Nephrin is expressed on the surface of insulin vesicles and facilitates glucose stimulated insulin release

Running title: Nephrin regulates insulin secretion

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**Objective.** Nephrin, an immunoglobulin-like-protein essential for the function of the glomerular podocyte and regulated in diabetic nephropathy, is also expressed in pancreatic β-cells, where its function remains unknown. The aim of this study was to investigate whether diabetes modulates nephrin expression in human pancreatic islets and to explore the role of nephrin in β-cell function.

**Research Design and Methods.** Nephrin expression in human pancreas and in MIN6 insulinoma cells was studied by Western-Blot, PCR, confocal microscopy, subcellular fractionation and immunogold-labeling. Islets from diabetic (n=5) and non-diabetic (n=7) patients were compared. Stable transfection and siRNA knockdown in MIN-6 cells/human islets were utilized to study nephrin function *in vitro* and *in vivo* after transplantation in diabetic immunodeficient mice. Live imaging of GFP-nephrin transfected cells was utilized to study nephrin endocytosis.

**Results.** Nephrin was found at the plasma membrane and on insulin vesicles. Nephrin expression was decreased in islets from diabetic patients when compared to non-diabetic controls. Nephrin transfection in MIN-6 cells/pseudoislets resulted in higher glucose-stimulated insulin release *in vitro* and *in vivo* after transplantation into immunodeficient diabetic mice. Nephrin gene silencing abolished stimulated insulin release. Confocal imaging of GFP-nephrin transfected cells revealed nephrin endocytosis upon glucose stimulation. Actin stabilization prevented nephrin trafficking as well as nephrin positive effect on insulin release.

**Conclusions.** Our data suggest that nephrin is an active component of insulin vesicle machinery that may affect vesicle-actin interaction and mobilization to the plasma membrane. Development of drugs targeting nephrin may represent a novel approach to treat diabetes.
In USA alone, diabetes mellitus affects more than 20 million people. Although advances have been made in the clinical care of diabetes, one of the major limitations for finding a cure is that the mechanisms regulating the function of insulin producing cells have not yet been fully characterized.

Nephrin is an immunoglobulin-like protein with important structural and signaling properties (1; 2). It was initially described in podocytes, highly specialized cells in the kidney glomerulus (3; 4). Nephrin is heavily down-regulated in human diabetic nephropathy (5), and nephrin mutations are responsible for the congenital nephrotic syndrome of the Finnish type (6).

Nephrin expression has been reported in pancreatic β-cells (7), where its function remains unknown. In immortalized human podocytes, the C-terminal portion of nephrin appears to bind VAMP-2, a vesicle associated membrane protein involved in exocytosis (8). The interaction of nephrin with VAMP-2, together with its well known interaction with the actin cytoskeleton (9-13), suggests that nephrin may play an important role in vesicles trafficking, a recently described feature of podocyte biology (14).

In pancreatic β-cells, glucose stimulation affects actin reorganization, and redistribution of cortical actin is essential for proper β-cell function (15). However, the pathways responsible for the regulated targeting of vesicles to the plasma membrane (PM) have not yet been fully characterized. The aim of this study was to understand the regulation of pancreatic nephrin expression in patients with type 2 diabetes, and the role of nephrin in the regulation of glucose-stimulated insulin release (GSIR).

RESEARCH DESIGN AND METHODS

Cell culture, islet culture and RT-PCR.

