Podocyte-specific GLUT4 deficient mice have fewer and larger podocytes and are protected from diabetic nephropathy.

Running title: Podocyte GLUT4 and proteinuria

Johanna Guzman¹,², Alexandra N Jauregui¹, Sandra Merscher-Gomez², Dony Maiguel¹, Cristina Muresan², Alla Mitrofanova², Ana Diez-Sampedro³, Joel Szust¹, Tae-Hyun Yoo²,⁴, Rodrigo Villarreal¹,², Christopher Pedigo², R. Damaris Molano¹, Kevin Johnson¹, Barbara Kahn⁵, Bjoern Hartleben⁶, Tobias B. Huber⁶, George W Burke III⁴, E. Dale Abel⁷, Frank C. Brosius⁸, Alessia Fornoni¹,².

¹Diabetes Research Institute, ²Division of Nephrology and Hypertension, ³Department of Physiology, ⁴Department of Surgery, University of Miami Miller School of Medicine, Miami USA; ⁵Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; ⁶Division of Nephrology, Freiburg University, Freiburg, Germany, University of Miami L Miller School of Medicine, Miami USA; ⁷Division of Endocrinology, Metabolism and Diabetes and Program in Molecular Medicine, University of Utah; ⁸Division of Nephrology, University of Michigan, Ann Arbor, MI.

Correspondence to:

Alessia Fornoni, MD PhD FASN
Associate Professor of Medicine
Diabetes Research Institute and Division of Nephrology and Hypertension
University of Miami Miller School of Medicine
1450 NW 10th Ave, Miami, FL, 33136
Tel: (305) 243-6558 (9945), Fax: (305) 243-3506
e-mail: afornoni@med.miami.edu

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ABSTRACT

Podocytes are a major component of the glomerular filtration barrier, and their ability to sense insulin is essential to prevent proteinuria. Here we identify the insulin downstream effector GLUT4 as a key modulator of podocyte function in diabetic nephropathy.

Mice with a podocyte specific deletion of GLUT4 (G4 KO) did not develop albuminuria despite having larger and fewer podocytes than wild type (WT) mice. Glomeruli from G4 KO mice were protected from diabetes-induced hypertrophy, mesangial expansion and albuminuria and failed to activate the mammalian target of rapamycin (mTOR) pathway. In order to investigate if the protection observed in G4 KO mice was due to failure to activate mTOR, we used three independent in vivo experiments. G4 KO mice did not develop lipopolysaccharide-induced albuminuria, which requires mTOR activation. On the contrary, G4 KO mice as well as WT mice treated with the mTOR inhibitor rapamycin developed worse adriamycin-induced nephropathy than WT mice, consistent with the fact that adriamycin toxicity is augmented by mTOR inhibition.

In summary, G4 deficiency in podocytes affects podocyte nutrient sensing, results in fewer and larger cells and protects mice from the development of diabetic nephropathy. This is the first evidence that podocyte hypertrophy concomitant with podocytopenia may protect from proteinuria.

INTRODUCTION

Ever since it was demonstrated that insulin infusion can induce an acute transient increase in albumin excretion rate (1), the possibility of a direct effect of insulin signaling in glomerular cell function has been suggested. In fact, insulin resistance correlates with the development of microalbuminuria in patients with both type 1 or type 2 diabetes (2-5), their siblings (6; 7) and in subjects without diabetes (8). Furthermore, impaired insulin sensitivity in diabetic patients is associated with altered renal cell glucose metabolism that may directly contribute to progressive renal damage independently of
hyperglycemia (5). The evidence that some of the patients with genetic mutations in the insulin receptor (IR) may develop a renal disease that resembles diabetic nephropathy (DN) (9), supports an important role for functional insulin signaling in renal disease and provides the rationale for interventions that target different elements of the insulin receptor signaling cascade.

Podocytes are glomerular cells of the kidney that depend on the integrity of their actin cytoskeleton to prevent the development of microalbuminuria (10). Podocytes have been reported to be a target of insulin (11) and to become insulin resistant prior to the development of microalbuminuria in animal models of diabetes (12). Mice with a podocyte-specific deletion of the IR gene develop a phenotype resembling DN in the absence of hyperglycemia (13; 14), suggesting that insulin signaling regulates podocyte function independently of blood glucose levels. Traditionally, the final step in insulin action is physiological modulation of glucose uptake and metabolism (15). Thus, disrupting glucose uptake by facilitative glucose transporters (GLUTs) might negatively affect podocytes similarly to what is observed in IR-deficient podocytes. However, glucose uptake and metabolism may also affect nutrient sensing pathways independently of insulin signaling (16). In particular, the AMP-activated protein kinase (AMPK) (17) and the mammalian target of rapamycin (mTOR) pathways (18; 19) are key direct modulator of podocyte function that can be affected by intracellular glucose.

Podocytes express several glucose transporters (1, 2, 3, 4 and 8) that are modulated by high glucose and by diabetes (11; 20-22). The overexpression of GLUT1 in mesangial cells leads to a phenotype resembling DN (23) and is associated with an upregulation of mTOR (24). This is not the case for podocytes, where podocyte-specific overexpression of GLUT1 prevents mesangial expansion (25), suggesting the presence of cell type specific functions of GLUTs. In this study, we hypothesized that podocyte GLUT4 deficiency mitigates mTOR dependent signaling independently of insulin signaling, thus protecting not only from the development of DN but also from other experimental models of proteinuria associated with mTOR signaling.
RESEARCH DESIGN AND METHODS

Patient cohort. Kidney samples, results of serology and urinalysis of the patients were made available through the organ procurement agency of our Institution and the Institutional Review Board approved their utilization. Briefly, kidney biopsies were collected by the organ procurement agency from three patients with type 1 diabetes, normoalbuminuria and high glomerular filtration rate, six patients with type 1 diabetes and microalbuminuria and six age and sex matched patients without diabetes. In addition, three patients with hypertensive nephrosclerosis were studied.

