Identification of Restoring Effect of Orai1 Inhibitor in Diabetic Nephropathy Using BTBR *ob/ob* Mouse

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Directed by Professor Minseob Eom

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ABSTRACT

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Diabetic nephropathy (DN) is a leading cause of chronic kidney disease (CKD) worldwide, accounting for approximately half of all cases. Renal fibrosis is the most critical pathological change in DN, and reversing advanced fibrosis is crucial for treating this irreversible and often fatal condition. Orai1, a key component of store-operated Ca^{2+} entry (SOCE), represents a major Ca^{2+} influx mechanism in non-excitable cells, including glomerular podocytes, and is proposed as a potential therapeutic target for DN. Recent studies yielded conflicting results regarding SOCE inhibition, with reports of protective effects against or exacerbating renal fibrosis. This study aimed to investigate the significance of Orai1 in human DN and to demonstrate the impacts of Orai1 inhibition in BTBR *ob/ob* mice, which exhibit morphological and functional changes similar to those observed in human type II diabetes mellitus (DM).

Immunohistochemical staining for Orai1 was performed on kidney tissues from 93 patients diagnosed with DN. The significance of Orai1 expression was evaluated in relation to the pathological and clinical parameters of DN using Pearson correlation, univariate logistic regression analysis, and multivariate logistic regression analysis. A 4-week intervention experiment was conducted on 14-week-old BTBR *ob/ob* mice by administering an Orai1 inhibitor. Essential parameter change, morphological, and functional analysis were performed.

In the results of the human study, Orai1 expression was significantly positively correlated with higher RPS grades of DN classification, increased interstitial fibrosis and tubular atrophy (IFTA) scores, and serum creatinine (Cr) levels and CKD stage. There was an inverse correlation between Orai1 expression and estimated glomerular filtration rate (eGFR). Logistic regression analysis showed a significant correlation between high RPS grades and advanced CKD stages. After adjustment, multivariate logistic regression analysis also revealed a strong association with advanced CKD stages. Based on these results, these findings were applied to animal experiments.

In the results of the animal study, baseline parameters of the mice were measured, and glomerular basement membrane (GBM) thickness, glomerular volume, and mesangial area were assessed. Podocyte density was quantified and analyzed through immunohistochemical staining for WT-1. Blood samples were collected to measure serum Cr levels, and the urinary albumin-to-creatinine ratio (ACR) was calculated. In the treated BTBR *ob/ob* mice, no significant effects on body weight, blood glucose, or kidney weight were observed. However, GBM thickness was reduced to the same level as that of the control group in the treated group. Additionally, a tendency for reduced glomerular volume was observed, and mesangial area significantly decreased. Podocyte density was restored, and the number of podocytes per glomerulus increased considerably. The urinary ACR was significantly reduced in the treated BTBR *ob/ob* mice.

In summary, this study demonstrated a strong correlation between Orai1 and clinical and pathological prognostic factors in human DN, indicating its involvement in the development and progression of DN. Based on these findings, the Orai1 blockade intervention experiment in BTBR *ob/ob* mice showed morphological and functional recovery of DN, suggesting that Orai1 is a meaningful therapeutic target for DN. Furthermore, Orai1 has potential value as a biomarker for the diagnosis, treatment, and prognosis prediction of DN.

Keywords: Store-operated Ca^{2+} entry, Chronic kidney disease, Estimated glomerular filtration rate, BTBR *ob/ob* mouse, Biomarker

I. INTRODUCTION

Diabetic nephropathy (DN) is a primary cause of chronic kidney disease (CKD) and a leading contributor to end-stage kidney disease (ESKD) worldwide, affecting 10–30% of patients with diabetes mellitus (DM) (1). Hallmark features of DN include early extracellular matrix (ECM) deposition and myofibroblast accumulation within the glomerulus, ultimately leading to glomerulosclerosis and ESKD (2). Renal fibrosis, characterized by α-smooth muscle actin (α-SMA)-expressing myofibroblast leading to excessive ECM production, is a critical pathological prognostic factor in DN (2).

Currently, dialysis or kidney transplantation are the only treatment options for DN, highlighting the urgent need for novel therapeutic approaches. Intracellular Ca^{2+} homeostasis plays a crucial role in the physiological processes of renal cells. Intracellular Ca^{2+} is regulated by two mechanisms: leakage from the endoplasmic reticulum (ER) and influx from the extracellular environment. When intracellular Ca^{2+} is depleted within the ER, Ca^{2+} influx occurs through the cell membrane, a process known as store-operated Ca^{2+} entry (SOCE). SOCE, one of the intracellular Ca^{2+} influx mechanisms, comprises the Ca^{2+} sensor stromal interaction molecule 1 (STIM1) in the ER and the Ca^{2+} channel Orai1 in the cell membrane (3).

Recent studies suggest that Orai1 could serve as a potential therapeutic target for inhibiting acute kidney injury and the progression to CKD through the blockade of SOCE (4). Furthermore, alterations in the expression and function of SOCE are closely associated with various types of cancers, particularly Orai1's correlation with the pathophysiology and prognosis of several cancer

types, including renal cell carcinoma, the most common kidney tumor (3).

In another study, Orai1 in mesangial cells has been reported as an endogenous protective factor in diabetes, inhibiting ECM deposition in the kidney (4). Additionally, when Orai1 in mesangial cells was inhibited using siRNA, it led to increased expression of ECM proteins in the renal cortex and glomerulus, inducing mesangial expansion. Conversely, other research has shown that inhibiting Orai1 can suppress renal fibrosis and protect the kidneys (2). Increased expression of Orai1 in kidney tissue is observed in advanced fibrosis compared to minimal change disease (MCD) (2).

However, mouse models suitable for DN study are limited and must meet three criteria proposed by the Animal Models of Diabetic Complication Consortium (AMDCC) in 2009: a lifelong reduction in glomerular filtration rate (GFR) by over 50%, a more than tenfold increase in albuminuria compared to age- and sex-matched controls, and pathological changes in the kidneys (5). The eNOS$^{-/-}$*db/db* mouse model, proposed as an ideal model of DN by AMDCC closely resembles human diabetes and exhibits obesity, hypertension, albuminuria, severe mesangial expansion, and mesangial matrix degradation, similar to advanced human DN after 24 weeks (6). However, it takes a long time (over 24 weeks) for typical changes of DN to manifest, and maintaining double gene mutations is challenging (7). The BTBR *ob/ob* mouse, which lacks leptin, exhibits morphological and functional changes almost identical to advanced type 2 DN in humans in a relatively short time (approximately 18 weeks), making it optimal for therapeutic model studies of DN (7).

To date, research on the association between DN and Orai1, including SOCE, has been limited to the studies mentioned above, and mainly, there has been no study on the significance of SOCE in human DN samples. Therefore, this thesis aimed to demonstrate the restorative effects of blocking Orai1 and other SOCE components on DN, both morphologically and functionally, using the BTBR

ob/ob mouse, which exhibits changes almost identical to human type II DM. Furthermore, this study sought to elucidate the significance of Orai1 and other SOCE components in human DN. Ultimately, this thesis intends to conclusively demonstrate that Orai1 and other SOCE components are novel targets for the treatment of DN.

II. METHODS AND MATERIALS

1. PART 1. Clinical study

1.1. Patients and tissue samples

Ninety-three patients diagnosed with DN at Yonsei University Wonju Severance Christian Hospital from 2009 to 2019 were enrolled in this study. Biopsy tissues from 10 patients diagnosed with MCD, characterized by histologically normal kidneys and a favorable clinical prognosis, served as the control group. Both paraffin-embedded blocks and fresh frozen tissues were utilized for pathological diagnosis. Slides stained with Periodic acid-schiff (PAS) and John methenamine silver (JMS), along with the accompanying pathological reports, were reviewed, reclassified, and quantified according to the Renal Pathology Society classification (RPS class) proposed in 2010 (9). The RPS class was launched by the Research Committee of the Renal Pathology Society and has since been used to predict the renal prognosis for DN (9, 10). In addition, electronic medical records were reviewed to collect the clinical parameters. This study was approved by the institutional review board of Yonsei University Wonju Severance Christian Hospital (CR320183). The requirement for informed consent was waived. All the procedures adhered to the principles of the Declaration of Helsinki.

