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MicroRNA-30c reduces hyperlipidemia and atherosclerosis by decreasing lipid synthesis and lipoprotein secretion

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Abstract

Hyperlipidemia is a risk factor for various cardiovascular and metabolic disorders. Overproduction of lipoproteins, a process critically dependent on microsomal triglyceride transfer protein (MTP), can contribute to hyperlipidemia. We show that microRNA-30c (miR-30c) interacts with the 3′-untranslated region of the MTP mRNA and induces degradation leading to reductions in its activity and media apolipoprotein B. Further, miR-30c reduces hyperlipidemia and atherosclerosis in Western diet fed mice by decreasing lipid synthesis and secretion of triglyceride-rich apoB-containing lipoproteins. Therefore, miR-30c coordinately reduces lipid biosynthesis and lipoprotein secretion to control hepatic and plasma lipids and might be useful in treating hyperlipidemias and associated disorders.

Keywords

MTP; apoB; lipoproteins; microRNA; miR-30; hyperlipidemia; atherosclerosis; triglyceride; cholesterol

High plasma lipids are risk factors for various cardiovascular and metabolic disorders such as atherosclerosis, obesity and metabolic syndrome. Cardiovascular disease itself is the leading cause of death in the US [1]. Statins reduce plasma lipids and lower incidence for some of these disorders in 20–40% individuals highlighting a need for new approaches to lower plasma lipids [2]. Dietary and endogenous lipids are transported in plasma by

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lipoproteins made mainly by enterocytes and hepatocytes [3, 4]. Microsomal triglyceride transfer protein (MTP) assists in lipoprotein assembly by interacting and lipidating nascent apolipoprotein B (apoB) to form precursors of LDL [5–8]. Therefore, MTP has been targeted to lower plasma lipids. But, these attempts have been hindered as MTP inhibitors cause steatosis and increase plasma transaminases, AST/ALT [9–12]. Recent studies have highlighted the importance of microRNAs (miRs) in the regulation of gene expression. miRs interact with the 3′-untranslated region (3′-UTR) of target mRNAs and reduce protein synthesis by enhancing mRNA degradation and/or by interfering with its translation [13, 14]. Their role in lipoprotein assembly and plasma apoB-lipoproteins has not been elucidated.

RESULTS

miR-30c reduces MTP activity and media apoB

TargetScan algorithm based on seed recognition identified several miRs that interact with MTP transcript (Fig S1a). However, evolutionary conservation studies among vertebrates narrowed this list to the miR-30 family (Fig S1a). To test whether miR-30 family regulates MTP, different members were introduced into human hepatoma Huh-7 cells. Increasing concentrations of miR-30b and miR-30e had no effect, but miR-30c significantly reduced (~50%) MTP activity compared with a control scramble (Scr) miR (Fig 1a). Different amounts of antimiR-30b and antimiR-30e had no effect, but antimiR-30c increased MTP activity (Fig 1a) suggesting that endogenous miR-30c might regulate MTP expression. Further, different amounts of miR-30c and antimiR-30c reduced and increased MTP protein, respectively (Fig 1b). Since MTP is critical for apoB-lipoprotein assembly, we studied the effect of miR-30c on apoB. Transfection with miR-30c increased its concentrations by several folds (Fig 1c). miR-30c reduced and antimiR-30c increased media apoB (Fig 1d); both miRs did not affect media apoAI (Fig 1d). Further, miR-30b and miR-30e had no effect on media apoB (Fig 1e). These studies indicate that miR-30c reduces cellular MTP and media apoB. Lack of miR-30e might be due to the absence of supplementary interactions (Fig S2). However, no effect of miR-30b suggests that optimum base pairing in the supplementary site might also be important for MTP mRNA degradation.

To determine whether miR-30c affects apoB synthesis or secretion, we performed pulse labeling studies in Huh-7 cells. ApoB and albumin syntheses were not affected by miR-30c and antimiR-30c (Fig 1f) compared to Scr. Pulse-chase studies revealed that intracellular disappearance of apoB and albumin was unaffected by miR-30c and antimiR-30c (Fig 1g). Further, appearance of albumin in the media was unaffected by miRs. However, secretion of apoB was lower and higher in cells transfected with miR-30c and antimiR-30c, respectively, compared with Scr. These studies indicate that miR-30c does not affect synthesis but reduces secretion of apoB100. It is likely that lower MTP levels in miR-30c expressing cells mainly affect posttranslational processing of apoB-containing lipoproteins.

miR-30c interacts with 3′-UTR of MTP and reduces mRNA levels

To understand how miR-30c regulates MTP, we measured mRNA levels. Increasing miR-30c concentrations decreased MTP mRNA by 60–70%, whereas the highest amounts of antimiR-30c increased MTP (Fig 2a). To explain how miR-30c reduces MTP mRNA,

transfected cells were treated with Actinomycin D to inhibit transcription. MTP mRNA disappeared faster from cells expressing miR-30c indicating increased degradation (Fig 2b). Effect of miR-30c on MTP mRNA translation was not studied. Mechanisms of MTP mRNA degradation were further evaluated in COS-7 cells deficient in MTP expression. MTP activity and mRNA (Fig 2c) were reduced when MTP expression plasmids containing 3′-UTR were co-transfected with miR-30c compared to Scr. In contrast, antimiR-30c increased MTP expression (Fig 2c). Next, we evaluated the importance of miR-30c target seed sequence located 90–94 bases after the stop codon (Fig S1b) using three approaches. First, mutagenesis of this sequence abolished the effects of miR-30c and anti-miR-30c on MTP (Fig 2d). Second, the luciferase activity in the psiCHECK2 plasmid encoding a luciferase gene fused with the 3′-UTR of MTP was decreased and increased in the presence of miR-30c and antimiR-30c, respectively (Fig 2e, left); these changes were not seen when the miR-30c binding site was mutated (Fig 2e, right). Third, effects of miR-30c and antmiR-30c on MTP activity and mRNA (Fig 2f) were not seen when co-expressed with MTP-FLAG plasmid lacking the 3′-UTR. These results suggest that miR-30c binds to the 3′-UTR of MTP mRNA and induces degradation.

