Studies on the pathogenesis of chronic kidney disease

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Chapter 6

Summary and General Discussion

In 2017, there were almost 700 million cases of chronic kidney disease worldwide [1,2]. Chronic kidney disease resulted in 1.2 million deaths in 2017, and it has been projected to rise to 2.2 million or might even up to 4.0 million by 2040 [3]. Diabetes is a major risk factor associated with chronic kidney disease, and it contributes to 30-50% of all chronic kidney diseases [4]. The global prevalence of diabetes is also rising [5], with a concomitant increase in diabetic nephropathy. The global epidemiology of diabetic nephropathy will result in severe social and economic ramifications if treatments are not improved. Currently, glycemic control is still the primary therapy for patients diagnosed with diabetes. However, this treatment does not reduce the risk of the clinical renal outcome of diabetic nephropathy [6]. Therefore, to address this worldwide epidemic issue, it is crucial to understand the underlying mechanisms of the development of diabetic nephropathy and to find novel preventative and therapeutic strategies.

Diabetic nephropathy

Oxidative stress results from the overproduction of reactive oxygen species in excess of the cell's antioxidant response [7]. This stress can damage all cell components, including DNA, proteins, and lipids [8,9]. For adaptation to oxidative stress, cells contain a bunch of antioxidant defences to protect them against reactive oxygen species. The sustained oxidative stress-mediated injury can lead to cellular apoptosis. It has been hypothesized that all mechanisms of diabetic complications are activated by one upstream event: the overproduction of reactive oxygen species [10]. The kidney is a highly metabolic organ, and it is vulnerable to oxidative stress. Growing evidence supports that increased oxidative stress may contribute to the development of diabetic nephropathy [11-13]. Nowadays, intensive glucose control, anti-lipid, and anti-hypertensive agents are used as treatments for patients with diabetic nephropathy. Since oxidative stress contributes to the pathogenesis of diabetic nephropathy, the availability of an effective and target-specific antioxidant agent is highly desirable [14].

Clusterin (CLU) has protective properties in diabetic nephropathy

A large body of evidence has shown that the *CLU* gene is an extremely sensitive biosensor to reactive oxygen species. It has a heat shock transcription factor-1 and an activator protein-1 element in its promoter sequence [15]. In **Chapter 2**, we demonstrated that, oxidative stress is the primary factor to induce *CLU* mRNA expression in podocytes under diabetic conditions. We also showed that recombinant clusterin protein could protect the podocytes against

oxidative stress-induced apoptotic cell death *in vitro*. This finding suggests that clusterin's antioxidant property might make clusterin a potential therapeutic agent to protect podocytes under oxidative stress. It has been reported that exogenous clusterin inhibits H_2O_2-induced reactive oxygen species generation and suppresses caspase-3 activity in the retinal pigment epithelium cells, and enhances the survival of cells mediated by the PI3K/Akt pathway [16]. Jun et al. demonstrated that the exogenous clusterin could protect cardiomyocytes against H_2O_2-induced oxidative injury via activating Akt/GSK-3β signaling pathway [17]. In accordance with these studies, we hypothesized that clusterin protects podocytes against oxidative stress-induced apoptosis by activating the Akt survival pathways. The detailed underlying molecular mechanisms of how clusterin protects podocytes against oxidative stress-induced apoptotic cell death need further elaboration. In *vivo* studies are required to confirm our hypothesis that clusterin may slow or even halt the progression of diabetic nephropathy.

