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## Role of tubular scattered cells of the kidney in disease and regeneration





# Role of tubular scattered cells of the kidney in disease and regeneration

Krzysztof Krawczyk



**LUND**  
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DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden.  
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<p><b>Abstract</b></p> <p>With well over 700 000 deaths every year worldwide, kidney disease constitutes an immense health problem for patients and society. The total global number of people with kidney disease regardless of severity amounts to a staggering 600 000 000. Not surprisingly, the total health care expenditure associated with kidney disease is second to no other costs, including oncology/cancer. If acute or chronic kidney disease is allowed to progress they may eventually lead to end-stage renal disease. This is unfortunately not an unusual development, since specific treatments for renal disease are virtually none, and current treatments mostly focus on symptoms rather than specifically altering disease progression. The end-stage renal disease is a lethal condition if renal replacement therapy is not amenable in the form of either dialysis or more seldom renal transplantation. Obviously, kidney injury only causes clinically relevant organ damage if the regenerative capacity of the kidney is overwhelmed. Surprisingly the basis for and regulation of kidney regeneration is still not unequivocally established. This is why it is so important to understand the processes behind kidney injury and regeneration.</p> <p>This thesis focuses on the cellular basis for kidney regeneration by investigating the so-called tubular scattered cells (TSCs). These are of central importance for renal injury and regeneration. Whether these cells are stem or progenitor cells or represent cellular reactions to injury is an unsettled issue, despite the intense investigation. First, we established a novel protocol based on fluorescence activated cell sorting (FACS) for rare cell isolation, allowing us to isolate good quality RNA from cell suspensions after fixation, permeabilization, and intracellular antibody labelling.</p> <p>Next, we focused on mechanistic aspects of the kidney injury and regeneration by studying the transcription factor MKL1. It was shown to control proximal tubular cell expression of caveolin-1, a protein not present in healthy kidney but highly expressed post-renal injury.</p> <p>In the third paper, we investigated the carrier protein polymeric immunoglobulin receptor (pIgR). In normal kidney, we showed this protein to be expressed by TSCs and expression increases significantly after kidney injury and we postulate that this serves to protect the kidney from, among others, ascending bacterial infections by facilitating excretion of IgA into the urine.</p> <p>Next, we analyzed the expression pattern of the transcription factor SOX9 in a normal and diseased kidney. We and others have found SOX9 to be associated with TSCs. We show that SOX9 expression is increased in injured kidney tissue and that the cellular expression is modulated by chemokines secreted from secondary inflammatory infiltrates attracted by tubular injury.</p> <p>Last, we investigated another aspect of TSC pathology. We suggest that papillary renal cell carcinoma (pRCC) is derived from TSCs and that pRCC might use regenerative programs associated with TSCs. We studied the most characteristic feature of the pRCC tumor, which is the presence of foamy macrophages in the papillary fronds. We identified a set of cytokines with the capacity to attract monocytes into the tumor tissue and convert these into M2 phenotype foam cells resulting in the characteristic histology.</p>		
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# Role of tubular scattered cells of the kidney in disease and regeneration

Krzysztof Krawczyk



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A kidney shaped word cloud with word size representing the frequency with which the words were used in the thesis.

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*“We’ve done studies, you know.  
60% of the time, it works every time.”*

Brian Fantana, *Anchorman*



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# List of Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

- I. Nilsson, H.\*, **Krawczyk, K. M.\***, Johansson, M. E. "High salt buffer improves integrity of RNA after fluorescence-activated cell sorting of intracellular labeled cells." J Biotechnol 192 Pt A: 62-65, (2014).
- II. **Krawczyk, K. M.**, Hansson, J., Nilsson, H., Krawczyk, K. K., Swärd, K., Johansson, M. E. "Injury induced expression of caveolar proteins in human kidney tubules - role of megakaryoblastic leukemia 1." BMC Nephrol 18(1): 320, (2017).
- III. **Krawczyk, K. M.**, Nilsson, H., Nyström, J., Lindgren, D., Leandersson K. and Johansson M. E. "Polymeric immunoglobulin receptor expression is induced by kidney injury related cytokines resulting in increased urinary levels of secretory IgA". (Manuscript in preparation).
- IV. **Krawczyk, K. M.**, Nilsson, H., Leandersson, K. and Johansson, M. E. "Kidney injury induces SOX9 expression in human proximal tubules by cytokine release from inflammatory cell infiltrates". (Manuscript in preparation).
- V. **Krawczyk, K. M.\***, Nilsson, H.\*, Allaoui, R., Lindgren, D., Arvidsson, M., Leandersson, K., Johansson, M. E. "Papillary renal cell carcinoma-derived chemerin, IL-8, and CXCL16 promote monocyte recruitment and differentiation into foam-cell macrophages." Lab Invest 97(11): 1296-1305, (2017).

\*these authors contributed equally to this paper

## Papers not included in this thesis

- I. Markowska, K., Grudniak, A. M., **Krawczyk, K.**, Wróbel, I., Wolska, K. I. "Modulation of antibiotic resistance and induction of a stress response in *Pseudomonas aeruginosa* by silver nanoparticles." *J Med Microbiol* 63(Pt 6): 849-854, (2014).
- II. Lindgren, D., Eriksson, P., **Krawczyk, K.**, Nilsson, H., Hansson, J., Veerla, S., Sjölund, J., Höglund, M., Johansson, M. E., Axelson, H. "Cell-Type-Specific Gene Programs of the Normal Human Nephron Define Kidney Cancer Subtypes." *Cell Reports* 20(6): 1476-1489, (2017)
- III. **Krawczyk, K. M.**, Matak, D., Szymanski, L., Szczylik, C., Porta, C., Czarnecka, A. M. "Culture in embryonic kidney serum and xeno-free media as renal cell carcinoma and renal cell carcinoma cancer stem cells research model." *Cytotechnology* 70(2): 761-782, (2018).

# Abbreviations

<b>AKI</b>	Acute kidney injury
<b>ATI</b>	Acute tubular injury
<b>ATN</b>	Acute tubular necrosis
<b>BMP</b>	Bone morphogenetic protein
<b>ccRCC</b>	Clear cell renal cell carcinoma
<b>CD</b>	Collecting duct
<b>chRCC</b>	Chromophobe renal cell carcinoma
<b>CKD</b>	Chronic kidney disease
<b>CTAL</b>	Cortical thick ascending limb
<b>DCT</b>	Distal convoluted tubule
<b>DT</b>	Distal tubule
<b>EGFR</b>	Epidermal growth factor
<b>ESDR</b>	End stage renal disease
<b>GFR</b>	Glomerular filtration rate
<b>HIF</b>	Hypoxia inducible factor
<b>HSC</b>	Hematopoietic stem cell
<b>HSP</b>	Heat shock protein
<b>IFN<math>\gamma</math></b>	Interferon gamma
<b>IgA</b>	Immunoglobulin A
<b>IRI</b>	Ischemia reperfusion injury
<b>ISC</b>	Intestinal stem cell
<b>LPC</b>	Liver progenitor cell
<b>LPS</b>	Lipopolysaccharides

<b>LRC</b>	Label retaining cell
<b>MKL1</b>	Megakaryoblastic leukemia 1
<b>MTAL</b>	Medullary thick ascending limb
<b>NHE3</b>	Sodium-Hydrogen exchanger 3
<b>NK-cell</b>	Natural killer cell
<b>PD1</b>	Programmed cell death protein 1
<b>PEC</b>	Parietal epithelial cells
<b>pIgR</b>	Polymeric immunoglobulin receptor
<b>pRCC</b>	Papillary renal cell carcinoma
<b>PT</b>	Proximal tubule
<b>RCC</b>	Renal cell carcinoma
<b>SGLT</b>	Sodium glucose linked transporter
<b>sIgA</b>	Secretory IgA
<b>SOX9</b>	(Sex-determining region Y)-Box 9
<b>TAL</b>	Thick ascending limb
<b>TAM</b>	Tumor associated macrophage
<b>TDL</b>	Thick descending limb
<b>TERT</b>	Telomerase reverse transcriptase
<b>TGFβ</b>	Transforming growth factor beta
<b>TNFα</b>	Tumor necrosis factor alpha
<b>TSC</b>	Tubular scattered cells
<b>VEGF</b>	Vascular endothelial growth factor
<b>VHL</b>	von Hippel-Lindau protein

# Abstract

With well over 700 000 deaths every year worldwide, kidney disease constitutes an immense health problem for patients and society. The total global number of people with kidney disease regardless of severity amounts to a staggering 600 000 000. Not surprisingly, the total health care expenditure associated with kidney disease is second to no other costs, including oncology/cancer. If acute kidney disease such as e.g. IgA nephritis or chronic kidney disease such as hypertensive nephropathy is allowed to progress they may eventually lead to end-stage renal disease. This is unfortunately not an unusual development, since specific treatments for renal disease are virtually none, and current treatments mostly focus on symptoms rather than specifically altering disease progression. The end-stage renal disease is a lethal condition if renal replacement therapy is not amenable in the form of either dialysis or more seldom renal transplantation. Obviously, kidney injury only causes clinically relevant organ damage if the regenerative capacity is overwhelmed. Perhaps surprisingly, the basis for and regulation of kidney regeneration is still not unequivocally established. This is why it is so important to understand the processes behind kidney injury and regeneration.

This thesis focuses on the cellular basis for kidney regeneration by investigating the so-called tubular scattered cells (TSCs). These are of central importance for renal injury and regeneration. Whether these cells are stem or progenitor cells or represent cellular reactions to injury is an unsettled issue, despite intense the investigation. Much work has been done using transgenic mice. Even though such models are common and generate very important insights, there are significant species differences between mouse and human kidneys regarding TSCs. For this reason, we have focused on human primary material and we have sought to develop new protocols to expand knowledge in the kidney field.

First, we established a novel fluorescence activated cell sorting (FACS) based protocol allowing us to isolate rare cells from cell suspensions. The protocol allows for the extraction of good quality RNA after fixation, permeabilization, and internal antibody labelling. We believe that this protocol will allow us and others to better analyze small cell populations such as TSCs.

Next, we focused on the mechanistic aspects of kidney injury and regeneration by studying MKL1. This transcription factor was shown to control PT cell expression of caveolin-1, a protein not present in healthy kidney but highly expressed post-

renal injury. In the third paper, we investigated the carrier protein polymeric immunoglobulin receptor (pIgR). In a normal kidney this protein is expressed by TSCs but expression increases dramatically after kidney injury and we postulate that this serves to protect the kidney from, among others, ascending bacterial infections by allowing IgA export into the urine of injured tubular segments. Next, we analyzed the renal expression pattern and regulation of the transcription factor SOX9, that we and others have found to be associated with TSCs. We show that SOX9 expression is increased in injured kidney cells and that the expression might be modulated by chemokines secreted from secondary inflammatory infiltrates attracted by tubular injury.

Lastly, we investigated another aspect of TSC pathology. We suggest that papillary renal cell carcinoma (pRCC) is derived from TSCs and that pRCC might use regenerative programs normally associated with TSCs. We studied the most characteristic feature of the pRCC tumor, which is the presence of foamy macrophages in the papillary fronds. We identified a set of cytokines with the capacity to attract monocytes into the tumor tissue and to convert these into the foam cells typical for pRCC.





# Popular scientific summary

## English version

The kidneys are essential in order to maintain electrolyte balance and blood pressure, to remove waste products from our body through filtration of blood and for the production of urine. They are extremely complex organs built of more than thirty different types of cells. The kidneys can be divided into two main parts: the outer part called the kidney cortex and the inner part, the medulla. Even though both are important for the function of the kidney this thesis is focused on the cortex, where the filtering function of the kidneys occurs, and which is mainly affected by kidney diseases. The functional unit of the kidney is the nephron. It consists of the filtration units called glomeruli, that are connected to the renal tubules. The tubules are a cellular conduit of high anatomic and histological complexity starting with the proximal tubules (PT). The PTs are followed by further segments such as Henle's loop, distal tubules, and collecting ducts. The function of the tubules is the reabsorption of water and small molecules from the primary urine. This is of utmost importance since the glomeruli produce around 200 liters of primary urine every day, but only about 1,5 liters are eventually voided. The PT cells perform most of the ATP dependent reabsorption, rendering these cells sensitive to nutrient and oxygen deprivation. In fact, the PT cells are among the most metabolically sensitive cells in the human body. It is a finely tuned system that can be easily affected by a disease.

Whereas kidney cancer is a well-known and feared disease, medical kidney diseases have often more serious consequences. According to a worldwide study from 2010 investigating causes of deaths, cancers of the kidney and other urinary organ caused around 160 000 yearly deaths. In the same period, other kidney diseases caused more than 730 000 deaths. In addition to primary kidney diseases, the kidneys are often affected secondary to the major diseases i.e. diabetes and hypertension. Specific treatments for kidney disease are lacking regardless of cause and therefore kidney disease is often progressive, resulting in a need for chronic renal replacement therapy such as dialysis or preferably transplantation. Dialysis requires spending many hours per week at the dialysis clinic, which is very time consuming and inconvenient for the patient. The cost of dialysis is also extremely high, placing it as one of the most expensive procedures overall for the health care system. The last

resort is kidney transplant, however, most of the patients are not suitable for transplantation, due to concurrent disease and a shortage of organs for transplantation.

This explains why it is of great importance to understand how kidney disease translates into renal injury and how the kidney can recover by regeneration. While the mechanisms and cells responsible for regeneration in other organs are relatively well established, this is not true for the kidney. Other epithelial organs such as intestine or skin are maintained by adult stem cell populations, cells that also play vital roles for regeneration. In contrast, normal kidney tissue is mitotically almost quiescent. The classical view on kidney regeneration is that stochastically surviving cells dedifferentiate, divide and then reform a mature epithelium. This model was recently challenged by the alternative hypothesis that the kidney harbors a distinct stem or progenitor cell population. Regardless, it is known that human kidneys contain 2-6% of scattered tubular cells expressing markers not present in the surrounding epithelial cells of the proximal tubules. These cells are thought to somehow be involved in the process of regeneration after injury in the kidneys. The two hypotheses could both be applied regarding the interpretation of the cell biology of these cells. As stated above, one suggests that these cells are resident stem cells present in the kidney cortex. Others argue that cells driving the regeneration process are randomly surviving cells that can acquire a more robust phenotype, and can be induced to express these markers. The aim of this thesis is to further study the function, origin and regulation of the scattered tubular cells found also in healthy kidneys.

