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Transcriptome profiling of visceral adipose tissue in a novel obese rat model, WNIN/Ob & its comparison with other animal models

Siva Sankara Vara Prasad Sakamuri[1], Uday Kumar Putcha[2], Giridharan Nappan Veettil[3] & Vajreswari Ayyalasomayajula[1]

Departments of [1]Biochemistry and [2]Pathology, National Institute of Nutrition (ICMR) & [3]Molecular Genetics Laboratory, National Center for Laboratory Animal Sciences (ICMR), Hyderabad, India

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***Background & objectives*: Adipose tissue dysfunction in obesity is linked to the development of type 2 diabetes and cardiovascular diseases. We studied the differential gene expression in retroperitoneal adipose tissue of a novel obese rat model, WNIN/Ob, to understand the possible underlying transcriptional changes involved in the development of obesity and associatedcomorbidities in this model.**

***Methods*: Four month old, male WNIN/Ob lean and obese rats were taken, blood was collected and tissues were dissected. Body composition analysis and adipose tissue histology were performed. Global gene expression in retroperitoneal adipose tissue of lean and obese rats was studied by microarray using Affymetrix GeneChips.**

***Results*: One thousand and seventeen probe sets were downregulated and 963 probe sets were upregulated (more than two-fold) in adipose tissue of WNIN/Ob obese rats when compared to that of lean rats. Small nucleolar RNA (SnoRNA) made most of the underexpressed probe sets, whereas immune system-related genes werethe most overexpressed in the adipose tissues of obese rats. Genes coding for cytoskeletal proteinswere downregulated, whereas genes related to lipid biosynthesis were elevated in the adipose tissue of obese rats.**

***Interpretation & conclusions*: Majority of the altered genes and pathways in adipose tissue of WNIN/Ob obese rats were similar to the observations in other obese animal models and human obesity. Based on these observations, it is proposed that WNIN/Ob obese rat model may be a good model to study the mechanisms involved in the development of obesity and its comorbidities. Downregulation of SnoRNA appears to be a novel feature in this obese rat model.**

Key words Microarray - obesity - small nucleolar RNA - visceral adipose tissue - WNIN/Ob obese rat

In mammals, adipose tissue stores energy in the form of triglycerides (TGs) and supplies energy to the system by delivering free fatty acids. It has been shown that adipose tissue acts as an endocrine organ by secreting hormones, cytokines, growth factors, acute phase proteins, complement-related proteins and

extracellular matrix (ECM) proteins[1]. In obesity, excess energy intake results in accumulation of large amounts of TGs in adipocytes leading to dysregulation of adipocyte metabolism and secretary function, resulting in the development of comorbidities such as insulin resistance, dyslipidaemia and hypertension, which can further lead to the development of chronic diseases such astype 2 diabetes and cardiovascular disease[2]. Expression profiling of various genes in the adipose tissue under obese conditions will lead to the better understanding of adipose tissue adaptation to high levels of TG accumulation, altered secretary function and also mechanisms involved in the development of obesity-associated comorbidities. Previous studies on microarray analysis of adipose tissue from animal models of obesity and human obesity have contributed to the understanding of genes that are altered in obesity and also the identification of candidate genes that play an important role in the development of obesity and its associated comorbidities[3,4].

WNIN/Ob obese rat model was developed by selective breeding of obese rat generated by spontaneous mutation in 80 yr old inbred Wistar rat colony[5]. The mutation is autosomal codominant, and the rat colony has three phenotypes (and also three genotypes) *i.e*., lean (+/+), carrier (+/-) and obese (-/-). Preliminary studies have shown no molecular defect in the open reading frame of the leptin or leptin receptor[6]. The WNIN/Ob obese rat shares several physiological and biochemical characteristics with genetic and diet-induced rodent models. They exhibit early-onset obesity with hyperphagia, hyperinsulinaemia and hyperleptinaemia along with dyslipidaemia[5].

In this study, the primary objective was to determine the differentiallyregulated genes in the visceral adipose tissue of WNIN/Ob obese rats to understand the possible underlying transcriptional changes involved in the development of obesity in this model. This would help in understanding the similarities/differences in the transcriptome composition of this novel obese rat model with already established obese rodent models and human obesity.

Material & Methods

Animals: This study was conducted by the Lipid Chemistry Division of Biochemistry Department in the National Institute of Nutrition (NIN), Hyderabad, India, during 2010-2011. Four month old, male WNIN/Ob lean and obese rats (n=6 for each phenotype) were obtained from the National Centre for the Laboratory Animal Sciences, Hyderabad. The animals were housed in cages and acclimatized for one week under controlled conditions of light (12 hof light/12 hof dark) and temperature (22°C ±2°C). Stock-diet and water were provided *ad libitum*. All experimental procedures were approved by the Institutional Animal Ethical Committee. After acclimatization, blood was drawn and animals were sacrificed by CO_2 asphyxiation. Retroperitoneal adipose tissue was immediately removed, frozen in liquid nitrogen and stored at -80°C.

Body composition: Body composition of WNIN/Ob lean and obese animals was assessed by Total Body Electrical Conductivity (TOBEC) small animal body composition analysis system (EM-SCAN, Model SA-3000 Multidetector, Springfield, USA). Lean body mass (LBM), fat-free mass (FFM) and total body fat percentages were calculated according to manufacturer's instructions.

Histology of adipose tissue: Retroperitoneal adipose tissue samples were fixed in 10 per cent neutral-buffered formalin, embedded in paraffin and 4 µ sections were taken for staining. Adipose tissue samples were stained with haematoxylin and eosin (H&E) to observe adipose tissue inflammation and determine adipocyte hypertrophy (calculated by number of cells per 16 mm^2). Images were taken with Nikon eclipse e800 microscope (Nikon Corporation, Tokyo, Japan) and analyzed with Image-Pro Plus software (Media Cybernetics, Bethesda, USA).

Global gene expression by microarray

RNA extraction: Total RNA was isolated, using the Trizol RNA isolation method (Invitrogen, USA), with slight modifications to increase the RNA purity and yield. Briefly, adipose tissue (500 mg) was homogenized in 0.5 ml Trizol reagent in 2ml Eppendorf tube and additional 0.5 ml Trizol was added and vortexed. Fat layer was removed after centrifugation of the homogenate for 10,000 *g* at 4°C for five minutes. Later steps were performed according to manufacturer's instructions. Chloroform wash was repeated three times. RNA was precipitated with isopropanol and washed with 75 per cent alcohol. After washing, alcohol was removed, and tubes were centrifuged at 2000 *g* for two minutes at room temperature to remove the traces of ethyl alcohol (which was the major contaminant, affecting the cRNA amplification step, during microarray standardization) and dissolved in autoclaved Milli-Q water. RNA concentration and quality were determined by reading the absorbance

at 230, 260, 270, 280 and 320 nm. Along with the RNA concentration (260 nm), protein contamination (260 nm/280 nm, ratio≥2), phenol contamination (260 nm/270 nm, ratio ≥1.2) and salt contamination (260 nm/230 nm, ratio ≥2) were checked. RNA integrity was confirmedon one per cen tagarose gel electrophoresis.

