

# miR-33a Modulates ABCA1 Expression, Cholesterol Accumulation, and Insulin Secretion in Pancreatic Islets

Nadeeja Wijesekara,<sup>1</sup> Lin-hua Zhang,<sup>1</sup> Martin H. Kang,<sup>1</sup> Thomas Abraham,<sup>2</sup> Alpana Bhattacharjee,<sup>1</sup> Garth L. Warnock,<sup>3</sup> C. Bruce Verchere,<sup>3,4</sup> and Michael R. Hayden<sup>1</sup>

Changes in cellular cholesterol affect insulin secretion, and  $\beta$ -cell-specific deletion or loss-of-function mutations in the cholesterol efflux transporter ATP-binding cassette transporter A1 (ABCA1) result in impaired glucose tolerance and  $\beta$ -cell dysfunction. Upregulation of ABCA1 expression may therefore be beneficial for the maintenance of normal islet function in diabetes. Studies suggest that microRNA-33a (miR-33a) expression inversely correlates with ABCA1 expression in hepatocytes and macrophages. We examined whether miR-33a regulates ABCA1 expression in pancreatic islets, thereby affecting cholesterol accumulation and insulin secretion. Adenoviral miR-33a overexpression in human or mouse islets reduced ABCA1 expression, decreased glucose-stimulated insulin secretion, and increased cholesterol levels. The miR-33a-induced reduction in insulin secretion was rescued by cholesterol depletion by methyl- $\beta$ -cyclodextrin or mevastatin. Inhibition of miR-33a expression in apolipoprotein E knockout islets and ABCA1 overexpression in  $\beta$ -cell-specific ABCA1 knockout islets rescued normal insulin secretion and reduced islet cholesterol. These findings confirm the critical role of  $\beta$ -cell ABCA1 in islet cholesterol homeostasis and  $\beta$ -cell function and highlight modulation of  $\beta$ -cell miR-33a expression as a means to influence insulin secretion. *Diabetes* 61:653–658, 2012

**G**luco- and lipotoxic stress are likely important contributors to pancreatic  $\beta$ -cell failure. Obesity is a prominent risk factor in the development of type 2 diabetes, and it is interesting that dyslipidemia, including elevated plasma cholesterol levels, can precede type 2 diabetes onset by several years. Recent data suggest that cholesterol may modulate both  $\beta$ -cell function and survival (1,2).

Human heterozygous carriers of loss-of-function mutations in the cellular cholesterol efflux transporter ATP-binding cassette transporter A1 (ABCA1) show  $\beta$ -cell dysfunction with impaired glucose tolerance (3). An R230C variant is also associated with early onset type 2 diabetes in the Mexican population (4).  $\beta$ -Cell-specific ABCA1 knockout (ABCA1BKO) mice have impaired glucose tolerance,

defective insulin secretion, and altered islet cholesterol homeostasis (5). Hypercholesterolemic apolipoprotein E (apoE) knockout mice also show a similar impairment in insulin secretion associated with reduced islet ABCA1 expression and elevated islet cholesterol (6). Therefore, cholesterol efflux via ABCA1 is a critical determinant of proper maintenance of both islet cholesterol levels and insulin secretion.

Accordingly, there is a need to identify potential mechanisms that may increase ABCA1 expression in pancreatic islets. Genome-wide screens recently showed that microRNA-33a (miR-33a) expression is differentially regulated in human macrophages during cholesterol depletion or enrichment (7), and additional studies show that modulation of miR-33a expression inversely correlates with ABCA1 expression (8–10). The 3' untranslated region of human ABCA1 consists of three highly conserved binding sites for miR-33a (Supplementary Fig. 1A). miR-33a-deficient mice have higher ABCA1 expression in their liver and macrophages and increased cholesterol efflux from the latter (11). We show here that miR-33a also regulates islet ABCA1 levels, thereby affecting cholesterol homeostasis and  $\beta$ -cell function.

## RESEARCH DESIGN AND METHODS

**Cell culture and mice.** MIN6 cells were cultured as described (12). ABCA1BKO mice were generated by crossing ABCA1 floxed mice with mice expressing Cre recombinase driven by the rat insulin 2 promoter (5,13). ABCA1 mRNA levels were described to be unchanged in extrapancreatic tissues, including the hypothalamus. All experiments were approved by the animal care committee at the University of British Columbia.

