REGULATION OF HUMAN GROWTH HORMONE RECEPTOR EXPRESSION BY MICRORNAS IN HEK293 CELLS

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DEDICATION

This thesis is dedicated to my roots and shoots in this world,

My dear parents, Abdullah and Souheila Elzein

and

My precious sons: Samer and Ali

With my love …

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LIST OF ABBREVIATIONS

3'UTR	3' untranslated region
3'RACE	Rapid amplification of cDNA 3'end
5'UTR	5' untranslated region
Ago2	Argonaut protein 2 (catalytic component of RISC complex)
AKT	A serine-threonine protein kinase , also known as protein kinase B.
B-CLL	B-cell chronic lymphocytic leukemia
BIC	B-cell integration cluster
C/EBPs	CCAAT/enhancer-binding protein
CGD	Constitutional growth delay
CIS	Cytokine-inducible SH2-containing protein
DGCR8	DiGeorge critical region 8 protein
ECD	Extracellular domain
EGR-1	Early growth response protein 1
ERK	Extracellular signal-regulated kinases
GH	Growth Hormone
GHBP	GH binding protein
GHD	GH deficiency
GHI	GH insensitivity
GHRH	Growth hormone releasing hormone
GHRHR	Growth hormone releasing hormone receptor
Grb2	Growth factor receptor-bound protein 2

HER2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia inducible factor 1, alpha subunit
ICD	Intracellular domain
IGF-I	Insulin-like growth factor-I
IGF-IR	Insulin-like growth factor-I receptor
IGFBP3	IGF binding protein 3
IRS1, 2 and 3	Insulin receptor substrate 1, 2 and 3
ISS	Idiopathic short stature
JAK2	Janus kinase 2
MAPK	Mitogen-activated protein kinase
MiRNA or miR	MicroRNA
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex1
NF-Y	Nuclear transcription factor Y
NF-κB	Nuclear Factor κB protein
PI3K	Phosphatidylinositol 3'kinase
PKB	Protein kinase B
PLA2	Phospholipase A_2
PTPs	Protein tyrosine phosphatases
RAF-1	Proto-oncogene serine/threonine-protein kinase-1
REM	Rapid eye movement
RISC	RNA-induced silencing complex
SH2	SRC homology domain 2

SMC4	Structural maintenance of chromosomes 4
SOS	Son of sevenless
SS	Somatostatin
STAT	Signal transducers and activators of transcription
TMD	Transmembrane domain
TNFα	Tumor necrosis factor α
Wnt	Wingless-related MMTV integration site family member

ABSTRACT

The pleiotropic actions of human growth hormone (GH) result from its binding to its receptor (GHR) on target cells and the downstream activation of multiple intracellular signaling pathways, leading to changes in gene expression, differentiation and metabolic activity. Previous studies have associated GHR deficiency with growth disorders while GHR overexpression occurs in a wide variety of cancers, suggesting the need to have tight regulation of the *GHR* gene.

Studies of what regulates *GHR* expression at the 5'UTR (promoter) and coding regions have been reported; however, investigations of regulation at the 3'UTR, particularly by microRNAs (miRNAs), has been overlooked. MiRNAs are small (19-21 nt) noncoding RNAs that play an important role in regulating gene expression mainly through targeting the 3'UTR of mRNAs and enhancing degradation or inhibiting translation. In the last decade, extensive biomedical research has demonstrated a critical role for miRNA in many chronic diseases, including cancer. In the present study, we have mapped the *GHR* 3'UTR for potential miRNA binding sites and investigated the role of miRNAs in regulating GHR expression.

We used multiple *in silico* prediction tools based on different algorithms to define several putative miR-binding sites within the *GHR* 3'UTR and prioritized a set of these sites based on conservation across several species, their hybridization energy, the presence of parallel sites in GH/IGF-I axis-related genes, and reports that link specific miRNAs to GHR-related physiological or pathophysiological activities. To test this set, we created a Luc-*GHR* 3'UTR luciferase reporter vector and screened for miRNA binding to the *GHR*-3'UTR and effects on the luciferase activity. Our studies identified miR-129-5p, miR-142-3p, miR-202 and miR-16 as

significant inhibitors of human *GHR* expression in HEK293 cells through binding to specific sites. A parallel decrease in endogenous *GHR* mRNA and protein by these miRNAs suggests that they act primarily by degrading *GHR* mRNA. This study paves the way for the development of novel miRNA inhibitors for GHR expression and their potential use as therapeutic agents in GH/GHR axis-related pathophysiologies.

RÉSUMÉ

Les actions pléitropiques de l'hormone de croissance humaine (GH) résultent de sa liaison à son récepteur (GHR) sur les cellules cibles et de l'activation en aval de multiples voies de signalisation conduisant à des changements au niveau de l'expression génique, de la différentiation et de l'activité métabolique. Des études antérieures ont associé une insuffisance du GHR à des problèmes de croissance alors que sa surexpression survient dans une grande variété de cancers, suggérant le besoin d'une étroite régulation du gène *GHR*.

Des études portant sur la régulation de l'expression de *GHR* au niveau du 5'UTR (promoteur) et des régions codantes ont été publiées; cependant, des recherches au niveau de la régulation du 3'UTR, particulièrement par les microARNs (miRNAs) ont été délaissées. Les miRNAs sont de petits (19-21nt) ARNs non codants qui jouent un rôle important dans la régulation de l'expression génique en ciblant essentiellement les 3'UTRs des ARNm donc en augmentant leur dégradation ou en inhibant leur traduction. Dans la dernière décennie, de nombreuses recherches biomédicales ont démontré un rôle critique des miRNAs dans de nombreuses maladies chroniques y compris le cancer. Dans la présente étude, nous avons cartographié des sites de liaison potentiels aux miRNAs dans le 3'UTR du *GHR* et étudié le rôle des miRNAs dans la régulation de l'expression de GHR.

Nous avons utilisé de nombreux outils de prédiction *in silico* basés sur différents algorithmes afin de définir plusieurs sites de liaison potentiels de miRNAs dans le 3'UTR de *GHR*. Nous avons priorisé une partie de ces sites en tenant compte de leur conservation entre plusieurs espèces, de leur énergie d'hybridation, de la présence de sites parallèles dans des gènes reliés à l'axe GH/IGF-I, et d'études reliant spécifiquement des miRNAs à des activités

physiologiques ou pathophysiologiques du GHR. Afin de tester ce groupe de sites, nous avons créé un vecteur rapporteur luciférase Luc-*GHR* 3'UTR et criblé la liaison des miRNAs au 3'UTR de *GHR* et leurs effets sur l'activité de la luciférase. Nos études ont identifié miR-129-5p, miR-142-3p, miR-202 and miR-16 comme étant des inhibiteurs significatifs de l'expression de *GHR* dans les cellules HEK293 par leur liaison à des sites spécifiques. Une diminution parallèle de l'ARNm et de la protéine de GHR endogène par ces miRNAs suggère qu'ils agissent principalement en dégradant l'ARNm. Cette étude ouvre la voie au développement d'une nouvelle approche utilisant ces miRNAs afin d'inhiber l'expression de GHR ainsi qu'à leur utilisation potentielle comme des agents thérapeutiques dans des pathophysiologies de l'axe GH/GHR chez l`humain.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Growth Hormone Synthesis and Secretion

Human Growth Hormone (GH) is a 191-amino acid, 20-22 kDa peptide hormone synthesized, stored and secreted by somatotrophs within the anterior lobe of the pituitary gland (Baumann, 2009). GH secretion occurs in a pulsatile manner throughout the day, with a major surge at night during REM sleep (Takahashi *et al.*, 1968; Hartman *et al.*, 1992; Van Cauter *et al.*, 1998). The synthesis and secretion of GH from somatotrophs is a calcium (Ca^{++})-dependent event during which an increase in cytosolic Ca^{++} is required for GH release (Mason *et al.*, 1993). The primary factors that control its synthesis and secretion are the two hypothalamic factors, Growth Hormone Releasing Hormone (GHRH) and somatostatin (SS) (**Figure 1.1)**. Under normal physiological conditions, GHRH promotes the synthesis and secretion of GH while somatostatin inhibits its release (Frohman *et al.*, 1992; McElvaine and Mayo, 2006). The GH releasing peptide, ghrelin, is the endogenous ligand for the GH secretagogue receptor and has a small stimulatory effect on GH secretion in humans (Arvat *et al.*, 2000). While ghrelin is produced predominantly by the stomach, it is also synthesized in the hypothalamus and appears to have a role in the integration of energy balance and growth (Wren *et al.*, 2000).

Negative feedback by members of the GH/Insulin-like Growth Factor-I (IGF-I) axis regulates GH synthesis and secretion: in many tissues, but especially in the liver, GH induces IGF-I synthesis and release which, along with GH itself, functions as a negative feedback inhibitor both at the hypothalamic and pituitary levels (Berelowitz *et al.*, 1981; Goodyer *et al.*, 1993) (**Figure 1.1)**. Other peripheral endocrine factors, such as glucocorticoids, estrogen, thyroid

hormone and leptin, have effects on GH production and release. Certain physiological factors, including sleep, feeding, stress and obesity, have also been shown to influence GH production, primarily by regulating gene transcription of GH as well as other genes in the GHRH/SS/GH/IGF-I axis (Hartman *et al.*, 1992). In addition to the established production of GH in the anterior pituitary, GH synthesis has been found to occur at a number of extra-pituitary sites, supporting the premise that this peptide hormone has important autocrine and paracrine as well as endocrine effects (Harvey and Hull, 1997; Le Roith *et al.*, 2001).

1.2 Physiological Roles of GH

GH is a major determinant of postnatal growth. In addition, it has significant regulatory effects on protein, carbohydrate and lipid metabolism at all stages of life (Veldhuis *et al.*, 2005; Lichanska and Waters, 2008b; Brooks and Waters, 2010). The biological effects of GH on growth and metabolism involve the simultaneous stimulation of anabolic processes and growth factors in multiple organs and physiological systems both directly and via IGF-I(Le Roith *et al.*, 2001; Lichanska and Waters, 2008b; Rotwein, 2012). GH, together with IGF-I, promotes longitudinal bone growth and enhances bone mineral density by stimulating chondrocyte precursor proliferation and differentiation and bone remodelling, respectively (Isaksson *et al.*, 1987; Yakar *et al.*, 2002; van der Eerden *et al.*, 2003). GH increases fasting serum glucose levels by stimulating gluconeogenesis and glycogenolysis in hepatocytes and also by decreasing the use of glucose in peripheral tissues by inhibiting glycogen synthesis and oxidation of glucose (Moller *et al.*, 1991; Cornblath and Ichord, 2000; Moller and Jorgensen, 2009).

Similarly, GH has anabolic effects on protein synthesis/turnover and is well known to increase the uptake of amino acids in liver, skeletal muscle and adipose tissue (Jorgensen *et al.*,

1989; Fryburg *et al.*, 1991; Copeland and Nair, 1994; Fryburg and Barrett, 1999). It increases nitrogen retention by increasing protein synthesis, inhibition of protein degradation or both (Rooyackers and Nair, 1997; Mauras and Haymond, 2005; Moller *et al.*, 2009). In the liver, GH promotes positive nitrogen balance by decreasing degradation of amino acids through the urea cycle, resulting in increased protein synthesis (Grofte *et al.*, 1997). As a result of these properties, GH has been used to treat protein loss in a variety of patients: for example, GH abrogates muscle wasting in AIDS patients, stimulates protein synthesis and promotes wound healing in patients with large burns and alleviates pain and stiffness in fibromyalgia patients (Gore *et al.*, 1991; Gilpin *et al.*, 1994; Low *et al.*, 1999; Hart *et al.*, 2001). In contrast, GH exerts catabolic effects on adipose tissues by stimulating lipolysis as well as lipid oxidation by increasing substrate availability, resulting in increased free fatty acids levels in the blood (Le Roith *et al.*, 2001; Moller and Jorgensen, 2009; Jorgensen *et al.*, 2010).

In addition to its important effects on growth and metabolism, GH serves diverse roles within the body including, but not limited to, the development and function of the immune system, especially B and T cells (Hattori *et al.*, 2001; Tesselaar and Miedema, 2008), promoting mammary gland development and lactation (Kleinberg, 1997; Kleinberg and Ruan, 2008), stimulating liver regeneration (Krupczak-Hollis *et al.*, 2003), and helping reconstitution of intestinal epithelial integrity following mucosal injury (Lal *et al.*, 2000). New research continues to reveal other potential roles of GH, including the regulation of cardiac function, mental agility and aging (Lanning and Carter-Su, 2006).

1.3 Pathophysiological Roles of GH

Because of the extensive effects of GH, any changes from its normal expression pattern can result in multiple acute and chronic endocrine and metabolic disorders (Kopchick and Okada, 2001). GH deficiency (GHD) leads to a reduction in the generation of GH-dependent factors, particularly IGF-I and its major binding protein, IGF binding protein 3 (IGFBP3), with auxological, clinical, and biochemical sequelae (Ayling, 2004). The majority of GHD clinical cases results from molecular defects in the GH gene or genes for different transcription factors that regulate the development of the anterior pituitary hormone secretory cells (Dattani, 2003; Bona *et al.*, 2004). GHD can also be acquired following head trauma or brain surgery. Patients born with GHD have a dwarf phenotype: they are short, accumulate fat around the waist area, and have reduced muscle mass and energy. Fortunately, the majority responds to exogenous GH, which leads to subsequent normalization of IGF-I levels, growth and metabolism.

In contrast, excessive production of GH by GH-secreting pituitary adenomas leads to two clinical disorders of GH that are distinguished by the age of onset. Pre-pubertal onset of GH overexpression is rare and causes gigantism, while post-pubertal onset results in acromegaly. The latter is characterized by the overgrowth of acral and soft tissues, severe debilitating arthritic features, skin thickening, thyroid enlargement, impaired cardiovascular function, sleep apnea, glucose intolerance and diabetes. In both cases, biochemical characteristics include elevated levels of GH and IGF-I. Trans-sphenoidal surgical and pharmacological interventions are presently the treatments of choice for both gigantism and acromegaly (Newman, 1999; Kopchick and Okada, 2001).

1.4 The Growth Hormone Receptor (GHR)

The ability of GH to exert its biological effects in target tissues is intimately linked to the number and functional status of its receptor, GHR. GHR is widely expressed but it is most abundant in major GH target tissues such as liver, muscle, bone and adipose. The human *GHR* gene is located on chromosome 5 at 5p13.1-p12, where it spans more than 300kb (Barton *et al.*, 1989; Godowski *et al.*, 1989; Goodyer *et al.*, 2001). It contains several non-coding 5'UTRs that give rise to 14 different mRNAs, each with a unique 5'UTR, but all of which code for a single protein through splicing into the same site upstream of the translation start site in the first coding exon (Pekhletsky *et al.*, 1992; Goodyer *et al.*, 2001; Orlovskii *et al.*, 2004; Wei *et al.*, 2006). The gene also consists of nine coding exons (2 to 10) (Ross *et al.*, 1997) (**Figure 1.2A**). The 4.5 kb *GHR* transcript is more than twice as large as the minimum 1.9 kb necessary to encode the 638 amino acids receptor/signal peptide molecule. The majority of the "excess" size of the *GHR* mRNA is due to the presence of an ~2.5 kb 3'UTR (Edens and Talamantes, 1998) (**Figure 1.2B**).

1.5 Regulation of *GHR* Gene Expression

Multiple studies, in our lab and elsewhere, have characterized regulation of the *GHR* gene by different transcription factors, which include, but are not limited to, Sp1 (Yu et al., 1999), MSY-1 (Schwartzbauer et al., 1998), C/EBPs, NF-Y, HES-1, CHOP, ETS1, GAF, GFI-1 (Goodyer *et al.*, 2001; Wei *et al.*, 2009; Kenth *et al.*, 2011), TNFα, HIF-1α and dexamethasone (Yu et al., 1999; Erman et al., 2011). These transcriptional factors regulate GHR expression by acting as downstream nuclear effectors, linking specific signaling cascades that are triggered by different growth factor-, developmental-, nutritional- or stress-related stimuli. Despite the

extensive regulatory studies of *GHR* at its 5'UTR, potential regulation at the 3'UTR has been overlooked. 3′UTRs of mRNAs are critical both for the targeting of transcripts to specific subcellular compartments and for translational control (Andreassi and Riccio, 2009).

1.6 The GH/GHR Signalling Pathways

The human GHR is an N-glycosylated homodimeric transmembrane protein. It is a member of the cytokine receptor superfamily, which includes receptors for prolactin, erythropoietin, thrombopoietin, and several interleukins, among others (Argetsinger and Carter-Su, 1996). Members of the cytokine receptor superfamily are single transmembrane proteins that lack intrinsic kinase activity and share certain structural motifs, including multiple paired cysteine residues in the extracellular domain and Box1, an eight residue proline-rich sequence located 10-20 amino acids below the transmembrane domain (Godowski *et al.*, 1989). There are two classes of cytokine receptors, I and II. GHR belongs to the class I group, which is distinguished by the presence of a conserved WSXWS motif just above the transmembrane region; the GHR is unique in that it contains a YGEFS motif (**Figure 1.2C**). Even though this motif does not directly interact with GH, alanine mutagenesis studies have shown that it is important for ligand binding and subsequent signal transduction (Baumgartner *et al.*, 1994).

Cloning of the *GHR* gene in 1987 opened the door to extensive investigations of how GH functions at the target cells. The *GHR* gene encodes a 638 amino acid protein (**Figure 1.2C**). In addition to the signal peptide (residues 1–18), the hGHR can be structurally divided into three domains: an extracellular domain (ECD), encoded by exons 2–7 (residues 19–264); a single transmembrane domain (TMD), encoded primarily by exon 8 (residues 265–288); and an

intracellular domain (ICD), encoded by exons 9 and 10 (residues 289–638) (**Figure 1.2C**). The ECD of the GHR consists of two functional subdomains: subdomain 1 (residues 19–141) is involved in GH binding, while subdomain 2 (residues 146–264) is involved in receptor dimerization and GH-induced receptor rotation (Behncken and Waters, 1999; Poger and Mark, 2010). The ICD contains the proline-rich motif termed Box 1, which is required for Janus kinase 2 (JAK2) association (**Figures 1.2C and 1.3**).

1.6.1 JAK-STAT pathway

GH binding to a dimer of GHR results in a structural conformation, inducing the rotation of one receptor subunit relative to the other (Rosenfeld *et al.*, 2007; Poger and Mark, 2010). This conformational change repositions the intracellular domains such that the Box 1-associated JAK2s transphosphorylate each other at specific tyrosine residues and phosphorylate several GHR intracellular tyrosines (Y534, Y566, and Y627), which then act as docking sites for signaling molecules containing Src homology 2 (SH2) domains (Rowlinson *et al.*, 2008; Derr *et al.*, 2011; Rotwein, 2012) (**Figure 1.3**).

The major pathways include the signal transducers and activators of transcription (STAT), the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and the phosphatidylinositol 3'kinase (PI3K) cascades (Behncken and Waters, 1999; Herrington and Carter-Su, 2001; Rosenfeld *et al.*, 2007; Rowlinson *et al.*, 2008; Xu and Messina, 2009). Collectively, activation of these signaling pathways mediates the biological effects of GH on growth and metabolism (**Figure 1.3**).

