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HORMONAL-INFLAMMATORY INTERFERENCE IN THE CONTROL OF *DE NOVO* GLUCOSE PRODUCTION BY THE LIVER

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"THE SEARCH FOR TRUTH IS MORE PRECIOUS THAN ITS POSSESSION."

Albert Einstein (Nobel Prize for Physics in 1921, 1879-1955)

Dedicated to my Parents

Zusammenfassung

Eine Voraussetzung für die Gesundheit und das Überleben von Säugetieren ist die Aufrechterhaltung des Blutglukosespiegels. Die Inhibition der stresskompensierenden *de novo* Glukoseproduktion in der Leber, Glukoneogenese, während akuter Entzündungprozesse ist eines der Hauptmerkmale eines veränderten Stoffwechsels und Haupttodesursache bei Sepsispatienten. Störung der hormonellen Signaltransduktion und die daraus resultierende Unterdrückung des Schlüsselenzyms der Glukoneogenese, der Phosphoenolpyruvate Carboxykinase (PEPCK), durch pro-inflammatorische Mediatoren trägt wesentlich zur schweren Hypoglykämie während einer Sepsis bei. Die molekularen Mechanismen der veränderten PEPCK Genregulation unter diesen Bedingungen sind jedoch noch unklar. Diese Arbeit stellt ein System für die Herstellung eines leberspezifischen adenoviralen Reportersystems vor, das die Identifizierung von dysfunktionalen cis-regulatorischen DNA Promoterelementen unter pathologischen Bedingungen in Mäusen erlaubt. Durch die Nutzung dieser *in vivo* Promoterkartierung konnte die Glukokortikoid responsive Einheit (*GRU) und das cAMP-responsabel Element (CRE) des PEPCK Gens-Genpromoters als wichtige regulatorische Elementen für die pro-inflammatorische Signalübertragung identifiziert werden. Die Deregulation der synergistischen Funktion dieser zwei Promoterelemente trägt dabei zur Inhibition der PEPCK Genexpression unter septische Bedingungen bei. Zudem konnte gezeigt werden, dass die Expression des Kernrezeptor Ko-Faktors PGC-1α, dem molekularem Vermittler des GRU/CRE-Zusammenspiels auf dem PEPCK-Promoter, spezifisch in der septischen Leber herunterreguliert ist. Die Verminderung der endogenen PGC-1α Expression verhinderte den durch Entzündungsmediatoren ausgelösten inhibitorischen Effekt auf die PEPCK Genexpression in primären Hepatozyten, während die Überexpression von PGC-1α die Expression von PEPCK unter den gleichen Bedingungen wiederherstellen konnte. Diese Ergebnisse identifizieren einen neuartigen *in vivo* - Mechanismus, der für die Suppression der glukoneogenetischen Genexpression in septischen Mäusen verantwortlich ist. Die Erhaltung der PGC-1α Aktivität könnte damit eine attraktive therapeutische Maßnahme zur Aktivierung der Glukoneogenese und zu Verhinderung von Hypoglykämie in septischen Patienten darstellen.

**Glucocorticoid response unit (GRU)*

Abstract

In mammals the maintenance of an appropriate glucose level in the blood is a prerequisite of good health and survival. The blockade of stress compensatory *de novo* glucose production by the liver, gluconeogenesis, during acute inflammation is one of the major characteristics of the aberrant metabolic state and a reason of death in septic patients. Interference with hormone signaling and the subsequent suppression of key rate limiting gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) through pro-inflammatory mediators importantly contribute to severe hypoglycemia in sepsis. However, the molecular mechanisms of aberrant PEPCK gene regulation under these conditions *in vivo* remain largely unknown.

In the present study we report the generation of a liver-specific adenoviral reporter system for the identification of dysfunctional gene cis-regulatory promoter elements under pathological conditions in mice. By employing *in vivo* promoter reporter technology, the glucocorticoid response unit (GRU) and the cAMP-response element (CRE) of the PEPCK gene were identified as critical promoter target sites of pro-inflammatory signaling. The disruption of the synergistic function of these two promoter elements was found to mediate PEPCK gene inhibition under septic conditions. Furthermore, the expression of nuclear receptor co-factor PGC-1α, the molecular mediator of GRU/CRE synergism on the PEPCK promoter, was found to be specifically repressed in septic liver. The depletion of endogenous PGC-1α with RNAi blunts the inflammatory suppressive effect on the PEPCK gene in cytokine-exposed primary hepatocytes while PGC-1α over-expression restores PEPCK expression under the same conditions.

These results provide an *in vivo* mechanism involved in the suppression of the key gluconeogenic gene PEPCK in septic mouse. The maintenance of PGC-1α activity might represent an attractive therapeutic defense for the rescue of gluconeogenic program repression and hypoglycemia in septic patients.

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1. INTRODUCTION

1.1 Metabolism and Metabolic diseases in XXI century

A highly integrated network of chemical reactions that occur in living organisms to maintain life is collectively known as metabolism. The chemical reactions of metabolism are organized into metabolic pathways, in which one chemical is transformed into another by a sequence of enzymes. All metabolic reactions can be divided into two major groups: catabolic that breaks down large molecules, e.g. nutritional substances, to provide energy and anabolic that uses the energy to construct components of cells such as proteins, lipids, nucleic acids (Alberts et al, 2002).

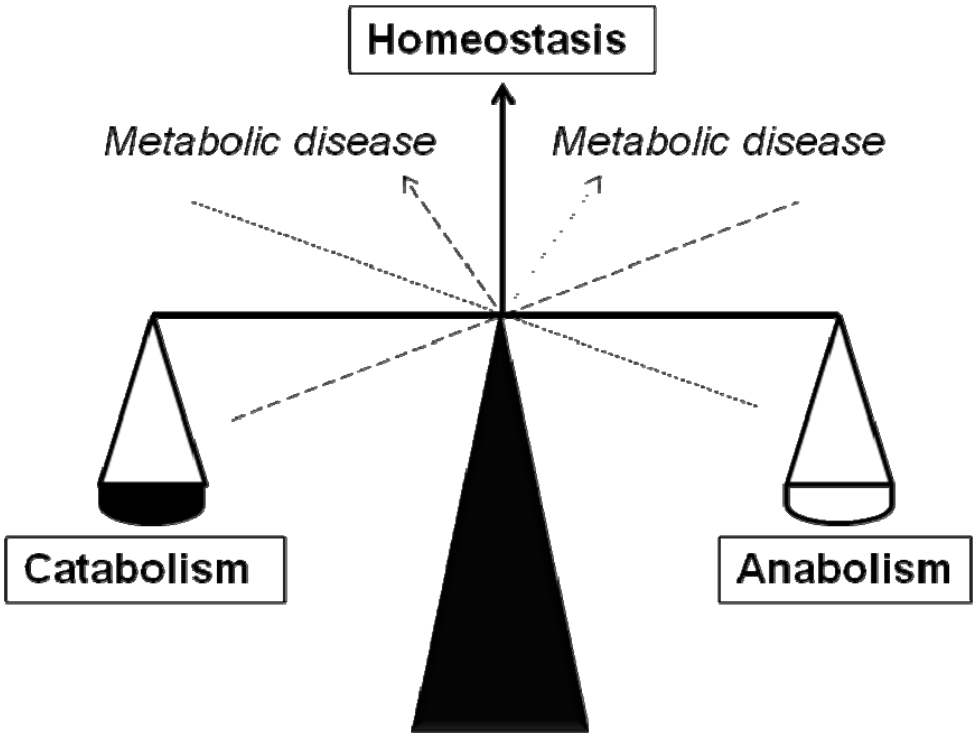


Figure 1 Metabolic pathways and the control of healthy physiological state.
All chemical reactions that occur in living organisms could be divided in two major pathways: catabolism that breaks down large molecules, e.g. nutritional substances, to provide energy and anabolism that uses the energy to construct components of cells such as proteins, lipids, nucleic acids. The equilibration between these two pathways, homeostasis, is a prerequisite of a healthy physiological state. The disruption of the homeostasis and the deviation of any of these pathways from its normal activation rate will result in generation of different pathological states named together as metabolic diseases.

Normally, the equilibration between catabolic and anabolic pathways, homeostasis, is tightly controlled and regulated. However once the equilibration is disrupted it will result in the

generation of a variety of malfunctioned processes named together as metabolic diseases (Figure 1). Over the last decades the incidence of metabolic diseases gained epidemic proportions and considered to be a serious problem for the people health and welfare in XXI century. By the latest reports of World Health Organization (WHO) more than 171 million people worldwide are affected only with type 2 diabetes and this number is predicted to be double by 2030. Not only diabetes but also the great number of other top-rated pathological states, i.e. obesity, cancer, autoimmune and cardiovascular diseases, infection were found to be associated with marked dysregulations of whole body homeostasis and metabolism (Benigni, Atsumi et al. 2000; Hsu, Kim et al. 2007; Halmos and Suba 2008; Luchsinger and Gustafson 2009). Recent data suggest that despite quite a different nature of metabolic diseases most of them have much in common. Since 2001 there was widely accepted the concept of 'clustering' metabolic disorders that gather different pathological states based on the term 'metabolic syndrome', that is a recognizable complex of symptoms and physical or biochemical findings for which a direct cause is not understood (Cornier, Dabelea et al. 2008). The metabolic syndrome that includes abdominal obesity, insulin resistance, dyslipidimia, hypertension is considered to be one of the important predictive and risk factors for the development of a variety of pathological metabolic states (Cornier, Dabelea et al. 2008; Bays 2009).

To date it is believed that genetic and environmental factors like aging, diet, lifestyle, changing ecological environment play a critical role in the etiology of metabolic disorders (Lusis, Attie et al. 2008; Lu, Lin et al. 2009), however, the complex molecular mechanisms involved in the governing of abnormal metabolic regulation in these states remain largely obscure.

1.2 **Inflammation as a hallmark of a variety of metabolic diseases**

Recently more and more evidences have emerged about the strong association of a variety of chronic metabolic diseases with a persistent, low grade inflammation (Handschin and Spiegelman 2008). For example, the development of insulin resistance and type 2 diabetes is closely correlated with immune cell infiltration and inflammation in white adipose tissue that is seen in obese patients (Stienstra, Duval et al. 2007), (Wellen and Hotamisligil 2005). In cardiovascular diseases, activated immune cells and inflammation plays a major role, particularly in the etiology of atherosclerosis (Haffner 2006). Tumor initiation, promotion and progression are stimulated by systemic elevation of pro-inflammatory cytokines (Lin and

Karin 2007), (Halmos and Suba 2008). Furthermore, the inflammatory cytokines such as a tumor necrosis factor α (TNF-α) and an interleukin 6 (IL-6) were shown to be involved in muscle and adipose tissue wasting during pathological syndrome cachexia, associated with the most aggressive types of cancer in gastrointestinal tract or lungs (Tisdale 2002), (Argiles, Busquets et al. 2005). A number of neurodegenerative processes, i.e. Alzheimer's and Parkinson's diseases are linked to a local inflammatory response in the brain (McCoy and Tansey 2008; Tansey, Frank-Cannon et al. 2008). On the other hand under some circumstances the chronic infection as such has been shown to cause insulin resistance, diabetes and atherosclerosis (Wellen and Hotamisligil 2005; Cani, Amar et al. 2007). And treatment of many of these diseases with anti-inflammatory agents, often give positive results to ablate or improve these metabolic malfunctions (Allary and Annane 2005; Glass and Ogawa 2006; Handschin and Spiegelman 2008).

The involvement of inflammation in the etiology of metabolic diseases is now understood to be quite deliberate. The nutrient and pathogen-sensing systems have evolved to be closely linked and interdependent. Many hormones, cytokines, signaling proteins, transcription factors can function in both metabolic and immune roles (Glass and Ogawa 2006) and the cross-talk between metabolism and immunity is not always deleterious. The normal inflammatory response relies upon metabolic support and energy redistribution, as exemplified by the immunosuppression characteristic of malnourished or starving individuals. In turn the inflammation favours a catabolic state and suppresses anabolic pathways, such as a highly conserved and powerful insulin signaling pathway that is ultimately beneficial for the energy balance and the maintenance of a good health (Wellen and Hotamisligil 2005). However the integration between metabolism and immunity which is useful under normal conditions can become deleterious under conditions of metabolic or inflammatory challenge where it can lead to interference with the function of these pathways alone. The inflammatory-metabolic interference represents a promising molecular mechanism that might be involved in a great variety of different diseases and it understanding might be crucial in fighting these pathological conditions.

1.2.1 ***Sepsis as a model of inflammatory-metabolic interference***

One of the models of such inflammatory-metabolic interference is sepsis. Sepsis is a pathological state, characterized by systemic inflammatory response syndrome (SIRS), initiated by infection in the blood. Recently it becomes evident that the development of septic phenotype largely belongs not to the detrimental action of the pathogen but rather to the activation of SIRS by the host immune system that characterized by elevated level of pro-inflammatory cytokines in the blood and leads to metabolic abnormalities, multiple-organ failure, hypoxia and death (Webster and Sternberg 2004). Metabolic changes in protein, fat, glucose and energy metabolism and associated physiological disturbances such us fever, anorexia, hypotension, transient hyperglycemia followed by severe hypoglycemia are important characteristics of sepsis (Blackwell and Christman 1996). The hypoglycemic shock, characterized by wasting of all glycogen stores and failure of liver for the compensatory *de novo* glucose production, gluconeogenesis, due to hormonal-inflammatory interference is one of the common septic outcomes resulting in patients mortality (McGuinness 2005), (Buras, Holzmann et al. 2005).

Different mouse models of sepsis and septic shock have been established for the reproducible study of septic pathophysiology. On the basis of initiating agent, septic mouse models can be divided into three categories: exogenous administration of a toxin (such as lipopolysaccharide (LPS), endotoxins or zymosan); exogenous administration of a viable pathogen (such as bacteria); or alteration of the animal's endogenous protective barrier (e.g. colonic permeability, allowing bacterial translocation) (Buras, Holzmann et al. 2005). Through these models the LPS mouse model of sepsis is particularly interesting for our study since in these animals there is no circulation of bacterial pathogens in the blood and the development of septic phenotype and subsequent metabolic dysregulations entirely belong to the detrimental activation of a host immune system and a metabolic-inflammatory interference.

1.3 **Liver in metabolism and immunity**

Liver represents a key metabolic organ maintaining protein, lipid and glucose homeostasis. It secretes amino acids, carbohydrates and fatty acids, lipoproteins, plasma proteins and vitamins, stores glucose and fat (Knolle and Gerken 2000).

In mammals liver serves as a key relay station for glucose and lipid between the periods of fasting and refeeding (Vegiopoulos and Herzig 2007). After feeding the pancreatic β-cell

hormone insulin triggers fast glucose uptake into peripheral tissues such as adipose tissue and skeletal muscles. The glucose is metabolized to C2-precursors that in the complex with special enzyme acetyl-Coenzyme A (acetyl-CoA) enter the Krebs cycle to be oxidized for energy needs (Figure 2). Via β-oxidation in the liver free fatty acids are also oxidized to the acetyl-CoA and NADH. Excess of acetyl-CoA in the liver is used for the synthesis of ketone bodies, which are secreted into the blood and serve as an energy source for peripheral tissues. Dietary fats, that are not consumed to generate energy are transformed into phospholipids and triacylglycerides and transported in lipoprotein particles to adipose tissue, where they are stored. Serum free fatty acids can bind to serum albumins secreted from the liver and be transported to skeletal muscle or heart, where they are directly metabolized.

In fed state insulin stimulates the storage of the glucose excess in the liver as a polysaccharide glycogen that is converted back into the glucose and released into the serum during fasting period. The maintenance of a blood glucose level under fasting or stress conditions is critical for some extrahepatic tissues such as brain, erythrocytes and renal medulla that specifically use the glucose as a carbohydrate and energy source. In response to low glucose level in the blood the insulin secretion is decreased while the concentration of counter-regulatory peptide hormone glucagon, secreted from pancreatic α-cells, catecholamines such as epinephrine and steroid hormones glucocorticoids, derived from adrenal glands, are released into the serum. These hormones activate different stress compensatory programs in which liver plays a critical role.

One of the prominent features of liver metabolism is the ability for *de novo* glucose synthesis, gluconeogenesis, when the glycogen stores are empty. In that case free fatty acids or amino acid precursors from adipose tissue and muscles are channeled to the liver where they enter gluconeogenic pathway that comprises various biochemical steps converting pyruvate to glucose (Vegiopoulos and Herzig 2007) as depicted in Figure 2.

The key enzymes in the gluconeogenic pathway are pyruvate carboxylase (PC), which converts pyruvate into oxaloacetate, phosphoenolpyruvate carboxykinase (PEPCK) promoting the decarboxylation of oxaloacetate to phosphoenolpyruvate, and, finally, glucose-6-phosphatase (G6Pase), hydrolysing glucose-6-phosphate into free glucose and inorganic phosphate (Vegiopoulos and Herzig 2007).

Since the gluconeogenesis uses the same substrates that are normally used for the tissue composition such as amino acids from muscles or fatty acids from the adipose tissue under conditions of glucose excess in fed state this pathway is blocked by insulin. The correct regulation of gluconeogenic pathway is important to maintain healthy homeostasis, since its

constant activation that is seen during insulin resistance leads to hyperglycemia and type II diabetes or to the severe muscle and adipose tissue wasting as it is seen in pathological syndrome cachexia, a side effect of some solid tumors such as gastrointestinal or lung cancer (Tisdale 2003).

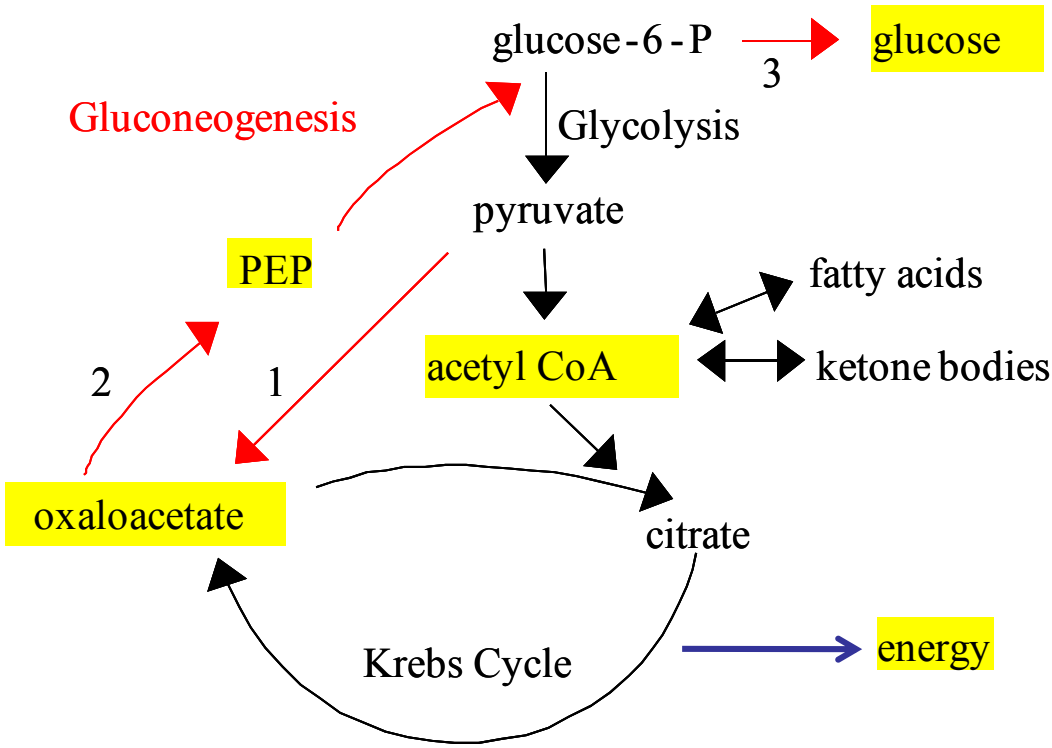


Figure 2 Gluconeogenesis and energy metabolism.
Some tissues or cells, i.e. erythrocytes, renal medulla and brain use specifically the glucose as carbohydrate and energy source thus the accessibility of this substrate is critical. Under fasting or stress conditions when the glucose in the blood decreases, the special compensatory mechanism for *de novo* glucose production by the liver, gluconeogenesis, maintains it at appropriate level. During gluconeogenesis glucose synthesized *de novo* from other substances such as amino acids from muscles, fatty acids from the adipose tissue or other intermediates such as ketone bodies, the product of fatty acids β-oxidation. Under normal feeding the gluconeogenic pathway is repressed and the dietary glucose and fatty acids are metabolized to C2-precursor acetyl-Coenzyme A (acetyl CoA) and oxidized in a Krebs cycle for energy production. The correct regulation of gluconeogenic pathway is highly important to maintain homeostasis, since its pathological repression i.e. in sepsis might lead to hypoglycemic shock and death in critically ill patients when over-activation leads to hyperglycemia and type II diabetes or tissue wasting as in patients with gastrointestinal or lung cancer.
The key enzymes in the gluconeogenic pathway are (1) pyruvate carboxylase, (2) phosphoenolpyruvate carboxykinase and (3) glucose-6-phosphatase. Key intermediates are: pyruvate, oxaloacetate, phosphoenolpyruvate (PEP), glucose-6-phosphate (glucose-6-P).

On the other hand the pathological repression of this pathway as in sepsis leads to hypoglycemic shock and death in critically ill patients.

Except the metabolic function liver is a key organ in organism immunity. First of all it is able to clear and detoxified waste or toxic products that are brought by the blood coming from the

gastrointestinal tract and from systemic circulation. The resident macrophage population, Kupffer cells, in the liver could phagocytose and eliminate particulate antigens or pathogens (Knolle and Gerken 2000). Furthermore, over the time of infection or injury the liver is responsible for the activation of crucial protective mechanisms collectively called as acute phase response. During this process liver secretes a large amount of acute inflammatory proteins that modulate different physiological functions and immune response. Some act to destroy or inhibit growth of microbes (C-reactive protein, complement factors, Serum amyloid A), other give negative feedback on the inflammatory response (serpins) or modulate blood coagulations (alpha 2-macroglobulin) (Ramadori and Christ 1999; Streetz, Wustefeld et al. 2001). Key liver metabolic transcriptional factors: glucocorticoid receptor (GR), liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPARs), effectively modulate immune function by repressing the expression of inflammatory genes or directly suppressing key pro-inflammatory transcriptional factors such as nuclear factor - kB (NF-kB) or activated protein 1 (AP1) (Ogawa, Lozach et al. 2005; Glass and Ogawa 2006). The PPARs and LXRs are nuclear receptors that play a critical role in fatty acid and cholesterol metabolism, when GR is involved in the glucose metabolism and is a key activator of the *de novo* glucose production, gluconeogenisis, by the liver. Under some pathological challenge with extreme activation of immunity such as in sepsis the overlapping function of the liver in metabolism and immunity could be detrimental and leads to a broad dysregulations of the whole body metabolism in which liver is critically involved.

1.4 Regulation of stress response. The control of PEPCK gene expression under physiological and septic conditions

In response to stress the bidirectional communication exists between the brain and immune system, in which the immune system signals the brain via cytokines and the brain response by regulating immune system, in part through the action of the hypothalamic-pituitary-adrenal axis (HPA) with resultant release of glucocorticoids (GC) (Webster and Sternberg 2004).

The action of GC in the cell is mediated through glucocorticoid receptor (GR), a transcriptional factor that could function in both DNA-dependent and independent manner. The GR could mediate stress response by trans-repressing the key pro-inflammatory transcriptional factors NF-kB and AP1 (1.3) and their dependent pro-inflammatory genes, by activating the apoptosis of immune cells, or by activating the stress compensatory processes such as *de novo* glucose production by the liver, gluconeogenesis (Figure 3). The last

mechanism is in particular important for the survival of patients with extreme activation of immune system such as in sepsis. The pathological repression of this pathway under these conditions leads to hypoglycemic shock and death in critically ill patients (Chajek-Shaul, Barash et al. 1990), (Chang, Gatan et al. 1996).

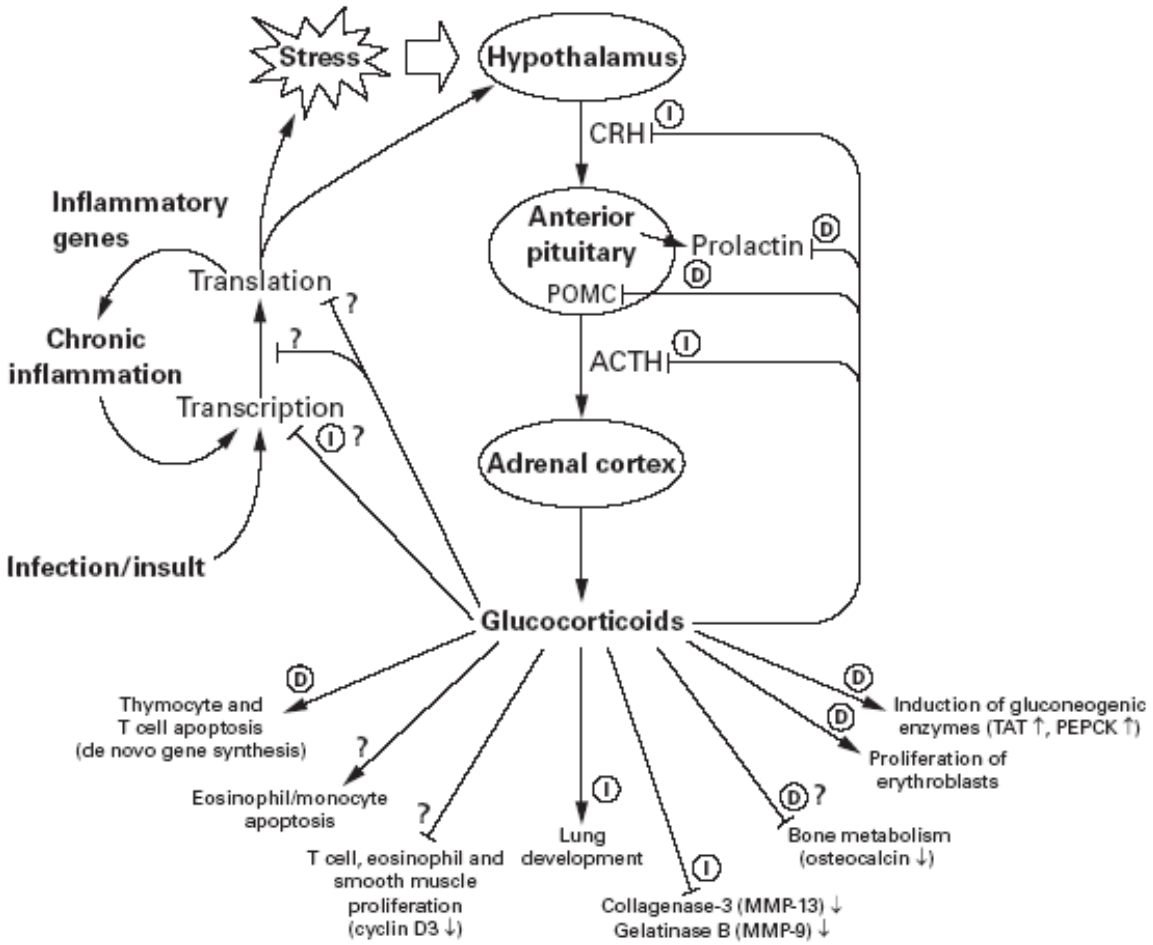


Figure 3 Effects of glucocorticoids on the hypothalamic-pituitary-adrenal (HPA) axis.
Inflammation, pain, infection or mental stress lead to activation of the hypothalamic-pituitary-adrenal (HPA) axis. These stimuli cause excitation of the hypothalamus, which responds by releasing corticotrophin releasing hormone (CRH) (also known as corticotropin releasing factor, CRF). CRH then acts on the anterior pituitary to induce enzymatic cleavage of a hormonal precursor Pro-opiomelanocortin (POMC) and subsequent synthesis and release of adrenocorticotropic hormone (ACTH). ACTH in turn stimulates the adrenal cortex to release glucocorticoids such as cortisol. The glucocosticoids mediate a broad range of metabolic and immune suppressive activities and function through glucocorticoid receptor (GR) in GR DNA binding dependent (D) or independent manner (I). Question marks indicate uncertainty as to the mechanism of action (Newton 2000). Abbreviations: tyrosine aminotransferase (TAT) and phoshoenolpyruvate carboxykinase (PEPCK).

Phosphoenolpyruvate carboxykinase (PEPCK) is considered to be the rate-limiting step in the gluconeogenic pathway, and its expression is largely regulated by gene transcription

(Vegiopoulos and Herzig 2007). Glucocorticoids, retinoic acid, and glucagon (via its second messenger, cAMP) stimulate PEPCK gene transcription, whereas insulin, phorbol esters, cytokines, and oxidative stress have an opposing effect (Waltner-Law, Daniels et al. 2000).

A number of important DNA elements and binding proteins that regulate the transcription of the PEPCK gene under normal conditions have been identified (Figure 4). Remarkably, the promoter sequence of the PEPCK gene from the mouse, rat and human has been shown to be highly conserved (greater than 95% sequence identity), thus the pattern of transcriptional regulation studied in rodent might be applied also to the control of the gene in mammalian species (Croniger, Leahy et al. 1998).

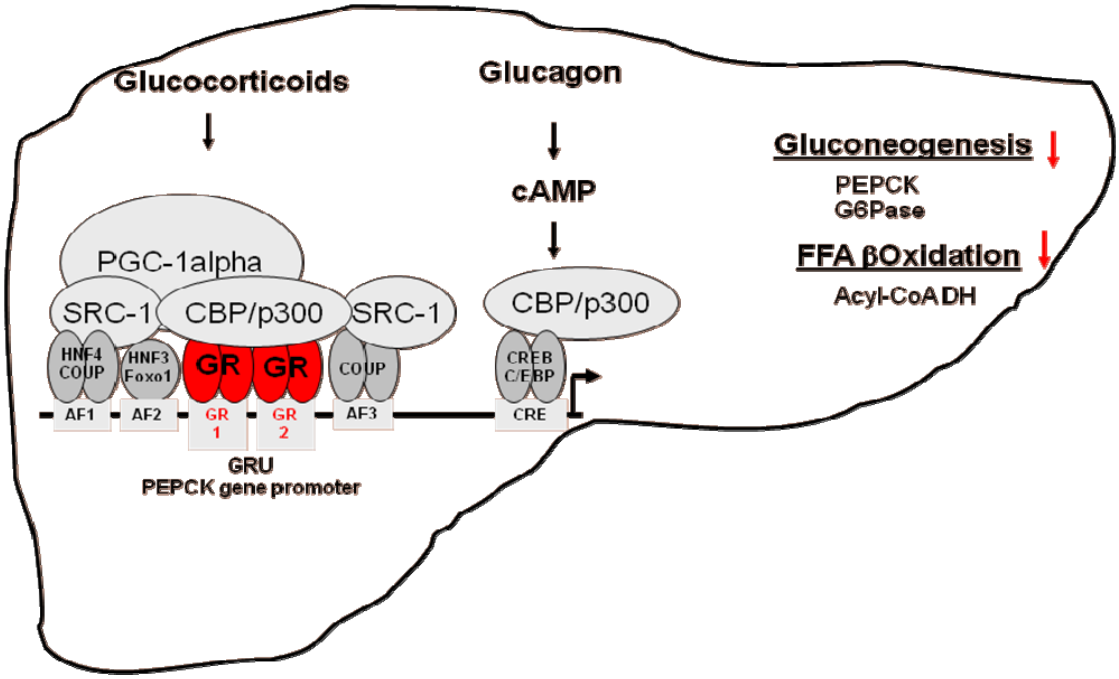


Figure 4 Gluconeogenic pathway and glucose homeostasis. Structure of the PEPCK gene promoter.

