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Activation of the Wnt/β-catenin pathway in pancreatic beta cells during the compensatory islet hyperplasia in prediabetic mice

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ABSTRACT

The Wnt/β-catenin signaling pathway, also known as the canonical Wnt pathway, plays a role in cell proliferation and differentiation in several tissues/organs. It has been recently described in humans a relationship between type 2 diabetes (T2DM) and mutation in the gene encoding the transcription factor *TCF7L2* associated to the Wnt/β-catenin pathway. In the present study, we demonstrated that hyperplastic pancreatic islets from prediabetic mice fed a high-fat diet (HFD) for 60 d displayed nuclear translocation of active β-catenin associated with significant increases in protein content and gene expression of β-catenin as well as of cyclins D1, D2 and c-Myc (target genes of the Wnt pathway) but not of *Tcf7l2* (the transcription factor). Meanwhile, these alterations were not observed in pancreatic islets from 30 d HFD-fed mice, that do not display significant beta cell hyperplasia. These data suggest that the Wnt/β-catenin pathway is activated in pancreatic islets during prediabetes and may play a role in the induction of the compensatory beta cell hyperplasia observed at early phase of T2DM.

1. Introduction

The pathogenesis of type 2 diabetes mellitus (T2DM) involves the development of an intolerance to glucose and a peripheral resistance to insulin that can be partially compensated by insulin hypersecretion and/or pancreatic beta cell mass expansion [1–3] The increase in beta cell mass occurs mainly during the prediabetes phase and as consequence of hypertrophy and hyperplasia of this cell type [1,4] Hyperplasia, in turn, is result of beta cell self-replication and/or neogenesis from other pancreatic cells [5,6]. The mechanisms linking obesity and insulin resistance to beta cell hyperplasia are not fully known.

The Wnt/β-catenin signaling pathway has been reported to be involved in cell growth and differentiation in several tissues/organs, including the endocrine pancreas [7–9]. Wnts, belonging to the Wnt family of secreted glycoproteins, interact with its receptor, known as Frizzled, and co-receptor, called LRP5/6 (low-density lipoprotein related receptor proteins 5 and 6), at the membrane surface of the target cell and trigger a cascade reaction resulting in β-catenin accumulation in the cytoplasm (reviewed by Refs. [8,10]). This cytoplasmic β-catenin pool is shifted to the nucleus where interacts with the transcription factor TCF/LEF (specific T Cell Factor/Lymphoid Enhancer-binding Factor) and activates the expression of several target genes related to cell proliferation and survival [8,10]. In the absence of Wnt stimulation, the cytoplasmic level of β-catenin is kept low by the interaction with the adhesion molecule cadherin at intercellular adhesion sites as well as by the proteosomal degradation triggered by the phosphorylation of β-catenin by the APC/Axin/CK1/GSK-3β complex as reviewed by Refs. [8,9].

Evidences for the implication of canonical Wnt pathway in beta cell proliferation and function come mainly from experiments *in vitro*, using beta cell lineages [11–13], or *in vivo* models, employing genetically modified rodents [14–16]. Nevertheless, *in vivo* studies, particularly those employing *Tcf7l2*-deficient mice, have gathered conflicting results concerning the role of this Wnt effector on beta cell function [14,16]. Yet, a putative role of the Wnt

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signaling pathway in the beta cell plasticity during type 2 prediabetes has not been directly addressed.

In this work, we investigated a possible activation of the Wnt/β-catenin signaling pathway in the compensatory beta cell hyperplasia in a model of type 2 prediabetes [4]. For that, C57BL/6 male mice were fed a high-fat diet (HFD) for different periods of time (30 or 60 days), which differ regarding the stage of the prediabetes (i.e. before and after the beta cell mass expansion is established, respectively). We firstly characterized metabolically and morphometrically our model and, then investigated the pancreatic islet cell distribution, content and gene expression of some proteins associated to the Wnt/β-catenin pathway (i.e β-catenin, *TCF7L4*, cyclins D1 and D2, c-Myc) in both control (fed a chow diet) and HFD mice.

