

# Pancreatic islets from cyclin-dependent kinase 4/R24C (Cdk4) knockin mice have significantly increased beta cell mass and are physiologically functional, indicating that Cdk4 is a potential target for pancreatic beta cell mass regeneration in Type 1 diabetes

N. Marzo<sup>1</sup> · C. Mora<sup>1</sup> · M. E. Fabregat<sup>1</sup> · J. Martín<sup>2</sup> · E. F. Usac<sup>1</sup> · C. Franco<sup>1</sup> · M. Barbacid<sup>2</sup> · R. Gomis<sup>1</sup>

<sup>1</sup> Endocrinology and Nutrition Unit, Department of Medicine, Clinic Hospital/Institute of Biomedical Research August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain

<sup>2</sup> Molecular Oncology (MB) and Biotechnology (SO) Programme, National Center of Oncologic Research, Madrid, Spain

## Abstract

**Aims/hypothesis.** Cyclin-dependent kinase 4 (Cdk4) is crucial for beta cell development. A mutation in the gene encoding for Cdk4, *Cdk4R24C*, causes this kinase to be insensitive to INK4 cell cycle inhibitors and induces beta cell hyperplasia in *Cdk4R24C* knockin mice. We aimed to determine whether this *Cdk4R24C* mutation also affects proper islet function, and whether it promotes proliferation in human islets lentivirally transduced with *Cdk4R24C* cDNA.

**Methods.** Our study was conducted on wild-type and *Cdk4R24C* knockin mice. Pancreases were morphometrically analysed. Intraperitoneal glucose tolerance tests and intravenous insulin tolerance tests were performed on wild-type and *Cdk4R24C* mice. We also did in vitro islet perfusion studies and islet metabolic labelling analysis. Human islets were transduced with *Cdk4R24C* cDNA.

**Results.** Pancreatic islets from *Cdk4R24C* knockin mice exhibit a larger insulin-producing beta cell area and a higher insulin content than islets from wild-type littermates. Insulin secretion in response to glucose is

faster and reaches a higher peak in *Cdk4R24C* mice without leading to hypoglycaemia. Conversion of pro-insulin into insulin and its intermediates is similar in *Cdk4R24C* and wild-type mice. Glucose utilisation and oxidation measured per islet were similar in both experimental groups. Insulin secretion was faster and enhanced in *Cdk4R24C* islets perfused with 16.7 mmol/l glucose, with slower decay kinetics when glucose returned to 2.8 mmol/l. Moreover, human islets expressing *Cdk4R24C* cDNA exhibited higher beta cell proliferation.

**Conclusions/interpretation.** Despite their hyperplastic growth, *Cdk4R24C* insulin-producing islet cells behave like differentiated beta cells with regard to insulin production, insulin secretion in response to glucose, and islet glucose metabolism. Therefore Cdk4 could possibly be used to engineer a source of beta cell mass for islet transplantation.

**Keywords** Beta cell replication · Cdk4R24C · Cyclin-dependent kinase 4 (mutation R24C) · Insulin secretion · Normoglycaemia.

Received: 7 October 2003 / Accepted: 9 January 2004

Published online: 1 April 2004

© Springer-Verlag 2004

R. Gomis (✉)

Endocrinology and Nutrition Unit, Department of Medicine,  
Clinic Hospital/Institute of Biomedical Research  
August Pi i Sunyer (IDIBAPS), University of Barcelona,  
C/. Villarroel 170, 08036 Barcelona, Spain  
E-mail: gomis@medicina.ub.es  
Tel.: +34-93-2279846, Fax: +34-93-4516638

**Abbreviations:** Cdk4, cyclin-dependent kinase 4 · CMV, cytomegalovirus · GFP, green fluorescence protein · IGTT, intraperitoneal glucose tolerance test · IITT, intravenous insulin tolerance test · LacZ,  $\beta$ -galactosidase

N. Marzo, C. Mora and M. E. Fabregat contributed equally to this paper.

## Introduction

Loss of beta cell mass is due to the negative balance between beta cell recovery and destruction in Type 1 diabetes (recurrent destruction due to autoimmunity) and in Type 2 diabetes (beta cell death unrelated to an autoimmune process). Beta cell mass increases by differentiation of precursor cells or by replication of pre-existing islet cells [1]. The increase in beta cell mass is determined by the number of cells entering the cell cycle (G1 phase) rather than by changes in the rate of cell cycle progression [2].

The transition between cell cycle phases is controlled by critical checkpoints, in which cyclins and cyclin-dependent kinases play an important role. Four main types of cyclin are involved in cell cycle control:

A, B, D and E [3]. D cyclins (D1, D2, D3) facilitate the entry of cells into the cell cycle (G1 phase) in response to growth-stimulating factors, by binding and activating the specific protein-kinases cyclin-dependent kinase 4 (Cdk4) and cyclin-dependent kinase 6 [4]. The activity of the Cdk-cyclin complex can be inhibited by phosphorylation or through binding to Cdk inhibitors such as the Ink4 and CIP/KIP families [5]. By binding to cyclin D1, moreover, Cdk4 mediates post-natal beta cell proliferation [2, 6]. However, Cdk4 deficiency has been shown to cause severe diabetes mellitus associated with pancreatic islet degeneration [4, 7]. Abnormal expression of the Cdk inhibitor p21 is associated with impaired beta cell proliferation in pancreatic islets, and this defect in beta cell proliferation is seen in some types of diabetes [8].

It has recently been shown that Cdk4R24C, the hyperactive mutated form of Cdk4 that prevents the binding of Cdk4 to INK4 [9], causes pancreatic islet hyperplasia specific for beta cells [4]. In the present study we aimed to characterise the functionality of pancreatic islets in response to glucose in *Cdk4R24C* mice, and to causally relate Cdk4 hyperactivation to increased beta cell replication in response to glucose in human islets.

## Materials and methods

**Experimental mice.** *Cdk4R24C* knockin mice (Panlab, Cornellà, Spain) have been previously described [4] in the mixed CD1/129Sv genetic background. Unless otherwise stated, 2-month old homozygous *Cdk4R24C/R24C* male or female mice were studied, and CD1/129Sv wild-type mice were used as a control group. The experimental mice were housed under a 12-h dark/light cycle with free access to a standard rodent diet (Panlab). All animal experimental procedures were reviewed previously and approved by the Institutional Ethical Committee of the University of Barcelona.

