Over-expression of Foxf2 in adipose tissue is associated with lower levels of IRS1 and decreased glucose uptake in vivo

Rickard Westergren¹, Daniel Nilsson¹, Mikael Heglin¹, Zahra Arani¹, Mats Grände¹, Anna Cederberg¹, Bo Ahrén² and Sven Enerbäck¹

¹Department of Medical and Clinical Genetics, Institute of Biomedicine, The Sahlgrenska Academy, University of Göteborg, SE-405 30 Göteborg, Sweden; ²Department of Clinical Sciences, Division of Medicine, Lund University, SE-221 84 Lund, Sweden.

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Correspondence to:
Sven Enerbäck, Department of Medical and Clinical Genetics, Institute of Biomedicine, Medicinaregatan 9A, The Sahlgrenska Academy, University of Göteborg, Box 440, SE 405 30 Göteborg, Sweden.
Ph: +46 31 786 33 34
Fax: +46 31 41 61 08
E-mail: sven.enerback@medgen.gu.se
Abstract

Many members of the forkhead genes family of transcription factors have been implicated as important regulators of metabolism, in particular glucose homeostasis e.g. Foxo1, Foxa3 and Foxc2. The purpose of this study was to exploit the possibility that yet unknown members of this gene family play a role in regulating glucose tolerance in adipocytes. We identified Foxf2 in a screen for adipose expressed forkhead genes. *In vivo* over-expression of Foxf2 in an adipose tissue restricted fashion demonstrated that such mice display a significantly induced insulin secretion in response to an intravenous glucose load as compared with wild type littermates. In response to increased Foxf2 expression, *Irs1* mRNA and protein levels are significantly down regulated in adipocytes, however the ratio of serine versus tyrosine phosphorylation IRS1 seems to remain unaffected. Furthermore, adipocytes over-expressing FOXF2 have a significantly lower insulin mediated glucose uptake compared with wild type adipocytes. These findings argue that Foxf2 is a previously unrecognized regulator of cellular and systemic whole body glucose tolerance, at least in part, due to lower levels of IRS1. Foxf2 and its downstream target genes can provide new insights with regard to identification of novel therapeutic targets.
Keywords:

Forkhead genes, Transcriptional regulation, Insulin signaling, Insulin resistance, Type 2 diabetes, Glucose uptake, Adipocyte metabolism.

Abbreviations:

MEF = Mouse embryonic fibroblast
ITT = Insulin tolerance test
IGTT = Intravenous Glucose Tolerance Test
2-DOG = 2-Deoxyglucose uptake
WAT = White adipose tissue
BAT = Brown adipose tissue
Introduction

In the world of today, approximately 170 million people suffer from type 2 diabetes and predictions postulate that by 2030, 366 million will be affected (21). The pathogenesis of type 2 diabetes involves insulin resistance in peripheral tissue such as skeletal muscle and adipose tissue. The insulin resistance syndrome or as it is also called the metabolic syndrome refers to several findings including glucose intolerance, obesity, dyslipidemia and hypertension. The importance of insulin receptor signaling pathway in development of insulin resistance has been shown by studies of genetic mouse models deficient in insulin receptor and its downstream signaling components such as Irs1 (Insulin receptor substrate 1) (3, 20). Even though mice lacking irs1 do not develop overt type 2 diabetes they have impaired glucose tolerance, hyperlipidemia and hypertension – a phenotype reminiscent of the prediabetic insulin resistant stage of type 2 diabetes (1, 18). In humans, some studies show that IRS1 mutations are linked to impaired insulin-stimulated signaling (2, 17) while others fail to show this (7, 8). In obese subjects an IRS1 G972R allele has been associated with a 50% reduction in insulin sensitivity (6) and in Pima Indians of Arizona, which have very high incidence of type 2 diabetes, an association exists between variants in IRS1 and type 2 diabetes (14). Furthermore, in human adipocytes low expression of Irs1 mRNA and protein has been shown to predict insulin resistance and type 2 diabetes (4).

