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DEVELOPMENTAL BIOLOGY REVIEW

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Angiopoietin growth factors and Tie receptor tyrosine kinases in renal vascular development

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Abstract Angiopoietin-1 (Ang-1) is a secreted growth factor which binds to and activates the Tie-2 receptor tyrosine kinase. The factor enhances endothelial cell survival and capillary morphogenesis, and also limits capillary permeability. Ang-2 binds the same receptor but fails to activate it: hence, it is a natural inhibitor of Ang-1. Ang-2 destabilises capillary integrity, facilitating sprouting when ambient vascular endothelial growth factor (VEGF) levels are high, but causing vessel regression when VEGF levels are low. Tie-1 is a Tie-2 homologue but its ligands are unknown. Angiopoietin and Tie genes are expressed in the mammalian metanephros, the precursor of the adult kidney, where they may play a role in endothelial precursor growth. Tie-1-expressing cells can be detected in the metanephros when it first forms and, based on transplantation experiments, these precursors contribute to the generation of glomerular capillaries. During glomerular maturation, podocyte-derived Ang-1 and mesangial-cell-derived Ang-2 may affect growth of nascent capillaries. After birth, vasa rectae acquire their mature configuration and Ang-2 expressed by descending limbs of loops of Henle would be well placed to affect the growth of this medullary microcirculation. Finally, preliminary data implicate angiopoietins in deregulated vessel growth in Wilms' kidney tumours and in vascular remodelling after nephrotoxicity.

Keywords Angiogenesis · Angiopoietin-1 and -2 · Glomerulus · Metanephros · Tie-1 and -2 · Vasa recta · Vasculogenesis

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Introduction

In the past decade, firm evidence has emerged that growth factors, generally signalling through cell-surface molecules called receptor tyrosine kinases (RTK), are critical for nephron formation and collecting duct maturation during development of the metanephros, the precursor of the adult mammalian kidney [1]. Currently, there is growing interest in molecules, including growth factors, which control the construction of renal endothelial cells (EC), not least because the adult kidney is highly vascular, receiving about 20% of cardiac output.

In this review, we focus on one such signalling system, mediated by a class of RTK comprising the Tie (tyrosine kinase containing immunoglobulin like loops and epidermal growth factor similar domains) genes, and the angiopoietin (Ang) ligands. First, however, we will address the anatomy of renal vascular development and also the evidence that a more familiar signalling system, that mediated by vascular endothelial growth factor (VEGF), is implicated in renal vascular development. As discussed below, the biological effects of the angiopoietins partly depend on the ambient levels of VEGF.

Vascular differentiation in the kidney

The mouse provides a precisely staged model of renal vascular development [2–7] (Fig. 1). At embryonic day 11 (E11), approximately equivalent to 5 weeks after fertilisation in humans, the metanephros is formed as nephrogenic mesenchyme condenses around ureteric bud epithelium, a branch of the mesonephric, or Wolffian, duct. At this stage renal mesenchyme is avascular as assessed by light and electron microscopy although capillaries are detected between the organ and the mesonephric duct, and also on the perimeter of the region of intermediate mesoderm which is condensing to form renal mesenchyme. Just 1 day later, when the ureteric bud has branched about twice, vessels are detected around the stalk of the ureteric bud in the metanephric hilum. By

