Diabetic Complications Consortium

Application Title: Harnessing renal blood flow and oxygenation to manipulate differentiation of nephrons

Principal Investigator: Sunder Sims-Lucas, PhD

1. Project Accomplishments:

During the course of this application we were able to map the developing renal vasculature and blood flow in the developing kidney. Further to this we were able to validate that oxygen concentration on its own in the absence of blood flow is able to drive nephron progenitor differentiation. We will utilize this information in our studies to control nephron progenitor differentiation.

2. Specific Aims:

Aim 1: To map the developing renal vasculature and blood flow in the developing kidney

Results:

Tomato-lectin injections to label intra-renal perfused embryonic vasculature:

Most embryonic tissues (including the kidney) contain a dense vasculature. This dense vascularity, however, does not prove that those tissues are also well perfused. To analyze the blood flow in the developing kidney we utilized a method of *in utero* embryonic intracardiac injection. Using a high-resolution ultrasound to identify the embryonic heart, and after extracting and exposing a single uterine saccule via a laparotomy, we were able to inject E11.5, E13, 15, and 17 embryos with 2.5ml of tomato-lectin (fluoresceine isothiocynate (FITC)-conjugated tomato lectin). As the embryonic heart continues to pump, the tomato-lectin tracer is pumped throughout the body to demarcate the vessels that are perfused throughout the embryos (Figure 1A). Once good perfusion was observed the kidneys were dissected and visualized to observe the 3-dimensional arrangement of the perfused vessels. At E11.5 we found that the perfused vessels surrounded the developing kidney in a web-like arrangement (Figure 1B). By E13.5 the major vessels were perfused with few of the smaller accessory vessels staining with tomato lectin (Figure 1C). Staining could also be seen throughout some of the ureteric epithelium, this may either be from glomeruli that are already perfused or due to the leakiness of the early embryonic vessels. By E15.5 the major vessels were perfused, however a large number of glomeruli could also be observed, suggesting that significant filtering is occurring (Figure 1D). Also at this time-point a number of the smaller vessels appear perfused, although a significant proportion of the outer nephrogenic zone appeared to be devoid of blood flow. However, by E17.5 the majority of the kidney was perfused (except for the very peripheral nephrogenic zone), smaller vessels contained the lectin and the number of perfused glomeruli had dramatically increased (Figure 1E).