It had previously been shown that several members of the forkhead gene family of transcription factors were involved in metabolism (5, 16, 22). Since Foxf2 has been reported to be expressed in adipocytes (9) we decided to investigate its putative role as a regulator of adipocyte metabolism. In this report, we show that mice with transgenic over-expression of the forkhead transcription factor Foxf2, in adipose tissue, have altered levels of mRNA encoding proteins involved in metabolism. In particular, mRNA as well as protein levels of
Irs1 are significantly down-regulated. Such mice also secrete significantly more insulin in response to an intravenous glucose load implicating Foxf2 as a negative upstream regulator of Irs1. Thus, negative Foxf2-mediated regulation of Irs1 mRNA/protein levels in adipocytes may contribute to development of glucose intolerance and support the view of low IRS1 protein levels as a risk factor for development of insulin resistance syndrome.
**Material and Methods**

*Cloning and DNA Construct* An incomplete human Foxf2 cDNA (Foxf2-pEVRF0) missing a part of the 5’ end was used (11). To complete the 5’ end a 978 bp PCR product, using a BamHI introducing forward primer (5’– ATAGGATCCCCAGATGACCACCGAGGGC–3’) and reverse primer (5’–GGACGAGGGTACCGGGCTGCT – 3’) on PAC clone RP4-668J24, was introduced into the BamHI-KpnI sites of Foxf2-pEVRF0. Full length Foxf2 cDNA was cloned in the BamHI-XbaI sites of pBluescript, and thereafter moved with HindIII-XbaI into pCB6+ vector. A 5.4 kb NotI-Smal fragment containing the aP2 promoter/enhancer from mouse was cloned upstream of Foxf2 in the Foxf2-pCB6+ plasmid, using NotI-EcoRV sites. After these procedures, the resulting 8.3 kb fragment, harbouring the aP2-Foxf2 construct with polyadenylation signal, was flanked by the unique sites NotI and AgeI. The plasmid was sequenced over ligation sites. Construct DNA, purified using Qiagen kit, according to manufacturer’s instructions, was injected into the male pronucleus of (C57BL/6J x CBA) F1 zygotes, cultured over night and transferred to pseudopregnant females. Tg founder lines were back-crossed to C57BL/6J for four generations.

*Cell culture* 3T3-L1 (American Type Culture Collection, Middlesex, UK) and mouse embryonic fibroblast (MEFs) were cultured in DMEM, 4.5 g/L glucose, 10% heat-inactivated calf serum, 100 U/ml Penicillin, 100 µg/ml Streptomycin (All from Invitrogen, Carlsbad, CA, US). For differentiation into adipocytes the MEFs were grown until confluence (day -2) and then stimulated (Day 0) with a differentiation media (DMEM, 10% FBS, antibiotics, 1 µmol/l dexamethasone, 0.5mmol/l isobutyl-methyl-xanthine, 10µg/ml insulin, 20 ng/ml FGF (all from Sigma Aldrich, Stockholm, Sweden) and 1µmol/l rosiglitazone (Alexis Biochemical, Lausen, Switzerland)). New differentiation media was added at day 3. At day 5 the
differentiation media was removed and the cells were cultured in a media containing DMEM, 10% FBS, antibiotics, 10µg/ml insulin, 1µmol/l rosiglitazone, and 20 ng/ml FGF. This media was changed every second day. From day 10 the cells were kept DMEM supplied with 10% FBS and antibiotics for 24 hours as a wash out period before initiating the experiments.

Intravenous Glucose Tolerance Test The mice were anesthetized with an intraperitoneal injection of midazolam (0.4 mg/mouse) and a combination of fluanison (0.9 mg/mouse) and fentanyl (0.02 mg/mouse) (Janssen Pharmaceutical, Beerse, Belgium). Thereafter, a blood sample was taken from the retrobulbar, capillary plexus in heparinized tubes, whereafter D-glucose 1g/kg (British Drug Houses, Poole, Dorset, UK) was injected rapidly intravenously. New blood samples were taken after 1, 5, 20, 50, and 75 min. Following immediate centrifugation at 4°C, plasma was separated and stored at −20°C or until analysis.

Insulin tolerance test Blood was withdrawn from the tail before a load of human insulin was administered (0.5 U/kg i.p.; Sigma-Aldrich, Stockholm, Sweden). Further samples were collected 15, 30, 60 and 90 min after the insulin challenge. Blood glucose levels were determined using an Accu-Check plus Compact Glucometer (Roche).

