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Article **Metabolism**

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Impaired pancreatic growth, β cell mass, and β cell function in E2F1$^{-/-}$ mice

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We evaluated the effects of E2F1 on glucose homeostasis using E2F1$^{-/-}$ mice. E2F1$^{-/-}$ mice show an overall reduction in pancreatic size as the result of impaired postnatal pancreatic growth. Furthermore, these animals have dysfunctional β cells, linked to impaired PDX-1 activity. Because of the disproportionate small pancreas and dysfunctional islets, E2F1$^{-/-}$ mice secrete insufficient amounts of insulin in response to a glucose load, resulting in glucose intolerance. Despite this glucose intolerance, E2F1$^{-/-}$ mice do not develop overt diabetes mellitus because they have insulin hypersensitivity, which is secondary to a diminished adipose tissue mass and altered adipocytokine levels, which compensates for the defect in insulin secretion. These data demonstrate that factors controlling cell proliferation, such as E2F1, determine pancreatic growth and function, subsequently affecting metabolic homeostasis.

Introduction

Type 2 diabetes mellitus (T2D) is a prevalent disorder of glucose homeostasis resulting from an imbalance between insulin secretion by pancreatic β cells and the sensitivity of peripheral tissues to insulin (1). Insulin secretion can be affected by signaling and transcription factors that are necessary for the proper differentiation and growth of the various pancreatic cell types (2). Characterization of human mutations and studies in knockout mice identified several transcription factors playing important roles in endocrine pancreas development, such as the insulin promoter factor 1 (IPF-1, also known as PDX-1) (3), Nkx2.2 or Pax4 (4, 5), neurogenin 3 (6), or NeuroD (7). The importance of transcription factors in the development of the pancreas was further underscored by the analysis of mutations in patients with a monogenic type of diabetes known as maturity-onset diabetes of the young (MODY), which is characterized by early age of onset, autosomal dominant inheritance, and impaired insulin secretion. To date five MODY genes have been identified, four of which are transcription factors. These include hepatocyte nuclear factor-1A (HNF-1A) (8), HNF-4A (9), and HNF-1B (10), which are responsible for MODY-1, -3, and -5, respectively, IPF-1/PDX-1, which underlies MODY-4 (11), and NeuroD, whose absence causes MODY-6 (12). The remaining gene that causes MODY-2 corresponds to the enzyme glucokinase (13). Furthermore, several signaling factors, such as FGFs (14) or EGFs (15), together with their respective receptors (sonic and Indian hedgehog) (16), as well as the notch-signaling pathways (17), have also been linked to pancreatic growth and morphogenesis.

Besides insulin secretion, insulin resistance, characterized by an impaired glucose uptake in response to insulin by peripheral tissues, is the other important determinant for the development of T2D. Several proteins have been implicated in the development of insulin resistance, including the insulin receptor (18–20), glucose transporter 4 (GLUT4) (21), the insulin-receptor substrate-1 (IRS-1) (22, 23) and IRS-2 (24), IGF-1 (25), protein tyrosine phosphatase 1B (PTP1B) (26), the p85a subunit of the PI3K (27), protein kinase C (28), and PPARγ (29, 30).

E2F transcription factors are the effectors of the pathway that controls the G1/S transition of the cell cycle. E2F DNA-binding sites were found to be critical in the promoters of genes involved in cell cycle progression, apoptosis, and DNA synthesis (for review see refs. 31–33). When bound to DNA, they exist either as free E2F/dimerization partner (E2F/DP) heterodimers or associated in larger complexes containing members of the retinoblastoma (RB) family (pRB, p107, p130) and members of the cyclin/CDK protein families. RB associates with all E2Fs except E2F5 and E2F6, whereas p130 associates specifically with E2F4 and E2F5 and p107 complexes exclusively with E2F4 (34). E2F complexes can act as repressors (large complexes) or as activators (free heterodimers) of their target genes. Depending on the promoter context, association of E2F with the pRB family members will either result in inhibition of E2F/DP transactivation or active repression through recruitment of histone deacetylases by the RB family members (35–37). The role of the individual E2F family members has been established based on both overexpression studies and the analysis of E2F-deficient mice and cells (reviewed in refs. 38, 39). These studies show that E2F1–E2F3 play a key role in the activation of E2F-responsive genes and therefore the induction of cellular proliferation. In contrast, E2F4 and E2F5 appear to be involved primarily in the repression of target genes and are particularly relevant for the transition between cell proliferation to differentiation (40–42).

We recently described that E2F1 favors adipocyte differentiation and fat formation (43). Since adipose tissue plays an important role in glucose homeostasis, we now evaluated the role of E2F1 in this process. E2F1$^{-/-}$ mice have impaired insulin secretion in response to a glucose challenge due to a defect in pancreatic growth and islet dysfunction. E2F1$^{-/-}$ mice are, however, protected

Nonstandard abbreviations used: dimerization partner (DP); dual x-ray absorptiometry (DEXA); hepatocyte nuclear factor-1A (HNF-A); insulin promoter factor 1 (IPF-1); insulin-receptor substrate-1 (IRS-1); intraperitoneal glucose-tolerance test (IPGTT); maturity-onset diabetes of the young (MODY); postcoitum day (dpc); protein tyrosine phosphatase 1B (PTP1B); retinoblastoma (RB); type 2 diabetes mellitus (T2D); white adipose tissue (WAT).

Conflict of interest: The authors have declared that no conflict of interest exists.