The STAT family of transcription factors is key in mediating GH/GHR biological effects on growth and the immune system. It consists of seven protein members, of which GH-activated

JAK2 phosphorylates at least four (STAT1, 3, 5a and 5b) to regulate a variety of genes. Although STAT5a and 5b are encoded by highly related genes that share 95% homology, it is STAT5b that has been implicated as the major mediator of growth through GH/GHR activation (Vidarsdottir *et al.*, 2006; Xu and Messina, 2009). JAK2 phosphorylates the docked STAT5bs on the activated GHR; they subsequently dissociate from GHR, form homodimers and translocate to the nucleus, where they drive transcriptional regulation of STAT5b-dependent genes (e.g. *IGF-I*, *IGFBP-3*, *ALS* and *SOCS1-3*) (**Figure 1.3**) (Shuai, 1999; Levy and Darnell, 2002; Rosenfeld *et al.*, 2007). Targeted disruption of STAT5a and b results in defects in liver gene expression, body growth and immune function similar to what is observed with GH or GHR deficiencies (Udy *et al.*, 1997; Vidarsdottir *et al.*, 2006; Rosenfeld *et al.*, 2007).

1.6.2 ERK/MAPK pathway

A second pathway that is important for GH regulation of gene transcription is the ERK/MAPK pathway that stimulates DNA synthesis and promotes cell cycle progression and survival (Chang and Karin, 2001; Chambard *et al.*, 2007). GH has been shown to activate this pathway by JAK2 phosphorylation of the protein SHC that, in turn, activates a cascade of downstream signaling molecules (GRB2, SOS, RAS, RAF-1, and MEK1/2) to ultimately activate the ERK1-2/MAPK1-3 proteins (**Figure 1.3**), (Zhu *et al.*, 2002; Rowlinson *et al.*, 2008). Interestingly, a targeted mutation of the Box-1 domain of GHR completely abrogates the JAK2-dependent signaling pathways involving STAT5b and PI3K/AKT but does not alter the ERK/MAPK pathway, suggesting that JAK2-independent signaling also occurs (Rowlinson *et al.*, 2008; Barclay *et al.*, 2010). The GH-activated MAPK pathway has been implicated in up-regulation of C-*FOS*, *EGR-1*, and *JUN-B* gene transcription and activation of other protein

kinases (e.g. Elk-1), cytoplasmic phospholipase A_2 and cytoskeletal proteins. These are thought to play an important role in regulating cellular growth and differentiation (Vanderkuur *et al.*, 1997; Hodge *et al.*, 1998).

1.6.3 PI3K pathway

Another major GH-dependent signaling cascade is the PI3K pathway. JAK2- dependent phosphorylation activates the adaptor proteins Insulin Receptor Substrate 1, 2 and 3 (IRS1, 2 and 3); these subsequently provide a binding site for the p85 regulatory subunit of PI3K (Yamauchi et al., 1998) **(Figure 1.3)**. Although PI3K is required for insulin-stimulated glucose transport, GH-induced glucose transport has been reported not to require PI3K (Sakaue *et al.*, 1997). However, inhibition of PI3K blocks GH-stimulated lipid synthesis and the anti-lipolytic actions of GH, suggesting that PI3K activity is important for the acute insulin-like actions of GH (Yamauchi *et al.*, 1998). In contrast, chronic effects of GH are anti-insulin-like, promoting insulin resistance and diabetes. Excess GH can lead to chronic activation of the IRS-PI3K pathway in the liver, reducing the insulin-related effects (Dominici *et al.*, 1999).

AKT, also known as protein kinase B (PKB) is the major downstream effector activated by PI3K. Once activated by phosphorylation, pAKT phosphorylates mammalian target of rapamycin (mTOR), another serine/threonine kinase **(Figure 1.3)**. It has been shown that the rapid activation of protein synthesis (minute to hour timescale) by GH involves mTOR complex1 (mTORC1), a key regulator of cell growth. Both GH and mTORC1 play key roles in regulating cell and tissue growth, and a key component of these effects is the stimulation of protein synthesis by activating both the initiation and elongation stages of protein translation (Hayashi and Proud, 2007). Defects in PI3K/AKT signaling have been implicated in many diseases,

including cancer (Feng *et al.*, 2005; Wong *et al.*, 2010) and type 2 diabetes (Farese *et al.*, 2005). Together, PI3K and AKT are major mediators of GH signaling, with roles in glucose metabolism, cell proliferation, and anti-apoptosis (Costoya et al., 1999) (**Figure 1.3**).

1.6.4 SRC kinase pathway

Although most GH/GHR downstream-signaling pathways are dependent on JAK2 activity, JAK2-independent pathways have also been identified (Argetsinger and Carter-Su, 1996; Manabe *et al.*, 2006; Brooks *et al.*, 2008). Experimental evidence indicates that GHR signaling can occur through direct activation of several members of the SRC family of protein tyrosine kinases (Manabe *et al.*, 2006; Zhang *et al.*, 2006).

The precise SRC binding site on the GHR remains unknown, but evidence to date suggests that it associates with GHR at the membrane proximal residues of the intracellular domain (Rowlinson *et al.*, 2008).

Members of the SRC family kinases are non-receptor tyrosine kinases that contain SH2, SH3 and tyrosine kinase domains. They are involved in the signaling of many cellular processes, including cell growth, proliferation, differentiation, motility, adhesion, angiogenesis and survival (Yarbro and Howards, 1987; Thomas and Brugge, 1997; Parsons and Parsons, 2004). There is substantial evidence of elevated levels of SRC kinase activity in solid tumors, implicating the SRC pathway in cancer progression and metastatic disease by facilitating the action of other signaling proteins (Homsi *et al.*, 2007).

1.7 Inhibitors of GHR Signaling

Multiple mechanisms are involved in terminating GH/GHR signaling pathways, including the actions of negative regulators, ligand-induced endocytosis and degradation of the GHR and signaling intermediaries. Protein tyrosine phosphatases (PTPs) and the suppressors of cytokine signaling (SOCS) proteins are the two major negative regulators involved in controlling GH/GHR activated signaling (Birzniece *et al.*, 2009). The PTPs function as negative regulators by rapidly dephosphorylating GHR, JAK2 and cellular substrates of JAK2, thus terminating the recruitment of signaling molecules to the receptor for activation (Herrington and Carter-Su, 2001). The SOCS family of proteins is rapidly induced by various cytokines, including GH (Vidal *et al.*, 2007). Out of the eight known members of SOCS family, GH induces the gene expression of *SOCS1*, *SOCS2*, *SOCS3* and *CIS* (cytokine-inducible SH2-containing protein) to exert negative feedback on GHR through blocking activation of STAT proteins (Greenhalgh *et al.*, 2005). SOCS proteins contain an SH2 domain able to bind phospho-tyrosine residues in target proteins (e.g. GHR and JAK2) (Ram and Waxman, 1999; Vidal *et al.*, 2007). SOCS2, SOCS3 and CIS also can inhibit JAK2 indirectly by first binding to tyrosine-phosphorylated GHR and then interfering with STAT5b binding (Uyttendaele *et al.*, 2007; Vidal *et al.*, 2007). In addition, evidence implicates the SOCS proteins as possible ubiquitin ligases regulating proteasomal degradation of GHR and JAK2 (Ram and Waxman, 1999; Ungureanu *et al.*, 2002; Vidal *et al.*, 2007). Mice deficient in SOCS2 display an excessive growth phenotype with elevated IGF-I levels, underscoring the significance of SOCS2 for terminating GH/IGF-I signaling (Metcalf *et al.*, 2000; Greenhalgh *et al.*, 2005).

At least two different mechanisms participate in the turnover of the cell surface GHR

protein: proteolytic cleavage and ligand-independent endocytosis (Flores-Morales *et al.*, 2006). The GHR proteolytic cleavage is exerted by two proteinases. A metalloproteinase (ADAM-17) cleaves the extracellular portion of the receptor at the membrane-adjacent part generating the GH binding protein (GHBP). The membrane-bound remnant is then degraded by a γ-secretase complex and targeted for proteasomal degradation (Zhang *et al.*, 2001; Flores-Morales *et al.*, 2006). This mechanism is largely responsible for GHBP release into circulation, but it is not expected to participate in direct termination of GH signaling since it is inhibited by ligand binding to the receptor (Zhang *et al.*, 2001; Flores-Morales *et al.*, 2006). In contrast, GHR endocytosis involves both clathrin-coated pits (Vleurick *et al.*, 1999) and caveolae (Lobie *et al.*, 1999) in a process that is accelerated by GH bound receptors. Internalized receptors are mostly directed to lysosomes and proteasomes for degradation, in a process that requires an intact ubiquitin-conjugating system (Sachse *et al.*, 2001; Flores-Morales *et al.*, 2006).

1.8 Disregulation of GHR Expression

1.8.1 Growth and metabolic disorders

Major growth disorders can occur as a result of abnormal GHR expression: Laron dwarfism, idiopathic short stature (ISS) and constitutional growth delay (CGD) are three classes of growth disorders associated with GH insensitivity (GHI), a term used to describe a clinical condition characterized by low to non-detectable levels of circulating IGF-I in parallel with normal or supra-physiological levels of GH (Rosenfeld *et al.*, 1994; Goh *et al.*, 1997). Mutations in the *GHR* locus can result in GHI by impairing GHR expression and/or function. At one

extreme, homozygous mutations or deletions within the GHR coding region that result in loss of functional GHR are linked to complete GHI (Laron syndrome). These individuals are diagnosed soon after birth because of metabolic instability and a rapid loss in growth rate; they have extreme short stature, very low serum IGF-I levels, increased body mass index, musculo-skeletal abnormalities and hypoglycemia (Savage *et al.*, 2006). In contrast, the majority of children with short stature are diagnosed with ISS. Usually their birth size is normal, but by the end of their first year they are growing below the 3rd percentile. Their clinical profile includes normal or elevated GH concomitant with normal to low serum IGF-I levels, a relatively high body mass index, with no evidence of hypothyroidism, malnutrition, intrauterine growth retardation, systemic disease or specific syndromes. Their circulating GH binding protein levels are often below the mean and 20% have levels >2SD below, suggesting that tissue levels of GHR are chronically low in many of these children (Goddard *et al.*, 1997). Approximately 50% of ISS cases show growth “catch up” at puberty and are reclassified as CGD. The cause of most ISS cases remains undetermined at the genetic level. Only rare cases (< 2%) of mutations in the GHR coding regions have been described in patients with ISS or CGD; thus the classification of these disorders remains "idiopathic" (Goddard *et al.*, 1995; Sanchez *et al.*, 1998; Savage *et al.*, 2002; Bonioli *et al.*, 2005; Hujeirat *et al.*, 2006).

1.8.2 Association of GHR with cancer

Normal growth requires a balance between the activity of genes that promote cell proliferation and those that suppress it, which ensures that healthy functioning cells survive in appropriate environments while damaged or non-functioning cells are eliminated. Cells acquire mutations in these genes as a result of spontaneous and environmentally-induced DNA damage.

Over time, these cells become increasingly resistant to the controls that maintain normal functions and, as a result, they start to grow out of control and a tumor can develop. The most important extracellular signaling molecules engaged in maintaining cell survival are growth factors.

The relevance of growth factors to the pathogenesis of human cancers has been long established. One mechanism that contributes to amplification of the signals of these growth factors is overexpression of their receptors and/or hyper-activation of their signaling mediators. GH is one of the growth factors that promote normal growth of tissues through pro-proliferative, anti-apoptotic and pro-angiogenic effects (Veldhuis *et al.*, 2005; Lichanska and Waters, 2008a; Lichanska and Waters, 2008b; Perry *et al.*, 2013). These actions are critical during childhood growth, especially for long bones, but they are also important throughout life for those tissues where there is continual turnover of cells (e.g. the lining of intestinal villi, skin). However, chronic overexpression of any member of the GH/GHR/IGF-I axis and/or hyper-activation of their downstream signaling effectors will promote cell hyperplasia and tumor progression (Sonntag *et al.*, 2000; Yakar *et al.*, 2005; Perry *et al.*, 2006; Johansson *et al.*, 2007; Kleinberg *et al.*, 2009; Brooks and Waters, 2010; Clayton *et al.*, 2011).

The link between GH/GHR/IGF-I axis and cancer goes back as early as 1950, based on clinical observations that hypophysectomy reduced breast cancer progression in women (Luft and Olivecrona, 1957). Multiple recent studies support this association in both human and animal models. For example, acromegalic patients show an increased incidence of colorectal tumors (Ispolatova, 1968; Kurosu *et al.*, 1984; Webb *et al.*, 2002), while studies of individuals with GH/GHR deficiencies have shown decreased incidences of cancer. In a study of a cohort (n=230; 1-75yr; mean=19yr) of Laron syndrome patients (with dysfunctional GHR) collected from all

over the world, not one individual had a clinical history of a tumor ($p<0.001$) while first-degree relatives had a tumor frequency of 8.3% (Steuerman *et al.*, 2011). In a similar study of an Ecuadorian cohort over a 25 year period, 99 individuals who carry mutations in the *GHR* gene that led to severe GHR deficiency, only one reported a non-lethal malignancy; in contrast there was a 17% cancer prevalence in first-degree relative controls (Guevara-Aguirre *et al.*, 2011). In addition, crossing $GHR^{-/-}$ mice with mice prone to prostate or breast neoplasias resulted in decreased incidence and/or progression of the tumors in their offspring (Wang *et al.*, 2005; Zhang *et al.*, 2007b).

Moreover multiple studies have reported GHR overexpression in diverse tumors. *GHR* mRNA and protein were increased 2-5 fold in human colorectal cancer tissues and cell lines relative to controls, suggesting a role for GHR in the progression of colorectal carcinomas (Dagnaes-Hansen *et al.*, 2004; Yang *et al.*, 2004; Yang *et al.*, 2005; Wu *et al.*, 2007). GHR over-expression has also been reported in gastric carcinomas: experimental results indicate that *GHR* mRNA expression is significantly higher in primary gastric adenocarcinoma than in normal gastric mucosa and is significantly correlated with tumor differentiation and tumor grade (Nagano *et al.*, 1995; Lincoln *et al.*, 2007; Lin *et al.*, 2011; Yang *et al.*, 2012; Ran *et al.*, 2013).

Together GH and PRL, through their receptors (GHR and PRLR), play a central role in mammary development (ductal and alveolar development and differentiation) and lactation (Feldman *et al.*, 1993; Kelly *et al.*, 2002; Xu *et al.*, 2011). In addition to its physiological role, several clinical observations and experimental studies have indicated a role for GHR in the development of human breast cancer. Clinical studies have revealed that *GHR* mRNA expression is up-regulated in malignant tissues compared to adjacent normal breast tissue, and its expression is inversely correlated with tumor grade and MIB-1 proliferative index (MIB-1, a

proliferative and prognostic biomarker for breast cancer, is an antibody that recognizes the proliferation marker Ki-67) (Lehr *et al.*, 1999; Gebre-Medhin *et al.*, 2001). Furthermore, in a large genome-wide association study, the GH/GHR signaling pathway was identified as the third most significant pathway correlated with susceptibility to develop a mammary carcinoma (Menashe *et al.*, 2010).

The first report about involvement of the GH/GHR/IGF-I axis in prostate cancer was by Kolle S. *et al*. in 1999; they detected the overexpression of *GHR* mRNA in 21 human prostatic carcinomas and 19 benign prostatic hyperplasias compared to controls suggesting that the growth hormone receptor may facilitate prostatic tumor cell growth and progression (Kolle *et al.*, 1999). In a later study, Chopin *et al.* detected the co-expression of both *GH* and *GHR* mRNA isoforms in the ALVA41, PC3, DU145 and LNCaP prostate cancer cell lines, suggesting the existence of an autocrine-paracrine pathway in the prostate that would be capable of stimulating prostate growth, either directly via GHRs or indirectly via IGF-I production (Chopin *et al.*, 2002). They also have shown that GH can increase the rate of proliferation in these prostate cancer cell lines. In a more recent study, Weiss-Messer *et al*. showed that GH induced rapid, time- and dose-dependent signaling events in LNCaP cells, including the phosphorylation of JAK2, GHR, STAT5A, p42/p44 MAPK and AKT/PKB in parallel with increased levels of androgen receptor. Collectively these results suggest a role for GH in the progression of prostate cancer in concert with other hormones and growth factors (Weiss-Messer *et al.*, 2004).

The GH/GHR axis has also been associated with hematopoietic malignancies, including leukemia. GHR is expressed widely in normal human lymphocytes, including T cells, B cells and many leukemic cell lines (Hattori *et al.*, 2001). Studies by Jeay et al. have suggested a dual effect of the GH/GHR axis on survival and proliferation of the pro-B Ba/F3 cell line (Jeay *et al.*, 2000).

When Ba/F3 cells were transfected with a GHR expression vector and treated with GH, a GH signaling pathway involving the activation of Nuclear Factor (NF)-κB and the expression of the anti-apoptotic protein, Bcl-2, was shown to mediate the anti-apoptotic effects. Increased concentrations of GH were shown to promote Ba/F3 cell proliferation, which was dependent upon the activation of PI-3K, leading to the induction of the proto-oncogene, c-myc (Dinerstein *et al.*, 1995; Baixeras *et al.*, 2001).

The above evidence strongly suggests an important role for the GH/GHR axis in the progression of multiple malignancies. Thus regulation of this axis at multiple levels must be tightly controlled. One mechanism is to block the deleterious effects at the earliest stage of the signaling, the GHR.

1.9 MicroRNAs

1.9.1 MicroRNA discovery

MicroRNAs (miRNAs or miRs) are newly emerging posttranscriptional regulators of gene expression. They are naturally occurring, ~19-22 nucleotides long, small noncoding RNAs that mainly bind to the 3'UTR of target genes, resulting in mRNA degradation and/or the arrest of mRNA translation (Chen *et al.*, 2004). Computational analysis indicates that more than 60% of protein coding genes may be directly modulated by miRNAs (Siomi and Siomi, 2010). Accumulating experimental evidence indicates that miRNAs extensively regulate gene expression in almost all eukaryotes, influencing numerous aspects of cellular biology, including development, metabolism, proliferation, differentiation, apoptosis, tumorigenesis and innate and

adaptive immune responses (Ambros, 2004; Chang and Mendell, 2007; Schickel *et al.*, 2008; Xiao and Rajewsky, 2009; Mendell and Olson, 2012).

MiRNAs were discovered relatively recently, in 1993, by the Ambros lab in MIT, when they found a noncoding small RNA, Lin4, transcribed from a gene known to control the timing of *C. elegans* larval development. Lin4 had the ability to bind to the mRNA of the *Lin 14* gene and repress its protein translation (Lee *et al.*, 1993). In 2000, the first miRNA was discovered in humans and other vertebrates (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). Recent advancements in high-throughput miRNA deep sequencing analysis have enabled the identification of a large number of novel miRNAs: the June 2013 release of miRNA database miRBase20 (http://www.mirbase.org/) contains 24521 entries, representing hairpin precursor miRNAs that express 30424 mature miRNA products in 193 species, and include more than 2500 mature human miRNAs (Kozomara and Griffiths-Jones, 2011).