Isolation of stromal-vascular fraction progenitor cells and adipocytes 6-moths-old female C57BL/6 mice were used to obtain adipocytes and stromal-vascular fraction (SVF) progenitor cells essentially as previously described (10). Briefly, the epididymal fat pads were removed, washed from the blood, minced, and digested using collagenase A (1 mg/g tissue) at 37 °C for 1 h in Dulbecco's modified Eagle's medium containing 4% bovine serum albumin (BSA). Samples were filtered through a 250 µM nylon mesh and adipose cells and SVF were separated by centrifugation at 700 g for 7 min at room temperature. Both
cells fractions were washed 3 times with 40 ml DMEM containing 4% BSA and directly used for RNA isolation. Total RNA was prepared using TRI reagent (Sigma Aldrich) according to manufacturer’s instruction and treated with Turbo-DNA kit (Ambion, Austin, TX, US).

Reverse transcription and quantitative Real-Time PCR Tissues were dissected and total RNA was isolated using Micro-to-Midi Total RNA Purification System (Invitrogen) according to manufacturer’s instructions and treated with Turbo-DNA. Similar isolation was also used for RNA preparation of MEF and 3T3-L1 cells. Reverse transcription of 1 μg total RNA was carried out using a 1st Strand cDNA Synthesis Kit for qrtPCR (Roche). For specificity, selected samples were analyzed twice with and without reverse transcriptase, and no amplification was seen in samples in the absence of reverse transcriptase. To scan genes of interest in WAT from Foxf2 transgenic animals and wild type littermates we used RT² profiler PCR Array (Superarray, MD, USA) according to manufacturer’s instructions. The following arrays were used. Mouse Insulin Signaling Pathway (PAMM-030) and Mouse Diabetes (PAMM-023). Based on arraydata specific genes were chosen and analyzed further using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, US). All expression was normalized to the expression of 36B4. For primer sequences see supplementary information.

Immunoprecipitation and western blotting WAT tissue were homogenized and MEFs differentiated to adipocytes were scraped in lysis buffer (50mM Tris-HCL, pH 8.0, 1mM EDTA, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 150mM NaCl, 1x Complete protease inhibitor (Roche), 1x PhosStop (Roche)). The samples were then centrifuged at 12,000 x g for 10 min, the supernatant was collected. The supernatant were
repeatedly centrifuged until totally clear and protein concentrations were measured using BSA protein assay kit (Pierce). The proteins were separated by SDS-PAGE (Invitrogen NUPAGE 4-12% Bis Gel), transferred to PVDF membrane (Amersham Biosciences), and the specific proteins were detected by immunoblotting with the indicated antibody using ECL plus western blotting detection system (GE Healthcare, Waukesha, WI, US).

In order to detect phosphorylation of the Irs1 protein we performed immunoprecipitation followed by western blot on isolated adipocytes from WAT from Foxf2 tg mice and wild type littermates in lysis buffer (25 mmol/l Tris-HCl, pH 7.4, 0.5 mmol/l EDTA, 25 mmol/l NaCl, 1% Nonidet P-40, 10 mmol/l NaF, 1 mmol/l orthovanadate, 0.01 mg/ml leupeptine, 1 mmol/l benzamidine, 2 mmol/l AEBSF) and equal amount (According to BCA protein measurement) of total lysate were precleared 3 times with protein A–Sepharose (Amersham Biosciences, Piscataway, NJ, US) and lysis buffer. Immunoprecipitation using anti-Irs1 (Upstate, Millipore) at 4°C over night followed. The beads were washed three times in lysis buffer and boiled for 5 min in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue). The solubilized proteins were separated by SDS-PAGE (Invitrogen NUPAGE 4-12% Bis Gel), transferred to PVDF membrane (Amersham Biosciences), and detected by immunoblotting with the indicated antibody using ECL plus western blotting detection system (GE Healthcare, Waukesha, WI, US). Antibodies used for detection were: anti-Irs1, anti-p307 Irs1, anti-p612 Irs1 and anti-Phosphotyrosine from Millipore and β-actin from Abcam. Some membranes were subsequently incubated at 55 °C for 30 min in stripping buffer (100 mmol/l mercaptoethanol, 2% SDS, 62.5 mmol/l Tris-HCl, pH 6.7) to prepare them for a second round of immunoblotting. The signal were quantified using LAS-1000 plus or LAS-4000 (FujiPhotoFilm, Tokyo, Japan).
2-Deoxyglucose uptake (2-DOG) 3H Deoxy-glucose (Perkin Elmer) uptake in differentiated mouse embryonic fibroblast (MEF) was measured, MEF cells were grown and differentiated in 12-well plates. The cells were serum starved for 3 h in Krebs Ringers Phosphate (KRP) buffer (12.5 mmol/l Hepes, pH 7.4, 120 mmol/l NaCl, 6 mmol/l KCl, 1.2 mmol/l MgSO₄, 1 mmol/l CaCl₂, 0.4 mmol/l NaH₂-PO₄, 0.6 mmol/l Na₂HPO₄) supplemented with 0.2% bovine serum albumin and then stimulated with 50, 100 or 200 nmol/l insulin for 15 min. Glucose uptake was determined in triplicates after addition of ³H-deoxyglucose (0.1 µCi per well) for 5 min. Non-specific glucose uptake was examined using addition of 25 μM Cytochalasin B and were subtracted from values for glucose uptake. Washing cells three times with ice-cold PBS terminated uptake. Cells were harvested using 1% Triton-X-100 in PBS for 15 min in RT. ³H was measures in a Liquis Scintillator (Beckman, LS 6000TA, Coulter, Fullerton, CA, US)

