Growth Hormone Receptor in Melanoma: A Unique Approach to Therapy

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Growth Hormone Receptor in Melanoma: A Unique Approach to Therapy

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ABSTRACT

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Human melanomas are one of the most therapy resistant types of cancer, expressing a repertoire of mechanisms for drug resistance. Intracellular signaling networks used by the melanoma cells for active proliferation, migration, invasion, therapy resistance and metastases happen to strongly overlap with those regulated by the human growth hormone (hGH). Indeed consistently high levels of GH receptor (GHR) expression have been observed in almost all melanoma cell lines in the NCI60 human cancer panel. There are no comprehensive studies investigating the effects of GH action or GHR antagonism on the GH-responsive intracellular signaling pathways and downstream effects in human melanoma. Here we report for the first time, a detailed analysis of the effect of siRNA mediated GHR knock-down (KD) and effect of hGH on the intracellular signaling and downstream phenotypes in human melanoma cells.

We report the existence of an autocrine loop of hGH-GHR in the aforesaid human melanoma cell lines, changes observed in relevant intracellular signaling pathways, RNA transcript level changes observed in multiple key modulators of the GH/GHR axis. Phosphorylation states of the JAK2, SRC, STAT1, 3, and 5, p44/42-MAPK, AKT and mTOR increased in a dose-dependent manner with hGH stimulation and were significantly attenuated by GHR-KD. Differential yet significant changes were observed in the relative mRNA transcript levels of prolactin (PRL), insulin and related growth
factors (IGF1, IGF2) and their receptors, following hGH treatment or GHR-KD. Thus, the GH/GHR interaction can influence the levels of the JAK2, SRC, STAT1, 3, and 5, p44/42-MAPK, AKT and mTOR in human melanoma cells and may provide leads as to potential targets for therapeutic intervention. In the second part of this dissertation, we report the effect of GHR-KD on the key mechanisms of drug resistance in human melanoma cells. We describe for the first time, a comprehensive list of ATP-binding cassette (ABC) transporter pumps involved in mediating resistance to cisplatin, doxorubicin, oridonin, paclitaxel and vemurafenib in each of the four human melanoma cell lines. We also report the variation in the levels of these drug efflux pumps and overall drug retention ability of the human melanoma cells following GHR-KD. Finally we show the effects on cell proliferation with sub-EC50 doses of the anti-tumor drugs in presence/absence of GHR-KD. Expression of specific ABC transporter pumps was found to be significantly reduced on GHR-KD. Intracellular drug retention was also found to be significantly higher following GHR-KD. We also report a critical downregulation of the transcription factor MITF and its target TYRP1 – important mediators of melanogenesis pathway - following GHR-KD. Additionally, we also report for the first time, the effects of GHR-KD on RNA and protein level expressions of markers of epithelial-mesenchymal transition involved in metastasis and drug-resistance in cancer. Thus, by virtue of its ability to sensitize melanoma cells to sub-EC50 doses of chemotherapy, we propose for the first time the mechanistic model by which GHR-KD can be highly beneficial as a combination therapy with several specific classes of existing and developing therapeutics against melanoma.
DEDICATION

To my friends, family and peers
ACKNOWLEDGMENTS

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demonstrated his marvelous analysis of science or his impeccable attitude towards peers
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mentoring students like me towards degrees and achievements but also by quietly being
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CHAPTER 1: GENERAL INTRODUCTION

Growth hormone (GH) in mammals has many functions. Endocrine GH secretion from somatotroph cells in the anterior pituitary of mammals is regulated by an interactive network of hypothalamic hormones [GH-releasing hormone (GHRH), ghrelin (releasing) and somatostatin (inhibiting; SRIH)] while multiple neurotransmitters and neuropeptides as well as feedback regulatory mechanisms contribute to additional levels of modulation. Secreted GH has distinct roles in childhood-adolescence (linear growth) and adulthood (post-completion of linear growth) and regulates normal development and maintenance of multiple organs (bone, muscle, liver, kidney, heart, and brain). GH interacts with multiple other hormones in circulation and is a major player in the homeostatic regulation of carbohydrate, lipid and protein metabolism. GH induced signaling is mediated primarily by binding to a pre-dimerized specific receptor [GH receptor (GHR)], and involves direct or indirect activation of well-known signaling pathways, such as the JAK2/STAT5 (also STAT1 and STAT3), the Ras/Raf/MEK/MAPK, the PI3K/AKT/mTOR and the IRS1/2 (Argetsinger et al., 1993; Vanderkuur et al., 1994; Harding et al., 1996; Carter-Su et al., 1996; Sotiropioulus et al., 1996; Campbell, 1997; Liang et al., 1999; Piwien-Pilipuk et al., 2002; Mimeault and Batra, 2010; Sedek et al., 2014; Waters and Brooks, 2015). GH induced secretion of insulin like growth factor 1 (IGF1) amplifies and contributes to a major portion of the observed GH-induced effects in tissues. GH action culminates in transcriptional regulation of several proteins, often in a tissue-specific manner. Specific members of protein tyrosine phosphatases (PTP) and suppressors of cytokine signaling (SOCS) families are known to be involved in the termination of intracellular GH/GHR
signaling. The dynamic role of GH in promoting cell proliferation and inhibiting apoptosis as well as in modulating immune cells is equally important in disease state (GH excess, GH deficiency, diabetes, aging, and cancer).

Cancer is one of the most difficult challenges in modern health science by virtue of its complex etiology, high genetic mutability, diverse prognosis, high relapse rates and significant variability in responses to existing chemotherapeutics. Existing therapeutics are thwarted with poor prognosis and limited life expectancies, while numerous candidate therapies including small molecules, immunotherapies and vaccines have met with limited success to control cancer incidence, progression and regression. New strategies in immunotherapy are still under investigation and combination drug therapy has met with mixed results. Elevated GHR levels have been consistently observed in melanoma and in tumors of breast, prostate, colon, and pancreas (Sustarsic et al., 2013). The role of the GH/IGF1 axis in cancer progression and metastasis has been repeatedly reported across some specific types of cancer (breast, prostate), by *in vitro, in vivo* and epidemiological studies. Yet, comprehensive studies on specific effects and relevant molecular mechanisms of GH action in melanoma and hepatocellular carcinoma (HCC) are few to none, although high GHR mRNA and protein levels have been reported in sporadic studies in both.

The specific objective of my research is to generate empirically validated information regarding the effects of GH action in melanoma and HCC, first *in vitro* and then *in vivo*. A high volume of work in GH and cancer in other tissues provides the scientific rationale for my proposed project. I hypothesize that antagonism of the GH
action in melanoma and HCC cells will negatively affect the tumor progression, proliferation and metastasis. Following observable effects of GHR antagonism, a comprehensive analysis of the underlying molecular mechanisms will be performed to arrive at a validated mechanistic model of GH effect in the selected cancer types.

1.1: Growth Hormone (GH)

The human (h) GH gene locus, spanning almost 47 kilobases, is located on the long arm of chromosome 17 (17q24.2). The gene cluster includes five genes – GH (GH1), placental-GH (GH2 or GH-V), chorionic somatomammotropin hormone 1 (CSH1), chorionic somatomammotropin hormone 2 (CSH2) and chorionic somatomammotropin-like pseudone (CSHP). In humans the GH gene family also includes prolactin (PRL). GH (and PRL) is secreted primarily from the somatotroph cells of the anterior pituitary under positive [GHRH, ghrelin, GH secretagogue (GHS)] and negative (SRIH, negative feedback by GH, IGF1) regulation (Figure 1). Almost half of the secreted GH is bound to GH binding proteins (GHBP). Human GH gene has five exons interspersed with four introns and the mature protein contains 191 amino acids (MW=22,129 Da; minus a 26 amino acid secretory signal sequence) with a number of post-translational modifications.

Alternative splicing of the GH primary mRNA transcript generates a number of splice variants, with the 22 kDa protein (GH1) coding transcript as the major product. A 20 kDa protein (GH1 variant; residues 32-46 deleted) also forms due to presence of a cryptic acceptor splice site in exon-3 and constitutes ~5% of the total pituitary GH
secretion. Post translational modifications (phosphorylation, glycosylation, amino-acylation, deamidation) of GH lead to its other hormonal isoforms.

Figure 1: Control of growth hormone (GH) secretion from pituitary gland. GH secretion from the anterior pituitary is controlled positively by hypothalamic GH releasing hormone (GHRH) and gastric ghrelin; and negatively by hypothalamic somatostatin (SRIH). GH-induced hepatic insulin-like growth factor-1 (IGF1) controls GH secretion by a negative feedback loop (Image courtesy: Kargi and Merriam, 2013).
The first crystal structure was obtained for porcine GH in 1987, while the human GH (hGH) structure was solved in 1992 (de Vos et al., 1992). The GH protein consists of four conserved alpha-helices and a conserved large loop formed by disulfide bridging, between the antiparallel stacking of the alpha-helices. hGH contains two (PRL has three) disulfide bridges from four Cys residues (amino acids 53, 164, and 181, 189) conserved across all GH family proteins. The third alpha-helix (amino acids 106-128) of hGH contains amphiphilic geometry and critical functional domains involved signal transduction (Figure 2).

Figure 2: Growth hormone (GH). Structure of hGH containing 191 amino acids, two disulfide bonds and the four alpha-helices (Image courtesy: Kaabi, 2012).
1.2. Growth Hormone Receptor (GHR)

hGH is known to bind to both human-GHR and human prolactin (PRL) receptor (PRLR) with almost equal affinity (Goffin et al., 1999; Kopchick and Andry, 2000; Longhi et al., 2003; Bartke and Kopchick, 2015). The hGHR (and hPRLR) is a member of the class-I hematopoietic cytokine receptor family (Goffin and Kelly, 1997). The hGHR gene is located in the short arm of chromosome 5 (5p13.1-12). The functional transcript consists of ten exons of which exon-2 codes for the secretory signal peptide and the first six amino acids of the extracellular domain (ECD) of hGHR; exons 3-7 codes for the remaining ECD; exon-8 codes for the transmembrane domain (TMD) and exons 9-10 code for the intracellular/cytoplasmic domain (ICD). The hGHR monomer consists of 638 amino acids with a molecular weight of ~71.5 kDa. One molecule of GH binds to a preformed GHR dimer on the cell surface (Ross et al., 2001; Strous and Gent, 2002). The monomeric and dimeric structures of GHR with its important domains are depicted in Figure 3.

Subcellular localization of GHR has been reported in the plasma membrane as well as in the nuclear membrane (Waters et al., 1994; Mertani et al., 2003; Swanson and Kopchick, 2003). The ECD of hGHR monomer consists of two fibronectin type-III domains (FnIII; amino-acids 1-123 and 128-238) containing critical sites for GH binding. The ICD of hGHR contains of Box-1 and Box-2 motifs, important for Jak2 association and a ubiquitination domain – important for internalization of the receptor (Kaabi et al., 2012). The GHR dimerization forms three distinct sites – site-1 (high affinity) and site-2 (lower affinity) – where GH physically associates with the GHR dimer inducing a 25
degree rotation – and a site-3 – which is critical for transactivation of the membrane-associated Jak2 by apposition of their kinase domains (Waters and Brooks, 2015). The GHR-antagonist (B2036) and corresponding drug (Pegvisomant), contains a lysine that replaces glycine at amino-acid 120 in hGH and is postulated to disrupt site-2 binding, thus blocking the induced rotational change in the receptor dimer, ultimately inhibiting GHR-mediated signal transduction (Chen et al., 1990, 1991; Kopchick et al., 2002).

Figure 3: Growth hormone receptor (GHR). A. The GHR monomer showing the Fibronectin-III like domains containing three disulfide bonds, the Box1 and Box2 motifs for JAK2 association and UbE motif for GHR internalization and the multiple Tyrosine (Y) residues which are phosphorylated for GHR signaling (Image courtesy: Kaabi, 2012). B. GHR dimer showing the high-affinity Site-1 and the low-affinity site-2 where one molecule GH physically binds to the dimerized GHR. The site-3 is critical for dimerization of the GHR monomers and critical for GHR signaling (Image courtesy: Waters and Brooks, 2015).
GH-GHR association activates the Jak2 which in turn phosphorylates receptor tyrosine residues on the ICD of GHR, providing scaffolds for adapter proteins of intracellular signaling molecules like SHC, and STAT5 (reviewed in Brooks and Waters, 2010) and repressors of GH induced signaling. Activated Jak2 in turn activates other signaling proteins like STAT1, STAT3, and STAT5, SHC, PI3K, etc. thereby amplifying the GH signal.

1.3. GH Action

GH is a critical regulator of postnatal growth by direct action on the stem-cell and differentiated cell populations in multiple tissues like bone, cartilage, smooth, skeletal and cardiac muscle, liver, kidney, adipose tissue, reproductive tract, nervous system and others (Mertani et al., 1995). A primary action of GH is longitudinal (somatic) growth. It is also to be noted that the GH action on somatic growth and gene expressions are often sex-dependent and regulated upstream by sexually predisposed pattern of GH secretion from the pituitary. Some of the well-known actions of GH on the body are listed below:

- GH has been known to be diabetogenic and to exert an anti-insulin effect on its target organs like liver and skeletal muscles (Tutweiler, 1976; Waldhausl et al., 1977; Adamson et al., 1981). Chronic exposure to elevated amounts or exogenous hGH produced hyperglycemia and glucose intolerance in mouse models (Kostyo et al., 1984). GH is believed to suppress muscle glucose uptake, increase endogenous glucose production, thereby increasing blood glucose levels, leading to increased insulin secretion (hyperinsulinemia) from pancreatic beta-cells (Moller et al., 1991). One of the possible mechanisms responsible for this effect
could be the GH-induced inhibition of insulin’s effect in activation of phosphatidylinositol (PI) specific phospholipase C (PLC) (Chou et al., 1990). These diabetogenic actions of GH are reported to be conserved across mammalian and non-mammalian species (Cameron et al., 1985).

- Primary site for GH action is the liver. At liver GH regulates expression of numerous sex-specific genes. In liver, GH promotes gluconeogenesis (anti-insulin effect), induces secretion of IGF1 (GH-induced hepatic IGF1 = >80% of total IGF1 in the body) and regulates cross-talk between liver and other tissues like adipose. Estrogen and GH work antagonistically in regulating hepatic gene expressions and hepatic lipid metabolism (Ho and Weissberger, 1992; Choi and Waxman, 2000; Fan et al., 2009; Fernandez-Perez et al., 2013; List et al., 2014).

- GH promotes osteogenesis by inducing hematopoietic and mesenchymal progenitor cell proliferation in bone marrow (Cool et al., 2005; Berger et al., 2007). GH also induces chondrocyte differentiation and proliferation in the growth plate of long bones (Zhou et al., 1997; Ohlsson et al., 1998; Sjogren et al., 2000).

- Lipolytic action of GH on adipose tissue has been well studied in several mouse models and in humans. GH improves body composition by decreasing fat mass, increasing free fatty acids (FFA) and ketone bodies, down-regulating glucose uptake (anti-insulin action), modulating adipokine (like leptin) levels, etc. (Berryman et al., 2004; List et al., 2013).
• GH action promotes skeletal muscle development via myoblast production and myofiber elongation, glucose uptake, and regulating metabolic cross-talks of muscle with other tissues (like adipose) (Masternak et al., 2005; Mavalli et al., 2010; Fuentes et al., 2013).

• GH action on kidney involves up-regulation of glomerular filtration rate, body fluid retention, maintaining phosphate-sodium-calcium homeostasis, and inhibiting renal gluconeogenesis (Grunenwald et al., 2011; Kumar et al., 2011; Kamenicky et al., 2014).

• GH promotes cardiovascular development by modulating aortic thickness, aortic contractibility and response to potassium, etc. (Egecioglu et al., 2007).

• Several mouse and human studies have indicated GH action in promoting insulin gene expression, biosynthesis and release from pancreatic beta-cells (Hauck et al., 2001; Coschigano et al., 2003; Liu et al., 2004; Wu et al., 2011).

• GH (directly and through IGF1) regulates reproductive performance, female puberty, fetal and placental growth as well as litter size, as seen from numerous mouse and human studies (Danilovich et al., 1999; Zaczek et al., 2002; Keene et al., 2002; Slot et al., 2006).

• In the brain and spinal cord GH action has established roles in axonal myelination and in maintaining neuronal and non-neuronal cell populations and differentiation of stem-cells. GH might also have a neuroprotective effect on the brain in sleep-deprivation states, as observed in rat models (Almazan et al., 1985; Morisawa et al., 1989; Nyberg, 2000; Frago et al., 2002; Kim et al., 2010; Aberg et al., 2010).
1.4. GH-GHR Induced Signaling

Direct action of GH on cells initiates via binding to the pre-dimerized GHR on the cell surface. Binding induced conformational change (discussed previously) results in phosphorylation and activation of the cytoplasmic JAK2, beginning the signaling cascade (Waters and Brooks, 2015). Activated JAK2 in turn can phosphorylate the GHR at multiple Tyr residues, providing sites of interaction for cytosolic components of STAT (STAT1, STAT3, and STAT5), Ras/Raf/MEK/MAPK (via SHC-Grb2-SOS and Src) and PI3K/AKT/mTOR (directly or via IRS1 and IRS2) pathways to directly interact with phosphorylated GHR. Activated GHR has also been reported to activate phosphokinase C (PKC) either via the IRS/PB-kinase pathway or by inducing DAG production via phospholipase C (PLC) (Figure 4). These intracellular signaling proteins execute the biological effects of GH by controlling gene expressions.

GH induced activation of the epidermal growth factor receptor (EGFR) signaling has been demonstrated in mouse hepatocytes using bovine-GH-overexpressed (bGH) and GHR-knock out (GHRKO) mice (Gonzalez et al., 2010). EGFR signaling overlaps with multiple GH/IGF1 signaling pathways (STAT1, STAT3, STAT5, MAPK, and AKT) and has widespread effects on cell proliferation differentiation and survival in tissues (Huang et al., 2003; Gonzalez et al., 2010). The active phosphorylated JAK2 phosphorylates the dimerized EGFR at distinct sites facilitating for its use as a scaffold for GH signal transduction (Yamauchi et al., 1997).
IGF1, secreted due to GH induction in target tissues like liver, is a major effector of GH induced somatic growth. GH mediated regulation of IGF1 levels also involve a GH induced expression of IGF binding proteins (mainly IGFBP3), which with ALS (also induced by GH and secreted by the liver), sequesters and transports circulating IGF1 as well as promoting IGF1 action at cell surface. IGF1 and the IGFBP expressions are also regulated by other hormones like progesterone, estradiol, LH and FSH. IGF1 and its
specific receptor IGF1R are ubiquitous to most tissues with well-studied autocrine/paracrine actions. The IGF1R is a receptor tyrosine kinase which mainly activates the MAPK (via SHC-Grb2-SOS Ras, Raf or Src), the mTOR (via IRS1-4), and the c-Jun N-terminal kinases (JNKs) downstream signaling pathways to effect transcriptional regulation via c-Fos and c-Jun. IGF1 acts as a mitogen, controlling cell cycle progression, and cell proliferation. IGF1 acts also as a cytokine by amplifying the differentiation and proliferation signals of the GH/GHR activation in most tissues of the body. A critical function of IGF1 also lies in inhibiting apoptosis in hematopoietic, cardiomyocytes and neuronal cell precursors. In addition to the above and more importantly, the GH/IGF1 axis, by virtue of its spectrum of anabolic and catabolic effects, plays a central role in systemic metabolism.

In a well-fed state, the GH/IGF1 axis along with insulin works in homeostatic regulation of body’s lipid, carbohydrate and protein reserves in a complex endocrine and autocrine/paracrine manner. Prolonged insulin effects down-regulate GH signaling (Xu et al., 2005). Yet a provocative test for GH secretion is the ITT where insulin is injected to induce GH secretion. Also, glucose will decrease GH secretion. Whereas in fasting (catabolic) stage, GH/IGF1 action exerts an anti-insulin (anabolic) effect in two main ways – (1) by preventing glucose uptake and oxidation while promoting gluconeogenesis – a diabetogenic effect of GH; and (2) by preventing amino acid oxidation and decreasing protein turnover rates. GH is known to induce lipolysis in adipose tissues and upregulate production of ketone and free fatty acids. GH, IGF1 and insulin together allow for distinct local and peripheral regulations. The somatic and metabolic effects of GH/IGF1
have been best characterized and found to be critically relevant in disease states, as discussed later.

Termination of GH signaling involves different members of the suppressors of cytokine signaling (SOCS) family (SOCS1, SOCS2, SOCS3 and CIS) and several protein tyrosine phosphatases (PTPH1, SHP1, and SHP2). Signal termination is brought about by different mechanisms like direct JAK2 kinase inhibition (SOCS1), inhibition of JAK2-GHR complex by binding to membrane-proximal Tyr-residues on GHR (SOCS3), inhibition by binding to GHR at its membrane-distal Tyr-residues (CIS, SOCS2), dephosphorylating the active GHR and/or JAK2 or by downregulating IGF1 production (Ram and Waxman, 1999; Christopher et al., 2005).

