Studies on renal progenitor cells and kidney cancer

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2016

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LUND
UNIVERSITY

DOCTORAL DISSERTATION
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To be defended at the main lecture hall, Medicon Village, Lund.
Friday 1st of April at 09.00 am.

Faculty opponent
Associate professor Nigel Mongan,
Faculty of Medicine & Health Sciences
The University of Nottingham
England

Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
Faculty of Medicine Translational Cancer Research Medicon Village, Lund	Date of issue: 1st of April, 2016
Author: Jennifer Hansson	Sponsoring organization

Title and subtitle: Studies on renal progenitor cells and kidney cancer

Abstract

Kidney cancer and renal injuries affect millions of people in the world resulting in high patient morbidity and mortality, as well as one of the most extensive medical costs for society. Upon renal injury the kidney has an endogenous ability to repair damaged tubules and restore kidney function, provided that the patient receives adequate supportive care in time. The molecular basis for this regeneration process is still not fully elucidated and the cellular origin for regeneration is extensively debated. Kidney cancer is characterized by vague clinical symptoms and pronounced resistance to common cancer therapies, like chemotherapy and radiation. Consequently, nearly 25% of patients present with spread disease already at diagnosis. For these patients a dismal prognosis awaits, with a 5-year survival rate of less than 10%. Thus, there is a desperate need for development of novel targeted therapies and diagnostic methods.

In this thesis we describe a new cell type intermingled between the proximal tubular cells of the nephron in normal human kidney. These cells share marker expression with regenerating tubules and display stem cell-like abilities as well as distinct morphological properties, such as low mitochondrial content and extensive structural and anchorage protein expression. By developing a novel human explant model of ATN ex vivo we also show that these cells are more resilient to injury. These cells were detected in normal chimpanzee and pig kidney, but not in mice, not even after induced renal injury. Additionally, the transcriptional profile of these cells is similar to that of papillary renal cell carcinoma (pRCC), and correlate to worse prognosis in clear cell RCC (ccRCC). These results indicate that these cells survive renal insults to a higher extent than bulk proximal tubule cells, and become activated to repopulate the tubule, but also suggest that pRCC might originate from oncogenic transformation of these cells.

In the second part of this thesis we show that the dopamine transporter SLC6A3 is highly expressed and functional in ccRCC, while only being expressed at very minute levels in normal kidney and other cancer forms. Additionally, we show that the SLC6A3 expression is affected by hypoxia inducible factor 2 alpha (HIF-2α), an important protein in the cellular oxygen sensing system, which is ubiquitously expressed in the constantly pseudohypoxic ccRCC tumors. We further demonstrate that hypoxia induces SLC6A3 expression in normal kidney cells, but not in cells from other normal tissues like breast or vessels. These results show that SLC6A3 is a highly specific biomarker for ccRCC and that the endogenous features of this dopamine transporter may be utilized for the development of novel treatment modalities in ccRCC.

Key words Renal regeneration, Renal progenitor cells, Kidney cancer, SLC6A3

Classification system and/or index terms (if any)

Supplementary bibliographical information | Language: English

ISSN and key title
1652-8220

ISBN
978-91-7619-262-7

Recipient's notes | Number of pages 88 | Price

Security classification

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Cover photo by: Jennifer Hansson

The photo shows a histological section of human kidney displaying a proximal tubule with a single proximal tubule progenitor cell (PTPC) positive for CD133 (red) while having low mitochondrial content, compared to the surrounding epithelial cells which are full of mitochondria (green). Nuclei are seen in blue.

Medical Faculty | Department of Laboratory Medicine, Lund

ISBN 978-91-7619-262-7
ISSN 1652-8220
Lund University, Faculty of Medicine Doctoral Dissertation Series 2016:36

Printed in Sweden by Media-Tryck, Lund University
Lund 2016

Till min familj

Content

Abbreviations

AACD	Aromatic L-amino acid decarboxylase
ABC	ATP-binding cassette
ADHD	Attention deficit hyperactivity disorder
AKI	Acute kidney injury
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
ARF	Acute renal failure
ARNT	Aryl hydrocarbon receptor nuclear translocator protein
ATN	Acute tubular necrosis
ATP	Adenosine triphosphate
BHD	Birt-Hogg-Dubé
BMSC	Bone marrow derived stem cells
CA9/CAIX	Carbonic anhydrase IX
cAMP	cyclic adenosine monophosphate
ccRCC	clear cell renal cell carcinoma
CD	Collecting duct
chRCC	chromophobe renal cell carcinoma
CKD	Chronic kidney disease
CKI	Chronic kidney injury
CNS	Central nervous system
CSC	Cancer stem cell
DAT	Dopamine transporter
DT	Distal tubule
EMT	Epithelial to mesenchymal transition
ESC	Embryonic stem cell
ESRD	End stage renal disease
FACS	Fluorescence activated cell sorting
FIH	Factor inhibiting HIF
GLUT	Glucose transporter
HIF	Hypoxia inducible factor
HR	Homologous Recombination
HRE	Hypoxia response element
HSC	Hematopoietic stem cell
IFN-α	Interferon alpha
IL-2	Interleukin-2
iPS cell	Induced pluripotent stem cell
IRI	Ischemia reperfusion injury

LOH	Loss of heterozygosity
mTOR	mammalian target of rapamycine
mRNA	messenger RNA
mRCC	metastatic RCC
NOD/SCID	Non-obese-diabetic/severe combined immunodeficiency
NTT	Neurotransmitter transporter
PD	Parkinson's disease
PDGF	Platelet derived growth factor
PD-1/PDL-1	Programmed death receptor 1/ ligand 1
PEC	Parietal epithelial cell
PHD	Prolyl hydroxylase-domain
PKA	Protein kinase A
PKC	Protein kinase C
pRCC	papillary renal cell carcinoma
PT	Proximal tubule
PTPC	Proximal tubule progenitor cell
PTRC	Proximal tubular rare cell
RA	Retinoic acid
RCC	Renal cell carcinoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SLC	Solute carrier
SPECT	Single photon emission computed tomography
TAAR1	Trace amine associated receptor 1
TCA	Tricarboxylic acid
TCGA	The cancer genome atlas
TERT	Telomerase reverse transcriptase
TKI	Tyrosine kinase inhibitor
TLR-2	Toll like receptor 2
TNM	Tumor-node-metastasis
TSC	Tuberous sclerosis complex
UUO	Unilateral urinary obstruction
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau

List of papers

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

I Isolation and characterization of progenitor-like cells from human renal proximal tubules.

Lindgren D, Boström AK, Nilsson K, **Hansson J**, Sjölund J, Möller C, Jirström K, Nilsson E, Landberg G, Axelson H, Johansson ME.

American Journal of Pathology. vol. 178 (2) pp. 828-37. 2011

II Evidence for a morphologically distinct and functionally robust cell type in the proximal tubules of human kidney.

Hansson J, Hultenby K, Cramnert C, Pontén F, Jansson H, Lindgren D, Axelson H, Johansson ME.

Human Pathology vol 45 (2) pp 382-93. 2014

III Species diversity regarding the presence of proximal tubular progenitor cells of the kidney.

Hansson J, Ericsson A, Axelson H and Johansson ME

European Journal of Histochemistry vol 60:2567. 2016

IV Overexpression of functional SLC6A3 in clear cell renal cell carcinoma.

Hansson J, Lindgren E, Nilsson H, Johansson E, Johansson ME, Gustavsson L and Axelson H

Manuscript

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Abstract

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transporter may be utilized for the development of novel treatment modalities in ccRCC.

The Kidneys

The maintenance of a relatively constant fluid volume and electrolyte composition is essential for homeostasis: the stable environment inside our bodies optimal for survival. Disruption of homeostasis, such as changes in ion concentrations, can have deleterious effects and can rapidly lead to severe illness or death. Homeostasis is maintained by several self-regulating biological systems, where many of the most central ones are localized to the kidneys, such as regulating the balance of electrolytes, blood pressure and acid base equilibrium.

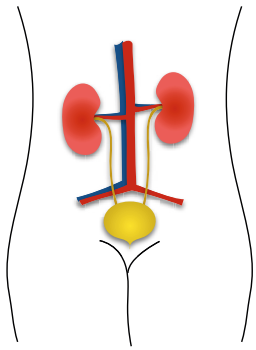

Figure 1. Anatomical location of the kidneys
The bean shaped kidneys are located in the retroperitoneal space of the body, on each side of the vertebral column. The kidneys are connected to the bladder via the urethra and to the aorta via the renal arteries.

The kidneys are two bean shaped organs located in the retroperitoneal space of the body, on each side of the vertebral column (Figure 1). For protection, the kidneys are surrounded by a tough fibrous cap, the fascia of Gerota underneath which a perinephric layer of fat resides. Interestingly, under conditions of starvation this fat compartment is the last that remains, reflecting the vital importance of protecting the kidney and thus maintaining kidney function. The kidneys are highly active organs that are estimated to consume 25% of the body's ATP in order to perform their daily tasks, despite their relatively modest weight (1). Apart from the aforementioned functions, the kidneys also serve important endocrine roles via renin and erythropoietin production. Erythropoietin is released in response to low oxygen tension and induce increased production of red blood cells in the bone marrow. The

enzyme renin is released from the juxtaglomerular apparatus of the kidney as a response to low arterial blood pressure, and exerts its function by activating angiotensin I and angiotensin II. The angiotensins induce general vasoconstriction and retention of salt and water, causing increased blood volume and a resulting increase of blood pressure. The kidneys also have a role in calcium homeostasis via the production of calcitriol, the activated form of vitamin D. Calcitriol stimulates the uptake of dietary calcium in the gut as well as inducing augmented calcium reabsorption in the kidneys, resulting in increased Ca2+ levels in the blood (2). However, the kidneys most apparent function is to filter the blood and eliminate metabolic end products and toxins through the production of urine. Each kidney is connected to the abdominal aorta via the renal arteries, which supply the kidneys with over 20% of cardiac output, by far exceeding the metabolic need of the kidney cells, while reflecting the kidneys essential role in plasma filtration (3). Each day, an astonishing 180 liter of primary filtrate is processed by the kidneys, where about 99,5% are reabsorbed into the body, generating approximately 1,5 liters of urine for excretion (1). This endless process of filtration and reabsorption is performed by the functional units of the kidneys, the nephrons.

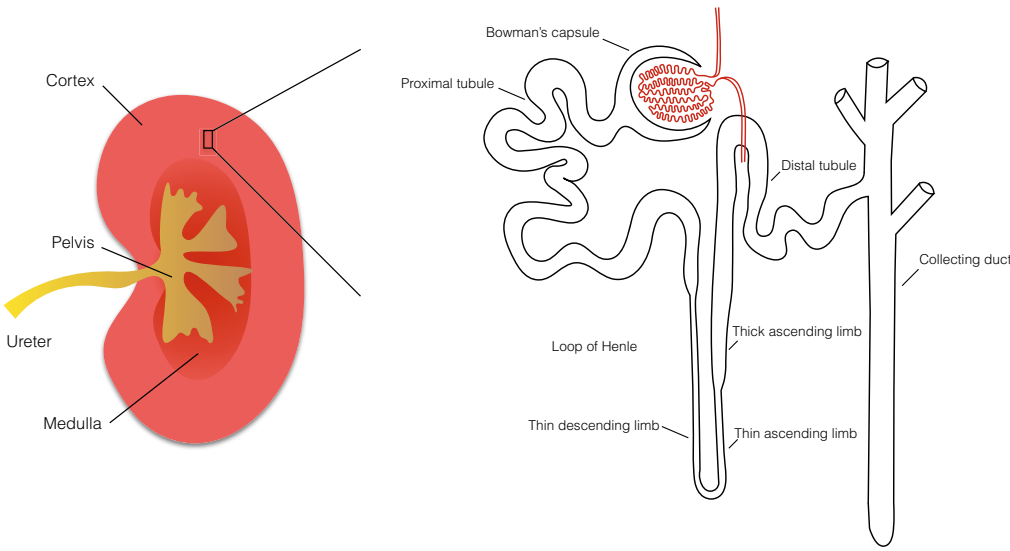

Figure 2. Schematic view of the kidney and the nephron
The nephrons are the functional units of the kidneys and the majority of nephrons are located in the kidney cortex. The nephrons are composed of the filtering glomerulus followed by the proximal tubule (PT), where the major part of the reabsorption takes place. The PTs leads the filtrate further on into the Loop of Henle continuing into the distal tubule that connects with the collecting duct, which transfers the urine down to the urethra.

The nephron

The nephrons are the functional units of the kidneys and each kidney contain about one million of these units, each organized into an intricate 3 dimensional architecture. The major part of the nephrons, roughly 90%, is located in the kidney cortex, the outer part of the kidneys (Figure 2). The nephrons are composed of a capillary tuft of the glomerulus, where filtration occurs. This is surrounded by Bowman's capsule were the primary filtrate is collected and guided into a complex tubular system. The renal tubules consist of the proximal tubule (PT), where most of the filtrate is reabsorbed, further leading into the loop of Henle in the renal medulla where it takes a sharp turn to then reach the distal convoluted tubules (DT) where a final modification of the primary filtrate takes place. The distal tubules connect to the collecting ducts (CD) ending in the renal pelvis where the urothelial tract originates and leads to the bladder where the end product urine is collected. The glomerulus is impermeable to larger proteins while salts, ions and all other organic molecules below a certain molecular size are freely filtered through the capillary tuft. The glomerular filtration barrier is composed of three layers: the fenestrated endothelial cell layer, the glomerular basement membrane of negatively charged proteoglycans and collagens, and the epithelial cell layer composed of podocytes with foot like protrusions that surround the capillary loops, allowing the filtrate of solutes and water to finally pass through their slit diaphragm into the Bowman's space (Figure 3).

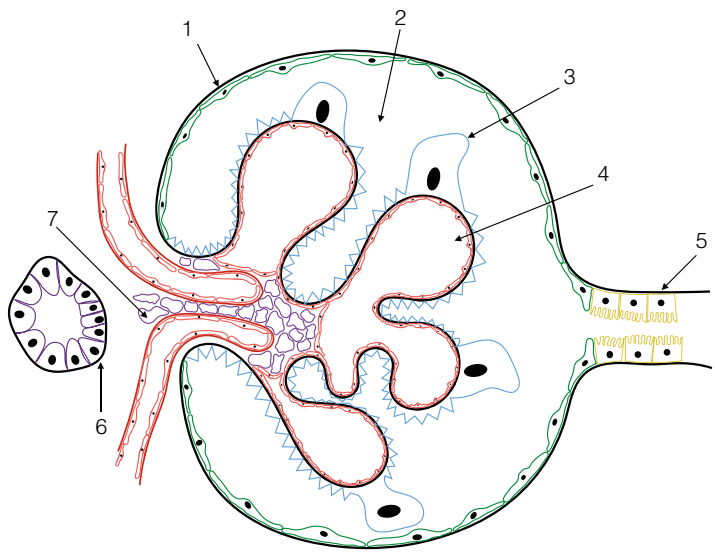

Figure 3. Schematic view of the glomerulus
1. Parietal epithelial cells (PEC) of the Bowman's capsule. 2. Bowman's space. 3. Podocyte. 4. Capillary loops. 5. Proximal tubule. 6. Macula densa. 7. Mesangial cells

The glomerulus also harbors mesangial cells, which besides having a phagocytic function also may regulate glomerular capillary blood flow due to their contractile ability. Following Bowman's space the filtrate enters the first tubular compartment, the PT, where essentially all glucose and amino acids are reabsorbed. The PTs also absorb 65% of the filtered water, sodium, potassium, chloride and bicarbonate. The principle for the reabsorption is vectorial transport and to accomplish this immense task the PT cells are endowed with an apical brush border in order to increase the surface area to the filtrate. Furthermore, numerous transporter protein systems are localized in the apical compartment, facing the tubular lumen, mainly performing secondary active transport of solutes down an electrochemical gradient for sodium. The foundation for this is basally localized Na^+/K^+-ATPases exporting intracellular sodium, thus creating the gradient. In order to fuel these Na^+/K^+-ATPases the PT cells are endowed with a massive abundance of mitochondria, accounting for some 60% of PT cell volume. In addition to reabsorption, the PT cells also actively secrete metabolic waste products, like organic acids and bases into the tubular lumen to ensure rapid excretion of potentially harmful substances. Further down the tubular system the filtrate encounters the loop of Henle that reaches deep into the medulla and have three distinctive segments. The cells of the thin descending segment have low metabolic activity with little active transport while being extensively permeable for water and urea. The water moves through the tubular layer by passive diffusion, due to the hyper osmotic pressure in the medulla caused by the active transport of Cl^- and accompanying passive transport of positively charged ions like K^+ and Na^+ in the ascending limb. The ascending limb is in turn virtually impermeable to water. Henle's loop in conjunction with the collecting ducts are the foundation for the counter current multiplicator system without which the essential property of urine concentration would be impossible. This property allows us to save water by decreasing urinary output during thirst or the reverse if water intake is plentiful (2).

