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Effects of Growth Hormone (GH) Overexpression in Signaling Cascades Involved in Promotion of Cell Proliferation and Survival

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1. Introduction

This chapter will describe the effects of long term exposure to growth hormone (GH) at the molecular level in the liver. Expression and activation of intermediates involved in GH-induced signaling were analyzed in transgenic mice overexpressing GH, as well as the influence of chronically elevated GH levels over Amend: epidermal growth factor receptor (EGFR) signaling. Several signaling mediators involved in cellular proliferation, survival and migration are altered in the liver of GH-transgenic mice. The molecular mechanisms underlying the pro-oncogenic pathology induced by prolonged exposure to elevated GH levels will be discussed.

1.1 Physiological actions of GH

Growth hormone (GH), also known as somatotropin, is the main regulator of postnatal body growth, but its actions are not limited to corporal stature. GH has important metabolic functions on carbohydrate, lipid and protein metabolism, as well as on tissue maintenance and repair, cardiac and immune function, mental agility and ageing. It exerts its actions both directly, and by means of endocrine and paracrine insulin-like growth factor (IGF) 1. At the cellular level, GH modulates proliferation, differentiation, motility and apoptosis.

GH secretion:

Growth hormone, part of the somatotrophic axis, is mainly synthesized in somatotroph cells from the anterior pituitary. Growth hormone secretion is regulated centrally, through the hypothalamic-portal circulation, by hypothalamic peptides: its synthesis and release are promoted by growth-hormone releasing hormone (GHRH) and inhibited by somatostatin, and it is also stimulated by stomach-derived ghrelin. Stress, exercise, malnutrition, and anorexia also promote its secretion. By means of negative feedback, GH inhibits its own secretion: in the short central loop, GH acts on somatotroph cells to generate IGF1 locally, that in turn inhibits the cell; and it acts at the hypothalamic level to inhibit GHRH synthesis and release and to stimulate both synthesis and release of somatostatin. In the long peripheral loop, GH acts on the liver to generate most of circulating IGF1, which inhibits GH secretion by a dual mechanism: direct inhibition of the somatotrophs and stimulation of somatostatin release. GH is also produced locally in many tissues, acting in an autocrine and

paracrine fashion. Central cholinergic stimulation increases the release of GH reducing the secretion of somatostatin. Glucocorticoids and metabolic substrates also affect GH secretion; treatment with glucocorticoids inhibits its release while fasting states with hypoglycemia, low circulating free fatty acids, and high circulating amino acid concentrations stimulate it. GH secretion presents sexual dimorphism: the frequency of pulses is higher in females. The difference is most striking in rats, where secretion profile in males is characterized by high amplitude pulses every 3-4 h, with almost non-detectable values between pulses. Female rats, on the contrary, have more frequent lower amplitude pulses, and thus present baseline GH levels (Waxman & Frank, 2000). Humans, on the other hand, present higher values at night, during the first hours of sleep, and lower values in the early morning. GH secretory pattern conditions the sexually dimorphic gene expression in the liver, particularly of proteins involved in steroid and drug metabolism.

GH secretion presents age-dependency: circulating GH levels progressively rise during childhood, to achieve a maximum towards the end of adolescence in humans, and slowly diminish thereafter. By the sixth decade of life, they are only 20% of maximum (Turyn & Sotelo, 2004).

GH function:

Somatic growth: GH acts directly on bone and indirectly, through endocrine hepatic derived IGF1, as well as the locally produced factor. These growth promoting peptides act on the epiphyseal cartilage, inducing chondrocyte proliferation, which leads to longitudinal skeletal growth (Tritos & Biller, 2009). Longitudinal growth ceases when the epiphyses of the long bones fuse to the diaphyses. Oversecretion before this instance results in *gigantism*, whereas oversecretion afterwards results in *acromegaly*, characterized by abnormal growth of hands and feet, and roughening of the facial features: protrusion of brow and lower jaw, and nose enlargement. Lack of GH or GHR in humans results in severe short stature, reduced muscle mass, increased fat storage, decreased cortical bone mineral density and decreased fertility in females (Lichanska & Waters., 2008a). In animals, basically the same growth outcome can be observed, either in spontaneous mutants or in genetically engineered models. Notably, mice lacking GH or GHR live longer than their littermates (Bartke & Brown-Borg, 2004).

Besides its effects on skeletal growth, GH regulates body composition, increasing muscle mass and decreasing adipose content. The relationship between GH status and body composition is evidenced in patients and animal models lacking GH, which become obese. In humans, symptoms of GH deficiency resemble those of ageing, when GH levels decline: loss of muscular mass and tone, loss of bone mineral density, loss of strength, abdominal obesity (Perrini et al., 2010).

Metabolic actions: GH has important metabolic actions on lipid and carbohydrate metabolism. It presents both insulin-like and insulin-antagonistic actions, presumably the first are IGF1-mediated while the latter are directly exerted by the hormone, since the effects of IGF1 on lipolysis and gluconeogenesis are contrary to those of GH (Kaplan & Cohen, 2010). GH exerts lipolytic effects, principally at the visceral adipose tissue, resulting in an increase of circulating free fatty acids, by increasing adipose tissue hormone-sensitive lipase activity; at the same time, it inhibits glucose uptake in adipose tissue. On the other hand, in liver GH promotes triglyceride (TG) uptake and storage, and in skeletal muscle it induces TG uptake and utilization. GH also presents anabolic actions on protein metabolism, since it stimulates protein synthesis and inhibits its proteolysis (Vijayakumar et al., 2010).

The contrasting effects of GH and insulin on substrate metabolism depend on the nutritional status and food intake. Endogenous GH secretion is down-regulated by food intake, allowing insulin action on storage of nutrients. In a fasted state, GH secretion favors lipolysis. Between these two states, GH and insulin may act concomitantly to promote IGF1 production and therefore, protein synthesis. Usage of fatty acids as the energy source during fasting instead of glucose protects against excessive protein break-down. Thus, GH is both anabolic and anti-catabolic in protein metabolism (Jorgensen et al., 2010).

