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Δ 40 Isoform of p53 Controls β -Cell Proliferation and Glucose Homeostasis in Mice

Charlotte Hinault,¹ Dan Kawamori,¹ Chong Wee Liew,¹ Bernhard Maier,² Jiang Hu,¹ Susanna R. Keller,³ Raghavendra G. Mirmira,² Heidi Scrable,³ and Rohit N. Kulkarni¹

OBJECTIVE—Investigating the dynamics of pancreatic β -cell mass is critical for developing strategies to treat both type 1 and type 2 diabetes. p53, a key regulator of the cell cycle and apoptosis, has mostly been a focus of investigation as a tumor suppressor. Although p53 alternative transcripts can modulate p53 activity, their functions are not fully understood. We hypothesized that β -cell proliferation and glucose homeostasis were controlled by Δ 40p53, a p53 isoform lacking the transactivation domain of the full-length protein that modulates total p53 activity and regulates organ size and life span in mice.

RESEARCH DESIGN AND METHODS—We phenotyped metabolic parameters in Δ 40p53 transgenic (p44tg) mice and used quantitative RT-PCR, Western blotting, and immunohistochemistry to examine β -cell proliferation.

RESULTS—Transgenic mice with an ectopic p53 gene encoding Δ 40p53 developed hypoinsulinemia and glucose intolerance by 3 months of age, which worsened in older mice and led to overt diabetes and premature death from \sim 14 months of age. Consistent with a dramatic decrease in β -cell mass and reduced β -cell proliferation, lower expression of cyclin D2 and pancreatic duodenal homeobox-1, two key regulators of proliferation, was observed, whereas expression of the cell cycle inhibitor p21, a p53 target gene, was increased.

CONCLUSIONS—These data indicate a significant and novel role for Δ 40p53 in β -cell proliferation with implications for the development of age-dependent diabetes. *Diabetes* 60:1210–1222, 2011

The maintenance of adult β -cell mass is critical for the appropriate regulation of glucose homeostasis in both humans and rodents. β -Cell replication is generally accepted as essential for β -cell expansion after birth but remains controversial in adult β -cells (1–7). Nevertheless, a recent study highlighted the ability of human β -cells to replicate in response to combined expression of two cell cycle regulators (8).

Cell replication is regulated by a balance between the active cyclin-cyclin dependent kinase (CDK) complexes and CDK inhibitors, which regulate phosphorylation of retinoblastoma protein, S-phase entry, and cell cycle progression

(4). Among the cell cycle proteins regulating the G1 to S phase transition, CDK4 and cyclin D2 are particularly important. Mice deficient in CDK4 develop β -cell hypoplasia and diabetes (9,10); conversely, overexpression of CDK4 in transgenic mice causes islet hyperplasia without hypoglycemia (9). Cyclin D2, the most abundant cyclin in the islet, has been reported to be critical for postnatal pancreatic β -cell growth (5,11). Global loss of cyclin D2 in mice causes loss of β -cell mass, glucose intolerance, and age-dependent diabetes (5,11), whereas absence of cyclin D2 prevents the expansion of β -cell mass in response to a spectrum of insulin resistance (12). Furthermore, transplantation of human islets transduced with cyclin D1 and CDK6 (expressed in humans but not rodent islets) into diabetic mice improved blood glucose (8). Other reports indicate that Akt is important for the regulation of cyclin D1, cyclin D2, and CDK4 (13), and confirm a link between cell cycle progression and insulin/IGF-1 signaling, a pathway critical for β -cell growth and function (14–17).

The tumor suppressor p53 is a transcription factor and a global suppressor of cell cycle genes, such as cyclins and CDKs (18), and also can actively block cell cycle progression by transactivating its main target gene *p21* (19). Mice deficient in p53 are developmentally normal, but show an increased risk of developing spontaneous tumors in diverse tissues as they age (20,21), but they do not develop insulinomas. Although most studies have focused on p53 action in the context of tumor formation and apoptosis, the key role of p53 in cell growth suggests that the protein may participate in β -cell cycle control and consequently contribute to glucose homeostasis.

The current study investigated the importance of p53 in β -cell growth using mice carrying an extra copy of p53 that codes for Δ 40p53, a 44 kD isoform of p53, also described as Δ Np53 or p44 (mouse)/p47 (human), that lacks the first 40 amino acids of the full-length protein (22). Indeed, up to 10 p53 isoforms have been recently identified indicating the complex regulation of p53 (23). Inappropriate expression of Δ 40p53 alters the balance that normally exists between the isoforms and stabilizes full-length p53. Stabilization of p53 in p44 transgenic (p44tg) mice confers increased tumor suppression, but also results in accelerated aging and is associated with abnormal insulin/IGF-1 signaling (22) and impaired cell proliferation (24). Because aging is an important risk factor for the development of type 2 diabetes (25,26), we used this model of accelerated aging to investigate the role of p53 in islet/ β -cell growth. We report that misexpression of Δ 40p53 in p44tg mice is associated with early hypoinsulinemia and glucose intolerance that worsens with age and leads to overt hyperglycemia and a dramatic decrease in β -cell proliferation. These data suggest that the coordinated activity of p53 isoforms, in particular that of Δ 40p53 and full-length

From the ¹Research Division, Joslin Diabetes Center and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; the ²Wells Center for Pediatric Research, Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana; and the ³Kogod Center on Aging, Mayo Clinic, Rochester, Minnesota.

Corresponding author: Rohit N. Kulkarni, rohit.kulkarni@joslin.harvard.edu. Received 17 September 2009 and accepted 20 January 2011.

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R.G.M. and H.S. contributed equally to this article.

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p53, is necessary for the maintenance of β -cell mass during aging, a finding that could have implications for the prevention of age-dependent diabetes.

RESEARCH DESIGN AND METHODS

Animals and physiological measurements. p44tg mice were generated as described previously (22). Data from male mice are reported. Body and pancreas weight were recorded, and blood glucose (Glucometer Elite, Bayer, Terrytown, NJ), plasma insulin (enzyme-linked immunosorbent assay), and glucagon (radioimmunoassay) levels (Joslin DERC Specialized Assay Core, Boston, MA) were measured before experiments. Glucose tolerance tests, acute glucose-stimulated insulin secretion (GSIS) tests, and insulin tolerance tests were performed as previously reported (27). Pancreata were harvested for immunohistochemical analyses and islet morphometry, and islets were isolated for RNA extraction and *in vitro* GSIS on batches of 15 size-matched islets as reported previously (28).

Islet morphology and immunohistochemistry. Pancreas sections were used to assess β -cell mass and size as described previously (27). β -Cell proliferation was assessed in pancreas sections as described previously (28), using anti-insulin (guinea-pig antibody, DAKO, Carpinteria, CA), anti-BrdU (rabbit polyclonal antibody, DAKO), or antiphosphohistone H3 antibodies (rabbit polyclonal antibody, Upstate Biotechnology, Lake Placid, NY). Other antibodies used include β -catenin (rabbit polyclonal antibody, BD Biosciences, Oxford, U.K.), glucagon (mouse monoclonal antibody, Sigma-Aldrich, St. Louis, MO), somatostatin (rabbit polyclonal antibody, Abcam, Cambridge, U.K.), p21 (mouse monoclonal antibody, DAKO), insulin (mouse monoclonal antibody, Biogenex, San Ramon, CA), and DAPI (Sigma-Aldrich) for nuclear staining.

Real-time RT-PCR. Quantitative real-time RT-PCR was performed on total RNA samples extracted from islets and processed as described previously (28). All primer sequences are available on request.

Western blot analysis. Western blotting was performed as described (3). Antibodies used include cyclin D2 (rabbit polyclonal antibody, Santa Cruz Biotechnology Inc., Santa Cruz, CA), FoxO1 (rabbit polyclonal antibody, Cell Signaling, Danvers, MA), pancreatic duodenal homeobox-1 (PDX-1) (gift from C.V. Wright, Vanderbilt University), p27 (mouse monoclonal antibody, BD Biosciences), p21 (mouse monoclonal antibody, DAKO), and tubulin (mouse monoclonal antibody, Abcam).

