Molecular pathways involved in the improvement of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis are components of the metabolic syndrome. Serum leptin levels are elevated in obesity, but the role of leptin in the pathophysiology of the liver involvement is still unclear. To identify the effects and mechanisms by which leptin influences the pathogenesis of NAFLD, we performed epididymal white adipose tissue (eWAT) transplantation from congenic wild-type mice into the subcutaneous dorsal area of *Lep^{ob/ob}* recipient mice and compared the results with those of the *Lep^{ob/ob}* sham-operated mice. The mice were followed for 102–216 days. During killing, the transplanted mice had significantly lost body weight and exhibited significantly higher leptin levels, improved glucose tolerance, and lower liver injury scores than the sham-operated mice. Liver microarray analysis showed that novel pathways related to GA-binding protein (GABP) transcription factor targets, pheromone binding, and olfactory signaling were differentially expressed in the transplanted mice. Our data also replicate pathways known to be involved in NAFLD, such as those involved in the regulation of microRNAs, lipid, glucose, and glutathione metabolism, peroxisome proliferator-activated receptor signaling, cellular regulation, carboxylic acid processes, iron, heme, and tetrapyrrole binding, immunity and inflammation, insulin signaling, cytochrome P450 function, and cancer. Conclusion: wild-type eWAT transplantation into *Lep^{ob/ob}* mice led to improvements in metabolism, body weight, and liver injury, possibly attributed to the production of leptin by the transplanted eWAT. These improvements were accompanied by the differential expression of novel pathways. The causal relationship between GABP downregulation and NAFLD improvement remains to be determined.

Key Words

- adipose tissue
- leptin
- liver
- microarray

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Introduction

The prevalence of obesity and insulin resistance (IR) is increasing worldwide: over 78 million Americans are obese, and one-third have high IR (Yanovski & Yanovski 2011). These factors have impacted the prevalence of non-alcoholic fatty liver disease (NAFLD), the most common form of chronic liver dysfunction in developed countries. While NAFLD could be considered a component of the metabolic syndrome, the liver involvement includes a spectrum ranging from simple steatosis (NAFLD) to non-alcoholic steatohepatitis (NASH) and cirrhosis (Neuschwander-Tetri & Caldwell 2003).

Approximately 23% of patients with NAFLD progress to NASH (Wong *et al.* 2010), which is histologically characterized by steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis (Kleiner *et al.* 2005). The mechanisms leading to NASH are poorly understood; the 'two-hit' hypothesis is a simple mechanistic explanation and has recently been modified to encompass a 'multiple-hit' hypothesis (Tilg & Moschen 2010). The 'first-hit' hypothesis is now considered to be related to the development of obesity and IR; these promote deposition of lipids in the hepatic parenchyma, causing a cellular stress response. According to the 'multiple-hit' hypothesis, NAFLD progression is due to the combined effects of a variety of insults, including direct hepatocyte lipotoxicity, oxidative and endoplasmic reticulum stress, and cytokine release. As a consequence, hepatocellular injury activates immune system-mediated necrotic and apoptotic cell death pathways (Malhi *et al.* 2006). Emerging views also question this NAFLD/NASH continuum and propose that these two entities may be independent, but akin conditions (Yilmaz 2012) because only a minority of liver steatosis cases progress to NASH, and NASH and NAFLD may be genetically distinct entities (Kawaguchi *et al.* 2012).

The absence of leptin, the adipokine produced mainly by white adipose tissue (WAT), is implicated in the pathogenesis of NASH in cases of lipodystrophy syndromes. Leptin therapy leads to significant reductions in steatosis, liver enzyme levels, and hepatocellular injury in human lipodystrophy (Javor *et al.* 2005, Simha *et al.* 2012). The leptin-deficient (*Lep^{ob/ob}*) mouse is a useful animal model of NAFLD, with severe obesity, hepatic steatosis, and IR. However, it does not spontaneously progress to steatohepatitis, requiring a second hit to trigger this progression (Faggioni *et al.* 1999). *Lep^{ob/ob}* mice have limited hepatic fibrosis capacity due to the suppression of innate and acquired immune responses, resulting in the depletion of natural killer T-cells and defective tumor

growth factor β -dependent genes, such as *pro-collagen type 1* (Leclercq *et al.* 2002). In *Lep^{ob/ob}* mice, short-term leptin treatment may not correct hepatocyte dysfunction and steatosis (Leclercq *et al.* 2006). Although WAT transplantation was effective for the treatment and prevention of obesity, as well as of endocrine/metabolic alterations associated with leptin deficiency, it exerted profound effects on multiple physiological functions in *Lep^{ob/ob}* mice: reduced body weight gain and IR and restored fertility (Klebanov *et al.* 2005, Sennello *et al.* 2006, Barros *et al.* 2009, Pereira *et al.* 2011). However, the assessment of NAFLD progression after WAT transplantation has not been evaluated yet.

In the current study, we transplanted congenic epididymal WAT (eWAT) to test whether leptin-producing eWAT influences the course of NAFLD in *Lep^{ob/ob}* mice. Furthermore, we characterized the liver gene expression profile and molecular pathways that accompanied these changes and employed computational enrichment analyses to direct follow-up experimental analysis.

