Shear stress regulated signaling in renal epithelial cells and polycystic kidney disease

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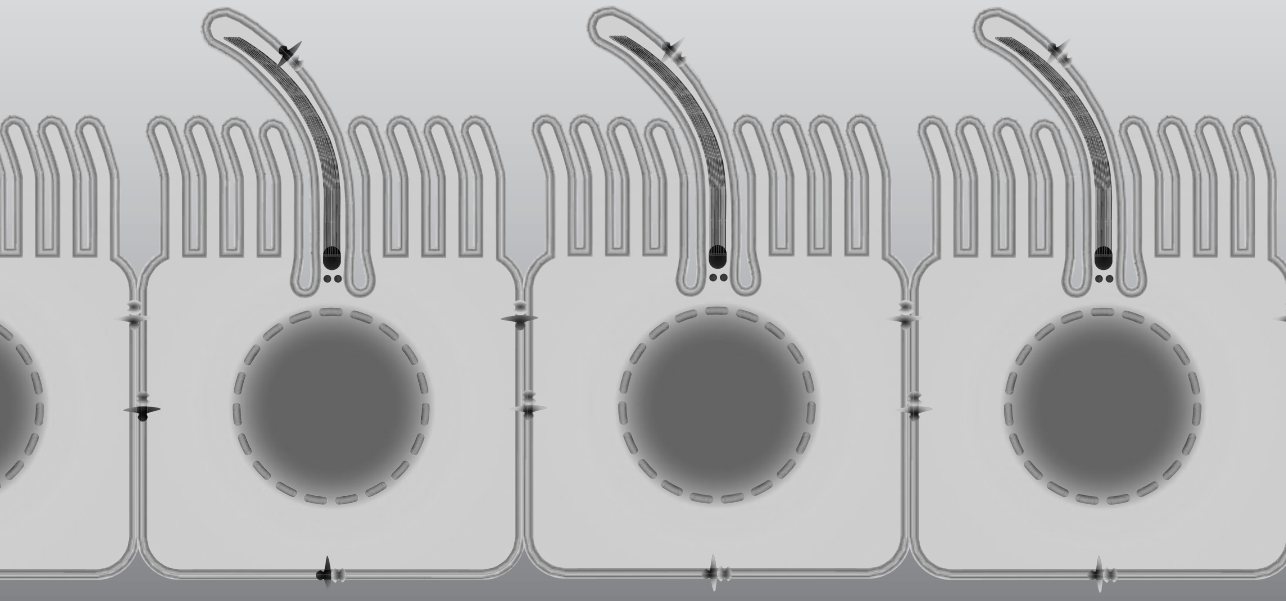
CHAPTER 1

Introduction



SHEAR STRESS AND MECHANOTRANSDUCTION

Mechanotransduction is the cellular biochemical response to mechanical or physical forces, like pressure, fluid shear stress, drag force/torque, mechanical load and circumferential stretch[1]. Not only is this mechanism important for hearing, touch experience and balance in animals, but cellular mechanotransduction plays a crucial role in embryonic and tissue development, as well as cell viability, cellular function and maintenance of organs[2-6]. A wide variety of cells types are exposed to different mechanical forces, which are detected by specialized mechano-sensors, thereby regulating the cellular response. The most well-known mechanical force is fluid shear stress on endothelial cells by blood flow[7,8]. The first publications of mechanical shear stress responses in endothelial cells were from the early 1980s and fluid flow exposure studies in osteoblasts followed roughly 10 years later[9,10]. Research on fluid flow in regulating ion, nutrients and water reabsorption in kidney epithelial cells was initially started in the 1960s using microperfusion techniques on renal proximal tubules[11-16]. However, it took until the beginning of this century before mechanotransduction in renal epithelial cells was receiving more attention. These studies show intracellular Ca^{2+} increase and morphology changes in renal epithelial cells upon shear exposure[17-19].

Several organs are subject to variations in fluid flow rate in response to physiological stimuli, which could be detected by mechano-sensing proteins or complexes. In kidneys, renal epithelial cells are exposed to mechanical forces due to fluid flow within the lumen of the nephron tubules (Figure 1). Urinary volume, diet and diuretics will expose the renal epithelial cells to variations in hydrodynamic forces including fluid shear stress, circumferential stretch, and drag force[20]. Several papers describe a relatively steady flow velocity and shear stress in renal epithelial cells in a physiological range between 0.05 - 1 dyn/cm^2, where proximal tubular epithelial cells (PTECs) experience the highest range of shear stress[18,21-24]. This is far lower than the shear stress experienced by endothelial cells caused by blood flow[2]. In addition, strong oscillatory flow conditions, which are seen in the vasculature, are not expected in nephrons, because the oscillations caused by the heartbeat are almost diminished in capillaries, like the glomerulus. Furthermore, renal auto-regulation and in particular tubuloglomerular feedback (TGF) are mechanisms to regulate renal blood flow and glomerular filtration rate (GFR) during changes in renal blood pressure, thereby keeping GFR stable and oscillations small[25,26]. Nevertheless, it is well known that high blood pressure or diabetes can cause renal hyperfiltration, resulting in a higher GFR, thereby increasing the shear stress in nephron segments. In addition, strong variations in hydrodynamic forces and shear stress are common in various kidney diseases due to tubular dilation, inflammation and obstruction, resulting in hyperfiltration in functional nephrons to compensate for lost glomeruli and tubules[27]. Depending on the cell type and the magnitude of the hydrodynamic forces, different responses will be activated and mutations in critical components may cause or accelerate kidney diseases[28].