Statistical analyses. All data are presented as mean \pm SEM and were analyzed using an ANOVA or unpaired, two-tailed Student *t* test as appropriate. A *P* value less than 0.05 was considered significant. A minimum of *n* = 3 was used for all studies unless indicated.

RESULTS

Glucose homeostasis and metabolic parameters. We compared the phenotype of mice at 3, 10, and 12–14 months of age. The phenotype of p44tg mice becomes evident at 3 months, and the animals begin to succumb at \sim 14 months of age (22). p44tg mice were lighter than their nontransgenic counterparts at all ages, as shown for 3 months of age in Fig. 1A. The animals had already developed hypoinsulinemia at this age, which persisted throughout life (Fig. 1C). Although the animals displayed normal glucose tolerance at 2 months (Fig. 2A), they had

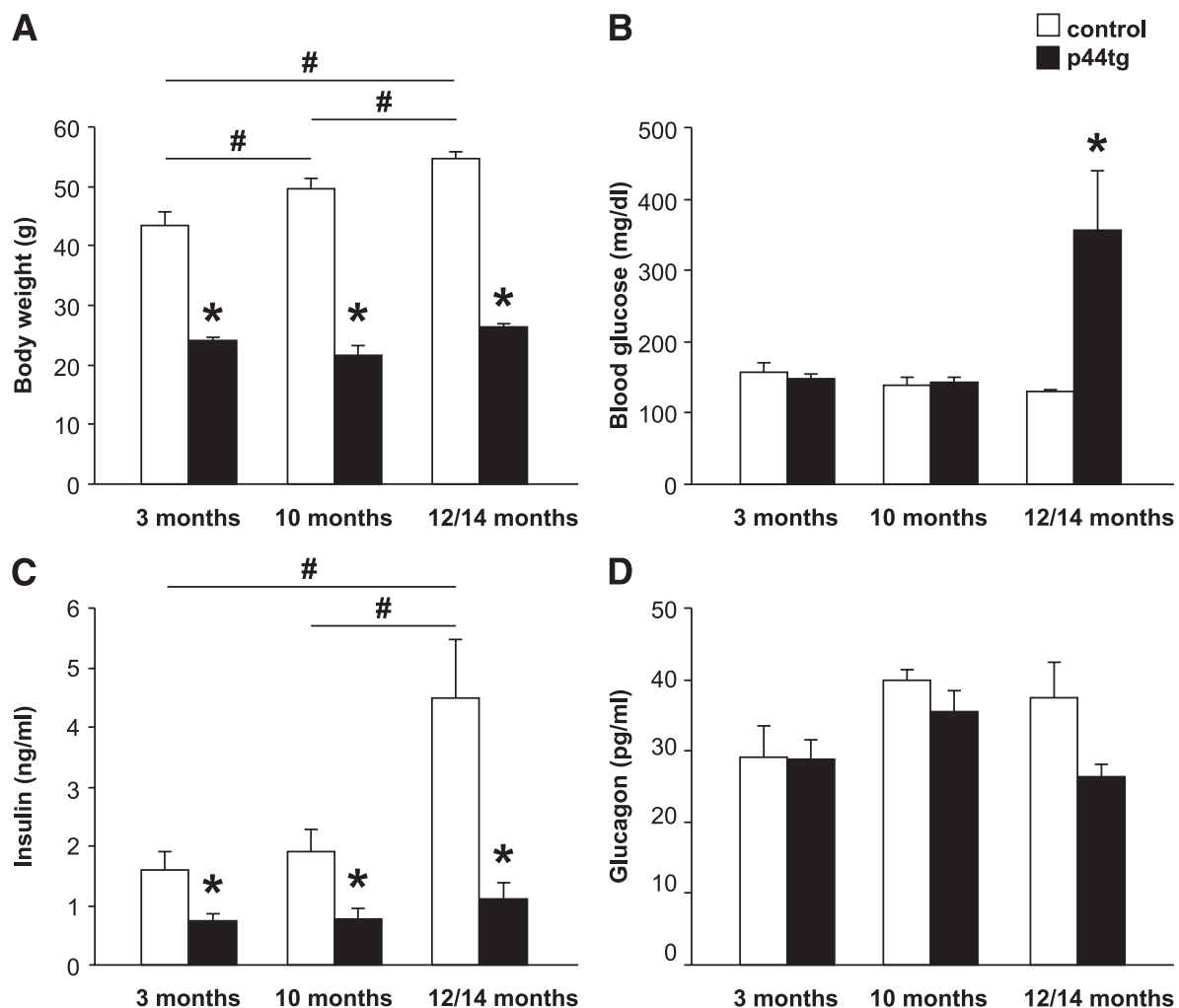


FIG. 1. p44tg mice exhibit hypoinsulinemia and hyperglycemia with age. Body weight (A), blood glucose (B), plasma insulin (C), and plasma glucagon (D) levels were measured at 3, 10, and 12–14 months of age from random-fed control (□) and p44tg (■) (*n* = 5–9) mice. **P* < 0.05 for p44tg vs. control mice. #*P* < 0.05 for control mice vs. different ages.