Foxf2 expression in diet induced obesity and insulin resistance C57BL6/J mice were divided into two groups (n=6), one given standard chow (4.8 % fat, D12310, Research Diets, New Brunswick, NJ, US) or high fat diet (35.9 % fat, D12309, Research Diets, New Brunswick, NJ, US)) for 8 weeks. White adipose tissue was dissected for further analysis.

Statistics All values are given as mean ±SEM. Student’s t-test was used for statistical analysis. A p-value of less than 0.05 was considered to be significant.
Results

Foxf2 is expressed in adipose tissue

In a screen for adipocyte expressed forkhead genes, we used the highly conserved DNA-binding domain of this gene family as probe to screen adipocyte cDNA libraries under non-stringent hybridization conditions. This lead to identification of Foxf2 as an adipose tissue expressed forkhead gene. To confirm this we used quantitative real time PCR (qrtPCR) enabling identification of significant Foxf2 mRNA levels in abdominal, inguinal and perirenal adipose depots (Fig. 1a). From this experiment it is also clear that only very low or background levels of Foxf2 mRNA are present in pancreas, skeletal muscle and liver. During differentiation of 3T3-L1 cells there is a gradual induction of Foxf2 mRNA levels during the first phase of adipogenesis. Approximately at day 10, after confluence, there is a reduction compared to day 4. Prolonged culturing of 3T3-L1 adipocytes demonstrates a second phase of induction around day 18 that appears to be constant at least to day 27. Thus, Foxf2 mRNA is significantly expressed in 3T3-L1 cells and display a biphasic profile during adipogenesis (Fig. 1b). Furthermore, Foxf2 mRNA is significantly more abundant in bona fide adipocytes as compared with adipose tissue stromal-vascular cells (Fig. 1c).

Systemic glucose tolerance

To study the role of Foxf2 in adipocytes, we generated transgenic mice with enhanced expression of Foxf2 in adipose tissue (see Methods). As can be deduced from figure 2a, tg mice have approximately 4-fold higher Foxf2 mRNA levels than wild type littermates. We found no significant difference in steady state mRNA levels for three unrelated adipocyte-
expressed forkhead genes; Foxa2, Foxc2 and Foxo1, indicating that increased Foxf2 expression did not affect expression of these genes (Fig. 2a). Foxf2 transgenic mice developed normally; there was no significant difference in food consumption, degree of adiposity, total body weight, adipocyte size or circulating adiponectin levels as compared with wild type littermates (not shown). However, while there was only very limited differences in how an intravenously given glucose load was handled the level of insulin secretion evoked in response to this was significantly different (Fig. 2b,c). Mice over-expressing Foxf2 in adipose tissue displayed several-fold higher levels of systemic insulin levels most pronounced in the early initial phase of insulin secretion (Fig. 2c). There is approximately a four-fold increase in insulin secretion during the first 20 minutes after an intravenous glucose load. To further investigate systemic insulin and glucose turnover we performed an insulin tolerance test demonstrating a significantly more rapid elimination of circulating glucose in wt mice as compared with tg (Fig. 2d). Thus, it appears that tg mice require more insulin to eliminate a given load of glucose (Fig. 2c) and that they also display a less pronounced sensitivity to insulin in terms of its glucose lowering capacity (Fig. 2d).

