Control of glucose homeostasis and insulin sensitivity by the *Let-7* family of microRNAs

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Diabetes mellitus is the most common metabolic disorder worldwide and a major risk factor for cardiovascular disease. MicroRNAs are negative regulators of gene expression that have been implicated in many biological processes, including metabolism. Here we show that the *Let-7* family of microRNAs regulates glucose metabolism in multiple organs. Global and pancreas-specific overexpression of *Let-7* in mice resulted in impaired glucose tolerance and reduced glucose-induced pancreatic insulin secretion. Mice overexpressing *Let-7* also had decreased fat mass and body weight, as well as reduced body size. Global knockdown of the *Let-7* family with an antimiR was sufficient to prevent and treat impaired glucose tolerance in mice with diet-induced obesity, at least in part by improving insulin sensitivity in liver and muscle. AntimiR treatment of mice on a high-fat diet also resulted in increased lean and muscle mass, but not increased fat mass, and prevented ectopic fat deposition in the liver. These findings demonstrate that *Let-7* regulates multiple aspects of glucose metabolism and suggest antimiR-induced *Let-7* knockdown as a potential treatment for type 2 diabetes mellitus. Furthermore, our *Cre*-inducible *Let-7*-transgenic mice provide a unique model for studying tissue-specific aspects of body growth and type 2 diabetes.

metabolic syndrome | insulin receptor | insulin receptor substrate 2 | β-cell

According to a World Health Organization estimate, diabetes mellitus affects 346 million people worldwide (1), and its prevalence continues to increase due to advancing age, inactivity, and increasing obesity rates (2). In industrialized countries, diabetes mellitus is the leading cause of blindness, renal failure, and lower limb amputations and is a major risk factor for cardiovascular disease. Diabetes mellitus encompasses a group of etiologically different metabolic diseases characterized by high blood glucose levels. Type 1 diabetes results in elevated blood glucose levels due to a lack of insulin-producing pancreatic β-cells. Type 2 diabetes (T2D), the predominant form of diabetes mellitus, is often part of the so-called "metabolic syndrome," which describes the co-occurrence of elevated blood pressure, dyslipoproteinemia, and obesity (2).

Patients with T2D have elevated blood glucose levels due to decreased insulin activity (i.e., insulin resistance) in peripheral organs, such as liver and muscle, with a consequent reduction in glucose uptake in these organs. Concomitantly, pancreatic insulin production/secretion is often reduced and is insufficient to meet the higher demand in T2D patients. Indeed, a number of lines of evidence, including genome-wide association studies, suggest dysfunction of pancreatic β-cells as a major pathogenic mechanism in T2D (3). T2D is often preceded by impaired glucose tolerance, which is characterized by abnormally increased postprandial blood glucose levels. Although fasting blood glucose levels often are only mildly elevated, impaired glucose tolerance is associated with increased cardiovascular mortality (4).

T2D can be initially treated with drugs that increase pancreatic insulin secretion and/or insulin sensitivity of peripheral tissues. Eventually, however, many patients require treatment with insulin to sufficiently control blood glucose levels. Novel therapies are needed to normalize pancreatic β-cell function and insulin sensitivity of peripheral tissues in patients with T2D.

MicroRNAs (miRNAs) are small, non–protein-coding RNAs that negatively regulate gene expression by promoting degradation and/or inhibiting translation of target mRNAs. MiRNAs have been shown to play important roles during development and disease, including T2D (5–7); for example, *miR-375* regulates pancreatic insulin secretion (8), and *miR-103* and *miR-107* influence insulin sensitivity in peripheral tissues (9).

Let-7, one of the first miRNAs discovered, was initially shown to control developmental timing in *Caenorhabditis elegans* (10), but recently has been implicated in cancer and pluripotency as well (11). In mice, 12 genes encode members of the *Let-7* family (Fig. S1), which includes nine slightly different miRNAs [*Let-7a*, *Let-c*, *Let-7f* (all encoded by two genes), and *Let-7b*, *Let-7d*, *Let-7e*, *Let-7g*, *Let-7i*, and *miR-98* (all encoded by one gene)]. All *Let-7* family members are believed to exert similar functions because they share a common seed region (nucleotides 2–8), which mediates miRNA interaction with target mRNAs. Processing of *Let-7* can be inhibited by LIN28, an RNA-binding protein that is highly expressed in embryonic stem cells (12). Interestingly, ectopic global LIN28a overexpression in mice was found to result in increased body size and crown–rump length, as well as increased glucose metabolism and insulin sensitivity (13). However, *Lin28a* transgenic mice showed down-regulation (up to 50%) of *Let-7* expression only in some adult organs, and whether the observed effects were mediated by *Let-7* is unclear. While the present paper was in preparation, Zhu et al. (14) reported that global overexpression of *Let-7g* in mice resulted in growth retardation and impaired glucose tolerance. Extrapolating from data in mice with muscle-specific gain and loss of function of Lin28, the authors concluded that *Let-7* regulates blood glucose levels at least in part via regulation of insulin signaling in muscle (14).

