Peer reviewed version

License (if available):
Other

Link to published version (if available):
[10.1152/ajprenal.00541.2017](#)

[Link to publication record in Explore Bristol Research](#)
PDF-document

University of Bristol - Explore Bristol Research
General rights

Modulation of APOL1-miR193a Axis Prevents Podocyte Dedifferentiation in High Glucose Milieu

Abheepsa Mishra[1], Kamesh Ayasolla[1], Vinod Kumar1, Xiqian LAN[1]. Himanshu Vashistha[2], Rukhsana Aslam[1], Ali Hussain[1], Sheetal Chowdhary[1], Shadafarin Marashi Shoshtari[1], Nitpriya Paliwal[1], Waldemar Popik[3], Moin A. Saleem[4], Ashwani Malhotra[1],Leonard G Meggs[2], Karl Skorecki[5], and Pravin C. Singhal[1]

[1]Immunology and Inflammation center, Feinstein Institute for Medical Research and Zucker School of Medicine at Hofstra-Northwell, New York, USA, [2]Ochsner Clinic, New Orleans, USA, [3]Meharry Medical College, Nashville, TN, USA, [4]Academic Renal Unit, University, Bristol, Bristol, UK, [5]Technion – Israel Institute of Technology, and Rambam Health Care Campus, Haifa, Israel.

Address for Correspondence
Pravin C. Singhal, MD
Nephrology Division
100 Community Drive
Great Neck, NY 11021
E-mail psinghal@northwell.edu
Tel 516-465-3010
Fax 516-465-3011
Running head: Podocyte dedifferentiation and high glucose

Abstract

The loss of podocyte (PD) molecular phenotype is an important feature of diabetic podocytopathy. We hypothesized that high glucose (HG) induces dedifferentiation in differentiated podocytes (DPD) through alterations in APOL1-microRNA (miR) 193a axis. HG-induced DPDs dedifferentiation manifested in the form of down regulation of WT1 and upregulation of PAX2 expression. WT1- silenced DPDs displayed enhanced expression of PAX2. Immunoprecipitation (IP) of DPD cellular lysates with anti-WT1 antibody revealed formation of WT1 repressor complexes containing Polycomb group proteins (PcG), EZH2, Menin, and DNA methyl transferase (DNMT1), whereas, silencing of either WT1 or DNMT1 disrupted this complex with enhanced expression of PAX2. HG-induced DPDs dedifferentiation was associated with a higher expression of miR193a, whereas, inhibition of miR193a prevented DPDs dedifferentiation in HG milieu. HG down regulated DPDs expression of APOL1. MiR193a-overexpressing DPDs displayed down regulation of APOL1 and enhanced expression of dedifferentiating markers; conversely, silencing of miR193a enhanced the expression of APOL1 and also preserved DPDs phenotype. Moreover, stably APOL1G0-overexpressing DPDs displayed the enhanced expression of WT1 but attenuated expression of miR193a; nonetheless, silencing of APOL1 reversed these effects. Since silencing of APOL1 enhanced miR193a expression as well as dedifferentiation in DPDs, it appears that down regulation of APOL1 contributed to dedifferentiation of DPDs through enhanced miR193a expression in HG milieu. Vitamin D receptor agonist (VDA) down regulated miR193a, upregulated APOL1 expression, and prevented dedifferentiation of DPDs in HG milieu. These findings suggest that modulation of the APOL1-miR193a axis carries a potential to preserve DPDs molecular phenotype in HG milieu.

58   Podocytes play a key role in the maintenance of slit diaphragm, a component of the

59   glomerular filtration barrier (5, 32, 33). Slit diaphragms are composed of several proteins

60   expressed by podocytes and prevent leakage of plasma proteins (32, 33). An optimal

61   expression of the slit diaphragm proteins is considered to be an integral part of podocyte

62   health. Since both parietal epithelial cells (PECs) and podocytes (PDS) are derived from

63   the same mesenchymal cells during embryogenesis (26, 37), injured adult podocytes go

64   into dedifferentiation mode- reverting to the expression of PEC markers such as paired

65   homeo box (PAX)-2 (31, 35, 39). High glucose milieu has been demonstrated to induce

66   dedifferentiation of PDs as a manifestation of PD injury (2, 16, 18, 37). However, the

67   mechanisms involved are not clear.

68   PAX2 is a transcription factor which plays an important role in the development of

69   kidneys (9-11, 38). In adult kidney, its expression is restricted to glomerular parietal and

70   tubular epithelial cells (10). However, ectopic PAX2 expression in podocytes is a common

71   finding in several pathological states including juvenile nephronophthisis (28), focal

72   segmental glomerulosclerosis (30, 31), collapsing glomerulopathy (7), and diabetic

73   glomerulosclerosis (2, 25). Since PAX2 is involved in cellular proliferation (43), this disease

74   state expression may be an attempt by podocytes to regenerate in adverse milieus. In a

75   mouse model of podocyte injury, evaluation of PDs dedifferentiation in podocyte reporter

76   mice demonstrated that PDs expressing PECs markers (PAX2/PAX8) far exceed PECs

77   expressing PD markers (33).

78   MicroRNAs (miRs) are small, non-coding RNAs that negatively regulate gene

79   expression at the post-transcription level (1). By an imperfect sequence complementation,

80   miRNAs recognize and bind to the 3'-untranslated regions (3'-UTR) of target mRNAs,

3

81 thereby inhibiting mRNA function through degradation, repression of translation, or both.

82 Recently, miR193a has been demonstrated to induce down regulation of WT1 in podocytes

83 (25). miR193a is tumor suppressor gene inducing apoptosis in podocytes through

84 generation of oxidative stress. However, the role of miR193a in high glucose-induced PD

85 dedifferentiation has not been reported (29). Since WT1 inversely regulate PAX2

86 expression (8, 34), we hypothesize that high glucose would induce PAX2 expression

87 through down regulation of WT1 and enhanced PD expression of miR193a.

88 APOL1 is a minor component of High-Density Lipoprotein (HDL) complex and is

89 expressed in kidney cells including podocytes, tubular cells, and other cell types (42). It is

90 predominantly secreted by liver cells and circulates in the plasma (42). The G1 variant is a

91 missense mutant haplotype (S342G:I384M), encoding two non-synonymous amino acids;

92 while the G2 variant is a 6 bp in –frame deletion resulting in loss of two amino acids (N388

93 and Y389) at the C-terminal helix of APOL1. Approximately 34% of African Americans

94 (AAs) carry one of the two risk variants and 13% have some combination of both coding

95 variants (41). Overt expression of APOL1G1 and G2 has been associated with podocyte

96 injury both *in vitro* and *in vivo* studies (3, 15, 23, 24).The trypanolytic activity of circulating

97 APOL1 (wild-type or G0) has been long appreciated and well characterized, though the

98 detailed molecular mechanism is not fully resolved (12). The function of APOL1G0 in

99 podocytes is not clearly understood. We hypothesize that high glucose induces PDs

100 dedifferentiation through down regulation of APOL1 and upregulation of microRNA (miR)

101 193a. We further hypothesize that modulation of APOL1-miR193a axis can be used as a

102 tool to preserve PDs differentiation in high glucose milieu.

103

4

Material and Methods

Human podocytes

Human podocytes (PDs) were conditionally immortalized by introducing temperature-sensitive SV40-T antigen by transfection (36). These cells proliferate at the permissive temperature (33˚C) and enter growth arrest after transfer to the non-permissive temperature (37˚C). The growth medium contains RPMI 1640 supplemented with 10%fetal bovine serum (FBS), 1x Pen-Strep, 1 mM L-glutamine and 1x ITS (Invitrogen). Undifferentiated (UND) PDs were seeded on collagen coated plates and differentiated through pre-incubation in normal RPMI (containing 11 mM glucose) for 10 days at 37°C (differentiated podocytes, DPDs). Prior to experimental protocols, DPDs were washed three times with glucose- and serum-free media. In experimental protocols, DPDs were incubated in media (glucose- and ITS-free RPMI) containing either normal glucose (5 mM) or high glucose (30 mM) for 48 h. DNA sequencing of these podocytes revealed APOL1G0 genotype.