During fasting or under stress conditions, glucose homeostasis is achieved by triggering expression of gluconeogenic genes such as phosphoenolpyruvate carboxykinase (PEPCK) or glucose-6 phosphatase (G6Pase) in response to glucagon acting via cyclic adenosine monophosphate (cAMP) signaling axis and glucocorticoids. These pathways act synergistically to induce gluconeogenesis by the liver. Suppression of the key gluconeogenic enzyme genes on the one hand and the decreased gluconeogenic substrate supply (reduced free fatty acids (FFA) βOxidation) on the other contribute to the blockade of compensatory *de novo* glucose production by the liver in sepsis. Schematic representation of the glucocorticoid regulatory unit (GRU) and associated factors/co-factors within the PEPCK gene promoter. Abbreviations: GR, glucocorticoid receptor; CREB, cAMP regulatory element binding protein; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; HNF-3 hepatocyte nuclear factor 3; C/EBP, CAAT/enhance binding protein; CBP/p300, CREB binding protein/p300; PGC-1α, peroxisome proliferator activated receptor γ coactivator 1α; SRC-1, sterol receptor coactivator-1, AF1, accessory factor 1; AF2, accessory factor 2; AF3, accessory factor 3; forkhead transcription factor; GR1 and GR2, glucocorticoid regulatory element; CRE, cAMP regulatory

elements (Vegiopoulos and Herzig 2007). PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; Acyl-CoA DH, acyl-CoA dehydrogenase.

One of the major PEPCK regulatory units is GRU (glucocorticoid responsive unit), (Figure 4). The GRU is a complex promoter region that consists of two glucocorticoid responsive elements (GRE) GR1 and GR2 to which the glucocorticoid receptor (GR) is bound, and few glucocorticoid accessory factor elements to which the accessory factors that are necessary for the full glucocorticoid response as well as glucagon induction are bound (Herzig, Long et al. 2001), (Hanson 2005), (Vegiopoulos and Herzig 2007).

Among these, the most prominent are hepatocyte nuclear factor 4 (HNF-4), peroxisome proliferator activated receptor γ2 (PPARγ2), retinoic acid receptor (RAR), retinoid X receptor (RXR) α (AF1), chicken ovalbumin upstream promoter transcription factor (COUP-TF) (AF1 and AF3) as well as forkhead transcription factor FOXO1 and hepatocyte nuclear factor 3 (HNF-3) (AF2). In this setting, the two intrinsic GR binding sites, GR1 and GR2, do not confer GC responsiveness to a heterologous promoter, demonstrating the requirement of a close functional interaction with their accessory factors on the PEPCK gene. In fact, accessory sites AF1 and AF2 have been shown to create a high affinity GR binding environment, thereby raising the low affinity of the GR to the non-canonical GR1 and GR2 PEPCK promoter sites (Scott, Stromstedt et al. 1998). The GR interacts with several non-steroid receptors in this context, resulting in a synergistic response of the PEPCK gene to GC exposure (Herzig, Long et al. 2001), (Hanson 2005). The second important promoter region is a cAMP responsive unit (CRU), that in turn consist of cAMP responsive elements (CRE), that binds (CRE)-binding protein (CREB) and CCAAT/enhancer-binding protein(C/EBP) α and β and additional elements PI to P4 contributing to cAMP response (Hanson 2005), (Yan, Gao et al. 2007). Importantly the remote promoter regions GRU and CRU have overlapping functions that necessary for synergistic PEPCK activation by counter-regulatory hormones glucagon and glucocorticoids (Herzig, Long et al. 2001), (Vegiopoulos and Herzig 2007). The synergism is mediated by a complex environment of transcriptional factors and co-activators that bridge proximal and distal transcriptional factors in a single unit.

During sepsis the increased level of glucocorticoids together with some other regulatory hormones, i.e. glucagon and catecholamines cannot properly fulfill its function in the regulation of glucose homeostasis. In particular, interference with hormone signaling and the subsequent suppression of PEPCK expression through pro-inflammatory mediators importantly contributes to the hypoglycemic phenotype and death in septic patients, the *in vivo* molecular mechanisms of which remain largely unknown.

1.5 Adenoviral gene delivery as robust tool to study the regulation of genes *in vivo*

Recombinant adenoviruses are replication-defective adenoviral vectors that found a high application for gene therapy, vaccine therapy and basic biology (Luo, Deng et al. 2007).

The adenoviruses have many advantages compare to other systems of gene delivery: they don't integrate into the host genome, they are able to infect a broad range of cell types, dividing and not dividing cells, have low morbidity, high virus titer and level of recombinant gene expression, high capacity for foreign DNA insert (Russell 2000). The most commonly used adenoviral vector is a human adenovirus serotype 5, which is a 36 kb double-stranded linear DNA with inverted terminal repeats (ITR). The recombinant vectors lack E1 gene, essential for virus replication and E3 gene responsible for evading of the host immunity. The deletion of these genes creates a large capacity for the insert: up to 7,5 kb of foreign DNA and let to control virus amplification after it enters the target host cell (Luo, Deng et al. 2007) . The infection with the adenovirus is transient, and the vector normally cleared from the tissue within one month.

Despite that the virus infect a broad range of different cell types, there are different strategies to target it to specific tissues. These are: the expression of the transgene under the control of a tissue specific promoter e.g. endogenous liver promoter, or the incorporation into the transgene of tissue 'silencer' elements in conjunction with ubiquitous promoters to drive tissue-specific expression e.g. in neuronal cells (Russell 2000). Another strategy to achieve tissue specific transgene expression is a way of virus inoculation. The injection of adenovirus via tail- vein in mouse leads to liver specific gene delivery since in that case the virus cleared up almost exclusively by the liver (Herrmann, Abriss et al. 2004).

In basic research, adenoviral vectors are often used to manipulate with gene dosage by gene over-expression or knockdown to address the function of the gene *in vivo* (Russell 2000; Luo, Deng et al. 2007). At current work we developed a novel liver specific system that let to use adenoviral vectors as a powerful analytical tool enabling to study the function of the gene cis-regulatory elements under physiological/pathological conditions *in vivo*.

2. AIM AND OBJECTIVES

The aim of this study was the identification of mechanisms of PEPCK gene dysregulation in sepsis due to hormonal-inflammatory interference through *in vivo* promoter mapping.

OBJECTIVES

1. Develop and characterize an adenoviral reporter system for *in vivo* promoter analysis

2. Map inflammatory responsive promoter elements of key gluconeogenic enzyme gene, PEPCK *in vivo* and thereby identify the mechanisms of its suppression during sepsis

3. RESULTS

Development of an adenoviral reporter system for gene promoter analysis *in vivo*

Despite the overall complexity of metabolic cellular signalling the end-point of pathways that interpret all these signals is gene transcription (Rosenfeld, Lunyak et al. 2006). Analysis of the gene promoter structure is of a great importance for understanding molecular mechanisms involved in the regulation of gene transcription (Vityaev, Orlov et al. 2002). To date, the function of different cis-regulatory promoter element has been identified primarily in cell culture by promoter deletion or mutation analyses. In this technique the 5'regulatory promoter regions of the gene are cloned downstream from the reporter gene such as luciferase in the way that it could direct the expression of a reporter enzyme. The expression of the reporter enzyme therefore will reflect the activity of the promoter in response to different cellular signals. By mutation or deletion of particular promoter elements the signal dependent promoter regulation can be studied in cell culture. This method is quite robust but restricted to limited number of signals and artificial physiological milieu that one can set in cell culture system. The way to address the function of gene regulatory promoter elements *in vivo* to date has been a generation of transgenic mouse with appropriate promoter deletion or mutations (Croniger, Millward et al. 2001; Cassuto, Kochan et al. 2005). However this technique is very laborious, time-consuming and expensive.

It the present study, based on an adenoviral gene delivery and major principles of *in vitro* technique for gene promoter study, we developed a novel robust system for DNA cis-elements analysis *in vivo*.

Since the expression of the key gluconeogenic gene PEPCK is fully regulated at the transcriptional level (1.4) and since this gene is a target of hormonal-inflammatory interference in sepsis (1.4) we chose PEPCK as a model for the development and the functional validation of this system.

3.1 Cloning strategy

To develop an adenoviral promoter reporter system the following cloning strategy has been used. The Firefly luciferase gene from pGL3-basic vector was introduced into the multiple cloning site of the adenoviral shuttle vector pAd-Track (Figure 5). To avoid cross-talk

between the target promoter PEPCK and the CMV that directs the expression of the GFP gene in original the pAd-Track vector, the CMV promoter was excised by restriction sites *Cla I* and *AgeI*. The plasmid was blunted with DNA-pol I (Klenow) and relegated.

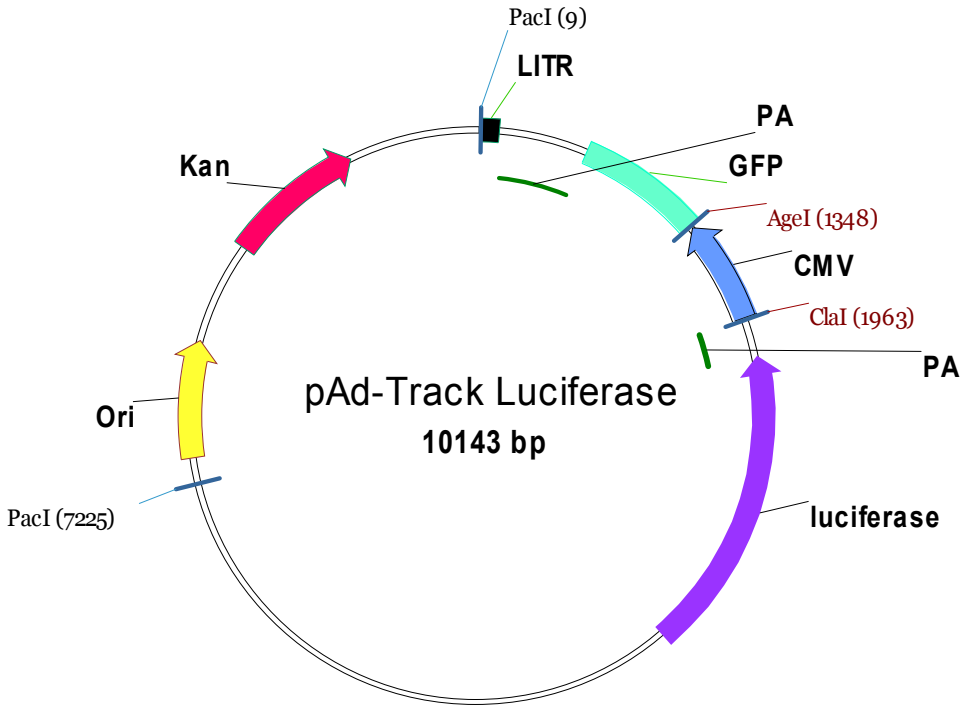


Figure 5 Map of the luciferase reporter adenoviral shuttle vector (pAd-Track luciferase).
This vector is designed for the analysis of the promoter of interest with luciferase readout *in vivo*. After the cloning of all genetic elements the vector has to be integrated in an adenoviral genome serotype V (pAd-Easy1 vector). The vector include the Origin of replication (Ori) for the vector amplification in bacterial cells, resistance to Kanamicin antibiotic (Kan) for the selective growth of positive bacterial clones containing the pAd-Track-luciferase plasmid, long inverted terminal repeats (LITR) for the integration in adenoviral genome, Polyadenylation sites (PA) for the correct maturation of the mRNA that expressed from genes of interest (e.g. GFP or luciferase), CytoMegalie-Virus promoter (CMV) that directed the expression of green fluorescent protein (GFP) gene in original pAd-Track vector and excised here by *Age* I and *Cla* I sites for the development of promoter reporter system, GFP gene sequence is used here for in tissue virus genome numbers determination (3.3), luciferase gene expression is used as a read out for the activity of a promoter of interest that has to be cloned upstream from luciferase. The original sequence of pAd-Track vector can be found elsewhere for commercially available ADEASY™ VECTOR SYSTEM

The different 5'-regulatory flanking regions of the PEPCK gene were cloned upstream from luciferase that they could direct the expression of the luciferase reporter enzyme in the adenoviral shuttle vector pAd-Track. PEPCK promoter elements were: -1330 bp (wt-promoter), -490 bp (includes most elements responsible for basal promoter activity and responsiveness to counter-regulatory hormones insulin, glucagon and glucocorticoids) and

-355 bp (lacking glucocotricoids response unit, GRU) as depicted in Figure 6. Based on the -490 PEPCK promoter construct we developed a series of point mutations of different regulatory responsive elements within the GRU or the CRE respectively that were also cloned in the pAd-Track vector later.

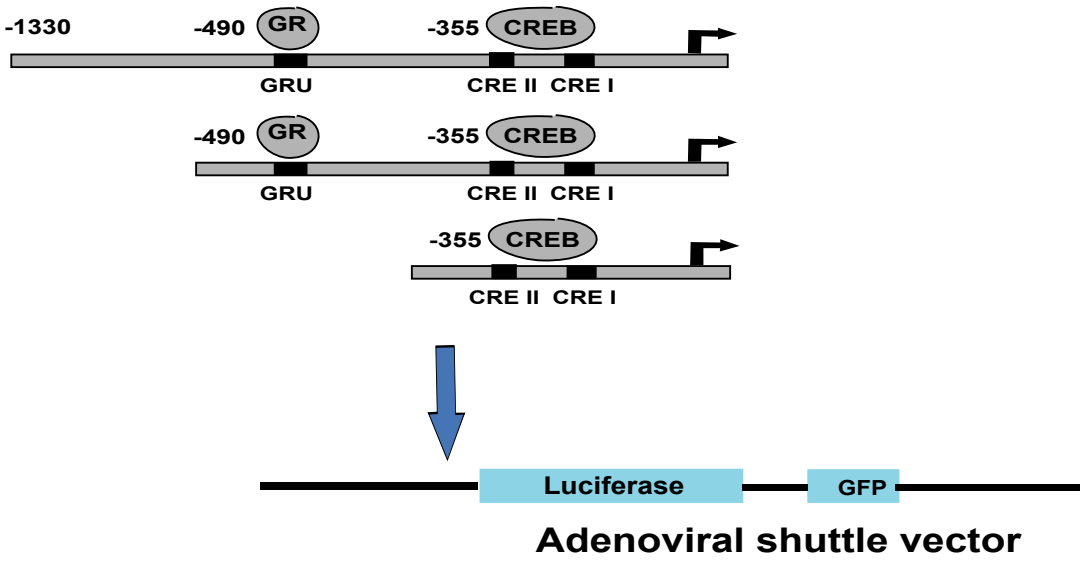


Figure 6 Cloning of the adenoviral promoter reporter shuttle vector.
Schematic representation of different phosphoenolpyruvate carboxykinase (PEPCK) promoter elements cloned upstream of luciferease gene in the pAd-Track Luciferase shuttle vector. Glucocorticoid response unit (GRU), cAMP response element I and II (CRE I and II), and associated transcriptional factors: glucocorticoid receptor (GR), CRE binding protein (CREB) are depicted. After excision of the CMV promoter the green fluorescent protein (GFP) is not expressed however the sequence of the GFP gene is still intact and is used for in tissue viral genome normalization (3.3)

The adenoviral shuttle vector pAd-Track-PEPCK Luciferase was linearised by *Pac I* digestion, and integrated within pAd-Easy1 plasmid containing adenoviral genome serotype V. The final scheme of adenoviral reporter virus is depicted in Figure 7. As a negative control, we also cloned a Luc-ctrl reporter virus that lacks any promoter element upstream of the luciferase and thus cannot specifically direct the expression of the reporter enzyme.

The virus is replication-deficient as E1 and E3 genes are both deleted from its genome. The *Pme I* linearised PEPCK promoter reporter viral vector was transfected into human embryonic kidney cells (HEK 293A) that complement for E1 and E3. Finally, the functional PEPCK reporter virus was amplified, purified and titered.

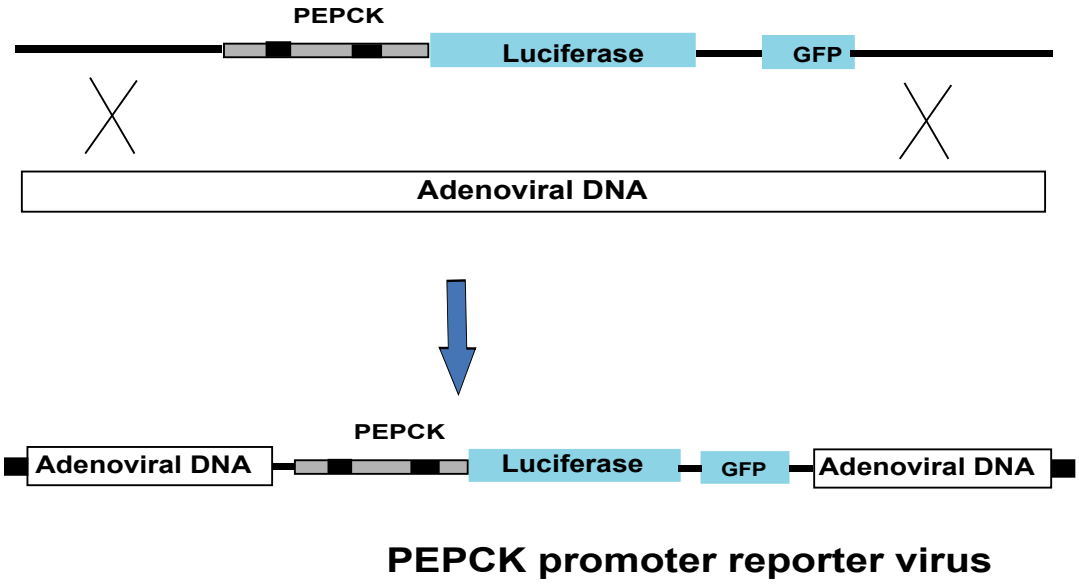


Figure 7 Cloning of the promoter reporter adenovirus.
Integration of the pAd-Track Luciferase PEPCK promoter shuttle vector into adenoviral genome (pAd-Easy1 vector). The recombination was performed in commercially available BJ5183 electrocompetent *Escherichia coli* cells containing Ad5 adenoviral genome. Abbreviations: phosphoenolpyruvate carboxykinase (PEPCK), green fluorescent protein (GFP).

3.2 Validation of the functional integrity of the system

Before utilizing the system for promoter deletion analysis *in vivo* we characterized it in hepatocyte cell culture and in C57Bl6/j wt-mouse.

3.2.1 Adenoviral promoter reporter system responds to key endogenous signals in hepatocytes

We first wanted to validate if the viral reporter promoter system was functional *in vitro*. The PEPCK gene is induced in the liver through glucocortigoid hormone via the glucocorticoid receptor (GR), through the glucagon via the cAMP axis, as well as synergistically via both stimuli (Imai, Stromstedt et al. 1990), (Herzig, Long et al. 2001).

The wt-PEPCK1330 adenoviral reporter infected cells were treated with glucocorticoid analogue dexamethasone (Dex, 10 nM), cAMP agonist Forskolin (Fsk, 10uM) or both. The readout from the PEPCK viral reporter was analyzed in luciferase assay.

As shown in Figure 8, the PEPCK promoter integrated in the viral genome had a basal transcriptional activity that could be further induced by regulatory hormones Dex, Fsk or both

together. The simultaneous promoter induction with Dex and Fsk gave synergistic effect on promoter activation as previously described (Herzig, Long et al. 2001). Thus, the adenoviral system responded to key endogenous signals in hepatocytes, validating its function in cell culture.

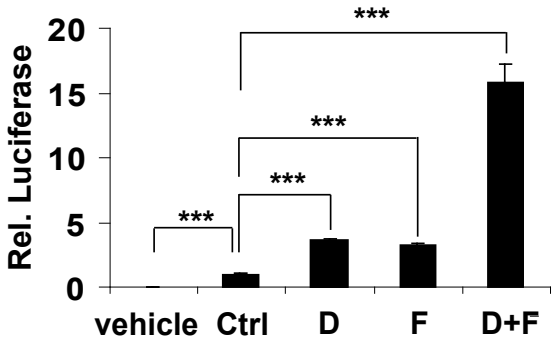


Figure 8 Functional validation of the viral reporter system in hepatocytes.
Wt-PEPCK-1330 reporter virus has a basal promoter activity (Ctrl) and response to key endogenous signals: glucocorticoid analogue dexamethasone (D), cAMP agonist Forskolin (F) or synergistically to both stimuli together (D+F) in H4IIE hepatocytes. The luciferase activity depicted as a relative value that for any experimental group is significantly above the background (vehicle). Data are presented as Mean ±S.E.M. of a 3 independent experiments (N=3), ***p≤0,001

3.2.2 *Adenoviral promoter reporter system specifically responds to physiological conditions, e.g. fasting*

To characterize the system *in vivo,* we first wanted to confirm that the promoter reporter virus responds to physiological conditions. As PEPCK is a key enzyme of gluconeogenesis in the liver, its expression is markedly induced in response to fasting (Herzig, Long et al. 2001).
The wt-C57BL6/J mice were injected with PEPCK1330-reporter virus or Luc. ctrl virus via the tail-vein, followed by fasting and refeeding of the animals (Figure 9). The viral toxicity was examined by measuring body weight, food intake and blood glucose level. Normally four days after post viral injection there were no significant changes in all of these physiological parameters.

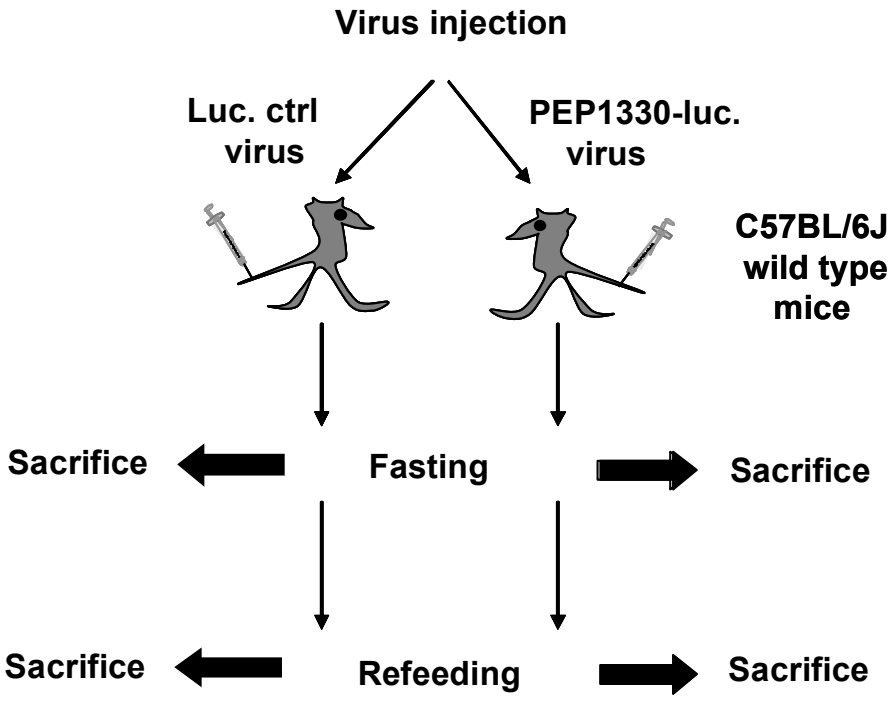


Figure 9 Examination of the wt-PEPCK1330 promoter reporter virus *in vivo*.
Since the gluconeogenic program is induced during fasting and is blocked after refeeding, we triggered these conditions to test the specific response of the system to physiological conditions. Two viruses: PEP1330-luc.virus, directing the expression of luciferase from wild-type PEPCK promoter, and Luc. ctrl virus, that has the same genetic background but lacks any promoter element upstream of luciferase gene and therefore cannot specifically express reporter enzyme, were injected through the tail-vein in a wt-mice. The experiment was performed 5 days after virus injection.

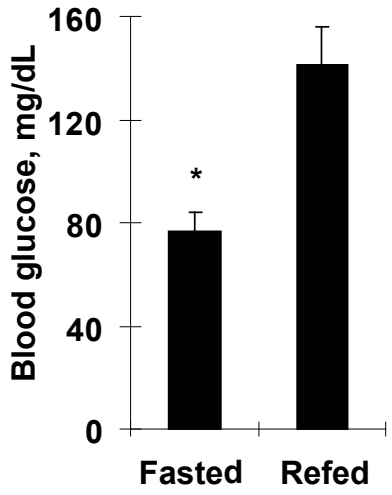


Figure 10 Control of fasting-refeeding conditions by the blood glucose.
Blood glucose level was measured in wt-mice after 24 hrs of fasting and refeeding, respectively. Experiment was performed as described in Figure 9. Data are presented as Mean ±S.E.M.; *p≤0,05 (N=3 animals per group)

We fasted mice for 24 hrs. Half of the mice were sacrificed and another half was refed for 24 hrs. The fasting and refeeding states were monitored by the blood sugar level (Figure 10).

[1]After fasting of animals, the endogenous PEPCK expression was strongly induced and suppressed in the refed state (Figure 11 A). As shown in Figure 11 B the luciferase readout from the PEPCK1330 reporter virus was also strongly suppressed thereby reflecting the endogenous pattern of the PEPCK gene regulation upon fasting. The Luc. ctrl virus did not respond specifically to fasting-refeeding and the reporter signal for both conditions remained at the level of the background.

These results demonstrate that the system specifically responded to physiological conditions *in vivo*.

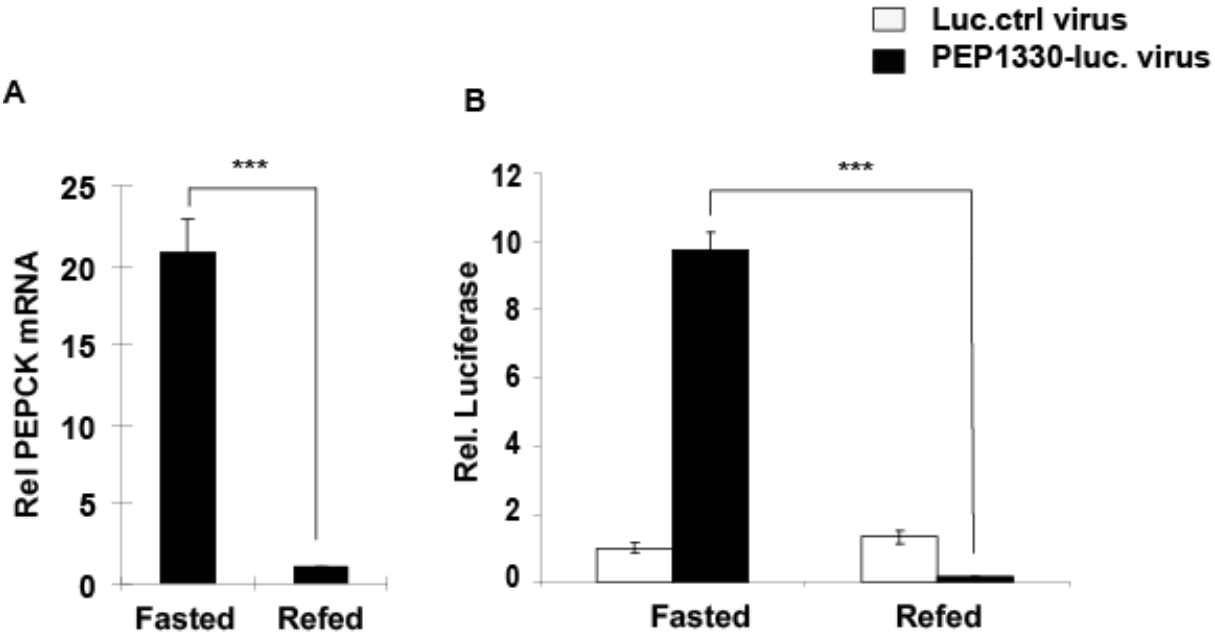


Figure 11 Luciferase readout of the promoter activity for the PEPCK reporter adenovirus reflects endogenous regulation of gene transcription upon triggering fasting response in the wt-mice.
Mice were fasted and refed for 24 hrs (A). The endogenous PEPCK mRNA level was assessed by real time q-PCR. Ct values for PEPCK are normalized against housekeeping gene TATA box binding protein (TBP). (B) The analysis of luciferase reporter signal for Luc. ctrl and wt-PEPCK-1330 reporter viruses. Data are presented as Mean ±S.E.M.; ***p≤0,001, (N=3 animals per group)

[1] The physiological manipulations were performed 4 days after tail vain virus injection.

3.2.3 *The system enables functional analysis of particular regulatory sites within the complex promoter structure in vivo*

To further characterize the system *in vivo*, we next decided to test the function of a well-known and fully-characterized PEPCK promoter unit such as the GRU. To this end, along with the -1330 wt PEPCK virus, we utilized a -490 PEPCK promoter deletion, which has been shown to be sufficient to drive basal and hormone-induced expression of the PEPCK gene (Hanson 2005), and the -355 PEPCK deletion reporter virus lacking the entire GRU (Figure 6), and therefore not responding to induction by glucocorticoids upon fasting conditions.

We fasted mice for 12 hrs and then refed animals for next 8 hrs, respectively. Despite that the time of fasting-refeeding had been shortened, it was sufficient to trigger gluconeogenesis and PEPCK promoter response (Figure 12 B). The reporter signals for three promoter viruses: -1330-wt, -490 and -355bp were analyzed (Figure 12 A). The blood glucose and endogenous PEPCK mRNA level was used to monitor fasting conditions and demonstrate that experiment technically worked (Figure 12 B, C).

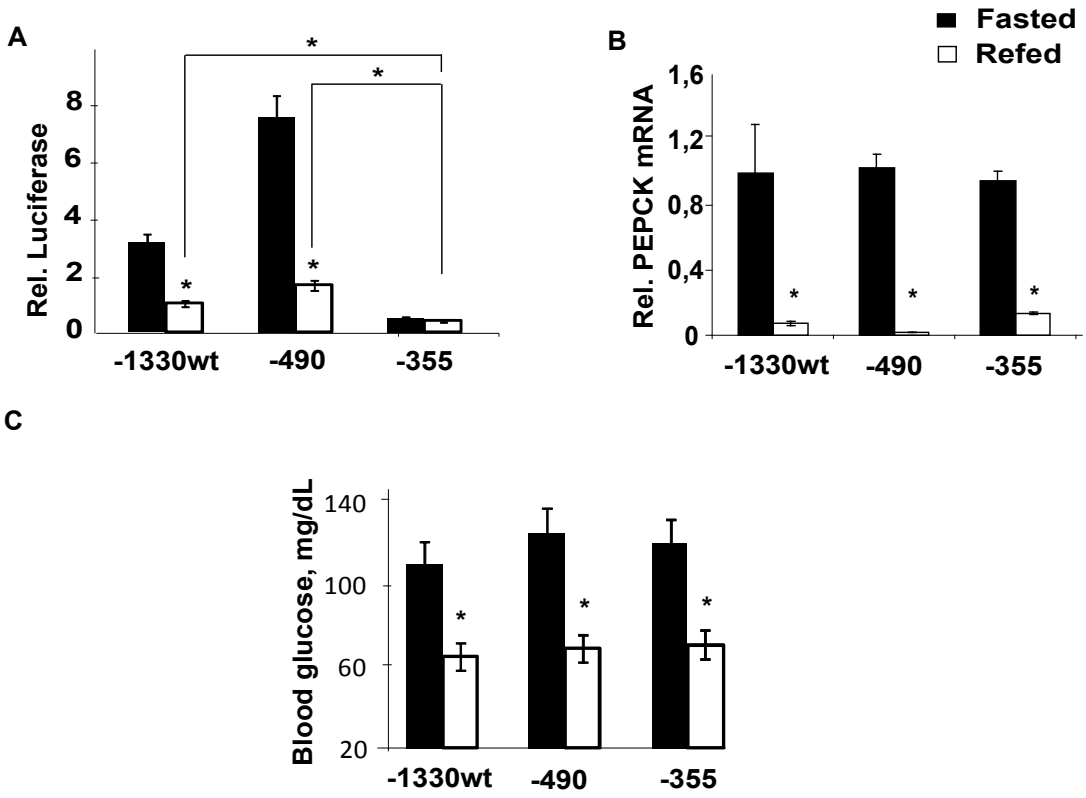


Figure 12 Adenovirus based promoter deletion technology enables the study of the function of a separate promoter unit within the entire 5' regulatory promoter region.

