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RAPID COMMUNICATION

Nucleobindin-2 is a positive regulator for insulin-stimulated glucose transporter 4 translocation in fenofibrate treated E11 podocytes

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Abstract. The physiology of insulin signaling under normal and disease conditions is well studied in classical insulin target tissues, but not in podocytes. To examine insulin stimulation of podocyte GLUT4 translocation, we established a protocol involving treatment with the PPARα agonist fenofibrate to induce E11 podocyte differentiation within 48 hours rather than 7-10 days, which is required for differentiation under the reported protocol. This allowed us to transiently introduce GLUT4 reporter cDNA and RNAi and thereby to examine the regulatory pathway involved. Here we demonstrate that treatment with 200μM fenofibrate for 36 hours following transfection had a dramatic effect on podocyte morphology, induced several podocyte specific protein expression markers (G protein-coupled receptor 137B, chloride intracellular channel 5, and nephrin) and resulted in insulin-stimulated GLUT4 translocation. In addition, Nucleobindin-2 was found to constitutively associate with Septin 7 (the repressor of GLUT4 translocation), and knockdown of Nucleobindin-2 was found to completely abrogate insulin-stimulated GLUT4 translocation. Together, these data suggest that Nucleobindin-2 may repress Septin7-induced inhibition of insulin-stimulated GLUT4 translocation in podocytes.

Key words: Nucleobindin-2, Podocyte, Insulin resistance, Glucose transporter 4 translocation, Insulin

NUCLEOBINDINS (Nucleobindin-1 [CALNUC, NUCB1] and Nucleobindin-2 [NEFA, NUCB2]) are a class of multi-domain Ca^{2+} binding proteins that share 62% amino acid identify [1]. The cellular localization of Nucleobindin-1 has been variously suggested to be a nuclear protein [2], secreted protein [3, 4], a resident endoplasmic reticulum protein [5] and as well as localized to the cytoplasm [6]. Similarly, Nucleobindin-2 (NEFA) was also reported as localized to the cytoplasm, on the plasma membrane, as well as being secreted in the culture medium [7].

Nucleobindin-2 contains 420 amino acids that can be further processed to generate an 82 amino terminal peptide termed Nesfatin-1. However the physiological action of Nucleobindin-2 is still poorly defined. It has been reported that Nesfatin-1 but not Nucleobindin-2 is anorexigenic as Nesfatin-1 blocked food intake whereas a Nucleobindin-2 mutant that could not be processed into Nesfatin-1 was without effect [8]. Recently Broberger *et al.* reported that Nucleobindin-2 co-localizes with insulin in rat and human pancreatic β cells [9]. Since islet Nucleobindin-2 content isolated from an animal model of type 2 diabetic rats was lower than that of non-diabetic control animal, Nucleobindin-2 was suggested to play a regulatory role in insulin secretion and as a potential contributor to diabetic pathology [9]. Recently centrally administrated Nesfatin-1 was reported to increase peripheral and hepatic insulin sensitivity by decreasing gluconeogenesis and promot-

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Abbreviations: Clic5, Chloride intracellular channel 5; VAMP2, Vesicle-associated membrabe protein 2; GLUT4, Glucose trasnporter 4; S.D., Standard deviation; NUCB2, Nucleobindin-2; PPAR, Peroxisome proliferator-activated receptor

ing peripheral glucose uptake *in vivo* [10]. In addition, Nesfatin-1, but not Nucleobindin-2, was reported to increase glucose uptake and GLUT4 translocation in cardiomyocytes [11].

Recently, it has been reported that insulin stimulates GLUT4 translocation of podocytes that it negatively regulated by Septin7 [12]. Disturbances of podocyte slit diaphragm structure has been defined as an important cause of proteinuria and proteinuria may directly result as a consequence of podocyte insulin resistance [13]. Thus a detailed understanding of normal and pathophysiologic insulin signaling is critical for our understanding of kidney dysfunction in diabetes and the metabolic syndrome. As the potential role of Nucleobindin-2 in podocyte biology has not been examined, we have determined that Nucleobindin-2 is a novel Septin7 binding partner and that Nucleobindin-2 negatively regulates insulin-stimulated GLUT4 translocation in podocytes.

