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Proteoglycan 4 deficiency protects against glucose intolerance and fatty liver disease in diet-induced obese mice

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ABSTRACT

Objective: Proteoglycan 4 (Prg4) has emerged from human association studies as a possible factor contributing to weight gain, dyslipidemia and insulin resistance. In the current study, we investigated the causal role of Prg4 in controlling lipid and glucose metabolism in mice.

Methods: Prg4 knockout (KO) mice and wild-type (WT) littermates were challenged with an obesogenic high-fat diet (45% of total calories as fat) for 16 weeks. To further stimulate the development of metabolic alterations, 10% fructose water was provided starting from week 13.

Results: Prg4 deficiency only tended to reduce diet-induced body weight gain, but significantly improved glucose handling (AUC: −29%; $p < 0.05$), which was also reflected by a tendency towards a reduced HOMA-IR score (−49%; $p = 0.06$ as compared to WT mice). This coincided with lower hepatic expression of glycolysis (Gck: −30%; $p < 0.05$) and lipogenesis (Acc: −21%; $p < 0.05$ and Scd1: −38%; $p < 0.001$) genes, which translated in significantly lower hepatic triglyceride levels (−56%; $p < 0.001$) in Prg4 KO mice as compared to WT mice. Prg4 KO mice likely had lower glucose utilization by skeletal muscle as compared to WT mice, judged by a significant reduction in the genes Glut4 (−29%; $p < 0.01$), Pfkm (−21%; $p < 0.05$) and Hk2 (−39%; $p < 0.001$). Moreover, Prg4 KO mice showed a favorable white adipose tissue phenotype with lower uptake of triglyceride-derived fatty acids (−46%; $p < 0.05$) and lower gene expression of inflammatory markers Cd68, Mcp1 and Tnfα (−65%, −81% and −63%, respectively; $p < 0.01$) than WT mice.

Conclusion: Prg4 KO mice are protected from high-fat diet-induced glucose intolerance and fatty liver disease.

1. Introduction

Obesity is recognized as a growing risk for global health. According to the World Health Organization, in 2016 13% of all adults worldwide were obese [1]. Obesity is associated with pathological conditions such as hepatic steatosis, type 2 diabetes and cardiovascular disease [2]. However the underlying etiology that links obesity with these pathologies remains to be elucidated. Proteoglycan 4 (Prg4) has emerged from human association studies, on both transcriptional and protein levels, as a possible factor contributing to weight gain, dyslipidemia and insulin resistance [3–5]. Furthermore, Prg4 is differentially expressed in delta and pancreatic polypeptide cell subsets located in the islets of Langerhans of type 2 diabetes patients, further associating this proteoglycan with this disease [6].

Proteoglycan 4 is a member of the proteoglycan family, a large group of macromolecules consisting of a protein core with negatively charged glycosaminoglycan side chains. Both the side chains as well as specific domains of the core protein can form local interactions with

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Abbreviations: Acc, acetyl-CoA carboxylase alpha; Apob, apolipoprotein b; CE, cholesteryl esters; Chop, C/EBP homologous protein; Cpt, carnitine palmitoyltransferase; Fasn, fatty acid synthase; FC, free cholesterol; G6pc, glucose-6-phosphatase; Gck, glucokinase; Glut4, glucose transporter type 4; HFD, high-fat diet; Hk2, hexokinase 2; Hsl, hormone sensitive lipase; KO, knockout; Ldlr, low-density lipoprotein receptor; Lrp1, LDL receptor-related protein 1; Mcp1, monocyte chemoattractant protein-1; Mttp, microsomal triglyceride transfer protein; OGTT, oral glucose tolerance test; Pepck, phosphoenolpyruvate carboxylase; Pfkm, muscle type 6-phosphofructokinase; Pk, pyruvate kinase; Prg4, proteoglycan 4; Scd1, stearoyl-CoA desaturase1; Sr-bi, scavenger receptor class B type I; TG, triglycerides; Trl4, Toll-like receptor 4; VLDL, very-low-density lipoprotein; WT, wild-type; Xiap, X-linked inhibitor of apoptosis protein

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other molecules like extracellular matrix proteins, receptors or signaling molecules [7]. Dependent on the tissue- and cellular localization, proteoglycans play a role in a diverse range of physiological and pathological mechanisms [8,9]. Contradicting roles have been found for proteoglycans in metabolic pathologies. For example, mice deficient for the chondroitin sulfate proteoglycan Neuron-glial antigen 2 develop adult-onset obesity, dyslipidemia and hepatic steatosis as a consequence of brown adipocyte dysfunction [10]. In contrast, syndecan 3 deficient mice are less susceptible to diet-induced obesity due to an altered melanocortin signaling in the central nervous system [11].

Despite its association with weight loss and metabolic parameters involved in obesity and insulin resistance, the function of Prg4 in energy metabolism has not yet been elucidated. Our aims were 1) to investigate whether the associations found between Prg4 and a metabolic phenotype can be of a causal nature and 2) to elucidate the role of Prg4 in metabolic processes.

2. Materials and methods

2.1. Experimental mice

Animal experiments were performed at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research in Leiden, The Netherlands. The mice were housed in a temperature (21 °C) and light cycle (12 h light/12 h dark) controlled environment. Food and water were supplied ad libitum. C57BL/6J mice, bred in-house at the animal facility of the Gorlaeus Laboratories, were maintained on a chow diet for 12 weeks or subsequently challenged with a high fat diet (HFD) containing 45% kcal lard (D12451, Research Diets Inc., New Brunswick, NJ, USA) for 12 weeks to determine the Prg4 gene expression in a subset of organs. A subcutaneous injection of the mixture of ketamine ($100\,\mathrm{mg\,kg^{-1}}$), xylazine ($12.5\,\mathrm{mg\,kg^{-1}}$) and atropine ($125\,\mu\mathrm{g\,kg^{-1}}$) put the mice under deep anesthesia. This was monitored by checking the hind leg withdrawal and eye reflexes. Subsequently, mice were killed by exsanguination. In preparation of harvesting the vital organs, the diaphragm was severed and the organs were perfused with PBS. The dissected organs were snap-frozen in liquid nitrogen and stored at −20 °C until RNA isolation.

