Dissertation

Posttranscriptional Regulation of Liver X Receptors: Specific Role for MicroRNAs

submitted by

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DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

The major part of work presented in this thesis has been summarized in the manuscript entitled ***"MIR-206 controls LXRα expression and promotes LXR-mediated cholesterol efflux in macrophages"*** *by Vinod. et al. submitted to BBA Molecular and Cellular Biology of Lipids.*

Graz ***Date and Signature***

Dedicated to my Family…

"Science is an imaginative adventure of the mind seeking truth in a world of mystery"

-Sir Cyril Herman Hinshelwood-

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ABBREVIATION

3'UTR	3' untranslated region
ABC	ATP binding cassettes transportes
AGO	Argonaute
CAR	Constitutive androstane receptor
CAT-1	Cationic amino acid transporter-1
Circulatory microRNA	cfmiRNAs
CrPV	Cricket paralysis virus
CYP	Cytochrome P450
EMCV	Encephalomyocarditis virus
FXR	Farnesoid X receptor
FXR1	Fragile-X mental retardation related protein 1
HCV	Hepatitis-C virus
IRES	Internal ribosome entry sites
KLF4	Krupple-like factor 4
LXR	Liver X Receptor
m7Gppp	7-methylguanosine cap
MI	Myocardial infraction
miRNP	microRNA ribonucleoprotein complexes
NPMI	Nucleophosmin I
PACT	Protein kinase R activating protein
P-bodies	Processing bodies
PPAR	Peroxisome proliferator-activated receptors
Pum1	Pumilio
PXR	Pregnane X receptor
RISC	RNA silencing complex
RMS	Rhabdomyosarcoma
RXR	Retinoid X Receptor
SMC	Smooth muscle cells
SXR	Sensing nuclear receptor
TRBP	Transactivation-response RNA-binding protein
YY1	Yin Yang 1

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KURZFASSUNG

Liver X Receptor (LXR) spielt als Transkriptionsfaktor im Cholesterol Metabolismus eine Schlüsselrolle. LXR reguliert nicht nur die Cholesterol Biosynthese und den zellulären Efflux, sondern auch den Gallensäure Metabolismus sowie die Ausscheidung aus der Leber und aus zahlreichen anderen Organen. In den Makrophagen moduliert LXR die Cholesterol Homeostase und beeinflusst maßgeblich inflammatorische stimuli, beides Stoffwechselwege, die in der Atherogenese eine wichtige Rolle spielen. Da regulatorische Stoffwechselvorgänge welche in der transkriptionellen Regulation von LXR involviert sind, hinlänglich detailliert untersucht wurden, konzentrierten wir uns in diesen Untersuchungen auf post-transkriptionelle Regulatoren der LXR-Aktivität. MicroRNAs (miRs) sind solche post-transkriptionelle Regulatoren von Genen und führen im canonischen Pathway zur mRNA Inaktivierung.

In silico Analysen führten zur Identifizierung von miR-206 als putativen Regulator von LXRα, jedoch nicht von LXR-ß. In unseren Studien konnten wir die kürzlich publizierten Ergebnissen bestätigen, dass miR-206 die LXRα Aktivität sowie die Expression der LXRα Zielgenen in Leberzellen reduziert. Hingegen konnten wir zeigen dass miR-206 in Makrophagen gegenteilige Effekte auslöst. Stabile Über-Exprimierung von miR-206 in humanen THP-1 Makrophagen führte zu einer Aktivierung, und miR-206 knockdown führte zu einer Inhibierung von LXRα sowie seiner Zielgene. Diese Befunde wurden dahingehend bestätigt als Knochenmarks-Makrophagen (BMDM) welche von miR-206 KO Mäusen isoliert wurden eine geringere Expression von LXRα aufwiesen als Makrophagen von Wild-typ Mäusen. Die physiologische Relevanz dieser Daten wurden durch "gain-und loss-of-function" von miR-206 bestätigt: Überexpression von miR-206 verstärkte den Cholesterol Efflux aus humanen Makrophagen und knock-out von miR-206 reduzierte den Cholesterol Efflux aus peritonealen Mäuse Makrophagen. Schließlich konnten wir noch zeigen, dass die Expression von miR-206 in Makrophagen durch LXRα Aktivierung reprimiert wird, währen oxidierte LDL sowie inflammatorische Stimuli die miR-206 Expression drastisch induzierten. Wir schlagen daher einen "feedback-loop" zwischen miR-206 und LXRα vor, welcher einen Teil der LXR-autoregulatorischen Maschinerie zum fine-tuning der LXR Aktivität darstellt.

ABSTRACT

Liver X receptors (LXRα and LXRβ) are key transcription factors in cholesterol metabolism that regulate cholesterol biosynthesis and cholesterol efflux as well as bile acid metabolism and excretion in the liver and numerous organs. In macrophages, LXR signaling modulates cholesterol handling and the inflammatory response, pathways involved in atherogenesis. Since regulatory pathways controlling LXR transcription are well understood, in the present study we aimed at identifying post-transcriptional regulators of LXR activity. MicroRNAs (miRs) are such post-transcriptional regulators of genes that in the canonical pathway mediate mRNA inactivation. *In silico* analysis identified miR-206 as a putative regulator of LXRα but not LXRβ. Indeed, as recently shown, we found that miR-206 represses LXRα activity and expression of LXRα and its target genes in hepatic cells. Interestingly, miR-206 regulates LXRα differently in macrophages. Stably overexpressing miR-206 in THP-1 human macrophages revealed an up-regulation and miR-206 knockdown led to a down-regulation of LXRα and its target genes. In support of these results, bone marrow-derived macrophages (BMDM) from miR-206 KO mice also exhibited lower expression of LXRα target genes. The physiological relevance of these findings was proven by gain-and loss-of-function of miR-206; overexpression of miR-206 enhanced cholesterol efflux in human macrophages and knocking out miR-206 decreased cholesterol efflux from MPMs. Moreover, we show that miR-206 expression in macrophages is repressed by LXRα activation, while oxidized LDL and inflammatory stimuli profoundly induced miR-206 expression. We therefore propose a feed-back loop between miR-206 and LXRα that might be part of an LXR auto-regulatory mechanism to fine tune LXR activity.

1. INTRODUCTION

1.1. MicroRNAs: Biogenesis and mechanism of action

MicroRNAs are one of the largest gene families accounting for about 1% of the human genome (1). MicroRNAs are evolutionarily conserved key post-transcriptional regulators of genes, which stringently fine tune cellular process and complex gene regulatory networks to facilitate normal coordinated regulation and functioning of complex multicellular organisms such as Eukaryotes (2). Extensive research in the field of microRNAs has revealed both negative regulation (sequestering, transcript degradation and translational suppression) and positive regulation (transcriptional and translational activation) (1, 3-8). This chapter will be dealing with the biosynthetic pathway of microRNAs and the mechanistic differences in microRNA mediated gene regulation.

1.1.1 ***MicroRNA Biogenesis***

MicroRNA Biogenesis includes a series of meticulously tailored biochemical events that result in biologically active mature microRNA from primary microRNA transcripts (Pri-miRNA) (9). MicroRNA coding genes in the chromosomes can be either exonic or intronic (10). MicroRNAs that originate from the intron of protein-coding genes are called intronic microRNAs (11). Intronic microRNAs do not require a specialized spicing machinery instead they are co-transcribed along with the protein-coding mRNA; the mRNA splicing of the protein is unaffected by the process of microRNA maturation (12). In contrast, microRNAs with independent transcriptional units on the chromosome are transcribed into Long Pri-miRNAs by RNA polymerase II (Pol II) (13). Figure-1, illustrates the canonical microRNA biogenesis pathway.

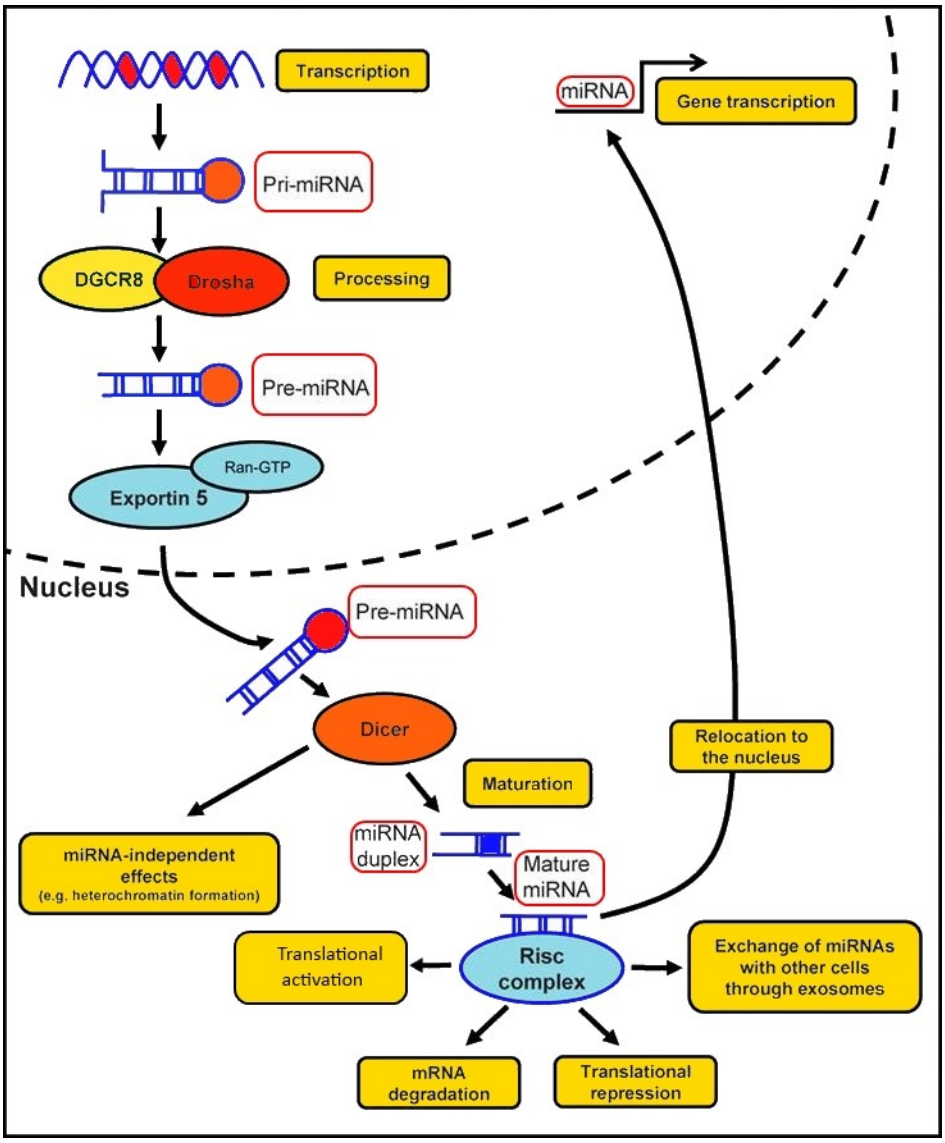


Illustration-1 MicroRNA Biogenesis. *Canonical microRNA biogenesis pathway consist of four main steps* ***1)*** *Pri-miRNA transcription from independent microRNA promoters facilitated by Pol II or from introns of protein-coding genes facilitated by standard transcriptional machinery.* ***2)*** *Pri-miRNA processing is carried out by Drosha-DGR8 complex to form Pre-miRNA.* ***3)*** *Pre-miRNA is exported out of the nucleus and further processed by Dicer to form duplex mature miRNA* ***4)*** *Duplex mature miRNA is loaded on to Argonaute, subsequent maturation step expels one miRNA strand to produce mature miRNA-RISC complex. Mature microRNA can be functional in many different ways suchs a translational repression, mRNA degradation, translational activation and miRNA export to other cells through exosomes or lipoproteins. It should be noted that this is a simplified cartoon that omits numerous accessory factors in the complex machinery of miR metabolism.*

Main steps in the microRNA biogenesis pathway:

a) **microRNA transcription:** most microRNA genes are transcribed from their independent promoters by Pol II into long primary transcripts (Pri-miRNA) (13). Pri-miRNA comprises of multiple hairpin loop like structures each composed of a terminal and a stem loop. More than one functional micoRNA can be generated from a single Pri-miRNA. Pri-miRNA consists of a 7-methylguanosine cap (m7Gppp) 5'- cap and a 3' poly (A) tail (14, 15). Transcription of several microRNAs can occur as a cluster under the control of a single promoter thereby resulting in mono-, di- or polycistronic microRNAs and such microRNAs have been reported to mediate the coordinated expression of several genes of the same pathway (9).

b) **Nuclear processing of Pri-miRNA:** In the nucleus, the Pri-miRNA is converted to Precursor microRNA (Pre-miRNA) by Drosha, an RNAase III enzyme (9). Drosha is part of a larger microprocessor complex. This complex is found in two forms, a larger complex of about 600KDa, with unkown function and as a smaller complex comprising of Drosha and it dsRNA binding partner- DGCR8 (16). Drosha cleavage results in a 60nt Pre-miRNA molecule with a 2 nt overhang with a hydroxyl group at the 3' end and a monophosphate at the 5' end. At this stage polycistronic microRNAs are also cleaved to produce single Pre-miRNAs (17). Most of the microRNAs are generated by this canonical pathway of micoRNA biogenesis but there exists alternate mechanisms that are Drosha-independent pathways.

Drosha independent pathways that were characterized recently include spicing derived mitrons or tailed mitrons (18-20) or RNaseZ mediated microRNA biogenesis (21) or SnoRNA- derived microRNAs (22).

c) **Pre-miRNA export to the cytoplasm:** Pre-miRNA, with its typical 2nt overhang is identified by exportin-5 and is exported out of the nucleus to the cytoplasm (15). This process is energy dependent and is mediated by Ran Protein bound GTP. In the cytoplasm RanGTP hydrolysis dissociates exportin-5 and releases the pre-miRNA for further processing mediated by Dicer (23).

d) Dicer mediated processing of Pre-miRNA

The exported Pre-miRNA is further processed in the cytoplasm to form a 22-nucleotide microRNA-microRNA* duplex. Dicer interacts with the 3' end of the Pre-miRNA heteroduplex and specifically excises the joining loop between the 3' and the 5' end. Alhough both the strands on the microRNA-microRNA* duplex can be functional only just one strand out of the duplex gets incorporated into the AGO proteins for target interaction (9). In mammals, Dicer has been found to be partnered with two proteins namely transactivation-response RNA-binding protein (TRBP) and protein kinase R activating protein (PACT). The role of these partner proteins is to provide Dicer with substrate specificity and specify Dicer cleavage site on the Pre-miRNA (9).

The 5' end of the microRNA incorporated along with AGO proteins acts as a domain (seed region), which consists of 6-8 nucleotides ~~are~~ incorporated into the RNA silencing complex (RISC). Complementarity between the seed and the microRNA can imitate miRNA mediated regulation of the target mRNA (9). miRNA guides AGO proteins to complementary or partially complementary mRNA sequences to facilitate post-transcriptional regulation. The functional consequence of the microRNA-mRNA interaction can greatly depend on the degree of complementarity between the microRNA and the target mRNA and also on the protein complexes interacting with the AGO proteins. There are different miRNA mediated post-transcriptional mechanisms reported in the literature out of which the main ones are described in detail in the forthcoming sections.

1.1.2 MicroRNAs as post-transcriptional regulators of genes- mechanism of action

MicroRNAs employ diverse mechanisms to post- transcriptionally regulate its target gene affecting ~~it~~ mRNA levels and also protein translation. MicroRNAs are operative as microRNA ribonucleoprotein complexes (miRNPs) or miRNA-induced silencing complexes (miRISCs). The most important and the best characterized protein in miRNPs are the Argonaute (Ago) proteins. In mammals, four Ago proteins are identified; Ago1 to 4, out of which except for Ago2 proteins have been shown to have endonucleolytic function cleaving the mRNA-siRNA/miRNA duplex thereby mediating siRNA mRNA degradation. In miRNA-mediated repressive mechanisms all Ago protein are shown to be involved. Several other proteins are thought to be involved in miRNA-mediated gene regulatory events but only few proteins in this elaborate machinery is functionally characterized. Interestingly, recent evidences suggest that miRNAs can also activate genes resulting in miRNA-mediated translational activation. However, the mechanism in which miRNAs can activate a gene has not been fully characterized. In the sections below, a brief description of the current understandings in microRNA mediated positive and negative gene translation are illustrated.

1.1.2.1 MicroRNA as negative regulators of gene translation

Translation is a complex highly regulated process comprising primarily three stages that involve multiple proteins: initiation, elongation and termination (24). The initiation step is elaborately regulated and is the rate limiting step in mRNA translation. From the available evidences it can be envisaged that miRNA can repress mRNA at both initiation and post-initiation stages.

a) MicroRNA regulation at translational initiation

Initially microRNAs were thought to repress translation at a post initiation stage. However, recent experimental evidences from mammalian cell cultures using either endogenous mRNAs or reporters have shown that repression at the stage of translational initiation as a possible mechanism (25). Polysome profiling is an established technique to monitor the translational status and the robustness of the protein translational machinery in a cell using a

sucrose gradient. Polysome binding to the mRNA is indicative of active translation and distributed towards the lower heavy fraction of the sucrose gradient. In the event of stalled initiation, majority of the mRNA is distributed at the top light fraction of the gradient (Figure-2). *Pillai et. al.*(26), demonstrated using endogenous let-7a and luciferase reporter constructs with three bulged let-7a target sites in the 3'UTR. Luciferase expression was markedly reduced up to 80-90% whereas mRNA abundance was only reduced up to 20%.

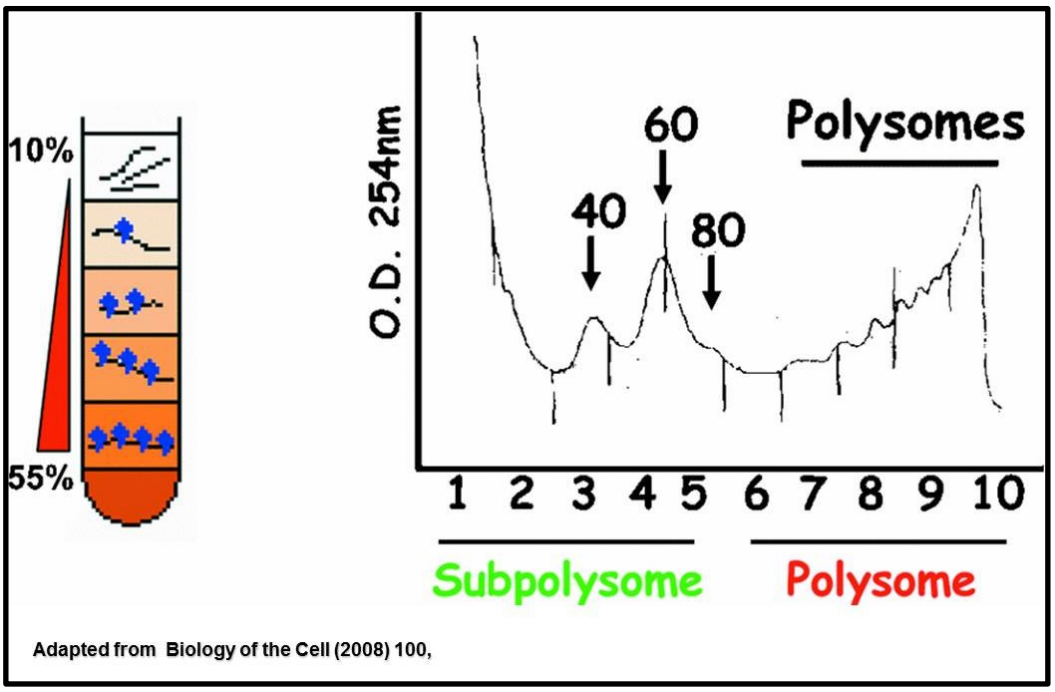


Illustration-2 Polysome Profiling: *Post-nuclear extracts derived from cell lysate are applied to 10-55% sucrose gradient (Left Panel);Standard polysome profiling results differentiating heavier mRNA associated polysomes from translationally inactive subpolysomal mRNA.*

Polysome profiling showed marked shift of mRNA towards the top lighter fraction peaking at the 40S disome region indicative of an effect at the translational initiation stage. Another experiment using miR-122 and its endogenous target cationic amino acid transporter (CAT-1) showed that under stress when miR-122 mediated repression on CAT-1 is relieved mRNA from the lighter fraction shifted to the higher polysomal fraction indicative of active translation (27). Several elements on the mRNA have been hypothesized to be involved in the process of miRNA mediated regulation of initiation. Numerous groups have attempted to identify possible regulatory factors and mRNA structural elements responsible for facilitating microRNA mediated repression of the target mRNA.

Pillai et. al., performed direct RNA transfections with 7-methylguanosine (m^7GpppG)-capped polyadenylated mRNA and microRNA let-7a. Six hours post transfection assays revealed that the presence or absence of Poly(A) tail had significantly minor or no impact on let-7a mediated repression of the transfected mRNA thus indicating that Poly(A) is not a critical requirement for microRNA mediated repression of translation initiation. Contrastingly, transfecting HeLa cells with m^7GpppG-capped polyadenylated reporter driven by internal ribosome entry sites (IRES) elements of encephalomyocarditis (EMCV) hepatitis-C (HCV) or cricket paralysis (CrPV) rendered them resistance to let-7a mediated repression further emphasizing the role of m^7GpppG mRNA cap structure on microRNA mediated translational repression. ECMV driven initiation events require all canonical translational initiation factors except eIF 4E and HCV IRES functions independent of all four initiation factors such as eIFs 4A, 4B, and 4G this result obtained by *Pillai et al.* indicates eIF4E be a crucial component in the repression mechanism. Similar observations were published by *Humphreys et al (28)*. Though in both the studies non polyadenylated ECMV IRES constructs were not repressed, adding a Poly (A) tail further increased the translational efficiency and also rendered it susceptible to siRNA mediated repression (25).