Materials and Methods

Reagents

GPR137B, Clic5, GLUT4, Nephrin, Syntaxin4, Septin7, and Nucleobindin-2 polyclonal antibody were obtained from Sigma-Aldrich. ECL and ECL+plus Western Blotting Detection System were obtained from GE Healthcare. The anti-mouse and anti-rabbit IgG-HRP were obtained from PIERCE. Cell culture media and reagents were from Invitrogen Life Technologies. ShRNA constructs against Mus. musculus Nucleobindin-2 was purchased from OriGene. All of other chemicals used in this study were purchased from Sigma-Aldrich.

Cell culture

Studies involved use of a conditionally mouse E11 podocyte cell line with modified culture condition. Usually the podocytes were cultured in RPMI-1640 media containing 10% fetal bovine serum, 100U/mL penicillin, and 100μg/mL streptomycin and were propagated in a medium containing 10U/mL mouse interferon-γ at 33^{o}C [12, 14]. To differentiation, cells were plated in collagen type I-coated flasks under the nonpermissive condition (37^{o}C without interferon-γ) for 7-10 days [12, 14]. However, under these conditions, we are not able to perform the experiments combined with transient transfection by electroporation because the expressed protein will start to diminish 48 hours after the transfection [15]. We therefore established an efficient transfection methodology (approximately 70%) for the E11 podocyte cell line as previously we reported [16].

However we have to maintain the cells at 37^{o}C and lift up the cells by trypsinization. Then we have to replate the electroporated cells in suitable plate and let the replated cells differentiate at 37^{o}C within the next 48 hours. To explore this we tested proliferator-activated receptor-α agonist fenofibrate because previous study reported that peroxisome proliferator-activated receptor-α is renoprotective and attenuates Doxorubicin-induced podocyte foot process effacement [17]. Also, According to FIELD study, DAIS study, and ACCORD Lipid study, fenofibrate was proved to reduce microalbuminuria in diabetic patients [18, 19, 20, 21]. Thus we speculated that fenofibrate might contribute to differentiate podocytes. In this manuscript we treated E11 podocytes with 200μM of fenofibrate suspended in dimethyl sulfoxide.

Transfection of E11 podocytes

E11 podocytes were suspended by mild trypsinization and electroporated with CsCl double banding plasmid under low-voltage condition (0.2kV, 950μF) [16]. The cells were then allowed to adhere to culture dishes for 30-48 h. Twelve hours following electroporation, either dimethyl sulfoxide (1:1,000 dilution) or 200μM of fenofibrate was added to the fresh culture medium to induce E11 podocyte cell differentiation.

Immunoprecipitation and Immunoblotting

Scraped frozen cells were rocked for 10 min at 4^{o}C with NP-40 lysis buffer (25mM Hepes, pH 7.4, 10% glycerol, 1% NP-40, 50mM sodium fluoride, 10mM sodium phosphate, 137mM sodium chloride, 1mM sodium orthovanadate, 1mM PMSF, 10μg/mL aprotinin, 1μg/mL pepstatin, 5μg/mL leupeptin). Insoluble material was separated from the soluble extract by centrifugation for 10 min at 4^{o}C, and the total protein amount in the supernatant was determined by BCA method. Immunoprecipitations were performed by using 2mg of the cell extracts incubated with 4 μg of a Septin7 polyclonal antibody for 2 hours at 4^{o}C. The samples were then incubated with protein A-Sepharose for 1 hour at 4^{o}C. Either immunoprcipitaed samples or whole cell lysates samples were resuspended in SDS sample buffer (125mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 100mM dithiothreitol, 0.1%

(w/v) bromophenol blue), and heated at 100 ^{0}C for 5 min. Samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes. The samples were immunoblotted with monoclonal or polyclonal specific antibody.

GST fusion protein precipitation

Cell lysates from E11 podocytes treated with 200μM of fenofibrate were incubated with either GST alone or with GST-Nucleobindin-2 fusion proteins immobilized on glutathione-agarose beads for 1 hour at 4^{o}C [22]. The beads were extensively washed three times with the lysis buffer. The retained proteins were eluted with SDS-sample buffer, heated at 100^{o}C for 5 min and separated by SDS-PAGE. The gel was stained by GelCode Blue Stain Reagent (PIERCE) following the manufacture's instruction.