16 Male Prg4 knockout (KO) mice of 13–16 weeks old and 16 age-matched male wild-type (WT) littermates (both C57BL/6J background) were bred in-house at the animal facility of the Gorlaeus Laboratories to determine the effect of Prg4 deficiency under HFD-induced obesity conditions [12]. Hereto, HFD (45% kcal lard; D12451) was supplied to both experimental groups for 16 weeks. During the last four weeks of the experiment, the drinking water was supplemented with 10% fructose (Sigma-Aldrich Corp., St. Louis, MO, USA). During the course of latter experiment, five Prg4 KO mice died of unknown causes. All animal experiments were performed in accordance with the principles of laboratory animal care and regulations of Dutch law on animal welfare guidelines and the ARRIVE guidelines. All experimental protocols were approved by the Animal Ethics Committee of Leiden University.

2.2. Glucose tolerance

Before the start of the HFD feeding and after 14 weeks of HFD feeding, which included 2 weeks of 10% fructose water supplementation, a randomly chosen subgroup of Prg4 KO mice (N = 6/7) and WT control mice (N = 7/8) were exposed to an oral glucose tolerance test (OGTT). An oral bolus of beta-D (+)-glucose in PBS ($2\,\mathrm{mg\,kg^{-1}}$ body weight) was administered to the mice. Blood was drawn from the tail at baseline and after 15, 30, 45, 60, 90, 120, 150 and 180 min after gavage for glucose measurements (Bayer Contour TS glucose meter, Leverkusen, Germany). Plasma was collected to determine insulin concentrations by ELISA (Alpco, Salem, NH, USA).

2.3. Plasma lipid analysis

The concentrations of free cholesterol and cholesteryl esters were determined in plasma obtained from blood drawn at baseline with the use of enzymatic colorimetric assays as described [13]. The concentrations of triglycerides and free fatty acids were quantified using commercially available kits (Roche Diagnostics, Mannheim, Germany; Sigma-Aldrich Corp., St. Louis, MO, USA).

2.4. Clearance study

After 16 weeks of HFD feeding, which included 4 weeks of 10% fructose water supplementation, a clearance study was performed. Glycerol tri[^{3}H]oleate-labeled very-low-density lipoprotein (VLDL)-like particles (average size 80 nm) were prepared as described previously [14]. Due to technical challenges, 12 WT and 10 Prg4 KO mice were injected with a mixture of the VLDL-like particles (1 mg TG) and [^{14}C]2-deoxyglucose intravenously after a 4 h fasting period. Approximately 30 μL of blood was drawn at baseline and at 2, 5, 10 and 15 min after injection using EDTA coated glass capillaries. Plasma was isolated, 10 μL of plasma was mixed with counting solution (UltimaGold, Perkin Elmer), and radioactivity was determined by scintillation counting (TRI-CARB, Perkin Elmer). Mice were killed by cervical dislocation, thereafter, blood was collected by orbital bleeding in EDTA coated tubes (Greiner Bio One, Kremsmünster, Austria). After perfusion using ice-cold PBS, organs were harvested and small samples (approx. 1,5 g) were dissolved using Tissue Solubilizer overnight at 56 °C (Amersham Biosciences, Rosendaal, The Netherlands). By subsequent scintillation counting of the samples the uptake of radioactivity was determined. Remaining parts of the organs were fixed for 24 h in formalin and stored in 0.1% sodium azide in PBS until histological analysis or snap-frozen in liquid nitrogen and stored at −20 °C until further analysis.

2.5. Gene expression analysis by quantitative real-time polymerase chain reaction

Total RNA was extracted from the organs using the guanidinium thiocyanate/chloroform/phenol extraction method according to Chomczynski [15]. RNA concentrations were determined using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and cDNA was synthesized from 1 μg of RNA using the RevertAid™ reverse transcriptase enzyme. mRNA levels were measured with PCR primers designed with Primer Express 1.5 Software (Applied Biosystems, Carlsbad, CA, USA) using real-time SYBR Green technology (Eurogentec, Seraing, Belgium). Primer sequences are available on request. Gene expression was normalized using ribosomal protein 36B4, peptidylprolyl isomerase A and ribosomal protein L27 as standard reference genes.

2.6. Histochemical analysis

Formalin-fixed liver specimens were embedded in Tissue-Tek® O.C.T. compound and a Leica CM3050-S cryostat was used to cut 8 μm cryosections. Liver cryosections were stained for neutral lipids with Oil red O (Sigma-Aldrich Corp., St. Louis, MO, USA) and hematoxylin (Sigma-Aldrich Corp., St. Louis, MO, USA) and eosin (Merck Diagnostics). Images were obtained using a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, UK).

2.7. Liver lipid extraction and quantification

Triglycerides were extracted from liver using Nonidet™ P 40 Substitute (Sigma-Aldrich Corp., St. Louis, MO, USA). Hereto, liver tissue was homogenized using the Nonidet™ P 40 Substitute, heated to 90 °C and chilled on ice. The heating and cooling procedure was repeated once more to solubilize all triglycerides. The concentration of triglycerides was determined with the use of an enzymatic colorimetric assay after a centrifugation step to remove remaining insoluble material. Cholesterol was extracted from liver tissue using the Folch extraction method [16]. The concentration of free cholesterol and cholesteryl esters was determined with the use of enzymatic colorimetric assays as described [13]. The concentrations of triglycerides, free cholesterol and cholesteryl esters were corrected for the total protein concentration as determined using a Pierce™ BCA Protein Assay Kit (ThermoFisher Diagnostics, Waltham, MA, USA).

