

Ectopic Expression of E2F1 Stimulates β -Cell Proliferation and Function

Gael Grouwels,¹ Ying Cai,¹ Inge Hoebeke,¹ Gunter Leuckx,¹ Yves Heremans,¹ Ulrike Ziebold,² Geert Stangé,¹ Marie Chintinne,¹ Zhidong Ling,¹ Daniel Pipeleers,¹ Harry Heimberg,¹ and Mark Van de Casteele¹

OBJECTIVE—Generating functional β -cells by inducing their proliferation may provide new perspectives for cell therapy in diabetes. Transcription factor E2F1 controls G₁- to S-phase transition during the cycling of many cell types and is required for pancreatic β -cell growth and function. However, the consequences of overexpression of E2F1 in β -cells are unknown.

RESEARCH DESIGN AND METHODS—The effects of E2F1 overexpression on β -cell proliferation and function were analyzed in isolated rat β -cells and in transgenic mice.

RESULTS—Adenovirus AdE2F1-mediated overexpression of E2F1 increased the proliferation of isolated primary rat β -cells 20-fold but also enhanced β -cell death. Coinfection with adenovirus AdAkt expressing a constitutively active form of Akt (protein kinase B) suppressed β -cell death to control levels. At 48 h after infection, the total β -cell number and insulin content were, respectively, 46 and 79% higher in AdE2F1 + AdAkt-infected cultures compared with untreated. Conditional overexpression of E2F1 in mice resulted in a twofold increase of β -cell proliferation and a 70% increase of pancreatic insulin content, but did not increase β -cell mass. Glucose-challenged insulin release was increased, and the mice showed protection against toxin-induced diabetes.

CONCLUSIONS—Overexpression of E2F1, either in vitro or in vivo, can stimulate β -cell proliferation activity. In vivo E2F1 expression significantly increases the insulin content and function of adult β -cells, making it a strategic target for therapeutic manipulation of β -cell function. *Diabetes* 59:1435–1444, 2010

The majority of adult β -cells is arrested in G_{0/1} cell cycle phases (1–5) and rarely replicates more than once even when stimulated to proliferate (6). Nevertheless, β -cell replication appears a major mechanism for postnatal formation of rodent β -cells (7,8). Increasing the number of β -cells that enter replication may provide new perspectives for cell therapy in

diabetes. The key factors controlling entry and progression through the cell cycle are not yet defined. The retinoblastoma (pRb) family proteins (termed “pocket protein” family) act as “ultimate brakes” of the G₁- to S-phase transition (for review see [9]). E2F1–3 transcription factors induce the expression of genes involved in DNA synthesis, cell cycle progression, and apoptosis but are inactivated by association with unphosphorylated pRb (10–13). The role of E2F transcription factors and pocket proteins in β -cells is not entirely clear (14–16).

E2F1^{-/-} mice have a reduced overall pancreatic size due to diminished growth of several pancreatic cell types. These mice are glucose intolerant because β -cell proliferation and function are impaired postnatally (14). E2F1^{-/-} E2F2^{-/-} mice show that individual E2F transcription factors have important nonoverlapping roles in regulating both β -cell proliferation and apoptosis (17,18), although it remains unclear whether the function of E2F1/2 is β -cell autonomous (17). Conflicting signals of E2F1 and cell cycle inhibitors such as p53 result in apoptosis (19,20). Accordingly, adenoviral delivery of E2F1 in primary cardiomyocytes increases apoptosis rather than proliferation. However, IGF-I efficiently rescues the cells and allows for E2F1-driven proliferation (21,22). In β -cells, environmental growth signals (insulin, IGF-1, epidermal growth factor) often inhibit apoptosis and downregulate cell cycle inhibitors by signaling through protein kinase B/Akt (for review see [23]).

In the current study, we overexpressed E2F1 specifically in β -cells to study its effect on β -cell replication and function in vitro as well as in vivo.

RESEARCH DESIGN AND METHODS

Transgenic strains. Rat insulin promoter (RIP)CreERT mice were provided by Yuval Dor (Hebrew University, Jerusalem, Israel) (8). R26E2F1 mice harboring the *Rosa26-loxP-LacZ-loxP-E2F1* conditional expression cassette (24) were from Ulrike Ziebold (Max Delbrück Center for Molecular Medicine, Berlin, Germany). Hemizygous RIPCreERT and R26E2F1 mice were crossed to double transgenic RIPCreERT × R26E2F1. Single transgenic R26E2F1 control mice and double transgenic experimental mice were littermates. Eight-week-old male double transgenic and single transgenic mice received subcutaneous tamoxifen injections (four injections of 4 mg, every other day). All procedures were performed in accordance with the Free University of Brussels Animal Studies Committee.

Cell isolation and culture. Rat pancreatic β -cells were isolated from 6-week-old male Wistar rats and cultured as described (25,26). Purity was always more than 90%. Mouse islets were isolated from 8-week-old BALB/c mice or transgenic mice as described before (27). BALB/c islets produced more than 75% β -cells after partial dissociation. See supplementary Methods (available in an online appendix at <http://diabetes.diabetesjournals.org/content/early/2010/03/25/db09-1295/suppl/DC1>) for culture. Viral transduction was as in (28). RNA and protein were extracted from freshly isolated transgenic islets.

Adenoviral transduction. AdE2F1, expressing human E2F1 under control of a cytomegalovirus promoter (21), and AdAkt, expressing myristoylated hemagglutinin-tagged constitutively active Akt1 (29), were gifts of, respectively,

From the ¹Diabetes Research Center, Vrije Universiteit Brussel, Brussels, Belgium; and the ²Max-Delbrück-Center for Molecular Medicine, Berlin, Germany.

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G. G. and Y.C. contributed equally to this study. I.H. is currently affiliated with the Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Mellebeke, Belgium.

All authors are members of the Juvenile Diabetes Research Foundation Center for Beta Cell Therapy in Diabetes, Brussels, Belgium.

