

# Statins Stimulate Hepatic Glucose Production via the miR-183/96/182 Cluster

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1    **Statins are the most common pharmacologic intervention in hypercholesterolemic patients, and**  
2    **their use is recognized as a key medical advance leading to a 50% decrease in deaths from heart**  
3    **attack or stroke over the past 30 years. The atheroprotective outcomes of statins are largely**  
4    **attributable to the accelerated hepatic clearance of low-density lipoprotein (LDL)-cholesterol from**  
5    **circulation, following the induction of the LDL receptor. However, multiple studies suggest that**  
6    **these drugs exert additional LDL-independent effects. The molecular mechanisms behind these**  
7    **so-called pleiotropic effects of statins, either beneficial or undesired, remain largely unknown.**  
8    **Here we determined the coding transcriptome, miRNome, and RISCome of livers from mice dosed**  
9    **with saline or atorvastatin to define a novel in vivo epitranscriptional regulatory pathway that**  
10    **links statins to hepatic gluconeogenesis, via the SREBP2-miR-183/96/182-TCF7L2 axis. Notably,**  
11    **multiple genome-wide association studies identified *TCF7L2* (transcription factor 7 like 2) as a**  
12    **candidate gene for type 2 diabetes, independent of ethnicity. Conclusion: our data reveal an**  
13    **unexpected link between cholesterol and glucose metabolism, provides a mechanistic**  
14    **explanation to the elevated risk of diabetes recently observed in patients taking statins, and**  
15    **identifies the miR-183/96/182 cluster as an attractive pharmacological candidate to modulate non-**  
16    **canonical effects of statins.**

17  
18  
19        Statins are competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase  
20    (HMGCR), the rate-limiting enzyme in the cholesterol biosynthetic pathway (1). The ensuing decrease in  
21    intracellular sterol levels triggers the proteolytic maturation of the sterol regulatory element-binding  
22    protein 2 (SREBP2), which orchestrates a transcriptional program that increases both *de novo* synthesis  
23    and uptake of cholesterol. Among the targets of SREBP2, the LDLR is critical in mediating the reduction  
24    in plasma LDL-cholesterol which reduces cardiovascular risk in patients taking statins (2). Meta-analyses  
25    of epidemiological data and studies in animal models and cells have demonstrated LDL-cholesterol  
26    independent effects of statins, both beneficial (improved endothelial function, decreased vascular

27 inflammation, reduced thrombogenic response) and undesired (myalgia, liver damage, memory loss) (3).  
28 Importantly, statin therapy is modestly, but significantly, associated dose-dependently with increased  
29 new-onset diabetes (reviewed in (4, 5)). Indeed, in 2012 the Federal Drug Administration mandated a  
30 modification of the safety label of statins to warn of increased HbA1c and/or fasting plasma glucose. This  
31 correlation might be the result of prescription bias, *i.e.* patients who are at increased risk of developing  
32 diabetes are also more prone to dyslipidemias and, consequently, are prescribed statins more frequently;  
33 however, different laboratories demonstrated decreased pancreatic  $\beta$ -cell insulin secretion (6), peripheral  
34 insulin sensitivity (7, 8), and skeletal muscle mitochondrial function (9, 10) following treatment with statins  
35 *in vitro* and/or *in vivo*, thus suggesting that statins *per se* do indeed impact glucose homeostasis. The  
36 role of statins on hepatic glucose production, however, has never been examined.

37         MicroRNAs (miRs) are small, noncoding 20-22 nt RNAs that bind to specific, partially  
38 complementary sequences in the 3' untranslated region (UTR) of target mRNAs and recruit them to the  
39 RNA-induced silencing complex (RISC) to promote their cleavage and/or translational repression (11).  
40 Over the last two decades, miRNAs have been recognized as key regulators of multiple  
41 (patho)physiological processes as their expression can be tissue-, developmental-, and disease-specific  
42 (12). In the context of cholesterol metabolism, we and others reported on miR-33a (reviewed in (13)): an  
43 intragenic miRNA encoded within intron 16 of SREBP2 that fine-tunes the expression of several  
44 transmembrane sterol transporters involved in high-density lipoprotein (HDL) lipidation (14-17), bile  
45 metabolism (18), and hepatic vesicular secretion (19). Since SREBP2 transactivates its own promoter,  
46 miR-33 expression is increased following treatment with statins in cells and *in vivo*. Jeon *et al.* reported  
47 that SREBP2 also induces the expression of the miR-183/96/182 cluster and that miR-96 and -182, in  
48 turn, target *FBXW7* and *INSIG2* thus providing feed-back regulation for SREBP2 expression and  
49 maturation (20). These studies relied on bioinformatic predictions of target genes and exogenous  
50 overexpression/inhibition of the microRNA to infer function. However, whether statins actually promote  
51 changes in the recruitment of specific mRNAs to the RISC *in vivo* has never been tested.

52

## 53    **EXPERIMENTAL PROCEDURES**

54    **Animal studies.** C57BL/6 mice were obtained from NCI, housed in Optimice racks (Animal Care  
55    Systems), maintained on a 12h/12h light/dark cycle with unlimited access to food (PicoLab 5353) and  
56    water, and bred in the SPF animal facility at Saint Louis University. All mice used for *in vivo* experiments  
57    were male at 10–12 weeks of age. Animals used for isolation of primary hepatocytes were male or  
58    female. Where indicated, mice were gavaged 100  $\mu$ L saline or 10 mg/kg atorvastatin (Cayman  
59    Chemicals) once a day for ten days, and on the day of sacrifice once at 8 am and fasted for 6 h prior to  
60    tissue collection. For overexpression experiments, animals were infused via tail vein with empty or miR-  
61    183/96/182 adenoviral vectors ( $2 \times 10^9$  pfu); pyruvate challenges and tissue collection were done 10- and  
62    12-days post-infusion, respectively. For antisense experiments, animals were gavaged daily with saline  
63    or 10 mg/Kg atorvastatin as above for 6 weeks, then kept on drug while injected *i.p.* on days 1, 2, 3, 9,  
64    and 12 with 100  $\mu$ L saline, scrambled, anti-miR-96, anti-miR-182, or anti-miR-183 oligonucleotides (5  
65    mg/kg) or combined anti-miR-96, -182, and -183 oligonucleotides (1.7 mg/Kg each). Pyruvate challenges  
66    were done on day 15 at 5 am after removing food at 6 pm the day before. Mice in the antisense  
67    experiments were not fasted before collection of blood and livers. For pyruvate challenges, baseline  
68    blood glucose was taken from tail snipping using a glucometer (Bayer Contour), then mice were injected  
69    *i.p.* with 2 g/kg pyruvate, as described (21), and sequential glucose measurements were taken 15, 30,  
70    and 60 min after pyruvate injection. All animals received humane care according to the criteria outlined in  
71    the “Guide for the Care and Use of Laboratory Animals” (NIH publication 86-23 revised 1985), and all  
72    studies were approved by the IACUC at Saint Louis University.

