MicroRNA Expression Is Required for Pancreatic Islet Cell Genesis in the Mouse

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OBJECTIVE—The generation of distinct cell types during the development of the pancreas depends on sequential changes in gene expression. We tested the hypothesis that microRNAs (miRNAs), which limit gene expression through posttranscriptional silencing, modulate the gene expression cascades involved in pancreas development.

RESEARCH DESIGN AND METHODS—miRNAs were cloned and sequenced from developing pancreata, and expression of a subset of these genes was tested using locked nucleic acid in situ analyses. To assess the overall contribution of miRNAs to pancreatic development, Dicer1, an enzyme required for miRNA processing, was conditionally deleted from the developing pancreas.

RESULTS—Sequencing of small RNAs identified over 125 miRNAs, including 18 novel sequences, with distinct expression domains within the developing pancreas. To test the developmental contribution of these miRNAs, we conditionally deleted the miRNA processing enzyme *Dicer1* early in pancreas development. Dicer-null animals displayed gross defects in all pancreatic lineages, although the endocrine cells, and especially the insulin-producing β-cells, were most dramatically reduced. The endocrine defect was associated with an increase in the notch-signaling target Hes1 and a reduction in the formation of endocrine cell progenitors expressing the Hes1 target gene neurogenin3.

CONCLUSIONS—The expression of a unique profile of miRNAs is required during pancreas development and is necessary for β-cell formation. ***Diabetes*** **56:2938–2945, 2007**

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LNA, locked nucleic acid; miRNA, micoRNA; Pdx-1, pancreatic duodenal homeobox-1; RIP2, rat insulin promoter 2.

Pancreatic organogenesis begins at embryonic day (e)9.5 in the mouse embryo with the budding of the dorsal anlagen from the prospective gut endoderm (1). Pancreatic duodenal homeobox-1 (Pdx-1) appears in the same area 1 day earlier and is expressed in all pancreas progenitors (2,3). As the pancreatic program continues, the expression of the basic helix-loop-helix factor neurogenin3 initiates the differentiation of the endocrine cells. Neurogenin3 expression peaks coincidently with the "secondary transition," a time of rapid endocrine cell generation that occurs between e13.5 and e14.5 (4,5). The cell-specific factors *Onecut1*, *Tcf1*, *Tcf2*, and *Sox9*, as well as the Notch signaling pathway, regulate *Neurog3* expression during development (5–9).

Many tissues of the developing organism express microRNAs (miRNAs), which are small (~20 nt) RNAs that mediate posttranscriptional gene silencing by interacting with the RNA-induced silencing complex and binding to the 3′ untranslated region of their cognate messenger RNA targets (10,11). Here, we hypothesize that miRNAs are expressed in the developing pancreas, that they are necessary for the normal development of pancreatic β-cells, and that they may be involved in regulating genes important for normal pancreas morphogenesis. Ultimately, an intimate understanding of how miRNAs govern pancreas development may be necessary for stem cell–based therapies for all forms of diabetes.

RESEARCH DESIGN AND METHODS

Mice were housed on a 12-h light/dark cycle in a controlled climate according to the University of California San Francisco regulations. Timed matings were carried out with e0.5 being set as midday of the day of discovery of a vaginal plug. Control mice in this study were double heterozygous for the *Dicer1* (12) and *Pdx1*-Cre (3) alleles. CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA).

Cloning of small RNAs from the developing mouse pancreas. A total of 118 embryos from 10 CD-1 mice were removed from pregnant mothers at e14.5, and embryonic pancreata were dissected and isolated from the spleen and other surrounding tissue. RNA was then isolated using Trizol and the manufacturer's protocols (Invitrogen, Carlsbad, CA), with the exception that the RNA pellet was not washed with 70% ethanol following isopropanol precipitation. We obtained 189 μg of RNA from 118 pancreata. miRNA cloning was carried out as previously described by Lau et al. (13). Briefly, linkers were then ligated onto the 3′ and 5′ ends of size-fractionated RNA, and RNA was reverse transcribed and amplified using PCR. The PCR products were restriction digested with *Ban*I and concatermized with T4 DNA ligase, and gel-purified fragments ranging from 600 to 800 bp were TOPO cloned (Invitrogen). Plasmid DNA was isolated from 100 colonies and sequenced using M13 primers. Sequences were deconvolved using a Java applet (available upon request from the authors). Sequences were then aligned against the miRNAome (available at http://microrna.sanger.ac.uk/sequences/), and unknown sequences were aligned against genome. Regions of high sequence homology were then folded using RNA shapes (14), and those that folded into hairpin loops were considered miRNAs.

Immunofluorescence analysis. Embryonic tissue was harvested and immediately fixed at 4°C with 4% paraformaldehyde. Tissues were then dehydrated through 50, 70, 95, and 100% ethanol and then through xylene. Tissues were then impregnated with paraffin (Paraplast; Fisher Life Sciences) at 60°C under a vacuum and then embedded in cassettes. Tissue was then cut into 5 μmol/l sections and adhered to glass slides (Superfrost Plus; Fisher Life Sciences). For immunoanalyses, tissue was first deparafinized with xylene and then rehydrated through a reverse ethanol series to ddH_20. Antigen retrieval was then carried out by microwave in a citrate buffer (Biogenex, San Ramon, CA). Tissues were then blocked at room temperature with 5% goat serum and goat anti-mouse IgG (1:30; MP Biomedicals/Cappel, Solon, OH) in PBS. Incubations with the primary antibodies were performed overnight at 4°C using standard techniques in PBS containing 1% goat serum with the following primary antisera: 1:2,000 guinea pig anti–pdx-1 (15), 1:3,000 guinea pig anti-glucagon (Linco, St. Charles, MO), 1:4,000 mouse anti-glucagon (Sigma-Aldrich, St. Louis, MO), 1:3,000 guinea pig anti-insulin (Linco), 1:2,000 guinea pig anti-neurogenin3 (15), 1:200 hamster anti-mucin (NeoMarkers, Fremont, CA), 1:200 mouse anti-synaptophysin (Biogenex), 1:1,000 rabbit anti-amylase (Sigma), 1:1,000 rabbit anti-Hes1 (a gift from T. Sudo, Toray Scienfic, Osaka, Japan [16]), 1:150 rabbit anti–activated caspase-3 (Cell Signaling, Danvers, MA), 1:200 rabbit anti-*MKi67* (Novocastra, Newcastle upon Tyne, U.K.), or 1:100 rabbit anti-Dicer (Santa Cruz Biotechnology). Secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA) and used at 1:200 (fluorescein isothiocyanate) to 1:400 (Cy3) dilutions, and coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Slides were imaged on a Leica TCS SL confocal microscope using sequential scanning of dyes. Morphometric analyses were done by serially sectioning the entire pancreas followed by staining and counting every 12th section for e18.5 embryos or every 8th section for e15.5 embryos. DAPI-stained cell nuclei were counted using the cell-counting application, and cytoplasmic staining was quantified using the integrated metamorphic analysis, both in the Metamorph suite of programs (version 7; Molecular Devices, Union City, CA). Statistical analyses were done using PRISM (version 4; Graphpad Software, San Diego, CA), and either *t* tests or nonparametric Mann-Whitney *U* tests were used. *P* values of <0.05 were considered statistically significant.

