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## IDENTIFICATION OF DIFFERENTIALLY REGULATED microRNAs IN A GENETICALLY OBESE RAT MODEL AND THEIR AFFINITIES TO POTENTIAL TARGET GENES

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#### **AUTHORS' CONTRIBUTIONS**

This work was carried out in collaboration among all authors. Authors SKK and AKB conceptualized the project, authors SKK and AKB designed and performed the experiments. Author AKB analyzed the data and helped in manuscript development. Author SG supervised the study and edited the manuscript. All the authors read the manuscript and consented.

#### Article Information

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#### ABSTRACT

Obesity and diabetes are some of the most prevalent non-communicable disease conditions that claimed a heavy toll on life in recent years. Understanding the complex molecular mechanism of onset and progress of obesity is important for developing appropriate therapeutics. Several studies on mouse and rat models established the essential and regulating roles of microRNAs (miRNAs) in the pathophysiology of obesity and diabetes. Using an indigenous obese rat model (WNIN-Ob), we aimed to identify differentially expressed miRNAs and possible targeting capability of those miRNAs for selected important genes associated with obesity and diabetes. Several miRNAs were found to be up-regulated and down-regulated in the adipose and liver tissue of WNIN-Ob rats. Simultaneously, miRNA-target gene network analyses revealed that ARNT2, mTOR, CAPN10, IRS1 were some of the most important selected genes that were targeted by the up-regulated and down-regulated miRNAs. The information reported herein may help in further understanding the mechanisms of development of obesity and future miRNA-based therapeutics.

Keywords: Obesity; microRNA; miRNA; Differential expression; miRNA-gene network.

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#### **1. INTRODUCTION**

Obesity and associated disorders like diabetes and cardiovascular diseases are among the most prevalent non-communicable disease conditions. Among different diabetes types, Type-II diabetes or diabetes mellitus is the most prevalent one [1].

MicroRNAs (miRNA) belong to a novel type of small non-coding RNA of 21-23 nucleotides. They function predominantly as post-transcriptional gene expression regulators. The miRNAs suppress protein synthesis by selective degradation of target mRNAs and thereby silences gene expression. miRNAs were reported to be functionally involved in homeostasis regulation and are also associated with disease conditions [2, 3]. Precise targeting capability and timely regulation of the target gene expression highlight the importance of miRNAs for their gene regulatory functions [4]. Understanding miRNA biogenesis and function revealed that apart from supporting homeostasis maintenance, miRNAs are also involved in the onset and progress of different disease conditions [5]. Prevalent and complex disease conditions such as cardiovascular disease [6], diabetes [7], neurological diseases [8], and many others were reported to have vital roles of miRNAs during the disease onset and progression.

Investigations suggest that multiple miRNAs are associated with the onset of obesity and diabetes as well as the progression of the conditions. Association of miRNAs has been discovered in gestational diabetes [9], Type-1 diabetes [10], and diabetes mellitus [11,12]. The role of miRNAs in metabolic processes has been connected to insulin resistance [13,14]. Another study reported that a miRNA related to the pancreatic islet, miR-375, negatively regulates insulin secretion by the pancreatic  $\beta$ -cells [15]. The miRNAs have been shown to promote adipogenesis, up-regulate expression of PPAR alpha, increase oxidation of fatty acids in the liver and decrease fatty acid synthesis [16]. In Goto-Kakizaki insulin-resistant rats, miR-29 was found to down-regulate insulininitiated signaling cascades, thereby, leading to the development of insulin resistance [17].

Therefore, miRNAs are important post-transcriptional modulators of various key biological processes including glucose homeostasis and obesity. Though there are several rat or mouse models of obesity and diabetes are available in the West and Japan, there is a limitation of such models in the Indian region. At the ICMR-National Institute of Nutrition (NIN), Hyderabad, a genetic model of obesity in rat was developed (WNIN-Ob) to accelerate the obesity or diabetes-associated studies. Unlike the models developed in the West, this model was developed from a spontaneous mutation in a pure inbred Wistar rat stock maintained at NIN over 90 years designated as WNIN [18]. WNIN-Ob shows all the major symptoms of metabolic degenerative conditions, making them ideal to study the metabolic syndromes at the molecular levels. However, the molecular etiology of this novel model is yet to be understood.

#### **1.1 Objective**

In this study, we aimed to explore the differential expression of miRNAs in adipose and liver tissues which play important roles in glucose homeostasis. Further, we intended to understand the probable targeting affinity of the differentially expressed miRNAs for selected genes that are known to be associated with obesity or diabetes.