Mice utilization and sacrifice. Twenty B6.Cg-m +/+ Lepr\(^{db}\)/Lepr\(^{db}\) (db/db) and sixteen B6.Cg-m +/+ Lepr\(^{db}\)/+ (db/) female mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All animal procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC). Podocyte-specific GLUT4 KO mice (G4 KO) were generated by breeding floxed GLUT4 mice (26) with a podocin-CRE mice (27). Wild type (WT) and Heterozygous (G4 Het) littermates were utilized as controls. Metabolic measurements were performed weekly until mice sacrifice. Systolic Blood Pressure (SBP) was measured at 32 weeks using a non-invasive tail cuff blood pressure unit and BpMonWin software (IITTC Life Science, Inc, Woodland Hills, CA) as previously reported (28). At sacrifice, mice were perfused with isotonic saline and tissue collected for histological analysis and glomeruli isolation.

Experimental models of proteinuria. For LPS injection, we used an intraperitoneal (IP) injection of 300 \(\mu\)g ultrapure LPS (Sigma) in mice that was shown in prior studies to induce proteinuria (29). Spot morning urine samples of all mice at time points 0h, 12h, 24h, 36h, 48h, 60h and 72h were collected and mice were scarified at 72 hours as described above. In a second experiment aimed at the collection of glomerular lysates and tissue sections after LPS injection, six mice per group were studied and tissue and glomerular lysates were collected at 36 hours, prior to the recovery from proteinuria. For the induction of diabetes, we performed intraperitoneal (IP) injections of streptozotocin (STZ, 50 mg/Kg daily on 5 consecutive days). For adriamycin experiments, 12 weeks old mice were challenged with a single injection of adriamycin (20 mg/kg). Four G4 KO and 4 WT
mice on a mixed background were exposed to adriamycin. Three mice per group served as controls. Urine were collected every other day for the first week and then weekly for four weeks. Mice were sacrificed as described above. For rapamycin experiments, adriamycin-treated G4 KO and WT mice were concomitantly injected with rapamycin (InvivoGen, 1 mg/kg/day IP), three times per week for five weeks. Fasting glucose levels were measured weekly from tail blood samples using a glucometer (Bayer, Pittsburgh, Pennsylvania). Albumin content was measured weekly by enzyme-linked immunoadsorbant assay (Bethyl Laboratories, Montgomery, TX). Urinary creatinine was assessed by an assay based on the Jaffe method (Stanbio, San Antonio, TX). Values are expressed as µg albumin/mg creatinine.

**Assessment of mesangial expansion, glomerular surface area, podocyte number and size.** Periodic acid-Shiff’s (PAS) staining of 4 µm thick slides were performed for the quantitative analysis of mesangial expansion calculated as the percentage of the total glomerular area that was PAS positive as reported (30). For podocyte counts, paraffin-embedded, paraformaldehyde-fixed tissue was sectioned at 3 and 9 µm and was performed as previously published (30). GV/P is a variable that incorporates the relationship between both podocyte number and glomerular basement membrane surface area, is the reciprocal of podocyte density, and is a useful measure of the degree of podocyte reserve (31). Measurements of the GLEPP1-positive area were performed in 50 consecutive glomerular tuft areas and the individual podocyte volume determined by dividing the mean GLEPP1-positive (podocyte) volume per glomerulus by the mean podocyte number as described (32).

**Immunofluorescence staining.** A standard immunofluorescence protocol was followed utilizing the following primary antibodies: polyclonal guinea pig anti-Nephrin antibody from Fitzgerald (Acton, MA), rabbit polyclonal anti-GLUT4 antibody (Millpore, Billerica, MA), rabbit polyclonal anti-GLUT1 antibody (Millpore, Billerica, MA), rabbit polyclonal anti-synaptopodin antibody (gift of Dr. Peter Mundel), mouse monoclonal anti-active RhoA antibody (New East Biosciences, Malvern, PA), rabbit polyclonal anti pS6 antibody (4858s Cell Signaling). Alexa-conjugated secondary antibodies from Invitrogen were utilized, images were acquired by confocal microscopy and quantified by Image J.
**Podocyte culture, western blotting and cell size analysis.** Primary podocytes were isolated as previously described (12). Protein concentrations of each sample prepared with CHAPS buffer were measured using the DC protein assay (Bio-rad, Carslbad, CA), and an equal amount of protein was loaded onto 4–20% SDS-PAGE gels (Biorad) and transferred to nitrocellulose membranes (Bio-Rad) for western blot analysis. The following primary antibodies were utilized: mouse monoclonal anti Glut4 and anti-RhoA and goat polyclonal anti-NPR-B (C-19) (Santa Cruz, Santa Cruz CA), Rabbit anti Glut1 (Millipore, Temecula CA), mouse monoclonal anti-Gapdh (Calbiochem), rabbit polyclonal anti synaptopodin and podocin (gifts of Dr. Peter Mundel), Guinea Pig anti-nephrin (Fitzgerald), rabbit polyclonal anti-ZO1 (Zymed), rabbit polyclonal anti-phosphorylated and total S6, P70S6K, AMPK, AKT, MAPK42/44, IKKα and MyD88 (Cell Signalling). Fixed and washed podocyte cells lines were imaged using Opera LX™ High Content Screening System (Perkin Elmer, Waltham, MA) with 10x air objective or 20x water objective. Cell area and cell number were determined using Acapella™ Software (Perkin Elmer, Waltham, MA). For the quantitative measurement of DNA, cells were lysed in T-PER reagent (Pierce, Rockford, IL) and DNA measurement performed using Pico-Green Kit (Invitrogen). Annexin V (Vybrant Apoptosis Assay, Invitrogen) staining was utilized to study apoptosis and quantitative assessment performed by flow cytometry with BD Diva 6.0 Software (BD LSRII System, BD Biosciences, San Jose, CA). Staurosporin (1mM for 2 hours, Sigma) was utilized as positive control.

