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Identification of differentially expressed microRNAs during lipotoxic endoplasmic reticulum stress in RAW264.7 macrophages

RAW264.7 makrofajlarında lipotoksik endoplazmik retikulum stres sürecinde ifadesi değişen mikroRNAların tanımlanması

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Abstract: Objective: Increased fatty acids in the circulation and their accumulation in non-adipose tissues play a significant role in the development of obesity related metabolic and inflammatory disorders such as insulin resistance, diabetes and atherosclerosis. While fat tissue has the ability to store excess fatty acids, uptake of excess fatty acids to other tissues burdens intracellular metabolic organelles such as mitochondria and endoplasmic reticulum (ER), leading to stress response and lipotoxic cell death. Unfolded protein response (UPR) is a key adaptation of the ER to stress. It is still not completely clear how lipids engage the UPR and how UPR manages both the adaptive and destructive consequences under its control. Increasing evidence point to the importance of miRNA regulation of the UPR as well as UPR's role in miRNA biogenesis. In order to understand how lipids engage the UPR, we set forth to identify microRNAs regulated by lipotoxic ER stress in macrophages.

Methods: We stressed the mouse macrophage cell line (RAW 264.7) with a saturated fatty acid, 500μM palmitate, reflecting the levels found in the circulation of obese patients. We analyzed the microRNAome profiles of this cell line using QRT-PCR based miScript miRNA PCR array which contained all known mouse microRNAs in miRBase release16 and performed pathway analysis for potential targets.

Results: 227 microRNAs showed altered expression levels; 43 microRNAs above 2 fold difference and 13 microRNAs 3-24 fold difference. Pathway analysis enriched the target mRNAs of these lipotoxic ER stress associated miRNAs.

Conclusion: When exposed to high concentrations of saturated fatty acids that can induce ER stress, macrophages display a dynamic range of changes in their microRNAome profiles. Our findings reflect the consequences of lipotoxic stress on circulating monocytes and tissue-associated macrophages in obesity. Further studies are needed to deliniate which UPR arm is reponsible for the microRNA changes reported here.

Keywords: Lipotoxic endoplasmic reticulum stress, microRNA, unfolded protein response, macrophage, QRT-PCR, pathway analysis, RAW264.7

Özet: Amaç: Kan dolaşımındaki serbest yağ asitlerinin artışı ve adipoz olmayan dokulardaki birikimi, insülin direnci, diyabet ve ateroskleroz gibi obezite ile ilişkili metabolik ve emflamatuvar hastalıkların gelişiminde önemli rol oynar. Yağ dokusu, fazla olan yağ asidini depolayabilme kabiliyetine sahipken, diğer dokulara ulaşan fazla miktarda yağ asidi, endoplazmik retikulum (ER) ve mitokondri gibi intraselüler metabolik organelleri zorlayarak stres cevabının oluşmasına ve lipotoksik hücre ölümüne neden olur. Katlanmamış protein yanıtı (KPY) endoplaz-

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mik retikulumun strese karşı önemli bir adaptasyonudur. Lipidler ile KPY arasındaki ilişkinin nasıl olduğu ve katlanmamış protein yanıtı ile adaptif ve destrüktif sonuçların nasıl yönetildiği halen tam olarak aydınlatılamamıştır. miRNA biyogenezinde KPY'nın rolünün yanı sıra, katlanmamış protein yanıtını düzenleyen miRNAların önemine işaret eden kanıtlar bulunmaktadır. Bu çalışmada lipidler ile KPY arasındaki ilişkiyi anlamak için, makrofajlardaki lipotoksik ER stresi sürecinde düzenlenen mikroRNA'ların tanımlanması amaçlanmıştır.

Metod: Fare makrofaj hücre hattına (RAW 264.7), serbest yağ asidi olan 500 μM palmitat -obez hastaların kan dolaşımındaki seviyede- uygulanarak stres oluşturulmuştur. miRBase sürüm 16'daki bilinen tüm fare mikroRNAlarını içeren QRT-PCR temelli miSCRİPT miRNA PCR array sistemi kullanılarak, hücrelerdeki tüm mikroRNAom profili analiz edilmiş ve potansiyel hedefler için yolak analizleri gerçekleştirilmiştir.

Bulgular: Lipotoksik ER stres sonucu, 227 mikroRNA'nın ifade seviyesi 2 kat üzerinde değişmiş ve 43'ü 2 kattan fazla,13'ü ise 3-24 kat değişim göstermiştir. Yolak analizi gerçekleştirilerek lipotoksik ER stresi ile ilişkili mikroRNA'ların mRNA hedefleri belirlenmiş ve gruplanmıştır.

