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DISSERTATION

The influence of glucocorticoids on osteoblast-derived factors and their impact on metabolism

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Abstract

Objective

Glucocorticoids (GC) administered at pharmacological dosage lead to significant side effects which often limit the otherwise successful therapeutic application of these drugs. GC affect bone turnover and cause secondary osteoporosis as well as inflicting metabolic dysfunction. Previous studies suggest an endocrinal involvement of the osteoblast participating in the regulation of energy metabolism. Therefore, the present study examined whether a relationship exists between GC-signalling in osteoblasts and GC-induced metabolic side effects. GC diminish osteoblast activity and in particular, osteocalcin secretion, regarding the role of this osteoblast-derived peptide was studied.

Methods

Experiments were performed employing a mouse model in which glucocorticoid signalling was disrupted solely in osteoblasts through transgenic over-expression of 11ß-hydroxysteroid-dehydrogenase type 2 (11ßHSD2) under the control of the osteoblast-specific 2.3kb collagen type Ia1 promoter (Col 2.3–11ßHSD2). The animals were treated with slow-release pellets containing 1.5mg of corticosterone or placebo, and compared to equally treated wild-type (WT) animals. Metabolic changes in response to GC-treatment were evaluated via total body fat mass measurements determined via DEXA scans, insulin tolerance tests (ITT), oral glucose tolerance tests (oGTT), metabolic parameters such as serum leptin, adiponectin, insulin and osteocalcin concentrations. An OCN construct was cloned utilising a commercially available vector called pLIVE. In addition, the method of hydrodynamic tail vein injections (hTVI) was established to induce sustained endogenous heterotopic expression of osteocalcin from hepatocytes. This technique was then performed to further examine the impact of osteocalcin on GC-induced metabolic adverse effects.

Results

In WT animals, glucocorticoid treatment caused insulin resistance and impaired glucose tolerance within 7 days of therapy. After 4 weeks of treatment, WT animals exhibited a significant increase in fat mass. Compared to WT animals, the effects of GC-treatment on body composition and metabolic parameters were significantly less pronounced or even absent in GC-treated trans-

genic mice. Serum insulin levels increased in response to GC administration regardless of genotype. Serum adiponectin concentrations were also increased in GC-treated animals, with significantly higher concentrations in treated tg animals compared to treated WT animals by day 21. GC-treatment induced a rapid fall in serum osteocalcin levels in both WT and tg animals, although levels remained higher in corticosterone-treated tg animals compared to WT littermates. Hydrodynamic tail vein injections (hTVI) using yellow fluorescent protein (YFP), were used to successfully establish the method of hTVI. The method of hTVI was then employed to transfect hepatocytes with a plasmid containing osteocalcin in order to replace GC-diminished osteocalcin concentrations. Osteocalcin replacement via hTVI leads to an amelioration of insulin responsiveness in WT treated animals.

Conclusion

Treatment of WT mice with pharmacological doses of glucocorticoids results in weight gain and diabetes-like changes in whole body metabolism. Targeted disruption of intracellular GC signalling in osteoblasts prevents or attenuates these metabolic changes. Thus, the osteoblast and most likely, osteocalcin secreted by the osteoblast, appears to play a role in the pathogenesis of GC-induced dysmetabolism. Substitution of osteocalcin lessens the insulin resistant state of GC-treated animals.

Keywords: Glucocorticoids, osteoblast, osteocalcin, energy metabolism

Kurzdarstellung

Hintergrund

Der therapeutische Einsatz von Glukokortikoiden wird regelmäßig von einer Vielzahl ernstzunehmenden Nebenwirkungen (NW) begleitet, welche häufig zur Beendigung der sonst sehr erfolgreichen immunsuppressiven Therapie führen.

Zu den Nebenwirkungen einer Langzeittherapie mit Glukokortikoiden gehört eine diabetische Stoffwechsellage, welche durch eine gestörte Glukosetoleranz und Insulinresistenz und der Zunahme an viszeralem Fettgewebe gekennzeichnet ist. Neben diesen metabolischen Komplikationen führt eine Therapie mit Glukokortikoiden zu einer Suppression von Osteoblasten und damit zur Entwicklung einer sekundären Osteoporose.

Da Osteoblasten in neuen Studien ein regulativer Einfluss im Energiehaushalt zugesprochen wird, ist es Gegenstand der vorliegenden Arbeit, den Einfluss der Osteoblasten auf die Entwicklung Glukokortikoid-induzierter, metabolischer Nebenwirkungen zu untersuchen. Mit besonderem Augenmerk auf das von Osteoblasten sezernierte Peptid Osteocalcin (OCN).

Methodik

Durch die transgene Überexpression des Enzyms 11ß–Hydroxysteroid–Dehydrogenase Typ 2 (11ß-HSD2) unter der Kontrolle des Kollagen Typ 1 Promoters (Col2.3–11ß-HSD2) im Mausmodell wurde der Glukokortikoid-Signalweg im Osteoblasten erfolgreich in vivo blockiert. Es erfolgte eine wöchentliche Implantation von 1.5mg Kortikosteronpellets oder Placebopellets in Wildtyp (WT) sowie in transgene (tg) Mäuse für eine Gesamtdauer von 28 Tagen. Im Verlauf der Experimente wurden die Gesamtkörperfettmasse mittels DEXA Verfahren und die Serumkonzentrationen von Insulin, Leptin, Adiponectin sowie OCN bestimmt. Darüber hinaus wurden Insulin- und Glucose Toleranz Tests durchgeführt.

Die Methodik der hydrodynamischen Schwanzveneninjektion (hTVI) wurde mit Hilfe eines gelb fluoreszierenden Proteins (YFP) in einem CMV Promotor erprobt. Damit mittels diesem Verfahren, eine kontinuierliche OCN Substitution in einem weiteren Experiment erreicht werden kann. In diesem wurde eine Gruppe WT Mäuse mittels hTVI mit dem pLIVE Expression Vektor injiziert, in welchem OCN via Cloning Prozedur inseriert wurde. Die anderen Versuchstiere erhielten den leeren pLIVE Vektor. Anschließend wurden die Mäuse mit Kortikosteron oder Placebopellets behandelt. Erneut wurden Insulin Toleranz Tests durchgeführt.

Ergebnisse

Die Verabreichung von Kortikosteron führte zu einer deutlichen Abnahme der OCN Serumkonzentration in WT Mäusen, wohingegen tg Mäuse signifikant höhere OCN Konzentrationen aufrechterhielten. Innerhalb von 7 Tagen nach Beginn der Kortikosteron Behandlung zeigte sich in WT Mäusen sowohl der Zustand der Insulinresistenz als auch eine gestörte Glucosetoleranz. Weiterhin war in allen mit Glukokortikoiden therapierten Tieren nach 4-wöchiger Behandlung ein erhöhter Körperfettanteil festzustellen. Im Vergleich zu WT Mäusen waren die tg Versuchstiere jedoch weniger stark von diesen metabolischen Nebenwirkungen der Kortikosteron Behandlung betroffen. Die Serumkonzentrationen von Leptin und Insulin wurden durch die Gabe von Glukokortikoiden im Verlauf der Studie sowohl in WT als auch in tg Tieren in gleichem Maß signifikant erhöht. Nach 21 Tagen Kortikosteron Gabe wurde in tg Mäusen im Vergleich zu WT Mäusen jedoch eine erhöhte Konzentration von Adiponectin festgestellt. Die Methode der hydrodynamischen Schwanzvenen Injektionen wurde erfolgreich etabliert, unter Zuhilfenahme eines gelb fluoreszierenden Proteins (YFP). Die gentherapeutische Überexpression von OCN mittels hTVI in WT Mäusen führte zu einer Verbesserung der Insulin Sensitivität während der Behandlung mit Glukokortikoiden.

Schlussfolgerungen

Es konnte gezeigt werden, dass die Blockade des intrazellulären Glukokortikoid Signalwegs in Osteoblasten transgener Mäuse vor der Entwicklung Glukokortikoid-induzierter, metabolischer Dysfunktionen schützt. Demzufolge kann den Osteoblasten, zumindest eine Vermittlungsrolle in den komplexen Regulationsvorgängen des Energiestoffwechsels zugesprochen werden. Während der Kortikosteronbehandlung können die transgenen Mäuse im Vergleich zu den WT Mäusen, höhere OCN Serumkonzentrationen aufrechterhalten. In einem zweiten Experiment konnte nun gezeigt werden, dass der gentherapeutische Ersatz von OCN in WT Mäusen, während einer Glukokortikoidbehandlung ebenso der Entwicklung einer Insulin Resistenz vorbeugt.

Schlagworte: Glukokortikoide, Osteoblast, Osteocalcin, Energie Stoffwechsel

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List of abbreviations

11ßHSD1	11ß-hydroxysteroid-dehydrogenase type 1
11ßHSD2	11ß-hydroxysteroid-dehydrogenase type 2
ACTH	Adrenocorticotropic hormone
CART	Cocaine-and amphetamine-regulated transcript
CRH	Corticotropin releasing hormone
Db/db mice	Mice with deficient leptin receptor activity
DEXA	Dual energy X ray absorptiometry
DNA	Desoxyribonucleic acid
Esp	Also known as Ptprv, a gene expressed in osteoblasts and Sertoli cells that encodes a receptor-like protein tyrosine phosphatase termed OST-PTP
ESI	Electrospray ionisation
ELISA	Enzyme linked immunosorbent assay
GLUT	Glucose transporter
GC	Glucocorticoids
GR	Glucocorticoid receptor
GIO	Glucocorticoid-induced osteoporosis
GREs	Glucocorticoid response elements
HPA axis	Hypothalamo-pituitary-adrenal axis
hTVI	Hydrodynamic tail vein injection
M	Molar
ITT	Insulin tolerance test
IR	Insulin receptor
IRMA	Immunoradiometric assay
MR	Mineralcorticoid receptor
mRNA	Messenger ribonucleic acid
NPY	Neuropeptide Y
NW	Nebenwirkungen
Ob/ob mice	Leptin deficient mice
OPG	Osteoprotegerin

OCN	Osteocalcin
OCT	Optimal cutting temperature compound
OST – PTP	Osteoblast protein tyrosine phosphatase
oGTT	Oral glucose tolerance test
pLIVE	Prolonged liver in vivo expression
PTP1B	Protein tyrosine phosphatase 1B
POMC	Pro-opiomelanocortin
PPARy	Peroxisome proliferator-activator receptor y
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
Runx2	Runt-related transcription factor 2
SEM	Standard error of the mean
SRM	Selected - reaction monitoring
TWIST2	Twist related protein 2
T2DM	Type 2 Diabetes mellitus
tg	Transgenic
TGF-ß	Transforming growth factor ß
TNF	Tumor necrosis factor
WT	Wild type
YFP	Yellow fluorescent protein

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1 Introduction

Glucocorticoids have been employed as therapeutic agents for over sixty years with major beneficial effects in terms of treating chronic inflammatory conditions such as rheumatoid arthritis, asthma or inflammatory bowel disease (Barnes 1993; Barnes 2006; Buttgereit, Doering et al. 2008; De Iudicibus 2011; Spies, Strehl et al. 2011). Glucocorticoids are also widely used as cancer therapeutics, for haematological conditions and in post-transplant management (Kassi and Moutsatsou 2011).

However, particularly when given at higher doses and for extended periods of time, glucocorticoids are associated with multiple detrimental side effects which are impossible to prevent. These include musculoskeletal pathologies such as osteoporosis (Sambrook, Birmingham et al. 1990; Saag, Emkey et al. 1998) and sarcopenia (Schakman, Gilson et al. 2008), metabolic disorders like glucose intolerance, diabetes mellitus and dyslipidaemia (Gounarides, Korach-André et al. 2008; de Oliveira, de Mattos et al. 2011) as well as excessive and abnormal fat accrual (Schäcke, Döcke et al. 2002; Gounarides, Korach-André et al. 2008).

The effects of exogenous glucocorticoids on bone and bone cells have been relatively well established (Weinstein 1998; Sivagurunathan 2005; Weinstein 2011). It is understood that glucocorticoids influence the function of all three cell types, osteoblasts, osteocytes and osteoclasts (Weinstein, Chen et al. 2002; Sivagurunathan 2005; Henneicke, Herrmann et al. 2011), with most of the evidence indicating that osteoblasts are the main skeletal target (Canalis 1983; Weinstein 1998; O'Brien, Jia et al. 2004; Henneicke, Herrmann et al. 2011).

In humans, glucocorticoids predominantly affect the osteoblast, manifested by a rapid and profound suppression of serum markers of bone formation, which correlates with bone loss over time (Prummel, Wiersinga. W.M. et al. 1991). For example, serum levels of osteocalcin, a marker of osteoblast activity, are reduced in patients receiving glucocorticoids (Calvo 1996; Woitge 2001).

In contrast the cellular and molecular pathways by which exogenous glucocorticoids exert their detrimental effects on energy and particularly glucose metabolism are still poorly understood (Besse 2005; Gounarides, Korach-André et al. 2008; Peckett, Wright et al. 2011).

Recent data suggests that osteocalcin may act as a hormone involved in the control of fuel metabolism (Lee, Sowa et al. 2007; Ferron, Hinoi et al. 2008; Ferron, Wei et al. 2010; Ferron, McKee et al. 2011; Kanazawa, Yamaguchi et al. 2011; Ferron, McKee et al. 2012).

These results provide evidence that the skeleton, through specific products such as osteocalcin, may have an active role in the control of fuel metabolism under normal physiological conditions. It has to be determined if osteocalcin is also involved in the onset of glucocorticoid–induced metabolic dysfunctions.

2 Literature review

2.1 Glucocorticoids

Glucocorticoids have long been established as potent therapeutic agents used to treat several divergent medical conditions due to their anti-inflammatory actions (Spies, Strehl et al. 2011). In 1948, Philip Showalter Hench treated his first patient with Compound E, more commonly known as cortisone, for rheumatoid arthritis (Kirwan, Balint et al. 1999). Through unknown mechanisms, cortisone was found to provide relief for patients suffering from this autoimmune disorder, decreasing inflammation and suppressing the immune response.

This was the beginning of a novel, effective and highly potent anti-inflammatory therapy for rheumatological diseases (Kirwan, Balint et al. 1999). Nobel laureates Kendall, Hench and Rechstein successfully isolated cortisone from the adrenal cortex. This allowed further investigation into the actions of cortisone (Mühl and Pfeilschifter 2003). Hydrocortisone (cortisol) is the principal glucocorticoid of the human adrenal cortex, and specifically produced in the *zona fasciculata*.

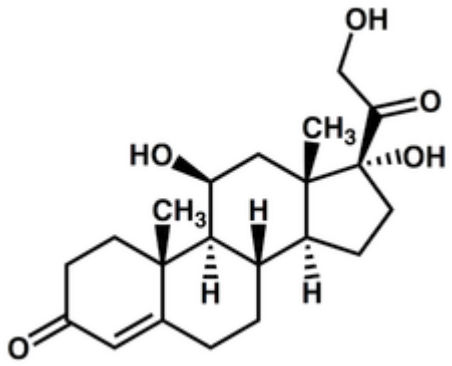

Figure 1: Structure of cortisol (Mutschler 2008)

The term glucocorticoid (**gluco**se + **cort**ex + ster**oid**) is derived from the role these steroid hormones play in the regulation of glucose metabolism, their synthesis in the adrenal cortex, and their steroidal structure. Secretion of cortisol is regulated by the hypothalamic–pituitary–adrenal (HPA) axis. Hypothalamic corticotropin-releasing hormone (CRH) leads to an activation of pituitary pro-opiomelanocortin (POMC) gene transcription resulting in an increase in adrenocorticotropic hormone (ACTH), which in turn induces GC production in the adrenal cortex. Serum

glucocorticoid levels represent a feedback signal to the HPA axis to limit glucocorticoid production. Glucocorticoids mediate their peripheral effects primarily through the glucocorticoid receptor (GR). The GR is a hormone receptor that translocates to the nucleus upon binding with its ligand and acts as a transcription factor, regulating GC responsive target genes either via direct DNA binding or through protein–protein interaction (Baschant, Lane et al. 2012).

It has been established that positive and negative regulation of gene expression is achieved by GC-induced activation of the GR. The positive regulation of genes is considered as transactivation and results in an increased gene expression rate contrasting transrepression, the downregulation of gene transcription. Recent findings suggest that certain adverse effects such as GC-induced diabetes are due to gene transactivation.

However, some deleterious effects of GC treatment are considered to be a result of both transactivation and transrepression (Vegiopoulos and Herzig 2007).

As therapeutic drugs, GC can be applied either systemically or topically. Topical application forms are used in dermatology, in the treatment of asthma or are applied to the gut against inflammatory bowel diseases. Systemically administered GC have more severe general side effects compared to topical application. However, topical therapy can also induce systemic adverse effects (Hengge, Ruzicka et al. 2006). It was later discovered that GC are involved in carbohydrate metabolism whilst mineralocorticoids, also produced by the adrenal cortex, regulate salt and water balance.

Cortisone became the drug of choice for many inflammatory conditions such as rheumatoid arthritis, Lupus erythematodes, rheumatic fever, inflammatory bowel diseases, sarcoidosis, asthma, hepatitis and for the replacement of GC in Addison's disease and other forms of adrenal insufficiency. They are also beneficially applied to treat malignancies as well as suppress the immune response to decrease the likelihood of transplant rejections.

Although highly effective in the management of inflammatory diseases, GC have been found to have serious side effects that reduce its possible range of application. Disruptions of the salt and water balance, secondary osteoporosis, visceral fat accrual, glucose intolerance and insulin resistance were observed in patients receiving GC treatment as well as iatrogenic Cushing syndrome and GC-induced suppression of the immune system, rendering the patient susceptible to infections (Rose, Vegiopoulos et al. 2010; Spies, Strehl et al. 2011; Baschant, Lane et al. 2012). Due to these adverse effects, a subsequent fear arouse also referred to as steroid phobia, which is still a considerable concern today, leading to decreased compliance by patients (Charman, Morris et al. 2000).

2.2 Secondary osteoporosis

GC induced osteoporosis is the most common form of secondary osteoporosis and 30 – 50 % of patients continuously treated with GC will experience a fragility fracture, thought to result from a disturbance in bone homeostasis (Canalis, Mazziotti et al. 2007).

Pharmacological GC exposure modifies the strictly regulated balance of bone metabolism. Secondary osteoporosis is caused by a relative increase of bone resorption over bone formation (Raisz 2005). Osteoporosis, as a severe adverse effect of glucocorticoid treatment, has been established to be a consequence of both transactivation and transrepression of GC targeted genes (Schäcke, Döcke et al. 2002). The result being reduced osteoblastic differentiation and osteoblastic activity by for example suppressing the synthesis of type I collagen, a major component of bone matrix, as well as lowering osteoblastic activity by accelerating apoptosis.

Depressed bone formation is considered to be the main skeletal target of GC action (Popp, Isenegger et al. 2006; Canalis, Mazziotti et al. 2007; Henneicke, Herrmann et al. 2011; Weinstein 2011). GC expedite bone resorption by influencing the differentiation, function and formation of osteoclasts. Also leading to an increase of the RANKL:OPG ratio, leading to further enhanced activity of osteoclasts.

GC reduce calcium resorption from the intestine as well as enhancing the excretion of calcium from the kidney inducing an increased production of parathyroid hormone (PTH). PTH in turn promotes osteoclastic activity, again leading to bone resorption and loss of bone substance.

A decrease of muscle mass, sex hormone levels and a suppression of the somatotrophic axis accelerate the onset of GC–induced osteoporosis. Also affected are, osteoblastic products such as osteocalcin, which is diminished by GC treatment (Meeran, Hattersley et al. 1995; Hozuki, Imai et al. 2010; Henneicke, Herrmann et al. 2011).

2.3 The influence of glucocorticoids on metabolism

Physiological GC levels play an important role in the delicate hormonal control of energy metabolism. However, excessive GC action has been linked to a range of metabolic diseases (Vegiopoulos and Herzig 2007; de Oliveira, de Mattos et al. 2011).

Glucocorticoid-induced Cushing Syndrome is the most prevalent adverse metabolic occurrence observed during long-term GC treatment. The common characteristics of this syndrome include the development of a moon face, buffalo hump, central obesity, glucose intolerance, os-

teoporosis, growth retardation and hirsutism (Guaraldi and Salvatori 2012). Notably, unphysiologically high GC levels in patients suffering from Cushing Syndrome have been detected to be of high significance for the central obesity observed in these patients (Walker 2006).

Both high levels of endogenous and exogenous GC lead to a redistribution of adipose tissue. In peripheral fat depots GC encourage lipolysis, whereas abdominal fat is induced to undergo hypertrophy compromising adipocyte cell functionality, which in turn is associated with insulin resistance. This results in an alteration of energy metabolism as a consequence of altered fat distribution.

GC modify insulin sensitivity by reducing glucose uptake, interfering with proximal insulin signalling and subsequent glucose transporter (GLUT 4) translocation as well as glycogen synthase activation (Rose, Vegiopoulos et al. 2010).

2.4 The effect of glucocorticoids on insulin sensitivity and insulin levels

The application of GC leads to impaired glucose tolerance or even full-blown type 2 diabetes mellitus (Besse 2005; Kauh, Mixson et al. 2011). Rodents receiving GC exhibit severe fasting hyperglycemia, hyperinsulinemia, insulin resistance and impaired ß–cell response following oral glucose application (Shpilberg, Beaudry et al. 2011).

GC have also been shown to directly influence adipocyte insulin sensitivity by interfering with components of the insulin signalling cascade (Caperuto, Anhê et al. 2006). In addition GC inhibit the relocation of GLUT 4 to the plasma membrane after insulin stimulation (Sakoda, Ogihara et al. 2000) which contributes to the state of impaired insulin sensitivity.