To summarize, two alternative pathways can be conceptualized; repression involving via 1) cap-dependent mechanism involving eIF 4E 2) disrupting the closed loop structure by promoting deadenylation of mRNA or by disrupting protein-protein/RNA interaction.

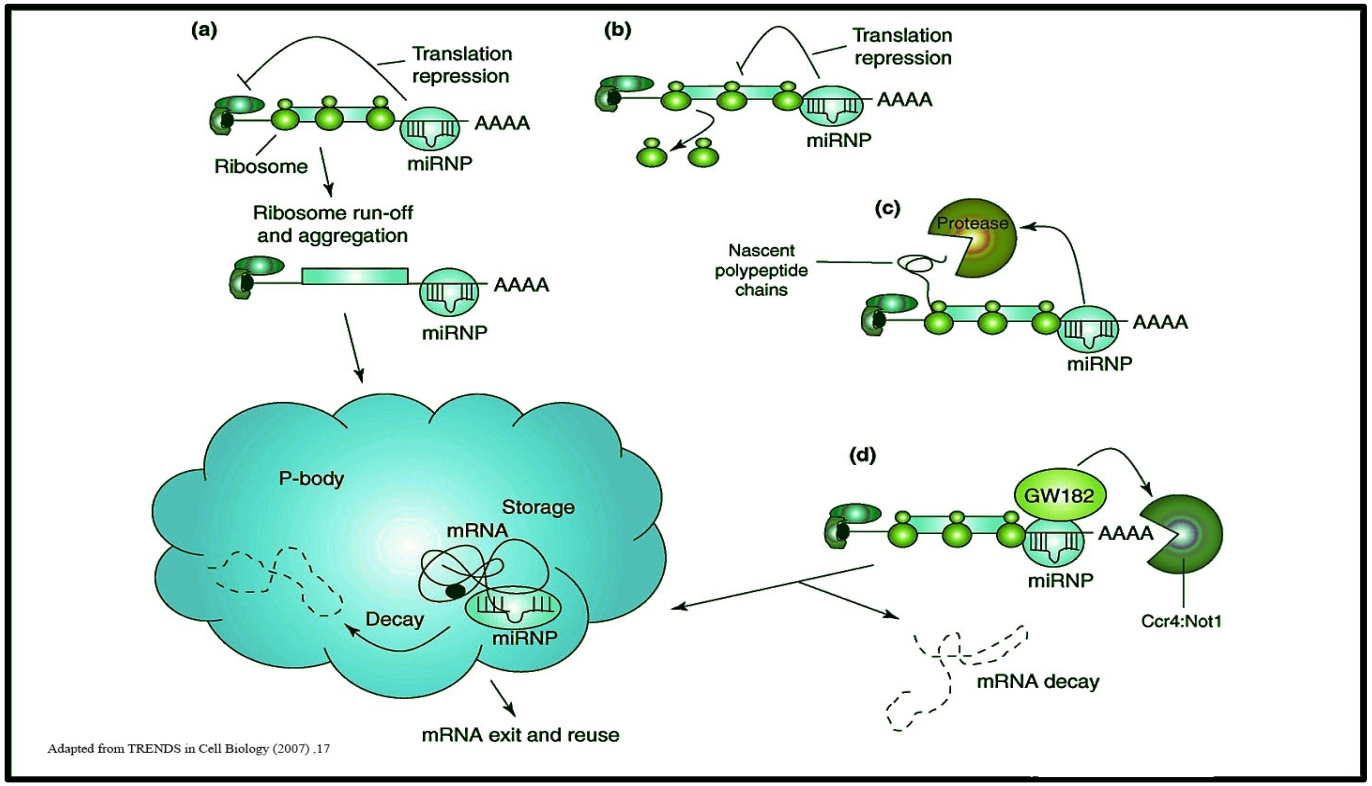


Illustration-3 Putative mechanisms of microRNA-mediated target mRNA translational repression

a) *MicroRNP mediated translational repression of the m^7 G-capped mRNA at the first step of initiation followed by a second step of ribosomal run off resulting in aggregation of mRNA into P-bodies for degradation or storage. Oval Green represents eIF4G interacting with eIF4E bound to the m^7 G-cap* ***b)*** *Repression of translation by microRNP at the elongation or termination step followed by premature dropping of ribosomes. The repressed mRNA is associated with ribosomes and might not translocate into P-bodies c) Protein lysis post-initiation by proteolytic enzymes recruited by the microRNPs* ***d)*** *microRNP mediated mRNA deadenylation followed by decay. Deadenylation mRNA might relocate to P-bodies for further decay or storage.*

b) MicroRNA regulation post-initiation

Experiments performed on mammalian cell cultures and *Caenorhabditis elegans* (*C. elegans*) evidence supporting microRNA-mediated mRNA repression post-initiation. During larval development of *C. elegans,* lin-4 microRNA is expression in the late L1 larval stage subsequently resulting in the repression of two heterochronic regulators lin-14 and lin-28 mRNAs while proceeding through further stages in development (L2 and L3) . Polysome profiling at L1, L2 and L3 stages remain unchanged under conditions of repression suggesting ribosomal loading onto the target mRNA and translational initiation, thus the repressive action of lin-4 should be at the post-initiation stage(29, 30). Similar results were obtained by *Peterson et al(31)*, in mammalian cells where they found that the repression of the transfected reporter mRNA was not associated with changes in the polysome profile thus supporting the above notion that initiation was uncompromised in these cells and repression of the target mRNA occurred post-initiation.

Cells pretreated with tRNA mimic, puromycin shifted the mRNA partially towards the lighter fraction the polysome profile, however ribosomes associated with the reporter, actively translated and not stalled in the elongation phase. This mechanism was active in both cap-dependent and independent translational machinery further supporting the role of microRNA in repressing the target mRNA post-initially. *Peterson et al*, proposed several models such as ribosome drop off model in which ribosomes from the target mRNA drop off prematurely resulting in termination of translation or a second model in which microRNPs associated with the target mRNA recruit proteolytic enzymes that would degrade the emerging nascent polypeptide (Figure-3b and d). MicroRNP mediated deadenylation of target mRNA has also been another proposed mechanism.

c) MicroRNA mediated target mRNA degradation

MicroRNAs also promote target mRNA degradation and is a conserved mechanism. However the decrease in mRNA abundance is relatively small when compared with the expression of the coded protein. Initial microarray analysis of HeLa cells transfected with exogenous microRNA revealed that microRNAs can facilitate target mRNA degradation. Consistently, anti-miR-122 transfection to mouse liver stabilized various miR-122 target mRNAs(32, 33).

The operative mechanism behind microRNA mediated mRNA degradation is ambiguous. Silencer-mediated cleavage mechanism like in the case of siRNA silencing mechanism requires A-form helical conformation at the center of the mRNA-siRNA/miRNA duplex. Mismatches and bulges in the animal microRNA-mRNA duplex is a limiting factor for the siRNA like silencing mechanism to function. Recent studies on mammalian cells and Drosophila cells demonstrate that mRNA decay is facilitated through normal pathways of deadenylation followed by decapping and 5'→3' exonuclease activity mediated mRNA degradation (Illustration-3d)(34, 35). The primary difference between normal mRNA decay pathways and microRNA mediated mRNA decay is that the binding of the microRNA at the 3' UTR region on the mRNA can act as a barrier during the 5'→3' exonuclease cleaving, thereby accumulating deadenylated decay intermediated with 3' end sequences (25).

Overall repression of mRNA by microRNA might be a combinatorial mechanism involving both translational inhibition and mRNA degradation and the relative importance of each mechanism might vary depending on the cell type and the stability of the microRNA-mRNA complex.

d) MicroRNA mediated mRNA target accumulation in P-bodies

P-bodies are cytoplasmic loci for translational repression and mRNA decay. P-bodies seem to aggregate mRNA and proteins associated with translational repression/decay machinery. However, the complete protein composition of P-bodies has not been characterized. Currently P-bodyies proteins have been classified broadly into three different categories; ***Conserved protein core***, consisting of mRNA decapping enzymes (Dcp1p/Dcp2p), decapping activators (Dhh1p/RCK/p54, Pat1p, Lsm1p-7p complex, Scd6p/RAP55 and Edc3p) and 5' to 3' exonuclease enzymes (Xm1p). ***MicroRNA repression factors,*** some species specific components such as proteins (Argonautes/ALG-1, GW182/AIN-1, TNRC6B, and MOV10) involved in microRNA function that affects subclasses of mRNAs. ***RNA binding proteins and translational repressors***, many RNA binding proteins found in P-bodies are also functionally involved in translational repression acting on cytoplasmic mRNA, targeting them to mRNA decay(36).

The mRNAs in the P-bodies seems to be a very crucial requirement for its integrity. Overexpressing untranslated mRNA in cells increased the P-body size and number indicating that P-bodies require mRNA for its assembly (37). Ribosomes and translational initiators except eIF4E are absent in P-bodies. It has been observed that in cells treated with cycloheximide, which blocks translational elongation, mRNAs are associated with ribosomes and the P-bodies decline in size and number. On the contrary, under conditions such as stress, translational initiation is inhibited, mRNAs dissociates from ribosomes subsequently P-bodies increase in size.

MicroRNAs along with the Ago proteins and target mRNAs have been shown to aggregate in the P-bodies. MiR-122 when expressed ectopically concentrates its target CAT-1 mRNA in P-bodies. MicroRNAs promote translational repression, deadenylation and mRNA decapping by recruiting factors involved in repression or decay machinery by interacting with Ago proteins. Potential components involved in microRNA induced mRNA accumulation in the P-bodies are GW182, an RNA-binding protein with glycine-tryptophan repeats, eIF4E binding protein 4E-T, RCK/p54 that has been reported to microRNA repression and Ccr4/Pop/Not complex critical for microRNA mediated deadenylation of target mRNA (36). It has also been reported that mRNA levels are reduced by microRNA in an Xm1p dependent manner. P-bodies may also function as temporary storage depots for mRNAs that are not actively translated and this repressed state can be reversed under specific conditions. Recently LSm1, a P-body protein has been reported to be involved in miR-122 mediated translational stimulation (38).

1.1.2.2 MicroRNAs as positive regulators of gene expression.

MicroRNA mediated transcriptional activation is less commonly observed than microRNA mediated transcriptional or posttranscriptional repression. MicroRNA induced post-transcriptional upregulation in eukaryotes is observed in some cell types such as germ cells under specific cellular conditions. In response to distinct cues, regulatory elements essentially repress or activate genes. MicroRNA mediated translational activation of a gene can occur directly by recruitment of distinct microRNPs or indirectly by relief of repression due to regulatory effects in microRNA-mediated repression (5, 30).

Though microRNAs are predicted to target over a third of the genome, not all predicted targets are subject to regulation in any given cell and environment. The 3'UTR regions on the mRNA are reported to be alternately polyadenylated to preclude or include certain regulatory sites or to have accessory sequences that can be occupied in a regulated manner which may facilitate or impede microRNA activity(39). The target specific and conditional activity of microRNAs can be understood from some of the interesting reports published recently. MiR-145 mediated upregulation of myocardin was observed during muscle differentiation. Conversely all other targets of miR-145 and miR-143 were down-regulated (40). KLF4 mRNA was differently regulated by miR-206 in confluent and noncancerous cells when compared to proliferating cancer cells. In proliferating cancer cells, KLF4 mRNA was canonically repressed by miR-206 and miR-344. In contrast, in noncancerous or confluent cancer cells KLF4 mRNA was upregulated by miR-206 (41). Another recent report suggests miR-122 mediated activation of IRES driven translation in HCV virus that is mediated through a P-body protein LSm1(38, 42).

Few studies also report microRNA mediated transcriptional regulation by binding of microRNA regulatory factors to response elements on the gene promoter (8, 43). Current mechanistic insights on microRNA mediated stimulation of gene regulation are briefly described in the sections below.

a) MicroRNA mediated activation

<u>Post-transcriptional activation regulated by cellular quiescence (G0)</u>

Quiescence is a stage in the cell cycle also called as the rest phase, where cells neither divide nor prepare to divide. This state can also be a unique adaptation in response to cellular stimuli which enables them to escape unfavorable conditions (5). Terminal differentiation in macrophages, muscle cells and nerves can also suspend cells in this quiescent state (44). Such cells remain in that state for a very long time and continue to perform their cellular functions normally. These cells are metabolically active and require specific gene expression to maintain the state, resist harsh conditions as well as respond to altered stimuli. Posttranscriptional gene regulatory mechanisms are quite likely to play an important role in these cells which may also include translational activation by microRNAs (44-46).

MicroRNA-induced translational activation reportedly is restricted to cells at the G0 states and not in cells at other stages of cell cycle. It is also intriguing that core AGO 2-associated microRNP factors such as GW182 involved in target repression are regulated cell cycle dependently (47-49). GW-bodies number increase in S and G2 phases significantly declines in G1 and G0 phase. GW182, as suggested earlier is involved in microRNA mediated deadenylation and repression and is marked by reduced GW-bodies Fragile-X mental retardation related protein 1 (FXR1-iso-a) isoform, was demonstrated to be associated with AGO2/miRNP in conditions such as quiescence and might contribute to reduced GW182-AGO2 association and therefore the lack of AGO 2 – GW182 association might lead to impediment of repression(6). Recruitment of FXR1-iso-a leads to translocation of mRNA to polysomes. It has been reported that overexpressing FXR1-iso-a increases translation (6). Another possible mechanism can be Poly (A) tail deadenylation or shortening which alters the PABP mediated interaction is increased in quiescent cells, suggesting a possible role in microRNA-induced translational activation.

Upregulation of translation can also be caused by activation of alternate translational mechanisms such as cap-independent IRES driven translational activation. HCV translation is induced by miR-122 in IRES driven mechanism through miR-122 target sites on the 5'UTR region (38, 42, 50). Translocation of miR-122 to 3'UTR region resulted in repression in normal cells but translation is activated in quiescent cells. MiR-122 binding at the 5'UTR region increases association of 40s subunits, 48s complex incorporation and enhanced polysome formation which also involves AGO2 and LSm1 proteins (38).

However, it has to be noted that in order to maintain G0 state several genes such as proteins controlling cell cycle and also other genes that are undesirable are both transcriptionally and posttranscriptionally repressed. These mechanisms might also involve posttranscriptional repression mediated by microRNAs targeting genes that are nonessential. As in the case of miR-143 and miR-145, microRNAs activating translation of a particular target gene required for G0 state might continue to repress other targets with are undesirable.

Several studies also demonstrate cytoplasm to nuclear migration of microRNAs (51) and sequence specific promoter binding and subsequent transcriptional regulation(8, 52, 53). There are also databases available that predict microRNA binding regions on gene promoters (54). E-cadherin and CSDC2 mRNA transcription is activated by sequence specific binding of miR-373 to the promoter (8).

MicroRNA mediated decoy of repressive proteins

Certain microRNAs have been reported to act as decoy, which decoys away the repressor thus preventing it from accessing its target mRNA and subsequently results in activation of the gene. In blast crisis chronic myelogenous leukemia, the repressor protein hnRNP E2 gets decoyed by miR-328 thus preventing it from binding to its target C/EBPα leading to activation (55).

Post-transcriptional activation by competition with decay factors

ARE decay pathway is an alternate pathway for mRNA degradation in which mRNA containing AU rich 3'UTRs are deadenylated and subsequently degraded by complex exonuclease action in the 3' to 5' direction. MicroRNAs have been reported to compete with protein complexes in the ARE decay mechanism to bind to the 3'UTR region. MicroRNA binding stabilizes by intercepting the interaction of ARE factors and thereby increasing the gene expression post-transcriptionally. MiR-125b has been reported to enhance the stability of κ B-Ras2 mRNA and miR-466l upregulates IL-10 mRNA levels by intercepting with TTP binding (an ARE decay factor)(56-58).

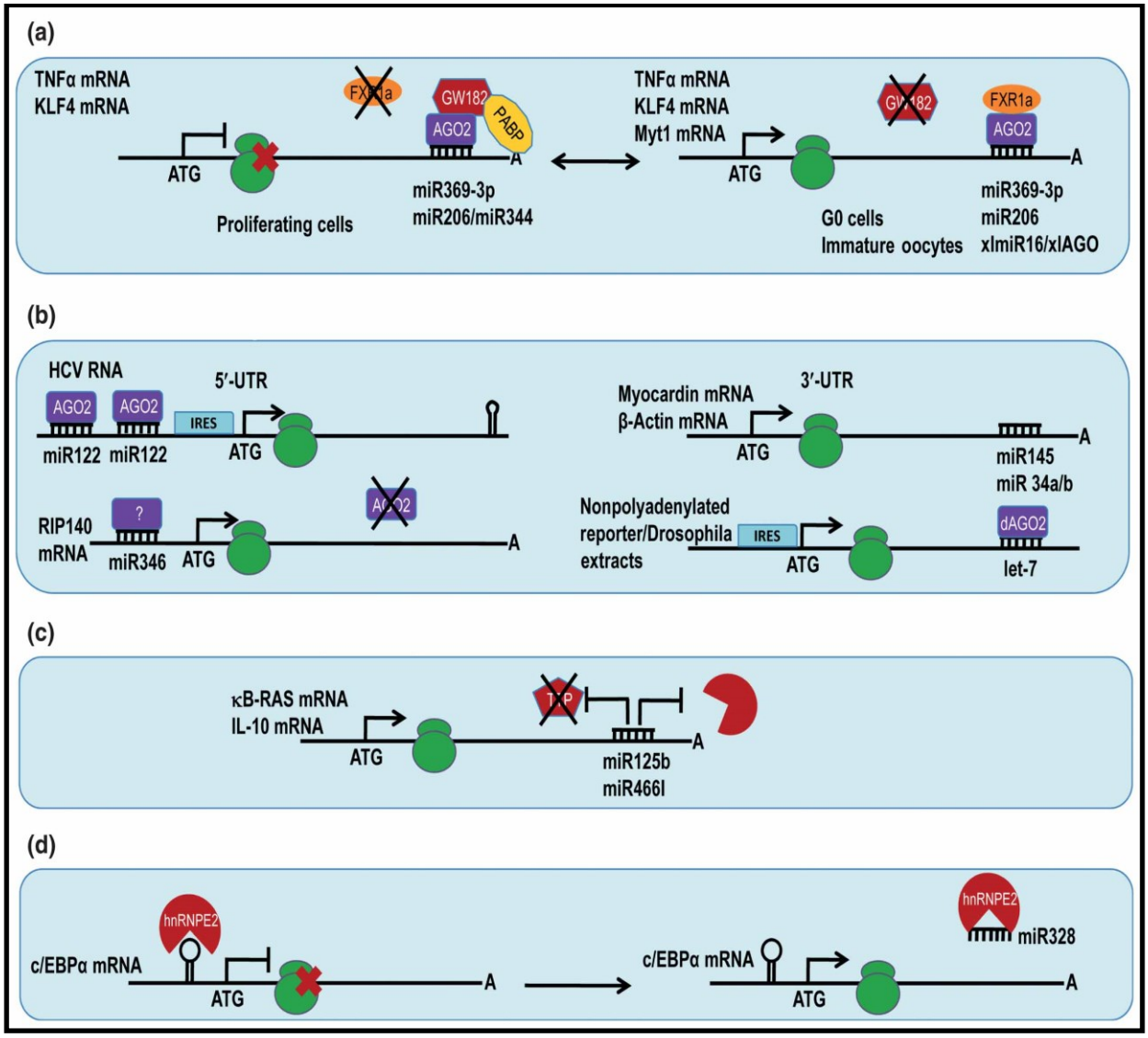


Illustration-4 Diverse mechanism of direct post-transcriptional gene activation by microRNAs
a*) Differential mechanism of transcript regulation in quiescent cells and proliferating cells owing to differences in the mRNP components b) Activation by alternate translational pathway driven through IRES by various mRNP factors c) Post-transcriptional activation by competition with decay factors d) MicroRNA mediated decoy of repressor protein. Adopted from Vasudevan WIRE RNA(2011)*

b) Relief of repression

Relief of repression by target mRNA manipulation

Negation of microRNA repression can be achieved by RNA-binding proteins or by changes in mRNA structure and localization. The repressive effect of miR-122 on CAT-1 mRNA was abrogated under conditions of stress by selective binding of HuR protein on the U rich regions on the 3'UTR (39). Similarly extracellular stimuli induced TOR pathway relives repression on Limk1 mRNA by miR-134. An interesting report on regulated and cell cycle specific alleviation of repression was published by *Kedde et al*. They found that binding of Pumilio (Pum1) protein specifically to P27KIP1 in cycling cells obscures microRNA access and relieves repression of P27 mRNA. However, in cell cycle arrested cells Pum1 is not phosphorylated and its stability is reduced. They observed that only phosphorylated Pum1 could bind to the 3'UTR region of P27(59).

Relief of repression by decoy mechanism

Psuedogene transcripts or non-coding RNAs and Viral transcripts can competitively decoy the microRNA binding resulting in relief of repression. Uncommon decoy machinery in *Arabidopsis* that can relieve microRNA mediated repression is by the action of AGO10 proteins. AGO10 proteins can identify and bind to the duplex structure of miR-165 thus decoying it away from binding to AGO1. This report suggests a differential role of Ago family of proteins in microRNA mediated mechanisms(60). Modification of AGO proteins such as poly-ADP ribose modifications can also impede with the functioning of the AGO protein resulting in alleviation of target repression (61). MicroRNA modifications such as uridylation or adenylation have been reported to cause functional consequences. It is reported that 3'-end modification by uridylation leads to instability (62). However, the mechanism behind dysfunction caused by modification of microRNA has not been characterized.

1.1.3. Extracellular communication via microRNA

MicroRNAs have been shown to be stable in extra-cellular environments such as body fluids, urine, saliva and ascites and have been classified as circulating microRNAs (cfmiRNA). Circulating microRNAs are normally stable as they are packaged into apoptotic bodies, lipoproteins, exosomes, microvesicles or bound to RNA binding proteins therefore they are protected from RNAases (63, 64). MicroRNAs get sorted out into microvesicles or exosomes during RISC disassembly in the cytoplasm (65, 66). Microvesicles and exosomes can easily enter the recipient cell by trans-locating across the cell membrane and thereby facilitating cell to cell communication through microRNA.