1.2. Immunohistochemistry

A Ventana Benchmark Ultra automatic immunostaining machine (Roche Diagnostics, Basel, Switzerland) was used for immunohistochemistry (IHC). Four ㎛ sections were deparaffinized in xylene, rehydrated in graded alcohols, and subjected to pretreatment with CC1 (formalin-fixed solution, Roche Diagnostics). The sections were washed with reaction buffer, followed by incubation with Orai1 antibody (Proteintech, Rosemont, IL, USA) at a 1:100 dilution. Bound antibodies were detected using the UltraView Universal DAB Detection Kit (Roche Diagnostics), and sections were counterstained with hematoxylin (Roche Diagnostics) according to the manufacturer's instructions. The positive and negative control stains were also tested.

This study divided the renal tissue into glomerulus and tubulointerstitium and categorized the expression of Orai1 IHC as semi-quantified as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong).

In this study, Orai1 expression was measured in the glomerulus and tubulointerstitium respectively. Furthermore, this study estimated the total expression of Orai1 by combining Orai1 expressions in glomerulus and tubulointerstitium, and expressed as a total score (T score).

1.3. Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from the tissue samples using a RiboEx Total RNA Kit (GeneAll Biotechnology, Seoul, Korea) and reverse-transcribed into cDNA using a cDNA Synthesis Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The abundance of mRNA was analyzed by quantitative RT-PCR with SYBR-green (Applied Biosystems, Foster City, CA, USA) using sequence-specific human primers: Orai1, F-5' TTGAGCCGCGCCAAGCTTAAA 3,' R-5' CATTGCCACCATGGCGAAGC 3'; 18S, F-5' CGGCGTTATTCCCATGAC 3,' R-5' GCCCTTCCGTCAATTCCT 3.' Experiments were performed in triplicates using a QuantStudio 6 Flex RT- PCR System (Applied Biosystems, Foster City, CA, USA). The 2-ΔΔCT method was used to analyze the data with 18S (18S ribosomal RNA subunit) as the reference gene.

1.4. Pathological parameters

Until recently, there was no alternative to the RPS class system utilized in this study for DN. Pathological factors incorporated into the RPS class include GBM thickening, mesangial expansion, nodular sclerosis, and global sclerosis. Additionally, this classification incorporates scores for tubulointerstitial and vascular lesions such as interstitial fibrosis and tubular atrophy (IFTA), inflammatory interstitial infiltrates, arteriolar hyalinosis (AH), and arteriosclerosis (AS) scores.

1.5. Clinical parameters

Collected data included age, sex, body mass index (BMI), serum creatinine (Cr), estimated GFR (eGFR), hemoglobin A1c (HbA1c), total 24-hour urinary protein, microalbuminuria, hypertension, duration of DM, and renal replacement therapy (RRT) status. Patients were stratified based on CKD stage by GFR and albuminuria as low-risk, moderate-risk, high-risk, and very high-risk based on the kidney disease improving global outcomes (KDIGO) 2012 guidelines (11).

1.6. Statistical analysis

The significance of Orai1 expression in human DN was assessed by examining its correlation with pathological and clinical parameters of DN. Pearson's correlation coefficient analysis was conducted to determine the relationships between the expression of Orai1 and individual pathological and clinical parameters. Associations between the expression of Orai1 and RPS class, eGFR, RRT status, and CKD stage were analyzed using univariate logistic regression. To overcome the limitations of semi-quantitative IHC analysis described above, this study compared the scores of each pathological and clinical factor to their mean values. Subsequently, factors showing significant relationships in the above analyses were re-evaluated according to the other clinical characteristics of each patient and included in the multivariate logistic regression analysis. The predictive accuracy of each model was determined using c-statistics, corresponding to the area under the receiver operating characteristic (ROC) curve. All statistical analyses were performed using SPSS (version 28.0, IBM Corp., Armonk, NY, USA). A p-value <0.05 was considered statistically significant.

2. PART 2. Animal study

2.1. Animal treatment

2.1.1. Mouse model

This study obtained approval from the Animal Experiment Ethics Committee of Yonsei University Wonju College of Medicine (YWC-200701-1). This study introduced 8-week-old male BTBR *ob/ob* mice and WT mice (No. 4824, Jackson Lab, Bar harbor, Maine, USA) and raised them until 14 weeks of age. Using 14-week-old male BTBR *ob/ob* mice and WT mice, we conducted intervention experiments blocking Orai1 with four experimental groups (Figure 1).

2.1.2. Intervention experiments

Among several Orai1 blockers, this study selected GSK-7975A (Sigma Aldrich, St. Louis, Missouri, USA) for the intervention experiment, as it showed clearer morphological effects in preliminary experiments, particularly in mesangial changes and some tubulointerstitial changes. This compound targets the Orai1 channel and prevents Ca^{2+} ions from entering the cell through the channel (8). GSK-7975A (hereafter referred to as GSK) was dissolved in dimethyl sulfoxide (Sigma Aldrich, St. Louis, Missouri, USA) at a concentration of 90mg/ml and injected into Alzet 1004 micro-osmotic pumps (Durect corporation, Cupertino, CA, USA), which were then inserted subcutaneously into the mice. The mice were sacrificed at 18 weeks of age.

Blood glucose levels were measured using tail vein blood at 14 and 18 weeks, and urine was collected for 6 hours using metabolic cages the day before the intervention experiment at 14 and 18 weeks. After the intervention experiment, blood was collected via the eyeball. Major organs (kidneys, heart, lungs, liver, pancreas, spleen, skin, and both eyes) were collected.

The collected organs were fixed in formalin solution and electron microscopy fixative, depending on the analysis method. Some kidney tissues were snap-frozen in OCT compound (Sakura Finetek, Torrance, CA, USA) and stored at -80℃ in a deep freezer.

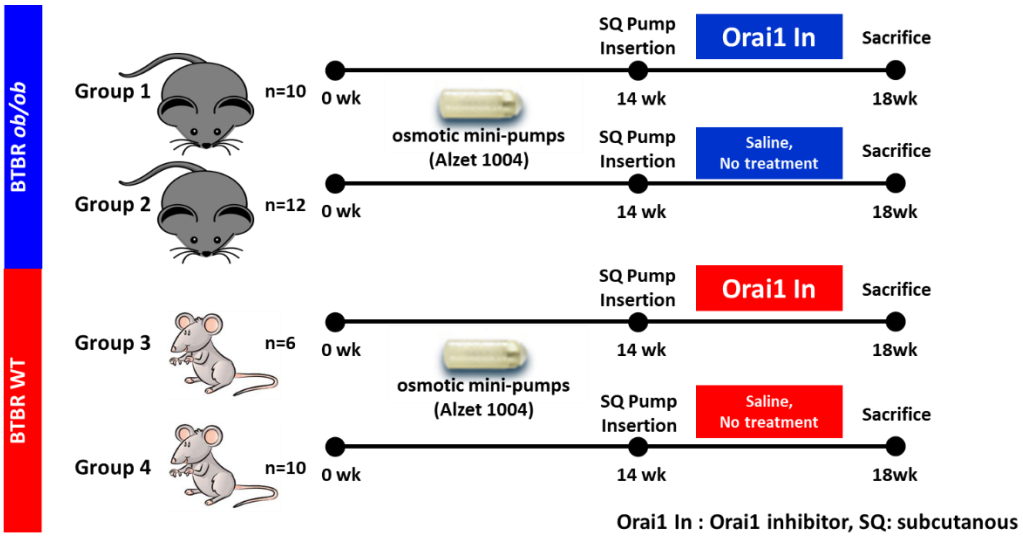

Figure 1. Intervention protocol for BTBR *ob/ob* mouse experiment

Orai1 inhibition intervention experiments were conducted using 14-week-old BTBR *ob/ob* and WT mice, divided into four groups. The Orai1 inhibitor (GSK) was administered via an osmotic mini-pump implanted subcutaneously in the experimental groups (Group 1, 3). For the control groups (Group 2, 4), either saline was administered via the osmotic mini-pump or no treatment was applied. The intervention experiments were concluded at 18 weeks of age.

2.2. Morphological analysis

2.2.1 Podocyte density and podocyte number per glomerulus

In this research, glomerular podocytes in mouse samples were stained immunohistochemically using WT-1 (ab267377, Abcam, Cambridge, UK), a marker for podocytes. Images were then randomly captured at 200x magnification under a light microscope. The podocyte density was measured using ImageJ (available at https://imagej.net), as described by Venkatareddy et al. (9).

2.2.2 Glomerular tuft area and Mesangial matrix

In this research, random images of slides stained with JMS at 100x magnification were captured using a light microscope. The glomerular tuft area (GTA) and the area stained black by JMS were then calculated using ImageJ.

2.2.3 Electron microscopy examination

In this research, an electron microscopy examination was conducted and the thickness of the GBM was compared by analyzing the findings.