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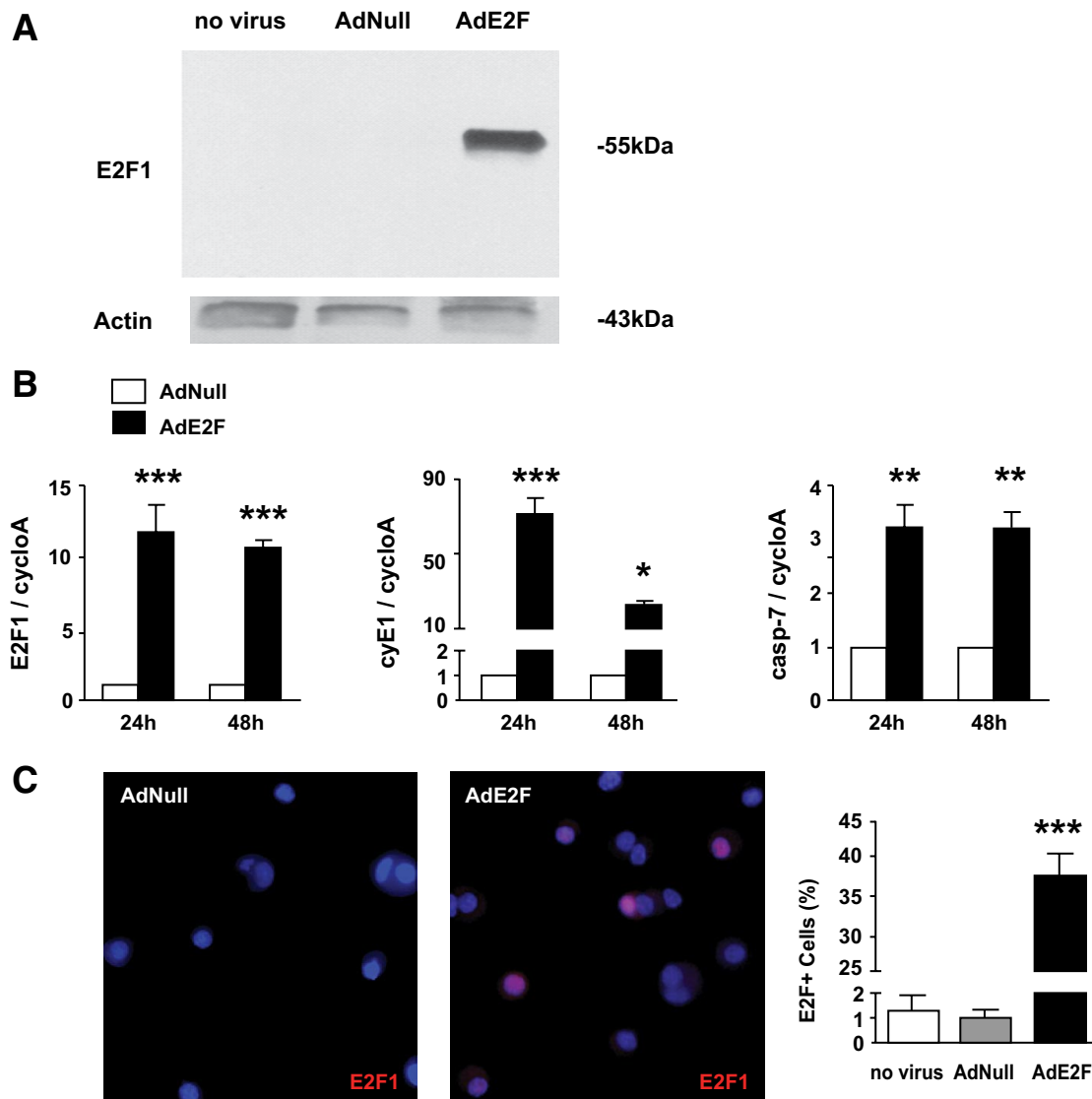


FIG. 1. Ectopic expression of active E2F1 in murine β -cells. Dispersed mouse islet cells were cultured in suspension and infected (MOI 20) with adenovirus AdE2F expressing human E2F1 or with AdNull without transgene. **A:** Immunoblot detection of E2F1 protein (± 55 kDa) 24 h after viral transduction. Actin signals indicate gel-loading efficiency. **B:** mRNA expression of endogenous E2F1 target genes E2F1, cyclin E1 (*cycE1*), and caspase-7 (*casp-7*) was assessed by quantitative RT-PCR, normalized to housekeeping gene cyclophilin A (*cycloA*) as described in the "Research Design and Methods" section, and expressed relative to the data obtained with AdNull. **C:** Immunocytochemical detection of E2F1 protein in rat β -cells (>90% purity) 48 h after viral infection. The percentage of E2F1⁺ cells in uninfected cultures, and in cultures infected with AdNull or AdE2F, was quantified. Data are presented as means \pm SEM ($n = 3-4$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. AdNull). (A high-quality digital color representation of this figure is available in the online issue.)

Rudiger von Harsdorf (University of Toronto, Toronto, Canada) and Mario Pende (Inserm, Université Paris 5, Paris, France). The control virus AdNull contained no transgene. All viruses were propagated and purified as described (30). Infection of β - or islet cells was as in (28).

Western blotting. Mouse endocrine cells or transgenic islets were used. Immunoblotting was performed as previously described (31). Antibodies were anti-E2F1 (rabbit; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-actin (goat; Santa Cruz Biotechnology Inc.), and anti-hemagglutinin (rabbit; Clontech, Mountain View, CA).

Real-Time PCR analysis. Real-Time PCR analysis was performed using predeveloped TaqMan assay reagents (Applied Biosystems) for mouse *E2f1*, *Ccne1* (cyclin E1), and *Casp7* (caspase 7), and for human *E2f1*. Expression levels were normalized to the expression of the housekeeping genes *Ppia* (peptidylprolyl isomerase A = cyclophilin A), *Gapdh*, and *Actb* using an adaptation of the $\Delta\Delta Ct$ method (32). Real-time PCR was further performed as described (33).

Immunohistochemistry and cytochemistry. For detection of bromodeoxyuridine (BrdU) incorporation in cells, see supplementary Methods. For immunocytochemistry, primary antibodies anti-Ki67 (rabbit; Novocastra Laboratories Ltd., Newcastle Upon Tyne, U.K.), anti-E2F1 (rabbit; Santa Cruz Biotechnology Inc.), anti-hemagglutinin for detection of recombinant Akt

(mouse; Cell Signaling Technology, Beverly, MA), anti-phospho-histone H3 (rabbit; Upstate Biotechnology, Waltham, MA), and anti-insulin (guinea pig; gift of Chris Van Schravendijk, Diabetes Research Center, Vrije Universiteit Brussel) underwent incubation either for 1 h at room temperature or overnight at 4°C.

Pancreatic tissue was fixed overnight in 4% formalin solution and embedded in paraffin using standard techniques. Staining for insulin, BrdU (mouse; Cappel, Cochranville, PA), Ki67, and activated caspase-3 (rabbit; Cell Signaling Technology) was performed on 5- μ m sections. Proliferation was assessed in cells costaining for insulin and BrdU or Ki67 on sections from transgenic mice intraperitoneally injected with BrdU (50 mg/kg) 16 h before killing. At least 3,000 insulin-positive cells were analyzed for each animal. Visualization and imaging are described in supplementary Methods.

Assaying β -cell number, death, and cycle. Total β -cell numbers in 96-well plates were determined using the CyQuant NF Cell Proliferation Assay Kit (Invitrogen), based on measurement of cellular DNA content via fluorescent dye binding, according to the manufacturer's instructions.