73

74    **MicroRNA vectors.** Precursor miRNAs for miR-96, -182, and -183 containing 140 bp upstream and 140  
75    bp downstream of the mature miRNA were amplified from mouse genomic DNA using Platinum Pfx  
76    polymerase (Invitrogen), and cloned into pCMV-miR (Origene). To generate replication-deficient  
77    adenovirus, a 4,100 bp containing the whole miR cluster (from 140 bp upstream of miR-183 to 140 bp  
78    downstream of miR182) was amplified from mouse genomic DNA as above and cloned into pAdTrack-

79 CMV; the vector was then recombined with pAdEasy1, and transfected into Hek293Ad cells to generate  
80 high titers of replication-deficient adenovirus, as described (22).

81

82 **Anti-miR oligonucleotides.** 15-mer locked nucleic acid anti-ctrl (5'- TCCTAGAAAGAGTAGA), anti-miR-  
83 96 (5'- ATGTGCTAGTGCCAA), anti-miR-182 (5'-TTCTACCATTGCCAA), anti-miR-183 (5'-  
84 TTCTACCAGTGCCAT) oligonucleotides were a kind gift from Miragen Therapeutics Inc. Antimirs were  
85 resuspended in sterile saline and stored at -20°C until used.

86

87 **Primary hepatocytes.** Cells were isolated from 8–10-week-old, male C57BL/6 mice fed chow, using  
88 Perfusion and Digest buffers (Invitrogen). Cells were resuspended in William's E Medium (Invitrogen)  
89 supplemented with Plating Supplements (Invitrogen), plated in 12- or 6-well BioCoat Collagen I plates  
90 (BD), and incubated at 37°C and 5% CO<sub>2</sub> for 6 h. Then, the media was switched to William's E  
91 supplemented with Maintenance Supplements (Invitrogen). Where indicated, cells were cultured in media  
92 supplemented with 1 μmol/L atorvastatin or pravastatin, 5 μmol/L simvastatin (Cayman), or 10 nmol/L  
93 siRNA-control or siRNA-Tcf7l2 (Dharmacon D-001810 and L-058892, respectively). Glucose production  
94 assays were performed as described (23). Briefly, cells were incubated 6 h in 1 mL glucose production  
95 buffer (glucose-free DMEM without phenol red supplemented with 20 mmol/L sodium lactate and 2  
96 mmol/L sodium pyruvate. Supernatants were collected and used to measure glucose with a colorimetric  
97 kit (Wako), and data were normalized to intracellular protein.

98

99 **Luciferase reporter assays.** HEK293 cells were transiently transfected with luciferase reporters and  
100 miR expression plasmids in triplicate in 24-well plates using the calcium phosphate method. Cell extracts  
101 were prepared 48 h later, and luciferase activity measured using the Luciferase Assay System  
102 (Promega). Data were normalized to β-galactosidase activity to correct for small changes in transfection  
103 efficiency.

104

105 **RNA analysis.** RNA was extracted from mouse livers and cells using Trizol (Invitrogen). RISC-  
106 associated RNA was immunoprecipitated from liver homogenates using an anti-AGO2 antibody (Wako,  
107 clone 2D4) and extracted as described (24). Bar-coded libraries for small and polyA-enriched large  
108 RNAs, and for RISC-associated RNAs were prepared for livers from saline (n=8) and atorvastatin (n=8)  
109 treated mice using Illumina kits, and transcriptional profiling by deep sequencing were analyzed as  
110 described (24, 25). For coding transcriptome, data from Saline-4 and Atorvastatin-2 libraries were  
111 discarded due to poor sequencing. For miRNome, all 16 libraries produced good quality data. For  
112 RISCome, libraries for Saline-1, -3, -4, -5, and 6, and for Atorvastatin-3, -4, -5, and -7 produced quality  
113 data. Annotated sequencing data are available in Supplemental Files 1–3. Predicted miRNA–mRNAs  
114 pairs/networks were evaluated with miRTarVis, as described (26). For all other experiments, cDNAs  
115 were generated from 1 µg of DNase1-treated RNA using Superscript III (Invitrogen) and random  
116 hexamers. Quantitative real-time PCR was done with Power SybrGreen reagent (Applied Biosystems),  
117 using a LightCycler-480 (Roche). Primer sets are available upon request. Values were calculated using  
118 the comparative  $\Delta\Delta C_t$  method and normalized to ribosomal *Rplp0*. Targeted miRNA expression was  
119 analyzed using miRCURY LNA reagents and assays (Exiqon) and normalized to U6.

121 **Protein analysis.** Protein were extracted from cells or livers using HEPES buffer (20 mmol/L HEPES pH  
122 7.4, 150 mmol/L NaCl, 0.2 mmol/L EDTA, 2 mmol/L  $MgCl_2$ , 1% Triton X-100, 10% glycerol) containing  
123 protease inhibitor cocktail (Pierce). Forty micrograms of protein were resolved in 4–12% polyacrylamide  
124 gels, transferred to PVDF membranes, and probed with antibodies for TCF7L2 (1:1,000 dilution, Cell  
125 Signaling C48H11), PCK1 (1:1,000 dilution; Abcam ab70358), G6PC (1:1,000 dilution; Novus NBP1-  
126 80533) and  $\beta$ -ACTIN (1:5,000 dilution; SCBT sc-130656) in TBS-Tween20 containing 4% non-fat dry  
127 milk. Immune complexes were detected with horseradish peroxidase-conjugated anti-rabbit secondary  
128 antibodies (1:5,000 dilution; Bio-Rad 1706515).