2. Material and methods

2.1. Animal treatment and metabolic evaluation

Male C57BL/6/JUnib mice were obtained from the breeding colonies maintained at the University of Campinas (UNICAMP, Brazil). The animals were housed at 25 ± 1 °C on a 12 h light/12 h dark cycle, and had free access to water and food. When aged 4–5 months (with a body weight mean of 30.1 ± 0.16 g), the animals were divided in two groups. The treated group was fed a high-fat and hypercaloric diet (HFD) for 30 days (HFD 30 d) or for 60 days (HFD 60 d) containing 21 g% lipids (mainly lard) (w/w), 50 g% carbohydrates and 20 g% proteins (4.7 kcal/g). The control group (CTL) was fed a standard rodent diet containing 4.5 g% lipids (w/w), 53 g% carbohydrates and 23 g% proteins (2.9 kcal/g) for the same periods of time. All animals were weighed before starting the diet period and at the end of it; values were expressed as percentage of body weight gain over the initial body weight. The measurement of fast and post-prandial glycemia, insulinemia and response to the insulin tolerance test (ITT; values expressed as area under curve (AUC)) were done as previously described [4,17]. Insulin concentration in plasma samples was measured using the Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem Inc.). All blood samples for all biochemical analysis were collected between 9:00 and 11:00 a.m. All experimental protocols used were approved by the Ethics Committee on Animal Use (CEUA) of UNICAMP under protocols #2815-1 and #3443-1).

2.2. Immunohistochemistry for insulin and pancreas morphometry

Pancreas were fixed for 18 h in 4% paraformaldehyde (in 0.05 M Tris-buffered saline, TBS, pH 7.4), sectioned in 3 fragments of similar sizes and separately embedded in paraffin. Two sections per block were randomly selected and processed for insulin immunoperoxidase reaction as previously described [4,17,18]. All islets and pancreas sections were photographed with a digital camera coupled to a Nikon Elipse E800 microscope. The *relative area of the beta cell* was determined by dividing the sum of the total areas of the insulin-positive cells (beta cells) by the total area of the pancreas section, that was measured using the free software ImageTool (http://ddsdx.uthscsa.edu/dig/itdesc.html).

2.3. Immunolocalization of active β-catenin in mouse pancreas cryosections

The cell localization of β-catenin was performed in −20 °C acetone-fixed pancreas cryosections by triple labeling of active β-catenin, insulin (by indirect immunofluorescence) and DAPI (4′,6-diamidino-2-phenylindole). For that, the pancreas sections were 2 h-incubated with a primary antibody that detects only the active (unphosphorylated) form of the β-catenin (see Table S1 in supplementary material) at room temperature (RT). After washings with TBS, the pancreas sections were incubated with their respective secondary antibody conjugated with FITC for 2 h at RT. For co-immunolabeling with insulin, the sections were incubated sequentially with an anti-insulin antibody followed by their specific secondary antibody conjugated with TRITC and DAPI (dilution 1:1000, Sigma) [19]. All sections were mounted in a commercial antifading agent (Vectashield, Vector Laboratories) and observed using an inverted fluorescence microscope coupled to an image capture system (Observer-Z1; Zeiss - AxioCam MRC). Pancreas sections from control and HFD-fed mice were analyzed and photographed during the same session using identical parameters of the fluorescence microscopy. The nuclear accumulation of active β-catenin in beta cells was determined by detecting the co-labeling of β-catenin and DAPI using the GIMP free software (http://www.gimp.org/). The values obtained were normalized against the area of the islet using the free software ImageJ (http://rsbweb.nih.gov/ij/), and expressed as pixels/islet area. Table S1 (Supplementary material) displays all the antibodies and the respective dilutions employed in immunohistochemistry and Western Blot (as described below).