To assay cell death, Hoechst 33342 (10 μ g/ml) and propidium iodide (10 μ g/ml) were added to the cultures. Dead, apoptotic, or living cells were identified as described (34). Three separate wells were examined (at least 400

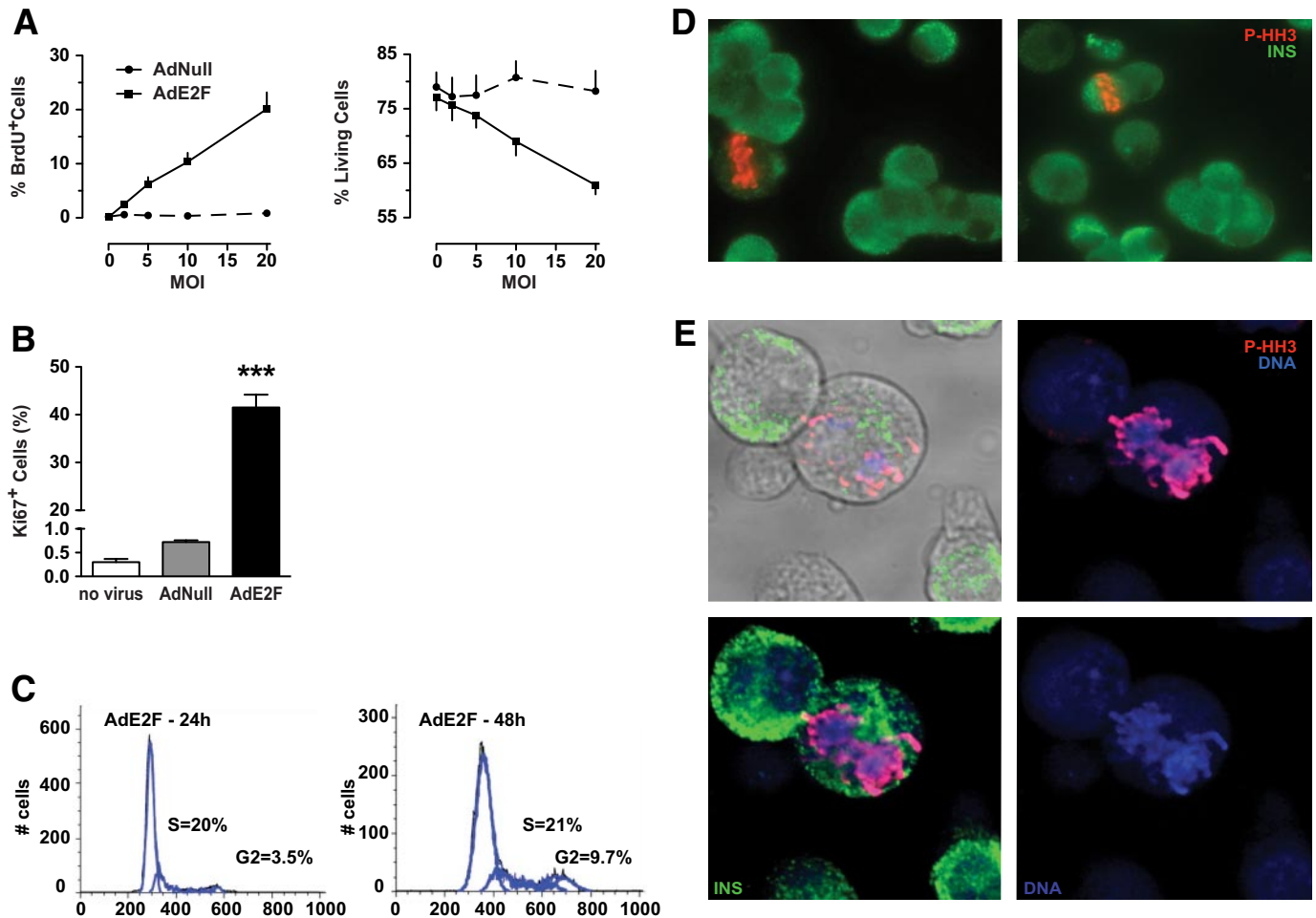


FIG. 2. E2F1 expression induces β -cell proliferation and death. Proliferation and survival were assessed in cultures of single rat β -cells (>90% purity) with overexpression of E2F1. β -cells were infected with AdE2F or AdNull and studied 48 h later by (immuno)fluorescent staining and microscopy. Data are presented as means \pm SEM. **A:** BrdU incorporation (16 h labeling, 100 μ mol/l) at the indicated viral MOI ($n = 3$) and percentage of cell survival assessed by exclusion of propidium iodide ($n = 4$). **B:** Ki67 expression induced by adenoviruses at MOI 20 ($n = 4$, *** $P < 0.001$ vs. AdNull). **C:** FACS cell cycle analysis on fixed and propidium iodide-stained cells 24 or 48 h after infection with AdE2F (MOI 20). **D:** Detection of the mitotic marker P-HH3 in INS-expressing β -cells. **E:** Confocal microscopy showing colocalization of P-HH3 and condensed DNA in INS⁺ β -cells in anaphase. (A high-quality digital color representation of this figure is available in the online issue.)

cells per well) for each experimental condition, and the percentage of dead and living β -cells was calculated.

For cell cycle analysis and sorting of live G_0/G_1 - and $S/G_2/M$ -phase β -cells, see supplementary Methods.

Insulin content measurement. Insulin content of β -cells cultured in 96-well plates, fluorescence-activated cell sorter (FACS)-sorted β -cells, or isolated pancreas of transgenic mice was determined by radioimmunoassay (33).

β -cell mass analysis. β -cell mass was determined on the basis of relative insulin-positive area, as measured in sections, and by pancreas weight (33). $4.7 \pm 0.1\%$ of the total pancreas volume was analyzed for each mouse.

Insulin release measurement. Insulin release by AdE2F1- and AdNull-transduced rat β -cells or by islets from transgenic mice (20 freshly isolated islets per condition) was measured in static incubations. Insulin biosynthesis was measured in adenovirus-transduced rat β -cells cultured in Ham F10 medium containing 10 mmol/l glucose. For more detail, see supplementary Methods.

Statistics. All data were expressed as means \pm SEM. Student *t* test, ANOVA (Bonferroni correction), or rank test (survival analysis) was used for comparison of data. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Adenovirus-mediated expression of human E2F1 in murine β -cells. Islets from mouse pancreas containing $\geq 70\%$ β -cells were dissociated, reaggregated to small cell clusters, and cultured for 24 or 48 h after infection with

adenovirus AdE2F1 (multiplicity of infection [MOI] 20) expressing human E2F1. E2F1 transcript was undetectable by quantitative RT-PCR (cycle threshold [Ct] > 40, $n = 3$) in AdNull-infected islet cells but clearly expressed 24 h after AdE2F1 infection (Ct 21.1 ± 0.4 , $n = 3$). E2F1 protein could be detected among immunoblotted proteins extracted from AdE2F1-infected islet cells after 24 h of culture (Fig. 1A). When cultured for 24 and 48 h, E2F1-transduced islet cells showed a significant increase in the abundance of endogenous transcripts encoding E2F1, cyclin E1, and caspase-7, all known E2F1 targets (Fig. 1B).

