Bio-engineering of insulin-secreting organoids: a step toward the bioartificial pancreas

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Résumé en français

Le diabète sucré est une maladie chronique et représente un problème de santé mondial avec plus de 460 millions de personnes atteintes en 2019. Le diabète de type 1 (DT1) représente à peu près 10% des patients et est le résultat de la destruction des cellules sécrétrices d'insuline (cellules ß), par un phénomène auto-immun. L'insuline étant une hormone régulatrice clé du taux de glucose dans le sang, la perte de ces cellules entraine chez les patients, une hyperglycémie responsable d'importantes comorbidités. L'injection quotidienne d'insuline exogène permet de maintenir une glycémie proche de la norme chez la plupart des patients. Cependant, elle s'accompagne d'un risque d'hypoglycémie pouvant parfois être sévère, nécessitant une hospitalisation en soins intensifs ou même être fatale. De plus, une faible proportion de patients présente une forme particulière de DT1, appelé diabète labile, qui se caractérise par une instabilité très importante de la glycémie, malgré un traitement insulinique intensif, ainsi que par de nombreux épisodes d'hypoglycémies sévères. Pour ces patients, le remplacement des cellules ß par la transplantation d'îlots de Langerhans est une alternative ayant fait ses preuves. Cependant, étant une greffe de type cellulaire et non pas d'organe entier, où la vascularisation peut être rétablie au moment de la transplantation, la transplantation d'îlots doit faire face à de multiples obstacles : (i) l'isolement des îlots entraine une destruction de leur vascularisation ainsi que de leur connexion avec leur matrice extra-cellulaire ce qui entraîne des phénomènes d'hypoxie et de nécrose durant la culture de ces derniers, (ii) une fois transplantés, la revascularisation des îlots peut prendre plusieurs semaines et (iii) les îlots doivent faire face à d'importantes réactions inflammatoires et immunitaires (allo-rejet, récidive de l'auto-immunité) une fois transplantés dans le foie. Tout cela entraine une mauvaise implantation des îlots raison pour laquelle, la greffe doit être répétée deux à trois fois, nécessitant plusieurs donneurs d'organes pour répondre à la demande métabolique et avoir un bon contrôle glycémique. L'amélioration de cette approche thérapeutique est nécessaire puisqu'elle permettrait à de multiples patients diabétiques d'en profiter sans pour autant épuiser le nombre de donneur potentiel.

Ce travail de thèse a tenté d'améliorer les résultats de la greffe d'îlots en augmentant l'implantation, la survie et la fonction des îlots à travers les mécanismes suivants : (i) en générant des organoïdes homogènes et de petites tailles afin de réduire le stress hypoxique une fois transplantés, (ii) en ajoutant des cellules endothéliales dans les organoïdes, dans le but d'apporter une source de cellules pour la création rapide de nouveaux vaisseaux et (iii) en ajoutant également des cellules amniotiques épithéliales humaines (hAEC) ayant des propriétés anti-inflammatoires et immunomodulatrices pour diminuer le stress inflammatoire lors de la transplantation.

La première partie de la thèse consiste en une introduction, composée de deux revues de la littérature. La première étude a permis de rappeler l'importance des interactions cellulaires ainsi que du micro-environnement au sein et autour des îlots de Langerhans. Cette revue a également permis de confirmer les bénéfices apportés par la génération d'organoïdes et de décrire les possibilités mais aussi les obstacles présents dans l'élaboration d'un pancréas bioartificiel. La deuxième revue a permis de faire une mise au point sur les bénéfices et les applications cliniques possibles des dérivés placentaires, plus particulièrement, des hAEC ainsi que de la membrane amniotique. **La deuxième partie** comprend deux articles démontrant les expériences réalisées durant cette thèse. La première est une étude méthodologique comparant les différentes techniques à disposition pour développer des organoïdes à partir de cellules d'îlots dissociées (pseudo-îlots). La deuxième étude représente la partie principale de la thèse avec le développement des organoïdes et leur transplantation dans un modèle murin, immunodéficient et diabétique. Nous avons pu observer une fonction supérieure des organoïdes par rapport aux îlots natifs et une meilleure revascularisation. **La dernière partie** consiste en une discussion globale sur les résultats observés dans les deux études précédentes et sur les perspectives de la greffe d'îlots et notamment, de l'importance du développement d'organoïdes dans l'élaboration d'un pancréas bioartificiel.

En conclusion, nos expériences montrent que l'élaboration d'organoïdes permet d'éliminer l'hétérogénéité de taille et de morphologie des îlots natifs et offre la possibilité d'ajouter des cellules ayant des caractéristiques bénéfiques pour les îlots tels que des cellules endothéliales et des cellules aux propriétés anti-inflammatoires et immunomodulatrices tels que les hAEC. L'explication des nombreux bénéfices apportés par ces hAEC n'est pas encore complètement comprise mais nous avons l'espoir que nos prochaines expériences permettront d'en élucider une partie.

English summary

With more than 460 million patients affected worldwide in 2019, diabetes mellitus represents a severe global health issue. Type-1 diabetes mellitus (T1DM) accounts for 10% of the cases and results from the autoimmune destruction of the ß cells, responsible for insulin synthesis and secretion. Being a key regulator of glucose metabolism, the loss of insulin leads to a dysregulation of blood glucose control, resulting in a hyperglycemic state, which is responsible for severe comorbidities. Exogenous insulin injection (insulin-therapy) is the treatment of choice for T1DM patients and allow to control as good as possible, blood glucose level. However, this therapy is accompanied by risks of hypoglycemia episodes that can be life threating. In addition, some patients present a severe form of diabetes called «brittle» diabetes, where glycemia control is very challenging even with intensive insulin-therapy. In addition, patients experience severe hypoglycemia episodes and can present an unawareness of those hypoglycemia. For those patients, ß cell replacement is a valid alternative and can be done by islet transplantation with satisfactory results. However, unlike whole organ transplantation where graft revascularization can be directly obtained during implantation, islets have to face several challenges that hamper transplantation outcomes : (i) islets lose their vascularization and their connections to the extra-cellular matrix during the isolation process which result in ischemic insults during culture, (ii) once transplanted, islet revascularization takes weeks and nutrients and O_2 are only obtained by diffusion, (iii) islets are transplanted inside the liver, through the portal vein and have to face multiple attacks by the innate and adaptative immune system (allo-rejection and auto-immunity recurrence). Altogether, this results in a poor engraftment of the transplanted islets and explains the need to repeat multiples islet infusions (two to three) from multiple deceased donors, in order to respond to the metabolic demand of the recipient. Knowing the actual issue of donor shortage, improving this therapeutic approach is a priority not only because it will decrease the number of needed donors but also because it will be possible to offer this treatment to many more patients.

This thesis aimed to improve islet transplantation outcomes by increasing the engraftment, the survival and the function of the transplanted islets through the following mechanisms: (i) by generating homogenous, controlled size organoids in order to reduce ischemic stress and necrosis until revascularization occurs, (ii) by adding an external endothelial cell source to improve graft revascularization, (iii) by adding human amniotic epithelial cells (hAEC) which will act as supporting cells to islet and endothelial cells through their anti-inflammatory and immuno-modulatory properties.

The **first part** of the thesis represents the introduction and is composed of two reviews. The first article aimed to present the islet as a whole mini-organ and to describe the importance of cell to cell and cell to matrix interactions within and around the islet. Also, we reported the benefices of organoid generation and the possibility of its usage as a building block for bioartificial pancreas creation. The second review aimed to summarize the benefices of amniotic derivatives, especially of the hAEC and the amniotic membrane, and to report their potential clinical application in regenerative medicine. The **second part** is composed of two articles containing the results of the

experiences performed during the thesis. The first article is a methodological comparative study of the available techniques for 3D-cell aggregation from single islet cells (pseudo-islet). The second study represents the main part of the thesis and reported the results of the generated pre-vascularized organoids and their increased function and vascularization after transplantation, in comparison to native islets. The **last part** consists of a global discussion about the observed results, the challenges remaining in islet transplantation and the importance of organoid generation in the elaboration of the bioartificial pancreas.

To conclude, our results demonstrated that the use of organoids allows to suppress the heterogeneity in term of size and morphology that is present in native islets and offers the possibility to add supporting cells such as endothelial cells and hAEC that can be beneficial to the islet cells. The improvement observed in our organoids remains partially unclear. However, a crucial role played by the hAEC is obvious and we are looking forward to our next experiments, where we will try to understand better the benefices offered by those cells.

Table of content

Abbreviations

ACs: Amniotic cells
AGE: Advanced glycation end-products
Ang-1: Angiopoietin-1
APC: Antigen presenting cell
ATP: Adenosine tri-phosphate
AUC: Area under the curve
bFGF: Basic fibroblast growth factor
BM: Basement membrane
BMI: Body mass index
BSA: Bovine serum albumin
CIT: Cold ischemia time
CM: Conditionned medium
CMV: Cytomegalovirus
CP: Chronic pancreatitis
DBD: Donor after brain death
DCs: Dendritic cells
DCD: Donor after cardiac death
DM : Diabetes mellitus
DMEM: Dulbecco-s modified Eagle's medium
DNA: Deoxyribonucleic acid
EC: Endothelial cells
ECM: Extra-cellular matrix
EFP: Epididymal fat pad
EGF: Endothelail growth factor
ELISA: Enzyme-linked immunosorbent assay
eNOS: Endothelial nitric oxide synthase
ENT: Ear nose throat
FDA: Fluorescein diacetate
GAD: Glutamic acid decarboxylase
GFP: Green fluorescent protein
GLP1R: Glucagon-like peptide-1 receptor
GSIS: Glucose stimulated insulin secretin
hAEC: Human amniotic epithelial cells
hAM: Human amniotic membrane
hAMSC: Human amniotic mesenchymal stem cell
HbA1c: Glycated hemoglobin
hESC: Human embryonic stem cells
HDL: High-density lipoprotein
HGF: Hepatocyte growth factor
HIF-1α: Hypoxia-inductible factor-1 alpha
HLA: Human leukocyte antigen
HUVECs: Human umbilical vein endothelial cells
IBMIR: Instant blood mediated inflammatory
IC: Islet cells
IEQ: Islet equivalent
IGF: Insulin growth factor
IGL-1: Institut Georges Lopez-1
IgM: Immunoglobulin M
IL: Interleukin
ILT: Immunoglobulin-like transcript

INF-ɣ: Interferon gamma
INS : Insulin
IP-10: Interferon gamma-induced protein 10
IPGTT : Intraperitoneanl glucose tolerance test
iPSC: Induced pluripotent stem cells
IT: Islet transplantation
KIR2DL4: Killer cell immunoglobulin like receptor 2 Ig domains and laong cytoplasmic tail 4
LPS : Lipopolysaccharide
LV: Lentivirus
MCP-1 : Monocyte chemoattractant
MHC: Major histocompatibility complex
MIF: Migration inhibitor factor
MIN6: Mouse insulinoma 6
MIP: Macrophage inflammatory proteins
MODY: Maturity onset diabetes of the young
MSCs: Mesenchymal stem cells
NADPH: Nicotinamide adenine dinucleotide phosphate
NF-κB: Nuclear factor-kappa B
NK: Natural killer
NKG2D: Natural killer group 2 member D receptor
NO: Nitric oxide
Oct4: Octamer-binding transcription factor 4
OGTT: Oral glucose tolerance test
ORT: Organ removal time
PAK: Pancreas after kidney
PBS: Phosphate-buffered saline
PD-L: Programmed death-ligand
PDX1: Pancreatic and duodenal homeobox 1
PEC: Pancreatic endodermal cells
PFA: Paraformaldehyde
PGE_2: Prostaglandin 2
PI : Pseudo-islet
PIO : Pre-vascularized islet organoid
PP: Pancreatic polypetide
PCSK: Proprotein convertase
PTA : Pancreas transplantation alone
qPCR: Quantitative polymerase chain reaction
RAGE: Receptor for advanced glycation end-products
RANTES: Regulated on activation normal T cell expressed and secreted
ROS: Reactive oxygen species
SI: Stimulation index
SMA: Superior mesenteric artery
SEM: Standard error to the mean
SOX2: Sex determining region Y- box2
SPK: Simultaneous pancreas and kidney
SSEA: Stage-specific embryonic antigen
STZ: Streptozotocin
T1DM : Type-1 diabetes mellitus
T2DM : Type-2 diabetes mellitus
Th cells: T helper cells
TIT: Total ischemia time
TNF-α: Tumor necrosis factor alpha
TRA: Tumor rejection antigen

Tregs: Regulatory T cells
UW: University of Wisconsin
VEC: Vascular endothelial cell
VEGF-A: Vascular endothelial growth factor-A

1 Introduction

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REVIEW ARTICLE

Generation of insulin-secreting organoids: a step toward engineering and transplanting the bioartificial pancreas

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Abstract

Diabetes is a major health issue of increasing prevalence. ß-cell replacement, by pancreas or islet transplantation, is the only long-term curative option for patients with insulin-dependent diabetes. Despite good functional results, pancreas transplantation remains a major surgery with potentially severe complications. Islet transplantation is a minimally invasive alternative that can widen the indications in view of its lower morbidity. However, the islet isolation procedure disrupts their vasculature and connection to the surrounding extracellular matrix, exposing them to ischemia and anoikis. Implanted islets are also the target of innate and adaptive immune attacks, thus preventing robust engraftment and prolonged full function. Generation of organoids, defined as functional 3D structures assembled with cell types from different sources, is a strategy increasingly used in regenerative medicine for tissue replacement or repair, in a variety of inflammatory or degenerative disorders. Applied to ß-cell replacement, it offers the possibility to control the size and composition of islet-like structures (pseudo-islets), and to include cells with anti-inflammatory or immunomodulatory properties. In this review, we will present approaches to generate islet cell organoids and discuss how these strategies can be applied to the generation of a bioartificial pancreas for the treatment of type 1 diabetes.

1.1 The pancreas

1.1.1 Anatomy

The pancreas is an abdominal organ situated in the retroperitoneum, crossing in front of the abdominal aorta between vertebra L1 and L2 and behind the stomach. It measures between 15 and 20 cm long and weights between 75 and 100g (1). Anatomically, it is divided into 4 different regions: the head, the neck, the body and the tail. The head of the pancreas is surrounded by the C-loop of the duodenum and lies on the inferior vena cava. This is the place where the pancreas is in communication with the digestive tube through the pancreatic duct. The neck of the pancreas is situated directly in front of the portal vein and the superior mesenteric artery (SMA). The body and the tail lie on the splenic vein and artery. The tail of the pancreas is ending inside the spleen hilum. Pancreatic blood supply is coming from several branches of the celiac trunk and the SMA. The duodenum and the head of the pancreas are vascularized by the anterior and posterior superior pancreaticoduodenal arteries and the anterior and posterior inferior pancreaticoduodenal arteries that branch from the gastroduodenal artery and the SMA, respectively. The body and the tail of the pancreas are vascularized by multiple branches coming from the splenic artery. Pancreatic venous drainage is portal and follows a similar pattern as the arterial vascularization. The duodenum and the head are drained by the anterior and posterior pancreaticoduodenal venous arcades. The superior anterior and posterior arcades drain in the portal vein and the inferior anterior and superior arcades drain in the superior mesenteric vein. The body and the tail drain in the splenic vein (Figure 1).

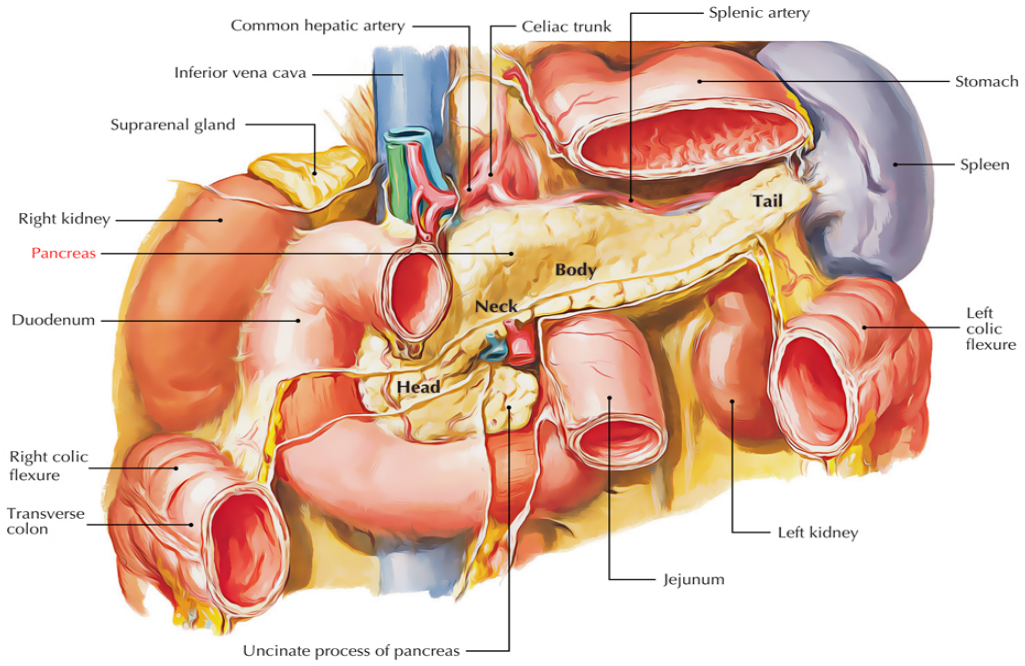

Figure 1. Anatomy of the pancreas. https://www.earthslab.com/anatomy/pancreas/.

Pancreatic parenchyma consists of 85% exocrine tissue, about 10% of extracellular matrix, vessels and ducts and by less than 5% of endocrine tissue, composed of islets of Langerhans (1). The exocrine tissue is composed of pyramidal-shaped, acinar cells organized in acini around tiny ducts. Acinar cells secrete enzymes responsible for food digestion and, in conjunction with ductal cells which secrete water and electrolytes, produce the pancreatic juice, released into the duodenum. The pancreas contains two excretion canals, the main pancreatic duct, known as the duct of Wirsung, which run across the whole pancreas in order to collect acini's secretion, and the accessory pancreatic duct, known as the duct of Santorini. In 60% of cases, both ducts fuse together and most of the pancreatic juice is excreted by the main duct through the greater papilla (composed of the ampulla of Vater and Oddi's sphincter). In 30% of the cases, the accessory duct ends up with a blind end and in 10% of cases, most of the pancreatic juice is excreted through the minor papilla (1) (Figure 2).

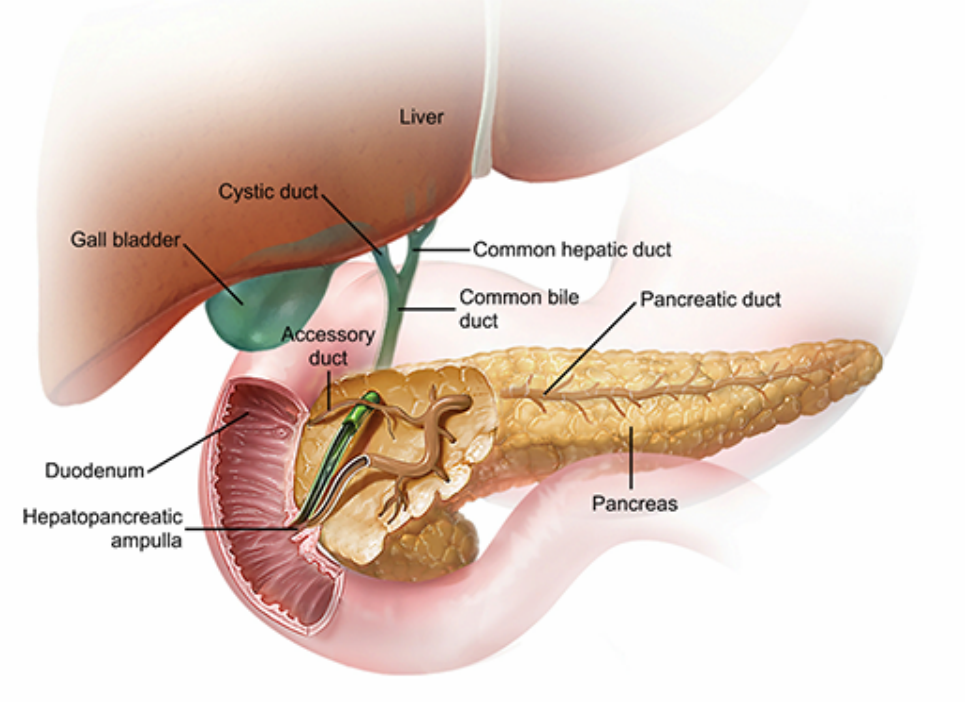

Figure 2. Pancreatic duct anatomy. https://www.visiblebody.com/

1.1.2 Function

1.1.2.1 Exocrine

The exocrine function of the pancreas is to digest the ingested nutrients. The primary digestion in the stomach will result in the release of peptides that will stimulate intestinal endocrine cells, which will induce pancreatic enzyme secretion through hormonal pathways. Acinar cells produce and release the following enzymes: amylase, lipase and protease for carbohydrate, fat and protein digestion, respectively. All acinar cells have the capacity to secrete all three enzymes but the concentration of each secreted enzyme can vary, depending on the type of ingested food. Despite amylase, which is the only enzyme secreted in its active form, the other enzymes are secreted as pro-enzymes in order to avoid pancreatic digestion and need to be activated once arrived in the intestinal lumen. Trypsinogen is present in the pancreatic fluid and is converted to its active form, trypsin, by the enterokinase present in the duodenum. Trypsin will then activate the different enzymes secreted by the pancreas. In order to avoid trypsinogen activation inside the pancreatic duct, inhibitors are secreted by acinar cells.

The pancreas is often visualized as two functionally separated organ with the exocrine and the endocrine function. In reality, they are in communication and influence each other.

Glucagon, pancreatic polypeptide (PP) and somatostatin are hormones secreted by islet cells that inhibit enzyme secretion by acinar cells. In addition, an insulino-acinar portal blood flow has been described, where islets are connected, through multiple venules, to acinar capillaries (2). Endocrine and exocrine pancreas work in concert and adapt the rate of digestion and the distribution of the digested nutrients. This is illustrated by the fact that patients can live without a pancreas by taking exogenous insulin and digestion enzymes replacement therapy. However, blood glucose control and digestion will not be normal. Another example of this crucial interaction is the appearance of endocrine dysfunction in patients with chronic pancreatitis (CP). It has been reported that 80% of patients with CP will develop diabetes, as a result of a multifactorial process of chronic inflammation, malabsorption leading to dysregulated incretin axis, surgical intervention and toxicity of anti-diabetic drugs (3).

1.1.2.2 Endocrine

The endocrine pancreas represents less than 5% of the pancreas and is composed of islets of Langerhans. Their main role is to control blood glucose level by secreting hormones in the bloodstream.

1.1.2.2.1 The islet of Langerhans: a connected object

1.1.2.2.1.1 Islet architecture

Islets are endocrine cell aggregates with a mean diameter of 100-150 µm (4-6). In humans, an islet equivalent (IEQ , defined as a standardized islet with a 150 µm diameter), contains approximately 1500 cells (7) and is composed of 60% insulin-secreting cells (ß cells) and 30% glucagon-secreting cells (α cells) (8, 9). The remaining 10% is composed of somatostatin-secreting cells (δ cells), pancreatic polypeptide-secreting cells (γ or PP cells) and ghrelin-secreting cells (ε cells) (8, 9). In addition to endocrine cells, islets contain stromal cells, macrophages, neuronal elements, endothelial cells (ECs) and pericytes, altogether representing less than 5% (7) (Figure 3). This indicates that more than a simple cell aggregate, the islet is a functional mini-organ with its own innervation (10) and complex intercellular communications (11). In order to exert their

endocrine functions, islet cells have to receive and process signals coming from the bloodstream and/or interstitial space such as nutrients, hormones, and neurotransmitters but also inputs from their innervation. Cell to cell contacts are therefore crucial for hormone release. In addition to autocrine, paracrine and endocrine pathways, cells communicate via inter-cellular connections using cell adhesion molecules (cadherins), gap junctions and ephrin receptors and ligands (12, 13). Cell adhesion molecules are important in the development of islet architecture and function. For example, lack of neural cell adhesion molecule (N-CAM) impairs islet cell organization and insulin secretion (14) and cadherin-mediated adhesion of ß cells promotes their function (15). Signals transmitted by E-cadherin play an important role in islet development, ß cell aggregation, viability and function (15-17). Gap junctions between ß cells allow to share small metabolites and cytoplasmic ions, such as calcium, which is essential for synchronized insulin release in response to glucose stimulation (18).

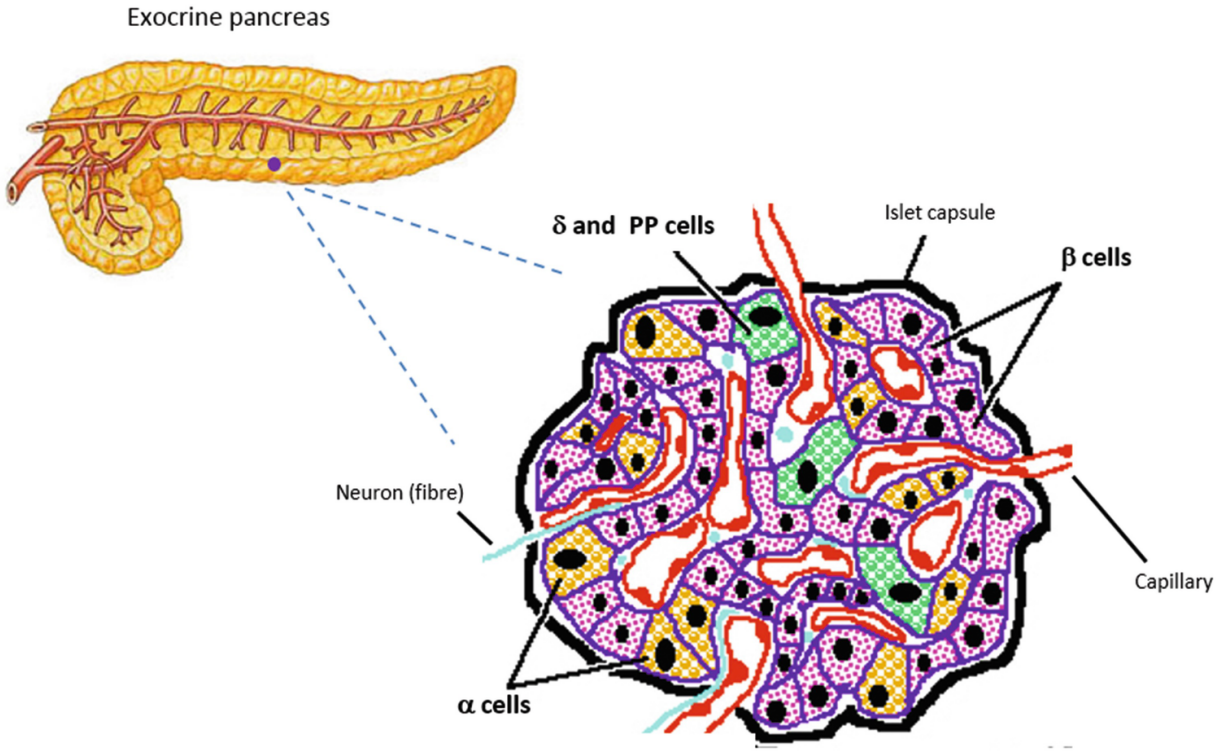

Figure 3. The islet of Langerhans (19).

1.1.2.2.1.2 Islet vascularization

In addition to cell-to-cell contacts, islet cell connections with their environment are also of great importance. Islets are well vascularized mini-organs, receiving 10 to 15% of the total pancreatic blood flow, with a vessel density five times greater than the exocrine part of the gland (20). Each islet is vascularized by one or several arterioles, depending on islet size, that branches into a highly developed fenestrated capillary network with a glomerular-like structure (Figure 4). This allows a rapid response of endocrine cells to achieve optimal control of blood glucose levels (21). Islet blood flow is tightly regulated and can largely variate, independently from the rest of the exocrine blood flow (22). Those variations are the result of afferent arteriole diameter modifications in response to neuronal (autonomous system), hormonal (somatostatin, PP, glucagon, glucagon-liked peptide, cholecystokinin), mediators (NO and ATP) and nutrient stimulations. It has been demonstrated that an overload of glucose increase the islet blood flow through parasympathetic innervation (23). In addition, hypoglycemia also increase islet blood flow, probably to facilitate glucagon delivery into the bloodstream (24).

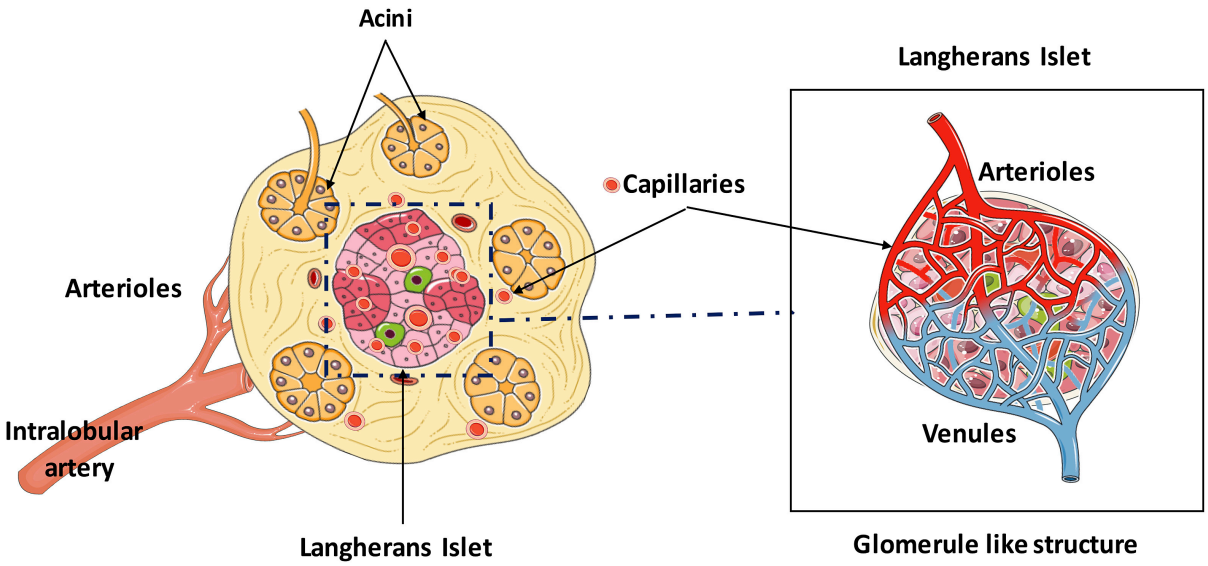

Figure 4. Islet vascularization (23).

1.1.2.2.1.3 Islet micro-environment

Endothelial and islet cell communications have mutual effects. Secretion of vascular endothelial growth factor (VEGF-A) and angiopoietin-1 (Ang-1) by islet cells promotes the development of a functional fenestrated capillary network (25). On the other hand, release of growth factors, such as hepatocyte growth factor (HGF), by ECs, stimulates insulin biosynthesis and secretion (26). In addition to their essential role in angiogenesis, intra-islet ECs synthetize ECM components, necessary for ß cell proliferation, differentiation, function and survival (27, 28). Islets are separated from the exocrine part of the pancreas by a peripheral capsule composed of fibroblasts and collagen fibers, entrapped between two basement membranes (BM) located beneath the exocrine and endocrine epithelium (peri-islet) (12). The peri-islet invaginates into islets along vascular channels to form a perivascular BM. Major components of the intra-islet perivascular BM are laminins, collagen IV and fibronectin (27). The importance of ß cell-ECM interaction has been intensively studied. The lack of vascular BM significantly impairs ß cell proliferation and insulin gene expression. Collagen IV binding to its receptor, the α1ß1 integrin, on ß cells not only augments insulin secretion (29), but also contributes to ß cell differentiation and survival (30). Signals transmitted through the α6ß1 integrin also play a major role in the regulation of ß cell survival (31). Laminin-332 is expressed in human islets and its interaction with the integrin ß1 sub-unit was shown to be essential for normal ß cell function *in vitro* (32, 33)**.** In addition, the vascular BM modulates cell behavior by acting as a source of growth factors and by trapping cytokines and others soluble signal molecules, necessary for maintaining ß cell phenotype and proliferation (34). The peri-islet BM is mainly composed of laminin and collagen IV and, to a lesser extent, of fibronectin, collagen I, III, V and VI (35, 36). Apart from functional support, the peri-islet BM is essential for regulation of ß cell survival as suggested by the improved viability and *in vitro* function of incompletely isolated “mantled islets” (37, 38). Of note, the isolation process not only disconnects islets from their peripheral BM, but also disrupts the intra-islet BM by the loss of intra-islet EC after isolation (39, 40). Altogether, isolated islets are subjected to anoikis, an integrin-mediated death signal resulting from the disruption of interaction between integrins and ECM proteins. This phenomenon is responsible for significant islet cell death in culture (41).

1.1.2.2.2 Blood glucose control

As mentioned above, islets are composed of 5 types of endocrine cells that participate in glucose regulation. The most abundant type of cells is the ß cells which synthetize and secrete insulin in response to an elevation of blood glucose level (hyperglycemia), after a meal for example. Blood circulating insulin will meet his receptor on target tissues and will induce the uptake of blood glucose by the liver, the muscle and the adipose tissue. The result will be a reduction of the glycemia to a normal level. The α cells secrete the glucose-mobilizing hormone glucagon, a counter-regulating hormone. A drop in blood glucose level (hypoglycemia), after an exercise or a fasting-period, will stimulate the secretion of glucagon by the α cells. Circulating glucagon will increase glucose concentration in the blood by inducing lipolysis, glycogenolysis, gluconeogenesis and by the inhibition of glycolysis and glycogenesis (42). The δ cells are responsible for the synthesis and the secretion of somatostatin, a hormone involves mainly in the inhibition of exocrine pancreatic secretion and endocrine hormone secretion such as insulin and glucagon. The γ cells secrete the pancreatic polypeptide which play a role in food intake, especially by reducing energy demands. It also reduces pancreatic exocrine secretion, insulin secretion, delays gastric emptying in order to slow down the digestion (43). On the contrary, ε cells secrete the ghrelin hormone, which stimulates the appetite and growth hormone release by the hypophysis and increases fat storage (44). Together, the ß and α cells are the key regulators of blood glucose control and are both implicated in the pathogenesis of the diabetes mellitus (DM).

1.2 Diabetes mellitus

1.2.1 Epidemiology

DM represents a major health issue with a prevalence of 463 million of adult people worldwide in 2019 and an expected prevalence of 578 million in 2030 (45). It was responsible for 1.6 million deaths in 2016 and 10% (760 billion USD) of global heath expenditure is used for DM (45, 46). With a number of adults affected that has tripled in last 20 years, DM represents one of the most challenging health issues (Figure 5). This impressive increase is mainly the result of the rising number of type-2 diabetes mellitus (T2DM), caused by the high prevalence worldwide of metabolic disorders and obesity, reflecting the lifestyle of our century.

