Ectopic expression of E2F1 stimulates beta cell proliferation and function.

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Objective — Generating functional beta cells by inducing their proliferation might open 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 beta cell growth and function. However, the consequences of overexpression of E2F1 in beta cells are unknown.

Research design and methods — The effects of E2F1 overexpression on beta cell proliferation and function were analyzed in isolated rat beta cells and in transgenic mice.

Results — Adenovirus AdE2F1-mediated overexpression of E2F1 increased the proliferation of isolated primary rat beta cells 20-fold but also enhanced beta cell death. Co-infection with adenovirus AdAkt expressing a constitutively active form of Akt (protein kinase B) suppressed beta cell death to control levels. At 48h post-infection, the total beta cell number and insulin content were respectively 46% and 79% higher in AdE2F1+AdAkt-infected cultures as compared to untreated. Conditional overexpression of E2F1 in mice resulted in a 2-fold increase of beta cell proliferation and 70% increase of pancreatic insulin content, but did not increase beta 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 beta cell proliferation activity. In vivo E2F1 expression significantly increases the insulin content and function of adult beta cells, making it a strategic target for therapeutic manipulation of beta cell function.
The majority of adult beta cells is arrested in G0/1 cell cycle phases (1-5) and rarely replicate more than once even when stimulated to proliferate (6). Nevertheless, beta cell replication appears a major mechanism for postnatal formation of rodent beta cells (7; 8). Increasing the number of beta cells that enter replication may open 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 beta 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 beta cell proliferation and function are impaired postnatally (14). E2F1/-/- E2F2/-/- mice show that individual E2F transcription factors have important non-overlapping roles in regulating both beta cell proliferation and apoptosis (17; 18) although it remains unclear whether the function of E2F1/2 is beta cell autonomous (17). Conflicting signals of E2F1 and cell cycle-inhibitors like p53 result in apoptosis (19; 20). Accordingly, adenoviral delivery of E2F1 in primary cardiomyocytes increases apoptosis rather than proliferation. However, insulin-like growth factor I efficiently rescues the cells and allows for E2F1-driven proliferation (21; 22). In beta cells, environmental growth signals (insulin, IGF-1, EGF) often inhibit apoptosis and downregulate cell cycle-inhibitors by signaling through PKB/Akt (for review see (23)).

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

MATERIALS 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 Delbrueck Center for Molecular Medicine, Berlin, Germany). Hemizygous RIPCreERT and R26E2F1 mice were crossed to double transgenic (DT) RIPCreERT x R26E2F1. Single transgenic (ST) R26E2F1 control mice and DT experimental mice were littermates. 8 week-old male DT and ST mice received subcutaneous tamoxifen injections (4 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 beta cells were isolated from 6 weeks old male Wistar rats and cultured as described (25; 26). Purity was always above 90%. Mouse islets were isolated from 8 week old Balb/c mice or transgenic mice as described before (27). Balb/c islets gave more than 75% beta cells following partial dissociation. See Supplementary Methods 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 CMV promoter (21), and AdAkt, expressing myristoylated HA-tagged constitutively active Akt1 (29), were gifts of, respectively, 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 beta 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., CA), anti-actin (goat, Santa Cruz), and anti-HA (rabbit, Clontech, Mountain View, CA).

**Real-Time PCR analysis.** Real-Time PCR analysis was performed using pre-developed 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 ΔΔCT method (32). Real time PCR was further performed as described (33).

**Immunohistochemistry and cytochemistry.** For detection of BrdU incorporation in cells: see Supplementary Methods. For immunocytochemistry, primary antibodies were anti-Ki67 (rabbit, Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK), anti-E2F1 (rabbit, Santa Cruz), anti-HA 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-VUB Brussels), 1 hour incubation 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-µm sections. Proliferation was assessed in cells co-staining for insulin and BrdU or Ki67 on sections from transgenic mice intraperitoneally injected with BrdU (50mg/kg) 16h before sacrifice. At least 3,000 insulin-positive cells were analyzed for each animal. Visualization and imaging is described in Supplementary Methods which is available in the online appendix at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org).

**Assaying beta cell number, -death and -cycle.** Total beta 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 3342 (HO, 10 µg/ml) and propidium iodide (PI, 10 µg/ml) were added to the cultures. Dead, apoptotic, or living cells, were identified as described (34). Three separate wells were examined (at least 400 cells per well) for each experimental condition, and the percentage of dead and living beta cells was calculated.

For cell cycle analysis and sorting of live G0/G1 and S/G2/M phase beta cells: see Supplementary Methods.

