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Wnt3a Regulates Proliferation, Apoptosis and Function of Pancreatic NIT-1 Beta Cells Via Activation of IRS2/PI3K Signaling

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ABSTRACT

Wnt-signaling pathway is implicated in pancreatic development and functional regulation of mature beta-cells. Wnt3a/Wnt pathway activation expands islet cell mass in vitro by increasing proliferation and decreasing apoptosis of beta-cells, thereby enhancing its function. However, the signaling pathways that mediate these effects remain unknown. By using a clonal beta-cell line (NIT-1), we examined the role of IRS2/PI3K in the mediation of Wnt3a-stimulated beta-cell growth. Real-time PCR and Western blot were employed to investigate the activity of Wnt/β-catenin and IRS2/PI3K signaling. Proliferation of NIT-1 cells was assessed by BrdU incorporation, and apoptosis was quantitatively determined by TUNEL and flow cytometry (FCM). Dkk1, an inhibitor of Wnt signaling, and wortmannin, an inhibitor of PI3K, were also used. Results showed that Wnt3a rapidly activated Wnt/β-catenin signaling, promoted IRS2 expression and Akt phosphorylation in NIT-1 cells. These effects were completely abrogated by Dkk1 or partially eliminated by wortmannin. Wnt3a also promoted NIT-1 cell proliferation, inhibited cytokine-induced beta-cell apoptosis, and increased insulin secretion. Both of these effects were also eliminated by Dkk1 or wortmannin. Our results demonstrated that Wnt3a regulates proliferation, apoptosis and enhances function of pancreatic NIT-1 beta cells via activation of Wnt/β-catenin signaling, involving crosstalk with IRS2/PI3K signaling, with the effect of Wnt signaling on beta-cells also being IRS2/PI3K/AKT dependent. J. Cell. Biochem. 114: 1488–1497, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: WNT3A; WNT SIGNALING; INSULIN RECEPTOR SUBSTRATE (IRS2); PI3K/AKT; BETA-CELL; PROLIFERATION; APOPTOSIS

Type 2 diabetes is one of the most common diseases of the middle-aged population, and has reached epidemic proportions worldwide. It is predicted that it will affect 325 million people in the coming 25 years in Western and Asian countries combined [Zimmet et al., 2001]. Type 2 diabetes is characterized by reduced insulin secretion complicated with development of peripheral insulin resistance [Bruning et al., 1998], but the exact mechanism is still not fully understood. Pancreatic beta-cells are the sole source of insulin in the body and total beta-cell mass changes with insulin demand [Weir et al., 2001]. In type 2 diabetes, an increased peripheral insulin resistance leads to a greater demand for insulin, compensatory expansion in functional beta-cell mass can prevent the progression of the condition to fully-developed hyperglycemia [Kloppel et al., 1985]. However, the beta-cells tend to functionally fail to compensate, with hyperglycemia/hyperlipidemia developing and eventually diabetes occurs. Thus, the deterioration of beta-cell dysfunction has been believed to be pivotal in development of type 2 diabetes, and, presumably, promoting the function and survival of beta-cells might be therapeutically meaningful for the treatment of type 2 diabetes.

Shuyan Gui and Gang Yuan contributed equally to this work.

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Wnt proteins are a family of highly conserved proteins. There are three known pathways of Wnt signaling; the Wnt/b-catenin (canonical), the Wnt/Ca2+, and the Wnt/polarity pathway [Haupt et al., 2010]. Activation of the canonical pathway occurs by binding of Wnt ligands to the frizzled receptor and its co-receptors, the low-density lipoprotein receptor-related protein (LRP) 5 and 6, leading to activation of an intracellular protein, disheveled (Dsh). This triggers an intracellular signaling cascade that leads to inactivation of GSK3b and preventing GSK3b phosphorylation of β-catenin. This enables accumulation and nuclear translocation of β-catenin. Once inside the nucleus, β-catenin acts in combination with the T-cell factor (Tcf) and lymphoid enhancer factor (Lef) families of transcription factors (which include TCF7L2) to stimulate transcription of Wnt responsive genes. The Wnt-signaling pathway has been shown to be linked with regulation of development and differentiation, and the abnormalities of the pathway are strongly associated with tumorigenesis [Akiyama, 2000]. The role of Wnt signaling in the pathogenesis of diabetes has become a subject of active investigations following the recognition that a transcription factor variant of TCF7L2, a component of the Wnt pathway, was a strong risk factor for type 2 diabetes [Grant et al., 2006]. This association of TCF7L2 variants with type 2 diabetes has been replicated in numerous study populations. Many of these studies have linked the predisposing variant of TCF7L2 with a decrease in insulin secretion, implicating beta-cells dysfunction as the underlying cause of diabetes in these patients [Dahlgren et al., 2007; Hattersley, 2007]. Studies rather than investigating the role of TCF7L2 polymorphisms in the risk of type 2 diabetes also were performed to assess the role of Wnt signaling or the role of the cross talk between Wnt and other signaling pathways (such as GLP-1 signaling) in the pancreatic beta-cells. Recent studies demonstrated that the Wnt-signaling pathway is implicated in the development of endocrine pancreas and functional regulation of mature beta-cells, including insulin secretion, and beta-cell survival and proliferation [Welters and Kulkarni, 2008]. Recently, in vitro and in vivo studies found that the canonical Wnt signaling pathway can induce beta-cell proliferation. Schinner et al. [2008] found that adipocyte-derived Wnt-signaling molecules were able to induce cyclin D1 transcription and proliferation of both beta-cell lines and primary murine beta-cells. In line with this finding, another study demonstrated that recombinant Wnt3a protein could induce cell cycle regulators such as cyclin D2 and Pitx2 and could increase the proliferation rate of cultured beta-cells. This study also confirmed that the over-expression of active β-catenin was sufficient to induce proliferation markers and could increase beta-cell mass in vivo [Rulifson et al., 2007]. Because beta-cell dysfunction is an essential component of type 2 diabetes, it is tempting to speculate the role and mechanism of Wnt signaling in diabetes. But, it remains largely unknown. Glucagon-like peptide-1 (GLP-1), an insulinotropic hormone, might preserve or restore pancreatic beta-cell mass. Liu and Habener found that GLP-1 and its agonist, exendin-4 (Exd4), induce Wnt signaling activation in pancreatic beta-cells, and Wnt signaling appears to mediate GLP-1-induced beta cell proliferation. Sunmin and Dong believe that the long-term effects of GLP-1 receptor agonists are mediated by the IRS2 branch of the insulin/insulin-like growth factor signaling cascade [Park et al., 2006].

Insulin receptor substrates (IRS) are evolutionarily conserved adapter proteins and are required for many biological processes such as growth control, apoptosis, differentiation, and regulation of fuel metabolism [Stocker and Hafen, 2000]. IRS proteins link various membrane receptors to intracellular signaling pathways [Pirola et al., 2003] and therefore, they, especially IRS2, are key molecules in the insulin signaling pathway. IRS2 serves as an adaptor protein linking to insulin receptors, it can activate downstream kinase cascades, including MAP kinase and PI3-kinase cascades. It is generally accepted that activation of downstream PI3K/PDK-1/PKB by IRS2 plays a crucial role in beta-cell survival.