1.2 GH-signaling

1.2.1 Growth hormone receptor

Structure: GH exerts its functions by binding to its cognate receptor, the GHR. This receptor is a single-chain transmembrane glycoprotein which belongs to class I cytokine receptors. It is composed of three domains: the extracellular ligand-binding domain, arranged as two fibronectin type domains connected by a short flexible linker; the transmembrane domain; and the intracellular domain (ICD). The ICD has two motifs that bind tyrosine kinase JAK2, Box1 and Box2, and several tyrosine residues, which are substrates of JAK2 and become docking sites for phosphotyrosine binding molecules (Brooks et al., 2008). JAK2 is critical for GHR-signaling since GHR lacks intrinsic kinase activity. GHR is a member of the cytokine receptor superfamily, and is therefore structurally related to other members of this family, such as the receptors for prolactin, erythropoietin, thrombopoietin, leptin, interleukin 3, 5 and 6, granulocyte/macrophage colony-stimulating factor and interferon (Rosenfeld & Hwa, 2009; Lanning & Carter-Su, 2006).

GHR loss of function mutations: The GHR mediates GH growth-related functions, as mutations in the receptor lead to severe stature deficit, similar to the lack of the hormone. Laron syndrome is a genetic disorder characterized by growth retardation and very short stature at adulthood (>5 SD), patients also present impaired muscle and bone development, as well as obesity and steatosis (Brooks et al., 2008). It is associated with deletions or mutations principally at the extracellular ligand-binding domain of the receptor; as a GH insensitivity syndrome, it is concurrent with high GH but low IGF1 circulating levels.

GHR levels: Liver exhibits the highest GHR concentration, but it is also highly expressed in muscle, bone, kidney, mammary gland, adipose tissue, heart, intestine, lung, prostate, pancreas, cartilage, fibroblasts, and embryonic stem cells; in fact, GHR is expressed in almost every tissue of the body, indicating the relevance of GH action in every organ. Several factors regulate GHR concentration, including nutritional status and developmental stage. GHR levels are down-regulated by under-nutrition and fasting, while their levels gradually increase from birth to adulthood (Tiong & Herington, 1999). GH is a principal modulator of GHR, indeed it induces the synthesis of its own receptor (González et al., 2001; González et al., 2007).

GHR turnover: at least two different mechanisms participate in the down-regulation of the mature form of the GHR at the plasma membrane: ligand-independent endocytosis and proteolytic cleavage (Flores-Morales et al., 2006). The protein break-down consists of two steps: a metalloproteinase, the tumor necrosis factor- α converting enzyme (TACE), cleaves the extracellular portion of the receptor, close to the insertion point at the membrane. This generates the soluble form of the receptor, known as growth hormone binding protein (GHBP). After this process, the membrane-bound remnant is degraded by a γ -secretase complex, and targeted to proteasomal degradation (Flores-Morales et al., 2006; Zhang et al.,

2000; Wang et al., 2002; Cowan et al., 2005). This mechanism is believed responsible for GHBP release into circulation, but it is not expected to participate in termination of GH signaling since it is inhibited by ligand binding (Flores-Morales et al., 2006; Zhang et al., 2001). GHR endocytosis, which involves both clathrin-coated pits and caveolae, does not require ligand-binding although receptor occupation accelerates the process. Internalized receptors are mostly derived to lysosome and proteasome degradation, as they are not recycled to the membrane (Flores-Morales et al., 2006; Sachse et al., 2001; Lobie et al., 1999). GHR-internalization requires an intact ubiquitin conjugating system, although actual ubiquitination of the receptor does not seem to be required (Lanning & Carter-Su, 2006; van Kerkhof et al., 2007).

GHBP: Growth hormone circulates in plasma bound to specific proteins known as growth hormone-binding proteins (GHBP). The most important of these proteins, high affinity GHBP, coincides with the extracellular domain of the GHR, and is generated by two different mechanisms. While in humans and rabbits it surges after proteolytic processing of the mature receptor, in rodents it is the result of alternative splicing of the GHR pre-mRNA (Baumann, 2001). Lower affinity and high molecular weight GH-binding proteins are not related to the GHR (Kratzsch et al., 1996).

GHR nuclear localization: Apart from its expected localization at the cell surface and at the endoplasmic reticulum, GHR has also been found at the nucleus of several cells. This localization is not unique for the GHR, since other transmembrane receptors have also been described in the nuclear compartment. Nuclear GHR was found in cells exhibiting high proliferative status, associated with transformation and tumor progression (Brooks et al., 2008; Campbell-Conway et al., 2007). Autocrine GH is key to the action of nuclear GHR on cell proliferation (Brooks et al., 2008).

1.2.2 Signaling pathways induced by GH

Hormone binding: Compelling evidence suggests GHR exists as a preformed dimer on the cell surface. Growth hormone binds to the extracellular domain of the GHR dimer via two asymmetrically-placed binding sites on the hormone: high affinity site 1, and lower affinity site 2. Specificity of the dimerization partner is conferred by the extracellular domain of the receptor, while union of the two moieties is attained at the transmembrane level through leucine zipper-like interactions (Lichanska & Waters, 2008a). The current model posits ligand binding to homodimerized inactive receptor induces relative rotation of the intracellular domains, leading to proper alignment of tyrosine kinase JAK2 (Brown et al., 2005). The repositioning of the GHR induces JAK2, constitutively associated to the GHR, to become activated and thus phosphorylate not only other GHR-associated JAK2 molecules by trans-phosphorylation, but also modifies key tyrosine residues on the GHR, which become docking sites for SH2 (Src homology 2)-domain containing proteins (Rosenfeld & Hwa, 2009; Brooks et al., 2008). Autophosphorylation of JAK2 tyrosine-residues involves both activation of the catalytic site as well as modification of regulatory residues which may enhance or diminish kinase activity (Lanning & Carter-Su, 2007; Feng et al., 1997; Argetsinger et al., 2004; 2010).

Activated signaling pathways: Different signaling mediators are already preforming complexes with inactive receptor or are recruited to phosphotyrosines on activated GHR complex and bind to the complex by means of their phosphotyrosine binding modules, the PTB and the SH2-domains. GH activates at least three major signaling pathways, the signal

transducers and activators of transcription (STATs), the mitogen activated protein kinase (MAPK) Erk1/2 and the phosphatidylinositol 3'-kinase (PI3K)/Akt pathways.

