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Mechanisms of epithelial development and neoplasia in the metanephric kidney

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ABSTRACT Recent studies on the mechanisms of normal epithelial development in the kidney, and on the aetiology of renal neoplasms, are converging to reveal remarkably close relationships between the phenotypes and behaviours of normally-developing and neoplastic cells. Normal renal epithelia arise from two sources; those of the collecting duct system develop by arborisation of an initially-unbranched ureteric bud, in a manner similar to the development of other glandular organs, while epithelial nephrons develop via an unusual mesenchyme-to-epithelial transition. Both types of development require controlled proliferation, cell-cell and cell-matrix interactions, protease activity etc., but of the two tissues, the development of the nephrons is arguably the more complex. It includes many defined stages, signals and checkpoints that ensure that events happen at the right time, and that processes such as proliferation, apoptosis and differentiation are properly balanced. Detailed investigation of renal neoplasms has revealed some to be caused by mutations in molecules with known roles in normal nephrogenesis (e.g. Wilms' tumour and the *WT-1* gene, renal cell carcinoma and the *c-met* receptor tyrosine kinase gene), some to be caused by mutations in genes expressed during normal development (e.g. renal cell carcinoma and the *TSC-2* gene, renal cell carcinoma of the clear cell variety and the *VHL* gene). Furthermore, these and other tumours of unknown aetiology re-express genes such as *Pax-2* that are expressed during the normal mesenchyme-to-epithelium transition but are shut off during terminal differentiation. Their re-appearance in tumours suggests that the cells have 'regressed' in an ontogenic sense, and their biology may therefore be understood most clearly by reference to the properties of normal developing cells rather than cells of a mature kidney.

KEY WORDS: *kidney, epithelium, Wilms', carcinoma, von Hippel-Landau*

Introduction to renal epithelia

Renal epithelia, of which there are many types in a mature kidney, arise from two distinct sources. The urinary collecting ducts develop, like the epithelia in most other ducted glands, by growth and repeated branching of an initially-unbranched epithelial bud, in this case the ureteric bud which is an outgrowth of the Wolffian duct. The excretory nephrons, on the other hand, develop by an unusual mesenchyme-to-epithelium transition induced by the growing collecting duct system. Work over the last two decades has revealed a wealth of molecular detail about the mechanisms by which both types of epithelium develop and, while we remain far from having a complete explanation of the kidney, we do at least know the expression patterns of literally hundreds of genes and the renal effects of over 40 mutations (source: the Kidney Development Database, Davies and Brändli, 1995). At the same time, work on the aetiologies of a number of congenital renal tumours has implicated genes known to play a role, often a transitory one, in normal epithelial development. The kidney therefore exemplifies the parallels between ontogeny and neoplasia, a common theme in current cancer biology. It is for precisely this reason that we shall discuss both normal development and neoplasia together in this review.

Development of the collecting duct system

The development of the collecting duct system is arguably simpler than that of the nephrons; the ureteric bud progenitor tissue is already epithelial and 'all' it has to do is to grow and

Abbreviations used in this paper: S-GAGs, sulphated glycosaminoglycans.

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branch in response to morphogenetic signals. Like the epithelia of other glandular organs, developing collecting duct depends on signals emanating from the mesenchyme surrounding it; isolated or combined artificially with most other mesenchymes, it fails to grow (Grobstein, 1955) though lung mesenchyme can support limited ureteric bud development (Sainio *et al.,* 1997).

