Unfolded Protein Response Regulated miRNAs in Lipotoxic Endoplasmic Reticulum Stress in Macrophages

A THESIS
SUBMITTED TO THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE OF BILKENT UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BY
ERDEM MURAT TERZİ
JULY 2014

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

_____

Assist. Prof. Dr. Ebru Erbay (Advisor)

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

_____

Assist. Prof. Dr. Özgür Şahin

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

_____

Assoc. Prof. Dr. Sreeparna Banerjee

Approved for Graduate School of Engineering and Science

ABSTACT

Unfolded Protein Response Regulated miRNAs in Lipotoxic Endoplasmic Reticulum Stress in Macrophages

Erdem Murat Terzi

M.Sc. in Moleculer Biology and Genetics

Supervisor: Assist. Prof. Dr. Ebru Erbay

July, 2014

The proper functioning and the development of the cell is essential to the fitness of the multicellular organisms - any significant disturbances in cellular mechanisms can lead to a multitude of diseases or death. Among these conditions, the global rise in metabolic diseases like obesity, diabetes and atherosclerosis draw significant research interest focus. Since the prevalence of metabolic disorders in the developed and underdeveloped world is expected to increase further in next decade; understanding the contributing cellular mechanisms is vital for the development of new and effective diagnostic and therapeutic tools against this devastating disease cluster.

Among the homeostatic cellular pathways important for health the Unfolded Protein Response (UPR) is highly conserved from yeast to mammals. Aside from most conserved UPR branch Inositol-requiring protein 1(IRE1), the mammalian UPR is composed of three different pathways regulated by IRE1, eukaryotic translation initiation factor 2-alpha kinase 3 (PERK), and activating transcription factor 6 (ATF6). The UPR signaling is activated in response to the accumulation of unfolded or misfolded proteins in ER that leads to endoplasmic reticulum (ER) stress. The goal of the UPR is to re-establish ER homeostasis via inhibition of further

protein translation and promoting protein folding. In the case of severe or unresolved ER stress, UPR instead triggers a programmed cell death.

Recent studies indicate that noncoding regulatory RNAs such as microRNAs (miRNAs) play important role in both upstream and downstream of the UPR. In this thesis, the regulation of miRNA expression by the different UPR arms are examined in macrophages under lipid-induced or lipotoxic ER stress conditions. The results of PCR array studies of RNA obtained from mouse macrophages stressed with a saturated fatty acid, palmitate (PA) , revealed multiple differentially regulated miRNAs. Among these miRNAs, significantly regulated ones were further examined for their regulation by the different arms of the UPR. Towards this end several complementary approaches were taken: First, significantly regulated microRNAs from microRNA PCR array results were analyzed. Next, macrophages were treated with palmitate after transfection with IRE1 and PERK silencer RNA (siRNA) to assess the role of UPR arms in lipid regulated miRNA regulation and the expression of relevant miRNAs was examined in treated macrophages. As an alternative method, macrophages were treated simultaneously with palmitate and specific inhibitors for IRE1's endoribonuclease or PERK's kinase activity. Then miRNA expressions were further examined in IRE1 knock-out mouse embryonic fibroblast (MEF) cell lines transfected with the wild type (WT) IRE or the endoribonuclease domain inactive (RD) mutant of IRE1 to verify the specific regulation of the miRNA by the IRE1's endoribonuclease activity. As a result, upregulation of miR-2137 expression by palmitate was determined as IRE1-endoribonuclease dependent. Next, potential target mRNAs were examined by the overexpression or knock-down of miR-2137 in macrophages. One possible target mRNA was found to be inositol polyphosphate phosphatase-like 1 (Innpl1) . Aside from miR-2137, miR-33 also showed significant alteration upon PA treatment in macrophages. Since the role of miR-33 in atherosclerosis, obesity and

insulin resistance is well established, its expression was studied further in RAW 264.7 macrophage cell line and bone marrow-derived primary macrophages after IRE1 and PERK knock-down with siRNA. ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1), a known target of miR-33, was investigated as down-stream target of miR-33 in PA treated macrophages, in an IRE1 dependent manner.

The results of this study uncovered new UPR regulated miRNAs under lipid stress in macrophages. Excess lipid is one of the prominent causes in metabolic diseases – obesity, atherosclerosis, insulin resistance – and these UPR regulated miRNAs may explain the underlying mechanism behind this set of diseases. Furthermore, the possible gene targets for these miRNAs could be responsible for progression of such conditions. Further studies are needed to reveal the exact mechanisms that can lead to the development of novel therapeutic approaches.

Keywords: UPR, microRNA, IRE1, macrophage, lipids

ÖZET

Makrofajlardaki Endoplazmik Retikulum Stresinde Katlanmamış Protein Yanıtı Tarafından Düzenlenen miRNAlar

Erdem Murat Terzi

Moleküler Biyoloji ve Genetik Yüksek Lisans

Tez Yöneticisi: Assist. Prof. Dr. Ebru Erbay

Temmuz, 2014

Hücrenin düzgün çalışması ve gelişimi, çok hücreli organizmalar için en temel gereksinimlerden biridir – hücre mekanizmalarındaki herhangi bir düzensizlik birçok hastalığın oluşumuna ya da ölüme neden olabilmektedir. Bu sağlık problemleri arasında, obezite, diyabet ve ateroskleroz gibi metabolik hastalıkların oluşumundaki küresel artış günümüzde önemli bir araştırma konusu oluşturmaktadır. Metabolik hastalıkların gelecek on yıl içinde hem gelişmiş hem de gelişmekte olan ülkelerde daha da yaygın hale geleceği beklendiği için bu hastalıkların oluşumunda etkili olan hücresel mekanizmaların daha iyi anlaşılması, bu tehlikeli hastalık grubuna karşı yeni ve etkili tedavi yöntemlerinin geliştirilmesi adına, önemli bir öncelik haline gelmektedir.

Sağlık için önemli olan homeostatik hücre yolakları arasında Katlanmamış Protein Yanıtı (KPY) fungilerden memelilere kadar evrimsel olarak korunmuş bir mekanizmadır. En korunmuş KPY dalı olan Inositol-requiring protein-1 (IRE1) dışında memeli KPY'si IRE1, eukaryotic translation initiation factor 2-alpha kinase 3 (PERK), and activating transcription factor 6 (ATF6) tarafından kontrol edilen üç koldan oluşmaktadır. KPY mekanizması katlanmamış ya da yanlış katlanmış proteinlerin endoplasmic reticulumda (ER) birikimi ile oluşan ER stresine yanıt olarak etkin hale geçmektedir. KPY'nin amacı protein üretimini

durdurarak ve protein katlanmasını arttırarak ER homestazını tekrar sağlamaktır. Çözülemeyen ER stresi durumlarında ise KPY hücreyi programlanmış hücre ölümüne yönlendirmektedir.

Yeni araştırmalar microRNA (miRNA) gibi kodlanmayan RNAların KPY mekanizmasının alt ve üst basamaklarında önemli rolleri olduğunu göstermektedir. Bu tezde, lipid tarafından oluşturulan ER stresin etkisi altındaki makrofajlarda miRNA ifadesinin farklı KPY kolları tarafından düzenlenmesi incelenmektedir. Doymuş yağ asidi, palmitat ile strese sokulmuş RAW 264.7 fare makrofajlarından elde edilen RNA ile yapılan PCR array sonuçunda, birçok farklı ifade edilen miRNA ortaya çıkarmıştır. Farklı KPY kollarının, bu miRNAlar arasından en fazla değişim gösterenlerin adaylar üzerindeki etkisi incelenmiştir. Bunun için çeşitli deney yaklaşımları kullanılmıştır. Öncelikle, PCR array sonuçlarından en anlamlı değişim gösteren miRNAlar incelendi. KPY kollarının doymuş yağ asitleri ile kontrol edilen miRNAlar üzerindeki etkisini incelemek için, macrofajlara IRE1 ve PERK silencer RNA (siRNA) ile transfekte edildikten sonra palmitat uygulandı ve alakalı genlerin ifadesi palmitat uygulanmış makrofajlarda ölçüldü. Alternatif bir method olarak palmitat, IRE endoribonükleaz ve PERK kinaz inhibitörleri ile birlikte uygulandı. Ayrıca miRNAların IRE1 endoribonükleaz tarafından regule edildiğini kanıtlamak için IRE1 ifade etmeyen fare embroyonik fibroblast (MEF) hücre hatlarında fonksiyonel ve endoribonükleaz aktivitesini kaybeden IRE1 mutantı ifade edildi. Sonuç olarak, palmitata bağlı miR-2137 artışının IRE1 endoribonukleaza bağlı olduğu belirlendi. Daha sonra miR-2137'nin potansiyel mRNA hedefleri miR-2137'yi yüksek oranda ifade ederek ya da inhibe ederek araştırıldı ve inositol polyphosphate phosphatase-like 1 (Innpl1) olası bir hedef olarak belirlendi. miR-2137'nin dışında, palmitat uygulanan makrofajlarda miR-33 de anlamlı bir değişim gösterdi. miR-33'ün ateroskleroz, obezite ve insülin direncindeki etkisi daha önce gösterildiği için, miR-33 ifadesi IRE1 ve PERK siRNA

transfekte edilmiş RAW 264.7 fare makrofaj hücre hattında ve kemik iliğinden elde edilmiş primer makrofajlarda incelendi. miR-33'ün bilinen bir hedefi olan ABCA1'nın ifadesi de kontrol edildi ve IRE koluna bağlı değişim gösterdiği doğrulandı.

Araştırmamızın sonuçları makrofajlarda yağ asidinin yarattığı stres sırasında KPY tarafından düzenlenen yeni microRNAlar olduğunu göstermektedir. Fazla oranda yağ; ateroskleroz, obezite ve insülin direnci gibi metabolik hastalıkların birincil nedenlerinden biridir. Bulduğumuz miRNAlar KPY'nin miRNA üzerinden rolünü gösterip, bu hastalık grubunun oluşumunun ardında yatan mekanizmaları açıklayabilir. Ayrıca, bu miRNA'ların bu tip rahatsızlıkların oluşumunda etkili olabileceğini düşündüğümüz hedeflerini gösterdik. İlerideki araştırmalar tam mekanizmayı açıklayıp, yeni terapatik yöntemlerin gelişmesine yol açabilecektir.

Anahtar Kelimeler: KPY, microRNA, IRE1, makrofaj, yağ asitleri

Acknowledgement

I would like to thank my thesis advisor Assist. Prof. Dr. Ebru Erbay for her invaluable support and supervision during my Master studies. She did not only help me in my thesis project, but I have also become a better researcher thanks to her. Before I even had a hard time to plan an experimental design, now I have confidence to lead my own project during my P.hd. I will be always indebted to her. I would like to thank Assist Prof. Dr. Arzu Atalay and her group members for our collebrative study for PCR array. I am also very thankful to Assoc. Prof. Dr. Sreeparna Banerjee, and Assist. Prof. Dr. Özgür Şahin for their contribution and advices as the members of the thesis committee.

In my thesis, I always inclined to use "we" instead of "I" because all my group members support me as much as possible, and their friendship helps me through all the hardships and problems. As a member of Erbay Lab I am always grateful for their help, it is very hard to express my appreciation with words. When I first come to Erbay Lab, there were only four members, four girls; Özlem Tufanlı, Seda Koyuncu, Büşra Yağabasan, Umut İnci Onat. They are always the core members of Erbay Lab in my heart. Their help were immense and I could never forget their friendship. I would also like to thank our post-graduates İsmail Çimen, Hamid Syed, Begüm Kocatürk. There is no way I could forget our lab manager, Buket Gültekin; our lab becomes much efficient and lively after she came. I also want to thank our seniors and undergraduates, especially ÇağlaCömert and Nil Aygün, for their support and friendship. I am also very grateful to my close friends Seçil Demirkol, Banu Bayyurt for their emotional support.

I would like to thank TUBITAK for the financial support I have received past two years. TUBITAK scholarships make it possible for many graduate students like me to continue their graduate education more easily; I can certainly say that it would be very difficult for me to complete my M.Sc degree without the support from TUBITAK.

This acknowledgement certainly would incomplete without thanking the most essential part of my life, my family. I know that they will always support me anytime regardless of wherever I am. Without them, I cannot accomplish all the things I have done.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1. 1. Endoplasmic Reticulum(ER) Function and ER Stress

Endoplasmic Reticulum (ER) is a continuous endomembrane system comprised of cisternae and microtubules and extending from the nuclear envelope to the plasma membrane. As such, ER membranes account for approximately half of all the cellular membranes (1). Aside from its large size, ER also exhibits a unique morphology. Heterogeneous and dynamic morphology of the ER reflects its many distinct functions. For instance; the rough ER, whose sheets include many ribosomes is connected to the biosynthesis and quality control of proteins destined for integral membranes or for secretion (1). ER is not only imperative in the protein biosynthesis, but it also assist in their correct folding (2). Another portion of the ER, the transitional ER, exports the correctly folded proteins to their proper cellular destinations or for secretion. On the other hand smooth ER, whose sheets are plain and void of ribosomes, is associated with cellular lipid metabolism. Since most key lipid metabolism enzymes reside on the smooth ER, its role in lipid biosynthesis and transport is indispensable (3). Moreover, the ER is the main site for intracellular Ca2+ storage and thus, important in Ca2+ mediated signaling (4).

Since the ER provides appropriate conditions for lipid and protein biosynthesis, protein folding and transfer, ER homeostasis is vital for cell's survival. Any disturbance of ER homeostasis promotes accumulation of unfolded or misfolded proteins in ER leading to ER stress. External or internal stimuli such as hypoxia, alterations in Ca2+ concentrations,

pathogens or inflammation can perturb homeostasis and cause ER stress (5). To maintain ER function and homeostasis, a highly specific pathway, called the Unfolded Protein Response (UPR) has evolved. The UPR mainly attempts to restore ER homeostasis via general translational inhibition and selective synthesis of proteins associated with protein folding and degradation of misfolded proteins. In the cases of severe or unresolved ER stress, the UPR instead initiates apoptosis (6). (Figure 1.1)

1. 2. Unfolded Protein Response (UPR)

The unfolded protein response was described first in Saccharomyces Cerevisiae as a signaling pathway that could be utilized to cope with the misfolded proteins that accumulate in the lumen of the ER. Inositol requiring protein 1 (IRE1) is the most the conserved and only UPR arm that is functioning in this organism (7). Mammalian UPR, on the other hand, has evolved more sophisticated mechanisms for the restoration of ER function. As an early response further protein influx into the ER is diminished via transcriptional or translational inhibition.

Subsequently, certain genes are selectively translated to enhance folding capacity and degradation of misfolded proteins. Nevertheless, restoration function is abandoned in prolonged ER stress and instead programmed cell death is mediated by the UPR arms (8). In mammals, three UPR branches have been demonstrated to regulate these processes with their proximal regulators sitting on ER membranes and known as IRE1, eukaryotic translation initiation factor 2-alpha kinase 3 (PERK), and activating transcription factor 6 (ATF6). (Figure.1.1)

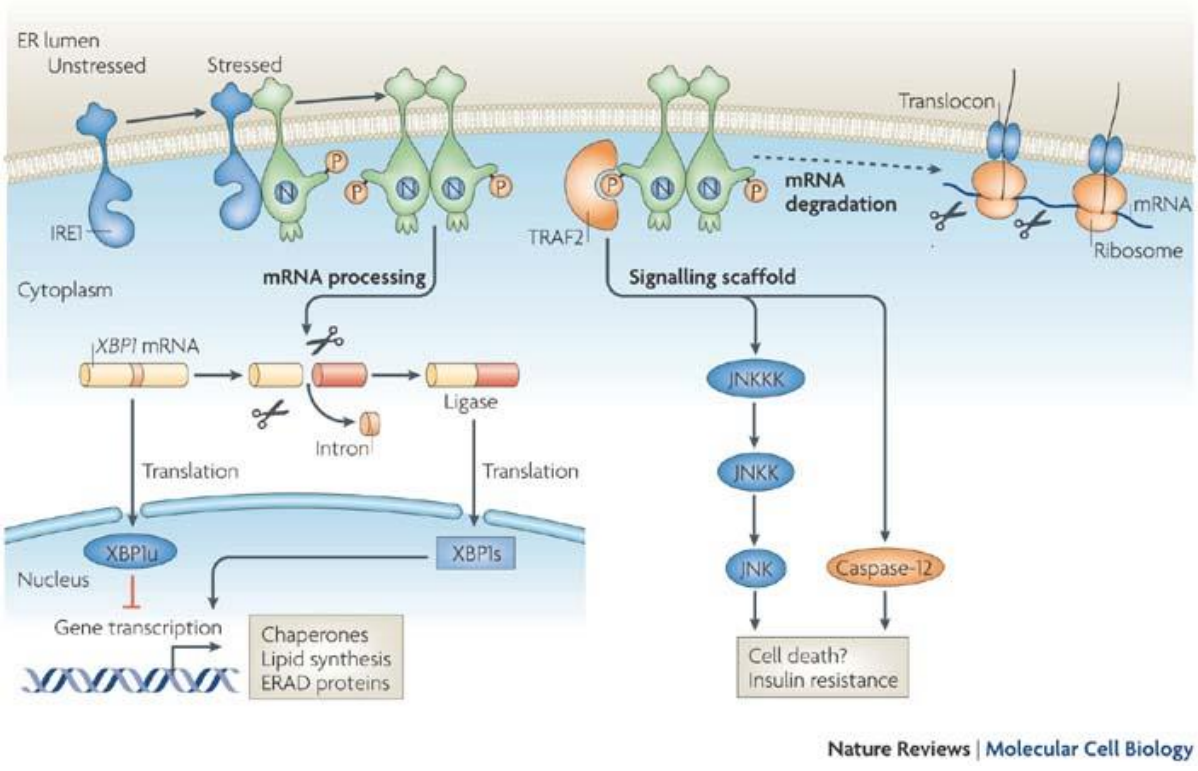

Figure.1.1. IRE1 arm of the UPR. The luminal domain of the IRE1 recognizes unfolded proteins. Upon ER stress, IRE1 forms oligomers and activates trans-autophosphorylation. Endoribonuclease activity of IRE1 is activated by this phosphorylation, leading to XBP1 mRNA splicing. IRE1 can also signal through JNK kinase and caspase-12 via binding to TNF receptor-associated factor 2 (TRAF2). ER-localized mRNAs can be targeted by IRE1 for degradation. Recent studies also demonstrate IRE1 can inactivate certain microRNAs via cleavage. (69)

The most conserved UPR arm is regulated by IRE1, which has two mammalian isoforms; IRE1α and IRE1β. IRE1α is ubiquitously synthesized unlike IRE1β that is expressed mainly in the gut. Furthermore, the lethality of IRE1α knock-out mice at embryonic stage implies that IRE1α has more central function than its isoform. IRE1 has two functional domains, Serine/Threonine protein kinase and endoribonuclease domains (9). In its active form, IRE1 is autophosphorylated and dimerizes upon sensing ER stress via its luminal domain.