Materials and methods

Animals

All animal procedures were performed under the established guidelines of humane care and use of animals and approved by the Australian National University Animal Experimentation Ethics Committee. All mice were fed standard chow and water *ad libitum* and housed in a facility that maintained a 12 h light:12 h darkness cycle and stress- and pathogen-free conditions. Heterozygous B6.V-*Lep^{ob}/J* mice (Jackson Laboratory, Bar Harbor, ME, USA) were bred with NZeg.EGFP (enhanced green fluorescent protein) transgenic mice to produce congenic NZegTg⁺ *Lep^{+/+}* (donors, referred here as *Lep^{+/+}*) and NZegTg⁻ *Lep^{ob/ob}* (leptin-deficient recipients, referred as *Lep^{ob/ob}*) mice. The B6.V-*Lep^{ob}/J* strain was backcrossed to the C57BL/6J background for more than 30 generations. The NZeg.EGFP strain on a C57BL/6 background is phenotypically normal, except for its intense fluorescence as it expresses a nuclear EGFP under a ubiquitous promoter (pCAGG) at very high levels in all cells (K Matthaei (2013), unpublished).

eWAT transplantation procedure

Seven- to twelve-week-old *Lep^{ob/ob}* mice were either sham-operated ($n=9$, six males) or transplanted

($n=12$, five males), under sterile conditions with eWAT harvested from multiple lean $Lep^{+/+}$ donors (two to four donors/recipients were used in order to transplant 2.0 g of eWAT for each recipient); eWAT was cut into small pieces and immediately loaded in a syringe through the plunger end, so that warm ischemia time was <10 min (Klebanov *et al.* 2005). $Lep^{ob/ob}$ recipients were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally. Their dorsal region was shaved, and a 0.5 cm skin incision was made, followed by the creation of a small subcutaneous pouch in which 0.5–2.0 g of donor $Lep^{+/+}$ eWAT were transferred with the help of a 16-gauge needle to minimize adipocyte damage during injection. Buprenorphine (0.5 mg/kg i.p.) was given before and after surgery. The sham-operated mice underwent the same surgical procedure and treatment, but no eWAT was transplanted. We transplanted eWAT from congenic mice to minimize the risk of rejection, and no immunosuppressive drugs were administered. To monitor rejection, the mice were frequently evaluated employing a structured score sheet assessing animal recovery. Weight was monitored weekly post-operatively for 150–200 days, followed by killing with truncan blood and tissue collection. The transplanted and sham-operated mice had access to the same diet.

Plasma hormone/metabolite measurements

Circulating leptin levels were determined at the end point using ELISA kits in duplicate (R&D systems, Minneapolis, MN, USA). Insulin levels were determined using an ultrasensitive ELISA (Alpco, Salem, NH, USA).

Intraperitoneal glucose tolerance test (IPGTT) was performed before killing. The mice were injected with a solution of 250 mg glucose/kg i.p. after 5 h of fasting. Blood was collected from the tail vein at 0, 15, 30, and 60 min after glucose injection. A standard glucometer (Accutrend, Roche Diagnostics) was used to measure blood glucose levels.

Histology

Liver samples from each mouse were fixed in a 4% formaldehyde solution for 24 h and embedded in paraffin wax. Sections of 4 μ m were cut and stained using standard protocols for hematoxylin and eosin and Masson Trichrome staining and observed using a Leica Stereo (1.210) microscope (Leica, Mannheim, Germany).

4'-6-Diamidino-2-phenylindole staining for EGFP cells

EGFP-expressing cells were identified by 4'-6-diamidino-2-phenylindole (DAPI) staining of the liver, spleen, and lung tissues. Small fresh tissue samples were extracted from the transplanted and sham-operated mice, embedded in $15 \times 15 \times 5$ mm cryomolds (Sakura Finetek, Torrance, CA, USA), and immersed in a cooling bath of acetone and dry ice. Tissue samples were embedded in the optimum cutting temperature compound (Sakura Finetek) and cut using a cryostat at -20°C to obtain 4–6 μ m cryosections on thin glass slides. Frozen sections were then air-dried for 1 h. For fixing of the slides, the slide racks were placed in pre-cooled acetone and fixed for 15 min. The slides were air-dried for 1 h and wrapped in an aluminum foil. For DAPI staining, the sections were rehydrated with PBS for 15 min and treated with DAPI solution diluted to 300 nM (Millipore, Billerica, MA, USA) for 15–20 min at room temperature. The slides were rinsed with PBS, and VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA) was used to prevent the rapid loss of fluorescence during microscopic examination. The slides were then mounted with cover slips and sealed with nail polish. The expression of EGFP-expressing cells in the liver, spleen, and lung sections of the transplanted and sham-operated mice was examined under a Leica TCS SP5 confocal microscope. The microscope was set at the laser setting of 405 nm for DAPI staining and 488 nm for GFP with 400 Hz scan speed.

Histological scoring

Liver sections were scored following the scoring system described by Kleiner *et al.* (2005), excluding the last item ('diagnostic classification'). This scoring system was complemented by the addition of macrovesicular steatosis grading (Sanyal 2002; Supplementary Table 1, see section on supplementary data given at the end of this article). Scoring was blinded (performed by G P-F and revised by M-L W).

RNA extraction

Liver samples were dissected, snap-frozen in liquid nitrogen, and stored at -80°C . Total RNA was extracted using the RNeasy Lipid Mini Kit including optional DNase digestion (Qiagen) and quantified using a Nanodrop ND-100 spectrophotometer (Thermo-Fisher Scientific, Scoresby, VIC, Australia), and its quality was assessed by a Nano-Chip assay (Agilent 2100 Bioanalyzer, Life Sciences Chemical Analysis, Foster City, CA, USA).

Quality control of RNA and microarray samples

The same amount of high-quality RNA from each of the samples was used for the transplanted and sham-operated groups. The assessment of RNA integrity on the Agilent Bioanalyzer 2100 indicated an average RNA integrity number of 9.5; thus, RNA samples provided for the microarray were of high quality. No samples were identified as biological or technical outliers; thus, all samples were included in the subsequent bioinformatic analysis. A gene expression profile was completed for the liver samples of the transplanted ($n=3$) and sham-operated ($n=3$) *Lep^{ob/ob}* mice.