Locked nucleic acid in situ hybridization. Embryos (e14.5) were harvested and fixed overnight at 4°C in 4% paraformaldehyde. Following fixation, embryos were briefly washed with PBS and transferred to 30% sucrose in diethylpyrocarbonate-treated PBS and kept for an additional 24 h at 4°C. Embryos were then mounted in Tissue-Tek O.C.T. (Sakura Finetek, Torrance, CA) and frozen on dry ice. Embryos were sectioned with a cryostat into 10-μm sections and adhered to slides (Superfrost, Fisher Scientific), and slides were kept at −80°C until use. Slides were removed from freezer and dried for 1 h at room temperature. Sections were then refixed for 10 min at room temperature in 4% paraformaldehyde and then washed with RNase-free PBS. Slides were then incubated for 2 min with 1 μg/ml proteinase K (Roche Applied Science, Indianapolis, IN) in 50 mmol/l Tris, pH 7.5, containing 5 mmol/l EDTA. Slides were then washed again in PBS and acetylated (1.2% triethanolamine, 0.018N HCl, and 0.25% acetic anhydride) for 10 min at room temperature. Slides were then washed again before prehybridization in 50% formamide, 5 × sodium chloride–sodium citrate, 0.1% Tween-20, 9.2 mmol/l citric acid, 50 μg/ml heparin, and 500 μg/ml yeast transfer RNA for 2 h at 60°C. Antisense locked nucleic acid (LNA) probes were ordered from Exiqon (Vedbaek, Denmark) and were subsequently labeled using the DIG Oligonucleotide 3′-End Labeling Kit (Roche Applied Science) and the manufacturer's protocol. Labeled LNAs were then diluted to 50 μl with RNase-free water and separated from unincorporated label using Microspin G-25 columns (Amersham Biosciences, Piscataway, NJ). A total of 1 pmol of DIG-labeled LNA was then diluted into 250 μl of hybridization buffer, briefly heated to 80°C, and then iced. Labeled probes were then applied to the slides and allowed to hybridize overnight at 60°C. Slides were then washed for 3 h at 60°C in 0.2 × sodium chloride–sodium citrate and incubated with alkaline phosphatase–conjugated sheep anti-DIG antibody (Roche Applied Sciences) in 100 mmol/l Tris, pH 7.5, 150 mmol/l NaCl with 1% goat serum (Invitrogen) overnight at 4°C. Slides were then washed extensively in the aforementioned buffer and then equibrated with 100 mmol/l Tris, pH 9.5; 100 mmol/l NaCl; and 50 mmol/l MgCl2 before alkaline phosphatase reaction in the same buffer with 1:50 dilution of NBT/BCIP stock (Roche Applied Sciences) for 1–3 days. For *Pdx1* staining, sections were then washed with PBS and endogenous peroxidases were quenched with a 15-min incubation in methanol containing 0.6% hydrogen peroxide. Slides were then incubated with a 1:3,000 dilution of guinea pig anti–Pdx-1 overnight at 4°C. Slides were then washed and incubated with 1:200 dilution of biotinylated goat anti–guinea pig secondary antibody (Vector Labs, Burlingame, CA). Slides were then incubated with ABC solution and then reacted with diaminobenzidine (VECTASTAIN; Vector Labs). Sections were dehydrated and coverslips mounted and imaged on a Zeiss Axio Imager.A1 equipped with an AxioCam MrC5 and using Zeiss AxioVision software (Thornwood, NY).

Quantitative PCR. Embryonic pancreata were harvested at e12.5 and immediately placed in 0.5 ml of Trizol (Invitrogen), and RNA was extracted using the manufacturer's protocols. A total of 250 ng of total RNA was subjected to reverse transcription using random hexamers, Superscript III (Invitrogen), and the manufacturer's suggested reaction conditions. Complementary DNA was then amplified in quadruplicate using Taqman with an intron-spanning primer–probe set for *Hes1* (forward, AAG GCA GAC ATT CTG GAA ATG AC; reverse, CGC GGT ATT TCC CCA ACA C; and probe, AGA TGA CCG CCG CGC TCA GC). *Hes1* expression was normalized to Taqman quantified mouse glucuronidase expression in the individual pancreatic cDNA samples (17).