Sonuç: Makrofajlar, ER stresini indükleyebilen yüksek konsantrasyondaki doymuş yağ asidine maruz bırakıldığında mikroRNAome profillerinde dinamik bir değişim gözlenmektedir. Bulgularımız, obezitedeki doku ilişkili makrofajlar ve kan dolaşımındaki monositlerdeki lipotoksik stres sonuçlarını yansıtmaktadır. Detaylı çalışmalar gerçekleştirilerek, lipotoksik ER stresi sürecinde ifadesinin değiştiğini rapor ettiğimiz mikroRNA değişimlerinden hangi KPY yolağının sorumlu olduğu belirlenebilir.

Anahtar Kelimeler: Lipotoksik endoplazmik retikulum stres, mikroRNA, katlanmamış protein yanıtı, makrofaj, QRT-PCR, yolak analizi, RAW264.7

Introduction

Endoplasmic Reticulum (ER) functions as a critical metabolic hub for protein, lipid and calcium metabolism [1]. Accumulation of unfolded proteins in the ER lumen, infections, toxins, hypoxia, excess food and energy deprivation trigger ER stress and activate the unfolded protein response (UPR) [2]. Upon ER stress, UPR functions as a switch between the adaptation of the cell against stress and the decision for apoptosis.

Chronic ER stress is harmful for cells and tissues and may lead to development of many metabolic diseases such as obesity, diabetes and atherosclerosis. One of the major known causes of these diseases is the accumulation of free fatty acids in non-adipose tissues like pancreas, liver and vascular wall, leading to cellular demise and death known as lipotoxicity [3,4]. The chronic inflammation observed in obesity can also arise from malfunctioning ER as UPR and the organelle itself is intricately linked to many immunological conditions [1]. Under normal conditions, UPR is an essential homeostatic mechanism for the management of stress associated with the accumulation of unfolded proteins in the ER [5]. UPR ensures the signal transfer to the nucleus for chaperone expression [6]. The unfolded or misfolded proteins can also be destined to ER-associated degradation pathways. If the ER cannot restore homeostasis in irremediable ER stress, UPR activates apoptotic pathways to initiate cell death [7].

The UPR is mediated by three different stress sensing pathways regulated by pancreatic ER kinase (PERK), inositol-requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6), all three transmembrane proteins located in the ER. PERK activation leads to phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) and inhibition of translation [7,8]. IRE-1 is unique in the sense that it has two distinct activities. Its kinase domain mediates autophosphorylation required for further oligomerization and activation. Whether IRE1 has other substrates than itself is not known. Its endoribonuclease activity is responsible for the splicing of XBP-1 (X box binding protein 1) mRNA resulting in production of active transcription factor XBP-1 [9–11]. The third arm is governed by ATF-6 cooperating with IRE-1 by upregulating the expression of XBP-1 mRNA and moreover, transcriptionally induces similar chaperone targets to XBP1 transcription factor. The expression and activation of XBP1s (XBP-1 spliced) as well as activation and translocation of ATF6 to nucleus leads to a complex transcriptional program that plays a central role in the UPR by upregulating mainly chaperone proteins promoting protein folding and production of essential components for protein degradation. Overall, these measures serve to re-establish homeostasis in ER [12,13]. In addition to protective responses, these UPR pathways can also induce important inflammatory signals when induced in cells of the immune system. Moreover, if ER homeostasis is not restored, ER activates apoptotic pathways [7].

ER stress is coupled to inflammation through several mechanisms [1]: IRE1-mediated activation of JNK induces expression of pro-inflammatory genes by directly influ-

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encing transcription factor activator protein 1 (AP1). Furthermore, activation of PERK triggers the degradation of inhibitor NFkB (IkB), which allows the translocation of NFkB into the nucleus and activation of pro-inflammatory genes. ER stress also leads to cleavage and activation of the transcription factor cyclic-AMP-responsive-element-binding-protein H (CREBH), which induces the production of acute phase proteins like C-reactive protein (CRP) and serum amyloid P-component (SAP). Additionally, reactive oxygen species can be produced during ER stress and lead to oxidative damage and activate many stress and inflammation signaling cascades.

