*Scientific paper*

# Transcriptome analysis revealed association of some P450 genes with obesity in a polygenic obese mouse model

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## Abstract

Superfamily of cytochromes P450 (CYP) have been extensively investigated in xenobiotic metabolism studies but only a handful of these genes (*e.g.*, *Cyp19a1* and *Cyp7a1*) have been associated with obesity. Examining these associations in animal models is relevant since obesity and metabolic syndrome in humans have become a prevalent health problem. In previous studies we identified four loci affecting obesity in mouse lines selected for high (Fat line) or low (Lean line) body fat content. Here we developed a Congenic line carrying a chromosome 15 obesity segment from the Lean line and compared its liver transcriptome and obesity traits with the original Fat line. Congenic segment exhibited therapeutic effect on several obesity-related parameters such as lowering fat depot size by up to 29% and lowering fasting glucose levels. Using the Steroltalk microarray focused on cytochromes P450 and cholesterol metabolism, we identified 9 differentially expressed genes including two *Cyp* genes (*Cyp26a1, Cyp2a4)*. Given the scarcity of literature reporting associations between obesity and *Cyps*, our results suggest that these genes might play a more pronounced role in regulating obesity than previously thought. Further functional studies are needed to explain the mechanism by which perturbations in these *Cyps* affect obesity in our polygenic model.

**Keywords:** *Cyp26a1*, *Cyp2a4*, obesity, cholesterol metabolism, quantitative trait locus, polygenic

## 1. Introduction

The cytochrome P450 genes have historically well documented role of participating in cellular functions such as metabolism of xenobiotics, cholesterol, bile acids, steroid synthesis, synthesis and degradation of vitamin $D_3$ and hydroxylation of retinoic acid. Mutations in some *Cyp* genes are also responsible for several clinically relevant diseases.[1] *Cyp* genes have already been extensively investigated in drug and xenobiotic designed studies but little information is available regarding their direct or indirect associations with obesity.[2,3] The nature of such questions is relevant since obesity and metabolic syndrome in humans is becoming a rapidly prevalent health problem in both western and developing world.[4] The rates of human obesity in the developed world have now reached epidemic levels with global prevalence of overweight individuals exceeding numbers of underweight individuals. A comprehensive review of the human obesity gene map lists hundreds of genes associated with obesity, including some of the cytochrome P450 genes (*e.g.*, *Cyp19a1* and *Cyp7a1*).[5]

Naturally occurring mutations in a single gene (*i.e.*, monogenic type of obesity) are severe but also extremely rare when compared to the more common form of obesity (called polygenic obesity), in which numerous genes make minor but accumulating contributions in determining the obesity phenotype.[6] Because of the fact that the obesity loci in this polygenic form have a relatively small phenotypic effect on their own, they are difficult to study in the human populations where genetic heterogeneity and environmental variation contribute to low statistical power of such studies. We therefore aim to examine polygenic obesity in the animal model that best represents this form

of obesity. Such a polygenic model has been developed in Edinburgh by divergent selective breeding for more than 60 generations resulting in lines that differ more than five-fold in fat content and have 22% (Fat line) or 4% (Lean line) body fat, respectively.[7] This process of gradual selection for high fat alleles in the Fat line and low fat (anti-obesity) alleles in the Lean line mimics the situation in humans, at least at the extremes of phenotypic distribution. Long-term selection or genetic drift in very fat and very lean people has likely resulted in a similar polygenic form of obesity and leanness as in our lines. Therefore, our selected mouse lines resemble well the situation in humans, where a large proportion of obesity cases are thought to have aforementioned polygenic basis.[5]

Earlier genetic studies on the crosses between the original selection Fat and Lean lines have identified several quantitative trait loci (QTL), which are the regions of the genome with significant effect on a quantitative trait such as obesity.[8] To study the effects of individual QTL regions on physiological parameters and obesity, so called Congenic lines need to be developed first. For example, in such a Congenic line only a segment carrying a particular QTL is transferred from one line (say from Lean line) to the Fat line by repeated backcrosses to finally generate a line that is essentially genetically similar to the Fat line but with a short QTL segment coming from the Lean line. One such line for a chromosome 15 QTL (called *Fob3b* QTL) has already been partially developed and a preliminary microarray experiment has indicated several differentially expressed genes in the cholesterol biosynthesis, gluconeogenesis and also some cytochrome P450 genes such as *Cyp7b1*.[9]

In our previous study a microarray was used with incomplete presence of genes from certain metabolic pathways such as the ones that showed potential differences (*e.g.,* cholesterol biosynthesis, cytochrome P450 superfamily).[9] For this reason, we tested in the current study if perturbations in expression of cholesterol biosynthesis and other cytochrome P450 genes are associated with the phenotypic differences between the Fat line and a Congenic line carrying *Fob3b* QTL. Analysis was carried out using Steroltalk v1 microarray, a focused array containing comprehensive targets of genes from the cytochrome P450 superfamily, nuclear receptor superfamily, genes involved in atherogenesis and other obesity related pathways.[10–11] We also performed a phenotypic analysis of both lines for several obesity-related parameters to link these phenotype data to the transcriptome results.