**Islet isolation and adenoviral infection.** Mouse and human islets were isolated, cultured, and dispersed as described (14,15). Islets/cells were infected with empty vector, miR-33a, and miR-33a inhibitor (miR-33a-Inh; ABM, Richmond, BC, Canada); green fluorescent protein (GFP; Vector BioLabs, Philadelphia, PA); or ABCA1 (16) adenovirus at 100 multiplicity of infection for 24 h in culture media, after which the virus was removed and fresh media was added. Experiments were performed after an additional 4 days (mevastatin experiments) or 24 h (all other experiments).

**RNA extraction and quantitative PCR.** Total RNA was extracted using miRNeasy Mini Kit (Qiagen, Toronto, ON, Canada). Reverse transcription was carried out using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). PCR reactions were performed using 7500 Fast Real-time PCR System and TaqMan MicroRNA Assay Kit (Applied Biosystems). The threshold cycle number was used to determine the relative quantities of mature microRNA. U6 small nuclear RNA was used as an internal control.

**Western blotting.** Lysates were resolved by 7.5% SDS-PAGE and immunoblotted with anti-ABCA1 (1:500) (17) or anti- $\beta$ -tubulin (1:1000; Sigma-Aldrich, Oakville, ON, Canada) antibodies. Immunoblots were scanned within the linear range of intensity and quantified using National Institutes of Health Image J software.

**Immunostaining and confocal microscopy.** Islets were immunostained as described (14) using anti-ABCA1 (1:100) primary and Cy3-conjugated anti-mouse secondary antibodies (Invitrogen, Burlington, ON, Canada). Islets were mounted on glass slides, and images were acquired using an Olympus Fluoview FV1000 confocal microscope. For live cell imaging, cells were seeded on glass-bottom culture dishes (MatTek Corporation, Ashland, MA) and loaded with 100  $\mu$ g/mL filipin (Sigma-Aldrich) with or without 5  $\mu$ mol/L FM1-43 (Invitrogen)

From the <sup>1</sup>Department of Medical Genetics, Centre for Molecular Medicine and Therapeutics, Child and Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada; the <sup>2</sup>Institute for Heart and Lung Health, St. Paul's Hospital, Vancouver, British Columbia, Canada; the <sup>3</sup>Department of Surgery, Child and Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada; and the <sup>4</sup>Department of Pathology and Laboratory Medicine, Child and Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada.

Corresponding author: Michael R. Hayden, mrh@cmmt.ubc.ca.  
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for 1 h at room temperature, and images were acquired using a Leica inverted confocal microscope system (18).

**Insulin secretion.** Insulin secretion was assessed as reported (6,19) from isolated islets stimulated with 0 mmol/L or 20 mmol/L glucose.

**Cholesterol measurement.** Neutral sterol isolation and total cholesterol measurement were performed as described (6). Total protein was measured using DC Bio-Rad Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada).