#### 2. MATERIALS AND METHODS

#### **2.1 Experimental Animals**

The present analysis was designed to understand the comparative expression profiles of the microRNAs in normal healthy rats and in the WNIN-Ob rat which is a naturally obese rat breed developed at the ICMR-National Institute of Nutrition, Hyderabad. Both the groups had 2 animals each for the present study.

Required approval for all experiments was obtained from the Institutional Animal Ethics Committee of the ICMR-National Institute of Nutrition (P8F/II-IAEC/2015/8/NIN/SG/WNIN-Ob/36M/Gr-Ob/36M). All the experimental animals were exposed to a 12hour light and dark condition daily with adequate light intensity and room temperature in the animal house of the National Institute of Nutrition. In this study, the 120 days old WNIN-Ob rats and their lean littermates were used and allowed to fast overnight before they were euthanized.

# 2.2 Tissue Sample Collections and RNA Extraction

Immediately after euthanization, the rat tissues that were important for glucose homeostasis, i.e., liver and adipose tissues were collected. The tissues collected were stored at -80°C for further analysis. Total RNA from the tissues was extracted using the Trizol method as per the protocol supplied by the manufacturer. Total RNA was isolated from 100 mg of tissue using 1 ml TRI reagent (Sigma Aldrich, USA). Briefly, 100 mg of tissue was homogenized in TRI reagent using zirconium beads in a bead beater homogenizer. The tissue debris was separated by centrifugation and to the clear supernatant, 200 µl of the chloroform (Sigma Aldrich, USA) was added, vortexed vigorously for 30 seconds followed by incubation at room temperature for 10 minutes. The upper aqueous layer was separated by centrifugation at 12000 g for 15 minutes in a refrigerated centrifuge and collected in a new tube followed by addition of equal volume of isopropanol (Sigma Aldrich, USA). After gentle mixing, the solution was centrifuged again at 12000 g in a refrigerated centrifuge for 10 minutes. The supernatant was discarded and the RNA pellet was retained. The RNA pellet was washed with 70% ethanol, air-dried and was suspended in RNAse free water. RNA concentration was estimated by using a Nanodrop spectrophotometer ND1000. RNA integrity was evaluated by 1% agarose gel electrophoresis.

#### 2.3 cDNA Synthesis and miRNA Profiling

was miRNA profiling carried out using а QuantiMirmicroNA profiling kit from System Biosciences (Mountain View, CA, USA). cDNA synthesis was done using 5 µg of total RNA using a quantimiR cDNA synthesis kit following the protocol supplied by the manufacturer for 20 µl miRNA cDNA. The miRNA cDNA was diluted in 380 µl of nuclease-free water. The master mix was prepared by adding 400 µl of 3' universal primer to the diluted 400µl miRNA cDNA and 1200µl of 2XSYBR reagent (TakaraBio, Japan). About 5 µl of master mix and 1 µl of miRNA-specific primer were used to set up the reaction in the Applied Biosystems 7900HT Real-time PCR System as per the instructions provided by the manufacturer. U1, U6, and RNU43 genes were used to normalize the miRNA expression.

#### 2.4 Computational Analyses of the miRNAs

Tissue-specific up-regulated and down-regulated microRNAs were identified and further computational prediction analyses were carried out using target gene sequences reported earlier [19] related to diabetes and obesity. Altogether, 32 genes were identified and collected from the ENSEMBL gene database for the rat. The obtained microRNAs were targeted to these 32 genes to understand the affinity of the microRNAs towards these genes and analyze the relevant multiplicity and cooperativity. The miRANDA algorithm in a Linux environment was used for this purpose. The parameters considered in this target prediction included threshold score and energy of 80 and -14.0, respectively, with a scaling factor of 2.0. During alignment gap opening and extension penalty considered were -2 and -8, respectively.

Further, mapping of the miRNAs and target gene network was analyzed using Cytoscape (version 3.9). Four different networks were separately developed for the up and down-regulated miRNAs obtained for adipose and liver tissue, respectively. Various analyses including the edges, nodes, centrality, betweenness, closeness among the nodes and edges were conducted using the Cytoscape network analysis environment.

#### **3. RESULTS**

#### **3.1 microRNA Profiling**

microRNA profiling using the Real-time PCR resulted in the identification of several significant (>3 fold) microRNAs with their respective fold changes in 120day old obese rats (WNIN-Ob) over their lean WNIN counterparts (Table 1, Table 2, and Table 3). Further, tissue-specific analysis was carried out to segregate the microRNAs based on the fold changes observed (Table 1, Table 2 and Table 3).