In the thick ascending limb the metabolic activity of the cells increase again, reflected by an increase in mitochondrial content. This segment reabsorbs an additional 25% of sodium chloride and potassium. The loop of Henle leads to the DT, which is highly convoluted and has similar reabsorptive properties to the thick ascending limb. The DT also contains parts of the juxtaglomerular apparatus, which is involved in feedback control of the glomerular filtration rate (GFR) and renal blood flow, and hence blood pressure. The distal tubules then connect to the CD. The late DT and the cortical CD contain two different cell types; the principal cells and the intercalated cells. The principal cells are responsible for NaCl reabsorption and K^+ secretion, while intercalated cells reabsorbs K^+ and have a role in controlling pH through the secretion of H^+ or HCO_3^-. Worth noting is that the medullary CD stems from the ureteric bud in contrast to the rest of the tubular system, which stems from the metanephric mesenchyme. Finally in the CD, the concentration of the filtrate is fine tuned by a controlled reabsorption of water, via the hormone levels of

vasopressin, before being discharged into the bladder. Ultimately, the formation of the final product, urine, is essential for the body's ability to remove metabolic waste substances like urea, bilirubin, uric acid, hormone metabolites and creatinine (1, 2, 4).

Stem and Progenitor cells

Stem cells are characterized by their ability to self-renew as well as their capacity to generate various differentiated cells. The stem cell can divide to generate one identical daughter cell as well as a progeny committed to differentiate into a mature specialized cell. Stem cells are classified as either embryonic or adult stem cells, also called tissue specific stem cells. Stem cells have several levels of differentiation potential. In mammals, the totipotent stem cell is found in the morula, the cluster of cells from the first cell divisions following oocyte fertilization. The totipotent cells can mature into all cells of the embryo as well as the extra-embryonic tissue, such as the placenta. The embryonic stem (ES) cells are derived from the inner cell mass of a blastocyst. These cells are pluripotent and can give rise to cells from the three germ layers: ectoderm, mesoderm and endoderm, comprising all the different specialized cell types in the body. Under the right culture conditions ES cells sustain their ability to self-renew indefinitely *in vitro*, while still maintaining their pluripotency (5, 6). This feature makes the cells excellent model systems for studying development, differentiation and function of different tissues

In contrast to the ES cells, the tissue specific stem cells can only give rise to a limited set of specialized cells within a given tissue. These cells can be multipotent or oligopotent, reflecting the potency for the amount of lineages they can give rise to (7). Tissue specific stem cells can be found in several organ systems in the adult body, most typically in tissues that need to continuously replenish their cells, as for instance in the bone marrow, where the hematopoietic stem cells (HSCs) endlessly generate new blood cells, or in the intestinal epithelium where the stem cells of the crypt rapidly replace the shed epithelial cells. Adult stem cells can also remain in a non-dividing state for a very long time until being activated by a need for normal tissue maintenance or by injury or disease, as in the case of stem cells in more quiescent organs like heart, liver and lungs (8-10). The concept of progenitor cells are often confused with stem cells, but in contrast to the latter, progenitor cells are restricted in their self-renewal capacity, that is, they can't divide indefinitely. Additionally, progenitor cells are more specialized than stem cells and they can in some cases be unipotent, that is, give rise to only one kind of mature cell, in which case they can also be termed precursor cells. However, similar to stem cells, some progenitor cells can give rise to several different specialized cell types and thereby be oligopotent (11).

Hematopoietic stem cells (HSC) provide a good example of a multipotent adult stem cell. The most primitive of the HSC is the long term hematopoietic stem cell (LT-HSC) that has a lifelong ability to self-renew and divide. The LT-HSC can give rise to the short term hematopoietic stem cell (ST-HSC) that has a shorter life span and a more restricted self-renewal. As the ST-HSC matures it gives rise to the multipotent progenitors, MPP, which differentiates into mature cells of the myeloid and lymphoid lineages (12).

The stem cell niche

Adult stem cells reside in a niche, which represents a microenvironment that helps to maintain the stem cells in an undifferentiated and often quiescent state. Stem cell niches have been thoroughly investigated with single cell resolution in invertebrates like Drosophila and C. elegans, while the study of human niches have been hampered by the vast size of their tissues, as well as by an uncertainty of reliable markers for the true primitive stem cells. In a niche, stem cells can be clustered together like the stem cells of the hair follicle that reside in the bulb, or scattered through the tissue as in the case of satellite muscle stem cells that reside underneath that basal lamina of myofibers. It is postulated that the niche supports the stem cells in several ways; both with supporting cells as the paneth cells in the intestinal crypt, which are required to sustain the Lgr5+ intestinal stem cells (13), and by extracellular signaling as in the case of HSCs, which require the cytokine CXCL12, angiopoitin and stem cell factor (SCF) for their maintenance (9). Also relatively low oxygen pressure (pO_2 1-8%) has been suggested o play a role for stem cell maintenance of HSCs, mesenchymal stem cells as well as neuronal stem cells (14). In this setting, lower oxygen levels (1%) decrease stem cell proliferation, suggesting a role in maintaining a quiescent state. However, substantial number of reports have ascribed niches to be located in close vicinity of vessels, such as in the bone marrow and spleen where HSC reside in the sinusoidal blood vessels (15). Additionally, what we define as hypoxia in cell culture conditions can be equal to physiologic normoxia, thus the role of oxygen tension in stem cell maintenance is not yet fully clarified.

Induced pluripotent stem cells

In 2006 the Yamanaka group revolutionized the field of stem cell research when reporting that fully differentiated cells could be converted into pluripotent stem cells by only four factors, Oct 3/4, Sox2 Klf4 and c-Myc (16). This suggested that stemness might not be as static as previously thought, but a more plastic state. Induced pluripotent stem (iPS) cells are important tools for the studies of the molecular mechanisms that maintain and control pluripotency. The full potential of iPS cells in human disease is still not clear, but they bring hope to the field of regenerative medicine, with the notion that each patient's own somatic cells could be used for patient specific therapies.

Due to their remarkable regenerating abilities, stem cells have been a hot topic of research in the hope of discovering new ways to treat regenerative and genetic diseases. They also hold enormous potential as candidates for drug screens and toxicity tests. However, deeper knowledge of the genetic pathways that govern the regulation of stem cells is essential in order to use these remarkable cells to their full potential. From a scientific point of view, the presence of adult stem cells in an organ influences the interpretation of the organs regeneration ability and pathophysiology. Furthermore it adds an important aspect to tumor biology, since it has been shown that several cancers and tumors may arise by malignification of organ confined stem cells (17).

Cancer stem cells

Cancer stem cells (CSC) represent a subpopulation of cells with the ability to self-renew and generate all different cells of a heterogeneous tumor, thereby replenishing the tumor with new cells and maintaining tumor growth. From a superficial point of view, the concept of cancer stem cells stands in contrast to another theory of cancer development, clonal evolution. This latter theory postulates that cancer will arise when a sufficient number of mutations have occurred in any given cell (18). This creates vicious cycle where mutations in the original cancer cell allows the progeny in the growing tumor to accumulate even more mutations, which may be different in individual progeny, thereby giving rise to intra tumor heterogeneity. The clonal evolution theory stipulates that all cells of a tumor harbor the same capacity to maintain and advance tumor growth, while the cancer stem cell theory postulate that only a small subset of cells has this ability However, there are also efforts to combine the two concepts that take into consideration aspects of both theories (19).

The cancer stem cell concept was first experimentally demonstrated in acute myeloid leukemia (AML), where a limited number of an isolated subpopulation of undifferentiated cells transplanted into NOD/SCID mice developed AML with fully differentiated tumor cells, while the remaining subpopulations were unable to give rise to tumor growth (20, 21). Whether or not the CSC originate from transformed normal stem cells or from more mature cells that gain the stem cell properties by mutations, is still not fully elucidated. An adult stem cell would require fewer mutations to gain CSC properties than a mature cell would, and there have been several reports suggesting that tissue stem cells are indeed the origin of many cancers (22-24). Nevertheless, several reports have also shown that CSCs can be created through dedifferentiation or epithelial to mesenchymal transition (EMT) of mature bulk cancer cells (25-27). Much like normal stem cells, CSC have been associated with the expression of multi drug resistance pumps of the ATP-Binding Cassette

(ABC)- family of transporters, contributing to therapy resistance in cancer treatment (28). Additionally, it is believed that conventional therapies like cytostatics affect dividing cells, while leaving the relatively quiescent CSCs unaffected, and that one thus need to specifically kill CSCs with targeted therapies to eradicate the tumor. However, since it has been shown that the bulk tumor cells can transition into CSCs it would be important to target both the bulk and the CSCs simultaneously to get rid of the tumor (18).

Identification and Isolation

Several techniques have been applied in order to search for, and isolate, stem cells. The use of specific surface markers is one method, like CD34 and Thy-1 that has been ascribed to hematopoietic stem cells or CD133, CD44 and Lgr5 as markers for intestinal crypt cells (29-31). "Label retention" has also been used to identify stem cells. This functional assay is based on the notion that stem cell divide more slowly than their progeny, but also on the "leading strand hypothesis". This hypothesis states that stem cells divide asymmetrically and that the stem cell always retain the original DNA strand, while the cells bound for differentiation receives the newly synthesized strands. Both of these notions imply that experimentally provided DNA labels, for example a BrdU pulse, would be retained in the stem cells while being washed out of their progeny after a long chase period, thereby allowing for identification of the stem cells by "lable retention". However, concerns have been raised for whether this technique really labels true primitive stem *in vivo*, partly because some mature cells have been shown to stay non-dividing for a long time, such as paneth cells in the intestinal crypt, (32), but also because not all stem cells divide asymmetrically as described in the leading strand hypothesis (33).

A conceptually different method to isolate stem cells is to use stem cell associated biological functions, as for instance to utilize their inherent and necessary ability to protect themselves against cytotoxic insults. Stem cells have been shown to express ABC-transporters, which allow the cells to efflux substances that can be potentially harmful. This capacity can be used to isolate a so-called side population, by identifying the cells that pump out Hoechst 33 342 dye (34). Another way that stem cells can protect themselves is through an enzyme based detoxification system, based on high aldehyde dehydrogenase (ALDH) activity. ALDH enzymes convert potentially toxic aldehydes to their corresponding weak carboxylic acids (35). An additional feature of ALDH, in association to isolation of stem cells, relates to its enzymatic function to catalyze vitamin A (retinal) into retinoic acid (RA). RA signaling is thought to be essential for cell fate determination and thereby stem cell maintenance. The levels of enzymatic ALDH activity in cells can therefore be used to

isolate subpopulation of cells with stem cell characteristics. Fluorescent activated cell sorting (FACS) based on ALDH activity has successfully been used to sort out stem cells of several origins, in particular HSC, but also breast cancer stem cells, intestinal mucosal stem cells and liver cancer stem cells (36).

Renal regeneration

Acute Kidney Injury

Acute kidney injury (AKI) is clinically characterized by a rapid decrease of urine output, oliguria, where between 50-400 ml urine is produced per day in contrast to the normal 1,5 liters of urine, as well as elevated creatinine levels, and/or decreased glomerular filtration rate (GFR) (37). AKI is a life threatening condition that leads to disturbed fluid and electrolyte balance, along with a decreased ability to eliminate metabolic waste products, uremia. AKI represent 3,2- 9,6% of causes for hospital admissions in industrialized countries, and the AKI incidence is estimated to be somewhere around 2000-3000 cases / million individuals / year (38). The most severe form of AKI is acute renal failure and around 6% of all AKI cases require renal replacement therapy and the mortality rates for these patients are as high as 60% (39). Additionally, patients who survive AKI are at higher risk do develop chronic kidney disease (CKD) (38). All in all, it is estimated that over 2 million people die of AKI each year (38, 40).

Acute Tubular Necrosis

One of the most common causes of acute kidney failure is acute tubular necrosis (ATN) (41). This condition can be caused by a number of insults, for example severe trauma, septicemia and acute pancreatitis (42). ATN can be divided into ischemic ATN and nephrotoxic ATN. Ischemic ATN is associated with hypovolemic shock, but can also be caused by hemolytic crisis, and is due to impaired renal blood flow and thereby diminished oxygen delivery to the tubular cells. Nephrotoxic ATN is less common and caused by intoxication, for example by heavy metals or antibiotics, but can also be caused by ingestion of poisonous mushrooms (41). Renal tubular cells, in particular the proximal tubular cells, are extremely energy dependent and thus very sensitive to disturbances in nutrient and oxygen delivery. The cells of the proximal tubule are packed with mitochondria and are virtually incapable of using non-oxidative glycolysis as their main source of energy (43). Impaired renal blood flow, or toxic renal insult, therefore leads to tubular cell dysfunction. The cells begin to loose their important epithelial polarization, and Na^+-ATPases shift from their baso-lateral

position to the apical side. This leads to disrupted vectorial transport capacity and consequently that the kidneys essential fluid reabsorption becomes impaired. Less sodium is thus reabsorbed in the proximal tubules, which leads to higher concentrations of Na^+ reaching the distal tubules where the tubuloglomerular feedback system responds by intrarenal vasoconstriction. This results in decreased glomerular filtration rate (GFR) and oliguria, clinically corresponding to AKI (44). The insult to the renal tubules can also cause the tubular cells to detach, morphologically seen as cells characteristically sloughing into the tubular lumen, leaving behind denuded basal membranes. The shedding of tubular cells can clog up the renal tubular system and in this way contribute to the decreased urine output. The obstructed urine flow can also cause increased intratubular pressure, resulting in leakage from the injured tubular cells into the interstitium, causing edema. However, remarkably, the serious condition of ATN can be reversible through the process of renal tubular regeneration (42).