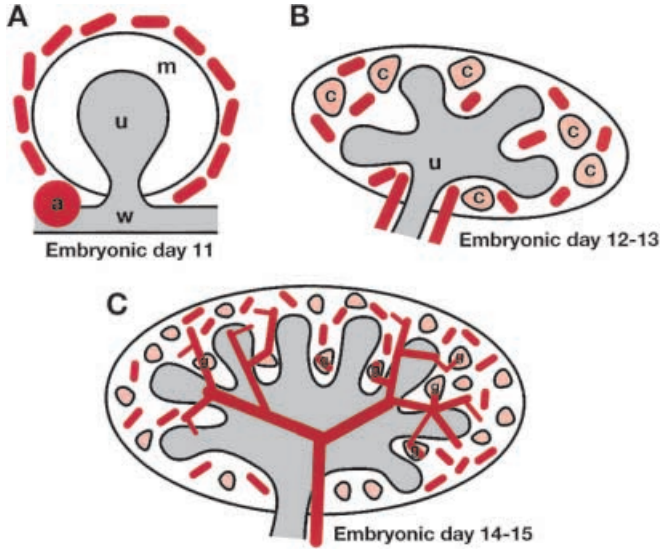

Fig. 1A–C The early steps of blood vessel formation in the mouse metanephros. *The panels*, which are not to scale, depict sections through the developing mouse metanephros. **A** At embryonic day 11, the metanephros forms. The ureteric bud (*u*, *grey*), a branch of the Wolffian duct (*w*), becomes enveloped by nephrogenic mesenchyme (*m*, *white*), derived from caudal intermediate mesoderm. A network of capillaries (*red*) surrounds the renal mesenchyme, which itself is avascular, and the dorsal aorta (*a*) is situated nearby. **B** Between embryonic days 12 and 13, the ureteric bud branches into primitive collecting ducts and primitive nephron condensates (*c*) form within the mesenchymal compartment. At this stage, capillaries surround the stalk of the ureteric bud, and a loose network of capillaries is detected in the mesenchymal compartment near to the primitive nephrons. **C** The first layer of vascular glomeruli (*g*) have formed by embryonic days 14–15. In the periphery of the organ, new layers of condensates form and are surrounded by a loose capillary network. By this stage, the main renal artery has formed and it branches serially deep in the organ. New layers of glomeruli continue to be generated until about 1 week after birth. Note that the vasa rectae microcirculation is not depicted in this figure, since it does not form until later in development

E13, patent capillaries surround nephron precursors, the most primitive of which are called condensates. More mature nephron precursors are called S-shaped bodies, each with EC in the cleft between maturing podocytes and the primitive proximal tubule. From E14 (approximately equivalent to weeks 9–10 of human gestation) arteries, sheathed by smooth muscle cells, run from the hilum of the mouse metanephros to the corticomedullary junction, where they form arcades from which small cortical arteries branch and terminate in afferent glomerular arterioles. At this stage, vessels destined to form the renal arterial system begin to be surrounded by specialised cells which differentiate into vascular smooth muscle cells or, in smaller vessels, pericytes [6].

From E15, the most mature nephrons, located toward the centre of the metanephros, acquire capillary loops supported by mesangial cells: the latter cells share many biochemical features with the smooth muscle/pericyte lineage. Thereafter, up to one postnatal week, new layers of glomeruli are generated. In the first three postnatal weeks, the medulla grows considerably, with maturation of loops of Henle flanking ascending and descending vasa rectae. In the normal adult kidney, there is thought to be a very low EC turnover, although both proliferation and apoptosis are upregulated in certain models of acute and chronic nephropathies [8, 9].

Growth factors and embryonic EC development

During development, there are two ways by which EC are formed [10, 11]. In vasculogenesis, or in situ vessel formation, precursor cells differentiate into EC. In angiogenesis, by contrast, EC sprout or delaminate from existing vessels and migrate to new locations to form new capillaries. The first embryonic EC, including those of the yolk sac and heart primordium, must form by vasculogenesis. Based on in ovo transplantation studies between chick and quail, the earliest vessels of some organs (e.g. brain and mesonephros) are considered to form by angiogenesis, whereas vasculogenesis operates in other sites (e.g. liver) [11]. Both angiogenesis and vasculogenesis occur in some tissues such as the limb bud.

As assessed by analysis of genetically engineered mutant mice, VEGF receptor (VEGFR) signalling is critical for embryonic EC differentiation in diverse vascular beds [10]. VEGFR-2 (also called flk-1 in mice and KDR in humans) initiates vasculogenic formation of EC while VEGFR-1 (also called flt-1) modulates subsequent embryonic assembly into vessels. Both receptors are expressed by E11 mouse renal mesenchymal tissues and subsequently by diverse EC later in metanephric development [3, 4, 12–14]. Moreover, VEGF mRNA and protein is expressed by renal mesenchyme from the inception of metanephrogenesis [3], with high levels of transcripts in nascent glomeruli [15].