Target preparation: Total RNA (100 ng) was taken and converted to antisense cRNA (complementary RNA) by *in vitro* transcription through single stranded and double stranded cDNA steps, using WT-cDNA synthesis and amplification kit (Affymetrix, USA). From antisense cRNA, single-stranded cDNA was synthesized, and cRNA was hydrolyzed using the same kit. cDNA was fragmented and labelled with phycoerythrin using WT Terminal Labelling Kit (Affymetrix, USA).

Hybridization and scanning: Hybridization cocktail containing the labelled probes was prepared using GeneChip Hybridization, wash and stain kit (Affymetrix, USA). Two hundred microliters of hybridization cocktail were loaded on to Rat Gene 1.0 ST Arrays (Affymetrix, California, USA) and incubated for 18 h at 45°C and 60 rpm in hybridization oven. After incubation, arrays were washed and scanned. Four chips were used for hybridization (two for lean and two for obese animals). The Rat Gene 1.0 ST Array consisted of 722,254 probes representing 27,342 well-annotated genes (covered 99.98% coverage of NM sequences present in April 3, 2007, RefSeq database).

Data analysis: Chip images were checked for artefacts and quality control analyses were performed. CEL files generated were pre-processed by robust multiarray analysis and normalized by quantile method, using Array Star software (DNASTAR, Inc., Wisconsin, USA). To determine the differentially expressed genes, probeset intensity values of two lean rat samples were averaged and compared against the averaged intensity values of two obese samples. Genes with more than two-fold differentially regulated (up- or downregulation) were taken into consideration. All the microarray work was done in compliance with MIAME (Minimum Information About a Microarray Experiment) guidelines[7] and submitted to Gene Expression Omnibus (GEO, Accession number GSE58575).

Validation of relative gene expression by reverse transcription polymerase chain reaction (RT-PCR): Ten micrograms of RNA was used to synthesize first strand cDNA. The reverse transcription (RT) reaction was carried out by incubating RNA with oligo dT primer (Sigma, USA) and Moloney murine leukaemia virus reverse transcriptase (Finnzymes, Espoo, Finland) at 37°C for 60 min.Total reaction volume used in RT was 20 µl. An aliquot of cDNA was amplified in a 20 µl reaction mixture. Polymerase chain reaction (PCR) conditions were as follows: denaturation at 94°C for one minute, annealing at 60 - 64°C for 45 sec and polymerization for 70°C for one minute with DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). A final extension was carried out at 70°C for seven minutes. The amount of RNA and the annealing temperature for different genes were standardized for linearity. Sequences of primers (self designed) used for amplification were stearoyl-CoA desaturase 1 (SCD1-NM139192.2): forward primer (FP)-5'-CGGC CCACATGCTCCAAGAGATCT-3' and reverse primer (RP) - 5'-GTCTTCTTCCAGATAGAGG GGCACC-3', malic enzyme (ME1-NM012600.2): FP-5'-ATAAAGTGACCAAGGGCCGTGCG-3' and RP-5'-ACAGGCCACTACCCCAAGAGCAA-3', lysosomal lipase (LIPA-NM012732.3): FP-5'-CGGTATCCAAAGAGACGGCTGCA-3' and RP-5'- ACAGGCCTCGATAAATTAGGGCCT-3', macrophage expressed gene (MPEG-NM022617.1): FP-5'-TCTTGCTGGTGAATGCCTGGGAC-3' and RP-5'-ATACCCGGGTCTCTGAGAGGCTTG-3', beta-3 adrenergic receptor (β3-AR-NM013108.1): FP-5'-ACTTTCGCGACGCCTTCCGT-3' and RP-5'-AGCCATCAAACCTGTTGAGCGGT-3', myotilin (MYOT-NM001106148.1): FP-5'-GATGTCACAGCCCGTCCAAACCA-3' and RP-5'-AGCTGCCAGACGCTGAAACTCTC-3', insulin-like growth factor binding protein 5 (IGFBP5-NM012817.1): FP-5'-GCATTTCCGAGCTGAAGGCCGA-3' and RP-5'-AGGGGCCTTGGTCAGATTCCTGT-3', calnexin (NM172008.2): FP-5'-GCAGCGACCTATGATTGACAACC-3' and RP-5'-GCTCCAAACCAATAGCACTGAAAG-3' (Bio-Serve, India). Calnexin was amplified as an internal control. After amplification, 8 µl of reaction mixture was subjected to agarose gel electrophoresis (2%) in Tris-acetate ethylenediaminetetraacetic acid buffer (pH 8.2). The ethidium bromide-stained bands were visualized by a ultraviolettransilluminator and analyzed densitometrically, using Quantity One software program (Bio-Rad, version 4.4.0, USA).

Statistical analysis: Data were analyzed by SPSS 11.0 software (Chicago, USA). For physical and TOBEC parameters, Student's t-test was used for calculation of significant changes (n=6). For gene expression

validation by semi-quantitative RT-PCR, Student's t-test was used for the calculation of significant changes (n=4). For parameters, where homogeneity of variance was significant, log-transformed data were used for Student's t-test or non-parametric Mann-Whitney test was used. All data were presented as a mean ± standard error of mean.

Results

Physical parameters, body composition and adipose tissue histology: Four month old, male WNIN/Ob obese rats had significantly elevated body weights as compared with those of age- and sex-matched lean rats (Table I). Weights of all visceral adipose tissue depots (retroperitoneal, omental and epididymal) and body fat percentage were significantly (P<0.001) elevated in obese rats as compared with those of lean rats (Table I). LBM and FFM were significantly lower in WNIN/Ob obese rats as compared with their age- and sex-matched lean rats (Table I).

H&E staining showed increased adipocyte size (hypertrophy) in obese rats as compared with that of lean rats (Fig.1). In adipose tissue of obese rats it also showed infiltration of inflammatory cells. No such changes were seen in the lean adipose tissue.

