ISSN 2472-1972

Na^+/Ca^{2+} Exchanger a Druggable Target to Promote β-Cell Proliferation and Function

Julien Papin,[1]* Francesco Paolo Zummo,[1]* Nathalie Pachera,[1,2] Claudiane Guay,[3] Romano Regazzi,[3] Alessandra K. Cardozo,[2] and André Herchuelz[1,2]

[1]*Laboratory of Pharmacology, Université Libre de Bruxelles, Faculty of Medicine, 1070 Brussels, Belgium;* [2]*ULB Center for Diabetes Research, Université Libre de Bruxelles, Faculty of Medicine, 1070 Brussels, Belgium; and* [3]*Department of Fundamental Neurosciences, University of Lausanne, 1005 Lausanne, Switzerland*

*These authors contributed equally to this study.

An important feature of type 2 diabetes is a decrease in β-cell mass. Therefore, it is essential to find new approaches to stimulate β-cell proliferation. We have previously shown that heterozygous inactivation of the Na^+/Ca^{2+} exchanger (isoform 1; NCX1), a protein responsible for Ca^{2+} extrusion from cells, increases β-cell proliferation, mass, and function in mice. Here, we show that *Ncx*1 inactivation also increases β-cell proliferation in 2-year-old mice and that NCX1 inhibition in adult mice by four small molecules of the benzoxyphenyl family stimulates β-cell proliferation both *in vitro* and *in vivo*. NCX1 inhibition by small interfering RNA or small molecules activates the calcineurin/nuclear factor of activated T cells (NFAT) pathway and inhibits apoptosis induced by the immunosuppressors cyclosporine A (CsA) and tacrolimus in insulin-producing cell. Moreover, NCX1 inhibition increases the expression of β-cell–specific genes, such as *Ins1, Ins2,* and *Pdx*1, and inactivates/downregulates the tumor suppressors retinoblastoma protein (pRb) and miR-193a and the cell cycle inhibitor p53. Our data show that Na^+/Ca^{2+} exchange is a druggable target to stimulate β-cell function and proliferation. Specific β-cell inhibition of Na^+/Ca^{2+} exchange by phenoxybenzamyl derivatives may represent an innovative approach to promote β-cell regeneration in diabetes and improve the efficiency of pancreatic islet transplantation for the treatment of the disease.

Copyright © 2018 Endocrine Society

This article has been published under the terms of the Creative Commons Attribution Non-Commercial, No-Derivatives License (CC BY-NC-ND; https://creativecommons.org/licenses/by-nc-nd/4.0/).

Freeform/Key Words: β-cell, calcineurein/NFAT pathway, diabetes, Na/Ca exchange

Type 2 diabetes is a complex disease characterized by insulin resistance, β-cell dysfunction, and reduction in islet and β-cell mass [1, 2]. Therefore, it is essential to find new approaches or targets to preserve, protect, and regenerate β-cells in type 2 diabetes.

Calcium (Ca^{2+}) is an important signaling molecule involved in key cellular functions, such as hormone secretion, cell metabolism, cell division, and death [3]. When stimulated by glucose, β-cells display a complex series of events, including the opening of voltage-sensitive Ca^{2+} channels that leads to Ca^{2+} entry into the cell with subsequent rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) that triggers insulin release [4]. After entering into the cell, the ion must be extruded to avoid its excessive accumulation, which may be toxic [5]. Two systems responsible for Ca^{2+} extrusion are present in the β-cell: the Na^+/Ca^{2+} exchanger (NCX), and the plasma membrane Ca^{2+}-adenosine triphosphatase (PMCA) [6].

Abbreviations: $[Ca^{2+}]_i$, free Ca^{2+} concentration; CsA, cyclosporine A; DMSO, dimethyl sulfoxide; E2F, E2 factor; FK-506, tacrolimus; HBSS, Hank's buffer salt solution; MEF2, myocyte enhancer factor 2; NCX, Na^+/Ca^{2+} exchanger; NCX1, isoform 1 of Na^+/Ca^{2+} exchanger; NFAT, nuclear factor of activated T cells; PMCA, plasma membrane Ca^{2+}-adenosine triphosphatase; PMCA2, isoform 2 of plasma membrane Ca^{2+}-adenosine triphosphatase; pRb, retinoblastoma protein; siRNA, small interfering RNA.

Received 14 September 2017
Accepted 22 May 2018
First Published Online 25 May 2018

July 2018 | Vol. 2, Iss. 7
doi: 10.1210/js.2017-00370 | Journal of the Endocrine Society | 631–645

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

In a recent work, we examined β-cell function in mice displaying heterozygous inactivation of NCX (isoform 1: NCX1; $Ncx1^{+/-}$ mice) [7]. The data showed that *Ncx1* inactivation elicits an increase in glucose-induced insulin release paralleled by an increase in β-cell proliferation and mass. The mutant mice also displayed augmented β-cell insulin content and glucose-induced Ca^{2+} uptake, and their β-cells were resistant to hypoxia. In addition, $Ncx1^{+/-}$ mice islets showed a four to seven times higher rate of diabetes cure than $Ncx1^{+/+}$ mice islets when transplanted into diabetic animals.

In more recent work, we showed that heterozygous inactivation of PMCA (isoform 2: PMCA2; $Pmca2^{+/-}$ mice) also increased glucose-induced insulin release and β-cell proliferation, mass, and viability [8]. Hence, inhibition of Ca^{2+} extrusion and subsequent intracellular accumulation of the cation is an approach to stimulate β-cell function, proliferation, and mass, and thus to preserve, protect, and regenerate β-cells in type 2 diabetes.

In the present work, we examined the effect of β-cell–specific small inhibitory molecules on β-cell function and proliferation. Moreover, the molecular mechanisms by which inhibition of Ca^{2+} extrusion by Na^+/Ca^{2+} exchange exerts its multiple favorable effects on β-cells was examined. Our data show that *Ncx*1 heterozygous inactivation increases β-cell proliferation in 2-year-old mice. Likewise, four small molecules of the phenoxybenzamyl family inhibit Na^+/Ca^{2+} exchange and increase β-cell proliferation both *in vitro* and *in vivo*. NCX1 inhibition activates the calcineurin/nuclear factor of activated T cells (NFAT) pathway and inhibits apoptosis induced by the immunosuppressors CsA and tacrolimus in β-cells. NCX1 inhibition also inactivates/downregulates the tumor suppressors retinoblastoma protein (pRb) and miR-193a and the cell cycle inhibitor p53. Hence, Na^+/Ca^{2+} exchange is a druggable target to stimulate β-cell proliferation. Specific β-cell inhibition of Na^+/Ca^{2+} exchange by phenoxybenzamyl derivatives may represent an innovative approach to promote β-cell regeneration, survival, and function in type 2 diabetes and improve the efficiency of pancreatic islet transplantation for the treatment of the disease.

1. Materials and Methods

A. Ethics Statement

Animals were treated according to the guidelines of the Belgian Regulations for Animal Care and with the approval of the local ethics committee. The principles of laboratory animal care (National Institutes of Health publication number 85-23, revised in 1985) were followed.

B. Animals

$Ncx1^{+/-}$ and $Ncx3^{+/-}$ mice were developed by our group, had F2 genetic backgrounds from 129/Sv and CD1 mice [7, 9], and were 12 weeks or 24 months old. They were backcrossed against C57BL6/N mice (10 generations). Control mice consisted of age-matched C57BL6/N littermate mice with two wild-type alleles at the *Ncx1* or *Ncx3* locus ($Ncx1^{+/+}$ or $Ncx3^{+/+}$). Of note, $Ncx1^{-/-}$ mice were not studied because they are not viable and die during embryogenesis [7].

C. Cell Culture

Two rat insulinoma cell lines, BRIN-BD11 and INS-1E, were used and cultured as previously described [10, 11]. The protocols for measuring Ca^{2+} were optimized by using the BRIN-BD11 cells, the siRNAs, miRNAs, and plasmid transfections were optimized in INS-1E cells. A total of 10 μM KB-R7943, 1 μM SEA0400, 10 μM SN-6, and 1 μM YM-244769 were used to block NCX1 activity, as previously described [12]. CsA and tacrolimus (FK-506) (Tocris Bioscience) were used at 10 μM, with dimethyl sulfoxide DMSO as control.

Pancreatic islets from $Ncx1^{+/-}$ and age-matched wild-type mice were isolated and cultured as previously described [7].