When single rat β -cells (purity $\geq 90\%$) were cultured in poly-D-lysine-coated wells and infected with AdE2F1 (MOI 20), $38 \pm 3\%$ of cells immunostained positive for E2F1 protein compared with $1.3 \pm 0.5\%$ in uninfected cultures (Fig. 1C). These results showed that infection of murine β -cells with adenovirus AdE2F1 increased the expression of active E2F1 transcription factor.

Adenovirus-mediated expression of human E2F1 induces proliferation and death of isolated rat β -cells. The effects of active E2F1 on β -cell proliferation were studied on isolated, single rat β -cells cultured in poly-D-lysine-coated wells. AdE2F1 (MOI 0–20) dose-dependently

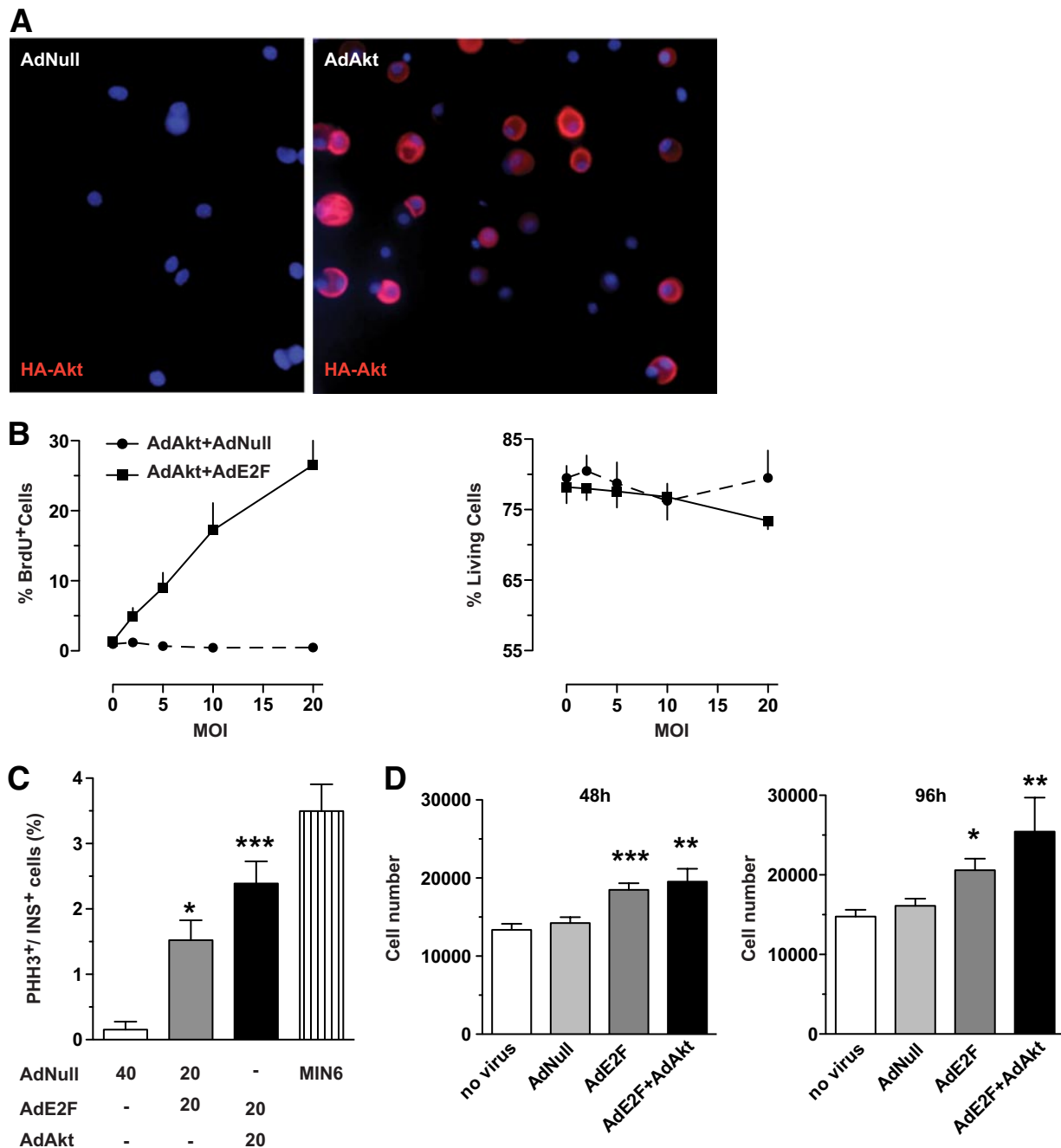


FIG. 3. Effect of Akt expression on cell death and proliferation in isolated β -cells overexpressing E2F1. Data are presented as means \pm SEM. **A:** Immunocytochemical detection of constitutively active (hemagglutinin [HA] tagged) Akt using a HA-specific antibody. Akt was localized at the cell membrane of rat β -cells after their infection with adenovirus AdAkt, suggesting myristoylation of Akt. **B:** BrdU incorporation ($n = 3$) and cell survival ($n = 4$) after co infection of β -cell cultures with both AdAkt and AdE2F1 at the indicated MOI, and as studied in Fig. 2. **C:** Quantitation of P-HH3⁺ cells in β -cell cultures infected with AdAkt and/or AdE2F as indicated ($n = 4$, * $P < 0.05$, *** $P < 0.001$ vs. AdNull). Results for MIN6 cell cultures are shown for reference. **D:** Total β -cell numbers determined by CyQuant assay at 48 and 96 h after viral infection with AdNull or AdE2F (MOI 20) or with AdE2F+AdAkt (both MOI 10). Assays were performed with $\sim 13,000$ cells as starting β -cell number ($n = 6$, ** $P < 0.01$, *** $P < 0.001$ vs. no virus). (A high-quality digital color representation of this figure is available in the online issue.)