In the adipose tissue, 29 microRNAs were found to be significantly up-regulated whereas 42 microRNAs were down-regulated (Table 1). Similarly, in the liver tissue, 26 microRNAs were up-regulated as well as down-regulated (Table 2). In the adipose tissue of obese rats (WNIN-Ob), miR-673, miR-25\*, miR-802, miR-295, and miR-349 showed >20 fold up-regulation. On the other hand, miR-200c, miR-183, miR-141 showed >20 folds down-regulation in the adipose tissue.

A similar investigation in the liver tissue showed that three miRNAs (miR-466c, miR-27a\*, miR-25\*) were up-regulated with >20 fold changes in obese rats whereas only 2 miRNAs (miR-24-1\* and miR-339-3p) were down-regulated with >20 fold changes.

However, the number of miRNAs with >3 fold changes were more in the down-regulated miRNA group in the adipose tissue, whereas, in the liver tissue, 26 miRNAs each were found to be upregulated and down-regulated (Table 3).

In addition, the significant (>3 folds) miRNAs that were either up-regulated or down-regulated in both the tissues were identified (Table 3).

The observation suggested that miR-18a, miR-27a, miR-802, and miR25\* were up-regulated in both the tissues whereas miR-224, miR-96, and miR-384-5p were down-regulated in both the tissues (Table 3). The normalized expression profiles of the adipose and the liver tissues are presented in Fig. 1.

#### 3.2 Target Gene Prediction for the miRNAs

All the target genes were considered as previously reported by Sripathi et al. [19]. Similar gene sequences for rats (*Rattus norvegicus*) were collected from the ENSEMBL database. However, the CANA1 gene was not present for the rat. In addition, considering the predominant role of Insulin Receptor Substrate3 (IRS3) [20, 21] and Hepatocyte Nuclear Factor 4 Gamma (HNF4G) genes in obesity and diabetes [22], they were included as target genes (Table 4).

All microRNAs in WNIN-Ob obese samples that were either up-regulated or down-regulated in the adipose and liver tissues, respectively, in comparison to the control rat tissue samples, were subjected to target analyses to the selected genes as mentioned in Table 4. Altogether, we observed 37 down-regulated and 27 up-regulated miRNAs, respectively, for the adipose tissue. Similarly, in liver tissue, 24 miRNAs each were observed in the down-regulated and up-regulated groups. The microRNA-gene target analysis revealed that the miRNAs that had maximum number of hits (>50) were rno-miR-370, rno-miR-125b-3p, rno-miR-330, rno-miR-327, rno-miR-672, rno-miR-375, rno-miR-125a-5p, rno-miR-181d, rno-miR-182, no-miR-125b-5p, rno-miR-204, rno-miR-382, and rno-miR-1et-7e in the adipose tissue down-regulated group (Fig.2A).

Table 1. The miRNAs significantly (>3 fold) up-regulated or down-regulated in the adipose tissue of WNIN-Ob rats

Up-regulated miRNAs		Down-regulated miRNAs	
miRNA	Fold Change	miRNA	Fold Change
miR-1*	19.1	miR-let-7c	3.26
miR-17-3p	3.03	miR-let-7e	3.3
miR-18a	5.6	miR-9*	3.8
miR-25*	41.26	miR-10a-5p	4.02
miR-27a*	12.64	miR-10b	3.1
miR-32	12.58	miR-34b	9.8
miR-122	12.1	miR-34c	8.47
miR132	7.11	miR-96	10.1
miR-142-3p	6.48	miR-124	15.54
miR-146b	3.1	miR-125a-5p	3.25
miR-147	5.08	miR-125b-3p	9.8
miR-207	3.35	miR-125b-5p	3.9
miR-221	5.74	miR-135a*	6.84
miR-300-5p	3.48	miR-141	25.76
miR-301a	3.56	miR-181d	4.42
miR-301b	4.78	miR-182	4.57
miR-349	20.48	miR-183	35.27
miR-363*	5.03	miR-191	4.21
miR-376c	3.88	miR-192	3.79
miR-384-3p	5.76	miR-194	3.68
miR-409-5p	7.42	miR-196a*	9.21
miR-451	5.49	miR-200a	5.91
miR-484	3.73	miR-200b	12.7
miR-501	6.77	miR-200c	47.32
miR-673	245.7	miR-204	9.08
miR-295	28.4	miR-224	10.83
miR-362	6.2	miR-327	5.23
miR-547	4.43	miR-330	3.27
miR-802	29.7	miR-340-5p	9.88
		miR-342-3p	4.38
		miR-370	12.72
		miR-375	4.78
		miR-382	3.53
		miR-384-5p	9.25
		miR-429	12.18
		miR-434	4.69
		miR-463	9.19
		miR-479	16.69
		miR-672	4.12
		miR-743b	19.15
		miR-513	13.05