**siRNA experiments.** Mouse podocytes were cultured as described. At day 10, cells were exposed to a pool of on target plus siRNA for GLUT4, GLUT1, AMPKα, ČČČ1 (Dharmacon, Lafayette CO), and on target plus non-targeting siRNA. After 6,12, 24 and 48 hours cells were collected in lysis buffer in the presence of protease inhibitors (Bio-Rad, Hercules CA) for protein analysis, in sample buffer for the analysis of apoptosis and fixed and stained with phalloidin to study the morphology of the actin cytoskeleton. Images were acquired by confocal microscopy as well as by light microscopy. Annexin V (Vybrant Apoptosis Assay, Invitrogen) staining was utilized to study apoptosis and
quantitative assessment performed by flow cytometry with BD Diva 6.0 Software (BD LSRII System, BD Biosciences, San Jose, CA). Staurosporin (1 mM for 2 hours, Sigma) was utilized as positive control.

**Statistical analysis.** All data are shown as mean and standard deviations. Four to eight independent experiments were performed for *in vitro* studies. Four to fourteen mice per group were used for *in vivo* experiments, which were repeated twice to allow for statistical analysis of western blots from glomerular lysates. Statistical analysis was performed with one-way ANOVA. When one-way ANOVA showed statistical significance, results were compared using *t*-test after Tukey’s correction for multiple comparisons. Results were considered statistically significant at *p*<0.05.

**RESULTS**

**Glomerular GLUT4 and GLUT1 expression are differentially modulated at different stages of human and experimental DN.**

In normal human glomeruli GLUT4 colocalizes with synaptopodin positive podocytes whereas only partial colocalization of GLUT1 with synaptopodin was detected (Figure 1A). GLUT4 and GLUT1 mRNA expression were studied in microdissected glomeruli from cadaveric patients. Six type 1 diabetic patients with microalbuminuria and normal creatinine (T1D-MA) and 3 type 1 diabetic patients with normoalbuminuria and serum creatinine <0.6 mg/dl (T1D-NA) were compared to 6 age, cold ischemia time and sex matched nondiabetic patients with normoalbuminuria and normal creatinine (Controls, Supplemental Table1). GLUT4 expression was significantly upregulated in glomeruli from T1D-NA patients with very low creatinine when compared to controls (Figure 1B). Glomeruli from T1D-MA were characterized by decreased GLUT4 expression (*p*<0.05) and increased GLUT1 expression (*p*<0.05) when compared to non-diabetic patients (Figure 1C) and to three patients with hypertensive glomerulosclerosis (data not shown). A similar pattern of GLUT4 and GLUT1 expression was observed in normoalbuminuric 12 week old db/db mice (Figure 1D) and microalbuminuric 22 week old db/db mice when compared to age matched db/+ (12) (Figure 1E).
order to understand if the increased GLUT4 expression in early nephropathy was of podocyte origin, we utilized primary culture of podocytes from db/db and db/+ mice of 12 weeks of age (12), and demonstrated that db/db podocytes are characterized by increased GLUT4 mRNA and protein expression (Figure 2A), unchanged GLUT1 expression, increased baseline glucose uptake (Figure 2C) but decreased ability of insulin to cause glucose uptake and GLUT4 translocation to the plasma membrane (Figure 2D and 2E).

Mice with a podocyte specific deletion of GLUT4 have no apparent renal phenotype at baseline. In order to determine if GLUT4 deficiency has a causative role in DN, we studied podocyte-specific GLUT4 knock out mice (G4 KO). While we were able to demonstrate effective recombination by both immunofluorescence and western blotting (Figure 3A and 4A), G4 KO mice did not develop any albuminuria (Figure 3B) or hypertension (Figure 3C) when compared to WT. Histological analysis of G4 KO kidney sections revealed unchanged mesangial expansion and glomerular surface area (Figure 3D, E, F). Western blot analysis of isolated glomeruli demonstrated that GLUT4 deficiency was not accompanied by a compensatory increase in GLUT1 (Figure 4A). Furthermore, glomeruli from G4 KO mice were characterized by increased nephrin and podocin expression while ZO-1 and synaptopodin were not modified (Figure 4A). Interestingly, glomeruli from G4 KO mice demonstrated almost undetectable S6 and p70S6 phosphorylation with unchanged AKT and increased AMPK and MAPK42/44 phosphorylation (Figure 4B), suggesting that the ability to suppress AMPK and to activate mTOR is impaired in podocytes of G4 KO mice, while insulin signaling through AKT is preserved.

G4 KO mice are characterized by decreased podocyte number and increased podocyte size. Although G4 KO mice were characterized by a generally normal renal phenotype at baseline, the number of podocytes/glomerulus was significantly lower in G4 KO mice when compared to WT (Figure 5A). This phenotype was already apparent at four week of age. This was accompanied by an
equal glomerular volume (Figure 5B), an increased glomerular volume per podocyte (GV/P, Figure 5C) as well as an increase in the Glepp1 positive area (Figure 5D). Primary podocyte cultures from three different G4 KO mice and three WT mice demonstrated increased podocin, ZO-1, nephrin expression with unchanged synaptopodin expression (Figure 5E), similar to what we had found on isolated glomeruli (Figure 5A). G4 expression was totally suppressed in primary podocyte cultures from G4 KO mice but was easily detected in cultures from WT mice, further confirming that effective recombination had occurred (Figure 5E). G4 KO cell lines were characterized by increased protein:DNA content (Figure 5E) and increased cell size (Figure 5F) when compared to WT cells. This was not accompanied by increased apoptosis (Figure 5G). The increased cell size was specifically associated with G4 deficiency, as siRNA for G4 in mouse podocytes resulted in increased cell size that was not observed in G1 siRNA-treated podocytes (Figure 6A and B). Increased cell size in G4 siRNA treated podocytes occurred in association with decreased P70S6K phosphorylation and increased AMPK phosphorylation (Figure 6C, D, E) but did not require AMPK or TSC1 expression (Figure 6F, G).