## 131 RESULTS

132 In an effort to understand the functional changes in the hepatic transcriptome that follow  
133 treatment with statins, we dosed C57BL/6 mice with saline or atorvastatin, and use RNA-seq to define  
134 total mRNAs (coding transcriptome), mRNAs associated with the RISC (RISCome), and miRNAs  
135 (miRNome) (Fig. 1a). The complete annotated sequencing data are available in Supplementary Files 1–  
136 3. Fig. 1b shows that ~10% of the coding transcriptome changed in response to the drug. As expected, at  
137 least 15 canonical targets of SREBP2 such as *Hmgcr*, squalene synthase (*Fdft1*), lanosterol synthase  
138 (*Lss*), 7- and 24-dehydrocholesterol reductases (*Dhcr7*, *Dhcr24*), proprotein convertase subtilisin/kexin  
139 type 9 (*Pcsk9*), and LDL-receptor (*Ldlr*) were induced in the livers of atorvastatin-treated mice, compared  
140 to saline (Supplementary File 1). Data in Figs. 1c and 1d, and in Supplementary Files 2 and 3 show that  
141 62 miRNAs and 1,504 RISC-associated mRNAs were differentially regulated by the statin. Our ultimate  
142 goal, however, was to identify transcripts that were both reciprocally regulated in total transcriptome vs.  
143 RISCome, and predicted as targets of miRNAs differentially regulated in the miRNome. Analysis of the  
144 three sets of data revealed several miR/mRNA pairs that fit those criteria (Fig. 1e and Supplemental Data  
145 Fig. 1). In the rest of this report, we focus on the miR-183/96/182 polycistronic cluster, because all 3  
146 miRNAs were upregulated in the livers of mice treated with atorvastatin, compared to saline (Fig. 1c),  
147 and 9 predicted targets for one or more miRNAs in that cluster were both RISC-enriched and  
148 transcriptome-depleted in the same livers (Fig. 1e). These targets are *Bicd2* (bicaudal D homolog 2),  
149 *Mkl2* (myocardin-like 2), *Nox4* (NADPH oxidase 4), *Pik3r1* (phosphoinositide-3-kinase regulatory subunit  
150 1), *Pxmp4* (peroxisomal membrane protein 4), *Sfxn5* (sideroflexin 5), *Slc22a23* (solute carrier family 22  
151 member 23), *Tcf7l2* (transcription factor 7 like 2), and *Tmem189* (transmembrane protein 189). Data in  
152 Figs. 2a and 2b show the relative abundance of miR-183, -96, and -182 in the livers of saline-treated  
153 mice, and the fold-induction following treatment with saline. We also highlight miR-33 in the same panels,  
154 as we and others have previously described this miRNA as regulated by statins. Data in Fig. 2c show the  
155 transcriptome and RISCome scores for the nine potential targets defined above. Importantly, both the  
156 induction of the miR cluster and the repression of the nine putative targets in response to statins were



157 validated by RT-qPCR in a second cohort of mice (data not shown) and in mouse primary hepatocytes  
158 (Fig. 2d). Collectively, data in Figs. 1 and 2 demonstrate that miR-183/96/182 is an *in vivo* hepatic sensor  
159 of exposure to statins, and that these miRNAs actively recruit specific mRNA targets to the RISC in  
160 response to statins.

161 In agreement with a report by Jeon *et al.* (20), we mapped two SREBP-responsive elements  
162 (SRE) upstream of the transcription start for the miR-183/96/182 cluster (Supplemental Data Fig. 2a).  
163 The proximal element is evolutionarily conserved between mice and humans, while the distal one is  
164 divergent (Supplemental Data Fig. 2b). Nevertheless, both sequences in the human and mouse  
165 promoters confer responsiveness to SREBP2 (Supplemental Data Fig. 2c, d).

166 Of the nine transcripts that originally fit our criteria (Figs. 1e and 2c), we decided to focus on  
167 *Tcf7l2*, as it has been identified as a key transcriptional regulator of hepatic gluconeogenesis, and  
168 polymorphisms in this locus have been associated with diabetes in several GWAS (27-31). We  
169 hypothesized that statins stimulate hyperglycemia, at least in some patients, by increasing hepatic  
170 glucose output via miR-183/96/182–TCF7L2. The miRNA target prediction algorithm Targetscan (32)  
171 identifies conserved sequences in the 3' UTR of *TCF7L2* that are partially complementary to miR-183, -  
172 96, -or 182 (Fig. 3a). These sequences, or the entire 3' UTR of mouse or human *TCF7L2*, were cloned  
173 downstream of a luciferase reporter and transiently transfected into HEK293 cells in the presence or  
174 absence of a plasmid encoding each miR. Data from these experiments in Fig. 3b show that each  
175 isolated conserved sequence (but not sequence 1 in human *TCF7L2*, which is not conserved in mouse),  
176 as well as the whole 3' UTR, were able to confer responsiveness to miR-183, -96, and -182. Additionally,  
177 point mutations in each predicted sequence to prevent interaction with the seed sequence of each  
178 miRNA abrogated the response (Supplemental Data Fig. 3). Importantly, data in Fig. 3c using anti-miR  
179 oligonucleotides demonstrate that in mouse primary hepatocytes the miR-183/96/182 cluster mediates  
180 the repressive effect of atorvastatin on *Tcf7l2*. Together, data in Fig. 3 identify conserved sequences in  
181 *TCF7L2* as functional statin and miR-183/96/182 responsive elements.