An increased inflammatory response in skeletal muscle and adipose tissue after GC treatment has also been linked to the occurrence of GC-induced insulin resistance (Nixon, Wake et al. 2012; Viguerie, Picard et al. 2012). Glucose intolerance and dysfunctional insulin response, also observed in Cushing patients, is partly due to a GC-induced failure of the pancreatic ß–cell and therefore diminished insulin secretion (Mazziotti, Gazzaruso et al. 2011; Shpilberg, Beaudry et al. 2011).

2.5 The influence of adipose tissue on metabolism

Adipose tissue serves as a reservoir for the storage of excess energy as well as a complex endocrine system, which produces hormones, named adipokines, immune-modulatory cytokines and chemokines which are actively secreted into the bloodstream. Adipocytes are involved in the well organised regulation of energy metabolism (Ahima and Flier 2000).

Two of the most exhaustively studied adipokines involved in the regulation of energy metabolism are leptin and adiponectin. Serum leptin levels are generally correlated to the amount of fat mass in the body whereas adiponectin levels are inversely correlated with body fat mass. Leptin is involved in the regulation of energy metabolism by means of adjusting the craving for food and increasing energy expenditure, acting through its receptor on the hypothalamus to suppress appetite. Leptin receptors have also been identified in other peripheral tissues; implying a peripheral leptin action (Hoggard, Hunter et al. 1997; Islam, Morton et al. 1997; Cohen, Yang et al. 2005; Papathanassoglou, El-Haschimi et al. 2006).

Adiponectin is considered an "antidiabetic" cytokine, which reduces gluconeogenesis and stimulates fatty acid oxidation in the liver and the skeletal muscle. A reduction of adiponectin expression and plasma levels may be related to obesity and insulin resistance (Groeneveld, Huang-Doran et al. 2012). Administration of pharmacological levels of adiponectin has been shown to ameliorate the insulin resistance and decrease glucose levels in diabetic and obese rodents (Groeneveld, Huang-Doran et al. 2012; Shehzad, Iqbal et al. 2012). However, no evidence for the development of insulin resistance was obtained in adiponectin knock-out mice when fed a normal diet (Maeda, Shimomura et al. 2002). Therefore additional convincing evidence needs to be generated to elucidate the role of adiponectin.

2.6 Glucocorticoid effects on the adipokines: leptin and adiponectin

It has been reported that exogenous GC administration results in increased mRNA and protein levels of leptin (De Vos, Saladin et al. 1995; Larsson and Ahrén 1996). The action of GC on adiponectin, however, is less clear.

Studies show in vitro and in vivo evidence suggesting a suppression of adiponectin expression and secretion after GC administration (Fallo, Scarda et al. 2004; Shi, Du et al. 2010; de Oliveira, de Mattos et al. 2011). However, other groups found increased plasma adiponectin levels after dexamethasone administration in rats and humans (Raff and Bruder 2006; Jang, Inder et al.

2008). Recent studies have described a biphasic response of adiponectin to glucocorticoids, which may explain the discrepancies (Cabanelas, Cordeiro et al. 2010; Sukumaran, Jusko et al. 2011).

2.7 Insulin, obesity and type 2 diabetes mellitus

Obesity and insulin resistance in humans is one of the greatest healthcare challenges of the 21st century. Metabolic abnormalities, such as diabetes, can lead to cardiovascular pathologies, which are the largest cause of death in the world (WHO, 2008). Humans suffering from T2DM mostly display a multifactorial disorder. Peripheral insulin resistance, impaired glucose production and declined β – cell function are components of this metabolic disarray (Mahler and Adler 1999; Mazziotti, Gazzaruso et al. 2011).

Insulin is a peptide hormone that is produced by the Langerhans cells of the pancreas. It is highly involved in the regulation of energy metabolism and known to be an anabolic mediator.

Insulin regulates glucose uptake in peripheral tissues, such as adipose tissue and muscle, by influencing the transcription, activation and deactivation of enzymes involved in glucose metabolism. After carbohydrate rich food intake, the pancreatic β–cell secretes the peptide hormone insulin, which in turn inhibits glucose production by the liver and stimulates glucose uptake from the bloodstream into skeletal muscle and adipose tissue.

Insulin signals through manifold cellular processes involving activation and deactivation of enzymes, protein synthesis and regulation of gene transcription. An important effect of insulin on glucose metabolism is the regulation of GLUT 4 receptors and the associated glucose uptake into skeletal muscle and adipose tissue. The translocation of GLUT 4 to the surface of the adipocyte and the muscle cell membrane has a pivotal role in the state of insulin sensitivity or, if impaired, guiding these tissues to increased of insulin resistance (Rowland, Fazakerley et al. 2011). Insulin resistance describes the decreased sensitivity of peripheral tissues to the metabolic actions of insulin.

The association between insulin resistance and high intra-abdominal adipose tissue is well established (Carey, Jenkins et al. 1996; Samocha-Bonet, Chisholm et al. 2012). Studies have found that insulin resistance is often acquired and related to obesity, physical inactivity, and glucose and lipotoxicity (Gerich and Dailey 2004). Furthermore, it was found that prior to the accruement of insulin resistance, deteriorated insulin secretion was detectable.

Impaired β–cell function therefore is likely to be the primary defect (Mahler and Adler 1999; Gerich and Dailey 2004; Szoke and Gerich 2005).When increasing glucose levels in T2DM patients, 80 % of type 2 diabetic patients react with decelerated, impaired insulin secretion compared to healthy individuals.

As a compensatory mechanism, the pancreatic ß–cell responsds to the abnormally high glucose levels with hypersecretion of insulin (Ward, Beard et al. 1986; Kruszynska and Olefsky 1996). If the state of hyperglycemia and hyperinsulinemia persists over an extended period of time, insulin secretion decreases, potentially due to toxic glucose intermediary metabolites accumulating within the pancreatic ß–cells.

Chronic hyperinsulinemia has been found to inhibit both insulin secretion and action (DeFronzo, Binder et al. 1981; Del Prato, Leonetti et al. 1994; Pullen and Rutter 2012). One particular model of genetically engineered mouse models uses a deletion of the Ptpn 1 gene, which encodes for the tyrosine-protein phosphatase 1B (PTP1B) (Elchebly, Payette et al. 1999).

PTP1B is known to inhibit insulin action due to dephosphorylation of the phosphotyrosine residues on the insulin receptor kinase. The deletion of the encoding gene results in increased insulin sensitivity, leading to improved glucose tolerance as well as refractory obesity and insulin resistance in mice fed a high fat diet (Elchebly, Payette et al. 1999).

These results were confirmed by independent laboratories (Ali, Ketsawatsomkron et al. 2009; Bakhtiyari, Meshkani et al. 2010). In 2009, Ali et al. described the protein tyrosine phosphatase as an insulin desensitising enzyme. They examined db/db mice, all of which were morbidly obese, insulin resistant and had elevated tissue specific PTP1B in muscle and adipose tissue compared to lean controls.

Contrary to their initial expectations, the deletion of PTP1B was found to improve glucose clearance through insulin receptor signalling resulting in improved glucose uptake.

2.8 Leptin control of bone metabolism

Leptin has been shown to influence and regulate physiological functions other than energy metabolism, such as controlling bone mass. The differentiation and function of bone cells are influenced by many different mechanical stressors such as exercise and body weight as well as biochemical stimuli like hormones.

Over the past decade, it was discovered that the metabolic system can influence bone development and remodelling (Karsenty 2006). Only very recently, it was suggested that the skeleton may play a role in the control of energy metabolism. This work contributes to that hypothesis.

The finding that obese mice were protected from osteoporosis led to the proposition that bone and energy metabolism could be controlled by the same hormones, and in particular, leptin (Ducy, Amling et al. 2000)

Support for this observation was provided by Turner et al. indicating that leptin, acting mainly through peripheral pathways, enhances osteoblast quantity and activity (Turner, Kalra et al. 2012).

In contradiction to this, it has been found that bone remodelling is partly regulated by leptin in an antagonistic manner through a central hypothalamic pathway (Ducy, Amling et al. 2000; Karsenty 2006). High serum leptin levels reduce bone mass (Ducy, Amling et al. 2000; Karsenty 2006). Leptin binds to its receptor located in the ventromedial hypothalamus, and induces sympathetic signalling via ß2 adrenergic receptors, resulting in inhibited osteoblastic proliferation and promoted receptor activator of nuclear factor kappa B ligand (RANKL), leading to enhanced bone resorption (Ducy, Amling et al. 2000; Takeda, Elefteriou et al. 2002; Elefteriou, Takeda et al. 2004).

Since leptin regulates bone remodeling, integrative physiologists proposed a bilateral or reciprocal connection between bone and energy metabolism (Lee, Sowa et al. 2007; Confavreux, Levine et al. 2009; Ferron, Wei et al. 2010; Kumar and Vella 2011).

2.9 Bone

The human skeletal system consists of bone and cartilage, attached tendons and muscles allowing locomotion, support and protection of the viscera. Haematopoiesis takes place within the central cavity of bones, more specifically within the bone marrow.

Bone also serves as a repository for calcium and phosphate ions, mainly in the form of hydroxyapatite. Calcium and phosphate ions can be recruited into the plasma to sustain serum mineral homeostasis. Bone is constantly regenerated to maintain bone mass within defined limits, meaning that bone is a living tissue in contrast to its inert appearance.

Bone is a specialised form of connective tissue made up of collagen type I fibres and inorganic layering of hydroxyapatite crystals which gives bone its characteristic tensile strength, and its compressive strength, respectively (John P. Bilezikian 2002).

The maintenance of bone mass relies upon the intimate coupling between bone resorption and bone formation. Osteoblasts, osteocytes and osteoclasts constitute the major cell types found in the bone microenvironment and play vital roles in bone growth, preservation and repair.

Osteoblasts, known as 'bone-forming' cells, originate from the same stromal mesenchymal stem cell lineage as fibroblasts and other connective tissue cells. These cells are responsible for secreting the organic components to their surrounding matrix. Various transcription factors including the bone specific core binding factor 1 (*Cbfa1*, otherwise known as *Runx2*) (Schinke 2002) and bone morphogenetic protein 2, as well as hormones, cytokines and other stimulatory agents act on osteoprogenitor mesenchymal stem cells in order to commit these cells to differentiate into osteoblasts.

Osteoblasts may undergo apoptosis or differentiate into relatively inactive lining cells or into osteocytes. These osteocytes are entirely implanted into mineralised matrix deposited around them. Cellular processes of the osteocyte membrane extend to both other osteocytes and to osteoblasts at bone surfaces, maintaining communication via gap junctions between these bone cells.

Osteoclasts are large, multinucleated cells recruited from the same haematopoietic lineage as monocytes and macrophages. These bone-resorbing cells secrete proteases and acid-forming protons via ATPase proton pumps located along their ruffled border situated adjacent to the site of resorption. Bone resorption and formation are linked processes, which are highly efficient and present at physiologically equilibrium. Bone mass is maintained at a constant level as these two processes renew the existing bone matrix.

2.10 Osteocalcin

Osteocalcin, a non collagenous, small osteoblastic protein, consisting of 46 amino acids. Osteocalcin contains three glutamic acid residues: Glu 13, Glu 17 and Glu 20, which can be post-translationally modified by the γ-glutamyl carboxylase. This carboxylation is vitamin K-dependent and is responsible for the high affinity osteocalcin has to mineral ions including hydroxyapatite crystals (Hauschka, Lian et al. 1989). Not all three glutamic acid residues are necessarily carboxylated at all times (Cairns and Price 1994).

The uncarboxylated or undercarboxylated forms of OCN are susceptible to being released into the blood stream (Berkner 2005). The undercarboxylated form is defined as being partially decarboxylated, ie decarboxylated at one of the glutamic acid residues. The concentration of OCN

in serum is closely linked to bone metabolism and serves as a biological marker for the clinical assessment of certain bone diseases.

2.11 Osteocalcin and its putative role in energy metabolism

Osteocalcin has been found to have several properties of a hormone: it is synthesised predominatly by osteoblasts as a prepromolecule and secreted into the bloodstream (Hauschka, Lian et al. 1989). Osteocalcin deficient mice also had an abnormally high amount of visceral fat tissue, suggesting a correlation between OCN and fat mass. The biological role of OCN has, until recently, only been connected to bone metabolism. However, OCN has now been implicated in the regulation of glucose and fat metabolism (Sarkar and Choudhury ; Lee, Sowa et al. 2007; Ferron, Hinoi et al. 2008; Ferron, McKee et al. 2011). Ferron, et al. claim that the first glutamic acid residue "13", if decarboxylated, plays a vital role in the activation of OCN as a hormone involved in energy metabolism (Ferron, Hinoi et al. 2008).

The *Esp* gene encodes for a protein tyrosine phosphatase (OST–PTP), exclusively to mice osteoblasts. Deletion of the *Esp* gene, therefore lacking the OST–PTP, resulted in a phenotype characterised by hyperinsulinemia, increased β–cell proliferation, higher insulin mRNA expression and secretion, hypoglycemia and high insulin sensitivity (Lee, Sowa et al. 2007; Ferron, Hinoi et al. 2008). These mice were also protected from obesity and glucose intolerance, suggesting that OST–PTP plays a critical role in glucose homeostasis.

Lee et al. were able to show that mice lacking OCN (OC-/-) present a contrary metabolic phenotype compared to *Esp*-/- mice, implying that OCN activity is essential for the regulation of metabolism. Hence, the proposition was made that OST–PTP influences the degree to which osteocalcin is post-translationally carboxylated, affecting its biological function.

Recent reports from two laboratories delved further into this connection, with Fulzele et al. and Ferron et al. describing a role for insulin receptor signalling within the osteoblast in this pathway (Ferron, Wei et al. 2010; Fulzele, Riddle et al. 2010; Ferron, McKee et al. 2011). Fulzele et al. showed that insulin receptor (IR) signalling is important for osteoblastic development and the expression of OCN. They found that insulin signalling enhances bone formation by suppressing the Runx2 inhibitor Twist2. Runx2 is a key transcriptional factor for the differentiation of osteoblasts (Fujita, Azuma et al. 2004).

The transcription factor Twist2 inhibits osteoblastic differentiation (Bialek, Kern et al. 2004). Fulzele et al. established a mouse model with a conditional deletion of the osteoblastic IR and

observed a phenotype almost identical to mice lacking OCN. These mice exhibited adiposity, hyperglycemia, glucose intolerance and insulin resistance. Mice lacking the osteoblastic IR have lower circulating undercarboxylated OCN levels, reduced bone formation and lower osteoblast numbers resulting in lower bone mass.

Fulzele et al. achieved an amelioration of these pathologies after a two week's infusion of undercarboxylated OCN. Ferron et al. 2010 established that the OST–PTP dephosphorylates the IR of osteoblasts, dephosphorylation results in the inactivation of the IR. Hence, osteoblastic insulin signalling results in the disinhibition of Twist2 and in turn Twist2 inhibits Runx2.

This series of events leads to a decrease in osteoblastic maturation and therefore a decrease in OCN expression. Ferron et al. hypothesised that osteoprotegerin (OPG) could also be regulated by insulin signalling and is responsible for the changes observed in bone resorption following the abolition of insulin receptor activity (Ferron, Wei et al. 2010).

Osteoprotegerin functions as a decoy receptor for RANK–Ligand, which is produced by osteoblasts and binds to the RANK receptor found on osteoclast precursors, osteoclasts and macrophages. When RANK–Ligand interacts with RANK, either osteoclastic differentiation is initiated or mature osteoclasts are stimulated to resorb bone tissue. This process is blocked by OPG (Lacey, Timms et al. 1998; Burgess, Qian et al. 1999; Feige 2001).

Insulin receptor activation decreased OPG levels and bone resorption was increased (Ferron, Wei et al. 2010).

It is accepted knowledge that former bound OCN can enter the circulation in high amounts when relieved from the binding agent hydroxyapatite. In contrast, mice with non-functional osteoclasts have been measured with less undercarboxylated osteocalcin in their bloodstream and exhibit a metabolic phenotype such as mice lacking OCN, high amounts of visceral fat tissue, glucose intolerance, insulin resistance and pancreatic insufficiency are parameters of that phenotype (Lee, Sowa et al. 2007; Ferron, Wei et al. 2010). Infusions or injections of osteocalcin into OCN-/- mice or WT mice fed a high fat diet improve these metabolic abnormalities (Lee, Sowa et al. 2007; Ferron, McKee et al. 2012).

Since GC treatment leads to similar metabolic side effects as the metabolic manifestation of OCN-/- mice, the substitution of OCN as a potential treatment option or prophylaxis to ameliorate or prevent GC-induced metabolic adverse effects was worth investigating. It would be of great significance to clinical practitioners if OCN could treat or at least improve GC-induced insulin resistance and glucose intolerance to prospectively prevent the feared metabolic derailment of these patients.

A possible relationship between the GC–decreased osteoblastic function and the abnormal metabolic function observed in GC-treated animals and humans needs to be investigated. It was of interest to examine whether the osteoblastic factor OCN is involved in the onset of GC-induced metabolic dysfunction and if a correlation between the diminished OCN levels and the earliest time point of detectable insulin resistance caused by corticosterone administration exists.

Research in the field of bone remodelling and associated disorders is of great importance in order to help uncover unknown molecular pathways and therefore develop new and more efficient treatment options. This also applies to work on GC associated disorders to support the effective therapy option and decrease unwanted deleterious side effects.

2.12 The Col2.3-11ßHSD2 transgenic mouse model

In these experiments the Col2.3-11ßHSD2 transgenic mouse model was employed (Sher, Woitge et al. 2004; Zhou, Mak et al. 2009). This model was previously used to examine GC-induced osteoporosis and the involvement of GC in fracture healing (Weber, Li et al. 2010).

GC signalling occurs via the classical steroid hormone receptor pathway (Cato 2002). Upon hormone binding, the glucocorticoid receptor (GR) translocates to the nucleus, where it acts as a transcriptional regulator of specific GC-target genes. The GR subunits homodimerise and bind DNA sequences, known as the glucocorticoid response element (GRE) (Schoneveld 2004). Transcription levels are modified either through protein–protein interactions or via direct DNA binding of the GC-GR complex (O. Kassel 2007). To study GC affects on bone, GR knockout mice are often administered in animal studies.

However, GR knockout mice have a very high rate of perinatal lethality (Cole, Blendy et al. 1995). Consequently, different approaches had to be developed to investigate the impact of GC on bone cells. Kream et al. generated a transgenic mouse model facilitating the GC inactivating enzyme, 11 ß-hydroxysteroid dehydrogenase type 2 (11ßHSD2). 11ßHSD2 disrupts intracellular GC signalling upstream of the receptor, therefore decreasing GC actions (Sher, Woitge et al. 2004).

The enzyme 11ßHSD2 and its isoform 11ß-hydroxysteroid dehydrogenase type 1 (11ß-HSD1) have the ability to inter-convert inactive and active glucocorticoids, thereby playing a major role in local modulation of agonistic concentration and activation of corticosteroid receptors in target tissues. 11β-HSD 1 is an NADPH-dependent enzyme highly expressed in liver, lung, adipose tissue, kidney, and brain, largely localised in cells expressing glucocorticoid receptors, but not mineralocorticoid receptors.

This suggests that 11ß-HSD1 modulates glucocorticoid access to glucocorticoid receptors. In these tissues, 11ß-HSD1 reduces cortisone to the biologically active hormone cortisol, whereas 11β-HSD2 catalyses the inactivation of cortisol (corticosterone in rodents) to cortisone (11-dehydrocorticosterone in rodents), all in an NAD+-dependent fashion. The enzyme 11β-HSD2 is most highly expressed in the distal nephron of the kidney, where 11β-HSD2 protects the mineralcorticoid receptor (MR) from activation by GC (Morris and Souness 1996).

Col2.3-HSD2, a construct in which the 2.3 kilobase fragment of the rat Col 1a1 promoter, was used to drive exclusive overexpression of 11 ßHSD2 in mature osteoblasts, avoiding complications of global transgene expression (Kream 2004). Tg mice were generated in a CD-1 outbred background using pronuclear injection. The transgene was inherited in the Mendelian ratio and litter sizes were normal (Kream 2004). The Col2.3–HSD2 tg mice were created to investigate the function of glucocorticoids on osteoblasts.

The transgene RNA was detected only in skeletal tissues such as calvariae, femurs, vertebrae, and tail, however, not in any non-skeletal tissue. To allow detection of the transgene RNA versus endogenous enzyme, a bovine GH polyadenylation region was integrated (see Figure 3).