To study the function of *Let-7* in mammals, we generated transgenic mice with *Cre*-inducible activation of *Let-7a*, *Let-7d*, and *Let-7f* expression, which enabled us to overexpress *Let-7* in a tissue-specific manner. Mice with global overexpression of *Let-7* were viable, but had reduced body size and weight. Furthermore, *Let-7* transgenic mice exhibited impaired glucose tolerance due to diminished glucose-induced insulin secretion. To test whether global knockdown of the *Let-7* family was sufficient to prevent or treat impaired glucose tolerance in mice, we developed a locked nucleic acid (LNA)-modified antimiR that could inhibit *Let-7* function. We found that antimiR-induced knockdown of *Let-7* improved blood glucose levels and insulin resistance in obese mice. We conclude that *Let-7* regulates glucose metabolism in multiple organs, and that antimiR-induced knockdown of *Let-7* could provide an approach to treating T2D.

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Results and Discussion

***Let-7* Regulates Adult Body Weight and Size.** Five of 16 (31%) loci that have been associated with body length in humans have been identified as predicted *Let-7* targets (15). Among these are confirmed *Let-7* target genes such as high-mobility group AT-hook2 (*Hmga2*) (16), cyclin-dependent kinase 6 (*Cdk6*) (17), DOT1-like histone H3 methyltransferase (*Dot1l*) (18), and *Lin28* (19). To study whether *Let-7* is sufficient to regulate body size, we sought to globally overexpress *Let-7* in mice. Because *Let-7a*, *Let-7d*, and *Let-f* (all of which are encoded by a single genomic cluster on chromosome 13) are among the most highly expressed *Let-7* family members in some organs, we used this genomic cluster to generate transgenic mice with *Cre*-inducible expression of *Let-7* (Fig. 1*A*). To achieve global overexpression of *Let-7*, we crossed transgenic mice carrying the inactive transgene construct (*Let-7* Tg OFF) with transgenic mice expressing CAG-*Cre*. Subsequent removal of the CAG-*Cre* transgene by backcrossing to C57BL/6 mice produced *Let-7* Tg ON mice. We performed Northern blot analysis to detect *Let-7* expression and observed overexpression of the members of the transgenic *Let-7* cluster in all tissues analyzed (Fig. 1*B*). The level of overexpression varied among tissues, with relatively stronger overexpression in organs with weaker endogenous *Let-7* expression, such as liver and muscle.

Let-7 Tg OFF mice did not demonstrate an overt phenotype and had comparable body weight to WT littermates (Fig. 1*C*). Transgenic mice globally expressing *Let-7* (*Let-7* Tg ON) were viable and exhibited only a mildly reduced fertility rate with smaller litter sizes; however, *Let-7* Tg ON mice were significantly smaller than their WT littermates (Fig. 1*D*). We observed this phenotype with 100% penetrance despite the mixed genetic background of the transgenic mice. The body weight of *Let-7* Tg ON mice was similar to that of WT mice in the first 2 wk after birth, but then increased slower than the rate seen in WT mice and was further diminished in adult mice older than age 10 wk (Fig. 1*E*). Nose-to-anus lengths of adult *Let-7* Tg ON mice overexpressing *Let-7a*, *Let-7d*, and *Let-f* were ~8% less than WT littermates at age 8 wk and 14 wk (Fig. 1*F*). Given that Zhu et al. (14) also found a growth-inhibiting effect of global overexpression of *Let-7g*, we assume that most, if not all, *Let-7* family members have a similar effect on adult body size.