2.4. Pancreatic islet isolation

Each pancreas was inflated by injecting 3 mL of 0.8 mg/mL type V collagenase (Sigma, Cat#C9263) in Hank's balanced salt solution (supplemented with 3 mg/mL bovine serum albumin plus 5.6 mM glucose, pH 7.4) and added to 2 mL of this collagenase solution (totalizing 4 mg of the collagenase/pancreas). Each pancreas was then incubated for 10 min at 37 °C for enzymatic digestion. Islets were then isolated in gradient of Histopaque®1077 (Sigma) and individually collected under a dissecting microscope. Pools of 300 isolated islets were homogenized in an anti-protease cocktail for immunoblotting or added to RNAlater® (Ambion) for Real Time-PCR as previously described [19,20].

2.5. Western Blot

Aliquots of islet homogenates containing 50 μg total protein were applied to 8 or 10% SDS-PAGE gel and then the samples were fractionated by electrophoresis as previously described [17,19]. Membranes were blocked with 5% dry skimmed milk in TBS containing 0.05% Tween20 (TTBS), overnight at 4 °C, and then incubated for 2 h at RT with one of the primary antibodies depicted in Table S1 (Supplementary material), followed by incubation with their respective secondary antibody conjugated with HRP. After washings, the membranes were revealed using an enhanced chemiluminescence kit (Chemiluminescent Substrate, Pierce), followed by autoradiography (Amersham) or detection using a chemiluminescence imaging system (GeneGnome XRQ, SynGene). Band intensities were quantified by optical densitometry using software ImageJ and normalized against β-actin band densitometry (internal control).

2.6. Quantitative real-time PCR

Total RNAs were extracted from pools of 300 isolated islets using the RNAqueous kit (Ambion) before being reverse-transcribed into cDNAs. Quantification of mRNAs encoding

Ctnnb1, *Ccnd1*, *Ccnd2*, *c-Myc*, *Tcf7l2*, *Ins2*, *Gapdh*, *Actb1* and *Rps29* was performed using the 7500 ABI system. The primers used in this study are shown in Table S2 in supplementary material. The specificities of amplifications were verified by size characterization of the amplification products on 2% agarose gel and by melting-curve analyses. The absolute quantities of target transcripts were normalized against the endogenous control *Gapdh* [21], that was demonstrated to be the best internal control for the experimental conditions tested in comparison with *Actb1* and *Rps29* (data not shown).

2.7. Statistical analyses

All numerical results were expressed as means ± standard error of the mean (SEM). For comparison between two groups, statistical significance was assessed using Student's *t*-test (two-tailed). The significance level was set at $p < 0.05$. All statistical analyses were performed using the GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, USA).

3. Results

HFD exposure for 30 d or 60 d induced significant weight gain above the control levels (HFD 30 d 17.7± 2.8% (25) *vs* CLT 30 d 5.3± 0.8% (25); HFD 60 d 43.3± 2.1% (63) *vs* CLT 60 d 9.1 ± 1.3% (52)); however, only treatment for 60 d with this diet resulted in metabolic alterations typical of prediabetes such as a marked insulin peripheral resistance (as revealed by the increased ITT AUC; Fig. 1j) associated with fast and fed hyperglycemia (Fig. 1f,g) and fast and fed hyperinsulinemia (Fig. 1h,i). Meanwhile, 30 d-exposure to HFD resulted only in an increased postprandial glycemia (Fig. 1b) and postprandial insulinemia (Fig. 1d) without significant changes in the other metabolic parameters analyzed (Fig. 1a,c,e).

The metabolic alterations induced by 60 d HFD exposure were accompanied by a significant increase in the relative volume of pancreatic beta cells (Fig. 3a) which was not observed in the pancreas from 30 d HFD-fed mice (Fig. 2a). This result is in accordance with previous works [4,17], which also showed that the beta cell expansion seen in 60 d HFD-fed mice was mainly result of hyperplasia due to beta cell replication.

In the next step of this study, we verified the degree of activation of the canonical Wnt pathway in pancreatic islets at these two distinct stages of prediabetes in mice: when the presence of hyperplastic pancreatic islets (Fig. 3a) associated with beta cell expansion (as seen after 60 d HFD exposure) is established and when no significative morphometric changes in beta cell mass are detected (as seen in 30 d HFD-fed mice) (Fig. 2a).