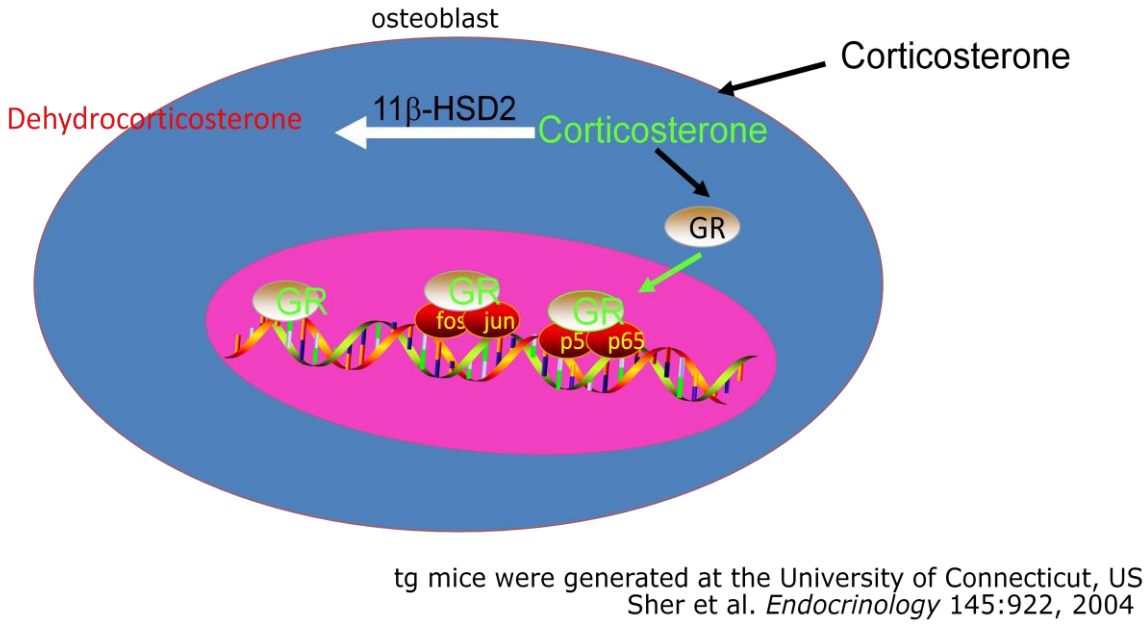

Figure 2: 11 ß HSD2 action within the osteoblasts of transgenic mice

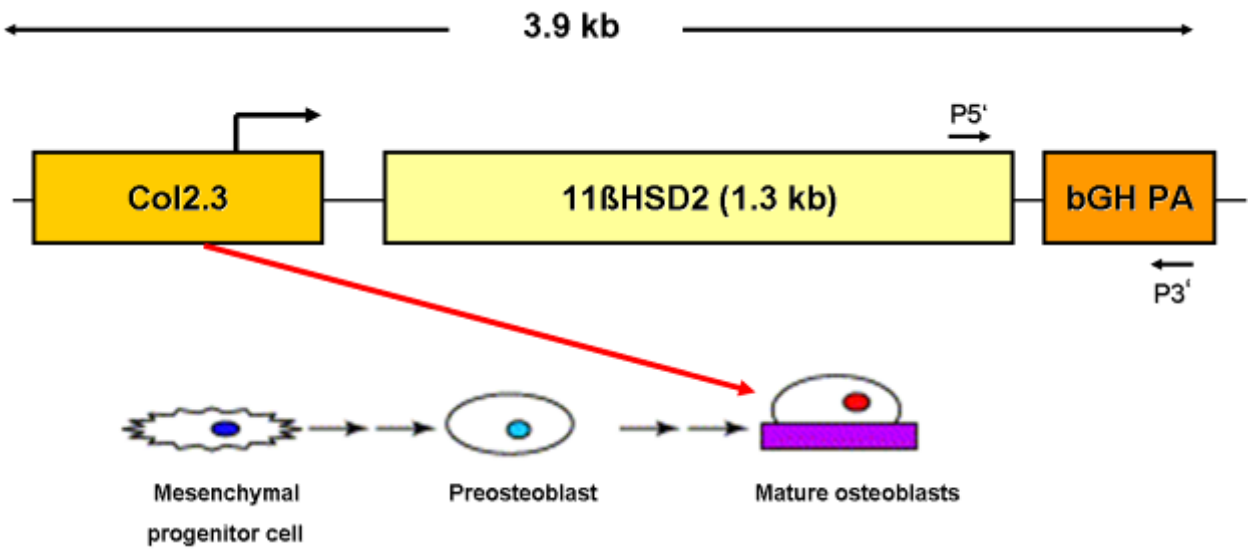

Figure 3: Col2.3-11ßHSD2 construct. The rat 11ßHSD2 cDNA was cloned downstream of the 2.3-kilobase fragment of the collagen type I (Col1a1) promoter and upstream of the bovine GH polyadenylation sequence (bGH PA) targeting mature osteoblasts (P5' forward primer; P3' reverse primer) (Woitge, Harrison et al. 2001; Sher, Woitge et al. 2004).
(picture adapted from: Woitge H et al., Endocrinology, 2001 (Woitge, Harrison et al. 2001)).

3 Hypothesis

Treatment with pharmacological doses of GC strongly affects osteoblast function, inducing a rapid and pronounced reduction in osteocalcin synthesis and secretion and bone loss. (Henneicke, Herrmann et al. 2011).

In addition, it is well established that GC can result in metabolic changes such as impaired glucose and fat metabolism. Prior work suggests that the osteoblast-specific product, osteocalcin, plays a central role in whole body energy metabolism (Lee, Sowa et al. 2007; Confavreux, Levine et al. 2009; Ferron, McKee et al. 2011).

The overarching hypothesis in the present investigation is therefore that GC-treatment induces a suppression of osteoblast function, which in turn leads to a reduction in osteoblast–derived signals involved in the control of whole body fuel metabolism.

A specific hypothesis was that osteocalcin is the major osteoblastic signal mediating the effects of systemic GC therapy on whole body energy metabolism, and that osteocalcin acts on peripheral insulin sensitivity by controlling adiponectin expression.

4 Animals and methods

4.1 Experimental animals and study design

Col2.3-11ßHSD2 tg mice were generated as described in chapter 2.12 and were provided as a gift by Dr. Barbara Kream (Department of Medicine, University of Connecticut Health Center, Farmington, CT, USA). Animals were kept at the animal facility of the ANZAC Research Institute (Sydney, Australia) in accordance with Institutional Animal Welfare Guidelines and according to a protocol approved by Sydney South West Area Health Services (SSWAHS) Animal Welfare Committee.

Mice were allowed access to food and water *ad libitum* and were exposed to a 12-hour light/dark cycle. Surgical procedures were performed on 8-week-old male Col2.3-11ßHSD2 tg mice and their wild type littermates.

A total of 96 mice from both groups were randomly assigned to four experimental groups. Mice in two of the groups as designated in Figure 4 were subcutaneously implanted with 1.5mg corticosterone 21–day release pellets into the nuchal fold to investigate influence of glucocorticoids on osteoblast–derived factors and their impact on energy metabolism.

Due to the decrease of corticosterone levels within the first week (Figure 9) after implantation the experimental animals were re-implanted every 7 days using 1.5mg corticosterone pellets.

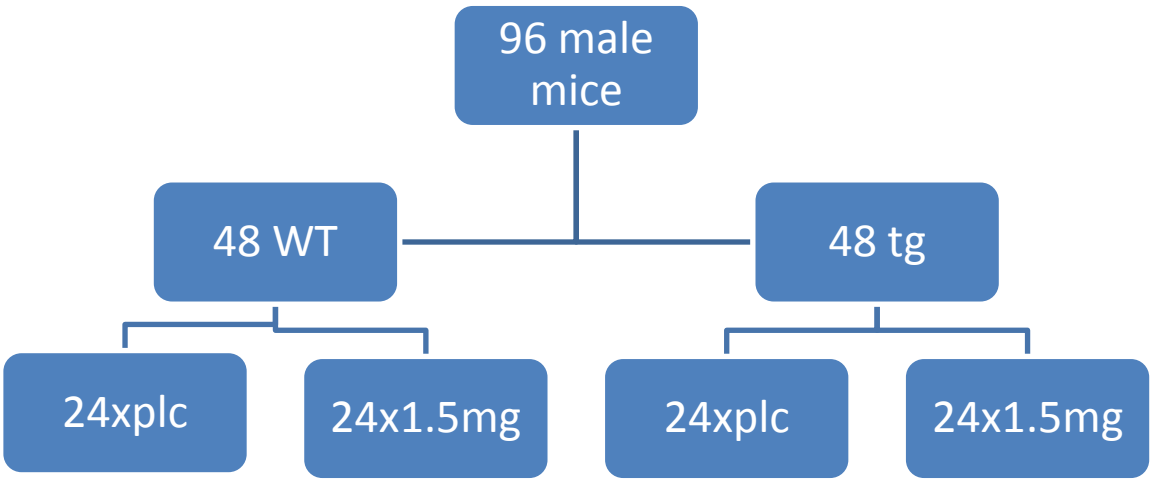

Figure 4: Study design

4.2 Genotype analysis

Genotyping was performed as follows: toe clips were collected between 7 and 12 days of age and their genomic DNA was isolated. Tissue samples were incubated with a lysis mixture containing 198.3µL Milli-Q water, 25µL $MgCl_2$ (20mM; Ajax Finechem Pty. Ltd., Taren Point, Australia), 25µL DNA Polymerase 10x Reaction Buffer (Fisher Biotec Australia, Wembley, Australia) and 17µL proteinase K (Roche Applied Sciences, Mannheim, Germany) per sample at 55°C for 2h. An incubation at 98°C for 15min followed to deactivate proteinase K.

5µl of lysed DNA sample in 20µL PCR reaction mix was amplified by PCR (Eppendorf Mastercycler ep, Eppendorf AG, Hamburg, Germany) using Mango Taq DNA polymerase enzyme (1000units; Bioline Pty Ltd., Randolph, MA, USA)

The forward oligonucleotide primer sequence 5'-ACC TTA GCC CCG TTG TAG-3' was part of the HSD2 gene and the reverse primer sequence was 5'-G AGG GGC AAA GAA GAA CAG ATG-3' within the bovine GH polyadenylation region. The PCR was activated with one cycle at 94°C for 5min and was continued with 30 cycles (94°C 30s, 60° 30s, 72° 45s). The reaction ended with a cycle at 72°C for 5mins.

PCR products were run on a 1.5% agarose gel, which were made up as follows. For 25ml, 0.375g agarose was dissolved in 25ml 1 X TBE buffer before the addition of 7µl SYBR safe (Invitrogen, Carlsbad, CA, USA). The mixture was poured into a mini sub tank with small well combs and allowed to set for ~40mins. PCR products were loaded into wells and run at 100V for ~30mins.

4.3 Anaesthesia

Preoperatively, general anaesthesia was performed by injecting ketamine (75mg/kg body weight; Cenvet Pty. Ltd., Kings Park, Australia) and xylazine (10mg/kg body weight; Cenvet Pty. Ltd., Kings Park, Australia) intraperitoneally.

4.4 Blood collection

4.4.1 Blood collection via retro–bulb plexus puncture

Blood was collected from retro-bulb plexus using heparin-filled micro capillaries on anesthetised mice. A maximum of 200µl was collected. The eyes were rinsed with a drop of saline solution to prevent dehydration.

4.4.2 Blood collection via cardiac puncture

Blood was collected by cardiac puncture on non-recovery anaesthetised mice using a 27G needle attached to a 1ml syringe. Mice were then appropriately killed by cervical dislocation.

4.5 Serum preparation

To prepare serum, whole blood was collected in heparin-filled micro capillary tubes or syringes. Blood was promptly centrifuged twice at 3500 x g for 20 minutes at 4 ± 2 ºC. The supernatant, now designated serum was carefully removed using a pipette, not to contaminate the serum with blood cells, and placed into fresh tubes. Samples were kept at -80ºC

4.6 Pellet implantation

1.5mg corticosterone pellets (Innovative research of America, 1.5mg, 21-day release) were subcutaneously implanted into the nuchal fold (in the interscapular space) every 7 days (Herrmann, Henneicke et al. 2009). Surgical procedures were performed on 8 week-old male Col2.3-11ßHSD2 tg mice.

Mice were intraperitoneally injected with the ketamine-xylazine solution to provide sufficient anaesthesia. The mouse was placed on a sterile underpad with its back facing upwards; the back of the mouse is carefully sprayed with 70% ethanol.

The scalpel blade was used to make a small incision into the skin on the back of the mouse. Through this incision a trocar was inserted which holds the pellet. The trocar was slid towards the neck of the mouse where a second metal pin is pushed through the trocar to ensure precise placement of the pellet.

The incision was sutured using 6–0 nylon suture (Ethicon Inc., Somerville, NJ, USA). A saline drop was delivered to the eyes to keep them from drying. Mice were observed until recovery.

4.7 Intraperitoneal micro pump implantation

The mice were intraperitoneally injected with the Ketamine-Xylazine solution to provide sufficient anaesthesia. The micro pump (Alzet osmotic pumps, Durect Corporation, Cupertino, CA, USA) implantation was started by placing the mouse on a sterile underpad with its abdominal side facing up.

The scalpel blade was used to make an approximately 1cm incision into the skin of the abdomen of the mouse. The cut was made in the middle of the abdomen, cutting away from the sternum. Firstly, the animal's skin was incised, followed by the abdominal muscle underneath it and last the peritoneum.

The micro–pump, held by forceps, was then placed into the abdominal cavity. The incision was sutured with at least 4 stitches using 6–0 nylon suture (Ethicon Inc., Somerville, NJ, USA). Every layer (peritoneum, abdominal muscle, skin/fur) needs to be sutured separately, the skin last, which ends the operative procedure.

The eyes were covered with a drop of saline solution to keep them from drying. Mice were kept either on a heating pad or under a heat lamp until full recovery.

4.8 Metabolic testing

4.8.1 Insulin tolerance test (ITT)

The ITT is a diagnostic procedure during which insulin is intraperitoneally injected to determine the peripheral response to insulin. Under normal conditions, the injected insulin acts to decrease blood glucose levels by stimulating organ (primarily adipose tissue and muscle) glucose uptake.

Blood glucose levels were therefore measured at intervals of 0, 15, 30, 60, 90 and 120 minutes post intraperitoneal insulin-injection using the Accu Check Performa system (Roche Applied Sciences, Mannheim, Germany).

The blood drop was obtained by a tail vein prick using a 25 gauge needle. Prior to insulin administration baseline glucose levels were measured (t=0) using the same method described above. The dose of insulin administered was 0.75 units per kilogram (kg) of body weight. Animals were fasted for 6 hours prior to the ITTs.

4.8.2 Oral glucose tolerance test (oGTT)

The oGTT is a diagnostic procedure which determines the uptake of glucose by peripheral tissues (muscle and adipose tissue) via the stimulation of endogenous insulin and provides insight into the physiological metabolisation of glucose.

Glucose was administered *per os* (by gavage using a 20G gavage needle with 2 ¼mm ball end for small laboratory animals) at a concentration of 2g/kg body weight. Blood glucose was measured as for the ITT and within the same time period of 2 hours. The oGTT also required a 6 hour fast (Andrikopoulos, Blair et al. 2008).

4.9 Body fat measurements via densitometer dual energy X-ray absorptiometry (DEXA)

To acquire total body scans to measure fat tissue mass and % fat of the experimental animals, the Lunar PIXImus II Mouse Densitometer (GE Medical Systems Model 51045; Madison, WI, USA) and a designated computer were used.

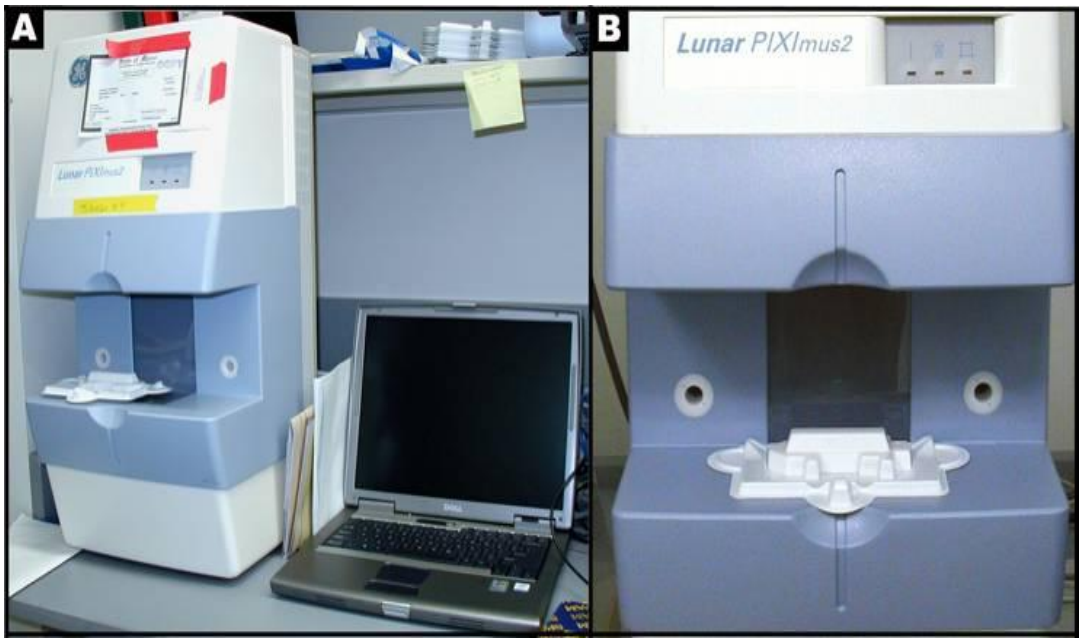

Figure 5: A: Lunar PIXImus2 densitometer with designated Laptop; B:. Close-up detail of the Lunar PIXImus2 densitometer with specimen tray.

4.9.1 Preparation for densitometry and collecting image scans

The PIXImus (small animal DEXA system, PIXImus™, Fitchburg, WI) was utilised to create full body scans and X-ray absorptiometry data were simultaneously processed with manufacturer supplied software. Before the system was used the PIXImus is calibrated daily with a "phantom mouse" according to manufacturer's protocol.

General anesthetic (ketamine 75mg/kg body weight; Cenvet Pty. Ltd., Kings Park, Australia, and xylazine 10mg/kg body weight; Cenvet Pty. Ltd., Kings Park, Australia, intraperitoneally injected) was used to guarantee absolute immobility.

Anesthetised mice were positioned back facing downwards with the tail positioned away from the body, the front legs extended to the side, and the neck and spine were gently straightened. Each mouse was placed on the provided specimen tray and carefully taped down on all four extremeties and then placed under the PIXImus beam path.

The X-ray process to obtain a single full scan took approximately 4 min. Disposable plastic trays were saved and re-used after cleaning and disinfection.

4.9.2 Measurement acquisition and image scan analysis

Based on PIXImus validation studies (Nagy and Clair 2000) DEXA estimated measurements of fat tissue correlate well with measurements obtained from chemical extraction. This is made possible by developing software versions with equations that adequately correct raw DEXA measurements. Following the completion of an image scan, the DEXA system automatically implements specialised software to distinguish bone tissue from either fat tissue or from lean tissue based on the resulting X-ray densities at two distinct energy levels (Pietrobelli, Formica et al. 1996).

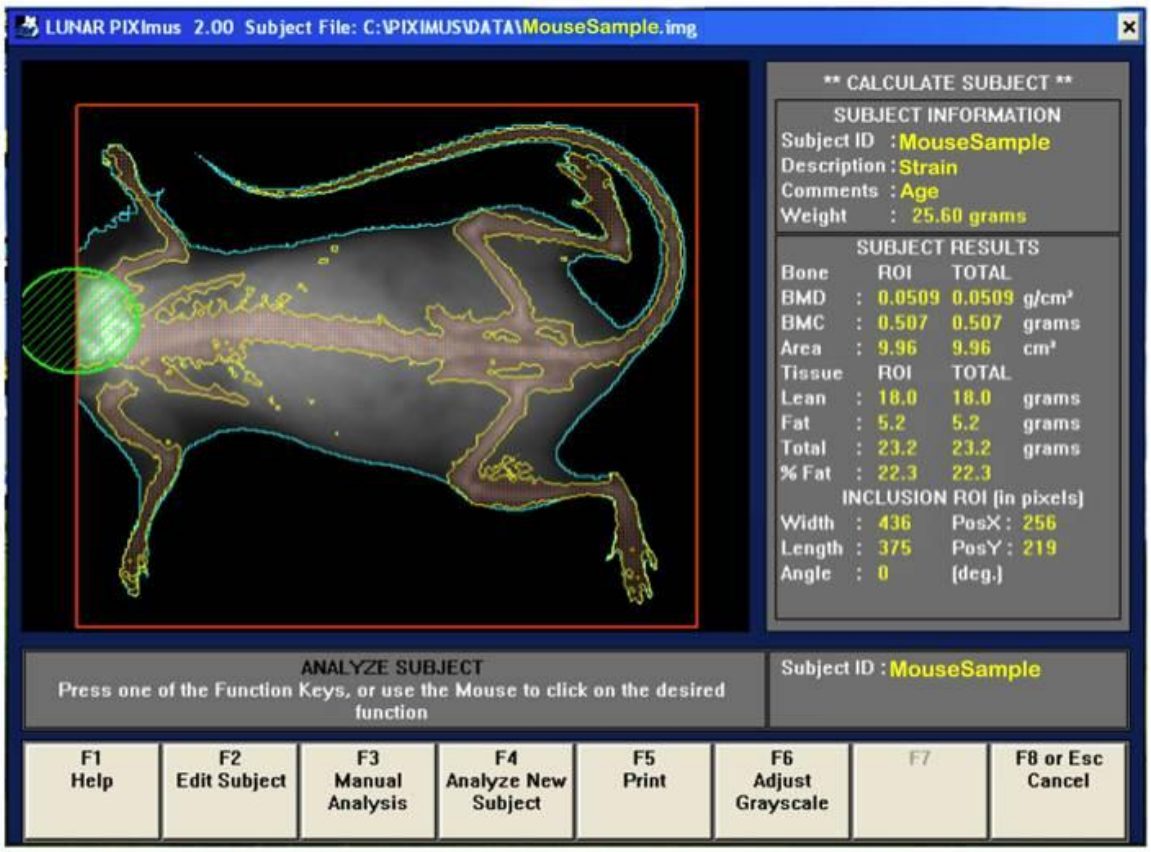

Figure 6: Screenshot Lunar PIXImus 2.00

4.9.3 Safety

For safety, gloves were worn at all times and radiation safety guidelines were strictly adhered too, such that 2m distance was kept from the PIXImus machine during scanning at all times.

4.9.4 Data collected by investigator

PIXImus data are from the whole body exclusive of the head. Percent body fat and fat tissue mass were measured.

4.9.5 Definitions and calculations

Fat tissue mass = all tissues with low density (x-ray scan)

T-area = Total body area (cm^2)

% Fat = (Fat tissue mass ÷ Total body tissue mass) x 100

4.10 Serum assays

4.10.1 Insulin ELISA

Serum insulin levels were measured using an ultrasensitive mouse insulin ELISA (Mercodia, Uppsala, Sweden). It is a solid phase two-site enzyme-linked immunoassay based on the sandwich technique. Calibrators and 25µL of each sample were pipetted onto a 96-well plate provided with the kit. Assay was performed according to manufacturer's instructions. The plate was read at 450nm and the x-and y-axes of the standard curve were represented in log scale.

