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8

The Development of the Kidney

Jamie A. Davies and Jonathan B. L. Bard

Centre for Developmental Biology and Department of Anatomy
University of Edinburgh, Edinburgh, United Kingdom

- I. Introduction
 - A. A Simple Introduction to the Mouse Kidney
 - B. Strategies and Tools: In Vitro, Markers, Knockouts, Cells, and Cell Lines, the Kidney Development Database
 - C. What This Review Includes and What It Sets Out To Achieve
- II. Growth and Differentiation
 - A. Growth and Development of the Kidney *in Vivo*
 - B. Growth and Death
 - C. Differentiated Cell Types That Develop in the Kidney
 - D. Kidney-Derived Cell Lines as Models for Development
- III. Congenital Disorders of Nephrogenesis and Their Significance
 - A. Wilms' Tumor
 - B. Congenital Cystic Kidney Diseases
 - C. Some Other Kidney Disorders with a Genetic Basis
 - D. Other Mutants Showing Defects in Renal Development
- IV. Experimental and Genetic Analysis
 - A. WT1 and the Early Stages
 - B. Lineage in the Developing Kidney
 - C. Collecting-Duct Growth and Bifurcation
 - D. Mesenchyme Induction
 - E. The Downstream Effects of Nephron Induction
 - F. Development, Renal Function, and Kidney Disease
- V. The Future
 - A. Where We Are Now
 - B. The Assay Problem
 - C. Likely Successes
 - D. Conclusions
- References

I. Introduction

This chapter sets out to describe what is known about the earlier stages of development of the vertebrate metanephric kidney [the development of the pro- and mesonephroi is reviewed in Vize (1997)]. We will concentrate mainly on the mouse and use descriptive morphology as a platform for considering both the molecular mechanisms underpinning kidney morphogenesis and differentiation and the ways in which these processes can go awry and lead to congenital kidney

disorders, particularly in humans. As will soon become apparent, however, it is not possible to consider any one of these aspects in isolation: Such has been the speed of progress over the past decade that the study of congenital kidney disease and the search for key genes that regulate nephrogenesis are as the two wheels of a bicycle. Moreover, the study of the molecular biology of the developing kidney is illuminating aspects of developmental morphology that have received very little attention in the past. Indeed, kidney development is turning out to be much more complicated than was suspected even 5 years ago, and we are nowhere near the position where the basic molecular mechanisms controlling kidney development are even approximately understood. This survey thus is to be seen as the view of the traveller.

Those who are in the field and who set out to read this chapter will already know why the kidney is such a wonderful model system for developmental biologists, especially for those who want to maintain a foothold in medical research. For the casual browser of these pages whose knowledge of nephrogenesis may be limited, we start with a brief summary that covers what goes on as the kidney forms and how its development can go awry and lead to congenital abnormalities and then list the tools for investigating these events and the problems that are under investigation. We hope that, having tasted this *hors d'oeuvre*, browsers from other fields will then want to stay for the rest of the meal.

To pursue this metaphor a little further, the reader of this chapter might expect to find each course weighed down with a substantial helping of molecular data, and, indeed, the past decade has produced an enormous wealth of information on patterns of gene expression in the developing kidney. We have chosen to be as light with this ingredient as possible, mainly because the detail makes heavy reading and will certainly quickly become out-of-date, and because much of it does not illuminate the processes of nephrogenesis in any profound way. Therefore, we have chosen to concentrate more on the biology of the system, but an up-to-date summary of the very great majority of gene expression patterns can always be found at the web site of the Kidney Development Database (Davies and Brandli, 1997); the reader is encouraged to use this resource to illuminate more fully the issues discussed in this chapter.

A. A Simple Introduction to the Formation of the Mouse Kidney

The mature kidney is a fairly complex organ attached to an arterial input vessel and two output vessels, the vein and the ureter. Inside, the artery and vein are connected by a complex network of capillaries that invade a large number of glomeruli, the proximal entrance to nephrons, which are filtration units that link to an arborized collecting-duct system that drains into the ureter. In addition, there are nerves and the juxtaglomerular apparatus, a set of specialized cells that produce renin. It should be said at the beginning that almost all of the work on the

development of the kidney has focused on how the nephrons and collecting-duct systems form, particularly in their early stages, but, as is now becoming clear, the other components play a more substantial role in development than was previously expected.

The essentials of kidney development have been known for many years and this review stands on many others, with the reader being directed to those of Saxén (1987) and Ekblom (1992) in particular for coverage of the classic experimental work on the emergence of the developmental phenotype. It is more difficult to recommend other general reviews that cover work on the underlying genetic regulatory mechanisms, not because they are wrong or dull, but because such is the speed of progress that even the most recent have been overtaken by the flood of molecular data. This, together with other useful information, should always be obtainable from the Kidney Development Database (Davies and Brandli, 1997), as mentioned previously.

Although the adult kidney is complex, its early morphogenesis seems relatively simple. The mouse metanephros starts to form just before embryonic day 11 when, on each side of the embryo, an epithelial tube called the ureteric bud grows from the nephric duct and extends some 200–300 μm into a small dense mass of about 5000 cells, called the metanephrogenic mesenchyme, which is located within the intermediate mesoderm at about the level of the middle of the hindlimb. A few hours after the bud invades the metanephrogenic mesenchyme, a reciprocal interaction takes place with two main results. First, the bud starts to bifurcate, eventually forming the collecting-duct system that will drain through the nephric duct into the bladder. Second, the metanephrogenic mesenchyme starts to differentiate, first into stem cells (E11.5), which are seen at the kidney periphery, and later into their descendants, the nephrons of the cortex (>E13) and the mature stroma of the medulla (Fig. 1). At about this time, the neural and vascular components of the kidney start to differentiate.

Developing kidneys show an unusual geographical feature: Those nephrons that are at the cortex–medullary border are the oldest formed and most mature, those within the cortex are younger, and those at the periphery, where the tips of the growing collecting-duct system are containing metanephrogenic stem cells, are new nephrogenic condensations that are still forming. Given that the kidney blood vessels enter and exit with the ureter, the kidney thus can become functional at its center while its periphery is still differentiating.

This brief summary, most of which could have been written 40 years ago, highlights the diverse set of developmental mechanisms involved in kidney morphogenesis. The more obvious of these are the following: budding of the nephric duct and its directed extension into the metanephrogenic mesenchyme; reciprocal inductions between the bud and the metanephrogenic mesenchyme; branching morphogenesis; control of stem-cell growth and differentiation; formation of mesenchymal condensations; mesenchyme-to-epithelial transition; epithelial morphogenesis and fusion; and patterning of the filtration capabilities

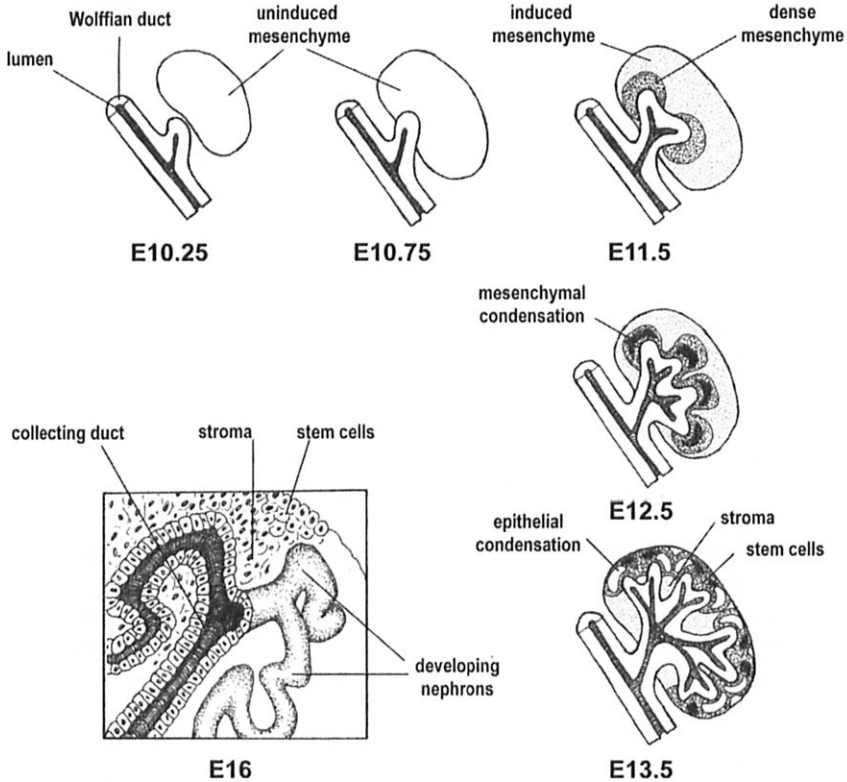


Fig. 1 The development of the mouse kidney. At E10.25 or so, the ureteric bud forms off the Wolffian duct and extends towards the uninduced mesenchyme of the metanephric blastema, reaching it some 12 h later. Over the next day, the bud invades the mesenchyme and starts to bifurcate while the mesenchyme is induced, with some of it condensing around the tips of the duct. A day later (E12.5), small dense (black) condensations form that will become nephrons. Over the next day, and as the bud continues to bifurcate to produce the collecting-duct system, a profound change takes place in the mesenchyme lineage. Here, existing condensations epithelialize (white) as new condensations (black) continue to form from the dense mesenchyme. This is now at the periphery of the rudiment where it acts as a population of stem cells, while the less dense mesenchyme has moved toward the center of the rudiment where it will form stromal cells. By E16, the epithelialized nephrons have fused to the collecting-duct system. (Drawings are not to scale and were produced by Amy Carless.)

along the length of the nephron. Thus, there are direct analogies between the kidney and many other tissues, and aficionados of embryogenesis will realize that this list covers all major developmental mechanisms. As will become clear later, kidney formation also requires most of the minor ones too!

One reason for studying the kidney is the belief that solving problems in this tissue will be easier than in other organs and, hence, that work on the kidney has

a greater significance than it might appear at first sight (We are aware that the same claim has been made for many other tissues but tend to find such claims unconvincing, as will be discussed). Another is that some important congenital kidney disorders in humans can be approached through mouse models (the best known being Wilms' tumor, in which growth and differentiation of the stem cells go awry); indeed, such disorders and others that can be manufactured through transgenic technology (Table I) are providing valuable keys to understanding normal kidney development (see later discussion).

B. Strategies and Tools: *In Vitro*, Markers, Knockouts, Cells, and Cell Line, the Kidney Development Database

The main reason for making the claim that we will understand the morphogenesis of the kidney before that of other equally complicated tissues is that many of the events already outlined will take place in relatively simple organ culture under well-defined conditions (one unexplored exception seems to be stem-cell maintenance and growth). In such cultures, branching morphogenesis and nephron formation occur, with each nephron extending, differentiating, folding to form a glomerulus, and fusing to a growing duct (Grobstein, 1955).

Table I Developmental Kidney Diseases^a

Disease	Pathology	Etiology (if known)
ADPKD	Cystic	Genetic: autosomal dominant mutation of polycystin
Alport syndrome	Glomerular defect	Collagen chain mutations
ARPKD	Cystic	Genetic: autosomal recessive, gene unknown (6p21 in human)
Beckwith-Wiedemann	Growth disorder	Genetic: autosomal, gene unknown (11p15 in human)
Meckel's syndrome	Cystic	Genetic: autosomal recessive, gene unknown (17q in human)
Medullary cystic disease	Cystic	Genetic: autosomal dominant
Nephronophtisis	Cystic	Genetic: autosomal recessive, gene unknown (2p in human)
Tuberous sclerosis	Cystic	Genetic: autosomal dominant mutation of tuberlin
von Hippel-Lindau	Cystic	Genetic: autosomal dominant, gene unknown (3p in human, probably cloned)
Wilms' tumor	Tumor	Genetic: autosomal recessive, ~15% due to mutation in WT1 (11p13 in human)

^aFor additional details, see Section III of the text.

The ability of the kidney and, indeed, isolated metanephrogenic mesenchyme (MM), to develop in culture (Fig. 8) means that the developing tissues can be subjected to a wide variety of experimental procedures designed to investigate their molecular and cellular properties and to test hypotheses about developmental mechanisms. The accessibility of the kidney to this type of investigation is underlined by the fact that expression patterns of some 250 genes are described in the Kidney Development Database, and the availability of this resource is a second reason for believing that we will understand the molecular interactions that underpin kidney development before those of other tissues.

Nevertheless, the problem with gene expression data in the kidney, as in every other tissue, is that knowledge of expression does not necessarily lead to an understanding of function (except under the guilt-by-association hypothesis). Knockout data using either transgenic mice *in vivo* or antisense technology *in vitro* can help to determine the function of key molecules, although the redundancy question remains as difficult to deal with here as elsewhere.

C. What This Review Includes and What It Sets Out To Achieve

What we would *like* to include in this review is a brief summary of the key steps in the development of the kidney followed by a terse analysis of the cellular and molecular mechanisms that underpin them. This would then be followed by a section showing how they go awry in congenital kidney disorders. Such a review is, unfortunately a decade or two into the future. Instead, this review proper starts with a slightly more detailed description of the various stages of normal kidney differentiation (Section II.A), which is followed by a summary of the roles played by growth and death (Section II.B) and a discussion of the extent to which kidney cell lines will emulate normal developmental processes *in vitro* (Section II.C). The following section covers kidney disorders, either natural [Wilms' tumor (Section III.A) and polycystic kidney disorders (Section III.B)] or induced through homologous recombination in transgenic mice. In a sense, these sections pose the detailed questions currently being investigated. The next and longest section considers the progress that has been made toward finding these answers, particularly with respect to competence in the metanephrogenic mesenchyme (Section IV.A), lineage relationships for the various cell types in the kidney (Section IV.B), ureteric bud induction and branching morphogenesis (Section IV.C), and mesenchyme induction (Section IV.D) and its downstream effects (Section IV.E). Accompanying these sections are tables that detail some of the core data about kidney development. It will soon become apparent that none of the obvious problems of kidney development as yet has a completely satisfactory solution. The cynic might say that the past decade of work has merely shown that things are far more complicated than the early workers ever expected; the cynic would, as ever, be correct, but would not be telling the whole truth. As a result of much clever work in the past few years, we not only have a far deeper insight into

the phenomenology of kidney development but are beginning to correlate it with the ever-increasing amounts of molecular data that are now available.

The study of kidney development is thus passing through interesting times as it seeks to make the leap from the phenotype to the genotype. It is not easy to see where the journey will lead, but the chapter ends with a view of the foothills that are likely to be surmounted in the next few years (Section V.A) and the mountains over whose tops we can hope to see in a decade's time (Section V.C). The developing kidney is small but complicated, accessible but opaque, and interesting but challenging. There is much to be done and work for as many as want to solve its problems.