Consistent with this notion, Wnt signaling and IRS2 were demonstrated to have an important role in the survival of beta cells, and both this two signaling were linked with GLP-1 signaling, so we hypothesized that there may be crosstalk between Wnt signaling and IRS2/PI3K/PKB pathways during the regulation of beta-cell survival. In this study, we examined the effects of Wnt3a on the survival and function of beta cells under conditions where IRS2/PI3K/PKB pathway was blocked or left unblocked to clarify whether the Wnt signaling cross-talks with IRS2/PI3K/PKB pathway.

MATERIALS AND METHODS

ANTIBODIES

Rabbit polyclonal antibodies against Akt and phosphorylated Akt (pAkt$^{ser-473}$), GSK3β, and phosphorylated GSK3β (Cell Signaling, Beverly, MA) [Almeida et al., 2005]; rabbit anti-IRS2 (Upstate USA, Chicago, IL); mouse anti-β-catenin (BD Transduction Laboratories, Lexington, UK), and rabbit anti-IRS1 (Santa Cruz Biotechnology, Santa Cruz, CA) were purchased as indicated. Secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were purchased (Santa Cruz Biotechnology).

CELLS AND CULTURE

The NOD/Lt mouse-derived pancreatic β-cell line NIT-1 cells were purchased from American Type Culture Collection (ATCC, 20–30 passages). NIT-1 cells are characterized by glucose-responsive insulin secretion and ultrastructural features of differentiated mouse beta cells [Hamaguchi et al., 1991]. Cells were grown and cultured in RPMI 1640 medium (Gibco Invitrogen) containing 11.1 mmol/L glucose, 10 mmol/L HEPES, 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified incubator gassed with 5% CO_2. In experiments involving serum-starvation, 0.1% BSA in RPMI 1640 without glucose was used instead of serum.

WESTERN BLOT

NIT-1 cells were grown in six-well plates to 60% confluence. Cells were pretreated with Dkk1 (160 ng/ml, R&D Systems) or wortmannin (100 nmol/L, Sigma) for 1 h then subsequently with or without 100 ng/ml Wnt3a for 24 h. Cells were lysed in buffer (Beyotime, Beijing, China) containing 1% Triton X-100 and a mixture of protease and phosphatase inhibitors. Protein content was measured by the Bradford assay (Bio-Rad, Hercules, CA), and protein samples (50 μg) were separated on an 8 or 10% sodium dodecylsulphate–

polyacrylamide gel electrophoresis (SDS–PAGE) and electrically transferred onto polyvinylidene difluoride filters (Bio-Rad). The target proteins on the blots were probed with the following specific primary antibodies against GSK3β, phospho-Ser^9-GSK3β, total Akt, phospho-Ser^{473}-Akt, β-catenin, IRS2, IRS1, followed by HRP-conjugated sheep anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG as the secondary antibody and visualized using electrochemiluminescent visualization (UVP Bioimaging Systems, Upland, CA). Protein band intensity on each blot from three experiments was densitometrically determined.

DETECTION OF DNA SYNTHESIS AND CELL PROLIFERATION

Proliferation of NIT-1 cells was evaluated by using a 5-Bromo-2′-deoxy-uridine (BrdU) labeling and detection kit (Roche, Indianapolis). In brief, cells were grown to 60% confluence in 24-well plates, serum-starved for 24 h and then cells were incubated with cytokines (IL-β, 10 ng/ml; TNF-α, 50 ng/ml; IFN-γ, 50 ng/ml; PeproTech Inc., NJ) for 18 h with or without 100 ng/ml Wnt3a for 24 h, Wnt3a-treated cells were pretreated with Dkk1 (160 ng/ml) or wortmannin (100 nmol/L) for 1 h. After addition of BrdU into the medium, cells were cultured for 1 h and then harvested. Afterwards, cells were then fixed and incubated with an FITC-conjugated anti-BrdU antibody by following the kit instructions. All nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (1:1,000; for 5 min at room temperature). An immunofluorescent assay was employed for the detection of BrdU incorporated into cellular DNA using an Olympus BX51 microscope. Proliferation was quantified by counting beta-cells positive for BrdU staining and calculating their ratios.

BRDU STAINING FOR FLOW CYTOMETRY

Cells were incubated with Wnt3a for 24 h and pretreated with Dkk1 or wortmannin for 1 h. The cells treated with Dkk1 or wortmannin alone served as controls. The medium was then removed and the cells were cultured in medium containing BrdU (10 μM) for 1 h. Then, the BrdU medium was aspirated and the cells were fixed with methanol fixative (80%, diluted with PBS) for 60 min at −20°C. The cells were washed and permeabilized by re-suspension in 100 μl of 0.1% Triton buffer at room temperature for 30 min. After washing and centrifugation, the cells were resuspended in 50 μl solution containing primary mouse anti-BrdU (1:100 dilutions) at 37°C for 30 min. After washing, the sample was resuspended in 100 μl of FITC-conjugated goat anti-mouse IgG (1:50 dilutions, Roche) in the dark at 4°C for 1 h. Then cells were washed twice, resuspended in 1 ml of PBS, and stored for flow cytometry by BD FACSCalibur System. FACS data were analyzed using Winlist 6.0 softwareFo.

DETECTION OF APOPTOSIS

TUNEL was used for the detection of cell apoptosis. Briefly, NIT-1 cells were seeded into 24-well plates pretreated in a similar way for cell proliferation assays. At the end of the treatment, cells were washed with PBS, fixed with 4% paraformaldehyde (for 20 min at room temperature) and permeabilized with 0.5% Triton X-100 (for 10 min at room temperature). The TUNEL assay, which detects DNA strand breaks formed during apoptosis, was performed by using an in situ cell death detection kit (Roche, Indianapolis) in accordance with the manufacturer's instructions. DAPI (5 μg/ml; for 5 min at room temperature) was used to stain nuclei. Results were expressed as percentage of TUNEL-positive cells among the total number of cells. For flow cytometric analysis, cells were grown to 60% confluence in 24-well plates, incubated with a mixture of cytokines for 18 h as aforementioned and treated overnight with or without Wnt3a at 100 ng/ml for 24 h. Inhibitor Dkk1 or wortmannin was then added. After the treatment, a minimum of $1–5 \times 10^5$ NIT-1 beta-cells were trypsinized and collected. Cells were washed twice with 1 ml PBS and centrifuged at 2,000*g* for 5 min. Cells were resuspended in 100 μl of binding buffer, and 5 μl of FITC-Annexin V was added. The mixture was then incubated for 5 min in the dark. Then, 5 μl of propidium iodide (PI) was added, and the mixture was incubated for another 5 min in the dark. Finally, the samples were put into 200 μl of binding buffer and subjected to flow cytometric assay within 1 h.