Generation of a stable cell lines expressing *APOL1G0* and Vector

A stable cell line expressing *APOL1G0* was generated by retroviral infection as described previously (27). Briefly, the open reading frame of APOL1G0 was cloned into the retroviral vector pBABE carrying resistance to puromycin. To generate retroviral particles, the viral packaging cell line HEK-GP was co-transfected with the pBABE construct of interest and the VSV gene. Undifferentiated podocytes (UNDPDs) were infected twice within 24 h with the viral-containing supernatant of HEK-GP cells. Selection with puromycin (1 µg/mL) was continued for a week, and expression of the sequence of the *APOL1G0* was

126 verified. Empty vector pBABE-eGFP was also transduced into UNDPDs to generate the

127 control cell line.

Transfection of miR193a inhibitor and miR193a expression plasmid

129 miR193a inhibitor (25 nM; Cat #4464084;Thermofisher, USA), miR193a expression

130 plasmid (25 nM; Cat #SC400232; Origene), and empty vector (25 nM; pCMV-MIR;

131 Origene) were transfected in the cells using Lipofectamine 3000 Transfection Reagent

132 (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. All miRNA

133 products were dissolved in nuclease-free water. Briefly, DPDs were transfected at 70 - 80%

134 confluence in 6 well plates. The Lipofectamine transfection reagent (7.5 µl) and plasmid

135 DNA were diluted in opti-MEM media (125 µl and 250 µl) (Applied Biosystems, Thermo

136 Fisher Scientific, USA) followed by addition of P3000 enhancer reagent (10 µl) to diluted

137 DNA. Diluted DNA (125 µl) was added to diluted Lipofectamine 3000 transfection reagent

138 (125 µl) in the ratio of 1:1 (v/v) and incubated for 10 min at room temperature (25°C). After

139 incubation, DNA-lipid complex was added to the cells and kept at 37°C in opti-MEM media

140 for 48 hrs. Control and transfected cells were harvested for protein and RNA analyses.

Vitamin D Receptor agonist (VDA) treatment

142 Vitamin D Receptor agonist (VDA; EB1089, 10 nM; Tocris, MN, USA) was used to

143 modulate the expression of miR193a. VDA (2.2 mM) was initially dissolved in 10% DMSO

144 (100 µl) and diluted further with sterile PBS buffer (pH 7.2) to achieve final working

145 concentrations of 10 µM and 1µM. The final concentration of DMSO was 0.1 % in the

146 vehicle in all the experiments. DPDs in the experimental conditions were treated with VDA

147 for 48 h and harvested for protein and RNA for further analyses.

Silencing of APOL1, WT1, and DNMT1

DPDs were transfected with scrambled siRNA (control) or APOL1 siRNA (20 nM; Santa Cruz), WT1 siRNA (25 nM; Santa Cruz), DNMT1 (25 nM; Santa Cruz) with Lipofectamine RNAiMAX transfection reagent according to the manufacturer's protocol (Thermo Fisher). Briefly, DPDs were transfected at 60-80% confluence in 6 well plates. Lipofectamine reagent (9 μl) and siRNAs (10 μM, 2-3 μl) were diluted in opti-MEM media (150 μl) (Thermo Fisher). Then, diluted siRNA (150 μl) was added to diluted Lipofectamine reagent (150 μl) in 1:1 ratio (v/v) and incubated for 5 min at room temperature (25°C). After incubation, the siRNA lipid-complex was added to cells and kept at 37°C in opti-MEM media for 48 hrs. The cells were harvested for protein and RNA analyses. Control and transfected cells were used under control and experimental conditions.

RNA isolation and qPCR studies

Total RNA was isolated from control and experimental DPDs with TRIzol reagent (Invitrogen, USA). A 20 μl reaction mix was prepared containing iTaq Universal SYBR Green reaction mix (2x) (10 μl), iscript reverse transcriptase (0.25 μl), forward and reverse primers (2 μl), RNA (4 μl), and nuclease free water (3.75 μl). Real-Time PCR was performed using one-step iTaq^{TM} Universal SYBR Green kit (BIO-RAD, USA) according to the manufacturer's instructions using specific primers obtained from Thermo Fisher Scientific, USA. *GAPDH* fw 5′ CCC ATC ACC ATC TTC CAG GAG ′3; rev 5′ GTT GTC ATG GAT GAC CTT GGC ′3, *WT1* fw 5′ CGAGAGCGATAACCACACAACG ′3; rev 5′ GTCTCAGATGCCGACCGTACAA ′3, *PAX2*fw 5′ GGC TGT GTC AGC AAA ATC CTG ′3; rev 5′ TCC GGA TGA TTC TGT TGA TGG ′3, *APOL1* fw 5′ ATC TCA GCT GAA AGC GGT GAAC ′3; rev 5′ TGA CTT TGC CCC CTC ATG TAAG ′3. The qPCR conditions were as

follows: 50°C for 10 min 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Quantitative PCR was performed using an ABI Prism 7900HT sequence detection system and relative quantification of gene expression was calculated using the ΔΔCT method. Data were expressed as relative mRNA expression in reference to the control, normalized to the quantity of RNA input by performing measurements on an endogenous reference gene (GAPDH).

MicroRNA assay

For miRNA quantification, the total RNA was isolated from control and experimental DPDs with miRVana miRNA isolation kit and 1 µg of RNA was reverse transcribed using miR193a and U6snRNA specific RT primers to generate first strand cDNA from mRNA using TaqMan microRNA Reverse Transcription kit (Thermo Fisher Scientific, USA) according to manufacturer's instructions. For cDNA, a 15 µl PCR reaction was prepared containing 100 mM dNTP mix (0.15 µl), multiscribe RT enzyme 50 U/µl (1 µl), 10X RT buffer (1.5 µl), RNase inhibitor 20 U/µl (0.19 µl), nuclease free water (4.16 µl), RNA (5 µl), and primers (3 µl). The PCR condition was as follows: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and 4°C until stopped. Real-time PCR was performed by using TaqMan-based PCR master mix and detection primers miR-193a and U6snsubRNA (Thermo Fisher) in ABI-7500, Applied Biosystems. For real-time PCR a 10 µl reaction mix was prepared containing TaqMan PCR master mix II (5 µl), cDNA (2 µl), nuclease free water (2 µl), and primer (1 µl). The qPCR conditions were as follows: 50°C for 2 min 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. U6 was used as an internal control. Relative quantification of gene expression was calculated using the ΔΔCT method and the results were normalized to U6-snuRNA expression.

8

Immunofluorescence detection of APOL1

Control and experimental podocytes were fixed and permeabilized with a buffer containing 0.02% Triton X-100 and 4% formaldehyde in PBS. Fixed cells were washed three times in PBS and blocked in 10% BSA for 60 min at 37˚C. Subsequently, cells were labeled with anti-APOL1 (Proteintech, IL). DAPI was used for nuclear localization. Control and experimental cells were examined under immunofluorescence microscope.

Western blotting studies

Western blotting studies were carried out as described previously (4, 17, 22). Briefly, control and experimental cells were harvested, lysed in RIPA buffer containing 50 mM Tris-Cl (pH 7.5), 150 mMNaCl, 1mM EDTA, 1% NP-40, 0.25% Deoxycholate, 0.1% SDS, 1X protease inhibitor cocktail (Calbiochem, Cocktail Set I), 1mM PMSF, and 0.2mM sodium orthovanadate. Protein concentration was measured using Biorad Protein Assay kit. Total protein lysed extracts (30 μg/lane) were loaded on a 10 % polyacrylamide (PAGE) premade gel (Bio-Rad) and transferred onto PVDF membranes were processed for immunostaining with primary antibodies against APOL1 (mouse monoclonal, 1:1000, Protein tech), WT1 (rabbit polyclonal, 1:1000, Abcam, MA), PAX2 (rabbit polyclonal,1:800, Abcam), Menin (mouse monoclonal, 1:1000, Santa Cruz), DNMT1 (mouse monoclonal, 1:1000, Santa Cruz), EZH2 (Goat polyclonal, 1:1000, Santa Cruz), RBBP4 (Rabbit polyclonal, 1:1000, Santa Cruz), and H3K27me3 (rabbit polyclonal, 1:1000, Cell Signaling Technologies, MA), podocalyxin (rabbit polyclonal, 1:1000, Thermo Fisher) and nephrin (rabbit polyclonal, 1:800, Abcam) followed by treating with horseradish peroxidase labeled appropriate secondary antibodies. The blots were developed using a chemiluminescence detection kit

9

216 (PIERCE, Rockford, IL) and exposed to X-ray film (Eastman Kodak Co., Rochester, NY).