Profiles of adipose tissue gene expression: Microarray analysis revealed that 1980 probe sets were differentially regulated (more than two-fold) in adipose tissue of WNIN/Ob obese rats as compared with that of age- and sex-matched lean rats (1017 probe sets were downregulated and 963 probe sets were upregulated). Of the 1017 downregulated probe sets, 359 probe sets coded for specific, known proteins. Three hundred and sixty five probe sets (approximately 35% of the downregulated probe sets) were specific for non-coding RNA. Small nucleolar RNA (SnoRNA) made a major percentage of the non-coding RNA and also the highly downregulated genes in adipose tissue of WNIN/Ob obese rats. MicroRNAs (miRNA) were also present in the downregulated non-coding RNA genes. Of the 963 upregulated probe sets, 787 probe sets had a code for specific, known proteins. Remaining probe sets had codes for non-coding RNA and hypothetical proteins.

From the down- (359) and upregulated (787) genes specific for known proteins, genes were selected and segregated into groups based on their cellular function and information as described in NetAffx and literature (Table II). Groups included genes involved in lipid and carbohydrate metabolism, electron transport chain, oxidative stress, transport, receptors and transcription factors. Majority of the upregulated genes (from 787 probe sets) were related to immune system and selected genes in this category are reported in Table III. Majority of the downregulated genes (from 359 probe sets) coded for structural proteins (Table IV). Other downregulated probe sets included SnoRNA, olfactory receptors, vomeronasal receptors and spetex proteins (data not shown). Predicted cellular, metabolic

Table I. Physical parameters of four-month-old, male WNIN/Ob lean and obese rats

Parameters	Lean rats	Obese rats
Body weight (g)	305±12	534±21**
Retroperitoneal adipose tissue (g)	1.9±0.12	17.9±1.2***
Omental adipose tissue (g)	0.5±0.3	2.0±0.2***
Epididymal adipose tissue (g)	2.2±0.2	13.0±0.1***
Fat (%)	10±1.0	56±1.0***
Fat free mass (g)	164±7	70±5***
Lean body mass (g)	300±10	225±10***

Values represent means±SEM of six rats per group. *P<0.05, **P<0.01; ***P<0.001 compared to lean rats

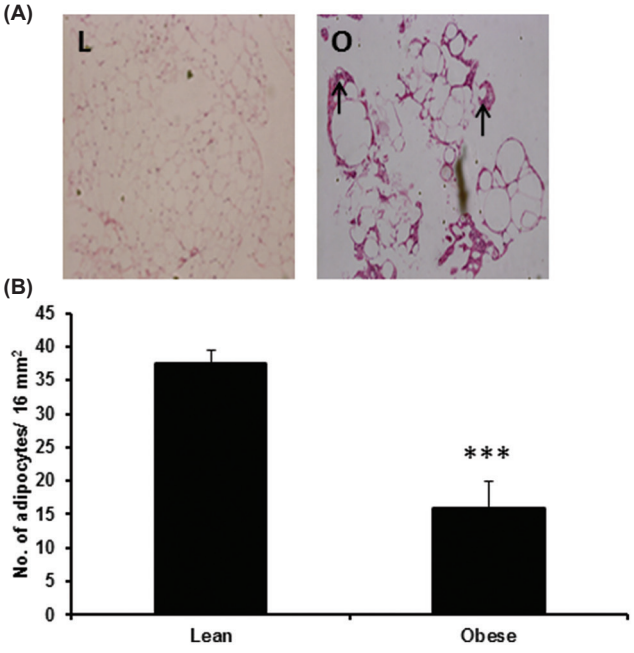

Fig. 1. Adipose tissue histology in four month old, male WNIN/Ob lean and obese rats. (**A**) Photographs of H&E stained retroperitoneal adipose tissue to study adipocyte size and inflammation. 'L' indicates lean sample and 'O' indicates obese sample. Arrow marks indicate the presence of infiltrated inflammatory cells. (**B**) Adipocyte size calculated as a number of cells/16 mm^2 (n=4/phenotype). *** indicates P< 0.001 compared to lean rats.

Table II. List of the selected differentially regulated genes in retroperitoneal adipose tissue of four-month-old, male WNIN/Ob obese rats

Gene symbol	Gene name	Fold change
	Fatty acid/TG synthesis	
Upregulated		
Scd1	Stearoyl-CoA desaturase 1	4.51
Elovl 1	Fatty acid elongase 1	2.44
Elovl 5	Fatty acid elongase 5	2.27
Elovl 6	Fatty acid elongase 6	13.0
Acaca	Acetyl CoA carboxylase alpha	3.88
Acly	ATP citrate lyase	4.61
Agpat3	1-acylglycerol-3-phosphate O-acyltransferase 3	2.55
Agpat5	1-acylglycerol-3-phosphate O-acyltransferase 5	2.49
	Fatty acid/TG breakdown	
Upregulated		
Lipa	Lipase A	11.3
Pnpla3	Patatin-like phospholipase domain containing 3	5.09
Acaa2	Acetyl-coenzyme A acyltransferase 2	2.09
Hadhb	Hydroxy acyl- CoA dehydrogenase/3ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional enzyme)	2.21
Cpt1b	Carnitine palmitoyltransferase 1b	2.24
Crot	Carnitine-O-octanoyl transferase	2.04
Gk	Glycerol kinase	2.68
Downregulated		
Lipe	Hormone-sensitive lipase	-2.37
	Cholesterol synthesis	
Upregulated		
Star	Steroidogenic acute regulatory protein	4.11
Insig1	Insulin-induced gene 1	2.71
Dhcr7	7-dehydrocholesterol reductase	2.39
Fdft1	Farnesyl-diphosphate farnesyltranserase 1	2.10
Sc4mol	Sterol-4-methyl-oxidase-like	3.12
Idi1	Isopentenyl-diphosphate delta isomerase 1	2.89
Hmgcs1	3-hydroxy-3-methylglutaryl-coenzyme A synthase	3.34
Lss	Lanosterol synthase	2.24
Cyp51	Lanosterol 14α-demethylase	3.43
Sqle	Squalene monooxygenase	3.69
	Glycogen metabolism	
Upregulated		
Gys2	Glycogen synthase 2	2.41
Downregulated		
Pygm	Glycogen phosphorylase, muscle	-14.3
Phkg1	Phosphorylase kinase, gamma 1	-3.45

Contd...

