SA1

## Glomerular permeability regulation by the podocyte foot processes

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Recent studies have emphasized the critical role of podocytes for the size-selective filtration barrier of the kidney and revealed novel aspects of the mechanisms leading to proteinuria, both in inherited and acquired diseases. It has been shown that a specialized cell junction, the slit diaphragm, connecting neighboring podocytes, is of critical importance to the development and the integrity of the glomerular filter. Several critical structural protein components of the slit diaphragm have been identified. In addition to their structural functions, these proteins participate in common signaling pathways. This talk will focus on what is known about the importance of the podocyte for the function of the glomerular filter of the kidney. It will provide a snapshot of our current understanding of the signaling properties of slit diaphragm proteins and project a framework for further studies necessary to delineate the function and dynamics of the slit diaphragm protein complex and the pathogenesis of nephrotic syndrome.

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SA2

## How cation entry into renal glomerular epithelial cells can regulate podocyte function

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Focal segmental glomerulosclerosis (FSGS) has been previously characterized as having primary (idiopathic), secondary and familial forms. In the latter category, both autosomal recessive and dominant inheritance patterns have been reported. Reports of familial forms of FSGS date back as far as 1956, with the observation of an autosomal recessive disease primarily within the Finnish population. The disease process is characterized by massive proteinuria *in utero*, with up to 20 to 30 grams of protein loss per day. *NPHS1* encodes a gene product termed 'nephrin', within which numerous mutations including deletions, insertions, nonsense, missense and splicing errors have been described. Nephrin localizes to lipid 'rafts' within the slit diaphragm of the podocyte. Steroid-resistant nephrotic syndrome (SRNS) is another human disorder that is characterized by autosomal recessive nephrotic syndrome. This disorder manifests between 3 months and 5 years of age, rapid progression to ESRD, and with few cases of recurrence after renal transplantation. The gene product is podocin (*NPHS2*), located on 1q25-31. Podocin most likely functions in the structural organization of the slit diaphragm and regulation of its filtration function. It has been shown to interact *in vivo* with both nephrin and CD2-associated protein (*CD2AP*), a cytoplasmic binding partner of nephrin. Mutations in the alpha-actinin 4 gene (*ACTN4*), which localizes to chromosome 19q13, have been associated with autosomal dominant FSGS, characterized by adult onset disease of variable severity and rate of progression to ESRD. Fractions of the mutant protein have been shown to form large aggregates within podocytes ultimately compromising the function of the normal actin cytoskeleton, both through its abnormal function and toxic accumulation.

Recently, a disease-causing mutation for hereditary FSGS has been localized to chromosome 11q 21-22, with the subsequent identification of transient receptor potential cation channel, subfamily C, member 6 (*TRPC6*) as the disease causing gene. The missense mutation causes a highly conserved proline in the first ankyrin repeat of *TRPC6* to become a glutamine at position 112 (P112Q). Additional work as reported by Reiser et al. has corroborated findings implicating *TRPC6* in the pathogenesis of familial FSGS. The TRPC6$^{P112Q}$ mutation is highly conserved and causes increased and prolonged calcium transients in transfected cells. The mutant channel also significantly enhances cation signals triggered by AT1 receptor activation. Biotinylation and immunostaining studies reveal that the mutation also appears to cause mislocalization of the ion channel to the cell surface. *TRPC6*–related FSGS suggests an additional mechanism for renal disease pathogenesis. Knowledge of *TRPC6*-mediated calcium entry into cells may offer unique insights into therapeutic options for glomerular diseases. Calcium as a second messenger affects many cellular functions such as contraction, apoptosis, vasoregulation and mechanosensation to name a few. We suggest that the exaggerated calcium signalling conferred by the *TRPC6*$^{P112Q}$ mutation disrupts glomerular cell function or may cause apoptosis. We further speculate that the mutant protein may amplify injurious signals triggered by ligands such as angiotensin II that promote kidney injury and proteinuria. Clinical manifestations of renal disease do not appear until the 3rd decade in individuals with the *TRPC6*$^{P112Q}$ mutation. This is in contrast to individuals with Finnish nephropathy and steroid-resistant nephrotic syndrome who typically develop proteinuria *in utero* or at birth. This delay may reflect the difference between these recessive disorders and the autosomal dominant mechanism of inheritance in our family, as such the presence of one normal *TRPC6* allele may postpone the onset of kidney injury. Patients with autosomal dominant FSGS due to mutations in the *ACTN4* gene also have a delayed onset of kidney disease. Because channels tend to be amenable to pharmacological manipulation, our study raises the possibility that *TRPC6* may be a useful therapeutic target in chronic kidney disease.

