

Glomerulogenesis and renal tubular differentiation: role of ${\rm HNF1}\beta$

Arianna Fiorentino

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Arianna Fiorentino. Glomerulogenesis and renal tubular differentiation : role of HNF1 β . Human health and pathology. Université Sorbonne Paris Cité, 2016. English. NNT : 2016USPCB121 . tel-02180581

HAL Id: tel-02180581 https://theses.hal.science/tel-02180581

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THESE DE DOCTORAT DE L'UNIVERSITE PARIS DESCARTES

Ecole doctorale « Bio Sorbonne Paris Cité », ED 562

Département Développement, Génétique, Reproduction, Neurobiologie et Vieillissement (DGRNV)

Spécialité : Développement

Présentée pour obtenir le titre de DOCTEUR de l'Université Paris Descartes

« Glomerulogenesis and renal tubular differentiation : Role of HNF1ß »

Par

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Soutenance le 13 décembre 2016

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(Le Petit Prince- Antoine de Saint-Exupéry)

Aknowledgments - Remerciements - Ringraziamenti

During this long adventure of the PhD, I was surrounded by many people that I will try to thank to in these pages.

In first place, I would like to thank the members of the jury that have kindly accepted to evaluate my work: Jean-Jacques Boffa, Yves Allory, Jean-Claude Dussaule and Rémi Salomon.

For the supervision and the precious advices, I would like to thank Evelyne Fischer and Marco Pontoglio that overviewed all my work. I thank Evelyne, my thesis director, for the scientific exchanges of ideas, for the guidance to complete my project and for her help in difficult moments. I thank Marco for the discussions, even for the heated ones, because the pressure in the environment not only helped me to work harder on science but more importantly on my character, to face the problems and solve them.

I would like to thank all members of Pontoglio's Lab. I am glad to meet them in my adventure of the PhD. Each one has a special place in my heart. Cecile that was technician in the lab when I arrived was the closest person to me, giving me all the information and support for the first days when I still did not understand and speak French. She was my first friend in Paris and I will never forget her. Serge always helped me with informatic problems, was patience with my bad French especially at the beginning and never complained about the thousands of stitching he was obliged to do with my immunofluorescence pictures. I thank Armelle for the help with the experiments and her understanding during all the period that we shared at the bench. Thanks to Magali for the help in the lab, especially with mice, but more for always finding a positive side of things, except on Mondays. I want to thank Alessia for the Italian help translating everything in English when I did not have the words, for the time spent together during the lunch break and for always being close to me. I thank John for the funny moments in the lab, but also for his advices about troubles of PhD life. I want to thanks Parla for the help with the English and for sharing her experience in work, at last for correcting the English of my thesis, but more for the faith that she always demonstrate to me as a friend. Finally, I thank Salvina for her presence on my side in the office, supporting all my good and bad moments and for her "comic" side that really help during working days. Last but not last, I want to thank Michel because of his presence and help in any situation. You are someone to lean on, always on my side. I discovered you later but when we really met each other, we built a

deeper friendship and work alliance. I thank all of you for all the help, for all the love and good/bad moments that we shared and for the support in all this time. You are really a wonderful group and my family lab. My PhD could be not the same without you.

During my thesis at the Cochin, I met a lot of kind people: I would like to thank Pierre Billuart for his support in this last period, not only to make me use is office during this long summer, but more for his friendship and empathy. Thanks to all the people of the histology, microscopy and animal house platforms for the technical support during all the work and all the students of the Jecco, in particular Rozen and Mariangela. A special thank goes to the best secretary ever, Maryse.

The experience of the PhD gave me another gift; I had the luck to be part of a Marie Curie Program in which I found wonderful people that shared with me both the science and the life. For this reason, I want to thank Chiara, Zoraide, Laura V, Aylin, Kanishka, Laura R, Martin, Alkaly and Tareq and all the PI that followed us during this journey. This kind of program are great for PhD to be in contact with other groups, for networking and exchange of experience.

During this three year far from home, I was lucky because I found very important people for my life. When I arrived in Paris, I found back my Italian friends Alessandra and Giuseppe that helped me from the first moment not to feel lost, to find a home and to be always close to me every time I need. I want to thank the first family that I had here "Casa Leonardo", a group of Italian students that became like a family in short time. I thank them for the nice time that we spent together in Paris and to be still present in my life as good friends. Two people of this group became my roommates and my second family, Anna and Claudia that I thank for their support in all my bad moments, to share lab problems and crisis of a PhD student, but also to be present in my life, for the fun and the love that we shared until now. I want to thanks all the friends that I met in Paris and with whom I spent good moments: Jean, Isabella, Lulù, Fabrizio, Luca, Lorenzo and Daria.

However, what really surprises me is that I was so lucky to find another big family, "Pariggini" group! Our friendship started like a joke when Chiara and Sara Zum invited me to sing in a gospel choir, I was so excited and happy for this experience, but I could not imagine that so quickly this could become more than a choir group. Thanks guys for all the fun, for the days and night spent together, between serious and stupid arguments, for your presence when I was happy but more when I was sad. I would like to thank you one by one

but probably these pages will be not sufficient to do it. Thanks to Matteo, Lorenzo, Amedeo, Chiara and Veronica for this crazy summer trying to move me out of the lab; Sara Cigna to share the anxiety of writing a thesis; Chiara (Sarto) and Marco to save me in hard moments and just to be present; Anthony my little French friend that I love so much, and Samantha my crazy side, supporter and motivator that always believed in me even when I felt lost. I thank all the people of this fantastic group: Araldo, Marchesini, Michele, Zum e Silvio, Fiask, Filippo e Luciana, Enrico, Leo, Sara Biz, Daniela, Diana e Giovanni, Debora, Elisa, Eugenio, Giancarlo, Giorgia and Angelo. I think this friendship will never finish even if part of us are already around the world.

All these wonderful people will always have a special place in my heart and my memories.

A special thanks go to friends in Italy that are still waiting for me to come back, and to the people that believed more than me in my capacity and pushed me to accept this challenge of the thesis: Daniele, Isabella and Silvia and all the other MBC friends.

In fine, voglio ringraziare la mia famiglia che per tutto questo tempo mi ha seguita a distanza dall' Italia, per il supporto, la fede nelle mie capacità e l'amore che mi dimostra ogni giorno. Il più grande ringraziamento va ai miei genitori e a mia sorella Ylenia, che mi hanno sempre spinto a seguire i miei sogni e ad affrontare questa avventura con forza, sebbene io sappia quanto gli costi stare lontani. La mia più grande soddisfazione è vederli orgogliosi di ogni mio passo, loro sono la mia forza e la mia vita.

These last three years were full of experiences, good vibes and life. I am glad to have this opportunity. PhD is not only an experience of work in science; it is an experience of life and a challenge with one's self. This experience makes me feel more confident and proud of my-self and I think it will be forever a part of me.

TABLE OF CONTENTS

INTR	RODUCTION	8	
CHA	PTER 1: KIDNEY MORPHOLOGY AND FUNCTION	9	
I.	Anatomy of Kidney	10	
II.	Anatomical description of the different nephron components	12	
III.	Renal tubular transporters	15	
CHAI	CHAPTER 2: KIDNEY DEVELOPMENT		
I.	UB outgrowth and collecting duct system development.		
II.	UB branching morphogenesis	25	
III.	Nephron formation.	27	
i.	. Comma and S-shaped body development.		
ii	i. Precapillary-loop precursor	31	
ii	ii. The development of the glomerulus	32	
	- Podocytes	32	
	- Capillaries and mesangial cells	33	
	- Glomerular filtration barrier	34	
	- Bowman capsule	35	
i	v. Tubular development	40	
	- Tubular expansion	40	
	- Tubular elongation.	40	
	- Apicobasal cell polarity.	45	
CHAPTER 3: HNF1 TRANSCRIPTION FAMILY AND MODY SYNDROMES			
I.	HNF1 Transcription Factor Family	50	
II.	HNF1 Mutations in Human	52	
i.	. MODY syndrome	52	
II.	MODY3 and MODY5 syndrome	54	
III.	Renal defects correlated to MODY3 and MODY5/HNF1B mutations	55	
i.	. Tubular dysfunction in MODY3 patients	55	
ii	i. Renal manifestations in patients carrying <i>HNF1B</i> mutations.	55	
	- <i>HNF1B</i> mutations	56	
	- Clinical presentation	56	
ii	ii. Other clinical manifestations observed in MODY3 and MODY5 patients	58	
CHA	CHAPTER 4: HNF1alpha AND HNF1beta EXPRESSION PATTERN		
I.	HNF1alpha and HNF1beta during embryogenesis	61	
II.	HNF1alpha and HNF1beta in liver, pancreas and intestine	62	

Ι	II.	HNF1alpha and HNF1beta in kidney	65	
	i.	HNF1beta in kidney early development	66	
	ii.	HNF1beta in tubular and glomerular specification	66	
	iii	. HNF1beta in tubular expansion	68	
CH	CHAPTER 5: TUBULAR DIFFERENTIATION AND FIBROSIS			
I.		Phenotypic modifications of tubular cells in renal fibrosis	74	
	i.	Partial Epithelial to Mesenchymal Transition (EMT)	74	
	ii.	Cell cycle arrest	76	
	iii	. Metabolic alterations	77	
II 	I.	Molecular pathways involved in tubular cells during fibrosis and their link with HNF11	oeta 79	
PR	OJ]	ECT DESIGN	82	
MA	TF	ERIAL AND METHODS	86	
RE	SU	LTS PART I	93	
R	Role	of HNF1beta in glomerular cyst formation	94	
S	Self	-strangling renal glomeruli in HNF1B-glomerulocystic disease	99	
F	IG	URES PART I	. 120	
RE	RESULTS PART II			
R	Role	e of HNF1beta in renal tubules	. 127	
Partial epithelial to mesenchymal transition in the absence of HNF1beta in mature renal tubules			. 131	
F	IG	URES PART II	. 156	
DIS	DISCUSSION			
I		The role of HNF1beta during glomerulogenesis	. 171	
Ι	I.	The role of HNF1beta during tubular maturation	. 173	
PE	RS	PECTIVES	. 177	
I.		GLOMERULAR MATURATION	. 179	
	Pe	erspective 1: Origin of the intercalating parietal cells	. 179	
	Pe in	erspective 2: Identification of the signaling pathways and the molecular cascades directly directly controlled by HNF1beta during glomerulogenesis	y or . 180	
Ι	I.	ROLE OF HNF1Beta IN TUBULAR MATURATION	. 183	
	Pe	erspective 3: Absence of homeostatic growth in <i>Hnf1b</i> deficiency	. 183	
	Pe	erspective 4: Tubular dilation in the absence of HNF1beta in the post weaning period	. 185	
	Pe	erspective 5: Identification of direct target genes bind of HNF1beta in proliferative versu	IS	
	qı	uiescent status	. 186	
BIE	BIBLIOGRAPHY			
Sur	nm	ary	. 208	

INTRODUCTION

CHAPTER 1:

KIDNEY MORPHOLOGY AND FUNCTION

KIDNEY MORPHOLOGY AND FUNCTION

I. Anatomy of Kidney

Kidneys are retroperitoneal paired organs located on each side of the vertebral column (Fig 1A). Macroscopically, kidneys are surrounded by a fibrous capsule, and from the outside towards the inside, are composed of the cortex and the medulla. In human, a mature and functional kidney is composed of 500000 to one million structural and functional units called nephrons. Each nephron consists of a glomerulus, the blood filtration unit, and a tubular component. The epithelial tubular portion of the nephron is composed of more than 20 subtypes of highly specialized cells, organized in successive subsegments. Starting from the glomerulus, the tubule of the nephron is composed of the Proximal Tubule (subdivided in S1, S2 and S3 segments), the Henle's Loop and the Distal Convoluted Tubule which is connected to the Collecting Duct system (Fig1C). Morphologically, S1 and S2 convoluted segments of proximal tubules, the glomerular globular structures and the collecting duct system. In the deeper part of the medulla, the collecting duct system will progressively form multipyramidal structures, renal calyces, pelvis and finally the ureter (Fig1B).

In the organism, kidneys have different important functions. They play a major role in the maintenance of whole body homeostasis by regulating the acid-base balance, electrolyte concentrations and extracellular fluid volume. They also participate to the elimination of wastes derived from the metabolism. Additionally, kidneys have endocrine functions and produce important hormones: erythropoietin which stimulates the production of red blood cells, and renin, an active actor in the blood pressure regulation via the renin-angiotensin conversion 25-hydroxycholecalciferol system. Finally, through of to 1,25dihydroxycholecalciferol, kidneys play a key role in the synthesis of the active form of vitamin D that participates to the homeostasis of calcium, important for bone morphogenesis and maintenance.

To maintain whole body homeostasis, the primary urine produced by the glomerular filtration is modified through the reabsorption or secretion of water and small molecules by the renal tubules. These functions are accomplished through the cellular complexity and peculiar spatial organization of the kidney. I will briefly describe the different characteristics and specific functions of the sub segments of the nephron.



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Figure 1. Anatomical structure of the kidney.

(A) Macroscopic representation of the kidney with its external connection to the urinary system and to the vascular network (adapted from https://media1.britannica.com). (B) Kidney is composed of two main parts: the cortex, containing the glomeruli and the convoluted portion of the proximal and distal tubules, and the medulla, composed of the straight portion of the proximal tubule, the Henle's loop and the collecting duct (C) Macroscopic representation of the nephron segments (adapted from https://www.cnx.org)

II. Anatomical description of the different nephron components

The mature renal glomerulus is a globular structure responsible for the filtration of blood. Its inner part is a cup-like structure composed of a layer of podocytes (visceral epithelial cells) that surround a network of capillaries supported by the mesangial matrix and contractile mesangial cells. The Bowman's capsule with its inner monolayer of Bowman's capsule cells (parietal epithelial cells) surrounds this capillary tuft. Glomerular capillaries arise from a single afferent arteriole that divides into capillaries and finally converges into a single efferent arteriole. The site where blood vessels enter the glomerulus is called vascular pole. The blood is filtered through a filtration barrier composed of three layers: a fenestrated endothelial layer, a basal membrane and the filtration slits created by the footprocesses of podocytes. This barrier is highly selective and allows only small molecules (low molecular weight proteins or ions) to pass. On the contrary, cells and high molecular weight proteins (for example, albumin) are normally retained in the circulation. The Bowman's capsule, surrounding the vascular tuft and delimiting the urinary space, collects the primary urine derived from blood filtration. Bowman capsular cells (or parietal cells) are flattened cells with a cytoplasm barely visible by light microscopy. They are in continuity with tubular cells at the urinary pole that represents the connection between the glomerulus and the proximal tubule (Fig 2) (Brenner and Rector's The Kidney 2012).



Figure 2. Structure of the glomerulus. The glomerulus is composed of a complex net of anastomosed capillaries surrounded by podocytes. The glomerular tuft is surrounded by the Bowman capsule, delimiting the urinary space. The macula densa cells are located between the afferent and efferent arterioles, associated to juxtaglomerular cells (adapted from (Human Anatomy and Physiology, 5th Edition by Marieb: Benjamin-Cummings, San Francisco, CA 9780805349894 Hardcover, 5th Edition. - a2zbooks n.d.))

The **proximal tubule** (PT) is subdivided into three segments. The S1 segment represents the initial portion starting from the urinary pole of the glomerulus and constitutes approximately two thirds of the pars convolute. The S2 segment consists of the last portion of the pars convoluta and the initial portion of the pars recta. The S3 segment represents the straight part of the proximal tubule located in the deep cortex and the outer stripe of the outer medulla. The function of each subsegment depends on the specific set of genes expressed by these tubular cells. The proximal tubule is lined by a cuboidal epithelium whose cells have several characteristic features: the presence of a brush border of microvilli at the apical side, which increases the surface area to reabsorb small molecules from the filtrate to the interstitium, and a high number of mitochondria (Brenner and Rector's The Kidney 2012).

The **Henle's loop** has a peculiar spatial organization, with a characteristic U shape. This tubular segment enters the medulla of the kidney and returns back to the cortex where it contacts its own glomerulus. Schematically, two functionally distinct limbs form the Henle's loop segment: the thin descending (TDL) limb and the thick ascending (TAL) limb of Henle. The descending thin segment is lined by simple squamous epithelium, whereas the ascending thick segment is lined by a cuboidal epithelium with scattered microvilli, high density of mitochondria and an interdigitated basal membrane.

The **Macula Densa (MD)** plaque is a group of epithelial cells located in the cortical thick ascending limb in close proximity with the vascular pole of each glomerulus (Fig 2). In association with the Juxtaglomerular cells ("J-G cells") in the wall of the afferent arteriole, they form the juxtaglomerular apparatus. This complex structure constitutes a unique anatomic arrangement where the vascular and the epithelial networks of the kidney come into contact. The recognized role of MD cells is to detect the increase of NaCl concentration in tubular luminal fluid and to transmit signals that modulate both the vascular tone of the afferent arteriole and the renin secretion from granular cells of the juxtaglomerular apparatus. These cells represent the critical link between renal salt and water excretion and glomerular hemodynamics, thus playing a key role in regulation of body fluid volume (Bell et al., 2003; Lapointe et al., 2003).

The **distal convoluted tubule** (DCT) and the short **connecting tubule** (CNT) are the portion of the nephron immediately downstream of the *macula densa*. These segments are

composed of a cuboidal epithelium that is structurally similar to the one of the thick ascending limb. This portion of the renal tubule continues to reabsorb useful solutes from the filtrate to the peritubular capillaries, actively pumping small molecules out of the tubule lumen into the interstitial space.

The DCT is then connected via the CNT to the **Collecting Duct** (CD) system. The collecting ducts are formed in the renal cortex by the connection of several nephrons. They go down within the medullary rays of the cortex, penetrate the outer and the inner medulla where they successively fuse together to bring the urine produced by the nephrons into the ureter and finally in the bladder. Based on their location within the kidney, the collecting ducts can be subdivided into cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD). The cells lining the CD can be divided into at least two cell types: principal cells and intercalated (IC) cells. The principal cells represent 70–75% of all cells, whereas IC cells represent the remaining 25–30% of cells.



Figure 3. Schematic representation of nephron components. Primary urine produced by the glomerulus pass through the different tubular portions of the kidney that reabsorb water and solutes and define the final urine composition. (Figures 44.14 and 44.19, page 879 and 884, Campbell's *Biology, 5thEdition*)

III. Renal tubular transporters

A complex set of tubular reabsorption and secretion mechanisms is activated in the kidney to maintain the whole body homeostasis. Approximately 2/3 of the water and Na+ and most of the electrolytes and amino acids filtered by the glomerulus are reabsorbed during their passage through the proximal tubule. Additional 25% of the filtered Na+ and water are reabsorbed in the Henle's loop. Final adjustments in the composition and volume of urine are made in the distal part of the nephron (distal and collecting duct) mainly under the influence of specific hormones (aldosterone and vasopressin, respectively).

In the next part, I will briefly describe the general mechanisms leading to tubular reabsorption in each tubular segment, and give more details concerning the molecular mechanisms involved in glucose, calcium and uric acid reabsorption.

The proximal tubule is responsible of the reabsorption of the 70% of the ultrafiltrate produced in the glomerulus. The driving force for this massive reabsorption is represented by the active sodium transport carried by the Na⁺-K⁺-ATPase present in the basolateral pole of the cells. The efflux of Na+ out of the cell creates a sodium gradient between the apical urinary medium and the intracellular environment. This gradient is favorable to an entry of sodium in the tubular cell. The transport of sodium is coupled to the transport of several dissolved substances and performed by specific transporters, which act as cotransporters or counter-transporters (Brenner and Rector's The Kidney 2012). For example, the reabsorption of Na⁺ can be coupled with glucose (Na⁺/glucose cotransporter) or exchange with H^+ (Na⁺/H⁺ exchanger). Glucose is an important metabolite that is reabsorbed entirely in the proximal tubule via the glucose cotransporters SLC5A1 (SGLT1) and SLC5A2 (SGLT2), which present different affinity and stoichiometry for glucose. The low affinity Na⁺/glucose co-transporter SLC5A2, that reabsorbs one molecule of Na⁺ per molecule of glucose, is located in the S1 segment, where glucose concentration in the urine is high. The Na⁺/glucose high affinity transporter SLC5A1 is located in the straight S3 segment where only few molecules of glucose have still to be reabsorbed (Kanai et al., 1994) (Kamiyama et al., 2012). The reabsorption of Na⁺ via Na⁺/H⁺ exchangers (NHEs) leads to the secretion of an H⁺ in the tubular lumen. This process is important for the acid-base homeostasis, since NHEs are responsible for 70% of the reabsorption of HCO₃⁻ (bicarbonate) in the PT, with the help of a luminal and intracellular carbonic anhydrase. Once the HCO₃⁻ has been reabsorbed, it is exported in the bloodstream

through a basal sodium/bicarbonate cotransporter, whose defect causes proximal renal tubular acidosis (Hamm et al., 2015).

The gradient generated by the reabsorption of solutes allows the passive reabsorption of the water, whereas the reabsorption of small molecules physiologically filtered by the glomerulus requires a receptor-mediated endocytosis system (Christensen et al., 2009; Nielsen, 1994).

In the convoluted part of the proximal tubule, 60% of the Ca^{2+} filtered by the glomerulus is reabsorbed in a passive and paracellular way. The passage of the calcium from the proximal tubular lumen into the blood occurs through the tight junction, via the membrane proteins Claudins which form the channels for the passage of the ions. Claudin-2, Claudin-1, Claudin-10a and -12 are involved in cations reabsorption in proximal tubule. As an example, it has been shown that inactivation of *Claudin2* in mice leads to hypercalciuria (Alexander et al., 2013) (Fig 4A).

Finally, uric acid is mainly reabsorbed in the PT through the apical URAT1 transporter (encoded by *SLC22A12*) and exchanged with anions that return into the lumen. On the other hand, the anion transporters, OAT1 and OAT3, present on the basolateral membrane of the proximal tubule are involved in the secretion of urate in the urine. Mutations in *SLC22A12* gene result in hypouricemia, with high levels of urate in the urine (Enomoto et al., 2002) (reviewed by Hediger et al., 2005).

The ability of the kidney to concentrate or dilute urine depends mostly on the generation of a cortico-medullary gradient. The active Na^+ transport in the thick ascending limb of **Henle's loop**, from the lumen to the surrounding interstitium, contributes to the formation of a hypertonic medullary interstitium. Thanks to the closely associated capillary network, reabsorbed osmoles are gradually accumulated to the deepest part of the kidney, via a countercurrent multiplier system. Thick and thin limbs of Henle's loop are characterized by different properties concerning solutes and water permeability. In the TDL portion, the reabsorption is limited to water, whereas the solutes are retained in the urine (Kokko, 1970). This process increases the concentration of the urine that reaches its maximum around the tip of the loop in the medulla. On the opposite the TAL is almost impermeable to water, but permeable to solutes. The energy for the reabsorption of Na⁺ in the TAL is again provided by the basolateral Na⁺-K⁺-ATPase. In these tubular cells, Na⁺ reabsorption is provided by SLC12A1 (NKCC2) and K⁺ is recycled back to the lumen through the apical ROMK channel. Mutations in ROMK channel result in defective fluid and salt

reabsorption, with a secondary excretion of potassium in the distal portion and a consequent hypokalemia (Lorenz et al., 2002; Brenner and Rector's The Kidney 2012) (Reviewed in Zacchia et al., 2016). Uromodulin, a protein expressed in the TAL, regulates the activity of the SLC12A1 (NKCC2) transporter, through its amino-terminal phosphorylation (Mutig et al., 2011). Mutations in *UMOD* reduced urinary concentrating ability, probably due to a defective sodium reabsorption in the TAL (Bernascone et al., 2010; Hart et al., 2002).

The TAL is also involved in HCO_3^- reabsorption, that is Na^+ dependent ($Na^+-HCO_3^-$ cotransporter, NBCN1), and in the paracellular transport of divalent cations such as Ca^{2+} and Mg^{2+} . Similarly to the situation in the proximal tubule, the passage of Ca^{2+} and Mg^{2+} is permitted by the presence of claudins in the tight junction between the epithelial tubular cells. In this subsegment, Claudin-16 and Claudin-19 form a cationic pore for the passage of Ca^{2+} and Mg^{2+} . In human mutations in the genes encoding for these two proteins are associated to familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC), characterized by progressive renal Mg^{2+} and Ca^{2+} waste. In this pathology, the nephrocalcinosis is caused by the increased waste of calcium in the urine, resulting finally in a chronic kidney disease (Alexander et al., 2013; Hou et al., 2008; Brenner and Rector's The Kidney 2012) (Fig 4B).

The reabsorption of sodium, chloride and calcium in the **DCT** and **CNT** is driven by the high Na⁺-K⁺-ATPase activity which generates the electrochemical gradient at the basis of ions transport. Mutations in *FXYD2*, encoding for the γ -subunit of the Na⁺-K⁺-ATPase pump, are involved in calcium-magnesium reabsorption defects and patients with *FXYD2* mutation have autosomal dominant renal hypomagnesaemia with hypocalciuria (Meij et al., 2000) (reviewed in (Hoenderop and Bindels, 2005)) (Fig 4C). The SLC12A3 (NCC), a sodium-chloride symporter, is located at the apical membrane of the DCT and benefits from the sodium gradient to transport Na⁺ and Cl⁻ from the tubular fluid into these cells. Defects in this transporter are associated with Gitelman syndrome, characterized by hypokalemia, hypomagnesaemia and hypocalciuria (Simon et al., 1996). The apical side of DCT and CNT cells carries also the active trans-cellular transporters for Mg⁺ (TRPM6) and Ca²⁺ (TRPV5). In the epithelial cells, Ca²⁺ is extruded into the blood through the Na⁺-Ca²⁺-exchanger (NCX1). In a similar way, Mg⁺ passes from the cytoplasm of cells into the bloodstream. Patients carrying *TRPM6* mutations suffer from hypomagnesaemia associated to Mg⁺ reabsorption defect (Walder et al., 2002), whereas animal model defective for Ca²⁺

transporter is characterized by calcium waste associated to polyuria (Hoenderop et al., 2003) (Zacchia et al., 2016). In addition, the presence of a K^+ channels at the basolateral side (KCNJ in the DCT) mediated the passage of the potassium in the interstitum (Woda et al., 2003). Defects of these channels lead to salt waste and hyperkalemia (Lorenz et al., 2002; Yang et al., 2010)(reviewed in Zacchia M et al 2016).

In the collecting duct, several transporters and channels are present at the apical membrane of principal cells, and are mainly regulate by the action of hormones, such as aldosterone and vasopressin. These transporters and channels play an important role in the final regulation of urine volume and concentration (Muto, 2001). In particular, water reabsorption is directly regulated by the vasopressin via the water channel Aquaporin2 (AQP2). Vasopressin induces the activation of the protein kinase A (PKA) responsible for the phosphorylation of AQP2 in intracellular vesicles (Katsura et al., 1997; Nishimoto et al., 1999). When it is phosphorylated, AQP2 is exocytosed to the apical side of the tubular cells (Fushimi et al., 1997). PKA is also important for the phosphorylation of transcription factors that control AQP2 gene expression, such as CREB-P and c-Jun/c-Fos. The up or down regulation of AQP2 expression in the CD determines the amount of water reabsorption and the urine concentration. For example, in experimental rat models of bilateral ureteral obstruction (BUO), the expression of aquaporin channels, sodium and urea transporters were strongly decreased, leading to a marked polyuria (Frøkiaer et al., 1996; Li et al., 2003). On the contrary, in unilateral obstruction, even if the expression of the transporters was altered, secretion of sodium and water was not changed probably due to compensatory mechanisms by the controlateral kidney (Frøkiaer et al. 1996)(reviewed by Nielsen et al 2007) (Figure 4D).



Figure 4. Schematic representation of the principal transporters/channels and their distribution in the different nephron tubular segments. A Na⁺-K⁺-ATPase pump is present all along the tubular compartments on the basolateral side of the epithelial cells, in order to provide the energy for electrolyte transport. (A) In the PT, there is a massive reabsorption of sodium, due for example to an exchange with H⁺ or to a cotransport with the glucose by SGLUT1 (in the S3) and SGLT2 (in the S1). (B) In the TAL, the reabsorption of sodium, chloride and potassium is achieved via the NKCC2. Potassium is then recycled in the lumen by the ROMK channel. (A,B) In both PT and TAL, Ca²⁺ and Mg²⁺ are reabsorbed in a passive way. (C) In the DCT, NCC transporter is responsible of the reabsorption of the sodium and chloride, and the active transport of the Mg²⁺ is mediated via TRPM6 (D) In the CNT, the active transport of the Ca²⁺ is mediated through TRPV5 whereas the K⁺ is excreted via the ROMK channel. In the CD, aquaporin channels reabsorb water and determine urine concentration (Adapted from (Dimke et al., 2010)).

CHAPTER 2: KIDNEY DEVELOPMENT

Kidney development

In the adult kidney, the functional unit, the nephron, is composed of highly differentiated glomerular and tubular cells, whose spatial organization is very complex. During renal morphogenesis, tubular and glomerular progenitor cells activate progressively the expression of a peculiar subset of genes to perform their specific functions in adulthood. Such high level of complexity is achieved through the action of several signaling pathways and crosstalk controlled mostly at the transcriptional level during kidney development.

During development, embryonic kidneys derive from the intermediate mesoderm (IM), one of the three mesodermal layers, and form continuum along the rostro-caudal axis. In mammals, three successive structures will develop in a distinct temporal sequence. In the anterior part, the two first primitive renal structures are represented by the pronephros and the mesonephros, which are transient structures. In mouse and human, the pronephros is represented by a group of primitive renal tubules, barely detectable, whereas the mesonephros is formed by well developed tubules with a glomerulus that is connected to the nephric duct. The mesonephros is a functional filtration unit during early embryonic life but starts to degenerate in concomitance with the metanephros development (reviewed in (Dressler, 2006)). The formation of the definitive kidney, the metanephros, starts at embryonic day 10.5 (E10.5) in mouse and around 5weeks in human, and is initiated by an outgrowth of the Wollfian duct, the ureteric bud (UB), which invades the mass of the metanephric mesenchyme (MM) (Figure 5).

Metanephros development depends on the reciprocal heterotypical interactions between the UB and the MM. Signals from the MM induce UB growth and branching that will ultimately form the urinary collective system. In parallel, the UB secretes factors to induce mesenchyme aggregation around the UB tips and mesenchymal to epithelial transition (MET) to generate the renal epithelial compartment of the nephron. This process of reciprocal induction is repeated many times during development in order to form the complex structure of the mature kidney (reviewed in (Dressler, 1999)).



Figure 5. Schematic representation of the development of renal structures. Two primitive structures, the pronephros and the mesonephros represent transient structures in mammals, degenerate and are replaced by the definitive renal structure, the metanephros (adapted from (Dressler, 2009))

I. UB outgrowth and collecting duct system development.

The **outgrowth of the UB** from the nephric duct (ND) is initiated by the interaction between the *Glial-cell-line Derived Neurotrophic Factor* (GDNF) peptide secreted by MM and its receptor Ret, a member of the receptor tyrosine kinase super-family, expressed by the UB (reviewed by (Krause et al., 2015)). The activation of the GDNF-Ret signaling modulates the expression of different genes in the UB. As an example, this signaling induces Wnt11 secretion by the UB which is crucial to maintain *Gdnf* expression in the MM, in a positive feedback loop (Pepicelli et al., 1997). In addition, two transcription factors *Etv4* and *Etv5* localized in the UB tip are also activated by GDNF-Ret signaling. These two factors induce the expression of target genes involved in proliferation and elongation of the UB, such as *CxCr4*, *Myb* and *Mmp14* (Lu et al., 2009). Any dysfunctions in the pathways regulating the emergence of the UB may lead to kidney agenesis, due to the absence of UB outgrowth.