1.5. Melanoma

Melanoma (a word derived from the Greek words - melas, "dark" and oma “tumor”) is an ancient disease, first reports dating back to 5th century BC and earliest physical evidence found in the 2,400 year old mummies of pre-Colombian era (Rebecca et al., 2012). Melanoma accounts for only 1% of all skin cancer cases and yet is the most aggressive form of skin cancer. Current statistics estimates diagnosis of about 76,380 new cases of melanoma (about 46,870 in men and 29,510 in women, 2880 cases in Ohio), contributing to about 4.5% of all reported new cancer cases in 2016. Additionally, approximately 10,130 death (about 6,750 men and 3,380 women) from existing melanoma is predicted in 2016, making up 1.7% of death by all cancer cases (http://seer.cancer.gov/statfacts, http://www.cancer.org/cancer/skincancer-melanoma). In general, occurrence of melanoma is far greater among males (28.2%) vs. females
(16.8%). Notably, predisposition towards melanoma is far greater among Caucasians compared to African Americans, as lifetime risk of getting melanoma is about 2.4% (1 in 40) for whites, 0.1% (1 in 1,000) for blacks, and 0.5% (1 in 200) for Hispanics (www.cancer.org). However, risk of melanoma is also modulated by various other factors, namely – age, UV exposure and previous family history of melanoma. Observed five-year survival rate for early stage (Stage IA) is about 97% while metastatic melanoma (Stage IVB), which often shows severe multidrug resistance, has poor five-year survival rate of 15-20%. Current understanding of melanoma initiation, development and metastasis, as well as the mechanism underlying the aggressive drug resistance, is not yet comprehensive and warrants further investigation into the disease mechanism for identification of novel therapeutic targets and therapeutic strategy development.

Melanoma usually occurs in the exposed parts of the body – face, neck, hands and feet – but can also be found in any anatomical site occupied by melanocytes, like the gastrointestinal, genitourinary and respiratory mucosa and the choroidal layers of the eye (see Figure 5). There are extensive reviews regarding the causes of melanoma identified through extensive research in the last 30-50 years. Below are summarized just a few of these causes.

- Exposure to ultraviolet (UV) radiation from the sun and/or artificial tanning beds (Cust et al., 2011).
- Genome wide association studies (GWAS) have identified several driver mutations (melanomagenic) in specific genetic loci, including BRAF (V600E and other SNPs), KIT, NRAS, MIT, Akt, PTEN, EGFR and several others
(extensively reviewed by Tsao et al., 2012; Shtivelman et al., 2014), which go on to disrupt the homeostatic activation states of signaling pathways like p44/42 MAPK (Erk1/2), PI3K/Akt/mTOR, STAT3, etc. (McCubrey et al., 2007; Woodman and Davies, 2010; Jazirehi et al., 2012; Lee et al., 2012; Ohanna et al., 2013; Kwong and Davis, 2014). Pro-apoptotic pathways are suppressed while cell survival and proliferation pathways are put on an overdrive in the altered cellular state in melanoma.

- Some population-based case-control studies have highlighted increased incidence of melanoma from prolonged estrogen use (hormonal replacement therapy or oral contraceptive) (Madhunapantula and Robertson, 2009; Koomen et al., 2009).

- Environmental risk factors, besides UV radiation, include lifestyle and geographical factors as well as drugs (NSAIDs, sulfonamides, etc.) and/or cosmetics that can sensitize the skin and activate melanocytes (Volkovova et al., 2010).

Diagnosis of melanoma is especially critical since early intervention can lead to cure. Presently, diagnosis depends on skin examination (for irregular margin, atypical moles, or expanding pigmentation) by a clinical practitioner and/or by a biopsy of the tumor. There is extensive investigation to identify a dependable prognostic biomarker for melanoma and increased levels of serum lactate dehydrogenase (LDH), Hsp90, Osteopontin, NCOA, were implicated with more aggressive and metastatic forms of the disease (reviewed in Gogas et al., 2009; Batus et al., 2013; Shtivelman et al., 2014). Diagnosis following metastasis requires whole body scans, including brain, by several
different techniques like computed tomography (CT) scan, positron emission tomography (PET) scan or magnetic resonance imaging (MRI). Most common metastatic sites for cutaneous melanoma are the lungs, liver, brain, and gastrointestinal tracts, with variable but a low survival prognosis (4-24 months) and high relapse rates (Batus et al., 2013).

![Figure 5: Different types of human melanoma. (Image courtesy: Tsao et al., 2012)](image)

Treatment options for melanoma target the biological ‘drivers’ of melanoma which are components of the intracellular signaling pathways, some of which have been briefly mentioned above. Available treatments include surgical resection of metastatic melanoma (metastasectomy), targeted therapy, chemotherapy (Dacarbazine, IL2, etc.) and most recently immunotherapy (reviewed in cure Batus et al., 2013). Of the
chemotherapeutic options, Dacarbazine, is cytotoxic (only cytotoxic drug approved by FDA) with less than 5% of patients attaining complete remission and ~4% survival rates. Combination regimens with Dacarbazine, Temozolomide, Carboplatin or Paclitaxel have had mixed responses in efficacy (4-23%) in practice.

Although a discussion on the available and developing treatments is beyond the scope of this dissertation, I briefly mention below a handful of promising results in different phases of clinical trials, since 2011. Detailed discussions are available in the aforementioned reviews.

- **Ipilimumab**, a fully humanized CTLA4 (cytotoxic T-lymphocyte antigen-4) monoclonal antibody, has shown de-repression of T-cell response and effect tumor regression in melanoma cases in patients with significantly longer survival rates. There were a few concerns like delayed clinical response, toxicity and adverse reactions such as dermatitis, colitis, drug-related hepatitis, etc. This immunotherapy was approved by FDA, for treatment of advanced melanoma, in 2011.

- **Programmed death-1 (PD1) and PD-ligand-1 (PDL1) interactions** are suppressors of T-cell activation and have emerged as promising drug targets in melanoma tumors which aberrantly express PDL1 on their surface. A fully humanized monoclonal antibody to PDL1 causes T-cell activation and tumor regression and was mostly well-tolerated in patients. Pre-clinical mouse studies also showed a synergistic effect of co-treatment with PDL1 antibodies and Ipilimumab.
• Vemurafenib was the first oral anti-melanoma targeted therapy, approved by FDA in late 2011. It specifically targets the mutated (V600E) BRAF, the critical oncogene in melanoma, which can constitutively activate the Erk1/2 pathway, irrespective of RAS activation. Although very successful in phase-I, -II and -III of clinical trials and approved as a drug, Vemurafenib has a significant toxicity profile in patients, which includes squamous cell carcinoma, hyperkeratosis, photosensitivity, rashes, nausea, diarrhea, etc. Another newer compound in the class - Dabrafenib, targeting specific BRAF mutation has shown early but better results and lower toxicity profiles in phase-III clinical trials and awaits approval.

• Imatinib, targets a mutated cKIT in melanoma cells and has shown dramatic results in phase-II clinical trials. A few other important developing approaches in melanoma therapy include oncolytic viral therapies, and adoptive cell transfers.

1.6. Mechanisms of Drug-resistance in Melanoma

Melanocytes are derived from neural crest cells and associate on the basement membrane with close interactions with the keratinocytes and Langerhans cells. The disruption of the homeostatic control exerted by the keratinocytes is a hallmark sign of initiation of melanoma. Hallmark features of this disrupted control include regression in expression of keratinocyte interaction mediators like E-cadherin and increase of inter-melanomal and fibroblast mediators like N-cadherin and vimentin. These are also markers of epithelial mesenchymal transition (EMT) – a process extensively reviewed to be implicated in cancer metastasis (Haass et al., 2005; Brychtova et al., 2011) (Figure 6). However there is a mounting body of evidence indicating a more intensive role of EMT
in mediating multi-drug resistance and generation of cancer cells with characteristics of stemness expressing drug-resistant pumps in multiple forms of cancer cells (Arumugam et al., 2009; Singh and Settleman, 2010; Kong et al., 2011; Lackner et al., 2012; Radvanyi, 2013; Fischer et al., 2015). EMT is normally employed in melanocytes during detachment from the neural crest and a subsequent reversal (MET) of the process allows anchorage to the basement membrane. Malignant forms of the tumor have been seen to have almost complete loss of epithelial markers following robust up-regulation of Snail1 transcription factors especially in states of BRAF mutation which is found in over 50% of the melanoma cases (Brychtova et al., 2011). The new-found role of EMT in acquisition of drug-resistance in melanoma cells is of significant importance.
Melanoma cells are intrinsically equipped with two other mechanisms aimed at chemotherapeutic refractibility – (i) multi-drug resistant ATP binding cassette (ABC) transporter pumps (Walsh et al., 2010) (Figure 7A), and (ii) inactivation of drug-effects by sequestering drugs in melanosomes by upregulating melanogenesis (Chen et al., 2006).
(Figure 7B). These are discussed in detail in subsequent chapters. Briefly melanoma cells particularly expresses a wide range of ABC transporters on its cell membrane as well as intracellularly on structures like melanosomes and the nuclear membrane to pump out various classes of xenobiotic compounds and sequester them within melanin containing packets called melanosomes for subsequent delivery out of the melanoma cells. The upregulation of the melanogenesis pathway is mediated by the critical melanomal transcription factor – microphthalmia associated transcription factor (MITF) and rate limiting enzymes in the melanogenic pathway like tyrosinase related proteins 1 and 2 (TYRP1, TYRP2) (Videira et al., 2013). GH is implicated in regulation of melanogenesis following recent reports of a significant loss of melanocyte stimulating hormone (MSH) levels in GHR knock out (GHRKO) mice brains (Sadagurski et al., 2015). Therefore the extent of GH regulation of the upregulated drug-resistance processes in melanoma needs immediate investigation.

Lastly, another important ligand-receptor pair active in several forms of cancer is known to play an important role also in melanoma – the hepatocyte growth factor (HGF) and its canonical transmembrane receptor MET (or c-MET), receptor tyrosine kinase. Both are expressed in various cancers and the interaction potentiates their subsequent expression levels, forming an autocrine loop (Yi and tsao, 2000; Blumenschein 2012; Guadino et al., 2014) which drives an aggressive form of the cancer. The intracellular signaling of HGF-MET overlaps with multiple oncogenic networks shared by the GH-GHR pair interaction, namely – the ERk1/2, STAT3, SRC and AKT (Figure 8).
Figure 7: Important mechanisms of drug-resistance in human melanoma cells. (a) Active efflux of drugs by ABC transporter pumps (Image courtesy: sanguinebio.com). (b) Sequestration of drugs in melanosomes (Image courtesy: Chen et al., 2006).
1.7. GH in Cancer

Several studies have shown that removal of GH action can significantly impede the development of cancer. For example, in recent studies, an Ecuadorian cohort of 99 Laron patients (insensitive to GH action due to mutant inactive GHR) had 1 malignancy (non-lethal) compared to 17% in control individuals (Guevara-Aguirre et al., 2011); while another cohort of 230 Laron patients exhibited zero cancer incidence (Steuerman et al., 2011). On the other hand, acromegalic patients (sustained hypersecretion of GH from a pituitary adenoma) have a two-fold higher risk of colorectal cancer and above-average
risk for developing thyroid and prostate cancers in their lifetime (Renehan and Brennen, 2008). Collectively this information aptly highlights the profound effect of GH action as it relates to cancer in the human body. The implication has been studied and investigated in several publications across the last 30 years or more. An excellent and current review of these developments has been authored by John J. Kopchick and group recently (Kopchick et al., 2014). Below is a summary of a few selected studies in GH-cancer relationship, which contributes to the rational backdrop of my subsequent project proposal.

- **Breast Cancer**: The expression of GHR on human breast cancer cells and tumor biopsies was first reported in 1995 (Decouvelaere et al., 1995). Around the same time, the tumor growth stimulatory effect of hGH (through both hGHR and hPRLR) and the anti-proliferative role of GHR antagonism *in vitro*, was also reported (Fuh and Wells, 1995). The GHR expression on various human breast cancer tissues was reported to be significantly higher than that in the normal adjacent mammary tissues (Gebre-Mehdin et al., 2001). The autocrine/paracrine effects of hGH (as well as hPRL and IGF1) in the breast cancer tissues has been repeatedly implicated (Gregoraszczuk et al., 2001; Perry et al., 2008; Wu et al., 2011) and has been found to have a greater proliferative and drug-resistant effect on breast cancer cells compared to exogenous hGH *in vitro* (Mojarred et al., 2009; Zatelli et al., 2009; Bougen et al., 2012). Pegvisomant, the GHR antagonist, successfully abrogated this effect and increased the drug sensitivity of breast cancer cells in these studies. Several important mouse strains of GH action had
been generated in our lab, viz. bGH [mouse transgenic for bovine GH (bGH)], GHA [mouse transgenic for mutated bGH (G119R) – a GHR antagonist], GHRKO (mouse lacking a functional GHR/GHRBP; GHR-/-) (reviewed in Kopchick et al., 2014), as well as different tissue specific knock-outs (KO) of the GHR. These mice have been used to study the effects of GH action in cancer. GHR antagonism was shown to provide significant protection from DMBA-induced mammary carcinogenesis where at the end of a 39-week study, two-third of the GHA mice remained tumor free, compared to only one-third in the littermate wild-type controls (Pollak et al., 2001). Similar effects of GH in active propagation and resurgence of mammary tumors were also reported in rats (Swanson and Unterman, 2002; Shen et al., 2007). Also, studies with the GHRKO mouse crossed with C3(1)/TAg mouse (mouse develops ERalpha-negative mammary cancers) showed TAg/GHR-/- mice too have delayed cancer latency, and lower tumor volume and multiplicity compared to the Tag/GHR+/+ littermates (Zhang et al., 2007). Athymic nude mice (bearing FOXN1 mutation) xenografted with MCF7 breast cancer cells, showed a steady decrease in tumor size on Pegvisomant treatment (Divisova et al., 2006). The collection of these studies highlighted the definite effect of GH action and GHR antagonism in breast cancer.

- **Prostate Cancer**: The presence of GH and GHR mRNA and protein in multiple human prostate cancers was reported in 2002 (Chopin et al., 2002). Soon afterwards, GH and GHR expression and GH-induced activation of
JAK2/STAT5, MAPK and AKT pathways, were reported in human samples of benign prostate hyperplasia and prostate adenocarcinoma tissues (Weiss-Messer et al., 2004; Bidosee et al., 2009). The possibility of GH acting in concert with gonadal hormones in prostate cancer progression was also reported by the group (Bidosee et al., 2011). Similar to a study described above in breast cancer, the TAg/GHR-/- mice showed 12.5% incidence of prostate cancer, compared to 87.5% in their TAg/GHR+/- littermates (Wang et al., 2005).

- **Colorectal Cancer:** GH and GHR expression has been observed to be present in human colorectal cancer tissues and at higher frequency and abundance than normal mucosa (Yang et al., 2004). In fact, GHR expression was particularly upregulated in the tumorigenesis phase suggesting an involvement of GH action in colorectal tumor development (Wu et al., 2007). Similar to other studies, Pegvisomant therapy on Colo205 xenografted athymic nude mice caused 39% reduction in tumor volume and 44% reduction in tumor weight than the saline-treated littermates (Dagnaes-Hansen et al., 2005). Also, siRNA mediated inhibition of GHR in SW480 human colon cancer cells xenografted in nude mice, exhibited >90% reduction in tumor volume (Zhou et al., 2013).

- **Meningial Cancer:** GHR expression in human meningioma tissues was reported in 1999 (Friend et al., 1999). The same study also showed an *in vitro* growth inhibitory effect of B2036 (non-pegylated GHR-antagonist) on meningioma cultures. A later study on nude mice xenografted with human meningioma tumors showed tumor growth inhibition and often tumor regression with 8 weeks of
Pegvisomant treatment (McCutcheon et al., 2001). The potential role of hGH in central nervous system (CNS) tumorigenesis was indicated in hGH treated mouse neuroblastoma cells (Grimbly et al., 2009).

- **Skin Cancer:** There are very few reports of GH action in skin cancer. The expression of GHR at levels higher than in normal skin was reported in non-melanoma skin cancers, especially in squamous cell carcinoma in human samples (Gunduz et al., 2013). Around the same time, a study from our lab reported very high GHR expression in human melanoma samples in the NCI-60 cell lines, compared to all other cancer tissue types (Sustarsic et al., 2013). The same authors also reported an increased cell proliferation and upregulated STAT5, AKT and Erk signaling in human melanoma cell lines treated with hGH. Till date no other studies are available, which investigate the involvement and action of GH in melanoma and other human skin-cancer.

- **Other Cancers:** The proliferative effects of endocrine and local (autocrine/paracrine) GH effects in endometrial cancer cells has been reported (Pandey et al., 2008). In human cervical cancer samples, majority of the tissues was found to express a nuclear localized GHR (Dehari et al., 2008). In human gastric cancer samples a significantly higher GHR expression compared to normal gastric mucosa, correlated with tumor differentiation and tumor grade; while hGH treatment on mice xenografted with human gastric adenocarcinoma cells showed significantly higher tumor growth compared to untreated controls (Yang et al., 2012; Yan et al., 2011; Nagano et al., 1995). A Thr495Pro polymorphism in
hGHR was reported to increase susceptibility to lung cancer in a Chinese population (Cao et al., 2008). The complex role of GH, IGF1 and somatostatin in the development and progression of hepatocellular carcinoma (HCC) has been recently reviewed (Pivonello et al., 2014). To the best of our knowledge no studies have been reported on mechanisms of GH action or GHR antagonism and effects in HCC.

PRLR expression in several of the cancer types mentioned above has been extensively reviewed (Lee et al., 2007; Fernandez et al., 2010) with many new studies bringing to focus the role of PRL and PRLR in breast and prostate cancer progression (Wen et al., 2014). The role of IGF1/IGF1R signaling as well as IGF2 and insulin in cancer development and progression are equally relevant and have been reviewed extensively (Grimberg, 2003; Grimberg, 2005; Bogorad et al., 2008; Neradugomma et al., 2014). The collection of these studies highlights a complex role of GH in physiological state in controlling tumor incidence and a critical role in the advancement of cancer state, often in tune with other local and systemic hormones. The important metabolic effects of GH/IGF1 axis in the normal and disease states need to be carefully resolved and studied to arrive at the big picture of cancer causation, development, metastasis and relapse.

1.8. References


Sustarsic, E. G., Junnila, R. K., & Kopchick, J. J. (2013). Human metastatic melanoma cell lines express high levels of growth hormone receptor and respond to GH treatment. *Biochemical and Biophysical Research Communications, 441*(1), 144–50. doi:10.1016/j.bbrc.2013.10.023


CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

Based on the above discussion on GH action in the body in physiological and disease states, it was reasonable to hypothesize an effect of GHR knock-down (KD) on the melanoma cell proliferation and sensitivity to anti-cancer therapy. Therefore we state our hypotheses as follows

_Hypothesis 1: A reduced GHR expression will decrease melanoma proliferation and affect multiple signaling pathways and processes involved in melanoma progression._

_Hypothesis 2: A reduced GHR expression will negatively impact the drug resistance property of melanoma cells._

In order to address these hypotheses, the following specific aims are proposed:

Specific Aim 1: Assess the *in vitro* effect of siRNA mediated GHR KD in cell-proliferation, clonogenicity, migration, invasion, wound-healing, and apoptosis of human melanoma cell lines. This addresses Hypothesis 1.

Specific Aim 2: Assess the *in vitro* effect of siRNA mediated GHR KD in relevant signaling pathways (JAK2/STAT, ERK1/2, AKT, mTOR, SRC) and GH-associated ligand- receptor pairs (PRL, IGF1, IGF2, insulin, PRLR, IGF1R, IGF2R, IR) in human melanoma cell lines. This addresses Hypothesis 1.

Specific Aim 3: Assess the *in vitro* effect of siRNA mediated GHR KD in reducing levels of drug efflux mediators (efflux pumps) in human melanoma cell. This addresses Hypothesis 2.
CHAPTER 3: THE EFFECTS OF GROWTH HORMONE RECEPTOR (GHR) KNOCK-DOWN ON INTRACELLULAR SIGNALING, LIGAND-RECEPTOR EXPRESSION AND TUMOR PHENOTYPES IN HUMAN MELANOMA CELLS

3.1. Abstract

A plethora of in vitro, in vivo, epidemiological and genome wide association studies (GWAS) have implicated a proliferative role of the human growth hormone (hGH) in different types of human cancers. Key oncogenic pathways are shared by the GH/GHR system and the ligand-receptor pair is expressed and utilized in breast, colorectal and prostate cancer in resisting apoptosis, accelerating proliferation and driving metastases. It was recently reported that melanoma, a rarer but most aggressive form of cancer of the skin, consistently expresses the highest levels of growth hormone receptor (GHR) transcripts amongst all other cancer types in the NCI60 panel. Following that report this is the first detailed investigation into the effects of hGH and GHR-KD on the shared intracellular signaling pathways and key downstream effects in tumor phenotype. We here report the results of hGH treatment or an siRNA mediated transient knock down of GHR expression in human melanoma cell lines. Significant attenuation of the phosphorylation states of multiple intracellular signaling molecules were observed, as well as differential, yet significant changes in transcript level expressions of prolactin, insulin and related growth factors and their cognate receptors was identified. The perturbations in the oncogenic intracellular signaling of the melanoma cells translated into regressive phenotypes in cell proliferation, migration, invasion, and colony formation on soft agar assays, which are also reported here. Together, these results unfold
for the first time the mechanistic details of GH/GHR action in human melanoma cells and propose a unique model of an autocrine ligand – receptor interaction of GH-GHR in melanoma, driving multiple critical cellular processes in the tumor. This dependence of the melanoma cells validates the GH/GHR interaction as an important pharmacological target of intervention in melanoma therapy.