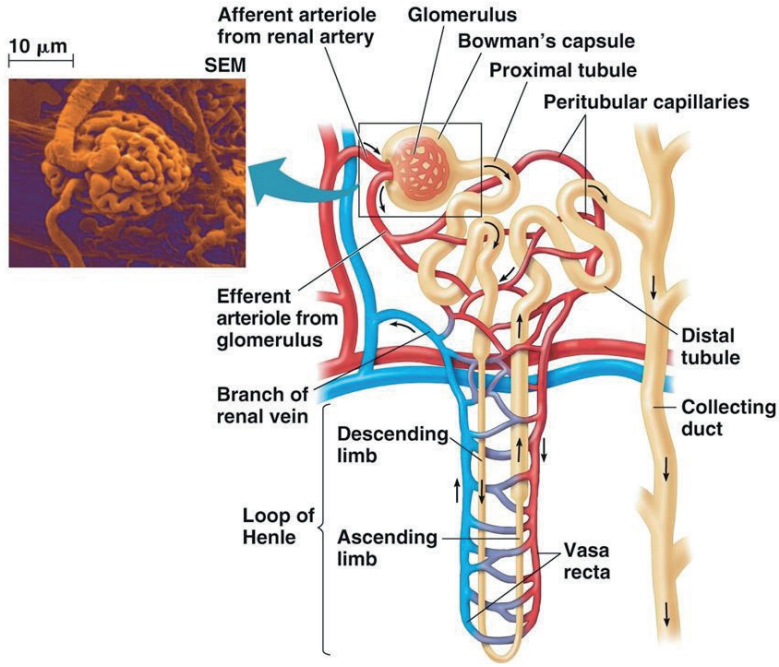


Figure 1. Structure of the nephron.

Blood enters the glomerulus from the afferent arteriole. A scanning electron microscope picture shows the glomerular structure at the top left. In the glomerulus, water, salt, nutrients and waste is filtered from the blood and is collected in the Bowman's capsule. From there the filtrate flows through the renal tubules, starting at the proximal tubule, where water, nutrient and salt molecules are reabsorbed and secreted to the efferent arterioles. Additional waste substances are excreted in the filtrate, including urea, creatinine, uric acid, potassium and hydrogen. The loop of Henle has the function to create a concentration gradient in the kidney medulla to reabsorb water from the filtrate and concentrate the filtrate. In the collecting duct the filtrate of several nephrons is collected. Image from Campbell *et al*.[29]

MECHANO-SENSING COMPLEXES IN THE KIDNEY

Fundamental in flow-sensing are a variety of proteins, called mechano-sensors, which are located throughout the cell membrane, primary cilium/ciliary base and the cytoskeleton. These include ion channels, G-protein coupled receptors (GPCRs), adherens junction proteins, focal adhesion proteins, components of the actin cytoskeleton, but also the glycocalyx and lipid rafts can act as mechano-sensing complexes to shear stress[30-32]. Activation of aforementioned sensors upon shear stress leads to alteration of cellular signaling. In the kidney, the primary cilium is the most extensively studied structure involved in flow-sensing, but it is likely that microvilli and the glycocalyx are involved as well[21,33,34]. These cellular

structures are present in proximal tubular epithelial cells (Figure 2). In contrast, collecting duct cells are devoid of microvilli and dependent on the primary cilium, glycocalyx and other flow-sensing complexes in the cell membrane or cytoskeleton[21,35].

