Regulation of ABCA1 Protein Expression and Function in Hepatic and Pancreatic Islet Cells by miR-145

Martin H. Kang,* Lin-Hua Zhang,* Nadeea Wijesekara, Willeke de Haan, Stefanie Butland, Alpana Bhattacharjee, Michael R. Hayden

Objective—The ATP-binding cassette transporter A1 (ABCA1) protein maintains cellular cholesterol homeostasis in several different tissues. In the liver, ABCA1 is crucial for high-density lipoprotein biogenesis, and in the pancreas ABCA1 can regulate insulin secretion. In this study, our aim was to identify novel microRNAs that regulate ABCA1 expression in these tissues.

Approach and Results—We combined multiple microRNA prediction programs to identify 8 microRNAs that potentially regulate ABCA1. A luciferase reporter assay demonstrated that 5 of these microRNAs (miR-148, miR-27, miR-144, miR-145, and miR-33a/33b) significantly repressed ABCA1 3′-untranslated region activity with miR-145 resulting in one of the larger decreases. In hepatic HepG2 cells, miR-145 can regulate both ABCA1 protein expression levels and cholesterol efflux function. In murine islets, an increase in miR-145 expression decreased ABCA1 protein expression, increased total islet cholesterol levels, and decreased glucose-stimulated insulin secretion. Inhibiting miR-145 produced the opposite effect of increasing ABCA1 protein levels and improving glucose-stimulated insulin secretion. Finally, increased glucose levels in media significantly decreased miR-145 levels in cultured pancreatic beta cells. These findings suggest that miR-145 is involved in glucose homeostasis and is regulated by glucose concentration.

Conclusions—Our studies demonstrate that miR-145 regulates ABCA1 expression and function, and inhibiting this microRNA represents a novel strategy for increasing ABCA1 expression, promoting high-density lipoprotein biogenesis in the liver, and improving glucose-stimulated insulin secretion in islets. (Arterioscler Thromb Vasc Biol. 2013;33:2724-2732.)

Key Words: ABCA1 protein • cholesterol efflux regulatory protein • lipid metabolism • microRNAs
MiR-33a is ubiquitously expressed \(^1\)\(^{7}\),\(^{18}\) and it can regulate the 3′-UTR (miRDB, FindTar) to identify miRNA binding sites in these 5 programs. This approach can increase specificity but may suffer from a high proportion of false-positives.\(^3^3\) Commonly used programs, such as TargetScan and PicTar, have a precision of \(\approx\) 6% and sensitivity between 6% and 12%.\(^3^2\) Prediction programs are based on different factors such as site conservation, nucleotide composition, and location of miRNA binding sites.\(^3^2\) We focused on the regulation of ABCA1 by miR-145 for 2 reasons: (1) the overexpression of miR-145 resulted in the second largest repression of 3′-UTR activity after miR-33a; and (2) the primary miR-145 binding site possesses features known to increase the efficacy of miRNA/mRNA target interactions. These features include a binding site ≥15 nucleotides away from the stop codon while also being located as far from the center of UTRs as possible.\(^3^4\) Of all the binding sites for the 5 miRNAs identified in our reporter assay as regulating ABCA1, miR-145’s primary binding site (site 1, 109–115) has a distance of >15 nucleotides from the stop codon while also being located furthest from the center of the ABCA1 3′ UTR (Figure II in the online-only Data Supplement).

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

**ABCA1 Transcript Has Numerous Binding Sites for miRNAs**

A luciferase reporter construct containing the 3′ UTR (3309 bp) of human ABCA1 was transfected into murine (MIN6 pancreatic cells, 3T3-L1 adipocytes) and human (HEK293 embryonic kidney, skin fibroblasts, HepG2 hepatocytes) cell lines, with activity measured 24 hours after transfection. Decreased activity was observed in all cell lines (Figure I in the online-only Data Supplement) and suggests that the 3′ UTR is a critical component in controlling ABCA1 expression levels.

We used an in silico approach to identify potential miRNAs that could bind the 3′ UTR of ABCA1.\(^3^2\) The lack of definitive miRNA target recognition patterns means most bioinformatic prediction programs have a high proportion of false-positives.\(^3^3\) Commonly used programs, such as TargetScan and PicTar, have a precision of ≥50% and sensitivity between 6% and 12%.\(^3^2\) Prediction programs are composed of unique algorithms that identify miRNA targets based on different factors such as site conservation, nucleotide composition, and location of miRNA binding sites.\(^3^2\) We used 5 programs (TargetScanHuman 5.2, PicTar, microRNA.org, miRDB, FindTar) to identify miRNA binding sites in the 3′ UTR of ABCA1 (Table I in the online-only Data Supplement). To narrow down the total number of potential miRNAs targeting ABCA1, we combined the results from these 5 programs. This approach can increase specificity but does so at a cost of decreased sensitivity.\(^3^2\) We also limited the miRNAs we investigated to those known to be expressed in the liver because of the critical role of hepatic ABCA1 in HDL biogenesis.\(^8\) By selecting miRNAs chosen by 4 of 5 programs, and those miRNAs known to be expressed in the liver, we identified 8 miRNAs that potentially regulate ABCA1: miR-17, miR-145, miR-19a, miR-96, miR-33a/33b, miR-27a, miR-148, and miR-144 (Table II in the online-only Data Supplement).