3.2. Introduction

Melanoma (a word derived from the Greek words - *melas*, "dark" and *oma* “tumor”) is an ancient disease, first reports dating back to 5th century BC and earliest physical evidence found in the 2,400 year old mummies of pre-Colombian era (Rebecca et al., 2012). In 2015, melanoma is considered the most aggressive and treatment-resistant form of human skin cancer, with an annual incidence of 73,870 in 2015 (SEER Stat Facts – National Cancer Institute) with a total of approximately 1,000,000 patients in the US. The estimated mortality from melanoma in US in 2015 is 9,970 and includes children, adolescents and adults. Fair skinned people have the highest propensity to acquire melanoma, with males (28.2%) having a higher predisposition than females (16.8%). The numbers of new cases have been rising steadily in the last 30 years, during which the five-year survival rates increased from 86% (1985) to 93% (2012), albeit with a poor quality of life. Melanoma usually occurs in the exposed parts of the body – face, neck, hands and feet – but can also be found in any anatomical site occupied by melanocytes, like the gastrointestinal, genitourinary and respiratory mucosa and the choroidal layers of the eye.
The presence of growth hormone receptor (GHR) RNA transcript in human skin cells, especially melanocytes, was reported more than 20 years ago (Tavakkol et al., 1992). The study supported the fact that by dint of the presence of the corresponding cognate receptors, the largest organ of the body – skin – was responsive to circulating GH and insulin-like growth factor – I (IGF-1). Autocrine levels of GH as well as IGF-1 was then reported in normal and basal cell carcinoma whole skin a few years later (Slominski et al., 2000). Since then there had been several reports of elevated GHR RNA transcript and proteins in human melanoma (Lincoln et al., 1999; Wyatt, 1999; Caldarola et al., 2010; Handler et al., 2012; Sustarsic et al., 2013). The melanoma cell cycle was recently reported to be under orchestrated regulation of endogenous GH, prolactin (PRL) and adrenocorticotropic hormone (ACTH) (Bhanu, 2012). Moreover, primary human melanoma specimens were found to have relatively high expression levels of growth hormone releasing hormone (GHRH) receptor (GHRHR) (Chatzistamou et al., 2008) while GHRH-analogs were successful in suppressing malignant melanoma growth in vivo (Szalontay et al., 2014). However, no definitive study exists investigating the existence and subsequent roles of an GH-GHR interaction in metastatic melanoma or the intracellular mediators involved therein.

GH induced signaling is mediated primarily by binding to a pre-dimerized specific receptor [GH receptor (GHR)], and involves direct or indirect activation of well-known signaling pathways, viz. the JAK2/STAT5 (also STAT1 and STAT3), the Ras/Raf/MEK/MAPK, the PI3K/AKT/mTOR and also the SRC family kinases (Argetsinger et al., 1993; Vanderkuur et al., 1994; Harding et al., 1996; Carter-Su et al.,
1996; Sotiropioulus et al., 1996; Campbell, 1997; Liang et al., 1999; Piwien-Pilipuk et al., 2002; Manabe et al., 2006; Barclay et al., 2010; Mimeault and Batra, 2010; Sedek et al., 2014; Waters and Brooks, 2015). The entire array of these signaling pathways are shared by multiple oncogenic drivers in melanoma and are often constitutively active in melanoma cells (reviewed in Tsao et al., 2012). The GH-regulated signaling pathways in normal cells are found to be also crucial in the interactions of melanoma with its microenvironment and progression to metastasis (reviewed in Brychtova et al., 2011). GH induced secretion of insulin like growth factor 1 (IGF1) amplifies and contributes to the observed GH-induced effects in tissues. IGF1 is found to be elevated in circulation of melanoma patients (Kucera et al., 2014) and its receptor (IGF1R) are implicated in an autocrine/paracrine regulation of melanoma growth (Yoshida et al., 2014). The network of insulin, associated growth factors (IGF1 and IGF2) their cognate receptors (IR, IGF1R, IGF2R) and binding proteins (IGFBPs) are important determinants of melanoma disease progression (Antoniadis et al., 2011; Capoluongo, 2011). The current facts readily indicate that GH putatively occupies a central regulatory role in melanoma cells and can offer a valuable insight into the control of oncogenic mechanisms of the melanoma cells.

In this study we addressed this impending need for a detailed investigation of the effects of GH excess as well as GHR-KD on human melanoma. Using four different human melanoma cell lines from the NCI60 panel of cancer cells (Sustarsic et al., 2013) we abrogated the GHR expression using GHR-specific siRNA to mimic a transient but acute inhibition and investigated the transcript expression levels and variations of key
components of the GH/IGF axis, as well as the activation states of multiple GH-dependent signaling pathways. We demonstrate that human melanoma cells have endogenous GH and GHR expressions, with the GH/IGF-1 axis affecting expression of multiple genes. We show that critical signaling networks in the melanoma cell are GH-dependent and were severely suppressed following GHR knock down. We further found that GHR-KD in melanoma significantly suppressed characteristic tumor phenotypes associated with proliferation and metastasis of cancer cells.

3.3. Methods

3.3.1. Cell culture and GH treatment

Human malignant melanoma cell lines SK-MEL-5 (#HTB-70), SK-MEL-28 (#HTB-72), MALME-3M (#HTB-64), MDA-MB-435S (#HTB-129) and normal human skin fibroblast cells MALME-3 (#HTB-102) cells were obtained from American Type Culture Collection (ATCC; Manassas, Virginia). SK-MEL-5 and SK-MEL-28 were grown and maintained in EMEM media (ATCC #30-2003), while MALME-3M and MDA-MB-435S were grown in IMDM (ATCC #30-2005) and RPMI-1640 (ATCC #30-2001) respectively. Complete growth media was supplemented with 5% fetal bovine serum (FBS; ATCC # 30-2020) and 1X antibiotic-antimycotic (Thermo Fisher Scientific #15240). MALME-3 cells were grown in McCoy’s medium (ATCC # 30-2007) supplemented with 15% FBS and 1X antibiotic-antimycotic. Cells were grown at 37C / 5% CO2 in a humidified incubator. Half the media was replaced every 48 hr. No hGH was present in the media or added externally unless specifically mentioned. Tissue culture treated sterile T-75 and T-25 flasks and 6-, 12-, 24-, and 96-well plates (Corning,
New York) were used. Trypsinization was done using 0.25% Trypsin/0.53 mM EDTA in Hank’s balanced salt solution (HBSS) without calcium or magnesium (ATCC # 30-2101) for 5 min / 37C / 5% CO2.

For hGH treatment, 16 hr. after seeding (or 24 hr. post-transfection), the cells were serum-starved for 2 hr. in serum free growth media and hGH (PBS was used as control where applicable) was added at the mentioned concentration. The concentration ranges tested were 0 - 500 ng/mL. For experiments, either or all of 0, 5, 50, 150 ng/mL concentrations were chosen. The hGH treatment was done for 24 hr. before evaluating RNA expression. Recombinant, active human growth hormone was purchased from Antibodies Online (#ABIN2017921, Atlanta, GA).

3.3.2. Transfection

Transfection was performed using siLentFect lipid reagent (Biorad #170-3360, Hercules, California) following manufacturers protocol. Pre-designed siRNA duplexes against human GHR (Origene #SR301794, Rockville, Maryland) at different concentrations were checked and 20 nM was found to be optimum for decreasing the GHR RNA by >85%. Mock transfection was done using universal scrambled negative control siRNA-duplex (Origene #SR30004). TYE-563-fluorescent labeled siRNA duplex (Origene #SR30002) was used as the transfection control. Cells between passages 4-8 were trypsinized, counted using a Countess automated cell counter (Life Technologies, Carlsbad, CA) and seeded at 25,000-30,000 cells/cm² and allowed to attach for 16-18 hr, followed by replacing the media with fresh antibiotic free complete growth medium just prior to transfection. A pre-incubated mix of 20nM siRNA duplex (scramble or GHR
specific) and siLentFect reagent at 1:1 molar ratio was added to the cells and incubated at 37°C / 5% CO₂. Media was changed to complete growth medium with antibiotics after 24 hr. RNA levels were analyzed 48 hours post transfection while protein levels were analyzed at 60 hr post-transfection.

3.3.3. RNA extraction and RT-qPCR

RNA extraction was done using the IBI-Trizol based total RNA purification kit (MidSci #IB47632, St. Louis, Missouri), and reverse transcription was performed using Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific #K1642, Waltham, MA) following manufacturers protocol. Real time-quantitative PCR and melt curve analysis were performed using Maxima SYBR-Green qPCR master mix (Thermo Fisher Scientific #K0241) and a T100 thermal cycler (Biorad #1861096, Hercules, CA). RNA and DNA concentrations were estimated using Nanodrop2000 (Thermo Fisher Scientific, Waltham, MA) spectrophotometer. Primers were obtained from Sigma-Aldrich for the following human genes and primer efficiency was confirmed: GAPDH, b-Actin, GH1, GHR, GHRHR, SOCS2, IGF1, IGF1R, IGF2, IGF2R, PRL, PRLR, Ins, InsR, IGFBP2, IGFBP3, EGFR, HGF, cMET and ERBB3. Each sample was a pool of two replicates per experiment; Experiments were done at least three times. Each qPCR for each gene and each treatment for every cell type was performed in triplicates.

3.3.4. Protein extraction

Total protein was collected 60 hr post transfection. The conditioned growth media for each treatment type were collected separately for subsequent analysis of secreted proteins. Total protein was extracted from the cells using RIPA buffer (Sigma-Aldrich
mixed with 1.5X Halt protease and phosphatase inhibitor cocktail (Thermo-Fisher #78442, Waltham, MA), following manufacturer’s protocol. Briefly, cells were washed twice with chilled sterile 1X phosphate buffered saline (PBS). Thereafter chilled RIPA buffer at 1 mL per million cells were added and incubated for 5 min / 4C. Then the cells were rapidly scraped with a cell-scaper to lyse residual cells. The cell lysate was clarified by centrifuging at 8,000 Xg / 10 min / 4C and the supernatant was collected and stored at -80C for subsequent use. Each sample was a pool of three replicates per experiment; Each experiment was done three times. Protein concentration was estimated in duplicates and two dilutions (1:2, 1:4) using Bradford reagent (Sigma-Aldrich #B6916) and 1 mg/mL bovine serum albumin as standard. Absorbance at 595 nm was measured using Spectramax250 (Molecular Devices, Sunnyvale, CA) and SoftmaxPro v4.7.1 software.

3.3.5. Western blot (WB)

The protocol closely follows the methods used in the laboratory (Sackmann-Sala et al., 2014) with few modifications (Akasimitiene et al., 2007). Briefly, intracellular proteins were subjected to gradient (4-16%) SDS-PAGE and transferred to nitrocellulose membrane by wet-transfer method at 70 mA / 14 hr / 4C, and blocked with 5% non-fat dry milk (NFDM) or 5% bovine serum albumin (BSA) in 1X TBS-T (Tris buffered saline, pH7.2 with 0.1% Triton-X100) for 12-16 hr / 4C with gentle rocking. Membranes were then incubated with primary antibody (at specific dilutions mentioned below) for 12-16 hr / 4C with gentle rocking. Membranes were then washed for 5 min / 3 times with 1X TBS-T and incubated with corresponding secondary antibodies (at specific dilutions
mentioned below) for 2 hr / 25C. Membranes were then washed for 5 min / 4 times with 1X TBS-T, treated with West Femto Chemiluminiscence detection reagents (Thermo Fisher Scientific) and chemiluminiscence signal was captured using a GelDoc (Biorad) fluorescence reader. Densitometric analysis of the blots was done by measured band-intensity from the area-under-curve using ImageJ software.

Primary antibodies were used to detect the following human proteins: GH (Rabbit, 1:100, Abcam #ab155276), GHR (Mouse, 1:300, SCBT #137185; Goat, 1:100, R&D Systems #AF1210; Rabbit, 1:200, Abcam #ab134078), STAT5 (Rabbit, 1:100, CST #9358S), P(Y694/Y699)-STAT5 (Rabbit, 1:100, ActiveMotif #39617, 39618), P(Y701)-STAT1 (Rabbit, 1:100, CST #7649), P(Y705)-STAT3 (Rabbit, 1:100, CST #9145), STAT3 (Rabbit, 1:200, CST #4904), STAT1 (Rabbit, 1:200, CST #9175), p44/42 MAPK (Erk1/2) (Rabbit, 1:2000, CST #9102S), P-p44/42 MAPK (Erk1/2) (Rabbit, 1:3000, CST #4370P), Akt (Rabbit, 1:2000, CST #4685S), P-Akt (Rabbit, 1:1000, CST #4058S), P-Jak2 (Rabbit, 1:200, GeneTex#61122; Rabbit, 1:100, CST #8082), JAK2 (Mouse, 1:200, Sigma Aldrich # SAB4200483), mTOR (Rabbit, 1:1000, CST #2983), P-mTOR (Ser2448) (Rabbit, 1:2000, CST #5536), P-mTOR (Ser2481) (Rabbit, 1:2000, CST #2974), Raptor (Rabbit, 1:500, CST #2280), Rictor (Rabbit, 1:500, CST#2114), GbL (Rabbit, 1:1000, CST #3274), Actin (Goat, 1:3000, SCBT #sc1616), GAPDH (Goat, 1:3000, SCBT #sc20357), P(S1524)-BRCA1 (Rabbit, 1:500, CST#9009), P(S139)-histone H2A.X (Rabbit, 1:1000, CST #9718), histone H2A.X (Rabbit, 1:1000, CST #2595), Caspase-3(Rabbit, 1:1000, CST#9665), cleaved (Asp175)-Caspase-3 (Rabbit,
1:1000, CST #9664), P(Y416)-SFK (Rabbit, 1:200, CST #2101), P(Y416)-SRC (Rabbit, 1:200, CST #6943), SRC (Rabbit, 1:500, AbcaM #47405).

Secondary antibodies used: anti-rabbit HRP-linked IgG (Donkey, 1:2000, CST #7074P2), anti-goat HRP-linked IgG (Donkey, 1:1000, SCBT #sc2020), anti-rabbit HRP-linked IgG (Donkey, 1:2000, GE #NA934), anti-mouse HRP-linked IgG (Rat, 1:1000, Antibodies Online #ABIN1589975).

3.3.6. Immunofluorescence (IF)

Cells were seeded at 10,000 cells/cm$^2$ in 8-well chamber slides and transfected was performed as described above. Transfection media was replaced with antibiotic containing complete growth media after 24 hours and cells were fixed after 36 hours more (a total of 60 hr post-transfection), and cells were washed twice with 1X PBS and fixed with 4% freshly-prepared formaldehyde (pH6.9) / 15 min / 25C (using 100% methanol for fixation gave equally good results). Cells were permeabilized with 0.2% Triton-X100 in 1X PBS / 15 min / 25C, followed by blocking with 1% BSA / 4 hr / 25C. Incubation time was 12hr / 4C for primary antibody and 2 hr / 25C for secondary antibody. Finally, the slides were washed four times with 1X PBS and the sample was mounted with Fluoroshield mounting medium containing DAPI (Abcam #ab104139, Cambridge, UK), covered with a 60 mm coverslip and the edges were sealed with nail-polish and stored at 4C for microscopy. Microscopic imaging was done using a Nikon Eclipse E600 compound fluorescent microscope fitted with a Nikon DS-Fi1CC camera (Nikon, Tokyo, Japan) and NIS-Elements BR3.2 imaging software. Antibodies used – Rabbit anti-human-Ki67 monoclonal antibody with AlexaFluor488 tag (Abcam
#ab154201, 1:300 dilutions); Rabbit anti-human GHR monoclonal antibody (Abcam #ab134078, 1:250 dilution); Goat anti-rabbit secondary antibody with AlexaFluor488 tag (Life Technologies #R37116, 1:500 dilution).

3.3.7. Cell proliferation assay

A 1% (w/v) resazurin (Sigma-Aldrich #R7017) solution in 1X PBS was made and filter-sterilized. The final concentration of resazurin in the assay was 0.004%. Inside the proliferating cells mildly fluorescent blue resazurin is reduced to a bright pink fluorescent product called resorufin (stable for 4 hr), which is a quantitative measure of the percentage of proliferating cells. In all cases, cells were incubated at 37°C / 5% CO₂ for 45-60 minutes for adequate sensitivity of detection. Briefly, cells were seeded at 10,000 cells/cm² into 96-well plates and transfected as described above. Resazurin assay was performed 60 hr after transfection (unless specified otherwise) and resorufin absorbance was measured at 570 nm (reference wavelength = 600 nm) using Spectramax250 (Molecular Devices, Sunnyvale, CA) and SoftmaxPro software.

3.3.8. Cell migration assay

Cell migration assays are standard methods of estimating the repair and regenerative properties of cells (Kramer et al., 2013). For our purpose, we used the Radius cell migration assay design from Cell Biolabs (Cell Biolabs #CBA-125, San Diego, CA) and experiments were performed as per manufacturer’s protocol. In this assay, a 24-well plate containing a non-toxic, 0.68 mm biocompatible hydrogel spot where cells cannot attach. siRNA treated cells were trypsinized 48 hr. after transfection,
counted and seeded at 5000 cells/well in a pretreated hydrogel spot containing 24-well plate. The hydrogel spot was gently removed after 24 hours incubation at 37°C / 5% CO₂. The cells were allowed to migrate for up to 48 hr at 37°C / 5% CO₂. Image was taken every 24 hr using a 4X objective (total magnification 40X) using an inverted Olympus IX70 microscope fitted with a Retiga 1300 camera (QImaging, Surrey, BC). Total uncovered area at the beginning and end of assay were quantitated using ImageJ software. Experiments were done in triplicates for statistical significance.

3.3.9. Cell invasion assay

The metastatic potential of a tumor is reflected by its ability to invade into surrounding tissues. Out of several invasion assay designs, we chose the 96-well 3D spheroid BME cell invasion assay (Trevigen, Gaithersburg, MD) since growing evidence indicates that a tumor spheroids are better representatives of tumors in-vivo, compared to tumor cells in a Boyden chamber, as is used in multiple invasion assay designs. Briefly, siRNA (scramble or GHR specific) treated melanoma cells were trypsinized 48 hr. after transfection, counted and seeded at 5000 cells/well in a 96-well spheroid formation plate and incubated for 72 hours at 37°C / 5% CO₂ to allow spheroid formation. Thereafter the invasion matrix was added, followed by 50 ng/mL hGH-containing culture medium as a chemoattractant. The invasive behavior of the cell was monitored every 24 hr. for up to 72 hr. Image was taken every 24 hr using a 4X objective (total magnification 40X) using an inverted Olympus IX70 microscope fitted with a Retiga 1300 camera (QImaging, Surrey, BC). Total pixels at the beginning and end of assay were quantitated using ImageJ software. Experiments were done in triplicates for statistical significance.
3.3.10. Clonogenicity assay

Colony formation on soft agar or anchorage independent colonization is considered to be a very stringent test for malignant transformation of cells and a hallmark of cancer. Ability of the tumor cell to develop colonies on soft agar reflects a reduced dependence for extracellular growth promoting factors, independence from the control of neighboring cells (like keratinocytes in the case of melanocytes) and infinite capacity to proliferate. For our purpose, we chose the CytoSelect 96-well format (Cell Biolabs #CBA-130, San Diego, CA), which provides a timely (one week) and quantitative (fluorometric) readout of the total colonies formed. Experiments were performed as per manufacturer’s protocol. Briefly, a 0.6% base agar medium containing 1X RPMI-1640 (10% FBS) was prepared and allowed to settle for 30 min / 4°C. siRNA treated cells were trypsinized 48 hr. after transfection, counted and seeded at 5000 cells/well in a 0.4% top agar layer also containing 1X RPMI-1640 (10% FBS) and allowed to settle for 15 min / 4°C. Finally, 100 uL of pre-warmed culture media containing 50 ug/mL hGH was added on top and incubated for 7 days at 37°C / 5% CO2. The media was then removed, the agar was solubilized and the cells were lysed in situ. Total DNA content was measured using the CyQuant GR dye (kit component) and fluorescence was measured at 485 (ex) / 520 nm (em) in a spectramax M2 fluorescence plate reader (Molecular Devices, Sunnyvale, CA) and SoftMax Pro v6.2.1 software. Experiments were done in quadruplicate for statistical significance.
3.3.11. Statistical analyses

Parametric and non-parametric statistical analyses for comparing RNA expression levels were done using R software (ver3.0.2). For RT-qPCR analysis of RNA expressions, the levels are to be normalized first against two reference genes (GAPDH and beta-actin) and the $2^{\Delta\Delta C_{T}}$ values were compared by Wilcoxon signed rank test for significance. A $p$-value less than 0.05 was considered as significant. The densitometric analyses, clonogenicity, migration and invasion, and resazurin based assays, are compared by a paired students T-test and ANOVA was performed (using GraphPad Prism software) to compare for significance ($p<0.05$ is considered significant).

3.4. Results

3.4.1. GHR expression in melanoma cells was abrogated by siRNA mediated knock-down

The four human melanoma cells selected for this study are reported to express GHR transcripts and are responsive to exogenous hGH treatments (Suatarsic et al., 2013). However the protein level expressions of GHR in these cell lines are not known. We optimized the siRNA and concentration as well as transfection efficiency for the melanoma cell lines first (Figures 9 and 10). The optimum siRNA concentration used in all our experiments is 20 nM.