The structure and function of the PEPCK promoter and its deletions: -490 and -355 was described previously in Figure 6. Wt-mice were fasted for 12 hrs and refed for 8 hrs respectively. (A) The analysis

of luciferase reporter signal from -1330wt, -490 and -355 PEPCK reporter viral constructs. The luciferase activity depicted as a relative value that for any experimental group is significantly above the background. (B) The endogenous PEPCK mRNA level, RT-qPCR. Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). (C) Blood glucose level.
Data are presented as Mean ±S.E.M.; *p≤0,05, (N=3 animals per group)

As shown in Figure 12 A, the wt-PEP1330 promoter construct was strongly induced in response to fasting. The fold induction for this construct was low compared with the 24 hrs fasting and 24 hrs refeeding experiment (Figure 11), since the time of treatment was reduced to 12 and 8 hrs, respectively. Similar to the wt-1330 promoter, the PEPCK-490 deletion showed a 3-4 fold induction upon fasting. The higher activity of the short PEPCK-490 promoter construct could be explained by the presence of some inhibitory regulatory elements upstream from PEPCK-490 promoter region (Hanson 2005). Since glucocorticoids are an important fasting stimuli, the PEPCK-355 promoter deletion virus significantly lost most of its response to fasting (Figure 12 A). Remarkably, the basal promoter activity in refed state was also significantly reduced compare to the wild type promoter, suggesting an important role of GRU for basal PEPCK transcription *in vivo*. Thus, the role of particular promoter regions for gene expression can be analyzed by adenovirus based promoter analysis *in vivo.*
We next wanted to test if the system could be used for more detailed promoter analysis, such as the analysis of particular regulatory cis-sites through point mutations. Since the GRU consist of two GR binding sites (GR1 and GR2) and several accessory elements (AF1, AF2 and AF3) (Fig 4), we next mutated GR1 and GR2 sites and thus specifically disrupted the binding of GR but not the binding of others accessory factors. The mutation of these elements has been done within the PEPCK-490 promoter region and was previously described and tested (Scott, Stromstedt et al. 1998). The mutation of glucocorticoid response elements (GRE) GR1 and GR2 resulted in a strong decrease in basal and fasting-induced PEPCK promoter activity (Figure 13) that is fully consistent with the deletion of the entire GRU unit in vivo (Figure 12 A). Therefore, these results demonstrated that the system is specific and that the function of a particular responsive cis-element can be identified within a complex promoter structure *in vivo*.

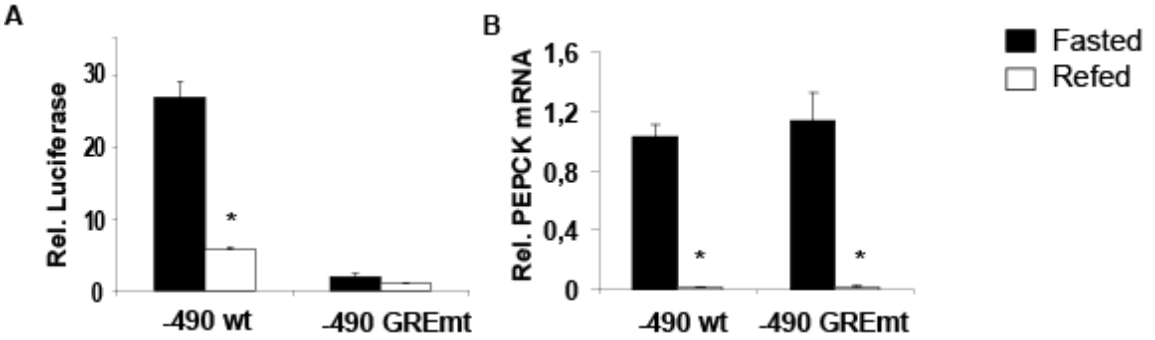


Figure 13 Adenoviral reporter system enables to identify the function of particular regulatory sites within the complex promoter structure in vivo.
The glucocorticoid response elements (GRE): GR1 and GR2 were specifically mutated within the -490 PEPCK promoter region integrated in adenoviral reporter system. Fasting-refeeding experiment was performed as described in Figure 12. (A) The analysis of luciferase reporter signal from wild type (-490wt) and mutated (-490 GREmt) PEPCK reporter viral constructs. The luciferase activity depicted as a relative value that for any experimental group is significantly above the background. (B) The endogenous PEPCK mRNA level, real time q-PCR. Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). Data are presented as Mean ±S.E.M.; *p≤0,05, (N=3 animals per group)

Taken together, we have demonstrated that the dissection of regulatory important blocks of the PEPCK promoter or the mutation of separate cis-regulatory elements within the promoter, integrated in an adenoviral reporter system provides an opportunity to study their role in signal-dependent regulation of gene expression *in vivo*, thereby further validating the functional integrity of the system.

3.3 Normalization and interpretation of results

The analysis of the *in vivo* adenoviral reporter system in response to fasting (3.2.1) revealed the significant difference for the luciferase activity in refed state between groups injected with wt-PEP1330 virus and with Luc. ctrl virus (Figure 14). Under refed conditions the endogenous PEPCK activity was blocked and therefore the reporter activity representing a background activity for the assay and should not differ between PEPCK promoter and control virus.

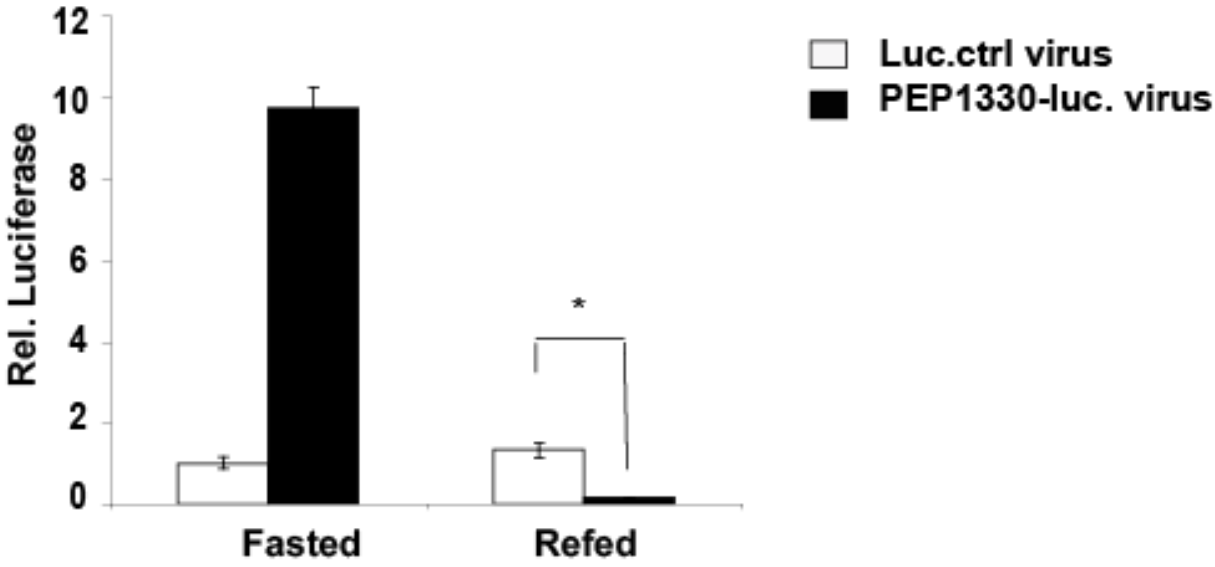
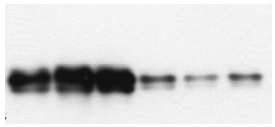


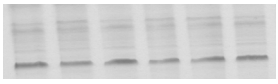
Figure 14 Normalization and interpretation of results presented in the first adenoviral test experiment (3.2.2).
Various background reporter activity of Luc. ctrl and wt-PEP1330 virus (in refed state) might indicate the unequal amount of viral reporter plasmid that was injected into these animals. Two viruses were used: PEP1330-luc.virus, directing the expression of luciferase from wild-type PEPCK promoter, and Luc. ctrl virus that has the same genetic background but lacks promoter elements upstream of luciferase gene and therefore cannot specifically express reporter enzyme. The conditions of the experiment and luciferase assay are the same as in Figure 11.

To this end we decided to examine the amount of virus in livers of Luc. ctrl and wt-PEP1330-virus injected animals. Since in that experiment we used the first generation of reporter adenoviruses[2] that constantly expressed GFP from CMV promoter, the amount of this protein in the liver was used to assess a relative number of viral particles that were injected in these tissues. The equal amount of total liver protein for every sample was used and western blot against GFP was performed.

[2] Next generations of viruses that were used for the functional validation of the system and promoter analyses in septic mice lacking CMV promoter as it was excised to avoid the crosstalk between the target promoter PEPCK and the CMV (Figure 5).



anti-GFP



PageBlue Protein staining

Luc.ctrl virus **PEP1330 virus**

Figure 15 Analysis of the viral dosage within liver tissues by anti-GFP western blot.
GFP (green fluorescent protein), liver samples and viruses are the same as in Figure 14.

The results demonstrated that the expression of the GFP in livers of Luc. ctrl injected animals was much higher than in PEPCK-1330 injected littermates (Figure 15), whereas the total liver protein did not differ between groups (Page Blue protein staining), suggesting unequal infection number for wt-PEP1330 and Luc. ctrl viruses in these animals.

The first virus titration method that was used at early steps of the system development (3.2.1) estimated the number of the viral particles by OD260 nm and could assess the total number of viral particles within the preparation (Herrmann, Abriss et al. 2004). We next assessed the viable virus number by a cell-based titration method and could see that the number of functional viral units varied significantly between different preparations compared to a total virus titer (data not shown). Since only functional viral particles are able to infect the liver for all next experiments we used a cell-based titration method, estimating only the titer of viable virus. The method that worked best in our hands was Tissue Culture Infectious $Dose_{50}$ ($TCID_{50}$) assay (see section 5).

Inexact tail-vein injection, differential redistribution of the virus within the liver with a blood stream could result in unequal distribution of the viral reporter plasmid within the analyzed tissue sample. To guarantee that the reporter enzyme signal depends only on the promoter activity for every experimental setting but not on the increased amount of the transduced plasmid expressing luciferase we had to design a method for in tissue virus normalization.

To this end, the same tissue piece that was taken for the luciferase assay was used for viral genome isolation (section 5). The total pool of cellular DNA that also includes adenoviral plasmid was first isolated. As it has been described above, the original pAd-shuttle vector had the GFP gene that was expressed from the CMV promoter. Despite that the CMV promoter was excised from the vector, the GFP gene sequence was still intact in all generations of reporter viral vectors that have been used for functional system validation and *in vivo*

promoter analyses in septic mice. Thereby for every analyzed liver sample we assessed quantitatively the relative number of viral genomes by highly sensitive real time q-PCR technique with Taqman probe against the GFP gene (see section 5). The obtained Ct value for the GFP gene between different samples in turn was normalized to the Ct value of housekeeping gene GAPDH, to guarantee the equal amount of total DNA pool, in which GFP-viral genome numbers were assessed for every sample.

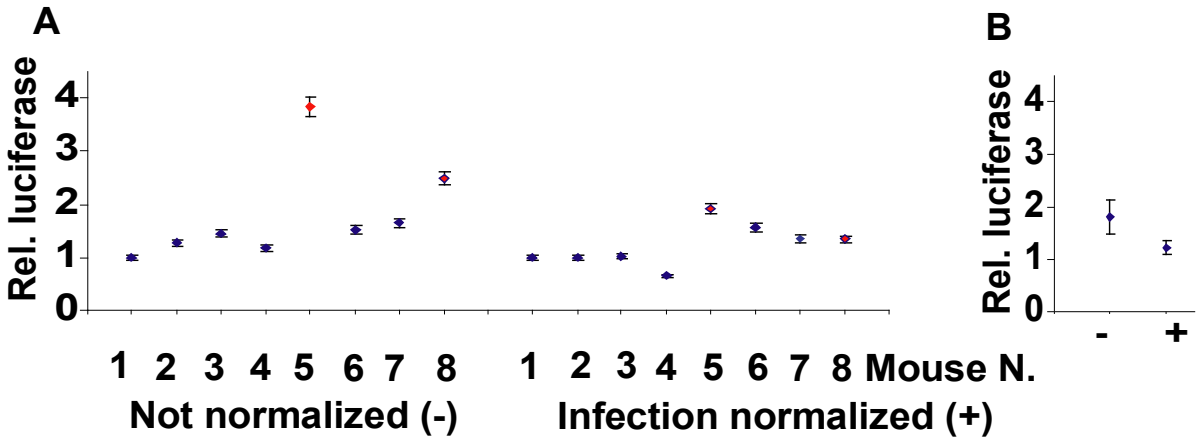


Figure 16 In tissue virus normalization could significantly reduce outliers (A) and a standard error (B) for the luciferase readout within the group of animals.
The luciferase readout of infection-normalized vs. non-normalized mouse samples for the same experiment is shown. Non-normalized are samples that were equilibrated only by the total protein amount used for the reporter assay and thus represent a raw luciferase activity. The infection-normalized are the samples where the raw luciferase activity was additionally normalized to relative infection rate assessed by q-PCR with a Taqman probe to viral specific GFP gene. The GFP Ct value for every sample is normalized against housekeeping gene GAPDH.

As shown for the raw data there were at least two mouse samples that lay much too far from the mid-line of the group (Figure 16). We next asked if these samples were natural outliers or that this was an artifact caused by increased plasmid DNA number for these samples. To this end, we normalized the raw luciferase activity to estimate by the GFP q-PCR infection factor. The results showed that the method significantly decreases the variation within the group by reducing outliers and drawing up samples of the group within one line. This suggests that in tissue virus normalization could substantially decrease the artifacts in the analysis of luciferase values for liver samples. Moreover, the analysis indicated that the method is sensitive even to very small differences enabling the accurate normalizing of reporter signals and significantly increasing the reliability of obtained data.

PEPCK and Inflammation

PEPCK gene expression in the liver plays a critical role in the maintenance of blood glucose level under stress conditions. During acute inflammation, interference with hormone signaling and the subsequent suppression of the PEPCK gene expression through pro-inflammatory mediators importantly contributes to the hypoglycemic phenotype and death in septic patients, the *in vivo* molecular mechanisms of which remain largely unknown (Yan, Gao et al. 2007).
In this study we aimed to identify molecular mechanisms involved in PEPCK gene dysregulation during sepsis *in vivo.*

3.4 Development of the LPS septic mouse model and characterization of the metabolic phenotype

Sepsis development is characterized by two stage kinetics: hypermetabolism associated with insulin resistance, wasting of glycogen stores in the liver and muscles and subsequent hyperglycemia, and hypometabolism characterized by the devastation of all glucose stores, blockade of the glucose compensatory pathway gluconeogenesis and subsequent severe hypoglycemia (Virkamaki and Yki-Jarvinen 1994; Sugita, Kaneki et al. 2002; Buras, Holzmann et al. 2005).

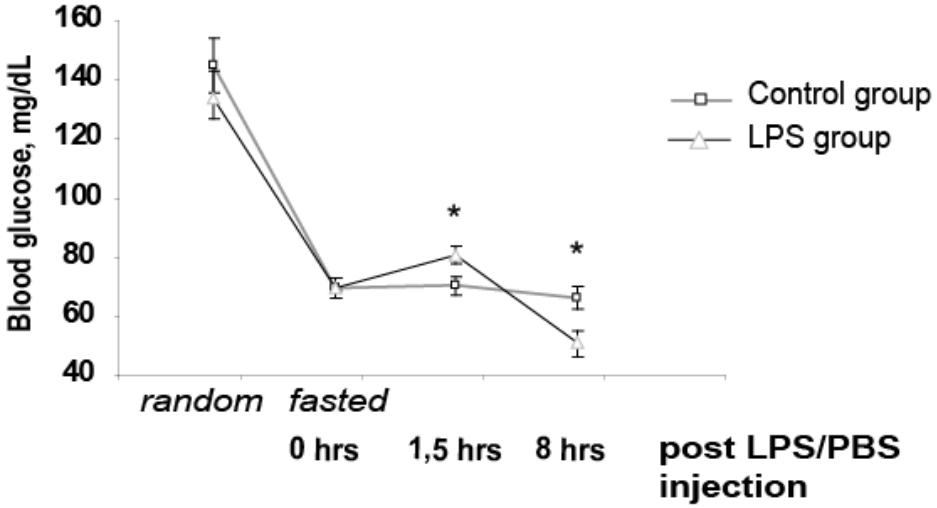


Figure 17 Kinetics of the blood glucose in LPS mice.
Random fed animals were fasted for 12 hrs and then injected intraperitonialy with either LPS (LPS group) or with PBS (Control group). The hyperglycemic stage after 1,5 hrs followed by severe hypoglycemia measured at 8 hrs post LPS injection, validating the development of a septic pathological phenotype.

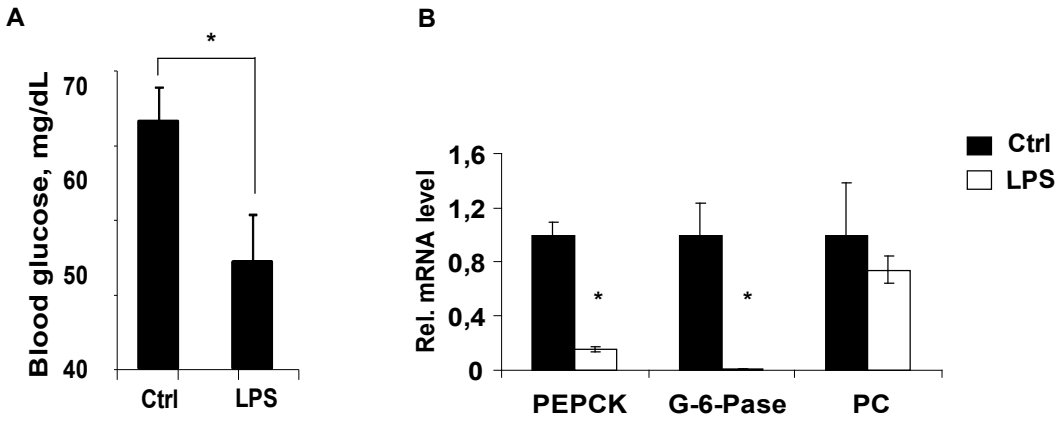


Figure 18 Hypoglycemia in septic mouse is associated with the suppression of key gluconeogenic enzyme genes.
The experiment was performed as in Figure 17. (A) Blood glucose level in septic animals at 8 hrs post LPS injection. (B) Endogenous mRNA level of key gluconeogenic enzyme genes: phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G-6-Pase) and pyruvate carboxylase (PC) measured by real time q-PCR. Ct value for every gene is normalized against housekeeping gene TATA box binding protein (TBP). Data are presented as Mean ±S.E.M.; *p≤0,05, (N=5 animals per group)

To mimic sepsis in animals we used the LPS-induced mouse model of sepsis. Wt C57BL6/j mice were fasted for 12 hrs to synchronize animals in term of food intake and gluconeogenic program activation, and injected interperitonealy with LPS (20 μg/ g body weight) or PBS as a control. The septic phenotype was monitored by different physiological parameters: blood glucose kinetics (hyperglycemic and hypoglycemic state), diarrhea and fatigue state. Since the septic group developed anorexia, all animals were maintained without food over the entire experiment. Mice were sacrificed at 8 hrs post LPS treatment.

As depicted in Figure 17 and Figure 18 A after endotoxin (LPS) injection mice developed hyperglycemia that peaked at 1,5 hrs followed by strong hypoglycemia measured after 8 hrs post LPS injection, validating the development of septic metabolic phenotype. We showed that the expression of key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-posphatase (G-6-Pase), but not pyruvate carboxylase (PC) were strongly suppressed (Figure 18 B), confirming the blockade of the gluconeogenesis.

We next analyzed the blood serum of septic mice. There was a trend for higher serum free fatty acids (FFA) suggesting the activation of a lipolysis program by peripheral tissues and diminished level of serum ketone bodies indicative of decreased hepatic β-oxidation (Figure 19 A, B).

Consistent with previous studies (Webster and Sternberg 2004; Allary and Annane 2005), the level of endogenous glucocorticoid hormone, corticosterone, the key activator of the gluconeogenesis in the liver, was significantly increased in septic animals (Figure 19 C), however it could not rescue the impaired PEPCK expression.

The inflammatory cytokines: Ifn γ, IL-1β, IL-6, TNF-α were strongly induced in the serum indicating the active inflammatory process (Figure 19 D). The abdominal fat mass was increased (Figure 19 E) probably due to enhanced water infiltration (Farand, Hamel et al. 2006).

Taken together, our results showed that 8 hrs LPS-triggered endotoxemia caused a complex metabolic phenotype characterized by dysregulations of glucose metabolism (hypoglycemia), increased blood serum concentration of regulatory hormones (corticosterone) and inflammatory cytokines, and a suppression of key metabolic enzyme genes PEPCK and G-6-Pase.

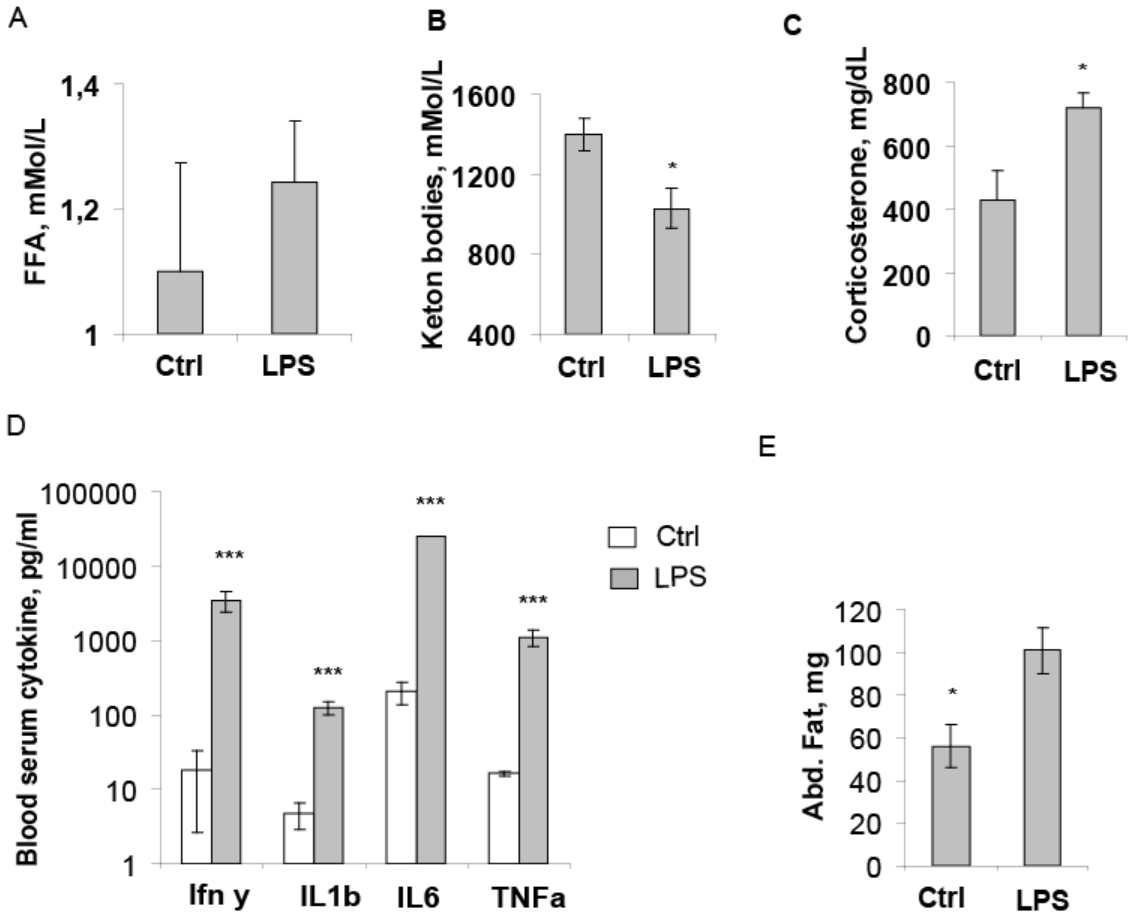


Figure 19 Analysis of blood serum components in septic mice.
(A-D) Free fatty acids (FFA), Ketone bodies, Corticosterone, Cytokines (E) Abdominal fat mass (Abd. Fat).The experiment was performed as in Figure 17. Abbreviations: Control group of animals (Ctrl),

Septic group of animals (LPS), Interferon γ (Ifn γ), Interleukin 1β (IL1b), Interleukin 6 (IL6), Tumor necrosis factor α (TNFa). Data are presented as Mean ±S.E.M.; * p≤0,05, ***p≤0,001, (N=5 animals per group)

3.5 Imitation of septic inflammatory conditions in cell culture

After characterization of the LPS septic inflammatory model *in vivo*, we tried to mimic the inflammatory environment in cell culture.

As we have seen from results above (Figure 19 D), the inflammatory process provoked the secretion of different cytokines by immune cells. Many of them were shown to suppress PEPCK gene expression (Waltner-Law, Daniels et al. 2000), (Yan, Gao et al. 2007). We assumed that multiple stimuli are responsible for PEPCK suppression and are working through mutual or separate regulatory pathways. To mimic inflammatory conditions in cultured hepatocytes, we used an inflammatory cocktail from LPS induced cultured macrophages (Raw 264.7 cells). The stimulation of Raw 264.7 cells with LPS results in cytokines secretion into the cultured medium (Samokhvalov, Bilan et al. 2009). This conditioned medium further was used as a trigger of inflammatory signaling in hepatocytes.

We first examined this system in isolated primary hepatocytes from 10 week old C57Bl6/j wt mice. To separate signaling pathways important for PEPCK gene induction we induced primary cells with Dex, Fsk or both hormones simultaneously followed by the treatment of cells with inflammatory conditioned medium (Raw) or LPS alone. As control for non-inflammatory conditions, we used the medium from cultured macrophages Raw 264.7 cells that had not been activated by LPS. The Raw inflammatory conditioned medium dramatically suppressed basal as well as hormone-induced expression of key gluconeogenic genes PEPCK, G-6-Pase and only to a small extent PC (Figure 20), suggesting cell-autonomous repressive effect of inflammatory environment on gluconeogenic pathway. Interestingly, under some conditions LPS alone also suppressed the expression of PEPCK and the suppression was even more pronounced for G-6-Pase. In the same hepatocytes, we could show a local production of cytokines, e.g. TNF-α under inflammatory conditions. The level of TNF-α expression induced in hepatocytes by LPS treatment was as high as for the complete Raw inflammatory conditioned medium (Figure 21). Therefore, through the stimulation of a local cytokine production in hepatocytes the LPS alone might additionally contribute to the gluconeogenic gene suppression. Taken together, the Raw inflammatory conditioned medium that includes LPS and a cytokine mixture from cultured macrophages suppressed gluconeogenic genes

(PEPCK, G-6-Pase and PC) in the similar pattern as did septic inflammatory conditions *in vivo* thereby representing a good cell culture model to mimic a septic phenotype.

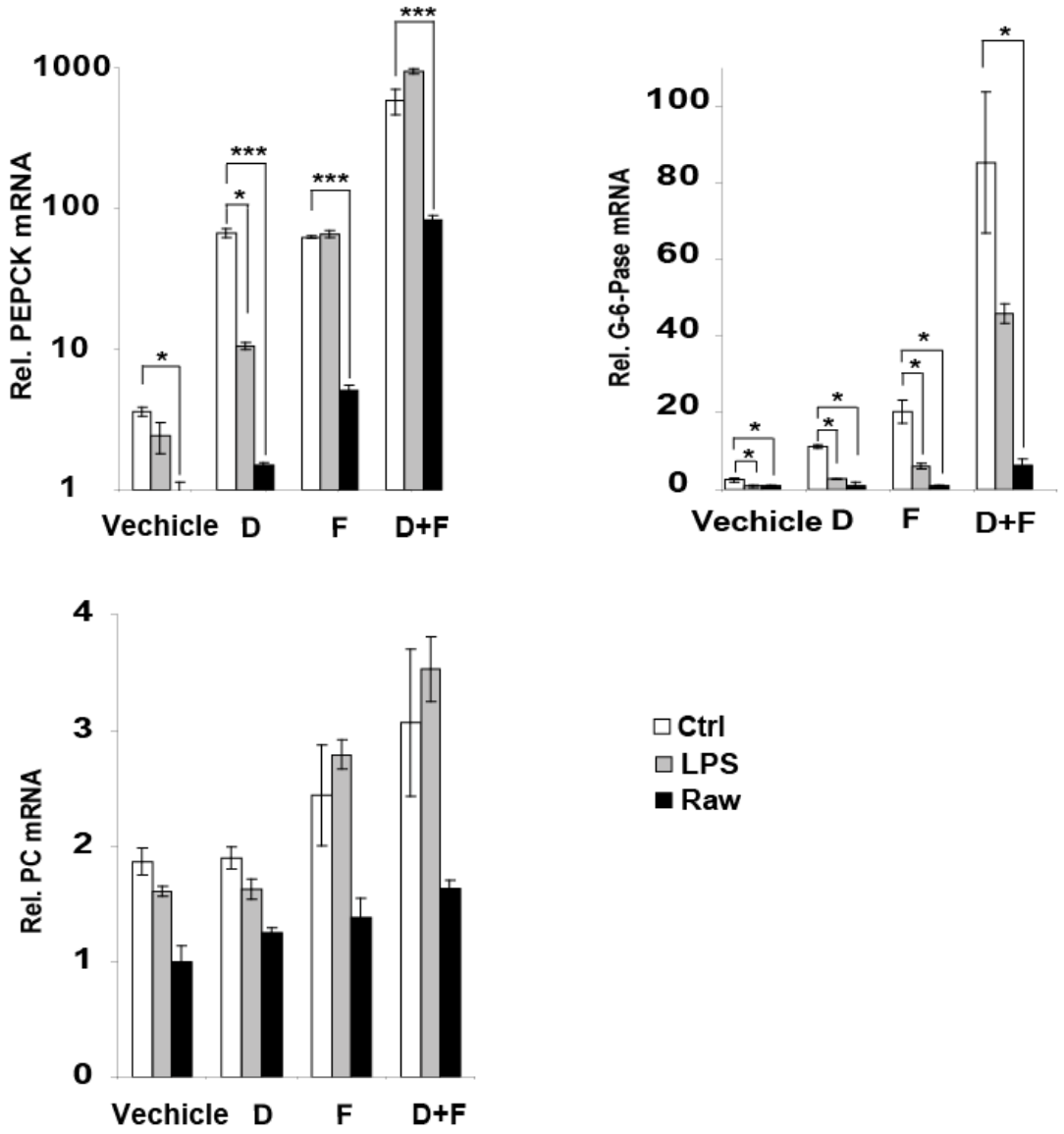


Figure 20 Mimicking of septic inflammatory conditions in cell culture.
The inflammatory conditioned medium from Raw 264.7 macrophages stimulated with lipopolysaccharide (LPS, 100ng/ml) for 4 hrs (Raw) was applied to isolated primary hepatocytes. The pattern of gluconeogenic genes suppression was assessed by real time q-PCR. Hepatocytes were treated with the cultured medium from Raw 264.7 macrophages that were not exposed to LPS (Ctrl), with lipopolisaccharide alone (LPS) or with Raw inflammatory conditioned medium (Raw) under basal (Vehicle), dexametasone (D), forskolin (F) or simulations (D+F) stimulated conditions. Abbreviations: phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphotase (G-6-Pase), pyruvate carboxylase (PC). Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). Data are presented as Mean ±S.E.M.; * p≤0,05, (N=3)

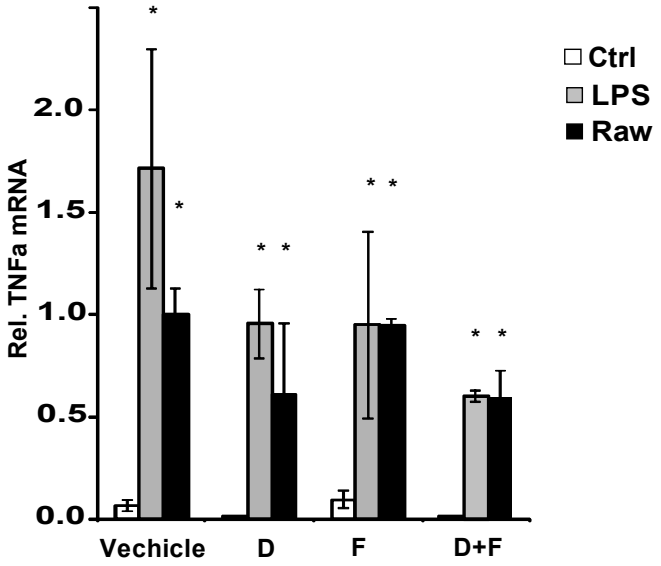


Figure 21 Cell- autonomous expression of the TNF-α in primary hepatocytes under inflammatory conditions.
The experiment and all treatments as in Figure 20. TNF-α expression was measured by real time q-PCR. Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). Data are presented as Mean ±S.E.M.; * p≤0,05, (N=3)

3.6 **Mutation of key PEPCK promoter response elements**

We next wanted to map inflammatory responsive sites of PEPCK gene promoter under of Raw inflammatory conditions in H4IIE cultured hepatocytes.