MIN6 cells, and the B1 and C3 subclones (gift of Dr A Thomas) were cultured in 25mM glucose DMEM (Invitrogen, Carlsbad, CA) (16), and pseudoislets generated in culture on a shaker incubator (17). To study the effect of acute versus chronic glucose exposure, cells were cultured in 2mM glucose for 7 days and then assigned to receive either 2mM or 20mM glucose culture media for 1 day (acute exposure) or 14 days (chronic exposure), a model we have utilized previously to mimic glucotoxicity in mesangial cells (18). Media supplemented with 5mM leucine and glutamine were utilized throughout the experiment to prevent apoptosis related to prolonged low glucose exposure (19), and cells were subcultured as necessary to prevent overconfluence. For selected experiments, cells were treated for 6 hours with 0 to 200nM cytochalasin D (Sigma) (20), 0.5µM jasplakinolide (21) or 200nM phalloidin (Invitrogen), and the change in G/F actin ratio was assessed by western blot (G-actin/F-actin Assay Kit, Cytoskeleton, Denver, CO). Human islets from cadaveric donors with research consent were obtained through the Islet Cell Resource Distribution system or were isolated at the local Human Cell Processing facility (22). B- cell content was determined as previously described by laser scanning cytometry (23). Nephrin and 18S ribosomal RNA expression were evaluated using the 7500 Real-Time-PCR System (Applied Biosystems). For standard PCR and sequencing, primers were designed at the intron-exon junction (5'-ATCTCCAAGACCCAGGTACACA and 3'-AGGGTCAGGGCCTGTAG for mouse, 5’-CCTGCTAGAGGTGAATTCCA and 3’-CCATTCTTCAGCCACTGCA for human). Additional human primers previously described were utilized to verify the presence of the two described splice variants of nephrin (24). Actin was used as housekeeping gene (5’-TCTGCGGTAGCTCCGTCAGG and 3’-TCTAGGCACCAAGGTG for human). A PCR product of 938bp including part of the extracellular nephrin domain was amplified
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and sequenced from human pancreas and the blast analysis matched completely the reported human nephrin mRNA sequence. **Western blotting (WB) and cell fractionation.** For WB, a polyclonal guinea-pig anti nephrin antibody (1:2000, C-terminal, Fitzgerald Laboratories, Concord, MA) and a monoclonal antibody against the IgG8-like domain of human nephrin (1:500, 50A9, gift of Dr. Tryggvason) were utilized. Both antibodies revealed a unique band of 180kD. Loading efficiency was verified by actin (1:5000, Abcam, Cambridge, MA). Plasma membranes (PM) were separated after biotinylation of cultured cells (Thermo Scientific, Rockford, IL). Unbiotinylated cells were utilized as control, and separation efficiency evaluated by WB for Na/K-ATPase (1:1000, rabbit polyclonal, Abcam). For sucrose gradient centrifugation, cells were glucose-starved for 1h and then exposed to either 0.5mM or 25mM glucose for 30 min, then homogenized in 250µl HEPES buffer. Pooled supernatants were loaded onto a 4.4 ml linear sucrose density gradient, centrifuged in a SW50 rotor (110,000 x g) for 18 h, and 15-16 fractions were collected from the top of the gradient. 20 µg of protein from each fraction was analyzed by immunoblotting; E-Cadherin was used as PM marker (1:1000, mouse monoclonal, BD Bioscience San Jose, CA); VAMP-2 (1:1000, rabbit polyclonal, Assay designs, Ann Arbor, MI) and insulin (1:3000, chicken polyclonal, Abcam) for vesicles fractions.

**Cell transfection and siRNA.** A full-length human nephrin-pCDNA3 vector was used to generate stable transfected MIN6 cells (Fugene-6, Millipore, Millilica, MA). The empty pCDNA3 vector was used as negative control (CTRL). Four separate clones were utilized to repeat experiments. An enriched population of MIN6 cells transfected with a GFP-nephrin construct was also obtained with a combination of antibiotic selection and fluorescence-activated cell sorting (FACS; Aria cells sorter with Diva 6.0 software). For nephrin siRNA in MIN6 cells, a pool of 4 siRNAs was transfected (On Target Plus SMART pool, Dharmachon, Lafayette, CO). siRNAs were tested separately for their efficiency to down-regulated nephrin without affecting four other randomly chosen genes. Nephrin expression after siRNA was verified by PCR and WB. A pool of non-targeting siRNA was utilized as control. In order to perform gene silencing in human islets, islet cells were dissociated by incubation at 37°C for 5 min in Accutase (Innovative cell technologies, San Diego, CA). 0.4x10^6 single cells in each well of a 6-well plate were cultured overnight in CMRL-1066 (Invitrogen) and then transfected with 3.5ug of a pool of 4 non-targeting siRNA and human nephrin siRNA (Dharmacon) using 6uL of DharmaFECT1 in 2mL final volume. After 3.5 days, 0.2x10^6 single islet cells were utilized for PCR and perifusion experiments.

