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Gavino Faa
Vassilios Fanos *Editors*

Kidney Development in Renal Pathology

Humana Press

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To our families for their constant and unique support.

Preface

So all my best is dressing old words new, spending again what is already spent

(W. Shakespeare, Sonnet 76)

The field of development of the human kidney is a complex and in part unknown process which requires interactions between pluripotential/stem cells, undifferentiated mesenchymal cells of the metanephros, epithelial and mesenchymal components, eventually leading to the coordinate development of multiple differentiated epithelial, vascular, and stromal cell types within the complex renal architecture.

In the last few years, the "old" embryology of mammalian kidney has been revisited and reinterpreted with new eyes, in the light of immunohistochemistry and molecular biology. Important stages in the progress of research are represented by the following passages: determining how the tree of the ureteric bud is formed; understanding the intimate reciprocal interactions between the two precursor tissues, the metanephric mesenchyme, and the ureteric bud; identifying the causes leading to kidney hypodysplasia; clarifying the factors influencing the end of nephrogenesis before birth; understanding and controlling the mediators which stimulate or inhibit kidney development and orient it either in the direction of normal or abnormal development.

Studies on perinatal programming are expanding the temporal horizon of precocious reduction in the number of nephrons at birth, which may have long-term effects on the renal function in adulthood.

This textbook will provide a comprehensive, state-of-the art review in the field of experimental and human nephrogenesis, and should serve as a valuable tool for pediatricians, neonatologists, nephrologists, gynecologists, pathologists, and researchers with an interest in kidney diseases.

The book will review new data on the effects on kidney development by neonatal asphyxia, obstructive uropathies, nephrotoxic drugs administered to the mother and/or to the neonate, malnutrition, underfeeding, overfeeding, and will provide all possible preventive measures to ensure the well-being of the kidney at birth, in order to assure health when the children reach adulthood and through the entire life cycle.

In this book, the authors will focus on the multiple cell types involved in nephrogenesis, which move from the mesenchymal toward the epithelial world and back, and will define the multiple factors that propel these cell types to differentiate during kidney development, rendering the notions of "mesenchymal" and "epithelial" identity more fluid than expected.

Finally, the possible implications between renal development and the insurgence of kidney disease in adult life, and the correlation with renal carcinogenesis will be discussed.

This textbook will provide a concise and comprehensive summary of the current status of the field of human nephrogenesis, and on the clinical consequences in adulthood of a block of nephron development in the perinatal period.

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Development of the Human Kidney: Morphological Events

1

Gavino Faa, Vassilios Fanos, Giuseppe Floris, Rossano Ambu, and Guido Monga

Introduction

The human kidney is a very strange organ, often acting on impulses that remain hidden, often reacting to endogenous or external stimuli with behaviors not completely comprehensible for us. Kidney development appears peculiar, even at panoramic view. Who is the mature human kidney? Why two kidneys? One was not enough, like for heart or liver? Why three kidneys during human development? One was not enough, like for the vast majority of organs? Why so many cell types participating to development of the mature kidney? Why so strict interrelationships between the mesenchymal and the epithelial world during kidney development?

Few cell types were not enough, like for liver, for suprarenal glands, for lungs, and for the vast majority of human organs? To these old questions, science has in recent years brought powerful tools and reams of data: in particular, genetics and molecular biology have opened a big window onto human kidney development [1]. Thanks to these new findings, a new morphological interpretation of human nephrogenesis is emerging, in which the process of epithelial–mesenchymal transition appears as the new main actor of kidney development [2]. But, the more we learn about kidney evolution and development, the more complicated the story becomes, even on morphological grounds [3]. New findings on the immunoreactivity of the pluripotential cells that give rise to the nephrogenic area located under the renal capsule are evidencing a previously unrecognized complexity of the nephrogenic zone, revealing the pres-

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ence of new cell types or, alternatively, of new differential stages of the renal progenitors during their trip towards the epithelial structures of the mature kidney, including glomeruli and tubules [4].

Here we will report the main known morphological steps of human nephrogenesis, with a particular attention to the process of epithelial–mesenchymal transition, the complex process originating with an indifferentiated metanephric mesenchymal cell and ending with the origin of the mature proximal nephron, and with its fusion with the collecting tubule. We will try to communicate our present view on the sequence of morphological events regulating human kidney development, and will analyze the multiple cell types until now known to be involved as characters of renal development, defining the known factors that propel these cells during their differentiation from a mesenchymal cell towards the multiple complex epithelial structures of the mature kidney. Sure that what we are here reporting is only a part of the story and that, in the next future, the application of immunohistochemistry and of molecular biology to the study of the developing human kidney will add new data, including new cell types or new differential stages of previously known renal cells, to the complex picture of the human nephrogenesis, with possible relevant consequences on the growth of regenerative renal medicine [5].

Morphological Sequence of Events in Kidney Development

Pronephros

Pronephros represents the first of three pairs of embryonic excretory organs, including mesonephros and metanephros, which appear in sequence during human embryogenesis. It appears early during the fourth week of human gestation, around the 21st–22nd day of gestation, and represents the simplest and early rudimentary kidney form, an obligatory precursor of the adult kidney. Pronephros is a much simpler organ than the mesonephric or metanephric kidney, consisting of three main components: (1) one blood filtering glomus/glomerulus; (2) the pronephric tubules, which connect glomus with the pronephric duct; (3) the pronephric duct, which transports the wastes to the cloaca. The filtration unit, represented by a capillary tuft, projects into a cavity defined nephrocoel, which is in communication with the coelom. The nephrocoel is joined through multiple pronephric tubules to the pronephric duct, which will give rise to the Wolffian duct, the structure along which the mesonephros and the metanephros will develop (Fig. 1.1). The epithelial cell of the pronephric tubules shows the first differentiation that will evolve into one of the most complex cells of our body: the proximal

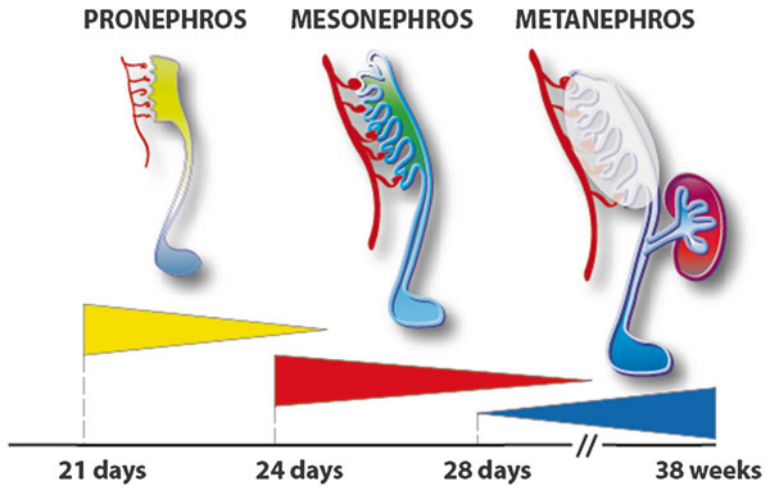

Fig. 1.1 Pronephros, mesonephros, and metanephros development in sequence during human gestation

tubular cells of the mature kidney. The epithelial cell of the pronephric tubules is ciliated: the coordinated movements of cilia move fluid towards the pronephric duct, experimenting some selective absorbing activity on the fluid secreted by the glomus. In the pronephric tubule, development of the epithelial cells occurs in concert with the organ function, being regulated and dependent on the nephron fluid flow. Migration of fully differentiated epithelial cells, epithelial proliferation, and differentiation, the main factors responsible for the elongation of pronephric tubules, are induced by nephron fluid flow, which orchestrates epithelial cell movements during tubulogenesis [3].

Despite its simplicity, the genetic programs regulating building of pronephros are evolutionarily conserved in all vertebrates. Patterning of pronephros appears as the result of the first approach of the human embryo to the construction of a so complex organ such as the mature kidney. For reaching the goal of building the metanephric kidney, embryo operates modelling renal architecture across development via a series of attempts, the pronephros representing the first attempt. In the pronephros project, the main functional structures of the mature kidney are present: the filtering unity, represented by one glomerulus, the collecting system, represented by the pronephric tubules, and the urinary excretory system, represented by the pronephric duct. The life of pronephros is very short: around the 25th day of gestation, it regresses and, at the same time, mesonephros begins to develop (Fig. 1.2).

Mesonephros

The mesonephros represents a much more complex project, as compared to pronephros, containing the majority of cell types and showing very similar stages of glomerular and tubular differentiation schemes observed in the metanephros [1]. For these reasons, historically the mammalian mesonephros has represented one of the most-utilized system for the study of kidney formation, complementary to the study of the developing metanephros, and its analysis yielded fundamental information about the mechanisms underlying nephrogenesis. The mesonephros develops via the reciprocal interactions between the Wolffian duct (WD), which represents the evolution of the pronephric duct and the mesonephric mesenchyme. Multiple, buds emerge from the WD and invade the mass of the mesonephric mesenchyme, inducing the mesonephric pluripotential mesenchymal cells to differentiate into epithelial cells through a process of mesenchymal–epithelial transition, in response to inductive signals from buds emerging from WD (Fig. 1.3). This process gives rise to the origin of a series of renal vesicles, that differentiate into S-shaped structures, which originate a variable number (up to 40) of glomeruli and proximal mesonephric tubules, which fuse with the WD. These simple rudimentary but functioning nephrons represent the first functioning filtration units, connected among them by the Wolffian duct, into which the mesonephric nephrons secrete the first urine. Mesonephroi appear as well developed excretory organs, consisting of a restricted number of glomeruli and tubuli, which function as "interim kidneys" for approximately four weeks. Mesonephron develop in a cranial–caudal wave, reaching the highest level of development around the 33rd day of gestation: at this time point, the highest number of functioning mesonephric nephrons is achieved, the mesonephroi representing the most prominent organ in the future abdominal region of the human embryo (Fig. 1.2a, b) [3]. At this gestational age, two events occur: (1) mesonephric nephrons initiate their regression, following a cranio-caudal wave paralleling the developmental wave; (2) the caudal region of the Wolffian duct gives rise to the ureteric bud that starts its invasion of the metanephric mesenchyme, giving rise to the origin of the metanephros.

For some weeks, from the 5th to the 9th week of gestation, both the mesonephros and the metanephros coexist in the human embryo, mesonephros progressively regressing and the metanephros enlarging. The process of involution of the mesonephros ends in different ways in the two sexes: in males mesonephric tubules form the efferent tubules of the testis; on the contrary, in females mesonephric nephrons regress completely, around the 10th week of gestation.

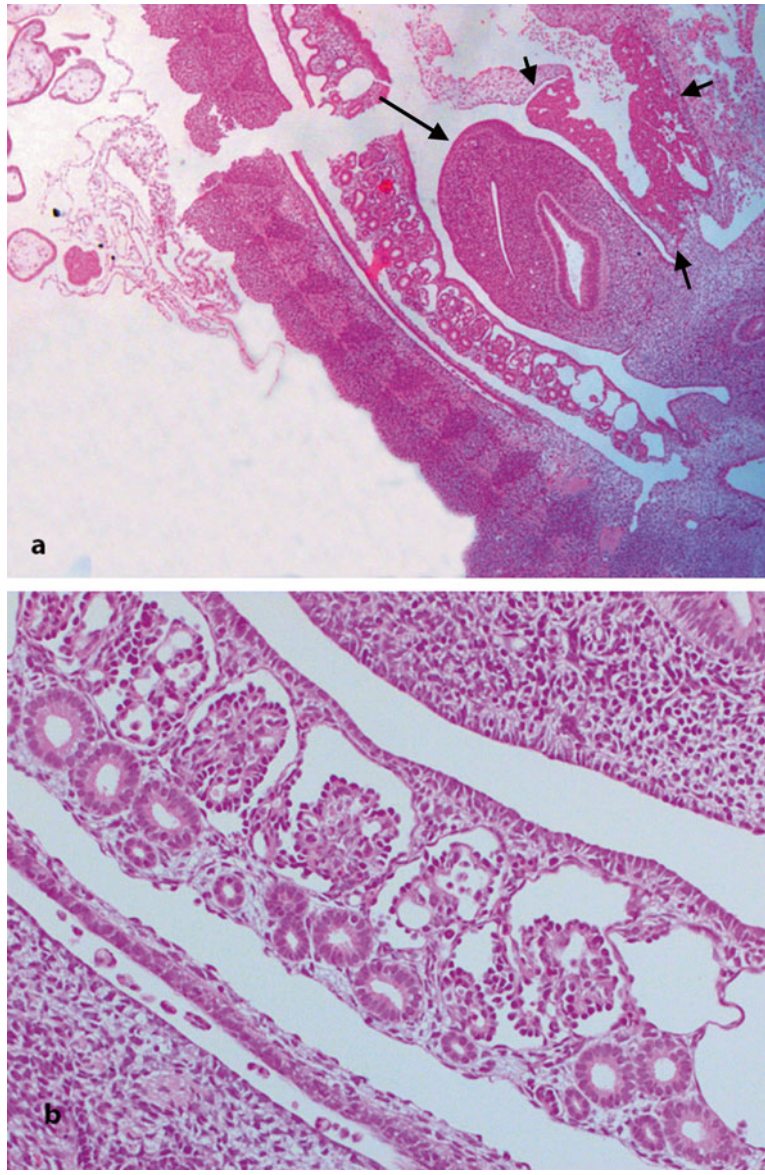

Fig. 1.2 Mesonephros in a human embryo. (**a**) A panoramic view showing mesonephros developing in a cranial–caudal wave, dorsal to the gut (*arrow*) and to the liver (*arrowheads*). (**b**) At higher power, mesonephric nephrons are formed by a rudimentary glomerulus from which a tubule emerges

Metanephros

The metanephric kidney develops via a series of reciprocal interactions between, the ureteric bud and the metanephric mesenchyme, both originating from the intermediate mesoderm. The metanephroi begin to develop early during the fifth week of gestation, and start its function during the ninth week with urine formation. The primary ureteric bud originates at the posterior end of the Wolffian duct as a solid aggregate of the epithelial cells that proliferate, migrate, and progressively invade the surrounding metanephric mesenchyme. The permanent human kidney develops through reciprocal interactions between these two precursor tissue cells: the epithelial cells originating

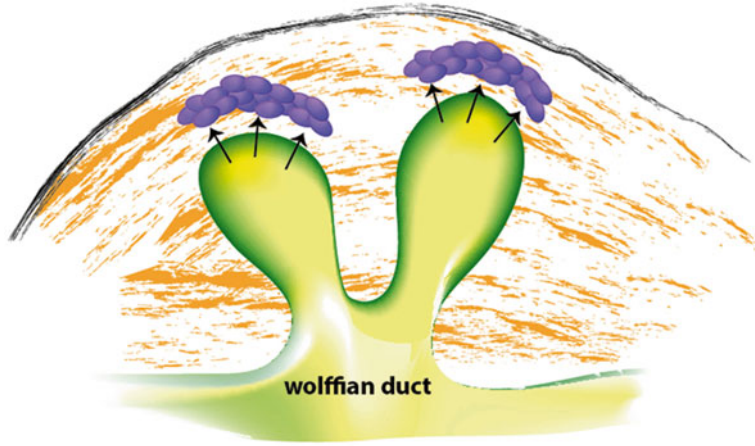

Fig. 1.3 Branching ureteric bud tip cells induce metanephric mesenchyme to condensate and differentiate giving rise to the cap mesenchyme

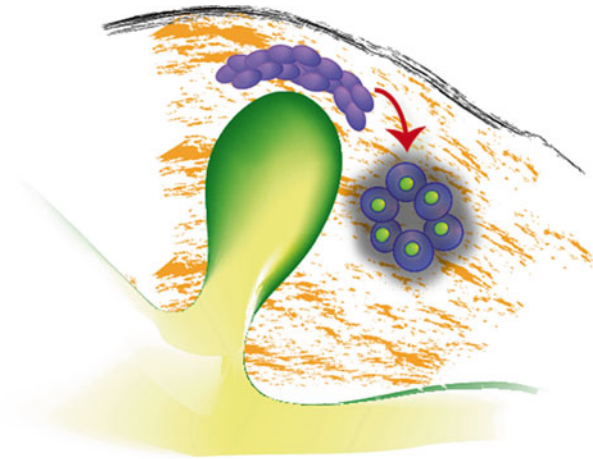

Fig. 1.4 Mesenchymal–epithelial transition of cap mesenchymal cells originates renal vesicles

from the Wolffian duct, and the mesenchymal cells of the metanephric mesenchyme, a mass of mesenchymal cells originating from the intermediate mesoderm which are programmed to make renal progenitors only in response to the inductive signals coming from the branching tips of the ureteric bud. Epithelial cords originating from the ureteric bud, branch into the metanephric mesenchyme, giving rise to the ureteric tree via branching morphogenesis and reaching its periphery, inducing nephron formation at each of its tips. Metanephric mesenchymal progenitor cells condense and aggregate around the tips of epithelial branches, transforming into the cap mesenchymal cells (Fig. 1.3). The pluripotential scarcely differentiated cap mesenchymal cells progressively undergo a process of mesenchymal-to-epithelial transition, which will form most of the epithelial cells of the mature nephrons. The epithelial cells of the tips of the ureteric tree induce cap mesenchymal cells to differentiate into pretubular aggregates, roundish groups of condensed cells which develop in their center a lumen, giving rise to the renal vesicles, the first simple epithelial structure and the precursor of each developing nephron. The renal vesicle is a simple tubule formed by small adherent cells polarized around a central lumen (Fig. 1.4) [1].

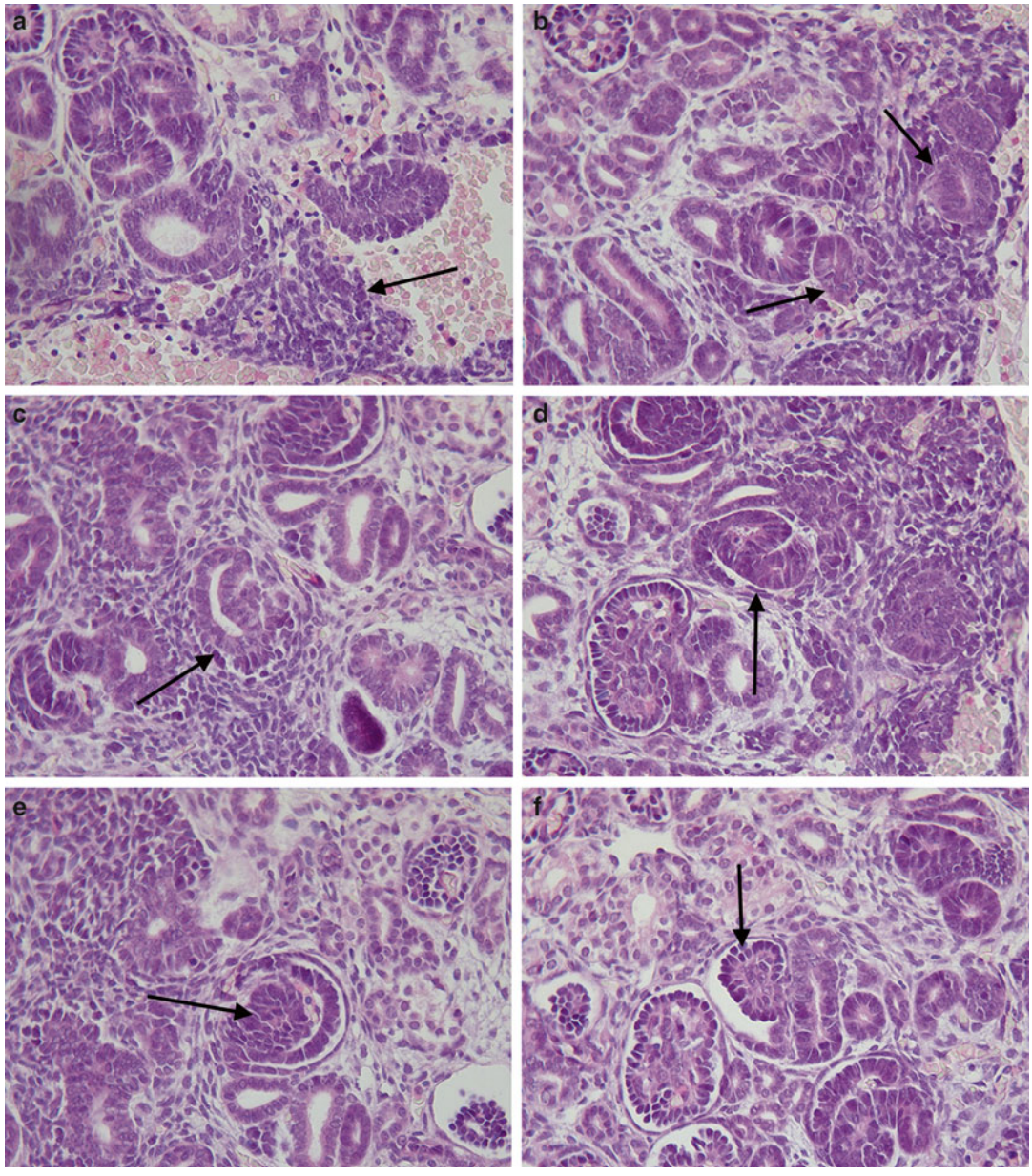

Fig. 1.5 Different steps of human nephrogenesis. (**a**) A cap aggregate (↑) gives rise to a renal vesical, to tubules, and to a rudimentary glomerulus (*top left*). (**b**) Renal vesicle (↑). (**c**) Comma-shaped body (↑). (**d**) S-shaped body (↑). (**e**) Vascular precursor cells (↑). (**f**) Glomeruli (↑) in the early phases of development

The transition from the renal vesicle towards the mature proximal nephron requires multiple sequential processes of segmentation and patterning (Fig. 1.5a–f). This process may be subdivided into four stages: stage I, in which the renal vesicle originates; stage II, in which two sequential segmentation events give rise first to the comma-body and subsequently to the S-shaped body; stage III, also defined as the capillary loop stage, in which angioblasts originate the vascular tuft; stage IV, characterized by the differentiation of the proximal tubule, elongation of the Henle loop, differentiation of the distal tubule, and differentiation of all the cells types that characterize the mature human kidney. The first stage is characterized by the differentiation of the solid pretubular aggregates,

formed by pluripotential cap mesenchymal cells, into roundish epithelial structures with a central lumen, the renal vesicles. This process, known as mesenchymal–epithelial transition, occurs in many developmental processes and is a multi-step process beginning with non-polarized cells embedded in the extracellular matrix and producing well-polarized and adhesive epithelial cells. The following sequential steps have been outlined: (a) cytoskeletal reorganization to actively drive cell adhesion, (b) acquisition of polarity markers, (c) expression of intercellular adhesion mediated by cadherins and adherens junctions, (d) basal membrane assembly. These steps are not all necessarily present in a given example of MET but, as a whole, they characterize the MET process. The second stage of differentiation is characterized by two sequential segmentations occurring in the renal vesicle: the first gives rise to the comma-shaped body, and the second originates the S-shaped body [3]. At the stage of comma-body, the developing nephron may be subdivided into a proximal and a distal segment, both characterized by the expressions of intercellular adhesion molecules such as E-cadherin. A second segmentation of the comma-body originates the S-shaped body, which is organized into three segments, proximal, medial, and distal. These three segments are, at this point, committed to originate different segments of the developing nephron: the cells of the proximal segment further differentiate to form the parietal epithelial cells of the Bowman capsule and develop into the precursors of podocytes; cells of the median segment of the S-shaped body differentiate into the proximal tubules; cells of the distal segment give rise to the distal tubules and drive the process of fusion with the collecting tubules [6]. The third stage is characterized by rapid developmental changes in the renal vasculature. The development of renal arteries and veins, with the origin of afferent and efferent arteries, the appearance of the glomerular capillary tufts in strict contact with the Bowman capsule, the differentiation of the endothelial cells of the corpuscle represent the most important changes in the developing nephron in this stage. Moreover, at the border between the median and the distal segment of the S-shaped body, the primitive loop of Henle originates, initiating its migration into the inner medulla that will be completed only later, during the fourth stage.

Stage IV is characterized by the differentiation and proliferation of the principal cell types that will give rise to the evolution of the mature renal corpuscles, by the differentiation of the different interstitial cells that characterized the cortical, the medullary, and the perihilar zones, and by the development of the juxtaglomerular complex. Inside the glomerulus, two main cell types undergo differentiation: podocytes and mesangial cells.

Podocytes originate from the inner layer of the proximal part of the S-shaped body. In immature glomeruli, they appear as roundish cells, characterized by a voluminous nucleus with dense chromatin and by a scant cytoplasm. These podocyte precursors envelop the developing renal corpuscle, and delimitate its borders, giving rise to a nest-like structure. During migration of the developing glomerulus from the subcortical zones towards the deep cortex, podocyte precursors develop an arborized structure within the glomerulus, progressively embracing the capillary structures with their foot-processes attached to the glomerular basal membrane. Finally, podocytes and glomerular capillaries undergo structural fusion, paralleled by slit diaphragms development among foot-processes, allowing filtration to occur.

Conflicting data have been reported on the origin of mesangial cells over the years. An origin from the multipotent self-renewing nephron progenitor population of the cap mesenchyme has been proposed by some authors [7].

Another hypothesis supporting the origin of the mesangium from the bone marrow has been proposed: according to this hypothesis, mesangial cell precursors might migrate into the developing glomerulus from outside, deriving from a hematopoietic stem cell [8].

The interstitial cells of the mature kidney take their origin from the cap mesenchymal progenitor cells through the non-nephron lineage, which parallels and intersects with the nephron lineage. A number of subcomponents of the renal interstitium progressively differentiate from the first actors in the non-nephron lineage, including angioblasts, the renal interstitial cells, putatively mesangium, pericytes, cells of the renal capsule, fibroblasts, muscle cells, and

resident macrophages [9]. Ontogeny of intrinsic innervations throughout the developing human kidney represents a new field of research. An abundance of adrenergic nerve fibers arise around the 20th week of gestation in the cortex in close proximity to arterioles and arteries, and in the medulla close to tubular cells [10].

The ureteric tree inside the metanephric mesenchyme will subsequently originate the collecting system, including collecting ducts, calyces, and the renal pelvis, whereas the part of the ureteric bud that does not enter the metanephric mesenchyme gives rise to the ureter and to the bladder trigone [11].

The sequential steps of kidney development here reported have been frequently reported in the literature as typical of all mammals. Recent studies have shown that renal development on pigs shows peculiar differences as compared to humans, suggesting that caution should be taken when comparing data on kidney development in experimental animals to human nephrogenesis, in health and disease [12].

The Newborn Kidney: A Checklist for a Developmental-Morphological Approach

The morphological study of the newborn kidney, and in particular of the premature human kidney, shows some relevant peculiarities, when compared to the study of the adult kidney. The neonatal kidney is often characterized by the presence of ongoing nephrogenesis, with multipotent stem cells giving rise to new nephron through the process of mesenchymal-epithelial transition and to new interstitial cells which cooperate with newly formed epithelial cells to originate new mature nephrons. In simple words, whereas the adult kidney is characterized by the presence of stable structures, the neonatal kidney is mainly characterized by developing and traveling immature structures. On the basis of these relevant differences, the morphological analysis of the newborn kidney necessitates a peculiar approach, focused on identifying the main cell types participating to nephrogenesis and their different differentiation levels. In order to simplify this morphological approach, mainly based on the knowledge that the neonatal kidney is a developing organ, we will try to give an answer to some questions.

Is Nephrogenesis Active in this Kidney?

The presence of ongoing nephrogenesis is characterized by the presence, in the subcapsular region, of pluripotential stem cells and of all the structures representing the multiple steps of mesenchymal-epithelial transition (Fig. 1.6). The pluripotential renal cells appear as small cells, with a roundish or elongated nucleus and a scant cytoplasm. The scarcity of the cytoplasm is at the basis, in H&E-stained sections, of the "blue" appearance of the nephrogenic areas located in close proximity of the renal capsule. Whereas the less differentiated pluripotential cells are not strictly aggregated, groups of these cells located in close proximity to the tips of the ureteric buds give rise to nest-like agglomerates, which are characterized by development of adhesion structures among them. The epithelialization of the cap mesenchymal aggregates is evidenced by the appearance of a central lumen. The number of the renal vesicles observed in the subcapsular area gives to the observer a semiquantitative indication regarding how many nephron are going to develop, each renal vesicle expressing one possible new nephron. The frequency of renal vesicles, of comma-shaped and of S-shaped structures, all taken together, may well represent the activity of the nephrogenic process in a neonatal kidney.

Did the Ureteric Bud Proliferate Correctly into the Metanephric Mesenchyme?

To give an answer to this apparently complex question, one should clearly evidence the collecting tubules, i.e., the component of the distal nephron that originates from the ureteric bud. In a normally developing kidney, ureteric bud tips may be observed in close proximity to the subcapsular nephrogenic zone, in strict contact with cap

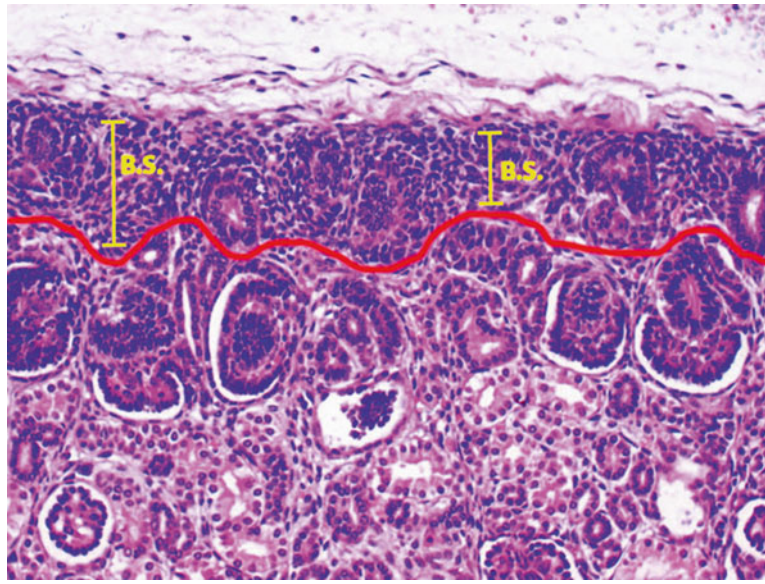

Fig. 1.6 The blue strip (BS) represented by stem/progenitor cells located in close proximity to the renal capsule

mesenchymal cells, nephrons originating upon induction by ureteric bud cells of the metanephric mesenchyme. Recently, a simple trick for identifying the ureteric bud tree in the neonatal kidney has been reported. An old histochemical method, the PAS stain has been recently reported to clearly evidence collecting tubules, which are characterized by high amounts of glycogen, appearing as coarse PAS-positive granules scattered throughout the cytoplasm of tubular cells [13].

Which Is the Nephron Burden of this Kidney?

This question regards the past nephrogenic activity in a newborn kidney. It can be easily determined by the count of the previously developed glomeruli and by the evaluation of their developmental stage. As for the quantitation of nephron number, a simple method has been developed, defined the "radial glomerular count" [14]. The radial glomerular count is based on the number of glomeruli detected along a straight line extending from the capsule and ending in the deepest cortex. The count should be made in well-oriented kidney sections, showing a well-defined corticomedullary boundary and complete renal pyramids (Fig. 1.7). The final count may be obtained by counting glomeruli in four different locations and averaging them. This semiquantitative datum may be considered as representative of the efficacy of the previous nephrogenesis during gestation.

Which Is the Nephrogenic Potential of the Kidney?

The multipotent metanephric mesenchymal cells located under the renal capsule represent the nephron progenitor population of a neonatal kidney, capable to give rise to all segments of new nephrons, except the collecting tubules. These scarcely differentiated multipotent cells appear as a hemtoxylinilofic (blue) strip at H&E-stained kidney sections, located in close proximity to the renal capsule. The width of the nephrogenic zone, i.e., the width of the blue strip, has been recently suggested to represent the residual nephrogenic potential of each neonatal kidney [15]. Measurement of the amount of pluripotential renal cells in a neonatal kidney could be considered as a simple and effective new tool for the evaluation of the residual potential nephrogenesis. The absence of the blue strip in a preterm newborn might indicate the early cessation of

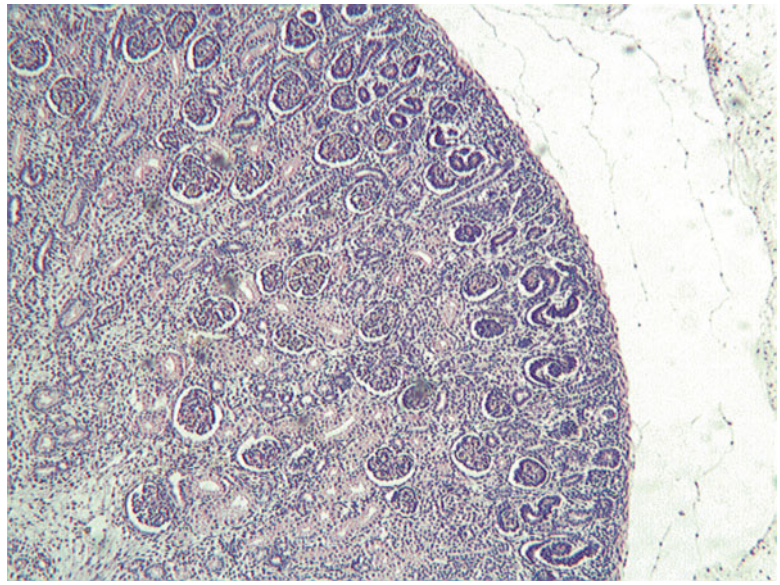

Fig. 1.7 The radial count in a fetal human kidney

nephrogenesis, following maternal or postnatal pharmacological treatments as recently demonstrated in baboons [16]. Alternatively, a reduction of the blue strip in a newborn kidney, as compared to the width normally expected at a certain gestational age, might suggest a modification of the complex factors regulating nephrogenesis in humans. The absence of the blue strip indicates that the glomerulogenic zone disappeared, and that no potential nephrogenesis could go on in that kidney.

