REVIEW

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

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SUMMARY

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.

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Introduction

Diabetes represents a major health issue with a current prevalence of 463 million affected adults worldwide and an expected prevalence of 578 million in 2030 [1]. In the long term, poor glycemic control puts diabetic patients at risk of developing micro- and macro-vascular complications leading to cardiopathy, neuropathy, retinopathy, and nephropathy [2]. Type 1 diabetes (T1D) is characterized by autoimmune destruction of insulin-producing ß cells and the current basis of treatment is by exogenous insulin administration. Despite recent improvements, insulin injection only imperfectly controls blood sugar levels, which may lead to two major clinical issues: the development of chronic complications, including end-stage renal failure and the need of kidney transplantation, and life-threatening problematic hypoglycemia. Beta-cell replacement by

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transplantation of the whole pancreas or isolated islets of Langerhans is an efficient way of restoring euglycemia, thus preventing the occurrence of severe hypoglycemia and protecting kidney grafts from the recurrence of diabetic nephropathy [3]. Pancreas transplantation, usually performed simultaneously with a kidney (SPK) in diabetic patients with end-stage renal insufficiency, is a major surgical procedure associated with significant morbidity [4]. Islet transplantation (IT) is a minimally invasive procedure, showing promising functional results that can be offered to a wider range of patients with T1D. However, the isolated islets have to face several challenges. The isolation procedure and engraftment process lead to a significant loss of insulin-producing tissue, due to isolation-related damage, loss of vascularization, loss of extracellular matrix, and an inflammatory microenvironment at the site of implantation [5]. These phenomena lead to the need for multiple donors in order to achieve insulin independence as well as to attrition of islet graft function over time [5] (Fig. 1). In the past decades, remarkable progress has been achieved in the enhancement of islet survival and engraftment, such as refinements in the islet isolation procedure, the design of steroid-free immunosuppressive regimens, and the development of anti-inflammatory strategies [6]. In the research field, further advances have shown promise in addressing the issues of immune protection and shortage of insulin-producing tissue. One strategy that has been widely studied, especially in tissue engineering, is the generation of organoids allowing to recreate organs from embryonic and/or adult stem cells [7]. This technology has been especially attractive in pancreatic islet research because of its ability to control the size and composition of the generated units, and the possibility to add supporting cells, such as endothelial or anti-inflammatory cells. The aim of this review is to highlight different approaches to improve the function and maturation of insulin-secreting organoids and discuss the perspectives and challenges of their clinical application.

The islet of Langerhans: a connected object

Islets of Langerhans are endocrine cell aggregates, representing less than 5% of the total pancreas volume and with a mean diameter of 100–150 μm [4,8,9]. In humans, an islet equivalent (IEQ, defined as a standardized islet with a 150 μm diameter) contains approximately 1500 cells [10] and is composed of 60% insulin-secreting cells (ß cells) and 30% glucagon-secreting cells (α cells) [11,12]. 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) [11,12]. In addition to endocrine cells, islets contain stromal cells, macrophages, neuronal elements, endothelial cells (EC), and pericytes, altogether representing <5% [10]. This indicates that more than a simple cell aggregate, the islet is a functional mini-organ with its own innervation [13] and complex intercellular communications [14]. 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 intercellular connections using cell adhesion molecules (cadherins), gap junctions, and ephrin receptors and ligands [15,16]. 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 [17] and cadherin-mediated adhesion of ß cells promotes their function [18]. Signals transmitted by E-cadherin play an important role in islet development, ß-cell aggregation, viability, and function [18-20]. 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 [21].

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 [22]. Endocrine cells are in close contact with a highly developed fenestrated capillary network allowing rapid responses to achieve optimal control of blood glucose levels. 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 [23]. On the other hand, release of growth factors, such as hepatocyte growth factor (HGF), by ECs, stimulates insulin biosynthesis and secretion [24]. In addition to their essential role in angiogenesis, intra-islet ECs synthetize ECM components, necessary for ß-cell proliferation, differentiation, function, and survival [25,26]. Islets are separated from the exocrine part of the pancreas by a peripheral capsule composed of fibroblasts and collagen