**Morphometric studies.** Mice were killed by cervical dislocation. Pancreases were then removed, fixed in 4% paraformaldehyde and dehydrated in 30% sucrose. For insulin immunostaining frozen sections were stained with an anti-insulin antibody (ICN, Costa Mesa, Calif., USA) detected by a peroxidated secondary antibody (Sigma-Aldrich Química, Madrid, Spain). AEC chromogen was used as peroxidase substrate. Toluidine blue was used for islet counter-staining. The beta cell area was quantified in a blinded fashion using optic microscopy and MicroImage software (Hamburg, Germany).

**Islet insulin content.** To obtain total islet protein, islets were isolated by collagenase digestion of total pancreases, using a modified version of a previously described procedure [10]. They were then immersed in an acid-alcohol solution, sonicated and left overnight at 4 °C. After centrifugation and protein purification from the supernatant, insulin content was determined by RIA (Insulin-CT, CIS bio International, Cedex, France). The results are expressed as insulin content per 10 islets.

**Proinsulin biosynthesis and conversion.** Isolated islets (60 islets per experimental condition) from wild-type and *Cdk4R24C*

mice were incubated in 16.7 mmol/l glucose while being pulsed for 10 min with <sup>35</sup>S-methionine (Amersham, Freiburg, Germany) and chased for 30, 60, 180 and 300 min respectively. Islets were lysed by sonication, pre-incubated for 1 h in Cowan solution (Sigma-Aldrich Química) at room temperature, and at 4 °C overnight with IMAD B37 (insulin immunoabsorbent) kindly provided by J. Hutton (Department of Clinical Biochemistry, University of Cambridge, UK). After washing with Low Background Solution (25 mmol/l Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, 1 mmol/l EDTA, 0.1% NaN<sub>3</sub>, 1% Tween-20, 6% BSA; 3 washes), Lysis Buffer (50 mmol/l TRIS pH 7.5, 150 mmol/l NaCl, 1% Triton X-100, 0.1% SDS, 5 mmol/l EDTA, 1% deoxycholic sodium salt; 2 washes), and water (1 wash), insulin and proinsulin were eluted with 25% acetic acid and dried overnight using speed vacuum at room temperature. Samples were resuspended in loading buffer (9 mol/l urea, 0.25 mol/l TRIS pH 8.3) and separated in an alkaline urea gel. Once electrophoresis had finished, the gel was fluorographed using PPO/glacial acetic acid and then dried, and exposed. Autoradiographs were quantified using densitometric analysis and normalised per total cpm incorporated.

Results were normalised according to the amount of radio-labelled protein. To quantify the insulin released, the supernatant of the incubation with <sup>35</sup>S-methionine was processed in the same ways as the pellets containing the islets.

**Insulin secretion.** The protocol was based on a modified version of one already described [11]. Isolated islets were perfused (33 islets per perfusion chamber) with 2.8 mmol/l glucose for 15 min. At this time point the glucose concentration was increased to 16.7 mmol/l for 40 min and then returned to 2.8 mmol/l. Supernatant was retrieved at various time points and insulin secretion determined by RIA.

**Metabolic parameters.** Blood glucose was measured using an automatic glucose monitoring device (Glucometer elite; Química Farmacéutica Bayer, Barcelona, Spain). Insulinaemia was measured by RIA. Two-month old male and female mice which had fasted for three hours were used to determine insulinaemia.

**Intraperitoneal glucose tolerance test.** Before the intraperitoneal glucose tolerance test (IGTT) mice were anaesthetised using sodium pentobarbital (60 mg/kg). After fasting for 16 h, they received an intraperitoneal injection of 150 mg glucose per kg body weight. Glycaemia and insulinaemia were measured at 0, 15, 30, 60 and 120 min after the injection. Insulinaemia was determined using an ultrasensitive rat insulin ELISA kit (Mercordia, Uppsala, Sweden). Glycaemia was determined as previously described.

**Intravenous insulin tolerance test.** Before the intravenous insulin tolerance test (IITT) mice were anaesthetised using sodium pentobarbital (60 mg/kg). After 16 h of fasting, 1 IU insulin per kg body weight (Regular Humulin; Lilly, Indianapolis, Ind., USA) was injected into the tail vein. Insulinaemia was measured at 0 min, and glycaemia at 0, 15, 30, 45 and 60 min after the injection. Insulinaemia and glycaemia were determined as previously described.

**Glucose oxidation and utilisation.** Groups of 15 islets per condition were incubated at 37 °C for 120 min in bicarbonate buffered medium (40 µl) (460 mmol/l NaCl, 96 mmol/l NaHCO<sub>3</sub>, 20 mmol/l KCl, 4 mmol/l MgCl<sub>2</sub>, 4 mmol/l CaCl<sub>2</sub>, 16.7 mmol/l glucose) containing D-(5-<sup>3</sup>H) glucose and D-(U-<sup>14</sup>C) glucose. Incubation was stopped by the addition of citrate-NaOH buffer (400 mmol/l, pH 4.9) containing an-

timycin-A (10  $\mu\text{mol/l}$ ) (Sigma, Madrid, Spain), rotenone (10  $\mu\text{mol/l}$ ) (Sigma) and potassium cyanide (5  $\text{mmol/l}$ ). Glucose oxidation was measured by the generation of hyamine hydroxide-trapped  $^{14}\text{CO}_2$  (Carlo Erba, Milan, Italy) after 60 min incubation at room temperature. Glucose utilisation was determined by measuring the amount of  $^3\text{H}_2\text{O}$  in 0.5 ml HCl (0.1  $\text{mol/l}$ ) after 20 h incubation at room temperature. The islet size was homogeneously distributed among both experimental groups, so both groups had the same number of large, average-sized and small islets.

**Lentiviral vector construction and human islet infection.** The non-replicative lentivirus particles were produced as previously described [12]. Briefly, three plasmid constructions were co-transfected in the 293T cell line by the  $\text{CaCl}_2$  precipitation method in order to obtain viral particles as follows: (i) the vector (env-coding plasmid) required for the formation of the Vesicular Stomatitis Virus capsid; (ii) the pCMV $\Delta$ R9, non-replicative, HIV-like vector encoding for the gag and pol lentiviral proteins required to encapsulate the viral particles into the Vesicular Stomatitis Virus capsid; and (iii) the third vector encoding the cDNA of interest, i.e. *Cdk4/R24C*,  $\beta$ -galactosidase (*LacZ*) or green fluorescence protein (GFP), under the cytomegalovirus (CMV) promoter (pHR'-CMV). The pHR'-CMV-*LacZ* vector, env-coding vector and pCMV $\Delta$ R9 plasmid were provided by B. Thorens (Institute of Pharmacology, University of Lausanne, Switzerland).

