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Invited Review:

***Biochimica et Biophysica Acta* – Molecular Basis of Disease**

Special Issue – The Role of TRP Channels in Disease

TRPC6 and FSGS: The Latest TRP Channelopathy

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Abstract

Focal and segmental glomerulosclerosis (FSGS) is a common cause of nephrotic syndrome in children and adults throughout the world. In the past fifty years, significant advances have been made in the identification and characterization of familial forms of nephrotic syndrome and FSGS. Resultant to these pursuits, several podocyte structural proteins such as nephrin, podocin, alpha-actinin 4 (ACTN4), and CD2-associated protein (CD2AP) have emerged to provide critical insight into the pathogenesis of hereditary nephrotic syndromes. The latest advance in familial FSGS has been the discovery of a mutant form of canonical transient receptor potential cation channel 6 (TRPC6), which causes an increase in calcium transients and essentially a gain of function in this cation channel located on the podocyte cell membrane. The TRP ion channel family is a diverse group of cation channels united by a common primary structure which contains six membrane-spanning domains, with both carboxy and amino termini located intracellularly. TRP channels are unique in their ability to activate independently of membrane depolarization. TRPC6 channels have been shown to be activated via phospholipase C stimulation. The mechanisms by which mutant TRPC6 causes an increase in intracellular calcium and leads to glomerulosclerosis are unknown. Mutant TRPC6 may affect critical interactions with the aforementioned podocyte structural proteins, leading to abnormalities in the slit diaphragm or podocyte foot processes. Mutant TRPC6 may also amplify injurious signals mediated by Ang II, a common final pathway of podocyte apoptosis in various mammalian species. Current evidence also suggests that blocking TRPC6 channels may be of therapeutic benefit in idiopathic FSGS, a disease with a generally poor prognosis. Preliminary experiments reveal the

commonly used immunosuppressive agent FK-506 can inhibit TRPC6 activity in vivo. This creates the exciting possibility that blocking TRPC6 channels within the podocyte may translate into long-lasting clinical benefits in patients with FSGS.

1. Introduction

Nephrotic syndrome is a clinical entity defined by the triad of edema, proteinuria, and hypercholesterolemia. Focal segmental glomerulosclerosis is a common cause of nephrotic syndrome, both in children, where it accounts for 7 to 20% of cases, and in adults, where it accounts for up to 35% of cases [1]. Studies performed at several large institutions have documented an increased incidence of FSGS in biopsies of adult patients and it is the leading cause of idiopathic nephrotic syndrome among black individuals [2]. Furthermore, despite aggressive therapy, idiopathic FSGS often leads to end-stage renal disease (ESRD). Classically, the clinicopathologic syndrome has been defined as either primary (idiopathic), secondary or familial. Well known medical maladies associated with secondary FSGS have been established, including human immunodeficiency virus infection, heroin abuse, sickle cell disease, and obesity [1,3-6]. Familial forms of FSGS include autosomal dominant and recessive patterns of inheritance and those associated with congenital syndromes such as Laurence-Moon-Biedl and Charcot-Marie-Tooth [7,8]. Over the past 50 years, significant advances have been made in the identification and characterization of familial forms of FSGS. Through the use of advanced molecular genetic cloning techniques, several mutations have been found in key podocyte proteins including nephrin, podocin, ACTN4, and CD2AP (figure 1). These proteins have typically been involved in podocyte signaling or the structural apparatus of the slit

diaphragm, the key filtration barrier of the glomerulus. One of the most recent advances in familial FSGS has been the discovery of a mutant variant of TRPC6 in a large New Zealand kindred [9]. This mutation causes an autosomal dominant form of hereditary FSGS which is particularly aggressive. It is characterized by high grade proteinuria by the third or fourth decade of life and ESRD in 60% of affected individuals. The TRPC6 mutation involves a proline to glutamine substitution at amino acid 112 in the protein, causing an increase in calcium transients. It appears that there is a mislocation of the ion channels to the podocyte cell membrane. This is the first ion channel identified as a cause of FSGS. Furthermore, these findings add to the growing spectrum of disease caused by abnormal calcium homeostasis, and more specifically, by mutations in TRP channels. FSGS joins hypomagnesemia with secondary hypocalcemia (TRPM6) [10], mucopolidosis type IV (TRPML1) [11], and polycystic kidney disease (TRPP1 and TRPP2) [12] in a fairly new category of diseases, the TRP channelopathies.