Regeneration

Neonephrogenesis denotes the ability to regenerate entire nephrons following injury. This capacity is present in invertebrates like the fruit fly (45), and in lower vertebrates like the zebrafish (46). In contrast, human and other mammals have lost this ability and the renal regeneration capacity is more limited, encompassing tubular, and to some extent, podocyte regeneration. The renal tubules are normally almost mitotically quiescent, in contrast to several other transporting epithelia. Nevertheless, upon injury, the nephrons demonstrate a pronounced capacity to regenerate damaged tubules, and ATN can be reversible if the patient receives supportive care in time. Maintenance of tubular structural integrity is essential for kidney function, and proper clinical management after ATN can thus be the difference between life and death. The regeneration of the renal tubules is based on: critical coverage of denuded basal membranes to ensure structural integrity, cellular proliferation to replace the cells lost by apoptosis or necrosis and finally differentiation to a fully functional tubular cell to regain transporting capacity. In the developing kidney, the cap mesenchyme harbors multipotent progenitors with the ability to give rise to all the cellular components of the nephron. However, the cellular source for renal regeneration in adult kidney is still debated (Figure 4). The classical view is that tubular regeneration is performed by the least damaged tubular cells, which subsequently dedifferentiate to repopulate the tubule (47, 48). This view has in recent years gathered supporting evidence through several animal studies using lineage tracing (49-51). Migrating bone marrow derived stem cells (BMSC) have also been suggested to take part in tubular regeneration (52). Although, animal models has shown that BMSC can ameliorate the symptoms after induced acute renal injury,

they do not seem to directly take part in the regeneration (53). The role of the BMSC might rather be production of protective paracrine factors that can facilitate the epithelial regeneration (54). Interestingly, a phase I clinical trial using bone marrow derived mesenchymal stem cells for therapeutic use, in patient undergoing cardiac surgery with high risk of developing AKI, indeed showed protective effects with regards to kidney injury (55).

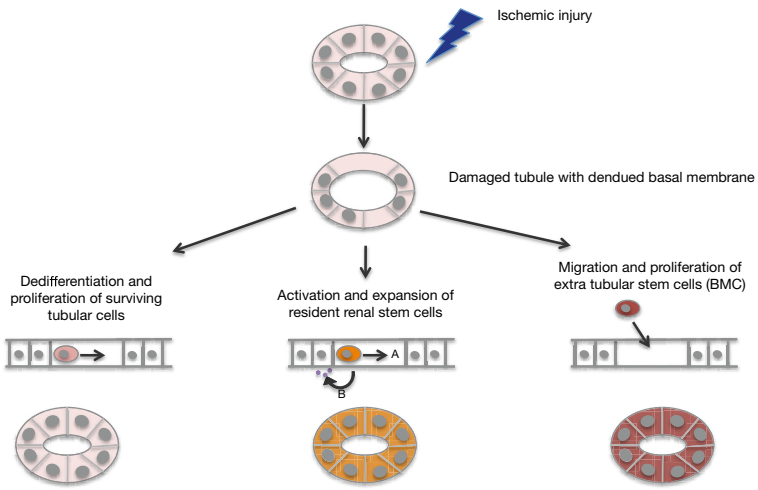

Figure 4. The hypotheses of the source for renal regeneration
Schematic image of the different hypotheses concerning renal regeneration. The classical view is that random surviving tubular cells dedifferentiate, divide and repopulate the tubule (left). Another view is that regeneration is performed by resident progenitor cells that become activated in the aftermath of renal injury and divide to repopulate the tubule (A) or that the progenitor cells secrete protective factors that help the bulk tubular cells survive the insult (B) (middle). A contrasting view is that extratubular stem cells like bone marrow derived stem cells (BMC) migrate to the tubule and integrate into the tubular epithelium and divide to repopulate the tubule (right). Modified from Anglani et al. Frontiers in Bioscience 2008.

Renal progenitor cells

An alternative hypothesis regarding tubular regeneration is that it is performed by adult resident renal stem cells (56). The localization of these putative progenitor cells have been ascribed to the renal papilla (57), renal interstitium (58, 59), the Bowman's capsule (60, 61), or to the renal tubules (62, 63). These cells may be more resistant to damage and become activated in the wake of renal damages. In human kidney, a progenitor cell population was described in the parietal epithelial cells (PEC) of the Bowman's capsule in 2006 (64). These cells were characterized by CD133 and CD24 expression, markers that have been ascribed to stem cells in several organs (65-68), additionally, CD24 is also expressed in the metanephric mesenchyme during renal development (69). Isolated human CD133+ CD24+ cells were shown to be multipotent and able to develop into several lineages *in vitro*, such as osteogenic cells

and adipocytes, as well as tubular cells. These cells were also able to integrate into renal tubules of SCID mice with glycerol induced renal injury, as well as to reduce the renal damage (64). The PEC progenitor cells were later subdivided into two groups based on their location in Bowman's capsule and on the expression of podocalyxin (PDX). PEC located close to the vascular pole were PDX positive and able to differentiate into podocytes, while PEC at the urinary pole were PDX negative and able to differentiate into both podocytes and tubular cells. The glomerular podocytes are post-mitotic, meaning that they are unable to divide and proliferate. Consequently, if podocytes are injured or lost, glomerular function is dependent on cellular replenishment from an alternative source. PEC of the Bowman's capsule has lately been suggested as the main source of podocyte regeneration (60, 61, 64), but also renin-producing cells in the juxtaglomerular apparatus have been shown to contribute to podocyte regeneration (70).

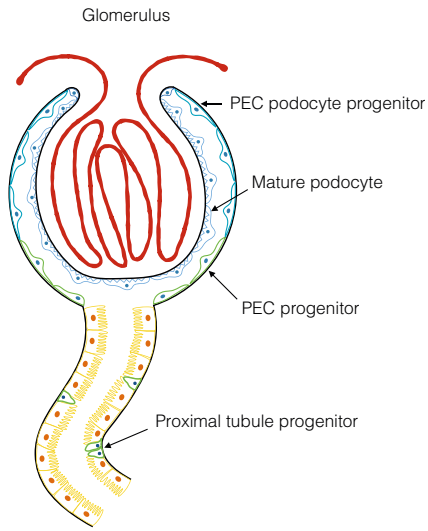

Figure 5. Location of the putative renal progenitor cells
The parietal epithelial cells (PEC) progenitor cells are located in the Bowman's capsule and are positive for CD24 and CD133. PEC located near the vascular pole are thought to give rise to podocytes, and PEC located near the urinary pole is thought to give rise to other PEC as well as to tubular cells. The PT progenitors are scattered among the tubular cells and are often located at plicae positions, where the PT make hairpin turns, and are sometimes found in duplets.

In 2011, our group described a rare population of divergent cells inconspicuously scattered among the differentiated cells of the PT in human kidney (Figure 5) (Discussed in detail in Paper I) (71). These cells were isolated based on high enzymatic activity of ALDH and displayed progenitor features such as clonogenic capacity and anchorage independent growth, while the bulk PT cells ($ALDH^{low}$) did not. Additionally, $ALDH^{high}$ cells displayed striking marker similarity to PEC

progenitor cells and expressed CD24, CD133 and vimentin along with many other markers that also were expressed by the PECs. We later showed that the scattered tubular cells have a distinct morphology indicative of increased adherence to the basement membrane, higher mechanical resilience as well as a dramatically lower mitochondrial content compared to the neighboring PT cells. Moreover, we developed a novel human explant culture, simulating ATN *ex vivo*, demonstrating that these cells were more resilient to insult that bulk PT cells (72) (Discussed in Paper II). This finding was strengthened by Angelotti et al. that showed that human renal CD133+ cells indeed were more resistant to death following exposure to nephrotoxic agents compared to other tubular cells. In xenograft experiments, it was also demonstrated that isolated human tubular progenitors, distinguished from the PEC progenitors via the lack of CD106 expression, were able to integrate into renal tubules of SCID mice following renal injury and to generate new epithelial cells that improved the recovery of renal function (73).

Sallustio et al. broadened the view regarding the functional role of these scattered tubular progenitor cells by demonstrating that the cells could protected bulk PT cells against cisplatin induced damage. This protection mechanism was Toll like receptor-2 (TLR2)-dependent and achieved via exosomal shuttling of protective factors from the tubular progenitors to the injured PT cells (74).

There is, however, an alternative interpretation of these scattered tubular cells. Based on the characteristic marker expression of these cells, which other than CD24 and CD133, also express Vimentin, CD44, KIM-1 and anexin-3 (75), some groups have interpreted these markers as, in themselves, signs of dedifferentiation, and that this is in fact an induced phenotype that any differentiated tubular cell could adopt upon injury (76). Thus they interpret the discovery of scattered cells in human kidney samples merely as signs of renal ware and tare, possibly due to old age of the patients from where the renal tissue is derived. However, we have demonstrated that these cells are present also in young healthy kidneys from humans of age 2 years and up (72). Additionally, these cells have a characteristic paucity of mitochondria in comparison to the massive mitochondrial load of bulk PT cells, of which the fully mature PT cells would quickly have to get rid of if this were indeed an induced phenotype. (These matters are discussed in detail in Paper II).

Lineage tracing and cell fate labeling in mice

In order to investigate which cells are responsible for regeneration, and simultaneously try to answer the question whether or not progenitor cells exist in adult kidney, several lineage tracing experiments have been performed. Lineage tracing is performed in transgenic mice that carry a specific promoter, which drives a reporter gene conveying a molecular mark, for example fluorescent proteins such as GFP (green fluorescent protein) or β-galactosidase, to the cell where the promoter is active. Since the activation of the marker gene is irreversible, the marked cells progeny then inherits this molecular mark. In this way, lineage tracing allows identification of all the progeny stemming from a defined single cell. The lineage tracing in kidney have been performed using a variety of promoters, some with the intention to mark a specific cell population, like the proposed renal progenitor cells, and other with a more wide approach intending to label all differentiated tubular cells. I will go through some of them here and also what critique that has been raised concerning the conclusions that has been drawn from the experiments.

Humphreys et al. used the Six2 promoter to mark all cells from the nephron (except collection duct). Six2 is expressed during embryogenesis and marks the stem cells of the metanephric mesenchyme that give rise to all differentiated cells of the mature nephron, except for the cells of the collecting duct, which instead are derived from the ureteric bud. Using this model in conjunction with ischemia reperfusion injury (IRI), which simulates human ATN, they could show that the injury-induced regeneration was performed by already labeled tubular cells and could thus exclude extra renal cells like BMSC as a potential cellular source for regeneration (50). However, in this experiment the labeling could not distinguish between fully differentiated tubular cells or a potential progenitor population, and thus no conclusion could be drawn of which intra-renal cell type that achieved the regeneration. The same group tried to resolve this issue using two different subsequent DNA-labeling thymidine analogue injections following IRI, CldU (5-chloro-2-deoxyuridine) 24h after induction of injury and IdU (5-iodo-2-deoxyuridine) 45h after IRI (49). They hypothesized that if a progenitor population indeed existed and were responsible for the regeneration, then the cells of the regenerated tubule would be double labeled. However, only a small fraction of tubular cells were double labeled and the researchers drew the conclusion that the regeneration was a purely stochastic event, performed by surviving differentiated tubular cells and not a progenitor population. These conclusions have been met with some criticism claiming that cells that are labeled with only one of the thymidine analogues not necessarily have completed cell division (77). Lombardi et al. argue that cell cycle labeling only indicate that the cell has entered the cell cycle but not that it has committed to dividing. This notion has been demonstrated in liver where most hepatocytes shows signs of cell cycle entry following partial hepatectomy, but the vast majority of these cells never undergo cell division (78). Additionally, pathological

investigations of injured renal tissue following mercury chloride injections in rats suggest that mitosis is a rare event (79).

In another lineage-tracing experiment Appel et al. used an exogenous promoter composed of a human and rabbit podocalyxin promoter, termed PEC-rtTA, to drive an inducible expression of LacZ (60). Podocalyxin is normally expressed in podocytes, while this model, quite surprisingly, the promoter marked PECs and also scattered cells within the PTs. In mice, nephrogenesis is active also after birth, and using this model on juvenile mice the researchers could show that PEC cells could migrate along the vascular tuft and mature into differentiated podocytes. In a subsequent study by the same lab the researchers investigated the marked scattered tubular cells further (75, 76). The scattered cells expressed several molecular markers common to the ones reported for the putative progenitor population in human such as anexin A3, CD44, KIM1 and src suppressed C-kinase. These cells were then followed after induced IRI. The frequency of marked scattered cells did not change following damage, however, if the mark was induced during injury more cells became positive. The authors thus drew the conclusion that these scattered tubular cells represent a phenotype that can be induced from any differentiated tubular cell following damage, rather than a resident progenitor population. However, no functional studies were performed using these cells, nor any other phenotypic description other than that some markers were shared with the human CD133+/CD24+ cells, although these markers are not the most characteristic for the human cells and they are also expressed in other mature renal cells. It has thus been suggested that the scattered cells marked using the PEC-rtTA promoter is not necessarily the same population that has been described in human kidney (77, 80).

In another study by Langworthy et al. it was demonstrated that mice heterozygous for NFATc1 are more sensitive to HgCl2 induced renal injury (81). Additionally, NFATc1 driven LacZ expression labeled cells that wore more resistant to the HgCl2 induced renal injury, also the NFATc1 positive cells progeny were responsible for 75% of the regenerated cells. The authors interpreted NFATc1 positive cells as a progenitor population. However, no NFATc1 positivity was detected in the uninjured kidney cortex, nor was NFATc1 expressed during kidney development. Taking these issues into account one may also interpret the findings as that NFATc1 is expressed by dedifferentiated surviving tubular cells that proliferate to restore the tubule.

Kusaba et al. took a different approach and used the promoter of the Slc34a1 (sodium dependent inorganic phosphate transporter) to mark all differentiated PT cells (51). Upon IRI they could not detect any dilution of the marker, suggesting that differentiated PT cells, rather than an unmarked population of stem cells, performed regeneration. However, a putative progenitor population might, to some extent, express the same markers as the differentiated cells that they are destined to become

(82-84). If this is indeed the case regarding Slc34a1, then this labeling tactic would not be able to discern between progenitor based regeneration or regeneration driven by dedifferentiation of mature cells. Additionally, the researchers reported that only 55% of the cells were targeted in the S3 segment of the PTs, which is the most sensitive segment for ARI (85), and also suggested to be the primary site for progenitors (73).

Since there is a lack of a definite progenitor marker in mouse, one cannot with certainty tag progenitor cells using a specific promoter segment. Another approach is to specifically label all cell and follow their clonal expansion following injury. This was recently performed by Rinkevich et al. that used "rainbow mice" where the confetti reporter was driven by the ubiquitous actin promoter. When expressed, the confetti reporter recombines in a stochastic way and then expresses one out of four fluorescent labels in a random manner. In this way, all single cells are individually marked and the expansion of a certain clone is seen as a cluster of unicolored cells, in this otherwise confetti-colored pattern of cells. This method allowed for an unbiased way to follow the clonal expansion of individual cells, both in renal development and following induced renal injury. The study demonstrated that cellular renewal and regeneration in the mouse kidney was performed by clonal expansion of fate-committed precursors, in a segment specific manner (86). This may suggest that unipotent progenitors are located along the different tubular segments of the nephron, and that these cells are responsible for, and restricted to, regeneration of the specific segment in which they reside.

Finally, the discrepancies regarding the cellular source for renal regeneration may be explained by the fact that mouse and human are not one and the same. They differ in longevity, as well as reproductive ability and also biological size and length of nephron. The shortage of convincing evidence for adult tubular progenitors in mice, does not exclude that such progenitors exist in humans, but may suggest that regeneration might be performed in different manners, and possibly by different cells dependent on the severity of the damage, in different species. (This matter is discussed more in Paper III in this thesis.) In the end, deeper knowledge of the mechanisms of regeneration as well as of the features of renal progenitor cells may provide important clues for the development of novel therapeutic strategies for AKI and CKD.

Kidney Cancer

Kidney cancers comprise all tumors stemming from the kidney, including transitional cell cancer of the renal pelvis and the childhood malignancy Wilms tumor. The most common of kidney cancers, embodying over 90% of all cases, are renal cell carcinomas (RCC). These tumors arise from the tubular epithelium of the kidney and represents 2,4 % of all cancers worldwide. Each year around 338.000 people are newly diagnosed with kidney cancer (87), and 143.000 people succumb to the disease, making it the 16th most common cause of cancer related death. RCC is more common in men than women, with a ratio of 2:1, for reasons that are not yet clarified. The disease also represents the ninth most common cancer in men, while it is the 14th most common cancer in women. Globally, the incidence of kidney cancer is rising, however, the prevalence varies greatly between countries. Less developed countries display a lower incidence although the mortality rates are similar to those of industrialized countries with more diagnosed cases (88). The growing rates of diagnosed RCC may partly be explained by the increase of accidentally discovered cases due to novel imaging modalities and abdominal scans for unrelated conditions.