182 We next tested the ability of statins, the miR cluster, and TCF7L2 to modulate glucose production  
183 in mouse primary hepatocytes. Data in Fig. 4 show that gluconeogenesis was increased in cells  
184 incubated with either atorvastatin, simvastatin, or pravastatin, compared to vehicle (Fig. 4a); in cells  
185 overexpressing miR-183/96/182 (via adenoviral transduction), compared to empty vector (Fig. 4b); and in  
186 cells where *Tcf7l2* was silenced using siRNA, compared to control oligonucleotides (Fig. 4c). Consistent  
187 with these data from cultured hepatocytes, the amounts of the key gluconeogenic enzymes  
188 phosphoenolpyruvate kinase 1 (PCK1) and glucose-6-phosphatase, catalytic subunit (G6PC) increased  
189 in the livers dosed with atorvastatin, compared to saline, and were inversely correlated to those of  
190 TCF7L2 (Fig. 4d). Collectively, these data demonstrate that statins, the miR cluster, and TCF7L2 are  
191 critical regulators of the gluconeogenic pathway in cultured hepatocytes.

192 To test the functional consequences of miR-183/96/182 overexpression on hepatic glucose  
193 production *in vivo*, we transduced mice with empty adenovirus or adenovirus encoding the miR cluster.  
194 After 10 days, animals were fasted overnight and then injected *i.p.* with pyruvate early in the morning.  
195 Hepatic gluconeogenic capacity was estimated from the conversion of pyruvate to glucose and its  
196 release into circulation, which was monitored with a standard glucometer. Data in Fig. 5a and  
197 Supplemental Data Fig. 4 show that, in two independent experiments, mice overexpressing the miR  
198 cluster were able to sustain increased glycemia in response to the pyruvate challenge, compared to  
199 control animals. These data demonstrate increased gluconeogenic capacity in the former mice. Animals  
200 were allowed to recover after the pyruvate test for 48 h, and then some were fasted overnight, and some  
201 allowed access to food. In agreement with the pyruvate challenge results, both mRNA (Fig. 5b and  
202 Supplemental Data Fig. 4b) and protein (Fig. 5c) panels show that miR overexpression in the liver  
203 resulted in the decrease of TCF7L2 and the concomitant increase in PCK1 and G6PC. These results are  
204 consistent with the previously reported suppressive effect of TCF7L2 on the gluconeogenic pathway via  
205 negative regulation of *PCK1* and *G6PC* expression (see discussion below). Interestingly, these changes  
206 were much more apparent in the livers of fed mice, and indeed PCK1 and G6PC proteins were as  
207 robustly expressed (if not more) in the livers of fed miR-overexpressing mice as in fasted control livers

208 (Fig. 5c). This latter observation suggests that the miR-183/96/182–TCF7L2 pathway can overcome the  
209 canonical nutritional regulation of these gluconeogenic genes.

210 Finally, we tested whether therapeutic silencing of each miR (or of all three) could revert the  
211 atorvastatin-induced increase in hepatic gluconeogenesis. Mice were randomized for daily gavage with  
212 saline or atorvastatin during 6 weeks, and then kept on treatment while dosed with one or more anti-miR  
213 oligonucleotides on days 1, 2, 3, 9 and 12 (see Methods for details). Data in Fig. 5d show that the statin  
214 (or statin plus scrambled anti-miR), raised the expression of all three miRNAs in liver, and that the anti-  
215 miRs were specific in silencing the expression of each cognate miR. Interestingly, silencing miR-96  
216 modestly reduced the expression of miR-183, and vice-versa. This is consistent with reports in other  
217 multi-cistronic miRNAs where altering the expression of one miR affects the levels of the others in the  
218 cluster. Nonetheless, *Tcf7l2* mRNA (Fig. 5d) and protein (Fig. 5e) levels were efficiently reduced in the  
219 livers of mice dosed with the statin, or statin plus scrambled anti-miR. Importantly, silencing miR-96 or  
220 miR-183, but not miR-182, negated the statin-induced reduction in *Tcf7l2* both at the mRNA (Fig. 5d) and  
221 protein (Fig. 5e) level. Silencing all three miRNAs did not provide further increase in TCF7L2 abundance.  
222 Supplemental Data Fig. 5a show that the expression of *G6pc* in the same livers largely opposed that of  
223 *Tcf7l2*: both gluconeogenic transcripts were induced by the statin, and treatment with anti-miRs  
224 abrogated the statin-dependent induction. Together, these data demonstrate that the miR-183/96/182  
225 cluster is the *in vivo* mediator of the statin-induced regulation of hepatic TCF7L2 and gluconeogenic  
226 enzymes. Interestingly, anti-miR182 was able to reduce the expression of *G6pc* (Supplemental Data Fig.  
227 5a), despite having no effect on *Tcf7l2* levels (Fig. 5c, d). No miR-182 binding sites are predicted in  
228 murine or human *G6PC*. Thus, the mechanism by which miR-182 modulates *G6pc* expression  
229 independent of *Tcf7l2* remains unknown. Supplemental Data Fig. 5a also shows results for the other 8  
230 transcripts initially identified in this study: data demonstrate that the miR-183/96/182 cluster does indeed  
231 control the liver expression of *Sfxn5*, *Tmem189*, *Slc22a23*, *Nox4*, and *Mkl2*; however, no changes in the  
232 mRNA amounts of *Pi3kr1*, *Pxmp4*, and *Bicd2* were noted. The reasons for the lack of response to  
233 atorvastatin of these latter genes are unclear, since both transcripts clearly were regulated in other *in*

234 *vivo* (Fig. 1) and in culture (Fig. 2d) experiments. It is possible that the nutritional status of the mice  
235 (animals were not fasted in Fig. 5d–f and Supplemental Data Fig. 5, as opposed to those in Figs. 1 and  
236 2) may contribute additional regulatory mechanisms, beyond SREBP2, for these genes. Nonetheless,  
237 Fig. 5f shows data from the same animals two days before sacrifice, when they were challenged with  
238 pyruvate to measure *in vivo* gluconeogenesis. Consistent with the data in Fig. 5a from miR-  
239 overexpressing mice, hepatic glucose production was induced in mice dosed with atorvastatin (or  
240 atorvastatin plus scrambled anti-miR), compared to animals receiving saline; importantly, treatment with  
241 anti-miRs abrogated the effect of the statin and returned glycemic kinetics to control levels. These latter  
242 results upon pyruvate challenge were reproducible in a second cohort of mice treated as above  
243 (Supplemental Data Fig. 5b). Collectively, data in Fig. 5 demonstrate that statins promote hepatic  
244 gluconeogenesis via miR-183/96/182.