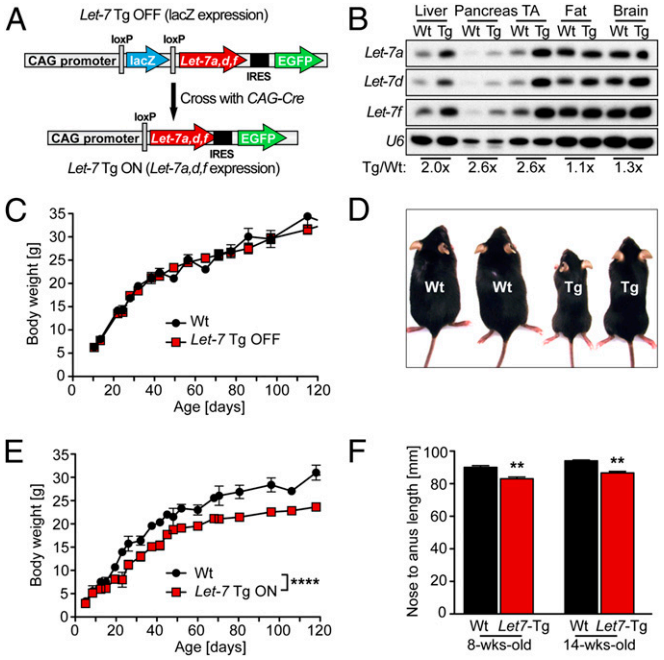

Fig. 1. Global *Let-7* overexpression results in reduced body weight and body size. (*A*) *Let-7* transgene. The "inactive" construct (*Let-7* Tg OFF) expresses lacZ globally under the control of the CAG promoter. After *Cre* mediated excision of the lacZ gene, the transgene expresses the chromosome 13 *Let-7* cluster comprising *Let-7a*, *Let-7d*, and *Let-7f*. Global overexpression of *Let-7* (*Let-7* Tg ON) was achieved by crossing the *Let-7* Tg OFF mice to CAG-*Cre* transgenic mice. *Let-7* Tg ON mice were then back-crossed to C57BL/6 mice to obtain *Let-7* Tg ON mice without the CAG-*Cre* transgene. Because of an internal ribosome entry site (IRES), *Let-7* Tg ON mice should also express GFP; however, the expression level was too low to enable detection by fluorescence microscopy. (*B*) Total RNA was isolated from multiple organs of 4-wk-old WT (Wt) and *Let-7* Tg ON mice and used for Northern blot analysis to detect *Let-7a*, *Let-7d*, and *Let-7f* expression. Detection of U6 was used as a loading control. The ratios of *Let-7* expression in Tg vs. WT organs are shown beneath each pair of lanes. (*C*) Body weight of WT mice vs. *Let-7* Tg OFF littermates at different ages. Values are mean ± SEM; n = 3–9 males per time point. (*D*) Two WT and two *Let-7* Tg ON male littermates at age 6 wk. (*E*) Body weight of WT mice vs. *Let-7* Tg ON littermates. Values are mean ± SEM; n = 3–14 males for each time point. $^{****}P < 0.0001$ for WT vs. *Let-7* Tg ON by ANOVA with Bonferroni posttest. (*F*) Reduced body length in *Let-7* Tg ON mice; n = 3 males per time point. $^{**}P < 0.01$ for WT vs. Tg by ANOVA with Bonferroni posttest.

***Let-7* Transgenic Mice Have Reduced Fat Mass.** Analysis of isolated individual organs from 6-wk-old *Let-7* Tg ON mice revealed that most organs were reduced in weight (or size) relative to WT littermates (Fig. 2*A*). Only liver and spleen were not reduced in weight, suggesting that *Let-7* does not control the size of these organs. One reason for the reduced organ size could be that transgenic overexpression of *Let-7* reduces proliferation and enhances differentiation of progenitor cell populations, which might express only low amounts of endogenous *Let-7*. Interestingly, body weight reductions seemed to be more pronounced than tibia or nose-to-anus length reductions in transgenic mice. This was most likely due to a disproportionate reduction in fat mass in the *Let-7* Tg ON mice, as measured by the weight of epididymal fat (Fig. 2*A*). Therefore, we performed body composition measurements using EchoMRI (Echo Medical Systems) and found that fat mass did not increase with age in the *Let-7* Tg ON mice as it did in their WT littermates (Fig. 2*B*). When fat mass was normalized to body weight, we found a 15% ± 4% lower fat mass in *Let-7* Tg ON animals vs. WT littermates at age 6 wk, a 32% ± 6% lower fat mass at age 8 wk, and a 60% ± 5% lower fat mass at age 14 wk. Lean mass was also lower in *Let-7* Tg ON mice, but it was proportionate to the decreased body size and exhibited normal age-dependent increases (Fig. 2*C*).

Given the important role of the hypothalamus in regulating body size and metabolism, we sought to generate transgenic mice with CNS-enriched *Let-7* overexpression. Toward this end, we crossed *Let-7* Tg OFF mice with transgenic mice expressing *Cre* under control of the *Nestin* promoter. *Let-7* Tg/*Nestin-Cre* double-transgenic mice showed a similar reduction in body length as

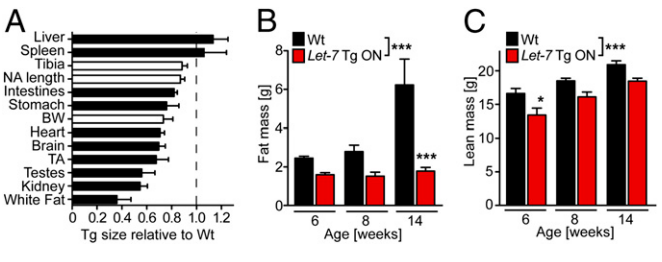

Fig. 2. Reduced fat mass in *Let-7* transgenic mice. (*A*) Relative organ weight or length [for tibia, nose-to-anus (NA) length, and intestine] of globally *Let-7*–expressing Tg mice vs. WT mice; n = 3. BW, body weight; TA, tibialis anterior; white fat, epididymal fat. (*B* and *C*) Body composition was measured in WT mice and *Let-7* -Tg ON littermates at age 6, 8, and 14 wk by EchoMRI; n = 3 or 4 for each time point. $^{*}P < 0.05$; $^{***}P < 0.001$ for WT vs. *Let-7* Tg ON by ANOVA with Bonferroni posttest.

that resulting from global *Let-7* overexpression at age 6 wk (Fig. S2). This might suggest that *Let-7* controls body size predominantly via regulation of neurohormones. Interestingly, *Let-7* Tg/*Nestin-Cre* double-transgenic mice exhibited only an attenuated reduction in body weight (Fig. S2), which suggests that fat mass was not affected, and that reductions in body size and fat mass by *Let-7* are independent events.

***Let-7* Transgenic Mice Exhibit Impaired Glucose Tolerance.** Given that changes in body composition can be caused by alterations in glucose metabolism, we investigated whether *Let-7* expression is regulated in high-fat diet (HFD)-induced obesity, a well-characterized model of obesity-induced glucose intolerance. We assessed *Let-7* expression by Northern blot analysis in multiple organs from mice on an HFD vs. mice on standard chow and found no major regulation of *Let-7* expression in the investigated organs (Fig. S3). However, we cannot exclude the possibility that cellular subpopulations such as pancreatic β-cells display altered *Let-7* expression on HFD. To study potential effects of global *Let-7* overexpression on glucose metabolism, we performed glucose tolerance tests (GTTs) in 8-wk-old *Let-7* Tg ON mice and WT littermates. Glucose stimulation produced abnormally elevated blood glucose levels in the *Let-7* Tg ON mice (Fig. 3*A*). Glucose levels in these mice were highest 30 min after glucose stimulation and decreased slowly thereafter, suggesting impaired glucose-induced pancreatic insulin secretion and/or insulin resistance of peripheral tissues. We observed this effect with 100% penetrance despite the mixed genetic background of the transgenic mice.