Paper I describes a new protocol for the isolation of high quality RNA from a low number of cells from a big pool of mixed cells. The protocol is based on one of the most commonly used techniques in such cases called fluorescent activated cell sorting (FACS). It allows to sort out a low number of cells from other cells based on specific fluorescently labeled antibodies. One of the main challenges in this field is the ability to extract intact genetic material for analysis from the sorted cells. In standard protocols, the genetic material is not protected well enough to be used for further studies. The protocol developed in Paper I allows us to avoid this obstacle and it will be used to isolate and study the tubular scattered cell population.

Papers II, III and IV focus on an analysis of kidney response to injury. Paper II investigates a protein called caveolin-1. We present evidence that the transcription factor MKL1 is a regulator of caveolin-1 – a protein not expressed in healthy kidneys but profusely expressed after injury. Results presented in this work shed light onto an important mechanism and open doors to potential manipulation of caveolin-1 levels that can be important for the regenerating kidney.

In paper III we study the polymeric immunoglobulin receptor (pIgR), another marker for the tubular scattered cells (TSCs). pIgR was originally discovered in the

breast glands and airways, where it is responsible for transporting immunoglobulin A (IgA) into mothers' milk and airway secretions respectively. It is also known to transport IgA across the intestinal epithelium. IgA plays an important role as the first line of defense against invading bacteria. Interestingly, we discovered pIgR to be present in the TSCs, where it plays the same role. In paper III we present how pIgR expression increases substantially after kidney injury and investigate the factors responsible for pIgR regulation. We propose that its role is to provide additional protection for the already injured kidney by preventing the ascension of injurious pathogens up the urinary tract and into the kidney.

Paper IV investigates a role of the transcription factor SOX9 in the TSCs. We established that SOX9 expression colocalizes with the other known TSC markers, and investigated the regulation of its expression in kidney cells. Our results suggest that SOX9 is one of the early responders after kidney injury and we hypothesize that it is recruited during the regeneration process.

Lastly, in paper V, we investigate the second most common kidney cancer type called papillary renal cell carcinoma (pRCC). The treatment options available for this cancer type are very sparse and in most cases are limited to radical nephrectomy. Originally our interest in this cancer type arose from our hypothesis that this cancer form arises from the scattered tubular cells. Little is known about the physiology of this tumor, hence we focused on its most characteristic property which is the presence of so called "foamy macrophages". In this study, we have established that pRCC cells secrete cytokines, chemicals responsible for cell signaling affecting the behavior of cells, that specifically attract macrophages. Depending on the macrophages' type, they can have pro- or anti-tumoral effects. We also established that their foamy appearance, believed to be caused by retention of lipids or glycogen in their cytoplasm, is caused by the accumulation of lipids. Our results give hope for new immune therapies that could alter the attraction of macrophages into the pRCC tumors.

These 5 papers attempt to broaden our knowledge about kidney regeneration after the injury by providing methods allowing for more precise research and helping to understand the complicated field of scattered tubular cells. They shed light onto the regulation of expression of genes present in TSCs and look into one of the cancers hypothesized to arise from them.

## Wersja polska

Nerki są niezbędne dla utrzymania odpowiedniego stężenia elektrolitów, ciśnienia krwi oraz wydalania zbędnych produktów przemiany materii z naszego ciała poprzez produkcję moczu. Charakteryzują się one skomplikowaną strukturą wewnętrzną i są zbudowane z ponad trzydziestu rodzajów komórek. Nerki składają się z zewnętrznej części nazywanej korą nerki oraz części wewnętrznej nazywanej rdzeniem nerki. Mimo iż obie struktury są niezwykle istotne dla funkcjonowania nerki, niniejsza praca skupia się na zagadnieniach dotyczących kory nerki, szczególnie wrażliwej na zmiany chorobowe.

Nefron jest podstawową jednostką funkcjonalną nerki. Składa się on z dwóch ważnych elementów jakimi są kłębuszek nerkowy oraz kanaliki nerkowe. Zadaniem kłębuszka nerkowego jest filtracja, podczas gdy kanaliki nerkowe, które podzielić można na kanalik proksymalny i dystalny, odpowiadają za resorpcję moczu pierwotnego. Kłębuszki nerkowe produkują około 200 litrów moczu pierwotnego dziennie. Ta ogromna objętość ulega redukcji dzięki kanalikom nerkowym, które resorbują 99% moczu pierwotnego, tym samym zapobiegając odwodnieniu organizmu. System ten, w którym nefron pełni funkcjonalną rolę, jest precyzyjnie regulowany, a jego praca może zostać łatwo zaburzona przez stany chorobowe.

Podczas gdy rak jest dobrze znaną wszystkim chorobą, nienowotworowe choroby nerki są często znacząco bardziej groźne dla ludzkiego zdrowia. Międzynarodowe badanie z 2010 roku analizujące przyczyny śmierci na całym świecie, wskazuje na raka nerki jako przyczynę 160 000 zgonów rocznie. W tym samym okresie czasu, choroby nerki i ich powikłania przyczyniły się do 730 000 zgonów. Jednymi z głównych przyczyn chorób nerki są cukrzyca, nadciśnienie oraz kłębuszkowe zapalenie nerki. Pomimo dostępności pewnej liczby terapii, choroby te postępują bardzo szybko i w większości przypadków jedyną deską ratunku pozostają dializy bądź przeszczep nerki. Pomimo stosunkowo wysokiej skuteczności tych metod należy pamiętać, iż dializy są bardzo wyczerpujące dla pacjentów ze względu na ilość czasu jaką pochłaniają. Przeciętnie zajmują kilka godzin i muszą być wykonywane kilka razy w tygodniu. Wiąże się to nie tylko z wysokim dyskomfortem dla pacjenta, ale również z ogromnym kosztem dla służby zdrowia. Głównym problemem związanym z przeszczepem nerki oprócz dostępności organów i procedury samej w sobie jest fakt, iż większość pacjentów dotkniętych cukrzycą, nie kwalifikuje się do tego zabiegu.

Z tego powodu zrozumienie procesów prowadzących do uszkodzenia nerki jak również jej regeneracji jest niezmiernie ważne. Komórki odpowiedzialne za regenerację większości organów zbudowanych z komórek nabłonkowych, takich jak skóra, jelito cienkie i grube oraz wątroba, są stosunkowo dobrze opisane. W przypadku tych organów najważniejszą rolę dla regeneracji pełnią komórki

macierzyste stale obecne wewnątrz organu. Sytuacja wygląda inaczej dla nerki. Zdrowa nerka prawie nie posiada aktywnie dzielących się komórek. W klasycznym ujęciu komórki, które losowo przeżywają chorobowe warunki, mogą ulec procesowi odróżnicowania, a następnie przyczynić się do regeneracji nerki. Stosunkowo niedawno uznanie części środowiska naukowego zaczęła zyskiwać teoria twierdząca, że nerka posiada populację komórek macierzystych stale obecnych w tym organie. Wiele publikacji naukowych wskazuje na obecność „rozsianych” komórek kanalików nerkowych (RKKN, ang. tubular scattered cells), a ich częstotliwość występowania szacowana jest na około 2-6% względem zwykłych komórek proksymalnych kanalików nerkowych. Komórki te charakteryzują się obecnością wielu markerów charakterystycznych dla komórek macierzystych, a nieobecnych w normalnych komórkach kanalików nerkowych. Obecnie naukowcy debatują nad dwiema hipotezami tłumaczącymi pochodzenie tych komórek. Pierwsza sugeruje, iż RKKN są stale obecne w rdzeniu nerki, podczas gdy druga teoria argumentuje, iż komórki te są zwykłymi komórkami nerki losowo opierającymi się śmierci komórkowej i wspomagającymi regenerację nerki. Celem niniejszej pracy jest dalsza analiza funkcji, pochodzenia i sposobu regulacji tych komórek.

Artykuł I opisuje nowy protokół mający zastosowanie podczas analizy FACS (ang.) – fluorescencyjnie aktywowanej cytometrii przepływowej. Metoda ta pozwala na sortowanie komórek na podstawie ich charakterystycznych markerów oraz izolację bardzo małych ilości komórek z dużej populacji. Jednym z głównych problemów w tej dziedzinie naukowej jest analiza materiału genetycznego wyizolowanego z sortowanych komórek. Przy zastosowaniu standardowych protokołów, materiał genetyczny a szczególnie RNA ulega nieodwracalnemu zniszczeniu. Protokół opisany w artykule I prezentuje metodę, pozwalającą na ominięcie tej przeszkody i będzie użyty w dalszych badaniach nad rozsiyanymi komórkami kanalików nerkowych.

Artykuły II, III oraz IV skupione są na analizie odpowiedzi nerki na stany chorobowe powodujące uszkodzenie komórek budujących ten organ. Artykuł II opisuje badania białka nazywanego caveolin-1 oraz jego białka regulacyjnego Mkl1, oraz dlaczego ich interakcja jest istotna dla chorobowo zmienionej nerki. W artykule tym prezentujemy dowód na to, że Mkl1 jest regulatorem caveolin-1 – białka, którego ekspresja nie jest obserwowana w zdrowej nerce, podczas gdy chore nerki wykazują jego wysokie poziomy. Wyniki opublikowane w tym artykule rzucają światło na ten istotny mechanizm jak również otwierają drzwi do potencjalnych manipulacji caveolin-1, które mogą mieć istotne znaczenie dla regeneracji nerki.

W artykule III analizujemy funkcję białka pIgR będącego polimerycznym receptorem immunoglobulin oraz jednym z markerów RKKN. Białko to zostało

pierwotnie odkryte w gruczole mlekowym oraz w nabłonku dróg oddechowych. Funkcją białka pIgR jest odpowiednio transport immunoglobuliny A (IgA) do mleka matki oraz wydzielin układu oddechowego. Proces ten występuje również w jelitach. Główną rolą IgA jest powstrzymanie bakterii przed wnikaniem w głąb naszego organizmu. Nasze badania doprowadziły do ciekawego odkrycia jakim jest fakt, iż białko pIgR obecne jest w RKKN gdzie pełni ono tą samą rolę. W artykule III prezentujemy jak ekspresja białka pIgR znacząco wzrasta po uszkodzeniu nerki. Nasze badania skupiają się również na czynnikach odpowiedzialnych za regulację ekspresji pIgR. Sugerujemy, iż rolą pIgR jest dodatkowa ochrona uszkodzonej nerki przed niekorzystnymi czynnikami zewnętrznymi a szczególnie bakteriami.

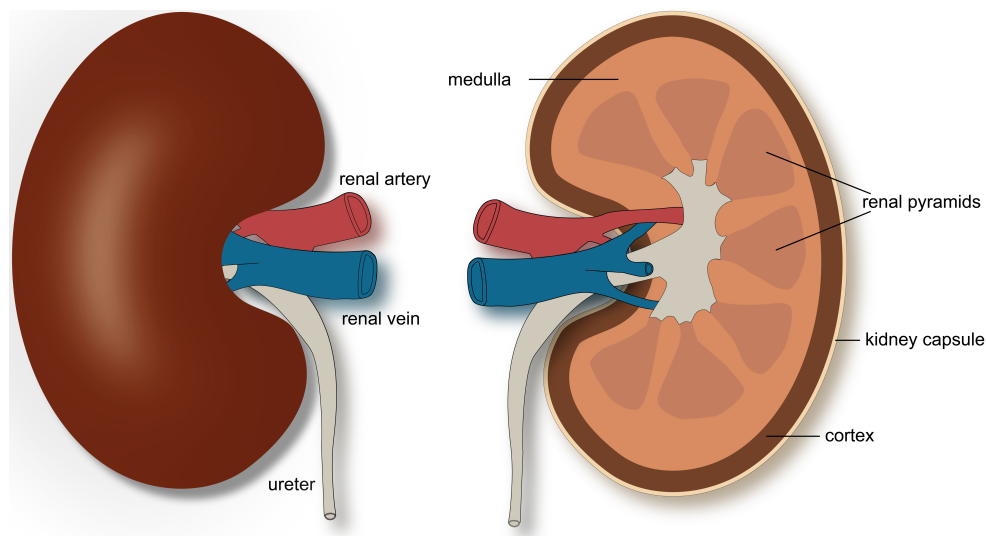
Artykuł IV analizuje rolę czynnika transkrypcyjnego SOX9 w RKKN. Na podstawie naszych badań donosimy, iż ekspresja SOX9 współwystępuje z innymi markerami RKKN. Nasze wyniki sugerują, że ekspresja SOX9 ulega zwiększeniu bardzo krótko po uszkodzeniu nerki, oraz że SOX9 jest bardzo istotne w procesie regeneracji uszkodzonego organu.

Ostatni artykuł skupia się na drugim co do częstości występowania rodzaju raka nerki nazywanego rakiem brodawkowatym. Terapie dla tego rodzaju raka są bardzo nieliczne i w większości przypadków jedynym rozwiązaniem jest chirurgiczne usunięcie nerki dotkniętej zmianami nowotworowymi. Pierwotnie nasze zainteresowanie tym rodzajem raka wynikało z publikacji naukowych sugerujących jego pochodzenie wywodzące się z RKKN. Jednak, ponieważ ten rodzaj raka jest bardzo mało zbadany, artykuł ten skupia się na fizjologii raka brodawkowatego, a w szczególności obecności „piankowatych” makrofagów (ang. foamy macrophages). W tej pracy ustaliliśmy, że komórki raka brodawkowatego produkują cytokiny, związki chemiczne odpowiedzialne za sygnalizację międzykomórkową zmieniające zachowanie komórek, specyficznie przyciągające makrofagi. W zależności od typu makrofagów, mogą one mieć właściwości promujące bądź hamujące rozwój raka. Nasze badania wykazują również, że piankowaty wygląd makrofagów spowodowany jest akumulacją lipidów w ich cytoplazmie. Nasze wyniki dają nadzieję na nowe terapie immunologiczne, które mogłyby wpływać na zmianę zachowania makrofagów infiltrujących guzy raka brodawkowatego nerki.