1.9.2 MiRNA nomenclature

MicroRNAs are named using the "miR" prefix followed by a dash and a number, the latter often indicating the order of naming. For example, miR-129 was named, and likely discovered, prior to miR-142. MiRNAs with nearly identical sequences differing in only one or two nucleotides are annotated with an additional lower case letter. For example, miR-15a would be closely related to miR-15b. To denote species-specific miRNA, a three-letter prefix is added, e.g., hsa-miR-129 is a human (*Homo sapiens*) miRNA while mmu-miR-129 is a mouse (*Mus musculus*) miRNA. When two mature microRNAs originate from opposite strands of the same pre-miRNA, they are denoted with a -3p or -5p suffix; for example, miR-142-3p (from the 3' arm) and miR-142-5p (from the 5' arm). The mature miRNA is designated miR-202 (with capital

R) in the databases and in much of the literature (Ambros *et al.*, 2003). The 5' end of the mature miRNAs is a particularly important determinant of miRNA function starting 2-8 nucleotides from the 5'end; this is known as the seed sequence (Stefani and Slack, 2008).

1.9.3 MiRNA biogenesis

MiRNA production begins in the nucleus and involves a series of RNA processing steps **(Figure 1.4**). MiRNA genes are dispersed throughout the genome, either within intronic sequences of protein-coding genes, within intronic or exonic regions of noncoding RNAs, or located between independent transcription units (intergenic). The majority of intronic miRNAs are transcribed from the same promoter as the host gene (Wahid *et al.*, 2010); however, approximately one-third of intronic miRNAs are transcribed from independent promoters, enabling separate control of their transcription (Ozsolak *et al.*, 2008; Wang *et al.*, 2009; Monteys *et al.*, 2010). Transcriptional regulation of miRNAs is dictated by the same properties as those regulating protein-coding genes: CpG islands, TATA box sequences, histone modifications, transcription factors, enhancers, silencing elements and chromatin modifications (Ghyka and Cernescu, 1975; Ozsolak *et al.*, 2008). The majority of miRNA genes are transcribed by RNA polymerase II, generating primary (pri-miRNA) transcripts **(Figure 1.4**) with 5'cap and 3' polyadenylation sequences. Following transcription, the pri-miRNA is cleaved by an RNase III-like enzyme, Drosha, and its cofactor, DiGeorge critical region 8 (DGCR8), to a 60-70 nt precursor (pre-) miRNA **(Figure 1.4**). The pre-miRNA is then exported by exportin5 protein into the cytoplasm where it is further processed by another RNase III enzyme, Dicer, that cleaves the stem loop to generate a ~22 nucleotide miRNA:miRNA* duplex. The double stranded- (ds-) miRNA then gets unwound and the passenger strand (miRNA*) degraded. The mature strand is

subsequently loaded into a pre-RNA-induced silencing complex (pre-RISC), which contains an Argonaut protein, mainly Ago2, as well as other proteins, giving rise to the mature miRISC complex. (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). The mature miRNA then leads the miRISC to its target mRNA. If the miRNA has 100% homology to its target mRNA, it results in mRNA target degradation, similar to the siRNA pathway. However, as the majority of animal miRNAs bind imperfectly to their mRNA targets, they instead destabilize the mRNA and/or repress its protein translation (Lee *et al.*, 1993) **(Figure 1.4**).

1.9.4 MiRNA functional analysis

The most significant challenge in miRNA studies is the identification of miRNA-targeted genes. Most known miRNA targets in animals have followed a canonical pattern, where target genes contain 7–8 bases in their 3′UTRs with perfect complementarity to the so-called seed region within the mature miRNA sequence (Bartel, 2009). A number of *in silico* target prediction tools have been designed to aid in the prediction of such 3′UTR targets: TargetScan 5.2 and later 6.2 (Lewis *et al*, 2003, www.targetscan.org) (Lewis *et al.*, 2005), PicTar (Krek *et al* 2005, http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi) (Krek *et al.*, 2005), miRDB (Wang *et al* 2008, http://www.mirdb.org/miRDB/) (Wang, 2008; Wang and El Naqa, 2008), Microcosm Targets (Rehmsmeie *et al* 2004, http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) (Rehmsmeier *et al.*, 2004) and MiRanda (John *et al* 2004, http://www.microrna.org/microrna/home.do) (John *et al.*, 2004). These target prediction algorithms use multiple criteria to determine whether a gene is a potential target for a miRNA, including complementarity to the seed site, evolutionary conservation among species, and the stability of hybridization energy between the miRNA and the predicted site on the mRNA of the

target gene (van Rooij, 2011). Such tools have proven to be invaluable resources for miRNA functional studies.

1.9.5 Roles of miRNAs

Since their discovery, research on the miRNAs has expanded exponentially, resulting in more than 20,000 published scientific papers within the last two decades (Xie *et al.*, 2013). Collectively these studies have shown that this class of small non-coding RNAs functions as an important regulator in a wide range of cellular processes. The biological effects of any miRNA depend on the cellular environment in which it is expressed, its turnover rate and the target sequence that the miRNA binds. In that manner, a small change in miRNA expression can lead to modest changes in the expression of multiple proteins directly or indirectly, and collectively these can add up to large changes in a biological system (Kim and Park, 1988; Baek *et al.*, 2008; Selbach *et al.*, 2008). As a consequence of their extensive participation in normal functions, their abnormalities have deleterious effects on a wide variety of human diseases (Kim and Park, 1988; Tang *et al.*, 2008; Fabian *et al.*, 2010). MiRNA expression can be regulated by a variety of stimuli and mechanisms, including direct transcriptional activators or repressors, genomic amplification or deletion, epigenetic modifications of the genome, cellular stress and inflammatory stimuli (Guo *et al.*, 2010; Tomankova *et al.*, 2010; O'Neill *et al.*, 2011). In turn, the effect of inducing or repressing miRNA expression can influence most biological processes, including cell fate specification, cell proliferation, DNA repair, DNA methylation and apoptosis and provide pro-inflammatory or anti-inflammatory stimuli (Pallante *et al.*, 2010).

1.9.6 Aberrant miRNA activities

The loss or gain of miRNA function can be caused by mutations in the miRNA gene or its promoter, in the miRNA itself or its target sequence. Given the importance of miRNAs in regulating cellular differentiation and proliferation, it is not surprising that their misregulation is linked to cancer. In fact, high-throughput analyses of miRNA expression in cancer have demonstrated that some miRNAs are over-expressed in cancer while others are markedly reduced (Soifer *et al.*, 2007; Lynam-Lennon *et al.*, 2009). Several of these miRNAs function as oncogenes or tumor suppressors and, thus, are termed oncomiRs or tumor suppressor miRs (Soifer *et al.*, 2007; Zhang *et al.*, 2007a; Osaki *et al.*, 2008). OncomiRs are overexpressed in cancers and promote cancer development by negatively regulating tumor suppressor genes and/or genes that control cell differentiation or apoptosis. In contrast, tumor suppressor miRNAs are underexpressed in cancers; they function as tumor suppressor genes and may inhibit cancers by downregulating oncogenes. Accumulating evidence from studies of miRNA profiling in cancers shows a direct functional link between aberrant miRNA expression and specific tumor types (Ambros, 2004; Chen *et al.*, 2004; Cheng *et al.*, 2005; Karp and Ambros, 2005; Hwang and Mendell, 2006; Shenouda and Alahari, 2009; Skalsky and Cullen, 2011). I will focus the remainder of my review on those tumors where abnormal overexpression of GHR was also detected, as described earlier in section 1.8.b: hematopoietic, colorectal, gastric, breast and prostate. Although the mechanisms underlying GHR aberrant overexpression are not known, dysregulation of potential GHR-regulating miRNAs is one possibility.

1.9.6.1 MiRNA association with hematopoietic cancers

Initial evidence for the involvement of miRNAs in cancers came from a molecular study characterizing the chr13q14 deletion in human chronic lymphocytic leukemia (CLL). It was observed that the genes for two miRNAs, miR-15a and miR-16, are located in a region of chromosome 13q14 that is deleted in more than half of B cell chronic lymphocytic leukemia (B-CLL) cases (Calin *et al.*, 2002). Detailed deletion analyses demonstrated that these two are the only two genes within the small (30 kb) common region which is lost in CLL patients, and expression analysis indicated that miR-15a and miR-16 were either absent or down-regulated in the majority (~ 68%) of CLL patients. MiR-15a and -16 are thought to exert tumor suppressor functions by targeting multiple oncogenes, including the anti-apoptotic protein, BCL2, which is overexpressed in the malignant B-cells of CLL (Calin *et al.*, 2002; Lerner *et al.*, 2009; Klein *et al.*, 2010).

On the other hand, overexpression of miR-155, a miR encoded by host gene *miR-155HG,* was observed in a long list of both hematologic and solid tissue cancers, where it was found to promote genomic instability, proliferation and survival of malignant cells. Such properties led to miR-155 being coined as the first oncomiR (Costinean *et al.*, 2006). In a more recent study by Kopp et al., they found that, while transient elevation of miR-155 levels is necessary for normal functioning of immune cells, a chronically increased expression of miR-155 is often detected in cancers; moreover, miR-155 expression alone is sufficient to trigger malignant transformation (Kopp *et al.*, 2013). They also showed that *miR-155HG* gene is a transcriptional target of STAT5; STAT5 directly binds to the *BIC* (B-cell Integration Cluster that includes the *miR-155* gene) promoter and induces miR-155 expression in T cell lymphomas. This links expression of an oncogenic miRNA to cytokine signaling of the JAK/STAT pathway and sheds light on the

aberrant role of STAT5 in the induction of oncogenic molecules, further suggesting a link between inflammation and cancer (Kopp *et al.*, 2013). As mentioned earlier, GHR overexpression was observed in multiple leukemic cell lines. STAT5 being the main mediator of GH/GHR signaling, the expression of the oncomiR-155 by STAT5 could be a mechanism by which GHR is involved in the tumorigenesis process.

1.9.6.2 MiRNA association with colorectal cancer

Both functional and profiling studies have established a connection between miRNAs and colorectal cancer. MiRNAs regulate many known oncogenic and tumor suppressor pathway members, such as Wnt/β-catenin, PI3K, KRAS and p53, that are involved in the pathogenesis of colorectal tumors (Slaby *et al.*, 2009). MiR-34b and c are epigenetically silenced in colorectal cancer cells but not in normal colonic mucosa (Toyota *et al.*, 2008). When Ng et al. investigated whether plasma miRNA levels could discriminate between patients with and without colorectal cancer, they found that five miRNAs, including miR-17-3p and miR-92, were up-regulated in both colorectal cancer tissue and plasma samples and that the plasma levels of these markers were significantly reduced after surgery in 10 colorectal cancer patients (Ng *et al.*, 2009). Further validation on an independent set of 180 plasma samples showed that miR-92 expression can differentiate colorectal from gastric cancer, indicating that miR-92 may be a non-invasive molecular marker for colorectal screening (Ng *et al.*, 2009).

A study by Bandrés *et al.* identified miR-129-5p (a known inducer of G1 phase arrest (Wu *et al.*, 2010)) as one of the significantly down-regulated miRNAs in both colorectal cancer cell lines and a set of tumor tissues compared to a human normal colon cell line and normal paired tissues (Bandres *et al.*, 2006). In a more recent study, Karaayvaz *et al*. found that the

level of miR-129-5p expression was significantly decreased in samples from 22 human colorectal tumor tissues compared to their paired normal controls (Karaayvaz *et al.*, 2013). Moreover, the same group studied a set of 61 colorectal specimens from different stages of the disease and found that the expression of miR-129-5p was significantly reduced in patients with stage 3 and 4 of the disease compared with normal or adenoma tissues (Karaayvaz *et al.*, 2013). Collectively these results suggest a tumor suppressor role for miR-129-5p, and that this may lead to novel therapeutic approaches for colorectal cancer.

1.9.6.3 MiRNA association with gastric cancer

Gastric cancer is diagnosed in nearly one million individuals each year and is regarded as one of the leading causes of cancer-related deaths in the world (Feng and Sheng, 2013). Gastric carcinogenesis is a complex multistep process involving genetic dysregulation of proto-oncogenes, tumor suppressor genes and miRNAs. For example, miR-19a was found to function as an oncogenic miRNA in gastric cancer by repressing expression of the tumor suppressor gene, SOCS1; its expression is inversely correlated with SOCS1 expression in gastric cancer cells and a subset of gastric cancer tissues. Moreover, ectopic expression of miR-19a dramatically promoted proliferation and tumorigenicity of gastric cancer cells both *in vitro* and *in vivo* (Qin *et al.*, 2013). Other studies have shown that the expression of miR-202 is down-regulated in gastric cancer, both in cell lines and tumor tissues. Moreover, overexpression of miR-202 in two gastric cancer cell lines, MNK-28 and BGC-823, markedly suppressed cell proliferation and induced cell apoptosis both *in vitro* and *in vivo* (Zhao *et al.*, 2013). Because malignant cells show dependence on the abnormal expression of miRNA genes, which in turn control or are controlled by the dysregulation of multiple protein-coding oncogenes or tumor suppressor genes, these

miRNAs provide important opportunities for the development of future miRNA-based therapies against gastric cancer (Kim and Park, 1988; Croce, 2009).

1.9.6.4 MiRNA association with prostate cancer

In developed countries, prostate cancer is the most common malignant tumor in men, and is the second highest cause of cancer mortality after lung tumors (Gronberg, 2003). As with other tumors, results from miRNA profiling experiments suggest that aberrant expression of miRNAs occurs in prostate cancer (Lu *et al.*, 2005; Volinia *et al.*, 2006; Porkka *et al.*, 2007; Ambs *et al.*, 2008; Tong *et al.*, 2009). For example, Volinia et al. studied RNA extracted from 363 solid cancers and 177 normal tissues, including 56 prostate tumours and 7 normal prostates; they identified a prostate cancer signature composed of 39 up-regulated miRs and 6 down-regulated miRs (Volinia *et al.*, 2006). More focused studies have revealed that alterations in expression of some miRNAs have been correlated with: (1) genetic alterations that eventually result in epigenetic reprogramming; (2) transcription factor activities; and (3) the acquisition of invasive features and/or androgen independence (Coppola *et al.*, 2010). For example, miR-15a/miR-16 genes reside in 13q14.3, a region deleted in 66% of prostate cancer patients without relapse and 73% with relapse (Brookman-Amissah *et al.*, 2007). MiR-15 and -16 expression levels were analyzed in 20 prostate tumour-derived primary cultures compared to their normal counterpart by qPCR and in 15 prostate tumour biopsies by in situ hybridization: there was a down-regulation of miR-15a and miR-16 levels in 85% of the tumor cases (Bonci *et al.*, 2008). Further experiments by Bonci *et al*. using a stable lentivirus-mediated inhibition of miR-15/miR-16 in RWPE-1 (normal epithelial prostate) cells resulted in an increase in proliferation and migration *in vitro* and the formation of small tumour masses in immunodeficient mice. In

contrast, lentivirus-mediated miR-15/miR-16 reconstitution in LNCaP (tumor prostate cell line) cells resulted in a dramatic apoptotic effect *in vitro* and a significant regression of tumour xenografts *in vivo*. Collectively, these lines of evidence suggest that miR-15/miR-16 loss may contribute to prostate cancer progression.

1.9.6.5 MiRNA association with breast cancer

Our understanding of the molecular classification of breast cancer stems from mRNA profiling studies which have broadly sub-classified breast tumors into five major groups: luminal A, luminal B, basal-like, HER2-positive and normal-like tumors (van 't Veer *et al.*, 2002; Kopp *et al.*, 2013). More recently, global studies of miR expression in normal breast tissues and tumors have generated a miR signature discriminating between normal and malignant breast tissues (Iorio *et al.*, 2005; Le Quesne and Caldas, 2010). For example, upregulation of let-7 and miR-342 occur primarily in ER-positive tumors, while increased expression of miR-135b and miR-18 occurs in ER-negative tumors. In fact, expression of two key players in miRNA biogenesis and actions, DICER1 and AGO2, is correlated with tumor subtype and may explain some of the changes in miRNA expression observed (Blenkiron *et al.*, 2007).

1.9.6.6 MiRNAs as cancer prognosis and therapeutic tools

The recent discovery of miRNAs has provided a new layer of complexity to the known mechanisms regulating gene expression and function. MiRNAs function by repressing specific target genes at the post-transcriptional level. The target mRNAs are often involved in the

regulation of diverse physiological processes ranging from developmental timing to apoptosis, very fundamental biological processes.

However, when miRNAs are aberrantly expressed they can contribute to pathological conditions, such as cancer. As such, they have been shown to be useful as biomarkers for diagnosis of cancer type and prognostic indicators of severity. For instance, Asuragen is the first pharmaceutical company to validate a first-generation miRInform Pancreas test, which analyzes the expression of two miRNAs, miR-196a and miR-217, in formalin-fixed and paraffin-embedded pancreatic tissues (Szafranska *et al.*, 2008). In 2012 the company launched an improved version of the test, which uses a broad panel of microRNAs from endoscopic ultrasonography fine needle aspirate specimens. This is an important advance since it is difficult to access tissue specimens in non-resected pancreatic cancer patients. This procedure makes it possible to screen high-risk individuals, establish a prognosis and predict the response to treatment in those numerous cases in which the tumor is not operable (Araki *et al.*, 1986).

In addition, miRNA therapy could be a powerful tool for multiple cancers, where miRNA mimics can be used to compensate for the aberrant loss of a tumor suppressor miRNA, or miRNA inhibitors to inhibit oncomiRs. Anti-miR-34 is the first miRNA therapy to advance into a human clinical trial for cancer. The Phase 1 trial is being conducted in patients with primary liver cancer or metastatic cancer with liver involvement. MiR-34 is termed a master regulator of tumor suppression. It is downregulated in multiple cancers and it inhibits malignant growth by repressing multiple members in different oncogenic signalling pathways: cell cycle, apoptosis/p53, wnt signaling/metastasis, cancer cell stemness, mitotic signaling, oncogenic transcription and metabolism (Bader, 2012; Di Martino *et al.*, 2012).

In addition, miR-122 is the most abundant miRNA in liver, and was the first anti-miR candidate for a non-cancer microRNA therapeutic. After successful preclinical experiments in both mice and nonhuman primates, clinical trials are now underway to investigate the use of miR-122 as an antisense target for the treatment of chronic hepatitis C virus infection in humans (Haussecker and Kay, 2010). The increasing evidence of significant involvement of miRNAs in almost every disease process suggests that, in the future, miRNA therapies will be used for treatment of multiple diseases, including cancer.

1.10 Hypothesis and Objective of the Study

There is a significant body of evidence suggesting a role for increased GHR expression in the progression of several cancers. However the underlying mechanisms behind the overexpression of GHR remain undefined. One possibility is a dysregulation at the post-transcriptional level by miRNAs. Therefore for my master's degree, I have tested the hypothesis that specific miRNAs regulate *GHR* gene expression using HEK293 cells as a model test system. Providing evidence for specific miR regulation of GHR will shed light on a new aspect of GHR regulation, may uncover mechanisms behind the role of GHR in progression of multiple cancers and, most importantly, may provide a therapeutic tool to block the tumorigenic effects of the GH/GHR axis in these tumors.