To assess insulin sensitivity of peripheral tissues, we performed insulin tolerance tests (ITTs) in 8-wk-old fed transgenic

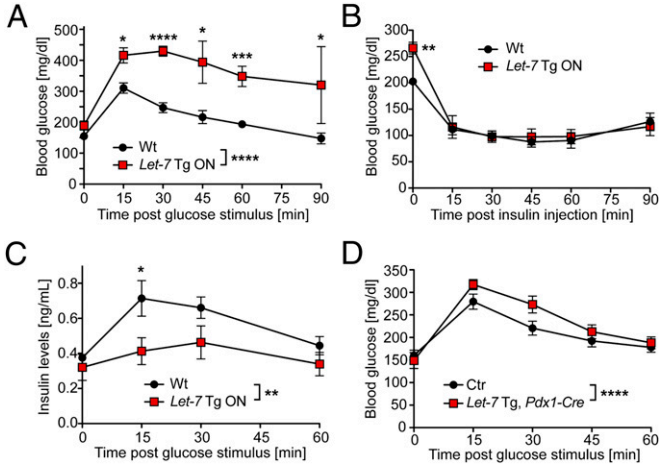

Fig. 3. Impaired glucose tolerance in *Let-7* transgenic mice. (*A*) A GTT was performed in 7-wk-old WT mice and *Let-7* Tg ON littermates after a 7-h fast. Glucose levels were determined at baseline and at the indicated times after an i.p. glucose stimulus (1.5 mg/g body weight); $n = 7$ (3 for 45 min and 90 min). $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$ for WT vs. *Let-7* Tg ON by ANOVA with Bonferroni posttest. (*B*) An ITT was performed in 8-wk-old fed *Let-7* Tg ON mice and WT littermates. Blood glucose levels were measured at baseline and 15, 30, 45, 60, and 90 min after i.p. injections of insulin (1.0 U/kg body weight); $n = 7$. $^{**}P < 0.01$ for WT vs. *Let-7* Tg at baseline by ANOVA with Bonferroni posttest. (*C*) 7-wk-old *Let-7* Tg ON mice and WT littermates were fasted for 7 h and then received a glucose stimulus (1.5 mg/g body weight i.p.). Blood was collected at baseline and after glucose stimulation and used for determination of insulin levels by ELISA; $n = 4$. $^{*}P < 0.05$; $^{**}P < 0.01$ by ANOVA with Bonferroni posttest. (*D*) *Let-7* Tg OFF mice were crossed to *Pdx1*-Cre mice to induce pancreas-restricted *Let-7* overexpression. A GTT was performed in 6-wk-old offspring after a 7 h fast to determine blood glucose levels after i.p. glucose stimulation (1.5 mg/g body weight). $n = 5$; $^{****}P < 0.0001$ for control (WT, *Pdx1-Cre* Tg, or *Let-7* Tg OFF) vs. *Let-7* Tg, *Pdx1-Cre* by ANOVA with Bonferroni posttest.

