1* E-box DNA element to participate in a common complex, similar to that suggested for HIF-1. It is noted that Bmal1/Clock complexes have significantly different chromatin binding properties at different times during the day, possibly due to post-translational

modification (Gallego and Virshup 2007). Thus, binding of these transcription factors to E-box elements follows a circadian pattern *in vivo* (Lee, Etchegaray et al. 2001). The Bmal1 ChIP analysis supports this notion; there was a difference between the levels of Bmal1 associated with the *hGHI* promoter region based on the time of euthanization (light versus dark cycle).

It is also noteworthy that Bmal1/Clock does not remain bound to chromatin throughout the day (Ripperger and Schibler 2006). Thus, the opportunity for other E-box associated factors to bind and perhaps modulate the promoter transcriptional activity is possible. As a result, a circadian exchange of different transcription factors binding to E-box elements would offer a target for a variety of signaling pathways perhaps reflecting environmental and/or intrinsic cellular cues to influence transcriptional activity depending on the physiological demands. Therefore, involvement of more than one E-box-related transcription factor in the regulation of *hGHI* is possible as there are multiple E-box factors and many of them are ubiquitous (Massari and Murre 2000, Ripperger and Schibler 2006, Biggs, Wan et al. 2007, Hu, Stiehl et al. 2011, Tang, Luo et al. 2011, Goriki, Hatanaka et al. 2014). As such, there is a potential for significant redundancy as well as cooperation and/or competition in terms of specific factor(s), (perhaps in different combinations through formation of heterodimers) and/or mechanism(s) associated with this region at different times, and/or under different experimental conditions. Collectively, and if the case, this suggests a remarkable role for the E-box DNA element in the regulation of hGH synthesis. This would be regardless of the nature of the E-box associated transcription factor serving as a target or perhaps an entry point to trigger an

effect on the *hGHI* in response to a HFD-induced hyperinsulinemia *in vivo* and insulin treatment *in vitro*.

Rhythmicity of biological functions is fundamental for optimal adaptations to environmental cues. GH is a major metabolic homeostatic factor, which is secreted in a circadian pattern, but it is not known whether it is produced rhythmically (Avram, Jaffe et al. 2005). The presence of an E-box DNA element as well as evidence suggesting association of Bmal1 with the *hGHI* promoter region *in situ*, supports the notion *hGHI* could also be a clock-controlled gene. As such, it is expected that *hGHI* transcription will show circadian oscillations and display circadian rhythms in its expression/production. Microarray studies of transcripts have revealed that 10-30% of the human genome is under the control of circadian molecular clocks (Bozek, Religio et al. 2009). Due to lack of access to human pituitary samples it is not known whether *hGHI* is a target for circadian rhythm. Thus, comprehensive *in vivo* approaches using the hGH/CS TG mice will be required to identify *hGHI* as a clock-controlled gene.

On the basis of observations made (Chapter 4), it is tempting to speculate that the hGH locus is compacted in a facultative heterochromatin state and, as such, can transit between euchromatin to a heterochromatin-like state every day in a cyclic manner. If so, local chromatin remodeling involving HS I/II and the proximal promoter regions might be expected to respond to or influence association of E-box transcription factor(s). The resulting complex(es) in turn might facilitate or inhibit recruitment of the general transcriptional machinery including RNA pol II in order to precisely orchestrate the

circadian transcription process. Although the focus has been on the potential contribution of an E-box element located at nucleotide position –264 to –259, it is also possible that multiple E-box elements play a role in any circadian oscillation of *hGHI*. Inspection of the hGH locus sequences reveal at least two putative E-box elements located within HS I/II sequences. Further studies will be required to rule out the contribution of the *hGHI* promoter E-box element in circadian regulation of *hGHI*.

### ***7.9. NCOR associated repression of hGHI in response to excess caloric intake***

The short-term excess caloric intake *in vivo* data also implicates induction and recruitment of the transcriptional co-repressor, NCOR, as part of the promoter complex in the decrease in *hGHI* expression. There was, however, no similar increase in NCOR association at the mGH gene promoter. Again, this is consistent with the lack of response of the mGH gene to acute excess caloric intake.