In order to verify RNA transcript levels and identify protein level expression of GHR in the tumor cells, we subjected the RNA collected 48 hr. post-transfection from the cells to RT-qPCR analysis using primers against GHR coding exons. Our results showed high levels of GHR RNA in all four melanoma cells which was inhibited by almost 90% following GHR-KD (Figure 11, top panel). We also analyzed the protein level expression
of GHR in the melanoma cell lines to confirm whether the marked decrease in RNA levels also translated to the protein expressions. Cell lysates collected 60 hr. post-transfection showed an almost complete inhibition of protein level expression of GHR, following the siRNA treatment, when compared to the corresponding scramble-transfected controls (Figure 11, bottom panel) in the WB analysis. Densitometric analyses of the blots confirmed a significant (70%-95%) reduction in the protein level expression of GHR in the melanoma cells. In order to further validate our results, we performed immunofluorescence staining for GHR on these cells, 60 hr. post-transfection. We observed differential yet high levels of expression of GHR in the cells, with the GHR protein levels being in an ascending order from SKMEL-5, MDAMB-435, MALME-3M and SKMEL-28. The trend was also seen in the WB from the cell lysates. Following siRNA mediated GHR-KD, we observed a dramatic decrease in the immunofluorescence levels in all the four cell lines, compared to the scramble-siRNA treated controls (Figure 12).

3.4.2. GH treatment or GHR-KD modulates RNA levels of multiple GH-associated genes in melanoma cells

GH-GHR action is known to overlap with or affect multiple signaling molecules in the cell, namely PRL, insulin (Ins), insulin-like growth factors 1 and 2 (IGF-1, and IGF-2) and their respective cognate receptors – PRL receptor (PRLR), insulin receptor (IR), IGF-1 receptor (IGF1R) and IGF-2 receptor (IGF2R). In addition, GH action is strongly correlated with IGF binding proteins (IGFBP) like IGFBP-2 and IGFBP-3.
Figure 9: siRNA transfection of SK-MEL-28 cells. SK-MEL-28 cells were plated at 10,000 cells/cm² and treated with either 20 nM GHR-specific siRNA (siRNA-A - 1b, 2b; siRNA-B – 1c,2c; siRNA-C, 1d, 2d) or scramble-siRNA (1e,2e). Untreated cells are treated with only transfection reagent (1a, 2a). Cells were photographed in grayscale (top row) and at 630 nm (bottom row). A Cy3-siRNA duplex at 10 nM was used as reporter. Red fluorescence indicates successful transfection. Identical results were obtained with SK-MEL-5, MALME-3M and MDA-MB-435 cells (data not shown here).

Figure 10: Optimizing siRNA effect of GHR knock-down (KD) on melanoma cell proliferation. SK-MEL-28 (a), MALME-3M (b) and SK-MEL-5 (c) cells were transfected with 20 nM of either GHR-siRNA or scramble siRNA for 24 hr. and cell proliferation was checked 60 hr. post-transfection using 0.04% resazurin (described in methods) and absorbance was read at 570 nm (600 nm = reference wavelength). Data for MDA-MB-435 (similar results) not shown here. [* , p < 0.05, Students t-test]
Figure 11: GHR RNA and protein levels are suppressed following siRNA mediated KD in melanoma cells. **Top panel** – RT-qPCR analysis of GHR RNA levels in SKMEL-28, SKMEL-5, MDAMB-435 and MALME-3M melanoma cells transfected with scramble or GHR specific siRNA. RNA was collected 48 hr. post-transfection. Almost 90% reduction in mRNA levels was achieved in all four cell lines. Expressions were normalized against expression of beta-actin and GAPDH as reference genes. [*, p < 0.05, Wilcoxon sign rank test, n = 6] **Bottom panel** – Densitometric analyses of the protein level expression of GHR in scramble or GHR-siRNA transfected samples, from western-blot analyses of cell lysates collected 60 hr. post-transfection. Significant decrease in GHR expression was achieved by siRNA-mediated GHR knockdown. [*, p < 0.05, paired t-test, n = 3]
Figure 12: GHR expression is abrogated following siRNA mediated KD in melanoma cells. Clockwise from top right – SKMEL-28, SKMEL-5, MDAMB-435 and MALME-3M melanoma cells transfected with scramble or GHR specific siRNA. In each of the four boxes, top row (A) shows melanoma cells transfected with scramble si-RNA while the bottom row (B) shows melanoma cells transfected with GHR-siRNA. In each box the left column shows cellular DNA stained with DAPI (blue) while the right column shows the same cells labeled with AlexaFluor 488 (green) -conjugated (goat) secondary antibody to rabbit IgG specific for hGHR. From the average of four pictures per cell, maximum GHR-specific fluorescent signal was in the order of SKMEL-28 > MALME-3M > MDAMB-435 > SKMEL-5 – a trend also seen in WB analyses. ICC/IF was performed on cells 48 hours after transfection.

In the context of the known importance of each of these molecules in tumorigenicity and cancer aggressiveness, we specifically chose to investigate their levels
following the perturbation of the GH-GHR axis by either addition of exogenous hGH or GHR-KD.

We found considerable expression of autocrine GH in all four melanoma cells tested. We not only detected GH at the RNA level (Figures 13a, 14a, 15a, 16a), but it was also detectable by WB analysis. We intended to look at both spectrums of GH/GHR action. Therefore on one hand we added recombinant active hGH to the melanoma cells at increasing doses of 0, 5, 50 and 150 ng/mL. On the other hand following GHR-KD, we separately treated the cells with 0 and 50 ng/mL hGH, to evaluate the effects of GHR-KD in absence and presence of exogenous hGH. The results are tabulated later as a heat-map format in Figure 17. We describe below the results of RNA expression analysis for each cell type individually.

- **SK-MEL-28**: With increasing concentration of exogenously added hGH, the autocrine GH transcript levels increased significantly (Figure 13a), while the PRL (Figure 13b) and IGF1 (Figure 13c) levels remained unchanged. We observed the intrinsic RNA levels of GH, PRL and IGF1 were low and in the order of GH > PRL > IGF1 (data not shown here). However, when the GHR was knocked down, we observed a significant rise in the levels of all three cytokines especially in presence of 50 ng/mL of added hGH (Figures 13a, 13b, 13c). We in fact observed a decrease in GH levels and no increase in the PRL levels following GHR-KD when the cells were only under autocrine GH control (Figures 13a, 13b, 13c).

Treatment with increasing concentrations of hGH induced an increase in the RNA levels of GHR, while the PRLR and IGF1R levels remained unchanged (Figures
13d, 13e, 13f). We observed the intrinsic RNA levels of GHR and IGF1R were high (IGF1R > GHR), while the PRLR expressions were quite low (data not shown here). When the GHR levels were knocked down by almost 90% (Figure 13d), we observed a concurrent increase in PRLR (also in absence of exogenous hGH) and IGF1R only in presence of externally added hGH (Figures 13e and 13f). We did not detect any RNA level expression of IGF2 or insulin in any of the four melanoma cell lines. However, we observed a very high level of IGF2R and also IR on SK-MEL-28 melanoma cells, indicating that they might be highly responsive to insulin and IGF levels – an important observation in the context of the fact that insulin resistance, which includes the presence of excess insulin in circulation, has been repeatedly associated with increased melanoma incidence (Haring et al., 1984; Antoniadis et al., 2011). We observed an hGH dose-dependent increase in IGF2R RNA levels but not in IR expression levels (Figures 13h and 13i). GHRHR presence in primary human melanoma cells has been reported (Chatzistamou et al., 2008) and GHRHR is known to drive accelerated proliferation of pituitary somatotrophs (Zeitler et al., 1998) and neuroendocrine tumors (Doga et al., 2001). We observed low yet consistent level of expression of GHRHR in all the four melanoma cell lines (Figures 13g, 14g, 15g, and 16g) in this study. In SK-MEL-28 cells, we observed an increasing trend in the GHRHR transcript levels in presence of exogenous hGH, which was significantly decreased on GHR-KD (Figure 13g). In our study, expression levels of suppressors of cytokine signaling -2 (SOCS-2) - a critical negative regulator of
GH action (Turnley, 2005) and IGF1R activity (LeRoith et al., 2005) – was found to rise significantly with increasing levels of hGH and also dropped drastically following GHR-KD (Figure 13i). IGF-binding proteins 2 and 3 were expressed at relatively high levels in SK-MEL-28 and are known to have differential roles in melanoma progression. Increasing IGFBP2 level has been correlated with progression to metastasis (Wang et al., 2003) and actively drives proliferation (Das et al., 2013) in melanoma. We here report an increase in IGFBP2 RNA transcript levels in SK-MEL-28 cells at high GH levels and a significant decrease following GHR-KD (Figure 13j). On the other hand, the IGFBP3 is known to have an anti-tumor effect in cancer and its concentration decreases markedly in circulation of cancer patients (Napsi et al., 2014). However, some also report IGFBP3 to have an oncogenic potential with drastic increase in expression in cultured human melanoma cells (Xi et al., 2006). In our investigation, SK-MEL-28 cells were found to have an upregulation in IGFBP3 levels following hGH addition, which remained consistently high in these cells even following GHR-KD (Figure 13k).

- MALME-3M: With increasing concentration of exogenously added hGH, the autocrine PRL RNA transcript levels increased in a dose dependent manner (Figure 14b), while the GH (Figure 14a) and IGF-1 (Figure 14c) levels remained unchanged in MALME-3M cells. Following GHR-KD, exogenous GH-stimulated increase in PRL transcript levels were significantly reduced (Figure 14b). However, similar to our observation in the case of SK-MEL-28 cells, we observed
a significant increase in IGF-1 levels following GHR-KD in MALME-3M, both in absence and presence of excess hGH (Figure 14c). When the GHR expression was knocked down by more than 90% (Figure 14d), we observed a significant increase in the level of PRLR (Figure S3e) and IGF1R (Figure 14f), albeit only at high concentrations of added hGH. The GHRHR expression levels significantly decreased on GHR-KD irrespective of the presence of excess GH (Figure 14g). We also observed a significant drop in the levels of the abundantly expressed IGF2R on GHR-KD (Figure 14i) while the IR levels increased only in presence of excess GH (Figure 14h). The IGFBP2 levels remained constant with increasing doses of hGH and decreased significantly following GHR-KD (Figure 14j). We had interesting observations with the RNA level expressions of IGFBP3 in MALME-3M cells. GHR-KD caused a significant reduction at basal GH levels (no hGH added) while addition of 50 ng/mL hGH reversed the effect (Figure 14k). The SOCS2 RNA levels in MALME-3M decreased significantly on GHR-KD irrespective of presence or absence of excess hGH (Figure 14l).

- MDA-MB-435: The endogenous GH and PRL levels in MDA-MB-435 cells were unchanged (Figures 15a and 15b) but IGF-1 levels increased significantly at high levels of added hGH (Figure 15c). Blocking GHR expression resulted in significant increase in the transcript levels of GH, PRL and IGF1, especially in presence of 50 ng/mL hGH (Figures 15a, 15b and 15c). The PRLR levels were significantly increased on addition of GH and remained unchanged even following GHR-KD (Figure 15e). Similar to our observations in the previous two
cases, GHR-KD resulted in a steep rise in the IGF-1 transcript levels which was higher in presence of exogenous GH (Figure 15c). PRLR increased in a dose dependent manner with excess GH (Figure 15e) and did not decrease appreciably when the GHR expression was decreased by almost 90%. Similar to our observations with SK-MEL-28 and MALME-3M, we observed an increase in IGF1R levels following GHR-KD in MDA-MB-435 cells in presence of 50 ng/mL hGH (Figure 15f). Also, similar to the other two cell lines discussed here above, MDA-MB-435 cells showed a marked decrease in the GHRHR transcript levels following GHR-KD (Figure 15g). GHR-KD also caused a significant drop in the abundantly expressed IR (Figure 15h) but not in IGF2R (Figure 15i). IGFBP2 expression levels increased significantly on GH addition and was unaffected by GHR-KD (Figure 15j). However, in absence of excess GH, GHR-KD caused a significant decrease in the IGFBP2 levels (Figure 15j). Similar to our observations in MALME-3M cells, the presence of excess GH led to a marked increase in IGFBP3 levels following GHR-KD (Figure 15k). The SOCS-2 levels significantly increased in response to added GH (Figure 15l). GHR-KD caused a downregulation of SOCS2 levels in MDA-MB-435 cells, which was absent when there was excess GH (Figure 15l).

- SK-MEL-5: In SK-MEL-5 cells, the endogenous levels of GH and IGF1 were unaffected by added GH (Figures 16a and 16c) but the PRL levels increased in a dose dependent manner (Figure 16b). Following GHR-KD, the GH and IGF1 levels remained unchanged but increased significantly in presence of excess GH.
(Figures 16a and 16c). The steep rise in IGF1 transcript levels with GHR-KD was thus consistently observed in all the four human melanoma cell lines in this study, which was significant mostly in presence of high levels (50 ng/mL) of added GH. The PRL and PRLR levels were significantly reduced on GHR-KD (Figures 16b and 16e) while the IGF1R levels did not change (Figure 16f) in SK-MEL-5 cells. The GHRHR levels increased on excess GH treatment but were significantly reduced following GHR-KD (Figure 16g). Following GHR-KD we also noticed significant increase in the IR levels (Figure 16h) but a decrease in the IGF2R levels (Figure 16i). Further, consistent with our observations in the above three cell lines, SK-MEL-5 cells also showed a significant drop in IGFBP2 levels following GHR-KD (Figure 16j). Also, similar to our observations in the case of MALME-3M and MDA-MB-435, we saw a significant increase in the IGFBP3 levels following GHR-KD irrespective of the presence or absence of exogenous GH (Figure 16k). The SOCS2 levels in SK-MEL-5 increased in a dose-dependent manner with GH addition while GHR-KD significantly reduced the same (Figure 16l).
Figure 13: Comparison of changes in RNA level expression of key components of GH/IGF-1 axis in SK-MEL-28 cells. Relative RNA expression was quantified for GH, PRL, IGF1, GHR, PRLR, IGF1R, IGF2R, GHRHR, IGFBP2, IGFBP3 and SOCS2 in SK-MEL-28 melanoma cells following addition of 0, 5, 50 and 150 ng/mL hGH or following GHR-KD, in presence or absence of 0 and 50 ng/mL hGH. In all cases, exogenous hGH treatment was for 24 hr. Expressions were normalized against expression of beta-actin and GAPDH as reference genes. [* , p < 0.05, Wilcoxon sign rank test, n = 4]
Figure 14: Comparison of changes in RNA level expression of key components of GH/IGF-1 axis in MALME-3M cells. Relative RNA expression was quantified for GH, PRL, IGF1, GHR, PRLR, IGF1R, IGF2R, GHRHR, IGFBP2, IGFBP3 and SOCS2 in MALME-3M melanoma cells following addition of 0, 5, 50 and 150 ng/mL hGH or following GHR-KD, in presence or absence of 0 and 50 ng/mL hGH. In all cases, exogenous hGH treatment was for 24 hr. Expressions were normalized against expression of beta-actin and GAPDH as reference genes. [* p < 0.05, Wilcoxon sign rank test, n = 4]
Figure 15: Comparison of changes in RNA level expression of key components of GH/IGF-1 axis in MDA-MB-435 cells. Relative RNA expression was quantified for GH, PRL, IGF1, GHR, PRLR, IGF1R, IGF2R, GHRHR, IGFBP2, IGFBP3 and SOCS2 in MDA-MB-435 melanoma cells following addition of 0, 5, 50 and 150 ng/mL hGH or following GHR-KD, in presence or absence of 0 and 50 ng/mL lGH. In all cases, exogenous hGH treatment was for 24 hr. Expressions were normalized against expression of beta-actin and GAPDH as reference genes. [* p < 0.05, Wilcoxon sign rank test, n = 4]
Figure 16: Comparison of changes in RNA level expression of key components of GH/IGF-1 axis in SK-MEL-5 cells. Relative RNA expression was quantified for GH, PRL, IGF1, GHR, PRLR, IGF1R, IGF2R, GHRHR, IGFBP2, IGFBP3 and SOCS2 in SK-MEL-5 melanoma cells following addition of 0, 5, 50 and 150 ng/mL hGH or following GHR-KD, in presence or absence of 0 and 50 ng/mL hGH. In all cases, exogenous hGH treatment was for 24 hr. Expressions were normalized against expression of beta-actin and GAPDH as reference genes. [* , p < 0.05, Wilcoxon sign rank test, n = 4]
Figure 17: Results of significant changes in GH/IGF gene expressions following GHR-KD or treatment with hGH. Results are tabulated based on Figures 13, 14, 15 and 16 and corresponding discussion in the main article for all the four melanoma cell lines used in this study.

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Corresponding discussion in the main article for all the four melanoma cell lines used in this study.
3.4.3. GH treatment enhances while GHR-KD suppresses expression levels of key oncogenic regulators in melanoma

Based on recent scientific reports, we estimated the presence and change in expression levels with perturbations in GH axis of four important drivers of melanomagenesis and progression – the autocrine system of hepatocyte growth factor (HGF) and its cognate receptor tyrosine kinase MET, and ERBB family members epidermal growth factor receptor (EGFR) and Erb-B2 receptor tyrosine kinase 3 (ERBB3 or HER3). All of them have been established to be critical drivers of aggressive disease progression and contributors to drug resistance in melanoma (Vergani et al., 2011; Wilson et al., 2012; Tsao et al., 2012; Girotti et al., 2013; Chattopadhyay et al., 2014; Cao et al., 2015; Zhang et al., 2016). We observed consistently high levels of MET and ERBB3 transcripts in all four melanoma cell lines and very low but detectable levels of endogenous HGF (Figure 18). We also detected EGF4RNA level expressions in all the four melanoma cell lines except in MALME-3M but found no significant variation with excess GH (upto 150 ng/mL or GHR-KD) (data not shown here). In SK-MEL-28 and MDA-MB-435 cells we observed dose dependent increase in HGF levels with increasing doses of hGH (Figures 18a and 18g). Both basal and increased levels of HGF were significantly suppressed following GHR-KD in all the four cell lines (Figure 18a, 18d, 18g, 18j). MET expression levels were significantly upregulated in a dose-dependent manner with added GH in three of the four melanoma cell lines (MET transcript levels decreased at maximum GH concentration tested – Figure 18k). We also observed a very consistent downregulation of MET levels following GHR-KD in both presence and
absence of excess GH, in all the four melanoma cell lines (Figures 18b, 18e, 18h, 18k). ERBB3 showed a significant rise in expression levels with increasing dose of GH in SK-MEL-28 and MDA-MB-435 cells, while the basal levels remained unresponsive to excess GH in the other two cell lines (Figures 18c, 18f, 18i, 18l). However, we observed a uniform and robust downregulation of ERBB3 transcript levels following GHR-KD in all the four cell lines (Figures 18c, 18f, 18i, 18l) irrespective of the presence of excess GH.
Figure 18: Comparison of changes in RNA level expression. Relative RNA expression was quantified for HGF, MET and ERBB3 in SK-MEL-28 (a, b, c), MALME-3M (d, e, f), MDA-MB-435 (g, h, i), and SK-MEL-5 (j, k, l) melanoma cells following addition of 0, 5, 50 and 150 ng/mL hGH or following GHR-KD, in presence or absence of 0 and 50 ng/mL hGH. In all cases, exogenous hGH treatment was for 24 hr. Expressions were normalized against expression of beta-actin and GAPDH as reference genes. [* p < 0.05, Wilcoxon sign rank test, n = 4]
3.4.4. *GH treatment enhances while GHR-KD suppresses phosphorylation states of key intracellular signaling pathway intermediates in melanoma cells*

In order to assess the effects of GHR-KD on the activation states of GH regulated shared oncogenic signaling pathways, we treated the scramble or GHR-specific siRNA transfected melanoma cells, 60 hr. post-transfection with 50 ng/mL of GH for 10 minutes, lysed and collected the total protein. In the WB analysis, we observed robust dependence of key signaling pathways on the activity of GH-GHR pair. In the absence of exogenous GH, we detected basal phosphorylation levels in AKT (Figure 19A), mTOR (Figure 19B), ERK1/2 (Figure 20A) and SRC (Figure 22). We observed base level phosphorylation also for JAK2 in absence of external GH (Figure 20B) indicating possible activity of the autocrine GH. On addition of 50 ng/mL hGH we observed significant increase in phosphorylation levels in all the four cell lines across the aforementioned signaling pathways, indicating robust response to GH-GHR activity. We also compared the phosphorylation states of STATs 1, 3 and 5 in this study (Figure 21). We observed moderate to high basal phosphorylation states of STATs 1 (Figure 21A) and 3 (Figure 21B) but not STAT-5 (Figure 21C), in absence of exogenous GH. Only MDA-MB-435 cells exhibited a strong basal STAT5 phosphorylation (Figure 21C). Addition of 50 ng/mL hGH significantly increased the phosphorylation levels of all three STATs within ten minutes.

GHR-KD had a drastic effect on the phosphorylation states of the above signaling molecules. Additionally, excess GH induced robust activation of AKT, mTOR, ERK1/2, SRC, JAK2, STAT1, STAT3 and STAT5 was significantly reduced to below basal levels
in all the cases (Figures 19, 20, 21 and 22). Only the p-STAT3/STAT3 ratio in MDA-MB-435 cells remained consistently high following GHR-KD (Figure 21B).

