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Origins and formation of microvasculature in the developing kidney

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Origins and formation of microvasculature in the developing kidney. Regulation of microvessel assembly in the developing kidney is not known and may occur through vasculogenic, angiogenic, or both processes. To examine this question, we grafted rat and mice embryonic (E) day 12 (E12) kidneys, which have only a rudimentary vasculature, into anterior eye chambers of mouse and rat hosts. Species-specific, monoclonal anti-basement membrane antibodies showed that glomerular basement membranes, mesangial matrices, and microvessel basement membranes were always derived from the graft. When wild-type E12 mouse kidneys were grafted into anterior chambers of ROSA26 mice, in which the β-galactosidase transgene is expressed ubiquitously, glomerular and microvascular endothelial cells stemmed from the graft, even after maintenance of kidneys in organ culture for 6 days before grafting. Immunolabeling with antibodies against the vascular endothelial growth factor (VEGF) receptor, Flk1, the EphB1 receptor, and its ligand, ephrin-B1, labeled discrete mesenchymal cells in embryonic and newborn kidney cortex, as well as developing microvessel and glomerular endothelium. In adult kidneys, Flk1 labeled glomeruli weakly, other vascular structures were unlabeled. When wild-type E12 kidneys were grafted under renal capsules of adult ROSA26 hosts, endothelium developing within the graft again came from the implanted kidney. In contrast, when E12 kidneys were grafted into renal cortices of newborns, glomeruli within grafts now contained host-derived endothelium. Similarly, when ROSA26 E12 kidneys were implanted into newborn wild-type hosts, chimeric vessels containing graft- and host-derived endothelium were seen in nearby host tissue. Our results indicate that cells capable of forming the entire microvascular tree of grafted metanephroi are already present in the E12 kidney. We hypothesize that Flk1/VEGF and EphB1/ephrin-B1 mediate renal endothelial mitosis-motility and cell guidance-aggregation behavior, respectively.

EMBRYONIC KIDNEY VASCULARIZATION

The renal microvasculature is an intricate, reiterated system of microvascular units that envelops each of the ~1-million nephrons in each human kidney. Each nephron microvascular unit consists of an afferent glomerular arteriole, a glomerular capillary tuft, an efferent glomerular arteriole, and a peritubular capillary bed. Once the full complement of nephrons has formed during kidney organogenesis, there is severely limited ability to repair injured glomeruli and no capacity whatsoever to generate new glomeruli to replace those that become irretrievably damaged. In addition, uncontrolled hypertension and diabetes invariably lead to renal arteriolosclerosis and glomerulosclerosis, which result in renal failure. Despite the importance of the renal microvasculature, the fundamental mechanisms accounting for its initial formation during organogenesis, and maintenance thereafter, are not known.

Development of the permanent, metanephric kidney in the mouse begins on embryonic day 11 (E11). At this time, the ureteric bud, which is an epithelial tube derived from the nephric duct, grows into the metanephric mesenchyme and undergoes a series of dichotomous branches. At the tips of each branch, mesenchymal cells are induced to aggregate [1, 2]. Each mesenchymal cell aggregate rapidly assumes an epithelial phenotype and becomes an early nephric figure referred to as a "vesicle" that subsequently develops into a nephron. On the lower aspect of the vesicle, a groove termed the vascular cleft develops, and endothelial precursors migrate into this cleft to begin forming the glomerular capillary tuft.