**Five miRNAs Decrease ABCA1 Activity in a Reporter Assay**

To identify the miRNAs that can regulate ABCA1, oligonucleotide mimics representing each of the 8 miRNAs were co-transfected with the ABCA1 3′-UTR reporter construct into U343 cell lines. Five miRNAs demonstrated significant inhibition of luciferase activity: miR-148 by 16%, miR-27a by 20%, miR-144 by 25%, miR-145 by 28%, and miR-33a/33b by 52%/48% (Figure I A). Studies by other groups have since validated miR-33a/33b,\(^1^5\)\(^-^1^9\) miR-144,\(^2^2\)\(^-^2^3\) and miR-27a\(^2^4\) as regulating ABCA1. Expression of miR-145 resulted in the second largest repression of activity after miR-33a. Two miR-145 binding sites are predicted in the 3′ UTR of ABCA1, a site downstream of the stop codon (site 1, 109–115), and a site upstream of the poly A tail (site 2, 3114–3120; Figure II in the online-only Data Supplement). Site-directed mutagenesis was performed on the 2 binding sites to identify which site(s) miR-145 binds to regulate ABCA1 (Figure 1B). Reporter constructs containing mutations to site 1 eliminated the inhibitory effect of miR-145 to the same extent as mutations to both sites (mutations to site 1 and mutations to site 2; Figure 1C), suggesting that site 1 is the primary binding site for miR-145.

**MiR-145 Regulates ABCA1 in HepG2 Cells**

We focused on the regulation of ABCA1 by miR-145 for 2 reasons: (1) the overexpression of miR-145 resulted in the second largest repression of 3′ UTR activity after miR-33a; and (2) the primary miR-145 binding site possesses features known to increase the efficacy of miRNA/mRNA target interactions. These features include a binding site ≥15 nucleotides away from the stop codon while also being located as far from the center of UTRs as possible.\(^3^4\) Of all the binding sites for the 5 miRNAs identified in our reporter assay as regulating ABCA1, miR-145’s primary binding site (site 1, 109–115) has a distance of >15 nucleotides from the stop codon while also being located furthest from the center of the ABCA1 3′ UTR (Figure II in the online-only Data Supplement).

To demonstrate that miR-145 can regulate ABCA1 in hepatic cells, we manipulated miR-145 expression in the HepG2 cell-line and assessed ABCA1 protein expression through immunoblotting studies and ABCA1 function through cholesterol efflux activity assays. Transfection of pre-miR-145 oligonucleotide mimics decreased ABCA1 protein levels (Figure 2A) and significantly reduced ABCA1 cholesterol efflux to apo A-1 in HepG2 cells (Figure 2B). In all our experiments, scrambled control miRNA was used as a
negative control, and miR-33a was used as a positive control. Measurement of miRNA expression levels after transfection of the mimics demonstrated that the effect of miR-145 on ABCA1 expression and function is specific to increased miR-145 expression, and not a result of changes in miR-33a expression (Figure 2C and 2D). Previous studies have suggested that miR-145 is cotranscribed with another miRNA, miR-143 that lies within 1.7 kilobases on chromosome 18.35 We examined whether miR-143 could regulate ABCA1, but observed no changes to ABCA1 protein expression or cholesterol efflux function after overexpression of miR-143 in HepG2 cells (Figure 2E and 2F). The specificity of miR-145 regulation on ABCA1 function was further demonstrated when we observed that miR-145* (passenger strand) does not decrease ABCA1 function (Figure IIIA and IIIB in the online-only Data Supplement). To validate the findings from our luciferase reporter assay, we analyzed the effect of miR-17 on ABCA1 expression and function. Although miR-17 was 1 of the 8 miRNAs predicted to target ABCA1 in silico (Table II in the online-only Data Supplement), our reporter assay revealed no significant changes to activity after the expression of miR-17. This was confirmed when our immunoblotting studies and cholesterol efflux assays demonstrated no changes to ABCA1 expression and function after miR-17 expression (Figure IVA and IVB in the online-only Data Supplement). Our findings validate miR-145 as a regulator of ABCA1 in HepG2 hepatic cells, a cell-type in which ABCA1 function is critical for HDL biogenesis.

Combination of miR-33a and miR-145 Does Not Result in Additive or Synergistic Effects on ABCA1

The majority of miRNAs have subtle effects on their predicted targets.36 A combination of miRNAs may additively or synergistically enhance the repressive effects over a single miRNA acting alone.37 However, we found that the combination of miR-33a and miR-145 did not enhance repression of ABCA1 protein levels (Figure 3A and 3B) or cholesterol efflux activity (Figure 3C) compared with either miRNA expressed singly.

Inhibiting miR-145 using single-stranded RNA inhibitor sequences in HepG2 cells increased ABCA1 protein levels (Figure 3D) and increased cholesterol efflux activity by 29% (Figure 3E). Again we did not observe an enhanced effect on ABCA1 protein expression or efflux function when combining the 2 miRNA inhibitors compared with each inhibitor expressed independently.