Endoribonuclease becomes active upon transautophosphorylation in the oligomerized IRE1 and regulates several downstream mechanisms (7). The most known function of the endoribonuclease domain is the splicing of the X-box binding protein 1 (Xbp1) mRNA. Only the spliced form of Xbp1 mRNA, after the excision of a 26bp segment, has the correct reading frame that can translate into a functional protein. As an adaptive response, the transcription factor XBP1 translocates into the nucleus and upregulates the proteins involved in in ER‑associated degradation (ERAD), protein folding and transfer (8). Aside from Xbp1, IRE1 endoribonuclease regulate various mRNA via Regulated IRE1‑Dependent Decay (RIDD) pathway, in which certain ER-located proteins are degraded to decrease unfolded protein accumulation in ER (8). IRE1 is also shown to activate JNK to induce macroautophagy, although the exact mediator of this is unknown. When adaptive responses have failed in irremediable ER stress, IRE1 can sensitize cells to apoptosis through c-Jun N-terminal kinase (JNK) activation and degradation of mRNA for chaperones (10). Another important element in ER stress-induced apoptosis, Thioredoxin-interacting protein (TXNIP), is shown to be the target of miR-17, which was shown in a recent study to be a target of IRE1. The same study demonstrated that IRE1 incites apoptosis via selective degradation of microRNAs (miR-17, miR-34a, miR-96, and miR-125b) targeting caspase-2 (11). (Figure.1.1)

The second arm of UPR, PERK, has a similar luminal domain to IRE1 for sensing unfolded protein response (12). PERK's cytosolic domain, on the other hand, only contains a serine/threonine protein kinase as a functional domain. Upon ER stress, PERK is autophosphorylated and dimerizes to achieve full catalytic kinase activity. The foremost function of PERK is the inhibition of general protein synthesis through phosphorylating the eukaryotic translation initiation factor (eIF2α). Phosphorylated eIF2α inhibits Guanosine

diphosphate (GDP) to guanosine triphosphate (GTP) conversation and attenuates translation as an early response to decrease protein influx (13). While phosphorylation of eIF2α diminishes general translation via inhibition of the recruitment of methionyl-tRNA to initiation complex, it also favors the translation of certain mRNAs with short inhibitory upstream regulatory elements, such as activating transcription factor 4 (ATF4). Through ATF4, PERK promotes the survival of cells by regulating certain pathways such as autophagy, amino acid metabolism and protein folding (8). Moreover, PERK is also implicated in the regulation of various microRNAs such as miR-211, which may fine-tune the expression of proteins important in ER homeostasis and UPR-dependent apoptosis (14). Furthermore, under irreversible ER stress C/EBP-homologous protein(CHOP), a target of ATF4, is upregulated by the PERK branch. As a pro-apoptotic factor, CHOP upregulates B cell lymphoma 2-interacting mediator of cell death (BIM) expression and down-regulate B cell lymphoma 2 (Bcl2) transcriptions. Eventually caspase cleavage is triggered via BCL2-associated X protein (BAX)/ BCL2-antagonist/killer 1 (BAK) dependent apoptosis (8). Another factor shown to have a role in this apoptotic pathway is p53, however, the exact link between p53 and ER stress is yet to be discovered (15). (Figure.1.2)

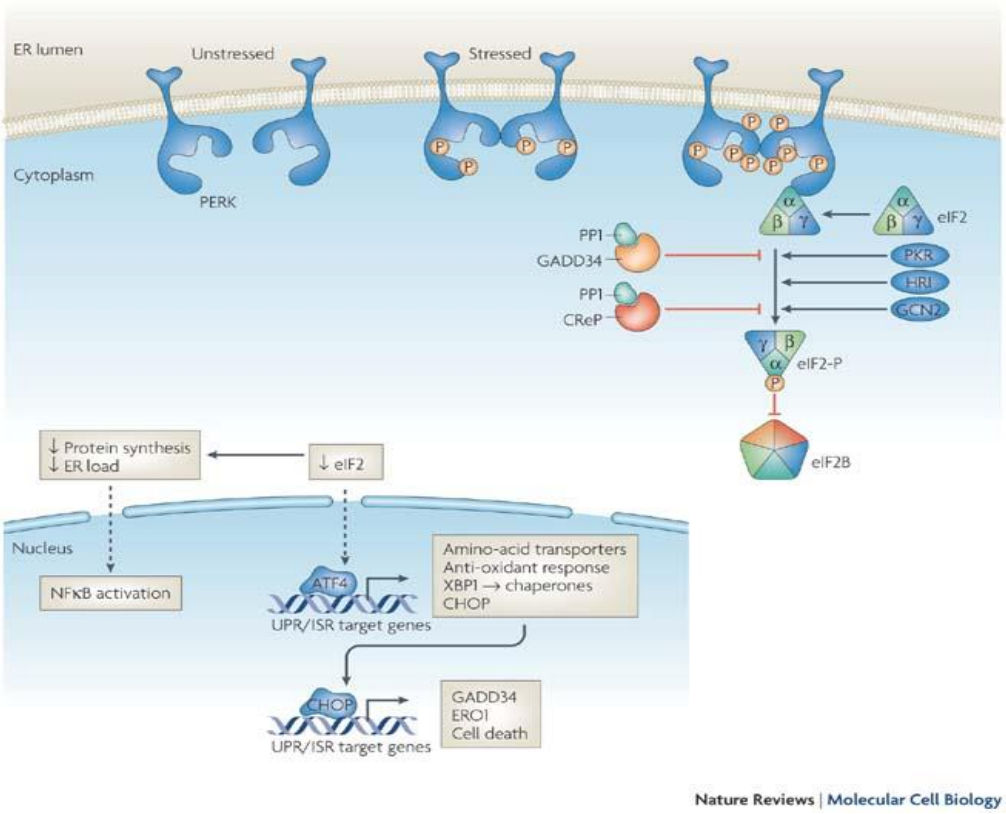

Figure.1.2 PERK arm of the UPR. Similar to IRE1, PERK form oligomers and trans-autophosphorylates after unfolded proteins are bound and recognized by luminal domain. Active PERK phosphorylates its target, eukaryotic translation initiation factor-2α subunit (eIF2α) leading to reduced activity of eIF2 complex and inhibition of general translation. The reduced general protein translation helps defray the protein load. On the other hand, specific genes are selectively up-regulated by eIF2 such as ATF4, which upregulates the transcription of UPR target genes. (69)

The third arm of mammalian UPR is regulated by activating transcription factor 6 (ATF6), a transcription factor which is synthesized as a transmembrane protein on the ER membrane. Although ATF6 also has a luminal domain, its sequence and structure is not related to IRE1 or PERK. Consequently the recognition of unfolded proteins by ATF6 is not known (16). Nevertheless, the downstream pathway has been deciphered. Upon stimulation by ER stress, ATF6 is transferred into small vesicles for transport into the Golgi apparatus. At the Golgi, ATF6 is processed by Site-1 and site-2 proteases (S1P and S2P) by cleavege of its luminal

domain by S1P and transmembrane domain by S2P, leading to the release of its cytosolic domain. The freed N-terminal cytosolic domain of ATF6 moves to the nucleous to function as transcriptional factor that regulate UPR related genes, such as chaperones, ERAD components and XBP1 (7, 8). (Figure.1.3)

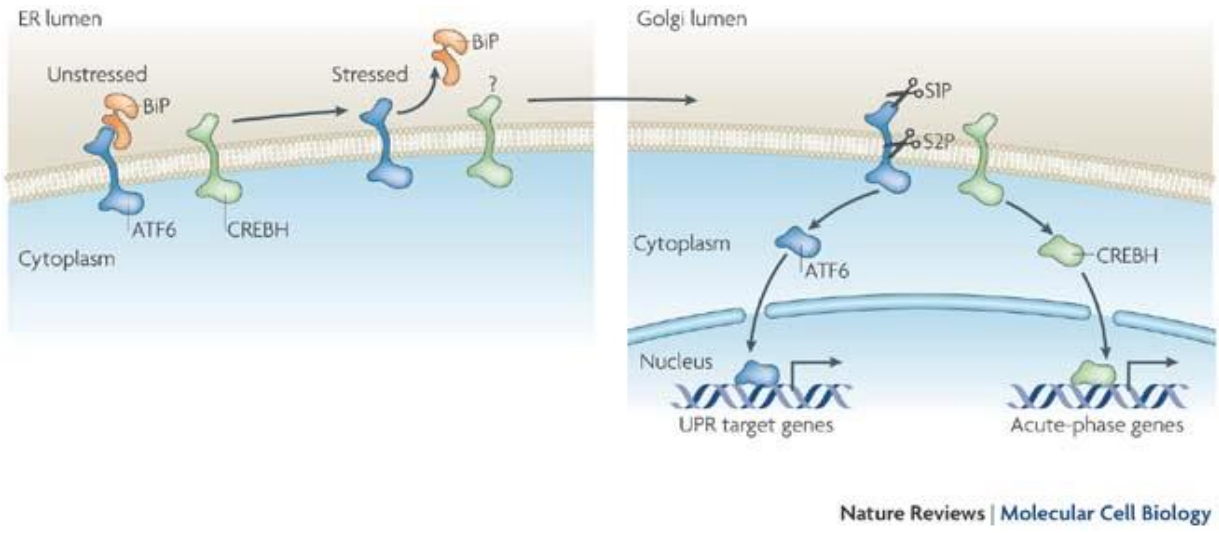

Figure.1.3: ATF6 arm of the UPR. In unstressed cells, ATF6 resides on the ER membrane. Disturbances in the ER lumen are recognized by ATF6, leading to its release and transfer to the Golgi apparatus. After ATF6 is located to Golgi, first it gets cleaved at the luminal domain by site-1 protease (S1P), followed by the cleavage of its transmembrane part by site-2 protease (S2P). Finally, the released cytosolic domain acts as transcription factor to upregulate UPR related genes. (69)

Overall, three UPR arms together buffer the ER against external or cellular stimulus via complex and only partially understood mechanism. One of the new and intriguing fuctions of the UPR is in the regulation of microRNAs. UPR is now shown to regulate a variety of microRNA via controlling their transcription, maturation or degradation. Moreover the UPR is also regulated by microRNAs (11). A better understanding of the relationship between microRNAs and the UPR is needed.

1. 3. MicroRNA; biogenesis, regulation and function

MicroRNAs are a large family of endogenous, 20-23bp long, single stranded RNAs that regulate the expression patterns of various genes in metazoans and plants (17). They have been shown to play a role in almost every known cellular mechanism and are estimated to regulate the expression of approximately half of the cellular proteins. A single microRNA can control many genes via transcriptional or translational inhibition and they themselves are regulated by many factors adding to the complexity of the miRNA functional network (17).

microRNAs are first synthesized as primary microRNA (pri-miRNA). RNA polymerase II or RNA polymerase III is responsible for their transcription. Many microRNAs are affected by inhibitors against RNA polymerase II, and enzyme involved in polyadenylation and capping (17,18). Upregulation of microRNAs can be accomplished by transcription factors. For example tissue-specific or developmental expression of many microRNAs are managed at this level. Aside from its targets, microRNAs can also regulate their own expression via effecting transcription factors in a feed-back loop (18). For example, miR-133b negatively regulate transcription factor paired-like homeodomain 3 (PITX3), which upregulates the transcription of its repressor, miR-133b (19).

After they have been synthesized as pri-miRNAs, a maturation process is necessary to obtain a functional microRNA. Cleavage of pri-miRNA by Drosha/ DiGeorge syndrome critical region gene 8 (DGCR8) complex is the first step in maturation. Drosha is responsible for cleavege, while DGCR8 is shown to be essential for double stranded RNA binding capacity of the complex (20) (Figure.1.4). A normal human pri-miRNA is composed of 33bp long hairpin stem and a loop between two flanking regions. After Drosha/DGCR8 complex recognize pri-miRNAs via stem and single standed regions primiRNAs are cleaved 11 bp away from the double stranded region. Drosha and DGCR8 is sufficient for cleavage of many miRNAs,

however, a larger complex with additional elements (such as RNA helicases and double stranded binding proteins) is also observed (20,21). Binding of specific elements to the complex can regulate their specificity, for instance transforming growth factor beta (TGF-β) can lead to the binding of ligand-specific signal transducers to the complex for enhancement of miR-21 maturation in muscle cells (22). Drosha/DGCR8 cleavage is not required by all microRNAs. Intron-derived microRNAs called mirtrons are readily cleaved in a way to resemble pre-miRNA, when host mRNA is spliced and they continue their maturation from the pre-miRNA step (23).

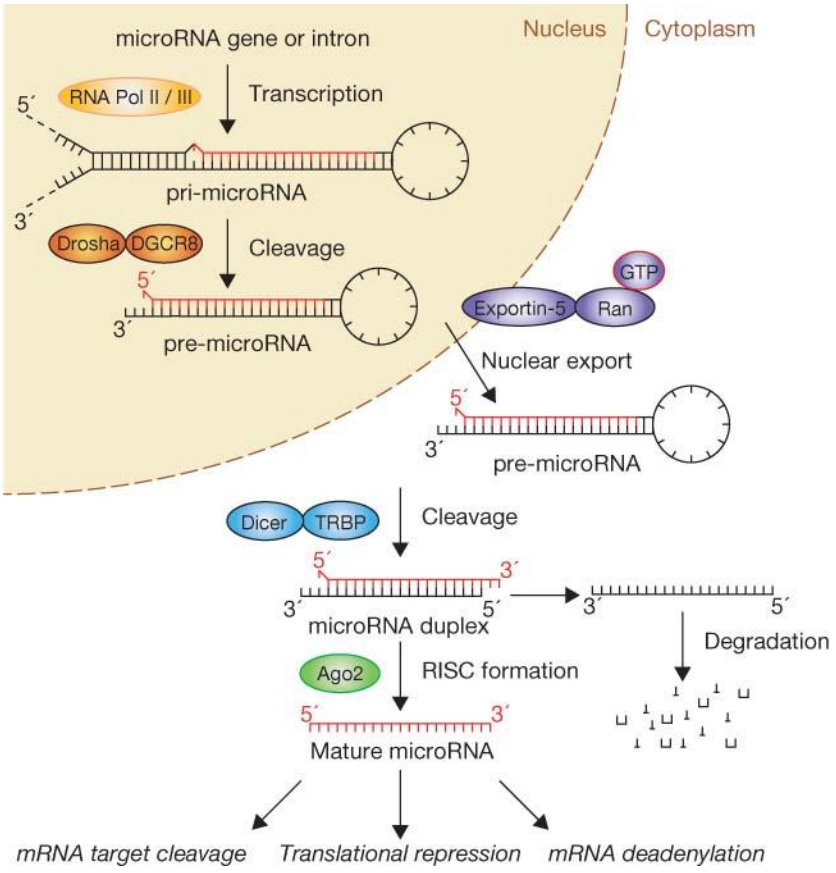

Figure.1.4: Canonical microRNA maturation. RNA polymerase II- or III is responsible for pri-microRNA transcription. 3’ and 5’ overhangs of pri-microRNA are removed by Drosha cleavage to obtain pre-microRNA, and then correctly processed pre-microRNA is transferred to the cytosol by exportin-5. In the cytosol, pre-microRNA is further cleaved by dicer 1, double-stranded RNA-specific endoribonuclease (DICER) and loaded on to the RNA-induced silencing complex (RISC) complex, involved in translational inhibition, mRNA cleavage or deadenylation (21).

After cleavage by Drosha/DGCR8 complex, precursor microRNAs (pre-miRNAs) are ready to be exported from the nucleous. Exportin-5 transports pre-miRNAs regardless of their sequence via formation of a comples with Rat sarcoma (RAS)-related Nuclear protein (RAN)-GTP (20). Instead of sequence recognition, Exportin-5 recognizes size and 3' overhang region to ensure transport of only correctly cleaved miRNAs (20, 21).

The sequences of both pre-miRNAs and pri-miRNAs can be changed by adenosine deaminases that act on RNA (ADAR). ADAR changes adenosine into inosine via deadenylation; resulting in base-pair or structural differences. These alterations can influence microRNA maturation or target specificity (21, 24). For instance, editing of pri-miR-142 diminishes its maturation via inhibition of its cleavage via Drosha (25).

MicroRNAs are loaded on to the RISC complex to find their matching mRNAs targets and down-regulate their expression. A loading complex called RISC loading complex (RLC) is crucial for loading of pre-miRNAs in RISC effector protein (17, 20) (Figure.1.4). RLC is composed of Dicer, argonaute RISC catalytic component 2 (Ago2), nuclear receptor coactivator 6 (TRBP), and protein kinase, interferon-inducible double stranded RNA dependent activator (PACT); the former two are obligatory while the latter two serve to enhance the process. Dicer subsequently cleaves the loop structure of the pre-miRNA in order to create the 20-23bp long miRNA. When complementarity is too high in the hairpin region, another cleavage from 3' end of the hairpin by Ago2 is necessary before Dicer can cleave. Ago2 cleaved pre-cursors can then be processed by Dicer. Inhibition of Dicer can diminish or eliminate maturation of microRNAs (21). For example, abnormal cell lineage protein 28 (Lin-

28) decreases lethal-7 (let-7) maturation via preventing Dicer cleavage, and consequently down-regulates mature let-7 expression (26).

After Dicer cleavege, the double stranded microRNAs are dissociated from the complex and separated by various helicases. One stand of the microRNA is usually loaded to RISC and used as a complementary sequence to bind target mRNAs, other stand is disposed (20) Thermodynamics is important for the selection of the guide strand, whose 5' end usually is less stable than the other strand. Selected strand is loaded on RISC-Ago complex to regulate target genes. Degradation of mature microRNAs is still largely an unexplored subject. Argonaute proteins seem to be able to stabilize mature microRNAs, indicating their importance in many steps of the microRNA function (21).

In mammalians, microRNAs recognize their target typically through partial complementation on the 3' prime end of the target mRNAs. A perfect complementary sequence called seed sequence usually located between 2nd and 7rd nucleotides at the 5′ end of microRNAs (27). After recognition of the target sequence, cleavage by Ago can prime target mRNAs for degradation (frequently observed in plants) but rare in animals. Alternatively, mRNA is destabilized via different processes such as deacetylation of polyadenosine tail to remove protection from exonucleases. In some cases, microRNAs down-regulate directly the protein levels without interfering with the mRNA levels, but through leading to translation inhibition. Although the exact mechanism for this is unknown, microRNA-RISC complex is shown to be involved in the inhibition of translational initiation or elongation (17, 20).