**Diabetic mice with a podocyte-specific deletion of GLUT4 are protected from glomerular hypertrophy and albuminuria.** As enhanced mTOR activity can induce glomerular hypertrophy in diabetes (18; 19; 33), we tested if G4 KO mice were protected from hyperglycemia-induced glomerular hypertrophy and albuminuria. Indeed, G4 KO mice were partially protected from the development of glomerular hypertrophy (Figure 7A and B). As glomerular hypertrophy in diabetes is a RhoA dependent phenomenon (34; 35), and RhoA activation is linked to mTOR (36), we tested if glomeruli from G4 KO mice were characterized by a decreased RhoA activity and/or expression. Active RhoA was increased in diabetic WT mice when compared to WT controls but was not modified by diabetes in G4 KO mice (Figure 7C). In addition, while diabetes increased total RhoA levels in WT glomeruli, G4 KO glomeruli showed decreased RhoA levels at baseline and RhoA did not increase after the induction of diabetes (Figure 7D). G4 KO mice were partially protected from the
development of albuminuria at 12 weeks (Figure 7E), and this trend was preserved at 24 weeks (Figure 7F) and at 32 weeks (Figure 7G). At 32 weeks, quantitative evaluation of PAS positive material demonstrated a significant reduction of PAS positive glomerular area in G4 KO diabetic mice when compared to diabetic WT (Figure 7H and I). Diabetes caused a further significant reduction in podocyte number in both WT and G4 KO mice (Figure 7J). Unlike WT mice, glomeruli from G4 KO mice were protected from increased S6 phosphorylation in response to hyperglycemia (Figure 7K). Blood glucose, body weight and kidney weight were not different between WT, G4 Het and G4 KO diabetic mice: mean body weights at sacrifice were 25, 24 and 26 g respectively; mean kidney weights 0.3 g in each group, and mean blood glucose 528, 570, 542 mg/dl respectively.

**G4 KO mice are protected from the development of LPS-induced albuminuria.** As LPS signaling requires activation of mTOR (33), we hypothesized that G4 KO mice would be resistant to albuminuria induced by LPS. As predicted, G4 KO mice were protected from the development of LPS-induced albuminuria (Figure 8A). In fact, while LPS increased myeloid-differentiation-primary-response-gene-88 (MyD88) in glomeruli from WT mice (Figure 8B), such increase was not observed in glomeruli from G4 KO mice. Deficient LPS signaling in G4 KO mice was also demonstrated by an inability of LPS to cause synaptopodin degradation (Figure 8B). Nephrin subcellular distribution from a linear physiological pattern to a predominantly granular intracytoplasmic pattern was also observed in WT mice but not in G4 KO mice (Figure 8C), and is consistent with the fact that mTORC1 activation results in nephrin mislocalization (19). While LPS caused an increase in the phosphorylation of S6 in WT mice, this phenomenon was not observed in G4 KO mice (Figure 8D).

**Mice with a podocyte specific deletion of GLUT4 are susceptible to adriamycin-induced nephropathy.** As the suppression of the mTOR pathway augments the toxicity of adriamycin in cancer cells (37), we tested the hypothesis that G4 KO mice would become susceptible to adriamycin induced nephropathy even on a background that is primarily C57BL6 and FVB, which are both known
to be resistant to Adriamycin-induced nephropathy (38). While WT mice developed a mild mesangial expansion and albuminuria over time, G4 KO mice demonstrated a much higher degree of albuminuria at 3 weeks and increased mesangial expansion at 28 days after adriamycin administration (Figure 9A, B and C). A significant reduction in podocyte number after adriamycin administration was observed in G4 KO mice but not in WT mice (Figure 9D). Overall, these data suggested that GLUT4 deficiency may affect the development of proteinuria through an intrinsic inability to activate mTOR. To further test prove this hypothesis in vivo, we administered rapamycin to adriamycin-treated WT and G4 KO mice. While rapamycin worsened albuminuria, mesangial expansion and podocytopenia in WT mice (Figure 9E, F, G and H), no changes were observed in G4 KO mice.

**DISCUSSION**

The ability of a cell to sense nutrients has emerged as a critical regulator of cellular homeostasis in several cell types including podocytes of the kidney glomerulus (17-19; 39). Several key pathways are involved in nutrient sensing, such as mTOR, AMP-activated protein kinase (AMPK) and sirtuin (SIRT) (16). Among these pathways, mTOR signaling is suppressed when deprivation of nutrients, such as glucose, occurs (40; 41). Clinical and experimental data support a role for nutrient sensing in the pathogenesis of diabetes and its complications (16). However, key signaling elements of the nutrient sensing pathway that may affect cell function even in the absence of diabetes or any metabolic disorders remain to be identified. It is possible that glucose uptake through facilitative glucose transporters (GLUTs) affects podocyte function through their effect on nutrient sensing, independently of extracellular glucose levels and of preserved insulin signaling. To address this question, we generated a podocyte-specific G4 KO mouse and investigated if GLUT4 deficiency modulates podocyte function at baseline and in experimental models of proteinuria.

