Generation and characterization of a pig model for Laron syndrome

von Arne Jens Hinrichs

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

Generation and characterization of a pig model for Laron syndrome

von

Arne Jens Hinrichs

aus Berlin

München 2018

Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Lehrstuhl für Molekulare Tierzucht und Biotechnologie

Arbeit angefertigt unter der Leitung von Univ.-Prof. Dr. Eckhard Wolf

Mitbetreuung durch: Dr. Barbara Kessler

Gedruckt mit Genehmigung der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.
Berichterstatter:	Univ.-Prof. Dr. Eckhard Wolf
Korreferent/en:	Priv.-Doz. Dr. Sonja Härtle Priv.-Doz. Dr. Karin Weber Univ.-Prof. Dr. Kaspar Matiasek Univ.-Prof. Dr. Heidrun Potschka

Tag der Promotion: 27.07.2018

Für meine Familie

During the preparation of this thesis the following paper has been published:

Arne Hinrichs, Barbara Kessler, Mayuko Kurome, Andreas Blutke, Elisabeth Kemter, Maren Bernau, Armin M. Scholz, Birgit Rathkolb, Simone Renner, Sebastian Bultmann, Heinrich Leonhardt, Martin Hrabě de Angelis, Hiroshi Nagashima, Andreas Hoeflich, Werner F. Blum, Martin Bidlingmaier, Rüdiger Wanke, Maik Dahlhoff, Eckhard Wolf (2018). "Growth hormone receptor-deficient pigs resemble the pathophysiology of human Laron syndrome and reveal altered activation of signaling cascades in the liver" Molecular Metabolism (accepted March 09, 2018).

DOI: 10.1016/j.molmet.2018.03.006

Parts of this study were presented at the following conferences:

DACH - Gemeinschaftstagung der deutsch, österreichisch und schweizer Endokrinologischen Gesellschaften; Munich, May 26-28, 2016

Februartagung des ReproZentrums, LMU Munich, February 15-17 2017; Abstract "Impaired weight gain and disproportionate organ growth, but preserved fertility in growth hormone receptor deficient pigs (Laron syndrome)" published in *REPRODUCTION IN DOMESTIC ANIMALS*. Vol. 52.

Gordon Research Conference *IGF & Insulin System in Physiology & Disease*, Ventura, California, March 12-17 2017;

Gene Center Retreat, LMU Munich, Abtei Frauenwörth, Chiemsee, June 26-28 2017

Final meeting COST Action BM1308 "Sharing Advances on Large Animal Models (SALAAM)", Leopoldina Nationale Akademie der Wissenschaften, Halle a.d. Saale, September 28-29 2017

TABLE OF CONTENTS

I. INTRODUCTION

Laron Syndrome (LS) is a very rare autosomal recessive hereditary disorder – just a few hundred people are affected worldwide (Rosenfeld, Rosenbloom et al. 1994). LS is caused by mutations of the growth hormone receptor gene (*GHR*) diminishing the binding or signal transduction of growth hormone (GH) action (Eshet, Laron et al. 1984). Therefore, LS patients exhibit low levels of circulating insulin-like growth factor 1 (IGF1) as its production is stimulated by GH action (reviewed in Laron 2004). In contrast, levels of circulating GH are elevated, as the negative feedback loop by IGF1 is diminished (Zhou, Xu et al. 1997). The phenotypic consequences of growth hormone receptor deficiency (GHRD) involve dwarfism, obesity and disturbances in glucose homeostasis (reviewed in List, Sackmann-Sala et al. 2011). The investigation of a very isolated cohort of LS patients in Ecuador (Guevara-Aguirre, Rosenbloom et al. 1993), draw special interest towards this syndrome as affected patients seemed to be protected against cancer and diabetes and effects associated with longevity were observed (Guevara-Aguirre, Balasubramanian et al. 2011). The Laron mouse, generated by targeted disruption of the *Ghr* gene (Zhou, Xu et al. 1997), resembles hallmarks of the phenotype of human LS and helped to develop therapeutic strategies and to investigate the function of the GH–IGF1 interaction. An interesting observation was the increased life expectancy, for which the Laron mouse model received the *Methuselah Mouse Prize* for life span extension (Bartke and Brown-Borg 2004). Nevertheless, the Laron mouse does not resemble all important aspects of the human phenotype of Laron syndrome due to taxonomic differences including the maturation of the endocrine growth axis, bone growth and transient changes in glucose homeostasis. As the pig has become a widely used animal model and provides the opportunity to close the evolutionary gap between mice and humans, we decided to generate a genetically tailored pig model for LS using the CRISPR/Cas technology.

The aim of this thesis was to characterize the phenotype of the *GHR*-knockout (KO) pig regarding growth impairment, endocrine alterations and effects on metabolism. Driven by the idea, that pro-aging effects resulting from GHRD might be associated with an increased insulin sensitivity and the absence of malignant factors, we investigated the hepatic signaling of the insulin receptor and GHR associated signaling pathways including mTOR.

II. REVIEW OF THE LITERATURE

1. The endocrine growth axis

Dwarfism is a main characteristic of the LS. Dwarfism can result from multiple causes such as skeletal disorders, chromosome alterations, metabolic and disorders affecting the endocrine growth axis (Rimoin 1976). In brief, the endocrine growth axis includes GH which is secreted by the pituitary gland under control of several feedback mechanisms involving circulating factors and the hypothalamus. GH promotes growth mostly via the stimulation of IGF1 production, but also by independent actions. The growth hormone receptor is required to maintain the actions of GH.

1.1. Growth hormone

Somatotrophic cells in the *pars distalis* of the pituitary gland release GH into the circulation. The secretion of GH is under the control of complex feedback mechanisms and occurs in a pulsatile manner, with a circadian rhythm involved leading to a nocturnal maximum of GH which was observed in human studies (Honda, Takahashi et al. 1969). The secretion pattern of GH is affected by both age and sex. GH promotes growth either directly or via the induced production of IGF1 by the liver (Kaplan and Cohen 2007), what is called the GH/IGF axis. The levels of circulating IGF1 and GH itself influence the GH – synonymous somatotropin - secretion on hypothalamic level. The hypothalamus controls GH secretion by growth hormone releasing hormone (GHRH; synonymous somatoliberin), which stimulates GH secretion by the pituitary gland, and somatostatin (synonymous growth hormone inhibiting hormone (GHIH)), which inhibits the secretion of GH. The interplay of those noncompetitive antagonists results in the pulsatility of GH secretion (Varela-Nieto and Chowen 2005).

Due to inflammation, trauma, neoplasia or idiopathic insults, hypopituitarism can be developed, which is one of the most important endocrine disorders leading to dwarfism. Facing hypopituitarism, GH secretion and its levels in circulation are diminished (Schneider, Aimaretti et al. 2007). In contrast, GHRD results in increased GH levels (reviewed in Laron and Kopchick 2011).

1.2. Insulin-like growth factors

The insulin like growth factors 1 and 2 are potent factors stimulating growth. They share 50% homology with insulin and are able to stimulate glucose uptake in muscle and fat cells (reviewed in Varela-Nieto and Chowen 2005). IGF1 is a potent growth factor throughout postnatal development, whereas IGF2 mostly stimulates intrauterine growth (Baker, Liu et al. 1993). In contrast to IGF2, the production of IGF1 by the liver depends on GH. In the circulation, IGFs are bound to six different high-affinity IGF binding proteins (IGFBPs). The IGFBPs bind IGF1 or IGF2 with different affinities, modulating the bioactivity of IGFs by providing a reservoir of IGF in circulation (IGFBP3), transporting IGFs from the circulation to peripheral tissues (IGFBP1; 2; 4) or inhibiting IGF-IGF receptor interactions (IGFBP2; 4; 6) (reviewed in Jones and Clemmons 1995, Rajaram, Baylink et al. 1997). Most of the IGF1 in the circulation is bound in a ternary complex with the acid-labile subunit (ALS) to IGFBP3. GH action is directly required for the production of both, IGF1 and ALS and indirectly for the production of IGFBP3 as its production is stimulated by IGF1 (reviewed in Cohick and Clemmons 1993). In LS, levels of IGF1, ALS and IGFBP3 are significantly reduced, indicating a disturbance in GH signaling through the GHR (Savage, Blum et al. 1993, Labarta, Gargosky et al. 1997).

1.3. The growth hormone receptor

The GHR is a 620-amino-acid transmembrane glycoprotein belonging to the family of class 1 cytokine receptors (reviewed in Waters 2015 and Brooks and Waters 2010). The human GHR is encoded by 9 exons, numbered 2 – 10, which are located on chromosome 5 (Godowski, Leung et al. 1989). Exon 2 provides a signal peptide and the first part of the extracellular domain, which is mostly encoded by exon 3 – 7. The transmembrane domain of the GHR is encoded by exon 8, whereas exon 9 and 10 encode the cytoplasmic domain. In humans and pigs, proteolytic cleavage of a part of the extracellular domain releases the growth hormone binding protein (GHBP) into the circulation, which prolongs the half-life of GH by protecting it from excretion and degradation (reviewed in Baumann, Shaw et al. 1994). Furthermore, the GHBP competes with the extracellular domain of the GHR in binding circulating GH, thereby modulating the activity of GH (reviewed in Amit, Youdim et al. 2000).

The GHR is present as a homodimer held together by strong interactions of the transmembrane helices, providing dimerization even at an inactive state (Gent, Van

Kerkhof et al. 2002). The dimerization of a GHR with a different cytokine receptor partner seems to be prevented by stereological hindrance of extracellular domains other than GHR (Lichanska and Waters 2008). The two distinct GHRs contain the same hormone-binding sites, but binding sites of GH are placed asymmetrically (Waters 2015). Binding of GH to the binding sites leads to positional readjustments of the extracellular domains involving rotation of the receptor molecules (Poger and Mark 2010). These conformational changes of the extracellular domain are transmitted via the transmembrane domain, switching to a crossover state, realigning catalytic subunits of the intracellular domain (Brooks and Waters 2010). Close to the cell membrane, the intracellular domain of the GHR contains the proline-rich box 1 motif – a common element of class 1 cytokine receptors – which binds the Janus kinase 2 (JAK2) (Tanner, Chen et al. 1995). The rotation of the GHR leads to a reorientation of the two box 1 motif-bound JAK2 molecules, increasing the distance between both, inducing the active state of JAK2 (Brooks, Abankwa et al. 2009). Basal JAK2 activity is inhibited by its own pseudokinase domain, holding the catalytic domain in an inactive state (Saharinen, Vihinen et al. 2003). Therefore, JAK2 activation requires the release of the tyrosine kinase from the pseudokinase domain. Waters *et al* hypothesized, that the pseudokinase domain of one inhibits the kinase domain of the other JAK2 (Waters 2015). The conformational changes, which increase the distance between the two box 1 motifs therefore remove the pseudokinase domain, bring the kinase domain into orientation and initiate the trans-activation of the JAK2 by each other (Brooks, Dai et al. 2014). Activated JAK2 proteins phosphorylate tyrosine residues of the cytoplasmic domain of the GHR forming binding sites for proteins containing phosphotyrosine binding domains (Herrington and Carter-Su 2001) such as the major substrate of GH action, the signal transducer and activator of transcription 5 (STAT5), which contains the src homology domain 2 (SH2) (Rawlings, Rosler et al. 2004). The SH2 domain of STAT5 interacts with the activated binding site of the cytoplasmic GHR domain, enabling the tyrosine phosphorylation of STAT5 by JAK2 (Ihle 1996). The SH2 domain also mediates the dimerization of two STAT5 proteins, what is hypothesized to trigger the dissociation from the complex with the GHR (Ihle 1996). Thereafter, the STAT5 dimer translocates to the nucleus and activates the transcription of target genes, such as *IGF1* (Herrington, Smit et al. 2000). An overview of the activation of the GHR is provided in **Figure 1**.

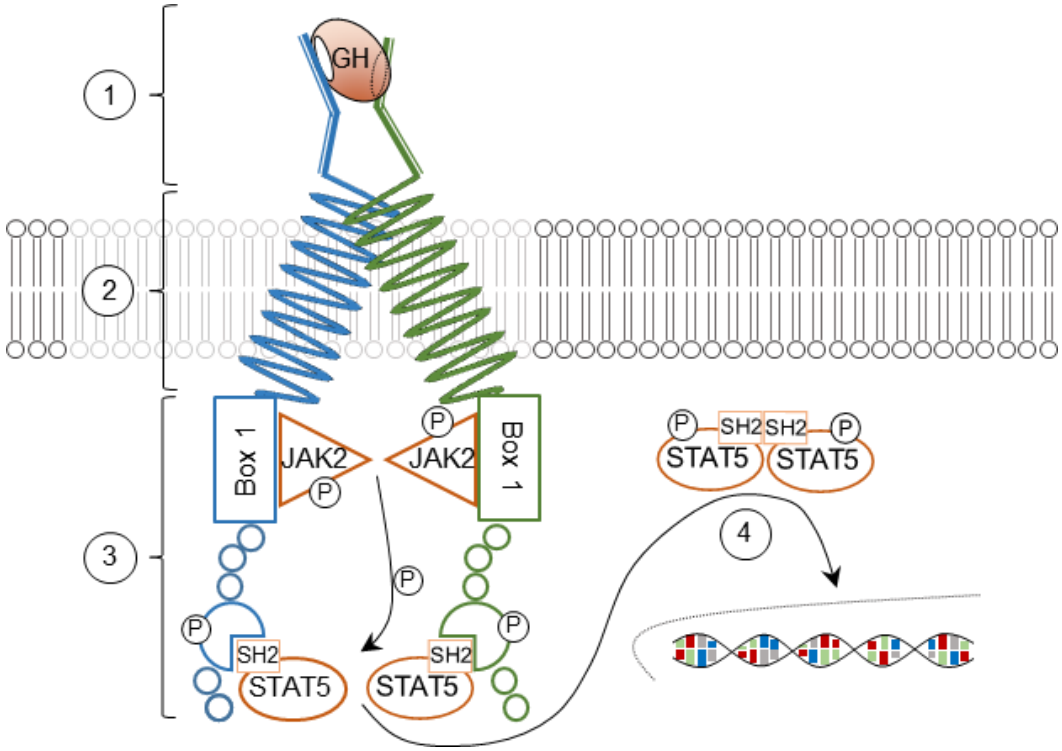


Figure 1. Activation of the growth hormone receptor. Binding sites on the GH are placed asymmetrically. Therefore, binding leads to rotation of the extracellular domain (1) of the GHR. These conformational changes are transmitted by the transmembrane domain (2) increasing the distance between the two box 1 motifs, allowing the catalytic activity of the JAK2 molecules at the intracellular domain (3). This leads to phosphorylation of tyrosine residues, forming a binding site for the phosphorylation of SH2-containing proteins as STAT5. The dimerization of two STAT5 molecules is provided by SH2 interaction, allowing the translocation to the nucleus and target gene transcription (4). (adapted from Brooks and Waters 2010).

2. Pathophysiology of the Laron syndrome

As mentioned, dwarfism in patients with LS cannot be due to reduced levels of GH as those are highly elevated. In fact, LS patients suffer from growth hormone receptor deficiency (GHRD), as liver samples derived from LS patients showed no binding capacity of human GH (Eshet, Laron et al. 1984). Under physiological conditions, GHR signaling - after activation by GH - induces the production of IGF1, IGFBP3 (Savage, Blum et al. 1993) and ALS (Labarta, Gargosky et al. 1997). IGF1 provides a negative feedback loop, decreasing the production of GH by the pituitary gland (reviewed in Brooks and Waters 2010). As GH signaling is diminished in LS patients, decreased levels of IGF1, IGFBP3, ALS and high levels of circulating GH are main characteristics of LS, which is commonly used synonymous to GHRD (Laron 2004).

GHRD is an autosomal recessive hereditary disorder. Most mutations leading to

GHRD appear in the region encoding the extracellular, hormone binding domain of the GHR (Rosenfeld, Rosenbloom et al. 1994). The first cohort of LS patients was described by Zvi Laron (Laron, Pertzelan et al. 1968) and is referred to as the *Israeli cohort*, as most affected patients derived from the Middle-East. Many patients from this cohort descent from consanguineous families with different mutations of the *GHR* gene (reviewed in Laron 2004). Just as the mutations, the phenotype of LS patients from the *Israeli cohort* shows a high diversity (reviewed in Laron and Kopchick 2011).

A more homogeneous cohort of LS patients was found in Ecuador by Guevara-Aguirre *et al* and is referred to as the *Ecuadorian cohort* (Guevara-Aguirre, Rosenbloom et al. 1993). In this consanguineous cohort, nearly all patients carry an E180 splice mutation that probably originated in the Mediterranean area (Rosenbloom and Guevara-Aguirre 1998) and was carried from Spain to South America by Jews fleeing from the Spanish inquisition (Gonçalves, Fridman et al. 2014, reviewed in Laron, Kauli et al. 2017).

Unique cases of LS patients have been described, where the mutation occurs in the region of the gene encoding the intracellular domain of the GHR (Milward, Metherell et al. 2004). When the box1 motive – the receptor binding domain for JAK2 – is preserved, JAK2 signaling appears, but STAT5 activation is diminished as its binding domain of the GHR is truncated. In contrast to LS patients affected by the common locations of *GHR* mutations, these patients have GHBP in the circulation. Nevertheless, the phenotype of these LS patients did not differ from the more common forms in the *Israeli* and *Ecuadorian cohorts* regarding short stature, elevated GH whilst decreased IGF1 levels (Laron, Klinger et al. 1993, Milward, Metherell et al. 2004).

While both cohorts show similarities in endocrine and auxologic hallmarks of the phenotype of LS, differences between the heterogeneous *Israeli* and more homogenous *Ecuadorian cohort*, regarding neurological findings and glucose metabolism are obvious (Kranzler, Rosenbloom et al. 1998, Guevara-Aguirre and Rosenbloom 2015) and a correlation between the differences in phenotype and specific mutations is hypothesized (Shevah, Kornreich et al. 2005).

3. Effects on metabolism

As the GHR is expressed in various tissues, nearly the whole organism is affected by GHRD. Furthermore, GH and IGF1 have autocrine and paracrine mechanisms, which play an important role during the development of an organism (Varela-Nieto and Chowen 2005).

3.1. Linear growth

The birth weight of LS patients does not differ from their reference population. Growth retardation becomes apparent already during infancy and patients reach a height of 116 – 142 cm in males and 108 – 136 cm in females (Laron and Kopchick 2011). No significant differences in growth patterns were observed between the *Israeli* and *Ecuadorian cohorts* of LS patients (Rosenbloom, Guevara Aguirre et al. 1990).

Regarding the endocrine regulation of linear growth, the maturation of the GH/IGF axis and its changing components and dependencies need to be considered. The maturation of the GH/IGF axis after birth is characterized by a shift in the relevance of specific growth factors (Hetz, Menzies et al. 2015). Intrauterine growth is stimulated by IGF2 and IGF1, and the production of both appears GH independent in this period of development (Baker, Liu et al. 1993), explaining why birthweights of LS patients remain unaffected. In contrast, postnatal growth is highly dependent on IGF1, whose production requires a stimulation by GH. The effects of GHRD on longitudinal growth cannot simply be explained by a lack of growth promotion by liver derived IGF1. In fact, the growth plate and endocrine actions are much more complex, including autocrine/paracrine interactions. The growth plate includes the cellular organization of specific layers whose dependence on GH and/or IGF1 differs. In general, GH stimulates the differentiation of cells of the germinal layer, whereas IGF1 promotes the clonal expansion of cells in the proliferative layer, leading to longitudinal bone growth (Isaksson, Lindahl et al. 1987).

Therefore, GHRD affects bone metabolism in a more complex manner, leading to a reduction in the width of the epiphyseal plate (reviewed in List, Sackmann-Sala et al. 2011), which is due to reduced proliferation of chondrocytes in the germinal layer (Wang, Zhou et al. 2004). Furthermore, bone mineral density is discussed to be reduced in LS patients, highlighting the effect of growth promoters on mineral homeostasis and skeletal health even after the termination of longitudinal bone

growth (reviewed in Laron and Kopchick 2011).

3.2. Lack of GH action results in severe obesity

GH is a potent regulator of lipid metabolism (reviewed in Vijayakumar, Novosyadlyy et al. 2010).

The mass of adipose tissue is strongly and negatively correlated with GH levels (reviewed in Laron and Kopchick 2011), as GH provides lipolytic action by stimulating the efflux of free fatty acids from adipose tissues by an activation of the adipose tissue hormone-sensitive lipase (HSL). Furthermore, GH functions as an antagonist to insulin action on adipose tissues. It reduces glucose uptake by a downregulation of glucose transporters (Tai, Liao et al. 1990).

Taking these manifold lipolytic actions provided by GH into account, it appears obvious that GHRD must increase the amount of adipose tissues in affected patients. Indeed, severe obesity starting at early childhood and increasing with age is a common characteristic in human LS patients (reviewed in Laron and Kopchick 2011). Investigations on LS patients revealed that the increasing obesity is not due to an increased caloric intake. The most promising explanation for the increasing obesity may be provided by the change in lipid concentration observed in LS patients. High levels of fasting triglycerides, cholesterol and lipoprotein were assessed for adult patients of both cohorts (Laron and Karmon 2010, reviewed in Guevara-Aguirre, Rosenbloom et al. 2015). An increase of adipose levels from childhood towards the high levels in adulthood, may indicate a disturbance in adipocyte lipid turnover leading to the increase in adipose tissues.

The function of adipose tissue includes more than simple energy storage, when taking its endocrine functions into consideration. Especially adipokines – such as leptin and adiponectin – have major endocrine actions (reviewed in Trujillo and Scherer 2006). Obesity is commonly associated with an increase of serum leptin levels, as seen in LS patients (Laron, Silbergeld et al. 1998). Leptin is a protein "signaling" the amount of adipose tissues and controlling energy intake and expenditure on hypothalamic level (reviewed in Jéquier 2002). Obesity induces a state of leptin resistance on hypothalamic level (El-Haschimi, Pierroz et al. 2000), as leptin induced weight loss is becoming diminished. When LS patients were compared to body mass index matched control relatives, a significant decrease in leptin levels was calculated (Guevara-Aguirre, Rosenbloom et al. 2015), allowing

to speculate that the obesity in LS patients is not associated with a developing leptin resistance. Adiponectin is known to decrease serum glucose and lipid levels by enhancing energy uptake and metabolism in muscles and reducing glucose secretion by the liver and increasing insulin sensitivity. While adiponectin levels are normally negatively correlated with obesity (Ryan, Berman et al. 2003), levels were constantly elevated in LS patients (Laron, Ginsberg et al. 2007).

3.3. Glucose homeostasis

Hypoglycemia is one of the first obvious symptoms of infants with LS – even with potential clinical complications - before growth retardation becomes obvious. Zvi Laron (Laron and Kopchick 2011) noted *"The parents of the first encountered patients (Laron, Pertzelan et al. 1966) related that from early age, the children wake up at night asking for sweetened drinks. Not being served or when breakfast was delayed, they sweated profusely and were subject to loss of consciousness."*

Interestingly, glucose levels of LS patients normalize when growing older. The investigation of underlying mechanisms focused on insulin homeostasis and responsiveness and revealed striking differences between the *Israeli* and *Ecuadorian cohorts*.

Laron et al (Laron, Avitzur et al. 1995) observed high levels of insulin accompanied with the low levels of fasting glucose in young LS patients. The high levels of insulin – and results from HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) values – indicate insulin resistance even when glucose values were low (reviewed in Laron and Kopchick 2011). GH is known to provide stimulatory effects on hepatic gluconeogenesis and to antagonize insulin signaling in the liver (reviewed in Vijayakumar, Novosyadlyy et al. 2010). The lack of these effects of GH may explain the hypoglycemia as seen in LS patients. The relation of glucose to insulin changed with age, leading to low levels of insulin and high levels of glucose in adulthood, suggesting an exhausting of pancreatic beta cells in face of developing insulin resistance. Glucose intolerance, insulinopenia and even cases of diabetes mellitus requiring insulin replacement therapy were reported for LS patients from the *Israeli cohort* (Laron, Avitzur et al. 1997).

Contrary development of carbohydrate metabolism is reported for LS from the *Ecuadorian cohort*. As mentioned earlier, juvenile hypoglycemia and transient changes of glucose levels reaching adulthood are a common symptom for all LS

patients. But, Guevara-Aguirre *et al* found patients from the *Ecuadorian cohort* being more insulin sensitive and no case of diabetes mellitus has been reported (Guevara-Aguirre and Rosenbloom 2015). This finding becomes even more astonishing, having the high degree of obesity in mind – which both cohorts have in common. Unfortunately, the differences in insulin sensitivity between the *Israeli* and *Ecuadorian cohorts* cannot be sufficiently explained by now (Guevara-Aguirre and Rosenbloom 2015). Differences due to the numerous mutations leading to GHRD in the *Israeli cohort* or different food supply and culture cannot be excluded.

3.4. Protective effects of GHRD

Insights from the *Ecuadorian cohort* of LS patients revealed a reduced incidence of cancer and diabetes. The same observation regarding cancer has been gained in the *Israeli cohort*.

The incidence of cancer was estimated by a comparison of causes of death in LS patients to their relatives. In the *Ecuadorian cohort*, the history of 53 dead LS patients was compared to 1606 unaffected relatives. Cancer was the cause of death in 20% of relatives, whereas no case of cancer was reported in LS patients (Guevara-Aguirre, Balasubramanian et al. 2011). For a survey in the *Israeli cohort*, 222 LS patients were compared to 338 unaffected relatives, gaining a similar result as from Ecuador: no LS patients died from cancer, whereas 9 to 24% of their relatives had a history of malignancies (Shevah and Laron 2007).

The involvement of IGF1 in the development of malignancies is a proven fact (reviewed in Pollak, Schernhammer et al. 2004). Its proliferative and antiapoptotic effects are putatively involved in cancer development. Furthermore, high levels of circulating IGF1 are commonly associated with a higher incidence of cancer (reviewed in Calle and Kaaks 2004). The conclusion that the lower IGF1 levels in LS patients lower the risk of cancer is obvious.

Besides the antiapoptotic effects of IGF1, a correlation between IGF1 signaling and increasing DNA damage was suggested by Guevara *et al* (Guevara-Aguirre, Balasubramanian et al. 2011). In a profound investigation, human mammary epithelial cells were incubated with serum derived from LS patients or their relatives. Just as expected, cells incubated with serum from GHR deficient patients showed fewer DNA brakes after treatment with H_2O_2. To evaluate the protective effects provided by GHRD serum, the expression rate of particular genes regulating

growth was evaluated. This analysis revealed a decreased expression of the mechanistic target of rapamycin (mTOR). The use of rapamycin in chemotherapeutic treatment of cancer draw the interest towards the target of rapamycin actions (reviewed in Sabatini 2017). Investigations revealed two protein kinase complexes – mTORC1 and mTORC2. mTORC1 contains the component raptor (regulatory protein associated with mTOR) and is inhibited by rapamycin, whereas mTORC2 is insensitive to rapamycin treatment as it contains rictor (rapamycin insensitive companion of mTOR) (reviewed in Saxton and Sabatini 2017). The mTOR complexes act as a link between the availability of nutrients and the regulation of anabolic or catabolic processes on the cellular level. Energy intake increases the availability of glucose in the blood stream and uptake by cells. This input leads to an activation of mTORC1, which induces the production of proteins, lipids and nucleotides, providing cell growth. mTORC2 is affected by insulin signaling and implicated in the regulation of ion transport, apoptosis, glucose metabolism, cell migration and cytoskeleton rearrangement (reviewed in Oh and Jacinto 2011). A chronic activation of mTORC1 – as seen in over-feeding of Western diet – results in inhibition of insulin receptor (INSR) signaling, contributing to an onset of diabetic states (reviewed in Zoncu, Efeyan et al. 2011). On the other hand, reduced activity of mTOR1 is known to increase life-expectancy in model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Sharp, Curiel et al. 2013). To distinguish between the activity of mTORC1 or mTORC2, downstream signal transducers specific for each complex were investigated. Just as expected, Guevara *et al* revealed a decreased expression of protein S6 kinase (S6K) – a key element of mTORC1 - in cells incubated with serum derived from LS patients (Guevara-Aguirre, Balasubramanian et al. 2011).

Unfortunately, no sufficient data for the life-expectancy of LS patients is available, as many cases are spread world-wide (Laron and Kopchick 2011) and socioeconomic consequences leading to high proportions of non-age related causes of death, such as alcohol toxicity, liver cirrhosis, convulsive disorders or accidents (Guevara-Aguirre, Balasubramanian et al. 2011). Nevertheless, an involvement of decreased mTOR signaling and protective effects associated with a lower activity of growth promotion providing protective effects was supposed and genetically engineered animal models were established for further investigations (Zhou, Xu et al. 1997).

4. Treatment of Laron syndrome

Laron syndrome occurs due to a disturbance in the growth axis involving GH and IGF1. A substitution of GH would not be sufficient, as no rise in serum IGF1 could be observed (Laron, Pertzelan et al. 1971) since the defect leading to LS affects the GHR. Since 1990, recombinant biosynthetic IGF1 is used as treatment for LS beginning at infancy (reviewed in Laron and Kopchick 2011). A long-term treatment starting at early ages is seen most sufficient, as the growth promoting effect of IGF1 administration is greater in young children (Laron 1993). Unfortunately, the half-life of exogenous IGF1 is reduced in LS patients compared to healthy subjects (Laron 1993). As mentioned earlier, most IGF1 in circulation is bound by IGFBP3 and ALS, which prolong the half-life of IGF1 by preventing its excretion and degradation. The production of IGFBP3 and ALS are both depending on GH signaling, which is diminished in GHRD, leading to low levels of both, decreasing the half-life of exogenous IGF1 (Rosenbloom 2016). Therefore, the administration of IGF1 has to be performed on a daily base until the final height is reached and cartilage epiphyses are closed. The long-term treatment of children with exogenous IGF1 leads to an increase in growth velocity, reduction in body fat and modifies the craniofacies (Carel, Chaussain et al. 1996). Adverse effects of IGF1 treatment are reported, involving hypoglycemia, headache, intracranial hypertension, swelling of lymph nodes and hyperandrogenism (reviewed in Laron and Kauli 2016). Luckily, most adverse effects are just due to over dosage or administration without food. The treatment of LS patients with exogenous IGF1 beginning at childhood leads to an increase of growth velocity, but patients still do not reach the estimated parental target height, as the direct effects of GH on the growth plate cannot be restored (Rosenbloom 2016). A beneficial effect of IGF1 treatment was observed in adult LS patients due to metabolic effects, for example reducing cholesterol levels and enhancing renal functions (Laron and Klinger 1994). Unfortunately, these positive effects in adults reverse once the administration of IGF1 is stopped.

5. Animal models for GH-deficiency and Laron syndrome

The Laron Syndrome provides a unique opportunity to study the various effects of GH signaling regarding growth and metabolism in humans. Furthermore, the possible protective effects on malignancies associated with diminished GH signaling in GHRD draw the interest toward further investigations of this syndrome. The study of human patients with LS rises several difficulties, as human cases are very rare and spread world-wide. The variety of mutations, especially in the *Israeli cohort*, and environmental factors complicate the comparability of findings in human patients and accentuate the need for suitable animal models.