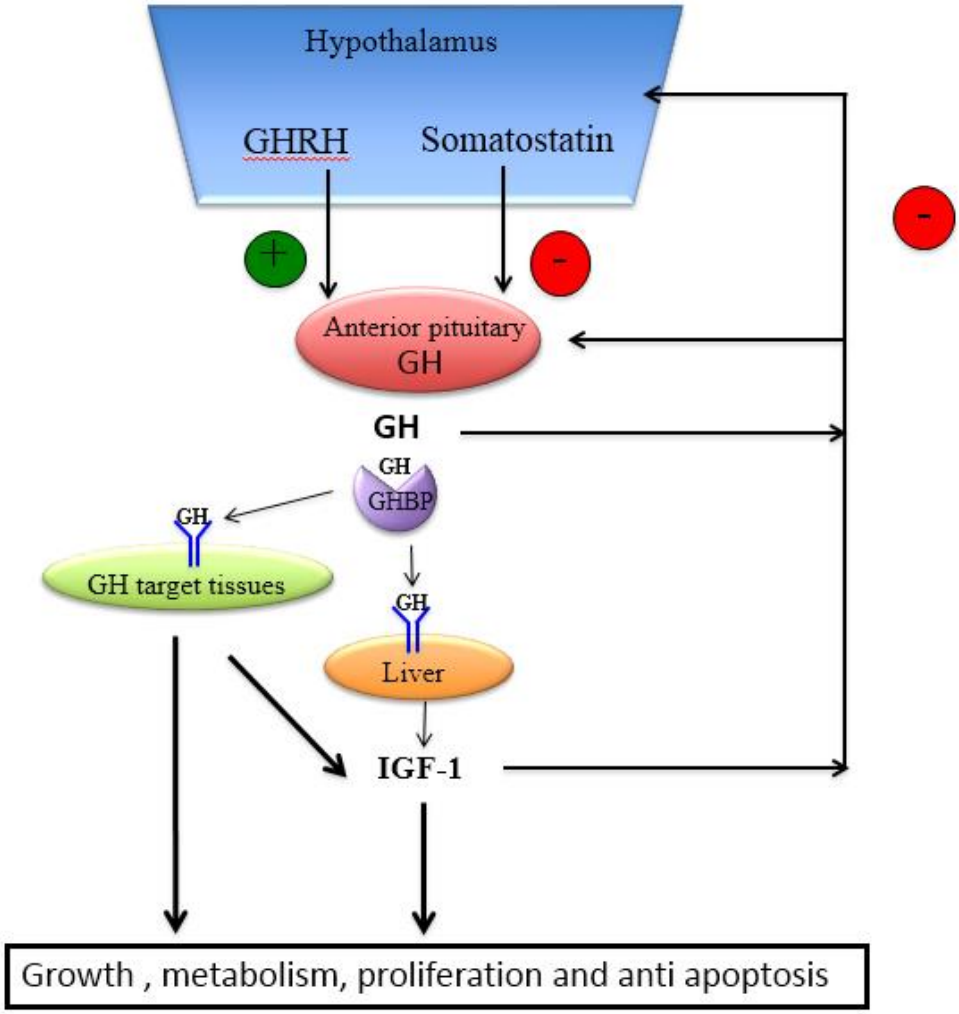

Figure 1.1: Regulation of Growth Hormone secretion

Hypothalamic GH Releasing Hormone (GHRH) and somatostatin regulate pituitary GH release into the blood where it circulates primarily bound to the GH binding protein (GHBP). GH binds and activates a dimer of its receptor (GHR) on target cells where it stimulates growth, metabolism, proliferation and anti-apoptosis, either directly or via the IGF-I system. Both GH and IGF-I inhibit GH secretion from the anterior pituitary. GH and IGF-I also work at the hypothalamic level, inducing the release of somatostatin, resulting in inhibition of GH secretion.

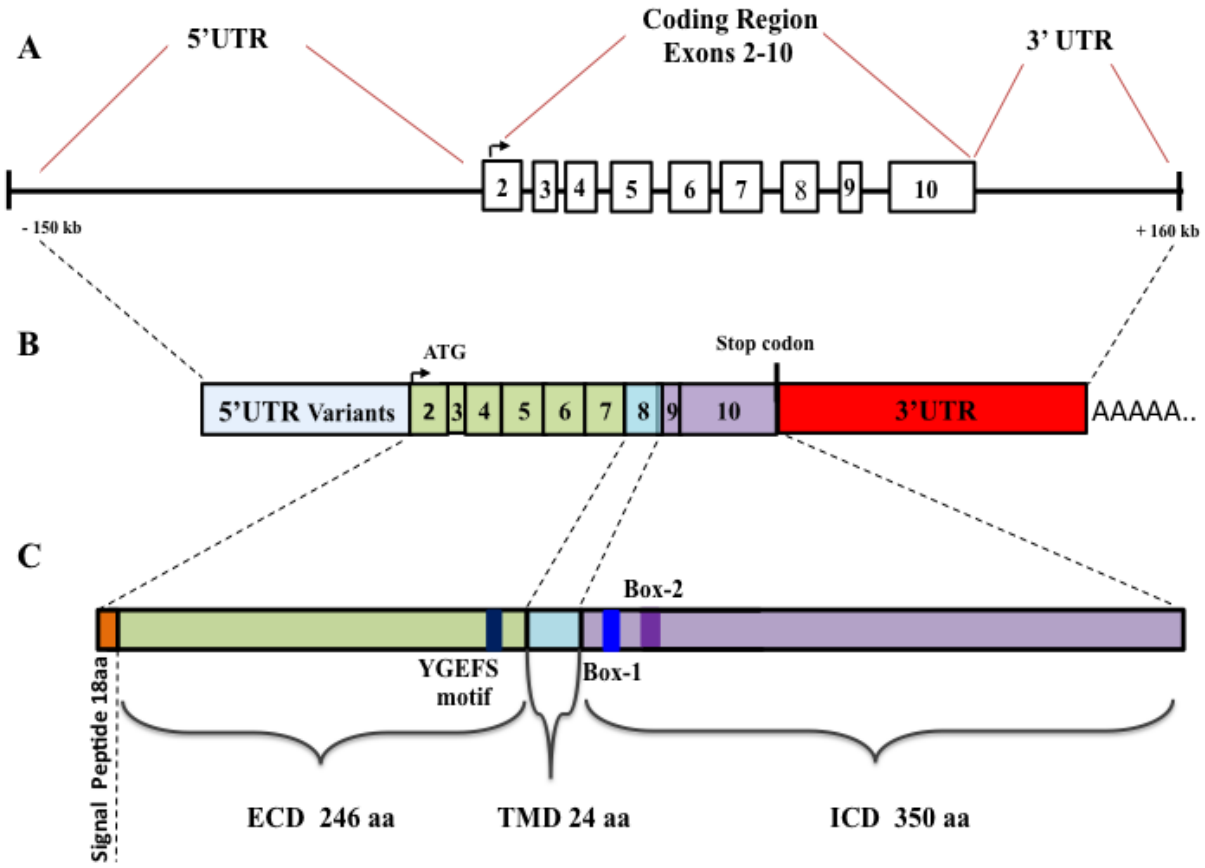

Figure 1.2: Schematic representation of the human *GHR* gene and protein

(A) The human *GHR* gene on chromosome 5p13.1-p12 which spans ~300kb. (B) *GHR* mRNA transcripts contain a non-coding 5'UTR with multiple splice variants giving rise to 14 different mRNAs and a ~2.5kb 3'UTR starting from the stop codon in exon 10. (C) The GHR peptide is comprised of an 18 amino acid signal peptide, 246 amino acid GH-binding extracellular domain (ECD), a transmembrane domain (TMD) of 24 amino acids, and a 350 amino acid intracellular signaling domain (ICD). The mature form of GHR after the signal peptide cleavage is 620 aa.

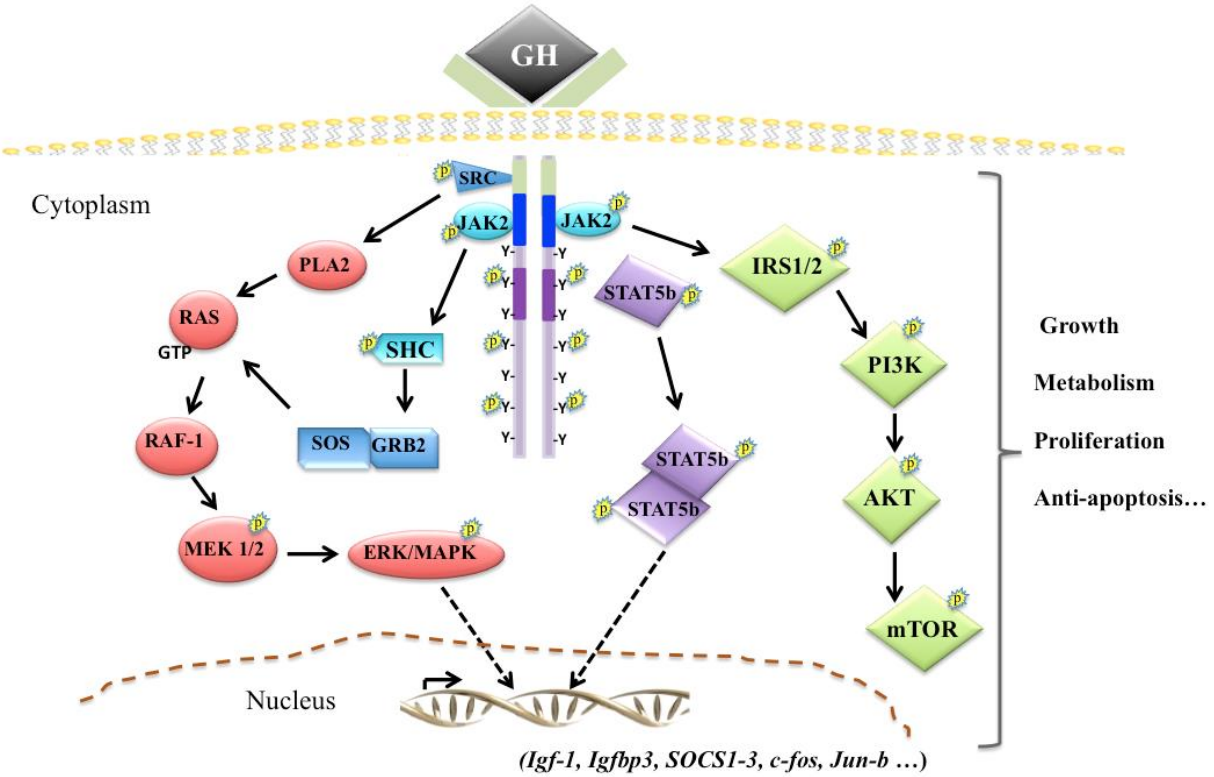

Figure 1.3: GH/GHR major intracellular signaling pathways

Schematic representation of the major GH/GHR signalling pathways. GH binds to a GHR dimer on the plasma membrane of target cells. This induces a conformational change in its intracellular domain, bringing the two JAK2s to a close proximity with each other, allowing for trans-phosphorylation of the JAK2s and activation of the GHR by phosphorylation of multiple tyrosine residues. JAK2 then, depending on the cell type, stimulates STATs, MAPK and/or PI3K/AKT pathways, which allows for divergence of the GH physiological effects. Members of the SRC family of kinases are also activated, leading to activation of the ERK/MAPK cascade.

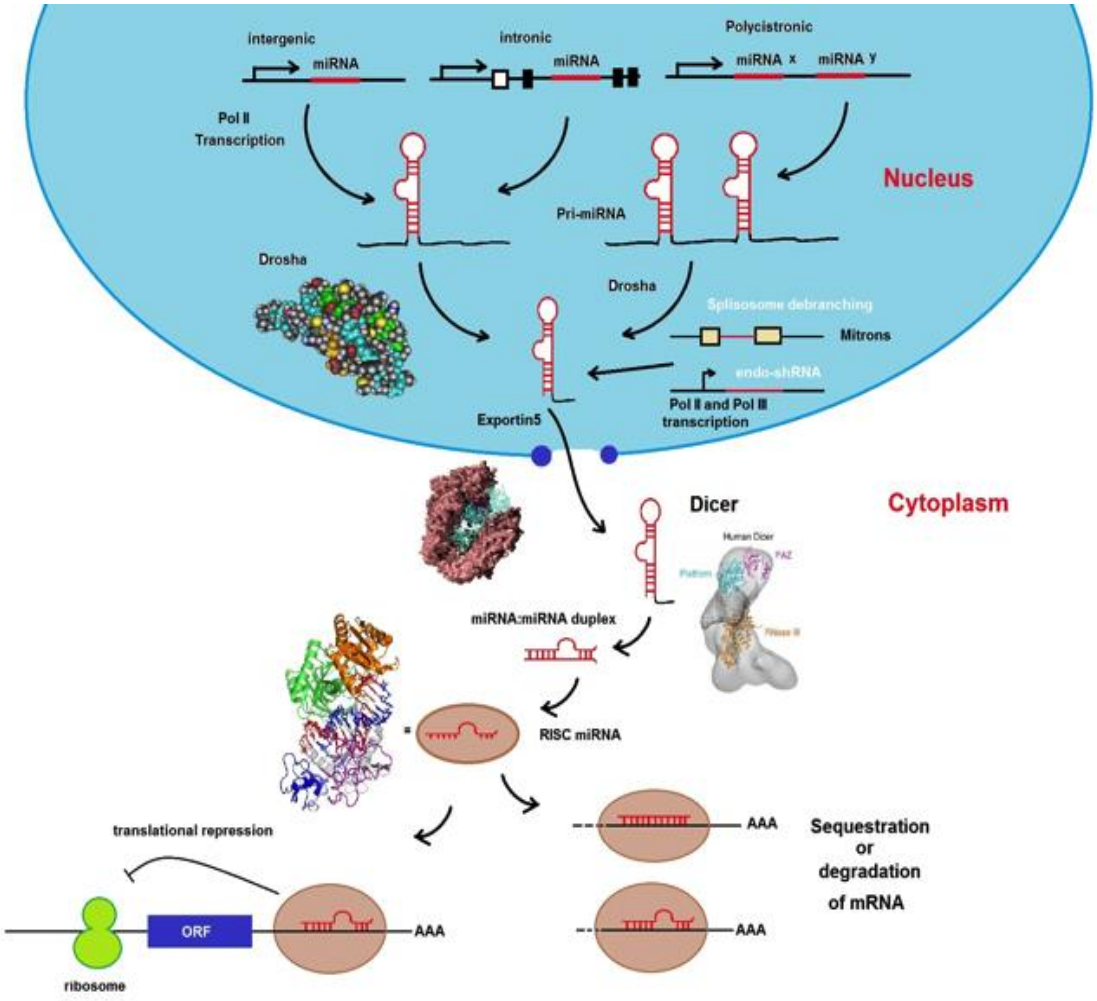

Figure 1.4: MiRNA biogenesis pathway

MiRNA genes in the nucleus are transcribed by Pol II resulting in a primary transcript which is then cleaved by an RNase III-like enzyme, Drosha, to a pre-miRNA. The pre-miRNA is transported to the cytoplasm by the exportin5 protein and further processed to form a mature miRNA. The mature miRNA enters the RISC complex, leading it to its target mRNA. This results in inhibition of protein translation and/or target mRNA sequestration and degradation. (Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews, Molecular Cell Biology. MicroRNAs in metabolism and metabolic disorders. Rottiers V, Näär AM , copyright April 2012)]

CHAPTER 2

REGULATION OF HUMAN GROWTH HORMONE RECEPTOR EXPRESSION BY MICRORNAS IN HEK293 CELLS

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Abbreviated Title: MiRNA Regulation of GHR in HEK293 cells

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2.1 Abstract

Human Growth Hormone (GH) binds to its receptor (GHR) on target cells and activates multiple intracellular pathways, leading to changes in gene expression, differentiation and metabolism. GHR deficiency is associated with growth and metabolic disorders while increased GHR expression has been reported in certain cancers, suggesting that the *GHR* gene requires tight controls. Several regulatory mechanisms have been found within its 5'UTR promoter and coding regions. However, the 3'UTR has not been previously examined. MicroRNAs (miRs) are small (19-22nt) noncoding RNAs that down-regulate gene expression mainly through targeting the 3'UTR of mRNAs and enhancing their degradation or inhibiting translation. In the present study, we investigated whether miRs regulate human *GHR* expression.

To define putative miR-binding sites in the *GHR* 3'UTR, we used multiple *in silico* prediction tools, analyzed conservation across species and the presence of parallel sites in GH/IGF axis-related genes, and searched for reports linking miRs to GHR-related physiological or pathophysiological activities. To test prioritized sites, we co-transfected a 'wildtype' Luc-*GHR* 3'UTR luciferase reporter vector as well as miR binding site mutants into HEK293 cells with miR mimics. Furthermore, we tested whether the miRs altered endogenous *GHR* mRNA and protein levels.

Our experiments have identified miR-129-5p, miR-142-3p, miR-202 and miR-16 as potent inhibitors of human *GHR* expression in HEK293 cells. A parallel decrease in *GHR* mRNA and protein suggests that they act primarily by degrading *GHR* mRNA. This study paves the way for the development of miR inhibitors of GHR expression as therapeutic agents in GH/GHR axis-related pathophysiologies.

2.2 Introduction

Human Growth Hormone is essential for normal musculoskeletal development in the child; in addition, it has important regulatory effects on protein, carbohydrate and lipid metabolism at all stages of life (Veldhuis *et al.*, 2005; Lichanska and Waters, 2008b). It functions by binding to a dimer of its high-affinity receptor (GHR) on target cells, leading to phosphorylation of associated JAK2 tyrosine kinases as well as the receptor itself. The subsequent activation of multiple intracellular signaling pathways culminates in the biological actions of GH: changes in gene expression, enhanced proliferation, blocking of apoptosis, differentiation and metabolic activity (Lichanska and Waters, 2008a).

The ability of GH to exert its biological effects is intimately linked to the number and functional status of GHRs in target tissues. Individuals with low GHR levels or a dysfunctional GHR do not respond normally to GH: they are not only short, they also have decreased bone mineral density and increased adiposity, with a greater risk of osteoporosis, lipid disorders and cardiovascular disease (Brooks and Waters, 2010). Persons with enhanced GH response, due to increased GH secretion or elevated functional GHR levels in target tissues, exhibit excessive growth and very abnormal metabolic activities, leading to an increased incidence of cardiomyopathies, hypertension, diabetes and several types of cancers: leukemia, breast, prostate, colorectal and gastric cancers (Savage *et al.*, 2006; Perry *et al.*, 2013). Thus, to prevent these major medical morbidities, GHR expression must be tightly regulated at every stage of life.

The human *GHR* gene is located at chromosome 5p13.1-p12, where it spans more than 300kb (Barton *et al.*, 1989; Godowski *et al.*, 1989; Goodyer *et al.*, 2001). It contains several noncoding 5'UTR exons with multiple splice variants that give rise to at least 14 different mRNAs, each with a unique 5'UTR but all of which code for the same protein due to splicing

into the same site upstream of the translation start site in the first coding exon, exon 2 (Pekhletsky *et al.*, 1992; Goodyer *et al.*, 2001; Orlovskii *et al.*, 2004; Wei *et al.*, 2006). Transcription of the *GHR* gene results in a ~4.5kb mRNA (Ross *et al.*, 1997). This *GHR* transcript is more than twice the minimum 1.9kb necessary to encode the 638 amino acid signal/receptor peptide molecule; the majority of the "excess" size is due to the presence of an approximately 2.5kb 3'UTR within the *GHR* mRNA (Edens and Talamantes, 1998).

