Cerebral Ischemic-Hypoxia Induces TRPC6 in the Glomerular Podocytes: A Novel Role for HIF1α-ZEB2 Axis in the Pathogenesis of Stroke-induced Proteinuria

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Running title: Ischemia-Hypoxia Alters Podocyte Permselectivity via ZEB2-TRPC6 Axis

Keywords: Hypoxia, Proteinuria, Podocytes, HIF1α, ZEB2, and Epithelial-Mesenchymal Transition, intracellular calcium, cytoskeleton reorganization, TRPC6.

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Abstract:

Podocytes are a key component of the glomerular filtration barrier (GFB), which plays a critical role in ensuing ultra-filtrated urine. The integrity of the GFB is compromised during ischemic stroke and is manifested by proteinuria. The mechanism by which glomerular permselectivity is compromised during stroke remains enigmatic. Hypoxia is a determining factor in the pathology of ischemic stroke. We investigated the mechanism of ischemia-hypoxia-induced proteinuria in a middle cerebral artery occlusion (MCAO) stroke model. Ischemia-hypoxia resulted in the accumulation of HIF1α in the glomerular podocytes, which further resulted in the elevated expression of ZEB2. ZEB2, in turn, induced the expression of transient receptor potential cation channel, subfamily C, member 6 (TRPC6) that has increased selectivity for calcium. Elevated expression of TRPC6 elicited increased calcium influx and aberrant activation of focal adhesion kinase (FAK). FAK activation resulted in the stress fibers reorganization and foot process effacement. Our study suggests that ischemia-hypoxia induced HIF1α-ZEB2-TRPC6 axis in podocytes resulted in increased intracellular calcium levels and consequently altered podocyte integrity and permselectivity.

Introduction:

Human physiology is often challenged with hypoxic conditions, a state of deficiency of oxygen in the blood and tissues. Both the continuous supply and adequate levels of oxygen are crucial for the normal functioning of the human body. The kidneys have a high oxygen demand, so as to facilitate energy dependent basic renal functions such as active salt absorption[1]. Kidneys carry out complex functions within a relatively narrow range of partial pressure of oxygen[2]. Kidneys possess low-resistance microvasculature that is exposed to both high volume and continuous perfusion[3,4]. The constraints of low oxygen supply, dictated by both renal architecture and high oxygen demand coalesce to make the kidney vulnerable to pathological stresses during hypoxia[1,5,6]. Limitations in oxygen supply susceptible the kidneys to hypoxia-induced adaptations and has been recognized as an important factor in the pathogenesis of renal injury and proteinuria[6-12].

The vertebrate kidney plays an essential role in the filtration of plasma, regulation of water, electrolyte and acid-base balance, thus maintaining the homeostasis. The function of the kidney is to ensure ultra-filtrated urine. In the absence of either infection or genetic abnormalities, proteinuric condition indicates renal injury, particularly aberrations in glomerular filtration barrier (GFB)[13]. The crucial components of GFB are glomerular capillary endothelium, basement membrane, and podocytes[13]. Proteinuria is often associated with conditions of decreased perfusion such as stroke, shock, trauma, and sleep apnea, wherein all these conditions are presented with moderate to severe hypoxia[12,14]. Accumulated evidence suggests that hypoxia does play a significant role in the pathogenesis and progression of chronic kidney disease (CKD)[6,7,10,15-17]. The

prevalence of CKD is more than 30% among stroke patients[18]. Renal dysfunction is associated with worse short and long-term outcomes in patients with ischemic stroke[19,20] and is an independent predictor of stroke mortality[18].

Epidemiological studies revealed that almost 1 in 10 adults in the USA has proteinuria[21]. Considering the magnitude of the proteinuric population and the association of proteinuria with the disorders wherein hypoxia prevails, it is imperative to understand the mechanism of renal dysfunction and to develop preventive or therapeutic strategies. In the present study, we investigated the mechanism of renal dysfunction in an ischemic stroke model. Ischemic stroke results from the blockage of blood vessels in the brain particularly in the cerebral region. Therefore, we employed the intraluminal monofilament model of middle cerebral artery occlusion (MCAO), which is commonly used to mimic human focal brain ischemia. In this study, we found that ischemic stroke and resultant ischemia-hypoxia injury resulted in the elevated expression of HIF1α in several organs including kidney. ZEB2, a downstream target of HIF1α is induced in glomerular podocytes, which in turn induced the expression of TRPC6 vis-à-vis calcium influx in podocytes. Calcium-induced FAK activation resulted in stress fibers rearrangement and manifested in impaired podocyte structure and function. Overactivity of the HIF1α-ZEB2-TRPC6 axis could be a mechanism by which systemic hypoxia provokes poor renal outcome.

Results:

Ischemic stroke is associated with systemic hypoxia and proteinuria: We performed Triphenyltetrazolium chloride (TTC) staining to assess the infarct size and injury following MCAO and reperfusion. Ischemic brain stained white color upon staining

with TTC indicates the infarct lesion (Fig 1A). In order to assess the impact of ischemia-stroke injury on renal function, we performed spot urine analysis and assessed urinary albumin to creatinine ratio. Rats subjected to ischemic stroke showed ~7 fold increase in albumin excretion when compared with control rats (Fig.1B). Silver staining revealed a significant amount of protein in urinary fractions from stroke-induced rats (Fig.1C). Hypoxia is a common occurrence following stroke and associated with poor clinical and functional outcomes[22]. Therefore to ascertain whether ischemic-reperfusion injury induces hypoxia, we analyzed the expression of HIF1α in several organs including brain and kidney and found that HIF1α is elevated in these organs (Fig.1D-G). In our earlier study, we demonstrated that HIF1α induces ZEB2 expression, which in turn transduces hypoxic effects in podocytes[23]. Therefore, we analyzed ZEB2 expression in tissues in which elevated HIF1α was observed and found elevated expression of ZEB2 is concomitant with HIF1α accumulation in stroke-induced rats (Fig.1D-F). The data suggests the ischemic stroke-induced rat's renal expression of HIF1α is associated with proteinuria.

To understand the renal biology of stroke-induced rats we assessed the morphology of glomerulus by various staining procedures. The PAS staining suggests glomerulosclerosis in stroke-induced rats (Fig 2A). Owing to the importance in glomerular filtration we would like to assess the podocyte morphology and their number in glomeruli from stroke-induced rats. WT1 staining revealed a decreased number of podocytes in stroke-induced rats compared with sham-operated rats (Fig.2B). Transmission electron microscope (TEM) images revealed that in podocytes from

ischemic-stroke rats foot processes become shortened and thickness of GBM was noticed (Fig.2C).

Ischemia-hypoxia induces ZEB2 and TRPC6 expression in podocytes: It was shown earlier that elevated HIF1α and ZEB2 expression was associated with epithelial-mesenchymal transition (EMT) of podocytes in rats exposed to normobaric hypoxia[24]. In the present study, as we noticed elevated HIF1α and ZEB2 expression in glomeruli from rats with ischemic stroke, we were interested to assess whether EMT phenomenon induced in podocytes from these rats. Cadherin switch is a characteristic feature of podocyte EMT[25-27]. Elevated expression of HIF1α and ZEB2 in ischemic stroke rat podocytes is associated with attenuation of E- and P- cadherin and increased N-cadherin expression (Fig.3A) and the phenomenon are known as cadherin switch. Human immortalized podocytes treated with FG-4592 (Hyx) also showed accumulation of HIF1α, ZEB2 and cadherin switch (Fig.3B). FG-4592 is a well-known HIF-α prolyl hydroxylase inhibitor, which stabilizes the HIF1α expression and ensures activation of HIF1α target genes. ZEB2 is a transcription factor and regulates expression of several proteins representing various cellular processes. The bioinformatics analysis revealed that TRPC6 (transient receptor potential cation channel, subfamily C, member 6) could be a downstream target of ZEB2[28]. We performed promoter analysis (TFSearch) and found that ZEB2 occupies E2-box1 region **(-430CAGGTG-425**) of human TRPC6 promoter. Interestingly, we found that TRPC6 expression was elevated in both stroke-induced rat podocytes and human podocytes exposed to FG-4592 (Fig.3A&B). Further, we found that HIF1α, ZEB2, and TRPC6 mRNA expression was also induced in stroke-induced rat podocytes and human podocytes treated with FG-4592 (Fig.3C&D).

Immunostaining data reveals that HIF1α, ZEB2, and TRPC6 are induced in glomeruli from stroke-induced rats (Fig.3E).

Hypoxia indcues HIF1α-ZEB2-TRPC6 axis and calcium influx in podocytes: To assess the elevated expression of TRPC6 is due to ZEB2 occupancy on TRPC6 promoter we performed ChIP assay. ChIP assay confirmed that ZEB2 occupies TRPC6 promoter at the E2-box1 region (Fig.4A). We utilized the interaction of ZEB2 with the promoter region of E-cadherin, which serves as a positive control. We also confirmed HIF1α occupancy on ZEB2 promoter (Fig 4A) wherein the interaction between HIF1α with VEGF promoter serves as a positive control. Further, we observed an increased TRPC6 promoter activity in HEK293T cells treated with FG-4592 (Fig.4B). Enhanced TRPC6 promoter activity was observed in HEK293T cells that ectopically express ZEB2 and either naïve or treated with FG-4592 (Fig.4B).