5.1. The sex-linked dwarf chicken

The aim to produce smaller breeding hens in the poultry industry without affecting the size broilers led to the establishment of a sex-linked mutation leading to dwarfism (SLD) in several strains of breeding hens in the mid of the 20th century (Mérat 1984). The recessive *dw* mutation of the *GHR* gene on the Z–chromosome (Agarwal, Cogburn et al. 1994, Boegheim, Leegwater et al. 2017) leads to a 30-40% reduction of body weight in hens, whereas heterozygosity does not affect male chicken. It was argued, that SLD chicken exhibit a form of inherited GH resistance due to GHR dysfunction, as no detectable GH binding activity, reduced levels of IGF1 and adiposity were observed (reviewed in Hull and Harvey 1999). Nevertheless, the SLD chicken never prevailed as a widely used model for LS due to the large taxonomic difference between birds and mammals.

5.2. Guinea pigs

Guinea pigs seem to be resistant to the growth promoting effects of GH. Studies with extracts derived from guinea pig pituitary glands revealed a growth promoting effect of hypophysectomized rats, whereas no effect on guinea pigs themselves was observed (reviewed in Hull and Harvey 1999). While a growth impairment in guinea pigs was suspected, it was revealed that their growth occurs independent from GH action. In contrast to other mammals, the IGF1 production in guinea pigs appears independent from GH (reviewed in Keightley and Fuller 1996), limiting its potential as a sufficient animal model for LS.

5.3. The miniature *Bos indicus* cattle

The study of Liu *et al* (Liu, Boyd et al. 1999) investigated the underlying defects of a line of miniature *Bos indicus* cattle. As the small size and high levels of circulating GH, whilst IGF1 levels were reduced pointed towards a defect similar to Laron syndrome, the endocrine conditions were investigated in detail. It was shown, that the condition was inherited as a recessive gene and exogenous GH administration did not restore the levels of circulating IGF1. Nevertheless, a genetic defect of the *GHR* gene - as essential for LS - was not found in those miniature cattle. The cause of this phenotype is a mutation of the *GH* gene affecting the GHR binding domain and thus leading to reduced binding of GH to its receptor (reviewed in Boegheim, Leegwater et al. 2017), decreased expression of IGF1, and reduced growth velocity of affected animals. Probability due to taxonomic and pathophysiologic differences, the miniature *Bos indicus* cattle has not been established as a proper animal model for LS.

5.4. The Laron / *Ghr* KO mouse

The first animal model for LS stablished by targeted mutation of the *Ghr* gene was the Laron mouse / *Ghr* KO mouse by Zhou *et al* (Zhou, Xu et al. 1997) at the lab of John Kopchick in 1997. By targeting exon 4 of the murine *Ghr* gene, the expression of both, GHR and GHBP was eliminated. The resulting GHR/BP KO mouse has been used in several GH-related studies (reviewed in List, Sackmann-Sala et al. 2011). In brief, the *Ghr* KO mouse mimics several hallmarks of the human phenotype of LS. As GH signaling is abolished, low levels of circulating IGF1 and increased levels of GH in circulation have been observed in all studies (reviewed in List, Sackmann-Sala et al. 2011). The lack of GH-related growth promotion leads to the characteristic dwarf phenotype due to post-natal growth failure, whereas birth weights remain unaffected. Coschigano *et al* observed improved longevity in the *Ghr* KO mouse, as speculated from the human phenotype and other GH insensitive mice models (Coschigano, Clemmons et al. 2000). A connection between improved insulin sensitivity and pro-aging effects was suggested. In fact, young *Ghr* KO mice have been found to be more insulin sensitive leading to hypoglycemia as in human patients of LS accompanied with hyperinsulinemia (Liu, Coschigano et al. 2004). The transient changes from hypo- to normoglycemia as seen in human LS patients also occurred in the *Ghr* KO mouse, but in contrast to finings of Laron *et al* (Laron, Avitzur et al. 1995), insulin

sensitivity of *Ghr* KO mice remains increased their entire life span, associated with decreased levels of fasting insulin. The improved insulin sensitivity and longevity gets even more impressive since the *Ghr* KO mouse is reported to be obese. Several studies focused on the body composition of the *Ghr* KO mouse, since severe obesity is reported to be a major characteristic of the human LS. While studies consistently showed, that the *Ghr* KO mouse has an absolute reduction in lean mass, the relative reduction is proportional to the growth retardation (reviewed in List, Sackmann-Sala et al. 2011). In contrast, an increase of percentage of body fat has been reported for the *Ghr* KO mouse. Whereas in human patients of LS, females are reported to have greater percentage of body fat than males, female *Ghr* KO mice showed lower values than males (Berryman, List et al. 2010). The authors avoid to call this phenotype a pronounced obesity, as the absolute fat mass of the dwarf sized *Ghr* KO mice does not differ from controls. Furthermore, the accumulation of fat mass in particular depots is dramatically changed in the *Ghr* KO mice, as a disproportional enlargement of subcutaneous fat has been observed, highlighting tissue-specific effects of GHRD.

The metabolic changes in the *Ghr* KO mouse are often compared to a state of caloric restriction (CR) (reviewed in List, Sackmann-Sala et al. 2011). As CR is associated with increased life expectancy and the prevention of cancer (Longo and Fontana 2010), similarities between *Ghr* KO mice and control animal raised under CR are interpreted as beneficial effects of GHRD. Similarities are further surprising, as *Ghr* KO mice show a high percentage of body fat, but Longo *et al* hypothesized, that the reduced levels of circulating IGF1 simulate a state of CR (Longo and Finch 2003) even when *Ghr* KO mice consume relatively more energy than control animals (reviewed in List, Sackmann-Sala et al. 2011). While it has been shown that CR increases the life-span of Ames dwarf mice (Bartke, Wright et al. 2001) - with diminished GH, thyroid stimulating hormone (TSH) and prolactin production – no increase in life expectancy was observed in *Ghr* KO mice under CR (Bonkowski, Rocha et al. 2006). It has been suggested, that the positive effects of GH deficiency combined with CR increase the life-span of Ames dwarf mice, while CR was not sufficient to further increase the already improved insulin sensitivity in *Ghr* KO mice (Bonkowski, Rocha et al. 2006, reviewed in List, Sackmann-Sala et al. 2011). Furthermore, no differences in specific gene expression in the liver due to CR have been observed in *Ghr* KO mice, while CR significantly altered the gene expression

in control animals under CR (Miller, Chang et al. 2002). It is further known, that CR results in a reduced activity of mTORC1, which contributes to the development of cancer (Longo and Fontana 2010). In line with an expected reduced activity of mTORC1 in *Ghr* KO mice, Dominick *et al.* showed a reduced activity of mTORC1 while mTORC2 activity was enhanced according to the investigation of downstream substrates (Dominick, Berryman et al. 2015).

Besides these positive effects of GHRD on the phenotype of the *Ghr* KO mouse, a reduced fertility was observed in several studies (reviewed in List, Sackmann-Sala et al. 2011). While in human LS patients, no fertility problems besides a delay in puberty and hypogenitalism have been reported and affected patients can reproduce (reviewed in Laron and Kopchick 2011), female *Ghr* KO mice are reported as subfertile, producing fewer pups due to a reduced ovulation rate and an increased postnatal mortality has been observed (Zhou, Xu et al. 1997, Danilovich, Wernsing et al. 1999).

It is undeniable that the *Ghr* KO mouse provided tremendous insight on the GH/IGF axis and the LS in particular. Nevertheless, mouse models have several limitations in common representing the phenotype of human disorders resulting from their small size, short life expectancy and physiological differences from humans. Regarding the LS and overall investigations of the GH/IGF1 axis, the different timing of maturation of the endocrine growth axis in mice and humans has to be considered. In contrast to humans, the maturation of the hypothalamic–pituitary–adrenal axis (HPA axis) of rodents occurs in the postnatal period (Symonds, Sebert et al. 2009). To overcome this limitation, a large animal model which is not born in this "immature" state but with a fully mature HPA axis at birth occurred necessary.

5.5. Wuzhishan minipigs with dominant-negative GHR

The approach to overexpress a dominant-negative GHR to generate a minipig model for LS was followed by Li *et al* (Li, Li et al. 2015). The idea was, that the overexpression of a non-functional GHR under the control of the ubiquitously active CAG promoter might bind large amounts of circulating GH, reducing sufficient signaling by the still functional wild-type (WT) GHR. Indeed, the expression of the dominant-negative GHR was about 100-fold increased compared to the WT GHR and GH levels in circulation were elevated 9-fold in transgenic pigs. In contrast to findings in humans and mice, an increase in fasting blood

glucose levels was estimated analyzing pooled samples of animals aged one, five and 77 days. It remains unclear, why the values of the different age groups were pooled and age-dependent changes in metabolism were investigated. Furthermore, IGF1 levels in transgenic pigs were not reduced in a significant manner, suggesting residual signaling via the residual WT GHR, making its use as a proper animal model for LS questionable.

5.6. The GHR deficient miniature pig

Cui *et al* (Cui, Li et al. 2015) generated the first miniature pig model with a knock out of the *GHR* gene by applying the zinc finger nuclease technology targeting exon 6. As expected, these GHR deficient pigs showed elevated GH levels in circulation, whereas IGF1 levels were decreased. Growth retardation became obvious after weaning, obesity was obvious by eyesight and high abundance of abdominal fat was observed at necropsy. Hypoglycemia has been described, but unfortunately the age of the corresponding pigs has not been clarified and a transiency as observed in human LS patients has not been estimated.

Miniature pigs are a highly recommended animal models in research, but since they are already growth impaired it may be questioned if a *GHR* KO on this background resembles all pathophysiological consequences of the human LS. Miniature pigs are dwarf breeds, characterised by "proportional dwarfism" of all body parts. As this type of dwarfism is seen to be associated with genetically caused dwarfism, such as IGF1 deficiency (Simianer and Köhn 2010), it has been speculated, that the selection for the dwarf phenotype led to an accumulation of alleles downregulating the GH/IGF pathway (Gärke, Ytournel et al. 2014). To maintain a pig population with undisturbed IGF1 function, we used the German landrace, since no selection for smaller body size occurred in this breed.

6. Methods for the genetic modification of pigs

According to the *from bench to bedside* principle in translational medicine, genetically tailored large animal models are required to bridge gaps between basic research in genetically modified rodent models and human diseases and syndromes (reviewed in Aigner, Renner et al. 2010, Dmochewitz and Wolf 2015). The usage of pigs as a large animal model steadily increases as it models the human situation more accurately (Lunney 2007). Several techniques for engineering of the pig genome are available, but they differ in their efficiency (reviewed in Dmochewitz and Wolf 2015).

6.1. Pronuclear DNA microinjection

The first approach to generate transgenic pigs was to inject DNA directly into pronuclei of zygotes (Hammer, Pursel et al. 1985, Selden, Springman et al. 1985). Unfortunately, the efficiency of this method is quite low and the risk to generate transgenic animals with mosaicism occurs (reviewed in Dmochewitz and Wolf 2015). As the integration of the injected DNA appears randomly, no target-site integration could be achieved, leading to different expression levels of the integrated transgene, as its expression can be influenced by effects of neighboring DNA (reviewed in Luo, Lin et al. 2012).

6.2. Sperm-mediated gene transfer

A further approach to increase the efficiency of gene transfer and to generate multigene transgenic animal models was the sperm-mediated gene transfer (SMGT) (Lavitrano, Busnelli et al. 2005). Using the intrinsic ability of sperm to take up exogenous DNA, a transfer into oocytes occurring during fertilization could be achieved (reviewed in Aigner, Renner et al. 2010). Nevertheless, just the same limitations as for DNA microinjections appear as no target-site directed gene transfer and no target knock-out of genes is possible (reviewed in Luo, Lin et al. 2012).

6.3. Lentiviral vectors

Belonging to the family of *Retroviridae*, lentiviruses can transfer RNA into cells via their infection. Lentiviral RNA is further transcribed into DNA by the reverse transcriptase and integrated into the host genome and transmitted through the germ line to the founder animal´s offspring (reviewed in Aigner, Renner et al. 2010). A

major advantage of lentiviral vectors is, that the transduction can appear in non-dividing cells, allowing the operator to reduce the risk of creating mosaicism in founder animals, when the integration appears in early embryo development (Pfeifer 2004). Limitations of lentiviral vectors occur, when larger inserts are transfected and furthermore, the possible case of multiple independent integration sites and their segregation in the founder animal´s offspring need to be considered (reviewed in Dmochewitz and Wolf 2015).

6.4. SCNT from genetically modified cells

As no porcine embryonic stem cells are available, the use of genetically modified somatic cells as donor cells for somatic cell nuclear transfer (SCNT) is a well-established method to generate genetically modified pig strains (reviewed in Dmochewitz and Wolf 2015). A detailed protocol is provided in (Kurome, Kessler et al. 2015). A wide range of somatic cells, like porcine fetal, ear or kidney fibroblasts, or mesenchymal stem cells are used. Usually, gene constructs are combined with resistance cassettes against a specific antibiotic, to enable a selection process after transfection of the cells. In case of gene targeting, the target specific introduction of the vector has to be verified. The possibility to screen for success of the genetic modification prior to the production of embryos and embryo transfer is a major advantage of this method. To generate SCNT embryos, *in vitro* matured oocytes are enucleated and single donor cell is inserted into the zona pellucida. Oocyte cytoplasm and donor cell are then fused and activated, and the reconstructed embryos are transferred into recipient gilts (Besenfelder, Modl et al. 1997).

6.5. Zinc finger nucleases

For targeted engineering of the pig genome, customized nucleases are available, such as the zinc finger nuclease (ZFN), offering the advantage that this technology can be applied via the cytoplasmic injection into zygotes, without requiring somatic cell nuclear transfer (SCNT) (reviewed in Dmochewitz and Wolf 2015). ZFNs are composed of a pair of fusion protein, containing the DNA cleavage domain (*Fok*I endonuclease) and a site specific DNA binding domain (reviewed in Luo, Lin et al. 2012). The binding domain is composed as “fingers” made up of 30-amino-acids coordinated by a zinc atom contacting 3bps of target DNA (Porteus and Carroll 2005). Each finger independently binds its target site. Therefore, the use of multiple fingers increases the specificity of target-site binding (Whitelaw, Sheets et al.

2016). ZFNs are used as pairs, binding the opposite DNA strands, as each binding domain is complexed with just one half of the dimeric endonuclease (Gaj, Gersbach et al. 2013). After the target-site binding, the endonuclease dimerization provides double stand breaks (DSBs) of the host DNA, inducing the cell´s intrinsic repair mechanisms such as non-homologous end-joining (NHEJ) or homology-directed repair (HDR) (Luo, Lin et al. 2012). The repair of DSBs by NHEJ contains the opportunity to generate mutations due to insertions or deletions, whereas a repair mechanism involving HDR enables the introduction of exogenous DNA, linked to templates homologous to host´s target region (Dmochewitz and Wolf 2015). The wide application of ZFNs increased the efficiency of gene targeting due to the site specific DNA binding domain, and allowed the site specific introduction of exogenous DNA. Nevertheless, their use is complicated as designing of specific DNA binding sites is time consuming and expensive (reviewed in Bogdanove and Voytas 2011).

6.6. TALENs

Just as ZFNs, transcription activator-like effector nucleases (TALENs) can be applied for target engineering of the pig genome. TAL effectors are known as virulence factors of the bacteria *Xanthomonas* as they mimic transcription factors of eukaryotic cells and thereby change the cell´s gene expression (Boch, Scholze et al. 2009). TAL effectors contain tandem amino acid repeats targeting promoter sequences of the host genome (Bogdanove and Voytas 2011). Target DNA binding can be achieved by generating artificial tandem repeats (Luo, Lin et al. 2012). TAL effectors can be equipped with the *Fok*I restriction enzyme creating TAL effector nucleases (TALENs) (Dmochewitz and Wolf 2015). As *Fok*I operates as a dimer, TALENS need to be designed as pairs, binding opposite DNA target sites to induce DSBs (Bogdanove and Voytas 2011). While the efficiency of TALENs is comparable with ZFNs, the architecture of TAL effectors as tandem repeats offers difficulties in designing new target binding sequences (Luo, Lin et al. 2012).

6.7. CRISPR/Cas

Derived from *Streptococcus pyogenes*, the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 endonuclease technology has been successfully used to generate genetically tailored small animal and pig models (Hai, Teng et al. 2014). In bacteria and archaea, the CRISPR/Cas system serves as a form of an

adaptive immune system. After infection by viruses or plasmids, short nucleic acid fragments are integrated as *Spacer* sequences in the host genome framed by a *repeat* motif, leading to the characteristic *repeat – spacer – repeat* architecture of the CRISPR sequence (reviewed in Gaj, Gersbach et al. 2013, Seruggia and Montoliu 2014). The CRISPR locus is transcribed into CRISPR-derived RNAs (crRNAs) containing the *spacer* sequences of foreign nucleic acids. At a further infection, the foreign nucleic acids - referred to as *protospacer* sequence - can be detected by the crRNAs and destructed by the CRISPR-associated (Cas) endonucleases which form a complex with the guiding crRNA (reviewed in Wiedenheft, Sternberg et al. 2012). An illustration of the general function of the CRISPR/Cas system is provided in **Figure 2**.

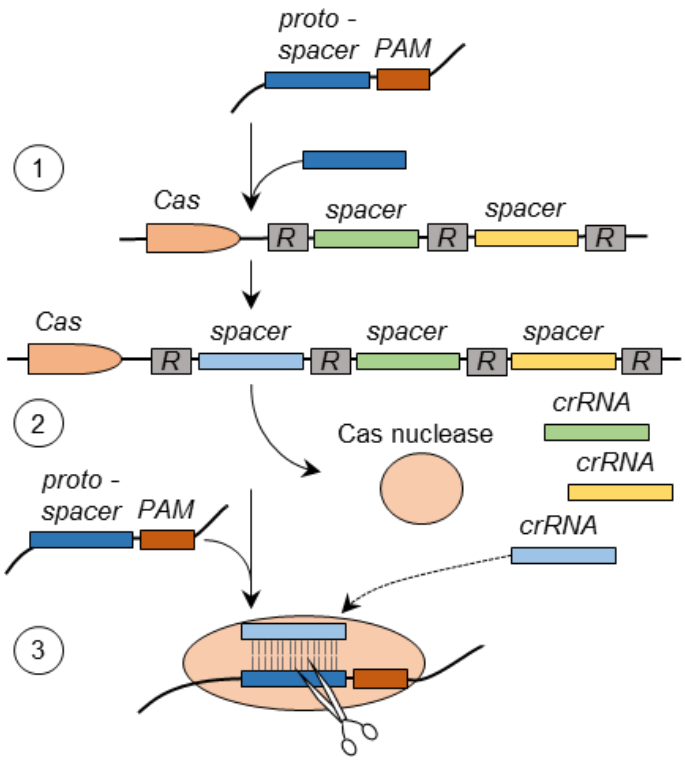


Figure 2. General function of the CRISPR/Cas system as a prokaryotic adaptive immune system. Foreign nucleic acids (*proto-spacers*) are acquired as *spacers* at the leader end of the host´s CRISPR array (1), characterized by the repetitive abundance of *spacer* and *repeat* sequences (indicated as grey boxes marked with "*R*"). *Spacer* sequences are transcribed as CRISPR – derived RNA (crRNA), providing a form of „*spacer* library" (2). At a further infection with an already known pathogen, the complementary crRNA binds to the intruder's *protospacer* domain close to the protospacer - associated motif (PAM) enabling the activity of Cas endonucleases (adapted from Bhaya, Davison et al. 2011).

Three types of CRISPR/Cas systems are known in bacteria and archaea. Type I and III just show little differences in the synthesis of crRNA and in required Cas endonucleases (reviewed in Bhaya, Davison et al. 2011). To adapt the CRISPR/Cas system as a tool for genetic engineering, the CRISPR/Cas Type II is used as the Cas9 endonuclease can perform DSBs even in eukaryotic cells. Homologue to the target gene sequence (*protospacer* sequence), a single guiding RNA (sgRNA) is

designed by the fusion of the target crRNA and the so-called trans-activating crRNA (tracrRNA) which is necessary for the activity of the Cas9 endonoclease (reviewed in Seruggia and Montoliu 2014). When several different sgRNAs are applied, it is even possible to target multiple loci in a single model (Wang, Yang et al. 2013). Injection of the sgRNA and RNA encoding for the Cas9 endonuclease in for example zygotes induces the assembling of the two components (Hai, Teng et al. 2014). DSBs are achieved by activity of two distinct Cas9 domains, where the Cas9 HNH nuclease domain cleaves the complementary and the Cas9 RuvC-like nuclease domain cleaves the non-complementary DNA strand (Jinek, Chylinski et al. 2012). It has to be noted, that the activity of the Cas9 endonuclease requires a protospacer-associated motif (PAM) (Anders, Niewoehner et al. 2014) close to the target *protospacer* sequence, wherein the DSB is induced (reviewed in Bhaya, Davison et al. 2011). The use of the CRISPR/Cas9 system as a tool for genetic engineering is illustrated in **Figure 3**. Targeted mutations are achieved by NHEJ or the introduction of genetic information by HDR, inserting donor templates containing homologous arms (reviewed in Dmochewitz and Wolf 2015).

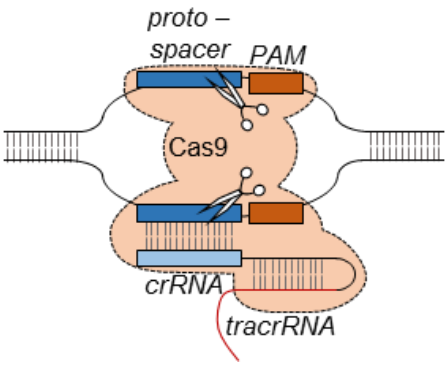


Figure 3. The CRISPR/Cas9 system as a tool for genetic engineering. An artificial single sgRNA containing the linked crRNA and tracrRNA is designed complementary to the protospacer sequence of the target genome inducing the activity of the Cas9 endonuclease. Being a part of the Type II CRISPR/Cas system, to separate domains of the Cas9 endonuclease cleave the complementary and non-complementary strand inducing a DSB (adapted from Jinek, Chylinski et al. 2012).

In summary, the use of the CRISPR/Cas9 system emerged rapidly due to the ease of designing guiding RNAs in contrast to protein engineering required for ZFN and TALEN applications, making it an ideal tool for the generation of genetically tailored pig models (reviewed in Dmochewitz and Wolf 2015) as used in our study.

III. PUBLICATION

MOLECULAR METABOLISM

Growth hormone receptor-deficient pigs resemble the pathophysiology of human Laron syndrome and reveal altered activation of signaling cascades in the liver

Arne Hinrichs[1,2], Barbara Kessler[1,2], Mayuko Kurome[1,2,3], Andreas Blutke[4], Elisabeth Kemter[1,2], Maren Bernau[5], Armin M. Scholz[5], Birgit Rathkolb[1,2], Simone Renner[1,2,6], Sebastian Bultmann[7], Heinrich Leonhardt[7], Martin Hrabě de Angelis[6,8], Hiroshi Nagashima[3], Andreas Hoeflich[9], Werner F. Blum[10], Martin Bidlingmaier[11], Rüdiger Wanke[4], Maik Dahlhoff[1,2,*], Eckhard Wolf[1,2,3,6,12,*]

[1]Chair for Molecular Animal Breeding and Biotechnology, Gene Center and Department of Veterinary Sciences, [2]Center for Innovative Medical Models (CiMM), Department of Veterinary Sciences, LMU Munich, [3]Meiji University International Institute for Bio-Resource Research, Japan; [4]Institute of Veterinary Pathology, Center for Clinical Veterinary Medicine, [5]Livestock Center of the Veterinary Faculty, LMU Munich, [6]German Center for Diabetes Research (DZD), Helmholtz Zentrum München, [7]Human Biology and Bioimaging, Faculty of Biology, Biocenter, LMU Munich, [8]Institute of Experimental Genetics, Helmholtz Zentrum München, and Chair of Experimental Genetics, Technical University of Munich, [9]Cell Signaling Unit, Institute of Genome Biology, Leibniz Institute for Farm Animal Biology; Dummerstorf, Germany; [10]University Children`s Hospital, University of Giessen, [11]Endocrine Laboratory, Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, [12]Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, LMU Munich,

*equal senior author contribution

Corresponding author: Eckhard Wolf, Gene Center, LMU Munich, Feodor-Lynen-Str. 25, 81377 Munich, Germany; phone: +49-89-2180-76800; e-mail: ewolf@lmu.de

Short running title: GHR deficient pigs resembling Laron syndrome

DOI: 10.1016/j.molmet.2018.03.006

ABSTRACT

Objective: Laron syndrome (LS) is a rare, autosomal recessive disorder in humans caused by loss-of-function mutations of the growth hormone receptor (GHR) gene. To establish a large animal model for LS, pigs with GHR knockout (KO) mutations were generated and characterized.

Methods: CRISPR/Cas9 technology was applied to mutate exon 3 of the GHR gene in porcine zygotes. Two heterozygous founder sows with a 1-bp or 7-bp insertion in GHR exon 3 were obtained, and their heterozygous F1 offspring were intercrossed to produce GHR-KO, heterozygous GHR mutant, and wild-type pigs. Since the latter two groups were not significantly different in any parameter investigated, they were pooled as the GHR expressing control group. The characterization program included body and organ growth, body composition, endocrine and clinical-chemical parameters as well as signaling studies in liver tissue.

Results: GHR-KO pigs lacked GHR and had markedly reduced serum insulin-like growth factor 1 (IGF1) levels and reduced IGF-binding protein 3 (IGFBP3) activity, but increased IGFBP2 levels. Serum GH concentrations were significantly elevated compared with control pigs. GHR-KO pigs had a normal birth weight. Growth retardation became significant at the age of five weeks. At the age of six months, the body weight of GHR-KO pigs was reduced by 60 % compared with controls. Most organ weights of GHR-KO pigs were reduced proportionally to body weight. However, the weights of liver, kidneys, and heart were disproportionately reduced, while the relative brain weight was almost doubled. GHR-KO pigs had a markedly increased percentage of total body fat relative to body weight and displayed transient juvenile hypoglycemia along with decreased serum triglyceride and cholesterol levels. Analysis of insulin receptor related signaling in the liver of adult fasted pigs revealed increased phosphorylation of IRS1 and PI3K. In agreement with the loss of GHR, phosphorylation of STAT5 was significantly reduced. In contrast, phosphorylation of JAK2 was significantly increased, possibly due to the increased serum leptin levels and increased hepatic leptin receptor expression and activation in GHR-KO pigs. In addition, increased mTOR phosphorylation was observed in GHR-KO liver samples, and phosphorylation studies of downstream substrates suggested the activation of mainly mTOR complex 2.

Conclusion: GHR-KO pigs resemble the pathophysiology of LS and are an interesting model for mechanistic studies and treatment trials.

Highlights:

Growth hormone receptor (GHR)-deficient pigs reveal postnatal growth retardation, disproportionate organ growth and an increased total body fat content, associated with markedly reduced IGF1 and IGFBP3 levels, but increased IGFBP2 activity.

Transient juvenile hypoglycemia in the presence of normal insulin levels and increased expression and phosphorylation of IRS1 in liver of adult GHR-deficient pigs suggest increased insulin sensitivity.

Increased phosphorylation of JAK2 in liver of GHR-deficient pigs may be explained by increased serum leptin levels and increased expression and phosphorylation of hepatic LEPR.

Abbreviations:

4EBP1, eukaryotic initiation factor 4E binding protein 1; aa, amino acid; AKT, serine-threonine protein kinase; AMPK, AMP-activated protein kinase; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR-associated; DAB, 3,3′-diaminobenzidine; DXA, dual-energy X-ray absorptiometry; eIF4E, eukaryotic translation initiation factor 4E; ELISA, enzyme-linked immunosorbent assay; GH, growth hormone; GHR, growth hormone receptor; GSK3B, glycogen synthase 3 beta; HDL, high-density lipoprotein; HOMA, homeostatic model assessment; HSL, hormone-sensitive lipase; IGF1, insulin-like growth factor 1; IGFBP, IGF-binding protein; IgG, immunoglobulin G; INSR, insulin receptor; IRS1, insulin receptor substrate 1; JAK2, Janus kinase 2; LS, Laron syndrome; LSM, least squares mean; LDL, low-density lipoprotein; LEPR, leptin receptor; LPL, lipoprotein lipase; MAPK, mitogen-activated protein kinase; MRI, magnetic resonance imaging; mTOR, mechanistic target of rapamycin; mTORC, mTOR complex; PCR, polymerase chain reaction; PI3K, phosphoinositide 3 kinase; PPARG, peroxisome proliferator-activated receptor gamma; RIA, radioimmunoassay; S6K, protein S6 kinase 1; SE, standard error; sgRNA, single guide RNA; STAT, signal transducer and activator of transcription; TBS, Tris-buffered saline.

1. INTRODUCTION

Laron syndrome (LS) is a rare autosomal recessive hereditary disorder caused by loss-of-function mutations in the growth hormone receptor (*GHR*) gene (https://www.omim.org/entry/600946), initially described as a syndrome of primary growth hormone (GH) resistance or insensitivity (1; reviewed in 2, 3). As a consequence, LS patients have low levels of insulin-like growth factor 1 (IGF1) and – due to the lack of feedback inhibition of GH secretion – high levels of GH [3]. A few hundred cases of LS have been reported world-wide, caused by a variety of *GHR* mutations (reviewed in 4). Among them is an isolated more homogeneous population of GHR deficient patients in Ecuador with only two distinct mutations of the *GHR* gene [5-7].

The main clinical feature is short stature. In addition, LS patients may exhibit reduced muscle strength and endurance, hypoglycemia in infancy, delayed puberty, obesity, and distinct facial features, including a protruding forehead, sunken bridge of the nose, and blue sclerae (reviewed in 3, 8). The standard treatment of LS is long-term application of recombinant IGF1, which increases growth velocity and improves adult height, but it may lead to a spectrum of side effects, in particular hypoglycemia (9, 10; reviewed in 11).

A particularly interesting observation in LS patients is their reduced incidence of malignancies (12, 13; reviewed in 8). In addition, LS patients from the cohort in Ecuador have been shown to be protected against the development of type 2 diabetes despite severe obesity [7].

Although mechanistic studies have been performed in cell lines derived from LS patients and healthy controls [7, 14], animal models are of pivotal importance for understanding the pathophysiology of LS *in vivo*. In particular, GHR-deficient mice [15] have provided new insights into the consequences of GH insensitivity for body and organ growth, body composition, endocrine and metabolic functions, and reproduction, as well as aging and life expectancy (reviewed in 16). More recently, inducible/tissue-specific *Ghr* knockout (KO) mouse models have helped to define the specific roles of GHR in liver, muscle and adipose tissue and revealed interesting differences compared with constitutive *Ghr* KO mice [17, 18]. However, due to their small size, short life expectancy and physiological differences compared with humans, findings from mouse models may be difficult to extrapolate

to the clinical situation of LS patients. In general, genetically tailored pig models are useful to bridge the gap between proof-of-concept studies in rodent models and clinical studies in patients (reviewed in 19, 20). Thus, we have developed a GHR-deficient (*GHR*-KO) pig model and show that it resembles important aspects of LS pathophysiology and reveals altered activation of signaling cascades in the liver.