Figure 1. A reporter assay validated 5 microRNAs (miRNAs) as regulating ATP-binding cassette transporter A1 (ABCA1) 3′-untranslated regions (3′UTR) activity. A, The ABCA1 3′UTR reporter construct was cotransfected into U343 cells along with 50 nmol/L of miR-17, miR-145, miR-19, miR-96, miR-33a/33b, miR-27a, miR-148, and miR-144. Dual-luciferase activity was measured with the error bars representing SD from measurements made in triplicate. The data are expressed as the percentage loss in activity with miRNA transfected cells to cells transfected with control miRNA. SC indicates scrambled control (Ambion). B, The human ABCA1 3′UTR contains 2 binding sites for miR-145 at nucleotides 109 to 115 and 3114 to 3120 downstream of the ABCA1 stop codon (predicted binding sites to the seed sequence of miR-145 by TargetScanHuman 5.2). Two nucleotides in both seed sequences were mutated using site-directed mutagenesis: mutations to site 1 (MU1)=109 to 115 (110:A→T, 113:G→A) and MU2=3114 to 3120 (3114:A→G, 3118:G→T). C, The luciferase reporter constructs containing either the wild-type (WT) or miR-145 binding site mutations (MU1 only or MU1+MU2) were cotransfected into U343 cells along with control (SC) or miR-145 miRNA mimics. The data are expressed as a change in luciferase activity after miR-145 transfection, compared with cells transfected with control (SC) miRNA. The error bars represent SD.
MiR-145 Regulates ABCA1 Expression in β-Cells and Islets

We generated adenoviruses expressing miR-33a, miR-145, along with their respective inhibitors. Transduction of HepG2 cells with adenoviruses resulted in similar effects on ABCA1 protein expression and cholesterol efflux activity as transfecting with their respective oligonucleotide counterparts (Figure VA and VB in the online-only Data Supplement).

We previously demonstrated that miR-33a influences islet cell cholesterol content and islet function through ABCA1.31 The identification of miR-145 expression in islets (Figure VI in the online-only Data Supplement) prompted us to investigate whether miR-145 regulates ABCA1 in MIN6 pancreatic β-cells. Because of low basal expression of ABCA1 in MIN6 cells, the cells were treated with 10 μmol/L of liver X receptor agonists (TO-901317) before expressing miR-145. Adenoviral-miR-145 expression in liver X receptor-treated MIN6 cells significantly decreased ABCA1 protein levels (Figure VIIA and VIIB in the online-only Data Supplement), while significantly elevating total cellular cholesterol content (Figure VIIH in the online-only Data Supplement). These findings suggest that miR-145 regulates ABCA1 protein expression and cholesterol content in β-cells.

To determine the effects of miR-145 on islet function, we used primary pancreatic islets isolated from mice. Murine primary islets were used rather than MIN6 cells because of the low levels of endogenous miR-145 in MIN6 cells (Figure 4A), the low basal levels of ABCA1 in MIN6 cells (Figure VIIA in the online-only Data Supplement), and primary islets representing a more physiologically relevant assessment of islet cell function than the MIN6 cell line.

In primary islets, adenoviral-miR-145 expression decreased ABCA1 protein levels, whereas adenoviral-inhibitors to miR-145 increased ABCA1 expression (Figure 4B and 4C). In a cell, miRNAs range in expression from <1 copy to >1000 copies.38 Expression analysis by reverse transcription-quantitative polymerase chain reaction (TaqMan) revealed that miR-145 is expressed in multiple human (Figure VIA in the online-only Data Supplement) and murine (Figure VIB in the online-only Data Supplement) tissues. The regulation of miRNA targets is partially dependent on the expression level of miRNA in cells and tissues, and high miRNA expression is expected to be associated with enhanced suppression of target transcripts.

We compared the concentrations of miR-145 with miR-33a in murine liver and islets, as well as in MIN6 cells, by absolute quantification reverse transcription-quantitative polymerase chain reaction using the standard curve method (Figure VIII in the online-only Data Supplement). After correcting for primer efficiencies, we found miR-145 and miR-33a to be equally expressed in murine liver and islets (Figure 4A). Based on their expression profiles, we expect that inhibiting miR-145 in islets could lead to similar increases in ABCA1 protein expression as inhibiting miR-33a. Indeed, we found that in primary murine islets, inhibiting miR-145 resulted in equivalent increases to ABCA1 protein levels as inhibiting...
miR-33a (Figure 4D and 4E). Our results identify miR-145 as a novel regulator of ABCA1 protein expression in islets.

**MiR-145 Regulates Total Cholesterol Content and Insulin Secretion in Murine Pancreatic Islets**

We next investigated the effects of miR-145 on islet cell function. Overexpressing miR-145 resulted in a significant increase to total islet cholesterol content (Figure 5A). However, changes in cholesterol were not observed after miR-33a inhibition (Figure 5A). This is similar to what we observed after miR-33a inhibition in islets in a previous study.31 In both cases, we speculate that inhibiting miR-145 or miR-33a results in localized reductions in cholesterol levels; however, these localized decreases are not detected in our total islet cholesterol measurements. In islets from mice lacking ABCA1 in the β-cell (ABCA1 β-cell−/−), no changes were observed in total cholesterol after miR-145 inhibition (Figure 5B). However, in a mouse model of hypercholesterolemia and reduced islet ABCA1 expression (ApoE−/−),39 primary islets treated with miR-145 inhibitors showed a significant reduction in total cholesterol content (Figure 5B). MiR-145 also influenced islet function, as adenoviral overexpression of miR-145 significantly decreased glucose-stimulated (20 mmol/L) insulin secretion (Figure 5C), whereas adenoviral inhibition of miR-145 significantly increased glucose-stimulated (20 mmol/L) insulin secretion (Figure 5D). These findings support a critical role for the regulation of insulin secretion and islet function by miR-145.

