Abstract Glomerular kidney disease is a major healthcare burden and considered to represent a sum of disorders that evade a refined and effective treatment. Excellent biological and genetic studies have defined pathways that go awry in podocytes, which are the regulatory cells of the glomerular filter. The question now is how to define targets for novel improved therapies. In this review, we summarize critical points around targeting the TRPC6 channel in podocytes.

Keywords Glomerular disease · Focal segmental glomerulosclerosis · Podocyte · Slit diaphragm · TRPC6 · Nephrin · Proteinuria

Introduction

Glomerular kidney diseases continue to affect an increasing number of patients around the world. An early sign of these disorders is loss of protein into the urine [1]. Studies on the mechanisms of proteinuria have focused in particular on podocytes, which are cells in the glomeruli that line the capillaries and constitute a major part of the filtration apparatus by providing foot processes with interconnected slit diaphragms (SDs). While it has become clear that proteinuria is almost inevitably associated with podocyte injury, as evidenced by disruption of the SD and effacement of foot processes, it is still not clear which best approaches would be to reconstitute normal podocyte function and to repair the damaged filtration barrier. When searching for the pharmaceutical targets in cells, the accessibility of the target cell, the target itself, and the signals that are modified by interfering with the target have all to be taken into account [2]. The TRPC6 ion channel has attracted special attention because not only are there gain-of function mutations in the TRPC6 gene in a subset of hereditary glomerular disorders, such as familial focal segmental glomerulosclerosis (FSGS), but it is also induced in its non-mutant form under a wide variety of acquired glomerular diseases [3]. In addition, TRPC6 is also an interacting protein at the podocyte SD [4] and thus located at an accessible site where drugs can reach TRPC6 before and after passing through the SD. Thus, TRPC6 in podocytes would appear to be the ideal target for small molecules or biological modifiers—once they become available.

Role of podocytes in the regulation of glomerular filtration

Renal function entails the ultra-filtration of blood in the glomeruli, which operates as a sieve that is permeable to water and small solutes while largely excluding macromolecules on the basis of size and charge by retaining them in the blood. The glomerular filtration barrier comprises several elements, including a fenestrated capillary endothelium, a triple-layered glomerular basement membrane (GBM), and a population of highly specialized cells called podocytes. Mesangial cells, which are inside the GBM, also play a role in regulating glomerular tuft stability and...
sustained glomerular function by participating in signaling exchange with podocytes [5].

Podocytes and in particular their adjacent foot processes are connected by specialized cell junctions referred to as slit diaphragms (SDs) [6–8]. These cells have attracted increasing attention because of their recognized role in glomerular pathology [9]. The biology of the adhesion and signaling molecule nephrin has turned out to be crucial for proper functioning of the SD [8]. This observation followed a series of important studies showing that defects in nephrin protein expression or in the nephrin gene can lead to severe proteinuria as part of an autosomal recessive congenital nephrotic syndrome. Nephrin is a transmembrane molecule with an intracellular signaling domain, a transmembrane domain, and a large ectodomain consisting of eight Ig motifs and one type III fibronectin domain. The ectodomain is long enough that two nephrin molecules can undergo a homophilic interaction in trans; consequently, they are sufficient to help keep the SD wide enough [8]. The genetic disruption of nephrin unravels its important role in keeping foot processes apart from each other. The results of nephrin inactivation are narrowed and dysfunctional filtration slits that soon thereafter lead to complete foot process effacement [10, 11]. Moreover, signaling through this system enables recruitment of a number of other molecules, including Nck and CD2-associated protein, which can then modulate the behavior of the underlying cytoskeleton [12]. Other critical components of the SD, such as Nep1, Nep2, podocin, and the ion channel TRPC6, have also been shown to play critical roles in glomerular filtration and in podocyte signaling by providing scaffold and mechanosensitive properties to the filtration site [4].