We focused our attention on GLUT4, because GLUT4 is one of the major GLUTs expressed in podocytes (11; 20), it has a predominant podocyte localization in normal human kidney (Figure 1A),
and it is the most profoundly regulated GLUTs after insulin stimulation (42). We found that while glomerular GLUT4 expression is up-regulated in human and experimental nephropathy with normoalbuminuria and glomerular hypertrophy (Figure 1B and 1D), it becomes down-regulated once microalbuminuria develops (Figure 1C and E). Up-regulation of GLUT4 in early nephropathy occurs in podocytes and is accompanied by increased baseline glucose uptake but decrease insulin responsiveness (Figure 2). Because insulin resistance appears to precede microalbuminuria (2; 12), and because podocyte-specific IR deletion causes proteinuria (13), we expected that the deletion of the final downstream effector of insulin action (GLUT4) would result in a similar phenotype. To our surprise, no apparent renal phenotype was observed at baseline after podocyte-specific deletion of GLUT4 in mice (Figure 3), suggesting that the phenotype of podocyte-specific IR deficient mice is independent of GLUT4 expression. However, podocyte-specific GLUT4 deficiency in vivo (Figure 4) resulted in the activation of AMPK and suppression of mTOR in isolated glomeruli. This is consistent with the observation that increased expression of GLUT4 in muscle fibers coincided with the activation of the mTOR pathway (43). Furthermore, there was a significant reduction in podocyte number (Figure 5A), which has been shown to be a major mechanism driving glomerulosclerosis (44). Interestingly, this inborn reduction in podocyte number did not result in any structural glomerular pathology or any increase in albuminuria. In fact, as opposed to what was shown in the rat model of acquired podocytopenia, decreased podocyte number in our model was found to be associated with increased podocyte size in vivo (Figure 5D) and in vitro (Figure 5F, 6A and 6B) that occurred independently of mTOR, P70S6K and AMPK. While primary mTORC1 activation has been clearly linked to podocyte hypertrophy (18; 45), our data suggest that podocyte hypertrophy in G4 KO mice is mediated by a mTOR-independent mechanism. The possibility of mTOR-independent cellular hypertrophy is strongly supported by the evidence that mice with the cardiac overexpression of non-functional kinase-dead mTOR develop the same degree of cardiac hypertrophy than compared to nontransgenic littermates (46). Moreover, the absence of albuminuria and glomerular pathology in podocyte-specific G4 KO mice demonstrates that inborn podocyte hypertrophy does not necessarily
cause functional and pathologic changes in the glomerulus as observed in forms of acquired maladaptive hypertrophy such as diabetic nephropathy. Increased podocyte volume in G4 KO mice was associated with increased synthesis of structural components (Figures 4A and 5E) and with the prevention of glomerular hypertrophy and glomerulosclerosis observed in WT mice in experimental models of diabetes (Figure 7). This suggests that a primary form of adaptive podocyte hypertrophy as observed in G4 KO mice may protect from glomerular hypertrophy, while concomitant maladaptive podocyte and glomerular hypertrophy occurred in the ageing rat (47) or in diabetic mice (18; 19; 45). Whether podocyte hypertrophy is the reason why G4 KO mice do not develop glomerular hypertrophy remains to be established. While mTOR inhibition and AMPK activation could explain the lack of glomerular hypertrophy observed in G4 KO mice, increased slit-diaphragm proteins might contribute to diminish albuminuria. It is possible that podocyte and glomerular volume in G4 KO mice are not related, and that G4 KO mice are protected from glomerular hypertrophy through similar mechanisms to those observed in calorie-restricted rats (32). Glomeruli from diabetic G4 KO mice also showed suppression of RhoA expression and activity (Figure 7D), which is consistent with the finding that mTOR inhibition in cancer cells suppresses RhoA expression and activity (36). Although cardiac myocytes in heart-specific G4 KO mice develop hypertrophy (26), this could result from the marked compensatory increase in GLUT1 and glucose uptake found in the cardiac G4 KO model, which does not occur in the podocytes from podocyte-specific G4 KO mice and which has been shown to cause up-regulation of mTOR in mesangial cells (24). The apparent discrepancy related to the fact that G4 KO mice and podocyte-specific GLUT1 overexpressing mice are both protected from DN could be explained by the significant down-regulation of GLUT4 expression in the latter model (25).