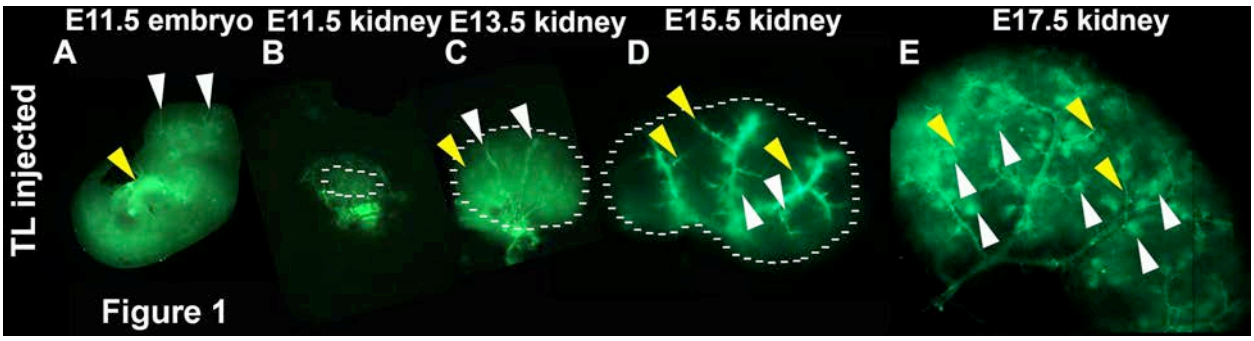

Figure 1: Kidney blood flow occurs in a sequential spatio-temporal pattern
A-E. Representative images of tomato lectin (green) injected embryo (A) and kidneys (B-E) at various developmental stages. A. E11.5 embryo showing perfused vessels throughout the head (white arrow) and body of the embryo, with the site of injection showing very bright staining (yellow arrow). B. Dissected E11.5 kidney showing perfused vessels surrounding the developing kidney in a honeycomb arrangement (as marked by dotted line). C. E13.5 kidney (as marked by dotted line) shows perfusion of the major renal vessels (white arrows), some lectin can also been seen sticking to the ureteric epithelium (yellow arrow). D. E15.5 kidney (as marked by dotted line) showing perfusion of the smaller vascular branches (yellow arrows) as well as several perfused glomeruli (white arrows). E. E17.5 kidney displaying significant perfusion throughout the developing kidney including numerous perfused glomeruli (White arrows) and smaller caliber vessels (yellow arrows).

Vascular formation precedes flow in the developing kidney
Our preliminary whole-mount assessment of embryonic renal blood flow suggested tomato lectin staining largely within the vessels, except at sites where potential filtering or leaking was occurring (Figure 1). Subsequently, we co-stained the vessels with a universal vascular endothelium marker (PECAM) to determine the relationship between the vessels and the branching ureteric epithelium, which was marked by a ureteric epithelial marker (Calbindin). From these whole-mount images we found that from E13.5 onward the major central blood vessels were perfused, however there were many smaller vessels that did not contain blood flow (Figure 2A). By E15.5, the developing vasculature could be seen nestled in between the branching ureteric epithelium. Many of the peripheral vessels now contained blood flow, except towards the periphery in the presumptive nephrogenic zone (Figure 2B). By E17.5 the majority of the smaller interdigitating vessels were now perfused, although the very peripheral nephrogenic zone was devoid of perfused vessels.

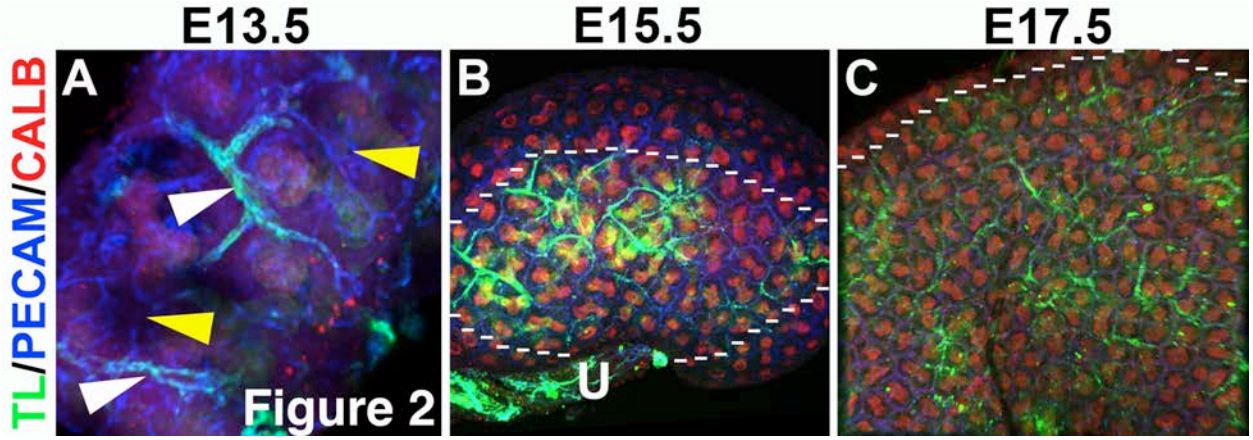

Figure 2: Whole mount images suggest that formation of the renal vessels precedes renal blood flow

A-C: Wholemount images of the developing kidney blood flow (green) at various developmental stages co-labeled with vascular (PECAM, blue) and ureteric epithelial (Calbindin, red) markers. A. Representative E13.5 kidney showing the major vessels (white arrow) that are perfused and peripheral areas of the kidney that are vascularized but lacking blood flow (yellow arrows). B. E15.5 wholemount kidney showing that the more centralized vessels (inside dotted line) that are closer to the ureter (U) and the angiogenic vessels are perfused and interdigitating between the ureteric epithelium (red), but the peripheral vessels (outside dotted line) likely of a vasculogenic origin are unperfused. C. Representative E17.5 kidney showing significantly more perfusion throughout the kidney interdigitating between the branching ureteric epithelium (below dotted line). However, in the presumptive nephrogenic zone the vessels are unperfused (above dotted line).