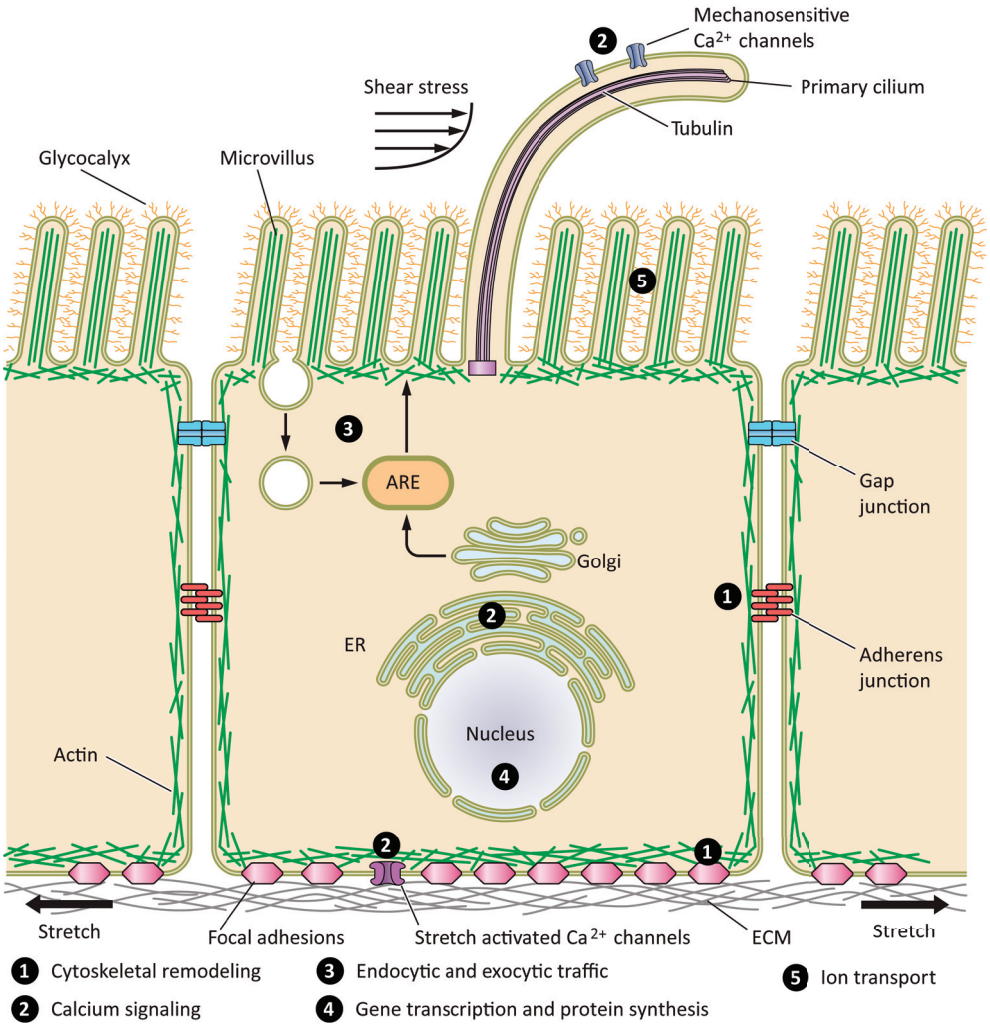


Figure 2. Mechano-sensing structures of a proximal tubular epithelial cell (PTEC).

There are 3 main structures involved in shear stress mediated mechanotransduction in PTECs: primary cilia, microvilli and the glycocalyx. Bending of cilia or microvilli by shear stress may activate mechano-sensing proteins, thereby modifying several cellular processes, including activation of Ca^{2+} and other ion transporters, cytoskeletal remodeling, endocytosis, and gene transcription. The glycocalyx may increase the frictional force of the fluid to amplify the bending of microvilli. Mechanical stretch can cause stimulation of stretch activated calcium channels and cytoskeletal reorganization, resulting in modulation of gene and protein expression. Image from Raghavan *et al.*[34]

Primary cilia

The primary cilium is a hair-like structure that protrudes from the cell membrane of almost every cell in the body[6]. It is reabsorbed during cell division and re-assembled when the cell exits mitosis. Immotile primary cilia can act as chemical sensor or bend under fluid flow, which mediates a cellular response and are therefore called sensory cilia. In contrast, motile cilia contain motor proteins to generate cilia movement and thereby creating a fluid current, which is crucial during embryonic development for left-right asymmetry[36]. The central axoneme of primary cilia is assembled from 9 + 0 microtubule doublets by anterograde intraflagellar transport (Figure 3). Motile cilia have a 9 + 2 axoneme, with an extra central pair of microtubules[37]. Several proteins are involved in ciliary trafficking, including dynein, kinesin, Bardet-Biedl syndrome (BBS) proteins and intraflagellar transport (IFT) proteins. Mutations in any of the proteins can impair cilia formation and function. This can have profound effects on the development of body pattern and the physiology of multiple organ systems, which is the cause of a wide variety of human diseases, called ciliopathies[5,38,39].

The primary cilium plays an essential role as mechano-sensing complex upon fluid shear, which regulates cellular signaling and homeostasis. Fluid shear regulated signaling by cilia will be discussed in more detail in later paragraphs. In addition, primary cilia act as a signaling platform for growth factor signaling. Ligands in the lumen of nephron tubules can bind to their receptors, inducing cellular responses through downstream signaling pathways, for instance affecting the Wnt, hedgehog (Hh), epidermal growth factor receptor (EGFR) and transforming growth factor β (TGF-β) pathways[37,38]. Although not exclusively, receptors involved in these pathways have been identified in the cilium of several cell types, including renal epithelial cells, suggesting that different signaling cascades are being regulated by this organelle[37,38,40-42]. For example, TGF-β signaling is mediated via clathrin-dependent endocytosis at the ciliary pocket[42]. The ciliary pocket is membrane domain found at the base of primary cilia that may act as platform involved in vesicle trafficking and signal transduction[43]. The aforementioned data indicate that primary cilia are essential signaling platforms that can sense and organize different environmental cues, and transmit the signals to the cell interior. Gene expression, protein activation and overall cellular physiology will be an integration of the different signals, triggered by fluid shear stress and by growth factor stimulation.

Microvilli

Proximal tubular epithelial cells have numerous (up to a few thousand) microvilli at the apical surface of the cell. Microvilli are actin filament-based protrusions of the cell membrane that increase the cell surface area and frictional force of fluid, thereby functioning in absorption, secretion, cell adhesion and mechanotransduction[44]. It has been suggested that brush border microvilli in PTECs are important in mechano-sensing, since drag forces on microvilli