Gene expression microarray

Microarray services were provided by the ACFR Biomolecular Resource Facility, Australian National University. All protocols were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. All samples selected for our microarray studies were obtained from male mice of a similar age at the time of the surgical procedure ($n=3$ shams, 79.3 ± 24.3 days old; $n=3$ transplanted mice, 79.7 ± 24.0 days old; $P=0.984$). During killing, body weights of the sham-operated mice were significantly higher than that at day 0 (D0). By contrast, the transplanted mice had lower weights during killing, when compared with their weights at D0. [Supplementary Tables 2 and 3](#), see section on [supplementary data](#) given at the end of this article, summarize data on the samples utilized for microarray.

Briefly, 100 ng of total RNA were reverse-transcribed, and cDNA was fragmented and biotinylated by terminal transferase and labeled. Three micrograms of labeled cDNA were added to probe the GeneChip Mouse Gene 1.0 ST Array (Affymetrix). All reactions were performed in biological triplicates and technical triplicates to minimize the effects of technical variability on true biological variability; we selected three age-matched male mice from each group.

Quantitative real-time reverse transcriptase PCR (RT-PCR)

A subset of differentially expressed genes was verified using RT-PCR, using liver samples from all the studied mice (nine sham-operated and 12 transplanted mice). Two micrograms of total RNA were reverse-transcribed to cDNA using the OMNISCRIPT RT kit (Qiagen) and oligo-dt primers (Invitrogen). Primers for each gene of interest were exon spanning, designed using the Primer Express

Software (Applied Biosystems); amplicon lengths were between 57 and 169 bp ([Supplementary Table 4](#), see section on [supplementary data](#) given at the end of this article). A standard curve of pooled, serially diluted cDNA was run for each gene and for housekeeping genes using the 7900HT Fast RT-PCR System (Applied Biosystems). cDNA samples were diluted between 1:20 and 1:200 and run in triplicate for both the gene of interest and housekeeping genes (*Gapdh* and *Rplp4*). All primer sets were tested for optimal dissociation curves with amplification efficiencies between 85 and 100%. The three SYBR cycle threshold values were averaged for each sample, and the RNA input for the curve was calculated from the standard curve. To check for the possibility of genomic contamination, we included a minus-reverse transcriptase control for each sample in a RT-PCR experiment run for the housekeeping gene *Gapdh* (primers targeted to the same exon). Fold changes (FCs) were expressed as a ratio of *Rplp4* expression for each sample.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. The Mann–Whitney *U* non-parametric test or Student's *t*-test was used when appropriate for comparison between the groups, using GraphPad Prism 5.00 (GraphPad Software, Inc., La Jolla, CA, USA), and differences were considered significant if $P \leq 0.05$. Pearson correlation analyses were also performed.

Microarray data analysis

Data files (.cel) were generated using default settings for scaling and detection call parameters in the GeneChip Operating Software (Affymetrix). Expression microarrays were normalized by quantile normalization and robust multi-array average summarization per gene ([Stratowa 2011](#)) with non-specific filtering of low variance genes (removed genes with an interquartile range (IQR) < 0.25 of overall IQR across the arrays). FCs were expressed in LogFC. LogFC and *P* values were computed by moderated *t*-statistic (using the Limma R package, [Smyth \(2005\)](#)). The *P* values were adjusted for false discovery rate (FDR; [Benjamini & Hochberg 1995](#)). Differentially expressed genes were ranked by the adjusted *P* values, and genes with an adjusted *P* value ≤ 0.05 were considered as differentially expressed genes at a statistically significant level. Only those genes were used for GO analyses.

Figure 1

Weight changes after sham operation and transplantation. Absolute individual weight changes after sham operation (a) and transplantation (b). (c) Differences between weights 136 days after transplantation or sham operation. * $P < 0.001$.

Enrichment analysis

Several programs were used to further analyze the lists of differentially expressed genes generated, with the significance level and FC settings mentioned above, to place them in a functional context. Enrichment analysis was performed using GoMiner. Differentially expressed genes were entered into GoMiner, which uses data in the Gene Ontology (GO) database (www.geneontology.org) to identify GO groups that are significantly overrepresented in gene lists. A cut-off $P \leq 0.05$ was selected for GO categories. Significant GO groups containing only one differentially expressed gene were disregarded to reduce the likelihood of false positives. Gene set enrichment analysis (Subramanian *et al.* 2005) was performed based on ranking by LogFC, using the digitally available R/Bioconductor (Luo *et al.* 2009).

Gene sets used were defined using the following resources: i) gene sets from Molecular Signature Database (MSigDB) v 3.0 (Liberzon *et al.* 2011), including miRNA and transcription factor (TF) target motif set gene sets as defined by Xie *et al.* (2005) and ii) GO (Ashburner *et al.* 2000) based on GO terms (gene_ontology_edit.obo, downloaded 1/25/2008) and their associations with human genes (gene2go, downloaded 1/22/2008). Only associations with the following evidence codes are included in the MSigDB gene sets: IDA IPI, IMP IGI, IEP ISS, and TAS.

Pathway analysis gene sets were obtained from BioCarta (www.biocarta.com), KEGG (www.genome.jp/kegg), and Reactome (www.reactome.org). Enrichment analyses were performed separately for FCs in one direction or two directions. Neighborhood gene sets were as defined by Subramanian *et al.* (2005): starting with a curated list of 380 cancer-associated genes (Brentani *et al.* 2003), and four expression compendium datasets (National Cancer

Institute 2010, Ramaswamy *et al.* 2001, Su *et al.* 2001, 2004) were used to find gene neighborhoods with ≥ 25 genes at a Pearson's correlation threshold of 0.8 (427 sets). A TF gene set was defined from Fulton *et al.* (2009). We also summarized gene set enrichments of the following specific pathways: sterol and fatty acid pathways, insulin-dependent regulation and sensitivity to insulin pathways, and immune/inflammatory mediator pathways. Pathways with an adjusted P value ≤ 0.10 were considered to be significantly differentially expressed.