Tissue expression of miR-30c

To study expression of miR-30c, we measured their steady state levels in human tissues. These studies showed that miR-30s were highly expressed in the heart, skeletal muscle and kidney (Fig 2g). Comparatively lower but significant amounts of miR-30c were also detected in other tissues (Fig 2g). In contrast, high MTP expression was in the small intestine and liver (Fig 2h). miR-30c is conserved in vertebrates (Fig S3) and resides in intron 5 of the human nuclear transcription factor Y subunit C (NFY-C) gene. NFY-C was ubiquitously expressed with high levels in the human heart and testes (Fig 2i) and this expression was different from miR-30c (Fig 2g). Therefore, we measured primiR-30c and found that its expression pattern was similar to NFY-C (Fig 2i) indicating that both are transcribed simultaneously and tissue levels of miR-30c might be regulated post-transcriptionally. To test this, we reduced the expression of Dicer that is critical for miR biogenesis [13, 15]. siDICER reduced DICER mRNA by 90%, had no effect on primiR-30c and NFY-C, reduced miR-30c and increased MTP mRNA in Huh-7 cells (Fig 2j). We interpret these data to suggest that NFY-C and primiR-30c might be co-transcribed and their tissue levels diverge due to differential post-transcriptional processing.

miR-30c lowers plasma cholesterol

Next, we explored physiological consequences of hepatic over expression of miR-30c. Male C57/Bl6 mice were transduced with lentiviruses expressing different miRs and fed *ad libitum* Western diet. After 3 weeks, hepatic miR-30c levels were approximately 4-fold higher in miR-30c expressing mice and these increases had no effect on endogenous miR-30b and miR-30e (Fig 3a). miR-30c levels did not increase in other tissues (Fig S4a). Expression of miR-30c and antimiR-30c reduced and increased hepatic MTP mRNA, activity and protein (Fig 3b), respectively, with no effect in other tissues (Fig S4b). Further, miR-30c had no effect on apoB (Fig S4d), apoA1 (Fig S4e), Abca1 (Fig S4f) and Abcg1 (Fig S4g) transcripts in different tissues. These studies indicate that miR-30c was mainly expressed in the liver and reduced hepatic MTP.

miR-30c significantly reduced (16–27%) while antimiR-30c increased (20–54%) plasma cholesterol due to changes in non-HDL apoB-lipoproteins (Fig 3c). However, miR-30c and antimiR-30c had no effect on fasting triglyceride (Fig 3d), AST (Fig S4h) and ALT (Fig S4i). These studies indicate that miR-30c reduces plasma cholesterol due to decreases in non-HDL lipoproteins without increasing plasma transaminases.

Attempts were then made to understand how miR-30c regulates plasma lipids. We hypothesized that lower plasma lipids in Western diet fed mice might be due to reduced hepatic lipoprotein production. Triglyceride production rates were significantly higher in antimiR-30c (372 mg/dl/h) and lower in miR-30c (119 mg/dl/h) compared with Scr (205 mg/dl/h) expressing mice that were injected with Poloxamer 407 [16] to inhibit lipases (Fig 3e). Therefore, hepatic over expression of miR-30c reduces triglyceride-rich lipoprotein production.

The above studies showed that miR-30c reduces hepatic lipoprotein production and plasma cholesterol. Hence, we measured lipids in liver homogenates anticipating them to increase. However, hepatic triglyceride and cholesterol (Fig 3f) were not increased in miR-30c expressing mice. To understand why miR-30c did not increase hepatic lipids, we studied hepatic β-oxidation, *de novo* lipogenesis, and triglyceride/phospholipid biosynthesis. miR-30c and antimiR-30c expression had no effect on β-oxidation (Fig 3g); but they, respectively, reduced and increased synthesis of fatty acids, phospholipids and triglyceride (Fig 3h). These studies demonstrate that miR-30c reduces hepatic lipid synthesis.

To explain reasons for decreased lipid synthesis, Gene Ontology analysis was performed for miR-30c target genes. This analysis revealed that miR-30c could affect several lipid synthesis pathways (Fig S5a) and genes (Fig S5b). We selected one gene from each pathway. Analysis of hepatic mRNA showed that miR-30c reduced Lpgat1, Elovl5, Stard3 and Mboat1 mRNA levels (Fig 4a). Expression of miR-30c in Huh-7 cells reduced mRNA levels of these genes while antimiR-30c increased their levels (Fig 4b). To ascertain their roles in lipid synthesis, we reduced (~80–90%) expression of individual genes using specific siRNAs (Fig 4c). Knockdown of these genes had no effect on MTP activity (Fig 4e), media apoB (Fig 4e) and apoAI (Fig 4f) suggesting that they might not play a role in lipoprotein secretion. In contrast, siMTP reduced MTP mRNA (Fig 4c), MTP activity (Fig 4d) and media apoB (Fig 4e), but had no effect on media apoAI (Fig 4f). Next, we determined the effect of reducing the expression of these genes on lipid synthesis. siMTP, siMBOAT1 and siStARD3 did not affect, but siELOVL5 and siLPGAT1 reduced *de novo* lipogenesis (Fig 4g). To evaluate whether these genes are targeted by miR-30c, cells were co-transfected with different siRNAs along with either Scr or miR-30c. miR-30c reduced lipid synthesis in control, siMTP, siMBOAT1, siELOVL5 and siStARD3 treated cells but not in siLPGAT1 treated cells (Fig 4h) suggesting that LPGAT1 might play a role in lipid synthesis and is targeted by miR-30c.

Next, we determined whether reduced expression of LPGAT1 would affect media apoB. To test this, Huh-7 cells were transfected with different siRNAs with either Scr or miR-30c (Fig 4i). siLPGAT1 had no effect on media apoB; however, siMTP significantly reduced media apoB. miR-30c reduced media apoB in all siRNA treated cells except those treated with

siMTP. These studies suggest that siLPGAT1 does not affect media apoB. Further, miR-30c does not lower media apoB in siMTP treated cells. Thus, miR-30c appears to reduce hepatic lipid synthesis and apoB secretion by targeting LPGAT1 and MTP, respectively.