As shown in Fig. 2, no alterations of the immunolocalization of active β-catenin (which was immunodetected mainly at the intercellular region and cytoplasm of islet cells) (Fig. 2b), neither in the protein cell content (c) or gene expression of β-catenin (d) were seen in islet homogenates of 30 d HFD-fed mice. In contrast, hyperplastic islets from 60 d HFD-fed mice showed nuclear translocation of active β-catenin (Fig. 3c) accompanied by an increase in the islet content of this protein (Fig. 3d) and β-catenin mRNA transcripts (Fig. 3f). In addition, homogenates of islets isolated from these prediabetic mice displayed an increment in protein islet content of cyclin D1/2 (Fig. 3e) as well as an increase in gene expression of cyclins D1 (Fig. 3g) and D2 (Fig. 3h), c-Myc (Fig. 3i) and insulin 2 (Fig. 3j), that have been shown to be target genes of the Wnt pathway [11,13,15,22], but not of TCF7L2, the transcription factor (Fig. 3k).

4. Discussion

Pancreatic beta cells play a critical role in the pathogenesis of T2DM being capable of an adaptive response that involves

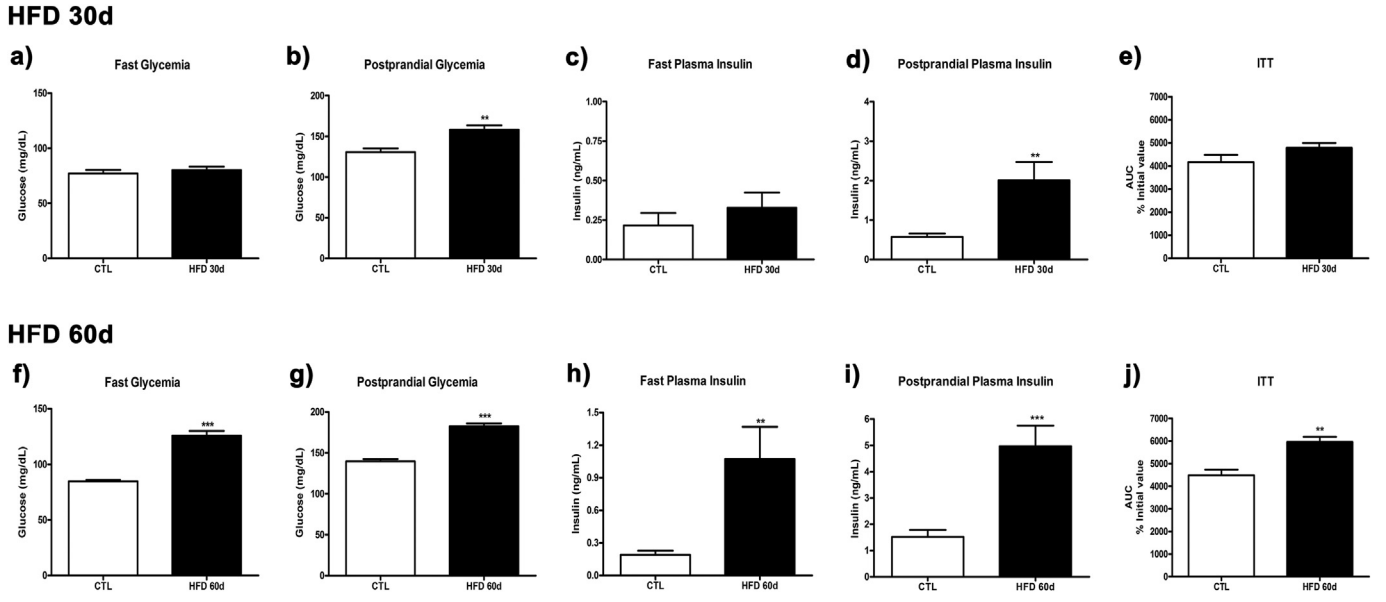

Fig. 1. Exposure to high-fat diet (HFD) for 60 days, but not for shorter period (30 days), induces metabolic alterations indicative of prediabetes. As metabolic parameters, we have measured the fast (**a** and **f**) and postprandial glycemia (**b** and **g**), the fast (**c** and **h**) and postprandial insulinemia (**d** and **i**), and the response to the insulin tolerance test (ITT) (shown as AUC of ITT curve, **e** and **j**). All values represent the means + SEM (n = 7–35 animals/group). **p < 0.001 or **p < 0.005 and ***p < 0.0001 as compared to its respective control (CTL) fed a regular diet (Student's *t*-test).