Upon confirming that in podocytes, HIF1α overexpression is concomitant with elevated expression of ZEB2 and TRPC6, we would like to understand the significance of elevated expression of TRPC6. Although, TRP family proteins have affinity for cation transport, TRPC6 has selectivity for calcium influx. Among glomerular cells, TRPC6 expresses predominantly in podocytes[29]. We found elevated intracellular calcium influx in FG-4592 treated podocytes as measured by calcium-sensitive fluorescent dye Fluo3-AM (Sigma-Aldrich, St. Louis, MO, USA) (Fig.4C). It is interesting to note that ectopic expression of ZEB2 increases calcium influx, while attenuation of ZEB2 expression by siZEB2 or by treatment with calcium channel blocker 2-aminoethyl diphenylborinate (2-APB) resulted in decreased intracellular calcium levels in podocytes (Fig.4C).

ZEB2 regulates activation of FAK via TRPC6: Auto phosphorylation of focal adhesion kinase (FAKpTyr397) increases with elevated calcium levels[30,31]. On the other hand, inhibition of FAK protects against proteinuria and Foot Process Effacement[32]. FAK is a central protein of focal adhesions and is known to regulate several cytoskeletal and other focal adhesion proteins[33]. Therefore, we measured pFAK levels in podocytes treated with FG-4592 or that ectopically express ZEB2. Podocytes that overexpress ZEB2, increased expression of pFAK is corroborated with elevated expression of TRPC6 (Fig 4D&E). Interestingly, in cells expressing siZEB2, attenuation of TRPC6 expression is concomitant with reduced pFAK levels (Fig.5A&B). Together, the data could suggest that ZEB2 regulates phosphorylation of FAK via TRPC6. Further, to ascertain the essential role of TRPC6 to ensure FAK activation in conditions of elevated HIF1α levels, we attenuated TRPC6 expression by siTRPC6 and measured the pFAK levels. pFAK levels decreased in relation to TRPC6 levels in cells treated with or without FG-4592 (Fig 5C&D).

Activated FAK is localized at focal adhesions. Focal adhesions serve as connections between cytoskeleton and the extracellular matrix whereas their formation is regulated by RhoA[33]. FAK suppresses RhoA activity and promote focal adhesion turnover[33,34]. In similar to the earlier reports, we also noticed that RhoA is decreased while FAK gets activated in podocytes treated with FG-4592 (Fig 5E). Upon treatment with either calcium channel blocker (2-APB) or FAK inhibitor 14, RhoA expression is restored in podocytes (Fig 5E).

HIF1α alters podocyte actin cytoskeleton: As we noticed activation of FAK and decreased RhoA expression in podocytes with elevated HIF1α, we further investigated

the cytoskeletal rearrangement, if any? Then, we assessed actin stress fibers (SFs) distribution in cells that are naïve or exposed FG-4592 employing Phalloidin staining. Differentiated podocytes naïve to hypoxia exhibit ample orderly arranged non-branching SFs (Fig. 6A-I). Upon exposure to hypoxia, the orderly arranged stress fibers of the podocyte actin cytoskeleton were disrupted (Fig. 6A-II). Stress fibers reorganization and altered morphology was observed in cells that ectopically express ZEB2 and treated with or without FG-4592 (Fig.6A-III&IV). Interestingly, ZEB2 knockdown prevented the SFs reorganization in podocytes exposed to FG-4592 (Fig 6A-V). We further tested the potential of calcium channel blocker (2-APB) or FAK inhibitor 14 to prevent altered cytoskeletal organization in podocytes exposed to hypoxic conditions. Phalloidin staining revealed that in podocytes that were exposed to FG-4592, or ectopically expressing ZEB2, treatment with 2-APB or FAK14 improved SFs reorganization (Fig.6A-VI-IX). Podocytes in which TRPC6 was knocked down, FG-4592 treatment did not alter the distribution of SFs (Fig.6A-X). We quantified the number of SFs per podocyte and the ratio of SFs to total cell size was calculated using Image J software (NIH). The data (Fig.6B) suggests that inhibition of TRPC6 channel thus calcium influx, or inhibition of FAK could prevent hypoxia triggered podocyte cytoskeletal rearrangements. The disruption of SFs of the podocyte actin cytoskeleton could probably explain the reason for altered cell morphology and FPE in podocytes from stroke-induced rats (Fig 2C vs. 6AII-IV).

Calcium Channel blockers (CCB) and FAK inhibitors prevented HIF1α induced podocyte permeability: We next examined the effect of 2-APB and FAK inhibitor14 on podocyte permeability employing paracellular albumin influx assay. Earlier studies

revealed that accumulation of HIF1α in podocytes increased podocyte permeability to albumin[23,24] and blunting the ZEB2 expression reduced the ability of hypoxia to elicit an increase in the albumin influx across podocyte monolayer suggesting that ZEB2 is necessary for transducing the effect of HIF1α on podocyte permeability. In this study, we showed that ZEB2 induces TRPC6 and calcium accumulation, and as a consequence, FAK is getting activated. Therefore, we assessed the permeability of podocytes treated with FG-4592 in the absence and presence of CCB (2-APB) and FAK inhibitor 14. Both 2-APB and FAK inhibitor 14 prevented the podocyte permeability to albumin (Fig 7A), suggesting that prevention of calcium influx and/or inhibition of FAK preserve the podocyte permselectivity during hypoxia.

Treatment with CCB improved proteinuria in ischemic stroke patients: The stroke patients admitted to the hospital were with and without hypertension. We collected the data from ischemic stroke patients without hypertension. These patients have reduced eGFR (Fig 7B) and increased UACR (Fig 7C). This data supports our animal experiment where we observed ischemic reperfusion injury is associated with proteinuria. Treating stroke patients with Dihydropyridine, a CCB along with other supplements of therapeutic regimen has been shown to reduce the albuminuria in proteinuric patients (Fig 7D).

Co-expression of HIF1α, ZEB2, and TRPC6 in kidney diseases: It is noteworthy that intrarenal hypoxic injury is considered to be the common cause of renal dysfunction and proteinuria in conditions such as hypertension, diabetes, and stroke[35,36]. We performed co-expression analysis for HIF1α, ZEB2, and TRPC6 in Nephroseq database (The Regents of The University of Michigan, Ann Arbor). We found the elevated expression

of HIF1α, ZEB2, and TRPC6 in Nakagawa CKD dataset and in Hodgin Diabetes Mouse Glomeruli datasets (Fig 8A&B). The data suggests in CKD of human origin and in diabetic mouse glomerular diseases these three proteins are elevated and co-expressed.

Discussion:

Podocytes are instrumental for contributing glomerular permselectivity and ultrafiltration. It has been known that ischemic stroke is often associated with proteinuria; we undertook the current study to investigate the cellular and molecular effects of stroked associated ischemia-hypoxia on podocyte biology. We show that after ischemic reperfusion, HIF1α and its down-stream target ZEB2 are elevated in glomerular regions and specifically in podocytes. Our results indicate a novel role of HIF1α, with the induction of TRPC6 in podocytes. This increase in TRPC6 is due to the HIF1α-dependent up-regulation of ZEB2 expression. TRPC6 ensure calcium influx into podocytes during ischemia-hypoxia, which elicits FAK activation and these events results in disruption of stress fibers (Fig. 9). In addition to altered morphology of podocytes, accumulation of HIF1α also resulted in the increased permeability to albumin across podocyte monolayer. Excess HIF1α-dependent induction of ZEB2 in the podocytes results in cadherin switch where expression of P- and E-cadherins decreased and N-cadherin increased. Cadherin switch is associated with both increased permeability and EMT of podocytes. In ischemic stroke patients, calcium channel blockers improved proteinuria, suggesting that inhibition of calcium influx preserves podocyte morphology and permselectivity. Our results establish that TRPC6

is a novel target of HIF1α/ZEB2 axis and TRPC6 transduces stroke-induced ischemia-hypoxia injury in podocytes (Fig 9).

MCAO model is the most frequently used experimental model of ischemic stroke in rats. Both, ischemic reperfusion injury in MCAO rats and humans with ischemic stroke were presented with significant proteinuria. Insufficient cerebral blood flow during ischemic stroke elicits hypoxia. The hypoxic condition results in reduced arterial oxygen pressure (AOP) in several organs including the kidney. Normally AOP is maintained at relatively stable levels by a functional interplay between renal blood flow, arteriovenous oxygen shunting, oxygen consumption, and glomerular filtration rate[2]. The fragility of this intricate interplay let the kidneys vulnerable to hypoxic injury[2]. It was reported that proteinuria is one of the major clinical outcomes following acute ischemic stroke[37]. Proteinuria refers to the impaired function of GFB; therefore, we investigated the effect of hypoxia in the glomeruli and in podocytes that are instrumental to ensure glomerular permselectivity.

HIF1α is a major transcription factor that transduces an array of cellular processes to let the cells adapt to hypoxic conditions that predominant during pathological and extreme physiological conditions. Our results establish that stroke- induced accumulation of HIF1α stimulates the expression of ZEB2. It was also shown earlier that ZEB2 is induced in podocytes exposed to normobaric hypoxia[24] and podocytes treated with GH[25]. ZEB2 is also known as smad-interacting protein 1(SIP1). ZEB2 belongs to the δEF1 protein family and these proteins possess a homeodomain flanked by two zinc finger clusters comprising a four-zinc finger N-terminal cluster and three-zinc finger C-terminal cluster[38]. Canonically, ZEB2 is considered as a

transcriptional repressor in C-terminal binding protein-dependent or independent manner[39]. ZEB2 has many targets including tight junction proteins and adherens junction proteins and owing to which elevated expression of ZEB2 could have pleiotropic effects on the podocyte biology. P- and E- cadherins are the canonical targets for transcriptional repression by ZEB2. P- cadherin is a part of a basic scaffold of podocyte slit- diaphragm, thus it is crucial for determining podocyte permselectivity. Decreased expression of P- cadherin is implicated in the pathogenesis of glomerulosclerosis and hypoxia-induced proteinuria[23,40,41]. On the other hand, decreased expression of E- cadherin is associated with the transition of podocytes from epithelial to mesenchymal phenotype[23,25] compromising their ability to offer epithelial coverage to glomerular capillaries.