2. MATERIALS AND METHODS

2.1. Generation of *GHR* mutant pigs using CRISPR/Cas

All animal procedures in this study were approved by the responsible animal welfare authority (Regierung von Oberbayern; permission 55.2-1-54-2532-70-12) and performed according to the German Animal Welfare Act and Directive 2010/63/EU on the protection of animals used for scientific purposes.

For CRISPR/Cas-assisted *GHR* gene disruption using a single guide RNA (sgRNA) specific for exon 3 sequence 5'-TTCATGCCACTGGACAGATG-3', a corresponding oligonucleotide was cloned into the pEX-A-U6-gRNA vector as described previously [21]. Cas9 mRNA and sgRNA were *in vitro*-transcribed using the Ambion Maxiscript SP6 kit (Thermo Fisher Scientific).

Porcine zygotes (German landrace background) were produced *in vitro* as described previously [22], and Cas9 mRNA (50 ng/µL) and sgRNA (100 ng/µL) was injected into their cytoplasm 8.5-9.5 hours after *in vitro* fertilization. Recipient gilts were synchronized in the estrous cycle by oral administration of 4 mL Altrenogest (Regumate®; MSD Animal Health) for 15 days, followed by intramuscular injection of 750 IU ECG (Intergonan®; MSD Animal Health) and 750 IU HCG (Ovogest®; MSD Animal Health) after an additional 24 and 104 hours, respectively. Embryo transfer was performed laparoscopically into one oviduct [23, 24]. Pregnancy was confirmed by ultrasonographic examination first on day 21 and again 4-6 weeks later.

Genomic DNA was isolated from tail tips of piglets using the Wizard DNA Extraction Kit (Promega). *GHR* mutations were detected by sequencing a *GHR* exon 3 PCR product obtained using primers GHR_Fw 5'-acc gct ctg aag ctg tga cc-3' and GHR_Rv 5'-cac cct cag ata ctc tca tgc-3'. Based on the detected mutations, an *Xcm*I restriction fragment length polymorphism assay was established, yielding fragments of 203 bp and 441 bp for wild-type *GHR* and a single fragment of 644 bp for the mutated *GHR* sequence.

Two female founder animals with different frameshift mutations were mated with wild-type boars to generate heterozygous F1 offspring. Heterozygous offspring of the same founder were intercrossed to obtain homozygous animals (*GHR*-KO) with the respective *GHR* mutations. The resulting pedigrees are shown in **Suppl. Fig. 1**.

2.2. Ligand immunostaining of porcine GHR

Liver and kidney tissue of *GHR*-KO and control pigs was fixed overnight in 4 % formalin and routinely embedded in paraffin. Paraffin sections were dewaxed, and endogenous peroxidase and biotin were blocked with 1 % H_2O_2 in Tris-buffered saline (TBS) for 15 min and by using the avidin/biotin blocking kit (no. SP-2001; Vector Laboratories), respectively. After blocking with 0.5 % fish gelatin for 30 min, the slides were incubated in 0.5 µg/mL recombinant rat GH (no. 16343667, ImmunoTools) in 0.2 % fish gelatin solution overnight at 4°C. They were then washed 3 times for 5 min in TBS and incubated in goat-anti rat GH polyclonal antibody solution (dilution 1:2,400, no. AF1566, R&D Systems) for 6 hours at room temperature. After 3 washing steps in TBS (10 min each), the slides were incubated in biotinylated rabbit-anti goat IgG solution (dilution 1:100, no. BA-5000, Vector Laboratories) for 1 hour at room temperature, washed 3 times for 10 min in TBS, and finally incubated with horseradish peroxidase-labelled avidin biotin complex for 30 min (no. PK-6100, VECTASTAIN Elite ABC-Peroxidase kit, Vector Laboratories). Immunoreactivity was visualized using 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB) (brown color). Nuclear counterstaining was performed with Mayer's hemalum (blue color). As specificity controls for the ligand immunohistochemistry assay, rat GH as well as rat GH plus primary antibody were omitted.

2.3. Blood collection

Animals were fasted overnight (16 hours) before blood collection from the jugular vein. After clotting for 30 minutes at room temperature, serum was separated by centrifugation (1200 × *g*) for 20 minutes at 6°C and stored at -80°C until analysis. For repeated blood sampling required to analyze GH secretion profiles, central venous catheters (Argon Careflow™; Merit Medical) were surgically inserted through the external ear vein. Blood samples were collected every 30 minutes for 9 hours, starting at 11 a.m., and processed for serum collection as described above. During the test, the animals were fed regularly and had free access to water.

2.4. Clinical chemistry, hormone assays, IGFBP ligand blot analysis

Clinical-chemical parameters in serum were determined using a Cobas 311 system (Hitachi) or an AU480 autoanalyzer (Beckman-Coulter) and adapted reagents from Roche Diagnostics or Beckman-Coulter, respectively. Serum GH concentrations

were measured by an ELISA for rat/mouse GH (EZRMGH-45K; Merck) that cross-reacts with porcine GH. To calculate the area under the GH curve, values below the quantification limit of the assay (<0.07 ng/mL) were arbitrarily set to 0.07 ng/mL. IGF1 levels in serum were determined by RIA after dissociation of IGF1 from IGFBPs by acidification and blocking the IGF1 binding sites with an excess of IGF2 [25]. IGFBP ligand blot analysis of serum samples was performed as described previously [26] using serial dilutions of recombinant human IGFBP3 (41/38 kDa), IGFBP2 (32 kDa), IGFBP5 (29 kDa) and IGFBP4 (24 kDa) for quantification. Plasma insulin was determined using a species-specific RIA (Merck Millipore) as previously described [27]. Blood glucose levels were determined immediately using a Precision Xceed® glucometer and Precision XtraPlus® test strips (Abbott) [28]. Serum leptin levels were measured using a multi-species leptin radioimmunoassay (Cat. # XL-85K; EMD Millipore Corporation) that has been validated for porcine samples [29].

2.5. Growth parameters and body composition

Body weight and body length (distance between tip of the snout and tail root in straightened animals) of *GHR*-KO and control pigs were determined at weekly intervals. Relative body length was calculated by dividing the body length by the cube root of the body weight to retain the same dimensions. Since not all animals could be weighed/measured at exactly the same ages, raw data were adjusted by linear interpolation to defined ages/time points.

The percentage of total body fat was determined in 6-month-old *GHR*-KO and control pigs using dual-energy X-ray absorptiometry (DXA; Lunar *i*DXA, GE Healthcare) as previously described [30]. In addition, magnetic resonance imaging (MRI; Magnetom Open, Siemens) [31] was performed to visualize and determine the muscle to fat ratio as the area of longissimus dorsi muscle divided by the area of its overlying back fat at the last rib.

2.6. Necropsy

GHR-KO and control pigs were euthanized at 6 months of age under anesthesia by intravenous injection of T61® (Intervet) and immediately subjected to necropsy. Organs were dissected and weighed to the nearest mg. Tissue samples were collected as described previously [32] and routinely fixed in neutral buffered formalin solution (4 %) for 24 h or frozen immediately on dry ice and stored at -

80°C for molecular profiling. Formalin-fixed tissue specimens were embedded in paraffin. Muscle sections were stained with hematoxylin and eosin (H&E).

2.7. Immunoblot analysis of signaling cascades

The concentrations and phosphorylation status of GHR-related signaling molecules in the liver were evaluated by Western blot analyses as described previously [33]. Briefly, liver tissue samples were homogenized in Laemmli extraction buffer, and the protein content was determined by the bicinchoninic acid protein assay. Forty micrograms of total protein was separated by SDS-PAGE and transferred to PDVF membranes (Millipore) by electro-blotting. Membranes were washed in TBS with 0.1 % Tween-20 and blocked in 5 % w/v fat-free milk powder (Roth) for 1 hour. The membranes were then washed again and incubated in 5 % w/v BSA (Roth) solution with the appropriate primary antibodies overnight at 4°C. The antibodies and concentrations used are listed in **Suppl. Table 1**. After washing, the membranes were incubated in 5 % w/v fat-free milk powder solution with the secondary antibody (donkey anti-rabbit; 1:2000; GE Healthcare) for 1 hour. Bound antibodies were detected using the ECL Advance Western Blotting Detection Kit (GE Healthcare) and appropriate films from the same supplier. Band intensities were quantified using the ImageQuant software package (GE Healthcare).

2.8. Statistical analyses

Longitudinal data for body weight and body length or relative body length respectively were analyzed using PROC MIXED (SAS 8.2), taking the effects of pig line (#2529; #2533), group (*GHR*-KO; control), sex, age, and interaction group*age into account. Least squares means (LSMs) and standards errors (SEs) of LSMs were calculated for group*age and compared using Student's t-tests. Data for glucose homeostasis and serum lipid concentrations were analyzed using PROC GLM (SAS 8.2), taking the effects of group, sex, age and the interaction group*age into account. LSMs and SEs were calculated for group*age and compared using Student's t-tests. Body composition, organ weight and clinical chemical data were analyzed using PROC GLM taking the effects of group and sex into account. LSMs and SEs were calculated for groups and compared using Student's t-tests. IGFBP ligand blot and Western immunoblot data were evaluated for significant differences between *GHR*-KO and control pigs using the Mann-Whitney U test.

3. RESULTS

3.1. Generation of a growth hormone receptor-deficient pig model

We employed CRISPR/Cas9 technology to generate *GHR* knockout (*GHR*-KO*)* pigs as a large animal model for Laron syndrome (LS). *In vitro*-transcribed RNA encoding Cas9 and sgRNA specific for *GHR* exon 3 was injected into *in vitro* fertilized porcine oocytes, which were transferred to recipient gilts. In total, 8 piglets were born, of which 3 showed monoallelic mutations in the *GHR* gene. Two female founder animals carried monoallelic insertions of 1 bp (#2529) or 7 bp (#2533) (**Fig. 1A**). The two founder animals were mated with wild-type boars to establish pedigrees for phenotypic analyses of GHR-deficient (*GHR*-KO) vs. GHR-expressing F2 animals (**Suppl. Fig. 1**). The two lines were kept separate except in one experiment to test the fertility of *GHR*-KO pigs. Wild-type, heterozygous and *GHR*-KO littermates were identified by PCR and restriction fragment length polymorphisms of the mutated and wild-type *GHR* alleles (**Fig. 1B**). Since we did not observe significant differences between heterozygous *GHR* mutant and wild-type animals (**Suppl. Fig. 2**), they were pooled and used as the GHR-expressing control group.

3.2. Homozygous frameshift mutations in *GHR* exon 3 result in GHR deficiency

The insertion of 1 bp (#2529) or 7 bp (#2533) leads to a shift in the reading frame in *GHR* exon 3. The mutant *GHR* transcripts encode the 18-aa signal peptide and 51 aa of the extracellular GHR domain, followed by an 18-aa or a 20-aa missense sequence and premature termination codon after 87 aa (#2529) or 89 aa (#2533) (**Fig. 1C**).

The presence of GHR was investigated in liver and kidney sections since these tissues naturally express high levels of GHR [34]. To evaluate GH binding, the sections were incubated with recombinant GH, and bound GH was detected using specific antibodies. This ligand immunohistochemistry approach showed strong GH binding in control tissues, while GH binding was absent in tissue sections from *GHR*-KO animals (**Fig. 1D**).

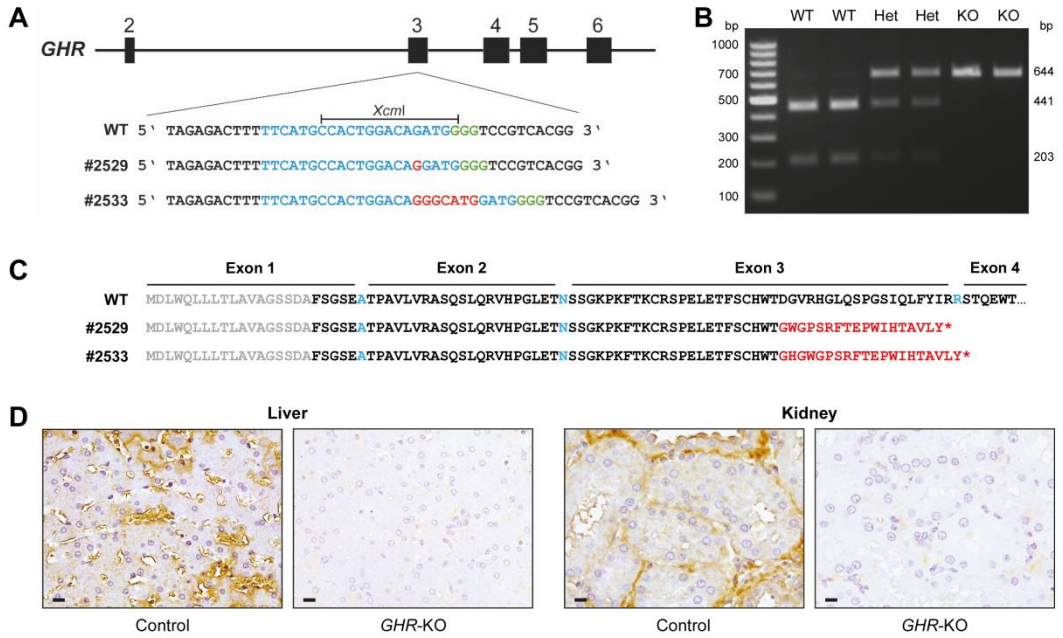


Figure 1. Generation of a GHR-deficient pig model using CRISPR/Cas technology. (A) Partial DNA sequence of *GHR* exon 3. The sgRNA binding site is indicated in blue and the protospacer adjacent motif (PAM) in green. Insertions (red) of 1 bp (founder #2529) or 7 bp (founder #2533) lead a shift of the reading frame. WT = wild type. (B) Restriction fragment length polymorphism analysis to detect the WT *GHR* sequence as well as monoallelic (Het) and biallelic (KO) mutations. (C) Partial amino acid sequences encoded by the WT and mutant *GHR* alleles. The signal peptide is shown in gray, WT GHR aa sequence in black (aa encoded by adjacent non-symmetrical exons in blue), missense aa sequence in red, and the premature termination codon as an asterisk. (D) Ligand immunohistochemistry demonstrating the absence of functional GHR (brown staining in control) in *GHR*-KO pigs. Chromogen: DAB; counterstain: Mayer's hemalum; bar = 10 µm.

3.3. Decreased serum IGF1 and IGFBP3, and increased IGFBP2 in *GHR*-KO pigs

GHR-KO pigs of all ages showed a marked reduction in serum insulin-like growth factor 1 (**Fig. 2A,B**). IGF binding proteins (IGFBPs) were evaluated by ligand blot analysis in 6-month-old animals using a dilution series of recombinant human IGFBPs for quantification (**Fig. 2C**). IGFBP3 was significantly decreased in *GHR*-KO pigs (426 ± 41 µg/L vs. 1775 ± 205 µg/L in control animals; $p < 0.0001$), while IGFBP2 was significantly increased (799 ± 53 µg/L vs. 607 ± 66 µg/L in control animals; $p = 0.0272$) (**Fig. 2D**).

3.4. *GHR*-KO pigs have high levels of circulating GH

To evaluate effects of GHR deficiency on the pulsatile secretion of GH, we collected serial blood samples at 30-min intervals over a period of 9 hours (starting at 11 a.m.) from five female and one male 9-month-old *GHR*-KO pig and from six

age-matched female controls. Serum GH levels of *GHR*-KO pigs were high with partially preserved pulsatility (area under the GH curve was increased 6.8-fold in *GHR*-KO compared with control pigs), indicating a disturbance in the negative feedback control of GH secretion (**Fig. 2E,F; Suppl. Fig. 3**).

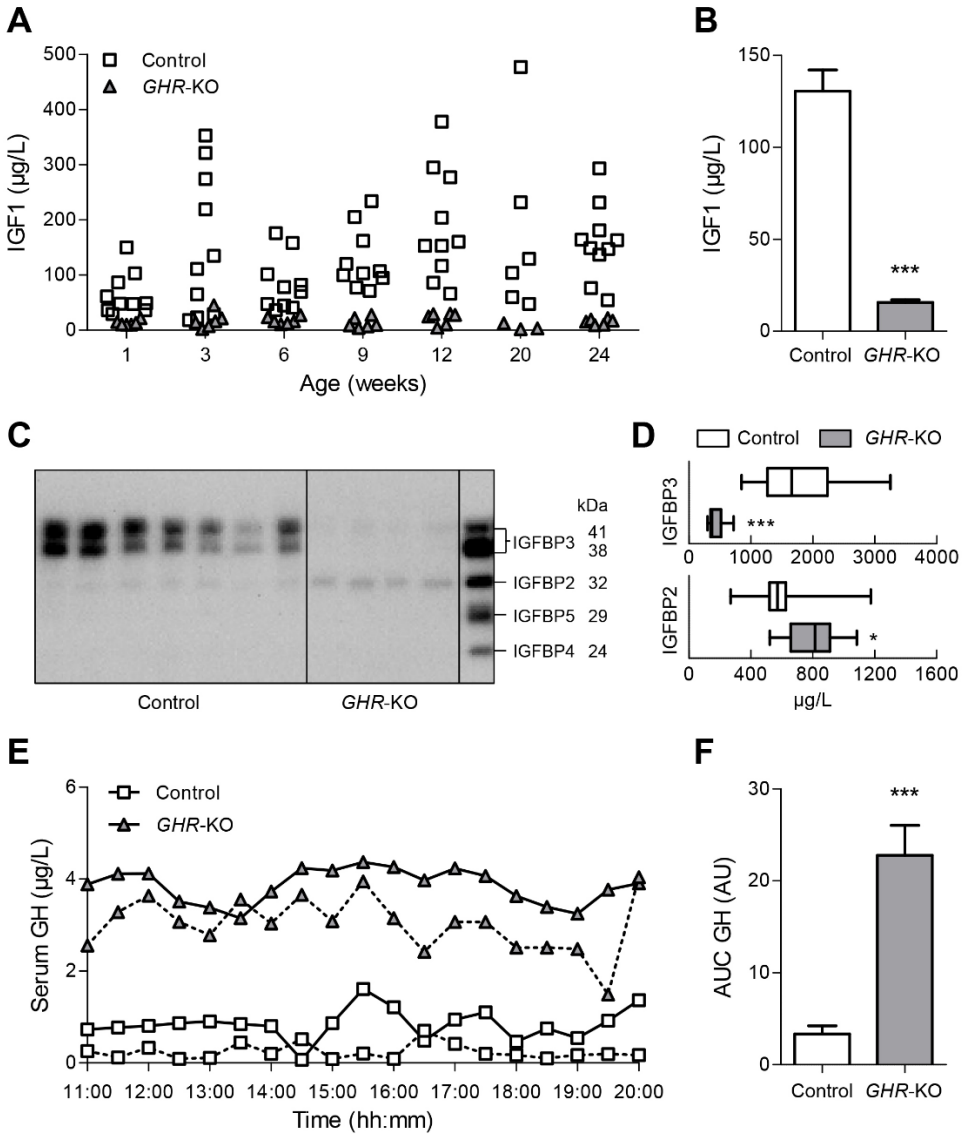


Figure 2. Serum IGF1, IGFBP and GH concentrations of *GHR*-KO compared with control pigs. (A) Scatter plot of serum IGF1 levels of *GHR*-KO and control pigs over time. (B) Means and standard deviations of all serum IGF1 values displayed in panel A (*GHR*-KO: n = 42; control: n = 69). (C) Representative IGFBP ligand blot. Right lane displays recombinant human IGFBP3 (41/38 kDa), IGFBP2 (32 kDa), IGFBP5 (29 kDa) and IGFBP4 (24 kDa). (D) Quantification of IGFBP3 and IGFBP2 in serum from *GHR*-KO (n = 10) and control pigs (n = 12). The figure shows medians, 25th and 75th percentiles (box), and extremes (whiskers). (E) Representative GH secretion profiles of two female GHR-KO and two female control pigs. (F) Area under the GH curve (AUC; means and standard deviations for 6 female *GHR*-KO and 5 female/1 male control pigs). AU = arbitrary units. *p < 0.05; ***p < 0.001.

3.5. *GHR*-KO pigs show severe growth retardation

The birth weight of *GHR*-KO piglets did not differ from control littermates. First significant growth retardation became obvious at five weeks of age ($p = 0.045$), leading to a 62 % reduction in body weight of 6-month-old *GHR*-KO pigs (33.0 ± 1.5 kg) compared with age-matched control animals (86.2 ± 1.1 kg; $p < 0.0001$) (**Fig. 3A,B**). Body length at birth did not differ between *GHR*-KO and control piglets (33.9 ± 1.4 cm vs. 37.3 ± 1 cm; $p = 0.4068$). Significant differences in body length appeared at four weeks of age (52.2 ± 1.4 cm in *GHR*-KO vs. 60.3 ± 1 cm in control animals; $p < 0.0001$). At six months of age, the body length of *GHR*-KO pigs was reduced by 27 % compared with control pigs (100.6 ± 1.6 cm vs. 138.1 ± 1.4 cm; $p < 0.0001$) (**Fig. 3C**). Up to an age of four months, weight gain was more affected than linear growth by GHR deficiency, as indicated by an increased relative body length (body length divided by the cube root of body weight) of *GHR*-KO pigs (**Fig. 3D**). No growth parameters exhibited significant sex-related differences.

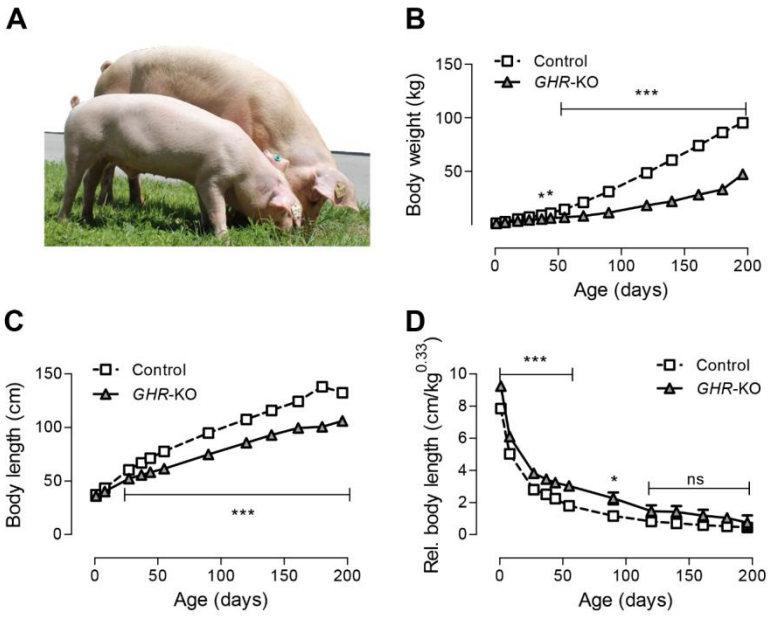


Figure 3. Body weight gain and growth of *GHR*-KO compared with control pigs. (A) *GHR*-KO pig (front) and control littermate aged 6 months. (B) Body weight gain. (C) Body length. (D) Relative body length (body length divided by the cube root of body weight. These parameters were determined in 12 *GHR*-KO and 25 control pigs. Panels A-D show least squares means (LSM) and standard errors of LSM estimated for group*age (see 2.8 for the statistical model). *p < 0.05; **p < 0.01; ***p < 0.001; ns = not significant.

3.6. *GHR*-KO pigs show an increased proportion of body fat and a reduced ratio of muscle to fat tissue

DXA analysis revealed a markedly increased percentage of total body fat in 6-month-old *GHR*-KO pigs (21.5 ± 0.7 % vs. 11.4 ± 0.5 % in age-matched control animals; $p < 0.0001$) (**Fig. 4A**). While female pigs in the control group displayed

significantly higher body fat content than male pigs (13.2 ± 0.7 % vs. 9.6 ± 0.7 %; p = 0.0009), no sex-related differences were observed in *GHR*-KO pigs (22.9 ± 1 % in males vs. 22 ± 1 % in females; p = 0.4530). To determine the ratio of muscle to fat tissue, MRI scans were performed at the location of the last rib, and the volume ratio of the longissimus dorsi muscle and its overlying back fat was calculated (**Fig. 4B,C**). *GHR*-KO pigs showed a significantly reduced muscle to fat tissue ratio compared with control pigs (3.0 ± 0.6 vs. 8.2 ± 0.4; p < 0.0001) (**Fig. 4B**). No sex-related differences were observed in this parameter (**Suppl. Table 2**).

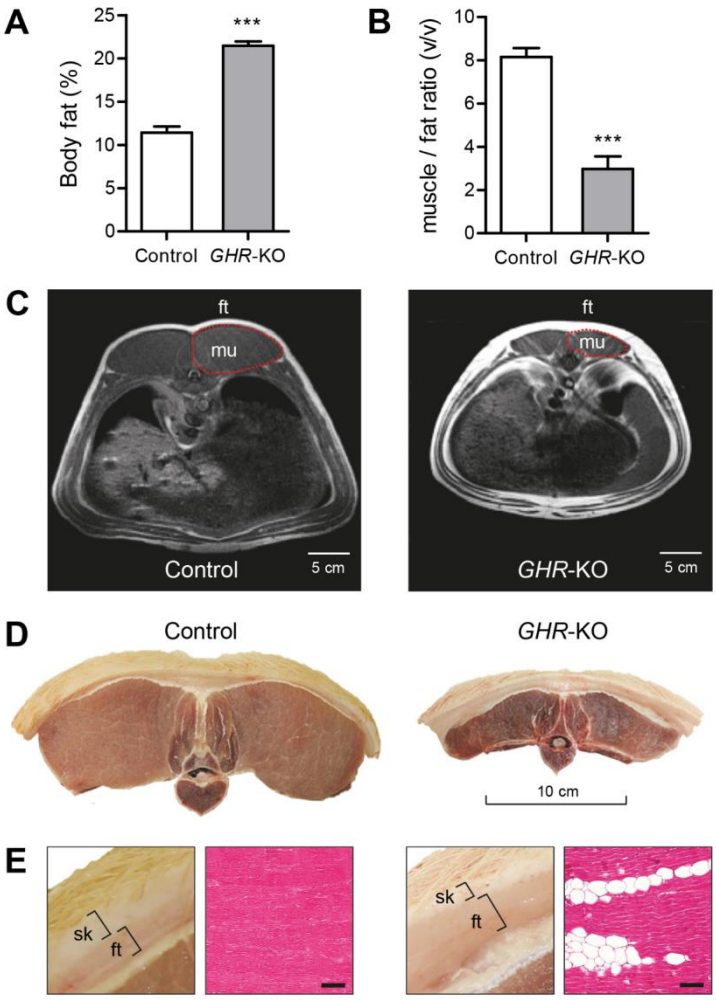


Figure 4. Body composition of 6-month-old *GHR*-KO compared with control pigs. (A) DXA analysis revealed a significantly higher amount of total body fat in *GHR*-KO pigs. (B) The calculated ratio of muscle to fat tissue from MRI images at the last rib revealed a significant shift towards fat tissue in *GHR*-KO pigs (*GHR*-KO: n = 12; control: n = 25; ***p < 0.001). Panels A and B show least squares means (LSM) and standard errors of LSM estimated for the 2 groups (see 2.8 for the statistical model). (C) Representative magnetic resonance images used to evaluate the volume of the longissimus dorsi muscle (mu) and its overlying back fat (ft) at the last rib in *GHR*-KO and control pigs. Note the larger subcutaneous and visceral fat depots in *GHR*-KO pigs. (D) Representative macroscopic cross-sections of the first lumbar vertebra, the two longissimus dorsi muscles and the overlaying back fat and skin. (E) Higher magnification of D showing an increased ratio of subcutaneous fat (ft) to skin (sk) thickness in a *GHR*-KO compared with a control pig. Histological section (H&E stain) showing an increased amount of intramuscular fat in *GHR*-KO pigs (bar = 100 µm).

The MRI findings were confirmed upon necropsy, showing a marked increase in the thickness of the subcutaneous fat tissue and a reduction in the size of the longissimus dorsi muscle in *GHR*-KO pigs (**Fig. 4D,E**). Histological sections of skeletal muscle samples from *GHR*-KO pigs revealed markedly increased numbers of adipocyte section profiles between muscle fibers (**Fig. 4E**).

3.7. Disproportionate organ growth of *GHR*-KO pigs

In 6-month-old *GHR*-KO pigs, absolute weights of all organs were significantly smaller than in age-matched control animals (**Fig. 5, Suppl. Table 3**). Most organ weights of *GHR*-KO pigs were reduced proportionally to body weight. However, the relative weights of liver (73 % of control animal relative liver weight;

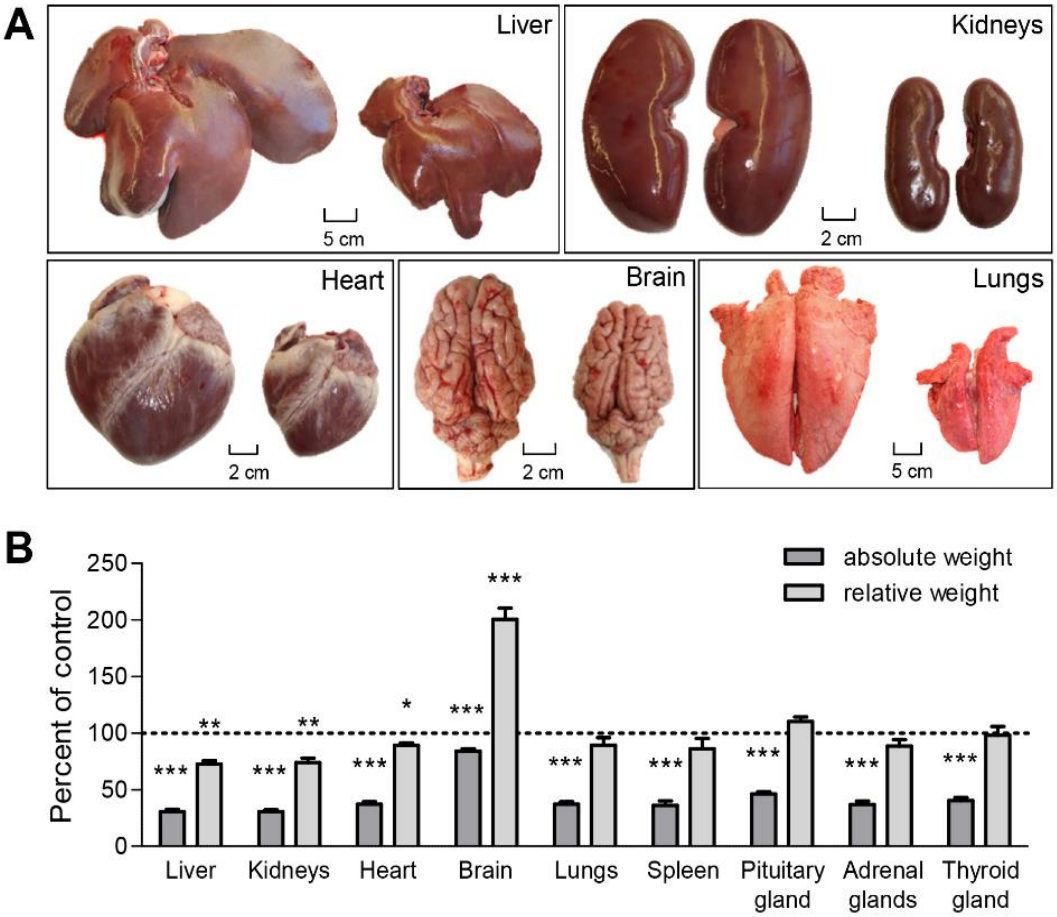


Figure 5. Disproportionate organ growth in *GHR*-KO compared with control pigs. GHR-deficiency led to a proportionate and disproportionate reduction in organ sizes. (A) Representative organs from control (left) and *GHR*-KO pigs (right). (B) Relative differences between *GHR*-KO and control pigs in absolute organ weights and in organ weight-to-body weight ratios (relative organ weights). These parameters were determined in 9 *GHR*-KO and 25 control pigs, and least squares means (LSM) and standard errors of LSM were estimated for the 2 groups (see 2.8 for the statistical model). *p < 0.05; **p < 0.01; ***p < 0.001.

p = 0.0005), kidneys (73 % of control animal relative kidney weight; p = 0.0002) and heart (87 % of control animal relative heart weight; p = 0.0119) were significantly reduced, while relative brain weight was doubled (200 % of control animal relative brain weight; p < 0.0001) (**Fig. 5, Suppl. Table 3**).