2.3. Functional analysis

This research measured serum Cr levels using the QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA) for blood samples. For urine samples, it utilized the Albuwell M Murine ELISA Kit (Ethos Biosciences Inc., Logan Township, NJ, USA) to measure albumin and Cr levels. The urine ACR for each group was calculated based on the results obtained. Additionally, albumin in 24-hour urine samples was measured using the Albuwell M Murine ELISA Kit and serum and urine Cr levels were measured using the QuantiChrom Creatinine Assay. In this research, the differences in ACR between the BTBR ob/ob and WT groups before and after GSK treatment were compared and analyzed.

2.4. Statistical analysis

Animal data analysis was performed using the GraphPad Prism software (version 9.5 GraphPad Software, San Diego, CA, USA). Multiple comparisons were conducted using one-way ANOVA followed by Brown-Forsythe or Bartlett's multiple comparisons test. p-values <0.05 were considered statistically significant.

III. RESULTS

1. PART 1. Clinical study

1.1. The expression of Orai1 is observed in diabetic nephropathy

Orai1 expression was elevated in DN patient samples. This research observed a significant increase in the relative mRNA levels of Orai1 in DN tissues compared to normal tissues (Figure 2). IHC staining demonstrated pronounced Orai1 expression in the cytoplasm of the tubular and interstitial stromal cells, unlike in the control group, where Orai1 expression was negligible in the tubulointerstitial area. Orai1 expression increased concomitantly with the RPS class in human DN specimens (Figure 3).

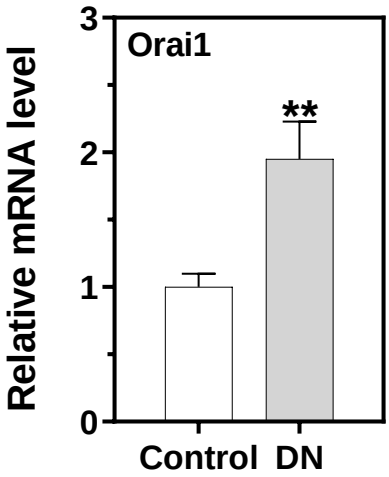

Figure 2. The result of relative mRNA level expression of Orai1 in diabetic nephropathy

Comparison of mRNA levels to SOCE revealed that the expression of Orai1 increased in the human diabetic nephropathy group compared to the control group.

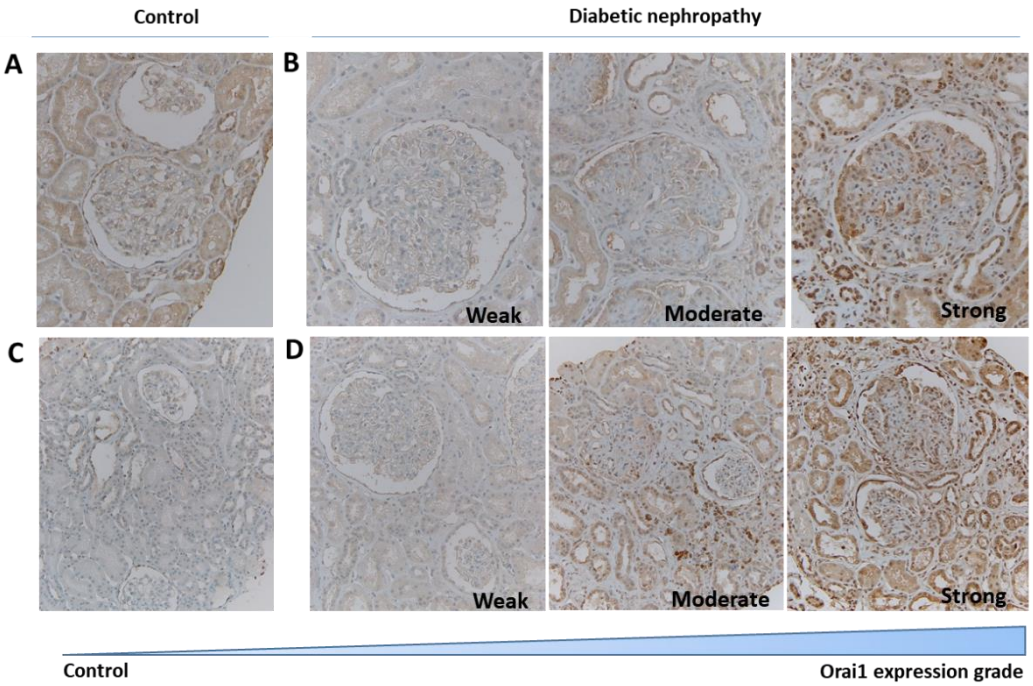

Figure 3. The result of immunohistochemical stain for Orai1

Immunohistochemical staining results for Orai1 are shown as glomerular control (A), glomerular DN (B), tubulointerstitium control (C), and tubulointerstitium DN (D). Orai1 is clearly expressed in the cytoplasm of tubules and interstitial stromal cells. The expression of Orai1 in each structure was categorized as weak grade expression (1+), moderate grade expression (2+), and strong grade expression (3+) (B&D) (x200).

1.2. The expression of Orai1 correlates with various pathologic parameters in diabetic nephropathy

This study found a significant positive correlation between Orai1 expression and AH in the glomerulus. In the tubulointerstitium, Orai1 expression demonstrated significant positive correlations with various factors, including RPS class, IFTA. Additionally, when examining the correlation between T score and pathology parameters, it was confirmed that significant positive correlations between Orai1 expression and RPS class, IFTA, and AH. (Table 1)

Table 1. Correlations between Renal Pathology Society Diabetic Nephropathy classification and Orai1 expression

Parameters	Pearson correlation coefficient (r)				
Orai1	RPS glomerular classification	IFTA	Interstitial inflammation	AH	AS
G	0.122	0.044	0.049	0.206*	-0.0057
T-I	0.278**	0.370***	0.153	0.157	0.058
T score	0.226*	0.231*	0.114	0.209*	-0.002

* p <0.05, ** p <0.01, *** p <0.001; RPS, renal pathology society; IFTA, interstitial fibrosis and tubular atrophy; AH, arteriolar hyalinosis; AS, arteriosclerosis; G, glomerulus; T-I, tubulointerstitium; T score, total score.

1.3. The expression of Orai1 correlates with clinical parameters

This study found that Orai1 expression in the glomerulus positively correlated with the CKD stage and negatively correlated with eGFR and HbA1c levels. In the tubulointerstitium, Orai1 expression also correlated positively with serum Cr level. Conversely, an inverse relationship was observed with eGFR levels, reaching statistical significance. This suggests that higher Orai1 levels are associated with lower eGFR values. The T score has a positive correlation with serum Cr level and CKD stage and a negative correlation with eGFR and HbA1c (Table 2).

Table 2. Correlations between clinical factors and Orai1 expression

Parameters	Pearson correlation coefficient (r)					
Orai1	Cr	eGFR	HbA1c	24h TP	24h Malb	CKD stage
G	0.047	-0.223*	-0.226	-0.011	-0.040	2.282**
T-I	0.340**	-0.508**	-0.66	-0.119	-0.155	0.449
T score	0.216*	-0.413***	-0.208**	-0.072	-0.109	0.407***

* p <0.05, ** p <0.01, *** p <0.001; Cr, creatinine; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; TP, urine total protein; Malb, urine microalbumin; CKD, chronic kidney disease; G, glomerulus; T-I, tubulointerstitium; T score, total score.

This study investigated whether there were differences in the values of pathological clinical factors based on the expression level of Orai1. The Orai1 high expression group in the glomerulus showed significantly higher RPS class and lower eGFR levels (Table 3). Similarly, the Orai1 high-expression group in the tubulointerstitium showed significantly higher RPS class, IFTA scores, interstitial inflammation levels, and serum Cr levels. However, eGFR levels were markedly lower in the high-expression group compared to the low-expression group (Table 4).