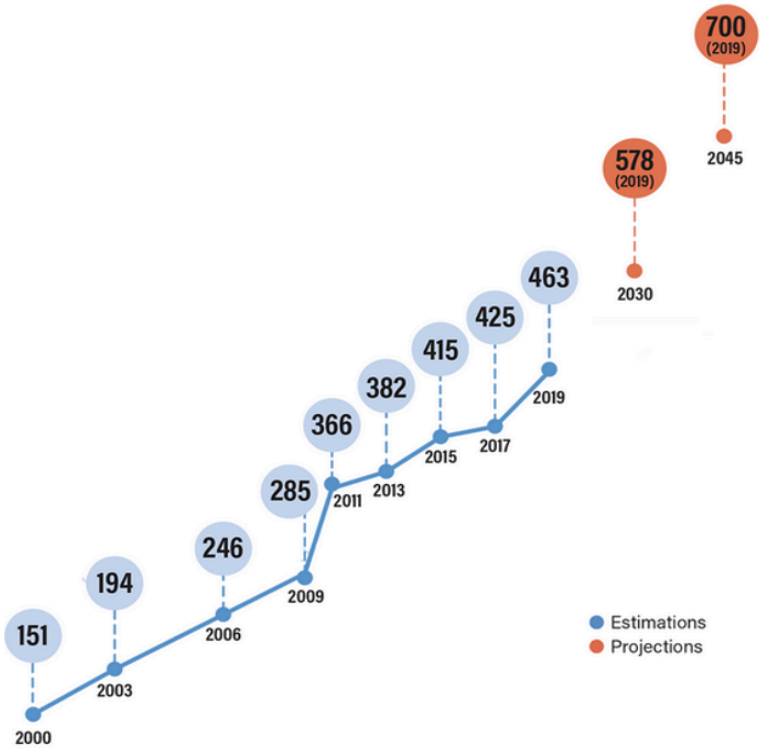

Figure 5. Estimated number of adults with DM (in million). Source: *IDF Diabetes Atlas* from 1st to 9th editions (45).

1.2.2 Definition

DM is characterized by a loss or an impairment in blood glucose control, resulting in a hyperglycemia state. It is defined by a glycated hemoglobin (HbA1c) ≥ 6.5%, a fasting blood glucose ≥ 7mmol/l (126mg/dl), a non-fasting blood glucose ≥ 11.1mmol/l (200mg/dl) and/or a glucose level 2 hours after an oral glucose tolerance test (OGTT) ≥ 11.1mmol/l (200mg/dl) (47) (Table 1). A pre-diabetes can be identified when HbA1c is comprised between 5.7 and 6.4%, a fasting glucose between 5.6 and 6.9mmol/l (100 and 125mg/dl) and a glucose value between 7.8 and 11mmol/l (140 and 199mg/dl), 2 hours after an OGTT. This intermediate state is more related to T2DM, where a resistance to insulin generally occurs before the loss of the hormone. Other than type-1 diabetes mellitus (T1DM) and T2DM who are the most common, other types of

diabetes have been defined such as the “maturity onset diabetes of the young” (MODY), which is a rare inherited form of diabetes and the gestational diabetes.

Test[a]	Threshold	Qualifier
Hemoglobin A_{1c} or	≥ 6.5%	Lab NGSP-certified, standardized DCCT assay
Fasting glucose or	≥ 126 mg/dL (7.0 mmol/L)	No caloric intake for at least 8 hours
2-hour glucose or	≥ 200 mg/dL (11.1 mmol/L)	After 75 g of anhydrous glucose
Random glucose	≥ 200 mg/dL (11.1 mmol/L)	Plus classic hyperglycemia symptoms or crisis

NGSP, National Glycohemoglobin Standardization Program; DCCT, Diabetes Control and Complications Trial.

[a] Results must be confirmed by repeated testing.

Table 1. Diabetes diagnostic criteria from the American Diabetes Association (47)

1.2.2.1 Type-1 diabetes mellitus

T1DM represents 10% of all cases of DM. Its onset occurs in 75% of the cases during childhood, with two identified peaks, between 4 and 6 years-old and in early puberty, between 10 and 13 years-old (48). A geographical distribution has been reported with a higher incidence in northern European (49). It is an auto-immune disorder characterized by the destruction of the ß cells in the islet of Langerhans, which leads to the lack of endogenous production and secretion of insulin (50). T1DM is the result of a complex, multifactorial process composed of genetic predispositions and environmental factors (51, 52). Major histocompatibility complex (MHC) class II genotype is recognized to be one of the most important factors to determine T1DM susceptibility, especially HLA DR3-Q2 or HLA DR4-Q8 (53, 54). Genetic predisposition plays an important role in the development of islet cell auto-immunity, as it has been demonstrated by an increased prevalence in patients with family history, especially in identical twin studies (55). However, it isn’t enough for T1DM development. Environmental factors are also involved, even though their participation isn’t fully understood. Virus infection, especially by enterovirus, during pregnancy and early

childhood has been associated with T1DM (56, 57). It was observed that 39% of newly diagnosed T1DM children had positive Coxsackie B virus-specific immunoglobulin M (IgM) in comparison to 6% in healthy children (58). This could be explained by the fact that this virus infects human ß cells *in vitro* and has been described as the most cytolytic enterovirus in islet infection (59). It could also trigger auto-immunity by molecular mimicry. Other viruses have also been implicated with T1DM such as cytomegalovirus (CMV), rubella, mumps and rotavirus (60). Several others environmental factors such as air pollution, vitamin D and gluten ingestion and gut microbiome have been studied and speculated to be implicated in T1DM pathogenesis. Cow's milk ingestion also has been described as a potential inducer of ß cell autoimmunity, whereas breastfeeding has demonstrated protective effect (61, 62). Altogether, T1DM auto-immunity seems to develop on a genetically predisposed background with an environmental trigger, early in life.

As a consequence, pancreatic islets are infiltrated by macrophages and lymphocytes which results in insulitis (51). The first autoantibodies can already be detectable at 2 years-old and are against insulin and glutamic acid decarboxylase (GAD65). Others autoantibodies directed against tyrosine phosphatase IA2, and zinc transporter ZnT8 appear later (51, 52). Reaching a certain percentage of ß cell loss (generally 80-90%), hyperglycemia occurs and T1DM is diagnosed (63).

1.2.2.2 Type-2 diabetes mellitus

T2DM is the most frequent form of DM, accounting for 90% of the prevalence. Unlike T1DM, T2DM pathogenesis is characterized by a progressive peripheral insulin resistance, a decrease in insulin sensitivity and ß cell function, and an impaired glucose regulation of hepatic glucose production, which finally results in ß cell failure (64). Initially, T2DM onset occurred in patients over 40 years of age. However, with the rising prevalence of obesity in children and young adults, T2DM is now observed in younger patients. Environmental risk factors of T2DM are the one responsible for the metabolic syndrome (abdominal obesity, high blood pressure, hypertriglyceridemia, low plasma level of high-density lipoprotein (HDL)), age, male gender, physical inactivity, alcohol and tobacco abuse, increase in fat and glucose intake and low education. Gut microbiome has also been described as a risk factor for T2DM development, especially the bacteria Prevotella copri and Bacteroides vulgatus (65). Finally, genetic

predispositions participated also to the pathogenesis of T2DM. Unlike in T1DM, predisposition don't involve HLA genes and has a greater heritability as demonstrated by identical twin studies, where both siblings were affected in >70% of the cases (66, 67). T2DM is a progressive disease that starts with peripheral insulin resistance, mostly induced by obesity. In addition, glucose uptake in adipose tissue and striated muscle is reduced and glucose metabolism in the liver is impaired, resulting in an increased glucose output. In order to overcome this, ß cells increase their insulin secretion resulting in hyperinsulinemia, maintaining a normal glucose tolerance and a normal glycemia. Progressively, an exhaustion of ß cells will occur and an impaired glucose tolerance will appear. At this point, patients are in a prediabetic state and a drastic lifestyle modification can still allow to return to a normal glucose tolerance. Otherwise, ß cell failure will appear, resulting in hyperglycemia and diabetes.

1.2.2.3 Complications

Complications related to diabetes are numerous and can be separated in acute and chronic complications.

1.2.2.3.1 Acute complications

Acute clinical symptoms resulting from hyperglycemia are the classic triad polydipsia, polyuria and polyphagia, often associated with loss of weight. In the absence of insulin, glucose is blocked in the bloodstream resulting in hyperglycemia. As a consequence, an alternative source of energy is needed for cell metabolism and is performed by free fatty acid catabolism in the liver with ketone bodies release. This will result in diabetic ketoacidosis. It occurs mostly in T1DM, accounting for 30% of initial T1DM presentation, and rarely in T2DM, because a presence of endogenous insulin is still present in most T2DM cases. Diabetic ketoacidosis is defined by hyperglycemia > 13.9mmol/l (2.5g/l), ketonemia/ketonuria, and metabolic acidosis. Patients usually present vomiting, abdominal pain, deep respiratory movements (Kussmaul respiration), polyuria, confusion and, if left untreated, can lead to coma and death. Administration of insulin and respiratory assistance is usually necessary to treat those patients.

Because endogenous insulin is still present in T2DM, patients don't usually experience ketoacidosis but can develop a hyperosmolar hyperglycemic syndrome, defined by hyperglycemia

>33mmol/l (>600mg/dl) and plasma hyperosmolarity (resulting from dehydration and hyperglycemia). This condition generally appears over several days and patients often present lethargy and confusion.

Diabetic patients, especially with T1DM are at risks of hypoglycemia episodes. Hypoglycemia is defined by a blood glucose level < 4mmol/l (0.7g/l) and occurs when there is an imbalance between food intake and exogenous insulin injection, for example when patients skip a meal, after physical activity or in case of insulin overdosage. Classical signs of hypoglycemia are sweating, shakiness, dizziness, hunger and fatigue. Most patients experience mild hypoglycemia and are able to correct it by themselves with glucose ingestion. However, hypoglycemia can be severe if prolonged and convulsions, loss of consciousness and in rare cases, death can occur. This especially happen in patients who are unaware of the onset of hypoglycemia as seen in an instable type of diabetes called “brittle diabetes”.

1.2.2.3.2 Chronic complications

Diabetic patients are usually treated with insulin injection for T1DM and with oral anti-diabetic medications and/or insulin injection for T2DM in order to maintain glycemia in a normal range. However, despite all improvements in diabetic therapies, physiological insulin release cannot be achieved and patients experience daily hyperglycemia episodes of different magnitudes, mainly depending on patient’s compliance. Repetitive hyperglycemic episodes on the long term will induce macrovascular and microvascular lesions.

1.2.2.3.2.1 Microvascular complications

Microvascular injuries are specific to diabetes. Capillary basement membrane thickening and increased permeability represent the structural hallmark of this pathology and are the results of four main factors (68):

1. Protein glycation with the formation of advanced glycation end-products (AGE). AGE can accumulate in the extracellular space inducing extra-cellular matrix (ECM) protein degradation, resulting into glomerulosclerosis and interstitial fibrosis in the kidney for example. In addition, it can penetrate cells and induce reactive oxygen species (ROS)

production or interact with its receptor (RAGE) and activate NF-κB transcription factor, generating pro-inflammatory cytokines.

2. Activation of the polyol pathway: glucose uptake by cells from retina, kidney and nervous tissue is insulin-independent. In case of hyperglycemia, the increase in glucose activates the polyol pathway, resulting in the conversion of glucose into fructose and sorbitol, which accumulate in the cell and increase ROS concentration.
3. Oxidative stress by ROS production from the polyol pathway, by the non-enzymatic glycosylation reaction, by the mitochondrial electron transport chain and by the membrane bound NADPH oxidase.
4. Activation of protein kinase C by hyperglycemia which results in (i) a decreased production of endothelial nitric oxide synthase (eNOS) and anti-oxidative molecule and (ii) an increase in VEGF secretion which can induce proliferative retinopathy.

Those microvascular injuries are responsible for 3 specific diabetes complications:

1. Nephropathy: which is one of the leading causes of end stage renal failure worldwide. It is a progressive process, starting with an increased glomerular pressure leading to a glomerular hyperfiltration. This will induce the development of fibrosis and glomerulosclerosis resulting in a reduced filtration and kidney failure.
2. Neuropathy: affecting the autonomic nervous system and the peripheral nerves, resulting in dysautonomia and hyposensitivity, especially in the lower limbs.
3. Retinopathy: represents the leading cause of blindness in developed countries and results from the damage of the small vessel of the retina. It is characterized by the loss of pericytes in retinal capillary leading to increased permeability, micro-thrombosis, new vessel formation and macula edema. Visual impairment and blindness result from retinal detachment secondary to fibrovascular proliferation, new vessel hemorrhages and neovascular glaucoma.

1.2.2.3.2.2 Macrovascular complications

Chronic hyperglycemia induces macrovascular lesions, mainly in the brain, the heart and in peripheral arteries, known as atherosclerosis. It results from the accumulation of lipid-containing macrophages (foam cells) in the intima. Over the time, accumulation of inflammatory and smooth

muscle cells, in addition to neo-vascularization leads to the development of fibrous plaques that will cause arterial stenosis. The consequences of those plaques will be (i) a hypoperfusion in the inferior limbs resulting in claudication, ulcer and amputation, (ii) ischemic cardiomyopathy, (iii) cerebral stroke and (iv) increased blood pressure secondary to the activation of the renin-angiotensin aldosterone system, which will deteriorate furthermore the renal function.

1.3 Diabetes treatment for T1DM

1.3.1 Insulin-therapy

For a long time, T1DM was a fatal disease, without any cure. However, in 1921, the Canadian doctors Frederick Banting and Charles Best discovered and isolated insulin, establishing a new era in the treatment of diabetes. The first diabetic patient was injected in 1922 and marked the beginning of insulin-therapy. During the next half century, injections were made with bovine or porcine pancreas extract with low purity and causing immune reactions. It is only in the early 1980s, that synthetic human insulin appeared with the discovery of DNA recombinant technology.

Type 1 diabetic patients require daily exogenous insulin, either by multiple injections or by continuous perfusion with the latest insulin pumps (69). However, perfect glycemic control cannot be achieved, even with intensive insulin treatment. Furthermore, the amount of exogenous insulin required by some patients puts them at risk of hypoglycemia, which can result in convulsions, coma and even death (51, 70). Insulin-therapy has significantly increased life expectancy and quality of life of patients. However, as a consequence, it has also unveiled another major issue, that is the long-term complications of diabetes, as previously mentioned. The development and progression of this complications are directly linked to glycemia control as it has been demonstrated that intensive insulin-therapy with optimal glycemia level significantly delays and reduces diabetes-related complications (70). Although a majority of patients respond well to insulin-therapy, a small percentage presents very unstable diabetes, such as brittle diabetes, with rapid progression of complications. For those patients, ß cell replacement therapies represent a valuable alternative, either with pancreatic or islet transplantation.

1.3.2 Beta cell replacement therapy

By replacing the lost ß cell mass, those therapies allow to restore a normal function in term of glucose homeostasis. It is superior to insulin-therapy in a way that physiological insulin secretion is present again and patients experience very few or no hypoglycemia episodes after transplantation (71-73). However, this beneficial effect has a heavy cost, as patients need lifelong immunosuppressive treatments, in order to avoid graft rejection and are at risk from surgical complications. In addition, pancreatic and islet transplantations are dependent on organs availability.

1.3.2.1 Pancreas transplantation

The first pancreatic transplantation was performed in 1966 in Minneapolis (74). Over the last 54 years, many progresses have been done in order to improve the surgical technique as well as immunosuppressive regimen, especially with the arrival of the ciclosporin in the 80s, followed by the introduction of tacrolimus and mycophenolate mofetil. This allowed to achieve an insulino-independence in 70% and an overall survival rate of 80% in T1DM patients, at 5 years (75). Pancreatic transplantation can be performed alone (PTA), after a kidney transplantation (PAK) or simultaneously with a kidney (SPK). Decision regarding which type of transplantation should be chosen is summarized in Figure 6.

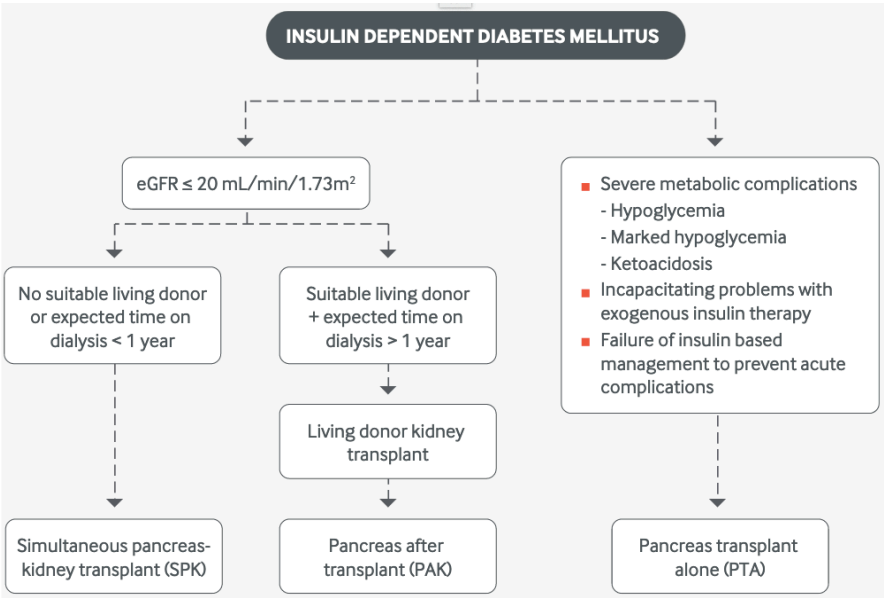

Figure 6. Algorithm for type of pancreas transplantation for T1DM patients (76).

Basically, if a patient has poor metabolic control but no renal failure, a PTA is indicated. In case of end-stage renal failure, a PAK or SPK is indicated depending of the availability of a living kidney donor and the time expected on dialysis. It has been reported that diabetic patients have a mortality rate of > 60% at five years on dialysis (77, 78). PAK is realized in order to minimize the time on dialysis, especially when waiting time for SPK may be long. SPK is the most performed transplantation and has the advantage, unlike PAK, that both organs come from the same donor. A large study recently published by a Dutch group reported a reduction of overall mortality in SPK patients of 44% and 31% at 10 and 20 years after transplantation, respectively, in comparison to kidney transplantation (79). This indicate that restoration of glycemic control by endogenous insulin has more beneficial effect than insulin-therapy, especially on kidney graft function. In the past, SPK has demonstrated better results than PTA and PAK in term of graft survival. The superiority of SPK isn't clearly understood but possible explanations have been proposed. First, early diagnosis of rejection using the kidney as a surrogate for control biopsies. Even though a discordance between kidney and pancreas rejection is seen in 21 to 33% of the time, renal biopsy is of great help in the treatment of pancreatic rejection (80). Knowing that 90% of pancreas rejection episodes, in patients with normoglycemia, are reversible makes understand how important early detection is important (81). Secondly, a higher immunocompetence in non-uremic patients could explained an inferior graft survival (82). Thirdly, an immunological effect of transplanting two organs at the same time has been proposed, as it has been observed that donor specific antibody was more strongly associated with graft failure in PTA rather than SPK (83). However, modifications in immunosuppressive regimen and usage of living kidney donor have improved PAK results over the last decade, making this transplantation modality, a valuable option (84, 85). The advantages offered by pancreas transplantation such as long-term survival and reduction in the progression of diabetes-related complications are indisputable (86-88). However, it still remains a major surgery with peri- and post-operative complications (massive bleeding, acute thrombosis, allo-rejection, pancreatitis) that can lead to early graft loss and patient deaths (5, 89). Therefore, pancreatic transplantation cannot be proposed to every diabetic patient, especially as they often present cardiovascular diseases, secondary to diabetes

complications. For those patients, islet transplantation is a good alternative, being minimally invasive.

1.3.2.2 Islet transplantation

1.3.2.2.1 Background

Islet transplantation (IT) is a minimally invasive method, where isolated islets are infused into the portal vein by interventional radiology. This technique presents minor risks, such as subcapsular hematoma of the liver, thrombosis and intraperitoneal bleeding, with a prevalence of less than 10% (72, 90). Islets are isolated from pancreases obtain from deceased-donors. Once procured, the pancreas is maintained in cold preservation solution until its arrival in the isolation laboratory. The isolation process consists of 4 main steps: (i) the enzyme perfusion, (ii) the digestion, (iii) the purification, (iv) the count of the islet and their culture as described by Ricordi et al. (91). The pancreas is initially dissected in the laboratory in order to remove the excessive tissue and fat. The main pancreatic duct is then cannulated for enzyme perfusion. The enzyme used, especially in our laboratory, is the Collagenase NB1, which will be perfused at 4°C for 10 minutes with controlled pressures. The perfused pancreas is then placed in a Ricordi chamber, heated at 37°C, containing metal beads, resulting in a mechanical and enzymatic digestion of the tissue. The digested tissue is then harvest and prepared for purification. Separation of the digested exocrine tissue from the islets is performed by centrifugation using a continuous gradient. Once the purification process is done, islet purity is evaluated and the final count of the isolated islet is performed either manually or by automated techniques (i.e. IsletNet; https://isletnet.com/). Finally, islets are placed in culture until transplantation or can be used directly depending on the transplantation center protocol. The day of the transplantation, islets are recovered from culture and conditioned in a transplantation bag. The transplantation procedure is performed by interventional radiology, where the portal vein of the recipient is catheterized. The islets are then connected with the portal catheter and infused by gravitation trough the portal vein, into the liver, where they will stay and secrete insulin (Figure 7).

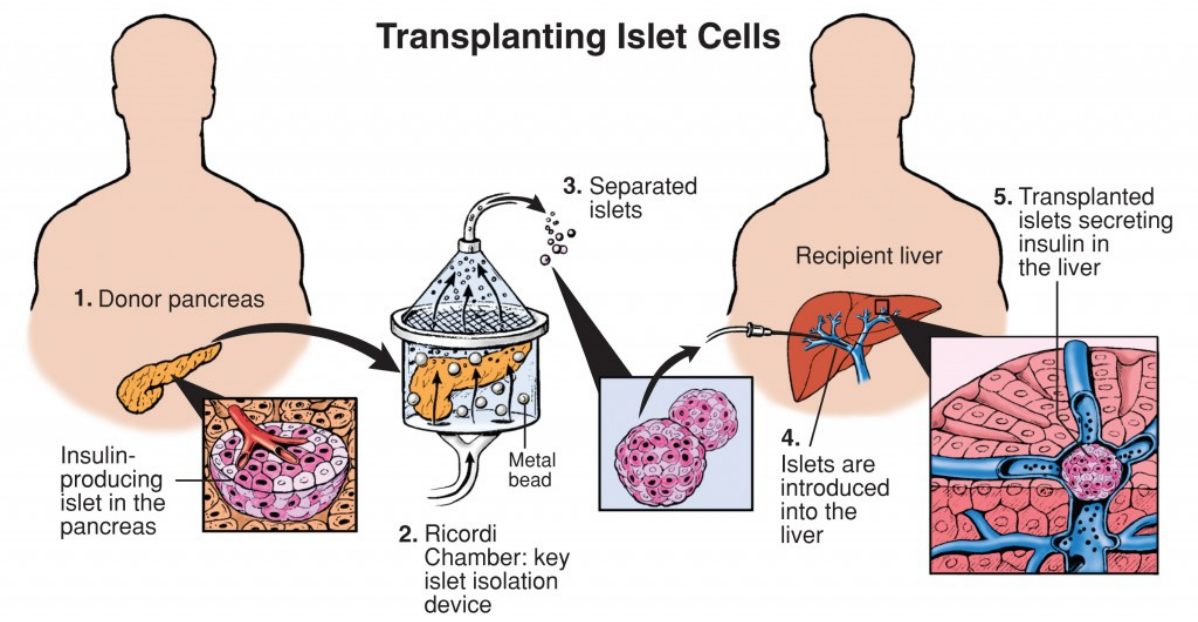

Figure 7. Islet isolation and transplantation process. This figure summarizes the big steps of islet isolation and transplantation into the liver. Source: Diabetes Research Institute, University of Miami, by Robert Margulies, 2005.

The first IT has been realized in Minneapolis, in 1974 (92). After more than two decades of poor results, a turning point was achieved with the publication of the Edmonton protocol in 2000, which successfully increased the 1-year insulin-independence rate from 10% to 80% (93). The main changes brought by this new protocol were: (i) a glucocorticoid-free immunosuppressive regimen combining sirolimus, tacrolimus and daclizumab, (ii) a larger transplanted islet mass (>4000IEQ/kg of recipient) harvested from several donors, and (iii) the application of good manufacturing practices in the islet production procedures. Since then, a lot of improvements have been done in the isolation procedure, the enzyme performance, the selection of donors and immunosuppressive protocols allowing to increase the insulino-independence to 50% at 5 years (94). The actual immunosuppressive regimen used for IT is composed of: (i) an induction treatment with anti-thymocyte globulin, for the first islet infusion, and an anti-IL2 (basiliximab) for the second or third injection and (ii) a maintenance treatment with a combination of mycophenolate mofetil and calcineurin inhibitor (tacrolimus).

1.3.2.2.2 Limitations of islet transplantation

Despite those improving outcomes, patients still require multiple islet infusions in order to achieve results that approach those from whole pancreas transplantation. This means that among all transplanted islets, only a fraction successfully engrafts while the rest is destroyed during and shortly after transplantation. In addition, it is most likely that the small number of engrafted islets cannot respond to patient metabolic demands on the long run, and will face exhaustion. The reason behind this poor islet engraftment is that IT, as a cellular transplantation, has to face several obstacles.

First, in contrast to whole organ transplantation, where donor and recipient vessels are connected at the time of the transplantation, islets lose their intrinsic dense vascularization during the isolation process and the remaining endothelial cells present within the islets, die in culture. Furthermore, once infused in the portal vein, into the liver, islet revascularization takes weeks (95). During that time, islets obtain nutriments and oxygen only by diffusion, which put large islets (>100 µm) at risk of ischemia, as it has been demonstrated by the apparition of a necrotic core in culture (96-98). In addition, islets act as emboli inside the liver which can result in hepatocyte necrosis and inflammation, further reducing the oxygen supply (99).

In addition to the disruption of their vascular bed, islets are disconnected from their ECM, which normally offer them mechanical protection and functional support. It has been demonstrated, that ECM-islet interactions are essential to islet function and survival (100). In fact, ECM proteins impact ß cell differentiation, proliferation, specific gene expression, survival and insulin secretion by modulating signaling pathways, among other things (101). Furthermore, isolated islets experienced anoikis, when remove from their ECM, which results in the death of many islet cells in culture (41).

The liver is at the moment the most efficient transplantation site for IT (102). However, many evidences showed that it is not the best suitable site for islet function and survival, especially because of its very pro-inflammatory microenvironment. Although transplanted islets can benefit in the liver from the oxygenation of the portal vein until revascularization from the hepatic artery occurs, their presence in the bloodstream triggers an intense inflammatory reaction known as the instant blood mediated inflammatory reaction (IBMIR) (103). This phenomenon has been well

studied and is described as an activation of the coagulation cascade resulting in thrombi formation and platelets depletion, followed by a large infiltration of inflammatory cells (104). The result is the destruction of 50 to 70% of the islet mass, rapidly after transplantation (105-107).

Once arrived in the liver, islets are exposed to many factors produced and secreted by cells participating in the inflammatory response, such as Kupffer cells, liver sinusoidal endothelial cells, stellate cells, resident lymphocytes and dendritic cells. As mentioned above, by embolizing portal branches, islets induce hepatocytes death which will results in local inflammation as well. In addition, since the liver is a key element of the gut-liver axis, islets are exposed to a large number of antigens and toxic agents coming from the gastro-intestinal drainage (103).

Finally, in addition to the innate immunity, islets are targeted by the adaptive immunity and face allo-rejection but are also vulnerable to a recurrence of the auto-immunity (108).

As a result from all those obstacles, the majority of isolated and transplanted islets don't survive. The limitations of IT are summarized in figure 8.

The need for lifelong immunosuppression in combination with organ shortage are the main reasons why currently, IT fails to cure T1DM patients on a large scale. Many research groups are currently and since many years working in order to improve islet revascularization, viability and function as well as to protect them from the immune system. Among all the different possibilities to achieve this goal, organoid generation is a very interesting and valuable option, by allowing size and composition control but also by offering the capacity to add supporting cells to islet cells.

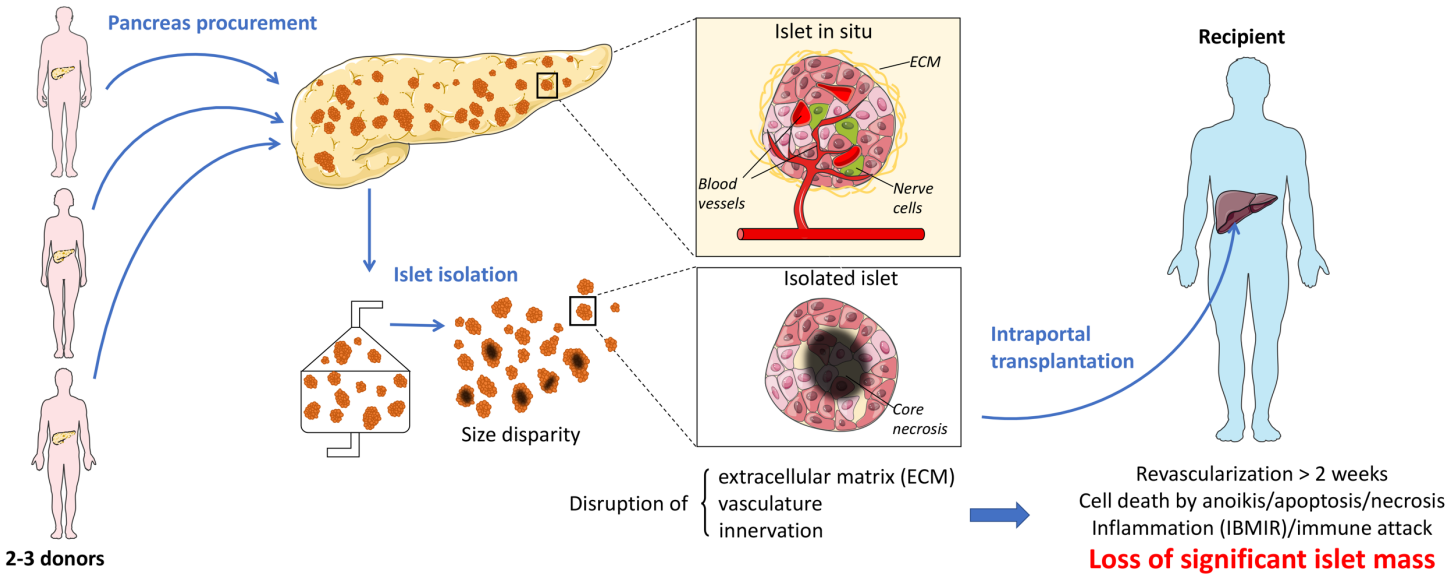

Figure 8. Limitations of clinical islet transplantation. The isolation process is responsible for the loss and disruption of the ECM, vasculature and innervation of the islets. In addition to the inflammatory and immune attacks, this process results in the loss of an important proportion of the islet mass. IBMIR: instant blood mediated inflammatory reaction.

1.3.2.2.3 Organoids: building blocks for bioartificial organ construction

Organoids are defined as 3D cell aggregates designed with the aim to reproduce *in vitro* the morphology and intrinsic function of organs *in vivo*. Organogenesis occurs as a result of programmed cell to cell contacts and close intercellular communications (109). In order to mimic this physiological condition, organoids have been initially generated from human embryonic stem cells (hESC) or adult mesenchymal stem cells (MSCs) and used as building blocks for tissue engineering and assembly into bioartificial organs. Numerous different methods have been developed to generate functional organoids, applying principles of cell self-assembly (110). Most of these approaches can be separated into microfluidic and non-microfluidic techniques. The microfluidic "organ-on-a-chip" method is defined by the application of a continuous, pressure-controlled, perfusion to the cells and has demonstrated good results in terms of cell aggregation and viability (111). However, while this approach represents a valuable system for high-throughput *in vitro* analyses, it is not designed for scaling-up. Non-microfluidic methods include the hanging drop technique (112), cell self-aggregation technique (113) and the use of microwell

culture plates (114). These methods can be adapted for large-scale production of organoids, like for example, the automated hanging drop method (115). The different techniques of organoid generation are summarized in Figure 9.

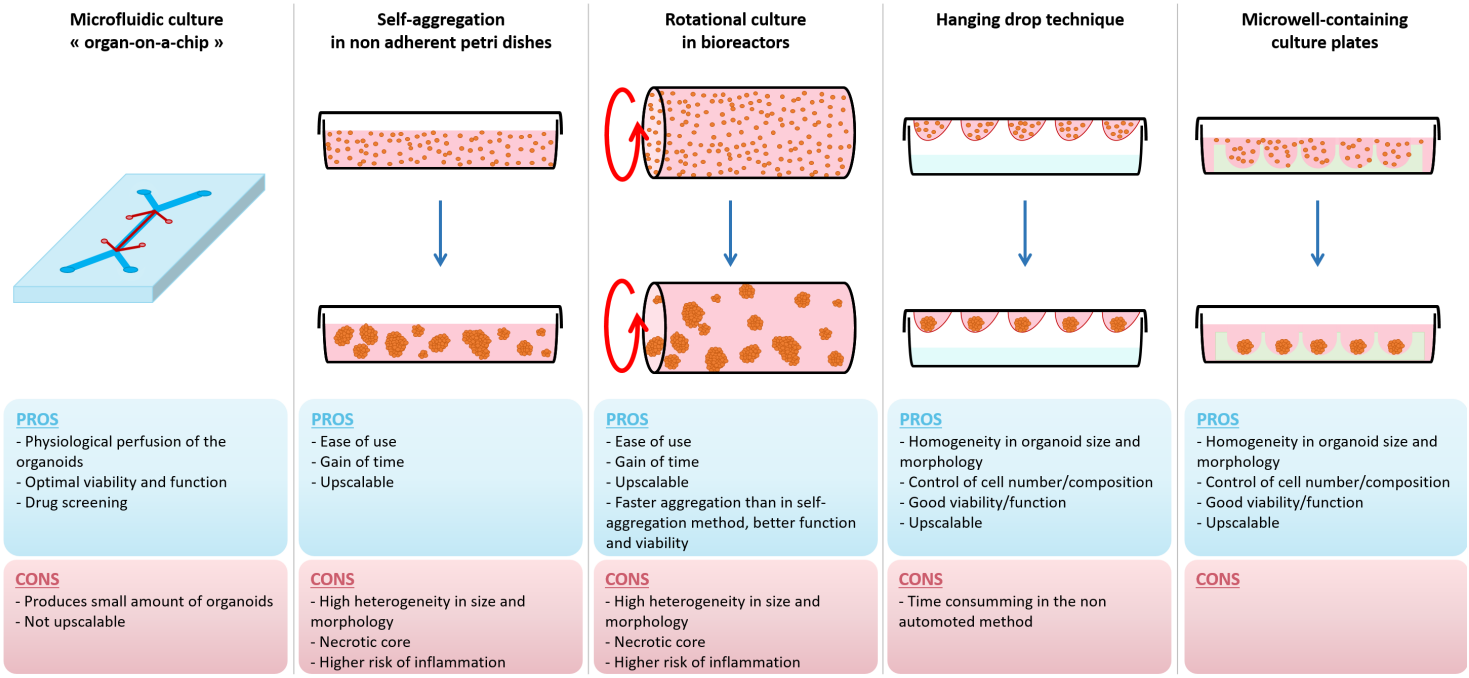

Figure 9. The different methods used for organoid generation. The upper panel of the figure describes graphically the different techniques; the lower panel describes the pros and cons of the different available methods using microfluidic or non-microfluidic techniques.

Over the last decade, the field of organoid science has developed considerably, notably for anti-cancer drug development (116, 117) and in regenerative medicine (118). The regenerative capacities of organoids can be further improved by modulating their cellular composition. Indeed, the combination of multiple cell types into organoids can better reproduce cellular interactions of complex tissues such as the liver, in which the aggregation of hepatocytes, stellate cells and fibroblasts allows to improve viability and function compared to monocellular cultures (119). It was demonstrated in studies where 3D aggregates were created using adipose stem cells (120), tumor cells (121), insulin secreting cells (122), intestinal stem cells and others that organoids

express the hypoxia inducible factor 1-α (HIF1-α) in response to decreased oxygen diffusion to their core, which stimulates secretion of angiogenic and anti-apoptotic factors. Finally, combining ECs or endothelial progenitor cells with other cell types allows the development of tubular and vessel-like structures sprouting within the organoids *in vitro* (123). In addition to ECs, others supporting cells, such as MSCs or other cells expressing anti-inflammatory mediators can also be incorporated into the organoids (124, 125).

