

Mechanisms of Angiogenesis Process after Pancreatic Islet Cell Transplantation: Role of Intra-islet Endothelial Cells

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Abstract

Angiogenic sprouting is a complex, multi-step process involving highly integrated cell behaviours, initial interaction with the environment and signalling pathways. Endothelial cells (ECs) are central to the angiogenic process, with recent insights establishing how these cells communicate with each other and with their microenvironment to form branched vascular networks. Using pancreatic islets as a model for vascularized tissue, this review will present a general overview of EC behaviour dynamics in sprouting angiogenesis, particularly focusing on the interplay between VEGF and Notch pathways. A better understanding of molecular mechanisms associated with intra-islet EC cross-talk and its micro-environment may present exciting new perspectives on islet graft to host revascularization and in supporting islet graft survival.

Keywords: Transplantation; Endothelial cells; Angiogenesis; Revascularization; VEGF; Notch signaling

Introduction

Pancreatic islets are highly vascularized and receive 10% of the pancreatic blood flow despite comprising of only 1-2% of the overall tissue mass [1]. Islets represent endocrine “island” clusters, embedded and scattered within large amounts of exocrine acinar tissue [2]. Most islets are irregularly shaped spheroids with a size distribution ranging from 50–200 μm , composed of 800–3,000 cells. In the context of islet studies and transplantation, 1 islet equivalent (IEQ) is often considered as a size of 150 mm, consisting of an average 2,500 cells. The cellular components of the islet include β -cells with the remainder of the islet comprised of other endocrine cells (including glucagon-secreting α -cells, somatostatin secreting δ -cells, pancreatic polypeptide-secreting γ -cells, and ghrelin-producing ϵ -cells), as well as ECs and support cells such as pericytes [3-12]. Species heterogeneity exists with respect to cellular composition of islets. Rodent islets are primarily composed of β -cells located in the center with other cell types in the periphery, human islets exhibit interconnected α - and β -cells [3-13,14]. β -cell, the central regulator of glucose homeostasis is the largest cellular component of islets in most species [12,13]. Vascular endothelial cells represent a major cell type present in islets and these cells are organized into a highly regulated and morphologically unique microcirculation. Studies using vascular corrosion casts have shown that 1-3 arterioles feed larger islets [15]. The capillary network within islets is about five times denser in comparison with exocrine tissue [16]. The capillary wall is composed of a permeable layer of ECs and contain ten times more fenestrae than ECs present in the exocrine pancreas [17,18]. Rapid and adequate revascularization is critical for survival and function of transplanted islets [19-21]. Unlike whole organ transplantation where revascularization occurs through surgical anastomosis of vessels, the revascularization of islets requires the

formation of vessel patencies either through inosculation of host and recipient microvessels or through neo-vessel penetration into the islet. The return of islet function depends on reestablishment of new vessels within islet grafts to derive blood flow from the host vascular system [22,23]. Transplanted islet grafts initially have a significant reduction in vascular supply and low oxygen tension in comparison to normal islets [24-26]. The human islet isolation technique completely severs the islet vasculature [20,27], the enzymatic digestion step contributing towards partially disrupting intra-islet ECs [22,28,29]. Revascularization is an important process for adequate engraftment of islets. Prevascularizing islets prior to transplantation could potentially improve islet survivability and function by aiding islet-to-host inosculation [30]. Studies involving cell and tissue engineering approaches have considered factors such as pancreatic islet size-dependency [31], use of stem cells [32-35], endothelial progenitor cell derived microvesicles [36], creating engineered vascular beds and hydrogels [37-39] and repurposed biological scaffolds [40] to improve islet revascularization potential. The angiogenic capacity of islet ECs has been previously determined [41]. These cells have been shown to support revascularization of fresh islets by participating in the early processes of vessel formation [30,42]. Unpublished data from our lab demonstrates that fresh islets, immediately after isolation, are capable of forming peri-islet vessels in a 3D-gel construct (Figure 1 & 2). The initial molecular events by which intra-islet ECs result in the formation of such vessels have not yet been explored. This review will focus on the VEGF-Notch signalling pathways and their associated molecular regulation which have been well characterized and shown to play key roles in endothelial crosstalk critical to proper vessel sprouting.