Saxén has reviewed the two probable embryonic sources for the glomerular endothelium [3]. First, metanephric mesenchymal cells may migrate into the vascular cleft and, by vasculogenesis, establish the glomeruli. Vasculogenesis is the process whereby dispersed, mesodermally-derived endothelial precursor cells (angioblasts) coalesce *in situ* to form a network of channels that then connect to larger vessels [4]. The possibility that glomerular endothelium stems from metanephric vasculogenic angioblasts was raised on the basis of earlier morphologic investigations, but until very recently, there was no direct experimental support. A second possible source for embryonic kidney endothelial cells is from the ingrowth of tubelike angiogenic sprouts derived from external vessels. Support for this second possibility comes from interspecific grafts of fetal

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kidneys onto avian chorioallantoic membrane (CAM) [6], a site that provides a rich vascular bed to sustain the graft. When E11 mouse kidneys are grafted onto chick CAM and labeled with species-specific anticollagen type IV antibodies, both antichick and antimouse antibody bind to the glomerular basement membranes (GBMs) of glomeruli forming in these grafts, but mesangial matrices only label with the antichick antibody [6]. Separate experiments showed that when mouse kidneys are grafted onto quail CAM, glomerular endothelial cells contain the prominent quail nucleolar marker [7]. Conclusions drawn from these studies are that both epithelial and endothelial cells contribute to the GBM and that there is an exogenous origin for renal endothelial and mesangial cells, which presumably stem from ingrowing angiogenic sprouts [6, 7]. Some problems acknowledged in these studies were that few mammalian glomeruli develop *in ovo* and that, in general, no full-blown glomeruli are seen [6, 7]. These earlier experiments also lack a method for the unambiguous detection of donor-produced endothelium, and they were carried out at a time when there was little awareness of angioblasts or the process of vasculogenesis and when markers for early endothelial cells were not available.

ANTERIOR CHAMBER GRAFTS OF EMBRYONIC KIDNEYS

To reexamine the embryonic origins of renal glomerular endothelial cells, we established interspecies grafts between rats and mice in which embryonic kidneys were implanted into anterior eye chambers of adult hosts [8]. After five to seven days, hosts then receive i.v. injections of species-specific monoclonal antibodies against either rat- or mouse-produced basement membrane proteins. In all cases, GBMs, mesangial matrices, and microvessel extracellular matrices within grafts label solely for donor- (kidney-) derived matrix [8]. To examine directly the origin of glomerular endothelial and mesangial cells, we grafted E11 to E12 kidneys from wild-type mice into ROSA26 mice, which are transgenic animals that express β-galactosidase in every cell [9]. When grafts are developed for β-galactosidase activity, host cells are seen in peripheral vessels, but the vast majority of glomerular and microvascular endothelial cells are of donor, not host, origin, and mesangial cells are always of donor origin [8]. Together, these results show that cells from the implanted kidney are almost entirely responsible for development of the renal microvasculature *in oculo*. External vessels from the host, although important for graft maintenance, are not major contributors to the glomerulus [8].

ORGAN CULTURE OF EMBRYONIC KIDNEYS

Although organotypic epithelial tubulogenesis occurs when kidneys are placed in culture, only avascular glomerular structures form *in vitro* under normoxic (20% O_2) conditions [2, 3, 10]. Nonvascularized glomeruli in these organ culture systems consist of differentiated podocytes replete with foot processes that surround lengthy basement membrane segments, but no morphologic evidence for renal blood vessel formation *in vitro* has been obtained [3, 10]. We cultured E11 to E12 mouse kidneys *in vitro* for up to 6 days and labeled the cultures with *Bandeiraea simplicifolia* isolectin B_4, which binds specifically to embryonic mouse endothelial cells [11]. Although lectin-labeled microvessels are seen in freshly harvested E12 kidneys, such structures are not observed after 6 days in organ culture [12]. To examine the fate of these organ cultures further, kidneys that had first been maintained *in vitro* for 6 days were then grafted into anterior eye chambers of adult ROSA26 hosts. Grafts were harvested 7 days after implantation and developed for β-galactosidase activity. These grafts contain numerous mature glomeruli perfused with erythrocytes, but the microvasculature within the grafts does not express the host transgene and is, therefore, of endogenous origin [12]. This evidence indicates that intrinsic endothelial precursors survive organ culture and, upon grafting, have the capacity to generate the entire kidney microvasculature *in vivo* [12].