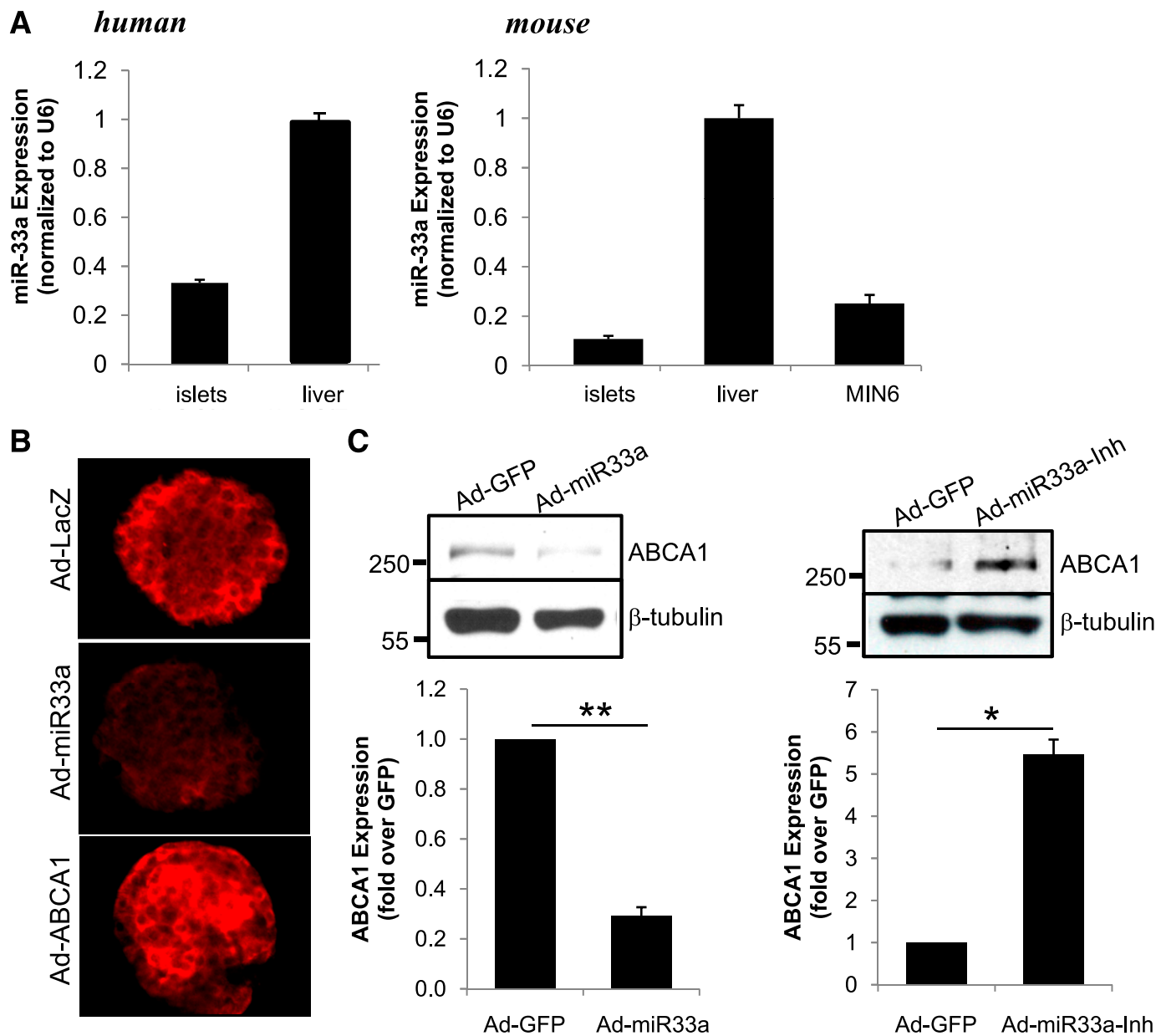
**Statistical analysis.** Data are expressed as mean  $\pm$  SEM. Significance was determined using Student *t* test or one-way ANOVA with Tukey–Kramer post hoc test. *P* < 0.05 was considered statistically significant.