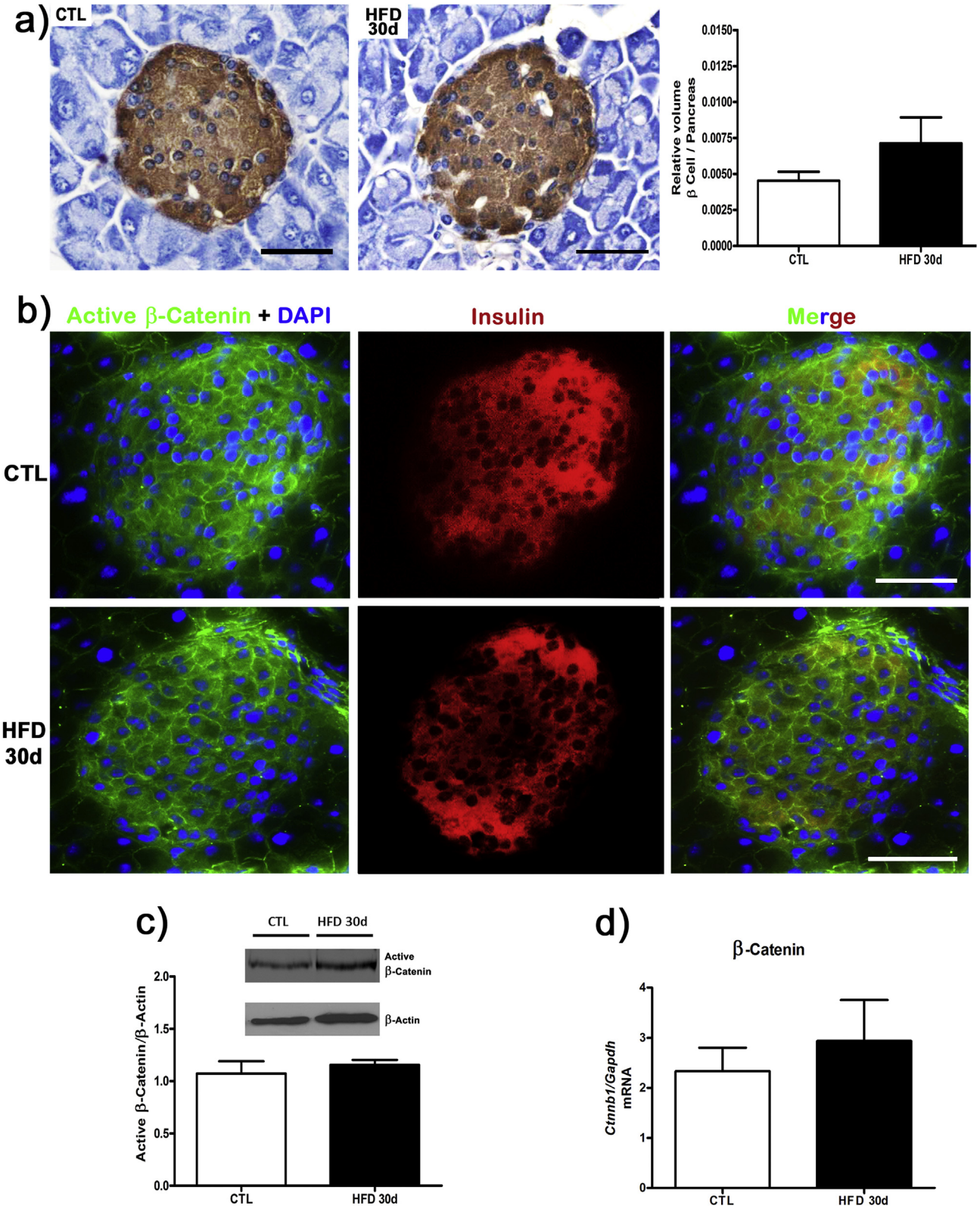

Fig. 2. Exposure to HFD for 30 days induces no changes in relative beta cell volume neither in β-catenin location and expression in pancreatic islets. Panel (**a**) depicts islet sections processed for insulin immunoperoxidase. Morphometric analysis showed no significant difference in relative beta cell volume between the control (CTL) and 30 d HFD groups. All values represent the means + SEM. Data were obtained from six pancreas sections from each animal (n = 4 animals/group) and a total of 240–259 islets were analyzed per group. Panel (**b**) shows photomicrographs of dual immunofluorescence for active β-catenin (green) and insulin (red) plus DAPI (blue) in CTL and HFD 30 d mice. Active β-catenin was found at intercellular region and in the cytoplasm of islet cells similarly in all groups. No differences in protein (**c**) and gene expression (**d**) of β-catenin were seen in islet homogenates from HFD 30 d group and the control. Data in **c** represent means + SEM of 5 membranes from 5 independent experiments. Data in **d** represent means + SEM of 5 islet pools/groups. Scale bars in **a** and **b**, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