II. Growth and Differentiation

A. Growth and Development of the Kidney *in Vivo*

1. Normal Development

All metanephric ("permanent") kidneys that have been examined initially develop in a similar way. Later development in larger organisms differs from that in smaller ones in the production of lobed kidney not present in the smaller organisms (this variant increases the surface:volume ratio as the diameter increases, thus allowing more nephrons to form from peripherally located stem cells). Here, we consider the mouse kidney because it has been subjected to the closest analysis, but the reader should not expect there to be major differences from the kidneys of other organisms, other than in timing.

Once induced, the kidney rudiment grows approximately exponentially (Fig. 2) until birth, initially doubling in volume every 8 hr or so, but slowing down to double in about 12 hr for the last few days of gestation. A very wide range of activities does, however, take place behind this envelope of regularity, and it is perhaps surprising that the net result is such uniform growth. It is convenient to divide kidney development into three distinct phases: (i) establishment, (ii) steady-state growth, and (iii) the mature state.

In the first phase that immediately follows induction (\sim E10.75), the ureteric bud extends and bifurcates while the MM segregates (\sim E11.5) into a pre-nephrogenic and a prestromal component, with the former adjacent to the bud and the latter occupying the periphery of the rudiment. One day later (E12.5 for the mouse), these components seem to have reversed locations and the nephrogenic stem cells have taken up their characteristic location at the cortex of the rudiment. It is probably at this point that the first nephrogenic condensations form and start to differentiate.

The second phase, that of steady-state development, starts when the essential features of the developing kidney are in place (\sim E13.5) and involves the ureteric bud-collecting-duct tree continuing to extend and bifurcate while the cortical stem cells divide rapidly, maintaining themselves and continuing to produce

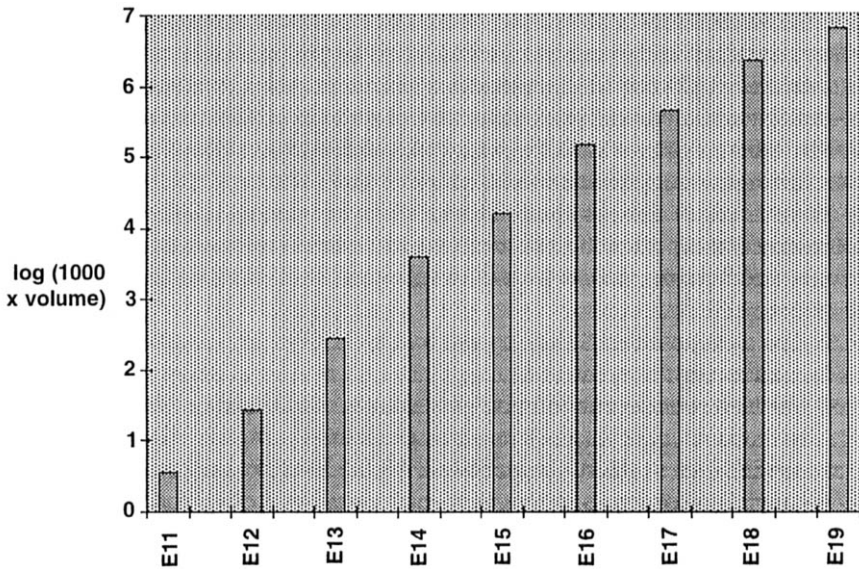


Fig. 2 The growth of the kidney. A log plot showing that the kidney volume (mm^3) grows exponentially, doubling in volume approximately every 9 h, up to about E16 when relative growth rates slightly decline (data courtesy of Anne Grattan).

small mesenchymal condensations adjacent to the tips of the arborizing collecting-duct system. The condensation and subsequent differentiation of the nephrons continue in the cortex until near birth, so that the farther a condensation is from the periphery, the older (and more differentiated) it is. At this stage, therefore, geography reflects history.

Each condensation undergoes a series of changes that leads to it forming a nephron: First, it becomes epithelial and forms a small cyst. The cyst then sprouts two tails. The first, on the side opposite the duct, gives it a comma shape (capillaries soon invade the space between the tail and the condensation to initiate the renal capsule), whereas the second (which will become the distal tubule) is near the collecting duct and gives the structure an S shape. The subsequent events are not entirely clear, but observations using markers specific for the duct system suggest that the domain of collecting duct adjacent to the distal rudiment extends and fuses with it (Davies, 1994). At the same time, the renal capsule differentiates and the proximal tubule forms. Differentiation to this stage probably takes about 2–3 days.

In this second phase, the medullary region of the developing kidney seems to include no more than the base of the collecting-duct tree, which is embedded in a very loose stroma of cells and a rich extracellular matrix and was, for many years, assumed to take a relatively passive role in the proceedings, merely providing space that the loops of Henle could invade. It is, however, worth mentioning for two reasons. First, the observations on the BFK $-/-$ mouse (Hatini *et*

al., 1996), which are discussed later, have forced us to revise the role of the medullary cells in mediating nephrogenesis, and, second, several hundred neurons together with a renal ganglion differentiate within this stroma, even *in vitro* (Karavanov *et al.*, 1995).

The third phase of kidney development reflects the end of nephron morphogenesis and the beginning of kidney function. There is, of course, no sharp border between these second and third phases because mature nephrons located at the corticomedullary border can be functional while the more peripheral nephrons are still differentiating. Nevertheless, on the basis of the morphology and emergence of differentiation markers in the nephrons and the behavior of the embryo, it seems as if the metanephros starts to function at around E16 when the mature nephron structures start to form and their loops of Henle descend into the medulla. Indeed, it is at about this time that the collecting-duct system starts to expand at its base and form calyces, the enlarged lumens into which urine flows on its way to the ureter.

By now, the great majority of nephrons that will form are in place, and one can get some sense of the growth that has occurred by considering the number of nephrons that are present. Although exact counts have not been made, two independent lines of analysis suggest that there are 1000–2000 nephrons in the mature mouse kidney. On the basis of relative size, the mature mouse kidney (~8 mm across) has about 0.1% of the volume of a human kidney (~10 cm across) with its 1.5 million nephrons (Fawcett and Raviola, 1994) and so should have ~1000 nephrons. Absolute size calculations give a similar figure: In the mouse kidney, each nephron is about 4 mm in length and about 100 μm in diameter apart from its larger, spherical glomerulus. If we assume that 50% of the kidney's volume is composed of nephrons, then there is space for about 2000 nephrons with each being composed of several thousand cells. This is an impressive figure for it means that, as the initial mesenchymal blastema itself had several thousand cells, each on average probably gave rise to one or two nephrons.

As birth approaches, the ability of the stem cells to produce nephrogenic condensations declines and these stem cells are lost soon after birth (in the human, small groups may be found that are called "rests," and mutations in these can lead to Wilms' tumor). Meanwhile, the stromal cells seem to be lost mainly because their division rates are so slow compared with the net growth in organ volume (Sainio *et al.*, 1994). With the loss of the stem and stromal cells and the filling of the medulla with loops of Henle, the metanephros acquires its adult form [for details, see, for example, Fawcett and Raviola (1994)].

B. Growth and Death

1. Mitosis

As has already been noted, the metanephros grows remarkably fast, doubling in size every 8 hr or so over the period E11–E16, and, as might be expected, many

of the cells express the proliferating cell nuclear antigen (PCNA), a marker for being within the S phase (L. McLaren and J.B.L. Bard, unpublished). The most remarkable feature is the degree of PCNA expression within the stem-cell population in the cortical rind, where almost every cell seems to express the antigen during the period E12–E16. A similar result is obtained with the use of BrdU where, at E16, more than one-half of the cortex is in the S phase at any moment as compared to about 12% for the medulla (Sainio *et al.*, 1994).

It is not clear, at first sight, whether the rapid growth of the kidney requires locally synthesized growth factor or whether it reflects the intrinsic ability of all of the kidney cell types to divide in the presence of nonspecific factors found ubiquitously in serum. The fact that kidney rudiments will develop, to some degree at least, in serum-free medium supplemented only with transferrin suggests at first that all necessary growth factors are synthesized by the kidney itself. However, things are not quite that simple. The growth of the kidney in culture is actually rather slow, and even after 4 days in the presence of serum it has only doubled in size twice (Bard and Ross, 1991). After that, there seems to be further differentiation, but no more growth. As cultured kidneys flatten in culture, this observation is compatible with the need for replacement of the kidney-synthesized factors that diffuse away from the rudiment.

The observations on two types of knockout mice provide positive evidence that the growth of the metanephric mesenchyme needs such factors (see Fig. 9). Loss of BMP-7 has no effect on early nephrogenesis, but, in its absence, growth soon slows and the mice are born with small kidneys and few nephrons (Dudley *et al.*, 1995), a result suggesting that BMP-7, which is synthesized by the cells of the collecting duct, is needed for the growth of the stem cells. This view is confirmed by the observations of Vukicevic *et al.* (1996), who showed that the addition of BMP-7 to isolated but induced metanephric mesenchyme *in vitro* enabled the cells to divide and differentiate. The second knockout is that of BF-2, a transcription factor expressed in the stromal cells of the medulla (Hatini *et al.*, 1996). In the BF-2^{-/-} mouse, however, it is the cortical cells that fail to grow or form proper nephrogenic aggregates (discussed earlier). It is thus clear that BF-2 controls the production, by the stroma, of factors that regulate growth and differentiation of the metanephrogenic mesenchyme. As the only growth factor known to be produced by the stroma is TGF β , and as its knockout has no effect on kidney development (Boivin *et al.*, 1995), it seems as if there are additional growth factors regulating growth and development and synthesized within the stroma that are still to be discovered.

As for the other cell types, things are clear only for the growth of neurons for whom the growth factor neurotrophin-3 seems necessary for survival and differentiation (Karavanov *et al.*, 1995). This factor generally is needed for neural crest cell differentiation and thus points to a neural crest source for the neuronal precursors.

2. Apoptosis

Much of the mitogenic effort is, in a sense, wasted: A large proportion, estimated to be as much as 50%, of the cells in the developing kidney are born to die (Camp and Martin, 1996). This is a surprisingly high number given the fact that the kidney doubles in size approximately every 8 hr (McLaren and J.B.L. Bard, unpublished). In p53-overexpressing mice even more cell death takes place (Godley *et al.*, 1996), but a small kidney still forms. Reference is made later to the fact that uninduced metanephrogenic mesenchyme shows strong suicidal tendencies, which are laid aside only when it is induced into the stem-cell state. However, it has become apparent that apoptotic death plays a role in development even after induction has taken place.

Assays for apoptosis based on DNA degradation, nuclear staining, and electron microscopy (Koseki *et al.*, 1992; Coles *et al.*, 1993) have revealed prominent apoptosis throughout all stages of renal development. One major wave is associated with early nephron formation, with many of the cells adjacent to newly forming nephric epithelia showing pyknic nuclei (Koseki *et al.*, 1992); this may reflect a mechanism to eliminate cells that were involved in the first stages of condensation, but were not included in the set that became committed to epitheliogenesis. A second wave is seen in the S-shaped body, where many cells in the region of the glomerular cleft die (Coles *et al.*, 1993). A third wave, occurring later, is seen in the medullary epithelia, including the ureteric bud.

The extent of apoptosis in a developing system generally is controlled by specific trophic factors (Raff, 1992). Coles *et al.* (1993) have shown that renal apoptosis can be dramatically reduced by treatment with excess epidermal growth factor (EGF), and it has been suggested that is possible that this and other growth factors are used to match the number of cells available for nephrogenesis to the length of the ureteric bud available for connection to nephrons (Camp and Martin, 1996). In transgenic mice lacking *bcl-2* (see Fig. 9a,b, below), there is too much early apoptosis followed by hyperproliferation, perhaps in an attempt to compensate for the abnormally high apoptosis. The result is the formation of epithelial cysts (Veis *et al.*, 1993). Whereas the precise mechanism of cyst formation is not yet understood, the phenotype of these *bcl-2*^{-/-} kidneys provides strong evidence for the importance of a proper balance between proliferation and death if normal anatomy is to develop.

C. Differentiated Cell Types That Develop in the Kidney

As morphogenesis takes place, differentiation markers are acquired and lost, and much of the work in the latter part of the 1980s was dedicated to documenting these changes [see, for example, Ekblom (1992)]. The development of the nephron can therefore be documented by changes in gene expression profiles, and it is

Table II Useful Markers for Different Tissue Types in the Developing Kidney

Tissue type	Useful marker
Ureteric bud–developing collecting duct	Calbindin-D-28K (unique to developing CD in normal culture conditions, but <i>in vivo</i> or in culture medium containing 1,21-dihydroxyvitamin D ₃ mature distal tubules also express it; Davies, 1994). Cytokeratin 7 (Moll <i>et al.</i> , 1991) IGFBP3 (Matsell <i>et al.</i> , 1993) KDN-1 (Burrow, 1993)
Uninduced metanephrogenic mesenchyme	Pax-2 not yet induced (Rothenpieler and Dressler, 1993); this tissue will only be present at the very early stages of renal development
Stem cells	11 (Svennilson <i>et al.</i> , 1995) with Pax-2 (Rothenpieler and Dressler, 1993)
Nephrogenic condensates	Strong NCAM (it is expressed weakly in MM and stem cells, but greatly up-regulated in condensates; Klein <i>et al.</i> , 1984) WT-1 also up-regulated (Armstrong <i>et al.</i> , 1992)
Early epithelium	No unique marker, but one could use Pax-8 (Polcev <i>et al.</i> , 1992) in the absence of glomerular, proximal tubule, and distal tubule markers
Developing glomerulus	α_3 -Integrin (Korhonen <i>et al.</i> , 1990, 1992) Desmin (some cells only; Bachmann <i>et al.</i> , 1983)
Proximal tubule	CD15 (Bard and Ross, 1991) Brush border antigen (Miettinen, 1986)
Distal tubule	Uromucoid (Tamm–Horsfall antigen) (Hoyer <i>et al.</i> , 1974)
Stroma	Tenascin (Aufderheide, 1987) BF-2 (Hatini, <i>et al.</i> , 1996)
Endothelium	PECAM (Baldwin <i>et al.</i> , 1994)
Juxtaglomerular apparatus	Renin (but earliest expression is more widespread; Jones <i>et al.</i> , 1990)
Neurons	Neurofilament 200 (Sainio <i>et al.</i> , 1994)

possible to identify many cell types by using specific markers (Table II). It will be helpful to list here the range of cells, with a key gene or two that it expresses and that can be used as a marker(s).

D. Kidney-Derived Cell Lines as Models for Development

1. Classical Renal Cell Lines

Kidney-derived cell lines, such as Madin–Darby canine kidney (MDCK) collecting-duct epithelium, have long been used in cell culture work for investigating problems as diverse as virus replication, cell polarization, induced scattering and cell–cell junction formation (e.g., Pasdar and Krzeminski, 1992; Timbs and Spring, 1996; Schultze *et al.*, 1996). However, their use to investigate the

mechanisms of kidney development has been much less frequent. The most promising results are probably those that have been obtained from the MDCK cell-cyst model system.