RESULTS

miRNAs are expressed in the developing pancreas. To determine their expression profile and identify novel miRNAs, small RNAs were cloned and sequenced from the pancreata of e14.5 mouse embryos using standard miRNA cloning and sequencing protocols (13). In total, 107 known mouse miRNAs were cloned, five of which were evolutionarily conserved but had not previously been found in the mouse (online appendix Table 1 [available at http://dx.doi.org/10.2337/db07-0175]). Unknown sequences were then aligned with the genome, and the surrounding, conserved genomic regions were folded into hairpins (online appendix Table 2). Using this approach, 18 novel mouse miRNAs were discovered, including some that lie in potential multicystronic miRNA transcripts (online appendix Table 2). In addition, significant differences in a number of miRNA sequences were observed from those annotated in miRBase (18). For example, multiple copies of mmu-miR-720 and mmu-miR-411 differed significantly from the sequences in miRBase (Table 1). Differences identified in the 5′ end of several miRNA are of particular significance because the majority of miRNA target prediction algorithms rely on Watson-Crick base pairing of nucleotides 2–7 from the 5′ end of the miRNA; thus, one – or two–base pair differences at the 5′ end could completely change the target profile of any miRNA (19,20).

Many of the miRNAs that were cloned here have not been previously detected at high levels in other tissues; therefore, we hypothesized that they might play specific roles in pancreas development. To assess the expression domain of these miRNAs during pancreas development, in situ hybridization with highly specific LNA probes was carried out for a few of these highly expressed miRNAs (Fig. 1) (21). We first tested mmu-miR-375 because it was overrepresented in our cloning experiment (online appendix Table 1) and has previously been demonstrated to be expressed in, and important for, pancreatic β-cell function. In situ analyses showed localization of miR-375 in the pancreatic ductal epithelium (i.e., the progenitor cells from which the endocrine and exocrine cells differentiate) at e14.5 (Fig. 1*A* and *B*). Furthermore, Fig. 1*B* demonstrates colocalization of miR-375 with Pdx-1 in the e14.5 pancreas; thus, in addition to regulating insulin secretion in mature β-cells (22), miR-375 expressed in the progenitor cells may also be important during normal development. We also performed in situ analyses for three other overrepresented miRNAs. Both miR-503 and miR-541 were expressed in a pattern similar to that in miR-375 in progenitor cells in e14.5 pancreata, while miR-214 was expressed throughout the gut (Fig. 1*C–H*).

miRNA expression is necessary for pancreas organogenesis. To determine the importance of miRNAs in pancreatic development, the second RNase III domain of

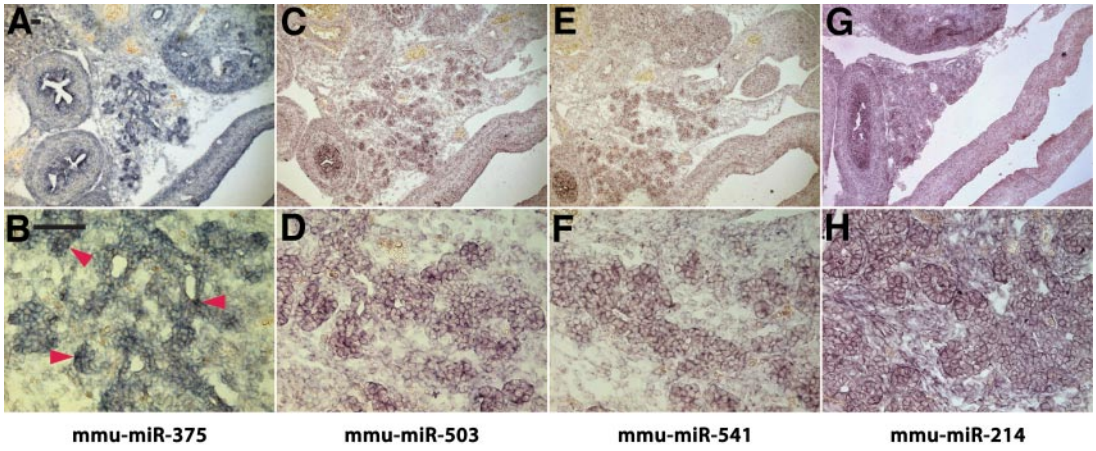

FIG. 1. In situ hybridization for developmentally expressed miRNAs. Embryonic pancreata were harvested at e14.5, fixed, embedded, and sectioned. Tissues were then subjected to in situ hybridization with DIG-labeled LNA probes. MiR-375 (*A* and *B*), miR-503 (*C* and *D*), and miR-541 (*E* and *F*) were specifically enriched within the ductal structures within the developing pancreas. In contrast, the intronic miR-214 was observed fairly uniformly across the gut and pancreatic epithelium (*G* and *H*). *A* and *B*: Pdx-1 immunostained following in situ analyses and intense Pdx-1 immunoreactivity (brown diaminobenzidine [DAB] staining) was observed in a subset of miR-375–positive cells lining the ducts (*arrowheads*). Scale bars = 50 μm. (Please see http://dx.doi.org/10.2337/db07-0175 for a high-quality digital representation of this figure.)

Dicer1 (3,12) was conditionally ablated in all pancreatic cells starting at e9.5 using *Pdx1*-Cre–mediated deletion (3). Biogenesis of miRNAs requires the sequential activity of two double-stranded RNA–specific endonucleases, Drosha and Dicer1, that act in the nucleus and cytosol, respectively (23). In mammals, removal of the premiRNA hairpin loop by a single Dicer homolog is a prerequisite for interaction of the mature sequence with RNA-induced silencing complex. Therefore, loss of the RNase III domain of *Dicer1* blocks the formation of miRNAs (12,24).

The pancreas-specific *Dicer1* knockout mice survived until birth but failed to grow and died by P3 (Fig. 2*A* and *B*). Pancreata from e18.5 null mice had a dramatic reduction in the ventral pancreas, as well as a reduction in the overall epithelial contribution to the dorsal pancreas (Fig. 2*D*). The remaining tissue in the *Dicer1*-null pancreata was comprised of clumps of disorganized epithelium interspersed with large vacuous areas and dilated ducts filled with impactions of debris (Fig. 2*F* and *H*).

