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Early nephron formation in the developing mouse kidney

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ABSTRACT

This paper reports 3-dimensional confocal microscopy observations on how nephrogenic aggregates form from the NCAM- and Pax2-positive caps (4–5 cells deep) of condensed metanephric mesenchyme surrounding the duct tips of the mouse kidney. Aggregates of 6–8 cells are first seen at ~E12.5–12.75 immediately proximal to this cap, closely abutting the duct surface. As the tip advances, NCAM expression is maintained in the cap but is otherwise restricted to aggregates whose cells rapidly epithelialise, forming tubules that invade the duct epithelium. Pax2 expression studies shows how the rind of nephrogenic blastemal cells forms: as duct tips extend towards the kidney surface, the associated Pax2+ cells form patches of cells on the kidney surface. These observations revise our knowledge of the timing and process of nephron initiation.

Key words: Confocal microscopy; kidney development; mesenchymal aggregate formation; induction; Pax2; NCAM; mouse kidney development.

INTRODUCTION

The formation of mesenchymal aggregates is an essential part of vertebrate development as it is the first step in the formation of many tissues that include somites, muscles, bones, teeth, hair follicles and nephrons. In most cases, the mechanisms by which cells come together to form these aggregates are not well understood: cell adhesion, chemotaxis, loss of extracellular matrix, cell traction and local growth are all possible candidate mechanisms (Bard, 1990). Many of these problems are highlighted in the developing kidney where, as has long been known, groups of cells derived from the metanephric mesenchyme (MM) are induced by the early collecting ducts to form small aggregates. These epithelialise, elongate and form early nephrogenic tubules that then interact with the local capillary systems at their 'proximal' end to form the renal corpuscle, and fuse to the adjacent duct at their 'distal' end (for review, see Saxén, 1987; Davies & Bard, 1998).

The formation of nephrogenic aggregates is a two-stage process: first, MM condenses around duct tubules to form primary condensates or caps (Saxén, 1987) and, second, small groups of nephrogenic aggregates emerge from these caps in a way that has not been easy to determine. Although there is now a considerable amount of molecular data on the process of nephrogenesis (for review, see Davies & Brandli, 1997), we still have very limited understanding of the mechanisms that cause small groups of MM cells to form functioning nephrons; indeed, it is only very recently that the signals that induce nephrons have started to be isolated and, even then, only in vitro (Barasch et al. 1999). We also do not know how blastemal cells reach the kidney periphery from their initial position within the early metanephros, how nephron polarity is assigned, or even whether induction requires separate signals to rescue MM cells from apoptosis (Kreidberg et al. 1993) and to induce them to form nephrons. One reason for this lack of knowledge is that it is hard to follow the early stages of nephrogenesis directly as they take place inside the developing kidney; in the case of the mouse, this process starts as about E12.75, when the kidney is abut 0.5 mm in diameter.

This paper reports a detailed study of early nephrogenesis using 3-dimensional (3D) confocal microscopy to follow the expression patterns of three early markers of MM induction: NCAM, Pax2, and laminin. The cell-surface adhesion molecule, NCAM, is expressed in the early stages of aggregate formation

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(Klein et al. 1988), while the transcription factor Pax2 is an early induction marker expressed in cells surrounding the tips of ureteric buds; this transcription factor is known, on the basis of gene targeting, to be a key factor in nephron differentiation and a marker of blastemal cells and early tubules (Rothenpieler & Dressler, 1993). Laminin is not only expressed in the basement lamina of the duct but also in that of the epithelialising aggregates (Klein et al. 1990), a process dependent on their expression of Wnt-4 (Kispert et al. 1998). The importance of 3D visualisation is partly that it allows events to be followed in their normal context, and partly that it ensures that unexpected observations are not artefacts resulting from a simple 2-dimensional view of the tissue such as would be seen in sectioned material.

The key observations reported here are that early nephrogenic aggregates form just proximal to the cap of induced MM that surround the growing duct tips, and in very close proximity to them; these aggregates epithelialise and fuse to ducts much earlier than hitherto supposed, and blastemal cells form at the metanephros periphery because local cells are induced there by the growing duct tips. The term 'blastemal cells' is used here because it is still not clear whether these cells are precursor cells destined to become nephrons or stem cells that have the choice of differentiating into either stromal cells or nephrons. The results as a whole provide additional resolution to the traditional view of early nephron formation; in particular, they clarify and explain some aspects of the developmental anatomy and amend some of the timing details.