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

D. $[Ca^{2+}]_i$ Monitoring

The effects of NCX1 inhibitors on Ca^{2+} fluxes in BRIN-BD11 cells were measured by using a plate reader (Victor X3; PerkinElmer). After 3 days of culture in complete medium, cells were detached and loaded with the Fluo-4 Ca^{2+} probe (Fluo-4 Direct™ Calcium Assay Kit, ThermoFisher Scientific). Cells were maintained at 37°C in suspension with stirring, and just before measurement, Fluo-4 loaded cells were injected in each well of a microplate containing normal or Na^+-free Hank's buffer salt solution (HBSS) with DMSO (control) or NCX1 inhibitors. Fluorescence was measured every second for 5 minutes. In some experiments, 10 μM cyclopiazonic acid was added as a control after 5 minutes to release Ca^{2+} from the endoplasmic reticulum.

E. Measurements of β-Cell Viability, Proliferation and Size

The viability of BRIN-BD11 and INS-1E cells was measured by using Hoechst 33342 and propidium iodide (Molecular Probes) [11]. For proliferation and size, tissue sections (5 μm thick) from paraffin-embedded pancreas or isolated islets pellets were prepared for immunofluorescence according to Montanya and Téllez [13]. The primary antibodies used were as followed: Ki67 (#ab16667; RRID: AB_302459; PMID: 24659141; rabbit monoclonal SP6; Abcam), phospho-Rb-Ser 780 (#3590S; RRID: AB_2177182; PMID: 28486108; rabbit monoclonal; Cell Signaling), and insulin (#A056401-2; RRID: AB_2617169; PMID: 23766132; guinea pig polyclonal, Dako). After incubation of primary antibodies at 4°C overnight, the proteins were detected by using dedicated secondary antibodies: donkey anti–guinea pig Alexa488 (RRID: AB_15085; PMID: 25192442) and donkey anti-rabbit Cy3 (#711-165-152; RRID: AB_2307443; PMID: 28803120; Jackson ImmunoResearch), diluted in blocking buffer and incubated for 1 hour at room temperature. In experiments where BRIN-BD11 or INS-1E cells were used, the cells were directly plated on coverslips and submitted to the same protocol without rehydration and antigen retrieval. *β*-cell apoptosis was measured by TUNEL assay (*In Situ* Cell Death Detection Kit; Roche Diagnostics). The method for TUNEL labeling and counting was similar to that for *β*-cell proliferation. All the sections or coverslips were analyzed by using an Axio Observer Z1 fluorescence microscope (Carl Zeiss), and the individual *β*-cell size was measured by using the associated image analysis software. The *β*-cell mass was quantified by point-counting morphometry of insulin-peroxidase immunostained pancreatic sections, as previously described [7]. For the measurement of *β*-cell proliferation, apoptosis, and mass, up to three sections per pancreas of three or four mice with a minimum of 1000 cells per condition were quantified. For the cell lines, three or four experiments, with a minimum of 1000 cells per condition, were analyzed.

F. Transfections

Islets were dispersed into single cells by dispase digestion (Roche Diagnostics) and cultured onto poly-D-lysine (Sigma-Aldrich)-coated glass coverslips in Ham's F-10, containing 5% fetal bovine serum, 0.5% fatty acid-free BSA, 10 mM glucose, 2 mM glutamine, 50 μM IBMX, 50 U/mL penicillin, and 50 μg/mL streptomycin. *β*-cells were identified by immunofluorescence using the anti-insulin antibody. siNcx1-1 (s132087) and siNcx1-2 (s73962) (Life Technologies) and the AllStars Negative Control (Roche Diagnostics) siRNAs were used at 30 nM. Cells were transfected by using Lipofectamine® RNAiMAX lipid reagent (Life Technologies) and cultured for a 48-hour recovery period before further analyses. Endogenous activity of miR-193a or miR-216a was inhibited by transfecting INS-1E or primary *β*-cells with miRCURY LNA Inhibitor hsa-miR-193a, hsa-miR-216a or negative control (Exiqon) using Lipofectamine® 2000 (ThermoFisher Scientific).

G. Measurement of mRNA, miRNA, and Protein Expression

mRNA extraction and reverse transcription were performed as previously described [14], and the real-time PCR amplification was done by using SYBR green, and compared with a

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

standard curve. Expression values were corrected for a housekeeping gene (*Gapdh)*. The primers used for mRNA expression analysis are listed in Supplemental Table 1. For miRNA microarray analysis, total RNA from wild-type and *Ncx1*$^{+/-}$ mice islets was extracted by using the miRNeasy Mini Kit (Qiagen). Global miRNA profiling was performed with miRNA Gene Microarrays (Agilent Technologies). The levels of individual miRNAs were quantified by performing a universal RT reaction with locked nucleic acid-enhanced PCR primers, followed by real-time PCR (Exiqon). The results were normalized by using cDNAs amplified with U6 primers in the same samples. Results were similar when the data were normalized to miR-7. For Western blot analysis, cells were washed with cold PBS and lysed in Laemmli buffer. Immunoblot analysis was performed by using monoclonal antibody against NCX1 (#R3F1; RRID: AB_2716744; PMID: 76922739; R3F1, Swant) [15] and mouse monoclonal anti-b-actin (#A1978; RRID: AB_476692; PMID: 213279; Sigma-Aldrich). Immunoreactive bands were detected by using a Molecular Imager ChemiDoc XRS+ with ImageLab software (Bio-Rad).

H. Luciferase Reporter Assays

INS-1E cells were plated in 24-well plates and transfected with siRNAs as described above. Cells were cotransfected with pRL-CMV encoding Renilla luciferase (Promega) and a firefly luciferase promoter-reporter construct specific for E2 factor (E2F), NFAT, myocyte enhancer factor 2 (MEF2), and p53 (Promega). After 24 hours of recovery, cells were incubated with or without 1 μM forskolin for 6 hours. The Cignal 45-Pathway Reporter Assay (Promega) was used to measure the activation of the following transcription factors: E2F, NFAT, MEF2, and p53. Luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega) and corrected for the luciferase activity of the internal control plasmid.

I. Drugs

KB-R7943, YM-244769, and SN-6 were synthesized by Takahiro Iwamoto (Fukuoka University, Fukuoka, Japan). SEA0400 was synthesized by Andras Toth (University of Szeged, Szeged, Hungary). All the drugs were dissolved in DMSO as stock solution. Final concentration of DMSO in culture medium was 0.1%.

J. Statistics

The results are expressed as means ± SEM. The statistical significance of differences between data were assessed by using Student *t* test or ANOVA, followed by the Tukey post-test. Statistical significance was accepted at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results

A. Heterozygous Inactivation of Ncx1 *or* Ncx3 *Increases Pancreatic* β*-Cell Proliferation in 2-Year-Old Mice*

The rate of β-cell proliferation decreases with age to reach almost null values in the elderly, and the decrease contributes to the development of diabetes [16]. As a first step, we examined whether heterozygous inactivation of *Ncx1* and *Ncx3* (another member of the NCX family) increased β-cell proliferation rate in very old mice (2 years). As expected, β-cell proliferation rate was almost null in islet from wild-type mice (0.026% ± 0.016%; n = 4) (Fig. 1a). In contrast, in *Ncx1*$^{+/-}$ and *Ncx3*$^{+/-}$ mice, the rate of proliferation averaged 0.28% ± 0.05% and 0.19% ± 0.06% ($P < 0.01$ and $P < 0.05$; n = 4) respectively, values similar to those observed in control adult (12-week-old) mice [7]. The β-cell size did not differ between the different types of mice (Fig. 1b). A tendency toward an increase (+45%) in β-cell mass in 2-year-old *Ncx1*$^{+/-}$ compared with *Ncx1*$^{+/+}$ mice (4.63 ± 1.54 vs 3.20 ± 0.68 mg; n = 4; $P < 0.1$) was observed.

