Contribution of the Long Noncoding RNA H19 to β-Cell Mass Expansion in Neonatal and Adult Rodents

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Pancreatic β-cell expansion throughout the neonatal period is essential to generate the appropriate mass of insulin-secreting cells required to maintain blood glucose homeostasis later in life. Hence, defects in this process can predispose to diabetes development during adulthood. Global profiling of transcripts in pancreatic islets of newborn and adult rats revealed that the transcription factor E2F1 controls expression of the long noncoding RNA H19, which is profoundly downregulated during the postnatal period. H19 silencing decreased β-cell expansion in newborns, whereas its re-expression promoted proliferation of β-cells in adults via a mechanism involving the microRNA let-7 and the activation of Akt. The offspring of rats fed a low-protein diet during gestation and lactation display a small β-cell mass and an increased risk of developing diabetes during adulthood. We found that the islets of newborn rats born to dams fed a low-protein diet express lower levels of H19 than those born to dams that did not eat a low-protein diet. Moreover, we observed that H19 expression increases in islets of obese mice under conditions of increased insulin demand. Our data suggest that the long noncoding RNA H19 plays an important role in postnatal β-cell mass expansion in rats and contributes to the mechanisms compensating for insulin resistance in obesity.

Pancreatic β-cells, located within islets of Langerhans, maintain the blood glucose level within a physiologically relevant range by precisely adapting insulin secretion to circulating levels of nutrients, particularly glucose (1). Insulin-secreting cells acquire a fully differentiated phenotype only after birth. Before weaning, neonatal rat β-cells produce and release insulin in response to amino acids but do not have the ability to secrete insulin in response to an increase in glucose level, a characteristic feature of fully mature β-cells (2,3).

Another hallmark of β-cells in newborns is strong proliferation that allows the number of insulin-secreting cells to expand massively during the postnatal period. After weaning, replication slows to maintain a more or less constant β-cell mass during the life span (4). Defective β-cell expansion early in life can result in an insufficient mass of insulin-secreting cells during adulthood, predisposing affected individuals to the development of diabetes. Under conditions associated with increased metabolic demands, such as pregnancy and obesity, β-cells increase their secretory activity and replication rate. Failure to compensate for insulin resistance linked to pregnancy or obesity can lead to hyperglycemia and eventually to the onset of gestational diabetes mellitus or type 2 diabetes, respectively (5).

So far, most studies investigating the mechanisms driving postnatal β-cell maturation have focused on protein-coding genes. However, high-throughput RNA sequencing revealed that most genome sequences generate RNA molecules that do not code for proteins (6). We now know that the noncoding transcriptome is involved in multiple biological processes influencing development,

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differentiation, and metabolism (7–9). Emerging evidence points to a role for noncoding RNAs in postnatal β-cell maturation as well. Indeed, we showed that the nutritional shift at weaning from the fat-rich milk from the mother to a carbohydrate-rich chow diet triggers vast changes in the microRNA (miRNA) profile of β-cells in newborns, which are instrumental in acquiring glucose responsiveness and a mature secretory phenotype (10).

In addition to small noncoding RNAs, the mammalian transcriptome includes a large number of long noncoding RNAs (lncRNAs) containing more than 200 nucleotides (11–13). Thousands of lncRNAs were recently identified in human and mouse pancreatic islets (14–16). Many of these lncRNAs are specifically expressed in islets and are induced during β-cell differentiation. These findings suggest a role for lncRNAs in the developmental programming and proper functioning and maintenance of pancreatic islet cells.

The aim of this project was to identify lncRNA-based mechanisms that contribute to the acquisition of a mature β-cell phenotype and that control the size of the functional β-cell mass. Screening for lncRNAs that are differentially expressed during postnatal islet maturation revealed the downregulation of H19, a maternally imprinted intergenic lncRNA generated from the Igf2 locus (17). We provide evidence indicating that H19 contributes to β-cell mass expansion in newborn rats and is re-expressed in adult islets under conditions of increased insulin demand. Moreover, we show that the level of H19 is lower in islets from pups born to dams fed a low-protein (LP) diet during gestation and lactation; these pups display a reduced β-cell mass and increased risk of developing diabetes during adulthood. Our findings provide new insights into the role of H19 in β-cells in newborn and adult rodents and unveil a potential mechanism explaining the increased susceptibility to diabetes of the offspring of mothers kept under deleterious dietary conditions during pregnancy and lactation.

RESEARCH DESIGN AND METHODS

Chemicals

Interleukin-1β, hexadimethrine bromide, and histopaques 1119 and 1077 were obtained from Sigma-Aldrich (St. Louis, MO). 3-Isobutyl-1-methylxanthine was purchased from Merck (Whitehouse Station, NJ); interferon-γ, from R&D Systems (Minneapolis, MN); and tumor necrosis factor-α, from Enzo Life Sciences (Farmingdale, NY).

Animals

Three-month-old male Sprague-Dawley rats were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France). Pups (postnatal day [P] 1 to P31) were obtained by collecting the offspring of pregnant Sprague-Dawley rats. Pregnant Wistar rats were fed during gestation and lactation a control diet (20% [w/w] protein) or an isocaloric LP diet (8% [w/w] protein) (Hope Farm, Woerden, the Netherlands) (18). C57BL/KsJ db/db and C57BL/6J ob/ob mice (13–16 weeks old) and age-matched lean control mice (db/+ C57BL/KsJ and −/− C57BL/6J, respectively) were taken from breeding colonies at the Garvan Institute of Medical Research. Male C57BL/6 mice (Charles River Laboratories, Raleigh, NC), aged 5 weeks, were fed a normal diet (ND) or a high-fat diet (HFD) for 8 weeks (F-3282, 60% energy from fat; Bioserv, Frenchtown, NJ). After eating an HFD for 7.5 weeks, the mice that weighed between 33 and 39 g were classified as low responders (LDR), whereas those weighing between 39 and 45 g were defined as high responders (HDR). The weight of and glycemia and insulinemia in these animals were reported previously (19).

Microarray Profiling

RNA was isolated from pools of islets from five pups (10 days after birth; P10) from three different mothers, and from three individual adult rats. Arraystar (Rockville, MD) performed mRNA and lncRNA profiling using the Rat lncRNA Array version 2.0 and analyzed the data. Differentially expressed transcripts were identified through volcano plot filtering (fold change ≥ 2, nominal $P \leq 0.05$). The data are available on the Gene Expression Omnibus GSE106919 record.

Culture of INS832/13 and 1.1B4 Cells

The INS832/13 rat β-cell line was provided by Dr. C. Newgard (Duke University) and was cultured as described previously (20). The cells were seeded at a density of 4×10^4 cells per square centimeter for immunocytochemistry and to assess cell death, at 7.5×10^4 cells per square centimeter to assess insulin secretion, and 1.1×10^5 cells per square centimeter to isolate RNA. The 1.1B4 human β-cell line was provided by Dr. P. Flatt (University of Ulster) and was cultured as described previously (21).

Isolation and Culture of Islet Cells

Rat pancreatic islets were isolated by collagenase digestion (22), purified on a Histopaque density gradient, and then cultured as described elsewhere (10). Islet cells were dissociated by trypsinization and seeded at a density of 5×10^4 cells per square centimeter for immunocytochemistry and at 10^5 cells per square centimeter for RNA isolation.

Fluorescence-Activated Cell Sorting

Dissociated islet cells from newborn and adult rats were sorted in a FACS on the basis of β-cell autofluorescence (23). Immunocytochemistry using anti-insulin antibodies (no. A0564; Dako, Basel, Switzerland) showed that $94 \pm 1\%$ of the cells in the purified fraction were insulin-positive.