The studies with siMTP indicated that miR-30c reduces lipid synthesis independent of MTP. To test the hypothesis that reduced lipid synthesis after miR-30c expression might be independent of MTP, we transduced liver specific *Mttp* deficient (Alb-Cre-Mttp$^{fl/fl}$, *L-MTP*$^{-/-}$) mice with lentiviruses expressing miR-30c. Increases in hepatic miR-30c by 3-fold had no effect on miR-30b and miR30e expression (Fig 5a), plasma triglyceride (Fig 5b) and cholesterol (Fig 5c), and hepatic cholesterol (Fig 5d). However, miR-30c expressing livers had lower hepatic triglyceride (Fig 5e). miR-30c had no effect on fatty acid oxidation but reduced fatty acid, triglyceride and phospholipid synthesis (Fig 5f–i). In a separate experiment, miR-30c and antimiR-30c had no effect on plasma triglyceride and cholesterol (Fig S6a) in different lipoproteins (Fig S6b) compared to Scr in *L-MTP*$^{-/-}$ mice. However, hepatic triglycerides, but not cholesterol (Fig S6c), were reduced in mice transduced with lentiviruses expressing miR-30c. For control, similar experiments were performed in *Mttp*$^{fl/fl}$ mice. Over expression of miR-30c by 3-fold (Fig S7a) had no effect on plasma triglycerides (Fig S7b), hepatic cholesterol and triglyceride (Fig S7c), and fatty acid oxidation (Fig S7d). However, it reduced plasma cholesterol (Fig S7b) and hepatic lipid synthesis (Fig S7e). These data indicate that miR-30c reduces hepatic triglycerides by targeting genes other than MTP.

miR-30c reduces atherosclerosis

Studies described so far indicated that over expression of miR-30c decreases *de novo* lipogenesis, reduces MTP activity, lowers plasma lipids, does not affect plasma transaminases without causing steatosis in Western diet fed wildtype mice. Next we evaluated the effects of miR-30c on plasma lipids and transaminases in female *Apoe*$^{-/-}$ mice transduced with lentiviruses expressing different miRs and fed a Western diet. We saw significant reductions in plasma cholesterol (Fig 6a) and triglyceride (Fig 6b) in miR-30c compared with Scr expressing mice. In contrast, plasma lipids were generally higher in antimiR-30c mice (Fig 6a–b). Similar reductions in plasma lipids were seen in another experiment (Fig S8a). However, there were no significant differences in plasma AST and ALT (Fig S8a) activities in Scr and miR-30c groups. Triglyceride secretion rates were higher and lower in antimiR-30c and miR-30c groups, respectively, compared with Scr controls (Fig 6c). Additionally, we measured apoB production. Plasma apoB100 and apoB48 (Fig 6d–e) were significantly lower and higher in miR-30c and antimiR-30c expressing mice, respectively, compared with Scr mice. These data indicate that miR-30c reduces production of triglyceride-rich apoB-containing lipoproteins.

Visual inspection of aortic arches showed less and more atherosclerotic plaques in miR-30c and antimiR-30c mice, respectively, compared with Scr (Fig 6f, Fig S8b). Hematoxylin/eosin staining of the cardiac/aortic junctions revealed that plaques were smaller in miR-30c group than in Scr and antimiR-30c groups (Fig 6g, Fig S8c). Macrophage staining at the cardiac/aortic junctions was higher in antimiR-30c group and lower in miR-30c group compared to Scr group (Fig 6h, S8d). Oil Red O staining of the whole aortas revealed

significantly reduced lesion areas in miR-30c compared with Scr (Fig 6i–j, Fig S8e–f). These studies indicate that miR-30c reduces atherosclerosis in *Apoe*$^{-/-}$ mice.

DISCUSSION

We have shown that hepatic over expression of miR-30c reduces MTP mRNA, protein and activity. Further, MTP mRNA is degraded faster due to the binding of miR-30c to its 3′-UTR. miR-30c lowers plasma cholesterol by reducing production of triglyceride-rich apoB-containing lipoproteins; a phenotype likely secondary to lower MTP expression. It also reduces *de novo* lipogenesis possibly by targeting other genes such as LPGAT1. Additionally, atherosclerotic plaques are smaller in *Apoe*$^{-/-}$ mice expressing miR-30c. Taken together, we provide evidence that high miR-30c levels reduce plasma lipids and atherosclerosis. Hence, our hypothesis is that hepatic over expression of miR-30c reduces lipid synthesis and lipoprotein production to lower plasma lipids and avoid steatosis by regulating different sets of genes (Fig S9).

Over expression of antimiR-30c had opposite effect than the expression of miR-30c suggesting that endogenous miR-30c might play a role in plasma and liver lipid homeostasis. Compared to heart, expression of miR-30c in the liver is low. But, in fact, there is significant expression of miR-30c in the liver and its expression is modulated by high fat diet and genetic perturbations. Trajkovski et al [17] showed that hepatic miR-30c levels were 1.3-fold higher in db/db and DIO mice compared to controls. In another study, Vickers et al [18] showed that miR-30c levels are higher than miR-33 in chow fed mice, and miR-30c levels are 3-fold higher than that of miR-33 in Western diet fed mice. This is a critical point since miR-33 antagomirs have significant effects on hepatic ABCA1 expression, plasma HDL and atherosclerosis [19–21]. Thus, there is ample evidence for the hepatic expression and regulation of miR-30c and increasing its expression might be relevant in controlling lipid metabolism.

The reductions in MTP protein in mouse liver were significantly higher than what is anticipated from changes in mRNA levels. Our studies in Huh-7 cells show that one mechanism for reductions in MTP mRNA involves posttranscriptional degradation. However, it is well known that miRs also interfere with protein translation [13, 15]. Hence, it is possible that miR-30c might also interfere with protein translation. More experiments are needed to address the role of miR-30c in MTP mRNA translation.

We have consistently seen that antimiR-30c increases apoB to a greater extent than is anticipated by the modest increases seen in MTP. It is possible that antimiR-30c reduces the expression of an unidentified protein that is perhaps involved in posttranslational degradation of apoB.