Another mode of extracellular communication can be routed through apoptotic bodies formed during cellular apoptotic. Apoptotic bodies have also been reported to be transporters of microRNAs and they differ from microvesicles and exosomes by the absence of certain surface markers (67). In this case processing of microRNAs into apoptotic bodies is a random event.

Some proteins and lipoproteins such as HDL, LDL and Nucleophosmin I (NPMI) have also been reported to be microRNA transporters (68-70). It has been reported that microRNAs transported by LDL can be delivered to the recipient cell by an SREBP mediated pathway and cellular microRNA packaging to HDL is reported to be mediated by ABC transporters (70).

However, the exact mechanism of microRNAs packaging and transported are unknown. Nevertheless, cfmiRNAs are now being extensively studied and have been proved to be useful diagnostic markers for various diseases such as cancer (64).

1.2 Nuclear receptors in lipid metabolism

Nuclear receptors are a large family of ligand-inducible transcription factors that play a multitude of essential roles in diverse biological processes such as reproduction, development, homeostasis, immunity and metabolism (71). The primary function of nuclear receptors is to mediate cellular transcriptional response to various metabolic ligands and hormones such as adrenal steroids, sex hormones, Vitamin D_3, thyroid and retinoid (72). In the human genome there are about 48 nuclear receptor family members (73). The physiological ligand and function of many of these nuclear receptors are not identified and they are often referred to as orphan nuclear receptors. All nuclear receptors are three important structural domains 1) transcription activation domain 2) DNA binding domain and 3) Ligand binding domain.

Ligand binding at the ligand biding domain dissociates the repressor of that particular nuclear receptor causing a conformational change in the protein, migrating to the nucleus, DNA binding, association with co-regulators and finally transcriptional activation or repression. Nuclear receptors identify and bind specific response elements on the promoter of the gene (74). Inappropriate nuclear receptor signaling is a key pathological determinant causing disorders such as diabetes, cardiovascular diseases, cancer, obesity and reproductive disorders.

Nuclear receptors can bind to DNA as homodimers or as heterodimers. The classic steroid hormone receptors bind to their target gene as homodimers and their ligands are produced exclusively by endogenous endocrine sources.

Conversely, the second set of nuclear receptors bind to the DNA as heterodimers with RXR (Retinoid X Receptor). The source of ligands for this class of nuclear receptors is from the diet. PPARs, LXRs, FXR, CAR, PXR and SXR are members of this class. Binding of ligands to any of these nuclear receptors activate a cascade of metabolic signaling events to maintain lipid homeostasis by transcriptionally regulating genes involved in lipid absorption, metabolism, storage and elimination. Three families of proteins involved in this cascade of feed forward metabolic signaling are 1) Cytochrome

P450 (CYP) family of enzymes that catalyzes redox reaction by acting on ligands to form metabolically inactive molecules for clearance 2) Intracellular lipid binding proteins that are involved in transport of hydrophobic lipids into the cell 3) ATP binding cassettes (ABC) transporters involved in shutting and packaging the precursors and lipid ligands out of the cell into lipoproteins (74).

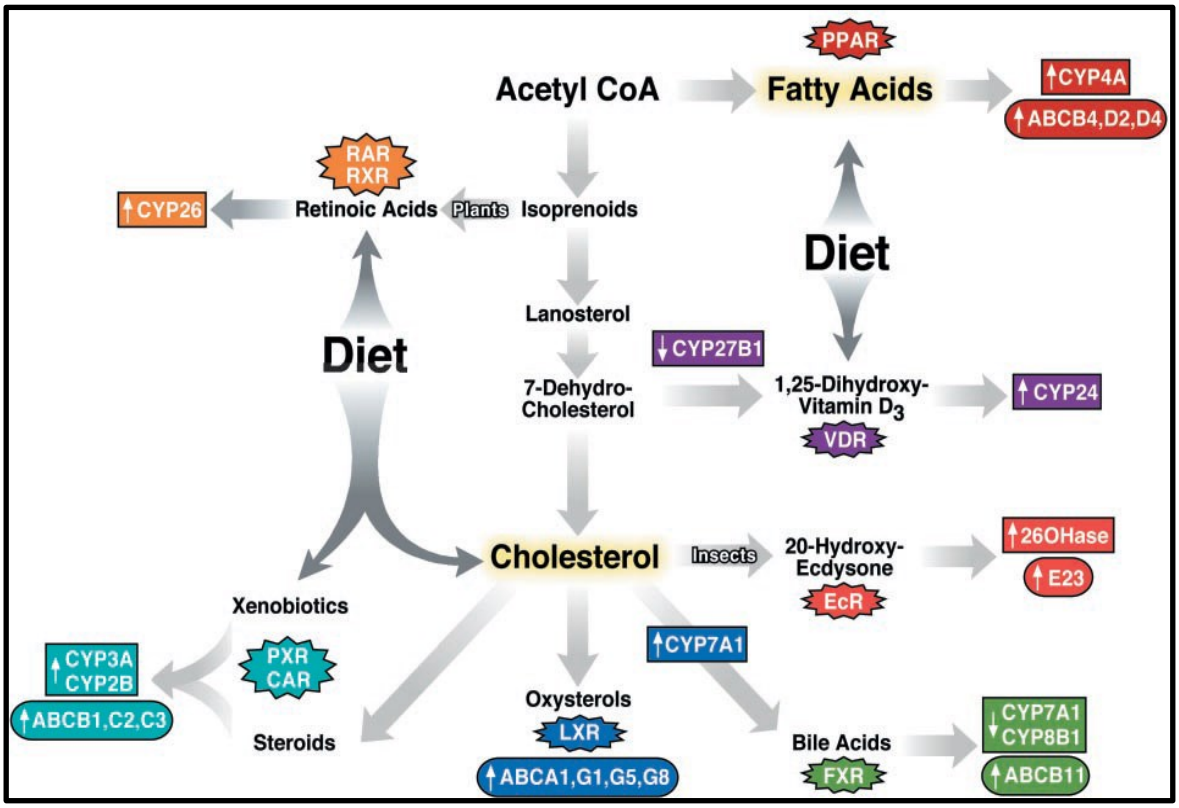


Illustration-5 Nuclear receptors, ligands and metabolic pathways

Adapted from Chawla et. al. Science (2001)294

1.3 LXR and atherosclerosis

Atherosclerosis, the leading cause for mortality and morbidity related to coronary and cerebrovascular diseases all though traditionally viewed as a lipid storage disorder is also an ongoing inflammatory syndrome. Recent advances in the field of cardiovascular research emphasize an unequivocal role of inflammation in mediating all stages of atherosclerosis such as initiation, development and thrombotic complications. Inflammation adversely influences both lipoprotein metabolism and the arterial wall biology leading to a completely altered pathophysiological state.

Physiologically atherosclerosis is an ongoing vicious cycle involving an altered lipid homeostasis and inflammatory response. The maintenance of this sensitive balance of lipid homeostasis and inflammation is synergistically carried out by a battery of transcription factors and nuclear receptors. Dysfunction or an altered mechanistic role of these transcription factors can lead to deleterious consequences.

Liver X receptor (LXR) is one such important nuclear receptor which plays a crucial role in transcriptionally regulating lipid metabolism and inflammation. LXRs act as "cholesterol sensors" that get activated by elevated intercellular cholesterol levels and in turn transcriptionally regulate genes that are involved in reverse cholesterol transport, cholesterol conversion to bile acid and intestinal absorption of cholesterol. Two isoforms of LXRs are LXRα and LXRβ, LXRβ is ubiquitously expressed whereas LXRα is expressed in metabolically active tissues such as liver, adipose, kidney, intestine, macrophages, lung and adrenal glands.

The role of LXR in macrophage cholesterol homeostasis and inflammation has been a topic of immense interest since a decade now considerable evidence links an altered macrophage function and LXR signaling to the progression of atherosclerosis.

The maintenance of cholesterol homeostasis in macrophages is a delicate balance between influx, de novo synthesis, esterification/hydrolysis and efflux. Activation of LXR in macrophages enhances the expression of ATP-binding cassette transporters ABCAs which

in turn increases the APOAI mediated cholesterol efflux and packaging to cholesterol into large HDL particles. LXR activation also increases the mobilization of free cholesterol to the plasma membrane there by increasing the availability of cholesterol to be effluxed. Recently LXRα has been shown to be post transcriptionally involved in LDLR degradation and there by reduction of cholesterol accumulation.

Macrophages by their innate ability to take up modified lipids are thought to accumulate ligands of LXR and release inflammatory mediators leading to a severe inflammatory microenvironment within the atherosclerotic lesion. Such anexcess in arterial wall inflammation is thought to increase atherogenesis and predisposes the individual to cardiovascular diseases.

Substantial evidences suggest that in addition to promoting reverse cholesterol transport, LXRs in macrophages also reciprocally repress the expression of pro-inflammatory mediators such as INOS, IL-6, COX2, MMP9. Recent studies also indicate a cross talk between macrophage inflammatory and LXR signaling pathways. TLR3 and TLR4 activation negatively influences the LXR induced cholesterol efflux pathway. However the signaling events related to this are currently unknown.

Endothelial cells and its importance in atherosclerotic plaque development is very well established however, the role of LXR and target genes in that cell types is elusive. It has been observed LXRs are differentially expressed in different parts of the aorta and the most arthro-prone region of the aorta expresses 5 fold less than the rest of the aorta.

Smooth muscle cells (SMCs) also play a crucial role in vascular and contractility. SMCs are involved in plaque stabilization, migration to form fibrous cap and rupture prevention. Both the isoforms of LXRs are expressed in very less amount in the SMCs.

LXR agonist on SMCs can influence proliferation, calcification and contractility however the physiological impact of it is ambiguous, as it is yet unclear whether LXR agonist induced vascular calcification can induce plaque rupture or stabilize the plaque.

Manipulation of LXR signaling pathways in treating atherosclerosis has been a topic of debate and interest. Though athero-protective the major drawback because of which the future of LXR agonist as a drug to control the progression of atherosclerosis is that LXR also increases hepatic lipogenesis thereby increasing plasma triglyceride (TG) levels and promote hepato-steatosis. Given the situation, it might be worthwhile to find alternative mechanisms to activate LXRs in a tissue specific way. It is also tempting to study the post transcriptional mechanisms that regulate the systemic LXR expression and also to observe any differential expression of these pathways under normal and diseased state.

1.3.1 MicroRNA and LXR

In the recent years there have been several attempts to delineate post-transcriptional pathways regulating LXR. In the liver, miR-613 represses LXRα and interestingly the expression of miR-613 was induced by LXR activation by promoter binding of SREBP (75). Concurrently, *Zhong et al* reported that in hepatocytes miR-1/206 repressed LXRα-induced lipid accumulation. Consequently several LXRα target genes involved in lipogenesis such as SREBP-1c, FAS, and CREBP were also downregulated (76). Although LXRα is expressed in many other tissues such as adipose, kidney, intestine, macrophages, lung and adrenal glands there has been no reports on post-transcriptional mechanisms in tissues other than liver. The fact that microRNA regulation is cell and cellular environment specific it would be important to study tissue specific differences in microRNA mediated LXRα regulation.

1.4 miR-206: Current understanding and perspective

miR-206 is considered to be a muscle specific microRNA involved in muscle differentiation and proliferation. The role of miR-206 has been implicated in rat models of myocardial infraction (MI) (77). MiR-206 is an intergenic microRNA located on chromosome 6 (Location: 6p12.2) in between the IL17A and fibrocystin genes. Mature microRNAs normally show cytoplasmic localization, interestingly miR-206 seem to shuttle between the nucleolus and the cytoplasm during different stages of muscle development (51). Previous papers also show that miR-206 is specially enriched in ribosomic regions both within the cytoplasm and the nucleus.

Recent papers show that miR-206 is down regulated in estrogen receptor positive breast cancer cell and over expression of miR-206 in turn resulted in down regulation of ERα, which indicates an auto regulatory feedback mechanism (78). Differential expression of miR-206 has been implicated in various diseases such as breast cancer metastasis, schizophrenia, MI, human rhabdomyosarcoma (RMS) and amyotrophic lateral sclerosis (ALS) (79-81). The expression of miR-206 is less abundant in the liver but lack of SHP expression further downregulated the expression of miR-206 in liver cells (82). This suggests a broader physiological function and tissue specific expression than originally anticipated.

Transcriptional activation of miR-206 is mediated by AP-1 binding to the AP-1 binding element at the promoter region. AP-1 is a heterodimeric protein composed of proteins belonging to the c-Fos, c-Jun, ATF and JDP families. AP-1 regulates gene expression in response to a variety of stimuli such as cytokines, stress, growth factors, bacterial and viral infection (82).

In contrast (Yin Yang 1) YY1 is a transcriptional repressor of miR-206 gene. YY1 is a multifunctional protein that plays a fundamental role in development, differentiation, replication and cellular proliferation. Depending on the context of the cell, YY1 can initiate, activate or repress transcription of a gene. The promoter region of miR-206 has YY1 binding region. It has been observed that YY1 could suppress C-jun activity by direct

protein- protein interaction. Over expression of YY1 in the absence of SHP resulted in repression of the miR-206 gene (82). Interestingly, miR-206 has also been reported to have cell cycle specific regulatory effects on KLF4 mRNA expression (41). Brown adipose tissue expresses miR-206 to a greater extend whereas there is no detectable expression in the white adipose tissue (83). MiR-206 has also been implicated to have important functions in the maintenance of the circadian rhythm (84).

In summary, miR-206 is a microRNA that has been reported to have important functions in several diverse cellular networks, have been reported to both repress and activate genes and is part of complex regulatory networks.

2. AIMS OF THE PhD THESIS

Major Aim:

- To identify microRNAs that potentially regulate LXRα expression and to study their impact on cellular cholesterol homeostasis.

Related to the major aim:

- To identify tissue specific differences in microRNA mediated regulation of LXRα
- To identify patho-physiological modulators of microRNA expression
- To identify regulatory loops in microRNA metabolism

3. METHODS

3.1 *Animals and cell culture*

Animal experiments: All animal experiments were carried out in accordance to the guidelines of the Division of Genetic Engineering and Animal Experiments, Austrian Federal Ministry of Science and Research (Vienna, Austria). MiR-206 knock out (KO) mice were obtained from the Dr. Eric N. Olson's laboratory (Department of Molecular Biology, University of Texas Southwestern Medical Center, TX). WT mice and miR-206 KO mice were on a mixed background (29SvEv-C57BL/6). All animals were fed ad libitum with normal chow diet (caloric Intake = 11.9% from fat; Ssniff®, Soest, Germany) and were maintained in a 12h light/dark cycle in a temperature controlled environment.

Cell lines*:* HepG2 *(ATCC NUMBER: HB-8065)*, THP-1 *(ATCC NUMBER: TIB-202)* and COS-7 *(ATCC NUMBER: CRL-1651)* cells used in this study were obtained from American type culture collection (ATCC) and were grown in DMEM supplemented with 10% fetal bovine serum and 1% pen-strip (pencillin-streptomycin). THP-1 cells were maintained in RPMI media (Gibco; Invitrogen, CA, USA) supplemented with 10% fetal bovine serum and 1% pen-strip. For differentiating THP-1 cells, the cells were treated with 100nM phorbol 12-myristate 13-acetate (PMA; Sigma# P 8139, St. Louis,USA) for 3 days.

3.2 *Primary cell culture: Isolation and differentiation*

3.2.1 Mouse bone marrow derived macrophages (BMDMs)

Mouse bone marrow derived macrophages were isolated from miR-206KO and control mice. Briefly, Mice were sacrificed by cervical dislocation. The abdomen and hind legs were sterilized by spraying 70% ethanol. Mouse femur and tibia were scrapped thoroughly to get remove skeletal muscles. Femur and tibia were briefly washed in sterile PBS and washed again with PBS containing 1% gentamycin. Femur and tibia from each group were pooled at this stage and were transferred into respective 50mL falcons containing 1X PBS supplemented with 1% Gentamycin. The bones were stored at 4 ºC.

Preparing L929 culture medium (LCM)

Batches of L929 cells were grown in 75cm^2 tissue culture flasks with 55ml of DMEM containing 10% fetal calf serum (FCS), L-glutamate and 1% gentamycin. Cells were grown for 10 days and the medium was collected. The collected media was initially filtered through 0.45µm filter and were stored at -80ºC until further use.

Preparation of lymphocytic L929 conditional Media (LCCM)

Before culturing mouse BMDMs LCCM was freshly prepared by supplementing 100ml of RPMI with 20% FCS, 30ml of LCM, 1ml of gentamycin and 1ml of L-glutamate.

Terminal ends of femur and tibia were cut with a scalpel and the bone marrow was flushed out with 5ml of LCCM using a 23G needle and syringe. Bone marrow from femur and tibia were collected in separate falcons and centrifuged at 1000G for 5 minutes. After centrifugation the supernatant was discarded and the pellet was thoroughly re-suspended in 10ml of media. Cells were counted using a heamocytometer and the concentration was adjusted to 2×10^6 per ml in LCCM. 10ml of the Cell suspension was plated on to 10cm bacteriological plates. Media was changed on day 1, day 3 and day 6. The cells were spilt on day 6 and were used for experiments after differentiating in LCCM media for 2 days. For M1 macrophages, the cells from day 8 were treated for 4 hours with 100ng/ml LPS.

For differentiating BMDMs to M2 macrophages, LCCM was complemented with 10ng/ml IL-4 during plating on day 0 subsequently media (LLCM +IL-4) was changed on day 1, day 3 and day 6.

3.2.2 Mouse peritoneal macrophages (MPMs)

Mouse peritoneal macrophages from miR-206KO and control mice were collected by intraperitoneally injecting 3ml of 3% thioglycollate per mouse. MPMs were collected after 3 days by flushing the peritoneum with 10ml of PBS-EDTA solution. MPMs from several mice were pooled at the stage and the cells were spun at 1000RPMs for 1 minute. After centrifugation, the pellet was re-suspended in RPMI containing 10% FCS, 1% gentamycin and 1% L-glutamate. Cells were counted and were plated at a density of 2×10^6 per ml and allowed to attach for 12-16 hours after which cells were used for various experiments as indicated.

3.2.3 Human peripheral blood mononuclear cells (hPBMs)

Human peripheral blood mononuclear cells were isolated from healthy normolipidemic individuals and were differentiated into monocyte-derived macrophages (MDMs) as described below.

Firstly, Buffy coats obtained from the donors were maintained overnight at 25ºC on a shaker. After overnight incubation, the buffy coats were transferred to 50ml falcon tubes and are diluted with 1X PBS+EDTA solution to make up the final volume to 50ml. 15ml of histopaque (room temperature) was aliquoted into fresh falcons and 20ml of the diluted buffy coat was overlaid on it. HPBMs were phase separated by centrifuging at 20 ºC on a speed of 2200 RPMs for 25 minutes. After centrifugation, the interphase was carefully transferred into a fresh falcon tube and was washed by adding enough 1XPBS+EDTA to make up the final volume of 50ml. The cells were pelleted by centrifuging at 1300 RPMS for 10 minutes after which the pellet was resuspended in 1ml of 1X PBS+EDTA. Volume was adjusted to 50ml and the cells were pelleted at 1200rpms for 10 minutes. The above washing step was repeated twice more and the cells were pelleted at 1200RPM (second wash)/900RPM (third wash) for 10 minutes. After the third wash the cells were re-suspended with 10ml of 1XPBS, the volume was adjusted to 50ml and the cells were pelleted at 900RPM for 10 minutes. The pellet was re-suspended in 10 ml of RPMI supplemented with 20% human serum, 1% L-glutamate and 1% gentamycin. The cells were counted using a heamocytometer and the concentration was adjusted to 2×10^6 per ml. The cells were plated in 6-well primera tissue culture plates or 60mm primera petri-

dishes. After 3 hours, the cells were washed with PBS and the media was changed to normal growth media (RPMI+20% FCS+1% L-glutame+1% gentamycin) for resident macrophage (RM) differentiation or M2-growth media (growth media + 10ng/ml IL-4) for alternative macrophage (M2) differentiation. The cells were allowed to differentiate for 7 days. For classically activated (M1) macrophage differentiation cells at day 7 were washed with PBS and were treated with 100ng/ml of LPS in growth medium (without serum) for 4 hours. After four hours the media was removed, the cells were washed with 1X PBS and the media was replaced with the normal growth media. The cells were allowed to differentiate into M1 macrophages for another 24 hours. After differentiation 7-8 days of differentiation MDMs were used as indicated.

3.3 *Computational prediction*

The microRNA that post transcriptionally regulated LXR was identified using publicly available prediction software suite, TargetScan (http://www.targetscan.org/). The software suite, RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) was used further to identify the microRNA that had the most favourable binding energy to the LXR mRNA. For predicting microRNA promoter interaction MicroPIR prediction software was used.

3.4 *miR-206 overexpression and knock-out*

HepG2 cells were transfected with 100nM of synthetic precursor miR-206 (Ambion) using MetafectinePro. The transfection was carried out as per the manufacturer's instruction.

For miR-206 overexpression or knockdown in THP-1 cells, pCDH-CMV-MCS-EF1-copGFP cDNA cloning and expression vector **(SBI # CD511B-1)** or miR-206KO vector **(SBI #MZIPPA/AA-1)** was co transfected with packaging vectors VSVG and PCMV (Clonetech) into 293T cells using standard calcium phosphate method.