**Expression of miR-145 Is Regulated by Glucose Levels**

We next investigated the regulation of miR-145 in the context of islet β-cell function. Previous studies demonstrated that miR-145 expression is induced after serum starvation in human colorectal carcinoma-116 and -8 cell lines.40 We verified this in cultured pancreatic β-cells where we found that reduced serum levels in cell culture media increased miR-145 expression levels by 38% in MIN6 cells (Figure IX in the online-only Data Supplement). We next investigated whether glucose levels could affect miR-145 expression. High glucose levels in cell culture media (25 mmol/L) resulted in a 35% decrease in miR-145 levels in MIN6 cells (Figure 6A). The effect of glucose...
and function. A luciferase reporter assay demonstrated miRNA regulators of ABCA1 were found. This included several miRNAs expressed in the liver, 8 potential binding sites for miRNAs. In this study, we identify miR-145 as a miRNA regulates a target mRNA. Although miR-145 has a role in the glucose homeostatic pathway. In HepG2 cells, a cell-line relevant for HDL biogenesis, miR-145 was found to regulate ABCA1 expression and function. In contrast, miR-143 and miR-145* did not affect ABCA1 activity, which demonstrates the specific regulation of ABCA1 by miR-145. MiR-143 was previously thought to be cotranscribed with miR-145 as part of a cardiovascular-specific miRNA cluster. MiR-145* is the complementary sequence (passenger strand) to miR-145 in the miRNA/miRNA* duplex that is generated when the RNAseIII enzyme Dicer processes pre-miRNAs. The addition of exogenous miR-145* may even inhibit miR-145 regulation of ABCA1 by binding endogenous miR-145 and inhibiting its ability to target and reduce ABCA1 expression and function.

### Discussion

Post-transcriptional regulation of protein expression occurs when miRNAs bind sites in the 3′UTR to inhibit translation or initiate mRNA degradation. ABCA1 contains a longer than average 3′UTR with multiple predicted and validated binding sites for miRNAs. In this study, we identify miR-145 as a novel regulator of ABCA1 in hepatic and pancreatic cell lines, and in pancreatic islets.

By combining the results from 5 bioinformatics programs and identifying those miRNAs expressed in the liver, 8 potential miRNA regulators of ABCA1 were found. This included several miRNAs recently validated as regulating ABCA1 expression and function. A luciferase reporter assay demonstrated that 5 of the 8 miRNAs decreased ABCA1 activity including miR-145. The features of the primary miR-145 binding site (site 1, 109–115; Figure II in the online-only Data Supplement) support a regulatory role for ABCA1 expression. These features include a location rich in AU nucleotides, being situated away from the middle of UTRs where occlusive interactions can form, being ≥15 nucleotides away from the stop codon where interference from bound ribosomes arises, and a location rich with binding sites for a variety of different miRNAs.

In HepG2 cells, a cell-line relevant for HDL biogenesis, miR-145 was found to regulate ABCA1 expression and function. In contrast, miR-143 and miR-145* did not affect ABCA1 activity, which demonstrates the specific regulation of ABCA1 by miR-145. MiR-143 was previously thought to be cotranscribed with miR-145 as part of a cardiovascular-specific miRNA cluster. MiR-145* is the complementary sequence (passenger strand) to miR-145 in the miRNA/miRNA* duplex that is generated when the RNAseIII enzyme Dicer processes pre-miRNAs. The addition of exogenous miR-145* may even inhibit miR-145 regulation of ABCA1 by binding endogenous miR-145 and inhibiting its ability to target and reduce ABCA1 expression and function.

Our studies demonstrated that miR-33a mimics decreased ABCA1 protein levels and efflux activity more than miR-145 mimics in HepG2 cells. Their effect may be explained by a unique feature of the miR-33a binding sites found in ABCA1. Multiple binding sites increase the likelihood that a miRNA regulates a target mRNA. Although miR-145 has 2 binding sites in the 3′UTR of ABCA1 at position 109 to 115 and 3114 to 3120 (TargetScanHuman 5.2), miR-33a has 3 binding sites at positions 134 to 140, 139 to 145, and 149 to 155 (TargetScanHuman 5.2) downstream of the ABCA1 stop codon. Binding sites can produce a synergistic effect when neighboring one another compared with 2 distant sites.

Although miR-33a has 3 binding sites within 20 bp of one
Another, miR-145 has 2 binding sites separated by 3000 bp (Figure II in the online-only Data Supplement).

Most biological processes are thought to involve multiple members of a miRNA family, unrelated miRNAs, or a combination of miRNAs and transcription factors. A slight additive effect between miR-758 and miR-33a on ABCA1 regulation has been reported previously. It was investigated the possibility that miR-145 and miR-33a could cooperate to enhance the regulation of ABCA1 in HepG2 cells. No significant difference in ABCA1 protein levels or function was observed after the combined expression of both miRNAs compared with either miRNA expressed alone. The lack of a combined effect by miR-145 and miR-33a may be because of an upper threshold in the regulation of transcripts by miRNAs or mechanisms that limit the effect of miRNAs on a gene.

miR-145 is expressed in several different tissues and cell lines, including in murine pancreatic islets (Figure 4A).

