Analysis on the mechanism of reduced nephron number and the pathological progression of chronic renal failure in Astrin deficient rats

Summary of Doctoral Thesis

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Chronic kidney disease (CKD) is a common disease exhibiting globally high morbidity rate. About 13.3 million people corresponding to 10-13% of general population is thought to have CKD in Japan. However, at present, there is no effective treatment for end stage CKD except for dialysis and kidney transplantation. Recently, the patients requiring dialysis therapy are increasing every year, because the treatment that can drastically improve quality of life in CKD patients has not been established. CKD ranks in the top 3 of the cause of death in dogs and cats, because the application of dialysis therapy and kidney transplantation is very limited in veterinary medicine. Therefore, the elucidation of pathogenesis in CKD, the identification of surrogate markers, the establishment of effective treatments for CKD is desired. In this situation, many challenging research has been performed to develop renal regeneration therapy. In renal development, however, three-dimensional architecture is intricately constructed with many types of cells, and many developmental events remained unclear. Thus, regeneration technique is delayed in kidney compared to other organs. Although CKD is caused by various congenital and/or acquired factors, there is a common pathway in which reduced number of nephrons causes overload to remaining individual nephron, irreversibly deteriorating renal damage (Brenner's theory). Progressively reduced number of nephrons result in reduced excretive function, renal anemia, and renal fibrosis at end stage of kidney disease. On the other hand, total number of nephrons has been reported to vary in humans and to be closely associated with birth weight. A congenitally reduced number of nephrons is considered an important risk factor related with pathogenesis and prognosis of CKD. Therefore elucidating mechanism for determining nephron number during renal development will provide important information useful for developing kidney regeneration therapy and estimating a risk factor for CKD. In addition, elucidating mechanism by which congenitally reduced number of nephrons causes CKD might provide

clues to understand pathological process common in different types of CKD by caused by various factors. In this thesis, I revealed the cause of reduced nephron number during embryonic development and the pathological progression of CKD in hypoplastic kidney (HPK) rats with congenitally reduced (-80%) number of nephrons resulting from loss-of-function type mutation of Astrin gene which is known to be related with the progression of mitotic metaphase and with the inhibition of hyperactivation of mTOR signaling. Finally, I discussed about possible therapeutic strategies and molecularly targets based on the results obtained in this study.

In chapter 2, I demonstrated that HPK rats show macrocytic erythropeina with the progression of CKD. In general, major source of erythropoietin (EPO) is considered fibroblasts located in juxtamedullary interstitium. EPO is secreted from fibroblasts in response to hypoxia and can induce erythropoiesis in bone marrow. Normocytic normochromic anemia accompanied by reduced response of EPO production to hypoxia is often observed in end stage of CKD. Recently, this pathological condition is considered to be caused by the transdifferentiation of EPO-producing fibroblasts into myofibroblasts with the progression of interstitial fibrosis. I found that HPK rats have normal level of plasma EPO concentrations under normoxic condition in despite of the appearance of CKD symptoms. Interestingly, EPO mRNA expression was decreased in kidney and increased in liver of HPK rats, indicating that increased hepatic EPO production might compensate decreased renal EPO production. Moreover, EPO mRNA expression were normally induced by hypoxic condition in both kidney and liver of KPK rats at 140 days of age, indicating HPK already affected by fibrosis still has potential to produce EPO in response to hypoxia. On the other hand, we found increased the fragility of erythrocyte membrane, the promotion of splenic hemosiderosis, and decreased serum transferrin concentration, normal level of plasma ion due to significantly increased transferrin saturation in HPK. These results suggested that, although erythropoiesis in HPK is maintained almost normal by hepatic compensative EPO production against renal reduced production, erythropenia is induced by hemolysis of red blood cells.

In chapter 3, I demonstrated that glomerular lesions are prior to interstitial alterations in renal fibrosis of HPK with 80% nephron reduction. Although it is known that a considerably reduced number of nephrons progressively induces renal damage via overload to individual nephron, it is still unknown how many nephrons are required for maintenance of normal renal function through life span. We found glomerular hypertrophy, discontinuous immunostaining of podocin along the glomerular basal membrane, and infiltration of inflammatory macrophages into glomerulus in HPK at 35 and 70 days of age. At 70 days of age and afterwards, accumulation of extracellular matrixes (ECMs), increase in mesangial cells, and glomerular sclerosis were gradually deteriorated in HPK. In accordance with these changes, glomerular PDGF- and TGF-ß-positive areas were increased in HPK. Glomerular PDGFr-ß-positive area was significantly increased in HPK, whereas α-SMA-positive myofibroblasts were rarely detected in glomerular tufts in spite of the appearance of myofibroblasts in glomerular parietal epithelium along Bowman's capsular walls of HPK. On the other hand, in interstitial tissue, age-related increase in accumulation of ECMs was accompanied by the age-related increase of myofibroblats by transdifferentiation from increased fibroblasts in the intrestitium of HPK after the progression of glomerular injury. In accordance with these alterations, we detected infiltration of macrophages into interstitium at 140 and 210 days of age. PDGF-positive area was also increased in tubulointrestitium with fibrosis. These results indicated that congenital 80% nephron reduction results in progressive CKD resulting in renal fibrosis. In HPK, moreover, glomerular lesions appeared early but progressed slowly without increased myofibroblasts, whereas interstitial fibrosis appeared later but progressed rapidly with increased myofibroblasts. These pathological changes might be mediated by growth factors including PDGF and TGF- β .