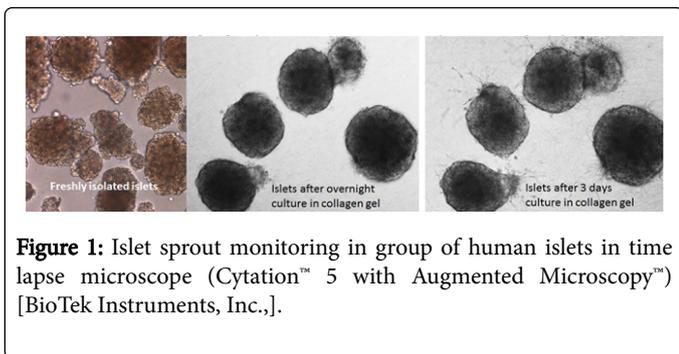


Figure 1: Islet sprout monitoring in group of human islets in time lapse microscope (Cytation™ 5 with Augmented Microscopy™) [BioTek Instruments, Inc.,].

Regulation of angiogenesis

VEGF family: critical regulators of angiogenesis

The family of VEGF (vascular endothelial growth factor) ligands and their receptors are major regulators of sprouting angiogenesis

[43-46]. VEGFs are critical, as they regulate vessel formation during embryonic development, play a major role in wound healing and in maintaining vessel homeostasis in adult organisms. In addition, impaired vessel function resulting from defects in VEGF ligands or receptors is the cause of many diseases. VEGF was originally described as vascular permeability factor (VPF), an activity released by tumor cells that promotes vascular leakage [43,47-56]. VEGF secretion is stimulated by tumor, hypoxia, low pH and many other factors. The VEGF binds to its receptor (VEGFR) located on the blood vessel ECs. The ECs upon activation produce enzymes and other molecules for EC growth and proliferation. Other effects include mobilization of endothelial progenitor cells from bone marrow, increased vascular permeability and tissue factor induction. The VEGF family comprises seven secreted glycoproteins that are designated VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor (PlGF) and VEGF-F [57-59]. VEGF-A, the most well studied factor within the VEGF family, is expressed in the extra-embryonic endoderm and mesoderm as blood islands, and within the intra-embryonic endoderm at E8.5 [60] (Table 1).

Type of VEGF	Role in regulating/modulating ECs	References
VEGF-A	Most potent pro-angiogenic protein described to date, implicated in both vasculogenesis and angiogenesis. It induces proliferation, sprouting and tube formation of ECs. Is a potent survival factor for ECs and has been shown to induce the expression of anti-apoptotic proteins in these cells. Causes vasodilation by inducing the endothelial nitric oxide synthase and so increasing nitric oxide production. VEGF-A binds many receptors on hematopoietic stem cells (HSCs), monocytes, osteoblasts and neurons; induces HSC mobilization from the bone marrow, monocyte chemo-attraction and osteoblast-mediated bone formation Many cytokines including platelet-derived growth factor, basic fibroblast growth factor, the epidermal growth factor and transforming growth factors induce VEGF-A expression in cells.	[49,57] [61,62] [63] [57,64] [65]
VEGF-B	Several reports suggest that VEGF-B may modulate cell proliferation and vessel growth. Conditioned medium from transfected cells expressing VEGF-B stimulates DNA synthesis in endothelial cells. Shown to play a central role in cardiac development.	[66] [67,68]
VEGF-C	The mature form of VEGF-C induces mitogenesis, migration and survival of ECs VEGF-C mRNA transcription is induced in ECs in response to pro-inflammatory cytokines (IL-β). Promote lymphatic vessel development and may also contribute to angiogenesis.	[69] [70] [71-73]
VEGF-D	The mature human VEGF-D is mitogenic, angiogenic and lymphogenic <i>in vivo</i> Stimulates growth of vascular and lymphatic ECs by signaling through the tyrosine kinase receptors (VEGFR-2, VEGFR-3) Promote lymphatic vessel development and may also contribute to angiogenesis.	[69] [74] [71-73]
VEGF-E	Highly specific isoform that acts only on the endocrine gland endothelial cells. VEGF-E is a potent angiogenic factor and data strongly indicates that the activation of VEGFR-2 alone can stimulate angiogenesis efficiently.	[75] [76]
PlGF	Originally identified in the placenta; occurs at low levels in the embryo and adult and has primarily been studied in pathological conditions where it is thought to stimulate angiogenesis in coordination with VEGF-A.	[77,78]

Table 1: Types of vascular endothelial growth factors (VEGFs) with evidence demonstrating their involvement in regulating endothelial cells.