TOF-Mass analysis

TOF-Mass analysis was submitted to Filgen, Inc. (Nagoya, Japan).

Quantification of insulin-stimulated GLUT4 translocation

Quantification of transfected GLUT4 translocation was determined using a qualitative colorimetric assay as previously described [16, 23]. Briefly, E11 podocytes were co-transfected with 200μg of eGFP-cMyc-GLUT4 plus 400μg of various other cDNAs as indicated in each figure. Following basal or hormonal stimulation, the cells were cooled to 4^{o}C and incubated with a myc antibody followed by an HRP-conjugated anti-myc antibody. The specific cell surface bound HRP was then determined by incubation with the o-phenylenediamine dihydrochloride peroxidase substrate.

Statistical analysis

All values are expressed as mean +/- standard deviation (S.D.). Data were evaluated for statistical significance by analysis of variance and *t* test using the InStat 2 program.

Results

Effect of fenofibrate on E11 podocytes

The FIELD, DIAS and ACCORD Lipid studies indicated that fenofibrate was effective at reducing microalbuminuria in diabetic patients [18, 19, 20, 21]. Fenofibrate is a selective peroxisome proliferator-activated receptor-α (PPARα) agonist that is renal-protective and attenuates doxorubicin-induced podocytes foot process effacement [17]. However there are no reports studyting whether PPARα agonist affects podocytes differentiation. To explore this possibility, we treated E11 cells with and without fenofibrate. E11 podocytes treated with 200μM of fenofibrate resulted in a dramatic morphological change after 8 hours and were substantially elongated with increased arborization by 24 hours. In contrast, the E11 cells in the absence of fenofibare did not undergo any significant morphological change (Fig. 1A b, c).

In Fig. 1B, we confirmed that fenofibrate treated E11 podocytes expresses podocytes specific markers as GPR137B, Clic5, Nephrin, and GLUT4 by western blotting (Fig. 1B). Having established transient transfection [9-16] and modified differentiation strategy, we estimated GLUT4 translocation of cultured podocytes with or without fenofibrate treatment using a GLUT4 reporter construct. As shown in Fig. 1C-a, insulin was an ineffective stimulator of GLUT4 translocation in undifferentiated E11 cells. In contrast, following fenofibrate treatment, insulin was capable of inducing an approximately 2-fold stimulation of GLUT4 translocation (Fig. 1C-b). This extent of insulin-stimulated GLUT4 translocation is similar to that previously reported using a conventional culture and differentiation protocol [12, 14, 16].

Nucleobindin-2 was identified as a binding partner for Septin7

To screen for potential Nucleobindin-2 binding partners in podocytes, we mixed either GST or GST-Nucleobindin-2 with lysates from differentiated E11 podocytes followed by SDS-PAGE and the gel was stained with GelCode Blue (Fig. 2A). The approximately 50 kDa band indicated by the arrow in the GST-Nucleobindin-2 lane was cut out and applied to TOF mass spectrometry analysis for sequence identification and multiple peptide sequences for Septin7 were found. Immunoprecipitaion of Septin7 from control and insulin-stimulated cells demonstrated that Septin7 directly binds to Nucleobindin-2, consistent with the mass spectroscopy identification. However, there was no significant effect of insulin on the Septin7/Nucleobindin-2 interaction (Fig. 2B). Having a high quality of transfection efficacy [16], we introduced Nucleobindin-2 RNAi that reduced Nucleobindin-2 protein levels by

