Embryonic mouse blood flow and oxygen correlate with early pancreatic differentiation

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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.

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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 activinins (Hembra 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of 21% oxygen and fully supplemented medium (serum, etc.), so a key in vivo constituent of blood flow might have been replaced by the culture conditions.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
We now present correlative evidence that enhanced blood circulation determine where the living embryonic mouse heart is pumping blood. Using the injection of vascular tracers, we are able to track the flow of blood 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 (Esnì 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); goat anti-amylase 1:500 (Santa Cruz); rat anti-E-Cadherin 1:200 (Zymed); rat anti-PECAM 1:50 (BD Pharmingen); 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-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 (Esnì et al., 2001).

Culture of pancreatic rudiments

Isolation and culture of E11.5 foregut containing pancreatic rudiments were carried out as previously described (Esnì 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 sac) 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 iodoacetamide (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 (CM) (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 evacuates 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 sac 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 isothiocyanate (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
Embryonic pancreas is vascularized early during development. Whole-mount immunohistochemistry analyses of E12.5 (A), E14.5 (B), and E17.5 (C) pancreas using antibodies against Pecam reveal that it is highly vascularized. (D) Ultrasound backscatter image of an E12.5 embryo with the echo-bright needle tip in the heart. (E) After in utero injection of the embryonic heart (at E12.5) with the endothelial tracer FITC-tomato lectin, a whole-embryo “angiogram” of perfused vessels is demonstrated. (F, G) Histologic sections of an E10.5 embryo demonstrate that there is scant perfusion with tomato lectin (TL) in the region of the dorsal pancreas (dp) and gut (g), but there are numerous unperfused vessels (Pecam+). The arrow in panel G indicates a glucagon-positive area in the dorsal pancreas that has significant perfusion. (H–M) At E12.5, there is a regionalization within the pancreas, with areas that receive detectable blood flow and that also show glucagon+ differentiation (K–M), and then other unperfused areas (H–J) that lack glucagon+ differentiation. (N–P) Perfusion of an early E14 pancreas is more diffuse compared with the regional perfusion at E12.5, but still only a subset of the Pecam+ vessels are perfused (O,P). Again, glucagon-positive cells are only in the vicinity of the tomato lectin perfused vessels (P), whereas numerous E-cadherin+ (Ecad) cells are in the vicinity of unperfused vessels (N, O).
as FITC-labeled dextran. The embryonic heart then pumps the tracer throughout the embryo to delineate which vessels are being perfused with blood. After the injection, the heart is observed for possible bleeding or contractile dysfunction. In some cases, the laparotomy is closed and the injected embryos are allowed to develop normally (Supplemental Fig. S3), even into adult life. Post-injection survival to birth is approximately 60–80%, depending on age (data not shown). The intracardiac injection of FITC-tomato lectin revealed a whole-embryo “angiogram” of perfused vessels (Fig. 1E, Supplemental Fig. S2), with large vessels and secondary branches throughout the embryo well visualized. By comparing FITC-tomato lectin-marked vessels (i.e., vessels receiving embryonic blood flow) with immunostaining for a general marker of vascular endothelium (Pecam), we identified numerous vessels early in gestation that were not receiving blood flow (non-perfused) (Fig. 1F–J).

In the early developing pancreas, there was a clear distinction between perfused vessels (FITC-tomato lectin+/Pecam+ double-positive) and non-perfused vessels (FITC-tomato lectin-negative/Pecam−) (Fig. 1). To confirm this regionalization of blood flow, with a general lack of flow to most of the mesenchyme, we injected 250,000-MW FITC-tagged dextran (smaller sized dextran molecules leaked out of the vessels, making interpretation of the images difficult), which showed a similar pattern as FITC-tagged lectin (Supplemental Fig. S4). The dextran signal was difficult to recover after fixation, and therefore, we used lectin imaging for the remainder of the studies. At E10.5 the vast majority of the Pecam− vascular endothelium was not double-stained with FITC-tomato lectin, and therefore was likely not perfused with a significant amount of blood (Fig. 1F, G). It is possible that trace amounts of blood are flowing to these tomato lectin-negative vascular regions, but this low level of flow would be below the sensitivity of our fluorescence imaging system. However, our system is definitely sensitive enough to detect normal capillary blood flow, since in other tissues (Supplemental Fig. S4) and in older embryos (E14–15), lectin-positive vessels, including capillaries, are seen throughout the vasculature (few Pecam−/lectin-negative blood vessels are present, see Fig. 2E–H).