ASSESSMENT OF GLUCOSE-STIMULATED INSULIN SECRETION (GSIS)

To measure the insulin secreted by NIT-1 cells, the cells were seeded into 24-well plates at a density of 1.5×10^5 cells per well and cultured for 2 days. After treatment with Wnt3a (100 ng/ml) for 24 h in RPMI 1640 in the absence or presence of inhibitor (either Dkk1 or wortmannin), the cells were washed with Krebs–Ringer bicarbonate buffer (KRBH; 130 mmol/L NaCl, 3.6 mmol/L KCl, 1.5 mmol/L $CaCl_2$, 0.5 mmol/L $MgSO_4$, 0.5 mmol/L KH_2PO_4, 2.0 mmol/L $NaHCO_3$, and 10 mmol/L HEPES, pH 7.4) supplemented with 0.1% (w/v) BSA, and then incubated at 37°C in 95% air and 5% CO_2 in 1 ml of the same KRBH buffer for 1 h in order to deplete glucose. After 60 min of glucose depletion, insulin secretion was elicited by incubation for 1 h in 0.5 ml of KRBH, containing either 2.8 or 16.7 mmol/L glucose. Medium was collected for insulin secretion measurements, transferred to Eppendorf tubes, centrifuged at 2,000*g* for 5 min to eliminate any insulin contained in cytoplasm. Afterwards, supernatants were collected and immediately frozen at −20°C. The insulin was determined by radioimmunoassay (Insulin RIA Kit, Beijing Institute of Atomic Energy, China).

ISOLATION OF RNA AND REAL-TIME PCR

Cells were grown in 24-well plates to 60% confluence and then treated for 24 h with purified Wnt3a (100 ng/ml), or wnt3a plus either Dkk1 (160 ng/ml) or wortmannin (100 nmol/L) as described before. After the treatment, RNA was isolated with TRIZOL (Invitrogen, Carlsbad, CA). By using the RetroScript kit (Toyobo, Japan), 1.0 μg of total RNA was then reversely transcribed into cDNA. The quantitative reverse transcription-PCR was carried out using the QuantiTect SYBR green PCR kit (Qigan, Inc. Valencia, CA) on an iCycler iQ real-time PCR detection system (Bio-Rad). The PCR conditions included an initial melting at 95°C for 5 min, followed by 40 cycles at 94°C for 15 s, 52°C for 20 s, and 72°C for 30 s, with specific primers (Table I) to detect mouse β-catenin, TCF7L2, IRS1, IRS2, Pitx2, CyclinD2, Bcl-2, glucose transport 2 (GLUT2), glucokinase (GK), PPARr, and Pdx-1. All transcript levels were normalized against β-actin. The number of amplifications was computed.

TABLE I. PCR Primers for β-Catenin, TCF7L2, IRS1, IRS2, Pitx2, CyclinD2, bcl-2, GK, GLUT2, PPARr, and Pdx-1

Target gene	Primers	Resultant length (bp)
Beta-catenin	Forward, 5′-GGCAACCCTGAGGAAGAAGAT-3′ Reverse, 5′-CCCTCTGAGCCCTAGTCATTG-3′	133
Pitx2	Forward, 5′-GAAAGCAAAGCAGCACTCCA-3′ Reverse, 5′-CGCACGACCTTCTAGCACAA-3	185
Cyclin D2	Forward, 5′-GGCGTGTTCGTCATCTGCTA-3′ Reverse, 5′-AGTCTGCGGGTTCTCCTGTAA-3′	291
Bcl-2	Forward, 5′-CGAGAAGAAGAGAGAATCACAGG-3′ Reverse, 5′-AATCCGTAGGAATCCCAACC-3′	133
IRS2	Forward, 5′-GGGGCGAACTCTATGGGTA-3′ Reverse, 5′-GCAGGCGTGGTTAGGGAAT-3′	139
IRS1	Forward, 5′-AGTGGTGGAGTTGAGTTGGGC-3′ Reverse, 5′-GAAGAGGCTGTGGAGGATGGA-3′	257
TCF7L2	Forward, 5′-AATCCTTGCCTTTCGCTTCC-3′ Reverse, 5′-TCTGTGACTTGGCGTCTTGG-3′	269
Pitx2	Forward, 5′-GAAAGCAAAGCAGCACTCCA-3′ Reverse, 5′-CGCACGACCTTCTAGCACAA-3′	185
Pdx-1	Forward, 5′-AGTGGGCAGGAGGTGCTTAC-3′ Reverse, 5′-CCGAGGTCACCGCACAAT-3′	297
GluT2	Forward, 5′-GCCAATTACCGACAGCCC-3′, Reverse, 5′-CAGCACAAGTCCCACCGA-3′	276
GK	Forward, 5′-GTGGTGGCAATGGTGAATGAC-3′ Reverse, 5′-TGAGCAGCACAAGTCGTACCA-3′	328
Beta-actin	Forward, 5′-CCGTGAAAAGATGACCCAG-3′ Reverse, 5′-TAGCCACGCTCGGTCAGG-3′	294

STATISTICAL ANALYSIS

All statistical analyses were conducted by employing SPSS 10.0 Software package. All data are presented as means ± SD calculated from at least three repeated experiments. The student's *t*-test was used for the assessment of differences. $P < 0.05$ was considered to be statistically significant.

RESULTS

EFFECTS OF WNT3A ON CELL PROLIFERATION AND APOPTOSIS OF MURINE INSULINOMA NIT-1 BETA CELLS

To test whether wnt3a promotes proliferation of NIT-1 cells, we measured the incorporation of BrdU, a proliferation marker, in the cultured cells. Compared with controls, Wnt3a-treated cells had a 58% and 75% increased BrdU incorporation in normal media or cytokine mixture, respectively (Fig. 1). TUNEL was used for the detection of cell apoptosis. Treatment of the NIT-1 cells with cytokines (IL-β 10 ng/ml, TNF-α 100 ng/ml, IFN-γ 100 ng/ml) for 18 h increased the number of apoptotic cells from 1.6% to 3.0% of total cells ($P < 0.05$; Fig. 2), however, treatment with Wnt3a dropped the number to 1.7% ($P < 0.05$; Fig. 2). Flow cytometric