On the other hand, the emergence of a unique UB is controlled by specific crosstalk between genes expressed in the nephric duct and in the MM. Misregulations in these crosstalk may lead to ectopic UB outgrowth and surnumerary kidneys. During morphogenesis, Pax2/8 genes have several important roles in metanephric kidney. In addition to their involvement in the maintenance of *Gdnf* in the MM, they regulate the expression of the *Gata3* gene in the ND, that inhibits the expression of *Ret* (reviewed by (Sharma et al., 2015)). Gata3 inactivation in the nephric duct leads to the formation of ectopic buds. In this model, the emergence of ectopic buds is due to presence of Ret positive cells all along the nephric ducts that response to the mesenchymal GDNF (Grote et al., 2008). In addition to *Gata3*, different genes are involved in the emergence of a unique UB and the inhibition of the ectopic branching, such as Sprouty1, Gremlin1, Bmp4 and *Slit-ROBO2*. Activated by the GDNF-Ret signaling, Sprouty1 (Spry1), a receptor tyrosine kinase antagonist, inhibits in a negative feed-back loop the expression of *Ret* in the nephric duct and in the UB. This inhibition of *Ret* is necessary to induce normal branching morphogenesis. When Spryl is inactivated, mutant kidneys present supernumerary buds and defective branching (Basson et al., 2006). As another example, the interaction between the ligand SLIT2, expressed in the nephric duct, and its receptor ROBO2, expressed in the MM, has an essential role in the unique emergence of the UB, via the definition of the boundary of *Gdnf* expression in the mesenchyme (Figure 6). Mutant mice for the either

Slit2 or *Robo2* developed ectopic buds along the stalk of the nephric duct, due to the persistent expression of the *Gndf* in the anterior nephrogenic mesenchyme (Grieshammer et al., 2004).

The transcription factor HNF1beta plays a crucial role in the definition of a unique emergence of the UB. Unpublished results obtained in our laboratory have shown that embryos deficient for this transcription factor display numerous and abortive ectopic UB outgrowth, due to the misregulation of the expression of *Spry1* and a decrease in ROBO-Slit signaling. In addition the expression of *Pax2* in the ND and UB epithelium has been shown to be directly regulated by HNF1beta. The cooperation of these two genes is necessary for the normal morphogenesis of the collecting duct system. Double heterozygous mice $Hnf1b^{-/+} Pax2^{-/+}$ are characterized by the formation of ectopic UBs in the caudal portion of the nephric duct and a reduced number of branching events (Paces-Fessy et al., 2012).



Figure 6. Principal signaling pathways involved in the UB outgrowth. The UB outgrowth is the results of the interplay between inductive and inhibiting signals in the MM and nephric duct. *Gata3/Spry1, Bmp4* and *Slit-ROBO2* inhibit the ectopic branching and define the correct position of the UB, whereas Grem1 inhibits *Bmp4* and promote UB outgrowth. This set of signals defines the correct position of the UB formation and its interaction with the MM through the activation of the GDNF-Ret cross talk (adapted from (Massa, 2012))

II. UB branching morphogenesis

GDNF/Ret signaling is important to maintain UB branching during kidney morphogenesis and to define the identity and fate of UB tip cells. During development of the UB tree, *Ret*expressing cells continue to be part of the tips and participate to each round of new branching, while cells that do not express *Ret* will form the UB trunk. The elongation of the UB trunk via proliferation will form collecting duct, renal medulla and papilla (reviewed by (Costantini and Shakya, 2006)). In addition, other signaling pathways involved in the outgrowth of the UB participate also to the formation of each new tip during the branching process. In particular, Slit-ROBO2 signaling is important to determine the interaction between the tip and the surrounding mesenchyme. In metanephroi culture, over expression of *ROBO2* inhibits normal branching due to a reduction in the number of condensing cells around each UB tip (Ji et al., 2012).

Intricate signaling pathways and molecular cascades are involved in the branching morphogenesis. As an example, the transcription factors Sox8/9 control the expression of crucial genes downstream of Ret, such as *Ert4* and *Spry1*. Deletion of one copy of *Sox9* in *Sox8* deficient mice inhibits the branching, probably due to a defective GDNF/Ret signaling (Reginensi et al., 2011). The transcription factor Emx2 (Empty Spiracles2) is involved in the branching morphogenesis but do not control the emergence of the UB. In fact, in *Emx2* mutant mice the UB emerges from the nephric duct, invades the MM but does not branch (Miyamoto et al., 1997). An important role in the branching morphogenesis is played also by the Fgf (Fibroblast growth factor) signaling, whose components are detected in both the UB and MM. In particular *Fgf10* and *Fgf7* ligands are expressed by the MM and interact during branching morphogenesis with the *Fgfr2IIIb* receptor expressed in the UB tips. Inactivation of either ligands or receptors cause defective branching and renal hypoplasia (reviewed by (Blake and Rosenblum, 2014)) (Figure7).



Figure 7. Schematic representation of the pathways involved in the UB branching. During UB branching the GDNF/Ret signaling is activated in the UB tip (marked in red), whereas the expression of *Spry1* in the ureteric bud stalk inhibits Ret signaling to avoid ectopic branching. In the surrounding MM, Grem1 inhibits *Bmp4* and promotes UB branching and invasion of the MM. At the UB tip the receptor Fgfr2 interacts with its ligands *Fgf7* and *Fgf10* express in the MM. Emx2 and Sox8/9 control branching morphogenesis. Emx2 maintains the expression of *Ret* in the UB tip (adapted from (Massa, 2012)).

III. Nephron formation

Around each UB tip, the metanephric mensenchyme starts to condensate and to form the cap mesenchyme. These condensed cells express *Six2* and *Cited*, and represent the nephrogenic progenitor pool that will give rise to all epithelial component of the nephron. In the next step of nephrogenesis, cap cells can either maintain the expression of both *Cited1* and *Six2*, forming the self-renewing progenitor population, or lose the expression of *Cited1*, and become engaged in the epithelial differentiation (Self et al., 2006; Kobayashi et al., 2008).

The initial and crucial event in nephron formation is represented by the mesenchymal to epithelial transition in the MM. This transition is initiated by the UB via the secretion of Wnt9b, a ligand involved in Wnt signaling (Carroll et al., 2005). In response to the Wnt9 signal, condensed cells will start an epithelial transition, form pre-aggregates and stop to express *Cited1*. The induced preaggregates start to express *Wnt4*, another Wnt ligand, whose binding to its receptor will activate the canonical Wnt signaling. In this context, the stabilization of the b-catenin will induce the activation of crucial genes involved in epithelial differentiation, including *Lhx1* and *Fgf8* (Dressler, 1997) (Figure 8).



Figure 8. Mesenchymal to epithelial transition. Cap mesenchyme is composed of two populations of cells: self renewing progenitor $Six2^+Cited1^+$, which remain in the mesenchyme around the UB tip, and the $Six2^+Cited1^-$, which are committed in the epithelial differentiation. These cells form the pre-aggregate in which the expression of *Wnt4* drives nephron morphogenesis (adapted from (Massa, 2012))

The next step of epithelial nephron precursors is the renal vesicle. This precursor is a small structure composed of approximately 70 epithelial cells. These vesicle cells surround a lumen that will fuse with the lumen of the UB, forming a continuity between the lumen of the collecting duct system and the lumen of the future nephron. The renal vesicle is a polarized structure, characterized by the expression of different set of genes along a proximal-distal axis (Figure 10A). For example, cells close to the UB will start to express *Lhx1*, *Brn1* and molecules involved in the Notch signaling (the receptor Notch 2 and the ligands *Dll1* and *Jag1*), which are part of a genetic program involved in the formation of the future tubular compartment of the nephron. Complementary, cells forming the proximal pole of the vesicle are characterized by the expression of *Wt1*, a crucial gene for glomerular development (Georgas et al., 2009). In the proximal portion of the vesicle the transcription factors *Pax2* and *Wt1* are co-expressed until the comma-shaped body, collaborating in the same regulatory pathway to allow a correct glomerular development (Ryan et al., 1995).

i. Comma and S-shaped body development. The polarized expression of specific markers of the different nephron segments, tubules and glomerulus, in the nephron precursors is maintained along a proximal-distal axis during the successive steps of nephrogenesis. Through a complex set of cell proliferation/cell rearrangement, the vesicle will give rise to comma-shaped body and then to S-shaped body (Figure 9). During morphogenesis these nephron precursors are characterized by the formation of two invagination processes: the first one takes place during the transition between the vesicle and comma-shaped body and a second one during the transition between the comma and Sshaped body. The first slit, the vascular cleft will separate the proximal part from the distal part of the vesicle. This process is characterized by a deep morphological cellular rearrangement. Few cells, at the junction of these two parts, reorganize their cytoskeleton and move the nucleus on the apical side, creating a notch on the vesicle surface. This notch will be invaded by vessels that will give rise to the future glomerular tuft. The second slit appears on the opposite side, close to the UB, and is formed by the expansion of the future tubular compartment. These two invagination processes will give rise to the typical Sshaped body, a nephron precursor characterized by the presence of three distinct subsegments, i.e. the distal, mid and proximal limbs, that will form different portion of the mature nephron (Saxén and Sariola, 1987). The distal portion will give rise to the distal tubule; the mid limb corresponds to the future Henle's loop and the proximal tubule and

the proximal limb will form the glomerular cup. At the S-shaped body stage, podocytes plate starts to invaginate and to form a cup that wraps the developing capillary network. At this stage, the position of the prospective urinary pole is contiguous to the prospective vascular pole, represented here by the rim of the cup (Figure 9).



Figure 9. Schematic representation of the transition between vesicle and S-shaped body nephron precursor stages. During nephrogenesis, the nephron precursors undergo two invagination processes. The first process occurs during the transition between the vesicle and comma-shaped body to form the vascular cleft. The second invagination process occurs during the transition between the comma and the S-shaped body. At the S-shaped body the prospective urinary pole is contiguous to the prospective vascular pole, represented by the rim of the podocytes cup. (Adapted from chapter 277 "Cellular and Molecular Biology of the Kidney", Harrison's Principles of Internal Medicine, 18e, 2012)

Recent analyses have characterized the molecular program expressed in the distinct segments of the S-shaped body and a set of genes that are selectively expressed in each limb of this nephron precursor (Figure 10C) (Brunskill et al., 2008). For example, the distal and medial portions express *Lhx1* (Karavanov et al., 1998; Kobayashi et al., 2005) and *Brn1* (Nakai et al., 2003). On the other hand, the transcription factors *Irx1*-3 (Nichane et al., 2006; Reggiani et al., 2007), important regulators of the intermediate tubule fate (Nichane et al., 2006), and ligands of the Notch pathway, required for the proximal tubule development (Cheng et al., 2007), are restricted to the mid limb. Interestingly, the expression of all these molecules is regulated by the transcription factor HNF1beta, a crucial player in the differentiation of the future tubular component. Its inactivation in the *Six2*⁺ cap mesenchyme cells or in *Wnt4* expressing cells in the pretubular aggregate leads

to defective morphology of the S-shaped body probably due to a concomitant downregulation of *Irx1-2* and the Notch ligands *Dll1* and *Jagged1* (Heliot et al., 2013; Massa et al., 2013). During normal development, the transcription factor *Pax2* continues to be express in the distal portion of the S-shaped body, but its expression is reduced in the proximal portion, where podocytes will be formed (Kreidberg et al., 1993) (Figure 9). A complex reciprocal control between these two genes depending on their spatial and temporal expression pattern has been shown: Pax2 activates the *Wt1* promoter, whereas Wt1 represses *Pax2* expression. As the renal differentiation proceeds, the expression of Pax2 is progressively downregulated in the glomerular structures.



Figure 10. Schematic representation of nephron precursors and gene pathways involved in nephrogenesis. During early nephrogenesis, the expression pattern of several genes is polarized along a proximal-distal axis (A) In the vesicle, the proximal part expresses Wt1 that starts to inhibit the expression of Pax2 whereas, the distal portion expresses Lhx1 and Notch ligands, represented by Dll1 and Jag1. (B) In the comma-shaped body, the proximal portion is committed to form the glomerular portion of the nephron. On the other side, in the distal portion the expression of genes specific for the diverse tubular segment starts to be delineated. (C) In the S-shaped body there are three different limbs: the distal limb that will form the distal tubule, the mid limb corresponding to Henle's loop and proximal tubule, and the proximal limb that will form the glomerulus. Each limb expresses already a specific set of genes (Adapted from (Desgrange and Cereghini, 2015)).

ii. <u>Precapillary-loop precursor</u>. The transition from the S-shaped body to the typical configuration of the precapillary-loop precursor is characterized by the expansion of the tubular compartment. In addition, the podocyte plate undergoes a progressive invagination process followed by a complex reorganization that will position the urinary pole on the external side of the glomerular cup (Potter, 1965; Saxén and Sariola, 1987). The mechanism at the basis of these latter structural changes is still unclear. I will describe in details the morphological processes occurring during the formation of the glomerulus, in the first part of my project.

At precapillary-loop stage, the elongating tubules give rise to the mature distal and proximal tubules connected by the Henle's loop. In parallel, cells that will differentiate into podocytes and Bowman's capsular cells will form the mature blood filtration unit, the glomerulus (Rak-Raszewska et al., 2015) (Pietilä and Vainio, 2014) (Boyle et al., 2014) (Figure 11). During this final step of nephron maturation, some embryonic pathways are downregulated, being progressively replaced by the genetic program leading to mature epithelial cells. As an example, the expression of Pax2 is gradually reduced in the maturing nephron precursors, until becoming restricted to the collecting duct. Down regulation of Pax2 is important to allow the terminal differentiation of the nephron. Indeed, its aberrant expression has been reported in several renal tumors in both human and rat, suggesting a state of undifferentiation or dedifferentiation of the epithelial cells (Davies et al., 2004).



Figure 11. Schematic representation of the transition between the S-shaped body and the mature glomerulus. At the precapillary loop stage the prospective urinary pole (UP) is close to the prospective vascular pole (VP). The transition between the capillary loop stage and the mature glomerulus is represented by the relative position of the urinary pole on the external side of the glomerular cup. (Adapted from chapter 277 "Cellular and Molecular Biology of the Kidney", Harrison's Principles of Internal Medicine, 18e, 2012)

iii. <u>The development of the glomerulus.</u> During the transition of the S-shaped body to mature nephron, important morphological processes will participate to the formation of the glomerulus. The early glomerulus in the more proximal portion of the S-shaped body is composed by the podocyte precursor plate (a thick columnar epithelium), the layer of Bowman capsule cells and the glomerular tuft, itself composed of glomerular capillaries and mesangial cells.

- **Podocytes.** During glomerulogenesis, podocyte precursors change their morphology. These changes are characterized by the expansion of the apical surface and the progressive movement of the initially apical junction belt towards the basal side. In the meantime, once the prospective vascular pole is colonized by capillaries, podocytes migrate and surround the developing capillary loops. At the end of this process, podocytes have developed large cytoplasmic projections, called foot processes that extend completely around the developing capillary loops (Schell et al., 2014). The normal podocyte development requires the expression of Wt1, a transcription four zinc fingers factor that can bind DNA and RNA. The high expression of Wt1 in mature podocytes that this factor could also play a role in the maintenance of normal podocyte functions throughout life. Wt1 controls the expression of key genes for podocyte function and specification, in particular:

- expression of *Nephrin* and *Podocalyxin*, involved in the organization of the specialized junctions of the slit diaphragm and podocyte foot processes;

- expression of actin cytoskeleton proteins, as *Synpo*, involved in podocyte polarity and cytoskeleton arrangement;

- expression of proteins involved in the cell-matrix adhesion of podocytes to the glomerular basement membrane (GBM) (reviewed by (Dong et al., 2015)).

Moreover, Wt1 is also necessary to maintain the paracrine and intercellular signaling between podocytes and mesangial cells and to regulate the expression of *Vascular Endothelial Growth Factor A (VegfA)* during glomerular development (Schell et al., 2014) (Gao et al., 2005) (Figure 12A). In mouse, podocyte-specific *Wt1* knock-out leads to defects in podocyte differentiation, whereas its inactivation later in already developed podocytes promotes foot processes effacement, proteinuria and glomerulosclerosis. In human, *WT1* mutations lead to glomerulopathies characterized by alteration in podocyte development or maintenance (reviewed by (Dong et al., 2015)). Mutations of *WT1* are also associated with diffuse mesangial sclerosis (DMS), characterized by increased deposition of extracellular matrix (ECM) in the vascular side of the GBM. In this pathology, the

defective expression of *WT1* and its target genes leads to an aberrant communication between podocytes, mesangial and endothelial cells that may control the deposition of ECM and the correct assembly of the glomerular tuft (reviewed by (Quaggin and Kreidberg, 2008)).

Another important transcription factor involved in the development and maintenance of mature podocytes is Tcf21 (Pod1). This transcription factor controls the expression of several key angiogenic factors, including *Vegfa*, necessary for the formation of the glomerular tuft. Mouse model of *Tcf21*-specific knock-out in podocyte showed retardation in glomerular maturation that results in simplified glomeruli with a decreased number of endothelial and mesangial cells. Around 50% of the mutant mice developed proteinuria and lesions similar to focal segmental glomerlosclerosis (FSGS) (Maezawa et al., 2014).

- Capillaries and mesangial cells. During glomerulogenesis, the secretion of VEGFA by podocytes precursors attracts angioblasts from the interstitium. This induces the formation of the capillaries by endothelial cells that invade the vascular cleft. Later on, VEGFA induces also the remodeling of the endothelial cells in capillary loop that becomes fenestrated (Eremina and Quaggin, 2004) (Figure 12B). Model of inactivation of Vegfa results in defective development of the filtration barrier and reduction of endothelial cells in the glomeruli (Eremina et al., 2003). Another gene involved in capillary loop formation is *Crim1*, a transmembrane cystein-rich repeat-containing protein. This gene is expressed in podocytes, mesangial cells, parietal cells and vascular smooth muscle, and regulates the delivery of VEGFA from podocytes to endothelial cells. This protein sequesters growth factors on the surface of the cells once they are secreted, in order to control the rate of maturation and the effective concentration gradient. Its mutation during kidney development leads to an abnormal VEGFA delivery to glomerular endothelial cells and results in increased activation of the VEGFA signaling pathway in these cells. This conditions leads to enlarged capillary loop, podocyte effacement and mesangiolysis (Wilkinson et al., 2007).

The second important event during glomerular maturation is the secretion of the PDGFB by podocytes that recruits mesangial cells via the activation of PDGFB/Pdgfrb pathway. In the mature glomerulus, mesangial cells are in contact with the endothelial cells in the glomerular tuft, and play a crucial role to maintain the capillary loop structure (Figure 12C). During glomerulogenesis, these cells derived from the stromal mesenchyme as

specialized vascular smooth muscle cells. They are activated by PDGFB and invade the developing glomerulus where they attach to the forming blood vessels. Normal structure and function of glomerulus depend on the interactions between endothelial cells, podocytes and mesangial cells (Lindahl et al., 1998) (Krause et al., 2015). The mature glomerulus is finally composed by mesangial cells at the basis of the capillary tuft that is enveloped by the GBM and the podocytes (Figure 12D).



Figure 12. Development of the glomerular endothelial and mesangial cells. (A) Podocytes (in blue) produce VEGF-A to attract endothelial cells (EC, in red), and PDGF-B to recruit mesangial progenitor cells (MC, in violet). (B) The endothelial cells organize the structure of capillaries whereas (C) the mesangial cells support endothelial cells to form the capillary loop. (D) Mature glomerulus: mature podocytes and basal membrane surrounded the fenestrated endothelium of capillaries, which are supported by mesangial cells (Adapted from (Schell et al., 2014))

- <u>Glomerular filtration barrier</u>. The filtration barrier is a multilayered structure that includes the fenestrated endothelium of the capillaries, the GBM and the slit-diaphragm, between podocyte foot processes. During development, capillaries and podocytes fuse their basal lamina in order to form a unique GBM. At the same time, specialized junctions

between the foot processes composed the slit diaphragm that connects podocytes one to each other. (Quaggin and Kreidberg, 2008) (Schell et al., 2014).

- **Bowman capsule.** The glomerular tuft is enveloped by the parietal epithelial cells (PECs) lying on the Bowman capsule. The PECs are characterized by a small and flat cell body that lines the urinary space of the glomerulus. PECs and podocytes derived from the same sub-compartment in nephron precursors and share a common phenotype until their mature differentiation at the precapillary loop stage. At this stage, in concomitance with the migration of endothelial and mesangial cells in the vascular cleft, podocytes start to express *Nephrin* and *Podocalyxin*, whereas the parietal cells are characterized by the expression of *Pax2* and *Claudin*-1.

In the adult kidney different subpopulations of PECs can be distinguished on the basis of the markers they express and on their localization in the glomerulus (Figure 13). For example, at the vascular stalk, a few cells called transitional cells are localized between podocytes and PECs. These transitional cells have phenotypical characteristics and marker expression common to both cell types. On the other hand, rare cells lining the Bowman's capsule express podocyte markers and are able to form foot processes. These cells are called ectopic or parietal podocytes (reviewed by (Shankland et al., 2014)).

In human, during glomerular disease, PECs can acquire an activated phenotype, characterized by increased proliferation, migration and matrix deposition. They are identified by the expression of CD44, a specific marker of aPECs, which is absent in other glomerular resident cells. CD44 is a typical marker of mononuclear cells, such as macrophages and monocytes (Fatima et al., 2012). As an example, during crescentic glomerulopathy, some PECs acquire a proliferating capacity and produce cellular crescents in Bowman's space. Activated PECs represent the predominant cellular type in this pathological multilayered neoformation, even if podocytes and macrophages have been identified as possible players (Bariéty et al., 2001, 2005). In glomerulosclerosis, the presence of adhesion between the Bowman capsule and the sclerotic lesions in the tuft facilitate the migration of aPECs into the capillary tuft, where they continue to deposit extracellular matrix leading to a global endocapillary glomerulosclerosis.

Seen the common genetic program shared by podocytes and PEC during renal development before their terminal differentiation, it has been hypothesized that PEC could trans-differentiate into podocytes under pathological situations and play a role as adult
podocyte stem or progenitor cells. In human adult glomeruli, subpopulations of cells expressing renal progenitor markers (CD24 and glycCD133) and podocyte markers have been identified at the urinary pole and/or close to the vascular pole. Moreover, in human pathologies characterized by a reduction of the number of podocytes, there is an increase of cells expressing both podocyte and PEC markers, supporting the hypothesis of a regenerative role played by this last subpopulation. However, several observations do not support this paradigm. First, there is no evidence that cells expressing podocyte markers during glomerular diseases acquire also the morphological and functional characteristics of mature podocytes. Second, cell-fate tracking studies have demonstrated that some parietal cells can move into the glomerular tuft to form functional podocytes, suggesting the presence of a pool of cells lining the Bowman capsule still committed to form podocytes. Nevertheless, this phenomenon is present only during glomerular development in new born mice. Turnover of podocytes or recruitment of PECs in the capillary tuft in adult mice, in ageing or after induction of compensatory glomerular hypertrophy have never being observed (reviewed by (Shankland et al., 2014)). Altogether, these studies suggest that PEC form a heterogeneous population, with some characteristics of podocyte progenitors, but their functional relevance in pathological conditions remains to be demonstrated.



Figure 13. Representation of the different cell populations in glomerulus. In mature glomerulus different PEC population are distinguished on the basis of expression of specific markers: **podocyte committed progenitors** expressing CD133⁺ CD24⁺PODXL⁺ line the Bowman capsule close to vascular pole, whereas **transitional cells** with an intermediate phenotype are present at the vascular pole between Bowman capsule and podocytes. However some podocytes could be observed lining Bowman capsule close to the vascular pole and are indicated as **parietal/ectopic podocytes**. Resident multipotent cells, which could give rise to parietal or tubular cells, line the Bowman capsule close to the urinary pole, express CD133⁺ CD24⁺, but are PODXL⁻, and are indicated as adult **parietal epithelial multipotent progenitors (APEMP)**. **aPEC** (in red) are PEC activated during glomerular disease with a different expression pattern and an increased capacity to proliferate and migrate (adapted from (Shankland et al., 2014))

During aberrant renal morphogenesis, some malformations include the formation of the **glomerular cysts**, characterized by a dilated Bowman capsule (Figure 14). This phenotypic trait is usually associated with other hereditary cystic disease, such as medullary cystic kidney disease, polycystic kidney disease, tuberous sclerosis complex, and have also been observed in patients carrying *HNF1B* mutations (Table 1) (Lennerz et al., 2010). The mechanisms at the basis of the glomerular cyst formation are not still clear. Some studies have suggested a potential role of the primary cilium in the pathogenesis of this cystic phenotype. This proposition was essentially based on the fact that most of the proteins encoded by the different genes mutated in glomerular cyst are normally part of this organelle. Interestingly, most of the glomerular cystic phenotypes develop during embryonic life. This observation suggests that an alteration of the morphogenetic process of the glomerular development could be at the basis of cyst formation (Bissler, 2010)



Figure 14. Schematic morphologic spectrum of glomerular cysts. Illustration of progressive changes in glomerular cysts development from left to right. Glomerular cysts can be spherical, oval or polygonal. The tuft is present on one side of the Bowman capsule either as separated dysplastic tuft (a,b,c,d) or as a flatted tuft-remnant (e,g) (Adapted from (Lennerz et al., 2010)).

Human disease	Mutated gene	Protein		
Autosomal dominant PKD	PKD1-2	Polycystin-1 Polycystin-2	ystin-1 ystin-2	
Autosomal recessive PKD	PKHD1	Fibrocystin		
MODY5 (RCAD)	HNF1B	Hepatocyte nuclear factor-1β		
Autosomal dominant GKCD	UMOD	Uromodulin		
PKDTS (Polycystic kidney disease and Tuberous sclerosis)	TSC2	Tuberin	in 3	
RHPD (renal hepatic pancreatic dysplasia)	NPHP3	Nephrocystin 3		
Urinary tract abnormalities			Obstructive GCK	
Ischemic GCK Or Drug-induced GCK			Sporadic GCK	

Table 1. Cystic kidney disease associated to glomerular cysts

(Adapted from (Lennerz et al., 2010)

iv. <u>Tubular development</u>. At the urinary pole, the Bowman capsule cells of the glomerulus are directly connected to the tubular portion of the nephron.

- Tubular expansion. Tubular expansion starts in the distal and mid limb of the S-shape body (SSB). The distal part of SSB (distal limb and distal part of the mid limb) is characterized by the expression of *Brn1*, a POU-domain transcription factor, which plays an essential role in the formation of the Henle's loop (HL), distal convoluted tubule (DCT) and the macula densa. It has been shown that mutant mice for Brn1 present a defect in the HL elongation and a decreased expression of typical genes of this subsegment, such as *Umod* and *Nkcc2* encoding a transporter essential for the reabsorption of NaCl in the TAL of the nephron (Nakai et al., 2003). The mid limb of the SSB is characterized by the expression of the Notch ligands Dll1 and Jag1(Georgas et al., 2008). Activation of Notch pathway starts with the binding of one of the ligands to Notch receptors. This binding induces a first cleavage by a metalloprotease ADAM10 (van Tetering et al., 2009). The cleaved receptor is then internalized and undergoes a second cleavage by the γ secretase enzyme, which releases the Notch intracellar domain (NICD) (De Strooper et al., 1999). The NICD is then ready to translocate into the nucleus and activate its relative target genes. Analysis of mouse mutant for Notch2 and Notch1 has shown that Notch2 plays a predominant role in proximal fate specification whereas Notch1 is more dispensable, although it participates to nephrogenesis (Cheng et al., 2007; Surendran et al., 2010). For example, in Notch2-deficient kidney, specific markers of proximal tubules, such as cadherin6, are not expressed, attesting a defective acquisition of the proximal tubular identity (Cheng et al., 2007). The Notch ligands, Dll1 and Jag1, expressed in the mid limb, have also an important role during proximal specification. The analysis of different models of compound mutant for *Dll1* and *Jag1* demonstrated that *Dll1* deletion in presence of *Jag1* leads to a mild defect in proximal tubules development, whereas the absence of Jag1 expression, even in presence of Dll1, leads to a more drastic defect. These results supported the predominant role for Jag1 in the proximal nephron specification (Liu et al., 2013).

- <u>Tubular elongation</u>. Tubular elongation is a particularly organized process that depends on the balance between convergent extension (CE) and oriented cellular division (OCD) in respect of the planar cell polarity (PCP) of the tissue (Goodrich and Strutt, 2011). The relation between these two events will determine the diameter and the length of the tubular compartment of the nephron. During embryogenesis, tubular elongation predominantly occurs through CE movements in which cells intercalate perpendicularly to the longitudinal axis with neighboring cells in the tubular wall in order to establish its diameter (Karner et al., 2009; Lienkamp et al., 2012). When tubular diameter is defined, cells can start tubular elongation through an OCD program without significant modification in the tubular diameter (Fischer et al., 2006) (Figure 15).



Figure 15. Mechanism of tubular elongation. Tubular diameter is determinate by an initial convergent extension (**b**,**c**). Once the diameter is defined cell division oriented along the proximal/distal axis allows tubular elongation without changing the diameter (**d**,**e**). (adapted from (Nigro et al., 2015)).

Tubular elongation is driven, at least partially, through the action of Wnt9 produced in the UB and collecting duct. This secreted molecule participates to the regulation of the planar cell polarity (PCP), by defining the mitotic spindle orientation and, as consequence, the orientation of the tubular cell division. Although this molecule acts initially during early morphogenesis through the b-catenin (canonical) pathway, Wnt9b regulates the tubular development predominantly through a non-canonical pathway that activates the Rho-GTPases and Jun kinase later during development (Karner et al., 2009). The switch between these two pathways has been suggested to be determined by the primary cilia, located at the apical side of the tubular cells. The primary cilium is composed by an axoneme of nine microtubules doublets. At the basis of the axoneme there is the basal body, composed by two centrioles. The primary cilium is organized via complex intraflagellar transport machinery and its basal body is connected with the cytoskeleton. This connection may allow the transmission of the movement of the primary cilium to intracellular signals (mechanotransduction) (Figure 16).



Figure 16. Primary cilium in renal tubular cells. (A) Primary cilium is located n the apical side of the epithelial cells in contact with the fluid flow. **(B)** Organization of the cystoproteins along the axoneme and the basal body of the cilium. All this proteins are involved in cystic kidney disease: PC-1 and PC-2, inversin, cystin, polaris (adapted from (Guay-Woodford, 2003; Zhang et al., 2004))

It has been hypothesized that tubular flow could represent one of the signals that control cellular orientation of the division. Inhibition of switch between canonical b-catenin signaling and non-canonical Wnt pathway induces increased proliferation and affects PCP (Simons et al., 2005). Planar cell polarity defect during tubular elongation interferes with normal tubule diameter and leads to tubular dilation. This pathological condition has been associated with the formation of tubular cysts, as it happens in human polycystic kidney disease (PKD) (Fischer et al., 2006; Karner et al., 2009; Luyten et al., 2010; Saburi et al., 2008)(Figure17). Many genes, whose mutations are involved in PKD, encode for proteins localized in the primary cilium, such as *PKD1* and *PKD2*, encoding for polycystin-1 (PC-1) and polycystin-2 (PC-2) (Table 2). Patients carrying heterozygous mutations in these genes develop an autosomal dominant PKD (ADPKD). In mouse models, germ line homozygous inactivation of *Pkd1* or *Pkd2* is lethal during embryogenesis, whereas cysts start to form around 13.5 (Lu et al., 1997; Wu et al., 2000). In Pkd1 deficient animals, cyst

derived mostly from tubular portion (Lu et al., 1997), whereas in the *Pkd2-/-* model glomerular cysts are predominant compare to tubular cysts (Wu et al., 2000).

Pkd1-/- mice are characterized by an increase in canonical Wnt signaling via an overexpression of *Wnt7b*, normally expressed during kidney development, and de novo expression of *Wnt7a*, normally absent in renal tubules. In addition an over-expression of *Pax2* has been observed in these mutant animals, possibly through a positive regulation via Wnt signaling (Qin et al., 2012). *Pax2* is normally expressed during nephrogenesis in the embryos, and it is turned off during kidney maturation, with the exception of a residual expression in the collecting duct. Several studies have shown that the level of *Pax2* expression is critical during cystogenesis. Genetic studies in mouse have shown that the deletion of one allele of *Pax2* in a context of *Pkd1* deficiency reduces cyst formation, due to an increase in apoptosis and a decreased proliferation. In addition, *Pax2* overexpression by itself induces cyst formation (Stayner et al., 2006; Ostrom et al., 2000). These studies highlighted the fact that genes expressed during tubular development but normally downregulated in the mature kidney are upregulated in cystic epithelium. Altogether, these results suggest that impaired tubular epithelial differentiation is observed in PKD, and could play a role in the formation or expansion of tubular cysts.