**RESULTS**

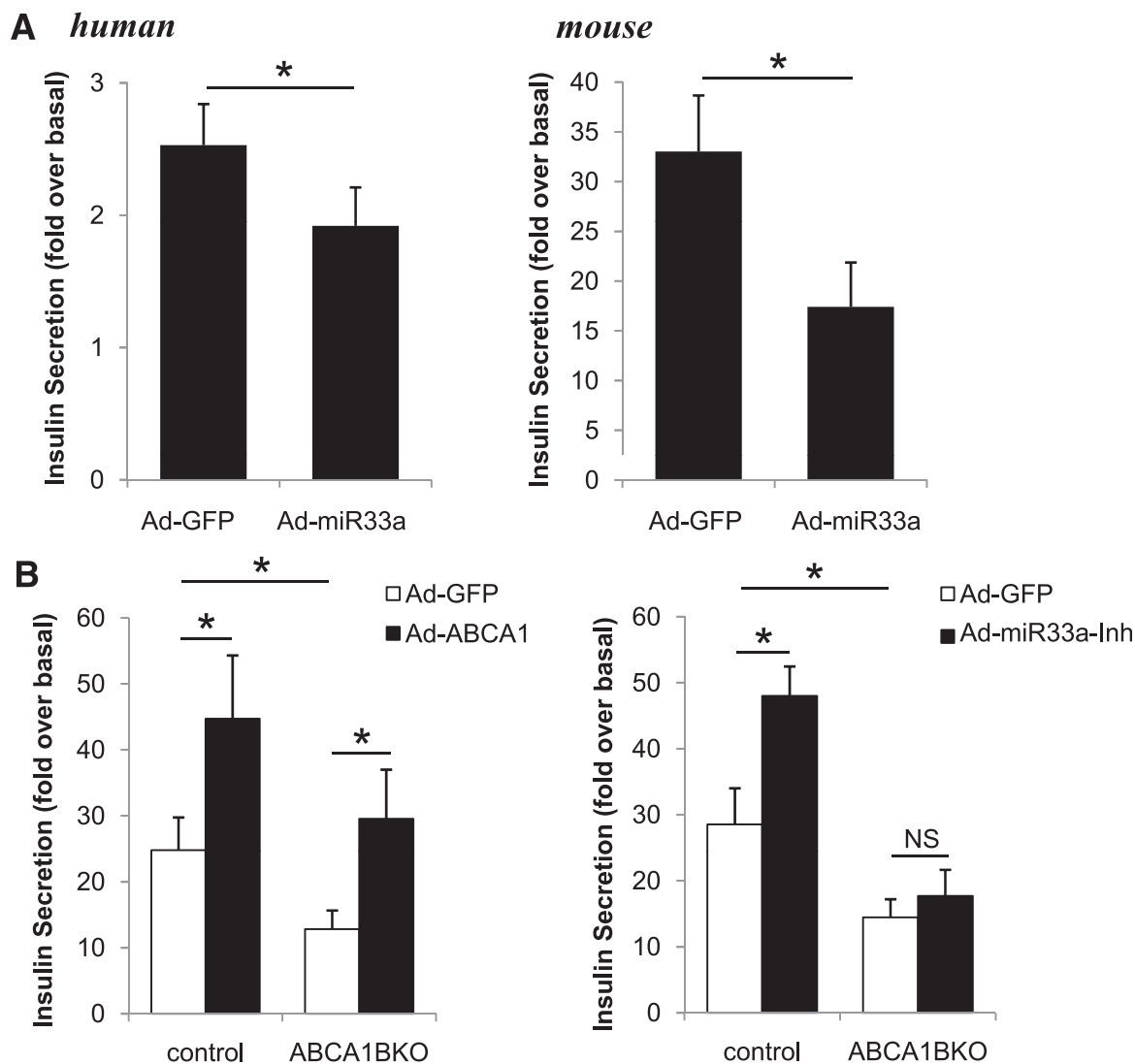
Quantitative RT-PCR analysis showed miR-33a expression in human and mouse pancreatic islets and MIN6  $\beta$ -cells (Fig. 1A), although to a lesser extent than in liver. Adenoviral overexpression of miR-33a in human (Supplementary Fig.

1B) and mouse islets (Fig. 1B and C) resulted in reduced ABCA1 protein expression by immunostaining and Western blot. Conversely, inhibition of miR-33a by expression of a recombinant miR-33a-Inh adenovirus significantly increased ABCA1 protein expression (Fig. 1C).

Overexpression of miR-33a in both human and mouse islets reduced glucose-stimulated insulin secretion (GSIS) (Fig. 2A). Conversely, inhibition of miR-33a led to a significant increase in GSIS in control islets (Fig. 2B), similar to that observed after direct ABCA1 overexpression. In ABCA1BKO islets, overexpression of ABCA1 led to an increase in GSIS, while miR-33a inhibition by expression of miR-33a-Inh adenovirus had no effect (Fig. 2B). No change in islet insulin content was observed under all conditions tested (data not shown).