Human islets were obtained by collagenase digestion from a deceased and previously healthy donor, with previous written consent from the donor or close relatives, following our established protocol [13] and cultured for 24 h in RPMI 1640 medium containing 11.1  $\text{mmol/l}$  glucose (BioWhittaker, Verviers, Belgium), glutamine, penicillin-streptomycin (BioWhittaker) and 10% FCS. The islets were then cultured for 24 h in RPMI 1640 supplemented with either 5.5  $\text{mmol/l}$  or 11.1  $\text{mmol/l}$  glucose. Next islets were partially disaggregated in EH  $\text{Ca}^{++}$  Free EGTA solution (30  $\text{mmol/l}$  NaCl, 5.4  $\text{mmol/l}$  KCl, 0.8  $\text{mmol/l}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 8.7  $\text{mmol/l}$   $\text{NaH}_2\text{PO}_4$ , 14  $\text{mmol/l}$   $\text{NaHCO}_3$ , 1.6  $\text{mmol/l}$  HEPES, 1  $\text{mmol/l}$  EGTA) and infected for 4 h with lentiviral particles (20 IU/ $\beta$  cell). After this, islets were cultured in the corresponding medium (5.5  $\text{mmol/l}$  or 11.1  $\text{mmol/l}$  glucose) for 2 days, and 12.5  $\text{mmol/l}$  hydroxyurea medium was added for 24 h. Islets were then washed in Hanks' Balanced Salt Solution and cultured in 5.5  $\text{mmol/l}$  or 11.1  $\text{mmol/l}$  glucose for 3 h, after which tritiated thymidine ( $^3\text{H}$ -thymidine, 370  $\text{kBq/ml}$ ) was added for 1 h. Next islets were counted and the same number of islets pelleted for each experimental condition. To quantify proliferation, beta cell radiation was measured.

**Statistical analysis.** All values are expressed as means  $\pm$  SEM. Statistical analysis was by Student's *t* test or ANOVA, depending on the case. Findings were considered to be statistically significant at a *p* value (*\*p*) of less than 0.05 or at a *p* value (*\*\*p*) of less than 0.005. The *p* values are given in the figure legends.

## Results

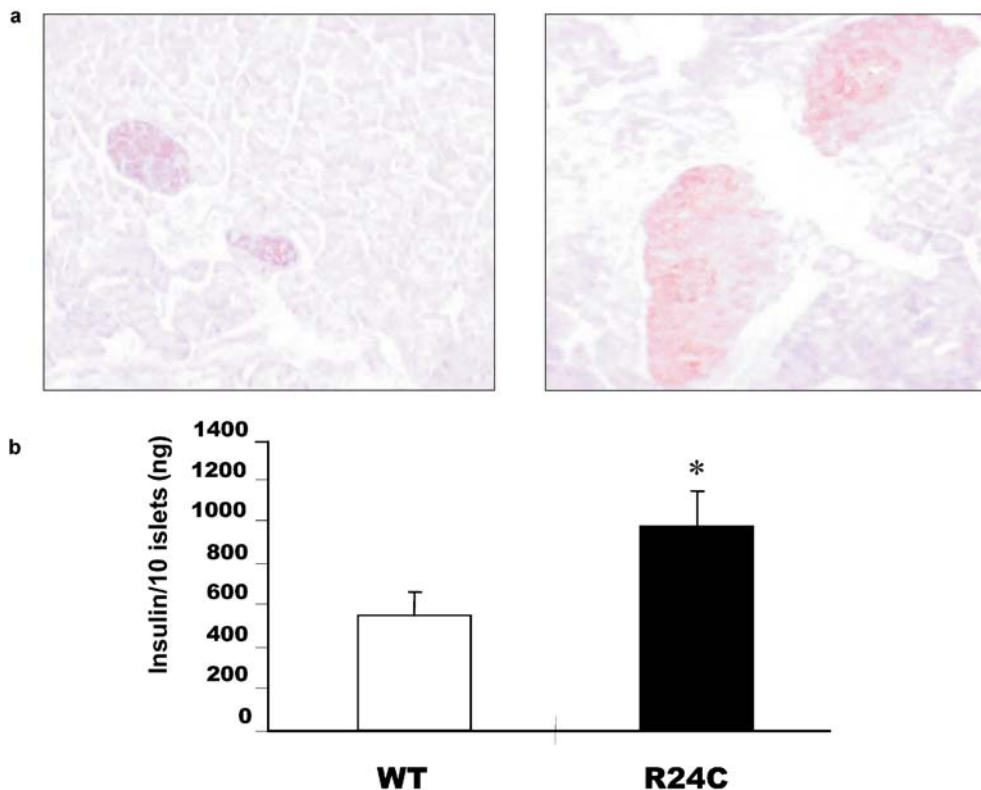
**Higher beta cell mass and insulin content in *Cdk4R24C* knockin mice.** As *Cdk4* is essential for attaining the steady-state beta cell mass that allows stable normoglycaemia, very probably by inducing beta cell proliferation, we characterised the insulin-produc-

ing beta cell mass and insulin content per islet in *Cdk4R24C* knockin mice and wild-type controls. Whole pancreases from 2-month old *Cdk4R24C* knockin mice or wild-type littermates were immunostained for insulin. The former exhibited a higher insulin-producing beta cell area per islet, approximately twice that of wild-type littermates (*R24C* knockin mice:  $47200 \pm 9135 \mu\text{m}^2$ ,  $n=180$  islets from six mice; wild-type mice:  $24800 \pm 2356 \mu\text{m}^2$ ,  $n=180$  islets from six mice) (Fig. 1a). Our observation of a significant increase in beta cell mass per islet when CDK4 is hyperactive explains the pancreatic islet hyperplasia observed in *Cdk4R24C* knockin mice [4]. Supporting these findings, the insulin content was also almost twice as high in islets from *Cdk4R24C* knockin mice (990 ng insulin/10 islets) as in wild-type mice (556 ng insulin/10 islets) (Fig. 1b). Interestingly, neither glycaemia nor insulinaemia differed significantly between the two groups at the two ages tested, i.e. 2 months (young mice) (Fig. 2), and 12 months (mature mice) (Fig. 3). At maturity, moreover, *Cdk4R24C* knockin mice had a normal response to exogenous insulin (similar to that in wild-type littermates) (Fig. 3c).

