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Vascular development in the vertebrate pancreas

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ABSTRACT

The vertebrate pancreas is comprised of a highly branched tubular epithelium, which is intimately associated with an extensive and specialized vasculature. While we know a great deal about basic vascular anatomy of the adult pancreas, as well as islet capillaries, surprisingly little is known about the ontogeny of its blood vessels. Here, we analyze development of the pancreatic vasculature in the mouse embryo. We show that pancreatic epithelial branches intercalate with the fine capillary plexus of the surrounding pancreatic mesenchyme. Endothelial cells (ECs) within this mesenchyme are heterogeneous from the onset of organogenesis. Pancreatic arteries take shape before veins, in a manner analogous to early embryonic vessels. The main central artery forms during mid-gestation, as a result of vessel coalescence and remodeling of a vascular plexus. In addition, we show that vessels in the forming pancreas display a predictable architecture that is dependent on VEGF signaling. Over-expression of VEGF disrupts vascular patterning and arteriovenous differentiation within the developing pancreas. This study constitutes a first-time in-depth cellular and molecular characterization of pancreatic blood vessels, as they coordinately grow along with the pancreatic epithelium.

1. Introduction

Blood vessel formation is essential to embryonic development and adult tissue function (see review Herbert and Stainier, 2011). During early embryogenesis, endothelial cell (EC) precursors called angioblasts arise in the mesoderm and arrange into cords. They then take on a squamous cell morphology, open lumens and give rise to vascular tubes. This initial *de novo* development of vessels from angioblasts is called *vasculogenesis*. Subsequently, emerging vessels change in various ways by processes collectively termed *vascular remodeling*. New vessels can develop via *sprouting angiogenesis*, where nascent sprouts branch from a pre-existing vessel. An existing vessel can also split into multiple vessels via *intussusceptive angiogenesis*. Conversely, smaller vessels can coalesce and fuse into larger ones. As these remodeling processes take place, arteries and veins differentiate under the influence of both genetic and hemodynamic mechanisms. The resulting mature vasculature is a hierarchical tree made up of arteries, arterioles, capillaries, venules and veins, each with different properties that enable vessels to serve specific functions. Arteries and veins have been shown to be established relatively early in the embryo, although they exhibit some plasticity for a time. Additionally, tissues develop distinct organ-specific capillary beds that carry out organ-specific functions. Therefore, the embryonic endothelium has to differentiate heterogeneously to form regionally adapted vessels throughout the body.

Once organ primordia emerge in the early embryo, their growth must be coordinated with vascular development. Indeed, blood vessels align with extending epithelial branches in branching organs, such as lungs and salivary glands (Lu and Werb, 2008). With respect to their ontogeny, blood vessels in many organs were long believed to arise as a result of angiogenesis; however, peripheral vasculogenesis has been shown to contribute significantly to organ-specific capillary beds (Drake and Fleming, 2000). In fact, a few studies have suggested that organ-associated vascular beds arise via a combination of vasculogenesis and angiogenesis to build functional organs (Sequeira Lopez and Gomez, 2011). Despite such studies, much remains to be understood

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about patterning of blood vessels and establishment of transcriptional heterogeneity during the building of organ-specific vasculatures.

Recent efforts have been directed to elucidate functional relationships between epithelia and endothelium in organs and tissues. Understanding this relationship is of great value to the fields of vascular development and tissue stem cells. In the pancreas, such a functional relationship is required for organ development. The dorsal pancreatic bud evaginates at embryonic day 9.5 (E9.5) from the dorsal gut endoderm towards the aorta, driven by epithelial stratification. At this time (E10.5), the bud consists of undifferentiated and largely apolar progenitor cells. As the epithelium remodels and branches, it undergoes de-stratification and cells regain apico-basal polarity (E10.5-E11.5). During these early stages (E9.5-12.0), termed the primary transition, very little cellular differentiation occurs. Around E12.0, the secondary transition begins, with rapid growth and branching of the epithelium coinciding with widespread cellular differentiation. Studies have shown that ablation of blood vessels associated with the pancreatic bud leads to failure in pancreas development: removal of the aorta prior to bud initiation, or blocking of vascular growth through VEGFR2 inhibition after budding, results in blockade of beta cell differentiation, or abnormal epithelial growth and differentiation, respectively (Lammert et al., 2001; Magenheim et al., 2011b; Pierreux et al., 2010; Sand et al., 2011b). Conversely, Pdx1-driven VEGF over-expression (Pdx1-tet-VEGF) and hypervascularization lead to pancreatic growth arrest, failed cellular differentiation and islet disruption (Cai et al., 2012; Magenheim et al., 2011b). Thus, pancreatic vasculature profoundly impacts development of the pancreatic progenitor epithelium.