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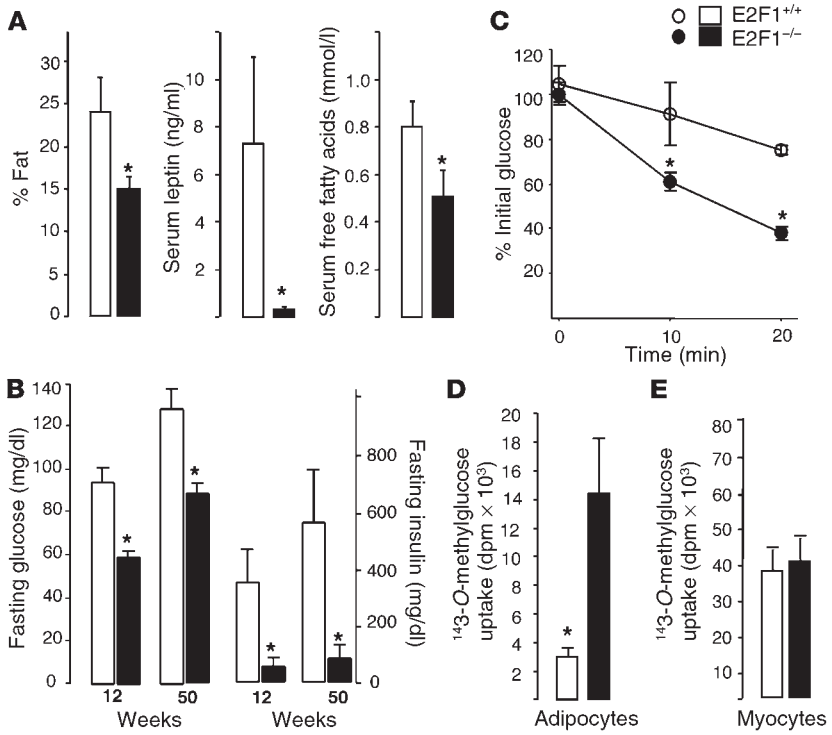

Figure 1
Insulin sensitivity in E2F1$^{-/-}$ mice. (**A**) Body fat mass, evaluated by DEXA scanning, and free fatty acid and leptin levels in the serum of E2F1$^{+/+}$ and E2F1$^{-/-}$ mice (six animals per group). Results are the average of three independent experiments. (**B**) Fasting plasma glucose and insulin in either 12-week-old or 50-week-old E2F1$^{+/+}$ and E2F1$^{-/-}$ mice. Results are the mean ± SD of 12 mice analyzed per group. The same number of mice were analyzed in subsequent experiments. (**C**) Glucose clearance after intraperitoneal injection of insulin (0.75 IU/kg) as a measure of insulin sensitivity in E2F1$^{-/-}$ and E2F1$^{+/+}$ mice. Glucose values are relative to the initial glucose levels. (**D** and **E**) In vitro ^{14}C-3-*O*-methylglucose uptake 10 minutes after insulin addition to isolated adipocytes (**D**) or myocytes (**E**) from the same amount of tissue from E2F1$^{+/+}$ and E2F1$^{-/-}$ mice. Results are normalized by the content of DNA. *Statistically significant differences (ANOVA, $P < 0.005$).

against the development of diabetes, because they are also insulin hypersensitive as a consequence of reduced adipose tissue mass.

Results and Discussion

Adipocyte differentiation is impaired in E2F1$^{-/-}$ mice, and we reported that this results in a reduced fat pad mass (43). Dual energy x-ray absorptiometry (DEXA) scan analysis performed in this study confirmed the reduction in total body fat mass in E2F1$^{-/-}$ compared with E2F1$^{+/+}$ mice (Figure 1A). Consistent with the decreased body fat mass, the E2F1$^{-/-}$ mice had lower serum leptin and free fatty acid levels (Figure 1A). Except in the case of lipodystrophic syndromes, body fat mass appears to be directly correlated with the development of insulin resistance. This prompted us to investigate whether the decreased fat mass in E2F1$^{-/-}$ mice could have an impact on glucose homeostasis. Both fasting glucose and insulin levels were statistically lower in E2F1$^{-/-}$ compared with E2F1$^{+/+}$ mice (0.6-fold and 0.2-fold, respectively; Figure 1B). This effect was independent of the age of the animals and suggested increased insulin sensitivity. Consistent with this hypothesis, glucose decreased 60% in E2F1$^{-/-}$ mice after insulin injection, whereas it decreased only 20% in E2F1$^{+/+}$ mice, indicating that the absence of E2F1 improved insulin sensitivity (Figure 1C). Furthermore, coherent with a better response to insulin, radioactively labeled glucose was more efficiently taken up by size-matched primary adipocytes from E2F1$^{-/-}$ than from E2F1$^{+/+}$ mice after 10 minutes of insulin challenge (Figure 1D), whereas no differences in glucose uptake were observed between cultured primary myocytes from E2F1$^{-/-}$ and E2F1$^{+/+}$ mice (Figure 1E). This finding suggested that the increased insulin sensitivity of E2F1$^{-/-}$ mice was not due to a defect in the muscle but rather was the result of the decreased adipose tissue mass in these animals.

We then analyzed the causes of the improved insulin sensitivity by performing quantitative RT-PCR analysis of a number of genes involved in metabolic control. The expression of the mRNAs encoding IRS-1, IRS-2, and PI3K, the proteins involved in insulin signaling, were increased 2.5-fold, 1.7-fold, and twofold, respectively, in the white adipose tissue (WAT) of E2F1$^{-/-}$ compared with E2F1$^{+/+}$ mice (Figure 2A). Furthermore, adiponectin mRNA expression was increased 2.2-fold, whereas leptin mRNA levels were decreased 0.2-fold in the WAT of E2F1$^{-/-}$ mice (Figure 2A). No differences in WAT mRNA expression for protein kinase B, GLUT4, or resistin were observed between the genotypes (Figure 2A). We also measured the expression of a number of genes in other insulin-sensitive tissues, such as muscle and liver. No differences in the expression of genes involved in the insulin-signaling pathway in the muscle were observed (Figure 2B). Genes controlling gluconeogenesis in the liver, such as phosphoenolpyruvate carboxykinase and glucose 6-phosphatase, also were not different between the two genotypes (data not shown).