Are Signs of Renal Injury Present?

The following elementary lesions should be checked in any neonatal kidney. The vast majority of renal lesions may be easily detected in H&E-stained sections. Periodic acid–schiff (PAS) method may be considered a simple ancillary stain, able to evidence the basal membranes inside the glomerular tuft. The following pathological changes should be checked:

- Vacuolization of the proximal tubular epithelium
- Interstitial edema
- Interstitial hemorrhages
- Tubular casts
- Tubular cell apoptosis
- Tubular necrosis
- Calcium deposits
- Increased glomerular matrix
- Glomerulosclerosis
- Cystic dilatation of the Bowman capsule
- Endothelial damage in renal vessels

Conclusions

According to our experience in the morphological interpretation of the newborn human kidney, our opinion is that morphology maintains a major role in the study of renal development. The recent acquisitions on genetic programming regulating nephrogenesis, the identification of progenitor/stem cells, the new correlations between signaling and morphogenesis in the neonatal kidney, taken together all these data allow morphologists to come back to H&E-stained renal section for a new interpretation. Moreover, the application of immunohistochemistry to the study of the developing human kidney may help researchers and

pathologists to better identify the multiple cell types involved in human nephrogenesis, evidencing their specific morphological modifications and, eventually, allowing their identification in routine histological sections.

Our experience of pathologists involved in the interpretation of neonatal kidneys and in the discussion of morphological data at the microscope with the neonatologist induces us to state that every kidney is morphologically different from the next. The marked interindividual variability in renal maturation in preterm infants, regarding the number of nephrons developed as well as the number of pluripotential/stem cells responsible for glomerulogenesis in the postnatal period, makes the interpretation of every neonatal kidney complex and difficult. Only the correlation with the clinical history, with pharmacological treatments of the mother and/or of the newborn in the postnatal period may allow a correct interpretation, correlating hypoxia or other pathological events with the peculiar behavior of nephrogenesis in a single case. A training in the interpretation of the neonatal kidney is absolutely recommended, even for expert renal pathologists involved in the study of the adult kidney. The interpretation of the different cell types involved in human nephrogenesis may easily lead to errors, given the complexity of the histological picture of the fetal kidney. The acquisition of the different steps of the process of mesenchymal-epithelial transition is fundamental, to build a morphological bridge between the elongated mesenchymal cells and the roundish adherent epithelial cells that originate the renal vesicles and the other epithelial structures of the proximal nephron.

Finally, a fascinating world may be found into each histological section of a neonatal kidney, and many answers may be given to the neonatology regarding the influence of multiple factors on the evolution of nephrogenesis during the intrauterine life. A question-based approach is mandatory, in order to give a functional significance to morphological changes and, more important, to give good answers to the questions of clinicians.

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Molecular Regulation of Kidney Development

2

Clara Gerosa, Daniela Fanni, Sonia Nemolato, and Gavino Faa

Introduction

The human kidney develops through reciprocal interactions between two precursor tissues: the ureteric bud and the metanephric mesenchyme. During the multiple steps of nephrogenesis, different morphogenetic molecules are reciprocally exchanged among the epithelial progenitor cells deriving from the ureteric bud and the mesenchymal cells originating from the metanephric mesenchyme. These molecules are at the basis of the complex and in part unknown cell-talking between stem/progenitor renal cells and progressively differentiated cells that regulate morphogenesis, ultimately leading to the development of the mature human kidney. Here the main molecular mechanisms involved in kidney development in different animal species will be described. The majority of molecular data regarding nephrogenesis will be relative to the developing mouse kidney, which is currently the best-characterized model of renal organogenesis at a transcriptional level [1].

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The Metanephric Mesenchyme

The physiological impact of molecular mechanisms regulating kidney development has been at least in part revealed by recent studies, demonstrating the role of multiple specific genes at particular stages of kidney development. The specification of the metanephric mesenchyme from the intermediate mesoderm represents one of the fundamental stages in human nephrogenesis. At the best of our knowledge, no single regulator has yet been identified to specify the insurgence of the metanephric mesenchyme within the intermediate mesoderm, the earliest step of metanephric kidney development and the molecular mechanisms controlling it are, at least in part, essentially unknown.

The transcription factor odd-skipped related 1 (**Odd1**) is one of the earliest known marker of the intermediate mesoderm whose expression defines the kidney stem/progenitor population, inducing the mesodermal precursors to differentiate into the metanephric mesenchyme (Fig. 2.1). Odd1 is localized to mesenchymal precursors within the mesonephric and metanephric kidney, where it plays an important role in establishing kidney precursor cells, and in regulating the initial steps of their differentiation into mature renal cells [2]. Odd1 expression is required for the activation of several other factors required for metanephric kidney formation, including Six2, Pax2, Eya1, Sall1 and Gdnf.

The chicken ovalbumin upstream promoter transcription factor II (**COUP-TFII**), a member

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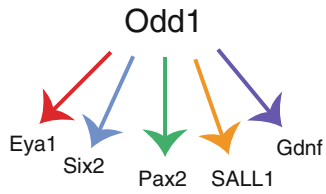

Fig. 2.1 Odd1 expression by mesenchymal precursors is required for the activation of several genes

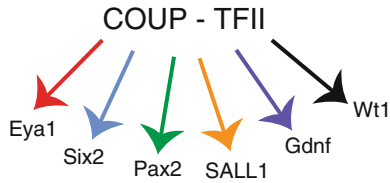

Fig. 2.2 The chicken ovalbumin upstream promoter–transcription factor II plays a central role in the expression of essential developmental regulators of nephrogenesis

of the steroid/thyroid hormone receptor superfamily, is required for the specification of the metanephric mesenchyme [3] (Fig. 2.2). COUP-TFII plays a central role in the specification of metanephric fate and in the maintenance of metanephric mesenchyme proliferation and survival by directly regulating Eya1 and Wt1 expression. COUP-TFII deletion causes the improper differentiation of the metanephric mesenchyme, due to the absence of essential developmental regulators, including Eya1, Six2, Pax2 and Gdnf [4].

Eya 1 is indispensable for the formation of nephric duct and mesonephric tubules, is a critical determination factor in acquiring metanephric fate within the intermediate mesoderm and is a key regulator of Gdnf expression during ureteric induction and branching morphogenesis. The principal role of Eya1 in nephrogenesis is shown by its loss that is associated with failure of metanephric induction ending with renal agenesis. Eya 1 probably plays an essential function at the top of the genetic hierarchy controlling kidney organogenesis, acting in combination with Six 1 and Pax 2 to regulate Gdnf expression during ureteric bud outgrowth and branching [5].

Pax 2 and **Pax 8** expression is necessary for morphogenesis and guidance of the primary nephric duct in the early phases of kidney development [6]. The Pax2 gene encodes a DNA binding, transcription factor whose expression is essential for the development of human kidney. During kidney development, the transcription factor Pax2 is required for the specification and differentiation of the renal epithelium [7]. Both gain and loss of function mutants in the mouse demonstrate a requirement for Pax2 in the conversion of metanephric mesenchymal precursor cells to the fully differentiated tubular epithelium of the nephron [8]. Decreased Pax2 protein levels have been associated with excessive amounts of programmed cell death (apoptosis) in the ureteric bud tips and in the deriving collecting tubules, ending with paucity of bud tip-derived collecting tubules and renal hypoplasia [9]. In humans, PAX2 haploinsufficiency causes the renal-coloboma syndrome (RCS) involving eye abnormalities, renal hypoplasia, and renal failure in early life [10].

Recently, a crosstalk between **p53** and **Pax2** has been hypothesized to represent a transcriptional platform in the metanephric mesenchyme that promotes nephrogenesis, the cooperation between p53 and Pax2 significantly contributing to nephron endowment [11] The following data have been reported to sustain this hypothesis: (a) peaks of p53 occupancy in chromatin regions of the Pax2 promoter and gene in embryonic kidneys; (b) p53 binding to Pax2 gene is significantly more enriched in Pax2-expressing than non-expressing metanephric mesenchyme cells; (c) Pax2 promoter activity is stimulated by wild-type p53 and inhibited by a dominant negative mutant p53; (d) p53 knockdown in cultured metanephric mesenchyme cells down-regulates endogenous Pax2 expression; (e) reduction of p53 gene dosage worsens the renal hypoplasia in Pax2(+/−) mice.

The glial cell-line derived neurotrophic factor (**GDNF**) is the major mesenchyme-derived regulator of ureteric budding and branching during nephrogenesis. GDNF is synthesized by metanephric mesenchymal cells and activates a receptor complex composed of Ret and GFRα1 on the ureteric bud epithelium. A Notch ligand, Jagged1 (Jag1), co-localizes with GDNF and its receptors

during early kidney morphogenesis [12]. The GDNF/c-Ret/Wnt11 pathway is generally considered the major positive regulator of ureteric bud development and branching [13]. Kidneys developing in the absence of GDNF display severe branching abnormalities [14]. The secretion of GDNF by the metanephric mesenchyme is under control of the transcription factor Pax2 [15].

The **Sall1** gene, encoding for a mesenchymal nuclear zinc finger protein, Sall1, is highly expressed in multipotent nephron progenitors in the metanephric mesenchyme [16]. Sall1 controls ureteric bud attraction and branching into the metanephric mesenchyme by regulating a novel kinesin, Kif26b. Moreover, Sall1-positive progenitor cells can partially reconstitute a three-dimensional structure in organ cultures following Wnt4 stimulation.

Recent studies indicate that renal stem/progenitor cells of the embryonic kidney are organized in a series of compartments, characterized by an increasing state of differentiation. The earliest progenitor compartment may be marked by expression of **CITED1**, and possesses greater capacity for renewal and differentiation than other compartments [17]. Signalling events governing progression of nephron progenitor cells through the very early stages of differentiation are poorly understood, and their knowledge will provide key insights into normal and dysregulated nephrogenesis, as well as into regenerative processes that follow kidney injury. The CITED1(+) stem/progenitor cell compartment is maintained in response to receptor tyrosine kinase (RTK) ligands that activate both fibroblast growth factor (FGF) and epidermal growth factor (EGF) receptors, the intracellular ligands mediating these growth factor effects [18]. FGF, EGF and its foetal form, transforming growth factor-alpha (TGF-α) are renal mitogens which induce epithelial hyperplasia and accelerate distal nephron differentiation in metanephric organ culture. Since RTK signalling function is dependent on **RAS** and PI3K signalling, the survival and developmental potential of cells in the earliest embryonic nephron progenitor cell compartment are dependent on FGF/EGF signalling through RAS [19].

Recently, the role of FGF receptor 1 and 2 in the maintenance of the metanephric mesenchymal stem/pluripotential cells has been confirmed. In particular FGFR2 signalling in the metanephric mesenchyme has been shown to promote Bmp4 expression, which represses Ret levels and signalling in the ureteric bud to ensure normal ureteric morphogenesis [20].

The Primary Ureteric Bud Origin

The ureteric bud emerges from a swelling of the caudal Wolffian duct epithelium as a result of Ret-dependent cell movements, renal agenesis occurring when the ureteric bud fails to emerge. Ureteric bud outgrowth during the initiation of metanephric kidney development is a complex process, regulated by the antagonism of multiple gene products, some stimulating and other halting ureteric epithelia proliferating and branching. Multiple gene regulatory networks have been reported to act either as inducers or inhibitors.

GDNF, a member of the transforming growth factor-beta (TGF-β), is secreted by the metanephric mesenchymal cells under the control of the transcription factor PAX2, and interacts with the GDNF receptors c-RET tyrosine kinase and GFRα-1 expressed by the ureteric bud tip cells, inducing their branching [21]. The GDNF/c-Ret/Wnt1 pathway is generally considered the major positive regulatory pathway responsible for ureteric bud development and branching [22]. The relevance of the role played by the c-RET signalling pathway in the development of the primary ureteric bud and in further kidney development is well evidenced in the RET knock-out mice which is affected by perinatal lethality due to bilateral renal agenesis [23]. GDNF/RET signalling is required for ureter and kidney development. RET regulates cell rearrangements in the caudal Wolffian duct, generating a specialized epithelial domain that later emerges as the tip of the primary ureteric bud. Wolffian duct cells compete, based on RET signalling levels, to contribute to this domain and to initiate ureteric bud morphogenesis,

whereas cells lacking Ret are excluded from the tips of the branching ureteric bud [24]. The pivotal role of GDNF in early kidney development has been well evidenced in knock-out mice, where lack of GDNF blocks development of the ureteric bud [25].

Ret signalling alters patterns of gene expression in UB tip cells, a critical event being up-regulation of the ETS transcription factors Etv4 and Etv5, that represent key components of a regulatory network downstream of Ret. Ret signalling via Etv4 and Etv5 promotes important cell rearrangements in the Wolffian duct, inducing the cells with the highest level of Ret signalling to preferentially migrate to form the first ureteric bud tip, giving rise to the branching morphogenesis in the developing kidney [26]. The specific function of the ETS transcription factors Etv4 and Etv5 remains unclear. They are known to be required for mouse kidney development and to act downstream of Ret. Recently, an autonomous role for Etv4 and Etv5 in the origin of the ureteric bud has been hypothesized. According with this hypothesis, Etv4 and Etv5 could play a broad role for cell rearrangements not restricted to the Ret pathway, but downstream of multiple signals, which are together important for Wolffian duct and ureteric bud morphogenesis [27].

Among the multiple factors modulating the ureteric bud branching, a major role is played by some members of the Bone morphogenetic protein (BMP) family, including multi-functional growth factors that belong to the TGF-β superfamily [28]. In particular, BMP4 and BMP7 are important signalling molecules throughout kidney development, and play an inhibitory role on the modulation of ureteric bud outgrowth and branching [29]. BMP7 is required for maintenance of the nephron progenitor cell population in the nephron progenitor niche, functioning as a mitogen in these cells by activating the MAPK signalling pathway or Smad pathway, and its expression is essential for appropriate nephrogenesis [30]. Recently, the expression of BMP2 has been reported in the proximal tubular cells of the adult healthy kidney, where BMP2 activates transcriptional responses in nephron epithelial cells [31].

Many antagonists act to restrict and negatively modulate the activity of secreted signals during progression of nephrogenesis. Among them, the extra-cellular BMP antagonist gremlin 1 (Grem1) plays a fundamental role at the stage of initiating ureteric bud outgrowth. Development of the primary ureteric bud requires the reduction of BMP4 activity in the metanephric mesenchyme in close proximity of the Wolffian duct. This down-regulation of BMP4 is operated by the antagonist gremlin 1, which in turn enables ureteric bud outgrowth and establishment of autoregulatory GDNF/WNT11 feedback signalling. Bmp4 is expressed by the metanephric mesenchymal cells enveloping the Wolffian duct and the ureteric bud and Grem1 is progressively up-regulated in the mesenchyme around the nascent ureteric bud prior to initiation of its outgrowth [32]. The mechanisms that regulate the GREM1–BMP4 signalling have been recently clarified. The expression of Grem1 in the metanephric mesenchyme is **Six1**-dependent, Six1 representing an upstream regulator of Grem1 in initiating branching morphogenesis and a crucial regulator of renal development [33]. Six1 mutations in human cause the branchio-oto-renal (BOR) syndrome and Six1(−/+) mice exhibit renal agenesis [34, 35].

The zinc finger protein Sall1 expression in metanephric mesenchymal cells is essential for ureteric bud attraction towards and into the metanephric mesenchyme, initiating and organizing the ureteric bud branching and inducing nephron formation [36]. Kif26b, a kinesin family gene, is a downstream target of Sall1. The disruption of this gene causes kidney agenesis because of impaired ureteric bud attraction. Kif26b is essential for kidney development because it regulates the adhesion of mesenchymal cells in contact with ureteric buds. In the Kif26b-null metanephros, compact adhesion between mesenchymal cells adjacent to the ureteric buds was impaired, resulting in failed maintenance of Gdnf, a critical ureteric bud attractant [37].

Fras1, a basement-membrane-associated protein, is physiologically expressed in the developing ureteric bud tip cells, where it favours interactions between the bud and the surrounding mesenchymal cells and regulates the expression

of key nephrogenic molecules. Furthermore, Fras1 may also be required for the formation of normal glomeruli [38]. Deficiency of the extracellular matrix molecule FRAS1 leads to bilateral renal agenesis in humans with Fraser syndrome as well as in the animal model of this genetic human disease, the blebbed [Fras1(bl/bl)] mice. Interestingly, introducing a single null allele into Fras1(bl/bl) mice resulted in downregulation in the ureteric bud of Sprouty1, an anti-branching molecule and antagonist of the GDNF/ RET pathway, able to prevent renal agenesis [39]. This study not only contributes to a better knowledge regarding molecular events occurring during nephrogenesis, but demonstrates rescue of renal agenesis in a model of a human genetic disease, and raises the possibility that enhancing peculiar growth factor signalling might be a therapeutic approach to ameliorate this devastating renal disease.

The homeodomain transcription factor PAX2 plays an important role in the early phases of urogenital development, maintaining a self-renewing nephron progenitor population during kidney development. In particular, PAX2 proper expression is fundamental for the emergence and guidance of the ureteric bud from the Wolffian duct [6].

Mdm2 (Murine Double Minute-2), a factor required to control cellular p53 activity and protein levels, is expressed in the ureteric bud epithelium and in the metanephric mesenchyme lineages. A critical and cell autonomous role for Mdm2 in the ureteric bud development has been demonstrated, (mdm2−/−) mice dying soon after birth displaying renal hypodysplasia due to defective ureteric bud branching and underdeveloped nephrogenic zone [40].

Angiotensin II AT2 receptor (AT2R) performs essential functions during UB branching morphogenesis via control of the GDNF/c-Ret/Wnt11 signalling pathway, ureteric bud cell proliferation, and survival. Embryonic mouse kidneys express AT2R in the branching UB and the mesenchyme. Treatment of embryonic metanephros with AT2R antagonists or by genetic inactivation of the AT2R has been shown to inhibit ureteric bud branching, decreasing the number of UB tips compared with control animals [41]. AT2R performs essential functions during ureteric bud insurgence and branching via control of the GDNF/c-Ret/Wnt11 signalling pathway [42].

The stem cell marker TRA-1-60 is linked to pluripotency in human embryonic renal stem cells and is lost upon differentiation. In 8- to 10-week human foetal kidney, TRA-1-60 is abundantly expressed by ureteric bud cells and derived structures, including collecting duct epithelium. Dual staining showed that TRA-1-60 positive cells co-expressed Pax-2 and Ki-67, markers of tubular regeneration. Given the localization in human foetal kidney, it has been hypothesized that TRA-1-60 may identify a population of pluripotential/stem cells contributing ureteric bud branching and to nephrogenesis [43]. The fibroblast growth factor receptor (Fgfr) signalling plays a relevant fundamental role in the context of embryonic kidney development. Fgfrs are receptor tyrosine kinases with four signalling family members and 22 known Fgf ligands in humans [44]. Fgfr1, Fgfr2 and Fgfr11 are the most relevant to renal development: Fgfr1 is highly expressed in the metanephric mesenchyme, in the cap mesenchyme, in renal vesicles and, at lower levels, in renal stromal cells; Fgfr2 is strongly expressed in the Wolffian duct cells, in the ureteric bud tips and at lower levels in the stromal mesenchyme; Fgfr11 is expressed in renal vesicles. Given its diffuse expression in the different embryonic compartments during kidney development, Fgrfr signalling should be considered critical for pattering of virtually all cell lineages during kidney development, as evidenced by the association of Fgfr2 loss in the metanephric mesenchyme with many kidney and urinary tract anomalies [45].

SLIT2 and its receptor ROBO2 are known primarily for their function in axon guidance and cell migration. Recently, a relevant role in the regulation of ureteric bud emerging from the nephric duct in response to GDNF secreted by the adjacent nephrogenic mesenchyme has been assigned to these two molecules. In particular, SLIT2/ROBO2 signal is transduced in the nephrogenic mesenchyme, and represents an intercellular signalling system that restricts, directly or

indirectly, the extent of the Gdnf expression domain. The restriction of Gdnf expression exerted by SLIT/ROBO2 signal is considered critical for correct positioning of ureteric bud development. Mouse mutants lacking SLIT/ROBO2 develop supernumerary ureteric buds that remain inappropriately connected to the nephric duct, due to the inappropriately maintained Gdnf expression [46].

Class 3 semaphorins are guidance proteins involved in axon pathfinding, vascular patterning and lung branching morphogenesis in the developing mouse embryo. Semaphorin3a (Sema3a) is expressed in renal epithelia throughout kidney development, including ureteric bud cells. Sema3a plays a role in patterning the ureteric bud tree in metanephric organ cultures, by inhibiting ureteric bud branching and decreasing the number of developing glomeruli. SEMA3A effects on ureteric bud branching involve down-regulation of GDNF signalling, competition with vascular endothelial growth factor-A (VEGF-A) and decreased activity of Akt survival pathways. Taken all together, these data suggest that Sema3a is an endogenous antagonist of ureteric bud branching and hence, plays a role in patterning the renal collecting system as a negative regulator [47]. Recently, another member of the semaphoring family, Sema3c has been shown to play a role in the primary ureteric bud domain and in ureteric bud development. Contrary to Sem3a, Sem3c is a positive regulator of ureteric bud and endothelial cell branching morphogenesis [48].

In summary, secreted semaphorins modulate ureteric bud branching, and vascular patterning, suggesting that they play a role in renal disease. Understanding the signalling pathways downstream from semaphorin receptors will provide insight into the mechanism of action of semaphorins in renal physiology and pathology.

Whereas the positive signals, including GDNF, regulating the development of metanephric kidney and the ureteric bud outgrowth from the Wolffian duct (WD) are better known, the negative regulation of this process remains in part unclear. Activin A, a member of TGF-beta family, has been recently shown to may cause inhibition of GDNF-induced bud formation. Activin A up-regulation was accompanied by inhibition of cell proliferation, reduced expression of Pax-2, and decreased phosphorylation of PI3-kinase and MAP kinase in the Wolffian duct, suggesting that activin A is an endogenous inhibitor of bud formation and that cancellation of activin A autocrine action may be critical for the initiation of this process [49].

The expression of transcription factors and growth factors in the developing foetal kidney may be significantly modified by external factors, including maternal conditions during intrauterine life (Figs. 2.3 and 2.4). Maternal food restriction altered gene expression of foetal renal transcription and growth factors, resulting in up-regulated mRNA expression for WT1, FGF2, and BMP7, whereas Pax2, GDNF, FGF7, BMP4, WNT4, and WNT11 mRNAs were down-regulated [50]. Ureteric bud branching morphogenesis is altered even by a high ambient glucose environment. High D-glucose (25 mM) specifically stimulates UB branching morphogenesis via Pax-2 up-regulation, resulting in the acceleration and alteration of branching morphogenesis but not nephron formation. Reactive oxygen species generation, activation of Akt signalling, and up-regulation of Pax-2 gene expression have been proposed as the possible intimate mechanisms underlying the effects of high glucose levels on nephrogenesis [51].

The Cap Mesenchyme

Development of the nephrons, the functional units of the kidney, requires the differentiation of a renal progenitor population of mesenchymal cells to epithelial cells, through the process of mesenchymal–epithelial transition. This process requires an intricate balance between self-renewal and differentiation of the renal progenitor pool. Recently, lineage tracing has confirmed that the portion of the metanephric mesenchyme closest to the advancing ureteric tips, the cap mesenchyme, represents the progenitor population for the nephron epithelia. In spite of recent advances in our knowledge regarding the role of cap mesenchymal cells during renal develop-

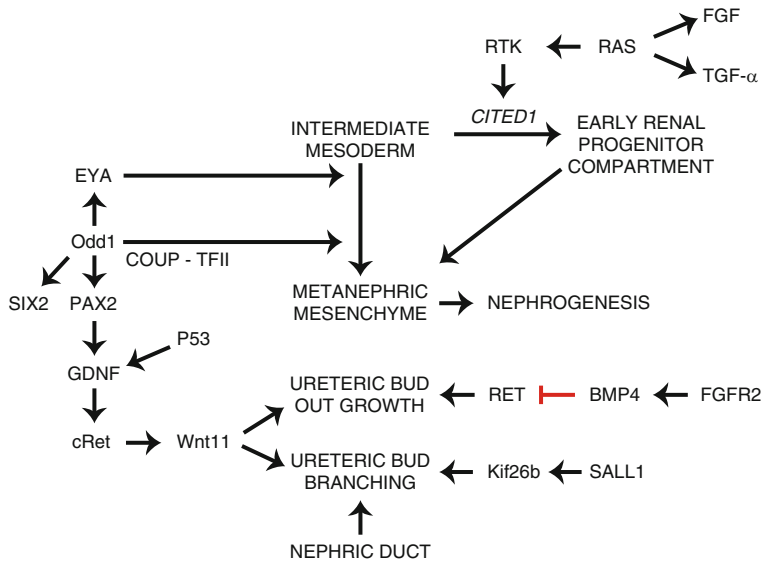

Fig. 2.3 Multiple factors whose expression is indispensable for acquiring the metanephric. Fate within the intermediate mesoderm and for ureteric bud outgrowth and branching

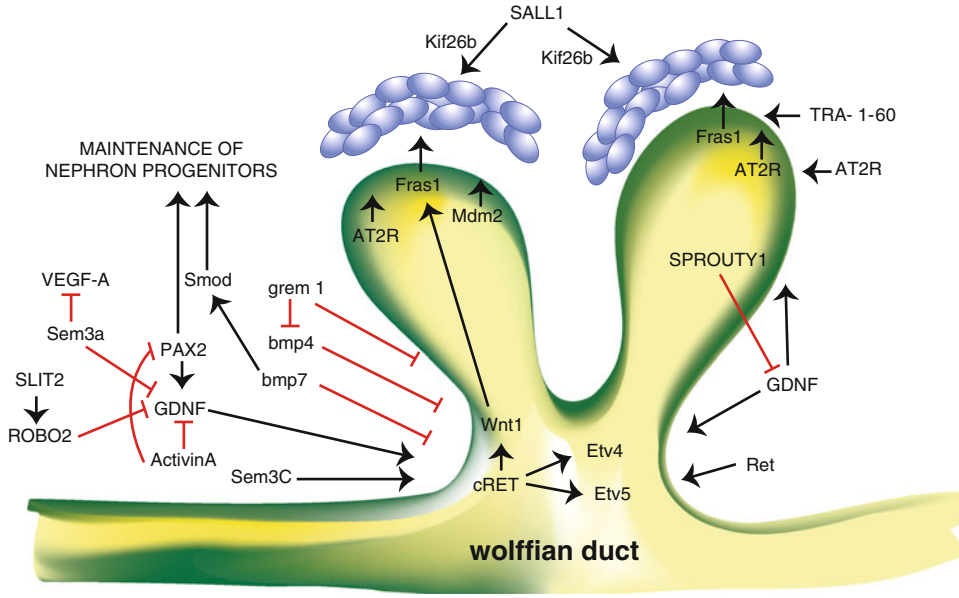

Fig. 2.4 Factors whose expression is necessary for ureteric bud origin and for induction of metanephric mesenchyme to differentiate into cap mesenchyme

ment, there remains a lack of clarity over the intrinsic and extrinsic regulation of cap mesenchyme specification (Figs. 2.5 and 2.6), self-renewal, and nephron potential. Maintaining a population of nephrogenic mesenchyme at the tips of the branching ureter and preserving this cell population conceptually requires three main signalling events: (1) the block of the tendency of metanephric mesenchymal cells to undergo apoptosis; (2) the induction of self-renewal in the

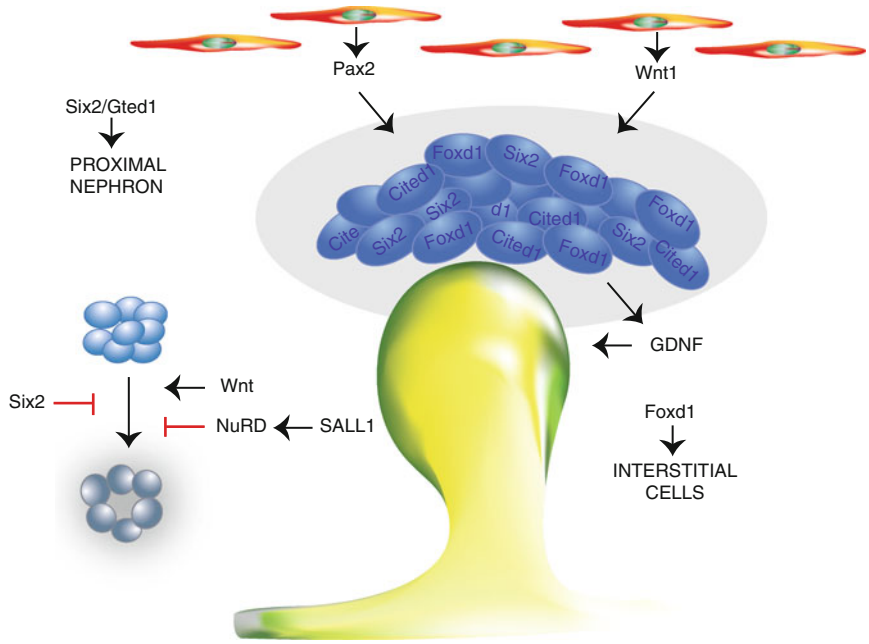

Fig. 2.5 Factors regulating cap mesenchyme specification

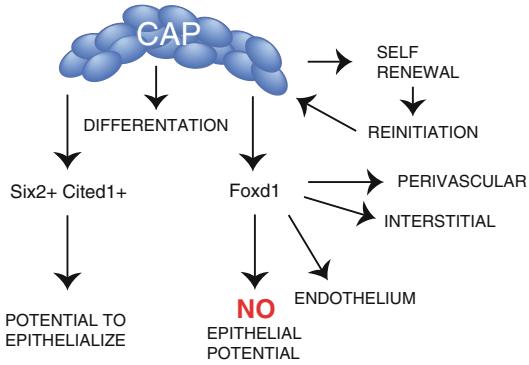

Fig. 2.6 Regulation of cap mesenchyme specification: Six2⁺ and Cited1⁺ cells undergo epithelial transition; Foxd1+ cells give rise to multiple non-epithelial renal cells

nephrogenic mesenchyme cell population, that is continuously depleted by differentiation and formation of new nephrons; (3) a signal is required to oppose tubulogenesis promoted by the branching ureter, reserving a subset of cells for future rounds of tubulogenesis.

It is also not known what regulates cessation of nephrogenesis: a better knowledge of molecular factors responsible for induction and cessation of cap mesenchyme differentiation into new nephrons might help researchers involved in the fascinating project of regenerative medicine recently defined "physiological renal regeneration", that might putatively give rise to kidney regeneration utilizing the renal stem/progenitors present in the newborn kidney [52]. Moreover, an increased understanding of the regulation of this population may better explain the observed variation in final nephron number and potentially facilitate the reinitiation or prolongation of nephron formation.

A program of repetitive reciprocal inductive interactions between the metanephric mesenchyme and the ureteric epithelium drives the assembly of the metanephric kidney. At the onset of metanephric development, the metanephric mesenchymal cells express GDNF, that induces the adjacent ureteric bud to invade and branch within the mesenchyme [15, 21]. The

metanephric mesenchymal cells largely consist of two sub-populations, an outer population of **Foxd1**$^+$ cells, termed the cortical interstitial mesenchyme, and an inner core of **Six2**$^+$ cells termed the cap mesenchyme [53]. Throughout the metanephric development, the cap mesenchyme maintains GDNF expression and remains closely associated with the tips of the branching ureteric epithelium in the cortical zone of the kidney. This expression domain ensures the outward growth of the ureteric epithelium and ultimately the establishment of the arborized network of the collecting duct system [54].