For many years the molecular carriers of these signals remained unidentified, but recently a combination of culture experiments and the analysis of transgenic mice has identified a number of candidate morphogens of the collecting duct system. One is Glial Cell-line-Derived Neurotrophic Factor (GDNF), which can drive arborisation in culture and can, if applied locally near the Wolffian duct, induce the production of supernumerary ureteric buds (Sainio *et al.,* 1997). GDNF is normally produced by the mesenchyme and its c-Ret receptor is expressed by the ureteric bud/developing collecting duct system. If either GDNF or its receptor is knocked out, the resulting animals show very poor or non-existent ureteric bud development (Pichel *et al.,* 1996). Neurturin and persephin also signal through c-Ret and have similar effects to GDNF, though persephin is less powerful (Milbrandt *et al.,* 1998; Davies *et al.,* 1999); unlike GDNF, neurturin appears to act in an autocrine rather than paracrine manner. Hepatocyte growth factor (HGF), made by the mesenchyme, also promotes collecting duct development in culture (Woolf *et al.,* 1995), and the tissue does express HGF's c-Met receptor, but HGF-/- mice show no renal abnormalities (Schmidt *et al.,* 1995). BMP-7 knockout mice show that BMP7 is required for normal collecting duct development (Dudley *et al.,* 1995; Luo *et al.,* 1995), but the molecule is expressed by both the ureteric bud and the surrounding tissue, so it is not yet clear whether the effect of the knockout on development of the bud reflects a primary effect or a secondary effect of abnormal development in the surrounding tissues.

It appears that the processes of elongation and branching of the developing collecting ducts are controlled separately. If kidneys developing in culture are deprived of their normal complement of sulphated glycosaminoglycans (S-GAGs), they become insensitive to physiological concentrations of many growth factors (one of the main functions of S-GAGs is presentation of certain growth factors to their high affinity receptors); under these circumstances, collecting duct development ceases unless an experimenter adds a high concentration of a relevant growth factor or second messenger modulator. Adding growth factors such as HGF 'rescues' tubule elongation but not branching, while adding protein kinase C activators 'rescues' branch initiation but not elongation (Davies *et al.,* 1995), suggesting that these two aspects of collecting duct development are controlled separately.

The actual cellular mechanisms that enable developing collecting duct to invade the mesenchyme surrounding it have received little attention to date, but recent work has shown that matrix metallo-proteinases, particularly MMP-9, are required (LeLongt *et al.,* 1997), as are specific integrin- extracellular matrix interactions (Kreidberg, 1996; Müller *et al.*, 1997).

Nephrogenesis

The induced mesenchyme-epithelium transition that gives rise to nephrons involves a large number of events which can conveniently be divided up into a series of stages (Fig. 1). The process begins when metanephrogenic mesenchyme receives an as-yet unidentified inductive signal from the ureteric bud/developing collecting duct that causes clumps of it to group together into tight aggregates of cells. These aggregates then undergo a mesenchyme-to-epithelium transition to form an epithelial sphere, which invaginates once to form a comma-shaped body and again to form an S-shaped body. One cleft of the S-shaped body develops into the glomerular cavity and the rest of the epithelium elongates and differentiates regionally into the specialised tissues of the proximal and distal convoluted tubules and the loop of Henlé. The distalmost part of the nephron fuses with the renal collecting duct.

A number of recent results have revealed that the idea of defined stages of nephrogenesis is not merely a descriptive convention; they are separated by a series of 'checkpoints' beyond which development will not proceed if a critical signal is missing. The first 'checkpoint' is the first signal from in-growing ureteric bud, which rescues cells from an otherwise apoptotic default fate and causes them to proliferate and form a population of stem cells, which persists at the outer edge of the renal cortex throughout development; without this signal, the metanephrogenic mesenchyme just dies (Koseki *et al.,* 1992). The stem cell population may be distinguished form the truly uninduced mesenchyme by differences in its expression of p75-NGFR, Trk B and Trk C (see Davies and Bard, 1998 for review). Its multiplication seems to be controlled in part by an autocrine loop by which hepatocyte growth factor (HGF), secreted by stem cells, acts back on them via its c-met receptor tyrosine kinase. Further inductive signals from the ureteric bud, or perhaps higher concentrations of the same signal, then recruit groups of stem cells into actual nephrogenic development (Fig. 1), this recruitment of groups of stem cells taking place throughout development as the growing collecting duct system continues to invade the outer cortex of the kidney.

Induction of cells into nephrogenesis is accompanied by changes in the expression patterns of several key transcription factors. One is *WT-1*, a gene that is already expressed to some extent in uninduced mesenchyme but is greatly up-regulated on induction; upstream regions of genes expressed during the aggregation stage of nephrogenesis, such as the proteoglycan, syndecan 1, contain putative binding sites for the WT-1 protein (Cook *et al.,* 1996). In addition to controlling the transcription of target genes, WT-1 can also (in a different splice form) associate with spliceosomes and presumably control the alternative splicing of downstream genes (Larsson *et al.,* 1995). WT-1 is absolutely necessary for response to inductive stimuli (Kreidberg *et al.,* 1993). Another transcription factor to be activated very soon after induction is Pax-2, which is also absolutely necessary for nephrogenesis.