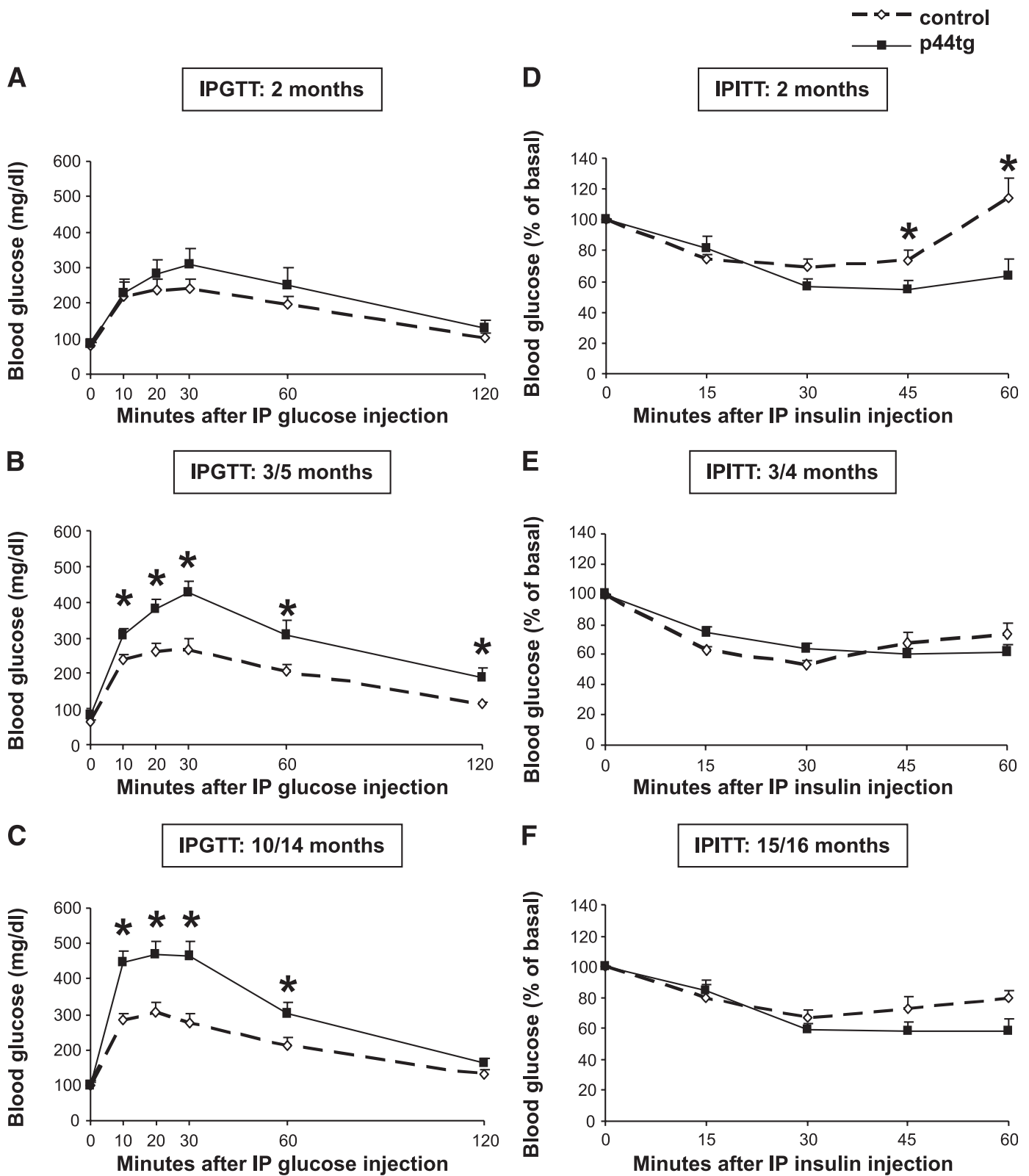


FIG. 2. p44tg mice exhibit age-dependent glucose intolerance and normal insulin sensitivity. Glucose tolerance tests performed at 2 months (A), 3–5 months (B), and 10–14 months (C) of age ($n = 6-8$). Blood glucose was measured at 0, 10, 20, 30, 60, and 120 min after intraperitoneal injection of glucose. Insulin tolerance tests were performed in each group at 2 months (D), 3–4 months (E), and 15–16 months (F) of age ($n = 6-8$). Glucose was measured at 0, 15, 30, 45, and 60 min after intraperitoneal injection of insulin. IP, intraperitoneal; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test. * $P < 0.05$ for p44tg vs. control mice.

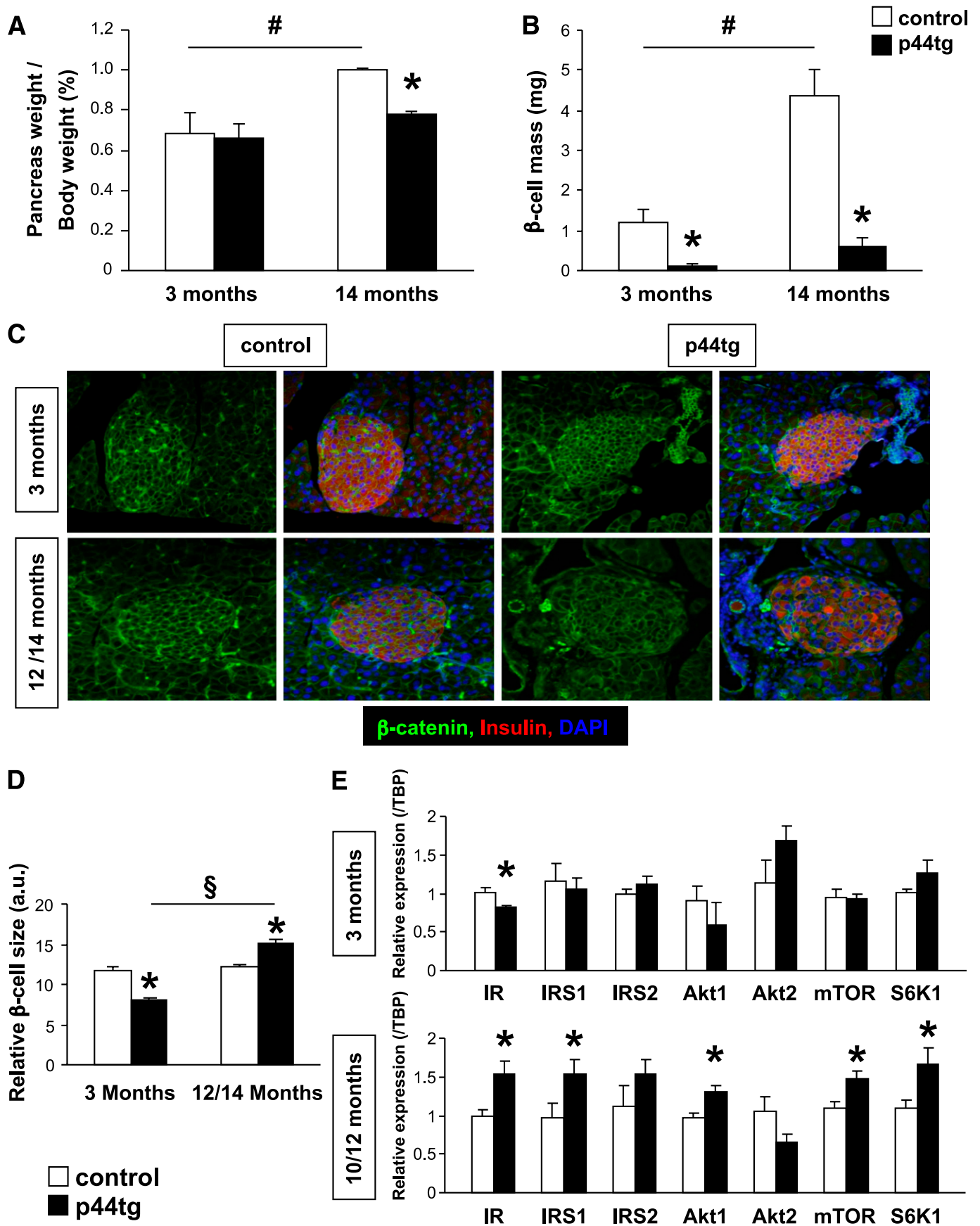


FIG. 3. Age-dependent alterations in β -cell size in p44tg mice. **A:** Pancreatic weight expressed as a percentage of the total body weight ($n = 3$). **B:** β -Cell mass was assessed as described in RESEARCH DESIGN AND METHODS. **C** and **D:** β -Cell size was assessed by coimmunostaining for β -catenin (green) and insulin (red) with DAPI (blue) in pancreas sections from control ($n = 5$) and p44tg ($n = 5$) mice at 3 and 12–14 months of age as

developed intolerance by 3 months (Fig. 2B) and were significantly hyperglycemic by 12–14 months (Fig. 1B and 2C), suggesting overt diabetes that could contribute, in part, to death at this age. In contrast, all controls exhibited fed blood glucose levels <150 mg/dL at 12 months of age (Fig. 1B). Glucagon levels were not significantly different between groups (Fig. 1D). Insulin sensitivity was unchanged in p44tg mice from 3 months of age (Fig. 2E and F) and even mildly improved in younger p44tg mice (Fig. 2D). These data indicate that increased dosage of $\Delta 40p53$ leads to hypoinsulinemia and glucose intolerance as early as 3 months and worsens with age.

Disordered islet morphology in p44tg mice. Although both pancreas weight and number of islets exhibited an age-dependent increase in control mice, the total number of islets was less in p44tg mice even when normalized to total pancreas weight (Fig. 3A and Supplementary Fig. 1). Compared with 3 months of age, the differences became more marked at 12–14 months. The reduced pancreatic weight and islet number in p44tg mice were associated with a dramatic decrease in β -cell mass (Fig. 3B). Indeed, β -cell mass was significantly lower in the p44tg mice and ranged between 11% (young) and 14% (old) in the age-matched control animals. Quantification of β -cell size revealed a 30% decrease in the p44tg mice compared with controls at 3 months of age but showed the opposite in the older p44tg mice (Fig. 3C and D). This represented a two-fold increase in β -cell size as the p44tg mice aged from 3 to 12 months. To explore the pathways that regulate β -cell size, we considered previous reports that proteins in the insulin/IGF-1 signaling pathways regulate β -cell size via Akt/mTOR/S6K1 (14–16). Quantitative real-time RT-PCR for these molecules did not reveal significant differences between groups at 3 months of age except for a decrease in insulin receptor (IR) expression in p44tg islets (Fig. 3E). However, in islets from older p44tg mice, we observed increased gene expression of *IR*, *IRS1* (IR substrate-1), and especially *Akt1*, *mTOR*, and *S6K1*, suggesting an attempt at compensation by increasing the β -cell size in the older mutant mice. This was not observed in the liver or white adipose tissue of p44tg mice (Supplementary Fig. 5).

