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CONDITIONS ASSOCIATED WITH INCREASED GROWTH HORMONE AND PROLACTIN SENSITIVITY

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Conditions Associated with Increased Growth Hormone and Prolactin Sensitivity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my beloved family

ABSTRACT

Growth Hormone (GH) and Prolactin (PRL) are critical regulators of body growth and metabolism. Secretion and actions of GH and PRL are regulated at several levels and by different factors. The biological actions of these hormones are initiated by their binding to the respective membrane bound receptors of GH and PRL (GHR and PRLR). Several hormone systems are characterized by changes in target tissue sensitivity. Key factors in hormone sensitivity include the number of particular receptors and the duration of receptor activated intracellular signals. A common theme concerning this is e.g. that tyrosine phosphorylated intracellular proteins become inactivated by tyrosine phosphatases or by proteasomal breakdown.

In this thesis a particular focus is put on two different proteins, Suppressors of Cytokine Signaling2 (SOCS2) and Tuberous Sclerosis Complex2 (TSC2) that uniquely impinge on JAK-STAT activation and on mTOR activation.

Study I. We explored the influence of SOCS2 on glucose metabolism by using a mouse model of diabetes induced by multiple low dose streptozotocin (MLDSTZ). Pancreatic islets from untreated SOCS2$^{-/-}$ mice appeared larger than in wild-type (WT) controls, which could explain the augmented serum insulin levels observed in SOCS2$^{-/-}$ mice. Pancreatic islets, derived from SOCS2$^{-/-}$ mice showed increased GHR and PRLR staining, which suggest a higher sensitivity to GH/PRL-STAT5 signals in SOCS2$^{-/-}$ than in WT-derived β-cells. Our results suggest that SOCS2 ablation can partly overcome β-cell destruction induced by MLDSTZ.

Study II and III. In these studies we aimed to investigate the relevance of PRL in two different human tumors, i.e.lymphangioleiomyomatosis (LAM) and glioblastoma (GBM), by analyzing features of the PRLR and the effect of a novel PRLR antagonist (PRLRA) in such tumors. Reduction of TSC2 (the disease causing gene in LAM) increased PRLR levels in LAM cells and PRL stimulated LAM cell proliferation; an effect that could be blocked with the PRLRA. In GBM, PRLR was detectable in cultured GBM cells as well as in tissue sections from patients with GBM. In cell culture GBM studies, PRL treatment increased STAT5 phosphorylation as well as cell invasion and both effects could be blocked by the PRLRA.

In summary, our studies indicate that the tissue sensitivity to GH/PRL is regulated by SOCS2 and TSC2 proteins. Since both SOCS2 and TSC2 have links to different disorders, an

increased GH/PRL sensitivity in such conditions could play a functional role. To block an increased PRL sensitivity we have developed a novel PRLRA and demonstrated its efficacy in cell cultures.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following studies referred to in the text by their roman numerals.

I. **Alkharusi A**[*], Mirecki-Garrido M[*], Ma Z, Zadjali F, Flores-Morales A, Nyström T, Castrillo A, Bjorklund A, Norstedt G, Fernandez-Pérez L. Suppressor of cytokine signaling 2 (SOCS2) deletion protects against multiple low dose streptozotocin-induced type 1 diabetes in adult male mice. Horm Mol Biol Clin Investig. 2015 Nov 12, ahead-of-print/hmbci-2015-0036.

II. **Alkharusi A**, Lesma E, Ancona S, Chiaramonte E, Nyström T, Gorio A, Norstedt G. Role of Prolactin Receptors in Lymphangioleiomyomatosis. PLoS One. 2016 Jan 14; 11(1): e0146653.

III. **Alkharusi A**, Yu S, Zadjali F, Davodi B, Nyström T, Gräslund T, Rahbar A, Norstedt G. Stimulation of prolactin receptor induces STAT-5 phosphorylation and cellular invasion in Glioblastoma Multiforme. *Submitted 2016*

*These authors contributed equally to the study.

CONTENTS

LIST OF ABBREVIATIONS

BS	Binding Site
CMV	Cytomegalovirus
DAB	Diaminobenzidine
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle's Medium
ECD	Extracellular Domain
ERK	Extracellular signal-Regulated Kinase
FBS	Fetal Bovine Serum
FSH	Follicle Stimulating Hormone
GBM	Glioblastoma Multiforme
GH	Growth Hormone
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
Grb2	Growth factor receptor-bound
HFD	High Fat Diet
HMB-45	Human Melanin Black antibody
HOMA-IR	Homeostatic Model Assessment and Insulin Resistance
HPV	Human Papillomavirus
IHC	Immunohistochemistry
IGF-1	Insulin-like Growth Factor-1
IL	Interleukin
ICD	Intracellular Domain
IRS	Insulin Receptor Substrates
ipITT	Intraperitoneal Glucose Tolerance Test
ipGTT	Intraperitoneal Insulin Tolerance Test
JAK	Janus Kinase
LAM	Lympangioleiomyomatosis
MAPK	Mitogen-Activated Protein Kinase
MLDSTZ	Multiple Low-Dose Streptozotocin
MSA	Murine Serum Albumin

mTOR	Mammalian Target of Rapamycin
OD	Optical Densities
ODC	Ornithine Decarboxylase
PI3K	Phosphoinositide 3-Kinase
PL	Placental Lactogen
PRL	Prolactin
PRLR	Prolactin Receptor
PTEN	Phosphatase and Tensin homologue
PVDF	Polyvinylidene Fluoride
STAT	Signal Transducer and Activator of Transcription
SOCS	Suppressor of Cytokine Signaling
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOS	Sons Of Sevenless
SH2	Src Homology domain 2
Shc	SH2 domain-containing transforming protein
TSC	Tuberous Sclerosis Complex
T1D	Type 1 Diabetes
T2D	Type2 Diabetes
TD	Trans-Domain
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WT	Wild-Type

1 INTRODUCTION

In multicellular organisms, distinct populations of cells coordinate with one another to accomplish roles, which no single cell could carry out on its own. A crucial aspect of this feature of multicellular organisms is inter-cellular communication, involving signals either from the environment or from other cells. In order to result in the appropriate cellular response, these signals must be transmitted across the cell membrane. Such signaling processes frequently involve receptor proteins that contact both the exterior and interior of the cell, and only cells that have the correct receptors on their surfaces will respond to any particular signal. These signals could either be from the cell itself (autocrine), from a neighboring cell (paracrine), or may travel through the bloodstream from another specialized cell in the body (endocrine). These particular signaling molecules are called hormones and cytokines.

1.1 PROLACTIN

A substance from extracts of bovine pituitary glands able to promote milk secretion was first described in 1928 by Stricker and Grueter. This substance was further purified and identified as prolactin (PRL) in 1933 by Riddle and Bates, and was named for its stimulatory action on lactation [1]. PRL, also known as lactogen or mammotropin, is a polypeptide hormone secreted by the acidophilic lactotroph cells located in the adenohypophysis of the pituitary gland. In addition to its stimulatory effects on mammary gland development and lactation, PRL is known to exert more than 300 different biological functions related to reproduction, development, growth, metabolism, immunomodulation, osmoregulation and behavior [2,3].

1.1.1 Gene, structure and variants

PRL is a member of the PRL/Growth Hormone (GH)/Placental Lactogen (PL) hormone family. All of these hormones share genomic, structural and biological features [4], and belong to an extended family of proteins known as hematopoietic cytokines [5]. The genes encoding PRL, GH and PL were derived from one common ancestral gene by gene re-duplication [6]. The gene encoding PRL is found in all vertebrates and is located on chromosome 6 in humans [7]; it consists of five exons and four introns [8] with an additional noncoding “exon1a” which is only transcribed in extra-pituitary tissues [9]. The mature form of the 23 kDa PRL protein consists of 199 amino acids in humans, and has four α-helixes arranged in an up-up-down-down style [10].

Unlike humans, rodents express several PRL-encoding genes, which are located on chromosomes 13 and 17 in mice and rats, respectively. In humans and other primates, but not in rodents, research has demonstrated expression of the PRL gene in several extra-pituitary tissues [11,12]. This is because of the presence of an alternative promoter located 5.8 kb upstream of the pituitary transcription start site which drives extra-pituitary PRL expression [13–16]. Although different promoters control PRL expression in pituitary and extra-pituitary tissues, the human PRL protein sequence is identical regardless of the site of production [17]. Extra-pituitary PRL production is well recognized, and although studies indicate a proliferative/anti-apoptotic role for this autocrine/paracrine-produced PRL, the role of extra-pituitary PRL production in humans requires further investigation [12].

In addition to 23kDa PRL, other larger forms of the protein (>100kDa) have been identified in the circulation. These include the 23kDa PRL complexed with IgG antibodies forming a big complex; however, this PRL possess lower biological activity compared to the monomeric mature form [18]. Macroprolactinemia is a heterogeneous condition characterized by the predominance of higher molecular mass PRL and the production of anti-PRL autoantibodies that reduce PRL bioactivity and delay its clearance [19]. Other well-characterized 14-, 16-, 22-kDa prolactin protein variants are generated by proteolytic cleavage of the 23kDa protein [10].

1.1.2 Synthesis and regulation of secretion

The lactotroph cells of the anterior pituitary gland are considered to be the primary source of PRL in the body. However, when pituitary-derived PRL synthesis and/or release is impaired, humans are not deprived from the extra-pituitary produced PRL since their transcriptional regulation is not similar. The production of PRL by lactotrophs is mainly regulated by the pituitary transcription factor Pit-1 [20]. The transcription and secretion of PRL hormone from the lactotrophs are controlled, to a large extent, by hypothalamus. In primates, this hypothalamic regulation is mainly inhibitory, in contrast to what is seen with other pituitary hormones. Dopamine, a neurotransmitter released into portal blood by hypothalamic neurons, acts as one major inhibiting factor on PRL secretion, through its binding to type-2 dopamine receptors, which suppresses both the synthesis and secretion of PRL from pituitary lactotrophs. PRL itself, along with other factors, acts in a short feedback loop to inhibit dopamine release and maintaining PRL homeostasis [21,22]. In addition to tonic inhibition by the hypothalamus, PRL production and release is positively regulated by several hormones,

including thyroid-releasing hormone, oxytocin, gonadotropin-releasing hormone, estrogen, prolactin-releasing peptide and vasoactive intestinal polypeptide [10,23].

PRL secretion in humans is primarily highest during sleep and is lowest during the waking hours, following a true circadian rhythm generated in a constant environment regardless of the sleep rhythm [24]. The secretion shows sexual dimorphism, having a higher amplitude and a more continuous secretion in females compared to males, who have a more intermittent secretion pattern [25]. This gender-specific difference in the mode of PRL secretion could at least partly be linked to sex-steroids. Other non-gender specific factors that regulate PRL release include light, stress, sex, suckling, odor and sound. PRL mediates its action on target tissues by binding to the membrane-bound PRL receptor (PRLR).

1.2 GROWTH HORMONE

As mentioned above, GH also known as somatotropin, somatropin or somatotrope hormone belongs to the same family as PRL. GH is a polypeptide hormone and in its most prevalent form has a size of 22 kDa, consisting of 191 amino acids in humans [26]. The primary site for GH biosynthesis is the somatotropic cells of the anterior pituitary gland, where its transcription is mainly regulated by Pit-1 [27]. The release of GH from the secretory granules in somatotropic cells is under a balanced control of two neuroendocrine peptides from the hypothalamus; growth hormone-releasing hormone (GHRH) and growth hormone-inhibiting hormone [28,29].

GH secretion, in primates, is pulsatile and occurs primarily during sleep [30]. The secretion is sexually dimorphic, with high nocturnal pulses and small daytime pulses in males but a more continuous secretory pattern or frequent pulses of similar amplitude in females [31]. These sex differences in the GH secretory pattern may explain the differences in growth between males and females. Once GH is released into the bloodstream and reaches its target organs, it binds to its receptor, the growth hormone receptor (GHR). GHR signaling triggers the expression and release of insulin-like growth factor-1 (IGF-1), which mediates some of the actions of GH [32].

1.3 PROLACTIN AND GROWTH HORMONE RECEPTORS

PRL/GH hormones exert their actions on target cells via specific membrane-bound receptors, PRLR and GHR. These, along with other receptors for ligands such as leptin, leukemia-inhibiting factor, and erythropoietin, are all members of the type 1 cytokine receptor family

[33]. PRLR and GHR share several structural and functional features despite possessing a limited sequence homology. Thus they contain three domains: an extracellular domain (ECD) that binds the ligand, a single trans-membrane domain (TD) and intracellular domain (ICD) [4,34–36]. Two separate binding sites on the ligand molecule each bind to one receptor protein in a two-step process in which site 1, on the ligand molecule, binds to one receptor molecule, after which a second receptor molecule binds to site 2 on the ligand. This leads to the formation of a homodimer consisting of one ligand molecule and two receptor molecules [35]. Initially, it was believed that PRL/GH dimerize two receptor molecules [37,38], while more recent studies have suggested that both PRLR and GHR are already present as preformed dimers in the membrane [39,40]; PRL/GH binding then only induces a conformational change in the receptor to activate the intracellular part of the PRLR/GHR and initiate signaling (Figure 1) [41].

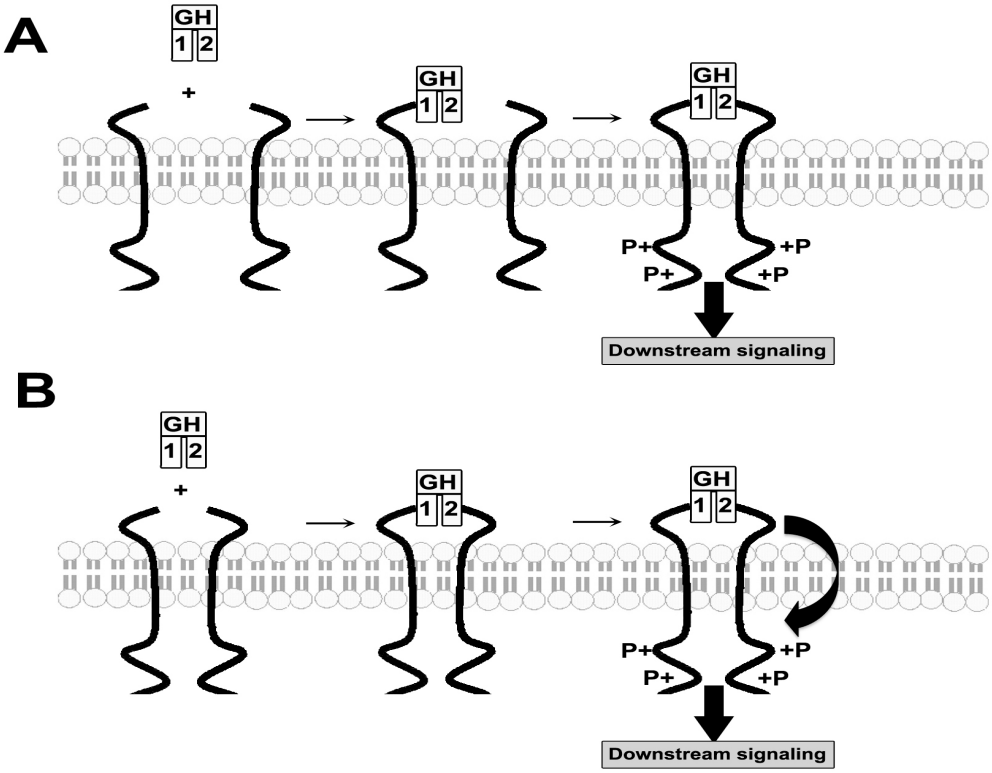

Figure 1. Models for PRLR/GHR activation. ***(A)*** *The early model for PRLR/GHR activation was based on ligand-induced receptor dimerization.* ***(B)*** *It is now believed that the receptor exists as a dimer in the membrane; this binds the ligand, causing a rotation in the receptor domains that triggers downstream signaling. Figure adapted from a review by Yahia A Kaabi [42].*

The structural and conformational changes induced by ligand binding to the receptor have been extensively reviewed by C.L. Brooks [43]. The GH binds to both GH and PRL receptors, whereas PRL only binds to the PRLR. [34].

PRLR is encoded by a single gene located on chromosome 5, and differential RNA splicing or posttranslational modifications lead to several tissue-specific variants or isoforms [2,35]. Basically, PRLR has three isoforms that differ in length to give long, intermediate and short forms of the protein. All isoforms share an identical ECD and TD but differ in the ICD [36]. In humans, one long, one intermediate and two short isoforms have been described, and in mice, one long and three short isoforms have been identified, while all of the major isoforms have been identified in rats [10]. Like all members of the class 1 cytokine receptor family, PRLR/GHR lacks intrinsic kinase activity; instead, the receptor molecule associates with an intracellular tyrosine kinase. Cytokine receptors are able to initiate signaling through several different intracellular signaling pathways.

1.4 BIOLOGICAL ACTIONS OF PROLACTIN AND GROWTH HORMONE

PRLR and GHR are expressed in several organs and tissues in vertebrates, and there is a tissue-specific variability in receptor expression levels. Many physiological actions have been attributed to GH and PRL, some of which will be listed below. In many cases, these biological functions are similar between different species, but particularly in the case of PRL's actions, large species-specific differences in biological functions have been observed.