MDCK cells grown on planar plastic substrates form polarized epithelial monolayers, but those grown in a three-dimensional suspension form small hollow cysts. If the three-dimensional matrix is an "inert" substrate such as agar, then the apicobasal polarity of the cells is opposite that in a normal kidney tubule—their basal surfaces are innermost and their apical ones outmost (like an early mammalian embryo). If, on the other hand, they are cultured in a matrix containing, for example, collagen, their polarity reverses so that they form the basal-out, apical-in structure typical of real kidney tubules (Wang *et al.*, 1994). This phenomenon underlines the importance of cell-matrix interactions for the establishment of epithelial anatomy, a subject that will be considered in more detail in Section IV.E.2.

MDCK cysts growing in these collagen gels show interesting morphogenetic responses that some researchers believe to reflect processes taking place in normal development (Fig. 3). For example, if they are treated with the growth factor HGF/SF, they extrude long cellular spikes in a process that has been likened to branching morphogenesis (Montesano *et al.*, 1991). It is, however, important to view these analogies critically as there is no strong evidence to suggest that the spikes formed from MDCK cysts are homologous to true branched epithelial tubules, and real ureteric bud cells do not behave in the same way (Sainio *et al.*, 1997).

2. The Search for Nephrogenic Cell Lines

While research into kidney development is made much easier by the facility with which organ rudiments will develop *in vitro*, it is hampered by one serious limitation of the system: So far it has proved impossible to establish a cell line that can be grown in bulk and then induced to undergo organotypic development. This is a nuisance to researchers who would like to use transfection techniques to generate specific mutants whose phenotypes could be characterized in culture [see Burrow and Wilson (1994) for a review]. Two strategies are being attempted to circumvent this: (a) immortalization of cells by transformation with temperature-sensitive SV40 large T antigen and (b) induction of multiplication of wild-type metanephrogenic mesenchyme cells without the induction of differentiation.

The immortalization strategy is based on the SV40 large T^{ts-58} transgenic mouse of Jat *et al.* (1991). This animal carries a temperature-sensitive allele of SV40 large T antigen under the control of a γ -interferon-inducible promoter. At 37°C and in the absence of γ -interferon, the antigen is inactive and the animal's cells are normal. If the cells are taken from an animal of this strain and cultured at 33°C with γ -interferon, the T antigen becomes active and forces the cells to multiply—at least in theory. Subsequent transfer to the nonpermissive tempera-

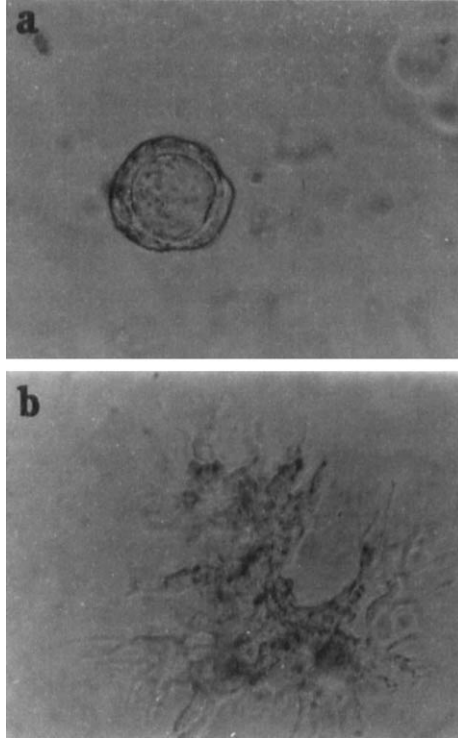


Fig. 3 HGF acts as a morphogen on MDCK cell cysts. These cells (derived from collecting duct) cultured in suitable extracellular matrix form simple cysts in the absence of HGF (a), but in the presence of HGF (b) the cysts push out extensions in a process that has been compared to the epithelial branching seen in normal organogenesis. Micrograph courtesy of Sarah Wallis.

ture will cause the cells, now far greater in number, to return to their previous behavior (again, in theory). Use of temperature-sensitive SV40 large T alleles has been effective with several types of cell, for example, neurons (Giordano *et al.*, 1993, 1996) and myocytes (Miller *et al.*, 1994; Benito *et al.*, 1993), but so far has failed to yield a nephrogenic cell line. It has, however, been useful with already epithelial renal cell lines (Piedagnel *et al.*, 1994; Hosoyamaad *et al.*, 1996). Thus far, the cell lines that have been produced seem to have already progressed beyond the stem-cell stage as they express cytokeratins.

The second strategy arises from the conclusion, which will be explained later in Section IV.D.2, that the induction of nephron development has two phases, the first of which switches cells from quiescence and apoptosis to vigorous growth, and the second of which causes differentiation. The aim is to induce the first without the second and thus create a multiplying population of nephrogenic stem cells. This appears to have been achieved by Barasch *et al.* (1996), whose elegant

technique combines the use of SV40 T^{ts-58} transgenic cells with exploitation of two-phase induction. Barasch *et al.* have created a cell line from the ureteric buds of SV40 T^{ts-A58} transgenic mice and have shown that conditioned medium from this line switches wild-type metanephrogenic mesenchyme cells into vigorous growth without compromising their ability to be later induced into nephrogenesis. The technique may enable researchers to transfect nephrogenic cells in the near future and so provide them with a key tool for investigating the molecular basis of nephrogenesis.

III. Congenital Disorders of Nephrogenesis and Their Significance

A. Wilms' Tumor

Wilms' tumor is the most common solid-tissue cancer of childhood, affecting 1 in 10,000 in the population and occurring in both sporadic and familial forms (although only about 1% of patients have a family history of the disease). Like most cancers, it presents with a somewhat variable histology, but "classical" (triphasic) Wilms' tumors show large numbers of proliferative blastemal cells, in which small islands of epitheliogenic and stromogenic differentiation are scattered that parody normal kidney development. The blastemal cells do not express Pax-2 (Dressler and Douglas, 1992) and therefore are unlike normal nephrogenic stem cells (see Section II).

Genetic study of Wilms' tumor family trees has resulted in the identification of a tumor-suppressor gene called *WT1*, which maps to the 11p13 region of chromosome 11 [for a review, see Coppes and Williams (1994)]. *WT1* is mutated in approximately 15% of tumors; the etiology of the remaining 85% remains mysterious, although there is one other site, closely linked to the *IGFII* gene on chromosome 11 in the 11p15 region, in the region of the Beckwith–Wiedemann locus that may account for some of them. A third gene is likely to be on chromosome 16 [for a review, see Tay (1995); Ward, 1997]. The role of *WT1* and its possible role in Wilms' tumor are discussed later.

B. Congenital Cystic Kidney Diseases

This set of disorders leads to the formation of large cysts within the epithelial tubules (see Fig. 4a), both nephric and collecting duct, in which normal physiological processes are inhibited or reversed. Although the mature kidney is a relatively robust tissue and can fulfill its filtration functions in the presence of considerable insult (and abnormal morphogenesis, e.g., horseshoe kidneys, Fig. 4b), the size and number of the cysts and the associated renal hyperplasia of these diseases usually lead to kidney failure.

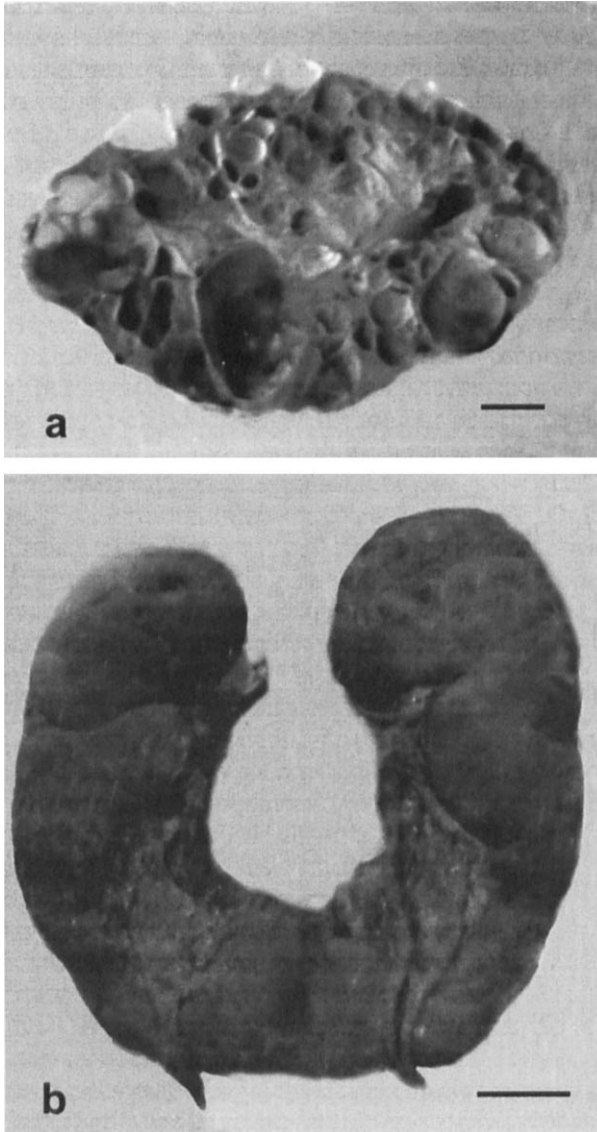


Fig. 4 Abnormal human kidneys. (a) a cystic kidney, with the cysts being up to several cm in diameter. (b) a horseshoe kidney. Bar = 2 cm. (Material from the Anatomy Museum, Edinburgh University.)

1. Autosomal Dominant Polycystic Kidney Disease

Mutations in three genes have been shown to lead to this phenotype. Polycystin (PKD1), the first of these, seems to be a membrane-traversing protein that may have a role in cell adhesion and is strongly expressed in fetal kidneys and less strongly expressed in adult kidneys (Palsson *et al.*, 1996). PKD2, the second, was identified by positional cloning and is similar to PKD1 in that it is a membrane protein, is related to the family of voltage-activated calcium channels, and contains a potential calcium-binding domain (Mochizuki *et al.*, 1996). There is also evidence for a third, ADPKD, gene (Daoust *et al.*, 1995), but it has not yet been cloned.

2. Autosomal Recessive Polycystic Kidney Disease

This version of polycystic kidney disease (ARPKD) is a rare but lethal inherited disorder resulting in the formation of cysts within the kidneys that are enlarged and dysfunctional. The gene responsible for ARPKD has been mapped to chromosome 6p21.1-cen (Zerres *et al.*, 1994).

3. Nephronophthisis (Medullary Cystic Disease)

This autosomal recessive disease is the most common form of early onset cystic disease that leads to renal failure. Although the underlying genetic defect has yet to be identified, the gene for juvenile nephronophthisis (*NPH1*) has been mapped by linkage analysis to chromosome 2q13. As a complete YAC contig of 7MB containing the region has now been constructed, it should not be too long before the gene is cloned and analyzed (Hildebrandt *et al.*, 1996).

4. Meckel's (or Meckel–Gruber) Syndrome

This monogenic, autosomal recessive disorder is recognized by three sets of abnormalities, neural tube closure defect, large polycystic kidneys, and polydactyly, and leads to the death of the fetus *in utero* or shortly after birth. Linkage analysis assigns the *MES* locus to chromosome 17q21-q24 (Paavola *et al.*, 1995).

C. Some Other Kidney Disorders with a Genetic Basis

Apart from Wilms' tumor and the polycystic kidney diseases, there are several rather obscure, but still serious congenital human kidney disorders that have been known for many years on the basis of their histology and symptoms. However, considerable effort has been put into elucidating their genetic and molecular bases, and the list that is briefly discussed here demonstrates the success of this strategy.

1. von Hippel-Lindau's Disease

This disorder, which may be familial (dominant) or sporadic, predisposes individuals to renal cell carcinoma, hemangioblastomas of the central nervous system, and pheochromocytoma. The disease now seems to be due to mutations in *VHL*, a tumor-suppressor gene on chromosome 3p that is expressed, *inter alia*, in the proximal tubular epithelium of the developing and adult kidney (Kessler *et al.*, 1995). It is thought to play a role in regulating the transcription of, as yet, unidentified downstream genes (Richards *et al.*, 1996).

2. Alport Syndrome

Alport syndrome (or hereditary nephritis) is an inherited kidney disease caused by irregularities and disruptions in the glomerular basement membrane and is associated with hematuria and sensorineural deafness. The common, X-linked form is associated with mutations in a gene encoding a novel basement membrane (type IV) collagen α_5 -chain (Zhou *et al.*, 1991), while mutations in the α_3 - and α_4 -chains have been reported for the rarer autosomal forms of the disease (Mochizuki *et al.*, 1994).

3. Denys-Drash Syndrome

Patients with this syndrome show early onset nephropathy, a high risk of Wilms' tumor (WT), and pseudo-hermaphroditism. Careful analysis of *WT1*, the Wilms' tumor gene, shows that the disorder is associated with mutations in the zinc-finger regions. *In vitro* studies of mutated genes show that they fail to bind to *WT1* target sites (Little *et al.*, 1995), although it is not yet clear how this failure manifests itself as a lesion in the glomerulus.

4. Beckwith-Wiedemann's Syndrome

In the context of the kidney, Beckwith-Wiedemann's syndrome gives a predisposition toward Wilms' tumor, and genetic analysis has shown that maternally expressed imprinted genes may be involved that are in the chromosome 11p15 region [for a review, see Ward (1997)]. Three such genes whose expression is altered in the disease are *IGFII* (Ogawa *et al.*, 1993), the human cyclin-dependent kinase inhibitor, p57KIP2 (Hatada *et al.*, 1996; Matsuoka *et al.*, 1996), and *KVLQT1*, which spans much of the interval between p57KIP2 and *IGFII* (Lee *et al.*, 1997).

5. Tuberous Sclerosis

This dominantly inherited disease of the skin, retina, and heart is characterized by the presence of small benign fibrous tumors; it is often manifested in the kidney as angiomyolipomas (~50%) and cysts (~30%). The disease seems to be caused

by a mutation in one of two genes that act as tumor suppressors. The first is on chromosome 9 and has yet to be cloned, whereas the second, tuberin (the *TSC-2* gene is on chromosome 16), is a widely expressed 180-kDa polypeptide that exhibits specific GTPase-activating activity *in vitro* toward Rap1, with which it colocalizes, and that functions as a Rab5 GTPase-activating protein (GAP) in modulating endocytosis (Wienecke *et al.*, 1997; Xiao *et al.*, 1997).

D. Other Mutants Showing Defects in Renal Development

Of the 30 or more knockout mice with deletion of genes expressed during normal kidney development, 22 have no recognizable renal defect. Examples of those with particularly interesting renal defects are shown in Table III.