Examination of islet morphology by immunohistochemistry (Fig. 4A) revealed the typical pattern of a core of insulin-containing cells surrounded by non- β -cells in islets from controls and in a majority of islets from p44tg mice at 3 months of age (Fig. 4A, top). However, $\sim 35\%$ of islets in the normoglycemic and hypoinsulinemic p44tg groups exhibited infiltration of α - and δ -cells within the islet core and a relative decrease in the number of β -cells (quantified in Fig. 4B), which correlated with the hypoinsulinemia at this age (Fig. 1C). These abnormal features, which were not observed at 2 months of age (Supplementary Fig. 2), were evident in $\sim 85\%$ of the islets by 12 months of age in the p44tg group (Fig. 4A, bottom). In parallel, islet hormone gene expression (Fig. 4C) revealed a significant increase in somatostatin gene expression that was consistent with a relative increase in δ -cells in p44tg mice (Fig. 4A), whereas glucagon gene expression (Fig. 4C) and circulating glucagon levels (Fig. 1D) were not different between groups at all ages. A significant increase in the

expression of insulin and GLUT 2 in 3-month-old transgenic mice suggested a compensatory effect at this younger age (Fig. 4C, top) that was not evident in the older hyperglycemic group (Fig. 4C, bottom). Because p44tg mice exhibit hypoinsulinemia and reduced β -cell numbers, we evaluated β -cell secretory function in vivo (Fig. 4D) and in vitro (Fig. 4E) by measuring insulin release in response to glucose stimulation. Although in vivo GSIS was impaired in p44tg mice (Fig. 4D) and likely contributed to the glucose intolerance (Fig. 2B), the GSIS in isolated islets in vitro was not different between groups (Fig. 4E), suggesting the absence of a cell autonomous insulin secretory defect.

Inhibition of β -cell proliferation in p44tg mice. Next, we determined whether the decrease in β -cell mass in p44tg mice (Fig. 3B) was due to changes in β -cell proliferation. Coimmunostaining of pancreas sections with antibodies against insulin and either one of two proliferation markers showed a decrease in the proliferation of β -cells with age in control islets (Fig. 5A and B) as reported previously (29). BrdU labeling demonstrated a virtual absence of replicating β -cells in young and old p44tg mice compared with controls (Fig. 5A). A decrease in pHH3 immunoreactive β -cells at 3 and 12–14 months of age in p44tg mice confirmed reduced number of cells in the M or late G2 phases of the cell cycle (Fig. 5B). Before 2 months of age, no significant difference was evident in β -cell replication between the two groups (Supplementary Fig. 2) and correlated with normal glucose tolerance (Fig. 2).

We next evaluated the effects of neogenesis (30) and apoptosis (31) in the maintenance of β -cell mass. Single and clusters (≤ 8) of insulin+ cells (markers of neogenesis) were virtually absent in pancreatic ducts in 3-month-old control and p44tg mice. Although some insulin+ cells/clusters were observed in 12- to 14-month-old mice, no significant difference was evident between groups (number/mm² pancreas: 0.3 ± 0 , control vs. 0.3 ± 0.2 , p44tg; $P = \text{NS}$, $n = 3$). Similarly, no significant differences were observed in insulin+ cells in extraductal pancreatic tissue (3 month; 14 ± 0.3 , control vs. 12.8 ± 3.4 , p44tg; 12–14 month; 8.3 ± 0.2 , control vs. 10.2 ± 2.4 , p44tg; $P = \text{NS}$; $n = 3$), suggesting that neogenesis is not altered significantly when $\Delta 40p53$ is misexpressed. Although p53 has been studied in the context of apoptosis in multiple tissues, it has not been causally involved in β -cell death during diabetes (32). Nevertheless, we performed the transferase-mediated dUTP nick-end labeling (TUNEL) assay and found no significant differences in TUNEL+ β -cells between groups (Supplementary Fig. 3). These data provide strong evidence that the misexpression of $\Delta 40p53$ led to a marked reduction of β -cell proliferation, consistent with reports for other cell types (22,24).

Altered expression of cyclin D2 and PDX-1. p53 regulates the G1 to S phase progression through cell cycle inhibitors such as p21, but recent reports indicate that p53 also targets integral cell cycle proteins, such as cyclin E and CDK4 (18). We observed a significant decrease in the gene expression levels of cyclin D2, CDK2, and cyclin E1, and a significant increase in the level of cyclin E2 in 3-month-old p44tg mice (Fig. 6A). At 12 months of age,

described in RESEARCH DESIGN AND METHODS. A representative islet for each group at magnification 40 \times is presented with the quantification of relative β -cell size (mean \pm SEM from $n \geq 200$ cells counted per mouse). E: Real-time RT-PCR on RNA extracted from islets of 3- and 10- to 12-month-old control ($n = 3$ –8) and p44tg ($n = 4$) mice. Results are normalized to TATA-binding protein (TBP) and expressed relative to control mice. * $P < 0.05$ for p44tg vs. control mice. # $P < 0.05$ for 3- vs. 12- to 14-month-old control mice. § $P < 0.05$ for 3- vs. 12- to 14-month-old p44tg mice. (A high-quality digital representation of this figure is available in the online issue.)