Unfolded protein and lipids trigger ER stress through different mechanisms [14]. In order to drive ER stress, saturated fatty acids need to bind intracellular lipid chaperones that shuttle them presumably to intracellular destinations such as the membranes of organelles and to the nucleus [14,15]. Moreover, unlike unfolded proteins, which bind the luminal domains of proximal ER sensors such as IRE1 and PERK to activate UPR signaling, saturated fatty acids can trigger UPR signaling in cells expressing luminal domain deletion mutants of IRE1 and PERK. These findings clearly demonstrate important differences in how unfolded and lipid stress signals engage the UPR and represent a window of opportunity for therapeutics designed with an understanding of these molecular differences in order to discriminate between adaptive UPR responses essential for ER homeostasis and destructive responses triggered by the excess of lipids in obesity.

MicroRNAs are small RNA molecules, which function during development, organogenesis, maintenance of stem cell status, cancer and stress responses; regulate 30-60% of all protein coding genes. MicroRNAs primarily play important role in post-transcriptional regulation of gene expression, making them potential targets for therapeutic applications.

Many high throughput screening studies have been performed to identify the lipotoxicity associated microRNAs in different cell types. In 2008, pancreatic beta cells were treated with free fatty acids and significant differences in the expression levels of 132 microRNAs -especially mmu-miR-34a and mmu-miR-146 were detected by microRNA microarray [16]. ER stress was generated in cells by using chimeric tRNA and differences in the expression levels of 200 microRNAs were observed upon activation of UPR [17]. In this study, we identified lipotoxic ER stress associated microRNAs in an immune system cell, namely macrophage. Our goal was to learn more about the role of miRNAs in coupling lipid stress to UPR signaling and outcomes in macrophages.

Materials and Methods

Cell culture and lipotoxicity assay

RAW 264.7 mouse leukemic monocyte macrophage cell line has been cultured in RPMI 1640 medium containing 10% Fetal bovine serum. In order to drive lipotoxic ER stress, cells were treated with 500 μM palmitic acid (Sigma, P0500) for 6 hours. Palmitic acid was dissolved in 1% fatty acid free BSA (Sigma, A8806) containing RPMI at 55°C. After 6 hours of administration, expression of spliced Xbp-1 transcript was detected by QRT-PCR. Cells were treated with 300 μM Thapsigargin as well as positive control for ER stress.

RNA isolation and cDNA synthesis

Total RNA from palmitate treated and untreated RAW 264.7 macrophages were purified according to total RNA isolation protocol using Trizol (Invitrogen). cDNA synthesis of microRNAs was done with miScript II Reverse Transcription kit (Qiagen) according to manufacturer's protocols.

MicroRNAome profiling assay

"Mouse miRNAome miRNA PCR Array" (Qiagen, MIMM-3216Z) was used in order to identify expression levels of miRNAs in different samples. This array contained all known mouse miRNA (940 miRNAs) primers in the miRBase (Release 16) on 384-well plate format with internal and normalization controls. The expression levels were identified on Roche Light Cycler 480 machine according to manufacturer's instructions. Results were analyzed on miScript PCR Array Data Analysis web sofware developed by Qiagen (http://pcrdataanalysis.sabiosciences.com/mirna/arrayanalysis.php). Results were calculated according to ΔΔCT method and displayed in different formats.

Target prediction of differentially expressed miRNAs and pathway analysis

Available potential mRNA targets of differentially expressed microRNAs were provided from microRNA.org webpage. For pathway analysis, gene lists from microRNA.org were transferred to publicly available GeneCodis 3.0 web software and genes were grouped according to their biological

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functions. Gene Ontology (GO) and KEGG enrichment analysis were performed for available gene lists.

Results

Total RNAs from two biological replicates of palmitate treated and untreated RAW 264.7 macrophages are shown in Figure 1. All ribosomal RNA bands observed to be intact on agarose gel electrophoresis. These biological duplicates were used for miRNAome study. The presence of ER stress was checked by detection of the spliced Xbp-1 transcript (Figure 2). Thapsigargin, a potent inducer of ER stressor by way of inhibiting SERCA (SarcoEndoplasmic Reticulum Calcium transport ATPase) was used as positive control for detection of spliced Xbp-1 transcript (lane 1). 257 bp spliced Xbp-1 transcript was observed in the palmitate treated macrophages (lane 3), while 283 bp unsliced Xbp-1 transcript was observed in untreated cells (lane 2).