Table 3. The difference in the mean value for pathological prognostic and clinical factors according to Orai1 expression (glomerulus)

Parameters	Orai1 (G)		
	Low expression (0&1) (Mean ± SD)	High expression (2&3) (Mean ± SD)	*p*-value
RPS glomerular classification	2.645 ± 0.95	3.07 ± 0.80	0.036
IFTA	1.95 ± 1.05	2.17 ± 0.85	0.387
Interstitial inflammation	1.23 ± 0.75	1.46 ± 0.58	0.123
Cr	2.79 ± 2.93	3.58 ± 3.07	0.28
eGFR	61.79 ± 50.50	36.23 ± 28.67	0.003

SD, standard deviation; RPS, renal pathology society; IFTA, interstitial fibrosis and tubular atrophy; Cr, creatinine; eGFR, estimated glomerular filtration rate; G, glomerulus

Table 4. The difference in the mean value for pathological prognostic and clinical factors according to Orai1 expression (tubulointerstitium)

Parameters	Orai1 (T-I)		
	Low expression (0&1) (Mean ± SD)	High expression (2&3) (Mean ± SD)	*p*-value
RPS glomerular classification	2.29 ± 0.83	3.09 ± 0.80	0.001
IFTA	1.36 ± 0.84	2.25 ± 0.84	<0.001
Interstitial inflammation	1.07 ± 0.83	1.47 ± 0.57	0.029
Cr	1.89 ± 2.76	3.66 ± 3.03	0.045
eGFR	87.86 ± 50.12	34.20 ± 26.59	<0.001

SD, standard deviation; RPS, renal pathology society; IFTA, interstitial fibrosis, and tubular atrophy; Cr, creatinine; eGFR, estimated glomerular filtration rate; T-I, tubulointerstitium.

Univariate regression analysis indicated that higher RPS class and CKD stages are associated with an increased likelihood of higher Orai1 expression. Subsequent multivariate regression analysis, adjusted for other relevant clinical factors, confirmed a robust association between the CKD stage and Orai1 expression. (Tables 5&6). The c-index values for Orai1 expression, serum Cr, eGFR, and 24-hour total urinary protein were 0.781, 0.905, 0.983, and 0.567, respectively, highlighting their predictive (Figure 4).

Table 5. Univariate logistic regression analysis of pathological or clinical factors for high expression of Orai1

Outcome	Parameters	Odds ratio	95% Confidence interval		*p*-value
			Lower limit	Upper limit	
RPS glomerular classification	Orai1 (G)	3.47	1.25	9.38	0.016
	Orai1 (T-I)	6.1	1.81	20.52	0.003
eGFR	Orai1 (G)	1.255	0.459	3.270	0.685
	Orai1 (T-I)	0.497	0.149	1.654	0.255
RRT	Orai1 (G)	2.007	0.648	6.216	0.227
	Orai1 (T-I)	2.824	0.727	10.973	0.134
CKD stage	Orai1 (G)	6.444	2.275	18.254	<0.001
	Orai1 (T-I)	13.2	3.611	48.251	<0.001

RPS, Renal Pathology Society; eGFR, estimated glomerular filtration rate; RRT, renal replacement therapy; CKD, chronic kidney disease; G, glomerulus; T-I, tubulointerstitium.

Table 6. Multivariate logistic regression analysis of pathological or clinical factors for high expression of Orai1

Model[†]	Parameters	Odds ratio	95% Confidence interval		*p*-value
			Lower limit	Upper limit	
CKD stage	Orai1 (G)	11.208	2.590	48.497	0.001
	Orai1 (T-I)	18.839	3.088	114.938	0.001

[†]Adjusted by age, sex, BMI, HbA1c, DM duration, and hypertension; G, glomerulus; T-I, tubulointerstitium.

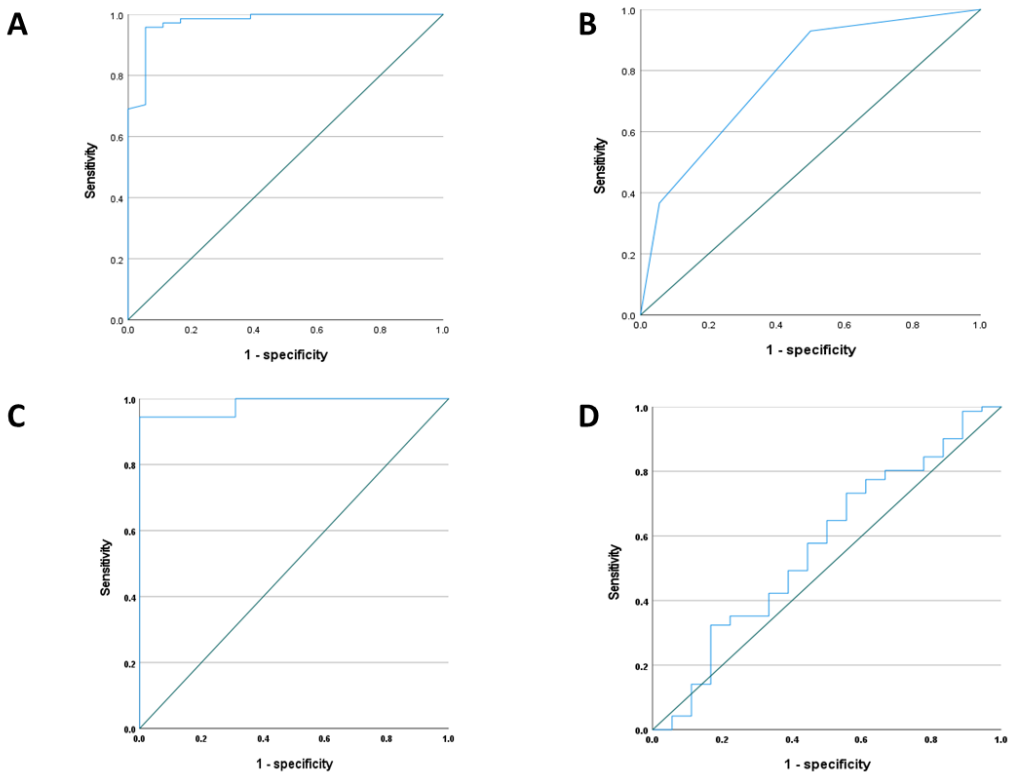

Figure 4. Receiver operating characteristic curve (ROC)

The ROC curves of Orai1 expression (A), creatinine (B), eGFR (C), and 24hr total protein (D) as predictors of CKD progression risk.

2. PART 2. Animal study

2.1. There was no difference in the primary parameter analysis of the BTBR *ob/ob* mouse experiment

In this study, body weight, blood glucose levels, and kidney weight were compared between BTBR *ob/ob* mice and WT mice (Figure 5). The body weight of the BTBR *ob/ob* mouse group was increased compared to the WT group. Regarding blood glucose levels, the BTBR *ob/ob* mouse group was higher than the WT group, but there was no difference between the GSK treatment group and the control group. Regarding kidney weight, similar to the increase in body weight observed in the BTBR *ob/ob* mouse group, kidney weight was also increased compared to the WT group; however, there was no difference between the GSK treatment group and the control group. Overall, no statistically significant differences in the primary parameter were observed between the GSK treatment group and the control group in BTBR *ob/ob* mice.

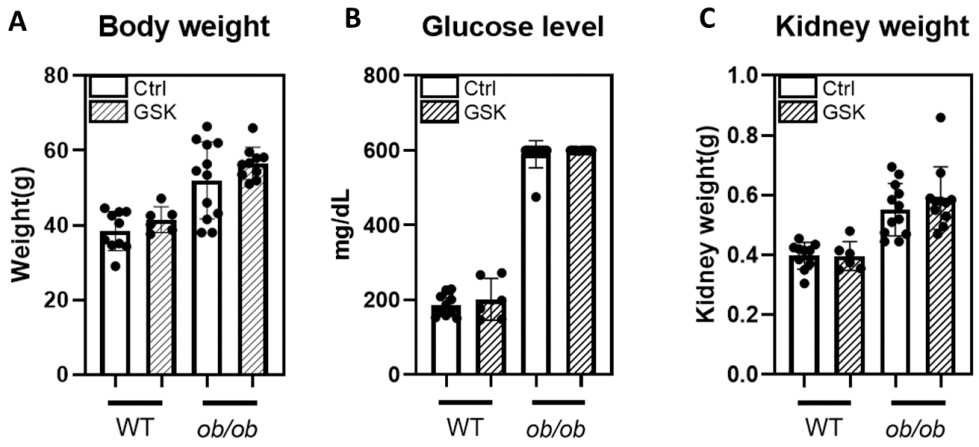

Figure 5. Effects of Orai1 inhibitor on body weight, glucose and kidney weight in a BTBR *ob/ob* mice

GSK treatment group had no effect on body weight in BTBR *ob/ob* mouse compared to control group (A). Glucose level was also unchanged with GSK treatment group (B). GSK treatment had no effect on kidney weight in BTBR *ob/ob* mouse (C).