The disproportionate decrease in fasting insulin relative to glucose levels (Figure 1B) encouraged us to explore insulin secretion from pancreatic β cells upon a glucose challenge during an intraperitoneal glucose-tolerance test (IPGTT). In contrast to expectations, intraperitoneally injected glucose was cleared at least as well in E2F1$^{+/+}$ mice as in E2F1$^{-/-}$ mice (Figure 3A). This observation seemed inconsistent with the decreased fasting glucose levels in E2F1$^{-/-}$ animals. Since glucose uptake was increased in isolated E2F1$^{-/-}$ adipocytes (Figure 1D) and the response of the E2F1$^{-/-}$ mice to insulin was improved compared with E2F1$^{+/+}$ mice (Figure 1C), the results of the glucose tolerance test pointed to an additional defect in insulin secretion in the E2F1$^{-/-}$ mice. In line with this, fasting insulin levels were significantly lower and the insulin levels remained almost flat upon glucose injection in E2F1$^{-/-}$ mice, which contrasted to the robust increase in insulin release in E2F1$^{+/+}$ mice (Figure 3A). We also performed a meal-tolerance test, consisting of feeding mice ad libitum after an overnight fast, to evaluate glucose disposal. Reminiscent to the

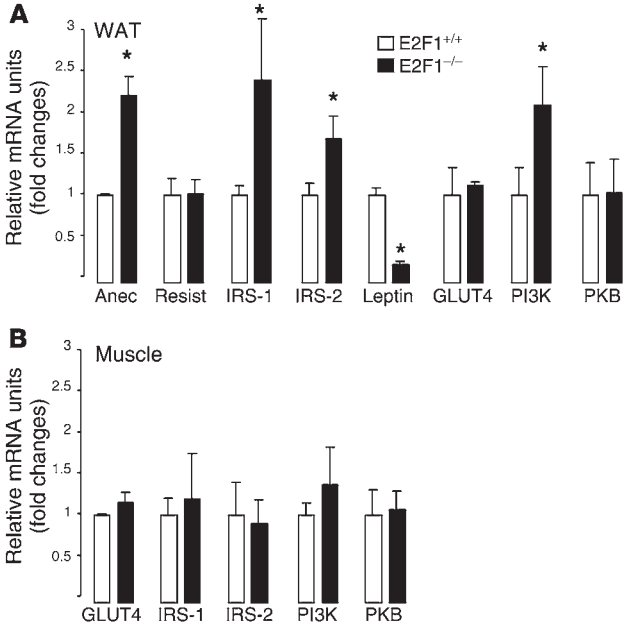

Figure 2
Gene expression analysis in different tissues of E2F1$^{-/-}$ or E2F1$^{+/+}$ mice. Quantification of the expression by real-time RT-PCR of relevant genes for the insulin-sensitivity pathway in WAT (**A**) and muscle (**B**). Results were normalized by the expression of the 18S ribosomal subunit RNA and expressed as fold changes relative to expression levels in E2F1$^{+/+}$ mice. Anec., adiponectin; resist., resistin; PKB, protein kinase B. *Statistically significant differences (ANOVA, $P < 0.005$).

results of the IPGTT, insulin levels were fivefold higher in E2F1$^{+/+}$ compared with E2F1$^{-/-}$ mice, whereas glucose levels were similar (Figure 3B). In combination, these data indicate that there might be an additional defect in insulin production/secretion in these E2F1$^{-/-}$ mice. A defect in insulin secretion results either from a reduction in size or the number of islets or a defect in insulin production by the β cells in response to glucose. Although the body weight of E2F1$^{-/-}$ mice was reduced compared with E2F1$^{+/+}$ mice (E2F1$^{+/+}$: 20.8 ± 1.7 g; E2F1$^{-/-}$: 18.6 ± 1.2 g), morphometric analysis of the pancreata of E2F1$^{-/-}$ mice showed a disproportionate reduction in overall pancreatic size, with a significant decrease in the ratio of pancreatic to total body weight (Figure 3, C and D). The ratio of organ to body weight was, however, similar in other tissues such as heart or kidney (Figure 3D).

A detailed histological analysis of pancreatic sections of E2F1$^{-/-}$ mice showed no differences in the ratio of the number of islets relative to pancreatic weight, suggesting that development of the endocrine pancreas was not affected in E2F1$^{-/-}$ mice (Figure 4, A and B; islet/pancreas ratio). The absolute number of islets was significantly reduced in E2F1$^{-/-}$ mice, however, when expressed relative to total body weight (Figure 4B; islet number). Similarly, the total area of pancreatic islets was not different between E2F1$^{+/+}$ and E2F1$^{-/-}$ mice if islet area was corrected by pancreatic surface, but decreased when related to their total body weight (Figure 4C).

The reduction of pancreatic size and islet number and surface could, in part, account for the decreased insulin secretion observed in these animals. This could be the consequence of either reduction in cell size or number of cells that compose the islets. Histological analysis of pancreatic sections indicated that the relative size of pancreatic cells was not affected by E2F1 deficiency, although other abnormalities, such as enlarged nuclei, were observed in the exocrine pancreas of E2F1$^{-/-}$ mice (44, 45) (Figure 4D). Pancreatic cell number is dictated by the proliferative rate of all pancreatic structures, including acini, islets, and, in particular, ducts, which constitute the main proliferative compartment of the pancreas (46–48). β cell growth could result from neogenesis or differentiation from ductal precursor cells (48, 49). Overexpression studies in cells and the analysis of E2F-deficient mice show that E2F1 plays a key role in the activation of genes involved in cell cycle progression and cellular proliferation (reviewed in refs. 38, 39). Consistent with a role of E2F1 in regulating pancreas cell proliferation, E2F1 was highly expressed in the pancreatic ducts of E2F1$^{+/+}$ mice (Figure 4E), but was absent in the ducts of E2F1$^{-/-}$ mice (data not shown).

