



Article

# TRPC6 Is Found in Distinct Compartments of the Human Kidney

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**Abstract:** In the context of renal proteinuric diseases, TRPC6 has been shown to play an important role in ultrafiltration associated with the slit diaphragm through the control of the intracellular Ca<sup>2+</sup> concentration in the podocytes of glomeruli. However, to date, the properties of TRPC6 have been studied mainly in cell lines or in animal models. Therefore, the aim of the study presented here was to investigate the presence and distribution of TRPC6 in human kidneys in order to possibly verify the applicability of the results previously obtained in nonhuman experiments. For this purpose, kidneys from nine cadavers were prepared for immunohistochemical staining and were supplemented with a fresh human kidney obtained by nephrectomy. TRPC6 was detected in glomeruli and in the parietal epithelial cells of Bowman's capsule. Larger amounts were detected in the tubular system and collecting ducts. In contrast to the peritubular capillary bed, which showed no immune reaction, the cortical resistance vessels showed mild TRPC6 staining. In conclusion, our studies on the expression of TRPC6 in human kidney tissue support the translational concept of the involvement of TRPC6 in various renal diseases and reveal new aspects of the distribution of TRPC6 in the human kidney.

**Keywords:** TRPC6; human kidney; glomeruli; tubular system



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## 1. Introduction

The functional unit of the kidney—the nephron—is composed of a renal corpuscle and a tubular system. A vascular network—the glomerulus—and the Bowman's capsule, which consists of a parietal and visceral layer, form the renal corpuscle. Its main functions include the regulated filtration of blood and the production of primary urine. A distinction is made between proximal, intermediate, and distal tubules. The proximal straight tubule, the descending and ascending thin limb, and the thick ascending limb form the loop of Henle [1]. The collecting duct, which is connected to the distal tubule via the connecting tubule, is responsible for the drainage of several nephrons. The primary urine undergoes a series of tubular modifications that result in the formation of concentrated excretory urine.

The visceral epithelial cells of Bowman's capsule—the podocytes—form so-called foot processes that interact with the glomerular basement membrane (GBM) on the urinary side of the renal filtration barrier. The slit membrane, a protein membrane that extends into the intercellular space between the foot processes, plays an important role in ultrafiltration by restricting passage for proteins with Stokes–Einstein radii similar to those of albumin [2–4]. The glomerular permselectivity also depends on the molecular charge and shape. Nephron

(NPHS1) and podocin (NPHS2) are important components of the slit membrane [5]. In one study, the transfection of podocytes with green fluorescent protein-TRPC6 constructs in combination with the staining of antibodies against nephrin, podocin, and CD2-associated protein (CD2AP) revealed the partial co-localization of TRPC6 with the aforementioned players of the slit membrane [6]. As a non-selective cation channel, TRPC6 regulates the intracellular  $\text{Ca}^{2+}$  concentration in podocytes. Actin cytoskeleton remodeling is critical for a functional ultrafiltration barrier and is thought to be mediated directly by the  $\text{Ca}^{2+}$ -dependent Rho GTPase and indirectly by the calcineurin pathway associated with adhesion protein expression [7]. Cytoskeletal remodeling is kept in balance by the contraction-promoting TRPC6/RhoA pathway and the motility-promoting TRPC5/Rac1 pathway [8]. TRPC6 gain-of-function mutations in the slit membrane may therefore lead to intracellular  $\text{Ca}^{2+}$  overload, which mediates pathophysiological contractile remodeling of the actin cytoskeleton. The latter is associated with changes in pore size and, subsequently, with renal proteinuria disorders [9–12].

The transient receptor potentials (TRP) superfamily consists of seven subfamilies, one of which is the TRPC 1–7 (canonical) subfamily. Only six of these are expressed in humans, with TRPC2 being a pseudogene [13,14]. Similar to several TRP channels, hTRPC6 mRNA is ubiquitously expressed, including in the kidneys [15]. They are tetrameric, non-selective  $\text{Ca}^{2+}$ -permeable cation channels. TRPC6 subunits occur in homomers and heteromers, allowing for a wide variety of homo/heteromeric TRPC channels [2,16]. Their subunits are composed of six transmembrane segments (S1–S6). The loop between S5 and S6 forms the cation-permeable pore, and both the COOH and the NH2 terminals are located in the cytosol [17]. Many cytosolic domains, such as ankyrin repeats, sinuous domains near the NH2 terminals or TRP domains, calmodulin and IP3 receptor-binding regions (CIRB region), and sinuous domains near the COOH terminals, often allow for interactions with numerous different players [2]. TRPC6 is transiently permeable and, because of its DAG sensitivity, can be induced by the activation of phospholipase C pathways [13]. Importantly, TRPC6 responds directly and indirectly via G-protein-coupled receptors to mechanical stress in podocytes [2,18,19]. Moreover, angiotensin II (Ang II) is known to induce the overexpression of TRPC6, which partly explains the nephroprotective effect of RAAS inhibitors [20].