Cell Transfection

Overexpression was achieved by transfecting a pcDNA3 plasmid harboring the sequences of the lncRNA; transfection was performed through the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in INS832/13 cells or Lipofectamine 3000 (Invitrogen) in islet cells. Downregulation was achieved by transfecting small interfering RNAs against green fluorescent protein (Eurogentec, Seraing, Belgium), H19 (Thermo Fisher Scientific, Waltham, MA),

Argonaute 2 (Ago2) or E2F1 (GE Healthcare Europe), or miRCURY LNA microRNA Inhibitors (anti-miRs) (Exiqon, Bedvaek, Denmark) with the use of Lipofectamine 2000 (INS832/13 cells) or Lipofectamine RNAiMAX (Invitrogen) (islet cells).

RNA Isolation and Detection

RNA was isolated with an miRNeasy Mini Kit (Qiagen, Basel, Switzerland) for islet cells and with the ZR RNA MiniPrep kit (Zymo Research, Irvine, CA) for INS832/13 cells. The samples were treated with DNase (Promega, Madison, WI) before retrotranscription using RNAse H minus (Promega), a Maloney murine leukemia virus reverse transcriptase. To quantify lncRNAs and mRNAs, real-time PCR was performed with the use of iQ SYBR Green Supermix. lncRNA and mRNA primers are listed in Supplementary Table 1. To quantify miRNA, real-time PCR was carried out by using a miRCURY LNA Universal RT microRNA PCR kit (Exiqon).

Immunocytochemistry

Glass coverslips were coated with poly-L-lysine alone for use with dissociated islet cells or with poly-L-lysine and laminin for use with INS832/13 cells. The cells were fixed with cold methanol and permeabilized with PBS supplemented with 0.5% (v/v) saponin (Sigma-Aldrich). The coverslips were incubated in blocking buffer (PBS supplemented with 0.5% [v/v] saponin and 1% [w/v] BSA; Sigma-Aldrich) and then exposed to primary antibodies at the following dilutions: 1:700 rabbit anti-Ki67 (no. ab15580; Abcam, Cambridge, U.K.) and 1:500 guinea pig anti-insulin (no. A0564; Dako). After being washed, the coverslips were incubated with dilutions (1:500) of goat antirabbit Alexa Fluor 488 or goat anti–guinea pig Alexa Fluor 555 (nos. A11008 and A21435, respectively; Thermo Fisher Scientific). Finally, they were incubated with Hoechst 33342 stain (Invitrogen), mounted on glass slides, and visualized with a Zeiss AxioVision fluorescence microscope. For BrdU immunocytochemistry, the cells were incubated with BrdU (no. ab142567; Abcam) for 48 h. After exposure to blocking buffer and before exposure to mouse anti-BrdU antibody (1:400; no. BD55627; BD Biosciences), DNA was denatured with 2 N HCl. BrdU-positive cells were visualized with a goat antimouse Alexa Fluor 555 antibody (1:500; no. A21422; ThermoFisher Scientific). Examples of Ki67- and BrdU-positive cells are provided in Supplementary Fig. 1. At least 1,000 cells were analyzed for each condition.

Insulin Secretion

Insulin secretion was induced in INS832/13 cells as described previously (10).

Cell Death Assessment

INS832/13 cells were washed with PBS and incubated with Hoechst 33342 stain (1 μg/mL). The fraction of cells displaying pyknotic nuclei was scored after visualization under a fluorescence microscope (AxioCam MRc5; Zeiss). As a positive control for apoptosis, a fraction of cells were exposed to a combination of cytokines (10 ng/mL tumor necrosis factor-α, 0.1 ng/mL interleukin-1β, 30 ng/mL interferon-γ) for 24 h. Approximately 500 cells were counted for each condition.

Protein Extraction and Western Blotting

INS832/13 cells were washed in cold PBS and incubated in lysis buffer (20 mmol/L Tris [pH 7.5], 2 mmol/L EDTA, and protease inhibitors [Roche]) for 15 min. The cells were then scraped, briefly sonicated, and centrifuged to eliminate nuclei and cell debris. Proteins (30 μg) were loaded on acrylamide gels and transferred to polyvinylidine fluoride membranes. The membranes were placed in blocking buffer (0.1% [v/v] Tween 20 and 5% [w/v] BSA) for 1 h, then incubated overnight at 4°C with primary antibodies diluted in blocking buffer. The following antibodies were used: rabbit anti–phosphorylated Akt (1:500; Thr308 [no. 9275S] or Ser473 [no. 4060S]; Cell Signaling Technology, Danvers, MA); mouse anti-Akt (1:500; no. 2920; Cell Signaling Technology); mouse anti–actin α (1:15,000; no. MAB1501; Merck & Cie, Schaffhausen, Switzerland). After 1 h of exposure at room temperature to horseradish peroxidase–coupled secondary antibodies (1:15,000; anti–rabbit IgG [no. 111-165-144] and anti–mouse IgG [no. 111-165-166]; Jackson ImmunoResearch Europe Ltd., Suffolk, U.K.), immunoreactive bands were visualized by chemiluminescence (Thermo Fisher Scientific) through the use of an ImageQuant LAS-4000 System.

Luciferase Assay

The luciferase construct with eight let-7 target sites in the 3′ untranslated region (UTR) of the Renilla gene was a gift from Yukihide Tomari (psiCHECK2-let-7 8x; plasmid no. 20931; Addgene). Luciferase activity was measured by using a dual luciferase reporter assay (Promega) and was normalized to the Firefly activity generated by the same plasmid.

Statistical Analysis

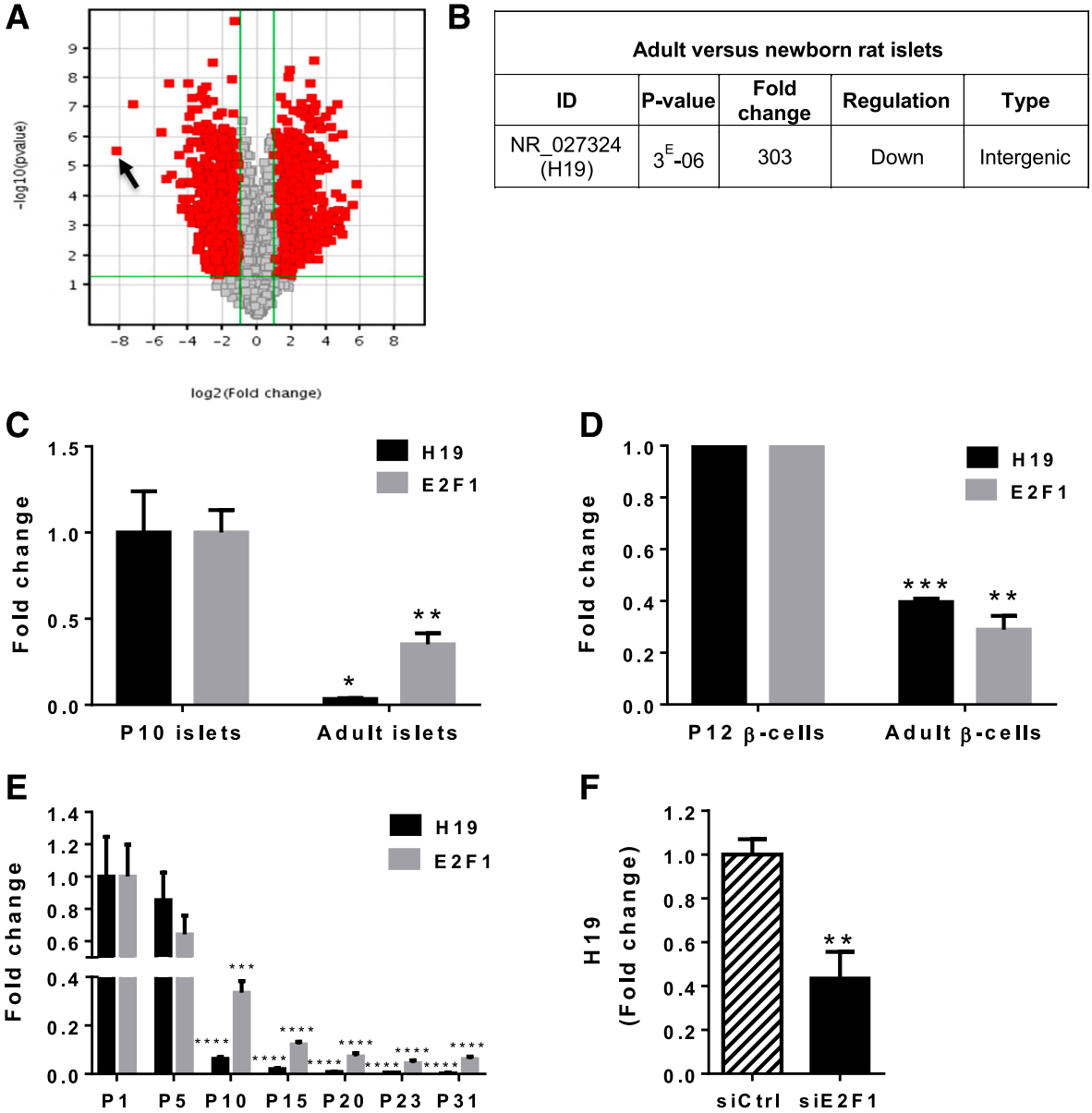
Data are presented as the mean ± SEM. Statistical significance was tested by using an unpaired or one-sample Student *t* test when two sets of data were analyzed, and one-way ANOVA followed by a multiple comparison test (the Dunnett or Tukey test) or the Kruskal-Wallis test with the Dunn multiple comparison test when the experiments included more than two groups. H19 expression and phenotypic characteristics of the mice were correlated by linear regression with the use of the *F* test (GraphPad statistical package; GraphPad Software, La Jolla, CA).