There have been extensive studies of how *GHR* gene expression is regulated at its multiple 5'UTR promoters by our lab (Goodyer *et al.*, 2008; Wei *et al.*, 2009; Erman *et al.*, 2011; Kenth *et al.*, 2011) as well as others (Yu *et al.*, 1999). However, potential regulation at the 3'UTR has not been examined. 3′UTRs of mRNAs are well-known to be critical for the targeting of transcripts to specific subcellular compartments and for translational control (Andreassi and Riccio, 2009). More recently, microRNAs (miRNAs or miRs) have been shown to be post-transcriptional regulators of gene expression, acting mainly via the 3'UTRs of mRNAs (Chen *et al.*, 2004; Xie *et al.*, 2005). MiRNAs are naturally occurring, 19-22 nucleotides long, noncoding RNAs; the #2-8 nucleotides at the 5'end are known as the 'seed sequence' while the remaining nucleotides are the 'flanking region' (Stefani and Slack, 2008). MiRNAs function in the form of ribonucleoprotein complexes known as miRISCs (miRNA-induced silencing complexes) (Chekulaeva and Filipowicz, 2009). The miRNAs direct the miRISCs to sites primarily in the 3'UTR of target mRNAs, the specificity of which is defined by both the miR seed sequence and the flanking region. The complex subsequently inhibits protein synthesis by mRNA degradation and/or the arrest of mRNA translation (Chen *et al.*, 2004; Meister and Tuschl, 2004; Fabian *et al.*, 2010). Computational analysis indicates that more than 60% of protein coding genes may be directly modulated by miRNAs (Siomi and Siomi, 2010) and accumulating evidence indicates

that miRNAs play a central role in controlling a broad range of biological activities including embryonic development, cell proliferation, metabolic homeostasis and apoptosis (Chen *et al.*, 2004; Poy *et al.*, 2004; Cheng *et al.*, 2005; Karp and Ambros, 2005; Skalsky and Cullen, 2011; Bhaskaran and Mohan, 2013).

To understand whether miRs play a role in regulating human GHR expression, we have undertaken an analysis of miRNA effects on the *GHR* mRNA 3'UTR.

2.3 Materials and Methods

2.3.1 PCR amplification and cloning of the wild type *GHR* 3'UTR into the pmiR-Luciferase vector

The following primers were used to amplify a 2293bp sequence of the human GHR 3'UTR using 10ng of genomic DNA as a template: forward primer (SacI) 5'-cgagctcaattgactggggcaataacg-3' and reverse primer (MluI) 5'-cgacgcgtaaactgccagacacaactagtca-3'. A two step PCR assay was run using the Phusion High Fidelity DNA polymerase kit (Thermo Scientific, Burlington, ON) under the following conditions: an initial denaturation at 98°C for 2min followed by 5 cycles of 98°C for 10sec, 60°C for 30sec and 72°C for 150sec, then 30 cycles of 98°C for 10sec, 60°C for 4min and 72°C for 150sec. The PCR product was inserted into the SacI and MluI sites of the pmiR-Luc vector (Applied Biosystems/Invitrogen Life Sciences, Burlington, ON) downstream of the luciferase gene under the CMV promoter. Prior to its use, the vector insert was sequenced at the McGill Genome Centre (Montreal, QC) to ensure no mutations or deletions had occurred.

2.3.2 Mutagenesis

The Quick Change Lightning site-directed mutagenesis kit (Stratagene, Santa Clara, CA) was used to mutate the seed sequence at each of the miRNA binding sites under study. The pmiR-Luc-GHR 3'UTR vector was the template and overlapping pairs of primers were designed using the Stratagene Primer Design Program (www.genomics.agilent.com/primerDesignProgram.jsp) (**Table 2.1**). To ensure that the mutated sequences did not form a new miR binding site, they were checked using the TargetScan Custom 4.2 in silico program (www.targetscan.org/vert_42/seedmatch.html) (Lewis *et al.*, 2005).

2.3.3 Cell culture conditions and transfections

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM medium with 10% heat inactivated (HI) FBS, 25mM HEPES, 50U/ml penicillin and 1.6 mg/ml gentamycin. Cells were grown at 37°C with 5% CO2 in air. For GHR RNA and protein studies, HEK293 cells were plated at 2.5-3x10^5 cells/well in 6-well plates in duplicates. The following day, the media were replaced with antibiotic-free DMEM supplemented with 10% HI FBS and 500nM miR mimics (Dharmacon, division of Thermo Scientific) were transfected using the Dharmafect-1 Transfection Reagent (Dharmacon), according to the manufacturer's protocol. Total RNA was isolated for qRT-PCR analysis 24h post-transfection while proteins were extracted from a parallel set of cells after 48h. For luciferase reporter gene assays, cells were plated at 50x103 cells/well in 24-well plates. 50 ng of pmiR-Luc vector harboring the wild type GHR 3'UTR or a mutant version in addition to 1-100nM miR mimics or 50-100nM miRCURY LNA microRNA family inhibitors (Exiqon) were co-transfected into cells using the Dual-Transfecting reagent (Dharmacon). 50ng of pmiR-β-galactosidase vector were co-transfected into all wells to

normalize for transfection efficiency. Experiments were performed a minimum of three times with each mimic or inhibitor.

2.3.4 Luciferase and β-galactosidase measurements

For luciferase experiments, 48h post-transfection cells were washed once with cold PBS and harvested in 200µl of lysis buffer (0.05% NP40, 0.01 DDT in 0.1M Tris [pH8]) for 15min at room temperature. 10µl of lysate were dispensed into 96-well microtiter plates and luciferase activity was assayed after injecting 100µl per well of 1x luciferin solution (0.1 mM coenzyme A, 2.5mM ATP, 1x luciferin in 5mM tris-HCl [pH 7.9]). For the β-galactosidase assay, 10µl of the lysate were dispensed into 96-well microtiter plates, mixed with 100µl of Tropix Galacton-Star substrate diluted to 1x in Galacto-Star reaction buffer diluent (Applied Biosystems) and incubated for 1h at room temperature in the dark. Luciferase and β-galactosidase activities were measured in a bioluminometer (GloMax, Promega, Madison, WI). Assays were performed in triplicates. Data were initially normalized to β-galactosidase activity and then expressed as a ratio of luciferase activity over empty pmiR-Luc vector values.

2.3.5 RNA and miRNA extractions and cDNA synthesis

Total RNA including small RNA (miRNA) was extracted from HEK293 cells using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. RNA quantity was assessed using the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific) and RNA integrity was verified using the Bio-Rad Bioanalyzer (Bio-Rad, Mississauga, ON). For cDNA amplification assays, 1µg of RNA from each sample was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD) according to manufacturer's instructions.

2.3.6 Quantitative PCR (qPCR)

GHR mRNA levels were measured in triplicate using the Syber Green PCR assay (Qiagen), proprietary human *GHR* primers (Qiagen) and the LC480 qPCR instrument (Roche, Laval, QC). No template and no RT controls were included in each assay. Human *B_2 microglobulin* (*B_2M*) mRNA was used as an internal control to normalize between samples. The fold expression of the *GHR* mRNA in HEK293 cells treated with specific miRNA mimics relative to negative control treated samples were determined using the $2^{\wedge -\Delta\Delta Ct}$ method. The final data represent a minimum of three experiments.

2.3.7 miRNA expression in HEK293 cells

Total RNA (1µg/20µl reaction) from HEK293 cells was converted to cDNA using the miScript RT-PCR assay (Qiagen) following the manufacturer's instructions. The miRNA miScript Primer assays (Qiagen) were then used to quantify specific mature miRNAs present in the HEK293 cells. Specific mature miRNA primers were purchased from Qiagen (**Table 2.2**). The PCR reactions were performed on the Roche LightCycler 480 under the following conditions: 15min at 95°C, followed by 35 cycles of 15sec at 94°C, 30sec 58°C and 30sec at 70°C. *U6B* (small nuclear) RNA was used as an endogenous control to normalize between samples. No RT and water were used as negative controls for the PCR reactions while RNA from cells transfected with miRNA mimics was used as a positive control for undetected miRNAs. The data represent 4 independent experiments using different passages of the HEK293 cells, with each performed in triplicate and presented as percent of $2^{\wedge -(\Delta Ct)}$, where ΔC_t = (C_t of target miRNA) minus (C_t U6B).

2.3.8 Immunoblotting

Two days after transfection with miR mimics, HEK293 cells were washed with cold PBS and lysed in ice-cold RIPA buffer containing 50mM Tris HCl (pH 8), 150mM NaCl, 1%NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease and phosphatase inhibitors (Roche). Protein concentration was measured by DC protein assays (Bio-Rad). 25μg of total proteins were separated on 8% SDS-PAGE gels at 100V for 2.5h. The proteins were then electroblotted onto PVDF membranes (Thermo Scientific) at 300 mA for 1h. Membranes were blocked in 1% Tween Tris-buffered saline (TBST) with 5% skim milk for 4h at room temperature or overnight at 4°C. For GHR detection, blocked membranes were immunoblotted with the primary antibody (H300 rabbit anti-GHR; Santa Cruz Biochemicals, Santa Cruz, CA) diluted 1:1000 in blocking buffer overnight at 4°C. After extensive washing with TBST, the membranes were incubated with a horseradish peroxidase-labeled anti-rabbit secondary antibody (Cell Signaling, Beverly, MA), diluted 1:25000 in blocking buffer, for 1h at room temperature. Signals were detected using the ECL Prime Western Blotting Detection kit (GE Healthcare Amersham, division of Thermo Scientific) and exposure on HyBlot CL® Autoradiography films (Denville Scientific, South Plainfield, NJ). The membranes were subsequently stripped using 0.1% SDS in Tris with 0.7% β-mercaptoethanol and reprobed using a primary antibody against calnexin (1:5000; BD Biosciences, Franklin Lakes, NJ) and the HRP-labeled anti-mouse secondary antibody (1:5000; Cell Signaling), as a control for protein loading. Developed films were scanned and a densitometric analysis of bands was carried out using the Image J program (National Institutes of Health, Bethesda, MD) (Schneider *et al.*, 2012).

2.3.9 Statistical Analysis

The results were expressed as mean ± SE from at least 3 independent experiments. All statistical analyses were performed with ANOVA; for subsequent group comparisons, the Tukey-Kramer Multiple Comparisons test was used. Data were considered statistically significant when $p < 0.05$. All statistical tests were performed using GraphPad InSTAT software (GraphPad, San Diego, CA).

2.4 Results

2.4.1 Mapping the human *GHR* 3'UTR sequence for potential miR binding sites

To identify putative miR target sites within the 3'UTR of the human *GHR* gene, multiple miR target prediction programs were used: TargetScan 5.2 and later 6.2 (Lewis *et al* 2003, www.targetscan.org) (Lewis *et al.*, 2005), PicTar (Krek *et al* 2005, http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi) (Krek *et al.*, 2005), miRDB (Wang *et al* 2008, http://www.mirdb.org/miRDB/) (Wang, 2008; Wang and El Naqa, 2008), Microcosm Targets (Rehmsmeie *et al* 2004, http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) (Rehmsmeier *et al.*, 2004) and MiRanda (John *et al* 2004, http://www.microrna.org/microrna/home.do) (John *et al.*, 2004). The miR sites were prioritized based on the fact that they were identified by two or more *in silico* programs, the sites are conserved across several species, there are homologous sites in GH/IGF1 axis-related genes, and there are reports linking specific miRs to GHR-related physiological or pathophysiological activities **(Figure 2.1 and Table 2.3)**. The final set included sites for: miR-129-5p, which has

three potential binding sites (denoted by A, B and C at 488-494, 873-880 and 914-920, respectively, counting from the first nucleotide after the stop codon in exon 10); miR-142-3p (located at 583-590); miR-202, a member of the Let7 family that is only present in mammals, has two sites (A and B at 713-719 and 2245-2252, respectively); the miR-15/16 family (at 2092-2099); and miR-135 (at 2294-2300, the most 3' site).

2.4.2 Endogenous levels of miRs in HEK293 cells

The endogenous expression levels of these miRNAs were determined in the HEK293 cells by qPCR; the data are presented as fold of ΔC_t values normalized to the *U6B* gene **(Figure 2.2)**. All of the miR-16 family members studied were expressed in HEK293 cells with miR-16 and -15b the highest, miR-129-5p was at the limit of detection, while miR-142-3p, -202 and -135 were not detectable. Cells transfected with specific mimics were used as positive controls to validate the assays for the low and undetectable miRs.

2.4.3 *In vitro* testing for miR regulation of the human *GHR* 3'UTR

To determine whether the five miRs were able to regulate the *GHR* 3'UTR, we created a pmiR-Luc-*GHR* 3'UTR reporter vector by cloning 2293nt of the possible 2454nt, spanning all of the putative miR sites **(Figure 2.1)**. Reporter assays were performed using transient co-transfections of the reporter vector along with miR mimics in HEK293 cells. MiR-129-5p, -142-3p, -202 and -16 showed significant dose-related inhibitory effects on the pmiR-Luc-*GHR* 3'UTR vector, while other members of the miR-16 family (miR-15, -103, -107 and -503), Let7-b and -e as well as miR-135 had very low to no effects **(Figure 2.3)**. Because it was possible that the lower effect of miR-16 was due to the high endogenous levels of members of the miR-15/16

family present in HEK293 cells **(Figure 2.2)**, we carried out reporter assays using the wild-type pmiR-Luc-*GHR* 3'UTR vector co-transfected with 50nM miR inhibitors against the three most highly expressed members: miR-15a, -15b and -16. Indeed, results from four independent experiments showed a significant (59%, $p<0.05$) up-regulation of luciferase activity when normalized to the negative inhibitor control.

2.4.4 Determining the specificity of the miRNA sites in *GHR* 3'UTR

To confirm whether the miRs that had inhibitory effects in the reporter assays were acting through their predicted sites, reporter vectors with mutations of the binding sites were tested in parallel with the wild type vector. Details of each mutation and the mutagenesis primers are in **Table 2.1**. In the case of multiple binding sites for a miR (miR-129-5p and -202), simple and compound mutations were created.

MiR-142-3p and -16: Both of these miRs have a single predicted "perfect seed site' (8mer) in the *GHR* 3'UTR (Diana miRNA prediction tools [DIANA-microT-CDS web server v5.0]), **(Figure 2.1)**. Mutating the corresponding potential binding sites resulted in full recovery of the luciferase activity, confirming that the observed repression in luciferase activity with the wildtype vector was due to effects of these miRs at their predicted sites **(Figure 2.4A)**.

MiR-129-5p: This miR has three putative binding sites in the 3'UTR of the *GHR* gene **(Figure 2.1)**. To test the contribution of each site to the total effect observed by the reporter assay, single mutants (miR-129-5p/A, -129-5p/B, -129-5p/C), double mutants (miR-129-5p/AB, -129-5p/AC, -129-5p/BC) and the triple mutant (miR-129-5p/ABC) were created and assayed with miR-129-

5p mimics along with the wild type vector. These assays showed that both sites A and B are the major contributors to the repression observed in the wild type, while site C has a minor contribution. However, it took the triple mutant to fully relieve the effects of miR-129-5p repression **(Figures 2.4A, 2.4B and 2.4C)**. Interestingly, the strength of the individual sites correlates with predicted seed weighting: sites A and B are 7mers, while site C is a 6mer **(Figure 2.4B)**.

MiR-202: MiR-202 belongs to the Let-7 family of miRNA. It has two predicted binding sites in the *GHR* 3'UTR denoted by A and B **(Figure 2.1)**. Single miR 202/A and 202/B mutants in addition to the double mutant (202/AB) were created and co-transfected into HEK293 cells along with miR-202 mimics. Mutating site A abolished the repression completely, while mutating site B only relieved the repression by ~37% **(Figure 2.4D)**. Interestingly, mutating the two sites simultaneously (202/AB) resulted in a ~50% increase in expression above controls **(Figures 2.4A, 2.4D and 2.4E)**. These results suggest that site A is sufficient and necessary for the inhibition of *GHR* expression by miR-202 while site B acts cooperatively with site A to enhance its inhibitory effects on the 3'UTR. Again the predicted seed weighting for these two sites (A is an 8mer and B is a 7mer) correlates with the effectiveness of each site **(Figure 2.4D)**.

2.4.5 The effect of individual miRs on endogenous *GHR* mRNA and protein levels in HEK293 cells

In order to test if miR-129-5p, -142-3p, -202 and -16 affect the endogenous levels of *GHR* mRNA in HEK293 cells, we carried out qRT-PCR assays after transfections with miR mimics *vs.* negative control mimics. Similar to the results from the reporter assays, the aforementioned

miRs significantly down-regulated the *GHR* mRNA levels compared to controls, by ~60% for miR-129-5p, -142-3p and -202, and ~30% with miR-16 **(Figure 2.5)**.

Since the ultimate functional effect of miRs on gene expression is the alteration of protein levels, we measured the effect of the same miRs on the endogenous GHR protein levels in HEK293 cells by western blotting. An antibody against the intracellular domain of GHR was used that recognizes a band of ~100-120kD, corresponding to the mature GHR. Densitometry results from the western blots were normalized to calnexin (representative blot in **Figure 2.6A**) and to negative control-treated cells. Composite results of four independent experiments showed statistically significant decreases in GHR protein levels of ~ 40% when treated with miR-129-5p and miR-142-3p and ~30% when treated with either miR-202 or miR-16 (**Figure 2.6B**).

2.5 Discussion

In this study, we identified a new mechanism by which human GHR expression in HEK293 cells is regulated: miRNAs. Our results show miR-129-5p, miR-142-3p, miR-202 and miR-16 to be significant inhibitors, acting through specific *GHR* 3'UTR sites. The parallel decrease in *GHR* mRNA and protein by these four miRs suggests that the primary mechanism by which they are acting is by degrading the *GHR* mRNA.

Initially, in order to prioritize a list of miRs that might regulate human GHR expression, we searched the literature for prior evidence of strong direct or inverse correlations between the expression of GHR and that of individual miRs in both physiological and pathophysiological contexts **(Table 2.3)**. While we were able to find a few links in relation to cell proliferation, apoptosis, angiogenesis and diurnal rhythms, the majority of miR papers have used high-throughput microarrays to identify cancer-specific miR 'fingerprints' in a spectrum of different

cancers, including breast (Iorio *et al.*, 2005), hepatocellular (Murakami *et al.*, 2006; Wang *et al.*, 2008), lung (Takamizawa *et al.*, 2004; Hayashita *et al.*, 2005; Yanaihara *et al.*, 2006), colon (Michael *et al.*, 2003; Schetter *et al.*, 2008) and gastric carcinomas (Petrocca *et al.*, 2008). Results from these studies have shown that the miRNA expression profile in malignant cells is significantly different from that in healthy control cells, suggesting that alterations in miRNA genes play a critical role in the pathophysiology of many human cancers (Aqeilan *et al.*, 2010). Thus, much of the discussion will focus on the potential relevance of our findings to GHR in cancer.