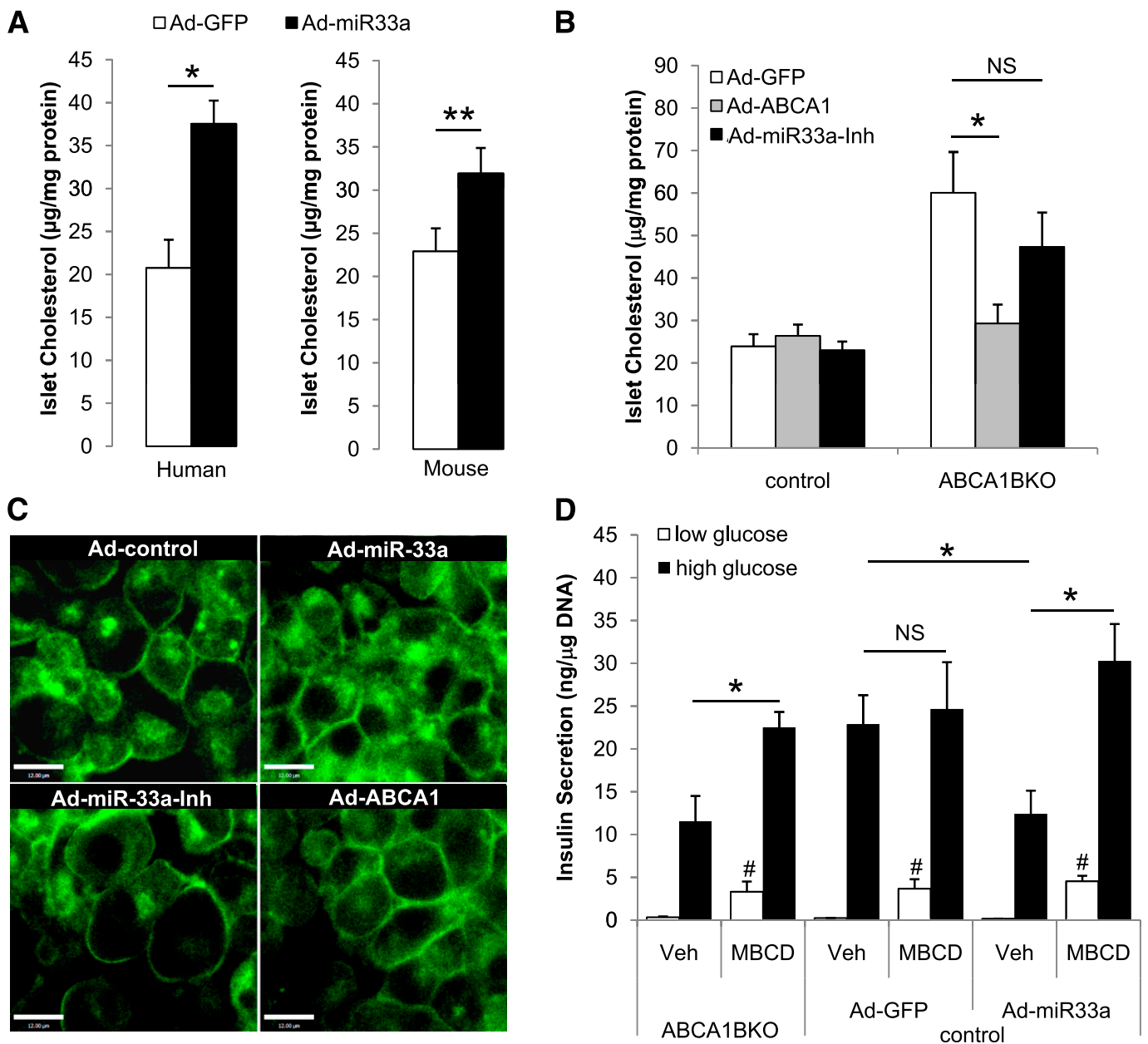
**FIG. 1.** miR-33a modulates ABCA1 expression in pancreatic islets. **A:** Quantitative PCR analysis of miR-33a expression in human and wild-type mouse islets and liver and MIN6 mouse insulinoma cell line. **B:** Immunostaining for ABCA1 (red) in fixed and permeabilized wild-type mouse islets infected with LacZ, miR-33a, or ABCA1 adenovirus (Ad). **C:** ABCA1 expression as assessed by Western blot in wild-type mouse islets infected with recombinant Ad-expressing GFP, miR-33a (*n* = 3), or miR-33a-Inh (*n* = 3). Quantified values are normalized to  $\beta$ -tubulin. \**P* < 0.05, \*\**P* < 0.01. (A high-quality digital representation of this figure is available in the online issue.)