245

246

## 247 **DISCUSSION**

248 The cardiovascular therapeutic benefits of statins are presumed to outweigh their diabetogenic effect.  
249 Yet, it is critically important to establish the molecular mechanisms behind this undesired side effect. This  
250 is especially important when considering preventive prescription to younger dyslipidemic patients who  
251 will presumably be taking statins for several decades. Clinical and *in vitro* studies showed that statins  
252 impair both pancreatic insulin secretion and peripheral insulin sensitivity (4, 5). Ours is the first report  
253 demonstrating a direct effect of statins on hepatic glucose production. We show that the SREBP2–miR-  
254 183/96/182–TCF7L2 axis controls the expression of gluconeogenic enzymes, and increases glucose  
255 production both in cultured hepatocytes and in mice (Fig. 6). Additionally, we show that therapeutic  
256 silencing of the miR cluster normalizes *Tcf7l2* expression and reverts statin-stimulated hepatic glucose  
257 production. TCF7L2 is a member of the Wnt signaling pathway (33), and modulates the transcription of  
258 target genes by interacting not only with  $\beta$ -catenin (its canonical partner), but also with the key metabolic  
259 transcription factors FOXO1 (34) and HNF4 $\alpha$  (35). The exact role of TCF7L2 on glucose metabolism has

260 been controversial for some time (36), and authors suggested that TCF7L2 acts as a transcriptional  
261 activator or repressor depending on its partner. In addition, at least 13 different transcripts and 2 different  
262 sized proteins are expressed from *TCF7L2*, and it is unclear whether these isoforms differ in their trans-  
263 activating/repressing properties. Four different *Tcf7l2*<sup>-/-</sup> models have been generated, all with perinatal  
264 lethality(34, 37-39). Oh *et al.* showed that *Tcf7l2*<sup>+/-</sup> mice display decreased glucose tolerance and insulin  
265 sensitivity, and exhibited increased hepatic expression of gluconeogenic genes (34). In contrast, Savic *et*  
266 *al.*(38) and Boj *et al.*(39) reported improved glycemic control using two other heterozygote mouse models  
267 and a BAC transgenic mouse, but did not report on hepatic glucose production. The reasons for the  
268 discrepancies among these different genetic models remain unknown. More recently, a liver-specific  
269 transgenic mouse expressing a dominant negative form of TCF7L2 showed progressive impairment in  
270 response to pyruvate challenge in the absence of hepatic insulin intolerance (40). Collectively, these  
271 studies show that TCF7L2 activity modulates hepatic and peripheral glucose metabolism, and whole-  
272 body glycemic control. In regards to gluconeogenesis, the literature strongly suggests that TCF7L2  
273 reduces hepatic gluconeogenesis likely by decreasing the transcriptional activity of positive regulators of  
274 *PCK1* and *G6PC* (34-36, 41, 42).

275 Interestingly, *TCF7L2* transcription is stimulated postprandially by insulin (41). One key  
276 observation in our studies is that the induction miR-183/96/182 overrides the canonical nutritional control  
277 of the gluconeogenic program (on during fasting, off postprandially). As a consequence, we demonstrate  
278 that both the mRNA and protein amounts of both PCK1 and G6PC are similar in fed miR-overexpressing  
279 livers than in fasted control livers. This has particularly worrisome implications when considering patients  
280 taking statins for extended periods of time, whom presumably will have persistently elevated expression  
281 of the miR cluster: our results suggest that these patients may have sustained activation of the  
282 gluconeogenic pathway. In an otherwise healthy individual, this accelerated glucose delivery from liver  
283 into circulation should trigger homeostatic insulin-dependent clearance in peripheral tissues to maintain  
284 euglycemia. However, it is tempting to speculate that the sustained unregulated hepatic glucose  
285 production in response to statins may over time elevate blood HbA1c and fasting glucose, and ultimately

286 contribute to new-onset diabetes in “vulnerable” patients. Meta-analyses of clinical trials and population-  
287 based studies revealed statin therapy increases the risk of new-onset diabetes up to 43%. However, why  
288 only some, but not all, patients taking statins develop diabetes remains a mystery. Additional research is  
289 necessary to determine whether these “vulnerable” patients, who are prone to develop statin-induced  
290 diabetes, have polymorphisms in the *miR-183/96/182* or *TCF7L2* loci that make them more sensitive  
291 (*i.e.*, hyper-responsive) to statins.

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403

## 404 **FIGURE LEGENDS**

405

406 **Figure 1. Atorvastatin-dependent changes in hepatic transcriptome, miRNome, and RISCome. a,**  
407 Experimental design. Mice were gavaged daily with saline or 10 mg/Kg atorvastatin. See Methods for  
408 details on RNA analysis. **b,** Summary of changes in total coding transcriptome in atorvastatin ( $n=7$ ) vs.  
409 saline ( $n=7$ ). **c,** Summary of changes in miRNome ( $n=8$ /group). **d,** Summary of changes in RISCome  
410 ( $n=5$ -saline;  $n=4$ -ATR). **e,** Node-link diagram of miRTarVis-predicted miRNA–mRNA networks changing  
411 in atorvastatin vs. saline; upregulated transcripts in red, downregulated transcripts in blue. See  
412 Supplemental Files 1–3 for complete annotated sequencing data and analysis.