Väkevä and colleagues reported that clusterin started to be deposited in affected regions of the heart soon after the onset of myocardial infarction. And clusterin binds to the components of the membrane attack complex and prevents the lysis of the cell [18]. In human kidney diseases, clusterin deposition was also found in association with glomerular membrane attack complex (MAC) formation in glomerulonephritis [19]. In a passive Heymann nephritis experimental model, clusterin deposits were detected along the glomerular capillary wall with an identical pattern to rat C5B-9. And clusterin's deposition was not observed in complement-depleted animals [20]. Rosenberg et al. demonstrated that ageing clusterin-deficient mice developed immune complex deposits in the mesangium, and moderate to severe mesangial lesions were found in these clusterin-deficient mice, while age-matched wild type controls have relatively few or no glomerular lesions [21]. A study by Bus et al. [22] and a review by Flyvbjerg et al. [23] suggest that complement activation contributes to the pathogenesis of diabetic nephropathy. Rastaldi et al. [24] demonstrated that recombinant clusterin protein can bind the podocytes via LDL receptor and suggested that binding of clusterin to the podocyte surface can prevent PKC activation by complement components. In **Chapter 2**, we showed that CLU expression was increased in micro-dissected glomeruli of patients with diabetic nephropathy compared to healthy subjects at both mRNA and protein levels. Therefore, we hypothesize that increased glomerular clusterin might also protect podocytes against complement activation in diabetic nephropathy. Therefore, an intriguing direction for future research is to investigate whether clusterin may offer protection by modulating complement activation in diabetic nephropathy.

The role of carnosinase-1 (CNDP1) overexpression in the progression of diabetic nephropathy

Carnosine is a natural antioxidant [25], which has been suggested to protect against diabetic nephropathy in diverse rodent models [26-29]. It has been shown under diabetic conditions that carnosinase-1 (CNDP1, also called CN1) overexpression lowers carnosine concentrations. Compared to non-transgenic *db/db* mice, humanCN1 (hCN1) transgenic *db/db* mice with carnosinase-1 overexpression had lower carnosine serum concentrations, more severe impaired renal function, and significant renal hypertrophy [30]. In **Chapter 3**, our findings also support the evidence that hCN1 overexpression results in lower renal carnosine and anserine storage and aggravates the development of diabetic nephropathy in BTBR *ob/ob* mice [31]. We detected an increase of glomerular hypertrophy and more mesangial matrix expansion in hCN1 transgenic BTBR *ob/ob* mice compared to non-transgenic BTBR *ob/ob* mice. Furthermore, we showed that the decline in renal carnosine and anserine levels correlate with different markers of diabetic nephropathy. These findings jointly suggested that low renal carnosine and anserine concentrations can aggravate the pathogenesis of diabetic nephropathy. In future studies, a direct comparison between the effect of hCN1 overexpression and carnosine supplementation on both renal carnosine and anserine levels and diabetic nephropathy need to be considered. Meanwhile, high CNDP1 enzyme activity in human serum can degrade carnosine, which hampers the efficacy of carnosine treatment in humans. Therefore, novel treatments should be investigated to address the adverse effects of the CNDP1 enzyme on carnosine supplementation.

Exercise could increase resting muscle carnosine levels [32]. A higher carnosine content in the circulation was also found in animals after physically activity [33,34]. Researchers have shown that aerobic treadmill training has a beneficial effect on nephropathy in a high-fat [35,36] or streptozotocin-induced [37] diabetic rat model and in *db/db* mice [38,39]. One hypothesis is that the induced muscle contractile activity by the exercise training can release carnosine and/or anserine from muscular storage into the circulation. The increased circulating carnosine and/or anserine levels subsequently reduces the progression of diabetic nephropathy. In contrast to previous studies, in **Chapter 3**, we did not find any significant protective effect of 20 weeks of chronic exercise training on the progression of diabetic nephropathy in these BTBR *ob/ob* mice in both genders. However, in other studies [35-39], animal models with milder renal damage were used in combination with 5 to 12 weeks of aerobic treadmill running as interventions. It has been shown in the literature that the BTBR *ob/ob* mice develop advanced diabetic nephropathy [31]. In **Chapter 3**, we kept all the BTBR *ob/ob* mice running for 20

weeks. We found that these BTBR *ob/ob* mice had very high glucose levels already, which possibly lead to severe ketoacidosis, lower exercise tolerance or dehydration during the chronic aerobic exercise training. Our data indicate that chronic aerobic exercise alone may not be sufficient to reverse the observed severe histological changes in BTBT *ob/ob* mice.