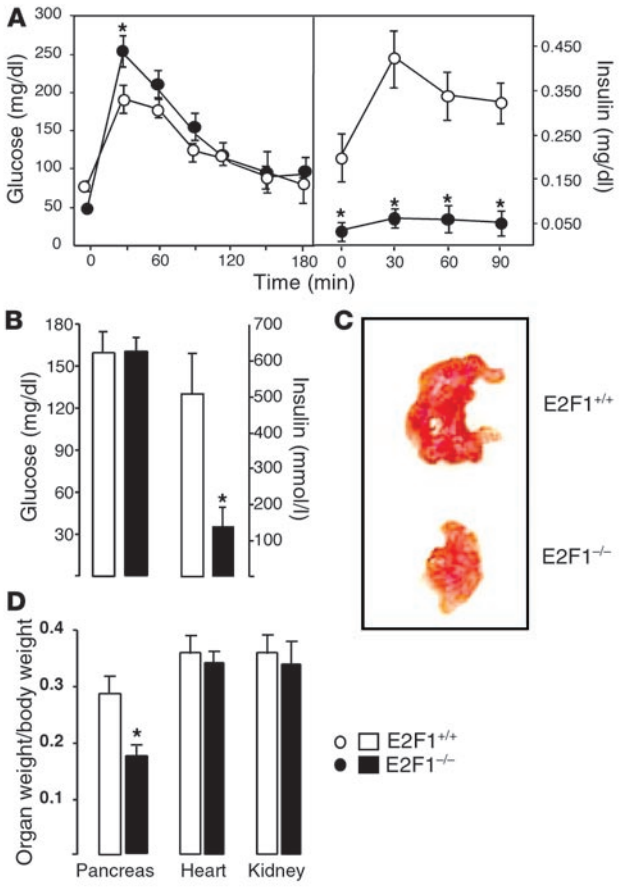

Figure 3
Glucose tolerance and macroscopic analysis of the pancreas of E2F1$^{-/-}$ or E2F1$^{+/+}$ mice. (**A**) IPGTT measuring the levels of glucose and insulin at different times after intraperitoneal injection of glucose in E2F1$^{+/+}$ or E2F1$^{-/-}$ mice. (**B**) Glucose and insulin levels upon a meal-tolerance test. E2F1$^{-/-}$ or E2F1$^{+/+}$ mice were fed ad libitum after an overnight fast. Ninety minutes after beginning the meal, plasma insulin and glucose levels were measured. (**C**) Morphology of the entire pancreas from E2F1$^{+/+}$ or E2F1$^{-/-}$ mice demonstrates the decreased size of the E2F1$^{-/-}$ pancreas. (**D**) Relative weight of three representative tissues of E2F1$^{-/-}$ or E2F1$^{+/+}$ mice. Weights are as a ratio to total body weight. *Statistically significant differences (ANOVA, $P < 0.005$).

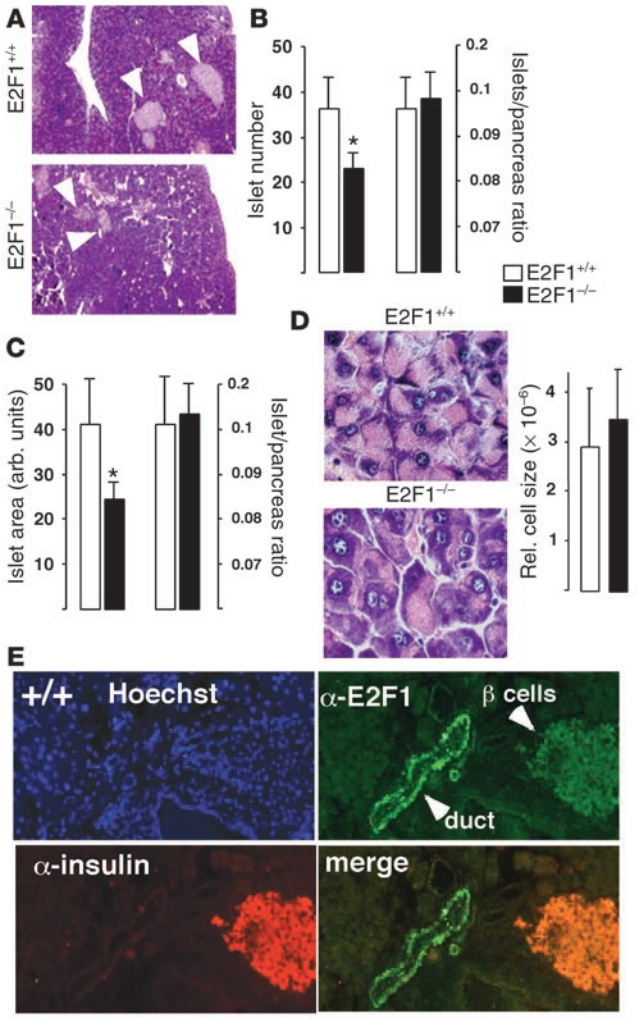

Figure 4
Pancreatic size and β cell area. (**A**) H&E staining of pancreatic sections of E2F1$^{-/-}$ or E2F1$^{+/+}$ 4-week-old mice. Pancreatic islets are indicated by arrowheads. (**B**) Pancreatic islet number in E2F1$^{-/-}$ and E2F1$^{+/+}$ 4-week-old mice. Summed islet numbers obtained from examining every fifth section of 20-μm thickness from a whole pancreas (left scale). The numbers were relative to pancreas weight to determine the islet/pancreas ratio (right scale). (**C**) Endocrine islet area calculated as arbitrary units (arb. units) from micrographs of pancreatic sections of E2F1$^{+/+}$ or E2F1$^{-/-}$ 4-week-old mice using the Canvas 7 software. The right scale represents the islet area relative to pancreas weight. (**D**) H&E staining of pancreatic sections of E2F1$^{-/-}$ and E2F1$^{+/+}$ mice. Quantification of the relative (rel.) cell size of exocrine pancreatic cells of 2-week-old E2F1$^{-/-}$ and E2F1$^{+/+}$ mice. (**E**) Immunofluorescence analysis of pancreatic sections showing expression of E2F1 in ducts and β cells (green), which are also positive for insulin (red). Nuclei are stained with the Hoechst reagent. *Statistically significant differences (ANOVA, $P < 0.005$).

A lower, but significant, level of E2F1 expression was also observed in β cells, suggesting an additional and direct role of E2F1 in regulating β cell function (Figure 4E).