In our WB analysis, we further evaluated change in expression levels of phospho-serine 139-histone H2A.X and phospho-serine 1524-BRCA1 – established markers of DNA damage (Sharma et al., 2012; Aleskandarany et al., 2015). We observed a significant increase in phospho-H2A.X to total H2A.X ratios following GHR-KD in all the cell lines (Figure 23), indicating that absence of GH action can lead to an accumulation of DNA damage. We also checked the amounts of total and cleaved caspase-3 as a marker of apoptosis but did not find significant changes in the levels in either of the cell lines (Figure 24). A separate detailed analysis of the apoptotic pathway in absence and presence of GH action is necessary to infer on the effect of GHR reduction on the apoptotic pathway in melanoma.
Figure 19: Comparison of GH-induced phosphorylation states of AKT and mTOR following GHR-KD. Changes in phosphorylation levels of (A) AKT and (B) mTOR in all four melanoma cells, ten mins after induction with 50 ng/mL hGH, in presence and absence of GHR-KD was compared 60 hr. post-transfection. WB was done against phospho–(serine 473)-AKT and phospho–(serine 2448)–mTOR antibodies. Blots were quantified and mean of three blots per sample was taken. Expressions were normalized against expression of B-actin. The p-AKT/AKT or p-mTOR/mTOR ratio was specifically compared as a true indicator of relative level of phosphorylation. [* , p < 0.05, Students t test, n = 3]
Figure 20: Comparison of GH-induced phosphorylation states of ERK1/2 and JAK2 following GHR-KD. Changes in phosphorylation levels of (A) ERK1/2 and (B) JAK2 in all four melanoma cells, ten mins after induction with 50 ng/mL hGH, in presence and absence of GHR-KD was compared 60 hr. post-transfection. WB was done against phospho–threonine 202 / tyrosine 204-ERK1/2 and phospho–tyrosine 1008–JAK2 antibodies as well as against total ERK1/2 and JAK2. Blots were quantified and mean of three blots per sample was taken. Expressions were normalized against expression of B-actin. The p-ERK/ERK or p-JAK2/JAK2 ratio was specifically compared as the indicator of relative level of phosphorylation. [*; p < 0.05, Students t test, n = 3]
Figure 21: Comparison of GH-induced phosphorylation states of STAT1, STAT3 and STAT5 following GHR-KD. Changes in phosphorylation levels of (A) STAT1, (B) STAT3 and (C) STAT5 in all four melanoma cells, ten mins after induction with 50 ng/mL hGH, in presence and absence of GHR-KD was compared 60 hr. post-transfection. WB was done against phospho–tyrosine 701-STAT1, phospho-tyrosine 705-STAT3 and phospho–tyrosines 694 and 699–STAT5 antibodies, as well as against total STAT1, STAT3 and STAT5. Blots were quantified and mean of three blots per sample was taken. Expressions were normalized against expression of B-actin. The p-STAT/STAT ratio was specifically compared as the indicator of relative level of phosphorylation. [*; p < 0.05, Students t test, n = 3]
Figure 22: Comparison of GH-induced phosphorylation states of SRC family kinases (SFK) following GHR-KD. Changes in phosphorylation levels of SRC kinases were evaluated using two different antibodies. We used Cell Signaling Technology antibodies (A) #2101; polyclonal and (B) #6943; monoclonal, both of which identify SRC only when phosphorylated at tyrosine 416. WB comparison was done in all four melanoma cells, ten mins after induction with 50 ng/mL hGH, in presence and absence of GHR-KD was compared 60 hr. post-transfection. WB was also done for total SRC. Blots were quantified and mean of three blots per sample was taken. Expressions were normalized against expression of B-actin. The p-SRC/SRC ratio was specifically compared as the indicator of relative level of phosphorylation. [*p < 0.05, Students t test, n = 3]
Figure 23: Change in markers of DNA damage following GHR-KD. Changes in phosphorylation levels of BRCA1 and histone H2A.X were evaluated using antibodies recognizing phospho-serine 1524-BRCA1 and phospho-serine 139- H2A.X. The p-H2A.X/H2A.X ratio was specifically compared as the indicator of relative level of phosphorylation. The p-BRCA1 values were significantly upregulated following GHR-KD but p-BRCA1/BRCA1 ratio was not evaluated. Cells were grown in presence of 50 ng/mL exogenous GH and protein level changes were estimated 60 hr. post-transfection. [* p < 0.05, Students t test, n = 3]
3.4.5. GH treatment enhances while GHR-KD suppresses melanoma cell proliferation

We next evaluated the response of the four melanoma cell lines to moderate to high doses (5, 50 and 150 ng/mL) of exogenous GH. We observed a consistent and significant rise in proliferation levels in all the cell lines at and above 50 ng/mL GH (Figure 25). When GHR was knocked down in presence or absence of 50 ng/ml GH, we observed a significant and consistent decrease in cell proliferation all four melanoma cells (Figure 26). The results indicate a consistent and robust response to perturbation of GH action as a characteristic of all the melanoma cell lines we tested in this study. In addition GHR-KD clearly had a profound inhibitory effect on the overall cell viability as measured by the metabolic assay.
Figure 25: Effect of increasing GH levels on melanoma cell proliferation. (a) SK-MEL-28, (b) MALME-3M, (c) MDA-MB-435 and (d) SK-MEL-5 cells were treated with increasing doses of hGH for 48 hr. and cell proliferation was estimated using a resazurin-based metabolic assay. A significant increase in cell proliferation was noted at and above 50 ng/mL hGH treatment. Averages of at least four independent experiments performed in quadruplicate were taken. [*: p < 0.05, Students t-test]
Figure 26: Effect of GHR knock down (KD) on melanoma cell proliferation. (a) SKMEL-28, (b) MALME-3M, (c) MDA-MB-435 and (d) SK-MEL-5 cells were transfected with 20 nM scramble or GHR-siRNA for 24 hr. and grown for 48 hr. in presence or absence of 50 ng/mL hGH. Cell proliferation was estimated using a resazurin-based metabolic assay. A significant decrease in cell proliferation was noted following GHR-KD. Averages of at least four independent experiments performed in quadruplicate were taken. [* p < 0.05, Students t-test]

3.4.6. GHR-KD suppresses melanoma cell migration and invasion

Migration and invasion are critical parameters in tumoral interaction with its microenvironment and cancer metastasis (Friedl and Wolf, 2003; Zijl et al., 2011). Several types of invasion assays are performed to evaluate invasive properties of cancer cells (Shaw, 2005; Vinci et al., 2015). To assay the migratory capacity of the four melanoma cell lines, we allowed the transfected cells to converge on a small circular area in the center of the culture well for upto 48 hr. The percentage free area at the end time
point was calculated using ImageJ and represented a decrease in migration. We observed significantly reduced migration levels of all the four melanoma cells following GHR-KD (Figure 27 and 28). In order to confirm the effects of GHR-KD induced by si-RNA treatment within a relevant time, we chose a commercially available 3-dimensional spheroid assay over 3 three day time-point to visualize and quantitate the invasion properties of melanoma spheroids into a basement membrane protein containing hydrogel matrix with SK-MEL-28 and SK-MEL-5 cells, starting 48 hr. post-transfection with scrambled or GHR-siRNA. We observed significant suppression in invasion capacity of the melanoma cells following GHR-KD compared to scramble siRNA treated cells (Figure 29), consistent with reduced migration levels.

3.4.7. GHR-KD suppresses anchorage independent colony formation in melanoma cells

We performed the colony formation on soft agar assay which is a gold standard method for evaluating the malignant transformation of cells. In our experiments with SK-MEL-28, MDA-MB-435 and SK-MEL-5, we observed a significant reduction in colony formation following siRNA-mediated inhibition of GHR expression compared to scramble siRNA treated controls irrespective of the presence of 50 ng / mL GH (Figure 30).
Figure 27: Effect of GHR knock down (KD) on SK-MEL-28 cell migration. SK-MEL-28 cells transfected with 20 nM scramble (2a, b, c) or GHR-siRNA (3a, b, c) as well as un-transfected controls (1a, b, c) were allowed to migrate into a 0.68 mm circular spot at the center of the well, in presence of 50 ng/mL hGH for upto 48 hr. The percentage free area was calculated using ImageJ software and reflected the decrease/inhibition in migration. A significant decrease in migration was noted following GHR-KD. [* p < 0.05, Students t-test, n = 3]
Figure 28: Effect of GHR knock down (KD) on melanoma cell migration. (1) MALME-3M, (2) MDA-MB-435, and (3) SK-MEL-5 cells transfected with 20 nM scramble (a, b) or GHR-siRNA (c, d) were allowed to migrate into a circular spot at the center of the well, in presence of 50 ng/mL hGH for upto 48 hr. The percentage free area at the end time point was calculated using ImageJ software and reflected the decrease/inhibition in migration. A significant decrease in migration was noted following GHR-KD. [* p < 0.05, Students t-test, n = 3]
Figure 29: Effect of GHR knock down (KD) on SK-MEL-28 cell invasion. SK-MEL-28 cells transfected with 20 nM scramble (1a, b, c, d) or GHR-siRNA (2a, b, c, d) were seeded onto U-bottom 96 well plates at 5000 cells/well and allowed to form a spheroid. A hydrogel invasion matrix was added above the spheroid and cells were monitored for upto 72 hr. in presence of 50 ng/mL hGH. Total pixels representing structural extensions from the spheroid were calculated using ImageJ software and reflected the invasive ability of the melanoma cells. A significant decrease in spheroid invasion was noted following GHR-KD. [*p < 0.05, Students t-test, n = 3]
Figure 30: Effect of GHR knock down (KD) on anchorage-independent growth of melanoma cells. SK-MEL-5, SK-MEL-28 and MDA-MB-435 cells transfected with 20 nM scramble or GHR-siRNA were allowed to form colonies on soft agar for 7 days in presence of 50 ng/mL hGH. The cells were lysed at the end time point and total DNA was quantitated using a fluorescent readout. A significant decrease in total number of colonies was noted following GHR-KD. [* p < 0.05, Students t-test, n = 3]

3.5. Discussion

The highly proliferative effect of an induced autocrine-hGH system in endometrial and mammary carcinoma as well as upregulated migration, anchorage-free growth and propensity to epithelial mesenchymal transition is known (Perry et al., 2006; Perry et al., 2008; Brunet-Dunand et al., 2009; Bougen et al., 2012). The autocrine/paracrine action of human GH in oncogenic incidents has been further established in autocrine GH-driven miRNA mediated upregulation of epithelial mesenchymal transition (EMT) in breast cancer (Zhang et al., 2015; Harvey et al., 2015). The presence of an autocrine GH-GHR loop is of particular interest in tumor biology
because of the reported presence of intracellular GHR on the nuclear membrane. Nuclear localization of GHR is known to be particularly relevant in upregulation of tumor proliferation (Conway-Campbell et al., 2007; Herington and Lobie, 2012). In this study we observed readily detectable levels of RNA and protein expressions of human GH and its specific reporter on human melanoma cells. Basal level phosphorylation of GH-dependent intracellular signaling networks like JAK2, STATs 1, 3, 5, ERK1/2, SRC, AKT and mTOR in absence of any externally added GH suggested the presence of an autocrine ligand-receptor loop existent and critical in melanoma. A ten minutes induction with excess GH showed robust increase in the phosphorylation states compared to PBS treated controls. Our observation was further confirmed by the drastic reductions in the phosphorylation states of all the above signaling components in spite of the presence of excess growth hormone in the media.

In this study we observed critical modulations of RNA levels of different cytokines and their cognate receptors associated with the GH axis. Although we did not detect any endogenous insulin or IGF2 levels in any of the four melanoma cells tested here, our RT-qPCR studies revealed low RNA levels of PRL and IGF1. PRL have been strongly implicated in breast and prostate cancers for a considerable time by dint of its mitogenic and angiogenic properties and expression of PRLR on tumor tissues (Goffin et al., 1999; Goffin et al., 2002; Jacobson, 2011). We found low but consistent RNA expressions of both PRL and PRLR and consistent marked rise in PRLR levels following GHR-KD in SK-MEL-28, MALME-3M and MDA-MB-435 cells. Presence of excess GH potentiated the effect. PRL-PRLR signaling engages intracellular mediators like JAK2,
PI3K, ERK1/2 and STAT5 which overlaps with GHR signaling pathway. Also human GH is known to bind and activate PRLR (Goffin et al., 1999). The siRNA mediated KD of GHR could lead to a compensatory non-canonical binding of GH-PRLR and downstream signaling. Future investigations with PRLR-inhibited models can provide more detailed information regarding the importance of the canonical PRL-PRLR and a putative salvage GH-PRLR interaction specifically in melanoma.

The role of insulin, related growth factor (IGF1, IGF2) and their cognate receptors are important regulators of multiple human cancers, including melanoma (Satyamoorthy et al., 2001; Pollak 2008; Capoluongo 2011; Haisa 2013; Chen et al., 2014; Subramani et al., 2014; Krudden et al., 2015). We especially found very high levels of RNA expressions of the corresponding cognate receptors – IR, IGF1R, and IGF2R. Recent reports have established a Wnt-mediated IGF2 secretion from keratinocytes which in turn strongly stimulated adipogenesis in 3T3-L1 cells (Donati et al., 2014). There is yet no known studies to characterize the role of IGF2 action in melanoma but some isolated studies have pointed at a tumor suppressing role of IGF2 in mammary carcinoma expressing IGF2R (Wise and Pravtcheva, 2006). In our analysis, we found significant suppression of IGF2R on all melanoma cells following GHR-KD and an inference can only be drawn following a better understanding of IGF2 functions on this type of cancers. The robust presence of IGF2R on melanoma cells which at a non-cancerous state remain under tight control of keratinocytes, suggests a possible paracrine/endocrine-IGF2 mediated signaling in melanoma. This would be of considerable value from the perspective of GH-regulation especially since it was reported that IGF2 secretion in
human hepatoma cells is regulated together by GH, insulin, glucagon and heparin (Zvibel et al., 1991). Except in MDA-MB-435 cells, all the other three cell lines showed a significant rise in IR RNA levels following GHR-KD especially at excess GH levels. It is reported that a state of insulin resistance, which includes a high level of circulating insulin, has been associated with a higher risk of melanoma incidence (Antoniadis et al., 2011). As we report here, the melanoma cells indeed appear to be in a state of heightened insulin/IGF sensitivity by dint of expressing abundant IR, IGF1R and IGF2R as seen in all four melanoma cells in this study.

Together with our and others’ observation of presence of GH and GHR, the melanoma cells are also reported to express important regulators of GH secretion – the growth hormone releasing hormone receptor (GHRHR) and somatostatin receptors (Ardjomand et al., 2003; Chatzistamou et al., 2008; Martinez-Alonso et al., 2009). Although somatostatin analogs have not been reported to be efficacious against melanoma cells (Martinez-Alonso et al., 2009), GHRH analogs had been shown to suppress growth in human malignant melanomas and triple-negative breast cancer (Seitz et al., 2013; Szalontay et al., 2014; Engel 2015). We observed RNA expression of GHRHR in all four melanoma cell lines. Significant upregulation in GHRHR levels following greater than 90% reduction in GHR levels, and the coincident upregulation of RNA levels of GH in all corresponding cases of GHR-KD, suggest a possible GHRHR mediated activation of compensatory GH release. We did not inquire into existence of GHRH in these melanoma cells, although neuroendocrine tumors reportedly secrete
GHRH (Doga et al., 2001). Specific investigations can be conducted to elucidate the regulatory mechanism of GH synthesis in melanoma further.

An important finding of our study was the identification of GH regulated autocrine hepatocyte growth factor (HGF) and its cognate receptor MET (or c-MET) on all the melanoma cell lines. Although intrinsic RNA levels of HGF were low, there was significant increase at and above 50 ng/mL hGH in SK-MEL-28 and MDA-MB-435 cells as well as a significant downregulation following GHR-KD. Moreover, there were very high RNA levels of the HGF receptor MET on all four melanoma cells. All the melanoma cell lines, exhibited a dose dependent rise in MET RNA levels with added GH. On the other hand, GHR-KD significantly suppressed the same, even in presence of excess GH. This set of results suggest a possible transcriptional control of MET and HGF expression by GH, which remains to be verified. The ERBB family members EGFR, ERBB1, ERBB2 and ERBB3 are known to be involved in driving several oncogenic processes in melanoma (Brychtova et al., 2011; Tsao et al., 2012; Zhang et al., 2015). We observed increased RNA levels of ERBB3 in response to excess GH in SK-MEL-28 and MDA-MB-435 and a consistent suppression of the same following GHR-KD. Both MET and EGFR are known to strongly activate the SRC signaling pathway (Vergani et al., 2011; Girotti et al., 2013). GH action is known to activate EGFR in liver regeneration (Zerrad-Saadi et al., 2011). Thus our results suggest the possibility of GH control of HGF, MET and ERBB3 expression in melanoma cells. The underlying mechanisms of transcriptional regulation can add to the value of the dependence of melanoma on GH-GHR axis.
We noticed significant basal phosphorylation of the ERK1/2 and AKT/mTOR components in all four melanoma cell lines which can be explained because of a constitutively active RAS, harboring the V600E mutation in these cell lines. However we further observed a significant decrease in ERK1/2, AKT, and mTOR in all cases below the basal levels, irrespective of stimulation with 50 ng/mL hGH. This significant downregulation does indicate a suppression of an active autocrine GH – GHR interaction which contributes significantly to the basal phosphorylation states of these signaling pathways. The residual phosphorylation observed following GHR-KD, although significantly low, could be contributed by GH binding and activation of PRLR as well as other shared signaling pathways.

The STAT3 activation in melanoma drives multiple critical transformations like EMT, angiogenesis and inhibition of apoptosis in the cell by increasing expressions of intrinsic oncogenic factors like microphthalmia associated transcription factor (MITF) and also cooperatively induces downstream factors like c-fos (Joo et al., 2004; Wang et al., 2008; Pensal et al., 2009; Brychtova et al., 2011). Robust GH-mediated STAT3 regulation is of further importance especially in melanoma following the recent report of the implication of the transcription factor in reprogramming senescent melanomal precursors towards tumorigenicity (Ohanna et al., 2013) and demands new studies investigating role of GH action in cellular reprogramming and cancer initiating cells. STAT3 is also a converging point in signaling networks for multiple different upstream regulators like SRC, JAK2 as well as ERBB family members like EGF4 and ERBB3 (Niu et al., 2002). Our results strongly corroborates with previous observations of
presence of constitutive activation of SRC and STAT1, 3, and 5 proteins in melanomal tumors. We found the known pattern of GH-induced activation of STAT proteins (Herrington et al., 2000) to be conserved and active in melanoma. The significant decrease of STAT activation below basal levels, even in presence of excess GH, with GHR-KD thus suggests (i) attenuation of the autocrine GH-mediated activation, as well as (ii) sensitivity and dependence of the melanoma cells on GH-GHR interaction and activation of either JAK2 or SRC or both. The presence of basal phosphorylation of both JAK2 and SRC kinases as well as their correlative changes with GHR-KD and/or excess GH as observed in this study, indicated both signaling mediators to be highly responsive in melanoma, to GH action. A separate analysis of the mutually independent roles of JAK2 and SRC in downstream signaling activation and cell fate, similar to studies done in preadipocytes and human hepatoma cells (Jin et al., 2008; Barclay et al., 2010), would be of plausible interest and value to resolve our observations further. The distinct upregulation of the basal STAT1 phosphorylation levels, reported to coincide with recurrent melanoma phenotypes (Lesinski et al., 2005), in response to excess GH or suppression due to GHR-KD clearly indicates a GH responsiveness and control of the STAT1 proteins in this tumor. The STAT5 dependence on GH-GHR induced activation as observed in this study is important because STAT5 is already an established oncogenic driver in melanoma and protects the cell against interferon-based immunotherapies (Wellbrock et al., 2005; Hassel et al., 2008). In melanoma cells, STAT5 acts to mediate resistance to apoptosis and is reported to be activated by both JAK2 and SRC kinases (Mirmohammadsadegh et al., 2006). Thus our results indicating significant basal
activation of JAK2, SRC, STATs 1, 3, and 5, in melanoma provoke further studies in the direction of resolving finer aspects of STAT1 vs. STAT3 as well as the enigmatic topic of cytokine-induced STAT mediated cross-talks between JAK and SRC pathways (Reddy et al., 2000; Manabe et al., 2006) using human melanoma cells as useful models.

The concerted effect of GH stimulation was evident on the upregulated proliferation of melanoma cells at above 50 ng/mL hGH. The robust decrease of GHR levels on the other hand, lead to a significantly lower proliferation rate as seen in our metabolic assay. A concomitant decrease in migration, invasion and clonogenic capacities of the melanoma cells following GHR-KD does present a strong argument in favor of identifying GH-GHR interaction in melanoma cells as a key regulator of melanoma progression and an important target for therapeutic intervention. The collection of our results will act as a springboard for identifying and investigating novel regulatory roles of GH in one of the most aggressive and disease-resistant forms of cancer.