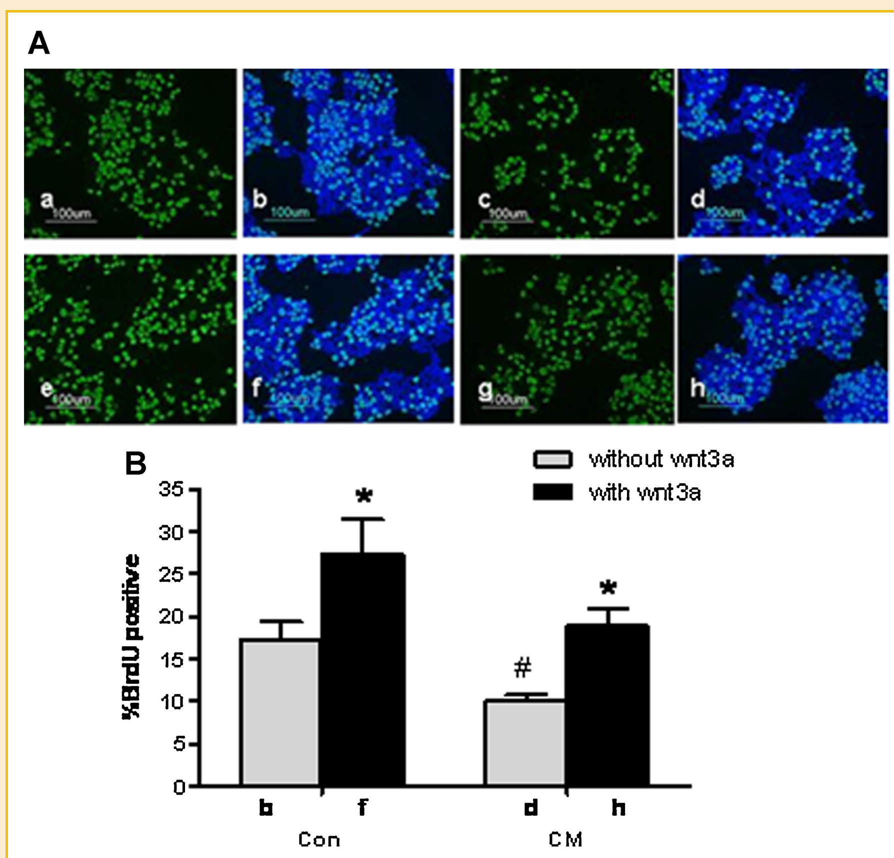

Fig. 1. Effects of Wnt3a treatment on pancreatic beta-cell proliferation. A: NIT-1 beta-cells under fluorescence microscope (original magnification, 200×) after incubation in different media (treatment groups). (a,b) normal control; (c,d) cytokine mix (CM); (e,f) normal control plus Wnt3a; (g,h) cytokine mix plus Wnt3a. In comparison with the normal control, less CM-treated cells were positively stained by BrdU. After treatment with Wnt3a, more cells were positively stained. Cells that were positive by BrdU exhibited green fluorescence in nuclei. B: The number of BrdU-positive cells compared with total cells is indicated as % BrdU-positive. Data are presented as the mean ± SD (n = 4). #$P < 0.05$ versus normal controls, *$P < 0.05$ versus cells treated without Wnt3a.

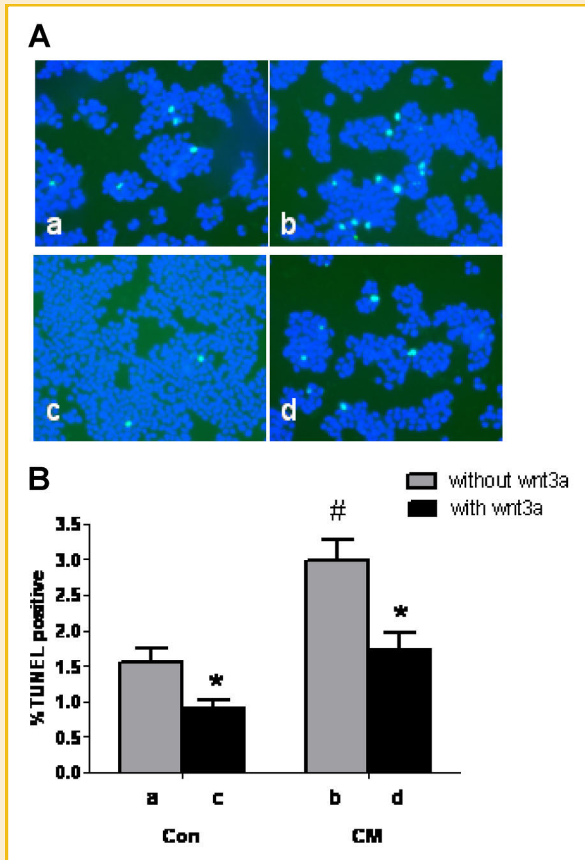

Fig. 2. Effects of Wnt3a treatment on apoptosis of pancreatic beta-cells. A: NIT-1 beta-cells under fluorescence microscope (original magnification, 200×) after incubation in different media (treatment groups). (a) normal control; (b) cytokine mix (CM); (c) Wnt3a; (d) cytokine mix plus Wnt3a. In comparison with the normal control, more CM-treated cells were positively stained by TUNEL. After treatment with Wnt3a, fewer cells were positively stained. Cells that were positive for TUNEL exhibited green fluorescence in nuclei. B: Percentage of TUNEL-positive cells. Data are presented as the mean ± SD (n = 4). $^{\#}P < 0.05$ versus normal controls; $^{*}P < 0.05$ versus cells treated without Wnt3a.

examination was used to evaluate the proliferation and apoptosis for further confirmation. The results also showed that Wnt3a increased proliferation (Fig. 3) and decreased the apoptotic rate (Fig. 4).

EFFECTS OF WNT3A ON GLUCOSE-STIMULATED INSULIN SECRETION IN BETA CELLS

In order to determine whether Wnt3a promotes GSIS in beta cells, NIT-1 beta cells were treated with Wnt3a protein for 24 h, and the amount of insulin secreted by glucose stimulation (16.7 mM, 1 h, and 2.8 mM, 1 h) was measured by insulin RIA. Figure 5 shows that pretreatment with Wnt3a significantly increased insulin secretion at 2.8 mM or 16.7 mM of glucose. These effects were blocked by Dkk1 and wortmannin. To determine which step, from glucose sensing to insulin exocytosis, is enhanced in Wnt3a-incubated NIT-1 cells, we compared the expression levels of insulin transcription factor Pdx-1, and glucose sensing molecules such as GLUT2 and GK. Semi-quantitative RT-PCR shows that the levels of Pdx-1 and GK mRNA experienced significant change following treatment with Wnt3a (Fig. 6). The treatment with Wnt3a did not affect the expression of GLUT2 (not shown).