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SA3

## Regulation of podocyte function by intracellular signalling

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In diseases resulting in proteinuria, the delicate architecture of the podocyte is remodelled in response to as yet unknown signals. Crucial to understanding this has been the discovery of podocyte molecules that are mutated in hereditary proteinuric diseases, and which are functionally linked to the actin cytoskeleton. These molecules include nephrin, podocin and TRPC6,

which are localised to the podocyte slit diaphragm. It is becoming increasingly clear that the slit diaphragm is a signalling complex, crucial to morphological integrity, though regulation of this process is little understood.

A number of observations suggest that the podocyte response to external ligands is unique. For example TRPC6 is a widely expressed cation channel, yet activating mutations only affect the glomerulus(Winn et al. 2005). In clinical practice, nephrotic syndromes such as FSGS are thought to be caused by circulating plasma 'factors' that are directly toxic to the podocyte. We have shown that the circulating plasma protease, hemopexin, causes dramatic remodelling of the podocyte actin cytoskeleton, but has no effect on glomerular endothelial cells or fibroblasts.

The slit diaphragm podocyte protein nephrin is restricted mainly to the podocyte, and recent data show that nephrin specifically signals to the actin cytoskeleton via the adaptor protein Nck(Jones et al. 2006). Our own studies now show that human plasma affects the podocyte in a unique way, affecting signalling to the actin cytoskeleton, subcellular localisation of nephrin and TRPC6 activation(Coward et al. 2005). These effects are dependent on the presence of nephrin, and we have evidence of a unique, nephrin-dependent, functionality of TRPC6 which switches this molecule between a plasma membrane ion channel and an intracellular calcium release channel. Additionally, the hemopexin effect is also dependent on nephrin.

Therefore we consider that nephrin is a central player in slit diaphragm signalling, and confers specificity to the way the podocyte responds to external ligands in health and disease.

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SA4

## Imaging ion fluxes in renal cortex *in vivo* using two-photon microscopy

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Multi-photon excitation fluorescence microscopy is a state-of-the-art confocal imaging technique ideal for deep optical sectioning of living tissues. It is capable of performing ultra-sensitive, quantitative imaging of organ functions in health and disease with high spatial and temporal resolution that other imaging modalities cannot achieve. Since the low cytotoxicity of multi-photon excitation allows continuous imaging of living tissues, real-time imaging of the tubuloglomerular feedback (TGF) and renin release mechanisms became possible. Novel, TGF-associated morphological findings include significant cell volume changes of the macula densa under isotonic or hypertonic conditions, the existence of bulk fluid flow in the JGA, a sphincter-like contraction of the terminal, intraglomerular afferent arteriole, and the TGF-associated contraction of not only the afferent arteriole, but the entire intraglomerular mesangium. Spreading of the TGF vasoconstrictor signal in the JGA and beyond involves an extracellular ATP-mediated purinergic calcium wave. This wave was directly visualized with confocal microscopy to propagate from the macula densa and extraglomerular mesangial area to the afferent arteriole, along the vasculature to adjacent glomeruli, and also to all cells of the glomerulus including the most distant podocytes. Propagation of the TGF calcium wave from afferent arteriole smooth muscle cells to the underlying endothelium was also observed in these studies. This phenomenon may provide negative feedback and helps to balance the TGF vasoconstriction by triggering endothelium-derived vasodilator mechanisms. These imaging studies further emphasized the roles of both gap junctional communication and extracellular ATP as integral components of TGF. In addition, these studies provided functional evidence that complementing the afferent arteriolar vasoconstriction, all cells of the glomerulus actively participate in TGF by contracting the glomerular tuft, thereby helping to reduce the rate of glomerular filtration. The unexpected finding that the calcium wave of TGF was mediated by extracellular ATP provided further support that ATP itself is directly involved in TGF and not only through its breakdown to adenosine.