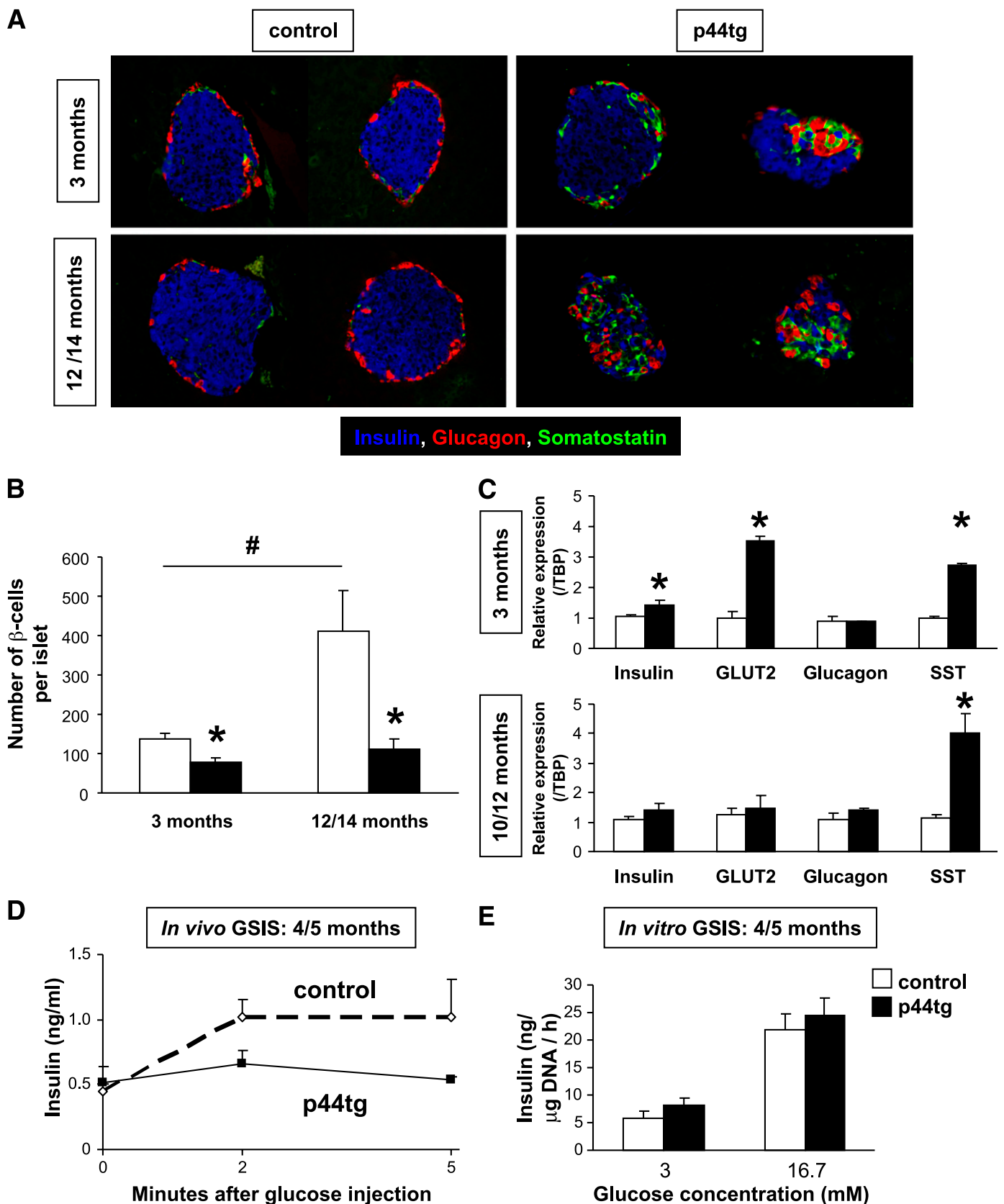


FIG. 4. Reduction in insulin-positive cells and increase in somatostatin-positive cells in islets from p44tg mice. **A:** Triple coimmunostaining for insulin (blue), somatostatin (green), and glucagon (red) on pancreas sections from control or p44tg mice at 3 and 12–14 months of age as described in RESEARCH DESIGN AND METHODS ($n = 5$). Two representative islets for each group at magnification 40 \times are presented. **B:** The number of insulin-positive cells was counted in at least 10 islets randomly selected from pancreas sections from each mouse ($n = 3$ mice at each age per group). **C:** Real-time RT-PCR on RNA extracted from islets of 3- and 10- to 12-month-old control ($n = 3$ –8) and p44tg ($n = 4$) mice. Results are normalized to TBP and expressed relative to controls. **D:** In vivo GSIS. Insulin was measured in plasma samples extracted from blood collected at

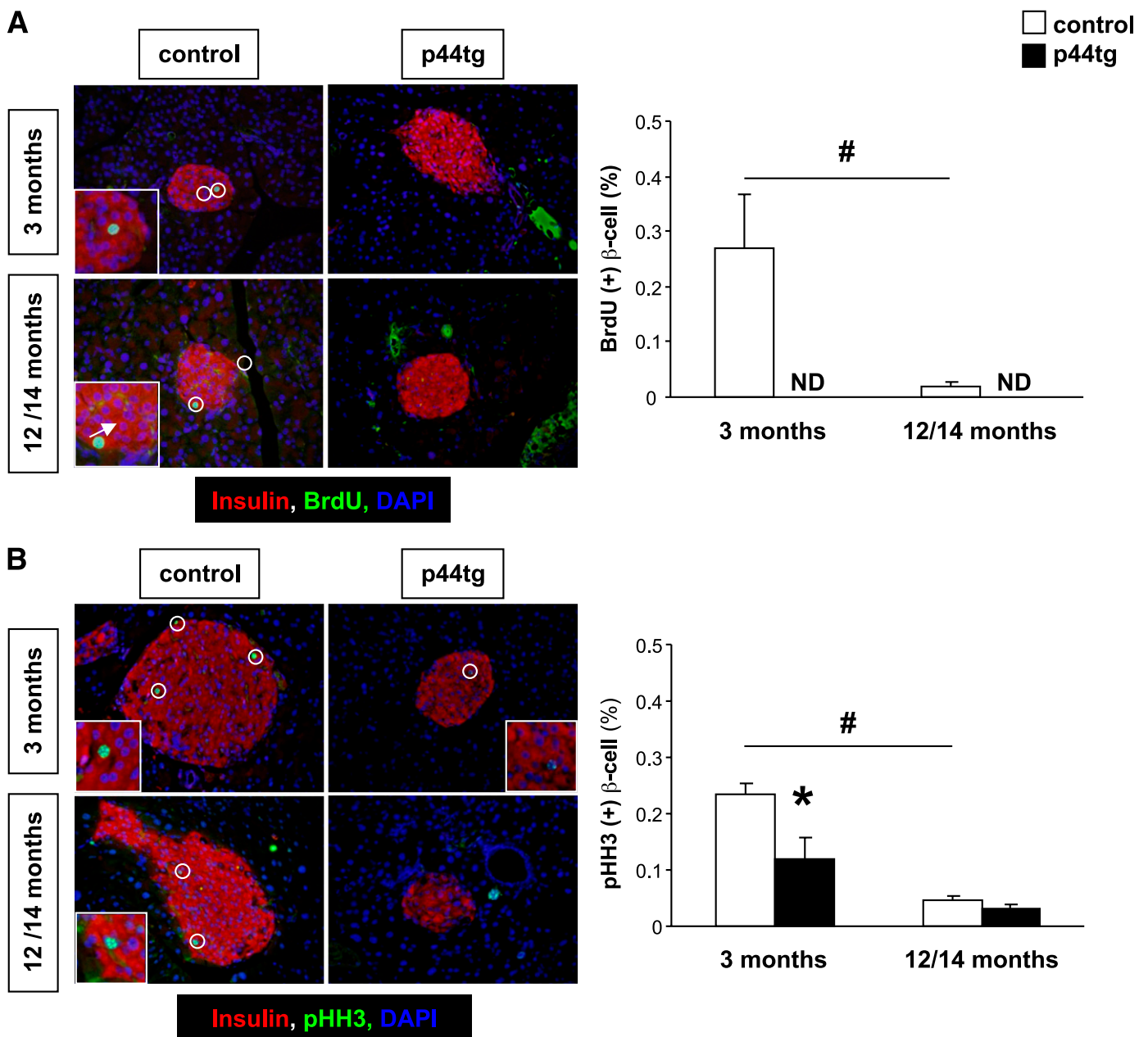


FIG. 5. Reduced β-cell proliferation in p44tg mice. β-Cell proliferation was assessed by coimmunostaining for BrdU (A, green) and pHH3 (B, green) with insulin (red) and DAPI (blue) in pancreas sections from control and p44tg mice at 3 and 12–14 months of age as described in RESEARCH DESIGN AND METHODS. A representative islet for each group at magnification 40× is presented ($n = 5$). Quantification is shown on the right. * $P < 0.05$ for p44tg vs. control mice. # $P < 0.05$ for 3- vs. 12- to 14-month-old control mice. (A high-quality digital representation of this figure is available in the online issue.)

only cyclin E1 gene expression remained low; at the same time, however, CDK4 levels became significantly increased. The decrease in cyclin D2 gene expression at 3 months of age correlated with lower protein expression in isolated islets (Fig. 6B, left). In old mice, despite normal levels of gene expression, cyclin D2 protein level was significantly decreased (Fig. 6B, right).

We next examined the expression of FoxO1, which regulates β-cell proliferation (33), and two of its target

proteins, PDX-1 (34) and p27 (35). Examination of gene and protein expression in whole islets revealed that FoxO1 expression was significantly lower at 3 months but unchanged at 10 months in p44tg mice (Fig. 7A and B). PDX-1 gene expression was consistently higher in p44tg mice (Fig. 7A); however, its protein level was consistently lower (Fig. 7B). This decrease was less pronounced in old mice and correlated with fewer β-cells in islets from the transgenic mice (Fig. 4B). p27 gene and protein expression

0, 2, and 5 min after intraperitoneal injection of glucose (3 g/kg body wt) from 4–5-month-old control and p44tg mice. E: In vitro GSIS. Islets isolated from 4–5-month-old control and p44tg mice were incubated for 1 h with 3 or 16.7 mmol/L glucose as described in RESEARCH DESIGN AND METHODS. Bar graph depicts data obtained from three to five individual batches of size-matched islets from at least three independent mice per group. * $P < 0.05$ for p44tg vs. control mice. # $P < 0.05$ for 3- vs. 12- to 14-month-old control mice. (A high-quality digital representation of this figure is available in the online issue.)