Two gene products are mainly involved in the differentiation of human embryonic stem cells of the metanephric mesenchyme towards kidney precursor cells of the cap mesenchyme, Pax2 and Wnt4 [55].

The cap mesenchyme, all Six2$^+$ cells lying between the ureteric tip and the cortical-most nephrogenic interstitium, is not a homogeneous population. Two main sub-populations have been identified in the cap mesenchyme: (1) the Six2(+) Cited1(+) population, that undergoes self-renewal throughout nephrogenesis while retaining the potential to epithelialize; (2) the Foxd1(+) portion of the cap mesenchyme, that shows no epithelial potential, developing instead into the interstitial, perivascular, and possibly endothelial elements of the kidney [56]. Gene expression stereotypically divides the Six2$^+$ nephron progenitor compartment into three sub-domains, the inner capping mesenchyme, the outer capping mesenchyme and the induced mesenchyme. The likely domain of uninduced nephron progenitors is refined to a sub-domain that is both negative for factors associated with nephron induction and also likely refractory to the primary inductive action of canonical Wnt signalling.

The homeobox transcription factor Six2 is expressed by a subpopulation of cap mesenchymal cells. Six2-expressing cap mesenchyme represents a multipotent nephron progenitor population that gives rise to all cell types of the main body of the nephron during all stages of nephrogenesis. The Six2-expressing population is maintained by self-renewal. Clonal analysis indicates that at least some Six2-expressing cells are multipotent, contributing to multiple domains of the nephron. Furthermore, Six2 functions cell autonomously to maintain a progenitor cell status, regulating a multipotent nephron progenitor population [57]. The major role of Six2 during kidney development should be related to its ability in maintaining the renal progenitor pool, by inhibiting the differentiation of renal progenitor cells: the action of Six2 is balanced by the opposite activity of Wnt, the gene responsible for the differentiation of renal progenitor cells, paralleled by their progressive disappearance [58]. Deficiency in Six2 during prenatal development is associated with reduced nephron number, chronic renal failure, and hypertension in adult mice, suggesting that proper levels of this protein during nephrogenesis are critical for normal glomerular development and adult renal function [59].

Sall1 is a transcription factor necessary for renal development which is expressed in renal progenitor cells of the cap mesenchyme. Sall1 recruits the Nucleosome Remodeling and Deacetylase (NuRD) chromatin remodelling complex to regulate gene transcription. The role of the NuRD complex in cap mesenchyme progenitor cells during kidney development has been highlighted in NuRD knock-out mice. These mutants displayed significant renal hypoplasia with a marked reduction in nephron number, whereas markers of renal progenitor cells, Six2 and Cited1 were significantly depleted and progenitor cell proliferation was reduced. Taken together, these data indicate that Sall1 and NuRD act cooperatively to maintain cap mesenchyme progenitor cells (D R D, M R). Mi-2/NuRD is required in renal progenitor cells during embryonic kidney development [60].

The Mesenchymal–Epithelial Transition

Epithelial differentiation occurs in the cap mesenchymal cells, the self-renewing progenitor cells located around the ureteric bud tips beneath the renal capsule. Morphological changes are

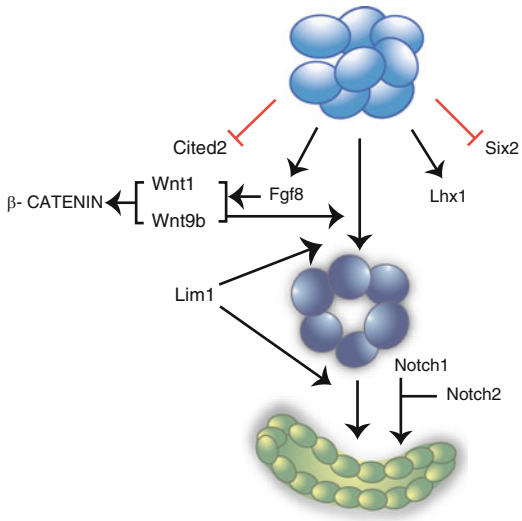

Fig. 2.7 Factors influencing the early phases of mesenchymal–epithelial transition: from cap aggregates to comma-bodies

guided by the activation of a large number of "epithelial" genes, including genes encoding for cytokeratins, desmosomal components, adherens and tight junctions, basement-membrane constituents, laminin types. Conversely, a large series of "non-epithelial" genes, including those encoding for vimentin and collagen, are progressively inactivated. During the progression from the cap aggregates towards the renal vesicle, Six2 and Cited2 are progressively silenced, whereas the expression of LhxI and Fgf8 increases [61] (Fig. 2.7).

Canonical Wnt signalling represents one of the major pathways in the organization of the mammalian urogenital system. In particular, Wnt signalling is the fundamental molecular pathway in the induction of epithelial transition in cap mesenchymal progenitor cells. The cap mesenchymal cells respond to Wnt-4 signalling by differentiating into the renal vesicle, a simple tube that undergoes segmentation, extensive growth, and differentiation. The process involves formation of proto-epithelial cell aggregates, conversion into epithelia, and proximal-distal patterning of the nephron. Two ligands from the Wnt family, namely Wnt9b and Wnt4, are required for nephron differentiation. Recent studies have addressed the downstream targets of these Wnt ligands and delineated the role of the canonical Wnt signalling pathway. The Wnt/β-catenin/TCF/Lef1 signalling pathway depends on the intracellular protein β-catenin and the T cell-specific transcription factor/lymphoid enhancer factor-1 (TCF/Lef1) family of transcription factors. Selective block of β-catenin signalling inhibits differentiation of cap mesenchymal progenitor cells, while forced activation triggers the progression towards proto-epithelial aggregates [62]. The Wnt4 expression is closely related to the Fgf8 expression, inactivation of Fgf8 in early mesoderm resulting in the absence of Wnt4 expression.

Ten molecular markers have been identified as specific of the renal vesicle: Dkk1, Papss2, Greb1, DII1, Pcsk9, Lhx1, Bmp2, Pou3f3, Tmem100, and Wt1 [63]. Recently, a linkage between DKK1 and β-catenin has been discovered: stabilization of β-catenin in the ureteric cell lineage before the onset of kidney development increased β-catenin levels, up-regulation of Dkk1, ending with renal aplasia or severe hypodysplasia [64].

The LIM-class homeobox gene Lim1 is expressed in the intermediate mesoderm, nephric duct, ureteric bud, and in particular in pretubular aggregates and their derivatives, including renal vesicles, comma- and S-shaped bodies. Lim1 has essential roles in multiple steps of epithelial tubular morphogenesis during kidney organogenesis: it functions in distinct tissue compartments of the developing metanephros for both proper development of the ureteric buds and the patterning of renal vesicles for nephron formation [65].

The Notch pathway regulates cell fate determination in numerous developmental processes. Notch2 is highly expressed in the early renal vesicle, where it acts non-redundantly to control the processes of nephron segmentation. Genetic analysis reveals that Notch2 is required for the differentiation of proximal nephron structures, including podocytes and proximal convoluted tubules [66]. Notch1 in concert with Notch2 contribute to the segmentation of the renal vesicle, of the comma-shaped body, and to the origin of the S-shaped body [67]. γ-secretase activity, probably through activation of Notch, is required for

maintaining a competent progenitor pool in the developing kidney, as well as for determining the proximal tubule and podocyte fates [68].

The Epithelial–Mesenchymal Transition During Nephron Repair

Understanding the mechanisms of nephron repair and of renal fibrosis is critical for the design of new therapeutic approaches to treat kidney disease. The kidney can repair after even a severe insult, but whether adult stem or progenitor cells contribute to epithelial renewal after injury and the cellular origin of regenerating cells remain controversial. In a mouse model of ischemia-reperfusion kidney injury, 48 h after renal injury more than 50 % of outer medullary epithelial cells have been shown to express Ki67, indicating that differentiated epithelial cells surviving injury undergo proliferative expansion. After repair was complete, 66.9 % of epithelial cells had incorporated BrdU, compared to only 3.5 % of cells in the uninjured kidney, suggesting that regeneration by surviving tubular epithelial cells is the predominant mechanism of repair after ischemic tubular injury in the adult mammalian kidney [69].

Renal epithelial cells may also undergo epithelial mesenchymal transition (EMT), during kidney development as well as in adulthood. The activation of the EMT is regulated by a complex transcriptional program, characterized by nuclear translocation of transcription factors Snail, Twist, and β-catenin, paralleled by loss of typical epithelial markers such as E-cadherin [70]. Most transcription factors involved in EMT, including Snail, Twist and β-catenin, are also known for their anti-apoptotic activity, hence protect tubular epithelial cells from death, suggesting EMT as a protective mechanism for the kidney [71]. EMT has been proposed as a highly efficient way for the kidney to fibroblast recruitment to local sites of tubulointerstitial injury [72]. Recently, conflicting results have been published regarding the ability of EMT to give rise to fibroblasts in vivo [73]. According with these studies, pericytes and perivascular fibroblasts should be considered the primary source of myofibroblasts and collagen-producing cells in the kidney, whereas their epithelial origin should be abandoned [74].

Glomerulogenesis and Tubulogenesis

Several genes are involved in regulating the complex process of glomerulogenesis.

The protease-activated receptor PAR2 and PAR3, members of the partitioning defective protein family, are required for normal differentiation of podocytes, and in particular for establishing and maintaining a polarized structure. Genetic deletion of PAR-3 impairs the nephroprotective effect of activated protein C, demonstrating the crucial role of PAR-3 for protein C-dependent podocyte protection [75]. Whereas podocytes play a central role in the organization of the mature glomerulus and in control of glomerular filtration, during kidney development podocyte precursors orchestrate the integration of the different cell types that will give rise to the functioning glomerular body. Different new molecules have been identified in podocytes, playing essential roles in the maintenance of podocyte integrity and in the control of vascular and mesangial integration in developing glomeruli. Of all of these, arguably the most pivotal is nephrin (NPHS1), a transmembrane receptor molecule located at the specialized podocyte cell–cell junction, termed the slit diaphragm. Recently, a role of nephrin as a signalling molecule in kidney podocytes has been identified: crucial functional properties, including insulin responsiveness and cytoskeletal reorganization depend on nephrin, indicating that nephrin should be considered a signature molecule required to define distinct podocyte characteristics [76]. Moreover, a role for NPHS1 has been identified in the control of vascular endothelial growth factor-A (VEGF-A), revealing an exquisite control from the podocyte on the developing vascular tuft, exerted by a crosstalk between the components of the glomerular filtration barrier [77]. Recently, Foxd1, which has generally been considered a marker of the kidney interstitium, or stromal lineage, showed extremely robust expression in podocytes [78].

Sem3a functions, during glomerulogenesis, as a negative regulator of endothelial cell survival, and plays a crucial role in podocyte differentiation. In particular, Sem3a up-regulation results in glomerular hyperplasia, whereas its down-regulation causes troubles in the glomerular vascular pattern, with an excess of endothelial cells [48].

The Interstitial Cell Fate

After making contact with the emerging ureteric bud, the metanephric mesenchyme divides into a nephrogenic lineage and a renal cortical stromal lineage.

The origins of the stromal lineage inside the metanephric mesenchyme remain not completely understood yet. The metanephric mesenchyme is believed to be composed solely of cells derived from caudal intermediate mesoderm, from transcription factor *Osr1*+ progenitors. *Osr1* is expressed in the intermediate mesoderm prior its subdivision into paraxial and intermediate domains. It remains unclear if the *Osr1*+ progenitor populations that generate nephron epithelial, stromal and endothelial progenitor populations all derive from the intermediate mesoderm [79]. Recently, the paraxial mesoderm has been shown to contribute renal stromal progenitor cells to the developing kidney, providing some insight into why known derivatives of the paraxial mesoderm such as cartilage and muscle, are ectopically expressed in dysplastic renal tissues. These data taken together suggest that renal morphogenesis is dependent on the integration of cells from both the intermediate and paraxial mesoderm into a single embryonic rudiment [80].

Foxd1-positive cells are generally considered the first actor of the non-nephron lineage in the metanephric mesenchyme, representing a self-renewing progenitor population that gives rise to medullary interstitium, the renal capsule, putatively glomerular mesangium, and renal pericytes [57]. The interstitial cell fate is repressed by PAX2, that probably represents a developmental boundary between the nephron and non-nephron lineages, maintaining and favouring the nephron lineage. The subcomponents of the renal interstitium are defined early during kidney development. HOX10 genes play a critical role in patterning of the stromal cell compartment, regulating the differentiation of different stromal cell types [81].

Origins of the vascular, mesangial and smooth muscle compartments are not fully resolved, though lineage-tracing studies suggest that the vasculature may arise intrinsically within the early metanephros or extrinsically by migration into the developing kidney [82]. A role for the tissue-type plasminogen activator (tPA) has been recently hypothesized during differentiation of renal interstitial fibroblasts. tPA is a potent mitogen that promotes interstitial fibroblast proliferation through a cascade of signalling events, including LDL receptor-related protein 1-mediated β1 integrin and FAK signalling [83].

The Ureteral Mesenchyme

The ureteral mesenchyme has been reported to derive from a distinct cell population that is separated early in kidney development from that of other mesenchymal cells of the renal system. The gene encoding the T-box transcription factor Tbx18 is expressed in undifferentiated mesenchymal cells surrounding the distal ureter stalk. A mouse model for congenital ureter malformation revealed the molecular pathway important for the formation of the functional mesenchymal coating of the ureter. In Tbx18−/− mice, prospective ureteral mesenchymal cells largely dislocalize to the surface of the kidneys. The remaining ureteral mesenchymal cells show reduced proliferation and fail to differentiate into smooth muscles, but instead become fibrous and ligamentous tissue. Absence of ureteral smooth muscles resulted in hydroureter and hydronephrosis at birth [84]. TBX18 acts synergistically with SIX1 in mediating the differentiation of ureteral smooth muscle cells. Six1 and Tbx18 genetically interact to synergistically regulate smooth muscle cell development and ureter function and their gene products form a complex in cultured cells and in the developing ureter [34].

After the basic shape of the mammalian ureter is established, a coat of smooth muscle cells differentiate around nascent urothelia. The ureter actively propels tubular fluid from the renal pelvis to the bladder, and this peristalsis, which starts in the foetal period, requires coordinated smooth muscle contraction. Teashirt-3 (Tshz3), a member of the Teashirt gene family, is expressed in smooth muscle cell precursors that form the wall of the forming mammalian ureter. A signalling pathway can be hypothesized, starting with sonic hedgehog secreted by the nascent ureteric urothelium and ending with ureteric smooth muscle cell differentiation, with Tshz3 downstream of bone morphogenetic protein 4 and upstream of myocardin and smooth muscle cell contractile protein synthesis. Null mutation of Tshz3 in mice leads to failure of functional muscularization in the top of the ureter, ending with congenital hydronephrosis [85].

Conclusions

Over the last years, major advances in the identification of the molecular mechanisms that direct kidney morphogenesis have been obtained, providing new insights for a better understanding of the abnormalities of kidney and urinary tract development. The molecular mechanisms that define kidney progenitor cell populations, induce nephron formation within the metanephric mesenchyme, initiate and organize ureteric bud branching, and participate in terminal differentiation of the nephron have been, at least in part, revealed [36].

Here, the most common signalling pathways that function at multiple stages during kidney development have been highlighted, including signalling via Wnts, bone morphogenic proteins, fibroblast growth factor, sonic hedgehog, RET/glial cell-derived neurotrophic factor, and notch pathways. Also emphasized are the roles of transcription factors Odd1, Eya1, Pax2, Lim1, and WT-1 in directing renal development.

The most relevant critical point in this review is that the vast majority of data regarding molecular control of nephrogenesis have been obtained in mutant and transgenic mice, or in zebra fish, or in other experimental models, whereas data from human nephrogenesis appear scant and occasional. Areas requiring future investigation include the factors that modulate signalling pathways to provide temporal and site-specific effects, with a new particular attention to data in the human kidney. The evolution of our understanding of the cellular and molecular mechanisms of kidney development may provide methods for improving diagnosis of renal anomalies and, hopefully, targets for intervention for this common cause of childhood end-stage kidney disease.

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Development of the Human Kidney: Immunohistochemical Findings

3

Daniela Fanni, Clara Gerosa, Peter Van Eyken, Yukio Gibo, and Gavino Faa

Introduction

The development of the human kidney is a complex process that requires interactions among multiple cell types of different embryological origin, including multipotential/stem cells, epithelial and mesenchymal cells: moreover, all these cell types undergo, during fetal kidney development, multiple steps of cellular differentiation, some of which have not well defined and characterized yet. The coordinate development of multiple highly specialized epithelial, vascular, and stromal cell types is a peculiar feature of the kidney architectural and functional complexity [1]. During renal development, all these cell types change their cellular shape, nuclear features and function, originating new differentiated cell types and/or inducing neighboring cells to differentiate into mature cells. A subset of multipotential cells deriving from the metanephric mesenchyme and located in the subcapsular zones undergo, under induction by the ureteric bud tip cells, the process of mesenchymal-to-epithelial transition (MET), and give rise to all the structures of the proximal nephron, including glomeruli, proximal and distal tubuli [2]. When solely based on morphology, the identification of the different cell types involved in human nephrogenesis may lead to errors in its interpretation, given the complexity of the histological picture that characterizes each fetal and newborn kidney. Here, the most recent works on the application of immunohistochemistry to a modern interpretation of the neonatal kidney are reported, with a particular emphasis on the contributions of immunohistochemistry to trace the fate of metanephric mesenchymal cells, from the initial renal stem cell(s) towards the differentiation into the multiple cell types that characterize the mature human kidney.

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The Process of Epithelial-to-Mesenchymal Transition

The Renal Stem/Progenitor Cells

The mature human kidney originates by the metanephric mesenchyme. In its original composition, the metanephric mesenchyme is formed by scarcely differentiated elongated cells, which

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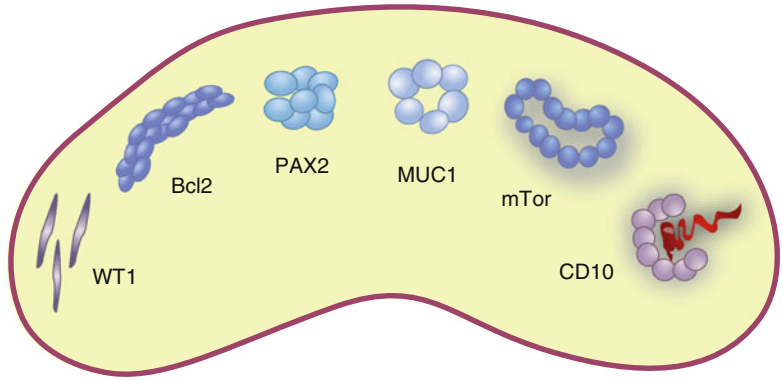

Fig. 3.1 Major immunohistochemical markers to be utilized in the interpretation of human kidney with active nephrogenesis

float in a loose intercellular mucoid matrix (Fig. 3.1). These multipotent/stem cells are also present in the neonatal kidney, particularly in preterm newborns, and are located in the subcapsular zone. The morphological appearance of these scarcely differentiated renal precursors is characterized by small size, scarcity of cytoplasm, and by a roundish or oval nucleus with dense hematoxylinofilic chromatin. Due to the small cytoplasm and to cell density, these renal precursors appear as a "blue strip" under the renal capsule. Immunohistochemistry has allowed us to differentiate the renal stem/progenitor cell pool, corresponding to the earliest stages of normal metanephric kidney development. Wilms Tumor 1 (WT1) has been one of the first tumor suppressor genes identified to play a relevant role in kidney development, being required for early kidney development [3]. WT1 is nowadays considered a master control gene that regulates the expression of a large number of genes that play a critical role in the early phases of kidney development, including the induction of angioblasts and the regulation of neoangiogenesis in the early kidney development [4]. Regarding immunohistochemistry, WT1 was first shown to be an immunohistochemical marker of stem/progenitor cells in the mouse embryo [5] (Fig. 3.2). Recently, a study from our group demonstrated that WT-1 is strongly expressed by stem/progenitor cells in the human fetal kidney [6]. Immunostaining for WT1 was detected in all the fetal kidneys examined, but not in the kidney of a newborn at term, in which active nephrogenesis was absent. In the fetal kidneys, WT1 appeared to be mainly localized in the undifferentiated stem cells located in close proximity of the renal capsule. These data suggest that WT1 plays a role in the active nephrogenesis in the human kidney, being involved in the maintenance of the stem/progenitor cell pool during kidney development, and playing an essential role in nephron progenitor differentiation [7]. Its absence in the mature newborn confirms this suggestion, paralleling WT-1 expression with active nephrogenesis and WT-1 silencing when nephron generation is halted, as physiologically happens in the at term neonate. From a practical point of view, WT-1 may be considered a marker of human renal stem/progenitor cells in their early phase of differentiation.

A recent study on immunoreactivity in the fetal human kidney for CD44, a transmembrane adhesion glycoprotein which participates in the uptake and degradation of hyaluronan, showed a marked reactivity for this glycoprotein restricted to undifferentiated mesenchymal cells in the renal hilum, probably representing the remnants of the metanephric mesenchyme [8]. Moreover, CD44 immunostaining was reported in isolate large cells inside the metanephric mesenchyme surrounding the newly formed renal vesicles, probably representing a subset of progenitor/stem cells involved in the early phases of kidney development [9].

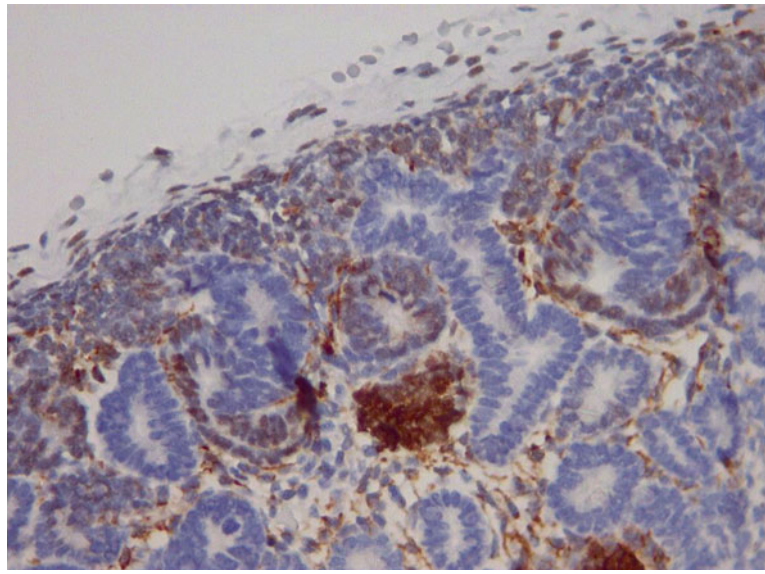

Fig. 3.2 WT1 reactivity is localized in the undifferentiated stem cells located in close proximity of the renal capsule

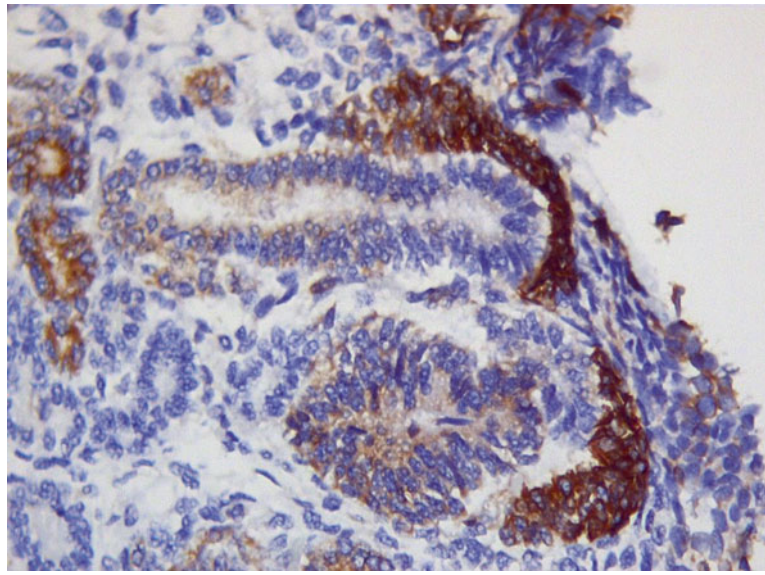

Fig. 3.3 Bcl2 immunostaining in cap mesenchymal cells

The Cap Mesenchyme

When the epithelial cords originating from the ureteric bud, after migrating and branching into the metanephric mesenchyme, eventually arrive in the proximity of the renal capsule, epithelial cells located at each of the ureteric bud tips orchestrate the progressive differentiation of the neighboring stem/progenitor cells. The initial morphological event demonstrating the starting process is the aggregation of a certain number of progenitor cells into roundish cell groups, each of them surrounding the corresponding ureteric bud tip. These aggregates have been defined as cap aggregates, and their constituents are the cap mesenchymal cells (Figs. 3.3 and 3.4). Cap-derived

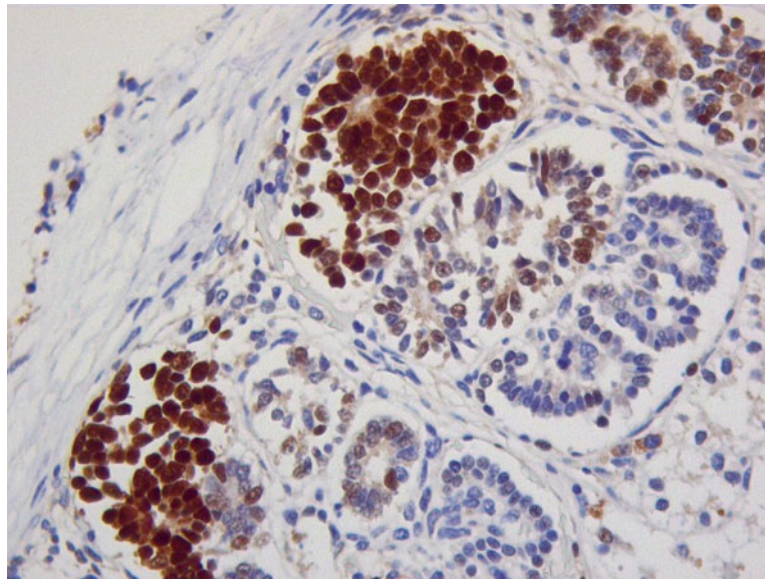

Fig. 3.4 PAX2 nuclear staining in close proximity of the ureteric bud tips, giving rise to the cap aggregates, probably one of the first steps of the mesenchymal–epithelial transition

cells are responsible for nephron formation and for invading the ureteric tips to form the connecting segment between the distal tubules and the collecting ducts, the only part of the human nephron originating from the ureteric bud [10]. Understanding factors that regulate the development, persistence, and self-renewing of this compartment and, on the other hand, factors that induce the premature unregulated epithelialization of cap mesenchymal cells, leading to premature loss of the progenitor field and cessation of nephrogenesis represents a key challenge for researchers involved in the fascinating field of physiological regenerative medicine [11]. To this end, recent studies from our group were focused on giving new data on the expression, at immunohistochemical level, of different markers in the different cell populations occupying the overcrowd theater of fetal nephrogenesis. Whereas in H&E-stained sections the identification of these aggregates may result difficult, due to their inclusion in the subcapsular blue strip, recent data by our group (Gerosa C, Unpublished data) showed that immunohistochemistry may help in the detection of cap mesenchymal cells. These progenitor cells, representing the most important nephron progenitor population of the human developing kidney, show a marked immunostaining for bcl-2 that allows their clear identification from the neighboring less differentiated progenitor renal cells (Fig. 3.2). The high expression of bcl-2 in the vast majority of cap mesenchymal cells underlines the relevance of this cell type in human nephrogenesis. The maintenance of homeostasis in normal tissues reflects a balance between cell proliferation and cell death, this balance necessitating the coordinate expression of both positive and negative regulators of cell growth. A key role in regulating cell survival is played by bcl-2. First identified at the chromosomal breakpoint of t (14; 18) bearing B cell lymphomas, the bcl-2 gene has proved to be unique among protooncogenes in blocking programmed cell death. The major role of bcl-2 is probably related to its ability to inhibit apoptosis and prevent oxidative damage to cellular constituents including lipid membranes in a variety of settings, including the developing kidney. The key role of bcl-2 in nephrogenesis and in renal development was well shown in bcl-2 knock-out mice: they were characterized by renal hypoplasia, due to a marked reduction in renal size at birth, a thinner nephrogenic zone, and few

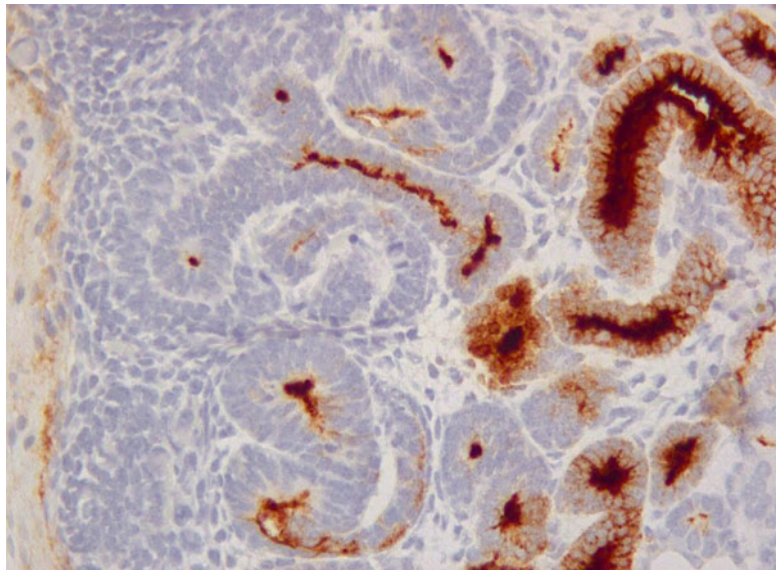

Fig. 3.5 MUC1 in pre-tubular aggregates, in the lumen of renal vesicles, comma- and S-shaped bodies

glomeruli as compared to bcl-2 (+/+) mice [12]. Moreover, the vast majority of bcl-2 (−/−) surviving mice undergo polycystic kidney disease [13]. Taken all together, these data clearly indicate bcl-2 as a useful marker for the identification of cap mesenchymal cells in paraffin renal sections, and lay stress on the relevance of the cap mesenchyme in the physiological development of human kidney. The finding that the intensity of immunoreactivity for WT1in kidney cells has been found to be different from one newborn to the next, according to the different gestational age [14], suggests that immunoreactivity for WT-1 might be utilized for a semiquantitative evaluation of the progenitor cell burden in a certain kidney, allowing a semiquantitative evaluation of the potential nephrogenic ability of a kidney. Recently, hCTR1, a high affinity membrane copper permease that mediates the physiological uptake of copper ions, was reported to be strongly expressed in the cap mesenchymal cells in the human developing kidney [15]. Immunoreactivity for this copper transporter in the nephrogenic zones in cells undergoing the process of epithelial-mesenchymal-transition suggests a role for copper and for its transporter in the early phases of human nephrogenesis.