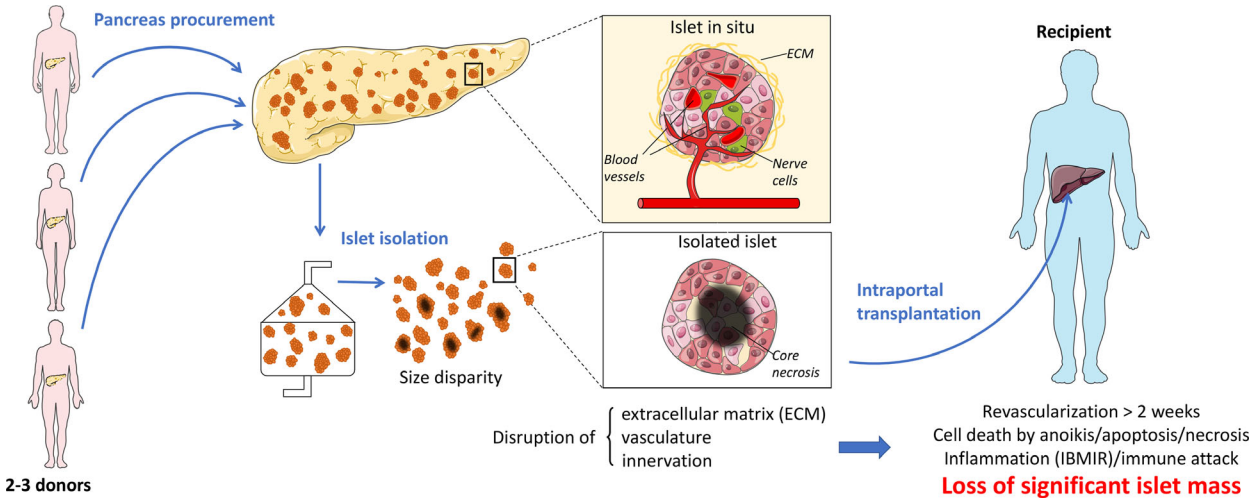

Figure 1 Limitations of clinical islet transplantation. The isolation process is responsible for the loss and disruption of the ECM, vasculature, and innervation of the islets. In addition to the inflammatory and immune attacks, this process results in the loss of an important proportion of the islet mass. IBMIR: instant blood mediated inflammatory reaction.

fibers, entrapped between two basement membranes (BM) located beneath the exocrine and endocrine epithelium (peri-islet) [15]. 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 [25]. 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 [27], but also contributes to ß-cell differentiation and survival [28]. Signals transmitted through the α6ß1 integrin also play a major role in the regulation of ß-cell survival [29]. 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* [30,31]. 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 [32].

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 [33,34]. 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" [35,36]. 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 [37,38]. 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 [39].

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 [40]. 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 [41]. Most of these approaches can be separated into microfluidic and nonmicrofluidic 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 [42]. However, while this approach represents a valuable system for high-throughput *in vitro* analyses, it is not designed for scaling up. Nonmicrofluidic methods include the hanging drop technique [43], cell self-aggregation technique [44], and the use of microwell culture plates [45]. These methods can be adapted for large-scale production of

organoids, like for example, the automated hanging drop method [46]. The different techniques of organoid generation are summarized in Fig. 2.

Over the last decade, the field of organoid science has developed considerably, notably for anti-cancer drug development [47,48] and in regenerative medicine [7]. 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 [49]. It was demonstrated in studies where 3D aggregates were created using adipose stem cells [50], tumor cells [51], insulin-secreting cells [52], 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. In addition, cellular 3D aggregates have shown the ability to express higher levels of stromal cell-derived factor 1 (SDF-1), in comparison to monolayer cultures. SDF-1 is a hypoxia-induced chemokine that recruits ECs for microvasculature development. 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* [53]. In addition to ECs, other supporting cells, such as MSCs, or other cells expressing anti-inflammatory mediators can also be incorporated into the organoids [54,55].

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 (PIs). In addition to primary dissociated islet cells, other cell sources can be used to generate PIs, such as ß-cell lines (e.g., MIN6 [56]), hESC [57], pancreatic stem cells [58], induced pluripotent stem cells (iPSC) [59], and other cell types using transdifferentiation such as insulin-secreting cells derived from other endocrine cell types (alpha cells) [60] or liver cells, for instance [61] (Fig. 3).