Our data suggest that miR-30c lowers plasma and hepatic lipids by targeting MTP and LPGAT1, respectively. To address the issue that VLDL production is due to targeting of MTP, we performed two sets of experiments. First, in the *L-MTP*$^{-/-}$ mice we observed that miR-30c does not reduce plasma cholesterol as it does in *Mttp*$^{f/f}$ mice and in wildtype mice. Second, we performed studies in Huh-7 cells. miR-30c reduces media apoB when MTP is

present, but not when MTP is reduced by siMTP. On the other hand, siLPGAT1 does not reduce apoB and miR-30c can reduce media apoB in siLPGAT1 treated cells. These studies show that miR-30c can reduce media apoB when MTP is present but not when MTP expression is reduced. We used similar approach to study the involvement of LPGAT1 in lipogenesis. While siLPGAT1 had no effect on media apoB, it reduced fatty acid synthesis. miR-30c did not reduce lipogenesis in siLPGAT1 treated cells but reduced media apoB. Thus, these studies indicate that MTP and LPGAT1 might be required for miR-30c to modulate media apoB and hepatic lipogenesis, respectively.

miR-30c reduced plasma cholesterol in wildtype and *Apoe*$^{-/-}$ mice. But, fasting plasma triglyceride levels were different in these mice; miR-30c had no effect in Western-diet fed wildtype mice but reduced plasma triglyceride in *Apoe*$^{-/-}$ mice. Nevertheless, miR-30c reduced triglyceride production in both wildtype and *Apoe*$^{-/-}$ mice. Therefore, the different effects of miR-30c on plasma triglycerides are most likely related to reduced lipoprotein lipase activity in the absence of apoE. Zsigmond et al [22] showed that heparin injection does not affect plasma triglyceride in *Apoe*$^{-/-}$ mice; unlike in wildtype mice where heparin significantly reduces plasma triglyceride. Purified lipoprotein lipase completely hydrolyzed lipoproteins from wildtype mice but not from *Apoe*$^{-/-}$ mice. They could see significant hydrolysis when lipoprotein lipase was incubated with lower amounts of lipoproteins from *Apoe*$^{-/-}$ mice. Based on these observations, they concluded that lipoprotein lipase activity is saturated in *Apoe*$^{-/-}$ mice and therefore is unable to hydrolyze lipoproteins as efficiently as in wildtype mice. Westerterp et al [23] have provided a different explanation. They advanced the idea that endogenous apoCI is sufficient to inhibit lipoprotein lipase activity in *Apoe*$^{-/-}$ mice. These studies clearly showed that lipoprotein lipase activity is diminished in *Apoe*$^{-/-}$ mice. Lower lipoprotein lipase activity might explain significant effects of miR-30c on plasma triglycerides in *Apoe*$^{-/-}$ mice but not in wildtype mice. Low lipase activity is expected to increase plasma triglyceride in Scr expressing mice.

miR-30 family was discovered during sequencing of short RNAs [24, 25]. Despite significant identity, distinct functions for different members have been identified. miR-30a plays a role in Xenopus kidney development [26]. miR-30e regulates apoptosis in breast tumor cells [27]. miR-30a and miR-30d stimulate adipogenesis [28]. miR-30c reduces adipocyte differentiation [29]. Thus, individual members appear to control different pathways. More experiments are needed to explain mechanisms that contribute to their specificity.

In short, these studies provide evidence that miR-30c reduces MTP transcript by binding to the 3′-UTR. Further, it reduces lipid synthesis and apoB-containing lipoprotein production by targeting different genes. Possibly, miR-30c coordinately decreases lipid biosynthesis and lipoprotein secretion to avoid steatosis and lower plasma lipids. Hence, higher levels of hepatic miR-30c might be anti-hyperlipidemic and anti-atherosclerotic and miR-30c mimics might have therapeutic potential.

METHODS

Methods and any associated references are available in the online version of the paper. Note: Supplementary information is available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

apoAI	apolipoprotein AI
apoB	apolipoprotein B
MTP	microsomal triglyceride transfer protein
miR	microRNA
Scr	scramble miR
UTR	untranslated region
ALT	alanine aminotransferase
AST	aspartate aminotransferase

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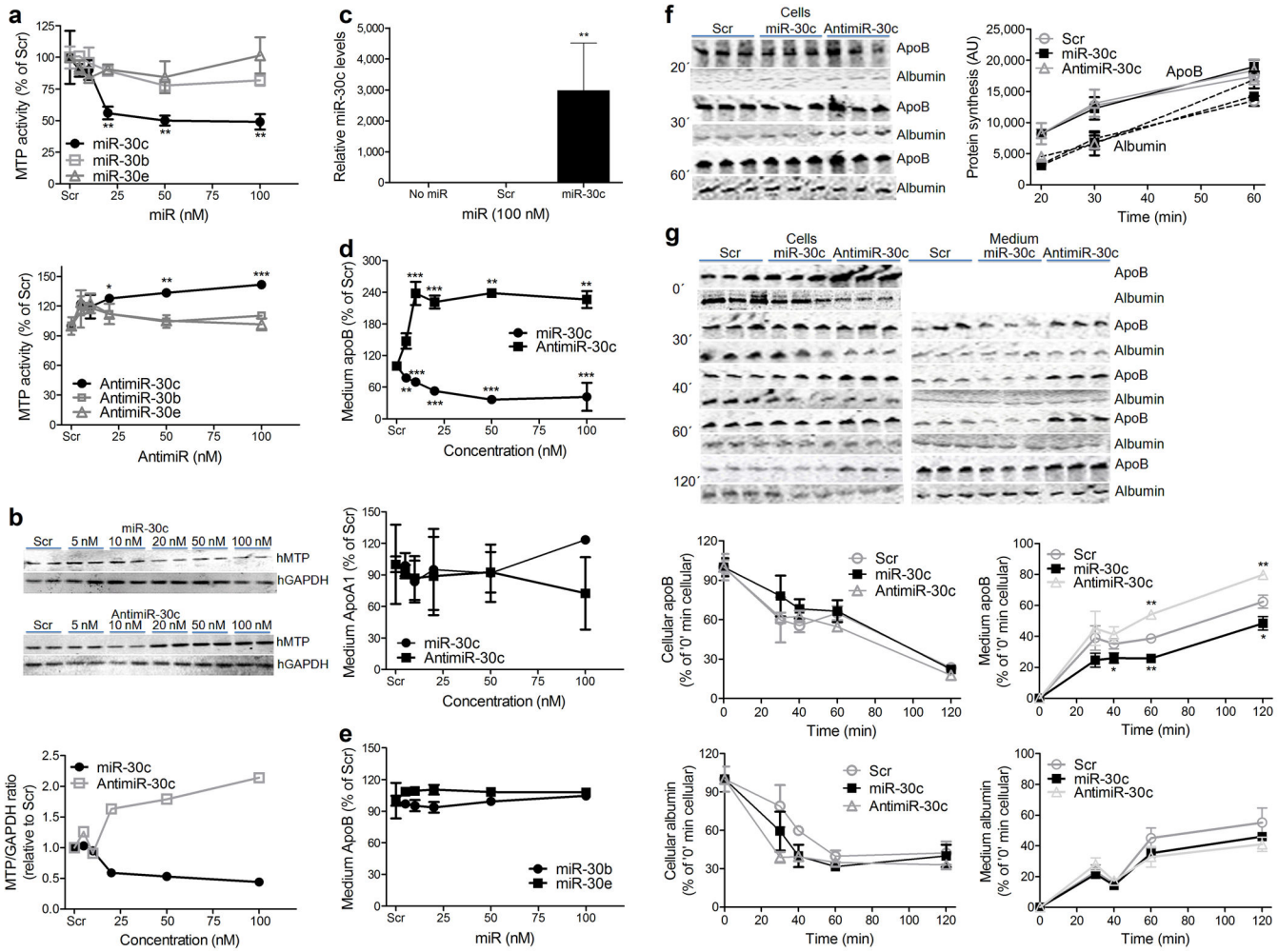