Figure 5. MiR-145 regulates total cholesterol levels and glucose-stimulated insulin secretion in murine pancreatic islets. A. Total cholesterol levels in primary islets transduced with adenovirus control (Cont) and adenovirus miR-145 or adenovirus control (Cont) and adenovirus miR-145 inhibitor at a MOI=100 for 48 hours. All cholesterol values are normalized to protein concentration (n=5). B. Total cholesterol levels in pancreatic islets isolated from mice with β-cell-specific deletion of ATP-binding cassette transporter A1 (ABCA1; ABCA1 β-cell−/−), and mice with a global deletion of apolipoprotein E (ApoE−/−) transduced with adenovirus control (Cont) and adenovirus miR-145 inhibitor at a MOI=100 for 48 hours. All cholesterol values are normalized to protein concentration (n=5). C. Glucose-stimulated (20 mmol/L) insulin secretion in pancreatic islets isolated from wild-type mice after transduction with adenovirus control (Cont) and adenovirus miR-145 at a MOI=100 for 48 hours. All insulin secretion values are normalized to total islet DNA concentration (n=6). D. Glucose-stimulated (20 mmol/L) insulin secretion in primary pancreatic islet cells isolated from wild-type mice transduced with adenovirus control (Cont) and adenovirus miR-145 inhibitor at a MOI=100 for 48 hours. All insulin secretion values are normalized to total islet cell DNA concentration (n=7). All data are expressed as the means±SD with the number of experiments performed indicated in parentheses. Significance is set at P<0.05.

Figure 6. Glucose regulates miR-145 in MIN6 cells. Relative expression levels (reverse transcription-quantitative polymerase chain reaction) of (A) miR-145 and (B) miR-33a in MIN6 pancreatic islet cells grown in medium containing 2.8 or 25 mmol/L glucose after 48 hours. All expression levels are normalized to U6 RNA (n=3). Significance is set at P<0.05.

ABCA1 plays a key role in maintaining islet cholesterol homeostasis and ensuring proper β-cell function, and we previously demonstrated that miR-33a can affect islet cholesterol homeostasis and mediate β-cell insulin secretion through the regulation of ABCA1.

The physiological role of miR-145 regulation of ABCA1 may be a response to the presence of nutrients. Human colorectal carcinoma cells grown without serum show a significant elevation of miR-145 expression. We found a similar increase in miR-145 expression in MIN6 β-cells grown in media containing low serum. MiR-145 plays a major role in the growth and differentiation of cells, and studies have validated several transcription factors and cell cycle genes as targets of miR-145. Increased levels of miR-145 decreases cell growth, induces cell cycle arrest, increases apoptosis, and decreases cell migration. Because of our demonstration that miR-145 has a regulatory role over islet function, we investigated the effects of glucose on miR-145 expression levels in cultured pancreatic β-cells. We observed a decrease in miR-145 expression when MIN6 cells were exposed to high glucose levels in the media after 48 hours. We propose a mechanism where increased glucose levels leads to decreased miR-145 levels in β-cells. This decreased expression of miR-145 can then increase the expression of miR-145 targeted genes involved in insulin secretion such as ABCA1.
These studies identify miR-145 as a novel post-transcriptional regulator of ABCA1 in both hepatic and pancreatic cells. ABCA1 in the liver is a critical determinant of plasma HDL levels. Future in vivo studies will be required to assess the impact of miR-145 on plasma HDL levels and atherosclerosis. Our studies also show that miR-145 is expressed in islets and that miR-145 regulates glucose-stimulated insulin secretion. Glucose levels also regulate miR-145 expression in β-cells. In conclusion, this study identifies inhibiting miR-145 as a novel strategy to increase ABCA1 expression, cholesterol efflux activity, and islet function.

Acknowledgments
We thank Dr Angel Baldan and Ryan Allen for their helpful discussions.

Sources of Funding
This work was supported by grants from the Canadian Institutes of Health Research (MOP-106684 and MOP-84437 to M.R. Hayden). N. Wijesekara was supported by postdoctoral fellowships from the Canadian Diabetes Association and the MSFHR. W. de Haan was supported by postdoctoral fellowships from the Canadian Institutes of Health Research and the Michael Smith Foundation of Health Research (MSFHR). M.R. Hayden is a University Killam Professor and holds a Canada Research Chair in Human Genetics. The other authors report no conflicts.

References
ABCA1 is an ATP-transporter protein that functions in the efflux of cholesterol from several different tissues. In the liver, ABCA1 cholesterol efflux to apolipoprotein A-1 generates high-density lipoprotein or good cholesterol. In the pancreas, ABCA1 maintains cholesterol homeostasis and proper insulin secretion function in β-cells. This work identifies miR-145 as a novel microRNA regulator of ABCA1 in both hepatic and pancreatic cells. Although transcriptional regulators can significantly increase ABCA1 levels, they also increase the expression of deleterious genes. Thus, there has been a focus on identifying novel post-transcriptional regulators of ABCA1. We demonstrate that inhibiting miR-145 in hepatic cells increases ABCA1 protein expression and cholesterol efflux function. The inhibition of miR-145 in islets increases ABCA1 protein expression and glucose-stimulated insulin secretion. Furthermore, we demonstrate that glucose regulates miR-145 expression. Inhibiting miR-145 represents a novel strategy for treating cardiovascular disease and diabetes mellitus.
Regulation of ABCA1 Protein Expression and Function in Hepatic and Pancreatic Islet Cells by miR-145
Martin H. Kang, Lin-Hua Zhang, Nadeeja Wijesekara, Willeke de Haan, Stefanie Butland, Alpana Bhattacharjee and Michael R. Hayden

Arterioscler Thromb Vasc Biol. 2013;33:2724-2732; originally published online October 17, 2013;
doi: 10.1161/ATVBAHA.113.302004
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/12/2724

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/10/17/ATVBAHA.113.302004.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
Materials and Methods

Cell Culture and Islet Isolation
HepG2 (ATCC), MIN6, and U343 cells (a kind gift from Dr Burton Yang) were maintained in DMEM (Gibco) media containing 25mM Glucose/10% FBS/4mM L-Glutamine. All cell lines were maintained at 37°C with a humidified 5% CO₂ atmosphere. Islets were isolated from two to three month old C57BL6/J male mice and cultured as previously described (1). All experiments were approved by the animal care committee at the University of British Columbia.