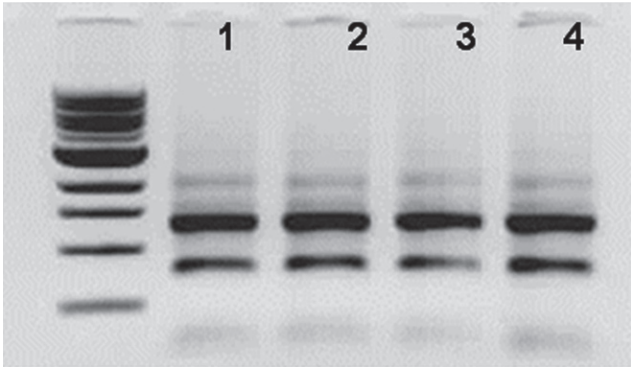

Figure 1: Total RNAs used in the study. 28S, 18S and 5S RNA bands are intact on agarose gel electrophoresis 1-3) Raw264.7, 2-4) Raw264.7+ Palmitate. Lanes 1- 2 and 3-4 are independent biological duplicates used in the miRNAome study. 1 kb ladder is from NEB (N3232).

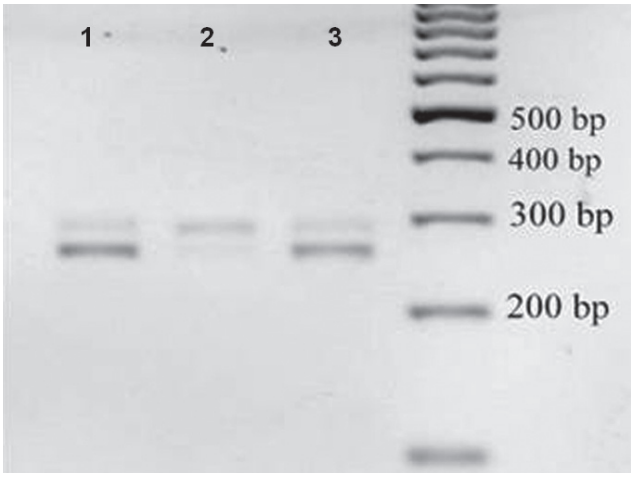

Figure 2: Xbp-1 splicing assay. Unspliced Xbp-1 gives 283 bp and spliced Xbp-1 gives 257 bp fragment. 1) Raw264.7 + Thapsigargin treatment, 2) Raw264.7 3) Raw264.7+ 500 µM palmitic acid.

MicroRNAome profiling assay was performed between palmitate-treated (test sample) and untreated macrophages (control sample) and out of the 940 known mouse mature microRNAs, 43 displayed differential expression above 2 fold (as calculated by miScript PCR Array Data Analysis web sofware). Figure 3 shows the changes in expression levels of these 43 microRNAs during lipotoxic ER stress. The results are mean of two biological replicates. Following palmitate treatment, expression levels of 18 microRNAs (Figure 3, blue bars) out of 940 mouse microRNAs increased at least 2 fold. Among these we were able to reach potential target mRNA lists of mmu-miR-486-5p, mmu-miR-2136, mmu-miR-132-3p, mmu-miR-1191, mmu-miR 542-5p and mmu-miR-449-5p. In addition, expression levels of 25 miRNAs decreased (Figure 3, red bars) at least two fold. Among these, we were able to reach predicted target mRNA lists of mmu-miR-294-3p, mmu-miR-125a-3p and mmu-mir-124-3p. A partial pathway analysis list of potential target mRNA numbers is shown in Table 1. For each microRNA, there were thousands of potential mRNA targets and by pathway analysis these genes were enriched in hundreds of groups according to their biological functions. Table 2 is a longer version of pathway analysis, genes were grouped according to gene ontology (GO). Out of approximately 350 GO terms, 45 of them are included which are related with ER stress, UPR, lipotoxicity, inflammation, fatty acid metabolism and so on.

Discussion

Since microRNAs play important roles in post-transcriptional regulation of gene expression during stress as well as many biological events, they have been very attractive potential targets for therapeutic approaches. To learn more about the role of microRNAs in coupling lipid stress to UPR signaling and outcomes in an immune cell, we stressed RAW264.7 mouse macrophages with a free fatty acid -thapsigargin- and analyzed the global microRNAome profiles with PCR array. Out of 940 known mouse miRNAs, expression levels of 18 miRNAs increased at least 2 fold and expression levels of 25 miRNAs decreased at least two fold. We were able to find downstream targets of 9 and performed pathway analysis in order to enrich predicted targets in gene groups according to their biological functions. Although we did not provide detailed gene lists here,