TRPC channels in podocytes

There are seven functional members of the TRPC channel family in mice (TRPC1–7) and six in humans, with TRPC2 being a pseudogene [13, 14]. The TRPC channels are members of a larger channel family known as TRP channels, which have a broad role in chemo- and mechanosensation and in allowing cells to sense changes in their local environment [13, 15]. TRPC channels can heteromerize and thereby have the potential to form a very large number of unique channels [14].

Minimally functional TRPC channels are tetrameric proteins composed of subunits with six transmembrane segments and NH2- and COOH-termini facing the cytosol [14] (Fig. 1). A series of ankyrin repeats are present in the NH2-terminal, and all of the TRPC channels have a highly conserved 25-residue TRP domain immediately attached to the COOH-terminal side of the last transmembrane segment, which includes a proline-rich domain known as TRP box 2. The ankyrin repeats and the TRP box 2 are domains for protein interactions. The presence of a calmodulin and inositol 1,4,5-trisphosphate receptor-binding region (CIRB region) has also been described in the COOH-terminal of TRPC channels [16, 17]. Calmodulin-binding domains have been identified in both the NH2- and COOH-terminals of some TRPC members, including TRPC6 [18]. The pore–loop domain is located between the fifth and sixth transmembrane segments [19], and TRPC channels are permeable to monovalent and divalent cations.

The mechanisms underlying the activation of TRPC channels are highly controversial and depend on whether or not the channels are part of a heteromeric complex containing other TRPC members, scaffolding proteins, or even members of other ion channel families [20].

Mutations in the TRPC6 gene cause FSGS

The discovery of TRPC6 mutations as a cause for familial FSGS has opened new areas of investigation [4, 21]. While TRPC6 gene mutations are inherited in an autosomal...
dominant fashion, the data suggest that TRPC6-related FSGS is not restricted to certain ethnic groups. TRPC6 mutations were found to cosegregate with kidney disease in families of African-American, Colombian, Polish, Mexican, and Irish/German descent alike [4, 21] and appeared to account for a significant number of inherited FSGS cases. In the study by Reiser and colleagues [4], 71 families with familial FSGS were tested, and five turned out to be positive for TRPC6 mutations, pointing to a relative frequency of TRPC6-related FSGS of approximately 7% of all cases of familial FSGS. The age range at disease presentation was broad, ranging from 16 to 61 years. As such TRPC6-related FSGS can be classified as a disease with a rather late onset, which is in contrast to other genetic glomerulopathies that often manifest in infancy, such as congenital nephrotic syndrome of the Finnish type [22], steroid-resistant nephrotic syndrome of the podocin type [23], or Wilms’ tumor protein 1 (WT1)-related diseases [24].

While the early onset in these diseases is due to the severe damage that is inflicted on the molecular architecture of the SD and the process of glomerular filtration, the question remains why the onset of kidney disease in patients with TRPC6 mutations occurs years into life. One possible explanation is that mutations in TRPC6 may produce subtle changes in intracellular function that lead to irreversibly altered cell behavior only after time and in the presence of other renal insults (second hit hypothesis) (Fig. 2). This would be similar to the adult onset and dominantly inherited form of FSGS due to mutations in the widely expressed protein α-actinin-4 [25]. In addition, podocytes express several TRPC channel subunits other than TRPC6 [20], and partial functional redundancy may also help account for the late onset of glomerular disease. Indeed, the ability of TRPC6 to form heteromers with other TRPC channels suggests a complex cellular regulation of calcium homeostasis [26].

Interestingly, patients with TRPC6-related FSGS do not present with any other pathological phenotype. TRPC6 protein is widely expressed in smooth muscle cells, where it is thought to contribute to the regulation of airway resistance and vascular tone [27]. However, TRPC6 mutations appear to exhibit their deleterious effects mainly in the kidney. These observations suggest that the glomerular phenotype is the consequence of a unique role of TRPC6 in the functional organization of the glomerular filtration barrier and the increased susceptibility of an imperfectly regulated filtration apparatus due to TRPC6 mutations. In this context, TRPC6 belongs to the same category of genes as α-actinin-4 and CD2AP. Mutations in both genes lead foremostly to glomerular kidney disease while being widely expressed and associated with a plethora of different cellular functions in many cells throughout the body [25, 28]. In the case of CD2AP, it is possible to rescue lethality in CD2AP−/− mice with podocyte-targeted expression of CD2AP, highlighting the particular role of CD2AP protein in podocytes [29].