MiR-129-5p: Bandrés *et al.* identified miR-129-5p (a known inducer of G1 phase arrest (Wu *et al.*, 2010)) as one of the miRNAs significantly down-regulated in 15 colorectal cancer cell lines compared to a human normal colon cell line (Bandres *et al.*, 2006). In a more recent study, Karaayvaz *et al*. found that the level of miR-129-5p expression was significantly decreased in samples from 22 human colorectal tumor tissues compared to their paired normal controls (Karaayvaz *et al.*, 2013). Moreover, the same group studied a set of 61 colorectal specimens from different stages of the disease and found that the expression of miR-129-5p was significantly reduced in patients with stage 3 and 4 of the disease compared with normal or adenoma tissues (Karaayvaz *et al.*, 2013). Although these miR studies did not examine GHR levels, multiple studies have reported the over-expression of GHR in human colorectal cancer tissues and cell lines, suggesting a role for GHR in the progression of colorectal carcinomas (Dagnaes-Hansen *et al.*, 2004; Yang *et al.*, 2004; Yang *et al.*, 2005). Our data suggest that the inverse relationship between miR-129-5p and GHR in colorectal cancers should be studied further, to determine if, in fact, overexpression of this miR in colorectal cells would down-

regulate GHR expression and halt or reverse progression of these tumors. Interestingly, in addition to *GHR*, miR-129-5p has putative binding sites on the *STAT5b* and *IGF-I* mRNA 3'UTRs, suggesting that it could affect multiple steps in the GHR signaling pathway **(Table 2.3)**.

MiR-142-3p: In a recent study by Shen *et al.*, miR-142-3p was shown to be down-regulated in colon cancer tissues; in addition, its overexpression acted as a tumor suppressor, inhibiting the growth of colorectal cancer cell lines (Shen *et al.*, 2013). As mentioned earlier, GHR has been strongly implicated in the progression of colorectal cancers. Our finding of a robust inhibitory effect of not only miR-129-5p but also miR-142-3p on GHR expression indicates that the interactions between both of these miRs and GHR in colorectal tumors now need to be verified and explored experimentally.

MiR-142-3p has also been shown to be involved in regulation of the circadian clock by targeting mRNAs of the clock gene, BMAL1. The expression level of miR-142-3p oscillates in a circadian rhythm in mouse NIH3T3 cells (Tan *et al.*, 2012) and the suprachiasmatic nuclei (Shende *et al.*, 2013). In addition, expression of the *MIR-142* gene is controlled by CLOCK/BMAL1 heterodimers, suggesting a negative feedback loop (Shende *et al.*, 2013). Studies by Itoh *et al*. and Zvonic *et al*. have reported that *GHR* mRNA levels in murine liver, skeletal muscle and calvarial bone are expressed in a diurnal manner (Itoh *et al.*, 2004; Zvonic *et al.*, 2007). While we have data demonstrating that the circadian regulators, D-site binding protein (DBP) and E4BP4, can regulate *GHR* expression through sites in at least two of the human *GHR* 5'UTR promoters (Kenth and Goodyer, unpublished), our present data suggest that it would be interesting to investigate whether miR-142-3p also plays a role in controlling the normal physiological circadian expression of GHR. Although miR-142-3p expression was not detected

in HEK293 cells under our experimental conditions, it was induced upon subjecting the cells to serum starvation (data not shown), suggesting a role for miR-142-3p in cell cycle regulation.

MiR-202: Expression of miR-202 (a known repressor of MYCN (Buechner *et al.*, 2011)) has been reported to be down-regulated in gastric cancer, both in cell lines and tumor tissues (Zhao *et al.*, 2013). Moreover, overexpression of miR-202 in two gastric cancer cell lines, MNK-28 and BGC-823, markedly suppressed cell proliferation and induced cell apoptosis both *in vitro* and *in vivo* (Zhao *et al.*, 2013). As with colorectal tumors, multiple studies have reported GHR over-expression in gastric carcinomas: experimental results indicate that GHR expression is significantly higher in primary gastric adenocarcinoma than in normal gastric mucosa and is significantly correlated with tumor differentiation and tumor grade (Lincoln *et al.*, 2007; Lin *et al.*, 2011; Yang *et al.*, 2012; Ran *et al.*, 2013). Our present data, showing a significant inhibitory effect of miR-202 on *GHR* mRNA and protein expression, indicate that further investigations of miR-202/*GHR* gene interactions in gastric cancers are in order. As observed for miR-129-5p, miR-202 may also target several members of the GH/GHR axis (*GHRHR, GHR, IGF-I, IGF-IR, CIS*) and, thus, could affect the axis at multiple levels **(Table 2.3)**.

MiR-16: MiR-16 belongs to the miR-15/107 group of miRNA genes. While all vertebrates express miR-15a, -15b, -16, -103 and -107, mammals alone are known to also express miR-195, -424, -497, -503 and -646. MiR-15a and -16 were the first miRNAs linked to a cancer, chronic lymphocytic leukemia (CLL) (Calin *et al.*, 2004), although their decreased expression was subsequently reported in multiple adenomas as well as prostrate and breast cancers (Dong *et al.*, 2001; Bhattacharya *et al.*, 2009; Rivas *et al.*, 2012). The *MIR15a/16-1* genes are located at chr

13q14.3, within the *DLEU2* (*Deleted in Leukemia 2*) locus. Deletions at 13q14.3 have been reported in more than 50% of CLL cases (Calin *et al.*, 2002). MiR-15a and -16 are thought to exert their tumor suppressor functions by targeting multiple oncogenes, including the anti-apoptotic protein, BCL2, which is overexpressed in the malignant B-cells of CLL (Bonci *et al.*, 2008; Lerner *et al.*, 2009; Klein *et al.*, 2010).

GHR is expressed widely in human lymphocytes, including T cells, B cells and many leukemic cell lines (Hattori *et al.*, 2001). Studies by Jeay *et al*. have suggested a dual effect of the GH/GHR axis on survival and proliferation of the pro-B Ba/F3 cell line. When Ba/F3 cells were transfected with a *GHR* expression vector and treated with GH, a GH signaling pathway involving the activation of Nuclear Factor (NF)-κB and the expression of the anti-apoptotic protein, Bcl-2, was shown to mediate the anti-apoptotic effect of GH (Jeay *et al.*, 2000). Increased concentrations of GH were shown to promote Ba/F3 cell proliferation, which was dependent upon the activation of PI-3 kinase, leading to the induction of the proto-oncogene, c-myc (Dinerstein *et al.*, 1995; Baixeras *et al.*, 2001). Interestingly, Calin *et al.* assessed the molecular basis for miR-15a/16 tumor suppression in leukemia by undertaking a high-throughput profiling of genes modulated by overexpressing miR-15a/16-1 in a leukemic cell line model, MEG-01(Calin *et al.*, 2008). The genome-wide transcriptome analysis showed a down-regulation of 3307 genes, including the *GHR* gene, which was significantly down regulated above the two fold threshold (p=0.018).

In addition to miR-129-5p and miR-202, miR-16 may also target several genes in the GH/GHR axis: *GHR, IGF1, IGF1R and IGF2R* **(Table 2.3)**. Also of interest is that the promoter of *miR-15b/16-2* genes has been shown to be negatively regulated by STAT5, a downstream effector of GH-activated GHR, resulting in decreased mature miR-15 and miR-16 expression

levels (Li *et al.*, 2010). These data suggest that there may be a coordinated regulation allowing for communication between the level of GH/GHR axis activity and miR15/16 expression via STAT5.

While our results suggest that four miRNAs may be important regulators of GHR expression, this does not exclude a role for other miRs. The limitation in miRNA prediction of true targets is due to the complex nature of the miRNA: mRNA interactions; as a result, the available prediction programs have many false positive results. Indeed, we found that other "predicted" miRNAs, such as miR-135, members of the Let7 family and other members of the miR-16 family (103, 107 and 503) **(Figure 2.1, Table 2.3, data not shown)** did not have any significant effect on the *GHR* 3'UTR luciferase activity. Thus, other determinants (e.g. flanking regions) likely contribute to the effectiveness of the "predicted" miRs. In addition, we used HEK293 cells as a model to test our hypothesis and, therefore, cannot rule out that these or additional miR binding sites will be active in a cell- or tissue-specific fashion. Developmental stages constitute another layer of miRNA gene regulation complexity whereby these four or other miRs could play a temporal role in the regulation of *GHR* expression. Finally, according to a new miRNA prediction tool (DIANA-microT-CDS web server v5.0; http:www.microrna.gr/microT-CDS) (Reczko *et al.*, 2012; Paraskevopoulou *et al.*, 2013), there are additional miRNA binding sites for the miR-16 and Let7 families, including miR-202, in the coding region sequence of exon 10 of the human *GHR* mRNA, which might play a role. Although miRs have been shown to have effects at sites outside the 3'UTR region of mRNAs (Reczko *et al.*, 2012), the exon 10 sites may be more important in regulating expression of the truncated forms of GHR: these represent up to 10% of total GHR and occur due to alternative splicing in exon 9, converting the entire exon 10 to 3'UTR status (Ross *et al.*, 1997).

In summary, our experiments have demonstrated, for the first time, significant inhibitory effects of miRNAs on human *GHR* mRNA and protein expression in HEK293 cells. This study paves the way for the development of miR inhibitors of GHR expression as therapeutic agents in GH/GHR axis-related pathophysiologies, including cancers.

Table 2.1: **Primers used to introduce mutations into the miR seed sequences in the wild type GHR-3'UTR luciferase vector.** The miRNA seed sequences are in bold, mutated nucleotides are italicized and deleted seed sites are underlined. S = sense and AS = antisense) and the numbers in brackets represent the position of the primer in the 3'UTR GHR sequence.

Primer	Primer Sequence (5' to 3')
129-5p.A (488-494) **S**	ctagaggtgagaaatttaaactataagcaagaag***cg*****a*****a*****t*****a*****t***tagtttggatatgtaaaacatttattttgacata
129-5p.A (488-494) **AS**	tatgtcaaaataaatgttttacatatccaaacta***a*****t*****a*****t*****t*****c*****g***cttcttgcttatagtttaaatttctcacctctag
129-5p.B (873-880) **S**	gatcaacttaccaggcaccaaaagaagtaaaa***cg*****a*****t*****a*****a*****t***agaaaacctttcttcaccaaatc
129-5p.B (873-880) **AS**	gatttggtgaagaaaggttttct***a*****t****tat*****c*****g***ttttacttcttttggtgcctggtaagttgatc
129-5p.C (914-920) **S**	acctttcttcaccaaatcttggttgatgc***gtt*****a*****t*****a**aaaatacatgctaagagaagtagaaatc
129-5p.C (914-920) **AS**	gatttctacttctcttagcatgtattttt***a*****t*****a*****a*****c***gcatcaaccaagatttggtgaagaaaggt
142-3p (583-590) **S**	taataatttagacttcaagcatggctattttatatt**a*****g*****a*****c*****a*****a*****t*****a**cactgtgtactgcagttg
142-3p (583-590) **AS**	caactgcagtacacagtg***t*****a*****t*****t*****g*****t*****c*****t**aatataaaatagccatgcttgaagtctaaattatta
202-A (713-719) **S**	caaactcgtttttacaaagccctttt**<u>tatacctc</u>**cccagactccttcaa
202-A (713-719) **AS**	ttgaaggagtctggg**<u>gaggtat</u>**aaaagggctttgtaaaaaacgagtttg
202-B (2245-2252) **S**	acatttctatagccaaaaatagctaa**a*****t*****t*****g*****c*****g*****c**aatcagtctcagaatgtcattttgg
202-B (2245-2252) **AS**	ccaaaatgacattctgagactgatt**g*****c*****g*****c*****a*****a*****t**ttagctatttttggctatagaaatgt
15/16 (2092-2099) **S**	ttgtaatagatgtttgatagattttctgctactt**<u>tgctgcta</u>**ggttttctccaagagc
15/16 (2092-2099) **AS**	gctcttggagaaaacc**<u>tagcagca</u>**agtagcagaaaatctatcaaacatctattac

Table 2.2: **Qiagen primers used to amplify the endogenous mature miRNAs in HEK293 cells.**

MiRNA	Mature miRNA Sequence 5’	Product Number
miR-15a	UAGCAGCACAUAAUGGUUUGUG	MS00003178
miR-15b	UAGCAGCACAUCAUGGUUUACA	MS00008792
miR-16	UAGCAGCACGUAAAUAUUGGCG	MS00031493
miR-195	UAGCAGCACAGAAAUAUUGGC	MS00003703
miR-424	CAGCAGCAAUUCAUGUUUUGAA	MS0000418
miR-497	CAGCAGCACACUGUGGUUUGU	MS00004361
miR-107	AGCAGCAUUGUACAGGGCUAUCA	MS00031255
miR-503	UAGCAGCGGGAACAGUUCUGCAG	MS00033838
miR-129-5p	CUUUUUGCGGUCUGGGCUUGC	MS00006643
miR-142-3p	CAUAAAGUAGAAAGCACUACU	MS00006671
miR-202	AGAGGUAUAGGGCAUGGGAA	MS0000903
miR-135	UAUGGCUUUUUAUUCCUAUGUGA	MS00008624

Table 2.3: Priority list of miRNAs to test for a functional role at the *GHR* 3'UTR based on # positive miRNA programs, if conserved, if sites in 3'UTRs of87678 related genes, as well as physiological and pathophysiological links.

Same seed sequence miRNAs	Number of prediction programs*	Parallel presence of miR binding sites in 3'UTRs of related genes	Physiological links	Pathophysiological links
15/16/103/ 107/195/424/ 497/503	4	*GHR* (human to chicken), *IGF-I, IGF-IR, IGF-2R*	• Ubiquitous expression in human tissues (Finnerty *et al.*, 2010) • Opposite effect of GH: pro-apoptotic (Le *et al.*, 2010; Rovira *et al.*, 2010), anti-proliferative (Aqeilan *et al.*, 2010; Biggar and Storey, 2011; Zhou and Wang, 2011), anti-angiogenic (Sun *et al.*, 2013) • 15/16 levels in bone inverse correlation with bone mineral density (BMD) (Reppe *et al.*, 2010) • 15/103 decrease adipocyte number but increase size (Andersen *et al.*, 2010; Trajkovski *et al.*, 2011) • 16: high expression in GI crypt cells + diurnal rhythm opposite to GHR (Balakrishnan *et al.*, 2010) • 15/16 ↓IGF1R in B lymphocytes (Klein *et al.*, 2010) • STAT5 regulates *15/16* expression (Li *et al.*, 2010) • ↑15/103/107 in livers of ob/ob mice (Trajkovski *et al.*, 2011)	• 15/16↓ in prostate cancer (Bonci *et al.*, 2008), parallel to ↑GHR + IGF-IR • 15/16/103↓ in prostate cancer (Porkka *et al.*, 2007; Porkka *et al.*, 2011), parallel to ↑GHR • 15/16↓ in somatotrope adenomas (Bottoni *et al.*, 2005) • 15/16/195↓ in hepatic and colon cancers (Chen *et al.*, 2004; Huang and He, 2011; Wang *et al.*, 2012), parallel to ↑GHR • 107↓ in lung cancer (Takahashi *et al.*, 2009) • 195/497↓ in breast cancer (Lehmann *et al.*, 2010; Li *et al.*, 2011) • 15/103↓ in AML (Dixon-McIver *et al.*, 2008)

202/(Let7 family)	4 (2 sites)	*GHR* (all species examined), *IGF-I, IGF-IR, GHRHR, CIS*	• Expression in human tissues (Dong *et al.*, 2010) • Strong negative regulator of MYCN (Buechner *et al.*, 2011)	• Let7↓ in hepatic, prostate and breast cancers (Dong *et al.*, 2001; Huang and He, 2011), parallel to ↑ GHR • 202↓ in lung cancer (Nymark *et al.*, 2011)
129-5p	3 (3 sites)	*GHR* (primate, dog, mouse), *IGF-I, STAT5b, ETS1*	• Induces G (1) phase arrest (Tsai *et al.*, 2011)	↓ 129-5p in gastric, CNS and colorectal cancers (Bandres *et al.*, 2009; Birks *et al.*, 2011; Tsai *et al.*, 2011)
142-3p	2	*GHR* (primate) *SH2B*1, *GFI-1*	• Role in circadian rhythms (Tan *et al.*, 2012)	↓ 142-3p in colon cancer (Shen *et al.*, 2013), parallel to ↑ GHR
135	2	*GHR* (primate, rat, opossum, chicken), *JAK2, STAT6*	• Inhibitory effects on osteoblastogenesis (Li *et al.*, 2008) • Down regulation of JAK2 (Navarro *et al.*, 2009)	↑ in colon cancer (Nagel *et al.*, 2008; Koga *et al.*, 2010) • Inhibitory effect on cell invasion (Golubovskaya *et al.*, 2013)

* MiRNA prediction programs used in analysis: TargetScan, PicTar, Miranda (micrRNA.org), miRDB, Microcosm.

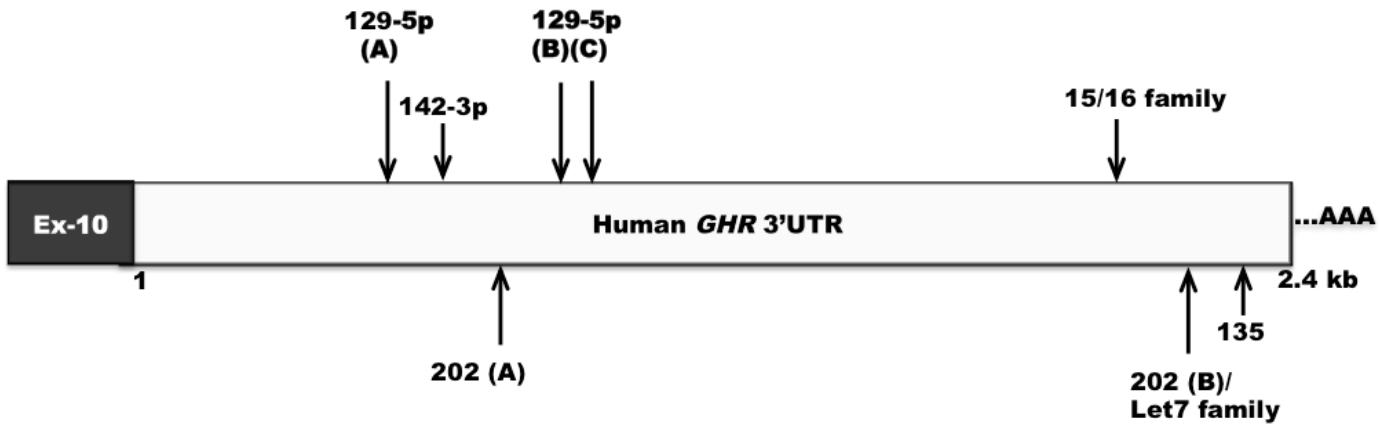


Figure 2.1: Putative miR binding sites in the human *GHR* 3'UTR

Schematic representation of the 3'UTR sequence from the human *GHR* mRNA indicating the potential miRNA binding sites within the sequence tested during the present study. A, B and C denote multiple binding sites for the same miR.