RCC is a slow growing disease, with an estimated growth rate of only 0.28 cm /year (89), and the mean age of diagnosis is at 64 years (90). The causes for kidney cancer are poorly understood, but age, smoking, obesity and hypertension are risk factors (91). For smokers, the relative risk for developing RCC is 1.38 compared to never smokers (92). It has been suggested that this increased risk is due to chronic hypoxia in the renal tissues as well as carbon monoxide exposure and DNA damage induced by tobacco associated N-nitrosamine (93). There is a strong correlation between being overweight and developing RCC, it is estimated that as much as 40% of all RCC cases in USA and 30% of cases in Europe are associated with high body mass index (BMI) (94). The reasons for this increase are not clear, however, it has been suggested that “adipokines” excreted from adipocytes may create a tumor-friendly environment and also induce angiogenesis (95). Additionally, chronic renal diseases increase the risk of developing RCC, possibly due to the persistent inflammatory microenvironment with high levels of inflammatory cytokines and/or due to genetic instability caused by disease associated hypoxia, uremia, and increased growth factor signaling (96). There are also hereditary causes for RCC, such as specific gene mutations, although they represent a minor part, and are estimated to constitute approximately 4% of all cases (97).

Renal cell carcinoma subtypes

Renal cell carcinoma is a collection of several histologically and phenotypically distinct subtypes that arise along different parts of the nephron. The classification of the subgroups is based on histological and genetic features; accordingly, RCCs are divided into clear cell RCC (ccRCC), papillary RCC (pRCC), chromophobe RCC (chRCC), oncocytomas, collecting duct (Bellini's) RCC or unclassified RCC. These represents the predominant subtypes although there are at least 16 different forms of RCC reported (98).

Clear cell renal cell carcinoma

ccRCC represent the vast majority of all RCC cases, 70-85% (99), and are thought to originate from the PT epithelium of the nephron. This subtype is histologically characterized by fat and glycogen accumulation, and as the name indicates, these features cause the cells to appear "clear" on hematoxylin & eosin (H&E) stained tissue sections (Figure 6).

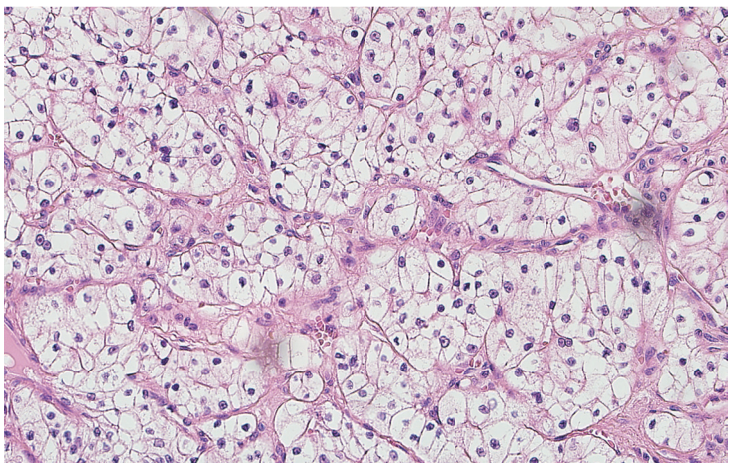

Figure 6. Clear cell renal cell carcinoma histology
Hematoxylin and eosin staining of a histological section of a ccRCC tumor, displaying characteristic "clear" tumor cells and extensive vasculature.

ccRCC are extremely well vascularized tumors, due to high excreted levels of the angiogenesis inducing protein, vascular endothelial growth factor (VEGF). The high VEGF levels are explained by loss of the von Hippel-Lindau (VHL) protein, which occurs in almost all cases of ccRCC. The VHL protein marks the hypoxia inducible factors (HIFs) for degradation at normal oxygen pressure. Loss of VHL causes stabilization of the HIFs and consequently transcription of genes of the *cellular*

adaptive response to hypoxia, such as *VEGF*. Additionally, in tumors with wild-type VHL, mutations in *TCEB1* can be found. *TCEB1* encodes the Elongin C protein, which is a part of the VHL-complex responsible for degrading HIF under normoxic conditions. These mutations are mutually exclusive from *VHL* mutations and chromosome 3p loss and occur in 1-5% of all ccRCC cases (100, 101). The fact that the HIF pathway is affected also in wild-type VHL tumors, further stresses the importance of this pathway in ccRCC tumorigenesis. Most tumors tend to rely on aerobic glycolysis, in combination with lactic acid fermentation, instead of oxidative phosphorylation to meet their energy demand, a phenomenon called the Warburg effect (102). By bypassing the tricarboxylic acid (TCA) cycle following glycolysis, and instead using lactate fermentation, significantly lower amounts of ATP is generated. However, this process also allows for the synthesis of membrane components and cellular building blocks that are important for cellular proliferation. Due to the loss of VHL and hence constitutive activation of the HIF, the Warburg phenomenon is especially prominent in ccRCC. Additionally, it has been shown that glucose metabolism is increased with higher grade in ccRCC and that the glutathione pathway is upregulated to inhibit potentially harmful reactive oxygen species (ROS) (103). In this study, an upregulation of tryptophan catabolism was also noted. Metabolites of the essential amino acid tryptophan has been associated with immune suppression in cancer (104).

Papillary renal cell carcinoma

Papillary RCC is the next most common variant of RCC, embodying 10-15% of all RCC cases and is, similar to ccRCC, thought to emanate from the PT. pRCC has been given its name due to the characteristic papillary growth pattern of the tumor cells. There are two histologically diverse subtypes of pRCC; the more benign type 1, which is characterized by cuboidal epithelial cells and uniform "low grade" nuclei, and the more aggressive type 2 that display a pseudostratified epithelium with "high grade" irregular nuclei (105). pRCC occurs in both hereditary and sporadic forms, which both can be connected to specific genetic mutations. Hereditary, and to a lesser extent, sporadic type 1 pRCC is associated with activating mutations of the proto-oncogene *MET*, a tyrosine kinase receptor, which in these cases becomes constitutively active and thereby increase cellular proliferation. The familial form of type 2 pRCC is associated with loss of the tumor suppressor fumarate hydratase (FH), which causes hereditary leiomyoma renal cell carcinoma (HLRCC). FH converts fumarate to malate in the TCA cycle and loss of this enzyme thus lead to accumulation of fumarate. The increased levels of this metabolite cause VHL independent HIF stabilization by inhibiting the prolyl hydroxylases (PHD), which marks HIFs for VHL recognition. Surprisingly, type 2 pRCC is phenotypically and genetically very different from VHL mutated tumors, highlighting that VHL has more functions than simply targeting the HIFs for destruction. Additionally, it has been shown that the fumarate accumulation lead to HIF independent oncogenic

effects by inhibiting Keap1 and thereby stabilizing the protooncogene Nrf2, a crucial transcription factor in the antioxidant response (106). FH mutations have not been detected in sporadic type 2 pRCC, however, the hereditary and sporadic forms both display upregulation of antioxidant genes such as *AKR1B10*, which may explain their shared phenotype (107). Other rare forms of hereditary pRCC can be attributed to mutations in the tumor suppressors *TSC1* and *TSC2*, encoding hamartin and tuberin respectively. These mutations cause tuberous sclerosis complex, autosomal dominant disease where patients have a predisposition to develop tumors in several organs such as brain, lung, kidney and skin (108). Additionally, these patients also have an increase risk of developing ccRCC and chRCC.

Chromophobe renal cell carcinoma

Chromophobe RCCs are low grade tumors, with a considerably better prognosis than the above mentioned subtypes, and represent 4-5% of all RCC cases (109). The cell of origin for chRCC is also suggested to stem from a different, more distal part of the nephron than ccRCC and pRCC does (110, 111). Also in this subgroup the name of the tumor is reflected by its histological appearance. In H&E-stainings chRCC tumor cells thus generally appear pale, but can also sometimes harbor eosinophilic granules in the cytoplasm. Expression of the proto-oncogene *KIT* at well as loss of chromosome 1, 2, 6, 10, 13, 17 and 21 is quite often seen in chRCC (112). A study using data from the cancer genome atlas (TCGA) showed that the most frequently mutated genes in chRCC are *TP53* (in 32% of cases) and in *PTEN* (in 9% of cases). The same study also showed that chRCC display recurrent genomic rearrangements affecting the TERT promoter and inducing elevated TERT expression (110). Hereditary chRCC is associated with loss of function mutations in the gene coding for follicullin, *BHD*, causing Burt-Hogg-Dube syndrome. Patients with BDH also develop lung cysts and oncocytomas, a benign form of RCC. Follicullin is suggested to play a role in the cells energy sensing mechanism via the AMPK and mTOR pathways (113), and in an animal model of BHD induced kidney cancer, the mice displayed prolonged survival after treatment with the mTOR inhibitor rapamycin (114).

The genomic landscape of ccRCC

The von Hippel-Lindau gene and hypoxia inducible factors

ccRCC is characterized by a virtually obligatory loss of VHL. In 80% of all cases of sporadic ccRCC, VHL is mutated with accompanying loss of heterozygosity due to chromosome 3p deletion (115). In an additional 5-10% of cases, VHL is silenced via methylation (116). Hereditary ccRCC represents approximately 2-3% of all cases and are also characterized by a loss of VHL, in this case due to a germline mutation of VHL, causing von Hippel Lindau disease. Apart from ccRCC, patients with VHL-disease also develop pheochromocytomas, renal and pancreatic cysts, neuroendocrine tumors, retinal angiomas and hemangioblastomas (113). These patients also have an earlier onset of ccRCC than sporadic ccRCC, with the mean age of diagnosis 37 years (90). The VHL protein pVHL is the substrate recognition subunit of a E3 ubiquitin ligase complex composed of Elongin B, Elongin C, cul-2 and rbx-1, which is responsible for the degradation of the HIF-1α and HIF-2α at normal oxygen levels (Figure 7). When enough oxygen is present PHDs hydroxylate HIF-αs on specific proline residues. This hydroxylation makes the HIF-αs recognizable for VHL that can bind the proteins and mediate ubiquitylation, which in turn signal for proteasomal destruction. Additionally, the HIF-αs are regulated by the enzyme factor inhibiting HIF (FIH) that, at normal oxygen levels, hydroxylate the proteins at an aspargine residue. This modification inhibits the HIF-αs from binding to their transcriptional co-factors, p300/CBP and thereby also impairs their capacity to initiate transcription. At hypoxia, no hydroxylation occurs and the HIF-αs become stabilized and translocate into the nucleus where they form a heterodimer together with HIF-β, also known as aryl hydrocarbon receptor nuclear translocator protein (ARNT). This complex subsequently binds specific DNA segments: hypoxia responsive elements (HRE) and recruit co-factors to initiate transcription of genes in the adaptive response to hypoxia. Without a functional pVHL, as in the case of ccRCC, the cells experience pseudohypoxia with transcription of the downstream targets of HIFs even at normal oxygen levels. Consequently, the cells express high levels of genes involved in angiogenesis, glucose uptake, acid-base balance and metabolism, such as *VEGF*, platelet-derived growth factor, (*PDGF*), transforming growth factor alpha (*TGF-α),* glucose transporter (*GLUT1*), carbonic anhydrase 9 (*CA9*) and fibroblast growth factor (*FGF*). At hypoxia, the expression of these transcriptional targets aims to maintain intracellular homeostasis, however, when constitutively expressed, as in the case of VHL loss, they create a tumor promoting setting favoring cellular proliferation and other aspects of tumorigenesis (97).

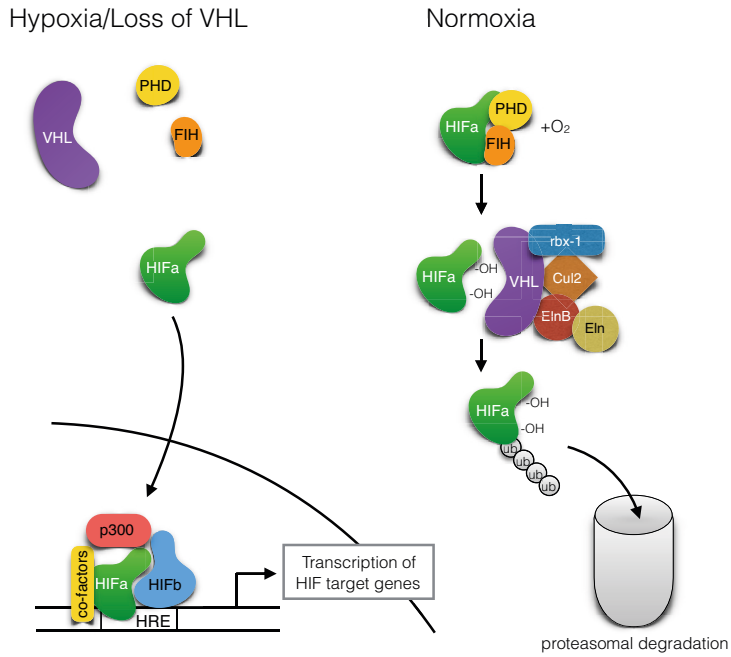

Figure 7. Regulation of the hypoxic machinery
The hypoxia inducible factors (HIFs) are continuously transcribed and translated and are instead regulated on the protein level. The HIF-αs are rapidly degraded in the presence of oxygen in a process mediated by hydroxylation by factor inhibiting HIF (FIH) and prolyl hydroxylases (PHDs) making the HIFs recognizable by the VHL complex that target the HIF-αs for proteasomal degradation. When the oxygen tension is low, the HIF-αs are instead stabilized translocated to the nucleus where they dimerize with HIF-β and together with co-factors initiate transcription of genes involved in the adaptive response to hypoxia. The VHL protein is lost in ccRCC causing the HIFs to be stabilized and initiate a hypoxic cellular response even at normoxia.

There are considerable functional similarities between HIF-1α and HIF-2α with overlapping transcriptional targets, but they are not functionally redundant and also display distinct exclusive targets. HIF-1α generally transcribes genes involved in glucose metabolism, while HIF-2α is more responsible for growth and angiogenesis. The specific HIF-1α and HIF-2α targets can even sometimes have opposing cellular effects. For example, HIF-1α can promote the expression of pro-apoptotic proteins like BNIP3, and it has even been suggested to be a tumor suppressor (117). HIF-1α is located at chromosome 14q, which is lost in 40% of ccRCC cases (118). In contrast, several reports have suggested that HIF-2α is the major contributor for the maintenance of tumorigenic features of ccRCC (117). For example, in patients with VHL disease HIF-1α expression was correlated with neoplastic lesions, while selective expression of HIF-2α was associated with advanced lesions (119). Furthermore, repression of HIF-2α in ccRCC has been shown to halt xenograft growth in mice (120). Nevertheless, it may be so that HIF-1α is important for the initiation of

ccRCC. Quite surprisingly though, so far only HIF-1α and not HIF-2α has been shown to promote renal carcinogenesis in conditional mouse models (121-123).