Our study also establishes that ZEB2 induces TRPC6 expression in podocytes. Although ZEB2 is most often considered as a transcriptional repressor, accumulated evidence suggests that it can act as a transcriptional activator[28,42]. It was reported that the upstream promoter of TRPC6 has several ZEB2 binding sites[28]. Together the data suggest that ZEB2 could be a dual transcription factor with both repressor and activator functions. Further, this study also identified that decreased ZEB2 levels resulted in the reduced TRPC6 expression. Human TRPC sub-family of channels is most closely related to TRP channels from Drosophila. These proteins have TRP box motif containing the consensus EWKFAR sequence at C-terminus and 3 to 4 ankyrin repeats at N-terminus. These channels are in general permeable to cations and the selectivity for calcium over sodium variable among different members, whereas TRPC6 has more affinity for calcium. TRPC6 is located on chromosome 11q and proline

to glutamine substitution at position 112 enhances TRPC6-mediated calcium signals in response to agonist such as angiotensin II[43]. This glutamine substitution in TRPC6 is associated with focal segmental glomerulosclerosis. Furthermore, calcium entry into cells by TRPC6 has been implicated in late-onset of Alzheimer's disease[44]. It is interesting to note that in patients with hypertension or stroke patients with hypertension calcium channel inhibitors improve proteinuria.

The importance of cytoskeletal and structural proteins in proteinuric kidney diseases has been emphasized in several studies and notably, podocyte cytoskeletal rearrangement is the common final pathway subsequently leads to podocyte FPE. The contractile actin filament bundles that are highly ordered and arranged parallel in the foot processes were converted into disordered, short, and branched under pathological conditions, thus ensuring podocytes to compromise their unique structure. The disruption of stress fibers of the podocyte actin cytoskeleton could probably explain the reason for altered cell morphology and FPE in podocytes from ischemic reperfusion injury. Disordered actin cytoskeleton was also observed in some transient proteinuric models such as the protamine sulfate infusion and lipopolysaccharide injection, where reversible FPE and proteinuria was observed[45,46]. FAK is a non-receptor tyrosine kinase, which is recruited to focal adhesions by paxillin and talin[47]. FAK plays an essential role in cell motility, maintenance of cell morphology, and also regulates podocyte cytoskeleton. Podocyte-specific deletion of FAK in mice protected from podocyte injury and proteinuria[32]. A recent study suggests that podocyte injury activates FAK phosphorylation that elicits increased FA turnover, FPE, and proteinuria[32]. FAK phosphorylation was observed during podocyte injury and it suggests that inhibition of

FAK signaling cascade may have therapeutic potential in the treatment of glomerular injury[32].

Previous studies have been shown that acute ischemic stroke induces the intracellular calcium accumulation[48]. The increased overload of intracellular calcium in stroke results in brain cell death, necrosis, cell catabolism[49]. Administration of CCBs improved renal function, GFR, renal blood flow, and electrolyte excretion[50]. Other reports have shown that CCBs are involved in exerting the positive hemodynamic function in diabetes and clinical trials demonstrated reduced urinary protein excretion[51]. Ischemic stroke with hypertension together with proteinuria is one of the predominant factors in contributing to the progression of CKD, which is a significant determinant of mortality and morbidity among stroke patients[52]. Stroke patients treated with CCBs appear to have reduced proteinuria, and UACR. In summary, our study identified TRPC6 as a bona fide target of ZEB2 and transduces ischemia mediated podocyte injury and proteinuria. TRPC6 mediated calcium influx possibly mediate the podocyte cytoskeletal abnormality and calcium blockers could be a therapeutic option to combat ischemia-hypoxia injury in podocytes.

Methods:

Intraluminal suture middle cerebral artery occlusion: Middle cerebral artery occlusion (MCAO) and sham surgery was performed in Male SD rats (aged 6-8 weeks) as reported earlier using the intraluminal monofilament technique[53]. Recirculation/reperfusion of cerebral blood flow was allowed by removing the monofilament carefully after 2h ischemia followed by 24h reperfusion. The rats subjected to stroke and sham-operated were transcardially perfused under deep

anesthesia with saline. Organs were removed carefully and then frozen immediately for further processing. To assess the success of the model and infarct volume, brains were sliced into 2-mm-thick coronal sections and stained with 1% Triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA). The institutional animal ethics committee of the University of Hyderabad approved experimental protocols.

Urine analysis: Urine samples from rats were subjected to 10% SDS-PAGE gel. Gels were fixed with 50% ethanol, 12% acetic acid, and 50ul formaldehyde for 1hr. The gels were washed three times with 50% ethanol and were sensitized with sodium thiosulfate for 1min. Gels were washed immediately with distilled water and exposed to 0.2% silver nitrate solution containing 0.037% formaldehyde for 30min at RT in dark. The gels were developed with 6% sodium carbonate solution containing 0.037% formaldehyde. We measured urinary albumin (#COD11573) and creatinine (#COD11502) using the commercially available kits (Biosystems, Barcelona, Spain).

Isolation of glomeruli and podocytes: The glomeruli from the kidneys were isolated by a series of stainless sieves as described earlier[23]. Primary podocytes from rat kidney were isolated as reported earlier[54].

Transmission Electron microscopy: Kidney sections were processed for TEM imaging as described earlier[23] and images were acquired on a JEM-1400TEM (Jeol, Peabody, MA) using an ultrascan CCD camera (Gatan Inc, Pleasanton, CA) at 2K × 2K resolution and 120kV.

Culturing of Podocytes and HEK293T cells: Human podocytes were cultured as described earlier[25]. Podocytes used for experimentation were from passages 10 to 16. HEK293T cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml

penicillin, 100 mg/ml streptomycin and incubated at 37ºC and 5% CO_2. HEK 293T cells or human podocytes were treated with 50µM FG-4592 and incubated for 24hr. Podocyte permeability was assessed by albumin influx assay as described earlier[23].

Immunoblotting: Equal concentration of protein from either glomerular or cell lysate was subjected to 8% or 10% SDS-PAGE gels and blotted onto nitrocellulose membrane. Immunoblotting and developing the blots was performed as reported[23].

qRT-PCR analysis: Isolation of RNA, preparation of cDNA, and quantitative RT-PCR was performed as reported earlier[24]. The expression levels of were normalized to either 18s rRNA or β-Actin and the expression levels were quantified as comparative (ΔΔCT) quantification method.

Immunohistochemistry: 5µm thick paraffin sections were made with Leica microtome on to pre-coated glass slides. Sections were allowed for deparaffinization, rehydration, followed by antigen retrieval. Following permeabilization and blocking, sections were incubated with respective primary antibody at 4ºC overnight. Further, incubation with secondary antibody and DAB staining with the kit method using Mouse/Rabbit PolyDetector DAB HRP Brown Detection System (Santa Barbara, CA, USA). Images were obtained with Leica Trinocular microscope 100X objective (Leica, Buffalo Grove, IL).

Immunofluorescence: Immortalized human podocytes were cultured on coverslips and allowed to differentiate. Following experimental conditions, these cells fixed with 4% paraformaldehyde and performed immunofluorescence protocol as reported earlier[23]. Imaging was done in Leica trinocular fluorescent microscope under 60x oil objective.

Calcium influx assay: Differentiated podocytes were treated with FG-4592 for 24 h and incubate with Fluo3-AM for 1hr and the intracellular calcium in podocytes was measured by the fluorescence intensity of Fluo3-AM as described earlier[55].

Transfection: The HEK293T cells or differentiated podocytes were used for transfection with Polyplus JetPEI transfection reagent (Illkirch, France). Cells were transfected with plasmid DNA or siRNA by mixing with NaCl-jetPEI complexes. After 48 hr of transfection, cells were washed twice with 1 X PBS and lysed with RIPA buffer containing protease inhibitor cocktail, the expression levels were measured by western blotting.

Phalloidin staining: Phalloidin staining was performed to visualize the distribution of stress fibers in differentiated podocytes. TRITC labeled phalloidin (Sigma-Aldrich) of 0.1mM final concentration was used for staining as described earlier[24].

ChIP Assay: Approximately 80% confluent podocytes were exposed to hypoxia. Chromatin immunoprecipitation (ChIP) was performed as described earlier[23].

Promoter-Reporter Assay: TRPC6 promoter was cloned into the PGL3 basic reporter vector with MluI and BglII restriction sites. The resultant pGL3-TRPC6 promoter construct was co-transfected with renilla plasmid into the HEK293T cells. Cells were then exposed to hypoxia or transfected with ZEB2 are scrapped with ice-cold PBS. Cells were collected after spin down at 5,000 rpm for 5min at 4^0C, followed by lysing the cells with passive lysis buffer. 20ul of lysate was used for measuring luciferase expression with LARII and renilla expression with stop glow reagents to quantify relative promoter activity.

Human Patients data: This study is approved by the ethical committee of Narayana medical college (Nellore, AP, India). A total of 50 Ischemic stroke patients (age: 54+/-11 yrs) urine samples and kidney function parameters were used in this study. Mean treatment regimen follow-up period was 3yrs.

Statistical analysis: All the experiments were performed at least three times, and representative results are presented. All data were showed as the mean ± SD of three samples. Significance was calculated using student's T-test and data represented as mean ± S.E. SPSS software (SPSS, Inc.) was used to apply Mann- Whitney and Kruskal-Wallis nonparametric tests to analyze statistical differences between the distributions of three or multiple independent samples, respectively. p values ≤ 0.05 were considered significant.