3.8. Male and female GHR-KO pigs are fertile

The ovaries of 6-month-old *GHR*-KO gilts did not show obvious morphological differences from control ovaries. Mating of an 8-month-old *GHR*-KO boar (line #2529) with a *GHR*-KO sow (line #2533) of the same age resulted in a litter of 6 healthy *GHR*-KO piglets (**Suppl. Fig. 4A**). Their birth weight tended to be reduced in comparison to *GHR*-KO piglets derived from heterozygote × heterozygote matings (on average 0.75 kg compared with 1.3 kg). However, the animals showed catch-up growth and achieved a higher body weight at 6 months of age than *GHR*-KO offspring from heterozygous *GHR*-KO parents (43.8 ± 1.3 kg vs 33.0 ± 2.2 kg; p < 0.0001; **Suppl. Fig. 4B**).

3.9. *GHR*-KO pigs show transient hypoglycemia, while insulin levels remain unaffected

Young *GHR*-KO pigs (12 to 15 weeks old) showed significantly reduced fasting blood glucose levels (41.5 ± 3.8 mg/dL vs. 63.1 ± 3.1 mg/dL in age-matched control animals; p = 0.0001), while this difference disappeared in older animals (23 to 27 weeks) (**Fig. 6A**). Fasting serum insulin levels of *GHR*-KO pigs did not differ from control pigs at any age (**Fig. 6B**). In addition, the homeostatic model assessment (HOMA) for evaluating insulin resistance was calculated from fasting glucose and insulin concentrations. In agreement with their low fasting blood glucose concentrations, HOMA-IR values of young *GHR*-KO pigs tended to be reduced (0.25 ± 0.20 vs. 0.76 ± 0.19 in age-matched control pigs; p = 0.0635), but increased to 0.76 ± 0.18 in the older age group (p = 0.0839), when controls had a HOMA-IR of 0.43 ± 0.20 (p = 0.2584; **Fig. 6C**). Analysis of variance revealed a significant (p < 0.05) interaction of group*age.

3.10. Serum lipid levels are reduced in young *GHR*-KO pigs

Young *GHR*-KO pigs (aged 12 to 15 weeks) revealed significantly decreased serum concentrations of triglycerides (23.2 ± 6.8 mg/dL vs. 42.3 ± 4.9 mg/dL in controls; p = 0.021; **Fig. 6D**), cholesterol (70 ± 3.5 mg/dL vs. 81.8 ± 2.8 mg/dL in controls;

p = 0.0129; **Fig. 6E**), low-density lipoprotein (LDL)-cholesterol (42.8 ± 2.7 mg/dL vs. 50.1 ± 2.1 mg/dL in controls; p = 0.0392; **Fig. 6F**) and high-density lipoprotein (HDL)-cholesterol (29.0 ± 1.6 mg/dL vs. 33.8 ± 1.3 mg/dL in controls; p = 0.0224; **Fig. 6G**). In older *GHR*-KO pigs (23 to 27 weeks), serum triglyceride, cholesterol and LDL-cholesterol concentrations were similar to those of control pigs, while serum HDL-cholesterol levels were increased (32.5 ± 1.3 mg/dL vs. 28.6 ± 1.0 mg/dL in controls; p = 0.0272) (**Fig. 6D-G**). Excluding triglycerides, all investigated lipid parameters were significantly (p < 0.0002) affected by sex, with higher levels in female than in male animals (**Suppl. Table 4**).

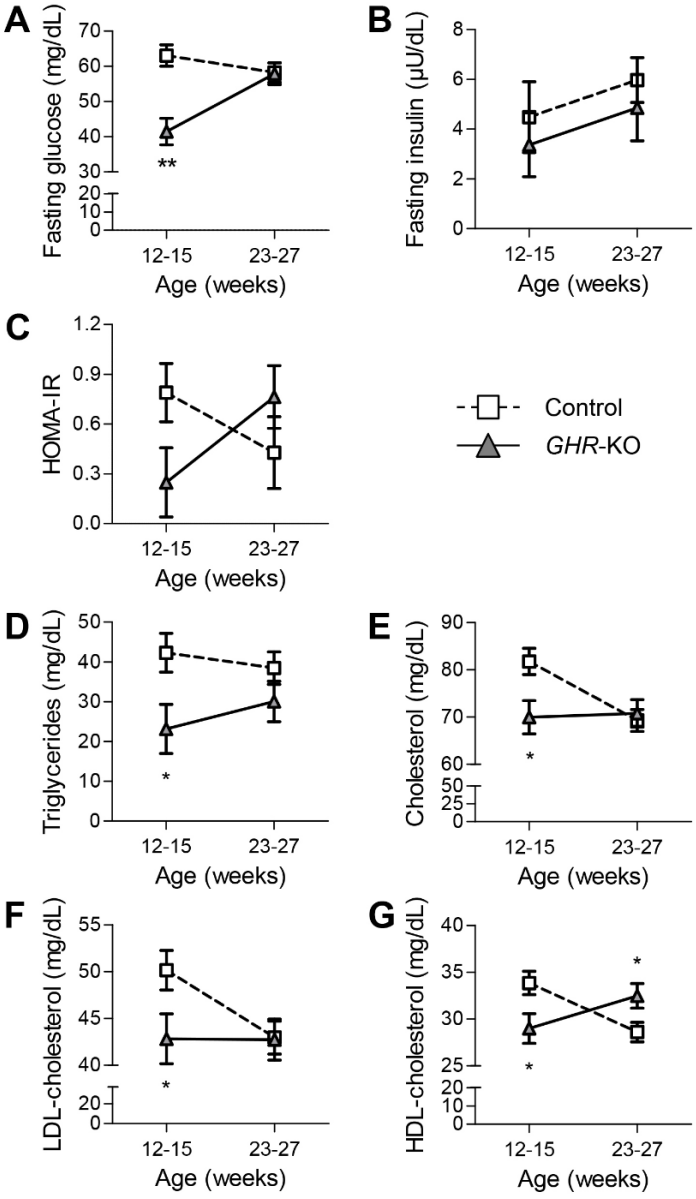


Figure 6. Age-dependent changes in glucose and lipid homeostasis parameters in *GHR*-KO and control pigs. (A) Transient juvenile hypoglycemia in *GHR*-KO pigs. B) Unchanged serum insulin concentrations. C) Initially lower, then higher HOMA-IR score (interaction group*age: p < 0.05). Serum concentrations of (D) triglycerides, (E) cholesterol, (F) low-density lipoprotein (LDL) and (G) high-density lipoprotein levels were significantly lower in young *GHR*-KO pigs than in age-matched controls, but normalized with age. HDL levels of 23- to 27-week-old *GHR*-KO pigs were even higher than in their control littermates. At least 6 animals per group and age-class were investigated. Panels A-G show least squares means (LSM) and standard errors of LSM estimated for group*age (see 2.8 for the statistical model). *p < 0.05; **p < 0.01.

3.11. Additional alterations of clinical-chemical parameters in *GHR*-KO pigs

To screen for changes in organ functions and metabolic pathways, a broad spectrum of clinical-chemical parameters in serum of 6-month-old *GHR*-KO and control pigs were analyzed. While all parameters remained within physiological ranges, *GHR*-KO pigs displayed lower levels of creatinine (91.2 ± 6.5 µmol/L vs. 126.4 ± 5.0 µmol/L in controls; $p = 0.0004$) but higher levels of urea (7.4 ± 0.5 mmol/L vs. 4.3 ± 0.3 mmol/L in controls; $p < 0.0001$) (**Suppl. Table 5**).

3.12. *GHR*-KO pigs display significant changes in the activation of hepatic signaling cascades

Since the liver is a major target tissue of insulin and GH, we evaluated changes in associated signaling cascades by Western blot analyses of liver samples from fasted 6-month-old *GHR*-KO and control animals.

The total amount of insulin receptor (INSR) and of phosphorylated INSR were unchanged in *GHR*-KO liver samples. In contrast, the concentrations of total and phosphorylated insulin receptor substrate 1 (IRS1) were significantly ($p = 0.0159$) increased in liver samples from *GHR*-KO vs. control pigs. The investigation of signal transducers downstream of the INSR revealed significantly increased phosphorylation of phosphoinositide 3 kinase (PI3K) and a trend ($p = 0.0635$) toward an increase in serine/threonine protein kinase AKT phosphorylation in *GHR*-KO liver samples. Furthermore, a trend ($p = 0.0635$) toward increased levels of total peroxisome proliferator-activated receptor gamma (PPARG) was observed (**Fig. 7A**).

GHR-KO liver samples showed significantly increased phosphorylation levels of Janus kinase 2 (JAK2). The phosphorylation levels of *signal transducer and activator of transcription 5* (STAT5) were significantly reduced, while STAT3 phosphorylation showed a tendency to increase ($p = 0.1111$). Phosphorylation of STAT1 was not significantly different between *GHR*-KO and control pigs, whereas significantly increased phosphorylation levels of mitogen-activated protein kinase (MAPK) were detected (**Suppl. Fig. 5**).

In addition, liver extracts from *GHR*-KO pigs showed a significant increase in phosphorylated mechanistic target of rapamycin (mTOR), which was not detected in control liver samples. To distinguish between the activation of mTOR complex

1 (mTORC1) and 2 (mTORC2), we analyzed several specific up– and downstream signal transducers for each complex [35, 36] (**Fig. 7B; Suppl. Fig. 5**).

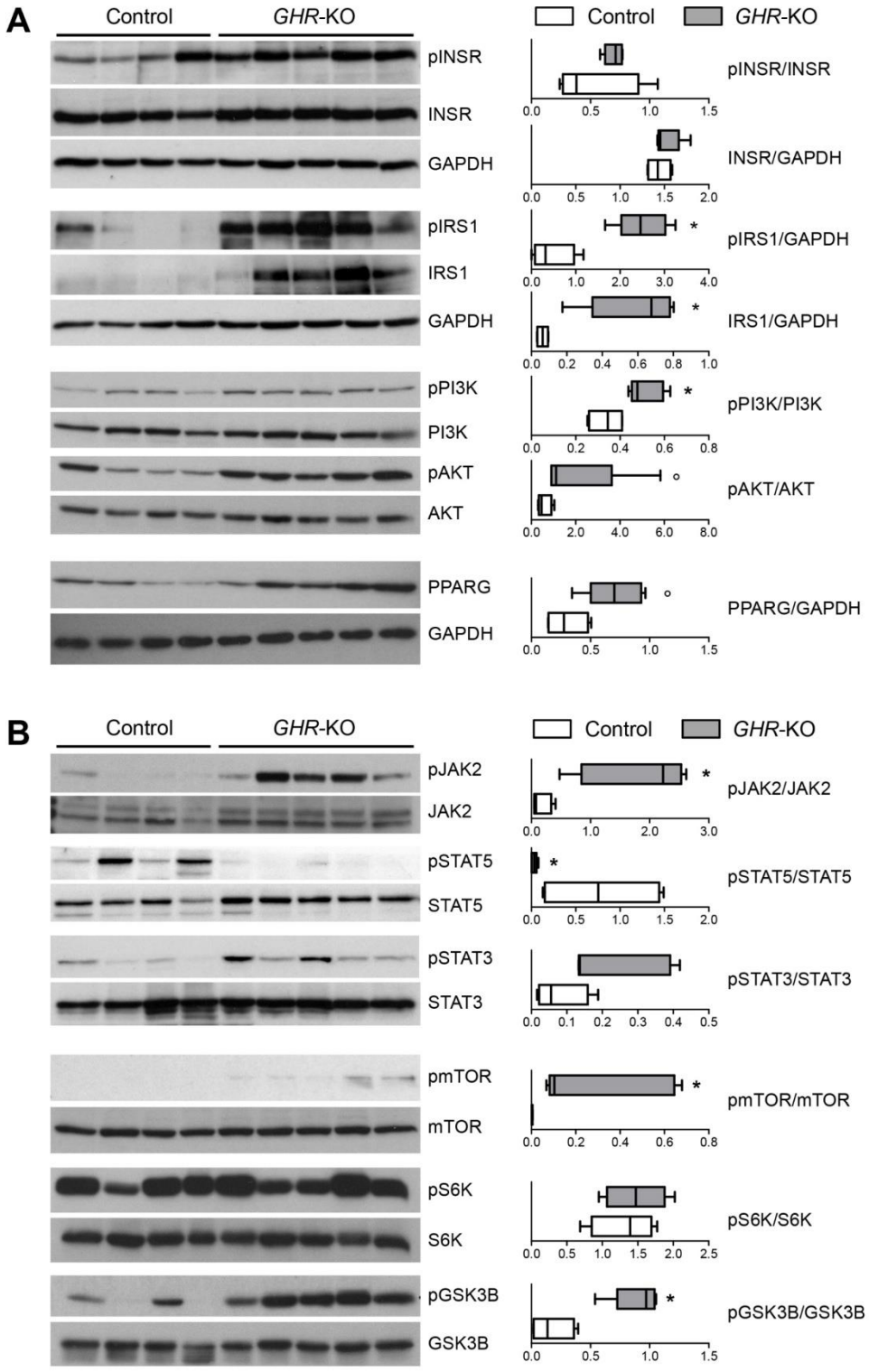


Figure 7. Western blot analysis of signaling cascades in liver samples of 6-month-old fasted *GHR*-KO (n = 5) and control pigs (n = 4). (A) Insulin receptor-related signaling pathway and PPARG. (B) GHR- and mTOR-related signaling pathways. The box plots show medians, 25th and 75th percentiles (box), and extremes (whiskers). *p < 0.05; °p = 0.0635; evaluated using the Mann-Whitney U test.

A key element of mTORC1 action is protein S6 kinase 1 (S6K), which phosphorylates S6. *GHR*-KO liver samples did not show increased phosphorylation of S6K (**Fig. 7B**). Phosphorylation of other effectors downstream of mTORC1 - eukaryotic initiation factor 4E binding protein 1 (4EBP1) and eukaryotic translation initiation factor 4E (eIF4E) - was unchanged in *GHR*-KO liver samples (**Suppl. Fig. 5**). Phosphorylation of AMP-activated protein kinase (AMPK), an inhibitor of mTORC1, was significantly increased in *GHR*-KO liver tissue (**Suppl. Fig. 5**). Collectively these data suggest that mTORC1 is not activated in liver of *GHR*-KO pigs.

In contrast, one of the most important downstream substrates of mTORC2 - glycogen synthase 3 beta (GSK3B) – showed significantly increased phosphorylation in *GHR*-KO liver tissue (**Fig. 7B).** Furthermore, the phosphorylation levels of potent regulators of mTORC2 action, PI3K and AKT, were also increased (**Fig. 7A**). These findings suggest that mTORC2 is activated in the liver of GHR-deficient pigs. A summary of these findings is provided in **Fig. 8A**.

3.13. Serum leptin levels and hepatic leptin receptor activation are increased in *GHR*-KO pigs

Considering the expected reduced STAT5 phosphorylation but unexpected increased JAK2 phosphorylation in the liver of *GHR*-KO pigs, we measured serum leptin levels and hepatic leptin receptor (LEPR) activation. LEPR signaling involves activation of JAK2 and STAT3, but not STAT5 (reviewed in 37). Overall, serum leptin concentrations of 6-month-old fasted *GHR*-KO pigs were significantly elevated compared with the controls (**Fig. 8B**), with higher levels in females than in males (**Suppl. Table 2**). In addition, LEPR phosphorylation was significantly increased in *GHR*-KO vs. control liver samples (**Fig. 8C**).

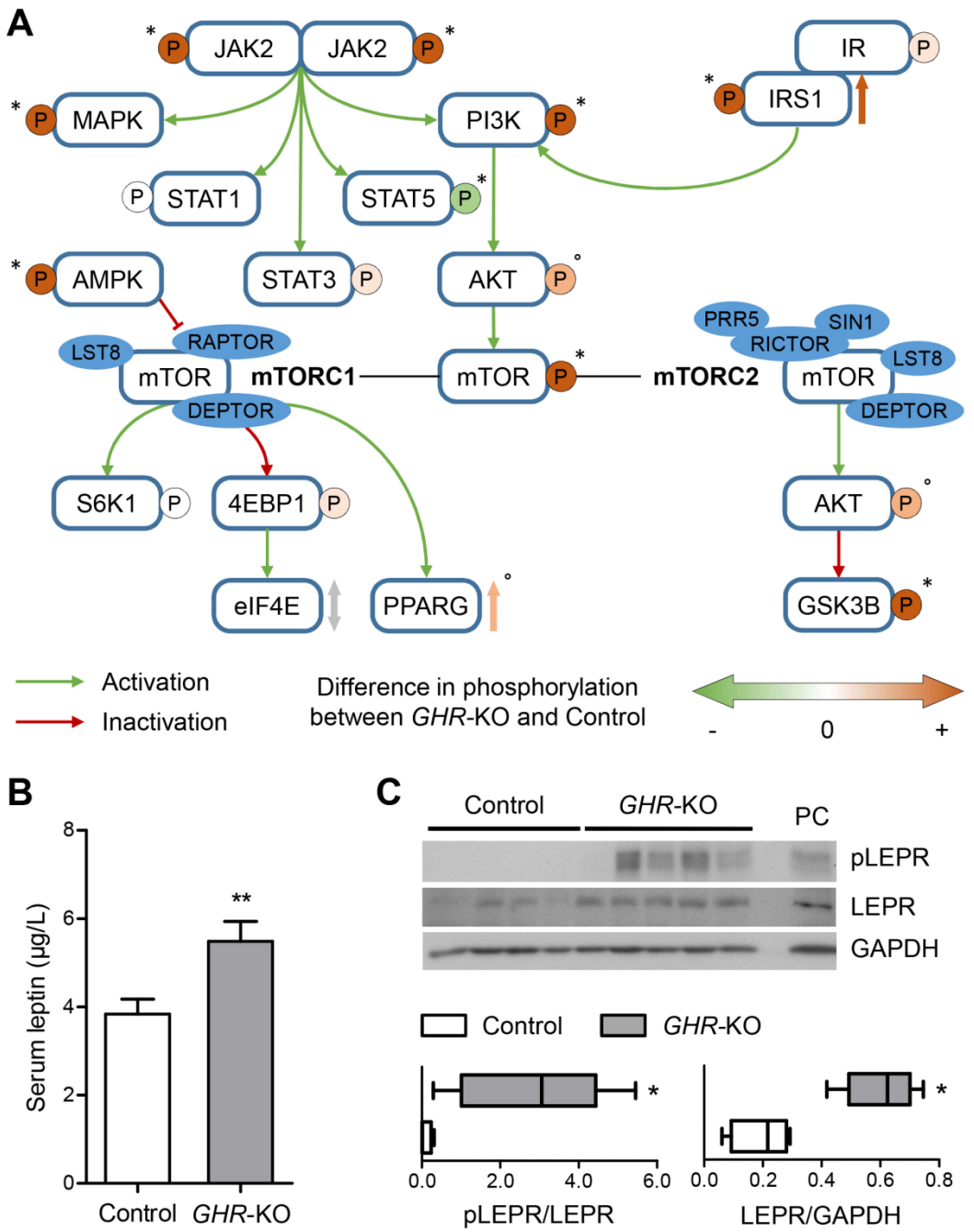


Figure 8. (A) Schematic summary of the changes in phosphorylation in INSR- and GHR-related signaling molecules in liver samples of *GHR*-KO compared with control pigs. *p < 0.05; °p = 0.0635; evaluated using the Mann-Whitney U test. (B) Significantly increased fasting serum leptin concentrations in 6-month-old *GHR*-KO vs. control pigs. The figure shows the estimated least squares means (LSM) and standard errors of the LSM for the two groups, taking into account the effect of sex (9 male/13 female control pigs; 6 male/6 female *GHR*-KO pigs). **p < 0.01 for the effect of group (PROC GLM). (C) Significantly increased expression and phosphorylation of LEPR in liver samples from *GHR*-KO compared with control pigs. PC = protein lysate from choroid plexus of a wild-type pig used as positive control. *p < 0.05; evaluated using the Mann-Whitney U test.

4. DISCUSSION

This study reports a new large animal model for GHR deficiency (Laron syndrome, LS). Previously described pig models with impaired GH function, which are either GHR-deficient [38] or express a dominant negative GHR [39], were established on a minipig background that is already growth impaired and may thus not resemble all pathophysiological consequences of human LS. Therefore, we established our GHR-deficient model on a German landrace background with the physiological growth potential of domestic pigs and performed a comprehensive characterization of body and organ growth, body composition, endocrine and metabolic changes, and signaling cascades in liver.

Our GHR-deficient pig model exhibits important hallmarks of human LS, such as insensitivity to GH, low circulating IGF1 concentrations, and reduced postnatal body and organ growth. Similar findings have been reported for minipig models with impaired GH function [38, 39]. Our study provides a more comprehensive phenotypic analysis, including serial GH measurements, effects on serum IGFBPs, age-dependent changes in glucose homeostasis and lipid profiles, and analyses of hepatic signaling cascades.

Circulating IGF1 originates in large part from the liver, as shown by liver specific *Igf1* KO mice [40], and is complexed by high-affinity IGFBPs, with IGFBP3 and an acid labile subunit (ALS) forming a 150-kDa complex, which is the main reservoir of IGF1 in the bloodstream (reviewed in 41, 42). ALS is directly regulated by GH, and reduced ALS levels are observed in GHR-deficient patients [43]. The production of IGFBP3 is also stimulated by GH [44], and GHR deficiency in mice and humans leads to reduced levels of IGFBP3 in the circulation [15, 45]. In agreement with these reports, serum IGFBP3 concentrations of *GHR*-KO pigs were significantly reduced, presumably leading to a decrease in the IGF1 reservoir in the circulation and thus to a shortened half-life. In addition, the concentration of IGFBP2 was increased in serum of *GHR*-KO pigs. Studies in transgenic IGFBP2-overexpressing mice have shown that IGFBP2 inhibits the growth of normal [46] and even of GH-overexpressing mice [47]. Therefore, the reduced growth of *GHR*-KO pigs most likely results from a combination of a decline in the production and half-life of circulating IGF1 and, possibly, IGF1 sequestration by inhibitory IGFBP2.

Circulating IGF1 is also responsible for the feedback inhibition of pituitary GH secretion (reviewed in 48). In accordance with this concept, *GHR*-KO pigs with low serum IGF1 levels showed high serum GH concentrations with partially preserved pulsatility. Serial blood samples for the analysis of hormone profiles can be obtained easily from pigs equipped with permanent central venous catheters [49], whereas serial blood sampling in mice is more difficult.

Interestingly, a growth deficiency phenotype of *GHR*-KO pigs was observed no earlier than postnatal week 5, which indicated that GH was not required as promoter of intrauterine and early postnatal growth. This finding is consistent with observations in infants with LS [50] and in neonatal *Ghr* KO mice [15]. Instead, intrauterine growth depends on IGF2 and IGF1, the latter of which acts independently of GH during this period of development [51]. An essential role of GH as a postnatal growth promoter has been established during the maturation of the endocrine growth axis, which is associated with an increase in expression of hepatic GHR [52]. In general, intrauterine maturation of endocrine functions in pigs is similar to humans, whereas rodents are born in a more immature state (reviewed in 53).

In agreement with observations in human LS patients [54] and *Ghr* KO mice [55], *GHR*-KO pigs displayed an increase in total body fat and a decrease in the muscle to fat ratio. Adipose tissue mass is determined by the storage and removal of triglycerides in adipocytes. A recent study in which human adipocyte lipid age was determined by measuring ^{14}C from nuclear bomb tests revealed that triglycerides are renewed up to 6 times during the average adipocyte life-span of 10 years [56]. In *GHR*-KO pigs, physiological adipocyte lipid turnover is apparently disturbed at different levels. First, the lipolytic action of GH via an increase in adipose tissue hormone-sensitive lipase (HSL) activity (reviewed in 16, 57) is lost in the absence of GHR. Second, the synthesis of storage lipids in adipocytes is likely to increase in *GHR*-KO pigs. This phenomenon requires hydrolysis by lipoprotein lipase (LPL) of the triglyceride component of circulating chylomicrons and very low-density lipoproteins (VLDL) into free fatty acids and 2-monoacylglycerol, which can be taken up by adipocytes. Adipose tissue LPL activity is increased by insulin but inhibited by GH and sex steroids (reviewed in 58). Reduced serum triglyceride levels in young *GHR*-KO pigs (12-15 weeks) with normal insulin levels, but lacking the counteracting effects of GH and sex steroids, may reflect an increased use of

triglycerides for lipid synthesis in adipocytes, leading to an increase in total body fat. In older *GHR*-KO pigs (23-27 weeks), the increased total body fat content may lead to decreased triglyceride turnover of adipocytes [56], limiting the use of circulating triglycerides for storage lipid synthesis in adipocytes. Furthermore, the animals became sexually mature, with sex steroids potentially inhibiting adipose tissue LPL and the hydrolysis of serum triglycerides, thus resulting in normal serum triglyceride levels. Decreased serum cholesterol levels, as observed in young *GHR*-KO pigs, have also been reported in human LS patients [3] and *Ghr* KO mice (reviewed in 16).

Measurements of organ weights in *GHR*-KO pigs revealed that growth of liver, kidneys and heart is particularly dependent on GHR/GH action, as their relative weights were significantly decreased in comparison to control pigs. Reduced relative liver and kidney weights have also been observed in *Ghr* KO mice [55], and interestingly LS patients have disproportionately reduced cardiac dimensions [59]. The important role of the GH/IGF1 system in the growth of these organs is supported by their disproportionate overgrowth in conditions of GH/IGF1 excess, as in GH-overexpressing transgenic mice [60, 61] and in patients with acromegaly [62]. In contrast, brain growth is less dependent on intact GHR signaling, as shown by a relatively moderate reduction in absolute brain weight and a marked increase in the relative brain weight of *GHR*-KO pigs. Based on similar observations in *Ghr* KO mice, Sjogren et al. [63] speculated that a large proportion of brain growth occurs relatively early in development in a GH-independent stage.

Patients with Laron syndrome exhibit delayed puberty, but they are able to achieve full sexual development and reproduce [2]. In *Ghr* KO mice, delayed puberty and reduced litter sizes have been observed. The latter has been attributed to reduced ovarian function due to the lack of IGF1 (reviewed in 16). To assess the fertility of *GHR*-KO pigs, a *GHR*-KO boar and a *GHR*-KO sow were mated, resulting in a litter of 6 healthy piglets. Although not significant, the mean birth weight of these piglets was 40 % lower than that of *GHR*-KO piglets from a heterozygote x heterozygote mating, most likely because of the limited fetal growth capacity of a smaller mother. Interestingly, *GHR*-KO piglets from homozygous parents showed catch-up growth and had a 33 % higher body weight at six months than *GHR*-KO piglets from heterozygous parents. An explanation for this increased growth performance may be hybrid vigor, potentially resulting from the mating of *GHR*-

KO pigs from two different lines (**Suppl. Fig. 1**).

GHR-KO pigs showed juvenile hypoglycemia that normalized when the animals reached sexual maturity. Hypoglycemia is a hallmark of juvenile age LS [3, 64] and has been mainly explained by the lack of stimulatory GH effects on hepatic gluconeogenesis (reviewed in 57). Contrasting observations have been obtained concerning the role of altered insulin sensitivity in the development of juvenile hypoglycemia (reviewed in 65). While no evidence for increased insulin sensitivity, and even some cases of insulin-resistant diabetes mellitus, have been reported in the Israeli cohort of LS patients [64, 66], individuals from the Ecuadorian LS cohort are more insulin-sensitive than their *GHR* intact relatives [67], and no cases of diabetes mellitus were reported [7]. The reasons for this discrepancy remain elusive [65]. Although HOMA-IR is not a routinely established parameter to assess insulin (in)sensitivity in pigs (reviewed in 68), the trend ($p=0.0635$) toward lower HOMA-IR scores in young *GHR*-KO vs. control pigs suggests that increased insulin sensitivity is a contributing factor to juvenile hypoglycemia, as also observed in some *Ghr* KO mouse models (69; reviewed in 16). While the HOMA-IR scores of *GHR*-KO pigs increased with age, and tended to be higher in sexually mature *GHR*-KO than in age-matched control pigs (interaction group*age: $p < 0.05$), there was no evidence for insulin resistance. Interestingly, we observed increased levels of total and phosphorylated IRS1 in liver samples from fasted adult *GHR*-KO pigs, which could represent a mechanism for increased insulin sensitivity. Similar observations and conclusions were obtained in GH-deficient Ames dwarf mice with increased insulin sensitivity [70]. Future studies involving state-of-the-art *in vivo* measurements of gluconeogenesis [71] and investigation of insulin sensitivity using hyperinsulinemic, euglycemic clamp studies in juvenile and sexually mature *GHR*-KO and control pigs will help to clarify the relative contributions of these mechanisms to juvenile hypoglycemia in LS and its normalization in adult LS patients. Such studies can be performed more accurately in pigs than in rodent models due to their larger size [49].

While all clinical-chemical parameters measured in the serum of *GHR*-KO pigs remained within the normal reference ranges for pigs, creatinine levels were reduced and serum urea concentrations were increased compared with the control pigs. The serum creatinine concentration correlates with muscle mass [72], which explains the reduced levels in *GHR*-KO pigs and in LS patients [3]. Increased serum

concentrations of urea, the end product of amino acid catabolism in mammals, have also been detected in *Ghr* KO mice [69] and are most likely due to IGF1 deficiency and the lack of its protein anabolic action (reviewed in 73).