**Confocal microscopy, electron microscopy and immunogold labeling.** The 50A9 anti-nephrin antibody was utilized for immunohistochemistry (Histostain-Plus, Zymed Laboratories, San Francisco, CA). For colocalization studies, immunofluorescence on frozen sections was performed with tyramide signal amplification (TSA kit, Invitrogen). Alexa-647-conjugated antibodies were utilized as secondary antibodies for other targets (1:200, Invitrogen). An irrelevant IgG2b antibody (R&D System, Minneapolis, MN) was utilized as negative control. Antibodies for insulin (1:1000, Mouse monoclonal, NeoMarkers), glucagon (rabbit polyclonal 1:500, Sigma), somatostatin (rabbit polyclonal 1:500, Sigma), CD31 (goat polyclonal, Abcam) and VAMP2 (1:500, mouse monoclonal, Synaptic System, Goettingen, Germany), were utilized. For immunocytochemistry, GFP-nephrin transfected cells were fixed with 4% paraformaldehyde, and counterstained with rhodamine-phalloidin and DAPI.
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When the same cells were utilized to study PM internalization upon glucose stimulation, cells were incubated with 2 or 20mM glucose medium for 20 min at 37°C, followed by a pulse incubation for 30 min with 5µM FM1-43 (Invitrogen) (25). Image acquisition was performed with a Leica SP5-confocal-DMI6000 microscope. For the quantification of vesicles number, cells were fixed in 2% glutaraldehyde/100mM Sucrose/0.05M PO₄. Slides were post-fixed with 2% Osmium, 0.1MPO₄, dehydrated and embedded in Epon-812. Image acquisition was performed with an electron microscope Phillips CM-10 (3,900x magnification). Double-immunogold labeling was performed as previously described (26) on human pancreatic sections. Stainings for insulin (5nm gold) and nephrin (10nm gold) were obtained with a rabbit polyclonal anti-insulin antibody (Dako, Glostrup, Denmark) and the mouse monoclonal anti-nephrin antibody 50A9.

Metabolic studies. In vitro and in vivo analysis of insulin production at baseline and after glucose stimulation were performed as previously described (27). Briefly, control, nephrin siRNA treated and nephrin transfected MIN6 cells were utilized for the in vitro analysis of the glucose stimulation index (expressed as the ratio of insulin secreted at 20mM glucose to 2mM glucose) after 30 min stimulation with 20mM glucose in static incubation experiments. Insulin content (cell lysates plus supernatants) and insulin secretion (cell supernatants) were studied by ELISA (Mercodia, SW) and normalized per DNA content (Quanti-iT PicoGreen dsDNA assay kit, Invitrogen). For perfusion studies in human islets, 200,000 dissociated cells were loaded on microcolumns connected to an inflow and an outflow port of a customized perfusion system (Biorep, Miami, FL). Cells were perfused with media of defined composition (3mM glucose, 11mM glucose and 25mM KCl), and samples collected every two minutes for insulin and DNA determination. For in vivo experiments, pseudoislets were transplanted under the kidney capsule of diabetic immunodeficient athymic nu/nu (nude) mice (Harlan Labs) under protocols approved by the University of Miami IACUC (27). Sampling for glycemia was consistently performed in the morning time during the first hour of light exposure. Intraperitoneal glucose tolerance test (IPGTT) after 5-hr fasting and after administration of a glucose bolus (2 g/kg) was performed to assess the role of nephrin expression on insulin secretion in vivo. Fasting serum prior to IPGTT was tested for the presence of insulin by ultrasensitive insulin ELISA (Mercodia, SW).

Data Analysis. Results represent the mean and standard deviation of 4 to 8 independent experiments. When one-way ANOVA showed statistical significance, results were compared using t-test after Tukey’s correction for multiple comparisons (Graph Pad Prism software). Statistical significance was set at p<0.05.

RESULTS

Nephrin expression is decreased in islets from patients with type 2 diabetes and is regulated by glucose. We have studied nephrin mRNA expression in human islets isolated from 5 patients with type 2 diabetes and 7 non-diabetic patients matched for age, sex, and cold ischemia time (Figure 1A). None of the diabetic patients had reached insulin dependence. Body mass index (BMI) was significantly higher in diabetic than in non-diabetic donors (p<0.05), and nephrin expression was inversely related to BMI (Figure 1A). β-cell content was not significantly different (42±11% in diabetic versus 49±14% in non diabetic patients). Confocal assessment of nephrin expression in human islets from the same patients was consistent with the down-regulation of nephrin expression observed by PCR (Figure 1C). To understand if glucose had a role in
the regulation of nephrin expression observed in diabetes, we studied nephrin expression after acute and chronic exposure to glucose. We found that an acute exposure of islet cells (both human islets and MIN-6 cells) to 20mM glucose causes up-regulation of nephrin expression (Figure 1D,E), while the opposite effect was observed with chronic exposure of MIN-6 cells to 20mM glucose (Figure 1E). Nephrin expression was absent in human islets after 14 days in culture (data not shown).