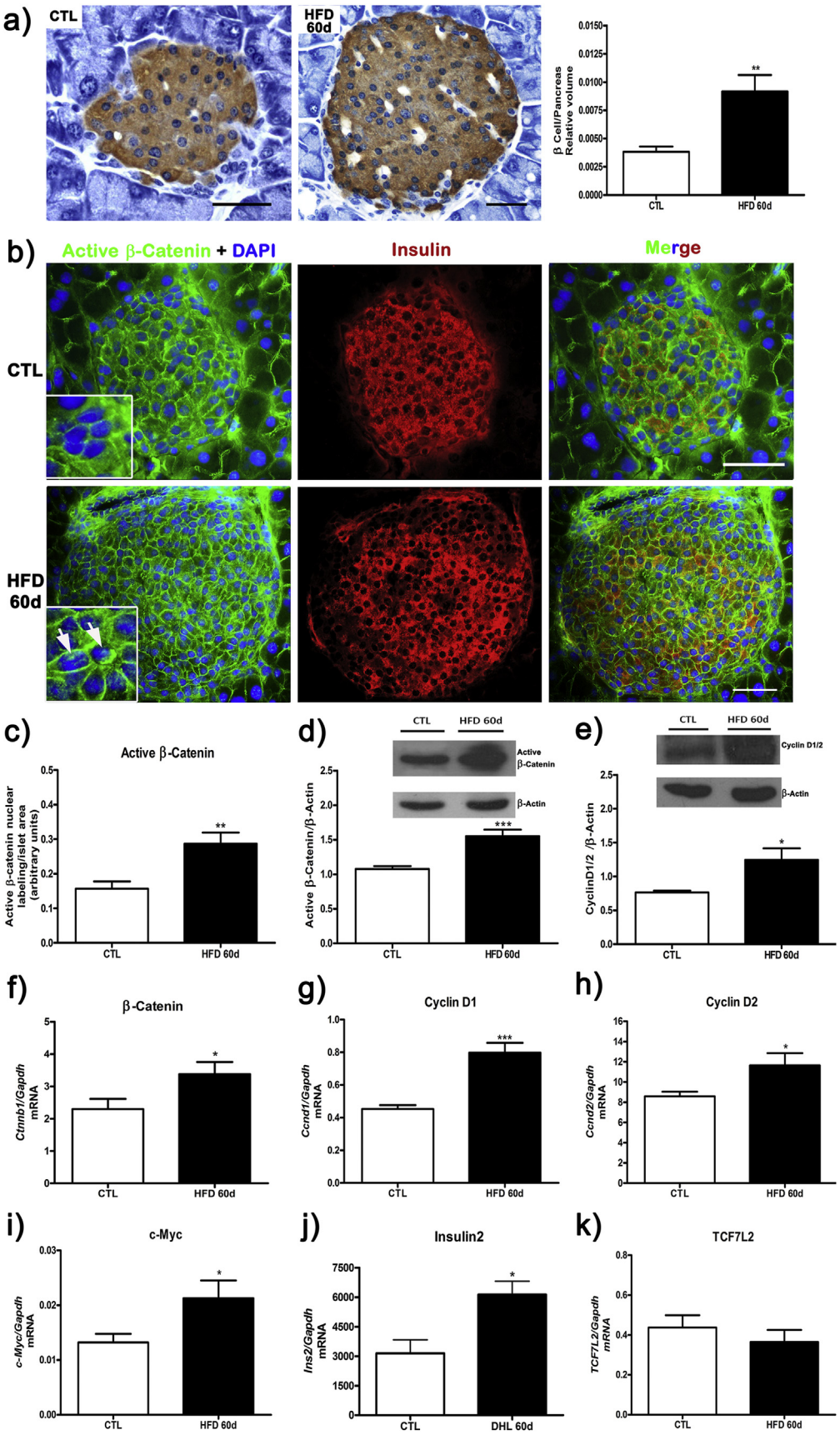

Fig. 3. Prediabetic mice, exposed to HFD for 60 days, display beta cell expansion associated with activation of the Wnt/β-catenin signaling pathway in pancreatic islets. Panel (**a**) depicts islet sections processed for insulin immunoperoxidase. Morphometric analysis showed significant increase in relative beta cell volume in HFD 60 d group, in comparison with the control group (CTL) (**$p < 0.008$), which is indicative of beta cell mass expansion. All values represent the means + SEM. Data were obtained from six pancreas sections

changes in their secretory function and mass to compensate for the peripheral resistance to insulin in rodents and humans [1–6,23–25]. Initially, beta cells increase the biosynthesis and release of insulin and then an expansion of beta cell mass usually occur to promote a further increase in insulin plasma concentration required to maintain normoglycemia [1–6,23,24]. Later, due to a continuously high insulin demand as result of the peripheral resistance to this hormone, beta cells gradually enter in functional failure and death, leading to a permanent hyperglycemic state which requires then exogenous insulin administration [1,3,23,25,26].

There has been an increasing interest in studying intracellular pathways involved in pancreatic beta cell proliferation and secretory function in order to apply this knowledge in molecular and cellular therapies for diabetes [2,24]. The canonical Wnt signaling pathway, well known as the Wnt/β-catenin pathway, has been investigated as inductor of cell proliferation and differentiation in several tissues/organs during embryonic development and in adults. Mutations in members of the Wnt pathway are invariably linked to human congenital defects and some diseases such as cancer [27,28].