GH binding to its receptor triggers activation of STAT1, STAT3 and STAT5. These SH2-containing transcription factors are latent on the cytoplasm, become activated by tyrosine phosphorylation in one critical residue, and thus dissociate the receptor, dimerize and migrate to the nucleus as active dimers, where they bind specific DNA sequences to regulate the expression of multiple genes. STAT5 a and b are essential for many GH functions related to metabolism, body growth and sex-dependent liver gene regulation (Lanning & Carter-Su, 2006), indeed, STAT5b is regarded as the most relevant signaling mediator for GH actions, both direct and IGF1-mediated, as it regulates the transcription of the IGF1 gene (Woelfle et al., 2003a, 2003b; Woelfle & Rotwein, 2004). While it is not clear if STAT1 and STAT3 require GHR phosphorylation or bind to phosphotyrosine residues on the receptor, STAT5 can bind to several phosphotyrosine residues at the carboxi-terminal part of the intracellular domain of the receptor (Rowland et al., 2005; Lichanska & Waters, 2008a).

To date, there is no sufficient evidence relating the PI3K or the MAPK/Erk pathways with GH-induction of IGF1 transcription. Among the STATs, STAT5b has been regarded as the GH-mediator of IGF1 expression, but also of other important contributors to IGF1 function, as the acid labile subunit (ALS) and IGF1-binding protein 3 (IGF-BP3), both in rodents and in humans (Rosenfeld & Hwa, 2009; Woelfle & Rotwein, 2004). These two proteins complex IGF1 in circulation to modulate its bioavailability. Mutations in STAT5b have been associated with severe short stature in humans and reduced size in rodents, suggesting this mediator is crucial for GH-dependent skeletal growth (Udy et al., 1997; Kofoed et al., 2003; Rosenfeld et al., 2005).

Fig. 1. Growth hormone signaling

GH activates the PI3K pathway, either by recruitment of insulin receptor substrates (IRS) 1, 2 and 3, or alternatively, by a CrkII-IRS1 interaction (Goh et al., 2000), or even by direct interaction of the PI3K to phosphotyrosine residues on the GHR (Lanning & Carter-Su, 2006; Moutoussamy et al., 1998). In either case, PI3K is recruited by binding of its p85 regulatory subunit to phosphotyrosine residues on its activated partners. This is a branching pathway, giving rise to multiple signals, which derive in protein and carbohydrate metabolism. Protein kinase B (PKB), a serine/threonine kinase most usually referred to as Akt, is one of PI3K principal downstream effectors. Akt activates mTOR (mammalian target of rapamycin), a protein-kinase that participates in the regulation of ribosomal protein translation, which leads to protein synthesis, among other substrates. Akt also participates in glucose metabolism, as it promotes glucose uptake and regulates glycogen synthesis. The IRSs/PI3K signaling cascade is the principal pathway activated by insulin and may therefore contribute to insulin-like actions of GH (Dominici et al., 2005).

The mitogen-activated protein kinase (MAPK) cascade participates in the control of cell proliferation, differentiation and migration. GH has been shown to activate the MAPK/Erk1/2 pathway by means of recruiting the adapter protein Shc (Src homology collagen) to the activated GHR-JAK2 complex, which becomes phosphorylated and thus binds Grb2, the guanine nucleotide exchange factor SOS, Ras, Raf, MEK (MAPK Erk kinase) and finally, the extracellular-regulated kinase (Erk) 1 and 2 (Lanning & Carter-Su, 2006). Recently, IRS-1 has been shown to act upstream of Shc/Grb activation of Erk1/2 (Wang et al., 2009). Alternatively, JAK2 was proposed to phosphorylate Grb2-binding site of the EGFR, allowing recruitment of Grb2 and activation of the pathway (Yamauchi et al. 1997; Lanning & Carter-Su, 2006). GH has also been shown to activate p38 and JNK/SAPK MAP kinases, other MAPK cascades (Zhu et al., 2001).

It is generally believed that activation of Erk1/2 rely on JAK2 activation, but it has also been reported to be activated by Src –another receptor-associated tyrosine kinase-, in a JAK2-independent fashion (Zhu et al., 2002; Rowlinson et al., 2008). GH-induced Src activation of Erk1/2 has been proposed to occur through an alternative mechanism, involving Ras-like small GTPases RalA and RalB and activation of phospholipase D (Zhu et al., 2002). The participation of JAK2 and Src in GH-mediated signal is dependent on cell type (Brooks & Waters, 2010), although the relative contribution of these cascades to GH signaling *in vivo* is controversial (Jin et al., 2008). Additionally, Erk1/2 have been shown to be activated by GH by another SKF (Src kinase family) member, Lyn, which signals through phospholipase C gamma and Ras (Rowlinson et al., 2008). Targeted mutation of Box1 –the JAK2 binding motif in GHR- in mice allows GH-induced activation of Src and Erk1/2 (Barclay et al., 2010) where JAK2 activation is abrogated, suggesting a minor contribution of this pathway to GH action. Moreover, it has recently been proposed that the conformational change the ligand impinges on the receptor conditions which signalling pathway becomes activated (Rowlinson et al., 2008).

1.2.3 Ending of the signal

GH is secreted episodically, thus the signaling elicited by each secretory burst must be readily counteracted to allow resensitization to a further pulse. Therefore, a critical balance between hormone signaling and its down-regulation is required. Described mechanisms of signaling attenuation involve blockage or removal of the phosphotyrosine residues in the activated GHR-complex by binding of inhibitory molecules or dephosphorylation, and ubiquitin-dependent GHR endocytosis (Lanning & Carter-Su, 2006).

Phosphatases: Since tyrosine phosphorylation is the primary event triggered upon ligand binding to receptor, it could be expected that removal of phosphorylation restores inactivated mediators. Several protein tyrosine phosphatases (PTPs) participate in GH-signaling termination, dephosphorylating not only GHR and JAK2 but also the STATs. Among these, the SH2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 were the first described. While SHP-1 is preferentially expressed in hematopoietic cells, SHP2 is ubiquitously expressed (Murphy et al., 2010). SHP-1 is considered as a negative regulator of GH signaling, whereas SHP-2 has been regarded as a dual modulator, serving both positive and negative effects on GH signaling. It may act as a dephosphorylating enzyme, but it may also act as an adaptor protein binding to phosphotyrosine motifs by means of its SH2 domains. Mutation of SHP-2-binding residues on the GHR prolongs GH-induced phosphorylation of GHR, JAK2 and STAT5, thus suggesting negative regulation, while overexpression of catalytically inactive forms inhibited GH action (Lanning & Carter-Su, 2006; Stofega et al., 2000; Kim et al., 1998).