Fig 1. Effect of different miR-30s and antimiR-30s on MTP activity and apoB secretion

(**a**) Huh-7 cells (n=3) were transfected with different amounts of miRs (top) or antimiRs (bottom). For control, cells received a non-specific Scramble miR (Scr) at 100 nM. After 48 h, cells were used to measure MTP activity (% triglyceride transfer/mg/h). Values in Scr exposed cells were normalized to 100%. MTP activities in non-transfected and Scr transfected cells were similar.

(**b**) Huh-7 cells were transfected with different amounts of miR-30c or antimiR-30c in duplicate. After 48 h, MTP and GAPDH were detected by western blotting (top). Bands were quantified and average MTP/GAPDH ratios in Scr treated cells were normalized to 1 (bottom).

(**c**) Huh-7 cells transfected with Scr, miR-30c, or only Lipofectamine RNAiMAX (No miR) were used to measure miR-30c.

(**d**) Media from Huh-7 cells transfected with various concentrations of miR-30c, antimiR-30c or Scr [100 nM] was used to measure (ng/mg cell protein) apoB (top) and apoAI (bottom).

(**e**) Huh-7 cells were transfected with various concentrations of miR-30b, miR-30e or Scr [100 nM] as in (d) to measure media apoB.

Representative of 4 experiments. * p<0.05; ** p<0.01, *** p<.001, **** p<0.0001;
(**f**) Huh-7 cells transfected with different miRs were radiolabeled with ^{35}S-methionine, apoB and albumin were immunoprecipitated, separated on gels and exposed to phosphor imager screen (left) and quantified (right).
(**g**) Cells were labeled with ^{35}S-methionine for 1 h and then chased in methionine supplemented media. ApoB and albumin were immunoprecipitated. Cellular values in Scr, miR-30c and antimiR-30c at '0' time were normalized to 100% (n=3/group). Representative of 2 experiments.

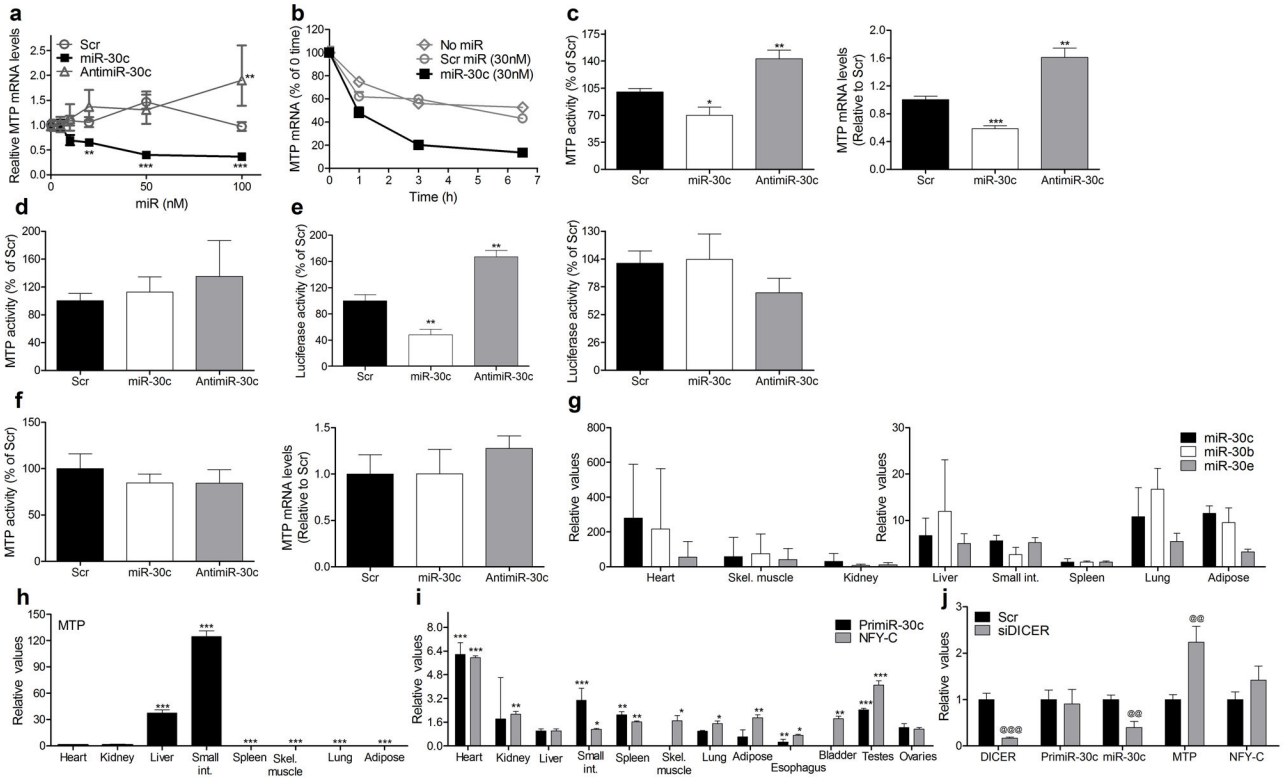

Fig 2. Regulation of MTP mRNA by miR-30c and expression of miR-30c in different tissues

(a) Huh-7 cells transfected with different miRs were used to quantify MTP mRNA. Values in cells not treated with miR were normalized to 1 and relative changes congruent with miR expression were plotted. Representative of 4 experiments.