The -490 promoter region has been described to be sufficient to maintain PEPCK basal and hormone induced gene expression (Hanson 2005). Within this promoter region we mutated several important PEPCK promoter cis-regulatory elements. These were GRE, CRE, AF1, AF2 and AF3 sites (Figure 4). The mutations were introduced based on the pGL3 PEPCK-490wt vector by site-directed mutagenesis. The sequences of all PEPCK promoter mutations have been described and tested previously by others (section 5). The cloning strategy enables further reintegrate the generated PEPCK promoter mutations in the adenoviral shuttle vector pAd-Track Luciferase by *Bgl II* and *Kpn I* restriction sites and develop corresponding promoter viruses.

3.7 Screening for the putative PEPCK promoter inflammatory responsive sites in cultured hepatocytes

To identify inflammatory responsive sites of the PEPCK promoter we next examined pGL3-PEPCK-490 promoter mutation constructs in H4IIE cultured hepatocytes treated with Raw inflammatory conditioned medium. To dissect pathways through which suppression of the PEPCK might work, cells were induced either with Dex or Fsk or with both hormones simultaneously. The culture medium from Raw macrophages that were not treated with LPS was used as a control. It was technically difficult to test all constructs for every condition at once, so we had to relate every construct to the wt PEPCK-490 promoter activity under control conditions. To this end, along with every mutated vector the reference wt PEPCK-490 plasmid was included. As shown in Figure 22, we had quite a strong variation between different experiments however the trend of regulation for every condition was quite consistent. For some mutations such as CRE, AF1 and AF3 the attenuation of the suppressive effect of the Raw conditioned medium referred to wt-PEPCK-490 was observed, in particular under Fsk stimulated conditions. However, none of the mutations could ablate the PEPCK suppression by inflammatory cocktail for all Dex, Fsk and Dex/Fsk conditions (Figure 22).
Since it was difficult to conclude from these results which pathway is suppressed under septic inflammatory conditions the application of the system for PEPCK promoter analysis *in vivo* was necessary.

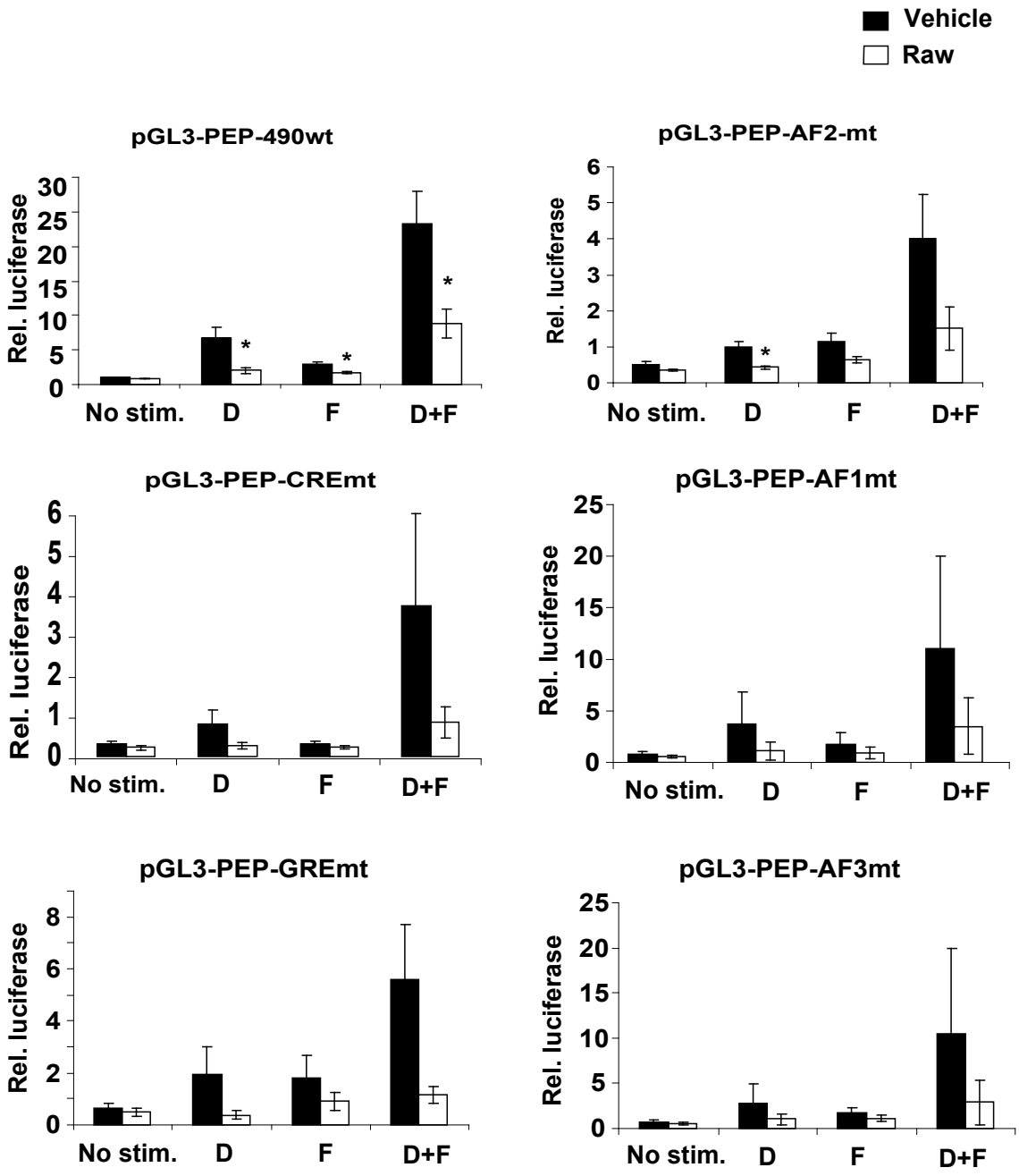


Figure 22 Mutation of any single PEPCK regulatory site does not ablate the inflammatory suppressive effect in H4IIE hepatocytes.

Cells were transfected with the wild type pGl3-PEPCK-490 vector and one of the pGl3-PEPCK-490 promoter mutations as described in 3.4.3. Cells were treated with the culture medium from Raw 264.7 macrophages that were not exposed to LPS (Ctrl) or with Raw inflammatory conditioned medium (Raw) under basal (Vehicle), dexametasone (D), forskolin (F) or simulations (D+F) stimulated conditions. All the luciferase values are significantly above background and are relative to -490 wt PEPCK not stimulated/vehicle group. The luciferase activities are normalized to β-Galactosidase that was constantly expressed from HRAS promoter vector, co-transfected for all conditions. Data are presented as Mean ±S.E.M.; * p≤0,05, (N=3) for at least 3 independent experiments.

3.8 Identification of the inflammatory responsive sites within the PEPCK promoter in septic mice

To address the question as to the mechanism of the PEPCK gene suppression during sepsis *in vivo*, we next applied the adenoviral PEPCK promoter reporter system in mouse model of LPS-induced sepsis.

3.8.1 PEPCK-490 promoter region is sufficient to mediate PEPCK suppression under inflammatory conditions in sepsis

We first asked if the -490bp promoter region is sufficient for PEPCK promoter suppression by *in vivo* septic inflammatory environment. To this end, the adenoviral wt-1330 PEPCK promoter construct was tested along with -490 PEPCK promoter deletion construct in a LPS septic mouse.

The10 week old C57Bl6/j mice (N=5) were injected with viral reporters 1x10(9) Ifu per mouse through the tail vein. Both experimental groups -1330 PEPCK and -490 PEPCK were divided in 2 subgroups (N=5): LPS-injected septic and PBS control animals. The LPS experiment was performed 5 days after the virus injection as described above. As shown in Figure 23 C in both groups -1330 PEPCK and -490 the significant hypoglycemia was observed under septic conditions (LPS). This phenotype was associated with an at least 3 fold suppression of endogenous PEPCK mRNA for both experimental groups as assessed by real time q-PCR, suggesting that the experiment technically worked for all conditions.

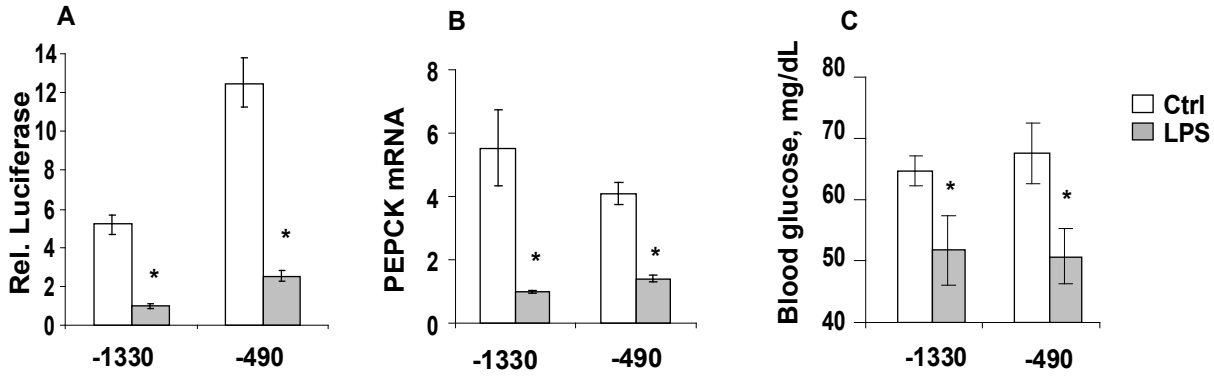


Figure 23 PEPCK-490 promoter region is sufficient to mediate PEPCK suppression under inflammatory conditions in sepsis.
The C57Bl6/J mice were injected with promoter reporter adenoviruses: PEPCK-1330 wt and PEPCK-490 through the tail-vein, 1x10(9) Ifu per mouse. After 5 days animals were fasted for 12 hrs and treated with either lipopolysaccharide (LPS) or with PBS (Ctrl). (A) Rel. luciferase activity, normalized to in tissue viral genome numbers (B) Endogenous PEPCK mRNA, real time q-PCR. Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). (C) Blood glucose. Data are presented as Mean ±S.E.M.; *p≤0,05, (N=5 animals per group)

The wt PEPCK-1330 reporter virus showed an up to five fold suppression of the luciferase activity upon LPS treatment. The same fold suppression was seen also for PEPCK-490 reporter virus (Figure 23 A). These results suggested that -490bp promoter region is largely responsible for the wt-PEPCK promoter suppression, allowing us to map the inflammation responsive sites of the PEPCK promoter within this region.

3.8.2 In vivo role of the GRE for PEPCK promoter suppression

It has been previously shown that mice with an increased glucocorticoid receptor (GR) gene dosage show enhanced resistance to stress and endotoxic shock (Reichardt, Umland et al. 2000). We could see that the increased serum corticosterone could not rescue PEPCK gene suppression (Figure 19 C). The dysregulation of the GR binding to its cognate responsive site could explain this effect and might represent a promising mechanism of PEPCK gene suppression. Therefore we chose to examine glucocorticoid response elements (GRE) GR1 and GR2, (Figure 4), as a first candidate target site for PEPCK gene suppression in sepsis.
We specifically mutated GR1 and GR2 sites within PEPCK-490bp promoter reporter adenovirus (see 3.2.3). This mutated viral construct was injected in mice along with PEPCK -490wt virus and the LPS treatment was performed as described above. The experimental conditions were monitored at the endogenous PEPCK expression and blood glucose levels (Figure 24 B, C).
The mutation of the GRE (GRE mt) specifically decreased PEPCK activation upon fasting. However, the diminished PEPCK GRE mt promoter activity was still significantly suppressed around 3 fold under septic conditions, thereby representing the same extent as the suppression of the wt PEPCK-490 promoter virus (Figure 24 A).
Taken together, these results suggested that the GRE is not directly mediating PEPCK gene suppression by inflammatory environment in LPS septic mice.

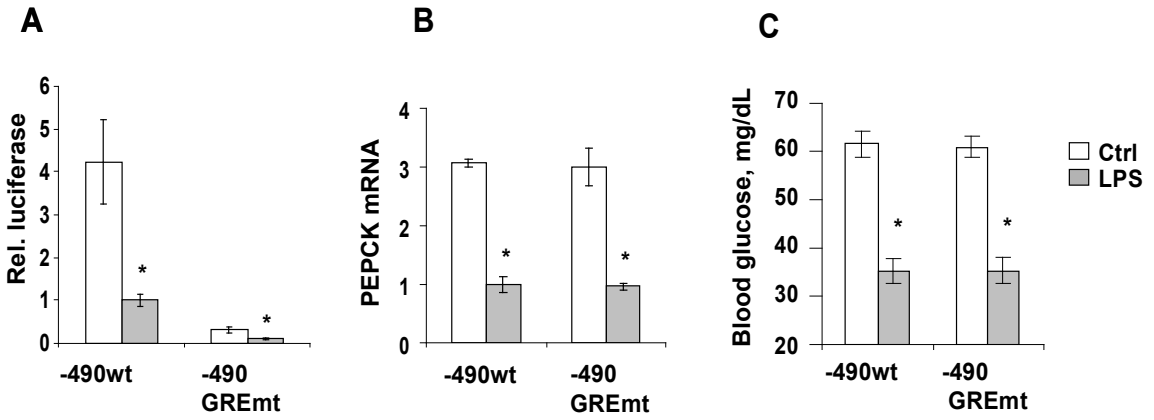


Figure 24 Mutation of PEPCK promoter glucocorticoid response elements (GRE) does not blunt PEPCK gene suppression in septic mice.
Mice were injected with wild-type PEPCK-490 promoter reporter (-490wt) or with the -490 promoter reporter carrying the specific mutation in GRE that blunts glucocorticoid receptor binding (-490 GREmt). Viruses were injected through the tail-vein, 1x10(9) Ifu per mouse and the experiment was performed as in Figure 23. (A) Rel. luciferase activity, normalized to in tissue viral genome numbers (B) Endogenous PEPCK mRNA, real time q-PCR. Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). (C) Blood glucose. Data are presented as Mean ±S.E.M.; *p≤0,05, (N=5 animals per group)

3.8.3 In vivo role of the CRE for PEPCK promoter suppression

The suppressive effect of TNF-α on cAMP-mediated PEPCK expression has been mapped to the cAMP response element (CRE) in H4IIE cell culture by others (Yan, Gao et al. 2007). Consistently, our own results demonstrated that the mutation of the CRE attenuated PEPCK gene suppression by the Raw inflammatory conditioned medium under cAMP-induced as well as under Dex-stimulated conditions. Therefore, we next explored the role of the CRE promoter element for the inflammation-triggered PEPCK gene suppression *in vivo*.

We first tested PEPCK-490 CRE-mt virus in fasting-refeeding experiment as described in 3.2. The mutation of the CRE strongly attenuated the PEPCK fasting response that was consistent with our previous results for the mutation of another key regulatory element GRE or the deletion of the entire GRU and emphasizes the important role of synergistic induction of these two promoter regions for the PEPCK activation (Figure 25).

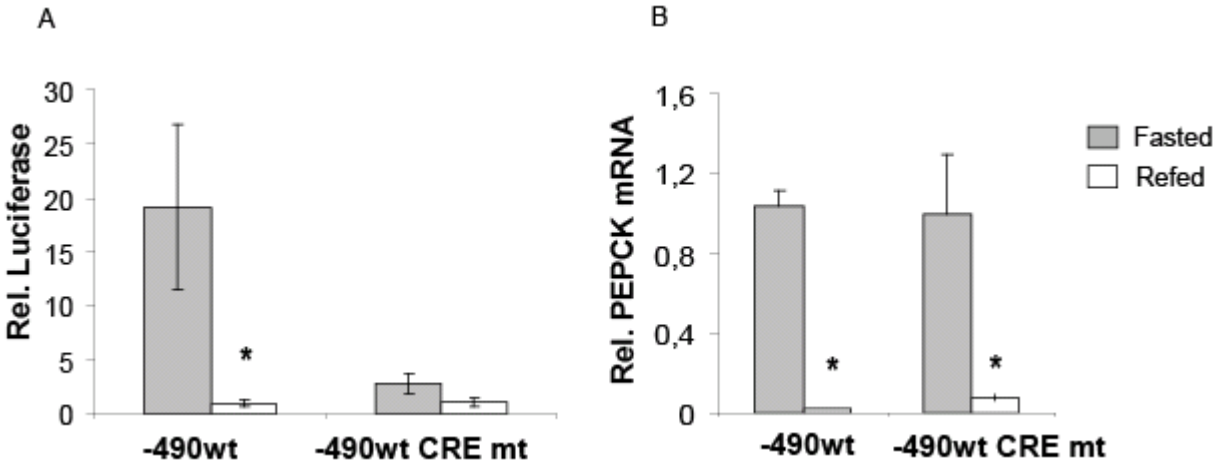


Figure 25 Testing the PEPCK-490 cAMP response element (CRE) promoter mutation virus in fasting-refeeding experiment.
Two PEPCK reporter viruses: the wild type (-490 wt) and -490 with mutated CRE element (-490 CRE mt) were injected through the tail-vein, 1x10(9) Ifu per mouse. The experiment was performed as in Figure 12. (A) Rel. luciferase activity normalized to in tissue viral genome numbers (B) Endogenous PEPCK mRNA, real time q-PCR. Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). Data are presented as Mean ±S.E.M.; *p≤0,05, (N=3 animals per group)

We next used PEPCK-490 CRE mt reporter virus along with a wt PEPCK-490 reporter construct in LPS mouse experiment. After triggering sepsis in mice the blood glucose and endogenous PEPCK mRNA levels were suppressed demonstrating that the experiment worked for both groups of animals injected with promoter viruses (

Figure 26 B, C). The mutation of the CRE significantly blunted the PEPCK suppression by the septic conditions *in vivo* (

Figure 26 A). Therefore the CRE represents a PEPCK inflammation responsive site *in vivo*.

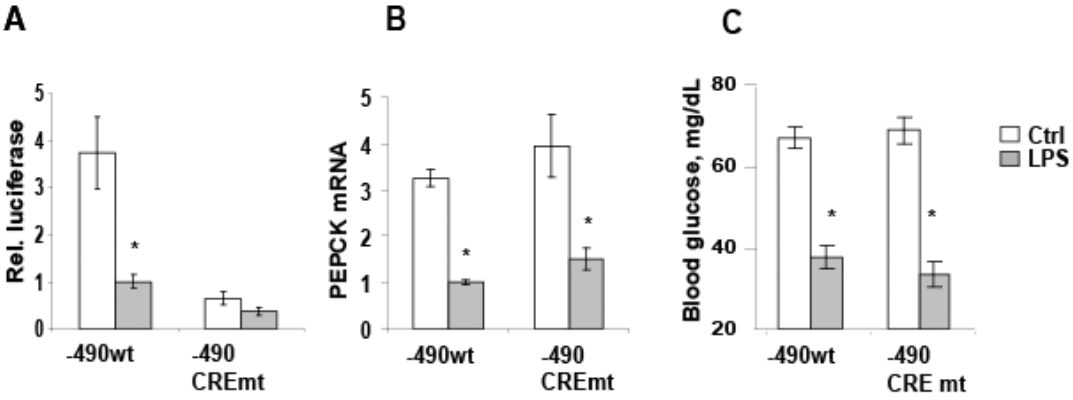


Figure 26 Mapping of the cAMP response element (CRE) as an *in vivo* inflammatory responsive site of PEPCK promoter in LPS septic mouse.
Two PEPCK reporter viruses: the wild type (-490 wt) and with mutated CRE element (-490 CRE mt) were injected through the tail-vein, 1x10(9) Ifu per mouse. The LPS experiment was performed as in Figure 23. (A) Rel. luciferase activity normalized to in tissue viral genome numbers (B) Endogenous PEPCK mRNA, real time q-PCR. Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). (C) Blood glucose level. Data are presented as Mean ±S.E.M.; *p≤0,05, (N=5 animals per group)

3.8.4 In vivo role of the GRU for PEPCK inflammatory response

To verify if the CRE is sufficient to mediate the suppression of PEPCK through inflammatory stimuli we next used a PEPCK-355bp promoter deletion virus that contains the intact CRE but lacks the entire glucocorticoid responsive unit (GRU), Figure 6. This viral deletion construct was tested along with full length wt PEPCK-1330 bp promoter virus in an LPS mouse experiment as described above. For both groups (-1330 and -355), we ascertained the suppression of blood glucose and endogenous PEPCK mRNA levels under septic conditions demonstrating that the experiment technically worked (Figure 27 B, C). Strikingly, the deletion of the PEPCK GRU completely blunted the PEPCK suppression in LPS septic mice (Figure 27 A). The suppression of a wt PEPCK-1330 promoter virus for the same experiment was around 5 fold. Therefore the deletion of the GRU could also blunt the inflammatory effect of sepsis demonstrating its role as a PEPCK inflammatory responsive site. These results suggested that CRE and its associated transcriptional complexes were not directly targeted by inflammation but rather represented a part of a more complex promoter regulatory organization, mediating the effect of acute inflammation on the PEPCK gene expression.

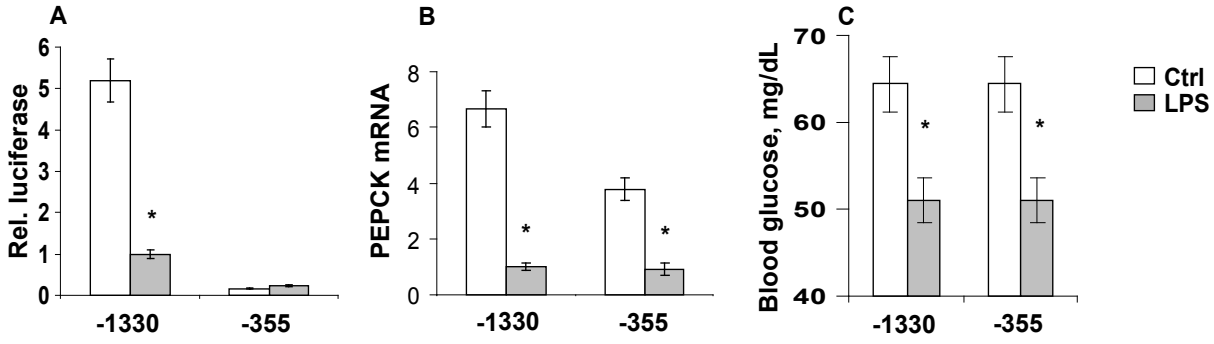


Figure 27 PEPCK glucocorticoid response unit (GRU) is identified as a second inflammatory responsive region of the PEPCK promoter in LPS septic mouse.
Two PEPCK reporter viruses: wild type (-1330 wt) and (-355) deletion that lacks entire GRU promoter region were injected through the tail-vein, 1x10(9) Ifu per mouse. The LPS experiment was performed

as in Figure 23. (A) Rel. luciferase activity normalized to in tissue viral genome numbers (B) Endogenous PEPCK mRNA, RT-qPCR. Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). (C) Blood glucose level. Data are presented as Mean ±S.E.M.; *p≤0,05, (N=5 animals per group)

3.9 Identification of the inflammatory targeted transcriptional complexes mediating PEPCK gene suppression

3.9.1 Expression profiling of key transcriptional factors regulating PEPCK gene expression revealed PGC1-α co-activator as a target of inflammatory signaling in septic mouse liver

Based on the mapping of the CRE and the GRU as sepsis-responsive sites, we next wanted to identify a protein or protein complex that could mediate PEPCK suppression by inflammation. To this end, we performed the expression profiling of the key PEPCK regulatory transcriptional factors and co-activators that bind to PEPCK-490bp promoter in the livers of septic mice. As shown in Figure 28, the inflammatory environment in the liver after 8 hrs of septic development did not change the expression of the majority of important DNA binding transcriptional factors except for Foxo1 and PGC-1α, suggesting that these proteins might be involved in PEPCK gene suppression in sepsis.

A

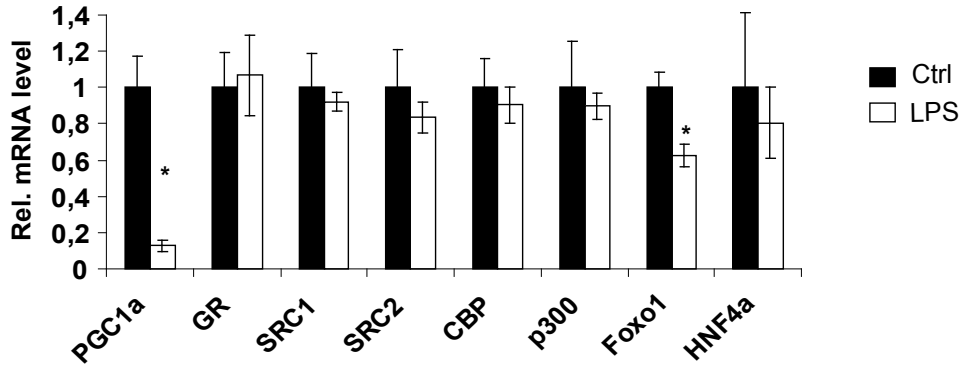


Figure 28 Gene expression profiling of key PEPCK transcriptional factors and co-activators in septic mouse liver.
The LPS experiment was performed as described in Figure 23. Gene expression was assessed by real time q-PCR. Ct values for every gene are normalized against housekeeping gene TATA box binding protein (TBP). Data are presented as Mean ±S.E.M.; *p≤0,05, (N=5 animals per group).
Abbreviations: Animal control group (Ctrl), animal septic group (LPS), glucocorticoid receptor (GR); CREB binding protein or p300 (CBP, p300); peroxisome proliferator activated receptor γ co-activator 1 alpha (PGC1a); sterol receptor coactivator 1 (SRC1), forkhead transcription factor 1 (Foxo 1); Hepatocyte nuclear factor 4 alpha (HNF4a).

3.9.2 *Cell-autonomous suppression of the PGC-1α expression with pro-inflammatory stimuli in primary hepatocytes*

Given the function of PGC-1α as an important co-activator that bridges different key PEPCK promoter regulatory units and is largely responsible for synergistic PEPCK activation by regulatory hormones glucagon and glucocorticoids (Herzig, Long et al. 2001), we decided to gain insight into the role of this protein in inflammatory signaling. To this end, we first checked the expression of the PGC-1α in primary hepatocytes treated with Raw inflammatory conditioned medium or LPS alone. Consistent with the suppression of the endogenous PGC-1α mRNA in septic mouse liver (3.9.1) we showed the cell autonomous suppression of PGC-1α gene with pro-inflammatory stimuli such as LPS or Raw conditioned medium (Figure 29), suggesting it as a target of impaired septic signaling.

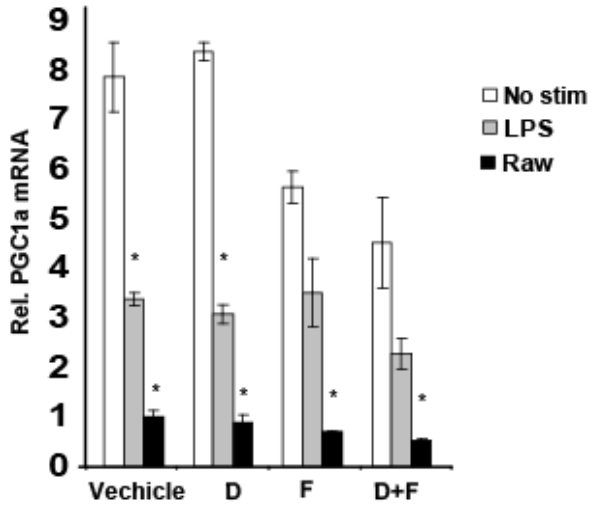


Figure 29 Cell-autonomous inhibition of the PGC-1α expression by pro-inflammatory stimuli in primary hepatocytes.
The effect of lipopolisaccharide alone (LPS) or Raw macrophage inflammatory conditioned medium (Raw) on PGC-1α expression is shown. The experiment was performed as in Figure 20. Real time q-PCR, Ct values are normalized against housekeeping gene TATA box binding protein (TBP). Data are presented as Mean ±S.E.M.; * p≤0,05, (N=3)

3.9.3 *PGC-1α transcription is directly repressed through inflammatory signaling*

We next asked if the decreased PGC-1α mRNA level under inflammatory conditions was due to the suppression of PGC-1α gene transcription. To this end, we transfected H4IIE cells with

a pGl3 vector carrying -1000bp of the PGC-1α promoter directing the expression of luciferase. The transfection was followed by the treatment of cells with a Raw inflammatory conditioned medium. As a positive control, a responsive promoter region of a key pro-inflammatory transcriptional factor NFkB (3xNFkB) directing the luciferase expression was used under the same conditions. The development of 3xNFkB luciferase reporter was described previously (Herzig, Long et al. 2001).

PGC-1α promoter activity was significantly diminished after the treatment of cells with Raw macrophage inflammatory conditioned medium while strongly inducing the activity of promoter carrying NF-kB responsive elements (Figure 30). These results indicated that at least in part the decrease in PGC-1α expression was due to the direct suppression of PGC-1α transcription. They also verified that the Raw conditioned medium indeed activate inflammatory signaling in particular the expression the NFkB that is known to be responsible for the induction of a variety of pro-inflammatory genes (Glass and Ogawa 2006).

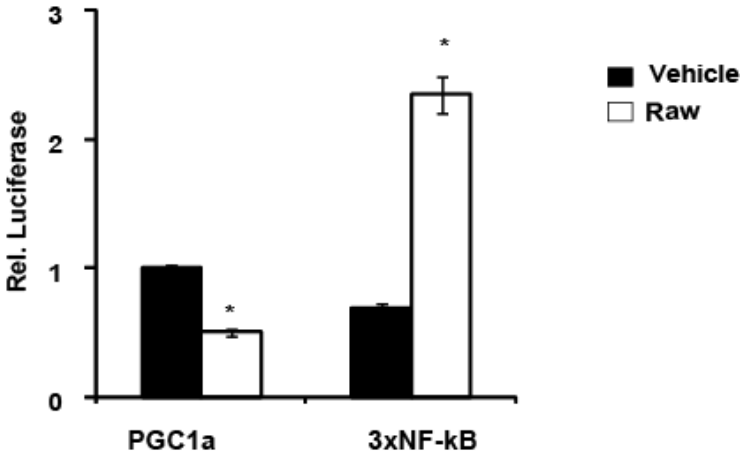


Figure 30 Suppression of the PGC-1α transcription by inflammatory environment.
PGC-1α -1000bp promoter construct directing the expression of the luciferase was transfected in H4IIE hepatocytes. 3xNF-kB luciferase reporter plasmid was used as a positive control for inflammatory conditions. Cells were treated with the culture medium from Raw 264.7 macrophages that were not exposed to LPS (Vehicle) or with Raw inflammatory conditioned medium (Raw). The luciferase activity is normalized to β-Galactosidase (β-Gal) activity. The β-Gal was constantly expressed from HRAS promoter in the vector that was co-transfected for every experimental condition. The reporter activity is depicted as a relative value that for any experimental group are significantly above the background. Data are presented as Mean ±S.E.M.; * p≤0,05, (N=3)

3.9.4 Transient over-expression of PGC-1α co-activator in septic mouse liver is not sufficient to rescue PEPCK suppression

We next decided to transiently restore the PGC-1α level in the liver of septic mice with PGC-1α expressing adenovirus (Yoon, Puigserver et al. 2001), (Herzig, Long et al. 2001). The

adenoviral construct expressing PGC-1α from CMV promoter was injected through the tail-vein to get liver specific PGC-1α over-expression. The control group of animals was injected with a GFP-expressing adenovirus that has the same genetic background but not express PGC-1α protein. After 5 days, mice tended to develop an increase in random blood glucose that became significant after fasting of the animals. The hyperglycemia in mice with PGC-1α over-expression is consistent with the function of PGC-1α to co-activate the gluconeogenic program especially under fasting conditions (Herzig, Long et al. 2001), thus confirming the functional integrity of the viral infection.