In order to investigate in vivo if impaired mTOR signaling protects G4 KO mice from the development of DN, we utilized three independent experimental models of proteinuria that were previously linked to the mTOR signaling pathway. As mTOR inhibition prevents LPS signaling in neutrophils (33), we challenged G4 KO mice with LPS. As expected, we were able to show that LPS failed to signal and to
cause proteinuria in G4 KO mice (Figure 8A, B). Among other experimental models of proteinuria, adriamycin has been extensively utilized and usually leads to a phenotype that is very mild in mice with a background that is primarily FVB and C57Bl6, which is the background of the G4 KO mice (38). Importantly, however, adriamycin has been demonstrated to have enhanced chemotherapeutic effect in the setting in mTOR inhibition (37). We therefore tested the hypothesis that G4 KO mice would develop a more severe renal phenotype than their WT littermates after exposure to adriamycin. Indeed, we were able to demonstrate that G4 KO mice were characterized by worsened albuminuria and mesangial expansion than WT mice after administration of adriamycin. In order to establish a link between mTOR suppression and adriamycin toxicity in our model, we performed a third in vivo experiment and demonstrated that addition of rapamycin to adriamycin worsened the renal phenotype of WT but not G4 KO mice (Figure 9), consistent with the finding that rapamycin treatment aggravates the loss of glomerular filtration rate in patients with FSGS (48). Our data suggest that inability to activate mTOR in GLUT4 deficient hypertrophic podocytes may influence the development of proteinuria in experimental animals. However, restoration of mTOR or suppression of AMPK in GLUT4 deficient podocytes does not restore cell size. Whether GLUT4 deficiency influences mTOR independent hypertrophic responses to natriuretic peptides, leucine or glutamate remains to be established. Furthermore, our data suggest a functional dissociation between insulin receptor and GLUT4 signaling. In fact, as insulin sensitizers improve albuminuria in clinical and experimental studies (49-51), one may expect that G4 deficiency would result in a disease phenotype due to the alteration of the physiological regulation of glucose uptake in response to insulin, as suggested based on prior studies from us and others (14). However, our findings obtained with the in vivo models do not support this hypothesis and add further complexity to the role of insulin signaling in podocyte function. Our in vivo observations are also consistent with the fact that insulin sensitizers of the class of thiazolidinediones (TZDs) prevent mTOR dependent signaling (52) while facilitating insulin signaling in several cells, including the podocyte (53). Additional studies are needed to investigate if the function of GLUT4 in podocyte is independent of insulin signaling. In this respect, it would be
interesting to determine if podocyte-specific deletion of GLUT4 is sufficient to restore the glomerular phenotype of mice with a podocyte-specific deletion of the IR. The evidence that GLUT4 binds aldolase, which is known to interact with actin, suggests that GLUT4 may directly regulate actin remodeling (54). Finally, our data suggest that GLUT4 may regulate podocyte function and that strategies that decrease GLUT4 expression and/or function may be beneficial in proteinuric kidney diseases.

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AUTHOR CONTRIBUTIONS. J. G. performed some of the in vivo experiments and the isolation of primary podocytes, A.J. performed some of the in vitro experiments and the siRNA experiments, S.M.G. designed the in vivo experiments and assisted in results interpretation, D.M. performed immunofluorescence studies, C. M. maintained the mice colony, A.D-S. studied protein content and glucose uptake, J.S. collected and analyzed human samples, T-H. Y., R.V. A.M. and C.P performed western blot analysis and urine analysis, R.D.M. assisted with mice sacrifice, K.J. processed tissue samples, B.K. and E.D.A provided the mice and assisted in results interpretation, B.H. contributed to the analysis of podocyte size, T.B.H. and G.W.B helped with result interpretation and manuscript preparation, F.B. and A.F. wrote the manuscript. Guarantor: Alessia Fornoni.

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FIGURE LEGENDS

**Figure 1. GLUT4 and GLUT1 expression in clinical and experimental DN.** (A) Confocal images of GLUT4 and GLUT1 expression in normal human glomeruli, showing the colocalization with synaptopodin utilized as a podocyte specific marker in the micrographs at higher magnification. Scale bar: 25 µM. (B) Glomerular GLUT4 mRNA expression is upregulated in patients with T1D, normoalbuminuria and low serum creatinine. (C) Once microalbuminuria is established, GLUT4 mRNA is down regulated when compared to non-diabetic controls. The opposite is true for GLUT1. (D, E) Bar graph analysis of GLUT4 and GLUT1 expression in glomeruli isolated from db/db and db/+ mice at 12 and 22 weeks of age. The number of mice utilized for each experiment is also shown. **p<0.001, *p<0.05.

**Figure 2. GLUT4 is upregulated in podocytes from db/db mice prior to the onset of microalbuminuria.** Bar graph representation of GLUT4 (A) and GLUT1 (B) mRNA and protein expression in primary podocytes isolated from db/db and db/+ mice at 12 weeks of age. Four
independent experiments were performed and a representative western blot is shown. *p<0.05, **p<0.01. (C) Bar graph analysis of three independent glucose uptake experiments performed in db/+ and db/db podocytes in the presence or absence of 100 nM insulin (INS) for 15 minutes. *p<0.05 when comparing Insulin to no insulin. #p<0.05 when comparing db/db to db/+. (D) Bar graph analysis of three independent experiments for the quantification of the percentage of cell membrane from db/db and db/+ podocyte positive for GLUT4 before and after insulin treatment (INS). *p<0.05 (E) Representative GLUT4 and GLUT1 immunofluorescence staining in db/+ and db/db podocytes at baseline and after insulin stimulation. Rhodamine phalloidin is utilized to stain actin fibers and DAPI to stain nuclei. Scale bar 50 µM.

**Figure 3. Podocyte specific G4 KO mice do not have an apparent renal phenotype.** (A) Representative confocal images demonstrating effective deletion of GLUT4 (green) in podocytes identified as synaptopodin positive cells (red). (B) Time course analysis of urinary albumin/creatinine ratios in morning urine collection in G4 KO and WT mice (N=6 each). (C) Bar graph analysis of systolic blood pressure (SBP) measurements at 32 weeks demonstrating no difference between WT and G4 KO mice. (D) Representative PAS staining of renal cortex of WT and G4 KO mice. Scale bar: 25 µm. (E) Bar graph analysis of mesangial expansion in G4 KO and WT mice (N=6 each). (F) Quantitative evaluation of glomerular surface area in WT and G4 KO mice (µm²).

**Figure 4. Glomeruli from G4 KO mice are characterize by increased synthesis of structural components and suppression of mTOR signaling.** (A) Representative western blot analysis and relative bar graph quantification of proteins isolated from glomeruli microdissected and pooled from four WT and four G4 KO mice (representative of two separate experiments performed in duplicate) showing markedly reduced GLUT4 expression, unchanged GLUT1 expression, increased nephrin and podocin expression, unchanged ZO-1 and synaptopodin expression. (B) Western blot analysis of glomeruli from G4 KO mice demonstrating almost undetectable S6 and p70S6 phosphorylation with
preserved AKT phosphorylation and increased AMPK and MAPK 42/44 phosphorylation when compared to WT. *p<0.05, **p<0.01, ***p<0.001.