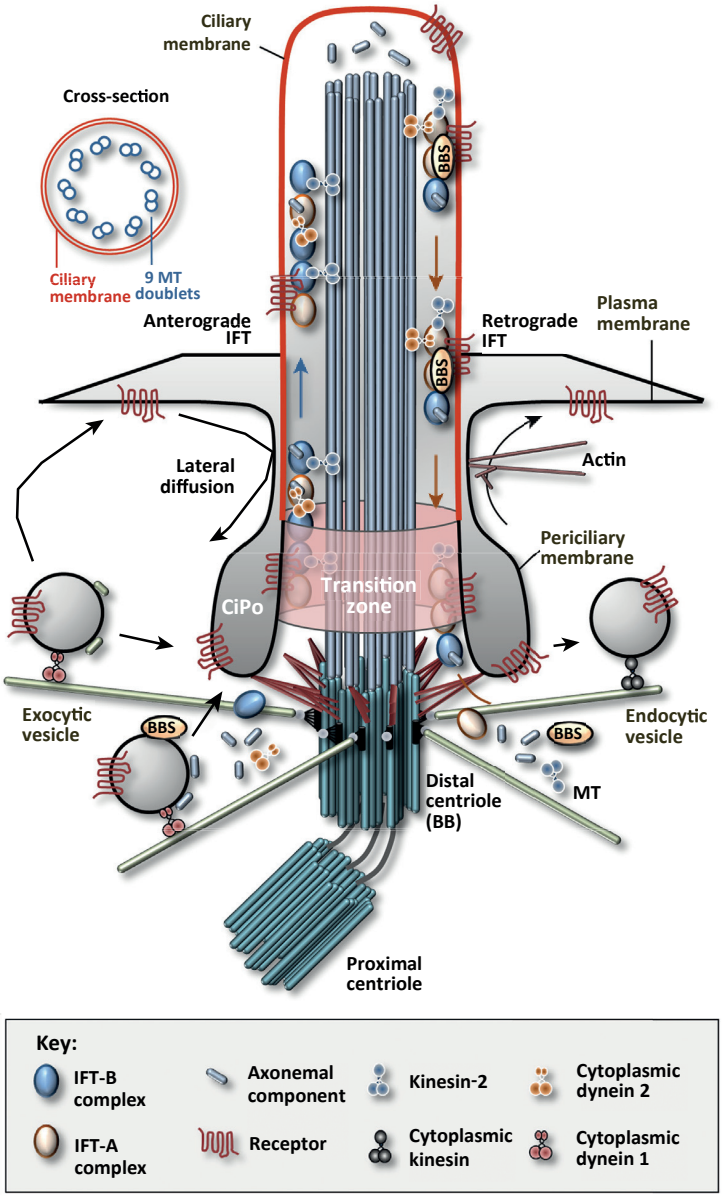


Figure 3. Primary cilium structure and assembly.

Illustration of the primary cilium structure, showing the basal body (BB), the transition zone, the ciliary pocket (CiPo) and the 9 + 0 microtubule (MT) doublets forming the axoneme. The axoneme is assembled by anterograde intraflagellar transport (IFT), executed by several IFT complexes, kinesin, and dynein. These protein complexes also play a role in transport of transmembrane receptor proteins, thereby facilitating growth factor signaling. Abbreviations: BB = basal body; BBS = the BBSome (complex of eight Bardet-Biedl syndrome proteins); CiPo = ciliary pocket; IFT = intraflagellar transport; MT = microtubules. Image from Pedersen *et al*.[37]

are much greater than on the apical membrane of PTECs[21,45]. Studies show that sodium transport and water reabsorption is increased upon shear stress mediated bending of microvilli in PTECs[46,47]. There is only one known inherited disorder affecting apical microvilli assembly, called microvillus inclusion disease (MVID). It is caused by mutations in the *MYO5B* gene, which is involved in membrane trafficking of apical and basolateral proteins causing impaired microvillus assembly[48]. Patients suffer from diarrhea and dehydration, likely caused by lack of water reabsorption in the intestine.

Glycocalyx

The glycocalyx is a layer of glycoproteins, glycolipids and proteoglycans at the surface of the cell and on the outside of microvilli in PTECs. It is involved in binding substances needed for uptake or as protection against harmful substances. In the kidney, the glycocalyx can act as barrier during glomerular filtration, preventing large proteins, like albumin, to pass the glomerular barrier[49]. In several vascular diseases, including atherosclerosis, the glycocalyx is degraded by digesting enzymes, as well as in patients with diabetes and kidney diseases[49,50]. The glycocalyx is known to play a fundamental role in mechanotransduction in endothelial cells due to its characteristic to increase the frictional force of fluid[51,52]. The glycocalyx is connected to the actin cytoskeleton inside the vascular endothelial cell. It bends upon blood flow exposure and transduces this force to the cytoskeleton to activate mechano-sensors and control endothelial cell function[34]. Shear induced nitric oxide (NO) production is dependent on heparin sulfate or hyaluronan groups in the endothelial glycocalyx[53]. However, it has barely been studied in renal epithelial cells as shear stress sensor. One study indicates that treatment of PTECs with the glycocalyx-digesting enzyme heparinase III did not modulate shear stress induced formation of adherens and tight-junction, while microvilli disruption did[54]. Nevertheless, it is likely that the glycocalyx can amplify the frictional force on the cell membrane of renal epithelial cells, as well as the bending moment of microvilli in PTECs, which may be important for other shear stress responses in the kidney.

SHEAR STRESS REGULATED SIGNALING IN THE KIDNEY

Renal epithelial cells are constantly exposed to fluid shear, which is needed to maintain cellular function and homeostasis. One of the main functions of renal epithelial cells is regulation of ion, nutrient and water reabsorption, which is regulated by fluid shear[11-16,46,47.] Although there are fluctuations in glomerular filtration rate and shear stress, proximal tubular epithelial cells are still capable to reabsorb 65-80% of water, ions and nutrients from the nephron lumen, which is needed to maintain the glomerulotubular balance[55]. The role of mechano-sensation in this process was demonstrated in a study showing increased Na+ and HCO_3^- reabsorption by shear stress in proximal tubular epithelial cells[56]. In addition, several

other signaling pathways and processes are modulated by shear stress in renal epithelial cells, including mTOR, STAT6/p100, Wnt, MAPK and TGF-β signaling, as well as endocytosis, cytoskeletal reorganization and Ca^{2+} influx[17,23,24,54,57-70].