# 2. Materials and Methods

## 2. 1. Animals and Maintenance

Inbred Fat line and U12 line (Congenic) were used in our study. Original selection lines were developed in Edinburgh by divergent selection experiments on high (Fat line) or low (Lean line) body fat content lasting over 60 generations.[7–9,12] A previously generated line $F^{chr15L}$ carrying the *Fob3b* QTL from chr 15 was developed further in our study by continued backcrossing scheme for further five generations.[13] The resulting Congenic line has L-line alleles between *D15Mit184* and *D15Mit107* on Chr15, and the rest of the genome is expected to be of the Fat line background. After 12 backcrosses, heterozygous individuals that possessed the desired genotype (successfully introgressed *Fob3b* QTL segment between *D15Mit184* and *D15Mit107*) were inter-se mated to generate homozygous individuals. Homozygous animals were founders of further brother-sister matings to develop genetically homogeneous Congenic line, which was used in the present experiment.

Animals were weaned at the age of 3 weeks and one pair of males (usually brothers) was housed per cage. Cages (height 12.5 cm, width 16 cm, length 33 cm, Techniplast, Buguggiate, Italy) were randomly distributed on the rack in order to minimize localized environmental effect. Throughout the entire experiment males were fed standard chow diet (Altromin 1324, Lage, Germany) containing 4% crude oil, 19% crude protein, 6% crude fiber, 7% ash, 13.5% moisture and 11.9 MJ/kg metabolizable energy. Animals were maintained at an ambient temperature of 21.5 °C (±0.5) with controlled 12 h light/dark cycle (lights on at 7.00 am). Body weights were recorded at 3, 6, 10 and 14 weeks of age. All procedures were approved by the Veterinary Administration of the Republic Slovenia and the Ethical committee for the laboratory animals following all the current Slovenian legislation that has been harmonized with the EU legislation on the use of laboratory animals in research.

## 2. 2. Mouse Tissue Collection

Animals were sacrificed at the age of 14 weeks (±3 days). Food was withdrawn at 7.00 am, 4 h prior to dissecting animals while water remained available *ad libitum.* Blood samples were collected into Li-heparin 1.3 ml micro tubes (Sarstedt, Germany) and animals immediately dissected – mice from both lines were dissected in parallel within 2 hours. In total, tissues from 42 Fat and 36 Congenic mice were collected. Samples of liver heart and left epididimal, left abdominal (perirenal and retroperitoneal), left femoral and mesenterial fat depots were promptly frozen in liquid nitrogen and then stored at –80 °C. Tissues were weighed on Mettler Toledo balance (PL 202-S) with 1/100 g accuracy.

## 2. 3. RNA Isolation

RNA from liver samples was extracted in »batches« (a pair of Fat and Congenic line samples simultaneously) from 10 Fat and 10 Congenic mice. Liver samples were homogenized for 10 s using Ultra-Turrax T8 homogenizer (IKA Labortechnik, Germany) using TRIzol reagent ac-

cording to the manufacturer's guidelines (TRIzol® reagent, Invitrogen, Life technologies) and total RNA was eluted in DEPC-treated water (Fluka, Biochemica). RNA concentrations were assessed using ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) and the integrity was estimated with Agilent 2100 bioanalyzer (Agilent Technologies, USA). According to the given RNA integrity number (RIN) a pair wise comparison between the Fat and Congenic line RNA samples was made in order to couple samples with the least quality divergence (RIN values of all samples ranged from 7.5–8.9). Maximal tolerated RNA quality divergence for coupled individual Fat and Congenic line samples co-hybridized on the same microarray was ±1 RIN.

## 2. 4. Microarray Procedures

The Steroltalk v1 cDNA microarrays were prepared as described previously.[11] Briefly, this is a custom array allowing a comprehensive analysis of genes from the cytochrome P450, nuclear receptor superfamilies as well as genes involved in atherogenesis, carbohydrate metabolism, different signaling pathways, transporters and inflammation. Total RNA from 10 Congenic and Fat line animals was individually labeled using cyanin-3 (Cy3) or cyanin-5 (Cy5) dye (Perkin Elmer Life Sciences, Boston, MA, USA). To 20 µg of total RNA sample from each line, control RNA we spiked in: 250 pg of Firefly Luciferase mRNA (Promega, Madison, WI, USA), and 0.5 µL of either test or reference spike mix from Lucidea Universal Scorecard kit (Amersham Biosciences, GE Healthcare UK limited, Little Chalfont, UK). mRNA was reverse-transcribed to amino-allyl cDNA using 2.5 µg of Oligo dT (Invitrogen, Carlsbad, CA, USA), 400U of SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and 1 µL of 10 mM amino-allyl dUTP (Sigma, St Louis, MI, USA) according to the manufacturer's protocol. Reaction was stopped after 2 hours by addition of 10 µL 0.5 M EDTA and 10 ml 1M NaOH followed by incubation at 65 °C for 15 min. 10 µL of 1 M HCl was added and cDNA was purified using MinElute PCR Purification Kit (Qiagen GmBH, Hilden, Germany) according to manufacturer's protocol with exception of using a phosphate buffer (5 mM $KPO_4$ pH 8.5 in 80% ethanol) for washing step and MilliQ water for elution. Purified amino-allyl cDNA was dried and resuspended in 4.5 µL of 0.2 M $Na_2CO_3$ (pH 9.0) and 4.5 µL of Cy3 or Cy5 dye in DMSO (Amersham Biosciences, GE Healthcare UK limited, Little Chalfont, UK). Labeling reaction was incubated at room temperature for two hours. Next, 35 µL of 0.1 M Na acetate (pH 5.2) was added and reaction purified using MinElute PCR Purification Kit (Qiagen GmBH, Hilden, Germany) according to manufacturer's protocol. Labeled cDNA was eluted in water and 1 µL was used for analyses using ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA).