We next performed LPS experiment as described above. The analysis of results showed that after 8 hrs post-LPS injection, the expression of endogenous PGC-1α in control GFP-virus group was strongly suppressed (Figure 32).

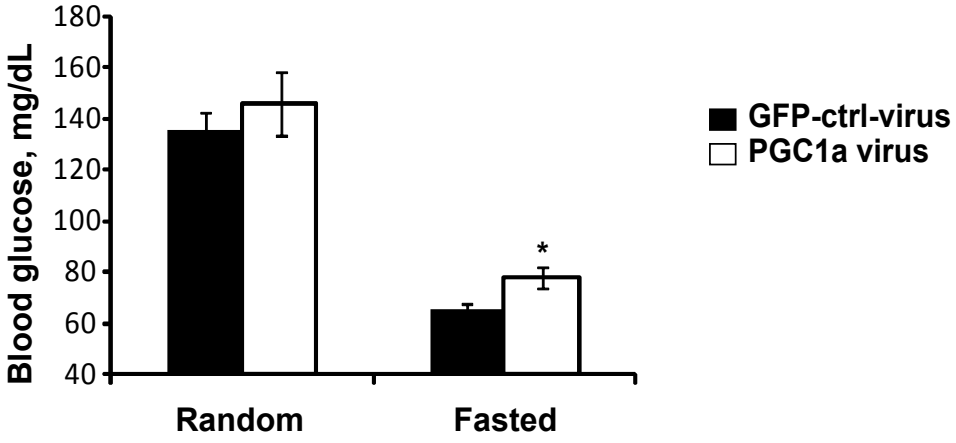


Figure 31 Transient over-expression of the PGC-1α in the liver of wild type mice results in fasting hyperglycemia.

The adenovirus expressing PGC-1α from CMV promoter (PGC1a virus) and the control virus expressing only GFP (GFP-ctrl virus) were injected through the tail-vein, 1x10(9) Ifu per mouse. After 7 days the blood glucose level under random and overnight fasted state was measured. Data are presented as Mean ±S.E.M.; * p≤0,05, (N=7 animals per group)

Animals in which PGC-1α was over-expressed through virus delivery had around 3 fold increased PGC-1α mRNA levels and after triggering of sepsis there was no significant decrease in PGC-1α expression (Figure 32 A). The PEPCK expression in both GFP virus-injected control and PGC-1α over-expressing group was suppressed. At comparable levels a slight increase in PEPCK expression in LPS mice upon PGC-1α over-expression was not sufficient to restore PEPCK mRNA to the level of control littermates (Figure 32 B). As shown in Figure 32 C, the PGC-1α over-expression did also not improve the hypoglycemic phenotype of these animals.

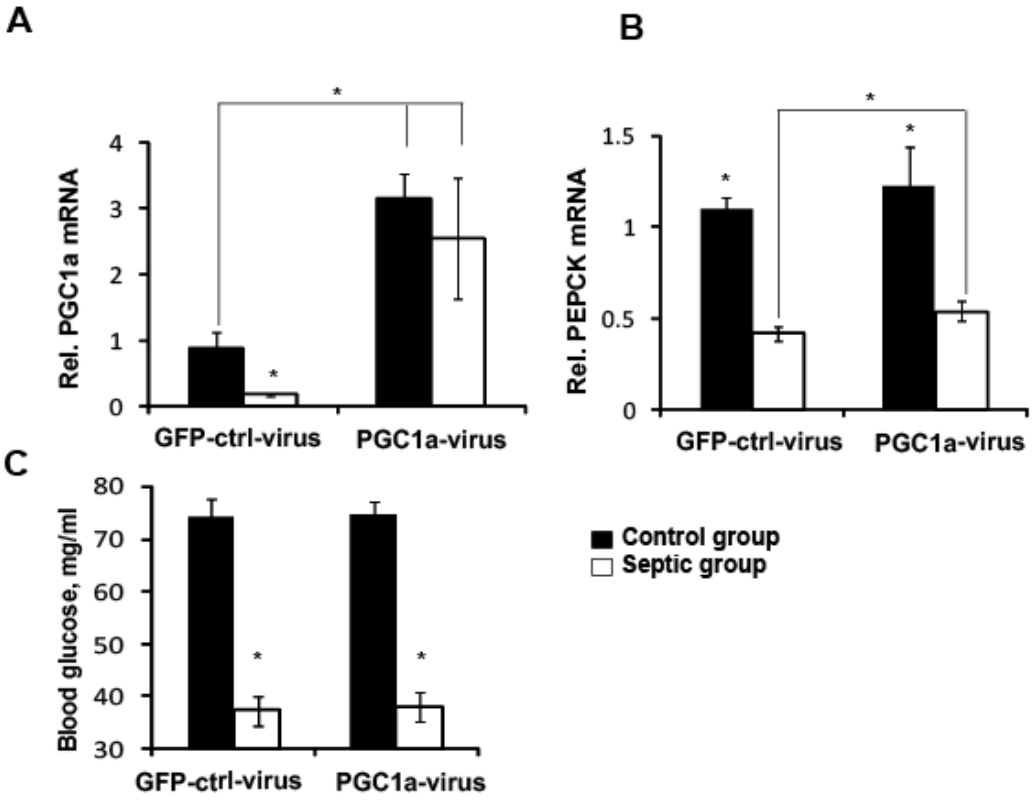


Figure 32 Transient over-expression of the PGC-1α in the liver is not sufficient to rescue PEPCK suppression and hypoglycemia in septic mice.
PGC-1α over-expressing and GFP-ctrl viruses were injected through the tail-vein, 1x10(9) Ifu per mouse. The adenovirus expressing PGC-1α from CMV promoter (PGC1a virus) and the control virus expressing only GFP (GFP-ctrl virus) were injected through the tail-vein, 1x10(9) Ifu per mouse. After 7 days the LPS experiment was performed as in Figure 18. (A) Rel. PGC-1α (B) PEPCK expression assessed by real time q-PCR. The Ct value for every gene is normalized against housekeeping gene TATA box binding protein (TBP), (C) Blood glucose level. Data are presented as Mean ±S.E.M.; * p≤0,05 (N=7 animals per group)

3.9.5 Inflammation affects the activity of PGC-1α protein in hepatocytes

To answer the question why PGC-1α over-expression was not sufficient to restore PEPCK expression in septic animals, we next checked if inflammation affects PGC-1α activity.

To this end, PGC-1α protein was fused with a Gal4 DNA binding domain (Gal4-DBD) that could bind to its cognate Gal4-cis element within the Gal4_luciferase reporter plasmid. The fusion Gal4-PGC-1α maintains co-activator function and can recruit other co-activators like CBP or SRC-1, all of which possess histone acetyltransferase activity (Spiegelman and Heinrich 2004). The assay does not depend on the expression of endogenous PGC-1α since only Gal4-PGC-1α fusion protein is able to induce luciferase expression. Moreover, since

Gal4-PGC-1α is constitutively expressed from a CMV promoter, luciferase readout reflects only changes in Gal4_ PGC-1α activity[3].

As a negative control, the expression of non-specific Gal4-DBD protein was used. H4IIE hepatocytes were co-transfected with Gal_4 luc reporter, Gal4_ PGC-1α or Gal4-DBD expressing plasmids followed by the treatment of cells with Raw conditioned medium. As shown in Figure 33, the over-expression of the Gal4_PGC-1α gave a strong increase of luciferase reporter activity over unspecific signal of the Gal4_DBD that was not differing from the assay background (data not shown). The Raw conditioned medium significantly decreased the Gal4_PGC-1α activity. Therefore, during inflammation along with gene expression, the PGC-1α is suppressed on the level of its transcriptional activity.

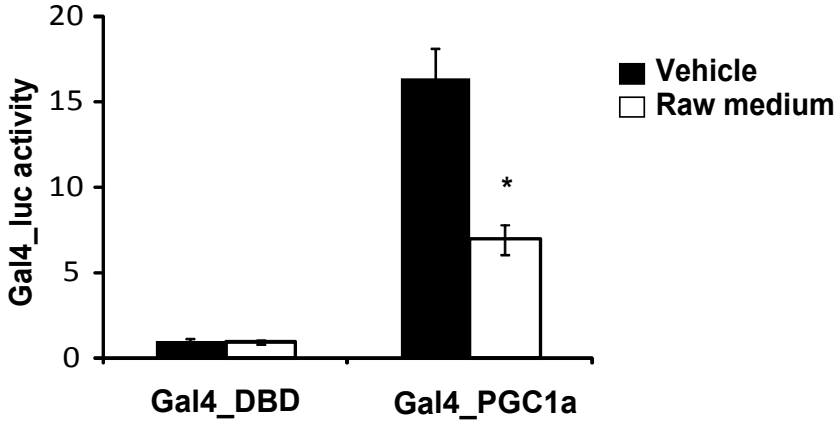


Figure 33 Inflammatory environment effects PGC-1α activity in hepatocytes.
H4IIE hepatocytes were co-transfected with the plasmid constantly expressing fusion protein Gal4_ PGC-1α and Gal4_luc reporter plasmid. The Gal4_PGC-1α fusion protein is able to bind Gal4_luc reporter plasmid and due to the PGC-1α co-activator activity activate the expression of reporter gene luciferase. The negative control protein Gal4_DBD that has Gal4 DNA binding domain but doesn't have any co-activator activity cannot induce luciferase expression at Gal4_luc plasmid. Since Gal4_luc cannot be affected by changes in expression of endogenous PGC1 α and since fusion Gal4_PGC-1α is constantly expressed from the CMV promoter, differences in luciferase activity reflect only the activity of Gal4_PGC-1α protein. Cells were treated with the culture medium from Raw 264.7 macrophages that were not exposed to LPS (Ctrl) or with Raw inflammatory conditioned medium (Raw). The luciferase activity is normalized to β-Galactosidase that was constantly expressed from HRAS promoter vector, co-transfected for all conditions. Data are presented as Mean ±S.E.M.; * p≤0,05, (N=3)

[3] The activity of CMV promoter is not affected by the Raw inflammatory medium (date not shown)

3.9.6 *Knockout of the PGC-1α in H4IIE hepatocytes ablates the PEPCK suppression by inflammatory environment*

As shown above, the inflammatory conditions affect both PGC-1α expression and activity. Moreover, it is difficult to assess to which extent it is necessary to over-express the PGC-1α protein to overtake both dysregulations together that PGC-1α could rescue its function to gluconeogenic gene activation. To ascertain that PGC-1α is indeed important for PEPCK suppression by inflammation we tried another approach that was independent from both expression and activity of the protein. We applied the RNAi that specifically depleted the endogenous PGC-1α protein from cells. The RNAi plasmid was provided by Herzig laboratory and was previously tested (data not shown). The efficacy of H4IIE cells transfection generally is quite low (Yan, Gao et al. 2007), around 10-20 %. Thus, it is difficult to detect the effect of RNAi on the expression of endogenous genes. Thereby we co-transfected PGC-1α –specific RNAi plasmid with the pGL3-PEPCK-490 plasmid, that enabled us to have a PEPCK gene expression readout for every cell that was efficiently co-transfected with RNAi. After transfection, H4IIE cells were treated with hormone stimuli Dex, Fsk or both together under normal or inflammatory conditions (Raw medium). As a negative control, a non-specific RNAi was used for all experimental conditions. As shown in Figure 34 the PGC-1α RNAi efficiently decreased the PEPCK promoter induction by hormones compared to samples transfected with non-specific RNAi. Importantly, despite the attenuation of the PEPCK expression due to PGC-1α depletion, the PEPCK induction through other transcriptional factors was still present in these cells.

The depletion of PGC-1α from hepatocytes dramatically ablated suppressive effect of the inflammatory conditioned medium on PEPCK for all Dex, Fsk and synergistic Dex/Fsk - stimulated conditions (Figure 34).

These results suggested that the presence of PGC-1α protein in hepatocytes is important for PEPCK gene suppression by inflammatory signaling and that the depletion of PGC-1α protein from cells could blunt the suppressive effect.

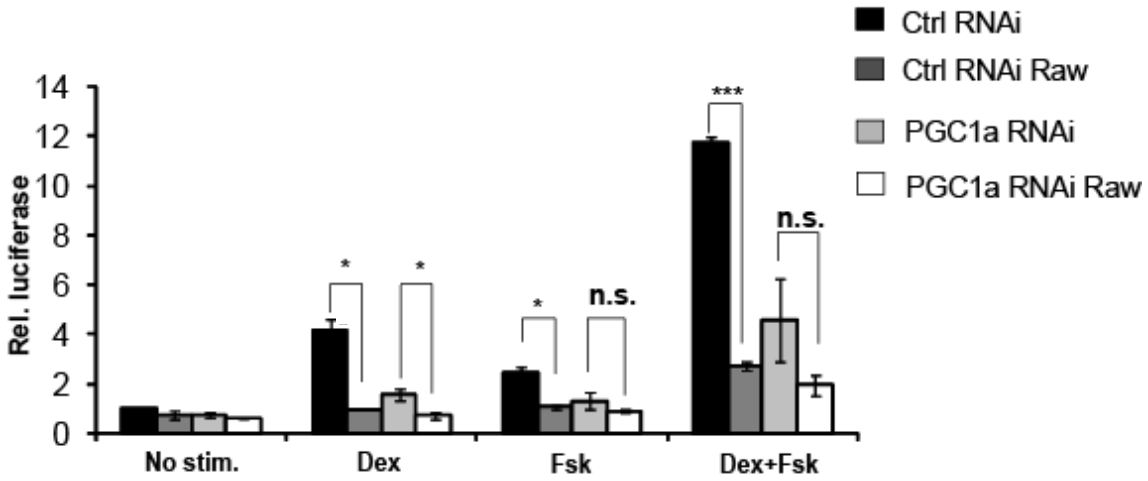


Figure 34 Depletion of the endogenous PGC-1α protein with RNAi blunts PEPCK suppression by inflammatory signaling in H4IIE hepatocytes.
The pGL3-PEPCK-490 wt reporter plasmid was co-transfected together with PGC-1α RNAi. Cells were treated with the culture medium from Raw 264.7 macrophages that were not exposed to LPS (Ctrl) or with Raw inflammatory conditioned medium (Raw) under basal (No stim.), dexametasone (Dex), forskolin (Fsk) or simulations (Dex+Fsk) stimulated conditions.. The luciferase activity is normalized to β-Galactosidase that was constantly expressed from HRAS promoter vector, co-transfected for all conditions. Luciferase activity depicted as a relative value that for any experimental group is significantly above the background. Data are presented as Mean ±S.E.M. of 3 independent experiments * p≤0,05, (N=3), not significant (n.s.)

3.9.7 *High fold adenoviral over-expression of PGC-1α rescues the PEPCK suppression by inflammatory environment in primary isolated hepatocytes*

The transient PGC-1α over-expression in mouse liver could not rescue PEPCK suppression; however we could show that not only the expression but also the activity of this protein might be affected (3.9.5). The activity of PGC-1α protein is regulated by phosphorylation and acetylation (Rosenfeld, Lunyak et al. 2006). We assumed that we might be able to saturate the inhibitory machinery by over-expressing of PGC-1α in fold higher dosage in primary isolated hepatocytes as compared with the animal experiment. To this end, cells were infected with GFP-expressing control adenovirus (MOI 20) and treated with Dex and Fsk under control or Raw inflammatory conditions. In addition, instead of GFP-control, the PGC-1α over-expressing virus (MOI 20) was given to cells under Raw inflammatory conditions. Cells were incubated under these conditions and harvested after 8 and 24 hrs. PEPCK expression in hepatocytes was strongly induced with hormonal stimuli Dex and Fsk and suppressed by the Raw inflammatory conditioned medium. The over-expression of PGC-1α under these

conditions significantly improved the suppressed PEPCK mRNA levels and even overtook the PEPCK suppression completely after 24 hrs of incubation (Figure 35 A, B).

Therefore, the over-expression of PGC-1α in a higher dosage could rescue PEPCK suppression by inflammation in primary hepatocytes, validating the role of this co-activator as a key target of inflammatory signaling under these conditions.

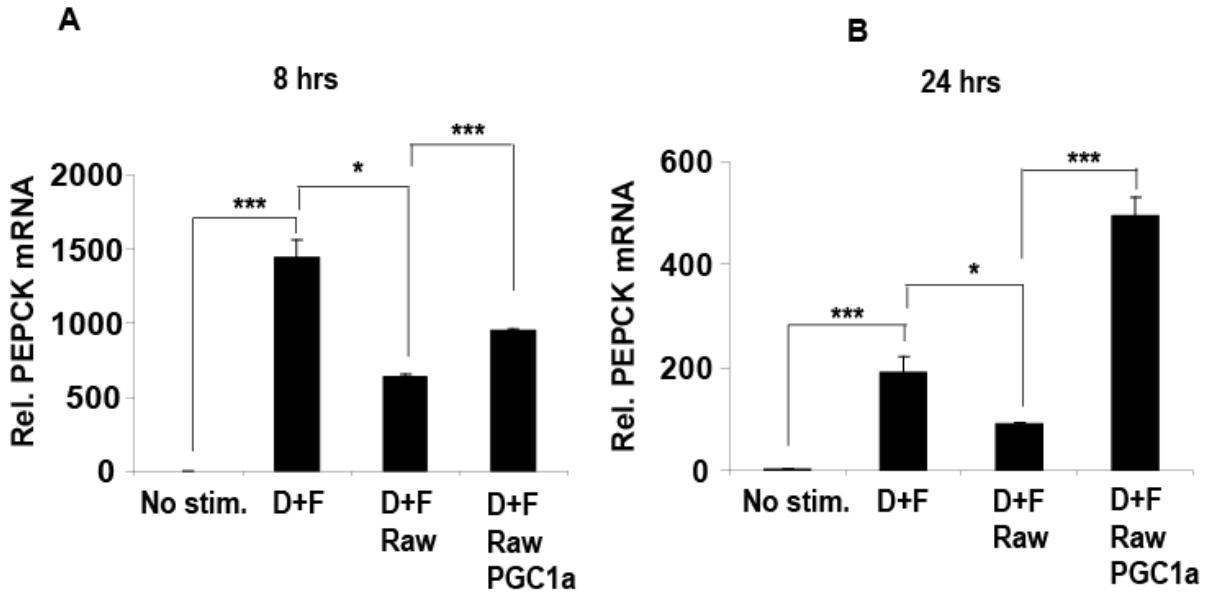


Figure 35 High fold PGC-1α over-expression could rescue the PEPCK gene suppression by inflammatory environment in primary isolated hepatocytes.

Cells were infected with GFP-expressing control adenovirus for all experimental conditions except of D+F /Raw/PGC1a group or with PGC-1α expressing adenovirus at MOI 20. Cells were treated with the culture medium from Raw 264.7 macrophages that were not exposed to LPS (Ctrl) or with Raw inflammatory conditioned medium (Raw) under basal (No stim) or hormone induced conditions (D+F). Abbreviations: Dexamethason (D), Forskolin (F), green fluorescent protein (GFP), phosphoenolpyruvate carboxykinase (PEPCK), peroxisome proliferator activated receptor γ coactivator 1α (PGC1a). PEPCK expression was assessed by real time q-PCR. The Ct values for PEPCK are normalized against housekeeping gene TATA box binding protein (TBP). Data are presented as Mean ±S.E.M.; * p≤0,05, (N=3)

4. DISCUSSION

4.1 Adenoviral reporter system as a novel system for promoter analysis *in vivo*

In the present study, we developed an adenoviral-based reporter system for the promoter analysis *in vivo*. This system was designed to investigate the function of cis-regulatory elements involved in the modulation of metabolic genes by inflammation in the liver. We chose to study the regulation of the phosphoenolpyruvate carboxykinase (PEPCK) gene since it represents a key rate limiting enzyme of gluconeogenesis in the liver and a prototypical gene dys-regulated in many metabolic diseases, such as insulin resistance and type 2 diabetes (Herzig, Long et al. 2001), (Beale, Harvey et al. 2007). Moreover, the expression of this gene entirely controls on transcriptional level and PEPCK has been used as a model for analysis of gene promoter regulation (Gurney, Park et al. 1994).

To date, the function of DNA cis-regulatory elements involved in the control of gene expression under physiological or pathological conditions has been primarily studied in cell culture. This approach is powerful but has it has limitations when one attempts to understand the physiological control of gene involved in metabolism. A step to understanding *in vivo* mechanisms of gene regulation was an introduction of the specific promoter mutations into transgenic animals and generation of a series of mouse lines (Hanson 2005). Indeed, most of the results elucidating regulation of PEPCK promoter *in vivo* were obtained by this technique (Hanson 2005). And to date, this approach remains to be the only choice if one has to confirm any cell culture cis-element analysis *in vivo* (Lechner, Croniger et al. 2001), (Cassuto, Kochan et al. 2005). The system that was designed in this study represents a novel technique for extensive and robust analysis of the gene promoter regulation under physiological as well as under pathological conditions, providing another application of the adenoviral gene delivery technology (see 1.5). In the present work we demonstrated that this approach could be successfully used on first steps of the investigation in particular for cases when it is difficult to mimic signaling environment in cell culture or when the endogenous stimuli are simply unknown.

Since adenovirus could infect a great variety of cell types such as skeletal muscles, liver, brain, heart (Russell 2000), the same adenoviral constructs could be applied to different cell culture systems, followed by it *in vivo* application in mouse models. Taken together, the

developed model represents a novel potent research instrument with a broad application spectrum.

4.2 **Functional validation of the system in hepatocytes and *in vivo***

We could show the integration of the PEPCK promoter gene into the adenoviral vector does not interfere with the proper promoter regulation by key endogenous stimuli such as glucocorticoids (Dex) or cAMP agonist (Fsk) in cultured hepatocytes. Moreover, the luciferase reporter expression reflects an *in vivo* regulation of genes at transcriptional level upon triggering physiological conditions such as fasting, that was confirmed by endogenous mRNA leves and associated physiological parameters i.e. blood glucose. Here we have to mention that the half-life of the luciferase protein is around 3 hrs which is much longer than the half-life of endogenous PEPCK mRNA (Thompson, Hayes et al. 1991). Therefore, one of limitations of the system is that it is not sensitive to short-term oscillations in gene expression and could reflect only a steady metabolic phenotype. This could also explain that outliers detected at the level of endogenous mRNA expression were not always coinciding with outliers detected by gene expression referenced to the luciferase readout (data not shown). However, the pattern of regulation for the entire group of animals was always the same when analyzing PEPCK gene expression by any of these methods. Moreover, the system can only address the regulation of gene expression at the transcriptional level, while changes at mRNA level are also affected by other mechanisms, e.g. mRNA stability.

To validate the *in vivo* system further, we developed a viral construct carrying deletions or specific mutations of some elements within the wild type PEPCK promoter. We mapped a fully characterized PEPCK promoter unit, GRU, demonstrating that disruption of a specific binding site for glucocorticoid receptor (GR) within this region could blunt the function of entire GRU unit *in vivo.* These results are fully consistent with the function of this promoter region described previously (Scott, Stromstedt et al. 1998), (Stafford, Wilkinson et al. 2001), and validate the functional integrity of the system, demonstrating that we indeed can study the role of particular cis-regulatory elements within the complex promoter organization *in vivo*.

4.3 In tissue virus normalization as a step in approaching quantitative results

The accurate determination of the adenovirus titer is crussial for the application of this system. First of all the underestimation of the absolute virus numbers may result in its toxicity for experimental animals. The empirically determined titer of adenovirus that does not cause any serious health problems is 1x10(9) infection units per mouse with a body weight of 20-25 g. This can be a general limitation of the system if the activity of adenoviral promoter construct is very weak. The same is generally true for other applications of adenoviral based systems for gene knockout or over-expression where the amount of over-expressed product might be not sufficient but cannot be compensated by the increasing dosage of the injected virus.

The second reason is an accuracy of the system for the quantitative analysis of the promoter activity. By transducing the promoter viral vector into mouse there are few steps that could contribute to the misinterpretation of obtained results. First of all, only functionally viable virus particles can successfully infect and therefore deliver an analyzed promoter to the liver. We could see the different viral stocks during its preparation may have a significantly different number of functionally viable virus particles as compared to a total viral titer determined by the OD260 nm method. The way to solve this problem is using the cell-based titering method that gives readout only for functional virus particles within the preparation. Since the absolute titer number for this method might depend on the current state of cells, e.g. passage number all the experimental viruses has to be tittered at once.

The next two obstacles on getting accurate results could not be controlled or improved before the experiment has started. These are a virus tail-vein injection that is performed manually and therefore cannot be absolutely exact and the distribution of the virus with a blood stream from the tail-vein to different parts of the mouse liver. We could see that overall infection rate of the liver is quite high and with the amount of virus 1x10(9) Ifu per mouse most of hepatocytes are infected, however the relative number of viral particles and therefore the promoter dosage could vary substantially within the liver (data not shown).

To get a higher reliability and better resolution of the obtained data we developed a method that enabled us to normalize for the number of viral genomes within the analyzed tissue. The designed method based on sensitive real time q-PCR technology and could significantly improve the accuracy of the approach. We showed that the method substantially decreased the

standard error for the group of animals by lining up samples of the group and strongly decreasing outliers.

Taken together, the in tissue virus genome normalization allowing to interpret our results as a function of promoter activity and not as an increased promoter dosage resulting in the enhanced reporter activity within the analyzed tissue.

4.4 Mimicking of inflammatory environment. What is the signal ?

The analysis of the blood serum in LPS mouse model of sepsis revealed that several different important pro-inflammatory stimuli i.e. Ifn γ, IL-6, IL-1β, TNF-α were elevated in the circulation after 8 hrs LPS injection. Most of these cytokines have been shown to suppress PEPCK gene expression in cell culture (Waltner-Law, Daniels et al. 2000), (Yan, Gao et al. 2007). In primary isolated hepatocytes we could also show that the cytokine mixture of the Raw inflammatory conditioned medium (Raw) could effectively suppress basal PEPCK expression as well as its separate or synergistic induction with hormone stimuli Dex and Fsk. Moreover we showed that, at least for some conditions, the LPS alone had suppressive effect on gluconeogenic genes mRNA levels, in particular on G-6-Pase. This effect might be mediated through activation of the receptor that recognizes LPS on the surface of hepatocyte i.e. Toll like receptor 4 (TLR4) and its subsequent pro-inflammatory signaling (Wong and Wen 2008). Indeed, we could show that under inflammatory conditions primary hepatocytes could trigger a local expression of cytokines such as TNF-α. All together, these results suggest that multiple inflammatory stimuli are involved in gluconeogenic genes suppression and could work through mutual or separate regulatory pathways. In most cell culture studies aiming to address mechanisms of hormonal inflammatory interference in the dysregulation of PEPCK gene the administration of a single cytokine has been applied (Hill and McCallum 1992), (Yan, Gao et al. 2007), (Kim, Sweeney et al. 2007). We assume that these models do not accurately mimic the entire inflammatory milieu that is why the conditioned medium of Raw macrophages stimulated with LPS was applied in our study. As we could see the Raw inflammatory conditioned medium suppressed key gluconeogenic genes in primary hepatocytes in the pattern similar to one for the septic mouse liver, however the suppression here was most likely caused by cumulative multiple mechanisms since most of pro-inflammatory stimuli are included into the medium.

4.5 Searching for the putative PEPCK inflammatory responsive elements in cell culture

The analysis of mechanisms of the inflammatory-metabolic interference in the suppression of PEPCK gene suggests quite a few cis-regulatory elements that might be involved in this process. These are glucocorticoid regulatory elements (GRE) to which GR is bound (Duma, Silva-Santos et al. 2004), (Reichardt, Umland et al. 2000), the cAMP responsive elements (CRE) (Yan, Gao et al. 2007) as well as GR accessory regulatory sites AF1, AF2 and AF3 for which the expression of cognate transcriptional co-activators i.e. SRC-1 or PGC-1α has been shown to be strongly reduced upon cytokine treatment (Kim, Sweeney et al. 2007). To this end, our strategy was to start from a broad screen of inflammatory responsive PEPCK promoter sites in H4IIE hepatocytes. Afterwards, we planned to validate the function of inflammatory responsive sites that were identified in cell culture with adenoviral reporter system in septic mice. To dissect through which pathway the suppression of PEPCK transcription might work, we investigated it under Dex, Fsk or synergistic Dex/Fsk stimulation. The cell culture analysis reveals that some mutations like CRE, AF3 and AF1 could attenuate cAMP-mediated PEPCK suppression by Raw conditioned medium. Consistently, the mutation of CRE has been shown to blunt the suppressive effect of TNF-α on cAMP-mediated PEPCK expression (Yan, Gao et al. 2007). However, none of the mutations could blunt the suppression of PEPCK gene completely for all Dex, Fsk and Dex/Fsk stimulated conditions. The explanation for these results could be that the PEPCK suppression is not mediated through the single promoter site and its cognate DNA binding transcriptional factors but rather through co-activators. One of the important functions of co-activators is to bridge distinct promoter elements in one unit. The organization of the promoter in complex unit is responsible for the synergistic PEPCK gene activation by multiple stimuli such as hormones GC and glucagon and is important for the full promoter response to fasting (Herzig, Long et al. 2001; Hanson 2005). The mutation of any of these single elements significantly attenuates synergistic promoter activation. However, the fact that we have still seen the residual promoter co-activation by simultaneous treatment with Dex and Fsk suggests that co-activators were still present on the promoter and bound to another intact transcriptional complex thus conducting its function on the promoter. To the end, we can speculate that none of the single mutations could cause a complete dissociation of co-activator complexes from the promoter. If the co-activators are the target of the inflammatory environment, then their dissociation under these conditions could result in a decrease in the

promoter activity that in fact was always the case for our single mutation promoter analysis. Indeed, it has been shown that the expression of some of the PEPCK co-activators such as SRC1 and 2, PGC-1α, CBP, RXR in hepatocytes is diminished upon cytokines treatment (Kim, Sweeney et al. 2007). These findings might differ however to an *in vivo* situation where we have seen the suppression of only PGC-1α co-activator expression.

Since cell culture experiments represent artificial conditions and it is difficult to assess which regulatory axis and to which extent is indeed activated at any stage of inflammatory process we can only speculate that the mechanism that we proposed might be valid *in vivo*. To this end the utilization of the adenoviral reporter system allowing studying gene regulation under complete signaling in septic mouse was necessary.

4.6 Metabolic phenotype of LPS inflammatory mouse model

Since we did not get conclusive results in cell culture we next proceed with a well-established *in vivo* LPS mouse model of sepsis (Buras, Holzmann et al. 2005).

We analyzed mRNA expression level of key gluconeogenic enzyme genes: PEPCK, G-6-Pase and PC. Interestingly, the dramatic suppression of PEPCK and G-6-Pase but not PC was seen in these animals. This could be explained by the fact that these two enzymes (PEPCK and G-6-Pase) are regulated similarly at the transcriptional level by hormones glucagon and glucocorticoids (Barthel, Scherbaum et al. 2003). These results are consistent with a pattern of gluconeogenic genes suppression in primary hepatocytes under Raw inflammatory conditions, where PC was also just slightly suppressed compared with the other two enzymes. This shows that the suppression of gluconeogenic genes by inflammatory stimuli represent a cell autonomous effect and independent of other signals that might cause gluconeogenic genes suppression, e.g. insulin. Remarkably, a macrophage derived inflammatory cocktail could suppress basal as well as hormone-induced expression of gluconeogenic genes in primary hepatocytes. These results suggest that the blockade of the entire gluconeogenic pathway is mediated through the suppression of particular enzyme genes that have similar regulatory pattern and not through the suppression of general transcription machinery. Thus, mechanisms of PEPCK gene suppression may also be extrapolated to other genes that are regulated in similar way (e.g. G-6-Pase) contributing to pathway suppression and might be important for the rescue of the entire physiological process.