VEGF family members interact with three main receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR in humans and Flk-1 in mouse) and VEGFR-3 (Flt4), all tyrosine kinase receptors and members of the PGDF receptor family. VEGF receptors possess an extracellular domain consisting of immunoglobulin repeats responsible for VEGF binding and intracellular tyrosine kinase domains. VEGF binding to its receptor leads to receptor dimerization and activation of receptor tyrosine kinases by autophosphorylation. This leads to several biologic

effects on endothelial cells. The VEGF receptor transmembrane tyrosine kinases, which upon binding of their ligands to the extracellular domain of the receptor, activate a cascade of downstream proteins after the dimerization and autophosphorylation of the intracellular receptor tyrosine kinases. VEGFR-2 appears to be the main receptor responsible for mediating the proangiogenic effects of VEGF-A [57,79,80]. VEGF-A and its receptors VEGFR-1 and VEGFR-2 are expressed early in embryonic development (Table 2).

Type of VEGFR	Role in regulating/modulating endothelial cells (ECs)	References
VEGFR-1	Expressed in ECs as well as osteoblasts, monocytes/macrophages, placental trophoblasts, renal mesangial cells and also in some hematopoietic stem cells (HSCs). VEGFR-1 expression is upregulated by hypoxia (HIF1 dependent mechanism). Has an active functional role and participates in monocyte migration, recruits EC progenitors and increases adhesive properties of natural killer cells.	[81] [82] [83-86]
VEGFR-2	Undergoes dimerization and strong ligand-dependent tyrosine phosphorylation in intact cells and results in a mitogenic, chemotactic, and pro-survival signal. Y1175 and Y1214 are the two major VEGF-A-dependent autophosphorylation sites in VEGFR-2. However, only autophosphorylation of Y1175 is imperative for VEGF dependent EC proliferation. In addition to the ECs, VEGFR-2 is also expressed on neuronal cells, osteoblasts, megakaryocytes and HSCs. It is down-regulated in the blood vascular ECs, and is again up-regulated in angiogenic blood vessels. Sequestration of VEGF-A results in down-regulation of VEGFR-2 and in apoptotic death of some capillary endothelial cells <i>in vivo</i> . It is an early marker of endothelial and hematopoietic precursor cells in blood islands.	[87] [88] [57,87] [89,90] [91,92]
VEGFR-3	Recently shown to be strongly modulated by Notch upregulating angiogenesis in absence of VEGF-VEGFR2 signalling. VEGFR-3 is up-regulated on blood vascular ECs in pathologic conditions such as in vascular tumors and in the periphery of solid tumors. Widely distributed in vascular tumors and can be considered as a marker of endothelial cell differentiation of vascular neoplasms. is down-regulated <i>in vivo</i> at sites of endothelial cell-pericyte/smooth muscle cell contacts; suggesting that VEGFR-3 signaling is important in nascent blood vessels, and it becomes redundant as the vessels mature. In humans, VEGFR-3 expression was upregulated in blood vessel endothelium in chronic inflammatory wounds.	[93] [89] [94] [95]

Table 2: An overview of vascular endothelial growth factor receptors and their roles in regulating endothelial cells.

Notch signaling

In addition to the VEGF receptor tyrosine kinases and their ligands, several recent studies demonstrate the importance of Notch signalling components such as ligands Dll4 (Delta-like ligand 4), Jagged-1 and Notch1 in EC specification during formation of a functional vascular network [96-99]. In mammals there are 5 DSL (Delta Serrate Lag-2) ligands: Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged-1 (Jag1) and Jagged-2 (Jag2). These ligands are type1 cell-surface proteins with multiple tandem epidermal growth factor (EGF) repeats in their extracellular domains (ECDs). DSL ligands bind to Notch receptors, which are large, single pass, type1 transmembrane receptors. There are 4 known Notch receptors, Notch1 to Notch4. Binding of a DSL ligand to the ECD of the Notch receptor (NECD) triggers a series of proteolytic cleavages of Notch, first by a member of the disintegrin and metalloproteases (ADAM) family within the juxta-membrane region, followed by γ -secretase within the transmembrane domain (Table 3). The Notch receptors, ligands, and several signaling pathway components have been identified in endothelial cells *in vitro* and *in vivo*, during development and tumor angiogenesis [100-102].

Notch pathway	Pathway component expressed by ECs	References
Receptors	Notch1 and Notch4	[101,103-105]
Ligands	DSL ligands Dll1, Dll4, Jag1 and Jag2	[101,103-105]
Key Notch signaling components	Rbpj, Hey1, Hey2, Maml1, Numb and Nrarp	[104,106-112]

Table 3: Notch pathway components expressed in endothelial cells.

Functional studies using gene targeting in mice, mutagenesis and knockdown in zebrafish, and biochemical analysis in cultured endothelial cells have demonstrated that Notch signaling plays a fundamental role in many aspects of endothelial cell biology during angiogenesis [113] (Table 4).