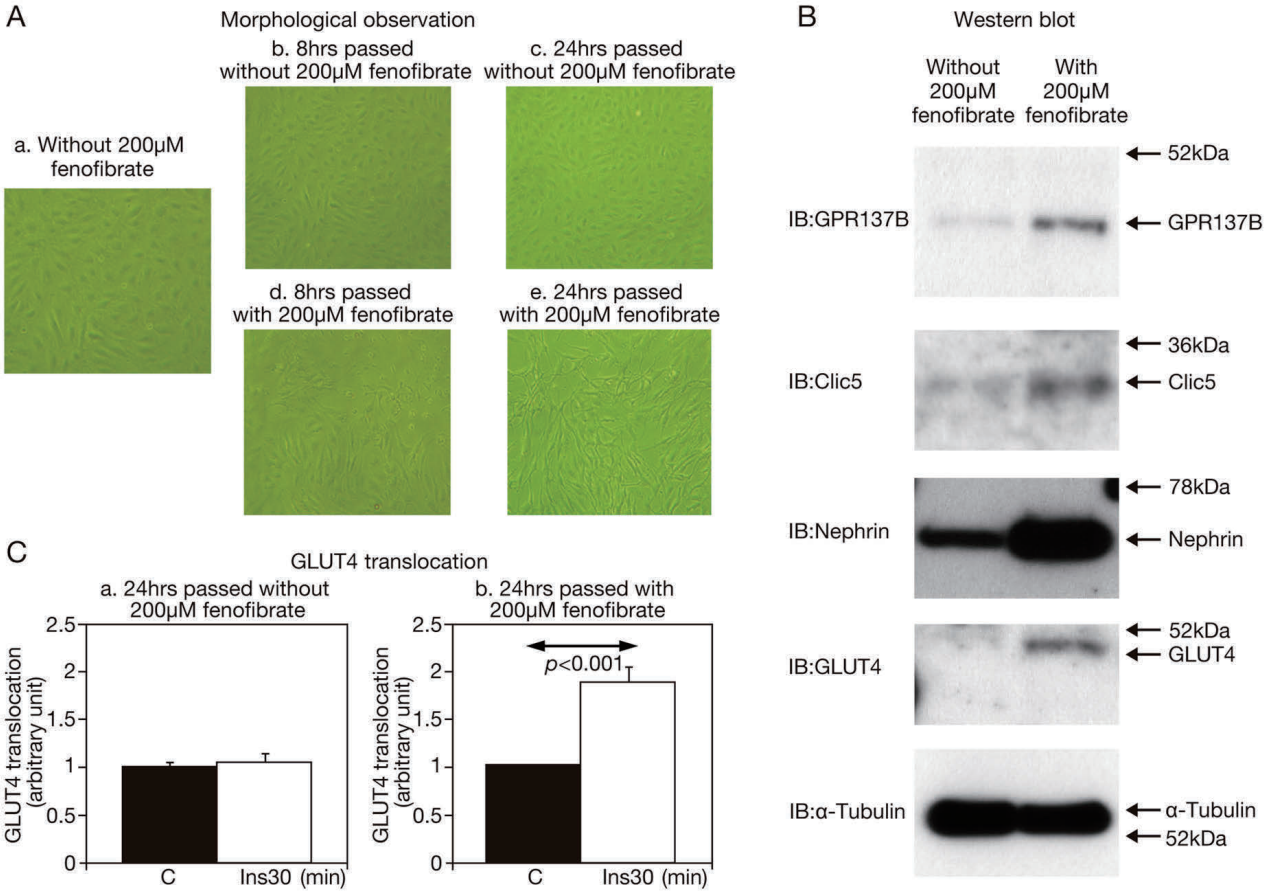

Fig. 1 Effect of fenofibrate on E11 podocytes

A) 200μM of fenofibrate started show morphological change 8 hours later (Fig. 1A-d) and completely changed E11 podocytes as shown Fig. 1A-e. As a control, vehicle alone did not make any change (Fig. 1A-b, 1A-c). Data show representative experiments independently performed and each experiment was repeated three times.

B) Estimation of quality about fenofibrate treated E11 podocytes is shown by western blotting. In this panel podocytes specific marker proteins such as GPR137B, Clic5, and Nephrin are compared between fenofibrate untreated (left lane) and treated (right lane) E11 cells. α-Tubulin blotting tells us each lane has equal protein loading amount. Data show representative experiments independently performed and each experiment was repeated three times.

C) Estimation of GLUT4 translocation of E11 podocytes without or with fenofibrate treatment is represented. On the left panel (Fig. 1C-a), GLUT4 translocation evaluated by colorimetric assay in E11 podocytes without fenofibrate is shown and on the right panel (Fig. 1C-b) GLUT4 translocation evaluated by colorimetric assay in E11 podocytes with fenofobrate is shown. Experiments were independently performed three times and expressed as the mean ± S.D.

approximately 60-70% (Fig. 2C, upper panel). The reduction in Nucleobindin-2 protein had no significant effect on Septin7 protein levels (Fig. 2C, lower panel). However, the loss of Nucleobindin-2 resulted in a near complete inhibition of insulin-stimulated GLUT4 translocation with no significant effect on the basal level of cell surface GLUT4 protein (Fig. 2D). Theses data suggested that endogenous Nucleobindin-2 is required for insulin-stimulated GLUT4 translocation and may function as an inhibitor Septin7.

