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Embryonic mouse blood flow and oxygen correlate with early pancreatic differentiation

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ABSTRACT

The mammalian embryo represents a fundamental paradox in biology. Its location within the uterus, especially early during development when embryonic cardiovascular development and placental blood flow are not well-established, leads to an obligate hypoxic environment. Despite this hypoxia, the embryonic cells are able to undergo remarkable growth, morphogenesis, and differentiation. Recent evidence suggests that embryonic organ differentiation, including pancreatic β-cells, is tightly regulated by oxygen levels. Since a major determinant of oxygen tension in mammalian embryos after implantation is embryonic blood flow, here we used a novel survivable *in utero* intracardiac injection technique to deliver a vascular tracer to living mouse embryos. Once injected, the embryonic heart could be visualized to continue contracting normally, thereby distributing the tracer specifically only to those regions where embryonic blood was flowing. We found that the embryonic pancreas early in development shows a remarkable paucity of blood flow and that the presence of blood flow correlates with the differentiation state of the developing pancreatic epithelial cells in the region of the blood flow.

Introduction

The embryonic mouse pancreas originates as two evaginations from the foregut. The early formation of the pancreas is thought to be under the control of multiple extracellular factors (Gittes, 2009). Commitment of endoderm to a pancreatic fate is thought to be controlled by notochord-derived FGF2 and activins (Hebrok et al., 1998). As development progresses, however, the paired dorsal aortae fuse in the midline, interposing themselves between the notochord and the dorsal pancreas. Interestingly, the aortic endothelial cells foster pdx1 and ptf1a expression (Lammert et al., 2001; Yoshitomi and Zaret, 2004), with subsequent insulin expression in the endoderm. These early embryonic recombination experiments of aortic endothelium and foregut were performed *in vitro*, without blood flow, suggesting that blood flow may be unnecessary for the endothelium-induced pancreatic differentiation. However, these *in vitro* experiments were performed in the presence of 21% oxygen and fully supplemented medium (serum, etc.), so a key *in vivo* constituent of blood flow might have been replaced by the culture conditions. Recent studies *in vitro* demonstrate that 21% oxygen (which may be a 4- to 6-fold higher oxygen tension than exists in the perfused regions of the embryo) may have supra-physiologic effects on pancreatic differentiation, and in particular enhanced endocrine differentiation (Fraker et al., 2007; Heinis et al., 2010).

Tissue oxygen tension in the early mammalian embryo represents a fascinating biological conundrum. The early mammalian embryo is located within the uterus, with a non-existent or immature cardiovascular system and blood supply. Despite this hypoxic environment, the embryo is still able to undergo rapid growth and organogenesis. It seems plausible that the inflow of blood, with the resulting increased oxygen tension, may be a control point for differentiation. Like the pancreas, many other developing tissues have been shown to use oxygen as a control point for differentiation (Fraker et al., 2007; Heinis et al., 2010). Thus, endothelial cells alone may not actually be sufficient to induce organ development *in utero* but may be dependent on additional signals from blood flow and oxygen. Such a specific dependence of organ development on blood flow rather than just endothelial cells has been shown for the developing zebrafish kidney (Serluca et al., 2002).

In the developing pancreas, we and others have shown that there are abundant PECAM-positive and VEGFR2-positive endothelial cells

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throughout the early embryonic mesenchyme (Lammert et al., 2001). Despite these numerous endothelial cells, however, the early epithelium does not undergo diffuse differentiation but rather shows a controlled, staged pattern of differentiation. In particular, between E9.5 and E13.5, there is mainly growth and branching of pdx1-positive pancreatic epithelium, with relatively little exocrine and endocrine differentiation (especially insulin-positive differentiation) (Gittes, 2009). Then after E13.5, a transition occurs wherein there is a rapid expansion of acinar cells and insulin-positive beta cells (Rall et al., 1973).

Here, we developed a new technique of ultrasound backscatter microscopy-guided *in utero* intracardiac injection in early mouse embryos. Using the injection of vascular tracers, we are able to determine where the living embryonic mouse heart is pumping blood. We now present correlative evidence that enhanced blood flow and oxygenation in the developing mouse pancreas may underlie the normal changes in differentiation kinetics seen during mouse embryonic pancreatic development.

Materials and methods

Mice

Mice were maintained according to the Animal Research and Care Committee at the Children's Hospital of Pittsburgh and the University of Pittsburgh IACUC.

Tissue preparation

For immunolabeling on cryopreserved sections, tissues were fixed, sectioned, and stained as previously described (Esni et al., 1999).