10 Congenic and 10 Fat line cDNA samples were co-hybridized in pairs and were all replicated in a dye swap experiment. In total, 20 microarrays were used for complete pair wise comparisons. Prior to hybridization, each labeled Congenic line cDNA sample was mixed with the Fat line cDNA sample. Using LifterSlip cover glasses (Erie Scientific Company, Portsmouth, NH, USA) samples were hybridized for 16 h at 65 °C in the buffer (final concentration 3xSSC, 0.2% SDS) using humidified hybridization chambers in a water bath (HybChambers, GeneMachines, San Carlos, CA, USA). After hybridization, slides were washed as described before and scanned using Tecan LS200 scanner (Tecan Group Ltd., Maennedorf, Switzerland).

## 2. 5. Microarray Data Analysis

Images were analyzed using Array-Pro Analyzer 4.5 (Media Cybernetics, Bethesda, MD, USA). The median feature and local background intensities were extracted together with the estimates of their standard deviation. Data analyses were done using Orange.[14] Data were filtered to exclude spots of low quality. Only features with foreground to background ratio higher than 1.5 and coefficient of variation (CV, ratio between standard deviation of the background and the median feature intensity) lower than 0.5 in both channels were used for further analysis. $Log_2$ ratios were normalized using LOWESS fit to spike in control RNAs according to their average intensity.[15] For normalization, two types of spike in controls were used: custom-made Firefly luciferase and commercial Lucidea Universal Scorecard. Data from Lucidea Universal Scorecard ratio controls, for which RNAs were added to each sample RNAs in different concentrations, were adjusted prior to fit of normalization curve according to the ratios between the concentrations. Differential expression of genes was assessed using Student's *t*-test for independent samples at a probability of type I error $\alpha = 0.05$.

## 2. 6. Plasma Lipids and Glucose

Blood samples were collected in Li-heparin tubes (35 I. U. Heparin/ml blood, Micro tube 1.3 ml LH, Sarstedt, Germany) and stored at +4 °C. Samples were centrifuged within 3–4 h of collection at 3000 rcf, for 10 min at +4 °C. Plasma samples were individually analyzed for total plasma cholesterol, HDL-cholesterol, plasma triglycerides and glucose. Plasma total cholesterol (TC) and triglyceride concentrations were determined by enzymatic assays adapted to microtiter plates using commercially available reagents (BioMerieux, Lyon, France). Plasma HDL-cholesterol levels were measured after precipitation of apolipoprotein (apo) B-containing lipoproteins with phosphotungstic acid/Mg (Roche Diagnostics GmbH, Mannheim, Germany). Non-HDL cholesterol was obtained by subtraction of HDL-C values from TC.

Lipoproteins were separated from 200 µl of pooled plasma by gel filtration chromatography using a Superose 6 HR 10/30 column (Pharmacia, Sweden). The gel was allowed to equilibrate with 10 mM phosphate-buffered saline (PBS) containing 0.01% (wt/vol) EDTA and 0.01% (wt/vol) sodium azide. Plasma was eluted with the buffer at room temperature at a flow rate of 0.2 ml per minute. The effluents were collected in 0.22 ml fractions. Cholesterol and triglyceride concentrations were determined in the eluted fractions as described above.

Cholesterol distribution among lipoproteins was obtained by separation of the major lipoprotein classes (VLDL, IDL+LDL, and HDL) by fast protein liquid chromatography carried on 200 ml of pooled plasma sample for Fat and Congenic line according to procedure previously detailed.[16] Cholesterol concentrations were determined in the eluted fractions. Plasma glucose levels were measured by an adapted method on microtiter plaques using glucose oxidase.

## 2. 7. Real-time Polymerase Chain Reaction (RT-PCR) Analyses

Selected genes (Table 1) were evaluated by RT-PCR method. Briefly, 1 µg of total RNA from individual animals pre-treated with DNAse I (Sigma, St Louis, MI, USA) was reverse-transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers (Promega, Madison, WI, USA) according to manufacturer's protocol. Analyses were done using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) and performed on the Stratagene MX3005P machine (Stratagene, Cedar Creek, texas, USA) according to manufacturer's protocols. Analyses were performed using internal control 18S rRNA. Relative transcript levels were calculated using the comparative Ct (cycle threshold) method and $-\Delta\Delta$Ct values were used for statistical analyses.[17]

**Table 1.** Primer sequences used in the RT-PCR assay.

| Gene | Sense primer (5'–3') | Antisense primer (5'–3') |
|---|---|---|
| *Hmgcr*[1] | cttgtggaatgccttgtgattg | agccgaagcagcacatgat |
| *Ldlr*[1] | aggctgtgggctccatagg | tgcggtccagggtcatct |
| *Sqle* | tcaaccccagtccagttctc | gactccttcaggtgctcagg |
| *18S* | cgccgctagaggtgaaattc | ttggcaaatgctttcgctc |

[1]Yang et al. 2001[40]
*Hmgcr,* hydroxy-3-methylglutaryl-coenzyme A reductase; *Ldlr,* LDL-receptor; *Sqle;* squalene epoxydase; *18S;* ribosomal 18S subunit

## 2. 8. Statistical Analysis of Phenotype and RT-PCR Data

Differences between the lines in all measured phenotypic parameters (fat depot sizes, plasma lipid parameters, RT-PCR data) were tested by Student's *t*-test for independent samples at a probability of type I error $\alpha \leq 0.05$ to determine statistical significance.