Classical studies based on organ culture of murine renal mesenchyme, and transplantation of metanephric rudiments onto avian chorioallantoic membrane, led to the conclusions that EC precursors were absent in the early metanephros and that glomerular capillaries arose by angiogenesis [16–18]. More recent studies, using VEGFR-1 and -2 as markers, confirmed that EC do not thrive when metanephric explants are grown in a standard, normoxic (i.e. 21% O_2), atmosphere in defined media [12–14]. However, hypoxic culture upregulates metanephric VEGF and EC proliferation [13], and addition of VEGF to explants grown in air also enhances vessel formation [14]. Moreover, transplantation of metanephroi to the ectopic location of the anterior eye chamber results in generation of implant-derived glomerular EC [12]. Collectively, these recent experiments suggest that EC precursors are indeed present in the early metanephros and their differentiation is enhanced by VEGF. Other studies, using genetic and immunological strategies to block VEGF in vivo, led to the conclusion that this factor is critical for glomerular capillary growth at later stages of nephrogenesis [19–21].

Intriguingly, after nephrogenesis is complete, podocytes continue to express VEGF [22], even though EC pro-

liferation is exceedingly low in this location in adults [8]. Here, the factor has been postulated to play a role in maintenance of EC fenestrae, based on the ability of VEGF to induce these structures in EC in vitro [23, 24]. In addition, since VEGF enhances capillary permeability [10], the molecule was considered a candidate for enhancing proteinuria in glomerular diseases. However, Gerber et al. [20] were unable to elicit histological changes in adult glomeruli despite up to 4 weeks of systemic VEGF blockade, and Ostendorf et al. [9] failed to modulate pathological glomerular proteinuria by inhibiting $VEGF_{165}$: instead, in the mature kidney, VEGF may have a role in EC survival and proliferation in acquired glomerular diseases [9].

The Tie-1 RTK in kidney EC differentiation

Many other growth factors expressed during nephrogenesis [1] affect the proliferation and migration of EC: these include fibroblast growth factor [25], hepatocyte growth factor [26, 27] and transforming growth factor-β1 [28]. However, the actions of these factors are not confined to EC, and they have effects on epithelial, and other metanephric lineages [1]. In contrast, the Ang/Tie signalling axis is much more specific for the endothelial lineage, and is the subject of the remainder of this review. Yet other EC-specific systems are likely to be important in renal vascular growth, such as those mediated by the ephrins [29], but are beyond the scope of this article.

As EC differentiate, the onset of Tie RTK expression postdates VEGFR-2 but precedes maturity: hence the role of these receptors is to modulate growth of precursors which have already entered the EC lineage [30–32]. Tie-1 is currently an 'orphan RTK', meaning that its growth factor ligand has yet to be defined. In mice genetically engineered so that both *Tie-1* alleles are ablated, the null-mutant embryos die in mid- to late gestation with impaired vessel integrity [30, 31]. Furthermore, mutant cells in animals derived from chimeras between –/– and normal cells (*Tie-1*^{lcz}/*Tie-1*$^{lczn-}$ chimeric mice) fail to contribute to renal vasculature, suggesting a nephrogenic role for this gene [32].

The Tie-1 gene is expressed in differentiating EC precursors in mouse metanephroi from E11 with transcript levels peaking in the first few weeks after birth [3–5]. We have exploited a *Tie-1/LacZ* transgenic mouse to follow kidney EC development [3, 4]: this otherwise normal strain expresses LacZ (bacterial β-galactosidase) driven by the *Tie-1* promoter, and hence gene activity can be visualised in situ by using a simple enzymatic (X-gal) colour reaction (Fig. 2). At E13, networks of Tie-1/LacZ-expressing capillaries are detected around developing nephrons (Fig. 2A), yet these delicate vessels regress when organs are cultured in air for a few days [4]. In contrast, E15 glomeruli in vivo have prominent Tie-1-expressing capillary loops (Fig. 2B). When kidney rudiments are explanted in hypoxic conditions, Tie-1 expression is maintained in large, but relatively unstructured, masses of EC precursors located between tubules [4] (Fig. 2C). Strikingly, when avascular E11 *Tie-1/LacZ* metanephroi are implanted into the nephrogenic cortex of wild-type neonatal kidneys [3], a site allowing the differentiation of nephrogenic precursors into filtering glomeruli [33], transgene-expressing glomerular and stromal capillaries develop within transplants (Fig. 2D). This demonstrates the possibility of in situ, vasculogenic, EC differentiation from Tie-1-positive precursors present at the inception of the metanephros.