MiRNAs up-regulated		miRNAs down-regulated	1
miRNA	Fold Change	miRNA	Fold Change
miR-9*	3.43	miR-9	10
miR-18a	5.25	miR-19a	3.4
miR-20b-5p	3.25	miR-19b	3.8
miR-25*	63.11	miR-24-1*	67.5
miR-27a*	63.9	miR-30a	6.34
miR-136	4.65	miR-30c-1*	7.77
miR-137	12.8	miR-30c-2*	4.51
miR-200b	3.6	miR-30d*	3.91
miR-297	14.6	miR-32	4.07
miR-299	4.75	miR-96	6.65
miR-323	3.59	miR-139-5p	5.8
miR-325-3p	3.76	miR-184	10.51
miR-329	13.48	miR-204*	3.65
miR-338	3.34	miR-224	3.72
miR-338*	6.43	miR-339-3p	18.52
miR-363	8.68	miR-363*	3.93
miR-376b-5p	6.74	miR-384-5p	15.1
miR-379	7.24	miR-450a	5.41
miR-380	5.9	miR-500	6.42
miR-423	3.06	miR-743a	5.3
miR-449a	3.6	miR-743	4.11
miR-463	5.49	miR-220	4.58
miR-466c	159.3	miR-294	3.0
miR-879	3.41	miR-653	4.28
miR-666	7.74	miR-678	3.33
miR-802	3.69		

	mIR-875	6.0			
Table 2. The miRNAs significantly (>3 fold) up-regulated and down-regulated in the liver tissue of the					
WNIN-Ob rats					

Table 3. The miRNAs significantly (>3 fold) up-regulated and down-regulated in both the adipose and
liver tissues of WNIN-Ob obese rats

MiRNAs up-regulated		miRNAs down-regulated			
miRNA	Fold Chang	ge	miRNA	Fold Chang	ge
	Adipose	Liver		Adipose	Liver
miR-18a	5.6	5.25	miR-224	10.83	3.72
miR-27a	12.6	63.9	miR-96	10.1	6.65
miR-802	29.7	3.69	miR-384-5p	9.25	15.1
miR25*	48.6	63.11	-		

In the up-regulated miRNAs of adipose tissue, the miRNAs that showed more than 50 targets altogether were rno-miR-1\*, rno-miR-673, rno-miR-146b, rnomiR-207, rno-miR-17-3p, rno-miR-484, rno-miR-27a\*, rno-miR-25\*, rno-miR-349, rno-miR-501, rnomiR-221, rno-miR-362, rno-miR-122, and rno-miR-147 (Figure 2B). In case of liver tissue, the downregulated miRNAs that showed more than 50 hits were rno-miR-678, rno-miR-30c-1\*, rno-miR-204\*, rno-miR-339-3p, rno-miR-500, rno-miR-30c-2\*, rnomiR-139-5p, rno-miR-184, rno-miR-24-1\*, and rnomiR-220 (Fig. 2C). Similarly, the up-regulated most frequently interacting miRNAs with the target genes were rno-miR-423, rno-miR-27a\*, rno-miR-25\*, rnomiR-666, rno-miR-325-3p, rno-miR-338\*, rno-miR-449a, and rno-miR-338 (Fig. 2D). The frequently interacting miRNAs with the target genes were found to be more in the adipose tissue compared to the liver tissue of the WNIN-Ob obese rat. The best 20 microRNAs based on the total scores for each group are provided in Supplementary Table 1.

The susceptibility of the considered genes as a preferred target for the miRNAs was analyzed. The genes that were found most susceptible as the miRNA target for the down-regulated miRNAs in the adipose tissue were ARNT2, ABCC8, and Phosphoinositide-3-Kinase Regulatory Subunit 5 (each with >100 hits) (Fig. 3A). In the case of the up-regulated miRNAs in the adipose tissue, such susceptible genes were CAPN10, mTOR(FRAP1), PKLR, and GCK(>75 hits each) (Fig. 3B). Similarly, for the liver tissue, the most susceptible genes for the down-regulated miRNAs were ARNT2 and CAPN10 (each with >75 hits) (Fig. 3C). In the case of the up-regulated miRNAs in the liver tissue, the most targeted genes were mTor (FRAP1) and ARNT2, each with more than 55 hits followed by IRS1 and

CAPN10 (each having 53 hits) (Fig. 3D). The observation suggests that ARNT2, mTOR(FRAP1), and CAPN10 are the most susceptible target genes for these up-regulated and down-regulated miRNAs in adipose and liver tissues of WNIN-Ob rats.