2. Hereditary Nephrotic Syndromes

2.1 Nephrin Mutation

Familial forms of FSGS have been known since the 1950s, but it has only been over the last 10 years that specific genetic defects have been discovered, largely due to advances in molecular biology and human genetics. The earliest gene known to be associated with a hereditary nephrotic syndrome is nephrin (NPHS1), whose mutations cause congenital nephrotic syndrome of the Finnish type, an autosomal recessive disease affecting approximately 1:10,000 newborns in Finland [13-15]. Of the fifty mutations discovered, the two most common are a frameshift mutation and a nonsense mutation,

Fin major and Fin minor, both of which cause a premature stop codon [16]. The nephrin protein is a 180 kDa transmembrane protein exclusively expressed by glomerular podocytes within the kidney and is predominantly localized to the glomerular slit diaphragm [17-19]. Nephrin is also expressed in brain and pancreatic tissue. Immunoelectron microscopy has revealed that nephrin is present in junctions with ladder-like structures between the differentiated podocytes [20]. These structures are absent if the mutated form of the gene is present.

2.2 *Podocin Mutation*

Podocin (NPHS2) mutations were next discovered as a cause of a hereditary nephrotic syndrome. Patients with mutations in podocin have an autosomal recessive, mostly steroid-resistant nephrotic syndrome [21]. These patients show disease onset in early childhood and rapid progression to ESRD. Podocin, a 42 kDa protein in the lipid raft-associated stomatin protein family, is predicted to form a membrane-associated hairpin-like structure with a cytosolic amino and carboxy terminal domain. Podocin has been shown to localize on podocyte foot process membranes at the insertion site of the slit diaphragm [22]. It accumulates in an oligomeric form in lipid rafts of the slit diaphragm. In vivo studies demonstrate that it interacts via its carboxy terminal domain with other podocyte proteins such as nephrin and CD2AP [23]. The interaction between nephrin and podocin is required for the proper initiation of nephrin signaling. Targeted disruptions of podocin inhibit both nephrin trafficking and nephrin-initiated signal transduction, thereby altering normal podocyte homeostasis [24].

2.3 *CD2AP and ACTN4*

Other relevant podocyte proteins associated with familial FSGS include CD2AP and ACTN4. CD2AP, a mouse homologue of the human p130(Cas) ligand, contains multiple SH3-binding domains that enhance CD2 clustering via its cytoplasmic tail. It is located in the membranes of T cells and natural killer cells where it facilitates T-cell adhesion to antigen-presenting cells [25]. CD2AP knock-out mice studies have shown compromised immune function and nephrotic syndrome with renal failure at 6 to 7 weeks of age. Post-mortem studies revealed that CD2AP is expressed primarily in glomerular epithelial cells within the kidney and that knock-out mice exhibit defects in foot processes, accompanied by mesangial cell hyperplasia and extracellular matrix deposition [26]. More recent mouse studies have revealed CD2AP's interaction with other podocyte proteins including fyn and synaptopodin. Huber et al., showed that combinations of CD2AP heterozygosity, and either fyn or synaptopodin heterozygosity, resulted in spontaneous proteinuria and in FSGS-like glomerular damage [27]. This genetic epistasis was followed by immunoprecipitation experiments which demonstrated a physical interaction between CD2AP and the two other podocyte proteins. The specificity of this interaction was shown by combining heterozygosity at Neph1, a transmembrane receptor closely related to nephrin and localized to the slit diaphragm, with CD2AP heterozygosity and showing no glomerular abnormalities or proteinuria. In humans, Kirsch et al. demonstrated that p130(Cas) ligand, which shares 86% homology with mouse CD2AP, is involved in vesicle formation and colocalizes with p130(Cas) as well as F-actin in cell membranes [28]. Putative actin-binding sites and a coiled-coil domain have been identified at the C terminus of the protein, as has a putative leucine zipper

motif. Kim et al. subsequently found that splice site variations within exon 7 of the p130(Cas) ligand gene interrupted protein translation and led to FSGS in 2 black individuals [29]. Thus, although the pathophysiology remains uncertain, it can be postulated that human p130(Cas) ligand mutations lead to altered actin-binding and abnormal podocyte cytoskeletal architecture, with resulting proteinuria and glomerulosclerosis.