1. 4. Relationship between UPR and microRNA

As microRNAs are involved in nearly all cellular mechanisms, they are also important in controlling the UPR. Several studies have shown that various microRNAs are both regulated by the UPR or can regulate the UPR arms. Most of the UPR regulation on miRNAs is observed at the transcriptional level. For example, miR-346 is regulated by XBP1. Subsequently, miR-346 directly targets ER antigen peptide transporter 1 (TAP1), which is associated with MHC class I-associated antigen presentation (28) (Figure 1.5)

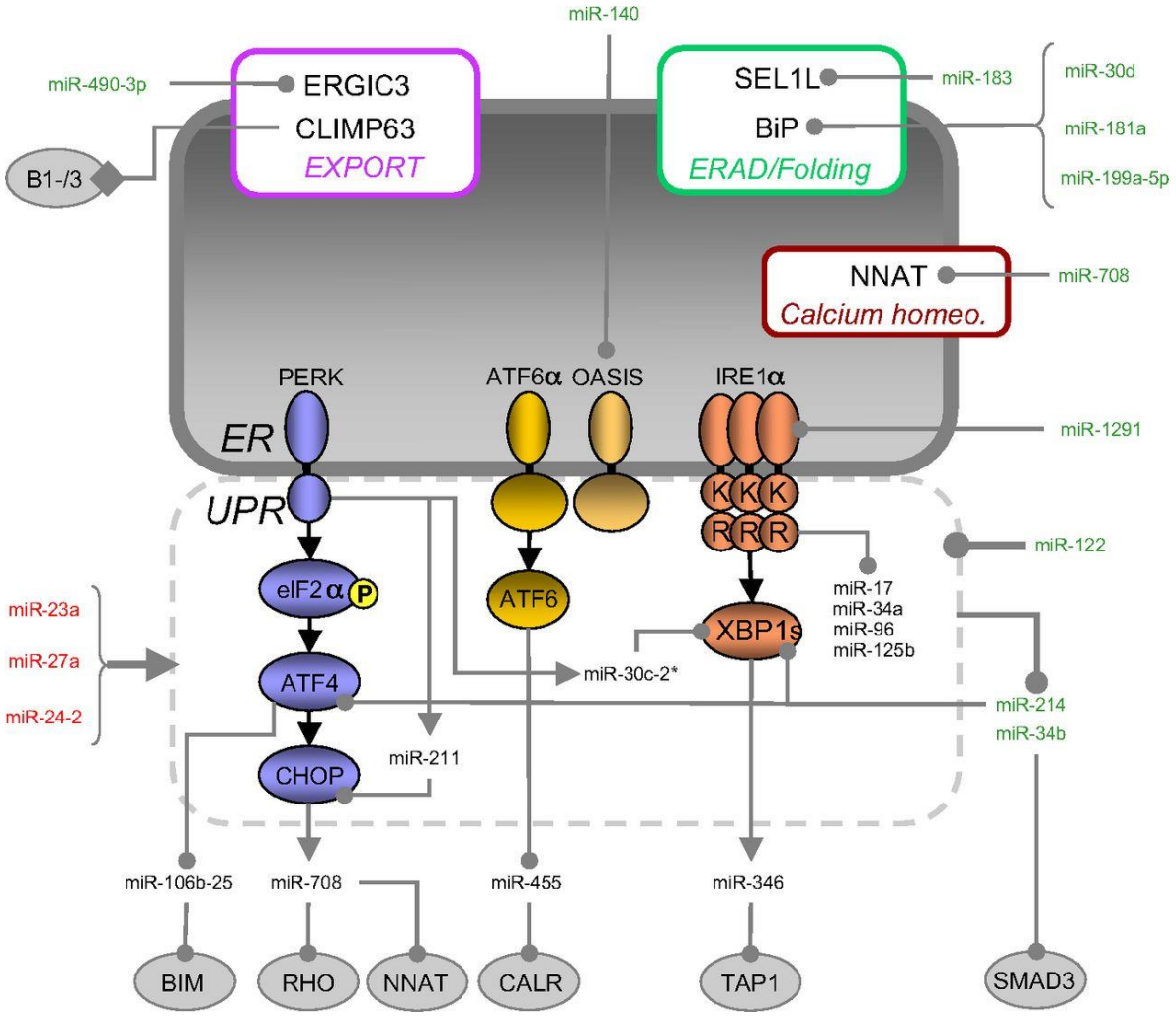

Figure.1.5: ER stress and microRNAs. MicroRNAs are known to be upstream or downstream of the four ER-regulated mechanisms- protein export and folding, ERAD and calcium homeostasis. Green indicates down-regulation while red indicates up-regulation by the upstream microRNAs. miRNAs that are regulated downstream of the UPR elements are depicted in black.(11)

A high-throughput study on ATF6 regulation on microRNAs also revealed a set of microRNA controlled by ATF6. The study found eight miRNAs are down-regulated and five are up-regulated by ATF6 in a miRNA array from ATF6 transgenic mice. One of the down-regulated microRNAs, miR-455, controls the expression of calreticulin, whose function is thought to be related to hypertrophic growth in pathologic heart (29).

ATF4, regulated by PERK arm of the UPR, is also implicated as an important transcription factor in microRNA regulation. In one study, CHOP regulated microRNAs were analyzed using genome wide microRNA profiling. Expression of miR-708, which is located in the intron of teneurin transmembrane protein 4 (TENM4) gene was shown to be controlled by CHOP, which in turn is regulated by ATF4 (30).

Aside from transcription factors, a recent study demonstrated a novel way for microRNA regulation. In this study, the endoribonuclease activity of IRE1α was shown to selectively degrade a set of microRNAs; miRs -17, -34a, -96, and -125b. These microRNAs were demonstrated to repress apoptosis by down-regulating caspase-2. Here, IRE1 cleavage of these target miRNAs (instead of Dicer) leads to their decay. Downregulation of miR-17 also stabilized thioredoxin-interacting protein (TXNIP), which can promote inflammation and apoptosis via formation of the nucleotide-binding oligomerization domain receptors (NLR) family, pyrin domain containing 3 (NLRP3) inflammasome.(31)

Several studies reveal microRNAs regulate the UPR. For example, miR-199a2/214 cluster, whose expression is down-regulated in many hepatocellular carcinoma samples, targets

XBP1. As an efficient promoter of survival, upregulated XBP1 is implied to contribute to cancer development (32). miR-30c-2-3p is another example of microRNAs targeting XBP1.

PERK is implied in the upregulation of miR-30c-2-3p, which in turn down-regulates XBP1, demonstrating a possible link between PERK and IRE arms of the UPR via a microRNA bridge (33). Another study that identified microRNA controlling the UPR involves the. miR-663, which downregulates ATF4 upon ER stress (34).

Understanding the relationship between microRNAs and UPR elements can illuminate many processes leading to pathologic conditions. Since both microRNAs and UPR are indicated in several diseases such as cancer, diabetes, obesities, atherosclerosis, it would not be a surprise to discover a functional link between miRNAs and UPR contributing to the pathogenesis of these diseases.

1. 5. ER stress and Chronic Diseases

Due to its various important functions in cellular processes (such as lipid and protein synthesis, calcium homeostasis, autophagy) ER has been always a vital element to understanding how cells function in normal or disease conditions. Therefore it is not surprising that ER stress is associated with many diseases, including cancer, diabetes, obesity, and atherosclerosis (35, 38, 41) (Figure.1.6). Understanding the underlying molecular mechanism that link ER stress to chronic and complex diseases could generate better diagnostic tools or treatment options for patients suffering from these diseases.

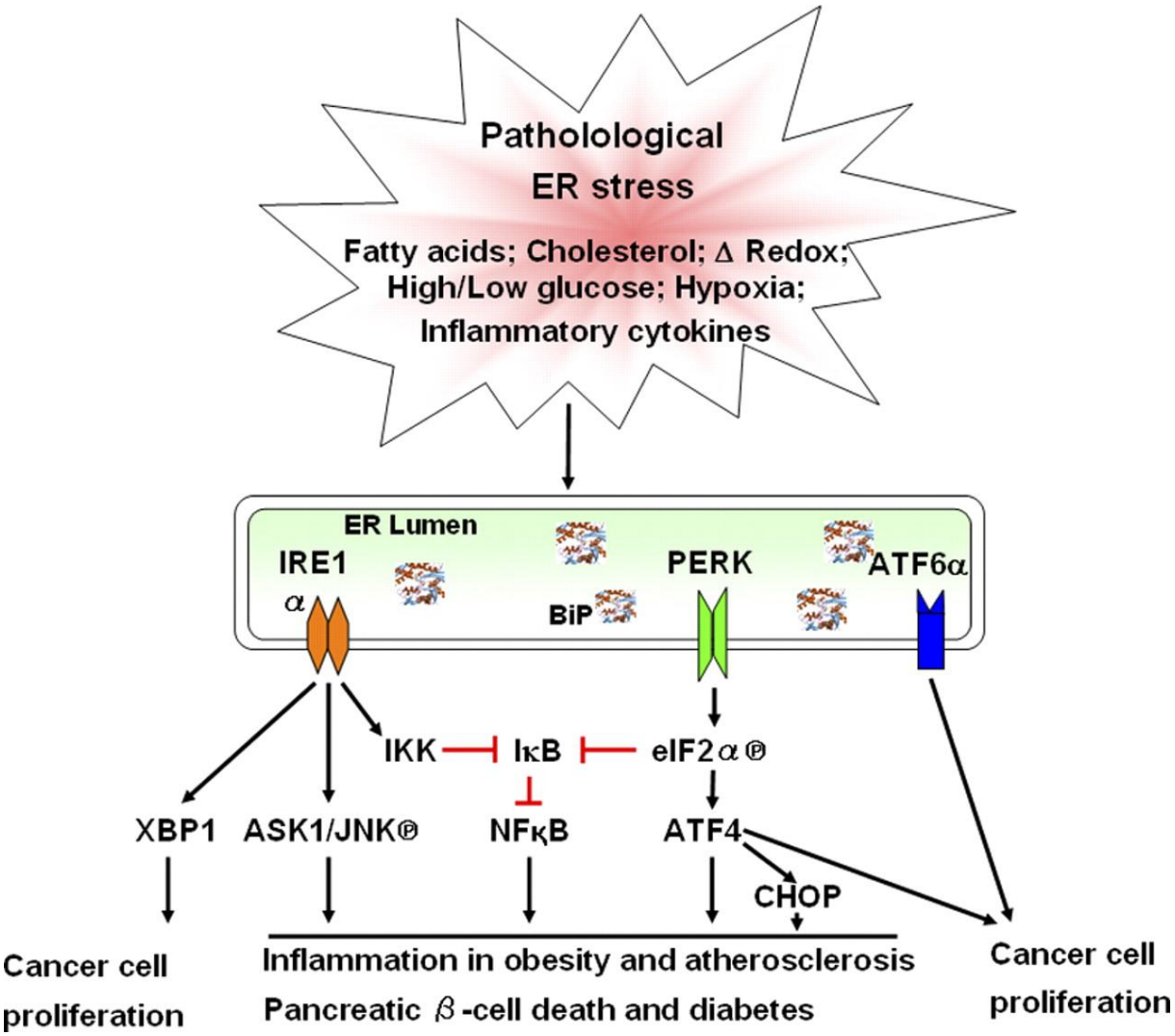

Figure.1.6: ER Stress and diseases. Various stimuli such as excess fatty acids, cholesterol, or reduced glucose, hypoxia and cytokines can activate the UPR, which is shown to contribute to complex diseases including cancer, obesity, atherosclerosis or diabetes. (68)

The relationship between UPR and cancer can be illustrative. Cancer cells are prone to divide without inhibition, hence requires a very high rate of protein production. Increase in the need for protein synthesis and folding is one of the first reasons for UPR activation in these cells (36). Moreover, cancer cells often experience nutrient deprivation and hypoxia, which are also strong stimulators of the UPR. Indeed, many tumors have demonstrated high ER stress and subsequent UPR activation. Early adaptive response to ER stress is thought to promote survival of cancer cells and help them to avoid apoptosis (35,36). For instance, chaperone

proteins like GRP78/BiP seems to be important for survival and drug resistance in several cancer types. Fibroblastoma cells with decreased GRP78 expression cannot form tumors in vivo, or heterozygous GRP78 background extends the latency and reduce tumor growth significantly (37). XBP1 and eIF2α also enhance survival of cancer cells via decreasing the protein load and restoring ER function (35).

Importance of ER stress in metabolic diseases such as obesity and insulin resistance is also demonstrated. Elevated ER stress markers are detected in obese mice, especially in hepatic and adipose tissues (38). In mutant mice bearing a non-phosphorylatable mutant of eIF2α, or lacking a copy of XBP1 (in the XBP1-deficient heterozygous mice) leads to increased susceptibility to insulin resistance after high fat diet. Similar upregulation of the UPR elements are also observed in humans – for example various ER stress markers including ATF6, PERK and XBP1 are activated in adipocytes of obese patients whereas weight loss is shown to be negatively correlated with ER stress and insulin resistance in obese patients after bariatric surgery (38, 39).

Although all cell types are affected by ER stress, β–cells in the pancreas are especially susceptible to ER dysfunctions and critical for the organism due to its role in secretion insulin. It has been shown that β–cell development is ceased in PERK knock-out mice or in the transgenic mice with non-phosphorylatable mutant of eIF2α (39, 40). As more insulin secretion is required during obesity; demand on the ER function is sharply increased, which cannot be compensated without PERK arm of UPR response. PERK mutations in humans also facilitate susceptibility to type-2 diabetes due to decrease in β–cell mass (40).

Accumulating evidence in the literature suggests that ER stress is also connected to atherosclerosis. Oxidized phospholipids trigger the UPR mechanism, which leads to

inflammatory cytokine secretion in these cells. Inflammatory cells sense these cytokines and infiltrate the surrounding plaque area (41). Decreased expression of ATF4 or XBP1 diminishes this UPR-dependent inflammation, which shows the atherogenesis promoting effect of ER stress in endothelial cells. For vascular smooth muscle cells, mechanism of the involvement of ER stress is not clear. In a study, bortezomib is used in aortic tissue of the Apolipoprotein E (ApoE) knockout mice as an ER stress inducer to display a correlation between UPR activation and decreased collagen and vascular smooth muscle cells (42). Most noticeable ER stress in experienced by macrophages during atherogenesis. Although apoptotic cells can be tolerated in early plaques, in advanced plaques, apoptosis is sharply triggered by chronic ER stress subsequently causing necrotic cores (43). The CHOP pathway is required for ER stress-triggered apoptosis in macrophages. Decrease in CHOP expression reduces necrotic core formation in advances plaques (41, 43). Other elements in the UPR such as IRE1 and JNK (downstream of IRE1) are also implicated in ER stress- triggered apoptosis. Both the inhibitions of JNK and IRE1 decrease apoptosis moderately in macrophages taken from ApoE knockout mice (43). Moreover, metabolic diseases like diabetes and obesity may contribute to severe ER stress in macrophages via increased exposure to saturated free fatty acid levels in the adipose tissue or in the circulation. Saturated fatty acids such as palmitate are known inducers of ER stress, expediting UPR activation, but the exact mechanism is unclear (42).

1. 6. MicroRNA in diseases

Since microRNAs are involved in regulation of almost all cellular processes, it is not surprising to detect microRNA deregulation in many pathological conditions. While their expression can be altered directly via genetic mutations or epigenetic modification, it is also possible to impair microRNA function by regulating activity of transcription factors or

microRNA maturation processes (21). Dysregulation of microRNAs cause distortions in gene expression profiles and these alterations can initiate or accelerate pathological conditions.

Cancer is one of the most studied examples of the diseases where the contribution of microRNA alters progression or initiation of a disease. MicroRNAs, can act as tumor suppressor or oncogenes (44). Tumor suppressor microRNAs hinder tumorigenesis and they are downregulated in cancer. miR-15a and miR-16 are examples for such microRNAs. They normally induce apoptosis and impede tumor formation by targeting B-cell lymphoma 2(BCL2), Wilms tumor 1 (WT1) and Ras-Related Protein Rab-9B (RAB9B) in chronic lymphocytic leukemia, prostate cancer and multiple myeloma (45). Oncogenic functions were also demonstrated for the others as well: let-7, miR-29, miR-145, miR-221, miR-222miR-34 and miR-17–92 cluster. Opposite to the tumor suppressor miRNAs, the mir17-92 cluster is known to stimulate proliferation by targeting BCL2-like 11 (BCL2L11), Phosphatase and tensin homolog (PTEN) and E2F transcription factor 1 (E2F1) and its expression is increased significantly in breast, stomach lung, colon, and pancreatic cancers (46).

MicroRNAs are also important regulators in metabolic diseases like diabetes and obesity. For example; miR-34a promotes programmed cell death of α–cells, miR-375 and miR-9 are important in insulin secretion, mir-122 regulates lipid biosynthesis, specifically cholesterol (47). The exact mechanism of microRNAs cannot be easily revealed and studies show a very wide selection of genes can be affected by these microRNAs. Some microRNAs are also observed in more than one disease type, such as mir-15a. Aside from its role as tumor suppressor, mir-15a is significantly associated with insulin biosynthesis (48, 50). Another example of a miRNA linked to metabolic diseases is the miR-17-92 cluster, oncogenic microRNA family. Expression of this microRNA cluster is increased in early stages of adipocyte differentiation, implying its role in adipogenesis. Adipose tissue, as triglyceride

storage and a regulator of energy homeostasis, is important organ in metabolic diseases like obesity in which fats accumulate excessively (49).

Specific microRNA expression can hasten or impede atherosclerosis development. Mice deficient in miR-143 and 145 display a higher rate of atherosclerosis due to disturbed smooth muscle homeostasis (51). miR-143 and 145 are important in smooth muscle cell differentiation, and display an anti-atherogenic affect. mir-126 promotes similar effects of endothelial cells by boosting repair and protecting against angiogenesis (51). miR-712 expression is stimulated by disturbed flow and increase the inflammatory response in endothelial tissues, and atherogenesis (52). Another pro-inflammatory factor, miR-155, inhibits Bcl-2, and consequently initiates inflammatory response via activation of nuclear factor-κB pathway. miR-342 regulates the expression of mir-155 via Akt1 pathway, representing a complex regulation pattern of microRNAs (53).

Understanding microRNA regulation during disease development can enable novel therapatic approaches to the diseases. For instance, several in vivo studies demonstrated the protective effects of miR-33 silencing. miR-33 regulates cholesterol homeostasis by targeting cholesterol transporters ABCA1 and ABCG1 and reducing cholesterol efflux (53). Silencing of miR-33 in the ApoE knockout mice prevents atherosclerosis (54). However, contradicting reports on the mir-33 involvement in atherosclerosis progression has been published in studies using the LDL-receptor knockout mice (55, 56). Nevertheless, a study in non-human primates shows that plasma high density lipoprotein levels increased after miR-33a/b inhibition, while very low density lipoprotein levels decrease, implying possible role of miR-33a/b in human atherogenesis (57). In these studies further regulation of targets like carnitine palmitoyltransferase 1A (CPT1A), hydroxyacyl-CoA dehydrogenase (HADHB), and carnitine

O-octanoyltransferase (CROT), important protein in fatty acid oxidation, by miRNA33 further underscore the complexity of miRNA-gene networks that must be carefully deciphered in order to develop miRNA-based therapeutics for the future (58).

1. 7. Macrophages in Disease

As a vital defense mechanism the immune system protects the organism from various pathogens in the environment, however, dysfunction in inflammatory responses may also lead to a variety of diseases. While it is clear that precise regulation of the innate and adaptive response is obligatory, it is still not entirely understood how distortions in immune mechanism cause such conditions. One of the best examined examples is atherosclerosis, whose initiation and progression is dependent on infiltration of macrophages to the lesion (59).