**FIG. 2.** miR-33a-regulated GSIS is mediated by ABCA1. GSIS (20 mmol/L) in human ( $n = 4$ ) and wild-type mouse islets ( $n = 4$ ) (A) and control ABCA1 floxed ( $n = 5$ ) and ABCA1BKO ( $n = 5$ ) islets (B) infected with GFP, miR-33a, ABCA1, or miR-33a-Inh adenovirus (Ad). Quantified values are normalized to DNA. \* $P < 0.05$ , NS: not significant ( $P > 0.05$ ).

Increased miR-33a expression led to an elevation of cholesterol accumulation in both human and mouse islets (Fig. 3A). No change in total cholesterol was observed in control mouse islets when either miR-33a was inhibited by expression of miR-33a-Inh adenovirus or ABCA1 was overexpressed (Fig. 3B). In ABCA1BKO islets, however, adenoviral rescue of ABCA1 expression led to a marked reduction in cholesterol accumulation. Although not significant, miR-33a inhibition also tended to reduce cholesterol levels in these islets. In live MIN6 cells, fluorescent staining for filipin, which binds to cholesterol with high affinity, was found at the plasma membrane with some segregation of fluorescence at the perinuclear region and perhaps secretory granules (Fig. 3C). Of interest, costaining with the granular marker FM1-43 (20) showed colocalization with filipin, generating a Pearson correlation coefficient of 0.912 (Supplementary Fig. 2A). Overexpression of miR-33a increased, whereas expression of miR-33a-Inh or ABCA1 overexpression reduced filipin fluorescence in the perinuclear region compared with cells infected with an empty adenoviral vector. Similar changes in filipin fluorescence were observed in primary  $\beta$ -cells (Supplementary Fig. 2B).

To address whether increased islet cholesterol levels are responsible for the miR-33a-induced decrease in GSIS, we treated islets with methyl- $\beta$ -cyclodextrin (MBCD) prior to stimulation of insulin secretion. Treatment of wild-type islets with 10 mmol/L MBCD for 30 min resulted in an  $\sim 23\%$  reduction in cellular cholesterol (Supplementary Fig. 3A) with no change in cell viability (data not shown). This led to complete recovery of GSIS inhibited by miR-33a but had no effect on GSIS from control islets expressing GFP (Fig. 3D). We also treated ABCA1BKO islets with MBCD and observed normalization of GSIS. It is interesting that MBCD treatment significantly increased basal insulin secretion from both knockout and control islets. Treatment of control islets with 10  $\mu$ mol/L mevastatin for 5 days in the presence of 250  $\mu$ mol/L mevalonate resulted in the complete recovery of GSIS inhibited by miR-33a, with no significant effect on those infected with GFP adenovirus (Supplementary Fig. 3B). Mevastatin treatment also caused an  $\sim 22\%$  reduction in cholesterol in wild-type islets (Supplementary Fig. 3A). As previously shown (5), mevastatin had no effect on insulin secretion from ABCA1BKO islets.



**FIG. 3.** miR-33a modulates pancreatic islet cholesterol levels. Total cholesterol in human ( $n = 3$ ), wild-type mouse islets ( $n = 4$ ) (A) and control ABCA1 floxed ( $n = 6$ ) and ABCA1BKO ( $n = 5$ ) islets (B) infected with GFP, miR-33a, ABCA1, or miR-33a-Inh adenovirus (Ad). Quantified values are normalized to protein. C: Filipin (green) staining in live MIN6 cells infected with empty vector control, miR-33a, miR-33a-Inh, or ABCA1 Ad. D: Insulin secretion in GFP or miR-33a Ad-infected control ABCA1 floxed ( $n = 3$ ) or ABCA1BKO ( $n = 4$ ) islets treated with or without 10 mmol/L MBCD for 30 min. Veh, vehicle. # $P < 0.05$  vs. untreated low glucose. Quantified values are normalized to DNA. \* $P < 0.05$ , \*\* $P < 0.01$ , NS: not significant ( $P > 0.05$ ). (A high-quality digital representation of this figure is available in the online issue.)