**Proinsulin biosynthesis and conversion in *Cdk4R24C* islets unaltered.** To investigate whether proinsulin was correctly processed in *Cdk4R24C* islets, we performed pulse-chase experiments on islets from wild-type and *Cdk4R24C* mice and determined proinsulin, intermediate molecules and insulin by fluorography (Fig. 4a). The ratio proinsulin : insulin plus intermediates was quantified densitometrically (Fig. 4b). Neither qualitative nor quantitative differences between *Cdk4R24C* and wild-type islets were observed in the processing of proinsulin into its intermediates and insulin (Fig. 4), suggesting that the increased insulin produced in *Cdk4R24C* islets is metabolically active. As *Cdk4R24C* mice were normoglycaemic and their fasting insulinaemia was similar to that of wild-type mice (Fig. 2, Fig. 3), our results show that insulin secretion rather than insulin production could be the key control point in preventing hypoglycaemia in *Cdk4R24C* knockin mice.

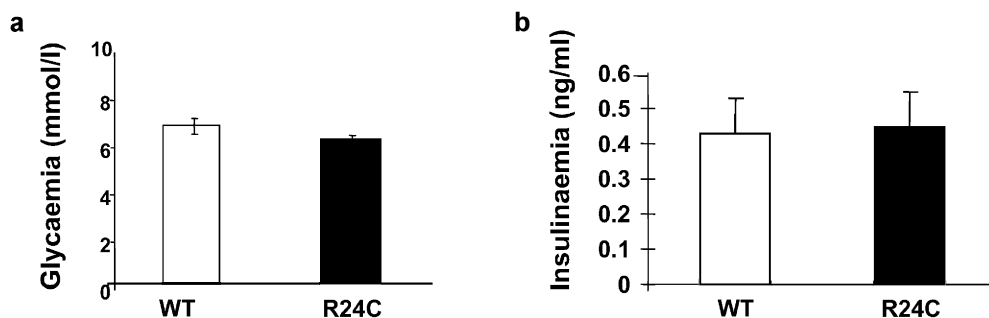
***Cdk4R24C* knockin mice have a more tightly controlled normoglycaemia due to increased insulinaemia in response to glucose stimulus.** We did IGTs on *Cdk4R24C* knockin mice and wild-type littermates to determine whether the physiological triggering of insulin secretion was abnormal in the former. Glycaemia and insulinaemia were measured in parallel from blood samples taken before (0 min) and at different time points (15, 30, 60 and 120 min) after an intraperitoneal injection of glucose. The glycaemic peak 30 min after the glucose injection was lower in *Cdk4R24C* knockin mice (8.7  $\text{mmol/l}$ ) than that in wild-type controls (11.15  $\text{mmol/l}$ ), as was glycaemia





**Fig. 1.** Insulin immunostaining of islets (a) from wild-type (left) and *Cdk4R24C* mice (right). Beta cell surface per islet was quantified. Insulin content (b) from wild-type (WT) and

*Cdk4R24C* (R24C) islets. Results are given as means  $\pm$  SEM of blinded observations on 6 different mice per experimental group (30 islets per mouse). \* $p < 0.05$

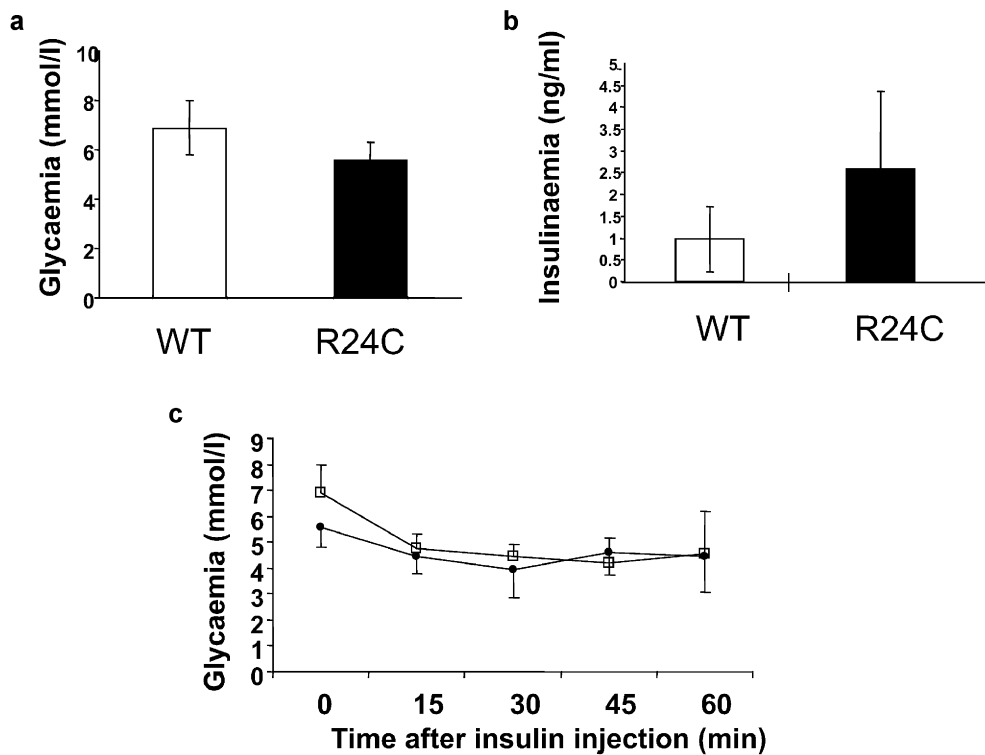


**Fig. 2.** Fasting blood glucose (a) and plasma insulin concentration (b) in 2-month old wild-type (WT) and *Cdk4R24C* (R24C) mice. Results are given as means  $\pm$  SEM. WT:  $n=7$ ; R24C:  $n=11$

vs 1.43 ng/ml respectively). Both groups had returned to basal insulin levels by 120 min after the glucose injection. These results suggest that *Cdk4R24C* knockin mice have more efficient glucose clearance because their pancreatic islets contain more insulin ready for release upon glucose stimulation.