Atherosclerosis is a chronic inflammatory condition which progress mainly by forming plaques in vascular areas experiencing disturbed laminal flow, such as arterial branching points. Initiation of the lesion occurs at endothelial damage sites where the accumulation of lipoproteins containing apolipoprotein B (with neutral lipids at the center) follows (59, 60). Accumulation and the modification of the lipoproteins in the plaque area is followed by the attraction of monocytes the injury site. Interaction of monocytes with factors like P-selectin glycoprotein ligand-1 supports attachment to the endothelium and infiltration (59, 60). Further chemokine secretion attracts more monocytes to the lesion. Following this infiltration, monocytes differentiate with the help of differentiation factors such as macrophage colony-stimulating factor (60). After differentiation, macrophages start to form lipid-loaded cells (also known as foam cells) as a result of hydrolysis of lipoproteins and their subsequent re-esterification to cholesteryl fatty acid esters. (Figure.1.7) Cholesterol efflux in macrophages at

this stage is quite important as defects in the ABCA1 and ABCG1 can accelerate atherogenesis (61). Inflammatory response originating from the infiltrating macrophages consequently escalates the lesion development. Factors like Nuclear Factor kappa B (NF-κB) signaling and regulation of anti- or pro-inflammatory cytokine secretion are parts of the inflammatory response worsening plaque stability. The inflamed and toxic environment leads to more macrophage apoptosis (62). The formation of the necrotic core, as a result of the macrophage death, and the weakening of the overlying fibrous cap are indicators of the advancing atherosclerotic plaque, which becomes vulnerable to rupture. UPR pathways are thought to be responsible for macrophage apoptosis in advanced plaques due to chronic ER stress in the macrophages (62).

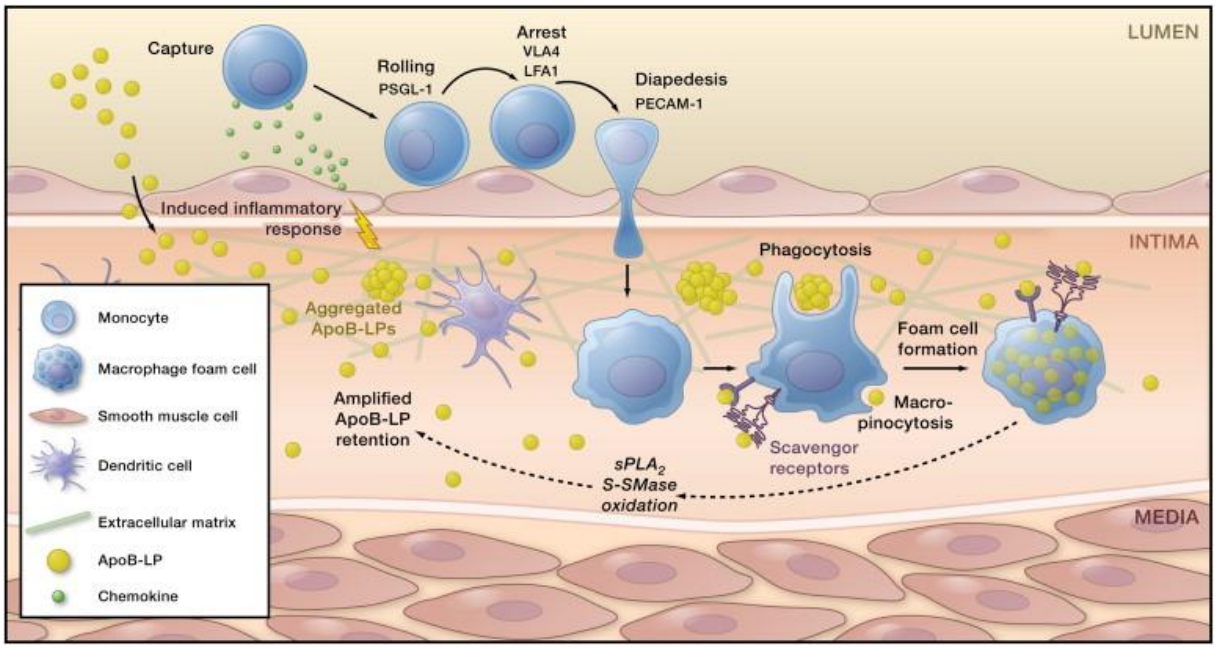

Figure.1.7: Macrophages in Atherosclerosis. ApoB containing lipoproteins aggregate and induce pro-inflammatory responses. Chemokine secretion attracts more monocytes to the site and the increased expression of adhesive molecules in the lesion endothelial cells promoters further infiltration of the plaques by the monocytes. Monocytes differentiate into macrophages and uptake lipoproteins via phagocytosis as they turn into lipid-laden foam cells. (43)

Aside from its established role in atherosclerosis, macrophages also contribute to obesity. Studies in obese mice models revealed a correlation between obesity and macrophage count in the adipose tissue. Accumulated macrophages secretes inflammatory factors to enhance inflammation (63). Chronic inflammation due to macrophage accumulation is demonstrated to play a central role in obesity related insulin resistance (64). During obesity, macrophages migrate into fat and change their morphology from M2 to M1, which increase the inflammatory capacity of the macrophages (65). Moreover, a recent study also showed that not only migration to adipose, but also emigration of macrophages from the tissue is regulated during obesity. Saturated fatty acids like palmitate can increase the secretion of factors from macrophages, which in turn decrease, their motility and cause the retention of macrophages in the tissue (66).

2. MATERIALS AND METHODS

2. 1. Materials

2. 1. 1. General Lab Materials and Kits

Methanol, Absolute Ethanol, Trizma Base, Sodium Chloride, Ampicillin, Bradford Agent, STF-083010, Phosphatase Inhibitor Cocktail 3 from Sigma-Aldrich (St.Louis, MO, USA), Isopropanol from EMD Millipore (Billerica, Massachusetts, USA), HEPES, Bovine Serum Albumin from Santa Cruz (Dallas, USA), DMSO, EDTA from AppliChem (Dermstadt, Germany). Tween from AMRESCO (Ohio, US) Biomax Agarose from Prona (The European Economic Community), Ammonium persulfate from Carlo Erba(Italy), Tryptone, Yeast extract, Bacto Peptone, Agar from Conda (Madrid, Spain), Amersham ECL Prime Western Blotting Detection Reagent from GE Healtcare(Buckinghamshire, UK), PureLink® Quick Plasmid Miniprep Kit, PureLink® HiPure Plasmid Midiprep Kit from Life Technologies, (Carlsbad, California, U.S.) Zymoclean™ Gel DNA Recovery Kit from Zymo Research

Corporation(CA, USA), HyClone® HyPure Molecular Biology Grade Water, PageRuler Plus Prestained Protein Ladder and Spectra Multicolor Broad Range Protein Ladder, PVDF Transfer Membrane, Neon™ Transfection System from Thermoscientific, RevertAid First Strand cDNA Synthesis Kit (Waltham, Massachusetts, USA), Acrylamide: Bis-Acrylamide 29:1 (40% Solution/Electrophoresis) from Fisher BioReagents (Waltham, Massachusetts, USA), LightCycler 480 Multiwell Plate 96, LightCycler 480 SYBR Green from Roche(Upper Bavaria, Germany), miScript SYBR Green PCR Kit and miScript II RT Kit, Hs_EIF2AK2_6 siRNA, Hs_ERN1_5 siRNA from Qiagen(Venlo, Netherlands)

2. 1. 2. Cell Culture Materials

Gibco Dulbecco's Phosphate Buffered Saline from Life Technologies, FBS, Tripsin, L-Glutamine (Carlsbad, California, U.S.), Costar® 6 and 12 Well Clear TC-Treated Multiple Well Plates, Cryogenic Vials, Costar® 5, 10, 25mL Stripette® Serological Pipets, Corning® 25cm, 75cm flask, Cell Lifter from Corning Incorporated (NY, USA), HyClone RPMI, DMEM from Thermoscientific(Waltham, Massachusetts, USA), CELLSTAR Cell Culture Dishes 100 and 145 from Greiner Bio One(Wemmel, Belgium)

2. 1. 3. Enzymes and Antibodies

Taq DNA Polymerase, DpnI, Phusion Hot Start II High-Fidelity DNA Polymerase, from Thermoscientific(Waltham, Massachusetts, USA), NotI, HindIII-HF from New England Biolabs(Ipswich, MA, UK)

Table.2.1: Antibodies used in this study.

Antibody Name	Catalog #	Company	Usage
IRE1 Phospho (pS724) (ERN1)	ab124945	Abcam	1:2000 for 1 hr

IRE1α (14C10) Rabbit mAb	3294S	Cell Signalling	1:2000 for 1 hr
Phospho-PERK(Thr980) (16F8)	3179S	Cell Signaling	1:1000 for O/N
β–Tubulin (H-235)	sc-9104	Santa Cruz	1:2000 for 1 hr
β–Actin (C-4)	sc-47778	Santa Cruz	1:1000 for 1 hr
Goat Anti-mouse IgG-HRP	sc2004	Santa Cruz	1:8000 for 1 hr
Goat Anti-rabbit IgG-HRP	Sc2005	Santa Cruz	1:8000 for 1 hr

2. 1. 4. Primers

For microRNAs, miScript primer assays for mmu-miR-16, mmu-miR-2137, mmu-miR-33, mmu-miR-146a, mmu-miR-486, mmu-miR-20, mmu-miR-17, mmu-miR-18, mmu-miR-129, mmu-miR-93, mmu-miR-322, mmu-miR-361, mmu-miR-674, mmu-let-7d from Qiagen(Venlo, Netherlands)

Table.2.2: List of primers used in this study.

Primer Name	Sequence
m_u/spc_XBP1_forw	TGAGAACCAGGAGTTAAGAACACGC
m_u/spc_XBP1_rev	CCTGCACCTGCTGCGGAC
m_spc_XBP1_qPCR_forw	TGAGAACCAGGAGTTAAGAACACGC
m_spc_XBP1_qPCR_rev	CCTGCACCTGCTGCGGAC
m_Lkb1_forw	CACACTTTACAACATCACCA
m_Lkb1_rev	CTCATACTCCAACATCCCTC
m_NOXA_forw	CCACCTGAGTTCGCAGCTCAA
m_NOXA_rev	GTTGAGCACACTCGTCCTTCAA
m_Inppl1_forw	ACTCTGCGTCCTGTATCAAAAG

m_Inppl1_rev	CAGGGCACAAACAAGACCC
m_Rgp1_forw	ACTGTCACTAATCCCCTTCCC
m_Rgp1_rev	GGCAGTGCTACTCGACTCTC
m_Fastk_forw	GGGGAGTCATGGTCTCCAC
m_Fastk_rev	GACTTGCTGGGTCCCAAACAA
m_Zfp185_forw	GAGGATCATACCATAGAGCCACT
m_Zfp185_rev	GACCTGGCATTTGATTCGTCT
m_Psd_forw	GGGCAGTGGCAACGAAGAT
m_Psd_rev	AGCCCAGAGTGAGGCATGT

2. 1. 5. Solutions:

50X Tris-Acetate-EDTA (TAE) : 242 g Trizma Base, 37.2 g EDTA (Tritiplex 3) and 57.1 ml Glacial Acetic Acid; completed to 1 lt with ddH2O, and autocleaved.

10X Phosphate Buffered Saline (PBS): 80 g NaCl, 2 g KCl and 15.2 g sodium phosphate dibasic dehydrate; completed to 1 lt with ddH2O, and autocleaved.

10X Tris Buffered Saline (TBS): 87.76 g NaCl, 12.19 g Trizma Base, pH 8.0 with HCl; completed to 1 lt with ddH2O, and autocleaved.

Luria-Bertani Medium (LB medium): 10 g NaCl, 10 g Tryptone, 5 g Bacto-yeast extract; completed to 1 lt with ddH2O, and autocleaved.

Agar medium: 10 g NaCl, 10 g Tryptone, 5 g Bacto-yeast, ; completed to 1 lt with ddH2O, and autocleaved. 1 ml 100 mg/ml Ampicillin is added in warm solution and poured on plates

100 mg/ml Ampicillin: 1 g Ampicillin in 10ml autocleave ddH2O; stored at -20 in aliquots

HBS Buffer: 21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM Glucose; filtered and kept in 4C.

1.5 M Tris-HCl (pH: 8.8): 54.45 g Trizma Base; pH: 8.8 with HCl and completed to 300 ml with ddH2O.

1.5 M Tris-HCl (pH: 6.8): 54.45 g Trizma Base; pH: 6.8 with HCl and completed to 300 ml with ddH2O.

10% Resolving Gel: 2,5 ml 40% Acrylamide mix, 2,5 ml 1.5 M Tris-HCl (pH: 8.8), 100 µl 10% SDS and 100 µl 10% Ammonium persulfate, 4 µl 0.08% TEMED; completed to 10 ml with ddH2O

5% Stacking Gel: 620 µl 40% Acrylamide mix, 630 µl 1.5 mM Tris-HCl (pH: 6.8), 50 µl 10% SDS and 50 µl 20% Ammonium persulfate, 5 µl 0.1% TEMED; completed to 5 ml with ddH2O

5X Running Buffer: 15 g Tris Base, 72 g Glycine, 5 g SDS; completed to 1 lt with ddH2O.

5X Transfer Buffer: 14.5 g Glycine, 29 g Trizma Base (29 g/liter), 1.85 g SDS; completed to 1 lt with ddH2O. 2%0 methanol is added when 1X Transfer is added.

Phospholysis Buffer: 50 mM HEPES, 100 mM NaCl, 4 mM Na4P2O7, 10 mM EDTA, 10 mM NaF, 1% Triton, 2 mM Na3VO4, 1 mM PMSF, Phosphatase Inhibitor Cocktail 3 and 1X Protease Inhibitor Cocktail.

Tris Buffered Saline with Tween: 50 ml 10X TBS, 500 µl Tween; completed to 500ml with ddH2O.

5 % Bovine Serum Albumin/Milk in TBS-T: 2 gr BSA/milk powder; completed to 40 ml with TBS-T.

2. 2. Methods:

2. 2. 1. Cell Culture:

Cell lines used in the study; RAW 264.7 macrophages, MEF WT, MEF IRE-/-, MEF PERK -/-, also bone marrow derived primary macrophage(BMDM) are used. RAW 264.7 macrophages are grown in 10% heat activated FBS, 1% L-glutamine supplied RPMI medium.

FBS is incubated in 55C for 1 hr for heat inactivation. MEF cell lines are grown in 10% FBS, 1% L-glutamine supplied DMEM medium. BMDM are incubated in conditional RPMI medium for 10 days before usage.

2. 2. 2. Cell Treatments:

Palmitate treatment is performed in no serum RPMI medium. First, 0,4 gr BSA is dissolved in no serum RPMI(1% BSA) and filtered. Before addition of palmitate, BSA is pre-warmed in 55C water bath. After BSA is added, medium is vortexed and incubated at 55C until palmite dissolved mostly. Ethanol is used as negative control.

Thapsigargin, PERK and IRE RNase inhibitors, 1NM-PP1, 1NA-PP1 directly solved in FBS supplied RPMI/DMEM medium. If treated together with palmitate, no serum RPMI is used. DMSO is used as negative control. In combined treatment with palmitate, both ethanol and DMSO are added as negative.

2. 2. 3. Electroporation:

Before electroporation, cells should cover around 80% and medium should be changed one day before to increase viability. If cells are good for electroporation, tripsin or scraper is used to take them in to a falcon. After cells are mixed, 10 µl is taken to find cell count. Required amount of medium of cells are separated into another falcon and precipitated at 500RPM for 4 min. Medium is sucked and precipitated cells are washed with pre-warmed dPBS two times at 500 RPM for 4 min. PBS is removes and cell are dissolved in HBS or R buffer. For each shot, total cell count should not pass 6 10^6 and 120 µl HBS/R buffer is used. In each well, 2,5 x 10^6 cell for RAW 264.7, 10^6 cell for MEF, 2 x 10^6 cell for BMDM is used with 1,5 or 2 ug of plasmid DNA. Optimized electroporation conditions for RAW 264.7 is 1680V, 20ms, 1 pulse; for MEF 1050V, 30ms, 2 pulse; for BMDM 1500V, 20ms, 1 pulse. Same samples are

collected in common pool and divided to separate wells to ensure homogeneity. 6-well plates are used for protein and 12-well plates for RNA. All RNA samples are prepared in quadruplicates.

2. 2. 4. Total Protein Isolation:

150- 200 μl phospholysis buffer is prepared for each well in 6-well plate and added to plates on ice. Cell are incubated for 5-10 minutes. Plates are shaken or scraped to deattach all cells and lysate is collected in pre-cooled 1,5 ml eppendorfs. Then, lysate is centrifuged at 13000 RPM for 12 minutes. Supernatant is collected in another set of eppendorfs and precipitated cell debris is discarded. 2 μl is taken from each sample and added to 48 μl grade water for concentration measurement. 450 μl Bradford solution is added and after 5 min incubation, absorbance values are read at 595A. 2 μl phospholysis buffer is used as blank. Measurements are done twice for each sample. After measuring absorbance, concentrations are calculated using BSA curve and protein samples are diluted to same concentration. 5x SDS loading dye is added to each well and samples are boiled at 95C for 5 min. After short spin down, proteins either loaded in SDS-polyacrylamide gel or stored at -20 C.

2. 2. 5. SDS-Polyacrylamide Gel Electrophoresis:

1,5mm glass and thin glass is placed on gel casting apparatus without any leakage. 10% resolving gel is prepared and poured between glasses and isopropanol is added on 10% gel. After gel is solidify, isopropanol discarded and 5% stacking gel is poured. Comb is placed on 5% resolving gel and prepared gel is stored at 4C.

Gels are placed in running cassette without any leakage and 1 running buffer is added. Each well is cleaned after comb is removed. 60-70 ug protein and ladder are loaded in wells and gel is run at 100V for 30 min and then 120V for 2-2,5hr.

2. 2. 6. Transfer to PVDF membrane:

Gels are removed from running cassette and glasses are separated. 3 sponges, 4 filter papers are soaked in 1 x transfer buffer. PVDF membrane is activated with methanol and wash with 1 x transfer buffer. 2 sponge, 2 filter papers, gel, PVDF membrane, 2 filter papers, 1 sponge are placed in order without any air bubble. They are placed in transfer cassette in correct way and pre-cooled 1 x transfer buffer is poured. Transfer is done in cold environment at 95V for 2 hr.

2. 2. 7. Blocking, Washing, Antibodies Incubation and Detection:

After transfer, membrane is washed with TBS-T and blocked in TBS-T supplied with 5% BSA or milk for 1 hr at RT or O/N at 4C. After blocking, primary antibody in same blocking solution is added and incubated as described in antibodies section. 5, 5, 15, 5 minutes wash with TBS-T is done after primary and membrane is incubate with suitable secondary antibody in same blocking solution for 1 hr. After secondary, membrane is again washed for 5, 5, 15, 5 minutes with TBS-T. Then, membrane is incubated with ECL solution for 5 minutes in dark. Excess ECL is removed and membrane is placed on glass. Films are developed to catch fluorescence and detect bound antibodies.