(**b**) Huh-7 cells transfected (16 h) with indicated miRs were incubated with Actinomycin D (10 μg/ml). MTP/ARPp0 mRNA ratio at 0 h was adjusted to 100%. Representative of 2 experiments.

(**c**) COS-7 cells were transfected with 1.5 μg of pRc-hMTP, a plasmid expresses human MTP with its 3′-UTR under the control of CMV promoter. After overnight incubation, cells were distributed equally, and transfected with Scr [50 nM], miR-30c [20 nM] or antimiR-30c [50 nM]. Cells were harvested 17 h later to measure MTP activity and mRNA. Representative of 3 experiments.

(**d**) Binding of miR-30c to 3′-UTR is necessary for MTP mRNA degradation. MTP seed sequence in the 3′-UTR was mutated (from GTTTACA in wild type to GAAAACA) in pRc-hMTP and transfected in COS-7 cells. After 17 h, cells were distributed into 6-well plates and transfected with Scr miR [50 nM], miR-30c [20 nM] or anti-miR-30c [50 nM]. Cells were assayed for MTP activity after 17 h. Representative of 3 experiments.

(**e**) Normal (left) or mutated (right) 3′-UTR sequences of MTP were cloned after the stop codon of *Renilla* luciferase in psiCHECK2 plasmid that also expresses firefly luciferase, and introduced into COS-7 cells. After overnight incubations, cells were transferred to different wells and transfected with Scr [50 nM], miR-30c [20 nM], or anti-miR-30c [50 nM]. After 16 h, ratios of firefly and *Renilla* luciferase activities were normalized to 100% in Scr transfected cells. Representative of 3 experiments.

(**f**) MTP lacking its 3′-UTR does not respond to miR-30c. COS-7 cells were transfected with 5 μg of hMTP-FLAG plasmid that lacks its 3′-UTR. Next day, cells were distributed equally, and transfected with different miRs to measure MTP activity and mRNA. Representative of 3 experiments.

(**g**) miR-30c, miR-30b and miR-30e levels were assessed in human tissues by qRT-PCR and normalized to SNORD44. Values in kidney and spleen were adjusted to 1 in the left and right panels, respectively. Representative of 2 experiments.

(**h–i**) MTP, primiR-30, and NFY-C was measured by qRT-PCR and normalized to ARPp0. MTP (h) and primiR-30c/NFY-C (i) values in spleen and liver, respectively, were adjusted to 1. Representative of 2 experiments.

(**j**) Huh-7 cells were transfected with 20 nM siDICER. After 18 h, Dicer mRNA, primiR-30c, miR-30c, MTP and NFY-C mRNA were measured. ARPp0 and U6 served as control for mRNAs and miRs, respectively. Representative of 2 experiments. * $p<0.05$; ** $p<0.01$, *** $p<.001$, **** $p<0.0001$;