Early pancreatic miRNA expression is necessary for normal pancreatic cell differentiation. Cell type–specific antisera were utilized to determine the effects of Dicer1 loss on specific pancreatic lineages. Costaining of the e18.5 pancreas for synaptophysin, an endocrine marker, and the exocrine enzyme amylase revealed reductions in both the number of stained cells and the intensity of staining for both markers (Fig. 3*A* and *B*). Immunoreactivity for the duct marker mucin1 remained unchanged in the knockout animal at this stage, but the debris filling the dilated ducts stained strongly for both mucin and amylase (Fig. 3*B* and *J*, *arrow heads*). Staining for specific hormones demonstrated a significant loss (94%) (Fig. 3*K*) of insulin-producing β-cells in the knockout animals and 79, 95, and 86% losses of α, δ, and pancreatic polypeptide cells, respectively (Fig. 3*K*), but no compensatory increase in ghrelin-producing ε-cells (Fig. 3*C*–*J*) (25). The differences in the reductions of the various endocrine cell types were significant, as demonstrated by significant changes in

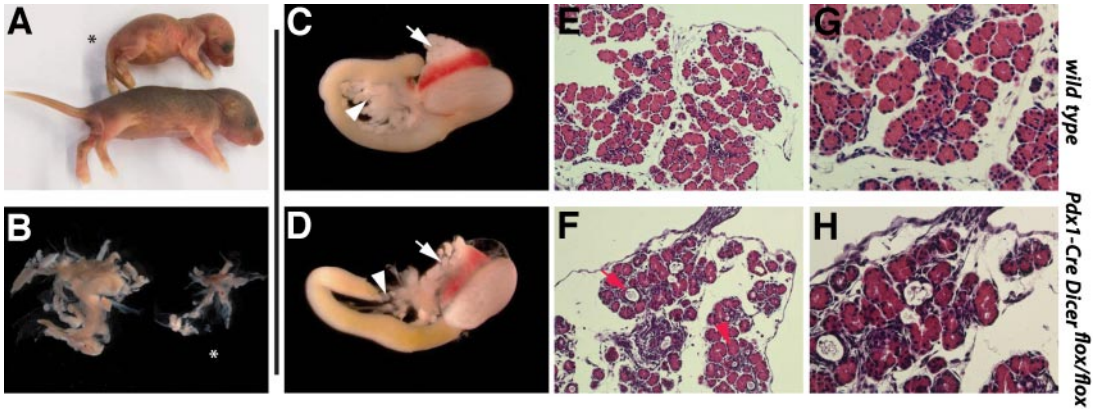

FIG. 2. The gross phenotype of the *Pdx-Cre Dicer1*$^{flox/flox}$ mouse. *A* and *B*: The runting of the mutant (*) animal and pancreas, respectively, at day P3. *Pdx-Cre Dicer1*$^{flox/flox}$ mice (*B*, *D*, *F*, and *H*) and their littermates (*A*, *C*, *E*, and *G*) were harvested at e18.5, and pancreas, stomach, attached spleen, and duodenum were removed (*C* and *D*). Tissues were then paraffin embedded, sectioned, and stained with hematoxylin and eosin (*E*–*H*). Dramatic reductions in both ventral (*arrowhead*) and dorsal (*arrow*) pancreas can be observed in the *Pdx-Cre Dicer1*$^{flox/flox}$ animals (cf. *C* and *D*). In addition, hematoxylin and eosin staining shows large vacuous spaces within the pancreas with general disorganization of the pancreatic morphology, including dilated ducts (*F*, *arrow*), a decrease in exocrine tissue (*F*, *arrowhead*), a decrease in eosin staining, and an increase in the centroacinar spaces (cf. *E*–*G*, and *H*). Scale bars (*E*–*H*) = 50 μm. (Please see http://dx.doi.org/10.2337/db07-0175 for a high-quality digital representation of this figure.)