Gene symbol	Gene name	Fold change
	Glycolysis/TCA cycle/Gluconeogenesis	
Upregulated		
Mdh1	Malate dehydrogenase 1, NAD (soluble)	2.05
Mdh2	Malate dehydrogenase 2, NAD (mitochondrial)	2.03
Pdhb	Pyruvate dehydrogenase (lipoamide) beta	2.37
Dlat	Dihydrolipoyltransacetylase	2.80
Eno1	Enolase 1	2.17
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	2.02
Downregulated		
Eno3	Enolase 3	-7.14
Pgam2	Phosphoglycerate mutase 2 (muscle)	-4.16
Pfkm	Pyruvate kinase (muscle)	-2.94
Fbp2	Fructose-1,6-bisphosphatase 2	-2.14
Ldha	Lactate dehydrogenase A	-3.33
	HMP pathway	
Upregulated		
Taldo1	Transaldolase 1	3.16
G6pd	Glucose-6-phosphate dehydrogenase	3.32
	Adipokines	
Lep	Leptin	2.93
	Receptors/nuclear receptors/transcription factors	
Upregulated		
Mc5r	Melanocortin-5 receptor	2.33
P2ry1	Purinergic receptor P2Y1	2.01
P2ry10	Purinergic receptor P2Y10	2.72
P2r×4	Purinergic receptor P2X4	2.65
Ptger2	Prostaglandin E receptor 2	2.48
Ptger4	Prostaglandin E receptor 4	2.18
Oxtr	Oxytocin receptor	2.12
C3ar1	Complement component 3a receptor 1	3.25
Ptafr	Platelet-activating factor receptor	2.42
Adora1	Adenosine A1 receptor	2.09
Downregulated		
Adrb3	Beta-3 adrenergic receptor	-3.03
Esr1	Estrogen receptor 1	-4.06
Ppara	Peroxisome proliferator-activated receptor alpha	-2.06
Nr4a1	Nuclear receptor subfamily 4, Group A, member 1	-2.51
Ar	Androgen receptor	-2.25
	Cellular stress	
Upregulated		
Gsr	Glutathione reductase	2.07
Gpx1	Glutathione peroxidase 1	3.34

Contd...

Gene symbol	Gene name	Fold change
Mt1a	Metallothionein 1A	2.37
Mt2a	Metallothionein 2A	3.48
Cyba	Cytochrome b-245, alpha polypeptide	2.37
Downregulated		
Gpx3	Glutathione peroxidase 3	-2.35
Hspb6	Heat-shock protein b6	-4.01
Hspb8	Heat-shock protein b8	-2.02
	Apoptosis	
Upregulated		
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	2.21
Aifm2	Apoptosis- inducing factor, mitochondrial	2.09
Siva1	Apoptosis-inducing factor	2.59
	Cell cycle	
Upregulated		
Pttg1	Pituitary-tumour-transforming gene 1	2.72
Pcna	Proliferating cell nuclear antigen	2.07
Ccna2	Cyclin A2	2.96
Ccnb1	Cyclin B1	3.16
Ccnb2	Cyclin B2	3.18
Ccne1	Cyclin E1	2.03
Ccne2	Cyclin E2	2.17
Ccnf	Cyclin F	3.14
	Proteasome degradation	
Upregulated		
Ctsa	Cathepsin A	2.25
Ctsc	Cathepsin C	2.65
Ctsd	Cathepsin D	3.39
Ctsk	Cathepsin K	2.20
Ctss	Cathepsin S	2.49
Ube2a	Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	2.26
Ube2f	Ubiquitin-conjugating enzyme E2F (putative)	2.39
Ufc1	Ubiquitin-fold modifier conjugating enzyme 1	2.01
Ufm1	Ubiquitin-fold modifier 1	3.07
Uhrf1	Ubiquitin-like with PHD and ring finger domains 1	2.13
Usp12	Ubiquitin-specific peptidase 12	2.87
Usp18	Ubiquitin-specific peptidase 18	4.36
Psma1	Proteasome (prosome, macropain) subunit, alpha type 1	2.03
Psma3	Proteasome (prosome, macropain) subunit, alpha type 3	2.33
Psmb4	Proteasome (prosome, macropain) subunit, beta type 4	2.04
Psmb6	Proteasome (prosome, macropain) subunit, beta type 6	2.32
Downregulated		
Ctse	Cathepsin E	-2.45

Contd...

Gene symbol	Gene name	Fold change
	Cell signalling	
Upregulated		
Adcy 7	Adenylate cyclase 7	2.54
Tgb1	Transforming growth factor b1	2.04
Map2k1	Mitogen-activated protein kinase kinase 1	2.51
Cav2	Caveolin 2	2.17
Downregulated		
Igf1	IGF-1	-2.33
Igfbp3	IGF-binding protein 3	-2.10
Igfbp5	IGF-binding protein 5	-6.50
Bmp4	Bone morphogenetic protein 4	-2.38
Bmp7	Bone morphogenetic protein 7	-2.57
	MicroRNA	
Downregulated		
H19, *Mir675*	H19, imprinted maternally expressed transcript (non-protein coding)	-4.74
Mir29c		-2.16
Mir143		-2.85
Mir145		-3.33
Mir23b		-2.94
Mir27a		-2.04
Mir297		-3.33
Mir7a2		-2.08

The fold changes were determined by microarray hybridization using pooled RNA samples from lean and obese rats (two animals from each phenotype). Total four chips were used (two each for lean and obese rats). Intensity values of probe sets from lean sample chips (two chips) were averaged and compared against the average values from obese sample chips (two chips). Genes that are differentially regulated by more than two-fold (up- and downregulated) were taken and grouped on the basis of their cellular function (information from NetAffx and literature was used to determine cellular function of each gene). NAD, nicotinamide adenine dinucleotide; IGF-1, insulin-like growth factor-1

and physiological changes based on the observed differentially regulated genes in the adipose tissue of WNIN/Ob obese rats are given in Table V. Predicted metabolic changes that can lead to the development of obesity and its associated comorbidities are depicted in Fig. 2. Some of the selected candidate genes that are well known to cause obesity and associated comorbidities are given in Table VI.

Differential expression of genes involved in lipid metabolism: Genes coding for enzymes involved in fatty acid biosynthesis (*Acaca*, *Acly*), elongation (*Elovl 1*, *Elovl 5* and *Elovl 6*), desaturation, (*Scd1*), TG (*Agpat3* and *Agpat 5*) and cholesterol biosynthesis (*Dhcr7*, *Fdft1*, *Sc4mol*, *Idi1*, *Hmgcs1* and *Lss*) were upregulated in the adipose tissue of WNIN/Ob obese rats as compared with those of age- and sex-matched lean rats (Table II). Genes coding for enzymes involved in TG breakdown (*Lipa*, *Pnpla3*) and beta-oxidation of fatty acids (*Hadhb*, *Cpt1b* and *Crot*) were elevated whereas TG breakdown enzyme, hormone-sensitive lipase (HSL) (*Lipe*) gene was lowered in the adipose tissue of obese rats (Table II).