2.8. Prg4 expression in a human obese cohort

RNA sequencing was performed to determine Prg4 expression levels in white adipose tissue samples from a cohort of obese women after bariatric surgery as described before [17]. Within this cohort, women with normal fasting glucose levels ($N = 17$) were compared to women with type 2 diabetes (classified according to WHO standards; $N = 15$), matched for age, weight and BMI. White adipose tissue samples were taken from both the omental and the subcutaneous fat depots.

2.9. Statistical analysis

All values represent means ± SEM. Statistical analyses were performed using GraphPad Prism Software (GraphPad Software, La Jolla, California, USA) on the raw data. Significance was calculated after the performance of a Gibbs's outlier test, using a two-tailed Student's *t*-test with Welch's correction where appropriate or one-way analysis of variance (ANOVA) with multiple comparisons and Bonferroni post-test. Pearson's r was calculated to analyze correlation. Probability values of < 0.05 were considered significant.

3. Results

In human proteomic and transcriptomic studies, Prg4 is associated with obesity and related metabolic changes. To get an indication whether Prg4 in mice associated to similar processes, we measured the Prg4 mRNA expression in a subset of murine organs. Prg4 expression could be detected in most metabolically active organs, i.e. white adipose tissue and muscle, but was highest in the liver (Fig. 1A). Post-hoc analysis on previously generated liver cell fractions from chow diet-fed rats [18] indicated that hepatocytes primarily contribute to total liver Prg4 expression. More specifically, relative Prg4 expression levels were > 50 higher (Fig. 1B) in hepatocytes as compared to those in liver endothelial cells and tissue macrophages (Kupffer cells). Importantly, as evident from Fig. 1C, challenging C57BL/6J mice with a lard-based high fat diet (HFD) significantly increased the expression of PRG4 in liver (+121%; $p < 0.01$), while leaving gonadal white adipose tissue PRG4 expression unaffected ($p > 0.05$).

To study the potential relevance of the HFD-associated increase in hepatic Prg4 expression in the modulation of body metabolism, Prg4 KO mice and age- and sex-matched WT littermates were fed the same HFD for 16 weeks supplemented with fructose in the drinking water for the last 4 weeks. Prg4 KO mice showed slightly reduced, but not-significantly different, body weight gain (Fig. 2A) as compared to WT littermates (−26%; $p = 0.16$). The average weight at baseline for both groups was 31 g. This increased in WT mice to 44 g, while Prg4 KO mice only weighed 39 g on average at the end of the experiment. Food intake was monitored for 4 subsequent days, showing a similar food intake in Prg4 KO mice versus WT mice (WT: 3.0 ± 0.1 g/day vs Prg4 KO: 2.9 ± 0.1 g/day; $p = 0.50$). Since Prg4 and lipid levels in human plasma correlated in the clinical studies, we investigated the effect of Prg4 deficiency on plasma lipid levels in mice under high fat diet feeding conditions. Plasma free cholesterol levels (−20%; $p < 0.001$) and triglycerides (−16%; $p < 0.05$) were lower in Prg4 KO mice than in WT mice (Fig. 2B). Plasma cholesteryl esters were not significantly different between both groups (Fig. 2B). As evident from Fig. 2C, fasting glucose values in the circulation were not significantly different between Prg4 KO mice and WT mice. However, fasting insulin showed a clear trend towards lower levels (−43%, $p = 0.07$) in Prg4 KO as compared to WT (Fig. 2C). This coincided with a trend towards improved HOMA-IR score, a measure of the degree of insulin resistance, in the Prg4 KO mice as compared to WT mice (−49%; $p = 0.06$). To further investigate the role of Prg4 in glucose handling, mice of both genotypes were subjected to an oral glucose tolerance test (OGTT). Prg4 KO mice displayed improved glucose tolerance, with lower peak glucose values and an overall lower area-under-the-curve as compared to WT mice (AUC: −29%; $p < 0.05$; Fig. 2D). Importantly, Prg4 KO mice and their WT littermate controls did not already display a difference in glucose tolerance before the HFD challenge, i.e. under regular chow diet feeding conditions (OGTT AUC: 1705 ± 101 for WT ($N = 7$) and 1845 ± 121 for Prg4 KO ($N = 7$), respectively). It can therefore be suggested that the apparent difference in glucose tolerance is perhaps secondary to other Prg4 deficiency-associated effects on metabolism that are specifically induced by the HFD trigger.