Fig. 1. Observed folds changes in the expression of microRNAs for (A) adipose and (B) liver tissue of the obese WNIN-Ob rat



Fig. 2. Predicted microRNA multiplicity for the considered target genes. (A) Down-regulated miRNAs obtained for adipose tissue of WNIN-Ob rat against the considered target genes. (B) Up-regulated microRNAs that were obtained for adipose tissue of WNIN-Ob rat against the considered target genes. (C) Down-regulated miRNAs obtained for liver tissue of WNIN-Ob rat against the considered target

#### genes. (D) Up-regulated miRNAs obtained for liver tissue of WNIN-Ob rat against the considered target genes. Asterisk (\*) refers to the low abundance of mature miRNA Table 4. Selected target genes for the mirre RNAs clong with their respective ENSEMPL gene ID and

Table 4. Selected target genes for the microRNAs along with their respective ENSEMBL gene ID and gene names

ENSEMBL ID	ENSEMBL ID	Gene Name	Gene Short
			Name
ENSRNOG0000001389	ENSRNOT0000001874.3	Insulin receptor substrate3	IRS3
ENSRNOG0000001821	ENSRNOT0000002492.6	Adiponectin, C1Q And Collagen	ADIPOQ
		Domain Containing	
ENSRNOG0000001849	ENSRNOT0000002533.8	Mitogen-activated protein kinase 1	MAPK1
ENSRNOG0000007668	ENSRNOT0000010084.3	V-maf musculoaponeurotic	MAFA
		fibrosarcoma oncogene homolog A	
ENSRNOG0000008839	ENSRNOT0000082969.2	Peroxisome Proliferator Activated	PPARG
		Receptor Gamma	
ENSRNOG0000008895	ENSRNOT0000089893.2	Hepatocyte Nuclear Factor 4 Alpha	HNF4A
ENSRNOG0000008971	ENSRNOT0000011890.7	Hepatocyte Nuclear Factor 4 Gamma	HNF4G
ENSRNOG0000009615	ENSRNOT0000014167.8	Mechanistic Target Of Rapamycin	Mtor(FRAP1)
		Kinase/Serine/threonine-protein kinase mTOR	
ENSRNOG0000011875	ENSRNOT0000015866.7	Solute Carrier Family 2 Member 2	SLC2A2
ENSRNOG0000012052	ENSRNOT0000016052.6	Insulin-1 precursor	INS1
ENSRNOG0000013017	ENSRNOT00000107318.1	Aryl Hydrocarbon Receptor Nuclear	ARNT2
		Translocator 2	
ENSRNOG0000013133	ENSRNOT0000017742.4	Forkhead box protein A2	FOXA2
ENSRNOG0000013397	ENSRNOT0000018244.6	Forkhead box protein O1	FOX01
ENSRNOG0000014597	ENSRNOT0000019579.6	Insulin receptor substrate1	IRS1
ENSRNOG0000015480	ENSRNOT0000021285.7	Protein Kinase C Zeta	PRKCZ
ENSRNOG0000015603	ENSRNOT0000020959.8	Protein kinase C Epsilon	PRKCE
ENSRNOG0000017226	ENSRNOT00000113573.1	Solute Carrier Family 2 Member 4	SLC2A4
ENSRNOG0000019073	ENSRNOT0000025851.5	Inhibitor Of Nuclear Factor Kappa B	IKBKB
		Kinase Subunit Beta	
ENSRNOG0000020155	ENSRNOT00000101766.1	Mitogen-Activated Protein Kinase 8	MAPK8
ENSRNOG0000020420	ENSRNOT0000027700.7	Pyruvate Kinase L/R	PKLR
ENSRNOG0000021128	ENSRNOT0000028685.4	Potassium Inwardly Rectifying Channel	KCNJ11
		Subfamily J Member 11	
ENSRNOG0000021130	ENSRNOT0000028696.8	ATP Binding Cassette Subfamily C	ABCC8
		Member 8	
ENSRNOG0000023428	ENSRNOT0000032785.6	Phosphoinositide-3-Kinase Regulatory	PIK3R5
		Subunit5	
ENSRNOG0000026842	ENSRNOT0000033627.4	Nicotinamide Nucleotide	NNT
		Transhydrogenase	
ENSRNOG0000029986	ENSRNOT0000067448.4	Insulin Receptor	INSR
ENSRNOG0000031174	ENSRNOT0000091681.2	Aryl Hydrocarbon Receptor Nuclear	ARNT
		Translocator	
ENSRNOG0000045623	ENSRNOT0000074160.2	Calpain 10	CAPN10
ENSRNOG0000046458	ENSRNOT0000071942.2	Pancreas/duodenum homeobox protein	PDX1
		1	
ENSRNOG0000049232	ENSRNOT00000100362.1	Transcription factor 7-like 2	TCF7L2
ENSRNOG0000061527	ENSRNOT0000086474.2	Glucokinase	GCK
ENSRNOG0000068183	ENSRNOT00000105464.1	Suppressor Of Cytokine Signaling 4	SOCS4
ENSRNOG0000070745	ENSRNOT0000001110.6	Tumor Necrosis Factor	TNF