Since the discovery that TRPC6 mutations lead to hereditary FSGS, research on TRPC6 has been a top priority, with TRPC6 becoming a potential target for the treatment of proteinuria kidney disease. Most studies on this topic are based on animal experiments or cell line culture. For example, human embryonic kidney cells (HEK293) have been used to study the functions and properties of TRPC6; however, cultured cells may undergo phenotypic changes and therefore differ from *in vivo* cells [2,21,22]. Although TRPC6 has occasionally been described in human kidneys, morphological characterization is lacking [22,23]. Therefore, the aim of the present study was to investigate the presence and distribution of TRPC6 in the human kidney to support the translational concept of TRPC6 in human kidney diseases.

## 2. Materials and Methods

### 2.1. Dissection

Kidney tissue was obtained during anatomical dissections from cadavers of the body donation program at Saarland University. Fixation was performed approximately 36 h post-mortem. All cadavers were preserved for 6–12 months with Basler solution (Otto Fischar GmbH, Saarbrücken, Germany). All cadaver donors had previously given their consent, and the Ethics Committee of Saarland also gave its approval (163/20). The mean age of the cadaver donors was  $76.3 \pm 17.8$  years (mean value  $\pm$  standard deviation). Of the nine cadavers, five were female and four were male. One specimen was freshly collected from a kidney that had been removed because of a renal tumor. The male patient was 64 years old at the time of examination. The healthy pole of the sample was fixed directly. The Ethics Committee of Saarland again gave its approval (141/14) on 28 July 2014.

## 2.2. Staining

Tissue blocks were fixed with 4% buffered formalin and embedded in kerosene. Hematoxylin and Eosin (H&E)-stained sections were prepared using routine procedures. For immunohistochemistry, the kerosene was removed, and antigen recovery was performed with citrate buffer (95 °C, 60 min). The primary antibody (polyclonal anti-TRPC6, knock-out validated, ACC017, 1:100, Alomone Labs, Jerusalem, Israel) and diluted rabbit serum were applied overnight at room temperature. A peroxidase-labeled secondary antibody (HRP-goat anti-rabbit, A10547; Invitrogen AG, Carlsbad, CA, USA) and diaminobenzidine (SK-4103, Vector Laboratories, Burlingame, CA, USA) were added, followed by counterstaining with hematoxylin. TRPC6 immunoreaction is characterized by brown staining. Diluted rabbit serum with a similar protein concentration to the diluted primary antibody was used as a negative control. All negative controls showed almost no immunohistochemically stained objects, confirming the method used.