Tych pięć artykułów jest próbą poszerzenia naszej wiedzy na temat regeneracji nerki występującej po jej uszkodzeniu. Artykuły te są źródłem nowych metod pozwalających na dokładniejsze badania oraz odpowiedzią na część pytań dotyczących skomplikowanej dziedziny rozsianych komórek kanalików nerkowych. Nasza praca rzuca światło na regulację ekspresji genów obecnych w RKKN oraz skupia się na jednym z rodzajów raka nerki, który może mieć w nich swoje początki.

# The kidney

The kidneys are bean shaped organs located in the back of the abdominal cavity on the left and right side of the body. Their size varies between sexes and individuals, with an average length of around 11 to 12<sup>1</sup> centimeters and a weight of 115/120 to 155/170 grams in men/women<sup>2</sup>. On the outside, the kidneys are covered with a capsule that adheres to the kidney (Figure 1).

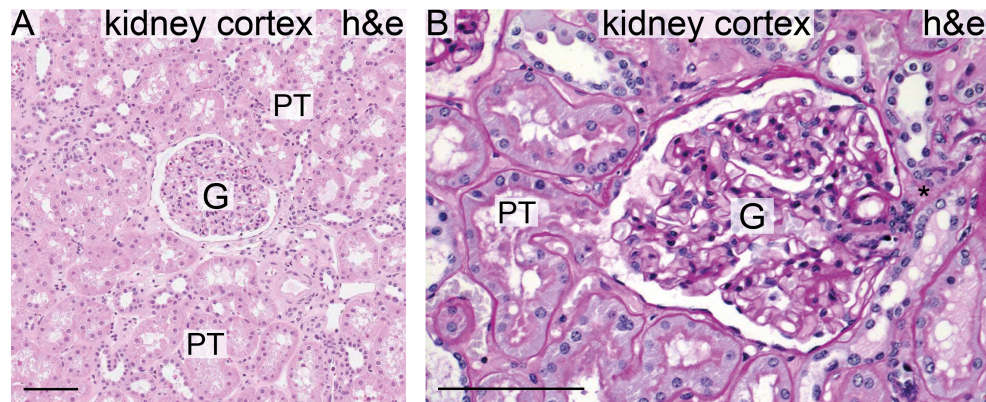


**Figure 1. Overview of the kidney gross anatomy.**

The kidneys are surrounded by perinephric fat and enveloped in the so called Gerota's fascia. The kidneys are essential for homeostasis, among other tasks they maintain electrolyte balance, regulate blood pressure and remove waste products from the body through the production of urine. The kidney is a complex organ built of around 30 different cell types. Anatomically, the kidney can be divided into two major parts: the renal cortex and the renal medulla. The kidney cortex is the outer, more granular part of the kidney, which is around one centimeter thick<sup>3</sup>. It is very important for the kidney function as this is where the ultrafiltration occurs. The



medulla is the much thicker, inner part of the kidney. It contains 8 to 18 renal pyramids having high concentration of loops of Henle and collecting ducts – nephron segments – arranged in a linear orientation. A histological section showing the main components of the kidney cortex is depicted in Figure 2.



**Figure 2. Healthy kidney cortex morphology.**

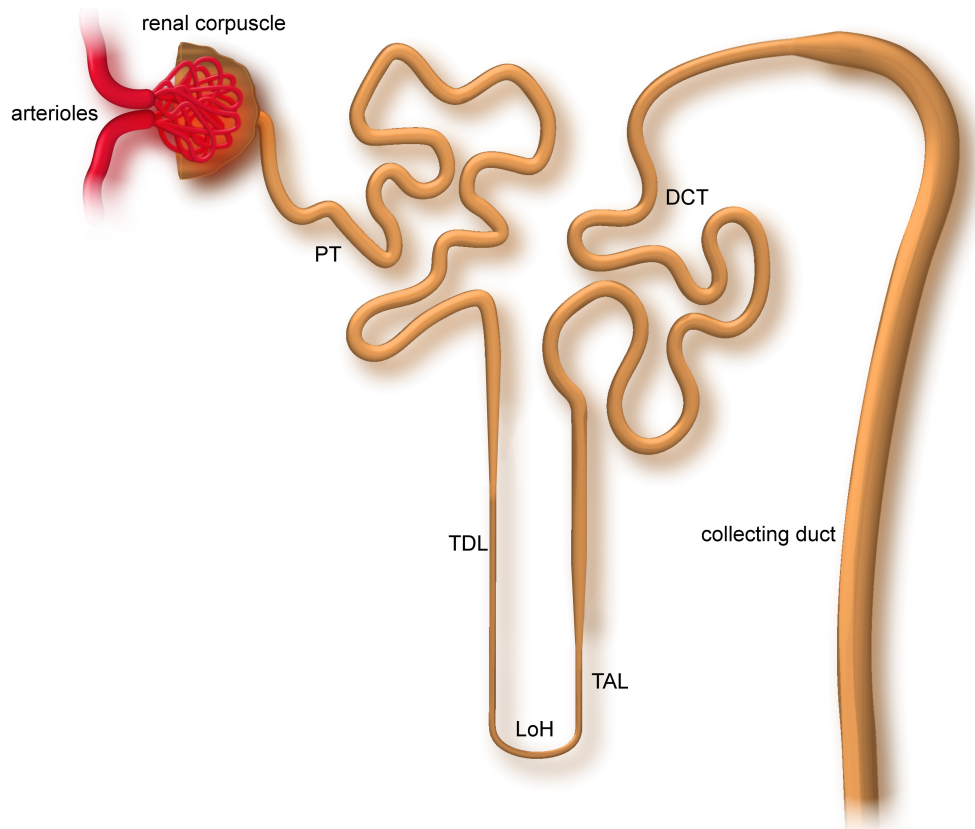
A, a representative image of healthy human kidney cortex stained with hematoxylin and eosin. A glomerulus and densely packed tubules can be observed. In B, higher magnification reveals a glomerulus with arterioles entering from the hilus side (marked with a \*) and the entry into the proximal tubule on the opposing side. Distal tubules characterized by a smaller diameter can also be seen. PT – proximal tubule, DT – distal tubule, G – glomerulus. Scale bars = 100  $\mu\text{m}$ .

## The nephron

The nephrons are the functional units of the kidney and there are around 1 million nephrons in each kidney. In short, each nephron is composed of a renal corpuscle and a renal tubule (Figure 3). The renal corpuscle contains a glomerulus, a round structure with a diameter of around 200  $\mu\text{m}$ , made of a tuft of capillaries enclosed by the Bowman's capsule. The capillary tuft is supported by the mesangium containing mesangial cells and their matrix. The mesangial cells contain actomyosin allowing them to contract and modulate the filtration rate. The largest cells in the glomerulus are the podocytes. They are characterized by prominent foot processes (pedicles), which cover the capillary walls. Adjoining foot processes are separated by small gaps, called filtration slits, creating a natural filter. Bowman's capsule is made of squamous epithelial cells called parietal epithelial cells (PECs) forming the parietal layer.

The renal tubule is a term describing a structurally complex cellular tube whose general function is to reabsorb important compounds and ions from the primary filtrate leaving toxins for excretion. About a quarter of the cardiac output passes

through the kidneys and the key principle behind the kidney's function is free filtration of all solutes up to a certain molecular weight, followed by re-uptake of 99,5% of the primary filtrate, leaving end-products and toxins for excretion. The blood is filtered through a process of ultrafiltration in the glomerulus. Ultrafiltration means that the blood pressure and the concentration difference lead to separation through the filter made of podocytes. High pressure created by the difference between afferent and efferent arterioles' diameter forces small molecules from the blood such as water, amino acids, glucose, urea and sodium chloride through this natural filter into the renal tubules.



**Figure 3. The nephron.**  
Schematic image of the general structure of the nephron. PT – proximal tubule, TDL – thin descending limb, LoH – Loop of Henle, TAL – thick ascending limb, DCT – distal convoluted tubule.

## **Proximal tubule**

The primary glomerular filtrate enters the proximal tubules (PTs). It consists of a convoluted part, being a continuation of the Bowman's capsule, followed by a straight part located in the medullary rays and in total measures around 14 mm. Even though the classification is not clear cut, in humans PTs may be subdivided into three segments: S1, S2 and S3<sup>4,5</sup>. The S1 segment consists of the initial and middle parts of the PT. The S2 segment comprises the end part of the proximal convoluted tubule and the beginning of the straight tubule and the S3 segment is the remaining part of the straight tubule. These segments are characterized not only by different location in the kidney but also by functional differences. Due to its characteristics, the S1 segments have much higher capacity to transport sodium, solutes, amino acids, and fluid than S2 or S3 segments. The PTs are characterized by the presence of a brush border localized to the luminal side of the tubular cells. The main role of the microvilli is to increase the surface area of the PT cells and in this way to facilitate the reabsorption. PTs reabsorb around 60% of the glomerular filtrate. Another characteristic feature of these cells is the presence of a great number of mitochondria, required by the cells to meet the energy demand created by active transport of the sodium ions. The transport is carried out in a two-step mechanism by specialized  $\text{Na}^+\text{-K}^+\text{-ATPase}$  cation transporters<sup>6</sup> localized to the basolateral plasma membranes. First, through the energy provided by the ATPase, sodium ions are exported from the basolateral membranes. This in turn allows for the passive re-entry of  $\text{Na}^+$  through numerous anti-porters and co-transporters in the apical membrane allowing for the reabsorption of the sodium ions. The electrochemical sodium gradient is thus coupled to secondary active co- or anti-transport systems. The sodium and hydrogen exchanger 3 (NHE3) is one of these important antiporters<sup>7</sup>. It exchanges luminal  $\text{Na}^+$  for hydrogen ions which are secreted into the lumen. Microvilli of the PTs contain high numbers of aquaporin 1, a water channel, allowing for water reabsorption. Other substances reabsorbed by the PTs are organic solutes such as glucose, transported by the sodium glucose linked transporter (SGLT), amino acids, potassium, urea, phosphate, and citrate.

## **Loop of Henle**

The next part of the nephron is the loop of Henle that penetrates deep into the medulla where it doubles back in a hair pin loop. The loop of Henle connects the PTs and the distal tubules (DTs) and it is characterized by selective permeability to water and ions. This feature is very important because it allows the generation of an osmotic concentration gradient in the vicinity of the loop of Henle which forces water to follow the gradient. In this way loss of high volumes of water is prevented and concentrated urine is created. The reabsorption of water and electrolytes in the

loop of Henle is achieved by different specialization of the cells in the descending and ascending limbs of loop of Henle. The descending limb of loop of Henle is permeable to water and not permeable to ions while the ascending limb is impermeable to water but actively pumps the ions out. This allows for reabsorption of water and important ions while reducing the volume of the urine.

### **Distal tubule**

The distal tubule is the next segment of the nephron connecting the loop of Henle and the collecting duct. It consists of three segments: thick ascending limb (TAL), the distal convoluted tubule (DCT) and the macula densa. The thick ascending limb can be further divided into medullary TAL (MTAL) and cortical (CTAL). Functionally MTALs lack aquaporins but they reabsorb sodium thanks to  $\text{Na}^+\text{-K}^+\text{-ATPase}$ . As the MTAL enters the cortex it turns into CTAL characterized by much lower activity of the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  but higher permeability to urea and calcium reabsorption. The DCT shows the highest  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity among the tubular segments<sup>8-10</sup>.

### **Collecting duct**

Finally, the nephron ends in the collecting duct (CD). It runs straight through the cortex into the medulla, perpendicular to the kidney surface, and opens into the renal papilla. It is made of two different types of cells: principal cells and the intercalated cells. Principal cells reabsorb sodium and water while secreting potassium. The function of these cells is controlled by the antidiuretic hormone (vasopressin), enhancing water reabsorption and aldosterone, increasing potassium secretion. Intercalated cells act to secrete hydrogen ions and reabsorb potassium by the  $\text{H}^+\text{-K}^+\text{-ATPase}$ , hence their higher mitochondria content. Presence of these highly specialized cells allows CDs to fine tune the contents of the urine and prepare it for the excretion from the body.

Eventually all components of the nephron combined together are a perfect system ensuring reabsorption of water and all the important solutes, while excreting the toxic metabolism waste products from the body in the form of urine. It is worth pointing out that the kidney is responsible for an astonishing daily shift of almost 200 liters of fluids, containing solutes measured in kilograms back into the body. This process requires high amounts of energy, making the kidney one of the chief resting state consumers of ATP. At the same time such high energy demand makes the kidneys, and especially the proximal tubules, susceptible to stress factors such as numerous kidney diseases, toxicity, hypoxia, and hypertension.

## Kidney development

Before looking into mammalian kidney development, it is important to know how the evolution of the kidney proceeded. Earliest in the development of primitive fish, the kidney was made of a single nephron called pronephros. Its function was to remove waste products from the body making elimination of excess water possible. More developmentally advanced fish are characterized by presence of mesonephros as the excretory organ. It consists of tens or hundreds of nephrons and additionally plays endocrine and hematopoietic functions<sup>11</sup>. As the life on earth progressed from water onto land, kidneys became more and more complex. Reptilians were the first big animals whose life cycle did not depend on water. Their excretory organs are called metanephros and are characterized by a higher number of nephrons. This adaptation was important not only due to their land based lifestyle but also because of their bigger body size. Finally, mammals and birds are characterized by the presence of a mature kidney. This organ is unique due to the presence of loop of Henle's connecting the proximal and distal tubule. The development of the loop of Henle allows for differential gradient creation, allowing for water reabsorption and concentration of urine<sup>12</sup>. Interestingly, mammalian embryological development of the kidney very closely resembles the evolution of the organ itself. First pronephros is developed, followed by mesonephros finally replaced by the mature metanephros.