Since the liver is a major target organ for GH, we investigated alterations in the activity of selected hepatic signaling pathways in adult fasted *GHR*-KO and control pigs. Upon binding of GH, the GHR homodimer undergoes a conformational change that brings the originally parallel receptor transmembrane domains into a rotated crossover orientation, thus separating the lower parts of the transmembrane helices. Thereby, the two JAK2 molecules that are associated with the membrane proximal Box1 motif of the GHR chains are separated, and the inhibitory pseudokinase domain of one JAK2 is removed from the kinase domain of the other JAK2 and vice versa [74]. The kinase domains of the 2 JAK2 molecules can then be transactivated and initiate tyrosine phosphorylation of the GHR cytoplasmic domains and STAT5, the key transcription factor mediating most genomic actions of GH (reviewed in 75), including stimulation of *IGF1* gene expression [76]. In agreement with this concept, the phosphorylation of STAT5 in liver of *GHR*-KO pigs was significantly reduced and the circulating IGF1 levels were markedly decreased compared with control pigs. However, unexpectedly, phosphorylation of JAK2 was significantly increased in liver of *GHR*-KO pigs, together with a significant increase in the phosphorylation of MAPK, PI3K, and mTOR, which are known to be – directly or indirectly – activated by JAK2 [77].

In addition to GHR, many other class I cytokine receptors also use the non-receptor tyrosine kinase JAK2 for signaling, including the receptors for erythropoietin, prolactin, interleukins 3, 5 and 6, granulocyte-macrophage colony-stimulating factor, interferon-gamma, thrombopoietin, and leptin (reviewed in 78). GHR is abundantly expressed in liver and can dimerize and associate with JAK2 in the absence of GH (reviewed in 75). Since GHR-bound JAK2 can only be activated in the presence of GH, elimination of GHR may increase the pool of JAK2 that can be phosphorylated by other class I cytokine receptors. In addition, the loss of GHR may alter the abundance of these receptors or their ligands and thus lead to increased JAK2 phosphorylation.

A candidate is leptin, since increased serum leptin levels have been detected in LS patients [79] and in the *Ghr* KO mouse model (reviewed in 16). Leptin is known to induce the expression of its receptor in liver [80], and leptin receptor activation

involves the phosphorylation of JAK2 and STAT3, but not STAT5 (reviewed in 37). Consistent with this hypothesis, we observed significantly increased serum leptin levels and increased expression and phosphorylation of leptin receptors in liver of *GHR*-KO pigs, providing a potential explanation for the significantly increased phosphorylation of JAK2, while phosphorylation of STAT5 was significantly reduced.

Phosphorylation of the serine/threonine kinase mTOR that forms the catalytic core of mTOR complex-1 (mTORC1) and mTORC2 was significantly increased in *GHR*-KO pigs. While mTOR phosphorylation was not directly investigated in *Ghr* KO mice, Dominick et al. [35] have evaluated the activities of mTORC1 and mTORC2 in fed and fasted animals based on the phosphorylation status of downstream substrates of these complexes. In fasted *Ghr* KO mice, the authors observed reduced phosphorylation of the ribosomal protein S6, while the phosphorylation status of S6K and 4EBP1 was unaltered compared with control mice. In fed *Ghr* KO mice, the phosphorylation status of all three indicators of mTORC1 activity was significantly reduced. In contrast, the phosphorylation level of several target substrates of mTORC2, including AKT, was significantly increased in fasted *Ghr* KO vs. control mice, while this difference disappeared after feeding [35]. The authors concluded that mTORC2 activity was increased in *Ghr* KO mice – at least in fasted animals - whereas mTORC1 activity was unaltered or reduced in fasted and fed *Ghr* KO mice, respectively. Our study of liver samples from fasted *GHR*-KO pigs did not reveal an increase in the phosphorylation of S6K or of 4EBP1 that would trigger its dissociation from eIF4E and allow cap-dependent mRNA translation (reviewed in 36). Moreover, we observed a significant increase in phosphorylation (= activation) of the mTORC1 inhibitor AMPK [81]. Collectively, these findings argue against a major activation of mTORC1 in liver of *GHR*-KO pigs. In contrast, a significant increase in GSK3B phosphorylation and a trend ($p = 0.0635$) toward an increase in AKT phosphorylation suggested a rise in the activity of mTORC2. While mTORC1 has profound effects on mRNA translation, metabolism and protein turnover, mTORC2 signaling is implicated in the regulation of ion transport, apoptosis, glucose metabolism, cell migration and cytoskeleton rearrangement (reviewed in 36). Future molecular profiling studies of liver and other tissues from *GHR*-KO and control pigs will clarify whether these biological processes are altered in the absence of GHR signaling.

Another interesting observation in liver samples from *GHR*-KO pigs was the trend ($p = 0.0635$) toward an increased abundance of PPARG. Discrepant findings have been reported regarding the consequences of increased PPARG activity in liver, ranging from the promotion of hepatic steatosis through the upregulation of genes involved in lipid uptake and storage, to the prevention of hepatic steatosis and fibrosis, possibly by sequestering fatty acids in adipose tissue and preventing hepatic stellate cell activation (reviewed in 82). Studies of *GHR*-KO and control pigs, e.g., after being fed a high-fat diet, may provide additional insight into these mechanisms.

One of the most striking effects of GHR deficiency is the increased life-span that has been observed in *Ghr* KO mice [83, 84]. Moreover, patients with LS show reduced incidences of cancer and diabetes, associated with reduced pro-aging signaling in cells incubated with serum derived from these patients [7]. While life expectancy studies in humans are difficult due to their long duration and to multiple confounding factors, they are possible in pigs, which can be maintained under standardized conditions and have a normal life expectancy of 15 to 17 years (reviewed in 85). Moreover, protective effects of GHR deficiency against tumors and diabetes can be evaluated by crossing the *GHR* KO mutation in existing pig models that are genetically predisposed to tumor development (e.g., [86]) or (pre-)diabetes [27, 87], thus bridging the gap between rodents and humans in longevity research with tailored large animal models. In addition to these long-lasting *in vivo* experiments, the effects of GHR deficiency on resistance to stress – such as UV light and heat – may be evaluated in primary cell cultures from *GHR*-KO pigs, similarly to studies that have been performed using cultured cells from dwarf mice [88].

Investigation of the neurological consequences of GHR deficiency is another interesting field, since mental retardation has been reported in a proportion of LS patients [2], while subjects from the Ecuadorian cohort display normal intelligence [89] or even enhanced cognitive performance [90]. As sophisticated methods for testing cognitive functions of pigs are available [91], the *GHR*-KO pig may also serve as a model to address these questions.

Finally, *GHR*-KO pigs are interesting animal models for developing and evaluating the efficacy and safety of new treatment options for LS, such as PASylated IGF1 with a prolonged plasma half-life [92]. In addition, it would be interesting to

determine whether the phenotype of *GHR*-KO pigs can be rescued by correction of the *GHR* mutation in a proportion of liver cells via gene editing. In a mouse model, hydrodynamic injection into the tail vein has been used to deliver components of a CRISPR/Cas9 system to correct a mutated fumarylacetoacetate hydrolase (*Fah*) gene in hepatocytes *in vivo* [93]. In pigs, refined techniques are available for direct application into the liver [94, 95].

In summary, G*HR*-KO pigs resemble important aspects of the pathophysiology of Laron syndrome and are thus an interesting model for mechanistic studies and treatment trials.

Acknowledgments. The authors thank Christina Blechinger, Luong Chau, Tamara Holy, Eva-Maria Jemiller, Sebastian Kaidel, Franziska Kress, and Tatjana Schröter for excellent technical assistance, and Christian Erdle, Sylvia Hering, and Harald Paul for expert animal management.

Funding. This study was supported in part by the German Center for Diabetes Research (DZD) and by the German Research Council (TRR127).

Duality of Interest. No potential conflicts of interest relevant to this article are reported.

Author Contributions. A. Hi., B. K., M. D. and E. W. conceived the experiments. A. Hi. and E. W. wrote the manuscript. All authors contributed to the manuscript and read and approved the final version. M. D., S. B., and H. L. developed the CRISPR/Cas system for *GHR* KO. B. K., M. K. and H. N. performed the *in vitro* fertilization, microinjection and embryo transfer experiments; A. Hi. and B. K. managed the breeding and performed the phenotypic characterization of *GHR*-KO and control pigs. A. Hi., A. B., and R. W. performed the necropsies. E. K. conducted the GHR ligand immunohistochemistry. M. B. and A. M. S. performed the DXA and MRI studies. A. Ho. carried out the IGFBP ligand blots analyses. W. F. B. and M. B. performed the IGF1 and GH assays. S. R. performed the insulin and glucose

measurements, B. R. and M. H. d. A. clinical-chemical measurements. M. D. performed the Western blot analysis of signaling molecules. A. Hi. and E. W. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

5. SUPPLEMENTARY MATERIAL

Supplementary Table 1. Antibodies employed for Western blot analyses.

Antigen	Antibody	Host	Dilution
pJAK2	Cell Signaling, Frankfurt, Germany #3776	rabbit	1:2000
JAK2	Cell Signaling, Frankfurt, Germany #3230	rabbit	1:2000
pMAPK	Cell Signaling, Frankfurt, Germany #4370	rabbit	1:2000
MAPK	Cell Signaling, Frankfurt, Germany #9102	rabbit	1:2000
pSTAT1	Cell Signaling, Frankfurt, Germany #7649	rabbit	1:2000
STAT1	Cell Signaling, Frankfurt, Germany #14994	rabbit	1:2000
pSTAT3	Cell Signaling, Frankfurt, Germany #9145	rabbit	1:2000
STAT3	Cell Signaling, Frankfurt, Germany #12640	rabbit	1:2000
pSTAT5	Cell Signaling, Frankfurt, Germany #9359	rabbit	1:2000
STAT5	Cell Signaling, Frankfurt, Germany #94205	rabbit	1:2000
pPI3K	Cell Signaling, Frankfurt, Germany #4228	rabbit	1:2000
PI3K	Cell Signaling, Frankfurt, Germany #4257	rabbit	1:2000
pAKT	Cell Signaling, Frankfurt, Germany #4060	rabbit	1:2000
AKT	Cell Signaling, Frankfurt, Germany #4691	rabbit	1:2000
pmTOR	Cell Signaling, Frankfurt, Germany #5536	rabbit	1:1000
mTOR	Cell Signaling, Frankfurt, Germany #2983	rabbit	1:1000
pAMPK	Cell Signaling, Frankfurt, Germany #2535	rabbit	1:2000
AMPK	Cell Signaling, Frankfurt, Germany #2532	rabbit	1:2000
pS6K	Cell Signaling, Frankfurt, Germany #9205	rabbit	1:2000
pS6K	Cell Signaling, Frankfurt, Germany #2708	rabbit	1:2000
p4EBP1	Cell Signaling, Frankfurt, Germany #2855	rabbit	1:2000
4EBP1	Cell Signaling, Frankfurt, Germany #9644	rabbit	1:2000
eIF4E	Cell Signaling, Frankfurt, Germany #9742	rabbit	1:2000
PPARG	Cell Signaling, Frankfurt, Germany #2435	rabbit	1:2000
pGSK3B	Cell Signaling, Frankfurt, Germany #9322	rabbit	1:2000
GSK3B	Cell Signaling, Frankfurt, Germany #9315	rabbit	1:2000
pINSR	Cell Signaling, Frankfurt, Germany #3024	rabbit	1:2000
INSR	Cell Signaling, Frankfurt, Germany #3025	rabbit	1:2000
pIRS1	Cell Signaling, Frankfurt, Germany #3203	rabbit	1:2000
IRS1	Cell Signaling, Frankfurt, Germany #3407	rabbit	1:2000
pLEPR	Merck, Darmstadt, Germany #07-1317	rabbit	1:500
LEPR	Bio-Rad, Munich, Germany #AHP1396	goat	1:1000

Supplementary Table 2. Factors affecting body fat content, muscle-to-back fat ratio, and serum leptin levels of 6-month-old control and *GHR*-KO pigs.

Parameter	Control			*GHR*-KO			Factors (p values)		
	Sex	mean	SD	Sex	mean	SD	Group	Sex	Group*Sex
Body fat (%)	male	9.6	1.1	male	20.9	2.2	< 0.0001	0.0108	0.1543
	female	13.2	2.5	female	22.0	4.1			
Muscle-to-back fat ratio (v/v)	male	8.6	2.3	male	2.9	0.5	< 0.0001	0.6289	0.4335
	female	7.7	2.3	female	3.1	1.3			
Serum leptin (µg/L)	male	3.0	1.0	male	4.8	2.7	0.0066	0.013	0.7437
	female	4.7	1.2	female	6.1	1.6			

The table shows means and standard deviations (SD) of means for body fat (%) and muscle to fat ratio calculated for 11 male/14 female control pigs and for 6 male/6 female *GHR*-KO pigs. Means and SD for fasting serum leptin were calculated for 9 male/13 female control and 6 male/6 female *GHR*-KO pigs. Data were analyzed using the General Linear Models Procedure (PROC GLM) as described in section 2.8 of the main manuscript.

Supplementary Table 3. Organ weights of control and *GHR*-KO pigs.

	Control (n = 25)	*GHR*-KO (n = 9)	% of Control	p value
Absolute weights (g)				
Liver	2315.5 ± 80.4	711.5 ± 133.9	30.6 ± 1.8	< 0.0001
Kidneys*	335.7 ± 11.0	102.4 ± 18.4	30.7 ± 1.4	< 0.0001
Heart	356.6 ± 9.9	133.0 ± 16.4	37.3 ± 2.1	< 0.0001
Brain	99.3 ± 1.5	83.4 ± 2.5	84.1 ± 2.0	< 0.0001
Lungs	686.2 ± 21.6	257.6 ± 34.9	37.1 ± 2.2	< 0.0001
Spleen	491.6 ± 28.9	177.6 ± 48.0	36.1 ± 4.1	< 0.0001
Pituitary gland	0.27 ± 0.01	0.13 ± 0.01	46.2 ± 1.8	< 0.0001
Adrenal glands*	5.56 ± 0.30	2.10 ± 0.50	37.0 ± 3.0	< 0.0001
Thyroid gland	7.45 ± 0.30	3.05 ± 0.60	40.4 ± 2.6	< 0.0001
Relative weights (% of body weight)				
Liver	2.53 ± 0.10	1.85 ± 0.20	72.8 ± 2.8	0.0005
Kidneys*	0.37 ± 0.01	0.27 ± 0.02	74.1 ± 3.8	0.0002
Heart	0.39 ± 0.01	0.34 ± 0.01	89.1 ± 2.1	0.0119
Brain	0.11 ± 0.005	0.22 ± 0.01	200.7 ± 10.0	< 0.0001
Lungs	0.75 ± 0.03	0.68 ± 0.05	89.3 ± 6.7	0.2304
Spleen	0.54 ± 0.03	0.46 ± 0.06	86.1 ± 9.2	0.2525
Pituitary gland	2.90 E-4 ± 0.09 E-4	3.30 E-4 ± 0.15 E-4	110.2 ± 4.0	0.0899
Adrenal glands*	6.10 E-3 ± 0.36 E-3	5.50 E-3 ± 0.65 E-3	88.7 ± 5.6	0.4267
Thyroid gland	8.20 E-3 ± 0.45 E-3	8.20 E-3 ± 0.80 E-3	98.2 ± 7.7	0.9384

Data are presented as least squares means (LSM) and standard errors (SE) of LSM estimated for the 2 groups (the statistical model used is described in section 2.8 of the main manuscript). LSMs were compared using Student's *t*-tests. *Cumulative weight of both organs.

Supplementary Table 4. Factors affecting parameters of lipid metabolism of control and *GHR*-KO pigs.

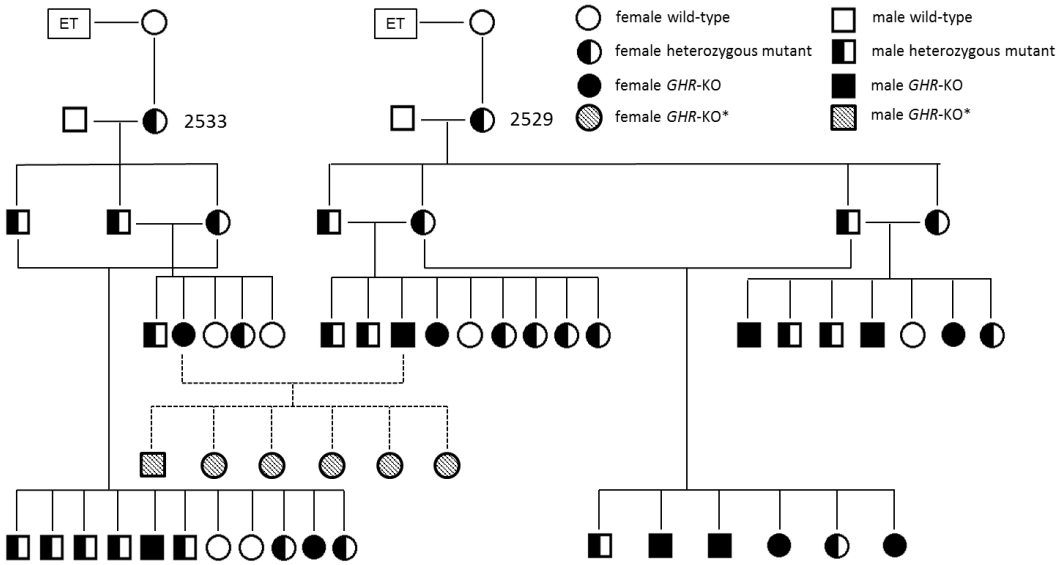
Parameter	Age	Sex	Control		*GHR*-KO		Factors (p values)				
			mean	SD	mean	SD	Group	Sex	Age	Group*Age	Group*Sex*Age
Triglycerides (mg/dL)	1	male	40.3	16.5	20.5	6.1	0.0113	0.2435	0.7666	0.301	0.9918
		female	44.3	20.5	25.8	10.9					
	2	male	34.8	14.6	26.2	8.1					
		female	42.1	20.3	33.9	11.0					
Cholesterol (mg/dL)	1	male	76.6	7.9	61.6	4.6	0.0851	< 0.0001	0.0535	0.0291	0.6255
		female	86.9	4.7	78.4	4.8					
	2	male	60.4	11.4	60.6	7.9					
		female	78.2	10.4	81.0	6.7					
LDL (mg/dL)	1	male	47.1	5.8	38.3	3.9	0.0971	< 0.0001	0.1097	0.1181	0.6526
		female	53.2	3.1	47.4	2.9					
	2	male	36.7	8.1	36.6	5.1					
		female	49.2	9.8	48.9	3.4					
HDL (mg/dL)	1	male	31.2	3.0	26.6	4.6	0.7111	0.0002	0.5071	0.0022	0.4577
		female	36.5	3.2	31.4	4.2					
	2	male	26.9	5.1	28.1	4.7					
		female	30.3	2.2	36.9	2.9					

The table shows means and standard deviations (SD) of means calculated for 6 male/4 female control pigs and 3 male/3 female *GHR*-KO pigs at Age 1 (12-15 weeks) and for 8 male/6 female control pigs and 5 male/4 female *GHR*-KO pigs at Age 2 (23-27 weeks). Data were analyzed using the General Linear Models Procedure (PROC GLM) as described in section 2.8 of the main manuscript.

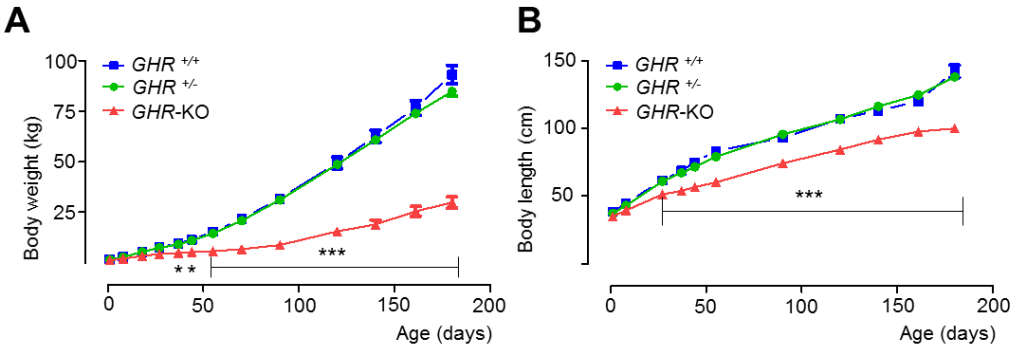
Supplementary Table 5. Clinical-chemical parameters of 6-month-old control and *GHR*-KO pigs.

Parameter [unit]	Control (n = 14)	*GHR*-KO (n = 9)	p value	Normal range
Urea [mmol/L]	4.3 ± 0.3	7.4 ± 0.5	< 0.0001	3.2 – 8.2
Creatinine [µmol/L]	126.4 ± 5.0	91.2 ± 6.5	0.0004	40 – 133
Total protein [g/L]	65.3 ± 2.2	63.6 ± 2.9	0.6381	55 – 85
Albumin [g/L]	40.1 ± 1.4	39.8 ± 1.8	0.8957	17.3 – 43.3
Bilirubin [µmol/L]	1.8 ± 0.1	1.5 ± 0.2	0.2351	< 4.3
GGT [U/L]	36.4 ± 1.8	32.5 ± 2.4	0.2131	0 – 40
Sodium [mmol/L]	141.5 ± 1.0	139.9 ± 1.3	0.3330	133 – 150
Potassium [mmol/L]	5.0 ± 0.2	4.2 ± 0.3	0.0602	4.4 – 6.7
Chloride [mmol/L]	101.8 ± 0.9	99.6 ± 1.2	0.1586	95 – 110
Calcium [mmol/L]	2.6 ± 0.04	2.7 ± 0.05	0.1744	2.4 – 3.0
Magnesium [mmol/L]	1.1 ± 0.04	1.1 ± 0.05	0.2740	0.5 – 1.2
Phosphorus [mmol/L]	2.4 ± 0.1	2.2 ± 0.04	0.1063	2.1 – 3.3
Iron [µmol/L]	17.7 ± 1.2	23.6 ± 1.6	0.0094	> 17.9

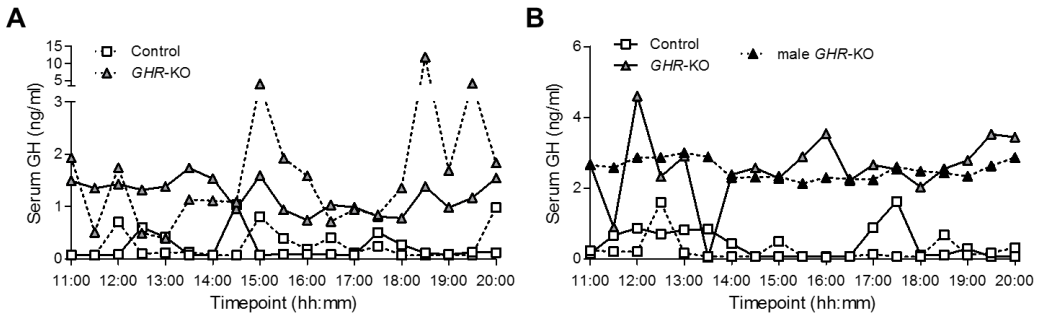
Data are presented as least squares means (LSM) and standard errors (SE) of LSM estimated for the 2 groups. The statistical model used is described in section 2.8 of the main manuscript. LSM were compared using Student's t-test.



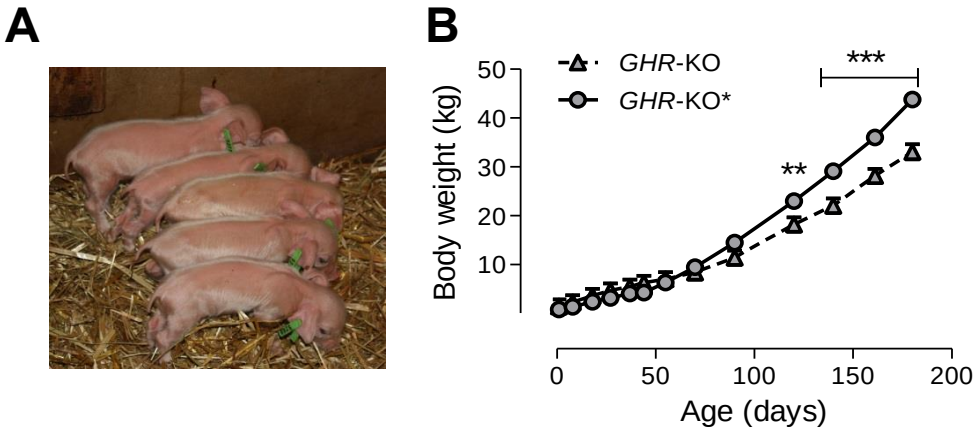
Supplementary Figure 1. Breeding scheme of *GHR*-KO animals. Heterozygous founder animals 2533 and 2539 were mated with wild-type boars and their heterozygous offspring were intercrossed to produce *GHR*-KO animals as well as wild-type and heterozygous *GHR* mutant littermates. The mating of two *GHR*-KO animals resulted in a litter of six healthy *GHR*-KO* piglets.



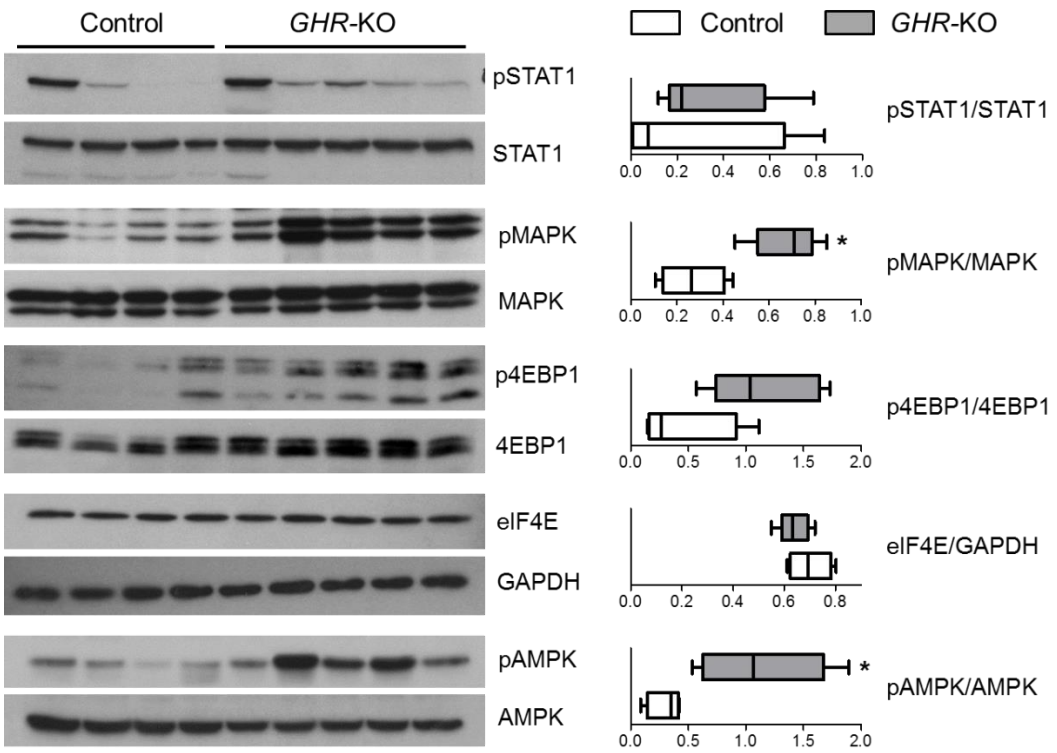
Supplementary Figure 2. Body weight gain (A) and longitudinal growth (B) of *GHR*-KO (n = 12) compared to wild-type (*GHR* $^{+/+}$; n = 6) and heterozygous *GHR* mutant (*GHR* $^{+/-}$; n = 19) pigs. Note that there was no significant difference in growth of *GHR* $^{+/+}$ and *GHR* $^{+/-}$ pigs. The two groups were thus pooled as GHR expressing control group.



Supplementary Figure 3. GH secretion profiles of additional *GHR*-KO (n=4) and control pigs (n=4). (A) Secretion pattern of two female *GHR*-KO and control pigs. (B) Secretion pattern of a female and male *GHR*-KO and two female control pigs.



Supplementary Figure 4. *GHR*-KO animals are able to reproduce. Mating of a *GHR*-KO sow and *GHR*-KO boar resulted in a litter of healthy *GHR*-KO* piglets. **(A)** Picture of *GHR*-KO* piglets at the age of one day. **(B)** *GHR*-KO* piglets had lower birth weights than *GHR*-KO piglets from heterozygote x heterozygote mating, but reached a higher weight at 6 months of age (*GHR*-KO: n = 12, *GHR*-KO*: n = 6). Data are presented as least squares means (LSM) and standard errors (SE) of LSM estimated for the 2 groups. The statistical model used is described in section 2.8 of the main manuscript. LSMs were compared using Student's t-test.



Supplementary Figure 5. Western blot analysis of GHR-related signaling molecules in liver samples of 6-month-old fasted *GHR*-KO (n=5) and control pigs (n=4). The box plots show medians, 25th and 75th percentiles (box), and extremes (whiskers). * $p < 0.05$; evaluated using Mann-Whitney U test.

6. REFERENCES

[1] Laron, Z., Pertzelan, A., Mannheimer, S. 1966. Genetic pituitary dwarfism with high serum concentation of growth hormone--a new inborn error of metabolism? Israel journal of medical sciences 2: 152-155

[2] Laron, Z. (2011) Clinical evidence of growth hormone resistance in patients with Laron syndrome. In: Laron Z, Kopchick JJ (eds) Laron syndrome - from man to mouse. Springer, pp 21-25

[3] Laron, Z. 2004. Laron syndrome (primary growth hormone resistance or insensitivity): the personal experience 1958-2003. The Journal of clinical endocrinology and metabolism 89: 1031-1044

[4] Laron, Z. 2016. Epilogue: The future of Laron syndrome — The need for changes. Growth Hormone & IGF Research 28: 79-80

[5] Guevara-Aguirre, J., Rosenbloom, A.L., Fielder, P.J., Diamond, F.B., Jr., Rosenfeld, R.G. 1993. Growth hormone receptor deficiency in Ecuador: clinical and biochemical phenotype in two populations. The Journal of clinical endocrinology and metabolism 76: 417-423

[6] Rosenfeld, R.G., Rosenbloom, A.L., Guevara-Aguirre, J. 1994. Growth hormone (GH) insensitivity due to primary GH receptor deficiency. Endocrine reviews 15: 369-390

[7] Guevara-Aguirre, J., Balasubramanian, P., Guevara-Aguirre, M., Wei, M., Madia, F., Cheng, C.W., et al. 2011. Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans. Science translational medicine 3: 70ra13

[8] Laron, Z., Kauli, R., Lapkina, L., Werner, H. 2017. IGF-I deficiency, longevity and cancer protection of patients with Laron syndrome. Mutation research Reviews in mutation research 772: 123-133

[9] Chernausek, S.D., Backeljauw, P.F., Frane, J., Kuntze, J., Underwood, L.E. 2007. Long-term treatment with recombinant insulin-like growth factor (IGF)-I in children with severe IGF-I deficiency due to growth hormone insensitivity. The Journal of clinical endocrinology and metabolism 92: 902-910

[10] Backeljauw, P.F., Kuntze, J., Frane, J., Calikoglu, A.S., Chernausek, S.D. 2013. Adult and near-adult height in patients with severe insulin-like growth factor-I deficiency after long-term therapy with recombinant human insulin-like growth factor-I. Hormone research in paediatrics 80: 47-56

[11] Cohen, J., Blethen, S., Kuntze, J., Smith, S.L., Lomax, K.G., Mathew, P.M. 2014. Managing the child with severe primary insulin-like growth factor-1 deficiency (IGFD): IGFD diagnosis and management. Drugs in R&D 14: 25-29

[12] Shevah, O., Laron, Z. 2007. Patients with congenital deficiency of IGF-I seem protected from the development of malignancies: A preliminary report. Growth Hormone & IGF Research 17: 54-57

[13] Steuerman, R., Shevah, O., Laron, Z. 2011. Congenital IGF1 deficiency tends to confer protection against post-natal development of malignancies. European journal of endocrinology 164: 485-489

[14] Lapkina-Gendler, L., Rotem, I., Pasmanik-Chor, M., Gurwitz, D., Sarfstein, R., Laron, Z., et al. 2016. Identification of signaling pathways associated with cancer protection in Laron syndrome. Endocrine-related cancer 23: 399-410

[15] Zhou, Y., Xu, B.C., Maheshwari, H.G., He, L., Reed, M., Lozykowski, M., et al. 1997. A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). Proceedings of the National Academy of Sciences of the United States of America 94: 13215-13220

[16] List, E.O., Sackmann-Sala, L., Berryman, D.E., Funk, K., Kelder, B., Gosney, E.S., et al. 2011. Endocrine parameters and phenotypes of the growth hormone receptor gene disrupted (GHR-/-) mouse. Endocrine reviews 32: 356-386

[17] Duran-Ortiz, S., Noboa, V., Kopchick, J.J. 2017. Disruption of the GH receptor gene in adult mice and in insulin sensitive tissues. Growth Hormone & IGF Research **38**: 3-7.