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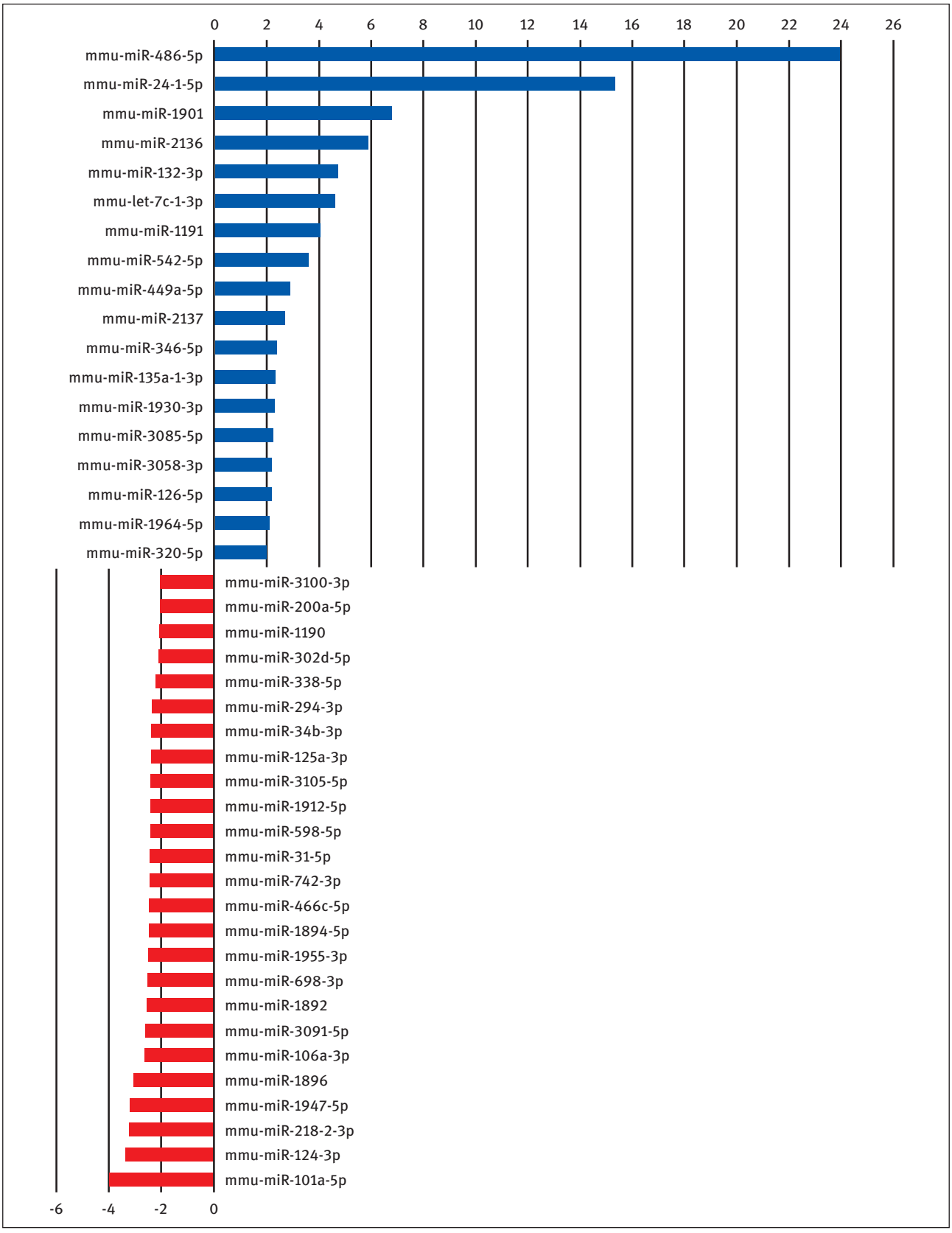

Figure 3: Differentially expressed miRNAs following lipotoxic ER stress. 43 miRNAs which are upregulated (blue bars, positive logarithmic fold change values) and downregulated (red bars, negative logarithmic fold change values) during lipotoxic ER stress. Palmitate treated Raw264.7 macrophage is the test sample and untreated Raw264.7 macrophage is the control sample

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Table 1: KEGG enrichment analysis of number of potential mRNA targets of the lipotoxic ER stress associated miRNAs (Partial list).

Biological processes (KEGG)	mmu-miR-1191	mmu-miR-542-5p	mmu-miR-48 6-5p	mmu-miR-132-3p	mmu-miR-294-3p	mmu-miR-124-3p	mmu-miR-449a-5p	mmu-miR-125a-3p	mmu-miR-2136
Ubiquitin mediated proteolysis	8		20	27	30	22	24		19
mTOR signaling pathway	5		8	9	13		9		7
Chemokine signaling pathway	13	8	15	39	25	41	30	31	
Insulin signaling pathway	11	11	15	23	24	35	26	32	14
ER protein processing	11		14	32	30	26	26	30	14
Adipocytokine signaling pathway	9	7			16		15		10
Apoptosis	8			18	18	21	12	19	
Type 2 diabetes		4						10	6

we were able to see enrichment of predicted target genes in lipotoxic ER stress related biological processes both in Gene Ontology (Table 2) and KEGG (Table 1) enrichments.