To better visualize blood flow to the embryonic pancreas, we used confocal 2-stack imaging and morphometric software to create a three-dimensional image of an E11.5 pancreas after FITC-tomato lectin intracardiac injection (Supplemental Fig. S5 and Video S2). Staining for glucagon, Pecam, and a marker of embryonic pancreatic epithelium (Sox9) demonstrate that blood flow (FITC-tomato lectin) appears to enter on one side of the pancreas and fans out to cover the epithelium. Most of this blood flow appears to be directed toward the epithelial region where glucagon is expressed (Supplemental Fig. S5A–D). At this E11.5 age, there are very few insulin-positive cells present. Despite the presence of a three-dimensional network of Pecam+ vessels, there appears to be minimal blood flow to the vessels in the vicinity of the mesenchyme (Sox9-negative/glucagon-negative) and the undifferentiated epithelium (Sox9+/) (Supplemental Figs. S4D–F and S5E–H), and thus much of the pancreatic vasculature is not receiving blood flow at E11.5 (yellow-only Pecam− cells in Supplemental Fig. S5I–L).

Interestingly, the region of the early pancreas (E10.5–E11.5) that was clearly perfused with blood was in the vicinity of the few glucagon-positive cells that are normally present at this early stage of pancreatic development. Here, it is important to note that there was diffuse Pecam− endothelium throughout the mesenchyme of the early embryonic pancreas, most of which was not receiving blood flow. Similarly, others have shown diffuse VEGFR2+ endothelium throughout the mesenchyme of the embryonic pancreas at this age as well (Lammert et al., 2001). This non-perfused endothelium did not, however, seem to induce the adjacent pancreatic epithelium to undergo differentiation. Between E10.5 and E14, the number of pancreatic vessels that were perfused with blood (FITC-tomato lectin+/Pecam+ double-positive vascular endothelium) slowly increased (Fig. 1H–P). During E12.5–E14 glucagon-positive cells tended to localize specifically near perfused blood vessels (Figs. 1M, P, and compare the non-perfused regions of the E12.5 pancreas in Fig. 1) with the perfused areas in Fig. 1M). By early E14, insulin-positive cells also tended to localize near blood flow, similar to the glucagon-positive cells (Fig. 2).

As late as E14, the percentage of Pecam+ vessels that were perfused (FITC-tomato lectin+) was still only around 50% (Fig. 2A–D, K). Interestingly, just a short time later (late E14/early E15), there was a substantial increase such that over 90% of the pancreatic vascular endothelium became FITC-tomato lectin+ (compare Fig. 2A–D with E–H, and see Fig. 2K). Early E14 embryos can be distinguished from late E14 by well-defined external characteristics of the embryo. This relatively sudden transition from selective regional blood flow in the earlier developing pancreas (E10.5 through early E14), to global pancreatic perfusion (starting at late E14/early E15), corresponds approximately to a time of rapid increase in the amount of amylase-positive exocrine differentiation (Pictet and Rutter, 1972; Rall et al., 1973; Zhou et al., 2007).

To assess any correlation between regional blood flow and pancreatic epithelial cell differentiation, examination of intracardiac-injected embryos from E11.5 to E15.5 confirmed that prior to E14.5, glucagon+ or insulin+ cells were generally localized to regions of the developing pancreas where blood was flowing (Figs. 1H–P and 2I–L). Some areas that were receiving blood flow did not have adjacent endocrine-positive cells, which could represent new blood flow to regions that had not yet undergone endocrine differentiation. Regions of the developing pancreas that had FITC-tomato lectin+/Pecam− (non-perfused) vessels were generally endocrine-negative, suggesting that the presence of endothelium alone, in the absence of blood flow, is insufficient to induce differentiation. Morphometric analyses of tissue sections showed that the distance between differentiated cells (endocrine-hormone-positive) and the nearest perfused vessel at early E14.5 was 3- to 4-fold less than the distance between undifferentiated non-mesenchymal pancreatic cells (E-cadherin+/endocrine-negative cells) and the nearest perfused vessels (Fig. 2L).