Nature of TRPC6 mutations

All known mutations map to the terminal domains of the TRPC6 protein (Fig. 1). Six N-terminal missense mutations (G109S, P112Q, M132T, N125S, N143S, and S270T) locate in or near ankyrin repeats and an adjacent lipid/trafficking domain. The ankyrin domains drive self-association of TRPC homomers [30], whereas the lipid-binding domain binds DAG and thus controls translocation of the channel to the plasma membrane [31]. A recently identified mutation L780P is near the EWKFAR motif that is conserved in all TRP channels, whereas four additional mutations (K874*, Q889K, R895C, and E897K) map to a predicted coiled-coil domain at the C-terminus [32].

In TRPC channels, the first ankyrin-like repeat is the minimum indispensable key structure for the functional assembly of homo- and heteromeric TRPC4/TRPC5 chan-
nels [30]. Mutations in this region may therefore compromise the ability of TRPC6 to oligomerize with other TRPC subunits or to interact with other binding partners. Ankyrin repeats have also been shown to be important determinants of the proper plasma membrane targeting of TRPC channels [33, 34]. Whereas not all ankyrin repeats seem to be crucial for the functional assembly of TRPC channels, a truncated variant of TRPC3 lacking all of the N-terminal ankyrin repeats was found to no longer mediate agonist-induced Ca\(^{2+}\)-entry in HEK293 cells [33]. Finally, mutations within the ankyrin repeats of TRPC6 may prevent the channel from being correctly inserted into the plasma membrane. This hypothesis is supported by findings of Cayouette et al. suggesting that TRPC6 levels at the plasma membrane are regulated by targeted membrane insertion [29]. Notably, no changes in peak current amplitude were detected for the N-terminal N143S and S270T mutations and for the K874Stop non-sense mutation [4]. These do, however, display an increased opening time and thus transport more current per opening [36]. Additional factors involved may include altered channel regulation (despite normal current amplitude), altered interaction with SD proteins, and/or altered protein turnover.

Among the three mutations at the C-terminus identified by Reiser et al. [4] two (R895C; E897K) are located very close to each other in a putative coiled–coil domain. The coiled–coil is an ubiquitous protein motif that is commonly used for oligomerization. There is one potential coiled–coil motif in each of the cytosolic – and C-termini of TRPCs. While no study has specifically investigated the importance of the C-terminal coiled–coil motif in the TRPC channel, the results of a recent study suggest that the C-terminal tail of TRPC4 and TRPC6 participates in channel oligomerization [37]. Importantly, the R895C and E897K mutations both resulted in TRPC6 channels with higher current amplitudes, suggesting that disruption of the C-terminal putative coiled–coil motif in TRPC6 has a direct consequence on channel activity. Whether this is indeed due to an interference with the ability of TRPC6 to oligomerize with other channel subunits will have to be addressed in future studies.

As is the case for the N143S and S270T mutations, the mutation identified by Winn et al. in a large family from New Zealand (P112Q) is also located at the N-terminal intracellular tail in the sequence region spanning the four ankyrin repeats [21]. Similar to the increased channel activity that is observed for the R895C and E897K mutations, Winn et al. found the current amplitude increased for the TRPC6 P112Q channel [21], indicating that interference with both the N– and C-terminal domains of TRPC6 may give rise to hyperactive channels. They also provide a possible explanation for the increased current amplitude caused by the P112Q mutation: based on the results of their surface biotinylation studies, they demonstrated that mutant TRPC6 channels were more abundant in the plasma membrane when expressed heterologously in HEK293 cells than their wild-type counterparts. Interestingly, Heeringa and colleagues described a mutation in the N-terminus of TRPC6 M132T that leads to FSGS disease in children. These authors found that the channel activity of M132T increased largely with augmented peak current as well as delayed inactivation [36]. This finding supports the notion that calcium-dose effects mediated by TRPC6 might play a role in determining the onset and severity of disease. Clearly, more studies are required to explore this concept.