These results, using Tie-1 as a marker for EC development, generally support the conclusions about the origin of kidney capillaries based on organ culture and in oculo transplantation studies using VEGFR-1 and -2 as markers [12–14]. However, it should be noted that neither the medullary (vasa rectae) microcirculation nor large vessels develop in organ culture or within transplanted rudiments, so the origins of these structures remain open to speculation.

Angiopoietins and vascular development

Tie-2 (previous called Tek) is a Tie-1 homologue and its ligands are the angiopoietins, an expanding family of secreted factors [34]. These ligands comprise an amino-terminal coiled-coil domain mediating the formation of dimers and higher order multimers between specific family members, while carboxy-terminal fibrinogen-like domains mediate the factors' differential effects on Tie-2 phosphorylation [35]. Angiopoietin-1 (Ang-1) binds to the Tie-2 RTK [36]: subsequent tyrosine phosphorylation transduces signals for EC survival and capillary sprouting [37, 38]. There is a multisubstrate docking site in the carboxy-terminal tail of Tie-2 (Tyr 1100), with diverse intracellular molecules, including Grb2, Grb7, Grb14, Shp2 and the p85 subunit of phosphatidylinositol 3'-kinase, interacting with the phosphorylated receptor through their SH2 domains, as assessed by the yeast two-hybrid system [39]. Other experiments demonstrate that Ang-1-induced bioactivities in EC are associated with activation of phosphatidylinositol 3'-kinase, Akt protein kinase B and focal adhesion kinase, as well as plasmin secretion [40, 41]. In relation to Ang-1-induced sprouting, synergy with VEGF has been documented [38].

Both *Ang-1* and *Tie-2* null-mutant mouse embryos have abnormal vascular networks with growth-retarded vascular smooth muscle and pericyte precursors [31, 42, 43]. In addition to the direct effects on endothelia, it has been postulated that Tie-2 activation also causes reciprocal, maturational effects on adjacent smooth muscle and pericyte precursors elicited by EC-derived factors such as platelet-derived growth factor B (PDGF-B) [10]. It has also been demonstrated that Ang-1 inhibits capillary permeability [44, 45], preventing plasma leakage in response to both VEGF and mustard oil, and the factor also has a role in blood formation [46].