NCOR is known to interact with the pituitary transcription factor Pit-1 (Scully, Jacobson et al. 2000, Voss, Demarco et al. 2005). Pit-1 binds to sequences in HS I/II within the hGH LCR as well as the proximal *hGHI* promoter region, and is essential for activation of the *hGHI* locus during somatotroph development, and for ongoing efficient postnatal expression (Jin, Surabhi et al. 1999, Ho, Elefant et al. 2002, Ho, Elefant et al. 2006, Ho, Shewchuk et al. 2013). An interaction between NCOR and Pit-1 was suggested as the mechanism to explain silencing of the GH gene in Pit-1-producing lactotrophs during embryonic differentiation of the anterior pituitary (Scully, Jacobson et al. 2000,

Voss, Demarco et al. 2005). Observations from this study suggest NCOR may also act as a co-repressor in the postnatal dynamic and transient transcriptional suppression of the hGH gene in somatotrophs.

Certainly, NCOR has been suggested as one of the key molecular mechanisms by which insulin signaling enhances transcriptional repression of its target genes which are highly involved in metabolic homeostasis (Yamamoto, Williams et al. 2011, Mouchiroud, Eichner et al. 2014). Furthermore, the metabolic role of NCOR has been elegantly demonstrated in tissue-specific knock out mouse models reviewed in (Mouchiroud, Eichner et al. 2014). The phenotypes caused by attenuated NCOR signaling in muscle, liver, and white adipose tissue are consistent with changes in adipokine secretion, increased systemic insulin sensitivity, enhanced ketogenesis, and reduced fat cell size, which are all reflective of an improved metabolic status (Yamamoto, Williams et al. 2011). As such, it has been suggested that NCOR is a useful target for future therapeutics in the treatment of type 2 diabetes as well as other insulin resistant diseases such as obesity (Li, Fan et al. 2011). These characteristics associated with NCOR are also consistent with its role in mediating *hGHI* repression by excess caloric intake as suggested in this study.

***7.10. A dynamic relationship between transcription factors and chromatin modifiers in insulin sensitive transcriptional repression of the hGHI gene***

Histone H3/H4 hyperacetylation is often associated with a more open and active chromatin conformation, and thus permissive for gene expression; in contrast, deacetylation facilitates gene suppression (Calestagne-Morelli and Ausio 2006). Involvement of NCOR as a component of transcriptional repression scaffolding platforms can connect co-repressor pathways through recruitment of histone deacetylases (HDACs) including class I (HDAC 3), class II (HDAC 4, 5, 7, and 9), and class III (sirtuins - SIRT1) (Perissi, Jepsen et al. 2010). NCOR and HIF-1 $\alpha$  are reported also to interact with members of class I, II, III HDACs and to recruit them to regulatory elements in target genes (Kato, Tamamizu-Kato et al. 2004, Qian, Kachhap et al. 2006, Lee, Lee et al. 2010, Perissi, Jepsen et al. 2010, Patsopoulos, Bayer Pharma et al. 2011, Rajendran, Garva et al. 2011, Du, Chen et al. 2012). Deacetylase inhibitors such as trichostatin A (TSA) can effectively block transcriptional repression mediated by NCOR (Heinzel, Lavinsky et al. 1997). In this context, treatment of primary pituitary cells from hGH/CS TG mice with TSA muted the insulin-dependent decrease in *hGHI* RNA levels (Vakili, Jin et al. 2012). Thus, class I HDAC is likely the mediator of transcriptional repression of the *hGHI* (Guenther, Lane et al. 2000, Watson, Fairall et al. 2012).

Even though the association of HDACs with the hGH locus was not explored, the data presented are consistent with a significant impact of alterations in energy homeostasis, through acute excess caloric intake, on H3/H4 hyperacetylation in the hGH LCR. Significant reductions in H3/H4 hyperacetylation were observed at HS I/II and the *hGHI* promoter regions with excess caloric intake *in vivo*. This reduction, however, did not have a significant impact on the overall “global” pituitary-specific pattern of