Discussion

Recently Nucleobindin-2 was reported to regulate insulin secretion, peripheral glucose uptake, and hepatic gluconeogenesis and decreased Nucleobindin-2 expression has been suggested to partly account for several metabolic consequences in diabetic animal models [8-11]. In addition to the regulation of insulin secretion and sensitivity in peripheral tissues, Nucleobindin-2/Nesfatin-1 proteins have also been shown to regulate hypothalamic function in the control of hepatic glu-

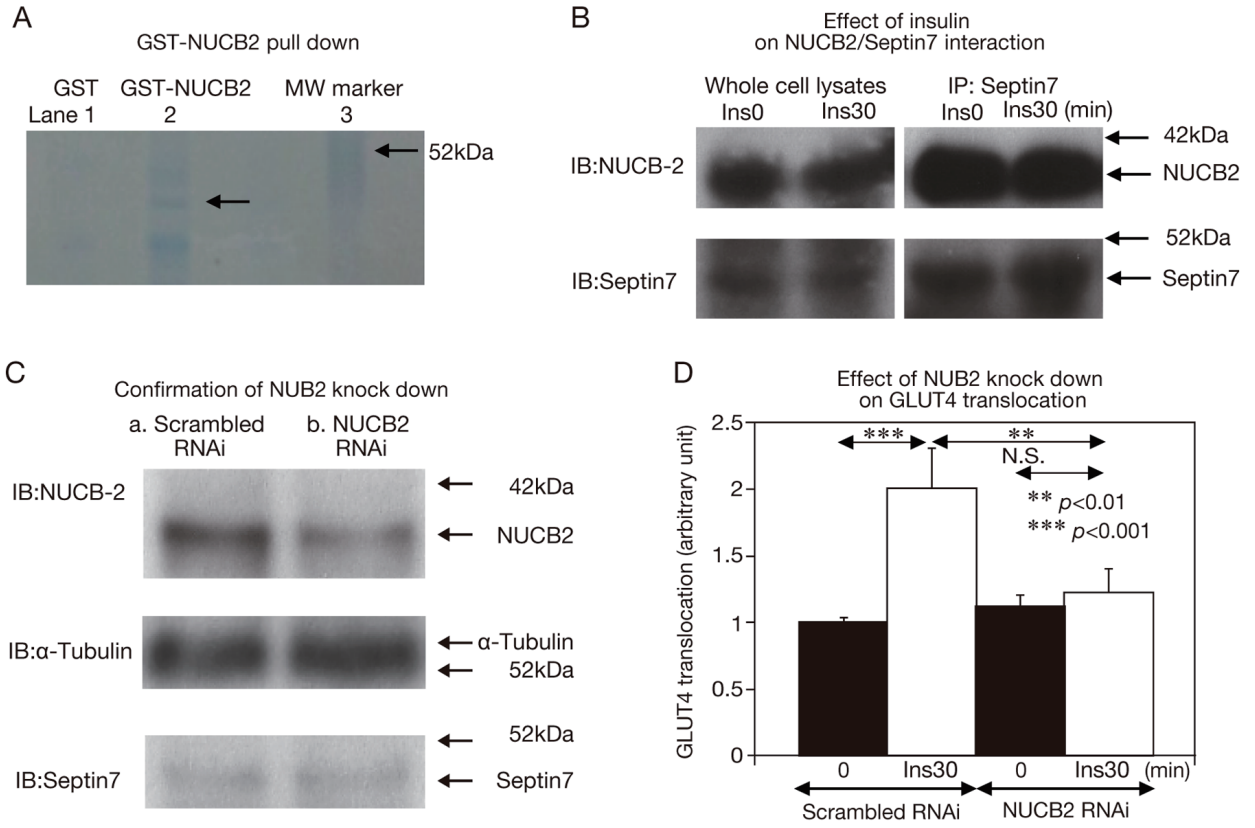

Fig. 2 Nucleobindin-2 was identified as a binding partner for Septin7

A) We mixed either GST (lane 1) or GST-Nucleobindin-2 (lane 2) with clear lysates from E11 podocytes with fenofibrate treatment and run on SDS-PAGE. The gel was conducted to GelCode Blue staining and the specific band (indicated by arrow in lane 2) for GST-Nucleobindin-2 around 50 kDa was cut out and applied to TOF Mass analysis to identify the molecules. Data show representative experiments independently performed and each experiment was repeated three times.