4.10.2 Adiponectin and leptin ELISA

Adiponectin and leptin serum concentrations were both obtained by using ELISA Kits manufactured by Millipore for mouse leptin and mouse adiponectin. The sample volume used for the adiponectin ELISA was 20 µL. The sample size used for the leptin ELISA totaled 10 µL per sample. Assays were performed according to manufacturers' instructions. The plates were read at 450nm and 600nm and subtracted from one another as the instructions advised.

4.10.3 Corticosterone measurements

Serum corticosterone levels were analysed from intact and corticosterone-treated males using a stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) method developed for this purpose. Briefly, 100 µl of serum was added to 700 µl Milli-Q H_2O fortified with d8-corticosterone, which is a special non-radioactive labelled form of corticosterone, so termed because it has eight heavy hydrogen (deuterium) atoms on it (CDN Isotopes, Quebec, Canada). The d8–corticosterone was used as the standard.

Samples were then loaded onto pre-conditioned (100% methanol followed by Milli-Q H_2O) Strata X 30mg/1ml cartridges (Phenomenex, Torrance, CA, USA), washed with 2ml 10% methanol and then eluted with 1ml 100% methanol into collection tubes. A 1µmol/l (1000 fmol/µl) working corticosterone (Sigma, St. Louis, MO, USA) stock solution was prepared in 50% methanol and then diluted to give a six-point standard curve (0, 25, 50, 100, 200, 400 fmol/µl). For preparation, both samples and standards were dried using a steady stream of nitrogen. Samples and standards were then resuspended in 100µl of 50% methanol and filtered prior to injection through the LC-MS.

The LC-MS system comprised of a Thermo Accela LC interfaced with a Finnigan TSQ Quantum Access triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA). Chromatographic separation was performed on a specialised column (C18, Thermo Hypersil Gold, 50×2.1 mm, 1.9 µm particle size) using a binary gradient.

Samples were run for 10 minutes. Detection and quantification of corticosterone and labeled d8-corticosterone was performed via selected reaction monitoring (SRM) using an electrospray ionisation (ESI). The collision energy, sheath gas and capillary temperature were set at 15, 20 (arbitrary units) and 300°C, respectively. The spray voltage was 3.5kV and SRM parameters were optimised by infusion of authentic material.

The most abundant fragment ions of corticosterone and d8-corticosterone were used for quantitation. All quantitative calculations were performed using peak area ratios relative to that of the internal standard, and based on the calibrated standards.

4.10.4 Osteocalcin immunoradiometric assay

Osteocalcin serum concentrations were measured using an immunoradiometric assay (IRMA) for the quantitative determination of mouse osteocalcin levels in serum. The IRMA was manufactured by Immunotopics. The assay was performed following the manufactures' instructions.

4.11 Cloning of OCN construct

4.11.1 Elution of OCN plasmid

The OCN construct was created and kindly donated by Yale Core Center for Muscular Skeletal Disorders (YCCMD). The construct was sent by blotting the DNA on a blotting paper (Clifton, NJ). Plasmid recovery from blotting paper was achieved by cutting out the circle containing the plasmid and placed into an eppendorf tube. 25µl of 10nM Tris ph7.5 was added and vortexed. The blotting paper was rehydrated for five minutes, followed by a brief centrifugation of 30 seconds, the remaining supernatant contained the plasmid. A 1% agarose gel was run to confirm presence and quality. The concentration of DNA was measured using UV spectrophotometry at 260nm.

4.11.2 Transformation

4.11.2.1 LB Broth preparation

10g of LB Broth powder (Amresco, Solon Ohio Miller) was added to a sterile bottle containing 400ml Milli-Q water. The lid was loosely screwed on and the bottle was autoclaved for 40 minutes.

4.11.2.2 Agar plate preparation

20g/l Bacto Agar (Bacto Laboratories, NSW, Australia) was dissolved by LB Broth and autoclaved. Kanamycin was added into the Bacto Agar/ LB medium at the final concentration of 50mg/ml after it was cooled down at 55ºC. The solution was poured into sterile 90mm petri dishes (Techno Plas, Dandenong, Victoria, Australia). The petri dishes were left to set at room temperature. Dishes were stored upside down.

4.11.2.3 Transformation of pLIVE and OCN vectors into competent bacteria cells (Topp ten E. coli, Invitrogen)

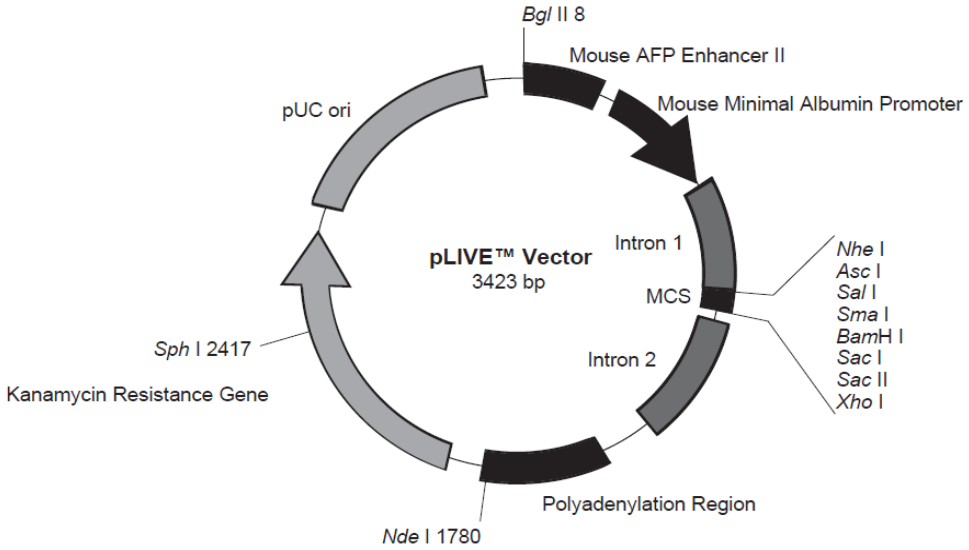

Figure 7: Map of commercial pLIVE vector

The process of DNA transformation in molecular biology is a method of genetic alteration resulting from the direct uptake, incorporation and expression of exogenous DNA. Transformation is commonly used to introduce recombinant plasmid DNA into recipient bacterial strains which can transform. These bacteria cells are then called competent.

The Topp ten E. coli cells were extracted from 80ºC storage and slowly thawed on ice until the solution was entirely liquefied. While working with the bacteria it was necessary to work close to a burning flame for sterilisation purposes. 1µl of DNA at a concentration of 0.1µg/µl (pLIVE, Mirus, Madison, WI, USA) was transferred into100µl of competent Topp ten E. coli cells.

The following steps included re-suspending of the cells, taking 100µl into a prechilled 15ml Falcon polypropylene tube and adding of 1.7µl of β-mercaptoethanol, diluted in distilled water. Contents (DNA, competent cells, β- mercaptoethanol) were gently swirled and cells were incubated for 5-10 minutes on ice. Heat shock for 45seconds in a 42ºC water bath was applied to the cells, followed by 2 minutes' incubation on ice. The accurate length of time of the heat shock is critical for obtaining the highest efficiency of transformation. 0.9ml of preheated (42ºC) LB Broth medium was added and the tubes were incubated at 37ºC for one hour on a shaker at 225-250rpm. Using a sterile glass spreader, 100µl of the culture containing the now transformed bac-

teria cells was spread onto agar plates containing the appropriate antibiotic and left to grow at 37ºC overnight.

4.11.3 Selection and purification of the plasmid DNA

4.11.3.1 Colonies selection

6 Colonies were selected from transformation plates using sterile techniques. The selected transformed bacteria cells were spread onto new agar plates containing the appropriate antibiotic and left to grow at 37ºC overnight.

4.11.3.2 Purification

On the following morning the LB-Broth was taken and the DNA was purified using the Wizard Plus SV Miniprep DNA Purification System. The company's protocol was followed.

4.11.3.3 Digest

Due to the specific recognition nucleotide sequences of the OCN inserts, the commercially available restriction enzyme S*alI* (Promega, 2800 Woods Hollow Road, Madison, WI 53711 USA) was used to release the OCN insert out of the plasmid. Since the pLIVE vector contained the same restriction side, it could also be recognised and released by the restriction enzyme S*alI* and was therefore used to linearise the pLIVE fragment. The purified OCN fragment is 500bp and the pLIVE fragment 2500bp in size. The following mastermix was used to complete the digest.

Mastermix:

- 0.5 µl of SAL 1 enzyme
- 0.5 µl of digestion buffer
- 10 µl of DNA → pLIVE plasmid
  → OCN plasmid

4.11.4 Confirmation of purified OCN and pLIVE fragments via 2% agarose gel

The 2% agarose gel was run incorporating a DNA ladder (DNA Hyperladder II) to confirm the size and therefore verify the product.

The 2% agarose gel was made up as follows. For 50ml, 1g of agarose was dissolved in 50ml of TBE buffer. One µl of SYBR safe (Invitrogen, Carlsbad, CA, USA) was added. The agarose solution was poured into a mini sub tank with a small well comb and allowed to set for 30 minutes. 300ml of TBE Buffer was added to the tank. The wells were loaded with 1µl of DNA dye, 3µl of plasmid DNA and 6µl of Milli-Q water. The gel was run at 150V for 40 minutes.

4.11.5 Purification

After verification of the correct amplified DNA, these bands were excised using a 22 blade scalpel, followed by DNA extraction using the QIA quick Gel Extraction Kit 50 (Qiagen, Hilden, Germany). The instruction manual was strictly followed. The purified DNA was stored at -80ºC.

4.11.6 Ligation of OCN and pLIVE

T4 DNA ligase catalyses the joining of two strands of DNA, the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides, in either a cohesive-ended or blunt-ended configuration. The enzyme has also been shown to catalyse the joining of RNA to either a DNA or RNA strand in a duplex molecule but will not join single-stranded nucleic acids. The mastermix used to ligate OCN and pLIVE fragments was compounded as followed.

Mastermix:

- 1 µl T4 DNA ligase
- 2 µl Ligase buffer
- 1 µl pLIVE vector
- 5 µl mOCN DNA fragment
- 11 µl Milli-Q water

Ligation took place at 15ºC for 4-18h. Additionally, Antarctic phosphatase was added to prevent the pLIVE vector from self-ligating.

The ligated product was then transformed into E.coli Topp Ten as described above and a new stock containing the ligated plasmid was employed. Six tip swabs were taken and placed into falcon tubes containing LBroth and the appropriate antibiotic. Half of these samples underwent the purification process using the Wizard Plus SV Miniprep DNA Purification System and confirmed by a 2% agarose gel with a Hyper Ladder II as a molecular weight marker and also com-

mercially sequenced. Glycerol stocks were also prepared containing 200µl bacteria cells per 1ml sterile glycerol and stored at –80ºC. The other three samples (10ml) were incubated at 37 ºC for two hours and poured into 1L of new antibiotic treated LB and incubated on a shaker overnight at 37ºC. These samples had to be prepared for injection into a vital mouse using an endotoxin free megaprep kit (Qiagen, Hilden, Germany).

4.12 Hydrodynamic tail vein injection (hTVI)

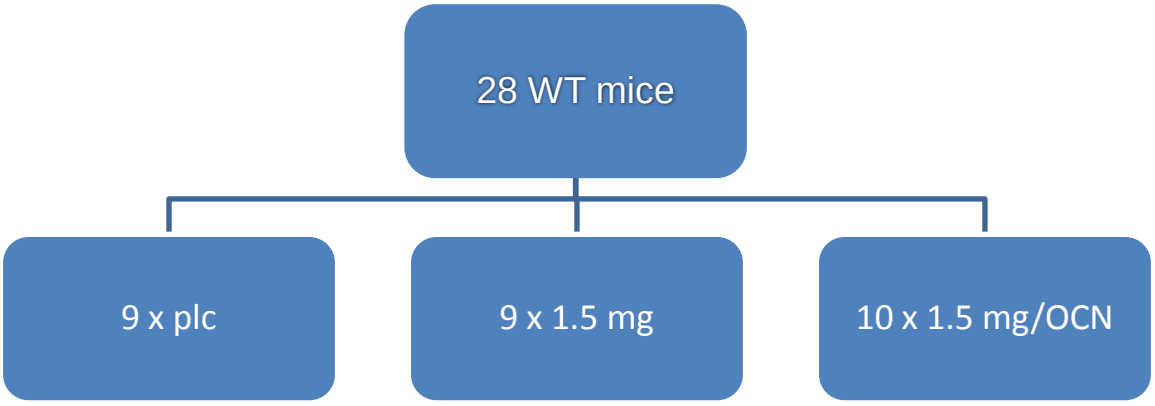

Figure 8 Experimental design

The preparation of DNA for *in vivo* delivery followed a sterile technique to provide the mice with high quality DNA, free of endotoxins and other contaminating proteins. 50µg of DNA plasmid was diluted into 10% volume of mouse body weight using the Trans IT delivery solution (Mirus Bio LLC, Madison, WI, USA). The required injection volume was determined using the following formula:

$$Total\ volume\ per\ mouse\ [ml] = \left(\frac{mouse\ weight\ [g]}{10g} + 0.1\right) \times Delivery\ Solution\ [ml]$$

The addition of the 0.1ml of delivery solution represents the void volume that remains in the syringe and needle after injection. (Example: a 30g mouse would require a total volume of 3.1ml).

Prior to injection the DNA was freshly prepared and used within 30min according to manufacture's recommendations. Animals were required to be conscious for the procedure to ensure

sufficient heart rate and blood pressure. A restraining device was employed to control movement of the mice. Tails were warmed in a water bath (37ºC-40 ºC) prior to injections for approximately 30 seconds to dilate the veins for visualisation and to optimise the injection.

The Hydrodynamic Delivery Solution/DNA mix remained at room temperature before injecting into the mouse. The injection was performed underneath a distinct light source. The needle was placed into the ventral tail vein at the distant end of the tail. The injection area was sterilised with an alcohol swab and allowed to dry. The needle was held nearly parallel to the tail vein, the bevel of the needle pointing up. Syringe was plunged once vein was successfully pierced, the needle was positioned correctly when the vein cleared of blood and no swelling occurred. The complete volume minus the 0.1ml void volume held within the syringe was dispensed into the tail vein within 4 to 7 seconds at a constant rate to achieve maximum *in vivo* delivery. Animals were observed until recovery.

4.13 Preparation of frozen liver sections of YFP injected mice

After hTVI using saline as a delivery solution containing plasmid DNA with a CMV driven YFP (kindly provided by Prof. Graham Robertson, Cancer Pharmacology Group, Anzac Research Institute, Sydney), livers were extracted from the euthanised mice 20 hours following the injection. The livers were carefully separated from the blood vessels and ligaments and the gall bladder and placed into a square plastic container and covered with optimal cutting temperature compound (OCT). The bottom of a styrofoam container was filled with dry ice and ethanol. The plastic squares were cautiously placed into the cold environment and frozen down. The frozen sections were properly labeled and stored at -20 ºC. The mouse liver sections were cut using a cryostat at microtome setting of 10 microns and placed onto a glass slide to be examined under a microscope.

4.14 Statistical analysis

Data are represented as the means ± standard error of the mean (SEM). Statistical analyses were performed with either a Student's t-test, two-way ANOVA or repeated measures ANOVA, with relevant post hoc tests using the Statview statistics program. A p-value of less than 0.05 was considered statistically significant.

5 Results

5.1 Determination of corticosterone levels

Administration of 1.5mg corticosterone 21-days slow-release pellets lead to an increase in serum corticosterone levels reaching a peak after 24 hours. Placebo animals remained at constantly low concentrations over the examined period of time. Corticosterone blood concentrations decline after day one and reached baseline levels by day seven as shown in figure 9 below. This data demonstrates an experimental design where mice were not reimplanted after seven days. Contradicting the company's measurements, corticosterone remained at baseline corticosterone levels after day seven. Consequently, new pellets were implanted in treated animals after day seven to successfully sustain high exogenous corticosterone levels.

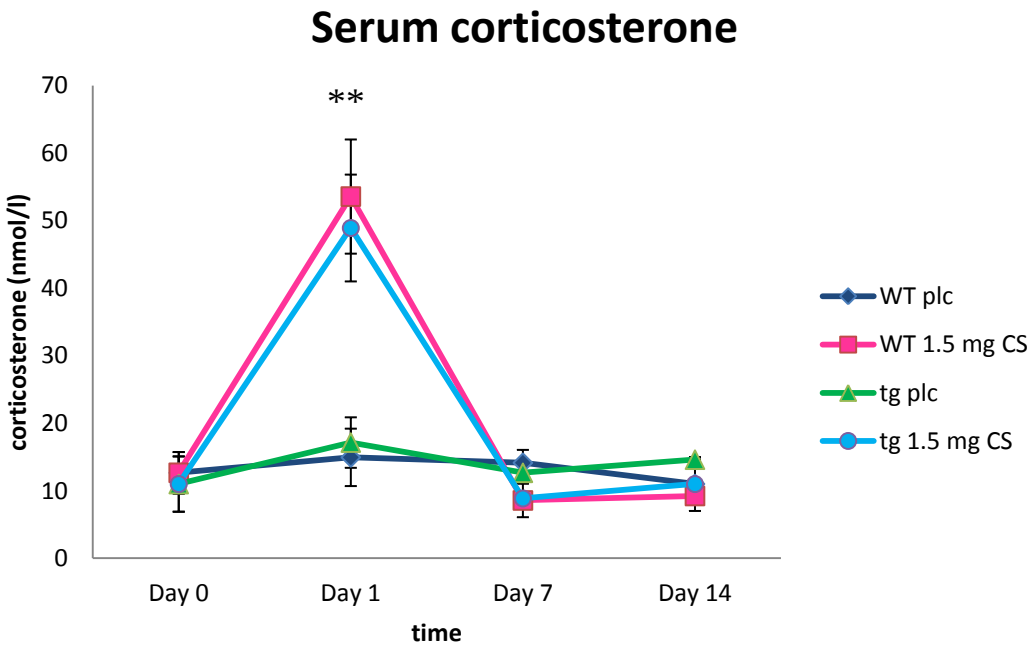

Figure 9: Corticosterone levels in serum in nmol/l. Measurements gained via mass spectrometry. Results are demonstrated in absolute values and expressed as means ±SEM. WT–wild type tg–transgenic ** $p<0.001$, n=12

5.2 OCN levels

OCN concentrations were measured over a time period of 28 days after corticosterone administration at day 0. OCN levels decrease only slightly in both placebo treated groups. Mice treated with 1.5mg corticosterone, however, suffer from a decline in OCN over the experimental time period of 4 weeks.

A significant difference in OCN concentration was detectable between GC treated WT and tg animals at day 14 and 28. OCN levels remained significantly higher in non-treated animalscompared to Gc-treated mice throughout the experiment.

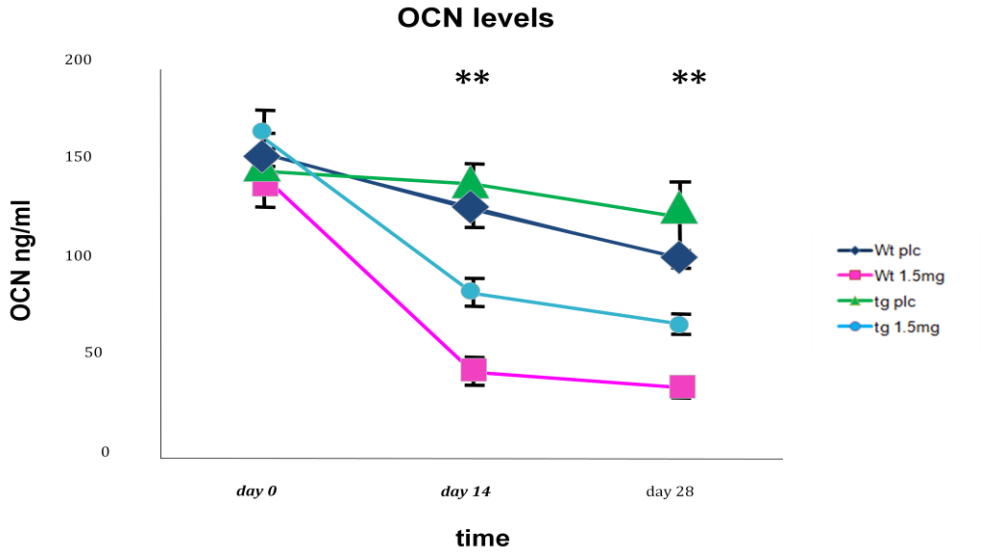

Figure 10: Osteocalcin levels measured at day 0 to day 28 via IRMA. Measurements from one experiment and expressed as mean±SEM. WT–wild type, tg–transgenic ** p<0.001,
n=8 per experimental group

5.3 Measurements of metabolic parameters

5.3.1 Insulin tolerance tests

Insulin resistance was not observed by day 1 in either genotype (see Figure 11). In fact, the data show no general pattern on day 1 and this is likely due to the process of recovery from the anesthesia (0.75ml Ketamin and 0.1 ml of Xylasil) utilised for implantation surgery on day 0.

By day 6 insulin resistance was observed only in WT animals treated with GC. All other groups including tg GC-treated mice remained sensitive to insulin (Figure 12).

However, WT animals treated with GC remained insulin resistant at day 14, tg mice showed a partial insulin resistance by day 14 (Figure 13).

The results gained from the ITT performed at day 21 (Figure 14) confirm the insulin resistance of WT treated mice and show that tg animals receiving GC become more insulin resistant as well compared to their non treated littermates.