217 Equal protein loading and the protein transfer were confirmed by immunoblotting for

218 determination of actin/GAPDH protein using a polyclonal β-Actin/GAPDH antibody (Santa

219 Cruz, CA) on the same (stripped) western blots.

Immunoprecipitation (IP)

221 Lysates from undifferentiated and differentiated PDs were first immunoprecipitated following

222 the addition of 5 μg of monoclonal antibody to WT1 (Santa Cruz Biotechnology). The

223 immune complexes were then collected using 25 μl of Protein-A + G sepharose beads (GE

224 Health Care, Life Science), in RIPA buffer. The IP was carried out at 4°C, for 4h, on a

225 rotating platform. Following this, precipitated A/G proteins were pelleted down by

226 centrifugation at 4,500 rpm for 10 min at 4°C. Next, the protein pellet was washed (3X)

227 each time with 1 ml of cold RIPA lysis buffer followed by centrifugation each time for 10

228 minutes at 2,500 rpm in a microfuge. After washings, beads were re-suspended in 100 μl of

229 lysis buffer to which SDS-PAGE sample buffer (50 μl) was added and samples were boiled

230 at 100°C, followed by SDS-PAGE and immunoblotted using specific antibodies as indicated.

Statistical analyses

232 Statistical comparisons were performed with the program PRISM using the Mann–

233 Whitney U test for nonparametric data and the unpaired t test for parametric data.

234 A P value<0.05 was accepted as statistically significant.

235

236

237

238

10

Results

High glucose causes dedifferentiation of podocytes

Dedifferentiation of PDs is characterized by enhanced expression of PAX2 and down regulation of WT1. To determine the effect of high glucose on PAX2 expression, Proteins and RNAs were extracted from control and high glucose treated DPDs (n= 3). Protein blots were probed for PAX2 and re-probed for actin. Gels from three different lysates are displayed in Fig. 1A (Upper panel). Cumulative densitometric data are shown in bar graphs (Lower panel, Fig. 1A). High glucose enhanced (P<0.05) expression of PAX2 in DPDs. cDNAs were amplified with a specific primer for PAX2. Cumulative data on mRNA expression of PAX2 are shown in Fig. 1C. High glucose also enhanced PAX2 mRNA expression in DPDs. Protein blots of the same lysate preparations were probed for WT1 and reprobed for actin. Gels from three different lysates are displayed in Fig. 1 B (Upper panel). Cumulative densitometric data are shown as a bar graph in the lower panel (Fig. 1B). cDNAs were amplified with a specific primer for WT1. Cumulative data are shown in a bar graph (Fig.1 D). High glucose down regulated (P<0.05) WT1 mRNA as well as protein expression in DPDs. These findings suggest that high glucose down regulates transcription and translation of WT1 but enhances transcription of PAX2 in DPDs.

We examined whether there is a causal relationship between high glucose-induced down regulation of WT1 and upregulation of PAX2 expression in DPDs. DPDs were transfected with either scrambled (SCR) or WT1siRNA (n=3). Subsequently, protein blots were probed for WT1 and reprobed for PAX2 and GAPDH. Gels from three different lysates are displayed in Fig. 1E. Cumulative densitometric data are shown in Fig. 1F. DPDs

11

silenced for WT1 displayed enhanced (P<0.05) PAX2 expression. These findings confirm that down regulation of WT1 causes upregulation of PAX2 in DPDs.

High glucose induces DPDs dedifferentiation through upregulation of miR193a

Since miR193a is a negative regulator of WT1 in PDs (11), we asked whether high glucose is down regulating WT1 via up regulation of miR193a. DPDs were incubated in media containing either normal glucose (control, 5 mM) or high glucose (30 mM) for 48 hours (n=3). RNAs were extracted and assayed for miR193a. As shown in Fig. 2A, high glucose enhanced expression of miR193a in DPDs.

To determine whether a specific miR193a inhibitor carries the potential to reverse effect of high glucose milieu, DPDs were incubated in media containing normal glucose (5 mM, control), high glucose (30 mM), empty vector (pCMV-MIR 25 nM) with or without miR193a inhibitor (25 nM, Applied Biosystems, Thermo Fisher) for 48 hours followed by RNA extraction. RNAs were assayed for miR193a. High glucose enhanced (P<0.01 the expression of miR193a in DPDs; however this effect of high glucose was attenuated by inhibition of miR193a (Fig. 2B).

We hypothesized that if high glucose induced dedifferentiation of DPDs through upregulation of miR193a, then inhibition of miR193a in high glucose milieu would preserve DPDs molecular phenotype. DPDs were incubated in media containing either normal glucose (control, 5 mM), high glucose (30 mM), empty vector (pCMV-MIR, 25 nM) with or without a specific inhibitor of miR193a (25 nM) for 48 hours (n=3). Proteins were extracted and protein blots were probed for WT1 and reprobed for PAX2 and actin. Gels are displayed in Fig. 2C. Cumulative densitometric data are shown as a bar diagram in Fig. 2D.

12

High glucose decreased (P<0.05) DPDs expression of WT1 and enhanced (P<0.05) expression of PAX2. However, miR193a inhibitor prevented upregulation of PAX2 in high glucose milieu. These findings confirm that high glucose induces dedifferentiation of DPDs through upregulation of miR193a.

High glucose down regulates DPDs expression of APOL1 through Upregulation of miR193a

To determine the dose-response effect of high glucose on APOL1 expression in DPDs, cells were incubated in media containing variable concentrations of glucose (5, 10, 20, 30, 35 mM) for 48 hours (n=3). Protein blots were probed for APOL1 and reprobed for GAPDH. Representative gels are displayed in Fig. 3A. Glucose down regulated APOL1 expression in DPDs at higher concentrations (30 mM and above).

To evaluate the APOL1s relationship with dedifferentiation markers, UNDPDs, DPDs-treated with RPMI containing either conventional glucose (11 mM) or HG (30 mm) for 48 hours were analyzed for dedifferentiation markers (n=4). Protein blots were probed for APOL1 and reprobed for WT1, PAX2, and GAPDH. Gels of three different lysates are displayed in Fig. 3B. Cumulative densitometric data are shown as a bar diagram (Fig. 3C). DPDs displayed the expression of APOL1 and WT1 but attenuated expression of PAX2; on the other hand, high glucose inhibited the expression of APOL1 and WT1 but enhanced the expression PAX2. These findings suggest that down regulation of APOL1 in DPDs is temporally associated with down regulation of WT1 and upregulation of PAX2 in high glucose milieu.

13

304   To determine whether miR193a is regulating the expression of APOL1, DPDs were

305   incubated in media containing either normal glucose (5 mM), high glucose (30 mM), empty

306   vector (25 nM; pCMV-MIR) with or without miR193a inhibitor (25 nM) for 48 hours (n=3).

307   Proteins and RNAs were extracted. Protein blots were probed for APOL1 and reprobed for

308   GAPDH. Gels are displayed in the upper panel of Fig. 3D. Cumulative densitometric data

309   are shown in bar graphs (Fig. 3E). High glucose down regulated (P<0.05) APOL1

310   expression in DPDs; however, miR193a inhibitor enhanced (P<0.05) APOL1 expression in

311   high glucose milieu. RNAs were extracted from the lysates of 3D and cDNAs were

312   amplified with a specific primer for *APOL1*. Cumulative data are shown as a bar diagram

313   (Fig. 3F). High glucose down regulated *APOL1* mRNA expression; however, inhibition of

314   miR193a stimulated *APOL1* mRNA expression both in control and high glucose milieus.

315   These findings suggest that miR193a negatively regulates APOL1 expression in DPDs

316   under control as well as in high glucose milieus.

317   DPDs grown on coverslips were incubated in media containing either normal

318   glucose (C, 5 mM), high glucose (30 mM) with or without a miR193a inhibitor (miR, 25 nM)

319   for 48 hours (n=3) followed by immuno-labeling for APOL1. Subsequently, cells were

320   examined under a confocal microscope. Representative fluoromicrographs are shown in

321   Fig. 3G. High glucose down regulated APOL1 expression (green fluorescence) in DPDs,

322   however this effect of high glucose was mitigated by inhibition of miR193a.

323   To determine the effect of overexpression of miR193a on APOL1 expression, DPDs

324   were transfected with either empty vector (EV) or miR193a plasmid (n=3). Proteins and

325   RNAs were extracted. Protein blots were probed for WT1, PAX2, APOL1, and re-probed for

326   GAPDH. Gels from three different lysates are displayed in Fig. 4A. Cumulative

14

327 densitometric data are shown as a bar diagram in Fig. 4B. MiR193a-overexpressing DPDs

328 showed down regulation of APOL1 and WT1 but upregulation of PAX2. cDNAs were

329 amplified for *APOL1*. Cumulative data are shown in a bar diagram (Fig. 4C). DPDs

330 overexpressing miR193a showed down regulation of *APOL1* mRNA. These findings

331 confirm that miR193a negatively regulates expression of APOL1 in DPDs.