PIs can be generated by self-aggregation in nonadherent petri dishes [62] or in bioreactors with rotational culture [63]. However, these techniques demonstrate a high heterogeneity in term of PIs sizes and morphology. Isolated human islets are not uniform in size, usually ranging 50–500 µm in diameter [64]. Larger islets are more prone to develop core necrosis after transplantation, until revascularization occurs [65]. Moreover, transplantation of large islets through the portal vein

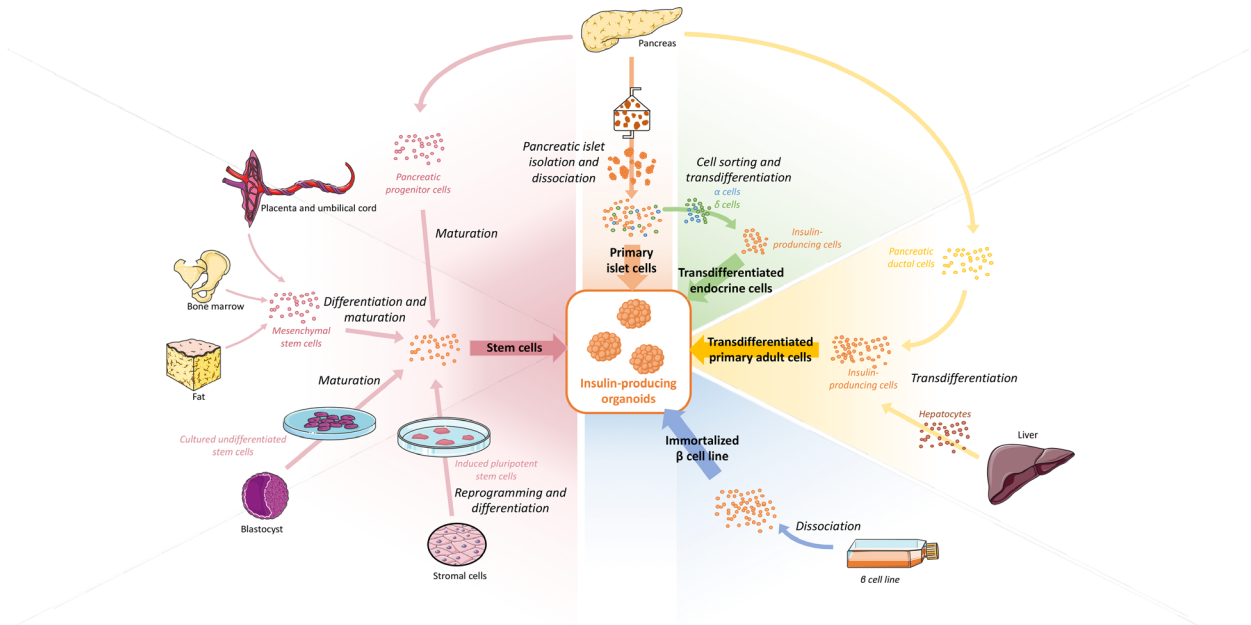

Figure 2 The different methods used for organoid generation. The upper panel of the figure describes graphically the different techniques; the lower panel describes the pros and cons of the different available methods using microfluidic or nonmicrofluidic techniques.