**Consequences of TRPC6 for the diagnosis of FSGS**

The finding that mutations in the TRPC6 gene are associated with familial forms of FSGS may be used for genotyping of candidate individuals. Polymorphisms of TRPC6 may act as a susceptibility factors for renal disease and may help determine which patients would benefit from early and intensified therapy. Whereas genotyping assays have been regularly used as a research tool to screen patients for the presence of mutations associated with FSGS and nephrotic syndrome [38], this approach is making it rather slowly into renal practice. However, with rapid, reliable, and affordable genetic testing becoming increasingly available, genotyping of candidate patients are likely to play an increasingly important role in clinical diagnostics. Tests for mutations in the NPHS1 (nephrin), NPHS2 (podocin), and TRPC6 genes are more frequently being used in daily clinical practice. Early knowledge on TRPC6-related FSGS risk may enable an earlier therapeutic intervention. It may also prompt the affected individuals to adopt a lifestyle aiming to preserve kidney function as long as possible, such as by adhering to a low-salt diet in an effort to control blood pressure. Probably even more relevant downstream mechanisms of dysfunctional TRPC6 channels will be unraveled, thereby facilitating the discovery of novel drugs that control TRPC6 function in the podocyte [5].

**Dual role for TRPC6 in genetic and acquired forms of proteinuric kidney disease**

TRPC6 mutations, although small in terms of numbers of affected families, provide important novel clues towards the understanding of hereditary forms of proteinuric glomerular disease. Moreover, a recent study showed that wildtype
TRPC6 expression was upregulated in a subset of acquired human proteinuric kidney diseases [39]. An induction of TRPC6 protein was also observed in experimental in vitro and in vivo models of acquired glomerular disease. These results are in line with the hyperactivity of mutated TRPC6 channels in genetic FSGS, pointing to a deleterious effect of too much TRPC6 activity, whether it stems from a mutation or from an induced wildtype channel, or both.

Of particular interest is the strong induction of TRPC6 protein in membranous glomerulopathy [39]. Membranous glomerular disease represents the most common cause of idiopathic nephrotic syndrome in white adults, accounting for about one fifth of all cases [40]. Eighty percent of cases are classified as idiopathic [41], and are often associated with auto-antibodies to M-type phospholipase A2 receptor (PLA2R) [42]. In membranous nephropathy, subepithelial immune deposits form in situ as a result of circulating antibodies against one or several antigens that remain yet to be identified in humans [43]. Hand in hand with the formation of immune deposits goes activation of the complement, leading to the assembly of C5b-9 membrane attack complexes on podocyte plasma membranes, which represents the main culprit for sublethal podocyte injury and the onset of proteinuria [44]. It has also been reported that the damage to podocytes mediated by the complement C5b-9 complex is associated with an activation of phospholipase C and an increase in intracellular calcium [45]. Finally, among the cellular mechanisms leading to proteinuria in membranous nephropathy are cytoskeletal changes [46] similar to those observed upon TRPC6 overexpression [39]. It will therefore be important to study possible mechanisms of TRPC6 regulation by the complement and to investigate the downstream effects of elevated TRPC6 activity in membranous disease—particularly now that the M-type phospholipase A2 receptor (PLA2R) has been discovered as an antigen for auto-antibodies found in a wide variety of patients with membranous nephropathy [42]. Since the influx of extracellular Ca\(^{2+}\) is tightly coupled to phospholipase A2 activation in some cell types, such as C62B glioma cells [47], it will be interesting to see if the binding of pathological antibodies to PLA2R facilitates signaling through TRPC6.

What does it all mean for treating patients?