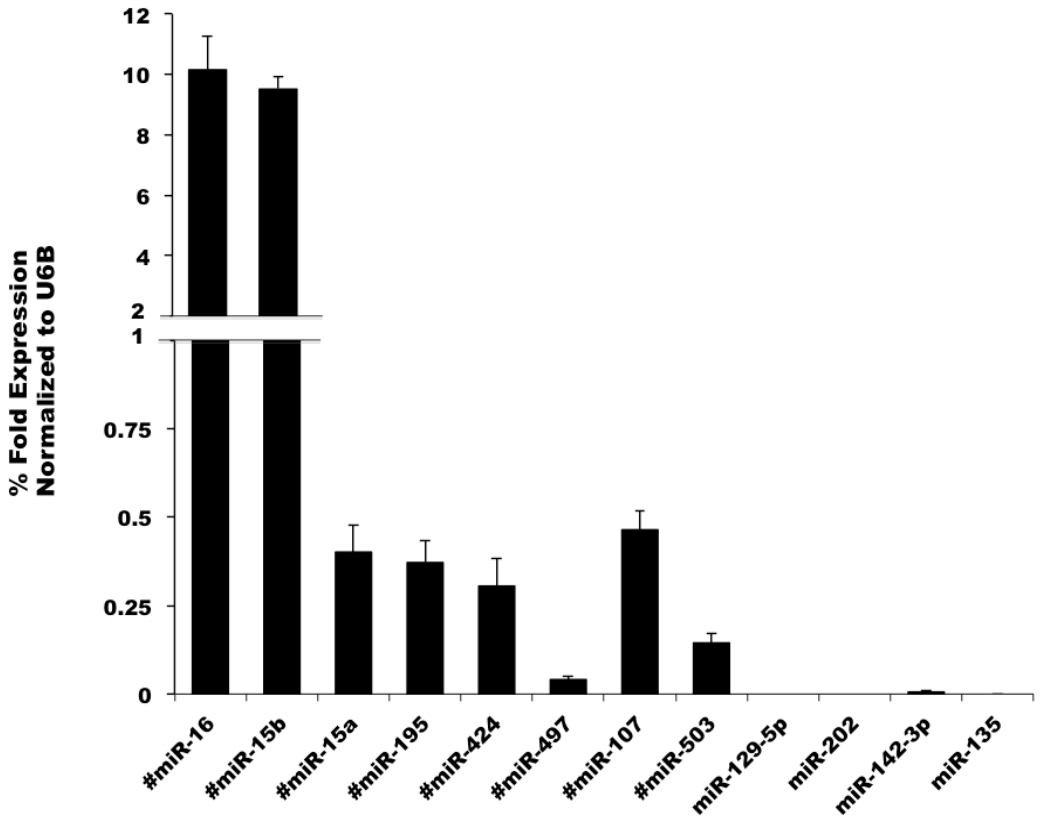


Figure 2.2: Endogenous levels of miRs in HEK293 cells

QPCR analysis of the expression of mature miRNAs in HEK293 cells; data are normalized to *U6B*. Results are the means of four independent experiments of different passages of HEK293 cells ± SE. # denotes members of the miR-15/-16 family.

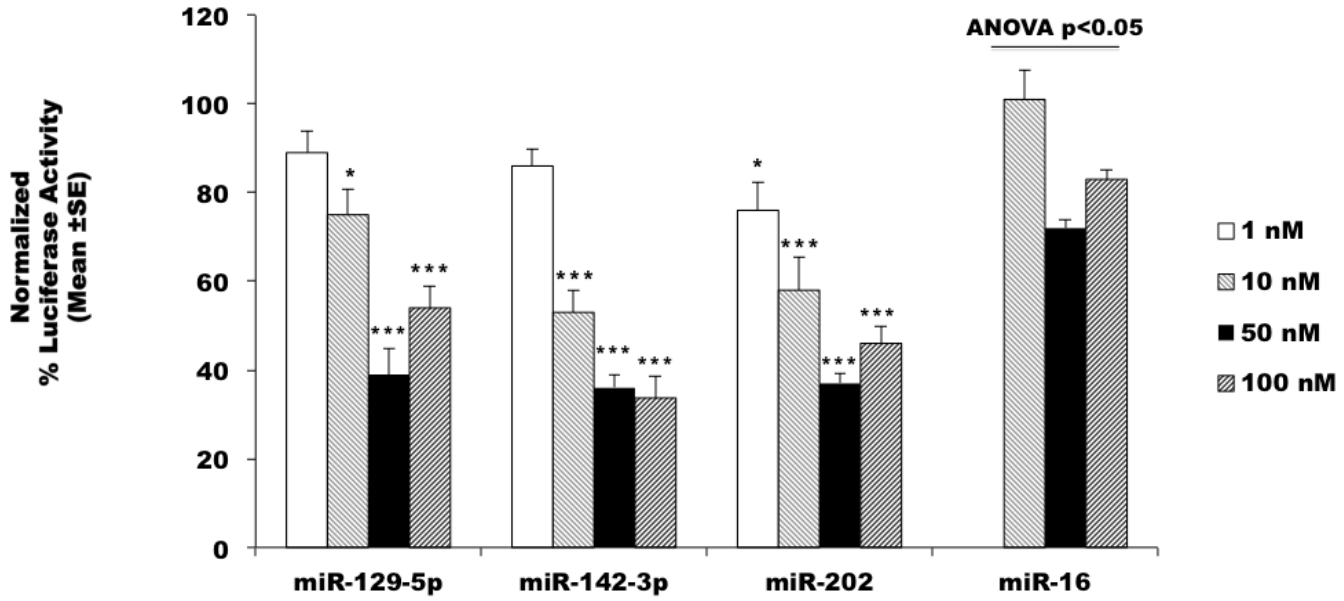
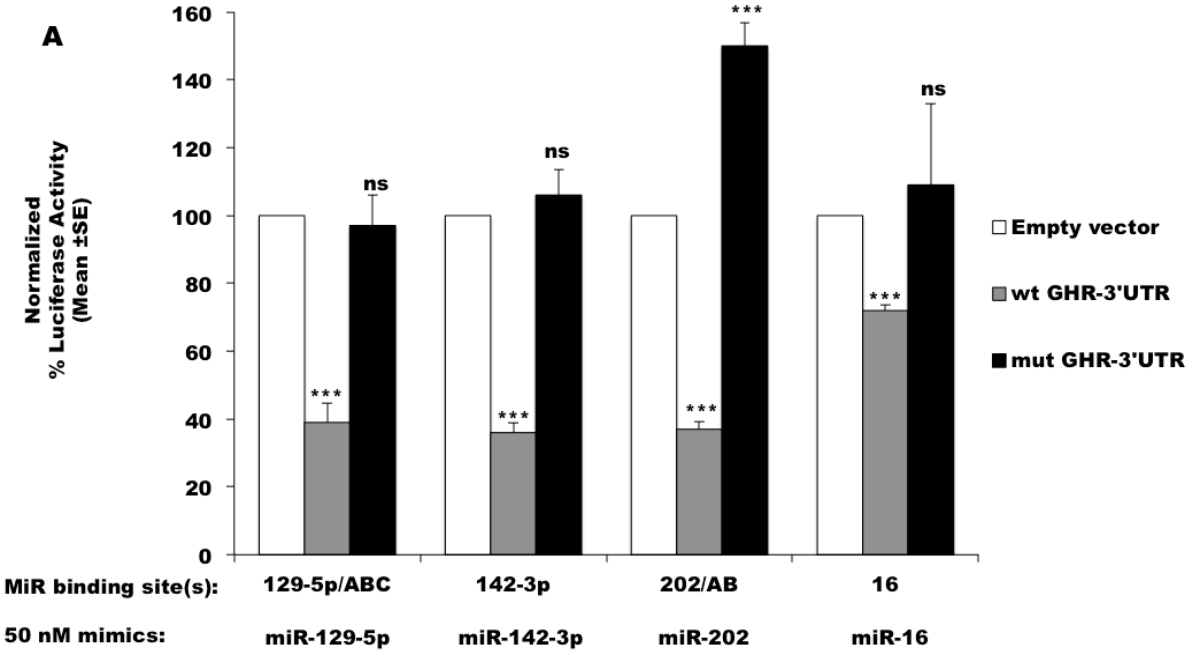
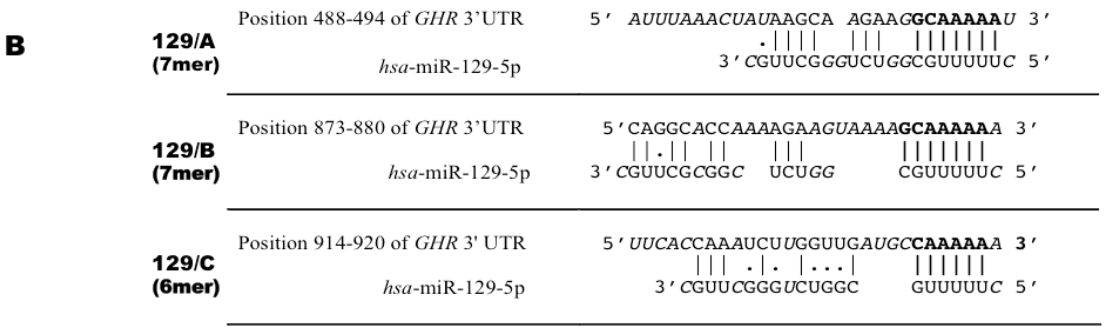
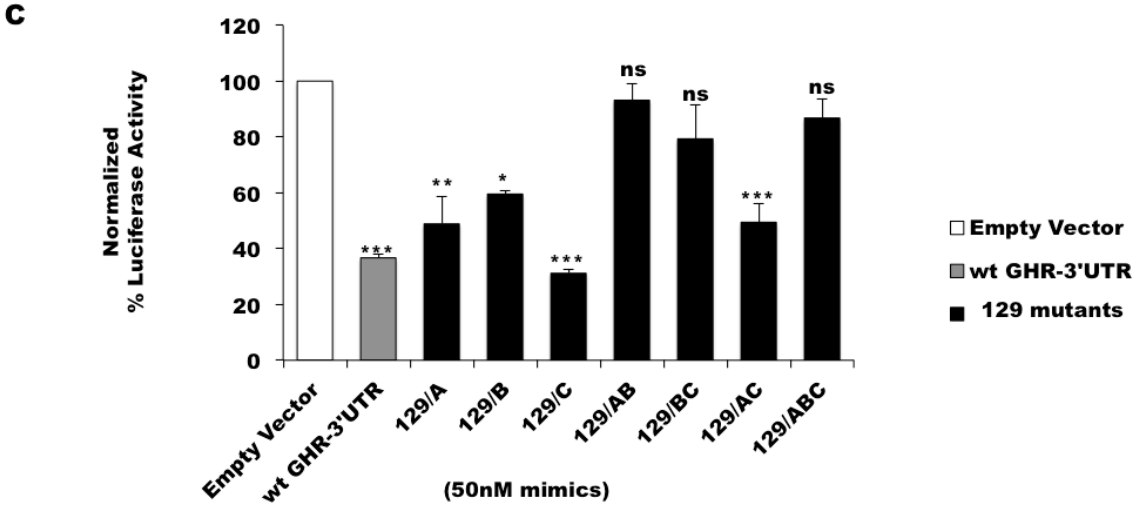


Figure 2.3: Effect of miRs on the Luc-*GHR* 3'UTR reporter vector

Effects of 1-100nM miR-129-5p, miR-142-3p and miR-202, and 10-100nM miR-16 on the pmiR-Luc-*GHR* 3'UTR reporter vector were examined in HEK293 cells. Data are presented as mean ± SE (n=5). All results were normalized to the empty Luc reporter vector and to negative mimic control treatment at each mimic dose followed by the calculation of luciferase/β-galactosidase ratios. Results for the first three miRs were highly significant by both ANOVA and Tukey group comparison tests, while the miR-16 data were significant for ANOVA ($p<0.05$) but not Tukey group comparisons. This is likely due to the relatively high level of endogenous miR-16 in the HEK293 cells. *$p\leq 0.05$ and ***$p\leq 0.001$ vs. negative mimics.


A
Normalized
% Luciferase Activity
(Mean ±SE)
160
140
120
100
80
60
40
20
0
***
ns
***
ns
***
***
ns
***
Empty vector
wt GHR-3'UTR
mut GHR-3'UTR
MiR binding site(s):
129-5p/ABC
142-3p
202/AB
16
50 nM mimics:
miR-129-5p
miR-142-3p
miR-202
miR-16


B
129/A
(7mer)
Position 488-494 of GHR 3'UTR
5′ AUUUAAACUAUAAGCA AGAAGGCAAAAAU 3′
hsa-miR-129-5p
3′CGUUCGGGUCUGGCGUUUUUC 5′
129/B
(7mer)
Position 873-880 of GHR 3'UTR
5′CAGGCACCAAAAGAAGUAAAAGCAAAAAA 3′
hsa-miR-129-5p
3′CGUUCGCGGC UCUGG CGUUUUUC 5′
129/C
(6mer)
Position 914-920 of GHR 3' UTR
5′UUCACCAAAUCUUGGUUGAUGCCAAAAAA 3′
hsa-miR-129-5p
3′CGUUCGGGUCUGGC GUUUUUC 5′


C
Normalized
% Luciferase Activity
120
100
80
60
40
20
0
***
**
*
***
ns
ns
***
ns
Empty Vector
wt GHR-3'UTR
129 mutants
Empty Vector
wt GHR-3'UTR
129/A
129/B
129/C
129/AB
129/BC
129/AC
129/ABC
(50nM mimics)

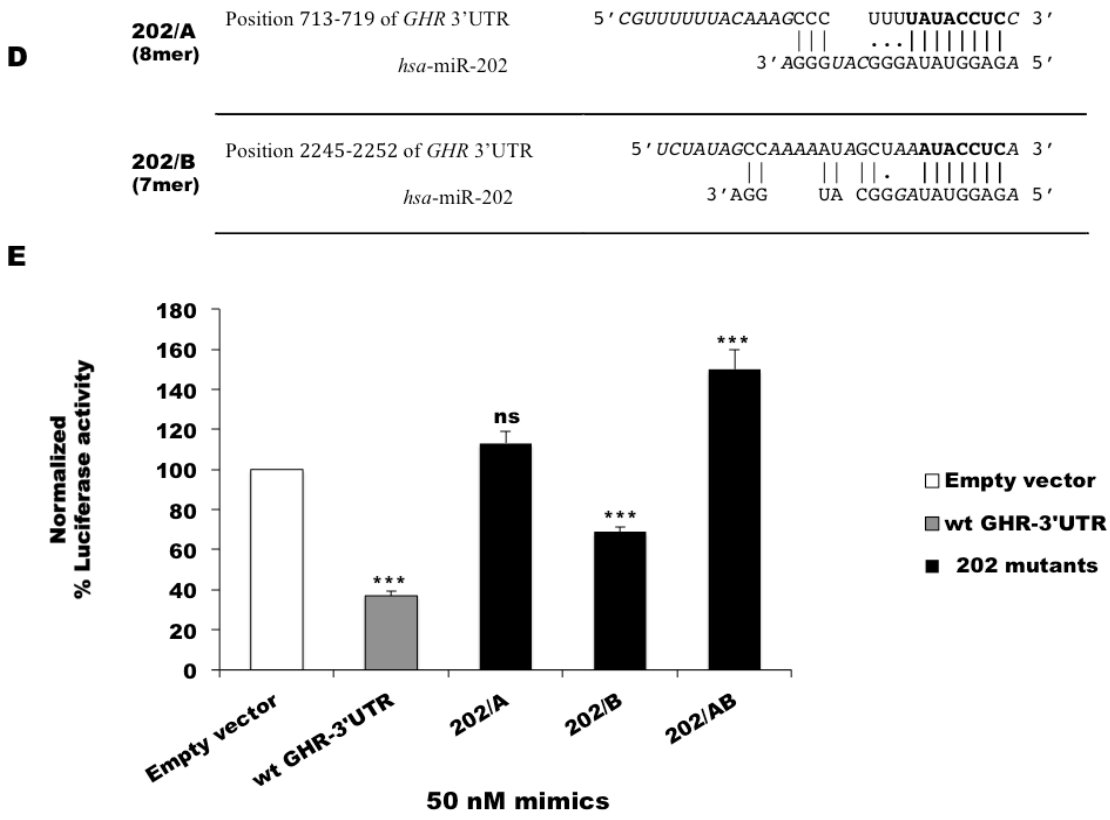


Figure 2.4: Reporter assays of wild type and mutant *GHR* 3'UTR vectors

(A) Effects of 50nM miR mimics on wild type *vs.* mutant *GHR* 3'UTR reporter vectors. Results are presented as mean ± SE (n=4 for all but miR-202/AB n=5). **(B)** Schematic representation of hybridisation between miR-129-5p and its three putative *GHR* 3'UTR binding sites (A, B and C): the seed sites are bold, non-complimentary nucleotides are italicized, lines represent Watson-Crick complementarity and dots represent U to G Wobble. **(C)** Reporter assays of wild type *GHR* 3'UTR and the predicted miR-129-5p single, double and triple mutants. Data are presented as mean ± SE (n=4). **(D)** Schematic representation of hybridization between miR-202 and its two putative binding sites in the *GHR* 3'UTR (A and B): the seed sites are bold, non-complimentary nucleotides are italicized, lines represent Watson-Crick complementarity and dots represent U to G Wobble. **(E)** Normalized data from luciferase assays of single and double mutants of miR-202 binding sites compared to the wild type vector. Results are presented as mean ± SE (n=5). ns = not significant, **p≤ 0.01, ***p≤ 0.001.

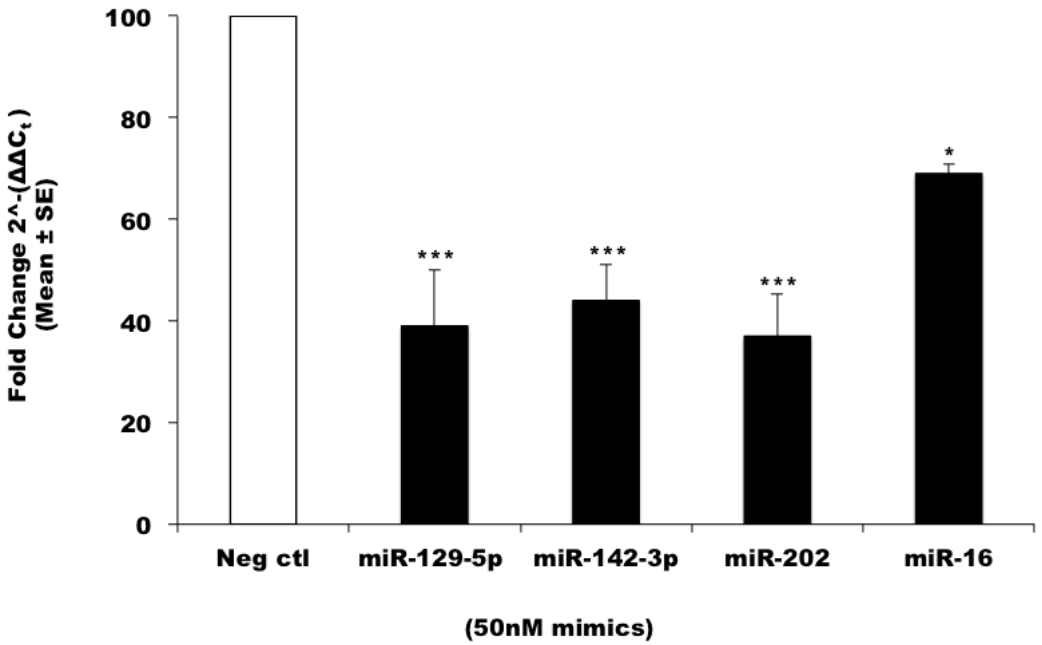


Figure 2.5: Studies of the effect of miRs on endogenous *GHR* at the mRNA level

Expression of human total *GHR* mRNA in HEK293 cells transfected with 50nM of miR-129-5p, miR-142-3p, miR-202 and miR-16 mimics. The data are presented as mean ± SE of three individual experiments for all but miR-142-3p (n=5); duplicates were assayed within each experiment. Statistically significant differences between the control and miR-treated cells were found for miR-129-5p, miR-142-3p, miR-202 (***$p \leq 0.001$) and miR-16 (*$p \leq 0.05$).