[18] Young, J.A., List, E.O., Kopchick, J.J. 2016. Deconstructing the growth hormone receptor (GHR): physical and metabolic phenotypes of tissue-specific GHR gene-disrupted mice. Progress in molecular biology and translational science 138: 27-39

[19] Rogers, C.S. 2016. Genetically engineered livestock for biomedical models. Transgenic research 25: 345-359

[20] Aigner, B., Kessler, B., Klymiuk, N., Kurome, M., Renner, S., Wünsch, A., et al. (2017) Genetically tailored pig models for translational biomedical research. In: Conn PM (ed) Animal Models for the Study of Human Disease. Academic Press, London, Oxford, San Diego, Cambridge, pp 671-701

[21] Dahlhoff, M., Gaborit, N., Bultmann, S., Leonhardt, H., Yarden, Y., Schneider, M.R. 2017. CRISPR-assisted receptor deletion reveals distinct roles for ERBB2 and ERBB3 in skin keratinocytes. The FEBS journal 284: 3339-3349

[22] Umeyama, K., Honda, K., Matsunari, H., Nakano, K., Hidaka, T., Sekiguchi, K., et al. 2013. Production of Diabetic Offspring Using Cryopreserved Epididymal Sperm by *In Vitro* Fertilization and Intrafallopian Insemination Techniques in Transgenic Pigs. The Journal of reproduction and development 59: 599-603

[23] Kurome, M., Kessler, B., Wuensch, A., Nagashima, H., Wolf, E. 2015. Nuclear transfer and transgenesis in the pig. Methods in molecular biology (Clifton, NJ) 1222: 37-59

[24] Besenfelder, U., Modl, J., Muller, M., Brem, G. 1997. Endoscopic embryo collection and embryo transfer into the oviduct and the uterus of pigs. Theriogenology 47: 1051-1060

[25] Blum, W.F., Breier, B.H. 1994. Radioimmunoassays for IGFs and IGFBPs. Growth regulation 4 Suppl 1: 11-19

[26] Wirthgen, E., Hoflich, C., Spitschak, M., Helmer, C., Brand, B., Langbein, J., et al. 2016. Quantitative Western ligand blotting reveals common patterns and differential features of IGFBP-fingerprints in domestic ruminant breeds and species. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society 26: 42-49

[27] Renner, S., Fehlings, C., Herbach, N., Hofmann, A., von Waldthausen, D.C., Kessler, B., et al. 2010. Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. Diabetes 59: 1228-1238

[28] Blutke, A., Renner, S., Flenkenthaler, F., Backman, M., Haesner, S., Kemter, E., et al. 2017. The Munich MIDY Pig Biobank - A unique resource for studying organ crosstalk in diabetes. Molecular metabolism 6: 931-940

[29] Papadopoulos, G.A., Erkens, T., Maes, D.G., Peelman, L.J., van Kempen, T.A., Buyse, J., et al. 2009. Peripartal feeding strategy with different n-6:n-3 ratios in sows: effect on gene expression in backfat white adipose tissue postpartum. The British journal of nutrition 101: 197-205

[30] Kremer, P.V., Fernandez-Figares, I., Forster, M., Scholz, A.M. 2012. *In vivo* body composition in autochthonous and conventional pig breeding groups by dual-energy X-ray absorptiometry and magnetic resonance imaging under special consideration of Cerdo Iberico. Animal : an international journal of animal bioscience 6: 2041-2047

[31] Kremer, P.V., Forster, M., Scholz, A.M. 2013. Use of magnetic resonance imaging to predict the body composition of pigs *in vivo*. Animal : an international journal of animal bioscience 7: 879-884

[32] Albl, B., Haesner, S., Braun-Reichhart, C., Streckel, E., Renner, S., Seeliger, F., et al. 2016. Tissue sampling guides for porcine biomedical models. Toxicologic pathology 44: 414-420

[33] Streckel, E., Braun-Reichhart, C., Herbach, N., Dahlhoff, M., Kessler, B., Blutke, A., et al. 2015. Effects of the glucagon-like peptide-1 receptor agonist liraglutide in juvenile transgenic pigs modeling a pre-diabetic condition. Journal of translational medicine 13: 73

[34] Fagerberg, L., Hallström, B.M., Oksvold, P., Kampf, C., Djureinovic, D., Odeberg, J., et al. 2014. Analysis of the Human Tissue-specific Expression by Genome-wide Integration of Transcriptomics and Antibody-based Proteomics. Molecular & cellular proteomics : MCP 13: 397-406

[35] Dominick, G., Berryman, D.E., List, E.O., Kopchick, J.J., Li, X., Miller, R.A., et al. 2015. Regulation of mTOR Activity in Snell Dwarf and GH Receptor Gene-Disrupted Mice. Endocrinology 156: 565-575

[36] Saxton, R.A., Sabatini, D.M. 2017. mTOR Signaling in Growth, Metabolism, and Disease. Cell 168: 960-976

[37] Rawlings, J.S., Rosler, K.M., Harrison, D.A. 2004. The JAK/STAT signaling pathway. Journal of cell science 117: 1281-1283

[38] Cui, D., Li, F., Li, Q., Li, J., Zhao, Y., Hu, X., et al. 2015. Generation of a miniature pig disease model for human Laron syndrome. Scientific reports 5: 15603

[39] Li, F., Li, Y., Liu, H., Zhang, X., Liu, C., Tian, K., et al. 2015. Transgenic Wuzhishan minipigs designed to express a dominant-negative porcine growth hormone receptor display small stature and a perturbed insulin/IGF-1 pathway. Transgenic research 24: 1029-1042

[40] Yakar, S., Liu, J.L., Stannard, B., Butler, A., Accili, D., Sauer, B., et al. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. Proceedings of the National Academy of Sciences of the United States of America 96: 7324-7329

[41] Wolf, E., Schneider, M.R., Zhou, R., Fisch, T.M., Herbach, N., Dahlhoff, M., et al. 2005. Functional consequences of IGFBP excess-lessons from transgenic mice. Pediatric nephrology (Berlin, Germany) 20: 269-278

[42] Hwa, V., Oh, Y., Rosenfeld, R.G. 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocrine reviews 20: 761-787

[43] Labarta, J.I., Gargosky, S.E., Simpson, D.M., Lee, P.D., Argente, J., Guevara-Aguirre, J., et al. 1997. Immunoblot studies of the acid-labile subunit (ALS) in biological fluids, normal human serum and in children with GH deficiency and GH receptor deficiency before and after long-term therapy with GH or IGF-I respectively. Clinical endocrinology 47: 657-666

[44] Laron, Z., Klinger, B., Blum, W.F., Silbergeld, A., Ranke, M.B. 1992. IGF binding protein 3 in patients with Laron type dwarfism: effect of exogenous rIGF-I. Clinical endocrinology 36: 301-304

[45] Savage, M.O., Blum, W.F., Ranke, M.B., Postel-Vinay, M.C., Cotterill, A.M., Hall, K., et al. 1993. Clinical features and endocrine status in patients with growth hormone insensitivity (Laron syndrome). The Journal of clinical endocrinology and metabolism 77: 1465-1471

[46] Hoeflich, A., Wu, M., Mohan, S., Foll, J., Wanke, R., Froehlich, T., et al. 1999. Overexpression of insulin-like growth factor-binding protein-2 in transgenic mice reduces postnatal body weight gain. Endocrinology 140: 5488-5496

[47] Hoeflich, A., Nedbal, S., Blum, W.F., Erhard, M., Lahm, H., Brem, G., et al. 2001. Growth inhibition in giant growth hormone transgenic mice by overexpression of insulin-like growth factor-binding protein-2. Endocrinology 142: 1889-1898

[48] Kaplan, S.A., Cohen, P. 2007. The somatomedin hypothesis 2007: 50 years later. The Journal of clinical endocrinology and metabolism 92: 4529-4535

[49] Kleinert, M., C. Clemmensen, S. M. Hofmann, M. C. Moore, S. Renner, S. C. Woods, P. Huypens, J. Beckers, M. H. de Angelis and A. Schürmann 2018. Animal models of obesity and diabetes mellitus. Nature Reviews Endocrinology.

[50] Laron, Z. 2000. The diagnostic and prognostic importance of neonatal length measurements. The Israel Medical Association journal : IMAJ 2: 84-85

[51] Baker, J., Liu, J.P., Robertson, E.J., Efstratiadis, A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75: 73-82

[52] Hetz, J.A., Menzies, B.R., Shaw, G., Rao, A., Clarke, I.J., Renfree, M.B. 2015. Growth axis maturation is linked to nutrition, growth and developmental rate. Molecular and cellular endocrinology 411: 38-48

[53] Symonds, M.E., Sebert, S.P., Hyatt, M.A., Budge, H. 2009. Nutritional programming of the metabolic syndrome. Nature Reviews Endocrinology 5: 604

[54] Laron, Z., Ginsberg, S., Lilos, P., Arbiv, M., Vaisman, N. 2006. Body composition in untreated adult patients with Laron syndrome (primary GH insensitivity). Clinical endocrinology 65: 114-117

[55] Berryman, D.E., List, E.O., Palmer, A.J., Chung, M.-Y., Wright-Piekarski, J., Lubbers, E., et al. 2010. Two-year body composition analyses of long-lived GHR null mice. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 65: 31-40

[56] Arner, P., Bernard, S., Salehpour, M., Possnert, G., Liebl, J., Steier, P., et al. 2011. Dynamics of human adipose lipid turnover in health and metabolic disease. Nature 478: 110-113

[57] Vijayakumar, A., Novosyadlyy, R., Wu, Y., Yakar, S., LeRoith, D. 2010. Biological effects of growth hormone on carbohydrate and lipid metabolism. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society 20: 1-7

[58] Wang, H., Eckel, R.H. 2009. Lipoprotein lipase: from gene to obesity. American journal of physiology Endocrinology and metabolism 297: E271-288

[59] Feinberg, M.S., Scheinowitz, M., Laron, Z. 2000. Echocardiographic dimensions and function in adults with primary growth hormone resistance (Laron Syndrome). The American journal of cardiology 85: 209-213

[60] Shea, B.T., Hammer, R.E., Brinster, R.L. 1987. Growth allometry of the organs in giant transgenic mice. Endocrinology 121: 1924-1930

[61] Wanke, R., Hermanns, W., Folger, S., Wolf, E., Brem, G. 1991. Accelerated growth and visceral lesions in transgenic mice expressing foreign genes of the growth hormone family: an overview. Pediatric nephrology (Berlin, Germany) 5: 513-521

[62] Lombardi, G., Colao, A., Ferone, D., Marzullo, P., Orio, F., Longobardi, S., et al. 1997. Effect of growth hormone on cardiac function. Hormone research 48 Suppl 4: 38-42

[63] Sjogren, K., Bohlooly, Y.M., Olsson, B., Coschigano, K., Tornell, J., Mohan, S., et al. 2000. Disproportional skeletal growth and markedly decreased bone mineral content in growth hormone receptor -/- mice. Biochemical and biophysical research communications 267: 603-608

[64] Laron, Z., Avitzur, Y., Klinger, B. 1995. Carbohydrate metabolism in primary growth hormone resistance (Laron syndrome) before and during insulin-like growth factor-I treatment. Metabolism: clinical and experimental 44: 113-118

[65] Guevara-Aguirre, J., Rosenbloom, A.L. 2015. Obesity, diabetes and cancer: insight into the relationship from a cohort with growth hormone receptor deficiency. Diabetologia 58: 37-42

[66] Laron, Z. 2002. Growth hormone insensitivity (Laron syndrome). Reviews in endocrine & metabolic disorders 3: 347-355

[67] Guevara-Aguirre, J., Rosenbloom, A.L., Balasubramanian, P., Teran, E., Guevara-Aguirre, M., Guevara, C., et al. 2015. GH Receptor Deficiency in Ecuadorian Adults Is Associated With Obesity and Enhanced Insulin Sensitivity. The Journal of clinical endocrinology and metabolism 100: 2589-2596

[68] Renner, S., Dobenecker, B., Blutke, A., Zols, S., Wanke, R., Ritzmann, M., et al. 2016. Comparative aspects of rodent and nonrodent animal models for mechanistic and translational diabetes research. Theriogenology 86: 406-421

[69] Liu, J.-L., Coschigano, K.T., Robertson, K., Lipsett, M., Guo, Y., Kopchick, J.J., et al. 2004. Disruption of growth hormone receptor gene causes diminished pancreatic islet size and increased insulin sensitivity in mice. American Journal of Physiology - Endocrinology and Metabolism 287: E405-E413

[70] Dominici, F.P., Hauck, S., Argentino, D.P., Bartke, A., Turyn, D. 2002. Increased insulin sensitivity and upregulation of insulin receptor, insulin receptor substrate (IRS)-1 and IRS-2 in liver of Ames dwarf mice. The Journal of endocrinology 173: 81-94

[71] Chung, S.T., Chacko, S.K., Sunehag, A.L., Haymond, M.W. 2015. Measurements of Gluconeogenesis and Glycogenolysis: A Methodological Review. Diabetes 64: 3996-4010

[72] Baxmann, A.C., Ahmed, M.S., Marques, N.C., Menon, V.B., Pereira, A.B., Kirsztajn, G.M., et al. 2008. Influence of muscle mass and physical activity on serum and urinary creatinine and serum cystatin C. Clinical journal of the American Society of Nephrology : CJASN 3: 348-354

[73] LeRoith, D., Yakar, S. 2007. Mechanisms of disease: metabolic effects of growth hormone and insulin-like growth factor 1. Nature clinical practice Endocrinology & metabolism 3: 302-310

[74] Brooks, A. J., W. Dai, M. L. O'Mara, D. Abankwa, Y. Chhabra, R. A. Pelekanos, O. Gardon, K. A. Tunny, K. M. Blucher, C. J. Morton, M. W. Parker, E. Sierecki, Y. Gambin, G. A. Gomez, K. Alexandrov, I. A. Wilson, M. Doxastakis, A. E. Mark and M. J. Waters 2014. Mechanism of Activation of Protein Kinase JAK2 by the Growth Hormone Receptor. Science **344**(6185).

[75] Waters, M.J. 2016. The growth hormone receptor. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society 28: 6-10

[76] Mukherjee, A., Alzhanov, D., Rotwein, P. 2016. Defining human insulin-like growth factor I gene regulation. American journal of physiology Endocrinology and metabolism 311: E519-529

[77] Rawlings, J.S., Rosler, K.M., Harrison, D.A. 2004. The JAK/STAT signaling pathway. Journal of cell science 117: 1281-1283

[78] Waters, M.J., Brooks, A.J. 2015. JAK2 activation by growth hormone and other cytokines. The Biochemical journal 466: 1-11

[79] Laron, Z., Silbergeld, A., Lilos, P., Blum, F.W. 1998. Serum leptin in obese patients with Laron syndrome before and during IGF-I treatment. Journal of pediatric endocrinology & metabolism : JPEM 11: 653-656

[80] Cohen, P., Yang, G., Yu, X., Soukas, A.A., Wolfish, C.S., Friedman, J.M., et al. 2005. Induction of leptin receptor expression in the liver by leptin and food deprivation. The Journal of biological chemistry 280: 10034-10039

[81] Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., et al. 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Molecular cell 30: 214-226

[82] Ahmadian, M., Suh, J.M., Hah, N., Liddle, C., Atkins, A.R., Downes, M., et al. 2013. PPARgamma signaling and metabolism: the good, the bad and the future. Nature medicine 19: 557-566

[83] Coschigano, K.T., Clemmons, D., Bellush, L.L., Kopchick, J.J. 2000. Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. Endocrinology 141: 2608-2613

[84] Coschigano, K.T., Holland, A.N., Riders, M.E., List, E.O., Flyvbjerg, A., Kopchick, J.J. 2003. Deletion, But Not Antagonism, of the Mouse Growth Hormone Receptor Results in Severely Decreased Body Weights, Insulin, and Insulin-Like Growth Factor I Levels and Increased Life Span. Endocrinology 144: 3799-3810

[85] Burgstaller, J.P., Brem, G. 2017. Aging of Cloned Animals: A Mini-Review. Gerontology 63: 417-425

[86] Flisikowska, T., Merkl, C., Landmann, M., Eser, S., Rezaei, N., Cui, X., et al. 2012. A porcine model of familial adenomatous polyposis. Gastroenterology 143(5): 1173-1175. e1177

[87] Renner, S., Braun-Reichhart, C., Blutke, A., Herbach, N., Emrich, D., Streckel, E., et al. 2013. Permanent neonatal diabetes in INS(C94Y) transgenic pigs. Diabetes 62: 1505-1511

[88] Murakami, S., Salmon, A., Miller, R.A. 2003. Multiplex stress resistance in cells from long-lived dwarf mice. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17: 1565-1566

[89] Kranzler, J.H., Rosenbloom, A.L., Martinez, V., Guevara-Aguirre, J. 1998. Normal intelligence with severe insulin-like growth factor I deficiency due to growth hormone receptor deficiency: a controlled study in a genetically homogeneous population. The Journal of clinical endocrinology and metabolism 83: 1953-1958

[90] Nashiro, K., Guevara-Aguirre, J., Braskie, M.N., Hafzalla, G.W., Velasco, R., Balasubramanian, P., et al. 2017. Brain Structure and Function Associated with Younger Adults in Growth Hormone Receptor-Deficient Humans. The Journal of neuroscience : the official journal of the Society for Neuroscience 37: 1696-1707

[91] Schuldenzucker, V., Schubert, R., Muratori, L.M., Freisfeld, F., Rieke, L., Matheis, T., et al. 2017. Behavioral testing of minipigs transgenic for the Huntington gene-A three-year observational study. PloS one 12(10): e0185970

[92] Schlapschy, M., Binder, U., Borger, C., Theobald, I., Wachinger, K., Kisling, S., et al. 2013. PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. Protein engineering, design & selection : PEDS 26: 489-501

[93] Yin, H., Xue, W., Chen, S., Bogorad, R.L., Benedetti, E., Grompe, M., et al. 2014. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. Nature biotechnology 32: 551-553

[94] Kamimura, K., Suda, T., Xu, W., Zhang, G., Liu, D. 2009. Image-guided, lobe-specific hydrodynamic gene delivery to swine liver. Molecular therapy : the journal of the American Society of Gene Therapy 17: 491-499

[95] Stoller, F., Schlegel, A., Viecelli, H.M., Rufenacht, V., Cesarovic, N., Viecelli, C., et al. 2015. Hepatocyte Transfection in Small Pigs After Weaning by Hydrodynamic Intraportal Injection of Naked DNA/Minicircle Vectors. Human gene therapy methods 26: 181-192

IV. DISCUSSION AND OUTLOOK

Beneficial effects resulting from GHRD are reported for human patients, regarding increased insulin sensitivity while facing severe obesity, protection against the development of malignancies as cancer and longevity-associated effects, the investigation of underlying mechanisms is promising. As human cases are spread world-wide and caused by different mutations and the use of the murine model is limited by physiological differences from humans, our *GHR*-KO pig can provide further insight into the consequences of GHRD. We show, that the generation of a *GHR* knockout was successfully performed applying the CRISPR/Cas9 technology in zygotes and that *GHR*-KO pigs resemble the endocrine, auxologic and metabolic phenotype of the human LS. Future investigations involving the *GHR*-KO pig overcoming limitations in mice are outlined.

1. *GHR*-KO was successfully generated by CRISPR/Cas

As outlined in detail in the review of the literature, the main difference of the pathophysiology of the Laron syndrome in contrast to other forms of dwarfism is the inability of the GHR to bind circulating GH or to signal due to mutations of the *GHR* gene (Eshet, Laron et al. 1984). We chose the CRISPR/Cas9 technology to induce mutations in exon 3 of the porcine *GHR* gene, as its application is widely emerging in the generation of transgenic animal models, providing target-site directed mutations which are transmitted through the germ-line (reviewed in Dmochewitz and Wolf 2015). By PCR and sequencing analysis of the *GHR* exon 3 of F2 generation offspring (**Figure 4**), we identified the target-site mutation. The sequencing analysis of the mutated *GHR* sequence revealed a shift of the reading frame leading to the establishment of premature termination codons predicting a non-functional GHR protein in *GHR*-KO animals. Our approach to investigate the direct binding capacity of exogenous GH proved that our *GHR*-KO animals exhibit a hallmark of the human LS, showing no binding capacity of GH. Furthermore, we can exclude any kind of remnant GH signaling, which is a limitation in the minipig model expressing a dominant negative GHR (Li, Li et al. 2015) with still functional GH signaling via residue wild-type GHRs leading to a form of intermediate phenotype.

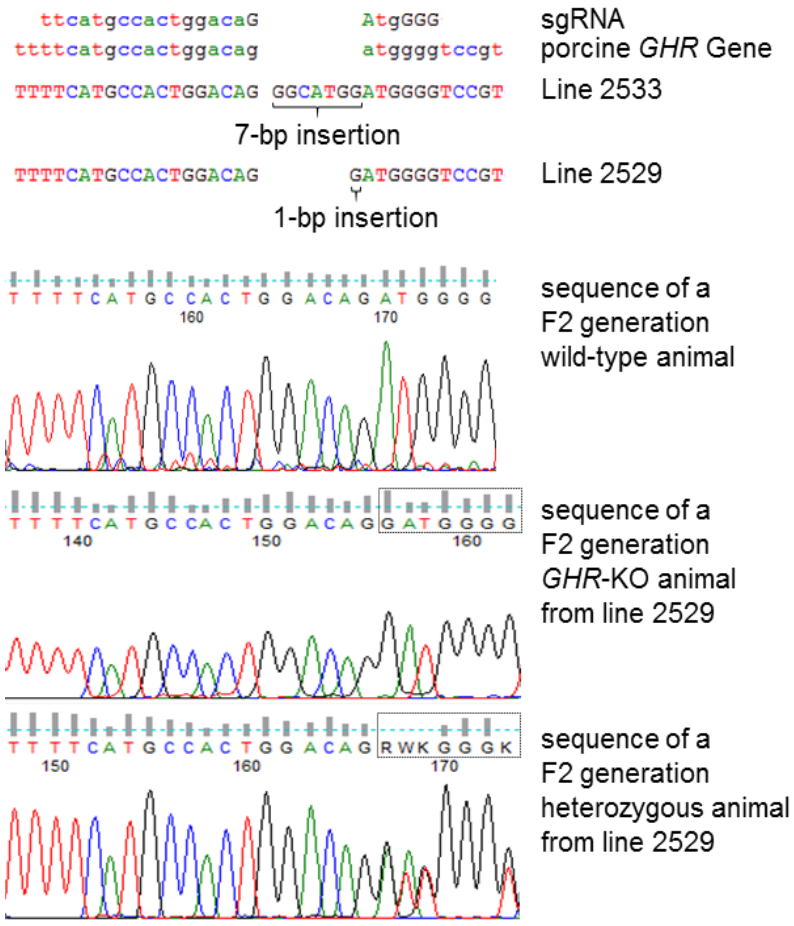


Figure 4. Applying the CRISPR/Cas9 technology, a 7-bp and 1-bp insertion has been introduced in exon 3 of the porcine *GHR* gene. F2 animals were obtained by breeding exhibiting the mutated *GHR* gene sequence specific for each line (marked as boxes).

2. *GHR*-KO pigs exhibit the phenotype of Laron syndrome

To overcome physiologic limitations in mice, we chose to generate our large animal model for LS on the genetic background of the German landrace pig breed. Other pig models for LS were established on minipig backgrounds (Cui, Li et al. 2015, Li, Li et al. 2015), where abnormalities of the endocrine growth regulatory axis may be suspected, as the proportional growth retardation of minipigs resembles the phenotype of pituitary dwarfism, such as GH or IGF1 deficiency (Simianer and Köhn 2010). In contrast, we chose a pig breed without a selection for dwarf size. As the maturation of the endocrine growth axis appears similar in pigs as in humans – in contrast to rodents, both are born with a fully mature endocrine growth axis involving the characteristic shift in growth promoting hormones (Savage, Blum et al. 1993) - *GHR*-KO pigs resemble the auxologic phenotype of the human LS (reviewed in Rosenfeld, Rosenbloom et al. 1994), showing no significant differences at birth but severe postnatal growth retardation (**Figure 5**) due to the shift from intrauterine GH independent growth promotion by IGF2 and IGF1 towards postnatal GH dependent growth promotion by IGF1 (Baker, Liu et al. 1993) (Hetz, Menzies et al. 2015). The lack of IGF1 is obvious in *GHR*-KO pigs of all ages and results in elevated levels of circulating GH. While other studies in GHR deficient animal models describe elevated GH levels by analyzing a single sample (Zhou, Xu et al. 1997, Cui, Li et al. 2015) disregarding the pulsatility of GH secretion, we evaluated the changes in GH levels over a longer period of time as performed in human

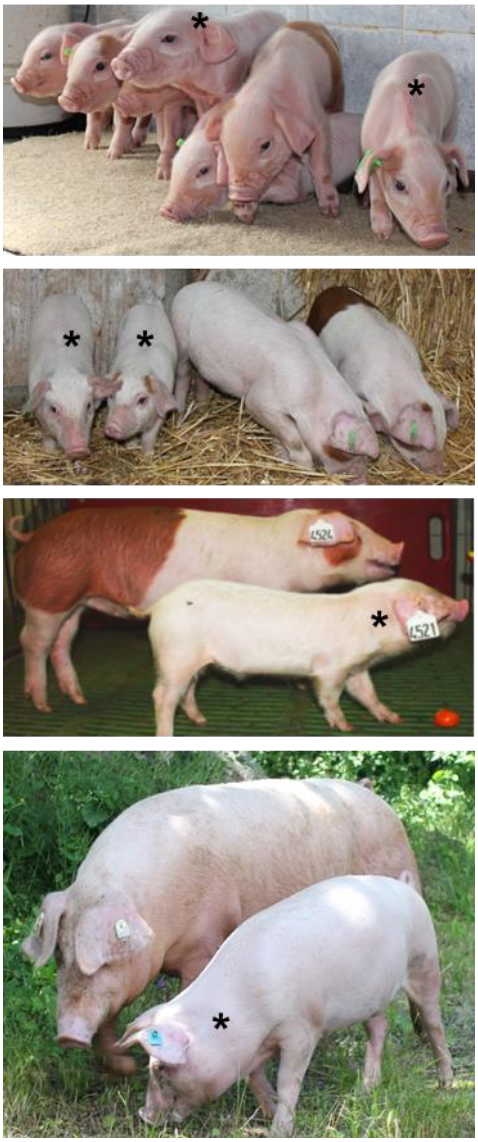


Figure 5. *GHR*-KO (marked by asterisk) and control littermates at top to bottom: 1 day; 1 month, 2 months, 6 months of age

patients with LS (Keret, Pertzelan et al. 1988). Long-term serum GH levels of *GHR*-KO pigs were significantly higher than control animal's values and displayed partially preserved pulsatility. It remains unclear, why the levels in *GHR*-KO pigs do not completely represent the exceeding peaks of GH secretion in human LS patients observed especially after meals or during sleep (Keret, Pertzelan et al. 1988). Nevertheless, a continuous blood sampling should be performed preferentially in pigs than mice as the placement of the central venous catheter takes place with minimal-invasive methods and the stress induced to the animals can be reduced by training (Kleinert, Clemmensen et al. 2018).

Regarding the growth of organs, *GHR*-KO pigs represent the phenotype seen in humans (Feinberg, Scheinowitz et al. 2000) (Backeljauw and Underwood 2001) and mice (Sjogren, Bohlooly et al. 2000, Berryman, List et al. 2006, Berryman, List et al. 2010), highlighting the different dependence on GH action of specific tissues resulting in proportional and disproportional reduction of organ weights.

A high proportion of body fat is a hallmark of the human LS and is referred to as obesity. While *Ghr* KO mice display a high abundance in total body fat, which was estimated in DXA analyses (Egecioglu, Bjursell et al. 2006), in depth investigations revealed an enlargement of specific fat depots, notably of subcutaneous fat (Flint, Binart et al. 2006), while epididymal fat pats, which are preferentially investigated in mice, were not enlarged (Berryman, List et al. 2004). The interpretation of the relative increase in adipose tissue in the *Ghr* KO mice remains complicated as it may be seen as the fat tissue being not affected by GHRD, while other tissues show growth impairment due to the lack of GH action (reviewed by Berryman in Laron and Kopchick 2011, List, Sackmann-Sala et al. 2011) or the lack of GH and/or IGF1 action leads to a progression of fat accumulation, referred to as obesity in human LS patients (Laron, Ginsberg et al. 2006). In line with the observations in humans and mice, *GHR*-KO pigs displayed a high percentage of body fat, leading to the investigation of effects on metabolism and especially glucose homeostasis, as differences between the human cohorts of LS patients are occurring.