During embryogenesis, mammalian kidney development starts from cells originating from the intermediate mesoderm, specifically speaking from masses of cells called nephrogenic cords. As mentioned before, pronephros, mesonephros and metanephros are formed, with the first two being transient organs. Pronephros consists of very few basic tubules. By day 25 of gestation, pronephros can no longer be identified. Development of mesonephros is characterized by formation of vesicles from the nephrogenic cord. Each of the vesicles give rise to a nephron containing glomerulus and a tubule that can be separated into proximal and distal fragments based on the thickness of the wall. Finally, the metanephros is formed, which can be divided into renal parenchyma originating from the metanephric mesenchyme and collecting ducts, calyces, renal pelvis and ureter originating from the ureteric bud<sup>13,14</sup>. Human nephron development and maturation stops around birth and no new nephrons can be created after that.

### **Kidney regeneration - a link to the development**

Knowledge regarding the signaling occurring during kidney development is of importance also during studies of kidney regeneration, especially when considering the possible involvement of a progenitor like cell, as will be discussed further in the following chapters. Through extensive studies PAX, SOX, SIX, HOX and FOX

transcription factor families have been identified to be key players during kidney morphogenesis. Of these, the first two are also found in studies of kidney regeneration. The PAX family, especially Pax2 and Pax8, have been shown to be involved in the formation of the nephric duct<sup>15</sup>. Pax2 has also been used in recent studies to specifically mark proximal tubular cells responsible for kidney regeneration<sup>16</sup>. Sox9 was used as a driver for Cre recombinase in two extensive studies on kidney regeneration in recent years<sup>17,18</sup>. Another example of embryonic kidney markers widely used for identification of progenitor like cells in the human kidney, and studies of kidney regeneration, are CD133 and CD24. This pair is expressed in embryonic renal vesicles and S-shaped bodies<sup>19</sup>. Homeodomain transcriptional regulator Six2, active during development, has been used in a lineage tracing study to understand renal development better<sup>20</sup>. Its role as a progenitor marker present in the cap mesenchyme giving rise to the nephron makes it very important for regeneration research. Extensive studies have shown that most of the genes expressed by the cap mesenchyme progenitor cells during development are re-expressed during kidney regeneration. Interestingly, injured kidney epithelium also re-expresses some of the mesenchymal markers such as vimentin and N-cam<sup>21</sup>. This and many other examples stress the importance of knowledge regarding kidney development processes in the studies of markers for renal regeneration. Some authors suggest that renal regeneration very closely resembles kidney development<sup>12</sup>. Others disagree with this point of view arguing that cell types present during the development of the kidney, and during kidney injury and regeneration, are very different. Hence, the reason for overlap in the expression of these genes could be caused by specific environmental conditions activating these specific pathways<sup>22</sup>. Regardless of which point of view is more correct, it is apparent that studies of kidney development are an important part to understand renal regeneration in humans.



# Kidney disease

Kidney diseases can be characterized as a heterogeneous group of disorders that affect the kidney both on structural and functional level<sup>23</sup>. The current state of our knowledge suggests that even mild abnormality in kidney function can lead to increased risk of developing problems in other organ systems. Diseases can be generally classified in terms of structure, cause, function, duration and outcome. In case of kidney disease, it is quite common to divide the diseases based on the affected structure of the kidney. The most common classification on structural level recognizes glomerular disease, tubular disease (which is the main focus of this book), interstitial disease and renal vascular disease. For clinicians, identification of the renal compartment primarily affected by the disease is one of the most basic and most important decisions. What complicates things is that the primary injury of one compartment very often causes secondary injury in the other. All these aspects are important in order to correctly categorize the disease and choose the appropriate course of action to help the patient. Another classification system focuses on the duration of the disease. In general terms, if kidney disease persists for less than 3 months, it is classified as acute and if it persists for over 3 months, it is classified as chronic. The relationship between acute and chronic diseases is complex, with acute kidney injury leading to chronic disease and chronic injury increasing the risk of developing acute syndrome<sup>24</sup>. One of the big challenges related to kidney disease is that clinical symptoms are often very mild and, in many cases, nonspecific.

During most forms of renal disease, the tubules, and especially the proximal tubules (PTs), sustain primary or secondary injury. The tubules are injured by a host of tubulointerstitial diseases and tubular injury causes about 85% of all the renal failure cases seen in hospitals. Being assigned the enormous task of reabsorption, especially the proximal tubules require large amounts of energy. This is the reason to why PT cells contain high numbers of mitochondria. Conversely, this renders the PT cells exceptionally sensitive to reduction in oxygenation or nutrient supply. A large number of diseases cause reduced oxygen supply to the proximal tubules, which results in massive cell death and leads to kidney loss of function, the histological equivalent of which is called acute tubular necrosis (ATN).

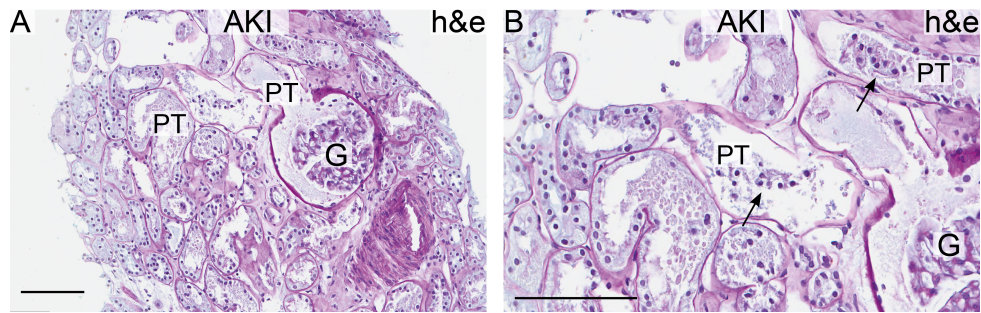


## Acute kidney injury

One of the forms of kidney disease dramatically affecting tubular function is acute kidney injury (AKI). Previously known as acute renal failure, it is relatively common among hospital admissions, with a frequency of almost 2% of all hospitalized patients<sup>25</sup>. It has a higher incidence rate especially among critically ill patients suffering from congestive heart failure, septic shock, chronic kidney disease, chronic lung disease and cancer<sup>25-27</sup>, and generally among intensive care patients<sup>28,29</sup>, with a frequency reaching 31%<sup>30</sup>. AKI is characterized by a loss of kidney excretory function and decreased glomerular filtration rate, and it usually occurs within hours or days<sup>31</sup>. Some of the consequences are increase in serum creatinine and inability to keep fluid and electrolyte homeostasis. From a pathologic point of view, AKI results in interstitial edema causing the kidneys to swell, resulting in a weight increase of around 25% to 30%. Vasa recta becomes congested and causes cortex to look pale and medulla to appear intensive red. The mortality rate is very high for AKI patients, exceeding 50%<sup>32</sup>. Worryingly, the incidence of AKI is increasing at a fast pace. Recent studies show that the higher prevalence of the disease noted in the past decades is not due to better diagnostic tools, but is in fact a true increase<sup>33</sup>. AKI is associated with a very high cost of care, as many patients require complicated therapy including dialysis and kidney transplantation<sup>34-36</sup>. AKI can be caused by various factors such as decreased blood flow (prerenal failure), impairment of the urinary collecting system (postrenal failure), or other diseases such as interstitial nephritis, glomerulonephritis or acute tubular injury.

### **Acute tubular injury**

Acute tubular injury (ATI) is one of the main results of acute kidney injury. Even though AKI can drastically decrease kidney function, patients with milder form of tubular injury sometimes show very subtle laboratory evidence of the injury. If loss of resorptive function appears, increase of many substances such as urea, glucose, and amino acids can be present in the urine. Higher degree of injury may cause appearance of tubular cells in the patient's urine, hematuria – presence of red blood cells in the urine, or oliguria – decreased urinary output. ATI can be divided into two main subcategories: nephrotoxic ATI and postischemic ATI. Nephrotoxic ATI associated injury is usually more pronounced. Epithelial cell damage is extensive and it affects mainly proximal tubules with more subtle damage to the distal nephron. The degree of nephrotoxic injury depends on the toxic agent and its dose. Most commonly observed pathologic changes in the tubules include loss of brush border, swelling of the cytoplasm, loss of individual tubular cells, flattening of the tubular epithelium, necrosis and apoptosis of tubular cells<sup>37-39</sup> (Figure 4).



**Figure 4. Acute kidney injury.**

A and B present low and high magnification images of acutely injured kidney respectively. Flattened PT cells can be observed as well as cell necrosis and sloughing (arrows). Scale bar = 100  $\mu$ m.

Among toxic agents causing ATI are antibiotics, antiviral agents, immunomodulatory agents, and chemotherapeutic agents. The main cause of the tubular injury by antimicrobial agents and especially aminoglycosides is the induction of apoptosis and necrosis of the tubular cells. Antibiotics are endocytosed and stored in lysosomes, Golgi apparatus and endoplasmic reticulum. When the capacity to store these agents is reached, the antibiotics are emptied into the cytosol where they react with mitochondria causing apoptosis and necrosis. Apart from this, aminoglycosides can inhibit numerous transporters present in the PTs, thereby affecting reabsorption<sup>40</sup>.

Antiviral agents in turn cause formation of crystals, especially in the collecting ducts, leading to tubular obstruction and swelling<sup>41-43</sup>. Other causes of tubular injury include interaction with mitochondria resulting in cell death. Blocking of the resorption processes causing proteinuria and downregulation of aquaporins or lowering responsiveness to vasopressin causing nephrogenic diabetes are among other risk factors<sup>44</sup>.

The mechanism behind nephrotoxicity caused by immunomodulatory drugs vary depending on the agent. Some of them, such as cyclosporine, cause vasoconstriction leading to hemolytic uremic like syndrome, while other cause cell cycle arrest and apoptosis<sup>13,45,46</sup>. Chemotherapeutic agents are overall cytotoxic, cause inflammatory response, disrupt mitochondrial function and lead to necrosis and apoptosis<sup>13</sup>.

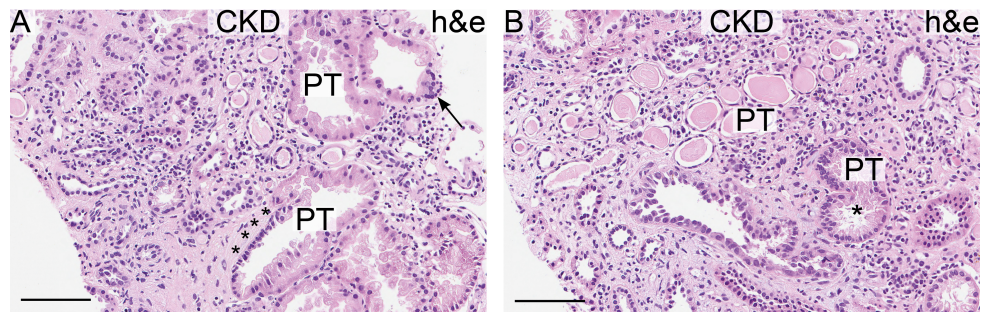
Ischemic ATI has particularly deleterious effects on tubular epithelial cells. The main reason behind it is the very high metabolic rate characteristic for the tubular component. As mentioned in the previous chapter, tubular epithelial cells have a very high energy demand due to their high level of transport activity. Ischemia and reduced oxygen supply disrupt the oxidative metabolism of these cells, thereby causing injury. When reperfusion occurs, generation of free oxygen radicals leads to further cell damage. The main reason for the tubular injury during ischemia is

ATP depletion. Polarity of the tubular cells is a critical factor for their proper function. To maintain cell polarity and correct placement of membrane bound proteins, the cytoskeletal network has to work properly. Even 5 minutes of ATP depletion causes cells to lose their polarity, disrupting their function<sup>47-49</sup>.

Ischemic tubular injury can be caused by hemodynamic factors, injury to the endothelium or epithelium, and immunological factors. Intra renal blood flow changes are very injurious given that the kidney function is dependent on an uninterrupted blood flow, especially to the smallest arterioles in the glomerulus. The inability of proximal tubule cells to switch to anaerobic metabolism, and almost hypoxic conditions in the medulla, make kidney an easy target for oxygen deprivation injury. Ischemic injury also leads to endothelial cells swelling and disruption, causing leakage of fluid into the tissue, creating edema. This in turn results in clotting and blockage of small vessels. The epithelium is the next component highly affected by ischemia. It can result in loss of the brush border, cell necrosis, basal membrane denudation and shedding of cells into the tubular lumen<sup>13,50</sup>. The immune response also plays a big role in ischemic tubular injury. Stressed and injured cells attract immune cells which in turn deepen the injurious insult. An initial response by neutrophils causes release of cytokines leading to cell injury, increased vascular permeability, and attraction of more immune cells<sup>51</sup>.

## Chronic kidney disease

Chronic renal failure or chronic kidney disease (CKD) is an enormous problem for healthcare systems. In a Global Burden of Disease study<sup>52</sup> CKD placed 18th on the global list of causes of death with a quick raise from the 27th place within 20 years. It is estimated that around 600 million people are affected by this disease worldwide with numbers increasing at rapid pace<sup>53</sup>. CKD is characterized by reduced glomerular filtration rate and increase in albumin content in the urine. High mortality rate is caused by complications leading to cardiovascular disease, anemia, bone disorders, and fractures. Among the most common causes for CKD are diabetes mellitus, hypertension, obesity, glomerulonephritis, infections, and toxins<sup>52</sup>. It is also worth noting that there is evidence pointing towards AKI as a risk factor increasing the chance of developing CKD<sup>54</sup>. Figure 5 presents a case of CKD where most tubules are injured and replaced by fibrotic tissue and immune cell infiltrate. Interestingly, some tubules show potential sites of regeneration.