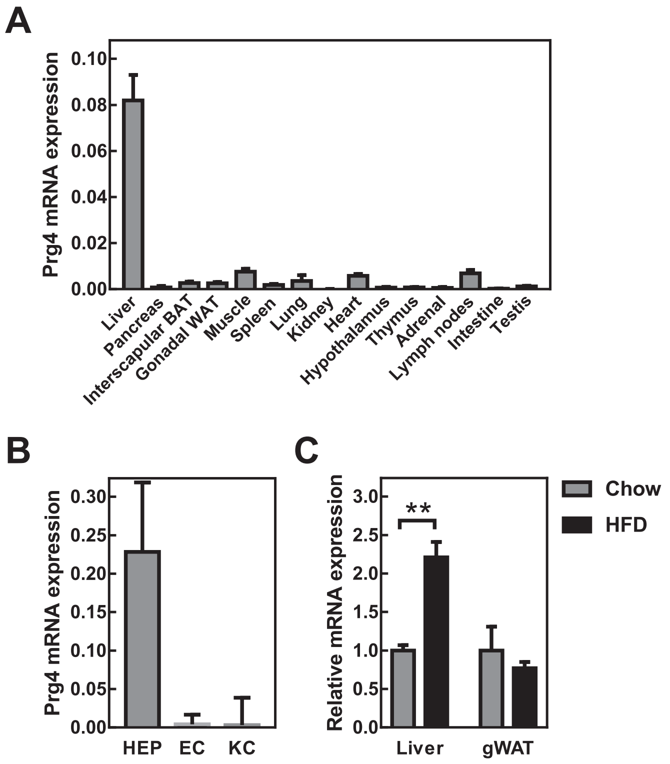


Fig. 1. Prg4 is expressed in metabolically active organs and subject to regulation in response to a high-fat diet challenge.
A) Organ distribution analysis in male C57BL/6J mice ($N = 4/5$) indicates that Prg4 expression is predominantly found within the liver. B) Gene expression profiling in isolated rat liver cells (2–5 different isolations; [18]) validates high expression in hepatocytes (HEP), but not liver endothelial cells (EC) and Kupffer cells (KC). C) Relative Prg4 expression levels are higher in livers from mice fed a high-fat diet (black bars; $N = 10/11$) as compared to those fed a chow diet (grey bars; $N = 4/5$). Data represent means ± SEM. $^{**}p < 0.01$.

Changes in plasma lipids and glucose handling often coincide with changes in liver metabolism. No significant change in liver weight was observed (1.63 ± 0.14 g for WT mice versus 1.38 ± 0.05 g for Prg4 KO mice, respectively; $p > 0.05$). Gene expression of the rate limiting enzyme in glucose utilization in the liver, glucokinase (Gck), was significantly lower (−30%; $p < 0.05$) in Prg4 KO mice than in WT mice (Fig. 3A). The glycolysis enzyme pyruvate kinase (PK) showed a similar trend towards lower gene expression levels (−21%; $p = 0.06$; Fig. 3A). Expression of the insulin-responsive genes glucose-6-phosphatase

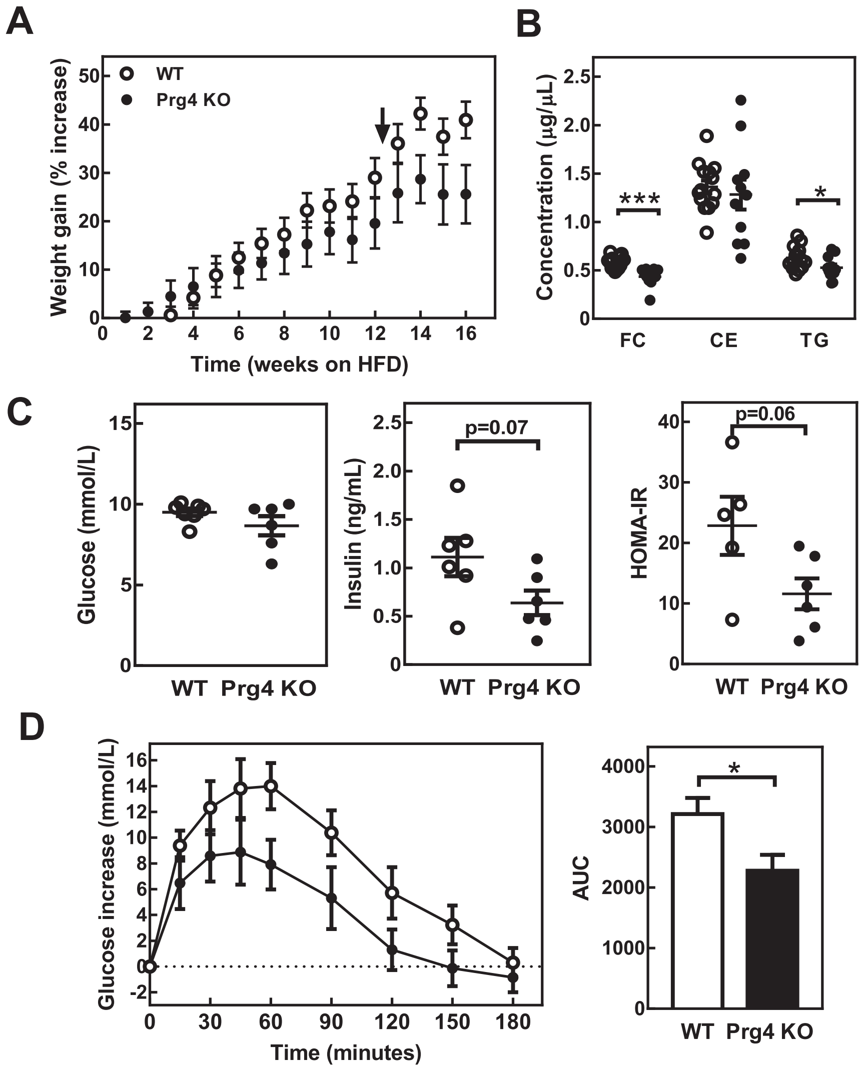


Fig. 2. Prg4 KO mice have a favorable diabetic phenotype as compared to WT mice.
A) Body weight gain during 16 weeks of high-fat diet feeding as a percentage compared to baseline in WT (open circles) and Prg4 KO mice (closed circles). Arrow indicates start of fructose supplementation. B) Plasma concentrations of free cholesterol (FC), cholesteryl esters (CE) and triglycerides (TG) after 16 weeks of high-fat diet feeding. C) Blood glucose and plasma insulin levels measured after 4 h of fasting. D) Glucose concentrations in plasma during an oral glucose tolerance test (*left*) and corresponding AUC values (*right*). Data represent means ± SEM of 8–15 WT and 6–11 Prg4 KO mice. $^{*}p < 0.05$, $^{***}p < 0.001$.