At this time, DNA sequencing and PCR assays are commercially available to identify TRPC6 mutations. These allow risk stratification for earlier management and possible delay of the progression of the disease. The standard treatment of TRPC6-associated FSGS is still not well defined, and efforts are mainly focused on the management of hypertension and proteinuria known to be classical risk factors predisposing to renal damage and scarring. On the one hand, blood pressure control has been shown to delay the progression of many glomerular diseases, especially when angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) are used, both of which also contribute to decreasing proteinuria, in part even independently of hemodynamics [48]. On the other hand, managing the complications of nephrotic syndrome includes the use of diuretics and lipid-lowering agents, such as statins [49]. Currently, there is no clinical consensus on routinely testing steroid-resistant FSGS patients for the known podocyte gene mutations, but this may change as new therapies for genetic FSGS become available.

In recent years, many groups have started experimenting with new methods to target overly active TRPC6. Since both overly active mutated TRPC6 or induced wildtype TRPC6 protein cause renal damage, multiple experimental hypotheses that focus on modifying TRPC6 expression or blocking TRPC6 channels specifically in podocytes are currently being investigated [2]. Novel TRPC6 siRNA coupled with a podocyte-specific delivery system (sham-porter = sheep anti-mouse podocyte transporter) has already been shown to significantly decrease TRPC6 protein expression in podocytes [50]. Other possibilities are currently being studied in experiments aimed at finding specific inhibitors of TRPC6 that will not affect other TRPC subunits such as, for example, TRPC5 or TRPC1, which are also expressed in podocyte foot processes [20]. This may be a very difficult task, and it might possibly be easier to utilize non-specific TRPC6 inhibitor with directed delivery. Profection methods have recently become available that can transport such inhibitory peptides or even entire proteins packed in synthetic lipid structures to specific cells, including podocytes [51]. The uptake of drug cargo to podocytes might be particularly promising given the capacity of podocytes for active endo- and macropinocytosis [52].

While the quest to find the right agent and approach is currently underway, we need to consider the multiple subcellular spaces in which TRPC6 operates (Fig. 3). While a known downstream target of TRPC6 is nuclear factor of activated T-cells (NFAT) signaling that originates in the cell body and carries on into the cell nucleus [53], the action of Ca\(^{2+}\) in foot processes might stem mainly from SD-associated TRPC6 protein, with the primary goal to regulate foot process dynamics. Given this possible spatial separation of TRPC6 function, a therapeutic goal might be to neutralize overly active TRPC6 in foot processes while leaving TRPC6 function in the podocyte cell body and nucleus unaffected. More studies will better clarify the possible multiple roles of TRPC6 in podocytes. While these studies will require some time, we should not forget to test existing drugs that might be re-purposed for blocking...
TRPC6 or TRPC6-mediated downstream signals in podocytes, such as calcineurin inhibitors. Since a non-T-cell inhibitory dose might be sufficient to positively affect podocytes, a low-dose treatment approach could spare many of the side effects of currently utilized calcineurin inhibitors in FSGS patients.

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**Questions**

Answers appear following the reference list.

1. Which is the key cell in the kidney that regulates filtration?
   a) mesangial cell
   b) proximal tubular epithelial cell
   c) podocyte
   d) parietal epithelial cell
   e) endothelial cell

2. The podocyte is most similar to?
   a) pericyte
   b) vascular smooth muscle cell
   c) adipocyte
   d) fibroblast
   e) none of the above

3. The glomerular slit diaphragm is?
   a) a microvillus
   b) an intracellular contact
   c) an extension of the GBM
   d) a modified adherens junction
   e) a classical tight junction

4. TRPC6 is potentially a good drug target because?
   a) it is involved in regulation of the kidney filter
   b) it is the main gene mutated in IgA nephropathy
   c) it is the TRP channel in polycystic kidney disease
   d) b + c are correct
   e) none of the above

5. Podocytes are druggable cells because?
   a) they turn over frequently
   b) they are exposed to blood and primary urine
   c) they display active endo- and macropinocytosis
   d) b + c are correct
   e) none of the above

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Answers
1) c
2) a
3) d
4) a
5) d

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