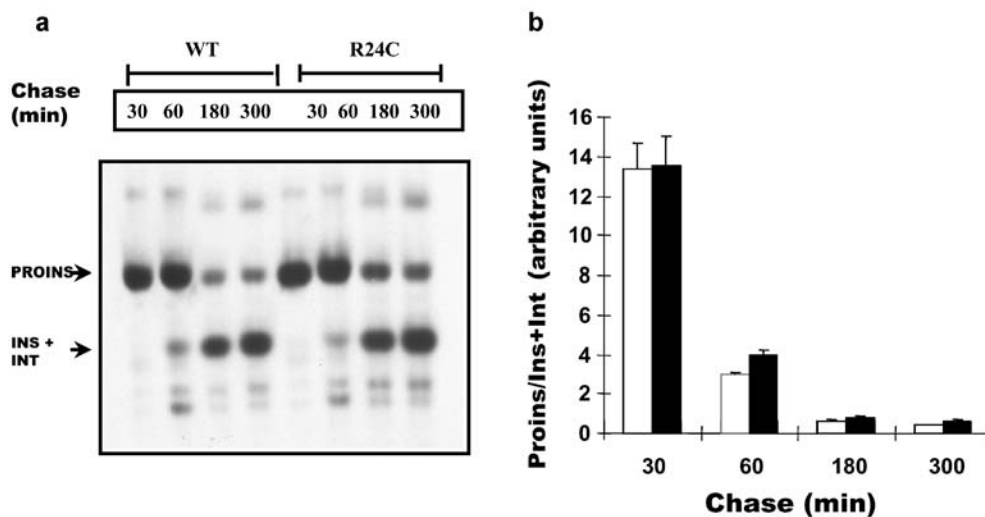
at 60 min, by which time *Cdk4R24C* knockin mice were already normoglycaemic (5.8 mmol/l vs 9.4 mmol/l in wild-type mice) (Fig. 5a). The wild-type group did not reach normoglycaemia until 120 min. The results for insulinaemia mirrored the glycaemia values (Fig. 5b), with *Cdk4R24C* knockin mice reaching a significantly higher peak of insulinaemia (1.77 ng insulin/ml) 15 min after the glucose injection, while the wild-type group was slower to peak (at 30 min). This peak was also significantly lower than the 30-min value for *Cdk4R24C* knockin mice (1.24

*Glucose utilisation and oxidation in Cdk4R24C knockin mice.* As glucose uptake, phosphorylation and ulterior metabolism in pancreatic islets trigger insulin secretion in response to blood glucose levels, we ascertained whether glucose utilisation in the glycolytic pathway and its oxidation in the Krebs cycle are altered in *Cdk4R24C* knockin islets. To obtain pancreatic islets, we killed the *Cdk4R24C* knockin and wild-type mice. We then incubated the islets ex vivo at 37 °C and two different glucose concentrations (5.5



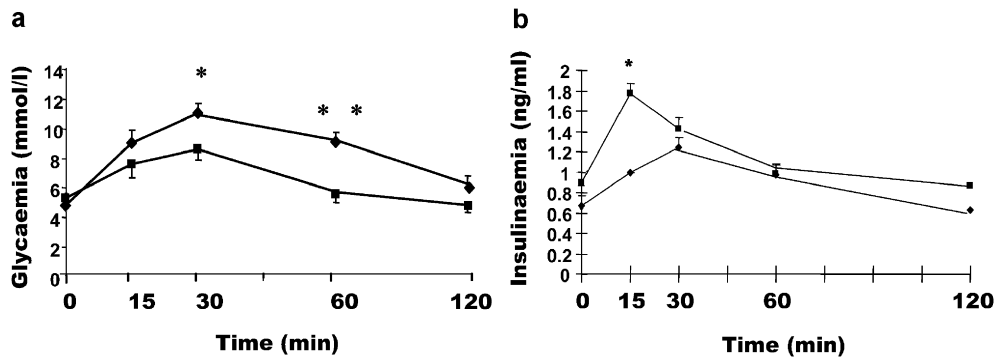
**Fig. 3.** Fasting blood glucose (**a**) and plasma insulin concentration (**b**) in 12-month old wild-type (WT) and *Cdk4R24C* (R24C) mice. Results are given as means  $\pm$  SEM using a minimum of 3 female mice for fasting insulinaemia and a minimum of 6 mice (male and female) for glycaemia measurements.

**c.** Intravenous insulin tolerance test. Mice (male and female) were intravenously injected 1 IU insulin/kg body weight and glycaemia values were determined at different time points. Results are given as means  $\pm$  SEM. White squares: WT mice ( $n=6$ ); black circles: R24C mice ( $n=6$ )



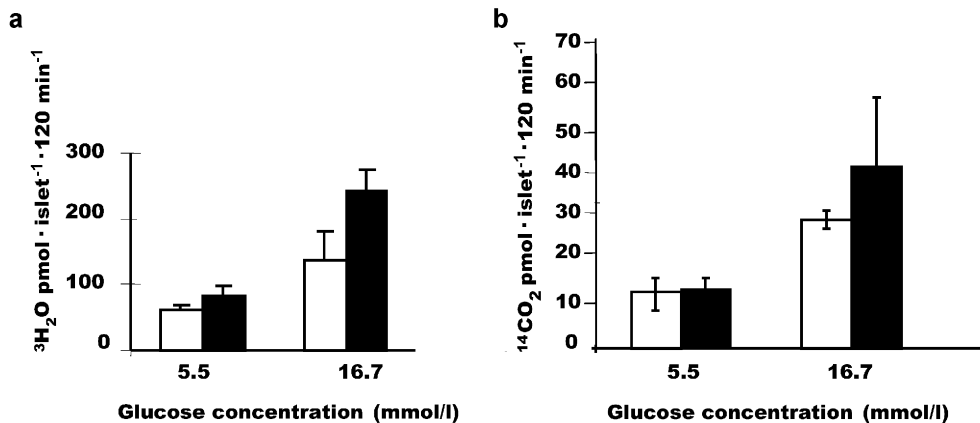
**Fig. 4.** Proinsulin biosynthesis and conversion. **a** Islets from wild-type (WT, left) and *Cdk4R24C* (R24C, right) mice were pulse-labelled with  $S^{35}$ -Methionine and chased at different times. Immunoprecipitation of insulin + intermediates (INS + INT) and proinsulin (PROINS) is shown. **b** Proinsulin/insulin + intermediates levels during chase. Values are expressed as means  $\pm$  SEM of 3 independent experiments. White bars: WT; black bars: R24C

and 16.7 mmol/l) for 2 h. This was done in the presence of either D-(5- $^3$ H) glucose (glucose utilisation) or D-(U- $^{14}$ C) glucose (glucose oxidation). After incubation was stopped, we measured either  $^3$ H $_2$ O (utilisation) or  $^{14}$ CO $_2$  (oxidation). No significant differences between the experimental groups were found (Fig. 6), when normalised per islet at the two concentrations tested. These results suggest that the physiological and tuned functionality of *Cdk4R24C* islets is comparable to that of wild-type counterparts at the ages analysed.