3.6. References


CHAPTER 4: GROWTH HORMONE RECEPTOR KNOCK-DOWN ATTENUATES KEY MECHANISMS OF DRUG EFFLUX AND SENSITIZES HUMAN MELANOMA CELLS TO CHEMOTHERAPY

4.1. Abstract

Melanoma cells express high levels of growth hormone (GH) receptor (GHR) and also happen to be one of the most therapy resistant forms of human skin cancer. With an increasing global incidence, melanoma is lethal following metastases and amounts to $3.3 billion globally in mortality cost burden in 2012. The biggest hurdle in treating melanoma is its intrinsic therapy resistance fueled largely by the abundant expression of a repertoire of xenobiotic efflux pumps of the ATP binding cassette transporter (ABC transporter) family as well as possible mechanisms of drug sequestration in melanosomes. Here we report for the first time a GH-GHR dependent mechanism of chemotherapeutic resistance in human melanoma cells. We found that treatment of melanoma cells with sub-EC50 doses of anti-cancer drugs, following siRNA mediated GHR knock down (KD), resulted in a significant suppression of RNA and protein expressions of different ABC-transporter pumps, significant downregulation of key modulators of melanogenesis pathway - the transcription factor MITF and its target TYRP, and significant reversal in the RNA and protein levels of markers of epithelial mesenchymal transition. Following GHR-KD, melanoma cells exhibited significantly longer drug retention and increased sensitivity to sub-EC50 doses of anti-cancer drugs, identified by loss of proliferation. Therefore by virtue of sensitizing melanoma cells to chemotherapy by suppressing multiple mechanisms of drug resistance, we propose that
GHR reduction can be a critical combination therapy with established and developing anti-tumor compounds. In areas of drug development, the approach of concomitant GHR antagonism can lead to newer as well as improved therapeutic intervention. Most importantly this approach can not only lead to a more effective treatment plan, but also might immensely benefit the patients by reducing the required dosage. This in turn can bring down the physiological side-effects and the associated cost burden.

4.2. Introduction

Human melanoma has remained the most aggressive form of skin cancer and one of the most therapy resistant forms of cancer worldwide while some recent reports have claimed an epidemic rate of incidence in some countries since the 1950s (Welch et al., 2005; Linos et al., 2009; Hery et al., 2010; Geller et al., 2013; Hallberg et al., 2013; Lowe et al., 2014). Studies indicate increased UV exposure, use of tanning-beds, hormone replacement therapies as well as improved and increased screening, as the underlying causes of heightened melanoma incidence (Berwick, 2010; Hery et al., 2010; Boniol et al., 2012; Handler et al., 2012). More than 10,000 men and women in the United States and 60,000 worldwide are expected to die of melanoma in 2016 (www.cancer.org), which globally claims about five lives an hour.

Chemotherapeutic interventions are strongly resisted by several mechanisms of drug resistance in the melanoma. Developing a melanoma vaccine have had limited success (Oza-Choy et al., 2014) whereas, in the last five years, a number of highly efficacious immunotherapies (anti-CTLA-4 antibody ipilimumab, PD-1/PD-L1 antibody pembrolizumab) and targeted therapies (V600E BRAF inhibitor vemurafenib) had been
approved by the FDA (Zitvogel and Kraemer, 2012; Khattak et al., 2013; Comfere et al., 2013; Srivastava and McDermott 2014; Luke et al., 2015; Rajakulendran and Adam, 2015) while several are in various stages of development (Russo et al., 2014). In spite of the hopeful scenario in melanoma therapy, there is need for a critical appreciation. Effective therapy with one of the most successful drugs pembrolizumab necessitates pre-existence of active cytotoxic T-cells in the system (Tumeh et al., 2014) while resistance to most other known chemotherapies, including ipilimumab and vemurafenib, have been repeatedly reported (Luke and Hodi, 2013).

Melanoma is unique among other types of cancers in possessing multiple robust mechanisms of chemotherapeutic resistance. This includes abundant expression of a repertoire of drug efflux pumps (Elliott and Al-Hajj, 2009; Chen et al., 2009; Walsh et al., 2010), melanosomal sequestration of drugs in melanosomes during melanogenesis (Chen et al., 2006; Videira et al., 2013) as well as upregulation of epithelial-mesenchymal transition (EMT) markers (Lackner et al., 2012; Fischer et al., 2015; Mitra et al., 2015). Clinicians and scientists are still grappling to identify the Achilles’ heel in melanoma drug-resistance.

Studies with MCF-7 breast cancer cells indicated an upregulated doxorubicin resistance in presence of supra-physiological doses (1 mg/mL) of GH (Zatelli et al., 2009) while high GH induced chemotherapy resistance in acromegaly patients was suggested (Perry et al., 2008). A number of clinical reports have long pointed towards a distinct association of human GH (hGH) with melanoma (Wyatt, 1999; Caldarola et al., 2010; Handler et al., 2012). Significantly elevated levels of the hGH receptor (GHR) was
observed by immunohistochemical staining of different stages of melanoma tumors and
34 of 37 tested cases were moderate to strongly positive for GHR expression (Lincoln et
al., 1999). Subcellular localization of GHR observed in that study hinted at the presence
of GHR at the nuclear membrane. In fact the GHR RNA transcript levels were reported to
be the highest specifically in human melanoma in the entire NCI60 panel of cancer cells
(Sustarsic et al., 2013). We recently performed (John J Kopchick, personal
communication) a comprehensive analysis of GH-GHR action in human melanoma cells
which exposed a definitive regulation of key intracellular signaling pathways like the
JAK, STAT(1, 3, and 5), SRC, ERK1/2, AKT and mTOR which are known to be critical
mediators of early gene activation and drug resistance in melanoma and other forms of
cancer (Vergani et al., 2011; Boone et al., 2011; Girotti et al., 2013; Goetz et al., 2014;
Chen et al., 2014; Kim et al., 2015; Zhao et al., 2016). We also observed robust GH-
dependent modulation of RNA expressions of HGF, MET, and ERBB3 in human
melanoma cells which indicate a possible profound involvement of GH on mechanisms
of therapy refractoriness in melanoma (Heynen et al., 2014; Ma et al., 2014; Fattore et al.,
2015; Cao et al., 2015). Further, the melanoma cells were found to express endogenous
GH as well as high levels of canonical receptors of the insulin family – IR, IGF1R and
IGF2R. In this study, we interrogated the effect of siRNA mediated GHR-KD on the drug
resistance property of human melanoma cells – SK-MEL-28, MALME-3M, MDA-MB-
435 and SK-MEL-5, which express both GH and GHR. We compared changes in RNA
expression of seven different ATP binding cassette (ABC) transporter family efflux
pumps, treated with sub-EC50 doses of selected anti-tumor drugs – cisplatin,
doxorubicin, oridonin, paclitaxel and vemurafenib - under basal and excess (50 ng/mL) GH levels as well as with and without decreased GHR expression. We report here significant GH dependence of the expression of the efflux pumps as well as marked reduction in RNA levels of important mediators of melanogenesis pathway. Additionally, GHR-KD was found to significantly attenuate and even reverse the pattern of expression of EMT markers in the melanoma cells. Our results strongly suggest that reducing GHR levels is an effective mechanism to curb drug efflux and markedly sensitize these highly therapy resistant melanoma cells to different classes of established and developing anti-cancer compounds. This is a novel finding in the area of drug resistance, GH action and therapeutic strategies in cancer.

4.3. Methods

4.3.1. Cell culture

Human malignant melanoma cell lines SK-MEL-5 (#HTB-70), SK-MEL-28 (#HTB-72), MALME-3M (#HTB-64), and MDA-MB-435S (#HTB-129) cells were obtained from American Type Culture Collection (ATCC; Manassas, Virginia). SK-MEL-5 and SK-MEL-28 were grown and maintained in EMEM media (ATCC #30-2003), while MALME-3M and MDA-MB-435S were grown in IMDM (ATCC #30-2005) and RPMI-1640 (ATCC #30-2001) respectively. Complete growth media was supplemented with 5% fetal bovine serum (FBS; ATCC # 30-2020) and 1X antibiotic-antimycotic (Thermo Fisher Scientific #15240). Human melanocyte ST-MEL (a kind gift from Dr. Lingying Tong) cells were grown in RPMI-1640 medium (ATCC # 30-2001) supplemented with 10% FBS and 1X antibiotic-antimycotic. Cells were grown at 37C /
5% CO₂ in a humidified incubator. Half the media was replaced every 48 hr. No hGH was present in the media or added externally unless specifically mentioned. Tissue culture treated sterile T-75 and T-25 flasks and 6-, 12-, 24-, and 96-well plates (Corning, New York) were used. Trypsinization was done using 0.25% Trypsin/0.53 mM EDTA in Hank’s balanced salt solution (HBSS) without calcium or magnesium (ATCC # 30-2101) for 5 min / 37°C / 5% CO₂.

4.3.2. Drug treatments

For treatment of melanoma cells the following five anti-tumor compounds were obtained from the sources mentioned – cisplatin (Calbiochem #232120, Darmstadt, Germany), doxorubicin (Sigma Aldrich #D-1515, St. Louis, MO), oridonin (Sigma-Aldrich #O-9639, St. Louis, MO), Paclitaxel (Sigma-Aldrich #C-7191), and vemurafenib/PLX4032 (ApexBio #A-3004, Houston, TX). We performed evaluation of EC50 values for each drug in each cell line and observed the following EC-50 ranges for the four melanoma cell lines – cisplatin (3-15 uM), doxorubicin (25 – 100 nM), oridonin (2-8 uM), paclitaxel (2-8 nM) and vemurafenib (2-20 nM). In our experiments we used the following drug concentrations unless specified otherwise – cisplatin (0.5 uM), doxorubicin (10 nM), oridonin (0.5 uM), paclitaxel (1 nM), vemurafenib (2 nM). Treatments were performed for 24 hr. starting 48 hr. post-transfection with siRNA.

4.3.3. Transfection

Transfection was performed using siLentFect lipid reagent (Biorad #170-3360, Hercules, California) following manufacturers protocol. Pre-designed siRNA duplexes against human GHR (Origene #SR301794, Rockville, Maryland) at different
concentrations were checked and 20 nM was found to be optimum for decreasing the GHR RNA by >80%. Three different siRNA duplexes were checked [siRNA-A: AGAAGGAAACCUCAGUUAAAGAAA; siRNA-B: AGCUAGAAUUGAGUGUUAAAGUTC; siRNA-C: UCAUGGUACAAAGAACCUAGGA-CCC] and finally siRNA-B was selected for maximum and most consistent GHR transcript suppression in all four melanoma cells. Mock transfection was done using universal scrambled negative control siRNA-duplex (Origene #SR30004). TYE-563-fluorescent labeled siRNA duplex (Origene #SR30002) was used as the transfection control. Cells between passages 6-10 were trypsinized, counted using a Countess automated cell counter (Life Technologies, Carlsbad, CA) and seeded at 25,000-30,000 cells/cm² and allowed to attach for 16-18 hr, followed by replacing the media with fresh antibiotic free complete growth medium just prior to transfection. A pre-incubated mix of 20nM siRNA duplex (scramble or GHR specific) and siLentFect reagent at 1:1 molar ratio was added to the cells and incubated at 37°C / 5% CO₂. Media was changed to complete growth medium with antibiotics after 24 hr. RNA levels were analyzed 48 hours post transfection while protein levels were analyzed at 60 hr post-transfection. For drug treatment, drugs at specified concentration were added to the cells 48 hr. post-transfection and treated for 24 hr. prior to immunocytochemistry and quantitation of RNA expressions.

4.3.4. RNA extraction and RT-qPCR

RNA extraction was done using the IBI-Trizol based total RNA purification kit (MidSci #IB47632, St. Louis, Missouri), and reverse transcription was performed using
Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific #K1642, Waltham, MA) following manufacturers protocol. Real time-quantitative PCR and melt curve analysis were performed using Maxima SYBR-Green qPCR master mix (Thermo Fisher Scientific #K0241) and a T100 thermal cycler (Biorad #1861096, Hercules, CA). RNA and DNA concentrations were estimated using Nanodrop2000 (Thermo Fisher Scientific, Waltham, MA) spectrophotometer.

Primers were obtained from Sigma-Aldrich for the following human genes and primer efficiency was confirmed: GAPDH, b-Actin, GH1, GHR, MITF, TYRP1, E-Cadherin, N-Cadherin, Vimentin, ABCB1, ABCB5, ABCB8, ABCG1, ABCG2, ABCC1, and ABCC2. Each sample was a pool of two replicates per experiment; Experiments were done three times. Each qPCR for each gene and each treatment for every cell type was performed in triplicates.

4.3.5. Protein extraction

Total protein was collected 60 hr. post transfection. The conditioned growth media for each treatment type were collected separately for subsequent analysis of secreted proteins. Total protein was extracted from the cells using RIPA buffer (Sigma-Aldrich #R-0278, St. Louis, Missouri), mixed with 1.5X Halt protease and phosphatase inhibitor cocktail (Thermo-Fisher #78442, Waltham, MA), following manufacturer’s protocol. Briefly, cells were washed twice with chilled sterile 1X phosphate buffered saline (PBS). Thereafter chilled RIPA buffer at 1 mL per million cells were added and incubated for 5 min / 4C. Then the cells were rapidly scraped with a cell-scaper to lyse residual cells. The cell lysate was clarified by centrifuging at 8,000 Xg / 10 min / 4C and
the supernatant was collected and stored at -80°C for subsequent use. Each sample was a pool of three replicates per experiment; each experiment was done three times.

Protein concentration was estimated in duplicates and two dilutions (1:2, 1:4) using Bradford reagent (Sigma-Aldrich #B6916) and 1 mg/mL bovine serum albumin as standard. Absorbance at 595 nm was measured using Spectramax250 (Molecular Devices, Sunnyvale, California) and SoftmaxPro software (ver.4.7.1).

4.3.6. Western blot (WB)

The protocol closely follows the methods used in the laboratory (Sackmann-Sala et al., 2014) with few modifications (Akasimitiene et al., 2007). Briefly, intracellular proteins were subjected to gradient (4-16%) SDS-PAGE and transferred to nitrocellulose membrane by wet-transfer method at 70 mA / 14 hr / 4°C, and blocked with 5% non-fat dry milk (NFDM) or 5% bovine serum albumin (BSA) in 1X TBS-T (Tris buffered saline, pH7.2 with 0.1% Triton-X100) for 12-16 hr / 4°C with gentle rocking. Membranes were then incubated with primary antibody (at specific dilutions mentioned below) for 12-16 hr / 4°C with gentle rocking. Membranes were then washed for 5 min / 3 times with 1X TBS-T and incubated with corresponding secondary antibodies (at specific dilutions mentioned below) for 2 hr / 25°C. Membranes were then washed for 5 min / 4 times with 1X TBS-T, treated with WestFemto Chemilumiscence detection reagents (Thermo Fisher Scientific) and chemiluminiscent signal was captured using a GelDoc (Biorad) fluorescence reader. Densitometric analysis of the blots was done by measured band-intensity from the area-under-curve using ImageJ software.
Primary antibodies were used to detect the following human proteins: GHR (Mouse, 1:300, SCBT #137185; Goat, 1:100, R&D Systems #AF1210; Rabbit, 1:200, Abcam #ab134078), Actin (Goat, 1:3000, SCBT #sc1616), GAPDH (Goat, 1:3000, SCBT #sc20357), Vimentin (Rabbit, 1:3000, CST #5741), E-cadherin (Rabbit, 1:1000, CST #3195), N-cadherin (Rabbit, 1:500, CST #13116), Vimentin (Rabbit, 1:3000, CST #5741), ABCG1 (Rabbit, 1:100, AbioCode #R0254), ABCB8 (Rabbit, 1:100, SAB #31025), ABCB1/MDR1 (Mouse, 1:100, SCBT #sc55510). Secondary antibodies used: anti-rabbit HRP-linked IgG (Donkey, 1:2000, CST #7074P2), anti-goat HRP-linked IgG (Donkey, 1:1000, SCBT #sc2020), anti-rabbit HRP-linked IgG (Donkey, 1:2000, GE #NA934), anti-mouse HRP-linked IgG (Rat, 1:1000, Antibodies Online #ABIN1589975).

4.3.7. Immunofluorescence (IF)

Cells were seeded at 10,000 cells/cm² in 8-well chamber slides and transfection was performed as described above. The cells were treated for 24 hours with 10 nM doxorubicin or 1 nM paclitaxel, 48 hours post-transfection. Subsequently cells were fixed after 36 hours more (a total of 60 hr post-transfection), and cells were washed twice with 1X PBS and fixed with 4% freshly-prepared formaldehyde (pH6.9) / 15 min / 25C (using 100% methanol for fixation gave equally good results). Cells were permeabilized with 0.2% Triton-X100 in 1X PBS / 15 min / 25C, followed by blocking with 1% BSA / 4 hr / 25C. Incubation time was 12hr / 4C for primary antibody and 2 hr / 25C for secondary antibody. Finally, the slides were washed four times with 1X PBS and the sample was mounted with Fluoroshield mounting medium containing DAPI (Abcam #ab104139, Cambridge, UK), covered with a 60 mm coverslip and the edges were sealed.
with nail-polish and stored at 4C for microscopy. Microscopic imaging was done using a Nikon Eclipse E600 compound fluorescent microscope fitted with a Nikon DS-Fi1CC camera (Nikon, Tokyo, Japan) and NIS-Elements BR3.2 imaging software.

Antibodies used – Rabbit anti-human-Ki67 monoclonal antibody with AlexaFluor488 tag (Abcam #ab154201, 1:300 dilutions); Rabbit anti-human GHR monoclonal antibody (Abcam #ab134078, 1:250 dilution); Goat anti-rabbit secondary antibody with AlexaFluor488 tag (Life Technologies #R37116, 1:500 dilution).

4.3.8. Cell proliferation assay

A 1% (w/v) resazurin (Sigma-Aldrich #R7017) solution in 1X PBS was made and filter-sterilized. The final concentration of resazurin in the assay was 0.004%. Inside the proliferating cells mildly fluorescent blue resazurin is reduced to a bright pink fluorescent product called resorufin (stable for 4 hr), which is a quantitative measure of the percentage of proliferating cells. Briefly, cells were seeded at 10,000 cells/cm² into 96-well plates and transfected as described above. The resazurin assay was performed 60 hr after transfection (unless specified otherwise) and resorufin absorbance was measured at 570 nm (reference wavelength = 600 nm) using Spectramax250 (Molecular Devices, Sunnyvale, CA) and SoftmaxPro software. In all cases, cells were incubated at 37C / 5% CO₂ for 45-60 minutes following resazurin addition, for adequate sensitivity of detection.

4.3.9. Drug retention assay

The presence of multiple drug resistance pumps along the cellular membrane is key to the resistance against chemotherapy in some cells like melanoma. ABC transporter pumps like MDR and MRP family members are involved in exclusion of xenobiotics
from inside the cells to outside. This reduces the retention time of drugs inside a cell and confers decreased sensitivity to the drug-effects. For our purpose, we chose the Vybrant multidrug resistance assay kit (Molecular Probes #V13180, Eugene, OR) which was developed initially by Tiberghein and Loor (Tiberghien and Loor, 1996). The assay uses the non-fluorescent calcein acetoxymethylester (calcein-AM) as a drug-mimic and a substrate for the efflux pumps on the melanoma cell. Calcein-AM is highly lipid soluble and permeates the cell membrane where it is converted to a fluorescent calcein by the intracellular eterases. In absence or decreased activity of the efflux pumps, the intensely fluorescent calcein is retained and can be measured as an indication of drug retention inside the cell. The assay was performed as per manufacturer’s protocol with some necessary optimizations. Briefly, the siRNA treated cells were trypsinized 48 hr. after transfection, counted and seeded at 50,000 cells/well in a black, clear bottom Costar 96-well plate (Corning #3603, Corning, NY), calcein-AM was added at a final concentration of 2 uM, and incubated at 37C for 2 hr. After thorough washing, the fluorescence was measured at 494 (ex) / 517 nm (em) in a spectramax M2 fluorescence plate reader (Molecular Devices, Sunnyvale, CA) and SoftMax Pro v6.2.1 software. Experiments were done in quadruplicate for statistical significance.

4.3.10. Statistical analyses

Parametric and non-parametric statistical analyses for comparing RNA expression levels were done using R software (ver3.0.2). For RT-qPCR analysis of RNA expressions, the levels are to be normalized first against two reference genes (GAPDH and beta-actin) and the 2^(-ddCt) values were compared by Wilcoxon signed rank test for
significance. A p-value less than 0.05 was considered as significant. The densitometric analyses, clonogenicity, migration and invasion, and resazurin based assays, are compared by a paired students T-test and ANOVA was performed (using GraphPad Prism software) to compare for significance (p<0.05 is considered significant).

4.4. Results

4.4.1. GHR knock-down significantly suppresses expression of ABC transporter pumps in human melanoma cells

The ATP-binding cassette (ABC) transporters are ATP dependent xenobiotic efflux pumps which are employed by various cancer cells as an important mechanism of lowering the intracellular accumulation of cytotoxic anti-cancer drugs (Fletcher et al., 2010). Melanoma expresses a number of ABC efflux pumps of which we specifically investigated the RNA levels of ABCB1 (Landreville et al., 2011), ABCB5 (Chartrain et al., 2012; Wilson et al., 2014, ABCB8 (Elliott and Al-Hajj, 2009), ABCC1 (Michaelis et al., 2014), ABCC2 (Liedert et al., 2003), ABCG1 (Sag et al., 2015), and ABCG2 (Ejendral and Hrycyna, 2002), based on reports of their presence and drug-resistance activity in human melanoma. We found RNA expression of these seven transporters on all four melanoma cell lines, in varying degrees. We found ABCB8, ABCC1 and ABCC2 had higher RNA expression levels while ABCB1, ABCB5, ABCG1 and ABCG2 had lower expression levels in these melanoma cells. Not all of these have been investigated altogether in any set of melanoma cell lines against as many as five different classes of anti-tumor compounds. We treated the melanoma cells with sub-EC50 doses (as described in Methods) of cisplatin, doxorubicin, oridonin, paclitaxel and vemurafenib
which have been extensively reported and used for their anti-tumor effects on different classes of cancer cells, especially melanoma. We report the results for each transporter separately below.