Expression of mmu-miR-486-5p was upregulated by 23.97 fold upon lipotoxic ER stress in RAW264.7 macrophages. Enriched GO terms in the predicted target genes of this miRNA were anti-apoptosis, apoptosis, cell communication, cell migration, cell-cell adhesion, cell-matrix adhesion, innate immune response, mRNA processing, negative regulation of apoptotic process, oxidation-reduction process, positive regulation of interleukin production, protein autophosphorylation and dephosphorylation (Table 2). According to our KEGG enrichment, some potential targets were clustered in biological pathways such as mTOR signaling pathway, ubiquitin mediated proteolysis, insulin signaling pathway, ER protein processing and chemokine signaling pathway (Table 1). These results were consistent with literature; although upregulation of mmu-miR-486-5p was not previously reported, this miRNA was shown to regulate many pathways in some diseases. During a study on global microRNA expression profile in myostatin knockout mice, mmu-miR-486 was identified as a positive regulator of IGF-1/Akt pathway, as a novel target of myostatin targeting [18]. Myostatin, also known as growth and differentiation factor-8, is a pivotal negative regulator of skeletal muscle mass and reduces muscle protein synthesis by inhibiting the insulin-like growth factor-1 (IGF-1)/Akt/mammalian target of rapamycin (mTOR) pathway. In myostatin knockout mice, the expression level of miR-486 in skeletal muscle was significantly increased. This study indicated miR-486 as one of the intermediary molecules connecting myostatin signaling and the IGF-1/Akt/mTOR pathway in the regulation of skeletal muscle size. In another study, stable expression of miR-486 ameliorated the disease progression in dystrophin deficient skeletal muscle of mice. Skeletal muscle-specific miR-486 overexpression in $Dmd^{mdx\text{-}5Cv}$ animals decreased levels of DOCK3, reduced PTEN expression, and subsequently increased levels of phosphorylated AKT, resulting in an overall beneficial effect [19]. Third study demonstrated FoxO1 to be a dominant mediator of chronic kidney disease-induced muscle wasting and miR-486 coordinately decreases FoxO1 and PTEN to protect against this catabolic response [20]. Chronic kidney disease accelerates muscle protein degradation by stimulating the ubiquitin proteasome system through activation of the E3 ligases. FoxO1 has a role in controlling ubiquitin proteasome system-related proteolysis. miR-486 decreased FoxO1 protein translation and increased FoxO1 phosphorylation by down-regulation of PTEN phosphatase, a negative regulator of p-Akt. Finally expression of the E3 ligases was suppressed and muscle mass increased despite the disease. Since our KEGG analysis enriched genes related with these biological processes, further studies are needed to understand the exact function of miR-486-5p during lipotoxic ER stress.

mmu-miR-2136 expression was upregulated by 5.89 fold upon lipotoxic ER stress. Pathway analysis identified GO terms such as apoptotic process, canonical wnt receptor signaling pathway, cell communication, cell migration, cell-cell adhesion, cytokine-mediated signaling pathway, lipid metabolic process, MAPK cascade, oxidation-reduction process, protein autophosphorylation and response to unfolded protein for mmu-miR-2136 (Table 2). Besides, KEGG enrichment identified biological processes such as ubiquitin mediated proteolysis, mTOR signaling pathway, insulin signaling pathway, ER protein processing, adipocytokine signaling pathway and finally type 2 diabetes.

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Table 2: Pathway analysis of number of potential mRNA targets of the lipotoxic ER stress associated miRNAs according to Gene Ontology (Partial list).