1.3.2.2.4 Pseudo-islet: the pancreatic endocrine organoid

As described above, islets of Langerhans are 3D clusters composed of several cell types. Islets can be easily dissociated into single cells and reaggregated. This allows to control their size and cell composition by manipulating cell number and types. Newly generated organoids are commonly named pseudo-islets (PI). In addition to primary dissociated islet cells, other cell sources can be used to generate PI, such as ß cell lines (e.g. MIN6 (126)), hESC (127), pancreatic stem cells (128), induced pluripotent stem cells (iPSC) (129) and other cell types using transdifferentiation such as insulin-secreting cells derived from other endocrine cell types (alpha cells) (130) or liver cells, for instance (131) (Figure 10).

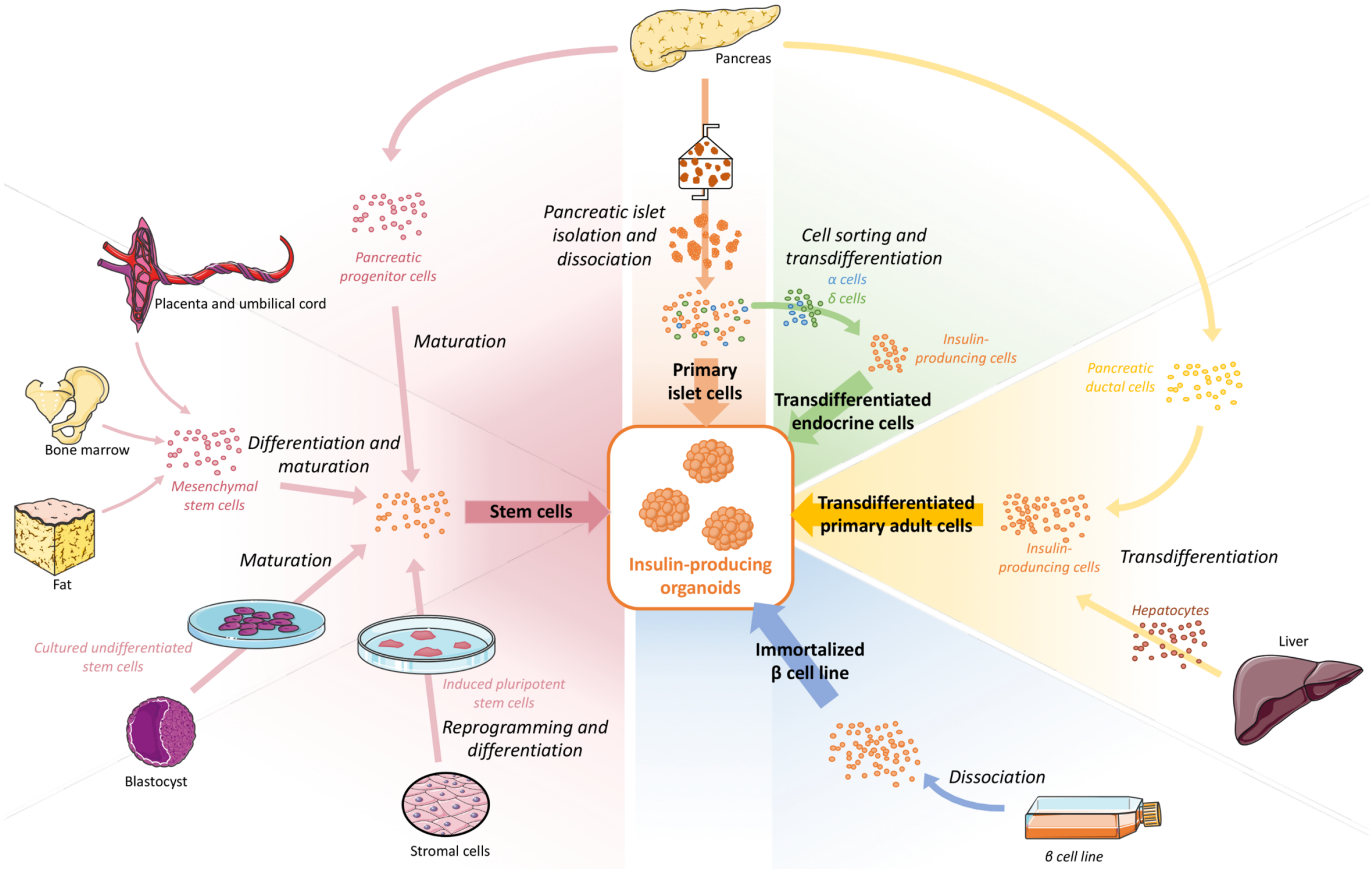

Figure 10. Sources of insulin-secreting cells for organoid generation

PI can be generated by self-aggregation in non-adherent petri dishes (132) or in bioreactors with rotational culture (133). However, these techniques demonstrate a high heterogeneity in term of PI sizes and morphology. Isolated human islets are not uniform in size, usually ranging 50–500 µm in diameter (134). Larger islets are more prone to develop core necrosis after transplantation, until revascularization occurs (96). Moreover, transplantation of large islets through the portal vein can elicit inflammatory reaction due to embolization of larger vessels causing liver damage. To avoid this, large scale generation of homogeneous, size-controlled PI can be achieved by using the hanging-drop method or microwell culture plates. PI have also demonstrated improved viability and function, both *in vitro* and *in vivo,* compared to native islets (98, 135), an observation attributed to the relatively small size of PI. These findings are in line with previous reports on better *in vitro* performance of smaller PI (136). Interestingly, once transplanted, morphology and cellular arrangement of PI changed to display a cell arrangement similar to that of native islets

(137). One step further, PI can serve as elements for bioartificial pancreas construction, implantable in extra-hepatic sites, thus avoiding the proinflammatory microenvironment found within the liver (138-140).

1.3.2.2.5 Validation criteria of newly formed pseudo-islets

Generation of PI can be considered as a novel and valuable strategy for the treatment of T1DM. Therefore, it is extremely important to develop a standardized validation system. In our opinion, PIs should meet at least three important criteria:

1. Morphology: PI should be small (< 150 µm-diameter) and uniform in size and shape. They should also respond to the definition of spheroids in the literature:" three-dimensional, compact, round shaped cell aggregates that do not disassemble easily and that can be easily manipulated" (98, 111, 141, 142).
2. Function: PI should be able to secrete insulin in response to glucose and other secretagogues, regardless of the insulin-secreting cell source. This can be assessed *in vitro* by static or perifusion secretion tests or, at the single PI level, by a reverse hemolytic plaque assay (143).
3. Viability: PI must exhibit and maintain cell viability over prolonged periods of time ("a lifetime"). Viability should be assessed before implantation by standardized assays. Unfortunately, there is currently no method available to measure islet or organoid longevity.
4. Non-tumorigenicity: PI must demonstrate the absence of risk of uncontrolled cell proliferation, especially if gene therapy techniques, or stem-cell-derived cells are used in their construction.

1.3.2.2.6 Improved pseudo-islets: the benefits of adding supporting cells into organoids

As mentioned above, organoid generation offers the possibility to combine several types of cells able to provide supporting functions (Figure 11). Several groups have used this approach and a large variety of cell types have been assessed to this end. For instance, ECs were used to improve islet function and revascularization (144, 145). Adding cholinergic neurons to islet cells

demonstrated an increased islet function and re-innervation *in vitro* (146). Jun et al. co-cultured islet cells with hepatocytes and, interestingly, albumin and insulin secretion were both increased in those hybrid organoids, in comparison to monocellular organoids made of hepatocyte or islet cells, respectively (147).

The inflammatory and immunological response against transplanted islets is detrimental for long-term graft function. Immediately after intraportal infusion of islets, IBMIR occurs, causing the destruction of a significant proportion of the islet mass. Multicellular spheroids combining islet cells with cells expressing anti-inflammatory and/or immunomodulatory factors could be protected from these phenomena. MSCs have been the main cell types used for this purpose (148). Co-culturing them with islets have enhanced revascularization, function and engraftment thanks to their angiogenic properties (149, 150). In addition, MSCs have differentiation capacity, which make them an interesting cell source for tissue regeneration. Over the last decades, MSCs have been used intensively, especially for inflammatory and degenerative disorders. However, MSCs harvesting is an invasive procedure, their numbers and properties decrease with donor's age, and they have a potential for tumorigenicity (151, 152). Human amniotic epithelial cells (hAECs) are an alternative source of cells with similar properties. Their origin, properties and clinical application are detailed in the next chapter.

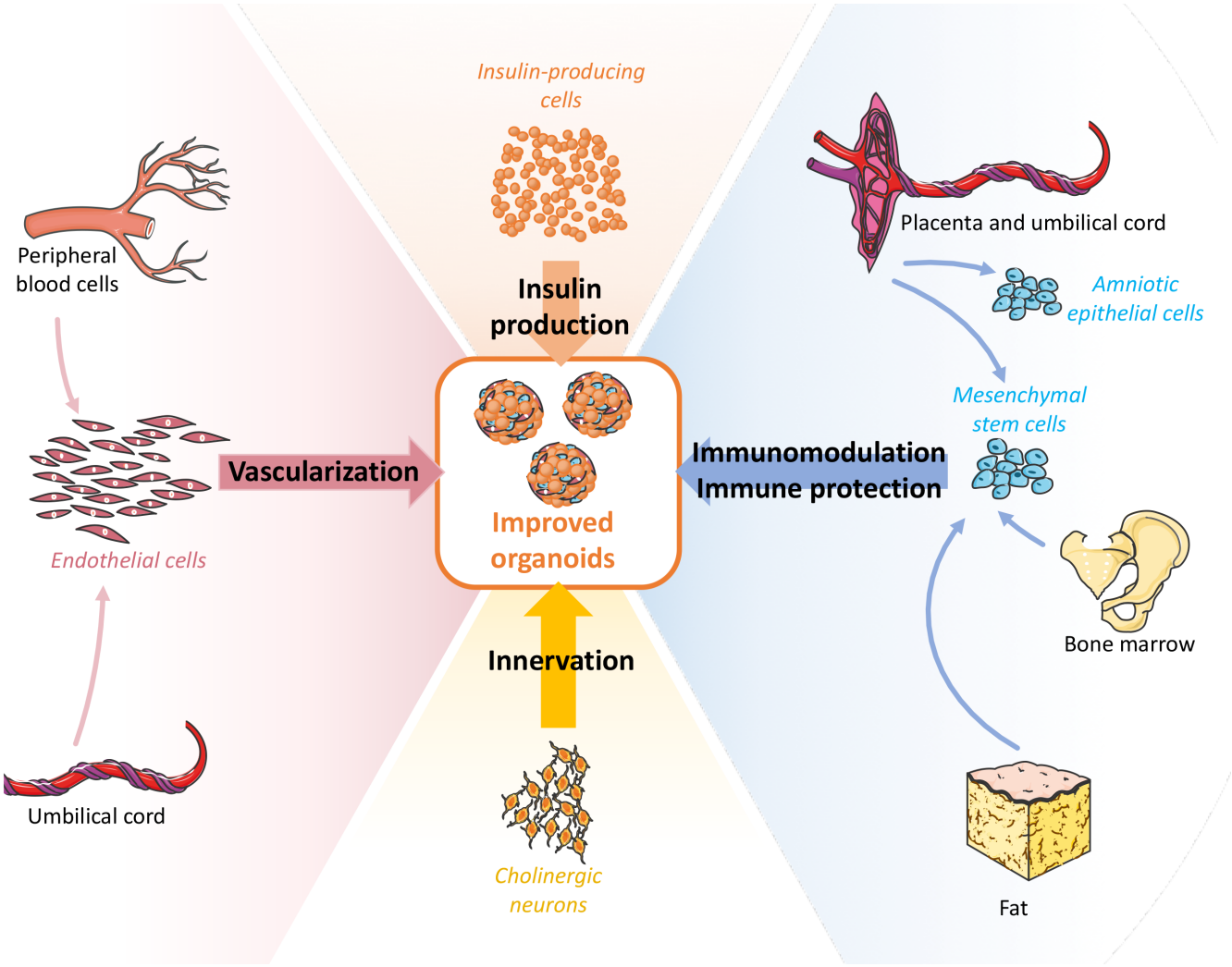

Figure 11. Supporting cells improving organoid function and engraftment.

1.4 Human amniotic epithelial cells as supporting cells

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IMMUNOLOGY, TRANSPLANTATION, AND REGENERATIVE MEDICINE (L PIEMONTI AND V SORDI, SECTION EDITORS)

Immunomodulatory Properties of Amniotic Membrane Derivatives and Their Potential in Regenerative Medicine

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Keywords: Amniotic membrane, amniotic epithelial cells, amniotic mesenchymal cells, regenerative medicine, immunomodulation

Abstract

Purpose of Review

During the last decades the field of regenerative medicine has been rapidly evolving. Major progress has been made in the development of biological substitutes applying the principles of cell transplantation, material science, and bioengineering.

Recent Findings

Amongst other sources, amniotic-derived products have been used for decades in various fields of medicine as a biomaterial for the wound care and tissue replacement. Moreover, human amniotic epithelial and mesenchymal cells have been intensively studied for their immunomodulatory capacities.

Summary

Amniotic cells possess two major characteristics that have already been widely exploited. The first is their ability to modulate and suppress the innate and adaptive immunities, making them a true asset for chronic inflammatory disorders and for the induction of tolerance in transplantation models. The second is their multilineage differentiation capacity, offering a source of cells for tissue engineering. The latter combined with the use of amniotic membrane as a scaffold, offers all components necessary to create an optimal environment for cell and tissue regeneration. This review summarizes beneficial properties of hAM and its derivatives and discusses their potential in regenerative medicine.

1.4.1 Background

Human amniotic membrane (hAM) and its derivatives express similar characteristics and advantages as MSC and exhibit a multilineage differentiation capacity. They are widely available, inexpensive, have limited ethical issues and have no risk of tumorigenicity (153). The hAM has been studied since many years and is used in the treatment of burns, skin defects and corneal injuries (154, 155). Because of their anti-inflammatory and immuno-modulatory properties, human amniotic cells have been considered as valid candidates for cell therapy in several degenerative disorders (156-159).

1.4.2 Placenta and maternal tolerance

Pregnancy is a unique state in which a semi-allogenic fetus coexists inside the mother without being rejected by the maternal immune system (160). This phenomenon of maternal tolerance is a complex process mediated by the restriction and modulation of leukocytes that permeate the maternal-fetal interface. Animal studies demonstrated significant reduction of T cell activation due to the indirect allorecognition of the fetus (161, 162). Furthermore, low numbers of dendritic cells (DC) have been found in decidua, in spite of the natural killer (NK) cell abundance. This was explained by the absence of local lymphatic vasculature in the endometrium (163). The effect of pregnancy and circulating fetal or placental antigens on T cell population has been also studied. It was shown that maternal T cells that can indirectly recognize the fetus are poorly primed and instead undergo clonal deletion (161). Furthermore, studies on mice have demonstrated recruitment and induction of fetal-specific T regulatory (Treg) cells at the maternal-fetal interface, thus inducing tolerance to fetal antigens. Fetal-specific Treg cells are capable of persisting beyond parturition while maintaining their functionality (164, 165).

1.4.3 Human amniotic membrane and its derivatives

The hAM is the innermost layer of the placenta and encloses the fetus in amniotic cavity. The hAM is an avascular tissue composed of five layers: a monolayer of epithelial cells, an acellular basement membrane, a compact layer containing proteins of ECM, a mesenchymal cell layer, and a spongy layer separating the amnion from the chorion (Figure 12A) (166). The compact layer and the fibroblast layer represent the amniotic mesoderm (167). Among all amnion components, the

hAM, the hAECs and the human amniotic mesenchymal stem cells (hAMSC) are the most studied for their anti-inflammatory and immunomodulatory properties.

1.4.4 Amniotic cells

hAECs and hAMSC can both be isolated from the hAM. hAECs reside on the first layer, directly in contact with the amniotic fluid and the fetus, while hAMSC are found deeper, in the amniotic mesoderm. Freshly isolated hAECs usually express CD324 (E-Cad), CD326, CD9, CD24, CD29, CD104, and CD49f as well as the stem cell markers stage-specific embryonic antigens 3 and 4 (SSEA-3, SSEA-4) and the tumor rejection antigen 1-60 and 1-81 (TRA-1-60 and TRA-1-81) (Figure 12B). Finally, they also express Oct4, Sox2, Nanog and Rex-1, members of the pluripotent stem cell transcription factor family (168, 169). hAMSCs possess similarities with BM-MSCs and express mesenchymal markers such as CD90, CD44, STRO-1 and CD105 (170). Like hAECs, they also express Oct4 and SSEA-4 (171). Moreover, hAECs and hAMSCs have common cell surface markers: CD73, CD29, CD49d, CD49e, CD166 and CD44 and are both negative for the hematopoietic makers CD34 and CD45 and the monocytic marker CD14 (166). By their potential to differentiate into the three germ lines (endoderm, mesoderm and ectoderm) and their capacity to downregulate innate and modulate adaptive immunity, hAECs and hAMSCs have been studied and used in the treatment of inflammatory and immune-based disorders.

1.4.4.1 Anti-inflammatory properties of amniotic cells

The downregulation of inflammation by amniotic cells (ACs) is the result of their action on several key role players of the innate immunity. These suppressive effects have been demonstrated in cell-cell contact studies between ACs and immune cells, but also without contact, in a transwell model, or even only with conditioned medium (CM) from AC culture. For instance, neutrophils and macrophages migration is inhibited *in vitro*, as the result of migration inhibitor factor (MIF) secretion by hAECs (172). A more recent *in vivo* study analyzed the ability of hAMSCs to improve corneal repair in a rabbit model and reported also a reduction of neutrophil migration to the injured site (171). Furthermore, ACs have demonstrated the capacities to inhibit NK cell cytotoxicity by downregulating NK-activated receptors (NKp30, NKp44, NKp46, NKG2D and CD69), and to reduce IFN-ɣ expression in a dose-dependent manner *in vitro* (173). This suppressive activity was partially explained by an increased production of IL-10 and prostaglandin

2 (PGE_2) by ACs when co-cultured with NK cells and was reversible when using anti-IL10 antibody or a specific PGE_2 inhibitor. An immunosuppressive activity toward monocytes was also observed in this study. LPS-stimulated monocytes showed a reduction of pro-inflammatory cytokine (TNF-α and IL-6) production when cultured with ACs. Magatti et al. demonstrated that amniotic mesenchymal cells and their CM shift differentiation of monocytes toward an anti-inflammatory M2 phenotype (174). Furthermore, they observed a reduction of pro-inflammatory cytokine secretion (IL-1α, IL-1β, IL-12, IL-8, TNF-α, MIP1α, MIP1β, MIG, Rantes and IP-10) by M2 macrophages, and an increased secretion of the anti-inflammatory cytokine IL-10. Finally, it was observed that M1 macrophages cultured with AC or their CM expressed less co-stimulatory proteins (CD80, CD86 and CD40) and induced a poor T cell response and a reduced number of IFN-ɣ-producing $CD4^+$ T cells. They also demonstrated an increasing number of activated Tregs when purified T cells were cocultured with either M1 macrophages exposed to CM during differentiation or M2 macrophages. The benefits of the shift toward the anti-inflammatory M2 phenotype was confirmed in several *in vivo* studies, for example in liver fibrosis, lung fibrosis and multiple sclerosis mouse models (175-178).

In summary, ACs strongly impair the development of an immune response by inhibiting neutrophil and macrophage migration, inducing M2 macrophage generation, reducing cytokine production by monocytes and NK cells and blocking the NK cytotoxicity.

1.4.4.2 Immunomodulatory properties of amniotic cells

It was thought for many years that one major characteristic of ACs was that they were not immunogenic and therefore under a state of immune tolerance. It has become clear that they are able to elicit immune responses, notably by expressing MHC class I (HLA-A, -B, -C) and II (HLA-DR), under certain conditions, for instance when cultured without serum or subjected to IFN-ɣ exposition (179). This was demonstrated by *in vitro* and *in vivo* studies, in which an immune response was triggered by AC (180, 181). This means that the immune protection of ACs is the result of an active mechanism of suppression or modulation of the immune system (Figure 12C).

In addition to downregulating the innate immune response, ACs have demonstrated their ability to suppress T cell proliferation in vitro in a dose-dependent manner (180-183). Suppression was observed after T cell exposition to alloantigen in the presence of ACs, either after CD3/CD28

stimulation or in classic mixed lymphocyte reaction models. As for innate immunity suppression, the ability to strongly suppress T cell proliferation was observed with cell-cell contact, in a transwell system and with CM.

DCs are essential for the initiation of an immune response (184). They present foreign or self-antigens to T cells, which can induce (i) $CD4^+$ T cells clonal expansion and polarization in the Th1, Th2 or Th17 phenotypes, (ii) $CD8^+$ effector T cell proliferation and activation or, depending on co-stimulation factors, shift T cell differentiation toward Treg cells (185-187). They also act on B and NK cells (188, 189) and are involved in the development of tolerance to self-antigens. Their interaction with immune cells in association with the environment will determine if the presented antigen will trigger a stimulatory of tolerogenic immune reaction. This critical role is obviously a target for cell-based therapy as tolerance can be induced by DCs manipulation (190). It has been demonstrated that ACs severely impair the function of monocyte-derived DCs by inhibiting their generation and maturation in vitro (172, 190). This phenomenon was observed not only in cell-to-cell contacts and transwell systems, but also when monocytes were just exposed to CM. Although a direct cell contact is not necessary for this inhibition to occur, it was demonstrated that the negative effect on DCs generation and function was stronger in cell-to-cell experiments. Furthermore, inhibition of DCs generation seemed to decrease when hAECs with higher numbers of passages were used, most likely resulting from hAECs epithelial to mesenchymal transition (190). In addition to impair DCs generation, it was observed that DCs exposed to ACs (in cell-to-cell or transwell systems) had significantly reduced capacities to stimulate $CD4^+$ and $CD8^+$ T cell proliferation. Finally, DCs exposed to ACs secreted higher level of anti-inflammatory cytokine IL-10 and reduced amounts of pro-inflammatory cytokines and chemokines (TNF-α, IL-12p70, IL-8 and MIP-1α) (190).

One key element responsible for the immunomodulatory properties of ACs is the expression of the tolerogenic HLA-G (122). This immunosuppressive molecule possesses 4 membrane-bound isoforms (HLA-G1, G2, G3 and G4) and 3 soluble isoforms (HLA-G5, G6 and G7). In addition to be present on hAECs, HLA-G expression can be induced on DCs when exposed to AC during differentiation (191). Furthermore, HLA-G expression is enhanced by IL-10 (192), IFN-α, -β and -ɣ (193, 194). The immunomodulatory properties of HLA-G result from the interaction with its

corresponding receptors (ILT2, ILT4 and KIR2DL4) present on immune cells. While ILT4 is present on monocytes and DCs, ILT2 can be found on most immune cells (NK, $CD4^+$, $CD8^+$, B cells, monocytes and DCs). HLA-G interaction with DCs was studied *in vitro* and *in vivo* by Liang et al. and resulted in the inhibition of DCs maturation and induced a differentiation toward the tolerogenic pathway (195). Furthermore, DCs function was altered by the reduction of MHC class II expression resulting in a decreased capacity to activate immune cells. It was also demonstrated that DCs exposed to HLA-G inhibited NK cell activation (196). HLA-G interaction with T cells results in inhibition of proliferation, shift toward a Treg phenotype, $CD8^+$ effector T cell inactivation and apoptosis of previously activated $CD8^+$ T cells (197). With regard to B cells, HLA-G inhibits proliferation, immunoglobulin secretion and chemotaxis. Finally, HLA-G also acts on innate immunity by suppressing NK cytotoxicity, through interaction with ILT2 and KIR2DL4 receptors, and by inhibition of ROS production and phagocytic capacity of neutrophils (198). Those results were also observed in clinical studies, where HLA-G was associated with better allograft acceptance in transplanted patients (199, 200). In addition to HLA-G, induction of tolerance by AC has been linked to their expression of the immune checkpoint proteins programmed death-ligands 1 and 2 (PD-L1 and PD-L2) (201). In the placenta, these molecules are present on hAMSCs and in the syncytiotrophoblasts, but they can be induced in hAECs by IFN-ɣ exposition (179). The interaction of PD-L1 and PD-L2 with their receptors will inhibit inflammatory cytokine secretion (IFN-ɣ, TNF-α, IL-2), and suppress T cell differentiation and proliferation (202).

In summary, ACs are able to block the initiation of an immune reaction by strongly altering the APC role of DCs. Furthermore, they inhibit $CD4^+$ and $CD8^+$ T cell proliferation, T cell cytotoxicity and induce the development and expansion of the Treg cell population. For these reasons, ACs have been implicated in numerous inflammatory and immune disease models. They also represent an interesting source of cells in regenerative medicine thanks to the anti-inflammatory and immunomodulatory properties they are able to confer.

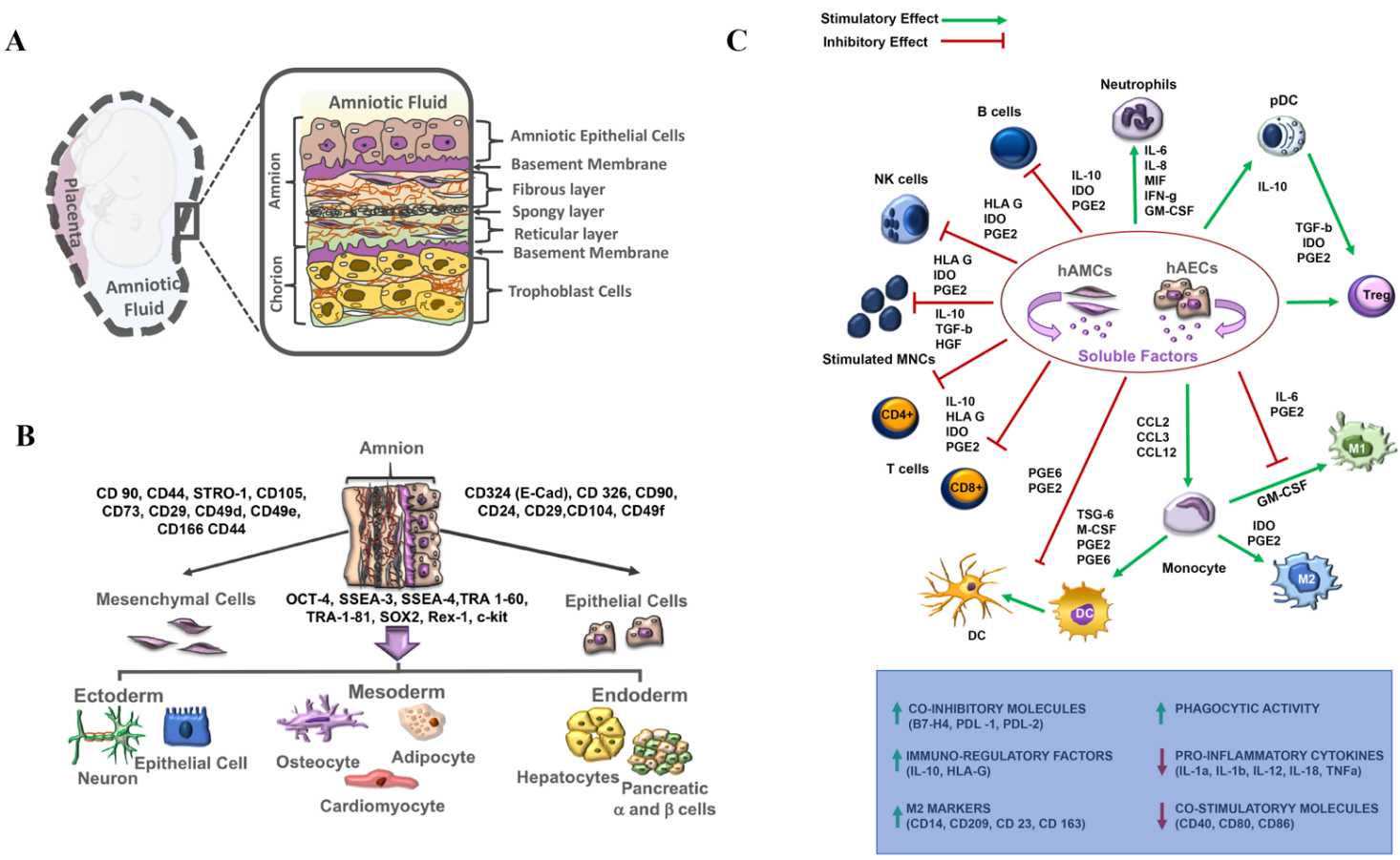

Figure 12. Amniotic membrane derivatives and their properties. A. Graphical representation of amniotic membrane. hAM is made up of two main parts, the amniotic epithelium and the amniotic mesoderm, separated by a basement membrane. hAECs (brown) are found in amniotic epithelium adjacent to the first ECM layer, basement membrane (purple). The amniotic mesoderm consists of fibroblast (beige), spongy (black) and reticular (light green) layers containing hAMCs (purple). **B**. Schematic diagram summarizing differentiation potential of hAECs and hAMCs into three embryonic germ layers, specifically ectoderm, mesoderm, and endoderm. **C.** Immunosuppressive/immunomodulatory properties of hAECs and hAMCs. ACs are known to suppress the proliferation, inflammatory cytokine production, and differentiation of T cells. At the same time, they stimulate generation of Treg cells. Soluble factors secreted by hAECs including PGE2, TGF-β, Fas-L, AFP, MIF, TRAIL, and HLA-G block dendritic cell and M1 macrophage differentiation and promote differentiation of monocytes into anti-inflammatory M2 phenotype. Moreover, ACs are known to be responsible for modulating host immune system, mainly through downregulation of TNF-α, IFN-γ, MCP-1 and IL-6 and upregulation of anti-inflammatory cytokines.

1.4.4.3 Application in regenerative medicine

There are actually more than 180 ongoing or completed clinical trials registered worldwide, in which amniotic membrane derivatives are utilized, in almost every field of medicine: ophthalmology, plastic surgery, dermatology, cardiology, neurology, urology, diabetology, nephrology, pneumology, hepatology, transplantation, dental surgery, gynaecology, orthopaedic surgery and ENT (ear nose throat).

1.4.4.3.1 Tissue engineering and cell-based therapy

ACs have been studied in several inflammatory diseases because of their anti-inflammatory properties but also for their potential to differentiate into many cell types, inducing tissue regeneration. Lung fibrosis can be idiopathic or secondary to chemical or physical insults. Several studies have demonstrated the benefit of hAECs transplantation in the bleomycin-induced mouse model. In addition to reduce fibrosis, inflammatory cell infiltration and cytokine production, hAECs showed the capacity to differentiate into alveolar epithelial cells *in vitro* and *in vivo*, making them a promising material for lung regeneration (176, 203).

Similar results were observed in a liver fibrosis mouse model in which hAECs transplanted intravenously decreased fibrosis, inflammation and apoptosis (204). The same results were observed in a recent study, using a murine model of steatohepatitis (158). The improvement was observed by injection of hAECs but also only with their CM. Furthermore, hAECs have been successfully differentiated into hepatocytes and cholangiocytes, *in vitro* and *in vivo*, improving tissue recovery (205, 206). It is noteworthy that hAECs were able to improve liver function in those studies without being rejected, despite the fact that animals were immunocompetent.

As mentioned previously, IT faces two major obstacles to generalize this therapy to the whole type diabetes patient population: (i) the scarcity of organ donors and (ii) the need for lifelong immunosuppression. ACs have been identified as a robust option to overcome these issues, by using two types of strategies. The first is to improve islet survival and engraftment by co-transplanting them with ACs as organoids. This has been successfully achieved by our group and others. Islets co-cultured with hAECs showed a better survival in hypoxic conditions and an increased functional potency as compared to unmodified islets (207). These results have been confirmed in immunodeficient (122, 207) and xenogeneic mouse models (208). In addition to

improved glycaemic control in vivo, histological assessments have demonstrated an increased vascularization of the grafts. The second is to use the stemness characteristics of amniotic cells as a source for differentiation into insulin-producing cells. hAECs have been successfully differentiated into cells with a β cell phenotype, with the capacity to control glycemia in streptozotocin-induced diabetic mice (209, 210).

1.5 Thesis aims

The application of IT in the treatment of brittle diabetes and the benefices on patient's quality of life, on the reduction of diabetes-related complications, on the resolution of severe hypoglycemia episodes and on the improvement in glycemic control are well established. However, there is a need to improve islet engraftment and survival in order to reduce the number of donors needed and to enhance long term graft function.

The generation of a bioartificial pancreas, immuno-protected, with enhance revascularization capacities and able to restore glycemic control, could resolve the challenges faced by IT. In addition, it would allow us to move from the intra-hepatic transplantation site to an extra-vascular location. The bioartificial pancreas should, therefore, be composed of:

- insulin-secreting cells for metabolic control. Those cells could be derived from primary islet, iPSC, ESCs, transdifferentiated cells
- endothelial cells for neo-angiogenesis of the transplanted construct
- hAECs for inflammatory and immune-protection
- biological scaffold for mechanical protection and functional support

Developing such a bioartificial organ requires to go through two main steps. The first one is to generate functional organoids composed of insulin-producing cells, hAECs and endothelial cells. The second step is to incorporate those organoids in a biological scaffold. In this thesis, we focused our work on the first step, the organoid generation.

The **first part** of this work was to determine the best method to generate organoids from single islet cells by comparing the different techniques available. In addition, this comparative study also aimed to offer a simple and clear explanation of which method to adopt, depending on the type of experiment that any researcher would perform.

The **second part** represented the main project with the development of functional pre-vascularized organoids. Our group previously performed experiments where either native islets or islet cells were co-culture with hAECs. They managed to demonstrate a protective effect of hAECs in hypoxic conditions *in vitro* and an improved engraftment *in vivo*. Those interesting and promising results were recently published (122, 211). We aimed to go further and to add

endothelial cells to the organoids. Our results demonstrated a good engraftment of the organoids with a better metabolic control *in vivo* in comparison to the controls. In addition, we successfully an external source of endothelial cells that participate to graft revascularization.

2 Experimental results and articles

2.1 Engineering of primary pancreatic islet cell spheroids for three-dimensional culture of transplantation: a methodological comparative study

Charles-Henri Wassmer, Kevin Bellofatto, Lisa Perez, Vanessa Lavallard, David Cottet-Dumoulin, Sanda Ljubicic, Géraldine Parnaud, Domenico Bosco, Ekaterine Berishvili and Fanny Lebreton

Abstract

Three-dimensional (3D) cell culture by engineering spheroids has gained increasing attention in recent years because of the potential advantages of such systems over conventional two-dimensional (2D) tissue culture. Benefits include the ability of 3D to provide a more physiologically relevant environment, for the generation of uniform, size-controlled spheroids with organ-like microarchitecture and morphology. In recent years, different techniques have been described for the generation of cellular spheroids. Here, we have compared the efficiency of four different methods of islet cell aggregation. Rat pancreatic islets were dissociated into single cells before reaggregation. Spheroids were generated either by (i) self-aggregation in non-adherent petri dishes, (ii) in 3D hanging drop culture, (iii) in agarose microwell plates or (iv) using the Sphericalplate 5D™. Generated spheroids consisted of 250 cells, except for the self-aggregation method, where the number of cells per spheroid cannot be controlled. Cell function and morphology were assessed by glucose stimulated insulin secretion test (GSIS) and histology, respectively. The quantity of material, labor intensity and time necessary for spheroid production were compared between the different techniques. Results were also compared with native islets. Native islets and self-aggregated spheroids showed an important heterogeneity in term of size and shape and were larger than spheroids generated with the other methods. Spheroids generated in hanging drops, in the Sphericalplate 5D™ and in agarose microwell plates were homogeneous, with well-defined round shape and a mean diameter of 90µm. GSIS results showed improved insulin secretion in response to glucose in comparison with native islets and self-aggregated spheroids. Spheroids can be generated using

different techniques and each of them present advantages and inconveniences. For islet cell aggregation, we recommend, based on our results, to use the hanging drop technique, the agarose microwell plates or the Sphericalplate 5D™ depending on the experiments, the latter being the only option available for large scale spheroids production

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Engineering of Primary Pancreatic Islet Cell Spheroids for Three-dimensional Culture or Transplantation: A Methodological Comparative Study

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Keywords : Islet, spheroids, 3D cell culture, type 1 diabetes, dissociation

Introduction

During embryonic development, cells undergo biological self-assembly to form complex tissues with 3D architecture and extensive cell–cell contacts, which are mandatory for maintaining intracellular functions (212). However, cells are mostly studied in two-dimensional (2D) monolayer models, since they provide a well-controlled and homogeneous environment, facilitate microscopic analysis and medium changes, and sustain cell proliferation for most cell types. This characteristic makes 2D platforms attractive for simplicity and efficiency considerations. Nevertheless, these methods are unable to mimic the complexity of the in vivo architecture and environment, which makes 2D-cultured cells different from cells growing in vivo in terms of morphology, proliferation, cell-cell and cell-matrix interactions, signal transduction, differentiation and other aspects (213). To better reproduce physiological conditions, there is a rapidly evolving trend towards the engineering of cell spheroids and their use as building blocks for functional tissue assembly. One of the main research fields using spheroids is oncology, where anti-cancer drugs are studied on 3D-cultured cancer cells (116, 117). Spheroids generation are also used for tissue regeneration using mesenchymal stem cells (MSC) (214). It has indeed been demonstrated that MSCs performed better when cultured in a 3D-manner (124). Among many other fields using spheroids, pancreatic islet transplantation benefits greatly from this 3D multicellular research tool (122, 207, 208).