Further, we have compared the microRNAs among these four groups, i.e., down and up-regulated microRNAs in adipose and liver tissue, to understand the presence of common miRNAs.

Initial comparison of the microRNAs from the experimental outcomes suggested that 3 miRNAs, such as, rno-miR-96, rno-miR-224, and rno-miR-384-5p were down-regulated in both the tissues, and

similarly, rno-miR-25\*, rno-miR-27a\*, and rno-miR-18a were commonly up-regulated in both adipose and liver tissue (Fig. 4A). Among the miRNAs which were down-regulated in the adipose tissue and upregulated in the liver tissue, rno-miR-200b and rnomiR-463 were common. In an alternative situation, i.e., miRNAs down-regulated in the liver and upregulated in adipose, rno-miR-32 and rno-miR-363\* were common. Fig. 4 shows the common miRNAs among various groups, such as, up-regulated and down-regulated miRNAs in adipose and liver tissue

along with their respective percentages.



Fig. 3. Presentation of the predicted target susceptibility of the considered genes by the microRNAs. (A) Analysis outcome of the genes' susceptibility as a preferred target by the microRNAs down-regulated in the adipose tissue. (B) Genes' susceptibility as a preferred target by the microRNAs upregulated in the adipose tissue. (C) Analysis outcome of the genes' susceptibility as a preferred target by the microRNAs down-regulated in the liver tissue. (D) Genes' susceptibility as a preferred target by the microRNAs upregulated in the liver tissue



# Fig. 4. Venn diagram representation for the down and up-regulated microRNAs in the adipose and liver tissue for the (A) miRNAs obtained by the experiment and (B) the predicted top 20 miRNAs based on the total target score

Similar analysis of the best 20 miRNAs based on the obtained predicted target total score, the down-regulated miRNAs in both adipose and liver were having rno-miR-224 as the common one and for the converse up-regulated condition, rno-miR-27a\* was the common miRNA (Fig. 4B). Thus, suggesting these two miRNAs have a possibly higher affinity towards the selected target genes along with the miRNAs having frequent hits for the target genes as mentioned earlier.

#### 3.3 Network Construction and Analysis

The predicted miRNA-gene interaction outcomes were further subjected to analyses of the gene-miRNA interaction network using Cytoscape (version 3.9). Separate networks were constructed for tissue-specific up-and-down regulated miRNAs and their respective gene interactions (Fig. 5 & Fig. 6). The miRNAs that were down-regulated in the adipose tissue and their respective gene interaction had 69 nodes and 2355 edges with an average number of neighbors of 22.17 (Fig. 5A). The observed multi-edge node pairs were 430. For the up-regulated miRNA-gene interaction in the adipose tissue, nodes were 59, edges were 1777, the average number of neighbors was 19.15, and 375 multi-edged nodes (Fig. 5B).

Correspondingly, for the down-regulated miRNAgene network for the liver tissue, the number of nodes was 56, edges were 1514, average neighbors were 18.35, and multi-edge node pairs were 304. For the up-regulated miRNA in the liver and the gene interaction, nodes were 56, edges were 1269, average neighbors were 17.17, and the multi-edged node pairs were 241 (Fig. 6A and Fig. 6B). Hence, the varying number of edges and the average number of the neighbors suggest the varying degree of interactions among the considered miRNA-gene interaction study groups. Higher complexity was observed in the miRNAs up-and-down regulated in the adipose tissue compared to the miRNAs of the liver tissue.