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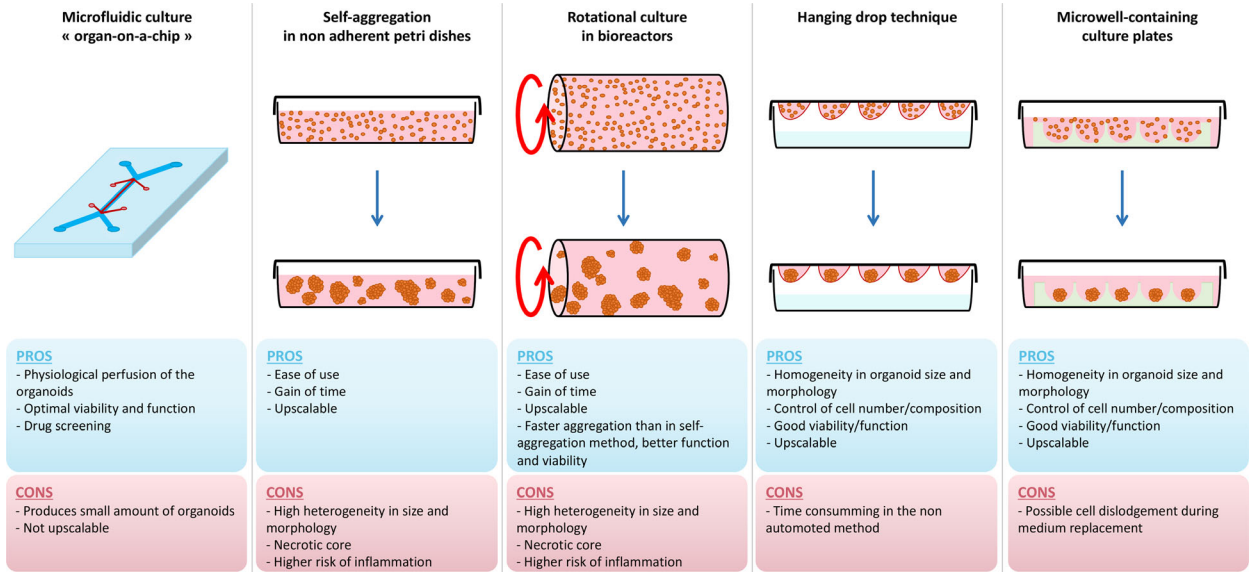

Figure 3 Sources of insulin-secreting cells for organoid generation.

can elicit inflammatory reaction due to embolization of larger vessels causing liver damage. To avoid this, large-scale generation of homogeneous, size-controlled PIs can be achieved by using the hanging drop method or microwell culture plates. PIs have also demonstrated improved viability and function, both *in vitro* and *in vivo,* compared to native islets [66,67], an observation attributed to the relatively small size of PIs. These findings are in line with previous reports on better *in vitro* performance of smaller PIs [68]. Interestingly, once transplanted, morphology and cellular arrangement of PIs changed to display a cell arrangement similar to that of native islets [69]. One step further, PIs can serve as elements for bioartificial pancreas construction, implantable in extra-hepatic sites, thus avoiding the proinflammatory microenvironment found within the liver [70–72].

Generation of uniform PIs from dissociated single islet cells has proven to be a very effective model for gene therapy experiments, allowing, for example, homogenous lentivirus transfection of the entire PI or gene modulation using shRNA (short hairpin RNA) [73]. Finally, ß-cell lines, usually cultivated and studied in monolayers, can be a useful cell source for PI generation and *in vitro* for functional studies and drug screening [74].

Validation criteria of newly formed pseudo-islets

Generation of PIs can be considered as a novel and valuable strategy for the treatment of T1D. Therefore, it is extremely important to develop a standardized validation system. In our opinion, PIs should meet at least three important criteria:

1. Morphology: PIs 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" [42,66,75,76].
2. Function: PIs 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 [77].
3. Viability: PIs 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. Nontumorigenicity: PIs 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.

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 (Fig. 4). 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 [78,79].