dently increased the number of BrdU-incorporating cells at 48 h after infection (Fig. 2A). Upon infection with AdE2F1 (MOI 20), $20 \pm 2\%$ of cells were BrdU⁺ and $42 \pm 3\%$ expressed the proliferation marker Ki67 (Fig. 2B). E2F1 was detected in $79 \pm 3\%$ (mean \pm SEM, $n = 9$) of BrdU⁺ cells and $78 \pm 6\%$ ($n = 3$) of the Ki67⁺ cells (not shown), indicating that increased E2F1 expression induced proliferation of primary β -cells. Staining with propidium iodide (labels nuclei of dead cells) revealed that E2F1 expression also caused cell death (Fig. 2A) through induction of

apoptosis (not shown). Cell cycle analysis by FACS using propidium iodide on cells that were fixed to label all nuclei showed that infection with AdE2F1 (MOI 20) induced S-phase in $20 \pm 4\%$ ($n = 3$) and G₂/M-phase in $3.5 \pm 0.9\%$ of the cells at 24 h after infection (Fig. 2C). At 48 h after infection, S-phase cells still represented $20 \pm 3\%$ ($n = 3$) of all β -cells, and the fraction in G₂/M-phase had increased to $9.5 \pm 1.5\%$ (Fig. 2C). At this time point, phospho-histone H3 (P-HH3)-positive mitotic β -cells were present (Fig. 2D). P-HH3 labeling colocalized with condensed chroma-

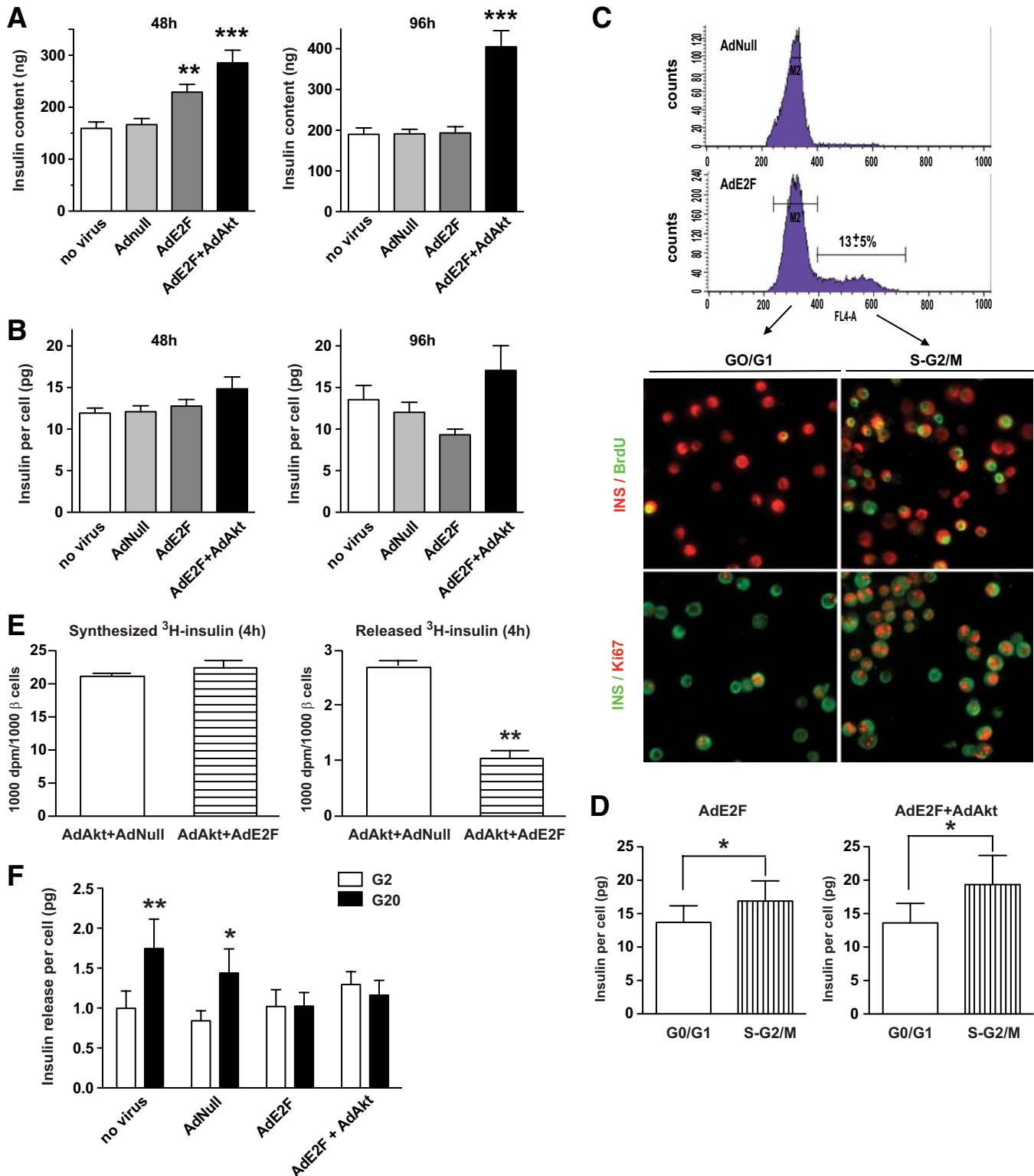


FIG. 4. Insulin content increases and insulin release decreases in proliferating β -cells. Data are presented as means \pm SEM. **A:** Total insulin (ng) and **(B)** insulin content per cell (pg) were determined in 48- or 96-h cultures of β -cells infected with AdNull or AdE2F (MOI 20) or with AdE2F+AdAkt (both MOI 10) ($n = 6$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. no virus). **C:** β -cells (150,000) were infected with AdE2F (or AdE2F+AdAkt); 24 h later, they were pulse-labeled with BrdU (1 h, 100 μ M), stained with Hoechst 33342, and then sorted into G₀/G₁ and S/G₂/M subpopulations using FACS on basis of Hoechst fluorescence, as indicated by the gating. Upon E2F transduction, an average of 13% of all cells were in the S/G₂/M-phase ($n = 6$). The G₀/G₁ and S/G₂/M subpopulations were collected on coverslips and stained for insulin and BrdU or Ki67. **D:** Measurement of the insulin content per cell for G₀/G₁ and S/G₂/M cells ($n = 6$, $^{*}P < 0.05$). In rat β -cell cultures infected with either AdE2F or AdE2F+AdAkt, S/G₂/M-phase cells contained more insulin than G₀/G₁ cells ($n = 6$, $^{*}P < 0.05$). **E:** Rat β -cells (25×10^3) were infected with AdAkt+AdNull or AdAkt+AdE2F (MOI 10 each). In the culture period between 20 and 24 h after infection, tritium-labeled tyrosine was present. At 24 h, both groups of cells were assayed for the total and the released tritium-labeled insulin ($n = 3$, $^{***}P < 0.01$). **F:** Cell cultures were infected as in **(A)** and **(B)** and cultured for 48 h. Insulin released into the medium during a 2-h incubation in 20 mM glucose (G₂₀) or in 2 mM glucose (G₂) was then measured ($n = 7$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. G₂). (A high-quality digital color representation of this figure is available in the online issue.)