ACTIVATION OF TCF7L2-DEPENDENT WNT SIGNALING BY WNT3A PROMOTED IRS2 EXPRESSION

To evaluate the mechanism for the Wnt3a effect on proliferation and apoptosis in NIT-1 cells, Real-time RT-PCR and Western blot were performed to evaluate several signaling pathways such as Wnt, cell cycle, apoptosis, and insulin signaling. The activation of downstream Wnt signaling requires the binding of TCF7L2 with active β-catenin (free β-catenin). TCF7L2 is a DNA-binding protein that, in the absence of β-catenin, serves as a transcriptional repressor. β-catenin promotes the transcription of TCF7L2 by binding to TCF7L2. Semi-quantitative RT-PCR analysis showed that the expression of TCF7L2 mRNA in Wnt3a treated cells was significantly increased compared to the control group (Fig. 6). No obvious change was observed in β-catenin mRNA level (not shown), but the protein level of β-catenin was significantly increased (Fig. 7). CyclinD2, as a cell cycle regulator, has been shown to modulate beta-cell proliferation in vivo [Rulifson et al., 2007]. Real-time RT-PCR results revealed that Wnt3a increased mRNA levels of cyclinD2 and Pitx2, a direct transcriptional regulator of cyclinD2. These effects were blocked by Dkk1 (Fig. 6). Furthermore, Wnt3a treatment of the cells for 24 h led to a significant increase in the level of Bcl-2 mRNA as compared with controls ($P < 0.05$). IRS2 plays a critical role in the growth, differentiation, and apoptosis of beta cells. To test if the activation of Wnt signaling increases the expression of IRS2 in NIT-1 cells, real-time RT-PCR was performed to measure the level of IRS2 mRNA in NIT-1 cells. Results showed that Wnt3a treatment increased IRS2 mRNA levels about twofold (Fig. 6). Immunoblotting revealed that tyrosine phosphorylation of IRS2 was also increased after Wnt3a treatment, which was in line with the increased expression of IRS2 mRNA (Fig. 7). By contrast, IRS1 expression was not increased by Wnt3a, and its level of tyrosine phosphorylation remained unchanged (not shown).

IRS2 MEDIATES WNT3A-STIMULATED AKT PHOSPHORYLATION

It is well established that the IRS2/PI3K/Akt signaling pathway is a crucial regulator of beta-cell mass and function. In this study, we used NIT-1 cells to see if Wnt signaling stimulates Akt activity and if IRS2 mediates the effects of Wnt3a upon Akt phosphorylation in β cells. Wnt3a (100 ng/ml) doubled the expression of IRS2 (Fig. 7) in NIT-1 cells without increasing the expression of IRS1 (not shown). Akt phosphorylation (p-Akt$^{ser\text{-}473}$) was increased about 1.6-fold, whereas its mRNA expression showed no change (not shown). Accordingly, Akt activity, measured by its ability to phosphorylate GSK3β, was much higher in Wnt3a treated cells than in control cells (Fig. 7). Furthermore, phosphorylation of GSK3β at Ser^{9}, which results in its inactivation, was higher in Wnt3a-treated cells than in control cells. These results suggest that Wnt3a promotes the IRS2/Akt cascade in NIT-1 cells. However, in NIT-1 cells pre-treated with Dkk1 (160 ng/ml), a specific potent inhibitor of Wnt signaling, the effect of Wnt3a on increasing IRS2 was blocked (Fig. 7), with complete abrogation of the phosphorylation of Akt and GSK3β.

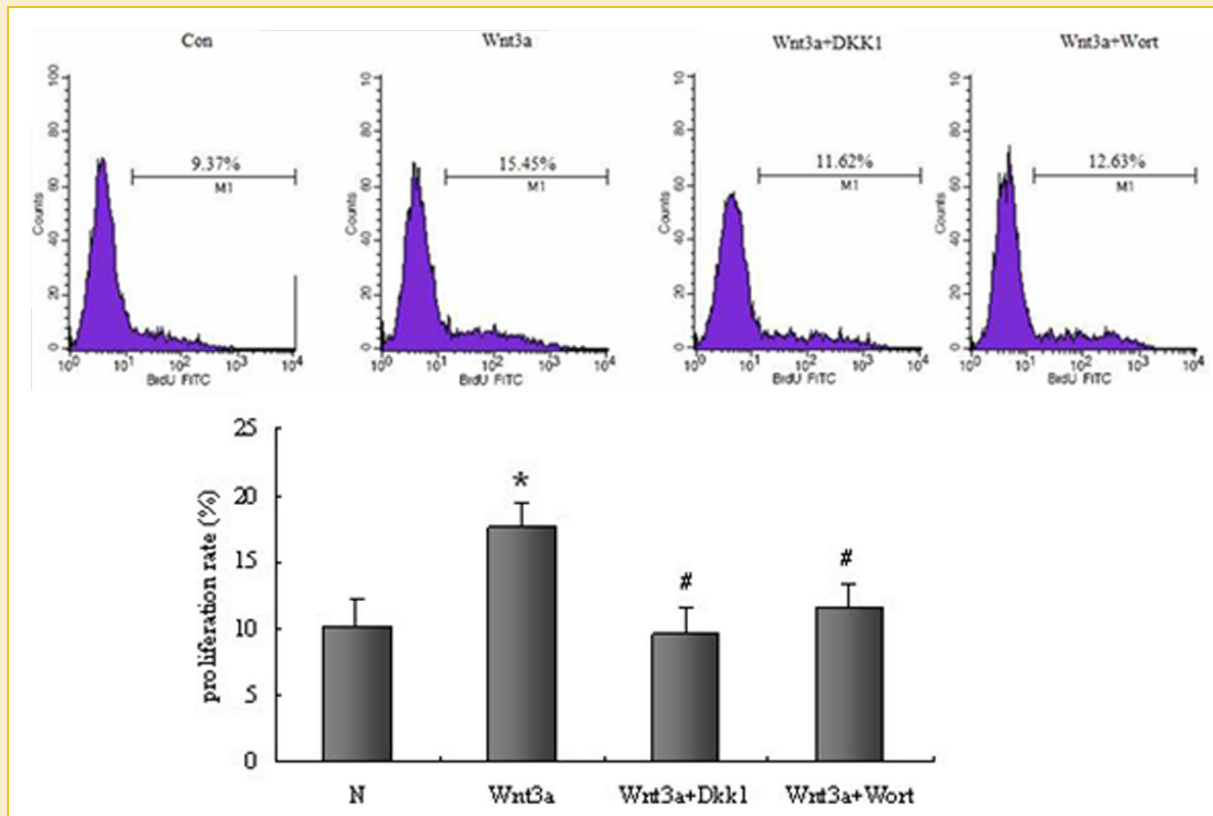

Fig. 3. Representative flow cytometric analysis of beta-cell proliferation after different treatments. Percentage of cells positive for BrdU reflects the proliferation rate of beta-cells. Wnt3a promotes the proliferation of NIT-1 cells, but Dkk1 or wortmannin abrogated or diminished the effect. The data are expressed as mean ± SD (n = 3). $^{*}P < 0.05$ versus controls; $^{\#}P < 0.05$ versus cells treated with Wnt3a alone.

There was, however, no effect on IRS1 expression, accompanied by a reduction in free β-catenin and TCF7L2, as shown previously. These results indicate that phosphorylation of Akt and GSK3β and changes of free β-catenin and TCF7L2 levels in NIT-1 cells, were dependent on Wnt3a protein, and Akt activation might be a consequence of Wnt signaling that takes place downstream of IRS-2.