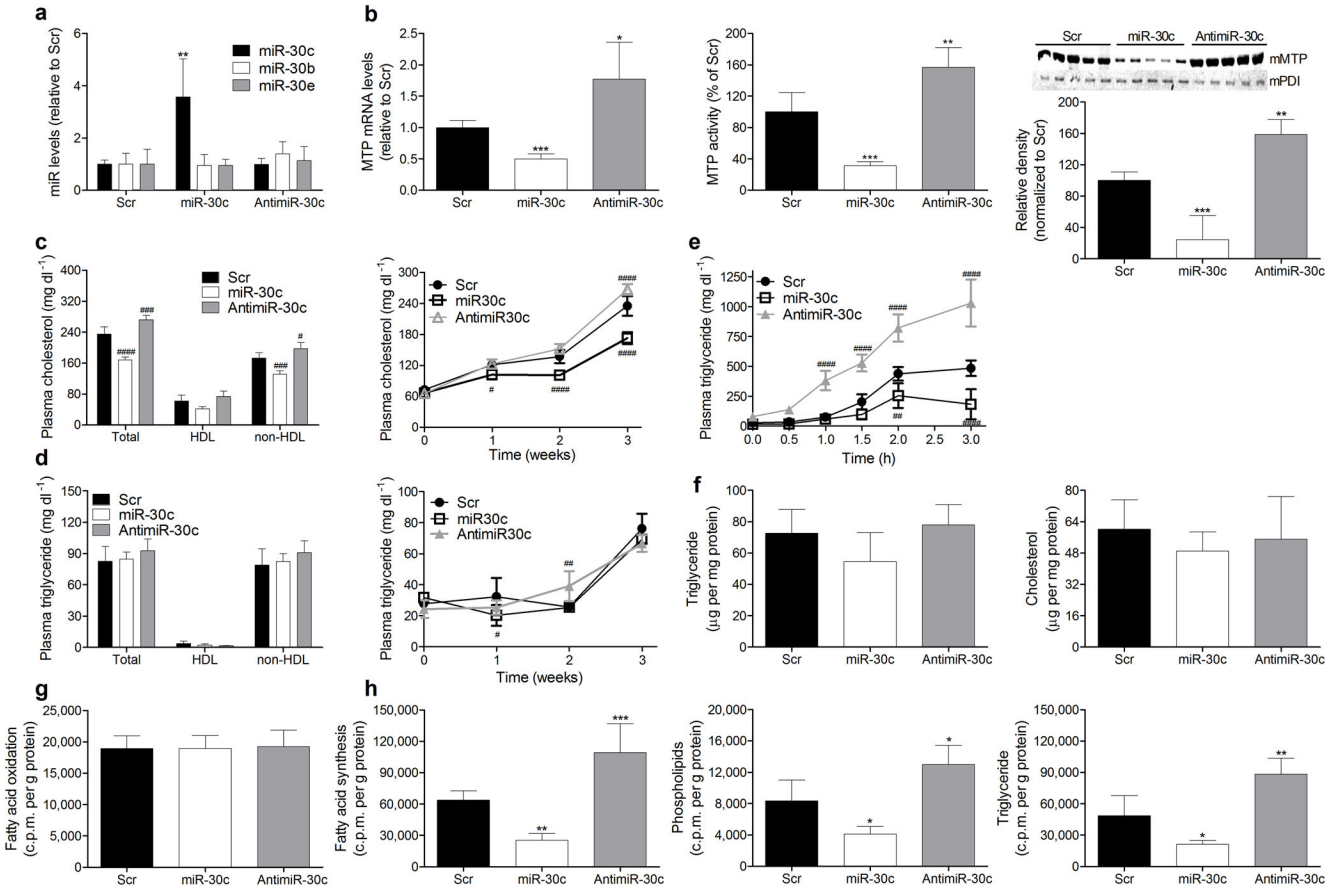

Fig 3. Mechanisms involved in the regulation of plasma and hepatic lipids by miR-30c

(**a–c, d–h**) Male C57/Bl6 mice (5/group; 8 weeks old) were injected (10^7 infectious units/mouse) retro-orbitally with lentiviruses expressing miR-30c, antimiR-30c or Scr and started on a Western diet. After 3 weeks, changes in hepatic miR-30c, miR-30b and miR-30e (a), MTP mRNA, activity and protein (b) were measured. In the right panel (b), bands are shown above the bar graphs that display quantification of mouse MTP (mMTP) normalized to mouse PDI (mPDI). Plasma collected at three weeks was used to measure cholesterol (c) and triglyceride (d) in total, HDL and non-HDL fractions. Plasma collected weekly was used to measure total plasma cholesterol and triglyceride. At 3 weeks, hepatic triglyceride and cholesterol (f) were measured. Additionally, liver slices were used to study β-oxidation (g), triglyceride and phospholipid synthesis (h). Representative of 3 experiments.

(**e**) In a separate experiment, C57/Bl6 mice (n = 5) were injected with different miRs. After week 5, mice were fasted overnight, injected with P407 and blood was collected to measure triglyceride. Slopes between one and 2 h were used to calculate triglyceride production rates. Data from 1 experiment. * p<0.05; ** p<0.01, *** p<.001, **** p<0.0001; significance calculated by one-way ANOVA.

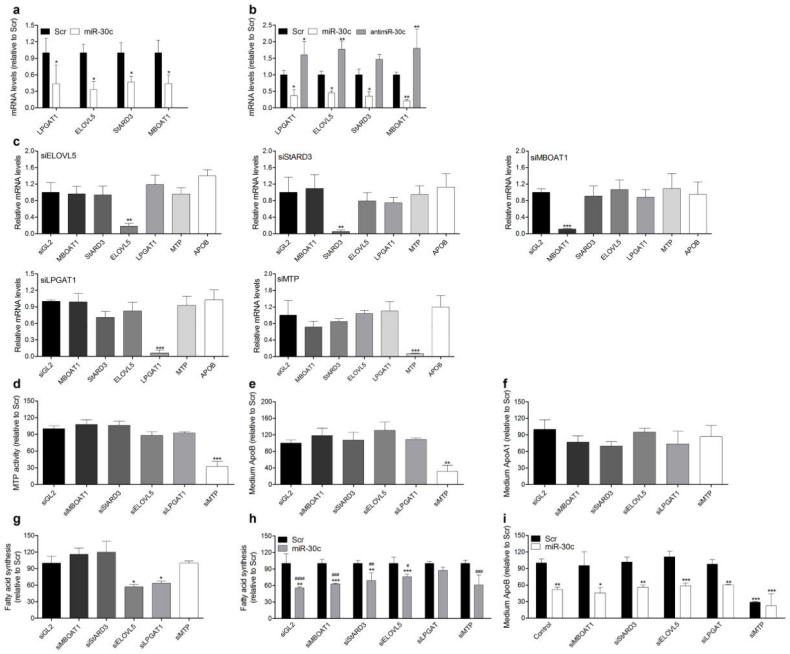

Fig 4. Regulation of hepatic lipid synthesis by miR-30c

(**a**) mRNA levels of various genes involved in lipid synthesis were measured in the livers of C57/Bl6 mice injected with different lentiviruses (n=5/group) and fed a Western diet for 3 weeks. Data representative 3 experiments.

(**b**) RNA from Huh-7 cells transfected with different miRs was isolated to quantify candidate genes and ARPp0 (endogenous control). All values are normalized to Scr group. Representative of 3 experiments.

(**c**) Specific siRNAs reduce their targets without affecting expression of other genes. mRNA levels were measured in cells transfected with 20 nM of different siRNAs. Values in siGL2 cells were normalized to 1. Representative of 3 experiments.

(**d–f**) Cells transfected with different siRNAs were used to measure MTP activity (d). Media were used to quantify apoB (e) and apoA1 (f). Representative of 2 experiments.

(**g**) *De novo* lipogenesis was measured in cells transfected with different siRNAs. Representative of 3 experiments.

(**h–i**) Cells co-transfected with indicated siRNA with either Scr or miR-30c were used to study fatty acid synthesis (h) or to measure apoB in the media (i). Representative of 2 experiments.