3’ UTR Luciferase Reporter Generation and Measurements
The 3’ untranslated region (3’UTR) of human ABCA1 (length: 3309 nucleotides, GenBank accession number NM_005502, position 17099-10412) was PCR amplified from human genomic DNA and cloned into the dual-luciferase expression vector pmirGLO (containing both Firefly and Renilla luciferases) at the XbaI and SalI restriction sites. All constructs were confirmed by sequencing. ABCA1 3’UTR-luciferase plasmid (0.5µg) was transfected into the U343 cell-line using Lipofectamine (Invitrogen) for 24-48 hrs. Dual luciferase (Firefly luciferase and Renilla luciferase as an internal control) protein/light was measured according to the manufacturer's instructions (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as relative light units (RLU). Measurements were plotted as the relative fold change in luciferase activity between the ABCA1 3’UTR construct compared to empty vector alone. Site-directed mutagenesis of the miRNA binding sites was performed using the Quick Change Mutagenesis kit according to the manufacturer's instructions (Stratagene).

MiRNA Mimic and miRNA Inhibitor Transfections
HepG2 cells were transfected with 50nM of Pre-miR™ miRNA precursor (Ambion) mimics, or 100nM of Anti-miR™ miRNA (Ambion) inhibitors using Lipofectamine RNAiMAX for 48 hrs (Invitrogen). Control miRNA oligonucleotides (Ambion) were simultaneously transfected in each experiment.

Adenovirus Generation
Adenoviral expression vectors (pHM10) containing miRNA precursor mimics or miRNA inhibitors were generated downstream of a CMV promoter (Applied Biological Materials and System Biosciences Inc). MiRNA-expressing adenoviruses were designed with a single copy of murine miR-33a or miR-145 sequence along with 150 bps of respective upstream sequence, and 150bps of respective downstream sequence. The inhibitor sequences to miR-33a and miR-145 (System Biosciences Inc), were sub-cloned into pHM10 vectors. All viruses were generated and amplified in HEK293A cells, and then purified by double cesium chloride gradient centrifugation (Applied Biological Materials).

Adenovirus Transduction
HepG2 and MIN6 cell-lines, and murine islets were transduced at a multiplicity of infection (MOI) of 100 in DMEM/10%FBS/4mM L-Glutamine, or RPMI 1640/10%FBS/antibiotics respectively. After 24 hrs the media was changed to fresh
DMEM/10%FBS/4mM L-Glutamine or RPMI 1640/10%FBS/antibiotics. After another 24 hrs (total = 48 hrs), the cells or islets were harvested and processed for experiments. All treatments were compared to a control virus expressing the pHM10 vector alone, or adenovirus expressing alkaline phosphatase (AP).

Protein Isolation

HepG2 cells, MIN6 cells, and murine islets were lysed in lysis buffer (10mM Tris pH8.0, 1% Triton X-100, Complete Protease Inhibitors [Roche]) for 30 mins on ice while vortexing every 10 mins. Five to one hundred micrograms of protein were loaded into separate lanes and separated on 7.5% acrylamide gels. Protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore).

Immunoblotting and Antibodies

ABCA1 was immunoblotted using an anti-ABCA1 monoclonal antibody generated to its C-terminus (2). Anti-GAPDH primary antibody (Millipore), and anti-mouse-HRP conjugated secondary antibody (Jackson IR Laboratories) were also used in immunoblotting analyses.

Cholesterol Efflux Assays

HepG2 cells were plated to near confluency in 24 well dishes in DMEM/10% FBS/4mM L-Glutamine. Cells were either transiently transfected with oligonucleotides or transduced with adenovirus. After 24 hrs, 1μCi/ml of [3H] cholesterol (Perkin-Elmer) were added to the cells. Sixteen hours following loading with [3H] cholesterol, cells were equilibrated in DMEM (no supplements) for 1 hr, followed by efflux to 10μg/ml of apo A-I (Lee Biosystems) for 4 hrs. Radioactivity was measured from the collected supernatant, and from cells lysed with 0.1N NaOH. Both the supernatant and cell lysates were diluted and measured in high flash-point scintillation cocktail fluid (Perkin-Elmer).

Relative and Absolute Quantification of miRNA Expression Levels

MiRNAs were isolated from cell lines or tissue samples using a MicroRNA Isolation kit (Qiagen). Reverse transcription – quantitative polymerase chain reaction (RT-qPCR) was performed with a TaqMan™ MicroRNA Assay kit (ABI) to detect miR-145 or miR-33a expression levels. Briefly, relative quantification of miRNA expression levels was performed with primers specific for mature miRNAs to generate cDNA by reverse transcription. This was followed by cDNA amplification using Real Time PCR with TaqMan™ probes specific for miR-145 or miR-33a. All miRNA expression levels were normalized to the expression of internal U6 RNA.

The comparison of miR-145 to miR-33a expression levels in murine tissues was measured by absolute quantification using the Standard Curve Method. Briefly, RT products (cDNA) from miRNA precursor (Ambion) mimics to miR-33a or miR-145 were serially diluted and qPCR was performed to generate the standard curves. Expression levels of miR-145 and miR-33a in RNA samples isolated from murine liver and islets were simultaneously measured. Actual miRNA expression levels of miR-145 and miR-33a were determined following correction for primer efficiencies.
**Cholesterol Measurements**

Islet cellular cholesterol levels were obtained and measured as previously described (3). Total protein was measured using a protein assay kit according to the manufacturer’s instructions (Bio-Rad).