The Pre-tubular Aggregates

The pre-tubular aggregates represent a further step of differentiation of the cap mesenchymal cells, previously aggregated around one ureteric bud tip. This peculiar embryonic structure plays a pivotal role in human nephrogenesis, representing a bridge between two worlds: the mesenchymal and the epithelial one. Cells of the pre-tubular aggregates are considered mesenchymal yet, but they undergo the initial steps of the MET, a process that will give rise to all, or at least to the vast majority, of cell types which form the proximal nephron [16]. Morphology is not able to differentiate pre-tubular aggregates in a steady state from those in which the process of MET is going on. Immunohistochemistry has been shown to may help to this end. MUC1, a transmembrane glycoprotein apically expressed in most epithelial cells has been demonstrated to be expressed in a subset of pre-tubular aggregates during nephrogenesis in human fetuses (Fig. 3.5). In particular, pre-tubular aggregates marked by MUC1 showed the initial features of MET, suggesting a major role for MUC1 in triggering the transition of pluripotent metanephric mesenchymal cells into epithelial cells [17]. The peculiar immunoreactivity

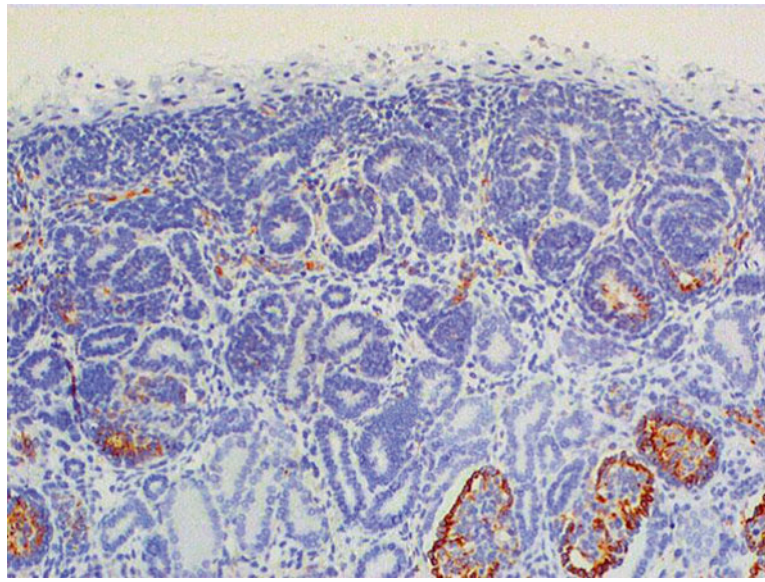

Fig. 3.6 CD10 in the cytoplasm of scattered pre-tubular aggregates of cap mesenchymal and in the lumen of renal vesicles and podocytes

of a typical epithelial marker, such as MUC1, in mesenchymal-appearing cells induced to hypothesize that MUC1 could change the fate of renal progenitors, facilitating their differentiation into epithelial cells. This hypothesis was subsequently confirmed by the same group in further studies, by enlarging the number of neonatal and fetal kidney immunostained for MUC1, confirming the strict association of this immunohistochemical marker with the epithelial differentiation of metanephric-derived mesenchymal cells [18]. In that study, MUC1 immunoreactivity was confirmed in all mesenchymal cells undergoing the initial phase of MET. Another immunohistochemical marker has been recently reported in pluripotential renal cells during human kidney development. At 25 weeks of gestation, CD10, a marker first identified in tumor cells of acute lymphoblastic leukemia, was detected in the subcapsular regions of the fetal kidney, mainly localized in the cytoplasm of scattered pre-tubular aggregates of cap mesenchymal cells [19] (Fig. 3.6).

The Renal Vesicle

Renal vesicles represent the first epithelial structure deriving from the cap mesenchymal cells through the MET process that is clearly detectable by morphology. Renal vesicles are characterized by MUC1 immunostaining, which is constantly detected in the lumen of renal vesicles [17]. Renal vesicle cells are also immunostained by CD10 in fetal kidneys characterized by active nephrogenesis [19].

The Comma-Shaped Body

The comma-bodies represent an evolution of the renal vesicle, due to the first segmentation and patterning process that transforms a roundish structure into a half-moon-like structure, better known as the comma-shaped body. At immunohistochemistry, these structures are characterized by a strong immunoreactivity for MUC1 at the apical border of epithelial cells [18]. Interestingly, in that study MUC1-immunostaining was not diffuse to the entire rudimentary lumen of the whole comma-body. On the contrary, only about half of these cells were immunostained by MUC1: this finding suggests that cells of the comma-bodies are probably more differentiated than previously thought, some starting their differentiation way towards the tubular structures and others towards the glomerular epithelium, in spite of their apparent identity on morphology. The complete absence of MUC1 immunoreactivity

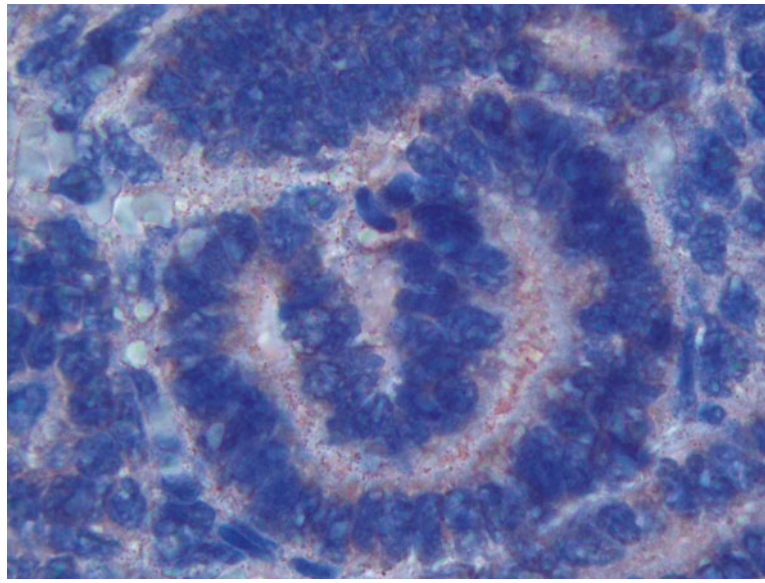

Fig. 3.7 mTor reactivity in a comma-body

inside the developing and the mature glomeruli suggests that MUC1 might identify the cells of the comma-body programmed to make tubules, whereas the absence of immunostaining for MUC1might allow to detect the cell pool programmed to differentiate into podocytes and epithelial cells of the glomerular Bowman's capsule. A recent study from our group (Gerosa C, unpublished data) evidenced the immunoreactivity of comma bodies cells to mTor (Fig. 3.7)

The S-Shaped Body

The second segmentation and patterning phase of the renal vesicle is at the basis of the transformation of the comma-shaped body into the S-shaped body. Even this renal developmental structure is characterized, at immunohistochemistry, by reactivity to MUC1 which, paralleling the type of reactivity found in comma-bodies, is restricted to one extreme and to the central area of the S-body and, in particular, to the segments which will give rise to proximal and distal tubules [18]. This finding confirms previous hypotheses regarding the complex organization of the S-shaped body into three segments, proximal, medial, and distal, each of them corresponding to cells programmed to give rise to a different nephron segment [2]. MUC1 has been shown to be able to mark selectively the medial and distal segments of the S-shaped body, i.e., the part of the body that will originate the tubular structures, whereas cells programmed to differentiate into podocytes and capsular epithelium are not immunoreactive for MUC1. According to these findings, MUC1 may be useful in neonatal kidney interpretation not only in identifying the different epithelial cell types, but even in the interpretation of the fate different cells will go towards, during the next differentiation steps of nephrogenesis.

The Glomerular Epithelial Cells

Glomerular epithelial cells develop from the proximal part of the S-shaped body, which is characterized by a half-moon shape. Cells localized in the inner part further differentiate to form the podocyte precursors, whereas cells localized in the external part of the body differentiate to form the parietal epithelium (Bowman capsule) of the mature glomerulus [10]. At immunohistochemistry, CD10 appears as a useful tool for the identification of visceral and parietal glomerular epithelium in all the different steps of their

differentiation [19]. Podocyte precursors and developing podocytes are also marked by WT1, a zinc finger protein expressed by human podocytes in the adult kidney, which probably plays a main role in multiple phases of nephrogenesis, including podocyte differentiation and maturation [6]. In this study, the intensity of reactivity for WT1 in podocytes changed from one developing kidney to the next, whereas immunostaining markedly decreased in at term newborns, suggesting a complex role for WT1 in different phases of kidney development and, in particular, in podocyte differentiation and maturation. Podocytes did not show any immunoreactivity for MUC1, Thymosin beta-4 and beta-10, nor for CD44 [20]. Interestingly, CD44 has been recently proposed as a marker of a subset of parietal epithelial cells in the glomeruli of adult kidneys, probably representing niche stem cells maintaining the ability, in adulthood, to differentiate into podocytes, replacing injured podocytes in the setting of focal segmental glomerular sclerosis (FSGS) [21].

The Proximal Tubules

The proximal tubules originate, in the human kidney, from the medial segment of the S-shaped body through a process of elongation and cellular proliferation [2]. Epithelial cells of the proximal tubules, at immunohistochemistry, may be easily marked by anti-CD10 antibodies [8]. Regarding the identification of different tubular segments in the human kidney, CD10 has been shown to allow the differentiation among different tubules, immunostaining for CD10 being restricted to the proximal tubules, but absent in distal as well as in collecting ducts [8]. The epithelium of the proximal tubules is also marked by thymosin beta-4, a small peptide member of the beta-thymosin family, which plays essential roles in many cellular functions including apoptosis, cell proliferation, and cell migration. In a study of thymosin beta-4 in the genitourinary tract of the human fetus, immunostaining for this peptide was detected in the proximal tubules, as well as in the distal tubules, with glomeruli completely spared [22]. Thymosin beta-4 was recently detected in the cytoplasm of a kidney proximal cell line derived from a newborn piglet, in normal conditions. After serum deprivation, thymosin beta-4 translocated from the cytoplasm into the nucleus [23]. Another member of the beta-thymosin family less frequently studied in human tissues, thymosin beta-10, has been recently detected in the vast majority of 22 human developing kidneys immunostained for this peptide [20]. In that study, immunostaining for thymosin beta-10 was mainly detected in the cytoplasm and occasionally also in the nuclei of proximal ductal cells. No reactivity for WT1, MUC1, and CD44 was detected in proximal tubular cells [8].

The Distal Tubules

Immunoreactivity for distal tubular cells is not frequent in our studies, this cell type being negative for WT1, CD10, CD44 and even for so-called typical epithelial markers such as MUC1[8]. On the contrary, an immunoreactivity for thymosin beta-4 and beta-10 has been constantly detected in cell of this nephron segment in the fetal human kidney [20, 22].

The Distal Nephron

The Collecting Tubules

Histochemistry surely represents the easiest way to mark the collecting tubules in the neonatal kidney. Tubular cells of the collecting tubules, deriving from the ureteric bud emerging from the Wolffian duct, are characterized by the presence in their cytoplasm of massive glycogen stores, revealed by PAS-stain [24]. At immunohistochemistry, collecting tubular cells are immunostained by antibodies against MUC1 and Thymosin beta-4, whereas immunoreactivity for WT1, thymosin beta-10, CD10, and CD44 has been shown to be absent [8]. MUC1-immunostaining in collecting tubules shows peculiar pattern, being restricted to the apical border of the cells, in close proximity to the tubular lumen [18].

The Stromal Cell Pool

The differentiation of the stem/pluripotential metanephric mesenchymal cells, occurring in the nephrogeneic zone in proximity of the renal capsule, progresses towards two main directions: (1) the nephron lineage, giving rise to the cap mesenchyme, giving rise to all epithelial cell types of the proximal nephron; (2) the non-nephron lineage, differentiating into the numerous non-epithelial cell types present in the mature kidney, including angioblasts, muscle cells of the arterial walls, cortical, medullary, and perihilar interstitial cells, connective cells of the renal capsule, nervous cells, cells of the juxtaglomerular complex including macula densa and, probably, intraglomerular and extraglomerular mesangial cells [25]. All these cells types normally share some immunohistochemical findings: they are all negative for cytokeratins, the typical marker of epithelial cells, and show a strong immunoreactivity for vimentin, the common marker of connective tissue cells. Unfortunately, in clinical histopathological practice, vimentin does not represent a useful tool in the study of the developing kidney: in fact, sections immunostained for vimentin show a diffuse and homogeneous dark stain, that is not useful for a better interpretation of the multiple non-epithelial renal cell types. Much more useful are the multiple immunostainings for the singular cell types that will be here reported.

Vascular Cells of the Glomerular Tuft

The migration, differentiation, and proliferation of angioblasts in close proximity of podocyte precursors in the segment of the S-shaped body that will give rise to the developing glomerulus is probably the result of a fascinating cross talk between different cells that will give rise to the glomerular filtration barrier. In particular, the development of the glomerular tuft is under exquisite control of vascular endothelial growth factor-A (VEGF-A) expression from developing podocytes [26]. CD31 and CD34 represent two useful markers for the identification of the vascular precursors' proliferation and differentiation inside the developing glomeruli. A recent immunohistochemical study carried out in human fetal kidneys showed the absence of vascular markers such as CD31 and CD34 in primitive developing nephrons [27]. In that study, CD31 and CD34 were detected only in the fourth stage of glomerular development characterized by the final differentiation of the main components of the renal corpuscle [25].

Mesangial Cells

Conflicting data have been published regarding the origin of mesangial cells that constitute approximately 30–40 % of the glomerular cell population. On the one hand, they have been proposed to share a common origin with the epithelial cells of the proximal nephron, originating from the non-nephron lineage of the cap mesenchymal cells [28]. On the other hand, glomerular mesangial cells have been hypothesized to originate in the bone marrow from hematopoietic stem cells [29], probably deriving from the granulocyte-macrophagic lineage [30].

Cortical Interstitial Cells

The differentiation and integration of stromal cells are necessary for the proper development of the human kidney. During organogenesis, among the cap mesenchymal cells, a subset of progenitor cells gives rise to the non-nephrogenic lineage that will differentiate into multiple cell types, including the cortical and the medullary interstitial cells [28]. A number of components of the renal interstitium are defined early during human kidney development, including fibroblasts and resident macrophages that are normal components of the mature renal cortex. HOX genes are required for the differentiation and integration of cortical stromal cells during kidney development: in particular, HOX_{10} genes have been shown to play a critical role in the development of the

cortical stroma compartment, whereas HOX_{11} genes are necessary for patterning the nephrogenic mesenchyme, suggesting a model whereby differential expression of HOX genes is critical for the integration of multiple different cortical stromal cells during kidney organogenesis [31]. The embryonic origin of fibroblasts is unclear as well, although some studies point to a neural crest origin of these cells [32]. The so-called renal fibroblasts are a heterogeneous population of mesenchymal cells with various essential functions during kidney development and in adult life. At immunohistochemistry, renal fibroblasts may be identified by the antibody TE-7 that recognizes growing and quiescent fibroblasts in paraffin sections [33]. Still, remarkable uncertainties exist in the nomenclature of renal mesenchymal cells (or renal fibroblasts), whereas their immunohistochemical characterization remains poor. The expression at immunohistochemistry of smooth muscle actin (SMA) marks the differentiation of fibroblasts into myofibroblasts, which most likely represent a stressed and dedifferentiated phenotype of fibroblasts that appears de novo in renal fibrosis, originating from renal fibroblasts [34].

Medullary Interstitial Cells

At birth, the medullary region of the newborn kidney is characterized by a peculiar histological appearance. The descending loops of Henle are separated from each other by a loose interstitial connective tissue that does not allow the efficiency of a counter-current mechanism indispensable for concentrating urine. As a consequence, a remodeling is required in the postnatal period for the medulla maturation. The putative actors of this remodeling have been identified in the stem cell population located in close proximity of each renal papilla, which could represent a niche for renal stem cells even in adults [35].

Ureteric Mesenchymal Cells

The specialized cell types that initiate and coordinate contraction of the smooth muscle cells at the pelvis-kidney junction, triggering ureter peristalsis remain, at the best of our knowledge, poorly characterized yet. Recent studies on ureter development revealed that the ureteric mesenchymal cells might derive from a distinct cell population that stem from the mesenchymal metanephric progenitors early in kidney development. The undifferentiated mesenchymal cells directly adjacent to the ureteric epithelium undergo differentiation into multiple cell lines, including smooth muscle cells, ureteric pacemaker cells, and the ureteric adventitial fibroblasts. Wnt signals from the ureteric epithelium pattern the ureteric mesenchyme proliferation and differentiation in a radial fashion by suppressing adventitial fibroblast differentiation and initiating smooth muscle precursor development in the innermost layer of mesenchymal cells [36]. At immunohistochemistry, scarcely differentiated mesenchymal cells undergoing differentiation into the ureteric smooth muscle layer cells may be identified with antibodies against SOX9, one of the several genes expressed in the periureteric mesenchyme, and whose loss may be at the basis of hydroureteronephrosis [37].

The urinary tract pacemaker cells are probably at the junction between the renal pelvis and the ureter and, in mouse, have been shown to express the hyperpolarization-activated cation (HCN_3), a calcium channel well known as a mediator of pacemaker activity in the heart [38]. These pacemaker cells have been recently shown to express at immunohistochemistry CD117 (C-kit), the typical marker of intestinal pacemaker cells, and as a consequence have been defined Cajal-like cells [39]. A better knowledge based on immunohistochemical stains on the origin and differentiation of renal fibroblasts in the newborn kidney during development could help to a better understanding of renal fibrosis, a central pathological process in kidneys of patients with chronic kidney disease and to the identification of effective treatments that might halt or reverse fibrosis. Further studies on the development of the periureteral mesenchymal cells will help to clarify the complex field of congenital urinary tract obstruction, a major cause of renal failure in infants and children [40].

The Macula Densa

The development of the juxtaglomerular complex, including macula densa, extraglomerular mesangium, and part of the afferent arteriole are typical events occurring in the developing kidney [41]. By immunohistochemistry, the extraglomerular mesangium may be easily identified by antibodies against connexins Cx37, Cx40, and Cx43, whereas renin-producing cells display strong immunoreactivity for Cx40 and Cx37 [42]. Since connexin is a component of gap junctions, the high expression of connexin in the juxtaglomerular cells suggests a major role of gap junctions in development of renin-producing cells and in their location in close proximity but outside of the glomerular tuft [43].

Conclusions

Immunohistochemistry, thanks to its ability to "give a certain name to cells," appears as a useful tool in the study of the initial phases of nephrogenesis as well as during the advanced steps of differentiation of the multiple cell types that characterize the mature human kidney. Immunostaining of fetal and newborn kidneys appears a certain source of interesting data, not only for a better comprehension of the complex and in part unknown processes that take place during renal development, but even for a better understanding of the pathological processes at the basis of renal disease, in childhood and in adulthood. The complex phases that characterize the MET at the basis of the proximal nephron development, and the multiple steps that characterize the epithelial-to-mesenchymal transition, typical reaction of tubular cells to a block in the urinary flow, are two clear examples of the utility of immunohistochemistry in defining the different cell types emerging during these differentiation processes.

Looking for the recent literature, characterized by the scarcity of articles utilizing immunohistochemistry in the study of fetal and neonatal kidney, some questions arise:

1. Why so many articles on zebrafish kidney and so few on human newborn kidneys?
2. Why so many articles on gene expression in the rat or mouse kidney and so few articles on immunohistochemistry?

According to data here presented, immunohistochemistry may have a relevant role in better defining the degree of differentiation of the multiple cell types involved in human nephrogenesis, and we are sure that, in the near future, multiple proteins will be added to the list of markers to be introduced in the routine study of fetal and newborn kidneys.

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Kidney Development: New Insights on Transmission Electron Microscopy

4

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Introduction

Electron microscopy has been extensively used in morphological studies of kidney to reveal ultrastructural details beyond the resolving power of the light microscope. Such studies carried out on human adult kidney are performed on autopsy, biopsy, or surgical samples. Because glomeruli usually are better preserved than are kidney tubules during processing for electron microscopy, studies tended to concentrate mainly on glomerular ultrastructure in the mature kidney [1–3], adding relatively little information on tubular fine structure [4].

Moreover, the focus of pathologists on glomerular dysfunction during renal disease [5–7] has resulted in inattention to kidney development, so that little ultrastructural data on nephrogenesis has been adduced [8, 9]. As a result, many questions on this matter remain to be answered. Recently, however, growing interest in renal regeneration has led to the emergence of ultrastructural investigations on mammalian kidney development [10]. Transmission and scanning electron microscopy, together with recent light microscopic insights, are highlighting the morphofunctional events that characterize the early stages of kidney development and new hypotheses are coming forth.

Although significant attention has been paid to the human kidney, more interest in specific experimental animal models is becoming manifest, mainly due to significant improvements in specimen preparation. Renal tissues are labile structures that undergo profound ultrastructural alterations if chemical fixation is not performed immediately after the tissue sample has been separated from its oxygen supply. Significant delays in fixation of human samples coming from autopsy or following biopsy often can produce severe artifacts, leading to great difficulty in interpreting morphological data. Whole body vascular perfusion or immersion fixation procedures in mouse and rat have given better results, preserving and resolving renal structures to a desirable degree. Moreover, well-characterized experimental animal models can be monitored in a timed fashion, so that electron microscopy analyses can be performed at each stage of the renal development process. The very early stages of nephrogenesis can be investigated in detail, permitting correlation between fine structure and involved molecular mechanisms. Although differences in the renal embryology have been

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described between several studied animal species (in rat and mouse, kidneys are not fully formed at birth and additional nephrons develop in the outer portion of the renal cortex during the first postnatal week), humans and the other mammals seem to share same molecular mechanisms and a similar sequence of renal morphogenetic events. The experimental animal models play a significant role in the study and understanding of the mechanisms that culminate in the formation of the adult kidney and may fill the existing gaps in knowledge of the molecular and morphological mechanisms involved in nephrogenesis. The aim of this chapter is to bring to the attention of the reader new insights provided by transmission electron microscopic studies of developing renal tissues in the mouse and man. It is not the last word on such matters, but shows a new way to look at forming renal structures, suggesting meaningful correlations with light microscopic observations and those of other investigative disciplines, including molecular biology, physiology, and pathology.

This is only the tip of the iceberg. We are approaching the *terra incognita* of kidney development and many intriguing features of this process are waiting to be discovered.

Fine Structure of Cap Mesenchyme in the Early Development Stages of the Mouse Nephrogenesis

To the best of our knowledge, no detailed studies have appeared on the fine structure of cap mesenchyme in the early phases of its origin from metanephric mesenchyme and during its transition to an epithelial phenotype. This chapter includes the latest findings concerning the very early stages of the sequence of the morphological events that lead to glomerulogenesis and tubulogenesis, using an "ad hoc animal model." The mouse renal tissues used in our studies were obtained from newborn mice housed in a pathogen-free environment in a local animal care facility. They were euthanized according to the guidelines for the Care and Use of Laboratory Animals (National Institutes of Health) and the European Communities Council Directive for the use of animals in scientific experiments.

As mentioned above, ultrastructural preservation of renal mouse tissue is at its best when fixation is performed right after the kidney excision, using a mixture of formaldehyde and glutaraldehyde. In our study, kidney specimens were fixed immediately after surgery. In general, for transmission electron microscopic analysis the fixed renal tissues are processed by standard methods for embedding in specific resins. One micrometer sections are cut and collected on glass slides for preliminary light microscopic observations. For ultrastructural investigation, ultrathin sections are collected on grids, stained, and observed in a transmission electron microscope (TEM).

At light microscopy level, the outer portions of the developing renal cortex are characterized by condensed cellular solid aggregates that are roundish or ovoid; these are the cap mesenchymal nodules. They are intermingled with scattered and isolated cells that represent the remnants of the metanephric mesenchyme (Fig. 4.1). At this stage of development the entire subcapsular region is reminiscent of downtown traffic flow, with the renal primordial constituents seemingly interacting under the control of specific rules [11]. At low power, cap mesenchymal aggregates are seen to envelop a branch of a single ureteric bud (UB) (Fig. 4.1). Their cells go through intense proliferation that reorganizes the cap mesenchymal aggregates to form spherical cysts, the so-called renal vesicles. Based on light microscopy, this early developmental stage was initially described as one of the early steps that occurs in the nephrogenic process. However, further developing stages can be observed between the two extremes of cap mesenchyme and renal vesicle.

With TEM, an extraordinary panorama becomes apparent to the observer. The higher resolving power of the electron microscope reveals details beyond those obtainable by light microscopy, accentuating the morphological changes that occur during the early stages of renal vesicle formation.

It is obvious that the role of the electron microscopy is not to gainsay but rather to find

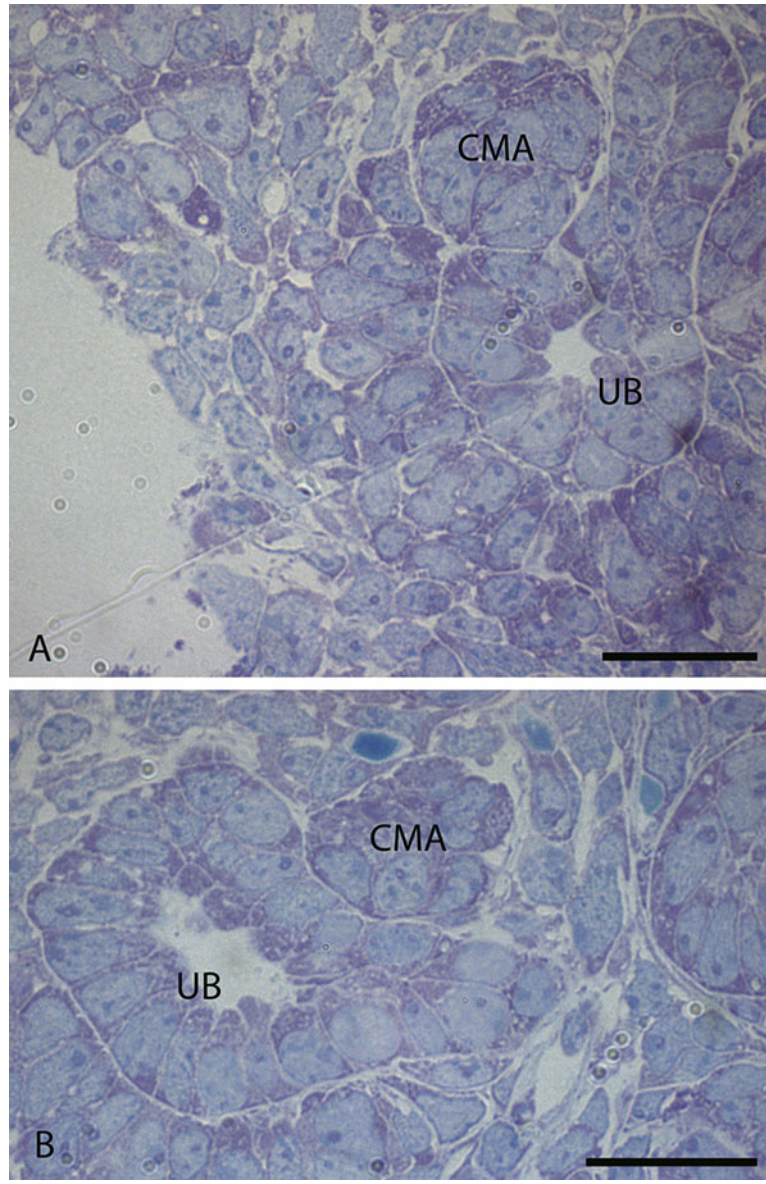

Fig. 4.1 (**a**, **b**) Light micrographs of the developing mouse renal cortex showing active nephrogenesis. Ureteric buds (UB) are surrounded by cap mesenchymal aggregates (CMA). Bars = 20 μm

significant correlations with earlier light microscopic observations [12–15], acquiring further ultrastructural informations concerning the specific morphological events occurring during the early stages of cap mesenchymal development and differentiation and highlighting the fine structure of cell organization in the cap mesenchymal aggregates. It's well known that the subsequent steps of nephron development are characterized by the mesenchymal-to-epithelial transition of cap mesenchymal cells, which eventually will form most of the epithelia of the mature human kidney [16, 17], however in the last years no extensive ultrastructural studies have been reported on the cap mesenchymal aggregates in the early phases of their origin from the metanephric mesenchyme and during their transition towards the renal vesicle. At higher magnification, their architecture is emphasized, showing variability in their morphological

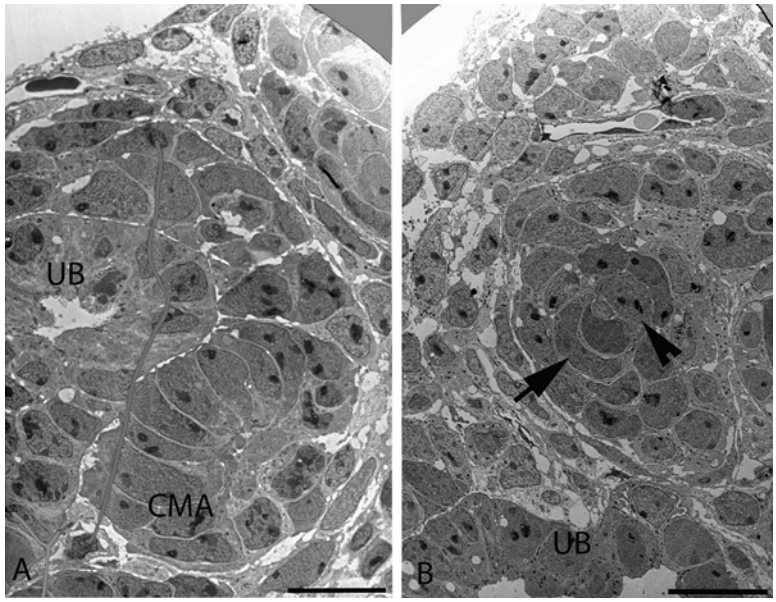

Fig. 4.2 Electron micrographs showing at higher magnification the outer portion of the mouse renal cortex. (**a**, **b**) Cap mesenchymal aggregates (CMA) with the adjacent ureteric buds (UB). (**b**) "Pine-cone body" characterized by a more conspicuous number of cells. Note the presence of the ovoid cell (*arrowhead*) in the central region surrounded by different thin curved shaped cells (*arrow*), resembling a pine-cone-shaped structure. Note the presence of evident nucleoli in most of the cellular constituents of the renal tissues. Bars = 10 μm

appearance and size. The cap mesenchymal nodules vary from small cellular solid nodules to bigger aggregates with a conspicuous number of cells. In general, all cellular constituents of cap mesenchymal nodules exhibit peculiar morphological features, being characterized by a scanty cytoplasm containing few cellular organelles and by a large nucleus that occupies most of the small cell body and contains prominent and pleomorphic nucleoli (Figs. 4.2 and 4.3). It is generally believed that the presence of prominent pleomorphic nucleoli indicates RNA and protein synthesizing and therefore increases cellular metabolic activity [18]. They are supposed to be tightly correlated with cellular differentiation processes that characterize the intermediate inductive events of nephrogenesis. Electron microscopic analyses reveal a degree of variability in cell shape and morphology among the cap mesenchymal constituents in the different nodules that populate the outer portion of renal cortex (Figs. 4.2 and 4.3). These changes may represent the various stages of cellular aging that take place in the growing cap mesenchyme and lead to the formation of renal vesicles. The bigger cap mesenchymal aggregates usually have thin curved cells in their outer areas that seem to twist around a fixed central cluster of a few roundish cells (Figs. 4.2 and 4.3), rather in the manner of a pine cone (Figs. 4.2b and 4.3a). During our investigation, we have speculated on the meaning of such morphogenetics events. The above data highlight the presence of a specific cap mesenchymal structure, the pine-cone body and show, at ultrastructural level, how each cap aggregate epithelializes proceeding in stages from a condensed mesenchymal aggregate to the renal vesicle, through the intermediate "pine-cone body" stage [19]. The peculiar architecture of the "pine-cone body" raises several interesting questions about the differentiation of its cellular constituents. Most of the curved cells detected in the outer regions of the cap mesenchymal aggregates might have evolved from the ovoid cells usually located in the central area of the same aggregate. Modifications of cellular shape can affect the area of contact between cells and could alter cell-to-cell cross talk [20, 21].

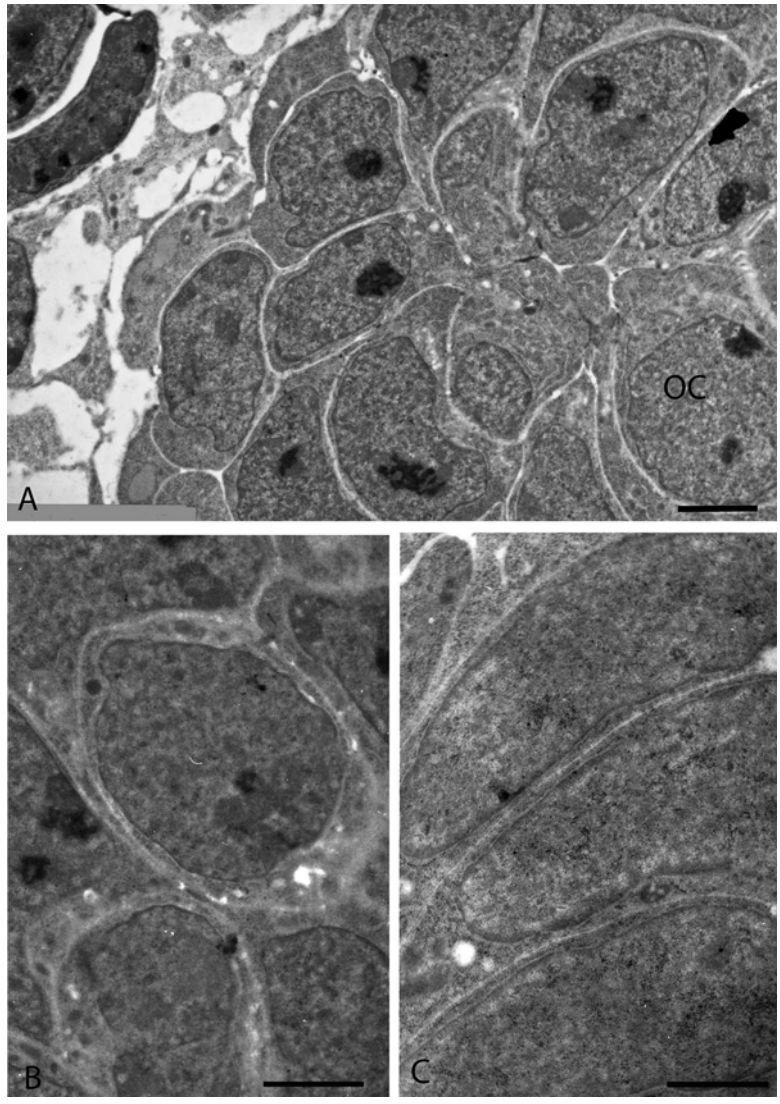

Fig. 4.3 (**a**) Portion of a pine-cone body. Note the presence of different shaped cellular constituents. The ovoid cells occupy the central region of the cap mesenchymal aggregate. (**b**) Details of the ovoid cells. (**c**) Details of the thin curved shaped cells. Bars = 2.5 μm

All these fascinating phenomena are initiated by the growing UB that induces the differentiation and proliferation process towards the surrounding mesenchyme [22, 23]. However if we focus more in depth on the early events of mouse nephrogenesis, that, starting from the cap mesenchymal induction, leads to the renal vesicle formation, a tight interaction emerges between cap mesenchymal induction and UB growing. Recent data suggest that nephrogenesis is initially based on the reciprocal induction between the UB and the metanephric mesenchyme. UB converts mesenchyme to an epithelium and, in turn, cap mesenchyme stimulates the growth and the branching of the UB. Although different gene products have been reported to regulate the early events of nephrogenesis [14, 16, 22, 24–27], most of the molecular mechanisms, that are supposed to control UB growth and cap mesenchymal induction, are still unknown.