**Figure 5. Chronic kidney disease.**

In A, most proximal tubules are chronically injured. Their lumens are small or non-existent, remaining proximal tubular cells are flattened. In remaining tubules protein casts can be observed, suggesting high degree of kidney injury. Stars mark area of very flattened tubular cells showing signs of mitotic activity with densely packed nuclei. Arrow points to a multinucleated cell. In B, another image presenting CKD injury to the tubules. Signs of potential regeneration can be observed in a tubule marked with \*, cells are densely packed and many nuclei can be observed. PT – proximal tubule. Scale bars = 100  $\mu$ m.

## Diabetes

Diabetes is the leading worldwide cause of CKD. Diabetic nephropathy is characterized by albuminuria, decreased glomerular filtration rate (GFR), and high blood pressure. There are multiple etiologies for diabetes related CKD. One of the most important ones is increased filtration rate in the glomeruli leading to damage of the nephrons<sup>55</sup>. Hyperfiltration can be caused by many mediators such as the renin-angiotensin system, nitric oxide, and vascular endothelial growth factor<sup>56</sup>. The precise reasons of increased GFR in diabetes are not known but there are several hypotheses explaining this phenomenon. General renal vasodilation and higher renal blood flow were observed in humans suffering from diabetes<sup>57</sup>. Especially damaging to the kidney is dilation of afferent arterioles with no dilation of efferent arterioles, causing increased filtration rate and intraglomerular pressure<sup>58</sup>. The cause behind it can be elevated insulin levels and increased nitric oxide signaling among others.

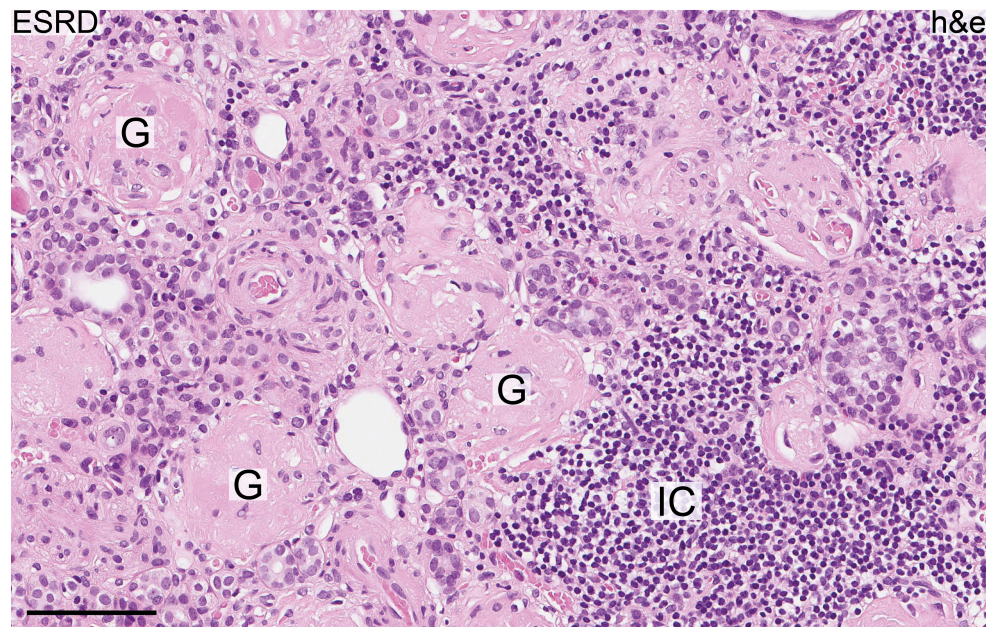
## Hypertension

Systemic hypertension is another big cause for kidney injury. Increased blood pressure is a big risk factor for developing proteinuria which in turn increases the chances of lowered GFR and kidney injury. Prolonged hypertension results in damage to kidney vasculature. Increased blood pressure causes several adaptive changes, such as increased blood vessel thickness as well as decreased lumen diameter. These changes allow blood vessels to withstand the increased blood pressure but at the same time lead to glomerulosclerosis and injury to the tubular

compartment<sup>59</sup>. Importantly, vasculature damage is also a direct cause of tubular cell death through lack of oxygen supply<sup>60</sup>.

### End stage renal disease

It is worth noting that CKD often leads to end stage renal disease (ESRD) – the terminal phase of CKD. When GFR is drastically decreased and patients go into chronic kidney failure, there is a highly increased risk that renal replacement therapy will be required. End stage renal disease is defined by a prolonged need for replacement therapy such as dialysis or kidney transplantation. The prognosis is very poor for ESRD patients and the most common causes of death are cardiac related deaths and infections. Mortality rates vary depending on the underlying cause of ESRD and are estimated to be around 17% for diabetes, 30% for hypertension, and almost 37% for chronic glomerulonephritis<sup>13</sup>. The severity of the condition is visualized in Figure 6.



**Figure 6. End stage renal disease.**

Image presents a highly advanced case of ESRD. Dense infiltration of immune cells can be observed. The tubular compartment is almost completely gone with no tubules visible. A high numbers of glomeruli pulled together by progressing fibrosis can be observed in the field of vision. G – glomerulus, IC – immune cells. Scale bar = 100  $\mu$ m.



## Immune cell infiltrate in kidney disease

Both acute and chronic kidney diseases attract immune cells during their course. The immune response can be divided into the innate immune response, characterized by lack of specificity and being the first responder to pathogens invasion, and the adaptive immune response. The latter can be described as specific, allowing for recognition and response to defined pathogens. Many cells involved in innate immunity are proven to further the progress of kidney disease, including the complement system, macrophages, natural killer cells, dendritic cells, toll like receptors and inflammatory cytokines<sup>61</sup>. The complement system is finely regulated and an important part of the innate immunity. Disruption in its regulation can have injurious consequences and lead to chronic kidney disease. One of the causes of lupus nephritis is infiltration of neutrophils due to incorrect complement regulation, leading to glomerular injury<sup>62</sup>. Similarly, even though macrophages play an important role in protection against pathogens and removal of cell debris, they can also have a detrimental effect on the kidney by causing inflammation and injury. Macrophages are commonly divided into M1 and M2 types. In this simplified concept, M1 macrophages are proinflammatory and can be activated by lipopolysaccharides (LPS), interferon gamma (IFN $\gamma$ ), or tumor necrosis factor alpha (TNF $\alpha$ ). M2 macrophages are generally associated with anti-inflammatory cytokines such as TGF $\beta$  and IL-10<sup>63</sup>. It has been shown that M1 or classically activated macrophages play a role in kidney injury by promoting oxidative stress and renal fibrosis<sup>64</sup>. Natural killer (NK) cells can also have an adverse effect by activating macrophages but also by their nonspecific response causing tubular cell apoptosis<sup>65</sup>.

The adaptive immune response can also promote renal injury. The main components of the adaptive immune response are T and B lymphocytes. In the case of B lymphocytes, the main cause of injurious action is production of autoantibodies that contribute to the development of lupus erythematosus, immunoglobulin A nephropathy, and Goodpasture's syndrome. T lymphocytes but, especially CD8<sup>+</sup> T cells, and CD4<sup>+</sup> T helper cell activity, play a big role in the progression of chronic kidney disease. These cells can cause inflammation and localized injury<sup>61</sup>. CD4<sup>+</sup> Th cells can be further divided into Th1, Th2 and Th17 cells. Their differential functions can lead to different types of kidney diseases. Th1 cells producing inflammatory cytokines are believed to be mainly involved in crescentic glomerulonephritis, a serious disease that can quickly progress to end stage renal disease. Th2 cells, even though often characterized as anti-inflammatory, can lead to chronic diseases such as membranous glomerulonephritis<sup>66</sup>. Th17 cells are associated with kidney inflammation and injury. Their role has been implicated in lupus erythematosus and in general in nephrotoxic nephritis<sup>67</sup>. It is also worth noting that diabetes, the leading cause of ESRD, is so strongly associated with immune

system activation that some propose classifying it as an inflammatory disease<sup>68</sup>. Kidney inflammation has very detrimental effects on the renal function as it negatively affects tubular transport. One of the important players in kidney inflammation during diabetes is TNF $\alpha$  and its production has been linked to hyperglycemia. TNF $\alpha$  has been shown to stimulate reactive oxygen species, cause sodium retention and renal hypertrophy, and to increase the expression of chemoattractants<sup>61,68</sup>.

# Regeneration of the epithelial organs

Regeneration under normal, healthy conditions or following an injury is relatively well described in the major epithelial organs, namely skin, small intestine, colon, liver and kidney. All of these organs but the kidney are proven to contain stem cells of at least one type, which ensure regeneration of the organ. In most of these organs, well described stem cell niches are present and the signaling involved in maintenance of the stem cells has been thoroughly studied. The kidney appears to be an exception where a heated debate persists on whether it contains resident stem cells or not. It is an interesting phenomenon where all of the epithelial organs are known to regenerate both during normal and injurious conditions, but only one of them lacks answers regarding not only the characteristics of the cells involved in the regeneration but also the nature of these cells.

## Regeneration of the skin

Skin is the largest external human organ and also the largest organ in general. Hence its regeneration is of utmost importance. Skin is a very complex organ comprised of epidermis, dermis and appendages such as hair follicles and sebaceous glands. The skin is constantly exposed to various injurious events like mechanical stress, UV radiation, and pathogens. Skin epidermis, as the outer most layer, is especially vulnerable to the injury. It is built of stratified squamous epithelium and its day to day preservation relies on constant proliferation and differentiation. These two processes occur in the lower part of the epidermis, while new cells migrate towards the surface<sup>69</sup>. Epithelial stem cells are the basis for skin regeneration. Adult stem cells usually reside in stem cell niches. These are characterized by specific microenvironments that can modulate stem cell fate and activity. In the skin there are three separate niches that has been identified so far: basal layer of the epidermis, the bulge of the hair follicle, and the sebaceous gland<sup>70</sup>. The slow cycling stem cells maintain the homeostasis and act to replenish the tissue. Stem cells are also involved in regeneration following injury. There are numerous lineage tracing studies showing that follicular stem cells can be converted into epidermal cells and help in regeneration<sup>71,72</sup>. Follicular stem cells are characterized by expression of various stem cell markers such as Sox9, Lgr5, Lgr6, Lrig1, or CD34<sup>70,73</sup>. It has been shown



that these stem cells can operate not only from their niche in the follicle bulge, but that they can also leave the site and function as self-sustaining stem cells outside of it. This mechanism takes place especially after mechanical injury to the skin. In such situation stem cells migrate to the wound site and can remain there for prolonged periods of time while maintaining their self-renewing and regenerating capacity<sup>74</sup>.

## Regeneration of the intestine

The epithelium lining intestines is rapidly proliferating and differentiating. The harsh conditions in the gastrointestinal tract require a fast turnover of the cells. Similarly to the skin, the basis for the regeneration is the presence of intestinal stem cells (ISCs). ISCs can be divided into two subpopulations, reserved stem cells that are quiescent for a long period of time, and primed stem cells that are actively cycling<sup>75</sup>. According to this division, regeneration under normal circumstances is achieved by the primed cells. These cells are believed to divide asymmetrically, creating one daughter stem cell and one committed daughter stem cell, that in turn can give rise to one of the four types of mature intestinal epithelial cells. This model is proposed to function during normal physiological conditions. During injury, ISCs can divide symmetrically to maintain the population of stem cells<sup>76</sup>. As in skin, intestinal stem cells reside in specific niches. There are two locations suggested as niches for ISCs. Active stem cells are located at the base of the crypt, hence their name crypt-base columnar cells. They are responsible for the majority of the regeneration during normal conditions. Located above them are label retaining cells or quiescent stem cells. They get activated in response to injury and can either duplicate or generate crypt-base columnar stem cells. These two types of cells are constantly stimulated by various factors keeping them in their respective states. Some of these factors are bone morphogenetic protein (BMP), BMP antagonists, and Wnt secreted by stromal cells, pericryptal myofibroblasts, and smooth muscle cells. The gradient of these factors created along the crypt-villus axis regulates the ISCs function. BMP signaling inhibits proliferation, and BMP concentration is lower at the crypt's base, allowing for higher cell proliferation rate of the stem cells located there. The opposite gradient is present for pro-proliferative Wnt, with the highest concentration at the crypt's base. These two factors are supported by BMP antagonists with concentration pattern following the one of Wnt<sup>77,78</sup>.

## Regeneration of the liver

The liver can regenerate in response to injury in two different ways. The first mechanism is by replication of mature hepatocytes. In the normal situation, if partial hepatectomy or chemical insult occurs, the liver mass is regained through this process. It has been shown in rodents that after resection of 2/3 of the liver, the liver mass returns to normal within a week. The same mechanism works also for smaller resections of less than 10% of the original liver mass<sup>79</sup>. Liver size is carefully regulated and is proportional to the whole body mass. Studies show that if a liver from a small dog is transplanted into a larger dog, the liver size will increase proportionally<sup>80</sup>. Similarly, if a small part of the liver is transplanted between human donor and recipient, the transplant increases in size proportionally to the recipient's weight within a few weeks after the surgery<sup>81</sup>. In all the above cases, liver regeneration occurs through hepatocyte replication with no involvement of progenitor cells.

In some cases of chemical injury, activation and replication of progenitor cells can occur<sup>82</sup>. This phenomenon takes place when hepatocyte replication is inhibited by exposure to viruses, drugs, or toxins. The role and origin of liver progenitor cells (LPCs) is not entirely clear and some data suggests their involvement in regeneration to be small<sup>83</sup>. These cells are known to be of oval shape, hence their alternative name used mainly in rat's nomenclature: oval cells. They can give rise to hepatocytes and cholangiocytes thus showing bipotential<sup>84</sup>. There are numerous theories regarding the origin of LPCs. It has been suggested that they are derived from bone marrow stem cells. This theory was supported by studies showing that female recipients of male bone marrow had hepatocytes characterized by XY chromosome configuration<sup>85,86</sup>. Further studies showed that transplantation of bone marrow stem cells can lead to complete restoration of liver function and the rescue of fumarylacetoacetate hydrolase deficient mice<sup>87</sup>. Other studies on the other hand point towards a fusion mechanism being responsible for liver regeneration. This hypothesis suggests that the liver regeneration occurs not through hematopoietic stem cell (HSC) differentiation into hepatocytes, but rather through a fusion between the HSCs and hepatocytes<sup>88,89</sup>.