An important checkpoint in the nephrogenic programme appears to control the transition between aggregation and epithelialisation phases, and presumably ensures that aggregates have grown large enough before they attempt to differentiate. During aggregation, induced cells express the signalling molecule Wnt-4 in an autocrine positive feedback loop so that Wnt-4 signals drive Wnt-4 expression further and the local concentration of Wnt-4 increases as aggregates grow (Stark *et al.,* 1994). It is possible, although not yet proven, that passage through the checkpoint between aggregation and epithelial differentiation depends on Wnt-4 concentration exceeding a certain threshold. Two observations support this idea; (1) transgenic deletion of Wnt-4 results in the failure of nephrogenesis to progress beyond the stage of

aggregation, as would be expected if the Wnt-4 threshold level could never be reached (Stark *et al.,* 1994); (2) mimicry of the Wnt signalling pathway by exogenous lithium ions (Klein and Melton, 1996) results in epithelial differentiation of aggregates that have unusually few cells, as would be expected if the perceived level of a Wnt-4 signal reached the threshold too early (Davies and Garrod, 1995).

Epithelial differentiation involves both the morphological change from a comparatively-disordered mesenchyme to an organised simple epithelium and also activation of a large number of 'epithelial' genes, e.g. cytokeratins, desmosomal components, adhaerens and tight junctions, basement-membrane collagen and laminin types etc., and inactivation of 'mesenchymal' genes e.g. vimentin, interstitial collagens etc., in a defined order presumably controlled by the new transcription factors, e.g. Hox B3, Hox B7, LFB-3 and Pax-8, also acquired at this time (for review, see Davies, 1996). The process depends for its completion on a number of signals that may be regarded as 'progress reports' because they arise from components synthesised during epithelialisation. One class of these signals, presumably transduced through integrins, apparently 'reports' on normal organisation and adhesion to the newly forming basement membrane. Nephrogenesis halts, for example, if the basement membrane-specific glycoprotein, laminin A, is present and interacting on the one hand with the cell via $\alpha 6\beta 1$ integrins and on the other with another basement membrane protein, nidogen (Ekblom *et al.,* 1994). It also halts if either cell-cell adhesion mediated by cadherin 6, or cell-basement membrane adhesion via $\alpha 8$ integrin, is inhibited (Müller *et al.,* 1997; Cho *et al.,* 1998). Only when epitheliogenesis is complete do nephrons progress to regional differentiation.