332 **WT1 repressor complex preserves DPDs molecular phenotype**

333 To characterize the molecular phenotypes of undifferentiated (UND) and

334 differentiated PDs (DPD), protein blots of UNDPDs (0 day incubation) and DPDs (10 days

335 incubation) were probed for PDs (nephrin, and podocalyxin), PEC (PAX2) markers, APOL1,

336 components of WT1 repressor complex (RBBP4, EZH2, Menin, H3K27me3, and DNMT1),

337 and actin. Gels from three different lysates are displayed in Figs. 5A (PD and PEC

338 markers) and 5B (components of WT1 repressor complex, input for IP data). Cumulative

339 densitometric data from the lysates of Figs. 5A and 5B are shown as bar diagrams (Figs.

340 5C and 5D). DPDs (10 days incubation) displayed higher expression of APOL1, nephrin,

341 and podocalyxin (PDX) but lower expression of PAX2 when compared to undifferentiated

342 PDs (0 day, Figs. 5A and 5C). Interestingly, DPDs (10 days incubation) displayed

343 enhanced expression of the components of WT1 repressor complex (Figs. 5B and 5D).

344 To confirm the composition of WT1 repressor complex, input lysates of UND and

345 DPD were immunoprecipitated (IP) with the anti-WT1 antibody. IP fractions were probed for

346 WT1, RBBP4 (Polycomb group protein), EZH2, Menin, H3K27me3, DNMT1, and IgG. Gels

347 from three different IP fractions are displayed in Fig. 5E. Cumulative densitometric data

348 from the lysates are shown in bar graphs (Fig 5F). IP fractions of DPDs displayed

349 enhanced expression of WT1, RBBP4, Menin, EZH2, H3K27me3, and DNMT1 when

15

compared to 0 day PDs. These findings confirm that WT1 repressor complex is composed of WT1, RBBP4, Menin, EZH2, H3K27me3, and DNMT1.

We asked whether the integrity of WT1 repressor complex is critical for the prevention of dedifferentiation of DPDs. DPDs were transfected with scrambled (SCR), WT1 siRNA, DNMT1 siRNA or WT1 + DNMT1 siRNAs. After 48 hours, protein blots were probed for PAX2, WT1, nephrin, podocalyxin (PDX), DNMT1, and reprobed for actin. Gels from three different lysates are displayed in Fig. 5G. Cumulative densitometric data are shown as a bar diagram in Fig. 5H. Lack of either WT1 or DNMT1 enhanced the expression of PAX2. Interestingly, combined silencing of WT1 and DNMT1 displayed additive effect on PAX2 expression. These findings suggest that disruption of WT1 repressor complexes de-represses the expression of PAX2.

Role of APOL1 in preservation of DPDs molecular phenotype

To determine the role of APOL1 in the preservation of the DPDs molecular phenotype, DPDs were transfected with either control (scrambled, SCR) or APOL1 siRNA. Proteins were extracted from control and transfected cells (n=3).Protein blots were probed for APOL1 and reprobed for PAX2, WT1, and GAPDH. Gels from three different lysates are displayed in Fig. 6A. Cumulative densitometric data are shown in a bar diagram (Fig. 6B). APOL1 silenced DPDs displayed attenuated ($P<0.05$) expression of WT1 and enhanced ($P<0.05$) expression of PAX2 when compared to control and SCR DPDs. These findings indicate that APOL1 expression is critical for the preservation of DPDs molecular phenotype.

16

371   To determine whether APOL1 would be preserving DPDs molecular phenotype

372 through alterations in miR193a expression, DPDs were transfected with either control

373 (scrambled, SCR) or APOL1 siRNA. RNAs were extracted from control and transfected

374 cells (n=3). RNAs were assayed for miR193a. Cumulative data are shown in a bar diagram

375 (Fig. 6C).

376   To establish a functional relationship between miR193a and APOL1, DPDs were

377 transfected with either control (scrambled, SCR) or APOL1 siRNA and incubated in media

378 with or without miR193a inhibitor (25 nM) for 48 hours (n=3). Protein blots were probed for

379 APOL1, WT1, PAX2 and GAPDH. Gels from three different lysates are displayed in Fig.

380 6D. Silencing of APOL1 in DPDs down regulated WT1 and enhanced the expression of

381 PAX2; however, inhibition of miR193a did not alter this effect of APOL1. These findings

382 suggest the importance of APOL1 expression to sustain the functionality of APOL1-

383 miR193a axis.

384   To determine whether, miR193a inhibitor is fully functional in APOL1-silenced PDs,

385 RNAs were extracted from the lysates of 6D. RNAs were assayed and cumulative data

386 (n=3) are shown in a bar diagram (Fig. 6E). miR193a inhibitor down regulated PDs

387 expression of miR193a in control conditions but could not do so in APOL1 silenced-PDs.

388 These findings suggest that APOL1 is required for the functionality of APOL1-miR193a axis

389 in DPDs.

390 **APOL1 negatively regulates miR193a expression in DPDs**

391 To confirm a relationship between APOL1 and miR193a, UNDPDs stably expressing vector

392 and overexpressing APOL1G0 were differentiated (incubation in RPMI containing 11 mM

17

glucose for 10 days). APOL1G0-expressing DPDs were transfected with either scrambled or APOL1 siRNAs (n=6). After 48 hours, proteins and RNAs were extracted. Protein blots were probed for APOL1 and reprobed for WT1, PAX2 and actin. Gels from three different lysates are displayed in Fig. 7A. Cumulative densitometric data (n=6) are shown as a bar diagram (Fig. 7B). DPDs overexpressing APOLG0 displayed enhanced expression of APOL1 and WT1 but down regulation of PAX2; however, silencing of APOL1 reversed this APOL1G0. RNAs were assayed for miR193a and cumulative data are shown as a bar diagram (Fig. 7C). DPDs overexpressing APOLG0 displayed down regulation of miR93a expression, however, silencing of APOL1 upregulated the expression of miR193a. These findings confirm that APOL1 negatively controls the expression of miR193a.

VDR agonist (VDA) preserves DPDs phenotype through modulation of miR193a-APOL1 axis in high glucose milieu

Vitamin D3 has been known to down regulate expression of miR193a in parietal epithelial cells (20). To determine the effect of VDA on miR193a expression in UNDPDs, UNDPDs were incubated in media containing either vehicle (0.1% DMSO) alone or different concentrations of VDA (EB1089, 0, 1.0, 10.0, and 100.0 nM) for 48 hours (n=3). RNAs were extracted and assayed for miR193a. Cumulative data are shown in a bar diagram (Fig. 8A). VDA down regulated miR193a in UNDPDs in a dose-dependent manner.

To determine the effect of VDA on high glucose-induced modulation of miR193a, DPDs were incubated in media containing either normal glucose (C, 5 mM), high glucose (HG, 30 mM), vehicle (0.1% DMSO) with or without VDA (EB1089, 10 nM) for 48 hours (n=3). RNAs were extracted and assayed for miR193a. Cumulative data are shown as a

18

bar diagram (Fig. 8B). High glucose enhanced (P<0.01) expression of miR193a in DPDs. However, VDA inhibited high glucose-induced upregulation of DPDs expression of miR193a.

To examine the effect of VDA on high glucose-induced down regulation of APOL1, DPDs were incubated in media containing either normal glucose (C, 5mM), high glucose (HG, 30 mM) with or without VDA (EB1089, 10 nM) for 48 hours (n=3). Protein blots were probed for APOL1 and reprobed for GAPDH. Representative gels are displayed in Fig. 8C. Cumulative densitometric data are shown in a bar diagram (Fig. 8D). High glucose down regulated (P<0.05) APOL1 expression in DPDs; however, VDA enhanced APOL1 expression in high glucose milieu. These findings suggest that VDA has potential to preserve DPDs expression of APOL1 in high glucose milieu.