# 3. Results

## 3. 1. Adiposity and Plasma Lipoprotein Profile

The male Congenic mice exhibited statistically significant lower body fatness at 14 weeks of age across all dissected fat depots (Table 2) when compared to the Fat mice. It is of note that successfully introgressed *Fob3b* Lean-line chromosome 15 segment into Congenic mice lowered adiposity by 20–29% in each of the four fat depots studied. A ratio of heart weight to body weight was significantly increased in Congenic mice (Table 2). Blood plasma was individually analyzed in Congenic and Fat males for fasting plasma glucose (Figure 1a), and lipids (Figures 1b–1e). Congenic mice were found to have significantly lower fasting plasma glucose (Figure 1a) compared to the Fat line. Ratios of HDL to total plasma cholesterol as well as nonHDL cholesterol to total plasma cholesterol were not statistically significantly different between the lines (Figure 1c and Figure 1e, respectively). Surprisingly, despite higher expression level of some genes involved in cholesterol biosynthesis in less fat Congenic mice (Table 3), measurements of total plasma cholesterol concentration resulted in *non*-significant differences between the lines (Figure 1b). Plasma triglyceride content also resulted in *non*-significant differences (Figure 1d). FPLC analysis

Fig. 1a

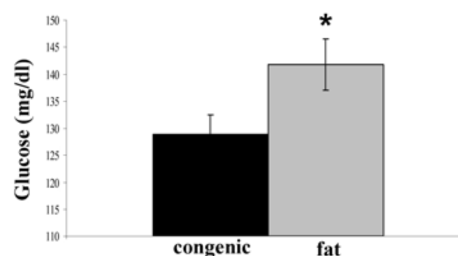
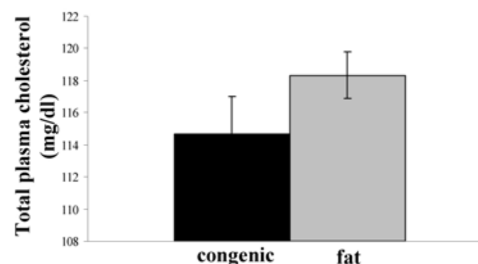