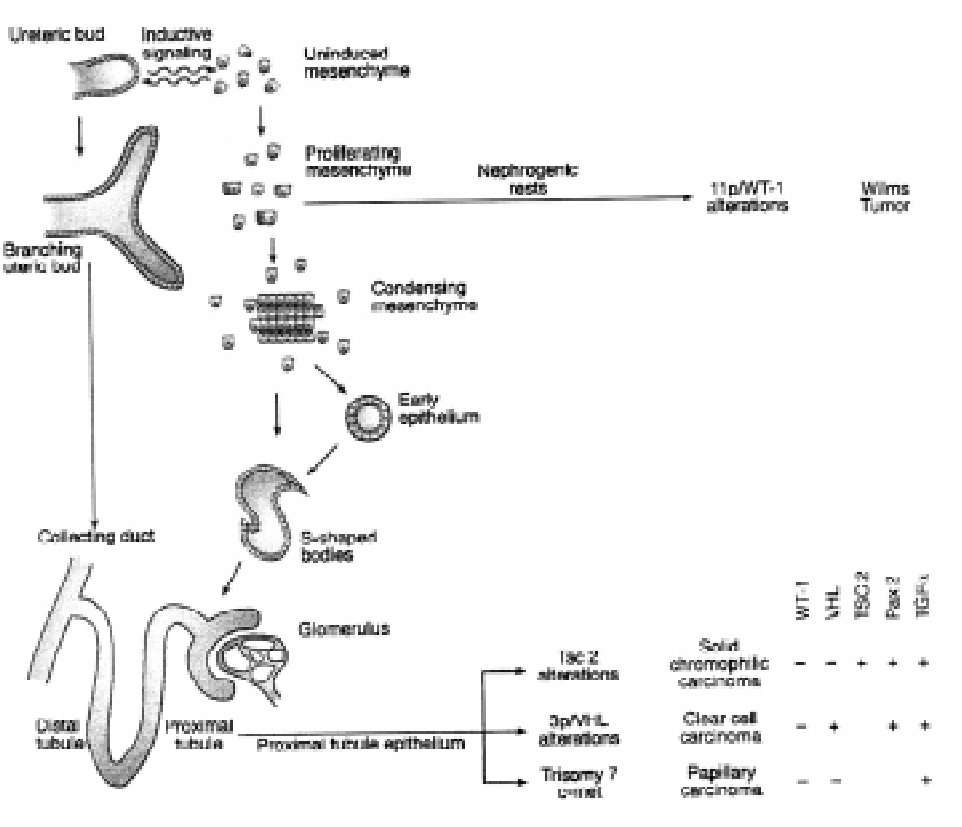

Fig. 1. Epithelial development in the kidney. *The left half of the diagram illustrates the stages of normal kidney development referred to in the text, during which the ureteric bud branches and forms a collecting duct system while metanephrogenic mesenchyme condenses and epithelialises to form the excretory nephron. The right half of the diagram summarises genetic alterations associated with neoplasia of particular tissues of the developing kidney.*

During normal nephrogenesis, it is possible and perhaps likely that not all cells of an aggregate will find a place in the newly-formed epithelium. The cells will 'know' of their exclusion because of the lack of signals reviewed above; what do they then do? One possible fate is apoptosis, of which there so much in developing kidney that an estimated 50% of all cells created are eliminated this way (Coles *et al.,* 1993). Elimination of cells not properly included in the epithelium may be very important; our preliminary experiments with cell-permeable caspase inhibitors, which block apoptosis by interfering with essential mediators of the apoptotic programme, show a marked inhibition of normal nephrogenesis (although collecting duct development is normal).

Regional differentiation and maturation of the tubules is accompanied by further changes in the activity of transcription factors, notably an increase in WT-1, particularly at the Bowman's capsule end of the nephron, disappearance of Pax 2 (possibly caused by the rise in WT-1), expression of Pax 8, and of course expression of region-specific genes such as ion transporters and brush-border proteins. Rather little is known about the control of maturation, but it is clear that some components that play a prominent role in earlier stages, for example Pax 2, have to disappear before maturation can proceed (Dressler *et al.,* 1992) .

Normal nephrogenesis, then, is a tightly controlled process orchestrated by a series of signals and checkpoints that keep processes of proliferation, apoptosis and differentiation in their proper sequence and balance. As more is learned about these normal signals and checkpoints, the loss of that balance seen in renal neoplasms can be increasingly well understood in terms of developmental mechanisms, particularly for the common cancers described in the next section.

Neoplasias of the developing kidney

Papillary renal carcinoma and the HGF/c-met system

Papillary renal carcinomas account for approximately 14% of all renal tumours and are found in both hereditary and sporadic forms. The cell type of origin of these tumours is less clear than for some other types of renal cell carcinoma. Kovacs (1993) has speculated that they may arise from undifferentiated mesenchyme that has for some reason avoided apoptosis and has instead survived as a 'nephric rest'. It could therefore, be reasoned that papillary renal carcinoma is the result of a derailment of normal developmental processes. Recent analysis of the *c-met* gene in the hereditary

papillary renal carcinoma has revealed mis-sense mutations in the tyrosine kinase domain of this gene (Schmidt *et al.,* 1997). These mutations result in the constitutive activation of the c-met receptor, and might therefore mimic inappropriately the autocrine loop that drives stem cell multiplication in normal development (see above) but which is switched off on differentiation. Interestingly, although c-met appears to be involved in the hereditary form of papillary renal cell carcinoma, its role in the sporadic form of this neoplasm is less clear (Schmidt *et al.,* 1997). Participation in hereditary but not sporadic disease suggests that the mutation may be required during nephrogenesis.