1.4.1 Mammary gland development

One important function of PRL is related to mammary gland development and lactation. During pregnancy, the mammary glands are under the control of multiple factors including insulin, glucocorticoids, GH, PRL, PL, estrogen and progesterone [44]. However, the final extensive and diffuse branching of the gland's ducts and alveoli is under the direct control of PRL and progesterone [45]. Knocking down of PRL and/or PRLR expression result in total absence of the lobulo-alveolar unit in mammary tissue [46]. PRL not only contributes to mammary gland development, but also stimulates lactogenesis (milk production) [47].

1.4.2 Luteal function

Studies on rodent models demonstrate that PRL has dual actions in the corpus luteum. PRL

has been recognized as a luteotropic and luteolytic hormone. The luteotropic action of PRL involves stimulation of progesterone production by luteal cells [48]. Progesterone is important for implantation of the fertilized ovum and maintenance of pregnancy. The luteolytic action of PRL involves induced programmed cell death in the corpora lutea [49]. In humans, studies show a correlation between high PRL levels and the inhibition of granulosa cell luteinization [49]. Furthermore, a direct PRL action on oocyte development and maturation has been reported [50].

1.4.3 Male sex organs

PRL is also produced in the male pituitary gland, despite the fact that PRL is primarily considered as a lactating hormone. PRLR is present on the plasma membranes of testicular interstitial cells including Leydig cells, as well as on Sertoli cells and the cells of the germinal epithelium in the testis. In Leydig and Sertoli cells, PRL has been shown to increase luteinizing hormone and follicle-stimulating hormone receptors expression levels, respectively [51,52], with a significant effects on the proliferation and metabolism of these cells [53]. In general, PRL stimulates testicular functions in mammals, stimulating both testosterone and estradiol secretion from Leydig cells [54], and increasing energy metabolism in epididymal cells and spermatozoa [55].

Another well-defined male-specific effect of PRL in rodents and humans concerns the prostate gland. In 1997, a research group confirmed the local production of PRL in human prostate tissues, and also reported the presence of PRLR in the secretory epithelium of the human prostate [56]. Several studies involving rodent models and human prostate cell lines support a role for PRL as a growth factor in the prostate [57].

1.4.4 Growth and cell proliferation

As mentioned above, cells in the anterior pituitary gland produce GH and PRL. These proteins belong to the same cytokine family, although it is clear that they have distinct and separate biological effects. One example of this is that the effect of GH on longitudinal growth does not involve PRL [58]. The role of GH to promote longitudinal growth depends on IGF-1 produced by the liver or produced locally in the growth zone [59]. IGF-1 is often used as a clinical marker for GH secretion. It is, however, clear that not all GH effects are dependent on IGF-1 [60].

On the other hand, PRL has a stimulatory role on certain tissues including the liver, ovary and uterus. PRL stimulates turnover of, and/or activates, a number of factors in liver cells, such as protein kinase C (PKC), diacylglycerol, mitogen-activated protein kinase (MAPK), and phosphoinositide turnover [61–64]. Another proliferative effect of PRL is on the β-cells of the pancreas [65,66]. A subsequent *in vitro* study using the β-cell line INS-1 showed that PRL triggers β-cell proliferation via various signal transduction pathways [67]. Moreover, PRL has been found to up-regulate ornithine decarboxylase (ODC) in a number of tissues including liver, heart, kidney, muscle, adrenal, gonads, and prostate [2,68]. ODC is a rate-limiting enzyme in polyamine biosynthesis that is classified as a growth mediator due to its effects on DNA and RNA synthesis in somatic cells [68].

1.4.5 Metabolism

Several endocrine and metabolic actions of PRL that are not related to reproduction have been reported. In adipose tissue, lipogenesis is suppressed by PRL to spare metabolites to the mammary gland during lactation [69,70], while another study also reported that low physiological PRL doses suppressed lipolysis in adipose tissue of non-lactating rats, suggestive of a physiological-status-specific action for PRL [71]. Via activation of signal transducer and activator of transcription (STAT) 5, PRL induces peroxisome proliferator-activated receptor-γ a known regulator of adipocyte differentiation [72,73].

PRL also regulates the β-cells in the pancreas, where it increases insulin secretion and decreases the glucose threshold for insulin secretion [74–76]. This may in turn be connected to a metabolic adaptation to the state of pregnancy. PRLR knockout ($PRLR^{-/-}$) mice develop reduced pancreatic islet density and showed a blunted insulin secretory response to glucose [77].

GH has more prominent actions on metabolism compared to PRL. Generally, GH has anabolic actions in muscle and bone that involve the stimulation of protein synthesis, while its actions in adipose tissue are catabolic [78]. In this tissue, GH promotes lipolysis and break down fatty acids instead of glucose, which partly explains its diabetogenic effects including hyperglycemia and decreased insulin sensitivity. In the liver, GH reduces glucose uptake and promotes gluconeogenesis, glycogenolysis and lipogenesis. Mice with a GHR deletion in the β-cells exhibit decreased glucose-stimulated insulin release and decreased β-cell hyperplasia in response to high fat diet (HFD), which indicates an important role of GH in β-cell

proliferation [79].

1.4.6 Inflammation and immunity

Beside their metabolic functions, PRL and GH also participate in regulating the immune system. Different types of immune cells express both PRL and GH, along with their respective receptors [80–82]. The function of PRL/GH in modulating the immune system is controversial. Studies suggest that GH promotes the production of pro-inflammatory cytokines such as interleukin (IL)-1α, IL-6 and tumor necrosis factor α in immune cells [83], while other studies demonstrate opposite actions [84]. Poor clinical outcome in patients with septic shock is also reported to be associated with high levels of GH [85]. Although GH affects immune function, its precise effects and their underlying mechanisms remain to be elucidated.

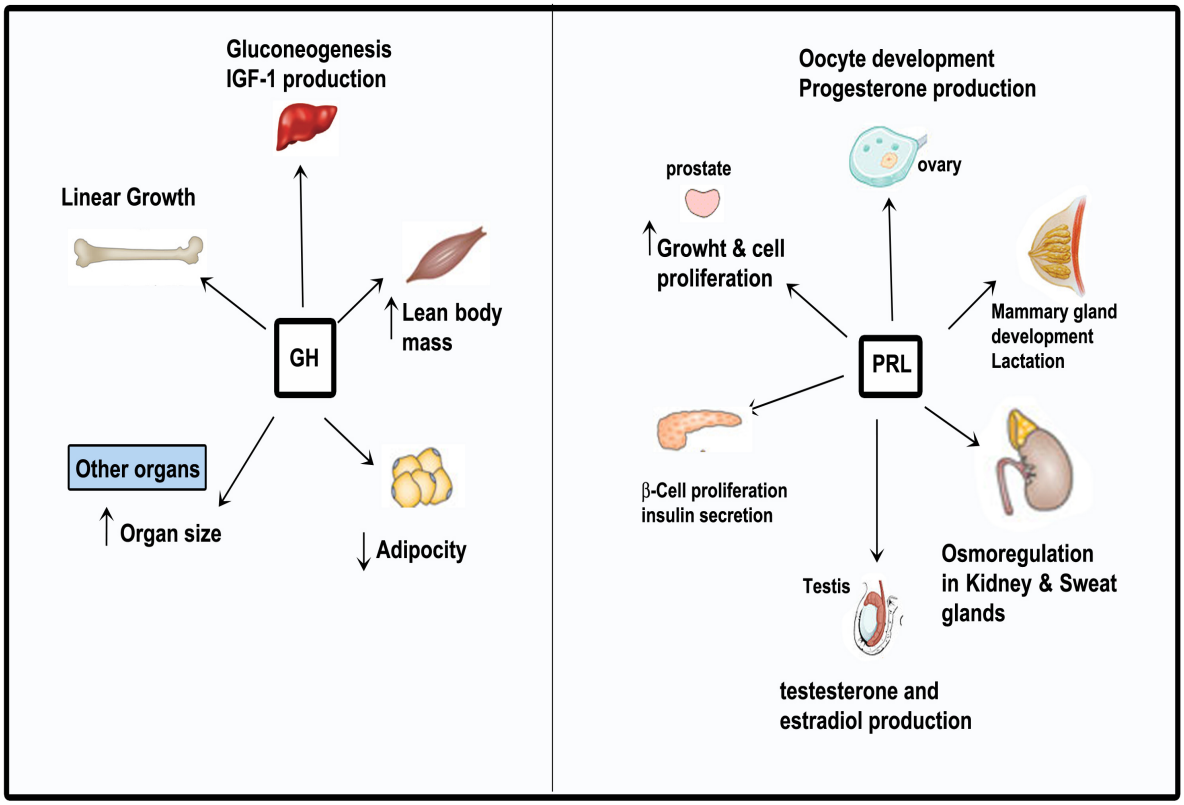

Figure 2. Summary of the different biological actions promoted by GH and PRL. *GH has more prominent actions on longitudinal bone growth and metabolism, while PRL exerts a prominent function on reproductive organs.*

1.5 INTRACELLULAR SIGNALING PATHWAYS INDUCED BY PROLACTIN AND GROWTH HORMONE

1.5.1 JAK-STAT pathway

Members of the class 1 cytokine family lack intrinsic kinase activity; instead, each receptor is bound to a specific tyrosine kinase, i.e. Janus Kinase (JAK). JAK phosphorylation leads to phosphorylation and nuclear translocation of STAT proteins. The JAK2-STAT5 pathway is the best-known signaling pathway utilized by both GH and PRL. When ligands bind to either GHR or PRLR, JAK2 tyrosine kinases bound to two receptor molecules become activated [86–88]. Activation of JAK2 promotes phosphorylation of STAT proteins [2,89,90]. A total of seven different STAT proteins have been identified in mammals [91]. GHR/PRLR signaling is primarily mediated by STAT5 proteins [92,93], but also by STAT1 and STAT3. Once STAT proteins are phosphorylated, they dimerize, and subsequently move to the nucleus, where they bind a specific DNA sequence, initiating GH/PRL-specific gene transcription (Figure 3) [94–96].

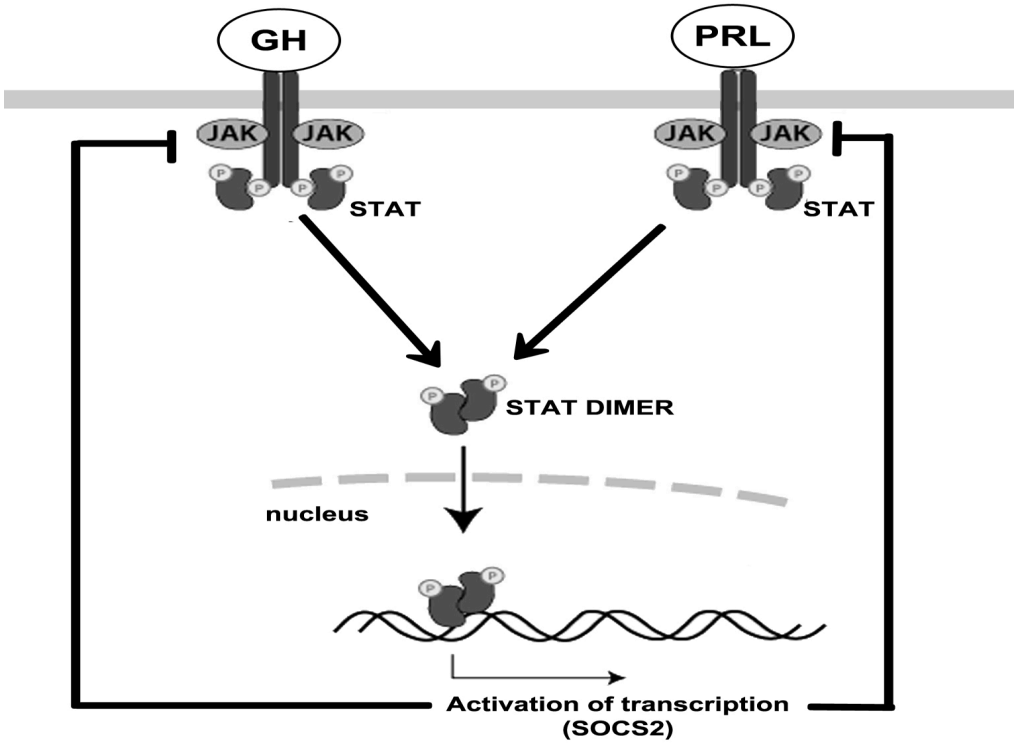

Figure 3. The cytokine-triggered JAK-STAT signal transduction pathway. *Phosphorylated STAT proteins dimerize, and migrate to the nucleus to act as transcription factors, initiating transcription of target genes.*

1.5.2 MAPK pathways

The JAK-STAT cascade is considered to be the most important signaling pathway evoked by cytokine receptors. However, other pathways like the MAPK, or extracellular signal-regulated kinase (ERK) pathway, is also involved in signal transduction by cytokine receptors, and plays an important role in gene transcription, cellular proliferation and prevention of apoptosis. The ligand SH2 domain-containing transforming protein (Shc) binds to the phosphorylated receptor, and is phosphorylated by JAK2. This activates Shc, which subsequently binds to and activates growth factor receptor-bound 2 (Grb2) protein. Grb2 interacts with Sons Of Sevenless (SOS), and this triggers further signaling by the downstream signaling proteins Ras, Raf and MEK. Both GH and PRL have been shown to activate MAPK through sequential activation of the Shc/Grb2/SOS/Ras/Raf/MEK/MAPK cascade, which then activates ERK 1 and 2 [97–99]. Unlike the STAT signaling pathway, GH/PRL activation of the MAPK pathway is independent of JAK2, since deletion of JAK2 has no effect on the activation of ERK1 and 2 kinases [100]. However, studies also suggest a cross talk between the JAK-STAT and the MAPK cascade pathways [101,102]. The activation of the ERK1/2 kinase-signaling cascade results in the phosphorylation of STATs, ribosomal S6 kinases and many other downstream transcription factors.

1.5.3 mTOR pathway

The mammalian target of rapamycin (mTOR) pathway responds to diverse factors including energy status, nutrients, amino acids, oxygen, and stress. mTOR is an atypical serine/threonine protein kinase that belongs to the phosphoinositide 3 kinase (PI3K)-related kinase family [103]; this kinase interacts with several proteins to form two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2) [104]. GH and PRL increase the level of protein synthesis in the cell, and AKT-mTOR-S6 kinase is an important signalling molecule involved in this effect [105]. PI3K activates the serine/threonine protein kinase AKT, which in turn activates mTOR. An important downstream effect of mTORC1 activation is increased protein synthesis, mediated by S6 kinase. This kinase activates mRNA biogenesis and facilitates protein translation [106]. In fact, all of these upstream signals, including those activated by growth factor binding, are integrated and transmitted through a heterodimer consisting of tuberous sclerosis complex (TSC) 1 and 2, which acts as an inhibitor for mTOR. The mTOR pathway kinases, notably AKT and ERK1/2, phosphorylate TSC to inactivate it, and thus activate the mTORC1. The mTOR-containing complexes also

have different sensitivities to the immune suppressive drug rapamycin, a well-known inhibitor of mTOR [107]. An anti-apoptotic function for PRL on rat Nb2 lymphoma cells has been reported to occur through activation of the PI3K/Akt pathway [108], and this action of PRL is further inhibited by rapamycin (Figure 4) [105].

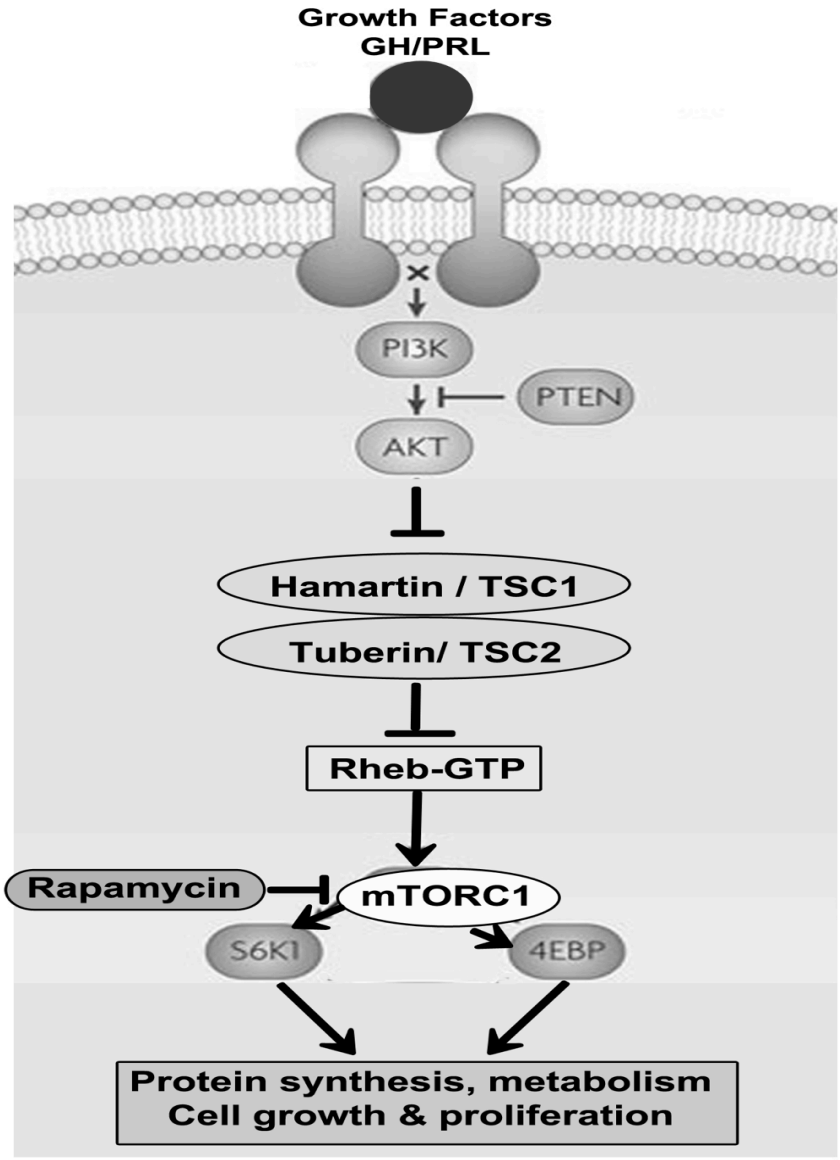

Figure 4. TSC2 complex is activated through the growth factor-PI3K-AKT signaling pathway. *Activation of the TSC2 complex overcomes its inhibitory effect on mTOR, and constitutively turns on cell growth, proliferation and protein synthesis.*

1.5.4 IRS and PI3 kinase pathway

Activation of the Insulin Receptor Substrates (IRSs) and PI3K pathways by insulin and IGF-1 is well studied. However, GH and PRL are also able to activate IRS-1, -2, -3 through JAK2 phosphorylation. Phosphorylated IRS proteins also constitute a binding site for the 85 kDa regulatory subunit (p85) of PI3K [109], which in turn, once activated, is involved in the regulation of glucose uptake into the cell by translocation of glucose transporter type 4 to the cell surface. Moreover, DNA synthesis, cell proliferation and cellular apoptosis can be induced through IRS phosphorylation evoked by PRL and GH [110,111]. Finally, GH and PRL also trigger other signaling pathways, which are out of the scope of this thesis.