(G6pc) and phosphoenolpyruvate carboxylase (Pepck), both essential in gluconeogenesis, was not changed (Fig. 3A). As glucose intolerance associates with hepatic steatosis [19,20], we subsequently investigated genes involved in hepatic lipid metabolism in these mice. Prg4 KO mice showed significantly lower gene expression levels of the insulin-responsive hepatic lipogenesis genes acetyl-CoA carboxylase alpha (Acc; −21%; $p < 0.05$) and stearoyl-CoA desaturase 1 (Scd1; −38%; $p < 0.001$; Fig. 3A) than WT mice. The lipogenesis gene fatty acid synthase (Fasn) showed a similar trend towards lower expression (−22%; p = 0.1) in the livers of Prg4 KO mice as compared to WT (Fig. 3A). The lower hepatic lipogenic gene expression coincided with lower hepatic triglyceride levels (−56%, $p < 0.001$; Fig. 3B). The Prg4 deficiency-associated decrease in lipid accumulation was confirmed histologically by H&E and Oil red O staining. H&E staining showed less macrovesicular lipid vacuoles in livers of Prg4 KO mice after 16 weeks of HFD challenge (Fig. 3C), which was confirmed by less intensive Oil red O staining for neutral lipids (Fig. 3C). These combined findings thus suggest that Prg4 KO mice exhibit a lowered susceptibility for the development of HFD-induced hepatic steatosis as compared to WT mice. Previous studies have shown that Scd1 quantitatively contributes to hepatic triglyceride levels [21]. In line, the Scd1 expression in the liver correlated significantly with the hepatic triglyceride levels ($r = 0.73$; $p < 0.001$; Fig. 3D). Furthermore, plasma and hepatic triglyceride levels correlated significantly ($r = 0.40$; $p < 0.05$). Notably, expression levels of microsomal triglyceride transfer protein (Mttp) and apolipoprotein b (Apob) were not changed (Fig. 3A), suggesting that the decrease in plasma triglyceride levels were not secondary to alterations in the ability of hepatocytes to generate/secrete very-low-density lipoprotein (VLDL) particles. From these combined findings we anticipate that the effects on hepatic and plasma triglyceride levels are primarily driven by a significant change in the hepatic lipogenesis rate. In further support, relative expression levels of carnitine palmitoyltransferase 1 and 2 were lower (Cpt1: −26%; $p < 0.05$; Cpt2: −30%; $p < 0.01$; Fig. 3A), excluding that the decrease in storage of fatty acids within the hepatic triglyceride pool in Prg4 KO mice was due to an increased fatty acid oxidation. Moreover, the relative expression levels of key genes involved in lipoprotein uptake, i.e. the low-density lipoprotein receptor (Ldlr), low-density lipoprotein receptor-related protein 1 (Lrp1), and scavenger receptor BI (Sr-bi), as well as the actual plasma clearance of VLDL-like particle-derived triglycerides labeled with glycerol tri[^{3}H]oleate by the liver was not significantly different between Prg4 KO mice and WT mice (Fig. 3A and E). Previous studies have suggested that the level of endoplasmic reticulum (ER) stress as well as hepatocyte toll-like receptor 4 (Tlr4) activity can influence the lipogenesis rate and associated liver steatosis extent [22–25]. However, a difference in these parameters also does not seem to underlie the Prg4 deficiency-associated protection against the metabolic disturbances as judged from the similar hepatic expression levels of Tlr4 and the ER stress markers C/EBP homologous protein (Chop) and X-linked inhibitor of apoptosis protein (Xiap) (Fig. 3A).

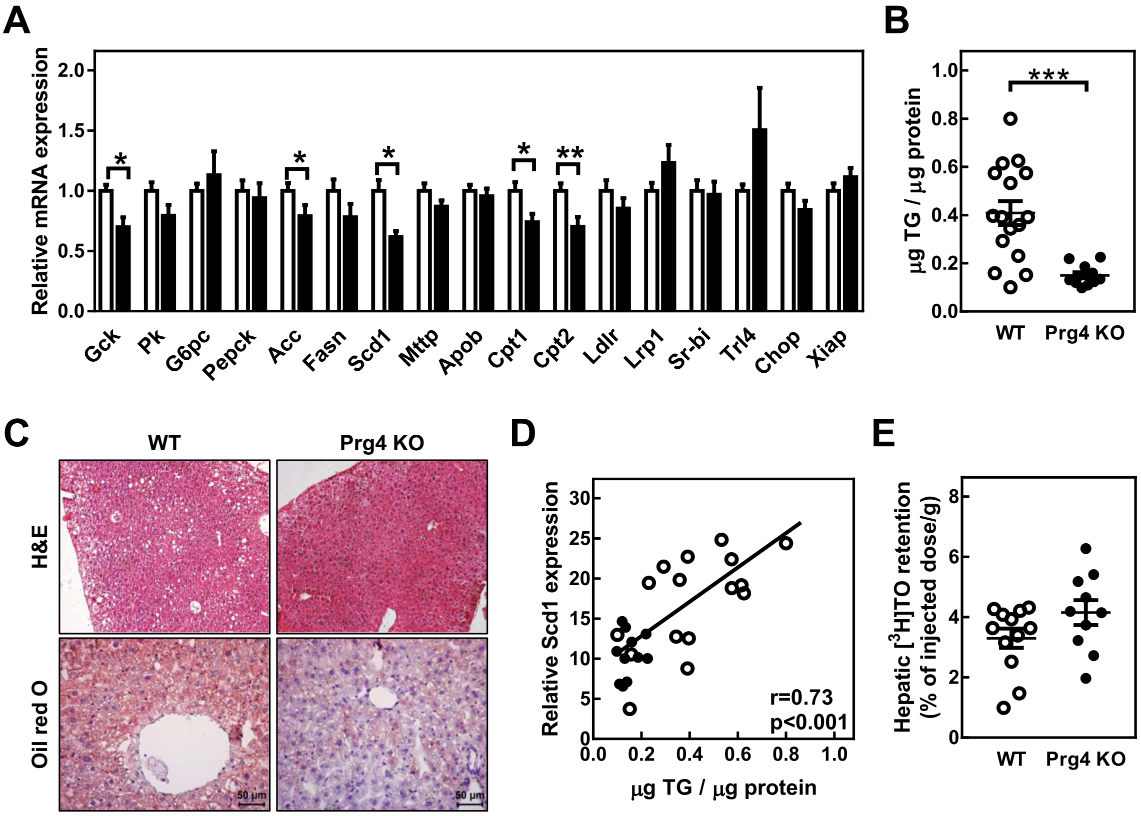


Fig. 3. Prg4 KO mice exhibit reduced liver steatosis after a HFD challenge.