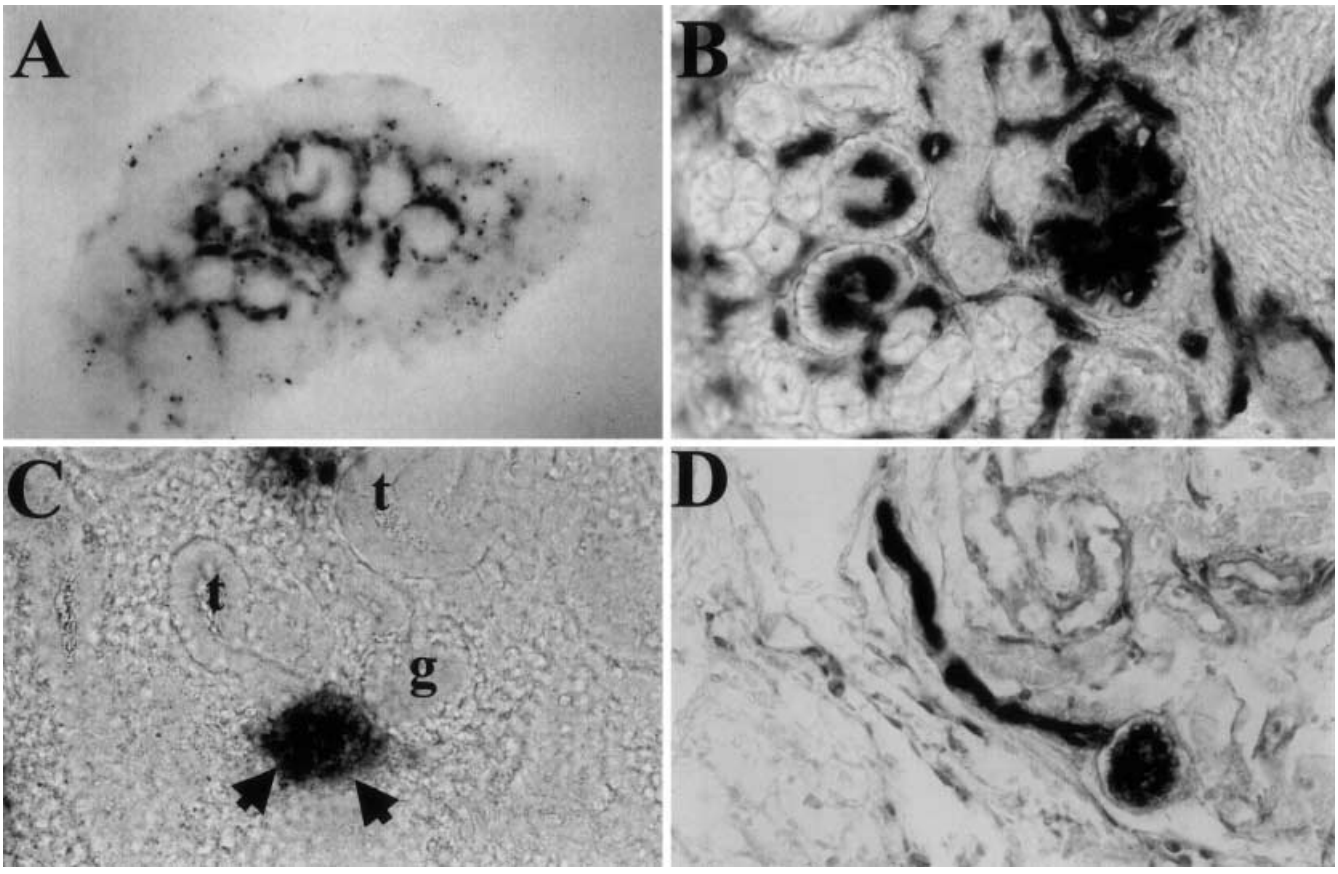

Fig. 2A–D Tie-1/LacZ gene expression in mouse nephrogenesis. **A** Promoter activity was detected in vivo at embryonic day 13 in a loose network of vessels around nascent nephron condensates: glomeruli have yet to form. Gene expression is also noted in the cortical renal mesenchyme in isolated cells, most likely EC precursors: these appear as 'dots' in the periphery of the organ. **B** Two days later, at embryonic day 15, Tie-2 expression is markedly upregulated in vivo in interstitial and glomerular capillaries. **C** In contrast, when the embryonic day 13 organ, depicted in **A**, is grown in hypoxic organ culture for a few days, normal vessel branching is lost, yet Tie-2-expressing EC precursors proliferate between tubules (*t*), forming unstructured masses (*arrows*). Glomeruli (*g*) remain avascular. **D** After transplantation of an E11 *Tie-1/LacZ* metanephros into the nephrogenic cortex of a wild-type mouse, EC precursors differentiate in situ and undergo normal patterning to form glomerular arterioles and capillaries, as well as interstitial vessels

Upregulated signalling though a constitutively active, mutant *Tie-2* receptor leads to the formation of cutaneous vascular malformations in humans [47], while transgenic Ang-1 overexpression in the skin of mice causes larger, more numerous and highly branched vessels compared to normals [48]. Peters et al. [49] demonstrated Tie-2 expression in breast tumour vasculature correlating with pathological angiogenesis, and Shyu et al. [50] reported that forced intramuscular expression of angiopoietin-1 enhanced revascularisation in rabbit ischaemic hindlimbs. Of note, Tie-2 gene expression is upregulated by hypoxia by endothelial PAS domain protein 1, an EC-specific transcription factor [51].

Ang-2, the second member of the ligand family to be described, binds Tie-2 without causing tyrosine phosphorylation in cultured EC [52]. Instead, it antagonises Ang-1-induced Tie-2 phosphorylation, while Ang-2 overexpression in vivo causes defects resembling Tie-2 and Ang-1 null mutants [52]. In the presence of abundant VEGF, Ang-2 is thought to destabilise vascular networks and facilitate sprouting, e.g. during tumour growth [53, 54]. Conversely, with low ambient VEGF levels, Ang-2 may cause vessel regression, e.g. in corpus luteum involution [52]. Hypoxia has been demonstrated to upregulate transcription of Ang-2 [55, 56].