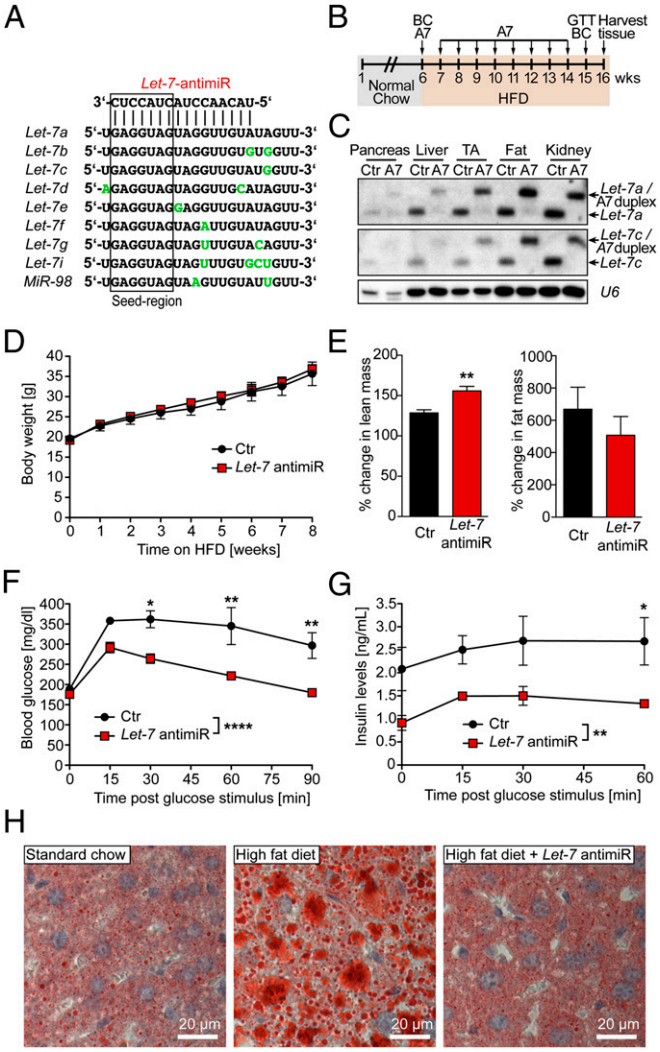

Fig. 4. AntimiR-induced repression of *Let-7* expression prevents impaired glucose tolerance. (*A*) Sequences of the *Let-7* family. Nucleotide differences to *Let-7a* are labeled with green. The box indicates the seed region (nucleotides 2–8). We designed a 16mer LNA-DNA antimiR with complimentarity to nucleotides 2–17 of *Let-7a* and *Let-7c*. (*B*) Design of the diabetes prevention study. The 6-wk-old C57BL/6 mice were maintained on an HFD and injected s.c. once weekly with saline as a control or a specifically designed *Let-7* antimiR (A7; 20 mg/kg body weight) for 8 wk. At 1 wk after the last A7 injection, a GTT was performed. Body composition (BC) was measured before the onset of HFD and at 1 wk after the last A7 injection. Tissues were harvested at 2 wk after the last A7 injection. (*C*) Tissues were harvested at the end of the prevention study, which was 2 wk after the last *Let-7* antimiR injection. Pooled total RNA of 4 Ctr and, respectively, antimiR-treated animals was used for Northern blot analysis to determine expression of *Let-7* family members. An upshift of mature *Let-7* was detected in tissues of *Let-7*-antimiR–treated mice, likely representing a *Let-7*/*Let-7* antimiR duplex. U6 was used as a loading control. (*D*) Body weight was determined weekly during the prevention study. (*E*) Body composition was assessed before the initiation of the HFD and at 1 wk after the last *Let-7* antimiR injection by EchoMRI. The percent changes in lean and fat mass during the study period are depicted. $n = 4$. $^{**}P < 0.01$, *t* test. (*F* and *G*) A GTT was performed at 1 wk after the last *Let-7* antimiR injection after a 7-h fast to determine blood glucose and insulin levels upon i.p. glucose stimulation (2 mg/g lean mass). (*F*) Blood glucose levels were measured at baseline and at 15, 30, 60, and 90 min after glucose stimulation. (*G*) For assessment of insulin levels, plasma was collected at baseline and at 15, 30, and 60 min after glucose stimulation. Consecutive insulin levels were determined by ELISA. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$ for control vs. *Let-7* antimiR–treated mice by ANOVA followed by Bonferroni posttest. (*H*) At the end of the prevention study, frozen liver sections were stained by Oil Red O to detect ectopic lipid deposition. Representative sections are shown.

mice (Fig. 3*B*). The *Let-7* Tg ON mice started with significantly higher blood glucose levels than their WT littermates (266 ± 11 mg/dL vs. 202 ± 7 mg/dL), but demonstrated reductions to similar levels as in WT littermates after insulin stimulation. This suggests that *Let-7* Tg ON mice are not resistant to insulin under basal conditions, but rather have insufficient insulin secretion. To study glucose-induced insulin secretion, we performed a GTT and collected blood samples at baseline and at 15, 30, and 60 min after i.p. glucose injection. Measurement of insulin levels by ELISA found similar baseline insulin levels (after a 7-h fast) in WT and *Let-7* Tg ON mice (Fig. 3*C*); however, the increase in insulin levels on glucose stimulation was severely attenuated and delayed in the *Let-7* Tg ON mice, likely accounting for the elevated glucose levels. Surprisingly, insulin levels returned to baseline levels by 60 min after glucose stimulation in the *Let-7* Tg ON mice, even though blood glucose levels were still severely elevated at this time (Fig. 3*A*). This suggests altered glucose sensing in pancreatic β-cells of *Let-7* Tg ON mice. Immunofluorescent staining of pancreatic slices for insulin and glucagon showed similar gross morphology of islets in WT and *Let-7* Tg ON mice (Fig. S4).

To study the specific effects of *Let-7* overexpression in the pancreas, we crossed *Let-7* Tg OFF mice with transgenic mice expressing *Cre* under control of the *Pdx1* promoter. These mice also displayed impaired glucose tolerance on the GTT, confirming that *Let-7* overexpression attenuates the pancreatic response to glucose (Fig. 3*D*). However, the observed effect was less pronounced in these mice than in *Let-7* Tg ON mice. This suggests that *Pdx1-Cre* was not sufficient to turn on *Let-7* in all pancreatic β-cells and/or that other cell types/organs also contributed to the impaired glucose tolerance. Thus, we activated *Let-7* specifically in muscle (*MCK-Cre*), liver (*Albumin-Cre*), neurons (*Nestin-Cre*), and adipocytes (*Fabp4-Cre*), but did not observe impaired glucose tolerance after glucose stimulation (Fig. S5 *A–D*). This finding might indicate that *Let-7* does not regulate glucose metabolism in these organs, or that other organs can counteract the effects of tissue-restricted overexpression of *Let-7* in a healthy mouse.

Mice with muscle-specific gain and loss of function of the *Let-7* inhibitor LIN28 display improved and diminished insulin sensitivity, respectively (14). Given that finding, the lack of insulin resistance of peripheral tissues in transgenic mice with global or muscle-specific *Let-7* overexpression in the present study is surprising. One reason for this could be that we studied blood glucose levels in only young transgenic mice (age 6–8 wk), whereas insulin resistance typically worsens with age. Furthermore, we did not stress the transgenic mice with a HFD, which might have revealed differences in insulin resistance. Transgenic mice with global *Let-7* overexpression also exhibited a significant reduction in fat mass, which might have reduced insulin resistance.