To further prove that E2F1 regulates pancreas cell proliferation, the in vivo cell proliferation index was analyzed by BrdU incorporation in the pancreata of mice. The proliferation index was 6% ± 0.8% in E2F1$^{+/+}$ mice, whereas only 2% ± 0.1% of E2F1$^{-/-}$ pancreatic cells proliferated (Figure 5A). Furthermore, the number of cells positive for phosphorylated histone H3, which is an additional marker of mitosis, was significantly decreased in the pancreas of E2F1$^{-/-}$ mice (Figure 5, B and C). Consistent with the observed decreased proliferation in pancreatic islets of E2F1$^{-/-}$ mice, mRNA expression of the E2F target gene cyclin E was decreased in isolated islets of these mice (Figure 5D), whereas no changes in mRNA levels of cyclin D1 and thymidine kinase were detected. Decreased proliferation could also correlate with increased apoptosis as was shown for thymocytes of E2F1$^{-/-}$ mice (50). No change in apoptosis rate was observed in pancreatic sections as measured by TUNEL assay (Figure 5E), however. These results indicate that reduced growth of E2F1$^{-/-}$ pancreata is the result of impaired proliferation of pancreatic cells.

Our results point to E2F1 as an important regulator of postnatal pancreatic growth. In contrast to early pancreas development, which is relatively well understood, little is known about postnatal pancreatic growth, which also participates in optimizing pancreatic insulin secretion and production. Proliferation studies have shown measurable levels of cell division in all pancreatic compartments, including acini, islets, and ducts (46–48), indicating that cell proliferation could account, at least in part, for the postnatal growth of the pancreas. Strikingly, inactivation of cyclin-dependent kinase 4, which is an upstream regulator of E2F1 function, also entails a defect in insulin secretion due to impaired proliferation of pancreatic β cells (51). In contrast to E2F1, which might stimulate β cell neogenesis from proliferating ductal cell precursors, the forkhead transcription factor foxo-1 is a negative effector of this pathway inhibiting the proliferation of these β cell precursors (52). Consequently, haploinsufficiency of *foxo-1* restores β cell proliferation in IRS-2$^{-/-}$ mice, which have impaired β cell proliferation (52), leading us to suggest that E2F1 could be a negative modulator of foxo-1 signaling. In support of this hypothesis is the fact that E2F1 favors adipogenesis (43), a process that is inhibited by a constitutively active form of foxo-1 (53).

An alternate possibility to explain the inadequate insulin response to glucose in E2F1$^{-/-}$ mice would be a specific defect in insulin-secreting β cells. Whereas at 4 weeks after birth no differences were observed between the proportional size, morphology, or distribution of exocrine and endocrine tissues of E2F1$^{-/-}$ and E2F1$^{+/+}$ mice (Figure 4), the ratio of glucagon-producing/insulin-producing cells was clearly elevated in E2F1$^{-/-}$ islets of 16-week-old mice as assessed by immunofluorescence analysis (Figure 5F). Furthermore, the total β cell area was reduced in E2F1$^{-/-}$ compared with E2F1$^{+/+}$ islets (Figure 5G). Consequently, the α cell/β cell ratio was increased in the islets of E2F1$^{-/-}$ mice (Figure 5G). Although many factors can influence circulating hormone levels, the increase in the serum glucagon/insulin ratio was consistent with the morphological alterations in the islets (Figure 5H). The lack of differences in β cell area in young animals indicates that E2F1 does not participate in pancreas development, but rather participates in postnatal growth. Consistent with this, we observed that E2F1 was highly expressed in embryonic pancreas at 16.5 postcoitum days (dpc's), a stage when the pancreas is already developed, but not in the developing pancreas at 12.5 dpc's, when PDX-1, a key player in pancreas development, was already expressed (Figure 6A). The lack of expression of E2F1 during the critical early phases of pancreas development demonstrates that it has no role in early pancreas development.

The fact that the ratio of glucagon-producing/insulin-producing cells, as well as the serum glucagon/insulin ratio, was increased in E2F1$^{-/-}$ mice suggested a specific defect in pancreatic β cell mass