- **ABCB1**: We observed significant upregulation of ABCB1 levels in response to cisplatin in SK-MEL-28 (Figure 31a) and MDA-MB-435 (Figure 31c) cells and in response to oridonin in SK-MEL-28 cells (Figure 31a). Interestingly, the intrinsic RNA level of ABCB1 was significantly downregulated following GHR-KD, in presence of cisplatin for all four melanoma cell lines. ABCB1 expression was markedly reduced following GHR-KD also on exposure to doxorubicin (in MDA-MB-435 and SK-MEL-5), oridonin (in MALME-3M and MDA-MB-435), and vemurafenib (in MDA-MB-435) (Figures 31a, b, c, d).

- **ABCB5**: RNA levels of ABCB5 were significantly upregulated following treatment with cisplatin, doxorubicin, oridonin and paclitaxel in SK-MEL-28 cells (Figure 32a) while the same was observed for doxorubicin and oridonin treatment in MDA-MB-435 cells (Figure 32b) indicating a role of ABCB5 in mediating multi-drug resistance specifically in these two cell lines. On the other hand, GHR-KD caused significant downregulation of ABCB5 expression compared to scr-siRNA treated controls, on exposure to cisplatin (in SK-MEL-28), doxorubicin (in MDA-MB-435), oridonin (in SK-MEL-28 and MDA-MB-435), paclitaxel (in SK-MEL-28 and MDA-MB-435) and vemurafenib (in MDA-MB-435) (Figures 32a, b, c, d). We observed a rise in ABCB5 levels in MALME-3M cells following GHR-KD in response to oridonin and vemurafenib treatments (Figure 32b) while
GHR-KD significantly decreased even the basal expression levels of ABCB5 in absence of any drug in MDA-MB-435 cells (Figure 32c).

- **ABCB8**: ABCB8 is known to induce doxorubicin resistance in human melanoma cells including MDA-MB-435 (Elliott and Al-Hajj, 2009). Consistent with previous observations, we found significant upregulation of ABCB8 levels following exposure to doxorubicin in MDA-MB-435 (Figure 33c) as well as in MALME-3M (Figure 33b) and SK-MEL-5 (Figure 33d) cells. A significant upregulation of ABCB8 was also noticed on treatment with paclitaxel in MALME-3M (Figure 33b) and SK-MEL-5 (Figure 33d) cells while vemurafenib induced a robust increase in ABCB8 in SK-MEL-28 cells (Figure 33a). When the GHR was knocked down, we observed robust downregulation of ABCB8, down to or below basal levels, following exposure to cisplatin (in SK-MEL-28 and MDA-MB-435), doxorubicin (in all four cell lines), paclitaxel (in MALME-3M, MDA-MB-435 and SK-MEL-5) and vemurafenib (in SK-MEL-28 and MDA-MB-435) (Figures 33a, b, c, d).

- **ABCC1**: ABCC1 levels were particularly upregulated in response to paclitaxel treatment in SK-MEL-28 and SK-MEL-5 cells (Figures 34a, 34d). However, following GHR-KD we saw significant suppression of ABCC1 RNA levels in response to doxorubicin (in MALME-3M), oridonin (in SK-MEL-28 and SK-MEL-5), paclitaxel (in SK-MEL-28, MALME-3M and SK-MEL-5), vemurafenib (in MDA-MB-435) (Figures 34a, 34b, 34c, 34d).
ABCC2: Paclitaxel significantly increased RNA levels of ABCC2 transporter in SK-MEL-28 (Figure 35a), MALME-3M (Figure 35b), and SK-MEL-5 (Figure 35d), while the same was observed for vemurafenib in SK-MEL-28 (Figure 35a) and cisplatin in MDA-MB-435 (Figure 35c) melanoma cells. GHR-KD caused robust decrease in ABCC2 levels on exposure to cisplatin (in MDA-MB-435 and SK-MEL-5 cells), doxorubicin (in MDA-MB-435), paclitaxel (in SK-MEL-28 and MDA-MB-435), and vemurafenib (SK-MEL-28, MDA-MB-435 and SK-MEL-5 cells) (Figures 35a, 35b, 35c, 35d).

ABCG1: In the four melanoma cell lines we tested, ABCG1 was significantly upregulated consistently for a number of drugs. ABCG1 levels surged in SK-MEL-28 in response to cisplatin, doxorubicin, paclitaxel as well as vemurafenib treatment (Figure 36a), in MALME-3M cells in response to doxorubicin (Figure 36b) and in MDA-MB-435 cells in response to cisplatin, oridonin and paclitaxel (Figure 36c). On the other hand, GHR-KD had an equally drastic effect in significantly downregulating ABCC2 following exposure to cisplatin (in SK-MEL-28 and MDA-MB-435), doxorubicin (in SK-MEL-28, MALME-3M and MDA-MB-435), oridonin (in SK-MEL-28 and MDA-MB-435), paclitaxel (in SK-MEL-28, MALME-3M and MDA-MB-435), and vemurafenib (in SK-MEL-28 and MDA-MB-435). Only in case of SK-MEL-5 did we observe a rise in ABCG1 levels following GHR-KD basally as well as in presence of all drugs tested (Figure 36d).
- **ABCG2**: In our study, ABCG2 levels were significantly upregulated in SK-MEL-28 cells on exposure to doxorubicin, paclitaxel and vemurafenib (Figure 37a); while the same was observed in MDA-MB-435 cells in response to cisplatin (Figure 37c) and in SK-MEL-5 cells in response to doxorubicin and oridonin (Figure 37d). GHR KD caused significant decrease in ABCG2 levels on exposure to cisplatin (in MDA-MB-435 and SK-MEL-5), doxorubicin (in SK-MEL-28, MALME-3M and SK-MEL-5), paclitaxel (in SK-MEL-28) as well as vemurafenib (in SK-MEL-28, MALME-3M and SK-MEL-5) (Figures 37a, 37b, 37c, 37d).

The results of GHR-KD on seven different ABC transporter pumps on all four melanoma cell lines on exposure to cisplatin (0.5 uM), doxorubicin (10 nM), oridonin (0.5 uM), paclitaxel (1 nM), and vemurafenib (15 nM) are listed in Table 1. This list summarizes a valuable set of information not only in the context of GH action on expression of ABC transporters mediating multi-drug resistance in human melanoma, but also identifying cell-specific and multiple drug-specific variations of seven different ABC transporters in melanoma.

WB analysis showed significantly lower ABCG1 levels in SK-MEL-28, MALME-3M and MDA-MB-435, (Figures 38a, 38c); while protein levels of ABCB8 were found to significantly lower in GHR-KD samples of MALME-3M, MDA-MB-435 and SK-MEL-5 cells (Figures 38b, 38c), consistent with their RNA level expression observed in this study. The collection of these results thus reveal a critical GH-dependent expression profile of several ABC transporter pumps in melanoma cells, on exposure to
specific anti-tumor compounds and establishes a novel role and regulation of GH in multi-drug resistance in melanoma.

Figure 31: Effect of GHR-KD on ABCB1 expression following drug treatment in human melanoma cells. Relative RNA expression of ABCB1 in (a) SK-MEL-28, (b) MALME-3M, (c) MDA-MB-435 and (d) SK-MEL-5 melanoma cells following scr- or GHR-siRNA mediated knock-down of GHR levels. Experiments were conducted in presence of 50 ng/mL hGH. In all cases, drug treatment was for 24 hr. starting 48 hr. post-transfection. Expressions were normalized against expression of ACTB and GAPDH as reference genes. [* p < 0.05, Wilcoxon sign rank test, n = 3]
Figure 32: Effect of GHR-KD on ABCB5 expression following drug treatment in human melanoma cells. Relative RNA expression of ABCB5 in (a) SK-MEL-28, (b) MALME-3M, (c) MDA-MB-435 and (d) SK-MEL-5 melanoma cells following scr- or GHR-siRNA mediated knock-down of GHR levels. Experiments were conducted in presence of 50 ng/mL hGH. In all cases, drug treatment was for 24 hr. starting 48 hr. post-transfection. Expressions were normalized against expression of ACTB and GAPDH as reference genes. [* , p < 0.05, Wilcoxon sign rank test, n = 3]
Figure 33: Effect of GHR-KD on ABCB8 expression following drug treatment in human melanoma cells. Relative RNA expression of ABCB8 in (a) SK-MEL-28, (b) MALME-3M, (c) MDA-MB-435 and (d) SK-MEL-5 melanoma cells following scr- or GHR-siRNA mediated knock-down of GHR levels. Experiments were conducted in presence of 50 ng/mL hGH. In all cases, drug treatment was for 24 hr. starting 48 hr. post-transfection. Expressions were normalized against expression of ACTB and GAPDH as reference genes. [* p < 0.05, Wilcoxon sign rank test, n = 3]
Figure 34: Effect of GHR-KD on ABCC1 expression following drug treatment in human melanoma cells. Relative RNA expression of ABCC1 in (a) SK-MEL-28, (b) MALME-3M, (c) MDA-MB-435 and (d) SK-MEL-5 melanoma cells following scr- or GHR-siRNA mediated knock-down of GHR levels. Experiments were conducted in presence of 50 ng/mL hGH. In all cases, drug treatment was for 24 hr. starting 48 hr. post-transfection. Expressions were normalized against expression of beta-actin and GAPDH as reference genes. [* p < 0.05, Wilcoxon sign rank test, n = 3]
Figure 35: Effect of GHR-KD on ABCC2 expression following drug treatment in human melanoma cells. Relative RNA expression of ABCC2 in (a) SK-MEL-28, (b) MALME-3M, (c) MDA-MB-435 and (d) SK-MEL-5 melanoma cells following scr- or GHR-siRNA mediated knock-down of GHR levels. Experiments were conducted in presence of 50 ng/mL hGH. In all cases, drug treatment was for 24 hr. starting 48 hr. post-transfection. Expressions were normalized against expression of beta-actin and GAPDH as reference genes. [* p < 0.05, Wilcoxon sign rank test, n = 3]
Figure 36: Effect of GHR-KD on ABCG1 expression following drug treatment in human melanoma cells. Relative RNA expression of ABCG1 in (a) SK-MEL-28, (b) MALME-3M, (c) MDA-MB-435 and (d) SK-MEL-5 melanoma cells following scr- or GHR-siRNA mediated knock-down of GHR levels. Experiments were conducted in presence of 50 ng/mL hGH. In all cases, drug treatment was for 24 hr. starting 48 hr. post-transfection. Expressions were normalized against expression of ACTB and GAPDH as reference genes. [* p < 0.05, Wilcoxon sign rank test, n = 3]
Figure 37: Effect of GHR-KD on ABCG2 expression following drug treatment in human melanoma cells. Relative RNA expression of ABCG2 in (a) SK-MEL-28, (b) MALME-3M, (c) MDA-MB-435 and (d) SK-MEL-5 melanoma cells following scr- or GHR-siRNA mediated knock-down of GHR levels. Experiments were conducted in presence of 50 ng/mL hGH. In all cases, drug treatment was for 24 hr. starting 48 hr. post-transfection. Expressions were normalized against expression of beta-actin and GAPDH as reference genes. [*; p < 0.05, Wilcoxon sign rank test, n = 3]
Table 1

List of ABC-transporter pumps with significantly down-regulated RNA expressions following 24 hr. exposure to anti-tumor compounds in GHR-siRNA transfected melanoma cells compared to corresponding scr-siRNA transfected controls.

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Efflux Pumps (expression level change, p &lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SK-MEL-28</strong></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>ABCB1, ABCB5, ABCB8, ABCG1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>ABCB8, ABCC1, ABCG1, ABCG2</td>
</tr>
<tr>
<td>Oridonin</td>
<td>ABCB5, ABCC1, ABCG1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>ABCB5, ABCC1, ABCG1, ABCG2</td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>ABCB8, ABCC2, ABCG1, ABCG2</td>
</tr>
<tr>
<td><strong>MALME-3M</strong></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>ABCB1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>ABCB8, ABCC1, ABCG1, ABCG2</td>
</tr>
<tr>
<td>Oridonin</td>
<td>ABCB1, ABCB5, ABCG1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>ABCB8, ABCC1, ABCG1</td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>ABCB5, ABCG2</td>
</tr>
<tr>
<td><strong>MDA-MB-435</strong></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>ABCB1, ABCB8, ABCC2, ABCG1, ABCG2</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>ABCB1, ABCB5, ABCC2, ABCG1</td>
</tr>
<tr>
<td>Oridonin</td>
<td>ABCB1, ABCB5, ABCG1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>ABCB5, ABCB8, ABCG1, ABCG2</td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>ABCB1, ABCB5, ABCC2, ABCC1, ABCC2, ABCG1</td>
</tr>
<tr>
<td><strong>SK-MEL-5</strong></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>ABCB1, ABCC1, ABCC2, ABCG2</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>ABCB1, ABCB8, ABCG2</td>
</tr>
<tr>
<td>Oridonin</td>
<td>ABCC1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>ABCB8, ABCC1, ABCC2</td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>ABCC2, ABCG2</td>
</tr>
</tbody>
</table>
Figure 38: Change in ABC transporter pumps following GHR-KD. Changes in protein expressions of ABCG1 and ABCB8 were analyzed. WB comparison was done in all four melanoma cells, 60 hr. post-transfection with GHR-or scr-siRNA. Blots were quantified using ImageJ software and mean of three blots per sample was taken. Expressions were normalized against expression of ACTB (B-actin). [* p < 0.05, Students t test, n = 3]
4.4.2. GHR knock-down significantly suppresses RNA levels of melanogenesis regulators in human melanoma cells

Recent studies in GHR knock-out (GHRKO) mice identified decreased levels of melanocyte stimulating hormone (MSH) in GHRKO mice compared to their wild-type littermates (Sadagurski et al., 2015). Since MSH being a potent regulator of melanogenesis in melanocytes as well as melanoma (Videira et al., 2013), it is reasonable to speculate a GH dependent variation in melanogenesis in these cells. We decided to inquire into the levels of two key regulators of melanogenesis – tyrosinase related protein 1 (TYRP1), a rate limiting enzyme in melanin synthesis pathway, and its transcriptional regulator the microphthalmia associated transcription factor (MITF) (Brychtova et al., 2011; Tsao et al., 2012; Videira et al., 2013). We had previously reported observing a potent downregulation of the phosphorylation states of ERK1/2 and AKT/mTOR pathways with GHR-KD as well as a dose-dependent increase with additional GH (Chapter 3). This is especially relevant with respect to the MITF regulation, following reports of an ERK1/2 binding domain in the MITF gene (Molina et al., 2005). MITF is the principal driver of melanocyte differentiation and development from neural crest cells (Tsao et al., 2012) and occupies a central role as a driver of melanoma and the tumoral interaction with its microenvironment and progress to metastasis. Therefore, identification of a GH-regulation of MITF could be of substantial importance.

We did observe a robust downregulation of MITF expression in all four GHR-KD melanoma cells compared to the scramble siRNA treated controls in absence and presence of added GH (Figures 39a, 39c, 39e, 39g). SK-MEL-28 cells also exhibited a
GH dose dependent increase in RNA levels of MITF (Figure 39a). We observed GH
dose-dependent increase in RNA levels also for the MITF target TYRP1 in SK-MEL-28
(Figure 39b) as well as in MALME-3M cells (Figure 39d). Additionally reduced GHR
expression correlated with a significant decrease in TYRP1 levels in all four melanoma
cell lines (Figures 39b, 39d, 39f, 39h). This finding strongly implicates GH action in
transcriptional control of the melanogenesis pathway used in the melanoma cell lines for
active sequestration of drugs via ABC transporters present on the cell membrane as well
as the melanosomal boundary (Chen et al., 2009). Specific detailed studies in this
direction can potentially unravel further details of countering drug resistance in
melanoma.
Figure 39: Regulators of melanogenesis pathway are influenced by GH action in melanoma cells. Relative RNA expression was quantified for MITF (a, c, e, g) and TYRP1 (b, d, f, h) in SK-MEL-28 (a, b), MALME-3M (c, d), MDA-MB-435 (e, f) and SK-MEL-5 (g, h) melanoma cells following addition of 0, 5, 50 and 150 ng/mL hGH or following GHR-KD, in presence or absence of 0 and 50 ng/mL hGH. In all cases,
exogenous GH treatment was for 24 hr. Expressions were normalized against expression of ACTB and GAPDH as reference genes. [*, p < 0.05, Wilcoxon sign rank test, n = 3]

4.4.3. GHR knock-down significantly modulates markers of EMT in human melanoma cells

EMT plays a physiological role in wound-healing, fibrosis as well as in progression of cancer (Brychtova et al., 2011). Melanomas break free from the homeostatic control of keratinocytes by loss in expression of E-cadherin, upregulation of expression of fibroblast interacting cadherins like N-cadherin and mesenchymal markers like vimentin (Haass et al., 2005, Brychtova et al., 2011, Brandner and Haass, 2013). Numerous studies have reviewed the importance of EMT in cancer metastasis (Voulgari and Pintzas, 2009; Heerboth et al., 2015). Recent publications also established a critical role of EMT as a more important regulator of drug resistance than metastasis in lung cancer using EMT lineage tracing studies (Fischer et al., 2015). Recent results also reported an autocrine GH mediated direct regulation of EMT via activation of the miRNA-96-182-183 cluster (Zhang et al., 2015). This provided a sound scientific rationale to investigate the effects of GHR-KD on the EMT markers in melanoma cells under study. In the present study we found increased levels of E-cadherin RNA levels following GHR-KD, normally found in very low levels in the human malignant melanoma cells (Figures 40 and 41). We saw a concomitant significant decrease in the RNA levels of N-cadherin (Figures 40a, 40d, 40g, 40j) and vimentin (Figures 40b, 40e, 40h, 40k) with GHR-KD consistently in all four melanoma cell lines in this study. Western blot analysis showed results consistent with the RNA level variations of E-
cadherin, N-cadherin and vimentin following GHR-KD (Figures 41a, 41b, 41c). This is the first report on the variations of EMT markers under decreased GHR or increased GH levels in human melanoma cells. Our observations thus bolster the role of GH as an important regulator of the aggressive phenotypes of melanoma multi-drug resistance and metastasis.

4.4.4. **GHR knock-down leads to significantly higher drug retention in human melanoma cells**

A significant suppression of expression of several efflux pumps (as we observed) should translate into a longer retention of xenobiotic compounds inside the GHR-KD melanoma cells compared to the scramble siRNA treated controls. In our fluorometric calcein retention assay, which is sensitive to ABCB1 and ABCC1 mediated drug efflux activity; we indeed observed a significantly lower concentration of retained calcein in the human melanoma cells compared to melanocyte (Figure 42b). Following GHR-KD, significantly higher concentration of calcein was retained inside GHR-siRNA transfected melanoma cells compared to the scramble siRNA transfected controls (Figure 42c). The result corroborated our above observations demonstrating the importance of the GH-GHR interaction in multi-drug resistance in melanoma.
Figure 40: Markers of EMT are strongly modulated by GH action in melanoma cells. Relative RNA expression was quantified for N-Cadherin, E-Cadherin, and vimentin in SK-MEL-28, MALME-3M, MDA-MB-435 and SK-MEL-5 melanoma cells following addition of 0, 5, 50 and 150 ng/mL hGH or following GHR-KD, in presence or absence of 0 and 50 ng/mL hGH. In all cases, exogenous hGH treatment was for 24 hr. Expressions were normalized against expression of ACTB and GAPDH as reference genes. [*: p < 0.05, Wilcoxon sign rank test, n = 4]
Figure 41: Change in markers of EMT following GHR-KD. Changes in protein expressions of vimentin (a), E-cadherin (b) and N-cadherin (c) were analyzed. WB comparison (d) was done in all four melanoma cells, 60 hr. post-transfection with GHR- or scr-siRNA. Blots were quantified using ImageJ software and mean of three blots per sample was taken. Expression levels were normalized against expression of ACTB (B-actin). [* p < 0.05, Students t test, n = 3]
4.4.5. GHR knock-down dramatically suppresses cell proliferation in response to sub-EC50 doses of chemotherapy in human melanoma cells

In order to evaluate the effects of decreased levels of drug efflux pumps and significantly higher drug retention times in GHR-KD melanoma cells, we performed an immunofluorescence analysis of the expression of Ki-67 – an abundantly expressed
marker of cell proliferation, routinely used to observe changes in cell viability. We compared the Ki-67 fluorescence levels in GHR-KD and scramble siRNA treated melanoma cells, following a 24 hr. treatment with cisplatin (0.5 uM), doxorubicin (10 nM), oridonin (0.5 uM), paclitaxel (1 nM), and vemurafenib (2 nM).

We observed dramatic decrease in Ki67 markers across all four melanoma cells following GHR KD at 2-10 folds lower than EC50 doses of the drugs. SK-MEL-28 cells showed particularly consistent decrease in Ki-67 levels following GHR KD when exposed to sub-EC50 levels of cisplatin and vemurafenib (Figure 43). MALME-3M cells showed the most drastic and consistent decrease in Ki-67 levels following GHR-KD when exposed to all five anti-tumor compounds we tested here (Figure 44). Similar reduction in cell proliferation was observed in MDA-MB-435 cells on exposure to cisplatin (Figure 45).