To evaluate the effect of VDA on high glucose-induced dedifferentiation, DPDs were incubated in media containing either normal glucose (C, 5mM), vehicle (Veh, 0.1% DMSO high glucose (HG, 30 mM) with or without VDA (EB1089, 10 nM) for 48 hours (n=3). Protein blots were probed for WT1, PAX2 and reprobed for GAPDH. Representative gels are displayed in Fig. 8E. Cumulative densitometric data are shown in a bar diagram (Fig. 8F). High glucose down regulated (P<0.05) DPDs expression of WT1 but enhanced (P<0.05) the expression of PAX2. However, VDA enhanced the expression of WT1 under high glucose milieu. Moreover, VDA down regulated high glucose-induced PAX2 expression in DPDs. These findings suggest that VDA carries a potential to preserve DPDs molecular profile in high glucose milieu.

19

Discussion

The present study demonstrated that high glucose (HG) induced dedifferentiation in DPDs. High glucose enhanced PAX2 expression, a marker of podocyte dedifferentiation, as a consequence of disruption of WT1 repressor complex. High glucose-induced DPDs dedifferentiation was associated with a higher expression of miR193a and inhibition of miR193a prevented DPDs dedifferentiation. DPDs overexpressing miR193a displayed down regulation of APOL1 and enhanced expression of dedifferentiating markers; conversely, silencing of miR193a enhanced the expression of APOL1 and also preserved DPDs phenotype. Interestingly, high glucose also attenuated DPDs expression of APOL1. Moreover, stably APOL1G0-overexpressing DPDs displayed the enhanced expression of WT1 but attenuated expression of miR193a; nonetheless, silencing of APOL1 reversed these effects. Since silencing of APOL1 enhanced miR193a expression as well as dedifferentiation in DPDs, it appears that down regulation of APOL1 contributed to enhanced miR193a expression in HG milieu. Vitamin D receptor agonist (VDA) down regulated miR193a, upregulated APOL1 expression, and prevented dedifferentiation of DPDs in HG milieu. These findings suggest a novel role of APOL1 in the preservation of molecular phenotype of DPDs in high glucose milieu.

Expression of parietal epithelial proteins such as Claudin 1 and PAX2 in the glomerular capillary tufts in diabetic nephropathy could be a consequence of the replacement of PDs by PECs or PDs reversal to PECs phenotype. Chen et al demonstrated that high glucose milieu enhanced PAX2 gene expression in mouse embryonic mesenchymal epithelial cells and kidney explants (6). In an experimental model of podocyte reporter mice, podocyte injury stimulated expression of PAX2 (34). Therefore,

20

460   expression of PAX2 by glomerular capillary epithelial cells may not be able to predict their

461   lineage.

462   WT1 has been reported to regulate PAX2 expression negatively through the

463   formation of a repressor complex (44).WT1 repressor complex containing PcG proteins,

464   EZH2, and Menin binds at PAX2 gene and has been demonstrated to decrease

465   transcription of PAX2 (40, 44). In the present study, high glucose down regulated WT1 and

466   decreased the transcription of PAX2 in DPDs. WT1 bound IP fraction revealed the

467   presence of PcG protein, EZH2, Menin and DNMT1. Silencing of WT1 or DNMT1 disrupted

468   the repressor complex and upregulated PAX2 expression in DPDs. These findings suggest

469   that high glucose-induced down regulation of WT1 and enhanced PAX2 expression

470   occurred through disruption of WT1 repressor complex. We have displayed proposed

471   composition of WT1 repressor complex on PAX2 in Fig. 9A. However, these observations

472   need to be confirmed *in vivo* studies.

473   MicroRNA193a has been demonstrated to regulate WT1 transcription inversely in

474   podocytes (14). miR193a transgenic mice displayed loss of WT1 by podocytes and

475   developed focal glomerular sclerosis (14). Notably, dedifferentiation of PDs in the form of

476   PAX2 expression was not studied in this model. In the present study, high glucose

477   enhanced expression of miR193a and down regulated WT1 expression in the podocytes.

478   Inhibition of miR193a caused upregulation of podocyte WT1 expression in high glucose

479   milieu, suggesting an inverse relationship between miR193a and WT1 in high glucose

480   milieu. Moreover, inhibition of miR193a upregulated PD expression of WT1 and down

481   regulated PAX2 expression in high glucose milieu. These findings suggest that modulation

21

of miR193a could be used as a therapeutic strategy to preserve podocyte molecular integrity in high glucose milieu.

In the present study, high glucose-induced upregulation of miR193a displayed a temporal relationship with down regulation of APOL1 expression in PDs. On the other hand, APOL1-silenced PDs displayed upregulation of miR193a and over-expressing APOL1-PDs showed down regulation of miR193a expression. These findings suggest a negative feedback relationship between APOL1 and miR193a in PDs. As noted below, this miR193a-mediated down regulation of APOL1 in high glucose milieu could provide an explanation for the low or absence of any association of APOL1 renal risk variants with diabetic kidney disease (13). On the other hand, down regulation of APOL1 was associated with dedifferentiation of DPDs both in high glucose milieu as well as under control conditions; while upregulation of APOL1 provided protection against dedifferentiation of podocytes in high glucose milieu. Therefore, enhanced APOL1 expression could be considered with caution as a strategy to preserve podocyte phenotype in high glucose or related adverse milieus. However, in the absence of such adverse milieus it is considered that APOL1 expression may be dispensable to kidney health (19).

Since LPS, TNF-α, HIV and IFN-γ have been reported to enhance expression of APOL1 in podocytes (23, 24, 27), these agents could be used to prevent down regulation of APOL1 in high glucose milieu. However, these agents are phlogogenic *de novo*, and would not be suitable in chronic kidney disease-carrying pre-existing inflammatory milieu. In our study, we observed that VDA not only down regulated miR193a but also enhanced PD expression of APOL1 in high glucose milieu. Therefore, VDA could be used to provide protection against dedifferentiation in high glucose milieu through enhanced PD expression

22

505 of APOL1. However, using VDA for increasing APOL1 in high glucose milieu would be

506 detrimental for PDs health if the host carries APOL1 risk alleles (3, 15, 23, 24). Therefore, it

507 would be mandatory to characterize the genetic profile of APOL1 before using VDA as a

508 therapeutic strategy to preserve PDs molecular phenotype in high glucose milieu.

509 Genetic epidemiology indicated that African Americans (AAs) carrying APOL1 risk

510 alleles (G1 and G2) are prone to develop chronic kidney diseases at higher rates with few

511 exceptions such as diabetic nephropathy when compared to European Americans (13, 21,

512 41). In the present study, high glucose milieu down regulated expression of APOL1 in

513 podocytes; therefore, high glucose milieu would also down regulate podocyte expression of

514 APOL1 risk alleles in Africans Americans carrying APOL1 risk alleles. Since enhanced

515 expression of APOL1 risk alleles has been reported to be cytotoxic to podocytes, down

516 regulation of APOL1 risk alleles in high glucose milieu is unlikely to modulate net outcome.

517 Therefore, our data are consistent with the epidemiologic observations (13).

518 We conclude that high glucose-induced up regulation of miR193a stimulated

519 attenuated expression of APOL1 manifesting in the form of DPDs dedifferentiation (Fig.

520 9B). This effect of high glucose could be prevented by VDA through the reversal of APOL1-

521 miR193 axis alterations.

23

References

1. Ambros V.microRNAs: tiny regulators with great potential. Cell. 107:823-6, 2001

2. Andeen NK, Nguyen TQ, Steegh F, Hudkins KL, Najafian B, Alpers CE.The phenotypes of podocytes and parietal epithelial cells may overlap in diabetic nephropathy.Kidney Int. 88:1099-107, 2015.

3. Beckerman P, Bi-Karchin J, Park AS, Qiu C, Dummer PD, Soomro I, Boustany-Kari CM, Pullen SS, Miner JH, Hu CA, Rohacs T, Inoue K, Ishibe S, Saleem MA, Palmer MB, Cuervo AM, Kopp JB, Susztak K. Transgenic expression of human APOL1 risk variants in podocytes induces kidney disease in mice. Nat Med. 23: 429-438, 2017

4. Chandel N, Husain M, Goel H, Salhan D, Lan X, Malhotra A, McGowan J, Singhal PC.VDR hypermethylation and HIV-induced T cell loss. J Leukoc Biol. 93:623-31, 2013.

5. Charest PM, Roth J. Localization of sialic acid in kidney glomeruli: regionalization in the podocyte plasma membrane and loss in experimental nephrosis. Proc Natl AcadSci U S A. 82:8508-12, 1985

6. Chen YW, Liu F, Tran S, Zhu Y, Hébert MJ, Ingelfinger JR, Zhang SL. Reactive oxygen species and nuclear factor-kappa B pathway mediate high glucose-induced Pax-2 gene expression in mouse embryonic mesenchymal epithelial cells and kidney explants. Kidney Int. ;70:1607-15, 2006.

