CD146$^+$ cells are essential for kidney vasculature development

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The kidney vasculature is critical for renal function, but its developmental assembly mechanisms remain poorly understood and models for studying its assembly dynamics are limited. Here, we tested whether the embryonic kidney contains endothelial cells (ECs) that are heterogeneous with respect to VEGFR2/Flk1/KDR, CD31/PECAM, and CD146/MCAM markers. *Tie1Cre;R26R^{YFP}*-based fate mapping with a time-lapse in embryonic kidney organ culture successfully depicted the dynamics of kidney vasculature development and the correlation of the process with the CD31$^+$ EC network. Depletion of Tie1$^+$ or CD31$^+$ ECs from embryonic kidneys, with either *Tie1Cre*-induced diphtheria toxin susceptibility or cell surface marker–based sorting in a novel dissociation and reaggregation technology, illustrated substantial EC network regeneration. Depletion of the CD146$^+$ cells abolished this EC regeneration. Fate mapping of green fluorescent protein (GFP)-marked CD146$^+$/CD31$^-$ cells indicated that they became CD31$^+$ cells, which took part in EC structures with CD31$^+$ wild-type ECs. EC network development depends on VEGF signaling, and VEGF and erythropoietin are expressed in the embryonic kidney even in the absence of any external hypoxic stimulus. Thus, the *ex vivo* embryonic kidney culture models adopted here provided novel ways for targeting renal EC development and demonstrated that CD146$^+$ cells are critical for kidney vasculature development.

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The structures performing renal functions are derived from Six2$^+$ nephron progenitors, FoxD1$^+$ stromal precursors, presumptive collecting duct cells of the ureteric bud, and endothelial cell (EC) progenitors.[1–3] Studies of nephron differentiation demonstrate a major role for Wnt signaling in kidney organogenesis. Wnt9b/β-catenin signaling induces Wnt4 synthesis in the Six2$^+$ cap mesenchyme, where the Wnt/calcium pathway leads to mesenchyme-to-epithelium transformation and the formation of renal vesicles. The Wnt9b/planar cell polarity pathway is involved in the maturation of epithelialized nephrons.[4] Pharmacological inhibitors of glycogen synthase kinase 3β, which is the phosphorylating part of the β-catenin destruction complex, activate the Wnt pathway downstream of β-catenin. The glycogen synthase kinase 3β inhibitors lithium and 5′-bromoindirubin-3′-oxime (BIO) both induce nephrogenesis in the embryonic kidney mesenchyme.[5,6]

Vascularization of the kidney occurs synchronously with nephrogenesis. Previous studies have shown that ECs expressing the platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31), kinase insert domain protein receptor (Kdr/VEGFR2/Flk1), and tyrosine kinase with immunoglobulin-like and epidermal growth factor (EGF)-like domains 1 (TIE/Tie1) are present in the early embryonic kidney.[7–9] In humans, transition of CD31$^+$/CD34$^+$ glomerular ECs into CD31$^+$/CD34$^-$ has been described.[10] Fate mapping of Flk1$^+$ and Tie1$^+$ cells shows that cells of these lineages constitute the vascular endothelium of the embryonic kidney.[8,11] In chimeric assay, the Tie1$^-$ cells are excluded from the renal vasculature.[12] Host-derived Flk1$^+$ ECs are implicated in vascularization of the grafted embryonic kidney.[13] Flk1 expression has also been reported in the ureteric bud (UB), where it may regulate branching morphogenesis.[13,14] The melanoma cell adhesion molecule (MCAM/Muc18/CD146), which functions as a coreceptor of Flk1 and is expressed in ECs, including the progenitors, has not been described in kidney development.[15–17] As far as the origins of the renal endothelium are concerned, the results of grafting experiments suggest that the murine embryonic kidney contains EC progenitors that are competent to vascularize the kidney,[13,18] but grafted embryonic kidneys also attract exogenous vasculature.[13,19] In any case, the EC

progenitors in the embryonic kidney have still not been characterized.

We show that the ECs in the mouse embryonic kidney are heterogeneous with respect to their surface markers and that the CD146$^+$ cells among them are necessary for microvascular network assembly. A novel approach involving fate mapping of the green fluorescent protein (GFP$^+$)/CD146$^+$/CD31$^-$ cells indicate that these cells differentiate into CD31$^+$ ECs. In addition, we show that vascular endothelial growth factor (VEGF) signaling is essential in order to maintain the EC, whereas the hypoxia response is not critical for EC survival *ex vivo*. The *ex vivo* models demonstrated here provide ways of targeting the mechanisms of kidney vasculature assembly.