Results

eWAT transplantation

eWAT transplantation led to significant weight loss in the *Lep^{ob/ob}* mice. During killing, the transplanted group had lost 1.84 ± 2.27 g and the sham-operated group had gained 21.44 ± 2.41 g of body weight ($P < 0.0001$). The weight differences between D136 and D0 were -2.32 ± 2.14 and

Table 1 Summary of body weight, liver histology, and serum biochemical results

Data	Transplanted (n = 12)	Sham-operated (n = 9)	P value
Male:female	5:7	6:3	0.256
Liver score	5.5 ± 0.4	9.9 ± 1.1	0.0004
Insulin (pmol/dl)	4.08 ± 1.51	156.90 ± 33.16	< 0.0001
Leptin (pg/ml)	365.7 ± 39.6	239.4 ± 14.2	0.01
Δ Weight (g; final-D0)	-1.84 ± 2.27	$+21.44 \pm 2.41$	< 0.0001
Δ Weight (g; D136-D0)	-2.32 ± 2.14	$+20.92 \pm 2.16$	< 0.0001
AUC glucose (mmol/l × 60 min)	34.41 ± 1.53	40.04 ± 2.33	< 0.05

AUC, area under the curve.

Figure 2
Changes in glucose tolerance after sham operation and transplantation. Glucose levels during IPGTT performed during killing (a). Area under the curve (AUC) for glucose (b). * $P < 0.05$.

+20.92 ± 2.16 g for the transplanted and sham-operated mice respectively ($P < 0.0001$; Fig. 1). No signs of rejection or inflammatory processes were observed after the transplantation procedure or sham operation.

Plasma hormone and metabolite measurements

Leptin levels were measured in seven mice of each group. The transplanted mice had significantly higher levels than the sham-operated mice (365.7 ± 39.6 and 239.4 ± 14.2 pg/ml respectively; $P = 0.01$; Table 1).

During killing, non-fasting plasma insulin levels were significantly lower in the transplanted group ($n = 12$; 4.08 ± 1.51 pmol/dl) than in the sham-operated group ($n = 9$; 156.90 ± 33.16 pmol/l; $P < 0.0001$). eWAT transplantation improved glucose sensitivity: the area under the curve for glucose measured during the IPGTT was significantly lower in the transplanted group (34.43 ± 1.53 mmol/l × 60 min) than in the sham-operated group (40.04 ± 2.33 mmol/l × 60 min, $P = 0.04$; Fig. 2).

Liver histology

Transplantation of eWAT from the $Lep^{+/+}$ donors improved liver histology. The histology of the sham-operated $Lep^{ob/ob}$ mice was similar to that of the non-operated $Lep^{ob/ob}$ mice (data not given), with micro- and macrovesicular steatosis, mild hepatocyte inflammation, few Mallory bodies, and mild fibrosis (Fig. 3A and B). Liver steatosis was improved in the eWAT-transplanted $Lep^{ob/ob}$ mice; their liver samples exhibited primarily microvesicular steatosis, very few/no macrovesicles, no visible injured hepatocytes (Fig. 3C), and few Mallory bodies (Fig. 3D). The liver injury score was significantly lower in the transplanted group (5.5 ± 0.4) than in the sham-operated group (9.9 ± 1.1 ; $P = 0.0004$). Table 1 summarizes the scores, hormone levels, glucose levels, and weights; individual values are given in Supplementary Tables 2 and 3. EGFP-expressing cells were identified in the sections of liver, spleen, and lung tissues of the transplanted mice (Supplementary Figures 1, 2 and 3, see section on supplementary data given at the end of this article).

Differential gene expression analysis

A total of 119 unique genes (significance-adjusted $P < 0.05$) were differentially expressed, by moderated t -test (Supplementary Table 5, see section on supplementary data given at the end of this article).

Figure 3
Liver histology of the sham-operated (A and B) and transplanted (C and D) mice. (A and C) Hematoxylin and eosin, 200×; (B and D) Masson Trichrome, 400×. Ma, macrovesicular steatosis; MB, Mallory bodies; Mi, microvesicular steatosis; F, fibrosis; I, inflammation.

Pathway analyses

Enrichment analyses showed that several pathways were differentially expressed in the same direction (either down- or upregulated). The pathways that were significantly upregulated (Adjusted *P* value <0.10) in the same direction included i) pathways involving cation channel activity and ii) pathways related to olfactory

signaling/transduction, odorant binding, and pheromone binding/response/receptor activity (Table 2).

Some of the pathways that were significantly down-regulated (Adjusted *P* value <0.10) were related to i) TF targets related to the GA-binding protein (GABP) motif and to miR-124A; ii) metabolic processes such as the biosynthesis of unsaturated fatty acids (FA) and sphingolipid,

Table 2 Downregulated and upregulated pathways on enrichment analyses. Only essential gene sets are included