ACTN4 is located on chromosome 19q13 and encodes for a 100 kDa protein [30,31]. The function of this protein is to crosslink and bundle actin filaments. Mutations in this gene have been associated with autosomal dominant FSGS, characterized by the adult onset of disease with variable severity and rate of progression to ESRD. Initial studies revealed that mutant ACTN4 binds actin filaments more strongly than wild-type ACTN4 *in vivo*. Recently, it was shown that mutant protein appears to form large aggregates within the podocyte [32]. One model of familial FSGS in these patients would be the development of podocyte damage as a direct effect of protein aggregation and the toxic effects associated with this phenomenon, such as is observed in severe degenerative neurologic conditions such as Alzheimer's, Parkinson's and Huntington's diseases [33,34]. Mutant ACTN4 was also found to have a decreased half-life in comparison to wild-type ACTN4 [32]. This would suggest another potential mechanism for familial FSGS in patients with ACTN4 mutations that would involve the loss of normal actin polymerization and abnormal cytoskeletal architecture.

2.4 TRPC6 Mutation

Recently, another genetic mutation was reported to be associated with hereditary FSGS. Through whole genome linkage analysis, fine-mapping and candidate gene screening, a mutated gene was localized to chromosome 11q21-22 and subsequently identified as the transient receptor potential cation channel, subfamily C, member 6 (TRPC6) gene [9]. The original missense mutation changed a highly conserved proline in the first ankyrin repeat of TRPC6 to a glutamine at amino acid 112 (P112Q). Subsequently, Reiser et al. have identified TRPC6 mutations in five other unrelated families of diverse ethnic origin. In each family, inheritance was consistent with an autosomal dominant pattern and the observed amino acid substitutions occurred in highly conserved residues throughout evolution. Two mutations predicted amino acid substitutions in the N-terminal intracellular domain of TRPC6; two predicted amino acid substitutions in the C-terminal intracellular domain; and one encoded a premature stop codon near the C terminus. In all families, TRPC6 variant and disease inheritance followed a pattern of cosegregation, with incomplete penetrance [35]. Fluorescent in situ hybridization studies in human and confocal microscopy of rat kidney sections show broad expression of TRPC6 throughout the kidney in tubules and glomeruli [9,35]. Recent studies have also shown that TRPC6 is expressed in podocyte foot processes. Labeling with gold particles revealed TRPC6 within the cell body of podocytes and in primary processes in close vicinity to the slit diaphragm. Colocalization studies showed that TRPC6 was associated with the aforementioned disease-causing slit diaphragm proteins nephrin, podocin, and CD2AP. However, immunoblotting showed direct biochemical interaction with only nephrin and podocin, but not CD2AP. It appears that when nephrin was specifically deleted, TRPC6 protein expression increased [35].

3. The TRP Ion Channel Family

3.1 TRP Ion Channels

The TRP ion channels are a large class of proteins in diverse mammalian species united by a common primary structure which contains six membrane-spanning domain polypeptide subunits, with both carboxy and amino termini located intracellularly [36]. Another feature in some TRP channels is ankyrin binding repeats at the N-terminus; ankyrin repeats fold into structures that determine molecular identification via protein:protein interactions. Recently, the crystal structure of the human TRPV channel subfamily, involved in integration of noxious stimuli, was delineated [37]. The 1.7 Å resolution crystal structure contained a six ankyrin repeat stack with multiple insertions in each repeat generating several unique features, including extended loops with an exposed hydrophobic patch and a prominent kink. The TRPV1 ankyrin repeat was shown to mediate interaction with two vesicular proteins, Syt IX and Snapin, which participate in SNARE-dependent exocytosis in excitable cells [38]. Interestingly, the TRPC6^{P112Q} mutation in the New Zealand cohort described by Winn et al., is located in the first ankyrin repeat [9]. The TRP channels mediate diverse biological functions such as mechanosensation, ion homeostasis, cell growth, and vasoregulation [36,39-41]. They are permeable to monovalent cations as well as calcium ions with a relative lack of selectivity. In addition, TRP channels can be activated independent of intracellular calcium concentration or membrane depolarization.