A role of the canonical Wnt signaling pathway in the endocrine pancreas development and physiology was firstly reported by Rulifson and co-workers [12]. They showed that perinatal activation of β-catenin in beta cells of bitransgenic RIP-Cre, β-cat^{active}mice, lead to an increased proliferation of this cell type while overexpression of Axin had an opposite effect in transgenic mice [12]. Several *in vitro* studies have reinforced this result [11–13]. Employing different beta cell lineages (i.e. MIN6, NIT-1beta, INS-1 cells) and isolated islets, they demonstrated that the activation of the Wnt/β-catenin pathway induced by exposure to synthetic Wnt3a or Wnt-enriched culture media resulted in significant beta cell proliferation as well as an enhancement of the glucose-stimulated insulin secretion [11–13]. However, loss-of-function studies in adult rodents have yielded some controversial data [14,16,29,30]. Conditional knocking-out of proteins associated to activation of the canonical Wnt pathway (i.e. LRP5, TCF4, β-catenin) was shown to have no repercussion in beta cell mass and insulin secretion [14,29] or to impact negatively on beta cell proliferation and secretory function [16,30]. Considering the problems underlying the use of gene knock-out and knock-in technologies, whose results should be therefore interpreted with some caution [31–33], we have taken a different approach to investigate whether the canonical Wnt pathway is activated during the compensatory beta cell mass expansion associated to type 2 prediabetes. By employing HFD-fed mice at two different stages of diabetes, i.e. before (30 days) and after (60 days) the establishment of a significant insulin resistant state, we decided to check whether an increased beta cell mass was associated with cell signs that are typically described in the literature as related to canonical Wnt pathway activation, such as nuclear translocation of β-catenin and increased expression of key proteins of this signaling pathway [34–37].