RESULTS

To assess the contribution of lncRNAs to the acquisition of a fully differentiated β-cell phenotype, we used a microarray to search for lncRNAs that are differentially expressed between adult and neonatal (P10) rat islets. The array includes 5,496 lncRNAs, 896 of which were upregulated and 1,018 downregulated during islet maturation (fold change ≥2; nominal *P* value ≤0.05) (GSE106919) (Fig. 1*A*). We decided to investigate in more

detail the role of the lncRNAs displaying the largest changes in expression, and in order to avoid possible interference from protein-coding genes, we focused exclusively on intergenic lncRNAs. It is interesting to note that the expression of H19 (National Center for Biotechnology Information [NCBI] reference sequence [NR_027324]), an lncRNA generated from a maternally imprinted locus, was 303 times lower in the islets of adult rats than in those of newborn rats (Fig. 1*B*). RT-PCR of whole islets (Fig. 1*C*) and of FACS-purified β-cells (Fig. 1*D*) confirmed these findings. A time course spanning the postnatal period showed that the expression of H19 is highest between P1 and P5 and strongly decreases thereafter (Fig. 1*E*).

Several transcription factors can regulate the expression of H19. The level of one of these, E2F1, is reduced both in islets (Fig. 1*C*) and in purified β-cells (Fig. 1*D*) upon



Adult versus newborn rat islets				
ID	P-value	Fold change	Regulation	Type
NR_027324 (H19)	3^{E}-06	303	Down	Intergenic

Figure 1—Comparison of lncRNA expression in islets from 10-day-old rats and adult rats and regulation of H19 expression. *A*: Volcano plot representing the lncRNAs that are differentially expressed between P10 and adult islets. Upregulated or downregulated transcripts are depicted in red (nominal *P* value <0.05; fold change ≥ |2|). The arrow points to H19. *B*: The *P* value, fold change, regulation, and type of lncRNA selected. The expression of H19 and E2F1 was measured by quantitative RT-PCR in P10 and adult islets (*C*) or in purified β-cells sorted with a FACS (P12 β-cells) (*D*). *E*: Expression of H19 and E2F1 in islets from rats at the indicated ages was determined by quantitative RT-PCR, normalized to 18S, and expressed as fold change versus P1. *F*: Dissociated islet cells from P10 rats were transfected with a small interfering RNA against green fluorescent protein (siCtrl) or E2F1 (siE2F1) for 48 h. The expression of H19 was measured by quantitative RT-PCR and expressed as fold change compared with expression of H19 in the control. The results are the mean ± SEM of three (*D*) or four (*C*, *E*, *F*) independent experiments per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Student *t* test or one-way ANOVA with the Dunnett post hoc test when more than two sets of data were analyzed).