Epigenetic regulators

Intra tumor heterogeneity is described in many cancer forms and seems to be exceptionally common in ccRCC, where the only shared clonal events are VHL loss/inactivation and chromosome 3p loss (124). Additional mutations occurring in ccRCC commonly affects epigenetic regulators such as polybromo 1 (*PBRM1*), SET domain containing 2 (*SETD2*) and BRCA1-associated protein-1 (*BAP1*), which are all located on chromosome 3p (125). *PBRMI* encodes BAF180, a subunit of the SWI/SNIF chromatin remodeling complex. This complex regulates DNA accessibility and thereby indirectly also transcription. Inactivating mutations of *PBRM1* is found in about 40% of ccRCC cases (126) and PBRM1 suppression has been shown to increase cell proliferation, migration and colony formation, and also to create alterations in pathways controlling chromosome stability (126, 127). *PBRM1* mutations appear to be an early event in ccRCC tumorigenesis and are quite often seen in all cancer cells within a tumor. There are reports suggesting that mutations are correlated to higher grade and worse outcome in ccRCC (128, 129), while other studies report that *PBRM1* mutation frequency is similar in all tumor stages and do not correlate to adverse patient outcome (130, 131). Thus the clinical effect of *PBRM1* mutations in ccRCC patient outcome is not entirely clear, however most studies suggest that *PBRM1* loss stimulate cellular proliferation (125, 126).

SETD2 is mutated in 10-15% of ccRCC cases and often occurs alongside *PBRM1* mutations. *SETD2* encodes a histone H3 lysine 36 trimethylating (H3K36Me3) enzyme (126). This histone mark is commonly associated with active transcription, but can also take part in alternative splicing and transcriptional repression. In contrast to *PBRM1* mutations, *SETD2* mutations are subclonal, meaning that they are present in some subclones of the tumor, but not in others (132). Additionally, different types of *SETD2* mutations can be found in the same tumor, suggesting a high selective pressure for SETD2 inactivation (125, 132). SETD2 is thought to contribute to genomic integrity by promoting DNA repair by homologous recombination and by reducing replication induced cellular stress (101). Mutations in *SETD2* have in some patient cohorts been correlated to low cancer specific survival (130).

Similar to *SETD2*, *BAP1* is mutated is 10-15% of ccRCC cases and also presents as a subclonal event. However, in contrast to *SETD2* mutations, *BAP1* and *PBRM1* mutations are virtually mutually exclusive. BAP1 is a histone deubiquitinase that is involved in HR repair and protects against chromosomal instability (101). Inactivating mutations of *BAP1* have been associated to tumor aggressiveness and worse prognosis in several studies (125, 126), and loss of BAP1 protein expression has

been shown to independently predict poor patient outcome in low-risk ccRCC (133). BAP1 loss has also been associated with mTOR pathway activation (134). The mechanism behind the tumor promoting effects of *BAP1* mutations is still no clear, but mutated tumors have been associated with metastatic spread at diagnosis and higher grade, as well as shorter recurrence free survival, suggesting that *BAP1* mutations might be used for stratifying patients into high risk groups and possibly guide treatment options. ccRCC also harbor additional mutation in genes affecting epigenetic regulation including the histone deacytelases JARID1C/KDM5A, which is mutated in 3-7% of tumors and UTX/KDM6A which is mutated at a low frequency, around 1% (126).

Clinical manifestation and prognosis

RCC is characterized by vague clinical symptoms such as blood in urine, persistent flank pain, weight loss, fever, fatigue and possibly also by an abdominal mass. As a consequence of these elusive symptoms, many RCC cases are discovered when the disease has already advanced, and as many as 20-25% of all patients have spread disease at diagnosis. If the disease is locally confined the patients have a very good prognosis, with a 5-year survival of 90% (135). However, approximately 1/3 of these patients later relapse, indicative of micrometastasis that were missed at primary diagnosis. Sadly, patients with metastatic disease await a dismal prognosis with a 5-year survival rate of less than 10%, and a median survival of 18 months (124). The most common metastatic sites are localized to the lung, soft tissue, liver, bone, skin and brain (135).

Great efforts have been made to find prognostic molecular markers in RCC, however the *tumor metastasis node* (TMN) staging system (Table 1) along with Fuhrman nuclear grade are still the most used prognostic indicators. They have together with Eastern Cooperative Oncology Group (ECOG) performance status, also known as the WHO score, been combined into the UCLA integrated staging system UISS (99, 136). The ECOG performance status assesses the general well being and functional impairment of cancer patients. According to the UISS, the 5-year survival rates for patients with localized disease are 92% for the low risk groups 67% for the intermediate risk groups and 44% for the high risk group, while the prognosis for patients with spread disease is more discouraging with 3-year survival rate of 37% for the low risk group, 23% for the intermediate group and 12% for the high risk group (99).

Table 1

The TMN staging system defines the localization and spread of the tumor, (T) primary tumor, (M) distant metastasis (N) lymph node involvment

TNM STAGING	
Primary Tumor	
T1a	Organ confined <4 cm
T1b	Organ confined 4-7 cm
T2a	Organ confined 7-10 cm
T2b	Organ confined >10 cm
T3a	Invades into perinephric tissue, renal sinus or renal vein
T3b	Invades into vena cava below the diaphragm
T3c	Invades into vena cava above diaphragm or into wall of vena cava at any level
T4	Invades beyond Gerota's fascia or directly into renal gland
Regional lymph nodes	
N0	No lymph node involvement
N1	Metastasis in one regional lymph node
N2	Metastasis in more than one regional lymph node
Distant metastasis	
M0	No distant metastasis
M1	Distant metastasis
Stage grouping	
Stage I	T1N0M0
Stage II	T2N0M0
Stage III	T1-T2N1 or T3N0-1
Stage IV	T4 (any N or M) or N2 (any T or M) or M1

Diagnosis and Imaging

If a patient is suspected to have a renal tumor, a computer tomography (CT) scan of the abdomen will be performed. If a mass is indeed detected, the scan will be extended to include the thorax, in order to assess possible metastatic spread (135). The CT scan is performed to evaluate the malignity of the tumor, as well as the tumor size, possible spread into surrounding tissue and/or adrenal gland, and also to assess lymph node involvement. Magnetic resonance imaging (MRI) investigations are less common, but can also be performed if venous tumor thrombus is suspected, or if the patient cannot tolerate the contrast injections that are included in the CT scan. Most cases of RCC can be correctly assessed following imaging analysis (136). However, if the diagnosis after imaging is uncertain, or if the patient is not suitable for surgery, a biopsy of the primary tumor may be performed and sent to the pathologist for assessment. If the patient presents with multiple metastasis, tumor core biopsies are also taken before starting systemic treatment. Biopsies are also

indicated in cases where the patient previously has had another form of cancer; in this case the biopsy will help to clarify whether the mass is in fact a RCC or a metastasis from the previous malignancy (135). Additional laboratory investigations should also be performed, such as measurements of serum creatinine, hemoglobin, leucocyte and platelet counts, and lactate dehydrogenase levels (136). The results of these tests will also give an estimation of the patient's kidney function, something that is crucial to consider when choosing suitable therapy.

Treatment of RCC

RCCs are characterized by poor response to common anti-cancer therapies like cytostatic drugs and radiation. At present, surgical resection is the standard treatment for localized RCC and still practically the only curative option for ccRCC patients. In the last couple of years, partial nephrectomy has become increasingly common for smaller tumors. This nephron sparing approach has been proven to be beneficial for patients by maintaining more of the kidney function, whereas radical nephrectomy has been shown to increase the risk of renal failure. Additionally, laparoscopic surgery is associated with decreased morbidity compared to open surgery, however there are no differences in recurrence or survival rates between the surgical approaches (91). There are cases when RCC is not operated on, for instances when the patients are considered to be too frail and/or have other co-morbidities that make them unsuitable for surgery. In these cases the patients can stand under active surveillance, where the disease is monitored closely via continual imaging. As an alternative, elderly patients may also undergo cryo- or radiofrequency ablation, which is considered a less invasive method than conventional surgery (89). For patients with a higher risk of recurrence T2-T4, (Table 1) radical nephrectomy is required, where the whole kidney is removed. For T3-4 tumors additional removal of perinephric fat and Gerota's fascia of along with the adrenal gland and surrounding lymph nodes is performed. These efforts result in a complete cure in 40-60% of RCC cases (89).

Cytokine therapy in metastatic RCC

For advanced disease the situation is different. In these cases nephrectomy is far from sufficient to halt the cancer and, as previously mentioned, metastatic RCC (mRCC) is considered to be one of the most chemotherapy resistant malignancies (95), making therapy exceedingly challenging. However, treatment with radiation and chemotherapy can be used in palliative care for example to reduce pain associated with skeletal metastasis. Traditionally, mRCC has been treated with immune system stimulators interleukin-2 (IL-2) and interferon-α (INF-α) that rely on the patient's own immune response to eradicate the tumor. However, these treatments have show relatively low response rates (20% and 5-15% respectively) and display severe

systemic toxicity and side effects, such as capillary leakage resulting in dangerously low blood pressure (89). Nevertheless, in some cases high dose IL-2 treatment resulted in complete curative outcome (137). This emphasizes the need for prognostic markers that can predict which patients will respond to a certain treatment. This is a pitfall for many cancer drugs, which may be discarded due to low response rates, while being beneficial for a subset of patients. It has been suggested that positive histological staining against the prototypical HIF target CA9, together with a favorable pathologic features may be predictive for IL-2 responders (138). Finally, retrospective studies have indicated that ccRCC responds better to immune stimulatory therapies, than other histological subgroups of RCC (139).

Targeted therapy in RCC

VEGF- and mTOR- targeting therapies

In the last 10 years targeted therapies have become increasingly common in the treatment against advanced RCC. These treatments mainly target the biological consequence of VHL loss, and essentially consist of angiogenesis inhibitors, affecting VEGF and mammalian target of rapamycin (mTOR) signaling pathways. The rational behind angiogenesis inhibitors is based on the notion that tumors cannot grow beyond a few millimeters without a blood supply, and that the tumor cells rely on the very same vessels for oxygen and nutrient delivery (140). Additionally, metastatic spread most often occurs through extravasation across the endothelial cells layer in the tumor vasculature. Thus, it was initially thought that by inhibiting the tumor-induced angiogenesis one would essentially starve the tumor, as well as inhibit metastatic spread (140, 141). However, for most types of cancer, antiangiogenic treatment have shown the best response in combination with for example chemotherapy and have been suggested to normalize the leaky tumor vessels, so that supplementary therapy would actually reach the tumor cells (142).

In 2007, clinical trials for mRCC showed that both a direct VEGF inhibitor, the monoclonal antibody *bevacizumab* and the tyrosine kinase inhibitor (TKI) *sunitinib* with multiple targets (VEGFR, PDGFR and c-Kit) displayed a doubling in progression free survival (10.2–11 vs. 5.4-5 months) compared to INF-α alone (95). Antiangiogenic therapy is now the mainstay of treatment for mRCC. Despite these initial encouraging results, resistance against VEGF targeted therapy is seen in 20-30% of patients trying it for the first time, and a majority of patients with primary response acquire resistance within 1 year (124). As an alternative to TKI inhibitors and treatment specifically targeting VEGF, inhibitors against mTOR have been developed. These drugs target both angiogenesis and tumor proliferation (135). mTOR is a key regulator of cellular homeostasis and is involved in nutrient sensing, energy status and cellular stress (143). The PI3K/Akt/mTOR pathway together

controls multiple cellular features such as proliferation, metabolism and differentiation (144). Additionally, a majority of ccRCC cases display increased mTOR activity, especially apparent in tumors of high grade and with poor prognostic features (143). For mRCC patients with particularly poor prognosis, treatment with the mTOR inhibitor *temsirolimus* was shown to be beneficial over INF-α treatment, or a combinatory treatment of both drugs. Temsirolimus treatment also increased the over all survival for these patients (10.9 vs. 7.3 months). In this study there were no difference in response between ccRCC and non-clear cell RCC (143).

For patients with mRCC that do not respond to treatment or develop resistance, there are alternative so-called second-line agents. *Sorafinib* is a multi kinase inhibitor of Raf1 serine/threonine kinase, b-Raf, VEGFR-2, PDGFR and c-Kit, which in clinical trials have been shown to prolong progression free survival with three months compared to placebo. However, there are no reliable data concerning over all survival, since the study allowed "crossover" from the placebo group arm to the treatment arm and an interim analysis shown that treatment with sorafinib was beneficial (135). As an alternative second-line treatment after failure with VEGF inhibitors, the mTOR inhibitor *everolimus* may be applied. This treatment showed a progression free survival of (4.9 vs. 1.9 months) in a patient cohort of metastasized ccRCC (143).

c-Met inhibitors

There are limited specific treatment options for metastatic pRCC, possibly owing to a lack of incentive for development of specific pRCC targeted drugs due the fact that pRCC only constitute 5-15% of RCC cases. However, an inhibitor of MET and VEGFR, *foretinib*, has recently been developed and a phase II clinical trial including patients with pRCC, showed an increased progression free survival compared to previous trials using sorafenib or sunitinib (9.3 months vs. 1.6 up to 6 months) (145).

The role of surgery in mRCC

Surgery may not be fruitless for mRCC patients. Radical nephrectomy in patients with metastatic disease, so called cytoreductive nephrectomy (CN) is not a standard procedure, but it has nevertheless been shown that INF-α treatment together with CN significantly prolonged the median overall survival time compared to INF-α treatment alone (13.6 vs. 7.8 months) (146). In line with this, there are now ongoing phase III clinical trials investigating the potential beneficial use of CN together with the standard care of VEGF inhibitors (95). The biology behind this potential beneficial effect is not clear, but it has been suggested that removing the primary tumor might reduce immunosuppressive cytokines, as well as growth promoting signals, creating a systemic effect also on metastasis (89). The possible benefit of surgical removal of metastasis, metastasectomy, is debated since no prospective

randomized studies have been performed and metastasectomy is therefore quite uncommon in routine practice (135). However, it may be employed in cases with solitary lung metastasis or if there has been a long duration between primary tumor and relapse, or between occurring metastasis (136).

Immune checkpoint inhibitors

RCC is considered to be an immunogenic cancer, based on the reports of spontaneous regressions of RCC in the 80-ties and 90-ties (147, 148), as well as the partial beneficial responses to cytokine therapies mentioned above. Efforts have therefore been made in order to develop novel immunomodulators for treatment of RCC. Programmed death receptor-1 (PD-1) is an immune checkpoint component expressed on T cells, B cells and NK cells, whereas its ligand PD-L1 is expressed on antigen presenting cells as well as tumor cells. When the ligand binds its receptor the cytokine secretion from the immune cell is inhibited. This is an important function in the normal immune tolerance, which prevents us from having an allergic reaction to all exogenous peptides. However, the PD-1/PD-L1 interaction is also suggested to be of major importance for the capacity of tumors to evade the immune response (149). A retrospective study showed that patients with ccRCC tumors staining positive for PD-L1, had a worse prognosis compared to patients with tumors not expressing PD-L1 (41.9% vs. 82.9% in cancer specific survival) (150). Additionally, PD-L1 expression has been correlated to worse outcome also in non-clear cell RCC, suggesting that these patients may also be aided by PD1/PD-L1 targeted therapy (151). Recent clinical trials, for mRCC patients resistant to first-line therapies, using targeted therapy against PD-1/PD-L1 pathways have displayed an overall survival benefit compared to treatment with mTOR inhibitors. Treatment with the PD-1 blocking antibody *nivolumab* prolonged the overall survival with 5 months compared to treatment with everolimus (25 months vs. 19.6 months) (152). Combination treatments with PD1/PD-L1 targeted therapy and other commonly used RCC treatments, such as angiogenesis inhibitors or cytokines, also improved response rates compared to single treatment, while however also displaying higher toxicity (149). These results indicate that treatment with nivolumimab will likely form a new standard of care for patients with mRCC. An additional immune checkpoint receptor, which is being investigated as a possible target for mRCC therapy, is CTLA-4. This receptor which is expressed on T-cells and functions in a similar way to PD-1, have been successfully targeted in metastatic melanoma using the blocking antibody *ipilimumab* (95). Future challenges for immune checkpoint based therapies will be to apprehend which patients that will respond to the therapy. Interestingly, there are contradictory data concerning the use of PD-L1 expression as a biomarker for response, since also negative tumors have been shown to respond to PD1/PD-L1

targeted therapy (149), which may suggest that this therapy has an additional general immune stimulatory effect, regardless of PD-L1 tumor status.