Immunolabeling

The following antibodies were used at the indicated dilutions for immunofluorescence analysis: guinea pig anti-insulin 1:1000 (Linco); guinea pig anti-glucagon 1:1000 (Linco); rabbit anti-glucagon 1:1000 (Linco); goat anti-amylase 1:500 (Santa Cruz); rat anti-E-Cadherin 1:200 (Zymed); rat anti-PECAM 1:50 (BD Pharmigen); goat anti-Pdx1 1:10,000 (AbCam); mouse anti-NGN3 1:2000 (Hybridoma Bank), chicken anti-β-galactosidase 1:1000 (Abcam); and goat anti-vimentin 1:50 (Santa Cruz). The following reagents were purchased from Jackson ImmunoResearch Laboratories: biotin-conjugated anti-rabbit 1:500, anti-rat 1:500, anti-goat 1:250; Cy2- and Cy3-conjugated donkey anti-guinea pig 1:300; donkey anti-rabbit 1:300; donkey anti-rat 1:300; donkey anti-mouse 1:300; Cy2-conjugated streptavidin 1:300; Cy3-conjugated streptavidin 1:1000; and Cy5-conjugated streptavidin 1:100. Images were collected on a Zeiss Imager Z1 microscope with a Zeiss AxioCam driven by Zeiss AxioVision Rel.4.7 software.

Whole-mount immunohistochemistry

Tissue preparation and whole-mount immunohistochemistry on embryonic pancreas were performed as previously described (Esni et al., 2001).

Culture of pancreatic rudiments

Isolation and culture of E11.5 foregut containing pancreatic rudiments were carried out as previously described (Esni et al., 2001, 2005), with BrdU added for the last 2 days of culture.

In utero cardiac injection of mouse embryos

Briefly, pregnant mice were anesthetized and subjected to a laparotomy, the uterus was exposed, and then a fenestrated dish was placed over the mouse and a single embryo (one uterine saccule) brought through the fenestration (Supplemental Fig. S1). The ultrasound microscope probe is used to guide the injection apparatus. A glass needle is used to inject the heart with fluorescein-conjugated tomato lectin (TL). The lectin is injected in a low volume and under low pressure to allow it to be passively carried in the blood wherever embryonic blood is normally flowing. Each embryonic heart was injected with 2.5–5.0 μl, depending on the age of the embryo. This procedure can be repeated for multiple embryos in the same pregnant mouse. The injected fluorescent-conjugated lectin is allowed to circulate for 10 min while it binds to the endothelial wall of the vasculature, after which the embryo is harvested, photographed, and fixed in 4% PFA for immunofluorescent analyses.

Tissue oxygenation measurement

Tissue oxygenation through oxidized thiol measurement was performed as described previously (Mastroberardino, et al., 2008). For tissue that was utilized for oxidized thiol detection, the free thiols in the harvested tissue were immediately blocked by performing all dissections in PBS with 100 mM N-ethylmaleimide (NEM) and 100 mM iodacetamide (IAM) (Sigma Aldrich). The isolated pancreas was then fixed in 4% paraformaldehyde containing 100 mM NEM and 100 mM IAM for 4 h. After embedding and sectioning the tissue, disulfides were reduced with 4 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma Aldrich) in PBS for 30 min. Reduced thiols were then labeled with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) (Sigma Aldrich) during a 30-min incubation period. After this step, routine immunohistochemistry was carried out on the tissue samples.

Results

Embryonic pancreatic vascularity and blood flow

Whole-mount immunohistochemistry of E12.5–E17.5 pancreas for the endothelial marker Pecam showed a dense vasculature throughout (Fig. 1A–C). This dense vascularity would suggest that the developing pancreas is well perfused with blood. However, in order to directly evaluate blood flow in the embryonic pancreas (as opposed to the mere presence of blood vessels and/or endothelium), we developed a method of survivable *in utero* embryonic mouse intracardiac injection. This embryonic intracardiac injection is guided by high-resolution ultrasound biomicroscopy (Fig. 1D, Supplemental Figs. S1, S2, and Video S1) and can be performed as early as E9.5, the time when the embryonic pancreas first evaginates from the foregut. Previous techniques have been used to access the cardiovascular system of mouse embryos, but either later in gestation (E14.5) for survivable injections (Peranteau et al., 2007), or else early in gestation (E11.5) but *ex vivo*, with immediate sacrifice of the embryo (Sugiyama et al., 2003). In our system, a midline laparotomy is performed on a pregnant mouse between gestational ages E9.5 and E17.5, and a single uterine saccule is exposed (Supplemental Fig. S1). Ultrasound biomicroscopy is then used to identify the embryonic heart through the uterine wall. A 30 μm drawn-glass beveled capillary pipette is inserted into the left ventricle of the embryonic heart (the E11 heart has an end-diastolic left ventricular volume of 160 nanoliters) (Tanaka et al., 1997), and then the heart is injected with 0.2–5 μl of a vascular tracer. We have used fluoresceine isothiocynate (FITC)-conjugated tomato lectin (volume depending on age of the embryo), an endothelial cell marker that labels vessels that are being perfused by the embryonic circulation (McDonald and Choyke, 2003), as well