**Insulin content measurement.** Insulin content of beta cells cultured in 96-well plates, FACS sorted beta cells or isolated
pancreas of transgenic mice was determined by radioimmunoassay (33).

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

**Insulin release measurement.** Insulin release by AdE2F1- and AdNull-transduced rat beta 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 beta cells cultured in Ham's F10 medium containing 10 mM glucose. For more detail, see Supplementary Methods.

**Statistics.** All data were expressed as means ± SEM. Student’s t-test, ANOVA (Bonferroni correction), or rank-test (survival analysis), was used for comparison of data. Differences were considered statistically significant when P values were < 0.05.

**RESULTS**

1. **Adenovirus-mediated expression of human E2F1 in murine beta cells -** Islets from mouse pancreas containing ≥70% beta cells were dissociated, reaggregated to small cell clusters and cultured for 24 or 48h following infection with adenovirus AdE2F1 (MOI20) expressing human E2F1. E2F1 transcript was undetectable by RT-qPCR (Ct>40, n=3) in AdNull-infected islet cells but clearly expressed 24h following AdE2F1 infection (Ct 21.1 ± 0.4, n=3). E2F1 protein could be detected among immunoblotted proteins extracted from AdE2F1-infected islet cells after 24h of culture (Fig. 1A). When cultured for 24h and 48h, 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 beta cells (purity ≥90%) were cultured in poly-D-Lysine-coated wells and infected with AdE2F1 (MOI20), 38 ± 3% of cells immunostained positive for E2F1 protein compared to 1.3 ± 0.5% in uninfected cultures (Fig. 1C). These results showed that infection of murine beta cells with adenovirus AdE2F1 increased the expression of active E2F1 transcription factor.

2. **Adenovirus-mediated expression of human E2F1 induces proliferation and death of isolated rat beta cells -** The effects of active E2F1 on beta cell proliferation were studied on isolated, single rat beta cells cultured in poly-D-Lysine-coated wells. AdE2F1 (MOI 0-20) dose-dependently increased the number of BrdU-incorporating cells at 48h post-infection (Fig. 2A). Upon infection with AdE2F1 (MOI20), 20 ± 2% of cells were BrdU+ and 42 ± 3% expressed the proliferation marker Ki67 (Fig. 2B). E2F1 was detected in 79 ± 3% (mean ± SEM, n=9) of BrdU+ cells and 78 ± 6% (n=3) of the Ki67+ cells (not shown), indicating that increased E2F1-expression induced proliferation of primary beta cells. Staining with propidium iodide (PI, 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 PI on cells that were fixed to label all nuclei, showed that infection with AdE2F1 (MOI20) induced S-phase in 20 ± 4% (n=3) and G2/M-phase in 3.5 ± 0.9% of the cells at 24h post-infection (Fig. 2C). At 48h following infection, S-phase cells still represented 20 ± 3% (n=3) of all beta cells and the fraction in G2/M-phase had increased to 9.5 ± 1.5% (Fig. 2C). At this time-point, phospho-histone H3 (P-HH3)-positive mitotic beta
cells were present (Fig. 2D). P-HH3-labeling co-localized with condensed chromatin in beta cells, some of which were in anaphase (Fig. 2E) indicating that the E2F-activated beta cells were cycling.

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

4. Beta 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 beta cells infected with AdE2F1 or AdE2F1+AdAkt. At 48 h post-infection, total insulin content of E2F1- and E2F1+Akt-transduced cells was respectively 44% and 79% higher than untreated cells, while the insulin content per cell was similar in all conditions (Fig. 4A,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 beta cells the total insulin content of E2F1+Akt-transduced cells increased by 113% and insulin content per cell increased by 26% (Fig. 4A, B). Thus, ectopic expression of active E2F1 and Akt increased beta cell numbers in vitro over extended periods of time, while preserving the cellular insulin stores.

To examine whether beta cells that are engaged in DNA synthesis also increase their insulin content, 150K cells/well were labelled with a short BrdU pulse (1h) at 24h post-infection, detached, and incubated with Hoechst (HO33342) and propidium iodide (PI). Live PI' cells were further sorted on the basis of HO-fluorescence reflecting DNA-content (Fig. 4C) into G0/G1-phase and S-G2/M-phase cell populations, the latter representing 13 ± 5% of AdE2F+AdAkt-transduced beta cells. BrdU was incorporated in the nuclei of 52 ± 4% (n=4) of the S-G2/M INS+ sorted cells versus 12 ± 2% (n=4) in sorted G0/G1 INS+ cells (Fig. 4C). In addition, in the S-G2/M population, 36 ± 6% (n=3) of INS+ cells stained positive for Ki67 (Fig. 4C). This indicated that proliferating and non-proliferating beta 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%, as compared to sorted G0/G1 cells from the same cultures (Fig. 4D). Thus, in proliferating beta 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 20h and 24h post-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 beta cells was examined at 48h following 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.