2.2. Glomerular podocytes recovered after GSK-7975A treatment

In the BTBR *ob/ob* mouse group, a significant increase in the number of podocytes per glomerulus can be observed in the GSK treatment group compared to the control group. Comparing podocyte density between the WT mouse and BTBR *ob/ob* mouse groups, there was a decrease in the BTBR *ob/ob* mouse group. In the BTBR *ob/ob* mouse group, there is a tendency for increased podocyte density in the GSK treatment group compared to the control group (Figure 6).

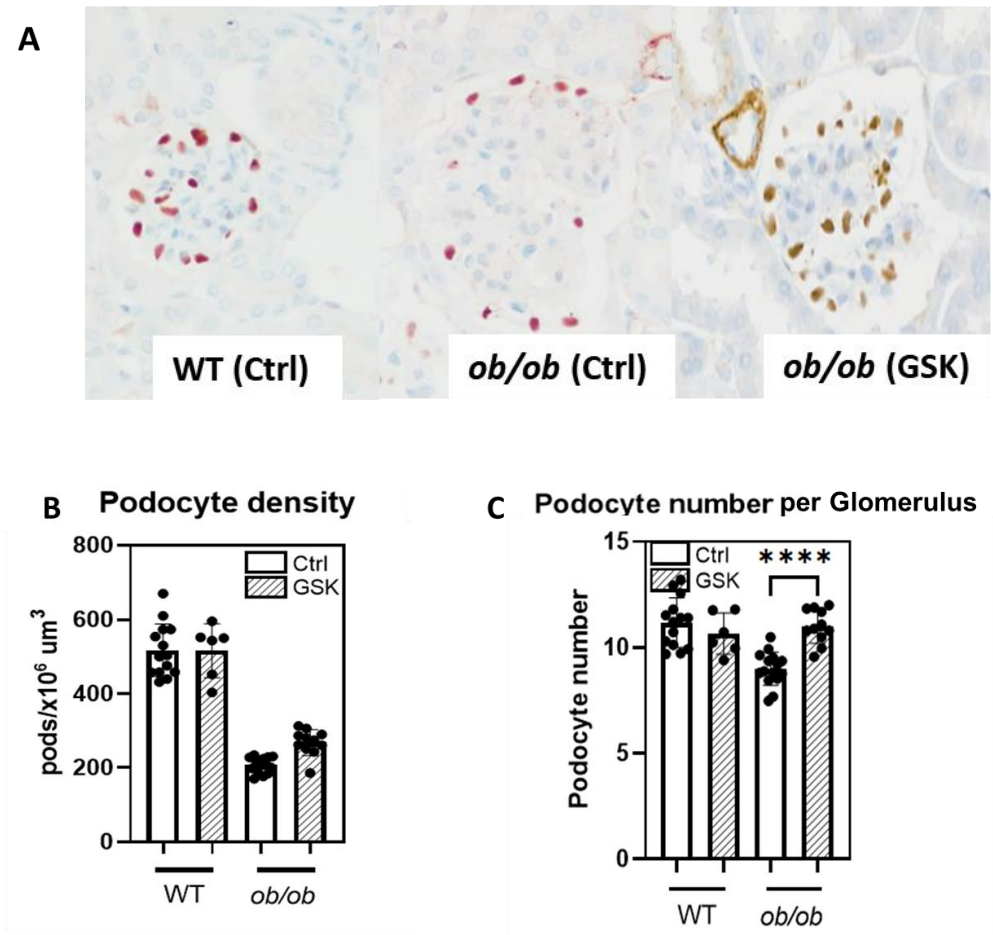

Figure 6. Glomerular podocytes recovered after GSK-7975A treatment

The representative glomerulus immune was stained with WT-1 (X200) (A). In the BTBR *ob/ob* group, the glomerular podocyte density was significantly restored in the GSK-treated group (B). There was also a similar trend of increase in the number of podocytes per glomerulus (C). $^{****}p$ <0.0001

2.3. GSK-7975A treatment results in a significant reduction of mesangial matrix in BTBR *ob/ob* mice

Comparing the WT mouse and BTBR *ob/ob* mouse groups, GTA and mesangial matrix increase can be observed (Figure 7). The BTBR *ob/ob* mouse group shows a noticeable trend towards reduced glomerular volume in the GSK treatment group. Furthermore, the area of the mesangium significantly decreased between the GSK treatment group and the control group in BTBR *ob/ob* mice.

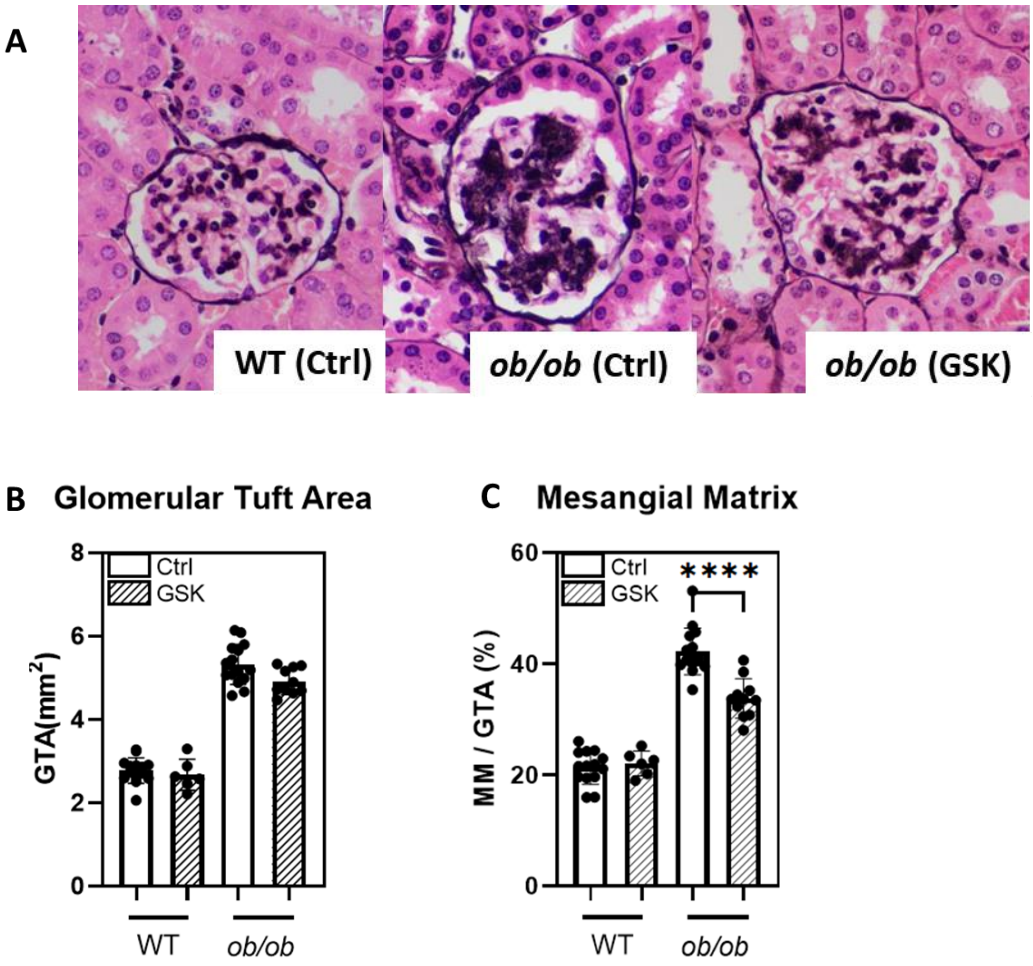

Figure 7. GSK-7975A treatment results in a significant reduction of mesangial matrix in BTBR *ob/ob* mice

The representative glomerulus showing mesangial matrix accumulation in BTBR *ob/ob* mice with GSK treatment significantly decreased compared with BTBR *ob/ob* control mice (X100) (A). Morphometric analysis of glomerular tuft area (GTA) stained by Jones methenamine sliver (JMS) of the mesangial matrix (B). The mesangial matrix significantly decreased in the GSK treatment group compared with a control group in BTBR *ob/ob* mice (C). $^{****}p$ <0.0001

2.4. GSK-7975A reduces the GBM thickness of BTBR *ob/ob* mice

When observed under an electron microscope, the BTBR *ob/ob* mouse group exhibited a significant decrease in GBM thickness in the GSK treatment group compared to the control group. Additionally, the foot processes of podocytes appeared to be well preserved compared to the control group (Figure 8).