Conclusions

In conclusion, electron microscopy adds new evidences concerning the early stages that characterize the nephrogenesis, trying to fill some of the gaps in our knowledge concerning the morphological events that take place during initial phases of kidney development. On the other hand, many questions remain to be ascertained and much work has to be done. As mentioned above we are at the very beginning of an exciting trip through a new and unknown world that waits to be revealed.

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The Human Kidney at Birth: Structure and Function in Transition

5

Robert L. Chevalier and Jennifer R. Charlton

Structure does not determine Function or vice versa, but both are simply different ways of regarding and describing the same thing.

—Jean R. Oliver, Nephrons and Kidneys 1968

The perinatal period is a critical transition for the fetus, shifting from a homeothermic aqueous environment with nutrition and excretory function provided by the placenta to a terrestrial environment with dependence on milk and renal excretory function. Human nephrogenesis is complete before term birth, and impairment of renal function in the healthy neonate is uncommon. However, maldevelopment of kidneys or urinary tract, fetal or perinatal stress, or preterm birth can result in a reduction of functioning nephrons at birth, placing the infant at risk. It has become clear that the consequences of reduced nephron number may not only impact the neonate, but also affect renal health throughout late adulthood. Noted first by British epidemiologist David Barker in the 1970s, adults dying of cardiovascular disease have a significantly lower birthweight than the rest of the population, and subsequent studies have extended these observations to reveal an increased incidence of hypertension and cardiovascular disease in individuals with lower nephron number [1].

Evolution of the Kidney and Its Relevance to Man

The development of the kidneys reflects a long evolutionary history, with sequential appearance in the embryo of pronephros, mesonephros, and metanephros; the metanephros serving as the functioning organ as of the 8th fetal week. Structure and function of the kidney are inseparable, as emphasized by the renal morphologist, Jean Oliver, in his magisterial atlas of human fetal kidney development, *Nephrons and Kidneys* [2]. Oliver builds on his predecessor, Sperber, who compared kidney morphology across many species, seeking a relationship between nephron size and number in each species [3]. He concludes that "the inefficiency of bigness … determines whether the kidney can provide adequate survival value" [3]. Following Poiseuille's Law, the length of renal tubules in mammals approaches a practical size limit. The evolutionary solution to this challenge is truly remarkable, ranging from the unipapillary kidney in small animals such as rodents, to the "crest" kidney of horses, and the "multirenculate" kidney of whales [2]. For the pig as well as primates (including man), the packaging of nephrons within the kidney is arranged in a multipapillary distribution. These species differences in assembly of nephrons within kidneys

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may be important in the choice of animal models of human disease. Whereas the rat and mouse have become the most widely used species for the study of most diseases, the sheep has the advantage of completing nephrogenesis prior to birth, and the multipapillary kidney of the pig more closely reflects the structure of the human kidney. Both have been used to advantage in the study of congenital obstructive uropathies [4].

How do these principles apply to the maximal size attainable by glomeruli and tubules following adaptive growth in response to reduced nephron number? No new nephrons are formed in response to a loss of renal mass, but in the human fetus with unilateral renal agenesis or multicystic kidney, adaptive nephron growth begins before birth [5, 6]. As demonstrated in animal studies by Brenner and his associates in the 1980s, reduced nephron number leads to maladaptive responses in hypertrophied nephrons, leading to injury to all components (glomeruli, tubules, vasculature, and interstitium) [7]. Damage to the proximal tubule appears to be central to this process, resulting in the formation of atubular glomeruli and aglomerular tubules [8]. The terminal events for these nephrons include the deposition of collagen in the glomerulus (glomerulosclerosis) and interstitium (interstitial fibrosis).

Nephron Number and Completion of Nephrogenesis

In obtaining accurate estimates of the number of glomeruli per kidney, the technique for arriving at the final count is of greatest importance. In 1930, estimates for an adult human kidney ranged from 560,000 to 5,700,000 depending on the approach used: counting the number of renal pyramids, counting serial sections, or counting glomeruli in aliquots of macerated kidney tissue following acid digestion [9]. All of these methods suffer inherent bias, as described by Bendtsen and Nyengaard [10]. This led to the application of the "disector" method, which is a stereologic approach unbiased by the size, shape, or tissue processing of the glomeruli [11]. Many pediatric texts reported an "average" number of 1,000,000 nephrons per kidney in man, ignoring data actually revealing significant variation in the normal population as early as 1928 and 1930 (Table 5.1) [9, 12]. Using the technique of counting glomeruli in aliquots of macerated kidneys, Vimtrup and Moore et al. counted nephrons in kidneys from subjects ranging in age from 1 to 74 years, reporting values from 600,000 to 1,200,000 and commenting, "the reason for the great variation probably lies in diversity of strain and heredity" (Table 5.1) [9]. By the late twentieth century, the more precise disector technique was developed, and has been applied in many studies over the past 20 years, with the largest series of subjects ($N=398$) having been reported by Bertram and his collaborators [13]. It is evident that using the disector technique in diverse populations reveals a dramatic 12-fold range in normal number of nephrons, from 210,000 to 2,700,000 (Table 5.1) [13]. These results should actually come as no surprise, since Darwin demonstrated that evolution cannot occur without variation [14], and our species is characterized by enormous variation in our metabolic as well as anatomic parameters [15, 16].

Table 5.1 Determination of the number of nephrons in the human kidney

		Subjects			
Author	Year	Number	Age	Technique	Number of nephrons
Vimtrup [12]	1928	4	1 child, 3 adults	Count glomeruli in acid digest	833,992–1,233,360
Moore [9]	1930	29	1–74 year	Count glomeruli in acid digest	600,000–1,200,000
Nyengaard and Bendtsen [48]	1992	37	16–87 year	Disector	331,000–1,424,000
Hughson et al. [23]	2003	56	11 children, 45 adults	Disector	227,327–1,825,380
Bertram et al. [13]	2011	398	Multiple races	Disector	210,332–2,702,079

Table 5.2 Determination of the timing of completion of nephrogenesis in the human kidney

Author	Year	Number	Gestational age	Technique	Termination of nephrogenesis (weeks)
Ferraz et al. [21]	2008	86	31–40 week	Nephrogenic zone thickness	32–36
Potter and Thierstein [18]	1943	1,000	20–40 week	Glomerular maturation	35
MacDonald and Emery [19]	1959	235	26 week–13.5 year	Glomerular maturation	36–44
Osathanondh and Potter [22]	1963	70	6–36+ week	Microdissection (acid digest)	36
Hinchliffe et al. [11]	1991	11 pairs	15–40 week	Disector	36–40

Now that preterm infants are surviving after birth prior to 25 weeks gestation (during a period of active nephrogenesis), the timing of completion of nephrogenesis has become more important. Most textbooks of pediatrics or nephrology define the completion of nephrogenesis as the disappearance of the nephrogenic zone at approximately 34–36 weeks gestation [17]. What are the actual data on which these conclusions are based? It is useful to review some of the techniques applied to this question. Early studies of nephrogenesis were based on morphologic transitions in the developing glomerulus following induction of metanephric mesenchyme by ureteric bud. The most notable of these was performed by Potter and Thierstein [18], and subsequently utilized by MacDonald and Emery [19] (Table 5.2). Potter and Thierstein described kidneys obtained at autopsy from 1,000 fetuses and infants (kidneys of malformed or macerated fetuses were excluded). If any incompletely developed glomeruli were visible, the nephrogenic zone was considered to be present [18]. They reported that the nephrogenic zone was present in nearly 100 % of fetuses at 30 weeks gestation, approximately 80 % at 34 weeks gestation, falling to 30 % at 36 weeks, and essentially zero after 40 weeks (Fig. 5.1). Based on these data, it is concluded that nephrogenesis in the majority of infants is complete by the 35th week of gestation [18]. Nearly 20 years later, Vernier and Birch-Andersen included electron microscopy in their study of 20 fetuses ranging from 1½ to 5 months gestation, and found that about 30 % of glomeruli contained adult-type foot processes at 5 months [20]. Immunohistochemical techniques were applied in the study of kidneys from 86 fetuses ranging

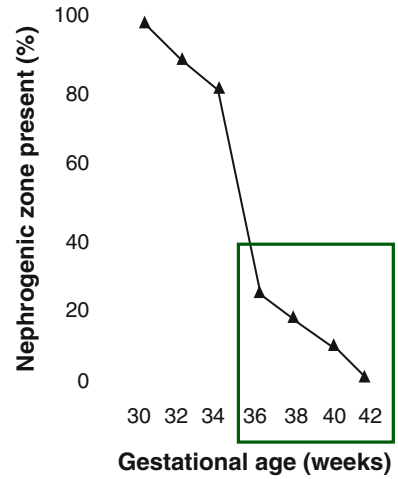

Fig. 5.1 Fraction of fetuses with identifiable nephrogenic zone (presence of developing glomeruli) in relation to gestational age. The nephrogenic zone has disappeared in over 70 % of infants after the 35th week (*green box*). Data from Potter and Thierstein [18]

from 15 to 40 weeks gestational age [21]. Using this approach, with the formation of the last layer of glomeruli (at 31–36 weeks), the nephrogenic zone was found to persist in about 50 % of subjects, but disappeared in the remaining 50 % (Table 5.2 and Fig. 5.2). This study confirms the variability in rate of maturation of nephrons between individuals.

In their report of 235 necropsy subjects spanning fetal life to 15 years of age, MacDonald and Emery classified developing glomeruli in six stages, ranging from the S-shaped glomerulus to the adult form with flattened podocytes and well-defined capillaries [19]. The number of glomeruli in each stage was counted along cortical columns lying between medullary rays. There was a marked decrease in Stage III glomeruli at 36 weeks, and

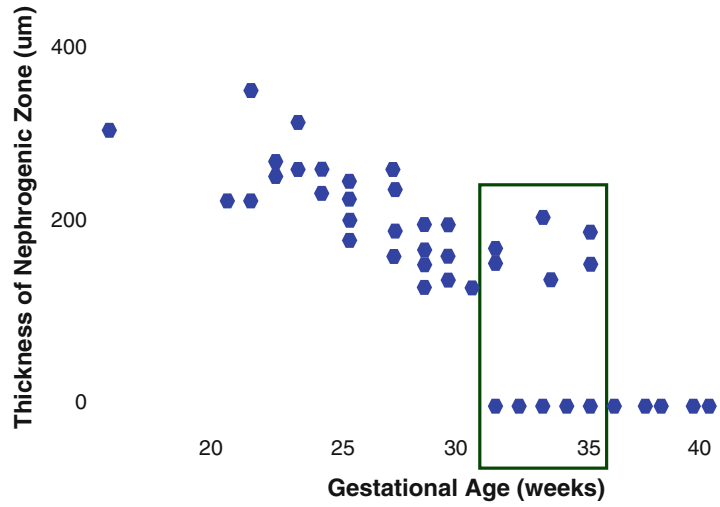

Fig. 5.2 Thickness of the nephrogenic zone in kidneys from human fetuses from 15 to 40 weeks of gestational age. With the formation of the last layer of glomeruli, the nephrogenic zone has disappeared in approximately half of the fetuses between 32 and 35 weeks (*green box*), and in all of the fetuses after 35 weeks. Adapted from Ferraz et al. [21]

the percentage of stage VI glomeruli increased from less than 10 % in the first 3 months of postnatal life to 50 % at 5 years, and 100 % at 12 years [19]. The authors suggest that the wide variation in persistence of immature glomeruli in childhood decreases the value of the Potter classification system as an index of developmental maturity.

Osathanondh and Potter analyzed fetal renal development using the microdissection technique in 70 normal individuals ranging from an 11 mm embryo to a 78-year-old man [22]. This allowed evaluation of branching morphogenesis, which ceases by 32–36 weeks, a range consistent with histologic analysis of glomerular maturation (Table 5.2). However, nephrons continue to form even after termination of branching, and this technique does not permit precise quantitation of the maturing nephron population [22].

Analysis of pairs of human kidneys from 11 normal spontaneous abortions and stillbirths (15–40 weeks gestation) yielded a coefficient of error of 8 % with intra- and inter-observer reproducibility of 98 and 94 % respectively [11]. There was a logarithmic increase in nephron number from 15,000 at 15 weeks to 740,000 at 36 weeks gestational age, with no additional increase from 36 to 40 weeks (Fig. 5.3). In a report of kidneys obtained at autopsy from 56 young adults, nephron number ranged from 227,000 to 1,825,000—an eightfold difference [23]. Importantly, there was a linear correlation between adult nephron number

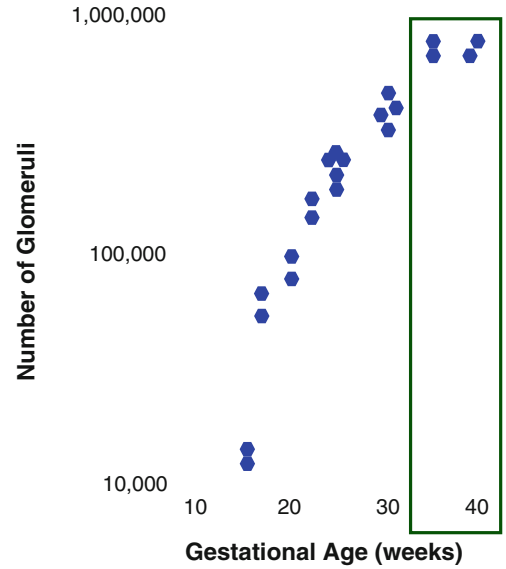

Fig. 5.3 Total glomerular number in paired kidneys from human fetuses from 15 to 40 weeks of gestational age, determined by unbiased disector technique. Note logarithmic scale of ordinate. The rate of increase of glomerular number is greatest at 15–17 weeks, and a plateau is reached at 36–40 weeks (*green box*). Adapted from Hinchliffe et al. [11]

and birth weight ($r=0.4$, $p=0.0012$), consistent with the predictions of Barker and Bagby [1].

Presumably because of the difficulty in measuring the dimensions of proximal tubules, there are few data regarding maturational changes in this nephron segment. Fetterman et al. described

changes in glomeruli and proximal tubules in microdissected nephrons from kidneys of 23 subjects varying in age from term neonate to 18 years [24]. Compared to older subjects, proximal tubules in the neonate are small in relation to corresponding glomeruli, and neonatal proximal tubular length ranges from 0.4 to 4.7 mm, an 11-fold variation [24]. However, by 1 month of age, the ratio of shortest to longest proximal tubule has decreased to 3.5, and proximal tubular length increases with age at a more rapid rate than increase in glomerular size [24]. This finding parallels a rapid maturation of proximal tubular function in the first year of life [25]. Taken together, available evidence suggests significant variation among individuals in the rate of nephrogenesis and in the timing of cessation of nephrogenesis: this clearly must be taken into consideration when interpreting data from preterm infants or from those with intrauterine growth restriction [26].

The Molecular Basis for Nephrogenesis

Over the past several decades, significant advances have been made in elucidating the molecular embryology of nephron morphogenesis and maturation, resulting in the identification of a number of key regulatory and structural genes and their interactions [27, 28]. The powerful techniques of genome-wide analysis using laser capture microdissection, fluorescence-activated cell sorting, and microarray profiling have yielded an atlas of gene expression in the developing mouse kidney [27]. Surprisingly, different developmental compartments demonstrate extensive overlap in gene expression patterns, suggesting an analog model of nephrogenesis. Thus, differences in the magnitude of gene expression appear to be more important than whether the gene is "on" or "off" [27]. Most importantly, this bioinformatics approach allows individual transcription factors to be connected with their targets by looking for evolutionarily conserved transcription factor-binding sites within promoters of expressed genes. Thus, expression of Hnf1 by developing proximal tubules is associated with Hnf1 binding sites in promoters of genes expressed by proximal tubules [27]. Analysis of global gene expression can also reveal points of transition resulting from genetic pathways activated during nephrogenesis. In a study of rat kidney development, global gene expression was examined as "self-organizing maps" which reduced more than 30,000 genes to 650 metagenes [28]. These maps revealed potential stages of development, suggesting points of stability/transition and candidate genes controlling patterning of nephron development. The patterning can be analyzed as macropatterned events (e.g., cortex and medulla) as well as micropatterned events (e.g., formation of glomeruli). Such an analysis can generate visual "portraits" of gene expression patterns, which reveal periods of transition at birth and at 1 week postnatal [28].

A question asked only recently is, "what factors determine cessation of nephrogenesis"? Whereas earlier studies were performed using a variety of mammalian species, most investigators currently utilize the mouse as a model of human renal structure and function because of the many murine mutants available. The alignment of equivalent developmental stages in mouse and man has been attempted, and human fetal maturation is not linearly related to that of the mouse [29]. Importantly, the mouse is a species in which nephrogenesis is completed after birth. Meticulous analysis of the completion of nephrogenesis in the neonatal mouse revealed a burst of nephron formation in the first two postnatal days, with complete cessation by the third day (Fig. 5.4) [30]. Since ureteric branch tips can still induce nephrons in culture, this was explained by depletion of the metanephric mesenchyme, rather than an increase in cell death (apoptosis) [30]. This work was further refined by the discovery that the last nephrons to be formed are clustered around ureteric bud tips rather than arising from individual tips [31], a phenomenon noted also in the late gestation human kidney by Osathanondh and Potter over 50 years ago [22]. The finding that cessation of nephrogenesis occurs when metanephric mesenchyme is depleted has significant clinical implications. If the mesenchyme is not

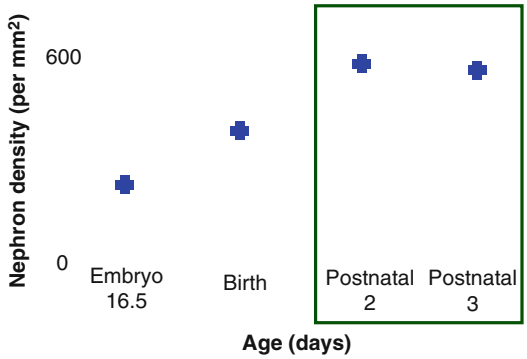

Fig. 5.4 Nephron density in mice in relation to late embryonic and early postnatal age. Nephron density continues to increase through postnatal day 2, but reached a plateau by day 3 (*green box*). Adapted from Hartman et al. [30]

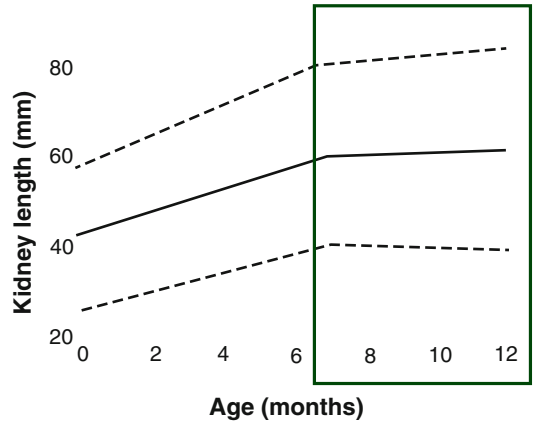

Fig. 5.5 Kidney growth in children during the first year of life, determined by renal ultrasound measurement. There is a rapid but slowly decreasing growth rate during the first 7 months, followed by a marked slowing from 7 to 12 months (*green box*). Adapted from Mesrobian et al. [33]

completely formed at the time of preterm birth, or if fetal stress leads to intrauterine growth restriction, there may be inadequate mesenchyme to produce an optimal number of nephrons [32].

Postnatal Renal Maturation: Growth and Function

To determine normal renal growth rate in the first year of life, 55 subjects underwent repeated renal ultrasound (2–8 times, median 3 per child) [33]. Growth rate decreased from 3.1 mm per month at birth to 0.25 mm per month at 7 months of age, remaining constant thereafter (Fig. 5.5) [33]. The growth rate transition at 7 months matches closely an analysis of glomerular filtration rate data (measured by polyfructose, Cr-EDTA, mannitol or iohexol) collected from eight studies (total 923 subjects) (Fig. 5.6) [34]. This study demonstrates the attainment of 75 % of adult GFR by 6 months of age, and approximately 90 % by 1 year of age (Fig. 5.6). Glomerular filtration rate measured at birth in preterm infants 28–34 weeks gestation is below 1 ml/min, whereas there is a significant increase at 36 and 40 weeks (Fig. 5.7) [35]. Notably, there is an acceleration in the rate of increase in GFR for preterm infants studied during later extrauterine life. Based on parallels with canine studies, the author concluded that the increase in GFR is signaled by the completion of nephrogenesis [35].

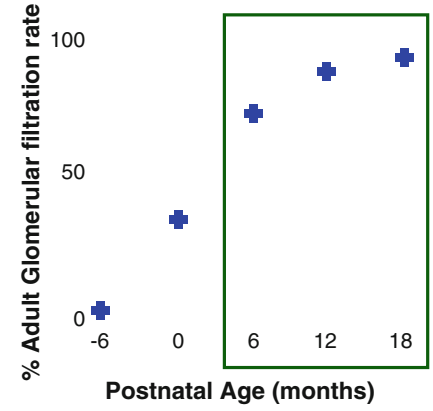

Fig. 5.6 Maturation of glomerular filtration rate expressed as the fraction of adult value (factored by 70 kg body weight). Data based on pooled published data from a total of 923 subjects ranging from preterm neonates (22 weeks postmenstrual age) to adulthood (31 years). 75 % of adult values are reached by 6 months, and >90 % by 18 months (*green box*). Adapted from Rhodin et al. [34]

For extremely preterm infants, however, postnatal nephrogenesis appears to be impaired, with cessation of nephrogenesis after 40 days of life [26, 36]. A more recent study demonstrated accelerated renal maturation following preterm birth, but an increase in the fraction of morphologically abnormal glomeruli in the outer cortex (those glomeruli formed in the extrauterine environment) [37]. Similar findings were reported in

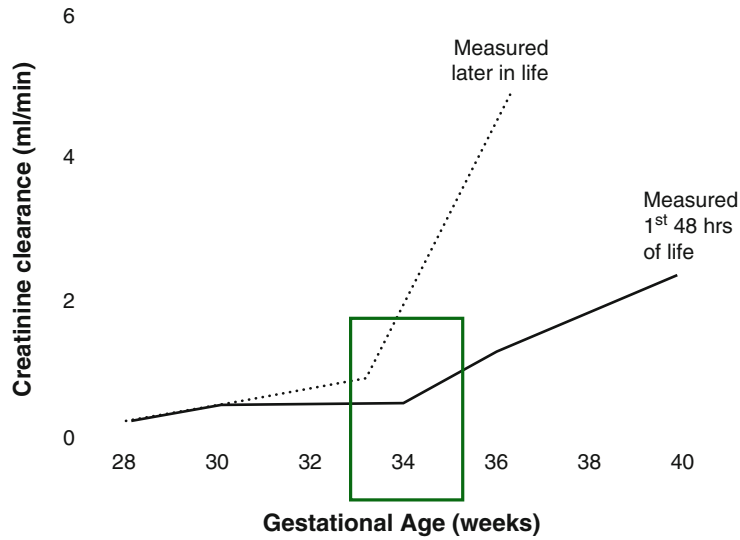

Fig. 5.7 Creatinine clearance in relation to gestational age for infants studied within 48 h of birth (*solid line*) and during later extrauterine life. After 34 weeks gestational age (*green box*), the rate of increase in glomerular filtration rate is greater for preterm infants whose function is measured at later postnatal ages (*dotted line*)

a non-human primate model of preterm birth [38]. There is accumulating evidence in support of an increased risk of chronic kidney disease in preterm and low-birth weight infants [39].

Biomarkers of Nephrogenesis

In addition to the conclusion that nephron number contributes significantly to long-term health outcomes, there is increasing evidence that acute kidney injury (particularly if recurrent) accelerates chronic kidney disease [40]. Plasma creatinine concentration, currently the most frequently used clinical marker of renal function, is insensitive and nonspecific as a marker of renal development or injury. There is an urgent need for biomarkers targeting renal development, renal injury, and repair mechanisms—particularly for the growing fetus, infant, or child. Cystatin C appears promising as a more sensitive marker of glomerular function, even when measured in amniotic fluid [41]. The excretion of CD24, a small glycosylated protein secreted in exosomes into urine and amniotic fluid, is produced by both glomerular and tubular cells, and may prove to be a useful marker of renal development and injury [42]. Trnka et al. suggest the term, "developmental injury" to distinguish the response to stress during fetal development, in contrast to "acute kidney injury" that occurs postnatally [43]. Charlton et al. have demonstrated that potential urinary biomarkers change dramatically with gestational and postnatal age, and caution that validation of any biomarker in the infant must take this into account [44].

The discovery of biomarkers reflecting nephron number is hampered by the absence of a gold standard to which each marker can be validated. There are currently no available techniques to determine nephron number in living individuals, but methods to determine nephron number in humans are currently under investigation. First, a prospective multicenter, observational cohort study in Japan is utilizing a combined method of glomerular density by renal biopsy and renal cortical volume by renal ultrasound or magnetic resonance imaging (MRI) to estimate nephron number in patients with chronic kidney disease [45]. Contrast enhanced MRI is a promising noninvasive approach to counting nephrons in vivo. Bioengineers have recently functionalized the highly conserved protein, ferritin, to provide a positively charged structure with iron at its

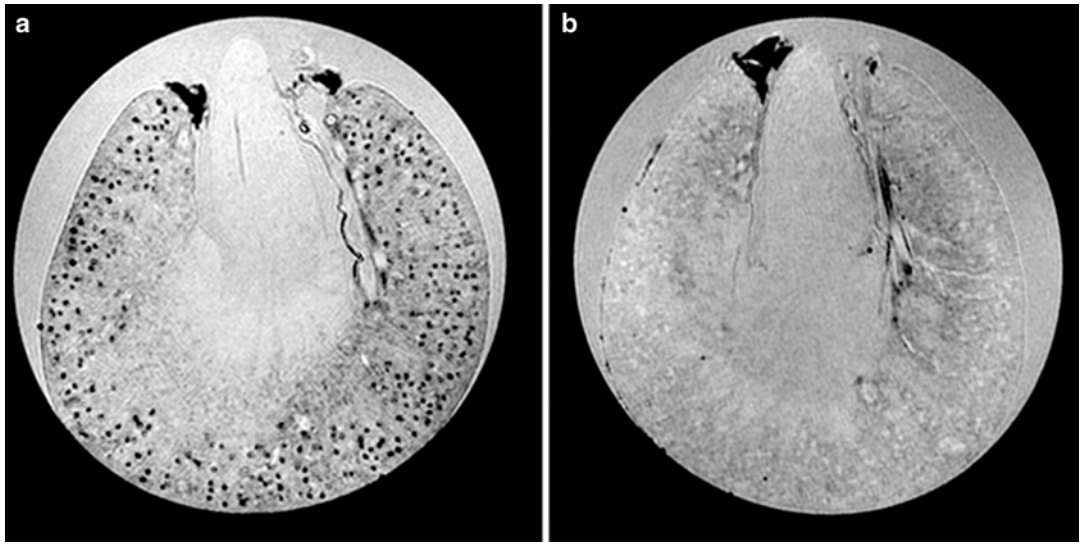

Fig. 5.8 MRI of rat kidney where each glomerulus is highlighted by the contrast agent, cationic ferritin (**a**). In the absence of cationic ferritin, the glomeruli are indistinguishable from the tubules (**b**). Images courtesy of Scott Beeman and Kevin Bennett

core (cationic ferritin), which has a high affinity to the anionic glomerular basement membrane. Cationic ferritin can reveal by MRI the otherwise concealed microstructure of the glomerulus. This technique has been utilized successfully in rodents, with ongoing efficacy and toxicity trials planned for larger animal species (Fig. 5.8) [46, 47]. In the future, if this technique is validated and deemed safe for humans, it could provide an accurate, individualized measure of glomerular number for both clinical and research purposes.

Conclusions

The transition from fetal to extrauterine life requires adequate renal function for maintenance of homeostasis, and adequate numbers of nephrons are required to maintain renal health into adulthood. There is significant inter-individual variation in the timing of completion of nephrogenesis, but the process should be complete in 90 % of infants by the 36th week of gestation. It appears that for infants with a final nephron number significantly below the median (900,000 nephrons per kidney) [13], hypertrophic growth can maintain adequate renal function for only a limited time before the onset of progressive chronic kidney disease [7]. Plasma creatinine concentration provides little information regarding nephron number or renal functional reserve. New biomarkers are needed to determine nephron numbers and their capacity for functional maturation. The growing population of very low-birth weight infants surviving the neonatal period has increased the urgency for progress in this field, and new advances are on the horizon.

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Perinatal Asphyxia and Kidney Development

6

Vassilios Fanos, Angelica Dessì, Melania Puddu, and Giovanni Ottonello

Introduction

Renal injury is a severe and extremely common complication that occurs early in neonates with asphyxia, occurring in up to 56 % of these infants [1].

The newborn presents in basal conditions compared to the adult, a state of relative renal insufficiency, including reduced renal blood flow and high renal vascular resistance (the neonate's kidney is halfway towards acute renal insufficiency). Many drugs are usually administered to sick newborns, especially preterm infants, and they may further worsen the renal function, thus leading to an amplification of the damage [2]. Moreover it is evident the specific role of hypoxia in determining functional and/or organic kidney damage. In absence of acidosis and hypercapnia, this role has been accurately studied only in experimental animal models [3, 4].

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The amount of damage depends, at least partially, on the degree and duration of the hypoxia and the neonate's capacity to respond to the condition [5]. In fact, in newborn piglets it has been demonstrated by the authors that there is a wide interindividual variability in the capability of the organism and in particular of the kidney to recovery after acute damages [6].

Severe injury may be the cause of acute tubular necrosis and acute renal insufficiency (the incidence may reach 10 % of cases), possibly associated with a picture of insufficiency in different organs [4].

Pathophysiology

Perinatal asphyxia is characterized by a variable period of hypoxia–ischemia, followed by reperfusion and reoxygenation. The term asphyxia derives from the Greek and means "the condition of being without pulse," which photographs the clinical aspect quite well.

Reperfusion injury has been suggested as the cause of kidney damage during resuscitation of neonatal asphyxia. Previous studies have demonstrated that postasphyxial serum from neonates with asphyxia may result in apoptosis of renal tubular cells. However, the mechanisms that mediate renal tubular cell apoptosis induced by asphyxia remain poorly understood. In a recent study Zhao et al. [7] investigate the intracellular signal transduction mechanisms that operate during injury of renal tubular cells induced by

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Table 6.1 The no-reflow phenomenon: causes

• Imbalance between vasoconstrictors/vasodilators
• Endothelial congestion injury
• Increased endothelial permeability
• Interstitial edema compressing the peritubular capillaries
• Increased leukocytes adherence
• Extra-vascular accumulation of leukocytes

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asphyxia in neonates. They concluded that postasphyxial serum may induce renal tubular cell apoptosis through the mitochondrial pathway and its intracellular signal transduction mechanism includes the activation of nuclear factor-kappa B.

Moreover, following an episode of renal ischemia, during renal reperfusion there are persistent reductions in renal blood flow up to 50 % (total and regional) [8, 9]. This is the so-called no-reflow phenomenon. The factors responsible for this phenomenon are presented in Table 6.1 [10]. There is a high sensitivity of the medulla and corticomedullary junction to a decreased supply of oxygen [10–12]. The causes are as follows: low amount of medullary blood flow (10 % of total renal blood flow); renal microvasculature serially organized; almost all descending vasa recta emerging from the afferent arterioles; shunting between descending and ascending vasa recta.