Foxf2 regulated genes

We used qrtPCR to quantify steady state levels of mRNA of genes known to regulate adipocyte metabolism according to commercially available primer sets (see Method section). As opposed to Foxc2, another adipocyte expressed forkhead gene, which in most instances act as a positive regulator of downstream target genes (5) we found the opposite to be true for Foxf2. As shown in figure 3, there are significantly lower levels of Irs1, Perilipin, Acrp30 (adiponectin) and Cpt2 (carnitoyl palmitoyltransferase 2) while Srebpl (Sterol Regulatory
Element Binding Protein 1) was induced in response to enhanced expression of Foxf2 in adipose tissue. Several of these genes are known to be involved in pathogenesis of insulin resistance. The rather isolated effect on the early phase of insulin secretion together with previous studies showing that low Irs1 mRNA levels in adipocytes are associated with increased susceptibility for type 2 diabetes and type 2 diabetes related phenotypes (4) made us particularly interested in irs1. To study this we used WAT from wild type and transgenic mice overexpressing Foxf2 in adipocytes. We also analyzed mouse embryonic fibroblasts (MEF) that were differentiated into adipocytes in vitro, comparisons were made using wt and Foxf2 tg MEF adipocytes cultured under identical conditions (see Methods). IRS1 protein level, as judged by Western blots, was significantly down regulated in response to enhanced levels of FOXF2, both in WAT (Fig. 4a) and in MEF adipocytes (Fig 4b).

To gain insight into phosphorylation pattern of IRS1 we used phospho-specific antibodies: pSer^{307} (Fig. 5b), pSer^{612} (Fig. 5c) and pTyr (Fig. 5d). IRS1-phosphorylation detected by theses antibodies was found to be reduced in proportion to the lowered level of IRS1 (Fig. 5a-d). This is compatible with a reduction in Irs1 transcription with reduced Irs1 steady state mRNA levels followed by similarly lowered IRS1 protein levels and unaffected phosphorylation pattern. Using western blot we analyzed levels of Glut1 and Glut4 in both MEF adipocytes and WAT without detecting any significant difference in expression levels of these proteins (not shown).

Reduced glucose uptake in Foxf2 MEF adipocytes as compared with wt

In order to measure, directly in adipocytes, the role of enhanced Foxf2 expression in terms insulin mediated glucose uptake we set up a 2-deoxyglucose assay using mouse embryonic
fibroblasts that had been *in vitro* maintained and differentiated to adipocytes (see Methods). In a range of physiological insulin values a significant reduction in glucose uptake was registered for adipocytes with increased FOXF2 levels (Fig. 6). This experiment supports the view that *in vivo* the most likely cell type to confer the Foxf2 induced reduction in glucose tolerance is the adipocyte (Fig. 2b,c).