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

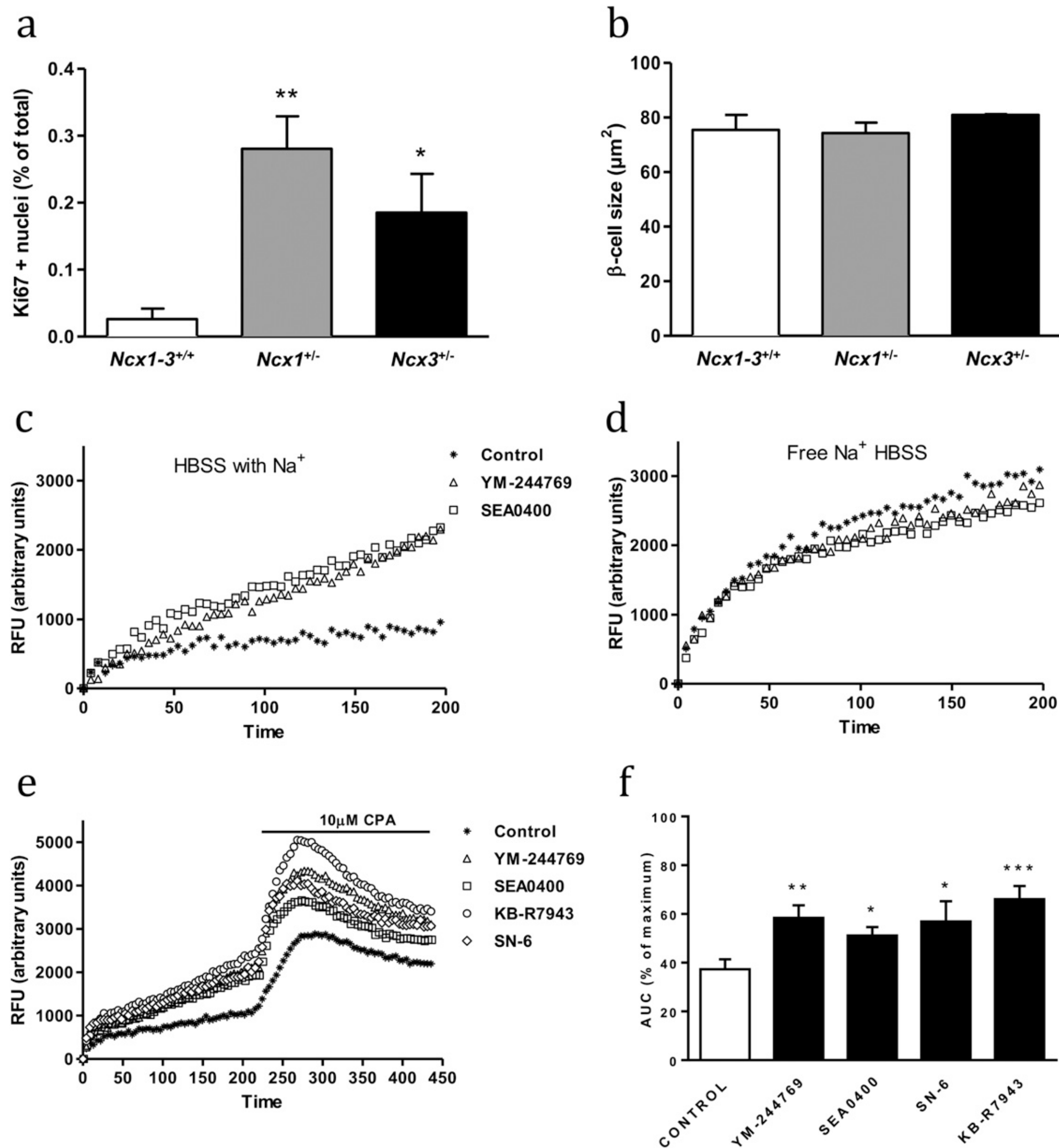

Figure 1. Effects of *Ncx1* and *Ncx3* heterozygous inactivation on β-cell proliferation and size in 2-year-old mutant mice and effects of NCX1 inhibitors on $[Ca^{2+}]_i$ in BRIN-BD11 cells. β-cell proliferation (a) and size (b) are shown in *Ncx1-3*$^{+/+}$ (white bars), *Ncx1*$^{+/-}$ (gray bars), and *Ncx3*$^{+/-}$ (black bars) mouse islets. Mean ± SEM values are shown for four independent animals in which at least three pancreatic sections and a minimum of 1000 cells per condition have been analyzed ($*P < 0.05$; $**P < 0.01$). Increases in $[Ca^{2+}]_i$ of BRIN-BD11 cells exposed to 1 μM YM-244769, 1 μM SEA0400, or DMSO (control) in the presence (c) or the absence (d) of extracellular Na^+. (e) Similar experiments as in part (c) but in the presence of 10 μM KB-R7943, 1 μM SEA0400, 1 μM YM-244769, 10 μM SN-6, or DMSO, and followed after 200 seconds by the addition of 10 μM cyclopiazonic acid. Fluo-4 fluorescence is expressed in relative fluorescence units (RFUs). (f) Area under the curve measured between 0 and 450 seconds ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Means ± SEM are shown for four independent experiments.

B. Monitoring Changes in $[Ca^{2+}]_i$ Induced by NCX1 Inhibitors

We then examined the effect of NCX1 inhibitors on $[Ca^{2+}]_i$ in BRIN-BD11 cells. For this purpose, $[Ca^{2+}]_i$ was measured in Na^+-containing HBSS, a condition in which NCX1 works in the forward mode (extrusion of Ca^{2+}), and in a Na^+-free HBSS, a condition in which

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

NCX1 works in the reverse mode (entry of Ca^{2+}). In Na^{+}-containing HBSS and in the absence of drug, the injection of Fluo-4 loaded cells induced a progressive increase in fluorescence (Fig. 1c). In the presence of NCX1 inhibitors, this increase was more marked; three- to four-fold higher values were observed at the end of the experiment ($P < 0.01$ for YM-244769 and $P < 0.05$ for SEA0400) because of the inhibition of Ca^{2+} extrusion from the cells by forward Na^{+}/Ca^{2+} exchange. In Na^{+}-free HBSS and in the absence of drug, a significantly higher rise in fluorescence than in Na^{+}-containing HBSS was observed ($P < 0.005$) (Fig. 1c and 1d). As expected, YM-244769 and SEA0400 tended to decrease the rise of fluorescence due to inhibition of reverse Na^{+}/Ca^{2+} exchange, but the differences were not statistically significant ($P = 0.46$ for YM-244769 and $P = 0.33$ for SEA0400). The latter data show that NCX1 inhibitors efficiently inhibit Na^{+}/Ca^{2+} exchange in its forward mode (50%). As a further step, we repeated the experiments in the presence of extracellular Na^{+} but prolonged their duration to allow the addition of cyclopiazonic acid to release Ca^{2+} from intracellular stores and hence to increase the efficiency of the method (Fig. 1e). A significant increase in the area under the curve measured in the presence of the four drugs (YM-244769, SEA0400, KB-R7943, and SN-6) compared with control was observed ($P < 0.05$) (Fig. 1f). Thus, the four drugs tested inhibited Na^{+}/Ca^{2+} exchange in insulin-producing cells.

C. NCX1 Inhibition Promotes β-Cell Proliferation in vitro *and* in vivo

We then examined the effect of NCX1 inhibitors on β-cell proliferation using Ki67 labeling (Supplemental Fig. 1). As shown in Fig. 2a, 24-hour incubation of BRIN-BD11 cells in the presence of SEA0400, SN-6, and YM-244769 led to a significant increase in proliferation rate and to a trend toward an increase for KB-R7943. In 12-week-old mice islets exposed to the inhibitors for 48 hours, SEA0400 doubled, whereas the other inhibitors tripled the proliferation rate of β-cells (Fig. 2b).

To examine the effects of long-term and *in vivo* treatment with NCX1 inhibitors on β-cell proliferation, we implanted micro-osmotic pumps containing two different concentrations of KB-R7943 or YM-244769 (and corresponding controls without inhibitors) in 12-week-old mice for 2 weeks. The mice were then euthanized, and pancreases were collected and prepared for analysis of β-cell proliferation and size. The results show that both drugs induced a dose-related increase in β-cell proliferation (Fig. 2c and 2d). No deleterious effect of the treatment on pancreases structure was seen, and β-cell size was similar in all the conditions (data not shown). Of note, none of the inhibitors were toxic for the rat β-cell lines or mouse islets (Supplemental Fig. 2).