Table III Selected Mutants with Interesting Renal Phenotypes^a

Mutant genotype	Phenotype	Reference
<i>bcl-2</i> -/-	Polycystic kidneys	Nagata <i>et al.</i> (1996)
<i>BF-2</i> -/-	Hypoplastic, underdeveloped collecting duct, few nephrons but many overlarge condensates	Hatini <i>et al.</i> (1996)
<i>BMP-7</i> -/-	1. Dudley and Luo mutants: severe hypoplasia; renal development seems to start properly but growth subsequently ceases 2. Jena mutant: adequate glomeruli formed but poor development of distal tubules; polycystic	Dudley <i>et al.</i> (1995); Luo <i>et al.</i> (1995) Jena <i>et al.</i> (1997)
<i>GDNF</i> -/-	Lack of ureteric bud; can be rescued <i>in vitro</i> by exogenous GDNF	Sanchez <i>et al.</i> (1996); Pichel <i>et al.</i> (1996); Moore <i>et al.</i> (1996)
p53 overexpression	Half-size kidneys	Godley <i>et al.</i> (1996)
<i>Pax-2</i> -/-	Failure of ureteric bud and nephron formation	Torres <i>et al.</i> (1995)
<i>PDGFB</i> -/- or <i>PDGF-R</i> -/-	Too few glomeruli	Soriano (1994); Leveen <i>et al.</i> (1994)
<i>c-ret</i> -/-	Variable failure of ureteric bud	Schuchardt <i>et al.</i> (1994)
<i>Wnt-4</i> -/-	MM condensates but does not epithelialize	Stark <i>et al.</i> (1994)
<i>WT1</i> -/-	Ureteric bud does not grow, metanephrogenic mesenchyme dies and cannot be induced by wild-type inducers	Kreidberg (1994)

^aAdditional mutants may be found in The Kidney Development Database (Davies and Brandli, 1997).

IV. Experimental and Genetic Analysis of Kidney Development

A. WT1 and the Early Stages

Renal development begins when a ureteric bud grows out from the Wolffian duct and toward the metanephrogenic mesenchyme. Without this early event taking place, all subsequent metanephric development will fail, so it is perhaps surprising that rather little is known about these critical early events. The first evidence of the metanephrogenic mesenchyme is the appearance of a domain of condensed mesenchyme ($\sim 250 \mu\text{m}$ in diameter) at the level of the midpoint of the hindlimb buds in each of the two bands of the intermediate mesoderm. It has recently become clear that these early stages of development absolutely depend on the activity of *WT1*, a gene cloned on the basis of its role in Wilms' tumor. *WT1* is a zinc-finger protein, with transcription and splice-regulating activity that is expressed in normal metanephrogenic mesenchyme but not in the Wolffian duct or ureteric bud. It has 10 exons and 2 of these (5 and the KTS domain of 9, part of zinc-finger 3) are under independent control, so that 4 isoforms are coexpressed.

Expression analysis of *WT1* shows that transcripts are present in the mouse in three distinct regions: Low-level expression is present in the uninduced metanephrogenic mesenchyme as soon as this tissue can be identified (Armstrong *et al.*, 1992). Following induction, the gene is expressed at noticeably higher levels in the metanephrogenic mesenchyme surrounding the ureteric bud and also in nephrogenic condensations. Its expression here is transitory, but the third and highest level of expression, in the podocyte layer of the differentiating renal capsule, is maintained until well after birth. The gene is also expressed elsewhere in the embryo (Armstrong *et al.*, 1992), particularly in regions where a mesenchyme-to-epithelial transition will take place (e.g., presumptive mesothelial cells and the gonad).

In *WT1*^{-/-} animals, ureteric bud outgrowth fails, and the condensed mesenchyme taken from the regions of the embryos that should be occupied by metanephrogenic mesenchyme cannot be induced, even by wild-type inducers in culture. Instead, it rapidly undergoes apoptosis (Kreidberg *et al.*, 1993). This phenotype carries two implications: (1) *WT1* function is required to establish a normal MM phenotype ("competence"); and (2) ureteric bud outgrowth is induced by the presence of normal MM.

As ureteric bud outgrowth takes place at a distance, *WT1* almost certainly has to act through the release of a diffusible inducing molecule. The production of normal and ectopic ureteric buds from the Wolffian duct can certainly be triggered by the experimental application of a growth factor gradient; for example, placement of beads soaked in high concentrations of GDNF near the Wolffian duct can produce supernumerary ureteric buds that grow toward the bead (Sainio

et al., 1997). The natural inducing molecule might be GDNF, which is expressed by uninduced metanephrogenic mesenchyme (Trupp *et al.*, 1995), but the observation that not all GDNF^{-/-} mice show complete absence of ureteric buds (Moore *et al.*, 1996) argues that there is some redundancy with other, so far unidentified, factors.

Sequence analysis of the *WT1* gene reveals it to be a transcription factor of the zinc-finger family with four such fingers; it also has a GC-rich region that is a potential target for trans-activating factors. Several laboratories have identified binding regions for *WT1* in the upstream regions of genes involved in nephrogenesis, such as *syndecan-1* (Cook *et al.*, 1996) and *EGR1* (see Maheswaran *et al.*, 1993). Transfection studies have pointed to possible stimulatory or inhibitory interactions between *WT1* and p53 (see Maheswaran *et al.*, 1993), the retinoic acid α -receptor (Goodyear *et al.*, 1995), *EGR1*, and *bcl-2* and *c-myc*, where loss of *WT1* led to the deregulation of these genes and contributed to tumor formation (Hewitt *et al.*, 1995). In the absence of *WT1*, normal changes in the expression of these genes fail to take place.

An additional function for *WT1* has emerged from expression analysis using antibodies to *WT1*. Larsson *et al.* (1995) showed that the presence or absence of an exon carrying the KTS amino acid sequence, between zinc-fingers 3 and 4, determines whether the WT1 protein is associated with nuclear DNA or with splicing factors in the spliceosomes, suggesting that the protein has two very distinct roles. In this context, it is intriguing that a search for *WT1* homologues across the vertebrates showed that the gene was present in chick, alligator, frog, and zebrafish as well as in mouse and humans (Kent *et al.*, 1995), with both the zinc fingers and the transregulatory domain exhibiting a high level of similarity. However, only one of the two alternatively spliced regions, the three-amino acid KTS insertion between zinc-fingers 3 and 4, is found in species other than mammals. The functional significance of this diversity is still obscure.

Although *WT1* was cloned almost a decade ago, only recently have there been substantial rewards for the large amount of effort that has been put into analyzing what controls its expression and how it exerts such a powerful effect on nephrogenesis. Although little is understood about the reasons for the *WT1* expression pattern, Hofmann *et al.* (1993) have shown that its upstream region contains at least four start sites and a range of other intriguing features, the most important of which may be that the *WT1* promoter region contains potential recognition sites for *WT1/EGR*, *Pax-8*, and GAGA-like transcription factors. The first of these points to a possible autoregulatory stimulation or inhibition of *WT1* expression (Rupprecht *et al.*, 1994) and the second to a way in which *WT1* is up-regulated in the podocyte layer soon after *Pax-8* starts to be expressed; this view is strengthened by the fact that transfected *Pax-8* isoforms can stimulate *WT1* expression in cells (Dehbi and Pelletier, 1996; Pelletier, 1996b). A similar and earlier role also seems to be taken by *Pax-2*, which is expressed by MM very soon after induction (see the following), and this activity may account for the initial up-regulation of

WT1 in the induced MM and early condensates (Dehbi *et al.*, 1996; Pelletier, 1996a).

Another intriguing and unexpected observation has been that there is a second gene, *WIT-1* (Gessler and Bruns, 1993) in the 11p13 region (in humans) some 2 kb upstream from the *WT1* gene and coexpressed with it, but transcribed in the opposite direction, apparently from the *WT1* promoter (Eccles *et al.*, 1994), albeit at some 10% of the level of *WT1* transcription. Hewitt *et al.* (1996) suggest that *WIT-1* may be an antisense regulator of *WT1*.

As a whole, these studies show that *WT1* is a gene whose expression can be regulated by a range of activators and that can, in turn, activate or repress a wide variety of proteins. Progress in understanding the *WT1* network has been rapid, and we can hope that we will soon have a solid genetic picture of how this gene is regulated and how it establishes the metanephric mesenchyme and guides its developmental responses as nephrogenesis proceeds.

B. Lineage in the Developing Kidney

Surprisingly little is known about lineage relationships in developing kidneys. It is clear that the comparatively few tissue types present in a very early kidney rudiment (ureteric bud, metanephrogenic mesenchyme, and perhaps some endothelial and neural crest cells) have to give rise to a much larger variety of cell types in the mature kidney (see Section II.B). Nonetheless, details of who gives rise to whom and in what order choices between developmental pathways are made remains, for the most part, uncertain. The classic story is that the ureteric bud develops into collecting duct and that metanephrogenic mesenchyme forms stroma and nephrons, but this is clearly inadequate as it provides no source for neurons (which grow in culture as well as *in vivo*) blood cells, and the juxtaglomerular apparatus.

1. The Collecting Ducts

The development of the ureteric bud has traditionally been thought to involve the least uncertainty over lineage: it develops as an outgrowth from the Wolffian duct. The bud, in turn, was believed to give rise to only the collecting-duct system, and the collecting-duct system was believed to arise solely from the ureteric bud. The work of Herzlinger *et al.* (1993) and Qiao *et al.* (1995) has, however, cast doubt on this nice, simple story. By using the techniques of retroviral cell marking and dil cell labeling, these researchers observed that the progeny of labeled ureteric bud cells could later be found in nephric epithelium. On this basis, they claimed that the cells of a labeled bud can leave it, undergo an epithelium-to-mesenchyme transition, and join the nephrogenic mesenchyme cells, where they then undergo a reverse mesenchyme-to-epithelial transition to become part of the nephrons.

Does this result mean that nephrons are made simply from cells that leave the top of the ureteric bud, migrate a short distance, and reepithelialize? Several observations suggest not, the most powerful of which is the well-established phenomenon of nephrogenesis in uninduced mesenchymes separated from ureteric bud and recombined with a completely different inducer (e.g., spinal cord) in culture (Grobstein, 1955). This works even for mesenchymes of mutant mice that have no ureteric buds (Schuchardt *et al.*, 1996). The potential contribution of nephrogenic cells by the ureteric bud observed by Herzlinger *et al.*, albeit intriguing, is therefore not necessary for nephrogenesis.

Similar work from the Herzlinger group has also provided evidence for cell traffic in the other direction; labeled mesenchyme cells appear to become incorporated into the growing ureteric bud (Qiao *et al.*, 1995). This two-way traffic between cell types, if a feature of normal kidney development, complicates cell lineage issues considerably and merits further investigation.

2. Mesenchyme Derivatives

The clump of cells grouped together as metanephrogenic mesenchyme gives rise to neuronal cells, vascular endothelium, renal stroma, cells of the juxtaglomerular complex, and excretory nephrons. Neuronal elements are widely assumed to develop from neural crest, a major source of migrating cells with neurogenic and other fates. Invasion of the metanephrogenic area by neural crest cells has been described in developing birds (Weston, 1963; LeDouarin, 1969; LeDouarin and Theillet, 1974), but their development at that site has not been followed in detail.

Vascular elements of the kidney have traditionally been thought to arise solely via angiogenic invasion of the organ rudiment by capillaries from elsewhere in the embryo [see Saxén (1987) for a review], a process that can be mimicked by culturing kidney rudiments on the chorioallantoic membranes of chick eggs (Sariola *et al.*, 1983). The idea that external vessels are the only source of renal capillaries is further supported by the observation that isolated kidney rudiments do not form capillaries in standard culture conditions. It has, however, become clear that isolated cells in the early (E10.5) metanephrogenic mesenchyme do express the endothelial markers VEGFR and Tiel, which are characteristic of endothelial cells (Landels *et al.*, 1994; Loughna *et al.*, 1996). If transgenically marked metanephrogenic mesenchymes of this age are transplanted into an unmarked host kidney, the capillary network that develops includes marked endothelial cells, confirming vasculogenesis from endothelial precursors endogenous to the metanephrogenic mesenchyme.

Early stromal cells are first seen soon after induction at the periphery of the kidney, where they express BF2 (Hatini *et al.*, 1996). Later, stromal cells expressing BF2⁺ are seen, mainly in the medulla, with small groups of them also appearing in the cortex, where they may later form the cells of the juxtaglomerular complex.

The apparently homogeneous metanephrogenic mesenchyme thus give rise to

four very different cell types: nephric epithelium, vascular endothelium, juxtaglomerular cells, and renal stroma. This may be explained by three distinct models: (1) MM consists of one cell type, the pluripotency of which embraces stromogenic, vasculogenic, and epitheliogenic fates, which diverge only after induction. (2) MM consists of a mixture of two morphologically indistinguishable cell types, one of which is restricted to vasculogenic growth even before induction and the other is pluripotent for both epitheliogenic and stromogenic fates. (3) MM consists of three or more cell types, restricted even before induction to epitheliogenic, stromogenic, juxtaglomerular, and vasculogenic fates. Discrimination between the models requires that cell-marking experiments be performed before commitment to epitheliogenesis takes place. The mesenchyme-marking experiments reported so far result in clones that are either purely epithelial or purely mesenchymal (Herzlinger *et al.*, 1994), but labeling was undertaken too late in development to rule out models 1 and 2. This issue urgently needs to be addressed, because each model implies the existence of quite different cell-signaling mechanisms to control subsequent differentiation (see Section IV.D.4).

Cell-labeling studies have provided good evidence that, before their nephrogenic differentiation, mesenchyme cells remain pluripotent with respect to the part of the nephrons to which their daughters can give rise; the clonal progeny of labeled mesenchymal cells can appear in multiple segments of a maturing nephron (Herzlinger *et al.*, 1994). Once differentiation of specialized nephron segments begins, the pluripotency of cells presumably is lost, although the order in which choices between fates become irrevocable is completely unknown.

The last segment of what is conventionally regarded as the nephron, the very short segment that attaches to the collecting duct and is called the connecting tubule, appears by its expression of markers and its response to signaling molecules to share the properties of collecting-duct cells rather than those of the nephron (Davies, 1994). This and the fact that connecting tubules have never been observed to form in the absence of a developing collecting-duct system suggest that this segment is in fact derived as an outgrowth from the collecting duct. The issue will be difficult to prove, however, because the apparent interconversions of ureteric bud and mesenchymal cells reported by Herzlinger *et al.* make the interpretation of conventional labeling experiments very difficult.