Recently, a third member of the ligand family, Ang-3, was cloned in mice: it is postulated to have a similar action as Ang-2 [34].

Angiopoietins and EC development in the kidney

Therefore, the Ang-1/Tie-2 signalling system appears to act as a stabilising influence during the later stages of capillary formation, while Ang-2 acts as a natural inhibitor of Ang-1. Expression studies in non-renal tissues are consistent with the hypothesis that Ang-1 and Ang-2 are expressed by smooth muscle cells, pericytes and their precursors and exert paracrine effects on Tie-2-expressing EC [36, 52]. However, certain types of EC [55, 56] can express Ang-2 as well as Tie-2, raising the additional possibility of an additional autocrine action.

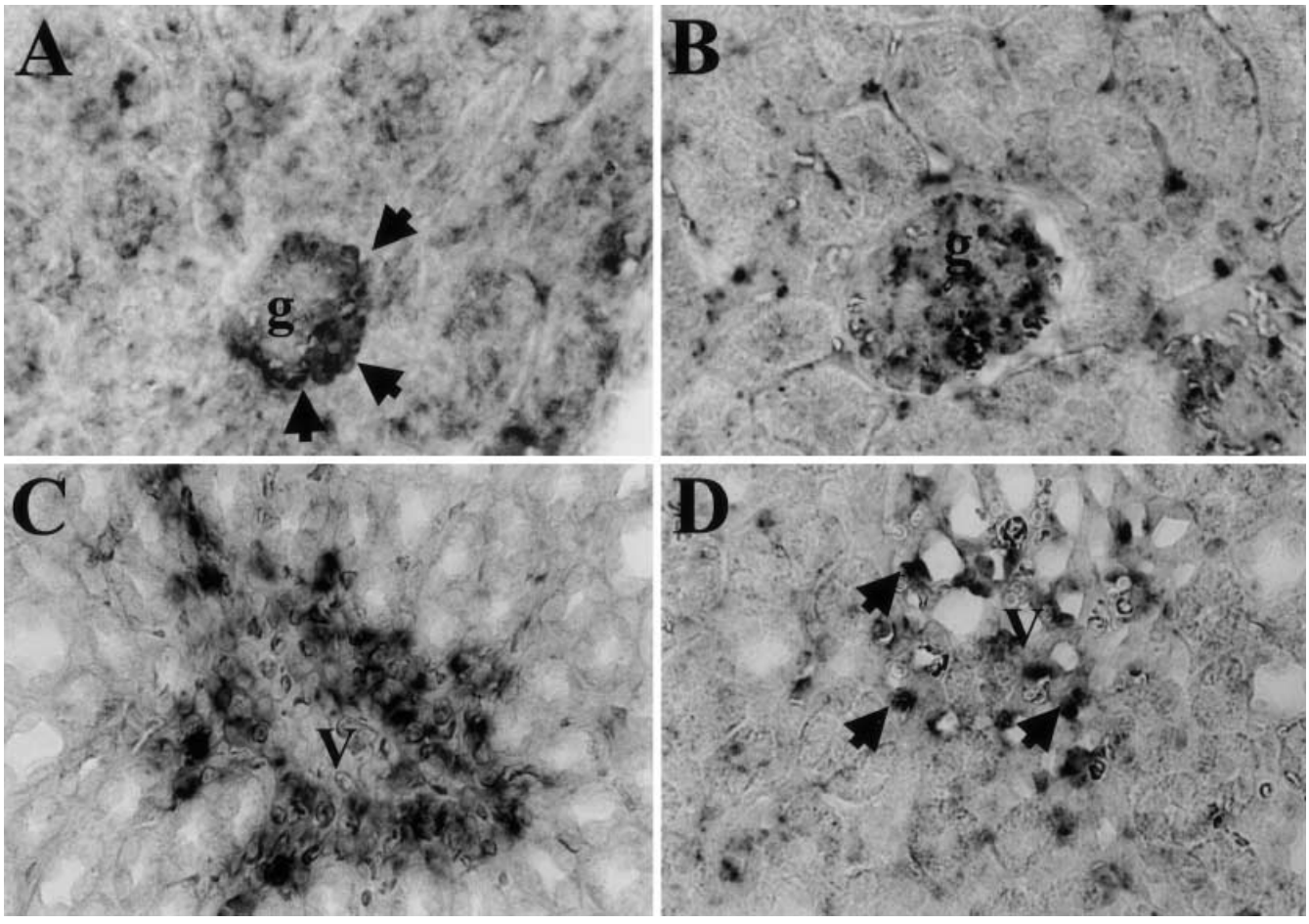

Fig. 3A–D Angiopoietin and Tie-2 gene expression during mouse kidney maturation. **A** Ang-1 expression in maturing cells (*arrows*) of a glomerulus (*g*) in a 1-week postnatal mouse kidney visualised by in situ hybridisation. **B** Tie-2 expression in glomerulus (*g*) and interstitial capillaries of a 1-week postnatal mouse kidney visualised by in situ hybridisation. **C** Ang-2 expression in tubules surrounding vasa rectae (*v*) of a 3-week postnatal mouse kidney visualised by in situ hybridisation. **D** Tie-2 expression in vasa rectae (*v*) capillaries (*arrows*) of a 3-week postnatal mouse kidney visualised by in situ hybridisation

Relatively little is known, however, about the expression or roles of these molecules during development of specific embryonic organs. Colen et al. [57] reported that Ang-1 and -2 were expressed during lung and pancreas vessel growth, and Akeson et al. [58] reported that subsets of lung precursor cells expressed these ligands as well as Tie genes: furthermore, after injection of these embryonic lung mesenchymal cells into blastocysts, they contributed to EC development within the developing lung and heart [58].

Recently, Yuan et al. [5] studied Ang-1, Ang-2 and Tie-2 expression from the onset of glomerulogenesis (E14) to adulthood in normal mouse kidneys. Using Northern blotting, these genes were expressed throughout the experimental period with peak levels in the first three postnatal weeks. Subsequently, these genes became downregulated, with low levels of mRNA persisting into adulthood. By in situ hybridisation, Ang-1 transcripts were found in condensing cortical mesenchyme, maturing glomeruli (Fig. 3A), proximal