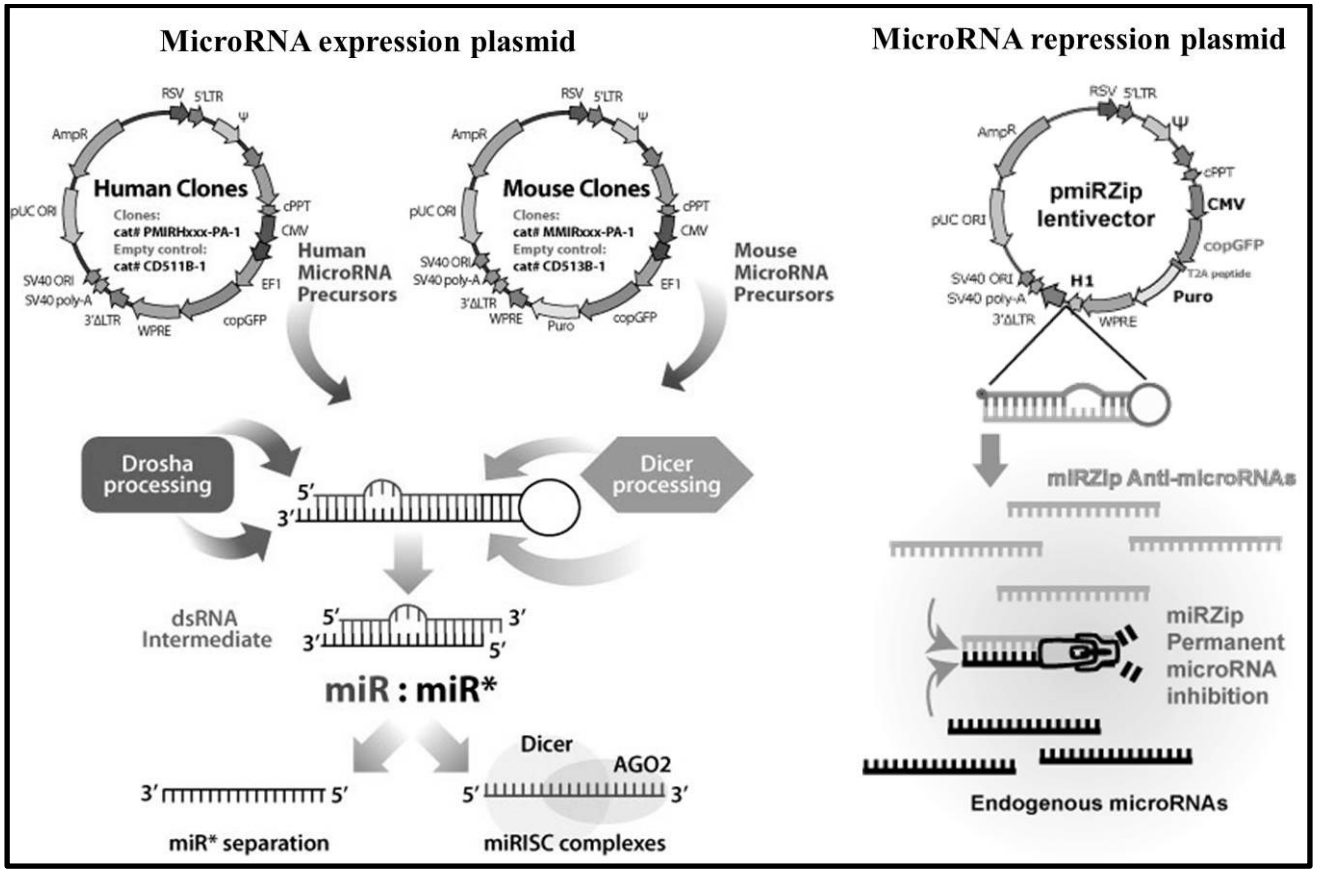


Illustration-6 ***Vector map for microRNA expression and repression plasmids.***

(Adapted from system biosciences)

Viruses were harvested by centrifuging the supernatant at 2000 g for 20minutes at 4^0 C. The supernatant was filtered and purified further and the viral stocks were aliquoted and maintained at -80^0 C until use. THP-1 cells were transfected using the viral particles and GFP positive cells were sorted out using FACS calibur.

3.5 RNA extraction and quantitative real time PCR analysis

Total RNA was extracted by Trizol (Peqlab Biotechnologies GmbH, Erlangen, Germany) extraction method. Using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), two micrograms of RNA was reverse transcribed and quantitative RT-PCR was carried out using gene specific primers (table-1) and QuantiFast SYBR Green PCR Kit (Qiagen, Germany) on LC480 (Roche diagnostics, Basel, Switzerland). All samples were normalized to cyclophilin mRNA expression as an internal control.

For mature miR-206 quantification the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Vadeak, Denmark) was used together with miR-206 or U6 (reference gene) specific predesigned LNA primers. All experiments were carried out in triplicates. The protocol and the cycling conditions were carried out in accordance to the manufacturer's instruction. Quantitative real time PCR was carried out using QuantiFast SYBR Green PCR Kit (Qiagen, Germany) on LC480 (Roche diagnostics, Basel, Switzerland). Data was analyzed using the open access software Relative Expression Software Tool – REST 2010.

Human primer sequences		
ABCA1	GAGTGAAGCCTGTCATCTACTG	GTGGAGGACACATAGGACTTC
ABCG1	CAGTCGCTCCTTAGCACC	TCCATGCTCGGACTCTCTG
APOE	CGCTGGGTTGCAGACACTGT	GGCCTTCAACTCCTTCATGGT
LXRα	GGGTAGCTGTTTAGCAAAGTCAA	CATGGCACCAGATCCCCATAG
SREBP-1c	AGCCATGGATTGCAGGTTTG	AGGCTTCAAGAGAGGAGCTC
hsa-miR-206	GCCACATGCTTCTTTATATCCCC	TTGCCGAAACCACACACT T
Cyclophilin	GCATACGGGTCCTGGCATCTTGTCC	ATGGTGATCTTCTTGCTGGTCTTGC
U6	GCTTCGGCAGCACATATACTAAAT	CGCTTCACGAATTTGCGTGTCAT
Mouse primer sequences		
ABCA1	ACACCTGACACACCAGCTACAAGGC	TGAGTTCTTCCCACATGCCCTCC
ABCG1	CGGATTTTGTATCTGAGGACGAA	TTCATCGTCCTGGGCATCTT
APOE	GAGCCGACATGGAGGATCTACG	CGAAGACGTCTAGTAGCCGTAGG
SREBP-1c	ATCGGCGCGGAAGCTGTCGGGGTA	ACTGTCTTGGTTGTTGATGAGCTGG
Cyclophilin	GCATACGGGTCCTGGCATCTTGTCC	ATGGTGATCTTCTTGCTGGTCTTGC

Table-1 Primer sequence used for quantitative real time PCR

3.6 Protein extraction and Western blotting

3.6.1 Protein extraction: Cells were lysed in ice cold RIPA buffer supplemented with protease inhibitory cocktail (PIC) at 1:2000 dilution. The samples were sonicated [Bransons ultrasonics (10 pulse; duty cycle 30)] and then centrifuged at 13,000 RPM for 15 minutes in cold condition (4°C) The pellet was discarded and the soluble fraction was separated and stored at -20 º C for future experiments.

3.6.2 Protein quantification: Bradford assay (BioRad) was performed to quantify the amount of protein present. A reaction mixture was made up of 800µL of MilliQ water and 200µL of Bradford Dye (Bio-Rad) to which1µL protein sample was added. Protein samples were quantified using a spectrophotometer reading absorbance at 595nm. The spectrophotometer was blanked on a sample containing 800µL of MilliQ water, 200µL of Bradford Dye and 1µL of lysis buffer instead of protein. Protein concentrations were measured from the BSA standard curve.

3.6.3 SDS-PAGE and immunoblotting: Based on the results obtained from the Bradford assay the samples were diluted in MilliQ water to bring to the final concentration of 50µg/mL. To make up the final volume of 20µL, 15µL of protein sample and 5µL of 4X loading dye was added[0.25M Tris HCl, 8% SDS, 40% Glycerol, 0.02 % bromophenol blue, and 10% 2-mercaptoethanol] and heated in a heat block at 95°C for 2 Minutes. After a brief centrifugation the sampled were loaded on a NuPAGE® 4—12% Bis-Tris Gel (Invitrogen) and were run for 45 minutes at 200 V in 1X MES [2- (N-morpholino) ethane sulfonic acid] buffer. Protein was transferred to a Hybond™-C membrane (Amersham Bioscience) in 1X ATM transfer [25 mM Tris, 192 mM glycine, 20% methanol] buffer at 40V for 45 minutes at 4ºC. After the membrane was blocked in Blotto [5% skim milk in PBS and 0.1% Tween-20 (Amresco®Polyoxoethylene-20-Sorbitan Monolaurate)] immunoblotting was carried out. The membrane was incubated with the respective antibody (table-2) overnight at the 4 ºC and was washed three times in PBS-T the subsequent day. The membrane was incubated for 2 hours on a rotating wheel (room temperature) with the respective Horseradish peroxidase conjugated secondary antibody.

Blot was washed three times in PBS-T to remove the traces of the secondary antibody and immunodetection was carried out using Immobilon ™ Western chemiliminescent HRP substrate (Millipore) and the blot was observed under Bio-Rad ChemiDoc MP imaging system.

Antibody	Product information	Dilution
Human LXRα	Abcam (ab-41902)	1:250
Mouse LXRα	SCB (SC-1202)	1:500
GAPDH	SCB (FL-335)	1:1000
ABCA1	Novus Biologicals (NB400-105)	1:1000

Table-2 Antibodies used for Western blotting

3.7 Luciferase construct and cloning

PremiR-Reporter constructs and synthetic Pre-miR206 used in this study were purchased from Ambion. The expression plasmid for miR206 was purchased from SBI. Briefly the sequence 3'UTR region of LXR, which is the putative miR-206 target for interaction, was cloned. LXR sequence flanking the restriction sites for Blp1 and BamH1 are as follows.

5'-AATGCACTAGTGACTGAGAAGGGCAAACATTCCTGCTCAGCAAGCTTAATGC-3'
5'- GCATTAAGCTTGCTGAGCAGGAATGTTTGCCCTTCTCAGTCACTAGTGCATT-3'

A scrambled negative control was also cloned to check for specificity in miR-206 LXR 3'UTR interaction. The plasmid and the sequence to be cloned were linearized and digested using the same fast digest Blp1 and BamH1 (Fermentas) using the manufacturer's

protocol. The digested products were run on 1.2% agarose gel and were extracted using Qiagen DNA Gel extraction kit.

The restricted plasmid and the sequence to be cloned were ligated using T4 DNA ligase (NEB), after which the resultant product was confirmed for the presence of the insert by a single digestion protocol employed Fast digest SpeI restriction enzyme, the site for which is located within the cloned region of the sequence. The plasmid was then transformed into Top10 competent cells using the standard bacterial transformation protocol. The plasmid was extracted using Qiagen Endotoxin Free Midi Plasmid extraction kit and the plasmids were sent out for sequencing.

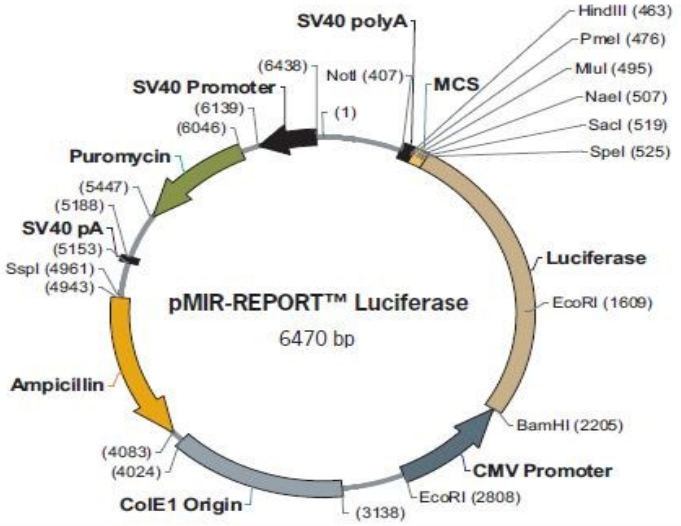


Figure 1. pMIR-REPORT Luciferase

CMV Promoter: 2210–2813
Firefly luciferase: 540–2210
MCS: 467–539
SV40 Poly(A): 404–467
SV40 Promoter: 6139–6438
Puromycin: 5447–6046
SV40 pA signal: 5153–5188
Ampicillin: 4083–4943
ColE1 Origin: 3138–4024

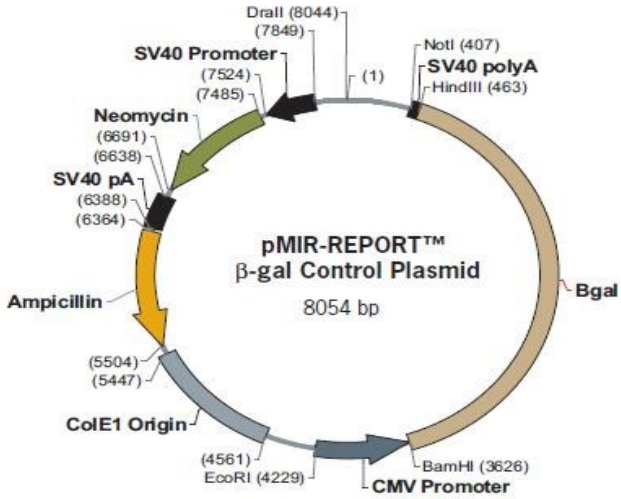


Figure 2. pMIR-REPORT β-gal Control Plasmid

CMV Promoter: 3627–4232
β-gal: 463–3627
SV40 Poly(A): 404–463
SV40 Promoter: 7524–7849
Neomycin: 6691–7485
SV40 pA signal: 6388–6638
Ampicillin: 5504–6364
ColE1 Origin: 4561–5447

Illustration-7 Vector map for pMIR-REPORT luciferase and β-gal control plasmids.
Top panel: Luciferase reporter construct with MCS on the 3'UTR
Bottom panel: Transfection control β-gal plasmid. (*Adapted from Life technologies)*

3.8 Luciferase assay

100ng/ml of the Pre-miR Reporter construct positive for the insert were co transfected with 25ng/ml β-Gal control plasmid and also with the 100nM synthetic precursor-miR206 strand or the 1μg/ml of miR-206 expression vector into COS-7 cells using Xtremegene siRNA transfection Kit (Roche). The cell were incubated after transfection for 36h and the cells were lysed in passive lysis buffer (Promega), 50μl of the lysate was used for β-Gal assay and 10μl (2X) were used for Luciferase Assay. β-Gal assay and Luciferase Assay (Promega) were carried out using the manufacturer's protocol.

3.9 LPS and TNFα treatment of macrophages

THP-1 cells were differentiated using 100nM PMA as described earlier. The cells were serum starved for 12h and treated with 10ng/ml TNFα and incubated for 24h before RNA extraction. Human peripheral blood mononuclear cells were isolated from normolipidemic individuals and were differentiated into monocyte-derived macrophages (MDMs) for 7 days as described earlier and were treated with 100ng/ml LPS in serum free DMEM for 24h before RNA extraction. RNA was extracted using the Trizol extraction method and the relative expression levels of miR-206 were quantified using SYBER green based quantitative real time PCR.

3.10 Lipoprotein and modified lipoprotein treatment

THP-1 cells were differentiated using 100nM PMA and allowed to differentiate as described earlier. The cells were serum starved for 12h and treated with native, acLDL, oxLDL or VLDL.

3.11 Lipoprotein isolation from human plasma

LDL, VLDL and HDL was isolated from human plasma using gradient ultracentrifugation. Human plasma was mixed with NaCl (5g/ 100ml of plasma) and sodium azide (1g/l of plasma) to adjust the plasma density to 1.06g/l. Plasma samples (approximately 40ml) were ultracentrifuged at 48000 RPM for 24 hours at 15ºC. After centrifugation, LDL and

VLDL were collected from the upper phase and HDL was collected from the lower phase. The separated phases were dialyzed using distilled water for 30 minutes after which the samples collected from the upper phase was subjected to further centrifugation at 24 hours at 15ºC after adjusting the density to 1.06g/l. HDL from the lower phase was separated into another tube, the density was adjusted to 1.063 g/l using 1.21g/l sodium bromide and centrifuges at 48000 RPM for 4 hours at 15ºC.

After the second step of centrifugation, LDL (lower) and VLDL (upper) were phase separated. The lower phase was transferred to fresh 3ml centrifugation tubes and was subjected to a third step of centrifugation at 48000 RPM for 24 hours at 15ºC. The yellow coloured band that separated out after the centrifugation was collected, sterilized by filter sterilization (LDL-0.2µm and VLDL-0.8 µm) and stored at 4 ºC until further use. HDL was collected from the upper layer after the second centrifugation and was dialyzed for 48 hours in PBS.

3.12 Acetylated LDL preparation

Native LDL collected from human plasma as described above was acetylated by adding diethyl ester. Briefly, equal volumes of native LDL and saturated NaCl were stirred continuously on ice and 5.4µl of diethyl ester was added to the mixture every 3 minutes till a total volume of 57.76µl of diethyl ester was added to the mixture. To avoid precipitation after the addition of 16.2 µl of diethyl ester, 100ml of PBS was added to the mixture. The mixture was stirred on ice for 45 minutes followed by dialysis for 2-3 hours in PBS at 4 ºC.

3.13 oxidative modification of LDL

Oxidized LDL was prepared as described earlier (85). To modify native LDL, 1 mg/ml of LDL in PBS was mixed with HOCl solution and was incubated for 60 minutes. Treating native LDL with HOCl at a concentration of 0.2 or 1.6mM resulted in 100:1 or 800:1 oxidant :lipoprotein. molar ratio respectively. Oxidized LDL was stored at 4° C for maximum 12 to 24 hours before use.

3.14 cholesterol efflux assay

THP-1 cells were serum starved in RPMI medium supplemented with 10% LPDS (lipoprotein deprived serum) and 1% Pen-strip for 16h after which 3H-cholesterol was added to the cells and incubated for 24h. After that, 2.8% apolipoprotein B–depleted serum from pooled human serum (HDL), 0.5% of pooled human serum or 20µg/mL apoAI were added as cholesterol acceptors for 6h. The cells were lysed in 0.3M NaOH at 85^0 C overnight. Efflux capacity was quantified using liquid scintillation to measure radioactive cholesterol effluxed from the cells (medium + intracellular lipids). The values were statistically analysed by the student's t-test. Cholesterol efflux assay was performed in MPMs after loading with [^{3}H]-cholesterol labelled ac-LDL as described earlier (86).

3.15 LXR and PPARγ agonist treatment

THP-1 cells were differentiated using 100nM PMA and allowed to differentiate as described earlier. The cells were serum starved for 12h and treated with 5µM, 10µM and 20µM concentrations of Rosiglitazone (PPARϒ agonist) or 1µM, 5µM or 10µM of TO-90137 (LXR agonist) and the cells were incubated for 24h before RNA extraction. RNA was extracted using the Trizol extraction method and the relative expression levels of miR-206 were quantified using SYBER green based quantitative real time PCR

3.16 Statistics

All statistical analyses were performed using GraphPad Prism 5.0. In cholesterol efflux assays the differences between groups were analyzed using two-tailed, unpaired Student's t-test. All qRT-PCR experiments were calculated using REST2009.

4. BUFFERS AND REAGENTS

4.1 Buffers used for Western blotting

RIPA Buffer:

- ✓ 50mM KH2PO4
- ✓ 150mM NaCl
- ✓ 0.5% Deoxycholate
- ✓ 1% Triton X-100

RIPA buffer was stored at 4°C. Protease inhibitory cocktail was at 1:2000 dilutions to RIPA buffer added just before using.

20X NuPAGE® MES SDS running Buffer:

To prepare 500ml of MES buffer the following ingredients were mixed.

MES	**97.6g**
TrisBase	**60.6g**
SDS	**10g**
EDTA	**3g**
MilliQ water	**500ml**

10X MES running buffer was stored at 4°C until further use. During western blotting 1X working solution was prepared by diluting 50ml of 10X running MES buffer with 950ml of MilliQ water.

20X NuPAGE® Transfer Buffer (pH 8.4):

To prepare 20X transfer buffer the following ingredients were mix in 125ml of MilliQ water.

Bicine	**10.2g**
Bis-Tris	**13.1g**
EDTA	**0.75g**
MilliQ water	**125ml**
pH	8.4

20X transfer buffer were stored at 4°C until further use. In order to prepare 1X transfer buffer 50ml of the 20x stock was dissolved in 950ml of MilliQ water.

NuPAGE® LDS sample buffer (pH 8.5)

To prepare 10ml of LDS sample buffer the following ingredient was dissolved in 8ml of MilliQ water.

Tris-HCL	**0.666g**
TrisBase	**0.682g**
LDS	**0.800g**
EDTA	**0.006g**
Glycerol	**4g**
SERVA® blue G250 (1%)	**0.75ml**
Phenol Red (1%)	**0.25ml**

After mixing the volume was adjusted to 10ml using MilliQ water. The buffer was stored at 4°C until further use.

Separating Gel buffer/ Buffer-1 (pH 8.8): To prepare separating gel buffer the following ingredients were dissolved in 80ml of MilliQ water.

TrisBase	**18.2g**
SDS (10%)	**4ml**
MilliQ water	**80ml**

After mixing the pH was adjusted to 8.8 with concentrated HCl and the final volume was made up to 100ml with MilliQ water. The buffer was stored at room temperature until further use.

Stacking Gel buffer / Buffer-2 (pH 6.8): To prepare separating gel buffer the following ingredients were dissolved in 90ml of MilliQ water.

TrisBase	**6g**
MilliQ water	**90ml**

After mixing the pH was adjusted to 6.8 with concentrated HCl and the final volume was made up to 100ml with MilliQ water. The buffer was stored at room temperature until further use.

10X washing buffer: To prepare 10X washing buffer the following ingredients were used.

Tween	**5g**
NaCl	**90g**
1M Tris HCl (pH 7.4)	**100ml**

The final volume was made up to 1000ml of MilliQ water. The buffer was stored at room temperature until further use.