Another important point is represented by endothelial injury and structural damage associated with increased vascular permeability, tissue congestion, vasomotor disorders, and inflammatory and hemostatic activation. This is due to: rapid loss of adherens junctions (V-E cadherin); leakage from the vascular bed to the surrounding tissue; endothelial cell swelling; channel dysfunction; and procoagulative response.

These events are followed by irreversible damage to the mitochondrial structures, thus causing downstream activation of apoptotic and other cell death pathways.

In fact experimental data by Zhang et al. [13] demonstrates that post asphyxial serum of neonate can induce apoptosis of human renal proximal tubular cell line HK-2 cells and translocation of Omi/HtrA2 from mitochondria into cytoplasm may play an important role in its intracellular signal transduction mechanism in induction of apoptosis.

Postasphyctic damage is characterized by imbalance of the delicate equilibrium between vasoconstrictor (kidney-aggressive) and vasodilatory (kidney-protective) factors (the so-called vasomotor nephropathy) [14, 15]. Among the most important vasoconstrictors are angiotensin II and endothelin; among the vasodilators are the prostaglandins E2. Adenosin presents a complex, physiology being a vasoconstrictor in the afferent arteriole and a vasodilator in the efferent arteriole.

Local activation of the renin–angiotensin system is particularly important because it can lead to the constriction of efferent arterioles, hypoperfusion of postglomerular peritubular capillaries, and subsequent hypoxia of the tubulointerstitium in the downstream compartment. In addition, angiotensin II induces oxidative stress via the activation of NADPH oxidase. Oxidative stress damages endothelial cells directly, causing the loss of peritubular capillaries, and also results in relative hypoxia due to inefficient cellular respiration. Thus, angiotensin II induces renal hypoxia via both hemodynamic and non-hemodynamic mechanisms [16].

In a recent paper Mao et al. [17] hypothesized that chronic hypoxia adversely affects renal development in the ovine fetus. It was demonstrated the adverse effect of chronic hypoxia on renal angiotensin II receptors (AT1R and AT2R) expression and functions in the fetus, suggesting a role of fetal hypoxia in the perinatal programming of renal diseases.

Endothelin (ET) is a potent peptide from vascular endothelium with vasoconstricting action and whose secretion increases during hypoxia. Tekin et al. [18] observed that urinary ET-1 levels during perinatal asphyxia were negatively correlated with 5-min Apgar scores and cord blood base excess levels.

Adenosine derives from the consumption of ATP: during an acute event, the consumption of ATP (assessed by Seidl et al. in experimental studies) is directly proportional to the duration of asphyxia and the greatest reduction in ATP takes place in the kidney (80-fold reduction compared to the basal value). In the brain the reduction is "only" 22-fold, in the heart fivefold [19].

Thus there is a conflict of interest: there is the "private" interest of the "tired" kidney which wants to stop filtering so as not to have to reabsorb, and a "public" interest of the entire organism which cannot allow the kidney to stop performing its institutional duties. At the beginning, a compromise is reached: the kidney must continue filtering, but must reduce reabsorbing (the FeNa increases). Adenosine is probably released into the renal medulla by thick medullary ascending limbs of Henle in response to the imbalance between transport activity and oxygen supply, and the released adenosine via adenosine receptor 1 (AR1) activation decreases sodium chloride absorption and oxygen consumption [20].

Chen et al. have investigated the variations of actin of newborn porcine renal tubular epithelial (RTE) cell during ATP deficiency and shed light on the possible mechanisms of renal deficiency during newborn asphyxia. It was found that the ATP deficiency time elongated, G-actin of the newborn porcine RTE cell decreased first and then increased, and the F-actin decreased step by step. This may destruct the cell bone-skeleton of the newborn RTE cell and maybe one of the important mechanisms of renal deficiency during newborn asphyxia [21].

Finally it has been demonstrated that the urinary ratio of uric acid (an important product of adenosine degradation) to creatinine can be used in the clinical diagnosis and grading of the severity of neonatal asphyxia [22].

Mohd et al. determined renal ultrasound findings among asphyxiated neonates and correlated this with uric acid levels and the severity of hypoxic encephalopathy. They concluded that kidneys are the most common organs involved in perinatal asphyxia and uric acid might be a causative factor for failure in addition to hypoxic insult. Routine use of kidney function test, along with abdominal ultrasonography form an important screening tool to detect any additional morbidity in these patients [23].

Prostaglandin E2 (PGE_2) belongs to a family of biologically active lipids derived from the 20-carbon essential fatty acids. Renal PGE2 is involved in the development of the kidney; it also contributes to regulate renal perfusion and glomerular filtration rate, and controls water and electrolyte balance. Furthermore, this mediator protects the kidney against excessive functional changes during the transition from fetal to extrauterine life, when it counteracts the vasoconstrictive effects of high levels of angiotensin II and other mediators. There is evidence that PGE2 plays an important pathophysiological role in neonatal conditions of renal stress, and in congenital or acquired nephropaties. In fact the perinatal kidney could be considered prostaglandin dependent [24–26].

Recent studies demonstrate that the loss of the eNOS function in the course of hypoxic/ischemic damage may precipitate renal vasoconstriction. Moreover there is an increase of production of toxic metabolites such as peronitrate which has been identified as a mediator of tubular damage in laboratory animals [14].

Rhabdomyolysis can also occur in newborns following severe asphyxia with consequent increase of myoglobinuria, which determinates direct and indirect tubular damage, especially in presence of dehydration [27, 28].

The main three events that happen in proximal tubular cells during an acute kidney injury and contribute to determine a complete cyto-architectural and morphofunctional upheaval are presented below: (a) "shaving" of the brush border; (b) shifting of the sodium/potassium pump from the antiluminal to the luminal side; (c) loss of intercellular ligands and those between cell and basal membrane (this phenomenon is called "homelessness," or "anoikis"). A schematic representation of these phenomena is presented in Fig. 6.1.

The tubular cells flake off in the cell lumen with consequent acute tubular obstruction of the lumen itself which has a diameter just double compared to that of the cells. The cell detritus linked together by the integrins, a kind of small hooks are essential elements in keeping the tubular cells attached to the basal membrane and the neighboring cells, assume a negative role with a boomerang effect, reducing the glomerular filtrate owing to the increase in intratubular pressure [29].

Recently, Yu et al. [30] recently investigated the role of beta-1-integrin in asphyxia followed by acute tubular necrosis in newborn rabbits: intrauterine asphyxia causes proteolysis of

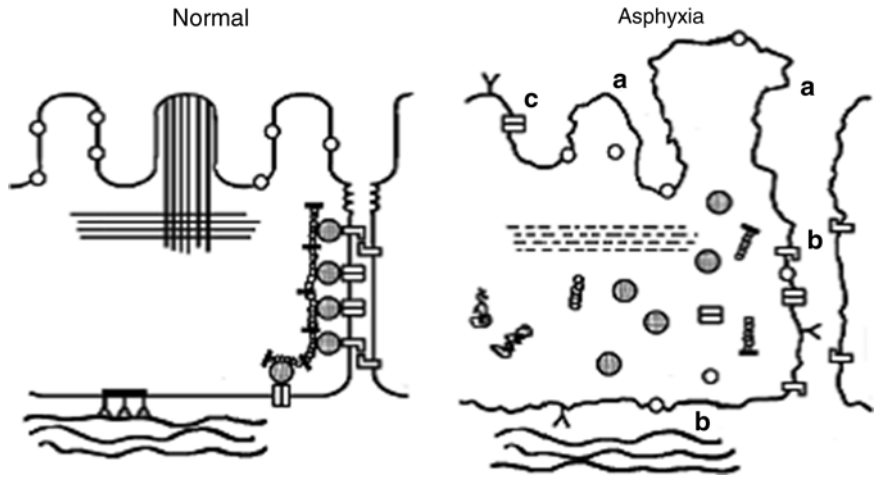

Fig. 6.1 Schematic representation of the three main processes in proximal tubular cells during asphyxia. (a) "shaving" of the brush border; (b) loss of intercellular ligands and those between cell and basal membrane; (c) shifting of the sodium/potassium pump from the antiluminal to the luminal side. Adapted from [10]

beta-1-integrin, with consequent depolarized distribution, leading to tubular lumen obstruction and renal tubule destruction. Damage to beta-1-integrin and the renal tubule is related to the activation of calpain, and the calpain inhibitor curtailed these effects.

Biomarkers

Acute kidney injury is one of the commonest manifestations of end-organ damage associated with birth asphyxia [31] and its diagnosis could be performed in the newborn with urinary biomarkers. They are presented in Table 6.1.

A "preclinical" tubular damage could be demonstrable only with tubular proteinuria dosage may be present, in particular α1 microglobulin (α1m), β2 microglobulin (β2m), retinol binding protein (RBP) or of enzymuria, especially alanine aminopeptidase (AAP) or *N-acetyl-β*-D-glucosaminidase (NAG). Normally it is said that when the urinary concentration of NAG increases it means that the cell "self-destruct button" has been pressed. During neonatal asphyxia the urinary excretion of β2m, α1m, and RBP increases 8-, 15-, and 20-fold respectively. NAG increased from 8- to 18-fold compared to normal values [2, 5].

Serum creatinine-based definitions of acute kidney injury are not ideal and are additionally limited in neonates whose serum creatinine reflects the maternal creatinine level at birth and normally drops over the first weeks of life dependent on gestational age. Recent studies confirm that urine and serum biomarkers may provide a better basis than serum creatinine on which to diagnose acute kidney injury [32].

In the last years the role of cystatin C determination has been underlined in several papers in the perinatal period. Its sperm concentration is not influenced by maternal values and normality data in the newborn are known [33–36].

A recent study by Sarafidis et al. has evaluated serum (s) cystatin C (CysC) and neutrophil gelatinase-associated lipocalin (NGAL) and urine (u) CysC, NGAL, and kidney injury molecule-1 (KIM-1) as markers of acute kidney injury in asphyxiated neonates. They concluded that sNGAL, uCysC, and uNGAL are sensitive, early acute kidney injury biomarkers, increasing significantly in asphyxiated neonates and their measurement from day of life is predictive of post-asphyxia-acute kidney injury [37].

A new marker useful for the prediction and diagnosis of perinatal asphyxia is represented by ischemia-modified albumin (IMA) a new

Table 6.2 The panel of altered metabolites in urine, blood, and brain in experimental models of asphyxia

Blood		Brain	
Anoxia			
Ratios of alanine to branched chained amino acids (Ala/BCAA) and of glycine to BCAA (Gly/BCAA)	↑	Phosphocreatine, ATP and ADP	↓
Reoxygenation		Urine	
Alpha ketoglutarate, succinate, and fumarate	↑	Urea Creatinine Malonate Methilguanidine Uric acid Hypoxanthine Malonylaldeide	↑

Adapted from [10] with permission

biomarker in identification of myocardial ischemia of myocardial necrosis. IMA may also increase in the ischemia of liver, brain, kidney, and bowel. Ischemia of these organs may also be seen in perinatal asphyxia as well. Reactive oxygen species, produced during ischemia/reperfusion which is essential steps of perinatal asphyxia, may generate the highly reactive hydroxyl radicals. These hydroxyl radicals modify the albumin and transform it into IMA [38]. We recently reviewed this matter in different papers [39–42].

In the next future the new holistic metabolomic approach (about 3,400 metabolites in biological fluids) may lead to an early diagnosis of asphyxia, predict mortality and neurologic outcome. Metabolomics has been studied in four experimental cases on animals [6, 43–45]: a synthesis of the discriminating metabolites is presented in Table 6.2.

Asphyxia and Kidney Development

If we analyze the acute effects of asphyxia to an organism, we find that this causes a quantitative reduction in the number of cells and a deficit in their functionality. Hypoxia and asphyxia-induced cellular hypodysplasia (fewer and less functional cells) is associated with reduced functionality of the organ which in the long run cannot perform its institutional functions and determines a mismatch between the requirements of the organism and the possibility of the organ to satisfy them. At the kidney level, this is associated with a reduced arborization of the ureteric bud [46].

Considering the relationship between differentiation of the cap mesenchymal cells during kidney development the major effect of fetal hypoxia is represented by a block in the process of the epithelial to mesenchymal transition occurring in the cap mesenchyme, mediated by the down-regulation of Wnt-4 (in some cases it is completely absent), leading to a lesser degree of UB branching and failure to develop nephron structures and ending in a reduction in nephron number and kidney size [47].

The epithelial marker E-cadherin is confined only to the UB, determining a reduced UB branching. These data must be taken into account when asphyxia intervenes in a preterm infants of GA <35 weeks, when nephrogenesis is not complete. It is credible that the block in the process of the epithelial to mesenchymal transition could be related to reduction of kidney size and nephrons number [47].

Very interestingly, not only asphyxia, but also neonatal oxidative injury causes long-term renal damage, important in the pathogenesis of hypertension. Sprague–Dawley pups were kept with their mother in 80 % O(2) or room air from days 3 to 10 postnatal, In male and female rats exposed to O(2) as newborns, systolic and diastolic blood pressures were increased (by an average of 15 mm Hg); ex vivo, maximal vasoconstriction (both genders) and sensitivity (males only) specific to angiotensin II were increased. Vascular superoxide production was higher; and capillary density (by 30 %) and number of nephrons per kidney (by 25 %) were decreased. These data suggest that neonatal hyperoxia leads in the adult rat to increased blood pressure, vascular dysfunction, microvascular rarefaction, and reduced nephron number in both genders [48].

Treatment

Concerning the treatment, the therapeutic hypothermia is standard treatment for asphyxiated infants. Several previous studies suggested that

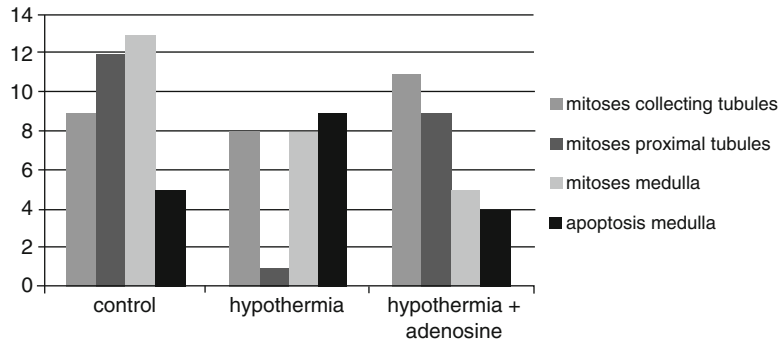

Fig. 6.2 Marked differences were observed among three groups regarding the mitotic activity and the apoptotic index. From [50] with permission

therapeutic hypothermia improves survival and neurodevelopment in asphyxiated infants without significant side effects. Little is known about renal changes in asphyxiated infants who underwent therapeutic hypothermia.

A recent study was performed to determine the effects of erythropoietin (EPO), moderate hypothermia, and a combination thereof on the kidneys of newborn rats damaged in an experimental animal model of perinatal asphyxia (Wistar rats). The conclusion of the paper is that EPO and hypothermia, as well as the combination thereof, have a protective effect on rats' kidneys damaged during perinatal asphyxia [49].

In an experimental model of hypoxia (rats) hypothermia was associated with a significant decrease in the mitotic index in proximal tubules. In this group, kidney also showed an increase in the apoptotic index in the medulla (Fig. 6.2). The association of adenosine to hypothermia resulted in a higher mitotic activity in proximal and in collecting tubules. No significant pathological changes were detected in kidneys from rats submitted to hypothermia and to adenosine treatment as compared to control rats [50].

In another study the authors aimed to determine if kidney structure and function were affected in an animal model (pregnant spiny mice) of birth asphyxia and if maternal dietary creatine supplementation could provide an energy reserve to the fetal kidney, maintaining cellular respiration during asphyxia and preventing AKI. AKI was evident at 24 h after birth asphyxia, with a higher incidence of shrunken glomeruli ($P<0.02$), disturbance to tubular arrangement, tubular dilatation, a twofold increase ($P<0.02$) in expression of NGAL (early marker of kidney injury), and decreased expression of the podocyte differentiation marker nephrin. Maternal creatine supplementation was able to prevent the glomerular and tubular abnormalities observed in the kidney at 24 h and the increased expression of NGAL [51].

Using a subacute swine model of neonatal hypoxia–reoxygenation (H/R), treating the piglets with *N*-acetyl-L-cysteine (NAC) significantly increased both renal blood flow and oxygen delivery throughout the reoxygenation period. NAC treatment also improved the renal function with the attenuation of elevated urinary NAG activity and plasma creatinine concentration observed in H/R controls (both $P<0.05$). The tissue levels of lipid hydroperoxides and caspase 3 in the kidney of NAC-treated animals were significantly lower than those of H/R controls. Conclusively, postresuscitation administration of NAC elicits a prolonged beneficial effect in improving renal functional recovery and reducing oxidative stress in newborn piglets with H/R insults for 48 h [52].

Finally, considering prevention, in the opinion of authoritative experts, theophylline does not at present have a definite place in the prevention or management of acute postasphyctic renal insufficiency except in controlled experimental studies [53, 54].

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Lessons on Kidney Development from Experimental Studies

7

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Introduction

The development of human kidney is a complex process requiring intricate cell and tissue interactions to assure the concerted program of cell growth, differentiation, and morphogenesis. Although the molecular and cellular nature of each of these interactions remains currently unclear, significant findings regarding nephrogenesis and its completion among different animal species have been reported over the last two decades. Research so far indicates that there are differences regarding the completion of the process of nephrogenesis among different animal species. In human, sheep, and spiny mouse, nephrogenesis is completed prior to birth, while in rat, mouse, and swine, nephrogenesis continuous after birth [1–7]. Nevertheless, the unrecognized morphological or functional peculiarities characterizing other animal species help the scientific community to reveal and understand the physiological mechanisms during nephrogenesis in human. This has been achieved mainly due to the increased use of animal models in renal basic science laboratories, as well as to the increased expertise of researchers who study kidney development. In the present chapter we aim at presenting and reviewing the existing knowledge on kidney development acquired from experimental studies.

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Novel Structural/Molecules Components that Extend Knowledge on Kidney Development

The Pine-Cone Body

The mature kidney of mammals is the final product of three embryonic excretory organs, the pronephros, the mesonephros, and the metanephros. The latest originates from two main components, the ureteric bud and the mesenchymal cells of the metanephric mesenchyme [7, 8]. Recent studies using light electron microscopy reported that in

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the subcapsular regions of the outer portions of renal cortex, characterized by active nephrogenesis, some cap mesenchymal aggregates showed variability in shape and morphology of their cells. The center of the cap aggregates was occupied by a roundish cell, while their outer regions were characterized by the presence of thin curved shaped cell types twisted around a fixed central cluster, resembling a pine-cone-shaped structure [9].

Although early studies on nephrogenesis speculated that the sequence of morphological events leading to glomerulogenesis and tubulogenesis might start with the outgrowth of the primary nephric duct and the ureteric buds, which invade the metanephric mesenchyme and induce the differentiation of the renal epithelial precursors [10, 11], similar changes in the size and appearance of developing renal cells may be correlated to the various stages of cellular differentiation occurring during cap mesenchymal development [9]. These curved cells which may evolve from the ovoid cells found in the central area of the same aggregate could account for changes in transmembrane signaling and consequently for changes of cellular metabolic activity [12, 13]. Moreover, the presence of prominent and pleomorphic nucleoli may indicate a significant increased cellular metabolic activity associated with cellular differentiation during cap mesenchymal development [14]. These findings suggest the "pine-cone body" formation as an intermediate stage between the condensed mesenchymal nodule to the renal vesicle during conversion of mesenchyme to epithelium. At cellular level, the entire cap developmental process seems to represent the final event of a complex balance between specific intercellular signals involved in the regulation of protein synthesis, cell proliferation, cell motility, and apoptosis [9]. However, further research is necessary in order to better investigate the intimate significance of this new developmental structure.

Wnt Glycoproteins

Wnt-4 belongs to the Wnt family of secretory glycoproteins that are implicated in signaling processes operating during metanephric development. Wnt-4 is expressed in pretubular mesenchyme cells shortly before their aggregation and transformation to simple epithelial tubules [15]. Kispert et al. [16] showed that mesenchymally derived Wnt-4 is not only required, but also sufficient for induction of tubulogenesis in the mammalian kidney and can elicit the complete program of tubular differentiation in isolated metanephric mesenchyme. Interestingly, the activity of Wnt-4 contrasts with other factors thought to regulate mesenchymal development but proved not sufficient or not essential for tubulogenesis [17–23].

Wnt-4 may have a later function in tubulogenesis which is masked in the earlier requirement to form a tubule as Wnt-4 expression in the metanephric mesenchyme is initiated in the aggregating mesenchyme and maintained in the comma shaped bodies before it is downregulated in S-shaped bodies. Wnt-4 probably acts as a trigger to start an intrinsic program in the mesenchymal cells which then proceed to form complex nephron like structures. Considering that a permissive signal from the ureter to the mesenchyme triggers survival and tubulogenesis in the mesenchyme, it can be concluded that kidney tubulogenesis is a multi-step process with a hierarchy of signaling systems. In general, the role of Wnt-4 in tubulogenesis reflects that additional signaling systems control the ratio between interstitial and metanephrogenic cells, between condensing and non-condensing cells, and the maintenance of the mesenchymal stem cells in the periphery [16].

Wnt-9b is another glycoprotein expressed in the Wolffian duct and its derivative that has been implicated in the induction of the mammalian kidney development. Wnt-9b is expressed in the inductive epithelia and is essential for the development of mesonephric and metanephric tubules and caudal extension of the Müllerian duct as it is required for the earliest inductive response in metanephric mesenchym [24]. In addition, Wnt-9b-expressing cells can functionally substitute for the ureteric bud in these interactions. Interestingly, Wtn-9b acts upstream of Wnt-4, demonstrating the major role of Wnt signaling pathway in the organization of the mammalian urogenital system. Wtn-9b-dependent activation of Wnt-4 expression in the metanephric mesenchyme plays

a central role in completing the process of tubule induction. Although Wnt-9b and Wnt-4 may act through distinct receptors, existing evidence suggest that Wnt-9b encodes a permissive signal, the region-specific response being governed by either the interplay of additional signaling factors or preprogramming of the target cell response by early patterning processes [24].

MUC-1

Although human MUC-1 mucin interest has mainly been focused on its role in carcinogenesis and tumor progression, its role in human and non-human embryogenesis was unclear until now. However, recent research in mouse embryos and neonates has shown, among other organs, increased MUC-1 expression in kidney as well [25]. In kidney, MUC-1 expression was mainly restricted to the apical part of the epithelial cells, in line with the characteristic pattern of MUC-1 in adult rat epithelial tissues [26, 27]. Although non-human studies related to MUC-1 have been mainly developed to obtain animal models useful for the comprehension of cancer, MUC-1 could play a relevant role during epithelia cellular differentiation and proliferation.

Glial Cell Line-Derived Neurotrophic Factor

Glial cell line-derived neurotrophic factor (GDNF) was shown to play a key role in kidney development through actions at the RET and GFR 1 receptor and coreceptor by initiating budding of the ureteric duct from the Wolffian duct, branching of the ureteric epithelium within the metanephric mesenchyme, and the formation of new nephrons at the branch tips [28–32]. In the late 1990s, knockout studies indicated that GDNF gene dosage influenced kidney development, with the loss of one allele being sufficient to cause a significant renal phenotype [33–40]. Recently, Cullen-McEwen et al. [41] found that the kidneys of GDNF heterozygous mice at 30 days of age were 25 % smaller than their wild-type littermates despite similar body weights, while stereologic estimates of nephron number identified a 30 % decrease in nephron endowment in young heterozygous GDNF mice compared with wild-type mice [42].

Although it was hypothesized that reductions in glomerular number lead to hypertrophy of the remaining glomeruli with time, evidence indicated that such hypertrophy also occurs when glomerular numbers are reduced genetically. Cullen-McEwen et al. [42] reported that by 14 months of age, glomeruli of GDNF heterozygotes were significantly hypertrophied such that the total glomerular volume was no longer different between wild-type and heterozygous littermates. Thus, the results found in this low nephron-number mouse are in accordance with the hypothesis of Brenner et al. [43] that a reduction in nephron number from birth leads to the development of hypertension and hyperfiltration.

Sodium Transporters

Although experimental studies have so far firmly established that the prenatal environment can modify the adult blood pressure [44–47], the mechanisms in humans are poorly understood. Nevertheless, several experimental models [44, 46–49] indicate that the various manipulations work through a common pathway.

Manning et al. [50] examined the expression of 4 key apical Na transport proteins that are critical for the regulation of Na balance and extracellular volume and found that upregulation of BSC1 and TSC, the apical Na transporters of TAL and DCT, respectively, occurs at both the mRNA and the protein level, reflecting increased Na reabsorption in these two segments. Moreover, NHE3 expression was not changed, suggesting that proximal tubule Na transport, at least the major fraction mediated by NHE3, is not affected by the prenatal programming; NHE3 may be upregulated by mechanisms not associated with altered protein abundance. Interestingly, the Na transporters were not downregulated after the hypertension became manifest, at 8 week of age. Considering that downregulation of TSC is an

important component of the pressure-natriuresis response designed to correct hypertension by increasing renal Na excretion [51], prenatal programming of the Na transporters may override the normal pressure-natriuresis mechanism. Although the signal(s) from mother to fetus that result in transporter upregulation remain unknown, the fetal overexposure to maternal glucocorticoids due to decreased placental activity of the 11β-hydroxysteroid dehydrogenase type 2 enzyme was implicated as a proposed explanation [52, 53]. Indeed, maturation of renal Na transport, measured as Na-K-ATPase expression, is regulated by glucocorticoids and, therefore, abnormal glucocorticoid exposure could therefore have a direct effect on the maturing kidney [54].

Influential Factors of Kidney Development

Maternal Nutrition

The relationship between nutrition and nephrogenesis has been adequately established on animal models with experimental studies showing that maternal nutrition may have an important influence on renal programming [55]. In rats, a restricted supply of nutrients to the mother during the critical window in which nephrogenesis occurs led to a reduced number of glomeruli per kidney, activation of the renin–angiotensin system, glomerular enlargement, and hypertension in later life [47], while in another study, early postnatal overfeeding increased the number of postnatal nephrons and decreased glomerular volume, suggesting that global filtration surface area remains unchanged [56]. Under these circumstances, glomerular hyperfiltration to meet excretory demands due to early postnatal overfeeding could contribute to elevated blood pressure, proteinuria, and progressive glomerulosclerosis in aging overfed males than overfed females. Although the reasons as to why the influence of postnatal nutrition on nephron endowment is limited to male gender are unknown, it has been speculated that hyperleptinemia associated with early postnatal overfeeding may influence renal functions through specific effects involving renal sympathetic hyperactivity and decreased sodium excretion, partially due to an upregulation of Na-K-ATPase [57]. In either case, altered nephrogenesis plays an important role in the early origins of cardiovascular and renal diseases in adulthood [58–61]. Considering that hypertension may be observed in the absence of glomerular number reduction, it is possible that mechanisms different from inborn nephron number deficit to be involved. Of note, early postnatal overfeeding during the suckling period has been demonstrated to induce obesity and cardiovascular and metabolic disorders in adult rats, such as hyperinsulinism and insulin resistance, impairing vascular dilatation capacity through endothelial dysfunction [62–64].

Vitamin A has been proposed as a determinant in fetal renal programming in rats in view of its capacity to closely modulate nephron number and vascular supply [65, 66]. Moreover, the role of vitamin A in renal formation is considered essential since null mice for these genes exhibited renal agenesis or rudimental kidneys [67], while recently, vitamin A supply restored nephron endowment to normal in offspring of rat mothers exposed to protein restriction [68]. In this study, offspring exposed to maternal protein restriction during pregnancy and lactation had a significantly reduced body weight, kidney size, and nephron endowment at weaning, suggesting that administration of retinoic acid during pregnancy, early in gestation, is able to stimulate nephrogenesis per volume of kidney tissue over and above control levels [67]. Although the mechanisms by which retinoic acid stimulates nephrogenesis are not fully understood, studies suggest that it mediates its effects on nephrogenesis by stimulating ureteric branching morphogenesis [69, 70]. The same investigators suggested that the likely molecular candidate mediating these early nephrogenic effects is GDNF, acting via its cell-surface receptor GDNF-α and subsequently activating the receptor

tyrosine kinase c-ret which is known to lead to increased branching morphogenesis of the ureteric bud and in turn enhance nephron formation [29, 30, 67, 71, 72]. Alternatively, administration of retinoic acid may mediate its effects on nephrogenesis via stimulation of the metanephric mesenchyme [73].

Previous studies reported that in male rats, exposure to maternal protein restriction either in utero or whilst suckling can have profound effects on kidney telomere lengths and on urine albumin excretion during much of adult life [74]. These rats appeared to be relatively protected against future nephron damage not only due to the absence of the nephrotoxic effects of urine albumin, but, also, because of their kidney telomere length. Telomere shortening has been implicated in renal diseases, while reduced renal telomere shortening is associated with increased levels of antioxidant enzymes, suggesting the beneficial effects of protein restriction on the development of kidney [75]. On the other hand, fetal exposure to a maternal low-protein diet is associated with disproportionate patterns of fetal growth and later elevation of blood pressure in the rat, suggesting that maternal undernutrition may program the renal nephron number and hence impact upon adult blood pressure and the development of renal disease [76]. Of note, in another study in rats exposed to a maternal low-protein diet in utero, renal morphometry and creatinine clearance at older ages were not influenced by prenatal diet, although blood pressure was elevated at all ages in the low-protein-exposed offspring [77]. However, blood urea N, urinary output, and urinary albumin excretion were significantly greater in low-protein-exposed rats than in control rats at 20 weeks of age, suggesting a progressive deterioration of renal function in hypertensive rats exposed to mild maternal protein restriction during fetal life. Although the mechanisms of protein restriction-induced adulthood hypertension are not well understood, Woods et al. reported that perinatal protein restriction in the rat suppresses the newborn intrarenal renin–angiotensin system and leads to a reduced number of glomeruli, glomerular enlargement, and hypertension in the adult [47]. Nevertheless, additional mechanisms may be involved in kidney development of protein-restricted mammals. Holemans et al. [78] investigated the hypothesis that malnutrition in pregnant rats may lead to altered cardiovascular function in adult female offspring and found that food restriction during the second half of pregnancy and/or lactation does not induce hypertension in adult offspring, but may effect subtle changes in vascular function. Interestingly, two other studies showed a very pronounced blunting of the response to acetylcholine in the neonatal vasculature from offspring of streptozotocin-diabetic rats on a high-fat diet and in the adult offspring of streptozotocin-diabetic rats [79, 80]. Brawley et al. [81] assessed isolated resistance artery function from adult male offspring of control and protein-restricted pregnant dams at two different ages and reported that dietary protein restriction in pregnancy induces hypertension and vascular dysfunction in male offspring. These disorders may be mediated via nitric oxide–cGMP pathway-induced abnormalities in endothelium-dependent and -independent relaxation, reducing vasodilation, and elevating systolic blood pressure [82]. Nevertheless, disturbances in the L-arginine–nitric oxide system and blastocyst abnormalities may contribute to the early appearance of hypertension in the offspring of mothers submitted to significant food restriction during pregnancy [83–87].

Intrauterine undernutrition also increases the oxidative stress by affecting the activity of various enzymes. In a study of pregnant rats submitted to intrauterine undernutrition, Franco et al. [88] tested the participation of certain enzymes on radical generation and found that NADPH oxidase inhibition attenuated superoxide anion generation and ameliorated vascular function. Indeed, release of the superoxide anion in the kidney can be deleterious as it inactivates NO, resulting in excess Na reabsorption and enhanced TGF feedback and thus hypertension [89–91].

In addition, inactivation of NO with oxygen radical forms peroxynitrite which can nitrosylate tyrosine residues, causing renal damage and increasing renal vascular resistance [92–97]. Furthermore, studies have also shown that oxygen radical causes direct vasoconstriction in preglomerular vasculature and in the renal cortical and medullary circulation, and increases intracellular calcium in vascular smooth muscle and endothelial cells, causing renal vasoconstriction and renal damage [98–104]. Accordingly, Franco Mdo et al. [105] reported that treatment with vitamins C and E reduced oxidative stress and high blood pressure levels, and improved vascular function in intrauterine-undernourished rats.

Studies in which oxidative stress was experimentally induced, caused increases in oxidative stress and hypertension, providing strong evidence for either an initiating or a sustaining role of reactive oxygen species in hypertension [94, 98, 106–116]. In Sprague–Dawley rats that received a high Na diet for 8 weeks, a period which is much longer than that in the above-mentioned studies, the arterial pressure increased significantly, and urinary albumin excretion and renal inflammation increased, suggesting that hypertension develops slowly when Na intake is increased in normotensive rats, and the blood pressure elevations are paralleled by increases in ROS and renal damage [117]. Based on the aforementioned data, it seems that long-term exposure to intrauterine oxidative stress may cause renal inflammation, renal damage, and arterial pressure postnatally. Oxidative stress, inflammation, and arterial hypertension participate in a self-perpetuating cycle which, if not interrupted, can lead to progressive cardiovascular disease and renal complications [118].