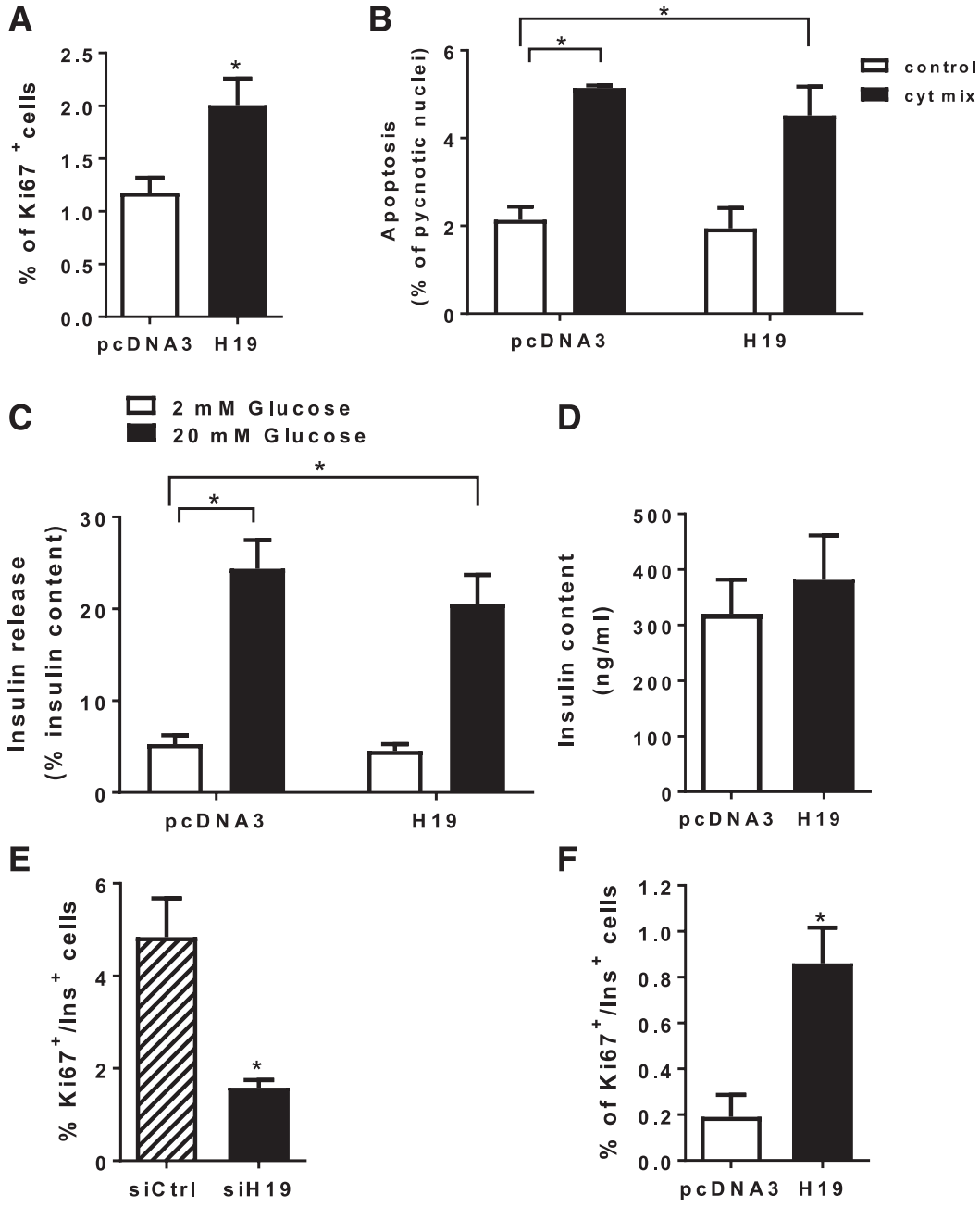


Figure 2— Functional role of H19 in β-cells. INS832/13 cells (*A–D*) or dissociated islet cells from adult rats (*F*) were transfected for 48 h with a control plasmid (pcDNA3) or a plasmid allowing the expression of H19. *A* and *F*: The fraction of proliferating cells was determined by scoring Ki67-positive cells. *B*: Cell death was assessed by scoring the cells displaying pyknotic nuclei. The cells were incubated for the last 24 h without cytokines (control) or with a mix of cytokines (cyt mix). *C* and *D*: Insulin secretion (*C*) and insulin content (*D*) were measured by ELISA after 45 min of incubation with either 2 or 20 mmol/L glucose. *E*: Dissociated islet cells form P10 rats were transfected with a small interfering RNA against green fluorescent protein (siCtrl) or against H19 (siH19). The fraction of proliferating insulin-expressing cells was determined by scoring Ki67-positive cells. The results are the mean ± SEM of three (*B* and *E*), four (*F*), five (*C* and *D*), or seven (*A*) independent experiments. Statistically significant differences from the control conditions were assessed by using the Student *t* test or one-way ANOVA with a Dunnett post hoc test when more than two conditions were compared. $^*P < 0.05$.

postnatal maturation, and E2F1 displays an expression pattern similar to that of H19 (Fig. 1*E*). Silencing of E2F1 in islets from P10 rats (Supplementary Fig. 2*A*) resulted in the downregulation of H19 (Fig. 1*F*), suggesting that this transcription factor contributes to the control of H19 expression during the postnatal period. By contrast, silencing of c-Myc, a transcription factor that regulates H19 expression in other cell types (24,25) and that is downregulated upon β-cell maturation, had no effect on the level of this lncRNA (Supplementary Fig. 3).

To elucidate the role of H19, we first investigated its function in the rat INS832/13 β-cell line. Overexpression

of H19 to reach levels comparable to those measured in neonatal islets (Supplementary Fig. 4*A*) led to an increase in proliferation (Fig. 2*A*) without effects on cell survival, as measured by scoring pyknotic nuclei (Fig. 2*B*) or by using a TUNEL assay (Supplementary Fig. 5). The increase in H19 expression also did not affect insulin content or secretion (Fig. 2*C* and *D*). Neonatal rat β-cells display a much higher proliferation rate than do adult rat β-cells (10). To assess whether H19 contributes to postnatal β-cell expansion, the level of this lncRNA was

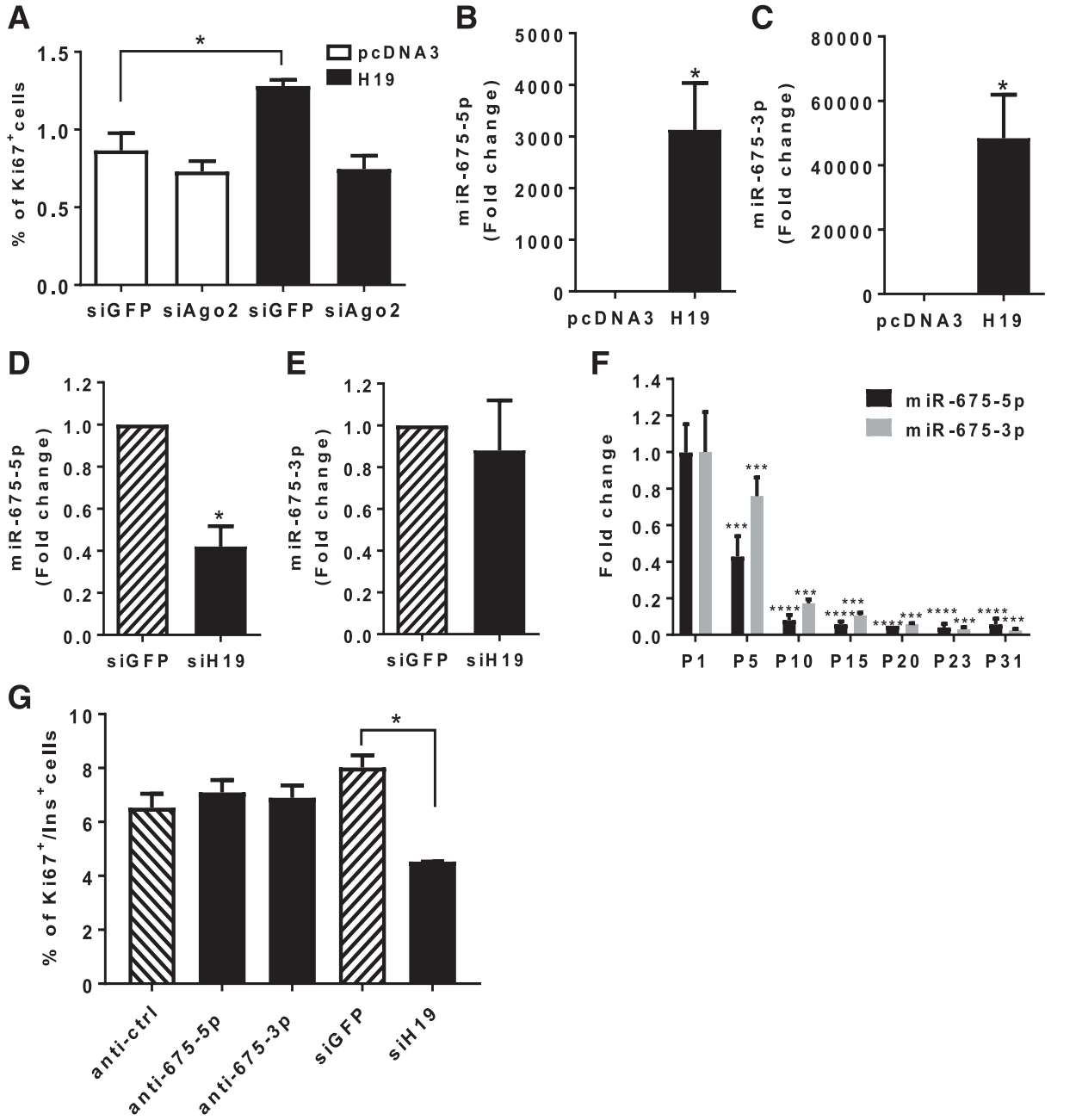


Figure 3—Involvement of miRNAs in H19 action. *A*: INS832/13 cells were cotransfected with a small interfering RNA (siRNA) against green fluorescent protein (siGFP) or Ago2 (siAgo2) and a control plasmid (pcDNA3) or a plasmid overexpressing H19. After 48 h, the fraction of proliferating cells was determined by scoring Ki67-positive cells. *B* and *C*: INS832/13 cells were transfected for 48 h with a control plasmid (pcDNA3) or a plasmid expressing H19. *D* and *E*: Dissociated islet cells from P5 rats were transfected for 48 h with siGFP or siH19. miR-675-5p and miR-675-3p levels were measured by quantitative RT-PCR, normalized to those of miR-130b-3p, and expressed as fold change vs. the control condition (siGRP column) or P1 levels for the postnatal islet time course expression (*F*). *G*: Dissociated islet cells from P5 rats were transfected with a control, with an miR-675-5p or miR-675-3p inhibitor, or with siGFP or siH19. After 48 h, the fraction of proliferating insulin-expressing cells was assessed by scoring Ki67-positive cells. The results are the mean ± SEM of three (*A*–*E*) or five (*F* and *G*) independent experiments. Statistically significant differences from the control conditions were determined by using the Student *t* test or one-way ANOVA with a Dunnett (*A* and *F*) or Tukey (*G*) post hoc test when more than two sets of data were analyzed. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