MATERIAL AND METHODS

Murine material

Kidneys were routinely dissected out from E11–E13.5 embryos of CBA mice (E12.5 mice were killed at 9.00 on E12; E12.75 kidneys came from mice killed at 15.00).

Kidneys were fixed in either −20 °C methanol (20 min) and then transferred to phosphate-buffered saline (PBS) for immunohistochemistry of extracellular markers or in 4% paraformaldehyde (2 h) and then transferred to PBS + 1% v:v Triton-X100 (PBST) for Pax2 immunohistochemistry.

Immunohistochemistry

Antibodies were made up in PBS or PBST as appropriate. Whole kidneys were incubated for 2 d at 37 °C in primary antibody made up in 5% normal goat serum (NGS) and 10^{-4}% w:v sodium azide (Sigma, UK), washed in NGS for 24 h, reincubated for 2 d in secondary antibody and a nuclear stain in NGS, and given a final NGS wash for 24 h before being mounted in 50:50 PBS:glycerol for confocal viewing. Controls received only secondary antibodies and showed minimal and nonspecific fluorescence.

Primary antibodies used were against NCAM (1:50, mouse monoclonal, SIGMA C9672), laminin (1:100, rabbit polyclonal, SIGMA L9393) and Pax2 (1:400, rabbit polyclonal, a gift from Dr Greg Dressler) (Dressler & Douglass, 1992). Secondary antibodies were labelled with Cy2 and ruthenium red (Jackson Laboratories), while nuclei were stained with TO-PRO-3 iodide (1:200, Molecular Probes) or propidium iodide in 100 μg/ml RNAse (although TO-PRO-3 iodide does not stain RNA, RNAse treatment seemed to sharpen nuclear morphology).

Pax2 and NCAM could not be co-viewed as the Pax2 antibody needs paraformaldehyde fixation and triton-X treatment to access this nuclear transcription factor. Unfortunately, the major form of NCAM in the kidney is anchored to the membrane by the lipid glycosylphosphatidylinositol (Lackie et al. 1990), and both are washed out by the detergent.

Confocal microscopy

Specimens were examined in a Leica TCS-NT 3-channel confocal microscope. For high-magnification work, a ×63 water-immersion lens was used with images digitised at planes separated by 1–2 μm. Otherwise, a ×20 lens was used and plane separation was 2–4 μm.

Two artifacts that are apparent in the micrographs should be mentioned. First, antibodies tend to get trapped within the lumina of tubules in specimens that have been treated with Triton-X, and, second, excessive amounts of fluorescence can be seen in regions of the field where the optical beam has to travel through minimal amounts of tissue. This is particularly apparent in specimens optically sectioned at a deep level; at and near the periphery of kidney rudiments there is little absorption, compared to more central regions where the emitted beam may have to traverse large amounts of tissues.

RESULTS

Early aggregate formation

In uninduced E11 kidneys, NCAM is not expressed, but, once the duct tip has started to enlarge (the first stage in bifurcation, E11.5), NCAM is expressed in a

domain of MM around the tips some 4–5 cells deep, but not in its more peripheral region (Klein et al. 1988). Confocal microscopy clearly demonstrates that by E12.5, the ureteric bud has bifurcated twice and all four tips are surrounded by a cap of condensed MM some 4–5 cells thick that expresses NCAM (Fig. 1).

No small aggregates can be recognised at this stage; these first become apparent in E12.75 kidneys. In the dozen or more kidneys that can be obtained from the embryos of a single pregnant female, a spectrum of aggregate morphologies can be seen. The smallest are observed just proximal to the tip and immediately adjacent to the basal lamina of the duct: they contain 6–8 cells and still express NCAM (Fig. 2). We have examined > 30 such kidneys and, in every case, the aggregates are in intimate contact with the basal lamina of the duct as assayed by the yellow colour shown by the overlap of the red NCAM and green laminin immunostaining; not a single aggregate has been observed that was not in such contact (Figs 2, 4).