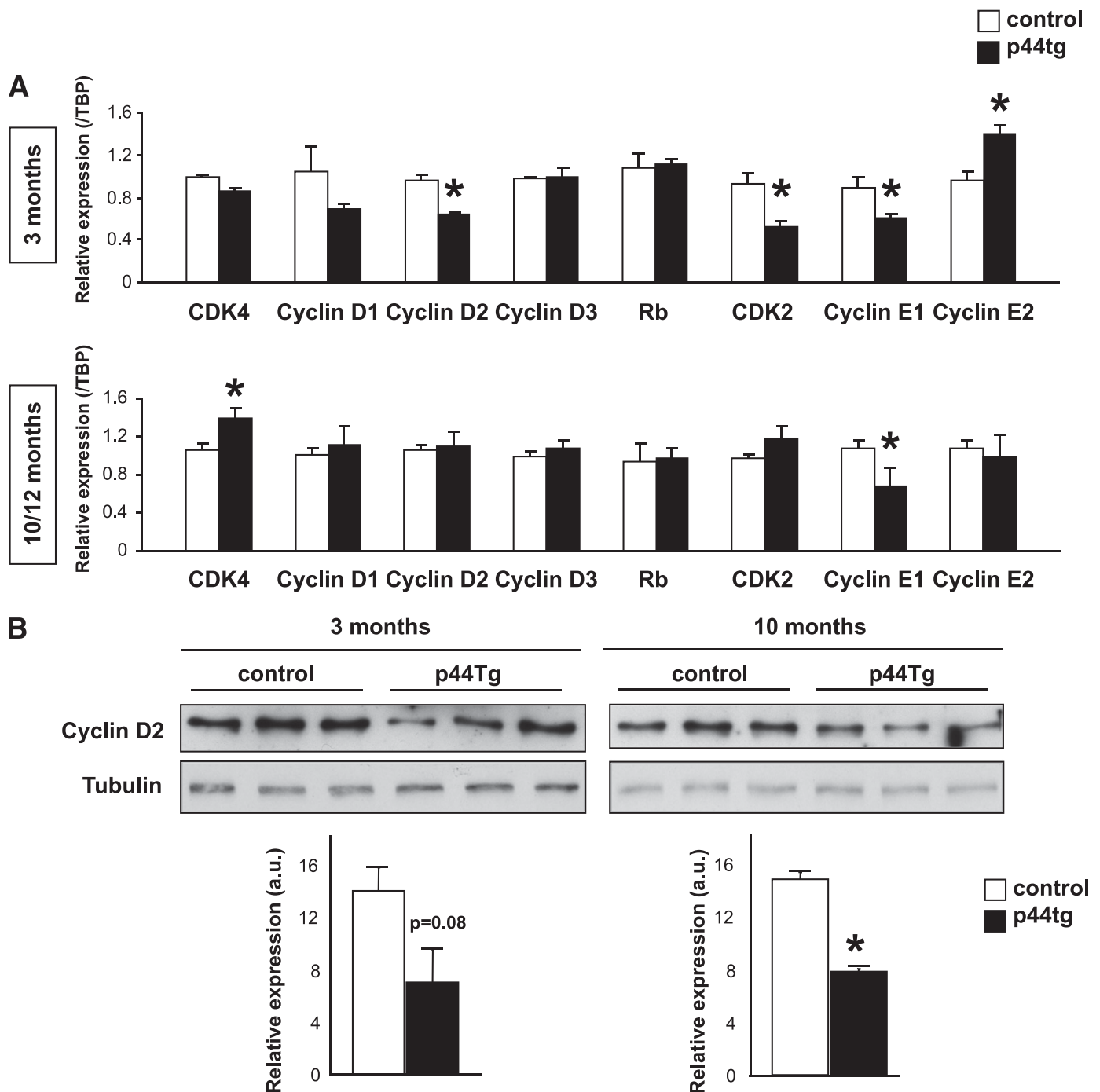


FIG. 6. Regulation of cyclin D2 expression in p44tg islets. **A:** Real time RT-PCR was performed on RNA extracted from islets of 3- and 10- to 12-month-old control ($n = 3-8$) and p44tg ($n = 4$) mice. Results are normalized to TBP and expressed relative to controls. * $P < 0.05$ for p44tg vs. control mice. **B:** Western blotting for cyclin D2 and quantification normalized to tubulin in islets from control and p44tg at 3 and 10 months of age. * $P < 0.05$ for p44tg vs. control mice.

remained unchanged (Fig. 7A and B). These data indicate that decreased β -cell mass and proliferation observed in p44tg mice (Figs. 3B and 5) are associated with decreased expression of cyclin D2 and PDX-1.

Increased p21 expression in p44tg islets during aging. Finally, we evaluated the gene expression of *p53* and its protein targets (19). To measure the level of transcription of both *p53* (endogenous) and $\Delta 40p53$ (endogenous and transgene) genes, we designed two sets of primers for quantitative RT-PCR because the use of specific primers to

distinguish both genes was not possible. Np53 primers targeted the 5' end of the *p53* transcript and amplified transcripts derived from the endogenous *p53* gene only, whereas Cp53 primers targeted sequences at the 3' end of RNA and amplified transcripts derived from both the endogenous *p53* gene and the transgene. We also measured the expression of several well-known *p53* target genes (19), such as *p21*, *14-3-3*, *Gadd45*, *Bax*, *MDM2*, *PTEN*, and the IGF-1 receptor (*IGF-1R*). We observed a significant decrease in endogenous *p53* gene expression (Np53