Others believe that there are resident LPCs localized in the liver. These cells are proposed to reside in a stem cell niche localized in the ductal plates in fetal livers and in the canals of Hering in adult livers<sup>90</sup>. The stem cell niche is very important for control and regulation of the progenitor cells. There has to be a balance between replication and differentiation of progenitor cells, and not only the localization of the niche is important but also the microenvironment in it<sup>91</sup>.

The role of progenitor cells in healthy liver is not well defined but the general consensus is that they are not very active and do not play a major role in liver under

physiologic conditions<sup>92</sup>. Regardless of the regeneration mechanism in play, liver has a great capability to regenerate itself in relatively short time.

Overall, the evidence suggests that skin, intestine, and liver are characterized by two mechanisms of regeneration. The first mechanism is based on the presence of a rapidly replicating cell responsible for maintenance of the tissue homeostasis under normal conditions. The second mechanism is through the presence of long term quiescent stem cell that can get activated during injury to help in the regeneration and maintain the number of stem cells.

## Regeneration of the kidney

Even though renal epithelial cells are mitotically quiescent, the kidney is well known to regenerate after injury. This phenomenon is especially well established after acute kidney injury described in the earlier chapter. Originally AKI was considered to be a fully or almost fully reversible disease, especially if the tubular injury was mild. Nowadays more in-depth studies suggest that even though the kidneys might recover functionally, some of the injury persist. This unresolved damage to the tubular compartment is associated with increased risk of chronic kidney disease and ESRD. It is important to remember that functional recovery does not mean full structural repair. After unilateral nephrectomies, patients present normal kidney function even though they are left with only half of the nephrons<sup>93</sup>. At the same time it is a fact that kidneys do regenerate their tubular compartment. One common reason for AKI is ischemia and regeneration after ischemic insult has been well studied. The first responders are the heat shock proteins (hsp). Among first to appear in the cells are hsp25 and hsp72. Their presence in the tubular cells can be detected after as little as 2h post injury<sup>94</sup>. Hsp72 localizes to various cell compartments showing its importance in the regeneration process. In the first stages the protein can be detected in the apical part of the tubular cell. This is where the first signs of injury, such as alterations to the brush border, loss of the brush border and re-localization of Na<sup>+</sup>-K<sup>+</sup>-ATPase to the apical membrane, occur. In the later time points hsp72 can be detected in the cytoplasm and finally in the vicinity of the basal membrane when the injury has been resolved<sup>94</sup>. Other hsp such as hsp70 has also been implicated in kidney regeneration. Their main role is protection against further cellular injury through prevention of apoptosis caused by ATP depletion. Other proteins such as caveolins and especially caveolin-1 were also shown to be important for protection against injury and for regeneration<sup>95</sup>.

Even though some protective and regenerative mechanisms of the tubular cells are known, as described above, the main question remaining in the kidney regeneration field is which cells are responsible for the regeneration process. As presented in the

previous chapters, all major epithelial organs are known to have resident stem cells residing in their designated niches. Although there is always need for more detailed characterization of these cells and the mechanisms by which they are regulated, scientists do agree that these are resident stem cells. In the kidney regeneration field this question still remains open, leaving room for a heated debate and more research.

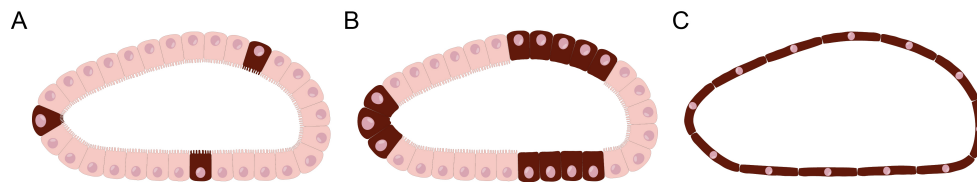
One of the early hypotheses regarding the cells responsible for kidney regeneration was that these cells were derived from bone marrow. A couple of independent research groups reported incorporation of previously isolated and labelled bone marrow cells into the proximal tubules (PTs) of the kidney<sup>96,97</sup>. However, other researchers argued that the observation that the bone marrow stem cells incorporate into tubular compartment of the kidney is false positive. It was argued that labelling methods were not fully optimized and reliable and that leukocytes rather than tubular cells were marked in these studies<sup>98</sup>. This phenomenon was even acknowledged by the authors themselves<sup>97</sup>. Further research suggested that the apparent restoration of kidney function after injection of bone marrow derived stem cells is due to their capability to secrete paracrine factors characterized by anti-apoptotic, proangiogenic, and immunomodulatory effects<sup>99</sup>. It was also noted that bone marrow cells are able to fuse with the resident cells. This phenomenon causes them to acquire the phenotype of the resident cells and in case of labeled cells gives a false positive result suggesting that the bone marrow cells differentiated into the resident cells<sup>100</sup>.

It is agreed that kidneys contain progenitor cells and that these are localized in the Bowman's capsule of the glomerulus. These cells called parietal epithelial cells (PECs) are characterized by the expression of acknowledged stem cell markers such as CD133 and CD24. PECs however are committed towards regeneration of podocytes localized in the glomerulus<sup>101</sup>.

The main question remains regarding the regeneration of the tubular compartment, which leads to regaining of the kidney function post injury. Many studies have shown the presence of tubular scattered cells (TSCs) in the proximal tubules of the kidney, characterized by presence of stem cell markers<sup>102-105</sup>. These cells seem to be present in the kidneys in normal, healthy state (Figure 7A). They were observed in both adults and infants and in many mammalian species such as pigs and primates. Interestingly, there is no convincing data showing presence of these cells in mice, the most widely used laboratory animal. Despite this, studies show that murine tubular cells have an intrinsic ability to regenerate<sup>106</sup>.

Currently two main hypotheses regarding the cells responsible for kidney regeneration remain. Both agree that tubular regeneration occurs within the tubules themselves, however the cell of origin remains the center of the debate. One hypothesis suggests that there are resident progenitor cells scattered in the PTs of

the kidney, while the other one claims that any PT cell can acquire stem cell like properties, survive the injury and regenerate the kidney (Figure 7B and C).



**Figure 7. Tubular scattered cells**

Schematic drawing of proximal tubules containing tubular scattered cells (TSCs) positive for vimentin in A and their expansion post injury in B. Panel C presents flattened tubular cells surviving the injury, preserving tubule's integrity and driving the regeneration.

### **Random survival and induction theory**

Lineage tracing studies are a great tool in following the fate of specific cells. This method is now being widely used in mouse models and has been adapted to help to answer the question of the origin of progenitor cells in the PTs. In a 2011 study, Humphreys et al. showed that repair of the proximal tubules does not require a specialized progenitor cell<sup>107</sup>. In this work, two thymidine analogs were utilized: CldU and IdU, that allow for accurate measurement of the proliferative activity of cells. To assess whether intrinsic progenitor cells are responsible for regeneration of the PTs, mice were injected with one thymidine analogue 24 h after unilateral ischemia reperfusion injury (IRI) and with the other 45h after IRI. The idea behind this experiment was that if only intratubular progenitor cells would proliferate during repair, they would contain both markers due to a fast division rate. In this case the percentage of double positive cells would be high. On the other hand, if any tubular cell could survive and duplicate, the fraction of double positive cells would be low due to the low proliferation rate characteristic for PT cells. The results of this experiment suggest that most or all PT epithelial cells can proliferate after injury<sup>107</sup>. Similarly, Kusaba et al. argue that cells expressing the SLC34a1 phosphate transporter, present only in fully differentiated cells, are responsible for the regeneration<sup>108</sup>. The authors suggest that PT cells can undergo transient dedifferentiation, and during this period express stem cell markers such as CD133 and CD24, and drive the regeneration. Smeets et al. came to a similar conclusion in their work based on human material and a rat model. They argue that since almost no cells expressing equivalents of human stem cell markers are present in healthy rat kidneys, but this population increases after injury, there are no resident progenitor cells. Instead they propose that randomly surviving cells can express these markers and fuel the regeneration<sup>102</sup>. The same conclusion is reached by

Berger et al. who used lineage tracing technique based on the PEC-rtTA mouse – an inducible model prepared to mark PECs. The authors suggest that since almost all markers are shared between PECs and scattered tubular cells, their model can also be used for TSCs studies. The data generated with this technique points towards a lack of preexisting progenitor cells and supports *de novo* formation of TSCs with regenerative properties<sup>109</sup>. In a recent study, Kumar and colleagues<sup>18</sup> used a lineage tracing approach to look into the contribution of TSCs to regeneration of mouse kidney. For this purpose the authors focused on the Sex-determining region Y box family member Sox9. SOX family members are involved in organ development<sup>110,111</sup> and SOX9 was found to be present in the developing kidney localizing in the ureteric bud<sup>112</sup> and to mark a population of progenitor cells in liver, intestine, pancreas, and lung<sup>110,112-115</sup>. An inducible Cre system driven by the Sox9 promoter was used to label Sox9 expressing cells in mice. The authors found a population of TSCs expressing Sox9 in PTs and distal tubules but not in thick ascending limb of loop of Henle or in the collecting ducts. A clear increase in Sox9<sup>+</sup> cells was observed after kidney injury, consistent with the hypothesis that Sox9 is an important player in the regenerating kidney. However, the authors found only a negligibly small expansion of scattered tubular Sox9<sup>+</sup> cells. At the same time, they observed a significant *de novo* Sox9 induction in other surviving cells and a big contribution of these cells to regeneration of the tubular compartment.

### **Progenitor cell theory**

On the other side of the spectrum is a collection of publications pointing towards existence of a fixed progenitor cell population. As mentioned earlier there is a clear histological evidence of the presence of TSCs in healthy kidneys<sup>102-105,116-120</sup>. As opposed to the theory presented above, there is also great number of publications calling these cells resident progenitors rather than induced or dedifferentiated cells. In this theory, TSCs are paused in a quiescent state and upon injury they can re-enter cell cycle to regenerate injured tubules<sup>104,117</sup>. In a simple study, Maeshima et al. identified label retaining cells (LRCs) in rat kidneys using BrdU labelling. In their work they show that this cell population significantly expands after injury, regenerates the tubular compartment, and thus represents a fixed progenitor-like cell<sup>117</sup>. Angelotti et al. characterize these cells by the expression pattern of CD133<sup>+</sup>CD24<sup>+</sup>CD106<sup>-</sup> when compared to CD133<sup>-</sup>CD24<sup>+</sup>CD106<sup>+</sup> expressing PECs – an established progenitor cell of the human kidney. The authors show in their work that CD133<sup>+</sup>CD24<sup>+</sup>CD106<sup>-</sup> cells show lower proliferative capacity than PECs and are more committed towards the tubular lineage. At the same time both types of the cells are more resistant to apoptotic stimuli and general injurious factors when compared to normal tubular cells. Even more importantly these scattered cells, upon injection into SCID mice with inflicted kidney injury, showed engraftment and

improved kidney function when compared to control<sup>104</sup>. In a more advanced study similar in design to Kumar's study<sup>18</sup>, Kang et al.<sup>17</sup> used a lineage tracing technique to pin down the cell of origin for tubular regeneration. As Kumar et al., Kang used an inducible Sox9 driven Cre mouse model. The authors found 6% of the cells to be Sox9 positive at birth and further expansion of these cells to 70% of kidney cells being Sox9<sup>+</sup> by day 42. These cells contributed to formation of proximal tubules, loops of Henle and distal tubules. Kang found that after induced injury, 85% of Sox9<sup>+</sup> cells were Ki67 positive, suggesting their involvement in regeneration. After isolation of Sox9<sup>+</sup> cells, the authors showed their progenitor properties, such as clonogenicity and ability to differentiate into adipogenic, osteogenic, and chondrogenic lineages. The authors concluded their work by stating that Sox9<sup>+</sup> cells mark segment-specific progenitors which regenerate the tubular compartment and their absence leads to an altered regeneration process and development of fibrosis.

As presented above, equally big pools of publications points towards opposing theories regarding the regeneration of the kidney. Advocates of both approaches agree on the presence of the TSCs in healthy human kidneys. There is evidence of existence of TSCs also in pig and primate kidneys, with a possible presence in the mouse kidneys. The final answer to the important question of the origin of TSCs appears to be difficult to find for a number of reasons. One big obstacle is the difference in expression of markers between humans and mice. Typical stem cell markers such as CD133 and CD24 are not present in mouse kidneys, making it difficult to compare expression pattern of TSCs between these two species. Some researchers suggested using qPCR technique to avoid this difficulty<sup>108</sup> while others pointed out that these genes are not homologs between humans and mice<sup>93</sup>. Other authors set out to look for other markers that could stain TSCs in mouse kidneys and suggested Sox9 to be a good choice. Even though this marker seems to mark a scattered population of cells in the mouse kidney, their prevalence of 0.05% in the PTs<sup>18</sup> is drastically different from the numbers reported in human PTs of 2% to 6%<sup>93</sup>. Considering all these limitations and difficult to solve arguments of suitability and similarity of a mouse model to human conditions, it seems wise to focus on regulation and induction of the TSCs phenotype, and plausible applications of this knowledge to pharmacologically improve kidney regeneration process as suggested by Lazzeri<sup>16</sup>.