Calcium and cAMP signaling

One of the first responses of renal epithelial cells to the onset of fluid flow is increased intracellular Ca^{2+} levels, which modulates several signaling cascades, including cyclic AMP (cAMP) signaling[17,66,71]. In addition, literature suggests that the ciliary polycystin1-2 complex, which is mutated in polycystic kidney disease (PKD), mediates fluid flow induced Ca^{2+} influx in kidney cells, followed by release of ryanodine receptors sensitive Ca^{2+} stores and subsequent cytosolic Ca^{2+} increase[67]. In models for PKD, inactivation of the polycystin complex causes lower intracellular Ca^{2+} levels, resulting in increased cAMP signaling, which induces MAPK/ERK mediated cell proliferation and trans-epithelial fluid secretion[71-73]. More recent studies showed that ciliary Ca^{2+} influx is regulated by homologous polycystin-like complexes (*Pkd1l2* and *Pkd2l1)*, whereas the polycystin1-2 complex was not directly involved[68,69]. However, the same researchers showed that fluid flow induced Ca^{2+} influx originates from the cell body after 10-20 sec of flow stimulation, which initiates a Ca^{2+} wave in the cytoplasm and propagates later into the primary cilium[70]. They concluded that ciliary Ca^{2+} influx was not directly mediated by mechano-sensation of cilia. Another group showed that trans-epithelial Ca^{2+} transport is increased by fluid shear in ciliated distal convoluted and connecting tubule cells via the apical TRPV5 channel[74]. They suggest that is fluid shear induced Ca^{2+} transport is mediated via increased TRPV5 and NCX1 channel expression, which is decreased upon cilia removal. However, other mechano-sensing complexes may be involved that are independent of primary cilia, since Ca^{2+} transport was lower but still present after cilia ablation. Despite numerous hypotheses about fluid shear mediated Ca^{2+} signaling and the relevance for development and disease, the mechanism how fluid shear stress regulates Ca^{2+} signaling is not entirely clear and still under debate, whereas the direct involvement of cilia and the polycystins is being criticized[33,68-70,75,76].

mTOR signaling

Renal epithelial cell-size is regulated by mTOR (Mechanistic Target Of Rapamycin) signaling, which is altered by primary cilia dependent shear stress sensing[77,78]. Several upstream signals and cellular stress factors can alter mTOR signaling, including growth factors, hypoxia, osmotic stress, energy and nutrient deprivation[79]. The mTOR complexes can modify several processes involved in transcription, translation, autophagy and cell volume control. mTOR signaling is also inhibited by fluid shear via LKB1-mediated AMPK activation[59,60,77]. These signal transducers can activate autophagy and thereby control the epithelial cell volume[80]. Defects in ciliogenesis decrease autophagy, showing the importance of cilia in the regulation of cell size[78]. Folliculin is suggested to be required for LKB1 mediated AMPK activation,

although it still remains unclear how fluid shear bending of the cilium activates Folliculin or downstream LKB1 and AMPK[60].

TGF-β signaling

The Transforming Growth Factor-β (TGF-β) superfamily proteins are multifunctional cytokines, including TGF-β's, activins and bone morphogenetic proteins (BMPs). TGF-β signaling modulates cell proliferation, differentiation, apoptosis, cell migration, cell adhesion and is believed to play a crucial role in fibrotic deposition[81], which is a hallmark of several diseases, like polycystic kidney disease[82]. TGF-β, as well as Activin and Nodal, binds to a pair of serine/threonine kinase transmembrane receptors, that mediate the phosphorylation of SMAD2 and 3 (Figure 4). These activated SMAD proteins, p-SMAD2 and 3, form a complex with SMAD4 and can enter the nucleus. Activity of SMAD transcription factors can be modulated by several co-factors, like yes-associated protein (YAP), to regulate the transcription of various genes, including plasminogen activator inhibitor type 1 (*Pai1* or *Serpine1*), fibronectin (*Fn1*), collagen type 1 alpha 1(*Col1a1*)[83,84]. Canonical (SMAD2/3) TGF-β signaling can interact with the non-canonical (MAPK/ERK) TGF-β pathways at different levels[85]. A direct link between TGF-β and MAPK/ERK signaling is the phosphorylation of ShcA by the activated TGF-β receptor complex[86]. ShcA competes with SMAD2/3 for binding to the TGF-β receptor, and stabilizes the TGF-β receptor complexes in caveolae, where it activates MAPK/ERK signaling[87]. Consequently, reduced ShcA expression results in increased levels of TGF-β receptor complexes in clathrin-coated pits, leading to enhanced SMAD2/3 activation. In addition, activated ERK1/2 can phosphorylate regulatory SMADs (R-SMAD) as well SMAD2/3 linker region, which modulate transcriptional activity of the SMAD complex[88,89] (Figure 4).

TGF-β signaling is involved in epithelial-to-mesenchymal transition (EMT), which is important during development and tissue repair, but it contributes to fibrosis and metastasis of several cancers[90]. Because of the multiple interactions between TGF-β signaling, MAPK/ERK and other cascades, the integration of these pathways is complex and biological context dependent.

In embryonic endothelial cells, shear stress mediated TGF-β/ALK5 signaling induced endothelial-to-mesenchymal transition, depending on the strength of shear and presence or absence of a cilia[91,92]. Similarly, in renal epithelial cells fluid shear stress dynamically regulated TGF-β gene expression and SMAD3 activation, depending on the magnitude of fluid shear, *i.e.* physiological versus pathological, and depending on ERK activation and NOTCH4 expression[23,24,47]. Moreover, several studies demonstrated that hypertension and pathological shear can induce TGF-β signaling and fibrosis, which is observed in a broad range of diseases, including renal diseases[93-98].