Biological processes (GO)	mmu-miR-1191	mmu-miR-542-5p	mmu-miR-486-5p	mmu-miR-132-3p	mmu-miR-294-3p	mmu-miR-124-3p	mmu-miR-449a-5p	mmu-miR-125a-3p	mmu-miR-2136
JAK kinase activation	2								
JUN kinase activation	6				9	7	9		
MAPK activation	9			125	12		12	13	
Adipose tissue development	4			187	6				
Anti-apoptosis	14		14	28	20		20	29	
Apoptotic process	43	22	52	364	93		76	94	42
Autophagy				70	14			11	
Canonical Wnt receptor signaling pathway	10			314			15		12
Cell communication			15	184					11
Cell migration	11		17	356	18		21	22	12
Cell surface receptor signaling pathway				336	19			22	
Cell-cell adhesion			10	277	14		18		11
Cell-matrix adhesion			9	348				12	
Cytokine-mediated signaling pathway							19		12
Cytoskeleton organization	7			30			18	18	
Endoplasmic reticulum unfolded protein response				189	8				
ER-associated protein catabolic process					9				
Fatty acid biosynthetic process				283			13	16	
Fatty acid elongation, polyunsaturated fatty acid	2								
Fatty acid metabolic process							16		
Immune response				214	26		25	22	
Inflammatory response				201	27				
Innate immune response			14	58				27	
JNK cascade	5	5			14		8		
Lipid biosynthetic process				59	18		21	21	
Lipid catabolic process				41			15		
Lipid metabolic process				238			35		23
Lymphocyte homeostasis				269	3				
MAPK cascade	7								9
mRNA processing	19		32	226	33		32	46	25
Negative regulation of apoptotic process	31	19	34	94	55		48	65	
Negative regulation of canonical Wnt receptor signaling pathway			11	126			13	20	
Negative regulation of interferon-gamma production		3		178			6		
Oxidation-reduction process			39	325	84		74	90	44
Phosphatidylethanolamine biosynthetic process	2			116					
Positive regulation of interleukin- production			6	280			6		
Positive regulation of NF-kappab import into nucleus				91					
Positive regulation of RNA splicing				245					
Positive regulation of Wnt receptor signaling pathway				359	20				
Protein autophosphorylation	12		17	290			30	27	23
Protein dephosphorylation	9		16	207			28	24	
Protein folding				56	13				
Response to unfolded protein				267			9		6

This study is the first one indicating mmu-miR-2136 to associate with a biological function.

mmu-miR-132-3p was upregulated 4.73 fold following lipotoxic ER stress in macrophages. According to our KEGG analysis, predicted target genes were enriched in groups such as ubiquitin mediated proteolysis, mTOR signaling pathway, chemokine signaling pathway, insulin signaling pathway, ER protein processing and apoptosis (Table 1).

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Associated GO terms for the predicted targets of mmu-miR-132-3p were numerous, as shown in Table 2. Among the groups it is not surprising to observe ER unfolded protein response, fatty acid biosynthetic process, lipid metabolic process, lipid catabolic process and inflammatory response genes. In a study investigating the expression levels of inflammation related miRNAs in white blood cells after 8 weeks of healthy diet, miR-132-3p expression was found to be associated with healthy diet [21]. In another study, mmu-miR-132-3p inhibited osteoblast differentiation by directly targeting EP300 (E1A binding protein p300) a type of histone acetyl transferase necessary for the acetylation of osteoblast differentiation factor Runx2, in simulated microgravity [22]. We also identified EP300 (Gene ID: 328572) with a mir-SVR score of -1.48, among predicted targets during our pathway analysis (data not shown).

mmu-miR-1191 expression was upregulated by 4 fold in thapsigargin treated macrophages. Our KEGG enrichment, grouped high number of genes into different biological process groups except type 2 diabetes (Table 1). Among the associated GO terms with this microRNA (Table 1) most remarkable ones were activation of kinases, adipose tissue development and apoptotic process.

mmu-miR-542-5p expression was upregulated by 3.6 fold upon lipotoxic ER stress. While our KEGG enrichment grouped genes related to chemokine signaling pathway, insulin signaling pathway, adipocytokine signaling pathway and type 2 diabetes (Table 1); GO terms were apoptotic process, JNK cascade, negative regulation of apoptosis and negative regulation of interferon gamma-production (Table 2), which are consistent with the previously reported tumor suppressor function. miR-542-5p was found to be directly targeting EGFR in non-small lung cancer and inhibiting growth of cancer cells [23]. More evidence demonstrated that mmu-miR-542-5p is a novel tumor suppressor in neuroblastoma [24]. This is the first report for the role of mmu-miR-542-5p during lipotoxic ER stress.

mmu-miR-449a-5p was upregulated 2.91 fold upon lipotoxic ER stress. Previous studies demonstrated that hsa-miR-449a inhibited expression of MAP2K1 directly by targeting its 3'UTR and its expression was downregulated in non-small cell lung carcinoma which suggested the use of this miRNA as a potential therapeutic target [25]. hsa-miR-449a inhibited liver cancer cell proliferation and induced apoptosis via suppression of Calpain6 and POU2F1 [26]. In another study, which also suggested the use of hsa-miR-449a as a therapeutic target in cancer, miR-449a suppressed epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma by multiple targets [27]. GO terms for the predicted targets not only identified cell proliferation and apoptosis associated groups consistent with literature, but also grouped these genes into cell migration, cell-cell adhesion, cytokine mediated signaling pathway, fatty acid biosynthetic process, fatty acid metabolic process, immune response, lipid biosynthetic process, lipid catabolic process, lipid metabolic process, oxidation-reduction process and response to unfolded protein that are related to inflammation, immune response, UPR, fatty acid and lipid metabolism as expected during lipotoxic ER stress in macrophages (Table 2). KEGG enrichment of predicted targets identified the role of these target genes in many biological processes as well (Table 1).