1.6 CONTROL OF PROLACTIN AND GROWTH HORMONE SENSITIVITY

Several hormone systems are characterized by changes in target tissue sensitivity. Key factors in hormone sensitivity include the number of cell-surface receptors, and the duration of receptor-activated intracellular signals. Many studies have addressed agents that control receptor number, and it is also recognized that tight control of receptor number is required to regulate the duration of hormone-activated signals. A common theme concerning the latter is the inactivation of tyrosine-phosphorylated intracellular proteins by tyrosine phosphatases. Alternatively, activated receptor signals can be processed using proteasomal breakdown, as described below. In this work, particular focus is given to two different proteins, SOCS2 and TSC2 that uniquely impinge on JAK-STAT and mTOR activation.

1.6.1 Suppressor of cytokine signalling

One prerequisite for cells to respond to GH/PRL is the cellular levels of GHRs/PRLRs, and one interesting regulator of the levels of GHR, and probably of PRLR, is suppressor of cytokine signalling 2 (SOCS2). This protein, which is able to regulate the intracellular signalling cascades (JAK-STAT), is essential to avoid the unwanted outcomes of excessive hormone stimulation, e.g. over-growth and cancer development. Knockdown of SOCS2 in primary cells from animals and humans increases the level of GHR, and this leads to increased GH sensitivity.

The SOCS protein family consists of eight different proteins (SOCS 1-7, and the cytokine induced Src-homology2 (SH2) protein [CIS]) [112,113]. All SOCS proteins contain a C-terminal SOCS-box, a central SH2 domain and an N-terminal domain of varying length.

These proteins are intracellular regulators of the cellular sensitivity to extracellular ligands. In particular, ligands activating cytokine receptors signaling through the JAK-STAT pathway have been connected to SOCS. SOCS proteins are the target recognition subunits of Culllin2/5-Rbx2 E3 ubiquitin ligase complexes, which transfer ubiquitin to tyrosine-phosphorylated proteins for degradation either in the lysosomes or proteasomes [114,115]. Four members of the SOCS-family have been shown to regulate GHR signaling, i.e. SOCS 1-3, and CIS, which act in a negative feedback loop to terminate the GH signal [116]. Figure 5 shows SOCS2 binding to GHR and its mediation of ubiquitination.

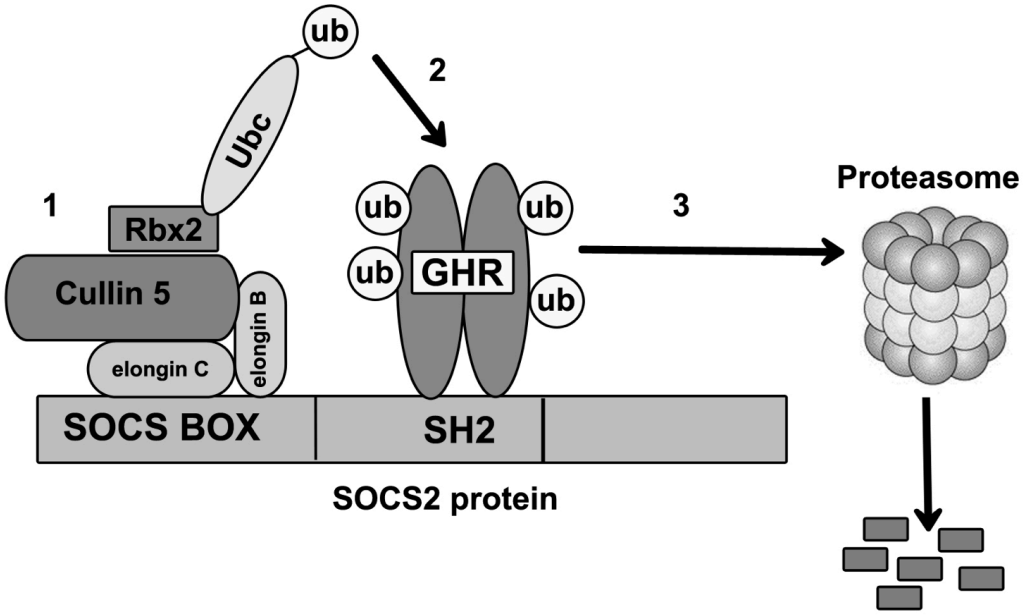

Figure 5. SOCS2 binds to the GHR and mediates its ubiquitination*. (1) SOCS2 assembles an E3 ubiquitin ligase complex together with Elongin B and C, Cullin5 and Rbx2. SOCS2 then binds to phosphorylated tyrosines on the GHR through its SH2 domain. (2) The GHR is poly-ubiquitinated by an E2 conjugating enzyme. (3) The ubiquitinated GHR is degraded by the proteasome.*

Experimental elimination of different SOCS proteins demonstrates that individual SOCS proteins have distinct functions in the contexts of inflammation, metabolism and growth. Elimination of certain SOCS proteins, e.g. SOCS1-3, results in increased inflammatory responses [117,118]. In addition, SOCS2 knockout ($SOCS2^{-/-}$) mice display gigantism compared to controls, without elevation of GH [119], a condition explained by increased GH sensitivity [120]. These mice are highly sensitive to GH because SOCS2 deficiency leads to a

reduced receptor breakdown, and consequently more GHRs [121]. SOCS2 has also been implicated in the negative regulation of PRL signaling. Absence of SOCS2 rescues the failure in lactation and reduction of mammary STAT5 phosphorylation in PRLR-heterozygous knockout mice [122,123]. In contrast to the GH related phenotype observed in $SOCS2^{-/-}$ mice, the gigantism in which presumably identifies SOCS2 as a down-regulator of GH function, the connection of any phenotype in mice missing a particular SOCS gene to PRL function has not yet been found.

1.6.2 Tuberous sclerosis complex

As mentioned above, a surge of research has shown that the TSC genes encode proteins that suppress the mTOR pathway. Molecular genetics research has identified two TSC genes, TSC1 and TSC2, the products of which form heterodimers and work together as one complex to control cell growth and proliferation through inhibition of mTOR. Loss of function or mutation in the TSC1 or TSC2 genes leads to similar phenotypes, although TSC2 mutations are more common [124]. Mutations in either the TSC1 or TSC2 genes cause an autosomal dominant genetic disorder known as TSC. This disease is characterized by development of multiple benign hamartomas in multiple organ systems [125]. Another abnormal phenotype associated with mutations in TSC2, which in turn results in dysregulated cell growth, is observed in lymphangioleiomyomatosis (LAM) disease [126–128]. LAM is a rare disease affecting females of reproductive age, and is characterized by an uncontrolled proliferation of lung LAM lesions consisting of smooth muscle cells, leading to pulmonary insufficiency and subsequent death if not treated [127]. Here we speculate that loss of the TSC2 gene's inhibitory action on mTOR may stimulate PRL synthesis and increase a cell's sensitivity level to PRL.

1.7 PATHOLOGICAL CORRELATES TO PROLACTIN AND GROWTH HORMONE

Endocrine disorders can be put into categories such as over-production or under-production of hormones, as well as in changes in hormone sensitivity. In the case of GH/PRL, all of these scenarios exist. Over the years, different pharmaceuticals have been developed to manage such conditions (Figure 6).

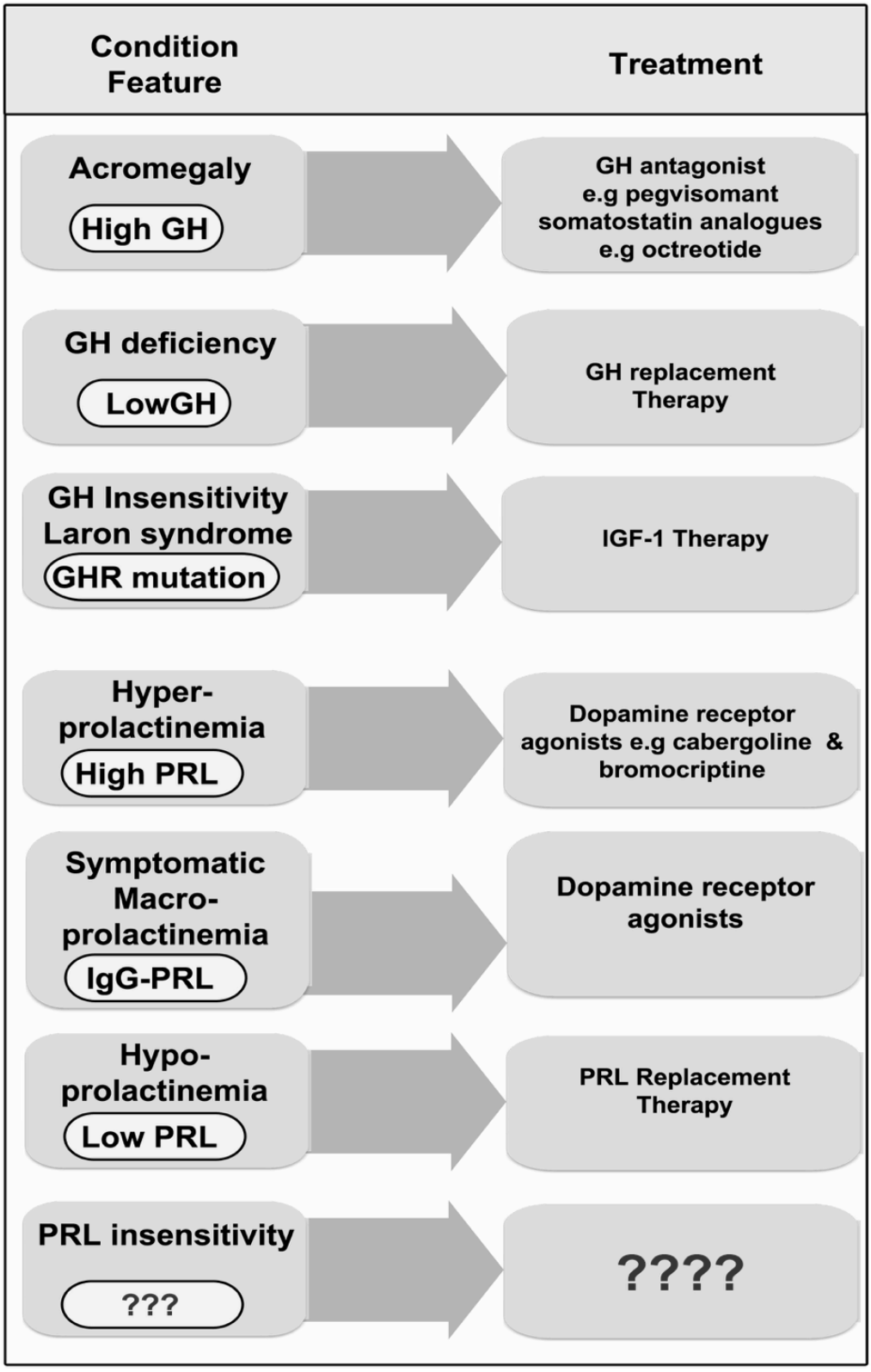

Figure 6. Different pathological conditions associated with GH/PRL*. There is no disease yet identified which involves insensitivity to PRL.*

1.7.1 Conditions associated with a disturbed prolactin and growth hormone axis

In humans and animals, excess amounts of GH lead to gigantism or acromegaly, depending upon when during life the hypersecretion begins. The term acromegaly was first coined in 1886 by Pierre Marie [129] to describe a condition caused by increased GH levels that appear after fusion of the epiphyseal plate at puberty [130]. More than 95% of patients with acromegaly harbour a GH-secreting pituitary tumor, which is clinically presented as an enlargement of extremities, prognathism and macroglossia [131]. Such patients are at a high risk to develop co-morbidities related to elevated GH levels, e.g. diabetes and cardiovascular disease [132].

Deficiency of GH leads to dwarfism, and such patients can be treated with exogenous GH treatment [133,134]. A particular form of dwarfism, i.e. Laron dwarfism, is caused by mutations in the GHR leading to complete GH insensitivity. People with Laron syndrome have mutations in the extracellular domain of GHR, commonly high levels of GH concomitant with low levels of IGF-1, and do not respond to exogenous GH treatment [135,136]. Besides Laron syndrome, other types of acquired insensitivity to GH exist, e.g. in kidney disorders and during glucocorticoid treatment [137,138].

Regarding the GH insensitivity in the Laron syndrome, there are no known genetic diseases associated with mutations in the genes coding for either PRL or PRLR in humans [139]. Beside PRL overproduction due to prolactinoma, a variety of other causes of hyperprolactinemia in humans have to be ruled out due to conditions such as psychotropic drug use, stress, chronic renal failure and some tumors [140,141]. The main clinical manifestation associated with elevated levels of PRL, in both sexes, is reproductive dysfunction resulting in hypogonadism, infertility and galactorrhea [142,143]. Moreover, insensitivity to PRL is not yet clinically known.

1.7.2 Growth hormone and prolactin role in cancer

There are many different causes of cancer including infection, chemical exposure, genetic mutations and local cytokine production. The hallmarks of cancer are self-sufficient growth signals, unlimited proliferation, permanent angiogenesis, failure of apoptotic mechanisms and insensitivity to anti-growth signals. One phenomenon in cancer cells is “the Warburg effect”, in which cancer cells favor the use of aerobic glycolysis, in preference to mitochondrial

oxidative phosphorylation that is used by normal cells to generate adenosine 5′-triphosphate [144,145]. The significance of cell metabolism in cancer is also suggested by findings that the drug metformin, a widely used drug for the treatment of type 2 diabetes, can protect from cancer. The anti-neoplastic effect of metformin remains elusive, although studies demonstrate a variety of mechanisms including attenuation of the Warburg effect [146]. In this context, researches have shown that metformin decreases the levels of circulating hormones such as androgens and estrogen that are known to be linked with postmenopausal breast cancer development [147–149].

Many human cancers develop as a result of exposure to viral infection. The most common one is human papillomavirus (HPV) infection, specifically by HPV-16 and -18, which contributes to a high risk of cervical cancer [150,151]. In this context, clinical screening programs for cervical cancer, i.e. tests for HPV, have been introduced in some countries. A new line of research in the last years suggest that human cytomegalovirus (CMV) infection can also contribute to several human malignancies including breast, colon, prostate, rhabdomyosarcoma, hepatocellular cancer, salivary gland tumors, neuroblastoma, and brain tumors [152]. In searching for treatments of malignancies, one way to control cell proliferation and transformation might be through the introduction of tumor suppressor genes into the cancer cell. One of the most commonly mutated tumor suppressor genes in humans is p53, a gene which is involved in almost 50% of all cancers, especially those of the lung, breast, ovary, colon, bladder and prostate [153].

Since hormones regulate cell growth and differentiation, they also have a role in cancer development. In fact, many tumors respond therapeutically to agents interfering with hormone action [154]. The influence of GH/PRL on different forms of cancers in humans has been widely discussed, and the realization that GH/PRL also have paracrine/autocrine actions is highly relevant in this context.

GH/IGF-1 act as potent proliferative agents, and IGF-1 is also a well known mitogen with anti-apoptotic effects that seem to contribute to the development of neoplastic lesions in colorectal tumor development [155]. It is interesting to note that loss of GHR creates an unfavorable situation for tumor growth both in animals and humans [156,157]. Overproduction of PRL has been reported to increase the aggression of colorectal cancer in humans [158], and to contribute to the pathogenesis and progression of human breast cancer [159]. Animal data suggest that PRL increases the rate of tumor cell growth and metastasis

[160]. Contradictory findings, however, also suggest that PRL has a favorable role in breast cancer prognosis [161]. Local production of PRL in myometrial smooth muscle tumors (uterine leiomyomas) indicates that the mitogenic activity of PRL participates in the progression of such tumors [162]. It has been suggested that PRL might be involved in the progression of LAM disease (see below), since only females are affected and because the disease is aggravated during pregnancy – a condition associated with elevation of lactogenic hormones [163]. In human ovarian cancer cells, studies also showed that lower levels of tumoral PRL/PRLR in clinical samples were associated with longer patient survival, suggestive of an important role for PRLR in the pathogenesis of such tumours [164].