**Insulin Secretion**

Insulin secretion measurements from islet cells were carried out as previously described (3). Islets were stimulated with 20mmol/l of glucose.

**Statistical Analysis**

Statistical significance was measured using the student’s t-test and corrected for using the Bonferroni method when there were more than two treatment groups. Significance is set at $p \leq 0.05$ for two comparisons, $p \leq 0.017$ for three comparisons, and $p \leq 0.0125$ for four comparisons.
References


Supplemental Material

Supplemental Figure I. The 3’ untranslated region (3’UTR) of ABCA1 may contain binding sites for microRNAs (miRNAs). The human ABCA1 3’UTR (3309bp) was PCR amplified from genomic DNA and cloned into a luciferase reporter plasmid. This reporter construct was transiently transfected into MIN6 beta cells, 3T3L1 adipocytes, 293 human embryonic kidney cells, primary human skin fibroblasts, and HepG2 hepatocytes. Following transfection, dual-luciferase (fire-fly luciferase, and renilla luciferase as an internal control) activity was measured. The relative fold change in luciferase activity for each cell line was plotted compared to pmirGLO plasmid lacking the ABCA1 3’UTR. The error bars represent standard deviation from measurements made in triplicate.
<table>
<thead>
<tr>
<th>miRNA Prediction Program (website address)</th>
<th>Number of miRNA binding sites predicted to reside in the 3’UTR of ABCA1 or the number of miRNAs predicted to target ABCA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TargetScan Human 5.2 (<a href="http://www.targetscan.org">www.targetscan.org</a>)</td>
<td>40 miRNA family target sites broadly conserved among vertebrates</td>
</tr>
<tr>
<td>PicTar (pictar.mdc-berlin.de)</td>
<td>37 miRNAs predicted to target ABCA1 in vertebrates based on conservation in mammals</td>
</tr>
<tr>
<td>microRNA.org (<a href="http://www.microrna.org">www.microrna.org</a>)</td>
<td>76 miRNAs predicted to target human ABCA1</td>
</tr>
<tr>
<td>miRDB (mirdb.org/miRDB/)</td>
<td>115 miRNAs predicted to target human ABCA1</td>
</tr>
<tr>
<td>FindTar (bio.sz.tsinghua.edu.cn/)</td>
<td>2889 predicted miRNA target sites in ABCA1. Many targets are redundant for a single miRNA. (miR33a predicted with 2 binding sites; miR-145 predicted with 4 binding sites; miR-17 predicted with 9 binding sites)</td>
</tr>
</tbody>
</table>

Supplemental Table I. The number of predicted microRNA (miRNA) binding sites in the 3’UTR of ABCA1, or the number of miRNAs predicted to target ABCA1 using five different bioinformatic programs: TargetScan Human 5.2; PicTar; microRNA.org; miRDB; and FindTar.
<table>
<thead>
<tr>
<th>miR</th>
<th>TargetScan 5.2</th>
<th>PicTar</th>
<th>microRNA.org</th>
<th>miRDB</th>
<th>FindTar</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-17</td>
<td>1 conserved site</td>
<td>2 sites</td>
<td>1 site</td>
<td>Not predicted</td>
<td>9 sites</td>
</tr>
<tr>
<td>miR-145</td>
<td>2 conserved sites</td>
<td>1 site</td>
<td>1 site</td>
<td>1 site</td>
<td>4 sites</td>
</tr>
<tr>
<td>miR-19a</td>
<td>1 conserved site; 2 poorly conserved sites</td>
<td>3 sites</td>
<td>1 site</td>
<td>2 sites</td>
<td>4 sites</td>
</tr>
<tr>
<td>miR-96</td>
<td>1 conserved site</td>
<td>1 site</td>
<td>1 site</td>
<td>1 site</td>
<td>5 sites</td>
</tr>
<tr>
<td>miR-33a/33b</td>
<td>2 conserved sites; 1 poorly conserved site</td>
<td>Not predicted</td>
<td>2 sites</td>
<td>3 sites</td>
<td>2 sites</td>
</tr>
<tr>
<td>miR-27a</td>
<td>1 conserved site; 1 poorly conserved site</td>
<td>2 sites</td>
<td>2 sites</td>
<td>2 sites</td>
<td>4 sites</td>
</tr>
<tr>
<td>miR-148</td>
<td>1 conserved site</td>
<td>1 site</td>
<td>1 site</td>
<td>1 site</td>
<td>3 sites</td>
</tr>
<tr>
<td>miR-144</td>
<td>2 conserved sites; 3 poorly conserved sites</td>
<td>5 sites</td>
<td>4 sites</td>
<td>4 sites</td>
<td>3 sites</td>
</tr>
</tbody>
</table>

**Supplemental Table II. Predicted number of miRNA binding sites in the 3’UTR of ABCA1 for eight different miRNAs.** A total of eight different miRNAs (miR-17; miR-145; miR-19a; miR-96; miR-33a/33b; miR-27a; miR-148; miR-144) were identified as potential regulators of ABCA1 by at least four out of the five programs. All eight miRNAs have been previously shown to be expressed in the liver or in hepatic cells. The number of binding sites predicted in the 3’UTR of ABCA1 for each of the eight different miRNAs are listed for each program in the Table above.
Supplemental Figure II. The predicted binding sites (TargetScanHuman 5.2) for the five miRNAs that repressed 3’UTR activity in our luciferase assay. ORF = open reading frame; AAAAAAAA = polyadenylation tail.