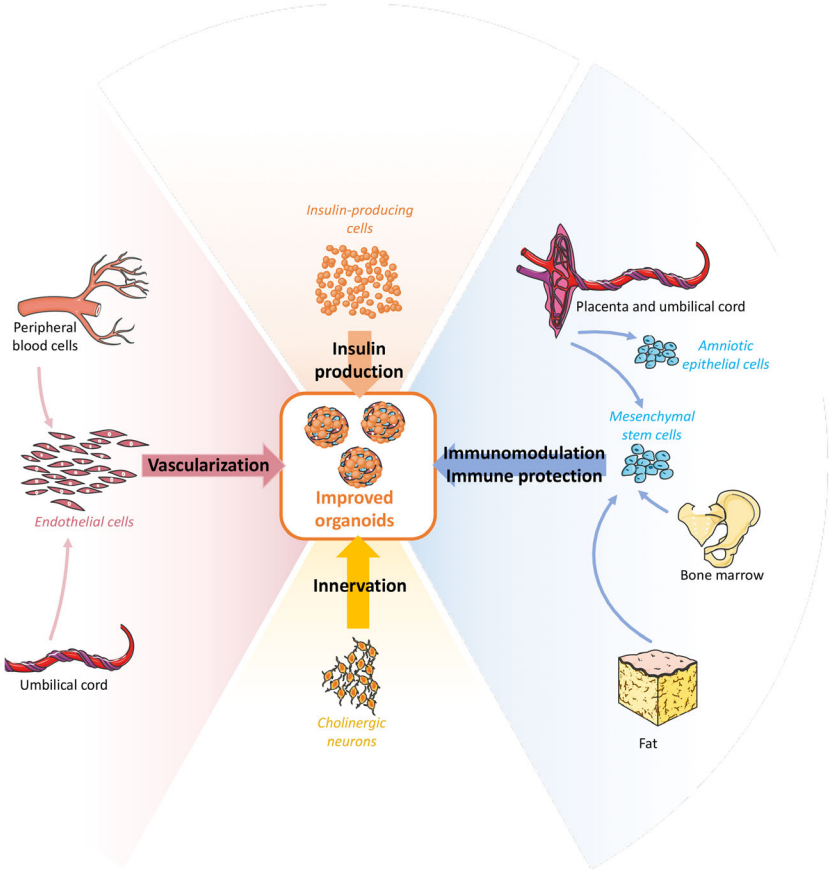

Figure 4 Supporting cells improving organoid function and engraftment.

Adding cholinergic neurons to islet cells demonstrated an increased islet function and re-innervation *in vitro* [80]. 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 [81].

The inflammatory and immunologic response against transplanted islets is detrimental for long-term graft function. Immediately after intraportal infusion of islets, an inflammatory cascade is activated, 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 [82]. Co-culturing them with islets has enhanced revascularization, function, and engraftment thanks to their angiogenic properties [83,84]. 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 [85,86]. Amniotic epithelial cells (AECs) are an alternative source of cells with similar properties [87]. These cells are derived from the amniotic membrane and are involved in the modulation of materno-fetal tolerance during pregnancy [88]. AECs have several advantages as a perennial source of active cells for organoid generation: They are easily accessible, inexpensive, and cause no ethical issue, since placentas are discarded after delivery; importantly, they have no tumorigenicity potential [89]. They have similar, or even more pronounced, angiogenic, antifibrotic, anti-inflammatory and immunomodulatory than MSCs [90]. Immune-modulatory abilities of AECs are mostly mediated by the expression and secretion of the nonclassical class I MHC antigens HLA-G and HLA-E that play an important part in materno-fetal tolerance [91]. We have recently reported on the effect of combining AECs with islets or dissociated islet cells. Shielding of whole islets with AECs markedly improved their secretory function in vitro and accelerated their revascularization *in vivo* [92]. Similar results have been

obtained *in vitro* and *in vivo* with insulin-secreting organoids, composed of dissociated islet cells and AECs [93]. Moreover, our studies have shown that AECs have a cytoprotective effect on islet cells under hypoxic conditions, mediated by HIF-1α. In addition to our promising results, others have demonstrated the ability of AECs to dampen the immune response against islets in both an allogenic model *in vitro* [94] and a xenogeneic model *in vivo* [95].

Taken together, these observations suggest that the inclusion of AECs inside insulin-producing organoids has translational potential as a therapy for T1D.

Assembling organoids into a bioartificial pancreas

As developed in this review, organoids can be used as individual units, without the need to seed them onto a scaffold. They can be generated either from an allogenic or a xenogenic source, or even recipient-derived, as described in Fig. 5. Once generated, regardless of their origin, insulin-producing organoids can be used as building blocks, assembled and incorporated into a scaffold, to construct a bioartificial pancreas. Over the past decades, a broad variety of naturally derived and synthetic polymers, collagen gels, with or without pores, and decellularized biological matrices have been proposed for scaffold construction [96–99]. While synthetic materials can be manufactured with consistent composition and can be easily fine-tuned according to needs [97], biological scaffolds are biocompatible, and can recreate the microenvironment of the islets due to the similar composition of the ECM. The major challenge with both synthetic and biological materials is providing sufficient immune protection and adequate vascularization to the islet graft. Ideally, cell-based tissue-engineered constructs should be able to simultaneously allow adequate nutrient delivery to the graft, with low-density cell loading, and be adaptable for scaling up from rodent to human dimensions [97,100]. Fig. 6 summarizes the main classes of biomaterials used in scaffold production together with their common advantages and disadvantages.