**Oxygen is a key factor in orchestrating pancreatic cell differentiation**

Enhanced oxygen delivery after the inflow of blood would seem a likely permissive factor for initiating differentiation. First, to confirm that tissue oxygenation is indeed enhanced in the vicinity of embryonic blood flow, we used a relatively new technique to detect oxidized thiols in histological samples (Mastroberardino et al., 2008). In states of higher tissue oxygenation, thiols exist as oxidized disulfides (Yang et al., 2007). We found that the region around perfused (FITC-tomato lectin+) vessels had more oxidized thiols compared to the rest of the embryonic pancreas, representing a higher level of tissue oxygenation (Fig. 3A–F). These findings confirm that the region of the pancreatic epithelium that is adjacent to what we identified as “perfused” vessels in the developing embryo was in fact less hypoxic than regions further away from those perfused vessels, implying that the observed tomato lectin staining is indeed indicative of where blood is flowing. Similar to the discussion in the last section, while not all perfused and oxygenated areas are endocrine, the endocrine areas tended to be areas with flow and higher oxygenation, although exact quantification here would be difficult. Some endocrine-negative areas are well-oxygenated, again suggesting perhaps recent onset of blood flow to cells that are still early in the differentiation process.

**Direct analysis of embryonic pancreatic differentiation in hypoxia**

To directly study the effect of oxygen tension on pancreatic differentiation, we cultured E11.5 pancreatic explants in vitro in hypoxia (1% oxygen, a level we estimate would exist in unperfused regions of the embryonic pancreas (Meschia, 2004 and see Discussion section). Here, proliferation was maintained (as measured by the
fraction of BrdU+ cells, data not shown) in 1% oxygen compared with 21% oxygen (Fig. 4, compare A with D), but with little exocrine differentiation and little or no additional endocrine differentiation beyond the number of glucagon cells that were present at the start of the culture (Fig. 4, compare E with H, and J with L, and see 4I). The endocrine cells that were present after 7 days in hypoxia were essentially all glucagon-positive, and the number of glucagon-positive cells present after the 7-day culture period was roughly the same as had originally been present in the E11.5 pancreas at the time of harvesting and initiating the culture (data not shown). Very few insulin-positive cells developed in 1% oxygen. Thus, the robust growth of endocrine and exocrine cells that occurs in 21% oxygen cultures does not occur when grown in hypoxia. The poor differentiation in hypoxia occurs despite normal numbers of Pecam+ vessels (Fig. 4K, M), again suggesting that vascular endothelium by itself is not sufficient to induce pancreatic differentiation. In addition, the number of ngn3+ cells appears not to differ in the different oxygen concentrations, suggesting that there is not a loss of endocrine progenitors in hypoxia (Fig. 4J, L).

We then altered the in vitro culture system to mimic the in utero changes in pancreatic tissue oxygenation that may occur during a transition from low or limited blood flow to more diffuse blood flow (which in the pancreas appears to occur at E14–E15). We first cultured E11.5 pancreatic explants for 3 days in hypoxic conditions (to mimic the period from E11.5 to E14.5 when there is no blood flow to the region of progenitor cells), followed by a switch to the supraphysiologic 21% oxygen for 4 days (to mimic the influx of blood flow that occurs at E14.5 (Fig. 4B, F, I)). Embryonic blood clearly does not reach a level of 21% oxygen, but since we currently do not know...
has been shown to be inhibited particularly, pancreatic differentiation, especially regulator of the differentiation of many embryonic tissue types. In rapid proliferation.

Mammalian embryonic tissues are well known to be able to undergo specification, no blood, but clearly providing less oxygen to tissues than in the post-natal state (Meschia, 2004). Despite this apparent obligate hypoxia, other nutrients, so in the actual in utero environment both endothelium and nearby blood flow together may be necessary for pancreatic differentiation.

Our findings suggest that blood flow plays an important permissive role in pancreatic differentiation in vivo, presumably by delivering oxygen and other nutrients, and that blood flow is not distributed evenly over the entire early embryonic pancreas. A role for blood-borne oxygen in regulating pancreatic differentiation (as opposed to vascular endothelium alone being sufficient to induce differentiation) is supported by findings in zebrafish where vascular endothelium was not necessary for pancreatic development (Serluca et al., 2002). Early developing zebrafish organs acquire oxygen by direct diffusion from the environment, without endothelium; in contrast, mammalian embryos require a functional cardiovascular system early in development to bring oxygen to the tissues (Baldessari and Mione, 2008; Zoeller et al., 2008). Our results also

**Discussion**

There is a fundamental lack of understanding of how organs in early mammalian embryos are able to form despite receiving little or no blood flow. The early embryonic tissues are relatively hypoxic since the early embryonic cardiovascular system is immature and is therefore presumably incapable of delivering high volumes of blood to all tissues. In addition, the embryonic blood only receives oxygen secondarily, by avidly removing oxygen from the mother’s placental blood, but clearly providing less oxygen to tissues than in the post-natal state (Meschia, 2004). Despite this apparent obligate hypoxia, mammalian embryonic tissues are well known to be able to undergo rapid proliferation.