In slightly more mature kidneys, aggregates are also seen some distance proximal to the duct tip. In these specimens, NCAM expression is maintained in the aggregates and condensed cap, but is very much lower in the intervening unaggregated MM that is proximal to the tip and surrounds the duct (Fig. 3). At this stage, the aggregates have enlarged to about 4–5 cells in diameter (giving a total number of up to 30 cells) and the first overt signs of epithelialisation can be seen. In such aggregates, cell morphology is still mesenchymal, but small amounts of laminin expression become visible on the external surface of the aggregate, while the basal lamina at the aggregate-duct interface is degraded (Fig. 4). In more mature aggregates, epithelialisation is more advanced as demonstrated by the columnar organisation of the cells and by the deposition of laminin on the surface of the aggregate not in contact with the duct, while laminin between the duct and the aggregate is lost (Fig. 4).

Specimens like that shown in Figure 4 also allow us to answer the question of how close nephrons can be. The nearest that we have observed 2 nephrogenic aggregates has been in a single plane almost orthogonal to the axis of the duct, but on roughly opposite sides (about 10 cell diameters apart around the periphery, Fig. 4). Because of the difficulties in visualising the interior of older kidneys and analysing the pattern of duct growth, it is not yet clear whether there is some regular spacing (or wavelength) for nephrons along the duct.

The laminin observations demonstrate that fusion between the nephrogenic and duct epithelia takes place before there are any obvious signs of nephron morphogenesis (e.g. the presence of the comma-shaped aggregate, the first stage of the morphogenesis of the renal capsule that takes place a day later). The extent to which fusion occurs as nephrons continue to develop can be observed because the aggregates maintain NCAM expression rather longer than hitherto supposed (Klein et al. 1998). Figure 5 shows an early nephrogenic tubule whose 'distal' end lies within the duct epithelium and whose body and 'proximal' end extends away from the duct. It is clear that continuity between the lumens of the duct and epithelialised aggregate takes place early in nephrogenesis and before the comma stage is reached.

Thus far, it has been assumed that the NCAM+ aggregates are nephrogenic on the basis that they epithelialise and fuse to ducts. Figure 6 shows a more mature aggregate fused to a duct in an E13.5 kidney (the stage at which comma-shaped bodies can be seen) and its morphology confirms that the NCAM+ region represents a developing nephron, with the adhesion molecule being expressed rather longer than hitherto supposed (Klein et al. 1988).

It is difficult to use confocal microscopy to discern fine detail of the morphology of the maturing nephrons (about E13.5) that lie deep within kidneys: once the beam's plane of focus is more than about 40 μm below the surface, the optical resolution deteriorates markedly. It is also not possible to estimate from these fixed specimens the speed with which aggregate formation and differentiation take place, or the speed with which the duct grows. Nevertheless, the fact that such a spectrum of morphologies can be seen in the kidneys from a single batch of embryos argues for these events taking place over but a few hours.

Pax2 expression and the location of blastemal cells

Pax2 is a transcription factor required for nephrogenesis; it is initially expressed in the ureteric bud and its derivatives, and later in nephrogenic tubules (Dressler & Douglass, 1992; Rothenpieler & Dressler, 1993). It is also expressed in MM and is initially localised to the cap of cells surrounding the tip of the ureteric bud within the central part of the kidney (e.g. Hatini et al. 1996), and is thus coexpressed with NCAM. From about E13 onwards, however, the Pax-2+ MM cells are located at the kidney periphery as nephron-forming blastemal cells. The way in which Pax2-positive cells reach the surface has remained unclear and we have investigated this question with confocal microscopy.