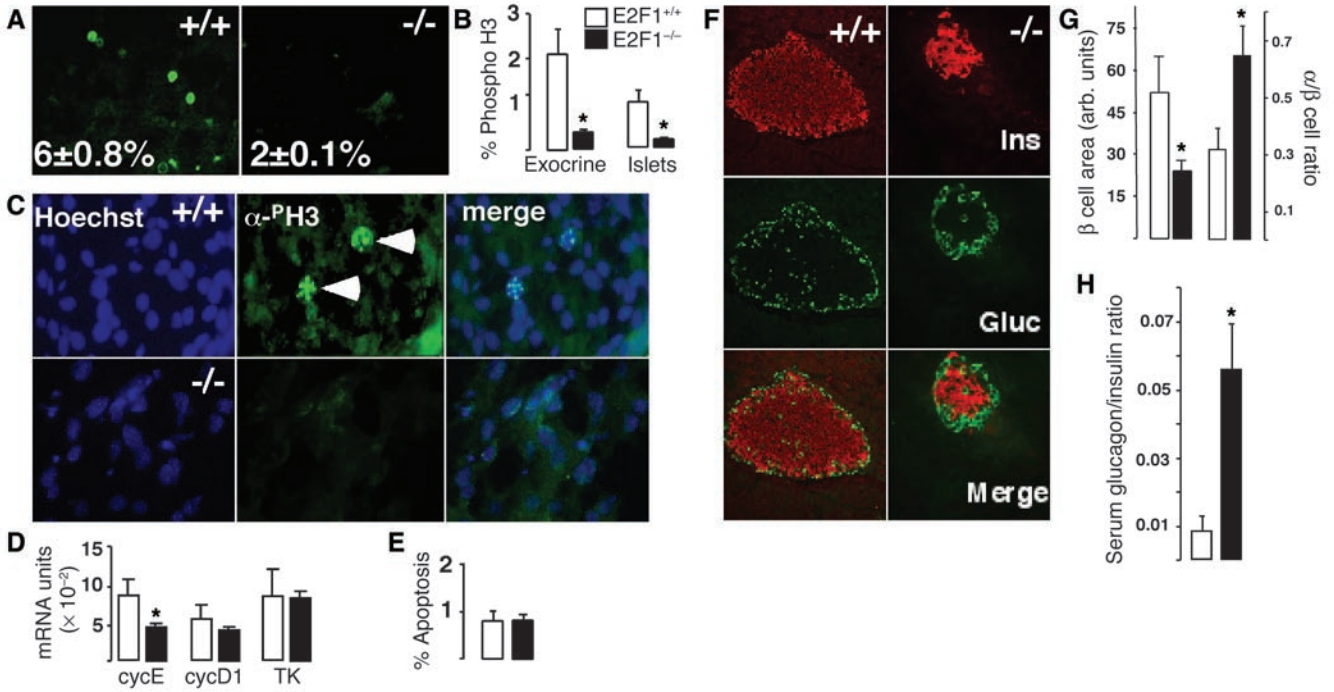

Figure 5

In vivo proliferation and apoptosis rates and expression of E2F1. (**A**) Representative micrograph showing BrdU immunostaining of pancreatic sections from E2F1$^{-/-}$ and E2F1$^{+/+}$ 2-week-old mice. The percentage of BrdU-positive cells is indicated. Results are the mean of the summed numbers from ten sections of each pancreas from five mice per genotype. (**B** and **C**) Phosphohistone H3 (^{P}H3) immunofluorescent labeling in pancreatic sections of either E2F1$^{+/+}$ or E2F1$^{-/-}$ mice. Nuclei are stained by Hoechst. Percentage of phosphohistone H3–positive cells is indicated (**B**). (**D**) Quantification of the expression by real-time RT-PCR of relevant E2F target genes in isolated islets. Results were normalized by the expression of the 18S ribosomal subunit RNA. Cyc E, cyclin E; cycD1, cyclin D; TK, thymidin kinase. (**E**) Percentage of apoptotic cells as measured by TUNEL assay in pancreatic sections of E2F1$^{-/-}$ and E2F1$^{+/+}$ 16-week-old mice. (**F**) Insulin (Ins) (red: β cells) and glucagon (Gluc) (green: α cells) immunostaining of pancreatic islets in E2F1$^{-/-}$ and E2F1$^{+/+}$ 16-week-old mice. (**G**) Quantification of the β cell area in arbitrary (arb.) units (left panel) and the α/β cell ratio (right panel) in pancreatic sections of E2F1$^{-/-}$ and E2F1$^{+/+}$ mice. At least 50 pancreatic sections from different mice were analyzed after immunostaining for insulin and glucagon. Areas were calculated using the Canvas 9 software in arbitrary units. (**H**) Quantification of the glucagon/insulin ratio in the serum of E2F1$^{-/-}$ and E2F1$^{+/+}$ male 16-week-old mice. *Statistically significant differences (ANOVA, $P < 0.005$).

and/or function. PDX-1/IPF-1 is a transcription factor that plays an important role, not only during pancreas development, but also in adult β cell function. We therefore evaluated the expression of PDX-1 in pancreatic sections of E2F1$^{-/-}$ or E2F1$^{+/+}$ mice by immunofluorescence analysis. Whereas 75% of insulin-producing cells were also positive for PDX-1 in E2F1$^{+/+}$ mice, only 30% of β cells were positive for PDX-1 in E2F1$^{-/-}$ mice (Figure 6, B and C). Interestingly, a significant number of E2F1$^{-/-}$ cells expressed PDX-1 in the cytoplasm, whereas PDX-1 was exclusively localized in the nucleus in E2F-1$^{+/+}$ cells (Figure 6D). Furthermore, PDX-1 mRNA expression was significantly decreased in isolated islets from E2F1$^{-/-}$ compared with E2F1$^{+/+}$ mice (Figure 6E). No further changes in the expression of other transcription factors involved in pancreas development or function, such as HNF-1, were observed (Figure 6E). The mRNA expression analysis also confirmed the increased glucagon-to-insulin ratio observed previously on the protein level (Figure 5, G and H). Taken together, these results are reminiscent of those observed in islets of IRS-2$^{-/-}$ mice, which showed both decreased expression and cytoplasmic localization of PDX-1 (52), and suggest that E2F1 could directly regulate the expression of PDX-1. The modified expression of PDX-1 and its altered cellular localization in the E2F1$^{-/-}$ mice clearly contribute to their β cell dysfunction.

To provide further arguments to support that the E2F1$^{-/-}$ islets were dysfunctional, we analyzed glucose-induced insulin secretion in isolated islets of E2F1$^{-/-}$ and E2F1$^{+/+}$ mice. Insulin secretion in response to glucose challenge was significantly reduced in the islets of E2F1$^{-/-}$ mice when compared with E2F1$^{+/+}$ islets (Figure 6F). The response of E2F1$^{-/-}$ islets was, however, still rather robust, suggesting that this β cell dysfunction was not sufficient to fully explain the hypoinsulinemia and glucose intolerance and further underscored the relevance of the decreased pancreatic mass to explain the phenotype. Similar results were obtained when arginine was used instead of glucose to stimulate insulin secretion (data not shown).

The fact that E2F1$^{-/-}$ mice did not develop diabetes over time, even after high-fat diet (Figure 1B), suggests that the increased insulin sensitivity is not just a compensatory mechanism to maintain glucose homeostasis. Rather, E2F1 seems to have two independent effects on glucose metabolism. First, E2F1 participates in the control of insulin secretion and production. E2F1 favors postnatal pancreatic growth by controlling the proliferation rate of pancreatic cells (Figure 5), and it directly controls β cell function through its regulatory role on genes such as PDX1 (Figure 6). This effect of E2F1 on β cell function is fully in line with an independent study that was published while this article was being reviewed and that