MECHANISMS OF VASCULAR DEVELOPMENT

Considerable recent evidence has implicated several growth factor/transmembrane receptor tyrosine kinase (RTK) systems in mediating early blood vessel formation. The intrinsic tyrosine kinase activity of these receptors is activated upon ligand binding at the external cell surface, which results in phosphorylation of the cytoplasmic tail of the receptor itself along with other intracellular proteins, and stimulates a cascade of cellular activities regulating growth and differentiation [13]. Several potentially relevant angiogenic growth factors, including acidic and basic fibroblast growth factor and platelet-derived growth factor and their respective RTKs, are expressed in the kidney [14, 15]. Precisely what roles these factors play in metanephric vascularization have not been firmly established, however.

Characterization of vascular endothelial growth factor (VEGF) and its two distinct RTKs, which in mice are referred to as fetal liver kinase (Flk1) and *fms*-like tyrosine kinase-1 (Flt1), has shown that this signaling system is involved directly in endothelial cell commitment and differentiation [16]. VEGF and Flk1 are expressed co-ordinately during kidney organogenesis, implying pivotal functions for this receptor/ligand pair in renal blood vessel development [17, 18]. VEGF is a potent endothelial cell-specific mitogen that enhances cell migration and induces serine proteases, all of which are important for angiogenesis and, probably, vasculogenesis. VEGF expression has been demonstrated by *in situ* hybridization in visceral epithelial cells of S-shaped bodies of human fetal kidneys and in glomerular epithelium of E15 mice [18, 19]. To assess the role of VEGF directly, VEGF gene expression