Nephrotoxic Agents

The administration of nephrotoxic agents may seriously affect renal development when performed prior to completion of nephrogenesis. Nathanson et al. [119] examined the potential adverse effects of ampicillin, amoxicillin, and ceftriaxone in rat kidney development and reported that both penicillins altered renal development in a dose-dependent manner, while ceftriaxone weakly impaired in vitro nephrogenesis; at a dose of 1,000 mg/ml kidney development is completely blocked. In young animals exposed to penicillins in utero, a mild oligonephronia was present and cystic tubule dilation was observed in newborn and in young animals as well. Gilbert et al. [120] analyzed, in vitro, the potential direct effect of gentamicin on early nephrogenesis and found that gentamicin induced a significant reduction in the number of nephrons in metanephric explants and that this effect was more important on less differentiated metanephroi. Smaoui et al. [121–123] studied the effect of gentamicin on the renal handling and transport of proteins in proximal tubular cells and reported that gentamicin, entering the proximal tubular cells via the endocytic pathway, decreased the tubular reabsorption of proteins, thus increasing urinary protein excretion and, consequently, nephrotoxicity.

Other drugs which probably have major embryo-fetal toxic effects are the nonsteroidal anti-inflammatory drugs (NSAIDs) which cross the placenta, reach the fetal circulation, and cause a spectrum of changes in the kidneys of the offspring [124]. Hasan et al. [125] examined the hypothesis that early postnatal ibuprofen has less adverse effects on neonatal rat renal prostanoids, COX-2 expression, and angiotensin II than indomethacin in newborn rats and found that indomethacin exhibited more potent suppressive effects on renal COX-2 and vasodilator prostanoids which are important regulators of renal development and function. Kent et al. [126] studied the type of renal changes found on light and electron microscopy following administration of indomethacin, ibuprofen, and gentamicin in a neonatal rat model and reported vacuolization of the epithelial proximal tubules, interstitial edema, intratubular protein deposition but no significant glomerular changes. Moreover, they found pleomorphic mitochondria and loss

of microvilli in the tubules and extensive foot process effacement and irregularities of the glomerular basement membrane, concluding that these drugs cause significant change in glomerular and tubular structure in the neonatal rat model.

A number of studies have demonstrated the effect of angiotensin-converting enzyme (ACE) inhibition on systolic blood pressure and renal and uterine blood flow. Olsson et al. [127] studied the effects of intravenous captopril in goats during the last months of pregnancy and lactation and reported a more pronounced fall in arterial blood pressure and a larger increase in plasma renin activity during pregnancy when compared with the lactating period or with the nonpregnant state. It is quite interesting though that in a study evaluating the effect of the Renin Angiotensin System inhibition on the blood pressure and the mesenteric arteriolar reactivity of the intrauterine-undernourished rats, use of Angiotensin System inhibitors normalized the cardiovascular alterations induced by intrauterine undernutrition [128]. Blood pressure may be elevated in young rats following intrauterine exposure to a maternal low-protein diet in order to maintain glomerular filtration rate against a background of fewer nephrons via the increased expression of AT(1) receptors, which may arise as a result of the direct effect of protein restriction or in response to the reported decrease in renal tissue angiotensin II concentration [129].

Anatomical/Congenital Malformations

A number of animal models have been developed to study the pathophysiology of congenital hydronephrosis. These include ureteral obstruction in the fetal sheep, as well as in the postnatal opossum, pig, rabbit, and rodent [130–134]. In addition, the renal cellular and functional consequences of complete unilateral ureteral obstruction in the neonatal rat and mouse have been examined, which bear many similarities to human obstructive nephropathy [135, 136]. Based on previous studies showing that impairment of renal growth is directly dependent on the duration of temporary complete unilateral ureteral obstruction in the neonatal rat [137], Thornhill et al. [138] have recently developed a new model of variable partial unilateral ureteral obstruction in the neonatal rat that will aid in the elucidation of the mechanisms underlying the renal consequences of congenital ureteropelvic junction obstruction. The authors concluded that renal growth is impaired by a critical degree of partial unilateral ureteral obstruction. Persistent partial unilateral ureteral obstruction progressively reduces the number of nephrons during the period of nephron maturation (after the completion of nephrogenesis). Fixed partial ureteropelvic junction obstruction leads to a progressive dilatation of the renal pelvis and proximal ureter, tubular atrophy and interstitial fibrosis that are correlated with tubular apoptosis and are developed before detectable pelvic dilatation. Persistent moderate partial unilateral ureteral obstruction leads to a marked reduction in ipsilateral glomerular filtration rate and increased protein excretion before significant impairment of renal growth. Partial unilateral ureteral obstruction has a delayed stimulatory effect on adaptive growth of the contralateral kidney when compared to complete unilateral ureteral obstruction, and finally partial neonatal unilateral ureteral obstruction impairs somatic growth (Fig. 7.1).

Conclusion

Although the exact mechanisms governing renal development remain unclear, the increased use of animal models in renal basic science laboratories has extended our knowledge on this remarkable process. These promising results not only clarify many of the dark areas of nephrogenesis, but also they will boost the scientific efforts towards the elucidation of this phenomenon.

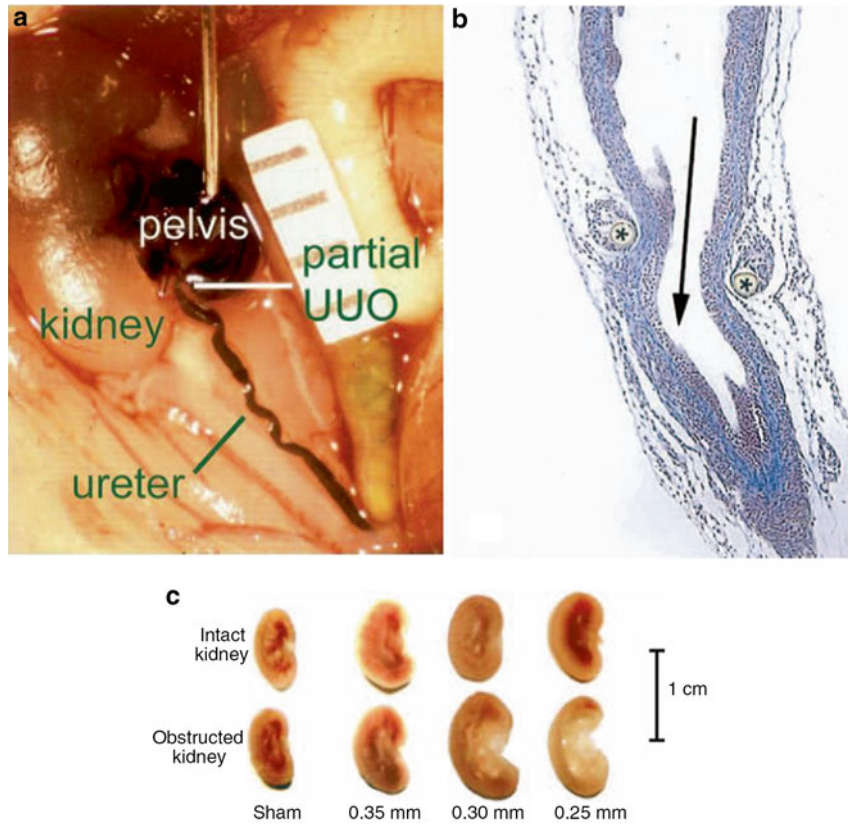

Fig. 7.1 (**a**) Fourteen-day-old rat with partial unilateral ureteral obstruction (UUO) at ureteropelvic junction (UPJ). Kidney is at upper left side, and renal pelvis has been filled with *India ink*, which traversed the obstruction and is visible in distal ureter (mm units on ruler). (**b**) Longitudinal section of ureter of 14-day-old rat showing the ureteral lumen (*arrow* indicates direction of urine flow) and cross-section of ligature (∗). The proximal ureter is dilated, and there is virtually no inflammatory response to the ligature. (**c**) Bisected representative kidneys from 14-day-old rats subjected to sham operation or partial UUO within the first 48 h of life. The contralateral kidney is shown on the *upper row* and the obstructed kidney on the *bottom row*. The luminal diameter of the partial obstruction is shown below each pair of kidneys (from [138] with permission)

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Do β-Thymosins Play a Role in Human Nephrogenesis?

8

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Introduction: The β-Thymosin Family

β-Thymosins are a family of ubiquitous peptides with a molecular mass of about 5 kDa and with a sequence of 40–44 amino acid residues [1]. The name thymosin derives from the first isolation of these peptides from calf thymus in 1966 by Goldstein et al. [2] among other lymphocytopoietic factors. Thymosins are subdivided into three main groups according to their different isoelectric points: α-thymosins, β-thymosins, and γ-thymosins with a pH below 5.0, between 5.0 and 7.0, and above 7.0 respectively. Hannappel and coworkers first isolated Tβ4 from vertebrate's and invertebrate's cells through different schemes of purification [3, 4]. More than 15 β-thymosins were described but Tβ4 is known to be the most expressed peptide in mammalians including humans [4, 5]. In water solution, β-thymosins are destructured and N- and C- terminal helixs are generated after addition in alcohol or binding to G-actin. Moreover, thanks to a flexible structure, β-thymosins can interact with different intra and extra cellular proteins [5].

Thymosin β4

Tβ4 is an ubiquitous peptide with very interesting multiple functions. The complete amino acid sequence of Tβ4 was described in 1981: it contains 43 amino acids, with a high proportion of lysyl and glutamyl residues [6]. The human Tβ4 gene (hTβ4) is located on chromosome X and comprises three exons and two introns [7]. The translation product is modified by removal of the N-terminal methionine and acetylation. Tβ4 plays pivotal roles in the cytoskeletal system as G-actin sequestering peptide, activity that can explain Tβ4 effects on regulation and differentiation of T lymphocytes [8], and inhibition of macrophage migration [9]. Tβ4 is leaderless: as a consequence, the mechanism of its release is completely unknown [1]. Tβ4 is considered the most abundant among β-thymosin peptides in mammalian tissues: its activity has been mainly related to the regulation of actin polymerization in living cells [10, 11].

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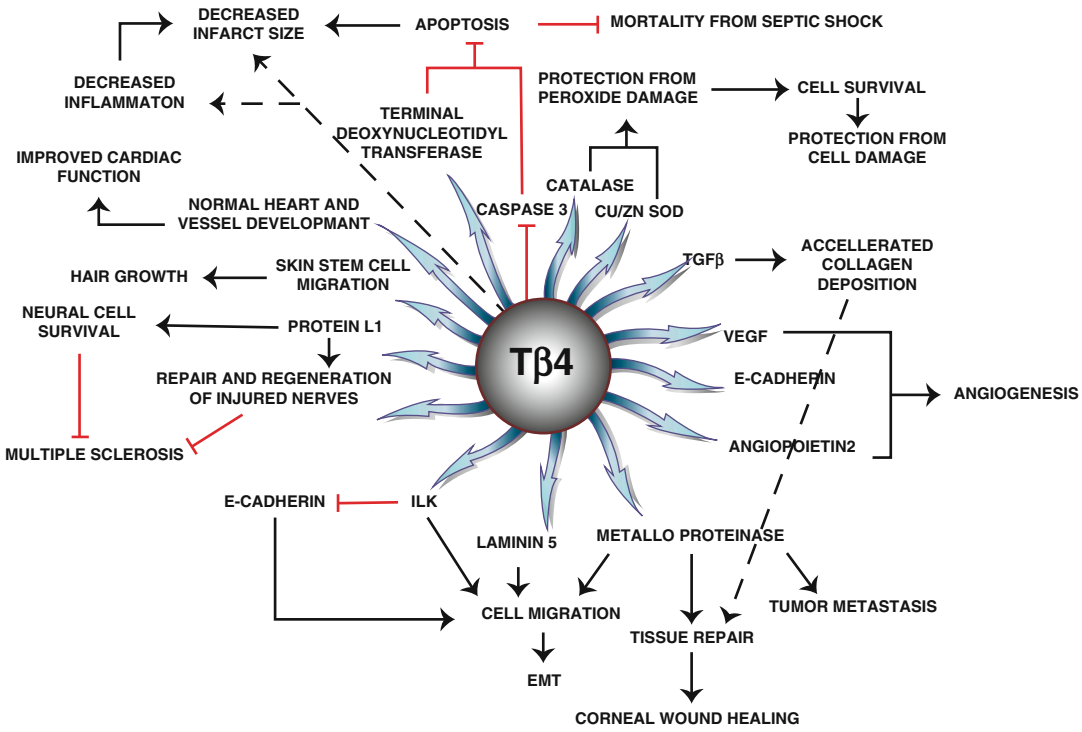

Fig. 8.1 Multiple biological functions of Tβ4

Tβ4 also exerts biological effects on hypothalamus and pituitary gland [12] and it is a potential precursor of seraspenide, the Ac-SPDK tetrapeptide corresponding to the N-terminal sequence of Tβ4 [13]. Seraspenide blocks hematopoietic pluripotent stem cells in the G0-phase, inhibiting their entry into the S-phase in vivo [14]. Tβ4 is also involved in many critical biological activities [15], including angiogenesis [16], wound healing [17], inflammatory response [18], and cell migration [19]. Furthermore, Tβ4 modifies the rate of spreading of endothelial cells on matrix components by inducing matrix metalloproteinases [20] and it is involved in the development and repair of heart [19] and brain damages [20] (Fig. 8.1). The presence of Tβ4 in human saliva and tears has been recently demonstrated by immunological techniques [21]: Tβ4 is highly expressed in saliva of human newborns, but not in saliva of adult subjects [22]. Tβ4 immunostaining was identified in acinar cells of the parotid, submandibular, and sublingual glands, as well as in minor salivary glands of fetuses, clearly indicating these cells as the source of Tβ4 in the saliva of human newborns [23].

Thymosin β10

Tβ10, a member of β-thymosins, was described for the first time in 1983 in mammalian tissues as a Tβ4 analogous [24, 25]. The Tβ10 gene is located on chromosome 2 and it consists in three exons and two introns. Tβ10 is a peptide with 43 amino acids and is located in the cytoplasm of different cell types. It plays a role in the modulation and organization of the cytoskeleton, by binding to G-actin. Thanks to this peculiarity, Tβ10 can interfere in cellular motility and proliferation [26]. There are many differences between Tβ4 and Tβ10: while Tβ4 promotes angiogenesis [27], Tβ10 inhibits it interfering with the Ras functions [28]. On the one hand, Tβ4 facilitates cellular migration through the production of metallopro-

teinases-2 and on the other hand Tβ10 inhibits endothelial cellular migration [29]. Tβ4 plays an important anti-apoptotic role preventing the apoptosis in cardiomyocytes [19] whereas hyperexpression of Tβ10 in cell lines of ovarian carcinoma enhances the process of apoptosis inhibiting tumoral growth [30]. Tβ10 has been reported to be over-expressed in human carcinogenesis [31], in carcinoma of the thyroid [32], in breast tumors [33], in lung carcinoma [34], in renal carcinoma [35], and in pancreatic tumors [36]. Tβ10 plays a critical role during human embryogenesis in multiple organs, including the central nervous system [22, 37, 38]. Interestingly, the high levels of Tβ10 found in human fetal brain were reported to drop rapidly after birth [39], suggesting a specific role for Tβ10 during human brain development [40, 41]. The role of Tβ10 during embryogenesis of neural cells was subsequently confirmed by studies showing its participation in neurite outgrowth [42]. Taken all together, these data suggest that Tβ10 is specifically implicated in the development of brain and nervous tissues. However, detailed expression patterns of Tβ10 in different cells and tissues of the human embryo and newborn, at the best of our knowledge, are not available.

Thymosin β10 in Fetal Salivary Glands

Tβ10 and Tβ4 are detectable in high concentration in whole saliva of human preterm newborns, while it disappeares in adults. On the basis of these data, it seemed of interest to study even the influence of Tβ10 during the development of the human salivary glands. To this end, we analyzed, using immunohistochemistry, the expression of Tβ10 in samples of the major and minor salivary glands obtained, at autopsy, from human fetuses and newborns, ranging from 13 up to 33 weeks of gestation. Tβ10 immunoreactivity was detected in all salivary glands examined, with marked differences from one gland to the next. The parotid glands showed the highest Tβ10 reactivity while the lowest reactivity was detected in the minor salivary glands. Marked changes were observed in Tβ10 expression and localization during embryogenesis. In particular, Tβ10 was mainly localized extracellularly in the youngest human fetuses (13 weeks), in the cytoplasm of immature duct cells at 20 weeks, in acinar cells, and in the duct lumen in 33 weeks old fetuses. For the first time we showed a strong expression of Tβ10 in the human salivary glands during the initial phases of the physiological development, Tβ10 being detected starting from the 13th week of gestation, and suggesting a role for the peptide in the salivary glands' organogenesis (37) (Fig. 8.2).

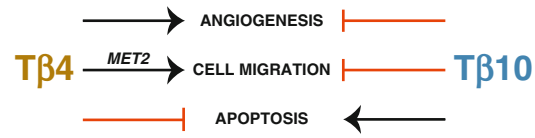

Fig. 8.2 Main antithetic functions of Tβ4 and Tβ10

The Role of β-Thymosins in Embryogenesis

First works on β-thymosins in human tissues focused on the role of Tβ4 in embryogenesis with the following aims: to identify whether the pattern of Tβ4 expression might change at different gestational ages during the intrauterine life, to control whether the Tβ4 expression pattern could change in neonates and adult subjects. To this end, we analyzed parotid, submandibular, sublingual, and minor salivary gland tissue samples obtained from human fetuses of different gestational ages. Immunohistochemical studies clearly demonstrated the presence of two main protein reactivity patterns: a granular pattern, observed in the cytoplasm of acinar cells, inside the ductal lumen, and in the connective tissues surrounding the epithelial structures; a diffuse pattern, characterized by the homogeneous staining of the entire cytoplasm, mainly detected in ductal cells [23]. We hypothesized that the granular immunoreactivity could be related to Tβ4 secretion in two ways: at the apical pole of acinar cells into saliva, in which the peptide is present in high quantities

[18, 44] and at the basolateral pole into the connective tissues, in which the peptide could have autocrine or paracrine functions. The homogeneous cytoplasmic pattern, mainly found in the ductal cells of adult salivary glands, was interpreted as characteristic of the binding of Tβ4 to G-actin monomers [23]. Moreover, when we analyzed immunoreactivity for Tb4 in tumors originating from salivary glands we detected the peptide in the vast majority of neoplasias studied. In particular, a strong expression for the peptide was detected in mixed tumors of salivary glands, being found in the cytoplasm of myoepithelial tumor cells and in Warthin tumor cells. Our data collectively suggest that Tβ4 expression in human salivary glands may be summarized by: (1) a strong reactivity in fetal glands; (2) marked decrease in expression in adult glands and (3) re-expression in tumor progression. Moreover, in some salivary gland tumors, a great number of intra- and peritumoral mast cells were observed, all characterized by a strong immunostaining for the peptide [45]. These findings indicate a role for Tβ4 not restricted to the physiological development of salivary glands, but also in cancer development and progression, likely due to the utilization of fetal programs by salivary gland cancer cells. In line with this hypothesis, the observation of Tβ4-rich tumor-infiltrating mast cells in salivary gland tumors underscores the hypothesis that this peptide could serve as a local paracrine mediator, with a relevant role in cellular cross-talking within the tumor microenvironment [46]. Concerning the function of Tβ4 in neoplastic cells, this peptide has been shown to have antiinflammatory and cytoprotective functions by suppressing secretion of the proinflammatory cytokine IL8 and by protecting cells against TNF-induced apoptosis [47] (Fig. 8.1) and by inhibiting neutrophil infiltration and decreasing the expression of proinflammatory cytokines [48]. Because of its multifunctional roles in protecting cells against apoptosis [22, 48] and in stimulating neoangiogenesis [49], Tβ4 released by tumor cells and/or by mast cells in the tumor microenvironment could significantly contribute to cancer cell survival and diffusion. As a consequence, Tβ4 might represent a new molecular target to be considered for future antitumor strategies in different human tumors [50]. The finding of strong expression of Tβ4 in the cytoplasm of tumor-infiltrating mast cells [45] extends our knowledge regarding the immunophenotypic profile of mast cells and contributes to our understanding of immune cells/cancer cells cross talk. Tβ4 has been suggested as the ideal actin monomer sequestering protein [51]. Its function was first restricted to regulate actin polymerization of non-muscle cells, with multiple effects on cell surface remodeling and motility [52]. Further data suggesting a role of Tβ4 in modulating stem cell migration [49], activation [53], and inhibition [54], as well as in regulating integrin signaling [55] prompted some authors to speak of the "thymosin enigma." [56]. The theory on the putative role of Tβ4 in the physiological development of embryos, as well as in vascularization and tissue recovery in acute and chronic ischemia, was reinforced by the discovery that Tβ4 is one of the most abundant factors secreted by embryonic endothelial progenitor cells [57]. Data suggesting a role for Tβ4 in the recruitment of stem cells in different organs, and in particular during the embryonic and fetal development, prompted us to investigate the expression of the peptide in human fetuses and embryos of different gestational ages, assessing a potential role of Tβ4 in the development of the different components of the gastrointestinal tract [58]. Moreover, we analyzed samples from gut, liver, and pancreas in order to study Tβ4 expression. Tβ4 was highly expressed in the epithelial cells during the early phases of the development, both in gut and pancreas, confirming previous studies indicating a possible relevant role of Tβ4 in the development of the gastrointestinal tract. For the first time, a marked heterogeneity of Tβ4 expression within the gastrointestinal tract was found, ranging from a diffuse immunoreactivity for the peptide in pancreas and gastrointestinal cells to the absence of the protein in the vast majority of fetal and newborn livers examined. Moreover, we detected marked differences in Tβ4 expression among different cell types within the single organs. The most striking differences were found in the fetal pancreas: Tβ4 immunoreactivity was

strong in the endocrine cells of the Langerhans islets, in the absence of any significant reactivity in exocrine acinar and ductal cells. Interindividual differences were also reported regarding the intensity of the immunoreactivity for Tβ4 and its subcellular localization, primarily related to the different gestational age of the subjects studied. The strong positivity of Tβ4 in multiple cell types of the developing gastrointestinal tract in humans suggests a relevant role for the peptide in human physiological development. When Tβ4 expression pattern was analyzed in the adult gastrointestinal tract, we observed a marked decrease in immunoreactivity for the peptide. In particular, enterocytes of the ileum and colon did not show any significant reactivity for Tβ4. The pattern of immunostaining for Tβ4 in the adult pancreas appeared similar to that described in fetal pancreas. On the contrary, significant changes were detected in the adult human liver: Tβ4 was highly expressed in the vast majority of adult hepatocytes, with a preferential localization in the hepatocytes bordering the terminal veins (zone3 of the acinus) [59].

Thymosin β10 Expression in Human Nephrogenesis

The report that the Tβ10 is expressed at high levels in embryonic human tissues as well in human kidney induced us to study Tβ10 reactivity in the preterm kidney in order to verify the immunoexpression of this peptide during renal embryogenesis [37]. To this end, we analyzed by immunohistochemistry, the expression of Tβ10 in samples of human kidney obtained, at autopsy, from fetuses and preterm infants ranging from 25 to 36 weeks of gestation and at term newborns. Tβ10 immunoreactivity was detected in the majority of kidneys examined. In all kidneys, immunostaining for the peptide was mainly restricted to proximal and distal tubules. Tβ10-positive tubular cells showed a diffuse cytoplasmic immunoreactivity, in the absence of significant intraluminal reactivity. Occasionally, even nuclei of tubular cells showed a mild reactivity for the peptide. No significant immunoreactivity was observed in the collecting ducts. The glomerular compartment was mainly excluded by Tβ10 localization with the vast majority of glomeruli being completely negative. In half of kidneys examined we detected scattered reactive cells inside the glomerular tufts. Nevertheless, in all preterms older than 29 weeks of gestation, glomeruli were completely negative. The extent and the intensity of immunoreactivity for Tβ10 in proximal and distal tubular cells changed from one case to the next. Immunostaining for Tβ10 was also observed in the subcapsular regions, in areas of active glomerulogenesis in half of cases observed. In this area, the reactivity for the peptide was mainly granular, and localized in the cytoplasm of the comma- and S-shaped bodies. Even in the zones of active glomerulogenesis, developing collecting tubules did not show any reactivity for the peptide. The adult kidney, utilized as a control biopsy, showed reactivity for Tβ10 restricted to the cytoplasm of proximal and distal tubules. No reactivity was detected in the glomeruli. In that study we added some new data, showing that Tβ10 is highly expressed in the developing human kidney, being localized in the "comma-shaped bodies" and in the "S-shaped bodies" during the earliest phases of glomerulogenesis and in ductal cells in mature nephrons. Interestingly, reactivity for Tβ10 disappeared in the "S-shaped bodies" when glomerulogenesis started, with the generation of the primitive vascular tuft by vascular cells. Immunostaining for Tβ10 was more often absent in the glomeruli during their maturation, only scattered positive cells being found in half of cases. These data confirm even in the human kidney the selective localization of β-thymosins during development and the restriction of their immunoreactivity to specific peculiar structures and cells, with marked differences from one organ to the next. In the developing kidney, the marked preference of Tβ10 for the proximal and distal ductal structures, from their origin from the "S-shaped bodies" to the developed proximal and distal ducts, observed in this study is peculiar and does not parallel any previously reported reactivity for the peptide in other organs. The reason for this localization and the intimate function of Tβ10 during the different phases of

kidney development remain, at the best of our knowledge, unknown. We showed, for the first time, a marked heterogeneity of Tβ10 expression among glomerular and tubular structures, ranging from a diffuse immunoreactivity for the peptide in the proximal and distal tubuli to the absence of Tβ10 immunostaining in the vast majority of glomeruli. Marked interindividual differences are also present in Tβ10 expression at tissue level, regarding the intensity of the immunoreactivity for Tβ10 and its localization, even in fetuses and newborn with the same gestational age, suggesting the presence of additional factors which might influence the expression of the peptide in the developing kidney.

Thymosin β4 Expression in Human Nephrogenesis

In order to verify if Tβ4 was involved in human nephrogenesis, immunoreactivity for this β-thymosin was performed in a series of fetal and newborn kidneys, ranging from 17 up to 38 weeks of gestation. The aim of our work was to verify if: (1) Tβ4 was expressed in the developing kidney; (2) Tβ4 was detectable in the same renal structures in which Tβ10 was previously observed; (3) the expression pattern of Tβ4 might change in the different phases of gestation. Here the preliminary results of our study are reported. These preliminary data show that, contrary to Tβ10, Tβ4 is not mainly expressed in the epithelial components of the developing kidney. In particular, in all kidney samples immunostained, Tβ4 reactivity was very weak or completely absent in all cell types of the nephrogenic zone in the subcapsular areas. Moreover, Tβ4 did not mark any cell component of developing glomeruli, of proximal tubules, and of collecting tubules (Fig. 8.3). Regarding the different segments of renal tubules, only in few cases anti-Tβ4 antibodies immunostained distal tubules (Fig. 8.4) and Henle loops (Fig. 8.5).

Contrasting with the absence of reactivity in the outer cortex, Tβ4 appeared strongly expressed at the renal hilum. In the perihilar regions the peptide appeared restricted to the mesenchymal/stromal cells, i.e., in the intersitium of the renal medulla (Fig. 8.6). Some peculiar zones appeared characterized by a stronger expression of Tβ4. The highest levels of Tβ4

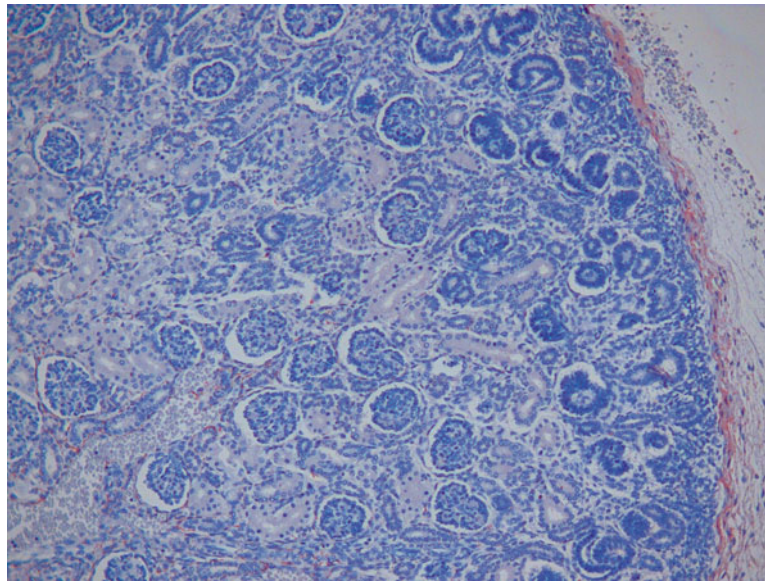

Fig. 8.3 A strong immunoreactivity for Tβ4 is detected in cells of the renal capsule. Developing glomeruli, proximal tubules, and collecting tubules are not reactive for the peptide

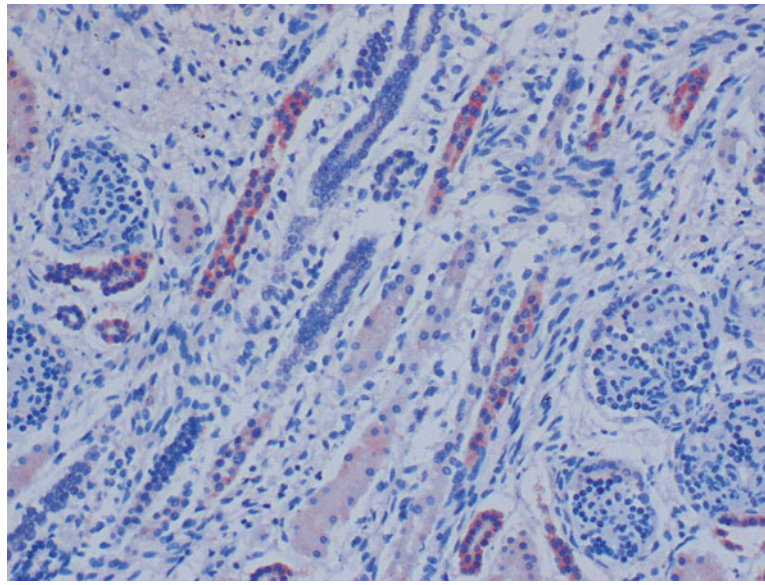

Fig. 8.4 Tβ4 shows a homogeneous immunoreactivity in the distal tubules

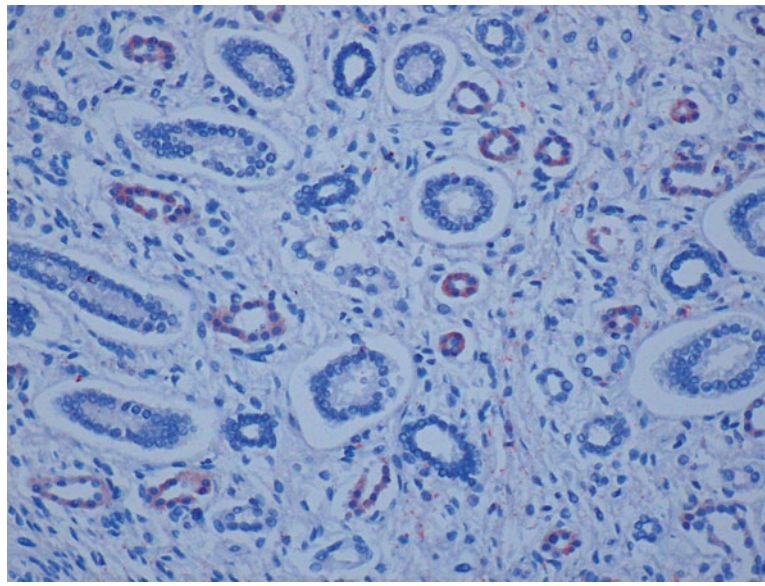

Fig. 8.5 A homogeneous activity for Tβ4 is observed in the Henle loops. Collecting tubules do not show any reactivity for the peptide

immunoreactivity were frequently found in the stromal cells encircling the ureter (Fig. 8.7). In the ureteral wall, the negativity of the transitional epithelium contrasted with the high levels of Tβ4 expression in the majority of cells giving rise to the ureteral wall (Fig. 8.8). The second preferential location of Tβ4 reactivity was the arterial wall: in particular, Tβ4 was highly expressed in cells of the outer layer of arteries (Fig. 8.9). Undifferentiated mesenchymal stromal cells of the renal medulla often showed Tβ4 immunoreactivity, appearing as small cytoplasmic

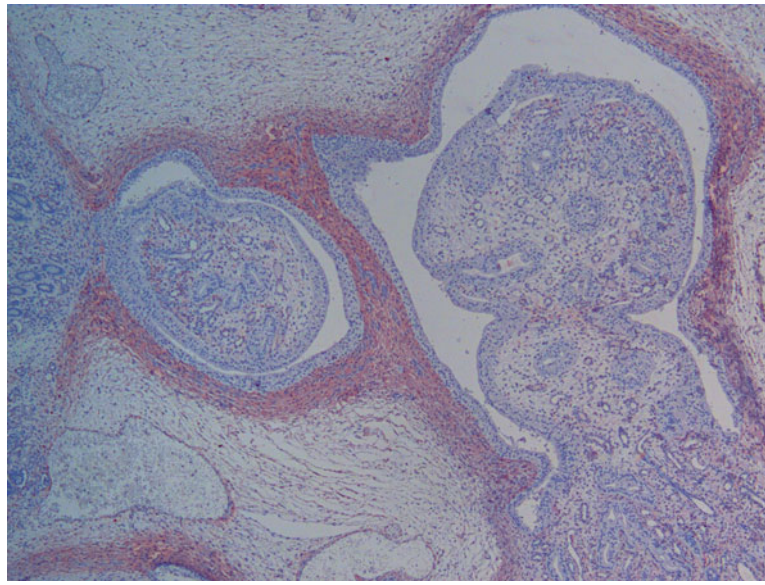

Fig. 8.6 A strong immunoexpression for Tβ4 is detected at the renal hilum

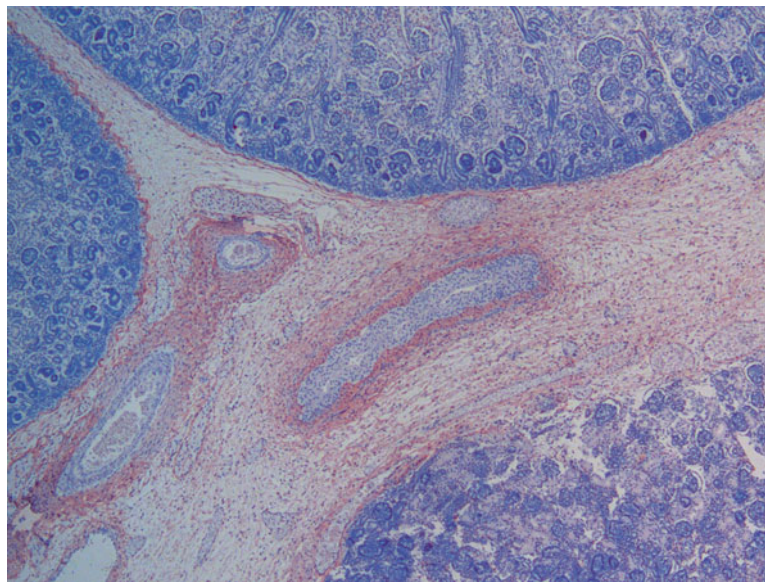

Fig. 8.7 An intense immunoreactivity for Tβ4 is observed in the cytoplasm of the stromal cells encircling the ureter and renal artery branches

granules, contrasting with the absence of any reactivity for the peptide in the collecting tubules (Fig. 8.10). The expression of Tβ4 in the renal cortex was less evident at panoramic views. At higher power, Tβ4 expression appeared restricted to the cortical-stromal interstitial cells.