tubules and outer medullary tubules. Using Western blotting, Tie-2 was detected from E14, with tyrosine-phosphorylated RTK evident from E18 into adulthood [5]. By in situ hybridisation and immunohistochemistry, Tie-2 was localised to capillaries in nephrogenic cortex, glomerular tufts (Fig. 3B) and vasa rectae (Fig. 3D). Furthermore, our preliminary data ([59]; A.S.W., H.T.Y. and M. Kolatsi-Jouannou, unpublished results) demonstrate that Ang-1 and Tie-2 genes are also expressed between E11 and E13 in the mouse metanephros, and that addition of Ang-1 to embryonic kidneys in organ culture leads to the formation of vascular channels.

In vivo, the highest levels of Ang-2 transcripts are detected in a subset of thin descending limbs of loops of Henle [5], a conclusion supported by the analysis of β-galactosidase reporter gene expression in *Ang-2/LacZ* heterozygous kidneys [6]. In this location in the outer medulla, Ang-2-expressing tubules show the striking configuration of a ‘fence’ which surrounds (Fig. 3C) ascending and descending vasa rectae capillaries. Furthermore, levels of this ligand increase postnatally at the same time as medullary expression of Ang-1 is declining [5, 6]. We speculate that the perpetual hypoxia which is known to occur in the medulla may tonically upregulate

Ang-2 in this locality, as demonstrated for other types of cells which express Ang-2 [7, 55, 56].

Ang-2/LacZ promoter activity and Ang-2 protein is also prominent in the walls of differentiating renal vessel walls [6, 7]. Some of these cells which express Ang-2 are likely to represent smooth muscle cells and pericyte precursors condensing from surrounding mesenchyme [60]. Hungerford and Little have described such precursors as 'fibroblast-like' cells, initially lacking myofilaments and basement membranes and appearing as aggregates near embryonic endothelium [60]. In this respect, it is interesting that Ang-2-expressing cells in nascent vessel walls appear to extend further than those which express alpha-smooth muscle actin [6]. The walls of the developing kidney vessels also show widespread expression of another secreted protein, renin [61]. However, while we found that Ang-2 expression was continuous through the fetal kidney arterial system to the level of small cortical arteries [6], albeit not in every cell in a single locus, renin-expressing cells were reported to be discontinuous, for example localised to branch points [61].