3.2 The TRPC6 (Canonical) Family

The TRPC (canonical) family is expressed in a wide variety of human tissues and can be divided into four subfamilies (TRPC1, TRPC 4,5, TRPC3,6,7 and TRPC2) on the basis of sequence homology and functional similarities. TRPC1 was the first member of the mammalian TRP family purported to form an ion channel [42]. Studies are conflicting with regards to TRPC1 activation based on intracellular calcium depletion, but it can be activated by DAG [43]. It has been shown to co-assemble with other TRPC subunits in vitro and in vivo, where it may be a component of different heteromeric TRP complexes [44]. Studies have also shown that TRPC1 channels co-localize with the autosomal recessive polycystic kidney disease protein PKD2 [45]. The second TRPC subfamily comprises TRPC4 and TRPC5, which both contain a carboxy terminal PDZ-binding motif not present in other TRPs [46]. PDZ domains are peptide binding domains that organize membrane proteins, particularly at cell-cell junctions, including neuronal synapses. For example, in drosophila eyes, TRP channels are organized in a supramolecular complex along with other phototransduction proteins, such as phospholipase C (PLC) and protein kinase C, through association with a multi-PDZ domain-containing protein, INAD (inactivation no afterpotential D) [47-49]. INAD contains five PDZ domains, each of which interacts with a particular target protein and thus serves as a scaffold to bring PLC, TRP, protein kinase C, and G-protein together in a signaling complex. Similar multi-PDZ domain-containing proteins exist in mammals and serve as important protein-protein interaction sites for clustering and organization of signaling molecules, particularly those involved in ion transport [50-52]. For example, murine TRPM4 and TRPM5 have been shown to bind the first PDZ domain of the Na⁺/H⁺ exchanger regulatory factor (NHERF). NHERF is a two PDZ domain-

containing protein that associates with the actin cytoskeleton via interactions with PLC isozymes and members of the ezrin/radixin/moesin family [46]. Thus, the scaffolding required for proper signaling of light perception in the drosophila phototransduction system may also be required for proper cytoskeletal architecture at the slit diaphragm of the podocyte in mammals.

Indeed, TRPC4 and TRPC5 are heavily expressed in the cerebral cortex of the mammalian brain [53]. Less information is available regarding TRPC2, it appears to be a pseudogene in humans [54]. TRPC2-deficient mice however, exhibit abnormal mating behavior and data have shown that this channel may have a role in pheromone signaling [55]. The TRPC3, TRPC6, and TRPC7 subfamily are approximately 75% identical and form a cationic non-selective channel that show both inwardly and outwardly rectifying cation currents. TRPC6 is the most selective of the TRP channels; the TRPC3,6,7 subfamily has selectivity on the order of P_{Ca}/P_{Na} 1.5 to 6:1 [36]. TRPC6 channels have been shown to be activated in response to phospholipase C stimulation. The GPCR pathway involves ligand binding to membrane receptors, activation of phospholipase C and the generation of inositol 1,4,5 triphosphate with binding to its receptor, and release of intracellular calcium from the endoplasmic reticulum. Recent studies using positional cloning have identified mutations in the PLC epsilon gene (PLC ϵ 1) as causing early-onset nephrotic syndrome with end-stage renal disease. Kidney histology of affected individuals showed diffuse mesangial sclerosis and immunofluorescence revealed an arrest in normal glomerular development [56]. Importantly, two children with truncating mutations in PLC ϵ 1 responded to therapy with corticosteroids or cyclosporine A, indicating that molecular causes of nephrotic syndrome may be amenable to treatment.

Currently, the physiologic role of TRPC6 channels is currently being studied through the use of TRPC6-deficient and transgenic mouse models. Thus far, TRPC6-deficient mice have rather surprisingly been shown to have elevated blood pressures and enhanced contractility of isolated aortic rings and cerebral arteries [57].

The TRPC6 Mutation Causes FSGS : Potential Mechanisms of Disease

4.1 Alteration in Podocyte Dynamics

The etiology of FSGS has focused on alterations in the structure or function of the podocyte, the visceral glomerular epithelial cell. The podocyte is a terminally differentiated cell that lines the outer aspect of the glomerular basement membrane, forming the final barrier to protein loss. Individual podocytes have foot processes which form tiny membrane bridging filtration slits 30 to 40 nm wide, termed the slit diaphragm. Abnormalities in podocytes may cause proteinuria when slit diaphragm function is altered. The normal function of the podocyte requires critical interactions between different proteins such as nephrin, podocin, and CD2AP. For example, extracellular immunoglobulin (Ig) domains of nephrin engage in homophilic interactions, and form heterodimers with the Ig domains of podocin [58-62]. As described earlier, mutations in some of these podocyte proteins leads to nephrotic syndrome.