Fig. 5. The miRNA and target gene network developed for (A) down-regulated and (B) up-regulated miRNAs in adipose tissue



## Fig. 6. The miRNA and target gene network developed for (A) down-regulated and (B) up-regulated miRNAs in liver tissue

Network interaction of the top 10 interactions based on the degree of interaction was studied further (Fig. 7). The obtained results strongly supported the earlier observations related to the gene susceptibility analysis done for the considered targeting miRNAs and confirmed the prediction outcomes. The prime genes and the interacting miRNAs are ranked based on the degree of interaction (Fig. 7) suggested susceptibility of ARNT2, CAPN10, mTOR (FRAP1), IRS1, PIK3R5, and ABCC8 genes. On the other hand, the miRNAs having a higher degree of targeting capabilities were miR-25\*, miR-27a\*, and others (Fig. 7).

#### 4. DISCUSSION

microRNAs potentially function as a gene expression regulator at the post-transcriptional phase in a normal cellular environment and are associated with various disease conditions as well [23, 24]. It has been found that an individual miRNA can target multiple mRNAs, on the contrary, an individual mRNA may be targeted by multiple miRNAs [23]. The specific interaction between a microRNA and an mRNA in animal species predominantly occurs in the vicinity to the 5' end of the mRNA sequence through the "seed" region. However, in plant species, the miRNA-mRNA interaction happens through sequence complementarity [25].

Apart from normal homeostasis, the relation of microRNAs with disease conditions is well established. miRNAs have been implicated in several non-communicable lifestyle disease conditions, such as diabetes [26, 27] and obesity [28, 29]. The involvement of microRNAs is reported in the differentiation of adipocytes and the secretion of insulin [30]. Metabolism of adipose and liver tissue is associated with obesity and also with the onset and progress of diabetes [31]. Similarly, obesity also affects the metabolism of the liver with relevance to adipocytes and hepatocytes [32].

Different hormonal productions occur from various cell types from the pancreatic islets. For instance, hormones such as insulin, glucagon, somatostatin, ghrelin, and pancreatic polypeptides are produced from different cell types of the pancreatic islands such as  $\beta$  cells,  $\alpha$  cells,  $\delta$  cells,  $\varepsilon$  cells. This specificity of the hormone-secreting cell types is possible due to this precise development and differentiation of the pancreatic cell types. Appropriate development of the pancreas is reported to have a crucial role by microRNA, specifically for the  $\beta$  cells that produce insulin [33]. Experiment on regeneration of the pancreas with relation to the expression of neurogenin-3 (ngn3) demonstrated the vital role of microRNAs, especially miR15 miRNA family, such as miR-15a, miR-15b, and miR-16 [34].



Fig. 7. The observed top 10 interacting hubs based on the degree of interaction for (A) down-regulated and (B) up-regulated miRNA-gene targets in adipose and (C) down-regulated and (D) up-regulated

## miRNA-gene targets in liver tissue. The ranking (1-10) is presented with the color gradient of red to vellow

Further, the real-time blood glucose level control requires the antagonistic actions of insulin and glucagon, such an on-and-off mechanism with realtime glucose level sensing requires differential regulation of the pancreatic hormonal secretion activity [35]. Different studies have established the relation of the real-time regulation of the pancreatic hormone secretion and expression of various microRNAs. Such reports included miR-124a, miR-30D, miR-107 [36], miR-375 (a microRNA directly associated with the expression of 3' phosphoinositidedependent protein kinase 1) [37], miR-9 [38], and many others. The miRNAs also have an influencing role in regulating the expression of the hormonesecreting genes such as the insulin gene. Significant upregulation of miR-124a2 was reported during crucial β cell differentiation. Similar miRNA was also reported to have other gene targets including foxA2 (forkhead/winged-helix transcription factor boxa2), a regulator of pancreatic duodenal homeobox 1 (pdx1) [39]. Such reports suggested the complex relation of hormone-producing gene expression and their association with differentially expressed miRNAs and relevant gene regulators.

Xie et al. [40] reported upregulation (miR-16, miR-24, miR-221, miR-222, miR-223, miR-146b, miR-23b, miR-27a, miR-27b, and miR-342-3p) and down-regulation (let-7d(\*), miR-103, miR-107, miR-145, miR-320, and miR-30a\*) of several miRNAs with relation to obesity in mouse adipocyte cells. Another similar report mentioned the role of miR-27 and miR-519d in adipocyte cells in relation to obesity. Interestingly, both these miRNAs are also having an association with peroxisome proliferator-activated receptor (PPAR) family members, responsible for fat cell development regulation [41].