413

414 **Figure 2. Treatment with statins induces the recruitment of miR-183/96/182 targets to the RISC. a,**  
415 Relative abundance of mature miR-183, -96, and -182 in the hepatic miRNome of saline-treated mice.  
416 For comparison, the previously reported statin-regulated miR-33 is shown in black. **b,** Relative fold-  
417 induction of mature miR-183, -96, and -182 in response to atorvastatin. **c,** Nine putative miR-183/96/182  
418 targets identified in Fig. 1e were simultaneously depleted in total transcriptome and enriched in the  
419 RISCome of mice dosed with atorvastatin. **d,** Validation by qPCR of changes in hepatic miR-183/96/182  
420 and their putative targets in a second cohort of mice ( $n=5$ ) dosed with saline or atorvastatin, as in Figs. 1.  
421 and 2a–c. Canonical SREBP2 targets (*Hmgcr*, *Ldlr*, *Pcsk9*, and *miR-33* are shown as positive controls).  
422 **e,** Validation of changes in miR-183/96/182 and their putative targets in mouse primary hepatocytes  
423 cultured 24 h in media supplemented with 1  $\mu$ mol/L atorvastatin (ator) or pravastatin (pra), or 5  $\mu$ mol/L  
424 simvastatin (smv) ( $n=3$ /group). Canonical SREBP2 targets *Hmgcr* and *miR-33* are shown as positive

controls. Dotted line represents normalized expression in dms0-treated cells. \* $p < 0.05$ , \*\* $p < 0.01$ , compared to dms0.

427

**Figure 3. Functional miR-183/96/182-responsive elements in *TCF7L2*.** **a**, Conserved sequences in the 3'UTR of *TCF7L2* with partial complementarity to miR-183/96/182 are color-coded as elements 1, 2, and 3. Distance from the stop codon are indicated. Alignments for mouse and human sequences are shown. Solid lines represent Watson-Crick complementarity; colon marks represent divergence between mouse and human sequences. **b**, Normalized luciferase activity in extracts from HEK293 cells transiently transfected with a reporter fused to the potential miR-responsive elements identified above or the whole 3'UTR, and expression vectors for each miR. As negative control, we used the empty pGL3Promoter vector. Data are mean  $\pm$  SE of 3 independent experiments in quadruplicate. \*\* $p < 0.01$ . **c**, miR-183/96/182 mediate atorvastatin-induced repression of *Tcf7l2*. Mouse primary hepatocytes were transfected with scrambled or anti-miR oligonucleotides, and 24 h later switched to media supplemented with vehicle or atorvastatin for 24 h. Data are mean  $\pm$  SE of 2 independent experiments in quadruplicate. \* $p < 0.05$ , compared to dms0;  $^{++}p < 0.01$ , compared to atorvastatin plus anti-scr.

440

**Figure 4. Statins, miR-183/96/182, and *TCF7L2* modulate hepatic gluconeogenesis.** **a**, Glucose production in mouse primary hepatocytes incubated in media supplemented with vehicle, 1  $\mu\text{mol/L}$  atorvastatin, 5  $\mu\text{mol/L}$  simvastatin, or 1  $\mu\text{mol/L}$  pravastatin. **b**, Glucose production in mouse primary hepatocytes after transduction with empty adenovirus or adenovirus encoding the miR-183/96/182 cluster. **c**, Glucose production in mouse primary hepatocytes after transfection with siRNA-control or siRNA-*Tcf7l2* oligonucleotides. **d**, Hepatic protein abundance of *TCF7L2* and gluconeogenic enzymes PCK1 and G6PC in mice dosed with saline or 10 mg/Kg/d atorvastatin for 6 weeks. Data in panels a–c are mean  $\pm$  SE of at least 2 independent experiments in quadruplicate. \*\* $p < 0.01$

449

**Figure 5. Therapeutic silencing of the miR-183/96/182 cluster abrogates statin-induced hepatic gluconeogenesis.** **a**, Blood glucose curves in response to pyruvate challenge (see Methods) show increased hepatic glucose output in mice transduced with adenovirus encoding the miR cluster, compared to empty adenovirus. Data are mean  $\pm$  SE;  $n=10$ /group;  $**p<0.01$ . **b**, Hepatic protein levels for TCF7L2, PCK1, and G6PC two days after the pyruvate challenge, in mice allowed access to food, or fasted overnight. Data are mean  $\pm$  SE;  $n=5$ /group;  $*p<0.05$ ,  $**p<0.01$ , compared to fed mice;  $^{\dagger}p<0.05$ ,  $^{\dagger\dagger}p<0.01$ , compared to empty adenovirus. **c**, Immunoblots of hepatic protein extracts in the same mice as (b). **d**, Mice were gavaged with saline or 10 mg/Kg/d atorvastatin daily for 6 weeks, then treated with control or anti-miR oligonucleotides, alone or combined, for 15 days (see Methods). Atorvastatin induces the hepatic expression of the 3 miRs and reduces that of *Tcf7l2*. Anti-miRs are specific in silencing the expression of their cognate miR. Silencing miR-96 or -183 abolished atorvastatin-induced repression of *Tcf7l2*. Data are mean  $\pm$  SE;  $n=6$ /group;  $*p<0.05$ ,  $**p<0.01$ , compared to saline;  $^{\dagger}p<0.05$ ,  $^{\dagger\dagger}p<0.01$  compared to atorvastatin plus anti-ctrl. **e**, Changes in TCF7L2 protein levels parallel those in mRNA. PCSK9 was used as positive control to monitor the efficacy of the statin. **f**, Hepatic gluconeogenic capacity in response to a pyruvate challenge was tested in the 4 groups shown, confirming the functional role of the miR cluster as the mediator to statin-induced hepatic glucose production. Area under the curve represented in arbitrary units. Data are mean  $\pm$  SE;  $n=6$ /group;  $*p<0.05$ ,  $**p<0.01$ , compared to saline;  $^{\dagger\dagger}p<0.01$ , compared to atorvastatin plus anti-ctrl.

**Figure 6. The SREBP-2–miR-183/96/182–TCF7L2 modulates hepatic glucose output.** During episodes of low-intracellular cholesterol, such as in response to statins, the expression of miR-183/96/182 is induced, which in turns reduces the levels of gluconeogenic repressor TCF7L2, ultimately promoting accelerated hepatic glucose production.

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481

482

483 **AUTHOR CONTRIBUTIONS**

484 T.J.M. and Á.B. conceived the idea, designed experiments, and wrote the manuscript. T.J.M. and R.M.A.  
485 performed experiments, and collected and analyzed data. M.R.C. analyzed data. G.W.D. and S.J.M.  
486 supervised library construction, sequencing, and bioinformatics analysis.