**Figure 5. Podocyte specific G4 KO mice have fewer and larger podocytes.** (A) Bar graph representation and representative image of the number of podocytes/glomerulus, that was significantly lower in G4 KO mice when compared to WT. G4 KO mice demonstrated no difference in glomerular volume (B), but increased glomerular volume per podocyte (GV/P)(C), when compared with WT. (D) Bar graph representation and representative image of G4 KO mice demonstrating significantly higher Glepp1 positive area when compared to WT. (E) Representative western blot analysis and relative bar graph analysis from primary podocyte cultures from three different G4 KO mice and WT mice. Undetectable GLUT4 confirmed effective recombination and increased podocin, ZO-1, synaptopodin and nephrin expression was observed in G4 KO podocytes when compared to WT. Protein:DNA content was also increased in G4 KO podocytes when compared to WT. (F) Quantitative analysis of cell size demonstrated increased cell size in each of three G4 KO cell lines when compared to the mean size of WT cell lines. (G) Bar graph analysis of Annexin V staining (% of positive cells) in G4 KO podocytes when compared to WT or staurosporin treated WT cells. *p<0.05, **p<0.01, ***p<0.001.

**Figure 6. Podocyte hypertrophy is specific to G4 deficiency and is independent of mTOR and AMPK.** (A) siRNA for G4 (siG4) in mouse podocytes resulted in increased cell size as demonstrated in bright field images (BF) and redistribution of F-actin as demonstrated by phalloidin staining (F-actin) 48 hours after siRNA treatment. (B) Quantitative analysis of cell size demonstrated increased cell size in siG4-treated cells when compared to siG1-treated cells and non-targeting siRNA treated cells (NT). (C) Representative western blot for phosphorylated and total P70S6K and AMPK in siG4, siG1 or siG4+G1 mouse podocytes. (D, E) Bar graph analysis of P70S6K and AMPK (phosphorylated over total) in siG4, siG1 or siG4+G1 mouse podocytes. *p<0.05, **p<0.01. (F) siRNA for TSC1 and for AMPKα in mouse podocytes was effective (representative western blot),
caused podocyte hypertrophy per se, but did not restore the hypertrophic phenotype of siG4 podocytes. (G) Bar graph analysis for the quantitative evaluation of mean cell area in siRNA treated mouse podocytes. *p<0.05, **p<0.01.

**Figure 7. G4 KO mice are partially protected from the development of diabetic nephropathy.**
(A) Bar graph analysis of glomerular surface area of WT, G4 Het and G4 KO mice with (DM+) or without (DM-) diabetes. (B) Representative PAS staining of glomeruli from WT, G4 Het and G4 KO with diabetes (DM) or without diabetes (Control). (C) Representative immunofluorescence staining showing increased active RhoA in glomeruli from WT diabetic mice; G4 KO glomeruli have less active RhoA at baseline as well as after the induction of diabetes. (D) Western blot analysis of lysates obtained from pooled isolated glomeruli of WT, G4 Het and G4 KO mice, demonstrating unchanged GLUT1 expression and increased RhoA expression in WT diabetic mice but not in G4 KO mice. (E) Bar graph analysis of urinary albumin to creatinine ratios in WT and G4 KO with (DM+) or without (DM-) diabetes. G4 KO mice were partially protected from the development of albuminuria at 12 weeks, and (F) this trend was partially preserved at 24 weeks and (G) at 32 weeks. Representative PAS images (I) and quantitative evaluation of PAS positive glomerular area (H) in WT and G4 KO after 32 weeks with (DM) or without (Control) diabetes. (J) Bar graph analysis of podocyte number in both WT and G4 KO mice with (DM+) or without (DM-) diabetes. ***p<0.001 and *p<0.05 when comparing diabetic mice to non diabetic mice of the same genotype. ##p<0.01 when comparing G4 KO diabetic mice to WT diabetic mice. (K) Representative immunofluorescence confocal images for pS6 (red), synpo (green) and DAPI (blue) in kidney sections from WT and G4 KO controls and diabetic (DM) mice.

**Figure 8. G4 KO mice are protected from LPS induced proteinuria.** (A) Time course analysis of albumin/creatinine ratios in morning urine collection in G4 KO (n=11) and WT (N=9) mice. The experiment was confirmed in a subsequent group of mice utilized to collect proteins from pooled microdissected glomeruli (B). Also shown in B is the western blot analysis of pooled glomeruli
collected after 36 hours of LPS injection at time of maximal albuminuria from 4 mice per group, demonstrated that while systemic administration of LPS increases MyD88 in WT mice, LPS signaling is impaired in G4 KO mice, where the LPS induced degradation of synaptopodin and nephrin is also prevented. (C) Representative confocal images of nephrin in controls (CTRL) and LPS treated mice. (D) Representative immunofluorescence confocal images for pS6 (red), synpo (green) and DAPI (blue) in controls (CTRL) and LPS treated mice. Scale bar: 25 μm. **p<0.01, ***p<0.001

Figure 9. G4 KO mice develop worse adriamycin nephropathy than WT. (A) Time course analysis of urinary albumin to creatinine ratios in WT and G4 KO mice after a single dose of adriamycin at time 0. (B) Representative low and high magnification images of PAS stained tissue sections from adriamycin treated WT and G4 KO mice. (C) Bar graph analysis showing significant increase in mesangial expansion in G4 KO mice after adriamycin. Four mice per group were utilized. (D) Bar graph representation of podocyte number showing significant reduction after adriamycin in G4 KO mice but not in WT mice. (E) Time course analysis of urinary albumin to creatinine ratios in WT and G4 KO mice after a single dose of adriamycin followed by rapamycin injections three times per week, during five weeks. (F) Representative images of PAS stained tissue sections, and (G) bar graph analysis of mesangial expansion in adriamycin and adriamycin-rapamycin treated WT and G4 KO mice. (H) Bar graph analysis showing significant decrease in podocyte number/glomerulus in WT after adriamycin plus rapamycin, and no changes in G4. *p<0.05, **p<0.01 when comparing treated mice to control mice of the same genotype. #p<0.05 and ##p<0.01 when comparing G4 KO mice to WT mice in the same treatment group.
Figure 1

A) Image showing immunofluorescence staining for GLUT4 and SYNPO in Type 1 DM with MA and normal GFR.