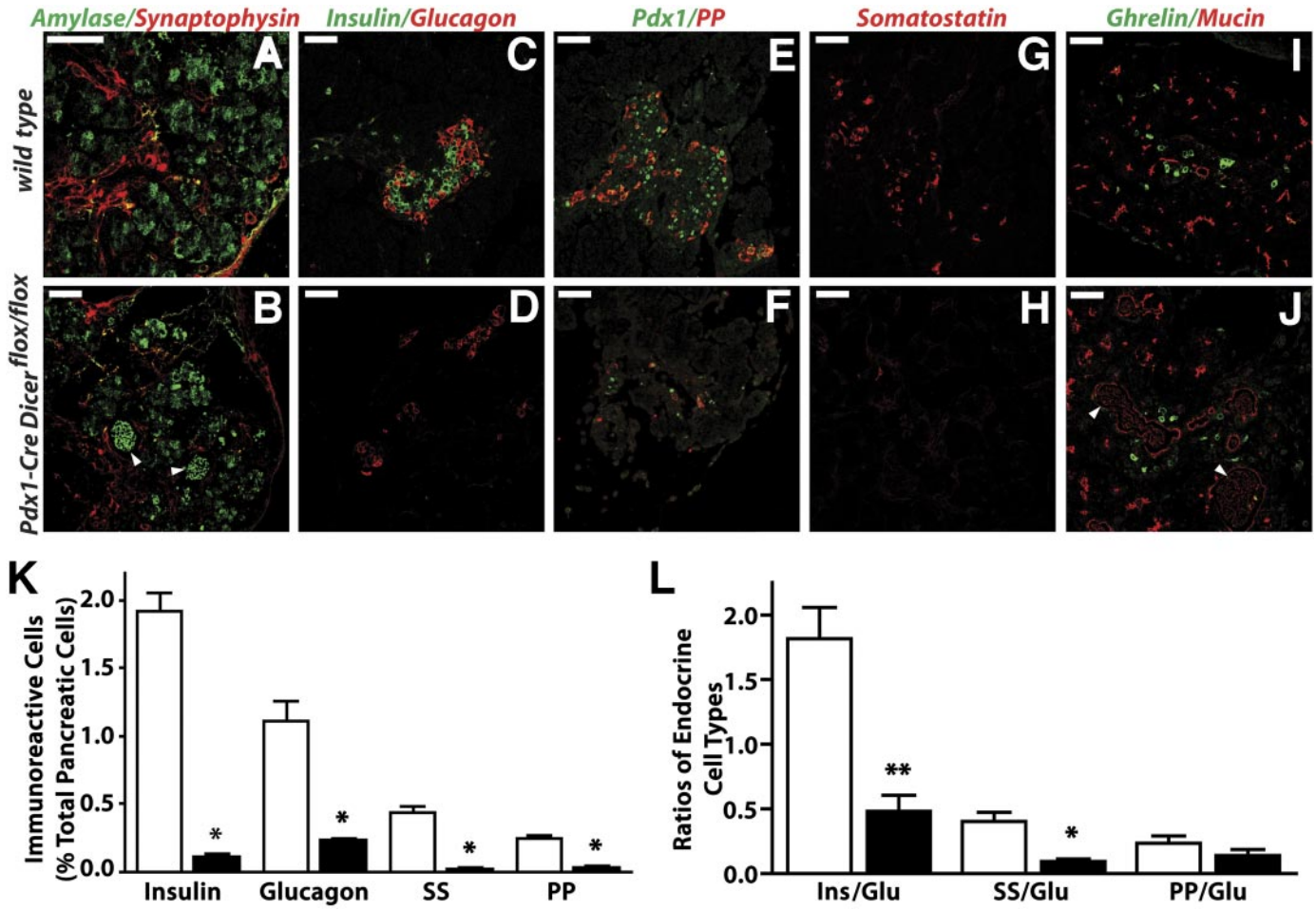

FIG. 3. Immunohistochemical characterization of the *Dicer1*$^{flox/flox}$ mouse. Pancreata from the *Pdx-Cre Dicer1*$^{flox/flox}$ mice displayed reductions in both synaptophysin (red) and amylase (green) immunoreactivity (cf. *A* and *B*). Pancreatic ducts were dilated in the mutant animals and filled with large amylase and mucin immunoreactive impactions (*B* and *J*, arrowheads). Endocrine differentiation was disrupted in the *Pdx-Cre Dicer1*$^{flox/flox}$ pancreas with a near-complete loss of insulin-producing β-cells (*C*, *D*, and *K*, green), a decrease in glucagon-producing α-cells (*C*, *D*, and *K*, red), a loss of PP cells (*E*, *F*, and *K*, red) and Pdx-1–positive cells (*E* and *F*, green), and a near-complete loss of somatostatin-producing δ-cells (*G*, *H*, and *K*, red). There was no change in ghrelin-positive ε-cells (*I* and *J*, green) observed in the mutant pancreas, and mucin staining (*I* and *J*, red) indicated the presence of a large number of secreting duct cells (*I* and *J*). Cells expressing distinct islet hormones were counted as described in the RESEARCH DESIGN AND METHODS section of the text and expressed as percent of total pancreatic cells (*K*) and ratios of islet cell types (*L*). Data are expressed as means ± SE. Asterisks indicate statistically significant differences between wild-type (□) and *Pdx-Cre Dicer1*$^{flox/flox}$ (■) littermates (P* < 0.05; ***P* < 0.005) (*n* = 4). Scale bars = 50 μm. (Please see http://dx.doi.org/10.2337/db07-0175 for a high-quality digital representation of this figure.)**

the ratios of distinct endocrine cell types in the *Pdx-Cre Dicer1*$^{flox/flox}$ relative to their wild-type littermates (Fig. 3*L*). Pdx-1 immunoreactivity, which is restricted predominantly to β-cells at this stage, was also decreased within e18.5 knockout pancreata (cf. Fig. 3*E* and *F*).

To distinguish between a role in β-cell genesis and in maintenance or proliferation of mature β-cells, we also specifically removed *Dicer1* from differentiated β-cells by crossing the *Dicer1*^{flox} mice with mice carrying a *Cre* allele driven by the rat insulin promoter 2 (RIP2) (26) (Fig. 4). β-Cell–specific Dicer1 knockout animals survived and their islets cells appeared morphologically normal at 8 months of age (cf. Figs. 4*A* and *B*). In addition, to exclude the possibility that Dicer1 loss specifically impacts the maintenance or proliferation of mature β-cells in the embryo, β-cell counting was performed also at e18.5. Control and RIP2-Cre Dicer$^{flox/flox}$ animals at this developmental stage contained equal numbers of insulin-immunoreactive β-cells (Fig. 4*C*). Finally, we also conditionally ablated *Dicer1* using a neurogenin3-Cre transgene, which causes recombination in all endocrine cells. These animals also exhibit normal islet cell hormone staining at e18.5 (data not shown).

To identify the origins of the Pdx-1–mediated Dicer deletion phenotypes that were observed at e18.5, earlier time points were examined. At e12.5 staining for Pdx-1 and glucagon, markers of undifferentiated pancreatic epithelial progenitors and the earliest differentiated cells in the pancreas, respectively, was not different between the mutant and wild-type pancreata (Fig. 5*A* and *B*). However, by e15.5 knockout embryos lacked Dicer1 immunoreactivity in the Pdx-1–expressing cells (online appendix Fig. 1) and had significant reductions in pancreatic size and ductal branching (Fig. 5*C–H*). At this stage in development, Pdx-1 antisera weakly stains the majority of cells lining the ducts and strongly stains the nascent β-cells, and neurogenin3 expression marks the committed endocrine progenitors. In the knockout animals, a decrease in the number of cells staining brightly for Pdx-1 (Fig. 5*D*) and a significant (89%) reduction in neurogenin3-expressing