5. Beta cell proliferation in mice with conditional expression of E2F1 is compatible with glucose-responsive insulin release - Given the capacity of E2F1 to induce beta cell proliferation in vitro, we investigated whether in vivo expression of E2F1 in mature beta 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 beta-Gal-encoding sequence (24), when given tamoxifen (TAM) (8). At 8 weeks of age, double transgenic (DT) RIPCreERT+/- x R26E2F1+/- mice and control single transgenic (ST) R26E2F1+/- littermates (Fig. 5A) were injected with TAM. This switched on human E2F1 in beta cells of DT mice and not in beta cells of ST mice (Fig. 5B). Two weeks later, the expression level of total (mouse + human) E2F1 in DT islets was 2.4-fold that of E2F1 in ST islets (supplementary Fig. 1); recombinant E2F1 protein was detected in DT islets (supplementary Fig. 2). The percentage of beta cells labeled with BrdU or Ki67 was increased in DT compared to ST mice (Fig. 5C,D). Beta cells of DT mice showed a decreased level of caspase-3 activation (apoptosis) (Fig. 5E). The beta cell mass in DT mice was slightly decreased (±12%) as compared to ST mice (Fig. 5F), however, their pancreas insulin content was increased by ±70% (Fig. 5G), and their fasting blood glucose level was decreased (6±0.3 mM vs. 7±0.4 mM, n=8, p=0.01). In addition, DT mice displayed an improved glucose tolerance in IPGTT (Fig. 5H), and secreted more insulin in response to glucose (Fig. 5I), while no significant difference in mean bodyweight was observed between DT and ST groups (Fig. 5J). Further, islets isolated from DT mice secreted more insulin than ST islets when exposed to 10 mM glucose, while both groups secreted equal amounts of insulin at 2 or 20 mM glucose (Fig. 5K). This suggests a leftward shift of the glucose response curve in E2F1-expressing islets. Together, these data show that beta 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, DT and ST littermates were subject to a single STZ dose of 200 mg/kg to induce selective loss of beta 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 DT mice survived the STZ-induced diabetes (Fig. 5L). From day 10 onward the random-fed blood glucose levels in DT mice remained significantly lower than in the ST littermates (Fig. 5 M).

DISCUSSION
Adult beta cells are growth arrested in G0/1 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 G1- 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 beta 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 beta 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) while increased E2F1/pRb dimer formation can lead to active repression of E2F target genes (35), all of which can contribute to G1 arrest. Our finding that several transcriptional targets (36-39) of E2F1 like E2F1, cyclin E and caspase-7 were activated in beta cells and that transduced beta cells labeled with BrdU and Ki67 suggests 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 non-immortal cells (10). Overexpression of E2F1 cannot enforce S-phase entry in in vitro cultures of primary cardiomyocytes in the absence of serum and concomitant presence of an anti-apoptotic factor (e.g. Bcl-2) or 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 24h in beta cells cultured in a minimal medium without growth factors or a feeder layer (26). Moreover, the E2F1-transduced beta cells progressively populated the G2/M phase between 24h and 48h post-transduction of the cells and a non-negligible fraction of those cells underwent mitosis as shown by confocal microscopy of beta cells with phosphorylated histone H3, a marker for initial stages of chromatin condensation in late G2 interphase until anaphase. This illustrates that overexpression of the cell cycle effector E2F1 can overcome inhibition of proliferation of primary beta cells. It is possible that autocrine stimulation by insulin helps to render cultured beta cells permissive to proliferation by ectopic E2F1 (42).