Endothelial function	Notch component(s) involved	References
Tip/stalk specification	cell	[97-99,114,115]
	Dll4	[97]
	Rbpja (zebrafish)	[98]
Proliferation	Dll4	[99,110,115-117]
	Notch1	[110]
	Notch4	[118]
	Rbpj/Rbpja (zebrafish)	[98,106]
	Mam1	[110]
Vessel stability	Hes1	[110]
	Nrarp	[112]
Motility	Dll4	[114,117]
	rbpja (zebrafish)	[98]
Filopodia protrusion	Dll4	[97,99,114,115]
	Notch1b (zebrafish)	[114]

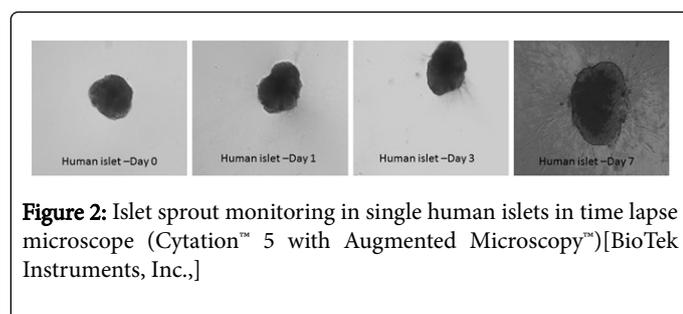
Matrix assembly and adhesion	production/ and cell	Dll4	[116,117,119,120]
		Notch1	[119]
		Notch4	[121]

Table 4: Evidence for the role of Notch components involved in endothelial cell function.

EC phenotypes: Interplay between VEGF and Notch signaling in regulating EC sprouting

An exciting breakthrough within angiogenic research in the past decade has been the identification of different EC phenotypes with different cellular fate specifications that are key in forming a vessel branch [122]. Leading the trail are 'tip cells' which sense and respond to guidance cues. 'Stalk cells' follow behind the tip cells and elongate the stalk of the sprout by proliferating, forming junctions, modulating the extracellular matrix and forming a lumen. 'Phalynx cells,' the most quiescent of the ECs, line vessels once new vessel branches have formed. These cells form a monolayer, are covered by pericytes, attached via tight junctions, and strongly held by a robust basement membrane. Phalynx cells are engaged in optimizing blood flow, tissue perfusion and oxygenation [123-125].

Specification of ECs into tip and stalk cells bearing different morphologies and functional properties is central to sprouting initiation [113,126]. Vessel networks, while expanding, require ECs to undergo frequent cycles of sprouting and branching. This results in dynamic transitions between the two cell phenotypes [113,126]. Tip cells express high levels of Dll4, platelet derived growth factor-b (PDGF-b), unc-5 homolog b (UNC5b), VEGFR 2/3 and has low levels of Notch signalling activity [98,99,103,127, 128]. Stalk cells produce fewer filopodia, are more proliferative, form tubes, branches and a vascular lumen, establish junctions with neighbouring cells and synthesise basement membrane components [113,129]. Tip cell migration depends on a VEGF gradient migrating outward from parent vessel whereas stalk cell proliferation is regulated by VEGF concentration [127,130]. VEGF stimulates tip cell induction and filopodia formation via VEGFR2 (abundant on filopodia), whereas VEGFR2 blockade is associated with sprouting defects [113]. VEGFR1 expression is induced by Notch signalling to reduce VEGF ligand availability preventing tip cell outward migration. VEGFR1 is predominantly expressed in stalk cells and is involved in guidance and limiting tip cell formation. Loss of VEGFR1 results in increased sprouting and vascularization [131,132].



Notch appears to act as a negative feedback mechanism to regulate VEGF signaling. This regulation may explain the observation that decreased VEGFR-2 allows for local differentiation of endothelial tip cells prior to sprout initiation with VEGF action on tip cells leading to increased Dll4 expression and activation of Notch signaling, which in turn downregulates VEGFR-2 in neighboring stalk cells [46]. Tip cells with higher VEGFR-2 expression will, therefore, readily respond to VEGF while stalk cells with fewer receptors will be less responsive. Interestingly, tip cells do not proliferate in response to VEGF, but rather form filopodia and migrate in the direction of the VEGF gradient. It is the stalk endothelial cells of the growing capillary branch that proliferate [127].