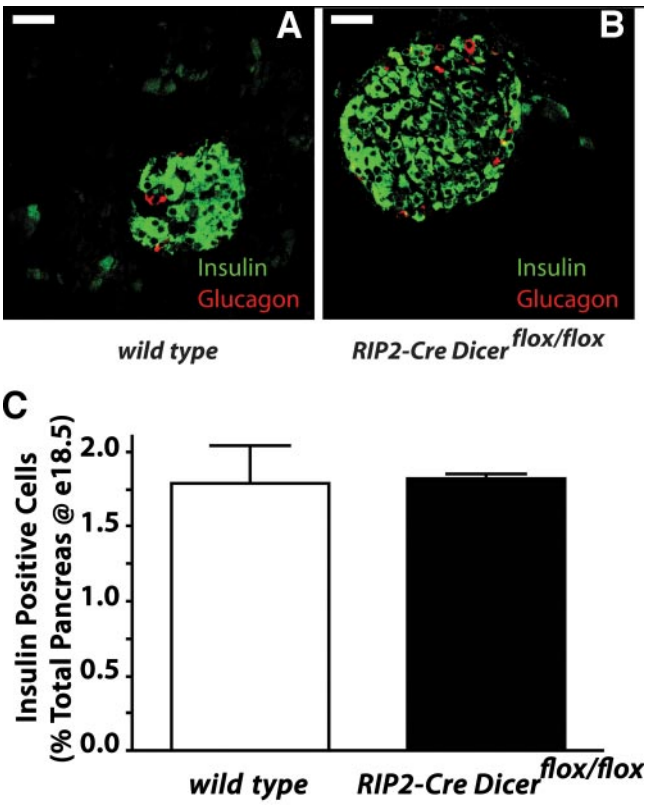

FIG. 4. *RIP2*-Cre *Dicer1*$^{flox/flox}$ mice show normal islet insulin (*A* and *B*, green) and glucagon (*A* and *B*, red) staining at 8 months of age and no difference in the number of insulin-positive cells at e18.5 relative to wild-type (*C*). Data are expressed as means ± SE (n = 3). (Please see http://dx.doi.org/10.2337/db07-0175 for a high-quality digital representation of this figure.)

cells was observed (Fig. 5*D*, *F*, *H*, and *I*), indicating a defect in the endocrine differentiation program. Ductal Pdx-1 and mucin1 staining was not grossly altered in the *Dicer1*-null animals at e15.5 (Fig. 4*D* and *H*).

miRNAs regulate the notch pathway and prevent programmed cell death during pancreatic development. Notch signaling inhibits the expression of neurogenin3 and thereby constrains endocrine differentiation in the developing pancreas in a manner analogous to lateral inhibition in the developing nervous system (5,27,28). The notch target gene *Hes1* is expressed in the majority of early pancreatic epithelial cells and strongly represses *Neurog3* gene transcription within those cells (8,16). We hypothesized that specific miRNAs may activate endocrine differentiation by inhibiting components of the Notch signaling pathway, thereby relieving the constraint on neurogenin3 expression. We tested this hypothesis by assessing *Hes1* expression using Taqman and found a modest increase in *Hes1* mRNA (35%) at e12.5 (Fig. 6*K*). Because Hes1 is expressed in the pancreatic mesenchyme as well as the Pdx-1–expressing epithelium, and because Hes1 may be a target for translational regulation, we also stained e12.5 pancreata for Hes1 and observed an increase in Hes1 expression specifically within the Pdx-1 expression domain in *Dicer1*-deficient pancreata (Fig. 6).

In addition to the loss of neurogenin3, starting at e15.5 there was an overall decrease in cells derived from the pancreatic epithelium in the knockout animals (Fig. 2 and data not shown). Since miRNAs can regulate apoptosis during organ development (12,29,30), rates of programmed cell death and cell proliferation were assessed in the pancreatic-specific *Dicer1* knockout animal, using specific antisera for activated caspase 3 and Ki-67 (*MKi67*), respectively. A clearly significant increase (4.6-fold) was observed in total apoptotic cells at e15.5 in pancreata from knockout animals, and this increase appeared to be limited to the epithelial cells, as apoptosis was unchanged in the surrounding mesenchymal cells, which still expressed Dicer (Fig. 6*H*–*J*). Cell proliferation was unchanged (online appendix Fig. 2; counting data not shown).

DISCUSSION

Here, we demonstrated that the developing pancreas expresses a unique complement of miRNAs and that Dicer1, the enzyme that generates these miRNAs, is essential for normal pancreatic development. Many of the miRNAs that were highly expressed in the developing pancreas have not been previously cloned in high numbers or from specific tissues (e.g., miR-503 and miR-541). High expression in the developing pancreas could suggest that these miRNAs play important roles in the developmental program; although, to date we have not identified any targets of these genes with known roles in pancreas development. In contrast, the novel miRNAs identified in this study all had fairly low expression levels as gauged by the number of clones obtained. Despite their low overall abundance, these miRNAs could still play important roles at specific target genes or in cell types at low abundance in the e14.5 pancreata.

Although exocrine and duct cells were also affected, loss of miRNA processing uniquely impaired the development of the endocrine lineage, especially the β- and δ-cells. Two lines of evidence suggest that miRNAs are important for β-cell genesis rather than β-cell maintenance. First, a dramatic reduction in the number of neurogenin3-positive endocrine progenitor cells from which all β-cells are derived was observed at e15.5. Consistent with a reduction in islet cell progenitors, glucagon-, somatostatin-, and pancreatic polypeptide–expressing endocrine cells were also reduced, although ghrelin-expressing cells were not. The relatively less severe α-cell phenotype may be solely a timing issue, as Pdx-1–mediated loss of Dicer1 may not be complete until after the first glucagon-positive cells differentiate, and Pdx-1–null animals do have glucagon-positive cells (31). Alternatively, miRNAs may act downstream of neurogenin3 to influence cell fate decisions, and, in the absence of miRNAs, the few remaining neurogenin3-expressing progenitors may preferentially differentiate into α- and ε-cells.