A) Relative hepatic expression levels of genes involved in glycolysis (Gck and Pk), gluconeogenesis (G6pc and Pepck), lipogenesis (Acc, Fasn and Scd1), lipoprotein synthesis (Mttp and Apob), fatty acid oxidation (Cpt1 and Cpt2), lipoprotein uptake (Ldlr, Lrp1, and Sr-bi), toll-like receptor signaling (Tlr4) and endoplasmic reticulum stress (Chop and Xiap). Respective values are relative to expression levels in WT mice. B) Hepatic triglyceride (TG) levels. C) upper panels: representative micrographs of H&E stained livers (10× objective). Lower panels: representative images of sections stained with Oil red O for neutral lipids (red; 20× objective). D) Correlation between hepatic Scd1 expression and hepatic triglycerides. F) Hepatic retention of glycerol tri[^{3}H]-oleate as a percentage of total injected dose. White bars/open circles: WT; grey bars/closed circles: Prg4 KO. Data represent means ± SEM of 12–16 WT and 10/11 Prg4 KO mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

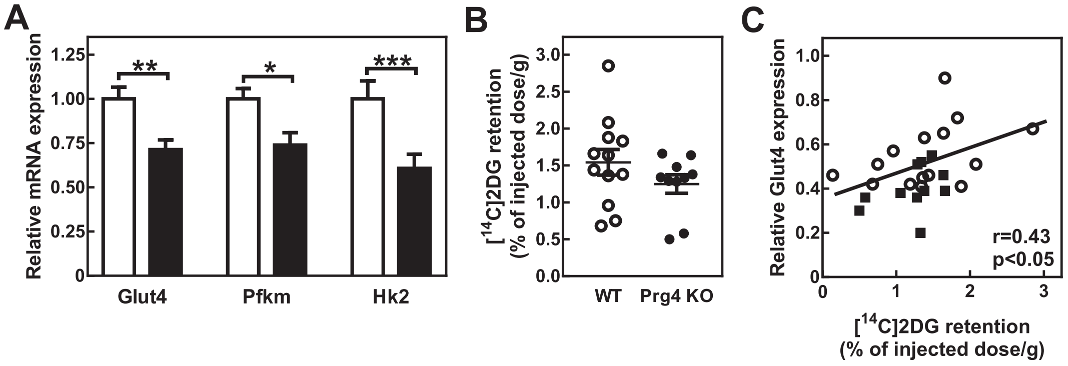


Fig. 4. Prg4 deficiency is associated with lower glucose utilization by muscles.

A) Relative expression levels of genes essential for glucose utilization in muscle: glucose transporter type 4 (Glut4), muscle type 6-phosphofructokinase (Pfkm), hexokinase2 (Hk2). Respective values are relative to expression levels in WT mice. B) The uptake of ^{14}C-labeled 2-deoxyglucose in the gastrocnemius muscle as a percentage of injected dose. C) Correlation between Glut4 expression and the uptake of ^{14}C-labeled 2-deoxyglucose in muscle. White bars/open circles: WT; black bars/closed circles: Prg4 KO. Data represent means ± SEM of 12/16 WT mice and 10/11 Prg4 KO mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In addition to the liver, muscle is a key target organ of insulin action as it stimulates the uptake and disposal of glucose to maintain blood glucose levels within the normal (physiological) range. Relative gene expression of glucose transporter type 4 (Glut4; −29%; $p < 0.01$), muscle type 6-phosphofructokinase (Pfkm; −21%; $p < 0.05$) and hexokinase 2 (Hk2; −39%; $p < 0.001$), key genes in the utilization of glucose, was significantly lower in Prg4 KO muscle than in WT muscle (Fig. 4A). This reduction in insulin-regulated Glut4 expression translated in only a minor, non-significant reduction of uptake of radiolabeled 2-deoxyglucose into skeletal muscle (Fig. 4B). The Glut4 expression correlated significantly with the uptake of the radiolabeled 2-deoxyglucose ($r = 0.43$; $p < 0.05$; Fig. 4C). It thus appears that Prg4 KO mice also display a reduced muscular insulin action, possibly related to the lower insulin levels in the Prg4 KO mice.

In white adipose tissue, the actions of insulin are bilateral, as it both decreases intracellular lipolysis by inhibiting hormone sensitive lipase

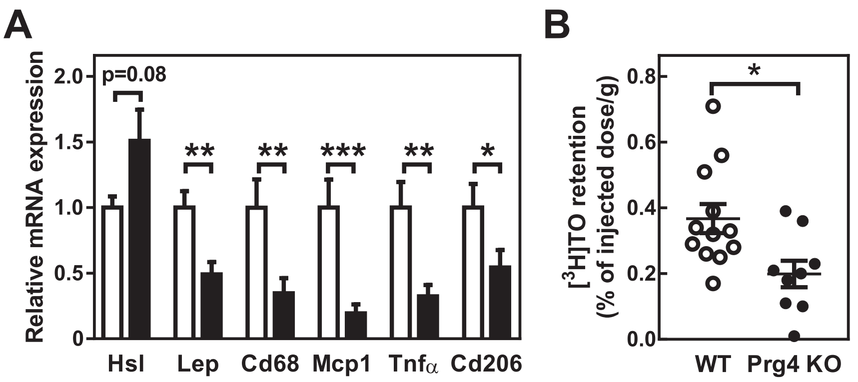
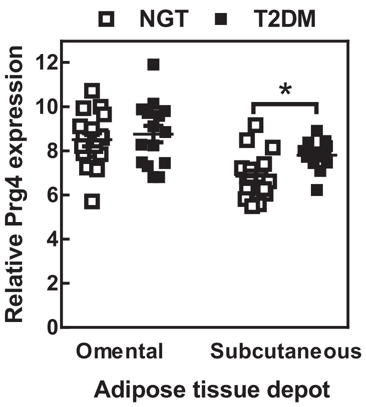