Other protein phosphatases have been shown to deactivate GHR-complex, namely, PTP-H1, PTP1, TC-PTP and PTP-1B (Pasquali et al., 2003); PTP-1B and PTP-H1 have also been shown to dephosphorylate STAT5 (Gu et al., 2003; Aoki & Matsuda, 2000). Catalytically inactive PTP-1B mutant mice present increased phosphorylation of JAK2 and STAT5-enhanced sensitivity to GH (Gu et al., 2003), while mice lacking the PTP-H1 catalytic domain show enhanced growth, with augmented hepatic mRNA IGF1 expression (Pilecka et al., 2007).

Suppressors of cytokine signaling (SOCS): SOCS proteins are regulators of the main signaling pathway induced by cytokines, the JAK/STAT pathway, therefore, they act to down-regulate the same signal that induced their expression, acting through a classical negative feedback loop (Flores-Morales et al., 2006). GH promotes transcription of four SOCS proteins, SOCS-1, -2, -3 and CIS (cytokine inducible suppressor), these proteins can be divided into two groups according to mechanism of action and to kinetics of appearance. SOCS-1 and SOCS-3 impede JAK2 activity once they are recruited to the GHR-JAK2 complex, whereas SOCS-2 and CIS bind to phosphotyrosine residues at the distal portion of the GHR, and thus interfere with STAT5 activation. SOCS-1 and -3 are rapidly but transiently induced after a GH stimulus, whereas CIS and SOCS-2 are induced after SOCS-1 and SOCS-3 mRNA levels diminish (Tollet-Egnell et al., 1999). Apart from the inhibitory mechanism, all SOCS proteins present a SOCS-box domain, by which they can associate an E3-ubiquitin-ligase complex that adds ubiquitin molecules to the SOCS-associated proteins, as JAK2 and GHR, to target them to proteasome degradation (Lanning & Carter-Su, 2006; Flores-Morales et al., 2006). SOCS-2 may also inhibit GH action indirectly, by limiting IGF1 signaling (Lanning & Carter-Su, 2006; Frutchman et al., 2005). Moreover, SOCS-2 was reported to target SOCS-3 for ubiquitination and protein degradation when it is associated to the signaling complex (Tannahill et al., 2005; Piessevaux et al., 2006), therefore contributing to cease the termination signal, and thus allow resensitization.

1.3 Therapeutical uses of GH

The use of growth hormone (GH) in clinical endocrine practice is expanding, and its role in the treatment of various clinical conditions is increasingly appreciated. GH has been used to treat children with GH deficiency (GHD) for more than 40 years. Human GH was originally obtained from cadaveric pituitaries and was available in limited quantities. From 1985, biosynthetic GH initially became available for prescription usage. Nowadays, human GH of

recombinant DNA origin with an amino acid sequence identical to GH of pituitary origin is produced commercially by several pharmaceutical companies. These GH preparations contain minimal impurities, are apparently safe, and are readily available in unlimited supply. As a result, use of the hormone both in children and adults has expanded.

GH is used in children for the following pediatric conditions: growth hormone deficiency (GHD), Turner syndrome, chronic renal insufficiency, children born small for gestational age or intrauterine growth retardation, Prader-Willi syndrome and when the height deficit continues at puberty. GH is indicated in adult patients with growth hormone deficiency caused by pituitary disease from known causes, including pituitary tumor, pituitary surgical damage, hypothalamic disease, irradiation, trauma, and reconfirmed childhood GHD (Medical Guidelines for Clinical Practice for GH use in adults and children, American Association of Clinical Endocrinologists-2003 Update). GH usage has also been approved for the treatment of AIDS-related wasting.

In addition to the generally accepted therapeutic uses of human growth hormone, considerable interest exists in using GH treatment in various other conditions. The usefulness of GH treatment in adults who have completed their statural growth derives from the role of GH in the following processes: increasing bone density, increasing lean tissue, decreasing adipose tissue, strengthening cardiac contractility, recovering mood and motivation, increasing exercise capacity. Considering the anabolic actions of human growth hormone, it has become attractive as a potential agent for catabolic problems in a wide range of clinical conditions, including patients in an intensive care environment, burns, cystic fibrosis, inflammatory bowel disease, fertility alterations, osteoporosis, Down's syndrome, and also for people wishing to reverse the effects of ageing and to improve the athletic condition (Hadzović et al., 2004). These last two potential uses have received most attention as abuse of growth hormone. The definitions of the word abuse include "improper or excessive use." The classic form of "abuse" of human growth involves its use by athletes or bodybuilders to gain an unfair advantage over their competitors. The use of human growth hormone to increase the height of children who are already of normal height would also be considered abuse (Hintz, 2004). Another common form of use of human growth hormone outside the established indication is in its supposed action of diminishing or slowing the effects of ageing (Rudman et al., 1990). In addition to the lack of evidence for effectiveness of human growth hormone in these proposed uses, it causes side effects such as diabetes, carpal tunnel syndrome, fluid retention, joint and muscle pain and high blood pressure. Considering the increased incidence of leukemia and certain types of tumors reported in acromegalic patients, cancer onset is also a risk of GH use and abuse.

1.4 GH association with cancer development

Normal growth of a living organism depends on the rate of cell division and cell death. There is a strictly controlled balance between these processes. Organ and body size are determined by three fundamental processes: cell growth, cell division, and cell death, each regulated both by intracellular programs and by extracellular signaling molecules that control these programs. The cell decides its fate depending on the balance between survival and cell death which ensures that healthy functioning cells survive in appropriate environments while damaged or non-functioning cells are eliminated by programmed cell death (apoptosis). If this equilibrium is disturbed, one of two disorders might occur: the cells divide faster than they die which

ultimately derives in tumor development or the cells divide slower than they die, which results in cell loss. One of the most important extracellular signaling molecules engaged in maintaining cell survival are the growth factors. When these proteins are overexpressed and when their receptors or signaling mediators are hyperactivated or overexpressed, cancer might arise.