7. Dijkman HB, Weening JJ, Smeets B, Verrijp KC, van Kuppevelt TH, Assmann KK, Steenbergen EJ, WetzelsJF.Proliferating cells in HIV and pamidronate-associated collapsing focal segmental glomerulosclerosis are parietal epithelial cells. Kidney Int. 70:338-44, 2006.

24

8. Discenza MT, He S, Lee TH, Chu LL, Bolon B, Goodyer P, Eccles M, Pelletier J.WT1 is a modifier of the Pax2 mutant phenotype: cooperation and interaction between WT1 and Pax2.Oncogene. 22:8145-55, 2003

9. Dressler GR, Deutsch U, Chowdhury K, Nornes HO, Gruss P.Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. Development. 109:787-95, 1990.

10. Dressler GR, Woolf AS.Pax2 in development and renal disease. Int J Dev Biol. 43:463-8, 1999

11. Eccles MR, Wallis LJ, Fidler AE, Spurr NK, Goodfellow PJ, Reeve AE. Expression of the PAX2 gene in human fetal kidney and Wilms' tumor. Cell Growth Differ. 3:279-89, 1992

12. Fontaine F, Lecordier L, Vanwalleghem G, Uzureau P, Van Reet N, Fontaine M, Tebabi P, Vanhollebeke B, Büscher P, Pérez-Morga D, Pays E.APOLs with low pH dependence can kill all African trypanosomes. Nat Microbiol. 11:1500-1506, 2017

13. Friedman DJ, Kozlitina J, Genovese G, Jog P, Pollak MR: Population-based risk assessment of APOL1 on renal disease. J Am Soc Nephrol 22: 2098–2105, 2011

14. Gebeshuber CA, Kornauth C, Dong L, Sierig R, Seibler J, Reiss M, Tauber S, Bilban M, Wang S, Kain R, Böhmig GA, Moeller MJ, Gröne HJ, Englert C, Martinez J, KerjaschkiD.Focal segmental glomerulosclerosis is induced by microRNA-193a and its downregulation of WT1. Nat Med. 19:481-7, 2013.

15. Hayek SS, Koh KH, Grams ME, Wei C, Ko YA, Li J, Samelko B, Lee H, Dande RR, Lee HW, Hahm E, Peev V, Tracy M, Tardi NJ, Gupta V, Altintas MM, Garborcauskas G, Stojanovic N, Winkler CA, Lipkowitz MS, Tin A, Inker LA, Levey AS, Zeier M,

25

573   Freedman BI, Kopp JB, Skorecki K, Coresh J, Quyyumi AA, Sever S, Reiser J. A

574   tripartite complex of suPAR, APOL1 risk variants and α integrin on podocytes

575   mediates chronic kidney disease. Nat Med. 23:945-953, 2017.

576   16. Herman-Edelstein M, Thomas MC, Thallas-Bonke V, Saleem M, Cooper ME,

577   KantharidisP.Dedifferentiation of immortalized human podocytes in response to

578   transforming growth factor-β: a model for diabetic podocytopathy. Diabetes.

579   60:1779-88, 2011

580   17. Husain M, Meggs LG, Vashistha H, Simoes S, Griffiths KO, Kumar D, Mikulak J,

581   Mathieson PW, Saleem MA, Del Valle L, Pina-Oviedo S, Wang JY, Seshan SV,

582   Malhotra A, Reiss K, Singhal PCInhibition of p66ShcA longevity gene rescues

583   podocytes from HIV-1-induced oxidative stress and apoptosis.JBiol Chem.

584   284:16648-58, 2009.

585   18. Imasawa T, Obre E, Bellance N, Lavie J, Imasawa T, Rigothier C, Delmas Y, Combe

586   C, Lacombe D, Benard G, Claverol S, Bonneu M, Rossignol R. High

587   glucoserepatterns human podocyte energy metabolism during differentiation and

588   diabetic nephropathy. FASEB J. 31:294-307, 2017.

589   19. Johnstone DB, Shegokar V, Nihalani D, et al. APOL1 null alleles from a rural village

590   in India do not correlate with glomerulosclerosis. PLoS One. 2012;7:e51546.

591   20. Kietzmann L, Guhr SS, Meyer TN, Ni L, Sachs M, Panzer U, Stahl RA, Saleem MA

592   Kerjaschki D, Gebeshuber CA, Meyer-Schwesinger C.MicroRNA-193a Regulates

593   the Transdifferentiation of Human Parietal Epithelial Cells toward a Podocyte

594   Phenotype. J Am Soc Nephrol. 26:1389-401, 2015.

21. Kopp JB, Nelson GW, Sampath K, Johnson RC, Genovese G, An P, Friedman D, Briggs W, Dart R, Korbet S, Mokrzycki MH, Kimmel PL, Limou S, Ahuja TS, Berns JS, Fryc J, Simon EE, Smith MC, Trachtman H, Michel DM, Schelling JR, Vlahov D, Pollak M, Winkler CA : APOL1 genetic variants in focal segmental glomerulosclerosis and HIV-associated nephropathy. J Am Soc Nephrol 22: 2129–2137, 2011

22. Kumar D, Plagov A, Yadav I, Torri DD, Sayeneni S, Sagar A, Rai P, Adabala M, Lederman R, Chandel N, Ding G, Malhotra A, Singhal PC. Inhibition of renin activity slows down the progression of HIV-associated nephropathy. Am J Physiol Renal Physiol. 303:F711-20, 2012.

23. Lan X, Jhaveri A, Cheng K, Wen H, Saleem MA, Mathieson PW, Mikulak J, Aviram S, Malhotra A, Skorecki K, Singhal PC.APOL1 risk variants enhance podocyte necrosis through compromising lysosomal membrane permeability. Am J Physiol Renal Physiol. 307:F326-36, 2014.

24. Lan X, Wen H, Lederman R, Malhotra A, Mikulak J, Popik W, Skorecki K, Singhal PC. Protein domains of APOL1 and its risk variants. ExpMol Pathol. 99:139-44, 2015 .PMID:26091559

25. Luna-Antonio BI, Rodriguez-Muñoz R, Namorado-Tonix C, Vergara P, Segovia J, Reyes JLGas1 expression in parietal cells of Bowman's capsule in experimental diabetic nephropathy.Histochem Cell Biol. 148:33-47,2017

26. Miesen L, Steenbergen E, SmeetsB. Parietal cells-new perspectives in glomerular disease. Cell Tissue Res. 369:237-244,2017.

27

27. Mikulak J, Oriolo F, Portale F, Tentorio P, Lan X, Saleem MA, Skorecki K, Singhal PC, MavilioD. Impact of APOL1 polymorphism and IL-1β priming in the entry and persistence of HIV-1 in human podocytes. Retrovirology. 13:63, 2016.

28. Murer L, Caridi G, Della Vella M, Montini G, Carasi C, Ghiggeri G, Zacchello G. Expression of nuclear transcription factor PAX2 in renal biopsies of juvenile nephronophthisis. Nephron. 91:588-93, 2002.

29. Nassirpour R, Raj D, Townsend R, Argyropoulos C.MicroRNA biomarkers in clinical renal disease: from diabetic nephropathy renal transplantation and beyond. Food Chem Toxicol. 98:73-88, 2016.

30. Ohtaka A, Ootaka T, Sato H, Ito S. Phenotypic change of glomerular podocytes in primary focal segmental glomerulosclerosis: developmental paradigm? Nephrol Dial Transplant. 17 Suppl 9:11-5, 2002.

31. Ohtaka A, Ootaka T, Sato H, Soma J, Sato T, Saito T, Ito S. Significance of early phenotypic change of glomerular podocytes detected by Pax2 in primary focal segmental glomerulosclerosis. Am J Kidney Dis.39:475-85, 2002.

32. Pavenstädt H, Kriz W, KretzlerM.Cell biology of the glomerular podocyte. Physiol Rev. 83:253-307, 2003

33. Reiser J, Kriz W, Kretzler M, Munde IP. The glomerular slit diaphragm is a modified adherensjunction.J Am SocNephrol. 11:1-8, 2000

34. Ryan G, Steele-Perkins V, Morris JF, Rauscher FJ 3rd, Dressler GR.Repression of Pax-2 by WT1 during normal kidney development. Development. 121:867-75, 1995

35. Miesen L, Steenbergen E, Smeets B. Parietal cells-new perspectives in glomerular disease. Cell Tissue Res. 369:237-244,2017.