hyperacetylation for the active *hGHI* locus, which extends over 32 kb from HS V upstream down to the *hGHI* promoter, peaking at HS I/II (Jones, Monks et al. 1995, Vakili, Jin et al. 2012, Ho, Shewchuk et al. 2013). Furthermore, data from primary pituitary cells indicate that the insulin response *in vitro* requires histone deacetylation via class I and/or II HDACs, as well as a decrease in H3/H4 hyperacetylation “locally” in the proximal promoter region. These observations are consistent with a previous assertion that target genes for both insulin and HIF-1 $\alpha$  are subject to histone modification by deacetylation in their regulatory elements via recruitment of class I and II HDACs (Kato, Tamamizu-Kato et al. 2004, Qian, Kachhap et al. 2006, Lee, Sohn et al. 2007, Lee, Lee et al. 2010). This suggests that a regulatory element(s) in the *hGHI* promoter is a target for insulin and HIF-1 $\alpha$  -mediated histone deacetylation. This would be expected to result in “inactive/repressed” chromatin, thereby decreasing accessibility to factors including RNA polymerase (Ronsch, Jager et al. 2011), and/or the pituitary specific transcription factor Pit-1. This limited accessibility of chromatin is more likely due to reduced hyperacetylation of the locus, which is reflected in the “expression loop” structure, as presented in Chapter 5 and will be discussed in the section below (Section 7.11).

### ***7.11. Chromosomal reconfiguration is a component of a dynamic transcriptional regulation of hGHI***

Significant reductions in H3/H4 hyperacetylation were observed at HS I/II and the *hGHI* promoter regions with excess caloric intake *in vivo* and in response to insulin treatment *in vitro*. These regulatory elements, even though distant from each other, are



implicated in the initial activation of the hGH gene locus during development and expression after birth under normal physiological conditions. These regions are found in a spatial proximity, perhaps through the formation of an “expression” loop comprised of ~14-15 kb of intervening chromatin. While the exact structure of this intervening chromatin is not known, its presence strongly correlates with efficient transcriptional activity of the *hGHI*. It is also not understood yet whether this structure forms as a consequence of transcriptional activation of this locus or perhaps as an active component of the activation process (Fleetwood, Ho et al. 2012, Ho, Shewchuk et al. 2013). Regardless of the cause or consequence, efficient postnatal *hGHI* expression requires maintenance of this intrachromosomal physical interaction between the hGH LCR at HS I/II and the proximal promoter region, which must be hyperacetylated to facilitate association with RNA pol II (Ho, Shewchuk et al. 2013).

It is known that the Pit-1 elements located in the HS I/II region when combined with Pit-1 binding sites located within the proximal *hGHI* promoter region are sufficient to mediate efficient as well as tissue/cell specific expression of *hGHI* *in vitro* and *in vivo*. Enhanced expression is observed when the upstream HS I/II (1.6 kb) sequences are linked to the *hGHI* proximal promoter region (~500 bp) in the absence of intervening sequences (Jin, Surabhi et al. 1999, Shewchuk, Asa et al. 1999). This result however raises an interesting question about the significance of the “expression” loop and intervening sequences. Perhaps, the presence of the chromatin loop provides the basis for further regulation, or flexibility of expression in response to environmental cues; the effect would be to influence the proximity of the critical Pit-1 binding sites in the distal HS I/II and proximal promoter regions under these circumstances. For example, this

might contribute to control of GH in cycles of feast and famine (as described in Section 7.2). As mentioned above, physical interaction between HS I/II and *hGHI* promoter regions appears to be facilitated by hyperacetylation of these regions (Ho, Shewchuk et al. 2013). Thus, as would be predicted, the decrease in *hGHI* expression with excess caloric intake correlates with decreased detection of the “expression” loop or HS I/II and *hGHI* promoter interaction, as well as a significant reduction in RNA pol II interaction with the proximal *hGHI* promoter region. These data support an important role for HS I/II in activation of the *hGHI* promoter but also suggest that these regions and perhaps the intervening sequences that are the basis for presence of an “expression” loop are targets for physiological influence that can regulate hGH synthesis after birth.