Other than adipose tissue, signal transducers, growth factors, and several transcription factor regulations in the liver have a pivotal role in maintaining the overall carbohydrate, protein, and lipid metabolism [42]. Investigators suggested the pivotal role of miRNAs in such metabolism regulation at the molecular level where important genes are regulated through the miRNAs.

The present study was designed to understand the crucial miRNA gene interactions based on the existing reports in the WNIN-Ob obese rat. Our investigations suggested some of the crucial miRNA-gene interactions as mentioned in the results section. We observed that in the down-regulated miRNAs in the adipose tissue, among the selected target genes ARNT2, PIK3R5, and ABCC8 were having a higher

affinity for interaction with the specific miRNAs such as miR-370, miR-125b-3p, miR-330, miR-327, miR-375, and others. Similarly, for the up-regulated miRNAs in the same tissue, interaction with CAPN10 and mTOR (FRAP1) genes with miR-673, miR-146b, miR-207, miR-173p were found significant. Investigation on the liver tissue data suggested that the down-regulated miRNAs (miR-30c-1\*, miR678, miR204\*, and others) were having a greater interacting affinity for CAPN10, ARNT2, and Mtor (FRAP1) genes. The up-regulated miRNAs (miR-423, miR-27a\*, miR-25\*, miR-666, and others) in the liver tissue demonstrated a higher degree of interaction with IRS1, mTOR (FRAP1), and ARNT2 genes.

The available literature supports these observations where important roles of these genes are associated with lipid metabolism, obesity, and diabetes directly or indirectly. IRS-1 is considered an important genetic determinant of insulin resistance for T2DM patients with hyperglycemia [43]. In a specific human population, the association of insulin resistance was established with insulin receptor substrate-1 (IRS-1) [44]. The mTOR gene expression is associated with diabetes and lipid metabolism. In diabetic rats, renal lipid metabolism was reported to be associated with mTOR where phosphorylation of mTOR was enhanced in the rat renal tubules [45]. Analysis of the cellular signaling suggested that mTOR activates mTOR complex 1 and 2 that influences autophagy and apoptosis and in turn affects the cell survival in diabetes mellitus [46]. ARNT2 dependent genes were reported to maintain the homeostasis related to the feeding response in mice where mutations in the ARNT2 gene showed significant hyperphagic obesity and diabetes in experimental mice [47]. Another recent study identified ARNT2 as a candidate gene responsible for monogenic obesity in humans [48].

The role of various miRNAs in regulating these genes are also reported, such as, miR-24 regulates the Aryl hydrocarbon receptor nuclear translocator (ARNT) in the human liver [49], miR-496 regulates the mTOR (Mechanistic Target Of Rapamycin Kinase) gene, a major controller of the anabolic and catabolic metabolic processes, in relation to the obesity and diabetes [50]. Interestingly, in the present study, we have observed these genes as major genes with higher degree of connectivity with the up-and-down regulated miRNAs in obese rats.

Apart from these reports, several other studies precisely mentioned the important and complex relationship of specific miRNAs with obesity or diabetes. Such reports indicated that either miRNA influenced the specific hormonal gene expression or targeted the important regulators of such genes. The miRNAs have precise targeting capability for most of the gene members of a cascading pathway related to obesity or diabetes.

In the present study, our findings predicted and suggested some plausible miRNA-gene interactions related to obesity. However, the present study is limited to the specific miRNAs and the selected genes. An overall holistic system-level analysis may reveal all the up-and-down regulated miRNAs and more specific genes that might be their respective targets.

#### **5. CONCLUSION**

In the present study we identified several up-anddown regulated miRNAs related to obesity in WNIN-Ob obese rats. Our predictions suggested probable specific interactions of the up-and-down regulated miRNAs with important genes that are associated with lipid metabolism, obesity, and diabetes. An integrated and system-level understanding is required to unravel the complete mechanism of miRNAdependent regulation of obesity and diabetes with reference to the healthy controls. Further, the role of miRNAs can be explored to monitor onset or progression of the disease conditions.

#### ETHICAL APPROVAL

Required approval for all experiments was obtained from the Institutional Animal Ethics Committee of the ICMR-National Institute of Nutrition (P8F/II-IAEC/2015/8/NIN/SG/WNIN-Ob/36M/Gr-Ob/36M).

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#### **COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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