Renin release is the first, and at least initially, the rate-limiting step in the activation of the renin-angiotensin system which helps to maintain body salt and water balance. Additional details of the renin release mechanism were also observed using the multi-photon imaging approach. Acidotropic fluorophores including quinacrine and LysoTracker dyes (Invitrogen) are highly membrane permeant weakly basic compounds that rapidly accumulate in acidic cellular organelles. They have been successfully used to label renin granular content both in vitro and in vivo, and even as a counter stain on histological sections. Imaging the entire granular content as opposed to labeling specific molecules of interest (renin itself) is of great advantage when studying the mechanism and regulation of renin granule exocytosis. For example, there is a renewed interest in the enzymatically inactive prorenin, which is part of the granular contents and therefore its release may also be visualized even though it cannot be detected by existing assays measuring renin activity. Renin exocytosis has been visualized in real-time and on the individual renin granule level in response to a number of physiological stimuli including beta-adrenergic activation, low perfusion pressure and the macula densa mechanism. Dimming and disappearance of the entire granular content (quantal release) was observed within 2-300 ms. A significant number of renin granules was released into the interstitial side of the JGA, in addition to the vascular lumen. Not only the degranulation process, but enzymatic activity of the released renin (angiotensin I generation) was visualized in real-time using a FRET-based renin substrate.

In vivo visualization of cellular variables like cytosolic calcium in the collecting duct, intracellular pH in the proximal tubule, cell-to-cell communication and signal propagation will be shown. Basic kidney functions that can be measured by in vivo quantitative multi-photon imaging include glomerular filtration and permeability, concentration, dilution, and activity of the intra-renal renin-angiotensin system.

New visual data challenge a number of existing paradigms in renal (patho)physiology. Also, quantitative imaging of kidney function with multi-photon microscopy has tremendous potential to eventually provide novel non-invasive diagnostic and therapeutic tools for future applications in clinical nephrology.

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SA5

## A molecular toolbox for studying glomerular function

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The glomerular filtration barrier separates blood from the urinary space. It is composed of 2 cell types: an outermost sheet of podocytes separated from the fenestrated endothelial cells by an intervening glomerular basement membrane. Crosstalk between intrinsic cell compartments of the glomerulus is essential for glomerular development and maintenance. Recent advances in mouse genetics make it possible to dissect the pathomechanisms of glomerular disease and function in vivo. Cre-loxP conditional gene targeting, siRNA in vivo and inducible gene targeting systems are all options readily available to the renal researcher. The development of numerous Cre-driver transgenic lines for the majority of cell lineages within the glomerulus allows precise control over genetic manipulation in specific cell types. An overview of available techiniques, together with a discussion of advantages and caveats will be provided. A number of examples of successful mouse models generated by conditional gene targeting within the glomerulus will be presented and include: the role of angiogenic signaling systems such as VEGF and angiopoietins, effects of stabilization of hypoxia inducible factors and the effect of genetic inhibition of mTor (the mammalian target of rapamycin).

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SA6

## Basolateral chloride channels in the mouse distal nephron: characterization and regulation

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Our group has investigated Cl- channels in microdissected fragments of the mouse renal tubule using the patch-clamp technique in several studies. The purpose was to elucidate elements of regulation and to establish the correspondance with ClC cloned channels. This has been achieved for one channel. Indeed, there is general agreement that the two ClC-Ks belonging to the ClC family of Cl- channels and transporters mediate the basolateral step of Cl- absorption in the thick ascending limb (TAL) and distal convoluted tubule (DCT). Mutations of the genes encoding ClC-Kb and regulatory Barttin subunit are responsible for Bartter syndrome (targeting TAL) or mixed Bartter-Gitelman syndrome (targeting TAL and DCT). In addition, ClC-K2 (the mouse equivalent for ClC-KB) is present in these segments and also in the intercalated cells of the collecting duct. We based our patch-clamp investigation on two properties of the ClC-Ks: anion permeabilities follow the sequence Cl- > Br- > NO3- > I- for ClC-K1, and that of Cl- > Br- = NO3- > I- for ClC-K2; the currents are enhanced by a basic external pH and by an elevated concentration of external calcium [1].

Forming patches on the basolateral membranes of the early DCT, we detected a Cl- channel of ~9 pS that exhibits comparable properties. Firstly, NPo increased 3-fold when the calcium in the pipette was increased from zero to 5 mM, and 15-fold when the pH was increased from 6.4 to 8.0. Secondly, setting aside I-, the permeability sequence Cl- > Br- ~ NO3- > F- observed for this channel matches that for ClC-K2 [2]. Single-cell RT-PCR experiments showed that CLC-K2 mRNA was largely predominant in the early DCT. Thus, we proposed that this channel corresponds to ClC-K2 [3]. We then were able to record a channel with similar conductive and regulatory properties in the late DCT, connecting tubule and cortical collecting duct. In the two last segments, the channel was never found together with potassium channels, suggesting that its presence in the intercalated cells only [3].