The TRPC6^{P112Q} mutation augments intracellular calcium influx into the podocyte, leading to FSGS through unclear mechanisms. One possibility is that increased intracellular calcium may modify the contractile structure of podocyte foot processes, resulting in an alteration of the ultrafiltration coefficient (Kf). It has been shown that TRPC6 is expressed at the podocyte cell membrane and partially colocalizes

with other podocyte proteins such as nephrin and podocin. Furthermore, these studies showed that disruption of the slit diaphragm architecture in nephrin-deficient mice leads to overexpression and mislocalization of TRPC6 in podocytes [35]. This suggests that TRPC6 may be a component of an organized signaling complex located at the slit diaphragm that mediates normal podocyte function. Very recently, studies by Reiser et al have also shown a relationship between the actin cytoskeleton and TRPC6 [63]. Cultured, differentiated podocytes with TRPC6 overexpression displayed loss of actin stress fibers. This suggests that abnormal TRPC6 expression may cause structural changes in the slit diaphragm that could lead to proteinuria and glomerulosclerosis. Another cause of abnormal foot process formation may be the loss of spatial cues within the podocyte. Li et al., have shown that brain-derived neurotrophic factor-induced (BDNF) chemo-attraction of axonal growth cones requires calcium signaling. Their studies in cultured cerebellar granule cells revealed that TRPC channels contribute to the BDNF-induced elevation of calcium at the growth cone and are required for BDNF-induced chemo-attractive signaling. In TRPC3 and TRPC6 deficient cells, calcium elevation and growth cone turning were abolished [64]. Analogously, foot process formation may require TRPC channels to act as molecular guideposts. The TRPC6 mutation may lead to abnormal podocyte polarity and an inability to adjust to changes in glomerular filtration pressure. A third mechanism of abnormal podocyte contractile function may be through altered mechanosensation. Spassova et al., have shown that TRPC6 is a sensor of mechanically and osmotically induced membrane stretch, independent of PLC activation [65]. The stretch responses were blocked by the tarantula peptide, GsMTx-4, known to specifically inhibit mechanosensitive channels by

modifying the external lipid-channel boundary. This study suggests TRPC6 mutations may cause altered hydrostatic pressure-driven ultrafiltration, with resultant proteinuria and glomerulosclerosis. Huber and colleagues have shown that TRPC6 interacts with podocin and both the MEC-2-dependent activation of mechanosensation and that podocin-dependent activation of TRPC channels require cholesterol. They have speculated that multiprotein complexes containing the transmembrane proteins such as Neph1, Neph2, nephrin, the cytoplasmic adaptor protein CD2AP and TRPC6 could form a sensor involved in monitoring glomerular pressure or filtration rate [66].

4.2 Podocytopenia

Another mechanism for the association between TRPC6 and familial FSGS is that an alteration in the intracellular calcium concentration may cause podocytopenia through a variety of mechanisms, with resulting glomerulosclerosis. There is a growing body of experimental and clinical literature that show podocyte number is a critical determinant for the development of glomerulosclerosis and that a decrease in podocyte number leads to progressive renal failure [67]. Wiggins et al., have shown that a single injection of puromycin aminonucleoside (PAN), a podocyte toxin, caused a marked decrease in podocyte number in rats and subsequent glomerulosclerosis [68]. Human studies by Meyer et al., have shown that a decrease in podocyte number in Type II diabetic Pima Indians correlated closely with microalbuminuria, the earliest manifestation of diabetic nephropathy. Follow up studies showed that decreases in podocyte number correlated with progression of diabetic nephropathy as well [69]. An increase in intracellular calcium may cause loss of podocytes either through apoptosis, detachment, or lack of