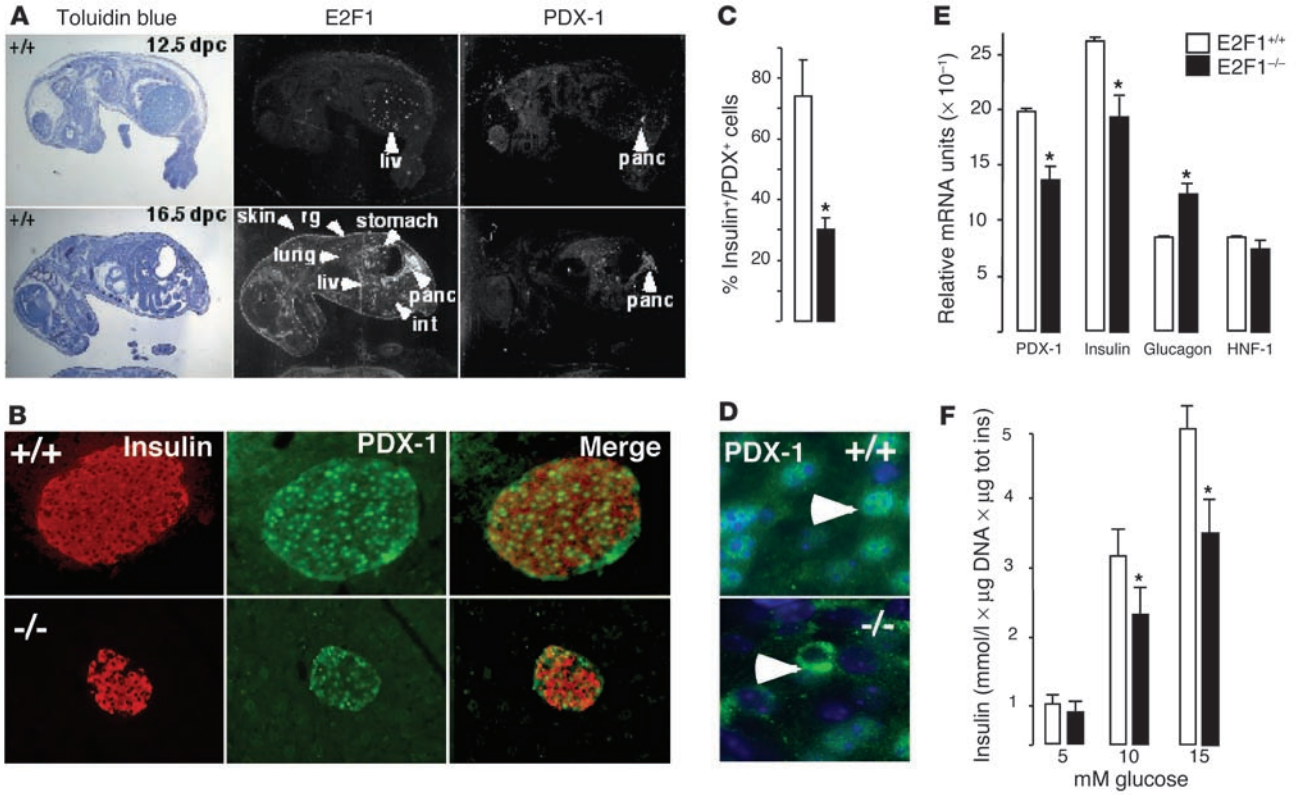

Figure 6

PDX-1 expression and insulin secretion of islets of E2F1$^{-/-}$ and E2F1$^{+/+}$ mice. (**A**) In situ hybridization of E2F1 or PDX-1 in sections of 12.5 or 16.5 dpc embryos. Location of liver (liv), stomach, skin, lung, pancreas (panc), intestine (int), and dorsal root ganglion (rg) in the embryo is indicated. (**B**) Immunofluorescence analysis of PDX-1 expression (green) in insulin-producing cells (red) of pancreatic sections of E2F1$^{+/+}$ or E2F1$^{-/-}$ 16-week-old mice. (**C**) Quantification (500 cells) of insulin-producing cells, which also expressed PDX-1. Results are relative to total number of insulin-producing cells. (**D**) Cellular localization of PDX-1 expression (green) in pancreatic β cells. Hoechst staining of nuclei is in blue. (**E**) Quantification of the expression by real-time RT-PCR of relevant genes for pancreatic islet development or function. Results were normalized by the expression of the 18S ribosomal subunit RNA. (**F**) Insulin secretion of isolated islets of E2F1$^{+/+}$ or E2F1$^{-/-}$ mice in the absence or presence of 5, 10, or 15 mM glucose. Islets were isolated from 16-week-old male mice. Results are relative to total DNA and insulin content. tot ins, total insulin.

characterized E2F1/E2F2 double-mutant mice (45). In contrast to the E2F1$^{-/-}$ mice, these E2F1/E2F2 double-mutant mice developed a more severe exocrine pancreatic atrophy that impacted on pancreatic β cell maintenance and function. Second, E2F1 negatively regulates insulin action, likely through regulation of the expression of genes implicated in this process. Interestingly, similar to E2F1$^{-/-}$ mice, S6K1-deficient mice are glucose intolerant and show low insulin levels due to a selective reduction of β cell size (54). These S6K1$^{-/-}$ mice do not develop diabetes, indicating that they are insulin hypersensitive (54) and suggesting an eventual crosstalk between S6K1- and E2F1-signaling pathways. Another putative E2F1 target in this context is the *PTP1B* gene, which dephosphorylates the insulin receptor and thus attenuates insulin signaling. Mice lacking PTP1B are, like the E2F1$^{-/-}$ mice (43), insulin sensitized and protected against diet-induced obesity (26). We speculate that in the case of the E2F1/E2F2 double-mutant mice, the disappearance of this negative effect of E2F1 on insulin action may be insufficient to compensate for the more severe pancreatic failure, ultimately causing these mice to become diabetic (45). It is at present also unclear whether E2F2 also regulates the expression of the same set of genes implicated in insulin sensitivity that are regulated by E2F1.