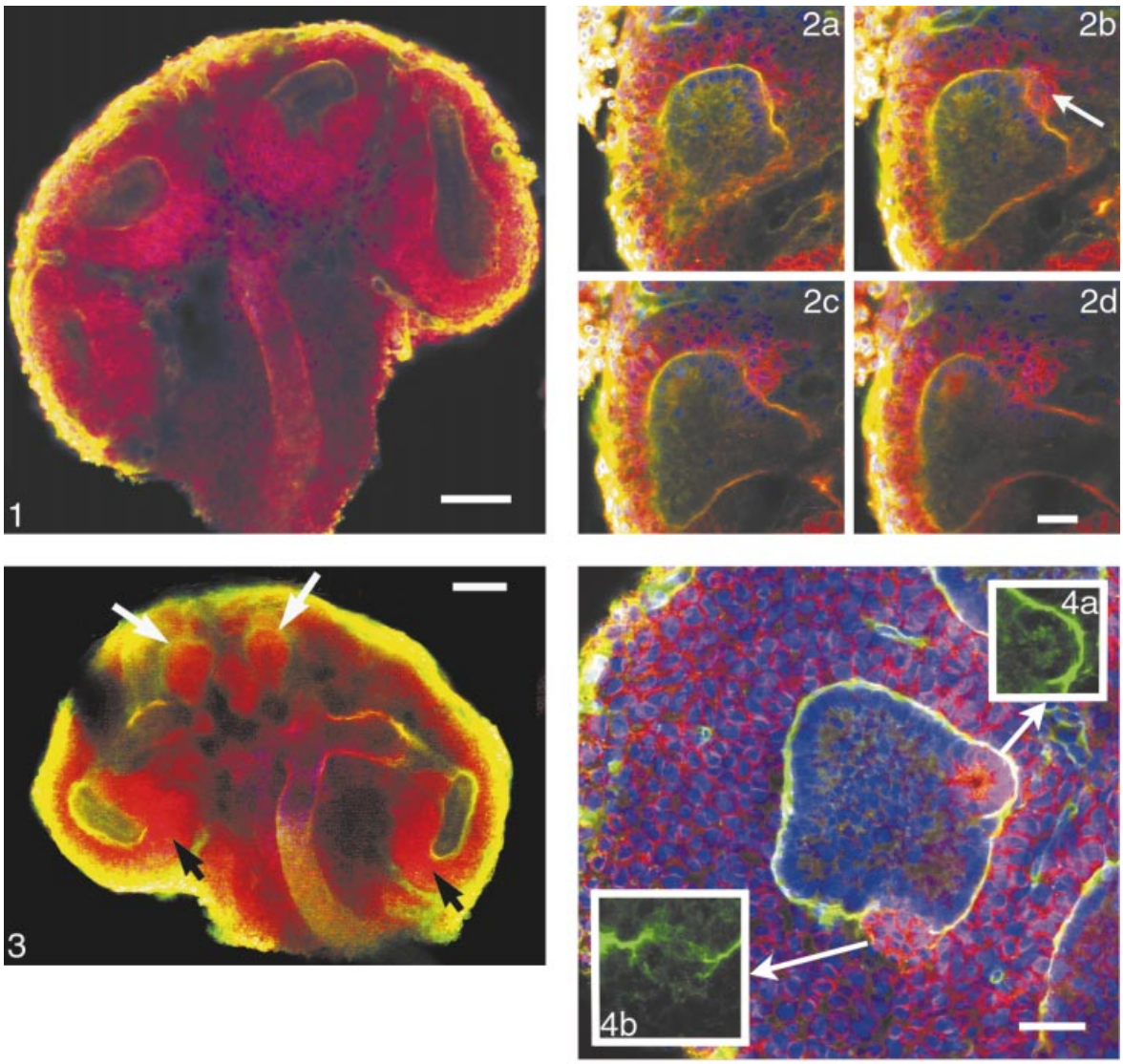

Fig. 1. Confocal micrograph of an E12.5 induced kidney in which the ureteric bud has bifurcated twice, stained with NCAM (red), laminin (green) and TO-PRO-3 nuclear (blue). NCAM is expressed in a domain of metanephric mesenchyme some 3–5 cells thick that surrounds each tip. Bar, 50 μm.

Fig. 2. A stack of 4 confocal images (vertical separation ~ 8 μm) near the surface of an E12.75 kidney showing a duct tip (*a*, *b*) extending into a duct (*c*, *d*). The cap of NCAM+ cells with their almost columnar morphology is clearly seen, and just proximal to it is the smallest aggregate of MM cells yet observed (arrow, *b*, *c*). The small aggregate is contained within the 4 optical sections and has ~ 8 cells. Note that the basal lamina is still present between the aggregate and the duct and there is no laminin staining on the outer surface of this small aggregate (NCAM (red), laminin (green) and TO-PRO-3 nuclear (blue); Bar, 25 μm.

Fig. 3. An optical section through the middle of an E12.75 kidney showing the ureteric tree with caps of NCAM+ cells (red, black arrows). Proximal to the ducts, NCAM staining is absent, apart from in the small growing nephrons (white arrows). The quality of this picture is low because the specimen is too thick at the centre for high resolution microscopy, while the variation of thickness across the specimen means that it is impossible to see internal detail without an excessive degree of fluorescent at the periphery. Bar, 50 μm.