Figure 17. Schematic representation of orientation of cell division in normal tubule *versus* defective oriented cell division in PKD. During normal development, oriented cell division in tubular cells allows the elongation of the tubule whereas disrupted planar cell polarity, as in polycystic kidney disease, leads to enlarged and short tubule. (Adapted from (McNeill, 2009))

Protein	Gene	Protein localization	Disease	Renal abnormalities
Polycystin1 (PC-1) Polycystin2 (PC-2)	PKD1, PKD2	Cilia, adherens and focal adhesions	Autosomal dominant PKD	Renal cysts, enlarged kidneys
Fibrocystin	PKHD1	Cilia and secreted	Autosomal recessive PKD	Renal cysts
Nephrocystin- 1,2 /inversin	NPHP1-2	Cilia, basal bodies and focal adhesions	Nephronophthis is	Renal fibrosis, renal cysts, tubular atrophy
BBS1-12	BBS1-12	Centrosomes and basal bodies	Bardet-Biedl syndrome (BBS)	Renal cysts
MKS1, meckelin, nephrocystin3,	MKS1, MKS3, NPHP3	Centrosome, cilia, plasma membrane	Meckel-Gruber syndrome (MKS)	Renal cysts
Uromodulin	UMOD	Cilia, basal bodies, centrosomes, secreted	Medullary cystic kidney disease (MCKD)	Small fibrotic kidney, medullary cyst

Table 2. Genes mutated in Human cystic kidney disease

Adapted from (Gascue et al., 2011)

- Apicobasal cell polarity. Epithelial cells have an apicobasal polarity defined as the cellular orientation along the vertical axis in respect to the matrix to which they are attached. In addition, some epithelial cells are also polarized according to the plan of the tissue, perpendicularly to the apicobasal axis. This polarity is called planar cell polarity (PCP). The acquisition of an apicobasal cell polarity is a fundamental hallmark during renal tubule development and maturation. The distribution of proteins along the cell membrane can be different during embryogenesis compared to mature tubules. In mature tubules, some proteins are localized in an opposite cellular position in respect to apicobasal polarity during development; some others have already reached their final position early during renal morphogenesis. The apical and basal cellular membranes express different set of proteins, which define specific functions of each side of the cell. These different sets of proteins are physical separated by the presence of specific complex of junctions at the lateral membrane: adherens junctions define the membrane asymmetry of tubular epithelial cells and are associated with tight junctions, controlling the paracellular permeability across the epithelia, in the apical junction complex (AJC) (Hartsock and Nelson, 2008). In addition, focal adhesions are formed at the basal membrane to connect the tubular cells to the underlying extracellular matrix. These junctions determine where different signaling and structural proteins, including PC-1, PC-2, fibrocystin and nephrocystins-1and -2, are activated/recruited. At adherent and focal adhesions, proteins are in contact with intracellular cytoskeleton elements and act in signaling pathways that control cell proliferation, cell survival, spreading and migration, whereas proteins localized in tight junctions control paracellular permeability in the renal epithelia. In particular, at the adherens junctions E-cadherins form a complex of proteins that includes PC-1, P C-2 and nephrocystin-2, and is involved in the activation of the canonical Wnt pathway. Renal cysts are the results of increased proliferation, luminal secretion of fluid and deposition of abnormal extracellular matrix. Interestingly, in ADPKD, E-cadherin is substituted by the embryonic isoform N-cadherin (Roitbak et al., 2004). In addition to this cadherin switch and the consequence disruption of the interaction with b-catenin and other actin-binding proteins, mutations in PC1 or PC2, in ADPKD, and in NPHP1 or NPHP2, in nephronophthisis, are at the basis of structural modifications of these proteins present at the cellular junctions. This leads to the consequent disruption of membrane junctions and a defect in the signaling pathways involved in the cellular polarity (Figure 18). In different mouse models of PKD, it has been observed that the apicobasal polarity was altered with a defective activation of the related signaling pathways (Table 3) (reviewed by (Wilson,

2011)). In PKD, the limit between the apical and basal membranes is lost. This defect induces an impaired sub-localization of cellular membrane proteins. For instance, in PKD, EGF receptor, involved in proliferative program and normally expressed at the basolateral membrane of mature tubular cells, is localized at the apical membrane (Du and Wilson, 1995). This receptor can therefore be activated by the EGF secreted in the lumen by tubular cells. The EGF activated signal promotes cellular proliferation, participating to the high proliferative level in cyst-lining epithelia (Sweeney and Avner 1998). In the same way, the misposition of the Na-K-ATPase pump on the apical side of cystic cell promotes Na secretion in the lumen with a consequent fluid secretion and expansion of the cyst (Wilson et al., 1991). The cell-cell junction proteins, whose abnormal expression is at the basis of the altered cell polarity, are important to maintain the separation between the apical and basal membrane and also to avoid the inappropriate intracellular passage of fluid and ions (reviewed by Wilson and Goilav, 2007).



Figure 18. Cellular junction organization in normal renal tubular cells and in ADPKD. (A) In normal renal tubular epithelium, PC-1 is collocated at the desmosomal (DJ) and adherens (AJ) junctions with E-cadherin. The AJ complex provides a linkage to the actin cytoskeleton, and DJ links together intermediate filaments (IF) of epithelial cells. PC-1 is also present at the focal adhesion complex, which connect the epithelial cell with is basal membrane. (B) In ADPKD, mutated PC-1 is located in cytoplasmatic vesicle and DJ and AJ are disrupted, whereas E-cadherin at the AJ is substituted by N-cadherin. (Adapted from (Ibraghimov-Beskrovnaya and Bukanov, 2008))

Gene	Protein	Mouse model	Phenotype	
Pkd1 Pkd2	Polycystin-1 Polycystin-2	Knock out (Lu et al., 1997; Wu et al., 2000)	Homozygous embryonic lethality	
		<u>Conditional</u> Tissue specific inactivation e.g. (Kim et al., 2009; Shibazaki et al., 2008)	Rapidly progressive cystic phenotype	
		<u>Inducible</u> Tissue specific inactivation can be induce by tamoxifen, interferon or doxycycline administration e.g. (Lantinga-van Leeuwen et al., 2007) (Kim et al., 2009)	The severity of disease depends on the age of inactivation and the extent of the inactivation	
Pkhd1	Fibrocystin	Knock out (Williams et al., 2008; Woollard et al., 2007)	Renal cyst formation in adulthood	
Hnf1b	HNF1beta	<i>Ksp</i> -Cre (Gresh et al 2004) <i>Inducible Mx</i> -Cre (Verdeguer et al 2010)	Renal cyst	
Nphp2 (Invs)	Inversin	<i>Inv</i> mouse (Mochizuki et al., 1998; Phillips et al., 2004)	Nephronophthisis	
Nphp3	Nephrocystin-3	<i>Pcy</i> mouse (Takahashi et al., 1986)	Nephronophthisis, Meckel- Gruber syndrome	
Bicc1	Bicaudal C	<i>Bpk</i> mouse (Nauta et al., 1993) <i>Jcpk</i> mouse (Flaherty et al., 1995)	Renal cystic dysplasia	

Table 3. Mouse model of polycystic kidney disease

Cysl	Cystin	<i>Cpk</i> mouse (Ricker et al., 2000)	Autosomal recessive PKD
Nek8/NPHP9	Nek8	Jck mouse (Atala et al., 1993)	Nephronophthisis
Tg737	Polaris	<i>Orpk</i> mouse (Moyer et al., 1994)	Cystic kidney disease

(Adapted from (Happé and Peters, 2014))

CHAPTER 3: HNF1 TRANSCRIPTION FAMILY AND MODY SYNDROMES

I. HNF1 Transcription Factor Family

During organogenesis, cell differentiation is progressively achieved by a complex set of events that result in specific patterns of gene expression in time and space. This set of events is mainly regulated at the transcriptional level. Transcription factors are distributed in a specific way in all tissues of the organism and can modulate the expression of genes in a specific cell type through their interaction or with other co-factors. The activation of the genes occurs by the binding of these proteins to specific short sequence in the promoter of the genes, or to more distal cis-acting elements, in the response to developmental and environmental stimuli.

The Hepatocyte Nuclear Factors 1 (HNF1s) family is a small homeoprotein family composed of two transcription factors, HNF1alpha and HNF1beta. HNF1alpha was first identified as a transcriptional regulator of gene expression in the liver (Courtois et al., 1988), and is encoded by a gene of 10 exons, whose locus is in the chromosome 12. *HNF1B* was discovered in a second time as its variant gene in the chromosome 17, composed of 9 exons. (Baumhueter et al., 1988). These genes share a high percentage of homology, suggesting that they could derive from the duplication of a common ancestor gene. These two factors bind to the same consensus DNA sequence (5'-GTTAATNATTAAC-3') as homo or heterodimers (Rey-Campos et al., 1991; Bach, 1992). Each of the two transcription factors is composed of three different functional domains (Figure19):

- 1. The dimerization domain is located at the N-terminal part. An α helical structure mediates the formation of a coiled coil structure between the monomers (Nicosia et al., 1990). This domain presents a homology of 72% between the two transcription factors.
- The DNA binding domain is composed by a homeodomain and POU domain, a 3 alpha helices structure. Due to the high level of homology in this region (91% of identity), the two proteins recognize the same DNA consensus sequence (Mendel et al., 1991) (Tomei et al., 1992).
- 3. The transactivation domain is located at the C-terminal part and is responsible for the interaction with co-regulators. In this region the two genes share only 47% of homology (Ingraham et al., 1988; Mendel et al., 1991; Sourdive et al., 1993).



Figure 19. Schematic representation of the transcription factor HNF1alpha and HNF1beta. The two transcription factors share high level of homology in the dimerization domain (72%) and DNA binding domain (92%), whereas they are diversified in the trans activation domain (47% of homology).

II. HNF1 Mutations in Human

HNF1A, a closely related gene to *HNF1B*, has been identified as a disease gene in a Maturity Onset Diabetes of the Young syndrome (MODY3) in 1996 (Yamagata et al., 1996). *HNF1B* was therefore an obvious candidate, and the first report of MODY associated disease with *HNF1B* (MODY5) was done in a Japanese family in 1997 (Horikawa et al., 1997). Interestingly, non diabetic renal diseases have been identified in all members of this family, and successively in other families with MODY5, leading to the term of renal cyst and diabetes (Bingham et al., 2001a). Since these initial descriptions, several studies have clarified that the most penetrating phenotypic trait in patients carrying *HNF1B* mutations is the presence of renal abnormalities, and diabetes is identified in only 50% of patients carrying *HNF1B* mutations. In addition, patient carrying *HNF1B* mutations usually present a large spectrum of extra renal phenotypic abnormalities. One other characteristic of the phenotypic alterations linked to *HNF1B* mutations is the high variability of the severity of the disease, even within the same family.

i. <u>MODY syndrome</u>

MODY syndrome is a monogenic form of non-insulin-dependent diabetes mellitus that appears early in life and is inherited in an autosomal dominant manner. This pathology is classified in different subtypes on the basis of the mutated genes involved in β -cells development and functions. MODY1, MODY2 and MODY3 (*HNF1A*) represent the 80% of all MODY cases, whereas, MODY5 (*HNF1B*) is a less common subtype (McDonald and Ellard, 2013) (Table 4).

Туре	Gene Name	Locus	Function	Primary Defect
MODY 1	Hepatocyte nuclear factor 4α (HNF4 α)	20q	Transcription factor	Pancreas
MODY 2	Glucokinase (GCK)	7p15-p13	Hexokinase IV	Pancreas/liver
MODY 3	Hepatocyte nuclear factor 1α (HNF1α)	12q24.2	Transcription factor (Homeodomain)	Pancreas/Kidney
MODY 4	Insulin promoter factor-1 (IPF1)	13q12.1	Transcription factor (Homeodomain)	Pancreas
MODY 5	Hepatocyte nuclear factor 1β (HNF1β)	17q12	Transcription factor (Homeodomain)	Kidney/Pancreas
MODY 6	Neurogenic differentiation 1 (NEUROD1)	2q	Transcription factor (bHLH)	Pancreas
MODY 7	Kruppel-like factor 11 (KLF11)	2p25	Transforming Growth Factor-Beta-Inducible- early growth response 2	Pancreas
MODY 8	Bile salt dependent lipase (CELL)	9q34.3	Pancreatic exocrine dysfunction	Pancreas
MODY 9	Paired Domain gene 4 (PAX4)	7q32	Transcription factor	Pancreas
MODY 10	Insulin (INS)	11p15.5	Beta cells of the Langerhans islets	NF-kappa-B
MODY 11	Tyrosine kinase B- Lymphocyte specific (BLK)	8p23-p22	Tyrosine kinase	Pancreatic β-cells
MODY 13	potassium channel, inwardly rectifying, subfamily j, member 11 (KCNJ11)	11p15	Potassium channel in pancreatic B-cells	Pancreas
MODY 14	Adaptor Protein, Phosphotyrosine Interaction, Ph Domain, And Leucine Zipper- Containing Protein 1 (APPL1)	3p14	serine/threonine kinase	Pancreas

Table 4. Description of Maturity Onset Diabetes of the Young (MODY) syndromes.

II. MODY3 and MODY5 syndrome

MODY3 is linked to heterozygous mutations of HNF1A gene and represents the most common subtype of diabetes with a young age of onset. Mutations of the dimerization/binding domain lead to an earlier onset of the disease compared to mutations in the transactivating domain (Timsit et al. 2016). HNF1A has an important role in development and function of pancreatic β -cells. This transcription factor regulates the expression of proteins involved in glucose transport, glucose metabolism and key enzymes in mitochondrial metabolism of glucose (McDonald and Ellard, 2013) (Timsit et al., 2016). In a model of *Hnf1a* inactivation, deficient islets present a decreased expression of genes involved in the glucose metabolism and a reduced glucose-stimulated insulin secretion, whereas an increased expression level of genes involved in glycolysis and gluconeogenesis was observed in the liver (Servitja et al., 2009). These global defects in gene expression result in hyperglycemia that in patients progresses rapidly from impaired glucose intolerance to overt diabetes. In most of the MODY3 cases, diabetes is diagnosed between 15 to 45 years of age. The high variability of the phenotype observed in MODY3 patients suggested the presence of modifiers genes that may control the onset of appearance of the disease (Bellanné-Chantelot et al., 2011). This hypothesis was recently confirmed in a mouse model study in which a single genetic locus suppresses diabetes in Hnfladeficiency (Garcia-Gonzalez et al., 2016).

Mutations in the *HNF1B* gene represent around 5% of the MODY, and diabetes is identified in only 50% of patients carrying *HNF1B* mutations. In some **MODY5** patients, it has been suggested that diabetes mellitus could result from a combined insulin secretion defect and insulin resistance. In these families, patients present hyperinsulinemia that could be a consequence of the combination of persistent insulin secretion in β -cells reserve, resistance to endogenous glucose, and reduced insulin clearance, principally performed by liver and kidney (Pearson et al., 2004). In MODY5 patients, diabetes mellitus is usually diagnosed before 25 years of age and mostly needs to be treated with insulin therapy (Chen et al., 2010). Nevertheless, in these patients, diabetes severity is variable, ranging from normal glucose tolerance at age 35 years to insulin-treated diabetes presenting with ketoacidosis (Bingham and Hattersley, 2004). In addition to endocrine pancreatic defects, some MODY5 patients also present abnormalities in the exocrine part of the pancreas, characterized by pancreas atrophy or hypoplasia, calcification and cysts (Chen et al., 2010).

III. Renal defects correlated to MODY3 and MODY5/HNF1B mutations

i. <u>Tubular dysfunction in MODY3 patients</u>

MODY3 patients present impaired proximal tubular functions, characterized by aminoaciduria, glucosuria and phosphaturia, that represent typical features of renal Fanconi syndrome. Glucosuria has been attributed to a reduced expression of *SLC5a2*, encoding a Na⁺/glucose cotransporter protein. Normally expressed in the S1 and S2 segments of the proximal convoluted tubule, this transporter is responsible of the reabsorption of the vast majority of the glucose present in the ultrafiltrate. Moreover, *Hnf1a* inactivation in mouse affects the expression of phosphate transporters *Npt1* and *Npt4*. The direct regulation of these transporters by HNF1alpha could explain the resulting phosphaturia (Cheret et al., 2002). In addition, in mutant *Hnf1a* animals some cotransporters involved in amino acids reabsorption are not properly expressed. This down-regulation could explain aminoaciduria in human, even if it has been suggested that this abnormality could be linked to the high level of glucose in the urine that leads to the dissipation of the electric gradient necessary to amino acid reabsorption (Bingham et al., 2001b). Moreover, in the proximal tubules of kidney in *Hnf1a-/-* mouse model, there is a defective expression of transporters responsible of reabsorption of bile acids (Shih et al., 2001).

ii. <u>Renal manifestations in patients carrying *HNF1B* mutations.</u>

As previously mentioned, the most frequent phenotypic trait in patients with *HNF1B* mutations is the presence of renal manifestations, mostly including the presence of renal cysts. On the other side, *HNF1B* mutations are frequently identified in various renal manifestations. *HNF1B* mutations represent the most frequent genetic cause of renal malformations in children (Heidet et al., 2010; Ulinski et al., 2006), and new algorithms have now proposed a *HNF1B* mutations screening in patients presenting with renal malformation (Faguer et al., 2014).

One of the characteristics of the renal involvement in MODY5 patients is its extreme heterogeneity. In this context, a large spectrum of renal abnormalities has been described, ranging from few cysts with tubular dysfunction to severe developmental pathologies during embryonic life (Table 5). Furthermore, an important phenotypic variability can be observed even between patients carrying the same mutations.

HNF1B mutations. Genetic modifications include base substitutions and small insertions and deletions in around 50% of adult and pediatric patients. Analysis of the nature of these mutations in a large cohort of patients identified the presence of two hot spot: the first in the exon 2 and the second in the exon 4, while mutations are sporadically distributed in the exon3. Mutations in the portion between 1-4 exons, encoding for the dimerization domain and DNA binding domain affect highly conserved amino acid residues(Bellanné-Chantelot et al., 2005; Chen et al., 2010; Edghill et al., 2006), and could therefore resulting in impaired ability to form homo/hetero dimers and to bind DNA specific sequence. Genetic alterations identified in the other half of patients are represented by deletions of the entire gene. Interestingly, in 50% of MODY5 patients, HNF1B mutations occur de novo in the absence of any family history. Genomic segmental duplications that surround the *HNF1B* gene may promote the unequal crossing over of this portion and explain the high instability of the locus (Bellanné-Chantelot et al. 2004; Carette et al. 2007). Despite the description of hot spots, and the identification of large genomic deletions, no direct correlation between the type of mutations and the severity of the disease has been observed. In particular, whole gene deletion was not demonstrated to give rise to a different phenotype compare to point mutations (Nakayama et al., 2010).

- <u>Clinical presentation</u>. In pediatric cohorts, *HNF1B* mutations represent the most frequent known genetic cause for Congenital Abnormalities of the Kidney and the Urinary Tract (CAKUT). CAKUT occurs in 1 out of 500 live births and is the most common cause of renal failure in childhood. Renal malformations associated to *HNF1B* mutations include renal agenesis, renal hypoplasia, and renal multicystic dysplasia, with the formation of glomerular cyst. These abnormalities are usually syndromic with a family history, but can also derive from de novo mutation or involve only the kidney or the urinary tract (Song and Yosypiv, 2011). Hypoplastic glomerulocystic kidney disease (GCKD), an autosomal dominant disorder has also been associated with *HNF1B* mutations. GCKD patients have small kidney, cortical cystic glomeruli with dilated Bowman capsule and primitive glomerular tuft, and abnormal calyces and papillae(Bernstein, 1993; Bingham et al., 2001a).

Renal manifestations in MODY5 children and fetuses are mostly characterized by the presence of hyperechogenic-enlarged kidney (Bingham and Hattersley, 2004). Two major phenotypes have been observed by ultrasounds in MODY5 fetuses: a bilateral abnormality in kidneys with or without cortical microcysts, or a unilateral affected kidney with diffuse

macrocysts and a large normal controlateral kidney (Decramer et al., 2007; Heidet et al., 2010; Ulinski et al., 2006). Progression to end stage renal disease (ESRD) seems to be rare in pediatric patients. In two large series of 27 and 71 patients, only three patients progressed to ESRD during the follow up period (Decramer et al., 2007; Heidet et al., 2010).

Mutations in *HNF1B* have been identified in 25% of in a large cohort of adult patients with renal cysts and unexplained renal disease (Edghill et al., 2006, 2008). Histological characteristics in patients with the same clinical presentation are represented by hypertrophy of the proximal and distal tubules, associated with focal areas of interstitial fibrosis and the presence of enlarged glomeruli (Sagen et al., 2003). Successively, several studies of large cohorts have confirmed these characteristics. In adult patients, nonspecific interstitial fibrosis and oligomeganephronia (reduced number of nephrons and enlarged glomeruli) were observed in hypoplastic kidneys. These histological abnormalities were associated with bland urinalysis in 75% of patients, low prevalence of hypertension and slowly progressive renal failure. All these clinical and biological manifestations are part of the typical presentation of tubulointerstitial nephritis. Renal function impairment in patients carrying *HNF1B* mutations can be deeply variable, even if a slow decline of renal function is the usual presentation. Faguer and colleagues have shown in their cohort of 27 patients that 4 patients progressed rapidly to end stage renal disease (ESRD) during a median period of follow up of 5.5 years. In the other patients, the median yearly estimated Glomerular Filtration Rate (eGFR) decline was low. Interestingly, in some cases the presence of sudden and severe glomerular filtration decline suggests an impaired repair after acute renal injury (Faguer et al., 2011).

In addition to these morphological abnormalities, tubular dysfunctions are frequently observed in children and adult patients carrying *HNF1B* mutations. In cohorts of children with *HNF1B* heterozygous mutation, hypomagnesaemia with hypermagnesuria and hypocalciuria are frequently reported (Heidet et al., 2010; van der Made et al., 2015). In these patients, urinary magnesium wasting has been linked to a distal convoluted tubule defect. It has been shown that HNF1beta directly controls *FXYD2* that encodes the γ subunit of the Na+-K+-ATPase, a transporter which represents the driving force responsible for ions transport in the distal tubule. Mutations of this subunit reduce the intracellular K+ and/or increase the intracellular Na+, inducing depolarization which finally leads to magnesium waste (Adalat et al., 2009).

In contrast to pediatric cohort, in adult patients, hypomagnesaemia and hypocalciuria are associated with hypokalemia. In this case, the potassium depletion depends on the fact that the potassium channel (ROMK) in the thick ascending limb is modulated by magnesium depletion (Yang et al., 2010) (Faguer et al., 2011).

Since HNF1beta represses the expression of the transporter *URAT1* in the proximal tubule, involved in the reabsorption of the uric acid, hypouricemia was expected in patients carrying *HNF1B* mutations. On the contrary, these patients were characterized by hyperuricemia. It has been previously reported that a decrease in uric acid excretion was associated to *UMOD* mutations, encoding uromodulin (Bingham et al., 2003; Heidet et al., 2010). Interestingly, *Umod* has been shown to be a direct target of HNF1beta in mouse (Gresh et al., 2004). Similarly, a down-regulation of *UMOD* could occur in *HNF1B* patients, which in this case could explain the tubular dysfunction observed in human.

The wide spectrum of phenotypes observed in patients carrying *HNF1B* mutations suggests a precise role of this transcription factor at different timing and in different compartments during kidney morphogenesis. The complete landscape of the molecular and cellular mechanisms leading to these renal abnormalities has not been yet completely elucidated.

iii. Other clinical manifestations observed in MODY3 and MODY5 patients

In MODY3 and MODY5 patients, some patients present liver abnormalities. Hepatic adenomas have been associated with biallelic mutations of *HNF1A*. In MODY5 patients, increased levels of liver enzymes represent the most common alteration in the liver function, although isolated cases of cholestasis with reduced number of bile ducts have been described in neonatal patients (Beckers et al., 2007; Bellanné-Chantelot et al., 2004). Others clinical manifestations in MODY5 patients include genital tract abnormalities, particularly in female, and neuropsychiatric alterations such as autism spectrum. This latter phenotypic trait has been described in patients with microdeletions in chromosome 17q12, but not with mutations restricted to the *HNF1B* gene itself (Bellanné-Chantelot et al., 2004; Clissold et al., 2016; Faguer et al., 2011).

Table 5. Summary of the clinical, biological and imaging characteristics, and family history of patients with HNF1B mutations

		Frequency (%)		
Symptoms	Specificity	Antenatal period	Children	Adult
Family history Antenatal renal abnormalities	Low Low	50-60	40-50 >80	50-60
Renal involvement Hyperechogenic kidneys Hypoplastic kidneys Renal cysts Solitary kidney Multicystic and dysplastic kidney Urinary tract malformation Hypomagnesaemia Hypokalemia Hyperuricemia and gout with early onset Glomerular cysts, oligomeganephronia	Intermediate Low Intermediate Low Low High Low Intermediate High	45 10 <10 10 15 15-20 	$ \begin{array}{r} 70\\ 10-20\\ 25-30\\ <10\\ 19\\ 10-15\\ 45\\\\ 5\\ \end{array} $	$\overline{30}$ 60 20 $\overline{<10}$ 60 45 <10
Pancreas involvement MODY-type diabetes mellitus Pancreas hypoplasia and/or exocrine pancreas insufficiency Liver involvement Liver test abnormalities	Intermediate High Low		<10	30-40 40-85
<i>Genital tract abnormalities</i> Female Male	High Intermediate	_	<10	45 10-30

"The data were pooled from published series, and concerned around 300 patients in total" (Faguer et al., 2014)

CHAPTER 4:

HNF1alpha AND HNF1beta EXPRESSION PATTERN

HNF1 alpha AND HNF1beta EXPRESSION PATTERN:

The HNF1 family of transcription factors was initially identified as homeoproteins that activate the expression of liver specific genes. Further characterization of the expression profile of *Hnf1a* and *Hnf1b* has shown that they are both expressed in epithelial cells of several organs, such as kidney, pancreas and intestine. In addition, *Hnf1b* is also expressed in lung.

These two transcription factors usually present an overlapping expression pattern in these different organs, with few exceptions: in liver, *Hnf1a* is expressed in hepatocytes, whereas *Hnf1b* is mainly restricted to the bile ducts. In the kidney, both transcription factors are expressed in tubular epithelial cells, but the expression pattern of *Hnf1b* is broader than the one of *Hnf1a*. HNF1alpha is restricted to proximal tubules, whereas *Hnf1b* is expressed in all cells of the tubular compartment and in Bowman capsule's cells. In addition, the timing of expression of these two transcription factors is also different, *Hnf1b* being turned on much earlier than *Hnf1a* during embryogenesis. This suggests that HNF1beta could play a role in the differentiation of polarized epithelia whereas HNF1alpha could be involved in the maintenance and specification of the tissues (Blumenfeld et al., 1991) (Lazzaro et al., 1992) (Cereghini et al., 1992).

I. HNF1alpha and HNF1beta during embryogenesis

Animals with a germline inactivation of *Hnf1a* survive only few weeks after birth (Pontoglio et al., 1996). Conversely, germline mutation for *Hnf1b* leads to a severe disorganization of all embryonic and extraembryonic tissues, already during the first days of embryonic life, and is responsible of an embryonic lethality around E6.5 (Embryonic day) (Barbacci et al., 1999; Coffinier et al., 1999a).

To follow the expression of those two genes during embryogenesis and in the adult animals, mouse models carrying a *LacZ* cassette under the control of the regulatory elements of the endogenous gene *Hnf1a* and *Hnf1b* have been generated (Coffinier et al., 1999a). The expression pattern of the β galactosidase, encoded by the *LacZ* cassette has been shown to be very similar to the one of the endogenous genes. Analysis of heterozygous *Hnf1a/LacZ* animals has shown that this gene starts to be expressed during embryonic development around E10.5 in the primordial liver, intestine, pancreas and stomach. In kidney *Hnf1a* starts to be expressed in nephron precursors around E13.5 (Blumenfeld et al., 1991). The analysis of β galactosidase expression in *Hnf1b* heterozygous embryos has shown that *Hnf1b* starts to be expressed in the extraembryonic visceral endoderm as early as E4.5 (Cereghini et al., 1992; Coffinier et al., 1999a). During organogenesis of both endodermal and mesodermal derivatives, *Hnf1b* is turned on in the epithelial cells of future tubular structures. *Hnf1b* starts to be expressed in the 2 somites stage in the epithelium of the forming gut and in the neural tube at E8, with a strong expression in the liver and pancreas primordia at E9.5. Later on, it starts to be expressed in the gallbladder and pancreatic buds (Coffinier et al., 1999b), and in pronephric duct.

In order to study the role of HNF1beta during organogenesis, and to circumvent the early lethality linked to its germline inactivation, chimera tetraploid morulae aggregation strategy has been used. In this model, the extraembryonic tissues are wild-type and develop normally, whereas the cellular component of the embryo proper derives from *Hnf1b* deficient ES cells. As an alternative strategy to circumvent the embryonic lethality, mice carrying a *Hnf1b* floxed allele (Coffinier et al., 2002), have been generated, allowing conditional inactivation via a Cre-LoxP strategy (Abremski and Hoess, 1984). I will briefly describe some of the results obtained with these strategies in different organs.

II. HNF1alpha and HNF1beta in liver, pancreas and intestine

Analysis of tetraploid/diploid compounds experiments showed that the absence of *Hnf1b* in embryonic tissues results in a drastic defect of liver and pancreas differentiation (Table 6). In these mutant embryos, which survive until E14.5-15.5, the ventral foregut endoderm differentiation is severely compromised and the liver bud does not form, due to the lack of expression of a set of specific transcription factors at the origin of hepatic differentiation (Lokmane et al., 2008). In addition, pancreatic morphogenesis is drastically affected, due to a defective pancreatic fate acquisition during embryonic development (Haumaitre et al., 2005). Using Cre-LoxP strategies, we and other groups have shown that HNF1beta plays also a role in subsequent processes during liver and pancreatic morphogenesis. Inactivation of *Hnf1b* in liver after the early steps of differentiation, specifically in hepatoblasts by using an *Alfp*-Cre mouse strain (Kellendonk et al., 2000), leads to a severe defect in intrahepatic biliary ducts development and metabolic abnormalities (Coffinier et al., 2002). *Hnf1b* early deletion during pancreatic morphogenesis has highlighted the crucial role of this transcription factor in multipotent pancreatic progenitor cells expansion, and the

generation of endocrine precursors, via a regulation of the Notch pathway. In addition, these *Hnf1b* deficient embryos also present an impaired pancreatic duct morphogenesis, with the presence of cysts (De Vas et al., 2015) (Table 6). Contrary to the important role of HNF1beta during liver and pancreas development, the lack of *Hnf1a* does not lead to a defective hepatic and pancreatic morphogenesis. Mutant mice for *Hnf1a* survive a few months after birth and display a strong growth retardation phenotype due to a wasting syndrome leading to cachexia. The lack of *Hnf1a* results in liver enlargement with steatosis and a severe hyperphenylalaninemia, due to the absence of Phenylalalnine hydroxylase, an enzyme whose expression is under the direct control of HNF1alpha. In addition to these liver defects, null *Hnf1a* animals suffer from diabetes, due to a defective glucose sensing leading to an abnormal insulin secretion, and kidney dysfunctions, that I will describe in the following paragraph (Pontoglio et al., 1996, 2000) (Table 6).

In intestine, single inactivation of *Hnf1a* or specific inactivation of *Hnf1b* in adult animals does not lead to severe defects. Nevertheless, the combination of germline inactivation of *Hnf1a* associated to a intestinal specific inactivation of *Hnf1b* (driven by the *Vil*-CreER^{T2} (El Marjou et al., 2004)) leads to an impaired terminal differentiation of the secretory and absorptive lineage, due to a defective Notch signaling (D'Angelo et al. 2010) (Table 6).