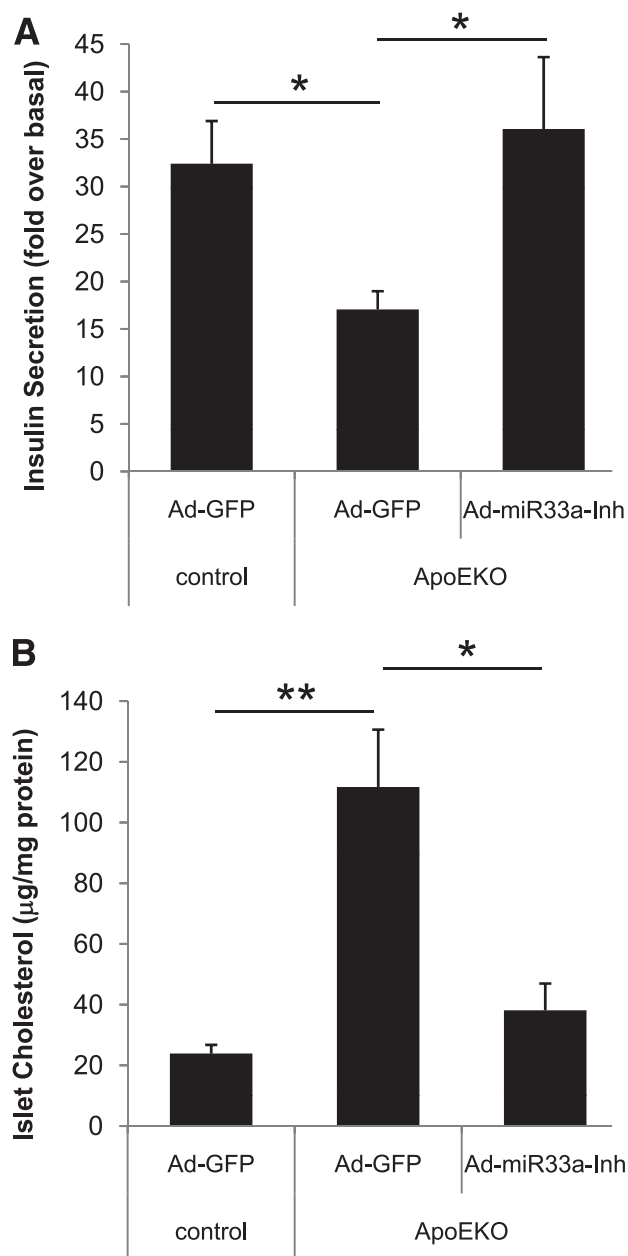
Whether miR-33a inhibition can improve islet function in a mouse model of impaired insulin secretion and hypercholesterolemia was assessed by expressing miR-33a-Inh in apoE knockout islets. As previously reported (6), GSIS from apoE-deficient islets was significantly reduced and associated with elevated islet cholesterol levels (Fig. 4). Adenoviral inhibition of miR-33a in apoE-deficient islets reduced islet cholesterol accumulation and rescued normal GSIS.

#### DISCUSSION

Elevated islet cholesterol levels associated with reduced expression of ABCA1 contribute to impaired  $\beta$ -cell function and glucose tolerance in mice and humans (3–6,21).

Our data show that miR-33a is expressed in pancreatic islets and  $\beta$ -cells and functions to modulate ABCA1 expression, thereby affecting cholesterol levels and insulin secretion from isolated islets. Because changes in miR-33a expression inversely correlate with changes in ABCA1 expression in islets, miR-33a can be described as an important regulator of islet ABCA1. Presence of miR-33a in MIN6 cells and islets indicates that it is likely expressed in primary  $\beta$ -cells. Furthermore, the decrease in ABCA1 immunostaining throughout islets overexpressing miR-33a suggests that miR-33a inhibits ABCA1 expression in  $\beta$ -cells.

Studies point to an important role for microRNAs in regulating islet function (22). In a similar manner, we show here that miR-33a regulates insulin secretion from islets.



**FIG. 4.** miR-33a inhibition improves insulin secretion and cholesterol levels in apoE knockout (apoEKO) islets. GSIS (20 mmol/L;  $n = 3$ ) (A) and total cholesterol ( $n = 3$ ) (B) in islets isolated from control wild-type or apoEKO mice infected with GFP or miR-33a-Inh adenovirus (Ad). Quantified values are normalized to protein (total cholesterol) or DNA (insulin secretion). \* $P < 0.05$ , \*\* $P < 0.01$ .