Insulin suppresses lipolysis. Consequently, patients with type 1 diabetes lacking insulin often have less adipose tissue, whereas those with T2D that receive insulin treatment often have more adipose tissue. Therefore, we wondered whether the decrease in adipose tissue in mice with global *Let-7* overexpression is secondary to disturbances in glucose metabolism. To test this possibility, we determined body composition by EchoMRI in transgenic mice with pancreas-restricted *Let-7* overexpression (*Pdx1-Cre*). These mice showed similar lean and fat mass as control mice (WT and single-transgenic mice) (Fig. S5*E*), suggesting that the loss in adipose tissue in mice with global *Let-7* overexpression is not secondary to impaired glucose metabolism. An alternative explanation would be that the milder disturbances in glucose metabolism in mice with pancreas-specific *Let-7* overexpression are insufficient to affect fat metabolism. The latter hypothesis is consistent with our finding that overexpression of *Let-7* only in fat tissue (*Fabp4-Cre*) also did not result in changes in fat mass (Fig. S5*F*). This suggests that the loss of fat mass with global *Let-7* overexpression is not due to a direct effect of *Let-7* in (FABP4-expressing) adipocytes, even though *Let-7* can regulate adipogenic differentiation in 3T3-L1 cells (20).

AntimiR-Induced Knockdown of *Let-7* Is Sufficient to Prevent Obesity-Induced Glucose Intolerance. As noted earlier, the *Let-7* family comprises a group of nine miRNAs encoded by 12 genes (Fig. S1), which poses a significant challenge to complete genetic deletion of this miRNA family. In an effort to inactivate the *Let-7* family in vivo, we designed a DNA/LNA antimiR with sequence complementarity to nucleotides 2–17 of *Let-7a/c* (Fig. 4*A*). This region contains the conserved seed region of all *Let-7* family members plus an additional eight nucleotides toward the 3′ end, which exhibit similar sequence in all *Let-7* family members. To test whether antimiR knockdown of *Let-7* was sufficient to prevent diet-induced glucose intolerance, we performed a prevention study in which C57BL/6 mice were placed on a HFD and injected once weekly for 8 consecutive weeks with *Let-7* antimiR (Fig. 4*B*). At 2 wk after the last injection, we harvested multiple tissues from these mice and assessed *Let-7* expression by Northern blot analysis. Mature *Let-7* was strongly reduced in all tissues analyzed, suggesting that antimiR-induced knockdown of *Let-7* is sustained for at least 14 d (Fig. 4*C*).

The *Let-7* antimiR–injected mice and control mice (saline-injected) demonstrated similar increases in body weight during the treatment period (Fig. 4*D*), which is not surprising given that we started the *Let-7* antimiR treatment only in adult mice (at age 6 wk). Furthermore, antimiRs do not cross the blood–brain barrier, whereas *Let-7* overexpression with *Nestin-Cre* suggests that *Let-7* regulates body size, at least in part, via gene regulation in neuronal cells (Fig. S2). As discussed earlier, the reduced body weight and fat mass in the *Let-7* Tg ON mice might result

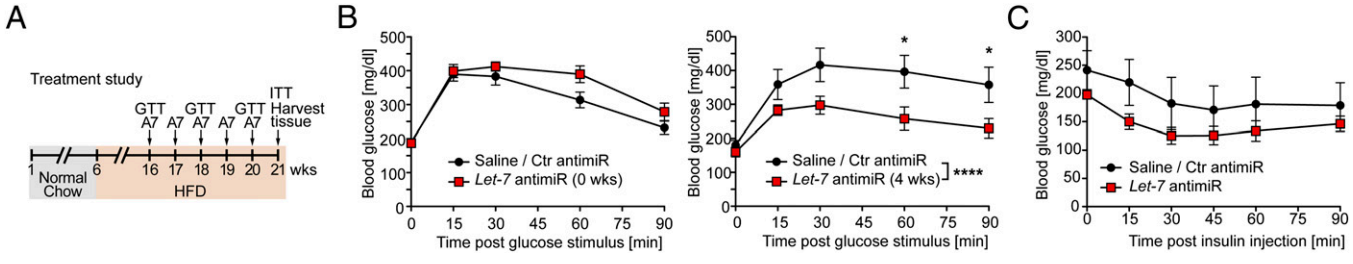

Fig. 5. AntimiR-induced repression of *Let-7* expression improves impaired glucose tolerance. (*A*) Design of the diabetes treatment study. Six-wk-old C57BL/6 mice were fed for 10 wk with HFD. Subsequently, the mice were maintained on an HFD for 5 more wk, but half of the mice ($n = 5$) were treated with *Let-7* antimiR (once weekly 20 mg/kg body weight) whereas control animals (Ctr) received either saline ($n = 3$) or control antimiR ($n = 2$). (*B*) GTTs (1.5 mg glucose/g body weight) were performed before initiation of antimiR treatment (*Left*) and at 4 wk after the first antimiR injection (*Right*). $^*P < 0.05$; $^{****}P < 0.0001$ for Ctr (saline + control antimiR) vs. *Let-7* antimiR– treated mice by ANOVA with Bonferroni posttest. (*C*) An ITT was performed at 1 wk after the last *Let-7* antimiR injection. Blood glucose levels were measured at baseline and at 15, 30, 45, 60, and 90 min after i.p. insulin injection (1.0 U/kg body weight).