It had been previously published that inflammatory cytokines IL1-β and TNF-α provoke marked alterations in lipid metabolism such as increased serum triglyceride levels and a

decreased hepatic fatty acid oxidation. Moreover, under these conditions, a marked decrease in an entire sets of key liver transcriptional factors and co-activators, i.e. RXR-α, PPAR-α and γ, LXR-α, PGC-1α and β, SRC-1 has been described (Kim, Sweeney et al. 2007). Analysis of these data has been performed in a culture of Hep3B hepatoma cells and in cytokine injected mice 14 hrs after injection. The characterization of the metabolic phenotype of our septic mouse model at 8 hrs post LPS injection revealed just a trend increase in the circulation of free-fatty acids and decreased ketone-body level, indicative of diminished hepatic fatty acid oxidation. On the other hand, mice had strong hypoglycemia, increased serum circulation of glucocorticoid hormone corticosterone and gluconeogenic gene suppression. The difference between results of two studies might be explained by the progression of the metabolic phenotype during sepsis development. As we could show the early stage of sepsis in LPS mouse untill 8 hrs affected mostly the glucose metabolism and only minor changes were seen for lipid metabolism. However, the long term inflammatory exposure might further expand marked dysregulation also at lipid homeostasis.

4.7 Disruption of cis-regulatory elements synergism as an *in vivo* mechanism of PEPCK gene suppression by inflammatory signaling

By applying our adenoviral promoter system for *in vivo* promoter analysis we could map PEPCK inflammatory responsive sites in LPS septic mice. We initially identified the CRE, the mutation of which could significantly attenuate promoter suppression by LPS in wild type mice. These data were consistent with previously described cell culture experiments, in which TNF-α suppression of PEPCK has been mapped to this cis-promoter element (Yan, Gao et al. 2007). However, we could show that CRE is not directly involved in PEPCK suppression in sepsis, since the promoter deletion PEPCK-355bp, carrying an intact CRE could no longer be suppressed in septic animals.

The deletion of the GRU responsive unit could completely blunt PEPCK suppression in septic mice. Remarkably, the mutation of the GRE within GRU unit did not rescue the PEPCK suppression. Given a central importance of GRE region for the GRU unit, as it is a site of GR binding to the promoter, we assumed that inflammatory suppressive effect is mediated through multiple elements within the GRU representing the interaction of the GR with accessory factors and co-activators. The CRE has been shown to be a part of the GRU unit through the overlapping functions and these remote regulatory elements are crucial for

synergistic promoter induction through glucocorticoids and cAMP signaling (Waltner-Law, Duong et al. 2003). Indeed we could show *in vivo* that mutation or deletion of any of these remote elements dramatically blunts the full PEPCK promoter activation in response to fasting. Thus, we propose a new mechanism of the PEPCK gene suppression in LPS septic mouse through the disruption of the synergism between remote promoter regulatory units GRU and CRE. Accordingly, we assume that co-activator proteins that bridge these regulatory units and therefore mediate this synergistic promoter activation are targeted by inflammatory environment. Our cell culture promoter analysis also supports a proposed model of PEPCK suppression by inflammatory stimuli.

4.8 From promoter to associated regulatory proteins. PGC-1α as a potential inflammatory target protein mediating PEPCK gene dysregulation in sepsis

In contrast to previously published data (Kim, Sweeney et al. 2007), the profile of key transcriptional factors and co-activators for our LPS mouse model revealed that the expression of the most important promoter associated proteins was not changed. However, the mRNA levels of PGC-1α co-activator were specifically reduced in LPS mouse liver. This is a remarkable finding since the PGC-1α co-activator is largely responsible for a synergistic PEPCK promoter activation through GC and cAMP signaling pathways (Herzig, Long et al. 2001), suggesting it as a target of hormonal-inflammatory interference in sepsis.

The best and direct approach to validate results that we obtained from promoter deletion analysis and test our hypothesis regarding PGC-1α as a key inflammatory target protein, would be the chromatin precipitation experiment (ChIP) for the endogenous PEPCK gene. However, since this technique critically depends on the quality of the antibody and some other technical factors we did not succeed with this approach (data not shown).

To gain insight into the role of PGC-1α in hormonal-inflammatory interference we continued with different descriptive approaches. We checked PGC-1α expression in primary hepatocytes treated with Raw conditioned medium and showed the cell-autonomous suppression of the endogenous PGC-1α expression by inflammatory stimuli. In the same system, we showed that, at least in part, the suppression of PGC-1α expression by inflammatory stimuli was mediated directly at the level of gene transcription. These results are consistent with previously published data, showing that TNF-α and IL-1could suppress PGC-1α expression at the transcriptional level (Kim, Sweeney et al. 2007). We could show that the expression of a

key pro-inflammatory transcriptional factor NF-kB was strongly activated upon Raw macrophage inflammatory conditioned medium in H4IIE hepatocytes. It has previously been published that NF-kB inhibits glucocorticoid and cAMP-mediated expression of the PEPCK gene (Waltner-Law, Daniels et al. 2000). Recently, it has also been discovered that NFkB/RelA transcriptional mediator is involved in the control of PGC-1α expression in human cardiac myocytes (Palomer, Alvarez-Guardia et al. 2008), therefore supporting the role of PGC-1α as a downstream target of inflammatory signaling that might further mediate PEPCK promoter suppression.

We tried to rescue PEPCK expression in LPS mouse by transient over-expression of PGC-1α with adenovirus. With this approach we could not substantially improve the impaired PEPCK gene expression in septic mice. We could show that inflammatory signaling affects not only the expression but also the activity of PGC-1α protein. Thus, the explanation for results of the mouse experiment could be that the fold over-expression of PGC-1α might not be sufficient to completely restore its co-activator function in animals. Consistently, in primary hepatocytes we could show that depletion of PGC-1α protein could dramatically blunt the inflammatory suppression of PEPCK gene for all Dex, Fsk and simultaneous Dex/Fsk stimulated condition, suggesting its role as a major target of inflammatory signaling on PEPCK promoter. Moreover, under the same pro-inflammatory conditions we could rescue PEPCK gene expression in primary hepatocytes by over-expressing the PGC-1α with adenovirus, which was consistent with our assumption that the high fold over-expression of PGC-1α could saturate inhibitory machinery (Rosenfeld, Lunyak et al. 2006) thereby overtaking the inflammatory suppressive effect. By technical reasons a high dose PGC-1α over-expression was not possible in mice however in accordance with all other experiments, PGC-1α is strongly involved in inflammatory-metabolic interference and represents a target protein mediating PEPCK gene suppression by inflammation in sepsis.

Furthermore, as PGC-1α is also known to control G-6-Pase promoter activity (Rhee, Inoue et al. 2003), its inhibition provides an amplification mechanism to severely block hepatic gluconeogenesis and to promote endotoxic hypoglycemia. Maintenance of hepatic PGC-1α activity might therefore represent an attractive therapeutic defense in the anti-hypoglycemic treatment of septic patients.

The transcriptional co-activator PGC-1α has been recently proposed as a key regulatory factor mediating anti-inflammatory effect in muscles (Handschin and Spiegelman 2008) and the reduction of PGC-1α mRNA levels in skeletal muscle of type II diabetic patients {Patti, 2003 #216} is thought to be tightly associated with the chronic inflammatory status of these

subjects (Choi, Befroy et al. 2008). Our study further demonstrates the involvement of the PGC-1α co-activator in the impaired control of metabolism by inflammatory signaling in the liver.

4.9 SUMMARY

- We developed and characterized a novel adenovirus-based system for *in vivo* promoter analysis

- We established and described an LPS mouse model with regards to hormonal-inflammatory interference causing gluconeogenic genes suppression

- We identified promoter inflammatory responsive sites of a key rate –limiting enzyme of gluconeogenesis, PEPCK, in LPS septic mice and proposed a new mechanism of PEPCK gene suppression in sepsis

- We showed that the PGC-1α co-activator represents a target protein mediating inflammatory suppressive effect of PEPCK. The impaired PGC-1α function might be involved in dysregulation of other key gluconeogenic enzymes such as G-6-Pase simultaneously contributing to the suppression of *de novo* glucose production by the liver and hypoglycemia in septic patients.

4.10 **OUTLOOK**

Complex, multi-factorable mechanisms that are involved in metabolic diseases make to create new powerful research techniques. In the present study, we developed a novel system for the analysis of gene promoter regulation *in vivo*. We could show the system might be applied to study mechanisms of different metabolic processes under physiological as well as under pathological conditions. By using this system we discovered and characterized an *in vivo* mechanism implicated in dysregulation of PEPCK, a rate-limiting gluconeogenic enzyme, under control of acute inflammatory environment in sepsis. The present study suggests PGC-1α as an important target of inflammatory-metabolic interference and favors the hypothesis that the maintenance of PGC-1α activity might represent an attractive therapeutic defense for the variety of diseases, associated with inflammatory complications. The adenoviral reporter system designed in this study represents a robust analytical tool that could further be applied fur the *in vivo* analysis of a variety of other genes under complex metabolic conditions.

5. MATERIALS AND METHODS

5.1 Equipment, Apparatus and Kits

Table 1 Equipment, Apparatus and Kits

Equipment	Manufacturer
Bacterial shakers	Infors, Switzerland
Bacterial incubators 37°C	Heraeus, Hanau, Germany
Blotting apparatus	Bio – Rad, Munich, Germany
Centrifuge (Biofuge fresco)	Heraeus, Hanau, Germany
Centrifuge (Biofuge pico)	Heraeus, Hanau, Germany
Centrifuge (Megafuge, 1.0R)	Heraeus, Hanau, Germany
Centrifuge (Mikro 22R)	Hettich GmbH & Co KG, Tuttlingen, Germany
Centrifuge (Super T 21)	Heraeus Sorvall, Langenselbold, Germany
CO_2 – Incubators for cell culture	Heraeus, Hanau, Germany
Dissection tools	Witegro, Taunusstein, Germany
Electrophoresis chambers	Steinbrenner, Wiesenbach, Germany
Electrophoresis Power supplies	Bio – Rad, Munich, Germany
Evaporation unit	Dr. Herzig´s Laboratory
Film Developer	Amersham, Freiburg, Germany
Film cassettes	Amersham, Freiburg, Germany
Freezer -20°C	Liebherr, Biberach, Germany
Freezer -80°C	Heraeus, Heilbronn, Germany
Gas burners, GASI	Schuett, Goettingen, Germany
Gel Documentation Unit	Bio – Rad, Munich, Germany
Haemocytometer	Carl Roth, Karlsruhe, Germany
Heating Block	VWR, Darmstadt, Germany
Hotplate / Stirrer	Berthold Technologies, Bad Wildbad, Germany
Horizontal Shaker, Duomax 1030	Heidolph, Kehlheim, Germany
Luminometer micropate LB 940	Berthold Technologies, Bad Wildbad,

	Germany
Microscope, Axiovert 40 CFL	Carl Zeiss, Jena, Germany
Nano Drop, ND-1000 Spectrophotometer	Peqlab Biotechnologies, Erlangen, Germany
One Touch Blood sugar measuring device	LifeScan, Neckargemuend, Germany
pH – Meter	VWR, Darmstadt
Pipettes single step	Eppendorf, Hamburg, Germany
Pipettes (2 – 1000 µL)	Gilson, Middleton, USA
Real Time PCR System 7300	Applied Biosystems, Darmstadt, Germany
Refrigerator 4°C	Liebherr, Biberach, Germany
Rotating wheel	Neolab, Heidelberg, Germany
SDS – gel chambers	Bio-Rad, Munich, Germany
Spectrophotometer	Ultrospec, Pharmacia
Tabletop Centrifuge (Mikro)	Neolab, Heidelberg, Germany
Tabletop Centrifuge (miniSpin plus)	Eppendorf, Hamburg, Germany
Thermocycler, PTC – 200	Biozym, Oldendorf, Germany
Thermomixer Comfort	Eppendorf, Hamburg, Germany
Tissue Lyser	Qiagen, Hilden, Germany
Tubes for ultracentrifugation	Beckmann, Munich, Germany
Ultracentrifuge XL-70	Beckmann, Munich, Germany
Ultraturrax	IKA Werke, Staufen, Germany
Vortexer, REAX 2000	Heidolph, Kehlheim, Germany
Water bath	Neolab, Heidelberg, Germany
Weighing balances	Satorius, Goettingen, Germany

Kits	**Manufacturer**
Mouse TNF-alpha/TNFSF1A Quantikine ELISA Kit	R&D Systems
Mouse IL-6 Quantikine ELISA Kit, 2 Plate, 2nd Generation	R&D Systems
Mouse IL-1 beta/IL-1F2 Quantikine ELISA Kit, 2 Plate	R&D Systems
MILLIPLEX™ MAP KIT (Mouse Cytokine/Chemokine)	Millipore

2-D Quantification Kit	Amersham Biosciences, Freiburg, Germany
AdEasy™ XL Adenoviral Vector System	Stratagene
Enhanced Chemiluminescence (ECL) Kit	Amersham Biosciences, Freiburg, Germany
First Strand cDNA Synthesis Kit	Fermentas, St. Leon – Rot, Germany
SuperScript™ II Reverse Transcriptase (10000U)	Invitrogen
Invitrogen Pure-Link ™ High Pure Plasmid	Invitrogen, Karlsruhe, Germany
Ketone bodies Determination Kit	Wako, Neuss, Germany
NEFA – C Determination Kit	Wako, Neuss, Germany
Platinum® Quantitative PCR Supermix	Invitrogen, Karlsruhe, Germany
QiaPrep Plasmid Miniprep Kit	Qiagen, Hilden, Germany
QiaQuick Gel Extraction Kit	Qiagen, Hilden, Germany
Triglycerides Determination Kit	Sigma – Aldich Chemicals GmbH, Steinheim, Germany
Triglycerides Liquicolour	Human GmbH, Wiesbaden, Germany

5.2 **Antibiotics, Chemicals and Enzymes**

Table 2 Antibiotics, Chemicals and Enzymes

Antibiotics	Producer
Ampicillin	Sigma – Aldich Chemicals GmbH, Steinheim, Germany
Chloramphenicol	AppliChem, Darmstadt, Germany
Kanamycin	Carl Roth, Karlsruhe, Germany
Chemicals and Enzymes	**Producer**
1 kb DNA Ladder	Fermentas, St. Leon – Rot, Germany
Generuler DNA Ladder Mix	Fermentas, St. Leon – Rot, Germany
Prestained Protein Marker	Fermentas, St. Leon – Rot, Germany
LB Medium, LB Agar, Acrylamide	Carl Roth, Karlsruhe, Germany
Agarose, Ammonium chloride, Acetic acid, Bicine, Bromophenol Blue, Dexamethasone, Dithiothreitol (DTT), Ethylendiamin-	Sigma – Aldich Chemicals GmbH, Steinheim, Germany

tetraacetat (EDTA), Ethylene glycol tetraacetic acid (EGTA), Guanidine Hydrochloride, Lipopolysaccharides (LPS) from E.coli (O55:B5), Orange G, 2-Nitrophenyl ß-D-galacto pyranoside (ONPG), Palmitic acid, Phenylmethanyl sulfonyl fluoride (PMSF), Ponceau S, Potassium Phosphate tribasic, Sodium sulphate anhydrous, Sucrose, Trizma base, Tween – 20, Urea	
QIAZOL Lysis Reagent	Qiagen, Hilden, Germany
Ammonium peroxide sulphate (APS), Ammonium sulphate, Caesium chloride, Glycerol, Potassium hydrogen phosphate, LB Agar, LB Medium, Milk of Magnesia, MOPS (3-[N-Morpholino]propanesulfonic acid), NZY Medium, Rotiphorese Gel 30 (37.5:1), Sodium Dodecyl Sulphate (SDS), TEMED (Tetramethylethylenediamine), Trypton/Peptone from Casein	Carl Roth, Karlsruhe, Germany
Bis-tris	Sigma – Aldich Chemicals GmbH, Steinheim, Germany
Boric acid	Riedel –de Haen, Germany
BSA Fraction V	Gerbu Biotechnik GmbH, Gaiberg, Germany
Chloroform	Fluka, Germany
Calcium chloride Dihydrate	AppliChem, Darmstadt, Germany
Dulbecco's Modified Eagle Medium (DMEM), Modified Eagle Medium (MEM), Non-Essential Amino Acids (NAA) Solution 10 mM (100X)	Gibco, Germany
DMSO (Dimethylsulfoxid) f. analysis	AppliChem, Darmstadt, Germany
Ethanol p.A.	Fluka, Germany

Ethanol vergällt, Formaldehyde 37%, Forskolin (25mg)	Sigma – Aldich Chemicals GmbH, Steinheim, Germany
Formamide	Calbiochem, Germany
Glycine	Gerbu Biotechnik GmbH, Gaiberg, Germany
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid))	Gerbu Biotechnik GmbH, Gaiberg, Germany
Igepal CA630 (Nonidet P40)	Sigma – Aldich Chemicals GmbH, Steinheim, Germany
Imidazole	Sigma – Aldich Chemicals GmbH, Steinheim, Germany
di-Potassium hydrogen phosphate	Neolab, Heidelberg, Germany
Lipofectamine Reagent, Lipofectamine 2000, Glycogen (RNase free, cat 10814-010)	Invitrogen, Karlsruhe, Germany
Magnesium chloride hexahydrate	Merck, Darmstadt, Germany
Magnesium sulphate Heptahydrate p.A.	Neolab, Heidelberg, Germany
Methanol	Sigma – Aldich Chemicals GmbH, Steinheim, Germany
PageBlue Protein Staining Solution	Fermentas, St. Leon – Rot, Germany
Phenol	AppliChem, Darmstadt, Germany
Sodium acetate anhydrous	AppliChem, Darmstadt, Germany
Sodium hydrogen carbonate	AppliChem, Darmstadt, Germany
di- Sodium hydrogen phosphate dihydrate	Neolab, Heidelberg, Germany
di-Sodium hydrogen phosphate	Neolab, Heidelberg, Germany
Sodium dihydrogen phosphate	AppliChem, Darmstadt, Germany
Sodium hydroxide	Gerbu Biotechnik GmbH, Gaiberg, Germany
2-Propanol	Riedel – de- Haen, Germany
Potassium Chloride	Acros Organics, New Jersey, USA
Potassium Hydroxide	Sigma – Aldich Chemicals GmbH,

	Steinheim, Germany
Sodium Chloride	Sigma – Aldich Chemicals GmbH, Steinheim, Germany
Triton X-100	AppliChem, Darmstadt, Germany
PageBlue™ protein staining solution	Fermentas, St. Leon – Rot, Germany
Yeast Extract	Gerbu Biotechnik GmbH, Gaiberg, Germany
Polyethylenimine (PEI MAX), 2g	Polysciences Europe

5.3 **Oligonucleotides.**

Oligonucleotides were designed with Vector NTI program and sensitised by Invitrogen Life Technologies, Germany. Stock solutions of 100 µM or 200 µM were prepared in RNAse – DNAse free water, stored at – 20°C.

Table 3 Primers applied for recombinant adenoviruses

Oligonucleotide	**Sequence (5´→ 3´)**	**Target DNA**
Adenovirus checking		
pAdPM13, for (1) *	AAAGGCGTCTAACCAGTCACAG	Adenovirus nts 9705-9726
pAdPM14, rev (1)	CTTACACCATCAACACGGGC	Adenovirus nts 10213-10194
Luc5 rev (2)	TTAGGCAGACCAGTAGATCC	Luciferase gene
Luc-1-for (2)	AAATCACAGAATCGTCGTATGC	Luciferase gene
pAd-Luc3 (3)	GCGGAGGAGTTGTGTTTGTGGACG	pAd Track (Luc-GFP)
pAd-GFP (3)	CGTCGCCGTCCAGCTCGACCAG	pAd Track (GFP-Luc)
Sequencing		
N-Luc-rev	CTTCATAGCCTTATGCAGTTGC	Luciferase N-terminus (Seq)
PEP-490 seq rev	AATAGTATACAGAAGGGAGGACAGC	PEPCK -50 to -490 rev (Seq)
PEP-490 seq for	GAAGTTTCACGTCTCAGAGCTG	PEPCK -490 to +1 forv (Seq)

** (X)-primers of the same PCR reaction*

Table 4 Primers for site-directed mutagenesis

Primers used for point mutations of PEPCK-490bp cis-promoter elements:

1	*GRE (GR1 &GR2)	For1: AGACTTTG GTACCTCTAGAAGTTTCACGTCTCAGAGC **Rev1:** CATACGCTGCTGGCTTCGTATTTTTGGTGCCAGTGGCTGCTG **For2**: AGCCAGCAGCGTATGAATACTAAGAGGCGTCCCGGCCAGC Rev2: AATTA AGATCTGCTTGGTAGCTAGCCCTCCT CG --- For1 (*KpnI*), Rev2 (*Bgl II*)-flanking; **Rev1 (GR1mt) , For2 (GR2mt)-**mutagenic
2	CRE	For: CTGCCCCTTACGGCAGAGGCGAGCCTC Rev: GGAGGCTCGCCTCTGCCGTAAGGGGCAG
3	AF1	For: AGCTGAATTTCCTTCTCATTCCCTTTGGCCGTGGGAGTGAC Rev: CACGGCCAAAGGGAATGAGAAGGAAATTCAGCTCTGAG
4	AF2	For: GACACCTCACAGCTGTGGGCTTTTGACAACCAGCAG Rev: CTGCTGGTTGTCAAAAGCCCACAGCTGTGAGGTG
5	AF3	For: CCAGCCCTGTCCTTAACACCCACCTGACAATTAAGGCAAGAG Rev: CTTAATTGTCAGGTGGGTGTTAAGGACAGGGCTGGC

All mutations has been described and tested previously.
1-(Scott, Stromstedt et al. 1998), 2-(Bokar, Roesler et al. 1988), 3-(Hall, Sladek et al. 1995), 4-(Scott, Mitchell et al. 1996)
5-(Scott, Mitchell et al. 1996)
*Site-specific mutagenesis by overlap extension
Others: In vitro mutagenesis using double stranded DNA templates: selection of mutants with *Dpn I.* // Sambrook, Mol.clonning, Vol.2

5.4 Taqman probes

Table 5 Taqman probes used for q-PCR experiments

Probe	Number
GAPDH	Mm99999915_g1
Glucokinase	Mm00439129_m1
G6Pase	Mm00839363_m1
Pyruvate kinase	Mm00443090_m1
PGC1 alpha	Mm00447183_m1
Liver glycogen phosphorylase	Mm00500078_m1
IFN gamma	Mm00801778_m1
Interleukin 6	Mm00446190_m1

Interleukin 6	Rn00561420_m1
IkBa	Mm00477798_m1
TNFά	Mm 00443258_m1
GR	Mm00433832_m1
PDK4	Mm00484152_m1
Interleukin 1	Mm00434228_m1
TBP	Mm00446973_m1
PEPCK	Mm00440636_m1
PEPCK	Rn01529014_m1
TLR4	Mm00445273_m1
	Rn-rat, **Mm-mouse

5.5 Antibodies

Table 6 Primary and secondary antibodies for immunoblot.

antibody	Source	species	Dilution
GR M-20	sc-1004 Santa Cruz	rabbit polyclonal	1:1000
HA-probe (Y-11)	sc-805 Santa Cruz	rabbit polyclonal	variable
GFP	A11122 Invitrogen	rabbit polyclonal	1:4000
VCP	ab11433 Abcam	mouse monoclonal	1:1000
PGC1 nr:6797	Herzig's Lab collection	rabit	1:1000
anti-mouse IgG, HRP	170-6516 BioRad	goat	1:1000 (to 3000)
anti-rabitt IgG, HRP	170-6515 BioRad	goat	1:4000 (to 10000)

5.6 Strains and Cell Lines

Table 7 Bacterial Strains

Genotype	Reference
E. coli XL1 Blue (chemical / electrocompetent competent cells)	Stratagene, La Jolla, USA
E. coli TOP10 (chemical competent cells)	Invitrogen, Karlsruhe, Germany

E. coli TOP10 (Electrocompetent cells)	Invitrogen, Karlsruhe, Germany
E.coli BL21(DE3)pLysS	Novagen ,Merck, Darmstadt, Germany
XL10-Gold Ultracompetent Cells	Stratagene, La Jolla, USA
BJ5183-AD-1	Stratagene, La Jolla, USA

Table 8 Cell Lines

Cell Line	**Source**
H4IIE, rat hepatoma cells	
RAW 264.7, mouse leukaemic monocyte macrophage cell line	
Human Embryonic Kidney (HEK) 293	ATCC, Wesel, Germany
HEK 293 A	Invitrogen, Karlsruhe, Germany
Hepa 1c1 wt, mouse hepatoma cells	ATCC, Wesel, Germany

5.7 **Buffers**

Table 9 Buffers

BBS (2x): 50 mM BES; 280 mM NaCl; 1,5 mM Na2HPO4; pH 6.95

Blocking Buffer: 1x PBS; 0,1% (v/v) Tween 20; 5% (w/v) Milk of magnesia

Destaining solution: 25% (v/v) Isopropanol; 10% (v/v) Acetic acid

MOPS (10x): 0.2 M MOPS; 50 mM Sodium acetate; 5 mM EDTA pH 7.0

ONPG-Buffer: 0.1 M NaP pH 7,5; 1 mM MgCl2; 10 mM KCl; 1 mg/ml ONPG; 100 mM ß-Mercaptoethanol (to be added fresh)

6x Orange G Sample buffer:10 mM EDTA; 70% Glycerol; Orange G (1 Pinch)
PBS (1x): 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na2HPO4; 1.47 mM KH2PO4
Protease-Inhibitor-Mix (50x): 50 mM PMSF; 50 mM NF; 0.5mg/ml Leupeptin; 0.5 mg/ml Aprotinin; 0.5 mg/ml Pepstatin A; make up volume with 10ml Ethanol

RNA-Sample Buffer: 0.5 µl Ethidium bromide; 0.5 µl 10x MOPS; 5 µl Formamide; 1.75 µl Formaldehyde; 1.7 µl; 6x Loading Dye and 0.55 µl RNase-free H2O)

SDS Running Buffer: 0.192 M Glycin; 25 mM Tris; 0.05% SDS

SDS Sample Buffer (2x): 120 mM Tris/HCl pH 6.8; 200 mM DTT; 4% SDS; 20% Glycerol; 0.01% (w/v) Bromphenolblau

TBE (1x): 45 mM Tris; 45 mM Boric acid; 1 mM EDTA

2xSDS sample buffer + 8M Urea for SDS PAGE

ml of Stock	Final
4,8g Urea	8M
1,2 ml 1M Tris HCl, pH 6,8	120mM
2 ml of 20% SDS	4%
2 ml glycerol (99%)	20%
100 ul 1% Brom phenol blue	0,01%
1,5 ml Water	to 10 ml final Vol.
After urea is dissolved	
0,31g DTT	200mM

Transfer Buffer (1x): 25 mM Tris; 192 mM Glycin; 0.01% (w/v) SDS; 20% (v/v) Methanol

Washing Buffer for Western Blots: 1x PBS; 0.1% (v/v) Tween 20

PBS-Tosh
30,8 mM NaCl, 120,7 mM KCl, 8,1 mM Na2HPO4, 1,46 mM KH2PO4, 10mM MgCL2

Gly-Gly working solution (for luciferase assay):
25 mM Gly-Gly pH 7,8,
4 mM EGTA,
0,1 mM DTT,
0,01% Triton X-100

Harvest Buffer (for luciferase assay): 100% GlyGly-working solution; 1% Triton X-100; 1 mM DTT

Luciferase-Assay-Buffer: 20 mM K3PO4 pH 7.8; 80% (v/v) Gly-Gly-Puffer; 1.6 mM DTT; 2 mM ATP

Luci Mix: 1 mM Luciferin; 10 mM DTT in Gly-Gly working solution.

5.8 MOLECULAR BIOLOGY

5.8.1 *Digestion of the plasmid DNA by restriction enzymes*

All restriction enzymes and buffers were obtained from New England Biolab (NEB) or Fermentas.

5.8.1.1 Analytical restriction digestion

200-500 ng of DNA was digested with 0,1-0,5 units of restriction enzyme for 2 hrs at 37º C. The total volume of restriction mixture was 20 ul. The digested DNA bands were analysed by agarose gel electrophoresis.

5.8.1.2 Preparative restriction digestion

When DNA fragment is digested for subsequent cloning, the higher concentration of DNA is necessary since some of the DNA could be lost over purification from the restriction mixture. 1000-5000 ug of DNA was digested with 5-50 unit of the enzyme depends of the efficiency of digestion in 50-100 ul of total volume 3 hrs, 37º C. The digested DNA was further purified by QIAquick PCR purification kit (QIAgen #28106) or by QIAquick gel extraction kit (QIAgen #28704), when digested fragment was extracted from the gel.

5.8.2 *DNA gel electrophoresis*

For routine applications (fragments from 500bp to 7kbs) 1% agarose gel was prepared. To detect bigger DNA fragments lower concentration of the agarose up to 0,5% was used when for the detection of smaller fragments (≤500bp), the higher concentration of the agarose up to 2 % was used. Normal agarose (Roth, Cat No. 12656) was dissolved in standard 1x Tris-borate-EDTA (TBE) buffer. Agarose was melted, cooled down, and 3-6 µl of ethidium bromide (10 mg/ml) were added per 100 ml of agarose solution. Depending on the gel size and concentration, gels were run at a voltage between 60 and 100 V with constant current. Plasmid DNA can exist in a number of different forms, each of which migrates differently in agarose gel electrophoresis. Generaly, supercoiled plasmid DNA exhibits the fastest migration rate, while open circular (nicked) plasmid DNA migrates more slowly. Linearized plasmid DNA generally migrates between supercoiled and open-circular.

5.8.3 *Extraction of DNA fragments from agarose gel*

DNA fragments were excised from the agarose gel using a UV lamp and purified with QIAquick Gel Extraction Kit (QIAgen #28704) according to the manufacturer's instructions. To decrease the harmful mutagenic effect of UV light the DNA was cut out on the glass tray that significantly improves the efficiency of subsequent cloning.

5.8.4 *Dephosphorylation of the restrictase-digested vector*

To avoid self-ligation of vectors that are cut with only one restriction enzyme or with two, where the double digestion might be not 100% it is recommended to dephosphorylate the vector but not the insert, before the ligation. In that case the amount of positive clones (with insertion in the vector) will be significantly increased compare to empty relegated vector clones. The enzyme Alkaline phosphatise (CIP) removes 5'phosphate group from DNA and RNA that makes the self-ligation not possible anymore. The insert has its phosphorylated 5' end intact so that ligase could use it to ligate vector and insert together.

Reaction conditions:

After restriction digestion CIP (NEB) was added directly to the restriction mix (the enzyme active in most of NEB buffers: NEB2, 3, 4, ECoRI, BamI) 0,5U per 1 ug of vector DNA. Mix was incubated for 1 hrs at 37 °C and inactivated at 75 °C for 10 min. I*mportant:* the dephosphorylated DNA has to be purified by gel purification, spin column purification or phenol extraction since the residual CIP activity can inactivate the insert.

5.8.5 *Blunting of the single stranded overhangs with DNA-pol I (Klenow)*

The restriction digestion that generates not complementary single-stranded ends could create problems for the subsequent ligation or may result in loss of these nucleotides and creating some mutation within the cloning DNA. To this end the single stranded overhangs has to be blunted by DNA-pol I (Klenow) or T4 DNA pol. DNA-pol I fill in of 5' overhangs and remove 3' overhang to form blunt ends ((for it function it needs 3' end (the enzyme can't use 5'end)). To fill in 3' overhangs T4 DNA-pol should be used.