EFFECT OF WNT3A ON PROLIFERATION AND APOPTOSIS VIA A PI3K-DEPENDANT PATHWAY

In NIT-1 cells pre-treated with wortmannin (100 nmol/L), a specific inhibitor of PI3K (at concentration of 100 nmol/L), there was no noticeable effect on basal Akt activity. However, addition of the inhibitor at the said concentration completely abrogated Wnt3a-stimulated Akt phosphorylation (Fig. 7), indicating that Wnt3a (IRS2) activates Akt in a PI3K-dependent fashion. Flow cytometric examination of DNA synthesis using BrdU incorporation showed that Wnt3a increased NIT-1 cell proliferation 1.7 ± 0.5-fold as compared with controls ($P < 0.05$; Fig. 3). In contrast, although wortmannin (100 nmol/L) alone did not affect cell proliferation, incubation of NIT-1 cells with Wnt3a in the presence of wortmannin inhibited the increase in proliferation, with levels being maintained at baseline throughout the incubation period. Treatment of the NIT-1 cells with cytokines for 18 h increased the ratio of apoptotic cells from 10.2% to 23.4% ($P < 0.05$) while Wnt3a decreased the ratio to 17.8% (Fig. 4), suggesting that Wnt3a can protect from cytokine toxicity. Inhibition of PI3K with wortmannin abrogated the inhibitory effect of Wnt3a on apoptosis ($P < 0.05$). These results showed that, as with Wnt3a-induced proliferation, the inhibitory effect of Wnt3a on cytokine-induced apoptosis is also PI3K dependent.

DISCUSSION

The purpose of this study was to gain insight into the role of Wnt signaling in proliferation, apoptosis and function of beta cells. We have showed that Wnt3a regulates proliferation, apoptosis, and enhances function of pancreatic NIT-1 beta cells via activation of the canonical Wnt/β-catenin-LEF/TCF7L2 signaling pathway. Further, the effect of Wnt3a also increased IRS2 expression and PI3K/Akt phosphorylation, indicating that there is crosstalk between Wnt and IRS2/PI3K signaling, and the effect of Wnt signaling on beta-cells is at least partly IRS2/PI3K/Akt dependent since inhibition of PI3K with wortmannin abrogated the effect of Wnt3a on proliferation, apoptosis and insulin secretion.

Recently, both in vitro and in vivo studies showed that Wnt signaling regulates the function and the survival of pancreatic beta-

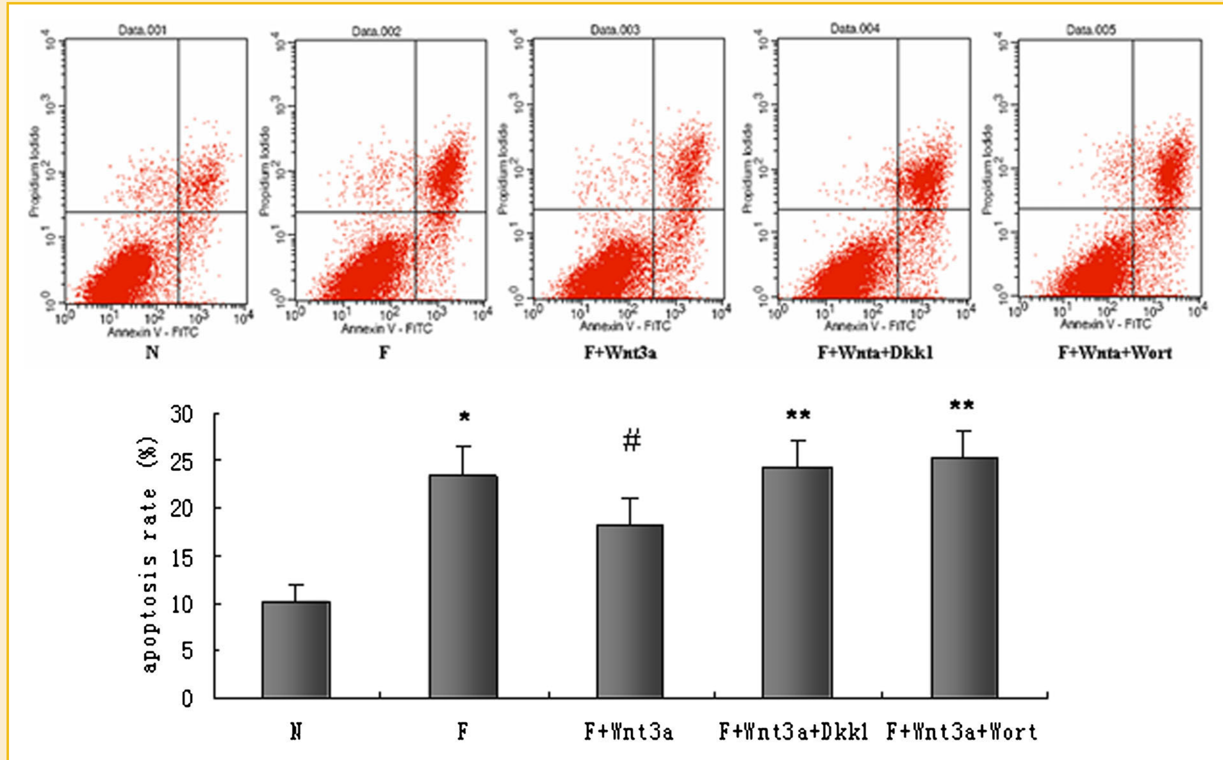

Fig. 4. Flow cytometric results of islet beta cell apoptosis. Incubation of beta-cells with cytokines (F) for 18 h increases the apoptosis of beta cells, $^{*}P < 0.05$ versus normal control cells (N), but Wnt3a can protect beta-cells from cytokine-induced toxicity, and reduced the apoptosis of the beta-cells ($^{\#}P < 0.05$ vs. NIT-1 cells treated with cytokines alone). Wortmannin (Wort), an inhibitor of PI3K, and Dkk1, an antagonist of Wnt signaling, can abrogate or diminish the protective effect of Wnt3a ($^{**}P < 0.01$ vs. cells treated with cytokines and Wnt3a). The results are expressed as mean ± SD (n = 3).

cells [Rulifson et al., 2007; Liu and Habener, 2008; Schinner et al., 2008; Shu et al., 2008]. This effect, in fact, was completely abrogated by Dkk1, a negative regulator of Wnt signaling [Niida et al., 2004], via induced internalization of the LRP/Kremen co-receptor complex [Patel et al., 2004]. However, the detailed mechanism by which this pathway modulates the proliferation and/or apoptosis of beta cells is not fully understood and further studies are warranted. In this study,

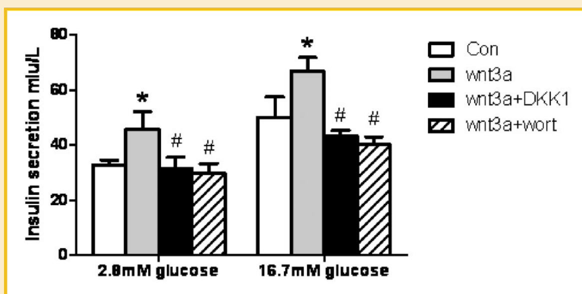

Fig. 5. Insulin secretion by NIT-1 cells in response to the stimulation of glucose. NIT-1 cells were cultured in control medium (Con) or Wnt3a-conditioned medium (Wnt3a) for 24 h in the absence or presence of inhibitor (either Dkk1 or wortmannin). Insulin secretion was measured by RIA at low (2.8 mM) or high glucose (16.7 mM) 1 h after incubation (n = 4). $^{*}P < 0.05$ versus cells treated without Wnt3a. $^{\#}P < 0.05$ versus cells treated with Wnt3a alone.