As outlined in the review of the literature, transient juvenile hypoglycemia is a common symptom of LS, but the *Israeli cohort* exhibits a progressing glucose intolerance and even cases of diabetes mellitus (Laron, Avitzur et al. 1997), while the *Ecuadorian cohort* remains insulin sensitive (Guevara-Aguirre, Rosenbloom et al. 2015). To get first insights into changes in insulin sensitivity of *GHR*-KO pigs,

we investigated the fasting blood glucose and insulin levels of animals aged 12-15 weeks representing young individuals with LS and animals aged six months, representing adults. Just as seen in the human and murine phenotype, low fasting blood glucose levels were observed at young ages, which increased and became similar to control animal`s values when reaching the age of six months. The measurements of insulin levels and the calculation of the HOMA-IR score revealed no pathologic alterations in insulin sensitivity, as the rise in HOMA-IR score represents the increase in glucose levels, and never reaches even the lowest values being seen critical for humans (reviewed in Tang, Li et al. 2015). These results indicate, that *GHR*-KO pigs do not represent the Laron phenotype of the *Israelicohort*, with a progressive decrease in insulin sensitivity resulting in diabetes mellitus. They rather exhibit the phenotype observed in the *Ecuadorian cohort*, including an increased insulin sensitivity and protection from the development of diabetes mellitus (Guevara-Aguirre, Rosenbloom et al. 2015). For further proof, we investigated the insulin receptor associated signaling pathways in liver tissue derived from fasted animals aged six months and revealed an increased expression and phosphorylation of IRS1 and downstream signal transducers of the insulin receptor, suggesting an increase in insulin signaling and sensitivity. While those observations point towards the "*Ecuadorian* phenotype" being represented by *GHR*-KO pigs, we are in need for further analyzes, which are outlined as future perspectives.

3. Altered activation of hepatic signaling cascades

Hepatic signaling studies were performed from tissue derived from fasted six months old *GHR*-KO and control animals, investigating insulin receptor associated, metabolic pathways and signaling associated with the GHR.

The observed activation of insulin receptor associated signaling cascades and transient juvenile hypoglycemia while insulin levels were normal point towards increased insulin sensitivity, which is a major positive effect of GHRD, protecting LS patients from developing diabetes even whilst facing severe obesity (Guevara-Aguirre, Rosenbloom et al. 2015)). As LS patients seem to be protected from the development of malignancies (reviewed in Laron, Kauli et al. 2017) and show increased longevity (Shevah and Laron 2007), a connection between our findings in the *GHR*-KO pig model and results from the *"Leiden longevity study"* seems

appropriate. In this study, nonagenarian siblings with a low mortality rate were compared to their offspring and partners (Westendorp, van Heemst et al. 2009). Investigations showed a correlation between a reduced insulin and IGF signaling and longevity but resulting in shorter stature (Van Heemst, Beekman et al. 2005), similar to findings in our animal model. Furthermore, the authors noted a tremendous effect of the activity of the mechanistic target of rapamycin (mTOR) signaling mediating lifespan extension (Slagboom, Beekman et al. 2011). As outlined in detail in the review of the literature, mTOR is seen as "sensing" the nutritional status of the organism and regulating anabolic or catabolic activity on cellular levels and a chronic activation of the mTORC1 resulting from over-feeding diets is seen to induce the onset of diabetes mellitus (reviewed in Zoncu, Efeyan et al. 2011). Similar to findings in humans, the analysis of downstream signaling pathways showed an increased activity of mTORC2, contributing to beneficial effects on metabolism (Guevara-Aguirre, Balasubramanian et al. 2011), while mTORC1 associated signaling showed no alterations in *GHR*-KO pigs.

Both findings, a suggested increase in insulin sensitivity and an increase in mTORC2 signaling, suggest beneficial effects on longevity of *GHR*-KO pigs, which raises the need for further investigations.

Results from hepatic GHR-associated signaling cascades appeared surprising at first sight. At a state of GHRD, it would have been expected, that the activity of STAT5 and JAK2 should be diminished, as those are the signal transducers of GH action (reviewed in Waters 2015). In line with that, the phosphorylation levels of STAT5 were significantly reduced in *GHR*-KO pigs, but levels of phosphorylated JAK2 were increased. In fact, investigations of LS patients and the *Ghr* KO mouse did not include the phosphorylation status of STAT5 and JAK2 on a routine base, neither was a significant decrease in STAT5 and JAK2 mRNA expression observed in other animal models for LS (Cui, Li et al. 2015). While an activation of JAK2 by residual functional GHRs could be excluded by ligand immunostainings of liver and kidney sections of *GHR*-KO pigs, its activation by other cytokine receptors seemed likely. A potential candidate was the leptin receptor as its activation induces the phosphorylation of STAT3 by JAK2 (reviewed in Rawlings, Rosler et al. 2004), whose phosphorylation levels were not decreased in *GHR*-KO pigs, and further increased fasting serum leptin levels were observed in *GHR*-KO pigs in line with findings in human LS patients (Laron, Silbergeld et al. 1998) and the *Ghr* KO

mouse (reviewed in List, Sackmann-Sala et al. 2011).

Showing that the phosphorylation levels of hepatic leptin receptors were in fact increased in *GHR*-KO and potentially lead to the observed JAK2 activation was a major finding of our study, since this connection in the context of GHRD has never been described before.

4. Future investigations

Our recent publication represents a first characterization of the *GHR*-KO pig model. While we gained insight into the effects of GHRD and successfully characterized endocrine alterations, it remains necessary to perform detailed investigations of effects on bone growth and formation of the epiphyseal growth plate. Further, in depth analysis of the pituitary gland as a target organ for endocrine feedback mechanisms seems promising. As alterations in glucose homeostasis and hepatic signaling provide potential beneficial effects of GHRD, an increase of insulin sensitivity in *GHR*-KO pigs needs to be proven and longevity associated analysis should follow. In contrast to the *Ghr* KO mice (Danilovich, Wernsing et al. 1999), no deficits in fertility of *GHR*-KO pigs were observed; thus large numbers of animals homozygous for the mutation can be maintained by mating of *GHR*-KO sows and boars for future experiments.

4.1. Bone growth

Investigating a dwarf phenotype, it appears obvious to perform detailed analyses of epiphyseal growth plates to determine the changes in bone formation leading to growth retardation. As longitudinal growth is physiological terminated by the epiphyseal closure, a porcine model appears more suitable than rodent models in representing human conditions. In mice and rats, bone growth persists throughout a large proportion of their life expectancy, after the animals are referred to as adults. In contrast, the growth plate is already closed in adult humans (reviewed in Kilborn, Trudel et al. 2002) induced by sexual maturity (Isaksson, Lindahl et al. 1987) Luckily, the pig is seen as the "species of choice" for representing human bone physiology, as close similarities are reported regarding anatomy, morphology, healing, remodeling and specifically bone mineral density (BMD) and mineral content (reviewed in Pearce, Richards et al. 2007), which is of particular interest in the context of LS. Besides a reduction of the width of the epiphyseal plate, reduced BMD is reported for *Ghr* KO mice (reviewed in List, Sackmann-Sala et al. 2011). In line with that, a reduction of absolute BMD is reported for human LS patients (Shaw, Fraser et al. 2003), but appears normal when corrected for bone volume (reviewed in Laron, Kauli et al. 2017). As expected, the length of all bones is reduced (**Figure 6**) and data from DXA analyzes of overall bone structure revealed reduced BMD (**Figure 7**) in *GHR*-KO pigs. A further investigation should include the comparison of data from *GHR*-KO pigs to control animals of the same weight and an analysis of the BMD of particular bones, which can easily be done with the existing images.

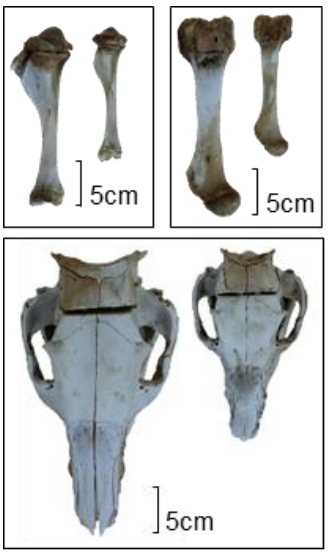


Figure 6. *GHR*-KO pigs exhibit shorter bones. Tibia, femur and skulls of control (left) and GHR-KO animals (right) aged six months

The determination of BMD of selected preserved bones by microCT scans as performed in the *Ghr* KO mouse (Berryman, List et al. 2010) can be useful. The increase in levels of serum alkaline phosphatase in *GHR*-KO pigs aged six months (**Figure 7**) can be of further interest, as increased levels can indicate active remodeling processes of bones involving the activity of osteoblast, which are usually seen at younger ages (Kraft 2005).

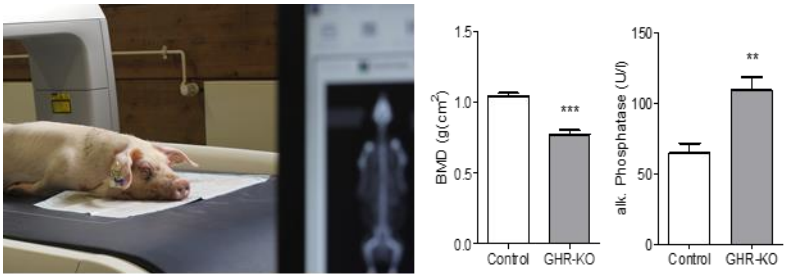


Figure 7. DXA analysis of a *GHR*-KO pig aged six months during the preparation of this study. BMD is significantly decreased while serum alkaline phosphatase levels were elevated.

A connection between bone remodeling processes and metabolism can be effecting the LS phenotype, as recent studies revealed the influence of osteoblast metabolism on energy utilization affecting the body composition (Fulzele, Riddle et al. 2010).

4.2. Alterations on pituitary levels

As the source of circulating GH and its involvement in complex endocrine feedback mechanisms (reviewed in Varela-Nieto and Chowen 2005), the pituitary gland is an organ of specific interest concerning GHRD. Besides the determination of the weight of pituitary glands of *GHR*-KO and control animals, we preserved manifold unpublished data and samples during the preparation of this study for future investigations, including pituitary diameters, formalin-fixed pituitary glands and hypothalamic tissues for histological and immunohistological analyses, tissue samples at -80°C for signaling studies, RNA sequencing or proteomic studies and tissue samples for electron microscopy. In line with observations in *GHR*-KO pigs, imaging analysis of pituitary glands of human LS patients revealed neither alterations in size, nor pathologic abnormalities (Kornreich, Horev et al. 2003). In spite of the impaired feedback inhibition of GH secretion in GHRD, the partially preserved pulsatility of GH secretion could be a reason why somatotroph hyperplasia was not observed (reviewed in Laron and Kopchick 2011). A suitable way to investigate this hypothesis, would be the investigation of compartments of

the pituitary glands of *GHR*-KO pigs using stereological methods (as described in (Albl, Haesner et al. 2016)), which of course cannot be performed in human patients. Similar investigations have been performed in the *Ghr* KO mouse revealing an increase in GH-immunoreactive cells and hyperplasia of granulated cells in electron microscopy (Asa, Coschigano et al. 2000). The immunostaining of GH performed in our study can easily be applied for the detection of GH-immunoreactive cells in the pituitary glands of *GHR*-KO pigs and a signaling studies can provide further insight on GHR-signaling associated alterations on pituitary and hypothalamic levels.

4.3. Longevity studies

A potential hindrance of the use of a pig model occurs when effects on longevity are studied. A simple estimation of the pig`s life expectancy as performed for the *Ghr* KO mouse (Bartke and Brown-Borg 2004) would consume way too much time. Potential approaches would be the *in vitro* estimation of stress resistance or the cross-breeding of *GHR*-KO pigs with genetically engineered pig strains harboring pathologic insults. The cellular resistance towards exogenous stress in *in vitro* studies is a well-established indicator for extended longevity (reviewed in Johnson, Lithgow et al. 1996). A suitable application to estimate longevity-associated effects in *GHR*-KO pigs would be the reproduction of the work of Murakami *et al.* (Murakami, Salmon et al. 2003), who found fibroblast derived from Snell dwarf mice being more resistant to cellular stress such as UV light, heat or H_2O_2. A study involving the cross-breeding of *Ghr* KO mice with a mouse strain developing prostatic neoplasia (called *Tag*) showed that while seven of eight *Tag* animals with intact GHR signaling showed neoplastic lesions, only one of eight *Tag* x *Ghr* KO mice exhibited those, suggesting impaired tumor growth in GHRD (Wang, Prins et al. 2005). As mouse models show several limitations in cancer research concerning longitudinal studies of tumor progression and remission, genetically tailored pig models developing neoplasia became more widely used (reviewed in Flisikowska, Kind et al. 2014). A possible candidate for the cross-breeding with *GHR*-KO pigs would be the APC^{1061}-pig (Flisikowska, Merkl et al. 2012), which develops adenomatous polyps in the colon and rectum, whose progression can be easily followed by colonoscopy.

4.4. Insulin sensitivity

The phenotype of the Ecuadorian cohort of LS patients is of specific interest, as it is characterized by increased insulin sensitivity in spite of severe obesity (Guevara-Aguirre, Rosenbloom et al. 2015). As obesity and diabetes are a major health problem in Western and developing societies (Ng, Fleming et al. 2014), the investigation of metabolic alterations of our GHR-KO pig seems promising, as it resembles the phenotype of this cohort of LS patients. Further, the pig is well established as an animal model for studying metabolic effects associated with diabetes (reviewed in Wolf, Braun-Reichhart et al. 2014) and a variety of methods generating meaningful data can be applied (reviewed in Renner, Dobenecker et al. 2016). Analysis should be performed in the young (12-15 weeks of age) and adult (six months of age) age group of *GHR*-KO pigs to address the phenomenon of transient juvenile hypoglycemia and reveal underlying mechanisms. For the investigation of insulin sensitivity, intravenous glucose tolerance tests (ivGTT) (Renner, Fehlings et al. 2010) or hyperinsulinemic, euglycemic clamp studies (Lang, Vaugelade et al. 1999) can easily be performed in large animal models. A tissue sampling for signaling analysis should include beside liver, muscle and fat tissue, due to their involvement in glucose metabolism and insulin sensitivity (reviewed in Saltiel and Kahn 2001). Muscle and fat tissue can easily be taken during the placement of central venous catheters for clamp studies, liver biopsies can be taken ultrasound-guided (Kessler 2000)). Besides signaling studies as performed in our recent publication including insulin receptor, mTOR and GHR associated signaling cascades, effectors of gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK) (reviewed in Chung, Chacko et al. 2015) should be included, as a lack of stimulatory GH effects on hepatic gluconeogenesis is expected (reviewed in Vijayakumar, Novosyadlyy et al. 2010).

4.5. Treatment trials

Data from both cohorts suggest, that LS patients do not benefit from positive effects of GHRD (Guevara-Aguirre, Balasubramanian et al. 2011, Laron and Kopchick 2011), as their appearance as growth retarded accompanied by obese physic and mental retardation in the *Israeli cohort* (Shevah, Kornreich et al. 2005) leads to socioeconomic marginalization, resulting in an increase of alcohol abuse (Junnila, List et al. 2013), making a suitable treatment desirable. As outlined in detail in the review of the literature, the treatment with recombinant biosynthetic IGF1 can

increase the growth velocity in infants and modify the physic appearance, but needs to be administered on a daily base due to short half-life and severe adverse effects are reported. As reviewed by Aigner *et al.* the use of pig models for the development of new therapies and drugs emerges, as moue models are limited in generating reliable data on efficiency and safety of new therapeutic approaches (Aigner, Renner et al. 2010). As the *GHR*-KO pig resembles the human phenotype of LS, it can be used to improve the treatment of the human syndrome. A major disadvantage of the existing treatment lies in the short half-life of recombinant IGF1, as most adverse effects are due to over dosage or administration without food. A suitable way to increase the half-life of recombinant IGF1 can be the PASylation technology (Schlapschy, Binder et al. 2013), where in brief the fusion of proline, alanine and serine (PAS) polypeptides to a therapeutic protein increases its half-life by a reduction of renal clearance due to increased hydrodynamic volume of the synthetic drug.

In conclusion, the *GHR*-KO pig represents the human phenotype, particularly of the *Ecuadorian cohort* regarding glucose homeostasis and provides a suitable model for the investigation of metabolic changes. To gain further insight on beneficial effects of GHRD from the *GHR*-KO pig and the development of new therapeutic options for the human syndrome is desirable.

V. SUMMARY

The Laron syndrome (LS) is an autosomal recessive hereditary human disorder with just a few hundred cases world-wide. Due to a mutation of the *GHR* gene, the binding capacity of circulating GH or the signal transduction is diminished, resulting in a decreased production of IGF1 by the liver and elevated serum GH levels due to the lack of regulatory feedback mechanism. Affected patients suffer from severe postnatal growth retardation leading to a dwarf phenotype. Furthermore, severe juvenile hypoglycemia and an increase in body fat are hallmarks of the human LS. Observations revealed a protection from malignancies such as cancer and increased insulin sensitivity is reported from a genetically homogenous cohort of LS patients from Ecuador.

A rodent model for LS, the *Ghr* KO / Laron mouse was established in 1997 and showed increased life expectancy resulting from growth hormone receptor deficiency (GHRD). While the *Ghr* KO mouse resembles hallmarks of the human phenotype, its usage is limited by evolutionary differences to humans concerning the endocrine growth stimulation and bone formation.

As porcine models are able to bridge (patho)physiological gaps between rodent models and human diseases and emerge as a useful tool in translational medicine, we generated a pig model for LS by target site mutation of the porcine *GHR* exon 3 applying the CRISPR/Cas9 technology. The *GHR*-KO pig resembles hallmarks of the human disease phenotype and our study revealed altered activation of hepatic signaling cascades. The endocrine changes in the *GHR*-KO pig result from a diminished binding of GH and include decreased levels of serum IGF1 and IGFBP3, while GH and IGFBP2 levels were increased similar to human LS patients. The characterization of the pig model further included auxologic and metabolic analyses and revealed postnatal growth retardation leading to a body weight of 60% of control animal´s weight at an age of six months. Furthermore, GHR-KO pigs displayed an increase in body fat and proportional and disproportional reduction in organ weights as reported for human LS patients and the murine model. The investigation of metabolic characteristics revealed transient juvenile hypoglycemia while insulin levels remained similar to control animal`s values.

To determine a state of increased insulin sensitivity, we investigated insulin receptor associated signaling pathways in liver samples derived from fasted animals aged six months and observed an increased abundance and phosphorylation of IRS1 and increased activity of other signal transducers. An increase in insulin receptor associated signaling in animals with normal fasting serum insulin levels suggests an increase in insulin sensitivity in *GHR*-KO pigs as seen in human patients from the *Ecuadorian cohort* who are protected from developing diabetes. As hyperactivation of the mTORC1 is commonly seen in association with the development of cancer, while mTORC2 is preferentially involved in cell homeostasis, the increase in mTORC2 but not mTORC1 activity in *GHR*-KO pigs can be seen as a positive effect of GHRD. As expected, the investigation of GHR associated signaling cascades revealed decreased phosphorylation STAT5. We were able to explain the increased phosphorylation of JAK2 by an increased signaling of the leptin receptor accompanied with elevated serum leptin levels.

In conclusion, we successfully generated a novel large animal model for LS, which can provide further insight on the molecular basis of the disease and can be used for the development of new therapeutic approaches.

VI. ZUSAMMENFASSUNG

Erstellung und Charakterisierung eines Schweinemodells für das Laron Syndrom

Das Laron Syndrom (LS) ist eine autosomal rezessiv vererbte Erkrankung, von der weltweit nur wenige hundert Personen betroffen sind. Die Mutation des Wachstumshormon-Rezeptor (*GHR*) Gens führt dazu, dass Wachstumshormon (GH) nicht gebunden werden kann oder dass die Signalkaskade gestört ist. Daraus resultiert eine verminderte Bildung von IGF1 durch die Leber und eine Erhöhung der Serum-GH-Spiegel aufgrund des Fehlens regulatorischer Feedback-Mechanismen. Die Patienten sind von einer postnatalen Wachstumsretardierung betroffen, die zu Minderwuchs führt. Des Weiteren sind eine dramatische Hypoglykämie bei Kindern und Zunahme von Körperfett charakteristisch für das Laron Syndrom. Untersuchungen ergaben, dass Betroffene vor einigen Erkrankungen, insbesondere Krebs, geschützt zu sein scheinen. Des Weiteren wurde in einer genetisch sehr homogenen Population von Laron Syndrom Patienten aus Ecuador eine erhöhte Insulin-Sensitivität beobachtet.

Im Jahr 1997 wurde das erste Nager-Modell für LS - die *Ghr* KO/Laron Maus - erstellt. An ihr wurde gezeigt, dass GHR-Defizienz (GHRD) die Lebenserwartung deutlich erhöht. Während die *Ghr* KO Maus die meisten Charakteristika des humanen Phänotyps wiederspiegelt, ist ihr Nutzen durch physiologisch Unterschiede zum Menschen begrenzt. Dies betrifft vor Allem die endokrine Steuerung des Wachstums und insbesondere die Physiologie der Knochen.

Da Schweine-Modelle in der Lage sind, Lücken zwischen Grundlagenstudien an Nager-Modellen und klinischen Studien zu schließen und im Rahmen der translationalen Medizin zunehmend an Bedeutung gewinnen, haben wir mittels der CRISPR/Cas9 Technologie ein Schweine-Modell für das Laron Syndrom erstellt, indem wir eine zielgerichtete Mutation des *GHR* Exons 3 erzeugt haben. Das *GHR*-KO Schwein gibt die Charakteristika des humanen Phänotyps wieder und Analysen von Leberproben ergaben eine veränderte Aktivität von Signalkaskaden. Die fehlende Bindung von Wachstumshormon bei den *GHR*-KO Schweinen führt zu weitreichenden Veränderungen auf endokrinologischer Ebene, die auch im humanen Phänotyp vorhanden sind. Während die Serum-Spiegel von IGF1 und

IGFBP3 reduziert waren, wurden erhöhte Konzentrationen von zirkulierendem Wachstumshormon und IGFBP2 gemessen. Die Charakterisierung der *GHR*-KO Schweine beinhaltete auxologische und metabolische Veränderungen. Unsere Studie zeigt, dass die postnatale Wachstumsretardierung dazu führt, dass das Gewicht der *GHR*-KO Schweine im Alter von 6 Monaten nur 60% des Gewichtes von Kontrolltieren erreicht. Des Weiteren wurde eine deutliche Zunahme an Körperfett und eine proportionale und disproportionale Reduzierung von Organgewichten registriert. Die Beobachtungen entsprechen dem menschlichen Phänotyp und Ergebnissen aus dem Maus-Modell. Junge *GHR*-KO Tiere zeigten eine deutliche Hypoglykämie, während die Plasma-Glukose-Werte von 6 Monate alten *GHR*-KO Schweinen im Bereich sich nicht von denen altersgleich Kontrolltiere unterschieden. Während der gesamten Entwicklung zeigten sich keine signifikanten Unterscheide der gefasteten Insulin Werte zwischen *GHR*-KO und Kontrolltieren.

Zur Bestätigung einer gesteigerten Insulin-Sensitivität untersuchten wir Signalkaskaden, die mit dem Insulinrezeptor in Verbindung stehen, in Leberproben von 6 Monate alten, gefasteten Tieren. Es zeigte sich eine erhöhte Abundanz und Phosphorylierung des IRS1 und weiterer Signalüberträger. Die erhöhte Aktivität von Insulinrezeptor-assoziierten Signalkaskaden bei normalen gefasteten Insulin-Werten weist auf eine erhöhte Insulin-Sensitivität der *GHR*-KO Schweine hin. Dies wurde auch bei ecuadorianischen Laron Syndrom Patienten beobachtet, die keinen Diabetes entwickeln. Als weiteren positiven Effekt von GHRD wurde bei den *GHR*-KO Schweinen eine verstärkte Aktivität von mTORC2 beobachtet, während die Aktivität von mTORC1, das häufig im Zusammenhang mit der Entwicklung von Krebs gesehen wird, weitestgehend ausgeschlossen werden kann. Wie zu erwarten zeigte die Untersuchung von Signalkaskaden im Zusammenhang mit dem GHR eine reduzierte Phosphorylierung von STAT5. Insbesondere waren wir in der Lage, eine erhöhte Phosphorylierung von JAK2 mit erhöhten Serum- Leptin-Werten sowie einer vermehrten Expression und Aktivierung von Leptin-Rezeptoren in der Leber in Zusammenhang zu bringen.

Zusammenfassend stellt das GHR-KO Schwein ein interessantes Großtiermodell für das Laron Syndrom dar, an dem weitere Einsichten in die molekularen Mechanismen der Erkrankung gewonnen sowie neue therapeutischer Ansätze entwickelt und getestet werden können.

VII. REFERENCES

Agarwal, S., L. Cogburn and J. Burnside (1994). "Dysfunctional growth hormone receptor in a strain of sex-linked dwarf chicken: evidence for a mutation in the intracellular domain." Journal of Endocrinology **142**(3): 427-434.

Aigner, B., S. Renner, B. Kessler, N. Klymiuk, M. Kurome, A. Wünsch and E. Wolf (2010). "Transgenic pigs as models for translational biomedical research." Journal of Molecular Medicine **88**(7): 653-664.

Albl, B., S. Haesner, C. Braun-Reichhart, E. Streckel, S. Renner, F. Seeliger, E. Wolf, R. Wanke and A. Blutke (2016). "Tissue Sampling Guides for Porcine Biomedical Models." Toxicol Pathol.

Amit, T., M. B. H. Youdim and Z. e. Hochberg (2000). "Does Serum Growth Hormone (GH) Binding Protein Reflect Human GH Receptor Function?" The Journal of Clinical Endocrinology & Metabolism **85**(3): 927-932.

Anders, C., O. Niewoehner, A. Duerst and M. Jinek (2014). "Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease." Nature **513**(7519): 569.

Asa, S. L., K. T. Coschigano, L. Bellush, J. J. Kopchick and S. Ezzat (2000). "Evidence for growth hormone (GH) autoregulation in pituitary somatotrophs in GH antagonist-transgenic mice and GH receptor-deficient mice." Am J Pathol **156**(3): 1009-1015.

Backeljauw, P. F. and L. E. Underwood (2001). "Therapy for 6.5–7.5 Years with Recombinant Insulin-Like Growth Factor I in Children with Growth Hormone Insensitivity Syndrome: A Clinical Research Center Study1." The Journal of Clinical Endocrinology & Metabolism **86**(4): 1504-1510.

Baker, J., J.-P. Liu, E. J. Robertson and A. Efstratiadis (1993). "Role of insulin-like growth factors in embryonic and postnatal growth." Cell **75**(1): 73-82.

Baker, J., J. P. Liu, E. J. Robertson and A. Efstratiadis (1993). "Role of insulin-like growth factors in embryonic and postnatal growth." Cell **75**(1): 73-82.

Bartke, A. and H. Brown-Borg (2004). Life Extension in the Dwarf Mouse. Current Topics in Developmental Biology, Academic Press. **63:** 189-225.

Bartke, A., J. C. Wright, J. A. Mattison, D. K. Ingram, R. A. Miller and G. S. Roth (2001). "Longevity: extending the lifespan of long-lived mice." Nature **414**(6862): 412.

Baumann, G., M. Shaw and K. Amburn (1994). "Circulating growth hormone binding proteins." Journal of endocrinological investigation **17**(1): 67-81.

Berryman, D. E., E. O. List, K. T. Coschigano, K. Behar, J. K. Kim and J. J. Kopchick (2004). "Comparing adiposity profiles in three mouse models with altered GH signaling." Growth Hormone & IGF Research **14**(4): 309-318.

Berryman, D. E., E. O. List, D. T. Kohn, K. T. Coschigano, R. J. Seeley and J. J. Kopchick (2006). "Effect of Growth Hormone on Susceptibility to Diet-Induced Obesity." Endocrinology **147**(6): 2801-2808.

Berryman, D. E., E. O. List, A. J. Palmer, M.-Y. Chung, J. Wright-Piekarski, E. Lubbers, P. O'Connor, S. Okada and J. J. Kopchick (2010). "Two-year body composition analyses of long-lived GHR null mice." The Journals of Gerontology Series A: Biological Sciences and Medical Sciences **65**(1): 31-40.

Besenfelder, U., J. Modl, M. Muller and G. Brem (1997). "Endoscopic embryo collection and embryo transfer into the oviduct and the uterus of pigs." Theriogenology **47**(5): 1051-1060.

Bhaya, D., M. Davison and R. Barrangou (2011). "CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation." Annual review of genetics **45**: 273-297.

Boch, J., H. Scholze, S. Schornack, A. Landgraf, S. Hahn, S. Kay, T. Lahaye, A. Nickstadt and U. Bonas (2009). "Breaking the code of DNA binding specificity of TAL-type III effectors." Science **326**(5959): 1509-1512.

Boegheim, I. J. M., P. A. J. Leegwater, H. A. van Lith and W. Back (2017). "Current insights into the molecular genetic basis of dwarfism in livestock." The Veterinary Journal **224**: 64-75.

Bogdanove, A. J. and D. F. Voytas (2011). "TAL effectors: customizable proteins for DNA targeting." Science **333**(6051): 1843-1846.

Bonkowski, M. S., J. S. Rocha, M. M. Masternak, K. A. Al Regaiey and A. Bartke (2006). "Targeted disruption of growth hormone receptor interferes with the beneficial actions of calorie restriction." Proceedings of the National Academy of Sciences **103**(20): 7901-7905.

Brooks, A., D. Abankwa, K. Tunny, K. Blucher and O. Gardon (2009). "Activation of growth hormone receptor by subunit realignment." ENDO **9**: 10-13.

Brooks, A. J., W. Dai, M. L. O'Mara, D. Abankwa, Y. Chhabra, R. A. Pelekanos, O. Gardon, K. A. Tunny, K. M. Blucher, C. J. Morton, M. W. Parker, E. Sierecki, Y. Gambin, G. A. Gomez, K. Alexandrov, I. A. Wilson, M. Doxastakis, A. E. Mark and M. J. Waters (2014). "Mechanism of Activation of Protein Kinase JAK2 by the Growth Hormone Receptor." Science **344**(6185).

Brooks, A. J. and M. J. Waters (2010). "The growth hormone receptor: mechanism of activation and clinical implications." Nat Rev Endocrinol **6**(9): 515-525.

Calle, E. E. and R. Kaaks (2004). "Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms." Nature Reviews Cancer **4**(8): 579.

Carel, J. C., J. L. Chaussain, P. Chatelain and M. O. Savage (1996). "Growth hormone insensitivity syndrome (Laron syndrome): main characteristics and effects of IGF1 treatment." Diabetes Metab **22**(4): 251-256.

Chung, S. T., S. K. Chacko, A. L. Sunehag and M. W. Haymond (2015). "Measurements of gluconeogenesis and glycogenolysis: a methodological review." Diabetes **64**(12): 3996-4010.

Cohick, W. S. and D. R. Clemmons (1993). "The insulin-like growth factors." Annu Rev Physiol **55**: 131-153.

Coschigano, K. T., D. Clemmons, L. L. Bellush and J. J. Kopchick (2000). "Assessment of growth parameters and life span of GHR/BP gene-disrupted mice." Endocrinology **141**(7): 2608-2613.

Cui, D., F. Li, Q. Li, J. Li, Y. Zhao, X. Hu, R. Zhang and N. Li (2015). "Generation of a miniature pig disease model for human Laron syndrome." Sci Rep **5**: 15603.

Danilovich, N., D. Wernsing, K. T. Coschigano, J. J. Kopchick and A. Bartke (1999). "Deficits in Female Reproductive Function in GH-R-KO Mice; Role of IGF-I." Endocrinology **140**(6): 2637-2640.

Dmochewitz, M. and E. Wolf (2015). "Genetic engineering of pigs for the creation of translational models of human pathologies." Animal Frontiers **5**(1): 50-56.