Protocol:

1 ug DNA, 2 mM dNTP mix, any 10x NEB buffer, 1U Klenow enzyme/1 ug DNA were incubated for 15' at 25 °C. The reaction was terminated by EDTA (10mM final) and 75 °C for 20'. The DNA was purified with PCR purification kit.

5.8.6 ***Ligation***

For the ligation the digested vector DNA and the insert were taken in the molar ratio 3:1 for sticky-ends ligation or 5:1 for blunt ligation. Molarities (M) were calculated as:

M (moles)=quantity of the plasmid (g) / size of plasmid (bp) x 325D x 2

1 base is equal 325 D, 1ng=$1x10^{-9}$ g, 1fmol=$1x10^{-15}$mol
The following protocol of the ligation mixture was used:
20-100 ng Vector, X ng Insert, 2 ul 10x ligase buffer, 2ul T4 DNA ligase, and water to 20 ul final volume. The reaction was incubated for 2-4 hrs at RT, and 1-2 ul of the mix were transformed into competent *E.coli* cells. The rest was kept at +4 °C, ON and re-transformed if necessary.

5.8.7 ***Transformation of bacteria for plasmid amplification***

5.8.7.1 Transformation of chemically competent cells

For routine plasmid amplification chemically competent *E.coli XL-1/blue* cells were used. For special applications e.g. site directed mutagenesis the commercially available *XL-10 GOLD* cells were chosen (Stratagene).
50 µl of chemically competent cells were thawed on ice and then transferred to a pre-chilled 15 ml Falcon round bottom tube. They were gently mixed with either 50-100ng plasmid DNA (0,5 to 1µl) or 1 to 3µl ligation reaction mixture and chilled on ice for approximately 30 minutes followed by a 30 second heat shock in a water bath at 42°C. The reaction was chilled again for 2 minutes on ice and 450 µl 42°C warm LB-medium was added. The reaction mixture was incubated 60 minutes at 37°C and 200 rpm.
Selection of positive clones was carried out by plating various amounts of the transformed bacterial culture onto LB-Agar dishes containing the appropriate selection antibiotic and incubation overnight at 37°C incubator.

5.8.7.2 Transformation of electrocompetent cells

Approximately 50 µl of electrocompetent *E.coli XL-1/blue* cells were used for the transformation. Generally, electrocompetent *E.coli XL-1/blue* cells have higher transformation efficiency then the chemical competent cells. Cells were thawed on ice and 1 to 2 µl ligation mix or plasmid DNA, 10-100ng was added. Mixture was gently stirred and then transferred to a chilled electroporation cuvette. Electroporation was done with 2,5 kV and 400 Ω. Immediately, 300 µl 37°C warm LB-medium were added to the cell suspension and the reaction was incubated for 1h at 37°C.

5.8.8 ***Plasmid purification from bacterial cells***

5.8.8.1 Plasmid purification with TENS lysis buffer

For checking of clones by restriction digestion the following method was used: 5 ml overnight bacterial culture in LB medium with selective antibiotic were spin down at 2000rpm for 10 min. Pellet was resuspended in 300 µl of TENS (1xTE, pH 8,0; 0,1M NaOH, 0,5%SDS). The solution was neutralised with 150 µl of 3M Natrium acetate, pH 5,2 and mixed with a pipette up and down several times. After 2 min of centrifugation at 13,000 rpm on table centrifuge supernatant was transferred to the fresh 1,5 ml tubes. 900 ul of chilled to -20°C 100% ethanol was added and the mixture and spined for 10 min, 13,000 rpm, 4°C. After discarding the super, pellet was washed with 200 ul chilled -20°C, 75% ethanol, and spined again for 2 min. Supernatant was discarded and pellet dried on ice for 5 min and diluted with 50 ul of sterile water.

5.8.8.2 Plasmid purification with miniprep Kit

For special needs of low-salt and well purified DNA, i.e. subsequent cloning, sequencing etc the plasmid purification Kits (QIAprep® Spin Miniprep Kit #27106, Invitrogen Pure-Link ™ High Pure Plasmid, Cat No K2100-26) was used according to manufacturer's instructions.
DNA concentration was determined by spectrophotometric measurement using a Nanodrop ND-1000 spectrophotometer (peqlab Biotechnology). Spectral absorbance at 260 nm was

used for calculation and ratio between of OD_{260nm}/OD_{280nm} served as purity control. Ratios obtained were in the range between 1,8 and 2,1.

5.8.9 ***Isolation of genomic DNA from murine tissue***

Approximately 5 to 20 mg of liver tissue were incubated in 500µl tissue lysis buffer (10 mM Tris pH 8.0, 100mM NaCl, 15 mM EDTA, 0,5% SDS, 0,5 mg/ml Proteinase K) at 56°C on the shaker overnight at 400 rpm.

Next day, the samples were mixed thoroughly until no cell clumps were visible and 500 µl PCI buffer (phenol pH 8.0: chloroform : isoamylalcohol 25:24:1 (v/v)) were added. The reaction mixture was gently mixed for 20 seconds and then spined at 13,000 rpm, for 5 minutes (Eppendorf centrifuge) at room temperature. The upper aqueous phase was transferred to a fresh tube and 500 µl PCI buffer were added again, mixed and spined at 13,000 rpm. The aqueous phase was transferred to a fresh tube.

500 µl CI buffer (chloroform: isoamylalcohol 24:1 (v/v)) were added, mixed well and spined for 5 minutes. Finally, the upper phase (around 500 ul) was transferred to a fresh tube and the DNA was precipitated with 500 µl 2-propanol, 15 min at 13000 rpm, 4°C. The supernatant was discarded and DNA pellet washed with 500µl 75% ethanol, 13,000 rpm, for 5 minutes at 4°C. Pellet was air-dried for 5 min and then re-solubilised in 30 to 100 µl Tris-EDTA buffer pH 8.0. For complete solubilisation samples were kept overnight at 4°C. Next day, after DNA dehydration the correct DNA concentration can be measured.

All pipetting steps should be carried out with care to avoid shearing forces. Buffers and materials used should be free of DNases.

5.8.10 ***Polymerase Chain Reaction (PCR)***

PCR is a technique that let to amplify a specific target region on the DNA template with heat stable DNA polymerase.

Table 10 The standard reaction mix used for PCR.

10x *Pfu*I DNA polymerase Buffer with MgCl 2	5 ul	1x
10 mM dNTP mix	1ul	0,2 mM
Forward primer (100 uM)	1,5 ul	3 uM

Reverse primer (100 uM)	1,5 ul	3mM
DNA template	2-5 ul	20-100ng
Pfu DNA Polymerase	3 ul	2,5U/100 ul
Double distilled water	to 50 ul	Total Vol

Alternatively the ready to use PCR master mix with *Taq* polymerase, dNTP and PCR-buffer can be purchased from Fermentas (#K0171). *Taq* polymerase is routinely used for the PCR reactions where the sequence accuracy of the amplifying fragment is not highly important e.g. the analysis of a positive bacterial clones, otherwise the high fidelity *PfuI* or Phusion DNA polymerase can be used.

Table 11 Standard protocol for PCR

Denaturation	95 ºC	5-10 min	
Denaturation	95 ºC	1 min	30-35 cycles
Primer annealing	55-65 ºC	30-45 sec	
Extension	72 ºC	1-3 min	
Extension	72 ºC	10 min	
Hold	4 ºC	∞	

For the analysis of bacterial clones, the colony can be picked and diluted in 10 uM $MgSO_4$ in water. Afterwards 1 ul of this bacterial suspension can be used directly for the PCR, and the rest 19 ul could be than used directly for mini or maxiprep.

5.8.11 ***PCR-mediated site-directed mutagenesis***

In our work two different methods of PCR- based site directed mutagenesis were used. The protocols for these methods are described by Sambrook and Russell / Molecular cloning / third edition, Vol.2. or in a QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene).

5.8.11.1 Site specific mutagenesis by overlap extension

This method was used for the mutation and amplification of some DNA region with their subsequent subcloning within the target plasmid. Four primers were needed to introduce site specific mutation. One pair of primers was used to amplify the DNA that contained the mutation site together with upstream sequences, when the second pair was used to amplify DNA with mutation and downstream sequences. Two sets of primers were used in two separate amplification reactions to amplify overlapping DNA fragments. The forward primer (FM) contained the mutation(s) to be introduced into the wt-template DNA, whereas the reverse primer R2 contained a wt sequence. The second pair of primers: the reverse primer (RM) contained the mutation(s) and the forward (F2) had a wt sequence. At least 15 bases of primer RM should be exactly complementary to primer FM, and the mutation(s) should be introduced in the middle of the primer sequence.

The overlapping fragments (resulting from the first and second round of amplification) were mixed, denaturated and annealed to generate heteroduplexes that can be extended and, in third PCR; amplified into a full length DNA using two primers (R2 and F2) that bind to the extremes of two initial fragments.

For flanking primers where restriction sites are incorporated, the 5-8 nt overhang should be included since the restriction enzyme needs it for effective recognition and binding to restriction site.

5.8.11.2 In vitro mutagenesis using double stranded DNA templates: selection of mutants with Dpn I

With this method the mutation can be introduced directly in any circular double stranded plasmid with a moderate size (≤7 kb) eliminating need for subcloning into special vectors. The two ologonucleotides (oligos) were used to prime DNA synthesis by high fidelity polymerase on a denaturated plasmid template. The two oligos both contain the desired mutation and have the same starting and ending positions on the opposite strand of the plasmid DNA. The entire length of both strands of the plasmid DNA was amplified in linear fashion during several rounds of PCR, generating a mutated plasmid containing staggered nicks on opposite strands. The *in vitro* amplified mutated DNA differs from the template DNA, which has fully methylated $G^{Me6}ATC$ sequences (as a characteristic of all bacterially amplified DNA). The digestion of the PCR products with *DpnI* enzyme which specifically

cleaves fully mutilated DNA let to enrich the mutated plasmid within this mixture. The subsequent transformation of *DpnI* resistant plasmid with antibiotic resistance in bacteria recovers mutation positive clones. The success of the method relies on the design of primers and the choice of the appropriate thermostable DNA polymerase.

The oligos:

- must anneal to complementary strands of the same target sequence
- must be equal length (between 25 and 45 nt) with melting temperature 78C or greater (high enough to suppress false priming and low enough to allow complete dissociation of primer-primer hybrids during denaturation)
- Should terminate in G or C residue

The high fidelity *PfuI* DNA pol. can be used. In our work the Phusion High-Fidelity DNA Polymerase (NEB, 100U cat F-530S or Finnzymes) was used successfully for generation of all mutations.
Reaction mix: 10 ul of 5x Phusion HF buffer, 1 ul 10 mM dNTP (Fermentas), 2,5 ul of 10 µM each For/Rew primers (0,5 µM final), 1 µL DNA (10-20 ng total), water to 50 µL and at last 0,5 µL Phusion DNA Polymerase. All preparations were done on ice and PCR was started immediately after addition of the enzyme (because of the high 3'-5'exonuclease activity of the enzyme that can modify mutagenic primers).

Table 12 PCR protocol for the site-directed mutagenesis.

Denaturation	98ºC	30 sec	
Denaturation	98 ºC	10 sec	25 cycles
Primer annealing	60-65 ºC	20sec	
Extension	72 ºC	2 min	
Extension	72 ºC	5 min	
Hold	4 ºC	∞	

5.8.12 ***Sequencing.***

Sequencing was done at AGOWA GmbH Berlin Germany:

10 ul of DNA (80-100 ng/ul) and 4 ul of (10uM primer) were used for analysis.

5.8.13 ***RNA interference.***

Oligonucleotides targeting mouse PGC-1α (5'- GGTGGATTGAAGTGGTGTAGA-3') were annealed and cloned into pENTR RNAi vector (Invitrogen, Karlsruhe, GER). Non-specific oligonucleotides (5'-GATCTGATCGACACTGTAATG-3') with no significant homology to any mammalian gene sequence were used as non-silencing controls in all experiments as described (Berriel Diaz, Krones-Herzig et al. 2008).

5.8.14 ***RNA isolation with Qiazol™ Lysis Reagent***

For most experiments the purity of the RNA obtained after Qiazol™ isolation was sufficient. If not, the RNA was purified further by DNase treatment and purification via the Qiazol RNeasy Mini purification kit.

5.8.15 ***RNA isolation from tissue samples***

Briefly, 10-30 mg of frozen tissue was transferred into a 2mL RNase/DNase-free reaction tube containing 1 ml Qiazol™ Lysis reagent (Cat No: 79306) and a stainless steel bead (Qiagen, 5mm, Cat No. 69989). The samples were lysed by vigorous shaking in the TissueLyser™ (Qiagen) for 90 sec at a frequency of 30 Hz. The lysate was incubated for five minutes at room temperature to release nucleoprotein complexes and transferred into a fresh RNAse/ DNase-free safety lock-1,5 ml tube containing 200 µl chloroform. The mixture was vortexed for 15 sec and then spined for 15 min at 12000 x g. The upper aqueous solution, containing mainly RNA, was carefully transferred into a fresh reaction tube. If the low amount of cell material (e.g. from 96 well plate) was used for RNA isolation the 1 ul of RNase free glycogen was added at this stage and mixed. Further the RNA phase was mixed with 500 µl 2-propanol and incubated for 10 minutes at RT, followed by 10 min centrifugation at 12000 x g. The supernatant was aspirated and the pellet washed once with 1 ml 75% ethanol. The solvent was discarded, and rest of the alcohol removed with a pipette. The pellet was briefly air-dried and re-solubilised in a suitable amount of water (30 -50 µl). To increase solubilisation, RNA solution was incubated at 55-60°C for 10 min on a Thermomixer (Ependorf). The samples were stored until further use at -80°C.

5.8.16 ***RNA isolation from cell samples***

Adherent cells were stimulated according to experimental needs. The medium was removed and the cell monolayer washed once with sterile PBS. The buffer was aspirated and 1mL Qiazol™ Lysis reagent was added per 10cm plate (or 500 µl per well on a 6-well plate). Cells were washed few times with the pipette and transferred into DNase/RNase-free reaction tubes. The cells were incubated for 5 min at room temperature and vortexed vigorously until no cell clumps were visible. The obtained cell lysate were stored at -80°C or used directly for RNA isolation.

5.8.17 ***RNA isolation with RNeasy Mini purification kit***

This protocol was used for applications, when genomic DNA contamination in RNA samples was needed to be removed e.g. for RT-PCR with Taqman probes that could not ignore genomic DNA.

Tissue samples were transferred into a 2 mL reaction tube equipped with 600 µl RLT buffer and 6µl β-mercaptoethanol and lysed for 2 min at 30 Hz using the TissueLyser. The obtained lysate was transferred to a Qiashredder column and spined at 13000 rpm for 2 min to get rid of the cell membranes and DNA contamination.

600µl 70% ethanol was added to the eluate and the solution was gently mixed by turning the reaction tube. The reaction mixture was transferred to a RNeasy column (maximum 700 µl at once) and spined for 1 min at 13000rpm. (RNA, isolated using Qiazol™ Lysis reagent can be used at this step). In this case the RNA sample was diluted with 350µl RW1 buffer and applied onto the RNeasy column. The flow-through was discarded and the column washed with 350 µl RW1 buffer for 5 min. The buffer was removed by centrifugation.

To the dry column 80 µl DNase buffer (10µl DNase and 70µl RDD buffer) was added directly and left for DNA digestion for 30 min at room temperature. Subsequently, 350 µl RW1 buffer was added and co-incubated for another 2 min. The column was washed twice with 500µl RPE buffer. Finally, the RNA was eluted in 30 µl RNase free water.

5.8.18 ***Evaluation of RNA quality and quantification***

To assess the quality of the RNA preparation, a 1% Agarose gel was poured with RNase-free reagents. RNA samples were denatured using RNA denaturation buffer (see below). To 10 µl RNA denaturation buffer 500 ng RNA sample were added followed by 10 min incubated at 65°C.

RNA denaturation buffer (per sample):

0,5 µl ethidium bromide (0,1%, Roth, Cat No. 2218.2)
0,5 µl 10 x MOPS buffer
5µl formamide
1,75 µl formaldehyde
1,7µl loading dye (Fermentas, #R0611)
0,55 µl RNase free water

After denaturation samples were loaded onto the agarose gel and separated for 30-40 min at 90-100 V. Since mRNA represents only 1% of total cellular RNA it is not possible to directly examine its degradation status. The quality of the RNA was determined visually by the ratio between 28S to 18S ribosomal RNA, which should be around 2:1.
The amount of RNA was determined spectophotometrically at 260 nm using the Nanodrop device. Furthermore, the ratio 260nm/280nm was determined to measure protein impurities. The value should be between 1,8 and 2,1.

5.8.19 ***cDNA synthesis***

5.8.19.1 Fermentas kit protocol

For the synthesis of cDNA 1000ng purified RNA was used. The RNA was adjusted to a volume of 9µl per sample with RNase/DNase-free water and 1µl oligo$(dT)_{18}$ primers (Fermentas #K1612) was added per sample. The reaction mixture was vortexed and incubated for 5 min at 70°C for primer annealing at the polyA tails. Samples were chilled for at least 2 min at 4°C and then a reaction mixture was prepared according to the manufacturer's instruction (Fermentas).
Reverse Transcriptase Reaction Mixture (per sample):

4 µl 5 x reaction buffer

2µl 10 mM dNTP mix

1 µl Ribolock ™ Ribonuclease Inhibitor

Samples were mixed with the buffer and incubated for 5 min at 37°C, followed by addition of 2µl M-MuLV reverse transcriptase (RT), 20u/µl. To assess DNA contamination a reaction without reverse transcriptase was performed. After 1 hour incubation at 37°C the cDNA synthesis was terminated by heat inactivation of the reverse transcriptase for 10 min at 70°C. The cDNA was stored at -20°.

5.8.19.2 Superscript protocol

For the cDNA synthesis of the low RNA amounts the Superscript cDNA synthesis kit were used. The superscript reverse transcriptase is able to amplify a total RNA of 50 to 1000 ng.
The protocol is similar to that in Fermentas kit and was performed as followed:
Reverse Transcriptase Reaction Mixture (per sample):

50-500 ng total RNA

1ul oligo$(dT)_{18}$ primers (500 ug/ml)

1µl 10 mM dNTP mix

Water to 12 ul, mix, incubated 65 °C, 5', and chilled on ice.

Add

4 µl First strand buffer

2 ul 0,1M DTT

1ul Ribolock (we used one from Fermentas kit)

Mix, incubate, 42 °C, 2'

Add 1ul Superscript (200U), and mix by pipetting
Incubate 42 °C, 50'
Inactivate the enzyme at 70°C for 15 min.

*5.8.20 **Quantitative Real-Time PCR***

The cDNA samples were diluted ten-fold with DNase/RNase free water and 5µl of this solution were used per reaction. A reaction sample mix included: 10µl Platinum® Quantitative PCR Supermix, (Invitrogen Cat. No. 11730-025), 3,6 µl DNase/RNase free

water, 0,4µl ROX dye (Invitrogen, Cat. No. 12223-012) and 1µl Taqman probe per individual reaction. Technical duplicates of all samples were performed. Taqman probes were obtained from Applied Biosystems and are listed in Table 5.
Water was used as a negative control and no RT (see 5.8.19.1) as a control for genomic DNA contamination. 20µl PCR reaction was transferred per well onto a MicroAmp™ Optical 96-well reaction plate (Applied Biosystems, Cat. No. 4316813) and quantitative PCR was done on a 7300 Real Time PCR System (Applied Biosystems).

5.9 CELL BIOLOGY

All cell lines were cultivated at 37°C, 95% humidity and 5% CO_2. Aseptic techniques were always used to maintain cells or for cell culture experiments.

5.9.1 *Cell line treatment and transfection*

In all experiments the amount of the transfected DNA between different experimental conditions were always equilibrated with an empty vector to maintain equal transfection efficiency in all samples. Before any stimulation cells were starved at the serum free medium for 5-6 hrs to bring gene expression profile to equal, not induced state.

5.9.1.1 Human Embryonic Kidney cells

Human embryonic kidney cells (HEK 293 cells) were maintained and extended in Dulbecco's Modified Eagle Medium with high glucose (DMEM) (Gibco Cat. No. 41966-052), 10% fetal calf serum (FCS) and 1 x penicillin/ streptomycin (P/S).
Transient transfections were done with the $Ca_3(PO_4)_2$-method according to Sambrook and Russell using a modified protocol: the cells were plated at a concentration of 1 x 10^5 cells/ ml the day before the experiment on a 12-well plate. Prior to transfection medium was exchanged and 500µl fresh medium/plate were applied again. Between 800ng and 2,5 µg total DNA were used for transfections per well and each condition was done in triplicates. For this purpose, the appropriate amount of DNA was diluted in sterile water and 12,6 µl 2,5 M CaCl2 were added to reach a final volume of the solution of 126µl. To facilitate an ion exchange on the DNA from Mg^{2+} to Ca^{2+} the reaction mixture was incubated for at least 5 min at 37°C.

Subsequently, 126µl HBSS buffer (pH 7.05) was added drop wise to the reaction while mixing the reaction. After incubation for exactly one minute 504 µl DMEM medium (37°C) were transferred to the solution, the transfection solution was mixed well and 190µl of it were transferred per 12-well immediately. After 3 to 4 hours $Ca_3(PO_4)_2$ crystals were visible microscopically.

Transfection was done overnight and the following day, medium was exchange to normal growth medium or stimulation was carried out.

5.9.1.2 H4IIE rat hepatocytes, Hepa1C1 wt cells and 293A cells

H4IIE cells were maintained in MEM medium (Gibco Cat No. 31095-052) with 10% FCS, 1% nonessential amino acids (NAA) and 1% penicillin/ streptomycin (P/S). 293A cells and Hepa1C1 wt cells were cultivated in D-MEM medium supplemented with 10% FCS, 1% NAA and 1% penicillin/streptomycin.

Transient transfections were done with method using linear polyethylenimine (PEI) described by Reed et al (Reed, Staley et al. 2006). The method works effectively in wide variety of mammalian cells has relatively low cytotoxicity. The optimal DNA amount that could be transfected by this method was 1 ug per well in a 12 well plate. After exceeding 1,5 ug the transfection efficiency decreased dramatically.

Briefly, H4IIE cells were plated at the concentration of 2 x 10^5 cells/ ml the day before the experiment on a 12-well plate. The amount of PEI (Polysciences Europe Cat. No. 24765) were calculated with respect to the amount of DNA that was used for transfection. Generally, 8 ul of PEI per 1ug of DNA. When 1 ug DNA per well was used: 8 ul PEI reagent was diluted with the water to 25 ul and mixed afterwards with 25 ul of NaCl (300mM) to keep a ratio of PEI: Salt as 1:1. The 1ug DNA was taken and the volume of DNA was brought to 25 ul and as previously diluted 1:1 with 25 ul NaCl (300mM). Finally, the DNA: Salt mix was added to the PEI: Salt mix and mixed thoroughly and incubated at room temperature for 10 min. Afterwards 1 ml of fresh MEM medium, FCS 10%, 1% P/S, 1% NAA was carefully mixed with PEI: DNA: Salt mixture and added to cells. Normally, the mixture was prepared as master mix and amount of medium was taken according to the number of repeats of the one sample. Cells were incubated overnight and next day the crystals were visible microscopically. Medium was changed to the serum free MEM for further stimulation.

5.9.2 *Harvest of transfected cells*

Cells were harvested 48 to 72h after transfection. The medium was discarded and cells were washed once with 1xPBS. Harvest buffer (25 mM Gly-Gly pH 7,8, 15 mM $MgSO_4$, 4 mM EGTA, 0,1 mM DTT, 0,01% Triton X-100) was added to wells and incubated for 5 min on a shaker to facilitate detachment from the dish. Strongly attached cell lines e.g. H4IIE were scratched from the plate on ice. Lysate was transferred to a 1,5ml reaction tube. Typically, for one well on a 12-well plate 200 µl of harvest buffer were used. Insoluble debris was removed by centrifugation for 3 min at 13000 rpm and the supernatant was transferred to a fresh tube. The lysate can be used directly for reporter gene assays or stored at -20°C.

5.9.3 *Measurement of luciferase activity*

Luciferase is an enzyme catalysing a reaction in which beetle luciferin is transformed into oxyluciferin by oxidative decarboxylation thereby emitting photons. Under assay conditions the substrate luciferin is available in excess, consequently the amount of light emitted is proportional to the amount of firefly luciferase in the lysate. Lucifirase were used as a reporter for all promoter studies.

For the determination of luciferase activity in lysates of transfected cells, 30µl of this lysate were transferred into a well on a black 96-well-plate. The lysate was diluted with 100µl assay buffer (25 mM Gly-Gly pH 7.8, 20mM K_3PO_4, 15 mM $MgSO_4$, 4mM EGTA, 2 mM ATP, 1,6 mM DTT) and the plate was measured by a luminometer (Mithras 940 Luminescence) equipped with a dispenser. Automatic injection of 100µl luciferin buffer (0,32 mg/mL luciferin, 25 mM Gly-Gly pH7,8, 15 mM MgSO4, 4 mM EGTA) starts the reaction.

Light emission was measured at a wavelength of 560 nm and as blank value harvest buffer was used. All samples were done in biological triplicates and each value was normalized against β-galactosidase values.

5.9.4 *Measurement of β-galactosidase activity*

Transfection efficiency was monitored by co-transfection of a plasmid constitutively expressing β-galactosidase from H-Ras promoter (H-RAS β-Gal). It is important to test whether any experimental condition e.g. stimulation has an influence on the expression of β-galactoside to exclude a false normalisation.

For the assay a buffer including the substrate ortho-nitrophenyl-β-galactopyranosid (ONPG) was prepared (1M Na_2HPO_4, 1mM $MgCl_2$,10 mM KCl, 1mg/ ml ortho-nitrophenyl-galactopyranosid). 5 ml of this buffer were mixed with 13,5 µl beta-mercaptoethanol prior to β-Gal assay. Into a clear 96-well plate (Costar Cat No. 13631) 50µl cell lysate/ well was pipetted and 50µl/ well ONPG buffer was added. As a blank value harvest buffer was used. The plate was incubated for 6-12 hrs at 37 °C until a clear yellow colour was visible. The absorption was measured at 405 nm, the maximum absorption of the ortho-nitrophenylat ion. The values were analysed with respect to the noise signal as well as to different experimental treatment where the β-Gal value should not varied more than a normal deviation.

5.10 **BIOCHEMISTRY**

5.10.1 ***Preparation of protein extracts from liver samples using PGC buffer***

Protein extracts for SDS-PAGE were prepared with PGC lysis buffer. A 2- ml reaction tube was filled with 1 ml PGC lysis buffer, protease inhibitors and a stainless steel bead and incubated on ice. Approximately 30 mg frozen liver tissue were transferred into the ice-cold buffer and the sample was immediately homogenized in the TissueLyser ® (Qiagen) for 2 min at 30 Hz. The extracts were incubated for 20 min on ice and the lysate was transferred to a fresh tube. The lysate was spined for 15 min at 13000 rpm. The fat layer was discarded without perturbing the lysate if it appeared at the surface after centrifugation. The lysate was transferred to a fresh reaction tube and a determination of the protein concentration was carried out. Samples were adjusted to a concentration of 1 mg/ml protein with 2 x SDS buffer and incubated for 5 minutes at 95°C for denaturation. Afterwards they were used directly for SDS-PAGE and immunoblotting. If not, stored at -20°C. The original lysates were be kept at -80°C before denaturation.

5.10.2 ***Preparation of protein extracts from liver samples using SDS lysis buffer***

20-50 mg snap-frozen liver tissue samples were transferred to a 2 ml SafeLock tube equipped with 1 ml 2xSDS lysis buffer with 8M Urea (at room temperature so that SDS cannot precipitate) and a stainless steel bead. The Urea additionally increases the protein denaturation and is very helpful for tissue samples. Protease inhibitors are not necessary when using this

buffer. The tissue sample was immediately homogenized for 2 minutes at 30 Hz employing the Qiagen Tissue Lyser, until no tissue clumps were visible. Afterwards, samples were incubated for 5 minutes at room temperature and the lysate was transferred to a fresh tube. The debris was removed by centrifugation for 10 minutes at 13000 rpm and room temperature. The supernatant was transferred to a fresh tube and incubated for 5 to 10 minutes at 95°C. The protein concentration was determined using the 2D-Quant Kit, since the high amount of SDS interferes with the protein determination of the BCA™ Protein Assay Kit.
A part of the original lysates was adjusted with SDS lysis buffer to 1 mg/ml. Original lysates and adjusted samples were stored at -20°C.

5.10.3 ***Preparation of Protein Extracts from liver tissue for luciferase assay***

Around 100-150 mg snap-frozen liver tissue sample was used and a small part of it (10-20 mg) was separated and kept in -20C for virus genome isolation (5.10.4). The bigger part was transferred to a 2 ml SafeLock ® tube equipped with 1 ml chilled tissue lyser buffer for luciferase assay (1mM DTT, 4mM EGTA, 16 mM EDTA, 1xProtease inhibitory mix (see below), 2,5M Potassium phosphate buffer, pH 7,8; 0,01% Triton X-100) and iron ball and lysed few times with Qiagen Tissue Lyser for 1,5 min, at 30 Hz until no tissue clumps were visible. The lysate was frozen and thaw once in liquid nitrogen and incubated on ice for 1 hour. Afterwards, the sample was spined at 13,000 rpm for 10 min. and supernatant transferred to a fresh 1,5 ml tubes. By transferring the supernatant, the lipid phase swimming on the surface has to be avoided. The protein concentration was determined using the 2D-Quant Kit, and the protein concentration between samples was adjusted to the smallest one (normally around 10-20 ug/ul) with a tissue lysis buffer. Further 30 ul of the lysate was used for a luciferase assay, see p 1.2.3. Original lysates and adjusted samples were stored at -20°C.

5.10.4 ***Adenoviral genome quantification in liver tissue***

The isolation of adenoviral genome from liver tissue was performed by the protocol described in 5.8.9. The viral DNA can be identified and relative viral genome numbers can be calculated with real time q-PCR with designed Taqman probes against GFP gene sequence. The GFP was cloned in virus together with pAd-Track vector with the gene of interest. Taqman probe assay with following primers (EGFP-ANYF:GAGCGCACCATCTTCTTCAAG, EGFP-ANYR: TGTCGCCCTCGAACTTCAC, EGFP-ANYM1 FAM: ACGACGGCAACTACA)

was ordered from Applied Biosystems. The relative amount of viral genome was used for the precise normalisation to the DNA amount used in luciferase assay in analysed tissue samples.

5.10.5 ***Protein determination with the BCA™ method***

The assay was done in a 96-well format to allow the simultaneous measurements of up to 42 samples. First a standard curve from 2µg to 10 µg protein in duplicates was transferred to the plate using BSA (2mg/ml, Pierce Cat. No. 23209). Then between 2µl and 8µl of protein lysates with unknown protein concentration were added to the separate wells. The protein concentration should be within the range of the standard curve. If necessary, dilutions of the original protein lysate can be prepared using 0,9% saline for dilution. Next working reagent was prepared by mixing BCA™ reagent A with BCA™ reagent B in a ration of 50:1 (v/v). Per sample 200 µl working reagent were transferred with a multistep pipette onto the plates and the reaction mixture was incubated for 5 min at 37°C.

5.10.6 ***Protein determination with the 2D-Quant Kit***

The 2D Quant Kit (Amersham Biosciences Europe, 80-6483-56) was used for determination of protein samples containing high detergents or SDS concentrations (e.g. 1% SDS and more). The procedure works by quantitatively precipitating proteins while leaving interfering substances in solution. The assay is based on the specific binding of copper ions to protein. Precipitated proteins are resuspended in a copper-containing solution and unbound copper is measured with a colorimetric agent. The colour density is inversely related to the protein concentration. Accurate estimation of protein concentration is achieved by comparing to a standard curve. For the standard curve BSA (2mg/ml) was used.

For the assay between 3 and 10µl protein lysates was used and proteins were precipitated following manufacturer's instructions. Samples were done in duplicates.