2. 2. 8. Total RNA isolation:

1ml Trisure is added to each well on ice and incubated for 5-10 minutes. Cells are collected into pre-cooled eppendorfs and 200 µl chloroform is added to each sample. Eppendorfs are shaken vigorously for 15 sec, and incubated for 3 min at RT. After incubation, eppendorfs are centrifuged at 14000 RPM for 15 min at 4 C. Three phases are separated after centrifuge and upper aqueous phase is taken to new eppendorfs carefully without disturbing other phases.

500 µl isopropanol is added to aqueous phase and mixed by inverting 3-5 times. After 10 min incubation at RT, samples are centrifuged at 13000 RPM for 10 min. Supernatant is removed with micropipettes and precipitated RNA is washed at 8000 RPM for 8 min with 75% and 100% ethanol in order. After 100% ethanol is removed, precipitate is air-dried for 5 min. 25 µl nuclease free water is added and dissolved. 1 µl is used in nanodrop to measure RNA concentration and samples are diluted to same concentration. Diluted RNAs are stored at -80.

2. 2. 9. cDNA prepration:

1000 nmol RNA is used to prepare cDNA. For microRNA analysis, miScript RT kit is used. 1 µl RT mix, 2 µl hiSpec Buffer, 1 µl nucleotide mix is added to 1000 nmol RNA and completed to 10 µl with nuclease free water. Mix is incubated at 37C for 1 hr and at 95C for 5 minutes. For mRNA analysis, RevertAid First Strand cDNA Synthesis Kit is used. 1000 nmol RNA is mixed with 1 µl oligodt and completed to 6 µl with nuclease free water. Mix is incubated at 65C for 5 min. After incubation, 0,5 µl RT, 0,5 µl ribolock, 1 µl nucleotide mix, 2 µl RT buffer is added and incubated at 42C for 1 hr, then 70C for 5 min. All cDNAs are diluted 1/10 after reaction with nuclease free water.

2. 2. 10. Semi-quantative Polymerase Chain Reaction (PCR):

2 µl of diluted cDNA is used in the reaction. 2,5 µl 10x Taq Buffer, 0,2 µl 25mM dNTP, 2,5 mM 25mM MgCl2, 0,75 forward and reverse primers, 0,5 Taq polymerase is added and completed to 25 µl with nuclease free water. PCR reaction; initial denaturation at 95C for 3 min, cycle 35, denaturation at 95 for 30 sec, annealing at 60 for 30 min, elongation at 72 for 45 sec, final elongation at 72C for 10 min. After reaction is completed, PCR products are run on 2% agarose gel at 100V for 1hr.

2. 2. 11. Real time quantitative Polymerase Chain Reaction (qPCR):

2 µl of diluted cDNA is used in the reaction. For microRNAs, miScript SYBR qPCR kit is used. 1 µl Universal primer, 1 µl primer assay, 5 µl SYBR mix added and completed to 10 µl with nuclease free water. qPCR reaction; initial hold at 95 C for 15 min, cycle 45, denaturation at 95 for C 15 sec, annealing at 55 C for 30 sec, elongation at 70 C for 30 sec., final hold at 95 C for 5 sec. Melting curve from 80 to 95; 1 degree for 5 sec.

For mRNAs, LightCycler 480 SYBR Green is used. 5 µl SYBR mix, 1 µl forward and reverse primers are added and completed to 10 ul. qPCR reaction; initial hold at 95 C for 15 min, cycle 45, denaturation at 95 for C 10 sec, annealing at 60 C for 30 sec, elongation at 72 C for 30 sec., final hold at 95 C for 5 sec. Melting curve from 80 to 95; 1 degree for 5 sec.

Significance of all results are measured with two-tailed student T-test.

2. 2. 12. Restriction Enzyme Digestion, Ligation:

500ng Empty pSUPER DNA is used for restriction. 0,3 µl NotI, 0,3 µl HindIII, 1 µl (10X)BSA, 1 µl (10X)NEB4 Buffer is added and completed to 10 µl with nuclease free water. Reaction mix is incubated at 37C for 3 hr. Restriction products are run on 0,5% agarose gel, at 100V for 1 hr. Uncut vector is used as negative control. Mir2137 insert is amplified from mouse genomic DNA isolated from RAW 264.7 cells and NotI and HindII restriction sites are added to each side. Insert is also restricted and run on the gel. 1 to 7 plasmid: insert ratio is used. 0.5 µl T4 Ligase enzyme, 1 µl T4 Ligase Buffer is added and completed to 10 µl with nuclease free water. Samples are incubated overnight at 16- 18°C. Ligated plasmid is cut and run on the gel to check the insertion.

2. 2. 13. Transformation, Colony PCR:

50 µl competent cells are thawed in ice. Ice cold 100pg ligated plasmid is added gently. The mixture is placed on ice for 30 minutes. Heat shock is applied at exactly 42°C for exactly 30 seconds. Then cells are placed on ice for 10 minutes. 250 µl of room temperature LB is added into the mixture near fire and incubated at 37°C for 60 minutes at 225 rpm. 225 and 25 µl of mixture is spread on warm selection plates to 37°C and incubated 16 hr at 37°C. Competent cell without DNA is used as negative. 6 colonies are selected from plate and colony PCR is done. For colony PCR, 2 µl of the 5X Phire enzyme buffer, 0.2 µl forward and reverse primers, 0.3 µl DMSO, 0.8 µl MgCl2, 0.2 µl dNTP, 0.2 µl Phire Hot Start Enzyme is added and completed to 10 µl with nuclease free water. Tips are put into ampicillin added LB. Colony PCR products are run on the gel and DNA from correct colonies are taken with miniprep.

2. 2. 14. Midiprepi, miniprep:

PureLink® HiPure Plasmid Midiprep and Miniprep Kit is used according to manufacturer's protocol. To solve the DNA, 35-50 µl nuclease free water is used for miniprep; 100-200 µl nuclease free water is used for midiprep.

3. EXPERIMENTAL RESULTS

3. 1. mmu-mir2137 and mmu-mir33 are IRE1-regulated miRNA during lipotoxic endoplasmic reticulum stress in macrophages

3. 1. 1. Verification of Palmitate regulated miRNAs

Palmitate is the most abundant saturated free fatty acid in serum and its levels are shown to increase significantly during metabolic conditions like obesity mostly due to increased dietary uptake. Increased exposure to palmitate can alter the expression profile of a cell significantly. Based on this assumption, we performed a collaborative study with Assist. Prof. Dr. Arzu Atalay group at Ankara Biotechnology Institute to analyze PA-regulated miRNAs in macrophages with high throughput miRNA PCR array. We selected highly regulated miRNA known to be relevant to metabolic conditions (30, 54, 67) and performed qPCR to validate the PCR array results and PCR results were normalized to SNORD61 and miR-16 levels(Figure 3.1). Since miR-16, often used as a normalization control, levels displayed more stable cT values across treatment, all miRNAs were measured relative to miR-16 levels. Among these miRNAs, two of them were found significantly upregulated – miR-2137 and miR-486 – and one downregulated – miR-33 (70).

miR-486 was shown to control expression of sirtuin 1 (SIRT1), a central regulator in mechanisms related to aging and metabolic disorder (67). miR-2137 was already shown to be regulated ER stress in other cell types, however, the exact mechanism has not been explored (30). miR-33 targets cholesterol transporters ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1), ATP-binding cassette, sub-family G member 1 (ABCG1) and shown to be important in development and progression of atheroschlerosis (57). Moreover, miR-33

antagomir treatment in both mice and non-human primates demonstrate beneficial effect in atherosclerosis models (54, 55).

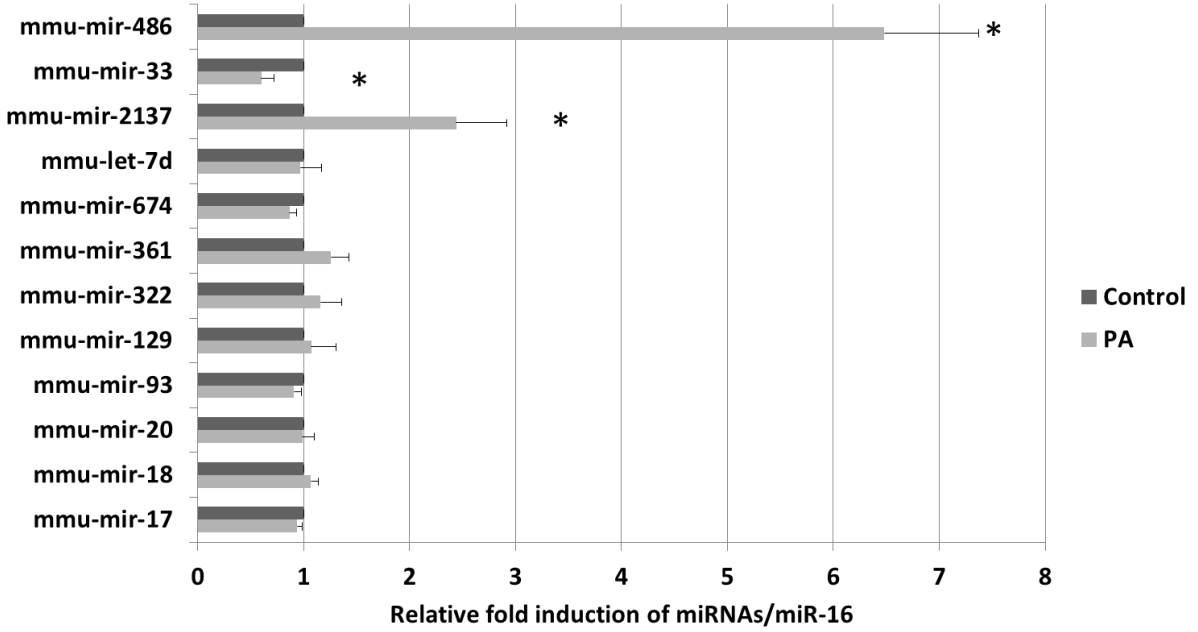

Figure.3.1: Verification of palmitate-regulated miRNAs. RAW 264.7 macrophages were treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for 9 hours. Following total RNA isolation, complementary DNA (cDNA) was generated with miScript reverse transcription kit. Mature miRNA levels were measured with qPCR and normalized to miR-16 levels (n = 3, two-tailed student T-test performed * = $p < 0.05$).

3. 1. 2. IRE1 or PERK Regulated mmu-miR-2137

After palmitate induction of miR-2137 was validated, we checked the possible role of UPR in this regulation. Since it is known that palmitate causes ER stress in macrophages and miR-2137 is upregulated by ER stress (6), we began to examine the contribution of the UPR arms using siRNA mediated knock-down approach in RAW 264.7 cell line. Western blot showed that IRE and PERK protein levels were suppressed considerably with siRNA treatment (Figure 3.2A). After both IRE or PERK knock-down, induction of miR-2137 by PA was markedly blocked compared to scrambled siRNA transfected samples (Figure 3.2B).

However, downregulation was not complete, suggesting that only knock-down of IRE or PERK was not sufficient to completely eliminate palmitate induced upregulation in miR-2137 levels and that both UPR arms contribute to PA induced upregulation of miR-2137.

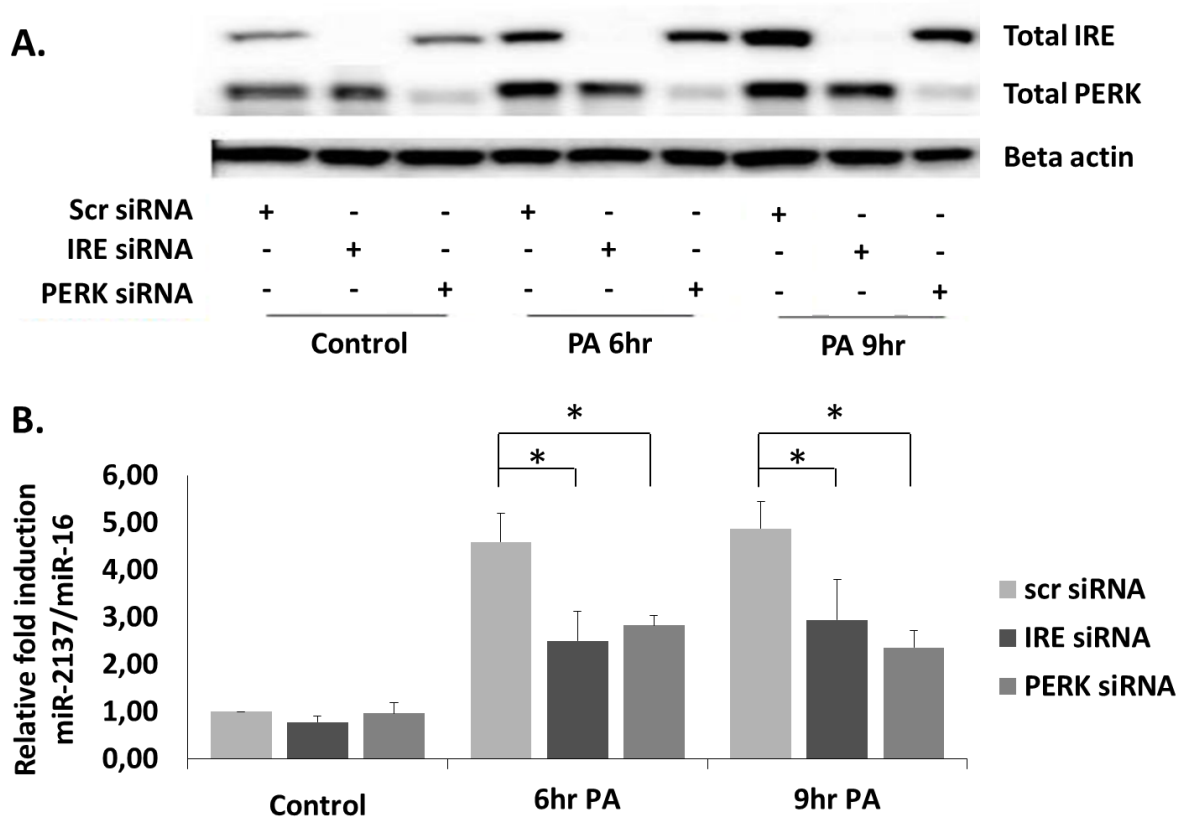

Figure.3.2: Mature miR-2137 levels are regulated by IRE1 and PERK in macrophages. RAW 264.7 macrophages were transfected with siRNA against IRE1(40nM), PERK(60nM) or scrambled siRNA(60nM, control). Then cells were treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for 6 or 9 hours. (A) Western blot analysis for total IRE and PERK proteins to show the efficiency of the knock-down. (B) Mature miR-2137 levels were measured with qPCR and normalized to miR-16 levels (n = 3, two-tailed student T-test performed * = $p < 0.05$).

Since we observed a significant decrease in palmitate induced miR-2137 upregulation in both 6 and 9 hours after IRE knock-down, we decided next to measure miR-2137 at the earlier hours for temporal regulation of miR-2137 expression by PA. We especially focused on IRE1 due to its endoribonuclease activity, which can regulate miRNA maturation directly (31). We treated RAW 264.7 cells with PA for 1, 2 and 4 hours after IRE and PERK knockdown via siRNA.IRE1 and PERK protein levels were decreased significantly with siRNA treatments (Fig.3.3.A). Mature miR-2137 levels started to increase at 2 hours after PA treatment and IRE1's inhibitory effect on miR-2137 rise became evident at 4 hours post PA treatment (Fig.3.3B). Another interesting observation was that the increase in mature miR-2137 levels started at the same time with the increase in spliced XBP1 mRNA, suggesting IRE1 endoribonuclease activity may be acting on both sXBP1 and mature miR-2137 at the same time (Fig.3.3B and Fig.3.3C). Further direct analysis is needed to confirm pre or pri-miR-2137 cleavage by IRE1 endoribonuclease domain.

Although we suspected that IRE1 endoribonuclease domain is responsible for miR-2137 regulation, the siRNA knock-down experiment down regulates both kinase and endorbionuclease activities. To assess the direct role of IRE1 endoribonuclease activity, we utilized specific inhibitor against this domain. Phosphorylated IRE levels (p-IRE) were unaffected by the inhibitor treatment, suggesting that kinase activity is intact and IRE1 can auto-phosphorylate itself (Figure 3.4A). Treatment with endoribonuclease inhibitor successfully diminished the splicing of the XBP1 mRNA, indicating that endoribonuclease domain was inhibited (Figure 3.4B). Inhibition of IRE1 endoribonuclease led to significant reduction in miR-2137 levels normally induced by PA demonstrating a role for IRE1's endoribonuclease domain (Figure 3.4C).