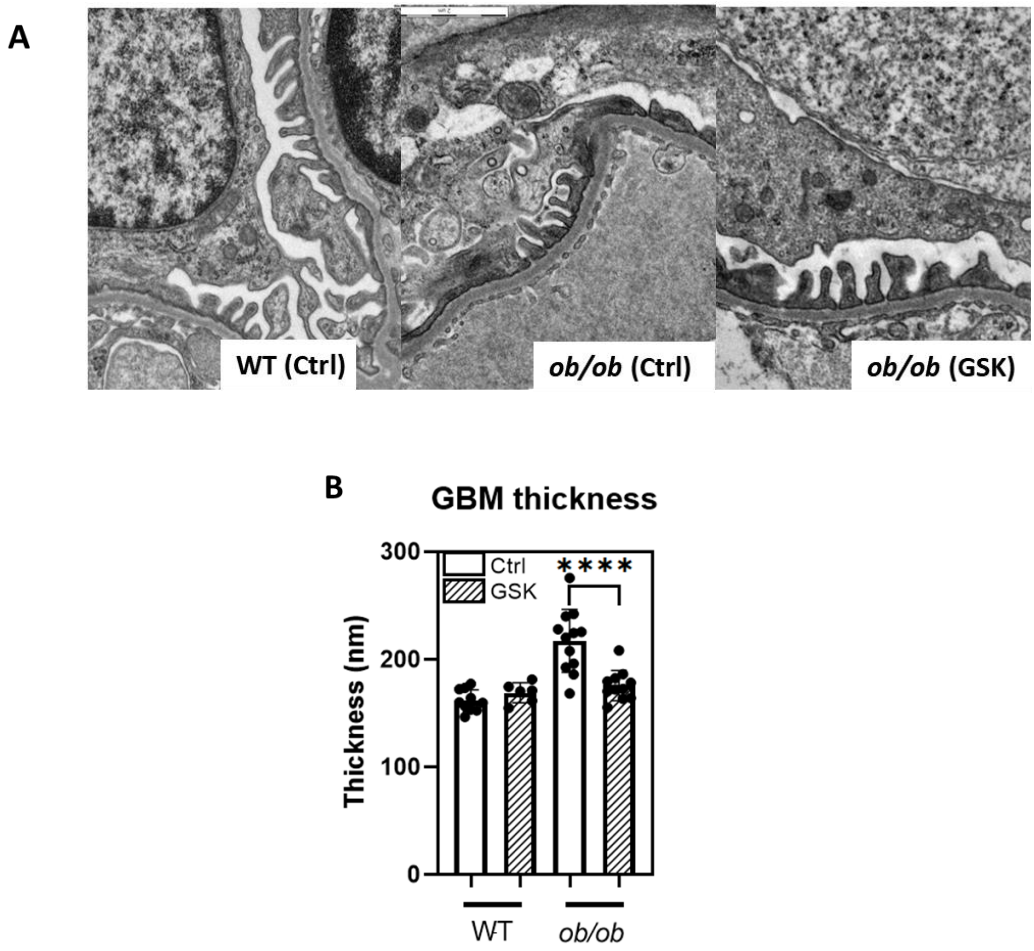

Figure 8. GSK-7975A reduces the GBM thickness of BTBR *ob/ob* mice

Electron micrographs of representative glomerulus show that the podocyte foot processes are better preserved in the GSK treatment group compared to the control group (A). The thickness of the GBM was significantly reduced in the GSK treatment group compared to the control group in BTBR *ob/ob* mice (B). $^{****}p$ <0.0001

2.5. Functional analysis of BTBR *ob/ob* mice showed a significant reduction in Albumin-Creatinine Ratio (ACR)

In the BTBR *ob/ob* mouse group, serum Cr levels appeared to be increased in the control group compared to the GSK treatment group. Conversely, in the WT group, serum Cr levels seemed to decrease in the GSK treatment group compared to the control group (Figure 9A).

When compared with the WT group, the urinary ACR in the control group of the BTBR *ob/ob* mouse group was significantly elevated. In contrast, a statistically significant decrease in the urinary ACR was observed in the GSK treatment group (Figure 9B).

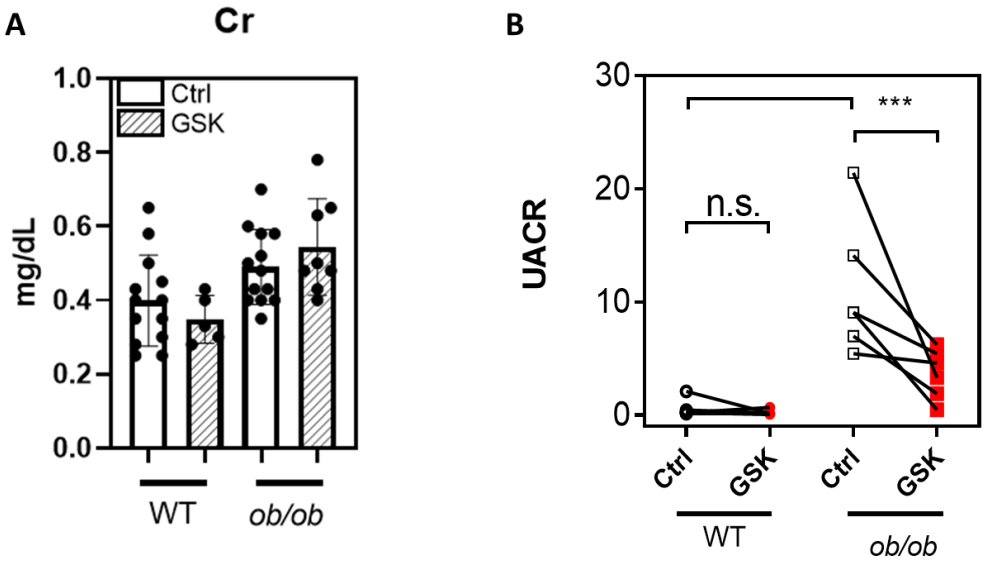

Figure 9. Functional analysis of BTBR *ob/ob* mice; Serum creatinine level, Albumin-Creatinine Ratio (ACR)

Analysis of serum samples to determine creatinine showed no significant differences in BTBR *ob/ob* mouse (A). The urinary ACR (UACR) showed a statistically significant decrease in GSK treated group (B). $^{***}p$ <0.001

IV. DISCUSSION

In human DN, Orai1 expression was increased in the DN group compared to the control group, and it was correlated with the RPS class, a representative pathological prognostic indicator for DN. A significant correlation with representative clinical parameters was observed, particularly a distinct correlation with the CKD stage. Additionally, Orai1 expression in the tubulointerstitium was found to have a very high correlation with the progression of DN and clinical and pathological prognostic factors compared to the result in the glomerulus. The therapeutic effects of Orai1 inhibition were analyzed on DN, using BTBR *ob/ob* mice, which exhibit morphological and functional changes very similar to those in human type II DM. This study showed that Orai1 blockade led to partial morphological and functional recovery of DN.

Ca^{2+} is a ubiquitous second messenger regulating various cellular functions (10). An increase in intracellular Ca^{2+} concentration has been found to play a crucial role in developing kidney diseases, including renal fibrosis (11). Additionally, the Orai1 Ca^{2+} channel is vital in regulating cellular Ca^{2+} oscillations in the kidney, as well as in cell migration, proliferation, and gene expression (3).

Podocytes are terminally differentiated epithelial cells. Their limited regenerative capacity and vulnerability to various diseases make podocyte injury particularly important in glomerular pathology. Loss of sufficient numbers of podocytes inevitably leads to glomerulosclerosis (GS) and eventual nephron loss (12). Podocytes are one of the several insulin signaling targets in the kidney (13). Insulin receptor signaling is pivotal for podocyte function, and perturbation of podocyte insulin signaling compromises the glomerular filtration barrier, causing proteinuria (14). Kim et al. (15) showed that podocyte damage and proteinuria coincided with increased Orai1 expression at the

hyperinsulinemic stage and that suppression of Orai1 Ca^{2+} signaling ameliorated the detrimental effects of elevated insulin. Additionally, Orai1 is the main component of SOCE in podocytes in vivo, and podocyte Orai1 is a crucial target for insulin's detrimental actions on the glomerular barrier (2).

Several studies have conflicting opinions on whether the overexpression of Orai1 protects or worsens kidney pathology (15, 16). In mesangial cells under diabetic conditions, Orai1 activation prevents the expression of matrix proteins such as fibronectin (17). In contrast, overexpression of Orai1 in proximal tubular epithelial cells exacerbates renal fibrosis (2). Furthermore, mesangial expansion generally occurs in the early stages of DN, while interstitial fibrosis is characteristic of the later stages of DN (2). Therefore, Orai1 in the kidney may function differently in tubular and mesangial cells in response to high glucose and lipid levels (2). This result suggests that the pathophysiological role of Orai1 and SOCE is cell type-specific and may vary depending on the stage of DN development.