Analysis	Pathway	Mean FCs	Unadjusted <i>P</i> value	Adjusted <i>P</i> value
KEGG	Cell cycle	-4.04	3.7×10^{-5}	0.0063
	Ubiquitin-mediated proteolysis	-3.02	1.4×10^{-3}	0.0873
	Chronic myeloid leukemia	-3.01	1.5×10^{-3}	0.0873
	Biosynthesis of unsaturated fatty acids	-2.99	2.1×10^{-3}	0.0891
	Glutathione metabolism	-2.78	3.4×10^{-3}	0.0928
	Regulation of actin cytoskeleton	-2.67	4.0×10^{-3}	0.0928
	Glycerolipid metabolism	-2.66	4.5×10^{-3}	0.0928
	Toll-like receptor signaling pathway	-2.62	4.7×10^{-3}	0.0928
	Limonene and pinene degradation	-2.76	5.0×10^{-3}	0.0928
	DNA replication	-2.61	5.6×10^{-3}	0.0938
	P53 signaling pathway	-2.53	6.4×10^{-3}	0.0981
	MGGAAGTG_V\$GABP_B (motifs corresponding to the transcription factor GABP)	-4.67	1.6×10^{-6}	0.001
Motif	TGCCTTA, MIR-124A (microRNA mir-124A)	-4.01	3.2×10^{-5}	0.0127
	Intracellular transport	-5.24	1.0×10^{-7}	0.0002
GO	Endosome	-4.45	5.7×10^{-6}	0.006
	Vacuole	-4.31	1.1×10^{-5}	0.006
	Cell cycle process	-4.23	1.0×10^{-5}	0.006
	Cytosol	-3.69	1.2×10^{-4}	0.0209
	Lipid binding	-3.02	1.3×10^{-3}	0.0986
	Protein folding	-3.02	1.4×10^{-3}	0.0986
	Reactome cell cycle mitotic	-5.58	1.9×10^{-8}	1.53×10^{-5}
	KEGG lysosome	-4.37	9.5×10^{-6}	3.84×10^{-3}
	KEGG leishmania infection	-3.62	2.1×10^{-4}	1.73×10^{-2}
	Reactome membrane trafficking	-3.33	5.4×10^{-4}	3.49×10^{-2}
KEGG and Reactome	KEGG glycerolipid metabolism	-2.96	2.0×10^{-3}	6.00×10^{-2}
	KEGG natural killer cell-mediated cytotoxicity	-2.86	2.4×10^{-3}	6.58×10^{-2}
	KEGG p53 signaling pathway	-2.81	2.9×10^{-3}	6.94×10^{-2}
	KEGG glutathione metabolism	-2.83	3.1×10^{-3}	7.07×10^{-2}
	KEGG vibrio cholerae infection	-2.67	4.5×10^{-3}	7.98×10^{-2}
	KEGG amino sugar and nucleotide sugar metabolism	-2.56	6.1×10^{-3}	9.39×10^{-2}
	BioCarta G2 pathway	-2.61	6.4×10^{-3}	9.39×10^{-2}
	KEGG biosynthesis of unsaturated fatty acids	-2.58	7.2×10^{-3}	9.47×10^{-2}
	KEGG endocytosis	-2.44	7.6×10^{-3}	9.47×10^{-2}
	BioCarta p53 hypoxia pathway	-2.57	7.6×10^{-3}	9.47×10^{-2}
	Reactome sema4d in semaphorin signaling	-2.49	7.8×10^{-3}	9.48×10^{-2}
	KEGG sphingolipid metabolism	-2.44	8.5×10^{-3}	9.83×10^{-2}
	KEGG <i>n</i> glycan biosynthesis	-2.42	8.9×10^{-3}	9.90×10^{-2}
	Cation channel activity	+4.09	2.6×10^{-5}	0.0571
	Odorant binding	+3.96	6.0×10^{-5}	0.0571
	Response to pheromone	+3.88	8.3×10^{-5}	0.0571
	Pheromone binding	+3.91	7.4×10^{-5}	0.0571
	Pheromone receptor activity	+3.80	1.1×10^{-4}	0.0626
	Reactome olfactory signaling pathway	+6.39	2.8×10^{-10}	2.25×10^{-7}
	KEGG olfactory transduction	+5.60	1.9×10^{-8}	7.55×10^{-6}
GO essential gene sets				
GO non-essential gene sets				
KEGG and Reactome essential gene sets				
KEGG and Reactome non-essential gene sets				

glutathione, and glycerolipid metabolism; iii) cellular processes such as cell cycle, intracellular transport, lysosome, DNA replication, p53 signaling, and ubiquitin-mediated proteolysis; iv) cancer gene sets; and v) immune/inflammatory processes and mediators such as toll-like receptor signaling pathway and natural killer cell-mediated toxicity (Table 2).

We also identified pathways that were differentially expressed whereby members of a gene set could be either up- or downregulated, but not necessarily in the same direction, such as i) drug and xenobiotic metabolism by cytochrome P450; ii) PPAR signaling pathway; iii) retinol, arachidonic/organic acid, and monocarboxylic acid metabolic processes; iv) heme, pyrrole, and iron binding; v) inflammatory response and immune system signaling; vi) monooxygenase, electron carrier, and oxidoreductase activities; vii) cell fractions; viii) cancer gene

sets; ix) lipid/lipoprotein metabolism; and x) biological oxidations (Table 3).

Gene set enrichments of specific pathways involved in the pathophysiology of NAFLD showed the significant downregulation in the same direction (Adjusted *P* value <0.10) of the following pathways: i) motifs corresponding to the GABP TF; ii) miR-124A; and iii) glucose regulation of insulin secretion. No pathway was significantly upregulated in the same direction. The following pathways were differentially expressed in either direction (Adjusted *P* value <0.10): i) metabolism of lipids/lipoproteins; ii) monocarboxylic acid metabolic process; iii) glutathione metabolism; iv) PPAR signaling pathway; and v) immune system signaling and inflammatory/defense responses (Table 4; full enrichment analyses summarized in Supplementary Table 6, see section on supplementary data given at the end of this article).