C. Collecting-Duct Growth and Bifurcation

1. Signals That Control Collecting-Duct Morphogenesis

Like the epithelia of other glandular organs (e.g., lung, salivary gland, mammary gland), development of the collecting-duct system (Fig. 5) from the ureteric bud depends completely on signals emanating from the mesenchyme that surrounds it. Isolated in culture, the ureteric bud fails to develop, and it also fails to arborize when recombined with most types of embryonic mesenchyme tested (although

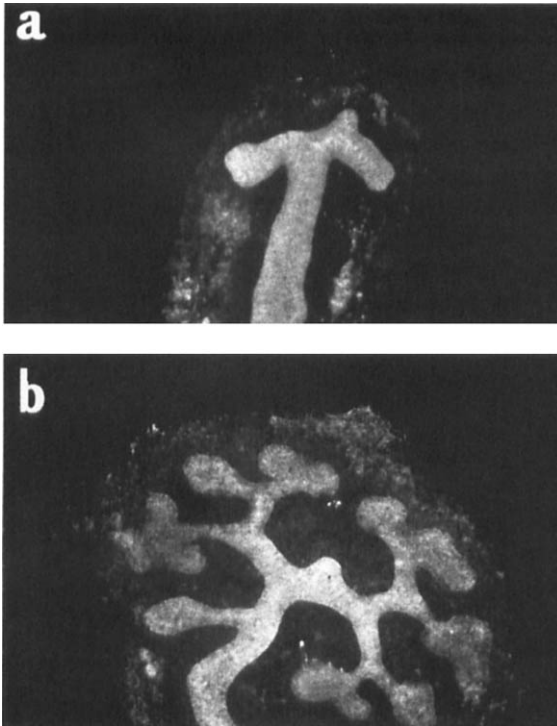


Fig. 5 Development of the collecting duct system; the initially unbranched ureteric bud bifurcates (a) then grows and undergoes more rounds of bifurcation to generate a tree-like epithelium (b) with, eventually, 250–500 tips (mouse).

some mesenchymes, such as lung, do support limited development; Sainio *et al.*, 1997).

The molecular identities of factors that renal mesenchyme uses to support and control ureteric bud development have been sought by three main techniques:

- Examination of the phenotypes of mutant mice showing abnormal ureteric bud development
- Specific inhibition of candidate molecules by antibody, antisense, transgenic, and biochemical techniques
- Testing the ability of candidate molecules to rescue the development of collecting-duct systems that has been experimentally inhibited in some way.

Four sporadic mouse and chick mutants show defective ureteric bud development: *Danforth's Short Tail (Sd)*, *limb deformity (ld)*, *fused (Fu)* and *Wingless (Wg)* (Gluecksohn-Schoenheimer, 1943; Maas *et al.*, 1994; Theiler and Gluecksohn-Waelsch, 1956; Ede, 1978). Three of these also cause defective limb develop-

ment, although the significance of this correlation is not understood: We therefore cannot exclude the possibility that the apparently linked effects are effectively byproducts of events elsewhere, and perhaps earlier, in the region. The molecules associated with *Fu*, *Sd*, and *Wg* have not yet been identified, although intensive mapping of the *Sd* region in the mouse (Alfred *et al.*, 1997) promises an early answer. *Limb deformity (ld)* arises from a defect in formins, which are nuclear proteins that are more likely to be concerned in the response to regulators than in the signaling system itself.

Specific inhibition of molecules in culture, or in transgenic mice, has yielded several signaling systems that are able to influence collecting-duct development (Table IV). The first of these is hepatocyte growth factor–scatter factor (HGF/SF), which is produced by the mesenchymal stem cells of developing kidneys (Sonnenberg *et al.*, 1993; Woolf *et al.*, 1995); its c-MET high-affinity receptor tyrosine kinase is expressed by the epithelium of the ureteric bud–developing collecting duct (Sonnenberg *et al.*, 1993; Woolf *et al.*, 1995). The HGF/SF–c-MET system was therefore an obvious candidate paracrine regulator, and its physiological function was tested by the addition of function-blocking antibodies to kidney rudiments growing *in vitro*; the antibodies strongly inhibited collecting-duct development (Woolf *et al.*, 1995). This result provided such firm evidence for HGF/SF having an essential paracrine rôle that the result of an HGF–/–

Table IV Candidate Regulators of Collecting-Duct Development

Candidate regulator of collecting-duct development	Evidence for	Evidence against
HGF	HGF expressed in mesenchyme and its receptor (c-met) expressed by ureteric bud Acts as a branching morphogen in the MDCK cyst model system Rescues growth (<i>not</i> branching) in S-GAG-deprived kidneys Anti-HGF blocks renal development in culture	HGF–/– mice show no renal defects
GDNF	GDNF expressed by mesenchyme and its receptor (c-RET) by ureteric bud GDNF beads elicit supernumerary ureteric buds GDNF rescues branching in S-GAG-deprived cultures GDNF–/– mice show little or no collecting-duct development GDNF receptor (c-RET) mutants show reduced collecting-duct development	GDNF also expressed by mesonephros (which does not support collecting-duct arborization) GDNF supports growth but not branching of isolated ureteric buds

transgenic knockout experiment came as a surprise—the kidneys seemed to develop normally (Schmidt *et al.*, 1995; Uehara *et al.*, 1995). This paradox has yet to be resolved.

A morphogenetic function for the signaling system based on a second growth factor, GDNF, and its high-affinity receptor tyrosine kinase, c-RET, is supported by both culture and transgenic knockout data. GDNF is synthesized by uninjured metanephrogenic mesenchyme and nephrogenic stem cells, whereas c-RET is expressed by the epithelium of Wolffian duct and ureteric bud. Transgenic knockout mice of the *GDNF*^{-/-} and *c-RET*^{-/-} types show marked inhibition of ureteric bud–collecting–duct development, although both show some variation in phenotype severity from mouse to mouse. Treatment of kidneys growing *in vitro* with beads soaked in high concentrations of GDNF causes an increase in collecting-duct branching in the vicinity of the beads, whereas the growth factor maintains the morphology of isolated ureteric buds in hanging-drop culture (Sainio *et al.*, 1997).

Another growth factor whose absence from transgenic knockout mice results in the failure of collecting-duct development is BMP-7 (Dudley *et al.*, 1995; Luo *et al.*, 1995). The *BMP-7* gene is transcribed by the ureteric bud–collecting duct itself, as well as by nephrogenic stroma and developing nephrons, and loss of BMP-7 also inhibits the development of nephrogenic mesenchyme into nephrons (see the following). It is not therefore clear whether the collecting-duct defect in BMP-7-deficient mice results directly from the developing collecting duct requiring BMP-7 or from a secondary effect caused by abnormal development in the surrounding mesenchyme.

A slightly different approach to understanding duct morphogenesis has been the use of purified growth factors to “rescue” growth-inhibited kidney rudiments in culture. Many growth factors bind to sulfated glycosaminoglycan (S-GAG) coreceptors on the surface of cells that serve to concentrate these factors (and thus increase their chances of binding neighboring high-affinity receptors) and may facilitate binding to the high-affinity receptors by inducing conformational change (Rapraeger *et al.*, 1991; Lyon *et al.*, 1994). The depletion of cultured kidney rudiments of their S-GAGs results in complete and reversible inhibition of collecting-duct growth and branching [Fig. 6a of Davies *et al.* (1995)]. This inhibition can be relieved by simultaneous treatment with concentrations of certain growth factors at concentrations high enough to compensate for the lack of S-GAGs. Under these conditions, HGF/SF restores growth to the developing collecting-duct system without activating branching morphogenesis, so that the epithelium develops into an abnormally long unbranched tube [Fig. 6b of Davies *et al.* (1995)]. GDNF, on the other hand, activates the branching program well [Fig. 6d of Davies *et al.*, (1995)], so well indeed that it can even induce branching from the “wrong” end of the ureteric bud (Sainio *et al.*, 1997). The implication of these results, that the growth and branching aspects of arborization seem to be controlled separately, may have important implications for the development of

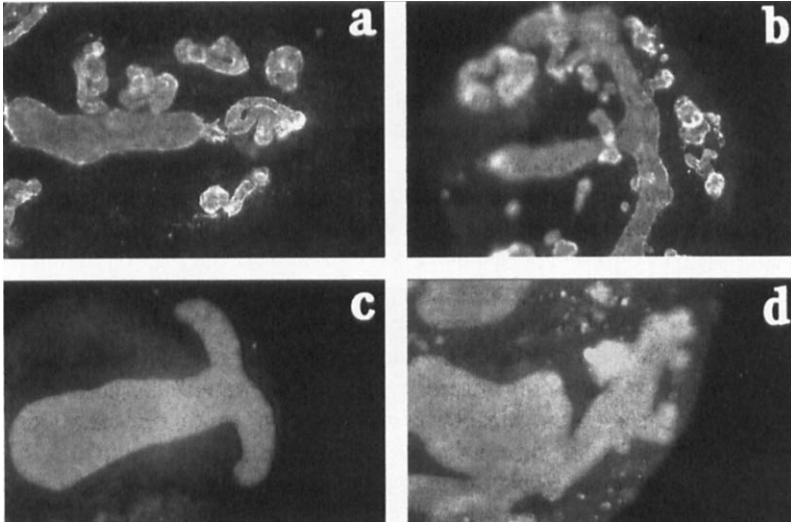


Fig. 6 Morphogenetic effects of HGF and GDNF in kidneys depleted of sulphated glycosaminoglycans. (a) Kidneys depleted of sulphated glycosaminoglycans (S-GAGs) show very limited collecting duct branching, compared with controls (e.g., Fig. 7, top panel), although they still form nephrons (panel (a)—nephrons are not visible in panels (c) and (d) because they are stained for a collecting-duct-specific marker). Treatment of the S-GAG-depleted kidneys with exogenous HGF causes their collecting ducts to extend but not to branch (b), while treatment with GDNF causes branching and swelling of the ducts without much extension (d—compare with the control culture (c)).

glandular organs in general. Each has a characteristic epithelial shape primarily governed by the surrounding stroma, so that, for example, salivary gland epithelium placed in lung stroma develops in the approximate shape of lung epithelium (Deucher, 1975). Different ratios of mitogenic and branch-generating signals might provide a simple explanation for the characteristic anatomies of glandular epithelia and the different degrees to which they fill three-dimensional space (their fractal dimensions).

2. Mechanisms of Collecting Duct Morphogenesis

Once it has received signals that induce it to grow and branch, the collecting-duct primordium must activate morphogenetic mechanisms that enable it to change its shape and mount a directed invasion of the surrounding stroma. The nature of most of these mechanisms remains mysterious, but some sketchy details are emerging from experiments.

Collecting-duct development traditionally has been described as involving terminal dichotomous branching. The situation may not, however, be that simple,

and two pieces of evidence, both obtained from culture work, suggest that internodal branching might also exist. A general property of trees that develop by terminal branching, first noticed by Leonardo da Vinci [see Long, (1994) for a review], is that the ratio of the diameter of a branch of generation n to that of a branch of generation $(n + 1)$ is a constant (da Vinci's number). This means that the ratio of the diameter of the trunk to that of the first main branches is the same as that of the first main branches to that of the secondary branches, and so on; in modern parlance, the tree shows self-similarity (fractal geometry). The arborization of many botanical trees, and also that of zoological "trees" such as canine airway epithelium, follows this general pattern (Nelson *et al.*, 1990). Attempts to calculate the da Vinci number (or the related fractal dimension) of the collecting ducts of kidneys developing in culture do not, however, work if one measures the branch generation number (primary, secondary, tertiary, etc.) by assuming that only terminal branching takes place. One can obtain a constant da Vinci number only by assuming that the internodes of branches of generation n can directly give rise to branches of generations $>(n + 1)$ (J. A. Davies, unpublished). Tantalizing as these results are, the question of whether internodal branching really takes place or whether branching is always terminal but can be very unequal will only be settled by the time-lapse studies underway. The second piece of evidence comes from restarting the arborization of ureteric buds whose branching development has been stopped; new branch tips arise along the lengths of old branches, not just at their termini (Davies *et al.*, 1995).

Whatever the choices of branch pattern available, investigation of their generation mechanisms is made simpler by the fact that branching seems to be independent of growth; ureteric buds of kidneys treated with concentrations of methotrexate sufficient to block DNA synthesis (and therefore cell cycling) still undergo branch initiation, although the branches fail to grow out (Davies *et al.*, 1995). This result is in accord with similar observations on the independence of branching from growth that have been obtained in the developing salivary gland (Nakanishi and Ishii, 1989).

Branching morphogenesis requires certain regions of a tubule wall to bend; in these regions, cells must become wedge-shaped, with a narrowed basal surface along the convex curve at the tip of the branch and a narrowed apical surface at concave surfaces where the branch leaves its parent tubule. One obvious potential mechanism for mediating this deformity of cell shape is cytoskeletal tension generated by actin and myosin; another is pressure of growth. Actin microfilaments are indeed concentrated at the sites of maximum cell deformation (J. A. Davies, unpublished, Fig. 7), but there is as yet no unambiguous evidence that the actin–myosin interaction is the main agent for branch initiation. Drugs such as cytochalasin D, which interfere with microfilament polymerization, do block branch initiation very effectively (B. Harron, L. Ramage, and J. A. Davies, unpublished), but far too many cellular processes are likely to be affected by this treatment to allow firm conclusions to be drawn.

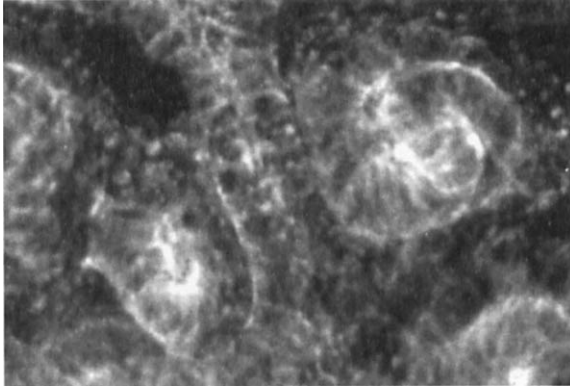


Fig. 7 Phalloidin staining of developing nephrons reveals a high concentration of filamentous actin where bending of the tubule is taking place, as predicted from a model in which localized “purse-string” contraction of apical microfilament networks drives bending morphogenesis.

In addition to changing its shape to initiate new branches, the growing collecting-duct epithelium has to invade the mesenchymal matrix that surrounds it. The mechanisms underlying this have not been studied in detail, but evidence implicates matrix metalloprotease 2, whose activity correlates with collecting-duct morphogenesis *in vitro* (McCormack and J. A. Davies, unpublished).

D. Mesenchyme Induction

1. The Inductive Signal—Evidence from Tissue Culture Experiments

The signals by which ureteric bud induces metanephrogenic mesenchyme to undergo further development have not yet been identified, although something is known of their general characteristics and a few candidate molecules now exist. The candidacy for each is supported by some experimental evidence. So far, however, no molecules meet all of the Slack criteria (Slack, 1993) for being renal inducers. In the case of the kidney, these criteria are as follows (Davies, 1996): (1) The molecule(s) must be present in the ureteric bud tips as long as induction takes place. (2) The candidate molecule(s) must be capable of inducing nephrogenesis or at least some aspect of the process in completely uninduced mesenchyme. (3) Inhibition of the molecule(s) should block induction in an intact kidney (provided there is no redundancy).