To investigate the possibility that Foxf2, with its negative effect on insulin mediated glucose uptake in adipocytes, could play a role in obesity induced insulin resistance we compared wt C57BL6/J mice which had received either standard chow or high fat diet (See Methods) for 8 weeks. We have previously shown that this regime renders the high fat fed mice obese and insulin resistant ((5, 13)). Using qrtPCR we could demonstrate a 4-fold induction of Foxf2 steady state mRNA levels in adipose tissue from high fat fed mice (Fig. 7). This is compatible with the view that induction of Foxf2 could play a role in mediating diet induced insulin resistance.
We demonstrate that Foxf2 is expressed in adipose tissue and 3T3-L1 adipocytes (Fig. 1). Interestingly, other tissues important for peripheral metabolism such as skeletal muscle, liver and pancreas display only very low or background levels of Foxf2 mRNA (Fig. 1a). Based on this and the fact that several other forkhead genes have been implicated as important regulators of adipocyte metabolism e.g. Foxc2 and Foxo1 (13, 16) we decided to investigate a mouse model with enhanced Foxf2 expression in adipose tissue. While mice transgenic for an aP2-promoter driven Foxf2 construct show no differences in terms of weight curves, degree of adiposity, adipocyte size, circulating adiponectin levels or food intake (not shown) they secrete significantly more insulin in response to an intravenous glucose load as compared with wt mice (Fig. 2b,c). Tg mice are also significantly less sensitive to insulin in terms of its glucose lowering capacity (Fig. 2d). We became particularly interested in the down regulation of Irs1 mRNA (Fig. 3a) since low Irs1 mRNA and IRS1 protein levels in human adipocytes has been proposed to predict insulin resistance and type 2 diabetes (4). This notion is also supported by the fact that mice lacking Irs1 have higher circulating insulin levels and display resistance to the glucose-lowering effects of insulin (18). Further analysis revealed significantly lower protein levels as judged by western blot in WAT from Foxf2 transgenic mice as compared with wild type littermates (Fig. 4a). MEF adipocytes generated from these mice show a similar pattern of Irs1 protein expression (Fig 4b). Immunoprecipitated Irs1 displayed a degree of significant reduction in phosphorylation on serines 307 and 612 (Fig. 5b,c) and total tyrosine residues (Fig. 5d), as one would expect due to lower total protein levels (Fig. 5a). This is most likely an effect of adipose tissue origin since the promoter used drives expression in adipose tissue. This view is also strengthened by the fact that Irs1 is down regulated and insulin mediated glucose uptake is decreased in Foxf2 tg MEF
adipocytes as compared with wt MEF adipocytes (Fig 4b, Fig6). We would like to point out that other gene regulatory events could contribute to the observed phenotype (15, 19). However, at 50 nM insulin we see approximately a 25% reduction in insulin mediated glucose uptake in MEF adipocytes while mice lacking \textit{Irs1} display approximately a 50% reduction at the same insulin concentration (12). Thus, data presented here supports the notion that a direct effect on insulin mediated glucose uptake is to be expected as a consequence of reduced IRS1 levels. It is interesting to note that high fat fed mice induce Foxf2 steady-state mRNA levels approximately four-fold (p < 0.001, fig 7). This indicates that diet induced alterations in Foxf2 levels could contribute to development of adipose tissue - and possibly also systemic - insulin resistance.

Even though human genetic studies in some instances show association between polymorphisms in the \textit{Irs1} locus and type 2 diabetes or related phenotypes others fail to do so. On the other hand, it appears that low levels of \textit{Irs1} mRNA/protein both in humans and animal models are associated with defects in insulin mediated glucose uptake, which leads to enhanced susceptibility to develop type 2 diabetes. While genetic studies using IRS1 related markers analyze the degree of involvement-derived form this particular locus such approaches fall short of identifying unrelated regulators of \textit{Irs1} expression levels. Epistatic interactions contributing to reduced levels of IRS1 through altered activation of genes regulating \textit{Irs1} expression could very well be linked to a particular phenotype such as type 2 diabetes even though the \textit{Irs1} locus as such is not. This gains support from a previous study in which human adipocytes from individuals with increased risk of developing type 2 diabetes were analyzed for \textit{IRSI} mRNA and protein levels. A significantly lower level of \textit{Irs1} mRNA and protein was found in those individuals with manifest insulin resistance. The authors demonstrate that lower \textit{IRSI} mRNA and protein levels in adipocytes predict insulin
resistance and type 2 diabetes (4). Interestingly, no association with the \textit{IRSI} G972R polymorphism was found in this study, emphasizing the possibility that genes regulating \textit{IRSI}, like \textit{Foxf2}, could play a role as regulators of glucose and insulin turnover.

Here we present data that supports a role for adipocyte expressed \textit{Foxf2} as a functionally important regulator of \textit{Irs1} expression and insulin mediated glucose uptake in adipose tissue and that this is of importance for systemic glucose tolerance. \textit{Foxf2} regulation and its downstream target genes may play a previously unrecognized role in systemic insulin mediated glucose uptake and possibly also in the pathogenesis of the insulin resistance syndrome.
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association of the insulin receptor substrate-1 G972R polymorphism with type 2 diabetes.


