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Research paper

Nutrition modulates *Fto* and *Irx3* gene transcript levels, but does not alter their DNA methylation profiles in rat white adipose tissues

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ABSTRACT

The *fat mass and obesity associated* (*Fto*) and *iroquois homeobox 3* (*Irx3*) genes have been recognised as important obesity-related genes. Studies on the expression of these genes in the fat tissue of human and mouse have produced inconsistent results, while similar data on rat are limited. Environmental factors such as diet, should be considered as potential modulators of gene transcript levels through epigenetic mechanisms including DNA methylation. The aim of this study was to evaluate transcription levels and DNA methylation profiles of rat *Fto* and *Irx3* genes in two white adipose tissue depots in response to high-fat and high-protein diets. The relative transcript levels of *Fto* and *Irx3* were shown to be tissue-specific with higher levels detected in subcutaneous fat tissue than in abdominal fat tissue. Moreover, negative correlations between the transcripts of both genes were observed for subcutaneous fat tissue. The identified interactions (*e.g.* diet × duration of diet regimen) indicated that the diet had an impact on the transcript level; however, this effect was dependent on the duration of the diet regimen. The high-fat diet led to upregulation of *Fto* and *Irx3* linearly with time across the two tissues. DNA methylation of the regulatory regions of the studied genes was very low and not related with the tissue, diet, or duration of diet regimen. Our study revealed that diet was an important factor modulating transcription of *Fto* and *Irx3*, but its effect is time-dependent. In contrast, the DNA methylation profiles of *Fto* and *Irx3* were not altered by nutrition, which may indicate that the feeding type, when applied postnatally, did not affect DNA methylation of these genes.

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1. Introduction

Obesity and obesity-related diseases are increasingly recognised as problems in human health. Studies on the genetic background of obesity have revealed that only a few genes are responsible for monogenetic obesity, while polygenic forms are determined by many genes and interactions with the environment. Different genomic approaches, such as candidate gene analysis and genome-wide association studies (GWAS), including meta-analysis, have been used to identify obesity-related candidate genes (for a review, see Apalasamy and Mohamed, 2015).

An important result from these studies was the discovery of the *fat mass and obesity associated* (*FTO*) *gene*, which encodes a protein responsible for nucleic acid demethylation (Hess and Brüning, 2014). This gene was identified by searching for type II diabetes susceptibility genes (Frayling et al., 2007), and subsequent GWAS studies confirmed that *FTO* was a BMI-susceptible locus across different ethnic groups (Loos and Yeo, 2014). The role of *FTO* in the pathogenesis of obesity was also demonstrated in a number of rodent models: knockout models as well as models in which this gene was overexpressed (Fischer et al., 2009; Church et al., 2010). Further studies showed the intronic SNP cluster of *Fto* contained an enhancer for the *iroquois homeobox 3* (*Irx3*) *gene*, which plays a role in adipocyte differentiation. The role of *Irx3* was confirmed in a mouse model, where knockout of this gene resulted in a decrease in body mass (Smemo et al., 2014). The obtained results indicated that *Irx3* should also be considered as a strong candidate for human adiposity. Recently the mechanism of how *Fto* variants influenced adipose tissue formation was described by Claussnitzer et al. (2015). It was shown that, depending on *Fto* polymorphisms, the expression levels of *Irx3* and *Irx5* were regulated through the AT-rich interactive domain 5B (ARID5B) repressor protein in adipocyte precursor cells, leading to formation of beige adipocytes (which are responsible for energy-dissipating thermogenesis) or white adipocytes (which are involved in lipid accumulation). The above results justify a detailed analysis of *Fto* and *Irx3* expression profiles in different fat tissue depots.

Environmental factors should also be considered as potential modifiers of *Fto* and *Irx3* gene expression. A number of dietary compounds have been identified to affect gene expression through several mechanisms: by regulating the activity of nuclear receptors/transcription

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Abbreviations

AFT	Abdominal fat tissue
CGI	CpG island
Fto	Fat mass and obesity associated
GWAS	Genome wide association studies
HFD	High-fat
HPD	High-protein
Hprt	Hypoxanthine-guanine phosphoribosyltransferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Irx3	Iroquois Homeobox 3
SFT	Subcutaneous fat tissue
Tbp	TATA box binding protein
XGal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

factors or through modification of DNA methylation levels (Bouchard-Mercier et al., 2013; Zhang, 2015). Among the different diet types tested, high-fat (HFD) and high-protein (HPD) diets have been studied intensively in relation to gene expression modulation. HFDs are associated with the development of obesity and the metabolic syndrome and have been found to alter the expression of genes involved in adipocyte differentiation, lipid metabolism, and inflammation (Xue et al., 2015). HPDs are known to reduce adiposity and have been shown to have an effect on the expression of hepatic genes (Schwarz et al., 2012). Moreover, HPDs may result in a high supply of the universal methyl donor, *S*-adenosyl-methionine, originating mainly from the conversion of methionine and resulting in an elevated DNA methylation process (Niculescu and Zeisel, 2002).