For many years, the induction of mesenchyme was believed to require direct cell–cell contact between the inducing and the induced cells. The evidence for this view came from transfilter experiments in which the mesenchyme was separated from the inducing tissue by a polycarbonate filter of sharply defined pore

size. Generally, the inducing tissue used was spinal cord rather than ureteric bud, because embryonic spinal cord is a much stronger inducer (Grobstein, 1955). When the filter pores were large enough to allow penetration by cell processes, transfilter induction took place (Fig. 8), but if the filter pores were too small to allow penetration by cell processes, then induction failed (Saxén *et al.*, 1976; Saxén, 1980). The obvious conclusion was either that the inducer was tightly bound to the cell surface, so that it could not diffuse away and act at a distance, or that a combination of its half-life in free solution and the concentration required for it to act limited the range of an effective concentration to a distance so small as to be indistinguishable from contact. Electron microscopy confirmed that contact takes place in the large-pore transfilter system and demonstrated that there were no morphological specializations at the sites of intercellular contact.

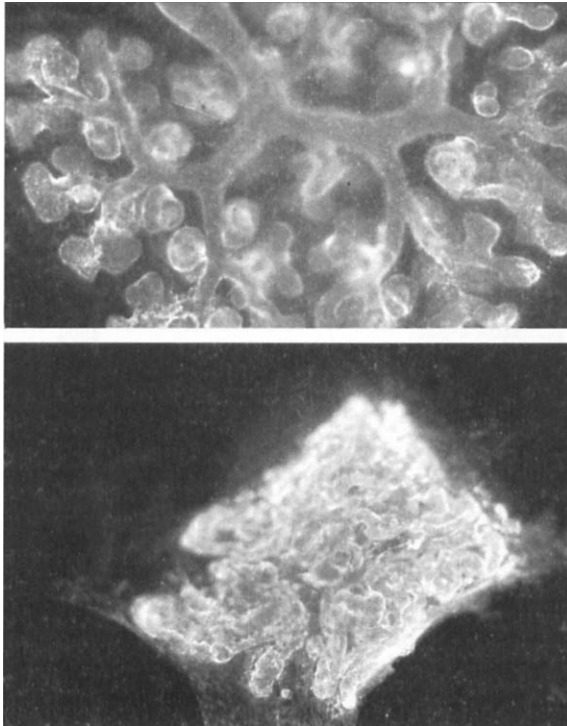


Fig. 8 Two commonly used methods of inducing nephron development in culture. In the top panel, a complete E10.5 murine kidney rudiment has been cultured on a filter at the gas-medium interface; its collecting duct system has branched and induced nephrons to form from the surrounding stroma. In the bottom panel, isolated metanephrogenic mesenchyme has been cultured on a filter underneath which is embryonic spinal cord; the spinal cord has induced the formation of many nephrons in the mesenchyme.

The classical transfilter experiments that led to the view that contact was a necessary feature of induction shared one methodological feature: A blob of agar or agarose was used to attach the inducing tissue to the underside of the filter (the mesenchyme was cultured on the filter's upper surface). This agar(ose) was regarded as a neutral "glue." However, it has become clear that many growth factors (e.g., FGF-2 and HGF) bind to sulfated glycans (particularly heparin sulfate). If the inducing molecule were to bind to the sulfated glycans present in agar, then the blob of glue may in fact have sequestered a diffusible inducing molecule before it was able to pass beyond the glue and through the filter pores. To explore this possibility, the classical transfilter experiments have been repeated but instead using a sandwich of filters instead of agar to hold the inducing tissue (again spinal cord) in some cultures. In the absence of agar, induction took place even across multiple layers of filters with pore sizes too small to admit cell processes; exclusion of these processes was confirmed by SEM (Davies and Bard, unpublished). These data lead to the view that the inducing molecules from spinal cord are capable of acting tens of cell diameters from their source. Unfortunately, transfilter induction by normal ureteric bud cannot be obtained even with large-pore filters, so that we still know nothing about the diffusibility or otherwise of signals from the natural inducer of nephrogenesis. However, induction of at least the first stages of development (see Section IV.D.2 for a discussion of multistage induction) can be performed by conditioned medium from a ureteric-bud-derived cell line, strongly suggesting that ureteric bud, too, induces at least some stages of the process by diffusible means (Barasch *et al.*, 1996).

2. Candidate Molecules

The implications of the preceding experiments—that the inducer is diffusible and interacts with sulfated glycans—are in accord with the results of one quest for the biochemical identity of the inducing molecule (see Table V). Perantoni *et al.* (1995) have identified the critical component of a cell extract that can induce nephrogenesis in the rat as FGF-2 and have gone on to show that very high concentrations of purified FGF-2 can induce nephrogenesis in isolated rat metanephrogenic mesenchyme in culture, albeit unusually slowly. In its favor, FGF-2 is present in the ureteric bud throughout the period of induction (Dono and Zeller, 1994), and it is present in the artificial inducers of nephrogenesis such as spinal cord. However, several facts argue against FGF-2 being the natural inducer. First, FGF-2 fails to induce mouse nephrogenic mesenchyme; this implies either that rats and mice induce kidney development very differently, which would be very surprising, or that FGF-2 by chance happens to mimic the natural inducer in rats, but itself is not that inducer. Second, FGF-2 is produced by tissues that are not inducers of nephrogenesis, including nephrons themselves. Third, treatments that would be expected (from other systems) to inhibit FGF-2 action do not inhibit nephrogenesis, at least in mouse. For example, the removal of cellular sulfated

Table V Candidate Inducers of Nephrogenesis

Candidate inducer	For	Against
FGF-2	Purified FGF-2 induces nephrogenesis in isolated rat mesenchymes FGF-2 is produced by ureteric bud	FGF-2 fails to induce mouse mesenchyme FGF-2 is also produced by tissues that do not induce nephrogenesis (including nephrons themselves)
BMP-7	Produced in the right place BMP-7 will induce isolated rat nephrogenic mesenchymes Inhibition of BMP-7 inhibits nephrogenesis BMP-7 ^{-/-} mice show a severe deficiency of nephrons	Produced in tissues that do not induce (mouse data)
Wnt proteins	Wnt-1-transfected cells induce mouse mesenchyme Lj ⁺ -mediated induction fits a Wnt model	No Wnt known to have the expression expected of an inducer

glycosaminoglycans by chlorate ions [which compete with sulfate ions in the synthesis of phosphoadenosine 5'-phosphosulfate, the sulfate donor utilized by sulfotransferases that mediate polysaccharide sulfation (Farley *et al.*, 1978; Rapraeger *et al.*, 1991)] or by heparitinase enzymes is known to inhibit the effects of FGF-2 in cell lines. These treatments do not, however, prevent nephrogenesis *in vitro* (Davies *et al.*, 1995). Treatment of kidney rudiments developing *in vitro* with function-blocking anti-FGF-2 also fails to block nephrogenesis (J. A. Davies, unpublished).

Another growth factor, BMP-7, is a second candidate for an inducing molecule. BMP-7 appears to meet the three criteria mentioned earlier. It is produced in the ureteric bud at the right time, it induces isolated rat mesenchyme (no mouse data are available), antibodies to BMP-7 block nephrogenesis, and transgenic BMP-7^{-/-} mice show a severe shortage of nephrons (Fig. 9e, f). These data, therefore, make it a very strong candidate for being the inducer. However, BMP-7 is also produced by developing nephrons themselves. In mouse, developing nephrons are known not to possess inducing activity (Saxén and Saksela, 1971), a fact that is difficult to reconcile with BMP-7 being the inducing molecule. An alternative explanation for the role of BMP-7, and one equally compatible with the data, is that BMP-7 is necessary for the subsequent nephrogenic development of cells induced by another molecule or for the maintenance of the stem-cell population.

Two strands of evidence implicate Wnt proteins in the induction of nephrogenesis. First, Herzlinger *et al.* (1994) have demonstrated that cells that are not normally inducers of nephrogenesis become inducers when transfected with

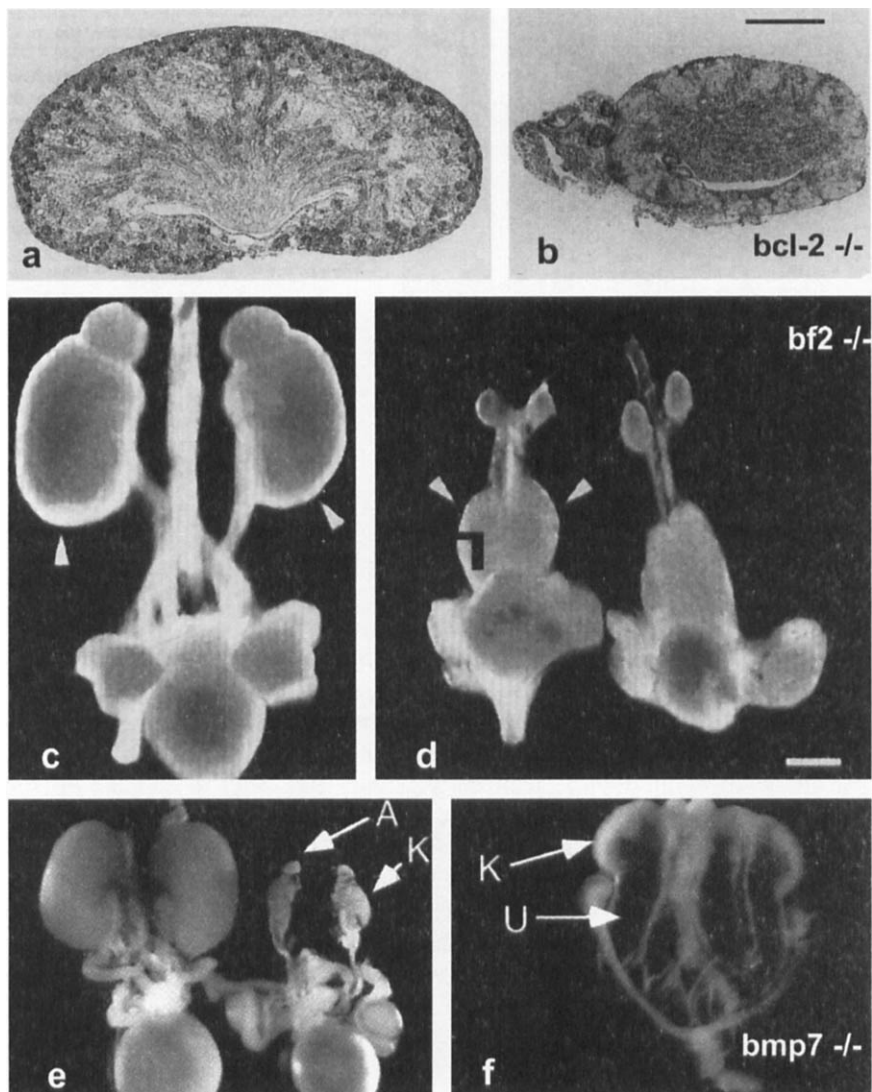


Fig. 9 Transgenic kidneys. *bcl-2* (a,b) BF-2 (c,d) and BMP-7 are three genes whose absence leads to kidneys that are much smaller than wild-type ones in adult mice. (a, b) Sections of a normal mouse kidney and the very much smaller kidney from a *bcl-2* $-/-$ mouse. Bar = 0.2 mm. (Courtesy of Nagata *et al.* (1996). *Am. J. Pat.*, **148**, 1601–1611.) (c, d) Kidneys (arrow heads) from a normal (c) and from a BF-2 $-/-$ (d) mouse. Bar = 1.5 mm. (Courtesy of Hatini *et al.* (1996). *Genes & Development*, **10**, 1467–1478.) (e) Kidneys (arrow heads) from an E19 BMP-7 $-/-$ mouse (A: adrenal, K: kidney) compared with those from a normal mouse. (f) The acute hydronephrosis phenotype (distended renal pelvis and ureter) shown by the majority of newborn BMP-7 $-/-$ mice. (Courtesy of Dudley *et al.* (1996), *Genes & Development*, **9**, 2795–2807.)

Wnt-1 cDNA. Wnt-1 itself is not present in developing kidneys (Wilkinson *et al.*, 1987) and so cannot be the inducer, but the promiscuous interactions within the Wnt signaling pathway suggest that another Wnt protein might be the inducer. So far, however, no Wnt proteins have been found to have the expected distribution of an inducer of nephrogenesis (present in ureteric buds but not in other renal tissues), although they are clearly involved in events downstream of induction (see Section IV.E.2).

The second strand of evidence for Wnt signaling comes from the observation that lithium ions induce the early stages of nephrogenesis in isolated mouse mesenchymes (Davies and Garrod, 1995). The primary biological effect of lithium ions is known to be inhibition of the enzyme, glycogen synthase kinase 3 β (GSK-3 β , the mammalian homologue of the *Drosophila* gene *shaggy/zeste-white 3*; Klein and Melton, 1996). GSK-3 β lies downstream of a Wnt signaling pathway, and binding of Wnt proteins to their receptors indirectly results in the inhibition of GSK-3 β . Li⁺-mediated inhibition of GSK-3 β therefore can be viewed as a means of mimicking Wnt-mediated signaling (though it might of course have other effects too). It therefore provides more circumstantial evidence in favor of Wnt proteins. Against the Wnt story, however, is the difficulty in reconciling it with the transfilter data described earlier that suggest that the inducer is a diffusible agent that binds to charged carbohydrate.

There are, then, at least three candidate inducers, each with some evidence in its favor and some evidence against. The confusion of the data and their interpretation probably reflects the complexity of the system and the fact that a succession of inductive and permissive factors appears to be necessary for complete nephrogenesis. The increasing evidence that there are, in fact, two stages of induction (see Section IV.D.3) and therefore perhaps two distinct inducers might help to clarify apparent contradictions in the data described earlier.

3. How Many Stages Are There in Induction?

The fate of isolated, uninduced metanephrogenic mesenchyme is to exist for a few days with very little cell division and then to die by apoptosis; this is true both in culture (Koseki *et al.*, 1992) and in mutants in which the ureteric bud fails to develop (e.g., WT1^{-/-} and c-ret^{-/-}; Kreidberg *et al.*, 1993; Pachnis *et al.*, 1994; Schuchardt *et al.*, 1994). The fate of mesenchyme that has been invaded by a ureteric bud is to proliferate sufficiently such that it can eventually produce 1000–2000 nephrons (discussed earlier). Once the metanephrogenic mesenchyme has been invaded by the bud, it has to set aside its death wish and multiply quickly.

A decade ago, it seemed likely that induction was a single-stage process, with the MM just growing while those cells in contact with the bud were induced to form nephrons. This view is no longer tenable as we know that all of the MM cells are rapidly induced to switch out of an apoptotic and into a growth phase, whether or not they are in contact with the bud (e.g., Koseki *et al.*, 1992). Only

later do small groups of cells then enter the nephrogenic pathway. Furthermore, this early change in growth pattern is reflected in changes taking place in the expression profile of the MM (e.g., the low-affinity NGF receptor is replaced by TrkB and -C; see below for more details).