proliferation [70]. Apoptosis may be caused by an ability of mutant TRPC6 to augment the deleterious effects of Ang II. Singhal et al., have previously shown that Ang II induces apoptosis in cultured rat podocytes and perhaps TRPC6 upregulates this pathway of programmed cell death [71]. Detachment is another mechanism of podocyte loss. Hara and colleagues have shown that cells obtained in the urine of patients with various glomerular diseases stained positive for the podocyte marker, podocalyxin [72-74]. Similar results have been shown in a puromycin aminonucleoside nephrosis (PAN) model of podocyte injury in rats [68]. The mechanisms of podocyte detachment remain unknown, but likely involve the abnormal function of specific integrins such as $\alpha 3\beta 1$ integrin [75] [76]. One speculation would be that possibly an increase in intracellular calcium affects integrin function and leads to podocyte detachment. A third mechanism of podocyte loss is lack of proliferation. Although podocytes are terminally differentiated cells, proliferation is a prerequisite for normal glomerulus formation. Proliferation is governed at the level of the cell cycle via cell cycle regulatory proteins [77]. To proliferate, cyclins must bind to and activate partner cyclin-dependent kinases (CDKs). In contrast, CDKs are inactivated by CDK-inhibitors, including p21, p27, and p57 [78]. Thus, it can be speculated that enhanced calcium entry may constitute a pathologic trigger, such as calcium overload of the podocyte that initiates cell death by apoptosis or causes dysregulation of cell cycle machinery that may lead to hypertrophy of the podocytes by altered levels of cyclins (E, A, B1) with concurrent changes in CDKs (p27, p57) thus causing an imbalance in the ratio of cell cycle progression and inhibiting molecules [79].

5. Therapeutic Manipulation of TRP Channels

5.1 Blocking TRP Channels

As stated earlier, TRP channels are a relatively new class of ion channels which can be activated by receptor binding and/or intracellular calcium depletion as opposed to strictly membrane depolarization. These so-called Receptor Operated Calcium channels (ROCs) represent a new molecular target for therapeutic manipulation. Previous work by Beech et al., has shown that blocking the TRPC1 channel inhibited the salient features of vascular disease which are smooth muscle cell proliferation and neointima formation [80]. Targeting TRPC1 may therefore represent a new therapeutic approach that avoids the decreased peripheral vascular resistance and cardiac output seen with the classical calcium channel blockers used as pharmacotherapy [81]. TRPC, TRPV, and TRPM channels have also been studied as potential drug targets in respiratory diseases such as chronic obstructive pulmonary disease and asthma. It has been suggested that ROCs are involved in airway smooth muscle contraction and specific TRP channels have been associated with mucus hypersecretion, airway inflammation, immunomodulation, and cough production [82].

5.2 Blocking TRPC6 Channels and Proteinuric Renal Disease

With regard to FSGS and other proteinuric renal diseases, TRPC6 may represent a new molecular target for blockade. Classically, a fundamental line of therapy in these diseases has been blocking of the renin-angiotensin system (RAS) system by angiotensin-converting enzyme inhibitors (ACE) or angiotensin-receptor blockers (ARBs). These medications may decrease proteinuria by altering podocyte morphology [83]. In

experimental diabetic nephropathy, podocyte foot process broadening was ameliorated by RAS blockade [84] and was associated with normalization of nephrin expression [85]. Given the close association of TRPC6 and nephrin [35], blocking TRPC6 channels may be beneficial. Furthermore, given the augmentation in Ang II-mediated calcium influx in mutant TRPC6 mice compared to wild-type [9], therapeutic blockade is biologically sound. This may represent a mechanism for targeted down-regulation of the harmful effects mediated by Ang II. Further evidence for the potential therapeutic blockade of TRPC6 arises from recent studies by Reiser et al. which have shown increased expression of wild-type TRPC6 in several non-genetic forms of human proteinuric kidney diseases, including FSGS, minimal change disease, and membranous nephropathy [63].

Currently, the most standard therapy for certain forms of proteinuric renal disease, such as idiopathic FSGS are glucocorticoids. It is also well-established that idiopathic FSGS responds poorly to such treatment [86]. Steroids have pleiotropic effects mediated by cytoplasmic receptors that translocate to the nucleus and activate the transcription of genes for cytokines, chemokines, eicosanoids, and other immunomodulatory substances [87-89]. Recent studies by Xing et al. have revealed that the glucocorticoid dexamethasone has direct effects on human podocytes. The authors used a conditionally immortalized human podocyte cell line transfected with a temperature-sensitive simian virus 40 transgene that when inactivated, causes the cells to adopt the phenotype of differentiated podocytes. Using immunocytochemistry, reverse-transcriptase-polymerase chain reaction, and Western blotting, direct effects of the glucocorticoid dexamethasone were studied at concentrations mimicking those achieved in clinical practice. Dexamethasone was shown to upregulate expression of nephrin and alpha-tubulin and