Our in vitro data suggest that the majority of beta 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 beta cell death may occur through p53-independent apoptosis (10). Growth factor signaling via Akt/protein kinase B has potent anti-apoptotic effects in beta cells (for review see (23; 43)). Furthermore, E2F1 mediated pro-apoptotic functions can be suppressed specifically by the PI3K/Akt pathway (44). Indeed, adenoviral co-expression of constitutively active (myristoylated) Akt reduced the E2F1-induced beta cell death to near control levels, and in addition increased E2F1-induced beta cell proliferation. Under these conditions the beta cell number and insulin content were clearly increased as compared to 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 beta
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 beta cells exhibit increased beta cell proliferation accompanied by overwhelming beta cell apoptosis, which rapidly leads to diabetes (48; 49). These observations do not apply to a beta cell specific conditional expression of E2F1, which increased their proliferation activity 2-fold while decreasing rather than increasing their death by apoptosis. Induced DT mice did not develop hyperglycemia over a period of three months (results not shown) also suggesting absence of long-term beta cell destruction or dysfunction. The effects on the percent BrdU-positive and active caspase-positive cells were however not associated with an increased beta cell mass; on the other hand the DT mice exhibited a 70% higher insulin content which appeared responsible for a significantly higher insulin release following 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 E2F knockout mice have also suggested that E2F is crucial for normal glucose tolerance and beta cell insulin secretion (50). Since the E2F -induced increase in pancreatic insulin reserve was not associated with an increased number of beta 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 E2F can increase the insulin content in normal beta 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 hE2F1 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 hE2F1 in AdE2F1-infected cells was >1000-fold the endogenous mE2F1 expression of uninfected cells (-ΔΔCt = 11.9±1.1, n=3, normalized to cyclo A). The in vitro death and impaired insulin release seen with AdE2F1 infection might be caused by unphysiological E2F1 expression levels highly inducing genes that interfere with beta cell survival (casp-7, this study) and function (Kir6.2, (50)), and are not necessarily caused by the high rate of beta cell proliferation. The importance of E2F1 for beta 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 beta cells.

In summary, previous loss-of-function studies in E2F-/- mice (14; 18; 50) pointed out an important role for E2F1 in controlling postnatal beta cell proliferation and function. We now report a gain-of-function study and demonstrate that ectopic expression of E2F1 in adult beta 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 beta 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 beta cell function as it is capable to increase physiologically regulated insulin release through elevating the hormone content of beta cells. Obviously, targeting this cell-cycle factor to increase beta cell growth/function would need to be very much controlled, to prevent oncogenesis.

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Figure Legends

FIG. 1. Ectopic expression of active E2F1 in murine beta cells. Dispersed mouse islet cells were cultured in suspension and infected (MOI20) with adenovirus AdE2F expressing human E2F1, or with AdNull without transgene. A: Immunoblot detection of E2F1 protein (±55 kDa) 24h 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 (cyclo A) as described in M&M, and expressed relative to the data obtained with AdNull. C: Immunocytochemical detection of E2F1 protein in rat beta cells (>90% purity) 48h 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 ± SEM (n=3-4, *P<0.05, **P<0.01, ***P<0.001 vs. AdNull).

FIG. 2. E2F1 expression induces beta cell proliferation and death. Proliferation and survival was assessed in cultures of single rat beta cells (>90% purity) with overexpression of E2F1. Beta cells were infected with AdE2F or AdNull and studied 48h later by (immuno)fluorescent staining and microscopy. Data are presented as means ± SEM. A: BrdU incorporation (16h labeling, 100µM) 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 MOI20 (n=4, ***P<0.001 vs. AdNull). C: FACS cell cycle analysis on fixed and PI-stained cells 24h or 48h after infection with AdE2F (MOI20). D: Detection of the mitotic marker phospho-histone H3 (PPH3) in insulin (INS) expressing beta cells. E: Confocal microscopy showing co-localization of PPH3 and condensed DNA in INS⁺ beta cells in anaphase.

FIG. 3. Effect of Akt expression on cell death and proliferation in isolated beta cells overexpressing E2F1. Data are presented as means ± SEM. A: Immunocytochemical detection of constitutively active (HA-tagged) Akt using a HA-specific antibody. Akt was localized at the cell membrane of rat beta cells following their infection with adenovirus AdAkt, suggesting myristoylation of Akt. B: BrdU incorporation (n=3), and cell survival (n=4), following co-infection of beta cell cultures with both AdAkt and AdE2F1 at the indicated MOI, and as studied in Fig. 2. C: Quantitation of phospho-histone H3⁺ (PPH3⁺) cells in beta 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 beta cell numbers determined by CyQuant assay at 48h and 96h after viral infection with AdNull or AdE2F (MOI20) or with AdE2F+AdAkt (both MOI10). Assays performed with approximately 13,000 cells as starting beta cell number (n=6, **P<0.01, ***P<0.001 vs. no virus).

FIG. 4. Insulin content increases and insulin release decreases in proliferating beta cells. Data are presented as means ± SEM. A: total insulin (ng), and B: insulin content per cell (pg), was determined in 48-h or 96-h cultures of beta cells infected with AdNull or AdE2F (MOI20), or with AdE2F+AdAkt (both MOI10) (n=6, **P<0.01, ***P<0.001 vs. no virus). C: 150K beta cells were infected with AdE2F (or AdE2F+AdAkt), 24 h later they were pulse-labeled with BrdU (1h, 100µM), 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 beta cell cultures infected with either AdE2F or AdE2F+AdAkt, S-G2/M phase cells contained more insulin than G0/G1 cells. E: Rat beta cells (25x10^3) were infected with AdAkt+AdNull or AdAkt+AdE2F (MOI10 each). In the culture period between 20h and 24h post-infection, tritium-labeled tyrosine was present. At 24h, 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 48h. Insulin released into the medium during a 2h 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).