**Nephrin expression in islets is predominantly in β-cells.** Nephrin immunostaining in normal human pancreas was present in islet cells regardless of islet size (Figure 2A). Immunofluorescence colocalization studies with nephrin, insulin, glucagon, somatostatin and CD31 showed nephrin expression primarily in β-cells (Figure 2B). Nephrin expression in adult and fetal human pancreas, human islets and murine insulinoma cells was confirmed by Western Blot (WB, Figure 2C) and standard RT-PCR (Figure 2D). A 938bp product of PCR amplification was sequenced to confirm specific nephrin amplification. Both transcript splice variants described for podocytes (24) were detected in pancreatic islets.

**Nephrin affects GSIR in MIN6 cells.** Nephrin overexpression was associated with significantly higher insulin content (Figure 3A) and improved GSIR in static incubation experiments when compared to controls (Figure 3B). To further characterize the role of nephrin in β-cell function, insulinoma cells were treated with a selected pool of 4 nephrin siRNAs with 85% efficiency. The depletion of nephrin expression was paralleled by a decrease in insulin content (Figure 3C) and in GSIR in vitro (Figure 3D). Nephrin expression was also compared between the glucose responsive and the glucose unresponsive B1 and C3 MIN6 subclones. MIN6 B1 cells had three fold higher nephrin expression than MIN6 C3 cells (3.1±0.5 versus 1 RQ in B1 versus C3 respectively, p=0.023). However, nephrin transfection of C3 cells was not sufficient to make them glucose responsive. Monolayers of nephrin transfected and empty vector transfected (Control) cells were analyzed by electron microscopy for vesicles quantification. Nephrin transfected cells had a significantly higher content of secretory granules and clusters of secretory vesicles than control cells (Figure 3E). The effect of nephrin overexpression on insulin production and glucose responsiveness was confirmed in vivo upon transplantation of nephrin and control pseudoislets in immunodeficient diabetic mice. Mice receiving nephrin-positive pseudoislets had a significantly lower glycemia starting at day 5 after transplantation (Figure 3F), and glucose tolerance tests performed at day 7 after transplantation showed a faster reversal to normoglycemia in mice that had received nephrin positive pseudoislets when compared to mice with pseudoislets transfected with the empty vector control (Figure 3G). All animals reverted to a glycemia of less than 100 mg/dl after 80 min of glucose administration. Serum insulin was detectable in nephrin-transplanted mice only (data not shown). All mice reverted to hyperglycaemia after nephrectomy of the graft-bearing kidney, suggesting that the transplanted pseudoislets were responsible for the correction of diabetes. No difference in the growth rate of nephrin transfected cells and cells transfected with an empty vector or untransfected cells was observed (Figure 3H).

**Nephrin affects GSIR in human islets.** To overcome the limitation of utilizing insulinoma cells to study the role of nephrin in insulin secretion, we applied nephrin siRNA gene down-regulation to dissociated human islet cells (from four different donors). A mean silencing efficiency of 62% was achieved (Figure 4A). Cell viability evaluated in siRNA treated dissociated cells by Trypan
blue exclusion was also conserved (data not shown). Nephrin siRNA affected both the first and the second phase of glucose response in perifused islet cells when compared to non-targeting siRNA (Figure 4B), resulting in diminished stimulation index (Figure 4C). Response to KCl was conserved (Figure 4B), suggesting that cell depolarization dependent insulin exocytosis is probably not under the direct influence of nephrin.

**Nephrin localizes to both PM and insulin vesicles of pancreatic β-cells.** Confocal microscopy of β-cells from human pancreatic sections revealed a prevalent granular cytoplasmic localization with partial insulin colocalization (Figure 5A). Double-immunogold labeling for nephrin and insulin of normal human pancreas revealed a prevalent localization of nephrin at the periphery of insulin vesicles (Figure 5B). When analyzing vesicles in the process of fusing to the PM, nephrin remains predominantly localized to the fused membrane (Figure 5B). Nephrin localization to the PM independent of insulin vesicles could also be observed (Figure 5B). Nephrin staining was also detectable in few empty vesicles (<5%). Immunofluorescence analysis of nephrin localization in cultured MIN6 cells revealed a partial colocalization of nephrin with insulin and VAMP2 (Figure 5C). Biotinylation experiments revealed a predominant cytosolic localization (unbound) and a limited PM localization (bound, Figure 5D). Proper separation was confirmed by reblotting the membranes for Na/K ATPase (Figure 5D).