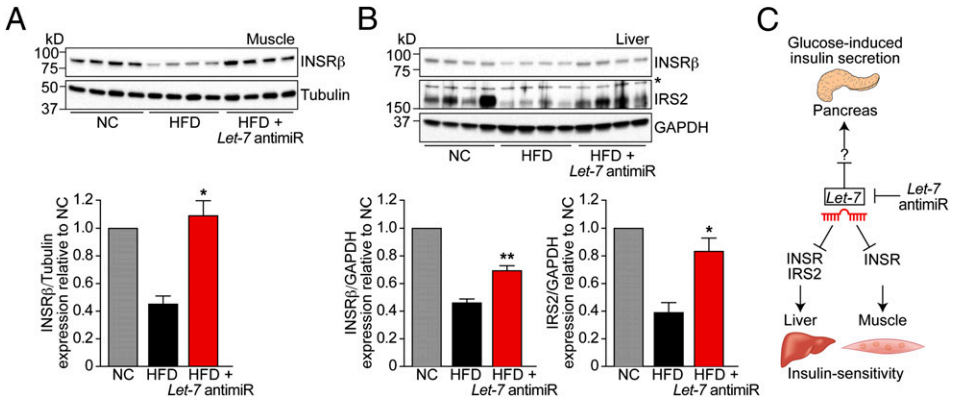

Fig. 6. *Let-7* regulates insulin signaling in liver and muscle. (*A*) Gastrocnemius muscle was harvested from mice at the end of the prevention study and from age-, sex-, and strain-matched mice fed with normal chow (NC). (*Upper*) Protein lysates from those mice were used to detect expression levels of the insulin receptor β (INSRβ) by Western blot analysis. Tubulin expression was used as a loading control. (*Lower*) Densitometric and statistical analysis. $*P < 0.05$ for HFD vs. HFD + *Let-7* antimiR, *t* test. (*B*) Detection of INSRβ and insulin receptor substrate 2 (IRS2) by Western blot analysis of liver lysates from the same mice. GAPDH expression was used as loading control. The asterisk indicates an unspecific band detected by the IRS2 antibody. $*P < 0.05$; $**P < 0.01$ for HFD vs. HFD + *Let-7* antimiR, *t* test. (*C*) Model of *Let-7*–induced modulation of glucose metabolism. *Let-7* inhibits glucose-induced insulin secretion from pancreatic β-cells as demonstrated in *Let-7* transgenic mice. In addition, antimiR knockdown of *Let-7* improves insulin sensitivity in liver and muscle, at least in part by restoring expression levels of INSR and IRS2.

secondarily from disturbances in glucose metabolism. We determined body composition before and after *Let-7* antimiR treatment by EchoMRI and found a significantly stronger increase in lean mass during *Let-7* antimiR treatment than during control treatment (Fig. 4*E*, *Left*). Consistently, individual muscles (gastrocnemius and tibialis anterior) also gained weight during *Let-7* antimiR treatment, whereas muscles of obese mice on control treatment lost weight (Fig. S6). In contrast, fat mass tended to increase more slowly in *Let-7* antimiR-treated mice (Fig. 4*E*, *Right*), accounting for the unchanged body weight. The smaller gain in fat mass might be secondary to higher energy consumption of the larger muscles of the *Let-7* antimiR-treated mice.

At the end of the prevention study, we fasted the mice for 7 h and performed a GTT. Because the *Let-7* antimiR-treated mice had greater lean mass compared with control mice, we adjusted the glucose injection to lean mass so that the *Let-7* antimiR-treated mice received slightly more glucose per gram of body weight than the control mice. As expected, the control mice displayed impaired glucose tolerance, with blood glucose levels still significantly above baseline at 90 min after glucose injection (Fig. 4*F*). In contrast, the *Let-7* antimiR-treated mice showed a normal response to glucose stimulation, with normalized blood glucose levels at 90 min after glucose injection. This finding suggests either that pancreatic insulin secretion is increased on glucose stimulation in *Let-7* antimiR-treated mice or that peripheral tissues of *Let-7* antimiR-treated mice are more sensitive to insulin. To distinguish between these possibilities, we measured blood insulin levels before and after glucose stimulation. We found significantly lower insulin levels in *Let-7* antimiR-treated mice (Fig. 4*G*), which points toward improved insulin sensitivity of peripheral tissues but does not rule out the possibility of improved β-cell function in the *Let-7* antimiR-treated mice.

Ectopic fat deposition in liver and other organs is one cause of reduced insulin sensitivity in obesity. Thus, we investigated lipid accumulation in the liver by Oil Red O staining. Although the saline-injected mice accumulated a significant amount of fat during the study period, the *Let-7* antimiR-treated mice had a similar liver fat content as age-matched control animals on a standard diet (Fig. 4*H*).

AntimiR-Induced Knockdown of *Let-7* Is Sufficient to Treat Obesity-Induced Glucose Intolerance. To test whether *Let-7* antimiR treatment is sufficient to treat impaired glucose tolerance, we put C57BL/6 mice on an HFD for 10 wk and then initiated weekly treatment with *Let-7* antimiR (20 mg/kg body weight) for 4 wk (Fig. 5*A*). Control mice received injections of either saline only or equal amounts of a control antimiR. GTTs were performed before antimiR treatment and at 2 and 4 wk after the onset of *Let-7* antimiR treatment. There was no significant difference in blood glucose levels at baseline (Fig. 5*B*, *Left*); however, blood glucose levels were significantly reduced in *Let-7* antimiR-treated mice compared with control mice after only 2 wk of treatment (Fig. S7). This effect was even more pronounced after 4 wk of *Let-7* antimiR treatment (Fig. 4*B*, *Right*). These data demonstrate that treatment with *Let-7* antimiR is sufficient not only to prevent, but also to treat impaired glucose tolerance. Analysis of insulin sensitivity of peripheral organs with ITTs showed that insulin injection reduced blood glucose to lower levels in *Let-7* antimiR-treated mice than in control mice; however, the control mice started with higher glucose levels (Fig. 5*C*).