In mouse and zebrafish angiogenesis, VEGFR3 is strongly expressed in the leading tip cell and is downregulated by Notch signalling in the stalk cell [98,133]. Notch1 and Notch4 and the three Notch ligands JAG-1, Dll1 and Dll4 are expressed in ECs for the induction of arterial cell fate and for the selection of endothelial tip and stalk cells during sprouting angiogenesis [134]. Activation of Notch signalling reduces while its loss induces sprouting. Notch-1 deficient ECs adopt tip cell characteristics [97,98,129] whereas in stalk cells, activation of Notch by Dll4 leads to downregulation of VEGFR-2 and -3 [101,135]. Cells dynamically compete for tip position utilizing differential VEGFR levels, as cells with higher VEGFR signalling produces more Dll4 and therefore inhibit their neighbouring cells. VEGF has been shown to induce the expression of Dll4 and Notch signaling [136]. Elevated Dll4 and VEGFR-2 expression was detected in tip cells compared to neighboring stalk cells [96]. Blockage of VEGF, in animal models, caused a decrease of Dll4 in vessels and inhibited sprouting [99] whereas administration of VEGF induced Dll4 expression [115].

Notch signaling also influences VEGF receptor expression, leading to the downregulation of VEGFR-2, as evidenced by decreased VEGFR-2 levels after Notch activation in ECs and in Dll4-deficient mice [99,109]. Endothelial Notch activation regulates the expression of different VEGFRs (VEGFR1, 2, and 3) as well as the co-receptor Nrp1 [46,93,97,98,103,114,115,137]. Dll4 activates Notch in adjacent cells, which suppresses the expression of VEGF receptors and thereby restrains endothelial sprouting and proliferation [98,99,113,138]. Notch activation in HUVECS leads to VEGFR1 mRNA induction [120,139]. In contrast, VEGFR2 and Nrp1 mRNA is markedly reduced by Notch activation in HUVECS [137,140,141], indicating that Notch signaling is able to regulate how the ECs respond to VEGF. The Notch and VEGF signaling appear to be intimately associated in angiogenesis. It has been shown that Notch signalling acts downstream of the VEGF pathway during physiological and pathological angiogenesis [115,140,142-144], suggesting that VEGF pathway controls expression of different Notch components (Table 5).

Conclusions and Future Perspectives

Significant progress has been made in our understanding of importance of angiogenesis in health and disease but our knowledge of coordinated events that result in vessel branching and inosulation remains incomplete. We are just beginning to appreciate the interplay of other signalling pathways such as Wnt and BMP in regulating vessel sprouting. Angiogenesis is a complex, multi-step process. Key to this process are ECs, which are pivotal to sprouting angiogenesis and have been implicated in many diseases [60,161-163].

Novel regulators/components	Recently identified for sprouting angiogenesis (VEGF/Notch pathways)	References
Deubiquitinases	Notch	[145]
Cholesterol	VEGF	[146-148]
MEF2 transcription factors	VEGF-Notch	[149]
Podosomes	VEGF-Notch	[150]
Adipogenic proteins	VEGF-Notch	[151,152]
Glucose regulators	VEGF-Notch	[152,153]
Foxo1 transcription factor	EC metabolism	[154]
Lactate	Angiogenesis	[155]
ROS and redox events	VEGF	[156-158]
Cilia	Angiogenesis	[159,160]

Table 5: Novel regulators recently identified to play a role in angiogenesis (ECs/VEGF/Notch pathways).

It has been shown that EC proliferative capacities can be stimulated by various inducers [41,42,164,165]. A variety of *in vivo* and *in vitro* models for understanding EC behaviour during angiogenesis at the cellular level have been derived from systems such as rabbit cornea [166], developing mouse retina [167], intersegmental vessel growth in zebrafish [168] and using ECs embedded in collagen or fibrin gels [169,170].

In the last two decades, focus has been paramount on the study of human pancreatic islets, its isolation techniques and in improving islet yield and function because of its critical involvement in debilitating diseases such as Type-1 diabetes and chronic pancreatitis. The dense vasculature within the pancreas is an important determinant in the physiology and disease of islets. The pancreatic islets is an ideal model 'tissue' to learn more about microvasculature and in this context the study of ECs within islets has potential benefits. The islet EC model represents an excellent platform to better understand molecular mechanisms associated with vessel sprouts, an important but greatly understudied area within islet research. Crosstalk of ECs with other islet cells, such as the β -cells has been evaluated [171-175] particularly in increasing β -cell mass and thereby insulin production. Moreover, a number of factors which may potentially improve islet transplantation involve ECs. Vascular ECs of the embryonic aorta have been shown to induce the development of endocrine cells from pancreatic epithelium in mouse [176,177] and overexpression of VEGF-A in transplanted mouse islets was shown to improve insulin secretion and blood glucose regulation in recipient mice [165,178]. Utilizing intra-islet ECs as a model to better understand mechanisms associated with sprouting angiogenesis is likely to generate exciting new hypotheses and offer new insights of how transplanted islets can reestablish vasculature more efficiently and successfully.

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