Separating Gel:

Reagents	10% SDS gel
Poly Acrylamide	2866.25 µl
Buffer-1	2712.5 µl
MilliQ water	5171.25µl
SDS (10%)	100 µl
TEMED	4.4 µl
APS (10%)	7.6 µl
Final Volume	87804.4 µl

Stacking Gel:

Reagents	10% SDS gel
Poly Acrylamide	326 µl
Buffer-2	770 µl
Glycerine	1770 µl
TEMED	3.8 µl
APS (10%)	38 µl

4.2 RNA buffers

DEPC water: equal volumes of DEPC and MilliQ water were added. The mixture was left open under the hood overnight. After which the DEPC water was autoclaved and stored at room temperature until further use

0.5M EDTA: 186.1g of EDTA and 20g of NaOH were dissolved in 900ml of MilliQ water. With 1N NaOH the pH was adjusted to 8.0. Final volume of 1000ml was made up with MilliQ water, the solution was autoclaved and stored at room temperature until further use

10X MOPS buffer (pH 7.0):

MOPS	**10.5 g**
Na-Acetate	**1.7 g**
EDTA (0.5M)	**5 ml**
DEPC water	**200ml**

The pH was adjusted with 10N NaOH and with DEPC water the final volume was made up to 250ml.

RNA sample buffer (3ml): The buffer was stored at 4°C until further use.

Reagents	10% SDS gel
Formamide (99.5%)	**1.44 µl**
10X MOPS	**280 µl**
Formaldehyde (37%)	**520 µl**
DEPC water	**400 µl**
Sterile Glycerol (80%)	**200 µl**
Ethidium Bromide	**10 µl**

5. RESULTS

5.1 miR-206 targets the conserved 3'UTR of LXRα in humans and several mammals.

To identify post-transcriptional regulators of LXRα, we performed in silico analysis of LXRα mRNA sequence using the Targetscan microRNA prediction tool. Figure-1 shows the stem miR-206 loop structure, the mature miR-206 sequence and the conserved binding site of miR-206 on the LXRα 3'UTR of human and several mammals. Targetscan analysis revealed miR-206 to be a putative miR that potentially targets the 3'UTR of LXRα mRNA (Figure-2).

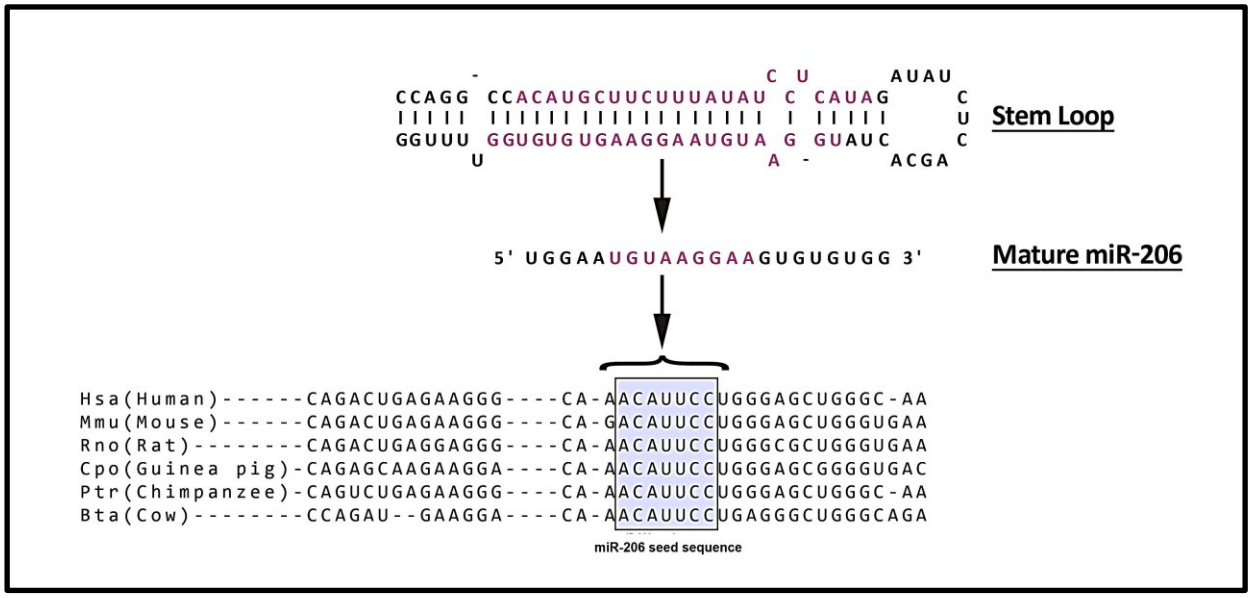


Figure-1 Conserved binding site for miR-206 on LXRα mRNA. *Top: Stem loop structure of miR-206 from which the mature miR-206 is formed. The seed sequence of miR-206 that can putatively bind to the LXRα 3' UTR region is represented in pink and the conserved miR-206 binding site on LXRα mRNA is highlighted in blue.*

We further examined the feasibility of the predicted interaction using the RNAhybrid program that predicts the minimum free binding energy (mfe) of miR206-LXRα interaction (Figure-3). This analysis revealed that the interaction between miR206-LXRα has a favorable mfe of (-) 23.5 kcal/mol. LXRβ lacked miR-206 binding sites and therefore was not among the predicted miR-206 targets.

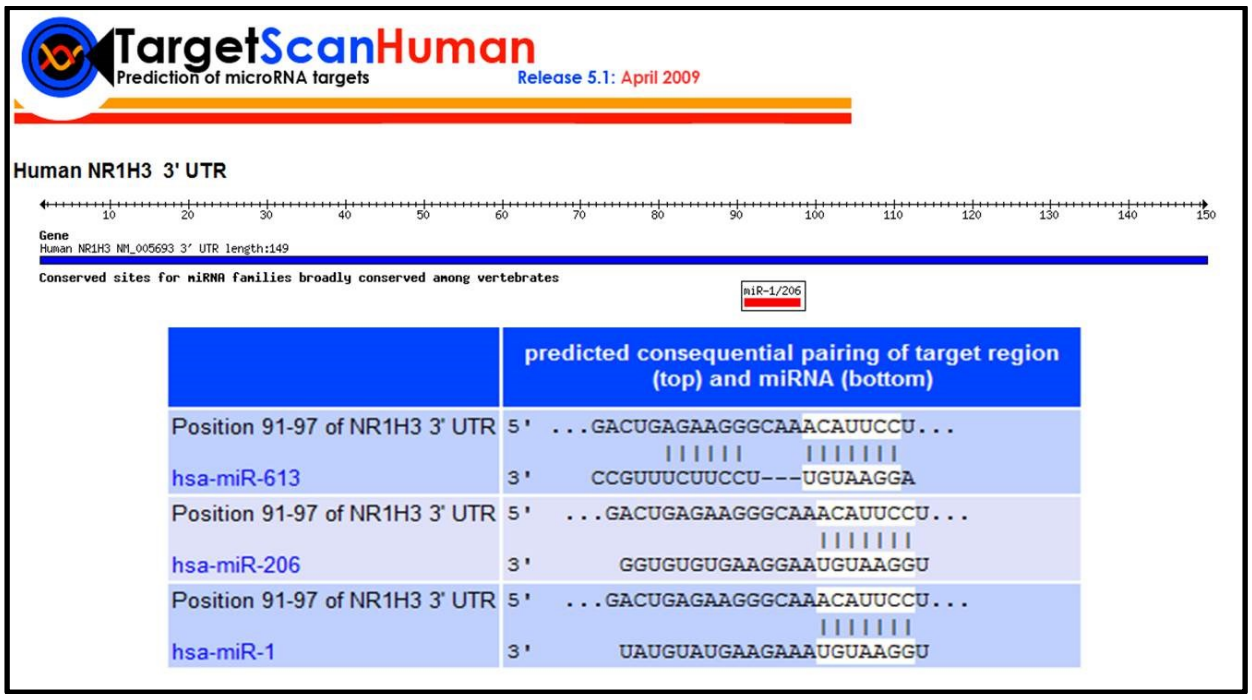


Figure-2 TargetScan analysis. *Results obtained from scanning of LXRα (NR1H3) mRNA sequence for microRNA binding sites. The software predicted three possible microRNA interactions namely miR-613, miR-1 and miR-206 on the 3'UTR region of LXRα.*

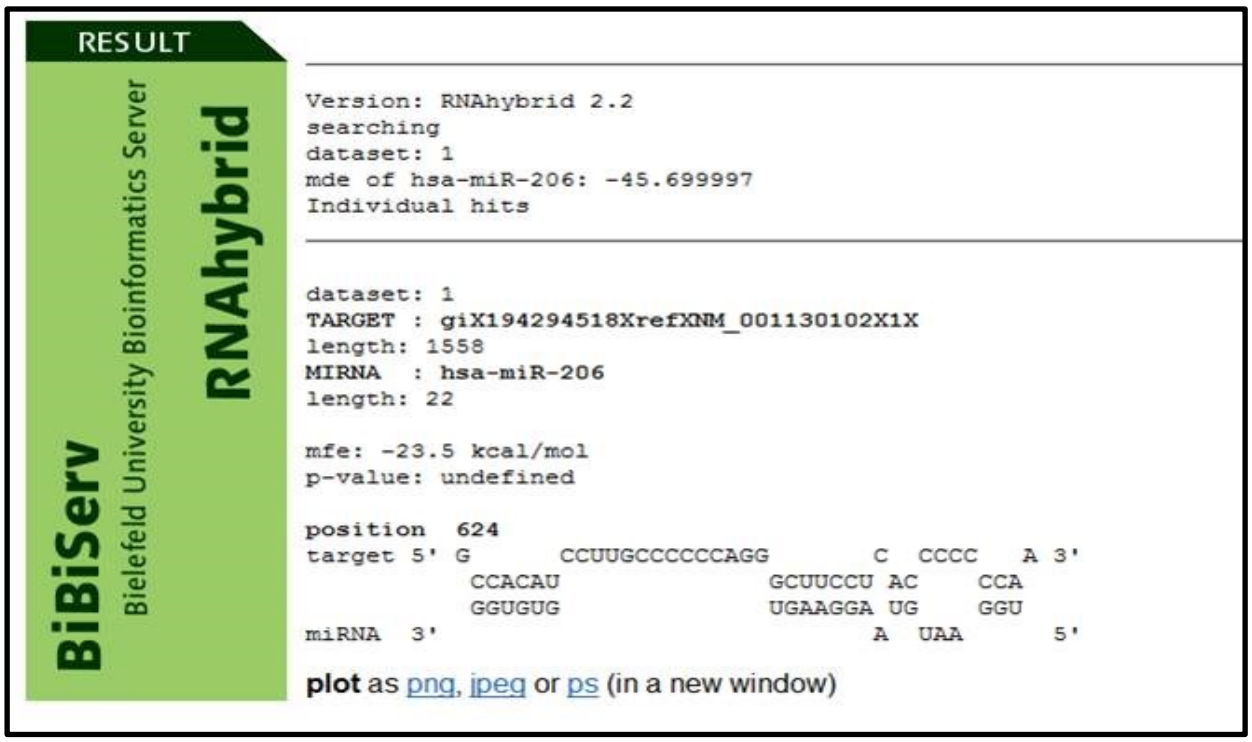


Figure-3 RNAhybrid analysis. *Results obtained from scanning of LXRα (NR1H3) mRNA sequence and miR-206 reveal a favourable minimum free energy of (-) 23.5 kcal/mol.*

To determine whether the predicted miR-206 binding site on LXRα 3'UTR was functional, we cloned the 3'UTR region of LXRα (LXR 3'UTR clone) or a scrambled seed sequence clone of the 3'UTR LXRα (scrambled control) into a miR-luciferase reporter construct. As shown in Figure-4, luciferase activity of the LXRα 3'UTR clone was markedly reduced in COS7 cells transfected with a miR-206 expression plasmid whereas the cells transfected with the scrambled control plus the miR-206 expression plasmid showed no reduction in the luciferase activity. As indicated in Figure-4, the basal luciferase activities of both the LXR 3'UTR clone and the scrambled control were comparable.

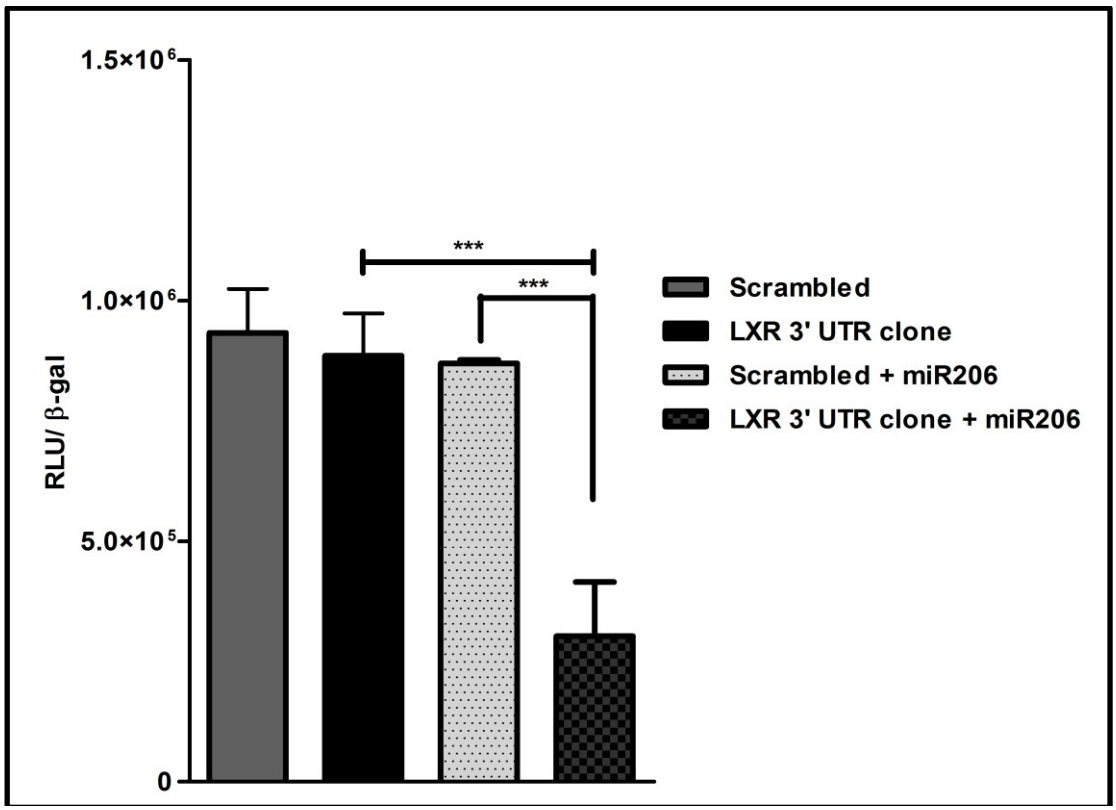


Figure-4 Luciferase reporter assay: *Relative luciferase activity of the LXR 3'UTR construct and the 3'UTR scrambled construct. Mean values ± SEM from three separate experiments is indicated;*** $p<0.001$*

5.2 miR-206 represses mRNA expression of LXRα and its target genes in HepG2 cells.

While this study was in progress, Zhong et al. reported that miR-206 reduces LXRα signaling in mouse primary hepatocytes and in human HepG2 cells(76). In order to confirm and extend these observations, we studied the expression of LXRα and its target genes in HepG2 cells transfected with pre-miR-206. Relative mRNA abundance of miR-206 gene revealed 20-fold increase in the expression level of miR-206 in HepG2 cells transfected with 100nM synthetic Pre-miR-206 (Figure-5).

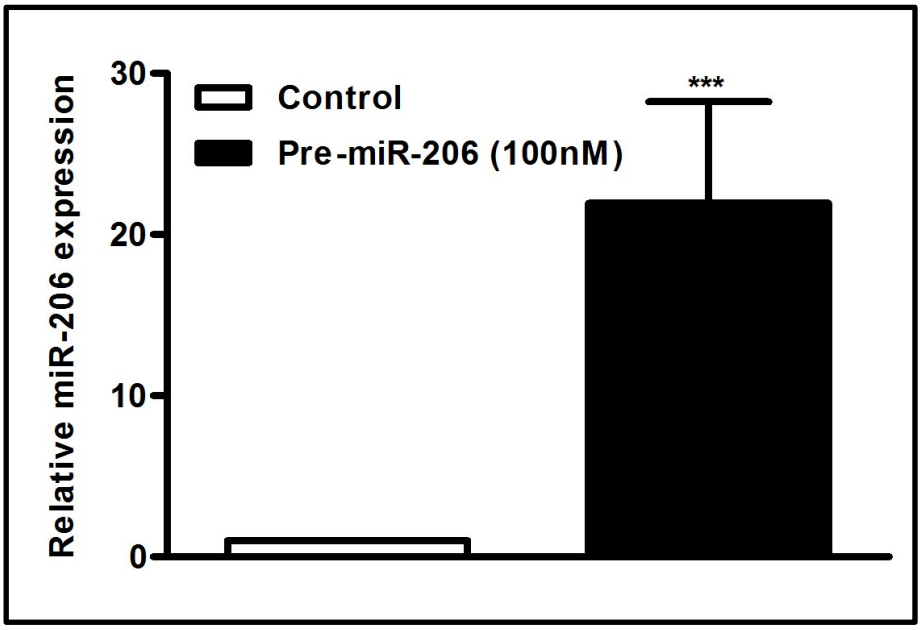


Figure-5 HepG2 transfection efficiency: *Relative mRNA abundance of miR-206 was measured 24 hours post transfection. Mean values ± SEM from three separate experiments is indicated;*** $p<0.001$*

LXRα protein expression in TO9-activated miR-206 expressing cells was also reduced by up to 50% (Figure-6). Figure-7, shows that in miR-206 overexpressing HepG2 cells treated with the LXRα agonist TO901317 (TO9, 10 μM), LXRα expression was reduced by 50%, and the expression of the target genes ABCA1, ABCG1, SREBP-1c and ABCG5 were reduced by 75 – 85%. ABCG8 expression was not significantly affected.

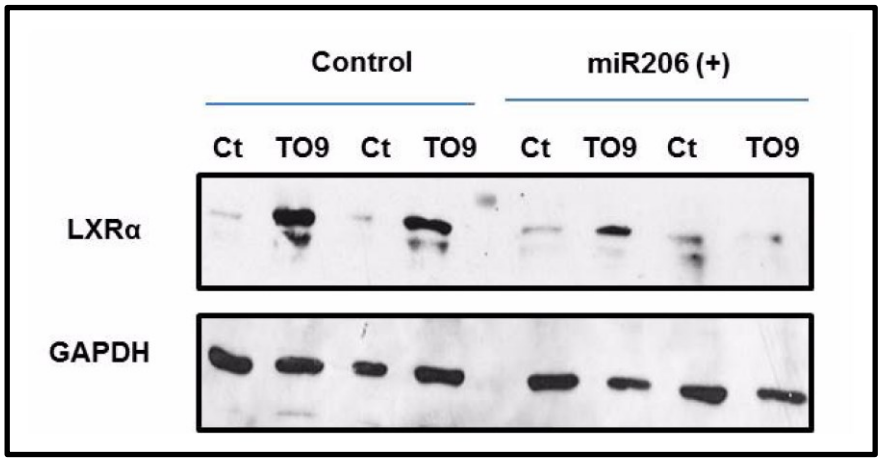


Figure-6 Effect of miR-206 overexpression on LXRα protein expression
Western blotting of LXRα protein expression in HepG2 transfected with miR-206 compared to HepG2 control cells (+/-TO9). Ct: Untreated TO9: LXR agonist TO9-induced.

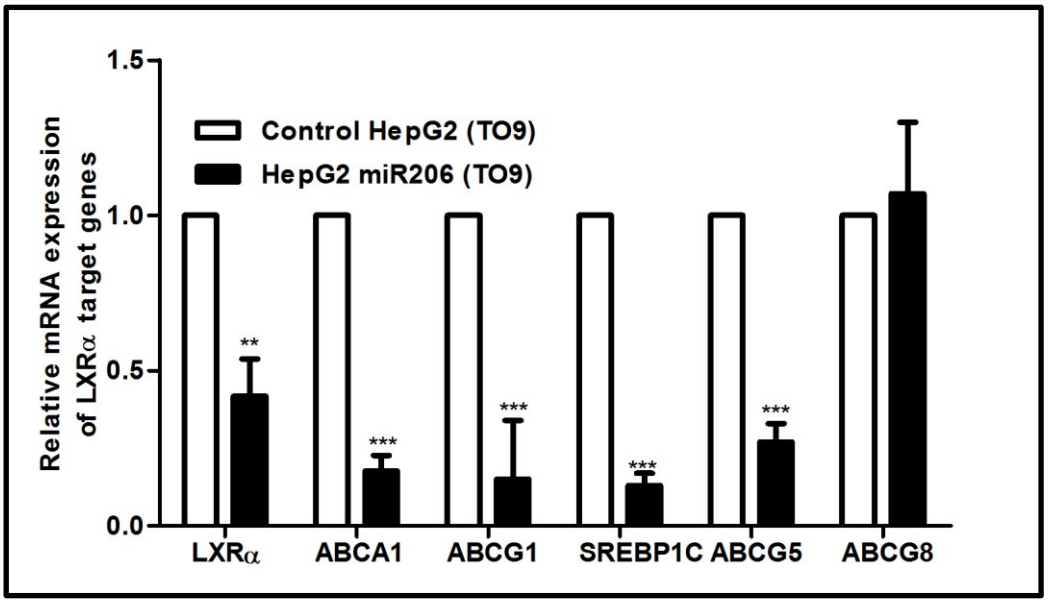


Figure-7 Effect of miR-206 overexpression on LXRα mRNA and its target gene in HepG2 cells: *mRNA abundance of LXRα and its target genes by qRT-PCR. Mean values ± SEM from three separate experiments is indicated; ** p<0.01; *** p<0.001.*

5.3 The expression of LXRα and its target genes is increased in miR206 KO liver.

In order to substantiate the physiological relevance of these findings, LXRα mediated pathways were investigated in miR-206 KO mice. In Figure-7a we show, that LXRα protein expression in miR-206 KO liver is markedly increased by up to 75%.