The compensatory mechanism through which E2F1$^{-/-}$ mice maintain their glucose homeostasis likely involves its role on adipose tissue development rather than a direct effect of E2F1 in insulin-sensitive target tissues such as muscle or liver. Supporting this hypothesis is the fact that, in contrast to what is observed in isolated adipocytes, isolated myocytes from E2F1$^{-/-}$ mice are not more sensitive to insulin than E2F1$^{+/+}$ cells. We have previously shown that E2F1 favors adipogenesis through the induction of PPARγ, the master controller of adipocyte differentiation (43). Total absence of adipose tissue, such as seen in lipoatrophic subjects (55) and animals (56–58), results in insulin resistance. Excess adipose tissue in obesity is also a risk factor for insulin resistance, since high levels of free fatty acids, which are the consequence of "spillover" from adipose tissue, are linked to the induction of insulin resistance (59). Furthermore, besides fatty acids, adipose tissue secretes several proteins (also called adipocytokines) that affect insulin signaling in other tissues, including TNF-α (60), resistin (61), and adiponectin (62–64). We hypothesize that the normal glucose tolerance and insulin sensitivity in E2F1$^{-/-}$ mice is most likely the result of the improved profile of adipose tissue–derived signaling molecules, as reflected by the increase in adiponectin and the reduction in free fatty acid levels. The reduced fat mass in the E2F1$^{-/-}$ mice could, therefore, through altered levels of these signaling factors (fatty acids and adipocytokines), contribute to the increased overall insulin sensitivity, a phenomenon reminiscent of that previously observed in mice heterozygous for mutations in the *PPARγ* gene (29, 30).

In summary, we have shown that the transcription factor E2F1 regulates pancreatic growth and islet mass and function. E2F1 achieves these effects through controlling cell proliferation and directly regulating islet function. The ensuing impaired insulin secretion does not, however, result in the development of diabetes, since E2F1$^{-/-}$ mice are more sensitive to insulin secondary to a reduced fat mass.

Methods

Materials. All chemicals, except as stated, were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

RNA isolation and quantitative real-time PCR. RNA isolation and reverse transcription has been described previously. Quantitative PCR was carried out by real-time PCR using a LightCycler and the DNA double-strand–specific SYBR Green I dye for detection (Roche, Basel, Switzerland). Reverse transcription of total RNA was performed at 42°C using the Superscript II reverse transcriptase (Stratagene, La Jolla, California, USA) and random hexanucleotide primers, followed by a 15-minute inactivation at 70°C. The cDNAs were then purified using a Qiagen Purification kit (QIAGEN GmbH, Hilden, Germany). Results were then normalized to GAPDH levels. The primer pairs used in this study are available upon request.

Histological and immunofluorescence analysis. Sections of pancreata were fixed in either Bouin's solution or in 4% formaldehyde. They were then stained with H&E or treated with 1.5 N HCl before incubation with specific Ab's directed against insulin (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), glucagon (a kind gift of G. Thomas, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland), phosphohistone H3 (Upstate Biotechnology Inc., Lake Placid, New York, USA), PDX-1 (65), or BrdU (Dako A/S, Glostrup, Denmark). Preparations were then incubated with an anti-mouse IgG and FITC-conjugated anti-rabbit IgG. For in vivo cell proliferation assays 2-week-old mice were intraperitoneally injected with BrdU (50 mg/kg body weight). After 18 hours, mice were sacrificed and pancreata were excised and fixed as described. Pancreatic sections were immunostained with an anti-BrdU Ab. BrdU-positive cells were counted. β cell and α cell areas were calculated as follows. Pancreatic sections were immunostained with both insulin and glucagon. Using Canvas 9 software (Deneba Software, Miami, Florida, USA), the area of α and β cells was quantified. At least 50 different pancreatic sections from different mice were analyzed.

Embryos from stages between 7 dpc's to 16.5 dpc's were directly embedded in cryomatrix (Shandon Inc., Pittsburgh, Pennsylvania, USA). In situ hybridizations were performed on 10-μm cryosections using ^{35}S-antisense RNA probes as described previously (66). Mouse mE2F1 cDNA was subcloned into the *Eco*RI site of pBS-SK^{+}, linearized by *Not*I, and antisense mE2F1 RNA was synthesized using T7 RNA polymerase (Promega Corp., Madison, Wisconsin, USA). Mouse PDX-1 cDNA was subcloned from pSP64-xβPDX-1 (67) into *Hind*III/*Xba*I sites of pBS-SK^{+}, linearized by *Hind*III, and antisense mPDX-1 RNA was synthesized using T3 RNA polymerase (Promega Corp.). Negative controls were performed in parallel using sense RNA probes for mE2F1 and mPDX-1 (data not shown).

Animal experiments. The generation of E2F1$^{+/+}$ and E2F1$^{-/-}$ mice has been described (44). These mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and were on a pure C57BL/6J background. Only age-matched (10- to 16-week-old) mice were used unless otherwise stated. Animals were maintained in a temperature-controlled (23°C) facility with a 12-hour light/dark cycle according to European Union guidelines for use of laboratory animals. Mice had ad libitum access to water and regular rodent chow (DO4; UAR, Epinay sur Orge, France). IPGTT insulin-sensitivity tests and glucose, free fatty acids, and insulin measurements were performed as described (68). Body fat mass was evaluated in anesthetized mice by dual energy x-ray absorptiometry (PIXIMU; GE Medical Systems, Buc, France).

Glucose uptake. Primary adipocytes and primary myocytes were prepared according to Rodbell (69) and Rando (70), respectively. Cells were incubated with 100 mM of radiolabeled ^{14}C-3-*O*-methylglucose for 10 minutes in the presence of 1 μM insulin. After that period, the cells were washed three times with PBS, lysed in NaOH 0.1 M, and radioactivity was measured.

Pancreatic islet studies. Islet isolation and insulin secretion studies were described previously (70). Briefly, small pieces of pancreas were digested by collagenase (3 mg/ml) and isolated in oxygenated Krebs-Ringer buffer. Approximately four islets per condition were handpicked and exposed to either 5, 10, or 15 mM glucose. Insulin released in the medium was measured 30 minutes later. Data were expressed as a ratio per DNA and total insulin content.

Statistical analysis. Data are presented as means plus or minus SEM. Group means were compared by factorial ANOVA. Upon significant interactions, differences between individual group means were analyzed by Fisher's protected least squares difference test. Differences were considered statistically significant at P values less than 0.05.

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