B) Bar graph showing GLUT4 and GLUT1 mRNA expression levels in Type 1 DM with NA and high GFR. * indicates statistical significance.

C) Bar graph showing GLUT4 and GLUT1 mRNA expression levels in Type 1 DM with MA and normal GFR. * indicates statistical significance.

D) Graph showing GLUT4 and GLUT1 mRNA expression levels in 12 weeks old db/db mice with NA. * indicates statistical significance.

E) Graph showing GLUT4 and GLUT1 mRNA expression levels in 22 weeks old db/db mice with MA. * indicates statistical significance.

ND: Normal diabetes; T1DM: Type 1 diabetes; NA: Normal albuminuria; MA: Macular albuminuria; GFR: Glomerular filtration rate; RQ: Relative quantification; **: p < 0.01; *: p < 0.05; NS: Not significant.
Figure 2

A. GLUT4 mRNA (RQ) and GLUT4/actin ratio for db/+ and db/db mice under control and insulin-treated conditions.

B. GLUT1 mRNA (RQ) and GLUT1/actin ratio for db/+ and db/db mice under control and insulin-treated conditions.

C. Glucose uptake (pmol/15 min) for db/+ and db/db mice under control and insulin-treated conditions.

D. GLUT4 localization (% of positive cell surface) for db/+ and db/db mice under control and insulin-treated conditions.

E. Immunofluorescence images showing GLUT4 and GLUT1 localization under control and insulin-treated conditions for db/+ and db/db mice.
Figure 3
Figure 4
Figure 5
### Figure 6

**Panel A**
- NT, siG4, siG1, and siG1+G4 showing changes in F-actin and BF (40x).

**Panel B**
- Graph showing Mean Cell Area (pixels²) with significantly increased values in siG4, siG1, and siG1+G4 compared to NT.

**Panel C**
- Western blots for pP70S6K, P70S6K, pAMPK, AMPK, and GAPDH.

**Panel D**
- Bar graph showing P70S6K (phospho/total) with significant differences in siG4 and siG1+G4 compared to NT.

**Panel E**
- Bar graph showing AMPK (phospho/total) with significant differences in siG4 and siG4+G1 compared to NT.

**Panel F**
- Images showing NT, siG4, siG1+G4, siG4+siTSC1, siAMPKα, siG4+siAMPKα, siG4+siTSC1, and siAMPKα.

**Panel G**
- Bar graph showing Mean Cell Area (pixels²) with significant differences in siG4, siG4+siTSC1, siG4+siAMPKα, and siG4+siAMPKα compared to NT.

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**Diabetes**
Figure 7

A) Glomerular surface area (mm²) measured by ImageJ software. Data are represented as mean ± SEM. ***p < 0.001 compared to WT DM, **p < 0.01, #p < 0.05 compared to WT DM.

B) Representative images of glomeruli stained with PAS dye. Scale bar: 100 μm.

C) Western blot analysis for Glut1, RhoA, and β-actin in the kidney of WT, G4 Het, and G4 KO mice under control and DM conditions. 

D) Western blot analysis for SYNPO, pS6, DAPI, and β-actin in the kidney of WT, G4 Het, and G4 KO mice under control and DM conditions.

E) Albumin/creatinine ratio (mg/mg) at 24 weeks measured by the standard amid method. Data are represented as mean ± SEM. ***p < 0.001 compared to WT DM, **p < 0.01, #p < 0.05 compared to WT DM.

F) Albumin excretion (μg/mg) at 24 weeks measured by the standard amid method. Data are represented as mean ± SEM.

G) Albumin excretion (μg/mg) at 32 weeks measured by the standard amid method. Data are represented as mean ± SEM.

H) PAS positivity (% glomerular area) measured by ImageJ software. Data are represented as mean ± SEM. *p < 0.05 compared to WT DM.

I) Representative images of glomeruli stained with PAS dye.

J) Podocytes/glomerulus ratio measured by ImageJ software. Data are represented as mean ± SEM. ***p < 0.001 compared to WT DM, **p < 0.01, #p < 0.05 compared to WT DM.
Figure 8

A) Alb/Creat (ug/mg) over time (hrs) for WT (n=9) and G4 KO (n=11).

B) Alb/Creat (ug/mg) over time (hours) for WT, G4 KO, WT LPS, G4 KO LPS.

C) CTRL and LPS images for WT and G4 KO.

D) SYTPO, pS6, DAPI images for WT and LPS.
Figure 9
SUPPLEMENTAL TABLE 1

**Donors’ characteristics.** Six diabetic microalbuminuric (T1D-MA), three diabetic normoalbuminuric (T1D-NA) and six non-diabetic (ND) cadaveric donors that were matched for age, sex, serum creatinine and mean cold ischemia time were studied. ACR=albumin to creatinine ratios on a spot urine. CIT=cold ischemia time.

<table>
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<th>ND (n=6)</th>
<th>T1D-MA (n=6)</th>
<th>T1D-NA (n=3)</th>
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<td><strong>Age</strong></td>
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<td><strong>ACR (mg/g)</strong></td>
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<td>130±89</td>
<td>21±7.8</td>
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