GH/PRL hypersensitivity is suspected in many tumors, e.g. in prostate cancer, since clinical studies reported increased levels of PRL in this cancer [165]. Furthermore, an increased expression of PRLR was demonstrated in dysplastic lesions of human prostate, as well as in low grade cancer, compared with normal tissues [166]. GH is also known to play a role in the normal physiology of the prostate gland, in addition to androgens, the main regulator of prostate function [167]. GHR mRNA levels were reported to be significantly higher in prostate carcinoma tissues, compared to benign prostate hyperplasia and normal tissues [168]. In summary, several studies suggest a potential role for PRL and/or GH hypersensitivity in different pathophysiological states related to tumor growth.

1.8 PROLIFERATIVE CONDITIONS ASSOCIATED WITH INCREASED SENSITIVITY TO GROWTH HORMONE AND PROLACTIN

GH and PRL also play roles in metabolism, regeneration and proliferation, and it is reasonable to believe that tissue sensitivity to GH/PRL might be important in this context. This project has investigated three different conditions, i.e. LAM, glioblastoma brain tumors and diabetes mellitus, to the relevance of GH/PRL tissue sensitivity in these disorders.

1.8.1 LAM

LAM is a multisystem disease, which affects almost exclusively women of childbearing age. It is manifested as pulmonary cystic destruction [126,169], and can be associated with extra-pulmonary tumour lesions, e.g. angiomyolipomas [170]. The disease affects the lungs, and results from uncontrolled proliferation of smooth muscle cells and melanocytes, which in turn infiltrate into the surrounding tissues and lead to airway obstruction [171]. Positive reaction to human melanin black antibody (HMB-45) is diagnostic of LAM [172]. Interestingly, a

potential biomarker for LAM is increments in serum vascular endothelial growth factor D (VEGF-D) [173,174]. The disease can occur as sporadic cases, or can be associated with an inherited autosomal dominant disorder (TSC), but in both cases is caused by mutations in the TSC1/TSC2 genes, although TSC2 mutations are more common [128,175]. The protein products of the TSC genes, Hamartin and Tuberin, together inhibit the mTOR signaling pathway, a major regulator of cell size and proliferation [176]. In LAM, mutations in TSC2 lead to mTORC1 activation and further phosphorylation of the downstream kinases which regulate cell growth and proliferation [176]. Until recently, LAM tumors were considered benign, but evidence for their neoplastic nature, e.g. invasiveness and metastasis, has accumulated. Similar TSC2 mutations from the same patient were detected in micro-dissected pulmonary LAM and in angiomyolipoma cells but not in normal cells, suggesting a common origin for both types of cells [177]. Moreover, recurrent LAM cells after lung transplantation share the same TSC2 mutations as those in native LAM cells, indicating that LAM cells can migrate and metastasize *in vivo* to the transplanted lungs [178]. The uterine or pelvic cavity organs have been proposed as the primary site of origin of LAM [179].

Clinical trials with inhibitors targeting mTORC, e.g. rapamycin (clinically known as Sirolimus) demonstrate a decrease in tumor progression and improved pulmonary function, in LAM patients [180–182]. Despite clinical improvements resulting from Sirolimus treatment in LAM patients, this treatment can cause serious adverse reactions. For this reason, patients need to be closely monitored; the current belief is that Sirolimus treatment only halts the progression of the disease and is not a cure [180]. Endocrine components of LAM have been suspected, because females are predominantly affected, and because the disease is aggravated during pregnancy – a condition associated with elevations of PRL. This is further supported by one previous study demonstrating that LAM lesions produce PRL, and showing that patients with LAM have elevated serum PRL levels [163]. Study II included in this thesis hypothesizes that LAM lesions are hypersensitive to PRL, which might be of relevance for an understanding of factors that regulate their growth. The scientific progress in LAM research is summarised in figure 7.

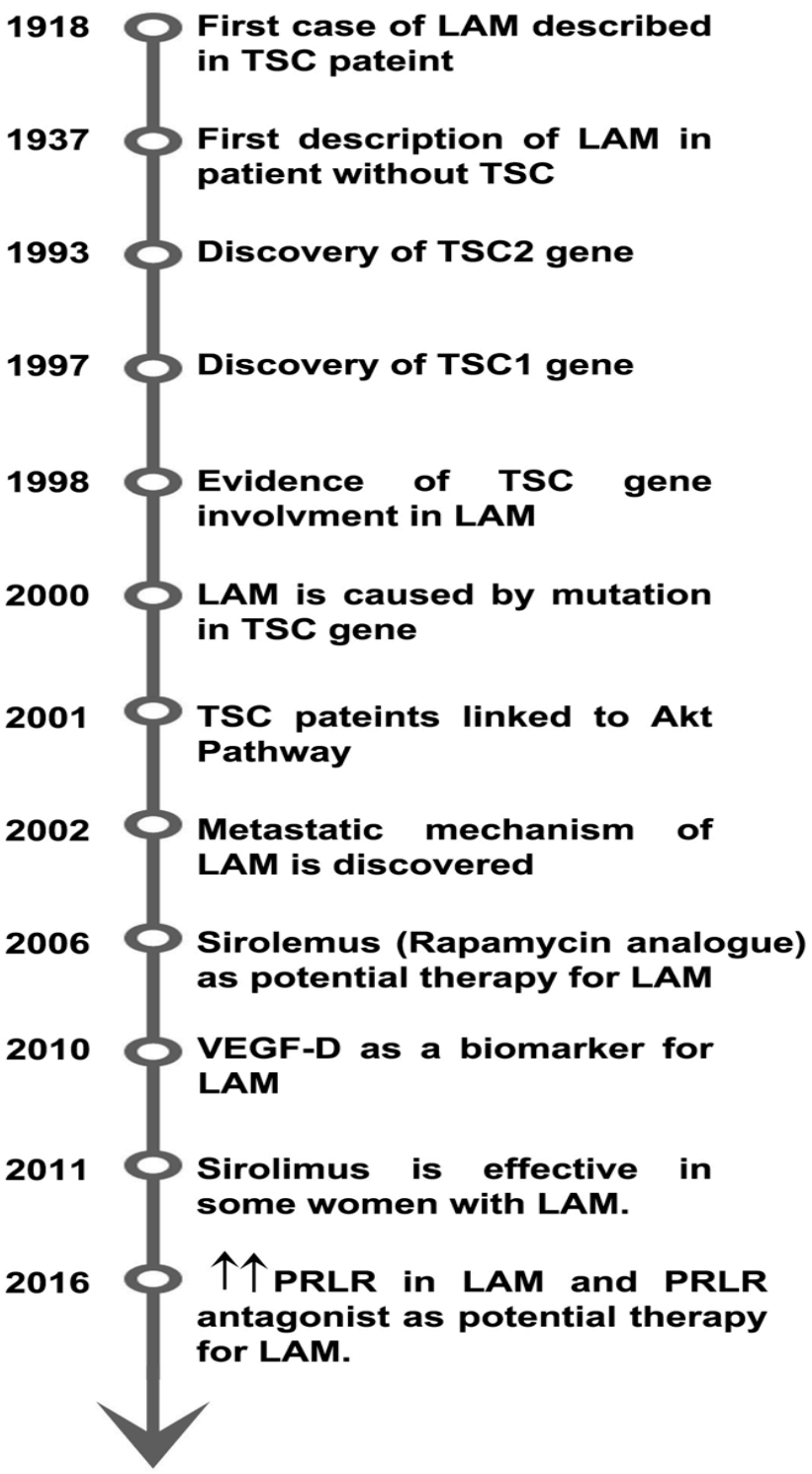

Figure 7. Historical representation of the scientific progress in LAM research.

1.8.2 Glioblastoma Multiforme (GBM)

GBM is the most common and aggressive primary brain tumor in humans, accounting for approximately 12-15% of all intracranial tumors, with a median survival of 15 months despite advanced cytotoxic therapy including surgical intervention [183]. Angiogenesis (the process of forming new vessels) is essential for tumor growth and metastasis. One main feature characterizing GBM is neovascularization, i.e. an imbalance between pro-, and anti-angiogenic factors [184,185]. A number of different alterations of cell signaling molecules have been found to promote angiogenesis in GBM, exemplified by increased signaling from VEGF receptors (VEGFRs), epidermal growth factor receptor (EGFR) [186] and platelet–derived growth factor [184]. On a genetic level, primary GBM is characterized by deletion or mutations of the tumor suppressors INK4a, p53 and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) [187,188].

SOCS2 and TSC2 are intracellular proteins that regulate the JAK-STAT and mTOR pathways respectively. Previous experimental and clinical studies have reported increased STAT and mTOR activity in GBM [189–193]. Studies of SOCS/TSC expression in brain tumors have shown that SOCS1 and SOCS3 are apparently expressed in GBM and play a significant role in tumor pathogenesis [194]. Of note, mTORC1 hyper-activation due to loss of TSC1 is not sufficient for gliomagenesis to develop but it accelerates malignant gliomagenesis when it is combined with oncogenic signals [195]. Patients with TSC have bi-allelic loss of TSC1/TSC2, and a few clinical cases have been reported the occurrence of GBM in such patients [196]. The limited success in the use of EGFR inhibitors to treat recurrent GBM opens the gates for new treatments such as combination therapy that also inhibit the mTOR pathway through PI3K.

With regard to PRL, it has been suggested that initiation and cessation of vascularization in the mouse placenta correlates with the sequential expression of proliferin and proliferin-related peptide [197,198]. These non-classical members of the PRL/GH family have been shown to act in an autocrine/paracrine fashion to either stimulate or inhibit various stages of angiogenesis. It is, however, interesting to note that anti-angiogenic factors induce local production of PRL, as a pro-survival response. A recent study showed that the combined application of the angiogenic inhibitors endostatin and tumastatin up-regulates the PRLR in GBM *in vivo*, and that the activation of PRLR signaling stimulates proliferation of tumor cells [199]. All of these observations suggest an important rule of PRL and its receptor in the

pathogenesis of GBM, and the targeting of this receptor (our study aim) may offer a new therapeutic approach in the field of GBM brain tumors.

1.8.3 Diabetes Mellitus

Diabetes mellitus (DM) is considered to be one of the most challenging diseases and health burdens to humans in the 21st century [200]. In general, there are two types of DM: Type 1 and Type 2 diabetes mellitus (T1D and T2D) that in common have the feature of high blood glucose levels that can cause serious health complications. T1D is caused by the destruction of the insulin-producing beta cells in the pancreas, while in T2D, the body is resistant to insulin, and this eventually leads to impaired insulin production by exhausted β-cells [201,202]. Accumulated data from different studies indicates a pivotal role for the β-cell mass in the pathogenesis of diabetes. β-cell growth, proliferation and differentiation, along with the regulation of insulin secretion, are influenced by different growth factors including GH, IGF-1 and PRL [203,204]. The best known states of increased β-cell mass are obesity [205,206] and pregnancy [207], as represented in Figure 8.

It is relevant to note that β-cells express GHRs/PRLRs [208,209], and that increased β-cell mass observed during pregnancy is suggested to be caused by the increased secretion of placental and pituitary hormones targeting such receptors [66,203,209]. This adaptive response of β-cells in pregnancy is associated with elevated levels of GH, PRL and placental lactogen [210,211], and is possibly linked to pathways of increased tissue sensitivity to these hormones. As mentioned before, DM can be a consequence of increased circulating GH levels, referred to as the diabetogenic effect of GH [60]. In relation to SOCS2, GH-associated diabetic features are not observed in SOCS2$^{-/-}$ mice under standard diet (hypersensitivity to GH). Yet GH levels are lower in SOCS2$^{-/-}$ mice, despite similar IGF-1 levels in SOCS2$^{-/-}$ mice and their wild-type (WT) littermates, indicating higher GH sensitivity in SOCS2$^{-/-}$ mice [212], and suggesting unique actions of SOCS2 in the regulation of glucose metabolism. Given the fact that SOCS2 also modulates inflammatory pathways [213], it is unknown whether its regulatory actions in the pancreas also impact on β-cell survival in models of T1D [214]. Important roles for other SOCS proteins in diabetes have been suggested, these intracellular regulators affect key pathways of relevance for diabetes development [215]. For example, it has been shown that elimination of SOCS1 increases the interferon response and potentiates islet cell death [216]. Furthermore, SOCS3 potently protects against T1D through suppressed production of IL-1β and TNF-β in pancreatic β-cells [217,218].

Changes in tissue sensitivity to other hormones like insulin that is involved in the development of diabetes have been reported after viral infection. Human adenovirus-36 infection has been associated with a lower occurrence of T2D and better insulin sensitivity, as reflected by reduced Homeostatic model assessment and insulin resistance (HOMA-IR) and increased levels of insulin-like growth factor binding protein-1 [219].

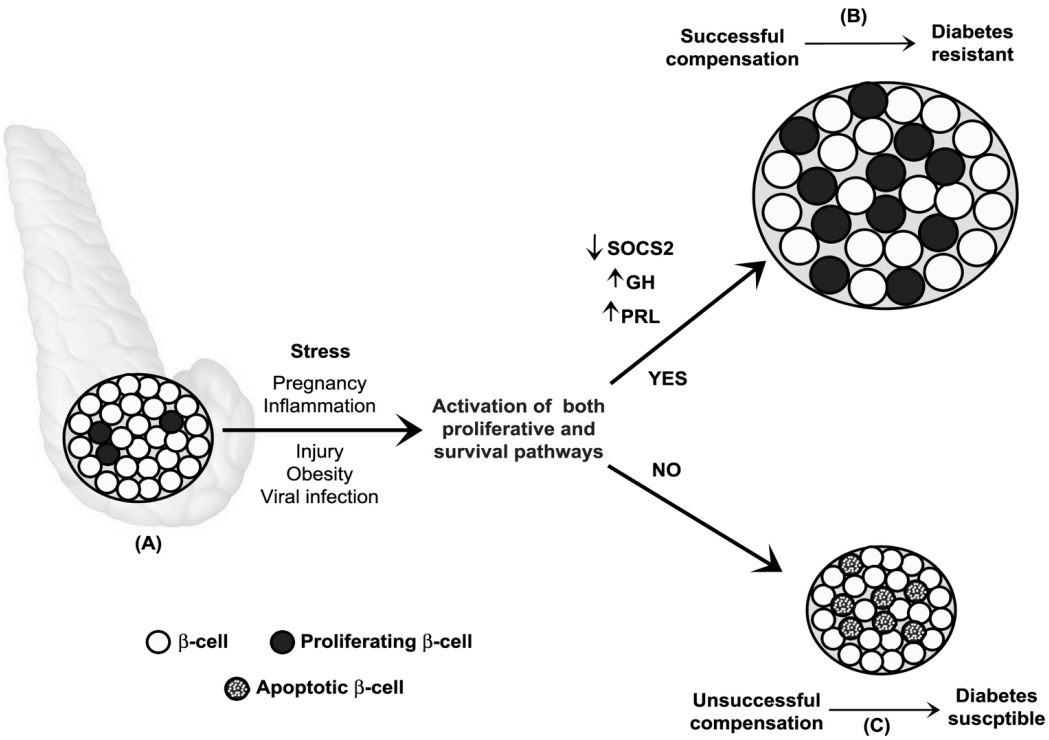

Figure 8. A model of β-cell expansion in response to increased metabolic loads*. (A) β-cells normally undergo very little turnover in adult life. Some physiological states like pregnancy and obesity stimulate β-cell proliferation in response to an increased systemic demand for insulin. (B) To accomplish this state, both proliferative and survival pathways are activated in β-cells, protecting against the onset of diabetes. (C) Failure of the β-cell mass expansion response to metabolic demands and continued β-cell apoptosis overcomes β-cell renewal mechanisms and leads to diabetes. Adopted from [207].*

1.9 DEVELOPMENT OF DRUGS ACTING ON THE GROWTH HORMONE AND PROLACTIN AXIS

There are several different pharmaceuticals that can affect the GH or PRL axis. In principal, these drugs are used to influence secretion, serve as hormone replacements or as receptor blockers. In this thesis, a particular emphasis is placed on PRLR antagonists.

1.9.1 PRLR antagonist development

As described in Figure 6, there are several compounds that target the GH/PRL system, with the exception of a PRLR antagonist (PRLRA). The development of a PRLRA (not yet in clinical use) is described below. The different utilities of such an antagonist can be considered since PRL production is not solely restricted to the pituitary gland, but also occurs in extra-pituitary tissues, e.g. the mammary gland, prostate and lymphocytes. Locally-produced PRL can take part in proliferation and in tumor development [159,220–223]. One may note that dopamine receptor agonists are clinically used as anti-PRL drugs. These drugs reduce production of PRL from the pituitary, but cannot target extra-pituitary PRL production.

Recently, a GHR antagonist has been developed by replacing a glycine at position 120 for an arginine; this interferes with the binding site of GH to its receptor [224,225]. This antagonist is used clinically under the name Pegvisomant® for the treatment of acromegaly [226]. This has stimulated efforts to develop PRLRA, based on a similar principle (Figure 9).