The relevance of growth factors to the pathogenesis of human cancer has long been established. Several recent studies have suggested that, in addition to its effects on growth and metabolism, GH is involved in tumorigenesis and tumor progression. GH overexpression has been associated with cancer in animal models as well as in humans; indeed, acromegalic patients show an increased incidence of this pathology (Webb et al., 2002; Jenkins, 2004; Siegel & Tomer, 2005). Studies focusing on cancer incidence or cancer prevalence in acromegaly report 1.5 to 4-fold increased relative risk for acromegalic patients to develop tumors, mainly of the colon, breast, prostate, thyroid and hematological system (Siobhan & Shereen, 2008). However, as the main causes of death are cardiovascular and respiratory events, patients with uncontrolled acromegaly might succumb before developing recognizable cancer. On the contrary, a decreased incidence of cancer in the absence of growth hormone has been observed. In a worldwide survey of individuals with growth hormone deficiency or growth hormone receptor mutation, not even a single case of malignancy was reported, whereas first and second degree relatives reported a 10-24% incidence of malignancies (Brooks & Waters, 2010). Moreover, another study revealed that GHR deficiency in humans is associated with an important reduction of pro-ageing signaling, diabetes and cancer development (Guevara-Aguirre J, et al., 2011). Cancer risks of GH replacement therapy implies three possible situations: (1) tumor recurrence in children with previously treated cancer; (2) second neoplasms (SNs) in survivors of childhood cancer treated with GH; and (3) de-novo cancer in non-cancer patients treated with GH. The general evidence suggests no increased risk in case 1, but several and complex studies concluded that there is a very modest increase in cancer risk in treated GH-deficiency patients in situations 2 and 3 (Renehan & Brennan, 2008).

Concerning animal models, a large body of evidence has implicated GH with tumor progression. Absence or low GH levels are associated with reduced tendency to develop malignancies spontaneously (Anisimov, 2001; Ikeno et al., 2003) or in response to carcinogen administration (Styles et al., 1990; Pollak et al., 2001). For example, when nitrosomethylurea was administrated to GH-deficient dwarf rats, none of them developed tumors; however, when the tumorigenic drug was administrated to normal and GH-supplemented dwarf rats incidence and latency to tumor development was similar in both groups. Hormone replacement in these animals increased the tumor incidence towards normal levels, whereas discontinuation of GH treatment resulted in tumor regression (Shen et al., 2007). On the contrary, transgenic mice overexpressing GH are more susceptible to develop cancer and they have an increased incidence of hepatocellular carcinoma at advanced ages (Orian et al., 1990; Wanke et al., 1991; Snibson, 2002; Bartke, 2003).

2. Transgenic mice overexpressing growth hormone as a model to study the pre-neoplastic alterations induced by high GH levels

2.1 General characteristics of transgenic mice overexpressing GH

Transgenic technology allowed the genomic incorporation of heterologous GH genes (rat, human, ovine, bovine or human placental variant) under the control of different promoters, allowing the development of multiple lines of GH-transgenic mice. Mice that exhibit high

levels of endogenous GH (pituitary mouse GH) were developed by transfection with the GH-releasing hormone (GHRH) gene (Bartke, 2003; Kopchick et al., 1999). Transgenic mice overexpressing GH have been extensively used to study the mechanisms of action of GH and as a model of acromegaly to investigate the effects of prolonged GH excess (Bollano et al., 2000; Colligan et al., 2002; Miquet et al., 2004; Dominici et al., 2005). Most of the GH-transgenic mice lines available were produced by standard microinjection techniques. Briefly, the DNA constructs (promoter fused to the coding sequences of GH gene) were injected in the male pronucleus of single cell embryos. Viable embryos were implanted into pseudopregnant females and the pregnancies were allowed to term. Offsprings positive for both integration and expression of the GH gene were used as founder animals for the development of individual transgenic lines. Mating of hemizygous transgenic males to normal females produced both normal and transgenic progeny, in approximately 1:1 ratio (McGrane et al., 1988, 1990; Bartke, 2003; Kopchick et al., 1999).

As the transgene is not controlled by its own promoter, GH is often expressed constitutively and in some cases ectopically, resulting in chronic exposure to high GH levels. The tissue and developmental stage expression of the GH transgene depends on the promoter used. For instance, genes under the control of the metallothionein I (MT) promoter are expressed in several tissues since fetal development, while the phosphoenolpyruvate carboxykinase (PEPCK) promoter leads to the expression of GH primarily in liver, kidney and adipose tissue just after birth. The expression of the transgene continues throughout the life of the animal and is not controlled by physiological mechanisms that modulate pituitary GH secretion (McGrane et al., 1988; McGrane et al., 1990; Kopchick et al., 1999; Bartke, 2003). The circulating GH levels vary depending on the transgenic line, but are usually extremely high and produce a consequent increase in IGF1 serum concentration (Kopchick et al., 1999).

Chronic overexpression of GH in transgenic mice leads to enhanced postnatal growth, achieving typically a 30-70% increase in adult body size compared to normal littermates, and in some lines transgenic mice almost double the size of their normal controls. Prolonged exposure to GH in these mice also leads to altered body composition, including reduced adiposity with increased lean body mass and organomegaly. Conditions of chronic elevation of GH, as seen in acromegaly, are often associated with hyperinsulinemia, insulin resistance and impaired glucose tolerance, which in some cases progress to diabetes; in fact, GH-transgenic mice have increased insulin levels with normoglycemia and insulin resistance. Prolonged exposure to high GH levels leads to several histo and physiopathological lesions in different organs in GH-transgenic mice, principally in the kidney, heart and liver (Quaife et al., 1989; Kopchick et al., 1999; Bartke et al., 2002; Bartke, 2003; Dominici et al., 2005).

The transgenic PEPCK-bGH line has been used to study the effects of prolonged exposure to high GH levels (McGrane et al., 1990; Valera et al., 1993; González et al., 2002; Dominici et al., 2005; Miquet et al., 2004). Transgenic mice present high GH and IGF1 serum levels, which result in an increment in both body and liver weight. While some organs are increased roughly in the proportion of the increase in body weight, the relative increase in liver weight is higher than the one observed for body weight, reflecting that transgenic mice exhibit hepatomegaly. Although transgenic mice displayed hyperinsulinemia, glucose levels were not altered, possibly reflecting a state of insulin resistance.