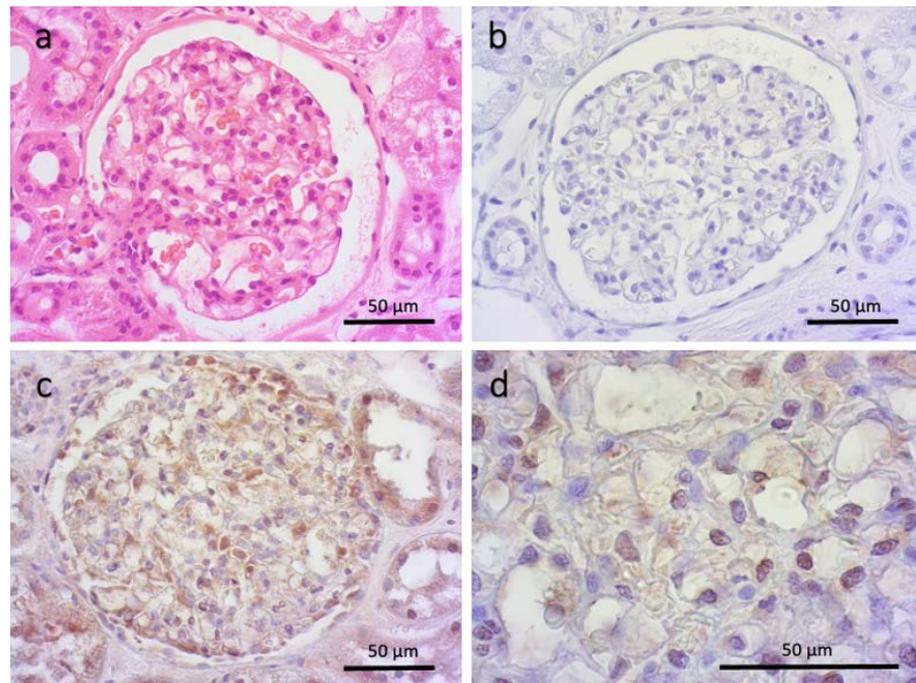
## 2.3. Analysis

An evaluation was carried out and photomicrographs were captured using a Leica DM100 system (Leica, Wetzlar, Germany) and a MikroCam SP 5.1 microscope camera (Bresser, Rhede, Germany) at 40× to 100× magnification (100× with oil). The description was based on an absolute and relative comparison.

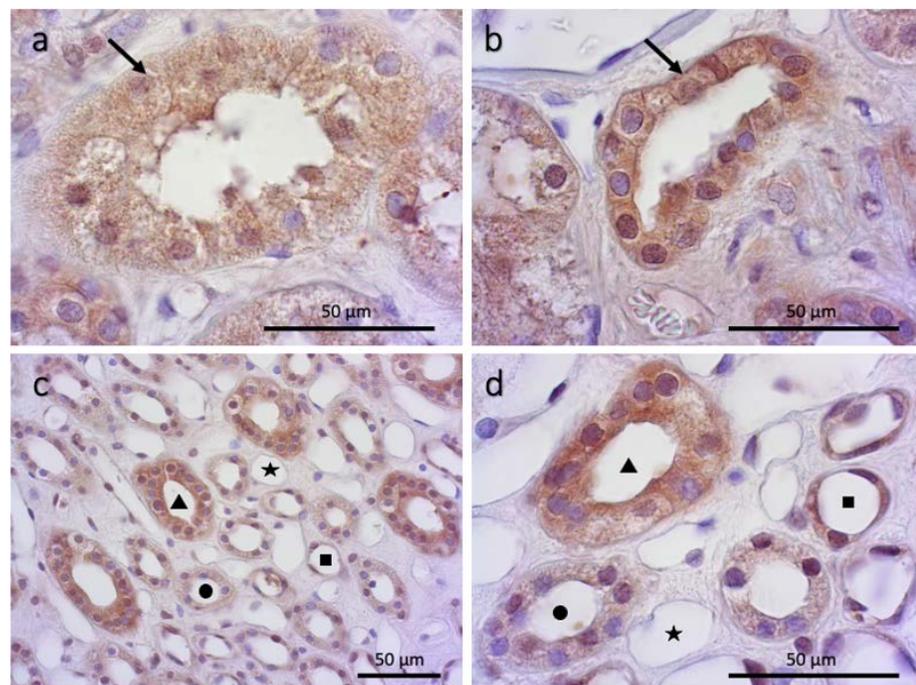
## 3. Results

In terms of immunoreactivity, both types of sections—those obtained from cadavers and by nephrectomy—were comparable.

Starting from the cortex, the glomeruli were interestingly rather weakly stained, although inter- and intraindividual differences were documented. Although the podocytes, endothelium, and inner mesangial cells could rarely be clearly distinguished, we observed that some cells of the glomerulus exhibited stronger immunoreactivity than others (Figure 1). The parietal layer of Bowman's capsule was lightly stained in many cases. Both the proximal tubules and distal convoluted tubules were overall strongly immunohistochemically stained. A clear distinction between the two was often possible only on the basis of the standard morphological description (Figure 2a,b). The medulla can be divided into an inner and outer zone, which in turn can be subdivided into an inner and outer stripe. Proximal tubules are found only in the outer stripe, whereas the thick ascending limb of the distal tubule begins in the inner stripe. Intermediate tubules, composed of thin descending and ascending legs, collecting ducts, and vessels that form the peritubular capillary bed, are found in both the inner and outer stripes. The intermediate tubules were immunoreactive and therefore expressed TRPC6, whereas the medullary vessels were negatively stained in most cases (Figure 2c,d). The distinction between both is made by the number of nuclei in a cross-section through the anatomic structure. Sections of medullary vessels should not contain more than one nucleus to allow for clear differentiation, whereas intermediate tubules should generally have more nuclei in the same section. In contrast, cortical vessels or resistance vessels showed some immunoreaction, which was weak compared to the staining of the tubule system. In particular, the thick ascending limb of Henle's loop was less reactive to TRPC6 immunostaining than the distal convoluted tubules. For their part, the collecting ducts were strongly immunoreactive (Figure 2c,d). The infiltration of immunocompetent cells was found in several sections.