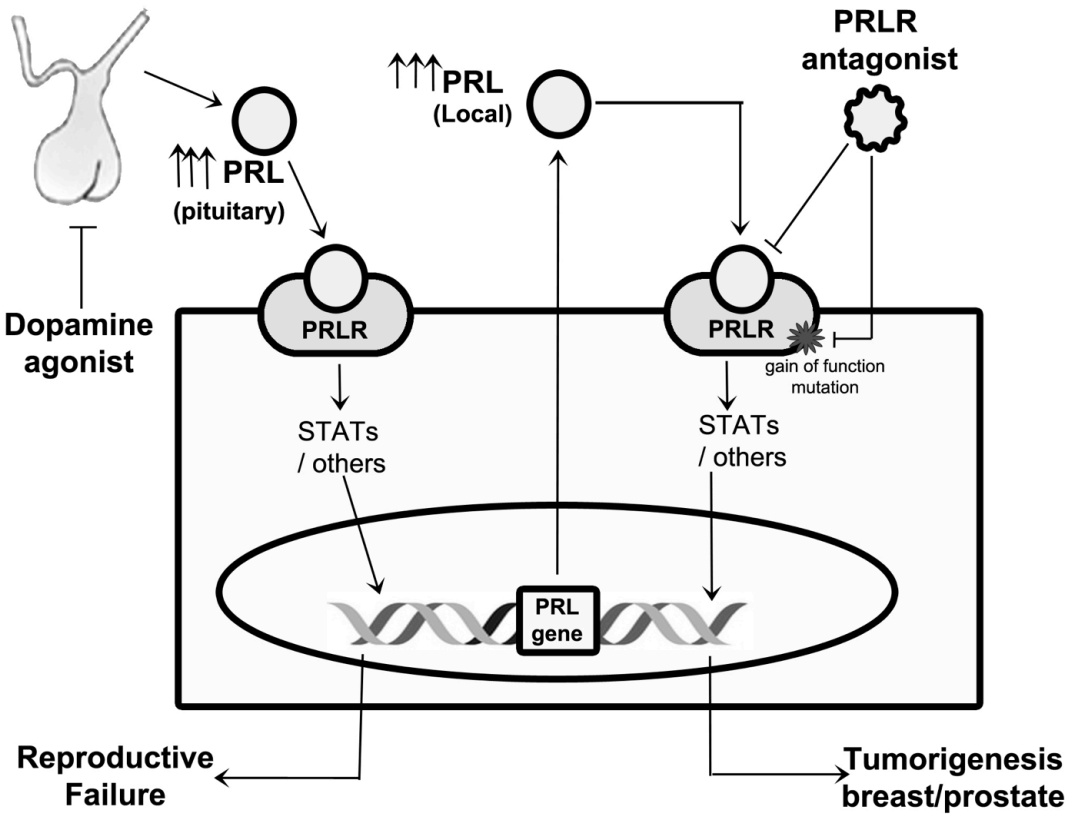

Figure 9. Development of a PRLRA that antagonizes PRL's action at the cellular level*. Dopamine agonists affect pituitary PRL production only, but have no role on local PRL production and actions in other body organs.*

1.9.2 Low-affinity PRLRA

PRL interacts through two binding sites (BS1 and BS2) with receptor dimer molecules and forms the PRL-PRLR homodimer [43,227]. In human PRL, 13 residues are involved in the process of receptor binding. Based on the two site-binding mechanism illustrated in (Figures 1 and 10), the first generation PRLRA were designed to impair BS2 [228]. By substituting glycine at position 129 in the conserved helix 3 for arginine, antagonists were created and named G129R-hPRL [229]. Multiple *in vitro* and *in vivo* assays proved the antagonistic activity of G129R-hPRL, but partial agonistic activity was also detected [230]. This stimulated efforts to develop a second-generation PRLRA by introducing N-terminal deletions (Δ1–9-G129R-PRL and Δ1–14-G129R-PRL) into the PRL sequence. Although this resulted in a pure antagonist, high concentrations were needed (>10-fold molar excess) to compete with endogenous PRL [231,232]. Other PRLRA variants have been reported, but they also have the drawback of residual agonist activity [232,233].

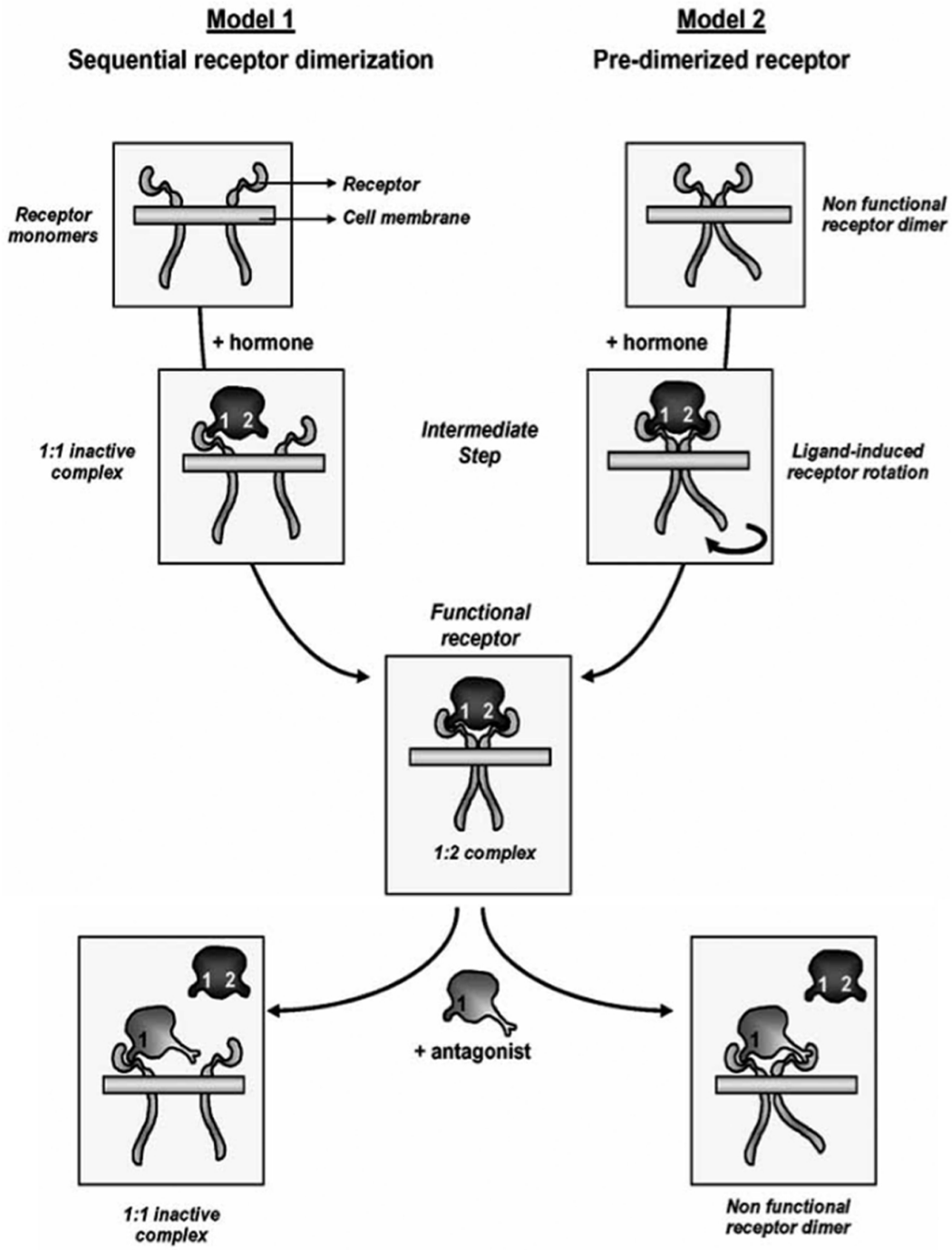

Figure 10. Models of PRLRA binding to PRLR. *Model 1 represents the old theory, in which PRLRA competes with PRL and binds to PRLR, preventing receptor dimerization. In model 2, PRLRA competes with PRL and binds to a pre-dimerized receptor. Adopted from [234].*

1.9.3 High-affinity PRLRA

It is clear that high receptor affinity is essential to generate a potent *in vivo* PRLRA. Different screening techniques have been used to identify amino acid changes in G129R-hPRL to increase the binding to BS1. A successful strategy to generate antagonists with significantly improved affinity to block the receptor has identified a series of single mutation sites, which enhanced BS1 affinity. By combining the single mutations, researchers succeeded in generating an antagonist with significantly improved affinity to block the receptor. We have used this antagonist, PRL-S33A, Q73L, G129R, K190R in our study [235,236].

2 AIMS

Factors controlling hormone sensitivity are an important aspect of several types of disorders. Regulators of GH and PRL sensitivity may be related to both metabolic and hyper-proliferative disorders. In this thesis, we hypothesize that SOCS2 and TSC2 uniquely impinge on JAK-STAT activation and on mTOR activation, and that this in turn regulates the cellular level of GH and PRL receptors. We also hypothesize that a PRLRA could offer a new therapeutic approach in the field of tumor treatment by blocking localized actions of PRL in cases of PRL hypersensitivity restricted to a particular organ site.

The present study had the following aims:

1. To experimentally investigate relationships between SOCS2 and diabetes.

2. To demostrate if increased GH/PRL sensitivity in SOCS2$^{-/-}$ mice will have any impact on β-cells.

3. To investigate if the mTOR suppressor TSC2 participates to regulate components of the PRL/GH system.

4. To evaluate the activity of a PRLRA in PRL-sensitive tumor cells.

3 MATERIAL AND METHODS

This section provides a brief summary of the major methods used in this thesis. For more detailed methodological descriptions, please refer to the individual papers.

3.1 MATERIALS

3.1.1 Animals

In Paper I, we have used the $SOCS2^{-/-}$ mice (C57BL/6J), as previously described [212]; littermates WT mice were used as controls.

3.1.2 Cell lines

All cells were obtained from the American Type Culture Collection, except for the human LAM/TSC cells, which were isolated from human LAM lesions. Before stimulation with PRL or GH, cells were washed and incubated overnight in medium lacking fetal bovine serum (FBS).

CRL-2620 cells were used to study the effects of WT and mutant versions of TSC2 on the PRLR and GHR (Paper II). CRL-2620 cells originating from a $TSC2^{-/+}$ mouse sarcoma were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO2. Superfect (Qiagen) was employed according to the manufacturer's protocol to carry out transfections.

LAM/TSC cells (used in paper II) were previously characterized as a homogenous population of α-smooth muscle-like cells. These cells have absence of TSC2 protein product for a TSC2 mutation of one allele and an epigenetic alteration of the second allele [175]. The cells were used to study the effects of TSC2 mutations on PRLR, and were grown in a 50/50 mixture of DMEM/Ham F12 (Gibco) supplemented with hydrocortisone (2.5 μg/ml), EGF (10 ng/ml), sodium selenite (8.6 ng/ml), insulin (25 μg/ml), transferrin 10 ug/ml), ferrous sulphate (0.445 μg/ml) and 15% FBS.

U251-MG cells were used in Study III to evaluate the activity of a PRLRA in PRL sensitive tumor cells. Cells were cultivated in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO2.

3.2 METHODS

3.2.1 Multiple low-dose streptozotocin model

We investigate the role of SOCS2 on DM induced by multiple low-dose streptozotocin (MLDSTZ) (Paper I). We used the MLDSTZ model to induce T1D in SOCS2$^{-/-}$ and WT littermates. Sixty-five days after MLDSTZ treatment was stopped, mice were sacrificed and tissues were collected for further studies.

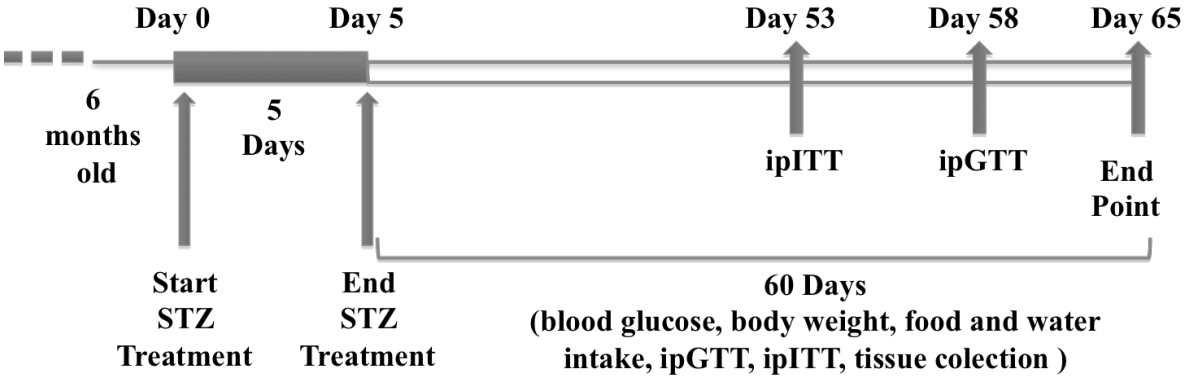

Figure 11. Schematic representation for MLDSTZ treatment in SOCS2$^{-/-}$ and WT mice that were used as a model of autoimmune diabetes and β-cell destruction in vivo.

3.2.2 Assessment of whole body insulin sensitivity

Insulin sensitivity (Paper I) was assessed *in vivo* by measurement of fasting blood glucose, fasting plasma insulin, intraperitoneal glucose tolerance tests (ipGTTs), intraperitoneal insulin tolerance tests (ipITTs), and HOMA-IR. Plasma insulin levels were measured using commercial ELISA kits. Blood glucose levels were measured using a glucometer (Roche Diagnostics, Basel, Switzerland).

3.2.3 Measurement of pancreatic insulin

To assess pancreatic insulin content (Paper I), one third of the pancreas mass from the tail was digested and homogenized in 1 mL acidic ethanol (70% ethanol and 37% HCl) for insulin quantification per unit weight pancreas. Insulin was determined using an ultrasensitive rat insulin ELISA kit (Crystal Chem, IL, USA). We also analyzed pancreatic insulin content before and after MLDSTZ treatment by immunohistochemistry (IHC).

3.2.3.1 Isolation of pancreatic islets

Islets of Langerhans were isolated from SOCS2$^{-/-}$ and WT control mice by collagenase digestion in Hank's balanced salt solution, followed by sedimentation, as described [237]. Islets were selected under stereomicroscope, and cultured free-floating in RPMI-1640 with 2 mmol/L glutamine, 10% (v/v) FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 11 mmol/L glucose. Lipotoxicity was induced by using 250 μM palmitate and hormonal treatment carried out as specified in paper I. Secreted insulin and islet insulin content were analyzed by RIA using ^{125}I-labeled insulin and anti-porcine insulin, generated at the Department of Endocrinology, Karolinska Hospital, Stockholm, Sweden.

3.2.4 Western blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate denatured protein samples, which were then transferred to polyvinylidene fluoride (PVDF) membranes and probed by antibodies to determine the presence and amount of proteins of interest (Paper I – III).

Western blotting is a robust and commonly used technique for the detection and quantification of proteins from various types of samples. It involves electrophoretic gel separation of the proteins in the sample, followed by transfer to a membrane, blocking and then probing with antibodies directed at specific proteins of interest [238,239].

3.2.5 Immunohistochemical analysis of PRLR expression

Immunohistochemistry (IHC) is a powerful method for localizing specific antigens in formalin-fixed, paraffin-embedded tissues. IHC is a commonly used technique that is based on an antigen–antibody interaction in various types of tissues; it contributes to the diagnoses of different diseases, and also differentiates several types of cancer.

Briefly, tissue sections were de-waxed in xylene and rehydrated in alcohol series, followed by blocking the endogenous peroxidase activity. Then tissue sections were stained with antibodies, and a colorimetric determination was performed with a three-step horseradish peroxidase detection system and the chromogen diaminobenzidine (DAB) (Papers II-III).

3.2.6 Assessment of cell proliferation

In Studies II and III, cells were seeded into 96-well culture plates at a density of 1000 cells per 200-µL well and allowed to attach. Then medium was replaced and cells were grown in low or high serum and exposed to different treatments. Then cells were fixed by 4% paraformaldehyde solution. Relative cell numbers were assessed after 72 h using the colorimetric crystal violet assay, in which the bound dye was dissolved with 1% (w/v) SDS, and the optical densities (OD) of the extracts were measured at λ= 600 nm.

3.2.7 PRLRA production and purification

All steps in the production and purification of PRLRA have been carried out by our collaborators in the Royal Institute of Technology, Alba Nova University Center, Dept. of Biotechnology, Stockholm, Sweden.

3.2.7.1 Production of prolactin constructs

The DNA of three PRL constructs was synthesized and inserted into pET vectors:

1.Human PRL: M-GSS- LPIC…PRL…NNNC-STOP.

2.Human PRLRA: M-GSS-RSQV…PRLRA*…NNNC-STOP. **This is used in our Studies II and III included in this thesis.**

3.Albumin-binding human PRLRA (ABD-PRLRA): M-GSS-ABD-RSQV…PRLRA …NNNC-STOP.

*PRLRA: RCQV….PRL (Δ1-9, S33A, Q73L, G129R, K190R)….NNNC

3.2.7.2 Protein extraction from inclusion bodies

High-level expression of PRL recombinant proteins in *Escherichia coli* (*E.coli*) leads to the formation of highly aggregated protein in what are commonly referred to as inclusion bodies. Inclusion bodies are normally formed in the cytoplasm; however, if a secretion vector is used, they can form in the peri-plasmic space. Inclusion bodies can be recovered from cell lysates by low speed centrifugation. In this study, *E. coli* BL21 was used for expression of the PRL constructs mentioned above, according to the protocol previously described [240]. After that, samples were loaded and run on SDS-PAGE gels (Figure 12).