Comparisons with Scr control group are identified with *, whereas comparison between Scr and miR-30c are noted as #. * p<0.05; ** p<0.01, *** p<.001, **** p<0.0001;

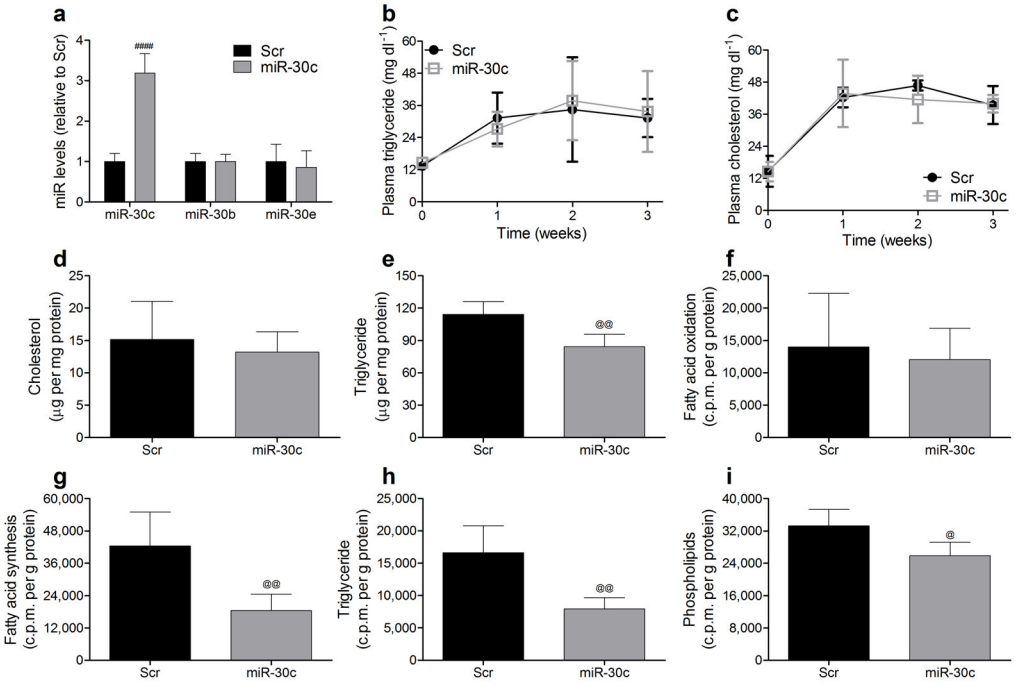

Fig 5. Effect of miR-30c in liver-specific MTP knockout mice

Male *L-MTP*$^{-/-}$ mice (5/group; 8 weeks old) were injected (10^7 infectious units/mouse) retro-orbitally with lentiviruses expressing miR-30c or Scr and started on a Western diet. After 3 weeks, changes in hepatic miRs livers were quantified (a). Plasma was used to measure triglyceride (b) and cholesterol (c). Livers were used to quantify cholesterol (d) and triglyceride (e). Additionally, liver slices were used to measure β-oxidation (f), *de novo* lipogenesis (g), and triglyceride (h) and phospholipid (i) synthesis. Representative of 2 experiments. * $p<0.05$; ** $p<0.01$, *** $p<.001$, **** $p<0.0001$;

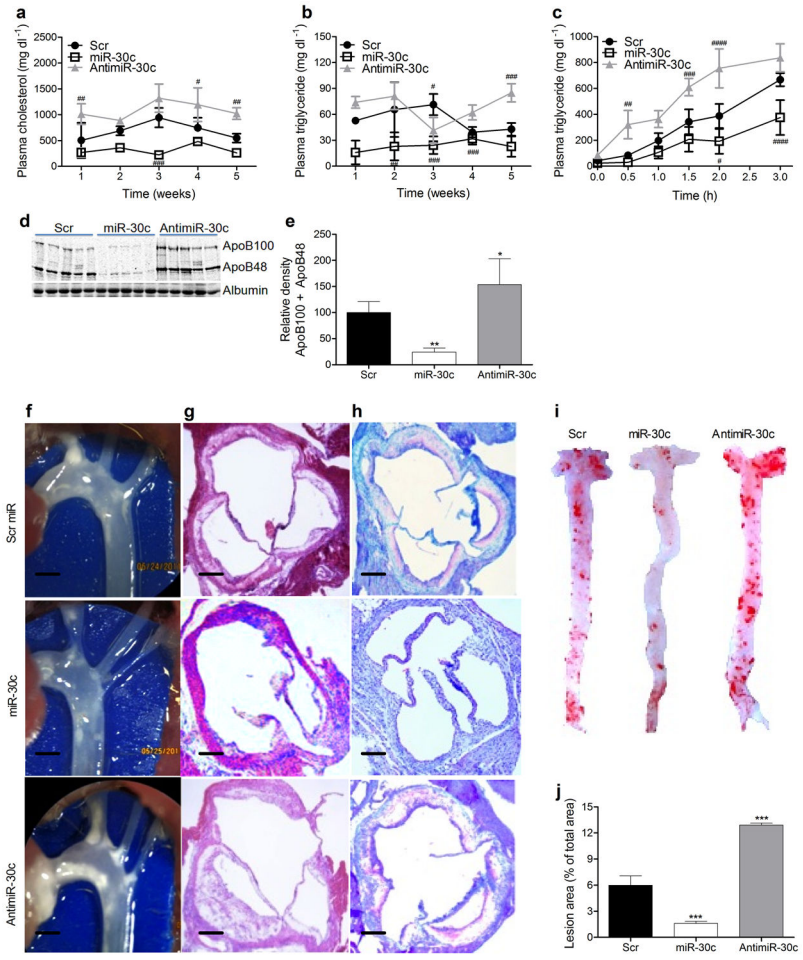

Fig 6. Effect of miR-30c on apoB production and atherosclerosis

(**a–b**) Female *Apoe*$^{-/-}$ mice (n=4/group) were injected with lentiviruses expressing miR-30c, antimiR-30c or Scr and started on a Western diet. Plasma was collected weekly to monitor changes in cholesterol (a) and triglyceride (b). Representative of 2 experiments.

(**c–e**) After 5 weeks of viral transductions, *Apoe*$^{-/-}$ mice (n=4) were fasted overnight and injected intraperitoneally with P407 and [^{35}S]Promix (0.3 μCi/mouse). Plasma was collected at different times to measure triglyceride (c). Plasma obtained at 2 h was used to immunoprecipitate apoB (d) and bands were quantified (e). Data from one study.

(**f**) Aortic arches were exposed and photographed.

(**g–h**) Aortic root sections were stained with Hematoxylin/eosin (g) or probed for macrophages (h). Scale represents 200 μm.

(**i–j**) Whole aortas (i) were isolated and stained with Oil Red O and quantified (j). * p<0.05; ** p<0.01, *** p<.001, **** p<0.0001;