# Kidney Cancer

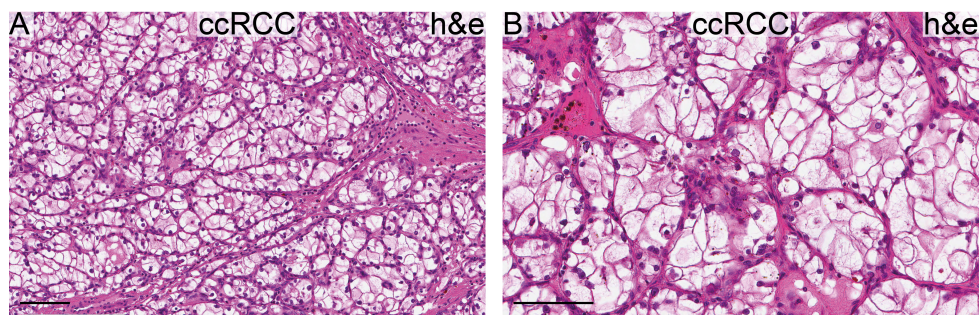
Kidney cancer is the 12th most common cancer type in the world, with 338 000 cases diagnosed in 2012 and 143 000 kidney cancer related deaths<sup>121</sup>. The most common type of kidney neoplasm is renal cell carcinoma (RCC), accounting for 90-95% of the cases<sup>122</sup>. The prevalence of the disease is higher in men, with a lifetime risk of developing kidney cancer of about 1 in 48, and lower for women with a lifetime risk of about 1 in 83. This accounts for 214 thousand cases in men and 124 thousand cases in women worldwide annually<sup>123</sup>. Treatment options are lacking and the economic burden is enormous, accounting for up to 1.6 billion USD in selected countries worldwide<sup>124</sup>. The prevalence of RCC is greatly increased in well-developed countries. Risk factors include obesity, hypertension, and cigarette smoking<sup>125</sup>. RCC is a slow growing tumor with very few cases diagnosed before the age of 40<sup>126,127</sup>, the median age at diagnosis is between 60 and 65 years old<sup>128</sup>. RCC is a group of neoplasms having the same origin, all arising from the epithelium. Cancers belonging to this group have very different morphologic and genetic background however. The three most common types of RCCs are clear cell RCC, papillary RCC, and chromophobe RCC, together accounting for more than 90% of all RCC cases<sup>129</sup>.

## Clear cell renal cell carcinoma

The most common type of RCC is clear cell renal cell carcinoma (ccRCC), representing around 75% of RCC cases<sup>130</sup>. Most of ccRCC cases appear sporadically, while the rest can be associated with hereditary syndromes such as von Hippel-Lindau's syndrome or tuberous sclerosis. The cell of origin for ccRCC is proximal tubular epithelial cells. Macroscopically the tumors can be characterized as solid, yellowish, with possible hemorrhage and varying level of internal necrosis<sup>130</sup>. The level of necrosis is linked to the grade and size of the tumor, where bigger size and higher grading score correlate with higher degree of necrosis<sup>131</sup>. The color of the lesions and their clear cell microscopic appearance is caused by high lipid and glycogen content of the cells<sup>132</sup>. Clear cell RCC is characterized by loss of the short arm of chromosome 3. Other common aberrations are also trisomy of chromosome 5, 12 and 20 and loss of chromosomes 8, 9, 13q and 14q<sup>133,134</sup>. One of



the characteristics of clear cell RCC is its high vascularization (Figure 8), caused by significantly increased levels of vascular endothelial growth factor (VEGF). The reason for this phenomenon is a loss of functional von Hippel-Lindau protein (VHL), leading to increased levels of hypoxia inducible factor 1 $\alpha$  (HIF1 $\alpha$ )<sup>135,136,137</sup>. Under normal oxygen levels HIF1 $\alpha$  undergoes rapid degradation by an E3-ubiquitin ligase of which VHL is a substrate recognition component<sup>138</sup>. Mutations in the VHL gene causes stabilization of HIF1 $\alpha$  and HIF2 $\alpha$  in both sporadic and hereditary forms of ccRCC<sup>139</sup>. With no HIF degradation, regardless of the oxygen concentration, cells enter a pseudohypoxic state resulting in a major metabolic shift inducing hundreds of genes among which the abovementioned increased level of VEGF is characteristic. VHL inactivation itself is not sufficient however to cause malignant transformation, resulting only in preneoplastic cysts<sup>140</sup>. Recent studies using whole exome and targeted sequencing have identified other mutations present in ccRCC tumors in genes involved in chromatin modifications such as PBRM1<sup>134,141</sup>. Mutations in the TCEB1 gene accompanied by chromosome 8 loss, detected in up to 42% of ccRCC cases, have also been found to be involved in VHL inactivation and ccRCC pathogenesis<sup>134</sup>. TCEB1 encodes the elongin C protein, a vital part of the VHL E3 ligase complex required for ubiquitination of its targets. Loss of TCEB1 therefore causes HIF accumulation as observed with VHL loss, increasing the risk of ccRCC occurrence.



**Figure 8. Clear cell renal cell carcinoma.**

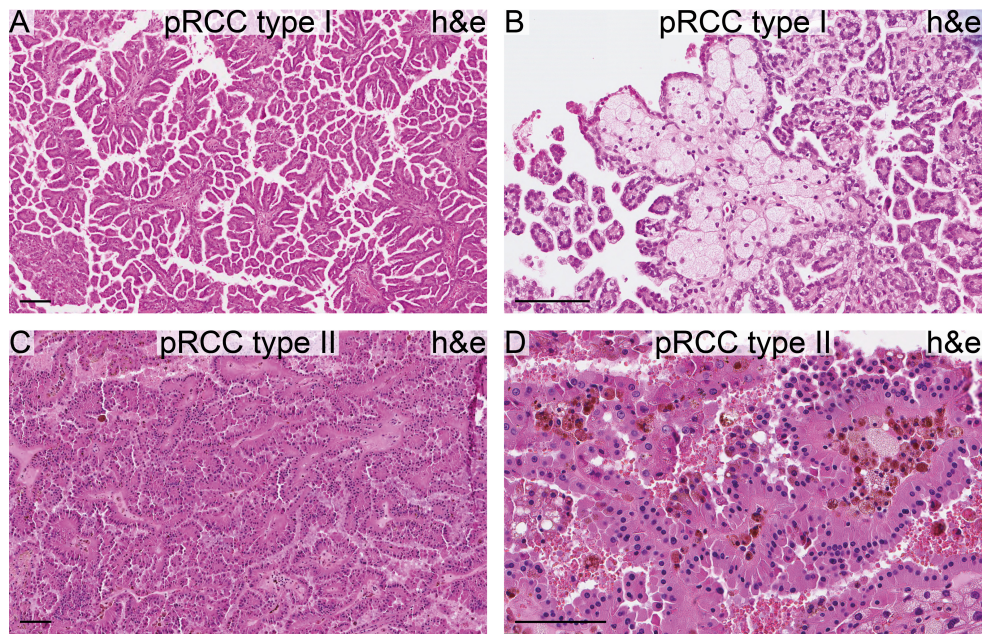
In A, overview of a ccRCC case can be seen. A dense network of vasculature is clearly visible. In B, high magnification image shows that almost every single cell has access to one of the vessels stressing out one of the very characteristic features of ccRCC. Clear cytoplasm filled with fat can be observed. Scale bars = 100  $\mu$ m.

The only curative treatment option for ccRCC is complete surgical resection of the tumor, often meaning full nephrectomy<sup>134</sup>. Other treatment options are also being used, including interferon  $\alpha$  treatment as well as VEGF and mTOR inhibitors, however their effectiveness is limited. More recent therapies include immune checkpoint inhibitors such as anti-programmed death receptor 1 (anti-PD1) and anti-PD1 ligand (anti-PDL1)<sup>142</sup>.

## Papillary renal cell carcinoma

Papillary renal cell carcinoma (pRCC) accounts for around 10 to 20% of all RCC cases<sup>130,143</sup>. In the majority of patients pRCC occurs sporadically, with only 4% estimated to be familial<sup>144</sup>. Similarly to ccRCC, the prevalence of pRCC is higher in males and the ratio to female cases is around 2:1. Cases are reported in patients in their early adulthood up to old age, however the mean range is between 50 and 55 years old. Mortality is calculated to be around 16% after 10 years<sup>145</sup>. The gross pathology of pRCC is characterized by globular tumors, often with necrosis and sometimes hemorrhage. Microscopically, in most of the cases the architecture of the tumor is papillary or tubulopapillary, hence the name. The papillae are characterized by fibrovascular cores with a single layer of carcinoma cells<sup>146</sup>. Papillae can have differential level of complexity, showing numerous branching or more simple, long arrays (Figure 9). Two subtypes of pRCC have been described over the years. Type 1 is usually characterized by small, single layered cells with sparse cytoplasm and small nuclei, while type 2 presents itself with large pseudostratified cells with eosinophilic cytoplasm and more prominent nuclear polymorphism. The histological distinction is supported by genetic studies of mutation profiling, RNA expression and proteomic analysis<sup>143,147</sup>. A very distinctive and almost pathognomonic feature of pRCC is the expansion of the cores by foamy macrophages<sup>148</sup> (Figure 9B and D). The foaminess is caused by accumulation of lipids in the cytoplasm of infiltrating macrophages<sup>149</sup>. A benign condition inseparable from pRCC is the so called cortical adenoma. Morphologically these cannot be separated from pRCC, but since they are much more common than pRCC they are considered benign. An arbitrary size cut-off has been set to separate cortical adenoma from pRCC<sup>150</sup>. We have previously presented extensive marker similarities between adenomas and pRCC<sup>105</sup>.

The cell of origin for pRCC has not been extensively characterized, but it is believed to arise from the proximal tubules, which is supported by recent data that shows a strong association between proximal tubular cells and pRCC<sup>151</sup>. Careful histological analysis of kidney tissue also points towards a proximal tubular origin, since on occasion proximal tubules may be filled with expanding dysplastic cells. If these arise from bulk tubular cells or PECs, or both, awaits formal verification, figure 10 (preliminary observation from our laboratory).



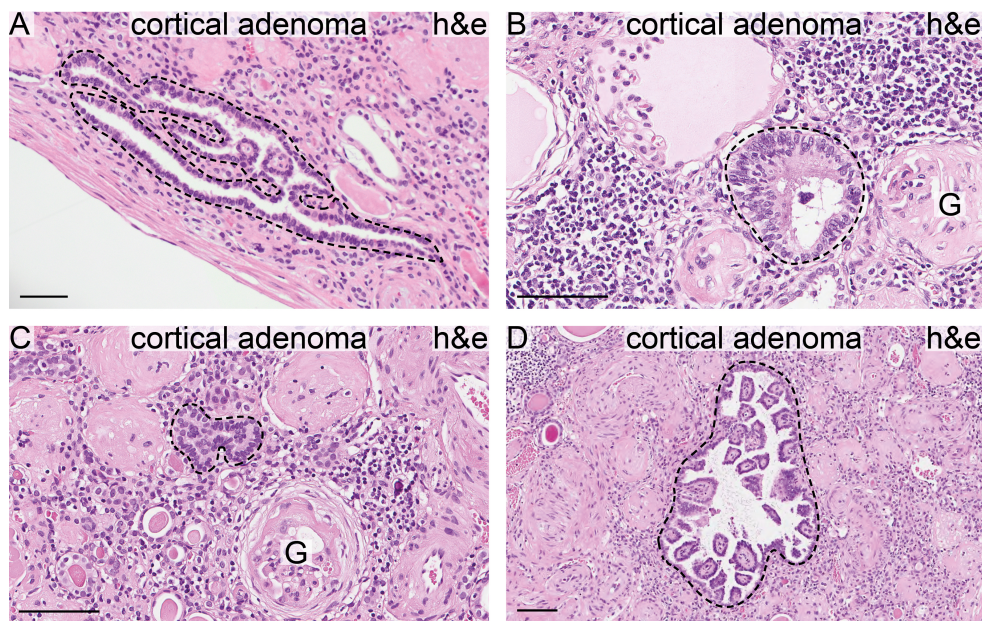
**Figure 9. Papillary renal cell carcinoma type I and II.**

In A h&e staining of pRCC type I with distinctive papillary growth pattern can be observed. Panel B shows characteristic foamy macrophages inside of the papillae. In C and D low and high magnification images respectively present pRCC type II with its characteristic morphology of large cells with more pronounced anisokaryosis. Scale bars = 100  $\mu$ m.

The genetic background of the disease varies between the two types. Type 1 pRCC can be associated with activating mutations in the MET oncogene. Its function is to activate pathways responsible for cell proliferation and survival, but also inhibition of apoptosis<sup>152</sup>. There are several mechanisms of MET expression deregulation, involving gene mutations, overexpression, gene amplification, and epigenetic changes<sup>153</sup>. One of the mutations discovered in some cases is a missense mutation in chromosome 7q31. It results in alteration of MET tyrosine kinase function and leads to constitutive activation<sup>154,155</sup>. Type 2 pRCC in hereditary form can be characterized by mutations of fumarate hydratase. This enzyme is involved in the Krebs cycle and interestingly, its mutation causes accumulation of HIFs, however the mechanism leading to cancer development is not well studied<sup>156,157</sup>. It is known that stabilization of HIFs, in this case HIF1 $\alpha$ , leads to a hypoxic response and increased expression of VEGF<sup>158</sup>, as mentioned above for ccRCC. This finding is important since it can be used in design of pRCC directed treatment. However, mutations of FH are rarely found in sporadic cases of pRCC. Type 2 pRCC is also often associated with mutations in genes implicated to have tumor suppressing functions such as CDKN2A<sup>159</sup> and SETD2<sup>160</sup>, as well as with abnormally high expression of the NRF-ARE pathway involved in oxidative stress regulation and tumor suppression<sup>143,161</sup>.



In general, type 1 pRCC is associated with better prognosis than type 2<sup>162,163</sup>. It is not well described why this phenomenon occurs, however some attribute it to the presence of tumor associated macrophages (TAMs)<sup>164</sup>. Current treatments options are far from perfect and include VEGF pathway inhibitors with reported response rate ranging from 3%<sup>165</sup> to 15%<sup>166</sup> and mTOR inhibitors with similarly low activity<sup>165</sup>. MET and epidermal growth factor (EGFR) inhibitors has also been subjected to clinical trials as potential candidates for pRCC treatment. Some researchers suggest combined treatment with these two inhibitors based on the knowledge that MET signaling can promote resistance to EGFR inhibition. Adding MET inhibitors could help overcome this problem and improve the therapeutic effect<sup>165</sup>.