Another interesting finding in **Chapter 3** is that hCN1-overexpression BTBR *ob/ob* mice had higher fasting cholesterol and triglycerides at week 14. This finding indicated that hCN1 overexpression stimulates hyperlipidemia in diabetic mice and suggested an adverse effect of the carnosinase-1 enzyme on dyslipidemia. Studies have shown that long-term carnosine supplementation could improve dyslipidemia in different diabetes rodent models [27,40,41] and diabetic patients [42]. Barski et al. found that carnosine can inhibit low-density lipoprotein (LDL) oxidation by chelating copper [43]. As the underlying mechanism of carnosine's anti-hyperlipidemic effect remains unknown, future studies using lipidomic and proteomics approaches might help us to unravel the role of carnosine in lipid metabolism.

Novel zebrafish models for chronic kidney diseases

To understand the pathogenesis of human disease and provide systems for testing novel therapeutics and developing effective treatments, animal models are an indispensable part of biomedical research. Recently, zebrafish have become an attractive model to study developmental biology, genetics and human diseases [44-46].

The role of leptin in the development of diabetes and diabetic nephropathy

Leptin is a hormone that functions in the regulation of energy homeostasis via suppression of appetite. Rodents lacking the *leptin* gene are commonly characterized by hyperphagia, obesity, insulin resistance and impaired glucose tolerance. For instance, leptin-deficient mice (*ob/ob* mice) exhibit the features of obesity and type 2 Diabetic Mellitus (T2DM) [47]. In **Chapter 4**, we found that *lepb*$^{-/-}$ adult zebrafish have an obese phenotype compared to the age-matched controls. We also demonstrated that, compared to control male adult zebrafish, *lepb*$^{-/-}$ male adult zebrafish have higher blood glucose, suggesting the leptin signaling regulates glucose homeostasis in zebrafish as well. A growing number of publications provide evidence that leptin has a glucose-lowering effect. Kamohara et al. showed that infusion of leptin into the brain could normalize the hyperglycemia in *ob/ob* mice [48]. It has been reported that leptin acts on glucose metabolism in both insulin-dependent and insulin-independent ways. Leptin can improve insulin sensitivity [49,50]. Reduced leptin in the mediobasal

hypothalamus leads to severe insulin resistance and glucose intolerance in Koletsky rats, and phosphatidylinositol-3-OH kinase signaling is a vital mediator of this effect in the hypothalamus [51]. Leptin can reverse hyperglycemia and ketosis by suppressing the action of glucagon on the liver and improving the utilization of glucose in the skeletal muscle in insulin-deficient diabetic rodents [52]. Our laboratory's earlier work demonstrated that *lepb* was significantly downregulated in zebrafish larvae in an insulin-resistant state [53]. Combining the previous study and the findings in **Chapter 4**, we propose that the diabetic phenotype of *lepb* mutants is caused by disruption of the insulin signaling pathway. We are currently performing transcriptomic analysis of *lepb*$^{+/+}$ and *lepb*$^{-/-}$ zebrafish larvae, which might give us new insights into the role of leptin in the insulin signaling pathway.

The initial aim of the study described in **Chapter 4** was to investigate whether *lepb*$^{-/-}$ adult zebrafish develop the features of diabetes and diabetic nephropathy. It is known that patients often develop diabetic nephropathy after a long period of diabetes (10~20 years) [54], and leptin-deficient mice develop only mild renal histological changes [55]. The mean lifespan of domesticated zebrafish is 42 months [56]. We therefore decided to investigate zebrafish at the age of 1.5 years, which is relatively old for this species. Our data indicate that 1.5 years old *lepb*$^{-/-}$ male zebrafish develop early signs of diabetic nephropathy. We assume that it might be more challenging to observe the kidney phenotype in younger fish regarding our findings in **Chapter 4**. Nevertheless, it is still interesting to look at earlier time points to further investigate the progression of these early signs of diabetic nephropathy in future studies. To accelerate or aggravate kidney damage, conducting other interventions in *lepb*$^{-/-}$ zebrafish can be considered, for instance, by combining the leptin mutation with other gene mutations associated with the development of diabetic nephropathy or by overfeeding these zebrafish.