Wilms Tumour and the WT-1 gene

The Wilms tumour suppressor gene *(WT-1)*, which has been implicated as a key player in the mesenchymal-epithelial transition (above), also plays a key role in the development of the childhood renal malignancy, Wilms' tumour (nephroblastoma), after which the gene is named. Wilms' is clinically very important, being the most common solid tumour of childhood. Most cases are unilateral and sporadic, but a small percentage are associated with a triad of congenital syndromes collectively known as the WAGR syndrome (WAGR standing for Wilms', Aniridia, Genital abnormalities and mental Retardation). In approximately 15% of familial cases, Wilms' tumour is associated with a mutation and/or loss/gain-of-function of WT-1 in the nephrogenic stem cell population, which allows continued growth but makes the cells unable or less able to respond to inductive signals. However, the precise molecular role of WT-1 in the genesis of tumours remains controversial. Although some sporadic Wilms' tumours are associated with loss of heterozygosity, as predicted by the idea of *WT-1* being a tumour suppressor gene, the majority of tumours are not associated with loss of heterozygosity and in fact show expression of both mRNA and protein, often at elevated levels. This has led to the suggestion that a dominant negative mutation in one of the alleles (gain-of-function) may be involved. In approximately 85% of cases, however, mutation analysis has revealed the *WT-1* gene to be wild-type, so mutations of different genes, perhaps those that act downstream of *WT-1*, are presumably involved. Detailed understanding of the role of *WT-1* in the aetiology of the tumour is still hampered by lack of knowledge about all of the functions of all of the splice forms of the gene during normal development; the fact that the gene is present, at differing levels, throughout the stages from uninduced mesenchyme to maturing epithelial nephrons suggests that it may play several different roles.

Clear cell renal cell carcinoma and the von Hippel-Lindau tumor suppressor gene

The development of both hereditary and sporadic clear cell renal cell carcinoma, a variant representing over 80% of renal cell carcinomas in humans, is a result of inactivation of the von Hippel-Lindau *(VHL)* tumour suppressor gene. Individuals carrying a germline mutation for this gene are also susceptible to a variety of other neoplasms including pheochromocytomas, hemangioblastomas of the CNS, islet cell tumours of the pancreas as well as pancreatic, renal and epididymal cysts (Melmon and Rosen, 1964). The *VHL* gene, located on chromosome 3p25 in the human, gives rise to a protein (pVHL) that is thought to have multiple functions, including roles that are linked to tumorigenesis and tumour progression. In this regard, there is strong evidence that pVHL may act as a tumour suppressor by negatively regulating the transcriptional activator Sp1. The failure of this inhibition of Sp1 has, for example, been blamed for overexpression of VEGF, driven via the Sp1 binding sites in its promoter, in many solid tumours (Mukhopadhyay *et al.,* 1997). The ubiquity of Sp1 as a regulatory entity predicts that the pVHL-Sp1 interaction may be important in transcriptional control of a variety of tumour associated genes. pVHL may also control the cell cycle more directly; the protein interacts with the elongin B and C complex to form a trimer that associates with the Hs-Cul-2, a member of the cullin family that regulates the ubiquitin-mediated degradation of cyclin-dependent kinase inhibitors. Recent evidence suggests that the inability of serum-starved clear cell renal cell carcinoma lines to stabilise p27 levels and exit the cell cycle is the result of the inability of the mutant *VHL* to inhibit Hs-Cul2 mediated degradation activity (Pause *et al.,* 1998). An ability to grow in the absence of exogenous growth factors, conferred on the cells in this case by loss of pVHL function, is shared by most cancer cells and may be a requirement for malignant transformation. Unlike the genes described above, the role of the *VHL* gene in normal nephrogenesis is uncertain. It is clearly expressed at high levels in the developing kidney and also in other organs (Nagashima et al*.,* 1996). Unfortunately, *VHL* deficient mice die at around the time of the onset of nephrogenesis due to deficits in placentation, so the precise functions of this gene during kidney development remain to be elucidated.