B) Co-immunoprecipitation experiment results by using Septin7 specific antibody is shown in lower panel. The samples were obtained from cells treated without or with 100nM insulin stimulation for 30 min. In the upper panel, Nucleobindin-2 (NUCB2) amount associated Septin 7 is shown. Theses experiments were independently performed three times.

C) We introduced Nucleobindin-2 RNAi by electroporation to knock down Nucleobindin-2 and reduced Nucleobindin-2 expression by 60-70% compared to control samples transfected with scrambled RNAi. Data show representative experiments independently performed and each experiment was repeated four times.

D) Estimation of Nucleobondin-2 knock down effect on insulin-stimulated GLUT4 translocation in E11 podocytes is shown. GLUT4 translocation was estimated by colorimetric assay in the presence of 200μM of fenofibrate in E11 podocytes. Theses experiments were independently performed three times and expressed as the mean ± S.D.

cose production [10]. Despite these studies, the functional role if any for Nucleobindin-2 in kidney function particularly podocytes insulin action has not been addressed.

Fenofibrate was reported to protect podocytes from doxorubicin-induced injury [17] but until today there are no reports to clearly state that fenofibrate alone can differentiate podocytes. We demonstrated that fenofibrate treated E11 podocytes showed morphological change resembling those reported for the differentiation of E11 podocytes by the conventional method [12, 14]. In addition, we observed the up regulation of several podocyte specific markers (GPR137B, Clic5, and Nephrin) indicating the formation of a podocyte phenotype [24]. It is important to note that the fenofibrate treatment required only 2 days of treatment and was as effective to the previous reported method that requires 7-10 days. In any case, our results are consistent with a previous large-scale clinical trial showing that fenofibrate administration protects against microalbuminuria in diabetic patients [18-21]. As PPARγ was also reported to have renal protective function [14], future

studies are needed to examine the relative contributions of PPARα and/or PPARγ agonist in the differentiation response.

In this report we also described a colorometric assay for the quantification of GLUT4 translocation in differentiated podocytes that was originally developed for differentiated cultured 3T3-L1 adipocytes. In this assay we need to express an exofacial myc tagged GLUT4 and to perform the assay within 48 hours after the transfection as differentiated podocytes due to the transient nature of myc-GLUT4 expression. The ability of fenofibrate to induce E11 podocyte cell differentiation within this time frame is necessary for the successfulness of this approach.

This report presents evidence that Nucleobindin-2 is expressed in podocytes and that Nucleobindin-2 is a Septin7 binding partner, although this interaction does not appear to be regulated by insulin. In several cell types the cellular localization of Nucleobindins appears to be variable with a distributions reported in most subcellular compartments [1-7] and the subcellular distribution within podocytes remains to be examined. A previous study indicated that Septin7 inhibits insulin-stimulated glucose uptake by blocking Nephrin-VAMP2 interactions [13]. Therefore Nucleobindin-2 expression status is potentially one mechanism to depress this inhibition. Interestingly, recently a podocyte specific GLUT4 knockout mouse phenotype (podocytes specific insulin resistance model) was reported to be protective from diabetic nephropathy. The report suggested that there might exist a compensatory mechanism to increase podocytes size (hypertrophy) despite a reduction in podocytes number [25]. In human being it is less likely to happen that GLUT4 null situation in podocytes but there might exist similar compensation mechanism exists and if so if it is impaired in diabetic patients, treatment of insulin resistance from the point of Nucleobindin-2 manipulation in podocytes would be a promising approach to potentially rescue the risk of diabetic nephropathy. In addition, future studies are needed to determine the effect of Nucleobindin-2 deficiency *in vivo* and whether Nucleobindin-2 expression is altered under various physiologic and pathophysiologic states.

Disclosure

All the authors declared no competing interests.

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