Dominick, G., D. E. Berryman, E. O. List, J. J. Kopchick, X. Li, R. A. Miller and G. G. Garcia (2015). "Regulation of mTOR Activity in Snell Dwarf and GH Receptor Gene-Disrupted Mice." Endocrinology **156**(2): 565-575.

Egecioglu, E., M. Bjursell, A. Ljungberg, S. L. Dickson, J. J. Kopchick, G. Bergström, L. Svensson, J. Oscarsson, J. Törnell and M. Bohlooly-Y (2006). "Growth hormone receptor deficiency results in blunted ghrelin feeding response, obesity, and hypolipidemia in mice." American Journal of Physiology - Endocrinology And Metabolism **290**(2): E317-E325.

El-Haschimi, K., D. D. Pierroz, S. M. Hileman, C. Bjørbæk and J. S. Flier (2000). "Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity." The Journal of clinical investigation **105**(12): 1827-1832.

Eshet, R., Z. Laron, A. Pertzelan, R. Arnon and M. Dintzman (1984). "Defect of human growth hormone receptors in the liver of two patients with Laron-type dwarfism." Isr J Med Sci **20**(1): 8-11.

Feinberg, M. S., M. Scheinowitz and Z. Laron (2000). "Echocardiographic dimensions and function in adults with primary growth hormone resistance (Laron Syndrome)." The American Journal of Cardiology **85**(2): 209-213.

Flint, D. J., N. Binart, S. Boumard, J. J. Kopchick and P. Kelly (2006). "Developmental aspects of adipose tissue in GH receptor and prolactin receptor gene disrupted mice: site-specific effects upon proliferation, differentiation and hormone sensitivity." Journal of endocrinology **191**(1): 101-111.

Flisikowska, T., A. Kind and A. Schnieke (2014). "Genetically modified pigs to model human diseases." Journal of applied genetics **55**(1): 53-64.

Flisikowska, T., C. Merkl, M. Landmann, S. Eser, N. Rezaei, X. Cui, M. Kurome, V. Zakhartchenko, B. Kessler and H. Wieland (2012). "A porcine model of familial adenomatous polyposis." Gastroenterology **143**(5): 1173-1175. e1177.

Fulzele, K., R. C. Riddle, X. Cao, C. Wan, D. Chen, M. C. Faugere, S. Aja, M. A. Hussain, J. C. Brüning and T. L. Clemens (2010). "Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition." Cell **142**(2): 309-319.

Gaj, T., C. A. Gersbach and C. F. Barbas III (2013). "ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering." Trends in biotechnology **31**(7): 397-405.

Gärke, C., F. Ytournel, A. Sharifi, E. Pimentel, A. Ludwig and H. Simianer (2014). "Footprints of recent selection and variability in breed composition in the Göttingen Minipig genome." Animal genetics **45**(3): 381-391.

Gent, J., P. Van Kerkhof, M. Roza, G. Bu and G. J. Strous (2002). "Ligand-independent growth hormone receptor dimerization occurs in the endoplasmic reticulum and is required for ubiquitin system-dependent endocytosis." Proceedings of the National Academy of Sciences **99**(15): 9858-9863.

Godowski, P. J., D. W. Leung, L. R. Meacham, J. P. Galgani, R. Hellmiss, R. Keret, P. S. Rotwein, J. S. Parks, Z. Laron and W. I. Wood (1989). "Characterization of the human growth hormone receptor gene and demonstration of a partial gene deletion in two patients with Laron-type dwarfism." Proceedings of the National Academy of Sciences **86**(20): 8083-8087.

Gonçalves, F. T., C. Fridman, E. M. Pinto, J. Guevara-Aguirre, O. Shevah, A. L. Rosembloom, V. Hwa, F. Cassorla, R. G. Rosenfeld and T. S. Lins (2014). "The E180splice mutation in the GHR gene causing Laron syndrome: witness of a Sephardic Jewish exodus from the Iberian Peninsula to the New World?" American Journal of Medical Genetics Part A **164**(5): 1204-1208.

Guevara-Aguirre, J., P. Balasubramanian, M. Guevara-Aguirre, M. Wei, F. Madia, C. W. Cheng, D. Hwang, A. Martin-Montalvo, J. Saavedra, S. Ingles, R. de Cabo, P. Cohen and V. D. Longo (2011). "Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans." Sci Transl Med **3**(70): 70ra13.

Guevara-Aguirre, J. and A. L. Rosenbloom (2015). "Obesity, diabetes and cancer: insight into the relationship from a cohort with growth hormone receptor deficiency." Diabetologia **58**(1): 37-42.

Guevara-Aguirre, J., A. L. Rosenbloom, P. Balasubramanian, E. Teran, M. Guevara-Aguirre, C. Guevara, P. Procel, I. Alfaras, R. De Cabo, S. Di Biase, L. Narvaez, J. Saavedra and V. D. Longo (2015). "GH Receptor Deficiency in Ecuadorian Adults Is Associated With Obesity and Enhanced Insulin Sensitivity." The Journal of Clinical Endocrinology & Metabolism **100**(7): 2589-2596.

Guevara-Aguirre, J., A. L. Rosenbloom, P. J. Fielder, J. F. B. Diamond and R. G. Rosenfeld (1993). "Growth hormone receptor deficiency in Ecuador: clinical and biochemical phenotype in two populations." The Journal of Clinical Endocrinology & Metabolism **76**(2): 417-423.

Hai, T., F. Teng, R. Guo, W. Li and Q. Zhou (2014). "One-step generation of knockout pigs by zygote injection of CRISPR/Cas system." Cell research **24**(3): 372.

Hammer, R. E., V. G. Pursel, C. E. Rexroad Jr, R. J. Wall, D. J. Bolt, K. M. Ebert, R. D. Palmiter and R. L. Brinster (1985). "Production of transgenic rabbits, sheep and pigs by microinjection." Nature **315**(6021): 680.

Herrington, J. and C. Carter-Su (2001). "Signaling pathways activated by the growth hormone receptor." Trends in Endocrinology & Metabolism **12**(6): 252-257.

Herrington, J., L. S. Smit, J. Schwartz and C. Carter-Su (2000). "The role of STAT proteins in growth hormone signaling." Oncogene **19**(21): 2585.

Hetz, J. A., B. R. Menzies, G. Shaw, A. Rao, I. J. Clarke and M. B. Renfree (2015). "Growth axis maturation is linked to nutrition, growth and developmental rate." Mol Cell Endocrinol **411**: 38-48.

Honda, Y., K. Takahashi, S. Takahashi, K. Azumi, M. Irie, M. Sakuma, T. Tsushima and K. Shizume (1969). "Growth Hormone Secretion During Nocturnal Sleep in Normal Subjects1." The Journal of Clinical Endocrinology & Metabolism **29**(1): 20-29.

Hull, K. L. and S. Harvey (1999). "Growth hormone resistance: clinical states and animal models." J Endocrinol **163**(2): 165-172.

Ihle, J. N. (1996). "STATs: signal transducers and activators of transcription." Cell **84**(3): 331-334.

Isaksson, O. G., A. Lindahl, A. Nilsson and J. Isgaard (1987). "Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth." Endocr Rev **8**(4): 426-438.

Jéquier, E. (2002). "Leptin signaling, adiposity, and energy balance." Annals of the New York Academy of Sciences **967**(1): 379-388.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna and E. Charpentier (2012). "A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity." science: 1225829.

Johnson, T. E., G. J. Lithgow and S. Murakami (1996). "Hypothesis: interventions that increase the response to stress offer the potential for effective life prolongation and increased health." The Journals of Gerontology Series A: Biological Sciences and Medical Sciences **51**(6): B392-B395.

Jones, J. I. and D. R. Clemmons (1995). "Insulin-Like Growth Factors and Their Binding Proteins: Biological Actions*." Endocrine Reviews **16**(1): 3-34.

Junnila, R. K., E. O. List, D. E. Berryman, J. W. Murrey and J. J. Kopchick (2013). "The GH/IGF-1 axis in ageing and longevity." Nature Reviews Endocrinology **9**(6): 366.

Kaplan, S. A. and P. Cohen (2007). "The somatomedin hypothesis 2007: 50 years later." J Clin Endocrinol Metab **92**(12): 4529-4535.

Keightley, M.-C. and P. J. Fuller (1996). "Anomalies in the Endocrine Axes of the Guinea Pig: Relevance to Human Physiology and Disease*." Endocrine Reviews **17**(1): 30-44.

Keret, R., A. Pertzelan, A. Zeharia, Z. Zadik and Z. Laron (1988). "Growth hormone (hGH) secretion and turnover in three patients with Laron-type dwarfism." Israel journal of medical sciences **24**(2): 75-79.

Kessler, B. (2000). Untersuchungen zur Sonographie der Leber und zur ultraschallgeführten Gallenblasenpunktion beim Schwein.

Kilborn, S. H., G. Trudel and H. Uhthoff (2002). "Review of growth plate closure compared with age at sexual maturity and lifespan in laboratory animals." Journal of the American Association for Laboratory Animal Science **41**(5): 21-26.

Kleinert, M., C. Clemmensen, S. M. Hofmann, M. C. Moore, S. Renner, S. C. Woods, P. Huypens, J. Beckers, M. H. de Angelis and A. Schürmann (2018). "Animal models of obesity and diabetes mellitus." Nature Reviews Endocrinology.

Kornreich, L., G. Horev, M. Schwarz, B. Karmazyn and Z. Laron (2003). "Pituitary size in patients with Laron syndrome (primary GH insensitivity)." European journal of endocrinology **148**(3): 339-341.

Kraft, W. (2005). Klinische labordiagnostik in der tiermedizin, Schattauer Verlag.

Kranzler, J. H., A. L. Rosenbloom, V. Martinez and J. Guevara-Aguirre (1998). "Normal Intelligence with Severe Insulin-Like Growth Factor I Deficiency due to Growth Hormone Receptor Deficiency: A Controlled Study in a Genetically Homogeneous Population1." The Journal of Clinical Endocrinology & Metabolism **83**(6): 1953-1958.

Kurome, M., B. Kessler, A. Wuensch, H. Nagashima and E. Wolf (2015). "Nuclear transfer and transgenesis in the pig." Methods Mol Biol **1222**: 37-59.

Labarta, J. I., S. E. Gargosky, D. M. Simpson, P. D. Lee, J. Argente, J. Guevara-Aguirre and R. G. Rosenfeld (1997). "Immunoblot studies of the acid-labile subunit (ALS) in biological fluids, normal human serum and in children with GH deficiency and GH receptor deficiency before and after long-term therapy with GH or IGF-I respectively." Clin Endocrinol (Oxf) **47**(6): 657-666.

Labarta, J. I., S. E. Gargosky, D. M. Simpson, P. D. K. Lee, J. Argente, J. Guevara-Aguirre and R. G. Rosenfeld (1997). "Immunoblot studies of the acid-labile subunit (ALS) in biological fluids, normal human serum and in children with GH deficiency and GH receptor deficiency before and after long-term therapy with GH or IGF-I respectively." Clinical Endocrinology **47**(6): 657-666.

Lang, V., P. Vaugelade, F. Bernard, B. Darcy-Vrillon, C. Alamowitch, G. Slama, P.-H. Duée and F. R. Bornet (1999). "Euglycemic hyperinsulinemic clamp to assess posthepatic glucose appearance after carbohydrate loading. 1. Validation in pigs–." The American journal of clinical nutrition **69**(6): 1174-1182.

Laron, Z. (1993). "An update on Laron syndrome." Arch Dis Child **68**(3): 345-346.

Laron, Z. (2004). "Laron syndrome (primary growth hormone resistance or insensitivity): the personal experience 1958-2003." J Clin Endocrinol Metab **89**(3): 1031-1044.

Laron, Z., Y. Avitzur and B. Klinger (1995). "Carbohydrate metabolism in primary growth hormone resistance (Laron syndrome) before and during insulin-like growth factor-I treatment." Metabolism **44**(10 Suppl 4): 113-118.

Laron, Z., Y. Avitzur and B. Klinger (1997). "Insulin Resistance in Laron Syndrome (Primary Insulin-like Growth Factor-I (IGF-I) Deficiency) and Effect of IGF-I Replacement Therapy." Journal of Pediatric Endocrinology and Metabolism **10**(Supplement): 105-116.

Laron, Z., S. Ginsberg, P. Lilos, M. Arbiv and N. Vaisman (2006). "Body composition in untreated adult patients with Laron syndrome (primary GH insensitivity)." Clin Endocrinol (Oxf) **65**(1): 114-117.

Laron, Z., S. Ginsberg, C. Pariente and H. Kanety (2007). Serum adiponectin levels in patients with Laron syndrome are unexpectedly high (Abstract# P2–67). 89th Annual Meeting of The Endocrine Society, Toronto, Canada, Program & Abstracts Book.

Laron, Z. and T. Karmon (2010). "Long-term changes in blood lipids and liver function in patients with Laron syndrome MD Thesis." Sackler Faculty of Medicine, Tel Aviv University, Israel Google Scholar.

Laron, Z. and R. Kauli (2016). "Fifty seven years of follow-up of the Israeli cohort of Laron Syndrome patients—From discovery to treatment." Growth Hormone & IGF Research **28**: 53-56.

Laron, Z., R. Kauli, L. Lapkina and H. Werner (2017). "IGF-I deficiency, longevity and cancer protection of patients with Laron syndrome." Mutation Research/Reviews in Mutation Research **772**: 123-133.

Laron, Z. and B. Klinger (1994). "IGF-I treatment of adult patients with Laron syndrome: preliminary results." Clinical endocrinology **41**(5): 631-638.

Laron, Z., B. Klinger, R. Eshet, H. Kaneti, A. Karasik and A. Silbergeld (1993). "Laron syndrome due to a post-receptor defect: response to IGF-I treatment." Israel journal of medical sciences **29**(12): 757-763.

Laron, Z. and J. J. Kopchick (2011). Laron syndrome-from man to mouse, Springer.

Laron, Z., A. Pertzelan and M. Karp (1968). "Pituitary dwarfism with high serum levels of growth hormone." Israel journal of medical sciences **4**(4): 883.

Laron, Z., A. Pertzelan, M. Karp, A. Kowadlo-Silbergeld and W. H. Daughaday (1971). "Administration of Growth Hormone to Patients with Familial Dwarfism with High Plasma Immunoreactive Growth Hormone: Measurement of Sulfation Factor, Metabolic and Linear Growth Responses." The Journal of Clinical Endocrinology & Metabolism **33**(2): 332-342.

Laron, Z., A. Pertzelan and S. Mannheimer (1966). "Genetic pituitary dwarfism with high serum concentation of growth hormone--a new inborn error of metabolism?" Israel journal of medical sciences **2**(2): 152.

Laron, Z., A. Silbergeld, P. Lilos and F. Blum (1998). "Serum leptin in obese patients with Laron syndrome before and during IGF-I treatment." Journal of Pediatric Endocrinology and Metabolism **11**(5): 653-656.

Laron, Z., A. Silbergeld, P. Lilos and F. W. Blum (1998). "Serum leptin in obese patients with Laron syndrome before and during IGF-I treatment." J Pediatr Endocrinol Metab **11**(5): 653-656.

Lavitrano, M., M. Busnelli, M. G. Cerrito, R. Giovannoni, S. Manzini and A. Vargiolu (2005). "Sperm-mediated gene transfer." Reproduction, Fertility and Development **18**(2): 19-23.

Li, F., Y. Li, H. Liu, X. Zhang, C. Liu, K. Tian, L. Bolund, H. Dou, W. Yang, H. Yang, N. H. Staunstrup and Y. Du (2015). "Transgenic Wuzhishan minipigs designed to express a dominant-negative porcine growth hormone receptor display small stature and a perturbed insulin/IGF-1 pathway." Transgenic Research **24**(6): 1029-1042.

Lichanska, A. and M. Waters (2008). "New insights into growth hormone receptor function and clinical implications." Hormone Research in Paediatrics **69**(3): 138-145.

List, E. O., L. Sackmann-Sala, D. E. Berryman, K. Funk, B. Kelder, E. S. Gosney, S. Okada, J. Ding, D. Cruz-Topete and J. J. Kopchick (2011). "Endocrine parameters and phenotypes of the growth hormone receptor gene disrupted (GHR-/-) mouse." Endocr Rev **32**(3): 356-386.

Liu, J.-L., K. T. Coschigano, K. Robertson, M. Lipsett, Y. Guo, J. J. Kopchick, U. Kumar and Y. L. Liu (2004). "Disruption of growth hormone receptor gene causes diminished pancreatic islet size and increased insulin sensitivity in mice." American Journal of Physiology - Endocrinology and Metabolism **287**(3): E405-E413.

Liu, J., C. K. Boyd, Y. Kobayashi, C. C. Chase, Jr., A. C. Hammond, T. A. Olson, T. H. Elsasser and M. C. Lucy (1999). "A novel phenotype for Lardon dwarfism in miniature Bos indicus cattle suggests that the expression of growth hormone receptor 1A in liver is required for normal growth." Domest Anim Endocrinol **17**(4): 421-437.

Longo, V. D. and C. E. Finch (2003). "Evolutionary Medicine: From Dwarf Model Systems to Healthy Centenarians?" Science **299**(5611): 1342-1346.

Longo, V. D. and L. Fontana (2010). "Calorie restriction and cancer prevention: metabolic and molecular mechanisms." Trends in pharmacological sciences **31**(2): 89-98.

Lunney, J. K. (2007). "Advances in swine biomedical model genomics." International journal of biological sciences **3**(3): 179.

Luo, Y., L. Lin, L. Bolund, T. G. Jensen and C. B. Sørensen (2012). "Genetically modified pigs for biomedical research." Journal of inherited metabolic disease **35**(4): 695-713.

Mérat, P. (1984). "The sex-linked dwarf gene in the broiler chicken industry." World's Poultry Science Journal **40**(1): 10-18.

Miller, R. A., Y. Chang, A. T. Galecki, K. Al-Regaiey, J. J. Kopchick and A. Bartke (2002). "Gene expression patterns in calorically restricted mice: partial overlap with long-lived mutant mice." Molecular endocrinology **16**(11): 2657-2666.

Milward, A., L. Metherell, M. Maamra, M. J. Barahona, I. R. Wilkinson, C. Camacho-Hübner, M. O. Savage, C. M. Bidlingmaier, A. J. L. Clark, R. J. M. Ross and S. M. Webb (2004). "Growth Hormone (GH) Insensitivity Syndrome due to a GH Receptor Truncated after Box1, Resulting in Isolated Failure of STAT 5 Signal Transduction." The Journal of Clinical Endocrinology & Metabolism **89**(3): 1259-1266.

Murakami, S., A. Salmon and R. A. Miller (2003). "Multiplex stress resistance in cells from long-lived dwarf mice." The FASEB journal.

Ng, M., T. Fleming, M. Robinson, B. Thomson, N. Graetz, C. Margono, E. C. Mullany, S. Biryukov, C. Abbafati and S. F. Abera (2014). "Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013." The lancet **384**(9945): 766-781.

Oh, W. J. and E. Jacinto (2011). "mTOR complex 2 signaling and functions." Cell Cycle **10**(14): 2305-2316.

Pearce, A., R. Richards, S. Milz, E. Schneider and S. Pearce (2007). "Animal models for implant biomaterial research in bone: a review." Eur Cell Mater **13**(1): 1-10.

Pfeifer, A. (2004). "Lentiviral transgenesis." Transgenic research **13**(6): 513-522.

Poger, D. and A. E. Mark (2010). "Turning the growth hormone receptor on: evidence that hormone binding induces subunit rotation." Proteins: Structure, Function, and Bioinformatics **78**(5): 1163-1174.

Pollak, M. N., E. S. Schernhammer and S. E. Hankinson (2004). "Insulin-like growth factors and neoplasia." Nature Reviews Cancer **4**(7): 505.

Porteus, M. H. and D. Carroll (2005). "Gene targeting using zinc finger nucleases." Nature biotechnology **23**(8): 967.

Rajaram, S., D. J. Baylink and S. Mohan (1997). "Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions." Endocrine reviews **18**(6): 801-831.

Rawlings, J. S., K. M. Rosler and D. A. Harrison (2004). "The JAK/STAT signaling pathway." Journal of Cell Science **117**(8): 1281-1283.

Renner, S., B. Dobenecker, A. Blutke, S. Zoels, R. Wanke, M. Ritzmann and E. Wolf (2016). "Comparative aspects of rodent and nonrodent animal models for mechanistic and translational diabetes research." Theriogenology **86**(1): 406-421.

Renner, S., C. Fehlings, N. Herbach, A. Hofmann, D. C. von Waldthausen, B. Kessler, K. Ulrichs, I. Chodnevskaja, V. Moskalenko and W. Amselgruber (2010). "Glucose intolerance and reduced proliferation of pancreatic β-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function." Diabetes **59**(5): 1228-1238.

Rimoin, D. L. (1976). "Hereditary forms of growth hormone deficiency and resistance." Birth defects original article series **12**(6): 15-29.

Rosenbloom, A. L. (2016). "A half-century of studies of growth hormone insensitivity/Laron syndrome: A historical perspective." Growth Hormone & IGF Research **28**: 46-50.

Rosenbloom, A. L. and J. Guevara-Aguirre (1998). "Lessons from the genetics of Laron syndrome." Trends in Endocrinology & Metabolism **9**(7): 276-283.

Rosenbloom, A. L., J. Guevara Aguirre, R. G. Rosenfeld and P. J. Fielder (1990). "The little women of Loja--growth hormone-receptor deficiency in an inbred population of southern Ecuador." N Engl J Med **323**(20): 1367-1374.

Rosenfeld, R. G., A. L. Rosenbloom and J. Guevara-Aguirre (1994). "Growth hormone (GH) insensitivity due to primary GH receptor deficiency." Endocrine reviews **15**(3): 369-390.

Ryan, A. S., D. M. Berman, B. J. Nicklas, M. Sinha, R. L. Gingerich, G. S. Meneilly, J. M. Egan and D. Elahi (2003). "Plasma adiponectin and leptin levels, body composition, and glucose utilization in adult women with wide ranges of age and obesity." Diabetes care **26**(8): 2383-2388.

Sabatini, D. M. (2017). "Twenty-five years of mTOR: Uncovering the link from nutrients to growth." Proceedings of the National Academy of Sciences.

Saharinen, P., M. Vihinen and O. Silvennoinen (2003). "Autoinhibition of Jak2 tyrosine kinase is dependent on specific regions in its pseudokinase domain." Molecular biology of the cell **14**(4): 1448-1459.

Saltiel, A. R. and C. R. Kahn (2001). "Insulin signalling and the regulation of glucose and lipid metabolism." Nature **414**(6865): 799.

Savage, M. O., W. F. Blum, M. B. Ranke, M. C. Postel-Vinay, A. M. Cotterill, K. Hall, P. G. Chatelain, M. A. Preece and R. G. Rosenfeld (1993). "Clinical features and endocrine status in patients with growth hormone insensitivity (Laron syndrome)." J Clin Endocrinol Metab **77**(6): 1465-1471.

Saxton, R. A. and D. M. Sabatini (2017). "mTOR Signaling in Growth, Metabolism, and Disease." Cell **168**(6): 960-976.

Schlapschy, M., U. Binder, C. Börger, I. Theobald, K. Wachinger, S. Kisling, D. Haller and A. Skerra (2013). "PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins." Protein Engineering, Design & Selection **26**(8): 489-501.

Schneider, H. J., G. Aimaretti, I. Kreitschmann-Andermahr, G. K. Stalla and E. Ghigo (2007). "Hypopituitarism." Lancet **369**(9571): 1461-1470.

Selden, R., K. Springman, J. Hondele, J. Meyer, E. L. Winnacker, H. Kräußlich, G. Brem, B. Brenig, H. Goodman and F. Graf (1985). "Production of transgenic mice, rabbits and pigs by microinjection into pronuclei." Reproduction in Domestic Animals **20**(4): 251-252.

Seruggia, D. and L. Montoliu (2014). "The new CRISPR–Cas system: RNA-guided genome engineering to efficiently produce any desired genetic alteration in animals." Transgenic research **23**(5): 707-716.

Sharp, Z. D., T. J. Curiel and C. B. Livi (2013). Chronic mechanistic target of rapamycin inhibition: preventing cancer to delay aging, or vice versa? Cancer and Aging, Karger Publishers. **38:** 1-16.

Shaw, N., N. Fraser, S. Rose, N. Crabtree and C. Boivin (2003). "Bone density and body composition in children with growth hormone insensitivity syndrome receiving recombinant IGF-I." Clinical endocrinology **59**(4): 487-491.

Shevah, O., L. Kornreich, A. Galatzer and Z. Laron (2005). "The intellectual capacity of patients with Laron syndrome (LS) differs with various molecular defects of the growth hormone receptor gene. Correlation with CNS abnormalities." Hormone and metabolic research **37**(12): 757-760.

Shevah, O. and Z. Laron (2007). "Patients with congenital deficiency of IGF-I seem protected from the development of malignancies: A preliminary report." Growth Hormone & IGF Research **17**(1): 54-57.

Simianer, H. and F. Köhn (2010). "Genetic management of the Göttingen Minipig population." Journal of Pharmacological and Toxicological Methods **62**(3): 221-226.

Sjogren, K., Y. M. Bohlooly, B. Olsson, K. Coschigano, J. Tornell, S. Mohan, O. G. Isaksson, G. Baumann, J. Kopchick and C. Ohlsson (2000). "Disproportional skeletal growth and markedly decreased bone mineral content in growth hormone receptor -/- mice." Biochem Biophys Res Commun **267**(2): 603-608.

Slagboom, P. E., M. Beekman, W. M. Passtoors, J. Deelen, A. A. M. Vaarhorst, J. M. Boer, E. B. van den Akker, D. van Heemst, A. J. M. de Craen, A. B. Maier, M. Rozing, S. P. Mooijaart, B. T. Heijmans and R. G. J. Westendorp (2011). "Genomics of human longevity." Philos Trans R Soc Lond B Biol Sci **366**(1561): 35-42.

Symonds, M. E., S. P. Sebert, M. A. Hyatt and H. Budge (2009). "Nutritional programming of the metabolic syndrome." Nature Reviews Endocrinology **5**: 604.

Tai, P.-k. K., J.-F. Liao, E. H. Chen, J. Dietz, J. Schwartz and C. Carter-Su (1990). "Differential regulation of two glucose transporters by chronic growth hormone treatment of cultured 3T3-F442A adipose cells." Journal of Biological Chemistry **265**(35): 21828-21834.

Tang, Q., X. Li, P. Song and L. Xu (2015). "Optimal cut-off values for the homeostasis model assessment of insulin resistance (HOMA-IR) and pre-diabetes screening: Developments in research and prospects for the future." Drug Discoveries & Therapeutics **9**(6): 380-385.

Tanner, J. W., W. Chen, R. L. Young, G. D. Longmore and A. S. Shaw (1995). "The Conserved Box 1 Motif of Cytokine Receptors Is Required for Association with JAK Kinases." Journal of Biological Chemistry **270**(12): 6523-6530.

Trujillo, M. E. and P. E. Scherer (2006). "Adipose Tissue-Derived Factors: Impact on Health and Disease." Endocrine Reviews **27**(7): 762-778.

Van Heemst, D., M. Beekman, S. P. Mooijaart, B. T. Heijmans, B. W. Brandt, B. J. Zwaan, P. E. Slagboom and R. G. J. Westendorp (2005). "Reduced insulin/IGF-1 signalling and human longevity." Aging Cell **4**(2): 79-85.

Varela-Nieto, I. and J. A. Chowen (2005). The growth hormone/insulin-like growth factor axis during development, Springer Science & Business Media.

Vijayakumar, A., R. Novosyadlyy, Y. Wu, S. Yakar and D. LeRoith (2010). "Biological Effects of Growth Hormone on Carbohydrate and Lipid Metabolism." Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society **20**(1): 1.

Wang, H., H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng, F. Zhang and R. Jaenisch (2013). "One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering." cell **153**(4): 910-918.

Wang, J., J. Zhou, C. Cheng, J. Kopchick and C. Bondy (2004). "Evidence supporting dual, IGF-I-independent and IGF-I-dependent, roles for GH in promoting longitudinal bone growth." Journal of Endocrinology **180**(2): 247-255.

Wang, Z., G. S. Prins, K. T. Coschigano, J. J. Kopchick, J. E. Green, V. H. Ray, S. Hedayat, K. T. Christov, T. G. Unterman and S. M. Swanson (2005). "Disruption of Growth Hormone Signaling Retards Early Stages of Prostate Carcinogenesis in the C3(1)/T Antigen Mouse." Endocrinology **146**(12): 5188-5196.

Waters, M. J. (2015). "The growth hormone receptor." Growth Horm IGF Res.

Westendorp, R. G., D. van Heemst, M. P. Rozing, M. Frolich, S. P. Mooijaart, G. J. Blauw, M. Beekman, B. T. Heijmans, A. J. de Craen and P. E. Slagboom (2009). "Nonagenarian siblings and their offspring display lower risk of mortality and morbidity than sporadic nonagenarians: The Leiden Longevity Study." J Am Geriatr Soc **57**(9): 1634-1637.

Whitelaw, C. B. A., T. P. Sheets, S. G. Lillico and B. P. Telugu (2016). "Engineering large animal models of human disease." The Journal of pathology **238**(2): 247-256.

Wiedenheft, B., S. H. Sternberg and J. A. Doudna (2012). "RNA-guided genetic silencing systems in bacteria and archaea." Nature **482**(7385): 331.

Wolf, E., C. Braun-Reichhart, E. Streckel and S. Renner (2014). "Genetically engineered pig models for diabetes research." Transgenic Research **23**(1): 27-38.

Zhou, Y., B. C. Xu, H. G. Maheshwari, L. He, M. Reed, M. Lozykowski, S. Okada, L. Cataldo, K. Coschigamo, T. E. Wagner, G. Baumann and J. J. Kopchick (1997). "A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse)." Proc Natl Acad Sci U S A **94**(24): 13215-13220.

Zoncu, R., A. Efeyan and D. M. Sabatini (2011). "mTOR: from growth signal integration to cancer, diabetes and ageing." Nat Rev Mol Cell Biol **12**(1): 21-35.

VIII. DANKSAGUNG

First of all, I would like to thank Prof. Dr. Eckhard Wolf for providing me the opportunity to do my doctoral thesis at the Chair for Molecular Animal Breeding and Biotechnology (LMU-Munich) in such an interesting topic and his continuous motivation and trust during the preparation of this study.

I would like to sincerely thank Dr. Barbara Kessler, Dr. Maik Dahlhoff, Dr. Elisabeth Kemter, Dr. Mayuko Kurome, Dr. Simone Renner and Dr. Nikolai Klymiuk for their support, input and help during my thesis.

I am thankful to all my colleagues at the Moorversuchsgut, and our technical assistants Eva, Christina and Tatjana. Further I would like to thank Franziska Kress, Juliane Ramisch and Maike Schäfer for their excellent work.

Special thanks go to the whole stable team, the veterinarians and especially the animal caretakers Harald Paul and Josef Bichler.

Furthermore, I would like to thank Dr. Andreas Parzefall, Prof Werner Blum and Dr. Martin Bidlingmaier for their great collaboration