Organ	Hnfla inactivation		Hnf1b inactivation	
	Malformations	Model	Malformations	Model
Liver	Hepatomegaly Hyperphenylalanine mia	- Germline inactivation (Pontoglio et al., 1996)	Drastic defect of liver specification	Tetraploid compound (Lokmane et al., 2008) Epiblast inactivation (unpublished)
			Defects of biliary duct	Alfp Cre (Coffinier et al., 2002)
Pancreas	Diabetes = insulin secretion defect	Germline inactivation (Pontoglio et al., 1998)	Pancreas agenesis	Tetraploid compound (Haumaitre et al., 2005)
	No phenotype	Germline inactivation (D'Angelo et al., 2010)	No phenotype	Villin Cre inactivation (D'Angelo et al., 2010)
Intestine	impaired terminal differentiation of the secretory and absorptive lineage	Germline inactivation associated to Villin Cre inactivation of Hnf1b (D'Angelo et al., 2010)	impaired terminal differentiation of the secretory and absorptive lineage	Germline inactivation associated to Villin Cre inactivation of Hnf1b (D'Angelo et al., 2010)

Table 6. Models of inactivation of *Hnf1a* and *Hnf1b* in mouse

(Adapted from (Massa 2012))

III. HNF1alpha and HNF1beta in kidney

In mouse, kidney development starts around E8, with the formation of the nephrogenic cord, which will give rise to the nephric duct, an epithelial structure, and to the nephrogenic blastema. The pronephros is formed by a unique primitive tubule connected to the nephric duct. Later on, the caudal part of the nephric duct elongates, in parallel with the migration of the nephric blastema. Induction of the mesonephric blastema by the nephric duct will give rise to the mesonephric tubules. These two primitive kidneys, that are transient in mammals, represent the functional kidney in other species. For example, the pronephros is functional in the amphibious tadpole and in the fish larvae, and the mesonephros is the definitive kidney for adult fishes and frogs. The study of these primitive kidneys has been very useful to identify some crucial genes and signaling networks. Indeed, several studies have highlighted the conservation of the molecular mechanisms in kidney development through evolution (reviewed by (Drummond and Davidson, 2010)).

Hnf1a expression during renal morphogenesis starts in the future proximal tubular cells at the S shaped body stage, a precise step of nephron precursors (Lazzaro et al., 1992). In adult animals, its expression is restricted to the proximal tubule compartment (Blumenfeld et al., 1991). Hnf1a is dispensable for the morphogenesis of the nephron, but its inactivation leads to severe functional defects mimicking the human renal Fanconi syndrome (Pontoglio et al., 1996). In the absence of this transcription factor, the massive reabsorption of low molecular weight proteins, glucose, phosphates and amino acids is impaired. This defect is due to the decrease of expression of specific transporters involved in glucose, aminoacid and phosphate reabsorption.

During kidney morphogenesis, in xenopus larvae, *hnf1b* starts to be expressed in the pronephros, and is particularly enriched in its distal portion (Raciti et al., 2008). Similarly, *hnf1b* is expressed in the pronephric tubules of the zebrafish. Interestingly, in this species, two *hnf1b* paralogues, *hnf1ba and hnf1bb*, are present in the genome, with a slightly different expression pattern: *hnf1bb* is expressed early in proximal and distal tubules, but its expression decreases later on in distal tubules, whereas *hnf1ba* expression persists in all tubular compartments (Naylor et al., 2013). In mammals, HNF1beta is already observed in the mesonephros, both in the nephric duct and the mesonephric tubules. In the metanephros, it is expressed in the ureteric bud as soon as it emerges from the nephric

duct, and its expression is maintained in the collecting duct system. In the metanephric blastema, *Hnf1b* is not expressed in the uninduced mesenchyme, but it is turned on in the first epithelial derivatives, in renal vesicles. In the adult kidney, it is expressed in all tubular epithelial cells and in Bowman's capsule cells, but is absent from the podocytes, the vascular and the mesangial cells of the glomerulus.

i. <u>HNF1beta in kidney early development</u>

To circumvent the early lethality observed in germline inactivation of *Hnf1b*, and to study the role of HNF1beta during kidney organogenesis, different strategies have been used (Table 7). Using an epiblast specific inactivation of *Hnf1b* (Mox2cre) or tetraploid and diploid chimera models, it has been shown that the lack of HNF1beta leads to severe defects in early steps of kidney morphogenesis. In this context, the emergence of the ureteric bud occurs normally but its further development is severely compromised. Only few branches form, characterized at the molecular level by a decreased expression of Pax2 and Wnt11 and a delayed expression of Ret. In addition, metanephric blastema cells do not correctly condense around the few UB tips, and mesenchymal to epithelial transition was not observed. This latter phenotype was due to the defective expression of Wnt9b by the UB, which is directly regulated by HNF1beta (Lokmane et al., 2010) (Massa 2012). In addition to this metanephric phenotype, mesonephric tubules are absent, and mesonephric duct display an abnormal tortuous morphology (Lokmane et al., 2010). Moreover, Hnflb inactivation in the epiblast results in the outgrowth of extranumerary buds along the nephric duct and in the ureteric bud. This defect is due to the downregulation of genes involved in the control of the unique and specific position of the ureteric bud, such as Sprouty1 that has a highly conserved binding site for HNF1beta (Massa 2012).

ii. <u>HNF1beta in tubular and glomerular specification</u>

The lack of HNF1beta in the ureteric bud prevents the formation of epithelial derivatives from the metanephric blastema. Mutations of HNF1B in children and fetuses are associated with dysplastic kidney suggesting a role of this transcription factor during nephron development. To elucidate this potential role, two different mouse models have been used, based on the selective inactivation of Hnf1b in the metanephric mesenchyme. In this

context, the UB is wild type, and able to send signals to the MM to perform the MET. Mouse strains carrying a Cre recombinase under the control of the promoter of the *Six2* gene (Kobayashi et al., 2008) or a *Wnt4* EGFPCre strain (Shan et al., 2010) have been crossed with *Hnf1bflox/flox* mice (Coffinier et al., 2002). The Cre recombinases are expressed only in the cap condensate cells or in early vesicle, respectively, and thus, will efficiently inactivate *Hnf1b* in all mesenchymal derived structures but not in the UB (Heliot et al., 2013; Massa et al., 2013).

Mutant mice died 2 days after birth and had smaller kidney compared to the control. These mutant kidneys are characterized by a severe reduction of all the tubular components. This defect was associated to a strong downregulation of the expression of genes specific for tubular subsegments, such as Nkcc2, Parvalbumin and Villin1. In order to understand the origin of this phenotype, early nephron precursors have been analyzed. In mutant mice, vesicle and comma did not present any morphological abnormalities and were normally polarized, whereas the S-shape bodies were drastically misshaped. Normally this precursor is composed of three domains: a distal limb, a mid limb and a proximal limb. A subsegment of the midlimb, called "bulge" is particularly enriched in *Hnflb* expression. This bulge of epithelial cells was absent in mutant mice. At the molecular level, the expression of a set of markers, normally observed in developing tubular structures, was strongly downregulated in this mutant precursor. As an example, Cadherin6, a gene expressed in early stages during nephrogenesis, was expressed in mutant vesicle and comma-shaped body but started to be downregulated at the S-shaped body stage. On the other side, some genes such as *Pax2* and *Lhx1*, whose expression is normally downregulated during tubular maturation, were overexpressed in mutant. Moreover, Sshaped bodies structures were characterized by an increased apoptosis in the distal and mid limbs (Heliot et al., 2013; Massa et al., 2013). In order to decipher the molecular mechanisms at the basis of this drastic tubular phenotype, the components of the Notch pathway have been analyzed. Indeed, the role of the Notch pathway in proximal tubular fate acquisition has already being illustrated in previous studies. Inactivation of *Hnf1b* in nephron precursor cells leads to a drastic reduction of *Dll1*, a ligand of Notch receptors, and Hes5, an effector gene activated by Notch signaling. This result suggests that HNF1beta plays a crucial role in tubular specification and expansion (Heliot et al., 2013; Massa et al., 2013), through the activation of the Notch pathway.

In addition to the strong tubular phenotype, mutant nephrons also develop glomerular cysts characterized by a dilated urinary space and an extended tuft connected to the collecting

67

duct via a short and primitive tubule. This phenotype reproduces the histological characteristics of *HNF1B* patients that present drastic reduction of proximal tubules and glomerular cysts (Massa et al., 2013). The cellular and molecular mechanisms at the basis of glomerular cyst formation are still unclear. The aim of my thesis was to elucidate part of these mechanisms, and I will describe in details the results I have obtained in the result part 1.

Interestingly, the role played by HNF1beta in tubular specification is conserved across the species. In xenopus larvae, the use of a Dominant Negative (DN) of HNF1beta interferes with normal pronephros development (Heliot et al., 2013). The C-terminal part of the protein was missing in the DN which was therefore unable to interact with coactivators (Barbacci et al., 2004). In the mutant pronephros, markers of the proximal and intermediate tubular segments, such as *Pax2* and *Nkcc2*, respectively were strongly downregulated. Moreover, the Notch signaling pathway was affected, as attempted by the decrease in *Dll1* expression, demonstrating that in this model, HNF1beta also acts through the Notch signaling to determine the proximal intermediate fate of pronephros (Heliot et al., 2013). In Zebrafish the intermediate and distal portion of the pronephros express high levels of *Hnflba* and *Hnflbb*, whereas it is excluded from the most anterior part of the pronephros that will form the prospective glomerulus. When Hnflba and Hnflbb are inactivated, all tubular segments are missing and the territory of Wt1, a glomerular marker, is extended ectopically in the region that normally forms the proximal tubule. Therefore, these last results suggest that HNF1beta restricts the podocyte fate and determines the region of proximal tubule development by defining the boundary of *Wt1* expression (Naylor et al., 2013).

iii. <u>HNF1beta in tubular expansion</u>

MODY5/*HNF1B* patients suffer from diverse renal manifestations, but one of the most penetrant phenotypic traits is the presence of renal cysts. Nevertheless, the molecular mechanism of this phenotype is still poorly understood.

Specific inactivation of *Hnf1b* has been carried out in already formed tubules with the use of a mouse strain expressing the Cre recombinase under the control of the kidney specific cadherin (*Ksp cadherin, Cadherin 16*) promoter (Shao et al., 2002). Using this strategy, inactivation of *Hnf1b* was obtained mostly in the distal part of nephron, in Henle's loop,

distal tubule and collecting ducts. Only a few (around 5%) of proximal tubules are deleted for *Hnf1b*. Mutant mice died few days after birth, due to the presence of massive formation of cysts in the medulla. Blood analysis has shown that mutant mice suffer from a severe renal failure, with a strong increase in serum urea and creatinine levels. Molecular analyses have shown that HNF1beta directly controls the expression of a group of genes: *Umod*, *Pkhd1*, *Pkd2*, *Nphp1* and *Tgf737/Polaris*. In human and mouse models, mutations or inactivation of most of these genes are responsible for polycystic kidney phenotypes. Altogether these results show that HNF1beta controls a critical network of genes essential for tubulogenesis (Gresh et al., 2004). In addition, Ma and coll have demonstrated that HNF1beta is able to directly repress *Socs3* expression. SOCS3 has been described to inhibit tubulogenesis induced by different growth factors through the decreased phosphorylation of ERK and STAT3. We could speculate that the overexpression of *Socs3* linked to the absence of HNF1beta could alter tubule formation and lead to defective kidney development.

As an alternative to the conditional inactivation of *Hnf1b*, Hiesberger and colleagues have use transgenic animals carrying a dominant negative form of HNF1beta to compromise the function of the endogenous transcription factor (Hiesberger et al., 2004). As mentioned before, the transcription factor HNF1beta is composed of three different domains: the dimerization domain at the N-terminal part, the DNA-binding domain and the transactivation domain at the C-terminal domain. Transgenic mice expressed mutant truncated HNF1beta proteins due to mutations already observed in MODY5 patients. These mutated proteins have lost their transactivating properties, due to the lack of the Cterminal part of the protein (Bingham et al., 2001a). The dominant negative (DN) proteins were expressed under the control of the *Ksp-(cadherin16)* promoter, which is specifically expressed in all the tubules (Shao et al., 2002). Transgenic mice develop kidney cysts, involving both tubules and glomeruli, surrounded by interstitial fibrosis (Hiesberger et al., 2004). In these animals, the expression of the DN-HNF1beta directly inhibited the expression of Pkhd1, a gene mutated in Autosomal Recessive Polycystic Kidney Disease (ARPKD). ARPKD is a monogenic disease characterized by the formation of cysts mainly in the collecting ducts. In addition, the analysis of transgenic animals carrying the Cterminal part deficient DN of HNF1beta helped to elucidate the molecular mechanism involved in the regulation of Pkhd1. Hnf1b- ΔC proteins can form homo or heterodimers with the wild type protein, and bind DNA. The C terminal portion is necessary to recruit coactivators (Barbacci et al., 2004) (Hiesberger et al., 2005). Two coactivators of HNF1beta, CBP and P/CAF, with a histone acetyl transferase activity, can remodel the chromatin in the *Pkhd1* promoter and induce its expression. The absence of the C-terminal portion inhibits coactivators' recruitment and expression of *Pkhd1* (Hiesberger et al., 2005).

During kidney morphogenesis, postnatal tubular elongation is characterized by a synchronized proliferation in large tubular segments mostly localized in the corticomedullary region. This massive proliferation is controlled in order to elongate tubules without any massive modification of their diameter. Retrospective clonal analysis using a mouse model, in which a Tamoxifen-inducible Cre recombinase was under the control of the *ROSA26* locus (crossed with RosaR26 mice), has shown that clones of tubular cells form a long stripe of cells, with only few intercalation between daughter cells. This result suggests that tubular cells have an intrinsic predetermined indication of the direction they have to divide. Measurements of mitotic angles have shown that tubular cells have an intrinsic planar cell polarization. This peculiar OCD is lost in *Hnf1b*-deficient tubules, participating to tubular dilation (Fischer et al., 2006). Several studies have shown that deregulated planar cell polarity (PCP) due to the defective expression of cystogenes participates to tubular enlargement in PKD (Fischer et al., 2006; Karner et al., 2009; Luyten et al., 2010; Saburi et al., 2008).

Once tubules have reached their final length, proliferation decrease and only few cells continue to divide, in order to accomplish the homeostatic growth of the kidney until adulthood. Verdeguer and colleagues demonstrated that the effects of Hnf1b mutations in tubular cells depend on the timing of inactivation of this gene. Using a mouse model expressing Mx-Cre, an interferon-inducible Cre recombinase (Kühn et al., 1995), they inactivated Hnf1b at different time points in postnatal life. When the inactivation was induced after birth at P0-P2, mice developed a kidney cystic disease. However, Hnf1b inactivation at P10, when tubules have completed their morphogenesis, did not give rise to any kidney phenotype. The analysis of gene expression pattern at these two time points showed that early postnatal inactivation induces the downregulation of cystogenic genes, whereas there is no modification in their expression when Hnf1b is inactivated later. This result suggests that during kidney development there is a key postnatal switch that defines the onset and severity of the PKD. When Hnf1b deficient cells were forced to proliferate

again in adult mice, through ischemia reperfusion injury, the expression of cystogenic genes was lost in tubular cells and cysts formed at the cortico-medullary junction, where *Hnf1b* was efficiently deleted. These data suggest that proliferation acts as a trigger event of the cystic phenotype. Our laboratory has shown that contrary to most of the transcription factors, HNF1beta remains attached to chromatin during mitosis. Altogether, these results suggest that HNF1beta acts as a bookmarking factor and is therefore responsible for target gene expression reprogramming after mitosis (Verdeguer et al., 2010).
Table 7. Mouse model of *Hnf1b* in kidney

Role of <i>Hnf1b</i> in kidney			
Mouse model	Phenotype		
Tretraploid compound	Drastic defect in kidney morphogenesis		
(Lokmane et al 2010)			
	Renal agenesis		
Epiblast inactivation (Sox2 Cre)			
(Pontoglio unpublished data)			
Inactivation during tubular elongation			
-(<i>Ksp</i> -Cre) (Gresh et al 2004)			
- (<i>Ksp</i> -Cre Hnf1b- Δ C) (Hiesberger et al 2004)	Polycystic Kidney Disease		
-(<i>Mx</i> Cre) (Verdeguer et al 2010)			
Inactivation in mature tubules			
-(<i>Mx</i> Cre) (Verdeguer et al 2010)	No phenotype		
Inactivation in the activated Metanephric Mesenchyme			
(Six2 Cre) (Massa et al 2013)	Absense of tubular specification and expansion		
Inactivation in the early ronal vasiala			
Wnt4 EGFPCre (Heliot et al 2013)			

CHAPTER 5:

TUBULAR DIFFERENTIATION AND FIBROSIS

TUBULAR DIFFERENTIATION AND HNF1beta : ROLE IN RENAL FIBROSIS?

The clinical spectrum of renal dysfunctions in patients carrying *HNF1B* mutations is very large, ranging from severe developmental pathologies with cysts to tubular dysfunctions.

Independently of the initial pathology, patients carrying *HNF1B* mutations have a tendency to progress to chronic renal failure. In a recent study analyzing a cohort of adult *HNF1B* patients, it was shown that renal phenotype involves chronic tubulointerstitial nephritis, with a slow and progressive decline in the glomerular filtration (Faguer et al., 2011). Another study has shown that kidney histology was characterized by interstitial fibrosis and to a lesser extent enlarged glomeruli or oligomeganephronia in adult patients carrying *HNF1B* mutations (Bellanné-Chantelot et al., 2004). All these reports suggest that the HNF1beta deficiency itself could play a role in the fibrotic process leading to chronic kidney disease. In mouse, an ischemia reperfusion injury in a context of HNF1beta deficiency in renal tubules leads to impaired tubular regeneration and to cyst formation (Verdeguer et al., 2010). These data support the idea of a possible role for HNF1beta in tubular repair after renal injury (AKI).

In the following part of this introduction, I will briefly describe the mechanisms of fibrosis after kidney injury, focusing on tubular damage. Considerable work has been done trying to elucidate the mechanisms at the basis of the response of tubular epithelial cells to injury and the role of this response for the perpetuation of the fibrotic process (Kaissling et al., 2013).

I. Phenotypic modifications of tubular cells in renal fibrosis

i. <u>Partial Epithelial to Mesenchymal Transition (EMT)</u>

After injury, epithelial cells in a damaged organ release cytokines that in turn activate myofibroblasts to repair the tissue. However, when trauma and inflammatory injury persist, the repair process turns into a maladaptive process with the consequent substitution of the parenchyma with fibrotic tissue. The general characteristics of tubulointerstitial fibrosis include: persistent inflammatory infiltrate, increased number of interstitial myofibroblasts, production of excessive extracellular matrix (ECM) and increased cell death with tubular atrophy (Norman, 2011, 2011). For a long time it has been hypothesized that EMT of tubular cells was the pathological process involved in kidney fibrosis. EMT, as well as the

reverse process (mesenchymal to epithelial transition (MET)), is involved in embryonic development, cell type differentiation and organ structural remodeling. During development, EMT starts with the disruption of cell-cell junction, loss of cell polarity and degradation of the basement membrane. Progressively, cells express mesenchymal markers and acquire motility and invasion ability. The high plasticity of epithelial cells allows the reactivation of a EMT-like process also during tissue regeneration of fully differentiated tissues in response to injury (Thiery et al., 2009).

Since tubular cells loose the expression of epithelial markers and start to express mesenchymal markers during tubular degeneration after kidney injury, it has been speculated for a long time that tubular epithelial cells (TECs) could give rise to myofibroblasts via an epithelial to mesenchymal transition and move into the peritubular interstitium (Iwano et al., 2002; Strutz et al., 1995). This hypothesis was based on the expression of *Fibroblast-specific protein-1 (FSP-1)*, considered as a marker of fibroblasts, in tubular epithelia (Strutz et al., 1995). In support of this hypothesis, labeled (LacZ) tubular epithelial cells, expressing *FSP-1*, have been observed in the interstitium in a cell fate tracing study (Iwano et al., 2002). However, Iwano's study raises different questions: first, the γGT promoter used to express the *LacZ* cassette is supposed to be specific for the proximal tubular brush border, but in this study all tubular cells expressed β -galactosidase were stained; second, *FSP-1* cannot be ascribed only as a fibroblast marker, since it is also expressed by interstitial inflammatory cells or in the endothelium of vessels (Kriz et al., 2011).

After Iwano's work, several studies reported the expression of mesenchymal markers by tubular cells during kidney fibrosis involving members of TGF- β 1 signaling pathway or interstitial collagen (type I and type III) (Rastaldi et al., 2002). Nevertheless, none of them showed the presence of true fibroblasts within the tubular basement membrane (TBM) or crossing the TBM (Kalluri and Neilson, 2003; Sato et al., 2003). A recent cell fate tracing study performed in Unilateral Ureteral Obstruction (UUO) demonstrates the absence of EMT contribution to renal fibrosis. In this work, tubular cells derived from ureteric bud and from the cap mesenchyme were genetically labeled using two different promoters, *Hoxb7* and *Six2*, respectively, in order to follow their behavior during kidney fibrosis. As positive control mesenchymal-derived cells were genetically labeled using *FoxD1* promoters (markers of future fibroblasts). This study has shown that during tubular degeneration TECs lose their fixation to the tubular basal membrane (TBM), but do not

delaminate. On the contrary myofibroblasts, producing the fibrotic tissue, were shown to derive exclusively from $FoxD1^+$ mesenchymal resident cells that normally produce the connecting matrix in the parenchyma (Humphreys et al., 2010). The old concept of EMT, considering tubular cells as a potential source of myofibroblasts, has been recently consensually replaced by the concept of partial EMT, illustrated by dedifferentiation of tubular cells during renal injury. Using cell fate tracing and co-staining, two additional recent studies demonstrated that tubular cells lose some of their specific epithelial markers, undergo a cell cycle arrest and increase inflammatory status of kidney tissue and fibrosis but remain attached to the TBM (Grande et al., 2015; Lovisa et al., 2015).

ii. <u>Cell cycle arrest</u>

After acute injury, the first mechanism involved in kidney regeneration is the tubule repair. The Acute kidney injury (AKI) is characterized by the irregular loss of TECs with focal area of tubular dilation and cellular regeneration (Devarajan, 2006). TECs can dedifferentiate, proliferate and differentiate in epithelial cells in order to restore renal morphology. During regeneration after AKI, different genes normally involved during renal embryonic development are reactivated or upregulated. Among the developmental genes, *HNF1B* plays a crucial role during tubular regeneration after AKI, regulating growth factor/cytokines signaling through the repression of *SOCS3* (*suppressor of cytokine signaling-3*) and the activation of the STAT3/Erk pathway involved in cell proliferation (Ogata et al., 2012). In the first hours after injury, *Hnf1b* is transiently downregulated, with a consequent upregulation of SOCS3 and a decreased expression of cystic disease-associated genes involved in the control of the renal tubule morphogenesis. A few hours later, *Hnf1b* expression is upregulated, in parallel with proliferation of dedifferentiated cells through STAT3/Erk pathway and the re-expression of epithelial markers in newly formed tubular cells during tubular repair.

When tubular regeneration cannot be achieved due to a persistent insult, TECs undergo a G2/M phase cell cycle arrest and remain in the tubule in an undifferentiated state, protected from cell death. Recently, it has been demonstrated that proliferation arrest is the first event of a partial EMT during kidney fibrosis. This process depends on the expression of the transcription factors Twist1 and Snail1 that are key regulators of the partial EMT conversion. In fibrosis mouse models, the inactivation of *Twist1* and/or *Snail1* reverts cell

cycle arrest and tubule can be repaired. Moreover, in *in vitro* assays, mouse tubular epithelial cells line, in which partial EMT is induced by TGF- β 1 stimulation, undergo a cell cycle arrest that can be reverted through inactivation of *Snail1* and *Twist1*. TECs in cell cycle arrest acquire mesenchymal markers but did not convert in fibroblasts and reside in the tubule (Lovisa et al., 2015).

iii. Metabolic alterations

During partial EMT conversion, TECs change their secretome profile and induce inflammation and immune recruitment that play an essential role in the induction and maintenance of kidney fibrosis. Loss of epithelial markers is accompanied by a decreased expression of VEGF (Vascular Endothelial Growth Factor) that is normally secreted by TECs and maintains microvascularization in kidney parenchyma. The consequent loss of capillaries, associated with the accumulation of ECM, increases intrarenal hypoxia and participates to the fibrosis process (Louis and Hertig, 2015; Yuan et al., 2003). In addition, partial EMT induces loss of expression in some transporters involved in absorption and secretion activities. In particular downregulation of solute transporter genes, such as Na⁺/K⁺/ATPase, AQP1 and OTA1 (SLC22A6), is typically present in renal fibrosis in mouse models and human biopsies (Poesen et al., 2013; Rajasekaran et al., 2010). Expression of these genes is restored with the inhibition of the partial EMT process through inactivation of Snail1 and Twist1 (Lovisa et al., 2015). Metabolic changes in TECs during fibrosis also include a defective fatty acid oxidation (FAO), whose depression is induced by TGF-B/Smad signaling. This event results into a high intracellular lipid accumulation, called lipotoxicity.



Figure 20. Schematic representation of the phenotypic modifications in tubular cells during fibrosis. Three major modifications occur in the phenotype of tubular cells during fibrosis: 1) G2/M phase arrest of the cell cycle; 2) partial EMT process and 3) metabolic alterations. The consequence of these events is the change in cell secretome profile with the induction of inflammation process and tubular atrophy (Lovisa et al., 2016).

II. Molecular pathways involved in tubular cells during fibrosis and their link with HNF1beta

Snail1 and Twist1 are two transcription factors (TFs) regulated by different convergent pathways. For example, they are activated by cytokines and inflammatory signals that interplay in the induction of partial EMT program. Among these pathways, canonical Wnt/b-catenin and Notch signaling have been shown to activate Snail1 expression (Hao et al., 2011; Timmerman et al., 2004). Binding of Wnt to its receptor and co-receptor on kidney cells results in the stabilization of β -catenin that can translocate in the nucleus and activate the transcription of different targets (Tan et al., 2014). On the contrary, Notch pathway requires cell-to-cell contact and is therefore a short range signaling. Wnt pathway has a critical role in the epithelial derivatives of metanephric mesenchyme during kidney development, whereas Notch pathway is involved in podocyte and proximal tubule fate acquisition. These two pathways are normally downregulated once the differentiation is achieved (Dressler, 1997) (Cheng et al., 2007) but can be reactivated during pathological process, such as fibrosis and cancer (reviewed by Lindsey and Langhans 2014). Interestingly, HNF1beta has been shown to directly regulate some key players of these signaling pathways. This transcription factor regulates the expression of Wnt9b (Lokmane et al., 2010) and *Dll1*, one of the Notch ligand (Heliot et al., 2013; Massa et al., 2013).

Another pathway regulating Snail1 expression is the TGF- β /Smad pathway. TGF- β , secreted by inflammatory cells and fibroblasts present in the parenchyma, binds to its receptor and induces the phosphorylation of downstream Smad3/4 in kidney cells. Smad3 and Smad4 form an oligomeric complex that translocates into the nucleus to activate the transcription of target genes, including *Snail1*. TGF- β participates to the partial mesenchymal conversion of the TECs, and in turn, TECs represent themselves a source of TGF- β . In addition, TGF- β promotes fibrosis process through the production of ECM and the inhibition of its degradation, and is involved in the differentiation of fibroblasts into myofibroblasts (Moustakas and Heldin, 2005). Snail1 activity also depends on Glycogen Synthase Kinase-3 β (GSK-3 β)-mediated post-transcriptional phosphorylation. NF-kB, Notch and Wnt pathways can interfere with GSK-3 β and increase Snail1 stability. Moreover, the pro-inflammatory cytokine, TNF- α , through the action of its transcriptional regulator NF-kB, is a potent inducer of both *Snail1* and *Twist1* (Li et al., 2012). Snail1 and Twist1 transcription factors bind E-box DNA sequences to repress epithelial gene expression and activates genes involved in the mesenchymal phenotype. During

embryogenesis Snail1 is actively expressed but its expression is silenced in the adult. However, in pathological processes, such as fibrosis and cancer, this TF could be reactivated and promotes partial EMT conversion. When Snail1 moves in the nucleus, it represses *E-cadherin* expression through the direct binding to its proximal promoter region. The lack of *E-cadherin* expression ends up with the disruption of epithelial cell-cell adhesion (reviewed in Thiery et al., 2009; Lamouille et al., 2014). Moreover, during the acquisition of a mesenchymal phenotype, Snail1 binds to HNF1b promoter and, represses its transcriptional activity that indirectly promotes the downregulation of the epithelial marker Cadherin-16 (Boutet et al., 2006). Others TFs binding E-box DNA sequences, ZEB1 and ZEB2, are involved in the acquisition of a mesenchymal phenotype. ZEB genes expression lies downstream of TGF- β 1 and WNT signaling pathways and, Snail1 in cooperation with Twist1 directly controls ZEB1 expression (Dave et al., 2011).

In a complex crosstalk of different signals, TGF- β 1/Smad pathway can also promote *ZEB* gene expression in an indirect way, through the downregulation of miR-200 family members. These miR-200 members have been identified as *ZEB* repressors during renal proximal tubule differentiation (Bracken et al., 2008; Oba et al., 2010). In an *in vitro* model of tubular TGF- β 1-induced partial EMT, miR-200 is downregulated with the consequent downregulation of *E-cadherin* and upregulation of *Acta2* in tubular cells. Those cells are also characterized by an increased activity of *ZEB1*/2. In this model, the re-expression of miR-200 reverts the phenotype with a consequent downregulation of *ZEB1*/2 and an increased expression of E-cadherin. During renal tubular dedifferentiation, degeneration and kidney fibrosis, miR-200 members are downregulated (Xiong et al., 2012).

Strikingly, by the combination of ChIP-seq and microarray analysis in epithelial renal cells, recent studies have demonstrated that members of miR-200 family are directly regulated by HNF1b. During kidney development HNF1beta and miR-200 are involved in tubular maturation and their expression pattern is overlapping. In cells expressing a dominant-negative mutant of *HNF1B* and in *Hnf1b* deficient mouse kidney, *miR200* is downregulated and *Zeb2* is upregulated. Moreover, *Pkd1*, which is mutated in ADPKD, is not a direct target of HNF1beta but it is post-transcriptional controlled by miR-200. These data introduce a novel aspect of the role played by HNF1beta in fibrosis and potentially in cystic kidney disease (Hajarnis et al., 2015).



Figure 21. Schematic representation of the major pathways involved in partial EMT of tubular cells. 1) Wnt signaling stabilizes b-catenin that go into the nucleus to activate *Snail1* transcription, 2) TNF- α , through the action of its transcription regulator NF-kB, promotes expression of both Snail1 and Twist1, 3) TGF- β /Smad signaling promotes both *Snail1/Twist1* and *Zeb1/2* expression, 4) Notch pathway control the expression of Snail. Notch, NF-kB and Wnt signaling together increase Snail stability, inhibiting GSK-3 β .

PROJECT DESIGN

PROJECT DESIGN

Hepatocyte Nuclear Factor 1B (HNF1B) is a member of a digenic transcription factor family, the Hepatocyte Nuclear Factor-1 family. This factor is expressed in the epithelia of different organs, such as liver, pancreas and kidney. During kidney morphogenesis, *Hnf1b* is expressed both in the ureteric bud (UB), which will give rise to the collecting ducts, and in the nephron precursors derived from the mesenchymal to epithelial transition of the metanephric mesenchymal cells (MM). In mature kidney, *Hnf1b* expression is maintained in all tubular epithelial cells.

In human, *HNF1B* mutations are responsible for a monogenic diabetic syndrome with early onset, the Maturity Onset Diabetes of the Young (MODY) type 5, but the most frequent phenotypic trait in these patients is the presence of renal structural and functional abnormalities (Bingham and Hattersley, 2004; Bingham et al., 2001a; Kolatsi-Joannou et al., 2001). Patients carrying *HNF1B* mutations have a wide spectrum of renal phenotypes. For example, in pediatric patients, renal malformations include kidney agenesis, renal hypoplasia and renal multicystic dysplasia. All these developmental pathologies belong to a genetic disorder called CAKUT, Congenital Abnormalities of Kidney and Urinary Tract (Chen et al., 2010; Heidet et al., 2010; Ulinski et al., 2006). In adult patients, kidney abnormalities can be represented by few cortical cysts to non-specific progressive interstitial fibrosis, leading to chronic renal failure that represents a serious issue of public health (Faguer et al., 2011).