Fig. 1b



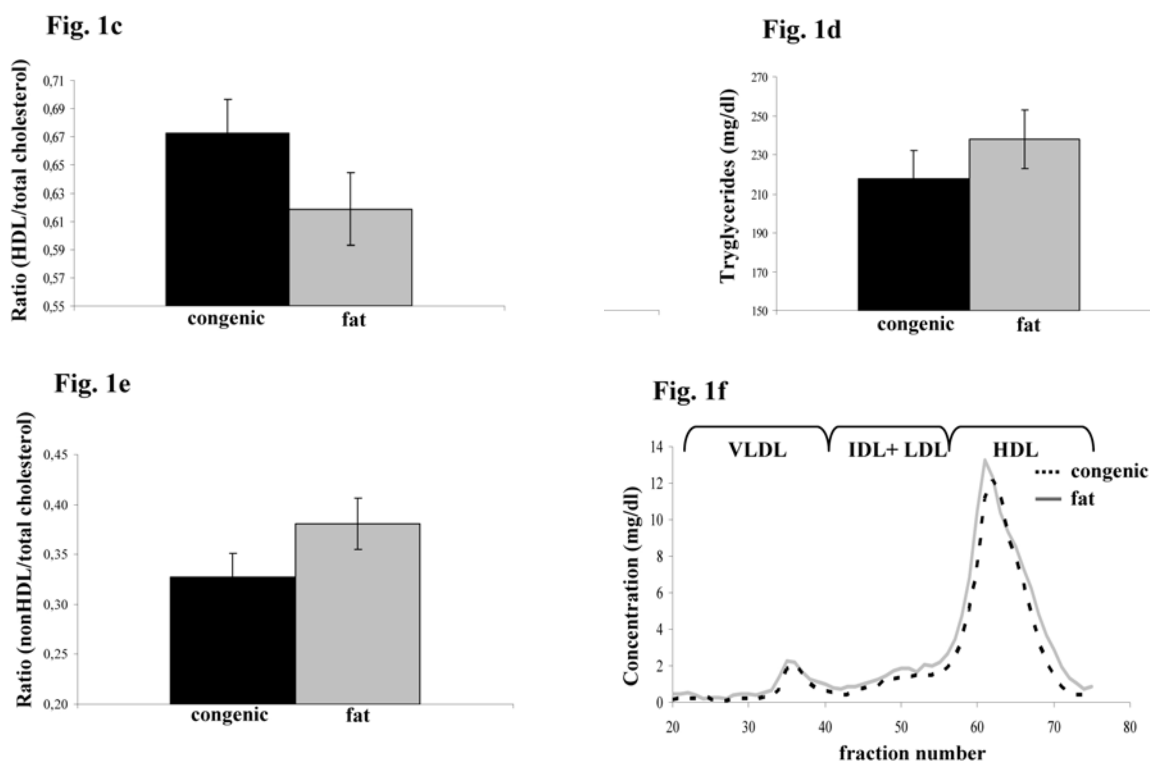



**Figure 1.** **(1a)** Mean fasting plasma glucose levels of Congenic and Fat mice (average +/–S.E.); **P* < 0.05; **(1b)** Mean total plasma cholesterol concentrations of Congenic and Fat mice (average +/– S.E.); **(1c)** Ratio of plasma HDL-cholesterol vs. total plasma cholesterol in Congenic and Fat mice (average +/–S.E.); **(1d)** Mean plasma triglycerides levels of Congenic and Fat mice (average +/–S.E.); **(1e)** Ratio of plasma nonHDL-cholesterol vs. total plasma cholesterol in Congenic and Fat mice (average +/–S.E.); **(1f)** FPLC analysis of total plasma lipoproteins from pooled sample of Congenic and Fat mice to determine the distribution of cholesterol (concentration on Y-axis) into plasma lipoprotein particles separated according to their size (X-axis). Ranges of the lipoprotein classes are indicated: fraction 20–40 for VLDL, 40–55 for IDL + LDL, and larger than 55 for HDL.

**Table 2.** Relative proportions (mg/g body wt) of fat depots and heart in Congenic and Fat mice (**P* < 0.05).

| Trait | Congenic line | Fat line | No. of individuals |
|---|---|---|---|
| Abdominal fat | 7.97 ± 1.65 | 9.97 ± 1.08* | Congenic = 36, Fat = 42 |
| Femoral fat | 14 ± 1.98 | 17.67 ± 2.32* | Congenic = 36, Fat = 42 |
| Epididymal fat | 16.60 ± 2.91 | 23.24 ± 3.12* | Congenic = 36, Fat = 42 |
| Mesenterial fat | 18.40 ± 2.63 | 22.33 ± 3.87* | Congenic = 29, Fat = 15 |
| Heart | 5.32 ± 0.88 | 4.58 ± 0.69* | Congenic = 35, Fat = 42 |

of pooled plasma samples for Congenic and Fat mice was carried out to determine the distribution of plasma lipoprotein particles separated according to their size. This analysis confirmed the results of the calculated nonHDL levels (Figure 1e) (based on subtracting total from HDL cholesterol) in that Congenic mice exhibit a tendency to lower concentrations of atherogenic (nonHDL) cholesterol (Figure 1f, see lower curve of fraction number from 20 to 55 on the X-axis for Congenic mice). No major differences were observed for the lipoprotein size (note peak overlaps in Figure 1f).

## 3. 2. Hepatic Transcriptome Analysis

The final list of genes detected as differentially expressed (*P* < 0.05) between the Congenic and Fat mice in the microarray experiment contains cytochrome P450 candidates, cholesterol biosynthesis genes, transcriptional factors and transporters of biliary cholesterol. The expression levels of two cytochrome P450s genes were found to be differentially expressed. Retionic acid hydroxylase (*Cyp26a1*) demonstrated the highest differential expression among all the candidates on the array and was upregulated in Congenic mice. *Cyp26a1* has a role in catalyzing degradation of all-*trans* retinoic acid (atRA). This points to the fact that hepatocytes of Congenic mice may more efficiently degrade atRA and that relative surplus of atRA might exist in liver of Fat mice. Another P450 candidate (*Cyp2a4*) was detected as being differentially expressed. *Cyp2a4, a* gene that encodes the *steroid 15α-hydroxylase* involved in catabolism of testosterone, was found to be significantly downregulated in Congenic mice. This indi-

**Table 3.** Differentially expressed genes obtained from microarray experiment ($P < 0.05$).

| Gene name (Gene symbol) | Genbank Accession | $log_2$ ratio (microarray) Congenic : Fat line |
|---|---|---|
| Retinoic acid hydroxylase (*Cyp26a1*) | NM_007811 | 0.53 |
| Cytochrome P450, family 2, subfamily a, polypeptide 4 (*Cyp2a4*) | NM_009997 | –0.186 |
| Sterol C5 desaturase (*Sc5d*) | NM_172769 | 0.212 |
| Sterolin 2 (*Abcg8*) | NM_026180 | –0.215 |
| Sterolin 1 (*Abcg5*) | NM_031884 | –0.31 |
| Multidrug resistance protein 3 (*Mrp3*) | NM_029600 | –0.231 |
| Nuclear receptor subfamily 1, group I, member 3 (*Nr1i3*) | NM_009803 | –0.344 |
| Peroxisome proliferator activated receptor alpha (*Ppara*) | NM_011144 | –0.165 |
| Farnesoid X receptor (*Fxr*) | NM_009108 | –0.145 |

cates that *Fob3b* QTL could directly or indirectly (*via* modulating obesity level) influence metabolism of vitamin A and testosterone.