Finally, it may be so that most of the above-mentioned drugs have limited effect as monotherapies, but appear to their full advantage as combination treatments. Avoiding severe adverse side effects in these cases however represents a major challenge. In a manner similar to Darwinian evolution, cancers have a high ability to adapt to their environment, possibly due to the high level of intratumor heterogeneity. Thus, hitting several functionally different targets at once may be necessary to eradicate the tumor. Still, there is a great need for predictive biomarkers in order to correctly guide the most suitable choice of treatment for each patient. The development of resistance remains a huge problem with current treatments and although recent therapies show prolonged survival, virtually no mRCC treatments have been curative. Thus there is a desperate need to develop novel effective targeted therapies for mRCC that may be used complementary to existing treatments. Additionally, there is a need for novel high-resolution diagnostic methods that allows for early detection of the primary tumor, before metastatic traits have been developed, as well as for the identification of early metastasis to ensure that the patients can be treated for mRCC as early as possible, i.e. before the tumor has developed a high level of heterogeneity.

Transporter proteins

Transporter proteins control vital parts of cellular homeostasis by regulating the import and export of essential substances like sugars, amino acids, solutes and drugs across the plasma membrane. It is estimated that 4% of our DNA codes for transporter proteins and virtually all cells depend on their function (153). However, transporters are deeply understudied, both in proportion to the sheer number of genes and to their importance in normal cellular physiology and disease (154). There are two major families of transporters, the solute carriers (SLC) and the ABC-transporters.

The ABC-transporters represent the second largest transporter family and consist of 48 members divided into 7 subfamilies. The ABC-transporters are intricate pumps that are driven by energy catalyzed from the hydrolysis of ATP in order to efflux molecules across cell membranes (155). The expression of ABC-transporters has been associated with stem and progenitor cells, which might be related to their capacity to eliminate harmful substances in order to protect their DNA and thereby their future progeny (156). Expression of ABC-transporters and have also been coupled to drug resistance in many human diseases, including chemotherapy resistance in cancer (28, 157).

The SLC family is composed of over 400 different members that are organized into 52 subfamilies and is thereby the largest group of cellular transporters. These transporters are mainly involved in molecular uptake (153), and are primarily driven by transmembrane ion gradients (158). The SLC families vary in substrate specificity; some have a very narrow range of substrates while others like the SLCO-family (OATPs), can handle a broad range of chemically diverse substrates (159). The transport of substrates via SLC can be driven in several ways. Passive transporters or facilitated transporters allow substances to diffuse down their concentration gradient. Active transporters on the other hand require energy to transfer substances against their concentration gradient. An example of a primary active transporter is the Na^+/K^+ ATPase, which is widely expressed in the kidney. Na^+/K^+ ATPase uses the hydrolysis of ATP to pump K^+ into the cell and Na^+ out of the cell, against their electrochemical gradients. Secondary active transporter uses the concentration gradients created by the primary active transporters to move a specific substrate, against its own gradient, together with Na^+ or K^+ along their electrochemical gradient. Secondary active transporters can be symporters, meaning that the specific substrate and the aid-solute,

for example Na^+, moves in the same direction, or antiporters meaning that the substrate and aid-solute move in opposite directions. (Figure 8)

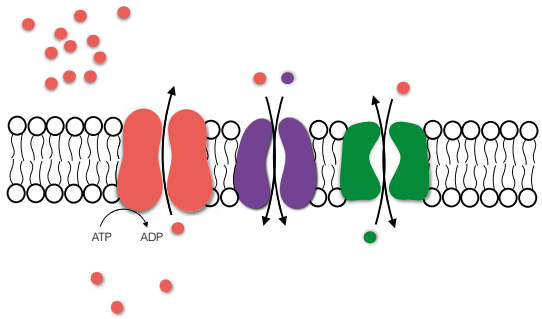

Figure 8. Secondary active transporters
Schematic figure of secondary active transporters that are driven by the flow of aid solutes down their electrochemical gradient, such as for example Na^+ (pink). This gradient is achieved by the active transport of, for example, Na+ out of the cell in a process requiring energy in the form of ATP hydrolysis (pink transporter). The secondary active transporter can be symporters (purple transporter or antiporters (green transporter).

Neurotransmitter transporters (NTTs) belong to the SLC6-family, which is one of the largest subgroups of the SLC-family consisting of 20 members. The SLC6-family handles the transport of amino acids or amino acid derivatives into the cells (160). The NTTs transport neurotransmitter substances such as serotonin, dopamine, norepinephrine, GABA and glycine into the cells. These transporters are primarily located to the central nervous system (CNS) in the brain, but can also be found in non-neuronal cells (161). NNTs use the electrochemical gradient of extracellular Na^+ to transfer their substrates via a symport of Na^+ into the intracellular compartment.

The dopamine transporter SLC6A3

Dopamine was first described as a neurotransmitter in the late 1950's by the Nobel Prize laureate Arvid Carlsson. The function of dopamine in the brain mainly involves control of voluntary movement via the basal ganglia. Degeneration of the dopaminergic neurons in the nigrostriatal pathway causes Parkinson's disease (PD) where patients have a decreased ability to execute smooth controlled movements. The contrary is seen in disorders associated with elevated dopamine levels such as Huntington's disease and Tourette's syndrome where patients display jerking involuntary movements (162, 163). Other than motor deficiencies, PD patients also present with other symptoms such as depression, hallucinations, cardiovascular symptoms and even cognitive impairment and dementia in later stages (164). These

symptoms may be due to other changes occurring in PD, affecting noradrenergic, serotenergic and cholinerig pathways (165) but may also reflect dopamine's non-motor functions. For example, in nucleus accumbens and striatum dopamine plays a part in the reward system, and is released upon pleasure stimuli like food or sex (166). Dopamine also has a role in the frontal lobe where it is involved in managing the information flow from other parts of the brain. Disorders affecting this system cause cognition deficiency and impaired attention, memory and problem solving (167, 168).

Dopamine release into the synaptic cleft results in binding to G-protein coupled dopamine receptors that convey excitatory or inhibitory signals through the post synaptic neuron, depending on which receptor that is activated. There are five dopamine receptors D_1-D_5 divided into either D_1-like family (D_1 and D_5) or D_2-like family (D_2, D_3 and D_4) (169). The D_1-like family is coupled to the stimulatory G-protein G_s that activates the enzyme adenylyl cyclase and thereby increases the levels of the common second messenger cyclic adenosine monophosphate (cAMP), while the D_2-like family is coupled to inhibitory G_i, which inhibits adenylyl cyclase and thus the formation of cAMP (170). The potency of the dopamine signaling also requires careful control of its levels at the synapse; this control is primary mediated by the clearance of dopamine from the synaptic cleft via the dopamine reuptake transporter SLC6A3. The *SLC6A3* or *DAT1* gene is located on chromosome 5p15 and encodes a transporter protein with 12 transmembrane domains that is dependent on a symport of sodium and chloride to execute its function (160). At the synapse, the extracellular concentration of Na^+ and Cl^- by far exceeds their intracellular concentration, the ions thus move through SLC6A3 down their electrochemical gradient, bringing dopamine along.

Pharmacological interaction with SLC6A3

SLC6A3 have been extensively studied from a pharmacological perspective, since drugs like cocaine and amphetamine primarily exert their function through SLC6A3 inhibition, thereby prolonging the dopaminergic signaling with molecular and behavioral effects as a result. SLC6A3s normal function of dopamine influx can in some instances be reversed, at least in some *in vitro* experimental settings. For example, if the electrochemical gradients of ions are reversed, with high intracellular concentration of Na^+ and Cl^-, the transporter can efflux dopamine out of the cells (171, 172). SLC6A3 can also be modified by phosphorylation via phosphokinase C (PKC) and phosphokinase A (PKA). Phosphorylated SLC6A3 causes decreased dopamine uptake due to either internalization of the transporter via PKC phosphorylation or an actual efflux of dopamine through the transporter via PKA phosphorylation (173). The PKA and PKC activities are mediated via the trace amine

associated receptor 1 (TAAR1) that can be activated by methamphetamine (174). Amphetamine is taken up via SLC6A3, inhibiting dopamine reuptake in several ways: in a competitive manner, through internalization (175), and also through activation of TAAR1 and subsequent induction of SLC6A3 dopamine efflux (176). Additionally, the action of amphetamine can cause calcium-induced depolarization, which activates Ca^{2+}/calmodulin-dependent protein kinase II (CAMII) that also can cause dopamine efflux by SLC6A3 phosphorylation (177, 178). Cocaine directly binds to SLC6A3 and inhibits the dopamine reuptake (179). This inhibition has been suggested to be caused by a stabilization of the transporter in an outward facing conformation (180, 181), however the exact binding sites of cocaine have not been exactly established (181).

SLC6A3 in disease

Mutations in SLC6A3 have been associated with infantile parkinsonism-dystonia, a rare autosomal recessive disorder that causes PD like symptoms and muscle contractions during infancy (160). The mutations cause either loss of function resulting in reduced levels of operative SLC6A3 (182), or severely decrease the binding affinity of dopamine to SLC6A3 (183). The symptoms may be explained by impaired replenishment of dopamine into synaptic vesicles. SLC6A3 harbors a 40 base pairs variable number tandem repeat VNTR section in its untranslated 3´ end. The VNTR has been suggested to affect SLC6A3 availability on the cell surface and variants of this VNTR have been linked to attention deficit hyperactive disorder (ADHD) susceptibility (184, 185). In humans, the most common forms are 10 or 9 repeats where the 9 repeat form has been associated with higher SLC6A3 activity (186). Other disorders that have been linked to malfunctioning SLC6A3 include ADHD, bipolar disorder, Tourette's syndrome and alcoholism (160, 187-189). Additionally, SLC6A3 knock-out mice display neurological deficiencies including hyperactivity resembling the symptoms seen in patients with ADHD (190).

Imaging using SLC6A3

As previously mentioned, PD is a progressive neurodegenerative disease where the dopaminergic cells die causing a variety of symptoms (191). The diagnosis of this disease can be aided using imaging modalities based on the presence of SLC6A3. Radioligands, such as ^{123}I-β-CIT or ^{123}I-Ioflupane also known as DaTSCAN are cocaine analogues that selectively bind to SLC6A3, which subsequently can be imaged using single photon emission computed tomography (SPECT) (192). This imaging allows for a quantitative measure of the dopaminergic neurons in striatum. In patients with parkinsonian symptoms these imaging techniques make it possible to differentiate PD from other neurodegenerative disorders such as essential tremor

(193) and additionally distinguish between Lewy body dementia and Alzheimer's disease (194).

Dopamine system in the kidneys

As previously mentioned, dopamine exerts several important functions in the brain in its role as a neurotransmitter. This vital role of dopamine is emphasized by the fact that disturbances in the its signaling causes several severe disorders. However, in the last 30 years it has become apparent that dopamine signaling also has various important physiological functions in non-neuronal tissues (195). In the kidney, dopamine has a natriuretic effect, resulting in decreased blood pressure and more sodium excreted with the urine. Disturbances in the renal dopamine system have therefore been associated with the development of hypertension (196). The renal tubular cells are equipped with amino acid transporters located on the apical and basal side that take up circulating or filtered L-dopa (197). Inside the cells, L-dopa is converted to dopamine via the enzyme aromatic L-amino acid decarboxylase (AADC). The kidneys express one of the highest levels of AADC in the body (198), and the activity of the enzyme is highly affected by sodium intake where it is upregulated by high levels of sodium and conversely downregulated by low sodium intake (199). The dopamine generated from the AADC conversion is subsequently secreted into the tubular lumen where it can bind dopamine receptors in an autocrine or paracrine manner. The D_1-D_5 receptors are all expressed in the kidney, although some of them at very low levels (200). Binding of D_1 receptors in the proximal tubules results in inhibition of Na^+/K^+-ATPase as well as the Na^+/H^+-exchanger, causing decreased sodium reabsorption. Animal studies have estimated that as much as 50% of sodium excretion is dependent on renal dopamine signaling (200). Apart from its role in blood pressure regulation dopamine is also suggested to be important in redox balance in the kidney. The dopamine receptors D_1, D_2 and D_5 can have antioxidant effects, for example by inhibiting NADPH oxidase and stimulation of superoxide dismutase (SOD) (201, 202). Additionally, dopamine has been shown to have a dampening role on inflammation by inhibiting immune cells from releasing interferon γ (INFγ), IL-12 and IL-4 (203, 204). Also, mice with malfunctioning renal dopamine system displayed increased infiltration of immune cells and increased oxidative stress (203). The transfer of dopamine through the apical and basal membranes of proximal tubule cells, both inwards and outwards, have been shown to be unaffected by cocaine or the specific SLC6A3 inhibitor GBR-12909 (198). Additionally, there are hardly any *SLC6A3* mRNA transcripts detected in the kidney (205), suggesting that SLC6A3 take very little, if any, part in the dopamine system in the kidney.

The present investigation

Overall Aim

The overall aim of this thesis was to shed light on kidney regeneration and the connection between different cell types of the kidney and renal malignancies.

Paper I-III

Aims and Results

Paper I: Isolation and characterization of progenitor-like cells from human renal proximal tubules

Specific Aims

The aim of this paper was to define and characterize a potential progenitor cell population in the kidney using an assay based on the stem cell associated aldehyde dehydrogenase (ALDH) activity.

Key Findings

In this paper we used the FACS-based ALDH assay to isolate human renal cortical cells with stem cell-like features. Transcriptional analysis of the two cell fractions $ALDH^{high}$ and $ALDH^{low}$ showed that the $ALDH^{high}$ cells were associated with a stem cell signature and cytoskeletal-, adhesion-, leading edge- profiles as well as with negative regulation of apoptosis. The $ALDH^{low}$ cells on the other hand, expressed genes involved in normal renal tubular functions, such as transmembrane transportation, solute carrier function and metabolic processes. We could also show that the $ALDH^{high}$ cells displayed stem cell like features like clonogenic capacity and anchorage independent growth while the $ALDH^{low}$ cells did not. Immunohistochemical examination of human kidney cortex showed that the genes upregulated in the $ALDH^{high}$ cells delineated a population of cells positive for vimentin, CD133, CD24, KRT7, KRT19 and BCL2 that were scattered within the

PTs. These cells were also negative for several characteristic PT markers such as XPNPEP and SUSD2. They were also immunophenotypically very similar to the parietal epithelial cells (PEC) of the Bowman's capsule, which previously has been described as a progenitor cell population. Additionally, regenerating tubules in kidneys with ATN, showed long stretches of cells positive for the markers of the $ALDH^{high}$ cells. Furthermore, we demonstrated that the transcriptional profile of the $ALDH^{high}$ cells showed a substantial overlap with that of pRCC. Collectively the findings of Paper I, suggest that the scattered progenitor-like cells may be more resistant to apoptotic stimuli associated with renal insult and that they may also be involved in renal tubular regeneration. Finally, we propose that pRCC might be derived from oncogenic transformation these cells.

Paper II: Evidence for a morphologically distinct and functionally robust cell type in the proximal tubules of human kidney

Specific Aims

The aim was to further characterize the progenitor-like cell discovered in Paper I both at an ultrastructural and functional level.