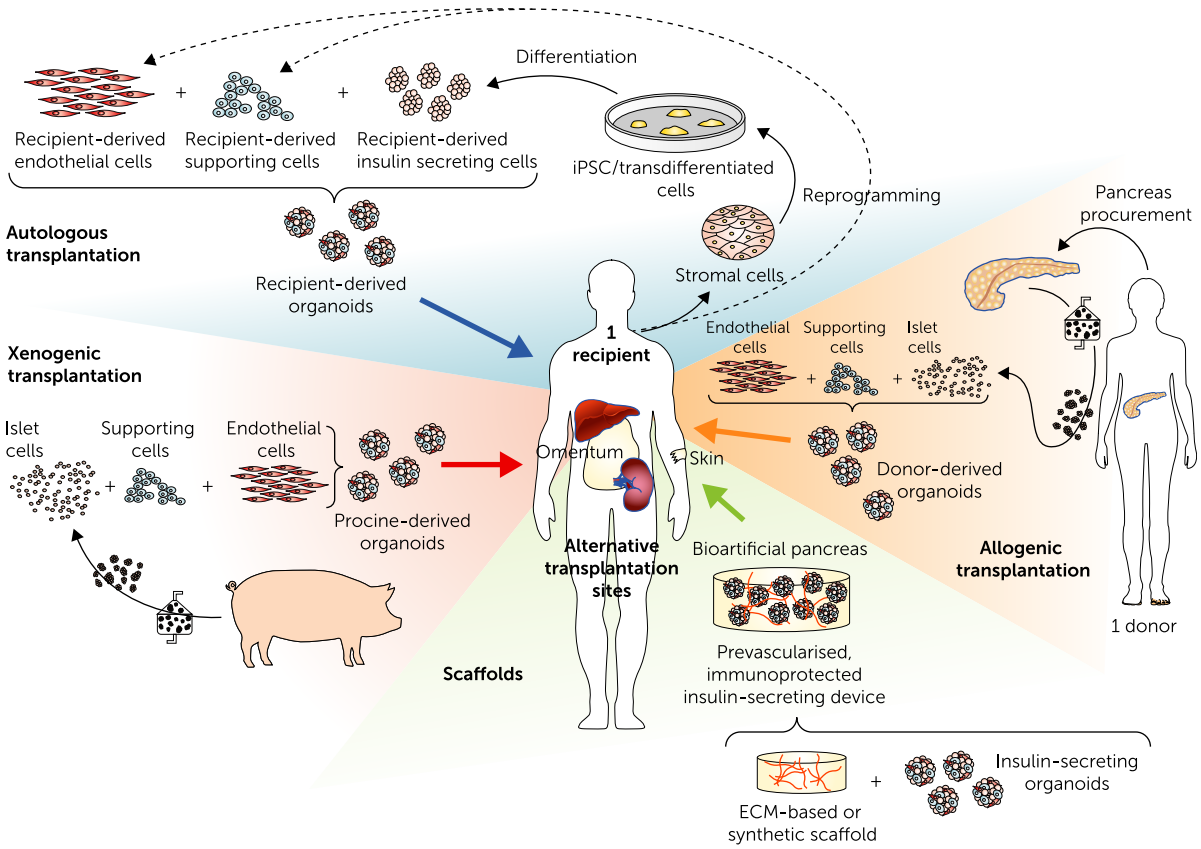

Figure 5 Perspectives for islet transplantation with the potential to develop either donor- or recipient-derived organoids, or xenogeneic-derived organoids. The lower panel describes the potential to incorporate those improved organoids in a scaffold, offering the possibility to explore new implantation sites.