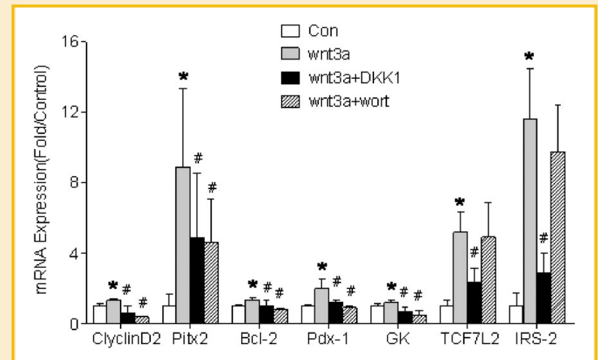

Fig. 6. Real-time RT-PCR. Wnt3a activated Wnt signaling and upregulated TCF7L2 mRNA by 5.2 ± 1.1-fold and IRS2 mRNA by 11.6 ± 2.8-fold. Dkk1, a Wnt antagonist, inhibits activation of the signal and expression of TCF7L2 and IRS2 downstream of it, and the PI3K inhibitor (wortmannin) does not substantially change the expression of TCF7L2 and IRS2. Wnt3a increases cyclin D2 mRNA expression by 1.3 ± 0.1-fold, Pitx2 mRNA expression by 8.9 ± 4.4-fold, and Bcl-2 mRNA expression by 1.3 ± 0.1-fold. mRNA expression levels of glucokinase (GK) and Pdx-1 are increased in Wnt3a-treated cells compared with controls without treatment of Wnt3a. No obvious change in GLUT2 and β-catenin mRNA was observed (data not shown). All RT-PCR results are normalized to β-actin and expressed as change from control, respectively. All the average values were obtained from three experiments. Significance was set at $P < 0.05$. $^{*}P < 0.05$ versus controls; $^{\#}P < 0.05$ versus cells treated with Wnt3a alone.

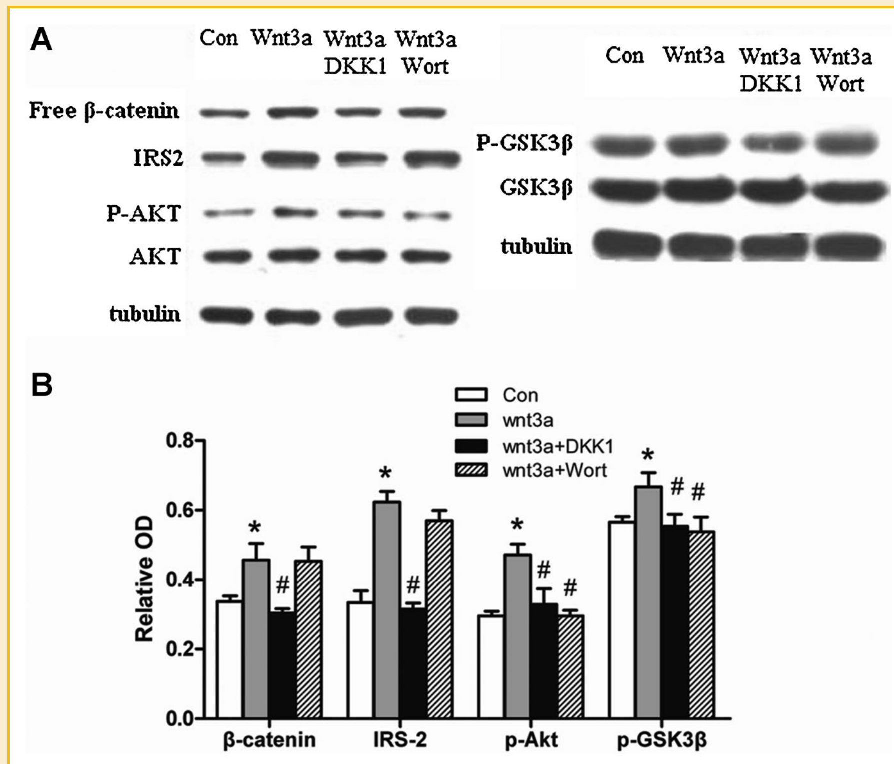

Fig. 7. Effects of Wnt3a on Wnt signaling, phosphorylation and activation of IRS2/PI3K/Akt in NIT-1 beta cells assessed by Western blot. NIT-1 cells were cultured in Wnt3a-conditioned medium (Wnt3a) or control medium (Con) for 24 h, which were also pre-incubated with Dkk1 (Wnt3a + Dkk1) or wortmannin (Wnt3a + Wort) for 1 h before the treatment. One hundred microgram of cell lysate were analyzed by immunoblotting with anti-β-catenin, anti-phospho-Akt (p-Ser473-Akt), anti-Akt, anti-IRS2, anti-phospho-GSK3β (Ser^9; p-GSK3β), and anti-GSK3β antibodies. Wnt3a activates Wnt signaling, increases free β-catenin levels, induces phosphorylation and activation of IRS2, and triggers Akt phosphorylation with GSK3β levels significantly increased. ($^*P < 0.05$ vs. control cells). Dkk1, a potent inhibitor of Wnt signaling, suppresses both Wnt signaling and IRS2/Akt phosphorylation ($^\#P < 0.05$ vs. cells treated with Wnt3a alone). Wortmannin reduces both phosphorylated Akt and GSK3β levels, but did not significantly decrease IRS2 and β-catenin expression ($^\#P < 0.05$ vs. cells treated with Wnt3a alone). All the average values were obtained from three experiments.