In recent years, many techniques have been developed to generate spheroids using the principle of self-assembly (110). Most widely used techniques can be separated into non-microfluidic and microfluidic methods. The first category is composed mainly of hanging-drop cultures (112), cultures on low-attachment substrates (215) and cultures using microwell-containing culture plates (135). Spheroids generation using microfluidic methods is a dynamic technique, where cells are exposed to a continuous, controlled pressure perfusion (216, 217). Although this technique demonstrated several improvements such as spheroid morphology and viability (111, 218) in comparison to non-microfluidic techniques, it is used in “organ-on-a-chip” models, in opposition to generation of large quantities of functional elements for cell therapy and will therefore not be addressed in this study.

An essential prerequisite is the high-yield fabrication of spheroids of controlled size and composition. Spheroid size is an important parameter, as cells in the core of the spheroid heavily depend on oxygen supplied by diffusion. Consequently, overall spheroid size should not exceed a few hundred micrometers to avoid necrosis. In the field of pancreatic islet transplantation, larger human islets showed an increasing percentage of necrosis when exposed to 24 hours of hypoxia in comparison with smaller islets (96). Furthermore, central necrotic cores appear in islets >100 µm, in normoxic culture condition, after 48 hours. In addition to an improved viability, it has been reported that small islets performed better in term of function in comparison to larger islets (97, 98, 135). Thus, in order to replace damaged organ function, thousands to millions of spheroids would be necessary. Therefore, there is a need to develop methods which simultaneously allows efficient generation of spheroids and their large-scale production.

Spheroids composed of dissociated islet cells are a typical example of multicellular spheroids. Engineering spheroids from dissociated islet cells allows to create small, homogenous neo-formed islets (135). Furthermore, this process offers the possibility to co-culture islet cells with stem cells, endothelial cells or any other cell types that can be beneficial to the islets (149).

Here we compare 4 different techniques commonly used to generate spheroids from islet cells in terms of morphology and function of the resulting spheroids, but also including considerations of technical handling and labor intensivity, in order to provide researchers with information that will allow to select the technique that best suits their planned experiments.

Material and methods

Animals

Pregnant female, 10-week-old Lewis rats were purchased from Janvier Laboratory (Le Genest St-Isle, France). Animals were kept and bred in our animal facilities at the University of Geneva school of medicine. All experiments were performed in compliance with the rules of Geneva Veterinary authorities and according to protocols reviewed and approved by the University of Geneva Institutional Animal Care and Use Committee.

Islet isolation and dissociation

Pancreas digestion was performed by collagenase perfusion (collagenase V, Sigma-Aldrich, Buchs, Switzerland) and islets were purified by a discontinuous Ficoll gradient as previously described (219, 220). Islets were cultured for 24 hours at 37°C, in a 21% O_2, 5% CO_2 atmosphere, in DMEM medium (ThermoFisher Scientific, Reinach, Switzerland) supplemented with 10% fetal bovine serum (FBS; Merck Millipore, Zug, Switzerland), 2 mmol/l L-glutamin, 100 U/ml penicillin, 0.1 mg/ml 1 mmol/l sodium pyruvate (Sigma-Aldrich) and 11 mmol/l glucose (Bichsel, Interlaken, Switzerland), hereafter referred to as complete DMEM medium. At day 1 post isolation, islets were dispersed into single cells with 0.05% trypsin-EDTA (ThermoFisher) as previously reported(28).

Generation of spheroids

For each experiment, 1500 spheroids, composed of 250 cells, were generated according to four different techniques:

1. *The self-aggregating technique (petri condition):* 375 000 dissociated islet cells were plated in a 35 mm non-adherent petri dish (Falcon) in 2.5 ml complete DMEM medium. The time needed for cell plating was about 30 seconds. Medium was changed every 48 hours.

2. *The hanging drop technique (drop condition):* 375 000 cells were resuspended in 45 ml complete DMEM medium in a 50 ml conical tube (Falcon). Eleven non-adherent petri dishes of 150 mm diameter (Falcon) were used for this condition. The bottom parts of the petri dishes were filled with 30 ml of phosphate buffered saline (PBS). After resuspension of islet single cells in the conical tube, 30 µl drops were plated on the internal side of the petri-dish lid. Lids were then rapidly turned upside-down upon the bottom part of the PBS-containing petri dish(221). In total, about 1500 drops containing 250 cells each were created. The time needed for drop plating was about 120 minutes when performed by one operator. Medium was not changed during the culture time.

3. *The agarose 3D microwell technique (mold condition):* 500 µl sterile agarose solution at a 2.5% concentration (Promega, Dübendorf, Switzerland), heated at 90°C, was distributed onto autoclaved silicon molds (Microtissues 3D Petri Dish; Sigma-Aldrich), to generate 256-microwell

casts (microwells: 300 µm diameter, 800 µm depth). Once solidified, agarose casts were removed from the molds and each cast was placed inside the well of a 12-well cell culture plate (Sigma-Aldrich). Before use, agarose casts were equilibrated for 1 hour in complete DMEM medium at 37°C. Equilibration medium was removed and 375 000 cells were seeded in 6 agarose casts (62 500 cells/agarose cast) in a final cell suspension volume of 150µl per cast. A resting period of 30 minutes was observed in order to allow the cells to sediment inside the microwells before adding 2 ml complete DMEM medium per well. Medium was changed every 48 hours. The time needed for plating was about 35 minutes (2.5 minutes for plating, 30 minutes for cell sedimentation and 2.5 minutes for medium addition). This time does not include the casting of the agarose microwells and their equilibration.

4. *Spheroids using the Sphericalplate 5D™ sphericalplates (Kugelmeiers, Erlenbach, Switzerland) (Kugel condition):* the Sphericalplate 5D™ is a 24-well plate containing 9000 pyramidal microwells (500 µm edge) distributed in 12 of the plate wells (750 microwell/well). The other 12 conventional wells were not used in this study. Complete medium (1 ml/well) was used to remove air bubbles and equilibrate the plate before seeding. A total of 375 000 cells was plated in two microwell-containing wells, in a total final volume of 2 ml complete DMEM medium per well. The time needed for cell plating was about 30 seconds. Medium was changed every 48 hours.

As a control, intact islets (300 IEQ) were plated in a 3.5 cm non-adherent culture petri dish (Falcon), in 2.5 ml complete DMEM medium, hereafter referred to as IEQ condition. The time needed for cell plating was about 30 seconds. Medium was changed every 48 hours.
For each condition, native islets or islet cell spheroids were cultured for 5 days at 37°C in a 21% O_2 and 5% CO_2 atmosphere.

Immunofluorescence staining:

Native islets and spheroids were recovered after 5 days in culture, fixed in 4% paraformaldehyde (PFA) during 60 minutes, and suspended in HistoGel (ThermoFisher) pre-warmed at 70°C. After centrifugation, HistoGels containing native islets or spheroids were left on ice for 15 minutes. Solidified HistoGels were then recovered and embedded in paraffin. Block sections of 5 µm were

cut and mounted on glass slides. Permeabilization was performed with 0.5% Triton X-100 in PBS for 30 minutes followed by 45 minutes incubation in 0.5% bovine serum albumin (BSA) in PBS at room temperature to block unspecific sites. Immunofluorescence staining was performed in 2 sequential steps: slides were first incubated overnight at 4°C with a rabbit anti-somatostatin primary antibody (1:100 dilution; DakoCytomation, Baar, Switzerland). The next day, slides were washed in PBS, and then exposed for 1 h to an anti-rabbit alexa 488 secondary antibody (Jackson ImmunoResearch Laboratories, Rheinfelden, Switzerland). After PBS rinsing, slides were incubated for 2 hours with a combination of primary antibodies: guinea-pig anti-insulin (1:100 dilution, DakoCytomation) and mouse anti-glucagon (1:4000, Sigma-Aldrich). Slides were washed in PBS before incubation for 1 hour with a combination of FITC goat anti-guinea pig and a Coumarin AMCA donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories). Both primary and secondary antibodies were diluted in PBS 0.5% BSA. Pictures were taken using a Zeiss Axioscan.Z1 slide scanner (Zeiss, Feldbach, Germany).

Morphology:

Native islets and spheroids were recovered after 5 days in culture. They were placed in non-adherent petri dishes and pictures were taken for morphology assessment. Diameters were measured on a minimum of 100 native islets or spheroids from 4 distinct preparations, using the ImageJ software (NIH, Bethesda, MD, USA).

Functional assessment

Glucose stimulated insulin secretion (GSIS) test was performed after 5 days of culture. Native islets and the newly formed spheroids from the different conditions were plated in triplicates in 24-well plates containing culture inserts (Millipore, Zug, Switzerland). A pre-incubation of one hour in Krebs–Ringer buffered HEPES (pH 7.4) with 0.1% BSA (KRB solution) containing 2.8 mmol/l glucose was performed. Native islets and spheroids were then exposed for one hour to a low glucose KRB solution (2.8mmol/l), followed by one hour in a high glucose KRB solution (16.7 mmol/l). Supernatants were recovered and insulin concentrations were measured using an ELISA kit (Mercodia, Uppsala, Sweden) for rat insulin. Native islet and spheroid capacity to respond to glucose was expressed as the ratio of insulin concentration in high to low glucose medium,

referred to as the stimulation index (SI). Finally, native islets and spheroids were incubated for one hour in acid ethanol for evaluation of total insulin content. Insulin secretion was further estimated as a percentage of total insulin contents.

Statistical analysis

Variables are presented as mean ± SD. Comparisons between two groups were performed with the Mann-Whitney U-test. Statistical analyses were performed using Prism software 8.0 (GraphPad, La Jolla, CA, USA), and a p value of < 0.05 was considered statistically significant.

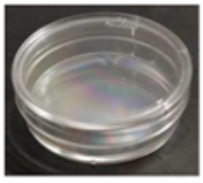
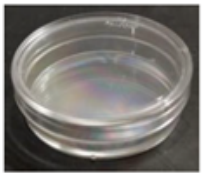
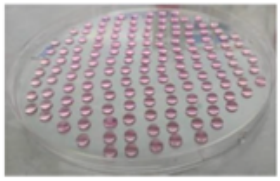
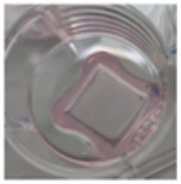
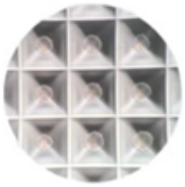
Conditions	IEQ	Petri	Drops	Molds	Kugel [a]
Number of islets or cells	300 IEQ	375 000 cells	375 000 cells	375 000 cells	375 000 cells
Number of spheroids	—	~1 500	~1 500	~1 500	~1 500
Substrate type	Petri 35 mm	Petri 35 mm	Petri 150 mm	256-well agarose molds in 12-well plate	Engineered microwells in Kugelmeier plate
Number of substrate	1 petri	1 petri	11 petri	6 molds	2 microwells of the 24-well plate
Culture medium volume	2.5–3 ml	2.5–3 ml	45 ml	12.9 ml	4 ml
Time for seeding	30"	30"	120'	2.5' seeding 30' sedimentation 2.5' adding medium	30"
Support devices					

Table 1 describes the amount of cells, materials, time needed to plate the cells in the five conditions, and the support device used for each condition. [a] Picture from https://www.kugelmeiers.com

Results

Spheroid generation

Plating data are summarized in Table 1. The self-aggregation technique in non-adherent petri dishes was the fastest and easiest method and required the fewest amount of material. Only one 35 mm non-adherent petri dish and about 4 ml of complete DMEM medium (2 ml for the initial seeding and one medium change) were necessary. By contrast, the hanging drop technique required the largest amount of material (eleven 150 mm petri dishes and 45 ml of complete medium) and took the longest time to plate the cells in order to form aggregates. Of note, in order to have a similar distribution of the cell types in the spheroids, cell preparations in the medium had to be homogenized regularly during drops generation. The mold technique was more labor-intensive than the others, except the drop condition, mainly because of the time needed to cast the agarose structures, and medium equilibration and cell sedimentation times. However, agarose casts can be prepared in advance and stored at 4°C in PBS or Hanks. In this study, the mold conditions required 6 wells of a 12-well culture plate, 24 ml complete medium and 6 agarose structures. The kugel condition was performed using two wells (containing 750 microwells/well) of a Sphericalplate 5D™ and required 8 ml complete DMEM medium (2 ml for the seeding and one medium change). In contrast to the mold technique where spheroid recovery required inverting the agarose structures upside down before spinning them to make the aggregates fall from the microwells, a simple resuspension of the aggregates with medium allowed to recover all spheroids from the Sphericalplate 5D™. Finally, the control IEQ condition required one 35 mm non-adherent petri dish and about 4ml complete DMEM medium (2ml for the initial seeding and one medium change). Figure 1 shows the plating substrates just after seeding for the 5 conditions. Importantly, 250-cell spheroids were created with the mold, kugel and drop techniques, whereas for the petri condition, the number of cells per aggregates could not be controlled.

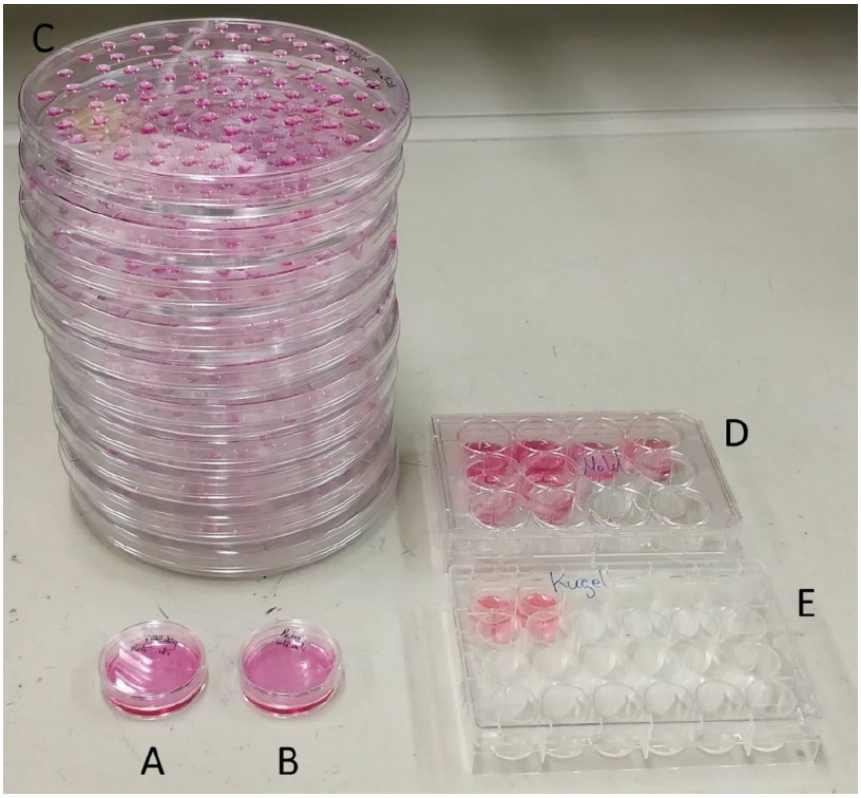


Figure 1. Picture of the results of the five conditions plated: (A) the IEQ condition, (B) the petri condition, (C) the drop condition, (D) the mold condition, and (E) the Kugel condition.

Morphology and immunohistological assessment

As shown in figure 2A, single islet cells showed a good aggregation after 5 days in culture regardless of the method chosen. Re-aggregated spheroids presented diameters ranging from 40 to 300 µm, with a mean diameter of 96,2 ± 44,3 µm. As expected, heterogeneity in term of size and shape was important in the IEQ condition (146,4 ± 52,2 µm) but also in the petri condition (96.17 ± 44.29 µm). In contrast, spheroids in the other three conditions showed a more homogenous morphology as observed by smaller standard deviations. Indeed, spheroid mean diameters were 94,5 ± 13,4 µm, 81,3 ± 17,1 µm and 102,3 ± 14,0 µm, for the drop, mold and kugel conditions, respectively. Diameter comparison is represented in figure 2B.

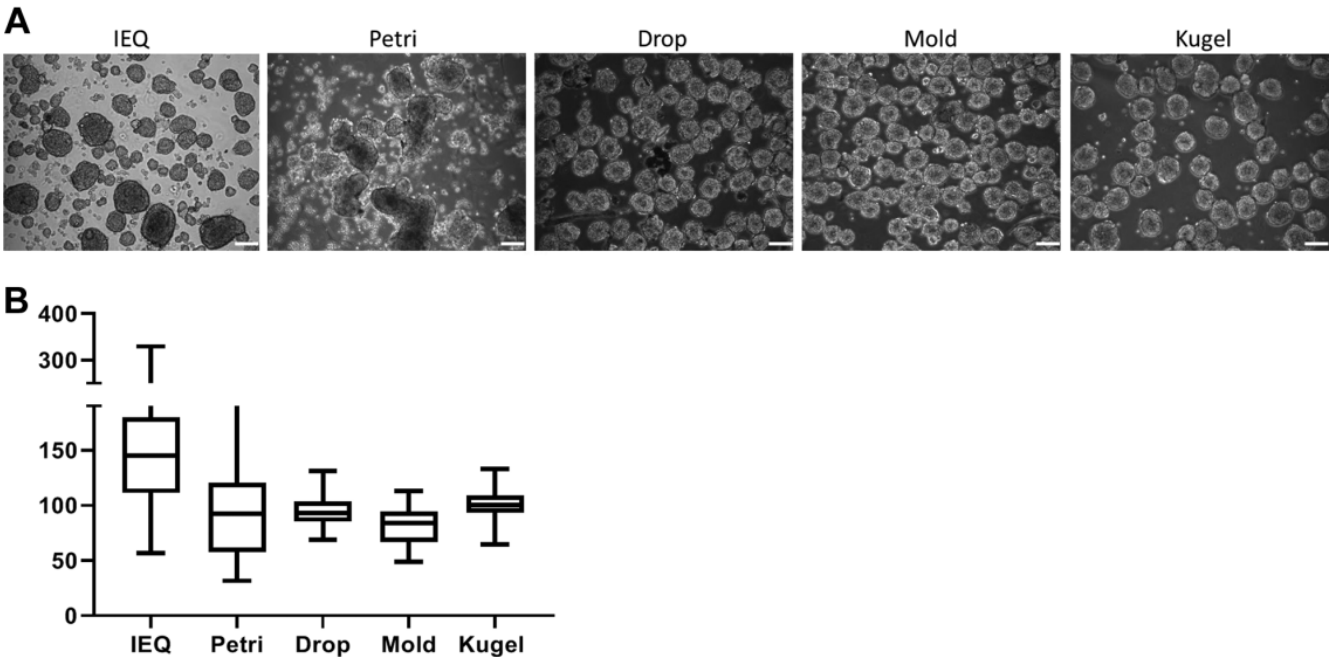


Figure 2. Spheroids morphology. (A) Phase-contrast microscopic images of native islets and generated spheroids after 5 days of culture. Scale bar = 100 µm. (B) Diameter values for each condition are presented as mean diameter with SD. SD: standard deviation.

Histology assessment by immunofluorescence is presented in figure 3 and demonstrates a similar distribution of the main endocrine islet cell types, with a majority of insulin-positive cells (in red), and the presence of glucagon-positive cells (in blue) and somatostatin-positive cells (in green). In summary, we succeeded to generate spheroids composed of the main endocrine cell types, and observed a better homogeneity in term of size and shape in the drop, mold and kugel conditions, in comparison to the IEQ and petri conditions.

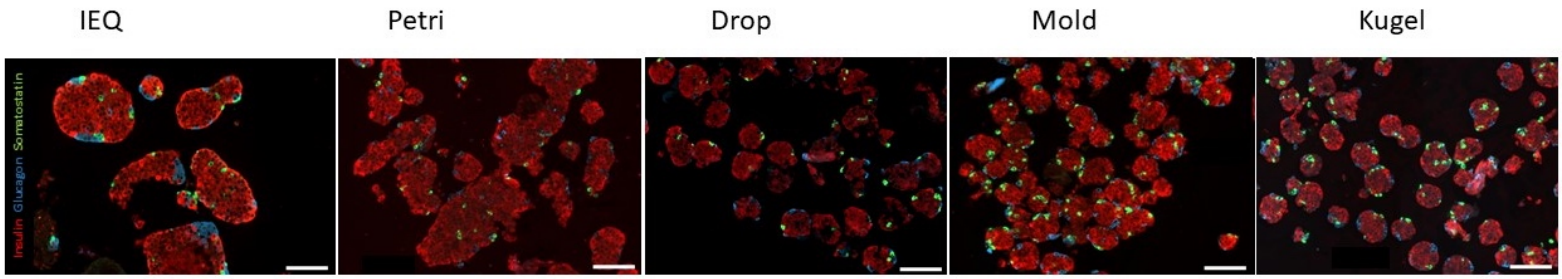


Figure 3. Immunohistology. Immunofluorescence staining of islets or spheroids from the five conditions. Insulin is stained in red, glucagon in blue, and somatostatin in green. Scale bar = 100 µm.

In vitro function

Function of islets and spheroids was determined by a glucose stimulated insulin secretion test. Stimulation indices (SI) from the different conditions are presented in figure 3A. In the petri condition, SI was 3.10 ± 0.64, a value quite similar to that of the IEQ control condition (3.22 ± 0.77; p=0.791). Spheroids formed by drop, kugel and mold techniques exhibited higher SIs with mean values of 4.99 ± 3.37, 5.22 ± 2.03 and 6.06 ± 2.80, respectively. A trend toward statistically significant differences when compared to the petri or IEQ conditions was observed for the mold (p = 0.076 and 0.085 respectively) and kugel (p = 0.078 and 0.085 respectively) conditions. Figure 3B presents insulin secretion during the basal and stimulated phases of GSIS expressed as a percentage of total insulin contents, determined by acid ethanol extraction. The drop condition showed higher insulin secretion during the basal and stimulated phases in comparison to the other conditions. Interestingly, this condition showed the lowest insulin content (figure 3C). The kugel and IEQ conditions showed the highest amount of insulin contents, but differences failed to reach statistical significance (figure 3C).

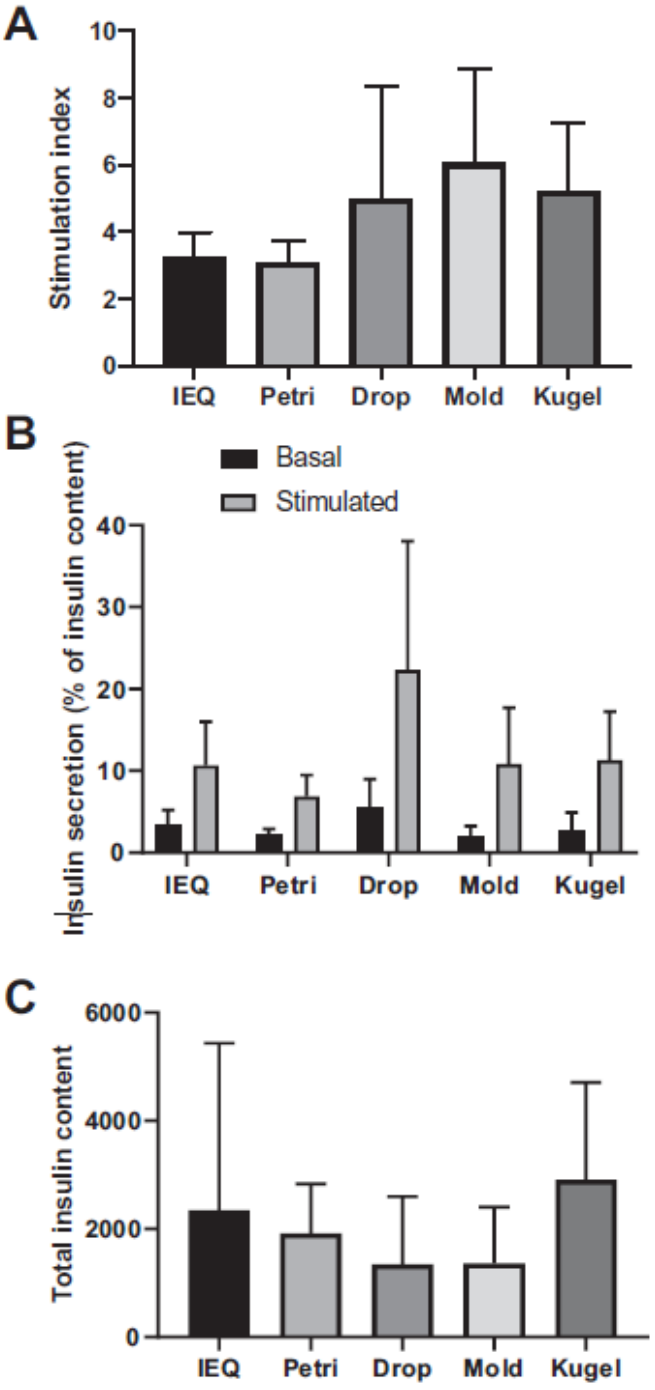


Figure 4. Functional assessment of native islets and newly formed spheroids. (A) Insulin secretion, expressed as the stimulation index, visualized as bar plot. (B) Percentage of inulin content secreted during basal and stimulated phase. (C) Total insulin content expressed as pmol/l. N=5.

Discussion

The generation of cell spheroids has been gaining increasing attention not only in the development of 3D culture systems, but also as part of organ bioengineering strategies (212). This is epitomized in the field of beta-cell replacement for type 1 diabetes, in which islet cell organoids with potential immunomodulatory features have been generated for transplantation (122, 207). In this study, we have assessed four different techniques of cell aggregation for the reconstitution of insulin-secreting spheroids from single islet cells. The different methods explored were assessed not only in terms of homogeneity and functionality, but first and foremost in terms of labor intensivity. The non-adherent petri dish technique was easy and fast but didn't allow controlling the size or the composition of the cell aggregates. For these reasons, this method cannot be recommended, especially if spheroids of predetermined size and structure, using different cell types, are needed. Furthermore, this technique showed the lowest secretory capacity in response to glucose stimulation.

The drop, kugel and mold conditions showed similar results in term of morphology (diameter, homogeneity) and function. Remarkably, insulin secretion was improved compared to native islets or spheroids generated by self-aggregation. Furthermore, these three techniques allow to control islet spheroid size by selecting the number of cells per aggregate, and offer the possibility of incorporating cells of different types. The drop method, although effective in terms of morphology and spheroid function, was much more labor intensive, and less cost-efficient given the additional materials and reagents required. Recovery of the newly formed spheroids was time consuming as well. Furthermore, this condition presented an intermediate result in term of in vitro function but with an important variability, as shown by high SDs. However, this technique can be useful when very small amount of spheroids are required.

The mold technique showed good results in term of morphology and function and the quantity of material required was low. It is important to mention that about 20% of the agarose structures presented defects or were damaged when they were removed from their casts. One of the main differences with the Sphericalplate 5D™ is the shape of the microwell bottoms. In the agarose structure, the bottom is flat whereas the Sphericalplate 5D™ microwells have conical bottoms. This potentially has a big impact because inter-cellular contacts are impaired in flat bottom wells

when cell density is low (<250cells/microwell), and aggregation can fail. On the contrary, the Sphericalplate 5D™ allows good intercellular contact regardless of the number of cells per microwell. Regarding cost considerations, Microtissues silicon molds can be used repeatedly, making this technique very cost-effective in the long run. The Sphericalplate 5D™ is very easy to use for cell plating as well as for spheroid recovery and presents good functional results. It allows good control of spheroid size and composition. Its major advantage is that it allows the large-scale production of substantial amounts of spheroids, with minimal effort in 750-microwell plates. This is of critical importance when large numbers of spheroids are needed (for instance in transplant experiments).

Bioengineering of cell aggregates has gained increasing interest in the field of cell transplantation, notably for the treatment of diabetes, but also in other areas of regenerative medicine and in cancer research(222). For example, Herrera et al. used cell re-aggregation after cell reprogramming of non-beta-cells into insulin secreting cells allowing to correct diabetes in rodents(223). We have recently published a study in which organoids composed of re-aggregated islet cells and amniotic epithelial cells were transplanted to diabetic mice and showed improved results in term of diabetes reversion when compared to native islets(122).

In this study, two methods stand out in terms of functional performance of the generated spheroids and minimization of labor intensivity. Islet cell spheroids generated either in locally produced silicon microwells (mold condition) or in the Sphericalplate 5D™ (kugel condition) provide highly functional insulin-producing constructs with minimal labor intensivity. From an economical perspective, the mold condition is more cost effective, but the Sphericalplate 5D™ is the only method that can be easily scaled up to produce large numbers of constructs, as would be required with a translational perspective to pre-clinical large mammal models or to the human. We believe that this study will also help researchers working in other fields than beta-cell replacement to select the best method to generate cells aggregates or engineer organoids, depending on the type of their experiments.

2.2 Bio-engineering of pre-vascularized islet organoids for treatment of type-1 diabetes

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Abstract

Lack of rapid revascularization and inflammatory attacks at the site of transplantation contribute to impaired islet engraftment and suboptimal metabolic control after clinical islet transplantation. In order to overcome these limitations and enhance engraftment and revascularization, we have generated and transplanted pre-vascularized insulin-secreting organoids composed of rat islet cells, human amniotic epithelial cells (hAECs), and human umbilical vein endothelial cells (HUVECs). Our study demonstrates that pre-vascularized islet organoids exhibit enhanced *in vitro* function compared to native islets, and, most importantly, better engraftment and improved vascularization *in vivo* in a murine model. This is mainly due to paracrine signalling between hAECs, HUVECs and islet cells, mediated by the upregulation of genes promoting angiogenesis (*VEGF-A*), ß cell function (*GLP-1R*) and ß cell survival (*Pdx1*). The possibility of adding a selected source of endothelial cells for the neo-vascularization of insulin-secreting grafts may also allow implementation of ß cell replacement therapies in more favourable transplantation sites than the liver.

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Keywords: islet transplantation, organoids, type-1 diabetes

Introduction

The development of the Edmonton protocol has drastically increased the rate of success of islet transplantation. However, due to the loss of a significant number of islets resulting from inflammatory attacks and impaired vascularization, long term graft function remains suboptimal (224).

Despite the fact that islets represent only 1-2% of pancreatic tissue volume, they receive 10 to 15% of the total pancreatic blood flow (20). Each islet possesses 1 to 3 pre-arterioles (21), depending on islet size, that rapidly branch out into a multitude of fenestrated capillaries and form an important intra-islet micro-circulation that is five time denser than in the exocrine tissue (25). The cross-talk between endocrine and endothelial cells is vital for proper islet development, configuration and vascularization. Islet cells secrete vascular endothelial growth factor-A (VEGF-A) and angiopoietin-1 in order to recruit endothelial cells (ECs) that are necessary for islet development, survival and function. On the other hand, ECs are involved in cell differentiation, insulin gene expression and cell segregation during embryogenesis (225, 226). In addition, they secrete components of the intra-islet basement membrane that are crucial for proper endocrine function (25).

Islet isolation and culture lead to the disruption of the islet capillary system, with significant loss of ECs due to de-differentiation or necrosis (227). In addition, islets vary in size, ranging from 50 to 400 μm in diameter. In the immediate post-transplantation period, avascular islets are supplied with oxygen and nutrients solely by diffusion until re-establishment of the blood flow, a process that can take about two weeks (2). Because of that, larger islets fail to engraft due to insufficient vascularization and subsequent necrosis (98). Significant efforts have been made to develop new strategies to minimize hypoxia-induced β cell death.

Several scientific groups, including our own, have demonstrated that re-aggregation of islet cells in combination with other cell types into homogeneous, round shaped and size-controlled spheroids leads to improvement of function and viability, thanks to heterotypic cell-to-cell interactions and reproduction of the complex natural morphology of the islet (122, 137, 207, 228, 229). In our previous studies, we have shown that incorporation of human amniotic epithelial cells (hAECs) into insulin-secreting organoids protected islet cells from oxidative stress in vitro,

subsequently improving ß cell viability, function and engraftment (122, 207). Here, we propose an improved approach, in which we engineer pre-vascularized organoids that provide both control over their size and composition, and prompt re-establishment of the cross-talk between ECs and islet cells, thereby facilitating graft revascularization after transplantation.

Materials and Methods

Reagents and antibodies

All reagents and antibodies used in this study are listed in supplementary tables 1 to 3 at the end of the manuscript

Animals

Animal experiments were performed in accordance with the Geneva veterinary authorities and approved by the Institutional Animal Care and Use Committee of the University of Geneva (Licence GE34/19). Ten-week-old, pregnant female, Lewis rats were purchased from Janvier Laboratory (Le Genest St-Isle, France) and bred in our animal facility at the Geneva University. Fifteen-to 21-week-old male rats were used for pancreatic islet isolation. Six- to 9-week-old male B6.129S7-Rag1^{tm1Mom}/J (abbreviated NOD–*Rag1*^{null} bred at Charles River Laboratories, Saint-Germain-Nuelles, France) mice were used as transplantation recipients. All animals were kept under conventional housing conditions with free access to water and food.

Human tissues

Studies involving human tissues were approved by the Commission Cantonale d'Ethique de la Recherche (CCER; protocol PB_2017-00101), in compliance with the Swiss Human Research Act (810.30).

Placentas were obtained from women undergoing elective caesarean section of uncomplicated, term pregnancies. Informed, written consent was obtained from each donor prior to tissue collection.

Isolation and culture of HUVECs and hAECs

Human umbilical vein endothelial cells (HUVECs) were isolated using a method adapted from a previously published protocol (230). Briefly, the umbilical vein was rinsed, then distended with Collagenase A solution (2mg/ml) and incubated at 37°C for 12 minutes. Released cells were then collected by flushing the vein with cold HBSS supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B. Isolated HUVECs were plated in a 75cm^2 flasks and cultured at 37°C, 21% O_2 and 5% CO_2 in M199 medium supplemented with 20% FBS, 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (1% of a L-Glutamin-Penicillin-Streptomycin stock solution), Fungin 0.1%, 30µg/ml endothelial cell growth supplement and 100µg/ml heparin. HUVECs from passage 2 to 7 were used in this study.

hAECs were isolated, cultured and characterized as described previously (10, 14). Freshly isolated hAECs were cultured in DMEM/F-12 medium, supplemented with 10% FBS, 2 mmol/l L-Glutamin, 100 U/ml Penicillin, and 0.1 mg/ml Streptomycin (1% of a L-Glutamin-Penicillin-Streptomycin stock solution, 1 mmol/l sodium pyruvate, 1% MEM NEAA 100X, 0.1% fungin, 0.05 mmol/l 2-mercaptoethanol, 10 ng/ml human recombinant epidermal growth factor (EGF). Only cells at passage 1 were used in this study.