Acknowledgments: This study was funded by grants from Life Science Research Board (LSRB-296) to AKP. Research fellowship by ICMR (to KMN) and UGC (to DM and RN) are acknowledged.

Figure Legends

Figure 1. Ischemic stroke alters kidney function: (A) TTC staining images of both sham and ischemic stroke-induced (MCAO) rat brain. (B) Estimation of albumin and creatinine levels in ischemic stroke-induced (MCAO) rats. Error bars indicate mean ± SE; n=3. **p<0.003. (C) Spot urine samples from sham (S) and stroke-induces rats (M) were subjected to SDS-PAGE and band intensities were visualized by silver staining, Mr, molecular weight marker (#1610374; Bio- Rad). (D) Expression of HIF1α and ZEB2 from the brain and (E) glomerular lysates from sham and stroke-induced (MCAO) rats was assessed by immunoblotting and (F) band intensities were quantified by image J (NIH). Error bars indicate mean ± SE; n=3. **p<0.003. (G) Steady state mRNA levels of HIF1α and ZEB2 from the brain and glomerular lysates was measured by qRT-PCR (G). Error bars indicate mean ± SE; n=3. *p<0.05.

Figure 2. Altered glomerular morphology in ischemic-stroke rats: (A) PAS staining of glomerular sections from ischemic stroke and sham-operated rats. The scale bar represents images of 50μm and images were captured with 63x objective Leica trinocular microscope. (B) Staining for WT1 in sham and ischemic stroke-induced glomerular sections. The scale bar represents images of 20μm and images were obtained with 100x objective Leica trinocular microscope. (C) TEM images of podocyte foot processes in sham and ischemic stroke-induced rat glomerular sections. In ischemic-stroke rats, podocyte foot-processes were small and thickness of GBM is more compared to sham operated rats.

Figure 3. Ischemia-hypoxia and HIF1α induce expression of ZEB2 and its targets: (A) Lysate from primary podocyte isolated from sham and ischemic-stroke induced rat

kidney was assessed for HIF1α, ZEB2, ZEB2 targets, and pFAK. (B) Differentiated human podocytes treated with FG-4592 (Hyx) and without FG-4592 (CTL) and analyzed the expression of HIF1α, ZEB2, ZEB2 targets, and pFAK (A&B) Densitometric analysis of band intensities was measured by image J normalized to β-actin and is shown below each blot. (C) We quantified mRNA levels of HIF1α, ZEB2, and TRPC6 from podocytes isolated from sham and MCAO (ischemic-stroke) rats and (D) human podocytes treated with or without FG-4592. Error bars indicate mean ± SE; n=3. *p<0.05. (E) Immunohistochemical analysis of HIF1α, ZEB2, and TRPC6 in glomerular sections from ischemic-stroke and sham-operated rat kidney tissue. The scale bar represents images of 20μm and images were captured with 100x objective Leica trinocular microscope.

Figure 4. Hypoxia indcued HIF1α-ZEB2-TRPC6 axis: (A) ChIP analysis with chromatin fractions from podocytes exposed to FG-4592 was performed as described in methods. Input DNA and DNA from each of the immunoprecipitated samples were PCR amplified for hypoxia response element (HRE) in VEGF promoter and ZEB2 promoter and E2-box region in both TRPC6 promoter and E-cadherin promoter. HRE in VEGF promoter fragment and E2-box in E-cadherin promoter serve as positive controls for HIF1α binding and ZEB2 binding respectively. (B) TRPC6 promoter activity was measured in HEK293T cells that ectopically expressing ZEB2 and exposed to FG-4592. Renilla luciferase was used as an internal control to normalize transfection efficiency. Error bars indicate mean ± SE; n=3. **p<0.003. (C) Intracellular calcium levels in podocytes were measured by Fluo3-AM following treatment with FG-4592 and treated with or without 2-APB. Podocytes expressing siZEB2 and extopically expressing ZEB2 were employed in this study. Error bars indicate mean ± SE; n=3. **p<0.003. (D) Immunoblot analysis of

ZEB2, TRPC6, and FAK phosphorylation in podocytes ectopically expressing ZEB2 (ZEB2OE) and treated with or without FG-4592. (E) Quantification of band intensities of ZEB2, TRPC6, pFAK was Image J analysis (NIH).

Figure 5. Essential role of ZEB2 in regulating TRPC6 expression: (A) Immunoblotting analysis of ZEB2, TRPC6, and pFAK expression in podocytes expressing siZEB2 and treated with or without FG-4592. (B) Quantification of band intensities of ZEB2, TRPC6, pFAK was performed with Image J software. (C) Immunoblotting analysis of TRPC6 and pFAK expression in podocytes in which TRPC6 expression was knocked-down and treated with or without FG-4592 (D) Quantification of band intensities of western blots was performed with Image J. (E) Immunoblotting analysis of TRPC6, pFAK, and RhoA expression in podocytes exposed to FG-4592 and treated with or without 2-APB (Calcium channel blocker) and FAK14 (FAK inhibitor).

Figure 6. HIF1α-ZEB2-TRPC6 axis regulates podocyte cytoskeleton reorganization: (A) Phalloidin staining was performed to visualize intracellular F- actin filaments in podocytes. Podocytes treated with FG-4592 (Hyx) compromised their morphology and presented with cytoskeletal reorganization (II). Ectopic expression of ZEB2 with and without FG-4592 treatment also resulted in the loss of typical SFs arrangement (III &IV). Knockdown of ZEB2 expression in podocytes by siZEB2 (V) or podocytes treated with calcium channel blocker; 2-APB or FAK inhibitor; FAK14 (VI &VII) improved SFs reorganization. Both, 2-APB and FAK inhibitor 14 ameliorated SFs organization in podocytes ectopically expressing ZEB2 (VIII&IX). Knockdown of TRPC6 expression in podocytes by siTRPC6 also ameliorated HIF-1α mediated SFs reorganization (X). The scale bar represents images of 50µm and images were captured with 63x objective

Leica trinocular microscope. (B) Quantification of the number of stress fibers per podocyte was analyzed by Image-J (NIH) and the ratio of stress fibers to total cell size was represented. Error bars indicate mean ± SE; n=3. *p<0.05.

Figure 7. Calcium channel blockers improved proteinuria in ischemic-stroke patients: (A) Quantification of albumin influx across podocyte monolayer was measured after 24 h exposure to FG-4592. Podocytes transduced with shZEB2 or treated with FAK14 or 2-APB showed improved permselectivity to albumin than podocytes exposed to FG-4592 alone. Error bars indicate mean ± SE; n=3. *p<0.05. Ischemic patients were presented with enhanced eGFR (B) and increased albumin creatinine ratio (C). 24h urinary microalbumin (mg/L) concentrations were measured in ischemic stroke patients with and without hypertension following treatment with dihydropyridine group calcium channel blockers (D).

Figure 8. Co-expression of HIF1α, ZEB2, and TRPC6 in glomerular diseases: Nakagawa CKD data set showing the elevated expression of HIF1α (2.6 fold), ZEB2 (2.7 fold), and TRPC6 (1.6 fold) in patients with CKD vs. control (A). Hodgin diabetes mouse glomeruli datasets showing the elevated expression of ZEB2 (1.55 fold), and TRPC6 (2.61 fold) in mouse with diabetic nephropathy vs. non-diabetic mouse models. Nephroseq (The Regents of The University of Michigan, Ann Arbor, MI) was used to analyze the data.

Figure 9. Proposed model for ischemic-hypoxia mediated podocyte injury. Ischemia-stroke rats develop systemic hypoxia that induces HIF1α accumulation in several susceptible sites including glomerular podocytes. HIF1α occupies ZEB2 promoter and drives its expression. ZEB2, in turn, induces TRPC6 expression. Elevated TRPC6

increases intracellular calcium accumulation. Calcium dependent phosphorylation of FAK elicits cytoskeletal rearrangements. These cytoskeletal rearrangements eventually manifest in effacement of podocyte foot- processes and compromise their permselectivity. The over activity of HIF1α-ZEB2-TRPC6 axis in podocytes mediates calcium accumulation vis-à-vis cytoskeletal abnormalities and proteinuria.