The following compartments were mainly immunoreactive for Tβ4:

1. The Bowman capsule cells were frequently encircled by a thin Tβ4-reactive line. Occasionally, Tβ4 was also expressed in the cytoplasm of capsular cells (Fig. 8.11).

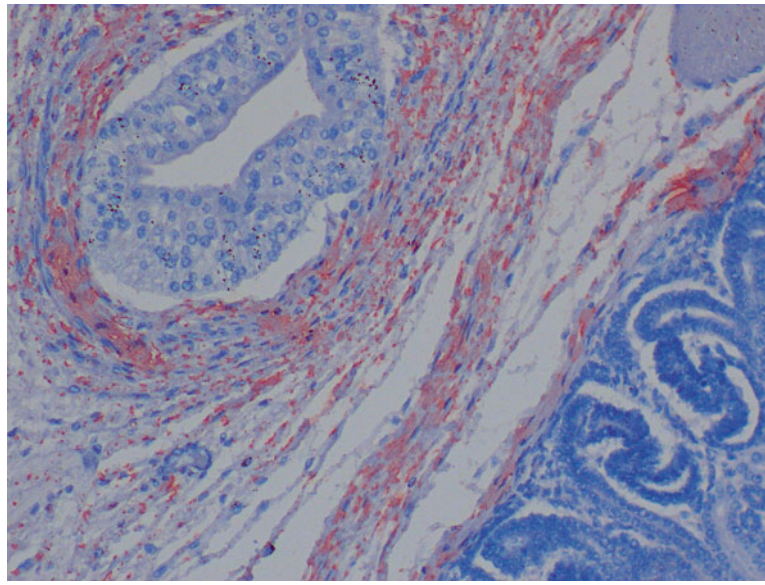

Fig. 8.8 Coarse cytoplasmic granules immunoreactive for Tβ4 are detected in the majority of cells of the ureteral wall. The transitional epithelium is negative. A diffuse immunoreactivity for Tβ4 is observed in the stromal cells surrounding the renal capsule

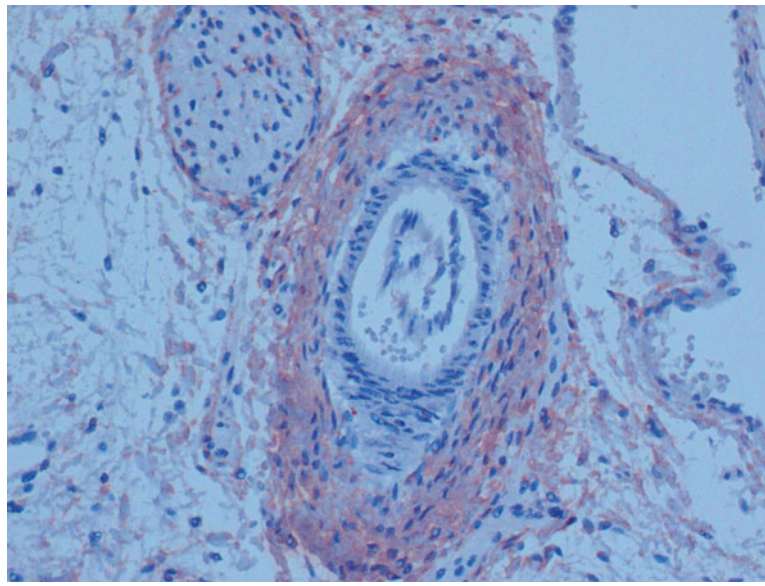

Fig. 8.9 A diffuse cytoplasmic reactivity for Tβ4 is detected in cells of the outer layer of the arterial wall. Immunoreactivity for Tβ4 is also detected in the stromal cells surrounding nerves and in the mesenchymal stroma

2. Tβ4 frequently marked the basal lamina of distal tubules, appearing as a thin line encircling epithelial tubular cells (Fig. 8.11).
3. Interstitial cortical cells, located among glomeruli and tubuli, frequently showed immunostaining for the peptide, appearing as granular deposits in the cytoplasm of stromal cells (Fig. 8.12).
4. A strong reactivity for Tβ4 was constantly detected in cells of the renal capsule.

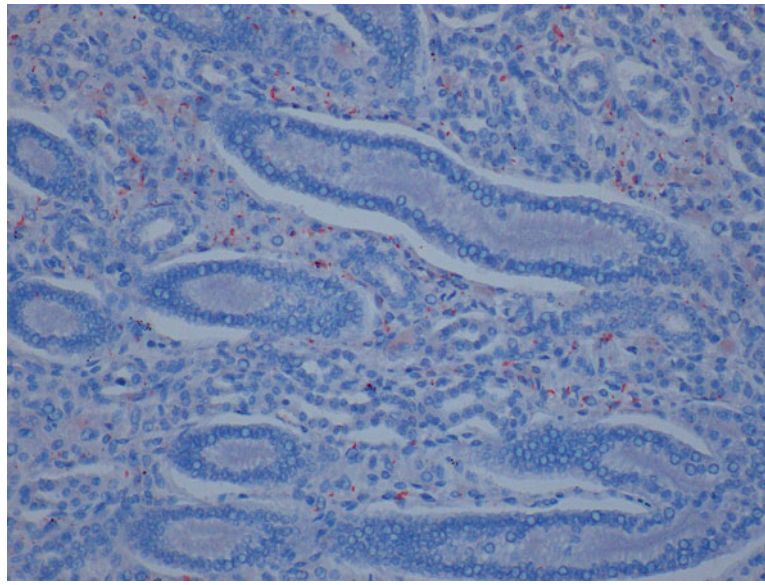

Fig. 8.10 Mesenchymal stromal cells of the renal medulla show immunoreactive for Tβ4. No reactivity for the peptide is detected in collecting tubules

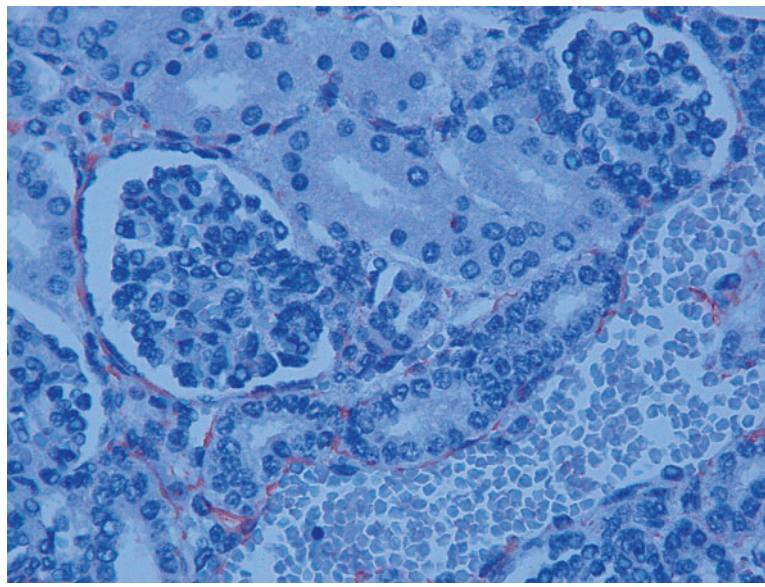

Fig. 8.11 Tβ4-reactive cells encircle distal tubules and Bowman capsule cells

Conclusions

Tβ4 and Tβ10 are both involved in human nephrogenesis, being detected in fetal and neonatal kidney at different gestational ages. The most interesting finding emerging from our immunohistochemical studies is represented by the restriction of these two β-thymosins to different kidney compartments. Tβ10 appears to be mainly involved in the early phases of differentiation of the proximal nephron lineage, being expressed in the S-shaped bodies.

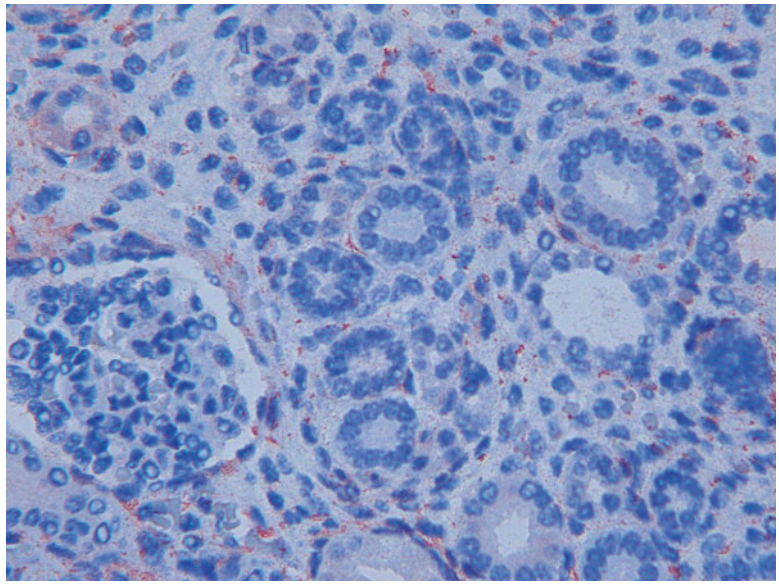

Fig. 8.12 Granular deposits in the cytoplasm of stromal cells are detected around glomeruli and tubuli

Moreover, Tβ10 was also expressed in proximal tubular cells. Contrasting with the prevalent "epithelial immunoreactivity of Tβ10, Tβ4 was mainly expressed in cells of the non-nephron lineage and, in particular, in the stromal–interstitial cells located in the cortex and in renal medulla. According with these data, Tβ4 appears as an important factor involved in the differentiation of the multiple (and in part unknown) cell types of the stromal lineage during kidney development. From a practical point of view, given the scarcity of immunohistochemical markers useful for the identification of cortical and medullary stromal cells, we suggest that Tβ4 might be utilized in the study of the interstitial component of the fetal and the newborn kidney. Expression of Tβ4 by two epithelial components, the cells of the Henle loops and the cells of the Bowman capsule, adds new data to confirm the "Thymosin enigma" [56]. In conclusion, our data evidence that Tβ4 and Tβ10 are both involved in human nephrogenesis but that their expression is restricted to different cell compartments: Tβ4 to the stromal/interstitial cells, and Tβ410 to the nephron lineage [57–59]. Further studies are needed in order to better clarify the relationships between these two β-thymosins during the different phases of kidney development, with the purpose to better defining the role of these peptides during human kidney development.

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Malnutrition and Renal Function

9

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The in utero environment which is extremely susceptible to maternal influence plays an important role in the fetal growth and development. Maternal metabolic and endocrine function placental function as well as maternal diet can have critical effects on various aspects of developing structures and functions of the fetus [1]. Both undernutrition and overnutrition can be classified as malnutrition because these two extremes of nutrition are commonly characterized by: (1) imbalances of nutrients (e.g., amino acids, vitamins, and minerals); (2) elevated levels of cortisol in blood; and (3) oxidative stress [2]. Malnutrition (nutrient deficiencies or obesity) in pregnant women adversely affects the fetal health by causing or exacerbating a plethora of problems, such as anemia, maternal hemorrhage, insulin resistance, and hypertensive disorders (e.g., pre-eclampsia/eclampsia). Maternal malnutrition during gestation impairs embryonic and fetal growth and development, resulting in deleterious outcomes, including intrauterine growth restriction (IUGR), low birth weight, preterm birth, and birth defects (e.g., neural tube defects and iodine deficiency disorders).

Undernutrition

In October 2010, the United Nations Food and Agriculture Organization reported that 925 million people worldwide, including a large proportion of women of reproductive age, suffered from hunger; nearly all of the undernourished reside in low- and middle-income countries [3]. Deficiencies of protein, vitamin A, iron, zinc, folate, and other micronutrients remain major nutritional problems in poor regions of the world. Furthermore, suboptimal nutrition may result from short interpregnancy intervals (<18 months) which are associated with miscarriage, IUGR, and preterm delivery [4, 5]. The human fetus is very sensitive to malnutrition. Because of ethical concerns, animal models have often been used to gain clarification on how the timing of exposure modifies the effects of maternal undernutrition on fetal development. Results from these studies indicate that deficiencies of energy or macronutrients during the first trimester of pregnancy have a greater detrimental effect on fetal development than during late gestation [6]. Regarding specific nutrients, the animal studies

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suggest that the embryo/fetus is most vulnerable to maternal deficiency of protein or amino acids during the peri-implantation period and the period of rapid placental development. Furthermore, the early to mid gestation is the critical period when a deficiency of micronutrients (vitamins and minerals) has greatest adverse impacts on fetal growth and development [7].

The development of the fetal kidney is strongly sensitive to malnutrition. Many studies have shown that perturbed maternal nutritional status alters renal renin protein and mRNA levels, as well as renal angiotensin II (AngII) concentration [8] and changes renal expression of AngII receptors and mitogen-activated protein kinase (MAPK) in the pups [9], resulting in higher blood pressure and structural changes in the kidney of adult offspring. Specific studies with maternal protein restriction during gestation have shown different modulations of RAS components. Sahajpal and Ashton [10] showed that offspring of mothers that received a low-protein diet during the entire gestational period presented, at 4 weeks of age, fewer glomeruli per g kidney weight and the AT1R protein level was 24 % greater in low-protein pups when compared with normally nourished pups. Mesquita FF et al. recently found that AT2R protein is down-regulated in kidneys of 16-week-old rats born to females that received a low-protein diet throughout gestation. These animals presented a total absence of AT2R in the glomeruli and this receptor was localized preferentially associated with intercalated cells of the distal and collecting segments. They also found that podocytes appear to be larger and crushed in low-protein rats, suggesting that changes in renal functions favor excess hydroelectrolyte reabsorption by the kidney, and as such might potentiate the programming of adult hypertension. These morphological changes could be attributed to an adaptation to the reduced nephron number and, consequently, to glomerular hyperfiltration and overflow in low-protein offspring, and could account for the breakdown in optimal glomerular filtration barrier function [11]. Alwasel and Ashton in a study of renal function and Na^+ transporters in 4-week-old male rats born to low-protein diet dams found that glomerular filtration rate (GFR) was unchanged, suggesting that single nephron GFR may be increased. In addition, these investigators reported that Na^+ transporter protein was unchanged, while the Na^+/K^+-ATPase-α1 subunit was absent in the kidney of low-protein rats. Interestingly, in a Western blot study, they found also that, at the end of nephrogenesis, the β1 subunit of the Na^+/K^+-ATPase protein was down-regulated in male low-protein rats, but that at 16 weeks of age the protein was increased in these animals when compared with the normal group [12].

The RAS balance is disrupted in kidneys from offspring of low-protein-fed mothers during different ages, and AngII receptors are expressed in all segments of the nephron, contributing to the increased sodium re-absorption in proximal tubules and to the final sodium excretion rate [13]. The low urinary sodium excretion data found by Mesquita et al., may also be explained by previous findings showing that excess fetal glucocorticoid, such as observed in maternal undernutrition status programs, reduced the expression of placental, central nervous system, and renal 11β-HSD. A programmed reduction in renal 11β-HSD would be expected to increase the ability of glucocorticoids to activate in many different tissues both glucocorticoid receptors and mineralocorticoid receptors, resulting in increased transcription of both, with a consequent increase in blood pressure.

We should consider that nephrogenesis is a complex process involving cell integration, cell growth, and apoptosis and that the rapid remodeling of structures requires massive apoptosis. Bcl-2 is an antiapoptosis protein that attenuates the effect of cytochrome *c* release from the mitochondria and counters the effects of the pro-apoptosis protein, Bax. Using real-time PCR in E13.0 metanephroi, Welham et al. [14] observed a step-wise increase in the expression of both Bax and Bcl-2 in the low-protein-fed mothers group. The increase was greater for the pro-apoptotic gene, Bax, versus the anti-apoptotic gene, Bcl-2, perhaps suggesting that a low-protein diet shifts the balance of expression to up-regulate the death of metanephric precursor cells. Alterations in DNA methylation and histone

acetylation in IUGR rats [15] suggest a molecular mechanism by which a low-protein diet and IUGR induce fetal renal apoptosis, with a resulting permanent loss of glomeruli.

Brennan et al. investigated on the effects of maternal nutrient restriction (NR) on the growth hormone–insulin-like growth factor (GH–IGF) axis because it is known to play an important role in kidney development [16]. Both the GH receptor and IGF-I are expressed at very high levels in the developing kidney: IGF-I is first detectable at approximately gestational day 11 in the rat, rising 8.6-fold during the subsequent 48 h and this timing coincides with that of metanephric differentiation, indicating that IGF-I may play a primary role. As well as a role in kidney development there is evidence to suggest that the GH–IGF axis can also affect kidney function. The fetal and adult GH–IGF axis is nutritionally sensitive. For example, NR affects IGF-I and -II expression in both the liver and skeletal muscle [17], whilst kidneys from growth-restricted sheep fetuses have greater sensitivity to GH infusion than normally growing controls [18].

Other important regulators of cell proliferation, differentiation, immune function, and apoptosis are vitamin A and its analogues (retinoids). Vitamin A is the determinant in fetal renal programming of rats in view of its capacity to modulate nephron number and vascular supply of the kidney [19]. Furthermore the role of c-ret in renal formation is considered essential since null mice for this gene exhibited renal agenesis or rudimental kidneys [20]. In conditions of vitamin A deprivation, proto-oncogene c-ret expression was decreased in the metanephron. However, vitamin A supply restored nephron endowment to normal in offspring of rat mothers exposed to protein restriction [21]. In a recent survey in a developing country of Africa, about 40 % of children with chronic renal failure had an uncertain etiology and environmental factors were advocated [22]. Close monitoring of renal function of children exposed to intra utero undernutrition emphasized the importance of nutritional programmes in populations with high risks of undernutrition [23].

Overnutrition

While maternal undernutrition has substantial implications for maternal and fetal health, overweight and obesity in pregnancy similarly increase the risk of poor health outcomes [24]. Currently one billion adults, worldwide, are overweight and more than 300 million are obese. Given the increasing epidemic of overweight and obesity in low-, middle- and high-income nations, overnutrition has emerged as a major public health problem globally. Approximately 60 % of women desiring pregnancy in the US are overweight by the body mass index (BMI) criterion, with incidence rising exponentially over the past 15 years [25]. Overweight and obese women experience poorer reproductive outcomes than normal weight women, including increased rates of infertility and pregnancy loss, as well as fetal and neonatal problems such as developmental delay and neurological deficits, respectively. Maternal obesity or overnutrition before or during pregnancy may result in IUGR and increased risk of neonatal mortality and morbidity, as well as adverse maternal health including insulin resistance, maternal hemorrhage, and hyperglycemia [26]. Currently one billion adults, worldwide, are overweight and more than 300 million are obese. Given the increasing epidemic of overweight and obesity in low-, middle-, and high-income nations, overnutrition has emerged as a major public health problem globally. Obese women at any period of gestation may experience a metabolic syndrome that is characterized by elevated levels of glucose, hyperinsulinemia, hyperlipidemia, hypertension, and insulin resistance [27].

With regards to timing of overnutrition, evidence shows that maternal obesity before or during pregnancy has negative impacts on fetal growth and development. However, the presence of obesity in the first trimester of gestation appears to be most detrimental to embryonic/fetal survival and growth. Interestingly, obese mothers who lose weight during part or all of the gestation have increased risk for IUGR. This apparent paradox may be explained by: (1) ketosis resulting from the mobilization of white fat stores and partial

oxidation of fatty acids in the liver; (2) deficiencies of nutrients (e.g., glutamine and micronutrients) in the fetus because of increased utilization by maternal tissues (e.g., kidneys) and impaired uteroplacental blood flow; and (3) elevations of cortisol that inhibit fetal protein synthesis [24]. IUGR and preterm birth contribute to high rates of neonatal morbidity and mortality. Major common mechanisms responsible for malnutrition-induced IUGR and preterm birth include: (1) abnormal growth and development of the placenta; (2) impaired placental transfer of nutrients from mother to fetus; (3) endocrine disorders; and (4) disturbances in normal metabolic processes.

Jugheim et al. demonstrated the adverse effects of diet-induced maternal obesity on oocyte maturation and embryonic IGF-IR expression, which manifest later as differences in offspring size, growth patterns, and metabolic parameters. The metabolic differences, specifically percent body fat, growth rate, glucose intolerance, and elevated cholesterol levels, suggest that the offspring, males in particular, are predisposed to develop metabolic syndrome [28].

Women who are overweight or obese have increased reduced rates of breast feeding initiation and earlier termination [29]. The role of extreme maternal BMI category, specifically women who have a BMI ≥35 or ≥40, should be further evaluated in future studies, particularly given the rising percentage of women in the United States and internationally who fall into this category [30]. Biological data suggest that adipose tissue changes as obesity becomes more severe, with histological changes as well as changes in endocrine and paracrine secretion. In some studies reviewed, obese and, in some cases, extremely obese women were more likely to have failure to initiate breast feeding or reduced duration of breast feeding in comparison with overweight or normal weight women [31]. Of the group of mothers with a BMI of 20–25, 89.2 % (95 % confidence interval (CI) 87.4–91.0) initiated breast feeding, compared with 82.3 % (95 % CI 77.6–87.0) of mothers with a BMI of 30 or more. There is also a significant difference between the mean and median duration of breastfeeding of obese and non-obese mothers (BMI 30 and over, <25, respectively) [32].

Intrauterine Growth Restriction

According to the 2001 classification of the American College of Obstetricians and Gynecologists, IUGR is defined as an estimated fetal weight (EFW) 10th percentile [33], while SGA is defined as birth weight and/or length at least 2 SD below the mean for gestational age [34]. Growth retardation in utero is not a specific disease caused by a single etiology, but the result of the simultaneous presence of multiple and diverse maternal and fetal abnormalities. Among the factors that determine fetal growth distinguish intrinsic ones, or fetal and extrinsic ones, both maternal and placental. In particular, IUGR is not a specific entity but it is the result of idiopathic, maternal, fetal, and placental disorders. The management and neonatal outcome depends in part on the etiology of delayed fetal growth and thus is always important to try to identify it, but this makes it possible in only 40 % of IUGR fetuses. In the remaining 60 % of cases the factors etiologic known to date do not appear to be involved: in this case idiopathic IUGR fetuses. Fetal factors may contribute up to 15–20 % of IUGR [35].

Maternal causes associated with a decrease in uteroplacental perfusion are responsible to account for 25–30 % of all IUGR cases [36, 37]. The placental factors explain 25–40 % of cases of growth retardation and include: inadequate trophoblastic invasion of the endometrium, multiple placental infarcts, and abnormalities in size or shape of the placenta. It was observed that the placenta of IUGR fetuses is smaller than the normality and that a ratio of placental weight greater than the value of 10 can be considered an index diagnosis of IUGR, irrespective of fetal weight. Other maternal causes are represented by nutritional abnormalities such as women with bowel inflammatory disease. Maternal substance abuse *i.e.* smoking, alcohol, and drug consumption is also correlated with IUGR.

Low birth weight, caused either by preterm birth or by IUGR, or a combination of both, has been associated with higher blood pressure (BP) in childhood, adolescence, and adulthood in a large number of epidemiological and clinical studies on cardiovascular risk [38, 39].

High BP is part of the metabolic syndrome, and because associated diseases, such as atherosclerosis, coronary heart disease, and stroke, have also been linked to low birth weight, it is generally conceived that these conditions share antenatal risk factors [40]. This fact is relevant, considering that low birth weight is a complication in 10 % of all pregnancies in the Western world. The mechanisms whereby slowed intrauterine growth confers vascular risk are not clearly understood, but fetal events might result in any arterial abnormalities early in the intrauterine life [41]. Newborn babies with growth restriction were found to have significant aortic thickening, a preclinical marker of atherosclerosis, [42] which suggested that prenatal events might predispose these infants to later cardiovascular risk. Therefore, early endothelial dysfunction and intima–media thickening may significantly contribute to the premature stiffening of the aortic tree, which ultimately predisposed these individuals to hypertension [43]. Zanardo et al. studied aortic Intima–Media Thickness (aIMT) in IUGR and AGA fetus at a mean gestational age of 32 weeks and at a mean postnatal age of 18 months. They found that systolic blood pressure was significantly increased in the IUGR subjects, and it correlated with the prenatal and postnatal aIMT values. The aortic wall thickening progression in IUGR fetuses and infants differed from AGA, which may predispose the infants to hypertension early in life and cardiovascular risk later in life. Evidence from this analysis indicates that monitoring in utero aortic wall thickness may be more important for long-term cardiovascular health. Persistent aortic thickening may predispose patients to later cardiovascular risk and may influence vasomotor tone and arterial compliance from early in life [44].

According to the fetal programming also the kidney appears to be extremely susceptible to IUGR and is often found small in proportion to body weight. Several studies in animals and humans have described a reduced number of nephron after IUGR [45]. This results in an inborn decreased glomerular filtration surface area, while renal blood flow per glomerulus is increased in attempt to maintain a normal overall GFR. According to the hyperfiltration hypothesis explained by Brenner et al. [46], this leads to glomerular hypertension and hypertrophy, which causes systemic hypertension and higher sodium reabsorption and glomerular damage resulting in albuminuria and glomerulosclerosis. Therefore IUGR can lead to impairment of renal function [47]. A kidney with a reduced nephron number has less renal reserve to adapt to dietary excesses or to compensate for renal injury. The pathway leading from small kidney to hypertension may include the renin–angiotensin system, which has been demonstrated to be altered in the early phase of primary hypertension. An increased activity of the renin–angiotensin system could be a compensatory mechanism in a decreased number of nephrons in order to maintain normal filtration. In the last 5 years more clinical data are available regarding maturation of renal function in IUGR infants. Keijzer-Veen and colleagues in 2005 identified a positive association of birth weight and GFR [48]. The study by Zanardo et al. [49] shows that fetuses with IUGR had significantly higher abdominal aortic intima–media thickness compared with age controls when measured both in utero and at 18 months. At 18 months, the median urinary microalbumine and median albumin–creatinine ratio were significantly higher in those infants who experienced IUGR compared to the controls. The higher risk of aortic intima–media thickening and microalbuminuria, markers of preclinical atherosclerosis and of glomerulopathy, respectively, may have clinical implications for IUGR infants at 18 months of age. Barker et al. [50] and other investigators [51] showed that small size at birth is linked to long-term adverse health effects. Recently, growth-restricted twin fetuses with velocimetry abnormalities seem to be associated with aIMT and higher albumin creatinine ratio (ACR) levels in amniotic fluid, which could be possible markers in utero of preclinical atherosclerosis and early glomerulosclerosis. The presence of higher amniotic microalbuminuria in IUGR twin fetuses with umbilical artery vasculopathy may have implications in pediatric and adult life [52]. Albuminuria is one of the first signs of renal disease, anticipating a decrease in GFR. In presence

of IUGR glomerulopathy, albumine appears in amniotic fluid in IUGR twins, suggesting a continuum of kidney and cardiovascular disfunction which begins in utero and worsens until the development of metabolic, hemodynamic, and renal failure in adulthood. The main source of amniotic fluid production is in fact, the fetal kidney, together with the fetal lung, while the intramembranous circulation is a secondary source. Baschat [35] states that amniotic fluid volume is determined by the concentration of oxygen and the renal vessels perfusion, which affect fetal urine production. Our group of study revealed the presence not only of albuminuria but also of sodiuria in urine of IUGR infants while lysozime urine excretion was unaffected in the same infants [53].

To explain the simultaneous presence of microalbuminuria and sodiuria that we found in children born with IUGR, it is necessary briefly to recall the histology of the kidney glomerulus and podocytes.

The cytoskeleton of the pedicels (cytoplasmic protrusions of podocytes) appears characterized by the presence of actin filaments highly organized which connects all layers of the glomerular basement membrane (GBM), which are: rare internal foil, dense foil, and rare outside foil. If a mutation occurs in a domain or a protein in one of these three layers (nephrin, CD2AP, podocin, α-actinin 4, α3-1-integrin, and laminin-2), profound changes will be remarked in the cytoskeleton of MBG with initial enlargement of the pores, also called windows filtration, and progressive disappearance of the pedicels, which is accompanied by proteinuria [54]. According to Menzel and Moeller glomerular filtration is normally done in two steps: first there is a electric potential difference due to the passage of water and ions (during the flow of plasma), then this same potential allows the passage of negatively charged albumin through the barrier; so that it might be considered both microalbuminuria and sodiuria as consequences of the same glomerular podocytes alteration [55]. The fact that both podocytes and the endothelial cells originate from the same embryonic layer could mean that these two cells also have a cytoskeleton, if not identical, at least similar, and the same applies to their membrane receptors. The conclusion may be that alterations in cytoskeleton of GBM (almost always at the base of pathologies characterized by proteinuria) and endothelial cells (whose dysfunction is the *primum movens* in the development of atherosclerotic lesions), both documented frequently in children born with IUGR may have a unique etiology. These observations support the contention that extrinsic vascular and renal injury is not a prerequisite for the initiation and perpetuation of renal injury and that certain intrinsic deficiencies in functioning renal mass, prenatally derived, may be sufficient to contribute to renal and cardiovascular functional decline occurring with advancing age.

Conclusions

In conclusion, studies published on maternal and fetal malnutrition highlight that both cardiovascular and renal fetal development are affected by nutritional state and that the most frequent complication is fetal IUGR with its frequent consequences: aortic intima–media thickening, reduction of functioning renal glomeruli with hyperfiltration of the remaining ones, resulting in the arterial nephro-vascular hypertension. All these data show the presence of ***continuum*** of fetal vascular-endothelial and glomerulus damage, which begins in utero, and these observations clarify and support the ***programming*** theory proposed by Barker and Brenner.

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