**Figure 1.** TRPC6 in glomeruli. H&E staining at 40-fold magnification (a). Negative control of immunohistochemistry at 40-fold magnification (b). Immunohistochemistry at 40-fold magnification (c). Immunohistochemistry at 100-fold magnification with oil. Immunoreactive and unreactive cell nuclei can be distinguished (d).



**Figure 2.** TRPC6 in the medulla. All images show immunohistochemical staining. 100× magnification with oil of a proximal tubule marked with an arrow (a). 100× magnification with oil of a distal convoluted tubule marked with an arrow (b). 40× magnification of the medulla. The triangle represents a collecting duct, the circle represents a thick ascending limb, the quadrate represents an intermediate tubule, and the star represents medullary vessels (c). 100× magnification with oil of the medulla. Again, the triangle represents a collecting duct, the circle represents a thick ascending limb, the quadrate represents an intermediate tubule, and the star represents medullary vessels (d).

#### 4. Discussion

To investigate the presence of TRPC6 in human kidneys, we performed immunohistochemical staining on tissue obtained from human cadavers and after nephrectomy.

Interestingly, TRPC6 was not always clearly detected in each glomerulus. The glomeruli that showed an immune response were characterized by a heterogeneous distribution of staining, suggesting that the three cell types identified in the glomeruli—the podocytes, endothelial, and mesangial cells—differentially express TRPC6. Given the existing TRPC6 podocyte studies and the morphology of our stained objects, it is highly likely that podocytes are involved in TRPC6 staining in the glomeruli. For example, Reiser and his colleagues attributed most of the TRPC6 expression in rat glomeruli to podocytes; however, they showed that glomerular endothelial cells also express TRPC6 [6]. Möller and colleagues even found the overexpression of TRPC6 in the podocytes of glomeruli affected by membranous glomerulonephritis or minimal change disease [24]. TRPC6 has been shown to attenuate podocyte damage when the complement system is involved in glomerular injuries, such as those occurring in FSGS or membranous nephropathy [25]. Other players in which TRPC6 expression has been reported are cultured human inner mesangial cells (MCs) [6,22,26]. Because of their contractile capabilities, MCs are important regulators of glomerular filtration rate (GFR) as they control glomerular blood flow and ultrafiltration barrier sieve size [27]. TRPC6 itself is involved in  $\text{Ca}^{2+}$  influx leading to the contraction of cultured human mesangial cells in response to Ang II stimulation [28]. The channel may even play a role in the pathophysiological increase in GFR in the early phase of diabetes mellitus. Indeed, the hyperfiltration described is attributed in part to a decreased  $\text{Ca}^{2+}$  influx, which occurs due to impaired TRPC6 expression. This, in turn, is induced by the increased production of reactive oxygen species (ROS; Nox-4/NADPH oxidase) in response to hyperglycemia [29]. In the outer layer of Bowman's capsule, the parietal epithelial cells (PECs), were interestingly immunoreactive for TRPC6 staining. Because both PECs and podocytes express TRPC6, it is plausible that PECs could be key elements in replacing podocytes, as suggested by promising results from animal experiments [30].

We assumed the presence of TRPC6 in the proximal tubule, as it was detected in our sections. However, TRPC6 has also been detected in cultured human proximal tubule cell lines (HK-2), and aspects of its pathophysiological function in ischemia reperfusion injury (IRI) have been previously elucidated [29,31,32]. The overexpression of TRPC6 in association with  $\text{Ca}^{2+}$  overload has been shown to be one of the major factors promoting apoptosis associated with IRI in tubule epithelial cells. TRPC6-driven  $\text{Ca}^{2+}$  influx inhibits autophagy and induces apoptosis via the activation of PI3K/AKT and ERK signaling pathways, ultimately leading to cytochrome c release in the cytosol and the high production of cytotoxic ROS. Indeed, in tubular epithelial cells from mice with TRPC6 knockdown, cytoprotective autophagy is activated, bypassing the apoptosis pathway [22,32,33].