Histone hyperacetylation as explored in this thesis is recognized as one of the main processes actively contributing to establishment of the somatotroph specific chromosome architectural of the *hGH* locus. The mechanism, however, as to how this long-distance interaction between HS I/II and the *hGH* promoter regions are formed or maintained is not well understood. It is also recognized that there are many other processes and chromatin remodeling events that might be cooperatively involved in formation and/or stabilization of chromatin loops. These include transcription of long non-coding RNAs encompassing the chromosomal interaction sites, as well as histone methylation, DNA methylation and association of nuclear architectural protein complexes (e.g., the CCCTC-binding factor (CTCF) and cohesin complex) (Maksimenko and Georgiev 2014, Zuchegna, Aceto et al. 2014, Kang, Song et al. 2015, Yoo, Brown et al. 2015). Additional experimental approaches would be required to assess the potential

contribution of these events in chromosomal reconfiguration of the hGH LCR in response to excess caloric intake.

The apparent reconfiguration of the hGH locus observed in response to an environmental cue, such as overeating, also supports investigation of systems where de-differentiation occurs as a result of physiological and/or pathological conditions. Under such conditions the three-dimensional nuclear organization of a cell type may change rapidly, and be stabilized in a reconfigured state, offering a potential explanation of the de-differentiation process.

#### ***7.12. A positive role for physical activity in counteracting the negative effect of excess caloric intake on hGH production***

Limited energy expenditure is central in the etiology of many metabolic diseases such as obesity, malnutrition, coronary heart disease, osteoporosis, and a variety of other chronic diseases (Takken, Stephens et al. 2010, Audelin, Savage et al. 2012, Henchoz, Bastardot et al. 2012). A review of studies using animal models of obesity and type 2 diabetes, such as the Zucker Diabetic Fatty model, is consistent with a beneficial impact of regular and moderate intensity physical exercise (training) in attenuation of insulin resistance, glycaemia control, dyslipidemia, and blood pressure reviewed in (Teixeira-Lemos, Nunes et al. 2011). In respect to obesity related GH insufficiency, many clinical reports reveal that the GH secretory response to physical activity is markedly impaired in obese individuals (Oliver, Hingorani et al. 2012). This may be explained by impaired

secretory signaling pathways and/or impaired GH production, and thus limited amounts of GH reserves to be secreted in response to the positive stimulation triggered by physical activity. The question of whether alterations in physical activity and energy expenditure could normalize the reduced levels of GH levels (synthesis and /or secretion) in response to the short-term (three days) HFD consumption was also investigated in hGH/CS TG mice. The results clearly demonstrate that prescribed physical activity can effectively block the repressive impact of excess caloric intake on hGH production both at the level of synthesis (RNA) and secretion (serum GH protein). Thus, increased energy expenditure in the form of forced physical activity is sufficient to restore reduced GH levels in mice fed a HFD to those detected in mice on the LFD.

One of the deficiencies with this study is lack of a valid measurement of energy expenditure during the swimming test (physical activity); specifically, assessment of oxygen consumption by indirect calorimetry, and increased heart rate (Speakman 2013). Despite the absence of these precise measurements, a significant and consistent decrease in body weight was detected in both (low fat and high fat) diet groups that also received the prescribed physical activity regimen, reflecting a decrease in fat mass. More importantly, the physical “swim” activity used in this study was adequate to exert a physiological function, specifically, a rescue of GH levels. Therefore, it is reasonable to conclude that swimming is a valid dynamic “physical activity” that can contribute to changes in the metabolic network and can affect GH production.