28

36. Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, Xing CY, Ni L, Mathieson PW, Mundel P. conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. J Am Soc Nephrol. 13:630-8, 2002.

37. Stieger N, Worthmann K, Schiffer M.The role of metabolic and haemodynamic factors in podocyte injury in diabetes. Diabetes Metab Res Rev. 27:207-15, 2011.

38. Suzuki T, Matsusaka T, Nakayama M, Asano T, Watanabe T, Ichikawa I, Nagata M.Genetic podocyte lineage reveals progressive podocytopenia with parietal cell hyperplasia in a murine model of cellular/collapsing focal segmental glomerulosclerosis. Am J Pathol. 174(5):1675-82, 2009.

39. Torres M, Gómez-Pardo E, Dressler GR, Gruss P.Pax-2 controls multiple steps of urogenital development. Development. 121:4057-65, 1995

40. Toska E, Roberts SG. Mechanisms of transcriptional regulation by WT1 (Wilms' tumour 1. Biochem J. 461:15-32, 2014

41. Tzur S, Rosset S, Shemer R, Yudkovsky G, Selig S, Tarekegn A, Bekele E, Bradman N, Wasser WG, Behar DM, Skorecki K. Missense mutations in the APOL1 gene are highly associated with end stage kidney disease risk previously attributed to the MYH9 gene. Hum Genet. 128:345-50, 2010.

42. Vanhamme L, Paturiaux-Hanocq F, Poelvoorde P, Nolan DP, Lins L, Van Den Abbeele J, Pays A, Tebabi P, Van Xong H, Jacquet A, Moguilevsky N, Dieu M, Kane JP, De Baetselier P, Brasseur R, Pays E: Apolipoprotein L-I is the trypanosome lytic factor of human serum. Nature 422: 83–87, 2003

43. Woolf AS, Winyard PJ. Gene expression and cell turnover in human renal dysplasia. Histol Histopathol. 15:159-66, 2000.

663   44. Xu B, Zeng DQ, Wu Y, Zheng R, Gu L, Lin X, Hua X, Jin GH. Tumor suppressor

664   menin represses paired box gene 2 expression via Wilms tumor suppressor protein-

665   polycomb group complex. J Biol Chem. 286:13937-44, 2011

666

667
668

669

670

671

672

673

674

675

676

677

678

679

680

681

682

683

684

685

686

Figure legends

688 **Fig. 1.High glucose causes dedifferentiation of podocytes**

689 A. Differentiated podocytes (pre-incubated in RPMI 1640 media containing glucose [11

690   mM] at 37°C; DPDs) were incubated in media containing normal glucose (C, 5 mM)

691   or high glucose (HG, 30 mM) for 48 hours (n=3). Proteins were extracted. Protein

692   blots were probed for PAX2 and re-probed for actin. Gels from three different lysates

693   are displayed (Upper panel). Cumulative densitometric data are shown in bar graphs

694   (Lower panel). *P<0.05 compared with C.

695 B. Protein blots from the lysate preparations of 1A were probed for WT1 and re-probed

696   for actin. Gels from three different lysates are displayed. Cumulative data are shown

697   in a bar diagram. *P<0.05 compared with C.

698 C. RNAs were extracted from the lysates of 1A. cDNAs were amplified with a specific

699   primer for PAX2. Cumulative data on mRNA expression of PAX2 are shown.

700   *P<0.05 compared with C.

701 D. RNAs were extracted from the lysates of 1A. cDNAs were amplified with a specific

702   primer for WT1. Cumulative data on WT1 mRNA expression are shown. *P<0.05

703   compared with C.

704 E. DPDs were transfected with scrambled (SCR, 25 nM) or WT1 (25 nM) siRNAs with

705   Lipofectamine RNAiMAX transfection reagent according to manufacturer's protocol

706   and left in opti-MEMmedia for 48 hrs (n=3). Subsequently, proteins were extracted

707   and protein blots from control and transfected cells were probed for WT1 and

708   reprobed for PAX2 and GAPDH. Gels from three different lysates are displayed.

709 F. Cumulative densitometric data from the gels of 1E are shown in a bar diagram.

31

710   *<0.05 compared with respective C and SCR.

711 **Fig. 2.High glucose induces PDs dedifferentiation through upregulation of mIR193a**

712   A. DPDs were incubated in media containing either normal glucose (control, 5 mM) or

713   high glucose (30 mM) for 48 hours (n=4). RNAs were extracted and assayed for

714   miR193a. Cumulative data are shown in a bar diagram. *P<0.05 compared with C.

715   B. DPDs were incubated in media containing either normal glucose (5 mM, control),

716   high glucose (30 mM), empty vector (25 nM; pCMV-MIR; using lipofectamine as a

717   carrier) with or without miR193a inhibitor (25 nM, plasmid-based inhibitor using

718   lipofectamine as a carrier)   for 48 hours (n=3). RNAs were extracted and assayed

719   for miR193a. **P<0.01 with other variables.

720   C. DPDs were incubated in media containing either normal glucose (control, 5 mM),

721   high glucose (30 mM) empty vector (25 nM) with/without a specific inhibitor of

722   miR193a (25 nM) (n=3). After 48 hours, proteins were extracted. Protein blots were

723   probed for WT1 and re-probed for PAX2 and actin. Gels are displayed.

724   D. Cumulative densitometric data from the protein blots of 2C. *P<0.05 compared to

725   other WT1/PAX2 variables; [a]P<0.05 compared to respective C.

726 **Fig. 3.High glucose Down regulates DPD expression of APOL1 through Upregulation**

727   **of miR193a**

728   A. DPDs were incubated in media containing different concentrations of glucose (5,

729   10, 20, 30, 35) for 48 hours (n=3). Protein blots were probed for APOL1 and re-

730   probed for GAPDH. Representative gels are displayed.

731   B. DPDs were incubated in media containing either conventional glucose (11 mM)

732   or HG (30 mm) for 48 hours. Proteins were extracted from UNDPDs and

733   experimental DPDs (n=4). Protein blots were probed for APOL1 and reprobed for

734   WT1, PAX2, and GAPDH. Gels of three different lysates are displayed.

735   C. Cumulative densitometric data of protein blots of 3B are shown in a bar diagram.

736   *P<0.05 compared to respective UNDPD and DPD/HG; **P<0.01 compared to

737   respective UNDPD and DPD/HG.

738   D. DPDs were incubated in media containing either normal glucose (5 mM), high

739   glucose (30 mM), empty vector (25 nM) with or without miR193a inhibitor (25 nm,

740   miR-Inh) for 48 hours (n=3). Proteins were extracted. Protein blots were probed

741   for APOL1 and re-probed for GAPDH. Gels are displayed.

742   E. Cumulative densitometric data are shown in bar graphs. *<0.05 compared with C

743   and EV; [a]P<0.05 compared with HG alone.

744   F. RNAs were extracted from the lysate preparations of 3D and cDNAs were

745   amplified for *APOL1* mRNA. Cumulative data are shown in a bar diagram.

746   *P<0.05 compared with respective C and EV; **P<0.01 compared with C, EV,

747   and HG alone; ***P<0.001 compared with C, EV, and HG alone; [a]P<0.05

748   compared with miR-Inh alone.

749   G. DPDs grown on coverslips were incubated in media containing either normal

750   glucose (C), high glucose with or without a miR193a inhibitor (miR, 25 nM) for 48

751   hours (n=3) followed by immuno-labeling for APOL1. Subsequently, cells were

752   examined under a confocal microscope. Representative fluoromicrographs are

753   shown.

754   **Fig. 4. Overexpression of miR193a down regulates APOL1**

33

A. DPDs were transfected with either empty vector (EV) or miR193a plasmid (n=3). Proteins were extracted. Protein blots were probed for WT1, PAX2, APOL1, and re-probed for GAPDH. Gels from three different lysates are displayed.

B. Cumulative densitometric data from the lysates of 4A.*P<0.05 compared to Control and EV; **P<0.01 compared to Control and EV.

C. RNAs were extracted from the lysates of 4A. cDNAs were amplified with a specific primer for *APOL1*. *P<0.05 compared with other variables.

Fig. 5. WT1 repressor complex preserves DPDs molecular phenotype

A. Protein blots of UNDPDs (0 day incubation) and DPDs (10 day incubation) were probed for PDs (nephrin, WT1, and podocalyxin) and PEC (PAX2) markers, APOL1, and actin. Gels from three different lysates are displayed.