Fig. 5. Prg4 KO mice display a favorable WAT phenotype after a HFD challenge as compared to WT mice.
A) Relative gene expression levels of the lipolysis enzyme Hsl, the adipocyte-derived hormone Lep, macrophage marker Cd68 and inflammatory mediators Mcp1 and Tnfα in gonadal white adipose tissue. Respective values are relative to expression levels in WT mice. B) Glycerol tri[^{3}H]oleate-labeled VLDL-like particle-derived fatty acid retention by white adipose tissue as percentage of total injected dose. Open circles/white bars: WT; closed circles/black bars: Prg4 KO. Data represent means ± SEM of 12/16 WT and 10/11 Prg4 KO mice. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and it stimulates the storage of circulating glucose and fatty acids [26–28]. To facilitate the latter, the lipoprotein lipase-mediated uptake of triglyceride-derived fatty acids from the circulation is increased in response to insulin [29]. Insulin-regulated gene expression of hormone sensitive lipase (Hsl), the rate limiting enzyme in adipocyte lipolysis, tended to be higher in Prg4 KO mice (+51%; $p = 0.08$) than in WT mice (Fig. 5A). Moreover, white adipose tissue of Prg4 KO mice took up significantly less VLDL-like particle-derived triglycerides labeled with glycerol tri[^{3}H]oleate (−46%; $p < 0.05$) than WT adipose tissue (Fig. 5B). Insulin also stimulates the transcription of leptin [30], an adipokine that reduces food intake and increases energy expenditure [31]. Leptin (Lep) gene expression was significantly lower (−51%; $p < 0.01$) in white adipose tissue of Prg4 KO mice than that of WT mice (Fig. 5A). Importantly, the gene expression of the macrophage marker Cd68 and monocyte chemoattractant protein 1 (Mcp1) in adipose tissue of Prg4 KO mice was significantly lower (−65% and −81%; $p < 0.01$) than that of WT controls (Fig. 5A). The decrease in Cd68 expression was paralleled by a decrease in the expression levels of both the M1 macrophage marker TNFα (−63%; $p < 0.01$) and the M2 macrophage marker Cd206 (−35%; $p < 0.05$) (Fig. 5A), suggesting that Prg4 deficiency was associated with a decrease in adipose tissue macrophage numbers in the context of an unchanged macrophage polarization state. White adipose tissue in Prg4 KO mice thus not only showed a more beneficial metabolic phenotype as compared to that of WT mice, but also exhibited a reduced inflammation extent.

From our aforementioned findings it appears that Prg4 KO mice have an improved metabolic profile as compared to WT mice, suggesting that the associations between Prg4 and metabolic disturbances previously found in humans are possibly causal. To provide further evidence for a role for Prg4 in the development of diabetes in humans, we analyzed Prg4 gene expression in a cohort of obese females with or without type 2 diabetes. The expression of Prg4 in the subcutaneous adipose tissue depot in female obese individuals with type 2 diabetes was higher as compared to glucose tolerant control individuals (+11%; $p < 0.05$). This effect seemed to be dependent on the adipose tissue depot, as omental adipose tissue Prg4 expression was not different between diabetics and controls (Fig. 6). Altogether, these data further suggest that Prg4 is likely to be clinically relevant in type 2 diabetes.



Source of Variation	% of total variation	P value
Interaction	1.673	0.2327
adipose tissue depot	23.89	<0.0001
diabetic phenotype	5.092	0.0397

Fig. 6. Prg4 expression is increased in subcutaneous adipose tissue in diabetic obese women as compared to non-diabetic obese women.
Relative gene expression of Prg4 in omental and subcutaneous adipose tissue of type 2 diabetic obese women (T2DM; N = 15) as compared to normo-glucose tolerant obese women (NGT; N = 17). $^*p < 0.05$.

4. Discussion

The current study is the first to show a role for the proteoglycan Prg4 in the regulation of glucose and lipid homeostasis. Under obesogenic and diabetic circumstances, Prg4 KO mice showed lower plasma lipids, significantly better glucose tolerance as well as a trend towards an improved HOMA-IR score. Prg4 KO mice furthermore showed an improvement of the characteristic pathologies that are associated with insulin resistance such as hepatic steatosis and inflammation of the white adipose tissue as compared to WT mice. Prg4 KO thus seem to be protected from the detrimental metabolic effects that are normally associated with HFD-induced obesity.

Importantly, various proteoglycans have previously been reported to be upregulated in murine models for obesity and type 2 diabetes and are therefore associated with the development of these pathologies [32,33]. Lower serum Prg4 levels specifically are associated with weight loss and improved plasma lipid profiles and ameliorated insulin resistance [3–5]. We here show a similar metabolic phenotype in mice, with HFD/fructose-challenged Prg4 KO mice. This study therefore points towards a causal role for Prg4 in these processes. Based on human association studies, we expected that Prg4 deficient mice would show a difference in body weight as compared to WT mice, which we did not observe. However, this could be a timing effect since the difference between the weights increased during the last weeks of the experiment. It would therefore be interesting to investigate the effect of Prg4 deficiency on body weight development under extended HFD feeding conditions.