One possible mechanism involved in this positive effect of swimming on GH

levels is through mitigating increased insulin levels. Consistent with this claim, studies with human subjects and mice suggest that exercise can suppress insulin secretion, and more importantly can be beneficial in terms of a response to the excess levels of insulin associated with overeating in healthy subjects (Karlsson and Ahren 1990, Dunning, Karlsson et al. 1991, Karlsson and Ahren 1991, Walhin, Richardson et al. 2013). Thus, if the decrease in *hGHI* expression reflects the ability of somatotrophs to act as a metabolic sensor (Childs, Akhter et al. 2011), and specifically to the hyperinsulinemic state, then prescribed physical activity is predicted to mitigate against this decrease. As such, support for suppression of a hyperinsulinemic state associated with acute excess caloric intake has been obtained through the studies described in this thesis (Chapter 6). In support of inhibition of insulin secretion by swimming exercise, Karlsson and colleagues demonstrated that adrenal gland function can be influenced by physical activity, since adrenalectomy as well as chemical sympathectomy could effectively prevent the inhibition of basal as well as glucose-stimulated insulin secretion, seen with mice after swim exercise (Karlsson and Ahren 1991). Thus, there is a role for both stress-associated neurotransmitters and hormones, adrenalin and glucocorticoids, and perhaps their elevated levels during exercise could account for the inhibitory and stimulatory effect of swimming on insulin and GH levels, respectively (Galbo 1982, Carlson, Marker et al. 1985, Winder, Fisher et al. 1991, Hill, Zack et al. 2008, Viru, Hackney et al. 2008). Adrenaline is a strong suppressor of insulin secretion (Peterhoff, Sieg et al. 2003). Adrenaline is also known as a positive stimulator of human GH secretion, however evidence supports an indirect regulation of GH secretion through changes in the hypothalamic factors such as GHRH and somatostatin (Terry, Crowley et al. 1982). The

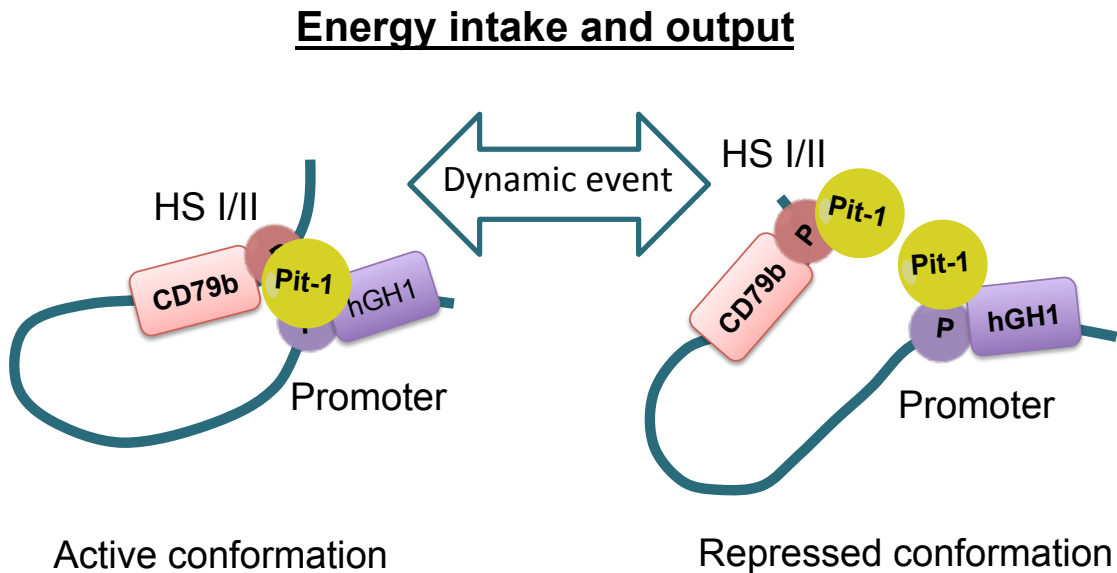
direct inhibitory effects of glucocorticoids on pancreatic  $\beta$ -cells function both *in vitro* and *in vivo* have been demonstrated (Lambillotte, Gilon et al. 1997, Ullrich, Berchtold et al. 2005). It is also possible that a rise in glucocorticoids associated with physical activity can directly prevent a reduction in GH levels in response to HFD-induced hyperinsulinemia. This could include a positive effect of glucocorticoids on somatotroph function and GH production (Vakili, Jin et al. 2011, Vakili and Cattini 2012).

### ***7.13. Flexibility of the hGH locus chromatin loop***

Intriguingly, incorporation of swimming into the diet regimen *in vivo* muted the negative impact of excess caloric intake on changes observed in chromosomal structure associated with the *hGHI* locus, including histone hyperacetylation and apparent recruitment of RNA pol II and, as a result, hGH RNA levels. Thus, these data provide the first evidence for a rapid and dynamic change in specific chromatin-chromatin physical interaction in response to physiological influences such as excess caloric intake and physical activity *in vivo*. Data indicate that the hGH locus is subject to dynamic structural changes that can accommodate homeostatic and/or adaptive transcriptional responses to energy homeostasis as shown schematically in Figure 7.3.