We did not observe similar drastic reductions in Ki-67 levels in SK-MEL-5 cells (Figure 46). When we quantified the cell proliferation levels of SK-MEL-28 and MALME-3M cells following exposure to EC-50 levels of cisplatin (10 uM) and paclitaxel (5 nM) with and without siRNA mediated KD of GHR, we observed drastic inhibition (>90%) in all cases (Figure 47). The results emphasized a net result of sensitization of the human melanoma cells to low doses of anti-cancer drugs following GHR-KD.
Figure 43: Effect of drug treatment on level of Ki-67-cell proliferation marker in SK-MEL-28 cells following GHR-KD. SK-MEL-28 cells were exposed to DMSO (a), 0.5 um cisplatin (b), 10 nM doxorubicin (c), 0.5 uM oridonin (d), 1 nM paclitaxel (e) or 15 nM vemurafenib (f) for 24 hr. Treatments were done 48 hr. post-transfection with either scr-siRNA (1 and 2) or GHR-siRNA (3 and 4). Panels 1 and 3 show cellular DNA stained with DAPI while panels 2 and 4 show fluorescence signals from AF488-tagged anti-Ki67 antibody. Picture was taken at 40X magnification; Scale bar represents 500 um.
Figure 44: Effect of drug treatment on level of Ki-67-cell proliferation marker in MALME-3M cells following GHR-KD. MALME-3M cells were exposed to DMSO (a), 0.5 um cisplatin (b), 10 nM doxorubicin (c), 0.5 uM oridonin (d), 1 nM paclitaxel (e) or 15 nM vemurafenib (f) for 24 hr. Treatments were done 48 hr. post-transfection with either scr-siRNA (1 and 2) or GHR-siRNA (3 and 4). Panels 1 and 3 show cellular DNA stained with DAPI while panels 2 and 4 show fluorescence signals from AF488-tagged anti-Ki67 antibody. Picture was taken at 40X magnification; Scale bar represents 500 um.
Figure 45: Effect of drug treatment on level of Ki-67-cell proliferation marker in MDA-MB-435 cells following GHR-KD. MDA-MB-435 cells were exposed to DMSO (a), 0.5 μM cisplatin (b), 10 nM doxorubicin (c), 0.5 μM oridonin (d), 1 nM paclitaxel (e) or 15 nM vemurafenib (f) for 24 hr. Treatments were done 48 hr. post-transfection with either scr-siRNA (1 and 2) or GHR-siRNA (3 and 4). Panels 1 and 3 show cellular DNA stained with DAPI while panels 2 and 4 show fluorescence signals from AF488-tagged anti-Ki67 antibody. Picture was taken at 40X magnification; Scale bar represents 500 μm.
Figure 46: Effect of drug treatment on level of Ki-67-cell proliferation marker in SK-MEL-5 cells following GHR-KD. SK-MEL-5 cells were exposed to DMSO (a), 0.5 um cisplatin (b), 10 nM doxorubicin (c), 0.5 uM oridonin (d), 1 nM paclitaxel (e) or 15 nM vemurafenib (f) for 24 hr. Treatments were done 48 hr. post-transfection with either scr-siRNA (1 and 2) or GHR-siRNA (3 and 4). Panels 1 and 3 show cellular DNA stained with DAPI while panels 2 and 4 show fluorescence signals from AF488-tagged anti-Ki67 antibody. Picture was taken at 40X magnification; Scale bar represents 500 um.
Figure 47: Effect of GHR-KD on cell proliferation following 24 hr. exposure to EC50 levels of cisplatin and paclitaxel. (a) SK-MEL-28 and (b) MALME-3M cells were exposed to DMSO (vehicle), or 10 µm cisplatin (Cis), or 5 nM paclitaxel for 24 hr. Treatments were done 48 hr. post-transfection with either scr-siRNA (green) or GHR-siRNA (red). Mean of three independent experiments performed in triplicate was taken. [*; p < 0.05, Students t test, n = 3]
4.5. Discussion

Melanomas have the unique property of resisting drug action by multiple processes involving active drug efflux, increased melanogenesis and concomitant packaging away of drugs in melanosomes as well as upregulation of the epithelial-mesenchymal-transition markers as means of decreased keratinocyte control and increased fibroblast interaction. We demonstrated distinct regressive effects of GHR KD on critical aspects of all the above processes in human melanoma cells. Significant reduction in expression of multiple different ABC transporter pumps following a decrease in GHR indicates a GH action dependent mechanism regulating drug efflux from melanoma. In fact we recently reported the existence of GH-GHR mediated regulation of the mTOR pathway in melanoma cells (John J Kopchick, personal communication) and GH induced activation of the pathway is known to be necessary for rapid activation of protein synthesis (Hayashi and Proud, 2007; Russo et al, 2004), as might be expected to be required in case of expression of transporter pumps in response to exposure to drugs. We are currently verifying our observations in vivo using appropriately designed mouse models. Further, the detailed effects of GHR on induction of apoptotic / necrotic cell death, and DNA damage can add significant value to our results.

Our present findings are valuable from several different perspectives. This study presents a first mechanistic model of GH action in mediating multi-drug resistance in human melanoma through possible transcriptional regulation of expression of multiple mediators. This study is in alignment with our observation of a significant GH-dependent
variation in transcription and protein expression of several intracellular mediators of oncogenic signaling pathways in melanoma and adds important and unknown information of the downstream effects of our earlier findings. Decreased drug efflux machinery, increased drug retention, a reversal in EMT markers and a markedly reduced cell proliferation at low doses of chemotherapy following GHR-KD strongly supports the idea of approaching GH-GHR interaction as a suitable chemotherapeutic target of intervention as a combination therapy for several classes of anti-tumor compounds. If effective in vivo, this approach can have several important downstream effects in cancer therapy – (i) a significantly lower drug dose applied in combination or following pretreatment with GHR antagonists can potentially lower the dose and duration of chemotherapy. This in effect should significantly reduce the harsh side-effects associated with chemotherapy. (ii) Having GHR inhibition as a means of sensitizing the tumor cells should be a valuable approach in the area of drug development. Combination of GHR inhibition and chemotherapy can not only markedly improve the efficacy of available anti-melanoma drugs but can also assist the development of candidate compounds under development. Decreased drug retention in tumors is a major hurdle in establishing efficacy of thousands of good drug candidates in pharmaceutical research and development. Our study directly indicates a breakthrough in this problem by establishing that GH-GHR interaction is a critical mediator of drug-resistance and targeting the same can successfully lead to improved drug action. Following our results in one of the most aggressively treatment refractory cases of cancer, we are presently extending the investigation of GH-dependent multi-drug resistance in other forms of GH-responsive or
GHR-expressing cancers (Kopchick et al., 2014). This would immensely benefit the development of new therapeutic approaches and millions of patients worldwide.

4.6. References


Liedert, B., Materna, V., Schadendorf, D., Thomale, J. & Lage, H. Overexpression of cMOAT (MRP2/ABCC2) is associated with decreased formation of platinum-DNA


CHAPTER 5: COMPOUND SCREENING

5.1. Introduction

This part of the dissertation summarizes screening of novel anti-tumor compounds developed by Dr. Stephen Bergmeier’s laboratory in the department of Chemistry and Biochemistry, Ohio University. The compounds were derivatives of a specific diterpenoid and synthesized by Rumita Laha, a graduate student in Dr. Bergmeier’s lab. The specific details of synthesis of these compounds will be documented and discussed in her dissertation. Therefore we will only briefly describe the results of compound screening in this section.

For two selected compounds we re-performed the cell proliferation assay in presence and absence of siRNA-mediated GHR knock-down.

5.2. Brief Methodology

5.2.1. Cell culture

Human malignant melanoma cell lines SK-MEL-5 (#HTB-70), SK-MEL-28 (#HTB-72), and MALME-3M (#HTB-64) cells were obtained from American Type Culture Collection (ATCC; Manassas, Virginia). SK-MEL-5 and SK-MEL-28 were grown and maintained in EMEM media (ATCC #30-2003), while MALME-3M were grown in IMDM (ATCC #30-2005) media respectively. Complete growth media was supplemented with 5% fetal bovine serum (FBS; ATCC # 30-2020) and 1X antibiotic-antimycotic (Thermo Fisher Scientific #15240). Human melanocyte ST-MEL (a kind gift from Dr. Lingying Tong) cells were grown in RPMI-1640 medium (ATCC # 30-2001) supplemented with 10% FBS and 1X antibiotic-antimycotic was used as a control for
testing cytotoxicity of the compounds in non-cancerous cells. Cells were grown at 37°C / 5% CO₂ in a humidified incubator. Half the media was replaced every 48 hr. No hGH was present in the media or added externally unless specifically mentioned. Tissue culture treated sterile T-75 and T-25 flasks and 96-well plates (Corning, New York) were used. Trypsinization was done using 0.25% Trypsin/0.53 mM EDTA in Hank’s balanced salt solution (HBSS) without calcium or magnesium (ATCC # 30-2101) for 5 min / 37°C / 5% CO₂.

5.3.2. Cell proliferation assay

A 1% (w/v) resazurin (Sigma-Aldrich #R7017) solution in 1X PBS was made and filter-sterilized. The final concentration of resazurin in the assay was 0.004%. Inside the proliferating cells mildly fluorescent blue resazurin is reduced to a bright pink fluorescent product called resorufin (stable for 4 hr), which is a quantitative measure of the percentage of proliferating cells. Briefly, cells were seeded at 10,000 cells/cm² into 96-well plates. Transfection was performed as described previously. 48 hr. following transfection, the compounds were added at either 50 uM (ester- and carbamate-derivatives) or 10 uM (amide-derivatives). Resazurin assay was performed 24 hr. after drug addition and resorufin absorbance was measured at 570 nm (reference wavelength = 600 nm) using Spectramax250 (Molecular Devices, Sunnyvale, CA) and SoftmaxPro software. In all cases, cells were incubated at 37°C / 5% CO₂ for 45-60 minutes following resazurin addition, for adequate sensitivity of detection.
5.3. Results

Single-point proliferation assay identified several ester and carbamate-derivatives to effect more than 50% reduction in cell proliferation at 50 uM concentration in one or more of the three melanoma cell lines used (Figures 48 and 49). The carbamate-derivatives were particularly efficacious in reducing cell proliferation to a marked extent in SK-MEL-5 (Figure 49a) melanoma cells. Selective amide-derivatives showed potent anti-proliferative effect on human melanoma cell lines (Figure 50) with particularly pronounced effect observed in MALME-3M cells (Figure 50c). We observed limited levels of cytotoxicity of the compounds in normal skin fibroblasts (Figures 51 and 52) and there was an apparent specificity for tumor cells compared to normal cells.

EC50 determination of compounds RLB-49 and RLB-76 was performed in MALME-3M cells transfected with either scr-siRNA or GHR-siRNA. We observed significant lowering of EC-50 values of both drugs following GHR KD. The EC50 of RLB-76 was 28 uM in MALME-3M cells but reduced to 14 uM following GHR-KD. On the other hand, RLB-49 had an EC-50 of 1.5 uM which was drastically reduced to as much as 22 nM following GHR KD (Figure 53).
Figure 48: Cytotoxicity of RLB-ester derivatives against human melanoma cells. (a) MALME-3M, (b) SK-MEL-28, and (c) SK-MEL-5 cells were seeded at 10,000 cells/well in a 96 well plate and treated with 50 uM compounds for 24 hr. Cell proliferation was measured by a resazurin-based metabolic assay. The plots show per cent inhibition compared with cells treated with only DMSO.
Figure 49: Cytotoxicity of RLB-carbamate derivatives against human melanoma cells. (a) MALME-3M, (b) SK-MEL-28, and (c) SK-MEL-5 cells were seeded at 10,000 cells/well in a 96 well plate and treated with 50 µM compounds for 24 hr. Cell proliferation was measured by a resazurin-based metabolic assay. The plots show per cent inhibition compared with cells treated with only DMSO.
Figure 50: Cytotoxicity of RLB-amide derivatives against human melanoma cells. (a) SK-MEL-5, (b) SK-MEL-28, and (c) MALME-3M cells were seeded at 10,000 cells/well in a 96-well plate and treated with 10 uM compounds for 24 hr. Cell proliferation was measured by a resazurin-based metabolic assay. The plots show per cent inhibition compared with cells treated with only DMSO.
Figure 51: Cytotoxicity of RLB-ester and RLB-carbamate derivatives against normal human skin fibroblast cells. MALME-3M – normal human skin fibroblast from the same patient from which MALME-3M melanoma cells were derived, were seeded at 10,000 cells/well in a 96-well plate and treated with 0.5, 5 or 50 μM compounds for 24 hr. Cell proliferation was measured by a resazurin-based metabolic assay. The plots show per cent inhibition compared with cells treated with only DMSO.
Figure 52: Cytotoxicity of RLB-amide derivatives against normal human skin fibroblast cells. MALME-3M – normal human skin fibroblast from the same patient from which MALME-3M melanoma cells were derived, were seeded at 10,000 cells/well in a 96-well plate and treated with 0.5, 5 or 50 uM compounds for 24 hr. Cell proliferation was measured by a resazurin-based metabolic assay. The plots show per cent inhibition compared with cells treated with only DMSO.
5.4. Conclusion

A total of 92 compounds were screened for anti-tumor activity against human melanoma cells. There were a number of positives with less than 10 uM EC-50. The quantitative details, analysis and discussion were not covered in this dissertation work. Our observation of significant reduction of EC50 values of two compounds following GHR KD supports the findings of this dissertation that reduced GHR sensitizes human melanoma cells to chemotherapy.
6.1. Conclusions

We had two distinct hypotheses (Chapter 2) at the beginning of this dissertation. By working along the stated specific aims, we arrived upon some valuable information regarding the role of a functional GHR and GH action in human melanoma cells.

**Hypothesis 1: Reduced GHR expression will decrease melanoma proliferation and affect multiple intracellular signaling pathways and processes involved in melanoma progression.**

A marked reduction of GHR expression in human melanoma cell lines brought about by siRNA-mediated KD allowed us to specifically investigate the effects of reduced GHR on several parameters of melanoma cell growth. Our observations reported above reveals a pronounced role of excess GH and/or GHR KD on multiple integral and essential ligand-receptor levels and activation of essential oncogenic intracellular signaling pathways. This study provides not only a first detailed analysis of the GH-GHR signaling pathway in human melanoma cells, but also establishes a central role of this ligand-receptor pair in melanoma biology on the whole. In addition, our findings will go on to act as a springboard for useful interrogations for further regulatory details of this pathway involving melanoma as well as its interaction with its microenvironment (discussed later). Importantly, it establishes the use of human melanoma cells harboring GHR and endogenous expression of GH, for the specific study of GH action in this form of cancer. Our results showing a significantly regressive tumor behavior in migration, invasion, clonogenicity and proliferation following GHR KD, highlights the global effect
exerted by the reduction of GHR in melanoma. This definitely argues for the fact that GHR-expressing human cancers like melanoma provide a plausible target, i.e. the GHR, for possible therapeutic intervention.

**Hypothesis 2: Reduced GHR expression will negatively impact the drug resistance property of melanoma cells.**

We identified a vital downregulation of RNA expression of a repertoire of multi-drug efflux pumps following GHR KD and exposure to five different types of anti-melanoma drugs. Additionally, we reported of critical findings regarding GH mediated regulation of EMT and melanogenesis mediators, both of which are known to be important processes involved in conferring the extremely high multi-drug resistance observed in human malignant melanoma. Sub EC-50 doses of cisplatin, doxorubicin, oridonin, paclitaxel, and vemurafenib were found to significantly reduce cell proliferation in GHR KD cells compared to the scramble siRNA treated controls. The GHR KD cells also showed a net increase in intracellular retention of calcein indicating a net decrease in drug efflux. Finally, we also exhibited a clear and drastic decrease in EC50 values of drug candidates following GHR KD in human melanoma cells. The collection of these results demonstrates a novel and valuable role of GH-GHR interaction in driving drug-resistance in the most aggressive type of skin cancer.

6.2. Significance

We answered several vital and longstanding questions in melanoma biology revolving multi-drug resistance and the criticality of the role of GH-GHR interaction in this type of cancer. Melanoma cells are now known to express GH, GHR, GHRHR as
well as somatostatin receptors in addition to IGF binding proteins (IGFBP2, IGFBP3) and cognate receptors of insulin, IGF1 and IGF2, thereby presenting a rich landscape of a neatly regulated system of GH action. We confirmed this indication by showing robust modulation of oncogenic intracellular pathways with reduced GHR expression or excess GH. Further, the significant upregulation of oncogenic processes and markers in presence of excess GH supported and highlighted the risk of melanoma incidence with GH therapy as had been discussed above. One of the most important findings of this entire study is possibly the revelation of distinct GH regulation on drug resistance in melanoma cells. In essence we incidentally demonstrated what a report had once aptly stated - ‘acquired or intrinsic mechanisms of resistance can reveal molecular vulnerabilities that may allow responses even to chemotherapy drugs that would have been considered ineffectual’ (Radvanyi, 2013). The consistent and significant suppression of various mechanisms of therapy evasion simultaneously due to GHR KD alone, establishes GH-GHR interaction as the possible Achilles’ heel in drug-resistant malignant melanoma. Following confirmation from in vivo studies, if a reduction in GHR leads to a more efficacious yet decreased concentration of chemotherapy, it might potentially translate into reduced side-effects of chemotherapy treatment. This would be invaluable for millions of cancer patients worldwide. In addition, we demonstrated the mechanism by which abundant GHR expression is possibly harnessed by melanoma to empower itself with drug-resistant capacity. Thus, sensitization of the melanoma cells by antagonism of GHR should be of great value in drug development whereby an expectedly higher number of
hits can be obtained by combining candidate compounds with GHR antagonists (e.g. Pegvisomant), not for an additive but a synergistic effect.

6.3. Future Directions

Our study revealed an array of information regarding GH action and its involvement in conferring multi-drug resistance in human melanoma. In the wake of those findings as discussed above, we briefly point towards the following future directions that are highly relevant and potentially valuable to add to the understanding of melanoma biology and tackling the disease effectively.

6.3.1. Effects of GHR antagonist

The laboratory of Dr. John Kopchick at Edison Biotechnology Institute at Ohio University have discovered a novel and only prescribed GHR antagonist – Somavert (Pegvisomant for injection) – which is used to treat acromegaly patients. Already established as a chemotherapeutic agent for a human disease, this GHR antagonist aptly suits the role of an inhibitor of GH-GHR interaction in the treatment of GHR expressing human melanoma. This also allows us to verify our observations by substituting siRNA mediated GHR KD with the GHR antagonist. We are in the process of acquiring the necessary reagents for undertaking this study using in vitro and in vivo approaches. Additionally the extremely high levels of IR, IGF1R and IGF2R and knowledge of the metabolic effects of pegvisomant in animals, prompt to design novel combinations of GHR antagonist with RTK-inhibitors like metformin and scores of others which have performed poorly as a monotherapy in different cancers (Pollak, 2008; Regad, 2015).
6.3.2. In vivo studies

The results described above were generated from extensive in vitro studies. The identification of novel regulatory roles of GH on melanoma cell culture models now should be verified on appropriate in vivo systems like mouse models. Our laboratory has generated extensively studied and widely used models of GH-excess and GHR disruption which are ideal for in vivo validation of the results described in this dissertation. The study is already underway.

6.3.3. Effect of GHR reduction on multi-drug resistance in other forms of cancer

The presence of GH-GHR interaction has been extensively reported for breast, colorectal, prostate and other forms of cancers as described above. However, the critical involvement of GH-GHR interaction in mediating widespread multidrug resistance mechanisms has not been reported in any other forms of cancer prior to this study. Therefore this study acts as a reference for interrogating other types of GH-responsive GHR-expressing human cancers for the extent and pattern of involvement of the ligand-receptor pair in conferring drug-resistance.

6.3.4. Effect of GHR reduction on apoptosis and DNA damage pathways

We observed significant increase in DNA damage markers following GHR KD in our study. However, detailed analysis of the apoptosis and DNA damage pathways are necessary following GHR KD or antagonism, as well as following treatment with excess GH. Both in vitro and in vivo studies in these critical pathways are vital especially because non-pituitary GH is known to exert anti-apoptotic effects (Chesnokova et al., 2013).
6.3.5. Effect of GHR reduction on the metastatic success of human melanoma

A highly preferred metastatic location for malignant melanoma is the bone. The role of GH in osteoclast differentiation and bone resorption is well documented (Kaji et al., 1996) and the osteoclastogenesis stimulation by metastasizing melanoma cells using SRC-mediated signaling (Perez et al., 2001). Following our report of the versatile role of GH in melanoma, including stimulation of SRC phosphorylation, it is reasonable to hypothesize a decreased metastatic success and stimulation of bone resorption by malignant melanoma following GHR antagonism.

6.3.6. GHR in cancer stem cells

Numerous reports have established a number of multi-drug efflux pumps like ABCB5, ABCG2, and ABCB8 as markers of cancer (as well as melanoma-specific) stem cells (Lee et al., 2014). We reported a profound regulatory effect of GHR KD on several ABC transporter pumps including the ones mentioned above. Further, we here reported GH-GHR action as a possible driver of EMT, which in turn is known to be a driver of drug resistance, cancer cell stemness and metastasis (Singh and Settleman, 2010). Although newly introduced immunotherapies (pembrolizumab) for melanoma have had an excellent success in CD8+ T-cell expressing patients, their use against GHR-expressing melanoma cells might require some added evaluation. In different forms of cancer, immunotherapy has been reported to drive the selection towards ‘stemness’ (Radvanyi, 2013; Restifo et al, 2016). It might be especially useful to evaluate the implication of GH-GHR interaction in driving cancer ‘stemness’ in order to target the
interaction to render immunotherapies more effective without selecting for any resistant population of cancer cells.

6.4. References