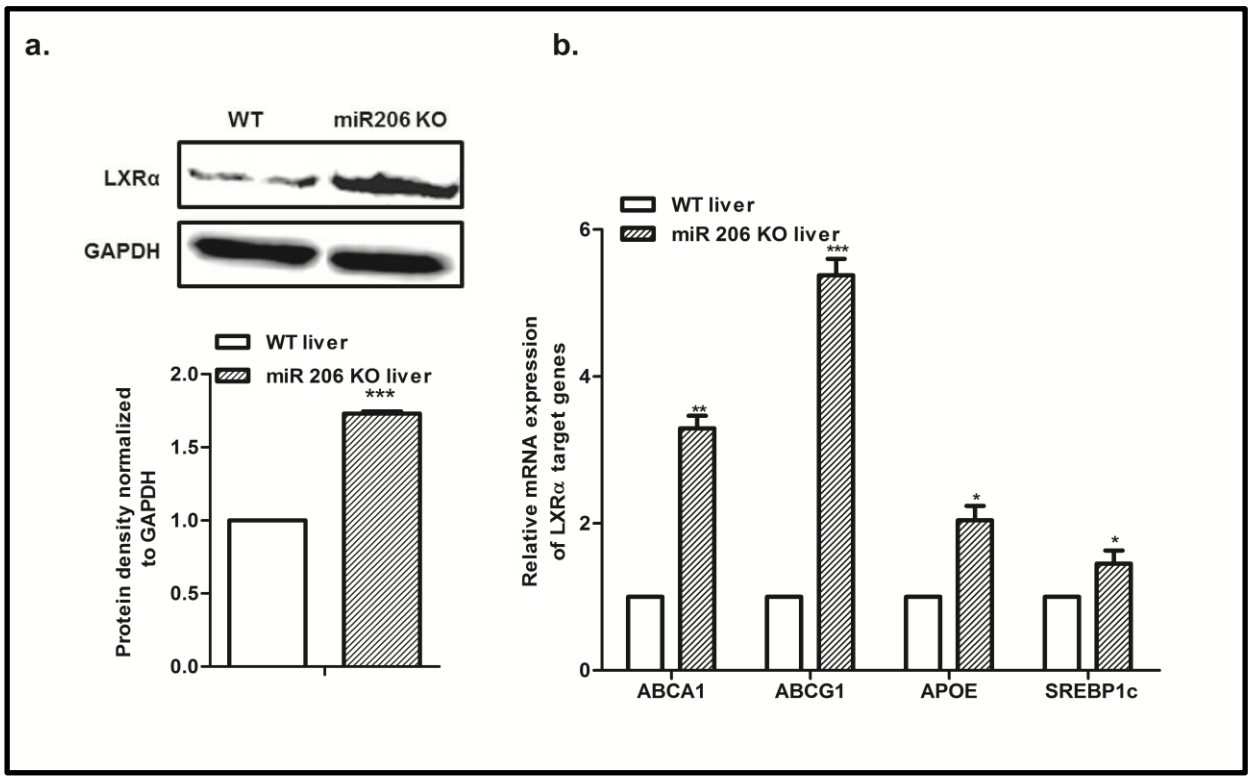


Figure-8 Effect of miR-206 knockout on mRNA and protein expression of LXRα and its target genes in the liver: ***a)*** *Western blot of LXRα from the liver of miR-206 KO and WT mice fed normal diet (n=6).* ***b)*** *mRNA abundance of LXRα and its target genes in WT and in miR 206 KO liver by qRT-PCR (n=4). Mean values ± SEM from three separate experiments are indicated;* p<0.05; ** p<0.01; *** p<0.001*

In addition, we noticed a significant 1.5 – 5 fold increase in the LXRα target gene mRNA expression (ABCA1, ABCG1, ApoE and SREBP-1c) in the liver of miR206 KO mice (Figure-7b).

In summary, we show that miR-206 directly interacts with the 3'UTR region of LXRα and represses its translation in HepG2 cells and in mouse liver and thereby interferes with LXRα mediated signaling pathways.

5.4 miR-206 regulates LXRα expression differently in liver and in human macrophages.

As LXRα plays a crucial role in regulating cholesterol efflux and homeostasis in macrophages (87), we were further interested to study the effect of miR-206 in macrophage cholesterol homeostasis. For this purpose, we stably overexpressed (THP-1miR206 OE) or knocked down (THP-1miR206 KO) miR-206 in the human macrophage cell line THP-1 using lenti-viral transduction. Transduction efficiency was measured using quantitative real time PCR. In THP-1miR206 OE miR-206 expression drastically increased (~ $1*10^7$ fold) compared to the control THP-1 cells (Figure-8). In THP-1miR206 KO cells miR-206 expression was undetectable (Figure-8). ABCA1 mRNA expression was measured as an mRNA control.

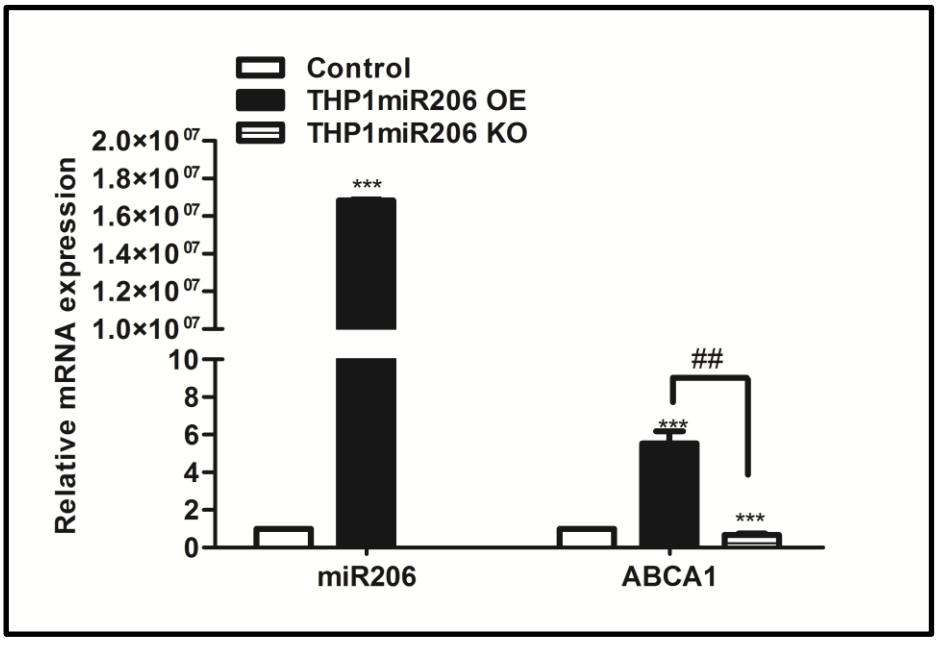


Figure-9 Expression of miR206 and ABCA1 in control, KO and OE THP-1 cells used for the cholesterol efflux: The expressions of genes were measured by quantitative real time PCR. Values are means ± SEM of 3 separate experiments. The experiments demonstrate the appropriateness of the used cells.

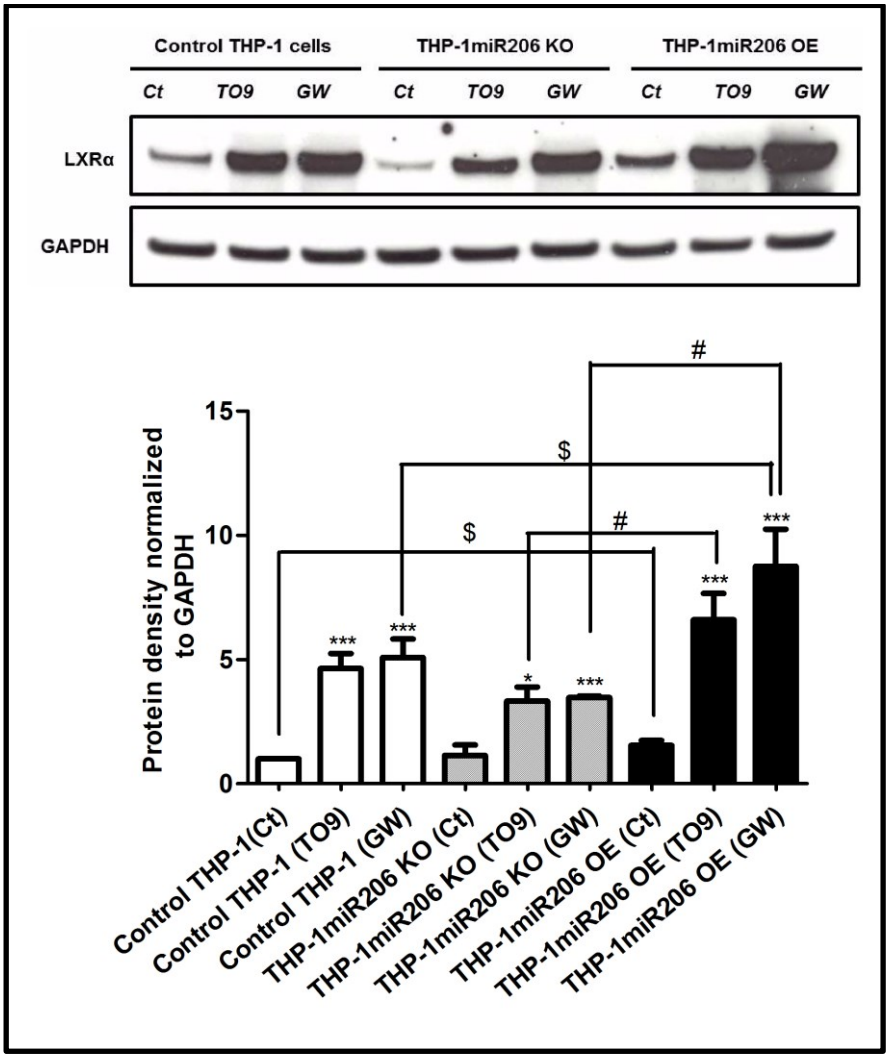


Figure-10 Regulation of LXRα protein expression by miR-206: *After differentiation, protein and mRNA expression of LXRα and its target genes were determined in control THP-1, THP-1miR206 OE and THP-1miR206 KO cells. a) Western blot of basal or TO9 or GW-induced LXRα protein from control THP-1, THP-1miR206 KO and THP-1miR206 OE cells. Western blots are from one representative experiment out of four experiments. (t test: THP-1miR206 OE Vs control and TO9 or GW-induced expression of LXRα in THP-1miR206 OE Vs control THP-1 cells. $ p<0.05 ; THP-1miR206 OE Vs THP-1miR206 KO and TO9 or GW-induced expression of LXRα in THP-1miR206 OE Vs THP-1miR206 KO. # p<0.05;Untreated control VS TO9 or GW-induced expression of LXRα in THP-1, THP-1miR2-6-KO and THP-1miR206-OE cells ; * p<0.05; *** p<0.001) After differentiation, protein and mRNA expression of LXRα and its target genes were determined in control THP-1, THP-1miR206 OE and THP-1miR206 KO cells. a) Western blot of basal or TO9 or GW-induced LXRα protein from control THP-1, THP-1miR206 KO and THP-1miR206 OE cells. Western blots are from one representative experiment out of four experiments. (t test: THP-1miR206 OE Vs control and TO9 or GW-induced expression of LXRα in THP-1miR206 OE Vs control THP-1 cells. $ p<0.05 ; THP-1miR206 OE Vs THP-1miR206 KO and TO9 or GW-induced expression of LXRα in THP-1miR206 OE Vs THP-1miR206 KO. # p<0.05;Untreated control VS TO9 or GW-induced expression of LXRα in THP-1, THP-1miR2-6-KO and THP-1miR206-OE cells ; * p<0.05; *** p<0.001)*

Surprisingly, Western blot analysis from THP-1miR206 OE cells revealed that LXRα protein expression was markedly induced in untreated as well as in LXR agonist-treated cells compared to control THP-1 cells. In contrast, though not significant both basal as well as the TO9 or GW-induced LXRα protein expression was moderately reduced in THP-1miR206 KO when compared to control THP-1 cells (Figure-9). In THP-1miR206 KO, TO9 or GW-induced LXRα protein expression is remarkably lower when compared to LXRα protein expression in THP-1miR206 OE cells.

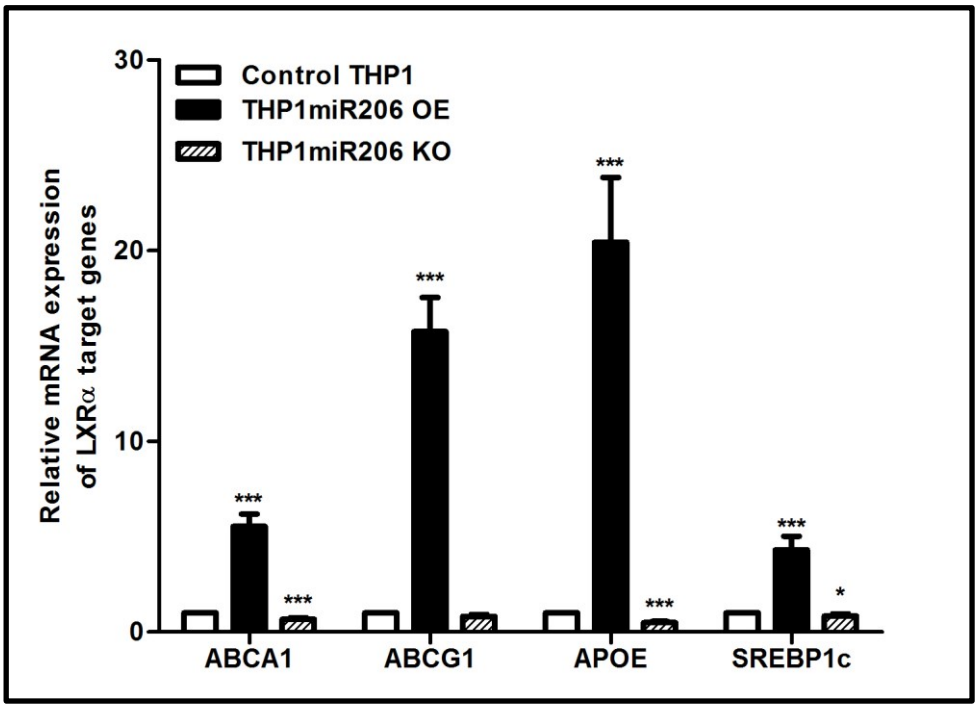


Figure-11 Effect of miR-206 overexpression and knock down in THP-1 cells: *Basal mRNA abundance of LXRα target genes in THP-1 cells by qRT-PCR Mean values ± SEM from three separate experiments are indicated; * p<0.05; ** p<0.01; *** p<0.001.*

qRT-PCR gene expression profiling of several LXRα target genes revealed that basal mRNA levels of ABCA1, ABCG1, ApoE and SREBP-1c were increased by 3- 18 fold in THP-1miR206 OE cells (Figure-10), whereas LXRα target genes were less expressed in miR-206 KO THP-1 cells (Figure-10), compared to control THP-1 cells. Comparable upregulation in THP-1miR206 OE cells and downregulation in THP-1miR206 KO cells was observed after treatment with TO9 or GW (Figure-11a & b).

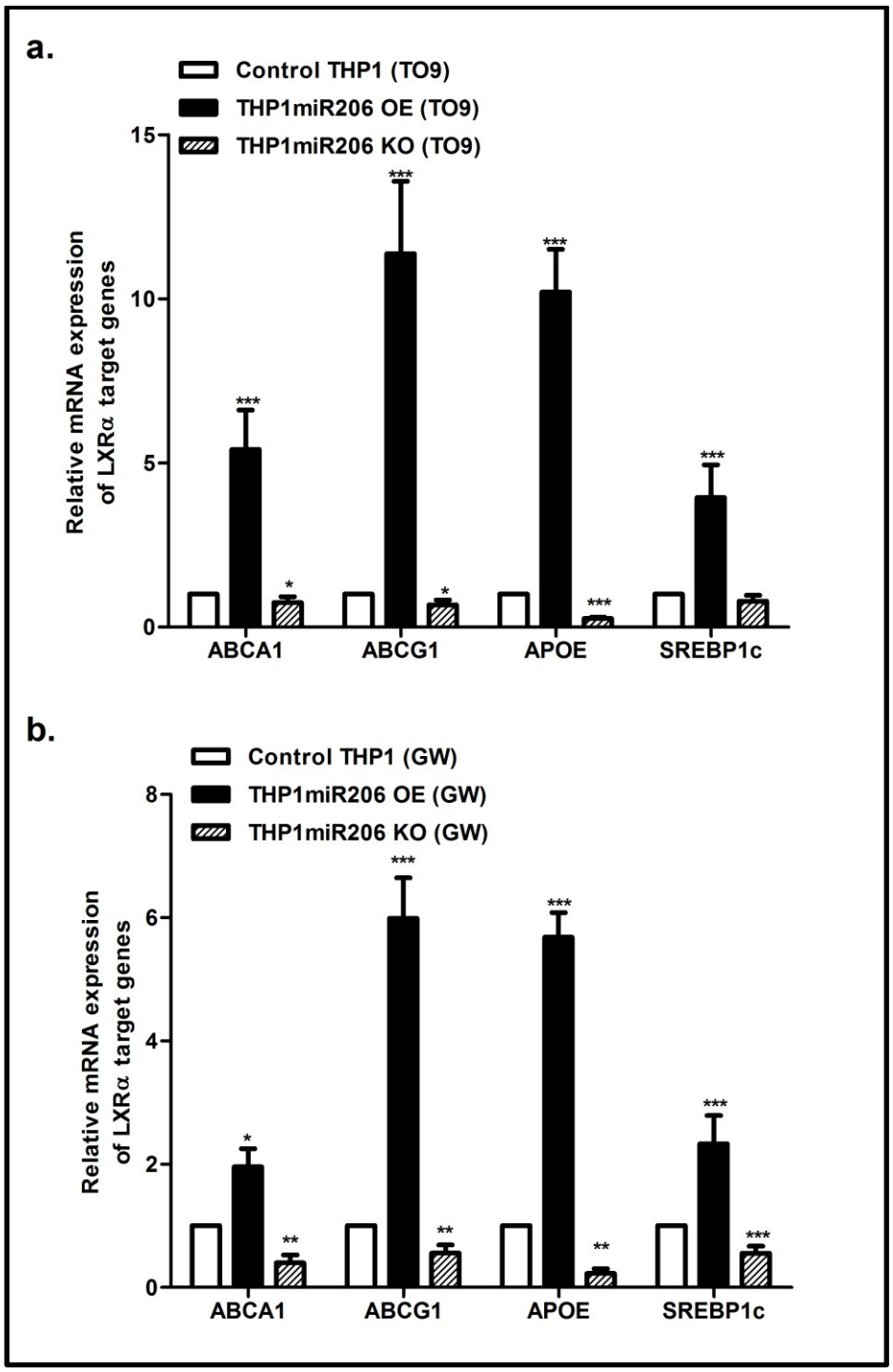


Figure-12 Agonist induced expression of LXRα target genes in Control THP-1, THP-1miR206 OE and THP-1miR206 KO cells:*TO9-induced mRNA abundance of LXRα target genes by qRT-PCR. GW-induced mRNA abundance of LXRα target genes by qRT-PCR. Mean values ± SEM from three separate experiments are indicated; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.*

5.5 LXRα target gene expression is reduced in miR-206 KO murine macrophages.

To examine whether this miR206-LXRα regulatory pathway is also operative in murine systems, we studied MPMs and BMDMs obtained from miR-206 KO and WT mice. Gene expression profiling of miR206 KO MPMs revealed that under basal conditions, the expression of LXRα target genes ABCA1, ABCG1, ApoE and SREBP-1c were about 40 - 80 % lower compared to WT MPMs (Figure-12).

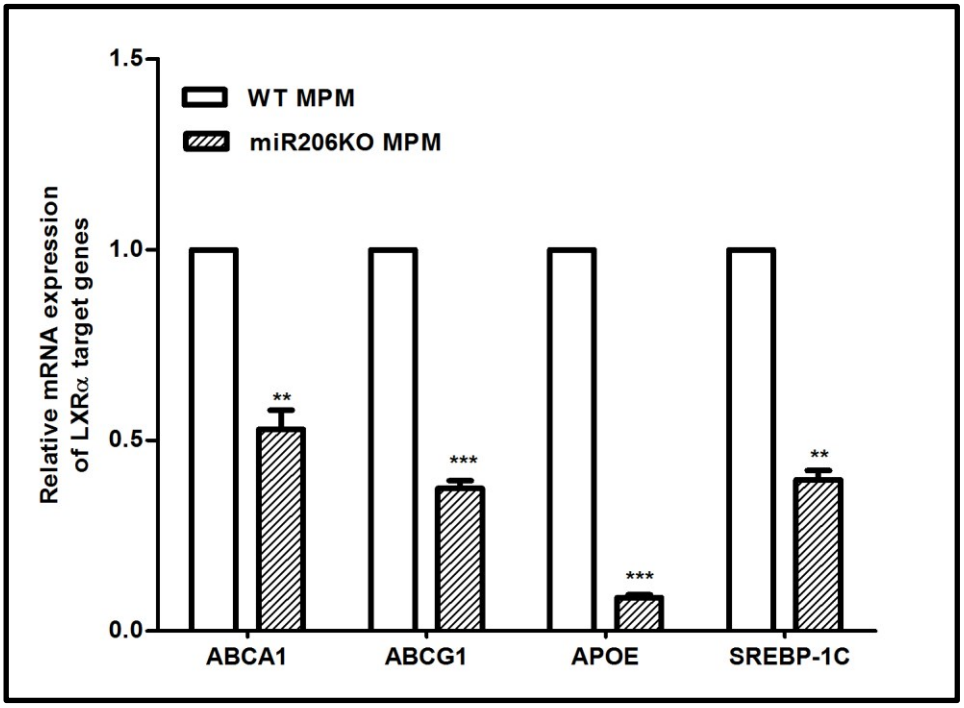


Figure-13 Basal mRNA expression levels of LXRα target genes in Control and miR206 KO MPMs :*Basal mRNA abundance of LXRα target genes in MPMs by qRT-PCR Mean values ± SEM from three separate experiments are indicated; * p<0.05; ** p<0.01; *** p<0.001.*

Comparable results were also observed after treatment of MPMs with GW (Figure-13), yet SREBP-1c expression was affected to a much greater extent as compared to untreated WT MPMs (90% vs. 55%, Fig.4a and 4b). TO9 treatment had lower effect on LXRα target

gene expression as compared to GW treatment, and no effect was seen on ABCA1 expression (Figure-14).