The change in behavior of the metanephrogenic mesenchyme cells upon being invaded by the ureteric bud is now regarded as a change in phenotype from uninduced metanephrogenic mesenchyme into nephrogenic stem cells and, hence, induction is a two-stage process. The first stage consists of an interaction that induces the metanephrogenic mesenchyme into becoming stem cells, and the second induces groups of stem cells to become nephrons. Alternative models in which a single inductive event causes a cell to divide into one daughter committed to nephrogenesis and another remaining mesenchymal to maintain the mesenchymal population are simply incompatible with the massive amount of growth that takes place.

The idea of multistage induction is gaining increasing experimental support. It has long been known that a rise in DNA synthesis precedes nephrogenesis by many hours (Saxén *et al.*, 1983). Following spinal cord induction, for example, there is a bout of DNA synthesis after some 8 hr of contact with an inducer, whereas the first signs of nephrogenesis can only be seen at 18 hr (Davies and Garrod, 1995). This pattern of timing is at least compatible with the idea of a first induction to stem cells followed by a second to nephrogenesis itself. Much more significant support for the model, however, has come from the observation that conditioned medium produced by immortalized ureteric bud cells can induce metanephrogenic mesenchyme into the stem-cell state (as assessed by the cessation of apoptosis and commencement of vigorous mitosis; Barasch *et al.*, 1996) without inducing progression into nephrogenesis itself. The second induction requires contact with the basolateral surfaces of the ureteric bud; cell-conditioned medium is not enough (Barasch *et al.*, 1996), although in the case of the rat, ureteric-bud-conditioned medium plus FGF-2 plus TGF α does induce nephrogenesis (Karavanova *et al.*, 1996). These two inductions could be mediated by different molecules. Alternatively, they could be mediated by a single molecule with threshold-dependent effects; a low concentration would effect the switch to stem cells, while a higher concentration would be required for nephrogenesis. This model would account for the fact that cells undergoing nephrogenesis are located close to the developing collecting-duct system whereas cells farther away multiply without differentiating.

An unexplained feature of nephron induction, so far observed with both living (e.g., spinal cord) and pharmacological (e.g., Li⁺) inducers, is the requirement that the inducer be present for at least 12–15 hr. Shorter exposures are insufficient (Davies and Garrod, 1995). This is a long time compared with that required for the transcription and translation of new genes (1–2 hr), for example, and raises the question of what the delay is for. The length of the period, 12–15 hr, is compatible with a connection to the cell cycle and perhaps suggests that cells must pass through a cycle before they become committed to epithelial differen-

tiation. There is, so far, no evidence that every induced cell passes through a cell cycle during the inductive phase, but it is known that inhibition of cell cycling (DNA synthesis) using mitomycin C blocks the induction of epitheliogenesis (Nordling *et al.*, 1978), although we do not know the precise stage at which the process halts.

Transition to the stem-cell state appears to be accompanied by the decline in apoptosis and the appearance of molecules absent from uninduced mesenchyme (I use the slightly vague phrase “appears to be” because the correlation of the molecules with the stem-cell state so far is based on distribution and timing rather than formal correlation with cell cycling). These include the transcription factors Pax-2 and Hox-C9 and the signaling molecules HGF, c-MET, and p75-NGFR. HGF and its receptor c-MET have the potential to form an autocrine loop because both are expressed in the same cells (Woolf *et al.*, 1995). The existence of such a loop is supported by the effect of adding antibodies that interfere with the HGF-c-MET interaction, which results in markedly increased apoptosis in what should be the stem-cell population. The antibodies also block subsequent differentiation, although it is not clear whether this results from a failure of earlier proliferation. There must be some redundancy *in vivo*, however, as HGF knockout mice have normal kidneys. Antisense inhibition of p75-NGFR synthesis in culture also blocks renal development (Sariola *et al.*, 1991), although once again the knock-out mouse shows no renal defect (Lee *et al.*, 1992).

Induction, or at least the progression of cells from the induction to condensation stages, can be blocked reversibly by the addition of the chemokine LIF to culture medium [Bard and Ross (1991)]. This blockage only works for ureteric-bud-mediated induction, however; induction by surrogate tissues such as spinal cord takes place normally. The effect of LIF therefore might be to abolish the inducing activity of the ureteric bud. Normally, data on the distribution of the LIF receptor could be used to indicate the likelihood of this, but the only known receptor for LIF does not appear to be expressed anywhere in the developing kidney, making the effect of the chemokine even more mysterious. LIF receptor knockouts have no reported renal phenotype (Ware *et al.*, 1995), nor do transgenic mice that lack IL6 or CNTF, both of which also bind to components of the LIF receptor (Dedera *et al.*, 1996; Masu *et al.*, 1993). The one deduction that can be made on the basis of the signal data at hand is that few receptors can be as promiscuous as those in the kidney!

4. The Relationship between Stromal and Nephrogenic Cells

In the most common model of lineage in the developing kidney (see Section IV.B), cells have to “decide” some time after their induction (one of their inductions) whether to follow a nephrogenic or a stromogenic course (or differentiate into the juxtaglomerular apparatus, etc.). So far, nothing is known about the mechanisms underlying this decision, although it is possible that almost any of the mutants that block nephrogenesis (Table III) may do so by shunting all of the

cells into a stromal fate; analysis of mutants tends not to include the use of molecular markers that will indicate stromal differentiation, partly because there are few of these. The same argument applies to the effects of factors that block nephrogenesis *in vitro*, such as LIF (see Section IV.D.3).

A mechanism that used to be thought feasible for explaining the splitting of mesenchyme into different fates was based on contact with the inducing tissue: If a mesenchyme cell made such a contact with it, it underwent mesenchyme-to-epithelial transition (MET), otherwise it remained mesenchymal and became stroma. Several experiments have, however, cast doubt on this view, particularly the discovery of pharmacological means to carry out induction (see Section IV.D). Isolated mesenchymes induced with either Li^+ ions or bFGF produce a mosaic pattern of developing nephrons and surrounding stroma, despite the fact that these small molecules will have reached all of the cells in the culture. If the nephrogenic and stromogenic cells arise from lineages that were separate even at the beginning of kidney development (model 2 in section IV.B), all that needs to be explained is the sorting out of a mixed population into a mosaic pattern. This may be explained rather easily by known changes in cell adhesion molecule expression (e.g., of NCAM; Klein *et al.*, 1984). If, as is usually assumed, the MM consists of one cell type that gives rise to both stroma and nephrons, we must explain how the choice of fate is made.

One possibility is a system of lateral inhibition mediated by signaling molecules in interacting feedback loops like that based on *Notch* and *Delta*, which divides ectodermal cells of *Drosophila* into neurogenic and epithelial fates (Simpson *et al.*, 1992). The vertebrate homologues of *Notch*, mouse *notch1* and *notch 2*, are indeed expressed in the developing kidney at about the right time and place (to the resolution of published data). Transgenic knockout of *notch1* results in lethality too early in development for any metanephric effects to be assessed; a system based on these molecules would involve only nearest neighbor interactions anyway and so would not be ideal for the establishment of islands of many tightly aggregated cells of similar fates. Partitioning between fates remains one of the most baffling problems of renal development.

E. The Downstream Effects of Nephron Induction

After they have been induced to begin their nephrogenic program, cells must aggregate, undergo an epithelial–mesenchymal transition, and, once epithelial, differentiate into the various specialized regions of a mature nephron. We now consider the limited data on these events.

1. Condensation

The first morphological consequence of nephron induction is the formation of tight aggregates of nephrogenic cells some 5–6 cells in diameter within the

already dense metanephric mesenchyme, and these soon undergo a mesenchyme-to-epithelial transition. In cultured kidney rudiments, condensation lasts approximately 6 hr, beginning some 18 hr after first contact with an inducer (Davies and Garrod, 1995). The mechanisms responsible for aggregation are not known, but work on other examples of mesenchyme condensation (see Bard, 1992) suggests the following possibilities (which are not mutually incompatible):

1. Increased intercellular adhesion caused by the expression of new adhesion molecules
2. Local disappearance of interstitial matrix
3. Directed migration
4. Localized cell division
5. Generation of tractional forces

The first model is supported by the fact that adhesion molecule expression does indeed change just before aggregation begins. For example, expression of the homophilic neural cell adhesion molecule, N-CAM, is strongly up-regulated, as is expression of the proteoglycan, syndecan-1 (Vainio *et al.*, 1992). Inhibition of N-CAM function, by antibody or transgenic knockout techniques (Klein *et al.*, 1988; Cremer *et al.*, 1994) does not, however, prevent normal nephrogenesis. The removal of syndecan-1's sulfated glycosaminoglycan side chains also fails to block nephrogenesis (Davies *et al.*, 1995), although there is as yet no information about the effects of inhibiting the synthesis of the syndecan-1 protein core. These few experiments cannot, of course, rule out the adhesion molecule hypothesis for aggregation, as the cells might also express additional, undiscovered adhesive systems. An explanation based on the specific and local expression of such adhesion molecules does imply the need for a prior mechanism specifying the population of cells that will undergo this change.

The evidence to support the second and third of these mechanisms is weak. Before condensation, mesenchymal cells are separated by a prominent interstitial matrix consisting of fibronectins, collagens I and III, and glycosaminoglycans, whereas in the condensates themselves cells are in very close apposition and all signs of interstitial collagens and fibronectins disappear (Ekblom, 1981; Ekblom *et al.*, 1981; Laitinen *et al.*, 1991). The rapid removal of these components suggest localized activity of degradative enzymes, but it is not clear that this activity is enough to account for condensation itself. In other systems, such as the condensation of somatopleuric mesenchyme to form cartilage, localized loss of extracellular matrix components seems to play an important role in bringing cells closer together to form the initial condensation (Toole, 1972), although N-CAM production can also be important here [for a review, see Hall and Miyake (1995)]. It will be interesting to determine whether there is any relationship between the formation of nephrogenic and cartilaginous condensations.

Evidence in favor of mitosis as the cause of condensation is also lacking. Localized cell multiplication without migration can produce an illusion of condensation as more and more cells fill a given space, and the necessity for cell

cycling in nephrogenesis referred to earlier is compatible with this mechanism. However, there is as yet no evidence that the condensation phase is associated with particularly high levels of mitosis, nor would mitosis provide a rapid enough increase in cell concentration to fit with the speed of condensation morphogenesis.

If the evidence supporting cell-adhesion molecules, migration, growth, and loss of extracellular matrix seems too weak to support them as mechanisms of condensation, what other possibilities are there? One that has yet to be excluded is cell traction, a mechanism based on the balance of two forces: the adhesions that cells make to one another and to their environment and the contractile abilities of the cells. Harris *et al.* (1984) have shown that these forces can cause uniform cell culture to break up into aggregates. One reason for suggesting that tractional forces may play a role in the formation of nephrogenic condensations comes from the following simple experiment (J.B.L. Bard, unpublished): If kidney rudiments are cultured on inert substrata, they adhere, spread, and form about 20–30 nephrons. If, however, such rudiments are cultured in hanging-drop culture where there is no substratum, no more than two or three nephrons form, even though other tissues such as salivary glands develop normally under these conditions. Such a result demonstrates the need for substratum adhesions if condensations are to form (Bard, 1990), but does not, of course, prove that this need is manifested through traction.

An article on the effects of knocking out the transcription factor BF-2 (Hatini *et al.*, 1996) has added a new perspective to the condensation story. This gene, one of the smallish family of “winged-helix” or “fork-head” transcription factors that has an evolutionarily conserved DNA-binding domain found in *Drosophila* homologues (Lai *et al.*, 1993), is expressed in two populations of mesenchymal cells: those that will form the medullary stroma and a second population in the cortex that does not participate in nephrogenesis and may become the juxtaglomerular cells that will make renin. Until this research was published there was no reason to suppose that the stromal cells had any effect on nephrogenesis, but, quite unexpectedly, the small kidneys of the BF-2^{-/-} mouse were found to contain relatively few but very large mesenchymal aggregates that failed to form nephrons (Fig. 9c, d). There was also an abnormally small number of branches in the collecting-duct system. The large mesenchymal condensates were up to 20 cells in diameter and expressed *wnt-4*, a marker of the late condensation stage (Gavin *et al.*, 1990), but their further differentiation seemed to be blocked.

The abnormalities in the BF-2 knockout show that signals from stromal cells play a role in the condensation process. It is not, however, clear whether these factors are the same as those whose loss restricts the general growth of the BF-2^{-/-} kidney. In short, we have no complete explanation as to how a balance of chemical signaling and physical forces leads to the formation of nephrogenic condensations within the relatively dense and adhesive metanephric mesenchyme, and this puzzle is one of the more intriguing facets of kidney develop-

ment. It is, however, clear that a great deal more needs to be done with the kidneys of the BF-2 knockout mouse to determine whether its expression patterns of known kidney growth factors are altered.

2. Epitheliogenesis

Once condensation has been completed, the cells in the aggregate undergo a mesenchymal-to-epithelial transition, a process that in culture lasts for about 24–36 hr (Davies and Garrod, 1995). During this process, markers characteristic of mesenchyme cells (e.g. fibronectin, vimentin) are lost, and those characteristic of epithelia (e.g., E-cadherin, desmoglein, cytokeratins) are gained according to a precisely timed sequence [see Davies (1996) for a summary of the timing].

There appears to be a checkpoint of development between condensation and epitheliogenesis, presumably to ensure that further development does not take place until condensation is complete. The signaling molecule Wnt-4 might be involved in this process. Wnt-4 is produced by condensing cells and is subject to positive feedback, so that it stimulates its own synthesis (Stark *et al.*, 1994). In a Wnt-4 knockout mouse, cells do not progress beyond the condensation stage, although the condensates themselves become rather large, suggesting that in the absence of Wnt-4 either a signal indicating that condensation has gone on long enough is missing or the cell multiplication that would normally take place to elongate nephrons takes place, even though nephrogenesis itself is blocked.

The molecular processes that regulate the large-scale change in gene expression accompanying mesenchyme-to-epithelial transition remain completely unknown. Several potentially significant transcription factors are first detectable around this time, including Hox-B3, Hox-B7, LFB-3, and Pax-8, but, on the basis of the timing of their expression or the behavior of $-/-$ mice, none seems to be a master regulator of epitheliogenesis. One of the functions of Pax-8 that is compatible with the binding-site data (discussed earlier) may be the down-regulation of WT1 in most of the condensate while allowing its upregulation in the podocyte layer (Pritchard-Jones *et al.*, 1990).