Medium was changed every 48 hours. Confluent cells were harvested by mild trypsinization and were cryopreserved for later utilization.

Rat islet isolation and dissociation

Rat islets were isolated by enzymatic digestion (collagenase V) and purified using a discontinuous Ficoll gradient (138-140). Isolated islets were cultured (37°C, 5% CO2) in DMEM medium supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin, 0.1 mg/ml 1 mmol/l sodium pyruvate and 11 mmol/l glucose for 24 hours. Islets were then dispersed into single islet cells (ICs) by incubation in 0.05% trypsin-EDTA (16).

Characterization of HUVECs and hAECs

HUVECs and hAECs were analysed for expression of previously reported endothelial cell surface markers or specific amniotic epithelial cell surface markers by flow cytometry.

For analysis, cells ($2.5x10^5$) were stained by incubation for 30 min with primary or isotype control antibody in 100 µl PBS with 0.2% BSA, washed twice with PBS, and analysed. Antibodies used for HUVECs were: AlexaFluor 657-conjugated anti-CD 144 (1:40 dilution), PE-conjugated anti-CD 31 and PerCP-Cy 5.5-conjugated anti-CD 45 (1:25 dilution). Antibodies used for hAECs were: FITC-conjugated anti- human CD105 (clone 266), BV421-conjugated anti-human CD326 (clone EBA-1), PerCP-Cy5.5 conjugated anti-SSEA4 (clone MC813-70) (1:50 dilution), PE-Cy7 conjugated anti-human CD90 (clone 5E10; 1:100 dilution), PE-conjugated anti-human HLA-E (clone 3D12) and APC-conjugated anti-human HLA-G (clone 87G; 1:20 dilution).

Flow cytometry analysis was performed on a Gallios cytometer using the Kaluza Analysis software. HUVECs were further characterized by immunostaining. Immunofluorescent assessment was performed on the cells cultured on gelatine-coated glass coverslips. Fixed cells were washed, permeabilized and stained with the following primary antibodies: mouse anti-CD 31 (1:50 dilution), rabbit anti-von Willebrand factor (1:100 dilution) and mouse anti-vimentin (1:50 dilution). Cells were then incubated with corresponding Alexa Fluor and FITC-conjugated secondary antibodies. For nuclear counterstaining samples were mounted with aqueous solution containing 4,6 diamidino-2-phenylindole (DAPI).

Functional assessment of HUVECs in vitro: tube formation assay

The tube formation assay was performed according to manufacturer's protocols of Corning® Matrigel® Matrix. Briefly, Matrigel thawed overnight at 4°C was mixed with VEGF (200ng/ml) and 250 µL of matrix was added to each well of 24-well plates. After 1 hour of incubation at 37°C, cells ($8x10^4$) were seeded onto the Matrigel and tube formation of HUVECs was observed and photographed using an inverted phase-contrast microscope during 6 hours.

Lentiviral (LV) transduction

HUVECs transduction was performed with LVs carrying green fluorescent protein (GFP) gene under the control of endothelial-specific promoter (vascular endothelial-cadherin promoter LV-VEC) using a multiplicity of infection of 10 (MOI=10). Transduction degree was confirmed and

considered successful when at least 80% of cells showed expression of GFP when assessed by fluorescent microscopy and flow cytometry.

Generation of pre-vascularized islet organoids

Pre-vascularized islet organoids (PIO) were generated on AggreWell^{TM}400 24-well plates by seeding mixture of ICs, HUVECs and hAECs at a ratio of 5:4:1 (800 cells/organoid). Undissociated native islets (NI), ICs spheroids (400 ICs/spheroid), hereafter referred to as pseudo-islet (PI), and IC: HUVEC spheroids (ratio 1:1, 400 cells/spheroid), hereafter referred to as IC+HUVEC served as controls. PIO, PI and IC+HUVEC were cultured for 4 days to allow cell aggregation at 37°C, 21% O_2 and 5% CO_2.

Culture medium for PIO was prepared by mixing equal volumes of complete DMEM, DMEM/F12 and M199 medium, hereafter referred to as organoid medium. IC+HUVEC were cultured in the mixture of complete DMEM and M199 medium at the ratio 1:1. Finally, PI and NI were cultured in complete DMEM medium. Culture medium was changed every other day. Mean diameter of NI, PIO and PI were calculated on the images taken on light microscope using ImageJ software.

In order to observe PIO composition and cell distribution during culture, fluorescent carbocyanine dyes CM-DiL (red) prelabeled hAECs and GFP transduced HUVECs were used. Pictures were taken using an epifluorescent microscope (DMi8 manual microscope).

Organoids sprouting assay

One hundred PIO were embedded in a hydrogel made of decellularized human amniotic membrane at a concentration of 5mg/ml. Hydrogel containing PIO were transferred into prewarmed 24-well plates and allowed to polymerize for 30 minutes. Next, 0.1 ml organoid medium supplemented with VEGF-A at the concentration of 200ng/ml was pipetted on top of each hydrogel containing PIO. The hydrogels were cultured for 24 hours at 37°C, 5% CO2, and 100% humidity. As control, one hundred IC+HUVEC spheroids and PI were cultured in the same way in the hydrogel.

In vitro functional assessment

To assess functional capacity, 300 NI and an equivalent number of PIO and PI, were incubated in duplicates for 1 hour in Krebs–Ringer solution. After a change of medium, islets and aggregates were incubated at 37°C for 1 hour in low glucose (2.8mmol/l), followed by 1h at high glucose (16.7mmol/l). Supernatants were collected and stored at −20 °C. Insulin concentration in supernatants was measured using a rat insulin ELISA kit and normalized to the total insulin content. Results are expressed as the ratio between insulin secreted in high glucose to low glucose, referred to as stimulation index (SI). In addition, total insulin content per IC was measured by dividing the total insulin content by the number of ICs present in the NI, PI and PIO.

Morphological assessment

Fully aggregated PIO were recovered, fixed in 4% PFA, permeabilized in Triton X-100/PBS for 4 h and incubated in 0.5% BSA/0.1% Triton X-100/PBS at 4 °C for 1 h to block unspecific sites. Immunofluorescence staining was performed in two sequential steps. First, PIO were incubated with a primary rabbit anti-pancytokeratin antibody (1:75 dilution) overnight at 4°C. PIO were then washed for 1 hour in PBS-0.5%BSA and incubated with a donkey anti-rabbit Alexa Fluor-conjugated secondary antibody for 4 hours at 4°C (dilution 1:300). Samples were washed again in PBS-0.5%BSA and a guinea-pig anti-insulin primary antibody (1:100 dilution) was applied overnight at 4°C. After washing in PBS-0.5%BSA for 1 hour, PIO were incubated with a donkey anti-guinea pig Coumarin secondary antibody (1:200 dilution).

Primary and secondary antibodies were diluted in PBS-0.5%BSA. Stained PIO were then transferred to Ibidi culture plates and subjected to optical sectioning 2,5-µm increments in axial (z) dimension using a spectral confocal microscope (Nikon A1R). Three-dimensional reconstruction of z-series was performed by the NIS-Elements Imaging Software.

Diabetes induction and xenogeneic transplantation

Three days before transplantation mice were subjected to intraperitoneal injection of STZ (180mg/kg). Non-fasting blood glucose levels were then checked daily using a portable

glucometer. Only mice with blood glucose levels over 18mmol/l for 3 consecutive days were used in this study.

A marginal mass of 300 islet equivalents (IEQ) for NI and 1200 PIO, PI and IC+HUVEC were transplanted. Number of organoids was based on the average number of islet cells per IEQ, previously estimated as 1560 ICs/IEQ (7).

At the day of transplantation, NI and engineered constructs were recovered from culture, packed in PE50 tubing and transplanted into the epididymal fat pad (EFP) of diabetic mice. Non-fasting glucose was assessed daily during the first week and 3 times per week thereafter. Normoglycemia was defined as two consecutive blood glucose levels under 11.1mmol/l. Overall blood glucose levels were expressed as the mean value over the 30-day period.

Graft metabolic function assessment

Graft capacity to clear glucose in vivo was assessed dynamically by intraperitoneal glucose tolerance test (IPGTT) at 30 days after transplantation. Mice were fasted for 6 hours and injected with 2g/kg glucose intraperitoneally. Blood glucose measurements were taken at 0, 15, 30, 45, 60 and 120 minutes.

Lectin injection

Functional graft vasculature was assessed by infusing fluorescein isothiocyanate-conjugated lectin into the beating left ventricle of mice hearts. Mice were injected with 100μl of undiluted lectin. Lectin was allowed to circulate for 1 minute. Then, the right ventricle was cut to allow blood flow decompression and a volume of 3ml of PBS was injected into the left ventricle, followed by 1ml of 4% PFA. The graft bearing EFPs were collected and fixed overnight in 4% PFA at 4°C. They were then maintained in 30% sucrose at 4°C until used for histology.

Immunohistological assessment of recovered grafts

Grafts were recovered, fixed in formalin and embedded in paraffin. Serial sections of 5μm were cut and processed for immunofluorescent staining. Tissue samples were permeabilized with 0.5% Triton X-100/PBS for 30 minutes, followed by 1-hour incubation in 0.5% BSA/PBS at room

temperature to block unspecific sites. Slides were then incubated with the following primary antibodies: guinea pig anti-insulin (1:100), rabbit anti-CD34 (1:2000), chicken anti-GFP (1:500), and rabbit anti-VEGF (1:100). The following secondary antibodies were then applied: donkey anti–guinea pig Alexa 555 Fluor-conjugated (1:300), donkey anti–guinea pig FITC-conjugated (1:200), donkey anti-rabbit Alexa 555 Fluor-conjugated (1:300) and goat anti-chicken Alexa Fluor 488 (1:500). Both primary and secondary antibodies were diluted in PBS-0.5% BSA. Finally, slides were mounted with aqueous mounting medium containing DAPI for nuclear staining. Slides were processed on a Zeiss Axioscan.Z1 slide scanner and a Zeiss Axiocam. To analyse vascularization, six pictures per condition were taken and the number of CD34+ cells were counted and normalized by the graft area.

Morphometric analysis was performed using Zen 2.3 Blue Edition software.

Real-time quantitative PCR

Graft bearing EFPs harvested at 3 and 30 days after transplantation were processed for PCR analysis. RNA was extracted using the RNeasy minikit and reverse transcribed with a High Capacity cDNA Reverse transcription kit. Gene amplification was performed by RT-PCR using TaqMan Fast Advance Master Mix. Primers used for amplification are listed in supplementary table 4. *RPLP1* was used as a housekeeping gene to normalize gene expression values. Data were calculated using the comparative cycle threshold Ct method ($2^{-\Delta Ct}$ method) and are expressed in arbitrary units.

Statistical analysis

Continuous variables are expressed as mean ± SEM. Comparison between groups were assessed with an unpaired Student's *t*-test. Cumulative number of animals reaching normoglycemia was compared using the log-rank (Mantel-Cox) test. A p value ≤ 0.05 was considered statistically significant. All statistical analyses were performed with the Prism software 8.0.

Results

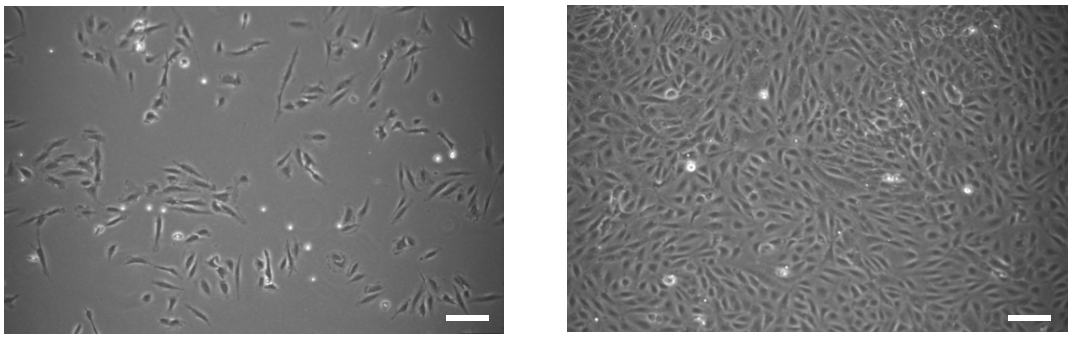
HUVEC characterization and transduction

HUVECs reached 80% confluence within 5 days with initial seeding density of 6000 cells/cm^2. Morphologically, cells displayed typical elliptic shape (Figure 1A) and were positive for von Willebrand factor and CD31 (Figure 1B). Endothelial origin of the cells was additionally confirmed by flow cytometry. Cells were positive for CD31 and CD144 (97.8% ± 0.7 and 98.1% ± 0.6 respectively) and negative for CD45 (95.8%) (Figure 1C).

When cultured on Matrigel, HUVECs formed well-shaped vascular-like structures over a period of 6 hours (Figure 1D).

To track HUVECs within organoids both *in vitro* and *in vivo*, cells were transduced with LVs carrying green fluorescent protein (GFP) gene under the control of the VEC promotor. HUVEC positivity for GFP was observed during culture and confirmed by flow cytometry with 86.6% of cells positive for GFP (Figure 1E right and left panel, respectively).

A



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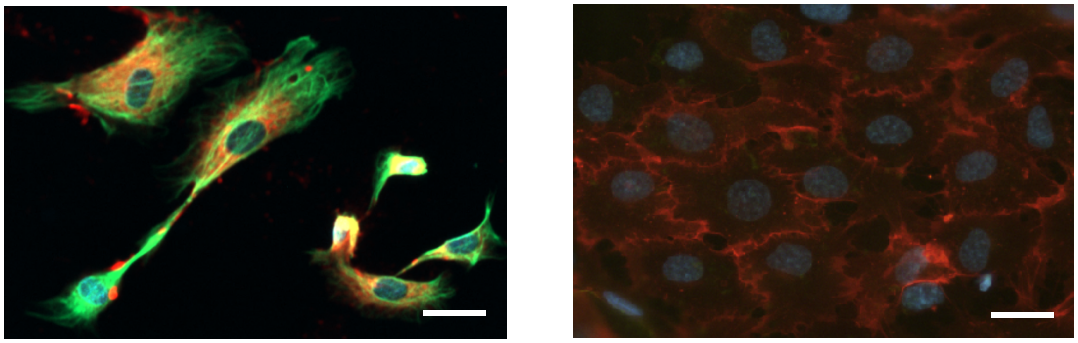


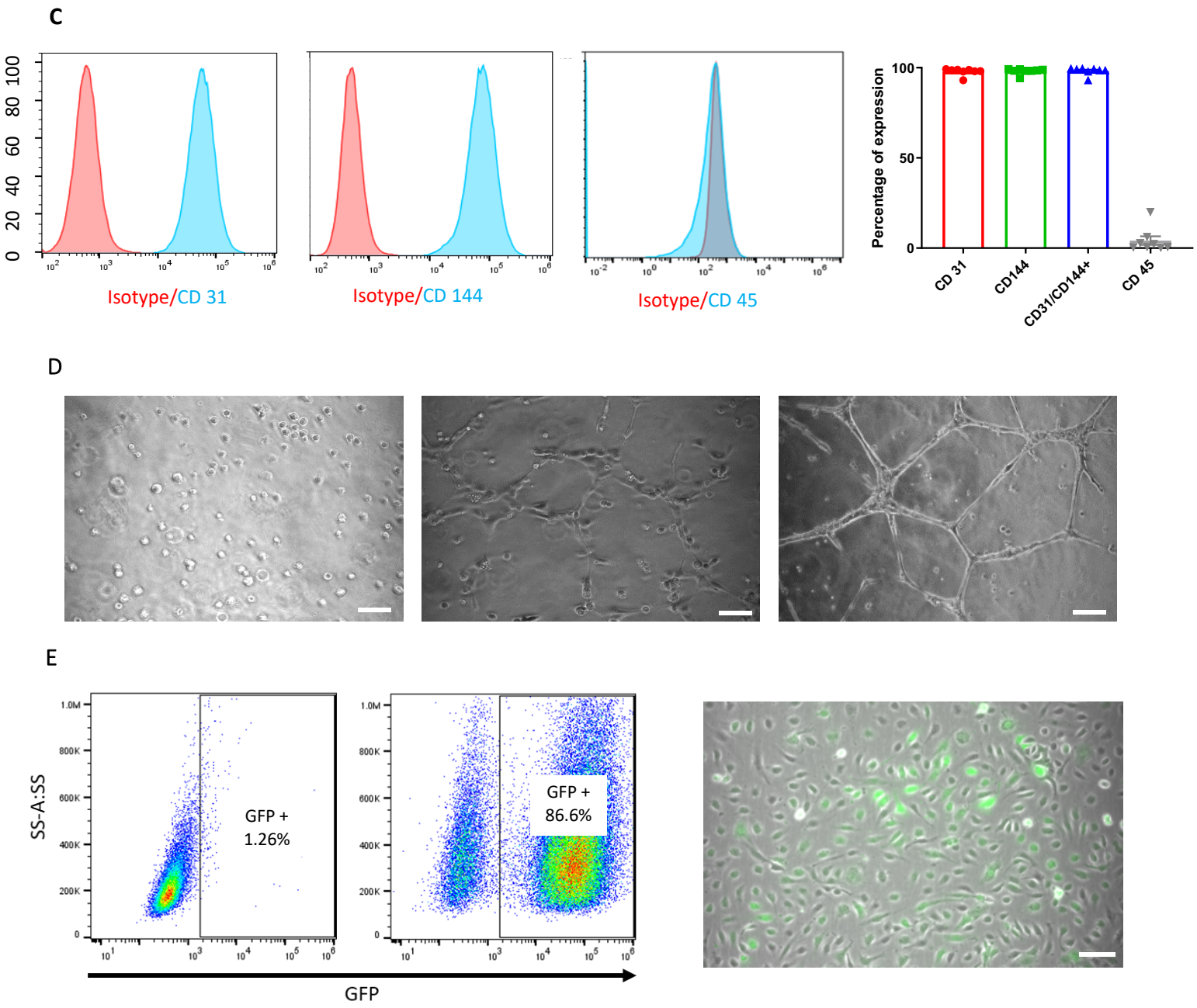


Figure 1. HUVEC characterization and *in vitro* functional assessment. **A**, Phase-contrast microscopic pictures of HUVEC in culture at day 1 and day 5. Scale bar = 50 µm. **B**, Immunofluorescence staining of cultured HUVEC with von Willebrand (red) and Vimentin (green, left panel) and CD31 (red, right panel). Nuclei are labelled with DAPI (blue). Scale bar = 25 µm. **C**, Flow cytometry analysis on HUVEC for CD31, CD144 and CD45 with their respective isotypes (left panels) and expressed as the percentage of positivity of expression on 8 consecutive preparations (mean ± SEM, right panel). **D**, Phase-contrast microscopic pictures of tube formation assessment on Matrigel at 0 hour, 2 hours and 6 hours. Scale bar = 50 µm. **E**, Assessment of GFP transduction success by flow cytometry analysis (left panel) and by phase-contrast microscopic images (right panel). GFP-positive cells are spontaneously green, scale bar = 50 µm.

hAECs characterization

hAECs used in this study were isolated from six different placentas. Flow cytometry analysis demonstrated strong positivity of hAECs for the embryonic cell surface marker SSEA-4 (88.4 ± 5.0%) and the epithelial cell adhesion molecule (CD326; 95.9 ± 1.3%). HLA-E and HLA-G were expressed in 16.9 ± 4.7% and 48.6 ± 12.3% of the cells, respectively. Finally, expression of CD105 and CD90 by hAECs were 17.6 ± 5.6%, 50.1 ± 7.1, respectively. The results of each hAEC preparation are described in Figure S1.

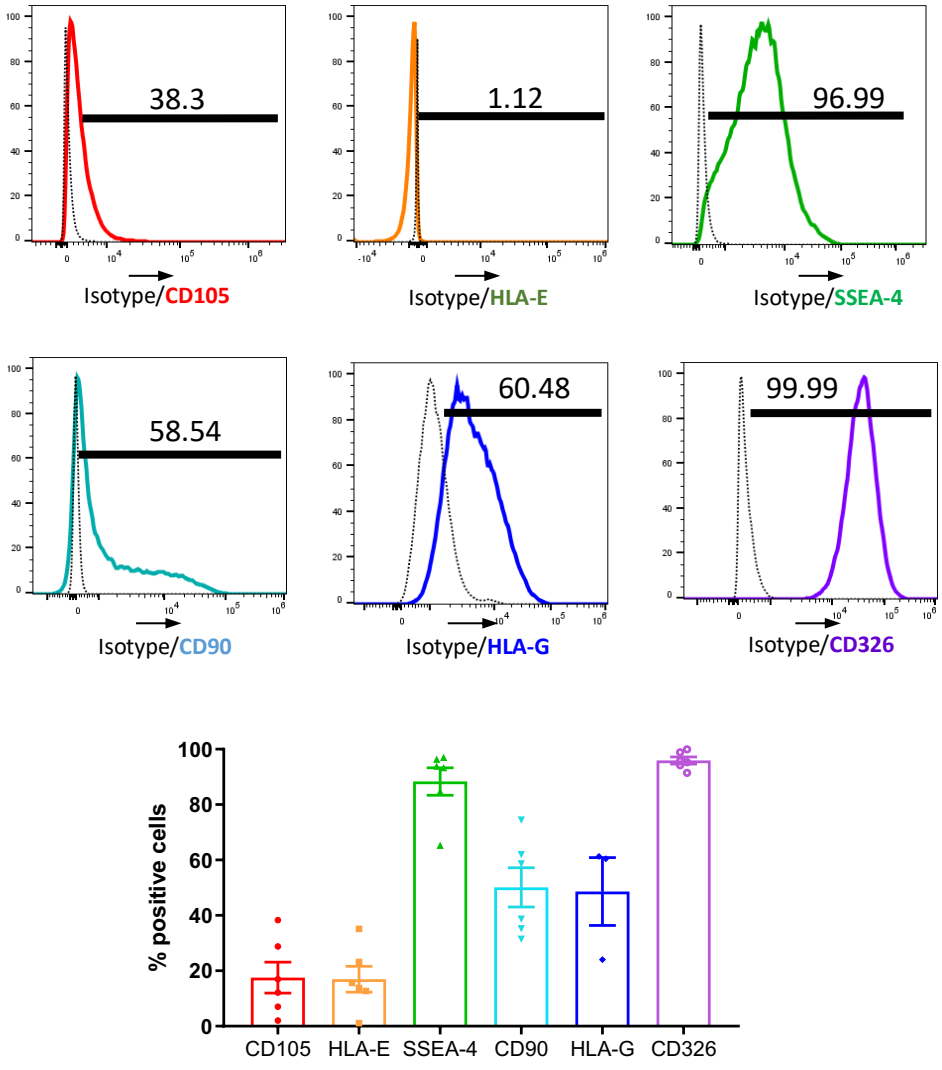
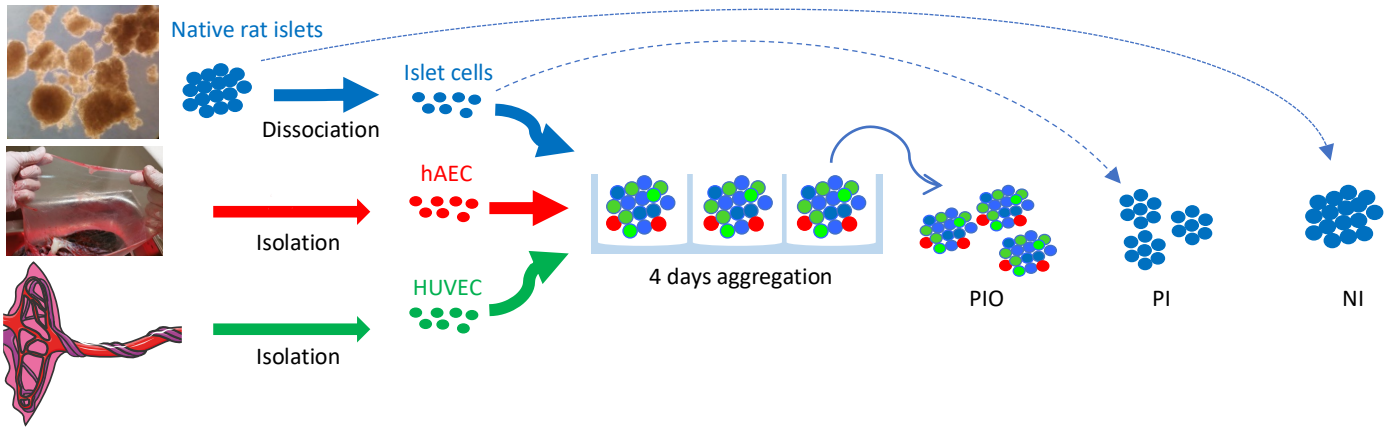


Figure S1. hAECs characterization by flow cytometry on 6 different placentas. Results are expressed as the percentage of positive cells for the following markers: CD105, HLA-E, SSEA-4, CD90, HLA-G and CD326. Three data are missing for HLA-G analysis.

Cellular composition, endocrine function and angiogenic activity of pre-vascularized islet organoids

Generation of PIO and PI is described in Figure 2A. Aggregation and incorporation of the different cell types occurred within 4 days (Figure 2B). Mean diameter of NI, PI and PIO was 144.4 ± 6.6, 105.8 ± 1.2 and 134.3 ± 2.3 μm, respectively (Figure 2C). NI showed the biggest heterogeneity in size. PI exhibited a significantly smaller mean diameter in comparison with PIO ($p < 0.001$), due to fewer cellular content. Cellular composition observed by confocal laser microscopy showed that all three cell types were present in the PIO (Figure 2D). The functional capacity of the constructs was evaluated by glucose-stimulated insulin secretion (GSIS) assay. PI and PIO demonstrated significantly improved insulin secretion in response of glucose stimulation (SI= 7.8 ± 1.5 and 7.7 ± 1.2), compared to NI (SI = 2.0 ± 0.5, $p=0.009$ and $p=0.004$, respectively). No significant difference was observed between PI and PIO (Figure 2E). In addition, total insulin content/IC was measured and compared between the three groups. PI and PIO demonstrated an increased insulin content/IC (0.01 ± 0.003 and 0.008 ± 0.002 pmol/l, respectively) in comparison with NI (0.002 ± 0.0004 pmol/l), however, only PI reached statistical significance ($p= 0.022$; Figure 2F).

A



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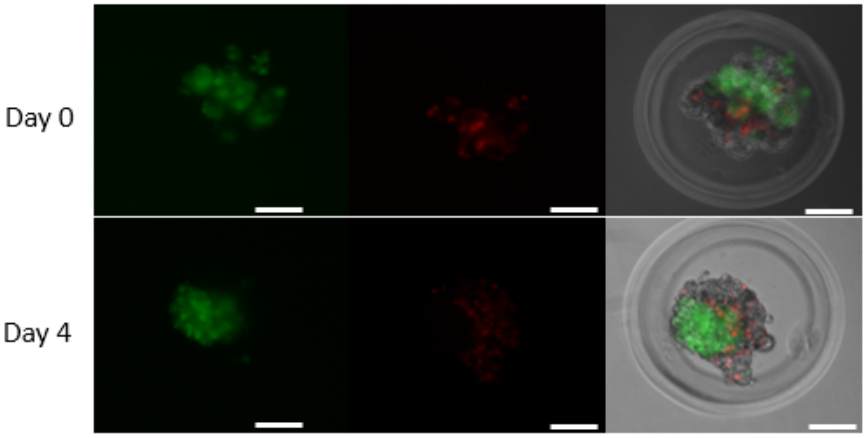
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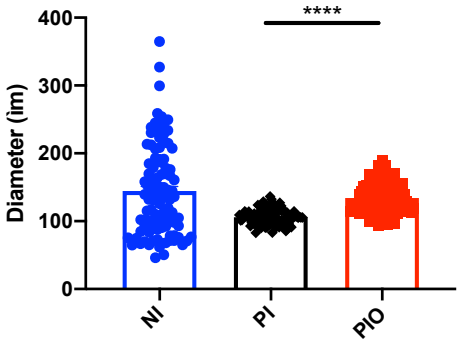
C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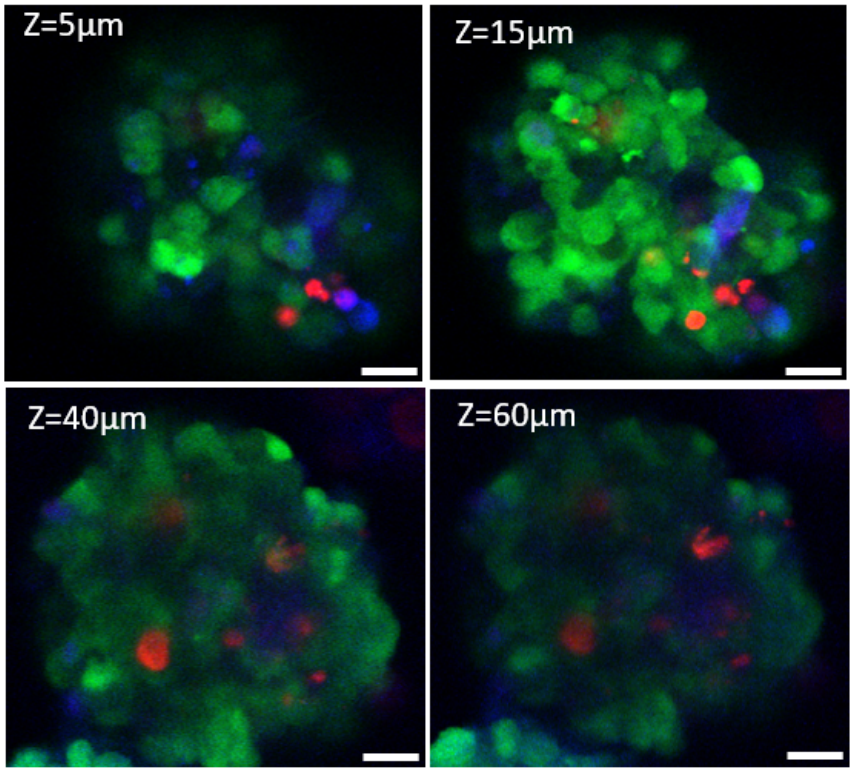
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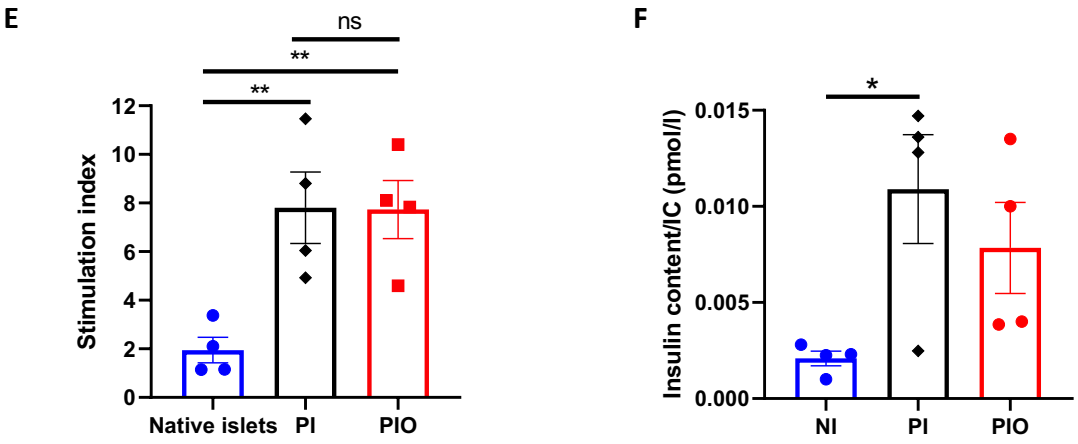


Figure 2. Organoids generation. **A,** Schematic representation of PI and PIO generation in culture. **B**, Light microscope pictures of PIO cultured in agarose mold at day 0 and day 4 with HUVECs previously transduced with GFP (green) and hAECs previously labelled with fluorescent carbocyanine dyes CM-DiL (red). Scale bar = 100 µm. **C**, Average diameter of each condition calculated at 4 days of culture (n=100/condition). **D**. Z-stacking images of PIO taken by spectral confocal laser. Islet cells are labelled in blue, HUVECs in green (GFP) and hAECs in red (CM-Dil). Scale bar = 25 µm. **E**. *In vitro* function assessed by GSIS and represented by the stimulation index (n=4). **F**. Total insulin content per IC in picomoles per liter. All data are expressed as mean ± SEM. *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, 2-tail unpaired Student *t* test.

To investigate the angiogenic potential of the PIO, collagen-based sprouting assays were performed. Our results demonstrated that PIO showed more extensive sprouting in surrounding matrix compared to IC+HUVEC (Figure S2). In contrast, no sprouting was observed from PI (data not shown). Furthermore, immunofluorescence revealed GFP positive cells confirming their endothelial nature.

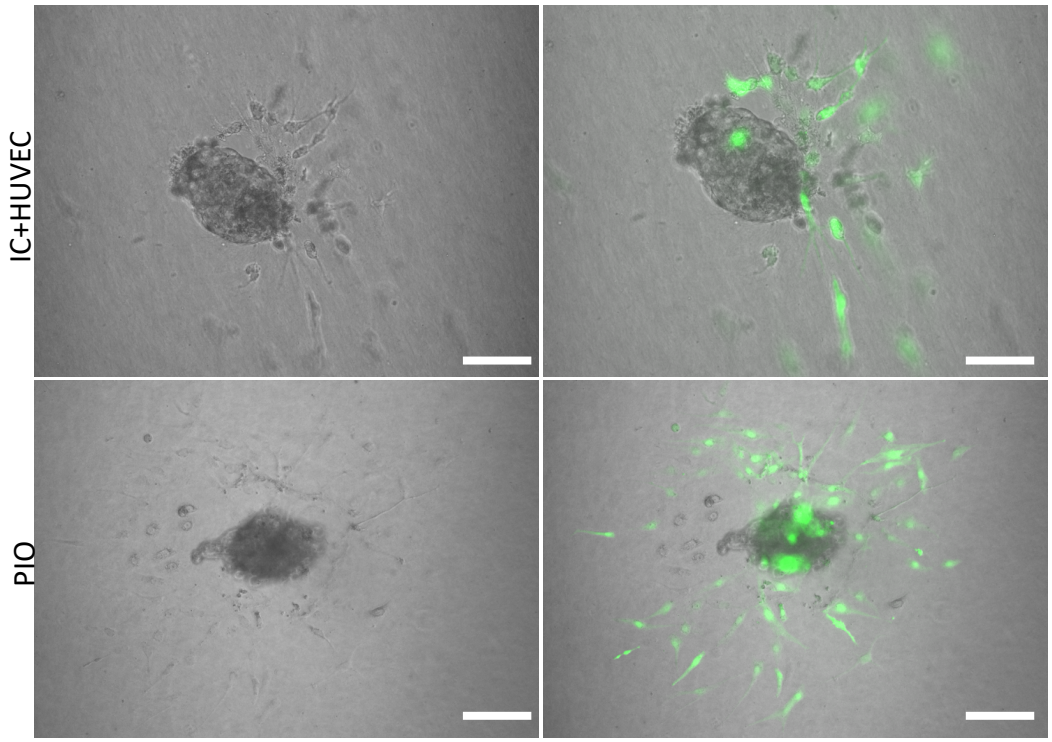


Figure S2. IC+HUVEC and PIO 3D sprouting assay. One hundred spheroids composed of IC + GFP-transduced HUVEC and 100 PIO were harvest after 4 days in culture and placed for 24 hours at 37°C in 5mg/ml placenta-derived hydrogel, supplemented with 200ng/ml of VEGF. Light microscope pictures of IC+HUVEC spheroids and PIO were taken inside the hydrogel. GFP-positive cells are spontaneously green. Scale bar = 75 µm.