Differential expression of genes involved in carbohydrate metabolism and electron transport chain: Glycogen synthesizing enzyme expression (*Gys2*) was upregulated, whereas glycogen breakdown enzyme expressions (*Pygm*, *Phkg1*) were downregulated in the adipose tissue of WNIN/Ob obese rats as compared with those of lean rats (Table II). Expression of genes coding for glycolytic enzymes was differentially regulated in adipose tissue of obese rats. *Eno1* and *Gapdh* gene expressions were higher, whereas *Eno3*, *Pgam2* and *Pfkm* gene expressions were lower in the adipose tissue of WNIN/Ob obese rats as compared with those of lean rats (Table II). Genes coding for

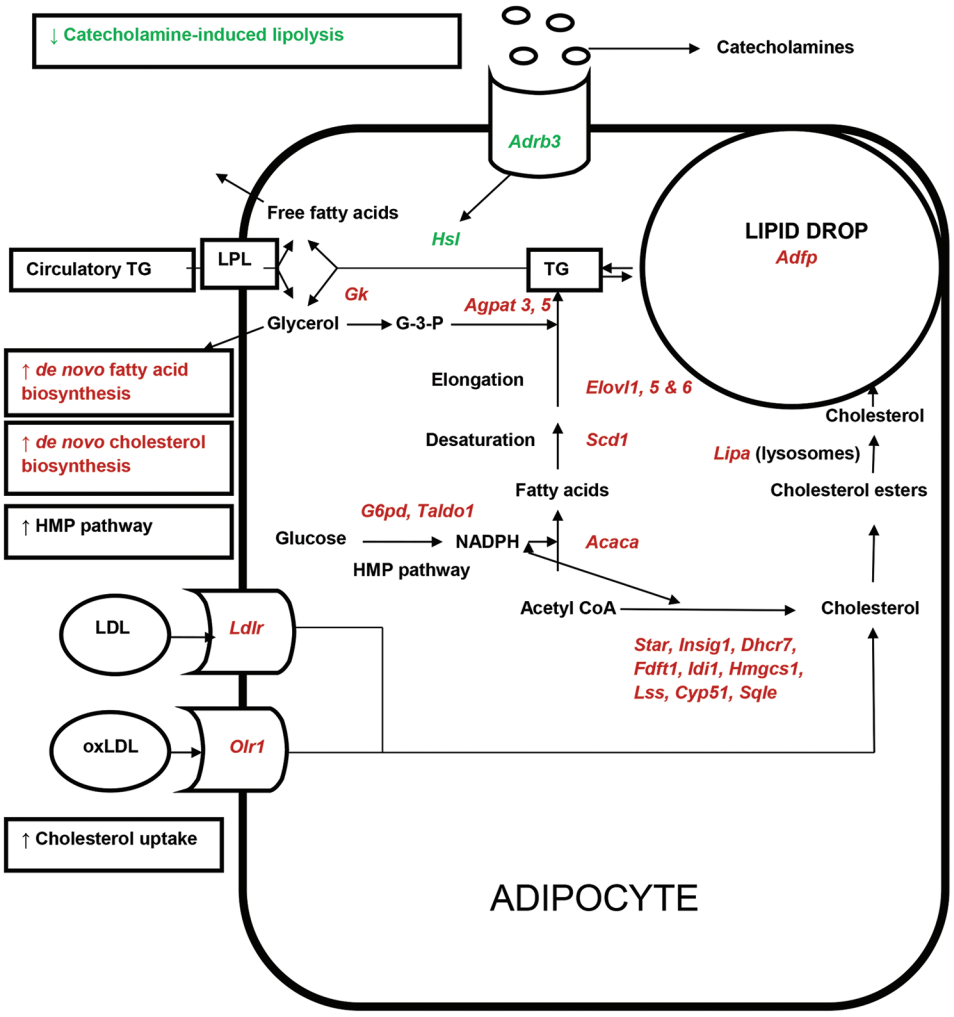

Fig. 2. Predicted metabolic changes in adipocyte of WNIN/Ob obese rat based on gene expression. Genes, pathways and metabolic processes indicated in green colour(downregulated) and red colour (upregulated). *Acaca*, Acetyl CoA carboxylase alpha; *Adfp*, Adipophilin; *Adrb3*, Beta-3 Adrenergic receptor; *Agpat3,5*, 1-acylglycerol-3-phosphate O-acyltransferase 3, *5*; *Cyp51*, Lanosterol 14α-demethylase; *Dhcr7*, 7-dehydrocholesterol reductase; *Elovl 1,5,6*, Fatty acid elongase 1,5,6; *Fdft1*, Farnesyl-diphosphate farnesyltransferase 1, *Gk*, Glycerol kinase; *Hmgcs1*, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1; *Hsl*, Hormone-sensitive lipase; *Idi1*, Isopentenyl–diphosphate delta isomerase 1; *Insig1*, Insulin-induced gene 1; *Ldlr*, low-density lipoprotein receptor; *Lipa*, Lipase A; *Lss*, Lanosterol synthase; *Olr1*, Oxidized-low-density lipoprotein receptor; *Scd1*, Stearoyl-CoA desaturase 1; *Star*, Steroidogenic acute regulatory protein; *Sqle*, Squalene monooxygenase; *Taldo1*, Transaldolase 1. LPL, Lipoprotein lipase; LDL, Low-density lipoprotein; OxLDL, Oxidized LDL; TG, Triglycerides.

enzymes involved in citric acid cycle (*Mdh1*, *Mdh2*, *Pdhb*, and *Dlat*) andhexose monophosphate (HMP) pathway (*G6pd* and *Taldo1*) and genes coding for proteins involved in electron transport chain (*Uqcrc2* and *Cox7a2*) and uncoupling (*Ucp2*) were elevated in the adipose tissue of obese rats as compared with those of lean rats.

Differential expression of genes coding for receptors/ transcription factors: Genes coding for various receptors including melanocortin receptor (*Mc5r*), purinergic receptors (*P2rx4*, *P2ry1* and *P2ry10*), Prostaglandin E receptors (*Ptger2* and *Ptger4*), complement receptor (*C3ar1*), oxytocin receptor (*Oxtr*), adenosine receptor (*Adora1*) and platelet-activating factor receptor (*Ptafr*) were upregulated in the the retroperitoneal adipose tissue of WNIN/Ob obese rats as compared with those of lean rats (Table II). Genes coding for sex hormone receptors (*Esr1* and *Ar*), adrenergic receptors (*Adrb3*), peroxisome-proliferator-activated receptor alpha (*Ppar1a*) and nuclear hormone receptors (*Nr4a1*) were lowered in the adipose tissue of WNIN/Ob obese rats as compared with those of lean rats (Table II).