Table 3 Differentially expressed pathway analyses in which gene expression changes were not necessarily in the same direction. Cancer gene sets included are given in the supplementary tables

Analysis	Pathway	Mean FCs	Unadjusted <i>P</i> value	Adjusted <i>P</i> value
KEGG essential gene sets	Drug metabolism by cytochrome P450	3.79	1.4×10^{-4}	0.0249
	Glutathione metabolism	3.39	6.0×10^{-4}	0.0331
	PPAR signaling pathway	2.77	3.3×10^{-3}	0.0944
KEGG non-essential gene sets	Metabolism of xenobiotics by cytochrome P450	3.34	7.0×10^{-4}	0.0331
	Retinol metabolism	3.23	1.0×10^{-3}	0.0331
	Arachidonic acid metabolism	3.21	1.0×10^{-3}	0.0331
GO essential gene sets	Cell fraction	5.01	3.6×10^{-7}	0.0005
	Monocarboxylic acid metabolic process	3.89	6.0×10^{-5}	0.0205
	Inflammatory response	3.62	1.7×10^{-4}	0.0394
GO non-essential gene sets	Insoluble fraction	4.96	4.76×10^{-7}	0.0005
	Membrane fraction	4.93	5.57×10^{-7}	0.0005
	Microsome	4.11	3.07×10^{-5}	0.0160
	Vesicular fraction	4.09	3.27×10^{-5}	0.0160
	Monooxygenase activity	4.11	3.49×10^{-5}	0.0160
	Electron carrier activity	3.94	5.62×10^{-5}	0.0205
	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	3.77	1.20×10^{-4}	0.0365
	Carboxylic acid metabolic process	3.60	1.73×10^{-4}	0.0394
	Organic acid metabolic process	3.59	1.74×10^{-4}	0.0394
	Response to wounding	3.57	1.92×10^{-4}	0.0394
	Defense response	3.56	2.01×10^{-4}	0.0394
	Iron ion binding	3.55	2.16×10^{-4}	0.0395
	Cellular alcohol metabolic process	3.52	2.39×10^{-4}	0.0409
	Lipid biosynthetic process	3.42	3.51×10^{-4}	0.0567
	Heme binding	3.31	6.38×10^{-4}	0.0921
	Tetrapyrrole binding	3.31	6.38×10^{-4}	0.0921
	Reactome metabolism of lipids and lipoproteins	4.54	4.0×10^{-6}	0.0032
	Reactome signaling in immune system	3.41	3.5×10^{-4}	0.0823
	Reactome biological oxidations	3.72	0.0001	0.0613
Cell lines essential	KEGG drug metabolism by cytochrome P450	3.50	0.0004	0.0823

FDR, false discovery rate.

Table 4 Gene set enrichments of sterol and fatty acid pathways, insulin-dependent regulation and sensitivity to insulin pathways, and immune/inflammatory mediator pathways

Analysis	Pathway	Mean FCs	Unadjusted P value	Adjusted P value
Sterol and fatty acid pathways	KEGG			
	Biosynthesis of unsaturated fatty acids	−3.0	2.1×10^{-3}	0.0891
	Fatty acid metabolism ^a	2.2	0.0160	0.2111
	Cell lines			
	Reactome metabolism of lipids and lipoproteins	−2.0	2.1×10^{-2}	0.1353
GO	Reactome metabolism of lipids and lipoproteins ^a	4.5	4.0×10^{-6}	0.0032
	Monocarboxylic acid metabolic process ^a	3.9	6.0×10^{-5}	0.0205
	Fatty acid metabolic process ^a	3.2	7.5×10^{-4}	0.1024
Insulin-dependent regulation and sensitivity to insulin Motif	MGGAAGTG_V\$GABP_B (motifs corresponding to the transcription factor GABP)	−4.7	1.6×10^{-6}	0.0012
	TGCCTTA, MIR-124A (microRNA mir-124A)	−4.0	3.2×10^{-5}	0.0127
	ACCAAAG, MIR-9	−3.1	9.6×10^{-4}	0.1270
	GCACTTT, MIR-17-5P, MIR-20A, MIR-106A, MIR-106B, MIR-20B, MIR-519D	−3.2	6.3×10^{-4}	0.1270
	KEGG			
	Insulin signaling pathway ^a	−2.1	1.8×10^{-2}	0.1307
	Glutathione metabolism ^a	3.4	0.0006	0.0331
	PPAR signaling pathway ^a	2.8	0.0034	0.0944
	Glycolysis/gluconeogenesis ^a	2.2	0.0137	0.2111
	Reactome			
GO	Reactome glucose regulation of insulin secretion	−2.2	1.5×10^{-2}	0.1232
	Glycolysis/gluconeogenesis	−1.8	4.0×10^{-2}	0.1588
Immune/inflammatory mediators	Reactome			
	Reactome signaling in immune system ^a	3.4	3.5×10^{-4}	0.0823
	GO			
	Inflammatory response ^a	3.6	1.7×10^{-4}	0.0394
GO	Defense response ^a	3.5	2.0×10^{-4}	0.0394
	Acute inflammatory response ^a	3.2	9.2×10^{-4}	0.1093

^aPathways that were differentially expressed whereby members of a gene set could be either up- or downregulated, not necessarily in the same direction.

RT-PCR

We confirmed the differential expression of 25 selected genes using RT-PCR (Table 5).

Discussion

NAFLD is a common complication of obesity and the metabolic syndrome and currently represents the most common form of chronic liver dysfunction. The pathogenesis of NAFLD and its progression to NASH are still not completely clear, and leptin is known to play a role (Garg & Misra 2002). In this study, the transplantation of perigonadal adipose tissue from leptin-sufficient mice into *ob/ob* mice led to increased serum leptin levels, significant weight loss, decreased insulin and glucose levels, and improvement of liver injury scores after 102–216 days of follow-up. Moreover, we observed that these changes were accompanied with the expected differential expression of pathways already known to be involved in liver metabolism and/or leptin function and of novel pathways related to *GABP* TF, pheromone binding,

and olfactory signaling, the biological significance of which in NAFLD pathogenesis remains to be determined.