**Glucose affects nephrin endocytosis.** Since nephrin was localized in both PM and insulin vesicles, and nephrin endocytosis has been reported in podocytes (28), we investigated if the glucose concentration affected the subcellular localization of nephrin. For this purpose, Green-Fluorescent-Protein (GFP)-nephrin transfected cells were utilized for confocal live imaging (Figure 6A). When cells were co-stained with the live membrane dye FM4-64 utilized to visualize vesicles, nephrin localization was primarily at the PM in cells cultured at 2mM glucose, and partially underwent endocytosis after stimulation with 20 mM glucose (Figure 6B). Subcellular fractionation experiments in untransfected MIN6 cells were utilized to confirm findings by immunofluorescence. Nephrin was clearly detectable in E-Cadherin-positive PM fractions from MIN6 cells cultured in 0.5mM glucose, but the nephrin/E-Cadherin relative intensity was markedly decreased in 25mM glucose (Figure 6C). On the contrary, when analyzing vesicle fractions (VAMP2-positive), nephrin and insulin-positive vesicle fractions were present in both 0.5 and 25mM glucose.

**Nephrin augmentation of insulin secretion is partially dependent on actin cytoskeleton remodeling.** Actin depolymerizing agents have been shown to affect insulin secretion by pancreatic β-cells (15; 29), while more complex may be the role of actin stabilizing/nucleating drugs such as jasplakinolide, which has been clearly shown to increase glucose responsiveness in MIN6 cells when utilized at high concentrations (30). Nephrin increases insulin secretion (Figure 3), and nephrin modulate actin cytoskeleton remodeling (9; 13). Therefore, we tested if nephrin trafficking from the PM to the cytoplasm can be prevented by actin stabilization with low dose jasplakinolide, and if the positive nephrin effect on insulin secretion could be abolished. Both glucose and cytochalasin D induced a redistribution of nephrin from the PM to the cytoplasm (Figures 7A, C), and jasplakinolide completely prevented this effect (Figure 7B), similar to what we observed with 200nM phalloidin treatment (data not shown). The augmentation of GSIR in nephrin-transfected cells was also prevented by jasplakinolide (Figure 7D), suggesting that nephrin-mediated increase in insulin secretion is partially
dependent on the polymerization status of actin. The actin remodeling function of cytochalasin D, jasplakinolide and phalloidin was studied by WB for G- and F-actin subcellular fractions. While untreated MIN6 cells are characterized by a content of F-actin of approximately 40% of total actin, cytochalasin D treatment resulted in a decrease F-actin content to 10% and jasplakinolide and phalloidin both increased F-actin content to 60 and 65%, respectively (Figure 7E).

**DISCUSSION**

The present study unveiled that nephrin affects the function of pancreatic β-cells and promotes insulin secretion in response to glucose. This function of nephrin is independent from its well known and described role in kidney podocytes, where it regulates structure and function of the glomerular slit diaphragm (10; 31; 32). The regulation of insulin secretion by nephrin is also different from a recently described function in skeletal muscles, where nephrin is responsible for secondary fusion of myoblasts into nascent myotubules (33). Despite such apparent discrepant functions of nephrin in different organs, a common mechanisms of action remains the ability of nephrin to modulate actin cytoskeleton remodeling (9; 13), a phenomenon that is equally important for the function of pancreatic β-cells, podocytes and skeletal muscles. The existing controversial data on nephrin expression in pancreatic islets (7; 34; 35) has prompted us to confirm nephrin expression in human pancreas. Here we clearly showed that nephrin is expressed in human pancreas predominantly in pancreatic β-cells (Figure 2). Of note, in β-cells nephrin is localized primarily on the surface of insulin containing vesicles (Figure 5B), suggesting a role for nephrin as a modulator of vesicle trafficking during insulin secretion. This vesicle associated intracellular localization was unexpected, because in podocytes nephrin has been described as a PM protein involved in cell-cell adhesion via homophilic (36; 37) and heterophilic interactions (38-40). As it stands, there are no reports of any endocrine dysfunctions in patients with gene mutations in the nephrin NPHS1 gene, and glucose tolerance tests performed in this subgroup of patients are normal (35). This however does not exclude that nephrin may be relevant to β-cell function, since truncated nephrin isoforms may still be functional in the pancreas. Interestingly, while nephrin in podocytes interacts with other proteins such as podocin and Neph1 to facilitate nephrin-mediated signaling, those proteins have not been detected in the pancreas (41). Together, these observations raise the intriguing possibility that nephrin serves a different function in the pancreas than in the kidney.