Blood flow does not extend into undifferentiated nephron progenitors

From the previous experiments there appeared to be a clear demarcation between the areas of renal blood flow and the nephrogenic zone where the renal stem cells exist. To specifically examine this issue, we studied blood flow (FITC-tomato lectin) and blood vessels (PECAM staining) in relation to the nephrogenic zone. From E13.5 to E15.5 the PECAM+ perfused vessels seemed to abut the border of the Nephrogenic zone (Figure 3).

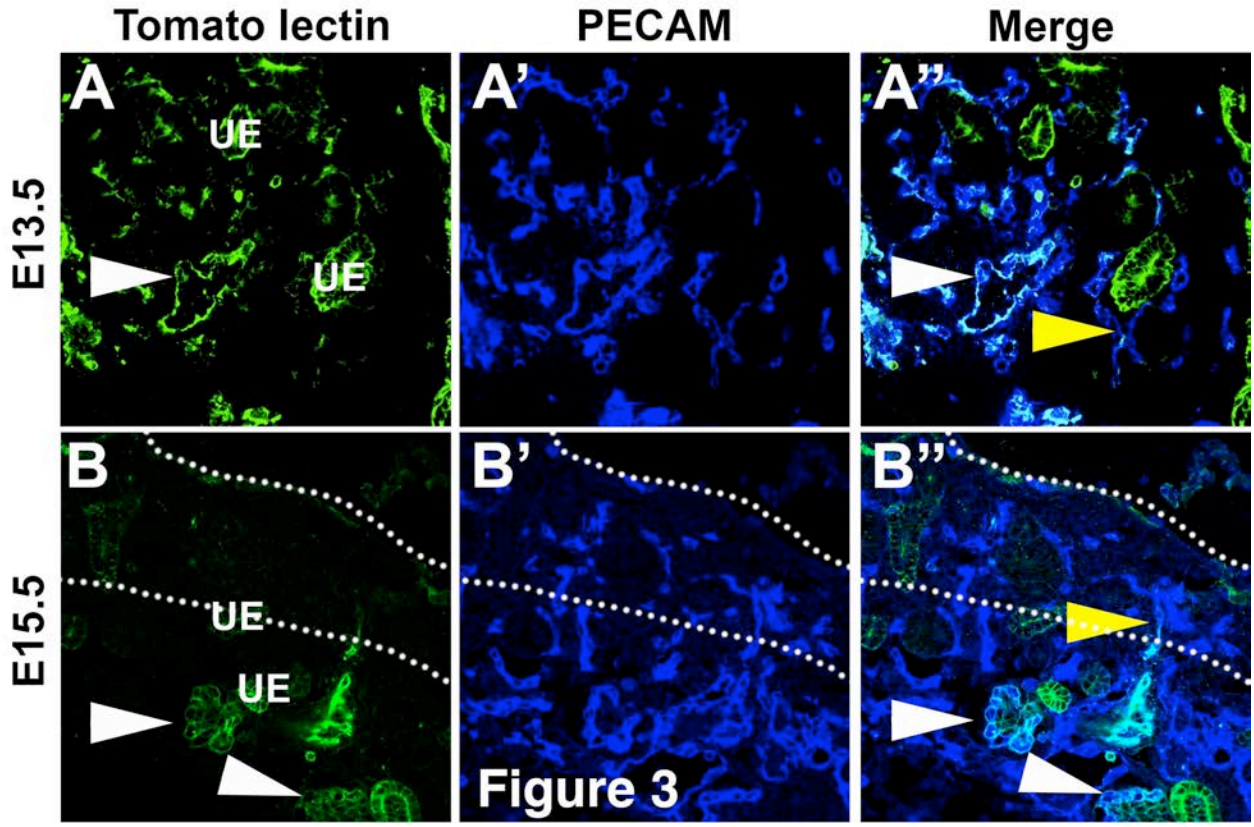

Figure 3: Blood flow does not extend into the undifferentiated nephron progenitors
A-B. Representative images of E15.5 kidneys that are perfused with FITC-tomato lectin and co-labelled for markers of nephron progenitors (Six2 and Pax2, red) and vasculature (PECAM, blue). A-A''. Six2 (red) is shown to mark the nephron progenitors, which reside in the nephrogenic zone (marked by the dotted line). Perfused vessels (yellow arrow) stained with tomato lectin (green) and PECAM (blue) can be seen all the way to the nephrogenic zone and then unperfused vessels (white arrow) are seen throughout the nephrogenic zone. B-B'''. A similar expression pattern is seen with Pax2 (red), although staining for Pax2 is also observed in the ureteric epithelium (UE) and developing glomeruli (G). Whereby perfused vessels (yellow arrow) marked by tomato lectin (green) abut the nephrogenic zone however the blood flow does not penetrate leaving the vessels marked with PECAM (blue) within the nephrogenic zone unperfused (white arrow).

Aim 2: To determine the role of oxygenation in driving nephron progenitor differentiation

Results:

Oxygen concentration drives nephron progenitor differentiation

From these series of experiments there seemed to be an intricate link between blood flow and nephron progenitor differentiation. So next we wanted to determine whether an increase in oxygen tension is sufficient to drive nephron progenitor differentiation in the absence of blood flow. We placed Six2-cre;tdTomato embryonic kidneys (permanently Tomato-labeled kidneys) into various oxygen concentrations; normal (21%), intermediate (5%) and hypoxic (1%) and grew the kidneys for 7 days. What we observed in the 7-day cultures was that the normoxic explants contained numerous differentiated structures (49.00±8.11), which

appeared adjacent to the presumptive nephrogenic zone. Conversely, under hypoxic conditions we observed little nephron differentiation (3.11±2.52; $p<0.0001$) and the nephron progenitor caps maintained their undifferentiated appearance (Figure 7). When we utilized an intermittent oxygen concentration (5%) some differentiation (19.67±1.52; $p<0.0001$) was induced but not backs to the normal oxygen condition (Figure 4). Furthermore when we correlate vascular growth in these explants using PECAM staining we see that under hypoxic conditions that the vessels are less organized as the degree of oxygen decreases (Figure 4G-I).