Table III. List of selected upregulated genes related to immune system in retroperitoneal adipose tissue of four month old, male, WNIN/Ob lean and obese rats

Gene symbol	Gene name	Fold change
Ccl9	Chemokine (C-C motif) ligand 9	12.6
Ccr1	Chemokine (C-C motif) receptor 1	4.07
Ccr2	Chemokine (C-C motif) receptor 2	4.77
Ccr5	Chemokine (C-C motif) receptor 5	4.75
CD166	ALKAM	5.88
CD18	Itgb2	10.5
CD180	Lymphocyte antigen 64	5.28
CD204	Macrophage scavenger receptor 1	5.50
CD244	NK cell receptor 2B4	4.90
CD4	Cluster of differentition 4	3.54
CD51	Integrin, alpha V (Itgav)	5.28
Clec4a3	C-type lectin domain family 4, member A3	5.54
Clec5A	C-type lectin domain family 5, member A	12.9
Clec7A	C-type lectin domain family 7, member A	11.9
Klra7	Killer cell lectin-like, subfamily A, receptor 7	3.43
Klra17	Killer cell lectin-like, subfamily A, receptor 17	6.29
Klra2	Killer cell lectin-like, subfamily A, receptor 2	3.50
Klra5	Killer cell lectin-like, subfamily A, receptor 5	3.30
Ly86	Lymphocyte antigen 86	4.19
Lyz2	Lysozyme 2	15.1
Mrc1	Macrophage mannose receptor c-type lectin	3.04
Mpeg1	Macrophage-expressed gene 1	6.55

The fold changes were determined by microarray hybridization using pooled RNA samples from lean and obese rats (two animals from each phenotype). Total four chips were used (two each for lean and obese rats). Intensity values of probe-sets from lean sample chips (two chips) were averaged and compared against the average values from obese sample chips (two chips). Genes that are differentially regulated by more than two-fold (up- and downregulated) were taken and grouped on the basis of their cellular function (information from NetAffx and literature was used to determine cellular function of each gene). ALKAM, activated-leucocyte cell adhesion molecule; NK, natural killer; Itgb2, integrin b2

Differential expression of genes involved in cellular stress: Expression of genes involved in defence against oxidative stress (*Gsr*, *Mt1a*, *Mt2a* and *Cyba*) was elevated while genes coding for protein involved in scavenging of free radicals (*Gpx3*) and heat shock proteins (*Hspb6* and *Hspb8*) were downregulated in the adipose tissue of obese rats.

Differential expression of genes involved in apoptosis and cell proliferation: Majority of the genes related to apoptosis (*Casp1*, *Bcl2l11*, *Aifm2* and *Siva1*) and cell cycle (*Ccna2*, *Ccnb1*, *Ccnb2*, *Ccne1*, *Ccne2* and *Ccnf2*) were elevated in the adipose tissue of obese rats when compared to respective lean rats (Table II).

Differential expression of genes involved in protein degradation: Most of the genes that are involved in protein degradation including cathepsin genes (*Ctsa*, *Ctsc*, *Ctsd*, *Ctsk* and *Ctss* except *Ctse*), proteasome subunits (*Psma1*, *Psma3*, *Psmb4* and *Psmb6*), ubiquitin conjugation (*Ube2a* and *Ube2f*), ubiquitin-specific peptidases (*Usp12* and *Usp18*) and ubiquitin-fold modifier proteins (*Ube2a* and *Ube2f*) were elevated in the adipose tissue of WNIN/Ob obese rats as compared with those of lean rats (Table II).

Differential expression of genes involved in cell signalling: Genes coding for adenylate kinase (*Adcy7*), transforming growth factor (*Tgb1*), mitogen-activated protein kinase kinase (*Map2k1*) and caveolin (*Cav2*) were elevated, whereas insulin-like growth factor signalling (*Igf1*, *Igfbp3* and *Igfbp5*) and bone morphogenetic protein signalling (*Bmp4* and *Bmp7*)

Table IV. List of selected downregulated genes related to cytoskeleton, cell-cell adhesion and extracellular matrix in four month old, male WNIN/Ob obese rats

Gene symbol	Gene name	Fold change
Acta1	Actin, alpha 1, skeletal muscle	-7.05
Actg2	Actin gamma 2, smooth muscle, enteric	-4.17
Actn2	Actinin alpha 2	-6.23
Actn3	Actinin alpha 3	-8.78
Cdh1	E-cadherin or uvomorulin	-2.57
Cdh19	Cadherin 19, type 2	-2.03
Cnn1	Calponin 1, basic, smooth muscle	-2.90
Des	Desmin	-2.32
Dmd	Dystrophin	-3.53
Dmpk	Myotonic dystrophy protein kinase	-2.33
Drp2	Dystrophin-related protein 2	-2.15
Fndc1	Fibronectin type 3 domain containing 1	-2.42
Krt15	Keratin 15	-2.52
Krt19	Keratin 19	-4.95
Krt7	Keratin 7	-2.38
Krtap31-1	Keratin-associated protein 311	-2.10
Myh1	Myosin heavy chain 1, skeletal muscle, adult	-11.3
Myh2	Myosinheavy chain 2, skeletal muscle, adult	-8.85
Myh4	Myosin heavy chain 4, skeletal muscle	-9.52
Myh7	Myosin heavy chain 7, cardiac muscle, beta	-3.76
Myl1	Myosinlight chain 1, alkali; skeletal, fast	-9.46
Myl3	Myosin light chain 3, alkali; ventricular, skeletal, slow	-4.67
Myot	Myotilin	-7.85
Myoz1	Myozenin 1	-5.86
Myoz2	Myozenin 2	-3.47
Nexn	Nexilin (F-actin-binding protein)	-5.17

The fold changes were determined by microarray hybridization using pooled RNA samples from lean and obese rats (four animals from each phenotype). Total four chips were used (two each for lean and obese rats). Intensity values of probe-sets from lean sample chips (two chips) were averaged and compared against the average values from obese sample chips (two chips). Genes that are differentiallyregulated by more than two-fold (up- and downregulated) were taken and grouped on the basis of their cellular function (information from NetAffx and literature was used to determine cellular function of each gene). E-cadherin, epithelial cadherin

genes were downregulated in the adipose tissue of WNIN/Ob obese rats when compared to their respective control lean rats (Table II).