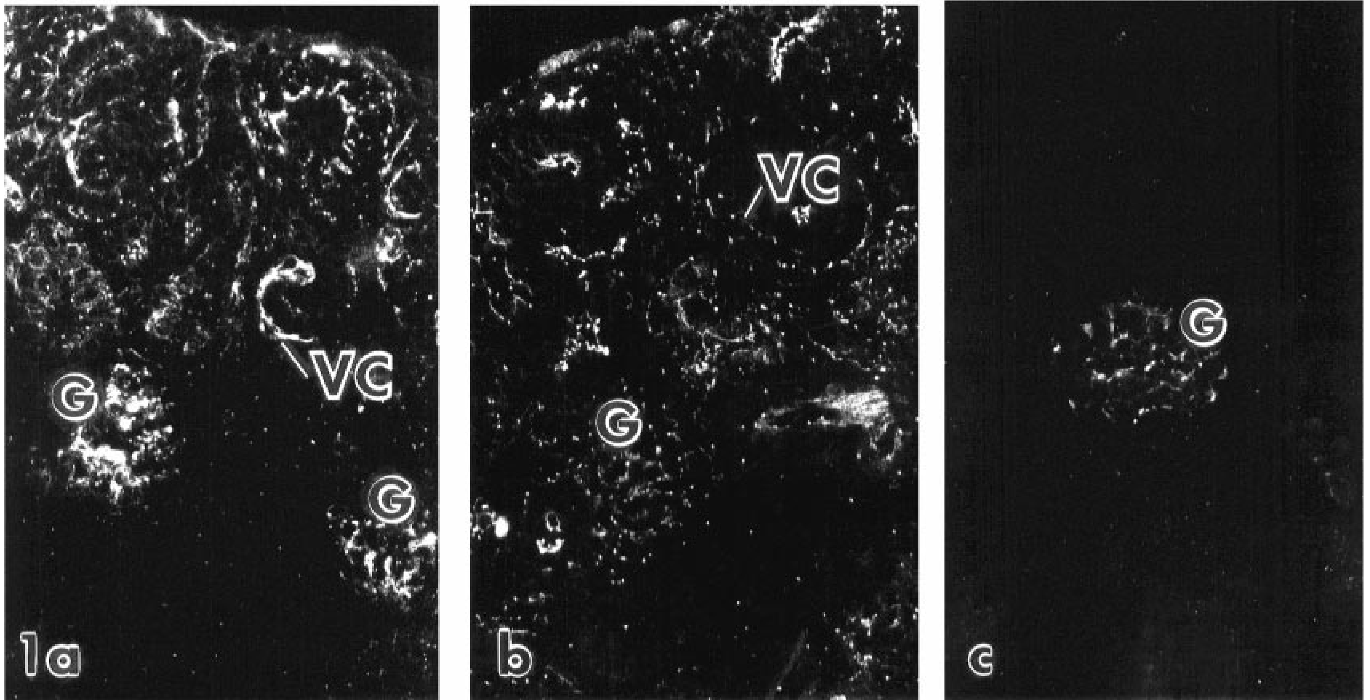

Fig. 1. Immunofluorescence micrographs of newborn mouse kidney cortex (*a and b*) and adult mouse cortex (*c*). Flk1 and EphB1 are distributed in similar patterns in vascular clefts (VC), developing glomeruli (G), and vessels and on discrete membrane domains on cortical mesenchymal cells in newborn kidney (a, b). In adult kidney (c), Flk1 only labels glomeruli (G); other vascular structures are negative (×300). See [12] and [27] for details.

has been disrupted in embryonic stem cells [20, 21]. Remarkably, the loss of a single VEGF allele is lethal in the ~E10.5—E11 mouse embryo, indicating a tight ligand-dose-dependent effect of VEGF on embryonic vessel development [20, 21].

Flk1 has been identified as a marker for some of the earliest endothelial cells and is first seen in mice at E7 on differentiating endothelial cells of the blood islands [16, 22]. Mice with targeted *Flk1* gene deficiencies die *in utero* by E8.5–E9.5 due to a failure of blood island formation, confirming a critical role for Flk1 in vascular development [23]. *In situ* hybridization studies have demonstrated Flk1 expression in most developing organs, including the kidney, in both human and mouse [18].

ERYTHROPOIETIN-PRODUCING HEPATOCELLULAR RECEPTORS SIGNAL CELLULAR RECOGNITION AND AGGREGATION RESPONSES

The erythropoietin-producing hepatocellular (Eph) RTK family includes at least 14 distinct receptors that are expressed in a highly tissue-restricted distribution [15, 24, 25]. What distinguishes Eph from many other RTK families is that rather than binding soluble growth factors, the ligands for Eph receptors (ephrins) are glycosylphosphatidylinositol-anchored proteins on cell surfaces (ephrin-A class) or transmembrane proteins (ephrin-B class). This unique receptor/ligand system is perhaps best understood in the developing nervous system, where it mediates nonproliferative organizational signals implicated in axonal targeting [25]. In addition, the EphA2 receptor expressed on vascular endothelial cells mediates chemotactic responses to its ligand ephrin-A1 [26]. This ligand is also angiogenic in a rabbit corneal angiogenesis assay [26], and ephrin-B1 induces cultured human renal microvascular endothelial cells to assemble into capillary-like networks *in vitro* [27]. Clearly, the temporal and spatial patterning that occurs in neural development has striking parallels with that of vascular development.

KIDNEY IMMUNOLOCALIZATION OF FLK-1, EPH, AND EPHRIN-POSITIVE CELLS

To examine further the distribution of cells expressing Flk1, we labeled sections from embryonic and newborn (developmentally active) and adult (developmentally quiescent) kidneys with antibodies against Flk1, EphB1, and ephrin-B1 [12, 27]. In developing kidneys, these proteins localized in remarkably similar patterns and were found on endothelial cells of forming glomeruli and immature microvessels (Fig. 1 a, b). Labeling was also seen on a dispersed population of mesenchymal cells in the nephrogenic, outer cortical zone, which in rodents is actively producing new nephrons for up to ~7 days after birth [1]. In marked contrast, anti-Flk1 antibody labeling was only weakly evident within glomeruli of adult kidneys, and all other vascular structures were negative (Fig. 1c). These