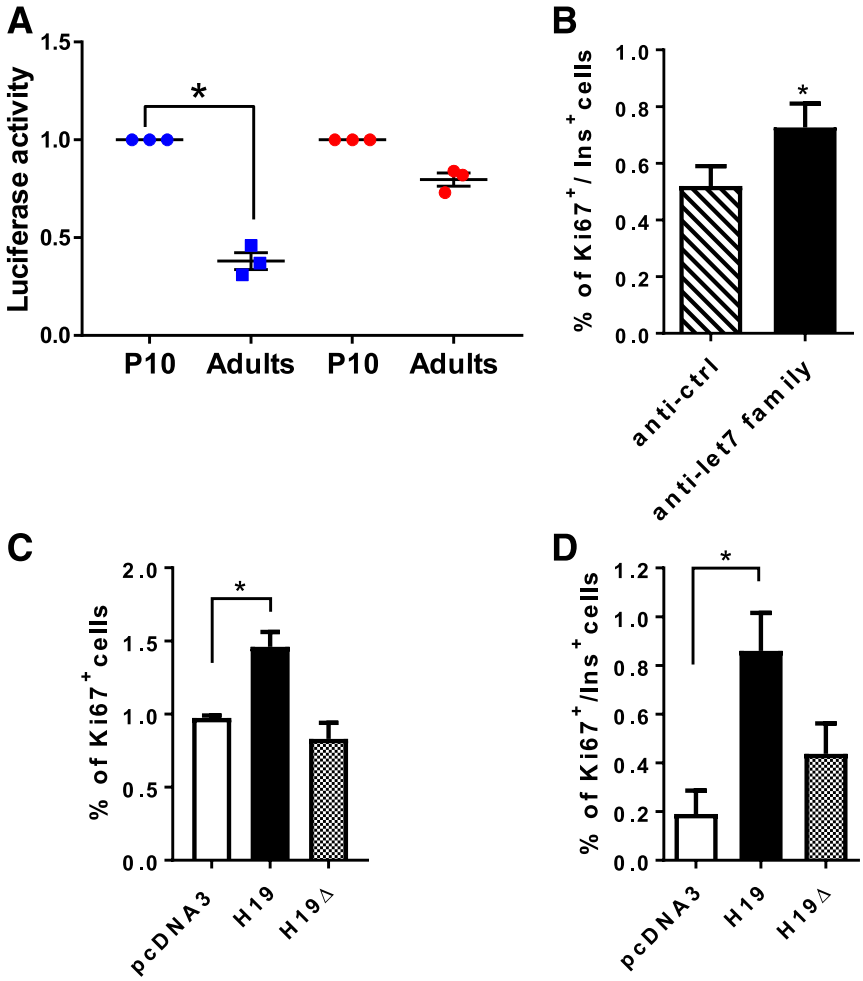


Figure 4—Let-7 family members participate in H19 action in β-cells. *A*: Dissociated islet cells from P10 or adult rats were transfected with a luciferase construct containing (blue dots) or lacking (red dots) eight target sites for let-7 in the 3′ UTR. Luciferase activity was measured 48 h later. *B*: Dissociated islet cells from adult rats were transfected with a control inhibitor (anti-ctrl) or with anti-miRNAs blocking all let-7 family members (anti-let7 family). INS832/13 cells (*C*) and dissociated islet cells from adult rats (*D*) were transfected with a control plasmid (pcDNA3), a plasmid overexpressing H19, or a plasmid overexpressing an H19 form without let-7 binding sites (H19Δ). Two days later the fraction of proliferating insulin-expressing cells was assessed by scoring Ki67-positive cells. The results are the mean ± SEM of three (*A–C*) or four (*D*) independent experiments. Statistically significant differences from the control conditions were calculated by using the Student *t* test or one-way ANOVA with a Dunnett post hoc test when more than two sets of data were analyzed. *$P < 0.05$.

reduced by RNA interference (Supplementary Fig. 2*B*). Silencing of H19 in neonatal β-cells resulted in a profound decrease in proliferation when assessed by scoring both the number of Ki67-positive cells (Fig. 2*E*) and the number of BrdU-positive cells (Supplementary Fig. 6). Moreover, restoration of elevated levels of the lncRNA in adult rat islets (Supplementary Fig. 4*C*) induced a fourfold increase in the number of proliferating β-cells (Fig. 2*F*). These effects were independent of Igf2, the levels of which were unchanged by modulation of H19 expression (Supplementary Fig. 7). Similar results were obtained upon overexpression of human H19 in the human 1.1B4 β-cell line (21) (Supplementary Fig. 8).

Other research has proposed that H19 exerts its action by affecting the level or the activity of different miRNAs (26–28). To investigate whether miRNAs mediate the regulation of β-cell proliferation, we assessed the effect of H19 in cells in which the expression of Ago2, a component of the RNA-induced silencing complex that is essential for miRNA action (29), is reduced by RNA interference (Supplementary Fig. 2*C*). We observed that H19-induced proliferation is lost in INS832/13 cells lacking Ago2 (Fig. 3*A*), indicating that miRNAs may mediate H19 action.

Two miRNAs, miR-675-5p and miR-675-3p, are produced upon processing of H19 (30). Indeed, overexpression of H19 in INS832/13 cells (Fig. 3*B* and *C*) led to corresponding changes in the level of these miRNAs and silencing of H19 (Fig. 3*D* and *E*), resulting in a decrease in miR-675-5p expression. Moreover, the expression profiles of these miRNAs throughout the postnatal period revealed a downregulation that parallels that of H19 (Fig. 3*F*), suggesting that these miRNAs may mediate the effect of