We have investigated several regulatory properties. Channel activity is gradually inhibited in the presence of PMA, an activator of PKC, but is not dependent on the adenylyl cyclase pathway: neither forskolin in cell-attached patches, nor the catalytic subunit of PKA in excised patches alter the NPo or channel frequency. Thus, this specific channel is not responsible for the cyclic-AMP dependent basolateral Cl- conductance in the medullary TAL [5]. In addition, we found that the channel is dependent upon voltage, being more active at positive than at negative voltages, and highly sensitive to the intracellular pH. We have now investigated the interrelationships between voltage and pH sensitivities, which are complex, and we have compared them to those described for ClC-0 and ClC-1. Our results suggest that the pH is a major regulator of the candidate ClC-K2 channel.

Table 1. Conductance and anionic selectivity of chloride channels

| | G (pS) | PBr | PNO3 | PI | pHi sensitivity |
|---|---|---|---|---|---|
| early DCT | 9.5 | 0.46 | 0.50 | 0.9 | + |
| late DCT | 8.8 | 0.41 | 0.48 | 0.62 | + |
| CNT | 10.6 | 0.44 | 0.56 | 0.86 | + |
| CCD | 9.8 | 0.60 | 0.54 | N.D. | + |
| CTAL | 9 | 0.62 | 0.64 | N.D. | + |

The table gives the unit conductance and permeabilities (relative to chloride) of the chloride channels detected in various segments of the mouse nephron. Data from refs 2,3,4 and unpublished results (CTAL)

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SA7

## Acid- and volume-sensitive potassium channels in renal function

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A variety of acid- and volume-sensitive $K^+$ channels have been described which are implicated in important cellular functions. In the kidney, many studies have explored the role of pH-regulated KCNJ1 (ROMK) channels in the straight and convoluted distal tubule and of volume-, pH-, and ATP-regulated $K^+$ channels in the basolateral membrane of the proximal tubule. Here, we have focused on two pH- and volume-regulated $K^+$ channels of the 2P domain channel family, TASK2 (KCNK5) and TASK1 (KCNK3). TASK2 is strongly expressed in proximal tubules and papillary collecting ducts. Patch-clamp experiments on proximal tubular cells indicated that the TASK2-specific $K^+$ current is activated during bicarbonate transport. In *in vivo* experiments, TASK2-/- mice displayed mild metabolic acidosis which was caused by an increased renal bicarbonate excretion. Activation of TASK2 by transport-induced cell swelling and basolateral export of bicarbonate appears to be an important mechanism to adapt membrane potential and osmolyte export to the needs (1,2).
Aldosterone regulates ion transport in the distal nephron which is critical for water/salt balance and the control of arterial blood pressure. TASK1 is probably the most abundant $K^+$ channel in human adrenal glands. This channel has been proposed to contribute to the background conductance whose inhibition by angiotensin II stimulates aldosterone secretion. We investigated the contribution of TASK1 for this $K^+$ conductance using a TASK1-/- mouse as a tool. Female TASK1-/- mice exhibited severe hyperaldosteronism independent of salt intake, hypokalemia, and arterial 'low renin' hypertension. The aldosterone phenotype was accompanied by a severe adrenocortical zonation defect. Aldosterone synthase was totally absent in the zona glomerulosa but abundant in the deeper zona fasciculata. Also young male TASK1-/- mice displayed a zonation defect. In contrast to females, at adulthood male TASK1-/- mice had acquired normal zonation patterns highlighting the dynamics of the process of adrenocortical zonation. Interestingly, the hyperaldosteronism of female TASK1-/- mice was fully remediable by glucocorticoids indicating that in those mice aldosterone secretion is under the control of ACTH. These findings are reminiscent of glucocorticoid-remediable hyperaldosteronism in humans (3).
In conclusion, proximal tubular TASK2 channels stabilize the driving force and cell volume during ongoing Na+ and bicarbonate transport. We propose TASK2 as a novel candidate to underlie the clinical manifestations seen in proximal renal tubular acidosis syndrome. The acid-sensitive TASK1 channel is a pivotal factor for normal adrenocortical zonation, aldosterone secretion and, thereby, regulation of renal function. The phenotype of TASK1-/- mice underlines the potential of $K^+$ channels to influence cell differentiation and development.