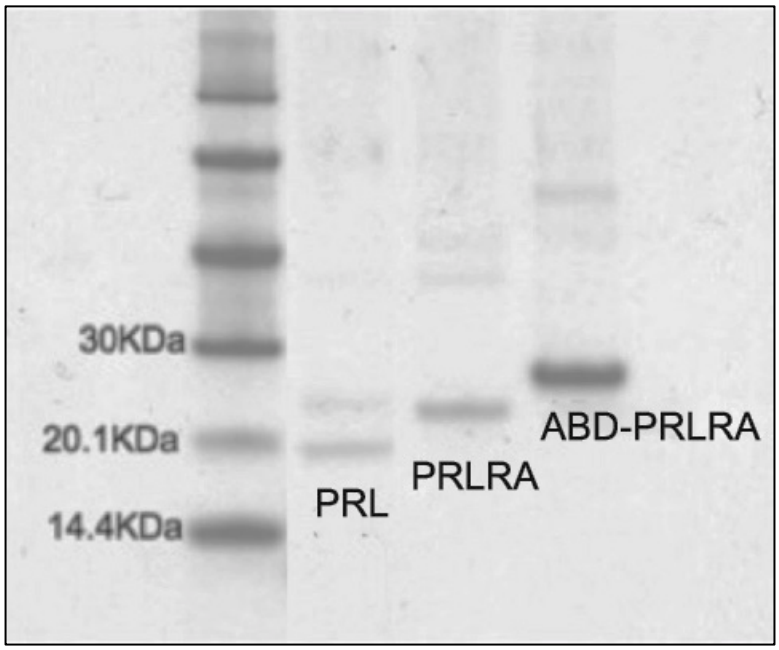

Figure 12. Extracted PRL constructs evaluated by SDS-PAGE gels.

3.2.7.3 Protein purification by ion exchange chromatography

Q-Sepharose Fast Flow resin was used for anion exchange purification of the three PRL proteins obtained above, using an ion exchange chromatography protocol. PRL, PRLRA and ABD-PRLRA were eluted in first elution peak, and 5 ml volumes were collected for each protein with OD_{280}=0.07, OD_{280}=0.06 and OD_{280}=0.1, respectively. After concentration, the three constructs were loaded on SDS-PAGE gels and pure protein obtained (Figure 13).

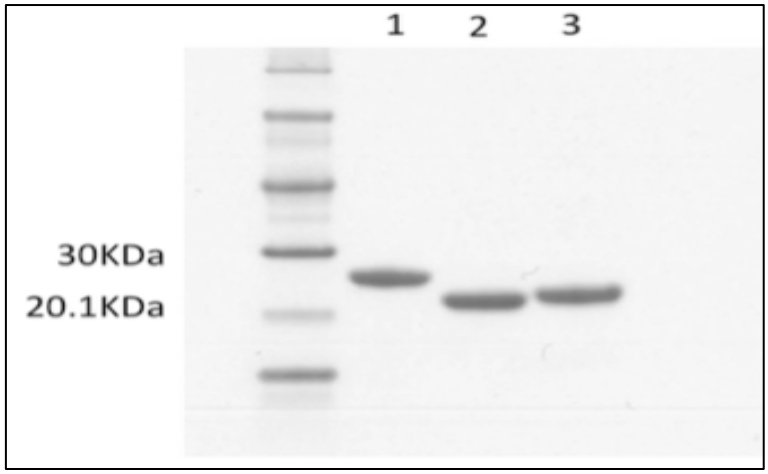

Figure 13. Pure proteins obtained and loaded on an SDS-PAGE gel. *Lanes 1: ABD-PRLRA (27.861 kDa), Lane 2: PRLRA (22.698 kDa), Lane 3: PRL (23.398 kDa).*

3.2.7.4 PRL construct binding affinity against PRLR

The interaction and binding affinity between human PRLR and murine serum albumin (MSA) and the purified constructs were measured using Biacore SPR Technology 3000 (GE Healthcare). PRLR and MSA were immobilized on separate flow cells on a CM5 chip at pH 4.6 in sodium acetate buffer. The immobilization level was approximately 300RU for PRLR and 600RU for MSA. A reference flow cell was created at the same time by activation and deactivation. HBS buffer was used for running the analysis and sample dilution, and the temperature was set at 25°C. 15 mM HCL was used for chip regeneration. Figure 14 shows that all three proteins bind to PRLR and that PRLRA used in this thesis has a 2x affinity to PRLR, compared to PRL.

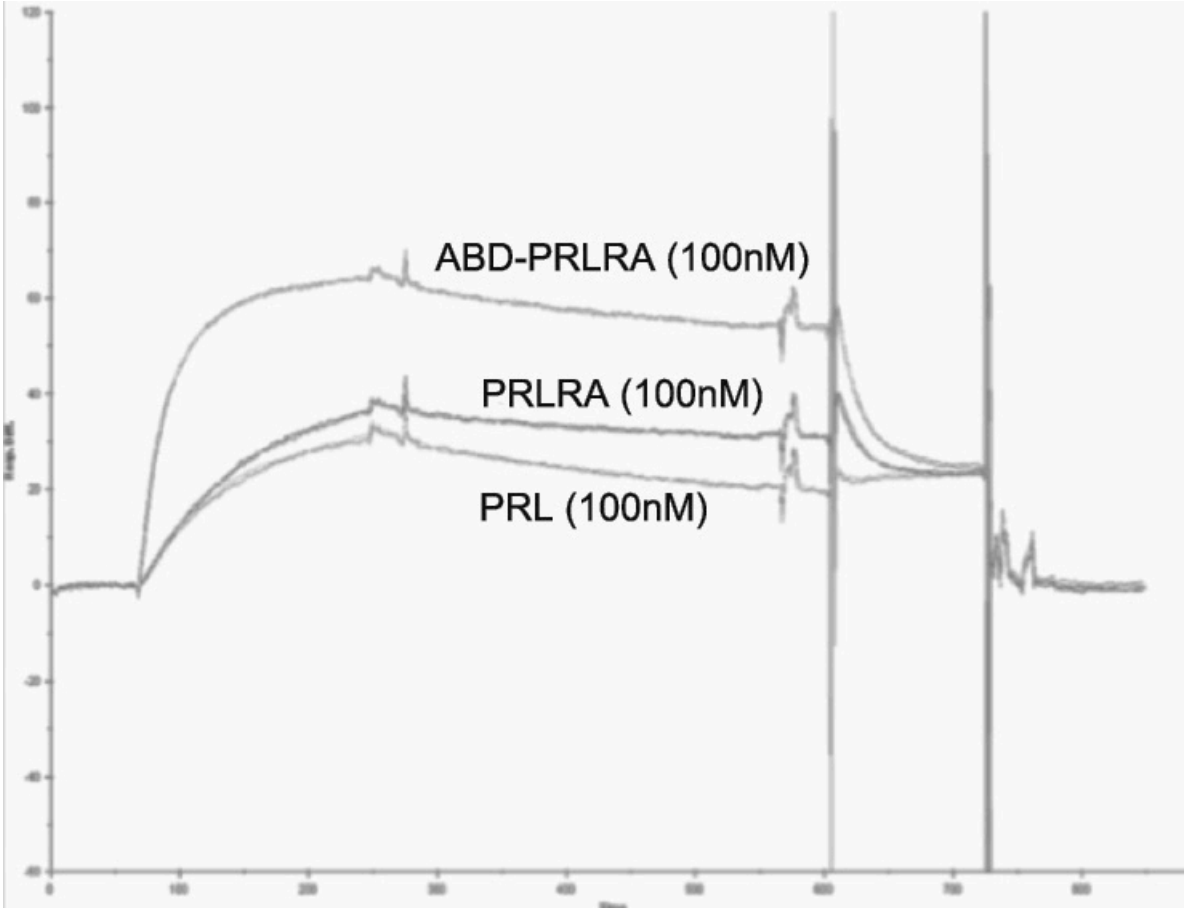

Figure 14. Biacore SPR Technology test*. ABD-PRLRA, PRLRA & PRL can bind to PRLR. 100 nM albumin binds PRLRA and shows the highest binding affinity. Human PRLR was immobilized on a biosensor chip in a Biacore 3000 instrument. The obtained curves fitted well to a 1:1 Langmuir interaction.*

3.2.8 Statistical analysis

In Study I, 2-way ANOVA was performed to test for significant differences in the multiple effects of different time points among the 4 mouse groups in each experiment. Student's t-test was used to test for significant differences between two groups. Cell culture experiments in all of the studies were performed in duplicates or triplicates in at least three independent experiments. Statistical significance of the differences was evaluated using unpaired, 2-tailed Student's t-test and ANOVA tests with post-hoc analysis, and was considered significant when the significance level of the test was $p < 0.05$.

3.2.9 Ethical considerations

All animal experiments were approved and carried out in strict accordance with the recommendations by the regional ethics committees. Animal experiments were held both at the animal facilities at the University of Las Palmas of Gran Canarias, Canary Islands, Spain, and at the Department of Microbiology, Tumor and Cell Biology at Karolinska Institutet, Sweden.

All cell lines obtained from American Type Culture Collection, and the brain tissue microarray, were provided from the company with the respective ethical statements. Human samples used in the LAM study were obtained from LAM patients who had given written informed consent according to the Declaration of Helsinki, and the study was approved by the Institutional Review Board of Milan's San Paolo Hospital.

4 RESULTS AND DISCUSSION

4.1 PAPER I

SOCS2 deletion protects against multiple low-dose streptozotocin-induced type 1 diabetes in adult male mice

In vitro and *in vivo* studies had showed that SOCS family proteins are relevant to the pathogenesis of DM. SOCS2 has been linked to metabolism and diabetes [215], although no clear metabolic phenotype has been described to date in SOCS2$^{-/-}$ mice. SOCS2 expression has been demonstrated in human pancreatic islets [241]. This prompted us to investigate more closely the metabolic phenotype of SOCS2$^{-/-}$ mice to investigate how SOCS2 ablation may influence the development of diabetes in a mouse model of autoimmune diabetes and β-cell destruction [242]. Our results demonstrate that, compared to controls, 6 month-old MLDSTZ-induced diabetic SOCS2$^{-/-}$ mice are less prone to develop diabetes (Figure 15).

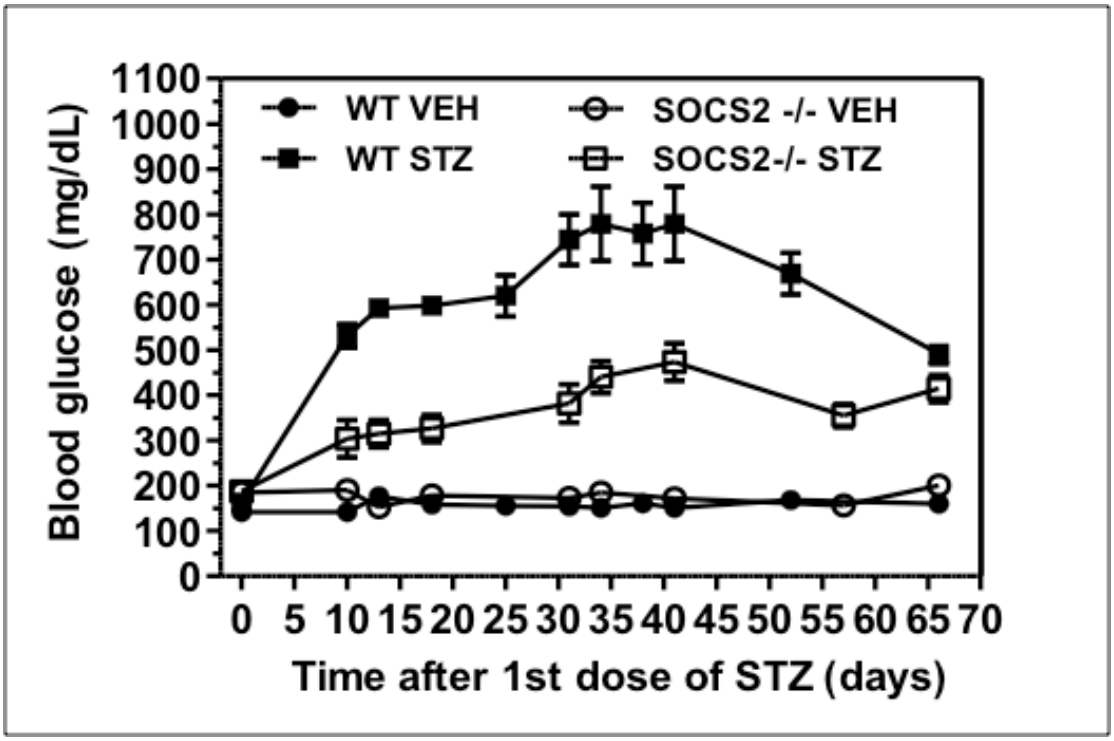

Figure 15. MLDSTZ induces diabetes in 6-month-old SOCS2$^{+/+}$ (WT) and SOCS2$^{-/-}$ male mice. *Blood glucose was recorded every 3-4 days, starting on day 9 after the first STZ injection.*

In MLDSTZ-treated WT mice, there was a clear reduction in the levels of serum insulin which was not observed in the SOCS2$^{-/-}$ mouse group (Figure 16A). In MLDSTZ-treated WT mice, there was an 88% reduction in pancreatic insulin content, whereas the corresponding change in pancreatic tissue from MLDSTZ-treated SOCS2$^{-/-}$ mice only was 20% (Figure 16B). Along with the analysis of pancreatic insulin content, analyses of pancreatic tissue obtained post-treatment showed significantly increased staining for insulin in MLDSTZ-treated SOCS2$^{-/-}$ mice, compared to control mice (Figure 16C). Therefore, 6 month-old SOCS2$^{-/-}$ mice appear partly to be protected from β-cell damage inflicted by MLDSTZ treatment. In line with the above findings, more marked glucose intolerance was seen in MLDSTZ-treated control mice compared to SOCS2$^{-/-}$ mice, confirming the greater severity of diabetes in the former group.

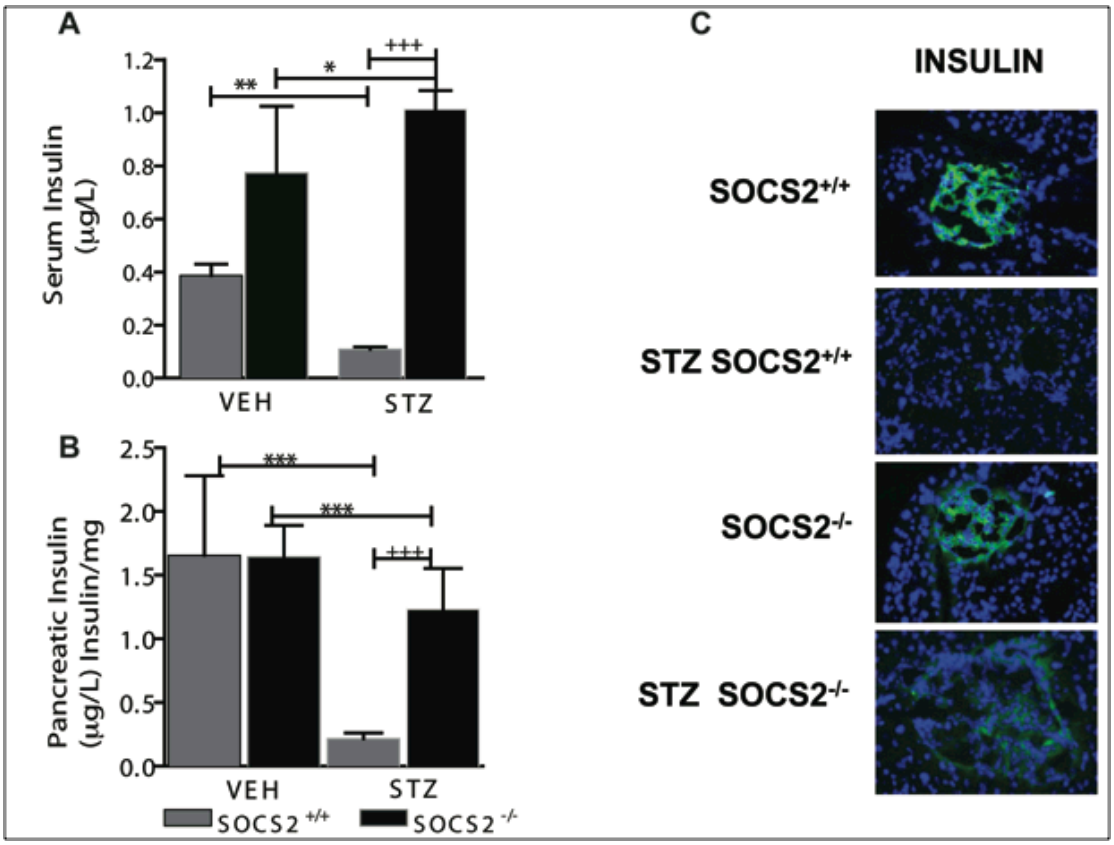

Figure 16. SOCS2$^{-/-}$ mice are partly protected from MLDSTZ-induced damage in β-cells. *(A) Insulin was measured after overnight fasting. (B) Insulin content per 100 mg of pancreas tissue. (C) Insulin fluorescence IHC in the pancreas showed that SOCS2$^{-/-}$ mice are protected against MLDSTZ-induced damage in β-cells. Green corresponds to insulin staining, blue corresponds to DAPI staining.*

One possible explanation for this observation might be due to the fact that SOCS2$^{-/-}$ mice are highly sensitive to GH and PRL. SOCS2 deficiency leads to reduced receptor degradation, and subsequently increased expression of GHR and PRLR [243]. The results from the immunostaining of pancreatic sections for GHR and PRLR obtained from both experimental groups support the proposed hypothesis that GHR and PRLR protein content are augmented in the mouse SOCS2$^{-/-}$ pancreas, compared to control mice (Figure 17).