**Figure 7.3: Schematic of dynamic structural changes of the hGH locus in response to energy homeostasis**

*Active conformation of the hGH locus is resulted from formation of the “expression loop” occurs due to physical interaction between the HS I/II (located within the promoter (P) region of CD79b gene) and the hGH1 promoter regions and looping out of the intervening chromatin (14.5 kb). This looping is associated with activation of the locus and efficient expression. Previous studies demonstrated that this physical interaction is mediated by pituitary specific transcription factor Pit-1 at cognate sites both at HS I/II and the hGH1 promoter (Hunsaker, Jefferson et al. 2012). It was proposed that interference with this loop would contribute to repression of hGH1 promoter activity. Data presented in Chapters 5 and 6 demonstrated that hGH locus chromatin loop is a dynamic structure and can rapidly transition from active conformation into repressed conformation during excess caloric intake as detected by loss of the “expression” loop integrity. More importantly physical activity can prevent this transition from one state into another as revealed by experiments using swimming test as a prescribed form of physical activity to counteract with negative effects of acute excess caloric intake (three days of high fat diet regimen).*



This flexibility of chromatin remodeling and formation of a particular cell-specific chromosomal architecture is consistent with a dynamic role for histone proteins. This reflects function not only as a scaffold providing support for the genomic materials but also as a modulator in the fine-tuning of the more complex transcriptional mechanism. This flexibility can be explained in part by reversible post-translational modifications of histones such as hyperacetylation to accommodate the different transcriptional/functional needs in response to the environmental cues; this includes excess caloric intake and physical activity as demonstrated (Chapters 5 and 6). The dynamic aspects of this chromatin folding and its implications at the structural and functional levels beyond increased accessibility to the transcriptional machinery complex are still poorly understood. This dynamic chromatin folding associated with reduced hGH1 transcript levels (presented in Chapters 5 and 6) is in contrast with what has been reported related to transcriptional regulation of the mammalian  $\beta$ -globin locus. Interestingly, when transcription of the members of the  $\beta$ -globin locus is pharmacologically silenced, the chromatin loops and chromosomal conformation persist, suggesting a very stable chromosomal conformation (Mitchell and Fraser 2008, Palstra, Simonis et al. 2008). Based on the observation regarding the stability of the chromatin loops of the  $\beta$ -globin locus, it was speculated that stability of this higher order chromatin organization contributes to a memory mechanism that facilitates a rapid re-activation and expression of a gene following a period of repression (Deng and Blobel 2010). Perhaps this controversy of a stable versus flexible chromosomal conformation and its association with the transcriptional status of the controlled genes can be explained in part by differences in the nature of repressive stimuli and impacted downstream targets that



facilitate gene repression with or without modulating the chromosomal architecture. There is some support for this based on the rapid transition of chromatin loops of the mammalian  $\beta$ -globin locus from one state into another, revealed by experiments using conditional “knock out” of transcription factors, which are implicated in activation of the  $\beta$ -globin locus and formation of chromatin loops (Drissen, Palstra et al. 2004, Vakoc, Letting et al. 2005, Jing, Vakoc et al. 2008). Collectively, the evidence from studies on regulation of the  $\beta$ -globin locus and studies of the hGH locus presented here, suggest that chromosomal loops exhibit characteristics of being both static as well as dynamic under different conditions and perhaps in different contexts. This would indicate that their regulation and contribution to gene expression is far more complex than expected. Further studies are required to identify specific transcription factor(s) involved in chromosomal reconfiguration of the *hGH* locus. This includes identification of key transcription factors and their involvement in formation as well as maintenance of the somatotroph-specific *hGH* locus chromosomal architecture. This would also include their contribution to *hGHI* expression under different physiological states. Any list of potential candidates will of course include the pituitary specific transcription factor Pit-1. The results of studies discussed here also implicate E-box related transcription factors including the circadian machinery complex Bmal1/Clock (Chapter 4) and/or other factors that can modulate the recruitment of chromatin modifiers such as histone acetyltransferases and/or HDACs.