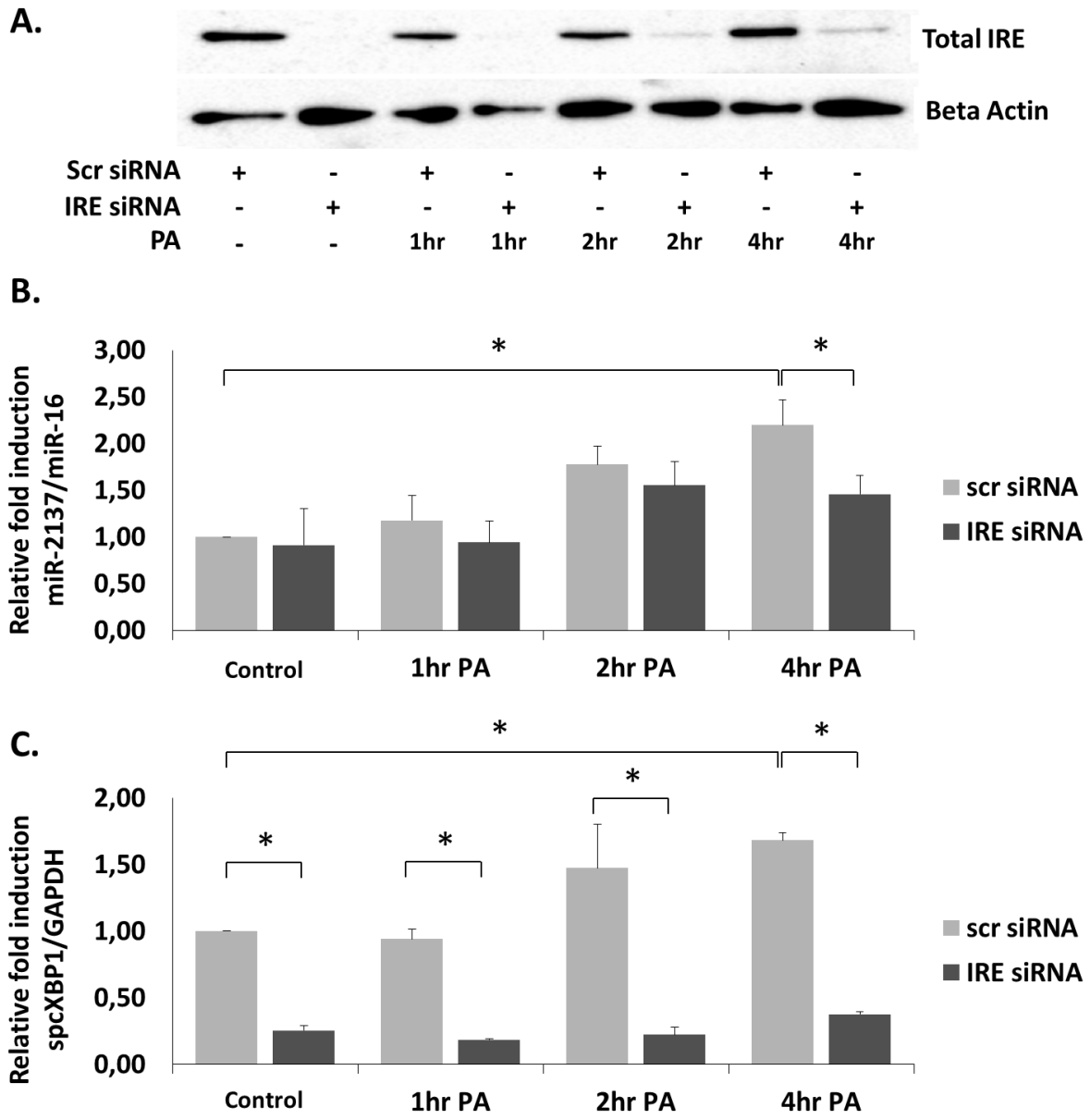

Figure.3.3: Mature miR-2137 levels are regulated by IRE1 in a time course. RAW 264.7 macrophages were transfected with 40nM siRNA against IRE. Then cell were treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for 1, 2 and 4 hours. (A) Western blot analysis for total IRE and PERK proteins shows efficiency of the knock-down. (B) Mature miR-2137 levels were measured with qPCR and normalized to miR-16 levels. (C) qPCR detects spliced XBP1 levels relative to GAPDH (n = 3, two-tailed student T-test performed * = $p < 0.05$).

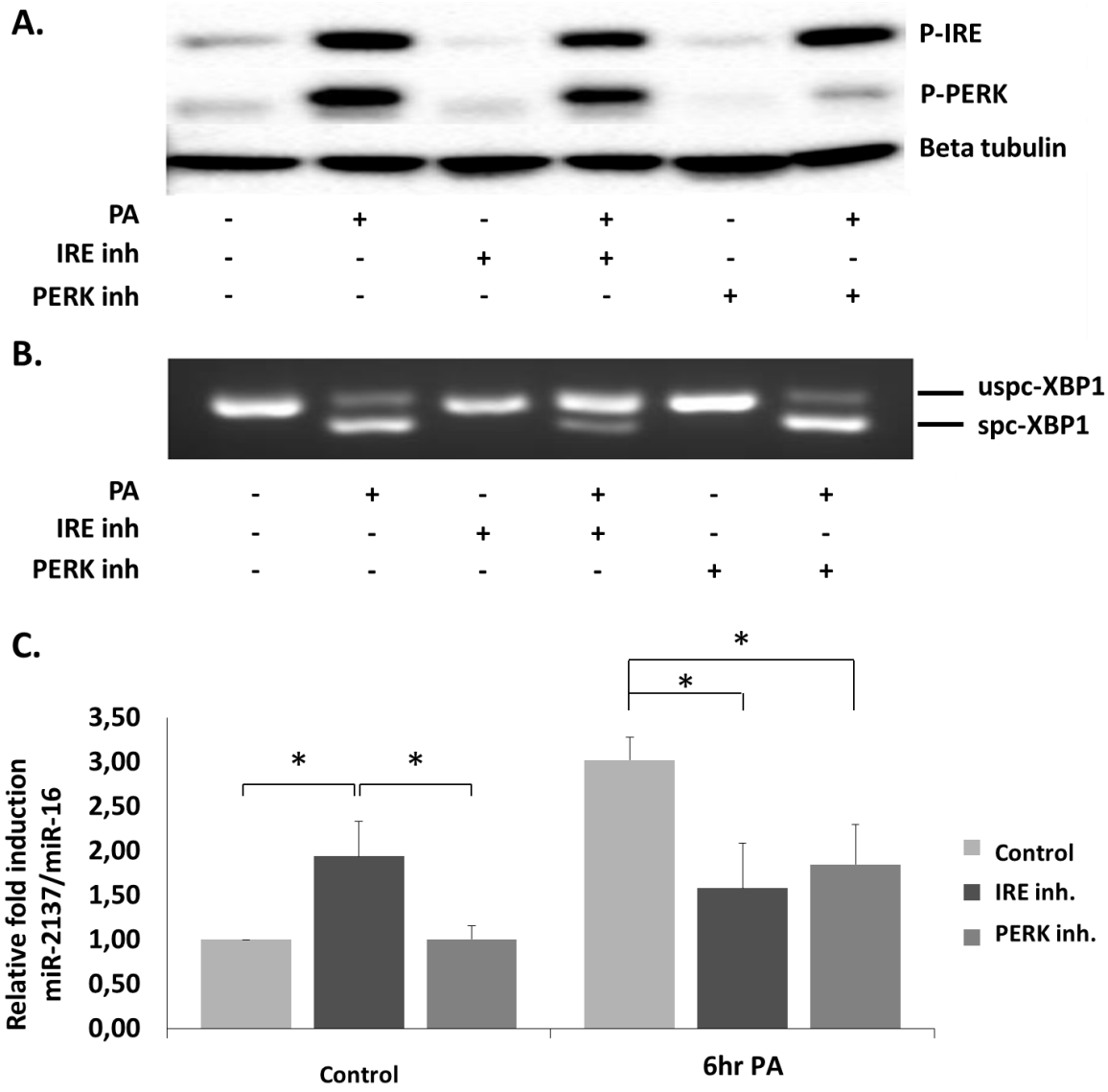

Figure.3.4: Regulation of mature miR-2137 levels by IRE endoribonuclease and PERK kinase activity in macrophages. RAW 264.7 macrophages were treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for 6 in combination with 300μM IRE endoribonuclease inhibitor or 1000nM PERK kinase inhibitor. (A) Western blot analysis of p-IRE and p-PERK shows efficiency of inhibitor treatment. (B) Semi-quantitative PCR detects both spliced(bottom) and unspliced(top) XBP1 mRNAs (C) Mature miR-2137 levels were measured with qPCR and normalized to miR-16 levels (n = 3, two-tailed student T-test performed * = $p < 0.05$).

PERK, unlike IRE1, has only a kinase domain. Hence, we expected that PERK kinase inhibitor would give similar results to PERK siRNA experiment. Indeed, the PERK inhibitor led to a marked inhibition of palmitate induced miR-2137 levels. (Figure 3.4A). Combined with our observations from the IRE1 inhibition experiments, both UPR arms contribute to the upregulation of miR-2137 upon PA treatment in macrophages.

To further validate the role of IRE1 endoribonuclease activity we transfected IRE knockout (IRE1 -/-) MEF with wild type (WT) or endoribonuclease inactive (RD/K907A) IRE plasmids. IRE1 protein was detected in both WT and RD transfected cells, while no IRE1 expression is observed in only vector transfected cells (Figure 3.5A). p-IRE was also observed in not treated endoribonuclease inactive overexpressed cells (Figure 3.5A). When IRE1 is abundant in the cell, they can oligorimerize without any stress; and treatment with palmitate increased the p-IRE level even further. Although kinase domain was intact in mutant IRE, only WT IRE had endoribonuclease activity as demonstrated by XBP1 splicing in WT IRE transfected cells (Figure 3.5B). Palmitate treatment increased the mature miR-2137 levels in vector transfected cells. Overexpression of WT IRE1 increased PA-induced miR-2137 levels significantly, while overexpression of the endoribonuclease inactive IRE1 mutant inhibited the upregulation by palmitate (Figure 3.5C). These results clearly demonstrated the role of IRE1 endoribonuclease domain in the maturation of miR-2137.

To complement our observation made in a macrophage cell line primary bone marrow derived macrophages (BMDM) were transfected with IRE siRNA. P-IRE1 levels were decreased significantly with IRE siRNA treatment (Figure 3.6A) Although the effect was not as strong as seen in the RAW 264.7 cell line, IRE1 knockdown also decreased the miR-2137 levels in primary macrophages (Figure 3.6B).

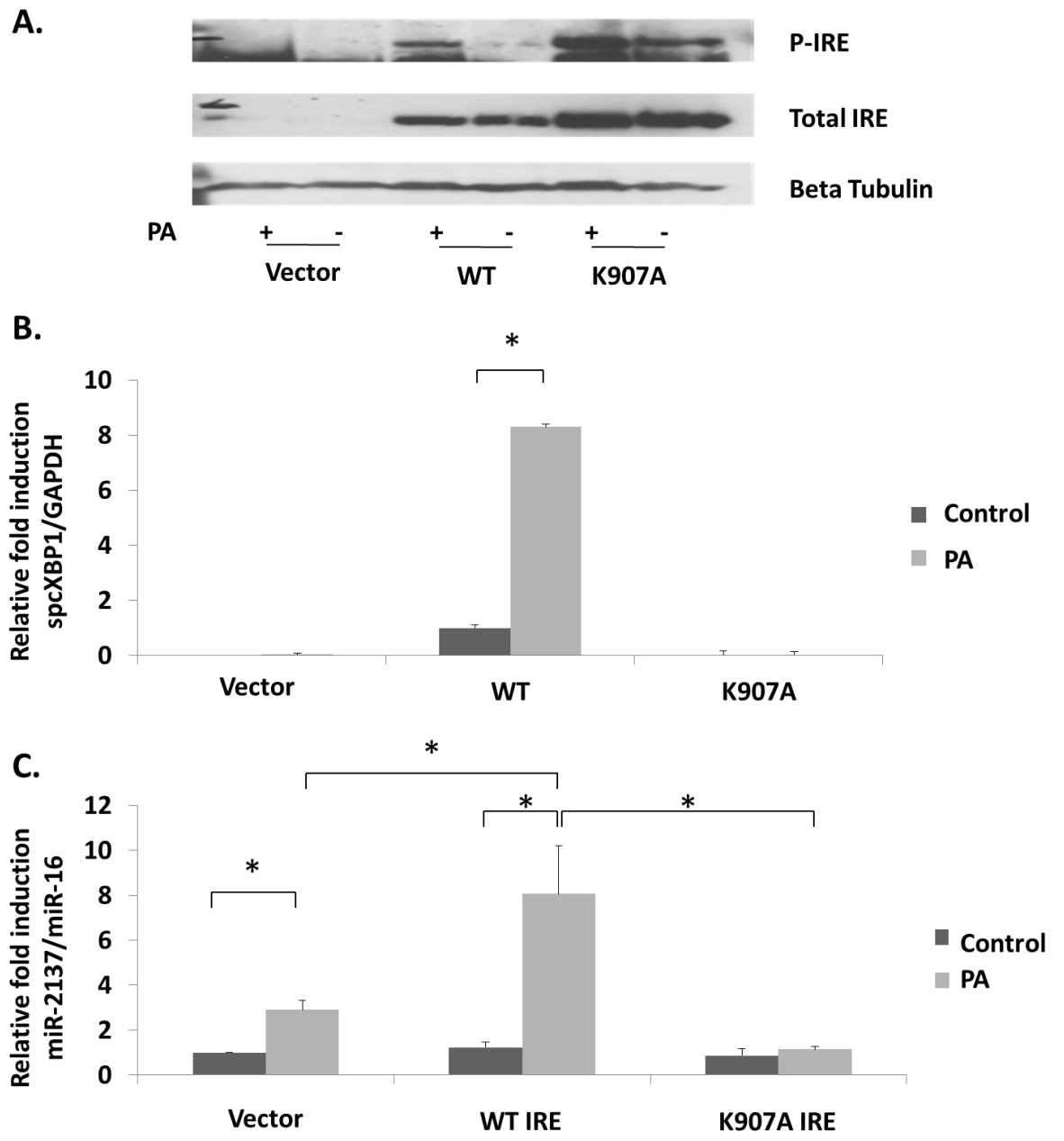

Figure.3.5: Mature miR-2137 levels are regulated my IRE1 in fibroblasts. IRE-/- MEFs were transfected with WT(2ug) or K907A(1,5ug) IRE1 plasmids. Then cells were treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for 12 hours. (A) Western blot analysis of total IRE shows the efficiency of the overexpression. (B) qPCR detects spliced XBP1 levels relative to b-actin (C) Mature miR-2137 levels were measured with qPCR and normalized to miR-16 levels (n = 3, two-tailed student T-test performed * = $p < 0.05$).

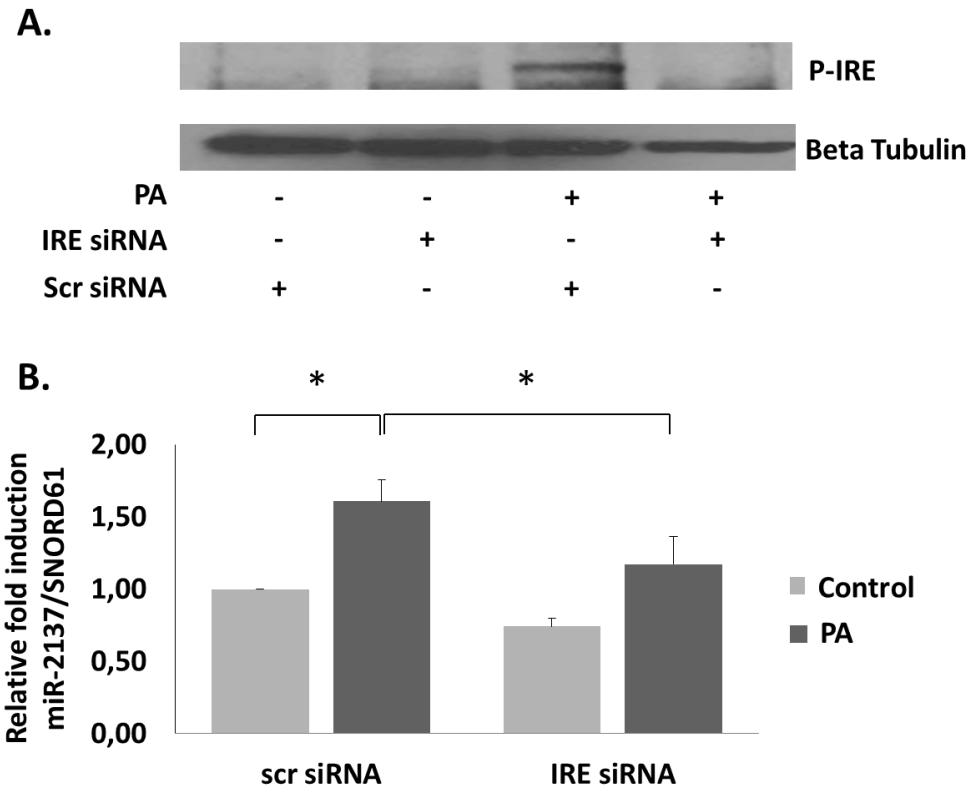

Figure.3.6: Mature miR-2137 levels are regulated by IRE1 in BMDM cells. BMDM cells were transfected with 40nm siRNA against IRE or scrambled siRNA. Then cell were treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for 9 hours. (A) Western blot analysis against p-IRE shows the efficiency of the IRE1 knock-down. (B) Mature miR-2137 levels were measured with qPCR and normalized to SNORD61 (n = 3, two-tailed student T-test performed * = $p < 0.05$).

3. 1. 3. IRE1 or PERK regulated mmu-miR-33

Since miR-33 is also regulated by palmitate, we also checked the possible involvement of IRE1 and PERK in this regultaion. When compared to scrambled siRNA treated samples, IRE1 knock-down with IRE1 siRNA decreased the miR-33 levels in 6 hours (Figure 3.7A). Consistent with RAW 264.7 cells, a decrease in miR-33 levels after both IRE and PERK siRNA transfection was seen in BMDM (Figure 3.7B).

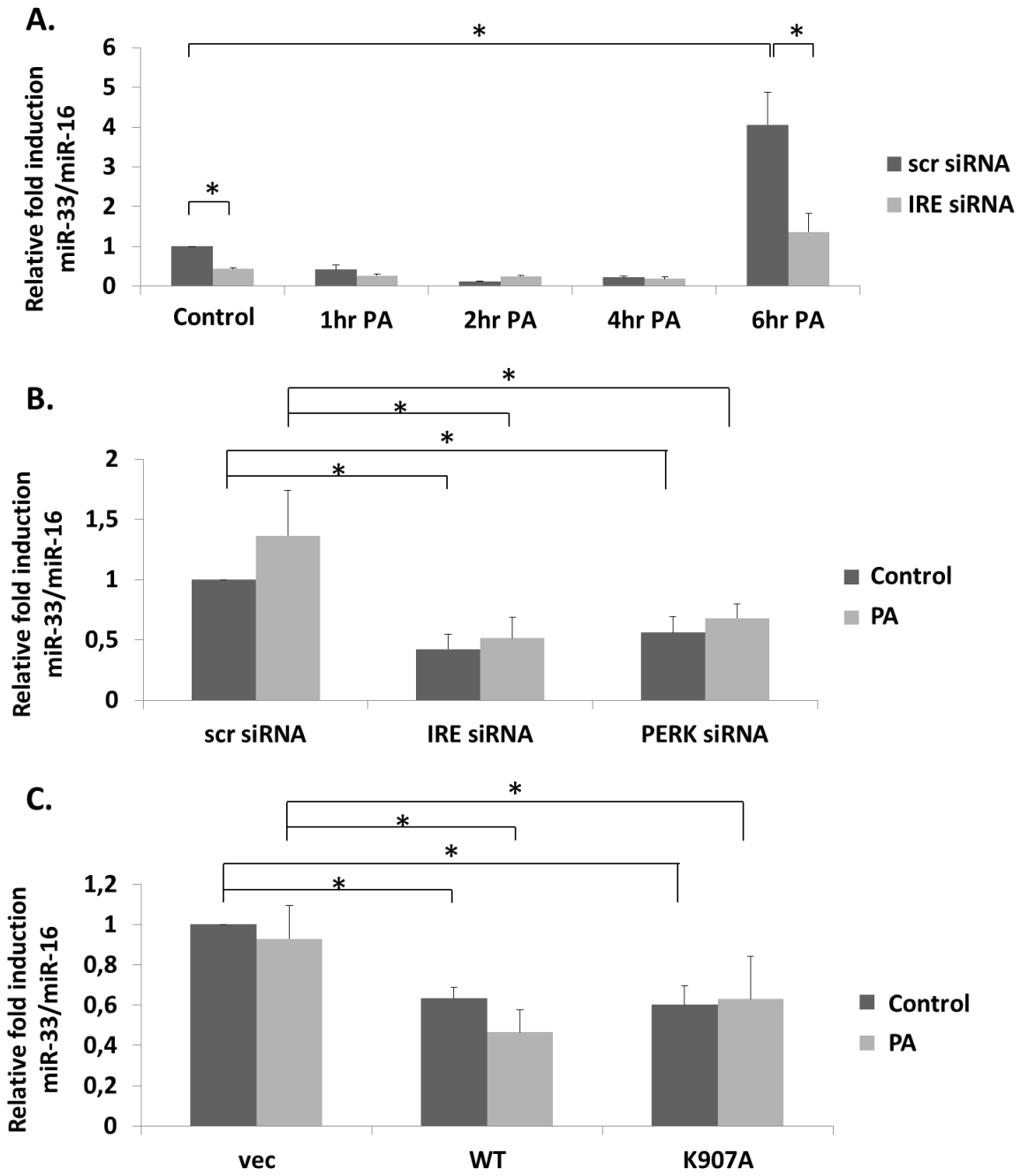

Figure.3.7: Mature miR-33 levels are regulated by IRE1 and PERK. Mature miR-2137 levels were measured with qPCR and normalized to miR-16. (A) RAW 264.7 macrophages were transfected with 40nm siRNA against IRE1 or scrambled siRNA. (B) BMDM were transfected with siRNA against IRE1(40nM) and PERK(60nM) or scrambled siRNA. (C) RAW 264.7 macrophages were transfected with WT(2ug) and K907A(1,5ug) plasmids. (A-C) Then cells were treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for (A) 1, 2, 4, (B) 6, 9 or (C)12 hours. (A-C) Mature miR-33 levels were measured with qPCR and normalized to miR-16 (n = 3, two-tailed student T-test performed * = $p < 0.05$).