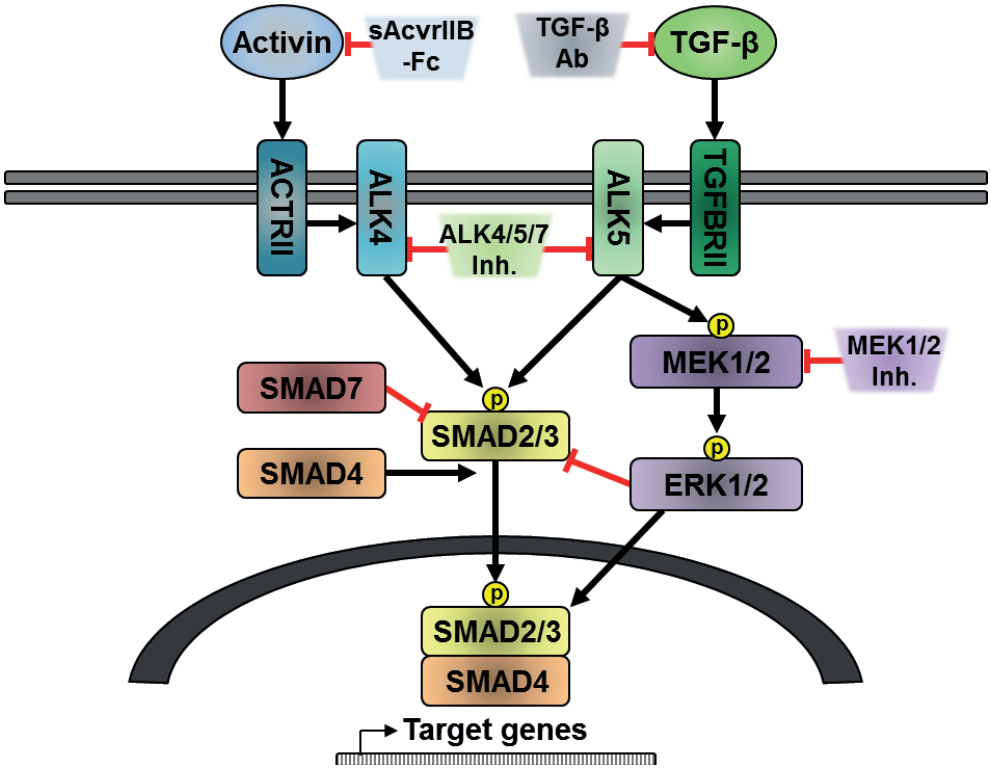


Figure 4. Representation of the TGF-β signaling pathway.

Activin or TGF-β ligands bind to their respective receptors, thereby recruiting and activating co-receptors, ALK4 (ACVRI) and ALK5 (TGFBRI), which can phosphorylate SMAD2/3 transcription factors. Activated SMAD2/3 proteins form a complex with SMAD4, which can enter the nucleus and thereby inducing target gene expression. The ALK5 receptor can also induce MEK1/2 and ERK1/2 phosphorylation via ShcA. Activated ERK1/2 can modulate transcriptional activity of the SMAD complex by cytoplasmic or nuclear SMAD retention. Several other proteins or compounds can modulate or inhibit TGF-β signaling (red lines).

Other shear regulated responses

Several studies report increased expression and reorganization of cytoskeletal components, cell adhesion and tight junction molecules under physiological shear stress, which is needed for differentiation and polarization of renal epithelial cells[18,54,99,100]. In contrast, another study showed loss of renal epithelial cell morphology during high levels of pathological shear stress (5 dyn/cm^2)[101]. Endocytosis was increased by fluid shear in proximal tubular epithelial cells as well[34,64,102]. Flow-induced endocytosis is mediated via cilium dependent Ca^{2+} increase, and subsequent calmodulin mediated activation of Cdc42[65]. Endocytosis is important for protein uptake and receptor internalization of several signaling pathways, indicating the importance of shear regulated endocytosis. Another cilia-dependent signaling cascade

affected by fluid flow is the canonical Wnt-signaling pathway, which is restrained by fluid-flow induced ciliary signaling in favor of non-canonical Wnt signaling[58]. This suggests that fluid flow act as central switch of canonical to non-canonical Wnt signaling, which may be important for normal kidney development and homeostasis. Finally, STAT6/p100-regulated transcription is negatively regulated upon flow-induced bending of the cilium, independent from flow-induced Ca^{2+} influx[61]. The numerous cellular processes and signaling pathways that are modulated by shear stress in renal epithelial cells demonstrate the importance of shear stress sensing for cellular homeostasis.

RENAL DISEASES ASSOCIATED TO SHEAR STRESS

Defects in shear stress sensing and mechanotransduction have been associated with various diseases, including diseases affecting cilia formation and function, called ciliopathies[5,38,39]. In kidneys, several physiological stimuli will expose renal epithelial cells to fluctuations in hydrodynamic forces, including fluid shear stress[20]. Depending on the cell type and the magnitude of the hydrodynamic forces, different responses will be activated and mutations in critical components may modulate, accelerate or cause (kidney) diseases[28]. In addition, strong variations in shear and other hydrodynamic forces are common in various kidney diseases due to tubular dilation, obstruction and hyperfiltration, which occur in functional nephrons to compensate for lost glomeruli and tubules, with diabetic nephropathy and Polycystic Kidney Disease as the most common examples[27]. Renal shear stress is increased after unilateral nephrectomy as well[103,104], which accelerates cyst formation in *Ift88*$^{-/-}$ and *Pkd1*$^{-/-}$ mouse models[105,106], indicating the role of shear in a ciliopathy and autosomal dominant polycystic kidney disease (ADPKD) model. Additionally, long-term high shear exposure may contribute to fibrotic deposition and tubulointerstitial lesions, which is commonly seen in renal epithelial cells upon pathological shear exposure, after renal mass reduction or during progression of renal diseases[19,47,107,108].