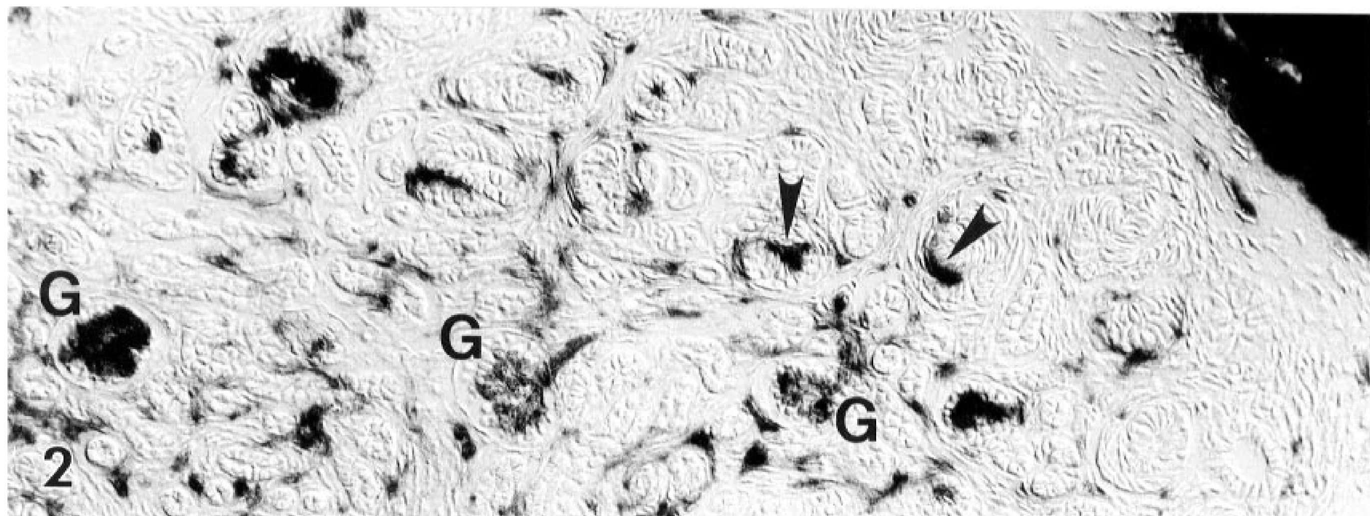

Fig. 2. Micrograph showing graft of embryo day 12 (E12) wild-type mouse kidney into newborn ROSA26 host, 7 days after grafting. Numerous microvessels throughout graft, as well as vascular clefts of S-shaped figures (arrowheads) and developing glomeruli (G), contain β-galactosidase—positive, and therefore host-derived, endothelium. Host kidney tissue is on upper right-hand corner (×300). See [12] for details.

protein immunolocalization results for Flk1 closely correspond to *in situ* hybridization studies carried out previously and that show relatively high levels of Flk1 expression in developing kidney and only very low levels of transcripts in adults [18]. EphB1 and ephrin-B1 protein persist in adult renal arteries, arterioles, and glomeruli but are not found elsewhere in the kidney [27].