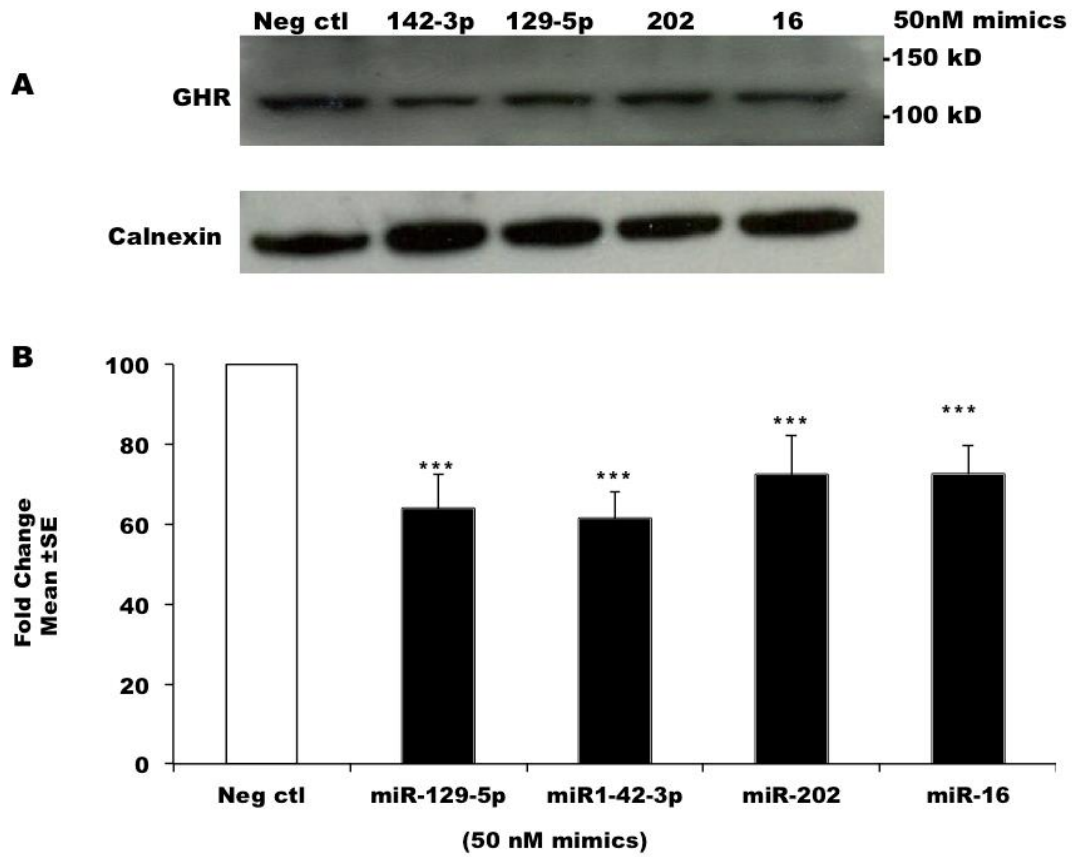


Figure 2.6: Studies of the effect of miRs on endogenous GHR at the protein level

(A) Representative Western blot of GHR and calnexin protein levels in HEK293 cells following transfections with 50nM of miR-129-5p, miR-142-3p, miR-202 and miR-16 mimics. **(B)** Composite results for n=4 individual experiments (M $\pm$ SE) showing statistically significant differences between negative control and miR-treated cells: ***$p \leq 0.001$ for all treatments for both ANOVA and Tukey group comparison tests.

CHAPTER 3

FINAL DISCUSSION AND FUTURE DIRECTIONS

3.1 GHR-Regulating MiRNAs

The effects of GH in its target tissues are dependent on the number and functional state of its receptor, GHR. Because the GH/GHR axis is involved in many significant cellular processes, including proliferation, differentiation, anti-apoptosis and cell migration, tight regulation of GHR expression is critical. Indeed, it is well recognized that faulty regulatory systems that lead to abnormal under- or over-expression of GHR result in multiple disorders in both humans and animals (e.g. severe short stature, obesity, cancer progression).

Although extensive studies of the human *GHR* gene have revealed complex transcriptional regulation within its 5'UTR (detailed in Chapter 1), regulatory effects via its 3'UTR have been overlooked. In the present study, we examined the possible role of miRNAs and identified that, in HEK293 cells, four miRNAs have potent posttranscriptional effects: miR-129-5p, miR-142-3p, miR-202 and miR-16. The ability of these miRNAs to regulate GHR expression was shown to be due to binding to specific sequences within the 3′UTR of *GHR* mRNA: site-directed mutagenesis of each site abolished the repressive effects **(Figure 2.5)**. Multiple *in vitro* and *in vivo* studies of the possible mechanisms by which miRNAs exert their suppressive effects on protein expression have revealed that they either block mRNA translation and/or promote mRNA deadenylation and subsequent degradation (Lee *et al.*, 1993; Wightman *et al.*, 1993; Olsen and Ambros, 1999; Chen *et al.*, 2004). Data from our experiments showed that endogenous expression of both *GHR* mRNA and protein was decreased in HEK293 cells transfected with miR-129-5p, miR-142-3p, miR-202 or miR-16 mimics, suggesting that the primary mechanism

by which these miRNAs act is by degrading the *GHR* mRNA **(Figures 2.6 A and B)**.

We used HEK293 cells as a model in which to test our hypothesis that miRNAs can regulate GHR expression. These cells have been widely used by others as well as ourselves for GHR studies because of their abundant GHR expression, their ability to activate known GH/GHR downstream signaling pathways and the fact that they are easy to grow and transfect. That being said, we cannot rule out that miRNA:GHR mRNA functional interactions are cell- or tissue-specific. Thus, our next experiments should be to examine whether the four miRs have effects in a variety of different cell types. In addition, to address if any of these miRs play a role in the pathogenesis of GHR expression in certain disorders, miRNA expression profiling of the tissues or cells where GHR under- or over-expression has been detected *vs.* their normal counterparts should be performed. Since miRNAs generally repress GHR expression, we expect to see an inverse correlation for levels of each miRNA *vs*. GHR. Subsequent experiments, introducing miR mimics or inhibitors into the cells, will help to determine the relative ability of the miRs to regulate GHR expression and may provide evidence for the efficacy of using miR therapy to reverse a GHR-related pathophysiological state.

MiR-129-5p*:* MiR-129 in humans is transcribed from two loci, *hsa-miR-129-1* and *hsa-miR-129-2*. *Hsa-miR-129-1* is located on chromosome 7, close to a fragile site region (FRA7H) that is frequently deleted in many cancers (Calin *et al.*, 2004), while *hsa-miR-129-2* is found at 11p11.2, embedded in a CpG island. Methylation of the *hsa-miR-129-2* CpG island has been frequently observed in colorectal cancer cell lines and in primary colorectal cancer tumors but not in normal colonic mucosa. In a study by Bandres et al., *hsa-miR-129-2* was found to be epigenetically regulated by aberrant DNA methylation and histone modification in colorectal

cancer cell lines (Bandres *et al.*, 2009). Furthermore, expression of hsa-miR-129 was upregulated when a demethylating agent or an inhibitor of histone deacetylases was used.

These findings suggest a tumor suppressor role for miR-129. Indeed miR-129-5p was found by multiple studies to be down-regulated in both colorectal cancer cell lines and tumor tissues from patients with colorectal cancers compared to controls (Bandres *et al.*, 2006; Karaayvaz *et al.*, 2013). In addition, Karaayvaz *et al.* found that the expression of miR-129-5p was significantly reduced in patients with stage 3 and 4 of the disease compared with normal or adenoma tissues. Recently miR-129 has been found to directly inhibit the expression of BCL2 leading to activation of the intrinsic apoptosis pathway; BCL2 is an antiapoptotic gene involved in an evolutionarily conserved intrinsic apoptosis pathway (Karaayvaz *et al.*, 2013).

Because a major role of the GH/GHR axis is also to block apoptosis, it is logical to test if regulation of GHR by miR-129, or more precisely up-regulation of GHR due to decreased expression of miR-129, contributes to the progression of colorectal cancer. Treatment of colorectal cell lines (e.g. HTC29, Caco-2, SW40) with miR-129 mimics should be followed by Real time PCR and western blotting analyses to determine the endogenous effects of miR-129 on both *GHR* mRNA and protein. We expect to observe down-regulation of GHR expression by miR-129 and, in parallel, a decrease in GH activation of its signaling pathways that promote proliferation and anti-apoptosis.

Furthermore, miR-129-5p has putative binding sites in the *STAT5b*, *IGF-I* and *IGF-IR* mRNA 3'UTRs, suggesting that it could affect multiple steps in the GHR signaling pathway. Thus, it would be interesting also to look at the effects of miR-129 mimics on the expression of these mRNAs and proteins, in both normal and tumor cells.

MiR-142-3p*:* *Hsa-miR-142-3p* is transcribed from a single gene located at chromosome 17q23, in a region that has been found to be susceptible to DNA alterations. For example, it is close to two translocation breakpoint regions (8 and 17), which cause an aggressive form of B cell acute leukemia. In addition, hsa-miR-142-3p is located near the FRA17B site, a target for HPV16 integration in breast and cervical cancers (Calin *et al.*, 2004). These findings suggest a critical tumor suppressor role for miR-142-3p in multiple tumor types.

In chapter 2, we demonstrated the regulation of GHR expression by miR-142-3p in HEK293 cells. Interestingly, according to multiple miRNA prediction programs, miR-142-3p is also a potential regulator of the prolactin receptor (PRLR). The human PRLR is structurally similar to the human GHR, especially within the ICD (Goffin and Kelly, 1997), and both receptors activate similar major signaling pathways, most commonly the JAK2/STAT cascade. Prolactin is a pituitary hormone mainly involved in milk protein production and, along with GH, it has an important role in development of the mammary gland (Wennbo *et al.*, 1997).

Several lines of evidence also support the involvement of both PRL and GH in breast cancer, including increased expression of their receptors (Peirce *et al.*, 2001; Clevenger, 2003; Meng *et al.*, 2004). In addition, GH has been found to bind and stimulate signaling pathways through homo- or heterodimers of GHR and PRLR in T47D breast cancer cell lines (Xu *et al.*, 2011; Xu *et al.*, 2013). Since the expression of both GHR and PRLR has been associated strongly with breast cancer, it would be extremely beneficial to determine if miR-142-3p, as a common miRNA repressor, might be useful as a therapy. To do this, several experiments need to be carried out. Initially, the regulation of PRLR by miR-142-3p needs to be validated experimentally by luciferase reporter assays, site-directed mutagenesis and mimic experiments, similar to what we have done for GHR in HEK293 cells. Second, expression profiling of miR-

142-3p, GHR and PRLR should be done in several breast cancer and control cell lines (e.g.: T47D, MCF7 and MCF10a) in order to determine the background profile for each *in vitro* cell model. Third, cultures of these mammary cells should be treated with miR-142-3p mimics and the expression of both GHR and PRLR at mRNA and protein levels monitored (by qPCR and western blots). Fourth, to determine the repressive effect of miR-142-3p on the oncogenic roles of both receptors, T47D and/or MCF7 cells treated with miR-142-3p mimics should be examined for changes in proliferation, apoptosis and migration compared to negative mimic treated control cells. Finally to assess, *in vivo*, the pathophysiological role of miR-142-3p on GHR and PRLR overexpression, miR-142-3p, GHR and PRLR expression should be investigated in variety of breast cancer tissues paired with normal control tissues from breast cancer patients. The goal would be to determine if specific subgroups would benefit most from the development of a new miRNA- based therapy.

In addition, miR-142-3p has been shown to be down-regulated in colon cancer tissues and, when overexpressed in multiple colorectal cancer cell lines, it acted as a tumor suppressor, inhibiting proliferation (Shen *et al.,* 2013). As mentioned earlier, GHR has been strongly implicated in the progression of colorectal cancer. Thus, the relationship between miR-142-3p and GHR expression in colorectal cancer needs to be verified and explored experimentally in experiments similar to the ones mentioned above, using colorectal cancer cell lines and tissues. Interestingly, as in breast cancer, higher expression of both GHR and PRLR has been observed in colon cancer tissues and cell lines compared to normal controls (Otte *et al.*, 2003; Neradugomma *et al.*, 2013). Therefore, there may be a beneficial role for miR-142-3p in multiple tumor types that overexpress GHR and/or PRLR.

MiR-202: The *miR-202* gene is located within chromosome 10q26: deletion of this region has been associated with endometrial and brain tumors. MiR-202 expression has also been reported to be down-regulated in many cancers, including breast cancer (Iorio *et al.*, 2005; Schrauder *et al.*, 2012), cervical cancer (Zhang *et al.*, 2009), colorectal cancer (Ng *et al.*, 2009), gastric cancer (Zhao *et al.*, 2013) and follicular lymphoma (Hoffman *et al.*, 2013). As the down-regulation of miR-202 occurs in multiple malignancies, the focus of future studies of its role in regulating GHR expression in cancers will depend on initial miRNA and GHR expression profiling in a spectrum of cancer cell lines representing the above mentioned cancers. As observed for miR-129-5p, miR-202 is predicted to target several members of the GH/GHR axis (*GHRHR, GHR, IGF-I, IGF-IR and CIS*). Further experiments looking at the effects miR-202 mimics on these targets could have the potential to validate a valuable new therapeutic tool to tightly regulate the activity of the GH/GHR/IGF-I axis at multiple levels. In addition, to determine the mechanism(s) behind the fairly common down-regulation of miR-202 in several types of cancers, genetic, epigenetic and molecular studies of the *miR-202* gene promoter should be carried out.

MiR-16: MiR-16 belongs to the miR-15/16 miRNA family, which contains 18 different miRNA members with strong influences on human health and disease (Finnerty *et al.*, 2010). MiR-16 is ubiquitously expressed at high levels in normal human tissues and is produced from two different gene clusters: *miR-15a/16-1* and *miR-15b/16-2* (Finnerty *et al.*, 2010). The *miR-15a/16-1* cluster is located at chr13q14.3 and is hosted by the noncoding *DLEU2* gene in a region that is deleted in more than 50% of CLL patiants (Calin *et al.*, 2002) and in 65% of prostate cancer cases (Brookman-Amissah *et al.*, 2007). On the other hand, the *miR-15b/16-2* cluster is located on chromosome 3 and hosted by the *SMC4* gene; the SMC4 protein forms a complex with other

proteins and regulates cell chromosome stability, assembly and segregation (Fujioka *et al.*, 2002; Hirano, 2002; Losada and Hirano, 2005). This cluster may also have roles in blood pressure, diabetes, prostate cancer and other diseases (Yue and Tigyi, 2010).

The mature miR-15a and -16 are thought to exert their tumor suppressor functions by targeting multiple oncogenes, including the anti-apoptotic protein, BCL2, which is overexpressed in the malignant B-cells of CLL (Bonci *et al.*, 2008; Lerner *et al.*, 2009; Klein *et al.*, 2010), cyclin D1, a well-known promoter of cell proliferation, and WNT3A, an activator of pivotal oncogenic pathways such as AKT and MAPK (Almeida *et al.*, 2005).

In a genome-wide transcriptome study of the molecular basis for miR-15a/16 tumor suppression in leukemia, the leukemic cell model, MEG-01, was modulated to highly express both miR-15a and -16 (Calin *et al.*, 2008). Interestingly, the transcriptome analysis showed a down-regulation of 3307 genes, including *GHR* ($p=0.018$). Our experiments showed the suppressive effect of miR-16 mimics on GHR expression in HEK293 cells. GHR is widely expressed in human lymphocytes, including T cells, B cells and several leukemic cell lines (Hattori *et al.*, 2001). In addition, experimental evidence has suggested a role for GH/GHR in survival and proliferation of the pro-B Ba/F3 cell line (Dinerstein *et al.*, 1995; Jeay *et al.*, 2000; Baixeras *et al.*, 2001). Based on these observations, we propose a role for miR-16 in regulating GHR expression in lymphocytes and that, escape from this regulation, by decreased expression or deletion of *miR-15a/16-1*, may lead to enhanced activation of the GH/GHR axis, with subsequent progression of the leukemic state. To further investigate the role of miR-16 on GHR expression in leukemia, experiments using MEG-01 or Ba/F3 cell lines and treatments with miR-16 mimics should be carried out, followed by assessments *GHR* mRNA and protein levels, proliferation and apoptosis.

It is also intriguing that the *miR-15b/16-2* gene promoter has been found to be negatively regulated by STAT5, a downstream effector of GH-activated GHR, resulting in decreased miR-15b and miR-16 expression levels (Li *et al.*, 2010). These data suggest that there may be a coordinated regulation allowing for communication between the level of GH/GHR axis activity and miR-15a/16 expression via STAT5.

3.2 Alternative MiRNA Regulatory Mechanisms

According to a new miRNA prediction tool (DIANA-microT-CDS web server v5.o; http:www.microrna.gr/microT-CDS) (Reczko *et al.*, 2012; Paraskevopoulou *et al.*, 2013), there are additional miRNA binding sites for the miR-16 and Let7 families, including miR-202, in the coding region sequence of exon 10 of the human *GHR* mRNA, which might play a role in miRNA regulation of GHR expression. Although miRs have been shown to have effects at sites outside the 3'UTR region of mRNAs (Reczko *et al.*, 2012), the exon 10 sites may be more important in regulating expression of the truncated forms of GHR: these represent up to 10% of total GHR and occur due to alternative splicing in exon 9, converting exon 10 to 3'UTR status (Ross *et al.*, 1997).

On the other hand, the usage of multiple polyadenylation signals within the 3'UTR of genes can result in changes in the length of the 3'UTR, which may result in loss of miRNA binding sites, causing escape from the specific miRNA regulation. To check if this mechanism is used as a mode of regulating the *GHR* gene, 3'RACE experiments should be carried out to determine the length and sequences of *GHR* 3'UTRs from cDNAs prepared from different cell lines as well as tissues originating from different organs at various developmental stages and from GHR-related

disorders. These investigations may help to uncover another layer of *GHR* gene regulation important for understanding changes in GH effectiveness due to tissue specificity, developmental stages and GHR-related pathophysiological processes.

In summary, our study has demonstrated significant inhibitory effects of miR-129-5p, miR-142-3p, miR-202 and miR-16 on human *GHR* mRNA and protein expression in HEK293 cells. These findings lay the foundation for unraveling novel mechanisms behind the roles of GHR in multiple pathophysiologies. In addition, they may permit the development of new therapeutic approaches to oppose the tumorigenic role of the GH/GHR axis in specific cancers.

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