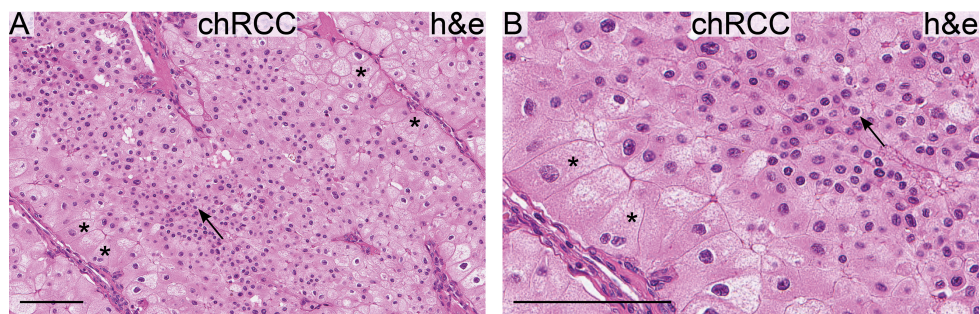


**Figure 10. Origin of pRCC.**

In A, early stage of cortical adenoma is presented (dashed line). The shape of the lesion resembles an enlarged proximal tubule suggesting potential origin of cortical adenoma and pRCC tumors. In B extensive division of cells (dashed line) possibly originating from parietal epithelial cells (PECs) of the Bowman's capsule supporting an alternative theory of cortical adenomas and pRCCs originating from PECs. C and D present progressing stages of developing cortical adenoma (dashed line), having potential to turn into malignant pRCC tumor. Scale bars = 100  $\mu$ m.

## Chromophobe renal cell carcinoma

Chromophobe renal cell carcinoma (chRCC) is the third most common RCC at 5% of the RCC cases<sup>130</sup>. In contrast to ccRCC and pRCC, chRCC has no predisposition in any of the sexes, with both males and females presenting with similar number of cases. The prognosis of chRCC is much better than the two previously described RCC types<sup>145</sup>. Typically, chromophobe tumors are solid and of spherical shape with size ranging from 2 to over 20 centimeters<sup>13,167</sup>. They are suggested to originate from the distal part of the nephron<sup>168</sup>. chRCC tumors are not well vascularized, as depicted in Figure 11. From a histological point of view, chromophobe RCC can be of two separate subtypes, typical and eosinophilic. The first type is usually attributed to solid tumors. Cells are usually large with abundant cytoplasm characterized by pale staining. Tumor cells of the eosinophilic variant contain finely granular cytoplasm with eosinophilic appearance. Cells of this subtype are smaller and tubular structures are observed more often. Based on these types, tumors can be separated into classic, containing over 80% of pale cells, eosinophilic, containing over 80% of eosinophilic cells, or mixed<sup>169</sup>.



**Figure 11. Typical morphology for chromophobe renal cell carcinoma.**

Panel A presents an overview of chRCC. Low degree of vascularization and cells of mainly two morphological types can be observed. Stars mark enlarged pale cells in proximity of blood vessels while arrow marks small eosinophilic cells typical for the low vascularized area. B presents high magnification of panel A showing clear difference in size between cells located in the proximity of the vessels when compared to cells located in non-vascularized area. Scale bars = 100  $\mu$ m.

Genetically, chRCC is characterized by loss of chromosomes 2, 10, 13, 17, or 21 in the majority of cases<sup>170</sup>. It has also been reported that in the familial form of the disease called Birt–Hogg–Dubé syndrome, around 30% of patients will develop chRCC lesions and 50% will be diagnosed with a mixed pattern of chromophobe RCC and oncocytoma<sup>171</sup>. This syndrome is characterized by mutations in the folliculin gene that is believed to be a tumor suppressor gene. Its loss causes mTOR upregulation and can lead to development of chRCC tumors. Another gene

implicated in chRCC signaling is the protooncogene KIT. Its product is a type III receptor tyrosine kinase involved in regulation of proliferation, cell differentiation, apoptosis, chemotaxis, and adhesion<sup>172</sup>. Yamazaki et al. described KIT overexpression in 88-100% of chRCC cases, showing its importance for both diagnosis and potential future treatment<sup>173</sup>. Other studies implicate mutations in the promoter region of telomerase reverse transcriptase (TERT), combined with TERT overexpression, as well as in the TP53 and PTEN tumor suppressor genes<sup>168</sup>. The remaining RCCs include cystic-solid (1-4%) collecting duct or Bellini (1%), medullary (1%), Xp11 translocation (rare), mucinous tubular and spindle cell (rare), associated with neuroblastoma (rare), and non-classified (4-6%) subtypes<sup>130</sup>.



# The present investigation

## Aim of this thesis

Kidney diseases cause substantially more deaths every year worldwide than kidney cancer. The incidence of kidney disease is constantly increasing, despite efforts being made to help affected patients. Treatment options are sparse and healthcare costs very high. The aim of this thesis was to better understand processes behind kidney injury and regeneration. A special focus was placed on the cells responsible for the regeneration occurring in the proximal tubules (PTs). We have previously published data showing the presence of tubular scattered cells (TSCs) with progenitor-like properties in the PTs of the kidney. Since only 2-6% of the PT cells show these properties it is a challenge to isolate and characterize them. At the same time not many TSC markers are characterized and very little is known about the regulation of their expression. This knowledge is of interest not only from scientific perspective but also because cancers like papillary renal cell carcinoma may originate from TSCs.

The main aims of this thesis were to:

- find a technique that would allow for better and easier isolation of low population cells and their subsequent analysis using molecular biology techniques
- identify new tubular scattered cell markers
- better understand how the tubular scattered cells' phenotype is regulated
- get insight into papillary renal cell carcinoma physiology

We believe that the papers presented below at least partially answer these questions and are good basis for further research.



# Paper I

## **Introduction**

In recent years molecular biology techniques and especially the ones used for collection of small samples of genetic material and subsequent bioinformatic analysis have progressed substantially. Single cell sorting and following transcriptomics or proteomics analyses are becoming more common. As presented recently by Park et al.<sup>174</sup>, single cell transcriptomics of almost 58 000 kidney cells can answer many important questions. The main remaining problem is RNA analysis after sorting of specific cells based on intracellular markers. Processes of fixation, permeabilization, and intracellular staining are known to substantially decrease RNA quality, making the genetic analysis of flow cytometry sorted cells almost impossible.

## **Results and discussion**

The main aim of this article was to develop a technique that would allow us to isolate RNA from sorted TSCs. In this paper, we present a simple but effective protocol for RNA isolation from fixed, permeabilized, intracellularly stained, and sorted cells. First, we established that while fixation and permeabilization steps are not very detrimental for RNA quality, blocking and staining with intracellular antibody steps were crucial. To maintain high RNA quality, we proposed 2 M NaCl high salt buffer to be used during blocking and labelling steps. We show that when the high salt buffer is used, high quality RNA can be isolated even after intracellular staining of cells. We also prove that using high salt buffer does not significantly decrease antibody binding to its targets and that it can be safely used in otherwise standard protocols. We propose that the main mechanism of action for the high salt buffer is inactivation of RNases and that our protocol can be successfully used throughout laboratories.

## Paper II

### **Introduction**

Caveolae are cell organelles characterized as membrane invaginations of 50-100 nm. They play important roles in cell signaling and survival. One important protein necessary for proper assembly of caveolae is caveolin-1. It is not expressed in proximal tubules of a healthy kidney but has been shown to be induced after renal injury. However, the role of caveolae in the kidney, as well as regulation of caveolin-1 expression, has never been well defined. Therefore, in this paper, we tested several hypotheses regarding caveolin-1 regulation.

### **Results and discussion**

In healthy renal cortex the expression of caveolins and cavins, the building blocks of caveolae, is limited to the parietal epithelial cells of Bowman's capsule, endothelial cells, and smooth muscle cells. No expression can be detected in proximal tubular cells, however, this pattern changes upon kidney injury. In this article, we present data proving that caveolar proteins are induced in cultured cells, as well as under injurious conditions in the kidney. In order to study the signaling events leading to the induction of caveolar proteins, we tested the effect of various stress signals on these proteins in cultured renal cells. We show that hypoxia and free radical stress have no effect, however, inhibition and viral overexpression of Mkl1 do. Mkl1 inhibition significantly reduced expression of caveolar proteins, while viral overexpression increased the mRNA levels of caveolin-1, caveolin-2, and cavin-2, together with increased levels of the archetypal Mkl1 target tenascin C. This data shows that Mkl1 is an important regulator of caveolar protein expression and probably plays an important role during renal injury.

## Paper III

### **Introduction**

Gene expression analysis indicated that the carrier protein polymeric immunoglobulin receptor (pIgR) is present in TSC. Given the main function of pIgR in other organs, which is protection against invading bacteria, we decided to evaluate its role in the kidney.

IgA is the most abundant immunoglobulin class produced in the human body with almost 2 grams produced every day. Most of the IgA is secreted in the form of secretory IgA (sIgA). The main sites of secretion are the airways, gastrointestinal tract, salivary glands and mammary glands, where sIgA contributes to the barrier preventing microbial and environmental agents from entering our bodies. A protein responsible for transcellular IgA transport and production of sIgA is the polymeric immunoglobulin receptor (pIgR).

### **Results and discussion**

In this article, we present a study of pIgR expression and regulation in human kidneys. We show immunohistochemical analysis proving pIgR co-expression with other tubular scattered cell markers such as vimentin. We also show that pIgR expressed by primary isolated tubular cells is able to transport IgA, and in the process turn it into sIgA. Our analysis of diseased kidneys shows that pIgR expression increases significantly post injury, together with sIgA urinary and blood levels. We show in vitro that inflammatory cytokines such as IFN $\gamma$ , IL-1 $\beta$ , and TNF $\alpha$  induce pIgR expression. We hypothesize that pIgR expression might be a part of the tubular injury response aimed at providing an additional line of defense for the injured kidneys.

# Paper IV

## **Introduction**

Even though mitotically quiescent in a normal state, proximal tubular cells of the kidney are able to regenerate vigorously post injury. The research regarding the cells responsible for this regeneration has been one of the important areas of interest in the kidney research field in the past years. SOX9 is a transcription factor important during kidney development and it has been identified as one of the genes necessary for the kidney regeneration process in mice. Little is known about SOX9 regulation and function in human kidney, however. Our work focuses on its expression pattern in healthy and diseased kidneys and attempts to find regulators of SOX9 expression.

## **Results and discussion**

In this article, we present evidence that SOX9 shows a similar expression pattern to other scattered cells markers. We also show evidence that it is co-expressed with one of them – vimentin. We show experimentally, using an improved explant model developed in our group previously, that following severe ischemia, only scattered SOX9 positive cells survive the injury. We also show that focal tubular injury attracts immune cells and is associated with SOX9 expression. Our in vitro experiments show that immune cell conditioned medium up-regulates SOX9 expression in cultured renal cells and that one of the cytokines responsible for this is transforming growth factor beta (TGF- $\beta$ ). Interferon gamma (IFN- $\gamma$ ) on the other hand, has an opposite effect, causing SOX9 downregulation. We hypothesize that even though TSCs expressing a distinctive set of markers are detectable in a healthy human kidney, proximal tubular cells can be induced to express the same markers in response to cytokines secreted by infiltrating immune cells favoring the view that the TSC phenotype is induced.

# Paper V

## **Introduction**

Papillary renal cell carcinoma (pRCC) is the second most common renal cell carcinoma, but the treatment options are very limited. It is believed that pRCC originates from the PTs. This was established when the PTs were thought to be cellularly homogenous. We have hypothesized that pRCC may originate from TSCs since they share many characteristics such as marker expression and that pRCC might be an example of TSC pathology. It could be that pRCC cells use the robust TSC cell biology during carcinogenesis. One of the most characteristic features of pRCC is the presence of foamy macrophages in the papillary stalks of the tumor. However, very little is known regarding the mechanism behind the attraction of macrophages and their potential role in the development and progression of the disease. The attraction of inflammatory cell infiltrates is central during renal cell injury and pRCC might recapitulate this injury response resulting in macrophage accumulation.

## **Results and discussion**

In this article, we present the presence of three cytokines, IL-8, CXCL16, and chemerin in conditioned medium from cultured primary human pRCC cells. We show that these cytokines attract human primary monocytes. Our bioinformatic analysis further shows that these cytokines are also expressed in pRCC tumors. We show in vitro that conditioned medium from primary pRCC cultures induces a shift of human monocyte phenotype towards the M2 inflammatory type. We also present data indicating that the foaminess of the macrophages is caused by accumulation of lipids in their cytoplasm, and show in vitro that this can be induced by factors secreted by pRCC cells. These results suggest that pRCC tumors secrete factors that cause attraction and differentiation of macrophages into M2 type.

## Overall conclusions

We believe that the results presented in this thesis in a satisfactory way fulfill the aims set at the beginning of this PhD project. The first paper, where we developed a new protocol allowing for fixation, permeabilization, intracellular antibody labelling, flow cytometry sorting, and isolation of high quality RNA from small populations of cells such as TSCs, helps to overcome one of the big issues in this field. In the past, only extracellular markers were used for fluoresce activated cell sorting if high quality RNA was required for further analysis. Our protocol makes use of intracellular markers possible while maintaining high RNA quality, allowing for more accurate studies. In the next three papers, we focused on specific markers characteristic for TSCs. In paper II we have experimentally proven that Mkl1 controls caveolin-1 expression. Caveolin-1 is an important structural protein and a tubular injury marker not expressed under healthy conditions. In paper III we focused on polymeric immunoglobulin receptor (pIgR) and its protective role in the injured kidneys. In paper IV we described a transcription factor SOX9 and its role and regulation in the kidney. Lastly, paper V investigated papillary renal cell carcinoma, a tumor type linked in its origin to TSCs, with a special focus on its characteristic physiologic feature of monocyte attraction.

Taken together these papers provide a new tool allowing for better analysis of tubular scattered cells, identify and investigate three important TSCs markers, their function and regulation, and provide new information about the physiology of a cancer type suggested to originate from TSCs.



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