Recent evidence indicates that oxygen tension is an important regulator of the differentiation of many embryonic tissue types. In particular, pancreatic differentiation, especially β-cell differentiation, has been shown to be inhibited in vitro by hypoxia (Heinis et al., 2010). A specific pro-insulin inductive role was shown for endothelial cells by Lammert et al. in which isolated early mouse endoderm, cultured in combination with structures containing endothelial cells (i.e., dorsal aortae or umbilical artery), led to Pdx1 expression and insulin-positive differentiation in the endoderm (Lammert et al., 2001). This inductive effect of endothelium on pancreatic differentiation was independent of embryonic blood flow since these inductive effects were seen in vitro in the absence of blood flow. However, since these in vitro studies were performed in normal culture media and 21% oxygen, a possible in vivo (in utero) need for blood flow to supply permissive factors (nutrients and oxygen) would not have been detected. Endothelial cells are clearly necessary for pancreatic differentiation in vivo. Yoshitomi and Zaret showed that dorsal endoderm, genetically depleted of endothelial cells (Flk1 null mutant embryonic mice), required recombination with endothelium-containing wild-type aortae to allow Ptf1a expression (Yoshitomi and Zaret, 2004). Although it is devoid of blood flow, the in vitro environment does provide oxygen and CO2 exchange, as well as other nutrients, so in the actual in utero environment both endothelium and nearby blood flow together may be necessary for pancreatic differentiation.

Fig. 3. Oxygenation and blood flow in the developing pancreas. (A–C) Oxidized thiols were used to identify areas of higher tissue oxygenation. Regions with perfused FITC-tomato lectin+ (TL) vessels (A) showed higher levels of oxidized thiols (B, C). (D, E) Pancreatic epithelium, including glucagon-positive differentiated cells, adjacent to FITC-tomato lectin+ vessels also showed higher levels of oxidized thiols as compared to the surrounding embryonic pancreas. (F) An intensity ‘heat’ map for oxidized thiol staining is a more sensitive method for assessing the gradient of tissue oxygenation, with decreasing oxygen tension seen with increasing distance from perfused vessels. TL–FITC-tomato lectin, Gluc–glucagon, Thiols–oxidized thiols.
support the *in vitro* data from Heinis et al. in which higher oxygen tensions led to greater \(\beta\)-cell differentiation in embryonic pancreatic explant cultures in a collagen gel (Heinis et al., 2010).

One concern with our analysis of vascular perfusion in the embryos is that the *in utero* injection process may “stun” the embryonic cardiovascular system due to direct manipulation of the heart, hypothermia during the injection, varying hemodynamic properties of the injectate (e.g., hyperviscosity), etc. Such altered physiology could lead to inadequate perfusion pressure of the embryonic vessels, and thus a falsely low perceived level of perfusion. Even with these supra-physiologic volumes of low viscosity injectate, we were not able to achieve significant additional flow to the “unperfused” areas, despite inducing numerous punctate hemorrhages, presumably due to high pressure (data not shown). These results suggest that decreased embryonic perfusion pressure during the injection is less likely to be the cause of any perceived lack of perfusion of existing vessels. In addition, the oxidized thiol data (Fig. 3) support that the regions demonstrating blood flow in our system were better oxygenated, implying that we were not seeing a “false-negative” for blood flow in other regions of the pancreas.