Ciliopathies

The ciliopathies are a wide range of genetic disorders caused by mutations in genes encoding ciliary proteins, which impair cilia formation or function[5,38,39]. Currently, there are 187 human genes associated to 35 ciliopathies, although the numbers are still rising because of the large quantity of genes involved in ciliary assembly and function[109]. Renal cyst formation is common clinical feature occurring in many ciliopathies. Autosomal dominant and autosomal recessive PKD are ciliopathies as well, since the affected proteins, polycystins (*PKD1* and *PKD2*) and fibrocystin (*PKHD1),* localize in primary cilia[110]. Other ciliopathy phenotypes include polydactyly, hepatobiliary disease, mental retardation, retinal degeneration, skeletal abnormalities and *situs inversus*.

Nephronophthisis (NPHP) patients develop corticomedullary cysts and tubulointerstitial fibrosis, which resembles the ADPKD phenotype, but in NPHP patients the kidneys are not enlarged[5]. In many NPHP patients, renal cyst formation and loss of functional nephrons leads to end stage renal disease within the first three decades. NPHP is an autosomal recessive disorder caused by mutations in more than 20 genes, *NPHP1-20* and *NPHPL1*[111]. Many of these gene mutations are classified as juvenile or adolescent NPHP, based on the age of onset, while *NPHP2* (inversin), *NPHP3*, *NPHP9* (*NEK8*) and *NPHP18* (*CEP83*), can cause infantile NPHP. The NPHP proteins interact with several cell adhesion, cytoskeletal and ciliary proteins to regulate various cellular signaling. One of the interaction partners of nephrocystin-1 (*NPHP1*) is *AHI1*, which is mutated in Joubert syndrome patients. Mutations in nineteen other cilia related genes (*BBS1-19*) have been associated with Bardet-Biedl syndrome (BBS)[112]. The BBS proteins are located at the primary cilia, basal body and the BBSome, which is a complex of several BBS proteins. BBS proteins are involved in ciliary membrane assembly and intraflagellar transport (IFT), which is crucial for cilia formation[113]. Several other proteins involved in ciliary trafficking have been implicated in other ciliopathies as well, including IFT, kinesin and dynein proteins. For example, genetic mutations of *IFT80* and *DYNC2H1* are the cause of Jeune syndrome (asphyxiating thoracic dysplasia)[114].

Oral-Facial-Digital Syndrome (OFD) is caused by mutations of the OFD1 gene, but several other causal genes are described as well[115]. The main clinical features of OFD are oral, facial and digital abnormalities, as its name already implies. In addition, polycystic kidneys are a common phenotype, as well as malformations of the central nervous system. Meckel syndrome (MKS) is a lethal autosomal recessive disorder leading to renal or respiratory failure. MKS1, 3-5 are identified as causative genes and are essential for centriole movement and ciliogenesis. Mutations in *CC2D2A* can also cause Meckel syndrome (MKS6 subtype) or Joubert syndrome, depending on type and location of the mutation or genetic modifiers[109]. This non-Mendelian type of inheritance is seen for various ciliopathy associated genes, likely caused by the multiple functions a gene/protein can have and the interactions with other proteins. The broad range of ciliopathy associated genes, as well as the disorder specific phenotypes and the overlapping clinical features between the ciliopathies, show the complexity and importance of the primary cilium function and its proposed role in mechanotransduction.

Autosomal Dominant Polycystic Kidney Disease

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disorder with a prevalence of 1:2,500 in European Union[116]. ADPKD is characterized by formation of many fluid-filled cysts and renal fibrosis, leading to deterioration or loss of renal function in adulthood[117,118]. Around 50% of the patients will develop end stage renal disease (ESRD) at the age around 55 years, requiring hemodialysis or renal replacement therapy[119]. In addition,

extra-renal manifestations are occurring as well, including cysts in the liver and pancreas, intracranial aneurisms, hypertension and cardiovascular abnormalities[120].

Germline mutations in the Polycystic Kidney Disease-1 (*PKD1)* gene are the cause of ADPKD in around 85% of the patients, while 15% of the patients carry a mutation in the *PKD2* gene[121,122]. The *PKD1* gene is located on chromosome 16 and has 46 exons, while there are several alternative splice variants described, including pathogenic splice variants[123]. *PKD2* is found on chromosome 4 and spans 15 exons. *PKD1* and *PKD2* genes encode polycystin-1 (PC-1) and polycystin-2 (PC-2). Somatic mutations in the unaffected allele of *PKD1* or *PKD2* can initiate cyst formation, called "second hit", but haploinsufficiency or stochastic fluctuations in gene expression can also lower PC-1 or PC-2 below critical levels[124-128]. Overall the probability of cyst formation is determined by functional polycystin protein levels and the biologic context[129]. For example, a number of studies indicate that renal injury can accelerate cyst progression and fibrosis[130-133]. In addition, the presence of existing cysts can trigger the formation of new cysts in the surrounding tissue[106,129].

Polycystin-1 is a large 450 kDa receptor-like trans-membrane protein consisting of 4303 amino acids, while polycystin-2 is much smaller with only 968 amino acids weighing 110 kDa[128,134]. PC-1 consists of a small intracellular C-terminal tail, eleven trans-membrane domains and a large extracellular N-terminal domain. PC-2 has six trans-membrane domains and a C-terminal calcium binding motif[135]. The PC-1 and PC-2 proteins interact via the C-terminal tails and co-localize throughout the cell membrane of renal epithelial cells, at cell-cell contacts, extracellular matrix (ECM) and primary cilia. PC-2 functions as a non-selective cation channel transporting Ca^{2+} in a complex with PC-1[67,136,137]. At the plasma membrane and in cilia, polycystins interact with diverse (mechanosensory) ion channels, signal transducers as well as cell-cell and cell-extracellular matrix junctional proteins[21,56,71,107,138,139]. Therefore, the polycystins are thought to play a role in differentiation and maintenance of the cell structure, mechanical force transmission and mechanotransduction[28,67,140]. Lack of the polycystin complex in primary cilia impairs epithelial differentiation and may play a role in cyst formation[141,142]. Moreover, mutations or deletions of other ciliary proteins can cause renal cyst formation in mouse models and patients, indicating the role of cilia during cystogenesis[39,143-146]. However, the cellular mechanism of cyst formations, caused by loss of functional PC-1 or 2 protein levels and the involvement of cilia and shear stress is still not completely understood.