To confirm that the increased β-cell proliferation rate induced by *Ncx1*$^{+/-}$ heterozygous inactivation and by the inhibitors was due to NCX1 activity inhibition, we examined the effect of NCX1 inhibition by siRNAs in INS-1E cells. The two siRNAs (siNcx1-1 and siNcx1-2) used reduced NCX1 protein level by ~40% and ~50%, respectively (Fig. 2e). Both siRNA induced a significant increase in INS-1E cell proliferation ($P < 0.05$ and 0.01) (Fig. 2f).

D. Ncx1*-Induced Cell Proliferation is Due to Activation of the Calcineurin/NFAT Pathway, and Its Inhibition Protects β-Cells Against Apoptosis Induced by Immunosuppressive Drugs*

We hypothesized that the effects of heterozygous inactivation of NCX1 on β-cell proliferation could be mediated by the activation of the calcineurin/NFAT signaling pathway [7]. To test this hypothesis, we assessed the transcriptional activity of NFAT using a reporter assay in β cells transfected with siRNAs against *Ncx1* or treated with the NCX1 inhibitors KB-R7943 and YM-244769. Figure 3a shows that under basal and forskolin-stimulated conditions, silencing of *Ncx1* increased calcineurin/NFAT activity in INS-1E cells. KB-R7943 (10 μM) and YM-244769 (1 μM) also increased NFAT transcriptional activity but only in the presence of forskolin (Fig. 3b). By releasing Ca^{2+} from intracellular stores, forskolin leads to a higher increase in $[Ca^{2+}]_i$ in the presence of the inhibitors and hence to a higher activation of the calcineurin/NFAT pathway.

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

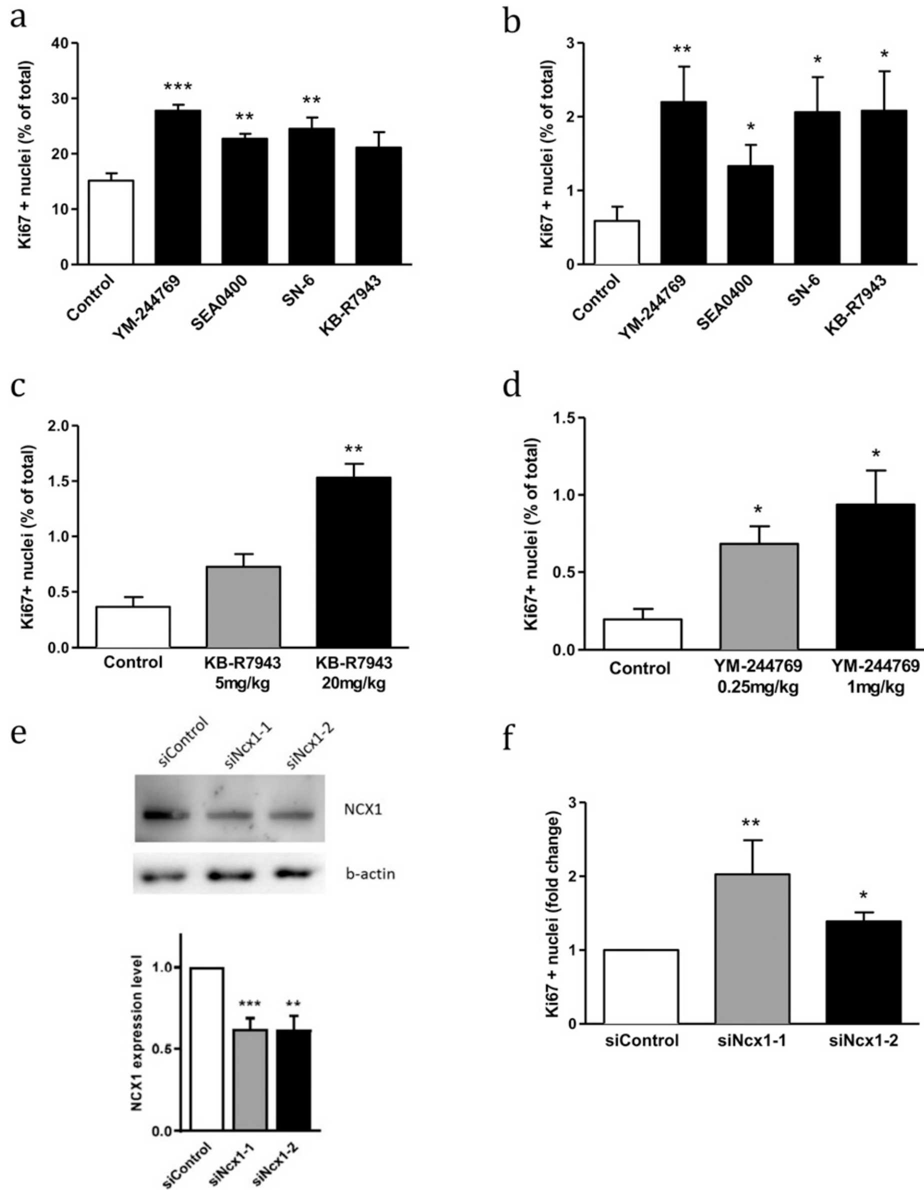

Figure 2. NCX1 inhibitors and *Ncx1* knockdown by siRNAs increase proliferation of BRIN-BD11 cell lines and primary β-cells *in vitro* and *in vivo.* Effect of 1 μM YM-244769, 1 μM SEA0400, 10 μM SN-6, and 10 μM KB-R7943 on the proliferation of the BRIN-BD11 cell line (a) or β-cells from mouse islets (b) is shown. Mean ± SEM values are shown for three to four independent experiments with at least 1000 cells per condition analyzed (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (c and d) Effects of 2-week *in vivo* treatment with 5 mg and 20 mg/kg per day KB-R7943 (c) and 0.25 and 1 mg/kg per day YM-244769 (d) on β-cell proliferation of 12-week-old mice are shown. Means ± SEM are shown of three different animals in which at least three pancreatic sections and a minimum of 1000 cells per condition have been analyzed (*$P < 0.05$; **$P < 0.01$). (e) Effect of siRNAs, siNcx1-1, and siNcx1-2 on NCX1 expression in INS-1E cells at the protein level as measured by Western blot analysis. Upper panel is representative figure of four individual experiments. Lower panel is Western blot quantification of four individual experiments (mean ± SEM; **$P <$ 0.01; ***$P < 0.001$). (f) Effects of *Ncx1* inactivation in INS-1E by siRNAs on cell proliferation are shown. Means ± SEM are shown of four individual experiments in which at least 1000 cells per condition have been counted (*$P < 0.05$; **$P < 0.01$).