From genes involved in the cholesterol biosynthesis, *sterol C5 desaturase (Sc5d)* gene was expressed at a higher level in Congenic mice. To support this, we further evaluated expression level of the gene *Hmgcr*, a rate limiting cholesterol biosynthesis enzyme, by RT-PCR quantification and also found statistically significant increase of mRNA levels in Congenic mice ($P = 0.013$). Since HMGCR enzyme is known to be allosterically downregulated by its own product cholesterol, we then tested the mRNA expression level of liver *Ldlr* known for uptaking substantial amount of plasma cholesterol into liver.[18] However, RT-PCR quantification of low density lipoprotein receptor (*Ldlr*) mRNA level failed to identify significant difference between the lines. On the basis of upregulation of two enzymes from cholesterol biosynthesis we conclude that this pathway might be upregulated in Congenic mice. In contrast, two genes involved in biliary cholesterol secretion (*Abcg5* and *Abcg8*) coding for heterodimeric transporter complex were found to be downregulated in Congenic mice compared to the Fat mice (Table 3). On the basis of these data we can speculate that Fat mice liver tissue produces less cholesterol but also has enhanced biliary cholesterol secretion.

From the group of genes encoding multidrug resistance proteins, *Mrp3* was detected as being downregulated in Congenic mice. *Mrp3* has a well documented role of basolateral transporter in hepatocytes that is able to excrete bilirubine-glucoronide and drug-glucoronide conjugates into blood. Constitutive androstane receptor (*CAR*, gene symbol also *Nr1i3)* known for its transcriptional regulatory role of *Mrp3*, was also downregulated in Congenic mice (Table 3).

Farnesoid X receptor (*Fxr*) as a potent nuclear receptor involved in glucose homeostasis was shown to be downregulated in Congenic mice. Beside its role in glucose metabolism, *Fxr* is also known to be involved in regulation of bile acid metabolism. No such candidate indicating differences in bile acid metabolism was detected. Array analysis uncovered downregulation of another gene in Congenic mice, a transcription factor Peroxisome proliferator activated receptor alpha (*Ppar*$\alpha$).

# 4. Discussion

Our previous microarray study[9] suggested that a QTL on chromosome 15 (*Fob3b*) identified in our polygenic mouse obesity model may affect expression of several pathways including some genes from the cytochrome P450 superfamily and cholesterol biosynthesis. By using a focused microarray Steroltalk containing a complete array of genes from the aforementioned and related pathways we tested if perturbations in expression of these genes and pathways could be associated with the phenotypic differences between the Fat and Congenic lines.[10–11] For this purpose, we developed a Congenic line carrying *Fob3b*-containing segment from the Lean line in the genetic background of Fat line by 12 successive backcrosses. We also phenotyped both lines for plasma and other obesity-related parameters as a basis for interpretation of the transcriptome results.

Analysis of four different fat depots revealed significant differences between the Congenic and Fat line. This is the first evidence that a chromosomal segment from the Lean line introgressed into the genetic background of the Fat line has been successfully transferred and that this QTL region can function independently of other Lean line QTL regions discovered previously.[8] The introgressed Lean line *Fob3b* QTL showed a therapeutic effect in Congenic mice resulting in significantly lowering the amounts of abdominal, femoral, epididymal and mesenterial fat depots by 20 to 29%. This effect is rather large given that QTL alleles are regarded as low to medium effect loci and do not reach the effect of major single gene mutations such as the *obese* mutation (lack of function of Leptin gene) or *diabetes* mutation (lack of function of Leptin receptor).[19,20] Altogether, our study provides the first evidence of reduced adiposity in all four major fat depots in the Congenic mice containing the *Fob3b* QTL region from mouse chromosome 15.

Apart from a significant effect on fat depot size, the introgressed *Fob3b* QTL containing region has other effects on obesity-related parameters. A ratio of heart weight to body weight was significantly increased in Congenic mice. This is an indication that Congenic mice might be more physically active or have increased oxidative metabolism thus increasing energy combustion. Some support for this offer our earlier results from energy budget analyses demonstrating that energy devoted to physical activity could indeed be an important part of the phenotypic differences between the Fat and Lean line.[21] We also found that Congenic mice have significantly lower fasting plasma glucose pointing to potential differences in insulin resistance between the Congenic and Fat mice. Future studies should be directed to test for plasma insulin and glucose tolerance test between the lines to provide a better understanding of this identified difference. Comparing total plasma cholesterol failed to identify a significant difference. Combining all the phenotypic measurements we can conclude that the Lean line allele(s) within the *Fob3b* QTL region have a therapeutic effect on several obesity-related parameters such as lowering fat depot size and lowering fasting glucose levels.

The main part of this study was devoted to microarray experiment comparing gene expression profiles in liver tissue between the Congenic and Fat mice on a custom array Steroltalk. The experiment was designed in a way that enabled high statistical power of detecting significant differential expression. Namely, 10 pairs of individual Congenic and Fat line mice were compared in duplicate (dye swap) totaling in 20-array hybridizations. This is a rather large size transcriptome experiment, which are often, due to the cost issue, performed at a small scale or based on the pools rather than individuals. The genes that showed statistical significance in our experiment can therefore be regarded as true differentially expressed genes rather than potential candidates. A relatively short list of genes was confirmed as differentially expressed between Congenic and Fat lines, which is not surprising given that Congenic and Fat mice are essentially genetically similar – they have the Fat mice genetic background and differ only for a segment of chromosome 15, that Congenic mice received from the original Lean mouse line. Therefore, a small number of differences between these two lines were expected, but the ones found should be highly physiologically related to the causative allele(s) residing in the differential chromosome 15 segment.