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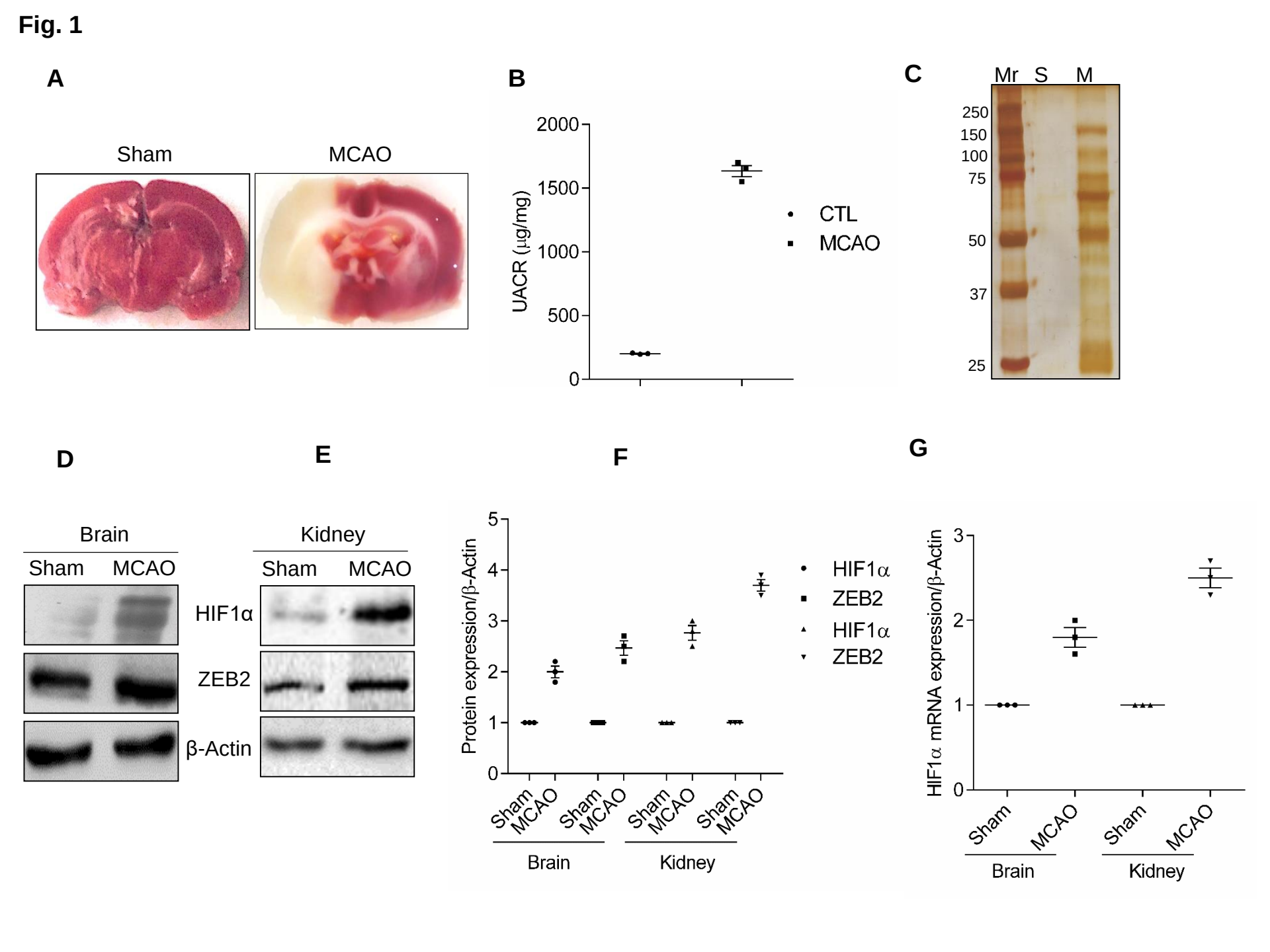
Fig. 1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Sham
MCAO
B
UACR (μg/mg)
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1500
1000
500
0
CTL
MCAO
C
Mr
S
M
250
150
100
75
50
37
25
D
Brain
Sham
MCAO
HIF1α
ZEB2
β-Actin
E
Kidney
Sham
MCAO
F
Protein expression/β-Actin
5
4
3
2
1
0
HIF1α
ZEB2
HIF1α
ZEB2
Sham
MCAO
Sham
MCAO
Sham
MCAO
Sham
MCAO
Brain
Kidney
G
HIF1α mRNA expression/β-Actin
3
2
1
0
Sham
MCAO
Sham
MCAO
Brain
Kidney

Fig. 2

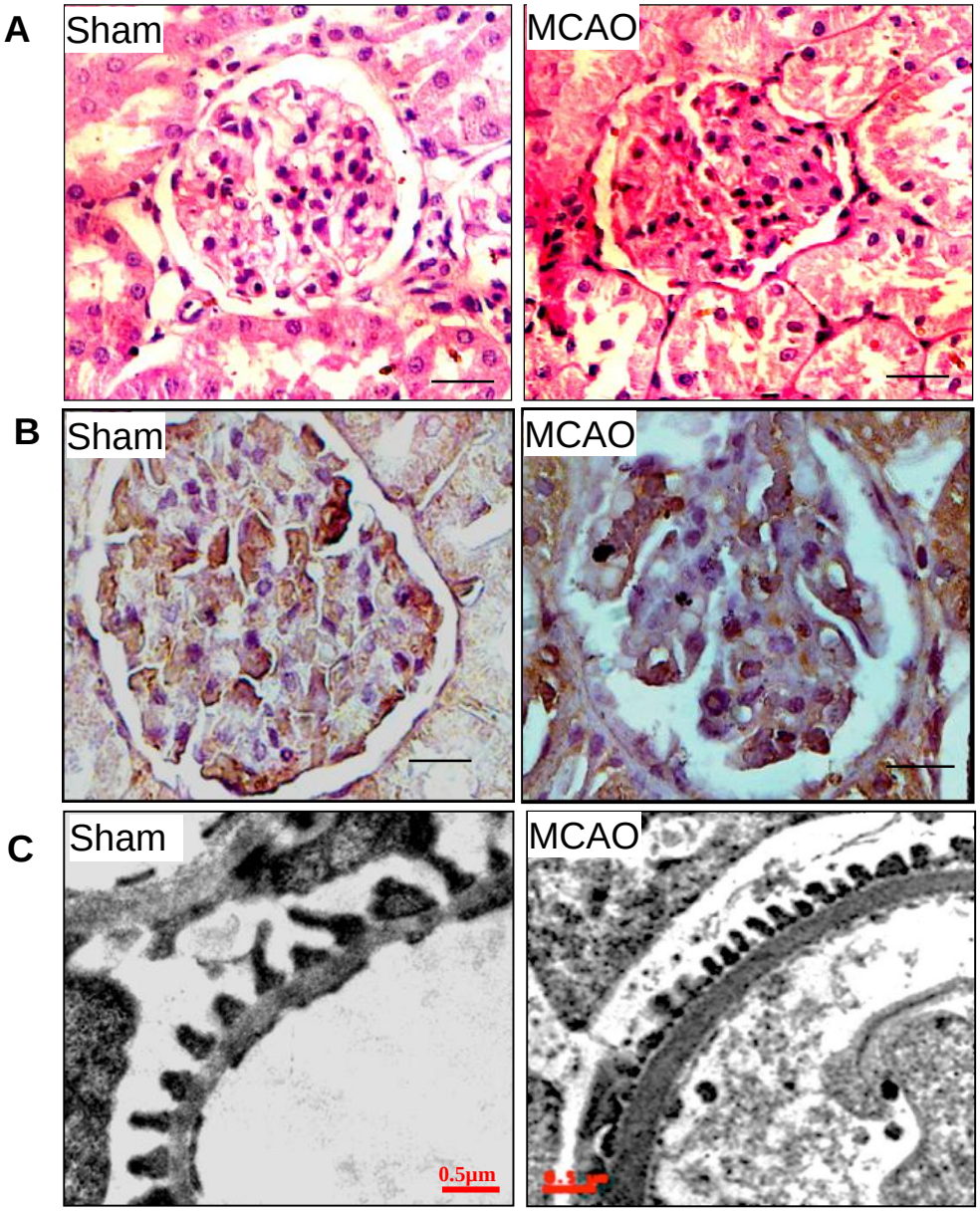
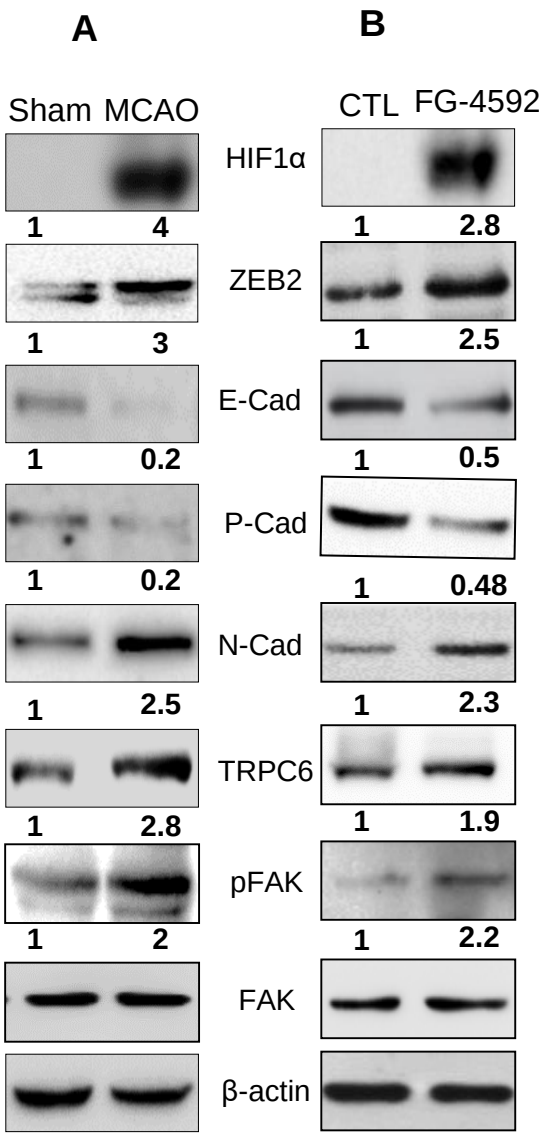
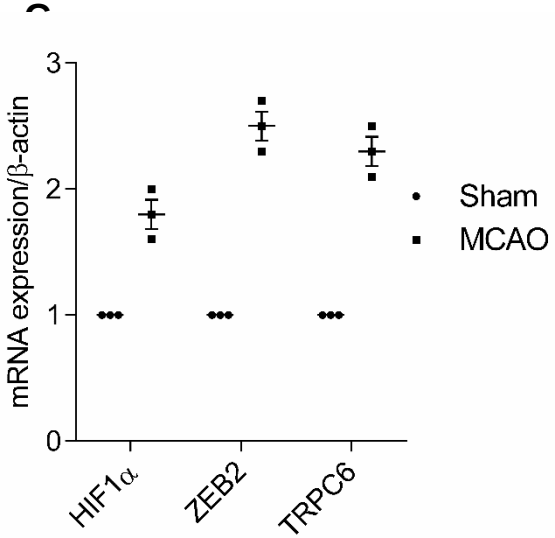