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

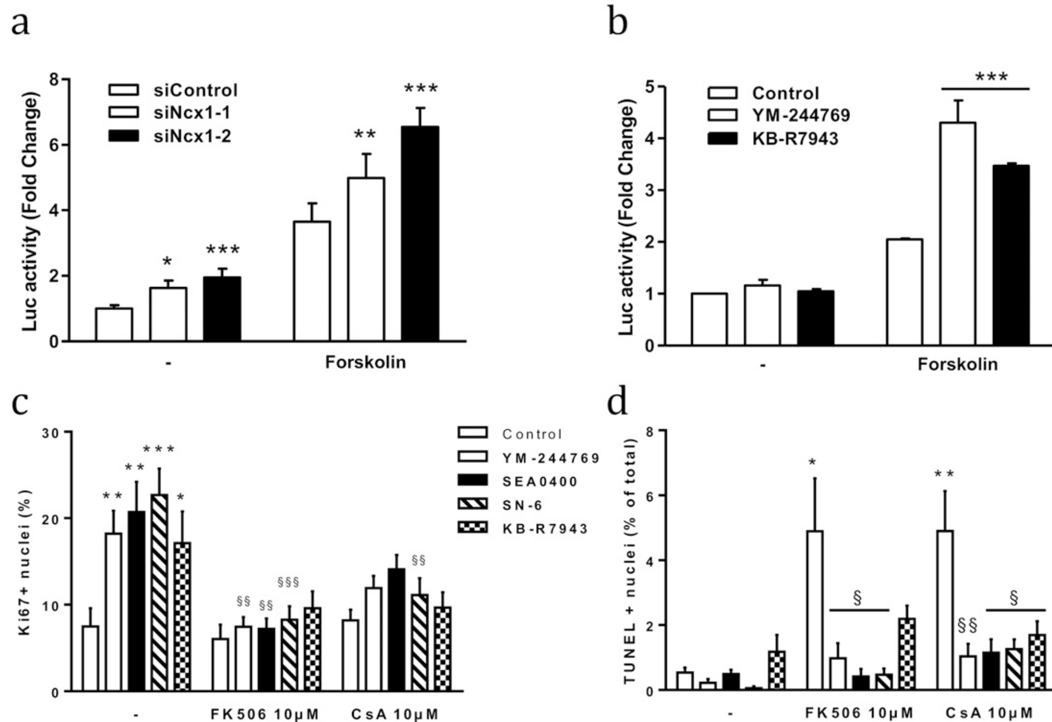

Figure 3. Proliferation and antiapoptotic effects of NCX1 inhibition are mediated via the calcineurin/NFAT pathway. (a and b) The transcriptional activity of NFAT assessed by Dual Luciferase Reporter assay is shown on INS-1E cells transfected with siRNAs targeting the *Ncx1* sequence (a) or treated with the NCX1 inhibitors KB-R7943 or YM-244769 (b) in the absence or presence of 1 μM forskolin. Means ± SEM are shown of four individual experiments (*$P < 0.05$, **$P < 0.01$***$P < 0.001$ vs respective control). (c) The effect of NCX1 inhibitors on BRIN-BD11 cell proliferation is shown in the absence or presence of 10 μM FK-506 or CsA. Means ± SEM are shown of three individual experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs respective control; §§$P < 0.01$ vs control in the absence of FK-506 or CsA). (d) BRIN-BD-11 cell viability measured by using the TUNEL method is shown in the absence or presence of CsA or FK506 and in the simultaneous presence of CsA or FK-506 and NCX1 inhibitors. Means ± SEM are shown of three individual experiments (*$P < 0.05$, **$P < 0.01$ vs respective control; §$P < 0.05$, §§$P < 0.01$ vs control in the presence of FK-506, or CsA). Luc, luciferase.

Immunosuppressive drugs such as CsA and FK-506 exert their immunosuppressive action by inhibiting the calcineurin/NFAT pathway in lymphocytes [17]. However, they are diabetogenic because they inhibit this pathway in neo-transplanted islets and promote β-cell apoptosis [17]. We next examined whether the NCX1 inhibitors could reduce such proapoptotic action. BRIN-BD11 cells were pretreated or not for 6 hours with different NCX1 inhibitors and further exposed for 48 hours to CsA or FK-506. As expected, all NCX1 inhibitors increased β-cell proliferation rate, an effect that was inhibited by FK-506 and CsA, although statistical significance was not reached in all cases (Fig. 3c). Both FK-506 and CsA increased the rate of apoptosis in these cells and all Ncx1 inhibitors reduced their proapoptotic actions (Fig. 3d).

E. Ncx1 *Inactivation Increases the Expression of* β*-Cell–Specific Genes and Increases pRb Phosphorylation*

To better understand the effects of *Ncx1* inactivation on β-cells, we analyzed gene expression levels of key regulators of β-cell proliferation and function in 12-week-old $Ncx1^{+/-}$ mice and wild-type littermates by quantitative RT-PCR. No significant changes were found for the key regulators of β-cell cycle [18] cyclin-dependent kinase 4 (*Cdk4*), cyclinD2 (*CcnD2*), and

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

cyclinD4 (*CcnD4*) (data not shown). However, heterozygous inactivation of *Ncx1* increased the expression of the insulin genes *Ins1*, *Ins2* [19], and *Pdx1*, the main transcription factors for endocrine pancreas development [20] (Fig. 4a). Treatment of INS-1E cells with KB-R7943 also increased the expression of *Ins1*, *Ins2,* and *Pdx1* (Fig. 4b).

Because none of the previously described regulators of the β-cell cycle were transcriptionally regulated by *Ncx1* downregulation, we investigated the phosphorylation of the tumor suppressor pRb [18] by immunofluorescence in 12-week-old mice islets. Figure 4c and 4d shows a higher level of phosphorylated pRb in insulin-positive cells among *Ncx1*$^{+/-}$ as compared with *Ncx1*$^{+/+}$ mice islets ($P < 0.05$).

F. Ncx1 *Heterozygous Inactivation Activates Transcription Factors Involved in* β*-Cell Function and Proliferation*

To confirm the role played by the calcineurin/NFAT pathway and pRb in the effects induced by *Ncx1* inactivation, we examined the activity of related transcription factors in INS-1E cells after transfection of a siRNA against *Ncx1* or after 24-hour treatment with KB-R7943. Figure 4e shows that *Ncx1* inactivation or NCX1 inhibition induced a two- to fourfold increase in E2F activity required for cell cycle progression [21] and decreased activity of the tumor suppressor p53 [21] (Fig. 4d). Moreover, as expected, there was an increased activity in NFAT and in its *trans*-acting factor partner, MEF2 [22].

G. Ncx1 *Heterozygous Inactivation Inhibits the Expression of Tumor Suppressors miR-193a and miR-216a*

miRNAs have been shown to play an important role in β-cell physiology [23]. To identify miRNAs potentially involved in the mechanisms by which *Ncx1* heterozygous inactivation increases β-cell function, proliferation, and mass in adult mice, a global miRNA expression profile analysis was carried out in pancreatic islets from 12-week-old Ncx*1*$^{+/-}$ mice and age-matched wild-type littermates. A total of 1190 miRNAs were analyzed, and the level of 10 miRNAs differed by more than 2.0-fold in *Ncx1*$^{+/-}$ compared with *Ncx1*$^{+/+}$ mice (Supplemental Table 2). However, only downregulation of miR-193a and miR-216a reached statistical significance (fold change: -2.42 and -3.48, respectively; $P < 0.05$) (Fig. 5a). None of the miRNAs already known to be involved in the control of β-cell function and diabetes pathogenesis were differentially expressed [23]. Downregulation of miR-193a and miR-216a was confirmed by quantitative RT-PCR, as shown in Fig. 5b and 5c. To determine whether miR-193a and miR-216a may indeed regulate β-cell proliferation, loss-of-function experiments were carried out in INS-1E cells and in primary β-cells by using specific anti-miR sequences. Both anti-miRs markedly reduced endogenous miRNA levels, as measured by real-time RT-PCR (Fig. 5d and 5e). To assess whether these two miRNAs alter the balance between proliferation and cell death, we first measured apoptosis by TUNEL assay. Repression of miR-193a and miR-216a did not affect apoptosis in INS-1E cells (Fig. 5f). Inhibition of miR-193a but not of miR-216a induced a significant increase in cell proliferation, as measured by using Ki-67 staining in INS-1E cells (Fig. 5g). The ability of anti–miR-193a to stimulate cell proliferation was also confirmed in primary mouse β-cells (Fig. 5h).

Discussion

In previous work, we showed that heterozygous inactivation of the Na^+/Ca^{2+} exchanger in mice leads to various changes in β-cell function that are opposite to the major abnormalities seen in type 2 diabetes [7]. An important effect observed in *Ncx1*$^{+/-}$ mice was an increased proliferation of pancreatic β-cells. We have now extended these experiments and observed that heterozygous inactivation of *Ncx1* and also *Ncx3*, [9] increases β-cell proliferation in very old mice. β-cell proliferation decreases with age in both humans and rodents [16], and there are only very few examples of induction of proliferation in very aged mouse β-cells [16].