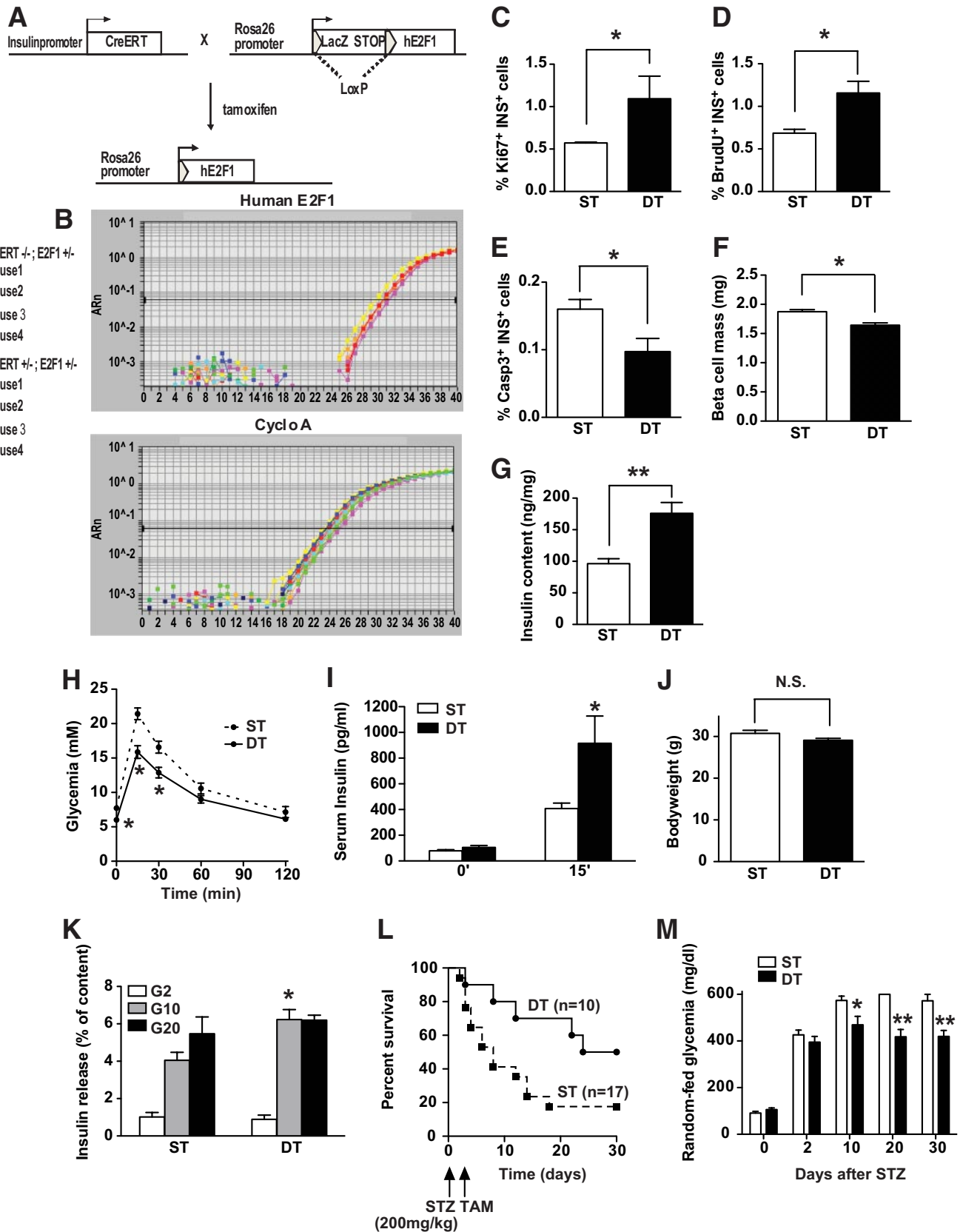


FIG. 5. Conditional expression of E2F1 in mice stimulates β -cell proliferation and function. **A:** Transgenic mice. Tamoxifen injection of bigenic RIPCreERT \times E2F1 mice results in nuclear translocation of CreER leading to excision of a loxP-flanked lacZ (serving as a stop sequence) and expression of the human E2F1 gene specifically in β -cells. **B:** Bigenic RIPCreERT \times E2F1 mice (double transgenic [DT]) and monogenic littermates (single transgenic [ST]) received 4×4 -mg injections (for 7 days). Two weeks later, the expression of human E2F1 and the housekeeping gene cyclophilin A (cycloA) was assessed by quantitative (within 7 days) RT-PCR on RNA isolated from their pancreatic islets. Only bigenic TAM-treated mice expressed the human E2F1 gene. **C:** β -cell proliferation assessed by Ki67 and insulin staining in the bigenic (DT) and monogenic (ST) mice ($n = 3$). **D:** β -cell proliferation assessed by BrdU (intraperitoneal injection 16 and 2 h before killing) and insulin staining

tin in β -cells, some of which were in anaphase (Fig. 2E), indicating that the E2F-activated β -cells were cycling.

Expression of active Akt inhibits E2F1-induced β -cell death and further increases proliferation. Purified rat β -cells were transduced with AdAkt to express constitutively active Akt/protein kinase B (Fig. 3A). In coinfection experiments with AdE2F1 and AdAkt, the E2F1-induced β -cell death at 48 h was nearly completely prevented (Fig. 3B). Infection with AdAkt alone did not increase β -cell proliferation (Fig. 3B), but its coinfection with AdE2F1 increased the E2F1-induced BrdU incorporation (Figs. 2A and 3B). The fraction of P-HH3⁺ β -cells also enlarged upon combined expression of E2F1 and Akt, compared with E2F1 alone (Fig. 3C). Expression of active E2F1 and Akt resulted in a net increase of β -cell numbers in vitro. The total cell number increased by 38 and 46% at 48 h, and by 39 and 72% at 96 h after infection with AdE2F1 and AdE2F1+AdAkt, respectively (Fig. 3D). Thus, in contrast to expression of E2F1 alone, combined expression of E2F1 and Akt continued to increase the absolute number of β -cells in vitro for more than 48 h.

β -cell proliferation increases insulin content but is incompatible with glucose-induced insulin release in vitro. The effect of proliferation on insulin stores was measured in isolated rat β -cells infected with AdE2F1 or AdE2F1+AdAkt. At 48 h after infection, total insulin content of E2F1- and E2F1+Akt-transduced cells was, respectively, 44 and 79% higher than untreated cells, whereas the insulin content per cell was similar in all conditions (Fig. 4A and B). At 96 h, the total insulin content of E2F1-transduced cells was equal to that of untreated cells, and insulin per cell was even 30% lower. In contrast, relative to untreated β -cells the total insulin content of E2F1+Akt-transduced cells increased by 113% and insulin content per cell increased by 26% (Fig. 4A and B). Thus, ectopic expression of active E2F1 and Akt increased β -cell numbers in vitro over extended periods of time, while preserving the cellular insulin stores.