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SA8

## Renal Na/Pi-cotransporter NaPi-IIa: a physiological and molecular overview

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Homeostatic balance of inorganic phosphate (Pi) has wide physiological implications. Plasma levels of Pi are kept constant by adjusting renal reabsorption/excretion to intestinal absorption. Both processes are mediated by members of the SLC34 gene family. NaPi-IIa/SLC34A1 and NaPi-IIc/SLC34A3 are responsible for renal reabsorption whereas intestinal absorption is mediated by NaPi-IIb/SLC34A2. NaPi-IIa and NaPi-IIc are expressed in the brush border membranes (BBM) of renal proximal tubules (PT) (1, 2). In the adult murine kidney, NaPi-IIa reclaims up to 80% of filtered Pi, with the 20% left attributed to NaPi-IIc (3). NaPi-IIa expression is reduced in animal models for X-linked hypophosphatemia (XLH), and in vitro studies indicate that similar defect could be involved in other phosphate wasting syndromes. However, such reductions on NaPi-IIa are probably secondary to defects on other factors collectively known as phosphatonins (FGF23, PHEX, FRP-4, and MEPE) (3). Recently, mutations in NaPi-IIc were linked to hereditary hypophosphatemic rickets with hypercalciuria. These findings indicate that

both cotransporters are critical for Pi homeostasis. Accordingly, the abundance of NaPi-IIa and NaPi-IIc in the proximal BBM is under strict hormonal and metabolic control.

Among the many factors that regulate renal reabsorption of Pi, parathyroid hormone (PTH), high dietary levels of Pi and FGF23 decrease the levels of NaPi-IIa and NaPi-IIc in the BBM, whereas they are upregulated in response to low dietary phosphate and 1,25 (OH)2 vitamin D (1-3). Both cotransporters are also overexpressed in mice homozygous for a mutated form of klotho (4). In particular, the phosphaturic effect of PTH has been analyzed in great detail regarding NaPi-IIa downregulation (1, 5). PTH binds to its G-protein coupled receptors located on both the apical and basolateral membrane of PT and via partially characterized steps promotes the fast endocytosis and lysosomal degradation of NaPi-IIa. Binding of PTH to apical receptors activates mostly PLC/PKC-dependent signaling whereas basolateral receptors signal preferentially via cAMP. This second messenger leads to downregulation of NaPi-IIa trough the classical PKA cascade, with not contribution of EPAC (6). In contrast to NaPi-IIa, endocytosis of NaPi-IIc upon PTH requires a prolong incubation time and degradation of the internalized cotransporter is not sensitive to leupeptine (7).

At the molecular level, regulation of the apical expression of NaPi-IIa depends on the association of the cotransporter with a complex network of interacting proteins (8). A cluster of such interacting partners is represented by the NHERF family. The four members of this family are PDZ-containing proteins expressed in renal PT. They interact via their PDZ-domains with the C-terminal PDZ-binding motif of NaPi-IIa. Truncation of this motif disturbs apical expression of the cotransporter in OK cells, a proximal tubular cell culture model. Several studies in OK cells and NHERF1-/- mice indicate a prominent role of this particular member of the NHERF family on the expression/regulation of NaPi-IIa. Thus, transfection of OK cells with dominant negative forms of NHERF1 hampered the apical expression of the endogenous cotransporter suggesting that NHERF1 is required for proper apical targeting/stabilization of NaPi-IIa. This hypothesis was confirmed in NHERF1-/- mice that are indeed characterized by high urinary excretion of Pi as consequence of reduced expression of NaPi-IIa in the proximal BBM (9). The absence of NHERF1 also interferes with PTH signaling: activation of apical receptors with 3-34 PTH (a fragment known to signal specifically trough the PKC pathway) failed to induce endocytosis of NaPi-IIa in NHERF1-/- mice. Since NHERF1 can bind simultaneously to PTH receptors and to PLC, the failing of 3-34 to exert an effect of NaPi-IIa in NHERF1-/- mice suggests a defective coupling of apical PTH receptors to PLC. Accordingly, the responsiveness to 3-34 PTH in NHERF1-/- mice was shown to be due to lack of activation of phospholipase C (PLC) in these animals. Unlike NaPi-IIa, the pattern of expression of NHERF1 remains unaffected upon PTH treatment, suggesting that the association between both partners is negatively regulated by PTH. Indeed, PTH reduced the amount of NaPi-IIa that coimmunoprecipitates with NHERF1 antibodies (10). In addition, PTH induced an increase in phosphorylation of NHERF1. In summary, NaPi-IIa is the major regulator of renal Pi handling. Therefore, its expression in the apical membrane is under tight control. The molecular mechanisms responsible for such control are under current investigation.

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SA9

## Anion exchangers in renal health and disease

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A variety of anion exchangers perform important vectorial transport function in the polarized epithelia that constitute the renal tubule. Normal anion transport function is important for acid-base balance, urinary calcium solubility and prevention of nephrolithisasis. This talk will focus on those transporters where malfunction or mistargeting are associated with a renal phenotype.

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