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

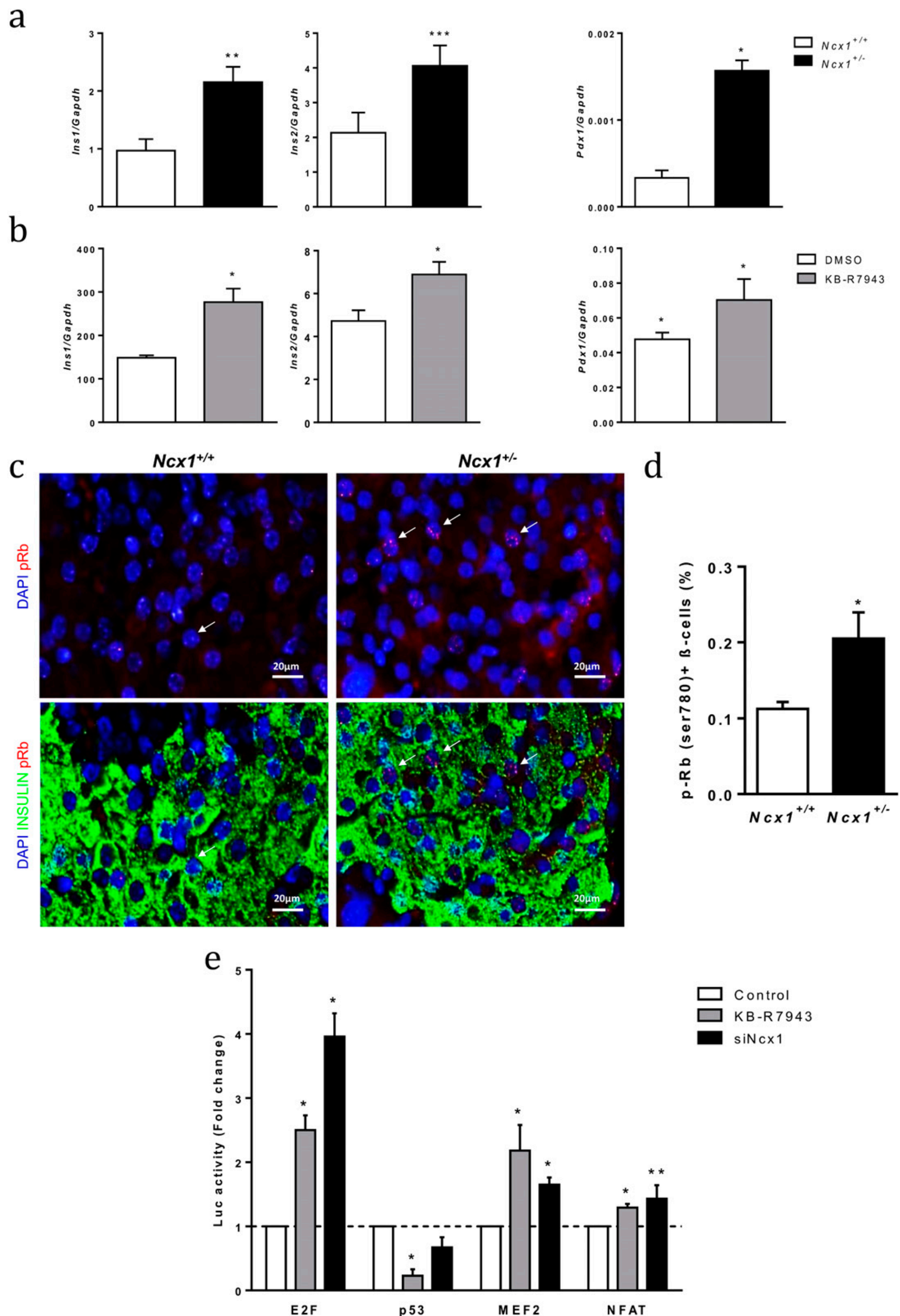

Figure 4. *Ncx1* inhibition/inactivation increases the expression of key regulators of β-cell function and regulates the activity of proteins controlling cell proliferation. (a) Gene expression of *Ins1*, *Ins2,* and *Pdx1* assessed by quantitative RT-PCR is shown in islets from 12-week-old $Ncx1^{+/+}$ and $Ncx1^{+/-}$ mice or (b) in INS-1E treated or not during 24 hours by KB-R7943. Means ± SEM are shown of four individual experiments in each case (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (c & d) The level of phosphorylated pRb assessed by immunofluorescence is

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

shown in insulin-positive cells in islets from 12-week-old *Ncx1*$^{+/-}$ and *Ncx1*$^{+/+}$ mice. (c) Representative image of four 12-week-old pancreas sections in each case. Arrows are showing pRb-positive cells. (d) Quantification of the number of insulin-positive cells where pRb phosphorylation is observed. Means ± SEM are shown of four individual experiments in each case (*$P < 0.05$). (e) Relative luciferase activities of E2F, p53, MEF2, and NFAT transcription factors in INS-1E cells treated with KB-R7943 or transfected with SiNcx1 are shown as means ± SEM of three independent experiments (*$P < 0.05$; **$P < 0.01$). DAPI, 4′,6-diamidino-2-phenylindole; Luc, luciferase.

In the current study we have identified other ways to inhibit the exchanger both *in vitro* and *in vivo* by using small molecules or siRNAs that lead to similar changes in β-cell function and proliferation than heterozygous inactivation of *Ncx1*. We also studied the mechanism of these improvements in β-cell function/proliferation.

Thus, we examined the effects of four NCX1 inhibitors of the benzyloxphenyl family on β-cells. One major potential advantage of this family of drugs over other types of drugs that could also stimulate β-cell proliferation is their specificity of action on β-cells over other tissues. Indeed, a recent study showed that one member of this family of drugs (KB-R7943) is 16 times more potent in inhibiting the Na^+/Ca^{2+} exchanger of the β-cell than that of the heart [24]. This is due to the presence in the splicing zone of the exchanger of the mutually exclusive exon B in β-cells and exon A in cardiac muscle [24]. All four agents inhibited β-cell Na^+/Ca^{2+} exchange in its forward mode. In agreement with our previous studies, the four molecules stimulated β-cell proliferation *in vitro* and *in vivo* without apparent toxicity, as examined in a β-cell line and in mouse islets. The effect of Na^+/Ca^{2+} exchange inhibition on cell proliferation was confirmed by the use of two anti-*Ncx1* siRNAs. Of note, because the four inhibitors have differential activity on NCX1 and NCX3 [12], their action may result from the inhibition of both NCX1 and NCX3.

We then examined the mechanism by which $Na^{+/}Ca^{2+}$ exchange inhibition may stimulate β-cell proliferation. The view that such effect could be mediated by the activation of the calcineurin/NFAT signaling pathway [7] was confirmed as follows. First, anti-*Ncx1* siRNAs and the inhibitors KB-R7943 and YM-244769 increased NFAT transcriptional activity in INS-1E cells. Second, with respect to both β-cell apoptosis and proliferation, Na^+/Ca^{2+} exchange inhibitors and the immunosuppressors CsA and FK-506 behaved as mutual antagonists. Whereas Na^+/Ca^{2+} inhibitors inhibited CsA and FK-506 induced β-cell apoptosis, CsA and FK-506 inhibited the proliferative actions of Na^+/Ca^{2+} inhibitors. As mentioned earlier, *Ncx1* heterozygous inactivation increases (by four- to sevenfold) the ability of mice islets to cure diabetes when transplanted into diabetic animals, probably by increasing their resistance to hypoxia [7], as recently confirmed by the beneficial effect of SEA0400 pretreatment on human islets grafted into NOD mice [25]. Thus, the use of NCX1 antagonists of the benzoxyphenyl family in islet transplantation could be beneficial not only by increasing the resistance of islets to hypoxia during the revascularization process [7] but also by reducing the diabetogenic actions of the immunosuppressive drugs CsA and tacrolimus.

We then looked at other factors that could be implicated in the increase in β-cell proliferation by measuring the activity of related transcription factors. As expected, NFAT activity increased after siRNA and KB-R7943 treatment, together with an increased activity of MEF2. MEF2 is an NFAT *trans*-acting factor partner [22] affecting cell proliferation and tissue differentiation in skeletal and cardiac muscle, neural, and hematopoietic tissues [26].