Anti-diabetic drugs which are effective for diabetic patients have been shown to ameliorate the hyperglycemia in overfed zebrafish, suggesting that the glucose homeostasis pathways are conserved between zebrafish and human [57]. In an experimental setting using zebrafish, potential anti-diabetic compounds to be tested can be easily put into the water for oral administration or be injected into embryos by using a robotic injection machine [58]. Screening efficient anti-diabetic drugs in zebrafish larvae have a high-throughput potential.

Ctns mutant adult zebrafish resembles the phenotype of human nephropathic cystinosis

Cystinosis is a rare lysosomal storage disorder. Our group generated *ctns* mutant zebrafish, which showed retarded development, cystine accumulation, and the signs of pronephric glomerular and tubular dysfunction at the larval stage [59]. Lysosomes are the sites of

intracellular digestion. They facilitate the degradation of foreign materials, and they are considered the vital metabolic coordinators of cells [60]. Sakarcan et al. observed notable swelling of lysosomes with numerous cytoplasmic vesicles in the cystine-loaded proximal tubule cells [61]. The hyaline-like eosinophilic droplets in the proximal tubules are suggested to represent lysosome [62]. Being a lysosomal storage disease, the key feature of cystinosis is the lysosomal cystine accumulation [63,64]. In **Chapter 5**, we observed that the cytoplasmic vacuoles are more frequently localized in the renal proximal tubular cells at 18 months old *ctns* mutant adult zebrafish than at 3 months and 6 months. Moreover, abundant cytoplasmic vacuoles were found with rectangular or polymorphous shape in the renal proximal tubules, suggesting cystine crystals' accumulation. Until now, the underlying mechanisms of how cysteine accumulation leads to nephropathic cystinosis are still unknown. In **Chapter 5**, the upregulation of cleaved-caspase 3 expression and nuclear fragmentation were found in the proximal tubular epithelial cells of *ctns* mutant adult zebrafish. It has been reported that lysosomal cystine release can activate protein kinase Cδ, resulting in a significant increase in apoptosis in cultured renal proximal tubular epithelial cells [65]. Sumayao et al. demonstrated that excessive lysosomal cystine accumulation reduced glutathione levels and enhanced intracellular reactive oxygen species production, which subsequently disrupted the mitochondrial integrity and augmented apoptosis in the renal proximal tubular epithelial cells [66]. The next step of research aims to unravel the precise mechanisms leading to apoptosis which could not be elucidated in **Chapter 5** due to materials limitation.

Nephropathic cystinosis, if untreated, will gradually progress to chronic renal failure. The administration of aminothiol cysteamine is the standard therapy for cystinosis [67]. Cysteamine also has undesirable side effects when it has to be taken lifelong. From this perspective, suitable animal models to test novel potential drugs could be useful. Our previous study demonstrated that *ctns* mutant zebrafish larvae showed a significant decrease in cystine levels upon treatment with increasing cysteamine concentrations [59]. It will be advantageous to use zebrafish larvae to screen potential drugs, followed by studying the curative effect in adult zebrafish in preclinical research.

As mentioned before, a defective cystinosin function results in the intralysosomal accumulation of cystine in all cells. Aside from the kidneys, also eyes, muscles, skin, and reproductive organs are affected in the course of cystinosis. Currently, we are investigating other organs in our zebrafish model to unravel this rare disease.

Summary

It has been reported that diabetic nephropathy occurs in the familial clusters, suggesting a genetic predisposition in diabetic nephropathy [68,69]. The advantage of studies of genetic predisposition is that it is free of preconceived hypotheses. These studies have offered robust tools to analyze the pathogenesis of this disease which has also been shown to have complex traits. In the studies described in **Chapter 2** and **Chapter 3** of this thesis, we investigated the role of *CLU* and *CNDP1* in the development of diabetic nephropathy.