using the glucose-responsive NOD/Lt mouse-derived pancreatic beta-cell line (NIT-1 cells) as a model for pancreatic beta-cells, we found that Wnt3a activates the canonical Wnt signaling pathway and modulates proliferation and apoptosis, accompanied by activation of various heterologous signaling proteins. Activated Wnt signaling strongly stimulates IRS2 gene expression and increases synthesis of IRS2 protein. Dkk1 inhibits the growth-promoting effects of Wnt3a on NIT-1 beta cells, with decreased IRS2 expression. This result suggests that IRS2 may be involved in the regulation of the beta cell by Wnt signaling. Studies indicate that IRS2 plays an important role in maintaining functional beta-cell mass. Mohanty et al. [2005] reported that overexpression of IRS2 is sufficient to induce proliferation in rat islets and to protect human beta-cells from D-glucose-induced apoptosis. Previous work with transgenic mice suggests that IRS2 provides one of the most potent regulatory signals for the PI3K cascade in beta-cells [Hennige et al., 2003; Suzuki et al., 2003]. There are two major downstream signaling pathways of IRS2, namely, PI3K/PDK-1/PKB and Grb2/mSOS/Ras/Raf/MEK-1/ERK pathways, respectively [Briaud et al., 2003]. PI3K activation in downstream of IRS2 plays a crucial role in beta-cell survival [Lingohr et al., 2003]. Recent studies demonstrated that signal transduction upstream via IRS2 and downstream via protein kinase B (PKB, also known as Akt) are critical to the control of beta-cell survival [Lingohr et al., 2002; Rhodes and White, 2002]. Our studies showed that Wnt3a activates Wnt-signaling, increases levels of phosphorylated Akt and promotes the growth of beta-cells, whereas wortmannin, an inhibitor of PI3K, diminishes these effects. This suggests that IRS2/PI3K/Akt pathway plays a role in mediating beta-cell survival/proliferation induced by Wnt3a.

The crosstalk between the Wnt/β-catenin and the ERK pathways in the regulation of cell proliferation was also reported previously. Yun et al. [2005] reported that Wnt3a activates the ERK pathway via the Ras-Raf-1-MEK-ERK cascade and β-catenin activates ERKs via activation of the MAP kinase cascade. Almeida et al. [2005] also reported that Wnt3a prolongs the survival of osteoblasts and uncommitted osteoblast progenitors via activation of the Src/ERK and PI3K/Akt signaling cascades. Axin, a negative regulator of Wnt/β-catenin signaling, was identified as an inhibitor of the Raf-1-MEK-ERK cascade in fibroblasts. Further, β-Catenin was identifiable as a mediator in the ERK pathway regulation by Axin [Jeon et al., 2007].

Several components of the canonical Wnt-signaling pathway are also members of other important signaling pathways in beta-cells. GSK3β, which efficiently modifies beta-catenin in Wnt signaling, can also be phosphorylated and, consequently, inhibited by the insulin-signaling cascade via PI3K and Akt activation [Patel et al.,

2004]. GSK3β is a downstream member of the Akt family. Phosphorylation of GSK3β Ser/Thr protein kinase by Akt inhibits GSK3β and promotes protein synthesis, resulting in increased size of beta-cells. Insulin/PI3K/Akt signaling can trigger proliferation in a wide array of cells and is required for compensatory beta-cell proliferation in response to peripheral insulin resistance [Okada et al., 2007]. A recent study demonstrated that treatment of gut endocrine cells with insulin increases levels of nuclear β-catenin and enhances TCF binding with β-catenin, indicating the existence of crosstalk between insulin and Wnt signaling [Yi et al., 2008]. Our data showed that Wnt3a induced-phosphorylation of GSK3β at Ser^9 and Dkk1 completely blocked the effect of Wnt3a. Interestingly, wortmannin, an inhibitor of PI3K, abrogated the effect of Wnt3a on phosphorylation of GSK3β, but did not inhibit the Wnt3a-induced β-catenin and IRS2 increases. This is a surprising result since GSK3β is an important part of the Wnt signaling pathway and normally regulates the levels of β-catenin. We hypothesize that the levels of β-catenin may be regulated not only by GSK3β but also by the interactions of β-catenin with various proteins such as AXIN, the gap junction protein connexin 43, the cell adhesion protein cadherin or the de-ubiquitinating enzyme Fam [Mao et al., 2001; Almeida et al., 2005]. However, the exact mechanism is unclear, and requires further study.

To further elucidate the mechanism by which Wnt3a affects proliferation and apoptosis in NIT-1 cells, cyclin D2 and Bcl-2 were also evaluated. Results showed that Wnt3a increased mRNA levels of cyclinD2 and Bcl-2. These effects were blocked by Dkk1 and wortmannin. This result suggests that cyclin D2 and Bcl-2 may be involved in proliferation and apoptosis induced by Wnt3a treatment. A previous study reported that GSK3β has been shown to phosphorylate cyclin D, promoting its degradation [Diehl et al., 1998]. Akt-mediated inhibition of GSK3β suppresses cyclin D phosphorylation and prevents its degradation, thus promoting progression of cell cycle and increasing mitogenesis [Sherr, 2001]. In line with our finding, another study also demonstrated that recombinant Wnt3a protein could induce cell cycle regulators such as cyclin D2 and Pitx2 and could increase the proliferation rate of cultured beta-cells, the overexpression of active β-catenin was sufficient to induce proliferation markers, increasing beta-cell mass in vivo [Rulifson et al., 2007].

The treatment of Wnt3a was found to improve GSIS in our study. Both wortmannin and DKK1 decreased the insulin secretion to basal level. This result indicates that the effect of Wnt signaling on insulin secretion may also be IRS2/PI3K/Akt dependent. Our study showed that incubation of beta-cells with Wnt3a protein increases GK and Pdx-1 mRNA expression in beta-cells. GK is crucial for glucose-sensing regulation in beta-cells, and is also a novel gene target for canonical Wnt signaling [Schinner et al., 2008]. Phosphorylation of GSK3β by Akt increases the expression of Pdx-1, which is a key regulator of insulin transcription, and growth and function of beta-cells. No significant difference in GLUT2, a specific glucose-sensing molecule in beta-cells, was observed in the study.

Further studies are needed to identify the mechanism by which Wnt3a activates IRS2 and to demonstrate the crosstalk between the Wnt/β-catenin and the IRS2/PI3K/Akt pathway in vivo and in vitro. A recent study reported that the expression of DKK1 is increased in a type 1 diabetes model associated with decreased IGF-1 and IGF receptor expression, reduced phosphorylated Akt, and low level β-catenin [Hie et al., 2011]. This suggests that IGF-1 and IGFR may contribute to inhibition of the phosphorylation of Akt and β-catenin proteins. Another possible explanation is that the Wnt pathway may induce the transcription of IRS2 directly. Yoon et al. [2011] reported that IRS-1 mRNA and protein were induced rapidly in C2C12 myocytes following Wnt3a treatment, suggesting that it is likely a direct transcriptional target. Norton et al. [2011] also demonstrated recently that TCF7L2 binds directly to the IRS2 gene.

In summary, although Wnt signaling has been extensively studied in many diverse contexts, the role of this pathway in beta cells has been largely ignored. The canonical pathway of Wnt activation is complicated, involving crosstalk and interactions with other signaling pathways. Our observations that Wnt/β-catenin signaling affects the proliferation, apoptosis and function of pancreatic NIT-1 beta cells via IRS2/PI3K/Akt pathway suggest that modulation of Wnt signaling in beta cells may have therapeutic value. Furthermore, given these observations, it will be of interest to further study the mechanism involved in crosstalk between the Wnt/β-catenin and the IRS2/PI3K/Akt pathway.

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