Fig. 3


A
Sham MCAO
1 4
1 3
1 0.2
1 0.2
1 2.5
1 2.8
1 2
B
CTL FG-4592
HIF1α
1 2.8
ZEB2
1 2.5
E-Cad
1 0.5
P-Cad
1 0.48
N-Cad
1 2.3
TRPC6
1 1.9
pFAK
1 2.2
FAK
β-actin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mRNA expression/β-actin
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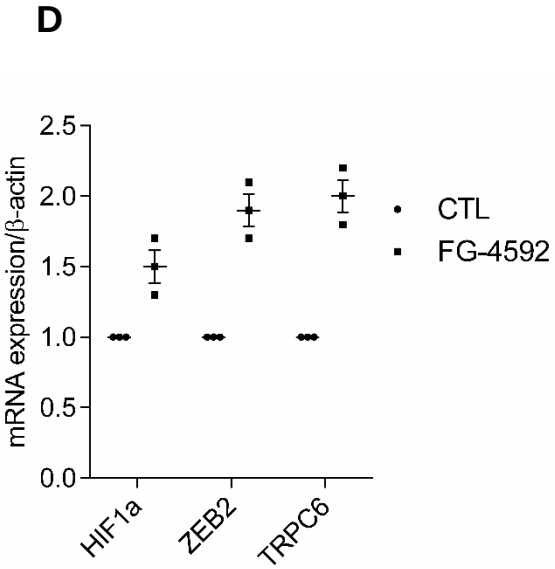
2
1
0
Sham
MCAO
HIF1α
ZEB2
TRPC6


D
mRNA expression/β-actin
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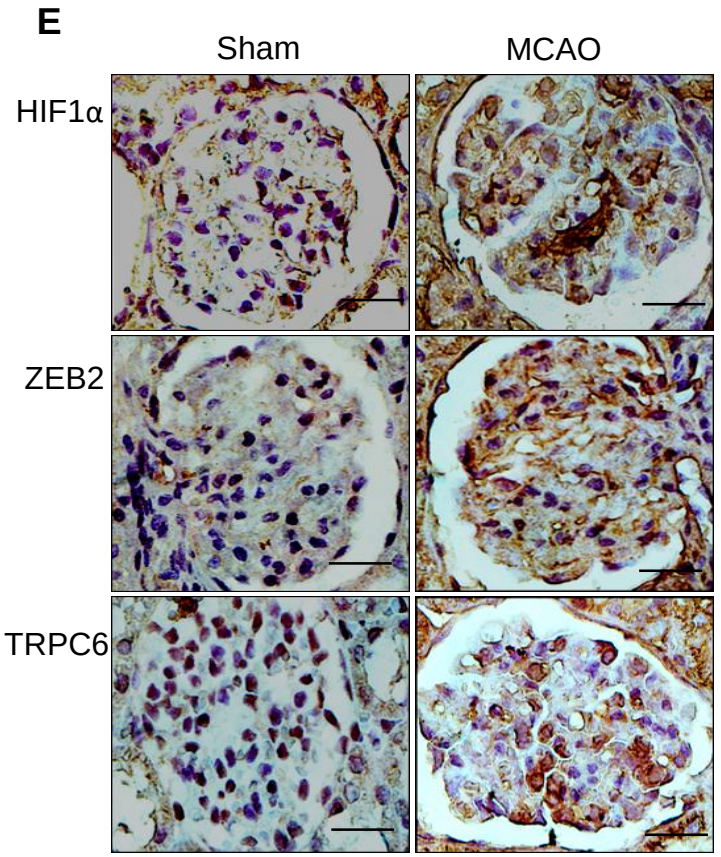
2.0
1.5
1.0
0.5
0.0
CTL
FG-4592
HIF1a
ZEB2
TRPC6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Sham
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HIF1α
ZEB2
TRPC6

Fig. 4

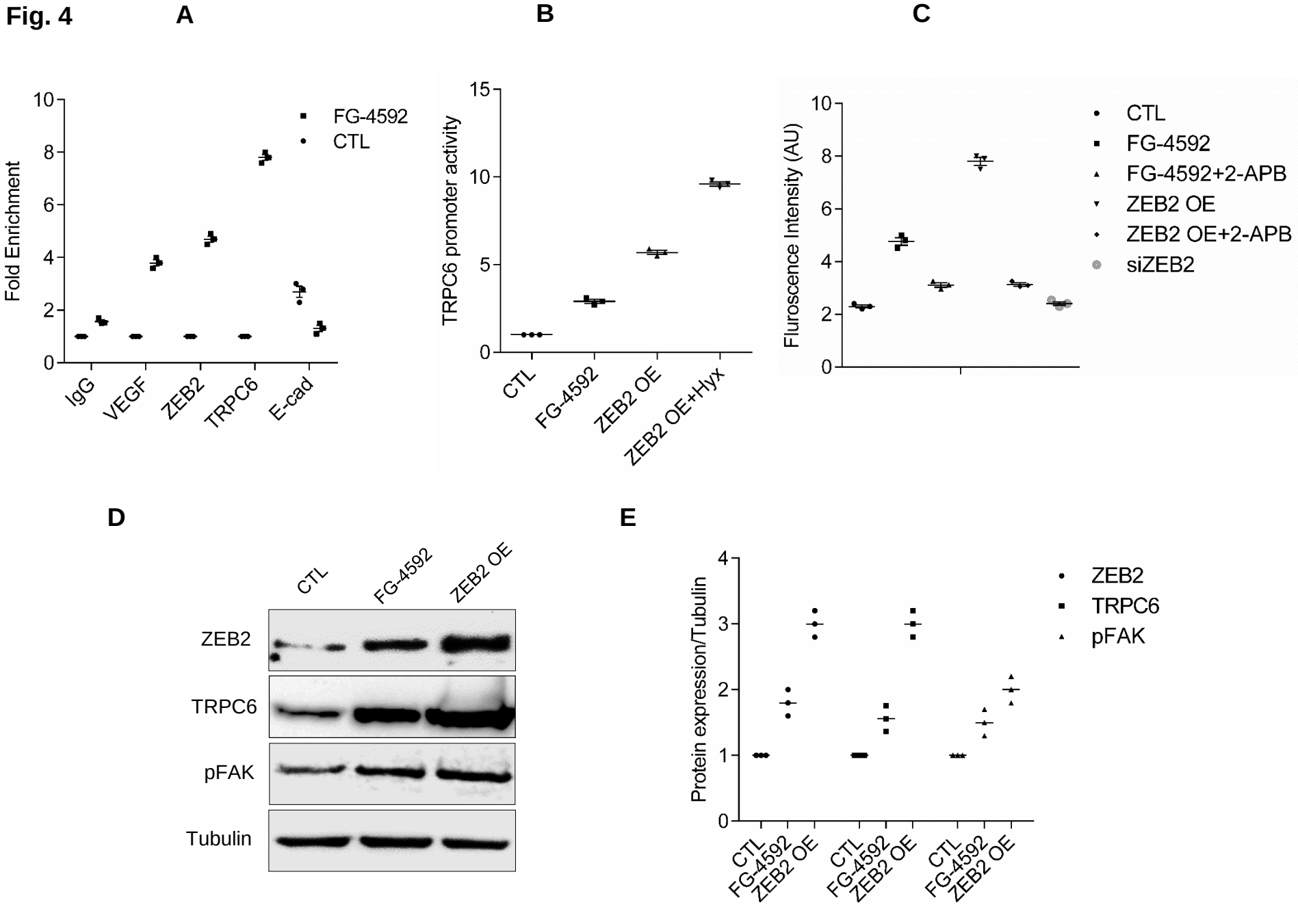

A
Fold Enrichment
FG-4592
CTL
IgG
VEGF
ZEB2
TRPC6
E-cad
B
TRPC6 promoter activity
CTL
FG-4592
ZEB2 OE
ZEB2 OE+Hyx
C
Fluroscence Intensity (AU)
CTL
FG-4592
FG-4592+2-APB
ZEB2 OE
ZEB2 OE+2-APB
siZEB2
D
CTL
FG-4592
ZEB2 OE
ZEB2
TRPC6
pFAK
Tubulin
E
Protein expression/Tubulin
ZEB2
TRPC6
pFAK
CTL
FG-4592
ZEB2 OE