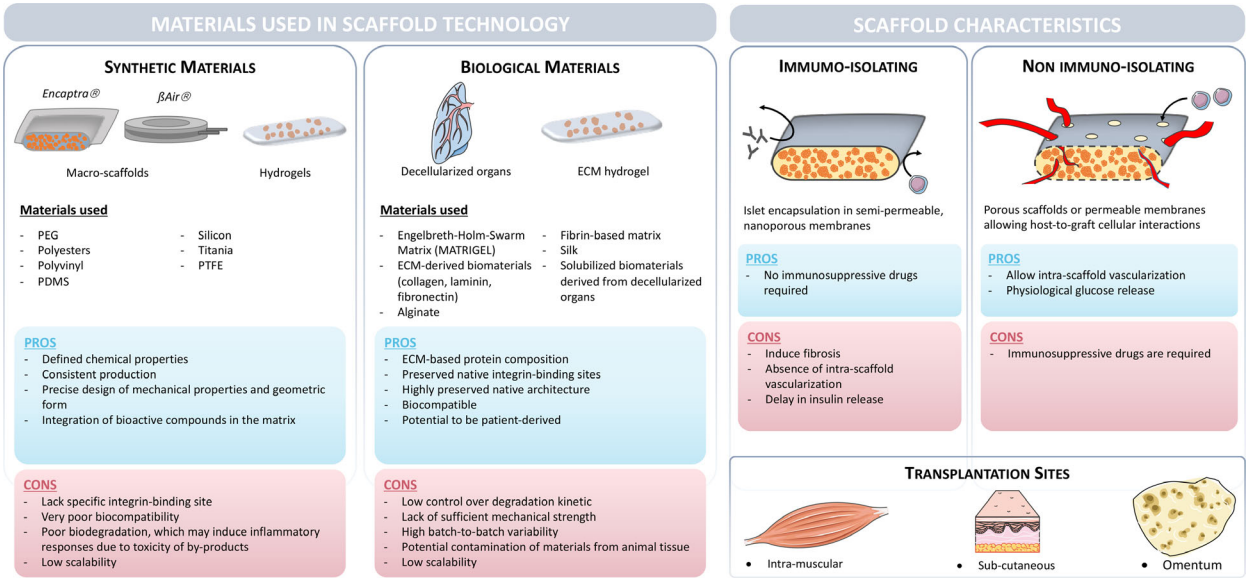

Figure 6 Scaffold generation. The first table shows the types of material available for scaffold generation, divided into synthetic and biological origins, with their advantages and disadvantages. The second table describes, according to scaffold sizes, the type of scaffolds, their advantages and disadvantages, the immunomodulation potentials and the possible sites of transplantation. PEG = polyethylene glycol, PDMS = polydimethylsiloxane, PTFE = polytetrafluoroethylene, ECM = extracellular matrix, MSC = mesenchymal stem cell, and hAEC = human amniotic epithelial cell.

Challenges and perspectives of insulin-secreting organoids

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, is the main reason 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 [101,102].

Beta-cell lines are useful mainly for *in vitro* investigation but uncontrollable proliferation characteristics limit their translation to clinical practice. Human embryonic stem cells (HESCs) have been successfully differentiated into pancreatic endodermal cells (PEC) and have shown great potential for offering an unlimited source of insulin-secreting cells [103,104]. However, PECs take several weeks to mature after transplantation. Recently, ViaCyte performed clinical trials using PECs encapsulated in the Encaptra® Drug Delivery System [105]. However, foreign body reaction hasbeen reported as a limiting factor for the engraftment of encapsulated cells [106].

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 [107]. 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 generates inhibitory and stimulatory signals affecting blood glucose homeostasis [108].

With the rapid development of genome editing, the CRISP-Cas9 or other systems have been used to "humanize" xeno-derived islets [109], to transfect nonislet cells with glucose-related promoters to express insulin [110], or to modulate immunity [111].

Another challenge of organoid generation for T1D 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. Of all the methods enumerated in this review, 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 [45]. 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 PIs. 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 T1D.

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 amount 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 microenvironment currently used.

Allorejection 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 T1D patients with severe disease. The modulation of the immune system, using cells such as MSCs or AECs, or utilizing gene therapy approaches, would potentially allow the reduction or even the elimination of the need for immunosuppressive drugs.

Finally, the use of induced pluripotent stem cells as a substrate for insulin-producing organoids could resolve the issue of organ shortage [112]. Ultimately, insulin-producing organoids, constructed with the approaches described in this review, could be used as building blocks for the bioengineering of a larger structure, and represent the first and major step toward the creation of a bioartificial pancreas.

Authorship

CW: designed, researched, and wrote the manuscript. FL and KB: contributed to manuscript contents and writing. TB and DB: critically revised the manuscript. EB: designed and supervised the writing of the manuscript.

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Conflicts of interest

The authors have no conflict of interest to declare.

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