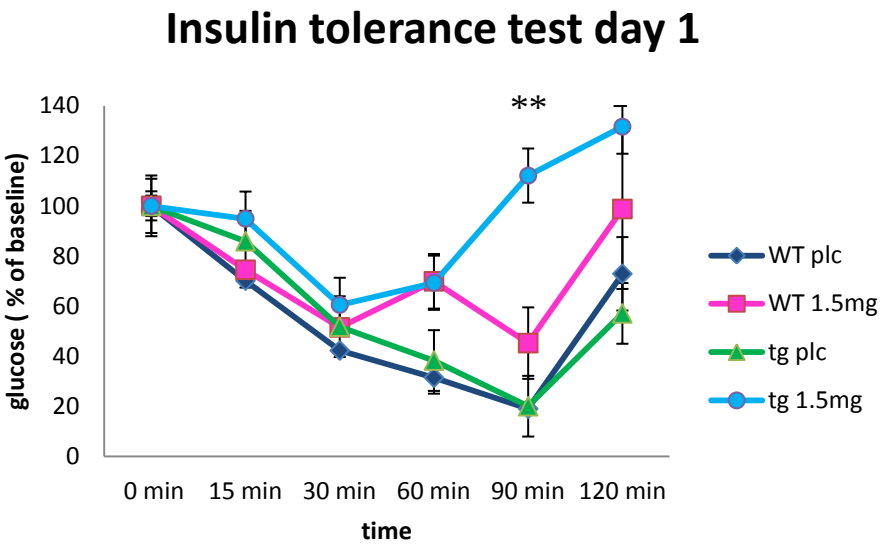

Figure 11: Insulin tolerance test day 1. Results are % of baseline glucose (pre-insulin administration) from two experiments and expressed as mean±SEM. WT–wild type, tg–transgenic.
** p<0.01, n=3 per experimental group

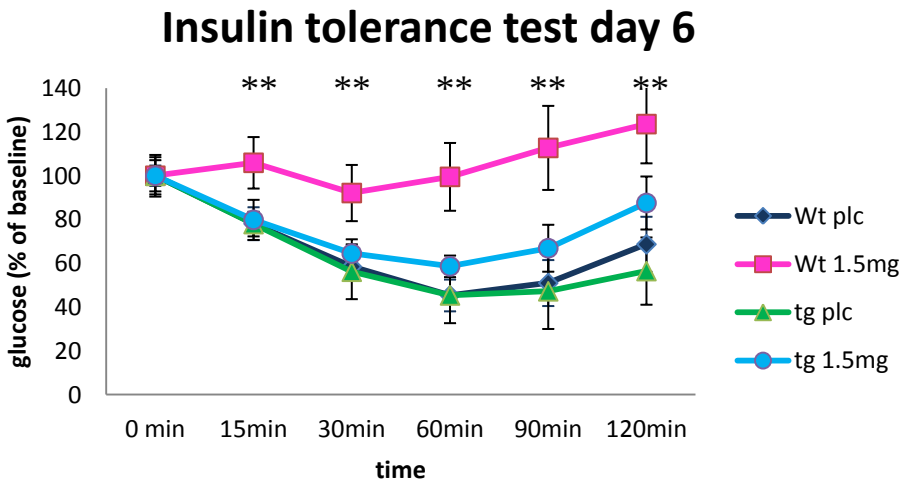

Figure 12: Insulin tolerance test day 6. Results are % of baseline glucose (pre-insulin administration) from one experiment and expressed as mean±SEM. WT–wild type, tg–transgenic.
** p<0.01, n=8 per experimental group

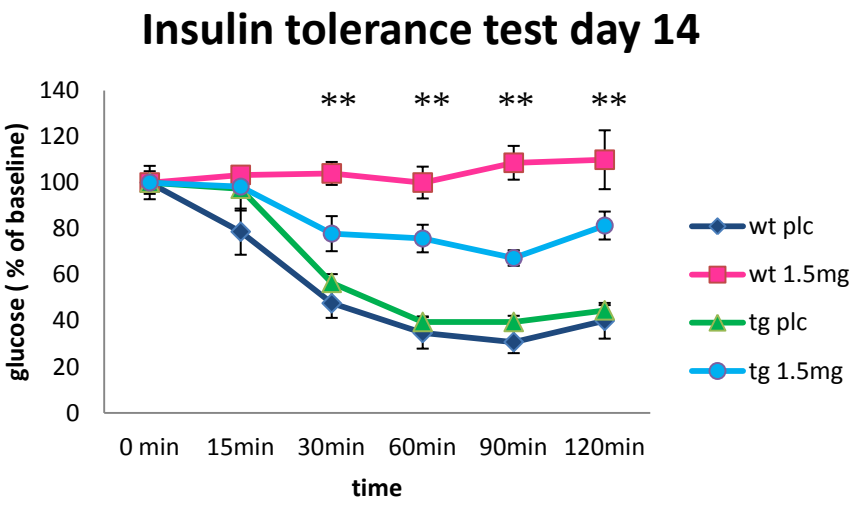

Figure 13: Insulin tolerance test day 14. Results are % of baseline glucose (pre-insulin administration) from one experiment and expressed as mean±SEM. WT–wild type, tg–transgenic. *p<0.05, ** p<0.01, n=8 per experimental group

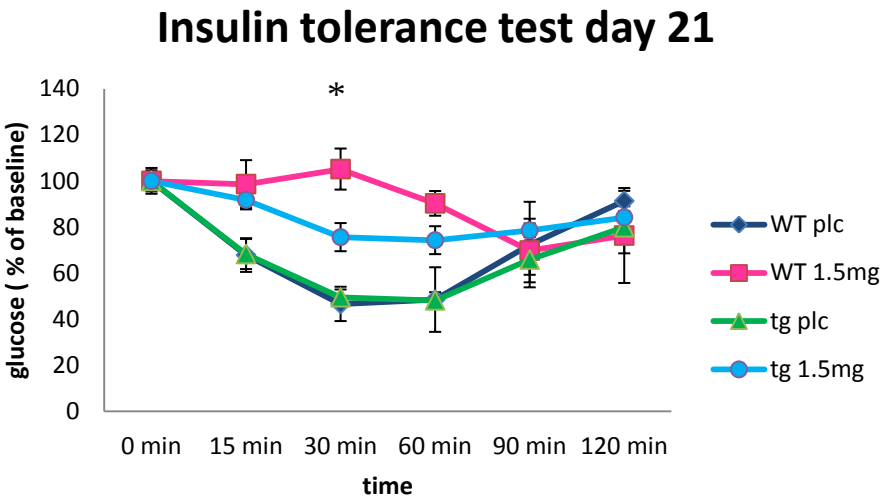

Figure 14: Insulin tolerance test day 21. Results are % of baseline glucose (pre-insulin administration) from one experiment and expressed as mean±SEM. WT–wild type, tg–transgenic. *p<0.05, n=8 per experimental group

5.3.2 Oral glucose tolerance tests (oGTT)

The results obtained at day 3 imitate a healthy response of glucose metabolism after administration of an oral glucose bolus (Figure 15). All experimental groups present no significant differences and lie within a normal schematic form of an oGTT. The data obtained at day 7 clearly show the GC-treated WT mice to be glucose intolerant whereas the transgenic treated mice stay glucose tolerant, with levels comparable to those of the untreated groups (Figure 16). The oGTT performed on day 15 clearly shows the glucose intolerance of GC treated WT animals whereas all other groups stay responsive to glucose (Figure 17).

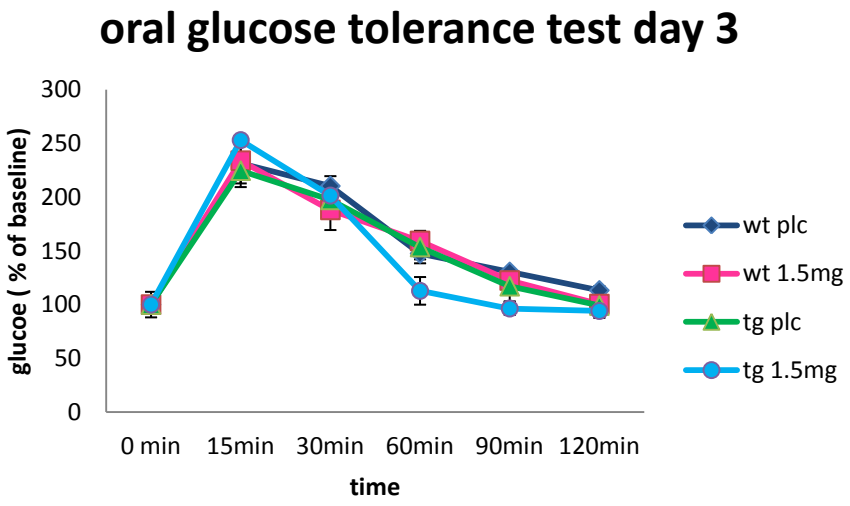

Figure 15: Oral glucose tolerance test day 3. Results are % of baseline glucose (pre-glucose administration) from one experiment and expressed as mean±SEM. WT–wild type, tg–transgenic. n=3 per experimental group

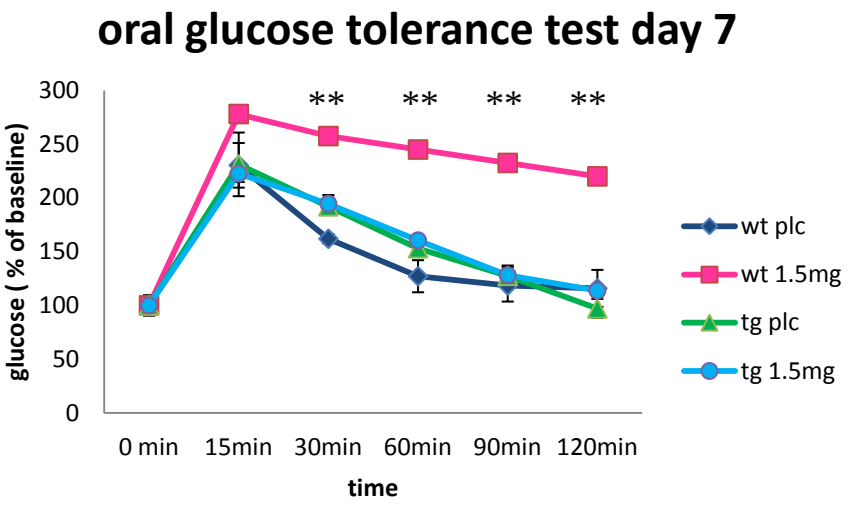

Figure 16: Oral glucose tolerance test day 7. Results are % of baseline glucose (pre-glucose administration) from one experiment and expressed as mean±SEM. WT–wild type, tg–transgenic ** p<0.01, n=3 per experimental group

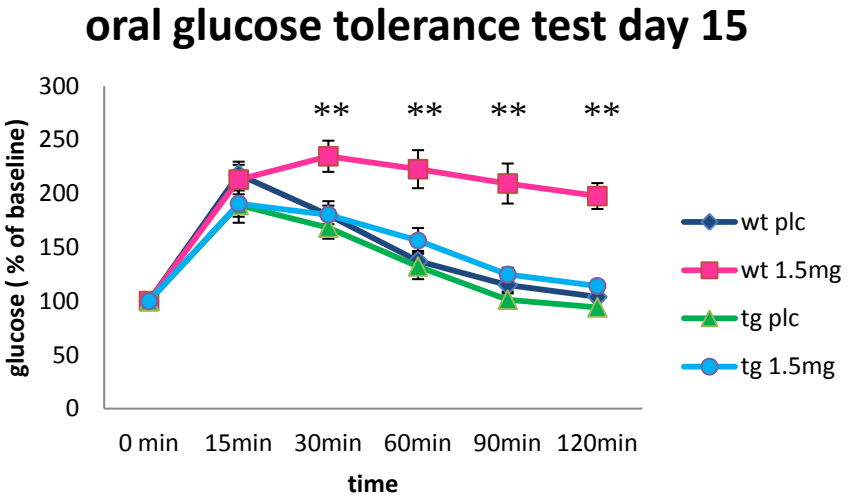

Figure 17: Oral glucose tolerance test day 15. Results are % of baseline glucose (pre-glucose administration) from one experiment and expressed as mean±SEM. WT–wild type, tg–transgenic ** p<0.01, n=3 per experimental group

5.3.3 Fat accrual

After four weeks of GC treatment the animals were sacrificed and the effect of corticosterone on the extracted fat pads was determined. The gonadal fat pads in mice are highly enlarged in mice treated with GC compared to non-treated animals (Figures 18,19,20). Interestingly, however tg animals gained significantly less fat compared to WT animals receiving the same amount of corticosterone over four weeks. To further quantify the fat accumulation, PIXImus scans were performed. The results clearly show that GC treatment leads to an increase of fat tissue in accordance to the weighed increase in fat pads.

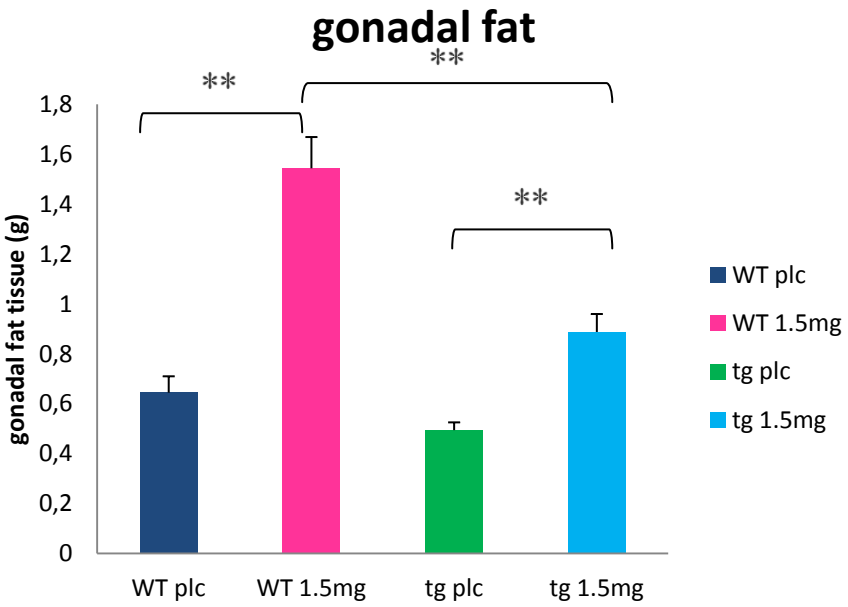

Figure 18: Gonadal fat tissue in gram eviscerated from the animals at day 28. Data shown as means ±SEM. WT–wild type, tg–transgenic ** $p<0.01$, WT plc n=20, WT 1.5mg n=22, tg plc n=21, tg 1.5mg n=23

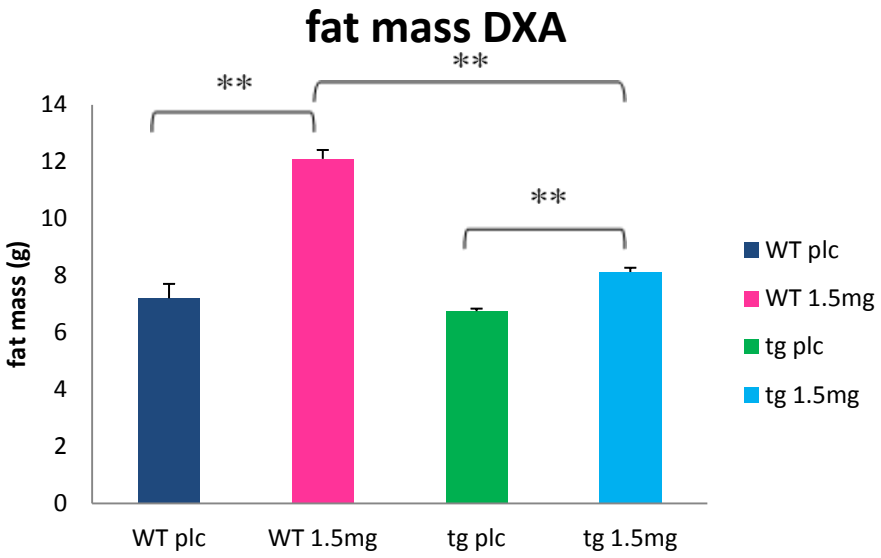

Figure 19: Absolute fat mass in g measured via PIXImus mouse densitometer in animals at day 28. Data shown as means ±SEM. WT–wild type, tg–transgenic ** p<0.01, WT plc n=20, WT 1.5mg n=22, tg plc n=21, tg 1.5mg n=23

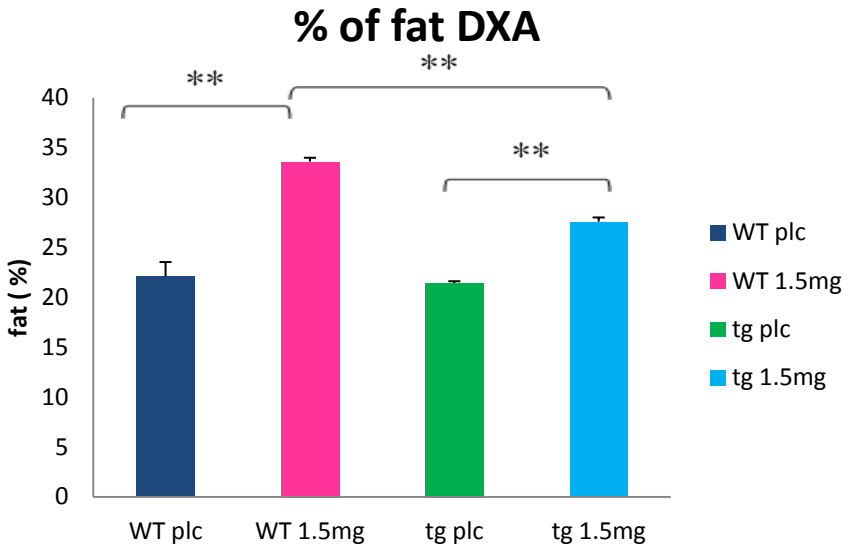

Figure 20: % fat content measured via piximus mouse densitometer in animals at day 28. Data shown as means ±SEM. WT–wild type, tg–transgenic ** p<0.01, WT plc n=20, WT 1.5mg n=22, tg plc n=21, tg 1.5mg n=23

5.3.4 Determination of serum insulin levels

Insulin is a vital metabolic hormone lowering glucose levels and inducing hyperphagia consequently, it was of interest to measure insulin concentrations in the experimental animals to study a potential difference between the genotypes and therefore providing a possible explanation or uncovering a new link to explain the difference in fat mass and insulin resistance and glucose intolerance. Insulin is secreted by the pancreatic ß-cell as a pre-pro-hormone. The determination of insulin serum levels would provide insight into whether GC abrogate solely the insulin secretion rates or if the peripheral function of insulin is affected and therefore leading to the divergent results obtained between WT treated and tg placebo treated mice in the ITTs.

Insulin levels obtained at day 7 show an increase in insulin by GC treatment (Figure 21). Whilst no significant difference in insulin levels could be observed between the two genotypes, a trend towards lower insulin levels in tg mice could be interpreted (Figure 21). Preliminary data obtained in 21 day old mice showed a significant difference between treated wild–type and transgenic mice favouring lower insulin levels in transgenic mice.