Animal models have provided powerful tools for understanding the different important roles of HNF1beta during nephrogenesis or in mature kidney. Conditional inactivation strategies of *Hnf1b* have recapitulated most of the abnormalities displayed in human, depending on the developmental timing and the tissue compartment in which this gene is inactivated. For example, the deficient expression of *Hnf1b* in the UB results in defective UB branching and absence of mesenchymal to epithelial conversion, leading to kidney aplasia (Lokmane et al., 2010) (Massa 2012). Conversely, *Hnf1b* inactivation only in the epithelial structures derived from MM, but not in the UB derivatives, produces a drastic reduction of all tubular structures. This study has highlighted the crucial role of HNF1beta in the specification and expansion of the tubular component of the nephron (Massa et al., 2013; Heliot et al., 2013). Specific *Hnf1b* inactivation during tubular elongation results in severe polycystic kidney disease (PKD). The analysis of this mouse model demonstrated

that HNF1beta controls the expression of cystic genes mutated in PKD, such as *Pkhd1*, *Pkd2* and *Umod* (Gresh et al., 2004) and plays a critical role in the maintenance of planar cell polarity in elongating tubules (Fischer et al., 2006). In a last model, the inactivation of *Hnf1b* in mature tubules demonstrates a critical role of HNF1beta as a bookmarking factor. Contrary to most of the transcription factors that are released into the cytoplasm during cell division, HNF1beta remains attached to the chromatin during mitosis (Verdeguer et al., 2010). This property could promote the rapid re-expression of crucial target genes after cell division.

Remarkably, renal abnormalities linked to *HNF1B* mutations are associated with a high prevalence of glomerular cyst formation (Bingham, et al., 2001). The mechanisms at the origin of the formation of glomerular cysts remain poorly understood. During glomerulogenesis, *Hnf1b* is expressed in the parietal cells of the Bowman capsule, but is excluded from the glomerular cup. Although it has been demonstrated that HNF1beta plays a critical role in the tubular compartment (Gresh et al., 2004; Heliot et al., 2013; Hiesberger et al., 2004; Massa et al., 2013), its involvement during glomerulogenesis is still unclear. **The aim of the first part of my work was to identify the role played by HNF1beta during glomerular development** whose dysfunctions is associated to glomerular cyst formation. For this purpose, I analyzed a mouse model in which *Hnf1b* is inactivated in all epithelial cells of the nephron precursors (*Six2*-Cre (Kobayashi et al., 2008) inactivation strategy) (Figure 22). The inactivation of *Hnf1b* in this model leads to a defective tubular specification and to the formation of glomerular cysts with an extended tuft and dilated Bowman capsule, connected to the collecting duct through a short and primitive tubule (Massa et al., 2013).

Inactivation of *Hnf1b* in mature tubules in mouse has highlighted the role of the status of tubular cells (proliferative vs quiescent) in the expression of HNF1beta target genes. When *Hnf1b* is inactivated in quiescent mature tubules, there is no renal phenotype, whereas when tubular cells are in a proliferative status (tubular elongation or tubular regeneration after injury) tubules form cystic dilations (Gresh et al., 2004; Verdeguer et al., 2010). At the molecular levels, the expression of *Hnf1b* target genes is lost during proliferation, whereas it is maintained in a quiescent cellular context. In the second part of my work, my objectives were to analyze and compare the molecular program controlled by *Hnf1b* in proliferative cells and in\ quiescent cells. In order to achieve this aim, I took advantage of the use of a *Ksp*-CreER^{T2} mouse line (Lantinga-van Leeuwen et al., 2006),

carrying a tamoxifen inducible Cre recombinase under the control of the *Ksp* promoter, to inactivate *Hnf1b* in all tubular compartments at two different phases of postnatal development (Figure 22). The first time point was the perinatal period (P0-P3), which is characterized by massive proliferative tubular elongation whereas the second step that we considered was the post-weaning period (P23), characterized by a much lower proliferative context.



Role of $Hnfl\beta$ during glomerulogenesis

Role of $Hnf1\beta$ in tubules

Figure 22. Schematic representation of the project design.

MATERIAL AND METHODS

Materials and Methods

Animals

Specific inactivation of *Hnf1b* in the metanephric mesenchyme was obtained using a Cre-LoxP strategy. *Six2*-Cre (Kobayashi et al., 2008), *Hnf1b LacZ/+* (Coffinier et al., 1999a) and *Hnf1bf/f* mice (Coffinier et al., 2002) were previously described. Since *Six2-Cre*; *Hnf1bf/+* or *Six2-Cre*; *Hnf1b LacZ/+* mice were indistinguishable from wild type mice, all these animals were used as controls (indicated in this study as "control"). Specific inactivation of *Hnf1b* in renal tubules was obtained using *Ksp*-CreER^{T2} mice, kindly provided by DJ.Peters (Lantinga-van Leeuwen et al., 2006). Animals were treated with Tamoxifen at concentration of 1mg/10g. For early inactivation, we injected the mother during the first two days of lactation, whereas, for the later inactivation, animals were gavaged for 3 days post-weaning. Cre -; *Hnf1bf/f* mice were used as controls (indicated in this study as "control"). Animals were maintained in an animal facility licensed by the French Ministry of Agriculture (agreement A 75-14-02). All experiments were conforming to the relevant regulatory standards.

Histological and Immunohistochemical Analysis

Animal samples

Mouse kidneys were dissected at different time points. Samples were fixed in HistoFix (phosphate-buffered formaldehyde solution) 4% (Carl Roth) for 24 hours and stored in PBS 1X, before to be cut with a vibratome, or stored in ETOH 70% before being embedded in paraffin.

For histological analysis, 5µm paraffin sections were stained with hematoxylin and eosin, Periodic acid–Schiff (PAS) and Picro Sirius Red. All these experiments have been performed at Histology platform at Necker Institute, Paris.

Immunofluorescence analyses were performed on 5 µm paraffin sections or 50-200µm vibratome sections. For paraffin sections, antigen retrieval was performed in boiling citrate buffer (Dako, S2369) for 15 minutes. Sections were then treated with the MOM Kit (Vector Labs, BMK-2202) according to the manufacturer's instructions. Sections were incubated with primary antibodies (see antibodies list) at 4°C overnight. After several

washes in PBS, samples were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen; 1:250) for 1 hour at room temperature. Vibratome sections were incubated with primary antibodies diluted in PBS1X Triton 0,1%, NaAzide 0,1% for 2 days, washed for 24 hours and incubated 24 hours with the secondary antibodies. The slides were mounted in liquid mounting medium VectaShield (Vector, ref H1000) and analyzed with a Leica microscope equipped with a Yokogawa CSU-X1M1 spinning disk or with the "Lamina"-Perkin Elmer slide scanner. Figures have been analyzed with ImageJ, Pannoramic Viewer and NanoZoomer Digital Patology (NDP) sofwares and produced with Above Illustrator. Experiments were performed on minimum three animals per genotype.

Human samples

Human fetuses carrying a *HNF1B* missense mutation c.494G>A (p.Arg165His) have been described in (Rasmussen et al., 2013). For immunofluorescence experiments, small pieces of paraffin blocks were deparaffinated, and sectioned with a vibratome to obtain thick sections (300 or 500 μ m). After antigen retrieval in boiling citrate buffer (Dako, S2369) for 15 minutes, sections were incubated with antibodies at 37°C for 5 days. After 3-4 washes in 0.1% Triton, 0.1% Na Azide in PBS at 37°C on a wheel for a total period of 3 days, samples were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen; 1:250) in 0.1 % Na Azide in PBS at 37°C on a wheel for 3 days.

For some experiments, thick vibratome sections or thick paraffin section of human fetuses were clarified using the BABB (Benzyl-alcohol; Benzyl-benzoate; 1:2) solution, as previously described (Kremer et al., 1996). Sections were analyzed with a Leica microscope equipped with a Yokogawa CSU-X1M1 spinning disk. Images were analyzed with ImageJ (NIH). IMOD software (<u>http://bio3d.colorado.edu/imod</u>) (Kremer et al., 1996) was used to create the 3D reconstructions from the confocal images.

Antibodies list

Host species	Target protein	Source	Dilution	
Rabbit	JAG 1	Santa Cruz (sc-8303)	1:200	
Rabbit	SYNAPTOPODIN	Santa Cruz (sc-50459)	1:200	
Rabbit	NOS1	Santa Cruz (sc-8309)	1:200	
Rabbit	CADHERIN 6	Homemade	1:200	
Rabbit	LAMININ	Sigma (L9393)	1:200	
Rabbit	PAX2	Invitrogen (71-6000)	1:200	
Rabbit	CLAUDIN-1	Abcam ab15098	1:200	
Rabbit	PDGF R β	Cell Signalling (#3169)	1:100	
Mouse	WT1	DAKO (M356101)	1:200	
Mouse	E- CADHERIN	BD transduction laboratories (610181)	1:200	
Chicken	BETA GALACTOSIDASE	Abcam (Ab9361)	1:300	
_	Peanut Agglutinin (PNA)	Vector Laboratories(RL1072)	1:250	
_	Lectin from Bandeiraea simplicifolia (Griffonia simplicifolia) (GSL I)	Sigma (L5264 TRITC conjugate)	1:500	
_	Texas Red-X Phalloidin	Invitrogen (T7471)	1:50	
Rabbit	HNF1B (v1.6)	Homemade	1:100	
Rabbit	AQUAPORIN2	Sigma (A7310)	1:400	
Rabbit	VIMENTIN	Abcam (ab 92547)	1:200	
Rabbit	NKCC2	ProteinTEch (18970-1-AP)	1:200	
-	Lotus Tetragonolobus Lectin(LTL)	Vector Laboratories (B-1325)	1:400	
-	Wheat Germ Agglutinin(WGA)	Vector Laboratories (FL-1021)	1:400	

Plasma and urine collection

Plasma and urine samples were collected from mice at P28, P39 and P53. For plasma collection we used heparinated syringe [Heparin 5000 USP/mol (Sigma H3393-10k) in physiological serum]. Blood was collected and centrifuged at 2500 rpm and RT for 10 minutes to obtain the plasma. Samples were analyzed by CRI – Plateforme de Biochimie UMR 1149 Université Paris Diderot.

Kidney weight and Body weight

Mice have been weighted before sacrifice. After dissection, fresh kidneys were weighted on high precision balance. We evaluated the ratio between kidney weight and body weight in mutant vs control mice.

Affymetrix

Expression patterning analysis was performed using the Affymetrix array MTA-1_0. The data obtained were analyzed with the Transcriptiomic analysis console 3.0 software.

RNA isolation and quantitative RT-qPCR

Collected kidneys were frozen in liquid nitrogen and stored at -80°C. RNAs were extracted from frozen samples in Phase lock gel tubes (5PRIME) using TRIzol as described by the manufacturer (Life Technologies). The quality of the isolated RNA was assessed on a Bioanalyzer 2100 (Agilent). For all samples, reverse transcription was performed on 2µg of RNA with the High-capacity cDNA reverse transcription kit (Applied Biosystems), with or without reverse transcriptase. Gene expression was analyzed by qPCR using the GoTaq®qPCR Master Mix (Promega) on a LightCycler Mx3005 qPCR system (Agilent). Primers were designed using Primer3 online software. All the genes were tested for 3 wild types and mutant animals at P7, P32 and P28, P53.

RT- qPCR primers

Zeb2	for	CCCAGGAGGAAAAACGTGGT
	rev	GTCCTGGTCCAGAGGGTTTG
Tafh1	for	CTGCTGACCCCCACTGATAC
1gj01	rev	AGCCCTGTATTCCGTCTCCT
Collal	for	CGATGGATTCCCGTTCGAGT
Collul	rev	GAGGCCTCGGTGGACATTAG
Vim	for	CAGGCCAAGCAGGAGTCAAA
v im	rev	CTCCAGGGACTCGTTAGTGC
Acta?	for	GTCCCAGACATCAGGGAGTAA
	rev	TCGGATACTTCAGCGTCAGGA
Tafh?	for	TCCCCTCCGAAAATGCCATC
1gj02	rev	ACTCTGCCTTCACCAGATTCG
	for	CGCCATCCTCCATGAGATTA
Aqp2		GGTCAGGAAGAGCTCCACAG
	rev	
Slc5a2	for	TGCGCTCTTCGTGGTGCTG
	rev	ACCAAAGCGCTTGCGGAGGT
Pkd2	for	CATGTCTCGATGTGCCAAAGA
	rev	ATGGAGAACATTATGGTGAAGCC
Pkhd1	for	TGCATCCTGCCTAATGAGTATAGC
	rev	AACTTGTTTTTGTCCTTTATCCCCA
SIc12a3	for	CGGCCTACGAACACTATGCT
	rev	GGCTACCTTCCTGCTTGAGG
SIc12a1	for	CTGGCCTCATATGCGCTTATT
51(1201	rev	AGATTTGGCATACGAGGCATG
Hnflb	for	CCATCCTCAAAGAGCTCCAG
	rev	GCTGTTGCATGTATCCCTTG
Hprt	for	AGTCCCAGCGTCGTGATTA
	rev	GGAATAAACACTTTTTCCAAATCC
Snai1	for	CCACACTGGTGAGAAGCCAT
5/10/1	rev	CACATCCGAGTGGGTTTGGA
LncmiR10Kb	for	GTATTTGGGATCCAGGCAAA
LICHINICIOND	rev	CTGGAGGCACGAAGAGGTAG
LncmiR14Kb	for	AAGGAAGCCCTCCAGACCTA
Diciniti Tho	rev	GCCCAGAGAAGTGGAGTTGA
LncmiR25Kb	for	ATGGTCATTGGGCACTTTTG
	rev	AGGAGGGAGGTTTTGCTACC

Proliferation assessment

To evaluate the index of proliferation, freshly dissected kidneys have been fixed in HistoFix 4% for 24hours and included in Agarose 4%. 80µm thick Vibratome sections were then postfixed for 10 minutes at RT and incubated with Lotus Tetragonolobus Lectin(LTL) (biotinylated, Vector, ref B-1325) and anti-Ki67 (rabbit, Novocastra Leica, ncl-Ki67p) antibodies for 48h in PBS-Sodium Azide 0.1%-Triton 0.01%. After this incubation, 3 washes were performed during 8 hours in total. The sections have been incubated with appropriated secondary antibodies and DAPI for 12 hours. Proliferation index was calculated by counting 3 areas of 1000 LTL⁺ cells for each sample, which were also co-stained for Ki67. The counting was done for 3 wild type and 3 mutant mice at P28.

Statistical analysis

Values are reported as a mean \pm s.d. for the Real Time qPCR analysis and median with interquartile range for the analysis of body and kidney weight, and proliferation index, unless otherwise noted. Statistics were calculated using the two-way, two-tailed Student t test. Differences were considered significant with a p-value <0.05.

RESULTS PART I

Role of HNF1beta in glomerular cyst formation

(Paper submitted)

Glomerular cysts are frequently observed in pediatric patients of foetuses carrying *HNF1B* mutations. *HNF1B* mutations represent a frequent genetic cause of glomerulocystic kidney disease (GCKD) (Bingham et al., 2001a). Nevertheless, the molecular and cellular mechanisms at the basis of this phenotype are still largely unknown.

In order to study the role of HNF1beta in the nephron precursors, I have used a mouse line carrying the Cre recombinase under the control of *Six2* promoter (Kobayashi et al., 2008). When crossed with *Hnf1b f/f* mice, this strategy allows the inactivation of *Hnf1b* only in the cap condensate cells without affecting the ureteric bud development (Massa et al, 2013). Previously, our lab demonstrated that these mutant embryonic kidneys showed a drastic defect in the development of all the tubular components. Mutant nephrons are composed of dilated glomeruli (Figure 23) directly connected to the collecting duct through a primitive tubule. In addition, we showed that fetuses carrying *HNF1B* mutations present a similar phenotype with a drastic reduction of proximal tubules and glomerular cysts characterized by an extended tuft and a dilated urinary space (Massa et al., 2013). However, the mechanisms of glomerular cysts formation were still elusive.



Figure 23. Immunofluorescence of a glomerulus in a *Six2-Cre⁺;Hnf1b f/f* **compared to a control.** Mutant glomerulus has a dilated urinary with an extended tuft directly connected to the collecting duct, whereas the control glomerulus has a normal developed tuft and urinary and vascular pole at the opposite side of the glomerular cup (Massa et al., 2013).

The aim of the first part of my study was to elucidate the role of HNF1beta during glomerulogenesis. During nephron development, the architecture of glomeruli is acquired through intricate remodeling steps. At the S-shaped body (SSB) stage the future podocytes and the future tubular cells are initially found adjacent to one another. During the transition from S-shaped body to precapillary loop stages, the epithelial podocyte precursors generate the podocyte cup through a drastic invagination process and become separated from the tubular component by the formation of an epithelial protuberance (Figure 24) (Saxén and Sariola, 1987). Through this process, the insertion of the prospective tubular cup (on the prospective Bowman capsule).



Figure 24. Formation of the epithelial protuberance during normal nephrogenesis. The transition from the S-shaped body to the precapillary loop (from left to right) is characterized by the formation of an epithelial protuberance (red arrow) that separate the podocyte plate from the tubular compartment.

By tridimensional (3D) reconstructions of nephron precursors, we have shown that nephron precursors that develop in the absence of *Hnf1b* are characterized by an aberrant connection of the urinary pole to the urinary space from the inner side of the glomerular cup, due to the absence of the formation of the epithelial protuberance (Figure 25).



Figure 25. Schematic representation of a precapillary loop stage glomerulus in control versus *Six2*Cre; *Hnf1b*^{ff} mutant kidney. During normal development, at the precapillary loop stage, urinary pole and vascular pole are still in a close contact on the same side of the glomerular cup, separated by the formation of the protuberance, whereas, in the *Six2*Cre; *Hnf1b*^{ff} mutant precursor, at this stage, tubule is inserted inside the glomerular cup.

These data demonstrated that tubular cells, normally programmed to be on the outer part of the glomerulus, are localized inside the glomerular cup in the absence of Hnflb. Interestingly, Hnflb itself is never expressed inside the glomerular cup, but its expression is restricted to the Bowman capsule's and tubular cells. In order to analyze the fate of cells that were programmed to express Hnflb in the absence of HNF1beta itself, we took advantage of a mouse model in which a *LacZ* cassette is under the control of the Hnflb endogenous promoter. Using a combination of markers specifically expressed in the tubular compartment or in podocyte precursors, we showed that the bilayered protrusion was characterized by the presence of podocyte precursors in the inner side, contiguous with the podocyte plate, and intermediate parietal-like cells on the outer side. In the absence of Hnflb, despite the presence of differentiating podocytes and intermediate- parietal-like cells, the protrusion never formed. This defect leads to the absence of the extrusion of the tubular component from the glomerular cup.

To better understand the morphological mechanisms driving the trapping of the tubule, we went back during nephrogenesis. It is known that, during development, early nephron precursors undergo two invagination processes: the first during the transition between vesicle and comma-shaped body nephron precursors, forming the vascular cleft, and the

second during the transition between the comma and the S-shaped body nephron precursors that will form the second cleft, accompanied by the expansion of the tubular structures. Here we showed that the second cleft was still formed in the mutant mice, but remained localized in close proximity to the vascular one, due to the absence of tubular expansion. The proximity of the two clefts and their further invagination induced the formation of a common cavity all around the tube. By immunofluorescence analysis, we showed that, in this conformation, capillaries and mesangial cells, which normally colonized the glomerular cup, surrounded the tubule that is trapped inside the glomerulus. The mesangial cells surrounding the tubule were accompanied by the organization of Factin filament rings that might provide the reason of the constriction of the tubular glomerular outflow. In the absence of *Hnf1b*, podocytes develop normally (Massa et al 2013), and filtration might start normally in the anterior part of the mutant glomerular cup. The production of the primary urine might induce the dilation of Bowman capsule and the formation of the cyst (Figure 26). In the last part of this study, we performed 3D reconstructions of precapillary loop stages from tissues of fetuses carrying HNF1B mutations. In these glomerular precursors, the urinary pole had an aberrant insertion in the inner layer of the glomerular cup, reminiscent of the defects observed in our mouse model. These results clarify the nature of the structural defects in these human nephron precursors. In conclusion, we disclose a novel role played by HNF1beta in the urinary pole remodeling during glomerulogenesis and provide a novel morphological mechanism of glomerular cyst formation in human patients.



Figure 26. Model of normal glomerular development vs glomerular development *Hnf1b* deficiency. (A) During normal development, in the transition between the SSB and precapillary loop stage, podocyte precursor plate under goes an invagination process and becomes separated from the tubular component by the formation of an epithelial protuberance. At this stage, the urinary pole is close to the vascular one, until it reaches the opposite side in the mature glomerulus. (B) In the absence of *Hnf1b*, the bulge of epithelial cells does not form and the vascular and second clefts are still in close proximity with the consequent formation of a common cavity around the tube that is trapped inside the glomerular cup. Successively, capillaries and mesangial cells colonize the full cavity and restrict the glomerular tubular outflow. In the anterior part of the mutant glomerular cup, podocytes develop normally and the starting of filtration with the production of primary urine might provide the reason for Bowman capsule dilation and glomerular cyst formation.

Self-strangling renal glomeruli in *HNF1B*-glomerulocystic disease

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ABSTRACT

The occurrence of glomerular cysts is one of the most characteristic traits that affect patients carrying Hepatocyte Nuclear Factor 1B (HNF1B) mutations. The cellular events and the genetic networks that lead to this developmental pathology are still poorly characterized. Our recent studies have shown that the lack of HNF1beta in nephron precursors leads to defective tubular expansion, and to extremely dilated glomeruli connected to collecting ducts with a short and primitive tubule. With the study of a mouse model for *Hnf1b*-deficiency, here we show that in the absence of HNF1beta the formation of glomerular cysts is linked to a specific dysfunction in the spatial remodeling of nephrons. During glomerulogenesis, the vascular and urinary poles, initially contiguous, are progressively separated by the formation of a protuberance leading to the restriction of the connection of the tubule on the external side of the glomerulus. In HNF1beta-deficient nephron precursors, this protuberance does not form, giving rise to a defect in the inside/outside patterning of the glomerulus. In the absence of HNF1beta, the tubuloglomerular connection is transiently trapped inside the glomerulus, concentrically surrounded by the vascular pole. In this aberrant configuration, the tubular outflow is constricted and strangled by mesangial cells leading to obstructive dilation and formation of glomerular cyst. Interestingly, our findings are relevant for humans since the same aberration is observed in the kidneys of fetuses carrying HNF1B mutations. Our studies have disclosed a novel and genetically programmed morphogenetic process whose dysfunction mechanistically leads to glomerulocystic disease in mouse models and humans.

INTRODUCTION

Nephron morphogenesis is based on a tightly controlled set of proliferative and differentiation processes that are responsible for the generation of very long tubular structures connected to a complex blood filtration unit, the renal glomerulus. This phenomenon is controlled by a set of regulatory circuitries where specific transcription factors play a crucial role. Dysfunctions in this morphogenetic process may lead to a variety of Congenital Abnormalities of the Kidney and Urinary Tract (CAKUT), a pathological condition that represents 20-30% of prenatal anomalies (see (Schedl, 2007)). Remarkably, CAKUT are responsible for about 40% of cases of end stage renal disease in children (Harambat et al., 2012). One of the most prevalent genetic defects responsible for CAKUT is represented by mutations in a transcription factor, Hepatocyte Nuclear Factor 1B (HNF1B) (Decramer et al., 2007; Heidet et al., 2010; Ulinski et al., 2006). *HNF1B* mutations lead to a wide spectrum of phenotypes including kidney agenesis, renal multicystic hypodysplasia and, remarkably, a high prevalence of glomerular cyst formation (Bingham et al., 2001a).

Mouse models for HNF1beta-deficiency have shown that the inactivation of *Hnf1b* with specific Cre recombinases may recapitulate the different phenotypes observed in patients. For instance, the lack of HNF1beta specifically in tubular precursors during their elongation/expansion phase leads to a polycystic phenotype (Fischer et al., 2006; Gresh et al., 2004; Verdeguer et al., 2010), whereas the inactivation of HNF1beta in early nephron precursors leads to nephrons that are formed by dilated glomeruli directly connected to collecting ducts via a primitive and short tubule. The lack of HNF1beta during nephrogenesis leads to a drastically compromised specification and expansion of the nephron tubular compartment (Heliot et al., 2013; Massa et al., 2013). The mechanisms responsible for the formation of glomerular cysts remain poorly understood.

Although the developmental sequence of events that normally shape nephron precursors has been disclosed long time ago, some of the morphogenetic events leading to the formation of mature glomeruli are still not entirely understood. In particular, the events, which lead to the remodeling of the urinary and vascular poles, are still poorly characterized. In the present study, we show the crucial role played by HNF1beta in this process. More importantly, we demonstrated that the disruption of this genetic program mechanistically leads to the development of glomerular cysts. Finally, our results are relevant for the human disease since we identified the same abnormalities in nephron precursors from glomerulocystic kidneys of fetuses carrying *HNF1B* mutations.

RESULTS

HNF1beta-deficiency leads to aberrant glomerulogenesis

The glomerulus is a complex structure derived from the lower limb of the S-shaped body. The inner layer of this limb gives rise to podocytes that surround capillaries and mesangial cells forming the glomerular tuft. The outer layer of this limb will form the Bowman capsule that will delimit the urinary space. In the mature glomerulus, the Urinary Pole (UP) is typically located at the opposite side of the insertion of afferent and efferent arterioles, also called the Vascular Pole (VP). We have previously shown that the vast majority of HNF1beta-deficient glomeruli are cystic, characterized by an aberrantly positioned UP, next to the VP (Massa et al., 2013). In order to characterize the morphogenetic mechanisms leading to this phenotype we analyzed the tridimensional aspect of the sequence of events that occurs during glomerulogenesis in the absence of HNF1beta.

In the wild-type embryos, at the S-shaped body stage, the prospective VP and UP are initially adjacent to one another, where the UP is directly abutting the rim of the podocyte precursor plate. During the transition from S-shaped body to precapillary loop stages, the epithelial podocyte precursor plate undergoes a drastic invagination that generates the podocyte cup. At the same time, a bi-layered epithelial protrusion (thumb) emerges from the rim precisely at the junction between the podocyte plate and the tubular component (Figure 1A and 1C, arrowhead; Movie S1-S3). An important topological consequence of this process is that the insertion of the prospective UP outflow is going to be restricted on the outer surface of the glomerular cup (on the prospective Bowman capsule). During the later stages of glomerular development, UP and VP progressively acquire their final diametrical opposite position.

Interestingly, the inactivation of *Hnf1b* has a profound impact on this complex set of events. We discovered that nephron precursors, in the absence of HNF1beta, give rise to topologically aberrant structures where the UP is systematically connected to the glomerular cup from the inner side (Figure 1B, arrows). Our results showed that this aberration is a direct consequence of the fact that the epithelial protuberance (thumb) is not generated in the absence of HNF1beta. Tridimensional (3D) reconstructions of nephron precursors showed that the spatial organization of VP and UP in precapillary loop stage precursors was structured in a very aberrant way. In this aberrant configuration, the UP

was inserted inside the glomerular cup, and was concentrically surrounded by the prospective VP (Figures 1D-1F; Movies S2-S4).

Altogether, these observations indicate that complex morphogenetic events are required to restrict the tubular component on the outside of the glomerulus and highlight the fact that HNF1beta plays a crucial role in this process (Figure 1G).

The aberrant glomerular morphogenesis is not due to any overt defective cellular specification.

In order to gain more insight on the mechanisms leading to this phenotype we decided to characterize the molecular marker signatures of the territories occupied by the different glomerular and tubular cell progenitor populations in the aberrant mutant nephron precursors.

During glomerulogenesis, one of the markers characterized by a peculiar expression pattern is represented by HNF1beta itself. This transcription factor is systematically expressed in all tubular and capsular precursor cells and, on the contrary, never expressed in podocyte precursors or in fully differentiated podocytes. In order to analyze the destiny of cells that are programmed to express Hnflb even in the absence of HNF1beta expression, we took advantage of a specific null knock-in allele ($Hnflb^{LacZ}$) (Coffinier et al., 1999a), and produced embryos carrying compound heterozygous alleles for Hnflb (Hnf1b^{LacZ/flox}) and inactivated Hnf1b with the Six2Cre (Kobayashi et al., 2008). As previously described (Coffinier et al., 1999a), the Lac-Z expression pattern perfectly correlated with the endogenous *Hnf1b* protein expression (Figures 2A and 2E). Podocyte precursors were characterized by a strong expression of *Wt1* (Figures 2A and 2E), and by the progressive expression of Synpo (Synaptopodin) during glomerular maturation (Figure 2C, arrow). The concomitant expression of low levels of both Wt1 and Hnf1b-LacZ was observed in prospective parietal (Bowman capsular) cells. In a similar way, at the junction between the podocyte plate and the adjacent tubular high Hnflb-LacZ positive cells, a specific set of cells (transitional cells) were characterized by the concomitant expression of low-*Wt1* and low-*Hnf1b-LacZ* (Figures 2A and 2E, arrowheads).

Hnf1b-deficient maturing glomeruli had an inner glomerular cup layer that was clearly patterned into anterior and posterior compartments. The posterior one, adjacent to the stalk of the ureteric bud, was composed of epithelial cells co-expressing low-*Wt1* and

low *Hnf1b-LacZ* (Figure 2B and 2F, orange arrowhead). On the most anterior portion, instead, the inner layer epithelium was characterized by cells that expressed a podocyte precursor signature (high levels of *Wt1* and *Synpo* (Figure 2D, arrow)). Finally, the trapped urinary pole was surrounded by transitional cells that expressed low levels of both *Wt1* and beta galactosidase (Figures 2B and 2F, white arrowheads).

A further characterization of the glomerular and tubular territories was obtained by the analysis of *Pax2*, *Jag1* and *Cldn1* expression patterns. In mutant glomerular precursors, similarly to the control, the expression of *Pax2* and *Cldn1* became progressively restricted to parietal cells (Figures S1A and in S1M, arrows) whereas *Jag1* was normally strongly expressed in tubular precursor cells and excluded from podocyte or parietal precursors (Figures S1G). Similarly to *Synpo*, *Cldn 1* and *Pax2* were also normally expressed in a few transitional cells immediately adjacent to the podocyte plate (Figures S1E and S1Q, arrows). In parallel, *Jag1* was expressed at low levels in transitional cells located between tubular and podocyte plate precursor cells (Figures S1K, arrow).

As previously mentioned the first step of the remodeling of the UP is characterized by the emergence of the bi-layered protrusion (thumb) that accounts for the transition between S-shaped bodies and maturing glomeruli. Our results showed that this protrusion is characterized by the presence of podocyte precursors (high Wt1) in the inner side (Figures 2G and 2K, arrow), and by transitional cells on the outer side (Figures 2I and 2K, arrow). Remarkably, in the absence of Hnf1b, despite the presence of differentiating podocytes and apparently normal transitional cells (Figures 2H, 2J and 2L, arrow) these cells never organize a protrusion. This defect leads to the absence of the restriction of the tubular component on the outside of the glomerular cup. At later stages of development, mutant glomeruli systematically undergo a severe dilation of the urinary space leading to the formation of a cystic structure.

The aberrant glomerular structure induced by the absence of *Hnf1b* indicates that some cells that are normally found outside the glomerular cup are transiently trapped inside the cup. The mechanism of this phenotypic abnormality is not linked to any apparent defective cell fate acquisition per se. In fact, the different glomerular and tubular progenitor populations are correctly specified and the frontier between the territories of these progenitors is maintained.

The nature of the observed malformations in *Hnf1b* deficient nephron precursors (the UP trapped inside the glomerular cup) highlights a novel aspect of the mechanisms that are at the basis of the morphogenesis of the glomerulus. When the epithelial podocyte

precursor plate starts its invagination, some cells will be destined to remain inside and form the inner glomerular cup (podocyte precursors) whereas others (parietal cells) become the outer layer of this cup. When *Hnf1b* is not expressed, the boundary of inner-fated cells is extended and includes the UP

The strangling of the tubule.

It is known that during nephrogenesis, renal vesicles undergo two successive invagination processes (Saxén and Sariola, 1987). The first one takes place during the transition from vesicles to comma shaped-bodies. This process creates a notch on the surface of the spherical vesicle, and initiates the formation of the vascular cleft (Figure 3A, arrowhead). The second invagination takes place at the opposite side of the vesicle (Figure 3A, arrow). In the mutant, the two clefts start to form normally. Nevertheless, due to the absence of the expansion of the tubular component the second cleft remains localized in close proximity to the vascular one. This leads to the formation of a common cavity that surrounds the trapped primitive tubule (Figure 3B, arrow and arrowhead).