Fig. 4. A confocal micrograph of a cross section of a collecting duct in an E12.75 kidney proximal to the tip and 2 immediately adjacent early nephrogenic aggregates. The main picture is stained with NCAM (red), laminin (green) and TO-PRO-3 nuclear (blue). Note that the 2 aggregates are in the same plane and only about 10 cells apart. The morphology of the cells in the aggregate to the right show clear signs of epithelialisation and this is confirmed by the laminin staining (insert). Here, the laminin of the basal lamina between the duct and the aggregate has almost gone, while at the periphery of the aggregate laminin staining is as substantial as that of the duct. In contrast, the other aggregate (bottom of picture) has only a fine basal lamina on its outer surface and the basal lamina between the aggregate and duct is clearly being degraded. Bar, 25 μm.

Examination of E12 Pax2-stained specimens has confirmed that this transcription factor is expressed in both the cap of condensed MM around the duct tip and in the ducts themselves, but not in rest of the MM. In E12.5-E13 kidneys, as the kidney enlarges and the ducts bifurcate and extend out from the centre

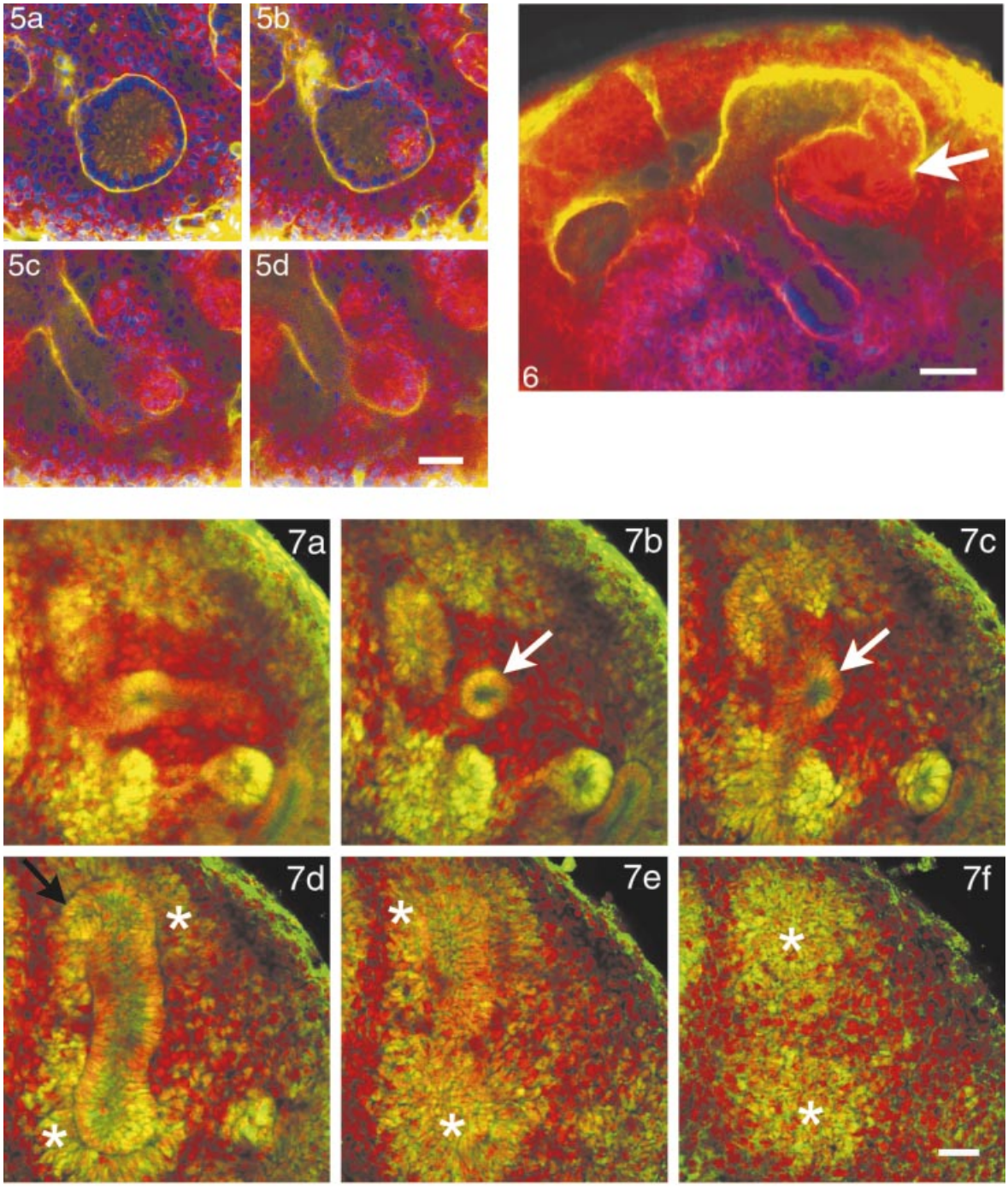

Fig. 5. A stack of confocal images (vertical separation 8 μm) showing an early nephron integrated into the duct epithelium. The first picture (*a*) shows the duct just distal to the site of integration, the next shows the NCAM+ region of the nephron within the duct. The successive pictures (*b–d*) show the primitive nephron extending away from the duct epithelium. Staining, NCAM (red), laminin (green) and TO-PRO-3 nuclear (blue). Bar, 25 μm.

Fig. 6. A confocal image of an extending ureteric duct within an E13.5 kidney that is connecting to an early nephron (arrow) at about the S-shaped stage. Bar, 25 μm.

Fig. 7. A stack of confocal images (separation about 10 μm) showing how Pax2+ cells (presumptive stem cells) reach the kidney periphery as duct tips extend towards its surface. (*a*) This section (the deepest) shows a duct extending towards the medial surface of the kidney; the basal part of a small duct (white arrow) is apparent. (*b*) and (*c*) show this small tubule (white arrow) connecting the duct (shown in *a*) to a more superficial T-shaped duct. (*c–e*) These sections shows the 2 domains of Pax2+ MM cells surrounding the tips of this duct, while an early Pax2+ epithelialising aggregate is visible in *d* (black arrowhead). In (*f*), the surface of the kidney and 2 patches (white asterisks) of Pax2+ cells are seen overlying the caps surrounding the duct tips. Staining, Pax2 (green) and propidium iodide (red); Bar, 50 μm.

of the rudiment, the duct tips are seen to approach the periphery of the kidney, still surrounded by their caps of Pax2-positive cells. Internal MM cells that had earlier expressed Pax2 appear to lose expression unless they are incorporated into a nephrogenic aggregate. By E13, Pax2-positive cells can, for the first time, be seen on the surface of the cortex adjacent to the extending tips.