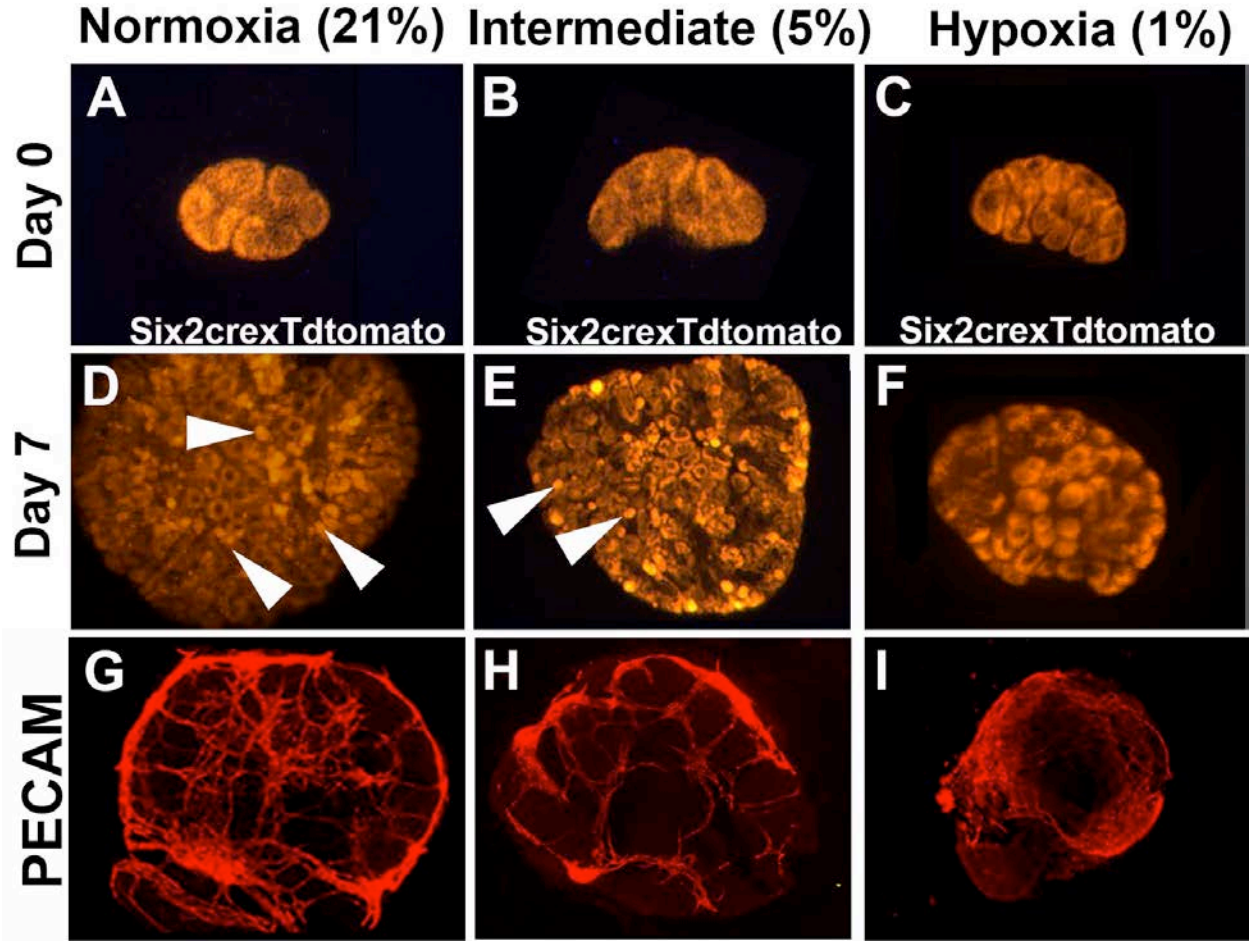

Figure 4: Varying oxygen concentration mediates amount of nephron progenitor differentiation

A-F. Six2cre mice bred with tdTomato reporter mouse (red) grown under varying oxygen concentrations for 7 days. A-C. E12.5 kidney at zero days of culture showing the kidneys placed in normoxia (A) intermediate hypoxia (B) and hypoxia (C) are at developmentally comparable stages. There is the presence of nephron progenitors but no differentiated structures. D-F. Following 7 days of culture in normoxia (C) the kidney displays numerous differentiated nephron structures (arrows), while a stepwise reduction in the number of glomerular structures is observed in the intermediate (E) and hypoxic (F) conditions. G-I. These are representative wholemount images of PECAM staining in explants at various oxygen concentrations. Under the normal oxygen concentration (G) significant differentiation and patterning of the vessels is observed. There is still relatively well-defined differentiation at 3% oxygen concentration (H) but a complete lack of vascular organization is observed in the 1% oxygen concentration (I).

3. Publications:

Rymer C, Paredes J, Halt K, Schaefer C, Wiersch J, Zhang G, Potoka D, Vainio S, Gittes GK, Bates CM, Sims-Lucas S. Renal blood flow and oxygenation drive nephron progenitor differentiation. Am J Physiol Renal Physiol. 2014 Aug 1;307(3):F337-45.