Lack of change in insulin secretion in the presence of miR-33a-Inh, but improvement in insulin secretion by ABCA1 overexpression in  $\beta$ -cell-specific ABCA1BKO islets, suggests that the effects of miR-33a on insulin secretion are dependent on ABCA1 expression. Upregulation of ABCA1 directly or via miR-33a inhibition appears to improve  $\beta$ -cell function primarily by affecting insulin secretion and not synthesis, as indicated by the unaffected islet insulin content.

Increased miR-33a expression also led to an elevation of islet cholesterol levels, confirming that reduced ABCA1 activity in islets results in disturbance of cholesterol homeostasis. Of interest, our data suggests that intracellular cholesterol may largely concentrate within secretory granules, likely in the granular membrane. Lack of change

detected in cholesterol levels in control mouse islets during miR-33a inhibition or ABCA1 overexpression is perhaps a consequence of small localized reductions in cholesterol levels that are not reflected as changes in total islet cholesterol. In support of this possibility, we observed only small changes in filipin staining within dispersed mouse  $\beta$ -cells as well as the perinuclear region of MIN6 cells overexpressing miR-33a-Inh or ABCA1. Nonetheless, treatment of islets overexpressing miR-33a with MBCD or mevastatin confirmed that effects of miR-33a on GSIS are indeed cholesterol dependent. In contrast, a significant reduction in islet cholesterol was apparent after ABCA1 overexpression in ABCA1BKO islets, which have elevated cholesterol accumulation. Normalization of insulin secretion in ABCA1BKO islets after MBCD treatment confirmed that the impaired insulin secretion in these islets is a result of islet cholesterol accumulation and not a direct effect of ABCA1. In contrast, mevastatin had no effect on insulin secretion from ABCA1BKO islets, which may result from the reduced hydroxymethylglutaryl-CoA reductase expression observed in ABCA1-deficient  $\beta$ -cells (5). miR-33a inhibition also partially reduced cholesterol levels in ABCA1BKO islets, perhaps as a result of elevated ABCA1 expression in non- $\beta$ -cells or its effects on the expression of ABCG1 cholesterol efflux transporter in  $\beta$ -cells (7). The significant increase in basal insulin secretion observed in both ABCA1BKO and control islets in the presence of MBCD suggests that under nonstimulatory conditions, MBCD may increase insulin secretion in a nonspecific manner unrelated to any changes in cell viability. A similar effect on basal insulin secretion was not observed with mevastatin treatment, suggesting that the MBCD effect on basal secretion may not be related to intracellular cholesterol levels. Furthermore, the lack of change in GSIS after MBCD or mevastatin treatment in GFP-expressing islets suggests that in stimulatory conditions, cholesterol depletion has no effect on GSIS unless islet cholesterol is already elevated.

In summary, we show that miR-33a inhibition can rectify normal insulin secretion by increasing ABCA1 expression and normalizing cellular cholesterol levels in pancreatic islets. As such, we observed in this study that in vitro upregulation of ABCA1 via modulation of miR-33a is a useful means to correct  $\beta$ -cell defects and improve cholesterol levels in a hypercholesterolemic mouse model of impaired insulin secretion. Although the direct functional role of miR-33a in some tissues is not yet completely understood, reports suggest that it modulates serum HDL cholesterol, triglyceride levels, insulin signaling, and fatty acid oxidation and synthesis, and its inhibition may be protective against atherosclerosis (11,23–25). Therefore, upregulation of ABCA1 via miR-33a may be a promising therapeutic strategy for individuals with combined defects in  $\beta$ -cell function and cholesterol homeostasis (3,4,21). The data also provide further proof of concept that modulation of islet cholesterol levels can influence insulin secretion and that upregulation of ABCA1 and reduction of islet cholesterol levels may be a therapeutic approach to increase insulin secretion in type 2 diabetes.

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No potential conflicts of interest relevant to this article were reported.

N.W. designed and conducted experiments, analyzed data, and wrote the manuscript. L.-h.Z. performed quantitative PCR experiments. M.H.K. provided the adenoviruses. T.A. acquired live cell images of filipin staining. A.B. provided general technical assistance. G.L.W. provided human islets. C.B.V. and M.R.H. supervised the project. M.R.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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