These events are shown in Figure 7, a set of optical sections through an E13 kidney displaying part of the duct system and stained with Pax2 and propidium iodide. The first picture (*a*) shows the central duct some 40 μm below the surface; off this duct extends a vertical spur (*b*, *c*) that bifurcates in a plane orthogonal to that of the original duct to give two terminal ducts whose tips extend towards the kidney surface (*d*, *e*). Pax2 stains all of the ducts, but only the MM which is in the caps of cells around the 2 duct tips (*d*, *e*) and in an early epithelialising aggregate than can be seen forming in one of these caps (arrow). Where these 2 caps abut the kidney surface, they form Pax2-positive clones (*f*) that will become the blastemal cells.

DISCUSSION

The traditional view of nephron induction is that the tips of the collecting ducts secrete some signal that induces nearby metanephric mesenchyme cells to condense and form aggregates; these then epithelialise, polarise and reorganise, with one end soon forming the renal corpuscle and the other eventually fusing to the duct (for review, see Saxén, 1987). The results presented here modify this view in several respects. First, all aggregates initially form in intimate contact with basal lamina of the duct tip rather than simply being nearby. Second, fusion of presumptive nephrons with duct epithelium is a far earlier event than hitherto supposed. Third, the localisation of the aggregates on the duct surface, just proximal to the tip of condensed MM suggests that the ureteric bud plays a more directive role in localising where nephrons form than would have been expected had the tip merely secreted an inducing signal into the adjacent extracellular milieu.

Early aggregate morphogenesis

The observation that nephrogenic aggregates form in intimate contact with the duct epithelium in the region of the duct tip does not explain the initial size of a nephrogenic aggregate. One possibility is that a local signal from the epithelium is attenuated as it induces adjacent MM cells and so limits the number of cells that can join the aggregate, but there are other mechanisms that might work here; it will be interesting to study this process in the BF-2 $-/-$ mouse, in which abnormally large nephrogenic aggregates form (Hatini et al. 1996). The mechanism by which epithelialisation takes place is also unclear and there are two obvious possibilities. The first is that the inducing signal not only causes aggregation but also its downstream effects. A more intriguing possibility, however, is that epithelialisation is an autonomous property of aggregated MM, mediated by the autocatalytic production of Wnt-4 (Kispert et al. 1998); the data presented here suggest that this would take place when the aggregate size is about 10–20 cells.