Second, when *Dicer1* was ablated using the *RIP2-Cre* (or the *Neurog3-Cre*) transgene (Fig. 4), we saw little effect on pancreatic islet cell morphology and no apparent effect on β-cell development (Fig. 4*C*) or β-cell maintenance. A previous study (22) demonstrated that miR-375 normally constrains β-cell insulin secretion in a mytrophin-dependent fashion. Our data here focused on the role of miRNAs in the formation of β-cells and are not at odds with these previous data on islet function. We have shown that islet and β-cell development remains unaffected in the *RIP2-Cre Dicer1*$^{flox/flox}$ mice, but we have not directly tested the glucose-stimulated insulin secretory response of these islets. It is possible that there could be a functional defect in the islets of these animals, although given the proposed inhibitory role of miR-375 on insulin secretion such defects might not result in glucose intolerance or

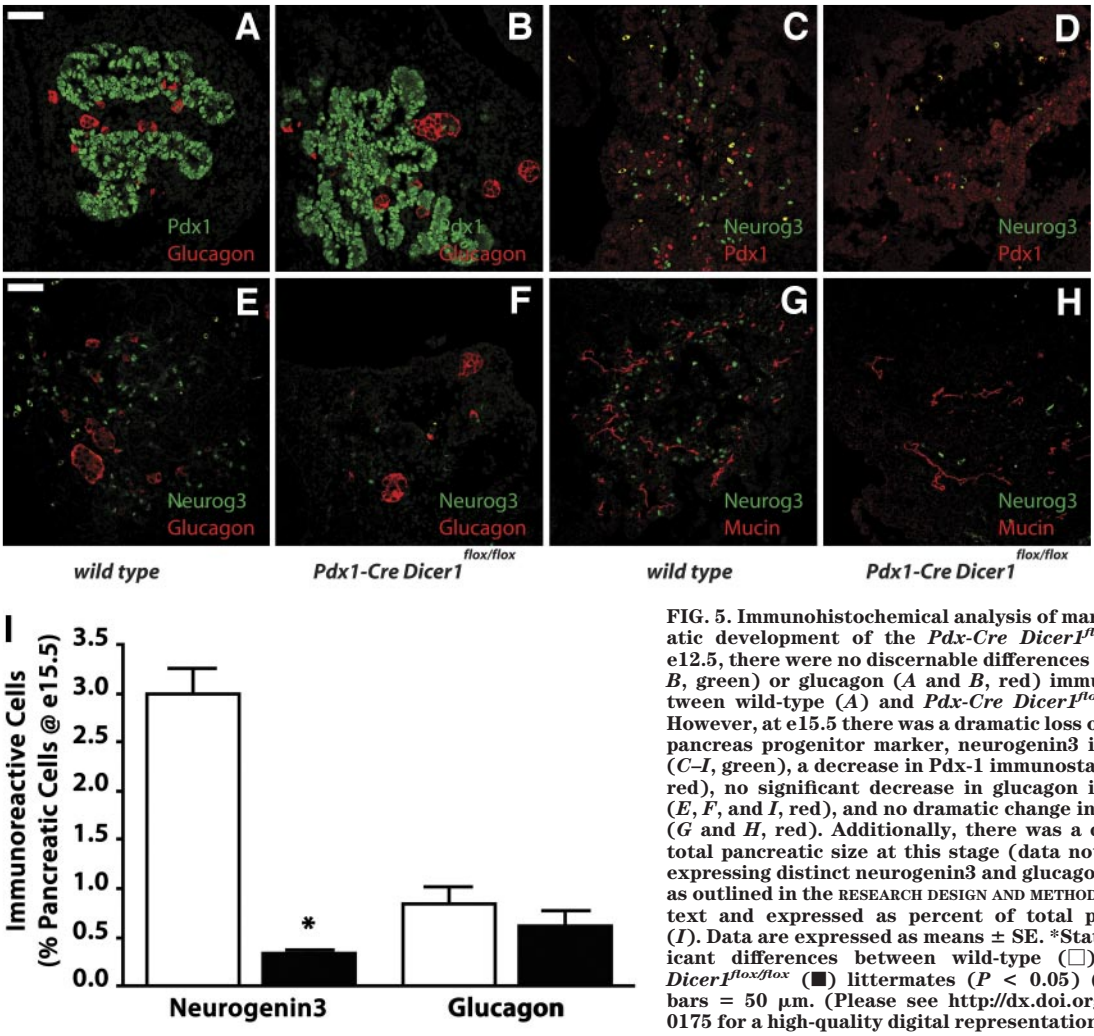

FIG. 5. Immunohistochemical analysis of markers of pancreatic development of the *Pdx-Cre Dicer1*$^{flox/flox}$ mice. At e12.5, there were no discernable differences in Pdx-1 (*A* and *B*, green) or glucagon (*A* and *B*, red) immunostaining between wild-type (*A*) and *Pdx-Cre Dicer1*$^{flox/flox}$ (*B*) mice. However, at e15.5 there was a dramatic loss of the endocrine pancreas progenitor marker, neurogenin3 immunostaining (*C–I*, green), a decrease in Pdx-1 immunostaining (*C* and *D*, red), no significant decrease in glucagon immunostaining (*E*, *F*, and *I*, red), and no dramatic change in mucin staining (*G* and *H*, red). Additionally, there was a decrease in the total pancreatic size at this stage (data not shown). Cells expressing distinct neurogenin3 and glucagon were counted as outlined in the RESEARCH DESIGN AND METHODS section of the text and expressed as percent of total pancreatic cells (*I*). Data are expressed as means ± SE. *Statistically significant differences between wild-type (□) and *Pdx-Cre Dicer1*$^{flox/flox}$ (■) littermates ($P < 0.05$) ($n = 4$). Scale bars = 50 μm. (Please see http://dx.doi.org/10.2337/db07-0175 for a high-quality digital representation of this figure.)

overt diabetes. Furthermore, glucose metabolism defects might be confounded by the expression of the *RIP2-Cre* transgene in cells in the hypothalamus that also impact glucose metabolism (26).

The large number of miRNAs expressed in the developing pancreas, and the large number of potential targets for each of these complicate the task of identifying the miRNAs that normally maintain neurogenin3 expression and endocrine cell generation, but the increase in the neurogenin3 inhibitor Hes1 suggests that Hes1 or upstream genes in the notch signaling pathway could be miRNA targets in pancreatic progenitor cells. Previous studies (32,33) have implicated Hes1 protein as a target of miR-23a, and miR-23a was expressed in the developing pancreas (Table 1). (Although, this article was withdrawn because the authors mistakenly used the wrong gene sequence for their analyses, the observation that miR-23a can influence the expression of Hes1 remains a possibility from this publication.) In addition, it seems likely that miRNA may target other notch pathway components upstream of Hes1, yielding an increase in Hes1 expression in the absence of Dicer1.