To validate a role for Prg4 in the white adipose tissue of obese humans, we checked Prg4 gene expression in a cohort of obese women with and without type 2 diabetes. Type 2 diabetic women had increased expression of Prg4 in subcutaneous white adipose tissue as compared to normoglycemic controls. However, this was not mirrored in the omental adipose tissue depot. It is known that adipose depots are functionally different based on their location [34]. The subcutaneous adipose tissue of obese women has increased capacity to take up circulating fatty acids as indicated by an increased lipoprotein lipase expression as compared to the omental adipose tissue depot [34]. Furthermore, the subcutaneous adipose tissue depot acts systemically to regulate glucose homeostasis in contrast to omental adipose tissue [35]. So the differentially expressed Prg4 might partially underlie these functional differences between the subcutaneous and omental adipose. Our observation that Prg4 expression levels in mouse liver were significantly altered (increased) in response to HFD feeding while those in murine gonadal white adipose tissue remained unaltered further supports the concept of functional tissue-specificity. These combined data suggest that Prg4 not only in mice, but also in humans plays a role in nutritional homeostasis.

To date, the metabolic function of Prg4 is unknown. Prg4 is ubiquitously expressed and secreted into the circulation. From our previous studies in the context of atherosclerosis, it has become apparent that systemic depletion of Prg4 levels is causally related to an increased atherosclerosis susceptibility [36]. However, Prg4 can also have local effects as judged from the finding that Prg4 produced locally by macrophages can affect the functionality of these cells [37,38]. Prg4 KO mice show an improved metabolic phenotype of the liver, muscle and white adipose tissue. These effects could be mediated via independent actions in these organs or via one central pathway. Interestingly, several genes that are regulated by insulin are differentially expressed in Prg4 KO mice as compared to WT mice. Genes in the glycolysis and lipogenesis pathways in the liver as well as genes involved in glucose metabolism in the muscle and genes in the white adipose tissue are all less activated. Further insight in the insulin signaling pathway in Prg4 KO mice might elucidate whether there is a common underlying mechanism.

We have shown that the gene expression levels of Prg4 are highest in the liver and that these levels are changed in response to a HFD challenge. The liver is also the organ where we found the most pronounced effects of Prg4 deficiency. Proteoglycans play a role in the cellular uptake of lipids from the circulation [39,40] and the clearance of remnant lipoproteins in the liver [41,42]. Olsson and colleagues found that both insulin and circulating fatty acids can alter the structure of secreted proteoglycans by the liver [43]. This structural change affected the capacity of hepatic proteoglycans to clear remnants of triglyceride-rich lipoproteins from the circulation, augmenting the dyslipidemia in insulin-resistant rodents [43]. Although it seemed that the absence of Prg4 had profound effects on plasma lipid levels and hepatic lipogenesis, the uptake of the fatty acids derived from the VLDL-like particles labeled with glycerol tri[^{3}H]oleate from the circulation was not significantly changed in our experimental setup. Specific studies tracking the cholesterol component of VLDL-like particles will be needed to provide more insight in a potential role of Prg4 in the uptake of the remnants by the liver.

Interestingly, Prg4 was recently identified as a specific marker for the pancreatic delta and pancreatic polypeptide cell subsets, which only comprise 3–15% of the cell population present in the islets of Langerhans [44,45]. The islets of Langerhans are clusters of endocrine cells that are affected in the pathology of diabetes. The islets consist of four main cell subsets, which closely work together in a paracrine fashion, regulating blood glucose by secreting insulin, glucagon and somatostatin [7,8]. Prg4 was one of the most differentially expressed genes in the pancreas of diabetic individuals versus healthy human controls [6]. Given the improved glucose handling in Prg4 KO mice and the contribution of the pancreas to the control of total body glucose metabolism, it would be highly interesting to perform further studies to elucidate the function of Prg4 in these cells. It could be proposed that Prg4, as one of the proteoglycans in the extracellular matrix, contributes to the interactions between islet cells and the extracellular matrix and therefore is essential for islet functionality [46]. Furthermore, intracellular proteoglycans are involved in the storage of secretory granules and the delivery and modulation of activity of the content of secretory granules in different cell types including hematopoietic cell types, pancreatic acinar cells and platelets [47,48]. Since the islets of Langerhans are specialized in the production and secretion of a range of hormones including insulin, glucagon and somatostatin, it can be proposed that Prg4 is involved in the functioning of the secretory vesicles that contain these products.

In conclusion, this study is the first to show a causal role for Prg4 in the regulation of energy metabolism in mice. Prg4 KO mice are protected from high fat diet-induced hepatic steatosis, which might be explained by the changes we see in hepatic lipogenic gene expression. Furthermore, Prg4 KO mice exhibit an improved glucose tolerance and a lower degree of white adipose tissue inflammation. Given that we observed an upregulation of Prg4 in obese women with type 2 diabetes, it can be suggested that Prg4 also plays a causal role in the development of metabolic disturbances in the human situation. Based upon our present findings and taking into account that Prg4 is a secreted protein that can act both locally as well as systemically, it is clear that follow-up studies are warranted to uncover the exact (molecular) mechanism driving the Prg4 deficiency-associated protection against glucose intolerance and fatty liver disease. In this respect, it will be of interest to generate mice with a tissue-specific Prg4 deficiency in for instance liver, adipose tissue, intestine, and/or pancreas.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflicts of interest

None.

Author contributions

J.E.N. contributed to the study design, acquisition, analysis and interpretation of the data and wrote the article. M.H. contributed to the study conception and design, acquisition and interpretation of the data and critically revising the intellectual content of the article. S.K., J.B.V.K. and V.V.H. contributed to the study design, acquisition and interpretation of the data and critical revision of the article. K.W.V.D, P.C.N.R and M.V.E. contributed to study concept, data interpretation and revision of the article. All authors approved the final version to be published.

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