The roles of the paired box genes Pax-2 and Pax-8 in renal neoplasias

Pax 2 is expressed during, and is necessary for, the staged of mesenchymal aggregation and mesenchyme-to-epithelial transition during nephrogenesis, and then disappears. Given that its disappearance correlates with terminal differentiation of nephric mesenchymes, it is perhaps not surprising that aberrant expression of Pax-2 is observed in a variety of renal tumours in both human (Dressler and Douglass, 1992; Gnarra and Dressler, 1995) and rat (C.L. Walker, unpublished data), indicating an undifferentiated or de-differentiated phenotype. Thus, consistent with the finding WT-1 is a negative regulator of Pax-2 during kidney development, altered function of this gene in Wilms' tumour results in high levels of Pax-2 expression. That clear cell renal cell carcinomas likewise show aberrant Pax-2 expression is an interesting finding; the demonstration that the VHL promoter has a putative Pax binding site suggests that there may exist a feedback loop between these two genes. Pax 8, another member of this transcription factor family, is expressed in the condensed mesenchyme of the developing nephron, slightly later than Pax-2. However, Pax-8 is also expressed at high levels in Wilms' tumour, suggesting that persistent expression of both members of this family may be required for development of this childhood tumour.

TGF-alpha/EGF-R in renal neoplasias

Transforming growth factor α (TGFα) and its receptor EGF-R have been shown to be expressed during nephrogenesis . In a normal mature kidney, the proximal tubules bear EGR-R but its ligand, TGFα, is expressed mainly by the collecting duct. In the vast majority of clear cell and chromophilic renal cell carcinomas, which are both thought to arise from the proximal tubules, however, the carcinomas overexpress both TGFα and EGF-R. It is thought that the inappropriate autocrine loop formed by this mitogen and its

receptor is one mechanism that drives the transformed epithelial phenotype. Studies in the Eker rat, a model that allows staged alterations in gene expression in renal carcinoma development to be analysed, has shown that the aberrant expression of TGFα is an early event in the development of this tumour. Its presence in pre-neoplastic, dysplastic tubules has made TGF-alpha the earliest marker yet identified for this disease.

Renal cell carcinoma in the Eker rat; the tuberous sclerosis-2 tumor suppressor gene

Eker rats provide an unique model for studying the aetiology of renal cell carcinomas; the animals carry a viral insertion in a gene called *TSC-2*, coding for tuberin, and loss of heterozygosity in the rats leads to renal cell carcinoma. The rat *TSC-2* gene , located on chromosome 10q12 in rat, has a human homologue on chromosome 16p13.3. A defect in a single allele of this locus in humans leads to tuberous sclerosis, a predisposition to develop usually benign tumours (harmartomas) of the brain, skin and kidney that become fatal usually because of renal cysts but occasionally malignant renal cell carcinoma develop in these individuals as well. In the Eker rat, the tumour suppressor function of *TSC-2* has been confirmed by the observations that loss of heterozygosity results in renal cell carcinoma while reintroduction of wild type *TSC-2* inhibits proliferation in Eker tumour cell lines. The precise role of tuberin, as with pVHL, seems to be complex. To date, tuberin is thought to act as a regulator of endocytosis via its action as a RAB5GAP, a cell cycle modulator via inhibition of cyclin D1 (Soucek *et al.,* 1997), and finally as a steroid receptor co-activator (Henry *et al.,* 1998). The latter has clear implications in a developmental context. Tuberin is indeed found in the foetal kidney (Geist and Gutmann, 1995), but a detailed analysis of its temporal and spatial expression during nephrogenesis has yet to be performed.

Of course, much work remains to be done on both normal development and on the aetiology of renal neoplasms, but the few examples described above are sufficient to illustrate that some tumours can arise directly by mutation of genes known to play roles in normal development (*WT-1, c-Met*), some arise by mutation of genes suspected of playing roles in normal development (*TSC-2, VHL*), and most result in the re-expression of genes that play a role in development but ought to be shut off in maturing tissues (e.g. *Pax-2*). The strong implication is that there are real parallels between the behaviour of developing and neoplastic cells in the kidney, and that of renal embryologists and oncologists therefore have much to gain from each other's efforts.

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