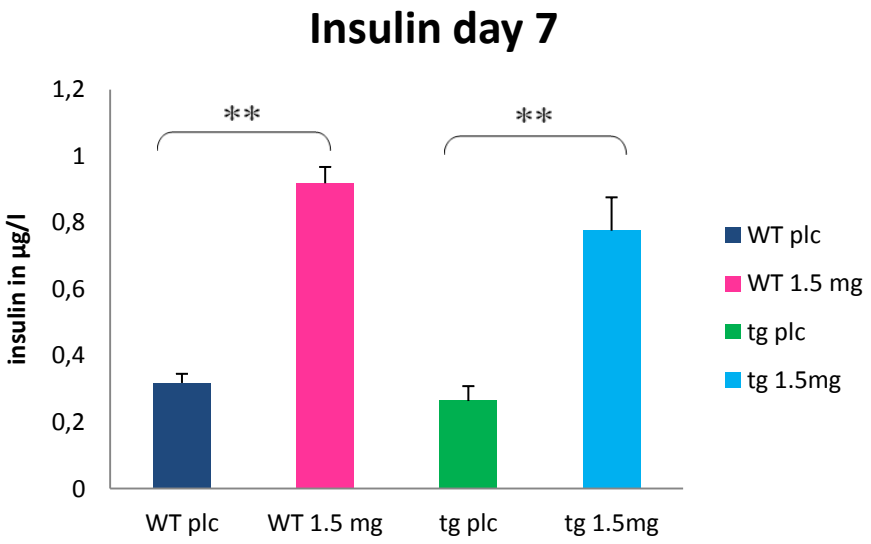

Figure 21: Serum insulin levels in µg/l. Data from 4 experiments at day 7 and expressed as mean±SEM. WT–wild type, tg–transgenic. ** $p<0.01$, WT plc n=9, WT 1.5mg n=7, tg plc n=7, tg 1.5mg n=8

5.3.5 Determination of serum Leptin levels

Higher leptin concentrations were found in corticosterone treated WT mice compared to placebo WT mice at all timepoints. Leptin levels continuously increased in all experimental groups by day 21. Animals treated with corticosterone had a significant increase in leptin concentrations compared to their placebo littermates. However, a significant difference between the two genotypes was not detected (Figures 22, 23, 24)

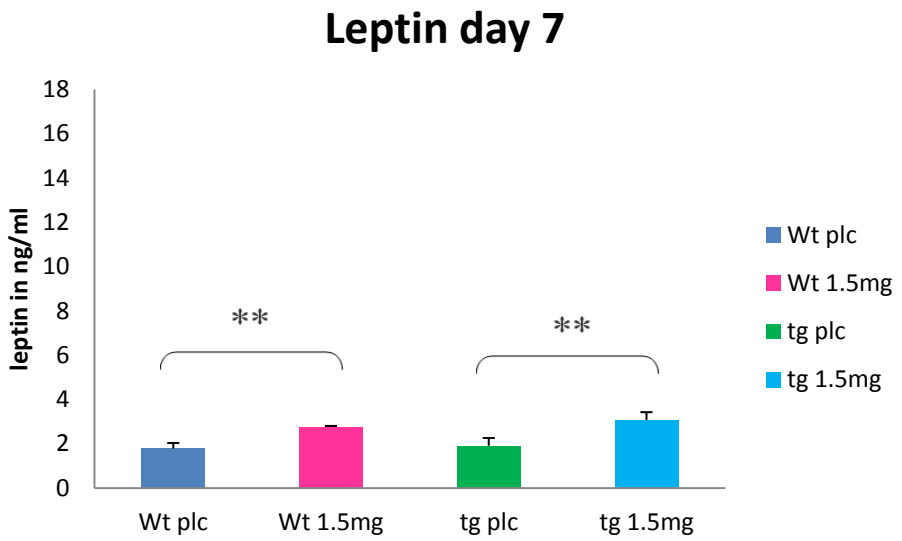

Figure 22: Serum leptin levels in ng/ml. Data from one experiment at day 7 and expressed as mean±SEM. WT–wild type, tg–transgenic * $p<0.05$, ** $p<0.01$, WT plc n=6 WT 1.5mg n=6, tg plc n=6, tg 1.5mg n=6

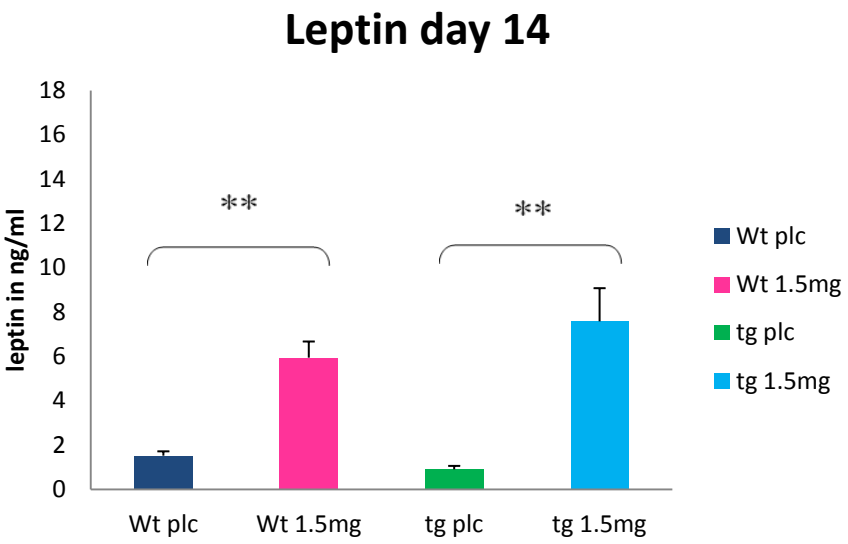

Figure 23: Serum leptin levels in ng/ml. Data from one experiment at day 14 and expressed as mean±SEM. WT–wild type, tg–transgenic * p<0.05, ** p<0.01, WT plc n=6, WT 1.5mg n=6, tg plc n=6, tg 1.5mg n= 6

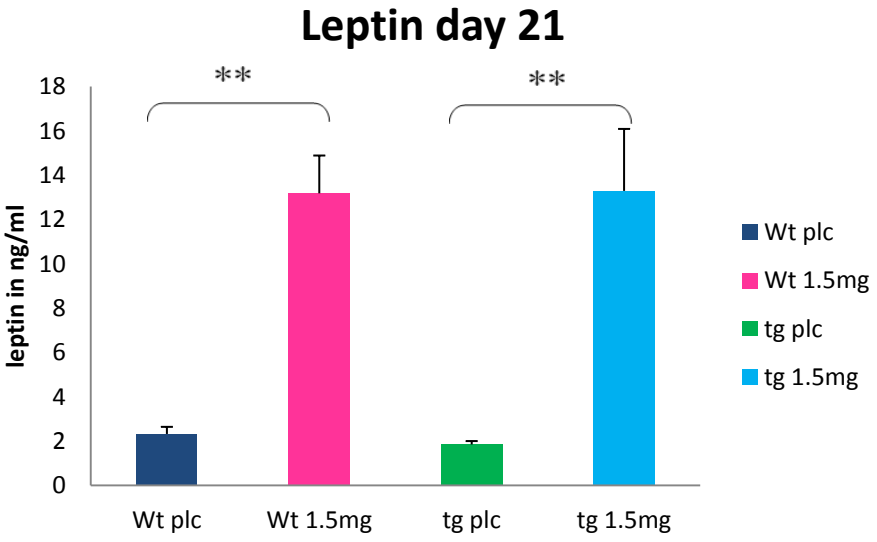

Figure 24: Serum leptin levels in ng/ml. Data from one experiment at day 21 and expressed as mean±SEM. WT–wild type, tg–transgenic ** p<0.01, WT plc n=6, WT 1.5mg n=6, tg plc n=6, tg 1.5mg n=6

5.3.6 Determination of serum adiponectin levels

In WT animals, GC treatment significantly increased adiponectin levels at day 7. The same significant pattern can be observed with tg animals. The adiponectin levels remained unchanged in the placebo groups. GC treatment significantly increased adiponectin levels compared to the placebo treated mice in both the WT and the tg groups (Figure 25).

By day 21, adiponectin levels in both the WT and tg GC treated mice were significantly higher than their respective placebo-treated controls and tg treated mice were also significantly higher than WT treated mice (Figure 26).

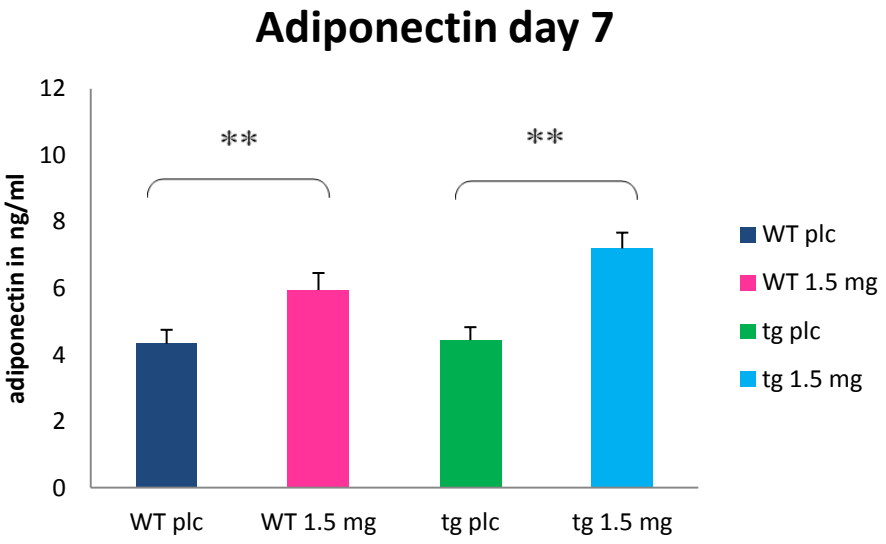

Figure 25: Serum adiponectin levels in ng/ml data from one experiment at day 7 and expressed as mean±SEM. WT–wild type, tg–transgenic ** $p<0.01$, WT plc n=8, WT 1.5mg n=8, tg plc n=8, tg 1.5mg n=8 (post hoc comparing WT 1.5mg and tg 1.5mg $p<0.09$)

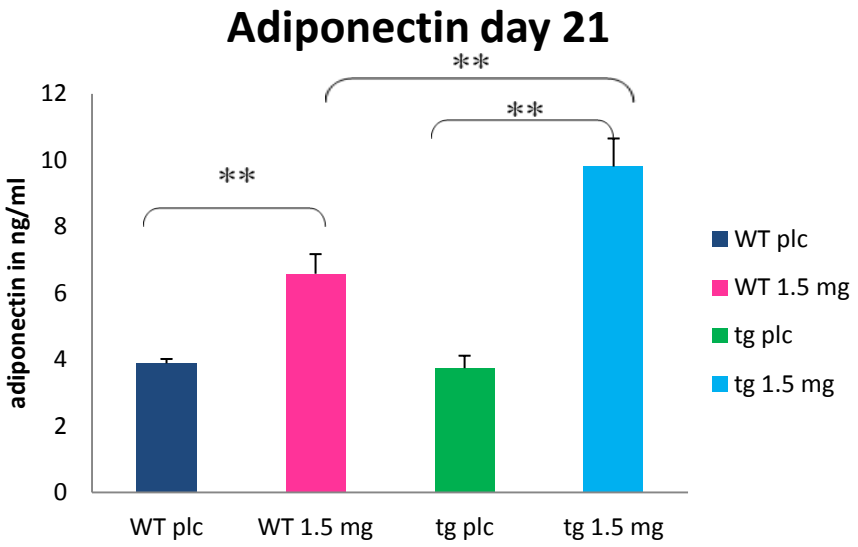

Figure 26: Serum adiponectin levels in ng/ml data from one experiment at day 21 and expressed as mean±SEM. WT–wild type, tg–transgenic * $p<0.05$, ** $p<0.01$, WT plc n=8,WT 1.5mg n=8, tg plc n=8, tg 1.5mg n=8

5.3.7 OCN measurements after pump implantation

As the osteoblast derived factor OCN has been identified as a potential factor to influence glucose metabolism, it was determined whether a substitution of the GC-diminished OCN concentration could reverse the adverse metabolic effects of GC. Therefore a replacement technique using osmotic micro–pumps was employed. The OCN protein utilised for this experiment was kindly donated by Dr. Caren Gundberg (Yale School of Medicine). The OCN protein was used in its purified form, therefore retaining the ability to be posttranslationally carboxylated.

Figure 27 displays OCN serum levels after six days of 1.5 mg corticosterone treatment compared to WT placebo and animals additionally treated with OCN. The OCN was administered via osmotic micro–pumps, which were implanted into the abdominal cavity. Six days after corticosterone application, OCN concentrations dropped significantly in animals treated with corticosterone compared to their placebo littermates. However, no significant distinction between the animals treated solely with corticosterone and the group receiving both corticosterone and OCN was detectable.

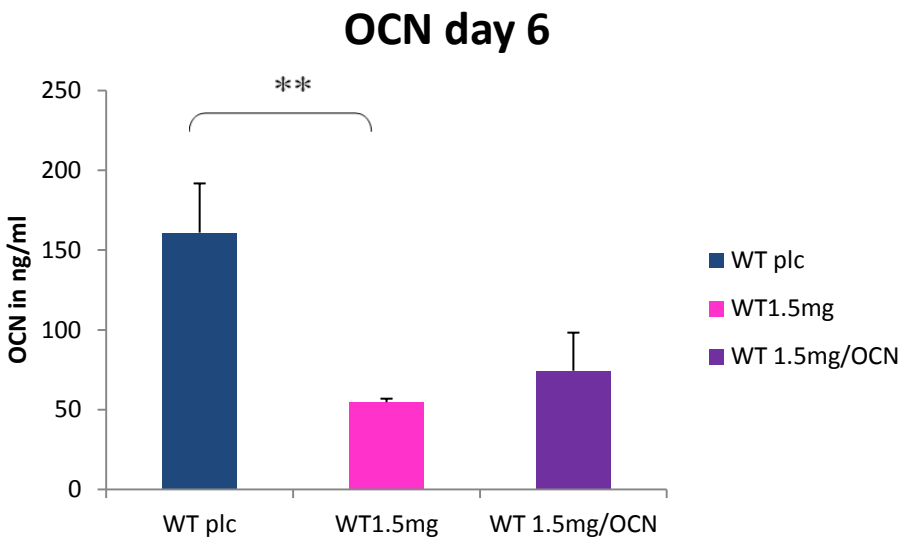

Figure 27: Osteocalcin levels measured at day 6 via IRMA. Measurements from one experiment and expressed as mean±SEM. WT–wild type ** p<0.01, WT plc n=3 , WT 1.5mg n=3, WT 1.5mg/OCN n=3

The ITT at day 6 was performed to test the influence of OCN on insulin resistance occurring due to corticosterone treatment. All animals received either sham surgery or implantation of corticosterone pellets as well as intraperitoneal implantation of the slow release pumps or intraperitoneal sham surgery. Animals receiving glucocorticoid treatment are insulin resistant compared to placebo WT animals. The intraperitoneal infusion of OCN altered the clearance of glucose from the blood stream after insulin injection. However, a significant difference between WT animals treated only with GC and animals treated with GC and OCN was only detectable at 60 minutes.

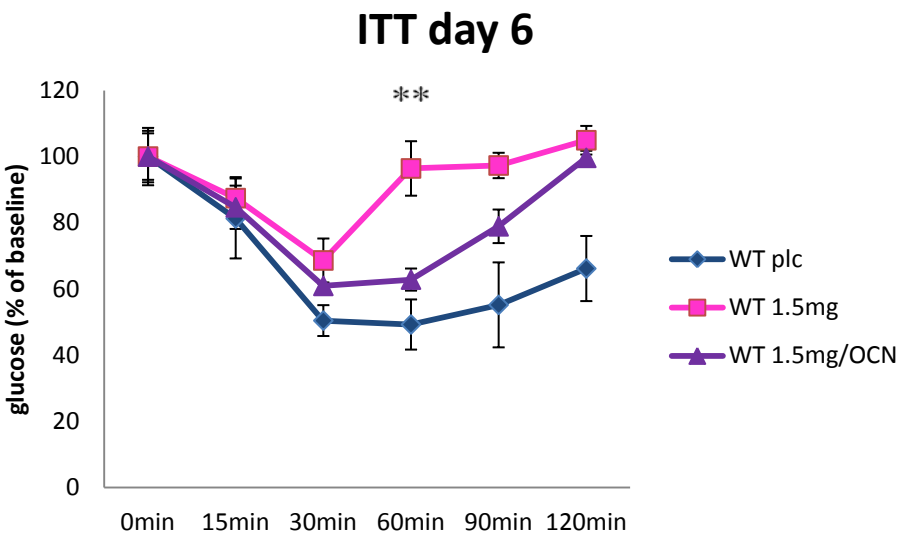

Figure 28: Insulin tolerance test day 6. Results are % of baseline glucose (pre-insulin administration) from one experiment and expressed as mean±SEM. WT–wild type, tg–transgenic.
* $p<0.05$, WT plc n=3, WT 1.5mg n=3, WT 1.5mg/OCN n=3

5.3.8 Cloning of the OCN construct into the pLIVE vector

Due to the insufficiency of the exogenous delivery method via osmotic micro–pumps to obtain high and sustained OCN levels, it was decided to employ a model of heterotopic expression. Therefore the technique of gene therapy using a vector comprising an albumin promoter and the OCN construct was employed.

To confirm the success of the cloning process and the accomplished ligation between OCN and the commercially obtained albumin vector pLIVE, a 2% agarose gel was run. A DNA molecular weight marker (HyperLadder II, Bioline GmbH, Biotechnologiepark TGZ 2, Luckenwalde, Germany) was incorporated to easily determine the band size of the cloning product. Each band corresponds to a precise quantity of DNA and therefore allows identification of the ligated product.

The gel in figure 29 displays the accurate product in column three from the left, which can be evaluated when the correct band size is known (OCN 500 bp, pLIVE 2500bp).

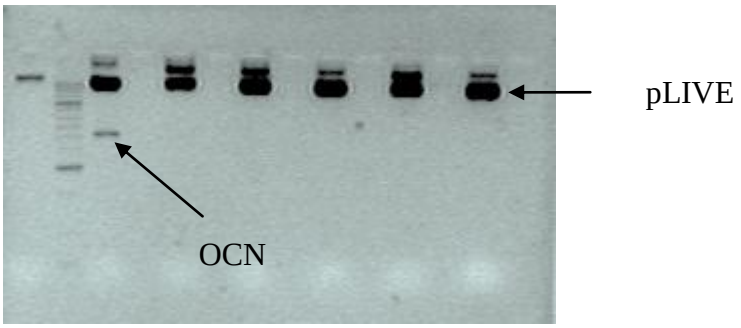

Figure 29: 2% Agarose gel, incorporating a DNA ladder left, OCN 500 bp, pLIVE 2500bp

5.3.9 Transfections via hydrodynamic tail vein injection (hTVI)

YFP Transfection to establish hTVI method

In an attempt to provide sustained OCN concentrations, the OCN construct was introduced into the hepatocyte via hydrodynamic tail vein injection (hTVI). This method needed to be established in our laboratory. While cloning the OCN construct into a commercial albumin promoter to gain a sustained long-term (at least four weeks) non-viral DNA plasmid for transfection, the method of hTVI was initially trialed with a different compound. Using a yellow fluorescent protein within a CMV promoter (kindly provided by Dr. Graham Robertson), the hTVI was performed on five mice to test and establish the method of hydrodynamic tail vein injection. The liver was eviscerated 20 hours after the hTVI and frozen sections were cut to determine the success of the transfection (Figures 30, 31).

The transfection was successfully established via hTVI. Protein expression was detected within the hepatocytes following hTVI after 20 hours using a standard light microscope and a yellow fluorescent filter. The detection was performed to prove the transfection of the hepatocytes with the YFP-marked CMV promoter. The central veins are marked due to their significance for the methodical distribution of the promoter via physical forces.

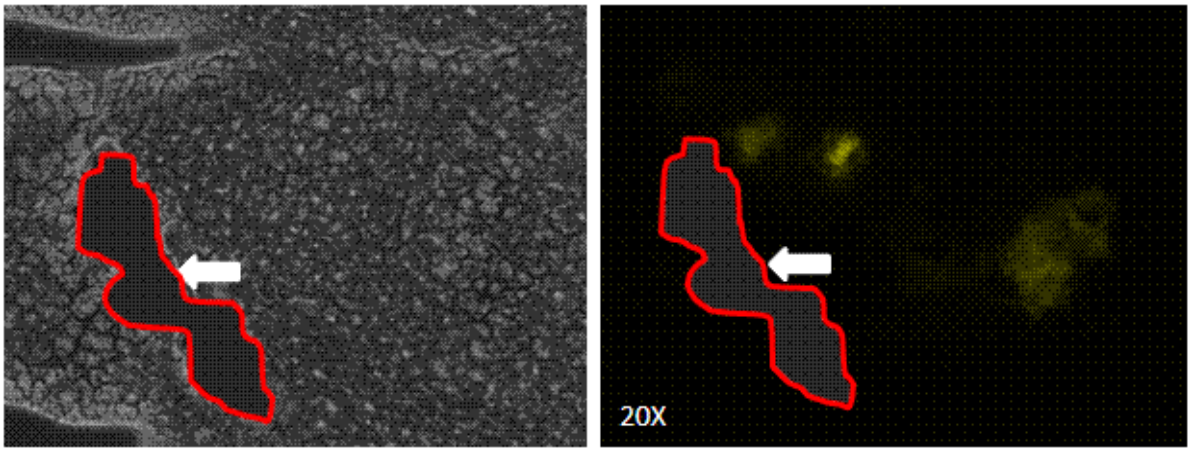

Figure 30: Frozen liver sections, Left: Standard light microscope images. Right: application of yellow fluorescent filter. Arrows and red marked areas indicate central veins of hepatic lobules. Magnification of both images x20

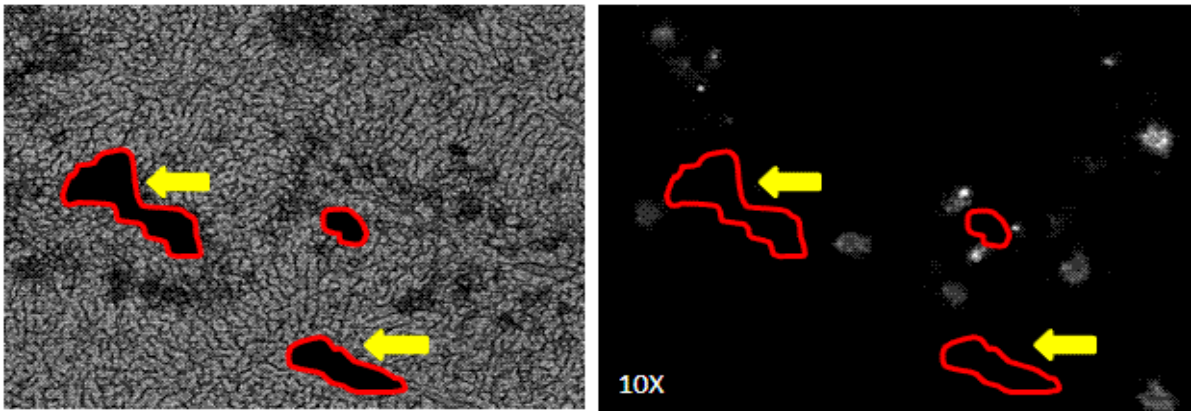

Figure 31: Frozen liver sections, Left: Standard light microscope images. Right: application of yellow fluorescent filter. Arrows and red marked areas indicate central veins of hepatic lobules. Magnification of both images x10

5.3.10 ITTs performed after hTVI with OCN in pLIVE

The ITT performed on day 7 after 1.5 mg corticosterone pellet implantation on day 0 and hTVI on day -7 shows a response to insulin injection in all animals. All three experimental groups received hTVI on day -7 either containing the OCN pLIVE vector or pLIVE in Trans IT delivery solution. Within the first 60 minutes no significant difference between the two groups

was detected. However at 90 min (p≤ 0.0427) and at 120 min (p≤ 0.0131) wild–type animals treated with corticosterone presented higher glucose levels compared to non treated WT animals.