Fig. 5

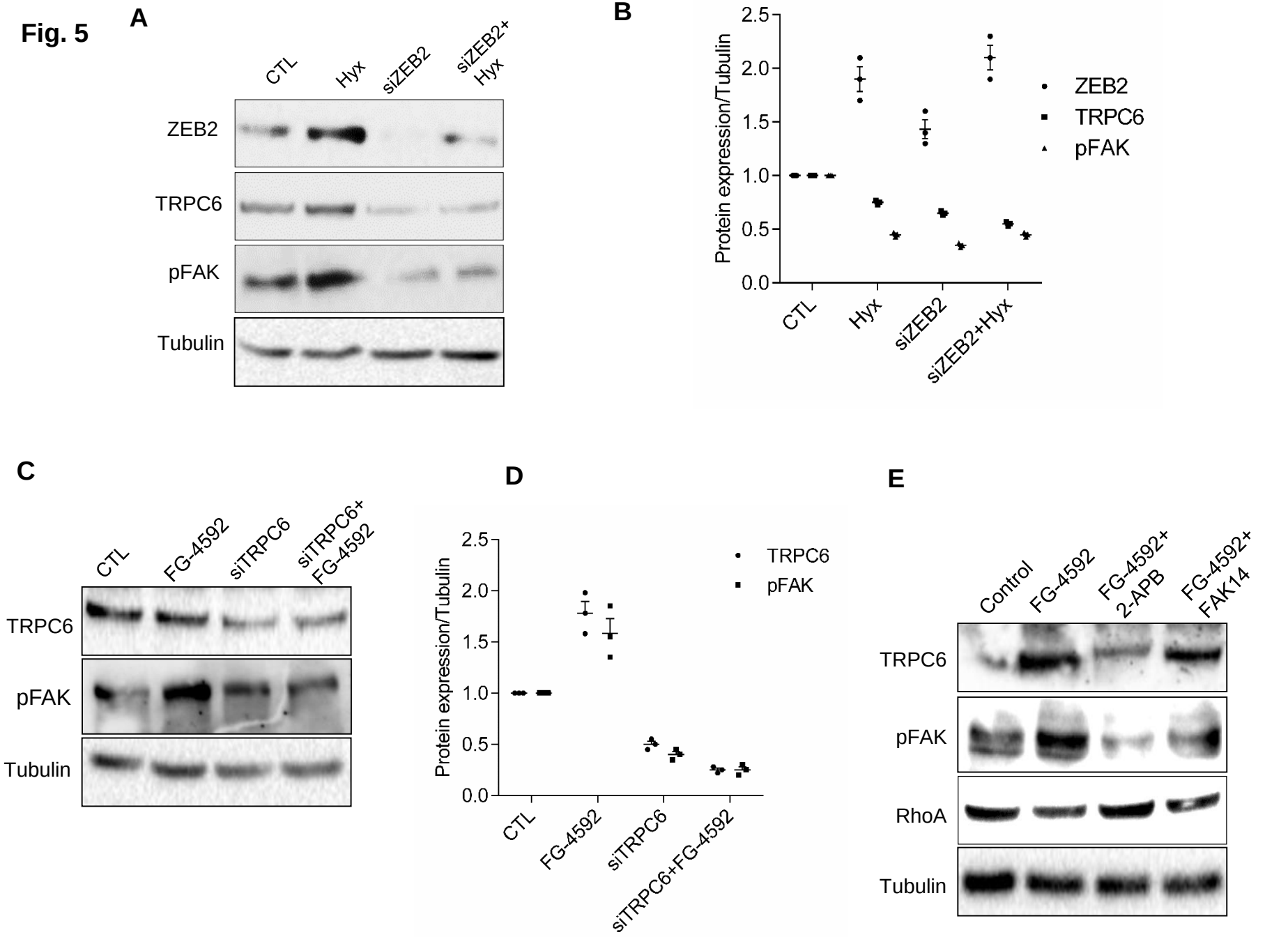
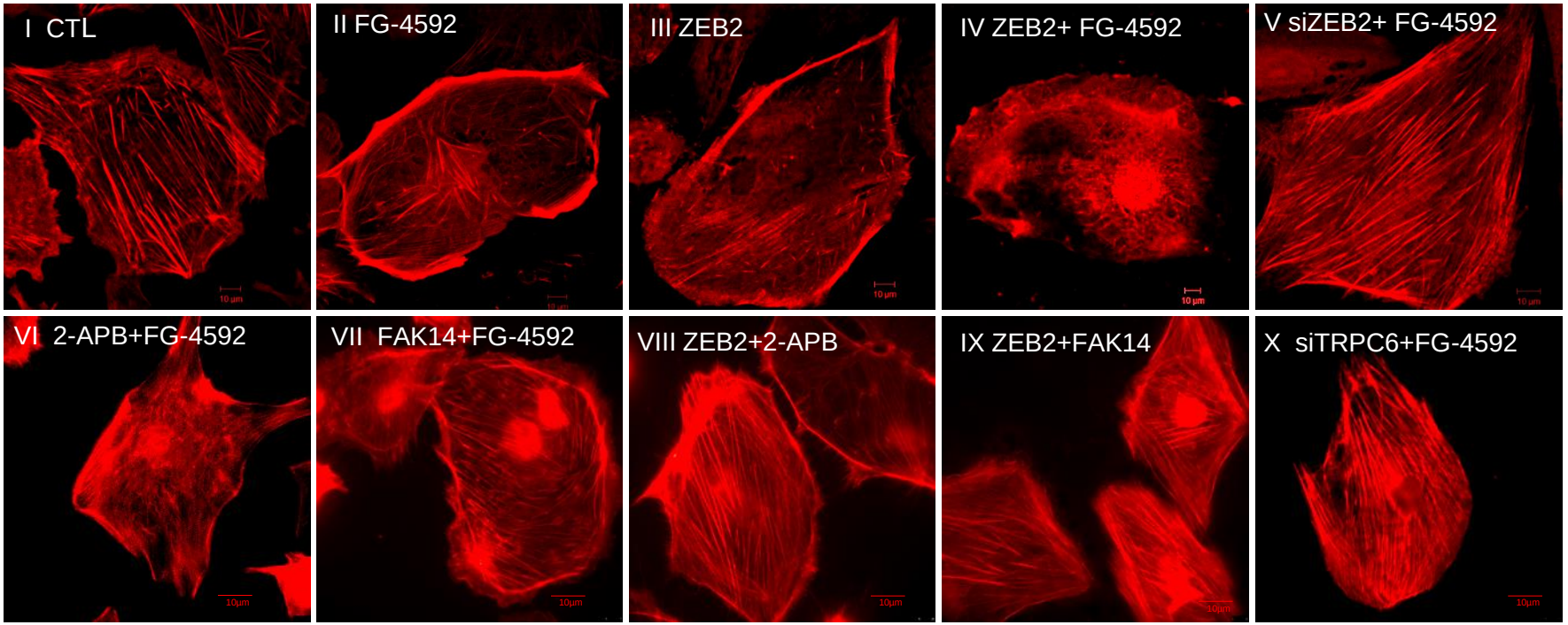

A
CTL
Hyx
siZEB2
siZEB2+ Hyx
ZEB2
TRPC6
pFAK
Tubulin
B
Protein expression/Tubulin
2.5
2.0
1.5
1.0
0.5
0.0
ZEB2
TRPC6
pFAK
CTL
Hyx
siZEB2
siZEB2+Hyx
C
CTL
FG-4592
siTRPC6
siTRPC6+ FG-4592
TRPC6
pFAK
Tubulin
D
Protein expression/Tubulin
2.5
2.0
1.5
1.0
0.5
0.0
TRPC6
pFAK
CTL
FG-4592
siTRPC6
siTRPC6+FG-4592
E
Control
FG-4592
FG-4592+ 2-APB
FG-4592+ FAK14
TRPC6
pFAK
RhoA
Tubulin

Fig. 6

A


I CTL
II FG-4592
III ZEB2
IV ZEB2+ FG-4592
V siZEB2+ FG-4592
VI 2-APB+FG-4592
VII FAK14+FG-4592
VIII ZEB2+2-APB
IX ZEB2+FAK14
X siTRPC6+FG-4592

B

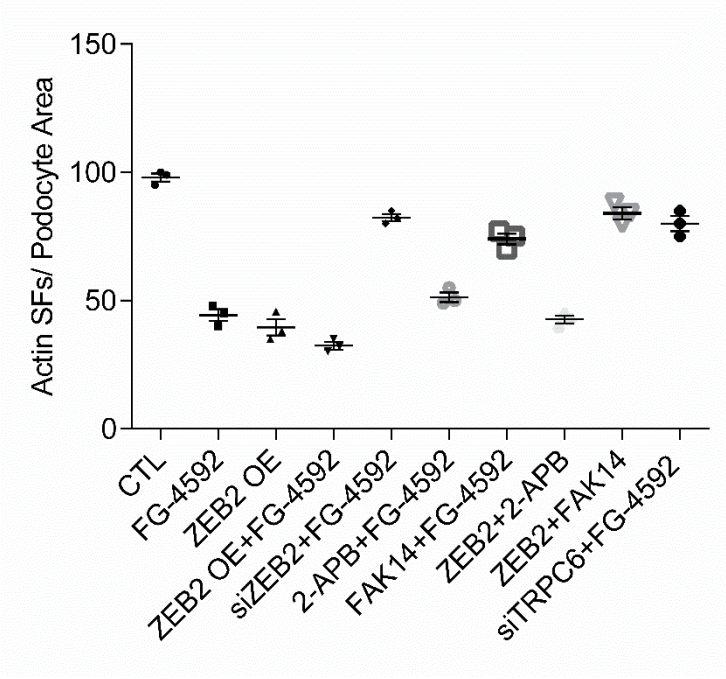

Actin SFs/ Podocyte Area
150
100
50
0
CTL
FG-4592
ZEB2 OE
ZEB2 OE+FG-4592
siZEB2+FG-4592
2-APB+FG-4592
FAK14+FG-4592
ZEB2+2-APB
ZEB2+FAK14
siTRPC6+FG-4592