Apical TRPC6/TRPC3 heteromers may be involved in transient  $\text{Ca}^{2+}$  reabsorption processes, as suggested by studies with distal tubule cell lines (MDCK cells) [34]. It was interesting to observe that TRPC6 expression varied along the distal tubule and was less detectable in the thick ascending limb, which is characterized by the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter, than in the distal tubule, which features the vital  $\text{Na}^+/\text{Cl}^-$  cotransporter [1,35]. The physiological background of this observation needs further investigation.

Collecting ducts (CDs) differ from the proper tubule system in their descent from the ureteral system and are responsible for draining multiple nephrons to which they are connected by the connecting tubule (CNT). Goel et al. found that TRPC6 did not co-localize with the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) in rats. Because NCX is highly expressed in CNTs but absent in cortical CDs, TRPC6 expression is thought to be high in CDs and low in CNTs. However, there are large differences in NCX expression in human and rat CDs, which is an additional fact that highlights the limitations in translating the results from animal studies to humans [36–38]. Unfortunately, we did not have clear access to CNTs in our sections. Nevertheless, we can confirm the expression of TRPC6 in CDs as observed in previous animal experiments. Interestingly, it has been found that antioxidant

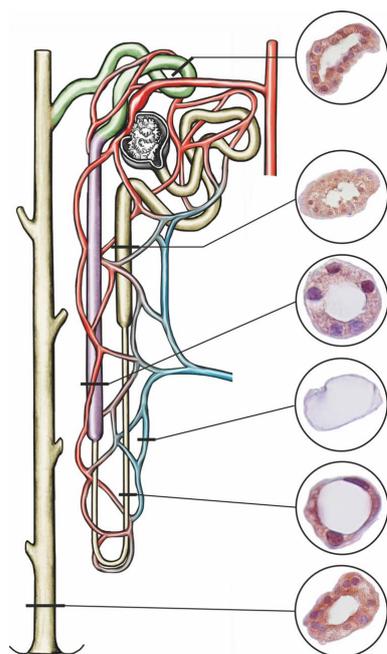
erythropoietin premedication in IRI increases ROS-induced low TRPC6 expression in CDs from male Sprague–Dawley rats, thereby improving clinical status [39,40].

The renal microvasculature, characterized by two capillary beds—one glomerular and one peritubular—is critical for the regulation of GFR in the cortex and for resorption and concentration processes in the medulla. Afferent and efferent arterioles, in which TRPC6 expression has been demonstrated, proceed and succeed the glomerular capillary network, respectively [6]. In animal experiments with male Sprague–Dawley rats, afferent arterioles were extracted and the presence of TRPC6 was detected in vascular smooth muscle cells (VSMCs) [41]. Similar results were obtained for rat total renal resistance vessels in which TRPC6 is abundant in VSMC, but almost absent in endothelial cells [42,43]. Our results also confirmed the absence of TRPC6 in endothelial cells, but of the peritubular capillary bed. Interestingly, we observed only low levels of TRPC6 in resistance vessels. Therefore, the question arises why the resistance vessels we stained showed only a weak immune response, when TRPC6 expression is widespread in renal vessels according to the relevant literature.

An important limitation of our study might be the restricted range of age, as the mean age of the investigated cadavers was  $76.3 \pm 17.8$  years at the time of death. Indeed, the distribution of TRPC6 might change along with the aging process of cells and consequently of the organs. An additional limitation of our research is the lack of functional analysis of TRPC6, which is quite difficult in human tissue. Therefore, future studies could focus on functional aspects and, for instance, investigate the colocalization of TRPC6 and TRPC3 as well as compare their distribution in kidney samples from non-kidney diseased cadavers with those from nephrectomy or biopsies with known kidney diseases in order to further elucidate the role of both channels in human kidney disease.

## 5. Conclusions

Glomeruli, tubules—proximal, intermediate, and distal—and collecting ducts undoubtedly express TRPC6 (Figure 3). Our morphological approach in this study did not largely challenge the findings on TRPC6 properties obtained in nonhuman models and raised some questions that should be addressed in future studies.



**Figure 3.** Nephron design showing the distribution of TRPC6 in the tubular system. From top to bottom: convoluted distal tubule, proximal tubule, thick ascending limb, peritubular capillary bed, thin descending limb or intermediate tubule, and collecting duct.

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**Institutional Review Board Statement:** This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Saarland (141/14 and 163/20).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Not applicable.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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