Unexpectedly, the overexpression of both WT and RD IRE1 in IRE1 deficient MEF cells increase also decreased the miR-33 levels significantly, suggesting complex feedback loops between IRE1 and miR-33 regulation (Figure 3.7C).

3. 1. 4. IRE1 regulation of mmu-miR-486

miR-486 levels were measured in RAW 264.7 macrophages transfected with IRE1 siRNA. miR-486 levels started to rise at 2 hours post PA-treatment, but IRE knockdown significantly reduced the mature miR-486 levels by 4 hours post PA-treatment. (Figure 3.8)

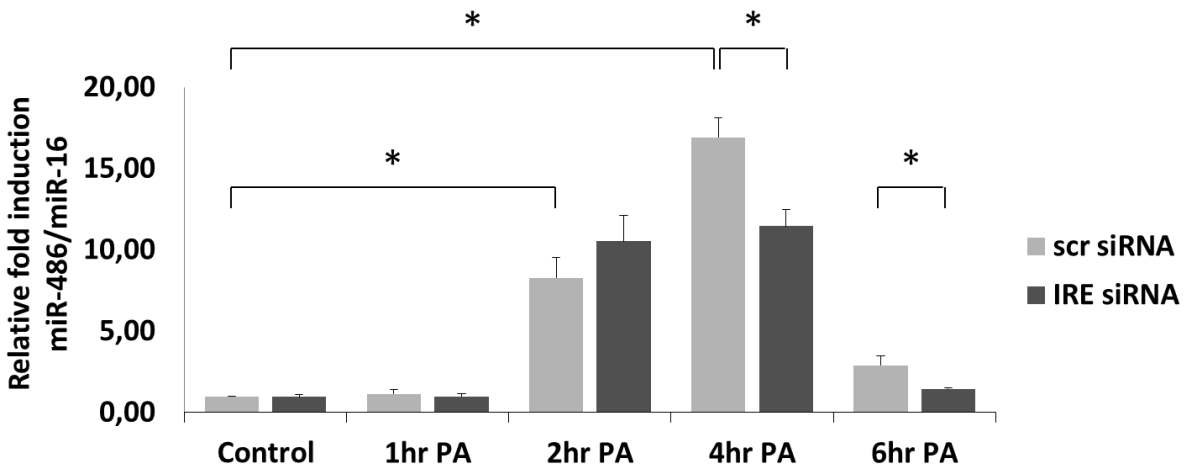

Figure.3.8: Mature miR-486 levels are regulated by IRE1. RAW 264.7 macrophages were transfected via electroporation with 40nm siRNA against IRE1. Then cell are treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for 1, 2, 4, or 6 hours. Mature miR-2137 levels were measured with qPCR and normalized to miR-16. (n = 3, two-tailed student T-test performed * = $p < 0.05$)

3. 2. mRNA targets of IRE1 regulated miRNAs during lipotoxic endoplasmic reticulum stress in macrophages

3. 2. 1. miR-2137 regulated mRNAs:

The results of our experiments demonstrated that miR-2137 is regulated by IRE1. There is no experimentally verified target for miR-2137. Three different target prediction databases are used to find candidate target genes for mi2137 (Table 3.1). Among these candidates, genes with high scores and relevant function were selected. mRNA levels of the selected genes were measured by qPCR from 9 hour PA-treated RAW 264.7 macrophages after anti-miR-2137 or control treatment. Mature mir-2137 levels are increased with palmitate treatement as expected and anti-miR-2137 treatment diminish the mir-2137 levels significantly. (Figure 3.9A) However, only inositol polyphosphate phosphatase-like 1 (Inppl1) showed significant upregulation with miR-2137 inhibitor. (Figure 3.9B)

Target gene		mirSVR	miTG	Database
Fastk	Fas-activated serine/threonine kinase	-0.86	0.814	microRNA, Diana
Inppl1	inositol polyphosphate phosphatase-like 1	-0.76		microRNA
Ltbp3	latent transforming growth factor beta binding protein 3	-0.61		microRNA
Lkb1	serine/threonine kinase 11		0.877	Diana
Rgp1	RGP1 retrograde golgi transport homolog	-0.48		Targetscan

Table.3.1: Selection of possible miR-2137 targets from three different databases. Possible targets are selected according to their miRSVR(microRNA, Targetscan), miTG scores (Diana) and their relevance to metabolic diseases. Lower miRSVR and higher miTG scores corresponds to higher accuracy.

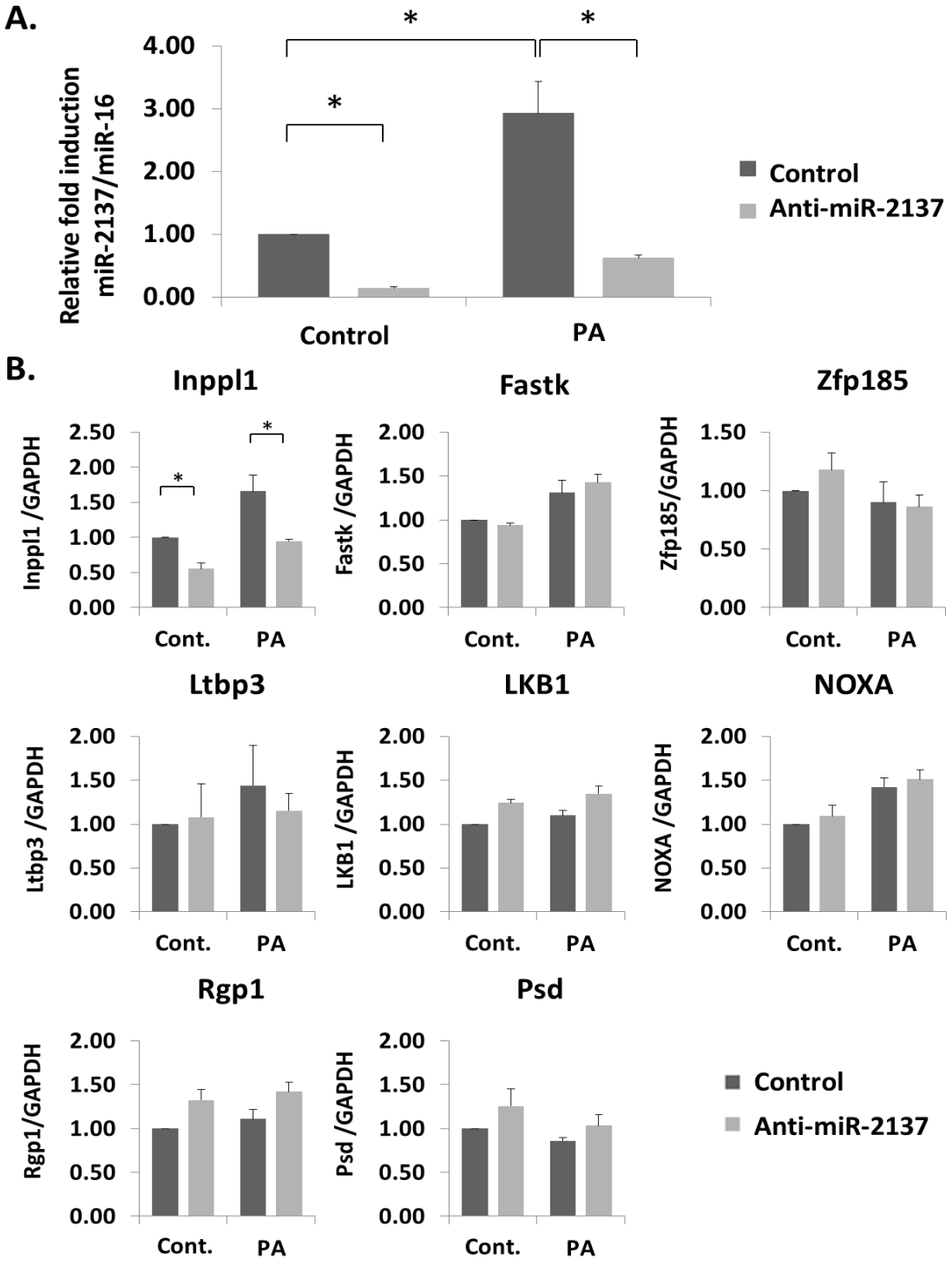

Figure.3.9: miR-2137 regulated mRNAs during lipotoxic ER stress in macrophages. RAW 264.7 macrophages were transfected with 200nm miR-2137 inhibitor or control. Then cells were treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for 9 hours. (A) Mature miR-2137 levels were measured with qPCR and normalized to miR-16. (B) RNA levels of candidate target genes were measured relative to GAPDH by qPCR (n = 3, two-tailed student T-test performed * = $p < 0.05$).

Next, miR-2137 was overexpressed n RAW 264.7 macrophages to validate mRNA regulation of the possible target genes with miR-2137 mimic. Dose-dependent increase in the mature miR-2137 levels were shown in Figure 3.10A. Although Inppl1 levels were effected by

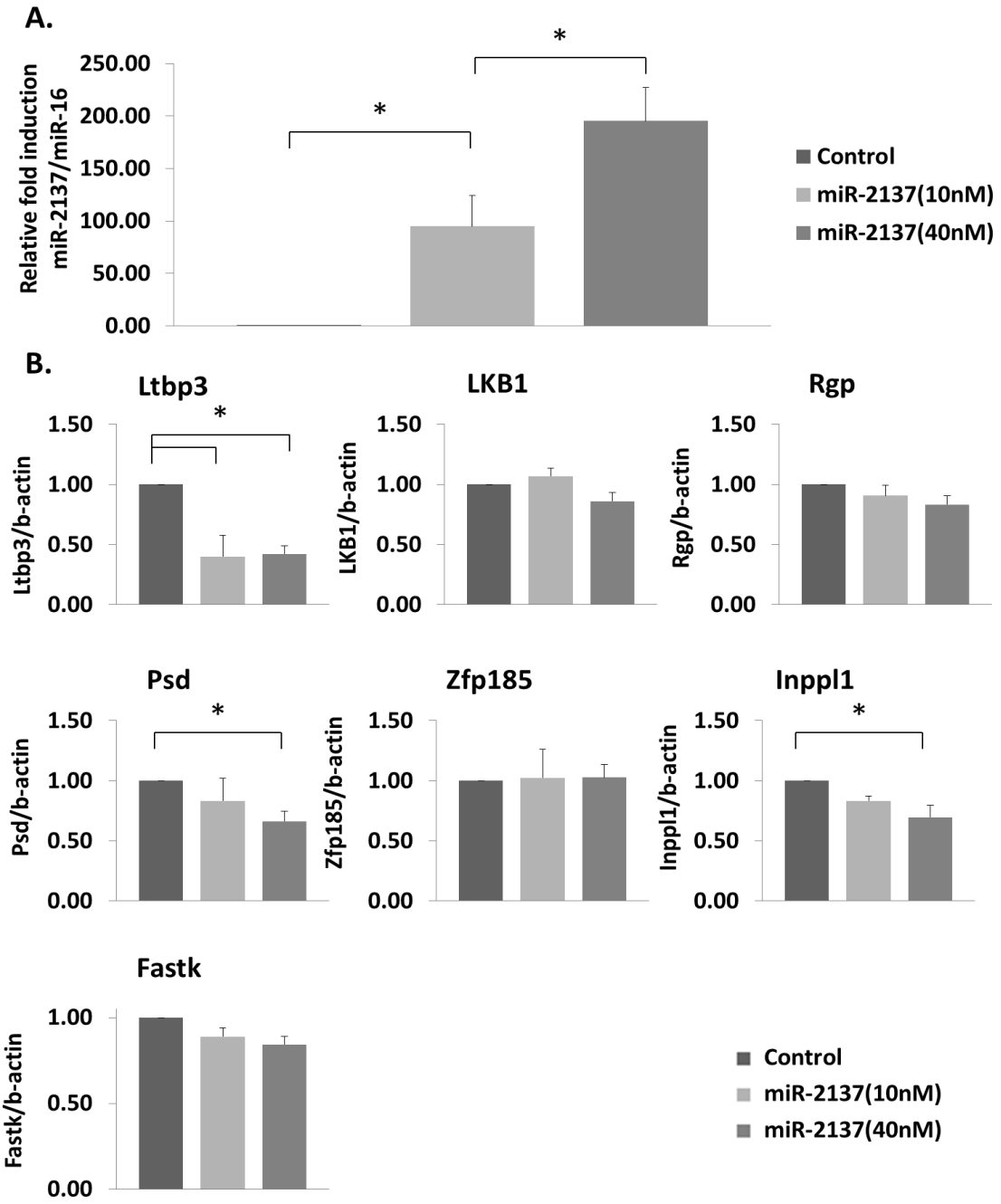

Figure.3.10: miR-2137 regulated mRNAs in miR-2137 overexpressed macrophages. RAW 264.7 macrophages were transfected with 10nM or 40nM miR-2137 mimic or control. (A) Mature miR-2137 levels were measured with qPCR and normalized to miR-16. (B) RNA levels of candidate target genes were measured relative to b-actin by qPCR (n = 3, two-tailed student T-test performed * = $p < 0.05$).

miR- 2137 inhibitor, change in Inppl1 mRNA levels was modest after miR-2137 overexpression and result were, not consistent with miR-2137 knockdown experiments. On the other hand, Ltbp3 mRNA levels decreased two-fold in miR-2137 overexpressed macrophages (Figure 3.10B). Ltbp3 levels were not regulated in miR-2137 inhibitor treated macrophages**,** however, miR-2137 levels were only reduced ten fold versus one hundred fold increase in the miR-2137 overexpression in cells.

3. 2. 2. Analysis of miR-33 target, ABCA1, in Macrophages and IRE1 or PERK -/- MEFs

ABCA1 is a well-known target of miR-33. Since our data suggests that IRE could regulate miR-33 expression, we analyzed the regulation of ABCA levels. ABCA1 levels were indeed higher in IRE1 siRNA treated macrophages compared to scrambled siRNA treatment. (Figure 3.11) However, no change in ABCA1 was seen in PERK siRNA treated samples suggesting a specific effect of the IRE1 branch on ABCA1 through miR-33 regulation.

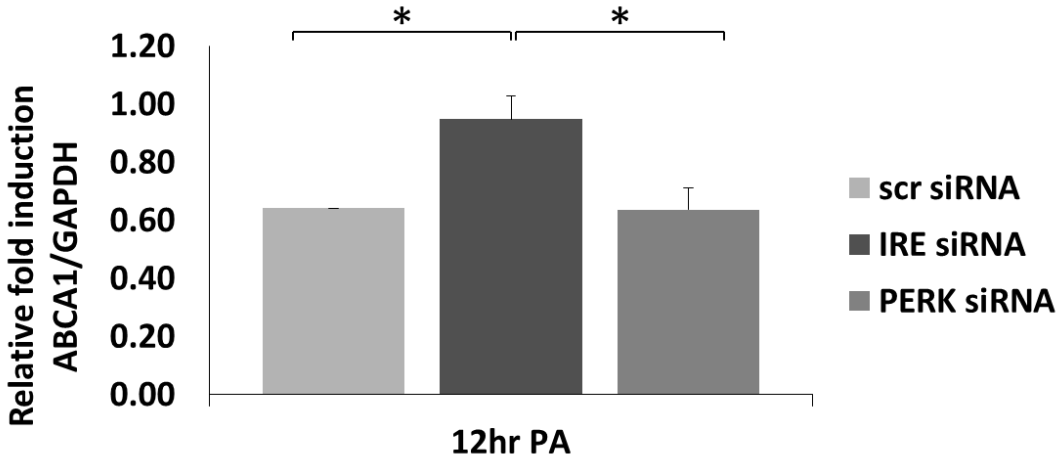

Figure.3.11 ABCA1 mRNA levels are regulated by IRE1. RAW 264.7 macrophages were transfected with siRNA against IRE(40nM) and PERK(60nM). Then cells were treated with or without 1000μM PA in serum free RPMI media supplemented with 1% free fatty acid free BSA for 12 hours. ABCA1 mRNA levels were measured relative to GAPDH mRNA with qPCR (n = 3, two-tailed student T-test performed * = $p < 0.05$).

4. CONCLUSION

The aim of this study was to uncover the functional links between microRNAs and UPR in macrophages exposed to lipid stress, such as seen in obese adipose tissue and in atherosclerotic plaques. Towards this goal, I analyzed miRNA PCR array results of palmitate treated RAW 264.7 macrophages to select highly regulated and metabolic disease relevant miRNAs based on literature. Only the most significantly regulated miRNAs with relevant functions to metabolic condition were further validated by qPCR upon palmitate induced lipotoxic ER stress. I found that miR-33 is downregulated while miR-2137 and miR-486 are upregulated by PA. miR-33 is known to control cholesterol efflux via regulating ABCA1 and ABCG1 transporters. miR-486 was interesting mainly due to its target SIRT1, which is important in aging and metabolic conditions. miR-2137 was previously shown as an ER stress regulated miRNA, however, neither the exact mechanism nor its mRNA targets are known.