To investigate nephrin function, we manipulated nephrin expression in insulinoma MIN6 cells and dissociated human islets and established a role for nephrin in both GSIR and in total insulin production (Figure 3,4). Although the increased total insulin content could result from a direct effect of nephrin on insulin transcription, it could also be the consequence of a well characterized feedback mechanism to increase insulin production and vesicle formation upon insulin secretion (43), as suggested by the electron microscopy quantification of the number of secretory vesicles in nephrin-transfected cells (Figure 3E). It is also possible that nephrin is involved in the machinery enabling glucose entry and metabolism, which may explain why nephrin down-regulation did not affect significantly KCl response. Decreased nephrin expression in the MIN6-C3 subline compared to the MIN6-B1 subline was not responsible for decreased glucose responsiveness. In this context, it will be interesting to test whether this is due to the decreased MIN6-C3 expression of gelsolin, another actin regulator that may play an
important role in glucose stimulated actin remodeling (29).

The localization of nephrin on both the plasma membrane and insulin vesicles, together with the known ability of nephrin to modulate actin cytoskeleton remodeling and to undergo endocytosis in podocytes (28), has prompted us to explore if nephrin endocytosis would occur as a consequence of glucose stimulation and would affect insulin release. Our finding that nephrin undergoes endocytosis upon glucose stimulation is in keeping with the hypothesis that nephrin relocation from the PM to the cytoplasm facilitates insulin release, probably though disruption of the dense web of cortical actin that prevents insulin vesicles fusion to the PM (44; 45). In fact, actin stabilization with phalloidin and jasplakinolide prevented nephrin endocytosis and abolished nephrin effect on increased insulin secretion. We have utilized a dose of jasplakinolide that specifically leads to actin stabilization, as the effects of jasplakinolide on the kinetics of actin polymerization are highly time and dose dependent (46). The opposite effect on insulin secretion and glucose responsiveness were reported in very elegant studies in whole islets and insulinoma cells where a 10-fold higher concentration of jasplakinolide was utilized (30), suggesting a multifaceted role of the actin cytoskeleton in the regulation of GSIR. Further studies will be needed to define the precise mechanism whereby nephrin augments insulin secretion.

Another interesting finding of the present study was the observation that nephrin expression is decreased in islets from diabetic patients when compared to age-matched non-diabetic controls, similar to what has been reported in the kidney (5). BMI and nephrin mRNA expression were inversely related in pancreatic islets, supporting a critical role of nephrin in GSIR. Consistent with these idea, prolonged exposure to high glucose led to a down-regulation of nephrin expression in MIN6 cells, while acute exposure to high glucose increased nephrin mRNA expression. Although the relevance of an in vitro model of acute and prolonged glucose exposure in insulinoma cells remains to be established, the opposite effect of acute and chronic glucose exposure on nephrin gene expression is consistent to what has been described in kidney cells (48), where chronic glucose exposure has been widely accepted as a model of glucotoxicity (18). One possible interpretation of our result is that nephrin allows a more dynamic vesicle mobilization and increased insulin secretion, which may be impaired in diabetes due to the down-regulation of nephrin in pancreatic β-cells.

In conclusion, our results identified nephrin as an important regulator of glucose stimulated insulin secretion in pancreatic β-cells. Our data suggest that nephrin is an active component of the vesicle machinery that guarantees the interaction of insulin vesicles with the actin cytoskeleton and thereby their mobilization towards the PM. Thus, interventions that lead to increased nephrin expression and/or function may delay the need for insulin therapy in type 2 diabetes. Whether an active role of nephrin in vesicle exocytosis in podocytes contributes to the integrity of the slit diaphragm remains to be explored.