What is known is that numerous signaling pathways are implicated in polycystic kidney disease, including mTOR, TGF-β, Wnt, Hippo, STAT, MAPK, PI3K-AKT, Hedgehog and cAMP signaling[131,147-174]. Remarkable is that several of these signaling pathways are being modulated by fluid shear as well, suggesting that implicated shear regulated signaling

may contribute to PKD. Increased mTOR signaling is suggested to be involved cell growth and proliferation, thereby accelerating cyst growth[147,148]. Activation of TGF-β signaling has been shown in several animal models for polycystic kidney disease and patient-derived tissues and is known to be involved in fibrosis, which is commonly seen in APDKD[82,146,175]. A recent study reports that Wnt/Ca^{2+} signaling is mediated by the polycystin complex, while canonical Wnt seems to be inhibited by polycystin-1[176,177]. Altered Wnt signaling is described in ADPKD models as well and is suggested to be involved in disoriented cell division, leading to cyst expansion[131,160,178,179]. Increased cAMP levels in ADPKD can activate MAPK/ERK signaling leading to induced proliferation. Tolvaptan and Sorafenib inhibit cAMP and ERK dependent cyst progression in ADPKD models[172,180]. Although several treatments have been tested successfully in PKD mouse models, the efficacy in human patients is sometimes minimal or absent, which was published for several mTOR inhibitors[152,181,182]. Therefore, it has been suggested to combine therapies and target multiple signaling pathways affected in ADPKD[174,183]. For example, the natural herb curcumin can modulate multiple signaling cascades, including mTOR, Wnt and Stat3, and was shown to inhibit cyst formation in ADPKD mice[162.] Initial experiments using a combination of mTOR and/or cAMP inhibitors showed promising results to inhibit proliferation in ADPKD cells and mice, but additional research is needed to evaluate the efficacy in ADPKD patients[184,185].

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to study fluid shear stress regulated signaling in renal epithelial cells and the relevance for ADPKD. Since several signaling pathways are regulated by fluid shear and are implicated in APDKD as well, we expect that impaired shear stress signaling is contributing to the ADPKD phenotype. We will compare altered cellular signaling upon physiological and pathological relevant levels of shear stress. Furthermore, we will evaluate the role of cilia in the shear response, since the polycystins localize in this organelle and renal cyst formation is a common feature in several ciliopathies. In **chapter 2-4** we analyze the cellular response of proximal tubular epithelial cells (PTECs) to fluid shear stress and the involvement of cilia and *Pkd1* expression. In **chapter 2** we focus on shear stress induced canonical TGF-β (SMAD2/3) signaling and the participation of MAPK/ERK signaling. We show that fluid shear induced activation of SMAD2/3 and epithelial-to-mesenchymal transition (EMT) processes are TGF-β/ALK5 dependent. The shear response in PTECs is modulated by *Pkd1* gene disruption and MAPK/ERK signaling. However, cilia ablation does not reduce SMAD2/3 target gene expression, suggesting that other mechano-sensing structures are involved.

In **chapter 3** we investigate shear induced alterations of the transcriptome in proximal tubular epithelial cells, using RNA-sequencing. We describe several pathways that are altered by shear stress and we validate these changes by qPCR. Many of these pathways are modulated by TGF-β/ALK5 and MAPK/ERK signaling. The role of cilia during the shear stress response is evaluated as well. We show that cilia only have a minor contribution to shear stress regulated signaling in PTECs. Finally, pathological levels of shear stress are compared to physiological controls, showing elevated shear induced expression of several genes under pathological conditions.

Shear stress dependent signaling in an *in vitro* ADPKD model was evaluated in **chapter 4** using RNA sequencing. The effect of fluid shear stress in PTECs without *Pkd1* expression was compared to *Pkd1*^{wt} controls. We show that *Pkd1* is not directly involved in shear dependent activation of many signaling pathways. In addition, differential gene expression in *Pkd1*$^{-/-}$ PTECs during shear is compared with *in vivo* transcriptome analysis of pre-cystic kidneys in a *Pkd1*^{del} mouse model, in which fluid flow is still present. Several signaling pathways that are known to be implicated in the renal cyst formation are altered in both the *in vitro* and *in vivo* models for *Pkd1* gene disruption. So, the data suggests that these processes are already altered at pre-cystic stage and may contribute to *in vivo* cyst formation.

In **chapter 5** we investigate the role of canonical TGF-β (SMAD2/3) signaling in cyst formation. Genetic disruption of TGF-β receptor type I (*Alk5*) in kidney epithelium doesn't reduce cyst progression in a *Pkd1*^{del} mouse model. Activin is another cytokine that can phosphorylate SMAD2/3 via the Activin receptors. Activin signaling was antagonized using a soluble Activin type IIB receptor (sActIIB-FC). Treatment with sActRIIB-Fc markedly reduced cyst progression in three different mouse models for ADPKD, suggesting that Activins drive the progression of PKD. Finally, the results of this thesis and future plans are summarized and discussed in **chapter 6**.

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