An intriguing observation is the fact that the lumen of the trapped primitive tubule was constricted just above its connection with the UP. In mutant precursors mesangial and endothelial cells colonized and proliferated in the glomerular cavity (Figures 3D, 3F and 3H). The fact that mesangial cells were distributed all around the tubule (Figures 3E and 3F; Movies S5-S6) might provide the reason for the constriction of the tubular glomerular outflow. Phalloidin-Texas-Red staining showed that the constriction of the tubule was generated by F-actin filament rings in the surrounding mesangial cells that in this way might provide an essential contribution to the restriction of the lumen of the tubule (Figures 3I to 3K, arrows; Movie S7).

Glomerular cyst formation in human *HNF1B* deficiency.

As previously mentioned, it is interesting to note that mutations in *HNF1B* are frequently linked to glomerulocystic lesions in patients (Bingham et al., 2001a; Heidet et al., 2010). We have previously shown that fetuses with *HNF1B* mutations have kidneys with focal lesions characterized by dilated glomeruli (Massa et al., 2013). In an attempt to extend our experimental observations to humans, we analyzed paraffin sections from two fetal siblings carrying an *HNF1B* missense mutation c.494G>A (p.Arg165His) (Rasmussen

et al., 2013). At histological level, the vast majority of the renal tissue was filled with focally distributed cystic structures, predominantly glomerular cysts, separated by a few areas of normally differentiated glomeruli and tubules (Figure 4A vs 4B, arrowheads). Just under the capsule, in restricted zones, a small number of nephron precursors were still present, represented by S-shaped bodies and glomerular precursors (Figure 4B, arrows). Most of these nephron precursors, when located in proximity of glomerular cysts, tended to have an abnormal configuration (example in Figures 4D vs 4C; Movie S9-S8). In particular, S-shaped bodies lacked the typical bulge in between the mid and the lower limbs, and their immature glomerular cups contained a tubular structure surrounded by capillaries and mesangial cells. 3D reconstructions starting from tissues samples of these affected fetuses showed that glomerular cup (Figures 4F vs 4E; Movies S11-S10). In addition, 3D reconstructions of S-shaped bodies showed that they were also aberrantly shaped, with a defective expansion of the prospective tubular component (data not shown). These results clarify the nature of the structural defects in these human nephron precursors.
DISCUSSION

During nephron development, the architecture of glomeruli is progressively acquired through intricate steps involving complex tissue remodeling processes. In our study, we have shown that the remodeling of the connection between the glomerulus and its tubular component plays a key role in this morphogenetic process. Initially described by Potter more than 50 years ago (Potter, 1965), this remodeling event has never drawn a particular attention. In one of the most renowned schematic models, illustrated by the Saxen's drawings (Saxén and Sariola, 1987), the S-shaped body is depicted with a podocyte precursor's plate in direct connection with tubular precursors. The immediate following scheme represents a glomerular precursor where the prospective UP is connected to the outer layer of the glomerular cup. Our study has focused on a so far neglected but key event in the transition between S-shaped bodies and the glomerular precursors. This event is characterized by the protrusion of a bi-layered epithelial structure (thumb) right at the junction between the podocyte plate and the tubular precursor cells. We have discovered that the formation of this epithelial outgrowth is genetically programmed since the formation of this bi-layered protuberance does not take place in the absence of *Hnf1b*. One of the most remarkable consequences of this defect is that the UP, instead of being inserted in the outer layer of the glomerulus, remains directly connected with the inner layer of the glomerular cup. This configuration leads to a structure where the outflowtubule is surrounded by capillaries and mesangial cells that could participate to the restriction of the caliber of the tubular lumen. We had previously shown by electron microscopy that podocytes developed normal foot process and fenestrated capillary structures are observed in Hnflb-deficient glomeruli (Massa et al., 2013). In addition, our experiments have shown that the expression of Synaptopodin is normally activated in differentiating podocytes localized in the anterior compartment of the glomerular cup. In this situation, filtration might start normally and the production of primary urine might occur in a normal way. However, seen the restriction of the lumen of the rudimentary tubule, the primary urine might not easily come out of the glomerulus, possibly provoking the observed obstructive glomerular dilation (Figure 4G).

Human fetuses carrying *HNF1B* mutations are often affected by severe abnormalities of the kidney and frequently present with prominent glomerular cysts. Our 3D analysis has demonstrated that maturing glomeruli are characterized by a short tubular segment that is directly inserted in the glomerular cup via the vascular pole. This aberration is reminiscent of the defective remodeling of the UP observed in mouse *Hnf1b* defective nephron precursors. These observations suggest that a common general mechanism (discovered in a mouse model and validated in human embryos carrying *HNF1B* mutations) is at the base of glomerular cyst formation in HNF1beta-deficiency. Our results demonstrate that a distortion of the program of glomerulogenesis may lead to glomerular cyst formation.

The nature of the mechanisms that lead to the remodeling of the urinary and the vascular pole during glomerulogenesis is still largely unknown. The first event that occurs during this morphogenetic process is the separation of tubular and podocyte precursors by transitional cells. A careful analysis of these cells and their neighboring environment (immediately adjacent podocytes or tubular cells) has led to the identification, at least partially, of their subtle genetic program. Their common molecular signature is the expression of low levels of *Hnf1b* and *Wt1* compared to the neighboring cells that express high Wt1 and no Hnf1b or vice versa no Wt1 and high Hnf1b in podocyte plate and tubular cell precursors, respectively. Interestingly, we observed that some parietal cells of this specific territory are characterized by the expression of a set of tubular/podocyte markers (Jag1 or Synpo as an example). Together, these observations underline a possible heterogeneity inside the parietal cell population during nephrogenesis. Depending on the morphogens/signaling that received these specific parietal cells, they could activate specific genetic programs leading to differentiate toward podocytes in certain conditions. However the potential of these cells to transdifferentiate into functional podocytes under pathological conditions remains to be characterized in more details.

In conclusion, our study has shed a novel light on the mechanisms that govern some crucial steps of glomerulogenesis. In particular, our results focused on the critical events at the basis of the remodeling of the UP. We discovered an unappreciated function of HNF1beta during glomerulogenesis, by showing its key role in the regulation of the genetic program controlling this remodeling. Altogether, these results show a novel mechanism of glomerular cyst formation, based on the developmental aberration of glomerulogenesis.

MATERIAL AND METHODS

Mice

Specific inactivation of *Hnf1b* in the metanephric mesenchyme was obtained using a Cre-LoxP strategy. Six2-Cre (Kobayashi et al., 2008), *Hnf1b LacZ/+* and *Hnf1bf/f* mice (Coffinier et al., 1999a, 2002) were previously described. Since *Six2-Cre*; *Hnf1bf/+* or *Six2-Cre*; *Hnf1b LacZ/+* mice were indistinguishable from wild type mice, all these animals were used as controls (indicated in this study as "control"). Animals were maintained in an animal facility licensed by the French Ministry of Agriculture (agreement A 75-14-02). All experiments were conforming to the relevant regulatory standards.

Samples preparation and immunofluorescence experiments

Mouse samples

Mouse kidneys were dissected from embryos at E17.5 and E18.5 and from newborn pups (P0-P1). Immunofluorescence analyses were performed on 5 µm paraffin sections or 100-200 µm vibratome sections. Paraffin sections were subjected to antigen retrieval in boiling citrate buffer (Dako, S2369) for 15 minutes. For the detection of the antigens sections were treated with the MOM Kit (Vector Labs, BMK-2202) according to the manufacturer's instructions. Sections were incubated with antibodies in PBS tween 0.1 % (Supplemental Experimental Procedures, Table 1) at 4°C overnight. After several washes in PBS, samples were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen; 1:250) for 1 hour at room temperature or overnight (for vibratome sections). Experiments were performed on minimum three animals per genotype.

Human samples

Human fetuses carrying a *HNF1B* missense mutation c.494G>A (p.Arg165His) have been described in (Rasmussen et al., 2013). For immunofluorescence experiments, small pieces of paraffin blocks were deparaffinated, and sectioned with a vibratome to obtain thick sections (300 or 500 μ m). After antigen retrieval in boiling citrate buffer (Dako, S2369) for 15 minutes, sections were incubated with antibodies at 37°C for 5 days. After 3-4 washes in 0.1% Triton, 0.1% Na Azide in PBS at 37°C on a wheel for a total period of 3 days,

samples were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen; 1:250) in 0.1 % Na Azide in PBS at 37°C on a wheel for 3 days.

For some experiments, thick vibratome sections or thick paraffin section of human fetuses were clarified using the BABB (Benzyl-alcohol; Benzyl-benzoate; 1:2) solution, as previously described (Kolesová et al., 2016). Sections were analyzed with a Leica microscope equipped with a Yokogawa CSU-X1M1 spinning disk. Images were analyzed with ImageJ (NIH). IMOD software (<u>http://bio3d.colorado.edu/imod</u>) (Kremer et al., 1996) was used to create the 3D reconstructions from the confocal images.

AUTHOR CONTRIBUTIONS

E.F. and M.P. conceived the project and supervised the work. M.R, M.R and MCG provided human kidney specimens. A.F., A.C. and F.M performed the experiments. A.F., A.C., F.M. and S.G. analyzed and interpreted the data. E.F and M.P. wrote the manuscript.

COMPETING FINANCIAL INTERESTS: The authors declare no financial competing interests

ACKNOWLEDGEMENTS

We thank all the members of Marco Pontoglio's lab, Luisa Dandolo, Jerome Rossert and Fabiola Terzi for helpful discussions and critical reading of the manuscript. We are very grateful to the platform of Imagerie Cellulaire, Morphologie et Histologie and the animal house facility of the Cochin Institute. This work was supported by the Fondation pour la Recherche Médicale (equipe FRM) and the Fondation Bettencourt-Schueller (Prix Coup d'Elan); European Community's Seventh Framework Programme FP7/2009 [agreement no: 241955, SYSCILIA]; and Agence National pour la Recherche. F.M. was supported by Société de Néphrologie, Association Polykystose France and Fondation pour la Recherche Médicale fellowships, Arianna Fiorentino was supported by EU FP7/2012 Marie Curie ITN [agreement n°: 317246 "TRANCYST"], Armelle Christophorou was supported by European Community's Seventh Framework Programme FP7/2009 [agreement n°: 241955, SYSCILIA] and Fondation pour la Recherche Médicale fellowships.

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A. Fiorentino

FIGURE LEGENDS

Figure 1: The absence of HNF1beta impairs the remodeling of the urinary pole.

A-B: Immunofluorescence on vibratome sections with an anti-laminin antibody. In wild type, the Urinary pole (UP) is separated from the inner layer of glomerular cup by an epithelial protuberance (arrowhead in A and C) whereas in mutant the UP (arrows in B) is inserted inside the glomerular cup. UP: urinary pole, VP: vascular pole. Scale Bars: 10 μ m. Green: Laminin A; Blue: Dapi. C-F: Tridimensional reconstruction of wild type (C, E) and mutant (D, F) nephron glomerular precursors. In mutant embryos, the UP is trapped inside the glomerular cavity (D, F). Blue: Tubular lumen and urinary space, Pink: Basal membrane of Bowman capsule of the glomerular cup, Red: Basal membrane of the tubular component. Scale Bars: 50 μ m. G: Schematic representation of some critical steps of normal and mutant glomerulogenesis.

Figure 2: Absence of formation of the epithelial protuberance in the aberrant mutant developing glomerulus.

Indirect immunofluorescence against WT1 (in Red), Synaptopodin (in White) and Betagalactosidase (driven by the endogenous *Hnf1b* promoter, in Green). **A-F**: In wild type, cells expressing low WT1 and low betagaloctosidase separate the tubular component from the filtration compartment (arrowheads in A and E). In the mutant, (B, D, F) the inner layer of the glomerular cup is patterned in an anterior and a posterior compartments. Low Wt1-low betagalactosidase cells (orange in Merge) are observed around the tubular junction (orange arrowheads in B and F), whereas High Wt1 and not expressing *Hnf1b*-betagal cells (red) are restricted to the anterior part. A comparable expression of Synaptopodin is seen in developing podocytes and the parietal cell immediately adjacent in mutant (arrow in D) and control (arrow in C). **G-L:** In wild type, the developing podocytes and the adjacent parietal cells develop an epithelial protuberance (arrow in G, I, K), whereas these cells remain flat in the mutant (arrow in H, J, L). Scale Bars: 10µm. **M:** Schematic representation of wild type and mutant immature glomeruli.

Figure 3: The mutant tubule is constricted and surrounded by capillaries and mesangial cells.

A, B: Immunofluorescence with Jag1 antibody and Phalloidin staining on vibratome sections. In spite of the defective expansion of the prospective tubular component, the second cleft is formed in the mutant (arrow in B) but its localization is closer to the vascular cleft (B, arrowhead) compared to the wild type situation (A, arrow and arrowhead, respectively). **C-H:** In the control embryos, capillaries (GSA-lectin, GSLI) and mesangial cells (expressing *Pdgfrb*) (C and E, respectively) colonize the vascular cleft whereas in the mutant, capillaries (D) and mesangial cells (F) surround 360 degrees the tubule that is inserted in the glomerular cavity. **(I-K):** Phalloidin staining shows that the constriction of the tubule (arrows in I, J and K) is accompanied by the organization of F-actin filaments. Scale Bars: 10μm

Figure 4: Abnormal UP remodeling in fetuses carrying HNF1B mutation.

A, B: In *HNF1B* fetuses, the renal parenchyma was mostly represented by cystic structures (arrowheads in B) and few nephron precursors (arrows in B) compared to control renal tissue (A). Scale Bars: 200 μ m. **C, D**: In a fetus with *HNF1B*-deficiency, the maturing glomerulus is characterized by an aberrant insertion of the tubule inside the glomerular cup (compare D to C) Scale Bars: 10 μ m.. **E, F:** Tridimensional reconstruction of *HNF1B* deficient nephron precursors showing abnormal tubular expansion and aberrant UP localization (F) compared to control (E). Scale Bars: 50 μ m **G**: Models depicting the glomerular cyst formation in the absence of HNF1beta. In HNF1B deficiency, mesangial cells are all around the tubule and might provide the reason for the constriction of the tubular glomerular outflow, and glomerular cystic expansion upon the onset of glomerular filtration.

SUPPLEMENTAL FIGURE

Supplementary Figure 1 (related to Figure 2): The patterning of cell types in developing glomeruli is not modified in the absence of HNF1beta. A-F: The expression of *Pax2* in control and mutant glomerular cup (arrow in A and B, respectively) was comparable, whereas the tubular expression of *Pax2* was extended to all the tubular compartment in mutant (B) whereas it was restricted to distal tubule in the wild type (A). Immunofluorescence with anti-Jag1 (G-L) and anti-Claudin1 (M-R) antibodies. Mutant glomerular and tubular cells showed restricted expression of *Jag1* in tubular precursors and *Cldn1* (Claudin1) in parietal glomerular precursors, similarly to control embryos. Arrows and arrowheads in (K,L) and (Q,R) showed the border of expression of *Jag1* and *Cldn1*, respectively. Scale Bars: 10µm.

SUPPLEMENTAL INFORMATION

Movie S1, related to the Figure 1C: Tridimensional reconstruction of a control precapillary loop stage

Movie S2, related to the Figure 1D: Tridimensional reconstruction of a *Hnf1b* deficient precapillary loop stage

Movie S3, related to the Figure 1E: Tridimensional reconstruction of a control maturing nephron

Movie S4, related to the Figure 1F: Tridimensional reconstruction of a *Hnf1b* mutant maturing nephron

Movie S5, related to the Figure 3G: Confocal stacks of Pdgf R β and GSL I staining of a control precapillary loop stage

Movie S6, related to the Figure 3H: Confocal stacks of Pdgf R β and GSL I staining of a *Hnf1b* mutant precapillary loop

Movie S7, related to the Figure 3I-J-K: Confocal stacks of Phalloidin staining of a *HNF1B* mutant precapillary loop

Movie S8, related to the Figure 4C: Confocal stacks of WT1 and PNA staining of a precapillary loop stage from a control human fetus

Movie S9, related to the Figure 4D: Confocal stacks of WT1 and PNA staining of a precapillary loop stage from a human fetus carrying a *Hnf1b* mutation

Movie S10, related to the Figure 4E: Tridimensional reconstruction of a precapillary loop stage from a control human fetus

Movie S11, related to the Figure 4F: Tridimensional reconstruction of a precapillary loop stage from a human fetus carrying a *HNF1B* mutation

Supplementary Table 1: List of antibodies

	Target protein	Source	Dilution
Rabbit	JAG 1	Santa Cruz (sc-8303)	1:200
Rabbit	SYNAPTOPODIN	Santa Cruz (sc-50459)	1:200
Rabbit	NOS1	Santa Cruz (sc-8309)	1:200
Rabbit	CADHERIN 6	Homemade	1:200
Rabbit	LAMININ	Sigma (L9393)	1:200
Rabbit	PAX2	Invitrogen (71-6000)	1:200
Rabbit	CLAUDIN-1	Abcam ab15098	1:200
Rabbit	PDGF R β	Cell Signalling (#3169)	1:100
Mouse	WT1	DAKO (M356101)	1:200
Mouse	E- CADHERIN	BD transduction laboratories (610181)	1:200
Chicken	BETA GALACTOSIDASE	Abcam (Ab9361)	1:300
_	Peanut Agglutinin (PNA)	Vector Laboratories(RL1072)	1:250
_	Lectin from Bandeiraea simplicifolia (Griffonia simplicifolia) (GSL I)	Sigma (L5264 TRITC conjugate)	1:500
_	Texas Red-X Phalloidin	Invitrogen (T7471)	1:50

FIGURES PART I



FIGURE 1





FIGURE 2



FIGURE 3



FIGURE 4





FIGURE S1

RESULTS PART II

Role of HNF1beta in renal tubules

(Paper in preparation)

HNF1B mutations are responsible for a wide spectrum of renal structural abnormalities frequently associated with tubular dysfunctions. Remarkably, recent studies highlighted the high prevalence of *HNF1B* mutations in adult patients with renal cysts and renal failure from unknown origin. In these patients, the most frequent clinical presentation is a tubulointerstitial nephritis, associated at the histological level with interstitial fibrosis (Faguer et al., 2011). The molecular and cellular mechanisms at the basis of this phenotype are not completely elucidated. Interestingly, analysis of mouse models with renal ischemia has shown that *Hnf1b* expression was significantly upregulated shortly after injury during the recovery phase (Faguer et al., 2013). In addition, our laboratory has shown that *Hnf1b* deficient quiescent tubules, when forced to proliferate again, developed tubular dilation, due to the absence of re-expression of HNF1beta target genes after cell division (Verdeguer et al., 2010). Altogether these results suggest a potential involvement of this factor in tubular regeneration.

In this second part of my project, my aim was to decipher the genetic program controlled by HNF1B in proliferative versus quiescent renal tubules. Previously, our laboratory has showed that the tubular phenotype elicited by the deficiency of *Hnflb* depends on the cellular status (proliferative versus quiescent status). Using an MxCre strategy to inactivate *Hnflb* in postnatal tubular structure, we demonstrated that the expression of HNF1beta target genes was strongly decreased during tubular elongation (proliferation) whereas target genes were still expressed in quiescent tubular cells (Verdeguer et al 2010). With this strategy, inactivation of *Hnf1b* was mainly achieved in the medullary region (Verdeguer et al 2010). To delete this gene in all tubular cells, I took advantage of a Ksp-Cre ER^{T2} mouse strain carrying a tamoxifen inducible Cre recombinase under the control of the *Ksp* promoter, which is known to target recombination guite efficiently in all tubular segments (Lantinga-van Leeuwen et al., 2006). I decided to analyze the effect of the deletion of *Hnf1b* at two distinct phases of postnatal kidney maturation. The first was the postnatal period (P0), which is characterized by massive proliferative tubular elongation; whereas, the second step that we considered was the post-weaning period (P21-23), characterized by a much lower proliferation context. For both periods of inactivation, we

A. Fiorentino

decided to sacrifice control and mutant mice, at 7 days and 1 month after tamoxifen treatment (Figure 27).



Figure 27. Strategies of the inducible *Hnf1b* inactivation. (A) In the post-natal inactivation approach, tamoxifen was injected in the mother during lactation for 2days after birth. Mice were sacrificed 5days and one month after tamoxifen administration respectively. (B) In the post-weaning inactivation of *Hnf1b* strategy, animals were gavaged for 3 days between P21-P23. Mice were sacrificed 5days, 2 weeks and one month after tamoxifen administration.

As a first approach, we verified the efficiency of *Hnf1b* inactivation. Already one week after treatment, at P7 or P28, the expression of *Hnf1b* was lost in the vast majority of tubular cells and only few scattered cells in the parenchyma escaped the inactivation. In particular at P28 tubular cells still expressing *Hnf1b* were mainly localized in the S3 portion of the proximal tubule. When inactivation was performed just after birth, mutant animals developed severe growth retardation and most of them have been sacrificed few days before the scheduled time (between P29 and P32). *Hnf1b* deficient kidneys were cystic and enlarged, and body weight in mutant mice was strongly reduced compare to control. Histological analyses showed that cysts were localized all over the renal

parenchyma, from the cortex to the medulla, and derived from all tubular segments. Remarkably, we discovered that the cystic lesions were surrounded by an intense interstitial fibrosis. When *Hnf1b* was inactivated at post-weaning period, mutant animals were small with a difference in weight that was already significant 2 weeks after inactivation. At the macroscopic level, mutant kidneys were undersized compare to control, and strikingly, their weight remained constant during the period of follow-up contrasting with the strong increase in weight observed in control kidneys. At the histological level, *Hnf1b* deficient proximal tubules were enlarged, lined by flattened cells with an impaired brush border. Analysis of tubular markers showed that large areas of renal parenchyma were devoid of proximal and distal markers, whereas Henle's loop and collecting duct markers were normally expressed. In addition, we discovered that in mutant kidney, the atrophic tubular lesions were surrounded by interstitial fibrosis, revealed by Picro Sirius Red staining, and the presence of vimentin in immunofluorescence. Interestingly, vimentin immunodetection was also observed in flattened tubular cells of enlarged proximal tubule. This aberrant expression of Vim was associated with a cytoplasmic delocalization of the LTL staining, an apical marker of proximal tubules in control kidneys. Altogether these results suggest that the lack of *Hnf1b* in tubules leads to a partial dedifferentiation of mutant cells.

In post-weaning inactivation, to determine if these morphological alterations could have an impact on renal function, we decided to monitor urea and creatinine levels at 5 days, two weeks and one month after tamoxifen treatment. Renal function was already slightly impaired two weeks after inactivation and plasma urea and creatinine levels became significantly increased at P53. These results showed that the lack of *Hnf1b* in tubules led to progressive renal failure, reminiscent of the renal manifestations observed in some adult patients carrying *HNF1B* mutations. Moreover, we showed that *Hnf1b* inactivation after weaning leads to tubular dysfunctions characterized by microalbuminuria, hypermagnesuria, hyperkaluria and glucosuria without hyperglycemia.

It has been recently shown that HNF1B controls indirectly the transcription factor *Zeb2*, involved in epithelial to mesenchymal transition, through the induction of the *miR-200*, its direct inhibitor (Hajarnis et al., 2015). We decided to monitor the levels of expression of this factor in our mutant mice. One month after the post-weaning inactivation of *Hnf1b*, the expression of *Zeb2* was increased, in parallel with a decrease in the expression of *miR-200*.

This result suggests that in the absence of *Hnf1b*, this misregulated molecular cascade could lead to a partial mesenchymal transition of mutant tubular cells.

In order to unravel the complete genetic program controlled by HNF1B during the two phases of postnatal development, we performed an affymetrix analysis at all time points of sacrifice (P7, P32, P28 and P53). The comparison gene expression at different developmental stages revealed that a considerable number of genes varied in their expression level. In particular, it is worth to note that in spite of an unchanged histology 5 days after inactivation at P7 and P28, there was already a massive deterioration in gene expression and the expression of the majority of genes tended to be decreased, indicating that HNF1beta predominantly acts as a positive regulator of transcription. Interestingly, a month later, the situation was inverted: at P32 the majority of genes become predominantly overexpressed whereas, at P53, one month after the post weaning inactivation, a massive change in gene expression was observed with a high number of genes upregulated and downregulated. Interestingly, Ingenuity pathway analyses performed on affymetrix data showed that two major pathways were particularly affected at both periods of inactivation, one month after tamoxifen treatment: nephron development and differentiation pathway and pro-inflammatory and fibrotic pathway.

In the second part of my study, I described a crucial dual role of HNF1B in the proliferative versus quiescent tubular cells. In a proliferative context, *Hnf1b* deficiency induces cystic dilations whereas in a quiescent status its absence is associated with a partial EMT process in the tubular epithelial cells. Surprisingly, at both phases of post natal development, the tubular deficiency of this transcription factor activates pro-fibrotic pathway in the kidney.

Classification: Biological Science

Partial epithelial to mesenchymal transition in the absence of HNF1beta in mature renal tubules

INTRODUCTION

Chronic kidney disease (CKD) is an epidemic pathology affecting around 10% of all adults in the world, and every year, millions of patients die prematurely of complications related to CKD. Both clinical and subclinical insults contribute to CKD development, including infections, xenobiotics, toxins, mechanical obstruction, and immune complexes resulting from autoimmune diseases or chronic infections, and genetic disorders. The most common causes of CKD in developed nations are, however, type-2 diabetes mellitus and ischemic/hypertensive nephropathy, which frequently coexist in the same kidney or complicate other diseases. More recently, several studies have highlighted the role of tubular damage or tubular dedifferentiation itself as a major player in interstitial fibrosis, a pathology that can progress to end stage renal disease. Interstitial nephropathies with renal failure have been recently associated with adult patients carrying mutations in Hepatocyte Nuclear Factor 1B (HNF1B) gene (Faguer et al., 2011). This gene encodes for a transcription factor that belongs to the POU family (from Pit1/Oct/Unc). In pediatric patients, mutations in HNF1B represent one of the most prevalent genetic defects responsible for Congenital Abnormalities of the Kidney and Urinary Tract (CAKUT) (Decramer et al., 2007; Heidet et al., 2010; Ulinski et al., 2006), which are responsible for about 40% of cases of end stage renal disease in children (Harambat et al., 2012). Most of the molecular mechanisms leading to these pathologies are still largely unknown.

Animal models have provided powerful tools for understanding the mechanisms of diseases. In this respect, mouse models for *Hnf1b*-deficiency have shown that the inactivation of *Hnf1b* with specific Cre recombinases may recapitulate most of the different phenotypes observed in patients (Gresh et al., 2004; Massa et al., 2013; Verdeguer et al., 2010). For instance, the deficiency of *Hnf1b* specifically in tubular precursors during their elongation/expansion phase leads to a polycystic phenotype whereas a postnatal inducible inactivation strategy 10 days later does not elicit any phenotype. At the molecular level, analysis of this paradoxical observation led us to discover that the expression of HNF1beta target genes was lost in proliferative tubular cells but was maintained in quiescent cells. This result has unraveled a previously undescribed aspect of gene regulation by HNF1beta. This transcription factor remains attached to chromatin during mitosis, suggesting that HNF1beta acts as a bookmarking factor that is

necessary for reopening the chromatin of target genes after mitotic silencing (Verdeguer et al., 2010).

The intriguing observation that the cellular status, proliferative versus quiescent plays a crucial role in the regulation of gene expression prompted us to decipher the complete landscape of the genetic program controlled by HNF1beta in elongating tubules versus quiescent tubules. In the present study, we show the crucial role played by HNF1beta in the molecular cascades regulating the maintenance of the differentiation in tubular cells. More importantly, we demonstrated that the disruption of this program mechanistically leads to chronic kidney disease that represents a serious issue of public health.

RESULTS

1) Postnatal inactivation of *Hnf1b* at different time points in renal tubular cells

Tubular elongation in early postnatal life is based on intense and synchronized proliferation. Around two weeks after birth, this intense phase of proliferation strongly decreases and affects a small proportion of asynchronous tubular cells, which continue to divide until kidneys reach their definitive size in adult animals.

In order to identify the genetic program controlled by HNF1beta in proliferating versus quiescent tubular cells, we crossed *Hnf1b f/f* mice (Coffinier et al., 2002) with mice carrying a tamoxifen inducible Cre recombinase under the control of the *Ksp* promoter (*Ksp*-CreER^{T2}) (Lantinga-van Leeuwen et al., 2006). This mouse strain is known to target efficiently recombination in most nephron tubular segments. We decided to analyze the effect of the deletion of *Hnf1b* in two distinct phases of postnatal kidney maturation. The first is the perinatal period, which is notoriously characterized by massive proliferative tubular elongation whereas the second step that we considered is the postweaning period, characterized by a much lower proliferation context.

As a first approach, we verified that already few days after tamoxifen administration, we had a prompt and significant decrease in HNF1beta protein. Immunofluorescence experiments performed at P7 and P28 demonstrated that 7 days after the beginning of tamoxifen administration, the majority of tubular cells lost the expression of the protein in mutant animals (Fig 1 A-B and C-D). At P28, few scattered cells escaped the genetic deletion, mostly localized in the straight part of the proximal tubule at the cortico-medullary region (Fig1 F-K). RT-qPCR experiments have shown that five days after the end of tamoxifen treatment (P7), there is a slight decrease in Hnf1b expression in the early inactivation strategy (Fig1E). The decrease in Hnf1b expression is more pronounced in mutant mice 30 days after early inactivation (P32) (Fig1E). In the post-weaning inactivation strategy, there is a significant decrease of Hnf1b expression already five days after tamoxifen treatment (P28), which is maintained 30 days after (P53) (Fig1L). We therefore decided to collect kidney samples either shortly after the deletion of Hnf1b (5 days), namely P7 and P28, or 30 days later, namely P32 and P53, respectively, to monitor the fate of kidneys deleted for HNF1beta for a longer period of time.

Our results have shown that few tubular cells escaped the recombination event at both perinatal and post-weaning periods. To verify if the escapers have a selective proliferative advantage, we performed qPCR analyses at P32 and P53, respectively. Our results showed that the residual levels of *Hnf1b* did not increase during this period suggesting that tubular structures were mostly composed of *Hnf1b* inactivated cells at these two time points (Fig1E and 1L).

2) Early deletion of *Hnf1b* leads to a polycystic phenotype

Early inactivation strategy led to premature lethality in juvenile mutant animals. Only few mutants survived until P32, and most of the mutant mice have been sacrificed few days before the scheduled time point, around P29, due to severe growth retardation (Fig2A).

Macroscopic analysis of the kidney showed that mutant kidneys were enlarged compared to control, with few irregularities on the surface of the kidney, due to the presence of cysts (Fig2 D-E). Kidney weight was significantly higher in mutant animals, representing twice the weight measured in control (Fig2B). In addition, the Kidney Weight/Body Weight ratio was increased in mutant, due to the combination of a lower body weight and a higher kidney weight in mutant compare to control mice (Fig2C).

Histological analysis at P7 demonstrated that despite the important loss of Hnflb expression in tubular cells, the renal parenchyma did not show major abnormalities in the tubular structure (Fig2 F-G). On the contrary, at P29-32, numerous cysts were scattered in the entire renal parenchyma, from the cortical to the medullary region (Fig2 H-I). As previously shown by numerous studies, the absence of HNF1beta or the expression of a dominant negative of this transcription factor during tubular elongation leads to a polycystic phenotype (Gresh et al., 2004; Hiesberger et al., 2004, 2005; Verdeguer et al., 2010). In contrast with the predominance of medullary cysts in these models, our results showed that cystic tubules derived from all segments of the nephron, such as proximal tubules stained by LotusTetraglobulus Lectin and collecting duct stained by AQP2 (Fig3 A-D; Fig3E-H). This apparent discrepancy could be due to the different Cre expression pattern in the different mouse lines. Indeed, the KspCreER^{T2} recombinase targets efficient recombination in the proximal tubules (Lantinga-van Leeuwen et al., 2006) in addition to its strong expression in the distal part of the nephron. Our results showed for the first time that inactivation of *Hnf1b* just after birth in cortical tubules leads to cyst formation. In addition to the presence of cystic lesions, some fibrotic areas surrounding the tubular cysts have been identified with Picro Sirius Red staining (Fig4 A-D) and immunoflorescence experiments using an anti-vimentin antibody (Fig4 E-H). The fibrotic process was associated with an increase in the mRNA levels of several markers of fibrosis, such as *Col1a1, Tgfb1, Acta2, Vim* at P32 (in affymetrix and RT-qPCR) (data not shown). These observations are in accordance with the description of interstitial fibrotic process observed around cystic lesions in numerous PKD mouse models. Nevertheless the molecular determinants involved in the formation of fibrosis remain to be clearly identified.