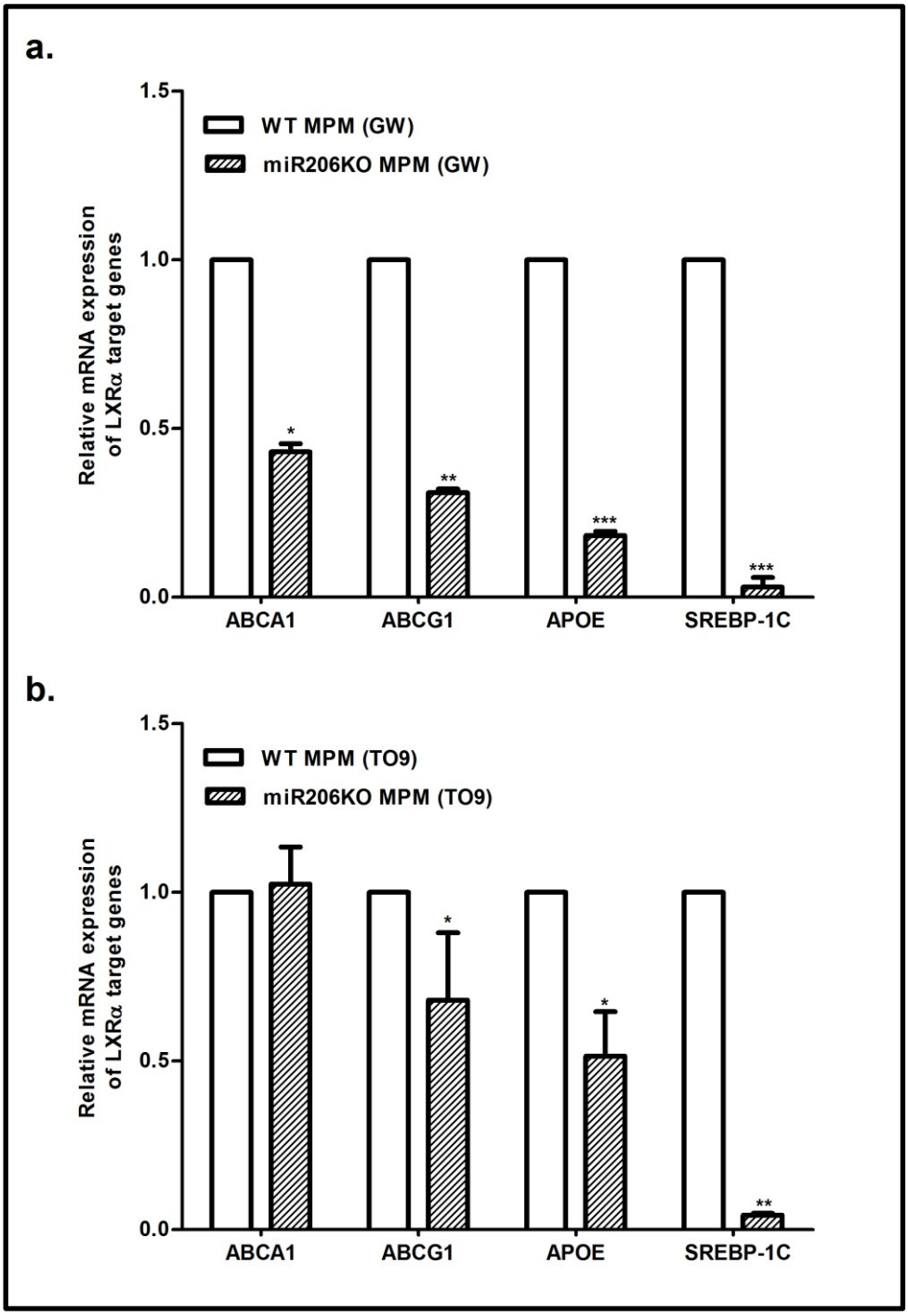


Figure-14 Agonist induced expression of LXRα target genes in control and miR206 KO MPMs: a) *GW-induced mRNA abundance of LXRα target genes by qRT-PCR.* ***b****)TO9-induced mRNA abundance of LXRα target genes by qRT-PCR Mean values ± SEM from three separate experiments are indicated; * p<0.05; ** p<0.01; *** p<0.001.*

Similar experiments were performed in BMDMs (Figure-14). Here, ABCA1 expression was not significantly different between WT BMDMs and miR206 KO BMDMs neither under basal conditions nor in the presence of the LXRα ligands TO9 or GW. ABCG1 and SREBP-1c expression were lower in miR206 KO BMDMs under basal conditions and also after TO9 and GW treatment, and ApoE expression was lower under basal conditions and after TO9 treatment (Figure-15 a & b). Notably, the expression of ABCG1, ApoE and SREBP-1c was affected in miR-206 KO MPMs to a greater extent than in miR206 KO BMDMs.

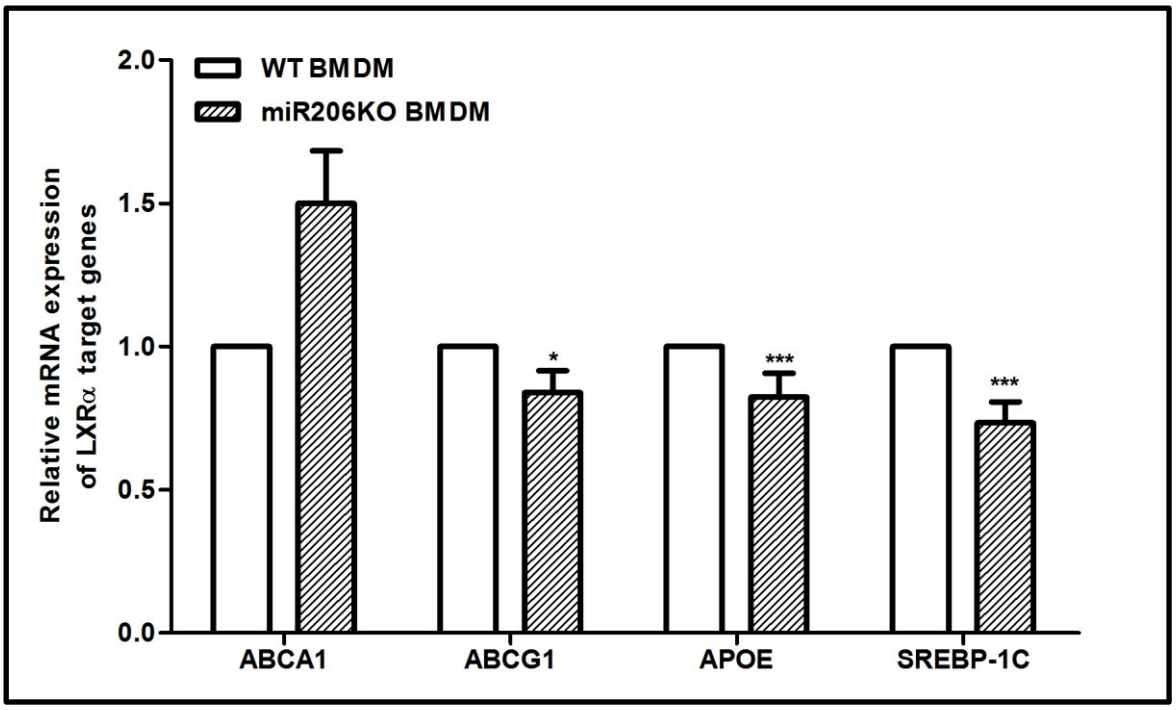


Figure-15 Effect of miR-206 KO on basal expression of LXRα target genes in BMDMs: *Basal mRNA abundance of LXRα target genes in miR206 KO and WT BMDMs by qRT-PCR. Mean values ± SEM from three separate experiments are indicated; * p<0.05; ** p<0.01; *** p<0.001.*

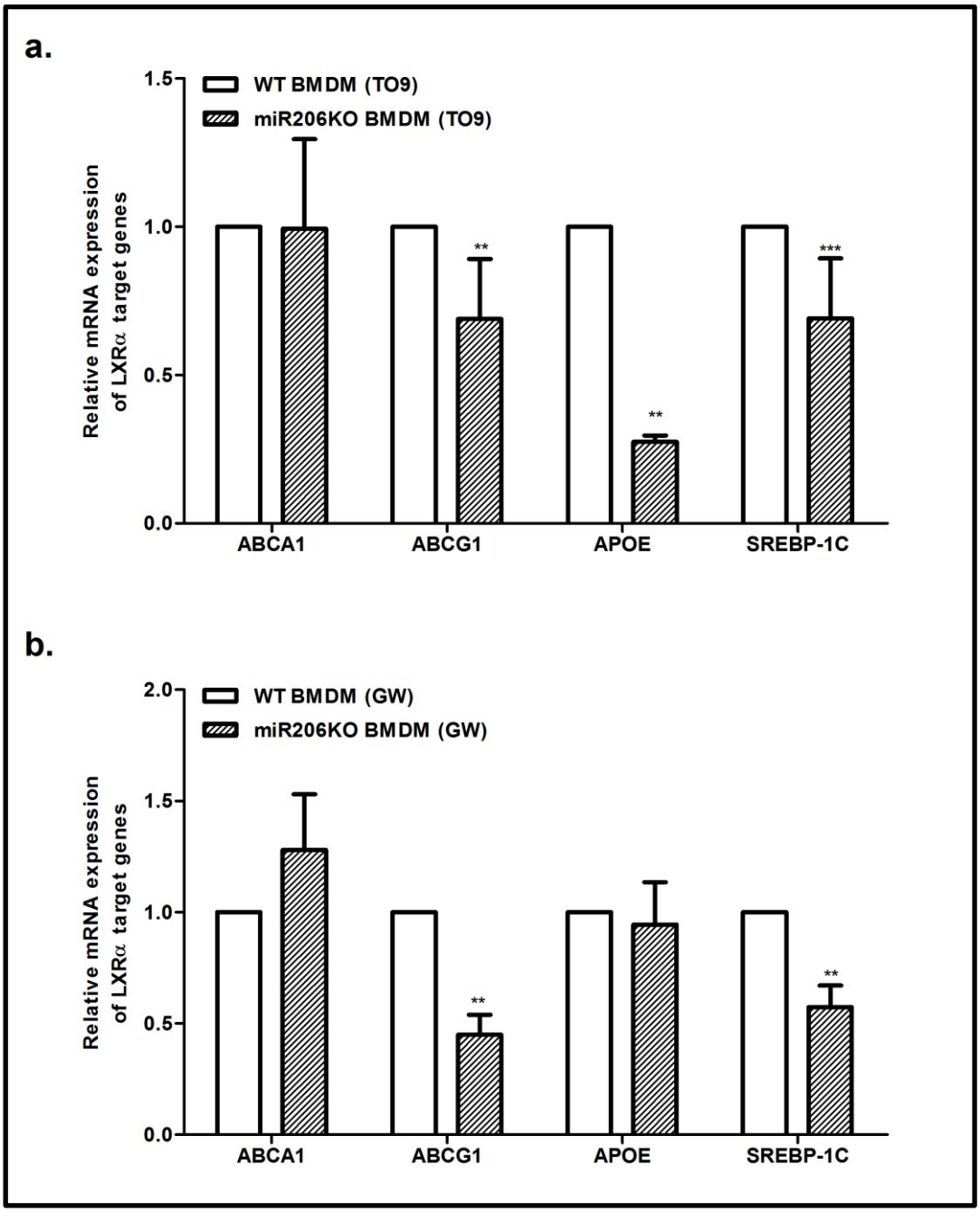


Figure-16 Agonist induced expression of LXRα target genes in control and miR206 KO BMDMs: a) *TO9-induced mRNA abundance of LXRα target genes by qRT-PCR Mean values ± SEM from three separate experiments are indicated* ***b)*** *GW-induced mRNA abundance of LXRα target genes by qRT-PCR.; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.*

5.6 *miR-206 overexpression in THP-1 cells increases cholesterol efflux to HDL and human serum*

To investigate whether the increase in LXRα and its target genes has functional relevance on human macrophages, we performed cholesterol efflux assays in THP-1miR206 OE, THP-1miR206 KO and control THP-1 cells. Briefly, cells were incubated with [3H] cholesterol for 24h, and the efflux to ApoA-I, HDL (ApoB depleted serum) and human serum was measured after stimulation with TO9.

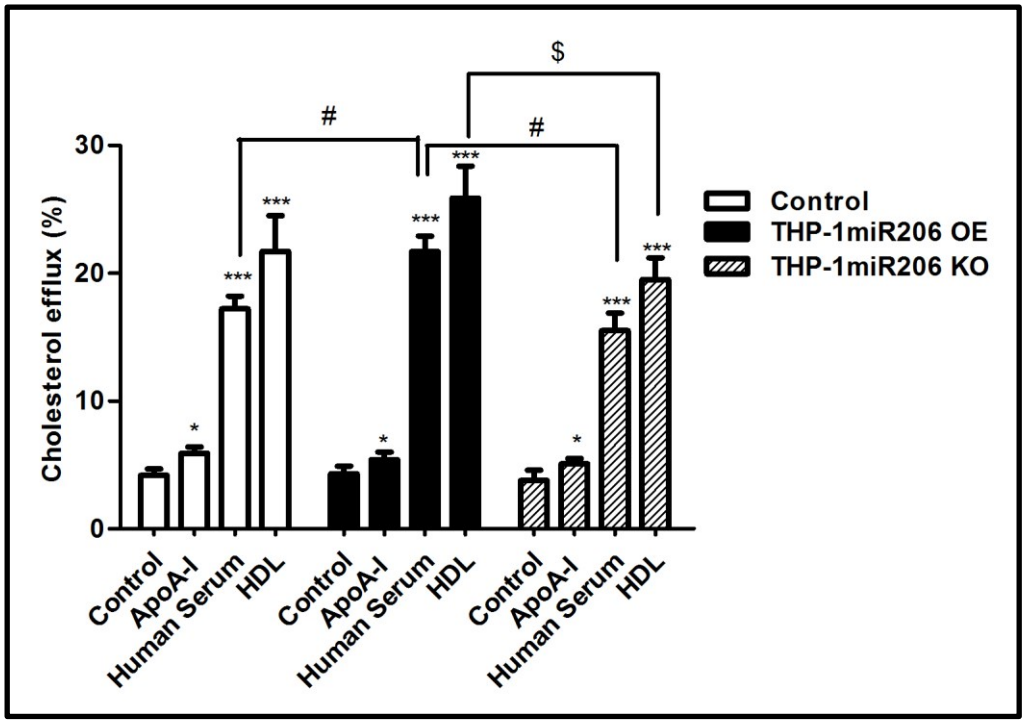


Figure-17 Cholesterol efflux assay in THP-1, THP-1miR206 OE and THP-1miR206 KO cells. *Control THP-1 cells, THP-1miR206 OE and THP-1miR206 KO were loaded with [^{3}H]cholesterol for 24 hours and cholesterol efflux to human serum, ApoB depleted serum (HDL), and ApoA-I was determined. a) Percentage of cholesterol efflux to ApoA-I, HDL and human serum was calculated relative to the unspecific cholesterol efflux to BSA. Statistical significance is indicated (t test: THP-1miR206 OE Vs control and THP-1miR206 OE Vs THP-1miR206 KO efflux to human serum #p<0.05 ; THP-1miR206 OE Vs THP-1miR206 KO efflux to HDL $ p<0.05; Efflux to ApoA-I, HDL and human serum compared to control-BSA; * p<0.05; ** p<0.01; *** p<0.001)*

Figure-16 demonstrates that all three cell lines effluxed cholesterol efficiently to ApoA-I, human serum and HDL. However, THP-1miR206 overexpression showed significantly enhanced cholesterol efflux capacity to human serum when compared to control THP-1 and THP-1miR206 KO cells. Though not significant, THP-1miR206 OE showed an increasing tendency to efflux cholesterol to HDL when compared to control THP-1 cells. In contrast to THP-1miR206 OE, THP-1miR206 KO showed a significantly lower cholesterol efflux to HDL when compared to control THP-1miR206 OE cells. Cholesterol efflux to ApoA-I and to BSA (control) was not significantly affected by miR-206 overexpression or knockout under these experimental conditions (Figure-16). Collectively, these data indicate that the miR206-LXRα pathway is functional and of potential physiological relevance in facilitating cholesterol efflux from human macrophages.

5.7 *miR206 KO mouse macrophages display reduced cholesterol efflux to HDL and ApoA-I*

In order to substantiate these findings and to examine the physiological relevance of the miR206-LXRα pathway in the murine system, cholesterol efflux was also investigated in MPMs from miR206 KO and WT mice. Figure-17, shows that the efflux of cholesterol to BSA, apoAI, HDL and human serum in the presence of TO9 were all significantly lower in miR206 KO MPMs as compared to WT MPMs underlining the physiological significance of miR-206 on LXRα target genes.

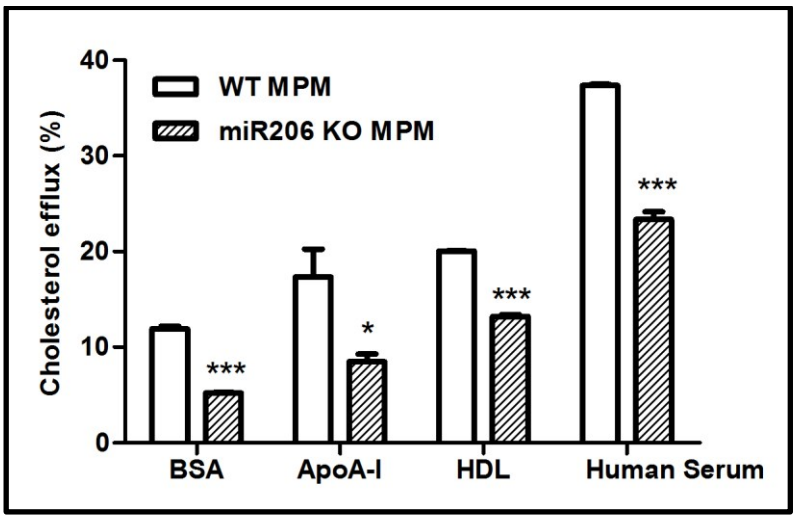


Figure-18 Cholesterol efflux assay in miR206 KO MPMs:
*Cholesterol efflux assay from WT and miR206KO MPMs to ApoA-I, HDL and whole human serum: MPMs were isolated and loaded with ^{3}H-acLDL for 24 hours and the cholesterol efflux assay was performed. Results are mean ± SEM of triplicate experiments. Statistical significance is indicated (t test; * $p<0.05$; *** $p<0.001$).*

5.8 *LXR activation represses miR-206 expression in THP-1 macrophages whereas ox-LDL, VLDL and other inflammatory stimuli induce miR-206 expression.*

To determine whether a feedback mechanism between LXRα and miR-206 may exist, we activated LXR in THP-1 cells with TO9 (100 nM, 500nM and 1μM) and measured the expression levels of miR-206. Notably, LXR activation by TO9 drastically repressed miR-206 expression (Figure-17). PPARγ activation at higher concentration of rosiglitazone such as 1 μM, 5 μM and 20 μM resulted downregulation of miR-206 conversely PPARγ activation at lower concentration of rosiglitazone such as 500nM and 100nM resulted in robust upregulation (~2 fold and ~5 fold) of miR-206 (Figure-18)

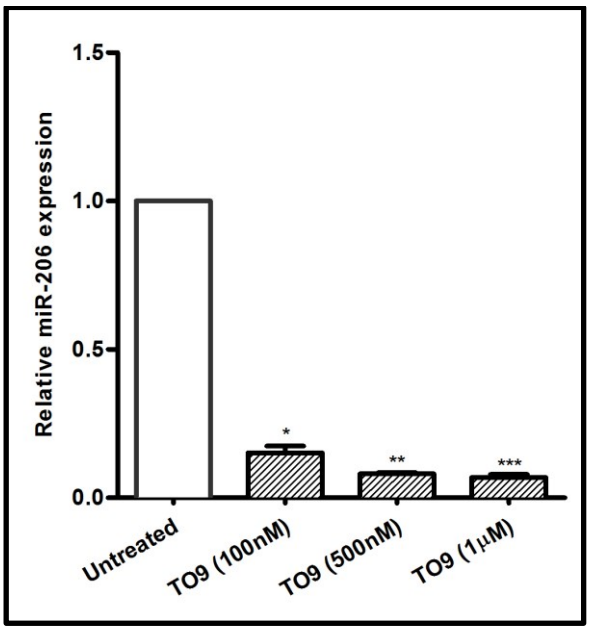


Figure-19 The Effect of LXRα activation on miR-206 expression: *THP-1 cells were differentiated and treated with 100nM, 500 nM or 1μM of TO9. The relative abundance of miR-206 was measured by qRT-PCR. Values are mean ± SEM of three separate experiments; * p<0.05 ** p<0.01; *** p<0.001.*

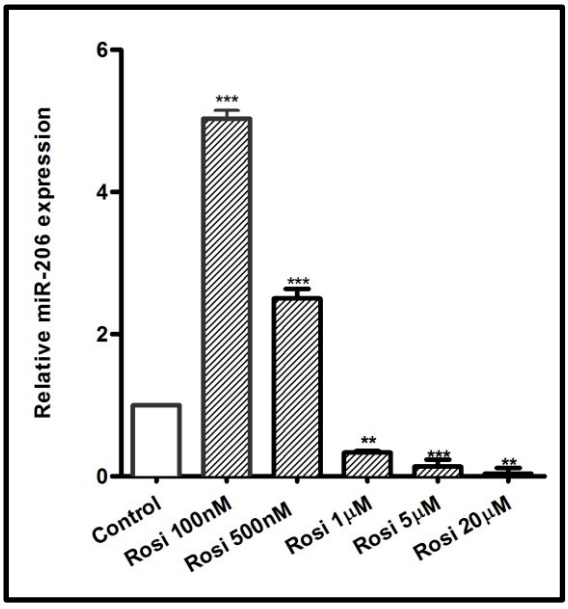


Figure-20 Concentration dependent effect of PPAR agonist treatment on miR-206 expression: *THP-1 cells were differentiated and treated with 100nM, 500 nM, 1μM, 5μM or 20μM of Rosiglitazone. The relative abundance of miR-206 was measured by qRT-PCR. Values are mean ± SEM of three separate experiments; ** p<0.01; *** p<0.001.*

We were also interested in identifying factors that may mediate miR-206 expression in macrophages. Thus, THP-1 cells were incubated in the presence of native LDL, oxLDL or VLDL. 50-100 µg/ml of oxLDL treatment or 50 µg/ml of VLDL treatment increased the expression of miR-206 in THP-1 cells by 1.5 – 2 fold compared to THP-1 cells incubated with native LDL (Figure-19).