Our study unequivocally shows that *Cyp26a1*, a member of the cytochrome P450 superfamily, was by far the most differentially expressed microarray candidate. In liver tissue *Cyp26a1* catalyses catabolism of all-trans retinoic acid (atRA) thus maintaining homeostasis of available atRA, which is markedly involved in various metabolic actions. Perturbation in retinoid homeostasis as demonstrated by *rdh1*-null mice (retinol dehydrogenase gene knock-out) provide evidence that *Cyp26a1* plays a major role in atRA homeostasis.[22] It was shown that availability of retinyl esters drives the expression profile of *Cyp26a1* gene. This study confirmed that the shortage of endogenously synthesized atRA leads to downregulation of *Cyp26a1* gene and the whole perturbation eventually results in increased adiposity. This is in line with our results demonstrating that *Cyp26a1* gene was downregulated in our Fat mice that are characterized by increased adiposity. Differences in vitamin A intake could potentially lead to differences in *Cyp26a1* expression, but we regard this possibility unlikely for two reasons: we found earlier that the founder lines (Lean and Fat) consume the same amount of diet[21] and in the present study were fed standard maintenance mouse chow diet that contains sufficient or surplus amounts of vitamin A. Therefore, differences in vitamin A intake seem to be less plausible as an explanation for differential expression of *Cyp26a1,* which we rather ascribe to genetic differences between the lines. Conclusively, we can speculate that perturbed atRA homeostasis governed by differential expression of *Cyp26a1* in our Congenic and Fat mice at least in part contribute to the divergent adiposity phenotype observed in this study.

Perturbed atRA mediated action due to observed differential expression of *Cyp26a1* could result in transcriptional activation of mitochondrial genes by atRA *via* atRA receptors in mitochondrial matrix.[23] Such a mechanism of retinoic acid action was shown in BHE/Cdb rats carrying a mutation in mitochondrial ATPase 6 gene. Rats were characterized with defective mitochondrial oxidative phosphorylation followed by insulin resistance and increased hepatic lipogenesis. When rats were fed additional amount of vitamin A the oxidative phosphorylation in liver mitochondria increased. It was also proposed that elevated blood and liver lipids might impair atRA synthesis.[24] This result can be extrapolated to our animal model as our Fat mice exhibited higher trends in triglyceride content in both liver and plasma. Moreover, current results showed hyperglycaemia in Fat mice supporting a previous study demonstrating that our Fat mice also develop insulin resistance.[25] In addition, we have recently demonstrated that Fat mice have significantly less active mitochondrial succinic dehydrogenase enzyme in skeletal muscle when compared to Lean mice (M. Simončič et al, unpublished data) indicating that the oxidative phosphorylation is obviously diminished in Fat mice. Altogether, the phenotype parameters observed in our current study and other studies emphasize the importance of *Cyp26a1* expression to the vitamin A status and its relation to mitochondrial gene expression in obesity. Future studies should be directed to elucidate the mechanism of *Cyp26a1* and its downstream effects in obesity determination in our model as well as in obesity genetic control in general.

Evaluation of the expression of another member of the cytochrome P450 gene subfamily *Cyp2a4* also result-

ed in differential expression between the Congenic and Fat mice. Congenic mice were found to have significantly reduced level of *Cyp2a4* mRNA. *Cyp2a4* encodes a steroid 15α-hydroxylase which catalyzes hydroxylation reactions of testosterone leading to further metabolism and/or excretion by the liver.[26] In addition, it was demonstrated that transcriptional factor CAR, also found as differentially expressed in our experiment, mediates induction of *Cyp2a4* expression and hence the growth hormone (GH) – CAR- *Cyp2a4* interplay was proposed.[27] Under physiological conditions pituitary GH in males is secreted in pulsatile manner (male-type of GH secretion), whereas in females pituitary gland constantly secretes GH (female-type of GH secretion) and it has been only recently demonstrated that GH with its sexually dimorphic plasma profile dictates the expression level of *Cyp2a4* in a sex-specific manner.[28] However, it was shown that male-type exogenous administration of GH in male mice resulted in decreased expression of *Cyp2a4* when compared to female-type administration of GH.[29] Perturbed GH secretion in relation to obesity is well documented in humans as well as in mice and it was evidenced that insulin resistance presents the causative factor of reduced pituitary GH secretion.[30] This indicates that our Congenic (normoglycemic) and Fat (hyperglycemic) mice might have different profiles of GH secretion subsequently affecting *Cyp2a4* expression profile. The result of this could be lower level of plasma testosterone in Fat mice as they exhibit more efficient metabolism and excretion by liver *Cyp2a4*. Conclusively, this is in accordance with findings from human studies where it was demonstrated that metabolic syndrome in men is associated with lower levels of plasma testosterone.[31]

Apart from the two cytochrome P450 genes discussed above, differential expression was found for some other genes on the Steroltalk array. Identified higher expression of *Fxr* in Fat mice might result as a defense mechanism to combat hyperglycaemia in these mice since *Fxr* activation results in suppressed expression of multiple genes involved in gluconeogenesis.[32] We have also detected decreased expression of some cholesterol biosynthesis genes (*e.g.*, *Scd5*, *Hmgcr)* which was not reflected in expected lower total plasma cholesterol in the Fat mice. This phenomenon can be explained by pronounced synthesis of cholesterol in larger fat depots in Fat mice which compensates for decreased liver production of cholesterol thus maintaining higher plasma cholesterol concentrations. One possible explanation is also offered by our result of differential expression of *Mrp3*. This gene is frequently induced in conditions of hepatocellular accumulation of toxic billiary constituents and provides an alternative excretory route of conjugated bile acids. As *Mrp3* showed elevated expression in Fat mice, this could be interpreted as an indicator of increased cholesterol catabolism in the liver (counteracting fat tissue derived cholesterol), as found also in other obese mouse models.[33,34] In line with this is our result of upregulation of *Abcg5* and *Abcg8* (two cholesterol transporters) in Fat mice liver providing a cue that hepatobiliary cholesterol excretion might indeed be enhanced in the obese models.[35,36]