Key Findings

In this paper we explored the ultrastructural features of the cells previously described in Paper I, using conventional electron microscopy (EM) and vimentin-immuno-EM. Structurally, we discovered that the cells were flask shaped, hade a dense nucleus and were smaller than surrounding PT cells. The cells were also endowed with an extensive filamentous mat and had drastically fewer mitochondria that surrounding bulk cells. The mitochondrial paucity was also confirmed using immunofluorescence co-stainings with the characteristic markers described in Paper I and the mitochondrial markers MTCO2 or VDAC1. To asses the functionality of the cells we also developed an *ex vivo* model of human ATN, where kidney pieces were subjected to ischemia in conjunction with nephrectomy and subsequently reoxygenated in cell culture conditions. Immunofluorescent analysis showed that the progenitor-like cells largely remained attached to the membrane in contrast to the bulk PT cells where 80% were lost. Additionally, we investigated the presence of these cells in renal injuries ranging from acute to chronic and demonstrated that they were present in regenerating tubules during all phases. We also showed that the cells could be detected in renal wedge biopsies from young healthy kidney donors. Collectively, these results show that the progenitor-like cells are morphologically distinct and indicate that these cells are more resilient to renal insult than bulk PT cells.

Paper III: Species diversity regarding the presence of proximal tubular progenitor cells of the kidney

Specific Aims

The aim was to investigate the potential presence of renal progenitor cells as defined in human kidney, in different species.

Key Findings

In this paper we investigated the presence of cells similar to human proximal tubular progenitors (PTPC) in kidneys from chimpanzee, pig, rat and mouse, using immunohistochemical markers that characterize the human PTPCs such as Vimentin, CD133, KRT7, KRT19, CLDN1 and low mitochondrial content ($MTCO2^{low}$). PTPCs could be detected in kidneys from chimpanzee and pig, however we were unable to find these cells in mouse kidneys. In rat we could find occasional, very rare cells with vimentin positivity and lower mitochondrial content. In an attempt to provoke the appearance of these cells in murine kidneys, we subjected mice to kidney injury in the form of unilateral urinary obstruction (UUO). Despite clear kidney damage we could not detect any vimentin positive cells within mouse kidney tubules. These findings have implications for the interpretation of the apparently contradicting results that prevail in the field of kidney regeneration.

Discussion

The kidneys are highly energy demanding organs and are consequently very sensitive to disturbances in renal blood flow causing hypoxia and decreased nutrient delivery. Ischemic or toxic insults can lead to acute tubular necrosis where the tubular cells detach and die. Normally, the cells of the kidney rarely divide, but following injury the renal tubules possess a remarkable regenerative ability. The cells or factors that control this regeneration process are still largely unknown.

Do renal progenitors exist?

In the field of regeneration there is a vivid discussion of whether renal progenitor cells exist, and if so, whether they have a role in the regeneration process. In this thesis we describe a previously overlooked cell population inconspicuously scattered within the epithelial cells of the proximal sections of the nephron. These cells displayed progenitor cell characteristics, such as clonogenicity and anchorage independent growth and were, as demonstrated by a novel explant model of human ATN, more resilient to renal insult than surrounding bulk PT cells. The cells also express a characteristic set of markers, among others, the stem cell associated CD24 and CD133, the antiapoptotic BCL-2 protein, and markers associated with cellular

structural integrity and attachment, which may contribute with mechanical resilience. These markers are virtually all shared with the population of PEC progenitors of Bowman's capsule. Additionally, we could find long stretches of cells positive for these characteristic markers in regenerating tubules of clinical samples of acute as well as more prolonged renal injury. The most striking morphological difference between the scattered cells and the bulk PT cells were the mitochondrial content. PT cells are packed with mitochondria, reflecting their normal function of endless active reabsorption and hence pronounced energy demand. In contrast, the scattered cells display remarkable mitochondrial paucity. Low mitochondrial content have been associated with stem cells and may contribute to DNA protection from reactive oxygen species (ROS) linked to mitochondrial respiration (206). Based on these findings we suggest that these proximal tubular rare cells, also called PTPCs, (which I will refer them to from know on) are better equipped to survive renal insults and may become activated to proliferate and repopulate the tubules in the wake of renal injury, thereby providing a cellular source for regeneration. On the contrary, the results from most lineage tracing studies performed in mice support the classical theory, that regeneration is not performed by a specialized progenitor cell population, but through dedifferentiation of randomly surviving cells. However, as we show in Paper III, human kidneys and mice kidneys are not mirror images of each other, at least with regard to the presence of PTPCs. The lack of PTPCs in mice does not exclude that progenitors exist, but may suggest that they have other characteristics and express other markers in mice. Recently, two papers applying lineage tracing techniques in renal epithelia have supported the notion of progenitor cells also in mice. Kang et al. demonstrated that Sox9 positive cells proliferated following damage and contributed to regeneration to all nephron segments except for Bowman's capsule, glomerular cells and collecting duct. Additionally, conditionally knocking out sox9 resulted in fibrosis (207). Rinkevich et al. revealed that Wnt responsive precursors gave rise to segment specific renal regeneration (86). Interestingly, we noted that *SOX9* is upregulated in the $ALDH^{high}$ fraction described in Paper I.

There are also a few alternative notions regarding the source for cellular regeneration. The group of Marcus Moeller has described a specific population of cells appearing *de novo* after damage. These cells are morphologically distinct and similar to the cells described by our group, most strikingly so with the reported lack of mitochondria and vimentin expression (75). This group reports a massive increase of these, so called scattered tubular cells (STC), upon AKI in mice and show that the STCs contribute to tubular regeneration. The STCs express KIM-1 and vimentin, which have been associated with kidney injury, this group therefore interpret the presence of STCs in normal human kidneys as signs of micro injury that possibly occur with age. However, we have found PTPCs in healthy human kidneys from ages 2 (which was the lowest age that we could get hold of) and up. Additionally, the PTPCs are immunophenotypically very similar to the PEC of Bowman's capsule, which are

widely accepted as a progenitor cell population and also express vimentin. It seems unlikely that these cells reside in a state of permanent injury. We thus propose that the PTPCs /STCs are indeed a resident progenitor population rather than an induced phenotype that could be adopted by any mature tubular cell, as suggested by the Moeller group (208). An alternative perhaps complementary hypothesis to the progenitor cell theory is presented by Sallustio et al. They propose that resident tubular progenitor cells aid in the regeneration process by protective paracrine signaling. They show that resident tubular progenitor cells secrete of protective factors such as MCP-1, C3 and IL-6 in response to TLR-2 activation signaled from the injured bulk cells (209), and that progenitor cell-secreted FGF2 and inhibin-A, as well as micro vesicle shuttled decorin and cyclin D1, could protect bulk epithelial cells from cisplatin induced damage via inhibition of apoptosis (74). Together these factors thus act on remaining bulk cells and protect them against injury and also induce proliferation, in this way minimizing the tissue damage. Thus resident renal progenitor cells may have several functions in AKI: to protect bulk PT cells from apoptosis and possibly stimulate their proliferation, as well as to regenerate tubules by proliferating to cover denuded basal membranes and subsequently differentiate to restore tubular function.

As indicated here, there is still confusion regarding the cellular origin of tubular regeneration. It may be so that the two main theories of renal regeneration, progenitors vs. dedifferentiation are mot mutually exclusive in mice, but work in concert to achieve tubular integrity after damage. In Paper III we did not see an induction of PTPCs even after damage. Our findings may shed some light regarding the discrepant results and conclusions concerning tubular regeneration in the field and may also suggest that tubular regeneration in mice and men are not orchestrated in exactly the same way. However, lineage tracing remains the current dogma for investigating cellular sources for biological processed *in vivo*, and this is for natural reasons not feasible in humans. Hence, the exact process of renal regeneration in humans is currently difficult to fully elucidate. Importantly though, one has to be careful when translating results attained in mice directly onto the human setting.

Anatomical location of PTPCs, a stem cell niche?

Adult stem cells are most often described to be located in a niche, which supports the optimal environment for the maintenance of these cells (9). In the process of characterizing the PTPCs we noted that they were often located in plicae positions, where the proximal tubules make hairpin turn. It is tempting to speculate that this localization may serve as a niche for the PTPCs. The plicae position could entail specific environmental features that might somehow support the PTPCs. One feature that may be specific for this anatomical location is the possible currents created from the flow of the filtrate, which may affect the PTPCs from the luminal side. Another specific feature may be the distance to the capillaries at this position. Low oxygen

tension has been suggested to be an important factor in maintaining quiescence in other adult stem cells (14). However the possible importance of the anatomical location of the PTPCs requires further investigation.

Connection to cancer and clinical impact?

In Paper I we found that the transcriptional profile of PTPC showed significant resemblance to that of pRCC. Additionally, two of the most characteristic PTPC markers KRT7 and KRT19, are used in the clinic to diagnose pRCC and are also expressed in renal adenomas, which are hypothesized to be a precursor lesion to pRCC. We thus propose that pRCC may be caused by a stepwise oncogenic transformation of PTPCs. This also relates to the hypothesis concerning adult stem cells as a plausible cell of origin in some cancers, as discussed in the stem and progenitor cell chapter of this thesis. Interestingly, the incidence of pRCC is higher in kidney transplant patients, both in native kidneys and in the allograft (210). Higher incidence of malignancies after organ transplants are often ascribed to the immunosuppressive treatment required upon transplantation (211) However, since the pRCC incidence is especially elevated, it is tempting to envision that this could be associated with PTPCs. In a retrospective study by Tillou et al. it was found that pRCC represented over 50% of RCC cases in comparison to around 10-15% in non-transplanted cases. Additionally, the proportion of pRCC in native kidneys of transplant patients has also been reported to be elevated, around 30% (210, 212). In the case of pRCC in allografts, could the transient hypoxic challenge in conjunction with the transplantation procedure cause erroneous activation of PTPCs, which in the long run could cause malignant transformation? Additionally, the incidence of pRCC is also elevated in patients with chronic kidney injury (96)), where PTPCs are also activated in regenerating tubules, as showed in Paper II. This may suggest a link between PTPCs, CKD and pRCC development.

A deeper knowledge of the process of regeneration and also the features of the putative renal progenitor cells may aid in the understanding of how AKI is best supported and possibly also how to exogenously induce the activation of the patients own progenitor cells. However, to much proliferation may lead to fibrosis and also have implications in the development of CKD, which is more prevalent in patients that previously have experienced AKI. So far, the molecular mechanisms of PTPC activation are unclear. Future studies are required to investigate which factors that activate the PTPCs and also what makes them differentiate. Knowledge of how to control this balance could be of clinical importance for both AKI and CKD, and possibly also hold clues to pRCC development.

Paper IV

Aims and Results

Paper IV: Overexpression of functional SLC6A3 in clear cell renal cell carcinoma

Specific Aims

The aim was to investigate transporter proteins differentially expressed in renal cell carcinoma compared to normal kidney.

Key Findings

In this paper we show that the dopamine transporter *SLC6A3* is highly expressed in ccRCC, while hardly being detected in normal kidney or other tumor types and thus bear the hallmarks of a highly specific biomarker ccRCC. We could show that ccRCC cells actively transported [^{3}H]-dopamine into the cells, an uptake that could be repressed using a specific SLC6A3-inhibitor, GBR-12909. Additionally, by knocking down HIFs using siRNA, we could see that the *SLC6A3* expression was maintained by HIF-2α expression. Furthermore, we could also show that *SLC6A3* expression could be induced in normal kidney cells grown in hypoxia. This effect was not seen in normal epithelial cells from breast (MCF10A), nor in normal endothelial cells (HUVEC). Altogether, this study shows that the high expression of *SLC6A3* in ccRCC is accompanied by a an enhanced uptake of dopamine and that the elevated expression level of the dopamine transporter can be linked to the main hallmark of ccRCC, i.e. a pseudohypoxic phenotype as a consequence of VHL-loss.

Discussion

Transporter proteins are understudied in the field of cancer, despite their important role in homeostasis and disease. Moreover, transporter proteins also hold great promise as potential targets for drug delivery. In this paper we performed an unbiased screen of transporter genes in several tumor types, compared to their corresponding normal tissues, including RCC and normal kidney. We found that RCC display a unique expression pattern of transporter genes compared to other tumor types, which most likely reflect their origin from the transporter-rich kidney. In general the tumor types reflected their tissue of origin and clustered together in the unsupervised hierarchical clustering analyses. However, there were examples of transporter genes that were present in the normal tissue but lost in the tumor samples. This may be due to dedifferentiation associated with tumorigenesis, but could also reflect the cell of origin. This is particularly evident when comparing normal kidney and the different

RCC subtypes. The kidneys are complex organs with advanced 3D structure and hence each normal biopsy contains many different cell types. Samples from the medulla and samples from the cortex will display different transcriptional profiles, reflecting their cellular composition. For example, the major part of the cortical samples will be composed of PTs. In the analysis of transporter gene expression, chRCC clustered away from the other RCC subtypes, and also away from the normal kidney samples. This may be explained by the notion that pRCC and ccRCC stem from the PT, while the cell of origin for chRCC is suggested to be located in the more distal part of the nephron, and that distal tubules represent a minor proportion of the cells in the normal samples. This highlights the importance of understanding the cellularity of normal samples, when comparing to tumors that originate from a distinct subset of cells within a given normal tissue.

When comparing RCC and normal kidney the most distinct differentially expressed transporter gene was the dopamine transporter *SLC6A3*. *SLC6A3* was highly expressed in ccRCC, while other tumor types, including pRCC and chRCC, barely express noticeable levels of this transporter. In normal tissue, *SLC6A3* expression is only seen in dopaminergic neurons in the brain, where it normally exerts its function of transporting released dopamine back into the neuron for later use. SLC6A3 plays a vital role for the dopamine signaling in the brain, and disturbances of its function cause marked neurological and behavioral effects. However, SLC6A3 has not been reported to be expressed at appreciable levels elsewhere. The exact reason for the upregulation of *SLC6A3* in ccRCC is not clear, but we could demonstrate that the expression is affected by HIF, in particular HIF-2α. We also show that *SLC6A3* is specifically induced by hypoxia in primary normal renal cells. These primary cultures are predominantly composed of PT cells, which are also suggested to be the cells of origin for ccRCC. As previously mentioned ccRCC has a very strong hypoxic drive due to the functional loss of VHL, and whereas HIF-1α has been reported to be lost in 40% of ccRCC cases, HIF-2α is continuously expressed (118). Collectively, this may suggest that the *SLC6A3* locus is specifically accessible in renal proximal tubule cells and that HIF-2α is responsible for its transcription, causing high expression in ccRCC exclusively.

Importantly, we also demonstrate that ccRCC cells can actively take up dopamine and that this is mediated by SLC6A3, providing evidence for functional SLC6A3 in ccRCC. The effect of the active dopamine uptake on the tumor cells is however not clear. In preliminary viability assays inhibition of SLC6A3 has not shown any significant effects, (unpublished data). However, long term treatment effects of SLC6A3 inhibition has not been studied. Additionally, the consequence of SLC6A3 expression may be different *in vivo* and *in vitro*. The environment of cell culture conditions is indisputably altered from the microenvironment for ccRCC tumor cells *in vivo*. As mentioned in the transporter chapter of this thesis, dopamine has been reported to have immune suppressive properties. One of the prerequisites for tumor

development is to evade the immune system, perhaps this could be an advantageous trait of the SLC6A3 expression? Dopamine is also involved in antioxidant protection, which may be beneficial in a tumor environment. Antioxidants have been implicated in promotion of metastasis in melanoma (213, 214), lung cancer (215) and breast cancer (216). Additionally, it may also protect the tumor cells from free radicals similar to the effect of antioxidants in normal cells. Antioxidant supplement in combination with radiation or chemotherapy has shown to have adverse effects on tumor progression in several cancer forms (217). Another possible usage for the dopamine uptake might be for metabolic purposes, perhaps for energy or building blocks. Dopamine can for example be catabolized to homovanillic acid (HVA) by the enzymes monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) (218). Dopamine is also the precursor for norepinephrine and epinephrine. Epinephrine has been shown to protect breast- and prostate cancer cells from apoptosis via indirect inhibition of the pro-apoptotic protein BAD (219). However, the metabolic pathways that could affect dopamine in ccRCC, and the possible consequence of the metabolites, require further investigation.