In summary, these data demonstrate for the first time that a unique set of miRNAs emerge during pancreas development and regulate pathways important for normal ductal, exocrine, and endocrine development. Exploration of the roles of individual miRNA will provide a more complete understanding of pancreatic development and the differentiation of the distinct cell types, especially β-cells, and help in developing methods for generating these cells for therapeutic uses.

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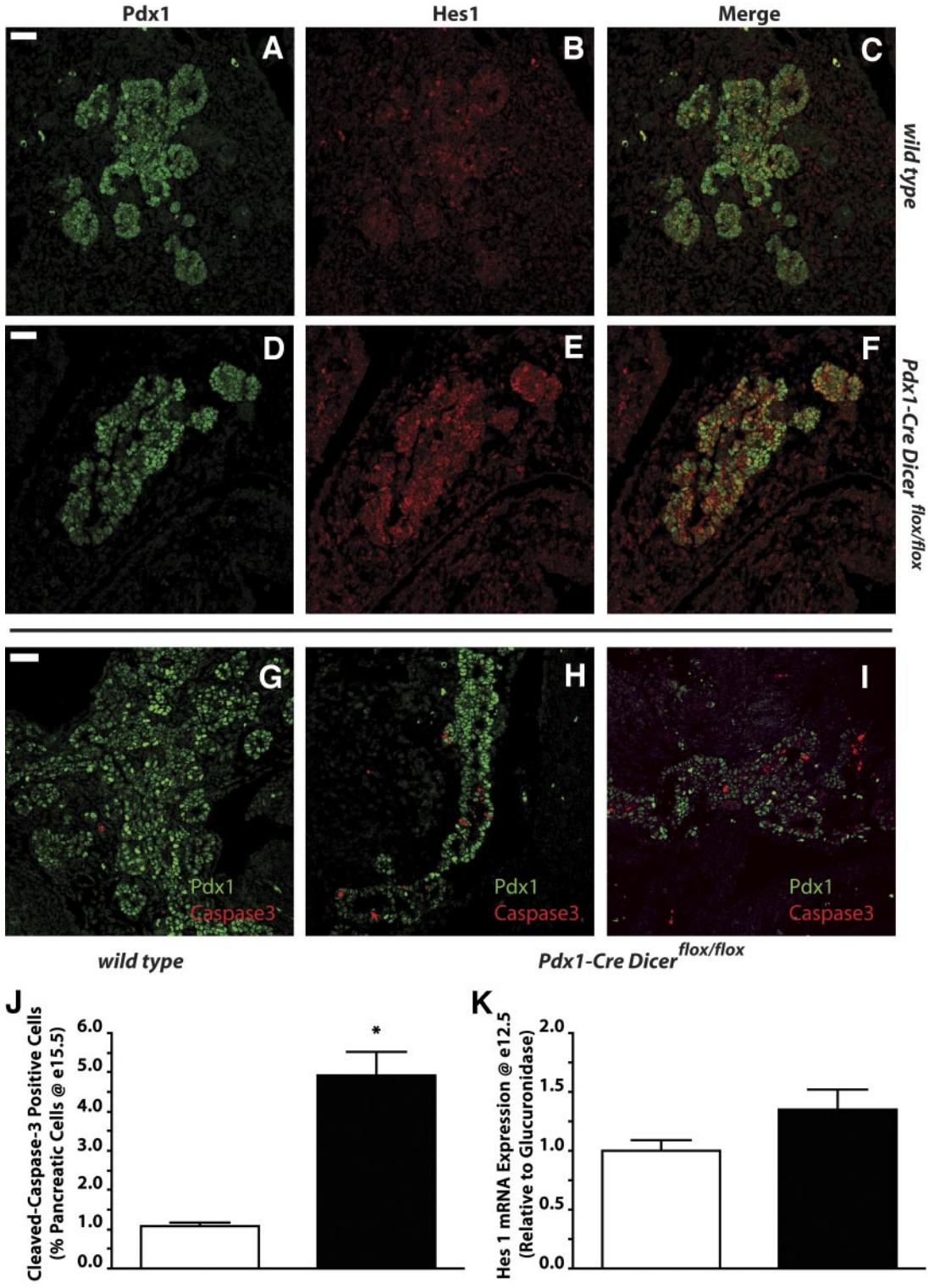

FIG. 6. Hairy enhancer of split 1 (Hes1) and activated caspase-3 immunostaining in the *Dicer1*-null pancreas. In an effort to define pathways by which miRNAs regulate pancreas development, staining for Hes1 was carried out. At e12.5, an upregulation of Hes1 (*B*, *E*, and *K*, red) was observed in the pancreatic bud from the *Pdx-Cre Dicer1*$^{flox/flox}$ animals when compared with the double heterozygous (wild-type) (*A–C*, and *K*) controls, while, as previously described, there was no difference in Pdx-1 staining (*A* and *D*, green). In an effort to determine why there was an overall decrease in pancreatic mass, e15.5 pancreas was stained for activated caspase-3 (*G–J*, red). There was an increase in activated caspase-3 immunoreactivity in the ductal cords (Pdx-1 positive; green) of the developing pancreas of the *Pdx-Cre Dicer1*$^{flox/flox}$ mice (*H–J*). Data are expressed as means ± SE. *Statistically significant differences between wild-type and *Pdx-Cre Dicer1*$^{flox/flox}$ littermates ($P < 0.05$) (*J*, $n = 4$; *K*, $n = 8$). Scale bars = 50 μm. *J* and *K*: □, wild type; ■, *Pdx-Cre Dicer1*$^{flox/flox}$. (Please see http://dx.doi.org/10.2337/db07-0175 for a high-quality digital representation of this figure.)

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