## Figure 1

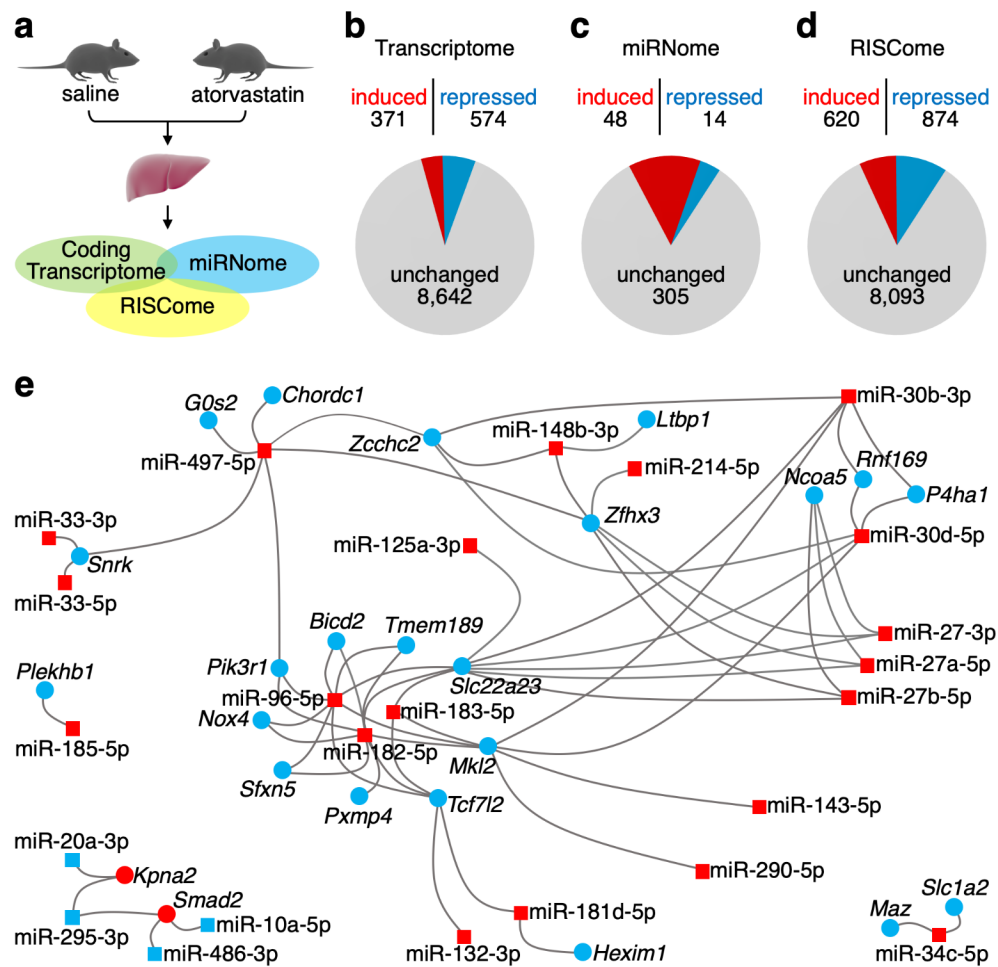


Figure 2

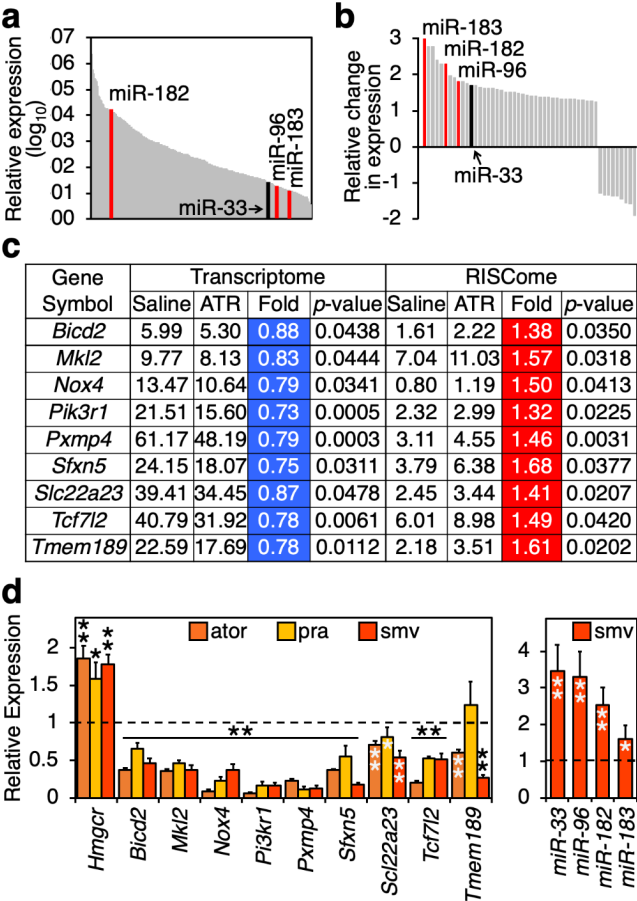


Figure 3

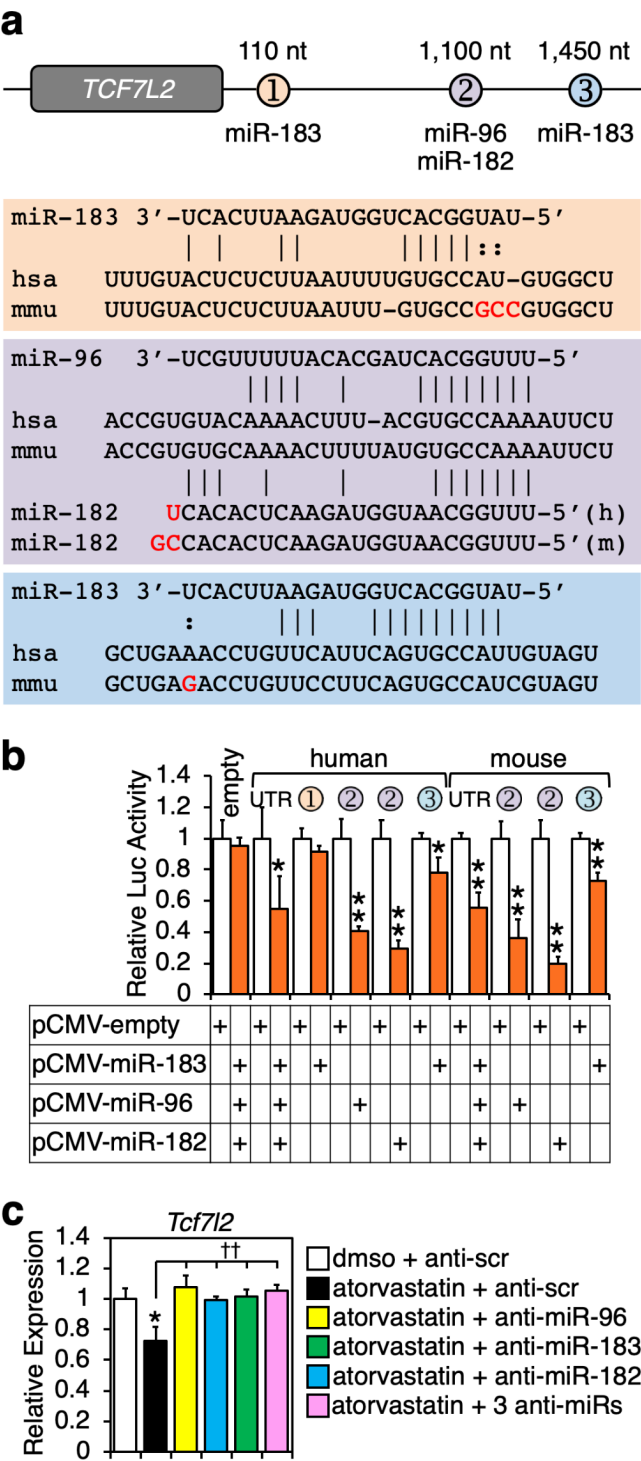