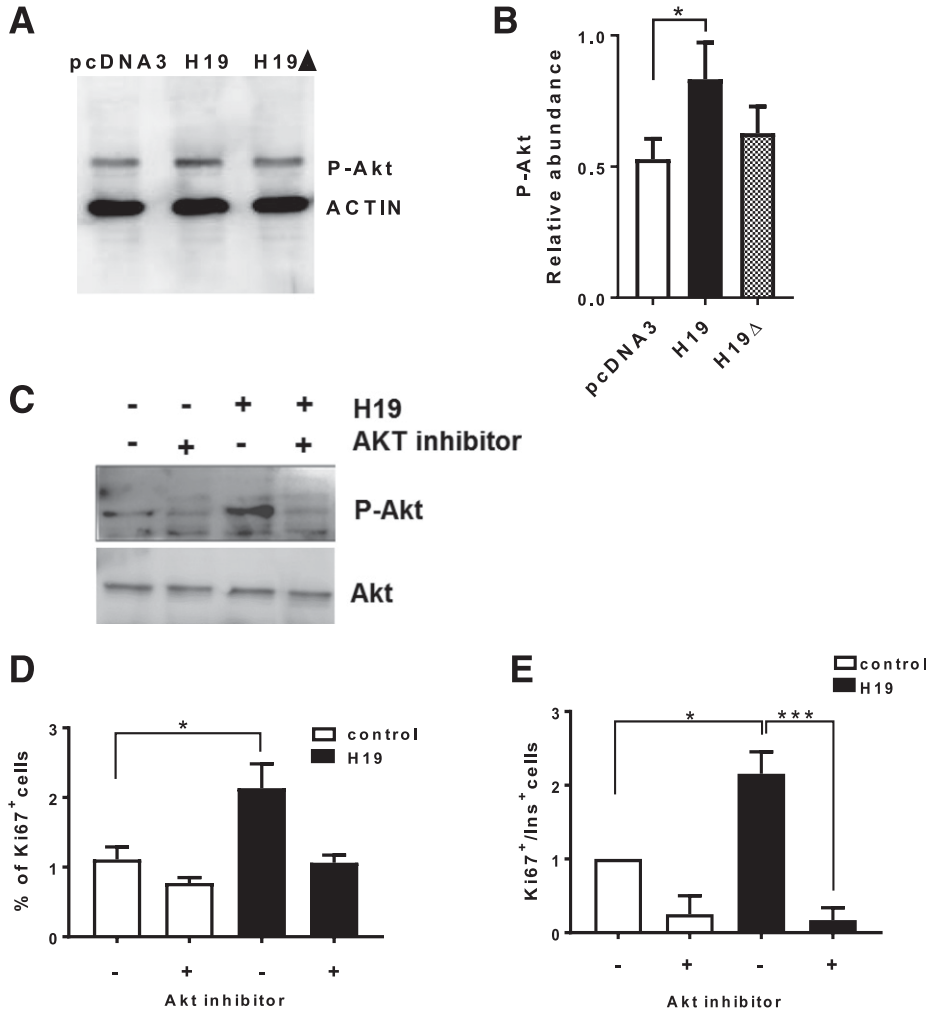


Figure 5—H19 induces β-cell proliferation through Akt activation. *A*: Representative Western blotting of Akt phosphorylation at Thr-308 (P-Akt) in INS832/13 cells transfected with a control plasmid (pcDNA3), a plasmid overexpressing H19, or an H19 form without two binding sites for let-7 (H19Δ). *B*: Western blotting was analyzed by using densitometry, and the abundance of P-Akt bands was normalized to that of actin α. *C–E*: INS832/13 cells (*C* and *D*) or primary rat islet cells (*E*) were transfected with a control plasmid (pcDNA3) or a plasmid expressing H19. After 48 h, cells were or were not exposed for 2 h to 10 μmol/L Akt inhibitor (ab142088). *C*: Representative Western blotting of AKT phosphorylation at Ser-478 (P-Akt) and Akt protein expression. *D* and *E*: The fraction of proliferating insulin-expressing cells was assessed by scoring those that were positive for Ki67. The results are the mean ± SEM of three (*D* and *E*) or four (*B*) independent experiments. Statistically significant differences from the control conditions were assessed by using the Kruskal-Wallis test with a Dunn post hoc test (*B*) or one-way ANOVA with the Dunnett post hoc test (*D* and *E*). $^{*}P < 0.05$; $^{***}P < 0.001$.

the lncRNA on β-cell proliferation. To assess this hypothesis, we transfected P5 islet cells with anti-miRs to block miR-675-5p or miR-675-3p (Supplementary Fig. 9*A* and *B*). As shown in Fig. 3*G*, silencing of H19 reduced β-cell proliferation, whereas blockade of miR-675-5p or miR-675-3p had no effect, indicating that the action of the lncRNA is likely to be independent of these miRNAs.

H19 expression is induced during myoblast differentiation and has been proposed to sequester the miRNA let-7 (31). The expression of most let-7 isoforms is not modified upon islet postnatal maturation (10), but to our knowledge, possible changes in the repressive activity of these miRNAs have not yet been investigated. To evaluate the activity of let-7 in P10 and adult islets, we transfected a reporter construct containing eight let-7 target sites in the 3′ UTR of the Renilla luciferase gene (32). We found that the luciferase activity of the let-7 sensor in adult rat islets is lower than that in newborn rat islets, whereas the activity of a reporter lacking the let-7 target sites was not significantly different between adult cells and newborn cells (Fig. 4*A*), confirming an increase in the activity of let-7 family members after postnatal maturation. Knockdown of let-7a by a specific inhibitor (Supplementary Fig. 9*C* and *D*) led to an increase in the proliferation of INS832/13 cells (Supplementary Fig. 10). A similar tendency was observed in primary adult β-cells, but the effect did not reach statistical significance, probably because of the presence of several other let-7 isoforms with overlapping functions.

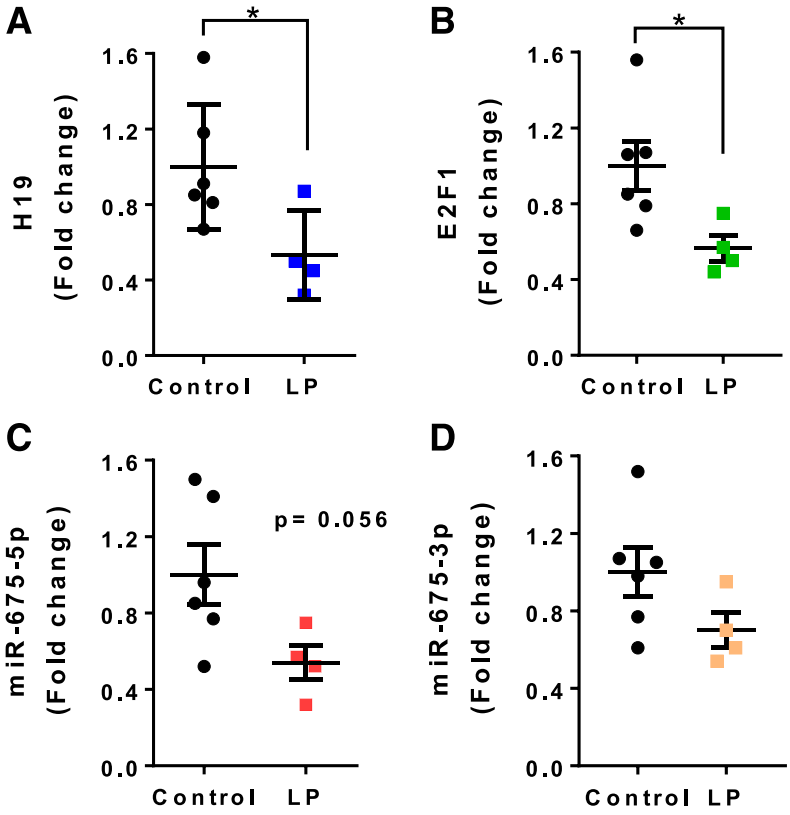


Figure 6—Expression of H19, E2F1, miR-675-5p, and miR-675-3p in islets from offspring born to dams fed an LP diet during gestation and lactation. H19 (*A*), E2F1 (*B*), miR-675-5p (*C*), and miR-675-3p (*D*) levels were measured by quantitative RT-PCR in P10 rats (Control) and P10 offspring born to dams fed an LP diet during gestation (LP) and normalized to those of Ppia (*A* and *B*) or miR-130b-3p (*C* and *D*); results are expressed as fold change. The results are the mean ± SEM of four or six rats. Statistically significant differences from control pups were calculated by using the Student *t* test. $^*P < 0.05$.

To circumvent this issue, adult β-cells were transfected with inhibitors targeting several let-7 family members (Supplementary Fig. 9*E*). This resulted in a significant increase in β-cell proliferation (Fig. 4*B*). In view of these findings, we hypothesized that H19 could induce β-cell proliferation by sequestering let-7, resulting in the de-repression of let-7 targets necessary for cell division. Using the RNAhybrid software (33), we identified several potential let-7 binding sites in the H19 sequence (Supplementary Fig. 11) and generated a mutant lacking the two most likely let-7 target sequences (Supplementary Fig. 12). To investigate whether H19 acts by sequestering let-7, we overexpressed wild-type and mutant H19 (Supplementary Fig. 4*B* and *D*) and observed that the construct lacking the let-7 binding sites fails to trigger proliferation of INS832/13 cells and of dissociated islet cells from adult rats (Fig. 4*C* and *D*). These findings suggest that preventing the interaction of let-7 with its endogenous target(s) may mediate, at least in part, the proliferative effect of H19.

The phosphoinositide 3-kinase (PI3K)–Akt signaling pathway is a critical regulator of β-cell mass (34). We measured the activation of Akt after overexpression of wild-type H19 or of its inactive mutant. We observed an increase in Akt phosphorylation after overexpression of wild-type H19 but not upon overexpression of the inactive mutant (Fig. 5*A* and *B*). Akt protein levels were unchanged (Supplementary Fig. 13). Moreover, blockade of Akt phosphorylation through the use of a pharmacological inhibitor (Fig. 5*C*) prevented H19-induced proliferation of INS832/13 cells (Fig. 5*D*) and of primary rat β-cells (Fig. 5*E*). Similar results were obtained upon inhibition of PI3K activity with LY294002 (Supplementary Fig. 14). These findings indicate that H19 is likely to trigger β-cell proliferation by activating the PI3K-Akt signaling cascade.