For pathway analysis, we were able to reach predicted mRNA targets of mmu-miR-124-3p, mmu-miR-125a-3p and mmu-miR-294-3p which are downregulated in thapsigargin treated RAW264.7 macrophages, 3.39 fold, 2.38 fold and 2.34 fold respectively. Previous reports identified that hsa-miR-124-3p targeted ROCK1 and inhibited cell migration and invasion [28]. Its downregulation was also implicated in gastric cancer tumorigenesis [29]. By targeting STAT3, this miRNA inhibited growth and metastasis [30]. Although pathway analysis did not reveal remarkable gene groups related to ER stress with mmu-miR-124-3p, we have noticed the consistency of miR-125a-3p involvement in such biological processes both with pathway analysis and literature. KEGG enrichment of predicted targets for this miRNA clustered some targets in biological processes such as ER protein processing, insulin signaling pathway, type 2 diabetes, apoptosis and chemokine signaling pathway, all being consistent with previous reports (Table 1). GO terms for mmu-miR125a-3p were adipose tissue development, apoptotic process, autophagy, cell migration, cytoskeleton organization, fatty acid biosynthesis, immune response and lipid biosynthesis (Table 2). rno-miR-125a-3p directly binds to the 3'UTR of p38 MAPK [31]. hsa-miR-125a-3p regulates the insulin signaling pathway and increased hsa-miR-125a-3p expression in omental adipose tissue might be a characteristic feature of insulin resistance in obese men [32]. Expression of rno-miR-125a-3p was upregulated during high fat diet following early leptin blockade [33]. hsa-miR-125a-3p promoted adipogenesis via suppressing the RhoA/ROCK1/ERK1/2 pathway and authors suggested novel therapies for obesity since they found the upregulation of this miRNA multiple symmetric lipomatosis patients [34].

mmu-miR-294-3p was found to be expressed in mouse embryonic stem cells [35]. It promoted induced pluripotency during reprogramming with Oct4, Klf4 and Sox2 [36]. miR-294/miR302 family promoted proliferation, suppressed G1-S restriction point and inhibited embryonic stem cell differentiation through Rb-dependent and -independent pathways [37]. mmu-miR-294-3p predicted targets

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were nearly in all biological processes in our KEGG enrichment results (Table 1). GO terms for this miRNA were activation for kinases, apoptosis, autophagy, cell migration, cell-cell adhesion, cell surface receptor signaling pathway, ER unfolded protein response, ER-associated catabolic process, protein folding, immune response, inflammatory response and lipid biosynthetic process (Table 2).

In this study, we have identified 43 microRNAs with at least 2 fold changes in expression level upon lipotoxic ER stress in macrophages. We restricted our pathway analysis results and mainly tried to focus on biological processes such as fatty acid and lipid metabolism, apoptosis, immunity, chemotaxis, cell migration and endoplasmic reticulum unfolded protein response. Among 43 miRNAs we were able to get the predicted target gene lists of 9 miRNAs. Our results were consistent with previous reported functions for mmu-miR-486-5p, mmu-132-3p, mmu-miR-542-5p, mmu-miR- 125a-3p and mmu-miR-294-3p. Although most information mentioned above originated from cancer studies, a few global microRNA profiling studies revealed functions for these miRNAs for ER protein processing, insulin signaling pathway, apoptosis, and inflammation and lipid metabolism. Since one of our questions was to figure out miRNA regulation of UPR as well as its role in miRNA biogenesis, it was not surprising to see "mRNA processing" in our GO term enrichment table for almost all differentially regulated miRNAs.

Our global miRNAome study identified expression level changes of many miRNAs during lipotoxic ER stress in an immune cell for the first time. High concentrations of saturated fatty acids - reflecting the levels found in the circulation of obese patients- induced ER stress, and macrophages displayed a dynamic range of changes in their microRNAome profiles. Our findings reflect the consequences of lipotoxic stress on circulating monocytes and tissue-associated macrophages in obesity. Further studies are needed to delineate which UPR arm is responsible for the microRNA changes reported in our study.

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