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FIGURE LEGENDS

Figure 1. Modulation of nephrin expression by diabetes and glucose. (A) Characteristics of pancreas donors with or without type 2 diabetes. BMI was significantly different among groups. (B) Correlation between the nephrin mRNA expression (RQ) in isolated islets and BMI among the 12 patients studied. An r² of 0.75 with p<0.001 revealed that higher BMIs were associated with lower nephrin expression. Circled in red are the 5 diabetic donors. N=2 denotes overlapping points (C) A representative immunofluorescence confocal image for nephrin, insulin and DAPI in human islets from diabetic and non diabetic donor is shown (20x magnification). A white scale bar of 50 µM is provided. (D-E) Quantitative analysis of nephrin mRNA expression in human islets (D) or MIN6 cells (E, black bars) cultured at either 2mM or 20mM glucose for 24 hours. Chronic exposure to high glucose concentration was tested in MIN6 cells only. Cells ‘starved’ in 2mM glucose for 7 days were assigned to either 2mM or 20mM glucose culture for an additional 14 days. A down-regulation of nephrin mRNA expression was observed (E, white bars). Mean and standard deviation of 4 independent experiments are shown. *p<0.05.

Figure 2. Nephrin is expressed in human islets and β-cells. (A) Immunostaining for nephrin on normal human pancreas revealed a predominant localization of nephrin to both small and large islets. (B) Immunofluorescence colocalization studies revealed a predominant localization of nephrin (red) to insulin-producing cells (green, first row), but not to glucagon, somatostatin, CD31 positive cells (green, subsequent rows). An irrelevant IgG2b was utilized as control and is shown in the last row. (C) WB analysis for nephrin. 1: COS7 (negative control), 2: human podocytes, 3: fetal human pancreas, 4-6: human islets from three different donors, 7: human kidney, 8: MIN6 cells. (D) Standard RT-PCR for nephrin and actin. Samples from 1 to 6 are of human origin, while 7 to 10 are mouse origin. 1: kidney, 2: fetal pancreas, 3,5: whole pancreas from two different donors, 4,6: islets from same donors, 7: kidney, 8: islets, 9: fetal pancreas, 10: MIN6 cells.

Figure 3. Nephrin affects insulin content and glucose responsiveness in MIN6 cells. Nephrin overexpression resulted in increased total insulin content (A), glucose response in static incubation experiments (B, graphic representation with mean and SD of 8 independent experiments) The effect of nephrin siRNA on total insulin content (C) and glucose response in static incubation experiments (D) are also shown. Representative blots showing nephrin protein level in transfected and siRNA treated cells are shown (A, C). Nephrin overexpression resulted in increased number of secretory vesicles (as evaluated by vesicles count in 20 separate electronmicrographs) when compared to empty vector transfected cells (Control) (E). (F) Graphic representation of non-fasting glycemia evaluated daily in diabetic nude mice recipients of nephrin-overexpressing pseudoislets (n=6) or control pseudoislets (n=5). (G) IPGTT profiles of recipients of nephrin-overexpressing or control pseudoislets performed one week after transplantation. (H): Growth curves of monolayer of untransfected cells (control) when compared to empty vector or nephrin transfected cells. *p<0.05, **p<0.01.

Figure 4. Nephrin affects glucose responsiveness in human islets. (A) Dispersed dissociated human islets exposed to either nephrin siRNA or non targeting siRNA (Control). PCR analysis
of nephrin siRNA efficiency from four independent experiments reached a mean value of 62% ***p<0.001. (B) Nephrin siRNA (open circles) or non targeting siRNA (control, filled triangles) were perifused with 3 mM glucose (G), 11mM glucose, 3 mM glucose, 25mM KCl and 3 mM glucose in the time and sequence described in the text box below the graph. A graphic representation of four independent experiments with mean and SD for each time point is provided. *p<0.05, **p<0.001.

**Figure 5.** Nephrin colocalizes with vesicles in β-cells. (A) 63x5x and 40x magnified confocal images of a human pancreatic β-cells and of human islets reveals a granular cytoplasmic nephrin localization (green) with partial overlap with insulin (red). (B) Double immunogold labeling for nephrin (10 nm gold) and insulin (5 nm gold) in human pancreatic sections. Enlarged are a single insulin vesicle (1), an insulin vesicle fused to the PM (2) and a PM without insulin vesicle (3), showing the localization of nephrin in the different phases of insulin secretion. (C) Confocal microscopy at 63x magnification of MIN6 cells showing a more intense nephrin staining (green) in insulin positive cells (blue) but not necessarily in VAMP2 positive cells (red). (D) Biotinylation of MIN6 cells and separation of PM (bound) and cytoplasmic (unbound) fractions revealed that only a modest fraction of nephrin was localized to the PM and that the molecular weight of PM and cytoplasmic nephrin are identical. Blotting for Na/K ATPase confirmed a proper membrane and cytoplasm separation. All experiments were repeated 4 times.