Differential expression of genes coding for microRNA: Genes coding for various miRNA (*Mir675*, *Mir29c*, *Mir143*, *Mir145*, *Mir23b*, *Mir27a*, *Mir7a2* and *Mir297*) were downregulated in the adipose tissue of WNIN/Ob obese rats as compared with those of lean rats (Table II).

Differential expression of genes coding for proteins involved in formation of cytoskeleton, cell–to-cell interactions and extracellular matrix: Majority of the downregulated genes in retroperitoneal adipose tissue of WNIN/Ob obese rats coded for structural proteins that were involved in the formation of cytoskeleton, cell-to-cell interactions and ECM (Table IV). Genes related to cytoskeleton included actin-related proteins (such as *Acta1*, *Actg2*, *Nexn* and *Xirp1*), myosin-related proteins (such as *Myh1*, *Myh2*, *Myl1* and *Myl2*), desmin (*Des*), proteins involved in the formation of cell junctions (*Cdh1* and *Cdh19*), ECM proteins such as keratins (*Krt7*, *Krt15* and *Krt19)* and collagen *(Col19a1*) were downregulated in adipose tissue of WNIN/Ob obese rats as compared with those of lean rats (Table IV).

Table V. Predicted cellular, metabolic and physiological changes in retroperitoneal adipose tissue of four month old, male WNIN/Ob obese rat based on the differentially regulated genes

↑ Fatty acid biosynthesis
↑ Triglyceride accumulation (endogenous synthesis and exogenous uptake)
↓ Catecholamine induced-lipolysis
↑ Cholesterol accumulation (endogenous synthesis and exogenous uptake)
↑ Cholesterol uptake
↑ Glycogen synthesis
↑ HMP pathway
↑ Leptin synthesis and secretion
↑ Molecular stress (protein misfolding)
↑ Oxidative stress
↑ Cellular proliferation
↑ Apoptosis
↓ Preadipocyte differentiation
↑ Protein degradation
Altered cytoskeleton
Altered cell-cell interactions
Altered extracellular matrix composition
Altered IGF-1 signalling
↑ Infiltration of macrophages, NK cells and T-cells

↑, increase; ↓, decrease; IGF-1, insulin-like growth factor-1; NK, natural killer; HMP, hexose monophosphate

Genes coding for proteins involved in remodelling of ECM such as matrix metalloproteinases (*Mmp12* and *Mmp19*) and tissue inhibitors of MMP (*Timp1*) were elevated in the adipose tissue of WNIN/Ob obese rats as compared with those of lean rats (Table II).

Differential expression of genes involved in immune system: Immune system-related genes made up to most of the upregulated genes in the adipose tissue of WNIN/Ob obese rats. These included clusters of differentiation (*CD*) genes, chemokine ligands (*Ccl*) and receptors (*Ccr*), natural killer cell receptors (*klra*), C-type lectin receptors (*Clec*) and integrins (Table III).

Validation of microarray data: Four upregulated genes (*Scd1*, *Mpeg1*, *Me1* and *Lipa*) and three downregulated genes (*adrb3*, *Igfbp5* and *Myot*) were selected for validation of microarray data by RT-PCR. All four upregulated genes showed similar observation as observed in microarray although the extent of fold change was different from microarray (*Scd1*, 3.6 vs. 4.52; *Mpeg*, 2.64 vs. 6.55; *Me1*, 2.17 vs. 4.6; *Lipa*, 4.0 vs. 11.35) (data not shown). Of the three downregulated genes, one showed significant decrease as observed in microarray (*adrb3*, 1.9 vs. 3.0). The fold change for other two downregulated genes (*Igfbp5* and *Myot*) could not be quantified as their expression was very low in lean samples and not detectable in obese samples under the given conditions (data not shown).

Discussion

Elevation of genes coding enzymes involved in TG biosynthesis predict the increased re-esterification of fatty acids to TG in the adipose tissue of WNIN/Ob obese rats. Endogenously synthesized fatty acids may also contribute to adipocyte TG accumulation, as fatty acid biosynthesis genes were also elevated in this model. Elevated expression of HMP pathway genes (*G6pd* and *Taldo1*) in the adipose tissue of WNIN/Ob obese rats could provide the required reducing equivalents for the increased fatty acid and cholesterol biosynthesis. Elevation of *Scd1* and *Elovl 6* genes in the adipose tissue of obese rats suggests that fatty acid desaturation and elongation are increased in the adipose tissue of WNIN/Ob obese rats[8,9].

Decreased catecholamine-induced lipolysis in adipose tissue is one of the well-characterized observations in obesity[10]. This is due to decreased expression of beta-adrenergic receptors and HSL[10]. In line with the observations in obese humans and animal models, WNIN/Ob obese rats had lowered expression of HSL gene expression which might have resulted in the increased fat accumulation in this model.

β3-AR gene expression is lower in the adipose tissue of obese rodent models, and its activation leads to fat loss and amelioration of obesity-induced insulin resistance[11]. Orphan nuclear receptor NR4A1 inhibits adipocyte differentiation, and it is underexpressed in the adipose tissue of obese Zucker rats and *ob*/*ob* mice[12]. Estrogen receptor alpha is implicated in the development of obesity, as knocking off this gene results in the increased adipose tissue mass[13]. AR knock-out mice develop late-onset obesity due to decreased energy expenditure[14]. WNIN/Ob obese rats had lower expression of genes coding for β3-AR, NR4A1, ERα and AR in the adipose tissue suggesting the role of these receptors in the development of obesity and insulin resistance in this model.

Glutathione peroxidases 1(GPx1) is expressed in the cytoplasm of majority of the cells with greater specificity towards hydrogen peroxide. GPx3, an extracellular enzyme, is the only isoform present in plasma and

Table VI. List of selected candidate genes differentially regulated in retroperitoneal adipose tissue of WNIN/Ob obese rats