We next performed siRNA-mediated knock-down experiments against IRE1 and PERK to understand the role of these UPR arms in the regulation of miRNAs altered by lipid stress. In the absence of IRE1 and PERK, palmitate induction of miR-2137 was diminished. This suggests that both the IRE1 and PERK arms of the UPR are responsible for miR-2137 upregulation upon lipid exposure to macrophages. Although decrease in palmitate induced miR-2137 upregulation is significant, miR-2137 levels were not completely reduced to basal level by either PERK or IRE1 blockage indicating that both arms contribute significantly to its regulation by PA. Knock-down of both PERK and IRE1 arms together may further downregulate miR-2137 level to basal levels. Mature miR-2137 levels increased as early as four hours, yet this increase was non-existent in IRE1 knock down in macrophages. I decided to examine IRE1's involvement in miR-2137 regulation more closely due to its

endoribonuclease activity, which can directly process mRNA and miRNA (31). When the endonuclease activity was inhibited with a specific inhibitor, palmitate induced upregulation was attenuated similar to what was seen in the IRE siRNA knock down, which suggests that the endonuclease activity but not kinase activity regulates miR-2137 expression. To validate this finding, endoribonuclease inactive (RD) and wild type (WT) IRE were transfected into IRE1 deficient MEF cells. WT IRE1, but not RD IRE1, further enchanced the PA induced miR-2137 expression significantly. Taken together, IRE endoribonuclease activity is required for palmitate induced maturation of miR-2137. Future studies are needed to show direct processing of pre- or pri-miR-2137 by IRE1.

Aside from miR-2137, miR-33 and miR-486 were also altered with palmitate treatment. The rise in miR-33 levels were blocked upon IRE1 siRNA transfection to RAW 263.7 cells and these results were also validated in bone marrow derived primary macrophages. However, overexpression of WT and RD-IRE in IRE knockout MEFs also decreased miR-33 levels, presenting a contradiction. Since MEFs and macrophages have different origin and background, miR-33 regulation may differ in these cell types. Another possibility is that PERK is over-activated in MEF IRE knockout cell lines due to compensation effect in the absence of IRE1. Introducing IRE1 into IRE1 deficient background can also affect PERK activity, which was shown to influence miR-33 in our BMDM data. PERK activity needs to be examined in these MEFs under PA stress.

PA induced miR-486 rise is also blocked by IRE knockdown similar to miR-2137 regulation. It is possible that both miR-2137 and miR-486 are regulated by similar mechanism led by IRE1 endoribonuclease domain and warrant further study.

miR-2137 has no known target gene. Therefore, we used three different target prediction databases to find possible targets for miR-2137. Overexpression and knock-down experiments were performed for miR-2137 in RAW macrophages to validate these potential targets. From the eight candidate targets analyzed, Ltbp3 and Inppl1 showed differential regulation dependent on with miR-2137 levels. Further confirmation is required to clarify contradicting findings from these experiments. Ltbp3 is shown to affect transforming growth factor beta (TGFβ) secretion, while Inppl1 dephosphorylates the phosphatidylinositol-3,4,5-trisphosphate(PIP3), which is an important factor in PI3-kinase pathway. If these genes are targeted by miR-2137, IRE1 may hinder or contribute the progression of metabolic diseases through these targets. Moreover, it is known that miRNAs can inhibit protein translation of their target gene without effecting protein levels. Therefore, certain mRNA targets can be missed by qPCR analysis alone. It is also possible that miRNAs can have an indirect effect on the regulated genes. Reporter assay can be utilized to check specific regulation of target genes by miRNAs.

ABCA1 is a well-known target of the miR-33 involved in atherosclerosis. Hence, I studied expression of ABCA1 in IRE1 siRNA transfected macrophages. IRE1 knockdown upregulated ABCA1 expression significantly, and in reverse to miR-33 levels, demonstrating ABCA1 is indeed a target of miR33 in an IRE1-dependent manner during lipotoxic ER stress.

In conclusion, our data revealed new UPR regulated miRNAs during lipotoxic ER stress in macrophages. Since these miRNAs can target genes with relevant roles in metabolic conditions, these miRNAs could provide the missing link between ER stress and these diseases. Further research is needed to reveal the exact mechanism and lead to discovery of a novel target to develop therapeutics against metabolic diseases.

5. FUTURE PERSPECTIVES

In this study, I showed that miR-2137 increases upon palmitate exposure in macrophages in an IRE1 endoribonuclase dependent manner. This study does not explain how IRE1 endonuclease domain regulates the mature miR-2137 expression. One possibility is that IRE1 increase the transcription of miR-2137. XBP1 is the downstream factor of IRE1 and a transcription factor that can alter transcription of microRNAs. Whether there is an effect of XBP1 on miR-2137 can be examined in XBP1 deficient MEF cell line. If miR-2137 is regulated similar in XBP1 deficient and IRE1 deficient cells, it would suggest that miR-2137 transcription is regulated dependent on XBP1. Another way to regulate mature miR-2137 levels is to control its maturation. As a next step, pre- or pri-miR-2137 levels can be measured to distinguish between these regulations. If mature miR-2137 levels increase, while pre- or pri-miRNA form does not change, this would suggest a potential role of IRE1 endoribonuclease activity in the maturation of miR-2137. If miR-2137 is regulated on pre- or pri-miRNA form, direct cleave of miR-2137 can be examined with isolated miR-2137 and Ire1 proteins in vitro.

I tried to determine possible targets for miR-2137. Protein levels of the targets should be determined to validate qPCR results. More candidates should be selected from different databases and possible targets should be further validated using a reporter gene with target 3'UTR. Predicted binding sites of miR-2137 can be mutated to demonstrate the specific regulation by miR-2137. Then functional studies can be performed to assess functional relevance of miR-2137 via target gene.

miR-33 is known to target cholesterol transporters, ABCA1 and ABCG1. The data presented in this thesis suggests that IRE1 blocks ABCA1 expression. Protein levels should be measured with western blot to confirm that IRE1 knock-down increased not only mRNA levels, but also protein levels of ABCA1. As a next step, cholesterol efflux can be measured in IRE knock-down macrophages to the see functional significance of these findings.

miR-486 is another IRE1 regulated miRNA. SIRT1 is a well-known target of the miR-486. SIRT mRNA levels and also protein levels can be measured to check whether IRE1 can effect SIRT expression via miR-486 regulation during lipid-induced ER stress. Protein levels should also be measured. If miR-486 seems to downregulate SIRT1 via IRE1, then function of SIRT1 should be further examined under lipotoxic ER stress in macrophages.

REFERENCES

1. Park SH, Blackstone C. Further assembly required: construction and dynamics of the endoplasmic reticulum network. *EMBO Rep.* 2010 Jul;11(7):515-21.
2. Braakman I, Bulleid NJ. Protein folding and modification in the mammalian endoplasmic reticulum. *Annu Rev Biochem.* 2011;80:71-99
3. Fagone P, Jackowski S. Membrane phospholipid synthesis and endoplasmic reticulum function *J Lipid Res.* 2009 Apr;50 Suppl:S311-6.
4. Giorgi C, De Stefani D, Bononi A, Rizzuto R, Pinton P. Structural and functional link between the mitochondrial network and the endoplasmic reticulum. *Int J Biochem Cell Biol.* 2009 Oct;41(10):1817-27
5. Engin F, Hotamisligil GS. Restoring endoplasmic reticulum function by chemical chaperones: an emerging therapeutic approach for metabolic diseases. *Diabetes Obes Metab.* 2010 Oct;12 Suppl 2:108-15.
6. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell.* 2010 Mar 19;140(6):900-17.
7. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science*. 2011 Nov 25;334(6059):1081-6.
8. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol.* 2012 Jan 18;13(2):89-102.
9. Hetz C, Martinon F, Rodriguez D, Glimcher LH. The unfolded protein response: integrating stress signals through the stress sensor IRE1α. *Physiol Rev.* 2011 Oct;91(4):1219-43.
10. Hetz C, Glimcher LH.Fine-Tuning of the Unfolded Protein Response: Assembling the IRE1α Interactome. *Mol Cell*. 2009 Sep 11;35(5):551-61

11. Maurel M, Chevet E. Endoplasmic reticulum stress signaling: the microRNA connection. Am J Physiol Cell Physiol. 2013 Jun 15;304(12):C1117-26.
12. Carrara M, Prischi F, Ali MM. UPR Signal Activation by Luminal Sensor Domains. Int J Mol Sci. 2013 Mar 21;14(3):6454-66
13. Cui W, Li J, Ron D, Sha B. The structure of the PERK kinase domain suggests the mechanism for its activation. *Acta Crystallogr D Biol Crystallogr*. 2011 May;67(Pt 5):423-8.
14. Chitnis N, Pytel D, Diehl JA. UPR-inducible miRNAs contribute to stressful situations. *Trends Biochem Sci.* 2013 Sep;38(9):447-52.
15. Li J, Lee B, Lee AS. Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. *J Biol Chem.* 2006 Mar 17;281(11):7260-70
16. Korennykh A, Walter P. Structural basis of the unfolded protein response. *Annu Rev Cell Dev Biol.* 2012;28:251-77.
17. Bushati N, Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol.* 2007;23:175-205.
18. Turner MJ, Slack FJ. Transcriptional control of microRNA expression in C. elegans: promoting better understanding. *RNA Biol.* 2009 Jan-Mar;6(1):49-53
19. Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E, Hannon G, Abeliovich A. A MicroRNA feedback circuit in midbrain dopamine neurons. *Science*. 2007 Aug 31;317(5842):1220-4.
20. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet. 2010 Sep;11(9):597-610.

21. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol. 2009 Mar;11(3):228-34.
22. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature*. 2008 Jul 3;454(7200):56-61.
23. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. *Nature*. 2007 Jul 5;448(7149):83-6.
24. Kawahara Y, Zinshteyn B, Sethupathy P, Iizasa H, Hatzigeorgiou AG, Nishikura K. Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. *Science*. 2007 Feb 23;315(5815):1137-4
25. Scadden AD. The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. *Nat Struct Mol Biol*. 2005 Jun;12(6):489-96.
26. Heo I, Joo C, Cho J, Ha M, Han J, Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol Cell.* 2008 Oct 24;32(2):276-84.
27. Shin C, Nam JW, Farh KK, Chiang HR, Shkumatava A, Bartel DP. Expanding the microRNA targeting code: functional sites with centered pairing. *Mol Cell.* 2010 Jun 25;38(6):789-802.
28. Bartoszewski R, Brewer JW, Rab A, Crossman DK, Bartoszewska S, Kapoor N, Fuller C, Collawn JF, Bebok Z. The unfolded protein response (UPR)-activated transcription factor X-box-binding protein 1 (XBP1) induces microRNA-346 expression that targets the human antigen peptide transporter 1 (TAP1) mRNA and governs immune regulatory genes *J Biol Chem.* 2011 Dec 2;286(48):41862-70.
29. Belmont PJ, Chen WJ, Thuerauf DJ, Glembotski CC. Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response *J Mol Cell Cardiol*. 2012 May;52(5):1176-82

30. Behrman S, Acosta-Alvear D, Walter P. A CHOP-regulated microRNA controls rhodopsin expression. *J Cell Biol.* 2011 Mar 21;192(6):919-27.
31. Upton JP, Wang L, Han D, Wang ES, Huskey NE, Lim L, Truitt M, McManus MT, Ruggero D, Goga A, Papa FR, Oakes SA. IRE1α cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. Science. 2012 Nov 9;338(6108):818-22.
32. Duan Q, Wang X, Gong W, Ni L, Chen C, He X, Chen F, Yang L, Wang P, Wang DW. ER stress negatively modulates the expression of the miR-199a/214 cluster to regulates tumor survival and progression in human hepatocellular cancer. PLoS One. 2012;7(2):e31518.
33. Byrd AE, Aragon IV, Brewer JW. MicroRNA-30c-2* limits expression of proadaptive factor XBP1 in the unfolded protein response. J Cell Biol. 2012 Mar 19;196(6):689-98
34. Afonyushkin T, Oskolkova OV, Bochkov VN. Permissive role of miR-663 in induction of VEGF and activation of the ATF4 branch of unfolded protein response in endothelial cells by oxidized phospholipids. Atherosclerosis. 2012 Nov;225(1):50-5.
35. Wang G, Yang ZQ, Zhang K. Endoplasmic reticulum stress response in cancer: molecular mechanism and therapeutic potential. *Am J Transl Res.* 2010 Jan 1;2(1):65-74.
36. Verfaillie T, Garg AD, Agostinis P. Targeting ER stress induced apoptosis and inflammation in cancer. *Cancer Lett*. 2013 May 28;332(2):249-64.
37. Dong D, Stapleton C, Luo B, Xiong S, Ye W, Zhang Y, Jhaveri N, Zhu G, Ye R, Liu Z, Bruhn KW, Craft N, Groshen S, Hofman FM, Lee AS. A critical role for GRP78/BiP in the tumor microenvironment for neovascularization during tumor growth and metastasis. *Cancer Res.* 2011 Apr 15;71(8):2848-57.

38. Cnop M, Foufelle F, Velloso LA. Endoplasmic reticulum stress, obesity and diabetes. *Trends Mol Med.* 2012 Jan;18(1):59-68.

39. Hummasti S, Hotamisligil GS. Endoplasmic reticulum stress and inflammation in obesity and diabetes. *Circ Res*. 2010 Sep 3;107(5):579-91.

40. Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. *J Hepatol*. 2011 Apr;54(4):795-809

41. Tabas I. The role of endoplasmic reticulum stress in the progression of atherosclerosis. *Circ Res*. 2010 Oct 1;107(7):839-50.

42. Scull CM, Tabas I. Mechanisms of ER stress-induced apoptosis in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2011 Dec;31(12):2792-7.

43. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell*. 2011 Apr 29;145(3):341-55.

44. Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet*. 2009 Oct;10(10):704-14.

45. Garzon R, Calin GA, Croce CM. MicroRNAs in Cancer. *Annu Rev Med.* 2009;60:167-79.

46. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med. 2012 Mar;4(3):143-59.

47. Kantharidis P, Wang B, Carew RM, Lan HY. Diabetes complications: the microRNA perspective. *Diabetes*. 2011 Jul;60(7):1832-7.

48. Ali AS, Ali S, Ahmad A, Bao B, Philip PA, Sarkar FH. Expression of microRNAs: potential molecular link between obesity, diabetes and cancer. *Obes Rev.* 2011 Dec;12(12):1050-62.

49. Heneghan HM, Miller N, Kerin MJ. Role of microRNAs in obesity and the metabolic syndrome. Obes Rev. 2010 May;11(5):354-61

50. Shantikumar S, Caporali A, Emanueli C. Role of microRNAs in diabetes and its cardiovascular complications. Cardiovasc Res. 2012 Mar 15;93(4):583-93.
51. Wei Y, Nazari-Jahantigh M, Neth P, Weber C, Schober A. MicroRNA-126, -145, and -155: a therapeutic triad in atherosclerosis? *Arterioscler Thromb Vasc Biol.* 2013 Mar;33(3):449-54
52. Son DJ, Kumar S, Takabe W, Woo Kim C, Ni CW, Alberts-Grill N, Jang I, Kim S, Kim W, Won Kang S, Baker AH, Woong Seo J, Ferrara KW, Jo H. The atypical mechanosensitive microRNA-712 derived from pre-ribosomal RNA induces endothelial inflammation and atherosclerosis. *Nat Commun.* 2013 Dec 18;4:3000
53. Wei Y, Nazari-Jahantigh M, Chan L, Zhu M, Heyll K, Corbalán-Campos J, Hartmann P, Thiemann A, Weber C, Schober A. The microRNA-342-5p fosters inflammatory macrophage activation through an Akt1- and microRNA-155-dependent pathway during atherosclerosis. *Circulation.* 2013 Apr 16;127(15):1609-19.
54. Horie T, Baba O, Kuwabara Y, Chujo Y, Watanabe S, Kinoshita M, Horiguchi M, Nakamura T, Chonabayashi K, Hishizawa M, Hasegawa K, Kume N, Yokode M, Kita T, Kimura T, Ono K. MicroRNA-33 deficiency reduces the progression of atherosclerotic plaque in ApoE-/- mice. J Am Heart Assoc. 2012 Dec;1(6):e003376.
55. Rotllan N, Ramírez CM, Aryal B, Esau CC, Fernández-Hernando C. Therapeutic silencing of microRNA-33 inhibits the progression of atherosclerosis in Ldlr-/- mice--brief report. Arterioscler Thromb Vasc Biol. 2013 Aug;33(8):1973-7.
56. Marquart TJ, Wu J, Lusis AJ, Baldán Á. Anti-miR-33 therapy does not alter the progression of atherosclerosis in low-density lipoprotein receptor-deficient mice. Arterioscler Thromb Vasc Biol. 2013 Mar;33(3):455-8.

57. Rayner KJ, Suárez Y, Dávalos A, Parathath S, Fitzgerald ML, Tamehiro N, Fisher EA, Moore KJ, Fernández-Hernando C. MiR-33 contributes to the regulation of cholesterol homeostasis. *Science*. 2010 Jun 18;328(5985):1570-3.

58. Gerin I, Clerbaux LA, Haumont O, Lanthier N, Das AK, Burant CF, Leclercq IA, MacDougald OA, Bommer GT. Expression of miR-33 from an SREBP2 intron inhibits cholesterol export and fatty acid oxidation. *J Biol Chem.* 2010 Oct 29;285(44):33652-61

59. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell*. 2011 Apr 29;145(3):341-55

60. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: a dynamic balance. *Nat Rev Immunol*. 2013 Oct;13(10):709-21.

61. Aiello RJ, Brees D, Bourassa PA, Royer L, Lindsey S, Coskran T, Haghpassand M, Francone OL. Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. *Arterioscler Thromb Vasc Biol*. 2002 Apr 1;22(4):630-7.

62. Tabas I. Macrophage apoptosis in atherosclerosis: consequences on plaque progression and the role of endoplasmic reticulum stress. *Antioxid Redox Signal.* 2009 Sep;11(9):2333-9

63. Weisberg SP1, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest*. 2003 Dec;112(12):1796-808.

64. Xu H1, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest.* 2003 Dec;112(12):1821-30

65. Oh DY1, Morinaga H, Talukdar S, Bae EJ, Olefsky JM. Increased macrophage migration into adipose tissue in obese mice. *Diabetes*. 2012 Feb;61(2):346-54

66. Ramkhelawon B1, Hennessy EJ1, Ménager M2, Ray TD1, Sheedy FJ1, Hutchison S1, Wanschel A1, Oldebeken S1, Geoffrion M3, Spiro W1, Miller G4, McPherson R4, Rayner KJ4, Moore KJ1. Netrin-1 promotes adipose tissue macrophage retention and insulin resistance in obesity. *Nat Med.* 2014 Apr;20(4):377-84.

67. Kim YJ1, Hwang SH, Lee SY, Shin KK, Cho HH, Bae YC, Jung JS. miR-486-5p induces replicative senescence of human adipose tissue-derived mesenchymal stem cells and its expression is controlled by high glucose. *Stem Cells Dev.* 2012 Jul 1;21(10):1749-60.

68. Wang S, Kaufman RJ. The impact of the unfolded protein response on human disease. *J Cell Biol*. 2012 Jun 25;197(7):857-67.

69. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol. 2007

70. McDonald JS. Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem. 2011