In view of the role of H19 in the control of β-cell proliferation, we investigated whether the expression of this lncRNA is modified under conditions associated with changes in β-cell mass. The administration of an LP diet to rats during pregnancy has a major impact on their offspring and results in reduced birth weight, impaired β-cell development, and a smaller β-cell mass (18,35). These features are associated with an increased risk of impaired glucose tolerance and type 2 diabetes during adulthood. We analyzed the expression of H19 and of E2F1 in the islets of 10-day-old pups born to mothers fed the control diet or an LP diet. We found that the levels of both transcripts are reduced in the islets of rat pups born to dams eating an LP diet (Fig. 6*A* and *B*), suggesting that altered levels of E2F1 and H19 may contribute to the

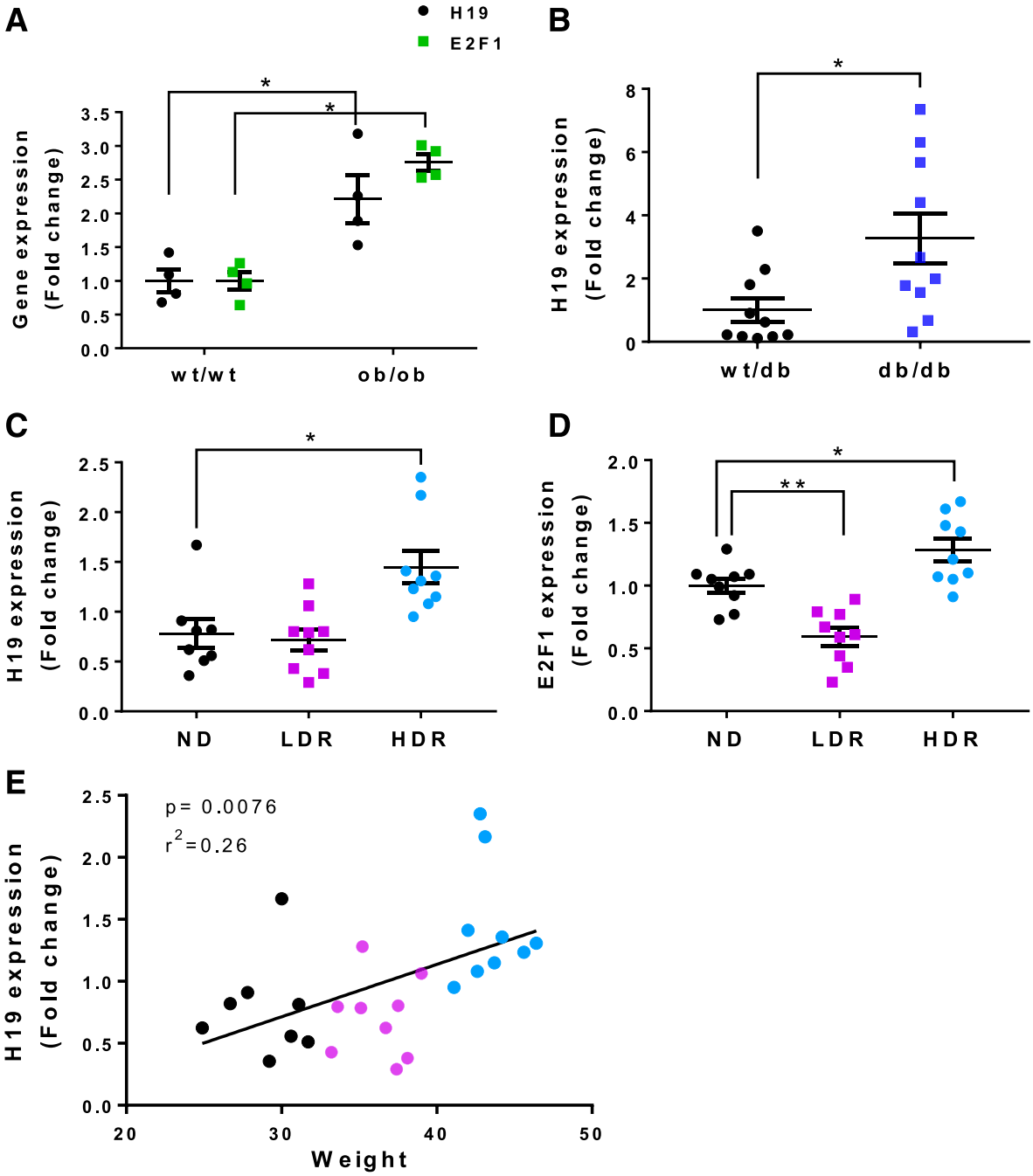


Figure 7—H19 participates in β-cell mass expansion under obesity-associated conditions of insulin resistance. RNA levels were measured by quantitative RT-PCR in islets from wt/wt and ob/ob mice (*A*), from wt/db and db/db mice (*B*), and from mice fed a normal chow diet (ND) or an HFD (LDR and HDR) (*C* and *D*). *E*: Linear regression analysis between H19 level and weight before we sacrificed mice fed the ND or the HFD. The black dots represent values in rats fed the ND; the purple dots, LDR; and blue dots, HDR. Statistically significant differences from the control mice (wt/wt, wt/db, or those fed the ND) were calculated by using the Student *t* test (*A* and *B*), the Kruskal-Wallis test with a Dunn post hoc test (*C*), or one-way ANOVA with a Dunnett post hoc test (*D*). The *F* test was performed to measure significance in *E*. $^{*}P < 0.05$, $^{**}P < 0.01$.

decrease in β-cell mass in these offspring. The levels of miR-675-5p and miR-675-3p also tended to be reduced, but the decrease did not reach statistical significance (Fig. 6*C* and *D*). Let-7a was, by contrast, slightly increased (Supplementary Fig. 15), potentially contributing to a reduction in neonatal β-cell proliferation.

Ob/ob mice, which are leptin deficient, become severely obese but remain normoglycemic as the result of a compensatory expansion of their β-cell mass (36,37). We found that the islets of ob/ob mice contain higher levels of H19 than do islets from their wild-type littermates (Fig. 7*A*). We also observed a similar increase in the expression

of this lncRNA in the islets of insulin-resistant and severely obese db/db mice that lack the leptin receptor and become diabetic starting at ~6–8 weeks of age (Fig. 7*B*). Note that an analogous trend in upregulation was found for E2F1 (data not shown) (38–40). To assess whether similar changes in H19 expression can be observed under conditions of acquired obesity, we measured the level of this lncRNA in the islets of mice fed the HFD for 8 weeks (19). The animals were classified in two groups according to their response to the diet: *1*) LDR, which presented mild obesity, insulin resistance, and normoglycemia, and *2*) HDR, which had much higher obesity, insulin resistance, and hyperglycemia, but also significant increases in β-cell proliferation and β-cell mass (19). We found H19 and E2F1 levels to be increased in the islets of the HDR group (Fig. 7*C* and *D*). Moreover, we observed a positive correlation between weight gain and H19 levels (Fig. 7*E*). Our data suggest that in the face of insulin resistance, re-expression of H19 may contribute to compensatory β-cell mass expansion during adulthood in order to achieve organismal glucose homeostasis.