2.2 Hepatic alterations in GH-transgenic mice

As previously mentioned, chronic exposure to high GH levels in transgenic mice produces hepatomegaly. Studies performed in different lines of transgenic mice overexpressing GH revealed that the disproportional increase in liver size is due to hypertrophy and hyperplasia, with hepatocytes presenting morphological alterations such as large cellular and nuclear size, intranuclear inclusions and invaginations of nuclear membranes. Throughout lifespan, transgenic mice present high levels of hepatocellular replication, followed by the onset of hepatic inflammation, fibrosis and cirrhosis, which may derive in hepatocarcinoma at advanced ages. The preneoplastic liver pathology observed in the GH-overexpressing transgenic mice resembles that seen in human patients at high risk of developing liver cancer, which turns this animal model suitable for studies of hepatic cancer. The liver of GH-transgenic mice develops various degrees of necroinflammatory, cirrhotic, fibrotic and regenerative changes, all of which are factors known to predispose human individuals to hepatocarcinogenesis (Orian et al., 1989; 1990; Snibson et al., 1999; Snibson 2002; Bartke 2003). Moreover, high GH levels are observed in patients with liver conditions associated with increased risk of liver cancer (Hattori et al., 1992; Kratzsch et al., 1995). The liver lesions observed in GH overexpressing mice would not be attributed to the liver being one of the major sites of GH production in transgenic mice expressing heterologous GH genes because comparable abnormalities were observed in GH-releasing hormone transgenic mice, in which GH is homologous and secreted by the pituitary (Bartke, 2003). The increased susceptibility of transgenic mice overexpressing GH to develop liver cancer is believed to be a consequence of the direct action of GH in this organ rather than secondary to the elevated IGF1 levels. This is supported by the fact that IGF1 binding to hepatocytes is barely detectable (Barreca et al., 1992; Santos et al., 1994) and, moreover, IGF1 has been described to induce discrete metabolic effects and only a slight increase of DNA synthesis in liver (Hartmann et al., 1990; Kimura & Ogihara, 1998; Grunnet et al., 1999). Moreover, transgenic mice overexpressing IGF1 do not show the hepatic histopathological alterations observed in the liver of GH-overexpressing transgenic mice (Bartke, 2003).

3. Growth hormone signaling-pathways induced in liver of GH overexpressing transgenic mice

The JAK2/STAT5 signaling cascade is the principal signaling pathway activated by growth hormone. High continuous GH levels *in vivo* produce desensitization of this pathway in the liver. In both Mt-GHRH and PEPCK-bGH transgenic mice this desensitization was evidenced as a lack of activation after a massive stimulus with GH and no increase in the basal phosphorylation of STAT5, in spite of the very high GH concentration in their circulation (González et al., 2002; Miquet et al., 2004). This lack of response to GH was associated with elevated levels of the negative regulator CIS, a member of the family of suppressors of cytokine-signaling (SOCS) proteins, which is proposed to be a major factor responsible for the down-regulation of STAT5 signaling in the liver (Ram & Waxman, 2000; Landsman & Waxman, 2005).

The liver is one of the principal target organs of growth hormone, and GH overexpressing mice exhibit phenotypic characteristics that indicate GH is indeed acting in this tissue. For instance, absolute and relative liver weight is higher in GH-transgenic than in control mice, accompanied by pathological alterations in the liver (Orian et al., 1989; Quaife et al., 1989; Snibson, 2002; Bartke, 2003). Circulating levels of IGF1 and hepatic levels of IGF1 mRNA, which are primarily regulated by GH action in the liver, are increased in GH transgenic

mice. Moreover, liver GHR expression is also increased, in accordance with the known ability of GH to upregulate its own receptor (Mathews et al. 1988; McGrane et al. 1990; González et al. 2001; Iida et al. 2004; González et al. 2007). As the JAK2/STAT5 signaling cascade is desensitized in the liver of GH-overexpressing mice, this pathway is probably not responsible for the proliferative effects of chronically elevated GH. Therefore, other signaling mediators induced by GH must be involved for the liver pathology observed in these animals. Studies performed in Mt-GHRH and PEPCK-bGH transgenic mice showed that these animals exhibit constitutive phosphorylation of STAT3 and upregulation of c-Src, FAK, EGFR, Akt, mTOR and Erk1/2 (Miquet et al., 2008). These molecules are all signaling mediators activated by GH that are involved in cell proliferation, differentiation, migration and survival, and their upregulation may represent alternative pathways to JAK2/STAT5 that are constitutively activated in the liver of transgenic mice overexpressing GH.

In several human cancers elevated protein levels and/or kinase activity of c-Src have been reported, and it was proposed that it could act by facilitating other signaling mediators action, including FAK and EGFR. A high proportion of breast cancers overexpress c-Src and members of the EGFR family, suggesting that they may interact to synergically promote cancer development and progression (Ishizawa & Parsons, 2004; Biscardi et al., 2000; Playford & Schaller, 2004). Importantly, EGFR upregulation was also found in hepatocellular carcinoma (Thomas & Zhu, 2005), and FAK overexpression was detected in several human tumor samples (Owens et al., 2005; Parsons, 2003; Ishizawa & Parsons, 2004; Playford & Schaller, 2004; Schlaepfer & Mitra, 2004). GH-transgenic mice show overexpression of c-Src, EGFR and FAK in liver, in accordance with the upregulation of these proteins that may be observed in human cancer (Miquet et al., 2008). Aberrant activation of STAT proteins is also related to cell transformation and oncogenesis. As mentioned before, GH-transgenic mice display increased basal activation of STAT3 in liver (Miquet et al., 2008). Constitutive activation of this protein has been found in many tumors with elevated activity of both c-Src and EGFR (Calò et al., 2003; Silva, 2004). Thus, higher STAT3 phosphorylation observed in transgenic mice, which also present elevated c-Src and EGFR levels, could be related to the increased kinase activity of c-Src they exhibit.

Akt is a crucial regulator of cellular proliferation, differentiation and metabolism, and has been implicated in the inhibition of apoptosis by several cytokines and growth factors. Akt is frequently activated or overexpressed in human cancers, probably cooperating with other oncogenic pathways to promote tumor progression by enhancing cell survival (Nicholson & Anderson, 2002). Signaling by the PI-3K/Akt/mTOR regulates mRNA translation, a crucial step for stimulation of protein synthesis (Proud, 2007). Altered mTOR signaling has been found in cancer, diabetes and obesity (Dann et al., 2007). Therefore, the upregulation of Akt and mTOR suggests that these kinases could contribute to the hepatic alterations transgenic mice exhibit. The MAP kinase proteins Erk1/2 are also involved in the control of the translational machinery and, thus, in the promotion of cell growth and proliferation (Proud, 2007). Erk1/2 were reported to be constitutively activated in many tumors and cancer derived cells (Chambard et al., 2007), so the increased levels of this kinase in transgenic mice liver could also contribute to the hepatic alterations observed.