Seven days later, at day 14 no significant distinction could be identified between the two groups. At day 21 however, animals receiving the OCN DNA injection via hTVI reacted with a decline in glucose levels after insulin injection. Unlike corticosterone treated WT animals, which were not injected with OCN DNA, they remained sensitive to insulin.

A significant difference between the two groups was found at 60 (p< 0.036), 90 (p< 0.0008).

The data obtained from the ITT performed at day 7 show significant difference in glucose elimination after insulin injections comparing placebo animals with GC treated animals. However, unlike animals treated only with corticosterone, mice receiving GC treatment and OCN hTVIs display an amended response to insulin but only at 30 minutes a significant difference was detected.

At day 14 a less pronounced but not significant insulin resistance was found in animals receiving OCN compared to treated WT mice, whereas at day 21 animals that received OCN and corticosterone were more insulin-sensitive compared to treated mice at 30 and 60 minutes.

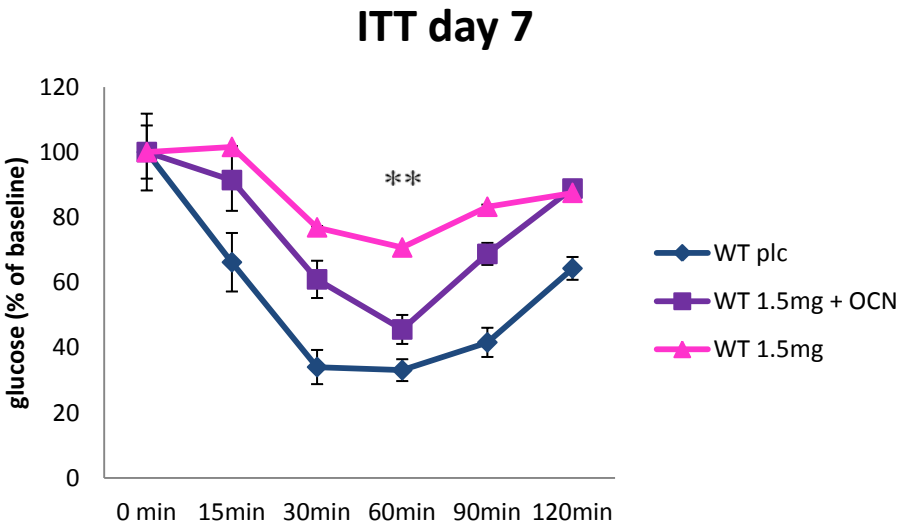

Figure 32: Insulin tolerance test day 7 of animals, which received OCN hTVI and corticosterone 1.5mg. Results are expressed as % of baseline glucose (pre-insulin administration) from two experiment and expressed as mean±SEM. WT–wild type ** p<0.01, n=4 per group

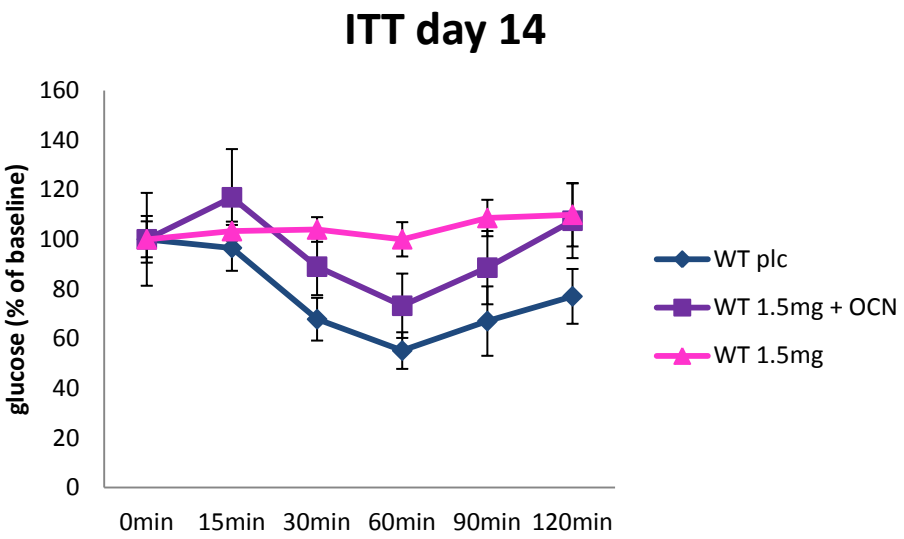

Figure 33: Insulin tolerance test day 14 of animals, which received OCN hTVI and corticosterone 1.5mg. Results are expressed as % of baseline glucose (pre-insulin administration) from two experiment and expressed as mean±SEM. WT–wild type n=4 per group

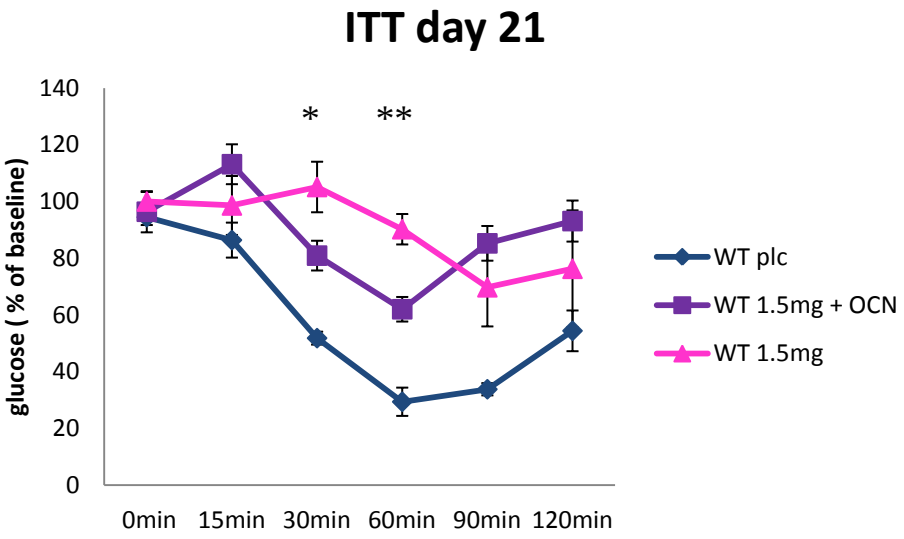

Figure 34: Insulin tolerance test day 21of animals, which received OCN hTVI and corticosterone 1.5mg. Results are expressed as % of baseline glucose (pre-insulin administration) from two experiment and expressed as mean±SEM. WT–wild type *p<0.05, **p<0.01 n=4 per group

6 Discussion

6.1 Discussion of methods

Fundamental to this investigation was the sustained administration of exogenous corticosterone. This was achieved via the application of corticosterone pellets and corticosterone levels quantified using mass spectrometry. The animals treated with 1.5mg corticosterone showed a 5-fold increase in corticosterone levels within the first 24 hours compared to their placebo-treated littermates. Corticosterone levels returned to baseline after 7 days, contradicting the company's findings and instructions for 21–day release pellets (Figure 9). Consequently, 1.5mg corticosterone pellets were renewed every 7 days to maintain pharmacological levels of corticosterone. This supports findings previously obtained by this laboratory (Herrmann, Henneicke et al. 2009).

The Col2.3-11ßHSD2 tg mouse model, in which GC signalling was exclusively disrupted in mature osteoblasts and osteocytes, was originally developed to investigate the role of endogenous GC effects on osteoblasts. Nevertheless, this mouse model lends itself also to examine the role of exogenous GC on osteoblastic function, shedding further light on the development of glucocorticoid-induced osteoporosis and determining the physiological effect of endogenous GC in osteoblast differentiation and function during fracture repair.

While working on these projects it became apparent that compared to their WT littermates, tg mice gained less fat tissue when exposed to exogenous GC. This posed the question whether the well-known effects of GC on fuel metabolism, such as fat accrual, insulin resistance and glucose intolerance, are somehow linked to the osteoblast.

The Col2.3-11ßHSD2 tg mouse model was employed to investigate whether the effects of GC on fuel metabolism were due to their actions on osteoblasts. While this model is well suited to study osteoblast-specific effects of exogenous GC, there are also some limitations that require consideration. Thus, the disruption of GC signalling is achieved through enzymatic inactivation of corticosterone to hydroxycorticosterone.

As mice were treated with relatively high pharmacological doses of corticosterone, the 11ßHSD2 enzyme could theoretically become overwhelmed, in particular during the first days of application when circulating GC concentrations are especially high. Consequently, the residual corticosterone may act on the GR, with apparent downstream transcriptional effects. This may explain why it was difficult to gain reproducible results for ITTs and blood glucose levels on days one to three of GC therapy.

It is also possible that the stress induced by surgery and the need for anaesthetics might have affected the mice and their metabolism within the first few hours of treatment. The mice were anaesthetised with ketamine and xylasine. Ketamine is known to affect blood glucose levels (Saha, Xia et al. 2005). Ketamine and xylasine increase blood glucose levels for at least 2 hours. It would be of interest to measure blood glucose levels on anaesthetised mice to verify this assumption. However, all animals were randomly allocated to the experimental groups and treated precisely according to protocol including sham surgeries. Accordingly, experimental stressors and environmental influences would be assumed to affect all animals equally.

Mouse models are widely used to investigate scientific questions. Especially if complex biological interactions are examined, *in vivo* studies are frequently performed using mice due to their short gestation period, low cost and the accessibility to mouse specific assays and experimental instruments. However, mice and humans are distinct species and extrapolation or even reproducibility of results between the two is often difficult or problematic. Consequently, results procured using mouse models pave the way for a better understanding of physiological, biochemical and molecular pathways, however, they cannot be completely translated onto humans.

Dysfunctions or diseases are often of multifactorial genesis and therefore impossible to research using only one mouse model. To investigate complex disorders, several different mouse models have to be employed to grasp every possible aspect of their origin. Results obtained in different studies have to be reviewed and only the consolidated aspects of several studies should be the foundation of new knowledge.

Furthermore, practical limitation such as small blood volumes and restricted blood collections often do not allow measuring of several parameters within the same experimental setup. The collection of complex data sets comprising several metabolic hormone measurements are difficult to obtain from the same animals. This can lead to variances within the measurements, since hormones are already of delicate regulation and only slight changes can affect their levels. Even with the greatest effort to keep experimental conditions identical, slight variations occur inbetween experimental setups leading to deviations within the results. However statistical analysis can account for most of them.

As the experimental procedures presented in this thesis were aimed at studying the role of hormones, it was of great importance to minimise hormonal fluctuations, therefore only male mice were employed in this study since female animals differ immensely in their hormonal makeup and regulation. Accordingly, the results gained can only allow conclusions about the functions within the male organism.

Unless stated otherwise, all blood collections, including ITTs and oGTTs were preceded by a fasting period of 6 hours (Andrikopoulos, Blair et al. 2008). However, it should be noted that food withdrawal is a significant stressor in mice which might affect the hypothalamic–pituitary–adrenal axis (HPA) and lead to increased corticosterone secretion, affecting the results, especially baseline glucose concentrations.

Blood collections were performed on several days throughout the experiments, by retro-orbital eye bleeds, to establish a coherent time line of metabolic changes. To avoid unnecessary stress, blood collections were spaced at least 72h apart to ensure complete recovery of the animals. While this also limited the number of potentially available blood samples and hence measurements, mice tolerated these interventions well and wound healing was rapid. All animals were observed closely to foresee any complications and to lessen physical stress.

We consistently observed that high GC levels lead to a suppression of serum OCN levels (Figure 10). Based on the hypothesis that GC treatment alters osteoblast-derived signals such as OCN, we suspected that the suppression in OCN level might contribute to the adverse effects of GC on fuel metabolism. We therefore aimed to sustain OCN delivery during GC therapy to determine if in fact this protein, when re-introduced into GC-treated mice, would prevent or at least ameliorate the metabolic effects observed following GC treatment in WT mice.

Thus far, the systemic introduction of exogenous OCN had proven very difficult. Prior experiments attempting to achieve sustained delivery of OCN via s.c. implanted micro-osmotic pumps failed to increase serum OCN concentrations, nor did they result in any metabolic changes. Similarly negative results, however, were seen with intraperitoneal micro-pump implantation, this was thought to be due to the degradation of OCN in the pump itself. Repeated OCN s.c. injections and OCN pellet implants showed to be ineffective as well.

The most promising approach was therefore to introduce OCN via gene therapy, using hydrodynamic tail vein injection, which results in the retrograde transfection of mouse hepatocytes. With this technique, hepatocytes organised around the central vein of the hepatic lobule are predominantly transfected. This pattern of distribution is due to a retrograde blood flow in the liver after the voluminous injection into the tail vein. The large liquid volume containing the DNA is drained by the inferior cava vein, and the blood volume drains into the hepatic veins and forces its way into the hepatocytes (Zhang, Basinski et al. 1997; Budker, Subbotin et al. 2006).

The inversion of the blood flow lasts approximately five minutes and is continued by a short period of haemodynamic stasis until the heart restores normal blood flow. The phase in which blood flow stagnates seems important for the transfer of DNA into the hepatocytes. It is unclear exactly how the DNA becomes transfected into the cells. Proposed mechanisms include sheer

physical force, endocytosis (due to the detection of intracellular vesicles after hTVI) or a formation of small pores within the cellular membrane (Budker, Subbotin et al. 2006). The innovative approach using gene therapy was then employed to introduce an OCN construct bound to a liver-specific promoter into hepatocytes, with the expectation that these cells would express and secrete OCN into the circulation (Pergolizzi, Jin et al. 2006).

Previous reports showed sustained high levels of secreted alkaline phosphatase from the hepatocytes for one year following hTVI delivery of the pertinent plasmid, equivalent to high levels gained from a cytomegalovirus promoter on day one (Wooddell, Reppen et al. 2008). OCN was successfully cloned into the pLIVE vector, then confirmed and verified using a 2% agarose gel in this laboratory and also confirmed by commercial sequencing. GFP was used as a control.

The problem of self-ligation was addressed by adding Antarctic phosphatase to the mixture. Antarctic phosphatase catalyzes the removal of 5´ phosphate groups from DNA and RNA. Since phosphatase-treated fragments lack the 5´ phosphoryl terminals as required by ligases, they cannot self-ligate (Sambrook 1989).

Before the plasmid DNA was introduced into the mice, it had to be purified using an endotoxin-free megapreparation kit in order to decrease any unwanted immune response or rejection of the plasmid DNA. To satisfactorily enhance transfection rates and reduce recovery time post injection, the endoxin-free *Trans*IT-QR (Quick Recovery) Hydrodynamic Delivery Solution was used.

The pilot study using WT animals that were injected with YFP integrated in a CMV promoter was performed to optimise the technique of hTVI. The frozen liver sections were examined 20 hours post injections and sufficient expression within the hepatocytes was detected within all animals. As expected the hepatocytes around the central veins of the hepatic lobules were primarily transfected, since the plasmid DNA reaches the cell plasma due to high pressure back flow of the inferior cava vein into the hepatic veins.

The procedure had to be done precisely and swiftly to prevent insufficient delivery and ensure maximal transfection rates. Insufficient injections and subsequent loss of delivery solution, which contained the plasmid DNA, could only be prevented with practice and experience. Therefore, the pilot study using YFP was performed to test the effectiveness of the procedure and to detect actual protein synthesis and expression within the hepatocytes surrounding the hepatic veins for verification. The procedure was performed without anaesthesia as it was necessary to maintain sufficient blood pressure and heart rate to ensure physiological responsiveness of the cardiovascular system to the increased blood volume. While animals always fully recovered, the procedure may have caused additional stress, although for both, control and intervention groups.

6.2 Discussion of results

The adverse effects of exogenous high-dose glucocorticoid treatment on systemic fuel metabolism have been reported and studied for years. This work provides insight into an innovative approach that the skeleton, specifically the osteoblast, might be involved in the onset of glucocorticoid-induced metabolic disorders. These findings provide a foundation for a deeper comprehension of the pathophysiology of glucocorticoid-induced insulin resistance, glucose intolerance and fat accrual. These symptoms are well known factors of diabetes and unwanted side effects of GC treatment, therefore of clinical relevance to a large number of patients worldwide.

Prerequisite for this study was the Col2.3-11β-HSD2 transgenic mouse model in which the enzyme 11β–hydroxysteroid dehydrogenase type 2 inactivates corticosterone, the active glucocorticoid in mice, at the pre-receptor level. Targeted overexpression of this enzyme results in a disruption of normal glucocorticoid signalling exclusively in mature osteoblasts and osteocytes (Sher, Woitge et al. 2004). The skeleton of tg mice is protected from the deleterious effects associated with long–term glucocorticoid treatment (Henneicke, Herrmann et al. 2011).

Now, the findings demonstrated in this thesis show that disrupted GC signalling averts or possibly even reverses metabolic side effects of exogenous high dose GC treatment.

Results of ITTs and oGTTs performed following exposure to exogenous GC demonstrated increasingly abnormal findings for both insulin sensitivity and glucose handling. The ITT performed on day 1 post pellet implantation is representative of the metabolic changes these mice experienced during the first 24 hours of GC exposure. During this time, mice remained completely insulin sensitive, however, tg mice treated with GC exhibited an initial decrease in blood glucose levels, which then increased to concentrations higher than their baseline levels at the 90 minute time point and continued to increase up to the last time point tested (120 minutes).

The increase in blood glucose levels following the insulin-induced decrease in blood glucose concentration could be due to a faster reaction of the metabolic system to initiate gluconeogenesis in order to restore physiological blood glucose levels. It should also be noted that the transgenic mice were smaller in total body weight on average than their WT littermates, which may also influence the initial glucose levels after insulin administration even when insulin injections were adjusted for body weight (Sher, Woitge et al. 2004).

By day 7 WT animals receiving 1.5mg corticosterone were less responsive to insulin, resulting in higher blood glucose levels when injected with insulin compared to all other groups, indicating the onset of insulin resistance in these animals. This implies that exposure to corticosterone over a period of 7 days results in beginning insulin resistance in mice not protected by the

expression of 11ß-HSD2 exclusively in the osteoblast. It is likely that GC leads to an inhibition of normal peripheral glucose handling by interfering with the insulin signalling cascade; however, the exact mechanism is not completely understood. It has been suggested that this involves downregulation of insulin receptor substrate (IRS) 1 and IRS 2, phosphoinositide 3 kinase (PI3K) and reduction of Akt phosphorylation resulting in a decrease of GLUT 4 translocation in adipose and muscle tissue (Buren, Liu et al. 2002; Brandon, Hoy et al. 2011).

Insulin resistance was also observed at days 14 and 21 in WT treated mice. Transgenic mice treated with GC also became less sensitive to insulin over time compared to their response to insulin on day 7. However, they were still significantly more insulin responsive compared to their GC-treated WT littermates.

To further confirm these findings, oGTTs were performed in all experimental groups. This procedure relies on glucose uptake by the intestine as well as upregulation or downregulation of metabolic liver enzymes for biochemical processing of glucose.

The potassium channel-dependent response of the pancreatic ß-cell to high glucose concentrations and the release of endogenous insulin are also stimulated by oral glucose administration. Compared to ITTs, which mainly test the peripheral tissue sensitivity to insulin, oGTTs involve several metabolic systems upstream of the endogenous pancreatic insulin release and subsequent glucose assimilation of designated tissues.

The incretin system including glucagon like peptide (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) is one of the affected physiological response systems (van Raalte, van Genugten et al. 2011). L-cells dispersed throughout the small intestine secrete incretins following food intake. GLP-1 is responsible for the augmentation of glucose mediated insulin secretion, enhances insulin gene expression and ß-cell growth and differentiation. GLP-1 normalises fasting hyperglycemia, which is thought to be partly due to reduced glucagon secretion from the α-cells of the pancreas and subsequently reduces hepatic gluconeogenesis (Chia and Egan 2008). It would be of great interest to study the metabolic interaction between OCN and the incretins GLP-1 and GIP since the oGTTs showed significant differences between the two genotypes after 7 days of GC treatment.

By day 3, all four groups showed normal clearance of an orally administered glucose bolus, suggesting that glucose resorption from the intestine and the pancreatic tissue as well as intestinal incretin response to absorbed glucose are intact. Furthermore, it can be noted that peripheral glucose uptake remained unaffected in all animals at day 3.

Glucose tolerance became significantly impaired in GC-treated WT animals at days 7 and 15. In contrast, GC-treated tg animals stayed responsive to glucose at all examined time-points.

These findings suggest that the tg mice with osteoblast targeted disruption of GC signalling are partly protected from the metabolic effects of GC on glucose metabolism.

Glucocorticoids are also known to affect adipose tissue. Indeed, the difference in body fat accrual between the two genotypes led to the initial hypothesis that osteoblasts are involved in mediating the effects of GC on fuel metabolism. Glucocorticoids promote the differentiation of preadipocytes to mature adipocytes and through this effect can cause adipose tissue hyperplasia. This process particularly affects abdominal fat pads (Vegiopoulos and Herzig 2007).

In contrast, peripheral fat stores undergo lipolysis due to an increase in hormone sensitive lipases and a reduction in lipoprotein lipase activity. Corresponding with this fact, WT mice treated with GC had significantly larger gonadal fat pads compared to both the tg GC-treated animals and the placebo-treated animals. Analysis of body composition by DEXA confirmed the dissection results, demonstrating a significant increase in both absolute and relative fat mass after four weeks of corticosterone treatment.

These observations indicate that compared to WT mice fat accrual, insulin resistance and glucose intolerance were significantly less pronounced or even undetectable in tg mice at several examined time points leading to the assumption that a factor, or several factors, originating from osteoblasts and modified by glucocorticoid signalling in these very cells, are at least in part responsible for the adverse effects of glucocorticoids on systemic energy metabolism and body composition. Current reports provide evidence that low serum osteocalcin levels are often found in patients with metabolic syndrome (Oosterwerff, van Schoor et al. 2012).