downregulate vascular endothelial growth factor (VEGF) . The effects on the podocyte cell cycle were complex with downregulation of p21 (a cell cycle inhibitor) expression and augmentation of podocyte survival, without any effect on apoptosis [90]. This study suggests that although the mature podocyte is highly differentiated, it can adapt in response to kidney injury. There may be an ability for the podocyte to release the ‘molecular brake’ and perhaps promote proliferation and repair in order to restore homeostasis. Although no direct connection was established between abnormal calcium homeostasis and steroids, it is possible that TRPC6 is involved in the adaptive response of the podocyte to stress through its interaction with nephrin and other podocyte proteins [90]. Given previous studies which revealed that nephrin-deficient mice have TRPC6 mislocalization and overexpression, it is plausible that blockade may ameliorate the abnormal slit diaphragm architecture in certain proteinuric renal diseases [35]. Ideally, blockade of TRPC6 could promote podocyte proliferation and complex formation.

Evidence also suggests that TRP channels may be involved in the immunosuppressant action of the calcineurin inhibitor FK-506, one of the latest therapies for steroid-resistant FSGS [91]. FK-506 has been determined to affect the interaction of TRP channels with immunophilins. Immunophilins are peptidyl-prolyl cis/trans isomerases which recognize specific XP dipeptides in target proteins and are mediators of the immune system [92]. Goel et al., have shown that TRPL channels interact with the immunophilin FKBP-59 and that this interaction can be abrogated by the addition of FK-506 [93]. Furthermore, TRPC3, TRPC6, and TRPC7 have been shown to co-localize with the immunophilin FKBP-12, another intracellular target of FK-506 [94]. Preliminary experiments have shown that the addition of FK-506 to cells overexpressing

TRPC6 caused an inhibition of channel activity. This work, although in its infancy, suggests a novel mechanism of action for FK-506 and suggests that impairing TRPC6 activity may also be immunosuppressive. The effects of FK-506 on steroid-resistant FSGS await a randomized-controlled trial where if beneficial, future focus on targeted blocking of the TRPC6 channel should be pursued.

In addition to a possible immunosuppressive effect of TRPC6 channel blockade, a very recent study of pathologic cardiac remodeling has further elucidated the intimate connection between TRPC6 and the calcineurin signaling circuit [95]. In this study, TRPC6 gene expression was shown to be upregulated in various mouse models of cardiac hypertrophy as well as human hearts with dilated cardiomyopathies. TRPC6 gene expression was also shown to be regulated by calcineurin and nuclear factor of activated T cells (NFAT) through a positive feedback loop. Transgenic mice overexpressing TRPC6 were shown to develop cardiomegaly, congestive heart failure, and premature death. Therefore, this study provides further evidence that TRPC6 overexpression may be pathologic and provides a strong rationale for therapeutic blockade.

5.3 Blocking TRPC6 Channels : Obstacles

The blockade of TRPC6 channels has many challenges. Firstly, since TRPC6 shares approximately 75% sequence homology with TRPC3 and TRPC7, creating a construct that is highly selective will be challenging. Secondly, since TRPC6 is expressed in a wide variety of human tissues, even if selective inhibition is achieved, there may be harmful side effects. As described earlier, the TRPC6 knockout mice produced by Dietrich et al., were found to be hypertensive [96]. This would be an

extremely untoward side effect of a potential anti-proteinuric medication. The goal would be to produce a molecule that is highly specific for TRPC6, a formidable task.

6. Conclusions

Studies of familial nephrotic syndromes have delineated the importance of the podocyte in normal glomerular function. Genetic abnormalities in podocyte proteins such as nephrin, podocin, ACTN4, and CD2AP lead to glomerulosclerosis, likely due to changes in the slit diaphragm or podocyte cytoskeleton. One of the latest advances in podocyte biology has been the unexpected association of familial FSGS with a mutant variant of TRPC6. The TRPC6 mutation causes an augmentation of inwardly rectifying calcium currents in the podocyte cell membrane. The mechanism by which abnormal calcium homeostasis leads to glomerular injury remains unknown. In the coming years, research will focus on the effects of intracellular calcium on the podocyte contractile apparatus and podocyte life cycle. Perhaps the increase in intracellular calcium in the podocyte leads to apoptosis, detachment, or cell cycle arrest during maturation, via either GPCRs or Ang II. Elucidation of the role of TRPC6 in glomerular function may also enable clinicians to better treat idiopathic FSGS, a disease with a generally poor prognosis. Ang II, a known mediator of kidney injury, has been shown to augment intracellular calcium currents in mutant TRPC6 mice, suggesting a therapeutic benefit to blocking TRPC6 channels. Furthermore, the calcineurin inhibitor FK-506, a potent immunosuppressive agent, has been shown to impair TRPC6 channel activity *in vivo*. In addition to therapeutics, the TRPC6 channel may also be useful in the genetic typing of patients. While far from the mythical “ESRD gene”, polymorphisms of TRPC6 may act