Category	Gene symbol	Gene name	Metabolic abnormality
Fattyacid/TG metabolism	*Scd1* (↑)	Stearoyl-CoA desaturase 1	Obesity, Insulin resistance
	Elovl 6 (↑)	Fatty acid elongase 6	Insulin resistance
	Lipe (↑)	Hormone-sensitive lipase	Catecholamine-induced lipolysis
Receptors/ transcription factors	*Adrb3* (↓)	β3-Adrenergic receptor	Catecholamine-induced lipolysis, Obesity, Insulin resistance
	Esr1 (↓)	Estrogen receptor α	Obesity
	Ar (↓)	Androgen receptor	Obesity
	Nr4a1 (↓)	Nuclear receptor subfamily group A, member 1	Obesity, Insulin resistance
	Adora1 (↑)	Adenosine A1 receptor	Hyperleptinemia
	P2rY1 (↑)	Purinergic receptor	Hyperleptinemia
Cellular stress	*Gpx3* (↓)	Glutathione peroxidase 3	Systemic oxidative stress
Cell signalling	*Igf1* (↓)	Insulin-like growth factor 1	Obesity
	Igfbp3 (↓)	IGF-binding protein 3	Obesity
	Igfbp5 (↓)	Insulin-like growth Factor-binding protein 5	Obesity
	Bmp4 (↓)	Bone morphogenetic protein 4	Obesity
Protein degradation	*Ctss* (↑)	Cathepsin S	Obesity
	Ctsk (↑)	Cathepsin K	Obesity
	Ctsl (↑)	Cathepsin L	Obesity
Immune system	*Ccr2* (↑)	Chemokine (c-c motif) receptor 2	Macrophage infiltration, Insulin resistance
Extracellular matrix	*Mmp12* (↑)	Matrix metalloproteinase 12	Obesity
	Mmp19 (↑)	Matrix metalloproteinase 19	Obesity
	Timp1 (↑)	Tissue inhibitor of matrix metalloproteinases	Obesity
Non-coding RNA	*Mir143/ Mir145* (↓)	MicroRNA 143/145	Obesity, Insulin resistance
	Mir27a	MicroRNA 27a	Obesity
	SnoRNA (↓)	Small-nucleolar RNA	Obesity, Fat-induced cell death
Olfactory receptors	*Olr1434* (↑)	Olfactory receptor 1434	Obesity

Candidate genes with respect to metabolic abnormalities were selected, if they satisfy any one of the given conditions: (*i*) transgenic/ knock-out studies; (*ii*) association studies; and (*iii*) similar observations in human obesity or animal models of obesity. ↑, increase; ↓, decrease; IGF, insulin-like growth factor

involved in reducing systemic oxidative stress[15]. Studies on obese humans and animals showed lowered plasma GPx3, due to the decreased expression of GPx3 in the adipose tissue[15]. GPx3 expression was low in adipose tissue of WNIN/Ob obese rats as observed in other obese animal models suggesting the possibility of increased systemic oxidative stress in this model. Elevated expression of GPx1 and glutathione reductase may be a protective mechanism against the elevated local oxidative stress in the adipose tissue of obese rats. *Cathepsin S*, *K* and *D* gene expressions were shown to be elevated in the adipose tissue of obese rats and animal models of obesity[16] which are also elevated in the adipose tissue of WNIN/Ob obese rats.

MMPs are one important class of enzymes involved in the remodelling of ECM. The expression of *MMP12* and *TIMP1* genes is higher in the adipose tissue of diet-induced obese mice[17]. *MMP 12*, *MMP19* and *TIMP1* genes show elevated expression in the adipose tissue of obese humans[17]. In line with these

observations,WNIN/Ob obese rats showed higher expression of genes coding for *MMP12*, *MMP19* and *TIMP1* in the adipose tissue, suggesting a possible role for ECM-remodelling and MMPs in the development of adipose tissue enlargement in this novel obese rat model.

Studies on animal models and human obesity showed elevated macrophage numbers in the adipose tissue[18]. Transcriptome analysis of the adipose tissue of WNIN/Ob obese rats revealed elevated expression of macrophage-specific genes such as *CD68*, *Mpeg1* indicating the possible role of macrophages in the development of insulin resistance in this model.

The expression of various cyclin genes was elevated in adipose tissue of WNIN/Ob obese rats suggesting increased cell division in adipose tissue. However, the expression of marker genes for preadipocyte differentiation such as preadipocyte factor 1 (*Pref1*), CCAAT-enhancer binding proteins and peroxisome proliferator-activated receptor γ (*PPAR γ*) was not altered in the adipose tissue of WNIN/Ob obese rats. From these observations, it may be possible that despite enhanced clonal expansion of preadipocytes, there is no subsequent commitment of these cells to adipocytes in the adipose tissue of obese rats and at this age, adipose tissue expansion in these obese rats may be due to hypertrophy than hyperplasia. Adipocyte apoptosis is reported to be higher in animal and human obesity and considered to be responsible for infiltration of macrophages and the development of insulin resistance[18]. Elevated expression of apoptotic genes in the adipose tissue of WNIN/Ob obese rats along with the enhanced expression of macrophage-related genes, suggested possibly increased apoptosis of adipocytes in these obese rats.

miR143 and miR145 cluster is highly expressed in the adipose tissue and shown to increase during adipose tissue differentiation and in adipose tissue of animal models of obesity[19]. Mice lacking these miR143 and miR145 are protected from the diet-induced insulin resistance[19]. The expression of *miR143* and *miR145* genes was downregulated in the adipose tissue of WNIN/Ob obese rats. *miR27a* has been shown to be a negative regulator of adipogenesis by decreasing the expression of PPARγ[20], and adipose tissue of WNIN/Ob obese rats had lower expression of *miR27a*.

SnoRNA catalyzes chemical modification of other classes of non-coding RNAs including rRNA, tRNA and small nuclear RNA (SnRNA). Involvement of SnoRNA has been shown in fat-induced cell death[21]. Except in the case of Prader-Willi syndrome, there are no studies linking SnoRNA with obesity. Probe-sets coding for snoRNA were downregulated to a major extent (more than 50-fold for some probe sets) in the adipose tissue of WNIN/Ob obese rats.

As microarray was done from whole adipose tissue, which possess majorly adipocytes along with preadipocytes, fibroblasts, immune cells, epithelial cells and nerve cells, contribution from cell types other than adipocytes to the reported gene expression changes cannot be ruled out. One of the major limitations of our study was the sample size as only two samples from each phenotype were used. Thus, careful interpretation is needed to analyse the results.

In conclusion, majority of the altered genes and pathways in the adipose tissue of WNIN/Ob obese rats were in line with the observations in other obese animal models and human obesity. Our findings indicated that WNIN/Ob obese rat model could be a good model to study the mechanisms involved in the development of obesity and associated comorbidities such as insulin resistance and dyslipidaemia. Downregulation of non-coding RNA such as SnoRNA appears to be a novel feature in this obese rat model.

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Reprint requests: Dr Vajreswari Ayyalasomayajula, Department of Biochemistry, National Institute of Nutrition (ICMR), Jamai Osmania (PO), Hyderabad 500 007, Telangana, India
e-mail: vaj_lipidresearch@yahoo.com