Interestingly, primary ccRCC cells, but only two of the tested ccRCC cell lines in Paper IV display elevated *SLC6A3* expression. The KMRC3 and SNU-349 cell lines show high levels of *SLC6A3* mRNA and are the only cell lines (apart form cultured primary ccRCC cells) that specifically take up dopamine mediated by SLC6A3. The growth characteristic as well as the morphology of these cell lines are also more in line with primary ccRCC cells than conventionally used cell lines, such as 786-O. This observation emphasizes the importance of using representative model systems for studying human diseases *in vitro*.

Just recently a paper was published on *SLC6A3* in ccRCC. Schrödter et al. report that *SLC6A3* mRNA levels are extremely high in ccRCC, but also that the protein levels are very low and conversely, that normal kidney samples have low mRNA levels but that the PTs express high protein levels of SLC6A3 (220). The authors also claim that the ccRCC cell lines Caki-1 and A-498 can be killed using an inhibitor of SLC6A3. However, they use *sertraline*, which is a selective serotonin reuptake inhibitor (SSRI). Moreover, this inhibitor was used at high concentrations, and appropriate controls using cells lacking SLC6A3 expression are missing. In light of our detailed transcriptional profiling showing exceedingly low levels of *SLC6A3* in normal kidney cortex, the results presented by Schrödter et al., are difficult to comprehend. We have also tried do detect the SLC6A3 protein in kidney tissue, both tumor and normal, using immunohistochemistry, but we have so far been unsuccessful. There are antibodies that work well on neuronal tissue, while no staining could be seen in kidney tissue. There could be several reasons for this discrepancy. Kidney tissue and neuronal tissue are of very distinctive natures and are usually handled differently prior to staining. Additionally, there might be differences in the post-translational modifications of SLC6A3 in ccRCC compared to dopaminergic neurons. Also,

kidney cells, and PT cells in particular, are crowded with transporter proteins reflecting their normal function, opening up for the possibility that the antibody used by Schrödter et al. not only detects SLC6A3 but also another transporter, since the structural homology between transporter proteins can be high (221, 222). This putative cross-reaction might not have been seen when validating the antibody in neuronal tissue, since dopaminergic cells and kidney cells do not in general share transporter expression.

ccRCC is exceptionally resistant to conventional cancer treatments such as radiation and chemotherapy, and patients with metastatic ccRCC await a dismal prognosis. Despite recent treatment advances using targeted therapy, virtually all patients with mRCC will succumb to the disease. There is thus a desperate need for novel targeted therapies and diagnostic methods. The observation that SLC6A3 is highly expressed and functional in ccRCC, may have noticeable clinical impact for the development of novel targeted therapies and diagnostics in ccRCC. It is tempting to envision a therapeutic approach that allows for SLC6A3-specific uptake of small toxic molecules into the ccRCC cells. Alternatively, one could use cocaine-analogues for delivery of targeted radionuclide therapy, which would specifically bind SLC6A3 and selectively kill the ccRCC cells. These approaches would target ccRCC features distinct from the pathways targeted with current therapies, and could perhaps be used in combination to achieve synergistic effects. Additionally, there are already diagnostic techniques that utilize the presence for SLC6A3 for detecting and quantifying dopaminergic neurons using imaging modalities. These are based on radiolabelled cocaine analogues and are currently in clinical use for the diagnosis of PD (223). We propose that these compounds could be used also for the diagnosis of ccRCC, and possibly allow for high resolution of lymph node involvement and early metastatic spread. We are currently in the process of initiating such a clinical trial. Since the prognosis of ccRCC is extremely different depending on whether the disease is spread or not, the proposed alternative diagnostic approach could possibly help to guide treatment options and initiate targeted therapies as early as possible.

Conclusion and Future perspective

In this thesis I have studied renal progenitor cells and kidney cancer and the findings of the included papers may lead to elevated insight to renal regeneration as well as to renal tumor development.

We herein present evidence for a novel progenitor cell type scattered among the tubular cells of the proximal part of the nephron. We propose that this cell type, PTPC, is involved in renal regeneration, and that it may represent the cell of origin for pRCC. Increased knowledge of the process of renal regeneration and progenitor cells has implications for treatment development for AKI and CKD, conditions associated with extensive morbidity and mortality as well as enormous medical cost for society. Further investigations of the regulation of PTPCs could perhaps be utilized for future cell based- or molecular therapies, to improve outcome following acute kidney damage as well as progressive kidney disease. Additionally, our findings regarding the species distribution of PTPCs could shed some light on the discrepant findings of the cellular source for regeneration within the field.

In this thesis we also present evidence for HIF driven upregulation of functional dopamine transporter SLC6A3 in ccRCC. The consequences of the *SLC6A3* overexpression in ccRCC are yet to be revealed, however the finding has implications for the development of novel specific drugs targeting ccRCC, which currently is essentially therapy resistant. Finally, since there are already diagnostic methods based on radiolabelled SLC6A3 ligands, I have high hopes for the usage of the same, or similar, imaging modalities for detection of ccRCC tumors and metastasis in the near future.

Populärvetenskaplig sammanfattning

Njurarna har en mängd viktiga funktioner i kroppen men har som främsta uppgift att filtrera blodet och föra ut gifter ur kroppen genom bildandet av urin. Njurarna filtrerar uppskattningsvis 180 liter primärurin varje dygn och 99,5% av all vätska pumpas tillbaka till kroppen genom njurarnas rörsystem, vilket slutligen resulterar i endast 1,5 liter urin. Detta konstanta pumpande är extremt energikrävande och medför att njurcellerna är mycket känsliga för störningar i sin energi- och syretillförsel från blodet. Om njurarna får syrebrist, till exempel vid en massiv blödning efter en olycka, kan cellerna i njurens rörsystem dö, vilket kan leda till njursvikt som är direkt livshotande. Om patienten snabbt får hjälp att rena blodet på konstgjord väg, via dialys, kan njuren dock laga sig själv via så kallad regenerering. Hur det går till och vilka celler som utför hoplappandet av rörsystemen i njuren är fortfarande inte helt klarlagt. Njurarna kan också, som så många andra organ i kroppen, drabbas av cancer, där tumörcellerna delar sig okontrollerat på grund av mutationer, felaktigheter i cellens DNA. Njurcancer drabbar cirka 1000 personer om året i Sverige. Symptomen för njurcancer är vaga och hos en stor del av patienterna så har sjukdomen redan spridit sig utanför njuren då man upptäcker sjukdomen. För dessa patienter är prognosen mycket dyster då njurcancer inte svarar på vanliga cancerbehandlingar såsom cellgifter och strålning och knappt 10 % av dessa patienter förväntas överleva i fem år. Behovet av nya behandlingsformer så väl som nya diagnostiska metoder är därför mycket stort.

I den här avhandlingen beskrivs en helt ny celltyp som ligger vilande mellan de vanliga cellagren i njurens rörsystem. Celltypen är stamcellslik och är utrustad med förankringsprotein som troligtvis bidrar till att den inte lossnar lika lätt vid skada. Vi visar även att celler i regenererande rör delar markörer med den nya celltypen. I en ny metod för att simulera njurskada utanför kroppen, i en cellodlingsskål, såg vi att den nya celltypen är mycket mer motståndskraftig mot celldöd än de andra cellerna i njurens rörsystem. Vissa har hävdat att ytmarkörerna som uttrycks av den nya celltypen endast är tecken på skada efter ett långt liv av blodfiltrering. Här visar vi däremot visat att cellerna finns i njurar från unga friska personer från 2 år och uppåt. Till vår förvåning så fann vi att de nya cellerna inte kunde hittas i njurar från möss, men att de fanns i njurar från schimpans och gris. Detta skulle kunna förklara den förvirring som råder inom njurregenerations-fältet om huruvida cellerna som ansvar för reparation är stamceller som finns på plats innan skadan eller om skadan repareras

av vanliga njurceller som omprogrammeras. Många av de rapporter som hävdar att njurregenerationen inte utförs av specialiserade stamceller grundar sig nämligen på studier i möss. Vi föreslår alltså att det finns en skillnad i hur njurregenereringen går till i olika arter. Baserat på de fynd som gjorts i denna avhandling så tror vi att de nya cellerna kan aktiveras efter skada och då börjar dela sig för att ersätta det förlorade cellagret i den mänskliga njuren. Men det finns även en helt annan sida av celltypen. Celltypens genuttryck var nämligen mycket likt en typ av njurcancer, så kallad papillär njurcancer. Det skulle alltså kunna vara just i dessa celler som denna typ av njurcancer uppkommer. Nästa steg är att ta reda på vad det är som aktiverar cellen och bidrar till läkning efter njurskada, men också vad som händer när cellen blir elakartad och ger upphov till cancer. På så sätt skulle denna upptäckt möjligtvis kunna bidra med idéer till nya behandlingar vid både njurskador och njurcancer.

I en annan del av avhandlingen har vi upptäckt att klarcellig njurcancer uttrycker mycket höga nivåer av dopamintransportören SLC6A3. I normala fall uttrycks endast denna transportör i dopaminproducerande celler i hjärnan, samma celler som för övrigt förloras vid Parkinsons sjukdom. Vi fann att andra tumörformer, och normal njure, uttrycker synnerligen låga nivåer av SLC6A3 och att det därför är en mycket specifik biomarkör för just klarcellig njurcancer. Vi visade också att njurcancercellerna aktivt kan ta upp dopamin och att detta upptag kan hämmas genom att sätta till en specifik SLC6A3-hämmare. Det verkade även vara så att SLC6A3-uttrycket i njure styrs av ett protein, HIF-2a, som normalt sett uttrycks specifikt när cellerna har syrebrist, så kallad hypoxi. När vi behandlade normala njurceller med lågt syretryck så inducerades uttrycket av SLC6A3, men detta skedde inte med celler från andra normala vävnader så som bröst och blodkärl. I klarcellig njurcancer förekommer en speciell mutation som gör att cellerna alltid befinner sig i ett tillstånd som om det rådde hypoxi, även vid normalt syretryck. Denna pseudohypoxi skulle kunna vara en av förklaringarna till det höga SLC6A3-uttrycket i klarcellig njurcancer. Genom att använda sig av dopamin transportörens egenskaper tror vi att dessa fynd skulle kunna leda till utveckling av nya riktade behandlingsformer och ny diagnostik för klarcellig njurcancer.

Acknowledgements

"Forskning är lätt när man har rätt"

Håkan Axelsson

I would like to express my sincere gratitude toward the people that have helped me during the course of this thesis work. Without some of you this thesis would not have been possible, and without some of you this thesis work wouldn't have been endurable!

I would like to thank my supervisors **Martin Johansson** and **Håkan Axelson** for your patience with me and for your never-ending enthusiasm about science and the kidney in particular, it's contagious. Thanks **Martin** for your endless brilliant ideas about how kidney regeneration really work, which made me look at our projects from a new point of view after almost every meeting. Also thanks for the pop culture-talks ranging from Håkan Hellströms musicians and Karl-Ove Knausgård to Game of Thrones, and for sharing my love of Bohuslän.

Håkan, thanks for your mentorship, pep talks and for believing in me, I don't think it is that common that supervisors praise their Ph.D. students in public, but you do, you always have our back! Thanks for Axelson dinners and for letting us visit your little gem in Bjäre and all the discussions about anything and everything from science to feminism. I really do think that you should initiate a "Nu kör vi"-campaign ;) I'm especially grateful for the opportunity to embark on the new Detego-adventure! Tipptopp!

Past and present Axelson-Johansson members (and step-members), **Anna-Karin, David**, **Kris**, **Emma**, **Elinn**, **Helén**, **Krzysztof**, **Sofie B**, **Sophie L** and last but certainly not least **Christina**! Thank you for nice lab meetings, journal clubs, lunches and discussions. **Christina**, thanks for all the invaluable help in the lab with cells and assays and moral support! **Anna-Karin**, thanks for introducing me to the group and for general life-advice and wisdom. **Sophie L**, thanks for lab- and thesis writing advice, for good company and pep talks. **Elinn**, thanks for company in the cell culture room and for always being in such a great mood! **David**, you bioinformatics-whiz, thanks for your meticulous reviews of manuscripts, for all your advice and support

and for sharing my love for cats ;) **Kris**, thanks for all the English-advice, Halloween parties, and for being an excellent office mate!

Lena Gustavsson, thanks for introducing me to the fascinating world of transporters, and for having such a positive spirit! **Johanna Asklin**, thank you for all your time and help and for being so enthusiastic about our project!

Everyone at **TCR** and old **CMP**, especially **Louise**, **Caroline B**, **Sofia**, **Greta**, "**Peppe**", **Tamae**, **Christian**, "**Karna**", **Rebecka**, **Caroline W** and **Matilda**, thanks for all the meetings, retreats, frukost and fika, lunches, dissertation parties and for creating such a great work environment! **Pietras members**, thanks for interesting journal club discussions and for really raising the spirit at TCR (and at retreats)! **Louise**, thanks for fantastic dinners, "bubbel" and the great coffee- and lunch company, I really miss you at TCR! **Sofie M**, **Arash** and **Marica**, thanks for fabulously entertaining skiing and conference company!

Margareta, **Siv** and **Christina** (again), thanks for the immense laboratory wisdom you are willing to share, for help with ordering and for the positive attitude you're spreading. **Elisabeth** and **Kristin**, TCR would crumble without your administrative help, thank you for keeping us afloat!

Biomedgänget, **Madde**, **Sara R** (kom hem!), **Tina**, **Helena**, **Sara S**, **Ewa**, **Mia, Hanna**, **Ida**! Alltså, vad skulle jag göra utan er! Lundatiden hade varit en helt annan, det är ett som är säkert! Tack för att vi har fått följas åt och dela samma våndor inför tentor och artikelsubmitteringar och samma glädje efter att det har gått bra! Tack för socialtnörderi, middagar, fester gin&tonic-kannor, skidresor. Så himla smarta och coola brudar, ni är bäst!!!

Annika, finaste ankan! Tack för att du tar med mig på franska-, brittiska- och göteborgska-äventyr och bara är ett telefonsamtal bort, je t'adore!

Lysekilsgänget! **Pernilla**, **Camilla**, **Emma** och **Josefine** tack för att ni finns där. Även om det går alldeles för länge mellan gångerna vi ses så är det som om inget har hänt när vi väl träffas, bara helt självklart, så himla gött!

Mamma **Eva-Britt** och pappa **Roger**, min största fanclub! Tack för allt ert stöd och nyfikna frågor om vad det är jag gör och hur det går med cellerna egentligen? Vad som än händer vet jag att jag kan lita på er, det känns så himla tryggt! Mamma, tack också för inpräntning av uttrycket "man ska göra så gott man lagom kan"! **Simon**, lillebror, (som inte är så liten längre) tack för att du äntligen slutade slåss ;) Skämt åsido, jag är så himla glad för att ha dig som bror! Du är så modig och det är inspirerande hur du sprutar ur dig idéer och ger dig huvudstupa in i nya saker.

Gleisners, vilken tur att ni är så himla trevliga hela bunten! **Annika**, **Peter**, **Ebba**, tack för barnpassning, stöttande ord och för fantastisk mat! **Tove**, **Patrik** (och **Edith**), tack för otaliga middagar och vardagshäng, det är så mysigt att ha er nära!

Christian, min klippa, min egen mästerkock och min stora kärlek! Tack för att du påminner mig om vad som egentligen är viktigt i livet. Jag är så oerhört tacksam för allt som du gör och för att du är världens bästa pappa till Siri. Utan ditt stöd hade inget av det här varit möjligt. **Siri**, fina du, tack för att du får mig att skratta varje dag och livar upp kvällarna med dansparty och världens bästa kramar! Jag älskar er min fina familj!

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