3) Late inactivation of *Hnf1b* leads to impaired tubular cell morphology.

Inactivation of *Hnf1b* in the post-weaning period did not compromise the survival of mice and all mutant animals survived until one month after *Hnf1b* deletion. Nevertheless, at P53, mutant animals were obviously smaller compared to their littermate control, with a difference in weight already significant 15 days after *Hnf1b* inactivation (Fig 5A).

Surprisingly, macroscopic examination of the kidney identified a severe defect in renal size in mutant mice (Fig 5D-E). During the experimental follow up after tamoxifen administration, kidney weight did not change in mutant whereas in control animals, kidneys harmoniously continue to grow (Fig 5B). This defect in kidney weight could not be related to the difference in global weight observed in mutant animals, since the KW/BW ratio was specifically decreased in mutant mice (Fig 5C).

In order to understand this phenotypic abnormality, we decided to analyze the mutant kidneys at the histological level. Hematoxilin-Eosin stained sections of mutant kidney did not show any specific alterations in the cortico-medullary organization of the kidneys at P28, P39 and P53 (Fig 5F-K). Nevertheless, the global tubular mass was strongly decreased in mutant one month after inactivation and tubular dilations, already detectable at P39, were clearly observed in the cortex at P53. A more careful analysis of the proximal tubular structures identified some morphological alterations. In particular, some proximal tubular cells presented a decreased differentiated brush border, had lost their typical cobblestone shape, and appeared flattened, (Fig 6A'-B'), suggesting a partial loss of the characteristics of terminal differentiation in these cells.

In order to determine the nature of the tubular structures affected by these abnormalities, we analyzed the expression of specific markers of tubular subsegment. Immunofluorescence experiments with LTL, specific for proximal compartment, and Wheat Germ Agglutinin (WGA), a lectin specific for glomeruli and distal tubules in adult kidney showed a strong decrease of these markers in large areas of the cortex (Fig 6C-H). On the contrary, the expression of Henle's loop and collecting duct markers, NKCC2 (SLC12A1) and AQP2 respectively, was preserved (Fig 7A-L). In addition, RT-qPCR experiments demonstrated that the mRNA of *Slc5a2*, a Na⁺/glucose cotransporter expressed in proximal tubular was strongly decreased at P53 (Supplementary Fig1C). Strikingly, its expression was already impaired at P28, even in the absence of any tubular defect at this time point. Conversely, several mRNAs normally expressed in the Henle's loop (*Slc12a1*), distal tubule (*Slc12a3*) and collecting duct (*Aqp2*) were increased, both at P28 and P53 in mutant mice (Supplementary Fig1 A-B-D).

We had previously shown that the absence of HNF1beta in mature tubules does not modify the expression of specific targets of HNF1beta, known to be decreased during tubular elongation. In particular, *Pkd2* and *Pkhd1* expression, in addition to the expression of other polycystic genes, were not affected using *Mx*-Cre inactivation strategy (Verdeguer et al., 2010). In the present model, the pattern of inactivation was different, including most of the tubules in the cortex with an impressive inactivation in proximal tubules. Similarly to the previous observation, most of the mRNA levels of HNF1beta target genes were comparable to the control, and only a slight downregulation of *Pkhd1* was observed at P53 (data not shown).

4) Interstitial fibrosis in post weaning inactivation of Hnf1b

In addition, the morphological defects, these *Hnf1b* defective tubules were surrounded by dense fibrotic deposits in the interstitium, stained by Picro Sirius Red, which underlined the presence of collagen type I and III (Fig 8A-F). In order to better characterize this fibrotic phenotype, we analyzed the expression of some markers usually upregulated during this process. RT-qPCR experiments have demonstrated that the mRNA levels of several markers of interstitial fibrosis were increased, including *Colla1, Acta2, Tgb1* and *Tgf2* (Supplementary Fig 2A-D). In addition we monitored the expression of *Vim*, usually observed in interstitial fibroblastic cells but not in epithelial cell. As expected, vimentin was detected in the focal areas of fibrosis, already at P39 (Figure 8I-J) and in more extended territories (Figure 8K-L) at P53. Interestingly, a de novo expression of vimentin was observed in proximal tubular epithelium already at P39, specifically in cells with an atrophic tubular brush border and a loss of their typical cuboidal epithelial shape. Co-

staining with LTL showed that vimentin positive cells presented a delocalization of LTL staining in the cytoplasm compared to its apical localization in control (Fig 9A-L). LTL recognizes α -linked L-fucose containing glycoproteins. The fucosyl transferase 9 (*Fut9*) is dramatically downregulated in mutant animals at all stages (data not shown).

The renal abnormalities displayed by mutant mice at P53 are represented by interstitial fibrosis and tubular atrophy. It has been recently shown that HNF1beta indirectly controls the transcription factor ZEB2, involved in epithelial to mesenchymal transition (EMT), through the induction of the miR-200, its direct inhibitor (Hajarnis et al., 2015). In our mouse model Zeb2 expression was increased at P28, and remained upregulated one month after the post-weaning inactivation of Hnflb (Fig 9N). In parallel we monitored the expression of the long non coding transcripts giving rise to miR-200. These LncmiR were downregulated by 2 folds at P53, and already showed a tendency to be decreased at P28 (Fig 9O-P). Interestingly, miR200 has also being showed to repress the expression of *Pkd1* (Hajarnis et al 2015). Several studies have highlighted the importance of a tight control of gene dosage of Pkd1 to conserve a normal tubular structure. Both inactivation (Lantingavan Leeuwen et al., 2007) and overexpression (Kurbegovic et al., 2010) of this gene in mouse give rise to a polycystic phenotype. In our model, the down regulation of miR200 could lead to an upregulation of *Pkd1* and thus to tubular dilation (data not shown). Altogether, these results suggest that the absence of HNF1beta during the postweaning period induces a partial epithelial to mechenchymal transition in tubular epithelial cells, which in turn triggers a fibrotic response in the interstitium surrounding these affected tubules.

5) Renal failure and tubular dysfunctions in postweaning inactivation of Hnflb

In order to determine if these morphological abnormalities could have functional consequences, we investigate renal function in these animals at different ages (P28, P39 and P53). Both plasma urea and creatinine concentrations were increased in mutant mice at P53, while renal function was still normal at P28 and P39 (Fig 10A-B). Given the observed loss of differentiation markers in mutant tubular cells, we wondered if mutant animals also suffer from tubular dysfunctions. Mutant animals suffered from glucosuria (without hyperglycemia) (Fig 10E-D) and microalbuminuria, already at P39 (Fig 10I). Calcium and potassium in the urine were increased, whereas the excretion of uric acid was decreased (Fig 1F-H-J). Unexpectedly, hypomagnesaemia was found in mutant animals (Fig 10C),

with an increased but not significant excretion of magnesium in the urine (Fig 10G). In addition, metabolic cages experiments showed that the 24h/Volume of urine in mutant at P53 was increased compared to wild type (Figure 10L). These results showed that the deficiency of HNF1beta in tubules after weaning leads to chronic kidney disease with renal failure and tubular dysfunctions. Interestingly, tubular dysfunctions preceded the increase in urea and creatinine levels observed at P53. They could therefore be attributed to the deficient of the tubular *Hnf1b* expression, and not be considered as secondary effect of the degradation of renal function. Most of these abnormalities are reminiscent of the biological abnormalities observed in adult patients carrying *HNF1B* mutations.

6) Genetic program controlled by HNF1beta during postnatal and postweaning period

In order to better understand the genetic program of this transcription factor at these two time points, during tubular expansion and homeostatic growth, we performed affymetrix analysis at P7 and P32 for the first model and P28 and P53 for the second time point.

Principal component analysis of gene expression levels led to a consistent repartition of samples. As expected the transcriptome of mice at P7 both wild-type and mutants were separated from the rest of the other samples since there is a large number of genes that are specifically differentially expressed at this stage. The comparison of the gene expression at different developmental stages revealed that a considerable number of genes varied in their expression level of the majority of genes tends to be decreased in the absence of HNF1beta, (103 vs 13 at P7) and (184 vs 36 at P28), indicating that HNF1beta acts predominantly with a positive effect on transcription. Interestingly, a month later, the situation is inverted at P32 with a majority of genes that becomes predominantly overexpressed in the animals that lacked the expression of HNF1beta (440 up vs 144 down). On the other hand, the kidney that were inactivated in a postweaning, non-proliferative context a month later showed a massive change in gene expression with 438 genes upregulated and 449 downregulated in the absence of HNF1beta.

Our results lead us to understand that at P7, in spite of a normal histology of the kidneys at this stage (Figure 2), there is an already consistent modification of gene expression with more than 100 genes differentially expressed (fold change >2 and a significance of P <0.05). The analysis of the results with the "Ingenuity Pathway" led us to realize that a

considerable and significant (P< 10^{-17}) number of genes were in common with the transcriptome of LHX1 kidney specific knockout. The embryos lacking the expression of *Lhx1 (Lim1)* are characterized by the defective development and differentiation of nephrons (Kobayashi et al., 2005), therefore the transcriptome of these embryos, compared to their controls, lacks the expression of all the genes that are normally expressed in nephrons. The overlap of this list of genes included *Umod, Tmigd, Slc5a8, Slc22a19, Pah, Mep1b, Kap, Hsd3b4, Fut4, Cyp2j5, Acsm2* and *Aadat*. All these genes are characterized by the fact that they are expressed in the nephron tubular structures. Another remarkable correlation at this stage is represented by the overlap of genes associated with the absence of PKD1 (P< 10^{-5}). All these results indicate that even before the manifestation of a clear histological phenotype, the differentiation state of renal tubular cells lacking HNF1Beta is already significantly affected.

Our results showed that at P32, the cystic epithelium of *Hnf1b*-deficient kidney is characterized by a transcriptome that presents with a drastic increase of T*gfb*1 activated pathway ($P < 10^{-29}$) and by the activation of pathways linked with several pro-inflammatory cytokines including TNF, IL1B ($P < 10^{-21}$) and IL13 ($P < 10^{-17}$).

Another remarkable finding is the considerable differential gene expression in kidneys following the inactivation at weaning and analyzed only 5 days later at P28. Also in this case, there is a significant overlap with the transcriptome previously mentioned (LHX1, P< 10^{-14}) and that of HNF1alpha (P< 10^{-12}), a close homologue of HNF1beta that is predominately expressed in proximal tubules. Therefore, similarly to what happens in kidney at P7, in spite of a normal histology, there is already a massive deterioration of gene expression. One of the surprising results that we observed is that the deletion of *Hnf1b* after weaning leads, in a matter of 30 days, to an even increased distortion of gene expression with globally almost 900 genes with a distorted gene expression level. The major significant "upstream effects" included *Lhx1* inhibited (P < 10^{-20}), Lipopolysaccharide activated (P< 10^{-15}); *Hnf1a* inhibited (P< 10^{-12}). Another remarkable pathway affected 30 days after the deletion of *Hnf1b* at weaning (P53 animals) is the defective LXR/RXR pathway (P< 10^{-10} , *Gc*, *Il33*, *IL1R1*, *Lbp*, *Lpl*, *Lyz*, *Nr1h4*, *Ptgs2* and *Serpinf2*.

Finally, the comparison of the canonical pathways affected by the inactivation of *Hnf1b* at different time points after birth is represented in the Figure 11. One of the most prominent features is the appearance of an Acute Phase Response that becomes evident only 30 days after the inactivation, either in the perinatal or the post weaning inactivation. This inflammatory response is accompanied by a massive activation of TGF- β and fibrosis in the renal interstitium.

DISCUSSION

By using a mouse line carrying a Cre recombinase that efficiently targets all renal tubular cells (*Ksp*CreER^{T2}, (Lantinga-van Leeuwen et al., 2006)), we have shown that the inactivation of HNF1beta during the intense proliferative phase of tubular elongation or in the post weaning period leads to different phenotypes that nevertheless share some common features. In the perinatal period, the lack of HNF1beta gives rise to cyst formation in all tubular segments, whereas its inactivation one month later leads to a partial loss of tubular differentiation. In both postnatal phases, the alteration of the genetic program controlled by HNF1beta in tubular cells triggers the formation of fibrotic areas around the mutant tubules.

During postnatal tubular elongation, several studies have already shown that the deficiency of *Hnf1b* in the distal part of the nephron is responsible for a polycystic phenotype (Gresh et al 2004; Verdeguer et al 2010). Here we show that proximal tubules, characterized by a more rare and scattered proliferation (personal observation) in postnatal period also develop cysts in the absence of HNF1beta.

Interestingly, HNF1alpha, a closely related transcription factor, is also expressed in proximal tubules. This transcription factor binds the same genomic consensus sequence of DNA (Baumhueter et al., 1988) and form homo or heterodimers with HNF1beta (Blumenfeld et al., 1991; Lazzaro et al., 1992; Rey-Campos et al., 1991). Nevertheless, in this tubular segment, the expression of Hnf1a, which is maintained in early postnatal inactivation, is not able to compensate for the absence of HNF1beta and to maintain a normal tubular structure. Conversely, the expression of Hnf1b in proximal tubules of Hnf1a-deficient mice is not sufficient to prevent the tubular dysfunction (Fanconi syndrome) observed in these mutant animals (Pontoglio et al., 1996). This suggests that in spite of an overlapping expression pattern in this specific sub portion of the nephron, and the property of these two transcription factors to bind the same genomic sequence, the

redundancy between them is not complete. These two factors have certain specificity in the regulation of their own targets, whose molecular nature is still largely unknown.

In addition to a severe tubular cystic phenotype, focal areas of fibrosis developed in renal parenchyma in mutant mice at P32, one month after inactivation. Several studies in human and animal models of PKD have also described an interstitial fibrosis associated with cysts formation. Interestingly, in some animal models, the deletion of the gene of interest (such as *Pkd1* or *Pkd2*) was restricted to the tubular compartment, similarly to our inactivation strategy. These observations suggest that the tubular damage itself could be at the origin of this fibrosis phenotype. In a recent study, these fibrotic processes have been proposed to be at the basis of the sudden burst of cyst formation in a model of late inactivation of *Pkd1* (Leonhard et al., 2015). Interestingly, in human, it has been proposed to use the degree of fibrosis as benchmark of disease progression in PKD patients. In fact the extent of fibrosis seems to represent a better predictive indicator of decline of GFR than the volume of the cysts (Caroli et al., 2011).

The inactivation of *Hnf1b* at post weaning period results in the absence of homeostatic kidney growth that normally leads to the increase in kidney size until adulthood. During the early postnatal period, around birth, the major morphogenetic process in the kidney is the formation and the maturation of the medulla, with an intense phase of tubular elongation at the corticomedullary junction. This process leads to the formation of Henle's loop, whose longer structures are at the basis of the formation of the cortico-medullary gradient. In contrast the increase in kidney size from juvenile to adult is mostly due to an increase in proximal tubular mass, which represent more than half of the total volume of the kidney. The phenotype observed at post weaning inactivation suggests that this increase of the proximal tubular component of the kidney did not take place in the absence of HNF1beta.

Blood analysis performed few days and one month after post weaning inactivation showed that mutant animals developed a progressive degradation of renal function. In addition, mutant animals also present with specific tubular functional alterations. These results are reminiscent of the biological abnormalities observed in some adult patients with *HNF1B* mutations. As an example, mutant animals presented a normoglycemic glucosuria due to defective reabsoption of glucose by the proximal tubule. This abnormality is due to the downregulation of *Slc5a2 (SGLT2)*, a tubular glucose/sodium cotransporter. Interestingly,

this cotransporter has already been shown to be a direct target of the closely related HNF1alpha transcription factor (Pontoglio et al., 2000). Interestingly, Fanconi syndrome with glucosuria has been recently described in two MODY5 patients (Faguer et al., 2011).

Our results have shown that the absence of HNF1beta during post weaning period resulted in a partial loss of tubular differentiation associated with interstitial fibrosis. Recent studies have highlighted the fact that tubular cells in the presence of a persistent injury can undergo a partial epithelial to mesenchymal transition (EMT) (Grande et al., 2015; Lovisa et al., 2015). This partial EMT can activate fibrosis through the release of effectors in the interstitial space that attract and activate the local fibroblasts. In our model, a *de novo* expression of mesenchymal markers normally present in interstitial cells was observed in proximal tubular cells concomitantly to the loss of some of their epithelial characteristics. All these data indicates that, HNF1beta is important for the maintenance of an epithelial differentiation during postnatal tubular maturation. In its absence, expression of *Zeb2* was upregulated via the down regulation of *miR200* that has been shown to be a direct target of HNF1beta (Hajarnis et al., 2015).

In conclusion, our study has shed a novel light on the mechanisms that govern some crucial steps of tubulogenesis. We discovered an unappreciated function of HNF1beta during homeostatic kidney growth and showed its key role in the regulation of the genetic program controlling the maintenance of the differentiation status of epithelial cells. Our results showed a novel molecular cascade between HNF1beta and profibrotic effectors in kidney fibrosis, which could play a major role in the progression to end stage renal disease in patients carrying *HNF1B* mutations.
Materials and Methods

Animals

Specific inactivation of *Hnf1b* in renal tubules was obtained using a *Ksp*-CreER^{T2} mice, kindly provided by DJ.Peters (Lantinga-van Leeuwen et al., 2006). *Hnf1bf/f* mice (Coffinier et al., 2002) were previously described. Juvenile mice (P23) have been treated with 1 mg tamoxifen/10g for three consecutive days, using a feeding needle. Weaning mothers received a similar dosage of tamoxifen for two days by intraperitoneal injection starting at postnatal day 1 of the progeny, and these newborn mice received tamoxifen via breast-feeding from the mother. Tamoxifen has been administrated to control Cre-; *Hnf1bf/f* mice (indicated in this study as "control").

Animals were maintained in an animal facility licensed by the French Ministry of Agriculture (agreement A 75-14-02). All experiments were conforming to the relevant regulatory standards.

Histological and Immunohistochemical Analysis

Mouse kidneys were harvested at P7 and P32 for the early inactivation strategy, and at P28, P39 and P53 for the post-weaning inactivation. Samples were fixed in 4% PBS diluted HistoFix (phosphate-buffered formaldehyde solution) (Carl Roth) for 24 hours and rinsed in PBS. After fixation samples were cut with a vibratome or embedded in paraffin. For histological analysis, 5µm paraffin sections were stained with hematoxylin and eosin, Periodic acid–Schiff (PAS) and Picro Sirius Red at the Necker's Hospital Histology Platform (Necker Hospital, Paris).

Immunofluorescence analyses were performed on 5 µm paraffin sections or 80-150µm vibratome sections. For paraffin sections antigen retrieval was performed in boiling citrate buffer (Dako, S2369) for 15 minutes. For the detection of the antigens, sections were first treated with the MOM Kit (Vector Labs, BMK-2202) according to the manufacturer's instructions, followed by an incubation with primary antibodies (see antibodies table) at 4°C overnight. After three washes in PBS, samples were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen; 1:250) for 1 hour at room temperature. Vibratome sections were incubated with primary antibodies diluted in PBS1X Triton 0,1%,

NaAzide 0,1% for 2 days, washed in PBS1X Triton 0,1%, NaAzide 0,1% for 24 hours and incubated 24 hours with secondary antibodies in the same solution. The slides were mounted in liquid mounting medium VectaShield (Vector H1000, Dako) and analyzed with Leica microscope equipped with a Yokogawa CSU-X1M1 spinning disk or with the "Lamina"-Perkin Elmer slide scanner. Figures have been analyzed with ImageJ, Pannoramic Viewer and NanoZoomer Digital Patology (NDP) sofwares and mounted with Above Illustrator. Experiments were performed on minimum three animals per genotype.

Host species	Target protein	Source	Dilution
Rabbit	Hnf1b (v1.6)	Homemade	1:100
Rabbit	Aquaporin2	Sigma (A7310)	1:400
Rabbit	Vimentin	Abcam (ab 92547)	1:200
Rabbit	Nkcc2	ProteinTEch (18970-1-AP)	1:200
_	Lotus Tetragonolobus Lectin(LTL)	Vector Laboratories (B-1325)	1:400
_	Wheat Germ Agglutinin(WGA)	Vector Laboratories (FL-1021)	1:400

Antibodies list

Plasma and urine collection

Plasma and urine samples were collected from mice at P28, P39 and P53. For plasma collection we used heparinated syringe [Heparin 5000 USP/mol (Sigma H3393-10k) in physiological serum]. Blood was collected and centrifuged at 2500 rpm and RT for 10 minutes to obtain the plasma. Samples were analyzed with Olympus AU400 Chemestry Analyzer (CRI – Plateforme de Biochimie UMR 1149 Université Paris Diderot).

Kidney weight and Body weight

Mice have been weighted before sacrifice. After dissection, fresh kidneys were weighted on high precision balance. We evaluated the ratio between kidney weight and body weight in mutant vs control mice.

Affymetrix

Expression patterning analysis was performed using the Affymetrix array MTA-1_0. The data obtained were analyzed with the Transcriptiomic analysis console 3.0 software.

RNA isolation and quantitative RT-qPCR

Collected kidneys were frozen in liquid nitrogen and stored at -80°C. RNAs were extracted from frozen samples in Phase lock gel tubes (5PRIME) using TRIzol as described by the manufacturer (Life Technologies). The quality of the isolated RNA was assessed on a Bioanalyzer 2100 (Agilent). For all samples, reverse transcription was performed on 2µg of RNA with the High-capacity cDNA reverse transcription kit (Applied Biosystems), with or without reverse transcriptase. Gene expression was analyzed by qPCR using the GoTaq®qPCR Master Mix (Promega) on a LightCycler Mx3005 qPCR system (Agilent). Primers were designed using Primer3 online software. All the genes were tested for 3 wild types and mutant animals at P7, P32 and P28, P53.

Statistical analysis

Values are reported as a mean \pm s.d. for the Real Time qPCR analysis and median with interquartile range for the analysis of body and kidney weight, and proliferation index, unless otherwise noted. Statistics were calculated using the two-way, two-tailed Student t test. Differences were considered significant with a p-value <0.05.

RT- qPCR primers

Zah?	for	CCCAGGAGGAAAAACGTGGT
	rev	GTCCTGGTCCAGAGGGTTTG
Tafh1	for	CTGCTGACCCCCACTGATAC
	rev	AGCCCTGTATTCCGTCTCCT
Collal	for	CGATGGATTCCCGTTCGAGT
Collai	rev	GAGGCCTCGGTGGACATTAG
Vim	for	CAGGCCAAGCAGGAGTCAAA
v im	rev	CTCCAGGGACTCGTTAGTGC
A ata ?	for	GTCCCAGACATCAGGGAGTAA
Acta2	rev	TCGGATACTTCAGCGTCAGGA
Tafh 2	for	TCCCCTCCGAAAATGCCATC
1gj02	rev	ACTCTGCCTTCACCAGATTCG
Slo5g2	for	TGCGCTCTTCGTGGTGCTG
SIC302	rev	ACCAAAGCGCTTGCGGAGGT
SI 12 #2	for	CGGCCTACGAACACTATGCT
SIC1203	rev	GGCTACCTTCCTGCTTGAGG
Sla12a1	for	CTGGCCTCATATGCGCTTATT
Sicizai	rev	AGATTTGGCATACGAGGCATG
Hufth	for	CCATCCTCAAAGAGCTCCAG
пијго	rev	GCTGTTGCATGTATCCCTTG
	for	CGCCATCCTCCATGAGATTA
Aqp2	rev	GGTCAGGAAGAGCTCCACAG
	0	
Hprt	for	AGICCCAGCGICGIGATIA
-	rev	GGAATAAACACTTTTTCCAAATCC
Snai1	for	CCACACTGGTGAGAAGCCAT
	rev	CACATCCGAGTGGGTTTGGA
LncmiR10Kb	for	GTATTTGGGATCCAGGCAAA
	rev	CTGGAGGCACGAAGAGGTAG
LncmiR14Kb	for	AAGGAAGCCCTCCAGACCTA
	rev	GCCCAGAGAAGTGGAGTTGA
LncmiR25Kb	for	ATGGTCATTGGGCACTTTTG
2110111125110	rev	AGGAGGGAGGTTTTGCTACC

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FIGURE LEGENDS

Figure 1. *Hnf1b* deletion detected by immunofluorescence and RT-qPCR at postnatal and post weaning time points: (A-D) (F-K) HNF1beta protein immunodetection (green) in kidneys from control and mutant mice at P7 and P28 one week after the tamoxifen administration. (B-D) Staining with anti-HNF1beta antibody was absent both in cortex and medulla in a large set of tubules at P7; few scattered cells in the parenchyma escaped the inactivation. (F-K) At P28, immunofluorescence signal was absent from the vast majority of tubular cells at P28. (H-I) Cells that escaped inactivation and continued to express HNF1beta were mostly localized in the straight part of the proximal tubule at the corticomedullary region. Scale bare 50µm

(E-L) Evaluation of *Hnf1b* deletion in wild type and mutant mice treated with tamoxifen at birth (perinatal) or in adulthood (after weaning), determined by RT-qPCR on genomic DNA after one week and 30 days after tamoxifen treatment. Data are means \pm s.d.; *n* = 3 per group. **P* <0.05, ** *P* <0.01, ****P* <0.005.

Figure 2. *Hnf1b* postnatal deletion leads to polycystic kidney phenotype: (A) Comparative analysis of body weight, (B) kidney weight and (C) kidney weight /body weight (KW/BW) ratios in control and mutant mice at P7 and P29. Mutant mice had larger kidneys and lower weight compared to control at P29. *P < 0.05, **P < 0.01, ***P < 0.005. Comparison done using non-paired t-test with the data presented as median with interquartile range.; n = 3-6 per group. (D-E) Representative macroscopic view of kidneys in control and mutant animals 30 days after tamoxifen administration. At P32 mutant kidney were enlarged and presented few superficial irregularities, compared to the control. (F-I) Representative histological sections of control and mutant mice at P7 and P32. Mutant kidneys at P32 were cystic. Cysts were localized all over the renal parenchyma. Scale bars represented 1000µm.

Figure 3. Cysts derive from all tubular segments of nephron in *Hnf1b* postnatal deletion: (A-D) Kidney paraffin sections from P7 and P32 mice stained with LTL (green), marker of proximal tubules, and (E-H) with AQP2 (red), a marker of the collecting duct.

At P32, cysts indicated with a star (*), derived from both proximal tubules and collecting duct. DAPI stained nuclei (blue). Scale bars represented 200µm.

Figure 4. *Hnf1b* postnatal deletion leads to kidney fibrosis: (A-D) Paraffin sections from mutant and control kidneys at P7 and P32, respectively. Sections were stained with Picro Sirius red, recognizing the type I and III collagens, specific of fibrotic tissue. Increased red staining is detected around the cystic lesions in mutant mice (D) compared to the control (C) at P32. (E-H) Immunofluorescence of kidney paraffin sections from P7 and P32 mice labeled with vimentin antibody (red). (G-H) Expression of vimentin is concentrated around the cystic lesions in mutant mice at P32. Scale bars 200µm.

Figure 5. *Hnf1b* post-weaning deletion leads to kidney growth arrest and tubular dilation: (A) Comparative analysis of body weight, (B) kidney weight and (C) kidney weight /body weight (KW/BW) ratios in control and mutant mice at 5 days (P28), at 16 days (P39) and at 30 days (P53) after tamoxifen treatment. (A-B-C) There was no increase in kidney weight from P28 to P53 in mutant mice compared to the control. Mutant mice were smaller at P39 and P53, with a reduction in the KW/BW ratio. *P < 0.05, ** P < 0.01, ***P < 0.005. Comparison done using non-paired t-test with the data presented as median with interquartile range; n = 15-29 per group. (D-E) Macroscopic view of kidney in control and mutant animals 30 days after tamoxifen treatment. (F-K) Representative histological sections of control and mutant mice at P28, P39 and P53. (I) tubular dilation were present in mutant kidney parenchyma from P39 onwards and (K-K') were more intense at P53. Scale bars 200µm (F-K) and 50µm (J'-K').

Figure 6. *Hnf1b* post-weaning deletion leads to morphological tubular defects: (A-B) Periodic Schiff Acid (PAS) staining of kidney paraffin sections at P53. (A'-B') Proximal tubular epithelial cells have partially lost their brush border and their typical rectangular shape in mutant compared to control (arrows in B). (C-H) Immunofluorescence staining of kidney paraffin sections from P28, P39 and P53 mice labeled with LTL antibody (green), specific for proximal tubules. At P39 onwards, a decrease of LTL staining was observed in the cortex and it was largely reduced in large cortical areas at P53. Scale bar 200µm (A-H) and 50µm (A'-B'). **Figure 7. Defective expression of tubular markers in** *Hnf1b* **post-weaning deletion:** (A-F) Immunofluorescence experiment on kidney paraffin sections from P28, P39 and P53 mice. AQP2 (red), a marker of the collecting duct, was normally expressed at all time points analyzed. (C'-F') At the cellular level, its expression was already increased (D') at P39 and even more upregulated (F') at P53. Scale bar 200µm. (G-L) Immunofluorescence with WGA (green), that specifically labels glomeruli and distal tubules, and NKCC2 (red), a marker of the Henle's loop. WGA staining was strongly decreased in distal tubules, whereas NKCC2 staining was normally expressed in Henle's loop. Scale bar 100µm

Figure 8. *Hnf1b* post-weaning deletion in renal tubules leads to interstitial fibrosis: (A-F) Picro Sirius red staining, specific for the type I and III collagens, on kidney paraffin sections from mutant and control kidney at P28, P39 and P53, respectively. (D) Increased red staining is detected in mutant kidney compared to the control already at P39 and (F) more clearly at P53. Scale bars 200µm. (G-L) Immunodetection of vimentin (red) on kidney paraffin sections from P28, P39 and P53 mutant and control mice. Vimentin expression is concentrated in focal lesions in the cortex of mutant kidney (J) at P39 onwards and (L) more clearly at P53. Scale bars 200µm.

Figure 9. *Hnf1b* post-weaning deletion leads to partial EMT: (panel A-L) immunofluorescence detection of (A-D) vimentin (red) and (E-H) LTL (green) on kidney paraffin sections of P39 mutant and control mice. Scale bars.10µm. T= tubule, V= vessel. (F-H) LTL was delocalized in the cytoplasm in mutant proximal tubules compared to (E) the normal apical staining observed in the control. (J-L) Vimentin is expressed de novo in mutant proximal tubules stained by LTL. (M-P) mRNA levels of EMT markers in control and mutant mice at P28 and P53, determined by RT-qPCR. (M-N) *Vim* and *Zeb2* mRNA were increased at P53 in mutant compared to the control. (O-P) *Long non-coding miRNA* (*LncmiR*) precursors 25K and 14K that will form *miR200* were downregulated. Data are means \pm s.d.; n = 3 per group. *P <0.05, ** P <0.01, ***P <0.005. (Q) Scheme of HNF1beta induction of miR200 and consequent inhibition of ZEB2

Figure 10. *Hnf1b* post-weaning deletion leads to tubular function defects: (A-J) urine and plasma dosage. In the plasma, (A) serum urea and (B) creatinine levels increased at P39 and became significantly different at P53. (C) Hypomagnesaemia was observed in mutant mice at P53 compared to the control. (E) In the urine, mutant animals suffer from glucosuria at P39 without (D) hyperglycemia; (F--I) increased levels of Ca²⁺, Mg⁺, K⁺ and microalbumina, and (J-K) decreased excretion of uric acid became significant at P53. Data are means \pm s.d.; n = 5-8 per group. *P < 0.05, ** P < 0.01, ***P < 0.005. (L) Comparison of volume urine collected in 24h with metabolic cages in control and mutant mice at P28 and P53. Increased urine volume produced by mutant mice versus control mice at P53. Data are means \pm s.d.; n = 4-6 per group. *P < 0.05, ** P < 0.01, ***P < 0.005.