We found that *in vitro* the hypoxic embryonic pancreas is still proliferative but does not differentiate. The recent findings by Heinis et al. that HIF1\(\alpha\) is present and active in hypoxic tissues, and then is presumably degraded upon increased oxygenation, could be an additional pathway involved in pancreatic differentiation. Growth of embryonic pancreatic explants in 1% oxygen may have led to an expansion of progenitor cells, since subsequent transfer to 21% oxygen led to a greater number of \(\beta\)-cells than explants kept in 21% oxygen for the entire culture period. Unlike the previous study by Heinis, where 3% oxygen was used as hypoxia, 1% oxygen may be more representative of regions of the embryonic pancreas that are remote from blood flow. Normal oxygenated mammalian maternal blood is typically in the range of 12% oxygen (Meschia, 2004). However, at best in the fetus ("best" meaning a late-gestation fetus, which has a mature cardiovascular...
system), the peripheral fetal arterial blood oxygenation is only about one-fourth that of the maternal blood, or an approximate oxygen concentration of 3%. This step-down between maternal and fetal blood oxygenation is due first to the obvious step-down that must occur with the oxygen gradient across the placenta, and then secondly and more importantly, because of the normal fetal/embryonic anatomical mixing (only a fraction of the fetal cardiac output goes through the placenta, as opposed to post-natal circulation where almost all of the blood flows through the lungs). With a fetal or embryonic arterial oxygen content of 3% at the most (and likely lower in a more immature fetus or embryo because of an immature cardioplacental circulation), then there will be a further step-down in oxygen concentrations in the surrounding tissues, with tissues of course being more and more hypoxic as the distance of the tissue from the blood increases. Thus, we chose the 1% oxygen level as our best estimate of an unfused area of the early embryonic mouse pancreas.

Interestingly, although insulin-positive cell numbers were greatly affected by oxygen tension in the explant culture experiments, the number of glucagon-positive cells was not, with no change in the number of glucagon-positive cells in the explant from the start of the culture period to the end, regardless of whether the explants were grown in 21% oxygen or 1% oxygen. These results suggest that our culture conditions are not conducive to new α-cell formation. In vivo, however, glucagon cells were primarily localized only to regions receiving blood flow, suggesting that new glucagon-positive cell formation in vivo, like β-cell formation, is dependent on oxygen.

The striking lack of perfusion of much of the vascular network in the early embryonic pancreas was unexpected. Several explanations are possible for the lack of perfusion of these vascular beds. First, early in development, there may be no anatomical connection between the main embryonic vessels (which carry blood flow) and the numerous small vessels that do not have blood flow. In order for blood flow to reach these smaller vessels, connections (anastomoses) would need to develop between the larger main vessels and the smaller branches. A second possible explanation is that there is some restriction of blood flow to the vast network of small vessels, perhaps through a sphenicteric mechanism. However, the ex vivo high-pressure injections described above would suggest otherwise. A third possible explanation for not detecting flow to these vessels is that there actually is blood flow to these small vessels, but at a very low level that is not detectable by lectin (or FITC-labeled dextran) labeling. Late in gestation (E14–15), however, we did see that all vessels, including small capillaries, were well demonstrated by lectin injections into the heart.

Teleologically, it makes sense that there are large vascular beds in the early fetus that are not receiving blood flow. Mammalian organogenesis has been shown to be closely coupled with vasculogenesis in several organs. This co-development of organ-specific cells and their accompanying vasculature would seem necessary for optimal alignment of the developing capillaries and epithelium to allow proper physiological function. For example, embryonic pulmonary capillaries develop in close association with the branching epithelium of the lung. This close association is critical for maximizing the interface of alveoli with pulmonary capillaries to optimize gas exchange. Similar to our findings in utero in the pancreas, dye injection studies have shown that the large embryonic pulmonary capillary network is, for the most part, unfused until later in gestation (deMello et al., 1997). This lack of perfusion of capillary beds may be common to many developing organs in the embryo, which may be advantageous because of the immature early embryonic heart that presumably has limited cardiac output. Such a selective capillary bed perfusion system could reserve the limited cardiac output and blood flow for only those tissues where it is needed.

Our studies also demonstrate survivable access to the early mammalian embryonic vascular system in utero. We can now deliver large reagents (that would not otherwise cross the placenta, including viral and plasmid constructs, large proteins, etc.) directly and systematically to the early embryo, without relying on trans-placentally transport from the mother (survivable injections can be done as early as E9.5). Such access may have important implications for future mammalian development studies, including direct manipulations of early organogenesis during or even before the actual process of organogenesis.

Our results show that vascular flow, rather than just vascular endothelium alone, may provide specific signals necessary for regional pancreatic differentiation in the early embryo. Vascular endothelium, blood flow, and blood-borne oxygen may also play a similar role in the development and differentiation of other organs.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.10.033.

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