Considering that limited data are available concerning the roles of *Fto* and *Irx3* in fat tissue of rat, the aim of the present study was to evaluate the expression and methylation profiles of these two genes in terms of different nutritional components (high-fat and high-protein diets). We analysed two white adipose tissue types (subcutaneous and abdominal fats) and two feeding types, namely HFD or HPD *versus* a control diet, as well as a duration of diet regimen where animals were fed for 30, 60, or 120 days.

2. Material and method

2.1. Animals and feeding types

The animals were kept in an animal house according to standard procedures approved by the local Bioethical Commission for Animal Care and Use in Poznan, Poland; (approval no. 7/2009). Male Wistar rats with initial weights of 225.2 ± 4.33 g were divided into three groups, which were fed with different diets after 10 days of adaptation. The following diets were tested: control (normal) diet: Labofed B (Kcynia, Poland) *ad libitum* (daily caloric intake: 2.75 kcal/g), HFD: normal diet + 20% triglycerides (daily caloric intake: 4.00 kcal/g), and HPD: normal diet + 20% soy protein (daily caloric intake: 3.53 kcal/g). The weight of each animal was measured every 10 days. White adipose samples were collected from two depots, subcutaneous fat tissue (SFT) and abdominal fat tissue (AFT). In all three feeding type groups, the animals received the particular diet for 30, 60, or 120 days. Each diet × duration of diet regimen group contained eight animals.

2.2. RNA extraction and real time PCR

Total RNA was isolated by a standard procedure using TriPure Isolation Reagent (Roche, Indianapolis, IN, USA). Then, 2 μg of RNA was transcribed into cDNA using a Transcriptor High Fidelity cDNA Synthesis kit (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Semi-quantitative PCR was performed on a LightCycler 480 II (Roche) using the SYBR Green detection system. Relative quantification of the mRNA level was performed in duplicate and calculated in relation to the results for the reference genes, *hypoxanthine-guanine phosphoribosyltransferase gene* (*Hprt*) and *TATA box binding protein gene* (*Tbp*) (Vandesompele et al., 2002). The primer sequences and PCR details are given in Supplementary Table S1.

2.3. Bisulfite sequencing and data analysis

DNA methylation was performed by sequencing after bisulfite conversion. The identification of CpG islands (CGIs) was performed using the CpG plot software (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/). Briefly, three potential CGIs were found in the *Fto* reference sequence (GenBank: NC_005118.4). Two of them were localised in intron 8, and the third one, which was studied in this work, was 510 bp long and overlapped the first exon spanning to approximately 0.4 kb upstream of the *Fto gene*. Five potential CGIs were found in the *Irx3* reference sequence (GenBank: NC_005118.4), which is located in close proximity to the *Fto* locus. The CGIs were detected in the 5′- and 3′-flanking regions of the *Irx3 gene*, as well as spanning the first exon. In this study, we analysed a fragment of the large CGI (812 bp) found in the 5′UTR of *Irx3* (Suppl. Fig. 1). Primer sequences were designed using the MethPrimer software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). The details are presented in Suppl. Table 1. In the *Fto* and *Irx3* amplicons (324 bp for *Fto*; 306 bp for *Irx3*), overlapped 23 and 19 CG dinucleotides, respectively.

DNA was isolated from two types of white adipose tissue (SFT and AFT) using a standard 25:24:1 phenol:chloroform:isoamyl alcohol acid mixture (Sigma-Aldrich, St. Louis, MO, USA) procedure. The concentration of isolates was measured on a Nanodrop spectrophotometer (Thermo Scientific, UK), and 1 μg of DNA was bisulfite converted according to the supplier's protocol (EZ DNA Methylation Kit, Zymo Research, Inc., Irvine, CA, USA). Touchdown amplification was applied for both fragments according to our previous protocol (Nowacka-Woszuk et al., 2015). The amplicons were ligated into the p-GEM T-Easy vector (Promega, Madison, WI, USA) following transformation of the DH5α competent *Escherichia coli* strain (Invitrogen). The bacteria were harvested overnight (37 °C) on selective agar plates with ampicillin, XGal, and IPTG (A&A Biotechnology, Gdansk, Poland). The white colonies were selected and plasmid DNA was amplified using a Illustra TempliPhi Amplification Kit (GE Healthcare, Piscataway, NJ, USA) for 4–16 h at 30 °C. The final step was clone sequencing using a Big Dye Terminator v.1.1 Sequencing kit (Thermo Scientific, UK) on a Genetic Analyzer 3130 system (Applied Biosystems). The obtained sequences were analysed with the QUMA software (http://quma.cdb.riken.jp/).

2.4. Statistical analysis

The transcript level data were analysed in a logarithmic scale. The statistical model for a gene transcript level included the fixed effects of tissue, diet, time class, all two-way interactions, and diet × time × tissue interactions (model 1). Analyses were also performed within a tissue under a model with diet, time class, and diet × time interactions (model 2). To examine the linear relationship between duration of diet regimen and gene expression a linear regression model (model 3) was used. Statistical significance was determined by ANOVA. To investigate the correlation between genes, Pearson's correlation coefficient was applied on data adjusted for experimental factors. All analyses were performed using the R software package (R Development Core Team).

3. Results

3.1. Impact of HFD and HPD on animal body weight

The average body weight of the animals at the beginning of the experiment was 225.2 ± 4.33 g. The rats fed the HFD showed increased

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