Currently, treatments aimed at ameliorating liver diseases should be limited to those with NASH, as patients with NAFLD without steatohepatitis have excellent prognosis from a liver standpoint (Chalasani *et al.* 2012). Treatment options include lifestyle intervention and use of insulin-sensitizing agents (such as metformin and pioglitazone) and the antioxidant vitamin E. Other therapies may be considered on individual bases or need further evaluation (Chalasani *et al.* 2012). To our knowledge, this is the first study to evaluate the effects of adipose tissue transplantation on leptin deficiency-associated NAFLD. Previous studies have described the effects of the procedure on parameters other than histological ones (Klebanov *et al.* 2005, Sennello *et al.* 2006, Barros *et al.* 2009). In our study, transplantation of leptin-secreting WAT led to weight loss, increased leptin levels, decreased insulin levels, and decreased IR, in concordance with previous studies. It was also an efficacious therapy for ameliorating NAFLD: sham-operated mice had significantly higher liver injury scores. Macrovesicular steatosis

Table 5 Genes differentially expressed by microarray and confirmed by RT-PCR. Liver samples from nine sham-operated and 12 transplanted mice were used for the RT-PCR experiment

Pathway	Confirmed genes	FCs	P value
Drug metabolism	<i>Cyp2b9</i>	−1.9558	0.033
	cytochrome P450 <i>Cyp2c54</i>	2.7124	0.001
Glutathione metabolism	<i>Gstm3</i>	−3.8226	0.0005
	<i>Gpx6</i>	5.0516	0.003
	<i>Gpx4</i>	−1.4507	0.0415
	<i>Gsta1</i>	−2.5128	0.0225
	<i>Gstp2</i>	3.8300	0.0089
PPAR signaling pathway	<i>Pparg</i>	−1.6606	0.0018
	<i>Cd36</i>	−1.9234	0.0005
	<i>Fabp5</i>	3.2580	0.0024
	<i>Cyp7b1</i>	11.0988	0.0012
Reactome biological oxidation pathway	<i>Cyp39a1</i>	−2.1529	<0.0001
	<i>Cyp2u1</i>	4.6404	0.0223
	<i>Cyp4f14</i>	3.5690	<0.00001
	<i>Cyp17a1</i>	−2.1795	0.0106
KEGG drug metabolism	<i>Mgst3</i>	−2.2831	<0.0001
	<i>Ugt2b1</i>	1.8220	0.0348
	<i>Adh4</i>	2.1911	0.0001
GABP	<i>Tfrc</i>	−2.0855	<0.0001
	<i>Rarb</i>	−1.6229	0.0003
MiR-124A	<i>Scd2</i>	−8.0906	0.0003
	<i>Anxa5</i>	−1.8316	0.0336
	<i>Abcc4</i>	−3.1400	0.0005
Glucose metabolism	<i>Airn</i> (iso-	−3.2089	0.0009
	forms 1/4, to		
	2, and 3) <i>Igf1bp2</i>	−1.3998 6.2809	0.0013 0.0057

was absent in only 22% of the sham-operated mice, whereas it was absent in 83% of the transplanted mice. Mild fibrosis was observed in a very small fraction of mice, including non-transplanted ones, possibly because leptin is an essential mediator of hepatic fibrosis in response to chronic liver injury (Leclercq *et al.* 2002). Similarly, only a mild degree of inflammation was observed in a few sham-operated mice, possibly due to the absence of the proinflammatory and fibrogenic effects of leptin. The introduction of leptin did not increase fibrosis/inflammation because it was followed by weight loss and a decrease in IR.

Novel pathways involved in NAFLD

We identified the differential expression of pathways that may play a role in the pathogenesis of NAFLD: pathways involving motifs corresponding to GABP TF targets and olfactory and pheromone physiology. To our knowledge, this is the first study to show the differential expression of GABP TF targets associated with NAFLD improvement. GABP is the only obligate multimeric TF of the *Ets* family,

intimately involved in critical cellular functions, including cell cycle control, cytoprotection, T-cell development, protein synthesis, cellular metabolism, embryogenesis, cellular differentiation, apoptosis, and carcinogenesis. It binds to DNA sequences that are rich in guanine and adenine and controls gene expression in several important biological settings (Rosmarin *et al.* 2004). It also regulates essential housekeeping genes important for cellular energy metabolism and ATP generation, including genes that control mitochondrial biogenesis and function.

In the liver, the GABP maybe a key regulator of mitochondrial function, detoxification, and antioxidative stress systems (Rosmarin *et al.* 2004), which are critical in ameliorating oxidative stress in different liver diseases. Loss of GABP exacerbates animal models of NASH (Chowdhry *et al.* 2010) by causing mitochondrial dysfunction, associated with the upregulation of cell death pathways via reactive oxygen species (ROS) and Ca^{2+} signaling (Pessayre 2007). In this setting, mitochondrial biogenesis is increased as a compensatory mechanism. By stimulating mitochondrial function and ameliorating oxidative stress, GABP activation could be a potential target for NASH treatment and prevention.

On the other hand, there is also evidence suggesting that lower GABP expression could also result in positive outcomes for metabolic functions. GABP deficiency may protect against diet-induced weight gain, obesity, and glucose intolerance; GABP also impairs adipogenesis, as it decreases adipose tissue mass and adipocyte size (Zhang *et al.* 2012) and modulates hepatic lipid homeostasis (Huang *et al.* 2010). Therefore, GABP downregulation could have improved insulin sensitivity and directly decreased fatty acid content of hepatic triacylglycerols of the eWAT-transplanted *Lep^{ob/ob}* mice. The higher GABP TF targets expression levels found in leptin-deficient mice that were sham-operated could also contribute to explaining why this mouse strain has relatively modest fibrosis and a benign NAFLD progression course, as it does not spontaneously progress to steatohepatitis.