Cyclins and cyclin-dependent kinases were not upregulated at the mRNA level, but as expected in a phenotype characterized by an increase in proliferation, there was a clear increase in pRb phosphorylation with subsequent rise in E2F activity. The latter was seen both after Ncx1 transcriptional and after translational inhibition. pRb is a tumor suppressor, namely a potent inhibitor of cell cycle progression, and its sequential inactivation by phosphorylation by cyclins/cyclin-dependent kinase complexes is required for the release of a small family of related transcription factors, the E2F transcription factors, which play a major role in the G1/S transition of the cell cycle and subsequent proliferation [18]. β-cells are

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

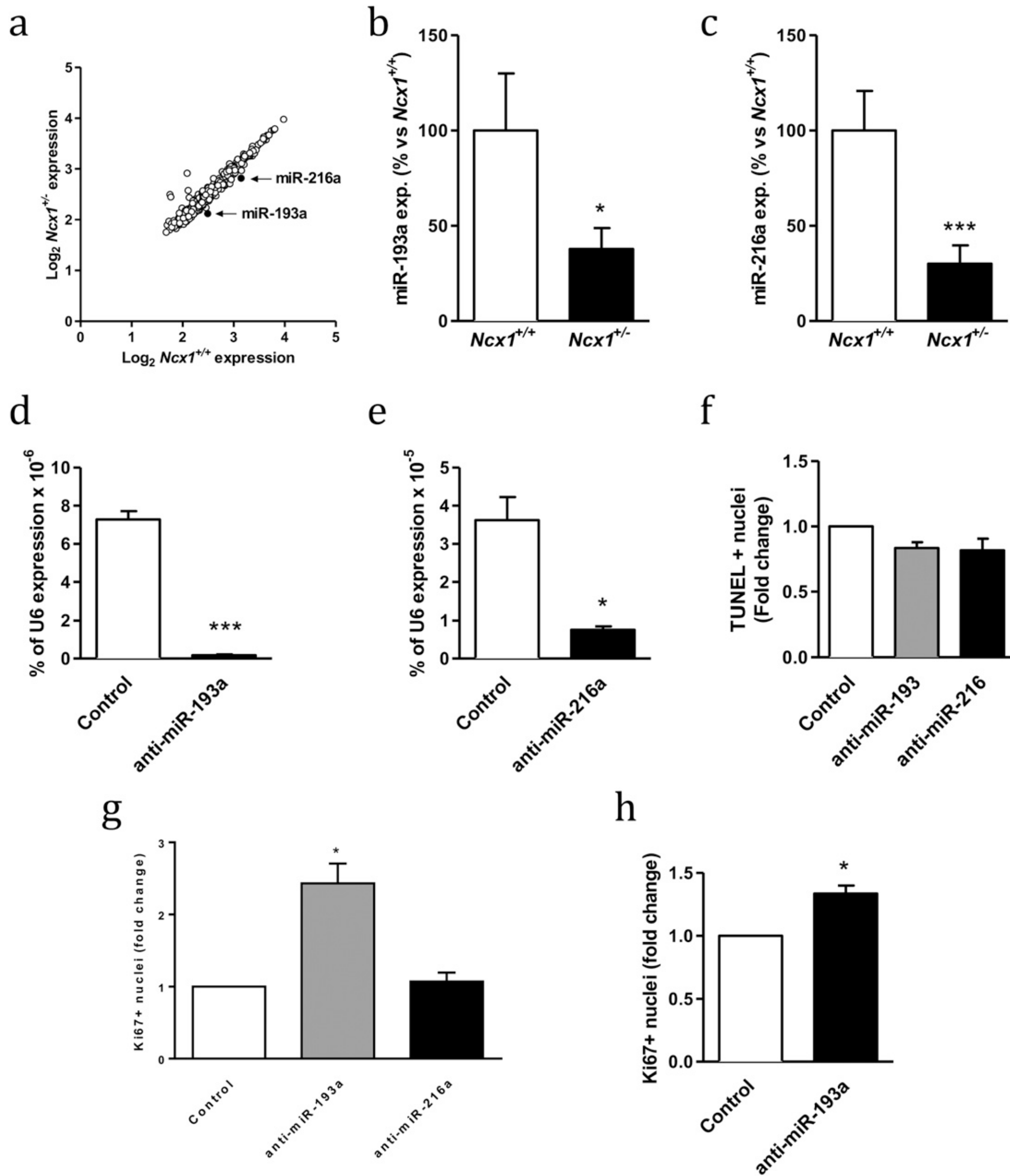

Figure 5. *Ncx1* heterozygous inactivation inhibits miR-193a, a miRNA controlling β-cell proliferation. (a) Global transcription analysis of miRs from islets of 4- to 12-week-old $Ncx1^{+/+}$ (control) and $Ncx1^{+/-}$ mice (n = 4). (b and c) Expression of miR-193a and miR-216a, respectively, was determined by quantitative RT-PCR in islets from 12-week-old $Ncx1^{+/+}$ and $Ncx1^{+/-}$ mice. Means ± SEM of five independent experiments are expressed as percentage of the level of each miR in $Ncx1^{+/+}$ mice (*$P < 0.05$, ***$P < 0.001$ vs $Ncx1^{+/+}$). (d–g) INS-1E cells were transfected with scrambled anti-miR (control) anti–miR-193a or anti–miR-216a. Forty-eight hours after transfection, the levels of miR-193a (d) and miR-216a (e) were assessed by quantitative RT-PCR, or cells were submitted to a TUNEL kit assay to measure apoptosis (f) or stained with a Ki67 antibody for proliferation analysis (g). Means ± SEM are shown of four independent experiments. (h) Dissociated mouse islets cells were transfected with control anti-miR or anti–miR-193a and double-stained for insulin and Ki67. Proliferating insulin-positive cells were counted by immunocytochemistry. Results are the mean ± SEM of four independent experiments (*$P < 0.05$, ***$P < 0.001$ vs control).

slow-proliferating cells with dephosphorylated and active pRb found predominantly into the nucleus [27]. Of note, in view of the effects seen on Rb and E2Fs, proteins that are regulated by upstream cyclins and cyclin-dependent kinases, an upregulation of the latter proteins

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

should be examined in future work by methods other than quantitative RT-PCR. Indeed, cell cycle proteins, notably cyclin-D2, are notorious for their post-transcriptional regulation [21]. There was also a decrease in the cell cycle inhibitor p53, which is an essential regulator of β-cell cycle progression [21]. Likewise, as expected in a phenotype characterized by an increase in β-cell function, there was an increased expression of the insulin genes *Ins1* and *Ins2* [19], and of *Pdx*1, the "master switch" of β-cell development [20].

Another factor that could be involved in the rise in β-cell proliferation was miR-193a, the expression of which was downregulated by *Ncx1* heterozygous inactivation. miR-193a affects cell proliferation acting as tumor suppressor and is known to negatively affect proliferation in different tumor models, including breast cancer, melanoma, oral squamous cell carcinoma, prostate cancer, hepatocellular carcinoma, and acute myeloid leukemia [28].

In summary, the present data show that Na^+/Ca^{2+} exchange is a druggable target to stimulate β-cell proliferation. *Ncx1* inactivation stimulates β-cell proliferation by activating the calcineurin/NFAT pathway, inactivating/downregulating the tumor suppressors pRb and miR-193a, and the cell cycle inhibitor p53. *Ncx1* inactivation also stimulates β-cell function by increasing the expression of *Ins1*, *Ins2*, and *Pdx*1. Hence, specific β-cell inhibition of Na^+/Ca^{2+} exchange by phenoxybenzamyl derivatives may represent an innovative approach to promote β-cell regeneration, survival, and function in type 2 diabetes and improve the efficiency of pancreatic islet transplantation for the treatment of the disease.

Acknowledgments

The authors thank Takahiro Iwamoto (Department of Pharmacology, School of Medicine, Fukuoka University, Fukuoka, Japan) for synthesizing and providing KB-R7943, YM-244769, and SN-6 and Andras Toth (Institute of Pharmacology, University of Szeged, Szeged, Hungary) for synthesizing and providing SEA0400.

A.H. is the guarantor of this study and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Support: The work in A.H.'s group was supported by grants from the Belgian Fund for Scientific Research (FRSM 3.4527.08), the Juvenile Diabetes Research Foundation International (award 17-2011-650), and the Swiss National Science Foundation (310030-146138). The work in A.K.C.'s group was supported by the National Funds from Scientific Research (FNRS; Belgium; T.0107.16), the Excellence of Science (EOS) Fonds National de la Recherche Scientifique/Nationaal Fonds voor Wetenschappelijk onderzoek Belgium (EOS 30826052), and ARC-Belgium 20063.

Current Affiliation: J.P.'s current affiliation is the Prof. Bornstein Group, Diabetes and Nutritional Sciences, King's College London, Rayne Institute, London SE5 9NU, United Kingdom. F.P.Z.'s current affiliation is Université Lille, INSERM, CHU Lille, Institut Pasteur de Lille, Lille F-59000, France.

Author Contributions: J.P., F.P.Z., N.P., C.G. researched data, contributed to discussion, and wrote the manuscript; R.R. and A.K.C. contributed to discussion and reviewed/edited the manuscript; A.H. researched data, contributed to discussion, and wrote the manuscript.

Correspondence: André Herchuelz, PhD, Laboratoire de Pharmacodynamie et de Thérapeutique and Université Libre de Bruxelles Center for Diabetes Research, Faculté de Medecine, CP-618, Route de Lennik 808, 1070 Brussels, Belgium. E-mail: herchu@ulb.ac.be.