**Fig. 5.** Intrapерitoneal glucose tolerance test on wild-type (WT) and *CdkR24C* (R24C) mice. Time course of blood glucose (a) and plasma insulin (b) after a glucose injection at

0 min. Results are given as means  $\pm$  SEM. Rhomb: WT ( $n=9$ ); square: R24C ( $n=7$ ). \* $p<0.05$ , \*\* $p<0.005$

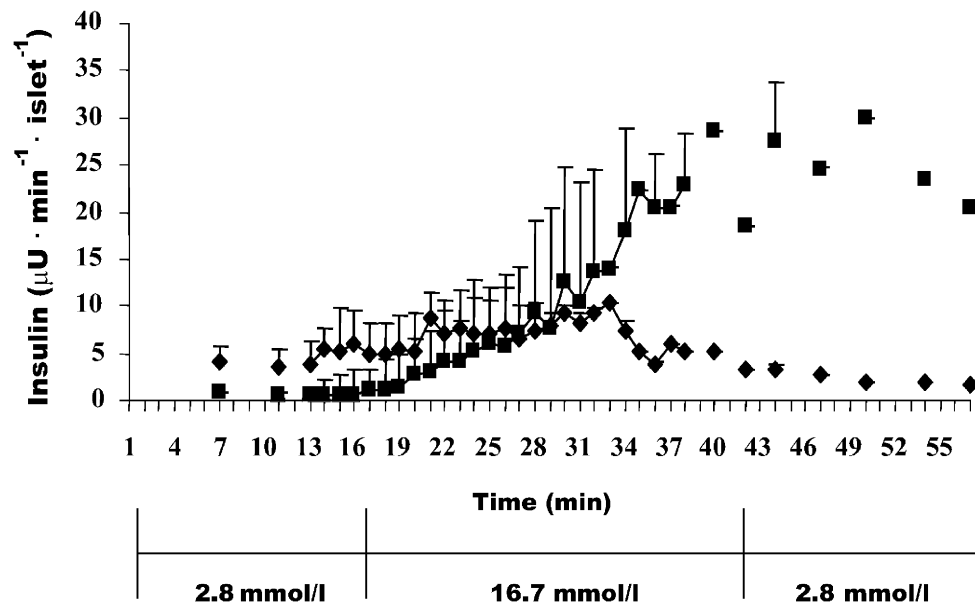


**Fig. 6.** Glucose metabolism in isolated islets of wild-type (WT) and *Cdk4R24C* (R24C) mice. **a** Glucose utilisation. Results are given as means  $\pm$  SEM of 8 observations from 2 separate experiments. **b** Glucose oxidation. Results are given as means  $\pm$  SEM of 12 observations from 3 separate experiments. White bars: WT; black bars: R24C

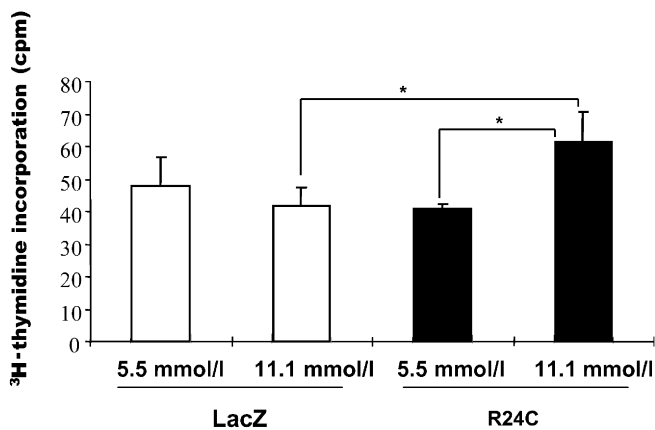
*Ex vivo* perfused *Cdk4R24C* islets exhibit higher and more lasting insulin secretion in response to high glucose concentrations than wild-type counterparts. Although the IGTT test revealed the close control of insulin release in *Cdk4R24C* knockin mice, it was an *in vivo* observation, and therefore did not discriminate between the intervention of various organs in glucose and insulin clearance from the blood. We therefore performed islet perfusion using isolated islets in the presence of a high glucose concentration and measuring insulin secretion to the medium at different time points. Aliquots were withdrawn from the incubation media (Fig. 7) at the time points indicated and insulin content assayed by RIA. Upon stimulation with the high-glucose solution, wild-type islets reached an insulin release peak at about 32 min, returning almost to basal levels of insulin secretion after changing to the low-glucose medium (55 min). Interestingly, the amplitude of the insulin secretion peak was greater in the *Cdk4R24C* islets, in agree-

ment with the IGTT test results. It also lasted longer, just starting to return to basal levels at 55 min (the end of the low-glucose medium incubation) (Fig. 7). This is surprising, for despite the *ex vivo* insulin secretion of *Cdk4R24C* islets in response to glucose, living *Cdk4R24C* knockin mice do not show fasting hypoglycaemia or hyperinsulinaemia. Moreover, in the IGTT test, using a single glucose injection, the rate of recovery of basal insulinaemia in *Cdk4R24C* and wild-type mice was similar.

*Cdk4R24C* confers higher proliferative capability on *Cdk4R24C*-expressing human islets. The R24C mutation in *Cdk4* could be used to generate an immortalised human beta cell line for *in vitro* studies. We therefore wondered whether *Cdk4* activation is sufficient on its own to promote human beta cell proliferation, thus providing a potentially unlimited source of physiologically functional human beta cells for use in islet transplantation. To answer this question, we infected human islets with non-replicative lentiviral particles expressing either *Cdk4R24C* or *LacZ* as control, and cultured them in 5.5 or 11.1 mmol/l glucose. The efficiency of infection was monitored by infecting several islets with GFP-expressing lentiviral vector (after every efficient infection high levels of GFP protein could be observed in the islet core). Measurement of  $^3\text{H}$ -thymidine incorporation showed that



**Fig. 7.** Insulin secretion from perfused islets. Data are from 2 independent experiments with islets from wild-type (rhomb) or *CdkR24C* (square) mice. Results are given as means  $\pm$  SEM of 6 independent observations from 2 different experiments



**Fig. 8.** Proliferation of human islets infected with lentiviral vectors carrying *Cdk4R24C* (R24C, black bars) cDNA or  $\beta$ -galactosidase (LacZ, white bars) as controls. Analysis was by ANOVA. Results are normalised per islet and expressed as means  $\pm$  SEM. This figure is representative of 3 independent experiments. \* $p < 0.05$

*Cdk4R24C*-infected islets proliferated at higher rates (61.23 cpm) than *LacZ*-infected controls (41.8 cpm) at 11.1 mmol glucose, and that proliferation increased significantly when the glucose concentration was raised from 5.5 mmol/l to 11.1 mmol/l in *Cdk4R24C*-infected islets (40.73 vs 61.23 cpm) (Fig. 8). This result suggests that Cdk4-induced proliferation in beta cells is related to changes in exogenous glucose concentration, which is a powerful physiological stimulus.