Human ABCA1 3’UTR (Length = 3309 base pairs)
Supplemental Figure III. The expression of the miRNA* strand (miR-33a* or miR-145*) does not repress ABCA1 cholesterol efflux activity in HepG2 cells. Cholesterol efflux activity to 10µg/ml ApoA-I in HepG2 cells transfected with control miR(100nM), miR-33a*(50nM) + control miR(50nM), miR-145*(50nM) + control miR(50nM), or miR-33a*(50nM) + miR-145*(50nM) for 48 hrs. Error bars represent standard deviation. Significance is set at p≤0.0125. (n=6).
Supplemental Figure IV. The expression of miR-17 does not repress ABCA1 protein expression or (A) Western blot analysis of ABCA1 in HepG2 cells transfected with 50nM of control miR, miR-17, or miR-33a for 48 hrs. The loading control is GAPDH. (B) Cholesterol efflux activity in HepG2 cells transfected with 50nM of control miR, miR-17, or miR-33a for 48 hrs. (n=6). All data are expressed as the mean ± standard deviation with the number of experiments performed indicated in parentheses. Significance is set at $p \leq 0.017$ for three comparisons or $p \leq 0.0125$ for four comparisons.
Supplemental Figure V. Adenovirus-mediated expression of miR-145 results in similar repressive effects on HepG2 ABCA1 protein expression and cholesterol efflux activity as seen using oligonucleotide precursor miRNA mimics in HepG2 cells. (A) Western blot analysis of ABCA1 in HepG2 cells transduced with adenovirus control, adenovirus miR-33a, adenovirus miR-145, adenovirus anti-miR-33a, and adenovirus anti-miR-145 at a MOI=100 for 48 hrs. (B) Cholesterol efflux activity to 10µg/ml Apo A-1 in HepG2 cells transduced with adenovirus alkaline phosphatase (control), adenovirus miR-33a, adenovirus miR-145, and adenovirus miR-33a+miR-145 in HepG2 cells at a MOI=200 for 48 hrs. Error bars represent standard deviation. Statistical significance is set at p≤0.0125. (n=10).
Supplemental Figure VI. Mature miR-145 is expressed ubiquitously in human and murine tissues. (A) Expression of miR-145 in human tissues using reverse transcription–quantitative polymerase chain reaction (RT-qPCR; TaqMan™) analysis. All results are normalized to U6 control RNA. (B) Expression of miR-145 in murine tissues using RT-qPCR (TaqMan™) analysis. All results are normalized to U6 control RNA. Error bars represent standard deviation. (Sk=Skeletal, S=Small)
Supplemental Figure VII

A. Untreated MIN6 cells

10μM LXR treated MIN6 cells

B. ABCA1 Protein Quantification

C. 10μM LXR treated MIN6

D. ABCA1 Protein Quantification

E. MIN6 Islet Cholesterol (μg/mg protein)

Control | miR-33a | miR-145
Supplemental Figure VII. The expression of miR-145 reduces ABCA1 protein levels and increases total cholesterol content in pancreatic MIN6 cells. (A). Western blot analysis of ABCA1 in MIN6 cells treated with and without 10µM LXR agonists (TO-901317). (B). Protein quantification of Western blot in Supplemental Figure 5A using BioRad Quantity1™. Error bars represent standard deviation. Significance is set at p≤0.05. (n=3). (C). Western blot analysis of ABCA1 in LXR-treated MIN6 cells transduced with adenovirus control, adenovirus miR-33a, and adenovirus miR-145 at a MOI=100 for 48 hrs. (D). Protein quantification of Western blot in Supplemental Figure 5C using BioRad Quantity1™. Error bars represent standard deviation. Significance is set at p≤0.017. (n=8). (E). Total cholesterol levels in LXR-treated MIN6 cells transduced with adenovirus control, adenovirus miR-33a, and adenovirus miR-145 at a MOI=100 for 48 hrs. Cholesterol values are normalized to protein. Error bars represent standard deviation. Significance is set at p≤0.017. (n=12).
Supplemental Figure VIII. Generation of Standard Curves. The standard curves were generated by performing reverse transcription (RT) on known concentrations of mature miR-145 or miR-33a oligonucleotides (Ambion). RT products (cDNA) were serially diluted and probe-based (TaqMan) qRT-PCR analysis was performed on triplicate cDNA serial dilutions. For the curves, the dark points represent the known concentrations of miR-145 or miR-33a. The light points are the samples including murine liver, murine islets, and MIN6 cells. All our samples are within the known concentration range used to generate the standard curve. For the miR-145 standard curve (top) the slope = -3.403, $r^2 = 0.996$, and efficiency = 96.721%. For the miR-33a standard curve (bottom) the slope = -3.591, $r^2 = 0.987$, and efficiency = 89.864%. The actual expression levels of miR-145 and miR-33a were calculated (Figure 4A) using these standard curves.
Supplemental Figure IX. Regulation of miR-145 by low serum levels in MIN6 cells. Relative expression levels (qRT-PCR) of miR-145 in MIN6 pancreatic islet cells grown in media containing 10% FBS or 0.2% FBS after 48 hrs. All expression levels are normalized to U6 RNA. (n=3). Significance is set at p≤0.05.