A reliable transcriptional regulatory process must be resilient to changes in environment (Lopez-Maury, Marguerat et al. 2008). The work presented in this thesis

indicates that the hGH locus is subject to structural changes that can accommodate homeostatic and/or adaptive transcriptional responses to energy homeostasis (Vakili, Jin et al. 2014). Moreover, the observations made support a fluid structure for the cell/tissue type-specific chromosomal architecture as opposed to a rigid structure, which can be accessed and subjected to postnatal transcriptional control. Thus, these rapid changes in *hGHI* expression as well as its associated chromosomal changes at the locus are targets for metabolism, which allow GH as one of the major endocrine/metabolic factors to rapidly adjust itself to cope with the modified metabolic environment.

It has been suggested that transcriptional re-activation of a gene (for the second time) following a period of repression might require a shorter time than the initial activation during embryonic development; this phenomenon is referred to as transcriptional memory (Deng and Blobel 2010). It can be anticipated that switching the HFD regimen (supplied to hGH/CS TG mice for a period of three days) to a LFD for three days would reverse the reduction in hGH levels and associated changes in the hGH locus (chromatin remodeling). This would likely involve elimination of repressive factors resulting from HFD consumption (e.g., correction of the hyperinsulinemic state associated with the acute excess caloric intake). Nevertheless, experiments testing the cause-effect relationships of chromatin loops and memory (reversibility of a repressive conformation) are required in order to comprehend this phenomenon at the molecular level.

A question is raised from these observations as how resilient the *hGHI* locus

chromosomal structure is. It is not known whether repeated and/or staggered overeating and exposure to associated metabolic fluctuations of excess caloric intake for a longer period would have an adverse impact on the chromatin structure of the *hGHI* locus. These repeated metabolic insults might result in a compromised recovery, if the condition of overeating is removed and/or physical activity is prescribed as a remedy. Although this is speculative, if this occurs, this may have a clinical relevance, such as an increased risk for the development of GH insufficiency in individuals that are exposed to repeated excess caloric intake during puberty. This is the stage at which the highest levels of GH production are expected. Another such stage would be pregnancy, a critical time point when the developmental activation of the *hGHI* locus occurs in the developing fetus. This aspect in regards to pregnancies complicated by gestational obesity will be further discussed and explored in Chapter 8.

#### **7.14. Final comments**

A major contribution presented in this dissertation is the characterization of a unique pituitary cell model to assess hGH expression and regulation *in vitro*, *in situ* and *in vivo*. The last provided the opportunity to begin to look at the effects of excess caloric intake, as the initial stage of obesity progression, on *hGHI* at the molecular level. These studies build on the work of others, notably from the laboratory of Dr. Nancy E. Cooke (University of Pennsylvania) as well as our own. They have increased our understanding of the molecular mechanisms, specifically the dynamic relationship between environmental cues that can be received by an intact endocrine system and the chromosomal structure of the hGH locus, responsible for activation, expression and regulation of the *hGHI*. The novel nature of this system has allowed insight into the contribution of regulatory domains involving “nearby” genes (lymphocyte *CD79b*) to the *hGHI*. The availability of the hGH/CS transgenic mice also offers the possibility to cross them with other mice of interest as will be described in the future directions (Chapter 8). It is vitally important for us to understand these basic regulatory mechanisms if we are to comprehend abnormalities in the processes of cellular growth and development, as well as exploit them therapeutically in the future.

## CHAPTER 8

### Future directions

#### *8.1. Generation of a humanized GH transgenic mouse model*

In response to the lack of a model system to study the *hGHI* gene, transgenic mice containing the intact *hGHI* gene and locus control region in a greater than 100 kb fragment of human chromosome 17 was used as a model system in this dissertation (Jin, Lu et al. 2009). The hGH/CS TG mouse is a powerful tool to assess regulatory mechanisms involved in *hGHI* gene expression in the presence of an intact endocrine system. In this model, however, the *hGHI* gene is co-expressed along with the intact endogenous mouse (m) GH gene in the same somatotroph cells of the anterior pituitary gland (Vakili, Jin et al. 2011). Thus, this system is limited in terms of the assessment of biological functions of hGH. This would include the consequences of physiological/pathophysiological regulation of hGH levels influenced by a variety of conditions, including obesity as well as overeating as was explored in this dissertation. Specifically the mouse GH counterpart may play a significant role in a compensatory or competitive manner and thus complicate the interpretation of any outcome.