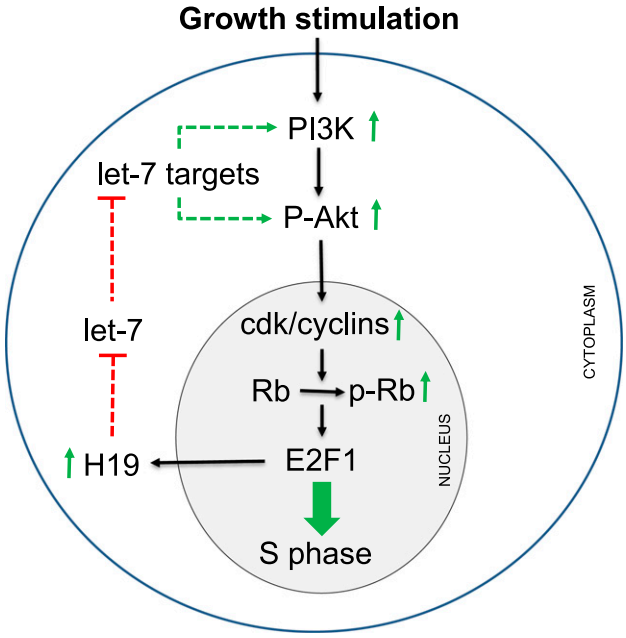


Figure 8—Model representing our view of the role of H19 in the control of β-cell proliferation. We propose a positive feedback loop, whereby growth stimuli activate PI3K and trigger Akt phosphorylation (P-Akt). This induces the activation of cyclin–cdk complexes that phosphorylate retinoblastoma tumor suppressor protein (Rb), causing the release and activation of E2F1. This results in an increase in the expression of H19, which binds let-7, relieving the repression of these miRNA targets and further enhancing the activation of the PI3K/Akt pathway and cell cycle entry. Solid arrows indicate proven interactions; dotted lines represent putative mechanisms inferred from our observations. p-Rb, phosphorylated retinoblastoma tumor suppressor protein. Green lines indicate stimulatory effects and red lines, inhibitory effects.

DISCUSSION

Increasing evidence points to lncRNAs as integral components of the transcriptional machinery controlling pancreas development and β-cell function (15,16,41,42). Although some lncRNAs have already been functionally linked to islet embryonic development (41) and to regulation of β-cell activities in adults (41), so far none of these transcripts have been formally linked to postnatal maturation of insulin-secreting cells, a critical process for the acquisition of adult β-cell function. A better understanding is needed of the events underlying the maturation of β-cells in order to unravel the developmental origins of a predisposition to diabetes and would be a leap forward in the engineering of functional insulin-secreting cells for use in the treatment of diabetes.

In this study we detected 5,496 lncRNAs expressed in newborn rat islets, 896 of which were upregulated and 1,018 downregulated upon postnatal islet maturation. H19, an lncRNA conserved between rodents and humans, is one of the most strongly downregulated transcripts during the postnatal period in islet and FACS-sorted β-cells.

The Igf2/H19 locus plays an important role in embryonic development and growth control (43). H19 is highly expressed during embryogenesis and is downregulated after birth in most tissues except skeletal muscle and the heart (44). We found that the transcription factor E2F1 is downregulated throughout β-cell maturation with kinetics superposing those of H19. E2F transcription factors play an important role in regulating cell survival and proliferation (45), and E2F1$^{-/-}$ mice display impaired postnatal β-cell proliferation and maturation, resulting in an overall reduction of pancreatic size and in glucose intolerance during adulthood (45). Moreover, ectopic expression of E2F1 in adult β-cells increases the proliferation of insulin-secreting cells both in vitro and in vivo (46). We found that silencing E2F1 causes a downregulation of H19 in immature islets, pointing to a prominent impact of this transcription factor on the control of H19 expression during the postnatal period.

Our data indicate that H19 is not involved in the regulation of insulin secretion but decisively affects β-cell proliferation. Indeed, silencing this lncRNA reduces β-cell proliferation in newborn rats, whereas restoring elevated levels of H19 promotes proliferation of adult β-cells. This proliferative effect necessitates the activation of Akt and of PI3K, but the events triggering this signaling pathway remain to be fully elucidated. H19 has been proposed to mediate all H19 effects by modulating the activity of a group of miRNAs, either by sequestering them (31), by controlling miRNA maturation (47), or by directly generating two miRNAs (miR-675-5p and miR-675-3p) encoded in the first exon of the lncRNA (30). The loss of the proliferative ability of H19 in the absence of Ago2, a core component of the RNA-induced silencing complex that is essential for miRNA function (29), corroborates the hypothesis of miRNA involvement in H19 action. These findings confirm a role for Ago2 in the induction of β-cell

proliferation (48). In these experiments we did not observe a significant decrease in proliferation under control conditions, probably because a transient reduction of Ago2 expression may not be sufficient to affect basal proliferation. The levels of miR-675-5p and miR-675-3p are tightly linked to the expression of H19 during the entire postnatal period. However, blockade of miR-675-5p or of miR-675-3p does not affect proliferation of β-cells from newborns, indicating that H19 acts via a different mechanism. H19 acts in skeletal muscle by sequestering let-7 (31,49). Several observations in this study suggest that a similar mechanism may operate in β-cells. Indeed, blockade of let-7 increases adult β-cell proliferation and removal of two let-7 binding sites in the sequence of H19 reduces the proliferative ability of the lncRNA. Our data do not exclude the interaction of let-7 with other domains of H19 or the binding of other miRNAs. The repressive activity of let-7 is higher in islets from adult rats, which contain much less H19 than do islets from newborn rats. Because the levels of most let-7 family members are not modified upon β-cell maturation (10), these findings suggest that H19 may control the availability of let-7. A general schema summarizing the proposed role of H19 in the regulation of β-cell proliferation is shown in Fig. 8. Our model does not preclude the existence of additional mechanisms through which H19 can modulate β-cell proliferation. Indeed, H19 may regulate enhancer and promoter activities, resulting in changes in the expression of neighboring genes such as *Igf2*, *Cdkn1c*, and *Kcnq1*, and H19 may play an important role in the control of chromatin structure and of imprinting regions.

Our data provide congruent grounds for the idea that H19 sustains β-cell expansion in newborns and is required to achieve adequate postnatal β-cell mass. Thus, exposure during gestation to environmental conditions that affect the expression of H19 could potentially result in a β-cell mass that is insufficient to cover the organism's needs. Indeed, we found that the level of H19 is reduced in the islets of offspring from dams eating the LP diet throughout gestation and lactation; these offspring display an increased risk of impaired glucose tolerance and type 2 diabetes during adulthood (35). Consistent with these observations, reduced H19 levels were previously observed in islets from offspring of dams with gestational diabetes mellitus (50), another adverse intrauterine condition linked to reduced β-cell proliferation and diabetes susceptibility during adulthood (51).

Adult β-cells undergo very little turnover and have low basal replication rates, which sustain metabolic homeostasis under normal conditions. However, pregnancy, obesity, and β-cell recovery after injury are examples of conditions in which the systemic demand for insulin increases. To successfully compensate for the relative insulin deficiency that occurs during these metabolically pressured conditions, both proliferative and survival pathways are activated in insulin-secreting cells in order to maintain blood glucose homeostasis and prevent the onset of diabetes (5). In this study we observed the upregulation of H19 in different mouse models characterized by compensatory β-cell mass expansion in response to obesity and insulin resistance. These findings suggest that restoration of higher levels of H19 during adulthood in response to obesity-associated insulin resistance may contribute to the expansion of the β-cell mass in order to counterbalance the diminished sensitivity of insulin target tissues and to prevent the onset of diabetes. Future studies involving the generation of gain-of-function and loss-of-function mouse models will be needed to corroborate this hypothesis.

Our observations uncover an important role for H19 in the control of β-cell proliferation in newborn and adult rodents and shed new light on the mechanisms that potentially contribute to diabetes susceptibility in the offspring of dams kept under deleterious dietary conditions during pregnancy and lactation. The level of H19 was reported to be higher in β-cells of young children than in those of adult donors, indicating that similar changes may occur in humans (52). Moreover, despite differences in the nucleotide sequence, we obtained evidence that human H19 can exert proliferative effects similar to those of rat H19. However, the mechanisms driving rodent β-cell replication are often challenging to translate to humans. Thus careful examination of the impact of H19 in primary human β-cells will be required before definitive conclusions can be drawn about the role of this lncRNA in humans.

Future efforts will be needed to fully capture the complexity of the events triggering compensatory β-cell expansion under physiological (pregnancy) and pathophysiological (obesity) conditions. This knowledge will contribute to the design of novel means to combat β-cell failure that occurs in type 2 diabetes.

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