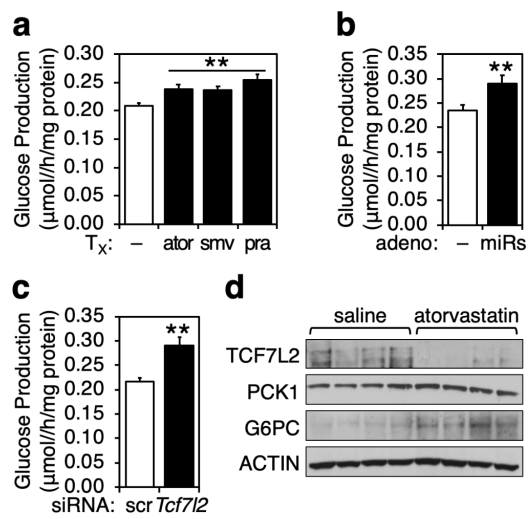
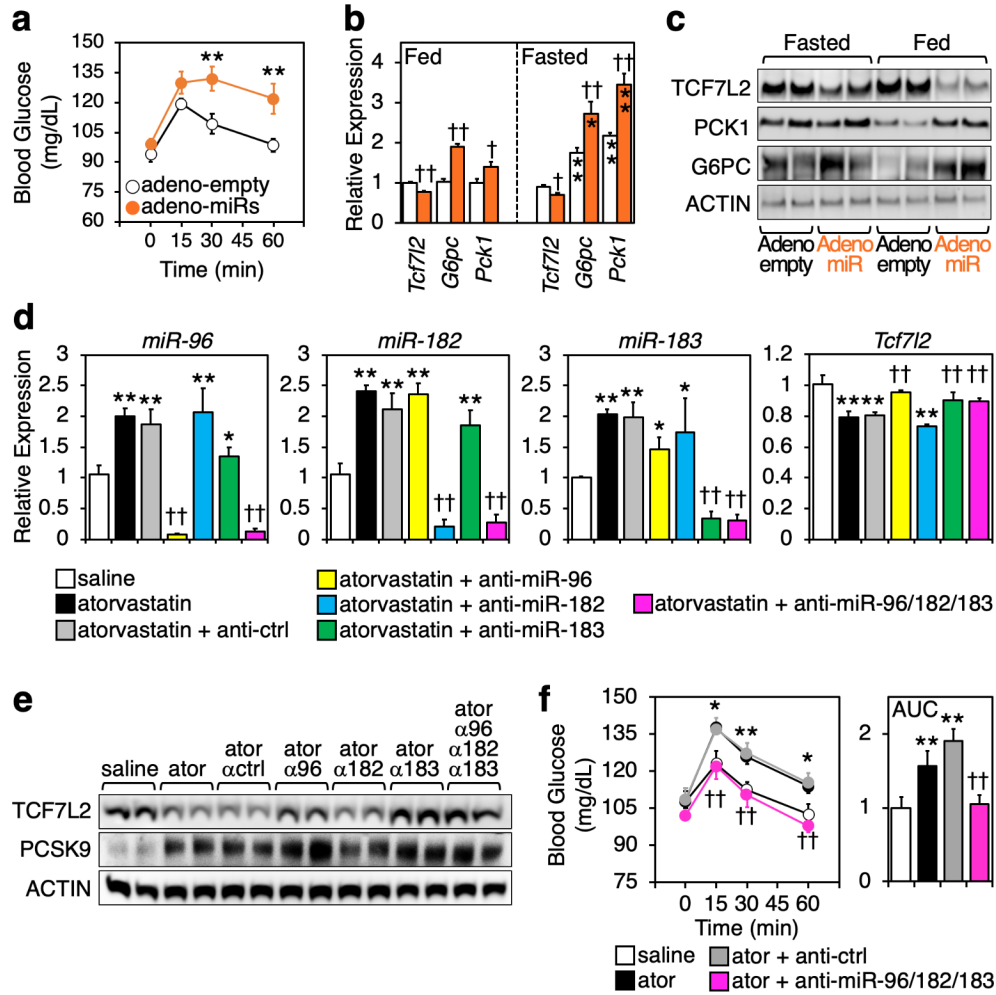


Figure 4





**Figure 5**



**Figure 6**