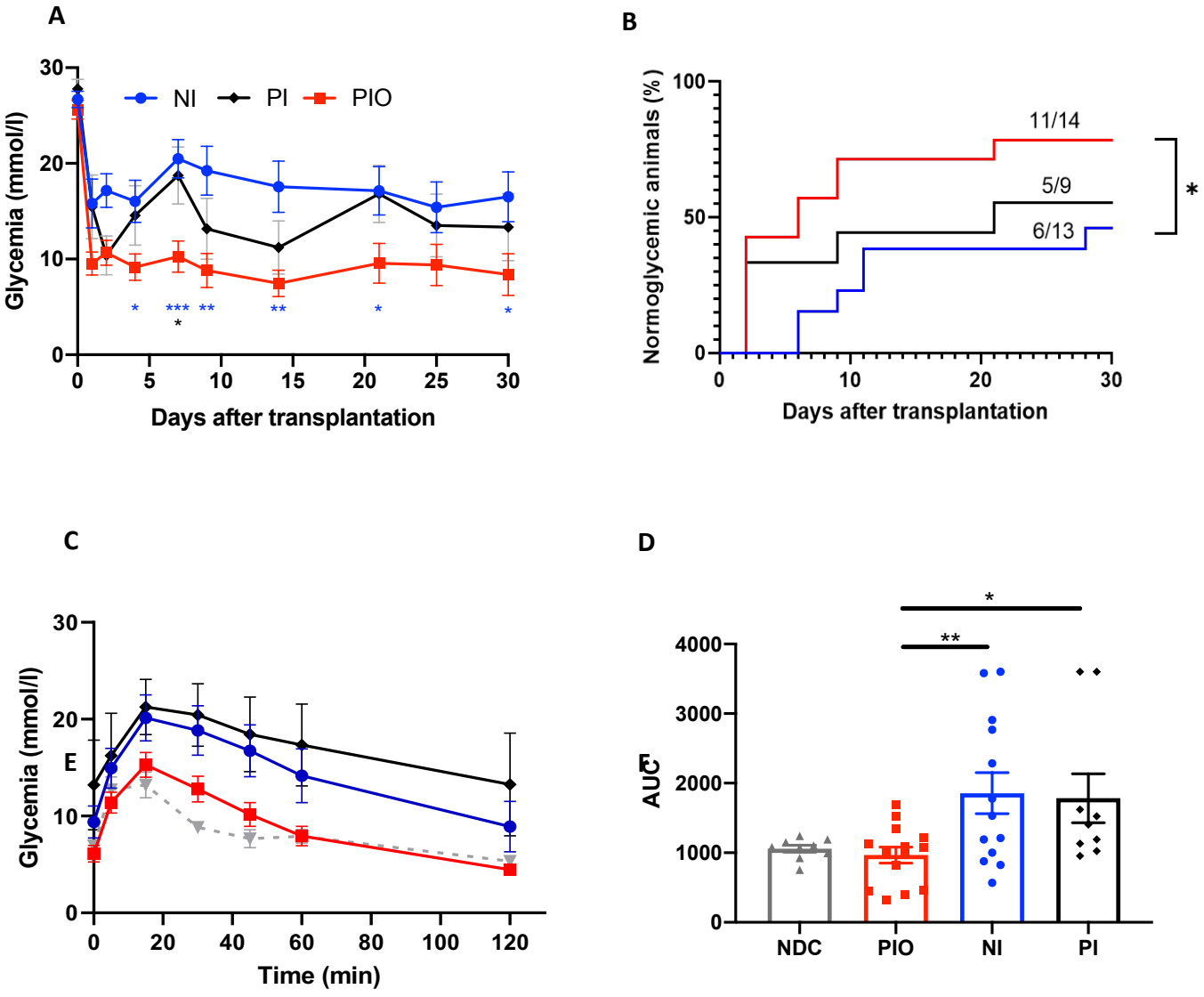
Pre-vascularized islet organoids improve glycaemic control in immunodeficient diabetic mice

To assess whether incorporation of hAECs and HUVECs into the islet organoids could promote engraftment and function *in vivo*, diabetic NOD–*Rag1*^{null} mice were transplanted with a marginal mass of PIO (n=14), NI (n=13) and PI (n=9). Mice transplanted with PIO demonstrated significant improvement of glycaemic control compared to both controls. Average blood glucose levels were significantly lower in the PIO group compared to NI at 4, 7, 9, 14, 21 and 30-days post transplantation (9.2 ± 1.4 vs 16.0 ± 2.2 mmol/L, p=0.013; 10.3 ± 1.6 vs 20.5 ± 2.0 mmol/L, p=0.0005; 8.8 ± 1.8 vs 19.2 ± 2.5 mmol/L, p=0.002; 17.6 ± 2.7 vs 7.5 ± 1.4 mmol/L, p=0.002; 17.1 ± 2.6 vs 9.6 ± 2.1 mmol/L, p=0.029 and 16.5 ± 2.6 vs 8.4 ± 2.2 mmol/L, p=0.024, respectively) and at 7 days in comparison to PI (10.3 ± 1.6 vs 18.7 ± 3.0 mmol/L, p=0.013) (Figure 3A). Normoglycemia was reached in 78.6% of animals (11/14) in the PIO group, in comparison with 55.6% (5/9) and 46.2% (6/13) for the PI and NI groups, respectively (PIO vs NI: p = 0.025; Figure 3B). Median time to achieve normoglycemia was 6 days in the PIO group, 21 days in the PI group and > 30 days in the NI group. To investigate secretory function of the graft, IPGTT was performed at 30 days post-transplantation. Mice transplanted with PIO and non-diabetic controls (NDC) showed lower blood glucose levels when compared to animals transplanted with PI and NI (Figure 3C). This is illustrated by the increasing AUC of the different groups, with PIO (966.8 ± 113.7), PI (1783 ± 351.1, p=0.016 vs PIO) and NI (1856 ± 294.5, p=0.008 vs PIO; Figure 3D).

We further investigated whether the improved glycemic control in the PIO group was associated with insulin production from the transplanted beta cells. Remarkable upregulation of rat insulin mRNA levels in the graft was found in the PIO group in comparison to controls (PIO vs. PI, p=0.0291, PIO vs. NI, p=0.0317; Figure 3E). These results were supported by insulin measurements in the serum taken from the same mice (Figure 3F). Although a statistical significance wasn't achieved, a ten-fold increase in insulin levels was detected in the PIO group (1259 ± 521pmol/l), in comparison to both controls (NI: 140.6 ± 22.1 pmol/l, PI: 159.8 ± 14.4pmol/l, p = ns)

GLP-1R, *Pdx1* are known to be critical for supporting β-cell survival and promoting insulin secretion (231-234). Therefore, we investigated whether these genes were involved in the improved secretory outcomes of PIO. Gene expression analyses revealed upregulation of genes

involved in β-cell survival (*Pdx1*, *PCSK1*, *PCSK2*) and function (*GLP-1R*) in PIO at 30 days post-transplantation, compared to controls (*Pdx1*: PIO vs PI, p=0.005, PIO vs native islet, p=0.005; *GLP-1R*: PIO vs PI, p=0.008, PIO vs native islet, p=0.008; *PCSK1*: PIO vs PI, p=0.039, PIO vs native islet p=0.043 and *PCSK2*: PIO vs PI, p=0.003, PIO vs native islet, p=0.004; Figure 3G). Interestingly, at an earlier time points (3 days), a similar increase in gene expression was observed in PI and PIO in comparison with NI group, although without reaching statistical differences (Figure S3). These data indicate that incorporation of accessory cells into the organoids supports long term survival and secretory function of β cells.



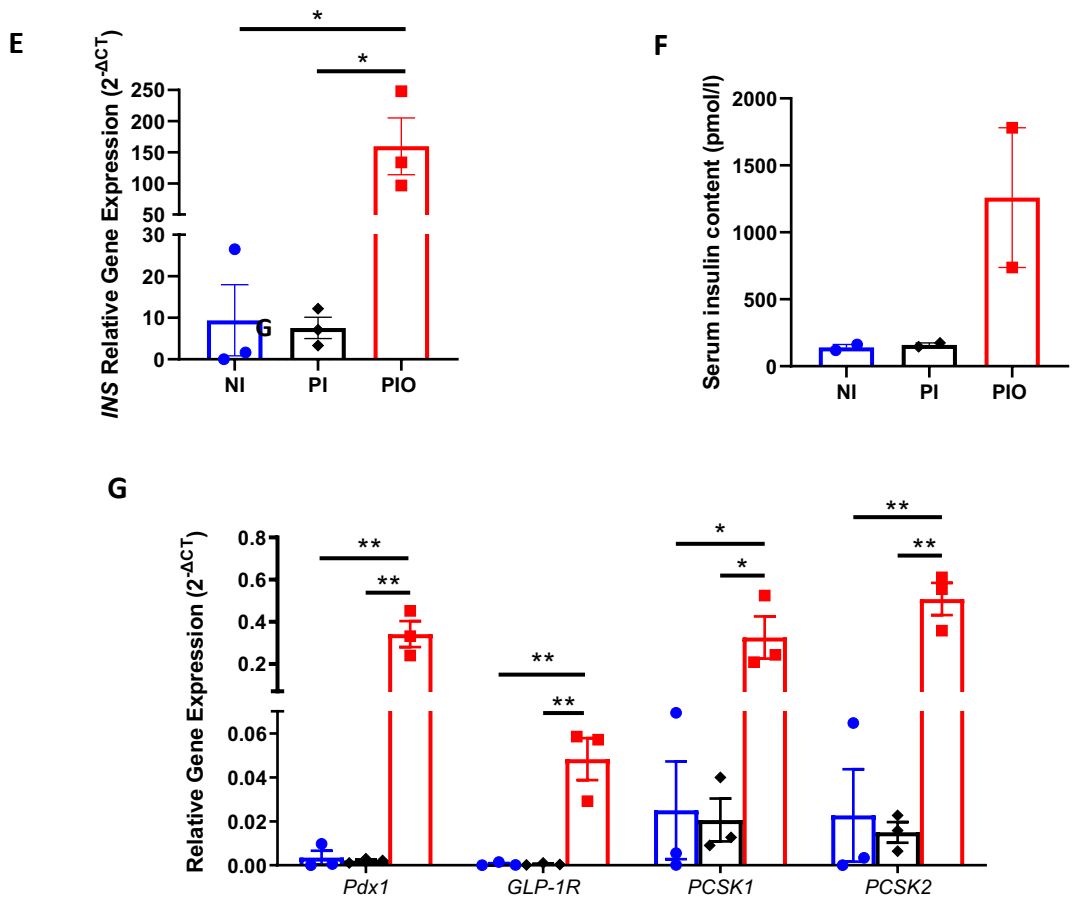


Figure 3. *In vivo* function of organoids in immunodeficient, diabetic mice. **A**. Glycemia level measured over 30 days in NOD-*Rag1*^{null} mice transplanted with 300 NI (n= 13, blue circle) and their equivalent number of PI (n=9, black diamond) and PIO (n=14, red square). Mean glucose level was compared at 4, 7, 9, 14, 21 and 30 days by a 2-tail unpaired Student *t* test. All data are expressed as mean ± SEM. *p < 0.05, ** p < 0.01, *** p < 0.001. **B**. Cumulative number of mice reaching normoglycemia over 30 days. Comparison made using the log-rank (Mantel-Cox) test, *p < 0.05. **C.** Glycemia level of each group during the intraperitoneal glucose tolerance test performed at 30 days post-transplantation (left panel) and their corresponding AUC values (right panel). Grey triangles represent the non-diabetic control (NDC) group (n=9). **E** and **G.** Gene expression by qPCR at 30-days post-transplantation of the following genes: *INS* (insulin), *Pdx1* (pancreatic and duodenal homeobox 1), *GLP-1R* (glucagon-like peptide-1 receptor), *PCSK1* (proprotein convertase 1) and *PCSK2* (proprotein convertase 2) in PIO, PI and NI (n=3 mice in each group). **F.** Insulin concentration measured by ELISA in mice serum at 30 days post-transplantation (n= 2 mice per group because of the unavailability of mice serum during one experiment). All data are expressed as mean ± SEM and comparisons were made using a 2-tail unpaired Student *t* test. *p < 0.05, ** p < 0.01.

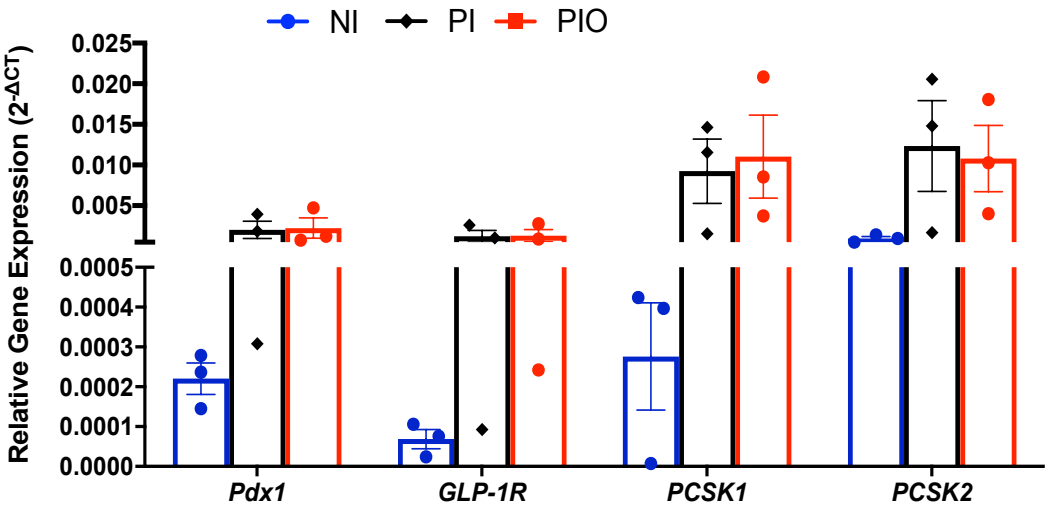


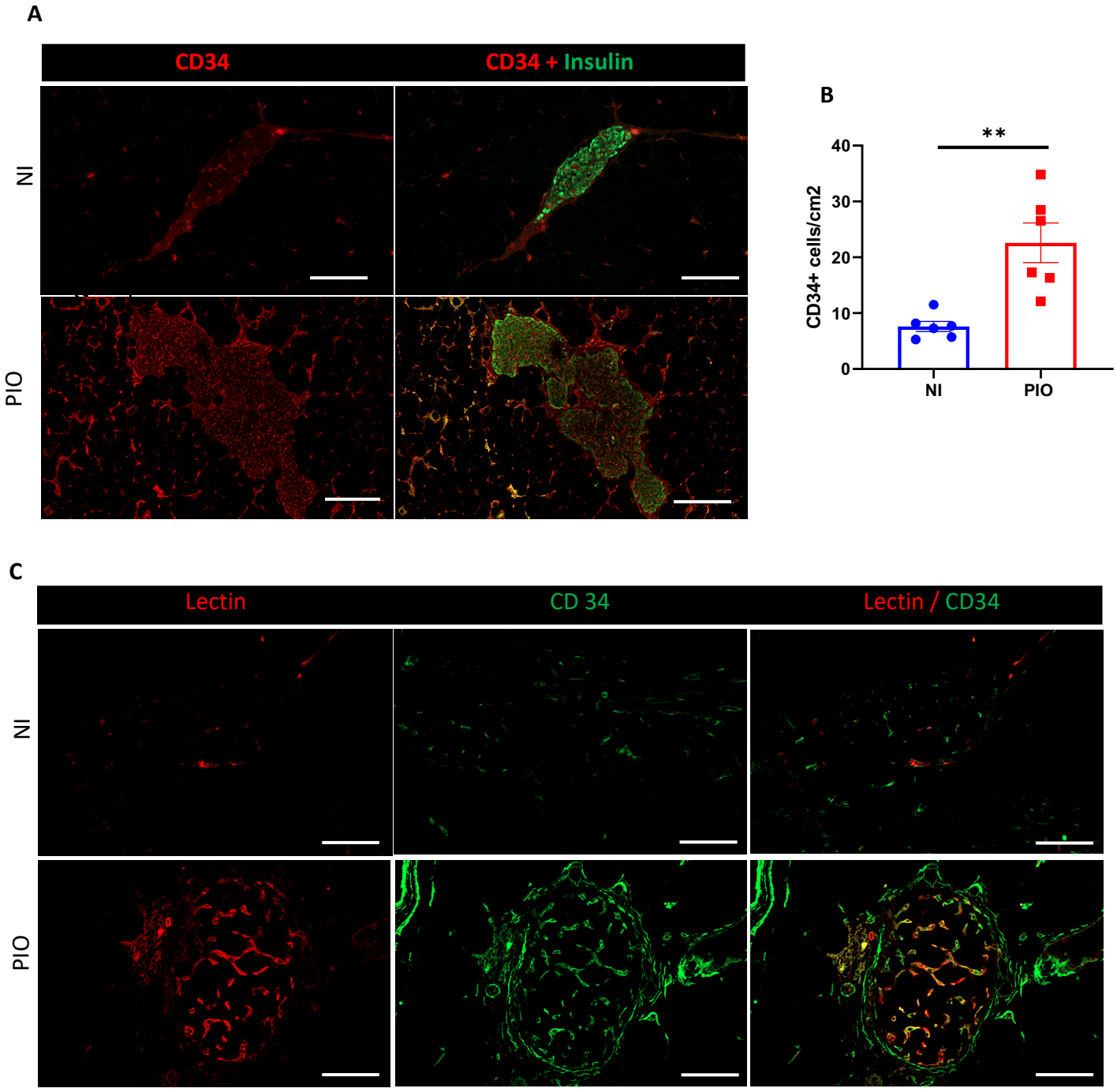
Figure S3. Gene expression by qPCR at 30-days post-transplantation of the following genes: *Pdx1* (pancreatic and duodenal homeobox 1), *GLP-1R* (glucagon-like peptide-1 receptor), *PCSK1* (proprotein convertase 1) and *PCSK2* (proprotein convertase 2) in PIO, PI and NI (n=3 mice in each group). All data are expressed as mean ± SEM

Transplantation of pre-vascularized islet organoids accelerates graft revascularization

To evaluate engraftment and revascularization, graft-bearing EFPs were removed at 30 days post-transplantation and processed for histology. Immunohistochemical staining for CD34, a marker for endothelial cells, showed that vessel density was significantly higher in the PIO samples (22.6 ± 3.5 CD34+cells/cm^2) than in the NI samples (7.6 ± 0.9, p= 0.002; Figure 4A-B). Furthermore, in the PIO group, vessels were observed not only around graft, but mainly within β cell positive area. To investigate whether the blood vessels formed within the engrafted tissue constructs become functional and contribute to graft perfusion, we used intravascular injection of fluorescently labeled Lectin. Histological assessment of the Lectin-perfused grafts demonstrated the presence of functional Lectin positive vascular network within the PIO, in contrast only few vessels were present within NI (Figure 4C).

Next, we examined the mechanisms by which supportive cells (HUVECs and hAECs) contributed to rapid neovascularization of the graft. To this end, we investigated whether these cells might induce the production of angiogenic factors, such as VEGF-A (Figure 4D). We observed, that rat

VEGF-A mRNA expression was significantly higher in PIO group (0.365 ± 0.033AU) compared to NI (0.038 ± 0.005 AU; p= 0.0006) group. This finding was further confirmed by immunohistochemical staining for VEGF of recovered samples, demonstrating higher fluorescent intensity in the PIO compared to NI (Figure 4E). These data indicate that incorporation of HUVEC and hAEC into PIO contribute to graft revascularization.



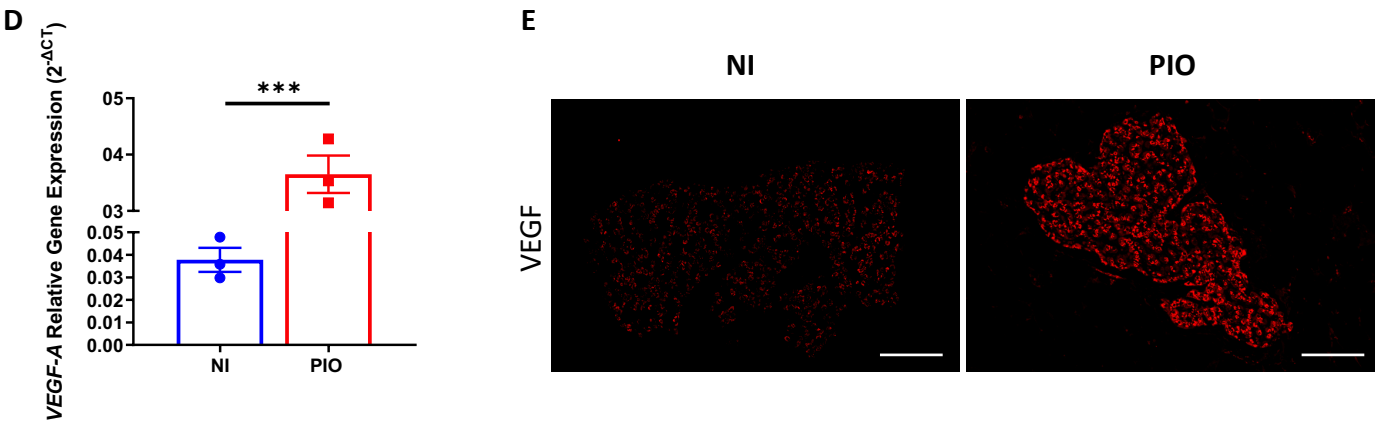


Figure 4. *In vivo* revascularization assessment by immunohistological analysis. **A.** The blood vessels of the graft detected at day 30 post-transplantation using CD34 (red) and insulin (green) immunostaining. Grafts Scale bar = 50 µm. **B.** Quantitative analysis of revascularization was achieved by calculating the number of CD34 positive cells in the insulin positive area and the result was divided by the graft surface area. This was realized in two graft regions per mouse and in 3 mice per group. Comparisons were made by a 2-tail unpaired Student *t* test. All data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$. **C.** Assessment of vessel functional capacity by mice injection of 100 µl of lectin. Capillaries are labelled in red and endothelial CD34 + cells in green. Scale bar = 50 µm. **D.** *VEGF-A* gene expression by qPCR at 30-days post-transplantation in PIO, PI and NI groups (n=3 mice in each group, at each time point). Comparisons were made by a 2-tail unpaired Student *t* test. All data are expressed as mean ± SEM. *** $p < 0.001$. E. The grafts stained for VEGF-A at day 30 after transplantation. Scale bars = 100 µm.

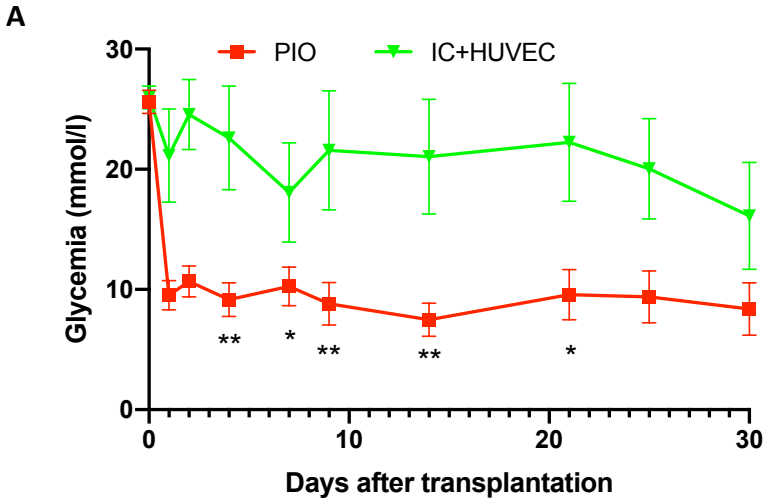
hAEC incorporation into organoids improve function and HUVEC-derived revascularization

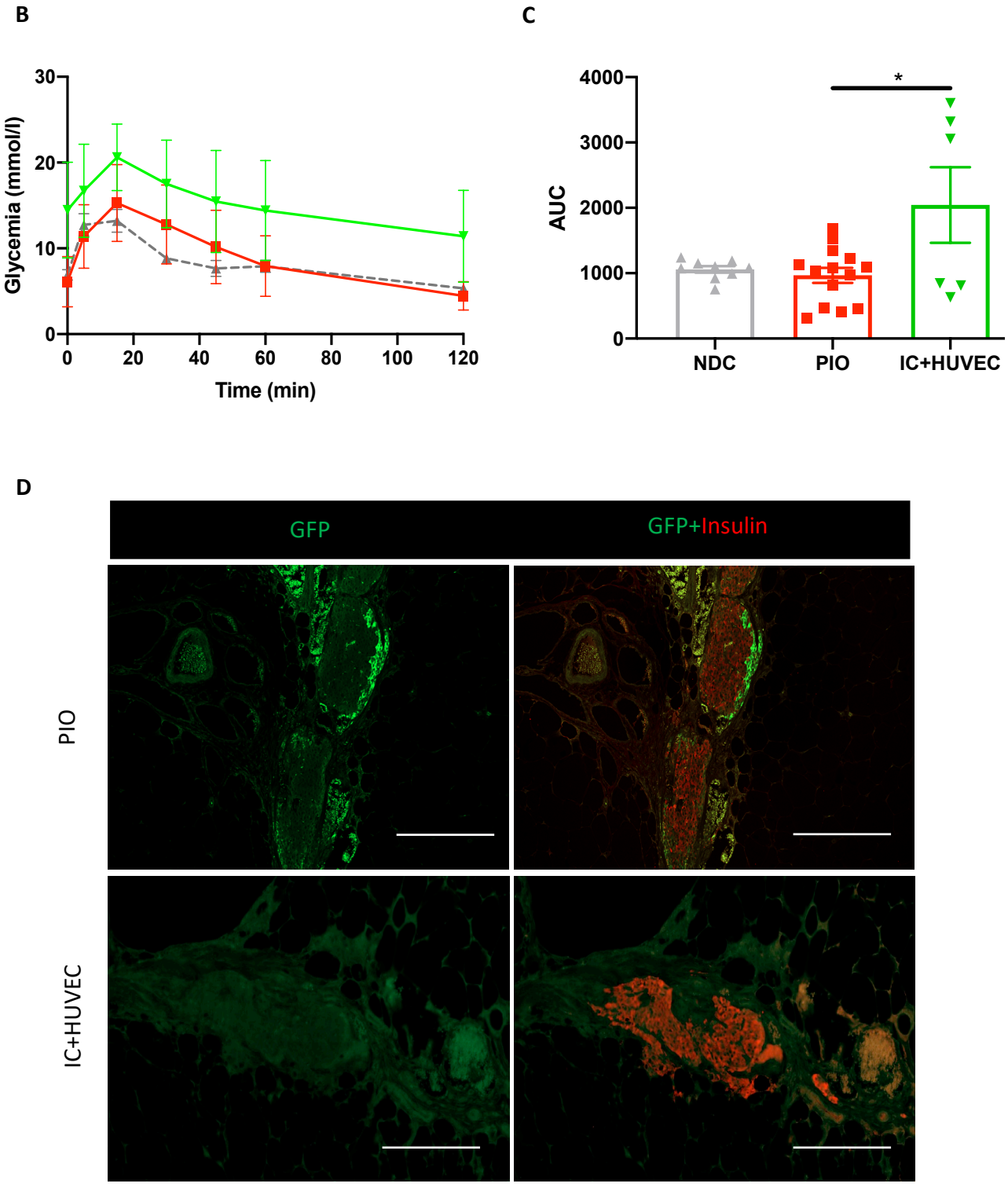
Finally, we evaluated whether incorporation of hAEC into the organoids was essential for the engraftment and vascularization of the PIO. To this end, we added an additional group of mice transplanted with spheroids composed of IC: HUVEC (1:1 ratio) to the three existing groups.

Figure 5 summarizes the results obtained with this group. Blood glucose control was significantly lower in the IC+HUVEC group in comparison to the PIO group at several time points (**day 4** : 22.6 ± 4.3 vs 9.2 ± 1.4 mmol/l, p=0.001; **day 7**: 18.1± 4.1 vs 10.3 ± 1.6 mmol/l, p=0.044; **day 9**: 21.6 ± 5.0 vs 8.8 ± 1.8 mmol/l, p=0.007, **day 14**; 21.1 ± 4.8 vs 7.5 ± 1.4 mmol/l, p=0.002; **day 21**: 22.2 ± 4.9 vs 9.6 ± 2.1 mmol/l, p=0.011, Figure 5A). The IPGTT performed at 30 days post-transplantation

demonstrated a poor glucose clearance in the IC+HUVEC group (Figure 5B). Response to increased blood glucose levels was significantly lower than for the PIO group as demonstrated by the AUC (2044 ± 578.1 vs 966.8 ± 113.7, p=0.016, respectively; Figure 5C).

After demonstrating that incorporation of supportive cells into the PIO improved graft revascularization, we investigated the degree to which these cells were contributing to new vessel development in the graft. To easily identify donor-derived new vessels, GFP-transduced HUVECs were incorporated into the PIO. Graft-bearing EFPs were recovered at 30 days post-transplantation and processed for immunohistological analysis. Interestingly, GFP positive cells were found inside the graft in the PIO group, while none was found in the IC+HUVEC group (Figure 5D). Both human and mouse vessels were positively stained by anti-CD34 confirming the establishment of anastomoses between donor derived HUVECs and mouse blood vessels. Furthermore, GFP/CD34 double positive endothelial cells were found at the graft periphery, inside capillaries containing erythrocytes, indicating that HUVECs were able to migrate and merge with a murine vascular system, forming functionally perfused blood vessels, as shown in Figure 5E. These data indicate that hAECs support HUVECs inside the organoids and thus contribute to accelerated revascularization.




B
Glycemia (mmol/l)
30
20
10
0
0
20
40
60
80
100
120
Time (min)
C
AUC
4000
3000
2000
1000
0
*
NDC
PIO
IC+HUVEC
D
GFP
GFP+Insulin
PIO
IC+HUVEC

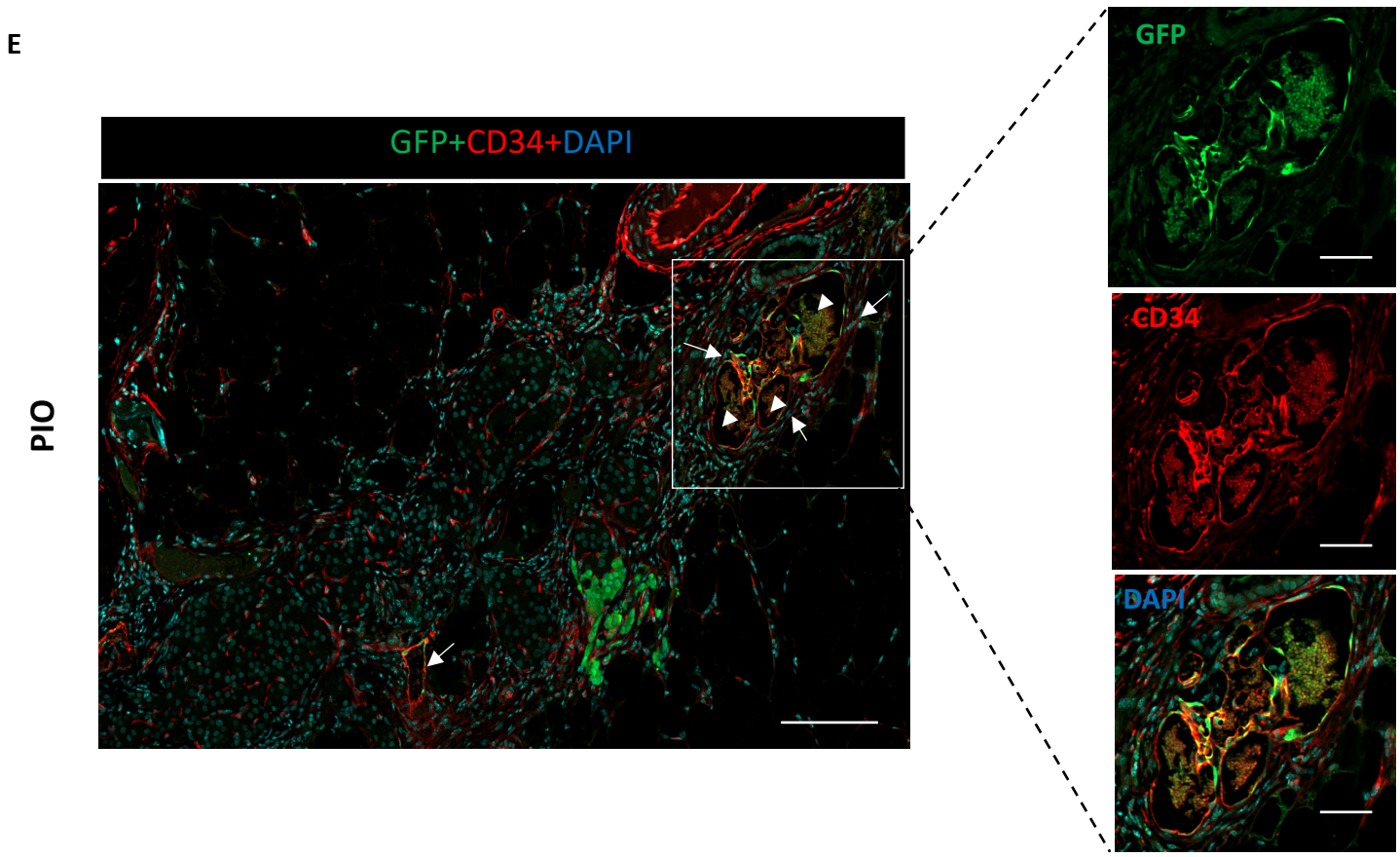


Figure 5. *In vivo* function of IC+HUVEC spheroids, in immunodeficient, diabetic mice. **A**. Mean glucose levels measured in NOD-*Rag1*^{null} mice transplanted with PIO (n=14, red square) and IC+HUVEC (n=6, green inverted triangle). Mean glucose level was compared at 4, 7, 9, 14, 21 and 30 days post-transplantation by a 2-tail unpaired Student *t* test. All data are expressed as mean ± SEM. *p < 0.05, ** p < 0.01. **B** and **C**. Intraperitoneal glucose tolerance test performed at 30 days post-transplantation and their corresponding AUC. Grey triangle represents the non-diabetic control (NDC) group (n=9). Comparisons were made by a 2-tail unpaired Student *t* test. All data are expressed as mean ± SEM. * p < 0.05, ** p < 0.01. **D.** Graft-bearing EFP recovered at 30 days post-transplantation and stained for GFP (green) and insulin (red). Scale bar = 100 µm. **E**. Immunohistological staining for GFP (green), CD34 (red) and DAPI (blue). The yellow color represents the GFP-HUVECs with positive staining of anti-CD34. Arrows indicate chimeric blood vessels. Arrowheads indicate red blood cells. Scale bar for top panel = 100 µm and for the 3 bottom panels, 20 µm.

Discussion

Impaired and delayed revascularization of the graft is a major issue in islet transplantation and represents a main limitation to the search for extrahepatic sites for islet transplantation. Common vascularization strategies focus either on the combination of accessory cells with islets (235) or incorporation of endothelial cells into islet-like constructs generated from embryonic stem cell-derived ß cells (21) or ß cell lines (22), and are mainly based on *in vitro* testing. In this study, we successfully generated functional pre-vascularized islet organoids using multiple cell types. The major finding of this study is that incorporation of hAECs and HUVECs into insulin-producing organoids hastens the rate of graft revascularization, and subsequently results in better engraftment of the β-cell mass.