B. Protein blots from 5A were reprobed for the components of WT1 repressor complex. Gels from three different lysates are displaced.

C. Cumulative densitometric data from the lysates of 5A are shown as a bar diagram. *P<0.05 compared to respective 0 day.

D. Cumulative densitometric data from the lysates of 5B are shown as a bar diagram. *P<0.05 compared to respective 0 day.

E. Lysates from 5A were immunoprecipitated (IP) with the anti-WT1 antibody. IP fractions were probed for WT1, RBBP4 (Polycomb group protein), Menin, H3K27me3, DNMT1, and IgG. Gels from three different IP fractions are displayed.

F. Cumulative densitometric data from the lysates of 5E are shown as bar graphs. *P<0.05 compared with respective 0 day.

34

G. DPDs were transfected with either scrambled (SCR), WT1 siRNA (25 nM), DNMT1 (25 nM), WT1+DNMT1 siRNAs with Lipofectamine RNAiMAX transfection reagent according to manufacturer's protocol and left in opti-MEMmedia for 48 hrs (in WT1 + DNMT1 experiments, cells were exposed to WT1 siRNA for 48 hours and DNMT1 siRNA for 24 hours). Subsequently, proteins were extracted. Protein blots were probed for PAX2, WT1, nephrin, podocalyxin (PDX), DNMT1 and reprobed for actin. Gels from three different lysates are displayed.

H. Cumulative densitometric data from the lysates of 5G are shown as a bar diagram. *$P<0.05$ compared to C, SCR, and siRNADNMT1 in PAX2 variables; **$P<0.01$ compared to C, SCR, and siRNADNMT1 in PAX2 variables; [a]$P<0.05$ compared with C and SCR in Nephrin variables; [b]$P<0.0.05$ compared with C and SCR in PDX variables; [c]$P<0.01$ compared C and SCR in WT1 variables; [d]$P<0.01$ compared with siRNADNMT1, C and SCR in DNMT1 variables.

Fig. 6.Role of APOL1 in preservation of DPDs molecular phenotype

A. DPDs were transfected with either control (scrambled, SCR) or APOL1 siRNA. Proteins were extracted from control and transfected cells (n=3).Protein blots were probed for APOL1 and re-probed for PAX2, WT1, and GAPDH. Gels from three different lysates are displayed.

B. Cumulative densitometric data of protein blots displayed in 6A. *$P<0.05$ compared with respective APOL1, WT1, and PAX2 in control and SCR variables.

C. DPDs were transfected with either control (scrambled, SCR) or APOL1 siRNA. RNAs were extracted from control and transfected cells (n=3) and assayed for

35

miR193a. Cumulative data are shown in a bar diagram.**P<0.01 compared with other variables.

D. DPDs were transfected with either control (scrambled, SCR) or APOL1siRNA and incubated in media with or without miR193a inhibitor for 48 hours (n=3). Protein blots were probed for APOL1, WT1, PAX2 and GAPDH. Gels from three different lysates are displayed.

E. RNAs were extracted from the lysate preparations of 6D and assayed for miR193a. Cumulative data are shown in a bar diagram. *P<0.05 compared with control and SCR; **P<0.01 compared with control, miR193a inh alone, and SCR; [a]P<0.05 with all other variables.

7. APOL1 negatively regulates miR193a expression in DPDs

A. UNDPDs stably expressing vector and overexpressing APOL1G0 were incubated in RPMI containing 11 mM glucose and 10% serum for 10 days at 37°C. APOL1G0-expressing DPDs were transfected with either scrambled or APOL1 siRNAs (n=6). After 48 hours, proteins were extracted from control (vector) and siRNA-transfected cells. Protein blots were probed for APOL1 and reprobed for WT1, PAX2 and actin. Representative gels from three different lysates are displayed.

B. Cumulative densitometric data (n=6) from the protein blots of 7A are shown in a bar diagram. *P<0.05 compared with V and G0 APOL1 siRNA in WT1 and all other variables in PAX2 proteins; **P<0.01 compared with V and G0 APOL1 siRNA in APOL1 protein.

36

C. RNAs were extracted from the lysates of the protocol 7A. RNAs were assayed for miR193a and cumulative data are shown in a bar diagram. *P<0.05 compared with vector; **P<0.01 compared with vector; ***P<0.001 compared with G0 and G0/SCR.

Fig. 8. VDR agonist (VDA) preserves DPDs phenotype through modulation of miR193a-APOL1 axis in high glucose milieu

A. UNDPDs were incubated in media containing either vehicle (0.1% DMSO) alone or different concentrations of VDA (EB1089, 0, 1, 10, and 100 nM) for 48 hours (n=3). RNAs were extracted and assayed for miR193a. Cumulative data are shown in a bar diagram. *P<0.05 compared with vehicle (VDA, 0 nM), VDA, 0 and1.0 nM;**P<0.01 compared with vehicle (VDA, 0 nM), VDA, 0 and 1.0 nM; [a]P<0.05 compared with VDA, 10 nM.

B. DPDs were incubated in media containing normal glucose (C, 5mM), high glucose (HG, 30 mM), vehicle (0.1% DMSO with or without VDA (EB1089, 10 nM) for 48 hours (n=3). RNAs were extracted and assayed for miR193a. Cumulative data are shown in a bar diagram. **P<0.01 compared with other variables.

C. DPDs were incubated in media containing either normal glucose (C, 5mM), high glucose (HG, 30 mM) with or without VDA (EB1089, 10 nM) for 48 hours (n=3). Protein blots were probed for APOL1 and re-probed for GAPDH. Representative gels are displayed.

D. Cumulative densitometric data from the lysates of 8C are shown in a bar diagram. *<0.05 compared to C; [a]P<0.05 compared to HG alone.

E. DPDs were incubated in media containing either normal glucose (C, 5mM), vehicle (Veh, 0.1% DMSO, high glucose (HG, 30 mM) with or without VDA (EB1089, 10 nM)

37

for 48 hours (n=3). Protein blots were probed for WT1, PAX2 and re-probed for GAPDH. Representative gels are displayed.

F. Cumulative densitometric data from the protein blots of 8E are shown in a bar diagram. *P<0.05 compared with respective all other variables.

Fig. 9. Proposed mechanistic schemes

A. Composition of WT1 repressor complex is shown in a cartoon. WT1 repressor complex binding to PAX2 promoter represses its transcription. Disruption of this complex would de-repress the expression of PAX2.

B. High glucose enhanced the expression of miR193a, which led to down regulation of APOL1 expression in DPDs. These alterations in miR193a-APOL1 axis induced DPDs dedifferentiation. VDA provided protection against this effect of high glucose through the reversal of miR193a-APOL1 axis alterations.

Fig.1.

Fig. 2

Fig. 3

G

Fig. 3

A

WT-1
PAX2
APOL1
GAPDH

C   EV   miR193a

Plasmid

B

☐ Control   ☐ EV   ■ miR193a

Protein/GAPDH

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

WT1   PAX2   APOL1

**

*

*

C

APOL1 mRNA

1.5

1

0.5

0

C   EV   miR193a

Plasmid

*

Fig. 4

A

APOL1
Nephrin
PDX
PAX2
Actin

0 day   10 days

B

WT1
RBBP4
Menin
EZH2
H3K27me3
DNMT1
Actin

0 day   10 days

C

Protein/Actin

□ 0 Day ■ 10 Days

Nephrin   PDX   APOL1   PAX2

D

Protein/Actin

□ 0 Day ■ 10 Days

WT1   RBBP4   Menin   EZH2   DNMT1   H3K273me

Fig. 5

Fig. 5

Fig. 6

A

APOL1

WT1

PAX2

Actin

Vector   G0   G0/SCR   G0/siRNA

APOL1

B

Protein/Actin

2.00

1.50

1.00

0.50

0.00

APOL1   WT1   PAX2

■ V
■ G0
■ G0 SCR
□ G0 APOL1 siRNA

**   **

*   *   *

C

miR193a (fold change)

4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

Vector   G0   G0/SCR   G0/siRNA

APOL1

** , ***

*   *

Fig. 7

Fig.8

A

EZH2 PcG Menin WT1 DNMT1 H3K27me3 Transcription PAX2

B

High glucose

VDA ⊣ ↑miR193a

↓↑

↓ APOL1

Dedifferentiation

Fig. 9.