The absorbance of samples and standards was read at 480 nm in spectrometer (Ultrospec, Pharmacia) using water as a reference. Subsequently the standard curve was generated by plotting the known protein concentration against the absorbance. Absorbance values of unknown samples should lie in the same range, extrapolation is not advisable.

5.10.7 ***SDS-PAGE***

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were diluted in 2xSDS sample buffer (120 mM Tris pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol,

0,01% bromophenol blue) and denatured by boiling for 5 min at 95 °C. If working with a liver tissue, 30-50 mg of it was lysed first with iron ball in corresponding lysis buffer on TissueLyser ® (Qiagen) for 1,5 min at 30 Hz. Afterwards lysate was mixed with 2xSDS sample buffer and boiled for 5 min. If no special buffer was necessary, tissue could also be lysed directly in 1 ml 2 x SDS sample buffer with tissue lyser, but SDS precipitation during the procedure has to be avoided.

Proteins were first focused in the low-percent collection gel and subsequently separated in the separation gel. For the separation gel different concentrations of acrylamide were used. Routinely, 10cm gels were poured. During polymerisation the gel was overlayed with 2-propanol. After 15-20 min on the top of the separation gel, 5 ml collection gel were added (625 ul 1M Tris-HCl, pH 6,8, 840 ul 30% acrylamide mix (37,5 acrylamide : 1 bisacrylamide), 25µl 20% SDS, 50µl 10% ammonium persulfate, 5 µl TEMED and water to 5 ml final volume). After 45-60 min polymerised gel could be stored in humid atmosphere at 4 °C for several weeks. Protein amounts between 10 and 50µg were subjected to separation on 10 ml gels in SDS electrophoresis buffer (1,92 M Glycin; 0,25 M Tris , 0,45% HCl; 30 mM SDS). Gel was run at 100V for 15 min and then 150V for 65-90 min.

Table 13 Composition of polyacrylamide gels with different concentration of acrylamide.

	8% gel	**10% gel**	**12% gel**
Water	4,6 ml	4 ml	3,3 ml
30% acrylamide mix	2,7 ml	3,3 ml	4 ml
1,5 M Tris (pH 8,8)	2,5 ml	2,5 ml	2,5 ml
10% ammonium persulfate	100µl	100µl	100µl
20% SDS	50µl	50µl	50µl
TEMED	6 µl	4µl	4µl

After electrophoretic separation gels were either stained with Coomassie or subjected to immunoblotting (see below). For Coomassie staining gels were briefly washed with water and then fixed in fixation buffer (25% 2-propanol, 10% acetic acid) for 1h and gentle shaking. The gels were washed twice with water and stained using PageBlue™ protein staining solution (Fermentas Cat. No. R0571) overnight. Excess staining was removed by washing the gel several times with water.

5.10.8 ***Immunoblotting***

Proteins were electrophroretically transferred to nitrocellulose membranes, which was equilibrated with transfer buffer for 15 min at room temperature (1,92 M Glycin; 0,25 M Tris, 20% methanol, 0,01% SDS), overnight at 30V and 4°C. The slow transfer facilitates especially efficient blotting of larger proteins. In fast protocol transfer could be performed also at 60V for 4-5 hrs. After transfer membranes were washed briefly in water and the transfer was examined by Ponceau S staining (Sigma, Cat. No. P7170) for 5 min. Ponceau S was removed and membrane was washed twice with water followed by 1 hour incubation in blocking buffer (5% non-fat powder milk (Roth #T145.2)) dissolved in TBST buffer (20mM Tris pH 7.5,140 mM NaCl, 0.1% Tween-20®) for 1 hour. Subsequently, membranes were washed three times with TBST buffer for 15 min each. Secondary antibodies (conjugated to horse radish peroxidase (HRP)) were used at a dilution of 1:2000 to 1:5000 and were applied in blocking buffer for 45 min to 1h. Membranes were rinsed with TBST buffer again three times for 15 min. To detect specific bands the enhanced chemiluminescence system (ECL™) Western Blotting Detection Reagent (Amersham Cat. No. RPN2106) was used, followed by the exposure to HyperfilmTM ECL films (Amersham Cat. No. RPN3103K). Exposure times varied between the different antibodies used.

5.10.9 ***Isolation of hepatic glycogen***

Frozen liver samples were cut into 100 to 200 mg big pieces and the exact weight was determined. The liver was transferred into a reaction tube equipped with 1 ml 30% (w/v) KOH and a stainless steel bead. Homogenization of the samples was facilitated in the TissueLyser® (Qiagen) for 2 minutes at 30 Hz. For complete tissue disruption the lysate was incubated for 30 minutes at 95°C with shaking in a Thermomixer (Eppendorf). The debris was removed by centrifugation for 5 minutes at 13000 rpm and the supernatant was transferred into a 15 ml reaction tube. The glycogen was precipitated by addition of 1,5 ml 95% ethanol and subsequent centrifugation at 3000 x g for 20 minutes. The pellet was washed once with 1 ml ethanol and after air-drying the pellet for 5 minutes it was re-solubilised in 0,5 ml water and incubated for 30 minutes at 37°C. After vigorous mixing, the insoluble material was removed by centrifugation for 5 minutes at 3000 x g. The supernatant was transferred to a fresh tube and can be stored at -20°C until further use.

5.10.10 ***Colorimetric Assays***

5.10.10.1 Digestion of glycogen with amyloglucosidase and determination of glucose deliberated from glycogen

A stock solution of Amyloglucosidase (Sigma, A7420) was prepared by dissolving 0,33 mg enzyme in 0,2 M sodium acetate pH 4.8. Samples with isolated glycogen were thawed and 50 µl of glycogen sample was mixed with 450 µl Amyloglucosidase stock solution. The mixture was incubated for 2 h at 37°C on a Thermomixer (Eppendorf) while shaking the samples. The reaction was stopped by neutralisation with 10 µl 30% (w/v) KOH.
The glucose determination was done with the Glucose (HK) Assay Kit (Sigma, GAHK-20). The kit uses the following reactions:

Glucose + ATP $\longrightarrow$ Glucose-6-phosphate + ADP (catalysed by Hexokinase)
Glucose-6-phosphate + NAD^+ $\longrightarrow$ 6-Phosphogluconate + NADH (catalysed by G6PDH)

The release of NADH was directly proportional to the amount of glucose in the reaction. NADH was measured sprectrometrically at 340 nm. A standard curve with known concentrations of D-glucose was prepared and the glucose content was determined according to the standard curve. The amount of sample used for the reaction varies; the obtained OD-values should lay within the range covered by the standard curve. The test was carried out according to manufacturer's instructions.

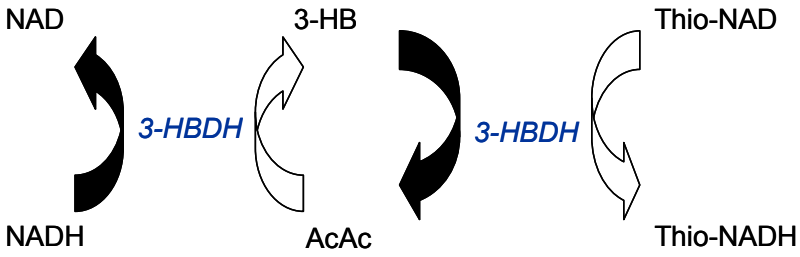
5.10.10.2 Determination of Free Fatty Acids

Free Fatty Acids were determined in serum samples with a colorimetric assay from Wako (NEFA C, Cat No: 999-75406).
The assay principle is based on the following reactions:
1) RCOOH + ATP + CoA-SH $\longrightarrow$ Acyl-CoA + AMP + PP_i
2) Acyl-CoA + O2 $\longrightarrow$ 2,3-Enoyl-CoA + H_2O_2
3) H_2O_2 + 4-Aminophenazon + 3-methy-N-ethyl-N(β-hydroxyethyl)-aniline $\longrightarrow$ Quinoneimine-color + 4 H_2O

R-COOH (any free carbonic acid). The reaction catalysed by (1) Acyl CoA synthetase, (2) acyl CoA oxidase and (3) peroxidase.
A standard curve using oleic acid (1mM, Cat No: 270-76499) and 4 µl sample (in duplicates) was used for the measurement. The procedure was carried out according to manufacturer's instructions. OD-values were determined at 550 nm and the amount of free fatty acid was calculated according to standard curve.

5.10.10.3 Serum ketone bodies measurement

The total ketone bodies could be determined in serum or plasma samples. The assay was performed with the commercial kit accordingly to the provided protocol (Autokit Total Ketone Bodies, Wako chemicals GmbH).
The principle of the assay is a cyclic enzymatic reaction where the rate of Thio-NADH production depends on the concentration of total ketone bodies in the sample and can be determined spectrophotometrically:



NAD: Nicotinamide adenine dinuclotide
NADH: Nicotinamide adenine dinuclotide reduced
3-HB: 3-Hydroxybutarate
AcAc: Acetoacetate
Thio-NAD: β-thionicotinamide adenide dinucleotide oxidized
Thio-NADH: β-thionicotinamide adenide dinucleotide reduced
3-HBDH: 3-hydroxybutarate dehydrogenase

The calibrator for standard curve was provided with the kit. All samples were measured in duplicates.

5.10.11 ***Radioimmunoassay for corticosterone***

Serum corticosterone levels were measured using the corticosterone RIA Kit from MP Biomedicals (MP Biomedicals, Cat No #07120102). Serum samples were slowly thawed on ice and briefly spined to sediment particles for 30'' at 13 krpm. Dilutions of serum were done in Seroid Diluent (included in Kit) depending origin of the sample, e.g. samples from fasted animals and db/db mice were diluted 1:200, serum samples with low corticosterone levels were diluted 1:25. The assay was carried out in Fiolax tubes (Roth Cat No: 223.1 8x70 mm) according to manufacturer's instructions. Safety rules valid for work with ^{125}I were applied. A standard curve was prepared with stock solutions provided in the Kit. Samples were done in duplicates.

After addition of all reagents following the kit instructions, samples were incubated for 2h at RT. Bound corticosterone was precipitated by centrifugation for 15 min at 2400 rpm at 20°C (Hereaus centrifuge). Supernatant was discarded and remaining radioactivity in residue was measured in a gamma Counter (Master 1277, LKB Wallac) with 1 min count time per sample. Corticosterone level was calculated based on the standard curve.

5.10.12 ***Chromatin Immunoprecipitation (ChIP-Assay)***

The ChIP assay is a method to test the occupation of a certain protein on a DNA sequence *in vivo*. The assay can be performed with cultured cells or with tissue lysates.

5.10.12.1 *ChIP assay from liver lysates*

Approximately 50 mg of snap-frozen liver tissue were homogenized in dry ice powder in a pre-chilled mortar using a pistil. The frozen tissue powder was transferred into a 50 ml reaction tube equipped with 1,5 ml DMA buffer (2,4 mg/ml DMA in triethanolamine pH 8.0) and wrapped with aluminium foil. Since the CO_2 powder leads to freezing of the DMA solution the whole mixture needs to thaw on ice. After complete thawing, the suspension was incubated at room temperature for 30 min to crosslink proteins with each other. The suspension was spined down for 3 min at 2000 rpm and the supernatant was discarded. The pellet was re-suspended in 5 ml 1% formaldehyde in PBS and incubated for 10 min at room temperature on a rotating wheel to facilitate DNA-protein cross-linking. The tissue was pelleted again by centrifugation for 3 min at 2000 rpm and washed once with 5 ml ice-cold

PBS. After removal of PBS the tissue pellet was re-suspended in 1 ml cell lysis buffer (10 mM Tris pH 8,0, 10mM NaCl, 0,2% NP-40) including protease inhibitors. The mixture was transferred into a pre-chilled Dounce Homogenizer (Size B) and homogenisation was done until no tissue clumps were visible. After incubation for 5 min on ice the solution was transferred to a 2ml reaction tube and spined down for 5 min at 13000 rpm. The pellet was re-suspended in SDS lysis buffer (50 mM Tris-HCl pH 8,1, 10 mM EDTA, 1% SDS) including protease inhibitors and incubated for 10 min on ice. The samples were subsequently sonicated 6 times for 15'' at Duty cycle 30, Output control 3. After another centrifugation step for 10 min at 13000 rpm and 4°C the lysate was transferred to a fresh tube and the pellet was discarded.

The protein concentration of each sample was determined using the 2D Quant Kit. Afterwards, all samples were adjusted to the same protein concentration using SDS lysis buffer. For the subsequent steps 200µg protein are used and 20µg protein are used for the input control samples. Alternatively, the samples could be de-crosslinked (see below) and the total DNA can be isolated and quantified. In that case the samples could be equilibrated by the equal DNA input and can be used for subsequent procedures.

The solution containing 200µg protein (or 2-5 µg total DNA) was diluted 10-fold with ChIP dilution buffer (16,7 mM Tris-HCl pH 8,1, 167 mM NaCl, 1,2 mM EDTA, 1,1% Triton X-100, 0,01% SDS) to obtain final SDS concentration of 0,1%. To each sample the 5µg antibody were added and antigen-antibody reaction was done overnight at 4°C on a rotating wheel.

Next day 60µl salmon sperm agarose (Upstate) were added per sample and incubated for 2h at 4°C on a rotating wheel. The suspension was gently centrifuged for 2min at 1000 rpm and the supernatant was discarded. The agarose was washed several times in the following sequence for 5 min per step and between each step washing solution was removed by centrifugation for 2 min at 1000 rpm.

First washing cycle was done once with 1 ml low salt buffer (20 mM Tris-HCl pH 8,1, 150 mM NaCl, 2mM EDTA, 1% Triton X-100, 0,1% SDS) per sample followed by washing once with 1 ml high salt buffer (20 mM Tris-HCl pH 8,1, 500 mM NaCl, 2mM EDTA, 1% Triton X-100, 0,1% SDS). 1ml immuno-complex buffer (10 mM Tris-HCl pH 8,1, 1% deoxycholic acid, 0,25 M LiCl, 1% Igepal-CA 630) were then added and finally, agarose was rinsed twice with 1ml cold TE buffer (10mM Tris pH 8,0, 1mM EDTA) per sample. After complete removal of TE-buffer, 100µl 10mM DTT per sample were added to the agarose and elution

was facilitated by incubation for 30 min at 37°C in a Thermomixr (Eppendorf) at 400 rpm. The elution was repeated and subsequently the eluates were combined.
Input samples containing 20µg protein were thawed and diluted with 100µl 10mM DTT. Samples were de-crosslinked by addition of 4µl 5M NaCl and 2µl 5M NaCl to the input samples, respectively and incubation at 65°C overnight.
Next day, samples were briefly spined down to collect the buffer and then a Proteinase K digest of DNA-bound proteins were performed by addition of 2µl 0,5M EDTA, 4µl 1M Tris-HCl pH 6,5 and 0,4µl proteinase K per sample. For the input samples the volumes were bisected. Protein digest was done for 1h at 45°C and the DNA was purified using the PCR purification Kit (Qiagen) following the manufacturer's instructions. DNA was eluted with 21µl TE buffer and stored at -20°C until evaluation with PCR.

5.10.12.2 ChIP assay from cultured cells

For each individual condition one 15cm plate is needed with 80 to 90% confluence. Per 25 ml medium 680 µl formaldehyde were used and cells were incubated for 10 min at 37°C. The medium was removed and cells were washed once with ice-cold PBS. Cells were harvested in 2ml PBS using a cell scraper and the suspension was transferred into a fresh tube. Cells were pelleted by centrifugation for 5 min at 3000 rpm and the supernatant was discarded. Re-suspension was done in 400µl SDS lysis buffer containing protease inhibitors.

5.10.12.3 Evaluation by PCR

After isolation of DNA protein complexes and subsequent proteinase K digestion DNA with an average size of approximately 500 to 2000 bp are available. To examine the amount of immuno-precipitated DNA complexes, a PCR reaction was performed.

Table 14 Specific primers used for the evaluation of ChIP experiments

Promoter	Species	Primer	Sequence 5'--3
PEPCK prox.1	Rat	Forward	GGCCTCCCAACATTCATTAAC
PEPCK prox.1	Rat	Reverse	GTAGCTAGCCCTCCTCGCTTTAA
PEPCK prox.2	Murine	Forward	TAACACACCCCAGCTAACTCAG
PEPCK prox.2	Murine	Reverse	ACTTCATATGTTGCTGGCTGC
PEPCK distal	Rat	Forward	TTCTCCTCCTCCATCATTGG
PEPCK distal	Rat	Reverse	TGCATCCCTGGAAGGTTAAG

Per individual PCR reaction 3-6µl DNA isolated in ChIP experiments, 12,5µl 2x PCR Mix, 7,5µl ultra-pure water, 1µl forward primer (1µM) and 1µl reverse primer (1µM) are used to reach a final volume of 25µl.

Table 15 PCR protocol for evaluation of ChIP experiment:

Initial denaturation:	10 min	95°C
35 cycles	30 sec	95°C
	30 sec	52°C
	90 sec	72°C
Final elongation	30 sec	95°C
	3 min	72°C
	cool down to 4°C.	

The PCR reaction was interrupted at cycle 28 to 30 and 10µl of the mixture were transferred to a fresh tube. The PCR was completed with the remaining reaction mixture, so that finally per individual reaction two samples at different times of the PCR reaction were available. The samples were diluted with Orange Dye (6-fold, Orange G dye, 50% glycerol, 10 mM EDTA)

and are run on a 1,5% agarose gel. The signal intensity obtained directly correlates with the amount of immuno-precipitated protein complex with the DNA and therefore is a measure for binding affinity under a given experimental condition. Alternatively with a set of PEPCK prox. primers 2 with a final concentration (300nM) the ChIP assay evaluation was performed my Cyber Green (MESA Green q-PCR MasterMix Plus (SYBR), 7.5ml, Eurogentec, cat RT-SY2X-03+WOU).

Table 16 SYBR GREEN protocol for quantification of ChIP experiment.

12,5 ul 2xReaction buffer (RB)
2,5 ul For.promer (300 nM final)
2,5 ul Rev. primer (300 nM final)
1,5 ul Water

6 ul eluted DNA.

_____

25 ul total.
Results were evaluated by dissociation curve.

5.10.13 ***Generation and production of adenoviruses***

5.10.13.1 *Cloning of adenoviruses*

All adenoviruses were generated based on pAd-Easy1 system from Stratagene. After cloning of the target genes into the pAd-shuttle vector it was leniarized with *Pme I* (Fermentas) and integrated into pAd-Easy1 adenoviral backbone in BJ5183 competent cells. The two types of colonies were detectable on *Kanamycin* –selective LB agar plates after 18-24 hrs incubation at 37 C: the empty pAd-shuttle re-circulated clones that are huge, and recombinant positive clones – that are small. The plasmid DNA from 5-10 positive clones was isolated and analysed by PCR with a primers pAd-PM13 and pAd-PM14 (that detect pAd-easy1) and primers for Shuttle vector with the target gene pAd-Luc3 and pAd-GFP. It is important to do the miniprep and DNA isolation from BJ5183 as soon as possible since they can modify the plasmid DNA by causal recombination. The isolated DNA for next steps should be re-transformed in normal *E.coli XL-Blue* competent cells. The positive clone was further verified by sequencing for the target gene, e.g. Luciferase and downstream PEPCK promoter element.

The 20 ug of positive DNA in total Vol. 150 ul was digested with *PacI* (NEB) and concentrated as following: 150 Restriction mix was mixed with 1 µL Glycogen followed by addition of 1/10-th of Vol. (15 ul) of Natrium acetate (3M), mixing and precipitated with2,5 Vol. 100% Ethanol at -80 C, for 20 min. The DNA was spined for 15 min at 13000 rmp, at 4 C and the pellet was washed with 500 ul 70% Ethanol. Finally the pellet was spined again for 5 min, air dried and re-suspended at 25ul. The high-concentrated DNA was then use for the transfection of HEK 293A cells with lipofectamine or PEI in 6 well plate. When transfected cells become confluent they was harvested and transferred to 10 cm plate in 2% FCS culture medium. After 6 to 8 days of transfection viral plaques were visible on the cell monolayer and cells start to detach from the cell culture dish. If the viral amplification took longer the fresh 2% FCS cultured medium was added. It is not advisable to change the medium completely after 4-5 days post-transfection. When plaques infect 60-90 % of cells the virus have to be harvested in PBS-Tosh medium, pH 7,2 to 7,5.

5.10.13.2 Virus harvest by Freeze-and-Thaw-Method

293A cells that were transfected with plasmids containing viral DNA or infected with functional viruses, were harvested when cells start to detach from the cell culture dish. For this purpose, the cell culture medium, in which the cells were maintained during viral infection, was used for washing cells from the plate to achieve high viral titers. Cell suspensions from up to 20x15cm plates were collected and the cells were pelleted for 10 min at 4000 rpm. The supernatant was discarded and the cell pellet was resuspended in 2 to 3 ml PBS-TOSH buffer, pH 7,2 and transferred to a 15 ml Falcon tube (only use polypropylene tubes!). The tubes were snap-frozen in liquid nitrogen for 2 min and then thawing was facilitated in a 37°C water bath. Thawing was accelerated by vigorous mixture of the suspension using a vortex. This step is crucial, since the ice crystals destroy the cell membranes thereby releasing the viruses. The Freeze-and-Thaw cycle was repeated three times. Subsequently, the cell lysate was centrifuged for 15 min at 4000 rpm and 4°C. The supernatant contains the virus particles and can be kept until cesium chloride purification at -80°C. The cell pellet was discarded.

5.10.13.3 Virus production

For the production of adenoviruses for mouse experiments 40 to 80 cell culture dishes with a diameter of 15 cm were used. 239A cells were maintained in D-MEM medium containing 10% FCS; 1% non-essential amino acids and 1 x penicillin/ streptomycin until they reach a confluence of 70 to 80%.
After transfection of adenoviruses and the first virus harvest it is advisable to infect a maximum of 10 x 15 cm plates with 10µl of crude virus lysate (obtained after Freeze-and-Thaw cycle). After three to four days cell detachment should be visible, otherwise, infection should be repeated.

5.10.13.4 Virus purification and concentration by Cesium chloride gradient

Crude virus lysates were thawed on ice and the viral solution was diluted with PBS-TOSH buffer to a final volume of 20 ml. This solution should contain the virus of 20 plates. After thawing and diluting the virus, spin 20 ml of viral solution for 10 min at 2000 rpm, to remove the rest of the cell breaks.
Before the gradient is poured the pH of all solutions should be adjusted to pH 7.2. 40 ml centrifuge tubes (Beckmann Polyallomer 25mm x 89 mm) were filled with 9 ml 4M cesium chloride and carefully covered by a second layer with 2,2M cesium chloride. Finally, the virus-containing solution should be added slowly to generate a third layer. In the end, three distinct layers should be distinguishable. Between each step gradient tubes should be weighed and balanced by adding the appropriate solution (different cesium chloride solutions have different density and therefore different masses). The virus was purified by ultracentrifugation (Beckmann ultracentrifuge XL-70) for 2h with a swing bucket rotor (SW28 rotor) at 24000 rpm and 4°C. The virus band appears between the 4M and 2,2M cesium chloride layer and was removed by carefully plunging the tube with 2ml syringe equipped with a cannula (BD Microlance 3, 18G x 1 ½’’). The obtained virus band was diluted with the same volume of a saturated cesium chloride solution and transferred into a 12 ml centrifuge tube (Beckmann Polyallomer 14mm x 89 mm). The maximum volume should not exceed 7 ml. The solution was gently overlayed by 4M cesium chloride (2-3 ml). Subsequently, 2 to 3 ml 2,2M cesium chloride were added dropwise to result in third distinct layer and the step gradient was centrifuged for 3h at 35000 rpm and 4°C in a swing bucket rotor (SW41 Ti rotor). After the 0

second purification step, a bluish-fluorescent band should appear between the 4M and 2,2M layers. The virus was isolated again by plunging the tube with a 18 gauge needle.
To remove the high salt concentration the virus was dialyzed three times against 1l PBS containing 10% glycerol (v/v) using a dialysis membrane with a 15 kDa cut-off (Spectra/Por® Biotech, MWCO 15'000, 10 mm diameter). The first two steps were done 1h each and the third dialysis were done overnight. The next day, the virus was transferred into a 2ml cryo-vial and aliquots for virus titration etc. were separately frozen to avoid repeated freezing and thawing.

5.10.14 ***Tittering of the adenovirus***

5.10.14.1 *OD260nm method*

The CsCl purified viral stock was first diluted as 1/10 and 1/20 in 0,1% SDS (minimal final volume of 100 ul and is dependent on the size of the quartz cuvette), and heated to 56 C for 10 min to free the DNA from the capsid. The sample was briefly centrifuged for 30 sec at 10000 rpm and the supernatant was transferred to a quartz cuvette for measurement of OD 260 nm. The 1 OD is considered to be equal to $1{,}1x10^{-12}$particles per ml. All measurements were performed in duplicates.

5.10.14.2 *Tissue culture infection dose 50 (TCID50) method*

The HEK293A cells were plated 1x10(4) cell per well in 96 well plate. All cells should attach after 2-4 hrs. For every viral stock the dilution was made in DMEM 2% Fetal calf serum (FCS), 1% penicillin/streptomycin. Virus first was diluted to 10^{-2} in 1 ml medium. Than 166 ul of this dilution was transferred to the 1,5 ml of medium in 5ml Polystyrene Round-Bottom Tubes 12x75 (BD Falcon) to obtain 10 fold dilution and mixed by vortexing. Afterwards every 166 ul of the next dilution was transfered and mixed to the 1,5 ml medium in Round-Bottom Tube till 10^{-12} final and 100 µl of a certain virus dilution was added per well with a multistep pipette. In 96 well formats 10 wells within the row was pipetted with particular virus dilution and 2 well in the row were used as a negative control. The first concentration applied to the plate was 10^{-5}. Cells were incubated for 10 days at 37°C, 95% humidity and 5% CO_2. Each virus titration was done twice on 96-well plates.

Ten days after infection wells were examined for plaque formation microscopically. The virus titter was calculated using the formula

Titter: $\mathbf{T_a=10^{1+(s-0,5)}}$ for 100µl; where **s** is the sum of all positive wells (that have at least one plaque) starting from the dilution 10^{-1}. Ten positive wells correspond the value of 1 for the individual virus dilution. To obtain the virus in Ifu/ml T_a has to be multiplied with 10.

5.11 ANIMAL EXPERIMENTS

Male 8-12-week old C57Bl/6J mice were obtained from Charles River Laboratories (Brussels, BEL) and maintained on a 12-hour light-dark cycle with unrestricted access to food. For sepsis experiments, animals were fasted for 12 h with free access to water and subsequently injected i.p. with LPS (20 µg/g body weight) or PBS. For virus injections, 1x109 plaque-forming units per recombinant virus were administered via tail vein injection. In each experiment seven animals received identical treatments. Septic phenotype was monitored by different physiological parameters: blood glucose kinetics (hyperglycemic and hypoglycemic state), diarrhea, fatigue development. Organs including liver, epididymal fat pads, kidneys, and were collected after the corresponding time periods, weighed, snap-frozen and used for further mRNA, protein or metabolic analysis. All animal procedures have been approved by local authorities and are in accordance with NIH guidelines.

5.12 STATISTICAL ANALYSIS

Statistical analyses were performed using a 2-way analysis of variance (ANOVA) with Bonferroni-adjusted post-tests, or t-test in one-factorial designs, respectively. The significance level was at $p = 0.05$.

5.13 APPENDICES

5.13.1 Abbreviations

Table 17 Abbreviations

Acetyl-CoA	Acetyl-Coenzyme A
Acetyl-CoA DH	Acyl-CoA dehydrogenase
ACTH	Adrenocorticotropic hormone
ADP	Adenosine nucleotide diphosphate
AF 1/2/3	Accessory factor 1/2/3
ATP	Adenosine nucleotide triphosphate
AP1	Activator protein 1
bp	Base pair
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
C/EBP	CAAT/enhance binding protein
CMV	CytoMegalie-Virus (promoter)
CO_2	Carbon dioxide
COUP-TF	Chicken ovalbumin upstream promoter-transcription factor
CRE	cAMP response element
CREB	cAMP regulatory element binding protein
CRH	corticotrophin releasing hormone
CRF	Corticotrophin releasing factor
CRU	cAMP response unit
DEPC	Diethylpyrocarbonte
DMEM	Dulbecco's Modified Eagle Medium
Dex	Dexamethosone
DMSO	Dimethylsulfoxide
dNTPs	Deoxyribonucleotides

DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
FFA	free fatty acids
FCS	Fetal Calf Serum
Foxo1	Forkhead transcription factor 1
Fsk	Forskolin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-6-Pase/ G6Pase	Glucose- 6-phosphatase
GFP	Green fluorescent protein
Gly – Gly	Glycine glycine
GR	Glucocorticoid receptor
GRE	Glucocorticoids response elements
GR1	Glucocorticoids response element 1
GR2	Glucocorticoids response element 1
GRU	Glucocorticoids response unit
GC	Glucocorticoid hormones (glucocorticoids)
HA	Hemagglutinin
HEK	Human Embryonic Kidney
HNF-3	Hepatocyte nuclear factor 3
HNF-4α	Hepatocyte nuclear factor 4 alpha
HPA	Hypothalamic-pituitary-adrenal axis
hr(s)	hour(s)
HRP	Horse radish peroxidase
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycans
Hz	Hertz (frequency)
Ifu	Infectious units
IL1/IL6	Interleukin 1/ Interleukin 6
Ifn	Interferon
IPTG	Isopropyl-beta-thio galactopyranoside
ITR	Inverted terminal repeats

Kan	Kanamicin antibiotic
KCl	Potassium chloride
KOH	Potassium hydroxide
LB medium	Luria Bertani medium
LITR	Long inverted terminal repeats
LPS	Lipopolysaccharide
Luc	Luciferase
LXR	Liver X receptor
MOI	Multiplicity of infection
MOPS	3-(N-morpholino)-propansulfonic acid
mRNA	Messenger RNA
mt	Mutant
NaCl	Sodium chloride
NADH	Nicotinamid-Adenin Dinucleotid-Hydroeen
NEB	New England Biolabs
NaOH	Sodium hydroxide
NFkB	Nuclear factor kB
OD	Optical density
ON	Over night
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside
Ori	Origin start site of bacterial DNA replication
PA	Polyadenylation site
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK4	Pyruvate dehydrogenase kinase, isozyme 4
PGC-1a	Peroxisome proliferator activated receptor γ coactivator 1α
PEP	Phosphoenolpyruvate
PEI	Linear polyethylenimine
PEPCK	Phosphoenolpyruvate carboxykinase
POMC	Pro-opiomelanocortin
PPAR	peroxisome proliferator-activated receptor
PC	Pyruvate carboxylase
q-PCR	Quantitative PCR

RAR	Retinoic acid receptor
Raw	Raw 264.7 macrophage inflammatory conditioned medium
RNA	Ribonucleic acid
RNAi	RNA interference
Rpm	Revolutions per minute
RT	Room temperature
RT q-PCR	Real time quantitative PCR
RXR	Retinoic X receptor
SDS	Sodium dodecyl sulphate
SDS – PAGE	SDS – Polyacrylamide Gel Electrophoresis
S.E.M.	Standard Error of Mean
shRNA	Short hairpin RNA
SRC1	Sterol receptor coactivator-1
TAT	tyrosine aminotransferase
TBP	TATA box binding protein
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
Tris	Trishydroxymethyl aminomethane
TNF- α	Tumor necrosis factor α
TLT4	Toll like receptor 4
UV	Ultraviolet
V	Volts
VCP	Valosin-containing protein
v/v	Volume by volume
wt	Wild – type
w/v	Weight by volume

5.13.2 *List of figures*

5.13.3 ***List of tables***

6. References

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Erklärung

Hiermit erkläre ich, Evgeny Chichelnitskiy, dass ich die vorliegende Dissertation selbst angefertigt habe und keine anderen als die angeführten Hilfsmittel und angegebenen Quellen benutzt habe. Ich habe die vorliegende Arbeit an keiner anderen in – oder ausländischen Fakultät eingereicht.

Heidelberg, den 25. März 2009

Evgeny Chichelnitskiy