Considering the well established association of the aforementioned signaling mediators with cancer, and taking into account their upregulation in the liver of GH-transgenic mice, it is reasonable to suggest that the described molecular alterations found in the liver of GH overexpressing transgenic mice may be implicated in the pathological alterations observed in these animals.

4. GH modulation of EGF signal

Among the growth factors and growth factor receptors that have been shown to be involved in the pathogenesis and progression of different carcinoma types is the epidermal growth factor (EGF) family of peptide growth factors and the EGF receptor (EGFR) (Ito et al., 2001; Normanno et al., 2001; 2006). EGF is a key regulatory factor in promoting cell proliferation and survival. Ligand binding to the receptor triggers several signaling pathways that activate different transcriptional programs in the nucleus which lead to the expression of proteins involved in cell cycle progression, apoptosis resistance, differentiation, adhesion, and cell migration.

4.1 The EGF family of receptors and EGF-induced signal transduction pathways

EGFR, also known as ErbB-1, belongs to a family of receptors, which comprises three additional proteins: ErbB-2, ErbB-3, and ErbB-4. These ErbB receptors are type I receptor tyrosine kinases that are activated by binding of growth factors of the EGF family. The ErbB receptors recognize different but structurally related growth factors and mediate processes in development, homeostasis and pathologies.

ErbB receptors consist of a heavily glycosylated and disulfide-bonded ectodomain that provides a ligand-binding site, a single transmembrane domain and a large cytoplasmatic region that encodes a tyrosine kinase and multiple phosphorylation sites. Upon ligand binding, the ErbB receptors form either homo or heterodimers (Jorissen et al., 2003). Except for certain constitutively active mutants, dimerization is induced by ligand binding and is essential for activation of their kinase domain. Dimerization and activation of the kinase domain results in trans-phosphorylation of the monomers. Subsequently, the activated receptor phosphorylates additional tyrosine residues on the C-terminal tail of the EGFR (Boeri Erba et al., 2005; Wu et al., 2006). Intracellular tyrosine kinases of the Src family such as c-Src and Abl are also capable of phosphorylating residues on the EGFR. Phosphorylated sites on the EGFR allow the association of proteins containing the Src homology 2 domain (SH2), like Grb2, Shc and Nck. These signaling mediators associate with additional proteins leading to their activation and the subsequent activation of other kinases or transcription factors. Post-receptor signaling by EGFR involves the activation of signaling pathways such as the MAPK Erk1/2 and p38, the PKC, the PI3K/Akt, and the STAT pathways (Jorissen et al., 2003; Henson & Gibson, 2006; Normanno et al., 2006). These signaling routes activate different transcriptional programs in the nucleus, leading to the expression of several genes involved in cell cycle progression, survival, differentiation, adhesion and migration.

As a consequence of ligand induced activation of the EGFR, it is endocytized and enters the endosomal pathway. In the absence of EGF stimulation, the receptor is recycled to the cell surface. On the contrary, after ligand binding, EGFR progresses from early to late endosomes and is subsequently degraded. EGFR was believed to trigger signal transduction pathways only when located at the cell membrane, however, recent studies suggest that signal is also propagated by internalized EGFR present in early or late endosomes (Burke et al., 2001). Moreover, it has been recently described that EGFR also acts at the nuclear level in response to stress and participates in cell proliferation and cell cycle and DNA repair processes (Dittmann et al., 2010).

Fig. 2. Signal transduction pathways triggered by EGF.

4.2 EGFR and cancer

EGF receptor family members were first related to cancer in the early 1980s (Stoscheck et al., 1986). Since then, activation of EGFR has been implicated in the progression of different types of carcinomas (Ito et al., 2001; Normanno et al., 2001; 2006). The complexity of EGFR signal transduction and its importance for cell growth and survival explains the potential role of EGFR alterations in the development and maintenance of cancer. EGFR undergoes different alterations including gene amplification, structural rearrangements and somatic mutations in human carcinomas. In addition, some types of tumors produce an excess of EGF that leads to an increased activation of EGFR (Henson & Gibson, 2006). Besides the alterations in EGF receptor expression and ligand production, intracellular signaling cascades are often altered in cancer cells. All these alterations in EGF-mediated survival signaling can promote tumor processes like angiogenesis and metastasis and contribute to cancer progression and resistance to cancer therapies (Salomon et al., 1995). EGFR is overexpressed or hyperactivated in a variety of solid tumors, including colorectal cancer, non-small-cell lung cancer, squamous cell carcinoma of the head and neck, and also in ovarian, breast, liver, kidney, pancreatic and prostate cancer (Baselga, 2002; Lurje & Lenz, 2009). Moreover, aberrant EGFR expression or activity has been associated with disease progression, resistance to radiochemotherapy and poor survival.

Considering the relevance of EGFR and ErbB receptors for the development and progression of cancer, several anti-ErbB targeted therapies have been developed and are still under study. Specifically, ErbB1 or EGFR-targeted therapies can be classified into two major classes: the anti-EGFR monoclonal antibodies (MoAbs) and the EGFR-specific tyrosine kinase inhibitors (TKI). The anti-EGFR monoclonal antibodies, such as cetuximab, bind to the extracellular domain of the EGFR on the surface of tumor cells, thus preventing EGFR ligands from interacting and activating the receptor and ligand-induced internalization of the receptor. TKIs such as gefitinib and erlotinib block the binding of adenosine triphosphate (ATP) to the intracellular TK domain of the EGFR, which results in the inhibition of the tyrosine kinase activity of the receptor. Besides the drugs that target the EGFR itself, there is a second group of small molecular inhibitors that interfere with the activation of signaling mediators downstream the EGFR. Such molecules mainly target crucial proteins involved in the Ras/MEK/Erk and PI3K/Akt/mTOR pathways. Additionally, inhibitors of the transcriptional regulation of pro- and anti-apoptotic proteins are used. These molecules include proteasome inhibitors like BAY 11-7085, BAY 11-7082, soy isoflavone genistein, flavopiridol, which affect the NF κ B signaling. Many of these substances are in various stages of clinical development and were proposed to be used in combination with standard chemotherapy (Lurje & Lenz, 2009). Antisense molecules that target the mRNA of the anti-apoptotic protein Bcl-2 like G3139 and Augmerosen interfere with pro-apoptotic pathways induced by EGF and could also be used as therapeutical agents. Moreover, peptides that mimic the BH3 only domain Bcl2 family members like ABT-737 interfere with anti-apoptotic pathways induced by EGF because they impede Bcl-2 association with pro-apoptotic Bcl-2 family members (Henson & Gibson, 2006).