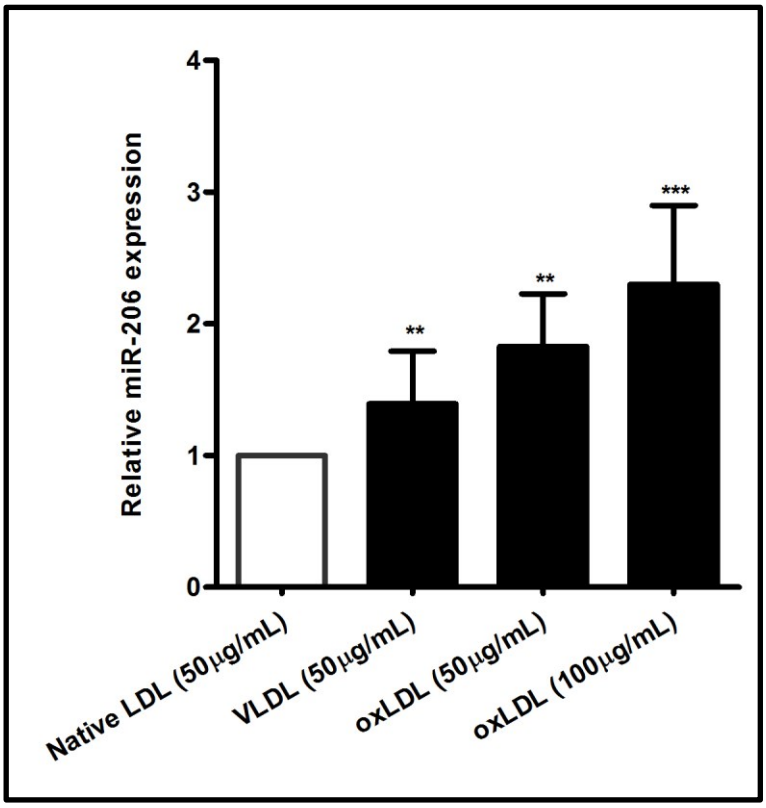


Figure-21 Effect of modified LDL treatment on miR-206 expression in THP-1 cells: *THP-1 cells were differentiated and treated with 50µg/ml of native LDL (control), 50µg/ml VLDL or 50 or100µg/ml oxLDL. The relative abundance of miR-206 was measured by qRT-PCR. Values are mean ± SEM of three separate experiments; ** p<0.01; *** p<0.001.*

As oxLDL robustly induced the expression of miR-206, we were further interested to know whether oxLDL-induced inflammatory cytokines such as TNFα or inflammatory mediators such as LPS might influence the expression of miR-206.

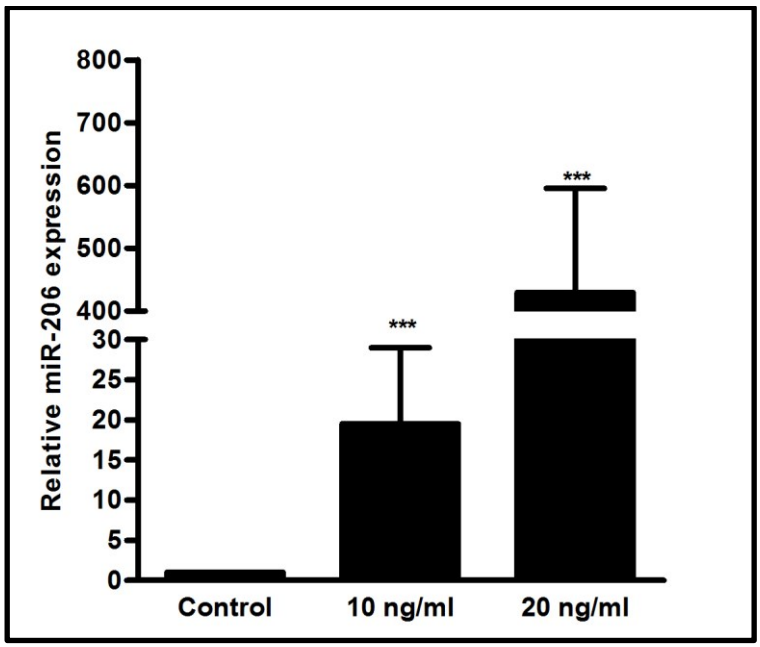


Figure-22 Concentration dependent effect of TNFα on miR-206 expression in THP-1 cells: *THP-1 cells were differentiated and incubation with 10 µg/ml or 20 µg/ml of TNFα. The relative abundance of miR-206 was measured by qRT-PCR. Values are mean ± SEM of three separate experiments;*** $p<0.001$.*

As shown in Figure-19 strong induction of miR-206 expression (20-fold) in THP-1 cells treated with 10ng/ml of TNFα. Consistently, LPS treatment of primary human monocyte-derived macrophages also resulted in a significantly increased expression of miR-206 (Figure-20).

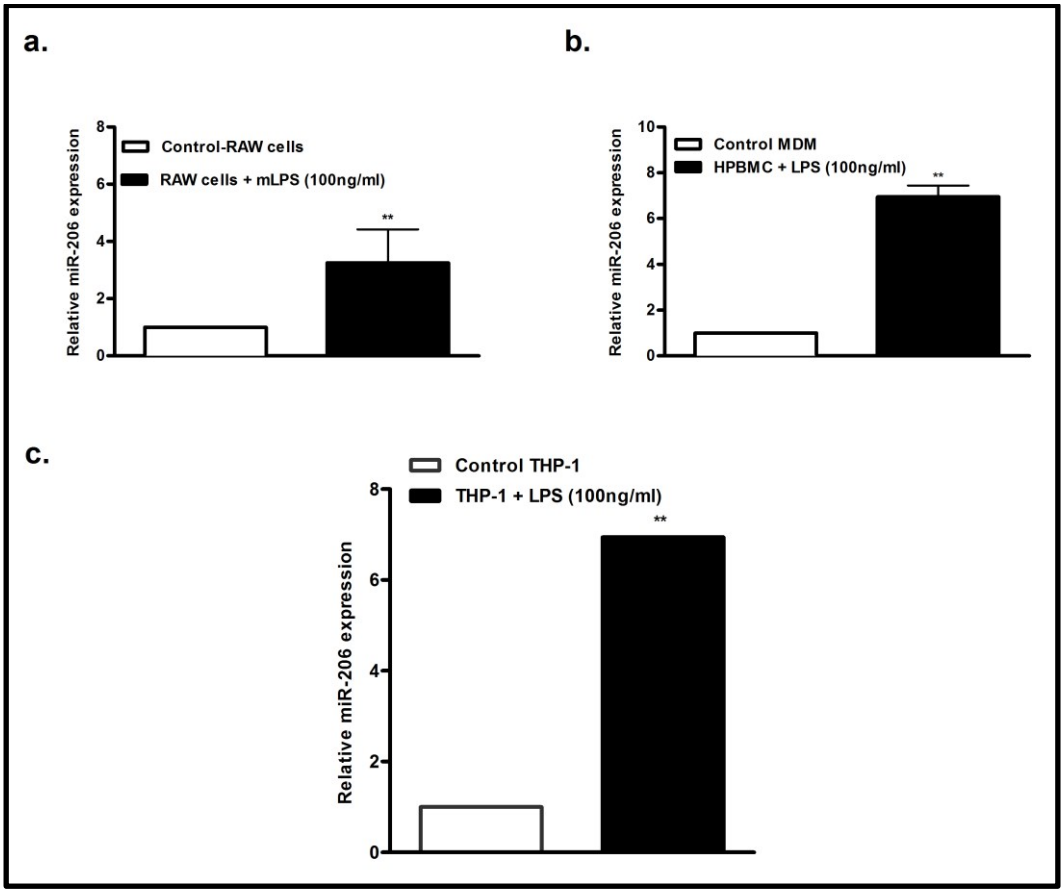


Figure-23 Effect of LPS treatment on human macrophages, human and murine macrophage cell lines ***a)*** *RAW cells were treated with 100ng/ml modified LPS for 24 hours.* ***b)*** *HPBMs were differentiated for 6 days and were incubated with 100ng/ml of LPS.* ***c)*** *THP-1 cells were differentiated and incubation with 100 ng/ml of LPS. The relative abundance of miR-206 was measured by qRT-PCR. Values are mean ± SEM of three separate experiments;** $p<0.01$.*

6. DISCUSSION

The major findings of this report are: **1)** miR-206 targets LXRα and affects signaling pathways differently in liver and macrophages of both mouse and human **2)** the identification of novel feedback regulation between LXR and miR206, miR-206 activates LXRα mediated pathway in cholesterol efflux and LXRα autoregulates its own expression by repressing miR-206 **3)** and the expression of miR-206 is induced by patho - physiological stimuli such as VLDL, oxLDL, LPS and TNFα.

MiRs play important roles in a diverse spectrum of events in development, cell growth and differentiation as well as in metabolism. The varying expression pattern and the tissue specific functionality further emphasizes the systemic importance of microRNAs in health and diseases (88). There is an increasing body of evidence illustrating the role of miRs in cholesterol homeostasis and atherosclerosis: Sun et al. (89) recently published that miR-26 directly targets ABCA1 and ARL-7 RNA and controls LXR- dependent cholesterol efflux from RAW264.7 cells. Ramirez et al. (90) found a similar effect of miR-758 on ABCA1 expression and concomitant reduced cholesterol efflux from human and mouse macrophages. Rayner et al (91, 92) and Horie et al. (93) reported that miR-33, that is co-transcribed with the SREBP-2 gene targets several LXR regulated genes and that miR-33 overexpression ameliorates cholesterol efflux from macrophages. Antagonizing miR-33 in mice promoted reverse cholesterol transport and regression of atherosclerosis (91). Recently two independent groups published a LXRα and farnesoid X receptor (FXR) induced expression of miR-144 that targeted ABCA1 mRNA and reduced ABCA1 mediated cholesterol homeostasis in the liver (94, 95). Importantly, miR-1/206 and miR-613 have been identified as negative regulators of LXRα expression and lipogenesis in the liver (75, 76). Notably, most of these metabolic miRs that have been identified are reported to repress their target genes.

In the present study we identified a cell-specific activity for miR-206, which acts as a repressor of LXRα signalling in the liver cells but intriguingly acts as an activator of

LXRα in macrophages. This differential change in function is operative both in the human and mouse macrophage system and affects cholesterol homeostasis.

In line with earlier reports, we also observe a decrease in LXRα expression in HepG2 cells transfected with miR-206 and a concomitant down-regulated expression of LXRα target genes, such as ABCA1, ABCG1 ABCG5 and SREBP-1c. In contrast, THP-1miR206 OE cells showed induction of LXRα and its target genes such as ABCA1, ABCG1, ApoE and SREBP-1c accompanied with an increased cholesterol efflux capacity. Exactly opposite effects were achieved with lenti-virus mediated knockdown of miR-206 in THP-1 cells, as well as in miR206KO BMDMs and miR-206KO MPMs, which further substantiates the activation of LXRα pathway by miR-206 in macrophages. Interestingly, the basal unspecific cholesterol efflux to BSA was also reduced in miR-206KO MPMs. Though purified albumin added to cell culture is a relatively poor promoter of efflux, numerous studies report that aqueous diffusion contribute significantly to the total cell cholesterol flux (96). Therefore, the reduction in efflux to BSA that we observe in miR-206KO MPMs might have contributed by the aqueous transfer mediated by albumin

As miRs were traditionally described as negative regulators of gene expression our results are quite surprising. Many recent reports suggest that miRs can not only repress but also activate translation by multiple mechanisms. Vasudevan et al.(5) recently reported that during cell cycle, miRs oscillate between activation and repression; in proliferating cells miRs repress the translation whereas in G1/G0 arrest miRs facilitate translational activation. One reported mechanism for activation is the preferential interaction of AGO2-FXR1 complex (part of the miR ribonucleoprotein complex) with the AU rich regions on the target mRNA during cell cycle arrest (6). They also report that under serum starved conditions let-7 interaction with the 3'UTR region of HMGA2 lead to activation; this mechanism also needed the interaction of AGO2-FXR1 complex with the HMGA2 mRNA (7). A recent report from the same group suggests that nuclear localization of AGO2-FXR1 complex along with the target mRNA and miR is a requirement for translational activation of the mRNA (4).

Interestingly, miR-206 has been reported to have both nuclear and cytosolic localization but the functional relevance of compartmentalized localization is unknown. miR-206 is also one out of the few miRs that were reported to have translational activation capabilities (51, 97). Lin et.(41) al., reported a feedback regulatory loop between KLF4-miR-206 and the context specific activation of KLF4 by miR-206 in terminally differentiated cells. In our experimental conditions, THP-1 monocytes were differentiated into macrophages with 100nM PMA for three days. PMA treatment initially induces cell cycle arrest and then terminal differentiation in macrophages. Thus after three days of differentiation, THP-1 macrophages are terminally differentiated cells which are thought to be incapable of further proliferation. As suggested earlier, it is conceivable that FXR1-AGO2-miR-206 complex mediated interaction on the 3'UTR of LXR is involved in transactivation of LXRα in THP-1miR206-OE cells.

Similarly, miR-206-KO BMDMs were differentiated for 10 days which also yields terminally differentiated macrophages. miR-206 KO MPMs are already differentiated macrophages which are activated in response to the injected thioglycollate stimuli. In miR-206-KO macrophages the modest reduction of LXRα expression and less pronounced induction of TO9 or GW mediated LXRα signaling further emphasize the native requirement of miR-206 in activating LXRα signaling. Interestingly, in Fig.3b and c, we observe a negative correlation in ABCG1 expression in THP-1miR206 OE and THP-1miR206 KO. Consistently, we also observed a reduction in ABCG1 expression in miR-206 KO BMDMs and MPMs (Panels Fig 4 & Fig 5). This may explain the pronounced differences in cholesterol efflux to HDL and complete serum in comparison to ApoA-I. It is intriguing to speculate that the observed up- and down regulation of ABCG1 by miR-206 overexpression or knock down might be at least in part independent of LXRα, i.e. the consequence of the direct regulation of ABCG1 expression by miR-206. However, further work is needed to experimentally address this possibility.

To examine the possibility of such an interaction we performed an in silico RNAhybrid analysis which also showed a favorable minimum free energy (mfe) of (-) 20.3 kcal/mol. MicroPIR (microRNA-promoter interactions resource) is a recently developed online

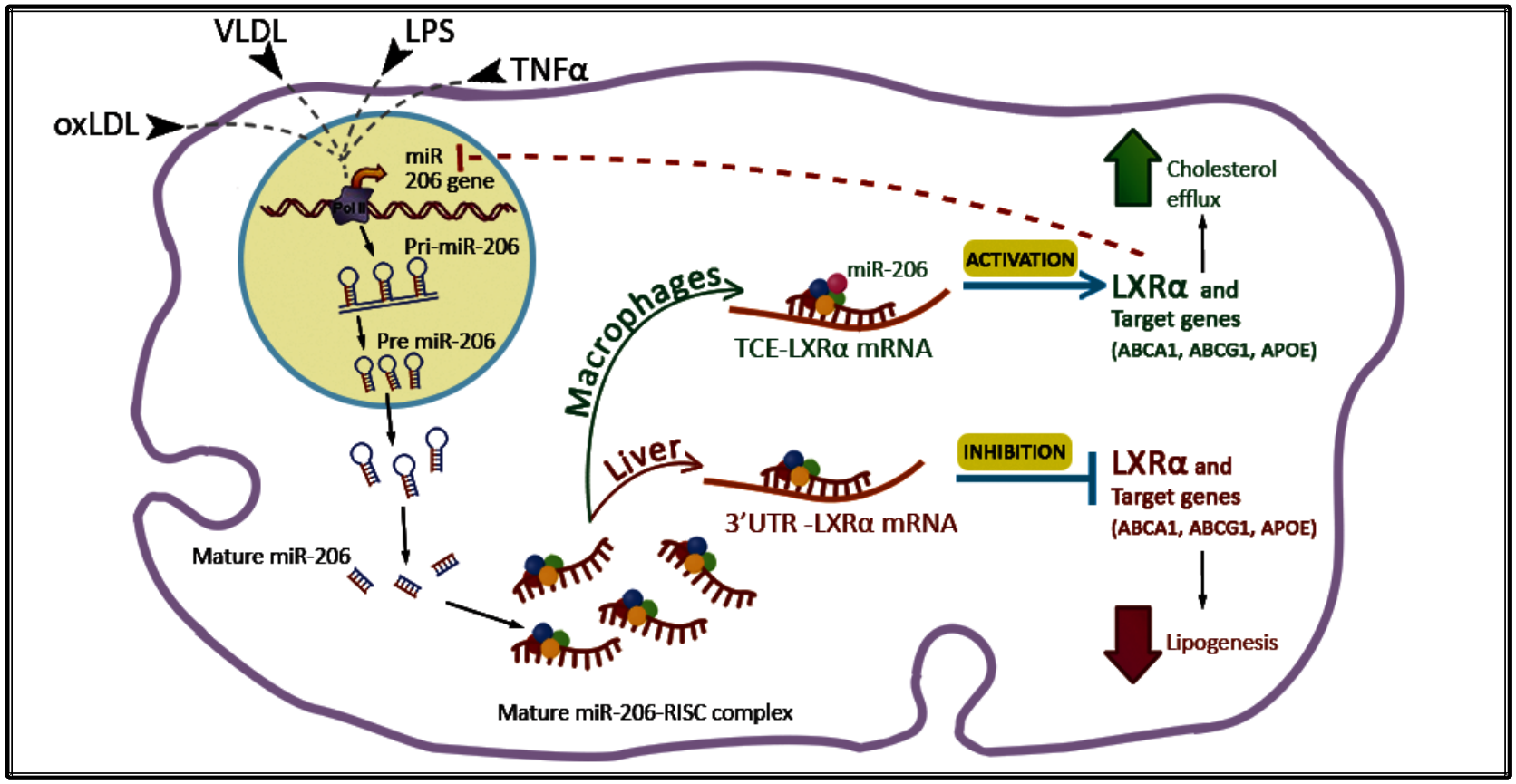
resource to predict putative miRs-promoter interactions. In fact, miR-206 is one among the predicted ones which can bind at position -4809th on the ABCG1 promoter with a mfe of (-) 25.50. MicroPIR also predicted miR-206 interaction with APOE (at -3937) and ABCA1 (at -907). The fact that miR-206 has been shown to localize also in the nucleus and can interact with the promoters of the above mentioned genes suggests that such a mechanism might also be operative in macrophages.

Lipoproteins have been recently shown to carry miRs either through ABCA1 mediated miR loading to HDL or binding to circulatory miRs(70). miRs carried by HDL get activated inside the cell and can regulate gene expression. Interestingly, miR-206 is also carried in the circulation by HDL (70). Physiologically, HDL molecules carrying miR-206 can deliver it to macrophages by SRB-1 mediated pathway and once inside the cells miR-206 can activate LXRα/ ABCG1 mediated signaling which can in turn initiate cholesterol efflux to HDL(70). This miR206-LXRα activation in macrophages might be part of the complex cholesterol homeostasis machinery. Another important finding is the negative feedback regulation of miR-206 by LXRα, which might be part of the LXR auto-regulatory mechanism to fine tune its own transcription and expression.

Our findings that VLDL and oxLDL as well as inflammatory cytokines and mediators such as TNFα and LPS induce the expression of miR-206 highlight the importance of this miR in diseases such as atherosclerosis and metabolic syndrome.

To summarize, we report a previous unknown pathway of miR-206 mediated LXRα activation in macrophages that influence macrophage cholesterol homeostasis. As LXRα is involved in various mechanisms of macrophage biology and functionality, it will be important to investigate in detail the influence of the miR-206 mediated LXRα signaling in a patho-physiological context such as atherosclerosis.

7. GRAPHICAL ABSTRACT AND CONCLUSION



Conclusion: *We have delineated an autoregulatory feedback-loop mechanism for LXRα, which is operative in both human and murine macrophages involving microRNAs, nuclear receptors, lipoproteins and inflammatory cytokines. Our study emphasizes on the cell and target specific functionality of microRNAs on genes and astounds us with the intricacies of cellular signaling. The tissue specific difference in gene regulation also calls out for the need of intense research and understanding of basic cellular mechanisms prior to the development of therapeutics to combat diseases*

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