To examine whether β -cells that are engaged in DNA synthesis also increase their insulin content, 150,000 cells/well were labeled with a short BrdU pulse (1 h) at 24 h after infection, detached, and incubated with Hoechst 33342 and propidium iodide. Live propidium iodide-negative cells were further sorted on the basis of Hoechst 33342 fluorescence reflecting DNA content (Fig. 4C) into G₀/G₁-phase and S/G₂/M-phase cell populations, the latter representing 13 \pm 5% of AdE2F1+AdAkt-transduced β -cells. BrdU was incorporated in the nuclei of 52 \pm 4% ($n = 4$) of the S/G₂/M INS⁺ sorted cells versus 12 \pm 2% ($n = 4$) in sorted G₀/G₁ INS⁺ cells (Fig. 4C). In addition, in the S/G₂/M population, 70 \pm 6% ($n = 3$) of INS⁺ cells stained positive for Ki67 (Fig. 4C). This indicated that proliferating and nonproliferating β -cells could be separated by FACS sorting.

At 24 h after transduction by E2F1 or E2F1+Akt, the insulin content of sorted S/G₂/M cells was increased by,

respectively, 23 and 44%, compared with sorted G₀/G₁ cells from the same cultures (Fig. 4D). Thus, in proliferating β -cells, the progression from G₀/G₁- to S/G₂-phase is associated with an increase of the cellular insulin content. During the culture period between 20 and 24 h after infection, E2F1+Akt-transduced and control cells synthesized equal amounts of insulin (Fig. 4E), but the release of newly formed insulin during this culture period was significantly lower in the E2F1+Akt-transduced cells (Fig. 4E). This suggests that in vitro proliferating cells in S-phase increased their insulin content by retaining newly formed insulin, rather than through increasing synthesis.

Next, glucose-regulated insulin secretion by in vitro proliferating β -cells was examined at 48 h after infection. Glucose-induced insulin release was blocked in cells transduced with either E2F1 or E2F1+Akt (Fig. 4F), indicating that E2F1-induced proliferation in vitro does not support regulated insulin release.

β -cell proliferation in mice with conditional expression of E2F1 is compatible with glucose-responsive insulin release. Given the capacity of E2F1 to induce β -cell proliferation in vitro, we investigated whether in vivo expression of E2F1 in mature β -cells was sufficient to induce their proliferation.

Rosa26-loxP-LacZ-loxP-E2F1 (R26E2F1) mice were crossed with *RIPCreERT* mice to conditionally express human *E2F1* from the *Rosa26* promoter upon excision of β -galactosidase encoding sequence (24), when given tamoxifen (TAM) (8). At 8 weeks of age, double transgenic *RIPCreERT*^{+/−} \times *R26E2F1*^{+/−} mice and control single transgenic *R26E2F1*^{+/−} littermates (Fig. 5A) were injected with TAM. This switched on human E2F1 in β -cells of double transgenic mice and not in β -cells of single transgenic mice (Fig. 5B). Two weeks later, the expression level of total (mouse + human) E2F1 in double transgenic islets was 2.4-fold that of E2F1 in single transgenic islets (supplementary Fig. 1); recombinant E2F1 protein was detected in double transgenic islets (supplementary Fig. 2). The percentage of β -cells labeled with BrdU or Ki67 was increased in double transgenic compared with single transgenic mice (Fig. 5C and D). β -cells of double transgenic mice showed a decreased level of caspase-3 activation (apoptosis) (Fig. 5E). The β -cell mass in double transgenic mice was slightly decreased (\pm 12%) compared with single transgenic mice (Fig. 5F), however, their pancreas insulin content was increased by \pm 70% (Fig. 5G) and their fasting blood glucose level was decreased (6 \pm 0.3 mmol/l vs. 7 \pm 0.4 mmol/l, $n = 8$, $P = 0.01$). In addition, double transgenic mice displayed an improved glucose tolerance in intraperitoneal glucose tolerance test (Fig. 5H) and secreted more insulin in response to glucose (Fig. 5D), although no significant difference in mean body weight was observed between double transgenic and single transgenic groups (Fig. 5J). Furthermore, islets isolated from double transgenic mice secreted more insulin than single transgenic islets when exposed to 10 mmol/l glucose,

in the same groups of mice ($n = 3$). E: β -cell apoptosis evaluated by staining for active caspase-3 (Casp-3) and insulin ($n = 3$). F: Measurement of total β -cell mass (mg) in pancreas of DT and ST mice ($n = 3$). G: Measurement of insulin content (nanogram of insulin per milligram of tissue) in pancreas of DT and ST mice ($n = 5$). H: Intraperitoneal glucose tolerance test performed in 16-h fasted DT and ST mice by blood glucose measurements at 0, 15, 30, 60, and 120 min after 2-mg/g glucose injection ($n = 8$). I: Insulin levels (pg/ml) measured by radioimmunoassay in serum obtained from 200 μ l tail vein blood just before (0') and 15 min after (15') intraperitoneal injection of glucose ($n = 8$). J: Body weight measurements for the DT and ST mice ($n = 8$). K: Glucose-induced insulin release (% of content) by freshly isolated mouse islets from DT and ST mice, during 2-h incubation at the indicated glucose concentrations ($n = 4$). In G10, insulin release from ST and DT islets differed significantly. L: Kaplan-Meier analysis of survival of DT ($n = 10$) and ST ($n = 17$) mice after one injection of STZ (200 mg/kg) at day 0, and administration of tamoxifen starting at day 2. The survival curves differ significantly (rank test, $P < 0.05$). M: Blood glucose concentration in random-fed STZ+TAM-treated mice ($n = 3$ –16) from (L). Data are presented as means \pm SEM. * $P < 0.05$, ** $P < 0.01$. NS, not significant.

whereas both groups secreted equal amounts of insulin at 2 or 20 mmol/l glucose (Fig. 5K). This suggests a leftward shift of the glucose response curve in E2F1-expressing islets. Together, these data show that β -cells from E2F1-expressing mice have a greater propensity to proliferate and to secrete insulin. This could yield a competitive advantage under conditions of hyperglycemia. To test this, double transgenic and single transgenic littermates were subjected to a single streptozocin (STZ) dose of 200 mg/kg to induce selective loss of β -cells, and 2 days later tamoxifen injections were given as before. Blood glucose of these mice was monitored for 1 month. Kaplan-Meier survival analysis showed that significantly more double transgenic mice survived the STZ-induced diabetes (Fig. 5L). From day 10 onward, the random-fed blood glucose levels in double transgenic mice remained significantly lower than in the single transgenic littermates (Fig. 5M).