4.3 EGFR and hepatocarcinoma (HCC)

Hepatocarcinoma (HCC) is a clear example of inflammation-related cancer because it mainly occurs in the context of persistent inflammation of the liver (Berasain et al., 2009). Indeed, the majority of HCCs slowly develop in a background of chronic hepatitis and cirrhosis, which are considered as preneoplastic conditions of the liver. A pro-inflammatory and proliferative microenvironment is a common feature of the preneoplastic liver regardless of the etiology. Upon tissue injury the liver triggers a defensive response to protect the organ and recover the lost parenchymal mass (Taub, 2004). This is a complex response that involves several cytokines and growth factors, among them the ErbB1 axis. Normal hepatocytes express high levels of EGFR, and EGFR ligands have a potent mitogenic effect on isolated or cultured hepatocytes. In the cases of acute and chronic liver injury and inflammation, the EGFR system plays an important role in liver regeneration and hepatocyte protection (Berasain et al., 2007). These pathological conditions are frequently associated with overexpression and overstimulation of the EGFR pathway (Berasain, 2009). Amplifications and mutations of the EGFR gene have been described in patients with HCC (Normanno et al., 2006) and sustained activation of EGFR was reported to induce the progression of HCC (Nalesnik et al., 1998; Ito et al., 2001). Moreover, pro-angiogenic roles of the EGFR have been described (Ueda et al., 2006). Currently, there are several studies in process concerning the use of anti-EGFR targeted therapies alone, or associated with other pharmacological agents, for the treatment of HCC (Berasain et al., 2007; Hu et al., 2011).

4.4 GH modulation of EGF signaling

EGF and GH signaling pathways share several signaling mediators. Both growth factors induce the JAK/STAT, MEK/Erk and PI3K/Akt pathways; however the degree in which each pathway is activated upon GH or EGF stimulus depends on the cell type and hormonal environment. In addition, GH regulates the expression of EGFR in the liver (Jansson et al., 1988, Johansson et al., 1989). Hypophysectomized and partially GH-deficient mutant mice showed reduced expression of the EGFR in liver (Johansson et al., 1989). When GH was administered to these animals, expression of the receptor was induced approaching EGFR levels found in normal controls (Johansson et al., 1989). Moreover, EGFR levels are increased in transgenic animals overexpressing GH, while its protein content is drastically diminished in mice lacking the GH receptor (GHR-KO mice) (Miquet et al., 2008; González et al., 2010).

GH has also been demonstrated to induce phosphorylation of the EGFR. EGFR phosphorylation at tyrosine residues 845, 992, 1068 and 1173 upon GH stimulation was described both in mice liver and in cell culture (Yamauchi et al., 1997; Kim et al. 1999, Huang et al., 2003). GH induced phosphorylation of the EGFR at tyrosine residue 1068 is mediated by JAK2 and allows Grb2 association and subsequent activation of Erk1/2 (Yamauchi et al., 1997). Another level of interaction between GH and EGF signaling involves GH-induced EGFR phosphorylation at threonine residues, which depends on Erk1/2 activity (Huang et al., 2004). Erk-dependent threonine phosphorylation of the EGFR reduces EGFR degradation, thus modulating EGFR trafficking and signaling (Frank, 2008).

Considering GH and EGF crosstalk and the relevance of EGFR in cancer, especially hepatocarcinoma, hepatic EGFR signaling was analyzed in two different *in vivo* models: GHR-KO mice, in which GH action is abolished, and the GH-transgenic mice. GHR-KO mice displayed diminished receptor activation due to EGFR down-regulation. Moreover, EGF-induced STAT5 and Erk1/2 phosphorylation was reduced in GHR-KO mice, while EGF did not activate STAT3 and Akt in these animals. On the other hand, overexpression of GH in transgenic mice induces EGFR up-regulation but this does not result in enhanced EGF signaling. Akt and Erk1/2 pathways showed diminished activation, while STAT3 and STAT5 activation was abrogated, indicating that GH differentially modulates EGF signaling pathways (González et al., 2010). The heterodesensitization produced by GH over EGF-induction of the STATs was related with diminished association between the EGFR and STAT3 or STAT5 in the liver from the GH-overexpressing transgenic mice. This suggested that recruitment of the STATs to activated EGFR might be inhibited. The increased association of STAT5 with SHP-2 phosphatase in transgenic mice could account for the observed desensitization of STAT5 signaling in this animal model, since this phosphatase is regarded as a negative regulator of STAT5 signaling (González et al., 2010).

5. Conclusion

Overexpression of GH has been associated with tumor promotion both in human and animal models. Chronic exposure to high GH levels induces hypertrophy and hyperplasia of hepatocytes, hepatic inflammation, fibrosis and even cirrhosis. These alterations comprise the preneoplastic liver pathology observed in the GH-overexpressing transgenic mice that might result in hepatocarcinoma at advance ages. Cancer cells show alterations in cytoskeletal organization, adhesion, motility, growth control and survival. Many of the signaling pathways implicated in these events are upregulated in the liver of mice that present high circulating levels of GH (Miquet et al., 2008) suggesting their role in the liver

pathology observed in these animals. Overexpression and/or hyperactivation of these signaling mediators provide a molecular basis for the oncogenic potential of GH.

GH also modulates the expression and signaling of a growth factor receptor relevant for cancer development, the EGFR. The relevance of EGF-induced signaling cascades for tumor development should be further investigated to determine if the silencing of only certain signaling cascades, therefore altering the normal balance between mitogenic and apoptotic signals, might facilitate the onset of tumor.

6. References

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