Several studies have shown that clusterin may have renal protective properties in various forms of kidney disease [21,70-72]. Microarray analysis revealed that *CLU* mRNA expression is increased in the glomeruli of patients with diabetic nephropathy compared to healthy subjects [73]. In the study described in **Chapter 2**, we investigated the role of glomerular clusterin in diabetic nephropathy. We first confirmed that clusterin expression was increased in glomeruli of patients with diabetic nephropathy both at the mRNA and protein levels in human biopsies. Furthermore, the immunohistochemical staining on both human and mice renal tissue revealed that glomerular clusterin is expressed in podocytes. In the *in vitro* experiments, we found that oxidative stress, not high glucose or angiotensin II, upregulates clusterin mRNA expression in podocytes, suggesting oxidative stress is an important factor that induces *CLU* mRNA expression under diabetic conditions. Lastly, we showed that recombinant clusterin protein enhances cell viability, reduces the elevated *Bax/Bcl2* mRNA ratio, and reduces the enhanced caspase 3/7 activity in podocytes under oxidative stress-induced damage *in vitro*. These findings indicate that clusterin can protect podocytes against oxidative stress-induced apoptotic cell death.

It has been shown that a polymorphism of the *CNDP1* gene strongly associates with the prevalence of diabetic nephropathy [74,75]. The *CNDP1* gene encodes for an enzyme that degrades carnosine, a dipeptide with multiple protective properties [25,27,76]. In humans, this enzyme is secreted by the liver into circulation. Carnosinase is present in human serum but not in that of rodents [77,78]. Compared to non-transgenic *db/db* mice, hCN1transgenic *db/db* mice had high CNDP1 serum levels resulting in low carnosine serum concentrations, a more severe impaired renal function, and significant renal hypertrophy [30]. Exercise increases circulating carnosine levels [32]. Besides, prolonged aerobic exercise prevents diabetic nephropathy in different rodent models [79-82]. In the study described in **Chapter 3**, we investigated the effect of two interventions, overexpressing the human carnosinase-1 enzyme and chronic aerobic exercise training, on the development of diabetic nephropathy in BTBR

ob/ob mice. Our study revealed that hCN1 overexpression further reduced the content of histidine-containing dipeptides (carnosine and anserine) in urine, kidney and gastrocnemius muscle, and significantly accelerated and aggravated diabetic nephropathy in BTBR *ob/ob* mice. Furthermore, we found that the amount of renal histidine-containing dipeptides was negatively correlated with mesangial matrix expansion and glomerular hypertrophy. We showed that hCN1-overexpression BTBR *ob/ob* mice had higher fasting cholesterol and triglycerides at week 14, indicating that hCN1 overexpression contributes to faster development of hyperlipidemia in diabetic mice. In contrast with previous studies [79,80], we did not find a protective effect of chronic exercise training on the progression of diabetic nephropathy. Our data suggest that the amount of renal histidine-containing dipeptides correlates inversely with the severity of diabetic nephropathy.

In recent decades, zebrafish have become an attractive model for studying developmental biology, genetics and human diseases [44-46]. In the studies described in **Chapter 4** and **Chapter 5** of this thesis, we investigated whether two novel zebrafish models could resemble the pathologic features of human chronic kidney disease.