Several other studies have postulated that osteoblasts and osteoclasts are important key players in the physiological control of murine energy metabolism and that OCN acting as a hormone might be of significance (Lee, Sowa et al. 2007; Ferron, Hinoi et al. 2008; Ferron, Wei et al. 2010; Fulzele, Riddle et al. 2010).

As expected and reported previously by Henneicke and colleagues (2011) and also shown in this study, serum OCN concentrations decreased significantly following corticosterone treatment of the animals (Henneicke, Herrmann et al. 2011).

However, pre-receptor inactivation of corticosterone in osteoblasts significantly attenuates the typical inhibition of osteocalcin synthesis and secretion observed after glucocorticoid administration. Since the OCN serum levels in tg mice were significantly higher compared to their corticosterone treated WT littermates (Figure 10), the role of OCN became the focus of further research.

A group of scientists have studied the reciprocal regulation of bone and energy metabolism orchestrated by leptin and osteocalcin over the past decade (Confavreux, Levine et al. 2009). Since starting to mark the skeleton as a participant in the regulation of fuel metabolism, they

propose a bone pancreas endocrine loop that possibly allows the metabolism to use energy resources more efficiently.

Their scientific process started with the discovery of a connection between fuel metabolism, the brain and bone mass (Lee, Sowa et al. 2007). Throughout their studies they employed several animal models. The identification of the *Esp* gene expressed in osteoblasts, which encodes for an intracellular tyrosine phosphatase (OST–PTP), provided the basis for the development of a gain of function model utilised to investigate glucose metabolism. Mice with intact *Esp* gene function and therefore adequate OST–PTP production were found to display impaired glucose metabolism (Lee, Sowa et al. 2007). Accordingly, Esp -/- mice exhibit an improvement of glucose metabolism. Several observations made by Karsenty et al. led to the conclusion that osteocalcin is involved in the regulation of glucose metabolism. In contrast to Esp -/- mice, osteocalcin -/- mice, a loss of function model, show glucose intolerance (Lee, Sowa et al. 2007). These findings can be compared to corticosterone treated WT mice examined in this study since GC administration leads to diminished OCN expression and secretion. Both GC treated WT mice and osteocalcin -/- show impaired glucose handling.

Karsenty et al. posed the question how an intracellular enzyme could affect the activity of an extracellular protein such as OCN. They found a connection between the dephosphorylation of the osteoblastic insulin receptors by the OST–PTP which results in an inhibition of the insulin signalling pathway and the activity rate of OCN. Mice lacking the insulin receptor (InsR osb -/-) solely in osteoblasts are glucose intolerant as are OCN -/- mice and GC treated WT mice.

An additional mouse model (mice lacking one allele of the insulin receptor in osteoblasts and one allele of OCN) was used to confirm the association between insulin signalling in osteoblasts and OCN activity (Ferron, Wei et al. 2010). It was discovered that insulin regulates bone resorption and leads to an acidic environment needed for the decarboxylation of OCN (Ferron, Wei et al. 2010; Ferron, Wei et al. 2010; Fulzele, Riddle et al. 2010). Undercarboxylated OCN is described as the biologically active form, which favors insulin secretion and sensitivity (Ferron, Hinoi et al. 2008).

Ferron et al. found that osteocalcin infusions via implanted osmotic pumps lead to ß-cell proliferation and upregulates insulin secretion. Insulin sensitivity increased in WT mice receiving osteocalcin administered via the implanted pumps. Insulin sensitivity was evaluated by performing ITTs (Ferron, Hinoi et al. 2008).

However, these findings could not be repeated in this experimental setup, also when using OCN filled osmotic pumps by Alzet (Figure 27 and 28) and after failing to introduce OCN into the circulation via injections. Ferron et al. implanted the osmotic pumps subcutaneously, unlike

the intraperitoneal implantation used in this experimental setting since the subcutaneous implanted pumps did not elevate OCN serum concentrations (results not shown). Osteocalcin levels obtained from the delivery solution after recovery of the pumps from the sacrificed mice suggested a degradation of osteocalcin within the pump itself.

Ferron et al. did not report OCN serum concentration in their paper, a fact conspicuous in most of the studies performed by Karsenty et al. Apart from Hinoi et al. 2009, who presented OCN levels in WT, ob/ob, Adrß2 osb -/- and control mice (Ducy, Desbois et al. 1996; Lee, Sowa et al. 2007; Ferron, Hinoi et al. 2008; Hinoi, Gao et al. 2009; Ferron, Wei et al. 2010; Ferron, Wei et al. 2010; Ferron, McKee et al. 2011). Unfortunately, the OCN levels are only presented as OCN in percent and the y–scale starts at 90% leaving the question regarding OCN percentage of the other two experimental unanswered, which makes it difficult to correctly interpret the graph. Nonetheless, Ferron et al. showed improved insulin sensitivity in animals implanted with OCN delivering micro-pumps. In accordance with increased insulin sensitivity, gene expression of adiponectin, discussed as an insulin sensitising hormone, was shown to be elevated in these mice, whereas adiponectin expression in osteocalcin -/- mice was down–regulated (Ferron, Hinoi et al. 2008). Adiponectin serum concentrations were measured at day 7 and day 21 in this study. An increase in adiponectin levels in animals treated with GC was observed.

On both days, GC-treated tg animals had higher concentrations of adiponectin compared to their GC-treated WT littermates (Figure 25 and 26). These results contradict findings by others reporting that GC treatment inhibits adiponectin expression and secretion, resulting in lower adiponectin plasma levels (Fallo, Scarda et al. 2004; Degawa-Yamauchi, Moss et al. 2005; Shi, Du et al. 2010).

This study found that adiponectin concentrations were raised in animals with higher insulin levels at day 7 also incompatible with findings by others who found that GC-induced hyperinsulinemia causes a decline in adiponectin plasma concentrations (Murdolo, Hammarstedt et al. 2009; Drapeau, Doucet et al. 2011; Sukumaran, Jusko et al. 2011).

At days 7 and 21 tg GC-treated animals present higher concentrations of adiponectin compared to their WT GC-treated littermates. The results contradict results published by Fallo et al., Shi et al. and Degawa-Yamauchi et al., who report that GC treatment inhibits adiponectin expression and secretion resulting in lower plasma levels (Fallo, Scarda et al. 2004; Degawa-Yamauchi, Moss et al. 2005; Shi, Du et al. 2010). Many clinical studies showed decreased adiponectin levels in obese patients, in patients with diabetes and cardiovascular diseases accompanied by low osteocalcin levels, again linking these two parameters (Kanazawa, Yamaguchi et al. 2008; Kanazawa, Yamaguchi et al. 2009; Kanazawa, Yamaguchi et al. 2011).

Since GC-treated tg animals display higher adiponectin levels compared to their GC-treated WT littermates, a connection to OCN can be proposed as GC-treated tg have sustained OCN levels, and a dose dependence between OCN and adiponectin could be assumed as suggested by Ferron at al. (Ferron, Hinoi et al. 2008). Since adiponectin is considered to enhance insulin sensitivity of adipose tissue and skeletal muscle, the raised levels in corticosterone treated tg mice could be responsible for the lesser degree of insulin resistance in these animals. However, placebo treated animals remained insulin sensitive at all time points and exhibit low adiponectin concentrations.

Interestingly, adiponectin has been reported to be involved in osteoblastic differentiation which in turn could be considered as a feedback loop resulting in higher OCN concentrations which would favour the adiponectin expression and secretion by adipocytes (Ferron, Hinoi et al. 2008; Lee, Kim et al. 2009).

Next to adiponectin the maintenance of glucose homeostasis and energy balance is regulated by several other adipokines one of which was also addressed in this work. Leptin is a hormone produced by adipose tissue known to regulate energy metabolism. Leptin levels are known to be proportional to fat mass (Schwartz, Baskin et al. 1996; Baudrand, Campino et al. 2011). It was therefore of interest to measure the concentration of this adipokine in the four animal groups investigated in this study. Over a time period of 21 days, leptin concentrations increased in all animals treated with GC, compared to their placebo-treated littermates. However, no significant difference was found between the two genotypes, suggesting that the known effects of GC on leptin are independent of osteoblast function (Kershaw, Morton et al. 2005; Cartmill, Thompson et al. 2006; Nishii, Takasu et al. 2006; Cimmino, Andraghetti et al. 2010).

While measuring serum insulin levels, it became apparent that GC treatment stimulated insulin production, but no significant differences between GC-treated WT and tg groups were detected by ANOVA at day 7. In keeping with the published literature, these results demonstrate that the development of hyperinsulinaemia is not influenced by genotype but is stimulated by the administration of GC. However, it would be interesting to measure insulin over a longer period of time, to detect possible changes, if any.

To further investigate the hypothesis it was of great importance to introduce exogenous OCN into the bloodstream of mice receiving corticosterone treatment.

After the hTVI method was successfully established, mice were injected with the custom made OCN construct and a second set of mice were handled as a control group injected with pLIVE in Trans IT delivery solution. The animals received hTVI with the DNA construct according to their experimental group and were implanted with 1.5 mg corticosterone pellets seven

days later. The ITTs performed on these animals showed improved insulin sensitivity in GC-treated animals harbouring the OCN construct, compared to GC-treated animals that received placebo. This effect was most pronounced at day 21. These findings agree with the hypothesis, which suggests that OCN acts as a hormone secreted by the osteoblast, and plays a role in the onset and development of GC-induced insulin resistance. Evidence for the therapeutic potential of OCN administration was also again reported by Ferron et al. this year (Ferron, McKee et al. 2012).

6.3 Future Directions

At all times left and right tibia, fat pads, livers, muscle tissue and blood samples were extracted at sacrifice to measure hormone levels and mRNA expression and qRT PCR. These experiments still need to be completed to determine the effects of the transgene in the osteoblast and the heterotypic expression of OCN in the liver on GC-induced metabolic disturbances.

Further studies are also required in order to clarify the molecular mechanisms by which the osteoblast mediates metabolic changes to the body, when treated with glucocorticoids. A gene array using Affymetrix GeneChip technology would be extremely useful in determining differences in gene expression between WT and tg mice. Micro arrays are a very effective method to examine the complex changes in gene expression within bone cells.

It was hypothesised that sustained high OCN exposure would ameliorate the adverse metabolic effects of GC treatment in WT treated animals. Thus far, improved insulin responsiveness in animals treated with OCN was achieved, however these preliminary data are only the beginning and confirmation of the role of OCN as a hormone involved in the regulation of energy metabolism is still necessary. It would also be of interest to show a reduction of fat accrual and a decrease of cholesterol levels. Furthermore, insulin levels at more time points to better understand the dynamics of the changes induced by GC and the possible differences between the two genotypes would be useful in this context. The pancreatic ß–cell function would offer a further site of investigation. The measurements of serum triglycerides and cholesterol levels could be another interesting assessment since these markers are elevated in most obese patients or patients with metabolic syndrome as well as in patients treated with GC (Anagnostis, Athyros et al. 2009; Baudrand, Campino et al. 2011). Osteocalcin serum concentrations were shown to be negatively

correlated with triglyceride levels in patients suffering from type 2 diabetes (Sarkar and Choudhury).

The incretin system is known for its antidiabetic actions and has now been proposed to affect bone turnover, again linking energy metabolism to bone metabolism (Nuche-Berenguer, Moreno et al. 2010; van Raalte, van Genugten et al. 2011). Due to these new developments it would be useful to measure GLP-1 and GIP concentrations, especially since OCN concentrations were raised after administration of the incretin mimetic, Exenatide, in insulin-resistant T2DM rats (Nuche-Berenguer, Moreno et al. 2010).

If successful, this will provide further evidence for the role of osteoblasts and osteoblastic derived products, specifically osteocalacin, as key players in the metabolic side effects seen following GC treatment. This may lead to future pharmacological solutions to minimise dramatic negative metabolic changes, when patients have to be treated with long term, high dosage GC.

7 Summary

Numerous severe side effects of GC treatment have been recognised for years. However, the precise molecular mechanisms through which GC exhibit their unwanted actions remain to be determined. More preclinical research is needed to further understand the molecular mechanisms and hormonal interactions leading to GC-induced side effects. One approach taken in this study was to examine the involvement of osteoblasts and their factors, with particular focus on OCN, in relation to metabolic adverse effects. This was based on recent findings by Karsenty et al. who suggest the skeleton to play a major role in the regulation of energy metabolism. The administration of exogenous GC profoundly suppresses osteoblast activity and function resulting in bone loss mostly due to less bone remodelling. Glucocorticoids also diminish the production and secretion of osteocalcin an osteoblastic peptide, which has been shown to be involved in the regulation of fuel metabolism via an endocrine pathway.

A well-established tg mouse model was employed in which intracellular GC-signalling was disrupted exclusively in mature osteoblasts and osteocytes through tg overexpression of 11 ß–hydroxysteroid dehydrogenase type 2 (11 ßHSD2) under the control of a collagen type Ia1 promoter (Col2.3–11ßHSD2). Insulin resistance and defective glucose metabolism was detected as early as seven days after the initial GC treatment in WT animals, though not in transgenic animals. Gonadal fat depots and total body fat mass determined at sacrifice showed a significant increase in GC-treated WT animals compared to their equally treated tg littermates ($p < 0.01$). Leptin levels were elevated by GC treatment in WT and tg mice, however they were unaffected by genotype at all time-points, displaying a well known GC action independent of osteoblast function.

Insulin concentrations were raised due to GC administration although not significantly different between WT and tg mice at day 7. Adiponectin levels were increased in all treated animals at all time-points, yet showed a significant increase at day 21 in tg GC-treated animals compared to their WT littermates. Osteocalcin concentrations were reduced by GC treatment as expected. These results support the hypothesis that the osteoblasts and their products are involved in the regulation of energy metabolism in animals treated with GC. It can be concluded that leptin is not affected by the osteoblast or its products, whereas adiponectin seems to be involved in the complex interaction between GC, osteocalcin and the onset of insulin resistance and glucose intolerance as well as fat accrual. To further examine the role of osteocalcin and confirm its in-

volvement in the complex interaction of metabolic players a separate method was established to re-introduce OCN into the circulation and therefore elevate OCN serum levels.

Hydrodynamic tail vein injection is a method through which plasmid DNA containing a gene of interest and a liver-specific promoter can be forced into hepatocytes and their intracellular enzymatic make-up used to produce and secrete OCN into the bloodstream. The method was set up using a yellow fluorescent protein (YFP) in a CMV promoter. The liver sections examined 20 hours after hTVI showed the desired perivenous accumulation of YFP, which was detected via flourescent light microscopy. ITTs performed on WT animals receiving the cloned OCN pLIVE construct showed ameliorated insulin sensitivity compared to WT GC-treated animals receiving the pLIVE control construct at days 7, 14 and 21.

Consequently, OCN could be shown to have positive systemic metabolic effects on insulin and glucose handling. Further evidence needs to be obtained to determine if the pharmacological elevation of OCN concentrations could be considered as a possible drug target to minimise or completely reverse the GC-induced side effects and therefore use the otherwise highly effective anti-inflammatory actions of GC to its full extend.

The application of OCN as a preventative drug to GC-induced adverse effects or as a possible treatment option for diabetic conditions would provide great relief to all patients suffering from the detrimental effects on health and lifestyle resulting in metabolic changes the body undergoes when treatment with GC becomes necessary or T2DM is apparent.

8 Zusammenfassung

Nach langjährigem erfolgreichen therapeutischem Einsatz von Glukokortikoiden (GC) zur Therapie von zahlreichen chronisch entzündlichen Krankheitsbildern, bleiben die vielen muskuloskeletalen und metabolischen Nebenwirkungen (NW) ein limitierender Faktor für eine suffiziente Langzeittherapie. Auch heute noch, sind nicht alle molekularen Mechanismen vollständig verstanden, durch welche GC zu unerwünschten NW führen.

GC supprimieren die Funktionalität von Osteoblasten und führen zu einer verminderten Sekretion von Osteocalcin. Möglicherweise beeinflusst das von den Osteoblasten produzierte Peptid die Regulation des Energiestoffwechsels über einen endokrinen Rückkopplungsmechanismus.

In dieser Studie wurde ein bewährtes Mausmodell genutzt, in welchem durch die transgene Überexpression des Enzyms 11ß–Hydroxysteroid Dehydrogenase Typ 2 (11ß-HSD2) unter der Kontrolle des Kollagen Typ 1 Promoters (Col2.3–11ß-HSD2) der Glukokortikoidsignalweg im Osteoblasten erfolgreich in vivo blockiert wurde. Die Behandlung mit 1.5mg Kortikosteron erfolgte an 8 Wochen alten männlichen transgenen (n=48) und Wildtyp Mäusen (n=48).

Es wurden zu verschiedenen Zeitpunkten Insulin Toleranz Tests und orale Glukose Toleranz Tests durchgeführt und verschiedene metabolisch aktive Hormone wie Insulin, Leptin, Adiponectin und Osteocalcin (OCN) gemessen, sowie die Ganzkörperfettmasse der Tiere nach 4 Wochen.

Während der ersten 7 Tage konnte eine beginnende Insulinresistenz und eine gestörte Glukosetoleranz in den behandelten Wildtyp Mäusen nachgewiesen werden. Die transgenen Tiere hingegen blieben insulinsensitiv und waren in der Lage Glukose nach oraler Applikation zeitgerecht aus dem Blutkreislauf zu eliminieren.

Nach 4 Wochen hochdosierter Kortikosterontherapie konnte bei den Wildtyp Mäusen ein signifikant vergrößertes viszerales Fettdepot und eine erhöhte Gesamtfettmasse im Vergleich zu den transgenen Mäusen festgestellt werden ($p<0.01$).

Die Leptin Blutkonzentrationen wurden durch die Gabe von Glukokortikoiden stark erhöht, allerdings konnte kein signifikanter Unterschied an allen untersuchten Zeitpunkten zwischen den Genotypen erkannt werden.

Weiterhin war in allen mit Kortikosteron therapierten Tieren die Adiponectinkonzentrationen ebenfalls erhöht. An Tag 21 wurde ein signifikanter Unterschied der Adiponectinkonzentrationen zwischen transgenen und Wildtyp Mäusen gemessen ($p<0.05$). Wie erwartet führte die Verabreichung von GC zu einer starken Verminderung der Osteocalcinkonzentration.

Insgesamt weisen die vorliegenden Ergebnisse daraufhin, dass die auftretenden metabolischen NW bei GC Gabe zum Teil durch den Osteoblasten bzw. dessen sezernierte Faktoren, wie Osteocalcin, vermittelt werden. Es lässt sich schlussfolgern, das Leptin nicht von dieser Ereigniskaskade beeinflusst wird, hingegen ist es wahrscheinlich, dass die Adiponectinkonzentrationen, welche möglicherweise von den OCN-Konzentrationen reguliert werden, an der Entstehung von Insulinresistenz und Glucoseintoleranz beteiligt sind. Damit eine genauere Einschätzung der möglichen Beteiligung von OCN stattfinden konnte, wurde eine Gentherapiemethode etabliert, um OCN-Konzentrationen in vivo zu erhöhen. Die hydrodynamische Schwanzveneninjektion ist eine Methode mittels welcher Plasmid DNA in den Hepatozyten angereichert wird und das zelluläre Enzymsystem das entsprechende Peptid produziert und sezerniert. Diese physikalische Methode wurde mit Hilfe eines gelb fluoreszierenden Proteins (YFP) in einem CMV Promotor erprobt und die gewünschte paravenöse Anreicherung des YFP in der Leber nach 20h gesichert. Anschließend wurden die hTVI mit OCN Plasmiden und Placebo an GC behandelten Tieren durchgeführt. Die ITTs zeigten eine Verbesserung der Insulinsensitivität in den Tieren, die im Vergleich zu den Mäusen mit Placeboinjektionen OCN erhalten haben. Weitere Studien sind nötig um zu evaluieren, ob OCN möglicherweise effizient und potent genug wäre, den glukokortikoiden metabolischen NW vorzubeugen oder diese möglicherweise zu therapieren, damit die hocheffektiven anti–inflammatorischen Wirkungen von Glukokortikoiden genutzt werden können. Die Möglichkeit eines Therapeutikums zur Vorbeugung von glukokortikoid-induzierten metabolischen NW und möglicherweise sogar zur Behandlung von Diabetes mellitus Typ 2 würde eine Erleichterung für die betroffenen Patienten und eine enorme Verbesserung der eingeschränkten Lebensqualität darstellen.

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10 Appendix

10.1 Publications

"Long-Term corticosterone treatment induced lobe-specific pathology in mouse prostate."
Simanainen U, Lampinen A, Henneicke H, Brennan TC, Heinevetter U, Harwood DT, McNamara K, Herrmann M, Seibel MJ, Handelsman DJ, Zhou H. (2011). The Prostate 71(3): 289-297. (IF=3.485)

"Corticosterone selectively targets endo-cortical surfaces by an osteoblast-dependent mechanism."
Holger Henneicke, Markus Herrmann, Robert Kalak, Tara Brennan-Speranza, Uta Heinevetter, Bertollo Nicky day, Robert E. Huscher, Dörte Buttgereit, Frank Dunstan, Markus Seibel, Hong Zhou, (2011). Bone 49(4): 733-742. (IF=4.463)

“Osteoblasts mediate the adverse effects of glucocorticoids on fuel metabolism.”
Brennan-Speranza TC, Henneicke H, Gasparini SJ, Blankenstein KI, Heinevetter U, Cogger VC, Svistounov D, Zhang Y, Cooney GJ, Buttgereit F, Dunstan CR, Gundberg C, Zhou H, Seibel MJ.(2012) J Clin Invest. 122(11):4172–4189. (IF=13.069)

10.2 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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10.4 Statement/ Erklärung an Eides Statt

„Ich, Uta Heinevetter, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „The influence of glucocorticoids on osteoblast-derived factors and their impact on metabolism“ selbst verfasst und keine anderen als die angegeben Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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