## Discussion

The transplantation of beta cells is vital for restoring normoglycaemia in Type 1 diabetic patients. Compared with whole pancreas transplantation, beta cell transplantation has much lower levels of associated morbidity and mortality [14]. Its success and feasibility, however, are severely limited by the relatively high number of viable human islets required (12000 islet equivalent/kg body weight) in each graft procedure [15]. Islet yield after the isolation procedure is not totally efficient and varies between laboratories, with two to three donor pancreases needed per Type 1 diabetic recipient to re-establish normoglycaemia [16]. For these compelling reasons, several groups have attempted to modify the number of islets transplanted by making them more resistant to cell death or inducing them to replicate, either in vitro, or after transplantation [12, 17, 18]. One approach involves the conditional transformation of a mouse beta cell line using the SV40 T antigen under the control of tetracycline-responsive element [19].

The present study describes a new potential target for inducing beta cell replication, namely Cdk4, which is specifically involved in post-natal beta cell proliferation. Hyperactivity of Cdk4, caused by the R24C mutation that inhibits the binding of INK4 inhibitors to Cdk4, promotes specific beta cell hyperplasia. The main function of the Cdk4/cyclin complexes could be to phosphorylate and inactivate the retinoblastoma (Rb) protein [20]. INK4 inhibitors associate with Cdk4 monomers in vivo [21] and prevent cyclin D1 binding to Cdk4, causing cell cycle arrest. The abrogation of INK4 inhibitors binding to Cdk4 promotes the entrance of the beta cell into the cycle, and an increase in beta cell mass. Against this background, we have characterised the *Cdk4R24C* phenotype physiologically and biochemically to ascertain whether insu-

lin secretion and glucose metabolism remain unaltered. In addition, the Cdk4 protein is naturally expressed in beta cells and not a non-self antigen expressed ectopically to induce replication [19]. As beta cell mass augmentation depends on cells likely to enter the G1 phase, and as the beta cell area at the age of 2 months was two times greater than in wild-type mice, we propose (and there is experimental evidence [22], as well as a personal communication by M. Barbacid and S. Ortega in support of our proposal) that more cells undergo replication in *Cdk4R24C* knockin mice than in wild-type mice.

We also wondered whether beta cell hyperplasia could cause sustained hypoglycaemia and hyperinsulinaemia in *Cdk4R24C* knockin mice. As noted, these mice showed no signs of fasting hypoglycaemia or hyperinsulinaemia. The *Cdk4R24C* knockin phenotype has tightly controlled *in vivo* insulin secretion in response to glucose, since the insulin secretion peak in the IGTTs was higher and faster to materialise, and paralleled by a lower glycaemic peak in response to an acute intraperitoneal glucose stimulus, without leading to “bounced” hypoglycaemia. As regards the other metabolic parameters analysed (glucose oxidation, utilisation, and proinsulin conversion), *Cdk4R24C* islets remained completely normal.

However, in our perfusion studies using islets from *Cdk4R24C* knockin or wild-type mice, the burst of insulin secretion after exposure to a high glucose concentration was greater in amplitude and longer-lasting in *R24C* islets than that in wild-type controls. Interestingly, *Cdk4R24C* knockin mice develop a wide spectrum of tumors at later ages, among them endocrine tumors (89% of the adenomas found in *Cdk4R24C* pancreases correspond to insulin-producing beta cells [23]). This could explain the larger amplitude and width of the insulin secretion peak found in *Cdk4R24C* islets in perfusion experiments. However, in our animal facility the *Cdk4R24C* colony has not been found to undergo hypoglycaemia at advanced ages. It is possible, therefore, that the *ex vivo* perfusion experiments do not completely reflect the *in vivo* scenario, in which insulin is metabolised in various tissues, especially the liver, thereby maintaining glucose homeostasis. Importantly, although the *R24C* mutation could confer an extreme phenotype on *Cdk4R24C* knockin mice (leading to tumor formation late in life), other milder ways of regulating Cdk4 activity might constitute a new approach to increasing beta cell mass. In this context, it is worth noting that *Cdk4R24C* mice heterozygous for the *R24C* mutation have a lower incidence of tumor formation than mice homozygous for the mutation.

It was essential to determine whether beta cell replication could be triggered by Cdk4 activation in human pancreatic islets, in order to be able to target Cdk4 and induce specific beta cell replication. Our and other recent observations using viral vectors show

the feasibility of expressing certain proteins in the islets [24, 25, 26, 27] without compromising their functionality and viability. We implemented the methodology required to obtain stable expression in islets infected with lentiviruses because this methodology is more efficient and allows the effects of increased replication to be evaluated in the mid and long term [26]. Our results show that *Cdk4R24C*-infected islets exhibit enhanced proliferation in response to glucose. Glucose has been shown to have mitogenic effects on beta cells [28, 29], and here we report that beta cell replication induced by *Cdk4R24C* depends on glucose stimulus, probably by changing the phosphorylation status of certain key molecules in the cell cycle and inducing cyclin D expression.

In conclusion, we have shown that *Cdk4R24C* mice are normoglycaemic, despite exhibiting higher islet insulin content than wild-type littermates, due to a selective beta cell hyperplasia. Moreover, pancreatic islets from *Cdk4R24C* knockin mice have normal insulin biosynthesis, glucose utilisation and oxidation, and secrete larger amounts of insulin in response to a glucose stimulus. Importantly, human islet cells expressing Cdk4R24C exhibit higher proliferation rates in response to a glucose stimulus than LacZ-expressing controls. Taken together, these results suggest that Cdk4 kinase is a key molecule in the regulation of normal beta cell replication, and that Cdk4 could play a key role in future therapeutic strategies for Type 1 diabetes.