Ang-2 is also expressed in cores of maturing glomeruli where mesangial cells reside [6, 7]. Since mesangial cells share biosynthetic and structural properties with vascular smooth muscle cells, Ang-2 expression is consistent with this relationship. Moreover, mesangial cells isolated from juvenile mice expressed Ang-2 and Ang-3 transcripts, but not Ang-1 or Tie-2, in culture: these mesangial cells upregulate Ang-2 mRNA and protein in response to hypoxia, concomitantly with an increase in VEGF [7]. Other studies have demonstrated that mesangial cells derive from mesenchymal-like precursors located near maturing glomeruli and that their differentiation is dependent on PDGF-B signalling [62].

There is currently little information about the expression of the Ang/Tie axis in renal diseases. Recently, however, we reported upregulated Tie-2 capillary immunostaining and mRNA expression in the stroma of Wilms' tumours, a childhood kidney cancer [63]. In the same study, epithelial elements of Wilms' tumours expressed Ang-1 transcripts but Ang-2 has yet to be examined. In addition, we have found that Ang-1 protein is markedly upregulated, assessed by Western blotting and immunohistochemistry, after folic-acid-induced acute renal failure in mice [64]: this is accompanied by increased expression of kidney VEGF. Here, it is possible that these growth factors have yet-to-be defined roles in the survival of kidney EC or vascular remodelling following renal injury.

Prosepctive roles of the angiopoietins and Tie genes in the kidney

Although formal proof for functional roles of the renal Ang/Tie axis requires data from functional experiments, we suggest that these genes may have the following roles in kidney development and disease:

1. Tie-1-expressing precursors in the early metanephros are capable of in situ differentiation into glomerular EC. In the early stages of metanephric growth, Ang-1 may enhance the survival and sprouting of Tie-2-expressing EC precursors, in concert with VEGF.
2. Later in fetal kidney vessel growth, Ang proteins secreted by cells surrounding nascent vessels would modulate Tie-2 activation in adjacent EC. As maturation proceeds, feedback occurs in which EC release factors enhancing the maturation of vessel wall cells.
3. Locally derived Ang-1 and Ang-2 would be well placed to modulate the growth of glomerular EC which express Tie-2. Again, the final actions on EC survival, proliferation and morphogenesis would be modulated by VEGF.
4. After birth, vasa rectae acquire their mature configuration and Ang-2 expressed by adjacent epithelia could maintain the structural integrity of this medullary microcirculation, in a yet to be defined manner.
5. Tie-2 signalling may be important in renal tumour angiogenesis and also in vascular survival and remodelling after renal injury.

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LITERATURE ABSTRACT

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Isolated hypercalciuria with mutation in CLCN5: relevance to idiopathic hypercalciuria

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Background Idiopathic hypercalciuria (IH) is the most common risk factor for kidney stones and often has a genetic component. Dent's disease (X-linked nephrolithiasis) is associated with mutations in the CLCN5 chloride channel gene, and low molecular weight (LMW) proteinuria was universally observed in affected males. We sought to identify mutations in CLCN5 or abnormalities in LMW protein excretion in a large group of patients with IH and in a rat model of genetic hypercalciuria.

Methods One hundred and seven patients with IH (82 adults and 25 children) and one asymptomatic hypercalciuric man with a known inactivating mutation in CLCN5 were studied. Secondary causes of hypercalciuria were excluded in all. The excretion of retinol-binding protein and beta2-microglobulin was measured by immunoassay in 101 patients with IH. Mutation analysis of the CLCN5 gene was performed in 32 patients with IH and in the genetic hypercalciuric stone-forming (GHS) rat strain.

Results LMW protein excretion was normal in 92 patients with IH, and only slight abnormalities were found in the other nine, none of whom had a mutation in CLCN5. One 27-year-old man who had a CLCN5 mutation was found to have isolated hypercalciuria without LMW proteinuria, renal failure, or other evidence of renal disease. Mutation analysis was normal in 32 patients with IH. The CLCN5 sequence was normal in the GHS rat.

Conclusions Inactivation of CLCN5 can be found in the setting of hypercalciuria without other features of X-linked nephrolithiasis. However, mutations in CLCN5 do not represent a common cause of IH.