In humans, patients lacking leptin have evident obesity and glucose tolerance impairments, the hallmarks of type 2 diabetes [83,84]. The congenital leptin-deficient (*ob/ob* mutant) mice [85,86] or leptin receptor-deficient (*db/db* mutant) mice [87] are the most widely used animal models in type 2 diabetes mellitus (T2DM) research. It has been reported that the basic structural features and intracellular signaling mechanisms of leptin and its receptor are conserved throughout vertebrates [88-91]. In a previous study, our group found that the *lepb* gene, but not the paralogous *lepa* gene, is significantly downregulated in zebrafish larvae under an insulin-resistance state, resulting from acute hyperinsulinemia [53]. Moreover, this finding indicated that *lepb* plays a vital role in insulin homeostasis in zebrafish. In the study described in **Chapter 4**, we investigated whether *lepb* deficiency results in the development of T2DM in adult zebrafish. We found that *lepb*$^{-/-}$ adult zebrafish have an increment in body weight and length compared to the age-matched control adult zebrafish. In addition, the quantification of magnetic resonance anatomical imaging (MRI) data showed a significant increase of visceral fat accumulation in *lepb*$^{-/-}$ adult zebrafish, compared to control adult zebrafish, in both genders. The increased body weight and length and body fat composition jointly indicate that *lepb*$^{-/-}$ adult zebrafish have an obese phenotype. Furthermore, we found that 2 hours postprandial blood glucose levels and fasting blood glucose levels in *lepb*$^{-/-}$ male zebrafish group were significantly higher compared to control male zebrafish group. However, we did not find this difference in the female group. Lastly, we observed that *lepb*$^{-/-}$ male zebrafish

develop glomerular hypertrophy and thickening of the glomerular basement membrane, indicating that *lepb*$^{-/-}$ adult zebrafish have the early signs of diabetic nephropathy.

Cystinosis is a lysosomal storage disease caused by several mutations of the *CTNS* gene encoding cystinosin. Most patients with cystinosis have kidney disease (nephropathic cystinosis), which is the most common cause of inherited renal Fanconi syndrome in humans [92]. Nephropathic cystinosis will eventually develop chronic renal failure when left untreated. In our previous study, we generated a *ctns* mutant zebrafish that displayed glomerular and tubular dysfunction at the larval stage. We also found that cystine expression level in the kidney of eight months old *ctns* mutant adult zebrafish was 20-fold higher than that of age-matched control zebrafish [59]. In the study described in **Chapter 5**, we further investigated the renal histopathology of *ctns* mutant adult zebrafish. We observed cytoplasmic vacuoles and hyaline-like eosinophilic droplets in the renal proximal tubules in *ctns* mutant zebrafish in both genders. Additionally, we found that these vacuoles in the renal proximal tubular cells of *ctns* mutant adult zebrafish have a rectangular or polymorphous shape on toluidine blue-stained slides and the images captured by the transmission electron microscopy. These findings indicate that cystine crystals were present. We also showed that *ctns* mutant zebrafish had glomerular hypertrophy, which is possibly related to hyperfiltration. Lastly, we observed that *ctns* mutant adult zebrafish have increased levels of cleaved caspase-3 and increased nuclear fragmentation in the renal proximal tubular epithelial cells, compared to control zebrafish. This finding suggests that apoptosis is involved in the pathogenesis of cystinosis tubulopathy in zebrafish.

Summarized, in this thesis, two potential therapeutic targets for diabetic nephropathy were identified and investigated. First, we show that glomerular clusterin is upregulated in diabetic nephropathy and demonstrated that recombinant clusterin protein can protect the podocytes against oxidative stress *in vitro*. Second, we reveal that hCN1 overexpression accelerated and aggravated diabetic nephropathy in BTBR *ob/ob* mice. We also studied two novel zebrafish models to investigate chronic kidney disease. We showed that *lepb*$^{-/-}$ adult zebrafish have the early signs of human diabetic nephropathy, and we demonstrated that *ctns* mutant adult zebrafish have the kidney pathologic features of human nephropathic cystinosis.