Despite the well-established role for developing vasculature in pancreatic morphogenesis and differentiation, how the pancreatic vasculature develops is poorly understood. While vascular development has been characterized in several organs, a comprehensive analysis of pancreatic vascular development has been missing (Coveney et al., 2008; Lazarus et al., 2011; Robert et al., 1998). Furthermore, it has become increasingly clear that blood vessels play different roles over the course of pancreatic development (see review Villasenor and Cleaver, 2012). Thus, a better understanding of pancreatic vascular development is needed to dissect the role of the vasculature during pancreatic growth and differentiation. Here, we provide an in-depth characterization of pancreatic vascular development. We explore when and how the pancreatic epithelium becomes vascularized, as well as how its vessels undergo arteriovenous differentiation. We show that the initially avascular pancreatic epithelium becomes integrated with blood vessels upon branching, combined with peripheral vasculogenesis. Our data suggest that the first central vessel of the pancreas, its main artery, is formed via coalescence and remodeling of capillaries. This occurs coordinately with recruitment of smooth muscle progenitors (or mural cells). We further demonstrate that pancreatic arteries and veins emerge at predictable and apposed locations within the bud. Additionally, we identify spatially distinct and molecularly heterogeneous capillary beds in the developing pancreas. Finally, we show that excessive epithelial VEGF in the Pdx1-tet-VEGF mouse model leads to patterning failure of the pancreatic vasculature, with defects varying from ectopic or expanded to non-hierarchical or coalesced networks depending on the vascular bed.

2. Materials and methods

2.1. Mice and tissue handling

Animal husbandry was performed in accordance with protocols approved by the UT Southwestern Medical Center IACUC. CD1, EphB4-lacZ, ephrinB2-lacZ, Flk1-lacZ, Flk1-GFP and Pdx1-tet-VEGF (Magenheim et al., 2011b) mice were used for experiments herein.

E9.5-E14.5 embryos were collected from pregnant mice. The tissues were dissected in ice-cold PBS buffer. Gut tubes or stomachs attached to pancreata were isolated and fixed in 4% paraformaldehyde (PFA) in PBS with gentle rocking as follows: at 4 °C overnight (o/n) for section or whole mount in situ hybridization, for 3 hours (h) at room temperature (RT) for section immunostaining, or for 1 h at RT for whole mount immunostaining. Tissues were washed three times in PBS for 5 minutes (min) each, and dehydrated to 70% Ethanol. Tissues were stored in 70% Ethanol at −20 °C. Postnatal tissues were collected and fixed in a similar manner.

2.2. Sectioning

For paraffin sectioning, tissues were fixed and dehydrated as described above. Then, the tissues were rinsed twice in 100% Ethanol for 5 min at RT, twice in xylene for 30 min at RT, then a mixture of 1:1 paraplast:xylene at 60 °C for 10 min, then a series of 100% paraplast at 60 °C (McCormick Scientific). The tissues were then embedded in paraplast and sectioned at 10 μm with a Biocut 2030 microtome. SuperfrostPlus glass slides (Fisher) were used.

For cryosectioning, tissues were fixed as described above. Then, the tissues were rinsed in PBS three times for 5 min each and incubated in 30% sucrose o/n at 4 °C for cryoprotection. Next day, the tissues were rinsed in OCT twice for 30 min each at RT. The tissues were embedded in OCT, snap-frozen on dry ice and sectioned at 10 μm using a Leica CM-3050S cryostat. SuperfrostPlus glass slides (Fisher) were used.

2.3. Immunostaining on sections

For paraffin sections, the sections were de-paraffinized with xylene washes twice for 5 min each. Sections were rehydrated in the following Ethanol series for 1 min each: 100% (x2), 95%, 90%, 80%, 70% and 40%. Slides were washed twice in PBS for 3 min each. Sections were treated with R-Buffer A or R-Buffer B in a 2100 Retriever (Electron Microscopy Sciences). Then, sections were permeabilized in PBS+0.1% TritonX for 30 min. After a quick dip in PBS, sections were blocked in CAS-Block (Invitrogen) for 2–3 h. Slides were incubated with primary antibody in CAS-Block o/n at 4 °C. Next day, slides were washed in PBS three times 10 min each, incubated with secondary antibody for 2 h at RT, and washed again in PBS three times 10 min each. For nuclear staining (when needed), slides were incubated in DAPI in PBS for 10 min at RT. Slides were washed in PBS three times 10 min each and mounted in Prolong Gold with or without DAPI (as needed).

For cryosections, slides were baked for 10 min at 55 °C and rinsed in PBS three times for 10 min each. Antigen retrieval was carried out using Electron Microscopy Sciences Buffer B in retriever (as above). Sections were blocked in 5% Normal Donkey Serum in PBS for 1 h at RT in a humidified chamber (with PBS). Slides were incubated with primary antibody in blocking serum o/n at 4 °C. Next day, slides were washed in PBS three times 10 min each, incubated in secondary antibody in blocking serum for 2 h at RT, and washed in PBS three times for 10 min each. Nuclear staining and mounting was performed the same way as for paraffin sectioning.

For DAB immunostaining on sections, paraffin sections were de-paraffinized and rinsed in PBS as described above. Then, slides were incubated in 3% H_20_2 in Methanol to quench endogenous peroxidase activity, and washed in PBS twice for 5 min each. Antigen retrieval and blocking were carried out as described for paraffin sections. Slides were incubated with primary antibody o/n at 4 °C (mouse α E-cadherin from BD Transduction, rabbit α VEGF from Abcam). Next day, slides were rinsed in PBS and incubated in secondary antibody at 1:500 for 2 h at RT (α mouse IgG-HRP, α rabbit IgG-HRP). Slides were then washed in PBS three times for 30 min each. For color reaction, slides were incubated in DAB staining solution (DAB Substrate kit from Vectorlabs: 2 drops stock buffer, 4 drops DAB, 2drops H_2O_2, 2 drops Nickel in 5 ml water) until staining develops as desired (2–10 min). Slides were then dehydrated in Ethanol series followed by xylene, and mounted with Permount (Fisher).