Figure legends

Figure 1. Foxf2 expression pattern in adipose tissue and 3T3-L1 adipocytes. (a) Using qrtPCR, relative expression of Foxf2 in abdominal (Awat), inguinal (Iwat), perirenal (Pwat) white adipose tissue and brown adipose tissue (BAT) was detected while pancreas (Pan), skeletal muscle (SM) and liver (Liv) showed no significant Foxf2 expression. Animals used were female C57Bl/6 mice approximately 4-6 month; n=3. (b) Foxf2 expression in differentiating 3T3-L1 cells. (c) Foxf2 expression is approximately 7 fold higher in the adipocyte (Ad) than stromal vascular (SV) fraction in Awat.

Figure 2. Mice over-expressing Foxf2 in adipose tissue display significantly elevated levels of insulin in response to a intravenous glucose load (a) Real time analysis showing mRNA levels for Foxf2, Foxa2, Foxc2 and Foxo1 in abdominal white tissue from Foxf2 transgenic animals (Black bars) and wild type littermates (White bars). (b) Wt (black circles) and Foxf2 tg mice (black squares) were fed a standard diet for 14 weeks. After intravenous (iv) injection of glucose (1 g/kg), blood samples were drawn immediately before and at 1, 5, 20, 50, and 75 min for analysis of glucose and insulin. Plasma glucose (b) and plasma insulin (c) levels from wt and tg mice (wt n=5, tg n=8) measured and plotted. For further details see methods. (d) Blood glucose levels at time=0, before a load of human insulin was administered (0.5 unit/kg i.p). Further samples were collected 15, 30, 60 and 90 min after the insulin challenge. n=4

Figure 3. Expression of genes in white adipose tissue of Foxf2 transgenic mice Gene expression analysis of Awat from Foxf2 tg mice (black bars) compared to wild type littermates (white bars) showed a significant change in gene expression for several important WAT expressed genes involved in adipocyte metabolism, differentiation and insulin
signaling (n=3). Lowered gene expression of Irs1 (a), Perilipin (b), Acrp30 (c) and Cpt2 (d) was detected while an increased expression could be observed for Srebp1 (e).

Figure 4 WAT from Foxf2 tg mice and Foxf2 tg MEF adipocytes expresses reduced levels of Irs1. Western blot analysis of protein levels of Irs1 in (a) WAT from wt and Foxf2 tg mice and (b) MEFs differentiated to adipocytes generated from Foxf2 tg mice and wild type littermates show a reduced expression of the Irs1 protein in the transgenic animals and MEF cells. β-actin was used as loading control.

Figure 5 Analysis of serine/tyrosine phosphorylation of IRS1 in WAT from wild type and Foxf2 tg mice. Irs protein levels and Irs serine/tyrosine phosphorylation was determined in WAT from Foxf2 tg mice (black bars) and wild type littermates (white bars) (n=3). (a) A significant reduction in IRS1 protein levels could be observed in Foxf2 tg WAT using immunoprecipitation/immunoblotting. (b-d) However no alteration in serine/tyrosine phosphorylation ratio could be observed in the Foxf2 tg relative to Irs1 protein levels.

Figure 6. Impaired insulin-stimulated glucose uptake observed in MEFs differentiated to adipocytes derived from Foxf2 tg mice. Differentiated mouse embryonic fibroblasts were incubated with different concentrations of insulin and 3H-deoxyglucose according to material and methods. MEF cells overexpressing the Foxf2 (black bars) show decreased insulin stimulated glucose uptake compared with the wild type (white bars) cells. All values are represented as fold induction from non-insulin treated cells. ** p<0.01

Figure 7. Foxf2 expression in response to high fat feeding. Foxf2 expression in WAT from mice fed normal chow (4,8 % fat, LF) and high fat chow for 8 weeks (35,9 % fat, HF).
Figure 1

(a) Relative Fox2 expression in different tissues (Awat, Iwat, Pwat, Bat, Pan, SM, Liv).

(b) Relative Fox2 expression during differentiation of 3T3-L1 cells (Days 0 to 27).

(c) Comparison of Relative Fox2 expression between Ad and SV.
Figure 3

(a) Irs1

(b) Perilipin

(c) Acrp30

(d) Cpt2

(e) Srebp1
Figure 4

(a) Irs1 and β-actin expression levels in wild-type (wt) and Foxf2 transgenic (tg) mice.

(b) Similar expression analysis as in (a) for another set of wild-type and Foxf2 transgenic mice.
Figure 5
Figure 6
Figure 7