Disclosure summary: The authors have nothing to disclose.

References and Notes

1. Utzschneider KM, Kahn SE. β-cell dysfunction in type 2 diabetes. In: DeFronzo RA, Ferrannini E, Keen H, Zimmet P, Eds. International Textbook of Diabetes Mellitus. Hoboken, NJ: John Wiley & Sons, Ltd; 2004:375–388
2. Rahier J, Guiot Y, Goebbels RM, Sempoux C, Henquin JC. Pancreatic β-cell mass in European subjects with type 2 diabetes. *Diabetes Obes Metab*. 2008;**10**(Suppl 4):32–42.
3. Brini M, Calì T, Ottolini D, Carafoli E. Intracellular calcium homeostasis and signaling. *Met Ions Life Sci.* 2013;**12**:119–168.

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

4. Wollheim C, Maechler P. β-cell biology of insulin secretion. In: DeFronzo R, Ferrannini E, Keen H, Zimmet P, Eds. International Textbook of Diabetes Mellitus. Hoboken, NJ: John Wiley & Sons, Ltd; 2004:125–138.
5. Brini M, Carafoli E. Calcium pumps in health and disease. *Physiol Rev*. 2009;**89**(4):1341–1378.
6. Herchuelz A, Nguidjoe E, Jiang L, Pachera N. β-Cell preservation and regeneration in diabetes by modulation of β-cell Ca^{2+} homeostasis. *Diabetes Obes Metab*. 2012;**14**(Suppl 3):136–142.
7. Nguidjoe E, Sokolow S, Bigabwa S, Pachera N, D'Amico E, Allagnat F, Vanderwinden J-M, Sener A, Manto M, Depreter M, Mast J, Joanny G, Montanya E, Rahier J, Cardozo AK, Eizirik DL, Schurmans S, Herchuelz A. Heterozygous inactivation of the Na/Ca exchanger increases glucose-induced insulin release, β-cell proliferation, and mass. *Diabetes*. 2011;**60**(8):2076–2085.
8. Pachera N, Papin J, Zummo F-P, Rahier J, Mast J, Meyerovich K, Cardozo AK, Herchuelz A. Heterozygous inactivation of plasma membrane Ca^{2+}-ATPase in mice increases glucose-induced insulin release and β-cell proliferation, mass and viability. *Diabetologia*. 2015;**58**(12):2843–2850.
9. Sokolow S, Manto M, Gailly P, Molgó J, Vandebrouck C, Vanderwinden JM, Herchuelz A, Schurmans S. Impaired neuromuscular transmission and skeletal muscle fiber necrosis in mice lacking Na/Ca exchanger 3. *J Clin Invest*. 2004;**113**(2):265–273.
10. Kamagate A, Herchuelz A, Van Eylen F. Plasma membrane $Ca^{(2+)}$-ATPase overexpression reduces $Ca^{(2+)}$ oscillations and increases insulin release induced by glucose in insulin-secreting BRIN-BD11 cells. *Diabetes*. 2002;**51**(9):2773–2788.
11. Cardozo AK, Ortis F, Storling J, Feng Y-M, Rasschaert J, Tonnesen M, Van Eylen F, Mandrup-Poulsen T, Herchuelz A, Eizirik DL. Cytokines downregulate the sarcoendoplasmic reticulum pump Ca^{2+}-ATPase 2b and deplete endoplasmic reticulum Ca^{2+}, leading to induction of endoplasmic reticulum stress in pancreatic beta-cells. *Diabetes*. 2005;**54**(2):452–461.
12. Takahiro Iwamoto. Na^+/Ca^{2+} exchange as a drug target. Insights from molecular pharmacology and and genetic engineering. In: Herchuelz A, Blaustein MP, Lytton J, Philipson KD, eds. Sodium-Calcium Exchange and the Plasma Membrane Ca^{2+}-ATPase in Cell Function. 5th International Conference. Ann NY Acad Sci. 2007;1099:516–528.
13. Montanya E, Téllez N. Pancreatic remodeling: beta-cell apoptosis, proliferation and neogenesis, and the measurement of beta-cell mass and of individual beta-cell size. *Methods Mol Biol*. 2009;**560**: 137–158.
14. Santin I, Moore F, Grieco FA, Marchetti P, Brancolini C, Eizirik DL. USP18 is a key regulator of the interferon-driven gene network modulating pancreatic beta-cell inflammation and apoptosis. *Cell Death Dis*. 2012;**3**(11):e419.
15. Van Eylen F, Bollen A, Herchuelz A. NCX1 Na/Ca exchanger splice variants in pancreatic islet cells. *J Endocrinol*. 2001;**168**(3):517–526.
16. Bender A, Stewart AF. Good news for the ageing beta-cell. *Diabetologia*. 2014;**57**(2):265–269.
17. Heit JJ. Calcineurin/NFAT signaling in the β-cell: From diabetes to new therapeutics. *BioEssays*. 2007; **29**(10):1011–1021.
18. Fiaschi-Taesch NM, Kleinberger JW, Salim FG, Troxell R, Wills R, Tanwir M, Casinelli G, Cox AE, Takane KK, Scott DK, Stewart AF. Human pancreatic β-cell G1/S molecule cell cycle atlas. *Diabetes*. 2013;**62**(7):2450–2459.
19. Poitou V, Stein R, Rhodes CJ. Insulin gene expression and biosynthesis. In: DeFronzo R, Ferrannini E, Keen H, Zimmet P, eds. *International Textbook of Diabetes Mellitus*. Hoboken, NJ: John Wiley & Sons Ltd; 2004:97–123.
20. Serup P, Nielsen JH. Development and life cycle of a β-cell. In: DeFronzo R, Ferrannini E, Keen H, Zimmet P, eds. *International Textbook of Diabetes Mellitus*. Hoboken, NJ: John Wiley & Sons, Ltd; 2004:59–78.
21. Kulkarni RN, Mizrachi E-B, Ocana AG, Stewart AF. Human β-cell proliferation and intracellular signaling: driving in the dark without a road map. *Diabetes*. 2012;**61**(9):2205–2213.
22. Demozay D, Tsunekawa S, Briaud I, Shah R, Rhodes CJ. Specific glucose-induced control of insulin receptor substrate-2 expression is mediated via Ca^{2+}-dependent calcineurin/NFAT signaling in primary pancreatic islet β-cells. *Diabetes*. 2011;**60**(11):2892–2902.
23. Guay C, Jacovetti C, Nesca V, Motterle A, Tugay K, Regazzi R. Emerging roles of non-coding RNAs in pancreatic β-cell function and dysfunction. *Diabetes Obes Metab*. 2012;**14**(Suppl 3):12–21.
24. Hamming KSC, Soliman D, Webster NJ, Searle GJ, Matemisz LC, Liknes DA, Dai XQ, Pulinilkunnil T, Riedel MJ, Dyck JR, Macdonald PE, Light PE. Inhibition of β-cell sodium-calcium exchange enhances glucose-dependent elevations in cytoplasmic calcium and insulin secretion. *Diabetes*. 2010;**59**(7): 1686–1693.

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018

25. Mera T, Itoh T, Kita S, Kodama S, Kojima D, Nishinakamura H, Okamoto K, Ohkura M, Nakai J, Iyoda T, Iwamoto T, Matsuda T, Baba A, Omori K, Ono J, Watarai H, Taniguchi M, Yasunami Y. Pre-treatment of donor islets with the Na^{+}/Ca^{2+} exchanger inhibitor improves the efficiency of islet transplantation. *Am J Transplant*. 2013;**13**(8):2154–2160.
26. Pon JR, Marra MA. MEF2 transcription factors: developmental regulators and emerging cancer genes. *Oncotarget*. 2016;**7**(3):2297–2312.
27. Sachdeva UM, O'Brien JM. Understanding pRb: toward the necessary development of targeted treatments for retinoblastoma. *J Clin Invest*. 2012;**122**(2):425–434.
28. Nakano H, Yamada Y, Miyazawa T, Yoshida T. Gain-of-function microRNA screens identify miR-193a regulating proliferation and apoptosis in epithelial ovarian cancer cells. *Int J Oncol*. 2013;**42**(6): 1875–1882.

Downloaded from https://academic.oup.com/jes/article-abstract/2/7/631/5003403
by Bibliotheque Cantonale et Universitaire user
on 15 August 2018