The process of acquiring an epithelial phenotype also involves the acquisition of a new set of cell–cell and cell–matrix adhesion molecules, including the E- and K-cadherins, α_6 -integrin matrix receptors, and laminin A in the basement membrane (Klein *et al.*, 1990; Xiang *et al.*, 1994; Ekblom *et al.*, 1990; Korhonen *et al.*, 1990, 1992; Sorokin *et al.*, 1990). Antibodies to E-cadherin fail to block nephrogenesis (Vestweber *et al.*, 1985), although this may simply reflect redundancy with K-cadherin. Antibodies that inhibit the interactions between α_6 -integrin and laminin A block nephrogenesis in culture (Klein *et al.*, 1988), suggesting a critical role for integrin-mediated attachment of cells to their basement membrane. Data have, however, shown that nephrogenesis apparently takes place normally in α_6 -integrin-deficient transgenic mice (Georges-Labouesse *et al.*, 1996).

3. Nephron Maturation

In the S-shaped body, the proximodistal polarity that is set up by unknown mechanisms at or before the comma stage becomes manifest in the differentiation states of the cells. Cells at the most proximal end flatten (Dorup and Maunsbach, 1982) and alter their gene expression, losing *c-myc*, *Hox-c9*, *LFB-1*, and *LFB-3*, but retain expression of *WT1*. Loss of these transcription factors is accompanied by what can be considered partial reversion to a mesenchymal phenotype: Glomerular podocyte cells express the "mesenchymal" markers α_3 -integrin and vimentin and cease to express c-MET and cytokeratins, but they continue to express the "epithelial" markers α_6 -integrin and desmosomal components (Holthofer *et al.*, 1984; Korhonen *et al.*, 1990, 1992; Garrod and Fleming, 1990; Sonnenberg *et al.*, 1993; Woolf *et al.*, 1995). The result of this is an arrangement of cells more organized into a sheet than a typical mesenchyme but more leaky than most epithelia, making an excellent primary filter for urine production.

The remaining cells of the S-shaped tubule remain classically epithelial but differentiate into regional segments, with segment identity being expressed (in terms of known molecular markers) in a proximodistal temporal sequence. All of the cells reduce their expression of *WT1* and also lose *N-myc*, *MFH-1*, and *Pax-2* (Lazzaro *et al.*, 1990; Poleev *et al.*, 1992; Mugrauer *et al.*, 1991; Miura *et al.*, 1993; Armstrong *et al.*, 1992; Dressler *et al.*, 1992; Rothenpieler and Dressler, 1993). If *Pax-2* loss is prevented in transgenic mice, differentiation of the tubule is inhibited and a pathological condition similar to nephrotic syndrome is produced (Dressler *et al.*, 1992).

At about this time, the developing nephrons interact with the vascular system of the developing kidney. Before they are of any physiological use, nephrons have to be connected to a blood supply at their glomerular end (the source of urinary fluid) and the collecting duct at their distal end. The first indications of a blood supply to the glomeruli are present very early in nephron development, when capillaries appear in the glomerular cleft (Loughna *et al.*, 1996). Most evidence indicates an angiogenic origin for these vessels (i.e., by the sprouting of preexisting vessels), the most significant observation being a lack of capillary development in kidney rudiments removed from E11 mice and cultured away from any external sources of endothelium (Bernstein *et al.*, 1981). Kidney rudiments elicit a strong outgrowth of capillaries if cultured on avian chorioallantoic membrane (Sariola *et al.*, 1983) and express vascular endothelial growth factor (VEGF; Landels *et al.*, 1994), which is known to be a major enhancer of angiogenesis in other systems (Breier *et al.*, 1992; Millauer *et al.*, 1992). VEGF is expressed by the glomerular epithelium, whereas its *flt-1* receptor is expressed by the endothelia themselves (Breier *et al.*, 1992; Simon *et al.*, 1995), suggesting a paracrine loop that might also be responsible for attracting growing capillaries to the correct site (although how they generate the complex glomerular architecture is still a mystery).

Connection to the collecting duct is achieved by fusion of the nephron and collecting duct epithelia, a process that again is not understood but that presumably includes the localized destruction of two basement membranes and the rearrangement of cell–cell contacts.

F. Development, Renal Function, and Kidney Disease

At this stage, when the basic structure of the kidney is in place, the reader might expect to find a section on the acquisition of the functional abilities of the organ that would in turn lead to an additional section on how these properties go awry in congenital kidney disorders. As almost nothing is known about the regulation of either of these two facets of the kidney story, these sections are conflated to this brief note.

We assume that the functional abilities of the various parts of the nephron derive from the pattern-formation processes that segment it into its proximal, loop, and other segments, but as yet have no knowledge of these events nor of their downstream effects. As for the congenital renal diseases, we should not confuse our success in identifying mutant genes with an understanding of why such mutations lead to abnormal kidneys. While we have a fair understanding of how mutated collagen IV genes help explain the abnormal glomerular filtration that characterizes the Alport syndrome, we have little idea as to how the other genes documented in Section III wreak their effects.

Here, it is interesting to look at Wilms' tumor, the first of the congenital renal disorders to be traced to a named gene, *WT1*. While it is true that mutations in this zinc-finger transcription factor can account for some 15% of the tumors, it is not obvious why a mutation here should lead to a single cell forming a 2-kg tumor composed of a disorganized mass containing what seem to be the appropriate cell populations. Moreover, a decade or so after the cloning of *WT1* we still await *WT2*, *WT3*, *WT4*, etc. (to account for the remaining 85% of the tumors). The only appropriate position to take is that a complex chain of events is involved in initiating kidney development and that the breakage of a single link can flip the system onto an abnormal trajectory that is recognized as a cancer.

The multigenic basis for renal disease is shown most sharply in PKD where at least six genes have been mapped or cloned, in which mutations can lead to cysts. Here, the use of gene targeting to make mouse models of the disease (e.g., Moyer *et al.*, 1994) is likely to provide the most profitable line of investigation in elucidating the molecular basis of the disorder. A further advantage of this approach is that by studying a congenital abnormality we learn a great deal about normal development.

Nevertheless, in spite of an approach that will, in due course, provide dividends to both the renal embryologist and the "real" nephrologist, an interesting tension remains between them. The former looks at the early stages of kidney

formation, particularly the emergence of the structures that define the organ, while the latter wants to know more about the ways in which mutations in kidney differentiation lead to congenital diseases. The practical nephrologist thus is likely to be disappointed with the general focus of contemporary work, which is mainly on early organogenesis rather than on later differentiation. A possible consolation, however, is that the study of the differentiation of the functional abilities of the kidney is empty, if not easily accessible, territory begging to be invaded by those seriously interested in medical research.

V. The Future

A. Where We Are Now

The increase in our knowledge about kidney development in the decade since Saxén's monograph (1987) has been dramatic and has demonstrated the remarkable power of molecular genetic technology in opening up a field in which progress was becoming frustrated. In 1987, we knew little more about the genetic control of kidney development than that the transcription factor *WT1* was important in regulating kidney development and that perhaps 15 or 20 regulatory genes of one sort or another were expressed during nephrogenesis. By now, researchers in the field have identified almost 300 genes that are expressed during the various stages of kidney development. We also know some of the signals and their downstream effects, we understand a great deal more about the various stages of kidney development and the interactions that underpin them, and we have mastered many new technical approaches to kidney development. The optimists among us can feel that a good job has been done over the past decade.

Nevertheless, there is a sense in which those dyed-in-the-wool pessimists who claimed that the advent of molecular technology would bring a surfeit of facts but little understanding can feel vindicated. They can point to the fact that we still understand almost nothing about what all of those genes do and that, even in a case like *WT1* where we know how much hangs on the activation of a single, key transcription factor, we still have little idea of what its downstream effects are and still await the cloning of those genes that account for the 85% of Wilms' tumors where *WT1* is not involved. It is even possible for them to have the gloomy satisfaction of saying that all of the millions of dollars spent on kidney research have done little more than demonstrate how much more complicated kidney development is than anyone expected.

We hold no truck with these views and think that those who hold them wanted to believe that elucidating the genetic basis of kidney organogenesis should be easy. In our view, the immense progress over the past decade has provided the study of kidney development with a far stronger factual and intellectual basis than it had then and has given it a substantial base from which we can properly and confi-

dently explore the processes that govern kidney development. Indeed, we confidently expect that the next decade will provide the answers to a whole set of questions and therefore use this last section to plot out the territory that we expect to be mapped during this period.

B. The Assay Problem

Progress in understanding the molecular and cellular underpinnings of any aspect of the developing kidney phenotype is, within limits, dependent on the assay systems available. Before going any further, it is probably sensible to remind readers of the practical context in which such work is going to be done.

The standard pathway for initiating change usually centers around a signal, a receptor, a signal transduction pathway if the receptor is not nuclear, and one or more transcription factors. The activation of these factors leads to a translation phase that, in turn, alters the cell phenotype, initiating a morphogenetic change that results in a new structure. There are many links in the chain of progress.

Given the wealth of molecules that have been identified as being involved in kidney development on the basis of their expression pattern, selection of a likely candidate gene for a particular role in many aspects of the process is not difficult. Proving that the gene has its hypothesized role is, however, turning out to be difficult, and here the Slack criteria [(i) expression at the right time and place; (ii) absence of the molecule blocks the phenotypes; (iii) addition of the molecule under these conditions restore the phenotype (Slack, 1993)] provide a first step in the analysis. Nevertheless, it is clear that the rules cannot always be adequate: They do not cover redundancy (where more than one molecule provides a single link in the chain), can only highlight a single link and not the whole chain, and may not always be practical to apply.

The expression test is the easiest and is always the baseline for selecting a candidate gene. Inhibition of expression is generally possible, in principle, through the use of transgenic technology, but the technique has severe limitations, even when it gives an abnormal phenotype. Although the abnormal phenotype confirms an important role for that gene, it gives little clue as to what that role is or even, without a great deal more work, the exact stage at which it executes that role. A finer level of resolution here, for the kidney at least, is provided by using antisense oligonucleotides to block translation in cultured rudiments or by adding antibodies to block the function of genes whose activity is external to the cell. However, in the absence of good downstream markers, it is still difficult to discern exactly what role a candidate gene has in a pathway. Moreover, as rudiments are small, it may not be easy to discern either quantitative changes in downstream markers or their presence if they are expressed only at a low level. The use of the remarkable ability of the kidney to grow in culture has limitations when the problem to be solved is that of identifying the function of a gene.

There are two other tools that are available, at least in principle. The first is the use of material from human congenital disorders, and much has already been made of Wilms' tumor. The second is the use of cell lines. If one could make a cell line that, for a short period at least, was able to display an appropriate phenotype for the problem under investigation, then it would be possible to block expression with antisense technology or antibodies, add excess protein, transfect the cells with appropriate genes, and up-regulate the expression of that gene, as well as recognize the presence of downstream genes expressed at low levels or quantitative change in their expression levels. These strategies would be particularly useful in the case of cell lines derived from the kidneys of knockout mice. In a sense, therefore, a set of problems in elucidating the genetic basis of kidney formation reduces to that of making highly state-specific cell lines. As has been discussed earlier, this approach has not been particularly successful so far for relatively early kidney development. Uninduced MM undergoes apoptosis, whereas induced MM progresses so far in culture that the cells are not useful for investigating the mechanisms that underlie that progress, and this is no matter what the genetic constitution of the MM cells.

Nevertheless, some of the tricks gleaned from studying kidney development *in vitro* may be helpful here. The use of various activators of induction (lithium, FGF, etc.) may enable early processes to be investigated, whereas the ability of LIF to block the differentiation of metanephrogenic mesenchyme at the stem-cell stage may provide stable cell lines for investigating slightly later signals and their effects. Even induced metanephrogenic mesenchyme is likely to be helpful as its downstream abilities are likely to be concentration- and substrate-dependent. In our view, the efforts required to make cell lines will be repaid many times over.

C. Likely Successes

One fortunate aspect of development in general is that its signals and receptors are used in many systems, and the tools for their investigation are readily accessible. It is, therefore, highly likely that we should soon know those molecules that signal inductive interactions in the kidney for both collecting-duct formation and MM stem-cell formation and differentiation. We also will probably know the signal from the stromal cells that helps regulate the formation of the nephrogenic condensation and that is probably upstream of Wnt-4. Knowledge of signals implies the appropriate receptor, and, given the availability of blocking antibodies that will work *in vitro*, we can look forward to substantial progress in our knowledge of the genetic pathways regulating the various interactions involved in kidney development before too many years have passed.

A second area in which progress is likely to be made in the short term is the elucidation of the various lineages within the developing kidney. As already

discussed, the work of Herzlinger and her colleagues has cast doubt on the traditional story that the duct gives rise to the collecting ducts while the MM forms nephrons, stroma, and (probably) the juxtaglomerular cells. It is also likely, but unproved, that neural crest and endothelial cells within the stroma form neurons and capillaries, respectively. The use of cell markers and confocal microscopy should provide the tools for confirming these lineage relationships and it should not be too difficult to do the experiments.

Progress in these areas will be relatively easy, and their exploration is also likely to clarify some aspects of the developmental phenotype that are still opaque. These include the early partitioning of the MM into stromal and stem-cell components, the origin of the juxtaglomerular complex, the morphogenesis of the nephron and neuronal organization within the kidney, mechanisms of growth, and reasons why stem cells are lost and nephron formation thus ceases around birth.

All of these problems fall under the rubric "reductionist," in that their solutions are simple facts. There are more such problems, but they are going to be much harder to solve. The most obvious of these are concerned with elucidating the downstream effects of cell signaling: These include the identification of the appropriate cascade of transcription factors and the genes that they cause to be expressed. This in turn will involve determining how these new genes change the cell phenotype. There is no shortage of such pathways to be elucidated, and this enterprise will certainly involve the whole repertoire of assays and approaches discussed in the preceding section, particularly the use of cell lines appropriate for each pathway. This will not be easy for any aspect of kidney development, but likely firsts here are the role of WT1 in effecting MM competence and the downstream effect on MM of the inducer from the ureteric bud.

Integration of these genes into regulatory pathways is the first step in shifting from the reductionist to the synthetic. Once we begin to see how signal pathways lead to new cell phenotypes, we will be able to work on the next set of such synthetic problems, those dealing with how these changes lead to new structures. Here, very little is known about any aspect of kidney morphogenesis, whether it be the localization of stem cells, formation of the bifurcations that generate the collecting-duct system, coalescence of nephrogenic cells into aggregates that undergo the mesenchyme-to-epithelium transition, or the first step in nephron formation or, indeed, any other step in their formation. These problems are not for the short term, partly because they are difficult and partly because they are unfashionable and therefore unlikely to attract much interest or financial support.

There is, however, one reason for supposing that the final set of such synthetic problems, those concerned with the generation of kidney function, may start to attract some attention soon: They are medically important, and the relatively pure research problems just discussed may provide some of the tools for investigating congenital kidney disease caused by either abnormal morphogenesis or differentiation.

D. Conclusions

Researchers whose field is kidney development find themselves living in interesting times. The past decade has provided us with a wealth of genetic and phenotypic information, questions are now well-defined, tools and assays are in place, and candidate genes abound. There is a clear program of work for the next decade that we are certain will lead to new insights into how the kidney develops and how the molecular basis of nephrogenesis goes awry in congenital kidney disease.

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