Figure 11. Comparison of transcriptomic analysis of kidney at different time points of *Hnf1b* postnatal and post-weaning deletion. 5 days after the deletion of *Hnf1b*, the expression level of the majority of genes tends to be decreased (103 vs 13 at P7) and (184 vs 36 at P28). A month later, at P32 (postnatal deletion) majority of genes becomes predominantly overexpressed (440 up vs 144 down). The kidney at P53 (postweaning deletion) showed a massive change in gene expression with 438 genes upregulated and 449 downregulated. N=4 per group.

SUPPLEMENTATY INFORMATION

Supplementary Figure 1. Defective expression of tubular marker in Hnf1b postweaning deletion (A-D) Expression levels of tubular markers in control and mutant mice at P28 and P53, determined by RT-qPCR. *Aqp2* (collecting duct) and *Slc12a1* (Henle's loop) expression is increased, whereas *Slc5a2* (proximal tubule) is decreased. *Slc12a3* (distal tubule) is increased at P53. Data are means \pm s.d.; *n* =3 per group. **P* <0.05, ** *P* <0.01, ****P* <0.005.

Supplementary Figure 2. (A-D) Expression levels of pro-inflammatory and fibrosis markers in control and mutant mice at P28 and P53, determined by RT-qPCR. *Tgfb1*, *Tgfb2*, *Acta2* and *Col1a1* expression was increased at P53 in mutant compared to the control. Data are means \pm s.d.; n = 3 per group. *P < 0.05, **P < 0.01, ***P < 0.005.

FIGURES PART II









P7

P29

Α



В





































SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2

DISCUSSION

Final Discussion

HNF1beta is a transcription factor that binds DNA as homo or heterodimer with the homolog HNF1alpha (Cereghini, 1996) and mostly acts as an activator of transcription. It is expressed in the epithelial cells of different organs, such as liver, pancreas and kidney. In particular, in kidney, *Hnf1b* starts to be expressed early during renal morphogenesis and its expression is maintained in all tubular epithelial cells in adulthood (Cereghini et al., 1992; Coffinier et al., 1999b; Lazzaro et al., 1992). In human, patients with *HNF1B* mutations are mostly characterized by the presence of renal malformations associated with Maturity Onset Diabetes of the Young (MODY) syndrome type 5 (MODY5) in one half of the patients. The most frequent phenotypic trait in *HNF1B* carriers is the presence of cysts, derived from tubules or glomeruli.

I. The role of HNF1beta during glomerulogenesis

Glomerular cysts are observed frequently in pediatric patients carrying HNF1B mutations (Heidet et al., 2010; Ulinski et al., 2006), suggesting that a developmental abnormality could be at the origin of this phenotypic abnormality. In order to understand the mechanisms at the basis of glomerular cyst formation, I used the Cre LoxP startegy to inactivate *Hnf1b* in developing nephrons. To study the role of HNF1beta during glomerulogenesis, I took advantage of the Six2-Cre mouse strain (Kobayashi et al., 2008) that when crossed with Hnflb f/f mice (Coffinier et al., 2002) induces results in the inactivation of *Hnf1b* in all epithelial nephron precursor cells. Using this strategy, mutant nephrons have a drastic defect in tubular specification and expansion, due to a defect in the Notch signaling pathway. In addition, the absence of *Hnf1b* in developing nephrons induces the development of glomerular cysts, reminiscent of the glomerular phenotype observed in children/fetuses carrying HNF1B mutations (Massa et al., 2013). However, the morphogenetic processes that drive glomerular cyst formation in the absence of *Hnf1b* are still unknown. In the first part of my thesis, I focused my attention on the role played by HNF1beta during glomerulogenesis. Taking advantage of a mouse strain in which the LacZ cassette is under the control of the *Hnf1b* endogenous promoter, we could follow the cells that were programmed to express *Hnf1b*, even in the absence of HNF1beta. During normal glomerulogenesis, at the S-shaped body level, the tubular precursors are contiguous to the podocyte precursor's plate and become progressively separated by the interposition of parietal cells. The transition to the precapillary loop stage is characterized by the formation

of an epithelial protuberance that leads to the extrusion of the tubular compartment outside the glomerulus (Saxén and Sariola, 1987). We showed that this protrusion was formed by two cell types, podocyte precursors in the internal side, contiguous to the podocyte precursor's plate, and intermediate-parietal like cells on the external side. By immunofluorescence staining, we showed that the transitional parietal cells of the protrusion expressed low levels of *Hnf1b* and *Wt1*, compared to the contiguous podocytes precursors, which were characterized by high expression of Wt1 and no expression of Hnflb, and the tubular cells, expressing high levels of Hnflb and no Wtl. In the absence of Hnflb expression, we discovered that this protrusion, in spite of the specification of the two cellular populations, did not form. In the mutant mice, this defect led to an abnormal connection of the tubule inside the glomerular cup. Due to the further invagination of the vascular and second cleft that formed a common cavity all around the tubule, mesangial cells and capillaries, which normally invaded the vascular cleft, colonized the whole cavity and surrounded the tube inserted in the glomerular cup. This atypical localization of mesangial cells possibly provided a reason for the restriction of the tubular glomerular outflow. In the anterior portion of the glomerular cup, podocytes differentiated normally, as showed before by electron microscopy (Massa et al., 2013). The presence of normal podocytes might provide the starting of filtration and the production of primary urine. However, due to the obstruction of the tubular outflow, urine might not come out easily provoking the dilation of the Bowman capsule and the formation of the glomerular cyst. The analysis of renal tissues from human fetuses carrying HNF1B mutations, characterized by multicystic dysplasia with the presence of glomerular cysts, also led to the identification of nephron precursors in which the tubule was inserted in the glomerular cup, which was reminiscent of malformations observed in our mouse model.

Our results demonstrate that an abnormal molecular program during glomerulogenesis could lead to glomerular cyst formation. In addition, we highlighted the crucial role of a so far neglected process, the remodeling of the urinary pole during glomerulogenesis, in the formation of glomerular cysts, in mouse and human.

II. The role of HNF1beta during tubular maturation

Inactivation of *Hnf1b* in the epithelial nephron precursor cells in the mouse model presented above (*Six2*Cre) prevents the specification and the expansion of the tubules. Beside this role during early tubulogenesis, our laboratory has previously showed that the inactivation of *Hnf1b* during tubular elongation in a proliferative context, leads to a severe polycystic kidney disease (Gresh et al., 2004; Verdeguer et al., 2010). On the other hand its inactivation at P10, in quiescent tubules, did not produce any renal abnormalities. This difference in the phenotype suggests that proliferation/quiescence of tubular cells plays a major role in the way HNF1beta controls the expression of its target genes (Verdeguer et al., 2010).

Our aim was to decipher the molecular program controlled by HNF1beta during tubular elongation and tubular maturation, in proliferative versus quiescent tubular cells. To do this, we took advantage of an inducible mouse strain (*Ksp*-CreER^{T2}) (Lantinga-van Leeuwen et al., 2006) to efficiently inactivate this transcription factor in all the tubular structures of the nephron at different times during kidney maturation. We decided to analyze the effect of the deletion of *Hnf1b* at two distinct phases of postnatal kidney maturation. The first was the postnatal period, which is notoriously characterized by massive proliferative tubular elongation; whereas, the second step that we considered was the post-weaning period, characterized by a much lower proliferation context. In both of above strategies, mice were sacrificed 5 days and 1 month after tamoxifen treatment.

One month after early inactivation, mutant animals developed a severe PKD. We demonstrated that cysts derived from all tubular segments, and that cystic lesions were surrounded by interstitial fibrosis. In several studies in human and animal models of PKD, inflammation and interstitial fibrosis were also observed in association with cystic lesions (Karihaloo, 2015). Interestingly, in some animal models, the deletion of the gene of interest (such as *Pkd1* or *Pkd2*) was also restricted to the tubular compartment, suggesting that the triggering event of fibrosis could be represented by the tubular damage itself. In addition, some groups suggested that the extent of fibrosis in PKD patients could be a predective indicator of decline of GFR (Caroli et al., 2011).

Post-weaning inactivation of *Hnf1b* did not lead to the formation of cystic lesions but tubular differentiation and function were impaired in mutant mice. The expression of some

proximal and distal tubular markers was decreased in large areas of renal parenchyma. As an example, the decreased expression of *Slc5a2*, encoding a glucose/sodium co-transporter SGLT2 (van den Heuvel et al., 2002; Santer et al., 2003), normally expressed in the proximal tubule and responsible of the reabsorption of the vast majority of glucose molecules in this compartment (Kanai et al., 1994), is responsible for glucosuria.

On the contrary, some other markers were up-regulated in P53 mutant mice compared to the control, such as Aqp2 or Nkcc2 that are specifically expressed in the collecting duct and the Henle's loop, respectively. The misregulation of these genes could be either a direct effect of the absence of *Hnf1b*, or it could be a secondary compensatory effect due to the dedifferentiation of the tubules. For instance, AQP2 is a water channel present on the apical side of the collecting duct and controls the reabsorption of water in this tubular segment under the regulation of vasopressin. It has been previously showed vasopressin levels are up-regulated in diabetic patients to compensate for the loss of water induced by glucosuria (Nejsum et al., 2001). In our model, one month after post-weaning Hnflb deletion, mutant mice had an increased volume of urine associated with a loss of high quantity of glucose in the urine. Therefore, the increased expression of Aqp2 transporter, at cellular level and without morphological defect of the collecting duct, could be a compensatory response to the loss of water due to glucosuria. Another gene whose expression is modified in our mouse model is *Slc12a1*, which encode for $Na^+/K^+/2Cl^-$ cotransporter (NKCC2) responsible for the NaCl transport across the thick ascending limb (TAL) epithelium in the Henle's loop. This transporter is activated by its amino-terminal phosphorylation. It has been previously shown that this phosphorylation is mediated by uromodulin (UMOD). In the absence of UMOD, NKCC2 is located in intracellular vesicles and is not efficiently addressed at the apical membrane (Mutig et al., 2011). Since Umod has been shown to be a direct target of HNF1beta (Gresh et al., 2004), and is downregulated in our mutant mice at P53, we could hypothesize that the increased expression of Nkcc2 is a compensatory response to its reduced activation. Moreover, Umod-/- mice presented reduced urinary concentrating ability (Bachmann et al., 2005; Mutig et al., 2011), which is reminiscent of the production of large volume of urine by mutant mice at P53.

As previously mentioned, recent studies have shown that *HNF1B* mutations in adult patients are frequently associated with non-specific renal interstitial fibrosis. Similar to the observations made in postnatal deletion, *Hnf1b* inactivation in a quiescent context leads to

a fibrotic phenotype. Fibrotic lesions are concentrated in the cortical part of the kidney in focal spots and branch out all around the tissue. The presence of fibrosis is supported by the up-regulation of genes such as Collal, Acta2 and Vim. Interestingly in a quiescent contest, at P39 in mutant mouse kidneys, vimentin staining was also observed in proximal tubular cells. It has been previously shown that in the presence of a persistent insult epithelial tubular cells can undergo a partial EMT. In this context, tubular cells express mesenchymal markers and activate fibrosis through the release of molecules in the interstitial space that attract and activate the local fibroblasts (Grande et al., 2015; Humphreys et al., 2010; Lovisa et al., 2015). Interestingly, in addition to its role in the phosphorylation of the NKCC2 transporter, UMOD plays a crucial role in the regeneration of the proximal tubule after acute kidney injury (AKI). Through a translocation from the apical side to the basolateral side of the TAL, UMOD communicates with the S3 portion of the proximal tubule and promotes the downregulation of pro-inflammatory cytokines (El-Achkar et al., 2013). In the absence of Hnf1b, Umod downregulation could participate to the inflammatory status in mutant renal tissues and promote the formation of fibrosis. Among the molecular pathways that are involved in the fibrotic process, TGF-β signaling promotes ECM production, inhibits its degradation and is involved in the differentiation of myofibroblasts (Moustakas and Heldin, 2005). This signaling pathway has also been involved in the partial mesenchymal conversion of tubular cells (Grande et al., 2015; Lovisa et al., 2015). In mutant mice in which *Hnf1b* was inactivated in a quiescent context, Tgfb1 and Tgfb2 were up-regulated one month after the deletion. Furthermore, we observed in these mutant animals an increased expression of Zeb2, a transcription factor involved in the acquisition of a mesenchymal phenotype. It is known that TGF- β induces the up-regulation of Zeb2 through the downregulation of the miR-200 family, which is a direct inhibitor of Zeb2. During renal tubular dedifferentiation, degeneration and kidney fibrosis, it has been shown that *miR-200* members are down-regulated (Xiong et al., 2012). Strikingly, recent studies demonstrated that HNF1beta directly targets the miR-200, inhibiting the Zeb2 expression and inducing the tubular differentiation and maturation during kidney development (Hajarnis et al., 2015). Our results have shown that the expression of miR-200 was downregulated in mutant mice at P53. Altogether these data could explain the complex phenotype observed in our mouse model. Moreover, the transcriptomic analysis performed at different time points of *Hnf1b* deletion, revealed that in both proliferative and quiescent contexts, *Hnf1b* deletion has a deep impact on gene expression with a drastic change only 5 days after administration of tamoxifen treatment.

At this point the vast majority of the genes were downregulated, indicating a prevalent positive role of HNF1beta on the transcription. Interestingly, the pathways involved in tubular development and differentiation, and pro-inflammatory and fibrotic processes, were mostly affected in both phases of postnatal tubular maturation.

In conclusion, my thesis project highlighted new aspects of the role played by Hnflb in post natal kidney. My results have identified a new morphological mechanism leading to glomerular cyst formation in the absence of HNF1beta in mouse and human. In addition, I elucidated the global gene expression modifications induced by the differential inactivation of this transcription factor in proliferative versus quiescent tubular cells. While its role in a proliferative context was already known (Gresh et al., 2004; Verdeguer et al., 2010), I showed here that HNF1beta is involved in the maintenance of tubular differentiation in mature kidney and that its absence leads to a partial EMT in renal tubular cells, which triggers the formation of interstitial fibrosis. The specific inactivation on different time/place of Hnf1b in our mouse models recapitulates some abnormalities observed in patients carrying HNF1B mutations.

PERSPECTIVES

PERSPECTIVES

During my thesis, I have identified novel important roles played by *Hnf1b* at different steps during kidney development. Inactivation of *Hnf1b* in all the epithelial cells of the nephron precursors leads to the formation of glomerular cysts and the absence of tubular specification and expansion. On the other hand, its deletion in the tubular segments of the nephron at different time points during the kidney maturation discloses a differential role played by HNF1beta on transcriptional networks involved in postnatal kidney. However, some questions concerning the function of HNF1beta during glomerulogenesis and in quiescent tubules remain open. In particular, the origin of the interposed parietal cells and the molecular networks regulated by HNF1beta during the glomerular maturation are still elusive. In addition, I will discuss some preliminary data and perspectives on the identification of the mechanisms leading to tubular dedifferentiation and kidney growth arrest in the mutant mice inactivated in the post weaning period. These future works could increase the knowledge of these fascinating and complicated processes.

A. Fiorentino

I. GLOMERULAR MATURATION

Perspective 1: Origin of the intercalating parietal cells

During normal glomerulogenesis, in the transition between the SSB and precapillary-loop stage, some parietal cells separate the podocyte precursor plate and the tubular compartment, defining the inner and the outer part of the glomerular cup (Saxén and Sariola, 1987). I have shown that this morphogenetic event leads to the formation of a bilayered epithelial horn, composed of the podocyte plate on one side, and the interposed parietal cells on the other. In turn, the formation of this horn leads to the extrusion of the urinary pole from the inner part of the glomerular cup. Nevertheless, the origin of interposed parietal cells is still not well understood. Interposed parietal cells between the podocytes and tubular precursor can originate from a subset of podocyte precursors or tubular precursors that activate a specific program to acquire parietal phenotypic features. An alternative hypothesis could be that parietal cells, which are already specified, migrate from the lower layer of the proximal limb of the S-shaped body.

To clarify the origin of these intermediate-parietal like cells, retrospective lineage tracing experiments could be performed, using Confetti animals. The Confetti mouse line (Schepers et al., 2012) carries a combination of four fluorochromes (RFP, YFP, GFP and CFP) at the Rosa 26R locus (Perspective figure 1). When Rosa26R-Confetti mice are crossed with a mouse strain carrying a Cre recombinase, the action of the Cre recombinase allows single cells to randomly adopt one of the four fluorescent colors encoded in the Rosa26R-confetti allele. In addition to this mutually exclusive expression of one fluorochrome, these different fluorescent proteins have different subcellular localizations (RFP and YFP are cytoplasmic, GFP is nuclear and CFP is expressed at the cell membrane).


Perspective Figure 1. Schematic representation of RosaR26-Confetti locus. At the RosaR26 locus of Confetti mice, the Cre recombinase activity will allow the expression of only one of the four colors (RFP, YFP, GFP and CFP) through excision or flipping of the LoxP sites.

In order to activate the expression of the fluorescent proteins, we could use mice expressing the Cre recombinase specifically in podocyte (*Nestin*-Cre) or tubular precursors (*Osr2*-Cre, a transcription factor whose expression is turned on at the S Shaped body stage). Using this strategy, two main scenarios can be envisioned: 1) Interposed parietal cells originate from cells that express *Osr2* (i.e. tubular precursors) or *Nestin* (podocyte precursors). In this case, intercalating parietal cells will share the expression of fluorescent protein with these precursors; 2) Parietal cells located on the lower layer of the proximal limb might migrate to interpose between tubular and podocyte precursors. In this case, interposed parietal cells will not express any fluorochromes in the two mouse models described above.

<u>Perspective 2: Identification of the signaling pathways and the molecular</u> <u>cascades directly or indirectly controlled by HNF1beta during glomerulogenesis</u>

The molecular programs that control the remodeling processes during glomerulogenesis are still largely elusive. Our aim will be to identify the molecular pathways whose defects lead to the phenotype that is observed in the absence of HNF1beta. To identify transcripts and cellular markers that are differentially expressed in wild-type and *Hnf1b*-deficient nephrons precursors, we aim to use a Next Generation Sequencing (NGS) mRNA sequencing strategy (Affymetrix GeneChip® Mouse Gene 1.0 ST Arrays).

Molecular signature of the different cell population during glomerular maturation

We will identify the molecular signature of the cells surrounding the urinary pole in mutant glomerular precursors, by laser capture microdissection experiments followed by massive scale mRNA sequencing on late nephron precursors. Using a laser capture microdissection, we will analyze cells (fated to express *Hnf1b*) that express β -galactosidase in the back compartment of the mutant glomerular cup. As a control population, we will microdissect Bowman capsule's cells, intercalating parietal cells and podocytes in wild-type animals (blue circles in Perspective Figure 2). In addition, in order to analyze the transcriptional program controlled by HNF1beta during the differentiation of parietal cells, we will compare this cell population in wild-type and mutant animals (red circles).



Perspective Figure 2. Scheme of cell populations that will be collected by laser capture microdissection from control and mutant mice. The blue circle in mutant nephron precursor indicate the cells expressing beta-galactosidase in the back compartment of the glomerular cup, whose transcriptomic analysis will be compared to control populations of podocytes, Bowman capsule's cells and intercalating cells (indicated with blues circles in the control nephron precursors). On the other hand, in red circles are indicated the cells from control and mutant nephron precursors that we will use to analyze the transcriptional program controlled by HNF1B during the differentiation of parietal cells

Specification of early nephron precursor cells toward glomerulogenesis.

In order to define the transcripts and cellular markers that are differentially expressed in early nephron precursors from wild type or *Six2*Cre *Hnf1b*-deficient animals, we will also use a massive-scale mRNA sequencing strategy (Affymetrix). To avoid the contamination of our samples with genes that normally become expressed in cells that are not developed in mutant animals, we will perform our experiments in whole embryonic kidneys at E12.75, and compare wild-type and mutant animals. Using this time point, nephron precursors are represented only by vesicle and comma shaped bodies on kidney sections, which do not show morphological differences between *Six2*Cre *Hnf1b*-deficient and wild type kidneys. To circumvent the possible imprecision in the dating of the embryos (the morning of the plug defines arbitrarily E0.5), we will test by Real Time q-PCR, the expression of a set of markers whose expression is turned on only at the S-shaped body stage (*Hnf4*, *Hnf1a*, *Synpo*, *Osr2*).

RNA sequencing and validation of the genes differentially expressed.

The Rna obtained in the two previous experiments will be analyzed by GeneChip® Mouse Gene 1.0 ST Arrays (a genome wide array with 27 543 probe sets with ENSEMBL support), to define differentially expressed genes in wild type and Six2Cre HNF1b-deficient kidneys and to compare the gene expression pattern of the different cell population at the precapillary loop stages. The comparison between wild type and mutant kidneys should lead to the identification of genes whose distorted expression is linked to the aberrant nephrogenesis in the absence of HNF1beta. This approach could also allow us to identify potential new genes/new signaling pathways important for the differentiation of glomeruli.

II. ROLE OF HNF1Beta IN TUBULAR MATURATION

Our perspectives in further understanding the role of HNF1beta in tubular maturation include the identification of molecular signaling pathways and transcriptional cascades involved during postnatal kidney maturation, which are not completely elucidated.

Perspective 3: absence of homeostatic growth in Hnf1b deficiency

In the model of *Hnf1b* deletion in a quiescent tubular context, homeostatic kidney growth is impaired. The weight of mutant kidneys remains constant during the month after tamoxifen administration, whereas the kidney weight harmoniously increases during this period in control mice. The molecular and cellular mechanisms at the basis of this phenotype are still unclear. This phenotype could be linked to a direct effect of *Hnf1b* deficiency on proliferation or apoptosis in tubular cells. To investigate this hypothesis I started to analyze the proliferation and apoptosis in kidney tissue at different time points after post-weaning *Hnf1b* deletion. Immunofluorescence experiments have been performed on kidney paraffin sections with an anti-Ki67 antibody, a marker of proliferation which labels cells in active phases of the cell cycle but not resting cells (G0). In parallel, proximal tubules were stained with LTL. By counting the Ki67 positive cells in proximal tubules LTL⁺, I could not observe any significant difference in the proliferation index between mutant and control mice at P28 (Preliminary results Figure 1).



Preliminary results Figure 1: No difference in proliferation index one week after the post-weaning *Hnf1b* deletion - Proliferation index at P28 in mutant and control mice: Ki67+ cells were counted in tubular cells stained with LTL. For each sample 3 areas of 1000 LTL cells have been counted. Comparison done using non-paired t-test with the data presented as median with interquartile range; n = 3 per group.

In the future, I will to analyze the proliferative index in mutant and control mice at P39 (18 days after *Hnf1b* deletion) when renal tubule dedifferentiation is effective. At this time point a significant difference in kidney size was already observed.

It has been recently demonstrated that tubular epithelial cells present a cell cycle arrest in G2 phase, as a consequence of the partial EMT after kidney injury (Lovisa et al., 2015). In order to analyze the number of the proliferating cells in the different phases of the cell cycle, I propose to perform in our model successive F-ara EdU (specific marker for S phase) injections (Perspective Figure 3). Kidney sections of treated animals will be co-stained with an anti-phosphoHistone-3 (pH3) antibody, specific marker for G2/M phase.



Perspective Figure 3. Scheme of F-ara EdU injection. Injection will be performed on control and mutant mice treated with tamoxifen between P21-23. Three injections will be performed between P28 and P35, mice will be sacrificed at P39

In addition, our lab has previously demonstrated that *Hnf1b* inactivation in the epithelial cells of the nephron precursors (*Six2*Cre) was associated with apoptotic events in the S shape-body stage (Massa et al 2013). In order to verify the possible involvement of apoptosis process in the growth arrest of mutant kidney, we will investigate the apoptosis index in mice sacrificed at P39.

Perspective 4: Tubular dilation in the absence of HNF1beta in the post weaning period

Our laboratory has previously shown that *Hnf1b* deletion with a *Mx*Cre recombinase mouse strain (Kühn et al., 1995) in early postnatal period induces a cystic phenotype, whereas its inactivation 10 days later, in a less proliferative context, does not produce any renal phenotype. based on this model, it has been shown that HNF1beta remains associated to chromatin during mitosis and that the loss of expression of HNF1beta target genes was observed only in a proliferative context, suggesting that HNF1beta acts as a bookmarking factor and is crucial for the rapid re-expression of its target genes after mitosis (Verdeguer et al., 2010).

During my thesis, I used a different mouse model in which Hnf1b was deleted using a Ksp-CreER^{T2}. In this case, when Hnf1b is deleted in a quiescent context, kidneys were characterized by tubular dilation and dedifferentiation of epithelial tubular cells that appeared flattened. The dilation observed could be directly due to the down regulation of cystic genes, even in the absence of cyst formation. At the molecular levels *Pkhd1*, a cystic gene normally controlled by HNF1beta, was downregulated with a fold change of two in mutant kidney one month after the deletion (P53). Other genes such as *Pkd2* were not significantly decreased (data obtained by RT-qPCR, preliminary results figure2). In the future, it will be interesting to investigate the molecular mechanism involved in this phenotype and follow up these mice during a longer period to analyze the evolution of this tubular dilation.



Preliminary results Figure 2: *Pkhd1*, but not *Pkd2*, was significantly down-regulated in mutant mice one month after *Hnf1b* post-weaning deletion.

Expression levels of cystic genes in mutant and control mice at P53. At P53, *Pkhd1* was down-regulated with a fold change of 2, whereas the expression of *Pkd2* was not significantly decrease. Comparison done using non-paired t-test with the data presented as mean \pm s.d.; *n* = 3 per group, ***P >00.5.

<u>Perspective 5: Identification of direct target genes bind of HNF1beta in</u> <u>proliferative versus quiescent status.</u>

By analysis of the Affymetrix data obtained from control or mutant kidneys from mice with an early inactivation (P7-P32) and late inactivation (P28-P53) of *Hnf1b*, we showed a deep change in the expression of genes involved into two principal pathways: differentiation development tubular and of epithelial cells (Lhx1)and fibrosis/inflammatory pathways (Tgfb1). In future, we want to investigate which genes in these two pathways are directly targeted by of HNF1beta As a first approach, we will perform an *in silico* approach, using a Hidden Markov Model based on 59 known HNF1beta binding sites, set up in the laboratory. This in silico approach allows the identification of putative HNF1beta binding sites conserved in a relative large number of species (Tronche et al., 1997) (Massa 2012). This approach should help to identify binding sites with high score and conservation in the group of genes involved in tubular differentiation and development pathway and in fibrosis/ inflammation pathway that are up (or down) regulated in the Affymetrix data. In the second time using ChIP-Seq (chromatin immunoprecipitation-sequencing) assay performed on kidneys, we want to verify the direct target genes identified through the in silico analysis. Altogether, these two approaches should help to unravel the complete molecular program controlled by HNF1beta during tubular maturation, whose defects induce partial EMT and fibrosis.

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Summary (English)

Hepatocyte Nuclear factor 1 beta (HNF1beta) is a transcription factor expressed in epithelial cells of different organs, such as pancreas, intestine and kidney. The most penetrant phenotypic trait in patients carrying HNF1B mutations is the presence of renal abnormalities, frequently associated with Maturity Onset Diabetes of the Young type 5 (MODY5). During my thesis, I analyzed the role played by this transcription factor in different compartments and at different time points in mouse kidney, using a Cre LoxP strategy. Our laboratory has previously shown that *Hnf1b* specific inactivation in the epithelial nephron precursors cells (Six2Cre) leads to defective tubular specification and expansion and to the formation of glomerular cysts. The analysis of the morphogenetic processes of glomerulogenesis in the absence of HNF1beta led me to propose a new mechanism of glomerular cyst formation. In mutant nephron, the connection between the glomerulus and the tubular component is abnormally trapped inside the glomerular cup. Due to this unusual configuration, the trapped tubule is surrounded by capillaries and mesangial cells, leading to a constriction of the urinary glomerular outflow. When filtration starts, the production of the primary urine might induce the dilation of the Bowman capsule and the formation of glomerular cyst. In the second part of my thesis, I analyzed the role played by *Hnf1b* in proliferative versus quiescent tubular cells, by inactivating this gene in renal tubules at different time points (inducible Ksp-CreER^{T2}). My results showed that Hnf1b inactivation during postnatal tubular elongation leads to polycystic kidney disease, as previously described in our laboratory. Analysis of this new inducible model of *Hnf1b* inactivation also highlighted for the first time a fibrotic phenotype in the tissue surrounding the cysts. The later inactivation in a more quiescent status is characterized by the presence of focal tubulointerstitial fibrosis and tubular dilation. Interestingly, in this context, Hnf1b-deficient proximal tubular cells present a partial loss of epithelial differentiation, characterized by a reduced expression of tubular markers (LTL) and de novo expression of vimentin. At the molecular level, the expression of Zeb2, a transcription factor involved in epithelial to mesenchymal transition, is increased. This is due to the downregulation of its inhibitor miR200, a direct target of HNF1beta. Finally, transcriptomical analyses in postnatal and postweaning periods showed that two major pathways are commonly affected. The development and differentiation of nephrons are severely compromised and pro-inflammatory and fibrotic signaling cascades are strongly upregulated. All together these results improved our knowledge about the role of HNF1 beta in glomerulogenesis and adult kidney.

Résumé (Français)

HNF1beta (Hepatocyte Nuclear Factor 1 beta) est un facteur de transcription exprimé dans les cellules épithéliales de différents organes tels que le pancréas, l'intestin et les reins. La présence d'anomalies rénales représente le trait phénotypique le plus pénétrant chez les patients porteurs de *HNF1B*, associées ou non à un diabète de type MODY (MODY5). Au cours de ma thèse, j'ai analysé le rôle de ce facteur de transcription dans différents compartiments du rein de souris et à différentes périodes, en utilisant une stratégie Cre LoxP. Notre laboratoire a précédemment montré que l'inactivation Hnflb spécifiquement dans les précurseurs du nephron (Six2Cre) provoque la perte de spécification et d'expansion tubulaires, et la formation de kystes glomérulaires. L'analyse des processus morphogénétiques pendant la glomerulogenèse en l'absence de HNF1beta m'a amenée à proposer un nouveau mécanisme de formation des kystes glomérulaires. Dans les néphrons mutants, la connexion entre le glomérule et le composant tubulaire est anormalement localisée à l'intérieur de la coupe glomérulaire. En raison de cette configuration inhabituelle, le tubule est entouré de capillaires et de cellules mésangiales, conduisant à un rétrécissement de la lumière tubulaire. Lorsque la filtration glomerulaire débute, la production de l'urine primitive provoquerait la dilatation de la capsule de Bowman entrainant la formation de kystes glomérulaires. Dans la deuxième partie de ma thèse, j'ai analysé le rôle de HNF1beta dans des cellules tubulaires en prolifération versus en quiescence, en inactivant ce gène dans les tubules rénaux à deux périodes post-natales (Ksp-CreER^{T2} inductible). Mes résultats ont montré que l'inactivation de *Hnf1b* pendant l'elongation tubulaire conduit à une polykystose rénale, comme déjà décrit précédemment dans notre laboratoire. L'analyse de ce nouveau modèle inductible d'inactivation de *Hnf1b* a également souligné pour la première fois un phénotype fibrotique dans le tissu entourant les kystes. L'inactivation plus tardive, dans des cellules quiescentes se caractérise par la présence d'une fibrose focale et de dilatations tubulaires. Par ailleurs, les cellules tubulaires proximales déficientes en HNF1beta présentent une perte partielle de différenciation épithéliale, caractérisée par une expression réduite de marqueurs tubulaires (LTL) et l'expression de novo de la vimentine. Au niveau moléculaire, l'expression de Zeb2, facteur de transcription impliqué dans la transition épithéliomésenchymateuse, est augmentée. Cela est dû à la régulation négative de son inhibiteur miR200, une cible directe de HNF1beta. Enfin, l'analyse des données transcriptomiques a montré que deux voies de signalisation majeures sont perturbées aux deux périodes d'inactivation. Les voies contrôlant le développement et la différenciation des néphrons sont gravement compromises et les cascades de signalisations pro-inflammatoires et fibrotiques sont fortement surexprimés. Dans leur ensemble, ces résultats ont amélioré nos connaissances du rôle de HNF1beta au cours de la glomérulogenèse et dans le rein adulte.