HUVECs are the most commonly used, robust source of human endothelial cells in regenerative medicine and tissue engineering (236). However, limited proliferative potential of these cells hinders their clinical application. hAECs isolated from the amniotic membrane of discarded placenta is considered a non-controversial stem cell source (153). These cells demonstrated profound anti-fibrotic, anti-inflammatory, non-tumorigenic and low antigenic properties (237). Furthermore, hAECs possess pluripotent stem cells characteristics, can be isolated in large quantities and are thus considered as an evolving therapeutic tool for the development of various clinical applications (237). Previously, we have shown that the generation of insulin-secreting organoids from primary IC in combination with hAECs improved islet engraftment and vascularization primarily by stimulating VEGF-A production from the graft via HIF1- α signaling pathway (122, 211). Therefore, in this study, we evaluated whether hAECs could accelerate the angiogenic potential of mature endothelial cells (HUVECs). Our results show that chimeric, pre-vascularized insulin secreting organoids are capable of establishing new vascular networks *in vitro* and *in vivo* when co-cultured with hAECs and HUVECs. The enhancement of the angiogenetic potential of HUVECs by hAECs can be explained by three possible mechanisms : (i) via the secretion of ECM-degrading proteases facilitating EC migration and sprouting (238), (ii) by up-regulating VEGF expression in endothelial and islet cells (239), and (iii) by the reduction or suppression of inflammatory responses (240, 241). Our *in vivo* experiments have demonstrated the superiority of pre-vascularized islet organoids for insulin secretion and revascularization.

Another important finding is the existence of a cross-talk between the islet, endothelial and amniotic epithelial cells associated within one organoid (summarized in Figure 6), and that this communication can be successfully employed for improving outcomes of islet transplantation. In terms of revascularization, we observe that both blood vessel density and number of functional vessels were significantly higher in the grafts explanted from mice transplanted with PIO in comparison to control groups. VEGF-A is a proangiogenic factor that recruits endothelial cells and circulating endothelial progenitors (25). Our results demonstrated significant upregulation of VEGF-A gene expression in the grafts explanted from mice transplanted with pre-vascularized organoids. Immunohistochemical analysis of the explanted grafts confirmed that the major producers of VEGF-A were islet cells. This finding was in agreement with our previous studies (10), demonstrating that hAECs markedly increase production of VEGF-A in islet cells via paracrine signalling. In addition, hAECs themselves are known to secrete VEGF-A (242), which on the other hand could also enhance performance of HUVECs within the organoids. To verify this hypothesis, we used GFP-HUVECs and tracked transplanted cells inside the graft. We found GFP- HUVECs both inside and in the vicinity of the graft. At the same time, GFP- HUVECs were also detected to be integrated into the peri-islet functional blood vessels containing red blood cells. This indicates that the donor derived endothelial cells anastomosed with the murine vascular system and formed functionally perfused blood vessels. Interestingly, the same was not observed in mice transplanted with IC+HUVECs, in which no GFP-HUVECs were found in the recovered grafts. In addition, almost no blood circulation was observed inside the graft area. This indicates that hAECs contribute to the process of endothelial cell remodelling and stabilization finally leading to mature vessel formation. Our findings are in agreement with previously reported data, demonstrating that hAECs enhance EC viability, function, proliferation, migration and blood vessel formation *in vitro* and *in vivo* (242). Furthermore, amniotic cells secrete additional factors that are critical for angiogenesis, such as EGF, HB-EGF, bFGF, HGF, IGF-1 (243). Taken together, these data suggest that hAECs promote revascularization both directly by secreting angiogenic factors and indirectly by stimulating VEGF-A secretion by islet cells.

Accelerated revascularization can also provide important survival cues to the islet cells. Another important challenge to islet transplantation is to achieve stable, long-term insulin independence,

preferably with single donor islet transplantation. In this study, improved revascularization was accompanied by prompt return of severely diabetic mice to a normoglycemic state after transplantation of minimal mass of pre-vascularized islet organoids. Mice transplanted with PIO showed significantly improved insulin secretion and better glucose clearance compared to mice transplanted with PI, NI and IC+HUVECs. Investigations of underlying mechanisms showed that superior function of β-cells in PIOs was mediated by the GLP-1R signalling pathway. GLP-1R has been found to regulate homeostasis of β-cell mass by inducing β-cell proliferation and protecting against apoptosis. On the other hand, activation of the GLP-1R leads to the activation of multiple downstream pathways, including EGF receptor signalling (244), which in turn stimulates proliferation of β cells (245). EGF has been shown to enhance glucose-dependent insulin secretion and upregulate PDX1 expression (20). Although the precise mechanisms underlying this pattern of increased gene expression in the PIOs are not fully understood, we speculate that growth factor expression profile of hAECs, mainly EGF, could stimulate upregulation of the expression of genes involved in β-cell function (GLP-1R) and survival (PDX-1).

In this study, we demonstrate a novel approach to generate pre-vascularized islet organoids by combining primary ICs with two additional supportive cell types, HUVECs and hAECs, and address some of the challenges of clinical islet transplantation such as donor supply scarcity, impaired islet engraftment and revascularization. Furthermore, our data demonstrate that hAECs not only promote cell viability and engraftment, but most importantly, play a primordial supporting role in the development of HUVEC-derived neo-vessels within the transplanted tissue. Taken together, these findings could be a basis for the design of novel extra-hepatic, extra-vascular islet transplantation sites.

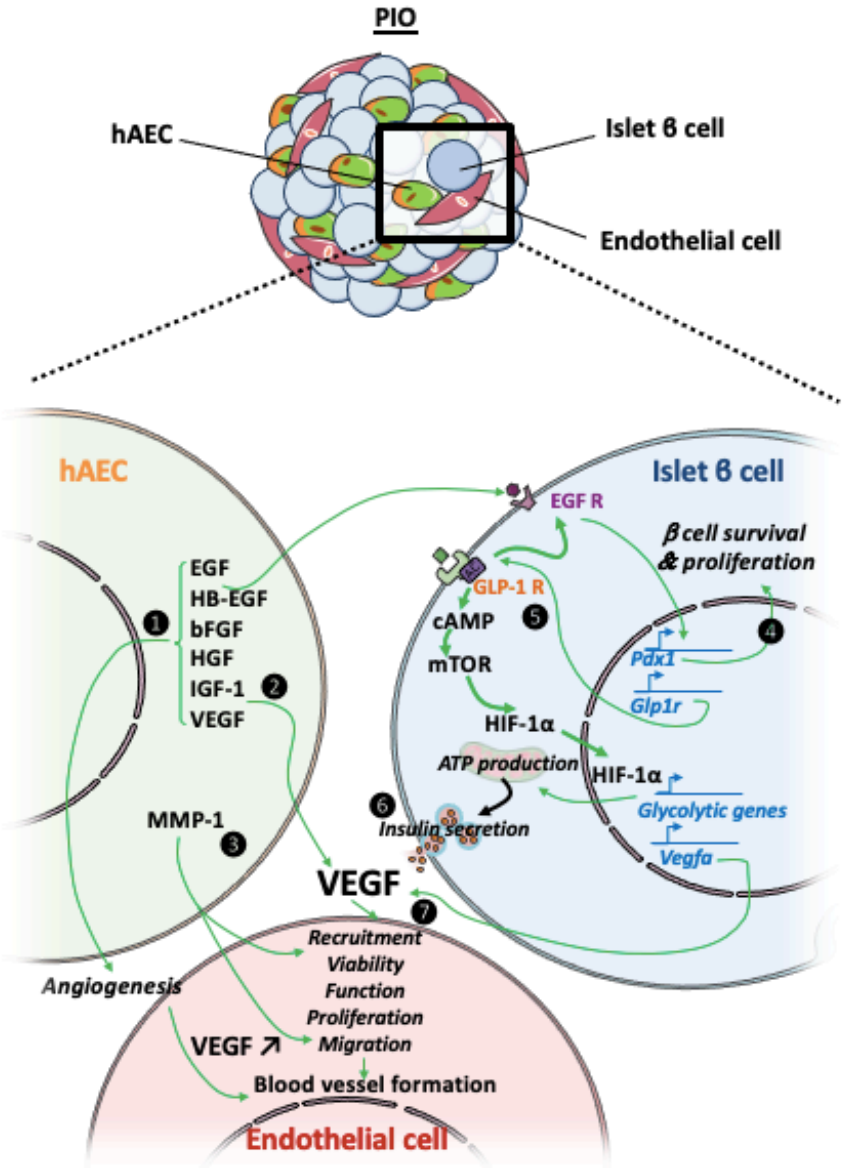


Figure 6. Crosstalk between the hAEC, the endothelial cell (EC) and the islet β cell (IC) within the PIO. hAEC enhances revascularization of the PIO in a direct manner by secreting ① angiogenic factors and ② VEGF that improve EC viability, function, proliferation and blood vessel formation, and ③ by producing ECM-degrading proteases (MMP-1) that facilitate EC migration and sprouting. Additionally, hAECs secrete EGF that ④ upregulates IC *Pdx1* expression, leading to higher IC survival and proliferation, as well as ⑤ GLP1-R expression, leading to an up-regulation of glycolytic genes and VEGF-A through the mTOR/HIF-1a pathway, resulting in ⑥ an improved insulin secretion and ⑦ a better revascularization of the PIO.

Supplementary tables

Supplementary table 1. Primary and secondary antibodies

	Antibody	Company	City	Country	Product number	Application	Dilution
Primary antibody	PerCP-Cy 5.5 Mouse anti-SSEA-4	BD Biosciences	Allschwil	Switzerland	561565	FC	1:50
	PE-Cy 7 Mouse anti-human CD90	BD Biosciences	Allschwil	Switzerland	561558	FC	1:100
	FITC Mouse anti-human CD105	BD Biosciences	Allschwil	Switzerland	561443	FC	1:50
	BV421 Mouse anti-human CD326	BD Biosciences	Allschwil	Switzerland	563180	FC	1:50
	PE anti-human HLA-E	Biolegend	London	UK	342604	FC	1:50
	APC anti-human HLA-G	Biolegend	London	UK	335910	FC	1:50
	PE anti-human CD31	Biolegend	London	UK	303106	FC	1:30
	AlexaFluor 657 CD144	BD Biosciences	Allschwil	Switzerland	561567	FC	1:40
	PerCP-Cy 5.5 anti-human CD45	Biolegend	London	UK	368504	FC	1:25
	PerCP-Cy 5.5 mouse IgG1, κ Isotype Ctrl	Biolegend	London	UK	400150	FC	isotype 1:60
	PE mouse IgG1, κ Isotype Ctrl	Biolegend	London	UK	400112	FC	isotype 1:40
	AlexaFluor 657 mouse IgG1, κ Isotype Ctrl	BD Biosciences	Allschwil	Switzerland	557714	FC	isotype 1:100
	PerCP-Cy 5.5 Mouse IgG3, κ Isotype Ctrl	BD Biosciences	Allschwil	Switzerland	561572	FC	1:50
	PE-Cy 7 Mouse IgG1, κ Isotype Ctrl	BD Biosciences	Allschwil	Switzerland	557872	FC	1:100
	FITC Mouse IgG1, κ Isotype Ctrl	BD Biosciences	Allschwil	Switzerland	555748	FC	1:50
	BV421 Mouse IgG1, κ Isotype Ctrl	BD Biosciences	Allschwil	Switzerland	562438	FC	1:50
	PE Mouse IgG1, κ Isotype Ctrl	Biolegend	London	UK	400112	FC	1:50
	APC Mouse IgG2a, κ Isotype Ctrl	Biolegend	London	UK	400222	FC	1:50
	Purified rabbit anti-human CD34	Abcam	Cambridge	UK	ab81289	FC, IF	1:50 (FC), 1:2000 (IF)
	Rabbit polyclonal anti wide spectrum Cytokeratin	Abcam	Cambridge	UK	ab9377	IF	1:75
	GuineaPig anti-insulin	DakoCytomation	Baar	Switzerland	A0564	IF	1:100
	Rabbit anti-human VEGF	Santa-Cruz biotechnology	Dallas	USA	SC-152	IF	1:100
	Mouse anti-vimentin	DakoCytomation	Baar	Switzerland	M0725	IF	1:50
	Mouse anti-human CD31	DakoCytomation	Baar	Switzerland	M0823	IF	1;50
	Rabbit anti human Von Wilderbrand Factor	DakoCytomation	Baar	Switzerland	A0082	IF	1:100
	Chicken anti-GFP	Abcam	Cambridge	UK	ab13970	IF	1;500
	Rabbit anti-GFP	Abcam	Cambridge	UK	ab6556	IF	1;500
Secondary	AlexaFluor 488 goat anti-mouse IgG	ThermoFisher Scientific	Reinach	Switzerland	A11001	IF	1:300
	AlexaFluor 488 goat anti-chicken IgG	Abcam	Cambridge	UK	ab150173	IF	1;500
	AlexaFluor 488 goat anti-rabbit IgG	ThermoFisher Scientific	Reinach	Switzerland	A11008	IF	1:300
	AlexaFluor 555 goat anti-rat IgG	ThermoFisher Scientific	Reinach	Switzerland	A21434	IF	1:300
	AlexaFluor 555 donkey anti-rabbit IgG	ThermoFisher Scientific	Reinach	Switzerland	A31572	IF	1:300

	AlexaFluor 555 goat anti-guinea pig IgG	ThermoFisher Scientific	Reinach	Switzerland	A21435	IF	1:300
	AlexaFluor 555 donkey anti-mouse IgG	ThermoFisher Scientific	Reinach	Switzerland	A31570	IF	1:300
	Donkey anti-guinea pig Fluorescein IgG	Jackson ImmunoResearch Laboratories	Rheinfelden	Switzerland	706-095-148	IF	1:200
	Donkey anti Mouse Rhodamine IgG	Jackson ImmunoResearch Laboratories	Rheinfelden	Switzerland	715-025-150	IF	1:200
	Goat anti GuineaPig Fluorescein IgG	Jackson ImmunoResearch Laboratories	Rheinfelden	Switzerland	106-095-003	IF	1:200

FC: Flow cytometry, IF: immunofluorescence, UK: United Kingdom

Supplementary table 2. Culture medium, reagents and materials

	Reagents	Company	City	Country	Product number	Concentration
HUVEC culture medium	Medium 199	ThermoFisher Scientific	Reinach	Switzerland	21180021	1X
	Fetal bovine serum (FBS)	Merk Millipore	Zug	Switzerland	s0115	10% v/v
	L-Glutamin-Penicillin-Streptomycin 10X	Sigma Aldrich	Buchs	Switzerland	G1146	Details of the product in the legend§
	fungin	Invivogen	San Diego	USA	ant-fn-2	0.1% v/v
	Endothelial cell growth supplement (ECGs)	Sigma Aldrich	Buchs	Switzerland	E2759	30ug/ml
	Heparin					100ug/ml
hAEC culture medium	Fetal bovine serum (FBS)	Merk Millipore	Zug	Switzerland	s0115	10% v/v
	Sodium Pyruvate	Sigma Aldrich	Buchs	Switzerland	s8636	1 mmol/l
	L-Glutamin-Penicillin-Streptomycin 10X	Sigma Aldrich	Buchs	Switzerland	G1146	Details of the product in the legend§
	DMEM/F-12	ThermoFisher Scientific	Reinach	Switzerland	21041-25	1X
	MEM NEAA 100X	ThermoFisher Scientific	Reinach	Switzerland	11140-035	1% v/v
	fungin	Invivogen	San Diego	USA	ant-fn-2	0.1% v/v
	2-mercaptoethanol	ThermoFisher Scientific	Reinach	Switzerland	21985-023	1 mmol/l
	human recombinant epidermal growth factor (hEGF)	Sigma Aldrich	Buchs	Switzerland	E9644	10 ng/ml
Rat islet culture medium	DMEM medium	ThermoFisher Scientific	Reinach	Switzerland	11966025	1X
	Fetal bovine serum (FBS)	Merk Millipore	Zug	Switzerland	s0115	10% v/v
	Sodium Pyruvate	Sigma Aldrich	Buchs	Switzerland	s8636	1 mmol/l
	Glucose 40%	Bichsel	Interlaken	Switzerland	32 923 373	11 mmol/l

	L-Glutamin-Penicillin-Streptomycin 10X	Sigma Aldrich	Buchs	Switzerland	G1146	Details of the product in the legend§
Reagents and materials for isolation / dissociation of islets and cells and spheroid characterization	Collagenase A	Sigma Aldrich	Buchs	Switzerland	10103578001	2mg/mL
	Collagenase V	Sigma Aldrich	Buchs	Switzerland	C9263-5G	1mg/mL
	0.05% (w/v) trypsin-EDTA	ThermoFisher Scientific	Reinach	Switzerland	25300-054	-
	HBSS	ThermoFisher Scientific	Reinach	Switzerland	14175-053	-
	Antibiotic/antimycotic solution	Sigma Aldrich	Buchs	Switzerland	A5955	100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B
	Dulbecco's Phosphate Buffer Saline (PBS)	Sigma Aldrich	Buchs	Switzerland	D8537	-
	Bovine Serum Albumine (BSA)	Sigma Aldrich	Buchs	Switzerland	A3733	0.1% w/v
	NaCl 0.9%	Bichsel	Interlaken	Switzerland	100 0 178	-
	CM-DiL	ThermoFisher Scientific	Reinach	Switzerland	C7000	0,736111111
	Streptozotocin	Sigma Aldrich	Buchs	Switzerland	s0130	12.6 mg/mL
	Paraformaldehyde (PFA)	Sigma Aldrich	Buchs	Switzerland	P6148-1KG	4% w/v
	DAPI ProTaqs MountFluor Anti-Fading	Quartett Biochemicals	Berlin	Germany	401603392	-
	Matrigel Matrix	Corning	New-York	USA	356234	-
	VEGF	ThermoFisher Scientific	Reinach	Switzerland	PHC9393	200ng/ml
	DyLight 649 Griffonia Simplicifolia Lectin-Isolectin B4	Reactolab	Servion	Switzerland	DL-1208-.5	undiluted

§ : 1% v/v (2 mmol/l L-Glutamin, 100 U/ml Penicillin, 0.1 mg/ml (mmol/l L-Glutamin, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin)

Supplementary table 3. Kits, instruments and softwares

	Materials	Company	City	Country	Product number	Application
RT qPCR	Rneasy minikit	Qiagen	Courtaboeuf	France	74104	
	High Capacity cDNA Reverse transcription kit	ThermoFisher Scientific	Reinach	Switzerland	4368814	
	TaqMan Fast Advance Master Mix	ThermoFisher Scientific	Reinach	Switzerland	4444557	
	Rat insulin ELISA kit	Mercodia	Uppsala	Sweden	10-1250-01	
Instruments/Software	Galios cytometer	Beckman Coulter	Indianapolis	Indiania (USA)	-	Flow cytometry
	Kaluza Analysis software (version 1.5.20365.16139)	Beckman Coulter	Indianapolis	Indiania (USA)	-	Flow cytometry
	DMi8 manual microscope	Leica Microsystems	Heerbrugg	Switzerland		-
	Nikon A1R	Nikon Imaging	Egg	Switzerland	-	IF
	NIS-Elements Imaging Software (version 4.20.00 Build 972)	Nikon Imaging	Egg	Switzerland	-	IF
	Zeiss Axiocam	Zeiss	Feldbach	Germany	-	IF
	Zeiss Axioscan.Z1 slide scanner	Zeiss	Feldbach	Germany	-	IF
	Zen 2.3 Blue Edition software (version 2.3.60.1000)	Zeiss	Feldbach	Germany	-	IF
	ImageJ software	NIH	Bethesda	Maryland (USA)	-	-
	AggreWellTM400 24-well plates	Stemcell Technologies	Köln	Germany	34415	Spheroid generation
	Ibidi microscopy culture chambers	Ibidi	Planegg	Germany	81158	IF
	Freestyle Precision glucometer	Abbott Diabetes Care	Baar	Switzerland	-	in vivo
	PE50 tubing	PhyMep	Paris	France	BTPE-50	in vivo transplantation
	Screw-drive syringe	Hamilton	Reno	Nevada (USA)	81341	in vivo transplantation
	Prism software 8.0	GraphPad	La Jolla	California (USA)	-	Statistics

Table 4. Rat primers used for gene amplification

Gene	Forward sequence	Reverse sequence
RPLP1	TCT CTG AGC TTG CCT GCA TCT ACT	CCT ACA TTG CAG ATG AGG CTT CCA
INS	AGC AAG CAG GTC ATT GTT CC	ACC AGG TGA GGA CCA CAA AG
Pdx1	TGC CAC CAT GAA TAG TGA GG	CAG GGG GAT TAG CAC TGA AC
GLP-1R	TGG GGG AGA TAC AAC AGA GG	CTC TGG GCT TCT CAA CTT GG
PCSK1	GCA AAG AGG TTG GAC TCT GC	TCT GGC CCT CCA TGT ATC TC
PCSK2	TGT CTC TGC CTC TCC TTG GT	TGA GAG CAA GCA AAG CTT CA
VEGF-A	GGT AAT GGC TCC TCC TCC TC	AAG CCA CTC ACA CAC ACA GC

3 Conclusions and perspectives

In this work we managed to generate homogenous, controlled sized, insulin-producing 3D-organoids. This technique allowed to control cell composition and to add supporting cells. The implementation of an external endothelial cell source as a substrate for neo-angiogenesis is crucial, especially in order to be able to transplant organoids or even a bioartificial pancreas in others extra-vascular sites such as the subcutaneous space.

One of the challenges of ß cell replacement is the insulin-secreting cell source. The high number of human islets needed per recipient in IT, together with the scarcity of available organs are the main reasons why this therapy cannot be proposed to more diabetic patients. Among alternative sources, xeno-derived primary islet cells have been intensively studied and have even been transplanted into humans without immunosuppression. Despite the persistence of a detectable C-peptide, none of the trials demonstrated significant graft function (246, 247).

Beta cell lines are useful mainly for *in vitro* investigation but uncontrollable proliferation characteristics limit their translation to clinical practice. HESCs have been successfully differentiated into pancreatic endodermal cells (PEC) and have shown great potential for offering an unlimited source of insulin-secreting cells (248, 249). However, PECs take several weeks to mature after transplantation. Recently, ViaCyte performed clinical trials using PECs encapsulated in the Encaptra® Drug Delivery System (250). However, foreign body reaction have been reported as a limiting factor for the engraftment of encapsulated cells (251).

iPSCs represent another valuable cell source. They can be obtained from the recipient and transplanted without risk of allorejection. However, the transplanted material would still face autoimmune destruction and the personalized production of a sufficient number of iPSCs is a significant challenge in terms of logistics and costs (252). In addition, hESC and iPSC are associated with a potential risk of teratogenicity that is not well characterized yet, and calls for caution with their use in humans.

We previously highlighted the importance of intercellular communications within the islet and the complexity of this micro-organ. It may therefore be crucial to re-establish those connections when engineering organoids. Beta-cells generated from hESCs, iPSCs or transdifferentiation processes are certainly promising cells sources for insulin-secreting tissue. However, it is important to take other islet cell types, especially α-and δ-cells, into consideration when

generating organoids. It has been demonstrated that the cross-talk between different types of islet cells generate inhibitory and stimulatory signals affecting blood glucose homeostasis (253).

Another challenge of organoid generation for T1DM treatment is managing the large-scale production in order to obtain the functional mass of tissue required to establish proper metabolic control in one individual, and further, to make this therapy accessible to as many patients as possible. The automated hanging drop technique and the use of microwell-containing culture plates seem to be the most versatile and fit for the necessary upscaling (114). Automated methods in combination with the use of 3D printing will most likely shape the future of tissue engineering. A key aspect will be the cell sources used for the development of organoids. The use of patient-derived insulin-secreting cells (autologous) is very interesting for immunologic reasons. This personalized medicine approach, in which the cell product is tailored to each individual patient as he needs it, is very attractive, but implies substantial costs and logistics.

On the other hand, deriving such constructs from hESCs, adult stem cells or from xenogenic origins would allow the continuous production of an off-the-shelf, universal cell product, which could be engineered in a limited number of dedicated, centralized facilities. The direction that ß-cell replacement will take in the future remains open, but the field has reached a stimulating point, where many opportunities are close to hand, with clear prospects of a breakthrough for cell-based therapies for T1DM. Figure 1 here summarized organoids generation using (i) xeno-derived, (ii) donor-derived or (iii) recipient-derived insulin-producing cells and the components of the bioartificial pancreas.

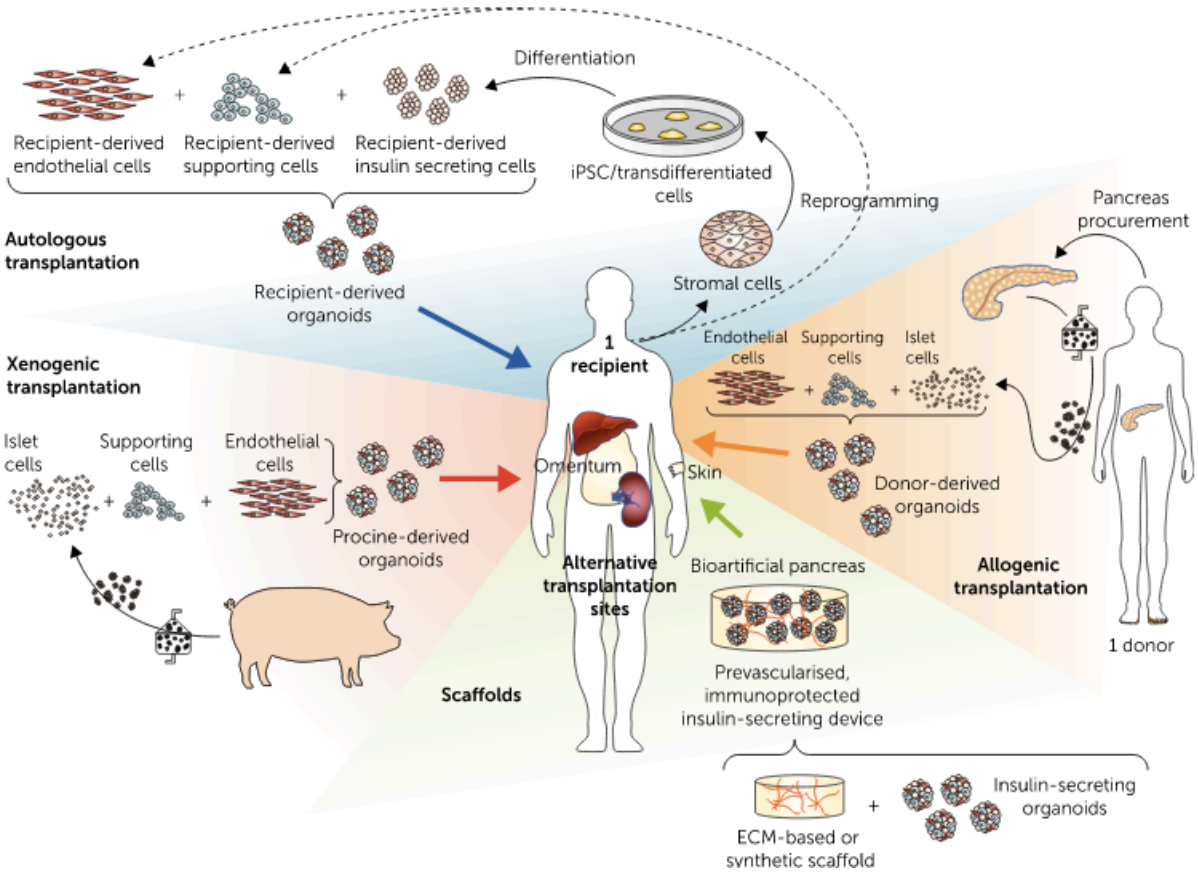


Figure 1. Perspectives for islet transplantation with the potential to develop either donor- or recipient-derived organoids, or xenogeneic-derived organoids. The lower panel describes the potential to incorporate those improved organoids in a scaffold, offering the possibility to explore new implantation sites.

In conclusion, organoid generation, with the possibility of incorporating supporting cells to an insulin-producing construct, represents a valuable strategy to overcome the hurdles faced by islet transplantation. By improving viability, function and engraftment, the number of islets required per recipient will be lowered, thus reducing the number of donors needed to achieve full glycemic control. Altogether, and in combination with the development of automated methods for industrial organoid generation, islet transplantation could become accessible as a therapy on a much larger scale. Furthermore, these advances will most likely open the path toward new transplantation sites, allowing to move away from the hostile liver micro-environment currently used.

Allo-rejection and auto-immunity recurrence are major issues in the development of islet transplantation. The need for systemic immunosuppression, which puts patients at risk of infection and neoplasia, makes this therapy available only to selected T1DM patients with severe disease. The modulation of the immune system, using cells such as MSCs or hAECs, or utilizing gene therapy approaches, would potentially allow the reduction or even the elimination of the need for immunosuppressive drugs.

Finally, insulin-producing organoids, represents the first and major step toward the creation of a bioartificial pancreas.

4 Appendix

4.1 Impact of ischemia time on islet isolation success and post transplantation outcomes: a retrospective study of 452 pancreas isolations

Charles-Henri Wassmer, Quentin Perrier, Christophe Combescure, Nadine Pernin, Géraldine Parnaud, David Cottet-Dumoulin, Estelle Brioudes, Kevin Bellofatto, Fanny Lebreton, Ekaterine Berishvili, Sandrine Lablanche, Laurence Kessler, Anne Wojtusciszyn, Fanny Buron, Sophie Borot, Domenico Bosco, Thierry Berney, Vanessa Lavallard

Abstract

Many variables impact islet isolation, including pancreas ischemia time. The ischemia time upper limit that should be respected to avoid a negative impact on the isolation outcome is not well defined. We have performed a retrospective analysis of all islet isolations in our center between 2008 and 2018. Total ischemia time, cold ischemia time and organ removal time were analyzed. Isolation success was defined as an islet yield ≥ 200 000 IEQ. Of the 452 pancreases included, 288 (64%) were successfully isolated. Probability of isolation success showed a significant decrease after 8 hours of total ischemia time, 7 hours of cold ischemia time and 80 minutes of organ removal time. Although we observed an impact of ischemia time on islet yield, a probability of isolation success of 50% was still present even when total ischemia time exceed 12 hours. Post-transplantation clinical outcomes were assessed in 32 recipients and no significant difference was found regardless of ischemia time. These data indicate that although shorter ischemia times are associated with better islet isolation outcomes, total ischemia time > 12 hours can provide excellent results in appropriately selected donors.

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Impact of ischemia time on islet isolation success and posttransplantation outcomes: A retrospective study of 452 pancreas isolations

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Keywords: type 1 diabetes, donors and donation, insulin/C-peptide, islet isolation, islet of Langerhans

Introduction

Pancreatic islet transplantation is a minimally invasive technique for beta cell replacement in type 1 diabetic patients (72, 254). Since the publication of the Edmonton protocol in 2000, allogeneic islet of Langerhans transplantation has made continuous progress, thanks to advances in isolation techniques, peri-transplant recipient management and immunosuppressive regimens, allowing to achieve insulin independence rates around 50% at 5 years in selected centers (93, 255-257).

Many studies have evaluated the impact of different parameters on islet isolation outcome. The variables shown to have an impact are donor characteristics (age, body mass index (BMI), gender, cause of death, duration of cardiac arrest, liver and pancreatic enzymes blood level, etc.), organ characteristics (weight, macroscopic appearance), procurement team, preservation solution used, pancreas fluid preservation microbial contamination and isolation process (258-263). Cold ischemia time (CIT), defined as the time between the pancreatectomy and collagenase perfusion, has also been studied and considered to play a role in islet isolation outcome variability (258, 260, 261). However, there is inconsistency in the literature on the effect of CIT on isolation success, and acceptable ischemia time (IT) is not well defined. Another important issue is that these studies have looked at the impact of these variables on islet yields, rather than on clinical functional outcomes after transplantation (264).

The Cell Isolation and Transplantation Center at the University of Geneva Hospitals has performed human islet isolation since 1991. Pancreases from Swiss and French multiorgan donors were obtained from hospitals participating to the GRAGIL network (265). In this context, we sometimes have to deal with long and not always predictable transportation times, and if maximum acceptable IT is set too strictly, it can become a contraindication for pancreas acceptance. In order to avoid unnecessary organ refusals, we have investigated the impact of IT on isolation success and post-transplantation clinical outcomes in our center over the last eleven years.

Materials and methods

This is a retrospective analysis of all islet isolations performed at the Cell Isolation and Transplantation Center between January 2008 and December 2018. Pancreases were obtained from Swiss and French multiorgan donors. Organs were perfused with cold preservation solution

right after aorta cross-clamping and then preserved and transported in Institut Georges Lopez-1 (*IGL*-1), University of Wisconsin (UW), or Celsior solution. For analysis purposes, we separated the preservation solutions in two groups, IGL-1 and others. We analyzed the impact of CIT, total ischemia time (TIT) and organ removal time (ORT), known also as secondary warm ischemia time. TIT was defined as time between aortic cross-clamping and initiation of collagenase perfusion in our laboratory. CIT was defined as time between pancreatectomy and initiation of collagenase perfusion. ORT was defined as time between aortic cross-clamping and pancreatectomy. Thus, TIT= CIT + ORT. Release criteria for transplantation in our center are the following: (i) ≥ 200 000 islet equivalent (IEQ) and/or ≥ 4000IEQ/kg of the recipient body weight, (ii) preparation purity ≥30%, (iii) viability ≥80%, (iv) tissue volume < 10ml. Therefore, islet isolation procedures were considered successful when islet yield was ≥ 200 000 IEQ in the final product, and failed when islet yield was < 200 000 IEQ. Analyzed variables related to donors were age, gender, BMI, type of donors (donor after brain death (DBD) or donor after cardiac arrest (DCD)), history of high blood pressure, the occurrence of a cardiac arrest, the number of vasopressors before procurement, history of smoking, alcohol and drug abuse. We also considered pancreas weight and isolation era (2008-2013 versus 2014-2018). Islets were isolated according to the automated method, with local modifications as previously described (91, 266). Over the observation period, no significant changes occurred in the isolation technique. In particular, the same collagenase (NB1; Nordmark pharmaceuticals, Uetersen, Germany) was used. When isolating islets from pediatric or juvenile donor pancreases, higher quantities of neutral protease were used (267). Islets were counted immediately after isolation and transplanted within 48 hours.

Results of islet isolation were expressed in IEQ and IEQ/g. For transplanted preparations, we analyzed the viability and function of the islets by FDA/PI assessments and the stimulation index, respectively. FDA/PI data were expressed as percentage of living cells. Insulin secretion data were expressed as stimulation index, which represented the ratio of stimulated-to-unstimulated insulin secretion during a glucose stimulating insulin secretion test (static incubation assay). Isolated islets were cultured separately according to their purity (>80%, 50-70%, >50%). For static incubation assay, 300 IEQ were collected from the pooled islets of the best purity. No hand picking was performed. Post-transplantation clinical outcomes were analyzed at 3 months after islet

transplantation. Islet auto-transplantations were excluded from analysis, and only first islet infusions were considered for clinical outcome analysis. In addition, patients who received a second islet transplantation within 3 months of the first infusion were also excluded. For clinical data, the pre- and post-transplantation C-peptide levels, HbA1c, insulin requirements and number of severe hypoglycemia episodes were analyzed, in order to assess graft success according to the Igls criteria (268). Our policy of slow insulin weaning after the first islet transplant made it difficult to calculate a reliable Igls score at 3 months, since most patients would have ended with a "marginal" score in spite of excellent glycemic control. Therefore, we attributed points for each Igls criterion, ranging from 0 to 3, for "failure", "marginal", "good" or "optimal" transplant outcomes, with a potential total score of 12. Each patient with a score > 6 was considered successful. In addition, correlation between CIT and the number of points obtained by patients was performed.

Successfully isolated preparations were separated for comparison at the following cut-off levels, determined by logistic regression analysis, as detailed in the results section: ≤ or > 8 hours for TIT, ≤ or > 7 hours for CIT, and ≤ or > 80 minutes for ORT.

Descriptive results are presented as mean ± standard deviation or frequency. Comparisons between groups were done using Student's *t*-test or X^2-test, wherever applicable. Associations with isolation success or failure were investigated by univariate and multivariate logistic regression models. In the multivariate model, the set of independent variables was defined a priori and no procedure of variable selection was used. The probability of isolation success according to the level of the independent variable was assessed using kernel estimates (269). Since this assumption was violated for some variables, segmented logistic regression models were used (270). The breakpoint was selected from a visual inspection of the relationship between the probability and the independent variables. With these models, the advantage is to capture a potential change in the association with the isolation success. The analyses were conducted with Prism software 8.0 (GraphPad, La Jolla, CA, USA) and software R (R Foundation for Statistical Computing, Vienna, Austria) (271). A two-sided risk of type 1 error of 0.05 was considered significant in all statistical analyses.