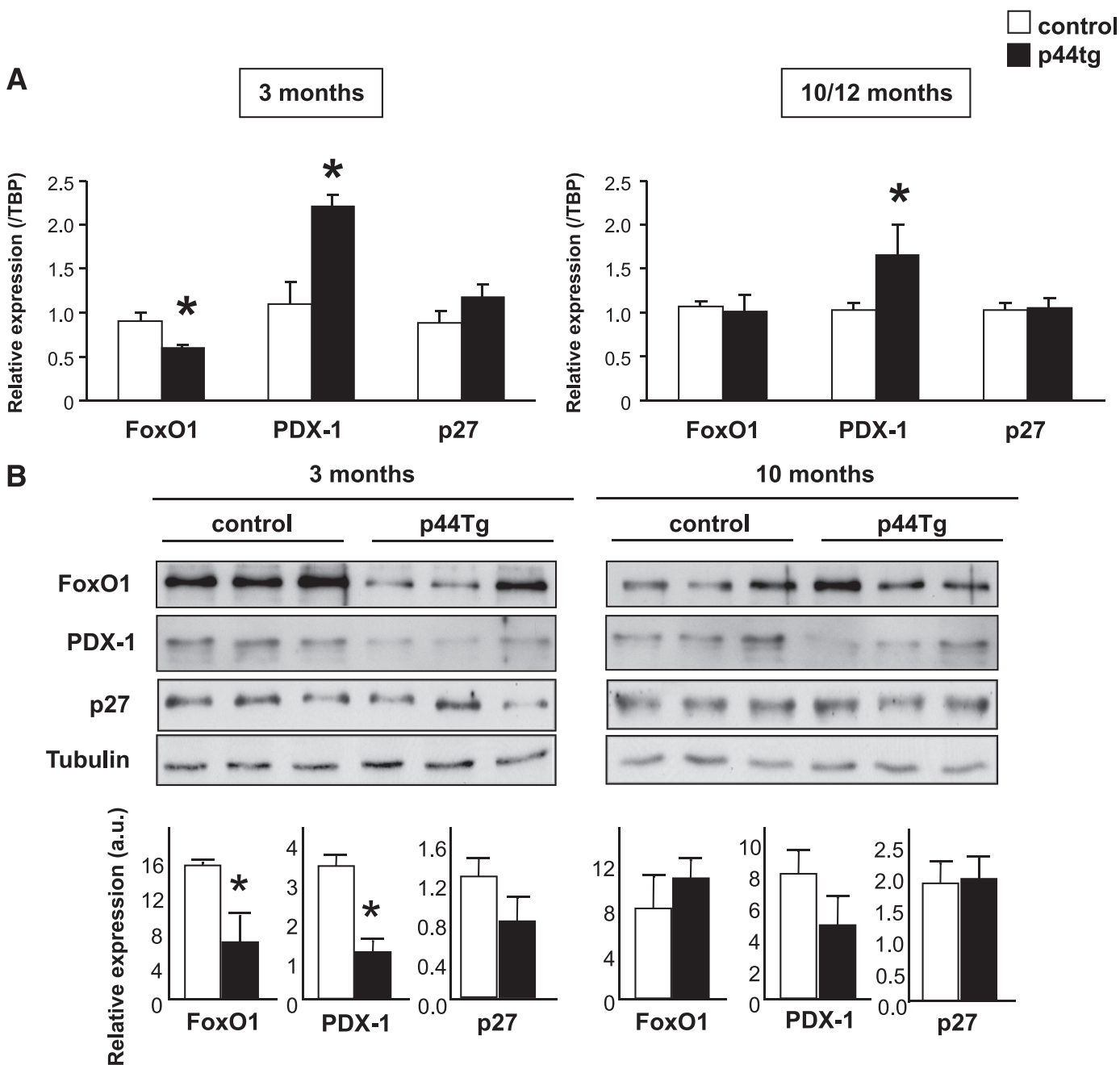


FIG. 7. Regulation of PDX-1 expression in p44tg islets. **A:** Real-time RT-PCR was performed on RNA extracted from islets of 3- and 10- to 12-month-old control ($n = 3-8$) and p44tg ($n = 4$) mice. Results are normalized to TBP and expressed relative to control mice. * $P < 0.05$ for p44tg vs. control mice. **B:** Western blotting for FoxO1, PDX-1, and p27 and quantification normalized to tubulin in islets from control and p44tg at 3 and 10 months of age. * $P < 0.05$ for p44tg vs. control mice.

primers) that correlated with reduced *Gadd45* gene in islets from 3-month-old p44tg mice (Fig. 8A, top). In islets from 12-month-old p44tg mice, we observed an increase in Δ40p53 expression (Cp53 primers) that was associated with an enhanced expression of p21 and MDM2 (Fig. 8A, bottom). Although *Bax* gene expression was significantly elevated in p44tg islets at both ages, we did not observe significance differences between groups in the TUNEL assay (Supplementary Fig. 3). The increase in gene expression of *IGF-1R* that was evident in young p44tg islets did not persist in older mice, and we did not observe alterations in *PTEN* gene expression at either age (Fig. 8A). However, we did find an increase in p21

protein by Western blot analysis of p44tg islets (Fig. 8B) and by immunohistochemistry in β-cell nuclei of p44tg islets (Fig. 8C). Thus, the strong inhibition of β-cell proliferation observed in p44tg islet cells correlated with a significant increase in p21, the main target of p53 in cell cycle regulation.

DISCUSSION

By using p44tg mice with an ectopic *p53* gene that encodes Δ40p53 (36), we have identified a role for the tumor suppressor p53 in the maintenance of normal β-cell mass and glucose homeostasis. Although Δ40p53 has virtually no

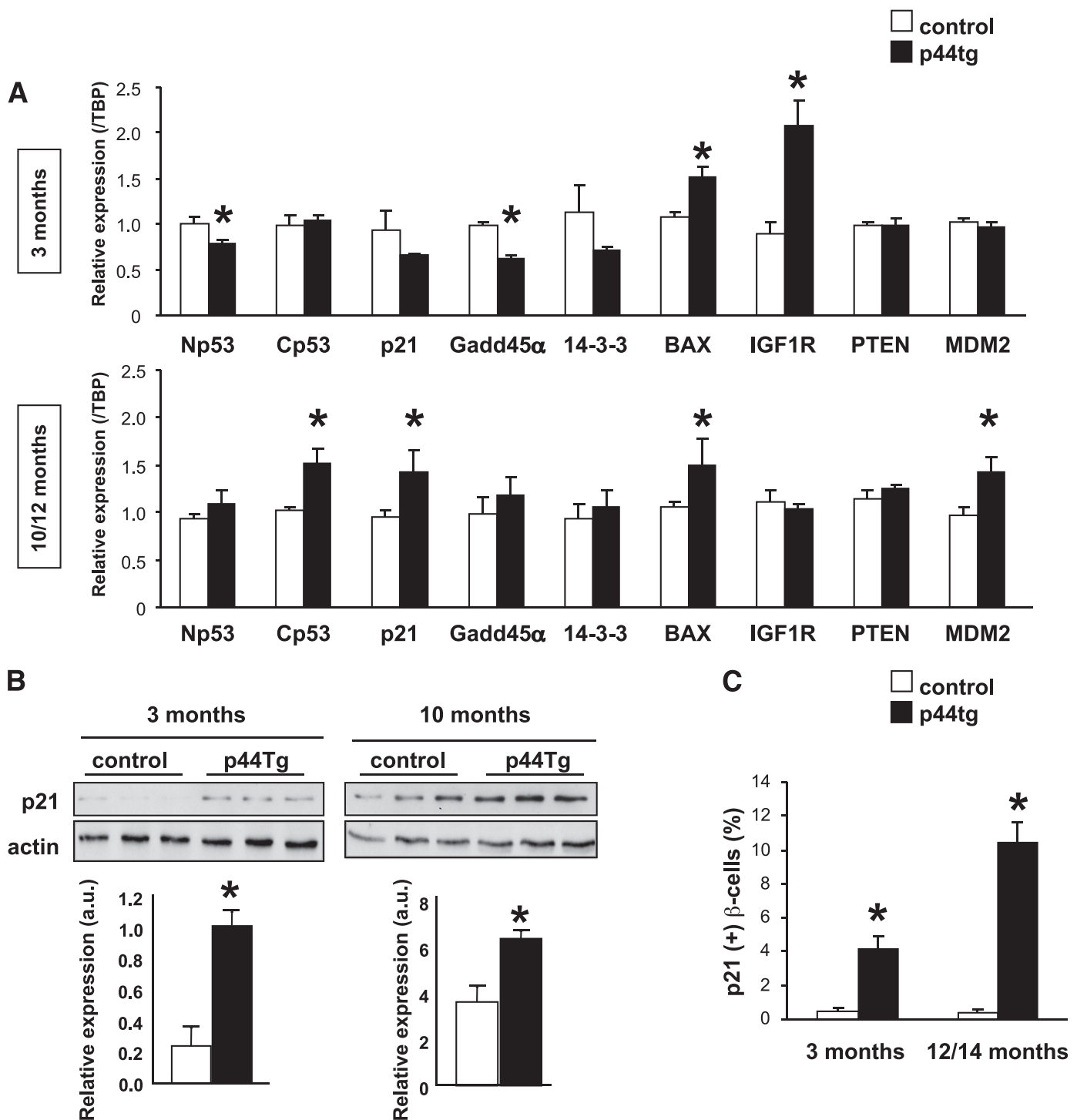


FIG. 8. Increased p21 expression in p44tg islets. **A:** Real-time RT-PCR was performed on RNA extracted from islets of 3- and 10- to 12-month-old control ($n = 3-8$) and p44tg ($n = 4$) mice. Results are normalized to TBP and expressed relative to control mice. $*P < 0.05$ for p44tg vs. control mice. **B:** Western blotting for p21 and quantification normalized to actin in islets from control and p44tg at 3 and 10 months of age. $*P < 0.05$ for p44tg vs. control mice. **C:** Quantification of p21-positive β -cell nuclei in pancreas sections from control and p44tg mice at 3 and 12–14 months of age ($n = 5$). $*P < 0.05$ for p44tg vs. control mice (at least 8–10 islets from five mice per group).