Our study showed that eWAT transplantation in leptin-deficient mice ameliorated liver injury and down-regulated hepatic GABP TF targets. It is unclear if the observed GABP TF targets downregulation in the transplanted mice was the cause of this mentioned amelioration or its effect. For instance, as a cause, leptin could have directly downregulated hepatic GABP TF targets expression, decreasing the fatty acid content of hepatic triacylglycerols and improving liver injury. As an effect, better metabolic performance could have decreased ROS and cell death, decreasing the compensatory

mitochondrial biogenesis. Mitochondrial biogenesis can be indirectly assessed by the expression levels of PPAR- γ coactivator-1- α (PGC-1- α), a transcriptional coactivator of GABP, which was not differently expressed in our study; therefore, we do not have enough data to support that mitochondrial biogenesis was affected by eWAT transplantation.

Pathways related to olfactory signaling/transduction, odorant binding, and pheromone binding/response/receptor activity were significantly upregulated in the transplanted mice. The relationship between the liver and pheromones is poorly defined, but it is known that the synthesis and release of pheromones are regulated by major urinary proteins (MUPs), synthesized in the liver (Tirindelli *et al.* 2009). It is possible that leptin had an effect on the synthesis of MUPs, determining the release of pheromones and the differential expression of pathways involving olfactory/pheromone physiology. Moreover, the reversal of the leptin-deficient state restores eugonadism and fertility, stimulating the synthesis and secretion of pheromones. However, it is possible that the upregulation of these pathways has no biological significance, since olfactory pathways are often differentially and non-specifically expressed due to the fact that mammals have a myriad of olfactory genes. In our study, these pathways are so intensely enriched that we cannot discard the hypothesis of these findings having true biological significance.

Known pathways relevant to NAFLD

We identified the differential expression of several pathways that are related to liver function and/or physiological processes that are related to the actions of leptin. These pathways are related to processes such as PPAR function, glutathione, insulin, and lipid/lipoprotein metabolism, immune system, cancer and p53 signaling, inflammatory and defense responses, and the cell cycle. Moreover, microRNA pathways were downregulated. Their expression is altered in the livers of NASH and NAFLD patients (Cermelli *et al.* 2011, Wang *et al.* 2012) and has also been implicated in the prevention/suppression of hepatocarcinogenesis via modulation of inflammatory signaling and induction of tumor-specific apoptosis (Hatziapostolou *et al.* 2011). It is unclear whether miR downregulation is an effect of decreased cell proliferation and apoptosis. In our study, cytochrome P450 pathways were upregulated, which could lead to a leptin-mediated improvement of liver injury, by decreasing the accumulation of polyunsaturated FAs (Gonzalez

et al. 2011). We observed a significant downregulation in the pathway related to the biosynthesis of unsaturated FAs, but our study design does not allow us to determine whether this was a direct effect of CYP450 upregulation.

The analysis of the differentially expressed genes showed the downregulation of many genes associated with a proinflammatory state, such as *Cxcl10*, *Fabp4*, and *Tlr12*. In comparison with a previous study where exogenous leptin was administered for 12 days, only *Cyp17a1* features among the top downregulated genes with FC being >2 in both studies (Sharma *et al.* 2010). In concordance with the study of Sharma *et al.*, *Gck* was downregulated, which is associated with decreased lipogenesis and decreased IR. Furthermore, *Igfbp2* was overexpressed in both studies, which has been associated with leptin-mediated decreased IR (Wheatcroft *et al.* 2007). These observations strengthen the hypothesis that the results obtained from a third group of leptin-treated animals would have been different from the results obtained from the WAT-transplanted mice. Nevertheless, future studies should compare the effects of chronic leptin administration.

In our study, although the transplanted mice had significantly higher leptin levels than the sham-operated mice, their serum leptin levels were less than one-tenth of the levels observed in WT mice. The procedure resulted in improvements in phenotype regardless of the low leptin levels, but it is unclear whether different results would have been observed had leptin levels reached normal levels. These improvements are likely to be attributed to the production of leptin by the transplanted eWAT, but the effect of other adipokines, such as adiponectin, cannot be ruled out. Also, the transplantation site might play a role in the phenotypic changes: intra-abdominal transplantation of small amounts of epididymal adipose tissue (100–150 mg) had significantly improved glucose tolerance when transplanted to areas close to the hepatic artery (Foster *et al.* 2011), with opposite results being observed when transplanted to areas with portal venous drainage (Rytka *et al.* 2011). In one study comparing the origin and the transplantation site, leptin-sufficient mice transplanted with visceral fat into the subcutaneous area did not lose weight, but further comparisons with animals submitted to transplantation into the visceral cavity were not pursued by the authors (Tran *et al.* 2008).

We studied a relatively small and heterogeneous sample, with three biological replicates for each condition (transplanted and sham-operated), which is typical and adequate for exploratory expression analyses. Donor mice that expressed nuclear EGFP were used, aiming at

localizing donor cells in the recipients. We found EGFP-expressing cells in the livers, spleens, and lungs of recipient mice (Supplementary Figures 1, 2 and 3); the nature of these cells was not determined, but our results indicate that these cells were producing leptin or other factors that were alleviating the symptoms of leptin deficiency. We hypothesize that WAT transplantation reduced systemic inflammation (corroborated by the observation that several inflammatory/immune pathways were downregulated in the non-essential gene set pathway analysis), leading to decreased hypoxia and hepatocyte injury and improving mitochondrial function. As a consequence, GABP TF targets expression was decreased. Further studies need to confirm the findings on GABP expression and to provide additional information on its role, such as chromatin immunoprecipitation sequencing, western blotting, and immunohistochemistry, to assess changes in the protein level and to locate the related genes. By establishing the causal relationship between GABP and NAFLD, GABP can become useful as a biomarker of liver injury improvement or as a therapeutic tool against hepatic steatosis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-13-0072>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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