as susceptibility or initiation factors for renal disease and may help determine which patients would derive benefit from early, aggressive therapy. Overall, the generous efforts of multiple families from diverse ethnic backgrounds have led to significant advances in our understanding of the podocyte and its effect on glomerular function. Hopefully, the next few years will see this molecular knowledge translate into long-lasting clinical benefits.

ACCEPTED MANUSCRIPT

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Legend

Figure 1. The unique shape of the podocyte is due to the actin cytoskeleton that contains a dense network of F-actin and myosin (M), besides several actin-binding proteins such as synaptopodin (S), and α -actinin-4 (ACTN4). The podocyte consists of three membrane domains namely, apical, basal, and junctional, based on the molecular anatomy of the site [97]. The junctional membrane domain proteins include nephrin whose cytoplasmic tail binds to podocin, while CD2AP co-localizes in the same domain with nephrin. Other proteins such as Neph-1, Neph-2, Neph-3, FAT1, ZO-1, densin and β -cadherin are also localized to this domain. TRPC6 associates with podocin and nephrin at the slit diaphragm. One of the key surface receptors, angiotensin receptor 1 (AT1) is shown. The apical membrane domain is negatively charged due to the presence of surface anionic proteins such as podocalyxin, podoplanin, podoendin, and GLEPP (glomerular epithelial protein 1) which effectively prevents the passage of albumin. The basal membrane domain consists of α 3 β 1 integrin and α - and β -dystroglycans to anchor the podocyte in the GBM (glomerular basement membrane) and also to help connect certain matrix proteins (such as agrin and laminin 11) within the GBM. The GBM is composed mainly of collagen IV (α 3, α 4, α 5) and other matrix proteins mentioned above. The α 3 β 1 integrin dimers connect the TPV (talin, paxillin, and vinculin) complex to laminin 11. The α - and β -dystroglycans connect utrophin to agrin. Other key molecules shown in various compartments include Cas (p130Cas), ezrin (EZ), focal adhesion kinase (FAK), integrin-linked kinase (ILK), Na^+ - H^+ exchange regulatory factor (N) and the non-selective cation channel (NSCC). G-protein coupled receptor (GPCR)- mediated signaling pathways play a major role in various functional activities of the podocytes (modified and updated from Pavenstadt et al., 2002 and Kriz, 2005 as well as other literature mentioned in the text and legend) [70,98].

Table 1. Currently known genes that cause inherited nephrotic syndrome and FSGS.

Name (OMIM*)	Associated Disorder	Chromosomal Location	Pattern of Inheritance	Clinical Features	Structure/function	Ref.
Nephrin (NPHS1)	Congenital nephrotic syndrome of the Finnish type	19q13	AR	Massive proteinuria in utero with high mortality rate	Transmembrane adhesion protein localizes to lipid rafts within the slit diaphragm of the podocyte	14-16
Podocin (NPHS2)	Steroid-resistant nephrotic syndrome	1q25-q31	AR	Proteinuria between 3 months and 5 years of age with rapid progression to ESRD	Structural protein that recruits nephrin and CD2AP to lipid rafts in the slit diaphragm	21
Alpha-actinin 4 (FSGS1)	Hereditary FSGS	19q13	AD	Adult onset FSGS with variable age of onset, severity, and progression to ESRD	Actin-binding protein that binds actin to the cell membrane of the podocyte	29
Transient Receptor Potential Cation Channel 6 (FSGS2)	Hereditary FSGS	11q21-22	AD	High grade proteinuria in 3 rd to 4 th decade with ESRD in 60% within 10 yrs of diagnosis	Relatively non-selective cation channel that associates with nephrin, podocin, and CD2AP at the slit diaphragm	9
CD2-associated protein (FSGS3)	FSGS	6p12	Haplo-insufficiency	FSGS	Scaffold protein that interacts with the cytoplasmic domain of nephrin	29

AR: Autosomal Recessive. AD: Autosomal Dominant.