DISCUSSION

Adult β -cells are growth arrested in $G_{0/1}$ and known to express high levels of cyclin-dependent kinase (CDK) inhibitors (p21, p27, and p57) as well as several members of the pocket protein family including pRb, the principal brakes of the G_1 -to S-phase transition (15,16). Manipulations that activate (or feed into) the pRb pathway, such as overexpression of large T antigen or CDK4/6 and/or cyclin D1 and D2, and downregulation of CDK inhibitors have been shown to stimulate β -cell proliferation (for review see [15]). In agreement with these findings, our study shows that ectopic expression of transcription factor E2F1, a direct effector of the pRb pathway, increased β -cell proliferation in vitro and in vivo. However, this finding is not trivial. First, E2F1 function provokes negative feedback loops involving ARF/p53 and repressor E2Fs (10), whereas increased E2F1/pRb dimer formation can lead to active repression of E2F target genes (35), all of which can contribute to G_1 arrest. Our findings that several transcriptional targets (36–39) of E2F1, such as E2F1, cyclin E, and caspase-7, were activated in β -cells and that transduced β -cells were labeled with BrdU and Ki67 suggest that overexpression of E2F1 resulted in increased levels of transcriptionally active E2F1 and activation of S-phase (35). Second, E2F1 overexpression in primary fibroblasts does not lead to S-phase entry but instead promotes senescence and apoptosis (40,41), a general observation in nonimmortal cells (10). Overexpression of E2F1 cannot enforce S-phase entry in in vitro cultures of primary cardiomyocytes in the absence of serum. Concomitant with E2F activity, expression of an anti-apoptotic factor (e.g., Bcl-2), or stimulation by the growth factor IGF-1, is necessary to achieve DNA synthesis (21). In contrast, our results show that adenoviral delivery of E2F1 ectopically stimulated traversal of S-phase within 24 h in β -cells cultured in a minimal medium without growth factors or a feeder layer (26). Moreover, the E2F1-transduced β -cells progressively populated the G_2 /M-phase between 24 and 48 h after transduction of the cells, and a nonnegligible fraction of those cells underwent mitosis as shown by confocal microscopy of β -cells with phosphorylated histone H3, a marker for initial stages of chromatin condensation in late G_2 interphase until anaphase. This illustrates that overexpression of the cell cycle effector E2F1 can overcome inhibition of proliferation of primary β -cells. It is possible that autocrine stimulation by insulin

helps to render cultured β -cells permissive to proliferation by ectopic E2F1 (42).

Our in vitro data suggest that the majority of β -cells that ectopically express E2F1 undergo proliferation and ultimately may undergo cell death. In view of the increased transcription of the effector caspase-7 gene in the cultured cells, part of the β -cell death may occur through p53-independent apoptosis (10). Growth factor signaling via Akt/protein kinase B has potent antiapoptotic effects in β -cells (for review see [23,43]). Furthermore, E2F1-mediated proapoptotic functions can be suppressed specifically by the PI3K/Akt pathway (44). Indeed, adenoviral coexpression of constitutively active (myristoylated) Akt reduced the E2F1-induced β -cell death to near control levels, and in addition increased E2F1-induced β -cell proliferation. Under these conditions, the β -cell number and insulin content were clearly increased compared with untreated cultures. The increased insulin stores in proliferating cells in S-phase appeared to result from reduced insulin release rather than increased synthesis; the insulin release from proliferating β -cells was found to be unresponsive to glucose.

The phenotypes of E2F1^{-/-} mice, and of transgenic mice overexpressing E2F1 either in the testes or in whole body, suggested that in vivo functions of E2F1 result in suppressing proliferation and/or promoting apoptosis (24,45,46). c-Myc, like E2F1, is a potent inducer of apoptosis in vitro (47). Transgenic mice activating c-Myc in mature β -cells exhibit increased β -cell proliferation accompanied by overwhelming β -cell apoptosis, which rapidly leads to diabetes (48,49). These observations do not apply to a β -cell-specific conditional expression of E2F1, which increased their proliferation activity twofold while decreasing rather than increasing their death by apoptosis. Induced double transgenic mice did not develop hyperglycemia during a period of 3 months (results not shown), also suggesting absence of long-term β -cell destruction or dysfunction. The effects on the percentage of BrdU-positive and active caspase-positive cells were however not associated with an increased β -cell mass; on the other hand, the double transgenic mice exhibited a 70% higher insulin content, which appeared responsible for a significantly higher insulin release after an intraperitoneal glucose bolus. This in vivo responsiveness is compatible with the leftward shift of the glucose dose-response curve in isolated transgenic islets. Recent findings in E2F1 knockout mice have also suggested that E2F1 is crucial for normal glucose tolerance and β -cell insulin secretion (50). Because the E2F1-induced increase in pancreatic insulin reserve was not associated with an increased number of β -cells, it can be attributed to an increased hormone content per cell. Direct proof for the latter requires direct measurements at the cellular level. Our data nevertheless strongly suggest that E2F1 can increase the insulin content in normal β -cells, and, more importantly, that this results in a more potent glucose-induced insulin release in vivo with a more rapid normalization of hyperglycemia.

Our in vivo data suggest that E2F1-driven cell cycle activity per se is not disruptive for insulin secretion. Whereas the mRNA expression level of recombinant human E2F1 was similar to that of endogenous E2F1 in the islets of transgenic mice used in this study (supplementary Fig. 1), the expression level of recombinant human E2F1 in AdE2F1-infected cells was >1,000-fold the endogenous murine E2F1 expression of uninfected cells ($-\Delta\Delta Ct = 11.9 \pm 1.1$, $n = 3$, normalized to cyclophilin A). The in vitro

death and impaired insulin release seen with AdE2F1 infection may be caused by nonphysiological E2F1 expression levels highly inducing genes that interfere with β -cell survival (*casp-7*, this study) and function (*Kir6.2* [50]), and are not necessarily caused by the high rate of β -cell proliferation. The importance of E2F1 for β -cell function as opposed to being secondary to nonphysiological overexpression is hard to determine. On the other hand, our data, both the in vitro and in vivo, support the notion that E2F1 increases cell cycle activity of β -cells.

In summary, previous loss-of-function studies in *E2F^{-/-}* mice (14,18,50) pointed out an important role for E2F1 in controlling postnatal β -cell proliferation and function. We now report a gain-of-function study and demonstrate that ectopic expression of E2F1 in adult β -cells can increase their proliferation activity in vitro as well as in vivo. Moreover, in vivo E2F1 expression was found to significantly increase the insulin content of adult β -cells that was associated with a more potent glucose-induced insulin release and subsequent correction of hyperglycemia. We propose E2F1 as a novel therapeutic target of β -cell function as it is capable of increasing physiologically regulated insulin release through elevating the hormone content of β -cells. Obviously, targeting this cell-cycle factor to increase β -cell growth/function would need to be very much controlled, to prevent oncogenesis.

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