In chapter 4, I demonstrated that loss of functional Astrin causes nephron reduction via decreased branching of ureteric bud (UB) associated with increased apoptosis of metanephric mesenchyme (MM). Normal renal development progresses through interaction between UB and MM. UB is the

first branch of Wolffian duct as a primordium of collecting duct, advances into MM, and interacts with MM surrounding UB. In HPK metanephros, increased apoptosis and decreased proliferation were observed in MM cell surrounding UB. In addition, population of MM cells expressing upstream interaction signals (Sall1 and Pax2) and mRNA expression of Sall1, Kif26b, and Pax2 were already decreased in HPK metanephros at embryonic day (E) 14.5, whereas decrease in mRNA expression of other interaction signals was not detected. In normal metanephric development, MM cells form Six2-positive cap cluster surrounding UB tips. I found decrease in metanephric size, thinning of Six2-positive cap cluster, and decrease in Six2 positive cap area around individual UB in HPK metanephros at E14.5, whereas reduced branching of UB was initially detected at E15.5 in HPK. Therefore, it was suggested that MM is mainly affected by loss of Astrin, secondarily causing reduced branching of UB. MM clusters are believed to differentiate into most of nephron components, and tubular epithelia and podocytes are differentiated through mesenchymal-epitherial-transition (MET). I observed normal progression of MET in HPK metanephros. It has been reported that the knockdown of Astrin in HeLa cells causes mitotic arrest at metaphase in cell cycle, resulting in apoptosis. In accordance with the phenotype in HeLa cells, abnormal mitotic metaphase and subsequent apoptotic cell death were observed in immature Sertoli cells of testicular dysplasia accompanied in male HPK rats. Therefore I assumed that decrease in MM of HPK metanephros would be caused by loss of Astrin function associated with cell cycle progression. Unexpectedly, increase of metaphase cells positive for phosphor-histion H3 was not detected in HPK metanephros at all embryonic days examined. Alternatively, as another function of Astrin, it has been reported that Astrin inhibits apoptosis through suppressing hyperactivation of mTOR signaling via recruiting Raptor, a component of mTOR complex 1, into stress granules in HeLa cells under stress condition. In my experiments regarding to mTOR signaling, increased mRNA expression of mTOR and it's downstream S6K1 and increased phosphorylation of S6K1 were detected in HPK metanephros, suggesting that Astrin is associated with the regulation of mTOR signaling in

developing metanephros.

In chapter 5, I tried to replicate metanephric phenotype using in vitro organ culture method and analyzed the phenotype of MM cells affected by Astrin deficient. The growth of E14.5 HPK metanephros cultured for 3 days was apparently delayed compared to that in normal metanephros. When metanephros were cultured with low dose (0.05ng/ml) of mTOR inhibitor (Evelorimus), metanephros derived from normal embryos were significantly decreased in size compared to vehicle control (DMSO), whereas metanephros from HPK embryos were almost comparable in size to control. In addition, the increment of metanephric size for 3 days culture was significantly lower in HPK than in normal in vehicle controls, whereas HPK metanephros exhibited significantly larger increment compared to normal in Evelorimus treatment. These results suggested that the regulation of mTOR signaling with Astrin is related with metanephric phenotype of HPK. Furthermore, Six2-positive MM cells were markedly decreased in HPK metanephros cultured for 3 days, indicating early loss of MM cells. In order to analyze the affect of Astrin defect in MM cells, I established culture system of isolated MM cells from E14.5 metanephros in individual rat embryo. Primary-cultured MM cells derived from normal metanephros expressed mesenchymal markers and Astrin but not epithelial and stromal markers. Passage 1 (P1)-MM cells derived from HPK metanephros showed decreased expression of mesenchymal markers and increased expression of a stromal marker. RT-PCR fragment including insertion mutation of Astrin transcript was hardly detected in HPK P1-MM cells. These results indicated altered stemness in HPK MM cells losing Astrin function. Furthermore, I observed increased apoptosis in Six2-positive HPK P1-MM cells, suggesting cultured HPK MM cells replicate similar phenotype as shown in vivo. Although both normal and HPK P1-MM cells formed immature podoplanin-positive clusters through induction with embryonic spinal cord, clusters in HPK MM cells look like immature and small compared to normal MM cells, suggesting it need more time to form glomerulus in HPK compared to normal.

In summary, the present study suggested that the loss of Astrin changes stemness in MM cells and decreases signals related with interaction between

MM and UB. It is also suggested that increased apoptosis and decreased proliferation in MM cells induce reduced branching of UB with thinning of nephron formation layer and early reduction of stem cells. These sequential events might finally lead to 80% nephron reduction. Interestingly my experiments also suggested that these defective processes are involved in hyperactivation of mTOR signaling under the loss of Astrin. In HPK rats, resulting 80% nephron reduction causes CKD at adult and leads to renal fibrosis at advanced age. The progression of CKD in HPK rats is characterized by early and slow progression of glomerular sclerosis, later appearance and rapid progression of interstitial fibrosis, and specifically macrocytic erythropenia. In last chapter, I described my collaborating research demonstrating that low dose of Everolimus treatment for long period attenuates renal dysfunction and fibrosis in HPK rats. These drastic effects might be mediated by preventing PDGF signaling by mTOR inhibition. Taken together, through a series of my studies, it was indicated that activation of mTOR signaling is associated with not only fibrosis via increased myofibroblasts but also early reduction of mesenchyme nephrogenic progenitors. I hope in future these evidences will contribute to reveal congenital risk for CKD, to improve renal regeneration therapies, and to develop molecularly targeted drag therapies against renal fibrosis.