**Acknowledgements.** This study was supported in part by grants from Instituto de Salud Carlos III, Red de Centros (RCMN) C03/08, Red de Grupos G03/212, Fundación Salud 2000 and a grant from Ministerio de Ciencia y Tecnología (CICYT SAF, ref. 2000/0053). N. Marzo has a pre-doctoral fellowship from the Institut de Investigacions Biomèdiques August Pi i Sunyer. C. Mora holds an advanced post-doctoral fellowship (ref. 10-2000-635) from the Juvenile Diabetes Foundation International. Our thanks go to Marta Julià for technical assistance in islet isolation and to B. Thorens for providing the pHR'-CMV-LacZ vector, env-coding vector and PCMVΔR9 plasmid.

## References

- Bernard C, Berthault MF, Saulnier C, Ktorza A (1999) Neogenesis vs. apoptosis as main components of pancreatic beta cell mass changes in glucose-infused normal and mildly diabetic adult rats. *FASEB J* 13:1195–1205
- Swenne I (1982) The role of glucose in the *in vitro* regulation of cell cycle kinetics and proliferation of fetal pancreatic  $\beta$ -cells. *Diabetes* 31:754–760
- Pines J, Hunter T (1995) Cyclin dependent kinases: an embarrassment of riches? In: Hames BD, Glover DM (eds) *Frontiers in molecular biology. Molecular immunology*. Oxford University Press, Oxford, pp 144–176
- Rane SG, Dubus P, Mettus RV et al. (1999) Loss of Cdk-4 expression causes insulin-deficient diabetes and Cdk-4 activation results in  $\beta$ -islet cell hyperplasia. *Nat Genet* 22:44–52



5. Morgan DO (1995) Principles of CDK regulation. *Nature* 374:131–134
6. Dunlop M, Muggli E, Clark E (1996) Association of cyclin-dependent kinase-4 and cyclin D1 in neonatal  $\beta$  cells after mitogenic stimulation by lysophosphatidic acid. *Biochem Biophys Res Commun* 218:132–136
7. Tsutsui T, Hesabi B, Moons DS et al. (1999) Targeted disruption of CDK4 delays cell cycle entry with enhanced p27<sup>kip1</sup> activity. *Mol Cell Biol* 19:7011–7019
8. Kaneto H, Kajimoto Y, Fujitani Y et al. (1999) Oxidative stress induces p21 expression in pancreatic islet cells: possible implication in beta-cell dysfunction. *Diabetologia* 42:1093–1097
9. Wölfel T, Hauer M, Schneider J et al. (1995) A p16<sup>INK4a</sup>-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* 269:1281–1284
10. Selawry HP, Mui MM, Paul RD, Distasio JA (1984) Improved allograft survival using highly enriched populations of rat islets. *Transplantation* 37:202–205
11. Fernández-Alvarez J, Hillaire-Buys D, Loubatieres-Mariani MM, Gomis R, Petit P (2001) P2 receptor agonists stimulate insulin release from human pancreatic islets. *Pancreas* 22:69–71
12. Dupraz P, Rinsch C, Pralong WF et al. (1999) Lentivirus-mediated Bcl-2 expression in betaTc-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo control of insulin secretion. *Gene Ther* 6:1160–1169
13. Conget S, Sarri Y, Novials A, Casamitjana R, Vives M, Gomis R (1994) Functional properties of isolated human pancreatic islets: beneficial effects of culture and exposure to high glucose concentrations. *Diabetes Metab* 20:99–107
14. Scharp DW, Lacy PE, Santiago JV, McCullough CS, Weide LG, Falqui L (1990) Insulin independence after islet transplantation into type I diabetic patients. *Diabetes* 39:515–518
15. Shapiro AM, Lakey JR, Ryan EA et al. (2000) Islet transplantation in seven patients with type I diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238
16. Bonner-Weir S, Smith FE (1994) Islets of Langerhans morphology and its implications. In: Kahn CR, Weir GC (eds) *Joslin's diabetes mellitus*. Lea & Febiger, Philadelphia, pp 15–28
17. Mellert J, Hering BJ, Hopt TU et al. (1992) Effect of local and systemic macrophage blocking on engraftment of allogeneic porcine islet. *Transplant Proc* 24:2847
18. Soria B, Roche E, Berná G, León-Quinto T, Reig JA, Martín F (2000) Insulin-secreting cells derived from embryonic stem cells normalize glycaemia in streptozotocin-induced diabetic mice. *Diabetes* 49:157–162
19. Fleischer N, Chen C, Surana M et al. (1998) Functional analysis of a conditionally transformed pancreatic  $\beta$ -cell line. *Diabetes* 47:1419–1425
20. Serrano M, Gómez-Lahoz E, DePinho RA, Beach D, Bar-Sagi D (1995) Inhibition of ras-induced proliferation and cellular transformation by p16<sup>INK4</sup>. *Science* 267:250–251
21. Serrano M, Hannon GJ, Beach D (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 366:704–707
22. Martín J, Hunt SL, Dubus P et al. (2003) Genetic rescue of Cdk4 null mice restores pancreatic  $\beta$ -cell proliferation but not homeostatic cell number. *Oncogene* 22:5261–5269
23. Sotillo R, Dubus P, Martín J et al. (2001) Wide spectrum of tumors in knock-in mice carrying a Cdk4 protein insensitive to INK4 inhibitors. *EMBO J* 20:6637–6647
24. Becker TC, BeltrandelRio H, Noel RJ, Johnson JH, Newgard CB (1994) Overexpression of hexokinase I in isolated islets of Langerhans via recombinant adenovirus. *J Biol Chem* 269:21234–21238
25. Naldini L, Blömer U, Gally P et al. (1996) In vivo gene delivery and stable transduction of non dividing cells by a lentiviral vector. *Science* 272:263–267
26. Naldini L, Blömer U, Gage FH, Trono D, Verma MI (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci* 93:11382–11388
27. Novials A, Jiménez-Chillarón JC, Franco C, Casamitjana R, Gomis R, Gómez-Foix AM (1998) Reduction of islet amylin expression and basal secretion by adenovirus-mediated delivery of amylin antisense cDNA. *Pancreas* 17:182–186
28. Del Zotto H, Gomez Dumm CL, Drago S, Fortino A, Luna GC, Gagliardino JJ (2002) Mechanisms involved in the beta cell mass increase induced by chronic sucrose feeding to normal rats. *J Endocrinol* 174:225–231
29. Trumper A, Trumper K, Horsch D (2002) Mechanisms of mitogenic and anti-apoptotic signaling by glucose-dependent insulinotropic polypeptide in beta (INS-1)-cells. *J Endocrinol* 174:233–246