FIG. 5. Conditional expression of E2F1 in mice stimulates beta cell proliferation and function. A: Transgenic mice. Tamoxifen injection of bigenic RIPCreERT x E2F1 mice results in nuclear translocation of CreER leading to excision a loxP-flanked lacZ (serving as a stop sequence) and expression of the human E2F1 gene specifically in beta cells. B: Bigenic RIPCreERT x E2F1 mice (DT) and monogenic littermates (ST) received 4 x 4mg injections (over 7 days). Two weeks later the expression of human E2F1 and the housekeeping gene cyclophilin A (cyclo A) were assessed by quantitative RT-PCR on RNA isolated from their pancreatic islets. Only bigenic TAM-treated mice expressed the human E2F1 gene. C: Beta cell proliferation assessed by Ki67 and insulin staining in the bigenic (DT) and monogenic (ST) mice (n=3). D: Beta cell proliferation assessed by BrdU (IP injection 16 h and 2h before sacrifice) and insulin staining in the same groups of mice (n=3). E: Beta cell apoptosis evaluated by staining for active caspase-3 (Casp-3) and insulin (n=3). F: Measurement of total beta cell mass (mg) in pancreas of DT and ST mice (n=3). G: Measurement of insulin content (ng insulin per mg 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 RIA in serum obtained from 200 µl tail vein blood just before (0') and 15 min (15') after IP-injection of glucose (n=8). J: Bodyweight measurements for the DT and ST mice (n=8). K: Glucose-induced insulin release (% of content) by freshly isolated mouse islets from DT mice and ST mice, during 2h incubation at the indicated glucose concentrations (n=4). L: Kaplan-Meier analysis of survival of DT (n=10) and ST mice (n=17) following one injection of streptozotocin (STZ, 200mg/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 panel L. Data are presented as means ± SEM. *P<0.05, **P<0.01.
REFERENCES

7. Georgia S, Bhushan A: Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. J Clin Invest 114:963-968, 2004
Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell* 132:197-207, 2008

**Figure 1**

A

![Image of Western blot analysis](image)

- No virus
- AdNull
- AdE2F

**B**

![Bar charts](image)

C

![Immunofluorescence images](image)
Figure 2

A

% BrdU cells (%) vs. MOI

- AdNull
- AdE2F

% Living cells (%) vs. MOI

B

Ki67 cells (%) vs. treatment

- No virus
- AdNull
- AdE2F

C

AdE2F - 24h

- S = 20%
- G2 = 3.5%

AdE2F - 48h

- S = 21%
- G2 = 9.7%

D

E

- P-H3
- IN5
- DNA
Figure 3

A

B

C

D

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Figure 4

A

Insulin content (ng)

48h

96h

no virus AdNull AdE2F AdE2F+AdAkt

B

Insulin per cell (pg)

48h

96h

no virus AdNull AdE2F AdE2F+AdAkt

C

13±5%

AdNull AdE2F

G0/G1 S-G2/M

D

E

Synthesized $^3$H-insulin (4h)

Released $^3$H-insulin (4h)

10000 dpm/1000 cells

E

Insulin release per cell (pg)

no virus AdNull AdE2F AdE2F+AdAkt

G2 G20

** *
Figure 5

A. 

Insulin promoter CreERT → X → Rosa26 promoter LoxP STOP hE2F1 → tamoxifen → Rosa26 promoter hE2F1

B. 

Human E2F1

C. 

% KI67 + INS + cells

D. 

% BrdU + INS + cells

E. 

% Caspase3 + INS + cells

F. 

Beta cell mass (mg)

G. 

Insulin content (mg/mg)

H. 

Glycemia (mM)

time (min)

I. 

Serum insulin (pg/ml)

J. 

Bodyweight (g)

K. 

Insulin release (% of control)

L. 

Percent fat mass

M. 

Blood glucose (mg/dL)

RIP-Cre-ERT +/− E2F1 +/−; 
mouse1 
mouse2 
mouse3 
mouse4 

RIP-Cre-ERT +/− E2F1 +/−; 
mouse1 
mouse2 
mouse3 
mouse4

Human E2F1

RIP-Cre-ERT −/−; E2F1 +/− 

RIP-Cre-ERT −/−; E2F1 −/−