**Figure 6.** Nephrin endocytosis occurs upon glucose stimulation. (A) GFP-nephrin transfected into MIN6 cells localizes both at the PM and in the cytoplasm. (B) MIN6 cells starved in 2mM glucose revealed that nephrin is predominantly localized to the PM and only partially to FM6-64 stained vesicles (red). Upon stimulation with 20mM glucose, nephrin disappears from the PM and localizes solely to the endocytosed vesicles compartment. (C) PM and vesicle fractions were collected from MIN6 cells cultured in either 0.5mM or 25mM glucose for 30 min. Human kidney (HK) was utilized as positive control for nephrin staining. While in 0.5mM glucose nephrin was present in both PM (E-Cadherin positive) fractions and insulin vesicle fractions (insulin positive and VAMP2 positive), in 25mM glucose nephrin almost disappeared from the PM. Shown are a representative blot analysis and the bar graph representation of the nephrin / E-cadherin ratios as well as the nephrin / insulin ratios (mean and SD of three independent experiments). **p<0.01.

**Figure 7.** Nephrin trafficking is dependent on actin and is essential for GSIR. (A) GFP-nephrin transfected into MIN6 cells is mainly localized at the sites of cell-to-cell contact in 2mM glucose. Upon glucose stimulation, nephrin undergoes a time-dependent endocytosis. (B) Actin stabilization with 0.5 µM jasplakinolide prevented glucose-induced relocation of GFP-nephrin from the PM to the cytoplasm. (C) Actin depolymerization with Cytochalasin D led instead to nephrin redistribution from PM to the cytoplasm in a dose dependent manner. (D) The positive effect of nephrin overexpression on GSIR was also totally prevented by pretreatment with 0.5 µM jasplakinolide. *p<0.05, **p<0.01. All experiments were repeated 4 times. (E) MIN6 cells exposed to Cytochalasin D (CyD), Jasplakinolide (Jasp) and phalloidin were analyzed for the change in F-actin content compared to control untreated cells. While an increased G/F actin ratio was observed with CyD (***p<0.001), both jasplakinolide and phallolidin treatment resulted in a 50% reduction of G/F actin ratios (*p<0.05).
Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>Diabetic (N=5)</th>
<th>Non diabetic (N=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>52 ± 12</td>
<td>49 ± 14</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>2/3</td>
<td>4/3</td>
</tr>
<tr>
<td>Cold ischemia (hours)</td>
<td>10.5 ± 3.4</td>
<td>9.8 ± 2.8</td>
</tr>
<tr>
<td>BMI</td>
<td>38 ± 2 *</td>
<td>30.6 ± 9.8</td>
</tr>
</tbody>
</table>

B

*Figure showing Nephrin expression (RQ) vs BMI with a regression line*.

C

*Images showing control and diabetes conditions with labels*.

D

*Bar graph showing Nephrin mRNA (RQ) for Human islets with glucose levels*.

E

*Bar graph showing Nephrin mRNA (RQ) for MIN6 with glucose levels with * symbols indicating significance*.
Nephrin regulates insulin secretion

Figure 3

A

CTRL Nephrin

nephrin

actin

B

Glucose-stimulation index

(28 mM/2 mM)

control

nephrin

C

CTRL siRNA

nephrin

actin

D

Glucoct-stimulation index

(28 mM/2 mM)

Control

nephrin siRNA

E

Control

Nephrin

F

glycemia (mg/dl)

Days after transplantation

G

glycemia (mg/dl)

time (min)

H

cell number

time (hours)

Control

Empty

Nephrin
Figure 4

A

Nephrin mRNA (RQ)

Control | Nephrin siRNA

***

B

Insulin / DNA (mU/µg)

Time (min)

0 10 20 30 40 50 60 70 80

3 G 11 G 3 G 25 KCl 3G
Figure 6
Nephrin regulates insulin secretion

Figure 7

A

0 min  2 min  5 min  15 min  30 min
GLU

B

0 min  2 min  5 min  15 min  30 min
Jasp + GLU

C

0 nM  0.2 nM  2 nM  20 nM  200 nM
Cy D

D

Glucose stimulation index (20 mM / 2 mM)

Nephrin - - + +
Jasplakinolide - + - +

E

G/F actin ratio

Control  Cy D  Jasp  Phalloidin

G F G F G F G F