One proposed solution for a functional improvement of the hGH/CS TG mouse model is to transform it to a “humanized” mouse model. A “humanized” mouse model is generally accepted as a biological system in which a mouse gene has been replaced by a

human gene. Thus, genomic surgery tools (nucleases) could be used to delete or silence the endogenous mGH gene in hGH/CS TG mice. These include zinc-finger nucleases (Carroll 2011), transcription activator-like effector nucleases (TALENs) and/or clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases (Pennisi 2013). It is anticipated that the resulting humanized GH TG mice would produce hGH solely and could be used to investigate the biological consequences of altered hGH levels at the different target organ levels. It is also possible to replace the coding region of the mGH gene with fluorescent reporter coding sequences. Development of tracers such as fluorescence dyes have been a powerful tool for *in vivo* and *in vitro* imaging in living animal models as well as live cells in culture (Shimomura, Johnson et al. 1962, Prasher, Eckenrode et al. 1992). This would allow the dynamic pattern of physiological changes to be captured at a molecular level following treatments and a variety of induced conditions, including diet-induced overeating and obesity, exercise, stress and other endocrine conditions known to affect somatotrophs and GH production. Once this “humanized” TG mouse model is developed and characterized, it can be further used as a background for somatotroph specific “knock out”, conditional “knock down” and/or over-expression models to look at factors implicated in regulation of *hGHI*, and/or factors implicated to be the target of hGH signaling.

## ***8.2. Epigenetic and chromosomal reconfiguration of the human GHI locus***

Unfortunately, the prevalence of obesity in women of reproductive age is a major health concern (Heslehurst, Ells et al. 2007, Heslehurst, Lang et al. 2007, Denison, Roberts et al. 2010). Pre-gestational and gestational obesity promotes exacerbation of insulin resistance during pregnancy and as a result can put mothers and offspring at a greater long-term risk for metabolic syndrome and associated problems (Desai, Jellyman et al. 2014). One of these disturbed metabolic parameters may include altered levels of pituitary GH. Findings presented in Chapter 3 support the notion that diet and physical activity can influence events that are crucial for normal embryonic development; specifically those events involved in activation of the *hGHI* locus and somatotroph development. This may have implications with respect to *trans*-generational epigenetic effects on offspring gene locus activation as well as regulation *in utero* in the case of pregnancies complicated by maternal obesity.

This topic requires a brief description of the term “epigenetics” and how it is used in this section. An epigenetic trait is defined as a stably heritable phenotype resulting from changes in a chromosome (chromatin structure through chemically modified DNA and/or histone proteins) without alterations in the DNA sequence (Berger, Kouzarides et al. 2009). Thus, the term “heritable” is most often used in the context of *trans*-generational inheritance or inheritance from mother to daughter cell, i.e., a form of memory that persists through mitosis or meiosis. Therefore, use of “epigenetics” in the case of an investigation of the effects of maternal obesity on the chromosomal

configuration of the hGH locus of an offspring is an appropriate terminology.

Investigation of this hypothesis that initial chromosomal configuration of the hGH locus of an offspring is a target for maternal obesity and its associated complications, requires a model system of maternal obesity and assessment of the offspring's pituitary in hGH/CS TG mice. Certainly, the hGH/CS TG mouse complements human studies by modeling pregnancy and allowing assessment of pituitary *hGHI* and placental hCS/GH-V gene expression in the presence of an intact endocrine system, with sampling as pregnancy progresses. This includes pregnancies with complications, such as HFD-induced maternal obesity and insulin-resistance. Recently, hGH/CS TG mice were used to investigate the regulation of placental members of the hGH locus by HFD-induced maternal obesity (Vakili, Jin et al. 2013). Thus, the model is accessible for epigenetic and *trans*-generational studies of the hGH locus, including the pituitary *hGHI* as well as placental hGH/CS gene members.



## CHAPTER 9

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