Whether Orai1 positively or negatively regulates DN features, including fibrosis and proteinuria, appears to depend on the type of diabetes (15, 18). Orai1-mediated SOCE in mesangial cells has been associated with negatively regulating ECM protein expression in a diabetic milieu (17, 19). In contrast, Orai1 overexpression has been observed in proximal tubular fibrosis induced by unilateral urethral obstruction or a high-fat diet and is positively correlated with interstitial fibrotic changes (2). From a proteinuria perspective, Orai1 isoforms are highly expressed in the proximal tubules but are downregulated in type I DM. Functionally, the Orai1 blockade accelerates proteinuria in type I DM (18). A recent study indicates that Orai1 is overexpressed in glomerular podocytes in a type II DM animal model and demonstrates that Orai1 inhibition ameliorates proteinuria and protects glomerular filter function (15). In this study, upregulated Orai1 was positively associated with the clinicopathological characteristics and CKD in the kidneys of patients with type II DM.

Accumulating evidence regarding the pathophysiological roles of Orai1 reveals bidirectional effects in rodent models of DN. Thus, this data from human patients with type II DM suggests that Orai1 may be a positive pathological mediator of interstitial fibrosis in the kidney.

Orai1 is intricately linked to key pathological prognostic factors of DN, especially the RPS class and IFTA scores, showing significant correlations. The RPS class standardizes the identification and scoring of DN lesions (20). DN is characterized by various changes, including vascular lesions (AH and AS), tubulointerstitial damage marked by tubular basement membrane (TBM) thickening and tubular cell hypertrophy, and progressive interstitial fibrosis in the later stages (21). Early DN stages are characterized by mesangial matrix accumulation (20). A notable positive correlation exists between Orai1 expression and serum Cr levels and CKD stage. Conversely, negative correlations were observed with eGFR levels, 24-hour total urinary protein, and microalbumin, with statistical significance. Orai1 was helpful for predicting the risk of progression of DN. These findings underscore the potential of Orai1 as a pathological and prognostic marker for DN and CKD. Based on the significance of Orai1 expression in human DN as previously described, this research conducted interventional experiments using the BTBR *ob/ob* mouse model, which is the most similar to human type 2 DM.

The BTBR *ob/ob* mouse model of DN meets nearly all the criteria proposed by the AMDCC (albuminuria, pathological changes) (7). Nephropathy in this model is characterized by podocyte loss, diffuse and focal nodular GS, mesangiolysis, glomerular capillary microaneurysm formation, thickening of GBM and TBM, absence of immune deposits, and mild to moderate interstitial fibrosis (22). Additionally, ACR shows a significant increase from 8 weeks of age and continues to rise, indicating functional changes (23). This data provides the advantage of shortening the time required for interventional studies aimed at improving DN, allowing for rapid progression and intervention

in developing this progressive disease (7). Although the eNOS-/-*db/db* mouse is an optimal model that closely resembles human diabetes, it takes a long time (over 24 weeks) for typical DN changes to manifest, and maintaining double genetic modifications is challenging (6, 7).

Maintaining the actin structure in podocyte foot processes is crucial for glomerular filtration (24). Podocyte actin changes can compromise the glomerular filter's integrity (15). Insulin alters this actin dynamics by regulating cellular Ca^{2+}, disrupting the cytoskeleton of the glomerulus and leading to albumin leakage (15). Orai1 is an essential target for the harmful effects of insulin on the glomerular barrier (15). Overexpression of Orai1 due to insulin administration leads to synaptopodin depletion and foot process effacement, exacerbating albuminuria. These results suggest insulin action targeting Orai1 in podocytes can further worsen the condition (15). Insulin-activated SOCE results in Ca^{2+} overload, causing actin remodeling, slit diaphragm disruption, and detachment of the GBM, leading to proteinuria (15). This study observed characteristics of BTBR *ob/ob* mice, including reduced podocyte density and a decreased number of podocytes per glomerulus, which are early changes in DN. After Orai1 inhibition, the recovery of podocyte density and the increase in the number of podocytes per glomerulus suggest that Orai1 is likely involved in maintaining podocytes. This supports the concept that the acute activation of Orai1 by insulin stimulation induces the transient disruption of the cytoskeletal structure in podocytes, leading to glomerular filter disruption and albuminuria (15). Therefore, to prevent proteinuria, it is necessary to regulate Orai1 in podocytes appropriately, and the key to this regulation is Ca^{2+}.

One of the early features of DN is the accumulation of ECM proteins in the glomerular mesangium (17). Excessive production of ECM by mesangial cells, including fibronectin and collagen type IV (Col IV), and the deposition of these proteins in the mesangium significantly contribute to mesangial expansion in the early stages of DN (25, 26). Orai1 protein expression in glomerular mesangial cells

has been shown to increase under high glucose or diabetic conditions (19, 27). Mesangial cells are crucial in mesangial matrix homeostasis (25, 28, 29). Dysfunction of mesangial cells is closely associated with various kidney diseases, including DN (30, 31). The mesangial cell function is regulated by intracellular Ca^{2+} like many other cell types (29, 32).

This study has several limitations. First, as this was a single-center study, the number of patients in each classification was not evenly distributed. Additionally, the study focused only on type II DM. Further analysis, including more cases, is necessary. Second, the lack of fresh kidney tissue limited our ability to explore Orai1 gene variations across RPS classes. If sufficient cases can be obtained, it is necessary to confirm these findings with an increased sample size. Lastly, due to the limitation of the BTBR *ob/ob* mouse model, which lacks fibrosis formation in the interstitium and tubules, it was difficult to observe the blocking effect of Orai1 resulting from fibrotic injury.

In this study, early changes in DN, such as GBM thickening and foot process effacement, were observed in BTBR *ob/ob* mice. These changes were found to be reversible by blocking Orai1. This result suggests that Orai1 blockade may protect the kidneys from diabetic damage in the early stages of DN. It was also found that inhibiting Orai1 in proximal tubular cells, rather than mesangial cells, can protect the kidneys from fibrotic damage during the progression of renal fibrosis (2). In this study, urinary ACR was improved by Orai1 inhibition. Orai1 inhibition may be considered to prevent renal dysfunction, consistent with improvements in renal structure.

V. CONCLUSION

In summary, this study demonstrated that Orai1 is highly correlated with clinical and pathological prognostic factors in human DN, indicating its involvement in the development and progression of DN. Based on these findings, the Orai1 blockade intervention in BTBR *ob/ob* mice showed morphological and functional recovery of DN, confirming that Orai1 is associated with the pathogenesis of DN and could be a potential therapeutic target. Ultimately, Orai1 could be considered a novel candidate biomarker for predicting DN's diagnosis, treatment, and prognosis.

REFERENCES

1. Stefan G, Stancu S, Zugravu A, et al. Histologic predictors of renal outcome in diabetic nephropathy: Beyond renal pathology society classification. Medicine (Baltimore). 2019;98(27): e16333.
2. Mai X, Shang J, Liang S, et al. Blockade of Orai1 Store-Operated Calcium Entry Protects against Renal Fibrosis. J Am Soc Nephrol. 2016;27(10): 3063-3078.
3. Kim JH, Lkhagvadorj S, Lee MR, et al. Orai1 and STIM1 are critical for cell migration and proliferation of clear cell renal cell carcinoma. Biochem Biophys Res Commun. 2014;448(1): 76-82.
4. Mehrotra P, Sturek M, Neyra JA, et al. Calcium channel Orai1 promotes lymphocyte IL-17 expression and progressive kidney injury. J Clin Invest. 2019;129(11): 4951-4961.
5. Brosius FC, 3rd, Alpers CE, et al. Mouse models of diabetic nephropathy. J Am Soc Nephrol. 2009;20(12): 2503-12.
6. Zhao HJ, Wang S, Cheng H, et al. Endothelial nitric oxide synthase deficiency produces accelerated nephropathy in diabetic mice. J Am Soc Nephrol. 2006;17(10): 2664-9.
7. Alpers CE, Hudkins KL. Mouse models of diabetic nephropathy. Curr Opin Nephrol Hypertens. 2011;20(3): 278-84.
8. Rubaiy HN. ORAI Calcium Channels: Regulation, Function, Pharmacology, and Therapeutic Targets. Pharmaceuticals (Basel). 2023;16(2).
9. Venkatareddy M, Wang S, Yang Y, et al. Estimating podocyte number and density using a single histologic section. J Am Soc Nephrol. 2014;25(5): 1118-29.