INTRARENAL GRAFTS INTO NEWBORN AND ADULT HOSTS

Having identified Flk1-positive, candidate angioblasts in the outer cortex of newborn kidneys, we sought to test the ability of these cells to form microvessels *in vivo*. For these experiments, E12 kidneys from normal donors were grafted under the *renal capsule* of both newborn and adult ROSA26 hosts [12]. When grafts were established in the outer cortex of adult kidneys, a site that does not contain dispersed Flk1, EphB1, or ephrin-B1-positive mesenchymal cells, the vasculature within these grafts was of intrinsic, donor origin [12]. In contrast, when kidneys were grafted into neonatal cortices, an environment rich in Flk1, Eph, and ephrin-positive cells, most glomeruli within grafts contained endothelium derived from the host neonatal kidney (Fig. 2). Large amounts of extraglomerular microvasculature within these grafts were also of host origin. Because the outer cortex of host newborn kidneys is also engaged in microvessel formation, we reasoned that angioblasts from the graft might be able to migrate from the graft and incorporate into host vessels forming in the vicinity. To test this premise, E12 kidneys from ROSA26 mice were grafted into neonatal renal cortices of normal, wild-type hosts. Indeed, cells expressing the graft-derived transgene were found intercalating into host endothelium, creating chimeric vessels (Fig. 3) [12].

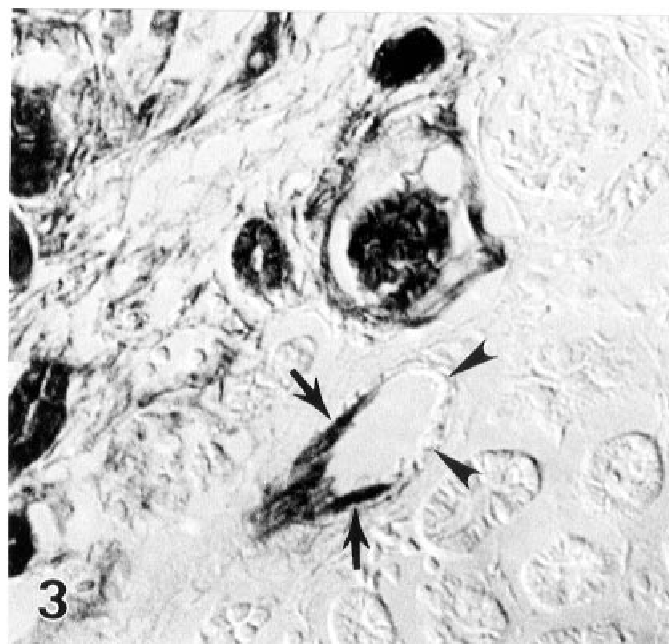

Fig. 3. Graft of an E12 ROSA26 kidney into a wild-type newborn host kidney cortex. Other details as in Figure 2. Note chimeric vessel containing β-galactosidase-positive (arrows = graft-derived) and normal (arrowheads = host-derived) endothelium (×550) [12].

To recapitulate, using antibodies against Flk1, the earliest known marker for endothelial cells, we have identified a discrete population of candidate angioblasts dispersed throughout the E12 kidney and located in the outer cortex of newborn mouse kidneys. Using anterior eye chamber and intrarenal grafts, we have demonstrated the vasculogenic potential *in vivo* of these dispersed Flk1-positive cells. Taken together, our data strongly suggest that at least some of the metanephric mesenchymal cells expressing Flk1 are

endothelial progenitors that form the microvasculature of the developing kidney, and cells expressing EphB1 and ephrin-B1 likely participate as well.

Our *in vitro* and grafting experiments summarized here have not definitively shown, however, whether renal microvessels normally arise by vasculogenesis exclusively. Indeed, some studies we have recently undertaken using heterozygote Flk-1^{tm1Jrt} animals with one functional Flk1 allele suggest that there is an important angiogenic component during early metanephrogenesis, and a combination of angiogenic and vasculogenic events probably occur thereafter [28]. Exactly how Flk1/VEGF and EphB1/ephrin-B1 mediate microvascular assembly is also not clear. Because these distinct receptor/ligand systems are expressed in similar patterns, our current speculation is that Flk1/VEGF activates endothelial progenitor mitosis and motility, and Eph/ephrin are involved with spatial guidance and aggregation of differentiating endothelial cells. Future studies in which selective components of these signaling systems are disrupted should help clarify these issues.

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