Fig. 7

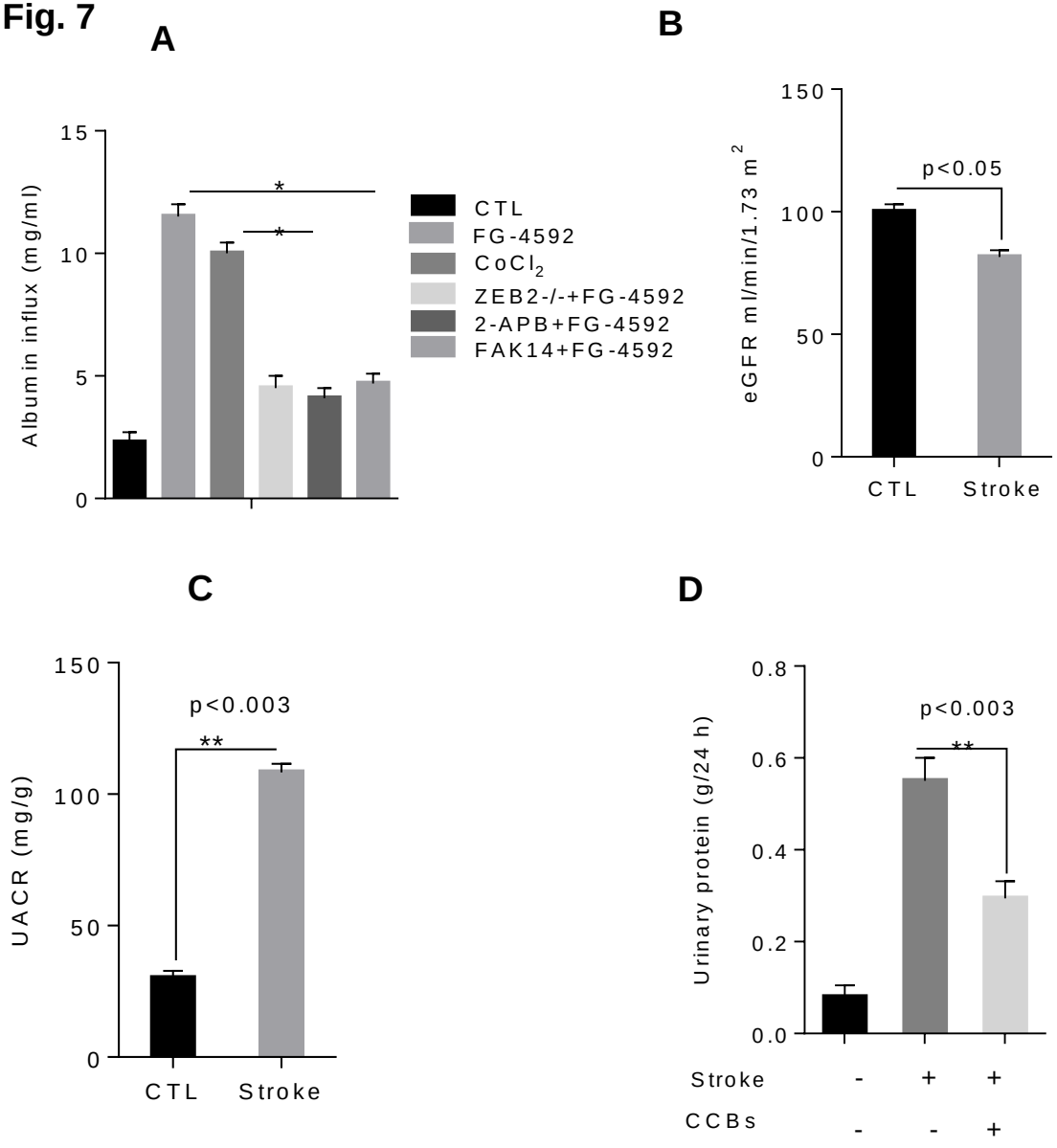
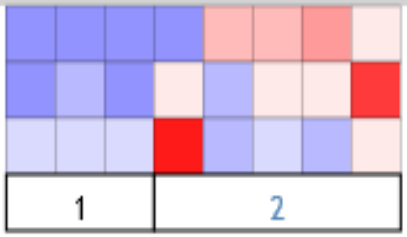

A
Albumin influx (mg/ml)
15
10
5
0
*
*
CTL
FG-4592
CoCl2
ZEB2-/-+FG-4592
2-APB+FG-4592
FAK14+FG-4592
B
eGFR ml/min/1.73 m2
150
100
50
0
p<0.05
CTL
Stroke
C
UACR (mg/g)
150
100
50
0
p<0.003
**
CTL
Stroke
D
Urinary protein (g/24 h)
0.8
0.6
0.4
0.2
0.0
p<0.003
**
Stroke - + +
CCBs - - +

Fig. 8

A

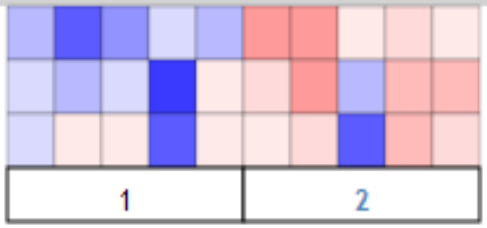
P-value	Fold Change	Gene	Reporter
0.010	2.62	HIF1A	A_24_P56388
0.014	2.70	ZEB2	A_24_P367454
0.180	1.61	TRPC6	A_23_P127547



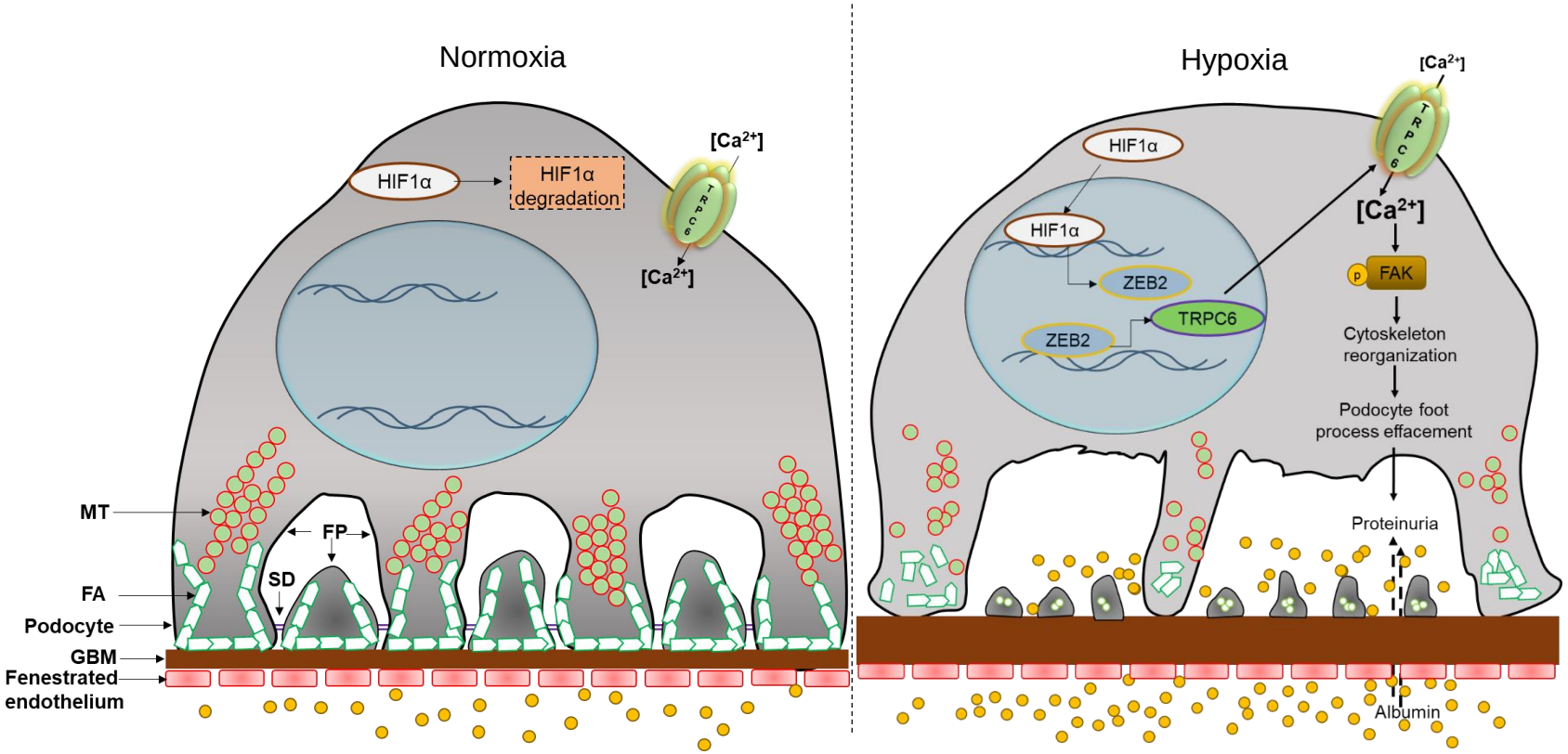
1.Normal Kidney (3) 2.Chronic Kidney Disease (5)

B

P-value	Fold Change	Gene	Reporter
1.60E-4	2.61	TRPC6	22068
0.015	1.55	ZEB2	24136
0.233	1.05	HIF1A	15251



1.Non-Diabetic Mouse Kidney (5) 2.Diabetic Nephropathy Mouse Model(5)


Normoxia
HIF1α
HIF1α degradation
[Ca2+]
TRPC6
[Ca2+]
MT
FP
SD
FA
Podocyte
GBM
Fenestrated endothelium
Hypoxia
[Ca2+]
HIF1α
HIF1α
ZEB2
TRPC6
ZEB2
[Ca2+]
p
FAK
Cytoskeleton reorganization
Podocyte foot process effacement
Proteinuria
Albumin