10. Leybaert L, Sanderson MJ. Intercellular Ca(2+) waves: mechanisms and function. Physiol Rev. 2012;92(3): 1359-92.
11. Saliba Y, Karam R, Smayra V, et al. Evidence of a Role for Fibroblast Transient Receptor Potential Canonical 3 Ca2+ Channel in Renal Fibrosis. J Am Soc Nephrol. 2015;26(8): 1855-76.
12. Tao Y, Mallet RT, Mathis KW, Ma R. Store-operated Ca(2+) channel signaling: Novel mechanism for podocyte injury in kidney disease. Exp Biol Med (Maywood). 2023;248(5): 425-433.
13. Rogacka D. Insulin resistance in glomerular podocytes: Potential mechanisms of induction. Arch Biochem Biophys. 2021;710: 109005.
14. Fornoni A. Proteinuria, the podocyte, and insulin resistance. N Engl J Med. 2010;363(21): 2068-2069.
15. Kim JH, Hwang KH, Dang BTN, et al. Insulin-activated store-operated Ca(2+) entry via Orai1 induces podocyte actin remodeling and causes proteinuria. Nat Commun. 2021;12(1): 6537.
16. Noel S. Orai1: CRACing the Th17 response in AKI. J Clin Invest. 2019;129(11): 4583-4586.
17. Wu P, Wang Y, Davis ME, et al. Store-Operated Ca2+ Channels in Mesangial Cells Inhibit Matrix Protein Expression. J Am Soc Nephrol. 2015;26(11): 2691-702.
18. Zeng B, Chen GL, Garcia-Vaz E, et al. ORAI channels are critical for receptor-mediated endocytosis of albumin. Nat Commun. 2017;8(1): 1920.
19. Chaudhari S, Wu P, Wang Y, et al. High glucose and diabetes enhanced store-operated Ca(2+) entry and increased expression of its signaling proteins in mesangial cells. Am J Physiol Renal Physiol. 2014;306(9): F1069-80.
20. Kim T, Kwak Y, Lee JY, et al. Pathological validation of the Japanese Renal Pathology Society classification and challenges in predicting renal prognosis in patients with diabetic nephropathy.

Kidney Res Clin Pract. 2022;41(5): 545-555.

21. Jefferson JA, Shankland SJ, Pichler RH. Proteinuria in diabetic kidney disease: a mechanistic viewpoint. Kidney Int. 2008;74(1): 22-36.
22. Pichaiwong W, Hudkins KL, Wietecha T, et al. Reversibility of structural and functional damage in a model of advanced diabetic nephropathy. J Am Soc Nephrol. 2013;24(7): 1088-102.
23. Hudkins KL, Pichaiwong W, Wietecha T, et al. BTBR Ob/Ob mutant mice model progressive diabetic nephropathy. J Am Soc Nephrol. 2010;21(9): 1533-42.
24. Pavenstadt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. Physiol Rev. 2003;83(1): 253-307.
25. Schlondorff D, Banas B. The mesangial cell revisited: no cell is an island. J Am Soc Nephrol. 2009;20(6): 1179-87.
26. Gorin Y, Block K, Hernandez J, et al. Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. J Biol Chem. 2005;280(47): 39616-26.
27. Wang Y, Chaudhari S, Ren Y, Ma R. Impairment of hepatic nuclear factor-4alpha binding to the Stim1 promoter contributes to high glucose-induced upregulation of STIM1 expression in glomerular mesangial cells. Am J Physiol Renal Physiol. 2015;308(10): F1135-45.
28. Abboud HE. Mesangial cell biology. Exp Cell Res. 2012;318(9): 979-85.
29. Stockand JD, Sansom SC. Glomerular mesangial cells: electrophysiology and regulation of contraction. Physiol Rev. 1998;78(3): 723-44.
30. Kashgarian M, Sterzel RB. The pathobiology of the mesangium. Kidney Int. 1992;41(3): 524-9.
31. Scindia YM, Deshmukh US, Bagavant H. Mesangial pathology in glomerular disease: targets for therapeutic intervention. Adv Drug Deliv Rev. 2010;62(14): 1337-43.

32. Ma R, Pluznick JL, Sansom SC. Ion channels in mesangial cells: function, malfunction, or fiction. Physiology (Bethesda). 2005;20: 102-11.

국 문 요 약

BTBR *ob/ob* 마우스에서 Orai1 차단제의 당뇨신장병 회복 효과 규명

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당뇨신장병은 전세계적으로 만성 신장병의 약 절반의 원인을 차지하는 대표적인 질환이다. 신장 섬유화는 당뇨신장병에서 가장 중요한 병리학적 변화로 알려져 있으며, 이로 인한 손상은 어려워 불가역적인 것으로 알려져 있다. 그러나 최근에는 이러한 불가역적인 변화를 회복시키는 시도가 진행되고 있다. 대표적인 저장조절 칼슘유입 기전(store-operated Ca^{2+} entry, SOCE) 인자인 Orai1은 사구체 발세포를 포함한 비흥분성 세포에서 주요한 칼슘유입 기전으로, 당뇨신장병의 잠재적 치료 표적으로 제시되고 있다. 최근 연구에서 SOCE의 차단이 신장섬유화에 대한 보호효과를 보이거나, 또는 악화시킨다는 상반된 연구결과가 보고되었다. 이에 본 연구에서는 인간 당뇨신장병에서 Orai1의 역할을 규명하고, 이를 바탕으로 인간의 제2형 당뇨신장병과 유사한 형태학 및 기능적 변화를 보이는 BTBR *ob/ob* 마우스 모델에서 Orai1 억제효과를 규명하고자 하였다.

당뇨신장병으로 진단된 93명의 신장 조직을 이용하여 Orai1에 대한 면역조직화학 염색을 시행하였다. Orai1 발현의 중요성은 당뇨신장병의 병리 및 임상적 인자와의 상관관계를 피어슨 상관계수 및 단변량 로지스틱 회귀분석을 통해 평가하였다. Orai1 발현은 높은 신장병리학회 당뇨신장병 분류(RPS 등급), 높은 간질 섬유화 및 세뇨관 위축 점수와 유의한 상관관계가 관찰되었고, 혈청 크레아티닌 수치 및 만성신장질환 단계와 양의 상관관계를 보였다. 추정 사구체여과율(estimated Glomerular Filtration Rate, eGFR)은 Orai1 발현과 역상관 관계가 있다. 로지스틱 회귀 분석에서는 높은 RPS 등급과 고등급 만성신장질환 단계와 유의한 상관관계가 있었으며, 다변량 로지스틱 회귀 분석에서는 고등급 만성신장질환 단계와 강한 연관성을 보였다.

BTBR *ob/ob* 마우스를 이용한 Orai1 억제제 주입에 의한 중재실험에서, 마우스의 기본 인자 측정 후 사구체기저막의 두께와 사구체용적, 메산지움 면적을 측정하였다. 면역조직화학 염색을 통해 발세포 밀도를 정량화하여 분석하였으며, 채취한 혈액에서는 혈청 크레아티닌 수치, 소변에서는 알부민-크레아티닌 비율을 계산하였다. BTBR *ob/ob* 마우스 치료군에서 체중, 혈당, 신장의 무게에는 뚜렷한 영향을 미치지 않았지만, 사구체 기저막 두께가 치료군에서 대조군과 동일한 수준으로 감소하였다. 또한 치료군에서 사구체 용적이 감소되는 경향을 보였고, 메산지움 면적은 유의하게 감소하였다. 발세포 밀도 회복과 사구체당 발세포 수의 유의한 증가를 확인하였다. 치료군에서 소변 알부민-크레아티닌 비율이 유의하게 감소하였다.

요약하면, 인간의 당뇨신장병에서 Orai1은 임상적·병리학적 예후 인자들과 높은 상관성을 보였으며, 당뇨신장병의 발생 및 진행과 밀접한 연관이 있음을 확인하였다. 이

를 바탕으로 BTBR *ob/ob* 마우스에서 Orai1 차단 중재실험을 통해 형태학적 및 기능적 회복을 보였으며, Orai1이 당뇨신장병 치료에 응용할 수 있는 표적으로 제시할 수 있었다. 나아가 Orai1이 당뇨신장병의 진단, 치료 및 예후예측이 가능한 바이오마커로서 가치가 있음을 강력히 시사한다.

핵심 되는 말; 저장조절 칼슘유입, 만성신장질환, 추정 사구체여과율, BTBR *ob/ob* 마우스, 바이오마커