Another aspect of nephrogenesis that is hard to follow is the actual speed with which the early events take place. Although it has not been possible to obtain direct data here, the wide range of early nephron morphologies seen in E12.75, but not E12.5, kidneys argues for these early stages being very rapid, perhaps taking only a few hours between aggregate initiation and full epithelialisation, with nephron growth being through recruitment rather than cell division. The mechanism by which the duct basal lamina adjacent to the epithelialising aggregate is lost as its own external lamina forms is also unclear, although MM is known to express metalloproteinases MMP2 and 9 (Lelongt et al. 1997). It is also worth mentioning that this difference in behaviour between the 2 surfaces of the aggregate marks the first overt sign of a polarity developing between what will become the distal and proximal ends of the nephron.

The breakdown of the intervening lamina may have a further implication: not only does it allow duct and nephron epithelia to become continuous, but it may also permit a degree of mixing between the cells of the 2 epithelia, should there be any movement. If so, it could account for the intriguing observations of Qiao et al. (1995) who showed that nephron formation was not only accompanied by the breakdown of the adjacent basal lamina, but that nephrogenic and duct epithelial cells could each be seen in the other tissue. Such a suggestion could be confirmed with the use of appropriate specific markers.

The formation of nephrogenic cells

One of the more intriguing problems in kidney development is how Pax2+ stem cells that form within the MM adjacent to the tip of the ureteric bud (e.g. Hatini et al. 1996) reach the kidney periphery

where they become the source of nephrons over the period E13.5-E19.5. There are 2 possible explanations for this phenomenon: either early-induced MM cells are carried outwards with the duct tip, or new MM cells continue to be induced by the tip as it extends, with more central cells losing the Pax2 that they had expressed earlier.

The observations on NCAM expression (which is essentially coexpressed with Pax2, and for longer than hitherto supposed; Klein et al. 1988) suggest that the latter is the more likely option. This is because the evidence (e.g. Fig. 3) shows that cells expressing NCAM when they are near the duct tip downregulate this marker once the tip extends past them, with only the aggregate cells maintaining expression. It should however be emphasised that the mode by which these markers are lost remains unknown.

Nephron induction

The results presented here suggest that nephrogenesis involves a series of inductive interactions controlled by the ureteric duct. The first clearly causes the MM to switch out of the apoptosis pathway and is WT1-dependent (Kreidberg et al. 1993). This is a relatively long range interaction extending over some 120 μm ($\sim$ 12 cells) and includes all of the MM (perhaps 10000 cells); it takes place at about E11, when the extending ureteric bud invades the MM. The second step produces caps of induced MM that surround duct tips and express NCAM, Pax2 and other markers such as syndecan (Vainio et al. 1989). The confocal evidence clearly shows that this cap extends out for some 5 cells (a few hundred cells in total). The third inductive event is manifested just proximal to this cap and is localised to specific domains on the duct surface that are a few cells deep and separated by at least ten cell diameters. The associated aggregates are about 2–3 cells across ($<$ 8 cells in all) when first apparent, but they soon enlarge, starting to epithelialise when they are perhaps 6 cells in diameter (perhaps 100 cells). The signals responsible for nephron induction in vivo have yet to be identified, but a mix of TGFβ2, FGF2 and LIF can cause uninduced MM to undergo nephrogenesis in vitro (Plisov et al. 2001), with LIF likely to be the key signal (Barasch et al. 1999). The status of these results is however still unclear as LIF $-/-$ mice have normal kidneys (Sendtner et al. 1996).

It thus seems that the process of nephrogenesis can be initiated via at least 2 routes. The easier one to assay requires only long-range signalling (e.g. via LIF) and is probably not the one actually employed in vivo. The other mode involves both a longer range disseminated interaction and a very short-range interaction (only a few cell diameters), with the latter signal possibly passing through direct cell-to-cell contacts, e.g. the notch-delta or *sevenless* systems (Hafen et al. 1994; Lendahl, 1998). In vitro evidence to support this view of 2 interactions being necessary for normal nephron induction comes from the recent observation (Godin et al. 1999) that isolated E11.5 MM expressing WT1 and Pax2 (and hence induced) will not generate nephrogenic aggregates in culture. This observation probably explains why a ureteric bud is incapable of inducing MM across a filter.

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