*PPARα* transcriptional factor was found to be elevated in Fat mice. It is possible that Fat mice try to withstand obesity by activating liver peroxisomal combustion of long-chain fatty acids. Subsequently, increased activity of peroxisomal acyl-CoA oxydase (AOX) results in disproportion between production and degradation of $H_2O_2$. Eventually, accumulation of $H_2O_2$ leads to profound oxidative DNA damage, hepatocellular proliferation and non-genotoxic hepatocarcinogenesis.[37,38] Interestingly, it has been only recently evidenced that oxidative stress induces transcription of *Mrp3* gene, aforementioned differentially expressed candidate in our study. This action results in maintaining glutathione (GSH) homeostasis in liver cells when exposed to oxidative stress.[39] The expression level of *PPARα* and *Mrp3* genes in our experiment is suggestive of a risk for increased oxidative stress which in older obese mice might provoke hepatocarcinogenesis.[38]

Therefore, the transcriptome results of some aforementioned non-P450 genes on the array do provide further explanation for the observed plasma lipoprotein profile between the Congenic and Fat mice. However, further more targeted studies are needed to fully explain the mechanism of action and how perturbations in these genes are linked to the *Cyp26a1* and *Cyp2a4* mediated effect on obesity in our polygenic model. Given the scarcity of association evidence in the literature between obesity and involvement of cytochrome P450 genes, our results indicate that these genes might play a more pronounced role in obesity than previously thought.

Finally, *Cyp26a1* and *Cyp2a4* and other identified differentially expressed genes do not map to the segment on chromosome 15 for which our Congenic line differs genetically from the Fat line. Therefore, it is likely that allelic differences in the identified differentially expressed genes are not the primary cause for the observed phenotypic differences but are most likely secondary effects. Our ongoing genetic studies involving fine mapping crosses as well as bioinformatics haplotype analyses should narrow down the list of positional candidate genes and eventually lead to identification of a mutation/gene that is causal. Once revealed, the results of the present study should provide crucial information necessary to unravel the mechanism by which this mutation on chromosome 15 causes all the downstream molecular events leading to the observed phenotypic differences. Such knowledge has potential application in therapies of obesity – as we have shown here that our Congenic line has 20–29% less weight of several fat depots, understanding the molecular basis for such an anti-obesity effect could be illuminating in designing novel targets and therapies for obesity.

## 5. Conclusion

Transcriptome and phenotype analysis of Congenic and Fat mice liver tissue was performed to advance our understanding of genetic basis of obesity in our polygenic model. Experimental set-up of the microarray experiment was designed in a way to reach a high statistical power to detect differential expression – 20 arrays were used in total to test 10 pairs of Congenic and Fat line individuals, respectively. Surprisingly, among the final short list of identified differentially expressed genes were also two cytochrome P450 gene family members (*Cyp26a1, Cyp2a4*) suggesting their involvement in determination of genetically predisposed obesity in our polygenic mouse model.

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## Povzetek

Proteini naddružine citokromov P450 (CYP) so dobro proučeni predvsem v povezavi s presnovo ksenobiotikov, le malo pa je znanega o vlogi genov te naddružine (npr. *Cyp19a1* in *Cyp7a1*) pri pojavu debelosti. Zaradi vse večjega števila bolezni, povezanih z debelostjo in metabolnim sindromom pri ljudeh, je preučevanje takih povezav na živalskih modelih v vzponu. V naših predhodnih genetskih študijah smo določili štiri lokuse, ki vplivajo na nalaganje maščevja v linijah miši, ki so bile selekcionirane na visok (debela linija) ali nizek odstotek telesnih maščob (suha linija). V tej študiji smo razvili kongeno linijo, ki vsebuje samo genomski segment kromosoma 15 iz suhe linije in primerjali njen transkriptom in druge fenotipske podatke z izvorno debelo linijo. Ugotovili smo, da ima kongeni odsek terapevtski učinek na večino z debelostjo povezanih lastnosti, kot so zmanjšanje količine posameznih maščobnih depojev ali zniževanje koncentracije glukoze v plazmi. Z uporabo Steroltalk mikromreže, ki je osredotočena na gene *Cyp* in gene v presnovi holesterola, smo uspeli določiti 9 diferencialno izraženih genov, ki vključujejo tudi dva citokroma P450 (*Cyp26a1, Cyp2a4*). Naši rezultati nakazujejo, da imajo ti geni pomembno vlogo pri uravnavanju debelosti, kar do sedaj še ni bilo opisano. Da bi lahko razložili, kako so motnje v delovanju genov *Cyp* povezane z uravnavanjem debelosti v našem živalskem modelu, bodo potrebne nadaljnje funkcijske študije.