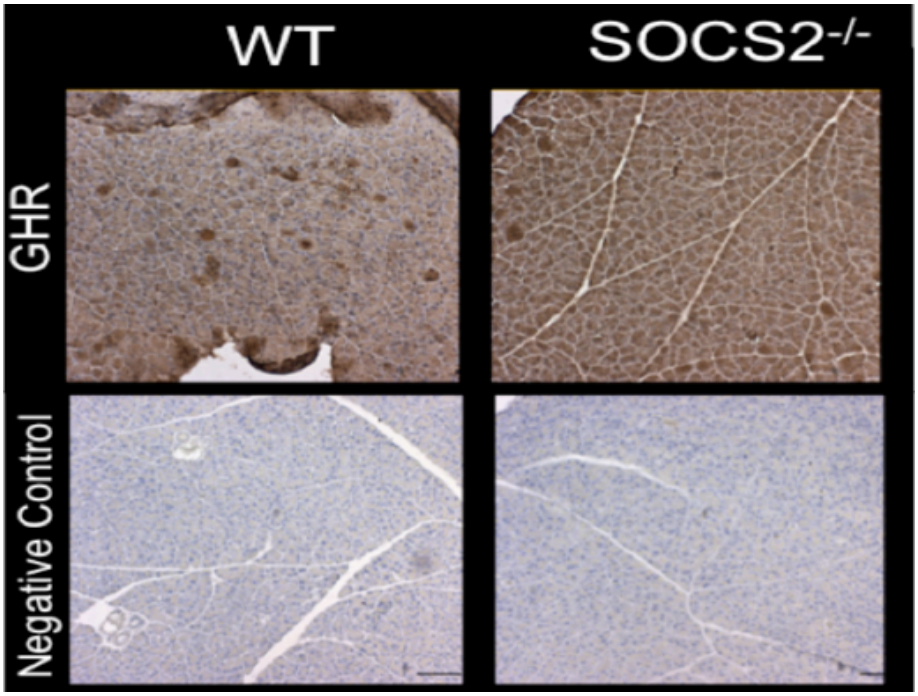

Figure 17. Immunohistochemical analyses of GHR in the pancreata from WT and SOCS2$^{-/-}$ mice.

Higher pancreatic GHR and PRLR content, in absence of SOCS2, leads to enhanced GH/PRL-induced JAK2/STAT5 signaling. Our study showed that both GH and PRL increased STAT5 protein expression to a greater extent in the pancreatic tissue of SOCS2$^{-/-}$ mice, compared to controls. We also analyzed STAT5 protein activation in isolated islets from control mice after treatment with ovine PRL or bovine GH, and both cytokines induced STAT5 activation in isolated islets. Other signaling pathways can also be affected by SOCS2 deficiency. However, the gigantism that has been observed in SOCS2$^{-/-}$ mice is not observed in other mouse models of SOCS inactivation, and therefore SOCS2 appears to be a key negative regulator of GHR signaling *in vivo*. In agreement with this, overgrowth in SOCS2$^{-/-}$ mice was attenuated in double SOCS2/STAT5b knockout mice, and in SOCS2$^{-/-}$ mice crossed with

GHRH signaling-deficient mice [244,245]. This further confirms that increased growth observed in SOCS2$^{-/-}$ mice is due to increased sensitivity to GH.

In line with our results, previous reports have shown that pancreatic β-cell growth, survival and insulin production are stimulated by GH through GHR/JAK2/STAT5b activation [246–248]. Furthermore, in our study, an increase in pancreatic islet size in SOCS2$^{-/-}$ mice was demonstrated vs. control mice. A physiological increase in β-cell mass is seen in pregnancy [249,250], and this is likely caused by the elevation of placental and pituitary hormones targeting GHR and PRLR [211]. Subsequently, SOCS2$^{-/-}$ mice, which possess a higher sensitivity to GH and PRL, may display similarities to the pregnant state in terms of β-cell function. Thus, our findings of augmented pancreatic GHR/PRLR levels in SOCS2$^{-/-}$ may explain the increase in β-cell size and insulin production observed in pancreata from the SOCS2$^{-/-}$ mice, in comparison with control mice.

Another possible explanation for the higher insulin levels observed in SOCS2$^{-/-}$ mice is that SOCS2$^{-/-}$ pancreatic islets are more resistant to the damage induced by MLDSTZ. It has been shown that PRL injections reduce the severity of STZ in mice [251], whilst other studies have demonstrated the ability of activated STAT5 to protect β-cells from various insults, including STZ and lipotoxins [252]. Our finding of increased STAT5 activity in SOCS2$^{-/-}$ pancreatic islets suggests that GH-, or PRL-mediated activation of STAT5 may protect pancreatic islets from the negative effects of MLDSZT-induced diabetes. Our findings are also in line with several other reports demonstrating the negative role for SOCS2 in insulin secretion, e.g. constitutive activation of SOCS2 in mouse or rat islets, or adenoviral-mediated overexpression of SOCS2 in MIN6 cells, all of which lead to decreased glucose-stimulated insulin secretion [253]. GH and IGF-I treatment augments β-cell growth and anti-apoptosis, both of which could be the ultimate manifestations of a synergistic activation of STAT5 and AKT in β-cells [254]. These findings support our hypothesis that increased GH and PRL sensitivity in SOCS2$^{-/-}$ mice is likely to explain the reduced damage caused by MLDSTZ.

GH excess can also affect insulin sensitivity, and may therefore be associated with the development of T2D. However, SOCS2 deletion generates a phenotype of increased GH sensitivity that is not identical to that due to GH overproduction [255]. Recently, we studied hepatic insulin resistance in SOCS2$^{-/-}$ mice and found that a normal control diet did not provoke glucose intolerance or insulin resistance in SOCS2$^{-/-}$ mice, whereas HFD caused a severe form of insulin resistance [256]. This suggests that the counteracting insulin actions of

GH are exacerbated by HFD-related mechanisms (e.g. insulin resistance) under SOCS2 control.

In this thesis, along with other regulators of GH and PRL sensitivity, we found two factors that might be involved in the regulation of β-cell mass, i.e. SOCS2 and TSC2. A critical role for the TSC2/mTOR pathway in regulating β-cells mass has been well reported, which further supports our hypothesis. Conditional deletion of TSC2 in pancreatic β-cells induced expansion of the β-cell mass, an action reversed by the mTOR inhibitor rapamycin [257]. We therefore hypothesized that TSC2 deletion increases tissue sensitivity to PRL, a hormone that regulates β-cell mass. This might open new doors in terms of the development of new approaches to expand the β-cell mass without a risk for oncogenic transformation induced by direct hormonal therapy.

To substantiate the current findings in this study, further studies are needed to elucidate the precise role of SOCS2 in the regulation of β-cell mass, e.g. by measurements of β-cell mass after $SOCS2^{-/-}$ restricted to the islets. In summary, $SOCS2^{-/-}$ mice are more sensitive to GH/PRL and exhibit higher pancreatic GHR and PRLR levels, compared to control mice. GH/PRL stimulates pancreatic β-cell growth, survival and insulin production through the GHR/PRLR-JAK2-STAT5 signaling pathway.

4.2 PAPER II

Role of PRLR in Lymphangioliomyomatosis

LAM disease affects 30–40% of women with TSC disease, and TSC2 is a known negative regulator of mTOR. Loss of TSC1/TSC2 function in LAM leads to hyperactivation of mTORC1 and inhibition of autophagy, and such effects can be observed in TSC2-deficient LAM cells [258]. In this study, we analyzed the relationship between TSC2 and the PRL system in CRL2620 cells in which TSC2 levels were reduced using siRNA; in addition, we studied cell lines derived from LAM patients. A strong immunofluorescence signal for PRLR was observed at intracellular locations including the nucleus in both mouse-, and human derived cells (see Fig 2 and 3; Paper II).

Activation of mTOR caused by TSC1/2 deficiency is one of several changes that occur in signaling pathways. Based on our finding that PRLR was increased in LAM/TSC cells, we

tested the short-term effects of PRL on intracellular protein phosphorylation by exposing human LAM/TSC cells to PRL. The most well studied PRL signaling pathway is the JAK2-STAT pathway. We could not detect any STAT5 activation in these cells, whereas STAT3 and ERK were activated by PRL (Figure 18).

The STAT3 regulation by PRL observed in this study confirms the observations in previous studies, and the activation of this transcription factor seems to be of particular relevance for LAM cell growth [259]. STAT3 is known to participate in many normal cellular actions including differentiation, proliferation, and cell survival after stimulation by cytokines, growth factors, and hormones [260]. Our findings show that PRLRA reduces STAT3 and ERK activation even without PRL stimulation and this finding that only blockage of PRLR reduces STAT3 and ERK activation needs to be further investigated. One possibility is, however, that LAM cells might produce PRL themselves [163]. In lesions from LAM patients, both PRL and PRLR mRNA were found to be higher, compared to the surrounding vascular tissues and similar findings have been reported for other tumor tissues such as cancers of the breast and colon [261,262]. Our findings, along with similar previously reported results from Eker rat $TSC2^{-/-}$ cells, are in line with the hypothesis that PRL signaling has an important role in LAM. JAK2-STAT3 signaling pathway can be activated by many other cytokines. However, PRL could have a critical function in LAM cells given the results using the PRLRA. Importantly, we could not find any activation of STAT5, a finding that supports the concept that STAT3 activation is of key relevance for the dysregulated cell growth observed in LAM cells.

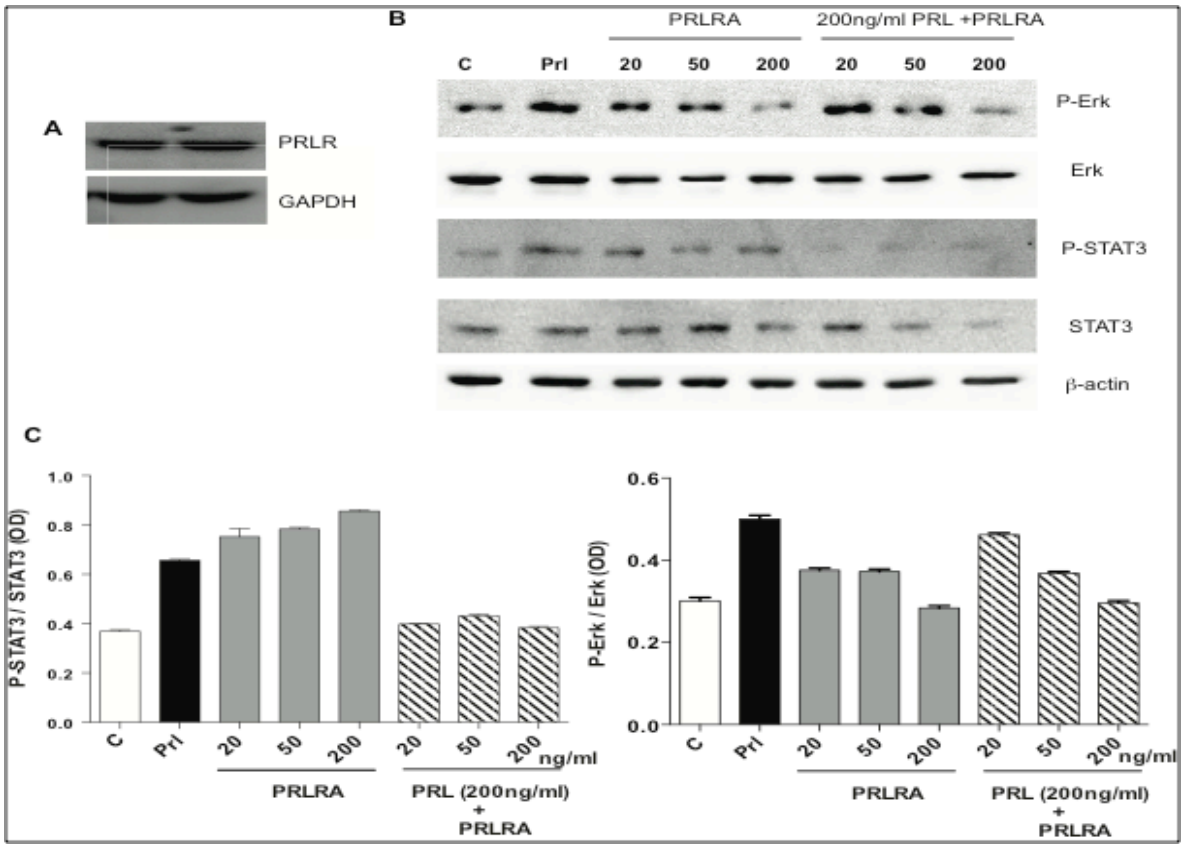

Figure 18. Human LAM cells express PRLR, and prolactin stimulates phosphorylation of STAT3 and ERK*. LAM/TSC cells were exposed to different doses of PRLRA (20, 50, 200 ng/ml) for 15 minutes, and subsequently the cells were exposed to 200 ng/ml PRL for 60 minutes, or to PBS as a control. Protein extracts were prepared for western blot analysis and probed with antibodies for P-STAT3, STAT3, P-ERK and ERK. (A) Western blots to analyze PRLR in LAM/TSC control cells. (B) Western blot, using antibodies directed against P-STAT3, STAT3, P-ERK and ERK in LAM/TSC cells. (C) Densitometric quantification of western blot signals, in which the Y-axis depicts the ratio between phosphorylated STAT3 and ERK to the total levels of each respective protein.*

STAT3 is a downstream mediator of Src. Src family kinases are tyrosine kinases which are key regulators of cellular proliferation, survival, motility and invasiveness. Reports have shown that accumulation of active Src kinase might lead to progression and metastasis of LAM cells, as for many other cancer cells [263]. It was recently shown that Src kinase activation promotes the migration and invasion of $TSC2^{-/-}$ cells, secondary to the upregulation of Snail transcription factor, which in turn suppresses E-cadherin expression [264]. Furthermore, we hypothesize that PRL may promote tumor growth in an autocrine/paracrine

manner in LAM lesions. Our study demonstrates the stimulatory dose-response effect of PRL on LAM/TSC cell proliferation *in vitro*, an effect blocked by PRLRA (Figure 19). In other tumor cells, antagonism of tumoral PRLR promotes autophagy-related cell death [164], supporting our findings, and suggesting that PRLRA might be a treatment option in LAM disease.

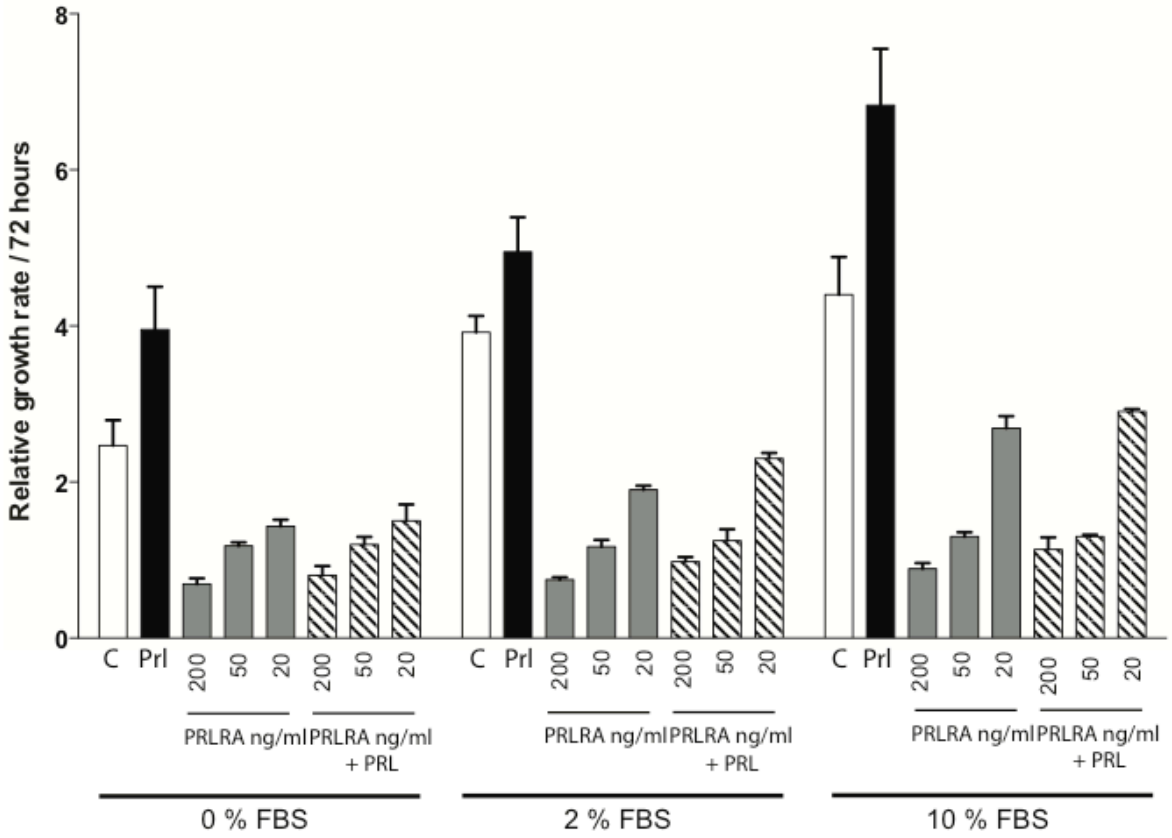

Figure 19. Addition of exogenous PRL stimulates cell proliferation in a manner that can be blocked by PRLRA. *LAM/TSC cells were cultured in different concentrations of serum (0%, 2%, 10%). Human PRL was added to sub-confluent cells at a concentration of 200 ng/ml, with or without PRLRA (20, 50, 200 ng/ml). After 72 hours, cells were subject to the crystal violet assay. The X-axis depicts serum concentration and the Y-axis shows relative cell proliferation, as determined by absorbance at 600 nm. Each data point represents a triplicate assay. All values are +/- standard deviation, P-value <0.05.*

Since the mTOR pathway is overactive in LAM disease, attempts have been made to treat LAM patients with rapamycin, a treatment recently approved by the US Food and Drug Administration (FDA). Treatment with rapamycin analogue improves lung function and reduces lesion size [180]. In concordance with these results, tumour re-growth and decline of lung function were associated with cessation of the same treatment [180]. An endocrine hormonal involvement (other than PRL) in LAM disease has been reported in several studies. LAM cells express both estrogen and progesterone receptors, and these cells seem to be estrogen responsive [265]. Estrogen is known to stimulate PRL secretion, and to stimulate ERK phosphorylation in $TSC2^{-/-}$ LAM cells. Due to this, estrogen antagonist treatment has been suggested and showed some efficacy in LAM; however this treatment was stopped due to the presence of side effects and the emergence of mTOR inhibitors [266]. Our study showed that PRL activates the MAPK pathway and phosphorylates ERK, and that this can be blocked with PRLRA. Taken together, it is tempting to hypothesize that PRLRA could provide an alternative to rapamycin, or could be used in combination for the treatment of LAM disease. Future studies are needed to explore this concept.