autonomous transcriptional activity, it can interact with full-length p53 to form tetramers that bind DNA and activate or suppress target gene expression (36,37). Expression of ectopic $\Delta 40p53$ in the mouse alters the balance between the full-length and short isoforms that normally exists and hyperactivates p53 (22,36,38). This increased activity of p53 can account for the higher levels of p21 transcripts, which we observed in β -cells

from 10–12-month-old p44tg mice. The expression of $\Delta 40p53$ in p44tg mice can also account for the higher level of mRNA encoding the IGF-1R, whose transcription is differentially regulated by wild-type and mutant p53 (39). An increased gene expression of *IGF-1R* in liver and white adipose tissue from p44tg mice (Supplementary Fig. 5) suggests a non-tissue-specific effect. Similar to embryonic fibroblasts derived from p44tg mice (22), islets from young

p44tg mice expressed significantly high levels of IGF-1R, indicating impaired *trans*-suppression activity. Both the increased *trans*-activation of p21 in old islets and the decreased *trans*-suppression of the IGF-1R in the young can be explained by differences in expression of the transgene encoding $\Delta 40p53$ with age, as detected by primers that amplify sequences encoding the COOH-terminal domain of the p53 protein. Younger p44tg mice expressed control levels of $\Delta 40p53$ and slightly lower levels of p53, whereas older mice expressed normal levels of p53 and higher levels of $\Delta 40p53$. Biochemical experiments have demonstrated that the stability and activity of the p53 tetramer are exquisitely sensitive to the dose of $\Delta 40p53$, with low doses of $\Delta 40p53$ activating and high doses inactivating p53 function (40,41). The differential effects of $\Delta 40p53$ on p53-dependent *trans*-activation and *trans*-repression with age could also explain how p21 levels increase in both age groups, but by different mechanisms. In old mice, the increase would be due to a direct effect of p53 on the p21 promoter, leading to increased transcription of the p21 gene. In young mice, on the other hand, increased p21 could be an indirect effect of higher IGF-1R expression and activation of IGF signaling due to impaired IGF-1R *trans*-repression. Stimulation of the IGF-1 signal transduction pathway can increase p21 (42), and further work is necessary to delineate the link among $\Delta 40p53/p53$, p21, and IGF-1R levels in β -cell proliferation.

β -Cell proliferation and glucose tolerance were impaired in 3-month-old p44tg animals and worsened to overt diabetes as the animals aged. Although random-fed blood glucose levels were normal in the transgenic mice at 3 and 10 months, the mutants displayed clear intolerance in response to a glucose challenge indicating deficiency in functional β -cells. Previous studies in rodents have reported normoglycemia even when β -cell mass is reduced. For example, Sreenan et al. (43) report a reduced β -cell mass before the onset of diabetes in the nonobese diabetic mouse, and Tavana et al. (44) describe the phenotypes of 1-month-old mice doubly mutant for p53 and non-homologous end-joining deficiency that exhibit an $\sim 50\%$ decrease in β -cell mass and yet manifest blood glucose levels that are not significantly different from controls. Further, obese and nondiabetic humans have been reported to express a wide range of β -cell mass that is adequate to maintain euglycemia up to a specific threshold, and crossing the threshold correlates with fasting hyperglycemia and overt diabetes (45). In our study, it is possible that p44tg mice that are significantly leaner than controls at all ages only cross this "threshold" at ~ 12 months of age when they begin to exhibit overt hyperglycemia. Additional longitudinal studies are necessary to investigate these observations. Thus, our data suggest that the balance between full-length and $\Delta 40p53$ isoforms plays a critical role in the maintenance of β -cell proliferation and glucose homeostasis, and may be important in regulating the cell cycle during aging. Further support for a role for p53 in glucose homeostasis is evident from recent studies. For example, Minamino et al. (46) reported a role for adipose p53 in the regulation of insulin resistance. In our study, circulating proinflammatory cytokines, which are potential effectors in insulin resistance associated with diabetes, showed no significant differences between groups (Supplementary Fig. 4). The cytokine expression profile in liver and white adipose tissue was also similar between groups. These data, along with the normal insulin sensitivity in the global p44tg mice, indicate that the

glucose intolerance is due to intrinsic effects of p53 on β -cell mass rather than to secondary effects of transgene expression in other insulin sensitive tissues. Indeed, Tavana et al. (44) reported that DNA double-strand breaks combined with an absence of p53-dependent apoptosis in mice leads to reduced β -cell replication and severe age-dependent diabetes, supporting our hypothesis that p53 plays a role in the regulation of β -cell proliferation.

Although the mechanisms that regulate the dynamics of β -cell turnover during aging are still being unraveled, several studies including our own support a role for replication as the major mechanism that underlies the compensatory growth response to insulin resistance (1–5,47). Two proteins that are important for this compensatory growth response are cyclin D2 (5,11,12) and PDX-1, the pancreatic duodenal homeobox domain protein (3). The lower expression of cyclin D2 in islets would potentially exacerbate the loss of β -cells and lead to glucose intolerance and overt diabetes. In this study, the expression of PDX-1 was also altered in p44tg β -cells. Although the gene expression of *PDX-1* was increased in both young and old p44tg mice, the protein expression in islets was low, suggesting compensatory effects in gene expression or more dominant regulation of the transcription factor at the protein level. In old p44tg mice, however, there were fewer β -cell nuclei in which PDX-1 could be detected. Immunohistochemistry (data not shown) also revealed that the localization of PDX-1 correlated inversely with that of FoxO1, a transcription factor that regulates expression of this homeodomain protein (34). Because FoxO1 transactivates cell cycle inhibitors (p21 and p27) (35) and represses cell cycle activators (cyclin D1 and D2), nuclear localization of FoxO1 could have a major impact on cell cycle progression in aging β -cells. Further subcellular fractionation approaches are necessary to directly address this question. It is also possible that FoxO1 and p53 interact with deacetylase sirtuin 1 and function in a cooperative manner in an aging environment (48). Thus, the alterations in both PDX-1 and cyclin D2, in our model of accelerated aging, underscore the concept that these two proteins are involved in β -cell replication during aging.

Two CDKIs, namely, p16 and p19, have been reported to be involved in β -cell regenerative failure in diabetes (49). Our observations of enhanced expression of *p16* and *p19* genes confirm the premature aging phenotype of p44tg mice (Supplementary Fig. 6). However, the regulatory link between p53 and the two aging markers in the maintenance or amplification of the phenotype requires further investigation.

Maier et al. (22) previously described that young p44tg mice displayed signs of aging in bone. Further, the osteoblast-secreted molecule, osteocalcin, has recently been reported to modulate insulin secretion and insulin gene expression that correlated with expression of *CREB* and *NeuroD* genes (50). In our study, we observed a mild but significant decrease in osteocalcin levels only in old p44tg mice; however, gene expression of insulin, *CREB*, or *NeuroD* was unaltered (Supplementary Fig. 7).

Finally, the significance of the presence of p53 in non- β -cell fractions obtained from dispersed islet cells (29) remains unclear. Although the relative increase in α -cells in p44tg islets was not associated with an increase in glucagon gene expression or circulating glucagon levels, we observed a significant increase in the number of somatostatin-secreting δ -cells and gene expression in the

mutant islets. The presence of a p53 response element in the somatostatin promoter (Genomatix) warrants further studies to evaluate whether the increase in somatostatin actually decreases insulin secretion or limits β -cell growth in a paracrine manner in the p44tg mice.

In summary, increased dosage of $\Delta 40p53$ in mice promotes hypoinsulinemia and glucose intolerance, ultimately leading to overt diabetes with age and early death. The suppression of β -cell proliferation secondary to changes in the expression of p21, cyclin D2, and PDX-1 in this mouse model of accelerated aging implicates impaired p53 function in the development of type 2 diabetes in the elderly.

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C.H. conceived and designed the experiments, performed the experiments, analyzed data, and wrote the article. D.K., C.W.L., and B.M. performed the experiments; contributed reagents, material, and mice; and analyzed data. J.H. performed the experiments and analyzed data. S.R.K. contributed reagents and performed experiments. R.G.M. and H.S. contributed reagents, material, and mice; analyzed data; and wrote the article. R.N.K. conceived and designed the experiments, performed the experiments, analyzed data, and wrote the article.

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