RESULTS

As judged by Flk1, CD31, and CD146 staining, the embryonic kidney contains a heterogeneous pool of ECs

At the initiation of kidney organogenesis on embryonic day (E) 11.5, the ECs are revealed by CD31-staining within the cortical interstitial stroma (Supplementary Figure S1A and B). They had then invaded the area adjacent to the Pax2$^+$ cap mesenchyme and its derivatives by E12.5 (Supplementary Figure S1C and D) and were present in the presumptive Bowman capsule assembling the glomerulus by E13.5 (Supplementary Figure S1E and F). Staining of sections of embryonic kidney at E11.5 with Flk1 and CD31 antibodies showed that the CD31$^+$ ECs made up a subset of the Flk1$^+$ cells (Figure 1).

Staining of whole mount embryonic kidneys with CD31 and CD146 antibodies at E11.5 demonstrated a CD31$^+$ EC network within the cortical interstitial stroma (Figure 2a and b, Supplementary Figure S2A–C, Supplementary Movie S1). Clusters of CD146$^+$/CD31$^+$ cells formed chain-like structures, but solitary CD146$^+$/CD31$^-$ cells were also observed. By E12.5, an extensive CD31$^+$ EC network had developed, whereas the proportion of CD146$^+$ cells had diminished (Figure 2c and d, Supplementary Figure S2D–F, Supplementary Movie S2). We conclude that the embryonic kidney contains a heterogeneous pool of ECs and that some of them are characterized by being positive for the CD146 marker.

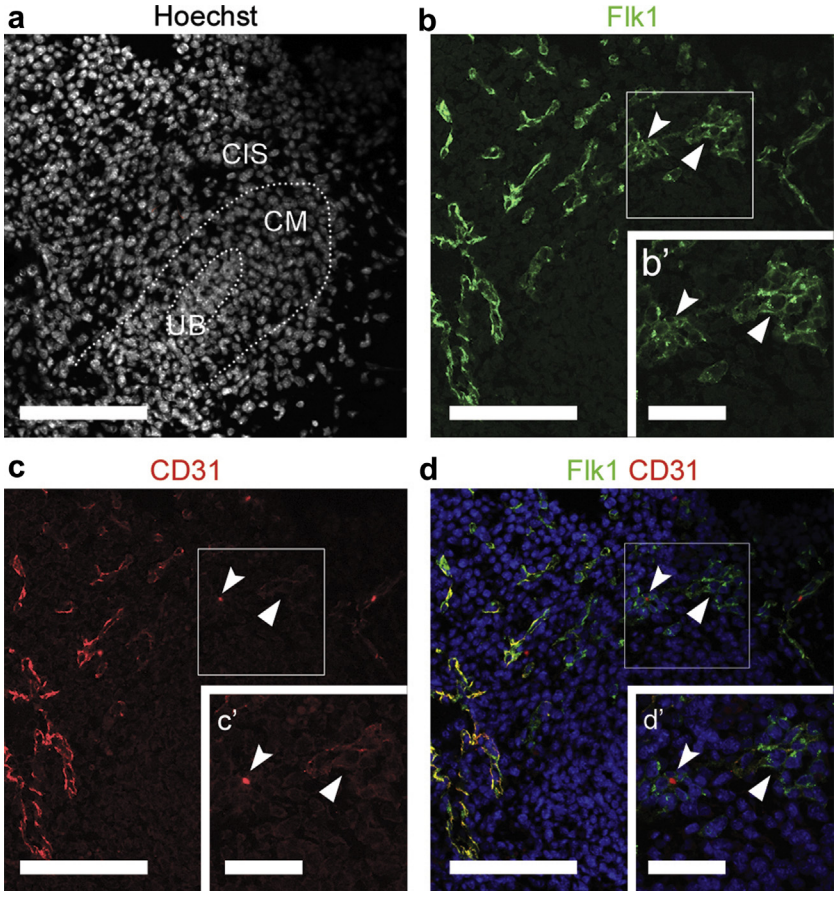

Figure 1 | Heterogeneous endothelial cells are present among the CIS. (**a**) Nuclei of an E11.5 embryonic kidney section containing the ureteric bud (UB), cap mesenchyme (CM), and cortical interstitial stroma (CIS). (**b**) Flk1 is located in the capillaries and cell clusters (concave arrowhead, arrowhead) among the CIS. (**c**) CD31-immunoreactivity is strong in the primitive capillaries but weak in the cell clusters (arrowhead). Occasional CD31$^+$ adhesions exist in the Flk1$^+$ clusters (concave arrowhead). (**d**) Merge. (*b′–d′*) Magnifications of the areas outlined in **b**–**d**. (**a**–**d**) Bars = 100 μm. (*b′–d′*) Bars = 50 μm. E, embryonic day.