In conclusion, we observed that reduction of TSC2 in LAM cells increases the PRLR levels, and that blocking this receptor by PRLRA may offer a new therapeutic angle for the treatment of LAM disease.

4.3 PAPER III

Stimulation of PRLR induces STAT-5 phosphorylation and cellular invasion in GBM

A number of different alterations of cell signaling components have been found in GBM, ranging from receptor signaling to the loss of tumor suppressors. Relatively few studies on GBM have concerned the involvement of PRL. Moreover, previous studies have reported increased STAT and mTOR activity in GBM [189–193]. In this study, we investigated components of PRL system in cell models of GBM, and in histological tissue sections obtained from GBM patients.

We found that PRLR is expressed at high levels in U251-MG cells. Immunofluorescence signals of PRLR expression were detected mostly in the perinuclear area. The phenomenon of perinuclear detection of PRLR has previously been seen in other cell types such as rat hepatocytes. A tissue microarray consisting of tissue samples from 32 GBM patients was

analyzed by IHC to detect the expression of PRLR. GBM lesions were found to express PRLR at different levels (negative, moderate, high-grade). Out of 32 sections, corresponding to 32 GBM cases, 66% were positive for PRLR. Expression of PRLR was not detected in the tissues of 34% (11 out of 32) of GBM patients, but it was detected at high-grade in GBM tumors of 50% (16 out of 32) of patients, and at moderate levels only in 16% (5 out of 32) of patients with the disease (Figure 20).

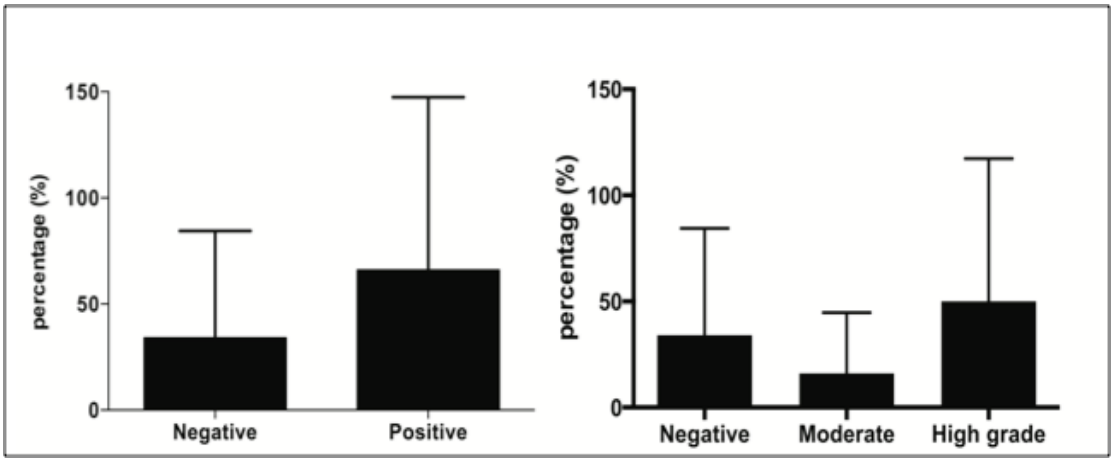

Figure 20. Percentages of PRLR-negative and PRLR-positive cells in 32 GBM patients. *Percentages of PRLR-positive cells were also shown in GBM patients with moderate and high-grade tumor. Percentages are shown with 95% confidence intervals*.

In line with our finding of PRLR expression in models of GBM, it is interesting to note that the choroid plexus is a rich source of PRLRs [267], and may have a role in the transport of PRL into the CNS. In addition, PRLR mRNA expression in human brain tissues has been reported in many gene expression databases. In recent yet unpublished work, we can corroborate such findings. In the present study, we found expression of PRLR in GBM cells and in brain tumor tissues. Taken together, evidences are accumulating that both PRL and its receptor are present in the brain, and we speculate that high-grade expression of PRLR could be related to the presence of GBM tumours.

We investigated the short-term effects of PRL on intracellular signaling in U251-MG cells, and found that PRL increased STAT5 phosphorylation after 10 min, with a peak response seen after 20 min. However, STAT5 phosphorylation remained elevated for the whole duration of the experiment, i.e. 60 min (Figure 21A). The capacity of the antagonist to block PRL-induced STAT5 phosphorylation was subsequently studied by exposing cells to different concentrations of the PRLRA, followed by the addition of 100 ng/ml PRL. As

shown in (Figure 21C), pre-incubation with 100 ng/ml PRLRA (a concentration equal to that of PRL) dramatically blocked STAT5 phosphorylation, and this was also the case when the concentration of the antagonist was reduced 10-fold. The regulation of STAT5 by PRL in U251-MG cells observed in this study confirms previous studies which showed that PRLR regulates STAT5 tyrosine phosphorylation in different cells [268,269], and so activation of this transcription factor seems of particular relevance for GBM cells. Previous studies have also shown that phosphorylated STAT5 mediates the oncogenic effects of EGFR [270] in different tumor tissues, including GBM [271,272].

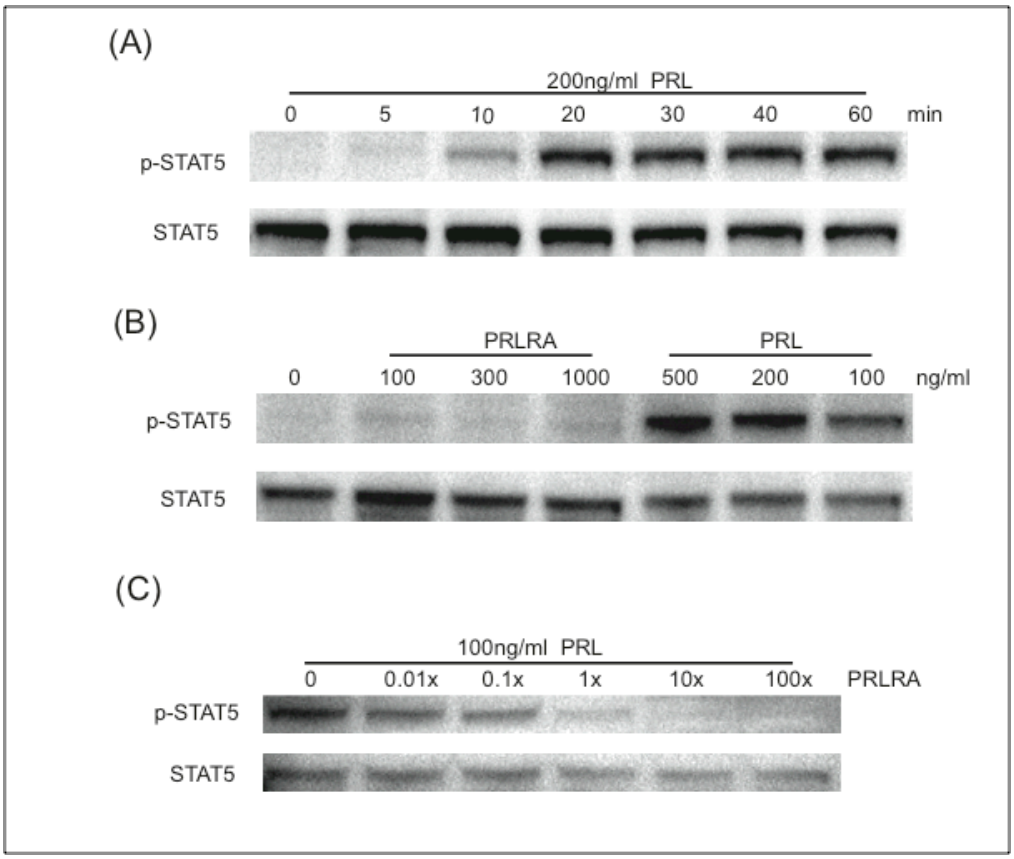

Figure 21. Effects of PRL and a PRLRA on STAT5 phosphorylation in U251-MG cells*. Following protein extraction and gel electrophoresis, phospho-STAT5 (p-STAT5) and total STAT5 (STAT5) was analyzed using Western blotting. (A) U251-MG cells treated with PRL only (B) U251-MG cells were exposed to different concentrations of PRL (100, 200, 500 ng/ml) for 20 minutes. To rule out any agonist activity of the PRLRA, cells were exposed to different concentrations of PRLRA (100, 300, 1000 ng/ml) for 15 min. (C) U251-MG cells were exposed to different doses of PRLRA for 5 min, and cells were then exposed to 100ng/ml PRL for 15 min.*

GBM cells have been reported to produce PRL locally [199], and this would be in congruence with the finding of extra-pituitary PRL production in primates. In the literature, one study reported detection of PRL mRNA in human central nervous tissues [273], whereas another study showed PRL expression in GBM which was detectable by IHC, but not with real-time PCR [274]. It is possible that GBM represents a state of either increased PRL production, or increased PRL sensitivity. For example, in study II, we showed that loss of TSC2 function leads to increased PRL sensitivity in LAM cells. The mTOR system is regulated by TSC, and seems to be over-active in GBM [192,275,276], and there are several clinical cases which report the occurrence of GBM in TSC patients [196]. However, mTOR inhibitors have a limited efficacy on human GBM, which could partly be explained by the limited ability of rapamycin to cross the blood brain barrier in humans [185]. Possibly there is a need for combined targeted treatments, e.g. using both mTOR inhibitors and EGFR inhibitors to treat recurrent GBM. There are studies showing that combining rapamycin with an EGFR inhibitor improves clinical outcome in a small number of patients with recurrent GBM [277–279].

In this thesis, we found that two regulators of GH/PRL sensitivity, SOCS2 and TSC2. In the context of GBM, as mentioned before that SOCS1 and SOCS3 are abnormally expressed in different GBM cell lines, and this leads to augmented cell signaling through the ERK-MAPK pathway [194]. To this date, no SOCS2-related defect has been reported in GBM, however; low levels of SOCS2 have been involved in oncogenesis and has been identified as a biomarker in human colorectal cancer [280]. Low SOCS2 gene expression has been reported in other tumors of the breast, ovary and prostate. Since SOCS2 has been associated with GH/PRL signaling, and GHR/PRLR turnover; we speculate that SOCS2 involvement in GBM should be understood in terms of increased PRL sensitivity in GBM tissue in relation to the involvement of SOCS2/TSC2.

It is further interesting to note that anti-angiogenic factors induce local production of PRL as a pro-survival response. As mentioned before, the combined application of the angiogenic inhibitors endostatin and tumastatin lead to the upregulation of PRLR in GBM *in vivo*, which in turn mediates tumor cell proliferation [199]. Another study has shown that PRL induces a dose-dependent increase in proliferation and survival of human U87-MG GBM cells [281]. Taken together, it is possible that GBM lesions can respond to agents that disrupt PRL

signals. Theoretically, a combination therapy of angiogenic inhibitors that up-regulate PRLR in GBM with a PRLRA could lead to therapeutic effects in GBM patients. The availability of a novel high-affinity PRLRA, employed in our study, that additionally lacks any residual agonistic properties, makes such studies feasible and of great interest.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis had the aim to increase our understanding of factors that control GH and PRL receptor sensitivity. The main conclusions are summarized below:

1. Deletion of SOCS2 partly protects against MLDSTZ-induced diabetes in mice.

2. SOCS2$^{-/-}$ mice are more sensitive to GH/PRL, and exhibit higher pancreatic expression levels of GHR and PRLR, compared to WT mice.

3. GH/PRL stimulates pancreatic insulin production through the JAK2-STAT5 signaling pathway and reduces palmitate induced lipo-toxicity in β-cells.

4. Reduction of TSC2 in LAM cells increases PRLR levels. Blocking this receptor may offer a new therapeutic approach for LAM treatments.

5. PRL is a potent mitogenic hormone that plays a critical role in LAM cell growth and invasion.

6. GBM cells respond to PRL by STAT5 activation, and expression of PRLR can be detected in histological sections from GBM patients.

The SOCS2-dependent increase of GH and PRL sensitivity in diabetes or other conditions raises questions on the importance of altered GH/PRL sensitivity, in addition to conditions related to over-, or under-production of these hormones. It may have a future potential to target GH/PRL sensitivity in cases where conventional hormone treatment fails because of reduced sensitivity. There are still many gaps in our knowledge that need to be filled, and this may in particular be the case for PRL, for which new data indicate that the hormone has a different profile of activity in humans, compared to animals. Future investigations, based on the findings in this thesis, are suggested by the following:

1. Further studies are needed to understand the role of SOCS2 in diabetes. The activity of GH and PRL, as well as of other cytokines, need to be examined. It is important to gain new knowledge about SOCS2 by creating tissue-specific $SOCS2^{-/-}$ mice.

2. The effects of $SOCS2^{-/-}$ on β-cell viability needs to be studied in islet transplantation models.

3. To understand more fully the molecular actions of SOCS2 and TSC2 on cytokine senstivity.

4. Cell culture studies have limitations, and studies of homone activity in more complex *in vivo* systems are needed.

5. The PRLRA is a new tool that will enable deeper investigations of PRL function in cells and animals. It is possible that the use of this antagonist can eventually lead to novel treatments in human PRL-dependent hyper-proliferative conditions.

6 GENERAL SUMMARY

Hormones mediate an important part of the crosstalk between different organs in the body. Hormones are secreted from specialized glands in the body and travel through the blood to reach their target cells to elicit the required responses through binding to respective receptors. Growth factors have a similar mode of action, but normally act in proximity to their site of production. In this study, we have focused on two hormones, GH and PRL. Both GH and PRL are secreted from the pituitary gland, but it is becoming increasingly clear that extra-pituitary PRL production occurs in many tissues. GH and PRL have distinct biological actions: GH regulates longitudinal growth, regeneration and ageing, whereas PRL is the main lactogenic hormone that also exerts actions on the gonadal axis. These hormones share structural similarities, and exert their action using similar signaling systems.

Given the overall general significance of hormone sensitivity, this thesis is primarily focused on the regulation of sensitivity for GH and PRL in different disease models. Altered sensitivity to hormones might be the cause and the mechanistic basis of disease. The sensitivity of a cell to GH and PRL is linked to the expression levels of GHR and PRLR in that cell. GH binds to both GH and PRL receptors, whereas PRL only binds to the PRLR. Binding initiates a signaling cascade inside the cell, which leads to the expression of hormone responsive genes. Regulation of GH and PRL sensitivity by two different genes, SOCS2 and TSC2, has been studied in this thesis. Alteration or deletion of these two genes leads to the reduced degradation of the GHR and PRLR, and thereby an increase in tissue sensitivity to these hormones.

In study I, the role of SOCS2 in diabetes was investigated. Mice in which the SOCS2 gene was deleted ($SOCS2^{-/-}$ mice) were injected with streptozotocin (a chemical that destroys the β-cells in the pancreas) in order to induce diabetes. We found that, compared to normal mice, $SOCS2^{-/-}$ mice were more resistant to streptozotocin. Subsequently, low levels of SOCS2 allow for a better survival of β-cells. It is tempting to speculate that increased GH/PRL sensitivity is an important factor to explain this observation.

In studies II and III, we studied the role of PRL in two different tumors, LAM and GBM. LAM is a benign destructive tumor affecting smooth muscle cells in fertile females, while GBM is an aggressive brain tumor. LAM and GBM tumor cells were found to be PRL-sensitive and to express PRLR, and activation of PRLR induced the JAK-STAT signaling

pathway. In addition, cell invasion of these tumor cells was stimulated by PRL. IHC was used to demonstrate the presence of PRLR in LAM and GBM. A reagent (PRLRA) that blocks the PRLR was produced and this compound reduced PRL's effects in cells.

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