This study was reviewed and approved by the state of Geneva Ethical Committee (2018-02288).

Results

Between 2008 and 2018, 452 pancreases were processed for clinical islet isolation in our facility. Criteria for successful isolation were met in 288 (63.7%), which were included in the success group. Isolation success was significantly higher between 2014 and 2018 when compared to the period 2008-2013 (p=0.0004). BMI was significantly higher in the success group in comparison to the failed group (p=0.0007). On the other hand, alcohol abuse was significantly more prevalent in the failed group (p=0.0048). Of all donors, 446 (98.7%) were DBDs and 6 (1.3%) were DCDs. Only 14.2% had experienced cardiac arrest with a median arrest time of 10 minutes (range: 1-55). Donor and pancreas characteristics are summarized in Table 1.

Table 1. Comparison of donor characteristics between successful and failed islet isolation

	Total	**Non transplantable**	**Transplantable**	**p-value***
All pancreases	452	164 (36.3)	288 (63.7)	
Donor characteristics				
Gender, n (%)				> 0.99
Male	256 (56.7)	93 (36.3)	163 (63.7)	
Female	196 (43.3)	71 (36.2)	125 (63.8)	
Age (year), mean ± SD	46.8 ±12.9	45.61 ± 14.8	47.4 ± 11.7	0.187
BMI (kg/m2), mean ± SD	25.9 ± 5.1	24.8 ± 5.0	26.5 ± 5.1	**0.0007**
Type of donor, n (%)				
DBD	446 (98.3)	161 (36.1)	285 (63.9)	0.673
DCD	6 (1.3)	3 (50)	3 (50)	
High blood pressure, n (%)				0.639
No	330 (73.0)	116 (35.2)	214 (64.8)	
Yes	103 (22.8)	39 (37.9)	64 (62.1)	
Missing data	19	9	10	
Cardiac arrest, n (%)				0.674
No	386 (85.4)	138 (35.8)	248 (64.2)	
Yes	64 (14.2)	25 (39.1)	39 (60.9)	

Missing data	2			
Number of vasopressors, n (%)				0.486
0	42 (9.3)	12 (28.6)	30 (71.4)	
1	372 (82.3)	136 (36.6)	236 (63.4)	
>1	34 (7.5)	14 (41.2)	20 (58.8)	
Missing data	4 (0.9)	2	2	
Smoker, n (%)				0.110
No	220 (48.7)	72 (32.7)	148 (67.3)	
Yes	209 (46.2)	84 (40.2)	125 (59.8)	
Missing data	23	8	15	
Alcohol abuse, n (%)				**0.0048**
No	362 (80.1)	121 (33.4)	241 (66.6)	
Yes	72 (15.9)	37 (51.4)	35 (48.6)	
Missing data	18	6	12	
Toxic abuse, n (%)				
No	434 (96.0)	154 (35.5)	280 (64.5)	0.131
Yes	18 (4.0)	10 (55.6)	8 (44.4)	
Pancreas characteristics				
Isolation period, n (%)				**0.0004**
2008-2013	247 (54.6)	108 (43.7)	139 (56.3)	
2014-2018	205 (45.3)	56 (27.3)	149 (72.7)	
Preservation solution, n (%)				0.085
IGL-1	286 (63.3)	95 (33.2)	191 (66.8)	
Others	160 (35.4)	67 (41.9)	93 (58.1)	
Missing data	6	2	4	
Pancreas weight (g), mean ± SD	100.8 ± 24.1	97.9 ± 25.3	102.4 ± 23.3	0.059
ORT (minute), mean ± SD	65.8 ± 23.9	63.7 ± 25.7	67.0 ± 22.7	0.157
Missing data	2	2	0	
CIT (hour), mean ± SD	6.8 ± 2.6	6.9 ± 2.8	6.8 ± 2.4	0.577

TIT (hour), mean ± SD	7.9 ± 2.6	8.0 ± 2.9	7.9 ± 2.5	0.774
Missing data	2	2	0	

BMI: body mass index, IGL-1: Institut Georges Lopez-1, SD: standard deviation, DBD: donor after brain death, DCD: donor after cardiac arrest. *Student t-test for continuous variables, X^2-test for binary or categorical variable

While the mean ORT, CIT and TIT were not significantly different between success and failure groups (Table 1), a non-linear relationship with the probability of success was observed (Figure 1): this probability tended to increase and then to decrease. The cut-offs were approximately 80 minutes for ORT, 7 hours for CIT and 8 hours for TIT. A non-linear relationship was also observed for age and pancreas weight but not for BMI (Figure S1).

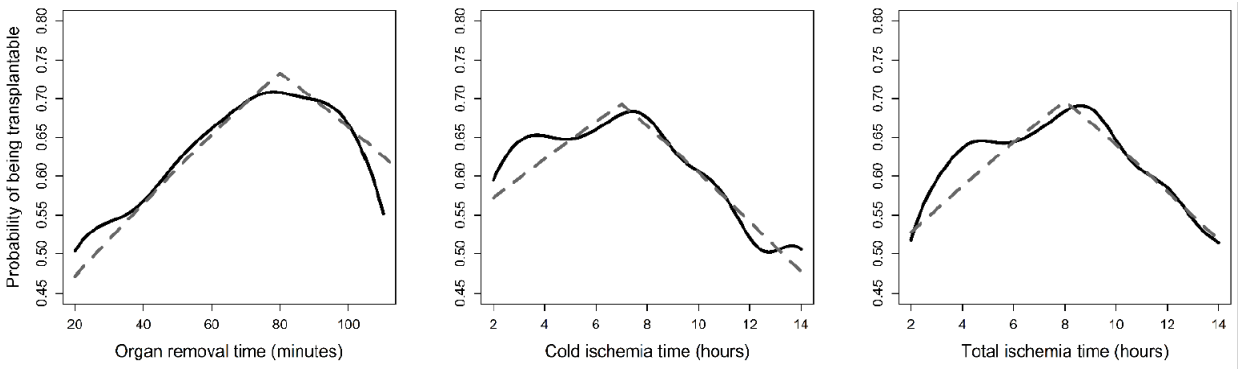

Figure 1. Transplantability according to organ removal time, cold ischemia time and total ischemia time. The smoothed black curves represent the probability assessed using kernel estimators. The grey dashed lines represent the probability modelled with univariate segmented logistic regression models.

Due to the non-linearity in some relationships, associations were assessed with segmented logistic regression models (Table 2). For ORT, the chance of successful isolation increased with the ischemia duration (OR=1.21 per 10 additional minutes, p=0.0047) up to an ORT of 80 minutes. After 80 minutes, the chance of success decreased (OR=0.85), albeit not significantly (p=0.09). Overall, ORT was associated with the probability of success (p=0.0161), even after adjustment by multivariate analysis (p=0.0369). For CIT, the trend was similar with a decrease in probability of

success after 7 hours of CIT. This association reached statistical significance after adjustment in the multivariate analysis (p=0.0201). For TIT, no significant overall association was observed, but a difference was detected when comparing the probability of success between before and after the 8-hour cut-off (p=0.04). Interestingly, 26 pancreases were isolated after more than 12 hours of TIT with a mean IEQ number of 283 620 ± 205 651. Among them, 13 (50%) were transplantable and 12 were actually transplanted (Figure S2).

Regarding the age, the probability of success increased until 45 years old (p=0.0003) before decreasing for older donors. However, this association disappeared after adjustment by multivariate analysis. The same observation was made with pancreas weight. Until 110g, the probability of success increased significantly (p=0.008) but this association didn't persist after adjustment. BMI was strongly associated with the probability of isolation success (p=0.0007) and this was confirmed in the multivariate analysis (p=0.0005). Alcohol abuse was significantly associated with isolation failure in univariate analysis (p=0.0043). However, this association disappeared after adjustment in the multivariate analysis. Finally, an association between the period and the isolation success was detected with a two-fold increase in the probability of successful isolation. On the other hand, no changes were observed in the association between isolation success and the other variables included in the multivariate analysis.

Table 2. Associations with the probability of success. Odds ratios (OR) assessed using segmented logistic regression models are reported with the 95% confidence intervals in brackets.

	n	Unadjusted OR	p-values*	p-values before vs after**	Adjusted OR	p-values*	p-values before vs after**
ORT (per 10 additional min)			**0.0161**			**0.0369**	
Before 80min	349	1.21 (1.06 - 1.37)	**0.0047**	**0.0126**	1.22 (1.05 - 1.42)	**0.0107**	**0.0364**
After 80min	101	0.85 (0.70 - 1.03)	0.0907		0.87 (0.71 - 1.08)	0.2140	
CIT (per additional hour)			0.1333			**0.0201**	
Before 7 h	253	1.10 (0.95 - 1.28)	0.1678		1.12 (0.93 - 1.35)	0.2254	**0.0241**
After 7 h	199	0.88 (0.78 - 1.01)	0.0676	0.0551	0.80 (0.69 - 0.94)	**0.0057**	
TIT (per additional hour) ***			0.1135				
Before 8 h	238	1.13 (0.97 - 1.31)	0.1080	**0.0400**			
After 8 h	212	0.88 (0.77 - 1.00)	0.0558				
Age (per additional year of age)			**0.0008**			0.1572	
Before 45 years	160	1.05 (1.02 - 1.08)	**0.0003**	**0.0005**	1.03 (1.00 - 1.07)	0.0547	0.1206
After 45 years	292	0.95 (0.92 - 0.99)	**0.0107**		0.98 (0.94 - 1.02)	0.3444	
Pancreas weight (per 10 additional grams)			**0.0265**			0.2406	
Before 110 grams	314	1.20 (1.05 - 1.37)	**0.0079**	0.0569	1.13 (0.96 - 1.33)	0.1525	0.0908
After 110 grams	138	0.94 (0.80 - 1.11)	0.4567		0.88 (0.73 - 1.06)	0.1806	
BMI (per unit)		1.08 (1.03 - 1.13)	**0.0007**		1.11 (1.05 - 1.17)	**0.0005**	
Gender, n (%)							
Female	196	1 (ref.)			1 (ref.)		
Male	256	1.00 (0.68 - 1.47)	0.98		1.00 (0.62 - 1.61)	0.9885	
Period, n (%)							
2008-2013	247	1 (ref.)			1 (ref.)		
2014-2018	205	2.07 (1.39 - 3.07)	**0.0003**		2.48 (1.53 - 4.02)	**0.0002**	
Preservation solution, n (%)							
IGL-1	286	1 (ref.)			1 (ref.)		
Others	160	0.69 (0.46 - 1.03)	0.069		0.96 (0.60 - 1.61)	0.8644	
Cardiac arrest							
No	386	1 (ref.)			1 (ref.)		
Yes	64	0.87 (0.51 - 1.51)	0.6099		0.82 (0.43 - 1.54)	0.5332	
High blood pressure							
No	330	1 (ref.)			1 (ref.)		
Yes	103	0.89 (0.56 - 1.41)	0.6163		0.83 (0.48 - 1.43)	0.5014	
Number of vasopressors							

0	42	1 (ref.)		1 (ref.)	
1	372	0.69 (0.33 - 1.37)	0.3080	0.81 (0.36 - 1.83)	0.6158
>1	34	0.57 (0.22 - 1.48)	0.2514	0.70 (0.24 - 2.08)	0.5270
Smoker					
No	220	1 (ref.)		1 (ref.)	
Yes	209	0.72 (0.49 - 1.07)	0.1087	0.82 (0.52 - 1.30)	0.3934
Alcohol abuse					
No	362	1 (ref.)		1 (ref.)	
Yes	72	0.47 (0.28 - 0.79)	0.0043	0.66 (0.35 - 1.21)	0.1799
Toxic abuse					
No	434	1 (ref.)			
Yes	18	0.44 (0.16 - 1.14)	0.0904		

*: for each odds ratio, a p-value is reported for testing the hypothesis that the odds ratio equals 1. For continuous factor with a potential non-linear relationship, this p-value is reported for both odds ratios before and after the cut-off. In addition, a p-value testing the global hypothesis that there is no association is also reported.
**: p-values for testing the hypothesis that the odds ratio is the same before and after the cut-off.
***: TIT was not introduced in the multivariable logistic regression model since it is the sum of ORT and CIT

Of all pancreases processed, 288 (63.7%) were successfully isolated but only 218 (48.2%) were transplanted. Seventy islet preparations were not transplanted for different reasons: medical contraindication in the recipient, bacterial or fungal contamination, insufficient IEQ number with respect to patient weight and positive cross-match.

We studied the effect of ischemia time, at the determined cut-off levels, on IEQ number, viability and *in vitro* islet function (Figure 2). No significant difference was observed for CIT (cut-off: 7 hours) and for ORT (cut-off: 80 minutes), except for islet viability in the CIT analysis. The percentage of viable islets was higher for CIT ≤ 7 hours as compared to > 7 hours (90.8% ± 3.7 vs 89.5% ±4.5, p=0.021; Figure 2B left panel).

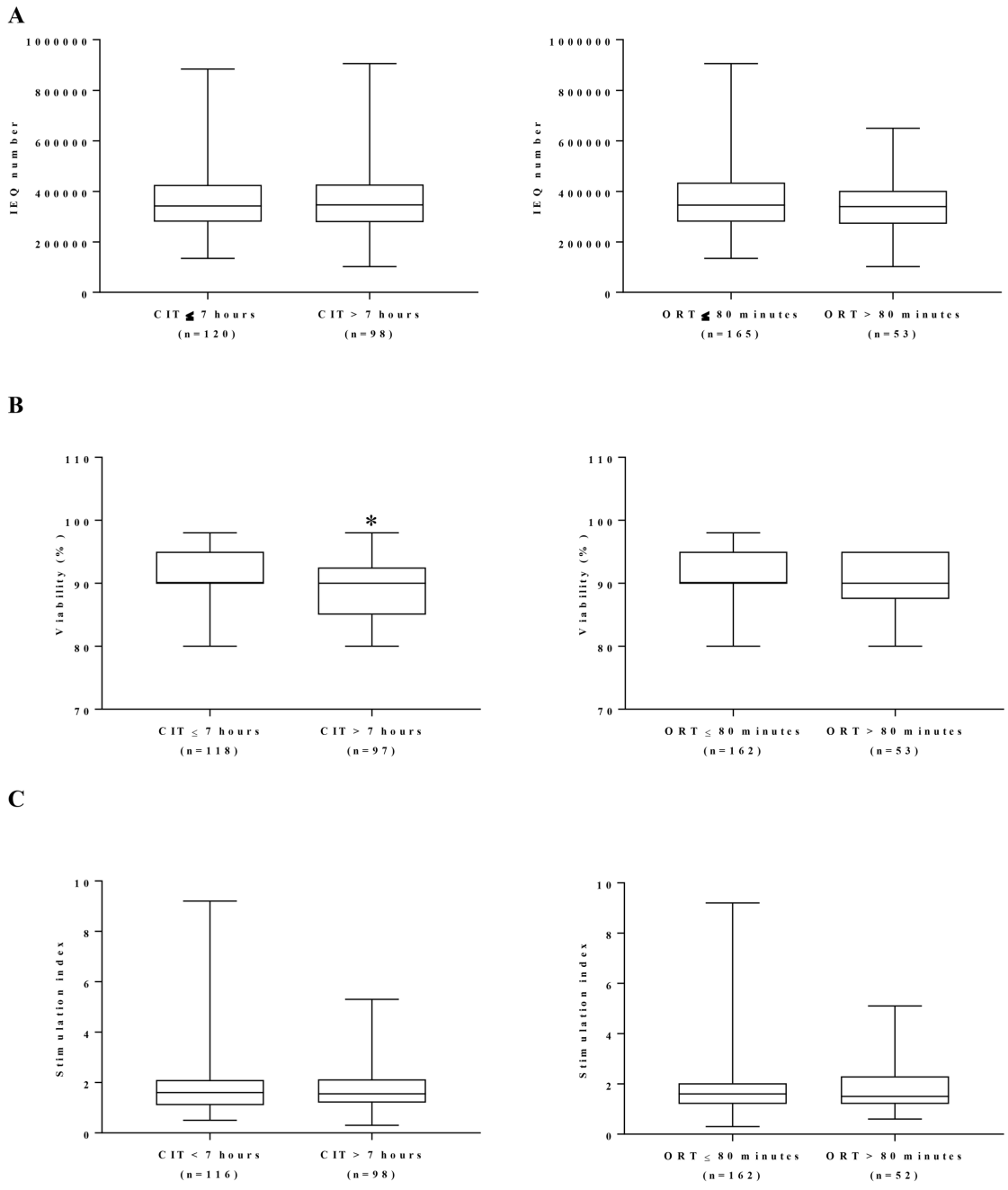

Figure 2. Number of IEQ (A), viability (B) and islet function (C) were analyzed as a function of cold ischemia time and organ removal time in 218 grafted islet preparations. Data are expressed as mean ± SD.

Finally, to determine whether CIT and/or ORT had an impact on clinical outcome, we assessed HbA1c and C-peptide levels and daily insulin requirement before and 3 months after the first islet transplantation in 32 recipients. Comparison was made between groups for CIT (Figure 3) and for ORT (Figure 4), at the same cut-off levels. We observed a similar decrease in the HbA1c level and daily insulin intake as well as a similar increase in C-peptide levels before and 3 months after transplantation in each group. In addition, we evaluated the transplantation success for each of the 32 patients, according to the Igls criteria. Twenty-eight patients (87.5%), were considered to have a successful transplantation 3 months after first islet infusion (Table S1). No correlation was found between CIT and the success rate according to the Igls criteria (r=0.05; Figure S3). These results indicate that, for successful islet isolation procedures, neither CIT nor ORT had an impact on early post-transplantation clinical outcomes.

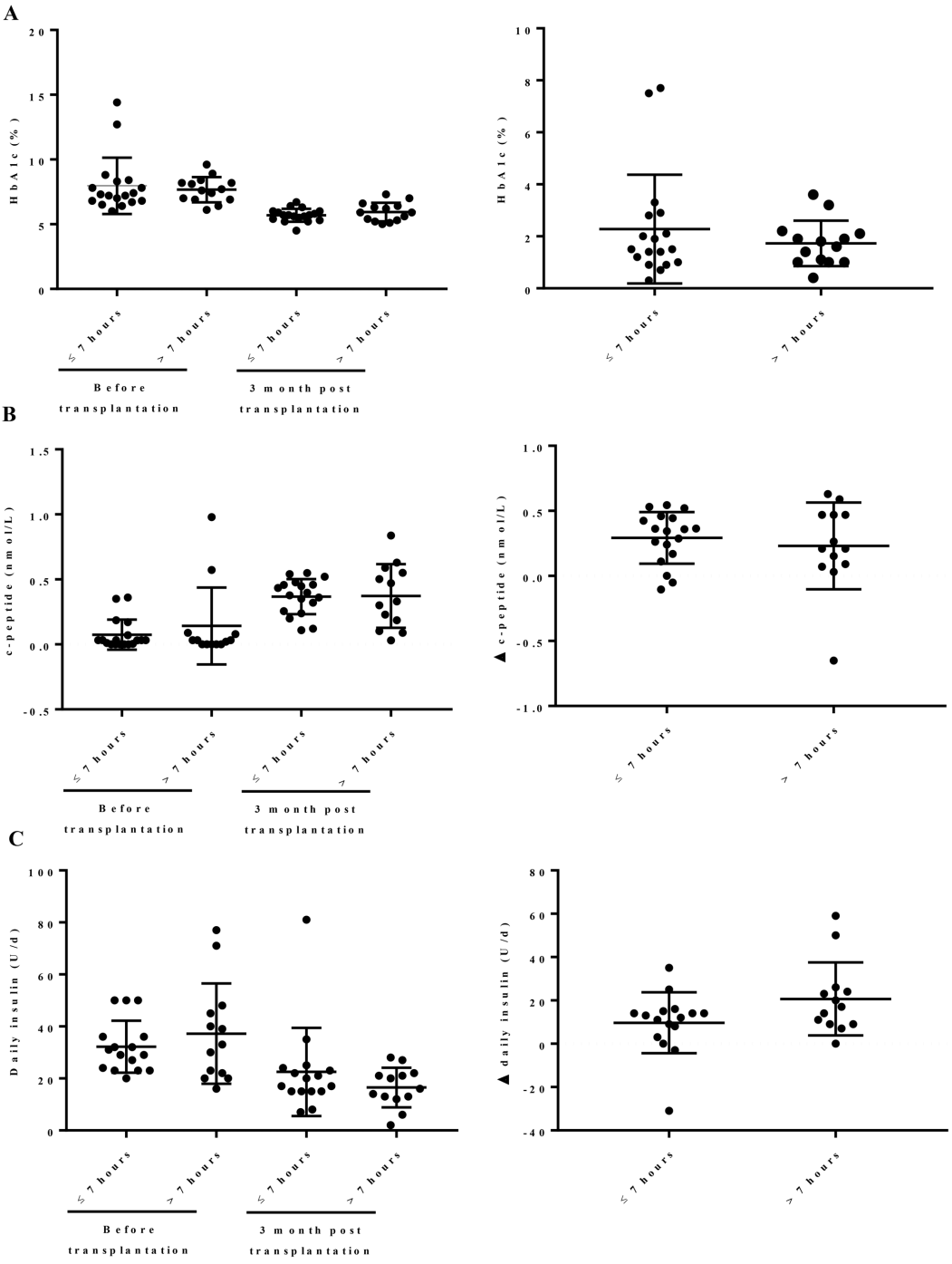

Figure 3. Clinical outcome as a function of cold ischemia time in 32 patients before and 3 months after transplantation. HbA1c values (A, left panel) and difference of HbA1c values (A, right panel) between pre and post-transplantation; C-peptide values (B, left panel) and difference of C-peptide values (B, right panel) between pre and post-transplantation; daily exogenous insulin intake (C, left panel) and difference of daily exogenous insulin intake (C, right panel) between pre and post-transplantation. Data are means ± SD. Missing data in 1 patient for C-peptide and 3 patients for insulin requirements. Data are expressed as mean ± SD.

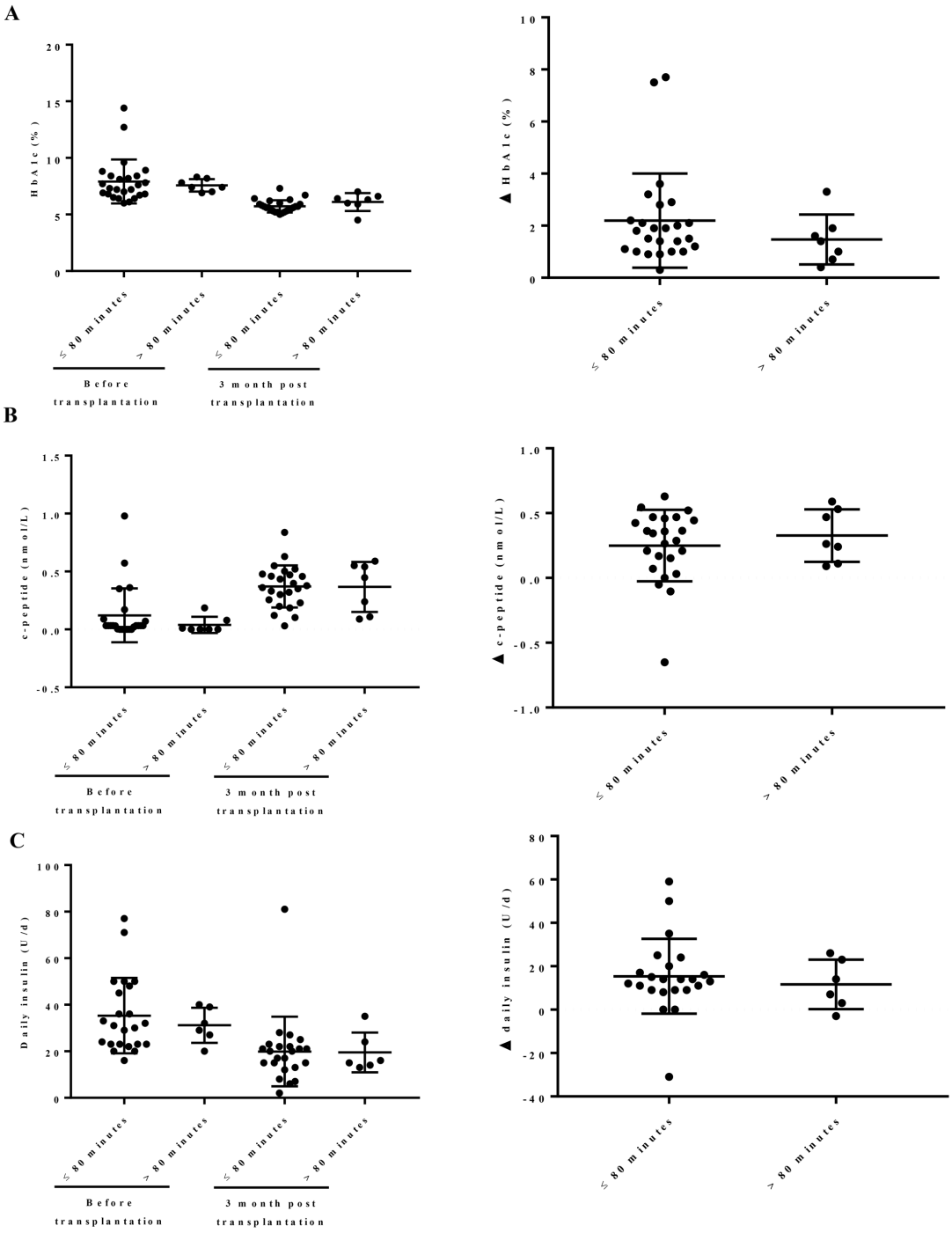

Figure 4. Clinical outcome as a function of organ removal time in 32 patients before and 3 months after transplantation. HbA1c values (A, left panel) and difference of HbA1c values (A, right panel) between pre- and post-transplantation; C-peptide values (B, left panel) and difference of C-peptide values (B, right panel) between pre and post-transplantation; daily exogenous insulin intake (C, left panel) and difference of daily exogenous insulin intake (C, right panel) between pre and post-transplantation. Missing data in 1 patient for C-peptide and 3 patients for insulin requirements. Data are expressed as mean ± SD

Discussion

Despite the constantly improving results of the islet isolation process, success remains unpredictable. Of the different variables with a potential impact on islet isolation outcome, defining reliable selection criteria for donor pancreases is crucial. Therefore, donor characteristics have been studied in-depth by many groups in order to identify and better define the relevant determinants of success. In 2005, the Edmonton group established a composite score combining donor and pancreas characteristics, and rating each organ from 0-100 donor points (263). The aim was to create a standardized score, allowing to evaluate the quality of a pancreas in the decision process of organ acceptance for islet isolation. Among the different variables used in the algorithm, reduced CIT was correlated with isolation success. In particular, CIT comprised between 8-12 hours, and more so CIT>12 hours, were allocated low donor points. This score was validated by Witkowski et al. in 2006 (272). Several other groups have studied the impact of CIT on islet isolation yields. Goto et al. reported significantly shorter CIT in successful versus failed isolations (260). Moreover, they observed that CIT >7 hours was associated with a significant decrease in the isolation success rate, number of isolated islets and islet function. Toso et al. reported that CIT > 8 hours and ORT (secondary warm ischemia time) > 30 minutes was associated with a reduced islet yield (261). These 2 studies were published over 15 years ago, and significant advances in the development of novel enzyme blends may have contributed to mitigate the impact of parameters previously shown as deleterious (266, 273). In a more recent study, Berkova et al. reported among other variables that a CIT > 8 hours had significant adverse effects on isolation success (258). Conversely, Nano and al. did not observe an impact of CIT on isolation yield when < 12 hours (274).

In our study, we observe for all 3 ischemia times that we have defined (TIT, CIT and ORT), a peak of probability of obtaining a transplantable preparation followed by a decrease showing the details of the association of ischemia time and isolation success in terms of time lapse. Better isolation outcomes with slightly longer CIT were already observed and integrated into the Edmonton donor point score (263). Interestingly, for ORT, the probability of being transplantable increased significantly from 50% to more than 70% in the first 60 minutes. This means that a short ORT could have a negative impact on isolation success. An explanation for this controversial result

could be that a very short ORT may reflect a rushed organ procurement, for example in case of surgical issues, potentially resulting in a damaged pancreas. On the other hand, during this early period of warm ischemia, a preliminary autodigestion process may occur in the pancreas, which can, if kept within certain limits, may be of assistance to the digestion phase of the isolation process.

Regarding the potential decrease in the probability of isolation success with time, we still observed a probability of success of 50% for ORT> 100 minutes and TIT and CIT>14 hours as described in the Figure S2.

Interestingly, at the determined cut-off values for CIT and ORT, we observed no impact on islet function, either in vitro in glucose-stimulated insulin release assays, or in vivo as determined by post-transplantation endocrine function. Four patients were considered to have a failed transplantation according to the Igls criteria. This is explained by our policy of slow insulin weaning, essentially preventing insulin independence by 3 months after a first islet infusion, and by the fact that none of these transplants was completed, all patients still expecting their subsequent islet infusions. However, those results seem to indicate that, after having passed the first hurdle of the islet isolation process, ischemia time has a limited impact on the short-term function of transplanted islets. A longer follow up period would of course allow to observe if this lack of impact is maintained in the long term. However, because patients generally receive a second or a third islet transplantation in the following months, long term results would be difficult to interpret. In addition, the lack of IT impact on short-term outcomes can result of a lack of power due to the small number of patients available for this analysis. Another limitation of this study is the cut-off values selected for the different ischemia times for statistical analysis. We used the values of the breakage of the ischemia time curves, and therefore, these analyses were data driven.

We detected a significant increase in isolation success over the last years when comparing with the period between 2008 and 2013. This result is in accordance with a recent report of the Collaborative Islet and Transplantation Registry (256), and is probably explained by improvements in the commercially available collagenase blends (266, 273). IGL-1 has become the most commonly used preservation solution in recent years. Even though a study performed in our

center showed no difference between the different preservation solutions (275), we observed in this study that more pancreases preserved in IGL-1 were successfully isolated when comparing with other preservation solutions, even-though it didn't reach statistical significance. This could also partially the improvement over the years.

The impact of ischemia times > 14 hours cannot be predicted from our small number of data at this threshold, but this study allows to conclude that a TIT or CIT between 8 and 12 hours is safely acceptable. In this context, when facing a decision to accept or not a pancreas, focus should be put more on other characteristics than ischemia time, if it is maintained under 12 hours. In this study, we assessed several donors' characteristics in order to detect new variables that impact the isolation outcome, others than the previously known such as BMI, age, history of alcohol abuse, time of cardiac arrest. In the recent years, we began to include DCD donors in Geneva for islet isolation. However, because of the small number of patients included, a conclusion about the impact of DCD donors on isolation success cannot be made. Among the others variables (history of high blood pressure, vasopressors requirement prior procurement, tobacco usage), no significant impact on isolation success was observed. Each donor is unique, but in case of a very good organ, defined by the classic donor characteristics mentioned above, and in view of our results, an ischemia time could in selected cases be pushed to > 12 hours. This should be taken in consideration when ischemia time during organ procurement becomes a limiting factor for the decision to proceed with a costly isolation procedure, especially when distances and transit times between the site of procurement center and the isolation center are important.

Supplementary data

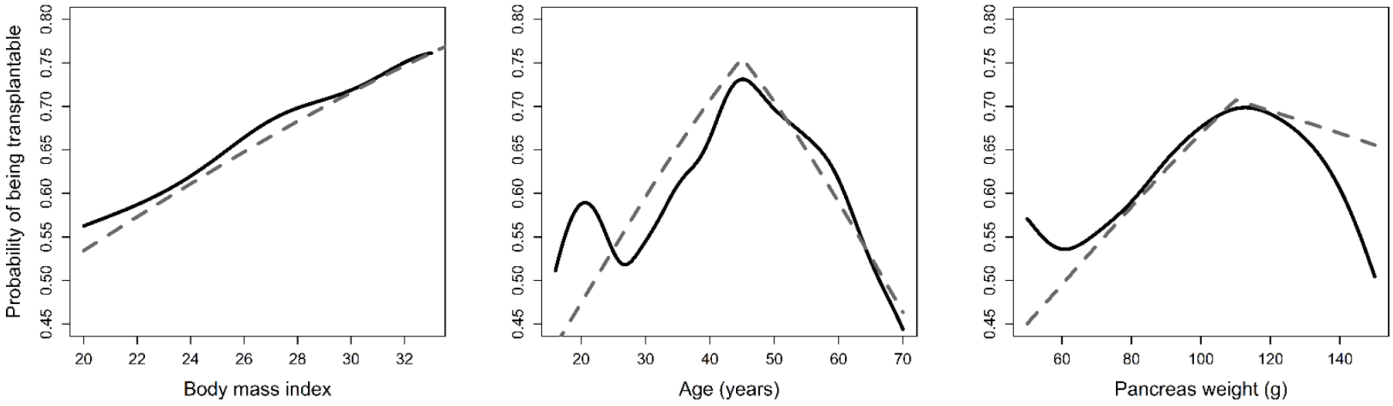

Figure S1. Transplantability according to body mass index, age and pancreas weight. The smoothed black curves represent the probability assessed using kernel estimators. The grey dashed lines represent the probability modelled with univariate segmented logistic regression models.

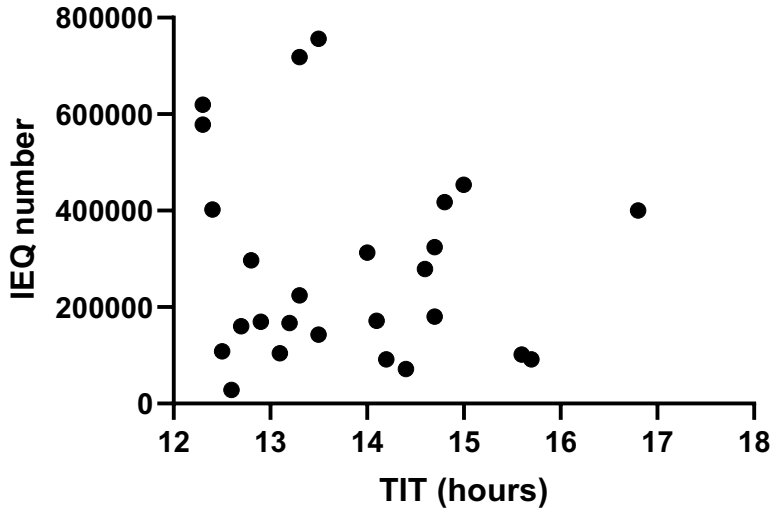

Figure S2. Correlation between the number of IEQ and TIT in pancreases with more than 12 hours of TIT.

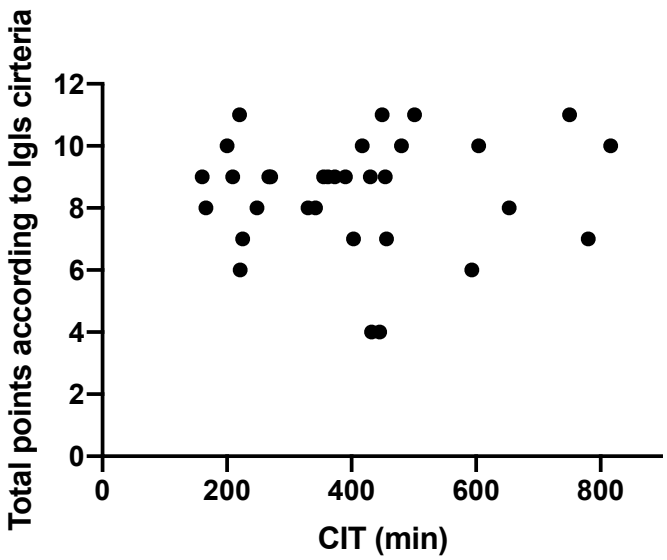

Figure S3. Correlation between CIT and the success of treatment according to Igls criteria

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