Let-7 AntimiR Treatment Restores Insulin Signaling in Muscle and Liver. Individual miRNAs can target multiple genes within biological pathways, which enables the miRNAs to modulate complex disease processes. Target genes can be identified using prediction algorithms, which rank potential targets based on the complementarity between miRNA and mRNA 3′UTRs and the degree of conservation of the 3′UTR target sequence across species. Using one of these programs (targetscan; www.targetscan.org), we found that *Let-7* is predicted to target the insulin receptor (*Insr*), which is responsible for insulin action in peripheral tissues. *Let-7* is further predicted to target insulin receptor substrate 2 (*Irs2*), which is phosphorylated by INSR on insulin stimulation and mediates the insulin signal to downstream effectors. Up-regulation of INSR and/or IRS2 by antimiR knockdown of *Let-7* could explain the enhanced insulin sensitivity that we observed during the *Let-7* antimiR treatment. Furthermore, because *Let-7* antimiR seems to work as a scaffold that binds *Let-7* rather than inducing its degradation, up-regulation of *Let-7* target genes serves as a necessary readout of *Let-7* inhibition. Therefore, we performed Western blot analysis of INSR and IRS2 expression in mice from our prevention study. As an additional control group, we used age-matched mice that were fed normal chow. The HFD reduced expression of INSR compared with normal chow in muscle and liver, but *Let-7* antimiR treatment restored INSR expression to levels found in healthy mice on normal chow (Fig. 6 *A* and *B*). In

addition, IRS2 expression was down-regulated in mice on an HFD, and *Let-7* antimiR treatment prevented down-regulation of IRS2 expression in the liver (Fig. 6*B*).

Potential Clinical Relevance. Our results demonstrate that knockdown of *Let-7* with an antimiR is sufficient to prevent and treat impaired glucose tolerance by improving insulin sensitivity in peripheral tissues. In addition, data from our transgenic mice indicate that *Let-7* can block glucose-induced insulin secretion from the pancreas, suggesting that knockdown of *Let-7* also might improve pancreatic β-cell function (Fig. 6*C*). Whether *Let-7* is causatively involved in the pathogenesis of T2D is unclear, given that *Let-7* expression was not altered in the mice on an HFD. Nevertheless, in principle *Let-7* antimiR treatment could provide a unique approach to treating T2D, for several reasons. First, *Let-7* likely regulates multiple components of glucose metabolism; second, antimiRs often result in long-lasting knockdown of miRNAs with sustained effects; and third, *Let7* antimiR decreased fat mass in obese settings, in contrast to most drugs currently used to treat T2D. However, it also should be noted that *Let-7* is associated with the occurrence of tumors, although we found no obvious signs of cancer during antimiR treatment periods of up to 10 wk. Furthermore, localized knockdown of *Let-7* in skeletal muscle (a tissue rarely affected by tumors) might be sufficient to improve glucose metabolism. Our *Cre*-inducible *Let-7*-transgenic mice provide a unique model for studying tissue-specific aspects of body growth and T2D and for testing the effects of drugs on the pathogenesis of diabetes.

Methods

Mice. For generation of the *Let-7* transgenic mice, a genomic region on chromosome 13 comprising the genes for *Let-7a*, *Let-7d*, and *Let-7f* was amplified from C57BL/6 DNA and cloned into the CAG-Z-EGFP 1.0 vector (Invitrogen), which allows activation of transgene expression by *Cre*-mediated deletion of an inhibitory DNA element. *Pdx1-Cre* mice were kindly provided by Doug Melton (Harvard University, Cambridge, MA). All experimental animal procedures were approved by the University of Texas Southwestern Medical Center's Institutional Animal Care and Research Advisory Committee.

***Let-7* Northern Blot Analysis.** Northern blots were performed as described previously (21).

Body Composition Analysis. Body composition was measured using an EchoMRI-100 body composition analyzer (Echo Medical Systems) at the University of Texas Southwestern Medical Center's Metabolic Core Facility.

AntimiR. The *Let-7* antimiR was synthesized by miRagen Therapeutics. 20 mg antimiR per kg body weight dissolved in 100 μL of saline was injected s.c. once weekly. The control antimiR had an identical chemistry and comprised a sequence directed against a *C. elegans*-specific miRNA (5′-TccTAgAAa-GAgtAgA-3′, with uppercase indicating LNA and lowercase indicating DNA).

Glucose and Insulin Measurements. For GTTs, mice were fasted for 7 h before i.p. glucose injection. Insulin levels in plasma were measured by ELISA (Chrystal Chem).

Western Blot Analysis. IRS2 and INSRβ antibodies were obtained from Cell Signaling and used at 1:1,000 dilutions as recommended by the manufacturer.

Statistical Analysis. All data are shown as mean ± SEM. Data were analyzed using the Student *t* test or ANOVA followed by a Bonferroni posttest as appropriate. Experiments are described in more detail in *SI Methods*.

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