Future perspectives

The use of primary cells and cell lines (*in vitro* model systems) has advanced our understanding of the mechanisms of disease. However, it is impossible to interpret the intact organism's biology by only using *in vitro* cell cultures [93]. Comparative studies using murine models (*in vivo* model systems) can give a bigger picture. Rodents are pre-eminent in modelling human diseases because of the high similarity between mammalian genomes [94]. Rodents have many similarities with humans with respect to anatomy and physiology [95]. However, murine models are usually time-consuming and expensive. Therefore, zebrafish is becoming an attractive animal model due to many advantages [96]. They have high fecundity and rapid development. The externally fertilized eggs make them well amenable to rapid genetic manipulation techniques. The optical transparency of the larvae makes screening with different fluorescent labels straightforward. Their small size makes zebrafish embryos highly suitable for large scale and cost-effective screening strategies. Nowadays, most of the existing studies related to zebrafish have been conducted with larvae. On the other hand, adult zebrafish are more appropriate to investigate chronic kidney disease because it takes a long time to develop. Besides the advantages mentioned before, there are also some drawbacks in the zebrafish model to study chronic kidney disease. Due to the small size of zebrafish, it is not easy to obtain sufficient blood to perform various physiological and biochemical measurements on one sample. Furthermore, it is sometimes difficult to obtain suitable antibodies to perform immunohistochemistry and western blot techniques.

It is a well-known fact that proteinuria is an important indicator to evaluate the glomerular filtration barrier function. However, this cannot be directly measured in zebrafish. Two approaches have been established to address this issue. One way is to establish reabsorption of high-molecular-weight FITC-dextran by proximal tubules as an expression of the increased permeability of the glomerular filtration barrier in zebrafish [97,98]. Naturally, the intact glomerular filtration barrier prevents larger sized molecules from leaking out. For instance, 40kDa dextran passes freely through the intact glomerular filtration barrier, but 70KDa dextran cannot. However, the 70kDa dextran can leak out of the glomerular filtration barrier and be resorbed into the renal proximal tubules when the glomerular filtration barrier is damaged. Zhou et al. generated a transgenic zebrafish line expressing green fluorescent protein (GFP)-tagged vitamin D-binding protein (VDBP) as a tracer for proteinuria [99]. The VDBP has a similar size as albumin. Therefore, when the glomerular filtration barrier is damaged, GFP resorption droplets can be observed in the renal proximal tubules by

histological imaging or by measuring VDBP in the swimming water. Outcrossing the gene mutant zebrafish with VDBP-GFP transgenic zebrafish or injecting the high-molecular-weight FITC-dextran can be very useful to evaluate the function of the glomerular filtration barrier of mutant zebrafish.

As mentioned before, the possibility of screening on a large scale is a significant advantage of the zebrafish larval model. Because of the short generation time, data obtained in the larval model can be efficiently translated to the adult level and *vice versa*. In this thesis, we focused on investigating the histopathology of zebrafish. Next, making use of the technological advances in the zebrafish models will generate extensive "omics" data, including transcriptomic, metabolomic, and proteomic data, to further obtain additional insights to understand the mechanisms of chronic kidney disease. For instance, spatial transcriptomics, a promising novel technique, allows us to measure genome-wide gene expression and display its spatially resolved expression on the tissue section level. Spatial transcriptomics will remarkably increase the depth and breadth of histopathological research. In the future, we can use the mentioned techniques to analyze the function of a large number of genes or proteins with our generated zebrafish model to get a comprehensive view of mechanisms underlying chronic kidney disease.

Another advantage of zebrafish is that they have the ability to regenerate kidney tissue [100], which cannot be found in mammals. In the future, it will be interesting to conduct comparative studies among patients, zebrafish, and mice to grab novel insights into renal regeneration and repair in chronic kidney disease. Although zebrafish are a valuable animal model for exploring the pathogenesis and therapy strategies for chronic kidney disease, they cannot fully replace cells culture techniques and murine models in fundamental research. Zebrafish models, embryos in particular, can fill the gap between the cell cultures and the highly organized murine models, especially in the light of drug screening or investigation of the gene functions. We believe that integrating the discoveries based on studies in cell cultures, zebrafish, and rodent models will deliver new biological insights that can drive the development of innovative treatments for chronic kidney diseases.

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