Our data clearly showed that the expansion of beta cell mass associated with the establishment of a prediabetic state, as seen in 60 d high fat diet-fed mice, was parallelled by β-catenin translocation to beta cell nuclei, as well as by a significant increase in β-catenin expression at gene and protein (in its active form) levels, which are indicative of canonical Wnt pathway activation. In contrast, mice treated for only 30 days with this high-fat diet did not show either significant changes in beta cell mass or in β-catenin location and expression in pancreatic islets. In addition, isolated islets from 60 d HFD-fed mice displayed increased expression of target genes of Wnt pathway related to beta cell proliferation and differentiation, such as *Ccnd1* [13,36,38], *Ccnd2* [11,12,15], *c-Myc* [35,38], and *Ins2* [22], but showed no significant changes in *Tcf7l2* expression, the major effector of the pathway. Although *Tcf7l2* seems to be a target gene of Wnt signaling *in vitro* [11], *in vivo* studies have reported a decrease in *Tcf7l2* islet expression at hyperinsulinemic state or after insulin exposure [39], indicating that the increase in *Tcf7l2* levels, usually used as a marker of Wnt pathway activation, should be revised.

Taken all together, our results indicate that Wnt/β-catenin pathway is activated in pancreatic islets during prediabetes induced by high-fat diet in mice. In accordance with our data, Mitchell and co-workers [16] have interestingly shown that conditional *Tcf7l2*-null mice fed a high-fat diet showed a significant reduction in beta cell mass in comparison with wild-type mice treated with the same diet. Nevertheless, the interpretation of their results allows the following considerations: 1) the authors have not compared these animals fed a high-fat diet with their respective control mice (fed a regular diet) to verify whether they were actually prediabetics and whether the beta cells were actively proliferating as consequence; and 2) they described that the *Tcf7l2*-null mice displayed significant decrease in the gene expression of GLP1r and insulin 2, that can interfere with beta cell mass independently of Wnt signaling [1–3]. To the best of our knowledge, our study for the first time addresses a possible role of the Wnt signaling in the induction of the compensatory beta cell hyperplasia observed at early phase of T2DM.

Future investigation will be necessary to determine the Wnt ligand(s) and its (their) source (autocrine, paracrine or endocrine) responsible for this beta cell expansion associated to the prediabetic condition.

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from each animal (n = 5 animals/group) and a total of 296–399 islets were analyzed per group. Panel (**b**) shows photomicrographs of dual immunofluorescence for active β-catenin (green) and insulin (red) plus DAPI (blue) in CTL and HFD 60 d mice. An increase in the co-localization of active β-catenin and the nuclear marker DAPI (inset), indicative of translocation of this protein to the nucleus (arrow), was detected in beta cells of prediabetic mice (HFD 60 d group) (**b**) as compared to the control (CLT), and confirmed quantitatively (**c**) (50–51 islets/group) (**$p < 0.008$). This was associated with a significant increase in active β-catenin islet content (**d**) (***$p < 0.001$) and in *Cnntb1* gene expression (**f**) (*$p < 0.05$) in HFD 60 d mice as compared to the control. In addition, HFD 60 d islets display significant increase in protein content of Cyclin D1/2 (e) (*$p < 0.02$) as well as in gene expression of *Ccnd1* (g) (***$p = 0.0002$), *Ccnd2* (h) (*$p < 0.05$), *c-Myc* (i) (*$p < 0.05$) and *Ins2* (j) (*$p < 0.05$), but not of *Tcf7l2* (k), relative to CTL. Data in **d** and **e** represent means + SEM of 5–6 membranes from 5 independent experiments. Data in **f** to **j** represent means + SEM of 5–7 islet pools/group. Scale bars in **a** and **b**, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.08.146.

Transparency document

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